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Publications arising from this thesis

1. **Lees, C.J**. Apostolopoulos, V., Acres, B.A., Ong, C-S., Popovski, V. and McKenzie, I.F.C. The effects of T1 and T2 cytokines on the cytotoxic T cell response to mannan-MUC1. *Cancer Immunology and Immunotherapy*, 48(11): 644-652, 2000.

2. **Lees, C.J**. Apostolopoulos, V. and McKenzie, I.F.C. Cytokine production from murine CD4 and CD8 cells after mannan-MUC1 immunization. *J. Interferon and Cytokine Research*. 19(12): 1373-1379, 1999.

3. **Lees, C.J**. Apostolopoulos, V., Acres, B.A., Ramshaw, I., Ramsay, A., Ong, C-S., and McKenzie, I.F.C. Immunotherapy with mannan-MUC1 and IL-12 in MUC1 transgenic mice. *Vaccine*, 19: 158-162, 2001.

4. **Lees, C.J**. Apostolopoulos, V., Ong, C-S., Smorodinsky, N., and McKenzie, I.F.C. In vivo evidence that a $MUC1^{+}$ metastatic mammary adenocarcinoma evades immune recognition by down regulating surface MHC class I expression. *J. Immunotherapy*. 2000 (In Preparation).

5. Apostolopoulos, V., McKenzie, I.F.C., **Lees, C.J.,** Matthaei, K. and Young, I. A role for IL-5 in the induction of cytotoxic T lymphocytes in vivo. *European Journal of Immunology*. 30:1733-1739, 2000.

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Declaration

In accordance with the regulations governing the preparation of a PhD thesis at Victoria University, I declare that all the experiments embodied in this volume constitutes original work performed by myself, unless stated otherwise. This thesis is less than 100,000 words in length exclusive of figures, tables and references.

Catherine Lees

The Austin Research Institute and
Victoria University

Dear Mum and Dad,

Thank-you for your constant support, love and encouragement always.

I would like to dedicate this thesis to two people who gave me a wonderful gift – the determination and passion to want to make a difference to the lives of people touched by cancer.

•

Sylvia Florence Ballingall

1910-1990

✞

•

Raymond Ernest LeRoy

1931-1993

✞

Cancer immunotherapy with Mucin-1 and cytokines

Thesis submitted for the degree of Doctor of Philosophy

by

Catherine Lees

The Austin Research Institute

and

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Thesis Summary

Cancer immunotherapy involves the manipulation of the immune response to combat tumour cells. The studies contained within investigated the role of cytokines in the anti-tumour immune response to the cancer antigen, MUC1, which is over produced in an altered form in cancers of the breast, pancreas, and ovary making an ideal target for immunotherapy. MUC1 coupled to oxidised mannan (MFP), forms a powerful immunotherapeutic reagent capable of inducing tumour regression and cell mediated immune responses that protect against tumour challenge in many *in vivo* tumour models. This thesis characterised the T1 cytokine profile induced from $CD8^+$ and $CD4^+$ T cells following immunisation with MFP and suggested a role for IL-12 in the MUC1 anti-tumour immune response. The addition of various combinations of T1 and T2 cytokines to MFP injections considerably increased the MUC1 CTLp response, and demonstrated a role for IL-5 in the induction of cytotoxic T cells. A MUC1 mammary carcinoma tumour was characterised to provide a more realistic model for MUC1 immunotherapy studies. However, of particular significance was the discovery that additional IL-12 included in MFP immunisations, considerably decreases tumour growth in MUC1 transgenic mice and significantly increases the MUC1 specific CTLp response. The use of IL-12 and MFP in cancer immunotherapy has now progressed into Phase I human clinical trials in cancer patients.

Thesis Introduction

Cancer affects the lives of one in three Australians. Despite the enormous advances made in the past century with genetic engineering and new emerging therapeutic drugs and technologies, cancer is still second only to myocardial infarcture (heart attack) as the leading cause of death in all Australians. While research continues to find the best way to treat existing cancers and minimise the impact of this disease on human lives, there is now much attention focused on both the early detection and prevention of cancer. The advent of successful early detection and screening programs such as BreastScreen, has seen a decline in the mortality rates from breast cancer in recent years. However, breast cancer still claims the greatest number of lives of Victorian women each year indicating that more research into the biology, treatment, early detection and prevention of cancer needs to be undertaken.

Cancer research in the late 1990s continued to focus on identifying new tumour antigens which may play a role in the early identification and therapy of cancer cells (e.g. immunochemical faecal occult blood tests for colorectal cancer screening). The identification of new tumour antigens, a greater understanding of the immune system and advances in adjuvant technology now sees scientists using immunotherapy to develop cancer vaccines that could ultimately be used to immunise high-risk members of the public from developing cancer in the future.

The development of a successful cancer vaccine requires the identification of a suitable tumour target, or antigen, which distinguishes malignant cells from normal cells within an individual, and the induction of an appropriate immune response for solid tumour immunotherapy is probably the induction of a T cell response. Mucins, particularly those found in breast, pancreatic, ovarian and colorectal cancer, are of interest as targets for immunotherapy because there is an increase in the amount of mucin present, an alteration in the distribution on the cell surface, and the exposure of new peptide epitopes in cancer which are not found in normal tissues. These MUC1 peptide epitopes are highly immunogenic in mice and are the target of monoclonal antibodies made against whole tumour cells, extracts and synthetic peptides. Human studies have shown cytotoxic T cell clones from the lymph nodes of patients with breast, pancreatic and ovarian cancers recognise not only mucin, but the same MUC1 peptide epitopes recognised by the murine monoclonal antibodies.

The conjugation of a MUC1 fusion protein containing antigenic peptide epitopes, to the oxidised sugar mannan (MFP) has been successfully shown *in vivo* to induce a cellular immune response characterised by the production of HLA-restricted cytotoxic T cells and little antibody production, and does indeed protect against MUC1 positive tumour challenges in mice. The MFP cancer vaccine developed by Dr Vasso Apostolopoulos in the mid-1990s has since been tested in Phase I human clinical trials in patients with breast and colon adenocarcinoma. The results from these trials indicate that the vaccine is clearly immunogenic in humans (producing high titres of antibodies) and does not induce toxicity or autoimmune responses (to other tissues expressing MUC1 protein) in cancer patients. To date no anti-tumour responses have been noted in humans immunised with MFP, although these have not been formally sought. Despite the finding that the vaccine is immunogenic in humans through its production of MUC1 antibodies, there is little evidence that a cellular immune response, the desired response for solid tumours, has been induced.

Based on these findings, it remained essential to investigate the effects of various adjuvants which had been shown to enhance the immune responses produced in other tumour models. Of particular interest was the vast array of newly discovered cytokines which play a crucial role in the distinction between the T1 cellular and T2 humoral immune pathways. Cytokines were quickly beginning to show much potential as they boosted the immune response to relatively weak immunogenic antigens and were therefore examined in the MUC1 tumour immunotherapy model.

The aim of this PhD thesis "Cancer Immunotherapy with Mucin-1 and Cytokines" was to investigate the relationship between cytokines and the MFP vaccine, and to investigate if the addition of recombinant cytokines to MFP could indeed boost the cellular immune response generated against MUC1 to ultimately decrease the tumour burden in humans. The experimental studies which comprise this thesis were performed at the Austin Research Institute, Australia, between January 1995 and April 1998.

Chapter one contains a literature review focusing on tumour immunotherapy and in particular looks at mucin-1 as a tumour antigen in breast, pancreatic and ovarian cancer, the role of mannan and other antigen delivery systems and the role of cytokines as adjuvants for enhancing both cellular and humoral immune responses *in vivo*.

Chapter two described the common materials and methods used throughout the thesis, including the production, purification and conjugation of the MUC1 fusion protein to mannan, the production and purification of vaccinia virus engineered with recombinant cytokines, the tumour models and transgenic mice used for the studies and the assays used to quantify immune responses such as cytotoxic T cell and T cell precursor assays and enzyme-linked immunosorbent assays (ELISA).

Chapter three described the results of the cytokine profile of $CD4^+$ and $CD8^+$ spleen T cells from mice immunised with various forms of the MUC1 tumour antigen (oxidised MFP, reduced MFP and varying combinations) to further characterise the immune responses to MUC1 and identify any key cytokines involved in the generation of an antitumour response.

Chapter four compared the effect of additional T1 and T2 cytokines from vaccinia virus on the cellular immune response to MUC1. CTLp frequencies were compared in mice immunised with MFP alone and MFP plus additional cytokines. The cellular immune response generated when oxidised MFP was injected into cytokine gene knock-out mice was also investigated. This study was used to determine if cytokines could enhance the antitumour immune response to MFP *in vivo*.

Chapter five characterised a MUC1 the aggressive $MUC1^+$ metastatic tumour, DA3-MUC1 because unlike other $MUC1^+$ tumour models, DA3-MUC1 is not spontaneously rejected in mice making it a better model for immunotherapy studies.

Chapters six and seven both focused on the enhanced effect of one additional cytokine on the cellular immune response induced to oxidised mannan-MUC1. Chapter six investigated the effect of IL-5 on oxidised MFP immunisations and its secretion patterns from $CD4^+$ and $CD8^+$ spleen T cells and chapter 7 investigated the role of IL-12, the cytokine shown to have the most potential for cancer immunotherapy studies, in a MUC1 transgenic immunotherapy model.

Finally chapter eight concludes this thesis with an overview of the results found from these studies and a look to the possible role cytokines may play in cancer immunotherapy in the future.

Abbreviations

aa	amino acid
ABTS	[2,2'-azino-di(3-ethylbenzthiazoline Sulphonate)]
BSA	bovine serum albumin
CASA	Cancer-associated serum antigen
CEA	carcinoembryonic antigen
^{51}Cr	$Na_2{}^{51}CrO_4$ - chromium
CO_2	carbon dioxide
CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T lymphocyte precursor
DAB	diaminobenzidine
DMEM	Dulbecco-modified Eagle's Medium
DTH	delayed type hypersensitivity
E.coli	*Escherichia coli*
ELISA	enzyme linked immunosorbent assay
FACS	fluoresence activated cell sorter
FITC	florescein isothiocyanate
g	acceleration due to gravity
G(M)-CSF	granulocyte (macrophage) – colony stimulating factor
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
HMFG	human milk fat globule membrane
h	hours
IFN	interferon
IL	interleukin
IPTG	Isopropyl-B-D-thiogalactopyranoside
kD	kilodalton
kB	kilobase
KLH	keyhole limpet hemocyanin
MFP	Mannan-MUC1 fusion protein conjugate
MHC	major histocompatibility complex
min	minutes
MPBS	mouse phosphate-buffered saline
NH_4Cl	Ammonium Chloride
NP-40	Nonidet P40
OD	optical density
PAGAL	phenyl N-acetyl-a-D-galactosaminide

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
RPMI	Roswell Park Memorial Institute Medium
UV	ultraviolet
VNTR	variable number tandem repeats

Table of one letter amino acid codes

Amino Acid	One letter Code
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Chapter 1:

Literature review

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Introduction

Patients diagnosed with cancer today have a greater chance of surviving than their predecessors, due to the continual advances being made by scientists. The 1900s have seen the development of cancer therapy technology such as chemotherapy and radiotherapy, newly designed surgical techniques involving microsurgery and laser treatments, new anti-cancer drugs such as taxol, and recently, advances in immunotherapeutic strategies which have seen many new approaches move from laboratories into human clinical trials. Surgery can be curative, but the treatment of metastatic disease (for solid tumours) is about as elusive today as it was decades (perhaps even centuries) ago. There are however some exceptions, as some metastatic diseases can at times be cured eg. melanoma. Thus for the majority of cancers, cytotoxic drugs and radioactivity have not cured metastatic disease and in this light, new therapies must be examined, including immunotherapy.

Immunotherapy for cancer involves the generation of an immune response specifically directed against one or more antigenic determinants displayed by a tumour and not by normal cells of the individual. Similar to chemotherapy, immunotherapy can be delivered systemically to combat a tumour however it has the added advantage of acting specifically against the tumour and therefore not damaging nearby healthy cells. The past decade has seen a vast increase in the amount of tumour immunotherapy research occurring and highlights the potential importance of this as a therapy for malignancies in the future.

This literature review focuses on tumour immunotherapy from the laboratory to the clinic. It discusses specific tumour antigens used in current immunotherapeutic studies and the strategies employed to direct and enhance the immune response against the tumour. The review concentrates on the tumour associated antigen Mucin-1, and the role of cytokines in improving the host's natural immune response against cancer.

Section 1: Tumour immunotherapy

1.1 The history of tumour immunotherapy

There have been various attempts over the decades to stimulate the immune response to kill cancer. Most have involved the use of irradiated or killed tumour cells together with an adjuvant in the hope that it would elicit an immune response that could eradicate malignant cells. The term 'vaccine' was introduced 200 years ago by Edward Jenner to name the prophylactic preparation of cowpox viruses which proved to be able to condition the human defence system to be able to recognise and fight smallpox. A hundred years later (in 1893) William B. Coley, the first tumour immunologist, used preparations derived from streptococcal cultures to treat human tumours (Coley 1893, 1906).

Coley discovered that this non-specific immunostimulant, erysipelas, had a powerful antagonistic effects against sarcomas (Coley, 1893). In a patient with inoperable sarcoma of the neck, he observed that the tumour completely disappeared after two consecutive attacks of erysipelas and that the patient was alive and in good health seven years later. His conclusion at that time was the curative action of erysipelas was systemic and most likely due to the toxic products of the streptocci. In fact, Dr Coley was at that very moment probably witnessing the effects of TNF induced by streptococci toxins which were released in the course of the erysipelas attack. After this observation, Coley started experiments in which he injected patients suffering from irresectable tumours with streptococci toxins (Coley, 1906). However, these toxin injections were harmful, so he continued with research aimed at reducing the toxicity of his so-called Coley's toxin – which even today has not been acceptable for use in humans due to its severe toxicity.

There were many attempts in subsequent years of scientists trying to treat established carcinomas *in vivo*, however it wasn't until the late 1950s that scientists were developing a greater understanding of the immune system and the mechanisms it employs to fight disease. One particular study claimed that the immune system was capable of detecting and combating tumour cells. In this study, Macfarlane Burnet (1957) and Lewis Thomas (1959), suggested that small masses of tumour could arise within a host, carrying new antigenic properties specific for the tumour which could easily be eradicated by the immune system without

leaving any clinical evidence of the tumour's existence (Burnet, 1957). Supporting this idea was that by Thomas, who suggested in 1959 that homograft rejection could be the primary defence mechanism for neoplasia (Thomas, 1959). Years later (1968), Rosenberg observed a patient who had been admitted to hospital with characteristic gallbladder complaints. He discovered that the patient was operated on 12 years earlier to remove a stomach cancer which had spread to the liver and was sent home without any further treatment. Remarkably, there was no sign of any tumour in the liver or anywhere else in his body (Rosenberg, 1990). It appears that this spontaneous recovery provides evidence that the human body is indeed capable of eradicating tumours.

Most immunotherapy studies of today have been directed towards melanomas with fewer focussing on renal cell, breast, prostate and colon carcinoma. However, despite the cancer being studied, the vast majority of immunotherapy studies are based on the assumption that tumours carry a specific cell surface component which could be antigenic, and which is needed to boost the immune system to make an appropriate immune response. Unfortunately, the description of these tumour antigens are few and while, apparently, specific antigens have been detected in some cancers, such as carcinoembryonic antigen (CEA) in colon cancer, in other cancers, such as brain and liver, there has been few recorded tumour specific antigens. It is this lack of tissue specificity of antigens that restricts potential immunotherapy of many cancer vaccines. The next section investigates the various tumour antigens which have been described to date.

1.2 The tumour specific immune response

A goal of cancer research has been to stimulate the immunological rejection of tumours. This goal is based on the hypothesis that malignancies express foreign antigens which can potentially serve as targets for their destruction by the immune system. Although it remains controversial to what extent spontaneous tumours can be recognised as foreign by the immune system (Hewitt et al., 1976), it is well documented that many experimentally induced tumours express antigens that can mediate tumour rejection (Hellström and Hellström, 1991; Boon, 1992). This review discusses the major tumour specific and tumour associated antigens used in tumour immunotherapy studies today.

Cellular immunity plays the key role in the rejection of tumour cells with both T helper cells and cytolytic T lymphocytes (CTLs) being involved (Greenberg, 1991; Melief, 1992). The generation of a tumour specific CTL immune response that can successfully eradicate cancer cells involves a complex series of interactions and signalling between various cells and effector molecules of the immune system (Figure 1.1). The activation of tumour-specific T lymphocytes and their subsequent differentiation into cytolytic cells is dependent on two signals from the antigen-presenting cell (APC). One signal is the antigenic peptide (from the tumour)-MHC complex that engages the T cell receptor (TCR); the other is a costimulatory signal, efficiently provided by B7, that binds to CD28 or CTLA-4 on the T cell (Leung and Linsley, 1994). Anergy rather than activation results when a T cell does not receive this costimulatory signal on recognition of antigen (Schwarz et al., 1990). The induction of anergy can however be prevented by providing IL-2 or cross-linking the gamma-chain of the IL-2 receptor (Boussiotis et al., 1994), indicating that one important function of the costimulatory molecule is to promote T cell cytokine production, especially IL-2, which leads to engagement of cytokine receptors that use the gamma-chain as a component. Dendritic cells and activated B cells are effective APCs of foreign protein antigens in part because of their expression of B7.1 (Leung and Linsley, 1994).

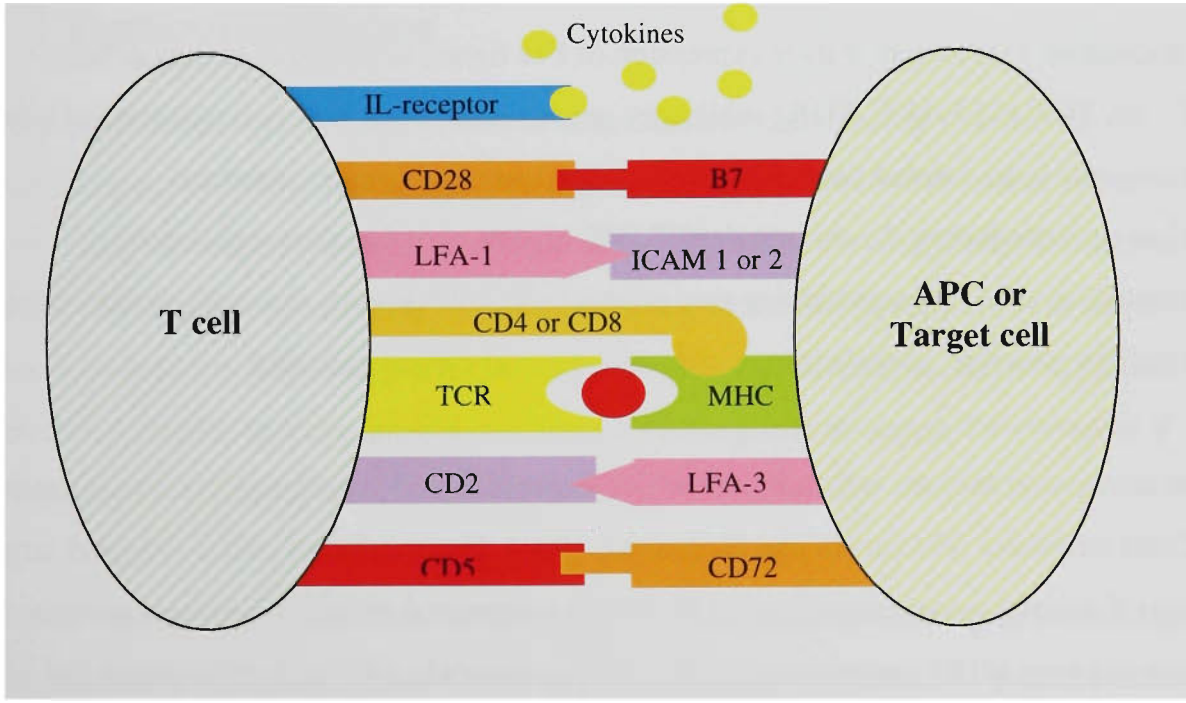


Figure 1.1: Schematic representation of some of the molecular interactions that occur during antigen recognition at the interface of a T cell and antigen presenting cell or target cell (Fraser et al., 1993).

During and after the engagement of these two signals, lymphokines such as interleukins, interferons, granulocyte-stimulating factors, tumour necrosis factors, etc are released. These

lymphokines act in many ways including to stimulate the clonal expansion of tumour specific cytotoxic T cells, B cells, and other haematopoietic cells such as NK cells and macrophages - all of which are thought to play a role in the tumour immune response. Most immunotherapy studies today focus on boosting one or more of these steps; whether it be by increasing the expression of the tumour antigen, MHC class I or II molecules, or the concentration of localised cytokines. This review will also discuss each of the immunotherapeutic strategies scientists have devised to overcome tumour tolerance in the quest to produce a cancer vaccine.

1.3 Evading the immune system

Tumours may effectively evade the immune system by several mechanisms which are not only confined to cancer cells, but may also be related to impaired function of the immune response in tumour-bearing hosts (Nawrocki and Mackiewicz, 1999). These include:

1. HLA absent or poorly expressed on tumour cells
2. Antigen processing defect
3. Outgrowth of antigen negative clones of tumour cells (lack of T cell recognition)
4. Inadequate expression of costimulatory molecules on cancer cells
5. Inadequate expression of adhesion molecules on cancer cells
6. Inadequate expression or over expression of Fas ligand expression on cancer cells
7. IL-10, TGF-β, SPARC, PGE_2, inhibitory neuropeptides (eg. vasoactive intestinal peptide, calcitonin gene-related peptide) secretion into tumour microenvironment
8. Host defence failure due to impaired T, NK or APC cell function (eg. decreased expression of signal transducing zeta chains in T and NK cells resulting in decreased cytokine response in melanoma, renal and cervical cancer patients).

There are several reasons why tumours which express rejection antigens can evade destruction by T cell immunity (Hellström and Hellström, 1991). Destruction of immunological targets requires T lymphocyte recognition via the T cell receptor of antigenic peptides presented in association with MHC molecules on APC (Björkman et al., 1987). Some tumours fail to adequately process and present antigens to T cells because of reduced expression of MHC Class I molecules (reviewed in Garrido et al, 1993). This may be rectified by transfection of tumours with MHC Class I genes, which leads to the rejection in some cases (Hui et al., 1984; Tanaka et al., 1984; Wallich et al., 1985). Another approach is transfection of cytokine cDNA,

in particular IFN-γ, into the tumours as it plays a role in the up-regulation of cell surface MHC Class I expression (Watanabe et al., 1989, Gansbacher et al., 1990a).

Lack of an effective antitumour response may also result from a deficiency in tumour-bearing hosts of T helper functions. This can be overcome with the transfection of tumour cells with the cDNAs from cytokines such as IL-2 or IL-4. This results in paracrine secretion of lymphokines which can substitute for T cell help, induce tumour-specific CTLs, and cause tumour rejection (Fearon et al., 1990; Gansbacher et al., 1990; Golumbek et al., 1991). Similarly, another reason why immunogenic tumours escape host immunity is that tumour-reactive T cells receive inadequate costimulation through the absence of the costimulatory molecules B7-1 and B7-2. This again can be over come through transfection with these molecules (Chen et al., 1992).

This thesis specifically investigates the role cytokines play in inducing effective antitumour immune responses in epithelial carcinomas expressing the tumour antigen, mucin-1 (MUC1), which will be described in more detail later.

1.4 Tumour antigens

Over the past decades, a major hurdle in understanding the immune response to tumours and in developing effective tumour-specific immunotherapy has been the lack of identifiable target antigens on tumour cells, apart from those expressed on viral induced tumours. Advances in antigen processing and presentation, and the identification of the major histocompatibility complex (MHC), associated with peptide antigen, have facilitated the search for tumour specific antigens that may be susceptible as targets for cytotoxic T lymphocytes (CTLs).

One of the earliest studies that showed tumours are capable of inducing immune responses was described by Prehn in 1965. Prehn demonstrated that a chemically induced carcinoma from the drug methylcholanthrene, could induce resistance to an implant of itself, but not an implant from another tumour in a syngeneic mouse induced by the same drug (Prehn, 1965). This study demonstrated that each tumour possesses individual antigens. Many studies searched to identify tumour specific antigens following Prehn's discoveries. One specific advance came with studies by Boon and colleagues who demonstrated that tumours passaged

within a single mouse strain, randomly mutated provoking strong transplantation antigens, hence causing rejection in syngeneic animals with healthy immune systems, to which they call *tum-* variants. Boon's laboratory developed a method for screening clones for the mutated gene using *tum-* specific cytotoxic T cell clones and eventually lead to the isolation of the *tum-* tumour antigen (Boon and Van Pel, 1978; Van Pel et al., 1979).

Numerous tumour antigens have since been identified, and a wide variety of strategies and techniques employed to use these antigens in the fight against cancer. Potential tumour antigens can be divided into two major groups,

a) *Tumour specific antigens:* which include mutant oncogene products such as ras proteins, bcr-abl, p53, HER-2/neu, BRCA1 and BRCA2, and
b) *Tumour associated antigens:* which include carcinoembryonic antigen (CEA), prostate serum antigen (PSA) , erb-B2, melanoma, human papilloma virus, Lewis antigens, and mucins.

a) Tumour Specific Antigens

A normal cell is transformed into a malignant cell through a series of characteristic and definable gene mutations which encode proteins that control cell growth and differentiation. During the course of malignant transformation, the cancer-related genes are altered by a variety of mechanisms including translocations, deletions and point mutations which commonly result in the expression of aberrant proteins. Cancer-specific proteins expressed by genetically altered cancer-related genes can function as tumour specific antigens. Tumour specific antigens are found only on malignant cells within the body, and are not present on normal cells.

i) *ras* proteins

Ras protooncogenes are activated by a point mutation in approximately 20% of human malignancies (Cheever et al., 1993). The mutations occur primarily at codons 12 or 61 and result in the expression of p21ras proteins with single substituted amino acids. Murine studies demonstrate that immunisation with p21ras synthetic peptides can elicit both MHC class I restricted $CD8^+$ cytotoxic T cell and MHC class II restricted $CD4^+$ T cell responses specific for the mutated p21ras protein (Cheever et al., 1993). In addition, the existence of *in vivo*

tumours expressing mutated ras proteins can be detected by assaying for T cell immunity to ras peptides.

Human studies show that some colon cancer patients have existing p21ras protein antibodies, implying the possible existence of T cell immunity to mutated ras protein (Cheever et al., 1993). Immunogenic CD4 and CD8 T-cell peptide epitopes reflecting codon 12 ras mutations have been identified. A HLA- A2 peptide epitope has been identified, which displayed specific binding to HLA-A2 by T2 bioassays as well as specific lysis of human cancer cell lines. In contrast, the wild-type ras peptide failed to bind to HLA-A2 (Bergmann-Leitner et al., 1998). In clinical trials, pancreatic adenocarcinoma patients were immunised with ras peptides and CD4 T cells restricted to HLA-DR6 were generated (Gjertsen et al., 1996). Furthermore, a phase I clinical trial in patients with advanced cancer were injected with a 13-mer mutated Ras peptide reflecting codon 12 mutations together with Detox adjuvant. These peptides corresponded to each of the patient's own tumour Ras mutation. 3/10 patients generated CD4 and CD8 T cell responses and were capable of lysing an HLA-A2-matched tumour cell line carrying the corresponding mutant but not the wild-type ras gene (Khleif et al., 1999). These studies are encouraging and form a basis for using ras peptide oncogenes for tumour immunotherapy.

ii) p53

In 1979, a 53kD protein was recognised by antisera prepared against BALB/c Meth A sarcoma in syngeneic mice. This protein was not detected in any normal tissues and subsequently, all transformed mouse cells including sarcomas, leukaemias, spontaneously transformed fibroblasts, and cells transformed by simian virus 40 and murine sarcoma virus, were found to express p53 (DeLeo et al., 1979). Since the identification of the p53 tumour antigen this protein has been shown to be expressed in a wide range of human malignancies including breast cancer.

The p53 gene has been described as an oncogene, a tumour suppressor gene (Levine et al., 1990) and as a tumour antigen (Davidoff et al., 1991); and on current evidence it may have all three activities. The p53 gene is commonly abnormal in sporadic breast cancer and a loss of normal p53 tumour suppressor function, has been implicated in up to 61% of breast cancers.

Mutated p53, which may behave as an oncogene, has been implicated in 15-46% of breast cancer cases (Thompson 1993).

p53 gene mutations occur in most human carcinomas and altered protein products accumulate within the cancer cell. Sera from cancer patients contain anti-p53 antibodies directed to the N- and C-terminal regions of p53 and in general do not distinguish the difference between wild type and mutated p53. These antibodies have not been found in normal individuals or cancer patients who do not express p53 mutations (De Leo 1998). In addition CTL have been obtained from patients with $p53^+$ cancers; these also proliferate to the wild type p53 protein (De Leo 1998). Mutated p53 or wild-type p53 overexpression could be targets for tumour immunotherapy and an HLA-A*0201 binding p53 peptide has been identified (LLGRNSFEV) which is detected by CTL from patients and a p53 peptide from HLA-A*0201 transgenic mice (STPPPGTRV) has also been identified (Theobald et al., 1995). In mice immunisation with plasmid full length wild type p53 DNA generates CTL responses and protection against mastocytoma cells transfected with mutant p53 (Hurpin et al., 1998). Mice have also received injections of dendritic cells pulsed with mutant p53 peptide and reduced tumour growth was evident, but only during treatment, tumours resuming growth after cessation of DC-peptide injections (Gabrilovich et al., 1996); IL-2 appeared to increase the immunogenicity of the DC-peptide (Gabrilovich et al., 1996). Although results are promising, an increase in the CTL activity is required; the problem does not appear to be with the peptide as the affinity of p53 binding peptides for class I is high, however, the CTL induced are of low avidity.

iii) *bcr/abl*

The bcr-abl tumour specific antigen is a chimera formed between a transformation of the human c-abl protooncogene from chromosome 9 and the specific breakpoint cluster (bcr) region on chromosome 22 (Cheever et al., 1993). The translocation results in the formation of a bcr-abl gene that encodes a 210kD chimeric protein expressed only by malignant cells. Murine studies demonstrate that immunisation with bcr-abl synthetic peptides can elicit class II restricted $CD4^+$ T cell responses to p210 bcr-abl proteins. Earlier studies demonstrated that bcr-abl peptides can bind in the groove of both murine and human class I MHC molecules and can elicit bcr-abl peptide-specific CTLs (Cheever et al., 1993). Recently, it was shown for the first time that *in vitro* immunisation with a 17mer peptide from the p190 minor bcr-abl fusion protein, resulted in HLA-DRB1*1501-restricted proliferative $CD4^+$ T cells using dendritic

cells as antigen presenting cells (Tanaka et al., 2000). A number of groups have tested peptides corresponding to the junctional region of the bcr/abl protein for their binding capacity to HLA class I molecules and have identified a few candidate epitopes. Peptides originating from the bcr/abl fusion protein have on the other hand so far been ignored. Using the reciprocal bcr/abl translocation product was recently tested for possible MHC class I epitopes using synthetic peptides (Berke et al., 2000). It was noted that the bcr/abl translocation product may be an even more important source of T cell peptide antigens demonstrating a number of HLA molecules (HLA-A1, -A2, -A3, -A11, -B7, -B27, -B35) which bind to the bcr/abl peptides, and thus may serve as candidates for T cell specific tumour antigens (Berke et al., 2000).

iv) HER-2/*neu*

HER-2/*neu* is an oncogene (c-erbB-2) which encodes a 185kDa transmembrane protein with homology to the EGF receptor (Linehan et al., 1995). The HER-2/*neu* oncogene is overexpressed in 20-40% of aggressive breast cancers and may be related to the formation of cancer, as it is present in 50-60% of ductal carcinomas *in situ*. Studies have shown that tumour associated CTL from ovarian and breast cancer patients can recognise a HER2/*neu* derived peptide epitope when presented in the context of HLA-A2 (Linehan et al., 1995). Some patients with cancer have natural humoral and cellular immune responses to HER-2/*neu*, and the aim of using HER-2/*neu* as a target for immunotherapy is to boost this preexisting immunity (Disis et al., 1998). Initially CTLs were identified in HLA-A*0201 patients which recognised the peptide p971-980; this peptide could stimulate CTL obtained from PBMC of patients with ovarian carcinoma (Ioannides et al., 1993). Other CTL epitopes have also been identified - p369-377 from the extracellular domain and p654-662 from the transmembrane region of HER-2/*neu* (Fisk et al., 1995; Peoples et al., 1995). Recently, HLA-A3 restricted CTL epitope (VLRENTSPK) was identified, and CTLs could lyse HLA-A3 and HER-2/*neu* positive tumour cell lines (147). Because of the cellular responses in patients, rats were immunised with peptide fragments of HER-2/*neu*, and $CD4^+$ T cells and antibodies were generated; GM-CSF was found to increase the immune response to HER-2/*neu* peptides (Disis et al., 1998). Phase I clinical trials have now begun using HER-2/*neu* peptides (Disis et al., 1998).

v) BRCA1 and BRCA2

In recent years, much interest has been generated by mutations identified in specific genes which carry a high risk for breast cancer, especially two genes called BRCA1 (which also carries an increased risk of ovarian cancer) (Miki et al., 1994) and BRCA2 (Wooster et al., 1994). The available data suggests that the cumulative risk of breast cancer in women with mutations in high-risk genes was about 80% to the age of 80 years and about 50% up to the age of 50 although these may be overestimates (Easton et al., 1993; McCredie et al 1995). The BRCA1 and BRCA2 genes encode large unrelated proteins that function as tumour suppressors in normal epithelial cells of the breast. However, the primary amino acid sequences of these proteins provide few insights into the mechanisms by which BRCA1 and BRCA2 inhibit tumour development. Nevertheless, recent studies have uncovered many similarities in the biological properties of BRCA1 and BRCA2, raising the prospect that these proteins may function in a common pathway of tumour suppression and that inactivation of either gene may represent an equivalent step in the development of breast cancer (Baer and Lee, 1998).

Several lines of evidence now suggest a role for BRCA1 and BRCA2 in the cellular response to DNA damage, where BRCA1 genes play a critical role in the regulation of apoptosis. Thus, since a wide variety of human malignancies like breast and ovarian cancers have a decreased ability to undergo apoptosis, this could be due to a lack or decreased levels of functional BRCA1 proteins (Shao et al., 1996). Also mutations in both genes show signs of a cellular proliferation defect associated with activation of the p53 pathway (Hakem et al., 1998). Accordingly, the loss of BRCA1 or BRCA2 function accelerates tumour development by allowing cells to accumulate DNA lesions that are potentially oncogenic (Baer and Lee, 1998)

Gene transfer of wild-type BRCA1 inhibits the growth of sporadic breast and ovarian cancer cells and suppresses growth of established breast and ovarian tumour models in nude mice (Holt 1997). A Phase I clinical trial of ovarian cancer patients treated with retroviral BRCA1 gene therapy reported a stable vector, minimal antibody response, and tumour reduction (Tait et al 1997). In a Phase II trial on patients with less extensive disease to evaluate vector pharmacokinetics, immune response, toxicity, and efficacy, patients showed no response, no disease stabilisation, and little or no vector stability. Because of vector instability and rapid antibody development, which differed dramatically from the Phase I trial data, the trial was terminated after treatment of six patients. Immune system status appears to have played a

major role in whether gene therapy was effective. Comparison of Phase I and II patients showed significant differences in tumour burden, immune system status, and response to BRCA1 gene therapy (Tait et al., 1999).

b) Tumour associated antigens

Tumour associated antigens are found on malignant cells, but are also present on normal tissues in an altered state. For example, the family of melanoma associated antigens, MAGE, which are expressed in melanoma cells, melanoma cell lines, and other tumours including small cell lung cancers and thyroid medullary carcinomas, are also expressed in normal tissues from the testis and retina (Van Der Bruggen et al., 1991) (Table 1.1).

i) Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a glycoprotein of molecular weight 180kD expressed in approximately 90% of human colorectal, gastric and pancreatic cancers and in some breast and non-small cell lung carcinomas (Oikawa et al., 1987; Thompson et al., 1991). Weak expression of CEA has been observed in some normal epithelial cells and strong expression has been observed in foetal gut tissues. The CEA gene has been shown to belong to the immunoglobulin superfamily and shares some homology with proteins expressed on some normal adult tissues (Thompson et al., 1991).

In the mid 1960s CEA stimulated renewed interest and hope that tumours contained components which were specific to human neoplasms. About 50% of breast cancers have elevated levels of CEA. However sensitive immunoassays showed that elevated serum levels of CEA occurred in other cancers and non-malignant conditions (hence, the term tumour-associated antigen) and has since been referred to as neither a sensitive nor a specific marker for breast cancer (Beard and Haskell, 1986).

Table 1.1: Tumour associated antigens that can be used for monitoring different types of cancers.

TUMOUR MARKER	CANCER
CEA	various carcinomas
c-erb-B2	breast
CA-125	ovarian
CA-19-9	pancreatic
CA-50	pancreatic
CA-549	breast
B72.3	colon
MUC1, DF3, CA15-3, CASA, MSA	breast
Lewis antigens	colon
Prostate Specific Antigen	prostate

Full length CEA cDNA plasmids have been injected in mice which developed antibodies and T cell proliferation, and protection against tumour challenge (Conry et al., 1998). The administration of a CEA recombinant vaccine to rhesus monkeys induced both a humoral and cell-mediated immune response directed against human CEA and no evidence of autoimmunity against normal cross-reactive antigen (NCA) - a protein which shares a high homology with CEA. This study indicates the potential use of this antigen in human cancer immunotherapy (Kantor et al., 1992a). Recently, phase I clinical trial results of a recombinant vaccinia virus encoding CEA in patients with metastatic adenocarcinoma were published. The results from this trial indicated that despite promising preclinical results, VV-CEA was unable to stimulate CEA specific lymphoproliferation, interleukin 2 release, delayed-type hypersensitivity, or antibody response and no clinical responses were observed (Conry et al., 1999).

CEA peptides have been identified which show promise in inducing specific T cell immune responses. The peptide YLSGANLNL is a CEA CTL (HLA-A*A0201) epitope recognised by CTL from rV-CEA vaccinated colon cancer patients. A single amino acid substitution N6D (YLSGADLNL) sensitised CTL 100-1000 times more efficiently than the native peptide. The enhanced recognition of the analogue was not due to increased binding to HLA-

A*0201 and therefore, the analogue, YLSGADLNL, is a CTL enhancer agonist peptide (Zaremba et al., 1997). Recently, HLA-A3 (HLFGYSWYK) and HLA-A24 (QYSWFVNGTF, TYACFVSNL) restricted CTL epitopes were identified in *in vitro* PBMC cultures, and the CTL so produced lysed CEA^+ tumour cell lines (Nukaya et al., 1999; Kawashima et al., 1999). The identification of such CTL peptide epitopes offers a greater opportunity for the design of peptide based immunotherapy of patients.

ii) Melanoma antigens

Human melanoma represents the major cause of death in patients with skin cancer in Australia. In 1988, Rosenberg adoptively transferred tumour infiltrating lymphocytes (TILs) and interleukin-2 into patients with metastatic melanoma which resulted in the regression of 35-40% of the melanomas in the patients (Rosenberg et al., 1988). Since this time, TILs recognising melanoma have been used to identify the major melanoma associated antigens MAGE, BAGE, GAGE, MART-1/Melan-A, gp100, tyrosinase, and gp75. The tumour specificity of these antigens makes them good target proteins for melanoma immunotherapy and vaccine design.

Table 1.2: Melanoma Specific Antigens and HLA CTL MHC restriction

	Antigens	HLA restricted epitopes
Overexpressed self proteins recognised by cytotoxic T cells:	MAGE-1,2,3 BAGE GAGE	HLA-A1 HLA-Cw* HLA-Cw6
Melanocyte specific antigens:	Tyrosinase MART-1/Melan-A gp100 gp75	HLA-A24 HLA-A2.1 HLA-A2.1 HLA-A3.1
Tumour specific mutated proteins	beta-catenin	HLA-A24
Others	p15	HLA-A24

MAGE

The MAGE family of melanoma genes (MAGE-1, MAGE-2 and MAGE-3) encode for a melanoma specific protein antigen, MZ2. MAGE antigens are expressed in melanoma cells, melanoma cell lines, and other tumours including small cell lung cancers and thyroid medullary carcinomas, and function as targets for tumour-reactive cytotoxic T lymphocytes (CTLs) in melanoma patients (Van Der Bruggen et al., 1994). The only reports of these genes being expressed in normal tissues is from the testis and retina. MAGE-1 was identified using T-cell clones from a melanoma patient in 1991, as the gene encoding the melanoma specific tumour antigen, MZ2-E; and is recognised by MHC restricted CTLs from melanoma patients (Van Der Bruggen et al., 1991). MAGE-3 is the gene encoding the melanoma antigen MZ2-D, and is recognised by MHC restricted melanoma CTLs.

BAGE

The BAGE gene codes for a 43 amino acid protein antigen recognised by autologous CTL from the human melanoma MZ2-MEL (Boel et al., 1995). BAGE gene expression occurs in 22% melanomas, 30% infiltrating bladder carcinomas, 10% mammary carcinomas, 8% head and neck squamous cell carcinomas and 6% non-small cell lung carcinomas and is only present on normal testis. CTLs against the BAGE protein are MHC restricted and recognise a 9mer peptide, AARAVFLAL (Boel et al., 1995).

GAGE

The melanoma family of GAGE genes (GAGE-1 and GAGE-2) encode the melanoma specific antigen MZ2-F, and were identified by CTLs against a human melanoma (MZ2-MEL) isolated from a melanoma patient (Van Den Eynde et al., 1995). Both GAGE-1 and GAGE-2 belong to the MAGE and BAGE family of melanoma antigens expressed only on tumours and not normal tissues except for testis. An antigenic peptide from GAGE-1 was identified (YRPRPRRY) which is recognised by anti-MZ2-F CTLs (Van Den Eynde et al., 1995). The GAGE melanoma genes are expressed in 24% of melanomas and 25% of sarcomas, however collectively, MAGE, BAGE and GAGE antigens are expressed on approximately 50% of all melanoma patients (Van Den Eynde et al., 1995).

MART-1/ Melan-A

MART-1/Melan-A (Melanoma Antigen Recognised by T-cells) encodes a putative transmembrane protein whose expression is limited to only melanoma, melanocyte cell lines, and human retina (Kawakami et al., 1994). MART-1 is MHC restricted and is expressed on 90-100% of all melanomas. A 9-mer peptide from MART-1 has been recognised (AAGIGILTV) by TILs from nine out of 10 HLA-A2 melanoma patients recognising MART-1 antigen (Kawakami et al., 1994a).

Tyrosinase

Tyrosinase is a transmembrane glycoprotein which has enzymatic activity associated with melanin synthesis (Halahan et al., 1993) and was identified in 1993 as the gene encoding the melanoma antigen from HLA-A2 patients (Brichard et al., 1993). CTLs from A2 melanoma patients were shown to recognise the protein product of the tyrosinase gene which was found to be active in melanoma cells, melanoma cell lines and normal melanocytes (Brichard et al., 1993). Tyrosinase is recognised by two different HLA-A2+, CD8+ TILs (Robbins et al., 1994) and is also the shared antigen recognised by one CD4+ TIL (Topalian et al., 1994). The tyrosinase antigen constitutes a useful target for A2 melanoma therapy, however the response against normal melanocytes of the skin needs to be considered before this antigen is trialed in human patients.

gp100

Melanocyte lineage-specific antigen glycoprotein (gp100) was identified as the target antigen for the melanoma specific antibody HMB-45, and is expressed on melanomas, melanocyte cell lines and retina (Kawakami et al., 1994b). gp100 was demonstrated as being a target antigen for TILs derived from a melanoma patient and CTLs against the antigen are MHC restricted (Bakker et al ., 1994). Nine peptides presented by HLA-A2.1 were recognised by TILs from melanoma patients in a study carried out by Cox et al (1994). One of these peptides, YLEPGPVTA, was the immunodominant peptide for the gp100 antigen, demonstrated as having an exceptionally high affinity for melanoma specific CTLs from five different A2 melanoma patients tested (Cox et al., 1994). The recognition of this peptide by multiple CTL lines implements its use in peptide-based melanoma vaccines.

gp75

The gp75 protein is a tyrosinase-related protein which has enzyme activity to melanin synthesis and is abundantly expressed in melanoma, melanocyte cells and normal retina (Thomson et al., 1988). gp75 was originally identified as a melanoma associated antigen when IgG antibodies from melanoma patients' serum recognised the protein (Mattes et al., 1983). The gp75 antigen is recognised by melanoma specific TILs and therefore constitutes a potential antigen for melanoma immunotherapy as both antibodies and CTLs react with the protein (Wang et al., 1995).

Beta catenin

A single amino acid mutation from the beta catenin protein involved in cell adhesion identified beta catenin as a melanoma associated antigen by Robbins et al in 1996 (Robbins et al., 1996). MHC restricted tumour-reactive T cells from a single melanoma patient reactive with a 9mer peptide (SYLDSGIHF) contained within the mutated beta catenin (Robbins et al., 1996), and abnormally high levels of beta-catenin expression in melanoma cell lines (Rubinfeld et al., 1997) demonstrate a role for beta catenin in the development of a malignant melanoma phenotype.

Clinical trials using melanoma peptide antigens

Several clinical trials have been completed using melanoma peptide antigens. In one clinical trial 45 HLA-A*0201 patients were injected with a multi epitope based peptide vaccine and many patients produced CTL responses to different combinations of 22 peptides when treated with a vaccine containing about 50 peptides derived from MAGE-3, Melan-A/MART-1, gp100, tyrosinase, melanocortin receptor, and dopachrome tautomerase (TRP-2) (Reynolds et al., 1998). In another study, the HLA-A2*0201 binding gp100 peptides were mutated to improve HLA binding affinity and used in incomplete Freund's adjuvant to immunise patients with advanced melanoma; patients induced a INF-γ secreting cells to a number of peptide combinations (Pass et al., 1998). In a separate study 17 patients were immunised intradermally (10^5-10^7 cells/injection) using tumour lysates pulsed autologous APCs (Nestle et al., 1998). One patient had a partial response of the tumour and 9 had a DTH response; no CTLs were evident, but after expanding T cells *in vitro* with autologous loaded APC, $CD8^+$ CTL were found in 5/9 (Nestle et al., 1998). Gp100 peptides binding to HLA-A*0201 have also been used to treat patients with metastatic melanoma. In this study, immunised patients developed an immune response and 13/31 patients receiving the peptide + IL-2 had tumour

responses, and 4 patients had minor responses (Rosenberg et al., 1998). Although all these immunisation protocols are promising further studies are necessary.

iii) Mucins

Mucins are of particular interest in cancer immunotherapy studies because they are produced by breast, ovary and pancreatic carcinomas in a different form compared to corresponding normal epithelial tissues (Gendler et al., 1987, 1989).

Mucins are present on the apical surface of normal tissues, where expression is concentrated predominantly around the secretory ducts. During breast, ovarian and pancreatic cancers, mucin expression is significantly upregulated (Gendler et al. 1989, 1991). This upregulation results in an ubiquitous overexpression of mucin covering the entire cancer cell surface, subsequently altering its normal distribution pattern (Langsdale et al. 1993). In addition, alterations in the carbohydrate side chains of the mucin glycoproteins result in the addition of shorter chains covering the protein core. The alteration in mucin structure exposes new carbohydrate and peptide epitopes in cancer that are not present in normal tissues or secretions. Furthermore, cell surface mucins on tumour cells exist as flexible rods which protrude relatively great distances from the cell surface and could be the first point of contact by immune effector cells and antibodies (Gendler et al. 1995). Taken together, the irregularities that exist between mucins on normal tissues and secretions and mucins in cancer, make this glycoprotein a useful antigen for tumour immunotherapy.

The next part of the literature review will bring together observations from studies that occurred independently but have since evolved to see patients with breast and colon epithelial carcinomas, immunised with synthetic peptides in human clinical trials to develop a vaccine for cancer. These studies occurred in several parts-

a) The knowledge of the structure of mucins, particularly the protein core, has rapidly advanced with the cloning of the cDNA for mammary and other mucins. Small peptide epitopes have been identified by antibodies (Xing et al. 1989).
b) These MUC1 peptide epitopes are highly immunogenic in mice and are the target of monoclonal antibodies made against whole tumour cells, extracts and indeed against synthetic peptides (Xing et al. 1992).

c) Human studies which led to the production of non MHC restricted CTL clones from the draining lymph nodes of patients with breast or pancreatic cancer and the isolation of non MHC restricted CTL from infiltrating lymphocytes in ovarian tumours, concluded that these T cells recognise mucin and the same mucin epitopes as detected earlier by murine monoclonal antibodies (Barnd et al. 1989; Jerome et al. 1990, 1993; Ionnides et al. 1993). In addition, specific mucin peptide antibodies have been found in patients with ovarian and breast carcinoma (Rughetti et al. 1993; Kotera et al. 1994).

d) Immunogenicity studies of mucin peptides in mice can therefore be applied to humans due to the recognition of the same peptide antigens in both human and murine studies providing a model for vaccine research.

These studies indicate that the immune system is capable of eliciting both CTL and antibody responses against epithelial carcinomas, and specifically, recognise mucin antigens; this review will examine these areas in detail.

Section 2: Mucins

Epithelial mucins are glycoproteins that exist in both membrane bound and not bound forms. Those not membrane bound are the major constituents of mucus, and provide it with its characteristic qualities of elasticity and viscosity (Sheehan et al., 1991). Major advances in mucin studies have occurred since the identification of twelve different mucin cDNAs (MUC1-MUC12) and in particular, the tumour associated antigenic properties of mucin-1 (MUC1). This section reviews the structure and functions of each of the mucin glycoproteins identified to date, and focuses on MUC1, and its use as a tumour associated antigen in cancer immunotherapy studies.

2.0.1 Expression and function

Mucins predominantly play a protective and lubricative purpose and are produced by normal epithelial surfaces of the respiratory, digestive and genitourinary tracts, sebaceous salivary and lactating mammary glands and in the example of mucin-1, as a major constituent of human milk fat globule (HMFG) (Arklie et al.1981; Swallow et al 1986). Bronchial mucins form a coating throughout the respiratory tract, protecting the airways from harmful microbes and particles by capturing them in the mucous membrane and removing them through mucous secretions in healthy individuals (Porchet et al., 1991a). In the respiratory airways of unhealthy individuals in diseases such as cystic fibrosis, chronic bronchitis and asthma, increased mucin production contributes to increased morbidity and mortality (Jany and Basbaum, 1991). In the gastrointestinal tract, mucins prevent dehydration of various surfaces, serve as a protective barrier against invading microbes and parasites attacking the gut wall, and protect the intestines from digestive proteases and the acidic conditions associated with gastric secretions (Young et al., 1991; Hilkens et al., 1992). Mucins are also known to have anti-adhesive properties due to their unique extracellular structure and a high density of mucins on epithelial cell surfaces may prevent cell adhesion by masking important adhesive molecules (Ligtenberg et al., 1992).

Despite previous findings that mucin reactive CTL and antibodies have been found in patients with breast and ovarian adenocarcinomas (described in more detail later), studies of mucins,

particularly mucin-1 (MUC1), suggest that tumour associated mucin may have another function - immune system evasion. Early studies showed an overexpression of mucins on tumour cell surfaces were able to inhibit natural killer cell, cytotoxic T cell and neutrophil cytotoxicity - originally believed to occur by steric hindrance whereby the mucin prevented the immune cells from reaching their target (Hayes et al. 1990; Gendler et al. 1991). A recent study by Paul et al (1999), investigated the immunosuppressive role of MUC1 based on the ability of supernatant from the MUC1 and MUC3-positive cell line T47D, to inhibit the proliferation of cells from various haematopoietic cell lines. The study found that the activity of T47D supernatents could be abrogated by the immunodepletion of MUC1 from the supernatant, however the immunopurified MUC1 from T47D was unable to inhibit cell proliferation. Similarly, supernatents from other MUC1 positive cell lines (3T3 and BHK-21), and purified MUC1 from bile or urine, were also unable to inhibit T cell proliferation. Interestingly, a crude mixture of bile mucins was able to suppress T cell growth suggesting that the immunosuppressive capabilities observed in the MUC1 positive T47D supernatant is attributed to the presence of mucin in combination with additional molecules such as other mucins or amino sugars. From these studies it appears that mucin glycoproteins in conjunction with other, yet to be identified, molecules have immunosuppressive effects which can be abolished by the use of purified mucin or mucin peptides, the later of which have been shown to be immunogenic *in vivo*.

2.0.2 Mucin structure

The identification of the cDNA clones encoding an identical protein core of a mammary and pancreatic mucin was a major breakthrough in the understanding of mucin structure, mucin epitopes recognised by mucin antibodies, and the production of monoclonal antibodies reactive with epithelial carcinomas. The isolation of these cDNA clones led to the sequencing of the protein core and later, the whole protein sequence of the first identified mucin, mucin-1 (MUC1) (Gendler et al. 1987; Siddiqui et al. 1988). Since the identification of MUC1 over 20 years ago, seven different mucin cDNAs (MUC1-7) coding for the protein core of mammary, intestinal, tracheo-bronchial, gastric and salivary mucins (Gendler et al. 1987; Siddiqui et al., 1988, Gum et al., 1989, 1990, Porchet et al., 1991b; Van Cong et al., 1990; Aubert et al., 1991; Toribara et al., 1993; Bobek et al., 1993) have been isolated and are described in more detail below.

Although the genes encoding these mucins are found on different chromosomes (except MUC2, MUC5 and MUC6 which are linked to chromosome 11) the structure of the mucin proteins are similar. Mucins are high molecular weight glycosylated proteins (250kD) composed of between 50%-90% carbohydrates, linked to a protein core rich in the amino acids serine, threonine and proline (Langsdale et al., 1993; Gendler et al., 1991; McKenzie and Xing, 1990). The carbohydrate side chains of mucins are predominantly O-linked, and surround a protein core that extends high above the glycocaylx (10nm) of the epithelial cell surfaces (200-500nm). The mucin protein core consists of a short cytoplasmic tail domain anchored within the plasma membrane by a hydrophobic transmembrane region spanning the membranes lipid bilayer. Following a C terminal domain, is a sequence of amino acids that is repeated a variable number of times giving this region its name of variable number of tandem repeats (VNTR), flanked by an N terminal domain. For example, MUC1 has a 20 amino acid sequence repeated 40-80 times depending on the individual. The VNTR region of the mucins appear to be the most immunogenic as both monoclonal antibodies and polyclonal antibodies to peptides within these regions led to the cloning of several mucin genes from various expression libraries (Gendler et al. 1987; Siddiqui et al., 1988, Gum et al., 1989, 1990, Van Cong et al., 1990, Toribara et al., 1993; Bobek et al., 1993).

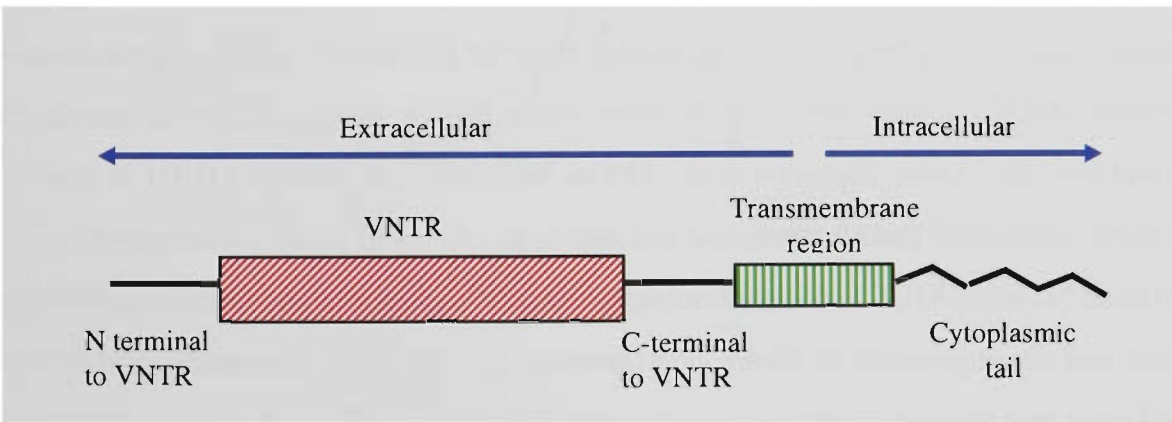


Figure 1.2: Schematic structure of the mucin protein core (Apostolopoulos et al., 1994a)

2.1 Characterising human MUC1

Mucin-1, or MUC1, was first identified by at least five different laboratories using antibodies that isolated numerous repeats found in the MUC1 VNTR from the λgtII expression library. The nomenclature for this high molecular weight glycoprotein has since been confused and it has been called many different names. MUC1 is also known as PAS-O (Shimizu and Yamauchi, 1981), Epithelial Tumour Antigen (ETA) (Hareuveni et al., 1990) DF3 (Gendler et al 1991), PUM (Young et al., 1991) episialin (Hilkens et al., 1992) and polymorphic epithelial mucin (PEM) (Price and Tendler 1993). For this thesis, the glycoprotein shall be referred to as MUC1.

In the search for tumour antigens, several monoclonal antibodies (DF3, SM3) prepared from tumour tissues were found to react specifically with cancer associated proteins encoded by the cDNA expression library, λgtII, prepared from the human mammary tumour cell line, MCF-7. The protein reactive with DF3 monoclonal antibody was cloned and the gene identified as pDF9.3, commonly known as MUC1, and mapped to chromosome 1q21.

MUC1 is present in normal breast, ovarian, pancreatic, gastrointestinal, genitourinary and respiratory tissues and breast milk and human milk fat globules (HMFG) (Apostolopoulos et al., 1993a). MUC1 is also expressed in cancer of the breast, ovaries, pancreas, cervix, kidney, lung and bladder (Apostolopoulos et al., 1993a; Willsher et al., 1993). MUC1 is often detected in colorectal carcinoma tissue and cell lines. A recent study investigated the correlation between MUC1 in intramucosal colorectal carcinoma and clinical-pathologic features and the expression of Ki-67, p53 proteins, and apoptosis (Tanimoto et al., 1999). The study found that the immunohistological expression of MUC1 in high-grade carcinoma and polypoid carcinomas was significantly more frequent than in low-grade carcinoma and superficial carcinomas, with no expression evident in adenomas. Interestingly in colorectal carcinoma development, MUC1 expression correlates with the overexpression of the p53 protein and prominent apoptosis (Tanimoto et al., 1999).

2.1.1 MUC1 structure

MUC1 is a transmembrane molecule with a relatively large extracellular domain varying between 1,000 and 2,200 amino acids and a cytoplasmic tail of 69 amino acids. The

extracellular domain extends at least 200-500nm above the cell membrane, far above all other membrane-associated proteins which are within the glycocaylx (10nm) (Hilkens et al.,1992).

The protein core of MUC1 consists of a:

a) cytoplasmic tail region which contains signal sequences (phosphorylation motifs) and is thought to be involved in microtubuli binding required for intracellular transport (Hilkens et al., 1992).
b) 28 amino acid (aa) transmembrane region
c) 228 aa C-terminal to the VNTR region
d) 20 aa variable number of tandem repeat region (VNTR) repeated 40-80 times producing the proteins high degree of polymorphism, and
e) 104 aa N-terminal to the VNTR region.

The 20 aa sequence repeated 40-80 times in the VNTR of MUC1 is;

S A P D T R P A P G S T A P P A H G V T

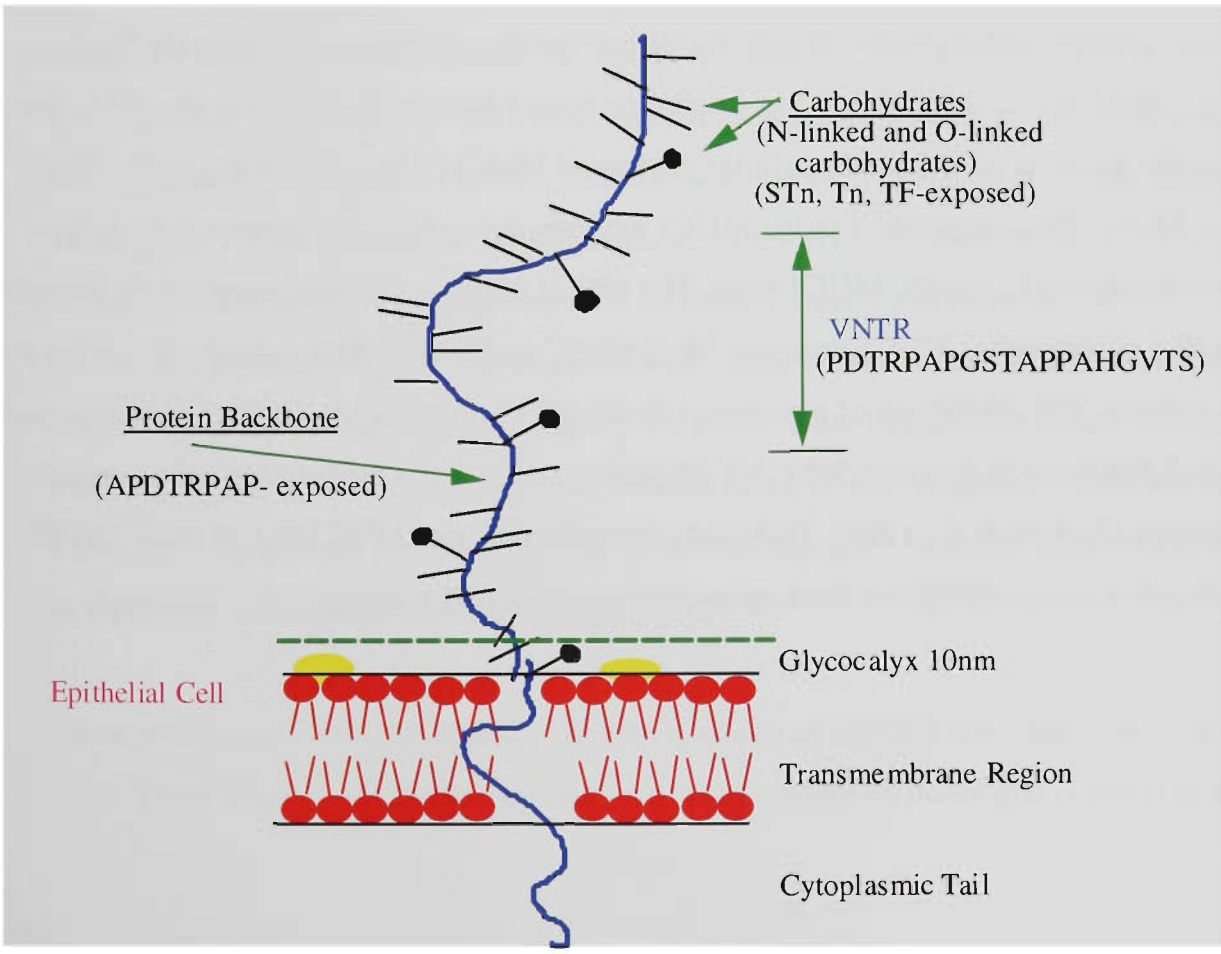


Figure 1.3. Comparative length and structure of mature cancer mucin (MUC1) molecule which extends 200-500nm above the plasma membrane (Apostolopoulos et al., 1996a).

In 1990, a secreted isoform of MUC1 was discovered which lacked a transmembrane and cytoplasmic tail domain (Wreshner et al. 1990). Nucleotide sequencing of MUC1 cDNA and genomic clones revealed two different mRNA species presumed to be generated by alternative splicing of a single precursor transcript. The alternate sequence of MUC1 was thought to encode a potential secreted isoform containing a short stretch of 10 hydrophobic aa located 48 aa upstream of the carboxyl terminus. As the length and hydrophilicity profile of the protein indicated this region was unlikely to act as a transmembrane region, it was concluded that a secreted form of MUC1 exists. The existence of a secreted isoform of MUC1 was later confirmed by anti-peptide monoclonal antibodies produced by Apostolopoulos et al. (1993c) against a unique amino acid sequence (S E M V S I G L S F P M L P) present only in secreted MUC1. The secreted isoform of MUC1 is present in secretions from normal and cancer serum of the breast, breast cancer cell lines and human milk.

MUC1 proteins, some of which contain a mucin-like domain and others lacking this region, can be generated from the human breast cancer associated MUC1 gene by alternative splicing. The MUC1/Y isoform is devoid of the mucin domain and is a cell membrane protein which undergoes transphosphorylation on both serine and tyrosine residues. Recently binding proteins, allied in characteristics to the membrane bound form of MUC1, was identified that specifically interacts with the extracellular domain of MUC1/Y (Baruch et al., 1999). The cognate MUC1 binding proteins target MUC1 expressed on mammary tumour cells *in vivo*, and are also derived from the MUC1 gene. The MUC1 binding proteins represent the secreted mucin-like polymorphic MUC1 proteins, MUC1/SEC and MUC1/REP, which contain tandem repeat regions. The interaction of membrane bound MUC1/Y with MUC1/SEC has important biological functions as it causes MUC1/Y phosphorylation and a pronounced effect on cell morphology (Baruch et al., 1999). This study demonstrates that MUC1/Y and MUC1/SEC form an active receptor/cognate binding protein complex that elicits cellular responses.

2.1.2 MUC1 carbohydrates

As previously described, mucins are high molecular weight glycoproteins composed of a protein core to which numerous carbohydrate side chains are attached. These carbohydrate side chains are O-glycosidic and N-glycosidic linkages. As many as 200 clustered carbohydrate chains per 250 kD of glycoprotein may be attached to the protein core through two possible types of linkages (Feizi, 1984).

The most numerous type of linkage is O-glycosidic linkages, and was shown by work carried out by Abe and Kufe (1987) when the MUC1 antigens PAS-O, DF3 and Ca1 were shown to be susceptible to alkali borohydride treatment, hence suggesting the presence of O-glycosidic linkages between carbohydrates and proteins within mucins. O-glycosylation occurs when the carbohydrate is attached to the oxygen atom of either serine or threonine protein residues, through a 'sugar linker' called N-acetylgalactosamine (Price and Tendler, 1993; Feizi, 1984).

The amount of carbohydrate side chains present in mucin molecules varies according to the tissue in which the mucin is found. For instance breast mucins are comprised of approximately 50% carbohydrates where as pancreatic mucins comprise approximately 80% carbohydrates (Layton et al., 1991). The composition of the carbohydrate side chains also varies greatly in the number of monosaccharides they contain hence producing a very heterogenous composition which are both difficult to purify and characterise (Feizi, 1984). To combat these problems, Feizi (1984) used well-characterised human monoclonal antibodies as reagents to study the carbohydrate chains of mucins. Anti-I antibodies which recognise short and long branch point sequences and anti-i antibodies which recognise long linear sequences were used to reveal the presence of linear and branched poly-N-acetyl-lactosamine sequences in high molecular weight mucins (Feizi, 1984).

Three structurally and antigenically distinct domains of carbohydrate side chains are known to exist. These are the core, backbone and peripheral regions of the sugars (Layton et al., 1991).

CORE REGION

Eight, possibly nine, types of core region structure are known (Feizi, 1984; Hanisch et al., 1993).

1 GalNAc-*O* -Ser/Thr

2 Galb1 - 3GalNAc

3 GlcNAcβ1 ⟍ 6 GalNAc
Galβ1 ⟋ 3

4 GlcNAcβ1 - 3GalNAc

5 GlcNAcβ1 ⟍ 6 GalNAc
GlcNAcβ1 ⟋ 3

6 GalNAcα1 - 3GalNAc

7 GlcNAcβ1 - 6GalNAc

8 GalNAcα1 - 6GalNAc

(9) Galβ1 - 6GalNAc

Biosynthesis of the core region does not proceed unless structure 1 is present (Feizi, 1984), after which elongation can give rise to any of 2-8 (possibly 9).

BACKBONE REGION

The backbone region of the carbohydrate side chains varies in length and branching pattern. They usually consist of alternating galactose and N-acetylglucosamine residues in two types of disaccharide units (Feizi, 1984). These are;

1 Galβ1 - 3GlcNAc

2 Galβ1 - 4GlcNAc

PERIPHERAL REGIONS

The best characterised structures in the peripheral regions of the carbohydrate side chains are the major blood group antigens H, A, B, Le^a and Le^b (Feizi, 1984).

2.1.3 Characterising murine Muc1

The cDNA for murine mucin-1 (Muc1) has been isolated and shows that both human and murine mucin-1 share a basic structural appearance with significant protein homology (Vos et al., 1991). There is an extracellular region of the molecule with 59-62% protein homology, a multiple amino acid repeat region with only 34% protein conservation, and highly conserved transmembrane (87%) and cytoplasmic (87%) domains (Vos et al., 1991). The cloning of murine Muc1cDNA has also led to the production of mouse mucin-1 knockout (-/-) mice by homologous recombination. These mice show no particular phenotypic differences to normal litter mates except for a delayed progression of transplanted mammary tumours and T cell abnormalities (Spicer et al., 1995).

To characterise murine muc1 and develop a model for cancer immunotherapy studies with a self antigen, anti-mouse Muc1monoclonal antibodies reactive with different regions of the mouse glycoprotein were produced (Xing et al., 1998). The antibodies include:

- M30 (IgM) and M70 (IgG1) produced to the extracellular repeat region using a synthetic peptide (Mp26).
- MFP25 (IgM) and MFP32 (IgG1) produced to a muc1 fusion protein of tandem repeats derived from a mouse cDNA clone, and
- CT91 (rat origin) and CT1.53 (IgG1) (from Muc1 o/o mice) produced to the cytoplasmic tail region using a human MUC1 peptide (CT-18) that has 87% identity with the murine cytoplasmic tail.

The antibodies to both the extracellular tandem repeat region and the cytoplasmic tail were found to react with mucin-containing murine tissues such as breast, stomach, colon, ovary, kidney and pancreas. In general, mouse Muc1 is widely distributed in epithelial cells, like human MUC1. The epitopes of the anti-Muc1 antibodies were mapped to LSGTSSP (MFP25); TSS (M30); and TAVLSGTS (M70), and of interest was the finding that some of the antibodies reacted with murine lymphocytes. The anti-human cytoplasmic tail peptide antibodies, CT90 (raised from rat) and CT1.53 (raised from 129SV/J o/o mouse) not only react with human tissues but also with mouse tissues, suggesting they can be used in both human and mouse mucin studies (Xing et al., 1998).

2.2 Characterising other mucins

Since the identification of human MUC1 over 10 years ago, 12 different mucin cDNAs (MUC1-12) coding for the protein cores of mammary, intestinal, tracheo-bronchial, gastric and salivary mucins have been isolated (Gum et al., 1989, 1990; Porchet et al., 1991b; Van Cong et al., 1990; Aubert et al., 1991; Toribara et al., 1993; Bobek et al., 1993; Shankar et al., 1997).

2.2.1 MUC2

MUC2 was cloned in 1989 by Gum et al, and is expressed in the normal small intestine, colon and respiratory tract and in carcinomas of the lung and intestines. Three cDNA clones for MUC2 (SMUC-40, SMUC-41 and SMUC-42) were cloned from a human intestinal λgt11 cDNA library using polyclonal antibodies reactive with deglycosylated LS174T mucin, and all were found to contain a 23 amino acid tandem repeat region, rich in threonine and proline, repeated 14 times (Gum et al., 1989). MUC2 is a polymorphic, high molecular weight (160-520kD) mucin which maps to chromosome 11p15.5.

The 23 aa sequence repeated 14 times in the VNTR of MUC2 is:
P T T T P I S T T T M V T P T P T P T G T Q T

Monoclonal antibodies reactive with the protein core of MUC2 were first produced by Xing et al. (1992), using a synthetic MUC2 peptide (M1-29) containing one repeat unit of the VNTR and four amino acids from the next repeat sequence. These antibodies, CCP31, CCP37, CCP58, 4F1 and 3A2, react by immunoperoxidase staining with MUC2 present in the intestines (Xing et al., 1992) whilst monoclonal antibodies produced by Devine et al (1993) stain MUC2 present in lung, intestine, and colon cancers and weakly in breast and ovarian cancers, similar in RNA binding studies (Jany et al., 1991). MUC2 is strongly overexpressed in tumours of the colon, breast, ovary and pancreas. In the corresponding normal tissue it is either not expressed (breast, ovary, pancreas) or it is expressed at considerably lower levels than in the tumours (colon). Four HLA-A2 binding epitopes have been identified and upon loading of these peptide onto dendritic cells, stimulation of HLA-A2 T cells was noted and lysis of target cells loaded with peptides was demonstrated (Bohm et al., 1998). This data

shows that the tumour-associated mucin MUC2 has the potential as a target antigen for CTL in patients with mucinous carcinomas.

2.2.2 MUC3

MUC3 (also known as SIB) was cloned in 1990 by Gum et al. and is expressed in the normal gastrointestinal tract and in colon cancer similar to MUC2. Two partial cDNA clones for MUC3 (SIB124 and SIB139) were identified from a human intestinal λgt11 cDNA library using polyclonal antibodies reactive with deglycosylated LS174T mucin; and consist of a 17 amino acid tandem repeat region, rich in serine and threonine, repeated 20 times (Gum et al., 1990). MUC3 is a polymorphic, high molecular weight (190-320kD) mucin which maps to chromosome 7q22. The cDNA sequence of carboxyl-terminal region of MUC3 indicated that rodent Muc3 contains two epidermal growth factor (EGF)-like domains, and putative transmembrane and cytoplasmic domains, whereas the sequence of human MUC3 predicted termination after the first EGF-like domain. The complete genomic sequence of human MUC3, has been recently published and it indicates that the gene is alternatively spliced, yielding a major membrane-bound form as well as multiple soluble forms (Crawley et al., 1999).

The 17 aa sequence repeated 20 times in the VNTR of the human MUC3 is:
H S T P S F T S S I T T T E T T S

Two monoclonal antibodies reactive with the MUC3 VNTR were produced by Apostolopoulos et al (1993) using a synthetic peptide containing two MUC3 tandem repeats (SIB35). The MUC3 antibodies react by immunoperoxidase staining with MUC3 present in the breast, colon, stomach, salivary glands and lungs.

2.2.3 MUC4

MUC4 (also known as JER 64) was cloned by Porchet et al. in 1991 and is expressed in the tracheo-bronchial tract. MUC4 was cloned from a human tracheo-bronchial mucosa λgt11 cDNA library using polyclonal antibodies reactive with bronchial mucin; and consist of a 16 amino acid tandem repeat region, repeated 39 times (Porchet et al. 1991). From this repeat region, eight amino acids remain constant whilst the remaining eight may vary. MUC4 maps

to chromosome 3 and whilst O-glycosylation is evident in the protein core, no potential N-glycosylation sites exist on the MUC4 protein core.

The 16 aa sequence repeated 39 times in the VNTR of MUC4 is:

T S S A S T G H A T P L P V T A

Note: The underlined aa represent the amino acids conserved within the repeat region whilst those not underlined may vary. Monoclonal antibodies reactive with MUC4 were produced by Xing et al., from a synthetic peptide reactive with the MUC4 protein core. Immunoperoxidase staining of these antibodies show MUC4 expression in cancers of the lung, breast and colon and also in normal colon, rectum, lung, salivary gland and prostate tissue. It was noted that MUC4 was present in lung carcinomas and not in normal lung tissues (Xing et al., 1997). *In situ* hybridisation experiments have demonstrated that MUC4 is expressed in mucous secretions of the lung, epidermoid metaplasia with complete squamous cell differentiation, and most of epidermoid lung carcinomas even well differentiated and keratinised (Copin et al., 2000). In squamous metaplasia and dysplasia, MUC4 gene expression was diffuse and less intense than in normal epithelium.

2.2.4 MUC5

MUC5 is a tracheo-bronchial mucin present in the lung and stomach and was identified by screening a human tracheo-bronchial λgt11 cDNA library using polyclonal antibodies reactive with bronchial mucin (Van Cong et al., 1990; Aubert et al., 1991). MUC5 contains a T-T-S amino acid sequence within the protein core indicating O-linked glycosylation sites and an eight amino acid VNTR repeat sequence consisting of four different repeat sequences.

The 8 aa sequences repeated in the VNTR of MUC5 are:

T T S T T S A P

T T D T T S A S

T A S T T S G P

G S T P S P V P

Note: The first sequence TTSTTSAP is most frequently repeated.

MUC5 has been identified as a 164 amino acid glycoprotein which maps to chromosome 11p15, and contains greater than 95% of the molecule within the VNTR. The VNTR of mucin

5 is comprised of 42% threonine (T), 25% serine (S), and 15% proline (P) and the predicted secondary structure of MUC5 consists essentially of β-turns.

Using antibodies against MUC5 has demonstrated that MUC5 is expressed in chronic bronchitic sputum as well as healthy respiratory secretions (Hovenberg et al., 1996). Using *in situ* hybridisation techniques, MUC5 has been found to be overexpressed in dysplasia as well as in mucous cell and basal cell hyperplasia of the lung. It was undetectable in squamous differentiated cells of the lung (Copin et al., 2000).

2.2.5 MUC6

MUC6 is a human gastric mucin isolated in 1993 by Toribara et al., and RNA studies indicate MUC6 expression in the stomach, trachea and gall bladder. MUC6 contains a 169 amino acid repeat sequence within the VNTR, which is twice as long as any other reported mucin repeating region, and there is 83-95% homology within each repeat and at least six repeats. Each repeat region of MUC6 is rich in the amino acids threonine, serine and proline, similar to other mucin repeat regions, and comprises 63.4% of the amino acids. MUC6 maps to chromosome 11p15.4-11p15.5 and is the third mucin gene, along with MUC2 and MUC5, to be mapped to this locus indicating a clustering of mucin secretory genes are on this chromosome (Toribara et al., 1993).

The MUC6 secretory mucin was originally isolated from a gastric cDNA library. *In situ* hybridisation, RNA analysis, and immunohistochemistry demonstrated that MUC6 is highly expressed in gastric mucosa, duodenal Brunner's glands, gall bladder, seminal vesicle, pancreatic centroacinar cells and ducts, and periductal glands of the common bile duct; focal expression is seen in basal endometrial and endocervical glands. Seven out of ten pancreatic and cholangiocarcinomas and four out of ten endocervical carcinomas expressed MUC6 (Bartman et al., 1998). The MUC6 mucin cDNA isolated from a human stomach cDNA library was shown to be expressed in a number of tissues in the gastrointestinal tract, including the gallbladder, pancreas, and parts of the ileum and colon as well as transiently expressed in the kidneys (Reid et al., 1999). To investigate the expression of MUC6 mucin in gastric carcinomas, a monoclonal antibody (CLH5) was generated using an MUC6 synthetic peptide. CLH5 reacted with the MUC6 peptide and with native and deglycosylated mucin extracts from gastric tissues. Staining was observed in normal gastric mucosa (pyloric glands

of the antrum), and 31 out of 104 gastric carcinomas were positive for MUC6 (Reis et al., 2000).

2.2.6 MUC7

MUC7 (also known as MG2) was cloned in 1993 by Bobek et al., and is expressed in human saliva. MUC7 was cloned from a human λgt11 submandibular gland cDNA library using polyclonal antibodies reactive with salivary mucin. MUC7 is a 39Kd protein which initially contains a hydrophobic sequence (the first 20 aa) followed by the remainder of the molecule which is hydrophilic. The VNTR of MUC7 consists of six tandem repeat units, the smallest of which is a 23 aa sequence. MUC7 maps to chromosome 4 and potentially has five N-linked glycosylation sites to the protein core.

The 23 aa sequence repeat region in the VNTR of MUC7 is:
T T A A P P T P S A T T P A P P S S S A P P G.

Antibodies to MUC7 have been generated using synthetic peptides in an attempt to characterise MUC7 further in tissue samples. Antibody, CpMG2, raised against the C-terminal peptide, recognised native MUC7 in saliva; glycosylation of MUC7 had no effect on the binding of CpMG2. MUC7 was found to be present in saliva from all seromucous glands, submandibular gland, sublingual gland, saliva, the palate and the lip (Bolscher et al., 1998). Using reverse transcription-PCR, MUC7 was found to also be expressed in bladder cancer cell lines, invasive transitional cell carcinomas, and not in superficial, non-invasive bladder tumours nor normal bladder urothelium (Retz et al., 1998). MUC7 transgenic mice have been generated. By RT-PCR, MUC7 gene was expressed in the salivary glands in three out of five mouse lines and by northern blot analysis, MUC7 was present in the sublingual glands in three out of five mouse lines (Bobek et al., 1998). Western blot analysis of protein extracts from different tissues of transgenic mouse line 3 showed that MUC7 gene product was produced in the submandibular-sublingual gland complex and not in the other tissues examined (Bobek et al., 1998).

2.2.7 MUC8

In 1994, the partial cDNA of MUC8 (also known as pAM1) encoding a major airway mucin glycoprotein with novel tandem repetitive sequence was cloned (Shankar et al., 1994). Additional new sequences from the stop codon, 3'-untranslated region of 458 bp, a polyadenylation signal, and poly A+ tail, (extreme carboxy terminus) of MUC8 were published 3 years later (Shankar et al., 1997). Using either a purified fusion protein or a synthetic peptide corresponding to the MUC8 repeat sequence (TSCPRPLQEGTPGS), polyclonal antibodies were raised in rabbits. The antibodies reacted with tracheobronchial mucin and submucosal glands in human trachea (Shankar et al., 1997).

Two tandem repeats have been identified for MUC 8:
TSCPRPLQEGTRV and
TSCPRPLQEGTPGSRAAHALSRRGHRVHELPTSSPGGDTGF

2.2.8 MUC9, MUC10, MUC11, MUC12

MUC9, (also known as oviductin), appears to be restricted to oviduct of the reproductive tract and is located on chromosome 1p13. Its relative frequency in pathologic cases was not statistically different compared with those found in normal fertile women (Lapensee et al., 1997). The biologic function of MUC9 is likely to include protection of the early embryo and of the fallopian tube itself.

MUC10 was isolated from a cDNA clone encoding mouse submandibular gland salivary mucin. The mucin cDNA encodes a protein of 273 amino acids with a molecular weight of 29,606 and is approximately 60% Thr, Ser, and Pro. This mucin shows considerable homology with the rat submandibular salivary mucin, but little overall homology with other mucins (Denny et al, 1996).

Using differential display, two novel mucin cDNAs MUC11 (dd34) and MUC12 (dd29), that are down-regulated in colorectal cancers have recently been identified. Northern blot analysis demonstrated that MUC11 and MUC12 is present on normal colon but absent or down-regulated in colorectal cancer. MUC11 cDNA is 2.8kB, and contains 35 serine/threonine-rich, 28 amino acid tandem repeats. The MUC12 cDNA contains two extracellular cysteine-rich,

EGF-like domains, a coiled-coil region, and a mucin-like domain consisting of 28 amino acid degenerate tandem repeats (Williams et al., 1999).

2.3 Establishing a link between human and murine studies

MUC1 is a suitable target for cancer immunotherapy studies because of findings made in several independent experiments in the late 1980s. Scientists found a common link between MUC1 reactive antibodies and cytotoxic T cells found in patients with breast, pancreatic and ovarian cancer, and MUC1 antibodies raised in mice. Both recognised the same MUC1 epitope present in both humans and mice. It took five to 10 years of experiments and several clinical trials for scientists to discover that in fact the murine model for human MUC1 was not a true reflection of the human immune response and new models were established for study (these are described in more detail later). However, significant preclinical data was obtained in these early MUC1 immunotherapy models in mice. The initial experiments that led to these findings are described below.

In the past, monoclonal antibodies to breast cancer antigens have been made by immunising mice with tumour cell lines or extracts such as human milk fat globule (HMFG) or carbohydrate stripped mucin (Arklie et al., 1981; McKenzie and Xing, 1990). These antibodies identified MUC1 as being over-expressed in cancer tissues (Stacker et al., 1985) and in the serum of patients with epithelial carcinomas of the breast, ovary, lung and pancreas (Arklie et al., 1981; McKenzie and Xing 1990). The MUC1 monoclonal antibodies SM3 and BC3 both bind to an epitope present on the first 15 amino acids of the VNTR region of the protein core (Gendler et al., 1988), and significantly inhibit the lysis of tumour cells by MUC1 CTLs (Barnd et al., 1989). This suggests that SM3 binds a determinant near, or similar to, the epitope recognised by the CTL (Apostolopoulos et al., 1993a). In addition, a synthetic peptide of five amino acids (APDTR) in an antigen presentation model could cause proliferation of the T cell line (Apostolopoulos et al., 1993a).

Studies showing the presence of MUC1-reactive CTL in the draining lymph nodes of breast and ovarian cancer patients (Barnd et al., 1989) and $CD8^+$ class-I restricted CTL in mice (Apostolopoulos et al., 1994, 1995a) have attracted much attention to MUC1 as a potential target for cancer immunotherapy. Draining the lymph nodes of patients with breast, pancreatic and ovarian cancer, produces tumour reactive cytotoxic T cells (CTL) that recognise mucins

(Ionnides et al., 1993; Barnd et al., 1988; Jerome et al., 1990). These CTLs kill breast, pancreatic and ovarian tumour cell lines in an unrestricted-MHC manner (Ionnides et al., 1993; Barnd et al., 1988). They are however not effective against colon tumour cell lines (Jerome et al., 1990). The T cell antigen receptor used by the CTL is the α/β heterodimer typically found on MHC-restricted T cells (Barnd et al., 1989). However, Apostolopoulos et al (1995b) found that MHC-restricted CTL are produced by MUC1 immunisation, and that these CTLs are specifically H2 restricted (discussed in more detail later).

The CTLs produced in breast cancer patients recognise an epitope present on the protein core of human mucin molecules. Further work by Jerome and Finn showed the successful establishment and functional analysis of mucin-specific CTL lines and clones derived from breast and pancreatic cancer patients, using either autologous or allogeneic B cells as antigen. These cells transfected with mucin gives them tumour Ag-presenting ability as well as susceptibility to lysis by mucin specific CTLs (Jerome et al., 1993).

A recent study by Snijdewint et al., (1999) showed that ovarian cancer patients have both cellular and humoral immune responses to MUC1 mucin and tandem-repeat peptides. The study investigated the proliferative T cell responses of peripheral blood mononuclear cells (PBMC) to MUC1 20-mer and 60-mer tandem repeat peptides in ovarian cancer patients. The results showed MUC1 specific PBMC proliferation in eight out of 15 (53%) of ovarian cancer patients and interestingly in five out of 13 (38%) pregnant women in their third trimester. MUC1 and free circulating MUC1 IgG and IgM antibody levels were detected in the plasma of ovarian cancer patients at significantly higher levels than healthy women. Although no significant correlation was found between MUC1 antibody levels and MUC1 T cell proliferation in ovarian cancer patients, a possible association may exist as both MUC1 humoral and cellular immune responses could be detected in ovarian cancer patients at a significantly higher level than in healthy women.

2.4 MUC1 antigenic epitopes

The differences in mucin molecules in normal tissue compared to malignant tissue resulted in the generation of new antigenic regions in the mucin molecule. These new antigenic sites exist primarily in,

a) the carbohydrate side chains with the generation of TF, Tn and STn carbohydrate regions, and

b) the protein core of MUC1, particularly in the VNTR.

Mucin-1 (MUC1) is present on the apical surface of normal epithelial tissues, where its expression is concentrated predominantly around the secretory ducts. During breast, ovarian and pancreatic carcinomas the expression of MUC1 is significantly up-regulated (Gendler et al. 1989, 1991). This upregulation results in an ubiquitous overexpression (10-40 fold) of mucin over the entire cancer cell surface, subsequently altering its normal distribution pattern (Langsdale et al., 1993). In addition, alterations in the carbohydrate side chains result in the addition of fewer, shorter chains covering the protein core. The alteration in mucin structure results in the production of new carbohydrate (Tn, STn, TF) and peptide epitopes, eg the sequence APDTRPA in the VNTR, exposed in cancer that are not present in normal tissues or secretions. Furthermore, cell surface mucins on tumour cells exist as flexible rods that protrude relatively great distances from the cell surface and could be the first point of contact by some immune effector cells and antibodies (Gendler et al., 1995). Taken together, the irregularities that exist between MUC1 on normal tissues and secretions and MUC1 in cancer, make this mucin a useful tumour antigen for immunotherapy in all MUC1 expressing tumours.

2.4.1 Mucin carbohydrate epitopes

Differences have been observed in the glycolipid profiles expressed by tumour cells, compared to those expressed by normal cells. In mammalian cells, the linkage sugar in glycoproteins carrying O-linked sugars is N-acteyl galactosamine (GalNac) and those carrying the N-linked sugars is N-acteyl glucosamine (GlcNAc). Alterations in the glycosylation of mucins results in the exposure of novel oligosaccharides that are highly expressed in a variety of cancers, whereas in normal tissues they are weakly exposed, presumably masked by further oligosaccharide chain elongation (Itzkowitz et al., 1989). The incomplete glycosylation is due to an alteration in glycosyl transferase activity (Zuang et al., 1991) leading to shortened carbohydrate side chains of normally O-linked core carbohydrate determinants such as Tn and TF, which are not normally exposed. The reduced activity of this enzyme therefore favours the synthesis of carbohydrate cancer associated antigens.

Four different carbohydrate antigens have been identified in cancer cells:

1. the Thompson-Friedenreich (TF) antigen
2. Tn
3. Sialated Tn (Stn), and
4. A10

a) Thompson-Friedenreich and Tn antigens

The Thompson-Friedenreich (TF) antigen is an O-linked disaccharide [βGal(1-3)αGalNAc-O-serine/threonine] found on all lymphocytes, erythrocytes and epithelial cells, which can be revealed by neuraminidase treatment. TF determinants and their monosaccharide precursors Tn, (αGalNAc-O-serine) are expressed on approximately 85% of human adenocarcinomas (Longenecker et al., 1989) particularly in breast and colon cancer. This finding is supported by the observation that TF and Tn monoclonal antibodies react with both primary and metastatic human carcinomas (Longenecker et al., 1989).

b) Sialated Tn

Sialated Tn (STn) is a core region structure of the mucin glycoprotein and represents one of the first steps in mucin glycosylation. In 1984, Springer et al., identified STn as a cancer associated antigen. Since this time, the expression of STn has been associated with a poor prognosis of colon cancer, and when present on circulating mucins, a predictor of a poor prognosis of also ovarian and breast cancers (Itzkowitz et al., 1989; Kobaayashi et al., 1992). Ovarian cancer patients with STn^- serum have a five-year survival rate of ~85% compared to patients with STn^+ serum who have a survival rate of only ~10% (Itzkowitz et al., 1989; Kobaayashi et al., 1992). This results suggest that the carbohydrate antigen STn may play an important role in cancer metastases thus making it an important target for cancer immunotherapy studies.

c) A10 epitope

Recently, a novel carbohydrate epitope was identified by a monoclonal antibody (A10) raised against murine Ehrlich tumour cell surface carbohydrates tested for reactivity on human normal and malignant tissues. A10 reacted strongly with a high proportion of adenocarcinomas arising from colon and other tissues, but not breast carcinomas or other malignant tumours (Medina et al., 1999). The A10 epitope involves O-linked carbohydrates in non-terminal reducing positions, and is carried on MUC1 derived from colon adenocarcinomas and probably on other mucin species although not MUC2. Deglycosylation studies indicated the involvement of core mucin glycans, and inhibition studies suggested the core 6 glycan [GlcNAcbeta(1-6)GalNAc] as being involved in the A10 epitope (Medina et al., 1999).

2.4.2 Mucin protein epitopes: antibodies reactive with mucin protein

Human mucin-1 has been extensively described as a tumour-associated antigen since the first description of monoclonal antibodies reactive with the mucin extracellular protein core (Arklie et al., 1981; McKenzie and Xing, 1990). The protein core of MUC1, in particular the VNTR, contains the most immunodominant epitopes present within the mucin glycoprotein. This immunogenicity is demonstrated by the large number of monoclonal antibodies reactive with, and studies mapping, MUC1 cytotoxic T cell epitopes to the VNTR protein core. This

increase in immunogenicity results from a reduction in the number of glycosylated carbohydrate side chains, which initially masked the protein core of normal MUC1, however during cancer, exposes previously unaccessible regions of the VNTR to cells of the immune system.

In the past, anti-MUC1 antibodies were produced against MUC1 by immunising mice with tumour cell lines or extracts such as human milk fat globule (HMFG) or carbohydrate stripped mucin. When cDNA clones were isolated and the MUC1 amino acid sequence obtained, existing antibodies were studies for their interaction with the peptide sequence and the antigenic peptide epitopes could be precisely mapped. Peptides from the 20 amino acid VNTR of MUC1 were synthesised and monoclonal antibodies known to react with breast cancer (BC1, BC2, BC3) reacted with the synthetic peptide A-P-D-T-R found in the protein core (Xing et al., 1989, 1990). These results were confirmed using overlapping 6 mer peptides synthesised on pins (Domenech et al., 1994). Most of the anti-mucin antibodies reacted with the peptide sequence APDTR of the VNTR demonstrating that APDTR is a highly immunogenic part of the mucin molecule.

SM3, the first monoclonal antibody described to react with $MUC1^+$ cancers and not normal tissue (Burchell 1987) reacts with the epitope, P-D-T-R-P, also located in the VNTR. It was of great interest when the anti-MUC1 antibodies SM3, BC2 and BC3 inhibited the lysis of tumour cells by CTLs while other antibodies that did not recognise the APDTR epitope did not, suggesting that these antibodies bind to a region in or near the epitope recognised by the CTLs (Jerome et al., 1990). Thus the target of these cells was the VNTR of MUC1 and it was considered that the repetitive nature of the VNTR led to crosslinking of the T cell receptor accounting for the lack of MHC restriction (Barnd et al., 1989; Jerome et al., 1990). However, it was recently demonstrated using peptide stabilisation assays that MUC1 can associate with MHC Class I molecules and are indeed MHC restricted (Apostolopoulos et al., 1997, 1998b). This is discussed in more detail later. Thus APDTR epitope, which was clearly immunogenic in mice, could be potentially used in humans, as CTL precursors already exist in lymph nodes of patients with breast cancer.

As APDTR was the most common epitope detected, second generation monoclonal antibodies were produced by immunising with synthetic peptides and fusion proteins in the hope that these antibodies would have enhanced specificity for mucin-expressing carcinomas, however,

the second generation antibodies were not substantially different from the first monoclonal antibodies (Xing et al., 1992, 1992a; Apostolopoulos et al., 1993). Also reacting with epitopes contained within the 20 amino acid repeat units of the VNTR are the antibodies HMFG1 and HMFG2 all produced against the immunogen HMFG, C595 produced against the MUC1 protein core, and VA1 and VA2 produced against a MUC1 fusion protein (Apostolopoulos et al., 1993). The protein epitopes recognised by some of these MUC1 monoclonal antibodies are summarised in Table 1.3 below.

In a recent study, high titre IgG and IgM MUC1 antibodies were produced by immunising breast cancer patients with a MUC1 30-mer peptide conjugated to keyhole limpet haemocyanin (KLH) plus the immunological adjuvant QS-21. These antibodies were shown to react with the MUC1 immunising peptide, but not with MUC1 positive tumour cells (Adluri et al., 1999). In an inhibition ELISA assay with these MUC1 reactive antibodies, only peptides containing APDTRPA at the C-terminal end were able to inhibit the 30-mer immunising peptide. Remarkably, sera from all six of the immunised breast cancer patients recognised, and were completely inhibited by, this same epitope. The specificity of the sera for this APDTRPA epitope at the C-terminal end of any tumour mucins, and the N-terminal alanine APDTRPA which is normally buried in the beta turn of MUC1 assumed in its secondary structure, explains the moderate to weak reactivity of these high titre sera against MUC1-positive tumour cells (Adluri et al., 1999).

Monoclonal antibodies reactive with other regions of the MUC1 protein core have also been generated. Recently a monoclonal antibody reactive with the MUC1 cytoplasmic tail was produced by immunising MUC1 -/- mice with an 18 amino acid peptide contained within the cytoplasmic domain of MUC1 (Xing et al., 1998). Antibodies recognising the N-terminal region of the MUC1 protein core also exist.

Table 1.3: Protein epitopes detected by some anti-mucin antibodies*

Antibody	Immunogen	MUC1 VNTR A P D T R P A P G S T A P P A H G V T S	Reference
3E1.2	Tumour tissue	Glycolylsialyl-Tn	Stacker 1985
OMI	Ovarian cancer cells	D T R P (A)	DKrester 1985
HMFG1	HMFG	P D T R (P A)	Swallow 1986
HMFG2	HMFG	D T R	Swallow 1986
SM3	Deglycosylated HMFG	P D T R P	Burchell 1987
BC2	Peptide	A P D T R	Xing 1992
BCP7	Peptide	A V T S	Xing 1992
BCP8	Peptide	D T R	Xing 1992
BCP9	Peptide	G S T A P	Xing 1992
BCP10	Peptide	R P A P	Xing 1992
VA1	Fusion protein	R P A P G S	Apostolopoulos 1993
VA2	Fusion protein	D T R P A	Apostolopoulos 1993

peptide sequence: PDTRPAPGSTAPPAHGVTSAPDTR
fusion protein sequence: (PDTRPAPGSTAPPAHGVTSAPDTR) x5: * (Apostolopoulos et al., 1994)

2.5 Cancer immunotherapy with MUC1

Cancer immunotherapy requires the presence of a tumour specific antigen which stimulates a cellular T1 immune response upon activation. The glycoprotein mucin MUC1 has been studied as an immunotherapeutic reagent in cancer studies because of differences noted in the structure and distribution of MUC1 in normal and cancer tissues, resulting in MUC1 developing antigenic properties making the glycoprotein a good target for cancer immunotherapy studies. Advances in cancer immunotherapy with the MUC1 molecule are rapidly underway with several areas of the MUC1 glycoprotein being used for its antigenic capabilities. Below is an overview of the work to date involving immunotherapy with MUC1, at both the experimental level, and clinical trials resulting from these studies.

2.5.1 MUC1 anti-tumour immunity studies in mice

A number of studies have been described which examine the immunogenicity of MUC1 in mice (Girling et al., 1989; Burchell et al., 1987; Xing et al., 1992). In early MUC1 cancer immunotherapy models, the gene for human MUC1 was transfected into 3T3 tumour cells and P815 mastocytoma cells in producing models for studying MUC1 antitumour immunity. In both of these models, the nontransfected tumours grew unremittingly in the strain of origin (3T3 BALB/c mice, P815 DBA/2 mice). However the transfected lines initially grew and were then rejected in varying times from 20-30 days indicating that human MUC1 is immunogenic in mice (as expected being a foreign antigen). Interestingly, the mice that rejected the $MUC1^+$ tumours were resistant to a subsequent challenge with $MUC1^+$ cells demonstrating the induction of immunological memory. Mice immunised with $MUC1^+$ 3T3 tumour cells generated a T1 immune response characterised by $CD8^+$ CTL, a high CTL precursor frequency (1/14,600), $CD4^+$ ($CD8^-$) delayed type hypersensitivity (DTH), tumour protection (up to doses of 5×10^7) and little MUC1 antibody (Apostolopoulos et al., 1994). These results were the first description of anti-human MUC1 CTL production in mice.

In two different MUC1 tumour models, the human MUC1 gene has been transfected into RMA cells (C57BL/6 T cell thymoma) and mouse mammary epithelial tumour cell 410.4 (Graham et al., 1995; Lalani et al., 1991). $MUC1^+$ RMA cells were used to immunise both syngeneic mice and mice transgenic for human MUC1 (see below), and MUC1 specific CTL were generated (Graham et al., 1995). Immunisation with $MUC1^+$ 410.4 resulted in a

reduction in tumour incidence following a challenge with 10^3 MUC1$^+$ 410.4 cells but not after 10^5 cells (Lalani et al., 1991). Such an approach could be used in humans using autologous cells expressing MUC1 for vaccination, however this strategy has been used in the past, and would clearly not be preferable to use. Scientists have therefore investigated the use of synthetic MUC1 reagents rather than MUC1$^+$ autologous tumours in future cancer immunotherapy experiments.

2.5.2 Murine immune response to MUC1 peptides

The protein core of MUC1 contains multiple tandem repeats of the 20 amino acid peptide (VTSAPDTRPAPGSTAPPAHG). The APDTRPA epitope is particularly immunogenic since it is recognised by a variety of murine monoclonal antibodies produced after immunisation with various MUC1 immunogens including synthetic peptides. To further investigate the immune responses produced to MUC1 peptides, Apostolopoulos et al made a MUC1 synthetic peptide (Cp13-32) containing the 20 amino acids of the repeat region (which spontaneously dimerise due to the formation of disulphide bonds), and linked this to either KLH or diptheria toxoid to immunise mice. As previously seen, antibodies were produced and cellular immunity demonstrated by CD4$^+$ mediated DTH reactions (Apostolopoulos et al., 1994). Anti-tumour immunity was examined using a MUC1$^+$ tumour in mice and some anti-tumour effects were observed with protection evident at a low dose tumour challenge (10^6 cells) (Apostolopoulos et al., 1994). This protection was no longer evident when the dose of tumour cells increased by five times. The immune response following immunisation with either the MUC1 peptide/KLH or MUC1 peptide/diptheria toxoid was characterised by weak antibody production and no CTLs. On the basis of the antitumour responses (albeit weak) after peptide immunisation, a clinical trial was performed.

Apart from the immunogenic VNTR of MUC1, significant CTL and CTLp can be induced to other regions of MUC1. Using the whole native MUC1 molecule, the human milk fat globule membrane antigen (HMFG) linked to mannan, CTLp can be generated in BALB/c, C57BL/6, transgenic HLA-A*0201/K(b) and double transgenic HLA-A*0201/K(b) x human MUC1 (A2 K(b)MUC1) mice. By immunising with HMFG and testing selectively on (a) extracellular (non-VNTR); (b) VNTR and (c) intracellular peptides, all three regions generate effective CTL. Further, the CTL responses to non-VNTR peptides were as strong as those generated to the VNTR. Thus, for CTL generation, the whole HMFG molecule is a powerful immunogen

when linked to mannan, especially as multiple peptide epitopes for presentation by many Class I molecules are contained within the one molecule. Furthermore, Class I restricted MUC1 CTL were generated in double transgenic A2 K(b)MUC1 mice by immunising with mannan-native mucin (HMFG), suggesting that tolerance to MUC1 can be overcome with mannan-HMFG (Pietersz et al., 2000).

2.5.3 Immunogenicity of human MUC1 peptides in primates

The MUC1 tandem repeat sequence was tested for its immunogenicity in chimpanzees following transfection into autologous Epstein-Barr virus (EBV)-immortalised B cells, to take advantage of their costimulatory molecules for T-cell activation. The vaccine was tested in chimpanzees because of the identity of the human and chimpanzee MUC1 tandem repeat sequence. EBV-immortalised B cells derived from two chimpanzees were transfected with MUC1 cDNA, treated with glycosylation inhibitor phenyl-N-acetyl-alpha-D-galactosaminide to expose tumour-associated epitopes, irradiated, and injected subcutaneously four times at three-week intervals. One vaccine preparation also contained cells transduced with the interleukin 2 (IL-2) cDNA and producing low levels of IL-2. After the first injection CTLps specific for under-glycosylated mucin were found in the peripheral blood. The highest frequency observed was after the last boost, in the lymph node draining the vaccination site. DTH reactions were also induced, whereas no appearance of mucin-specific antibodies was seen. Long-term observation of the animals yielded no signs of adverse effects of this immunisation. Autologous antigen-presenting cells, like EBV-immortalised B cells, expressing tumour-associated antigens are potentially useful immunogens for induction of cellular anti-tumour responses *in vivo* (Pecher et al., 1996).

MUC1 immunotherapy studies described thus far have shown the potent antitumour effects induced by $CD8^+$ cytotoxic T cell responses. However a different approach seen to elicit potent immunity to $MUC1^+$ tumours, may be in generating MUC1-specific T-helper cell responses, which to date, have not been reported in cancer patients. Studies have shown that a synthetic vaccine representing five copies of the MUC1 tandem repeat peptide can be used to prime MUC1-specific human CD4+ T cells *in vitro*. These studies were extended to test the immunogenicity and safety of the tandem repeat peptide in the chimpanzee. To promote the induction of T1-type responses, the novel adjuvant LeIF, a Leishmania-derived protein that is known to stimulate human peripheral blood mononuclear cells (PBMCs) and antigen-

presenting cells, was used to produce a T1-type cytokine profile. Results from this study showed that MUC1 tandem repeat peptides administered with LeIF elicited positive, albeit transient, proliferative T-cell responses to MUC1 in the PBMCs from four out of four chimpanzees. Immunisation induced MUC1-specific IFN-gamma but not interleukin 4 expression in $CD4^+$ T cells from PBMCs and draining lymph nodes. MUC1-specific CTLs were also generated that did not induce detectable autoimmune dysfunction during the one year of observation. From this study it was concluded that the MUC1 tandem repeat peptide can be used to elicit both T-helper and cytotoxic cell responses to MUC1 in the primate and holds promise as a safe and effective cancer vaccine (Barratt-Boyes et al., 1999).

2.5.4 Immunogenicity of human MUC1 peptides in humans

A phase I clinical trial in 13 cancer patients was performed using increasing doses of MUC1 peptides (150-1,000 micrograms) to determine the toxicity, autoimmunity and immunogenicity of MUC1 peptides in humans. For these trials, diptheria toxoid was used as a carrier and the only toxicity observed was the severe DTH reactions to the carrier. There was no evidence of autoimmunity, which could occur due to the presence of MUC1 in normal salivary gland, lung, kidney and pancreas. No antitumour responses were noted, however there was weak immunogenicity evident by a small amounts of antibody production and T cell proliferation in some patients (Xing et al., 1995). This clinical trial confirmed the MUC1 peptides are weakly immunogenic and future studies sought to improve the immunogenicity of these peptides with the various use of adjuvants, in particular mannan (which shall be discussed in more detail below).

In a separate phase I clinical study, a 105 amino acid synthetic mucin MUC1 peptide that has five repeated immunodominant epitopes was used to evaluate the toxicity and detect mucin-specific immune responses in patients with adenocarcinoma (Goydos et al., 1996). The enhancement of these responses was also examined by vaccinating patients with the synthetic mucin peptide admixed with BCG. All patients were able to tolerate vaccination, with most experiencing local ulceration at the vaccination site. Only three patients experienced DTH responses to the long peptide. Examination of 55 biopsies showed intense T-cell infiltration in 37 patients and lesser infiltration in seven. Seven of 22 patients tested had a 2- to 4-fold increase in mucin-specific CTLp. From this study it was again concluded that mucin

vaccination is safe and might serve to enhance specific responses to tumour antigens (Goydos et al., 1996).

In a recent MUC1 clinical trial, six breast cancer patients were immunised with a MUC1 30-mer peptide conjugated to keyhole limpet haemocyanin (KLH) plus the immunological adjuvant QS-21. Patients produced high levels of IgG and IgM antibodies reactive with the MUC1 immunising peptide and specifically, the APDTRPA epitope (Adluri et al., 1999). Remarkably, sera from all six of the immunised breast cancer patients recognised, and were completely inhibited by, this same epitope (Adluri et al., 1999).

2.5.5 MUC1 vaccinia virus recombinants

The potential use of recombinant viruses to elicit immune responses to MUC1 have been tested in a number of experimental models. MUC1 cDNA was first cloned into the vaccinia virus (VV) genome in the early 1990's and was used to successfully immunise rats challenged with MUC1-bearing syngeneic tumour cells (Hareuveni et al., 1991). In these initial experiments, 60-80% of rats immunised with VV-MUC1 rejected the $MUC1^+$ tumours (Hareuveni et al., 1990). Furthermore, a lower yet significant proportion of DBA/2 mice immunised with VV-MUC1 have been shown to be protected from a challenge of $MUC1^+$ P815 tumours (approximately 30% rejection) (Acres et al., 1993). In these studies, no evidence of MUC1 CTL activity was found after immunisation with VV-MUC1 however anti-MUC1 IgG antibodies were produced despite these being considered subsequent to the cause of tumour rejection (Acres et al., 1993). In recent studies, VV-MUC1 was used to immunise MUC1 transgenic mice and showed that VV-MUC1 is able to generate an immune response to a cancer-related antigen without damaging normal tissues expressing MUC1 (Acres et al., 2000). These studies demonstrate that recombinant VV can present MUC1 antigen to the immune system providing protection from some $MUC1^+$ tumours.

In addition VVs have also been constructed to co-express MUC1 antigen and other adjuvants eg cytokines and costimulatory molecules, in an attempt to increase *in vivo* tumour protection. VV-MUC1 constructs co-expressing recombinant cytokines have been successfully used to deliver cytokines to tumours in MUC1 immunotherapy models (Acres et al., 1994; Balloul et al. 1994). VVs co-expressing the MUC1 antigen and various cytokines: IL-2, IL-4, IL-5, IL-6, IL-7, have been used on the on the basis of their previously described

anti-tumour activities. The immunotherapy of cancer VV and recombinant cytokines forms an integral part of this thesis and will be described in more detail later.

In 1997, Akagi et al hypothesised that the recombinant VV-MUC1 constructs met with limited success in protecting animals from MUC1-expressing tumours because the vaccinia genome was being subject to high-frequency homologous recombination. This was due to the polymorphic nature of the mucin molecule and therefore VV-MUC1 was unstable in the expression of the tandem repeats. In light of this, two concurrent recombinants were used to improve MUC1 immune responses: VV containing a modified 'mini' MUC1 gene with only 10 tandem repeat sequences (rV-MUC1), and a mixture of rV-MUC1 and a recombinant VV containing the gene for the murine T-cell costimulatory molecule B7-1 (rV-B7) (Akagi et al., 1997). Experimental results in C57BL/6 mice demonstrated that immunisation with rV-MUC1 was indeed stable and protected mice (>90%) from establishing pulmonary metastases and induced MUC1-specific cytolytic responses which were enhanced by admixture with rV-B7. However rV-MUC1 was not successful at treating mice with existing metastases, although when tumour-bearing mice were primed with an admixture of rV-MUC1 and rV-B7, followed by two boosts with rV-MUC1, there was a significant reduction in pulmonary metastases which correlated with 100% survival (Akagi et al., 1997). This study demonstrates that the coexpression of MUC1 antigen and B7 was essential at treating established pulmonary metastases and has implications in the clinical setting for cancer treatment.

2.5.6 Immunotherapy with dendritic cells expressing MUC1

Dendritic cells differentiated *in vitro,* from blood and other sources using cytokines, hold particular promise as immunotherapeutic agents in cancer. MUC1 has been stabely expressed on human dendritic cells (DCs) through retroviral transduction of $CD34^+$ progenitor cells and their subsequent cytokine-induced differentiation (Henderson et al., 1996). The transduced DCs were shown to be potent stimulators of allogeneic $CD4^+$ T cells (Henderson et al., 1996). Following on from these studies, a retroviral vector (MFG-MUC1) expressing a 22-tandem repeat of MUC1 cDNA was used to stably transduce an immortalised murine DC line and showed that immunisation of mice with the transduced cells elicits MUC1 specific immune responses (Henderson et al., 1998).

However, adenoviral vectors transduced with the gene encoding MUC1 and used to immunise mice, produce MUC1 specific CTL and tumour protection against $MUC1^+$ tumour (Gong et al., 1997). Similarly, dendritic cells fused with MC38 carcinoma cells stimulate naive T cells and induce MC38 tumour-specific CTLs *in vivo.* Immunisation with the fusion cells induces rejection of established metastases (Gong et al., 1997a). Immunisation of adult MUC1 transgenic mice with these MUC1-DC fusions result in the rejection of established metastases and no apparent autoimmunity against normal tissues (Gong et al., 1998).

In a primate model using dendritic cells differentiated by culturing with cytokines, the immune response to MUC1 was examined and showed antibody responses to the mucin peptide could be induced while no T cell proliferative responses to mucin peptides were detected in the draining lymph node of any animal. (Barratt-Boyes et al., 1998). In a separate study, it was reported that glycosylated MUC1 tumour antigen circulating as soluble protein in patients' serum is not processed by dendritic cells and does not elicit MHC-Class II-restricted T helper responses in vitro (Hiltbold et al., 1999). Furthermore, three different forms of MUC1, ranging from glycosylated and underglycosylated protein to unglycosylated synthetic peptide, were able to elicit MUC1-specific, class-I-restricted CTL responses, and the more efficiently processed 100 mer peptide primed a broader repertoire of CTL than the glycosylated protein (Hiltbold et al., 1999).

Perhaps the most promising of the immunotherapy studies with DC and MUC1 come from a clinical trial in patients with metastatic renal cell carcinoma. In this trial, patients were

vaccinated with hybrids of autologous tumour and allogeneic dendritic cells that presented antigens expressed by the tumour in concert with the costimulating capabilities of dendritic cells. After vaccination, four out of seventeen patients completely rejected all metastatic tumour lesions, one presented a 'mixed response', and two had a tumour mass reduction of greater 50%. The response was characterised by the induction of HLA-A2-restricted cytotoxic T cells reactive with the MUC1 tumour-associated antigen and recruitment of CD8+ lymphocytes into tumour challenge sites (Kugler et al., 2000). This study indicates that hybrid cell vaccination is a safe and effective therapy for renal cell carcinoma and may provide a broadly applicable strategy for other malignancies with unknown antigens.

2.5.7 Studies in MUC1 transgenic mice

MUC1 transgenic mice have been generated by several groups in order to investigate MUC1 immune responses and mechanisms for breaking tolerance to a self antigen without generating autoimmunity in MUC1 expressing tissues. The earliest report of the generation of MUC1 transgenic mice was from Peat et al (1992) who showed the expression of MUC1 on tissue was very similar to the profile of expression seen in human tissues. Similarly, the antibody SM-3 which is directed to a core protein epitope, exhibits a distribution pattern in the tissues of the transgenic mice similar to that seen in humans (Peat et al., 1992).

One of the concerns of using MUC1, a self antigen, in human studies is the possible threat of developing an autoimmune response to normal MUC1 expressing tissues. While results from primate studies and clinical trials shown this not to be the case, studies in MUC1 transgenic mice also dispel any concerns of autoimmunity. Adoptive transfer of immune cells from wild-type mice primed *in vivo* with B16.MUC1 tumour cells into MUC1 transgenic recipients result in significant increases in the survival of MUC1 transgenic recipients compared with unmanipulated control mice challenged with B16.MUC1 tumour cells. No gross or histologic evidence of autoimmunity was observed in recipient MUC1.Tg mice, indicating that tumour immune responses mediated by MUC1-specific CD4+ lymphocytes spare non-transformed MUC1 expressing tissues (Tempero et al., 1998, 1999).

Mice transgenic for MUC1 were generated on a C57Bl/6 background to investigate the effect of endogenous MUC1 expression on the ability of mice to generate anti-MUC1 tumour immune responses (Rowse et al. 1998). Immunohistochemical staining revealed MUC1

expression levels and staining patterns in transgenic mice to be similar to that observed in humans. The MUC1 cytoplasmic tail polyclonal antibody CT1, and the monoclonal antibody HMFG-2 showed MUC1 expression in the lung, mammary gland, pancreas, kidney (distal convoluted tubules and collecting ducts), gall bladder, salivary gland, stomach and uterus. No MUC1 was evident in tissue from the colon, heart, liver, muscle and spleen (Rowse et al., 1998). Further evidence demonstrating MUC1 tolerance in MUC1 transgenic mice was provided by the finding that sera from transgenic mice challenged by the MUC1 tumour contained no MUC1 reactive IgM or IgG antibodies whilst wild-type mice rejecting the MUC1 tumour challenge expressed both IgM and IgG MUC1 antibodies in their sera (Rowse et al., 1998).

Immunisation of MUC1 transgenic mice with fusions of MUC1-positive tumour and dendritic cells (FC/MUC1) reverses MUC1 unresponsiveness and results in rejection of established MUC1-positive pulmonary metastases. Lymph node cells from immunised mice proliferate in response to MUC1 antigen by a mechanism dependent on the function of CD4, MHC class II, B7-1, B7-2, CD28, CD40 and CD40 ligand. $CD8^+$ T cells recognise peptides presented in the context of MHC class I molecules $H\text{-}2K^b$ and $H\text{-}2D^b$ and protect mice against MUC1-positive metastases (Gong et al., 2000)

A recent study in MUC1 transgenic mice examined breaking tolerance by immunising with either a fusion protein comprising MUC1 and glutathione S-transferase (MUC1-GST), MUC1-GST fusion protein coupled to mannan (MFP) or with a recombinant vaccinia virus expressing both MUC1 and interleukin-2. Transgenic mice immunised with MUC1 were observed to be partially tolerant, in that the MUC1-specific antibody response was lower than that observed in syngeneic mice. However, a significant MUC1-specific CTLp response to all three vaccines was observed, indicating the ability to overcome T cell, but to a lesser extent B cell, tolerance to MUC1 in these mice (Acres et al., 2000). In another study investigated the role of retroviral vectors encoding interleukin (IL)-2, IL-4, IL-12, or IFN-gamma to evaluate the effect of cytokine-secretion on the immunogenic properties of the cells in the MUC1 transgenic mice. *In vitro* analyses confirmed the presence of T cell-mediated cytotoxicity toward the breast cancer cells in MUC1 transgenic mice immunised with the IL-12-secreting cells (Carr-Brendel et al., 2000), while the immune response to MUC1 immunisation in MUC1 transgenic mice can also be induced by coexpression of B7.1 (Smith et al., 1999).

2.6 Cancer immunotherapy with mannan-MUC1

The MUC1 synthetic peptides used in early immunotherapy studies were generally poor immunogens despite being able to induce weak antibody responses and T cell proliferation. A range of carrier proteins and immunological adjuvants have been shown to enhance the immunogenicity of synthetic peptides in *in vivo* studies. One such carrier is mannan, a polysaccharide made up of mannose. Mannan has been shown to be a potentially useful carrier when coupled to peptides via an aminocaproic acid spacer (Okawa et al., 1992) and so was subsequently coupled to a MUC1 fusion protein (MFP) for investigation. The following section described the immunotherapy of cancer using mannan conjugated to a MUC1 fusion protein.

2.6.1 Murine immune responses to MFP

After using a number of methods to couple MUC1 to a potential carrier, the conjugation of a MUC1 fusion protein (five repeats of the 20 amino acid repeat region from the VNTR) to mannan, under oxidising conditions using periodate, induced tumour regression in mice challenged with $MUC1^+$ tumours (Apostolopolous et al., 1995a and 1996). Immunisation with oxidised mannan conjugated to the MUC1 fusion protein (MFP), induces a cellular immune response characterised by the production of MHC class I restricted $CD8^+$ cytotoxic T cells, a high CTL precursor frequency (1/6,900), T1 cytokine secretion (high IFN-γ and no IL-4) and little IgG2a antibody production (Apostolopolous et al., 1995a). Mice given three injections at weekly intervals were completely protected against a tumour challenge of up to 5×10^7 cells, whereas with previous immunogens mice were susceptible to only 5×10^6 tumour cells (Apostolopolous et al., 1995a).

The conditions with which the mannan is conjugated to MUC1 are crucial to the establishment of an effective antitumour immune response. Under oxidising conditions mannan forms Schiff bases and aldehydes which are crucial to the induction of a T1-cellular immune response and tumour protection. Conversely, the reaction of mannan with sodium borohydride reduces the Schiff bases and aldehydes to amines and alcohol, and results in a T2 immune response characterised by the production of high titres of IgG1 MUC1 antibodies, little CTL induction, IL-4 secretion and no tumour protection from $MUC1^+$ tumours

(Apostolopolous et al., 1995a). A complete summary of the various MUC1 immunogens, including MFP, can be found in Table 1.4.

2.6.2 Parameters of MFP immunisations

A study by Pietersz et al. (1998), investigated the parameters necessary to achieve optimum MFP cellular immune responses *in vivo*; antigen dose, route, number of immunisations and adjuvants. In dose/response studies measured by cytotoxic T cell precursor (CTLp) frequencies, MFP given intraperitoneally (IP) with doses of between 1-7 micrograms of antigen, was clearly a better inducer of cellular immunity compared to higher doses (7-150 micrograms) which induced humoral immune responses. The most favoured route of administration for MFP antigen was either IP or intradermal, which in turn were successively better than intramuscular, intravenous, and subcutaneous which was the worst. Three immunisations were necessary for a maximum cellular response to be achieved, with additional immunisations decreasing the CTL precursor frequency and therefore increasing the humoral immune response (Pietersz et al. 1998). These findings have enormous implications for vaccine development in humans as they highlight the various immunological responses which can be obtained using the same immunogen with varying immunisation protocols.

Table 1.4: Immunogenicity of various MUC1 immunogens in mice[a].

	MUC1 Tumour	MUC1 peptide Fusion Protein or HMFG	Oxidised MFP	Reduced MFP
Immunogenicity				
Antibody Production	+	+++	±	++
DTH	+++	+++	+++	+++
CTL	+ (after PAGAL)	-	++	-
Tumour protection	++	±	+++	±
Mediators				
CD4	-		-	
CD8	+		+	
Cytokine Secretion after immunisation				
IL-2		-	+	-
IL-4		+	-	+
IL-12		-	+	-
IFN-γ		-	+	-
CTL responses				
MHC-restricted			Yes	
MHC-non restricted			No	
Responding H-2 haplotypes			b, d, k, s, z	
Non-responding H-2 haplotypes			nil	
HLA responses			HLA-A2 (others not tested)	
Enhancement of responses to mannan MUC1				
Cyclophosphamide			Yes	
Adjuvants				
CFA			No	
IFA			Yes	
Aluminium hydroxide			Yes	
GM-DP			Yes	
M-DP			Yes	
Macrophage ($F4/80^+$, $33D1^-$) presentation by adoptive transfer				
in vivo			Yes	
in vitro			Yes	
Dendritic cell ($F4/80^-$, $33D1^+$) presentation by adoptive transfer				
in vivo			in progress	
in vitro			in progress	

[a] +++ excellent; ++ very good; + good; ± weak; - nil.
PAGAL, phenyl N-acetyl-a-D-galactosamide

(Apostolopoulos et al., 1996b)

2.6.3 MUC1 T cells are MHC restricted in mice

Finn et al. reported the production of non-MHC restricted CTLs after immunisation with MUC1 antigens. Contrary to this report, Apostolopoulos et al. (1995b) found that MFP induces MHC restricted CTL responses. In all the murine models studies so far, MFP induces only H-2 restricted responses and indeed, nine strains of five different haplotypes could all be immunised. By using inbred, congenic, recombinant and mutant mice, the responses are clearly H-2 restricted and this was confirmed using cells transfected with selected MHC Class I genes and in stabilisation studies using RMA-S cells.

These studies have also been extended to map the epitopes presented by five different MHC class I molecules. The epitopes were defined in CTL assays using peptide-pulsed phytohemaglutinin blasts or MHC class I-transfected L cells as targets; in addition, peptide binding assays and t cell proliferation studies were performed. Within the 20-amino acid VNTR of MUC1, nine potential epitopes could be defined. The epitopes for the four MHC Class I molecules [Kb (three epitopes), Dd. Ld and Kk] were closely related, all containing the amino acid sequence PDTRPAP. For Db, three epitopes were identified, all containing APGSTAP. Most of the epitopes did not contain a consensus motif for the particular MHC class I allele, and bound with 'low affinity', compared with known high-affinity peptides. When conventional anchor residues were introduced into these peptides, peptide binding increased, whereas CTL recognition was either retained (K^b) or lost (D^b) depending on the epitope (Apostolopoulos et al., 1997). Of note, is that these findings show that the epitopes do not fit the usual binding rules, but nonetheless, molecular modelling studies clearly demonstrate that MUC1 is firmly bound to Class I molecules, and furthermore, provides the only example where peptides presented in the groove of Class I are accessible to anti-MUC1 antibodies (Apostolopoulos et al 1998b) -this phenomenon is described in more detail below.

2.6.4 Enhancement of anti-MUC1 responses

In the murine tumour models investigated thus far, MFP immunisations have proven successful at eradicating most established tumours through the induction of a cellular immune response. While these preclinical studies of MUC1 tumour immunotherapy have proven promising for the clinical setting, immunotherapy studies incorporating adjuvants into the immunisation protocol with numerous other antigens including gp100 (Takahashi et al 1990), malaria proteins (Rickman et al 1991) and other tumour antigens (Monte and Szoka, 1989), have been shown to boost the immune responses generated. In a similar way, several studies have employed the use of adjuvants to boost the immune response generated to MFP immunisations, with some findings having exciting implications for future human clinical trials.

Cyclophosphamide, a chemical drug, was used in a study by Apostolopoulos et al (1998a) to boost the cellular immune response generated to MFP. The study found that cyclophosphamide plus MFP substantially increased the MFP CTL precursor frequency from 1/84,900 without cyclophosphamide to 1/8,100 with cyclophosphamide. Furthermore, in the presence of cyclophosphamide, established tumours were rapidly eradicated, a finding not evident in the absence of the drug (Apostolopoulos et al., 1998a). In a similar study, six adjuvants [incomplete Freund's adjuvant (IFA), complete Freund's adjuvant (CFA), Alum, Adjuprime, muramyl dipeptide (MDP) and glutaminyl-muramyl dipeptide (GMDP)] were tested in conjunction with MFP for their potential to boost the MUC1 specific cellular immune response (Pietersz et al., 1998). Alum, GMDP, MDP and CFA all moderately increased the CTLp frequency when combined with MFP, with the best results seen upon the addition of IFA (Pietersz et al., 1998).

Ex vivo targeting of the macrophage mannose receptor using MFP conjugates has recently been demonstrated to induce high specific $CD8^+$ CTL and tumour protection in mice after one such immunisation as compared to 3 MFP immunisations (Apostolopoulos et al., 2000). Targeting the mannose receptor was crucial in this study to obtain high frequency CTL; in the absence of oxidised mannan, the CTLp frequency was much lower. While MFP targets the mannose receptor, it can also directly stimulate IL-12 production *in vitro* by macrophages (Apostolopoulos et al., 2000a).

These results clearly indicate that tumour immunotherapy with oxidised MFP can be significantly enhanced upon the addition of the adjuvants cyclophosphamide, Alum, GMDP, MDP, CFA, IFA or by *ex vivo* targeting of the macrophage mannose receptor. These preclinical studies have formed the basis for these reagents, in combination with MFP, to be trialed in phase I and II clinical trials (this will be discussed in more detail below).

2.6.5 Presentation of MUC1 by MHC class I, H-2 and HLA molecules

Our laboratory has identified 9 different MUC1 peptide epitopes recognised via five different H2 molecules (Kb, Db, Kk, Dd, Ld). The majority of these peptide epitopes do not carry the classical motifs for peptide binding to the individual alleles. The MUC1 peptides were found to bind poorly compared with known high affinity binding peptides. Substitution of anchor amino acids increased the affinity of peptide binding (Table 1.5) (Apostolopoulos et al., 1996). Furthermore, HLA-A2/Kb transgenic mice were immunised with MFP and strong $CD8^+$ CTLs to MUC1 peptides, presented by HLA-A2, were generated. The 9-mer MUC1 peptide sequences (APDTRPA and STAPPAHGV) were found to be presented by HLA-A2. These CTLs could specifically lyse only $HLA\text{-}A2^+$ $MUC1^+$ MCF7 breast cancer cells *in vitro*, and not control $HLA\text{-}A2^-$ $MUC1^+$ BT20 cancer cells (Table 1.5) (Apostolopoulos et al., 1997).

a) Anti-MUC1 antibodies react directly with MUC1 peptides presented by Class I H2 and HLA molecules.

Peptides bound in the groove of MHC class I molecules and detected by cytotoxic T cells are not normally accessible to antibodies. However, in the MUC1 system, MUC1 peptides bound within the groove of the MHC class I molecules (H2 and HLA), which are detected by CTLs, can also be detected by anti-MUC1 peptide antibodies. Studies by Apostolopoulos et al (1998b) indicate that CTL activity could be blocked by antibodies reactive with the C-terminal and middle regions of the MUC1 peptide bound to H2Kb and HLA-A*0201 and only to the mid-region of $H2D^b$. Molecular modelling confirmed these findings that the N-terminal of the MUC1 peptide was buried deep within the MHC class I groove (and therefore not accessible to antibody binding) whereas the mid-peptide residues form a loop and the C terminus is free, making these two areas accessible to antibody (Apostolopoulos et al 1998b). Crystal structure of MHC class I H-2Kb in complex with SAPDTRPA peptide, has revealed some unusual and novel features of peptide binding (Apostolopoulos et al., manuscript in preparation).

It has also been clearly demonstrated that the peptide SAPDTRPAP from MUC1, is presented by H-2Kb, however it has recently been demonstrated that it can also be presented as 5, 6, 7 or 8 mer peptides (deletion only from the C terminus) [Apostolopoulos V, McKenzie I unpublished observations]. These observations are clearly outside the normal structural guidelines for tight binding of class I peptides, as deduced from the crystal structures of many peptide-MHC complexes. In another study based on structural studies, a peptide mimic of MUC1, SAPDTRPAP, has been described. The sequence of the mimic (DAHWESWL) being entirely sequence unrelated to MUC1 was found using a phage display library and by structural similarities of the two peptides. MUC1 specific CTL could be generated against DAHWESWL and protection against MUC1+ tumours (Apostolopoulos et al., 1998). It was found that the mimic peptide was presented by H-2Dd in mice. Furthermore, DAHWESWL was mutated to contain the HLA-A2 anchor binding motifs and, DLHWASWV was found to generate CTL in HLA-A2 transgenic mice and lysis of human breast cancer cell line, MCF7 (Apostolopoulos et al., 1998). This peptide is being prepared for a clinical trial, and it would be of interest to see if MUC1 mimics are more potent than MUC1 peptides.

Table 1.5. Protein epitopes detected by anti-MUC1 CTLs

	MUC1 VNTR
H2 Allele	T S A P D T R P A P G S T A P P A H G V
$H2K^b$	A P D T R P A P G
	S A P D T R P A P
	S A P D T R P A
	T S A P D T R P A
$H2D^b$	A P G S T A P P A
	P A P G S T A P P
	R P A P G S T A P
$H2D^d$	S A P D T R P A P
$H2L^d$	A P D T R P A P G
$H2K^k$	P D T R P A P G S
HLA-A2	S A P D T R P A P
	S T A P P A H G V
H-$2D^d$ MUC1 mimic	D A H W E S W L
HLA-A2 MUC1 mimic	D L H W A S W V

(Apostolopoulos et al., 1997, 1997a)

2.6.6 Clinical trials with MFP in monkeys and in patients

Recently, our laboratory immunised cynomologous monkey with MUC1 fusion protein conjugate to oxidised mannan. It had been shown in mice that oxidised mannan linked to MUC1 induces strong $CD8^+$ CTL and tumour protection (see below). Immunised monkeys generated anti-MUC1 antibodies, MUC1 specific $CD4^+$ and $CD8^+$ T-cell proliferative responses and specific cytotoxic precursor cells, but not MUC1 specific CTL. There was no toxicity of mannan MUC1 immunisations in the monkeys (Vaughan et al., 1999).

The immune responses in 25 patients with advanced metastatic carcinoma of the breast, colon, stomach or rectum were investigated in a phase I clinical trial with increasing doses of MFP (Karanikas et al., 1997). After 4 to 8 injections, large amounts of IgG1 anti-MUC1 antibodies were produced in 13 out of 25 patients (with antibody titres by ELISA of 1/320-1/20,480). Most of the antibodies reacted to the epitopes STAPPAHG and PAPGSTAP. In addition, T cell proliferation was found in 4 out of 15 patients, and CTL responses were seen in 2 out of 10 patients (Karanikas et al., 1997). From this study it remained to be seen whether such responses have antitumour activity. Recently it was demonstrated that, using the flow cytometry, intracellular cytokines IL-2, IL-4, IFN-gamma, and TNF-alpha were produced by CD4+CD69+ and CD8+CD69+ activated T cells after MUC1 antigen stimulation, from peripheral blood mononuclear cells of immunised patients (Karanikas et al., 2000). It was concluded that, in MUC1-immunised patients, the measurement of TNF-alpha and IFN-gamma in activated CD69+CD8+ T cells may be indicative of their immune status.

From these studies, it can be concluded that mannan-MUC1 can successfully immunise patients, particularly for antibody formation and to a lesser extent, cellular responses and importantly, does not induce autoimmune reactions to normal MUC1 expressing tissues making this immunogen safe for use in humans.

Clinical studies with mannan MUC1 immunotherapeutic agents indicate that patients predominantly produce antibody responses despite the induction of cellular immune responses in murine models. This immune deviation is attributed to the presence of naturally occurring, anti-Gal alpha(1,3)Gal, antibodies in all humans. These anti-Gal antibodies have been shown to react directly with the mucin component of the mannan-MUC1 immunogen, and act as carbohydrate-peptide mimics blocking the cellular immune response. The immune deviation

can, however, be overcome by *in vitro* sensitisation of antigen-presenting cells in the absence of anti-Gal antibodies in mice, which may provide useful information for future clinical trials in humans (Apostolopoulos et al., 1999).

2.6.7 Mechanism of MFP

Mannose receptors are predominantly found on macrophages and dendritic cells; they cycle rapidly through the endocytic pathway, and as the binding to the mannose receptor is of low avidity, incoming antigens are released at the early endosomal stage and do not usually reach lysosomes where they would be rapidly destroyed. This pathway is efficient and has been demonstrated that the presence of mannose on an antigen leads to 100-10,000 fold enhanced potency to stimulate MHC Class II presentation to T cells (Tan et al., 1997). However, with oxidised MFP, there is a selective passage to the Class I and not Class II pathway. It has been demonstrated that for oxidised MFP there is a rapid uptake by the mannose receptor, entry into early endosomes, leakage or transfer into the cytoplasm and finally uptake via proteosomes prior to TAP dependent entry to the endoplasmic reticulum (ER), where there is complexing with MHC Class I heavy and light-chains prior to passage through the Golgi apparatus and presentation on the cell surface (Apostolopoulos et al., 2000b). Reduced MFP is also taken up by the mannose receptor, however remains in the endosomes, which become lysosomes where MUC1 is available for MHC class II molecules. It was clearly demonstrated that, aldehydes cause rapid entry into the Class I pathway, and direct the subsequent immune response (Apostolopoulos et al., 2000b).

2.6.8 Future vaccine studies with mannan-MUC1

Mannan MUC1 (MFP) produced under oxidising conditions has clearly been demonstrated to be the most potent immunogen described for inducing CTL T cell immunity in mice. It gives rise to much greater responses than those found with synthetic MUC1 peptides alone, and also greater than any other carrier or adjuvant used. However it needs to be emphasised that the majority of studies performed are measuring the murine immune response to human MUC1, a mere model and an indication of what could be happening in humans. As shown, patients immunised with human MUC1 produce predominantly humoral, rather than cellular, immune responses indicating the difference between the human and murine systems.

To overcome these problems and provide more realistic models for MUC1 immunotherapy studies, human MUC1 transgenic mice are being studied, along with the immunogenicity of mannan conjugated to autologous MUC1 in monkeys (by cloning cynologous monkey MUC1) and also mannan-murine MUC1 in mice (Apostolopoulos et al., 1996b). As reported earlier, MUC1 transgenic mice can be successfully immunised against MUC1, although the CTL precursor frequency is substantially less than obtained in cross species experiments. Recently, mice were immunised with mannan-macaque-MUC1 and CTL responses were induced (as has been found with human MUC1 in mice), however, cynomolgus monkeys (Macaca fascicularis), immunised with mannan-macaque MUC1 produced a humoral responses, with no T cell proliferative, cytotoxic responses or CTLp found. In spite of the presence of anti-MUC1 auto-antibodies, there was no toxicity or induction of autoimmunity (Vaughan et al., 2000).

Yet despite the short-comings of the MUC1 models studied so far, the antitumour immune responses produced in mice are encouraging and form the basis of further clinical trials investigating MFP and other adjuvants. It remains to be seen the role of mannan-MUC1 therapy in the treatment (or vaccination) of cancer patients in the future, however with continuing research into enhancing the mannan-MUC1 immune response, the creation of more realistic tumour models and the commencement of new clinical trials, the outcomes will quickly become evident.

Section 3: Manipulating the immune response

The primary substance used to manipulate and stimulate the immune response to a specific antigen is the use of adjuvants. Immunological adjuvants are substances which when mixed with antigen potentate an immune response (Audibert and Lise, 1993). Adjuvants are used to amplify either or both the humoral and cell-mediated immune responses to an antigen. The most frequently used adjuvant in experimental animals is complete Freund's adjuvant (CFA), which contains heat-killed *Mycobacteria tuberculosis* in a water-oil emulsion, that activates macrophages due to the mycobacterial cell wall. Although very effective in evoking long lasting immune responses, CFA is not suitable for use in humans because it induces granulomas, fever and inflammation due to the oil and mycobacterium. Incomplete Freund's adjuvant (IFA) is a similar adjuvant to CFA which contains antigen in an aqueous solution of mineral oil and emulsifying agent without the *Mycobacteria tuberculosis* (Kuby, 1992). Incomplete Freund's adjuvant has been used to elicit a $CD8^+$ CTL response in Rhesus monkeys with simian immunodeficiency diseases as a model for an AIDS virus vaccine (Yasutomi et al., 1993).

There is only one registered human adjuvant (aluminium hydroxide or aluminium phosphate) which is currently used in diptheria, tetanus and hepatitis B vaccines. Aluminium salt adjuvants are limited in their use, in that they preclude freezing, they are not effective with all antigens and they do not stimulate cell-mediated immunity. Other adjuvants used in tumour immunotherapy studies include Bacille Calmette-Guerin (BCG), immunostimulating complexes (ISCOMs), liposomes, Ribi adjuvant, viral vectors and cytokines.

3.1 Bacille Calmette-Guerin

Bacille Calmette-Guerin (BCG) is a live attenuated bovine tubercle bacillus used to immunise against tuberculosis, which is also used as a vaccine vehicle for delivering protective antigens to hosts. It is the most widely used vaccine in the world because it can be given at any time after birth, is unaffected by maternal antibodies, can be administered as an oral vaccine, is heat stable and is inexpensive to produce (Stover et al., 1991).

BCG as an adjuvant can be used in conjunction with cells or incorporated proteins where it persists *in vivo* and continually stimulates the immune system with its recombinant antigen, producing both humoral and cellular immune responses (Stover et al., 1991). For example, Bloemena et al. used BCG as an adjuvant in 1993, where it was used to incorporate an autologous tumour cell for vaccination in colon carcinoma patients. The outcome of this study was the production of DTH and T-cell proliferation in the cancer patients (Stover et al., 1991) indicating that BCG used in this context shows bias towards cellular immunity.

3.2 Immunostimulating complexes

Immunostimulating complexes (ISCOMs) are vaccine vehicles composed of the adjuvant Quil A, a semipurified product isolated from the bark of a tree found in the Andes (Morein, 1988). ISCOMs form micelles which have regions accessible for hydrophobic interactions with molecules such as membranous proteins (Morein et al., 1987), and is held together by the Quil A matrix, lipids and amphipathic antigens (Morein, 1988).

ISCOMs have been used as immunological adjuvants to induce cellular immunity in several *in vivo* studies. $CD8^+$ MHC class 1 restricted CTLs have been induced against non-infectious antigens (Audibert and Lise, 1993), and used HIV (Takahashi et al 1990) when ISCOMs were used in immunisations. Takahashi et al. showed that mice immunised with ISCOMs containing either purified intact gp160 envelope glycoproteins from the HIV virus or influenza haemaglutinin, priming of HIV specific and influenza specific $CD8^+$, MHC class 1 restricted cytotoxic T cells are produced.

3.3 Liposomes

The immunoadjuvant properties of liposomes were first described by Allison and Gregoriades in 1974. Liposomes are concentric spheres which consist of a phospholipid bilayer separated by aqueous compartments, which are able to trap proteins and other antigens inside them and deliver them into the cell (Allison and Gregoriadis, 1974). The association of protein antigens with liposomes has been shown to potentiate the antibody response to that antigen when injected into animals (Monte and Szoka, 1989). This work has been demonstrated with various antigens including diptheria toxoid, hepatitis B surface antigen, tetanus toxoid and tumour cell antigen (Monte and Szoka, 1989).

When antigen is incorporated into pH-insensitive liposomes, it is degraded in the lysosomes, recycled to the endosomes, and presented to T cells in association with MHC class II molecules. However, when antigen is incorporated into pH-sensitive liposomes, they disrupt and become fully active on exposure to the low pH of the endosomes. Here they release their contents into the endosome and partly into the cytosol where it can be processed and presented in association with a MHC class I molecule (Reddy et al., 1991; Zhou et al., 1992).

3.4 Ribi adjuvant

Ribi has been clinically tested with a Plasmodium falicparum circumsporozite malarial protein vaccine, and has been compared to responses to the same immunogen administered with aluminium hydroxide adjuvant (Rickman et al 1991). Patients administered with Ribi adjuvant malarial vaccine produced higher antibody titres when compared to the antibody levels produced in patients who were administered the aluminium hydroxide adjuvant vaccine. The data support the evaluation of the Ribi complex as an adjuvant for other vaccines (Rickman et al 1991).

Ribi adjuvant has also been used for a potential synthetic breast cancer vaccine (Henningson et al 1987). Mice and humans injected with the vaccine elicit both humoral and cellular (DTH) immune responses (Fung et al., 1991), and the Ribi adjuvant is non-toxic in humans (Longnecker et al 1993). Ribi is therefore a promising candidate to be used as an adjuvant for human vaccines.

3.5 Gene therapy

Gene therapy is a rapidly evolving concept for the treatment of different forms of cancer. There are two elements to gene therapy which must be considered for tumour immunotherapy studies. The first is the choice of vector for the efficient transfer and expression of the transgene in the cancer cell, and the second is the therapeutic gene to be expressed. The most popular choice of vectors used in preclinical studies are vaccinia virus, avipox viruses and adenoviruses (one of most advanced vectors ready for clinical use) because they are easy to manipulate and infect rapidly dividing cells. The choice of transgene that can be delivered to the tumour cell and directly effect its tumourigenicity is varied. Many studies delivered faulty or missing genes such as the p53 tumour suppressor gene to the tumour, while others have focussed on stimulating the immune system by delivering tumour specific antigens, costimulatory molecules, major histocompatibility molecules, and cytokines. Whatever the combination of antigen and delivery vehicle chosen, cancer gene therapy holds great promise to become an important addition to the multimodality in the therapy of some forms of cancer. The choice of viral vectors and various transgenes for tumour immunotherapy studies are described in more detail below.

3.5.1 Viral vectors

The ideal vaccine for cancer is a live attenuated derivative of the tumour cell which can induce protective immunity to tumour specific antigens on the malignant cell without causing any side effects. There are however, certain barriers to the development of such cancer vaccines including the ethical problems associated with the delivery of live -albeit attenuated- tumour cells to healthy individuals and the difficulties that give rise to growing tumour cells and attenuating in the laboratory. One strategy of overcoming these barriers is to insert the tumour antigen, or other therapeutic genes, into a nonpathogenic organism. The non-virulent organism (recombinant virus) serves as a vector for the expression of the genes encoding for the antigens (Perkus et al., 1985). This has the advantage that the recombinant virus simultaneously synthesises the foreign antigen and delivers it to the immune system of the host. Several vectors have been tested in the development of vaccines and are summarised below.

a) Vaccinia virus

Vaccinia virus is a complex DNA virus of the pox family which replicates within the cytoplasm of infected cells independently of the nucleus. The virus contains all the enzymes necessary for mRNA synthesis and translation (Moss et al 1991) and has been used successfully as a live vaccine for the eradication of smallpox (Fenner et al., 1988).

Poxviruses, such as vaccinia, have several advantages as vectors because homologous DNA recombination occurs naturally during the viral replication (Fenner and Comben 1958) thus allowing the insertion of relatively large fragments of DNA (Smith and Moss 1983) into a choice of sites. VV are easy to manipulate and purify. The Copenhagen strain of vaccinia has been shown to be 5000 times less virulent than the laboratory strain WR when injected intracranially into 3 week-old mice, and it is also better tolerated by nude mice (LD_{50} has been shown to be > 10^9 PFU as opposed to 422for WR) (Tartaglia et al., 1992). Furthermore, mutations in the thymidine kinase gene activity of vaccinia drastically reduces virulence probably by making the virus much less infective for quiescent cells (Buller et al., 1985; Taylor et al., 1991). Taken together, these factors make vaccinia virus an ideal vector for tumour immunotherapy studies.

Recombinant vaccinia vaccines are now being used in immunotherapy studies for cancer and infectious diseases because the co-presentation of potential immunogens with the highly immunogenic vaccinia proteins, have been shown to elicit strong immune responses against the inserted gene products. Thus in tumour immunotherapy, recombinant vaccinia viruses have been used with a vast array of tumour antigens including c-erbB-2 oncogene (Bernards et al., 1987), melanoma antigens (Hu et al 1988, Estin et al 1988, Kim et al 1998), CEA (Kantor et al 1992, Tsang et al 1995, McLaughlin et al 1996, Conry et al 1999), beta-galactosidases (Carroll et al 1997) and MUC1 (Acres et al., 1993).

Vaccinia viruses have also been used to deliver other therapeutic genes to tumours in an effort to decrease tumour burdens and stimulate a protective immune response *in vivo*. Several experiments have shown the therapeutic effects of delivering VV-costimulatory molecules directly to malignant cells. Recently, poxvirus vectors such as vaccinia and avipox (see below) were used as tumour vaccines in a murine colorectal cancer model. Hodge et al (1999a) compared the use of a retroviral vector versus a poxvirus vector (vaccinia) in whole tumour-cell vaccines. The transgene used was the T-cell costimulatory molecule B7-1, and the

tumour was the weakly or nonimmunogenic MC38 murine colon adenocarcinoma. This study demonstrated that a whole tumour-cell vaccine (either live or X-irradiated) containing a vaccinia transgene is at least as efficient, and sometimes more efficient, in inducing antitumour effects compared with the same vaccine using a retrovirus to express the transgene (Hodge et al 1999a).

This thesis focuses on the effect of cytokines delivered to MUC1 expressing tumours by recombinant VV. There have been many tumour immunotherapy studies examining the delivery of recombinant cytokines to various tumour models *in vivo*. These shall be reviewed in more detail later.

b) Avipox virus

A variety of avian viruses have been evaluated as potential vaccine vectors. For example, the genome of the fowlpox virus (Webster's mild vaccine strain) is composed of a single, double-stranded DNA molecule with covalently linked terminal hairpins and approximately 300 kb in length (Coupar et al 1990). There are several advantages of using avipox viruses over vaccinia viruses. Firstly, similar to vaccinia virus, avipox viruses can be engineered to express the gene of interest and can successfully infect dividing cells. However, the size of the fowlpox virus genome is more than 100 kB larger than that in vaccinia, indicating that avipox viruses have the potential to code for more proteins than other groups of poxviruses (Coupar et al 1990). Secondly, unlike vaccinia virus, avipox viral infections do not replicate in mammalian cells and therefore do not spread to other organisms because the avian virus is host-restricted (Taylor and Paoletti, 1988).

Recombinant avipox viruses have also been used to deliver antigens in several viral models, and have demonstrated promising effects in the immunisation against the human immunodeficiency virus (HIV-1). The successful immunisation of many viruses, such as HIV-1, depends upon the roles of both antibody and CTL responses. Canarypox and fowl pox (both avipox) viruses have been used as vectors to express the envelope glycoproteins of HIV-1. Mice immunised with the vaccine produced antibody responses to HIV gp120 and high levels of primary and memory $CD8^+$ CTL responses (Cox et al 1993). Rabbits immunised with the vaccine also produce env-specific humoral and cell-mediated immunity capable of inhibiting specific HIV-1 functions (Radaelli et al 1994 and 2000). Recombinant canarypox and fowlpox viruses that contained two forms of the HIV-1 (SF2 strain) env gene were also

engineered and successfully expressed in chick, simian, human (Radaelli et al 1994a) and macaque cells (Macaca nemestrina). The hosts were vaccinated with complex recombinant fowlpox virus (rFPV) vaccines expressing both HIV-1 antigens and type-1 cytokines (IFN-gamma), and were shown to be safe, and enhanced T cell proliferative responses to HIV-1 antigens following IFN-gamma expressing vaccinations (Kent et al., 2000).

Further studies with avipox viruses include the construction of an attenuated canarypox virus expressing the measles virus and produced high levels of neutralising antibodies against the measles virus in canines (Taylor et al 1992). Recombinant avipox viruses containing the Japanese encephalitis virus (Konishi et al 1994), rabies virus (Taylor et al., 1991), Newcastle disease virus (Boursnell et al., 1990) and feline leukemia virus (Tartaglia et al 1993) have also been constructed.

Avipox viruses have also been used to deliver the carcinoembryonic (CEA) tumour antigens in immunotherapy studies in both mice and human clinical trials (Hodge et al., 1997, Marshall et al 1999). Canarypox recombinants expressing the human CEA gene were used in a murine colorectal cancer model and results showed that vaccination with the avipox recombinant induced antibody, lymphoproliferative and cytolytic T-cell responses as well as tumour inhibition (Hodge et al., 1997). Interestingly when mice were immunised with a diversified immunisation scheme using a recombinant VV-CEA followed by recombinant avipox-CEA, CEA-specific T-cell responses were at least four times greater, and far superior to those achieved with avipox alone. Multiple boosts of avipox-CEA following VV-CEA immunisation further potentiated anti-tumour effects and CEA specific T-cell responses, demonstrating an advantage of diversified immunisation protocols employing both recombinant VV and avipox vectors (Hodge et al., 1997).

Avipox viruses have since been used to deliver the CEA tumour antigens in a human clinical trial (Marshall et al 1999). The first phase I clinical trial in patients with advanced carcinoma was conducted using a replication-defective avipox vaccine containing the gene for human CEA. The canarypox vector was able to infect human cells but could not replicate. When injected into advanced cancer patients, the recombinant CEA vaccine was safe and elicited CEA-specific CTL responses (Marshall et al 1999). The CEA-specific derived T cells were capable of lysing allogeneic and autologous tumour cells in a MHC-restricted manner (Zhu et al 2000). These studies thus form the basis for the further clinical exploration of the avipox-

CEA recombinant vaccine in phase I/II studies in protocols designed to enhance the generation of human T-cell responses to CEA.

c) Adenovirus

Adenoviruses are linear double-stranded DNA viruses which have also been used as vaccine vectors. The common serotypes 2 and 5 have been used as the base vector and by deleting parts of the virus genome, the virus becomes a replicating deficient vector capable of transferring the exogenous DNA to cells such as those found in the airway epithelium (Mastrangeli et al 1993). The vector interacts with the cell through a specific unidentified receptor, enters an endosome, breaks out of the endosome and moves to the nucleus where it functions in an epi-chromosomal fashion to express the new gene (Crystal 1995).

Adenovirus vectors have been used extensively in gene therapy studies to transfer genes to malignant cells *in vivo*. The adenovirus-mediated gene transfer of functionally active human p53 and p21 tumour suppressor genes has shown promise in several preclinical experiments. The transfer of p21 into a p53-deficient mouse prostate cancer cell line (Eastham et al 1995) and human papilloma virus (HPV)-positive cervical cancer cells (Tsao et al., 1999) resulted in an increased survival of tumour-bearing host animals mediated through the apoptosis of infected cells. Similarly, the transfer of the p53 gene into a p53-deficient mouse prostate cancer cell line (Eastham et al 1995) and a peritoneal tumour model of ovarian cancer in nude mice (Kim et al., 1999) also produced encouraging results.

In addition to being able to deliver genes that may be altered or missing in tumour cells, adenoviruses can also deliver tumour antigens to malignant cells making them more susceptible to immune invasion. In melanoma immunotherapy studies, adenovirus vectors have been used to deliver the melanocyte differentiation antigens, gp75 and gp100. Adenovirus-mediated gene transfer of gp75 can successfully induce cellular immune responses capable of rejecting B16 melanomas (Hirschowitz et al., 1998). Adenoviral delivery of gp100 to melanoma cells induced limited protective immunity in C57BL/6 mice, however a subcutaneous injection of dendritic cells infected with adenoviral gp100 resulted in a potent T-cell-mediated protective immune response and greater than 80% reduction of established tumours when administered to B16 tumour-bearing hosts (Wan et al 1999).

Adenovirus vectors have also been used to deliver recombinant cytokines to tumour-bearing hosts as another major immunotherapeutic strategy to combat cancers. These studies will be discussed in more detail later. Taken together, these results demonstrate that adenoviruses can successfully transfer various genes into tumour cells that can ultimately suppress the growth of peritoneal tumours, and prolong the survival of tumour-bearing mice indicating the potentially useful therapeutic properties of adenoviral vectors in the treatment of human cancers.

3.5.2 Costimulatory molecules

In 1970 it was proposed that two signals were required to activate T cells (Bretscher and Cohn, 1970). The first signal being provided through the interaction of the T cell receptor with the MHC Class-1 antigen on the APC and the second through the interaction of B7-1 on the APC with its ligands CD28 and/or CTLA-4 on the resting T cell (Bluestone 1995). Costimulation of T cells by B7-1 plays an important role in eliciting antitumour immunity. The failure to deliver a costimulatory signal enables a tumour cell to evade the immune system, even if the tumour expresses normal levels of MHC class I antigens and presents tumour-specific peptides. Consequently, several models, include murine melanoma (Chen et al., 1992), have focussed on increasing the expression of B7-1 (by transfection) in tumours with antigens that induce specific antitumour immunity leading to tumour eradication in immunocompetant hosts.

In two independent tumour immunotherapy models, the intratumoural administration of an adenoviral vector vaccine containing either B7-1 or B7-2 transgenes with IL-2, caused a complete regression of mice bearing adenocarcinomas. Regressed mice were all protected from a rechallenge in both models and antigen-specific CTL were identified (Emtage et al., 1998 and 1998a). Interestingly, the administration of adenoviral-B7-1 or B7-2 alone were not satisfactory at inducing tumour regression (38%) demonstrating the need for more than the second signal in some murine tumour models.

Another model investigated the role of three costimulatory molecules in the activation of T cells. Two poxvirus (vaccinia and avipox) vectors were used because of their ability to efficiently express multiple genes. Murine cells were initially stimulated with a primary signal and the second signal was supplied through an infection with either recombinant vaccinia or

avipox vectors containing three costimulatory molecules (B7-1/ICAM-1/LFA-3, designated TRICOM). The TRICOM induced the activation of T cells to a far greater extent than cells infected with any one or two costimulatory molecules, yet despite this T-cell 'hyperstimulation' using TRICOM vectors, no evidence of apoptosis above that seen using the B7-1 vector was observed (Hodge et al 1999). This study demonstrated the ability of vectors to introduce three costimulatory molecules into cells, and activate both CD4+ and CD8+ T-cell populations to levels greater than those achieved with the use of only one or two costimulatory molecules. This new threshold of T-cell activation has broad implications in future vaccine design and development.

3.6 DNA vaccines

DNA vaccination or genetic immunisation is a rapidly developing technology that offers new approaches for the prevention of disease and in particular, cancer. This method of vaccination provides a stable and long-lived source of the protein vaccine, and it is a simple, robust, and effective means of eliciting both antibody- and cell-mediated immune responses. The observation that naked DNA injected into mice could be expressed was first observed back in 1962 (Atanasiu 1962), however it was not until the observation that plasmid DNA injected into the muscles of mice and could be expressed as protein by the muscle cells (Wolff et al., 1990), that this new technology expanded. Two reports in 1993 indicated the potential value of genetic vaccines against infectious agents by demonstrating that plasmid DNA-encoding proteins from influenza virus could induce immune responses (Robinson et al., 1993; Ulmer et al., 1993). Since these initial observations, DNA vaccines have been studied in many infectious diseases models including herpes (Manickan et al., 1995), influenza (Donnelly et al., 1995), rabies (Xiang et al., 1994), hepatitis B (Kuhober et al., 1996) and HIV (Wang et al., 1993) and have been reviewed by Manickan et al. (1997).

Initially, DNA vaccines were administered intramuscularly, taken up and the protein expressed by myocytes (Wolff et al., 1990). The expression of the new protein can persist for many months, perhaps even years (Yankauckas et al., 1993), and may indeed represent a considerable advantage to DNA vaccinology, because the outcome might be the sustained maintenance of effector cells and immunological memory. The plight of antigenic DNA injected intramuscularly into animals undoubtably leads to the nucleus, gene transcription and protein production in myocytes, however what remains unclear despite earlier studies

suggesting otherwise, is if the mycoytes act as antigen presenting cells to achieve CTL induction because these cells lack the expression of costimulatory molecules (Pardoll and Beckerleg, 1995). Other studies have suggested the APC role for resident dendritic cells or other cells invading in response to muscle cell damage (Pardoll and Beckerleg, 1995).

The most effective method of DNA delivery shown to date has been achieved through the use of a 'gene gun' in influenza studies (Fynan et al., 1993). The gene-gun delivers DNA covered gold-particles as micro-projectiles into the epidermis through an 'accell' instrument that generates an electric spark discharge as the motive force. The success of the gene gun vaccinations may be attributed to the fact that lymphoid tissues are as rich as epidermal Langerhan cells and are capable of presenting antigens to the T-helper component of the immune system (Steinman et al., 1991).

Furthermore, DNA vaccines have a number of potential advantages such as they can address several diseases in one vaccine, they are cheap and easy to produce and have no special cold storage requirement because they are extremely stable (Tuteja 1999). It has proven to be a generally applicable technology in various preclinical animal models of infectious and noninfectious diseases (Manickan et al., 1997), and in models of cancer immunotherapy, including multiple myeloma (Stevenson et al., 1999), lymphoma (Spellerberg et al., 1997; Benvenuti et al., 2000), melanoma (Park et al., 1999; Zhou et al., 1999; Steitz et al., 2000), HPV induced murine tumours (Chen et al., 1999; Ji et al., 1999), colorectal cancer (Conry et al., 1998) and mammary carcinomas (Chen et al., 1998; Charo et al., 1999).

Vaccination with DNA constructs expressing specific tumour antigens can trigger immune recognition and the destruction of tumour cells. While there are still hurdles that need to be overcome on the road to the use of DNA vaccines for cancer immunotherapy, the future holds much promise.

3.7 Cytokines

Since their identification some 40 years ago, cytokines have been used extensively to manipulate immune responses in animal models and as a result, many have progressed into clinical trials and use in humans eg. IL-2 in metastatic renal cell carcinoma and melanoma (Danheiser 1997). Many of these studies have focused on the use of

recombinant cytokines, administered either locally or intratumourally by repeated low-dose injections including IL-1, IL-2, IL-4 and IFN-γ (Musiani 1997), or on cytokine gene therapies whereby murine tumours have been transfected to secrete cytokines *in vivo* including IL-2, IL-4, IL-6, IL-7, IL-12, GM-CSF, TNF, IFN-γ etc. (Colombo 1994; Tepper 1994; Dranoff 1995). The use of cytokines in tumour immunotherapy studies is discussed extensively in the next section.

Section 4: Tumour immunotherapy using cytokines

As demonstrated thus far, successful immunotherapeutic strategies may involve either localised treatment with immune-active adjuvants or the use of whole tumour cells or tumour cell fragments as vaccines. Another therapeutic approach to the induction of anti-tumour cell immunity is with the systemic administration of cytokines, either alone, or delivered in conjunction with tumour antigens.

The induction of an anti-tumour cellular immune response requires tumour specific antigens to be processed and presented by either the tumour, professional antigen presenting cells (eg. B cells, dendritic cells and macrophages), or both, in association with MHC class I and costimulatory molecules to activate naive T cells into tumour specific cytotoxic cells, under the influence of locally secreted cytokines. Early in the immune response, cytokines such as IL-1, IL-2, IL-4 and GM-CSF act to recruit APCs and $CD4^+$ T cells to the site of antigenic challenge. After presentation, a cascade of various cytokines including IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF and IFN-γ are secreted to activate $CD8^+$ T cells and other cells involved in tumour rejection to eliminate tumour cells and ensure T cell memory is established to protect against recurrences. With an increasing understanding of the requirements for the development of an immune response, immunotherapeutic strategies have focused on providing mechanistic requirements, such as tumour or accessory antigen expression and now a new wave of immunotherapy focusing on cytokine-based 'immune help'.

4.1 The cytokines

With the emerging technology of molecular biology, it is possible to produce a range of pure recombinant cytokines that could be used to manipulate specific immune responses. Cytokines are attracting attention for the treatment of cancer and their applications in tumour immunotherapy studies are described below. The cytokines IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IFN-gamma, GM-CSF, TNF-α have been used in this thesis and have been

reviewed in more detail. Table 1.6 summarises cytokines (IL-1 to IL-18), interferons and granulocyte colony-stimulating factors.

4.1.1 Interleukin-2

IL-2 was the first hormone of the immune system to be recognised (Morgan 1976). It is a polypeptide produced by activated T cells which promotes T cell division, and activation of other cells in the immune system such as NK and B cells (Smith 1990). Human IL-2 was first purified from the culture supernatants of mitogen or alloantigen-activated T cells and the leukaemic cell line, Jurkat, and was shown to have a molecular weight of 14-17 kDa on SDS-PAGE. IL-2 has many biological functions including the stimulation of T cell proliferation, B cell growth and differentiation, generation of lymphokine-activated killer (LAK) cells, the activation of macrophages and stimulation of T cells to produce other cytokines (Smith 1990).

IL-2 interacts with cells by binding to a receptor with two differing affinities; a high affinity receptor (K_d $1x10^{-11}$M) which mediates the physiological response of T cells to IL-2 and a low affinity receptor (K_d $1x10^{-8}$M) which does not. The IL-2 receptor is comprised of three components, an α–chain, a β-chain and a γ-chain. The α–chain of the IL-2 receptor is a 55kDa protein that binds IL-2 with a low affinity (Kondo et al., 1986). The β-chain is a 75kDa protein that has an intermediate binding affinity for IL-2. However when combined, both the α–chain and β-chain receptors bind IL-2 with a high affinity (Teshigawara et al., 1987). A third chain of the IL-2 receptor has been cloned (IL-2Rγ), and is a 64kDa protein with protein kinase activity involved with signal transduction (Takeshita et al., 1992). Each subunit of the IL-2 receptor can independently bind IL-2 with lower affinity and may interact with a different region of the IL-2 molecule, however the binding of IL-2 to both proteins produces the biologically active high affinity receptor (Wang and Smith, 1987).

Tumour immunotherapy studies with IL-2 have demonstrated additional IL-2 is effective at inducing tumour rejection and the establishment of memory. Numerous IL-2 transfection studies into non-immunogenic tumour models show that continual low dose secretion of IL-2 provides sufficient cytotoxicity necessary to reduce the tumourigenicity and metastatic potential of tumour models including melanomas (Karp 1993), fibrosarcomas (Gansbacher 1990), bladder carcinomas (Conner 1993) and neuroblastomas (Corrias 1998).

Table 1.6: Principle cellular sources and targets of cytokines

Cytokine	Molecular Form	Cellular Source	Cellular Target
IL-1-α	Monomer 159aa. membrane bound, (17.5kD)	Monocytes, macrophages, fibroblasts, neuronal cells, epithelial cells, T and B cells	Involved in inflammatory and immune responses. Acts on cells to produce IL-2 receptors
IL-1-β	Monomer 153aa. membrane bound, (17.3kD)	Monocytes, macrophages, fibroblasts, neuronal cells, epithelial cells, T and B cells	Involved in inflammatory and immune responses. Acts on cells to produce IL-2 receptors
IL-2	Monomer 133aa, (15-20kD)	T cells (cytotoxic and helper)	Stimulates T cell proliferation and differentiation, enhances cytolytic activity of NK cells and LAK cells
IL-3	Monomer 133aa, (14-30kD)	T cells (helper), thymic epithelial cells, neuronal cells, mast cells	Stimulates the production of macrophages, neutrophils, eosinophils, basophils and megakaryocytes.
IL-4	Monomer 129aa (15-19kD)	T cells (helper), macrophages, mast cells, basophils, B cells, bone marrow stromal cells	Differentiates naive CD4 cells into T2 cells. Induces proliferation and differentiation of B cells.
IL-5	Monomer 115aa (22.5kD)	T cells (helper), mast cells, eosinophils	Stimulates growth and differentiation of eosinophils. Activates eosinophil function
IL-6	Monomer 184aa (26kD)	T cells, monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, mast cells	Induces growth of T and B cells, hepatocytes and nerve cells
IL-7	Monomer 152aa (20-28kD)	Bone marrow stromal cells, foetal liver cells	Pre / Pro- B cell growth factor, supports proliferation and generation of CTL and LAK cells
IL-8	Dimer (N-terminal variants: 69,72,77 or 79aa) (8-10kD)	T cells (helper), monocytes, fibroblasts, endothelial cells, epithelial cells, neutrophils	Chemoattractant for T cells, basophils, neutrophils *in vitro*. Activates neutrophils
IL-9	Monomer 126aa (32-39kD)	T cells (helper)	Enhances *in vitro* survival of T cells, mast cell activity

Cytokine	Molecular Form	Cellular Source	Cellular Target
IL-10	Homodimer 160aa (19kD)	T cells (helper), macrophages, keratinocytes, B cells	Immunosuppressant of macrophage functions. Enhances Ig secretion and B cell proliferation
IL-11	Monomer 180aa (23kD)	Stromal fibroblasts	Together with IL-3, increases the size and number of magakaryocytes
IL-12	Heterodimer of 197aa and 306aa (35kD and 40kD)	T cells, B cells, macrophages	Initiates cell mediated immunity (induces T1 cells), and initiates growth and activity of NK and T cells
IL-13	Monomer 132aa (16kD)	T cells (helper)	Induces B cell growth and differentiation. Inhibits cytokine production by macrophages
IL-14	Monomer 468aa (60kD)	T cells	Induces proliferation of activated B cells and inhibits Ig secretion of mitogen-activated B cells
IL-15	Monomer (400aa) (~40kD)	Activated monocytes and macrophages, epithelial cells, fibroblasts, PBMC	Enhances NK cytotoxicity. Stimulates the proliferation of T cells (T_H and CTL)
IL-16	Homotetramer 14-17kD chains (56kD)	CD8 T cells	Chemoattractant for lymphocytes, CD4 and CD8 T cell growth factor, induces IL-2 receptor expression on T cells
IL-17	Homodimer 150aa (20kD)	T cells	Stimulate NF-κB and IL-6 secretion in fibroblasts, costimulate T cell proliferation, epithelial and endothelial secretion of cytokines
IL-18	157aa (18kD)	Osteoblastic stromal cells	Induces IFN from T cells, NK cells, macrophages and Kupffer cells. Induces T cell proliferation and the production of T1 cytokines from T cells

Cytokine	Molecular Form	Cellular Source	Cellular Target
M-CSF	Homodimer 522 or 224aa (40-90kD)	Fibroblasts, monocytes, endothelial cells	Stimulates macrophage proliferation, increases MHC class II and Fc receptor expression
G-CSF	Monomer 174aa (18-22kD)	Macrophages, fibroblasts, endothelial cells, mesothelial cells, T cells	Stimulates granulocytes, enhances antibody-dependant cytotoxicity, enhances tumour lysis by granulocytes
GM-CSF	Monomer 127aa (22kD)	T cells, mast cells, macrophages, neutrophils, eosinophils, endothelial cells, fibroblasts	Stimulates all cells in the granulocyte, macrophage and eosinophil lineage, enhances killing by macrophages and granulocytes, stimulates cytokine production from macrophages
IFN-α	Monomer 166aa (16-27kD)	T and B cells, macrophages	Has anti-viral activity, enhances MHC Class I expression, enhances NK cell activity
IFN-β	Monomer 166aa (20kD)	T and B cells, macrophages, fibroblasts, epithelial cells	Has anti-viral activity, enhances MHC Class I expression, enhances NK cell activity
IFN-γ	Homodimer 143aa (20-24kD)	T cells (CTL, helper), NK cells	Stimulates macrophages and has an anti-viral activity, enhances MHC Class I and II expression, enhances NK cell activity
TNF-α (also called TNF)	Trimer 157aa, also memberane bound 17aa (157kD)	NK and LAK cells, activated lymphocytes, macrophages, T cells, B cells, smooth muscle cells, neutrophils	Involved in host resistance of tumour growth to infection. Also involved in a wide variety of effects
TNF-β (also called lymphotoxin, LT)	141aa and 171aa (20kD and 24kD)	T cells	Involved in host resistance of tumour growth to infection. Also involved in a wide variety of effects
TGF-β	Homodimer 112aa (12.5kD)	Platelets, activated macrophages, T cells, mesothelial cells, thymocytes	Endothelial cells, T cells, B cells.

Abbreviations used: aa – amino acids, kD – kilodalton, PBMC – peripheral blood mononuclear cells. (IRL Press, 1993, Cytokines and cytokine receptors, 2nd edn., Oxford University press; R&D Systems Inc., Cytokine release from human leukocytes – Poster)

4.1.2 Interleukin-4

Interleukin 4 cause the activation, proliferation and differentiation of B cells, is a growth factor for T cells and mast cells, and exerts other effects on granulocytes, megakaryocytes, erthrocyte precursors and macrophages (Lee 1986; Yokota 1986; Paul and Ohara, 1987; Paul 1991). IL-4 regulates B cell growth and expression of cell surface antigens such as CD23 and MHC Class II which it upregulates. It is a switch factor for IgE and IgG1 by lymphocytes. The major source of IL-4 is activated T2 subsets of $CD4^+$ T cells in mice (Mosmann and Coffman 1989) and the equivalent population in humans. The biological effects of IL-4 are mediated by high-affinity IL-4 receptors. The IL-4 receptor consists of an IL-4 specific ligand binding chain (α) and a signalling chain (γc) that is shared with several other cytokines including IL-2 and IL-7 (Kondo 1993; Russel 1993). The receptor protein binds IL-4 with a single high affinity and has a molecular weight of around 130kDa. The two-chain receptor is found on most cells of the hematopoietic lineage as well as some non- hematopoietic cells (Lowenthal 1988; Park 1987). Receptors for IL-4 are found on virtually every cell tested so far including T and B cells, monocytes, fibroblasts, myeloid epithelial and endothelial cells. Receptor numbers increase on cell activation, and approximately 300 receptors have been identified on B and T cells but more on B cell lymphomas and T cell lines (Miyajima 1992; Paul 1991; Galizzi 1990).

Many studies indicate that IL-4 gene transfer into tumour cells confers a certain extent of protection from subsequent lethal challenges of tumour, however this protection is generally attributed to nonspecific effector cells and not always $CD8^+$ CTLs required for complete tumour rejection (Tepper 1989, 1992, Li 1990, Hock 1993, Pericle 1994). IL-4 has been shown to inhibit the development of cytotoxic T cells while inducing the proliferation of T2 cells (Erard 1993; Brown 1997). Even in syngeneic mice, IL-4 transfected tumours are not completely rejected in all animals, perhaps due to the level of IL-4 expression in the respective tumour model (Tepper 1989, Dranoff 1993, Hock 1993, Pericle 1994). This suggests that the T cells are not optimally activated by IL-4 cytokine gene-transfected tumours. One method used to successfully overcome the lack of activation of T cells induced via IL-4 stimulation is with the cotransfection of the T cell costimulatory molecule B7.1. Cayeux et al (1996) showed that complete long term tumour eradication was induced in all of the syngeneic mice injected with J558L cells cotransfected with both IL-4 and B7.1, whereas only 73-82% mice injected with tumour cells transfected with either IL-4 or B7.1, were

protected. Other studies investigating the role of IL-4 in tumour immunotherapy studies are described in more detail later.

4.1.3 Interleukin-5

Murine IL-5 was originally discovered as a soluble T cell-derived factor, called T cell replacing factor (TRF); that induced T cell-depleted, activated B cells to secrete immunoglobulin (Schimpl et al., 1972; Takatsu et al., 1980, 1980a). Subsequently, IL-5 was also independently discovered as a B cell growth factor (BCGFII) that exhibited growth promoting activity on dextran sulphate-stimulated normal B cells and the murine leukemic B cell line, BCL1 (Swain et al., 1982), and as a T cell-derived eosinophil differentiation factor (EDF) that promotes the proliferation and differentiation of eosinophils (Sanderson et al., 1986). Protein purification and molecular cloning and expression studies eventually established that a single protein, renamed IL-5, was responsible for all these independently discovered activities (Yokota et al., 1987; Kinashi et al., 1986). Human IL-5 is a potent eosinophil differentiation and activation factor, but is reported to have little or no TRF or BCGFII factors towards human B cells (Clutterbuck et al., 1987).

IL-5 is a glycosylated, disulfide-linked homodimer with an apparent molecular mass of 45kDa. It causes B-cell activation, growth and differentiation, acts as a differentiation factor and regulator for eosinophils and is involved in B cell growth and antibody production (IgA, IgM and IgE). Cells known to express IL-5 include activated $CD4^+$ T cells, eosinophils, and mast cells (Van Strattan et al., 1994; Dubucquoi et al., 1994; Plaut et al., 1989). IL-5 exerts its activity on target cells by binding to specific cell surface receptors. Whereas only a single class of high affinity receptors for IL-5 is detected on human eosinophils or eosinophilic lines (Plaetinck et al., 1990; Ingley et al., 1991) both high and low affinity receptors for IL-5 have been detected on murine IL-5 dependent B cell lines (Mita et al., 1989). The IL-5 high affinity receptor is composed of at least two membrane bound proteins, an α-chain (p60) that binds murine IL-5 independently with low affinity and a β-chain (p130) that does not bind murine IL-5 by itself, but associates with the murine IL-5R α-chain to generate the high affinity receptor complex and transduce the IL-5 signal (Takaki et al., 1990, 1990a; Devos et al., 1991).

4.1.4 Interleukin-6

Il-6 is a terminal differentiation factor for B cells, a hybridoma growth factor and pro-inflammatory factor (39). IL-6 is a glycoprotein with a molecular weight of between 21-28kD and is synthesised by a number of cells including fibroblasts, T cells, macrophages, endothelial cells, keratinocytes and mast cells. IL-6 has many biological functions including acting as a differentiation factor for B cells, a growth factor for B cell hybridomas and a role in the activation of T cells (Van Snick 1990). IL-6 plays an active role in stimulating the proliferation of mature $CD8^+$ T cells and cytolytic T cell responses (Lotz 1988) and for these reasons has shown potent antitumour activity when transfected into several tumour models (Mullen 1992, Porgador 1992, Tanaka 1997, Mullen 1996).

The IL-6 receptor is found on both lymphoid and non-lymphoid cells although expression may depend upon the state of differentiation or activation of the cells e.g. normal resting B cells do not express IL-6Rs while activated B blasts do. Like the IL-2, IL-3 and IL-5 receptors, the IL-6 receptor consists of at least two chains - an α-chain of molecular weight 80kDa and a β–chain of 130 kDa (Shrader et al., 1988). The α-chain has features of the hematopoietin family with an amino-terminal loop characteristic of the immunoglobulin supergene family and a small intracellular domain (Yamasaki et al., 1988). In contrast, the β–chain is a member of the hematopoietin receptor family that has a lrage intracellular domain involved in signalling. The β–chain does not bind IL-6 unless the α–chain is present (Taga et al., 1989).

4.1.5 Interleukin-7

IL-7 is a 25kDa glycoprotein that was originally isolated from a murine bone marrow stromal cell line (Namen et al., 1988). Murine and human IL-7 are 81% homologous at the DNA level and 60% homologous at the amino acid sequence level (Goodwin et al., 1989). Receptors for IL-7 have been found on myeloid cells, macrophages and monocytes (Park et al., 1990; Alderson et al., 1991). IL-7 was first described as having a proliferative effect on B cell precursors *in vitro* (Namen et al., 1988), and has since been shown to stimulate the growth of pre-B cells, thymocytes, and mature T cells and enhances the generation of CTL and LAK cells (Namen et al., 1988; Morrissey et al., 1989; Grabstein et al., 1990; Armitage et al., 1990; Chazen et al., 1989, Alderson et al., 1990; Lynch and Miller 1990; Park et al., 1990).

Numerous *in vivo* studies have shown the therapeutic effects IL-7 exerts by increasing $CD8^+$ T cell responses (Lynch et al., 1994; Komschiles et al., 1994). Studies examining VVIL-7 have shown enhanced T cell proliferation during viral infection whereby both $CD4^+$ and $CD8^+$ T lymphocytes are increased 2-3 fold, and both antigen-specific and antigen-non-specific immune activity characterised by the production of anti-viral CTL, natural killer cells and lymphokine activated killer cells is enhanced (Leong et al., 1997). In tumour immunotherapy studies, recombinant IL-7 increased the number of B and T cells in tumour bearing mice and significantly reduced the number of pulmonary metastases in mice challenged with Renca renal carcinoma cells (Komschlies et al., 1994). In a separate study, IL-7 and B7.1 induced antitumour immunity when delivered to mammary adenocarcinoma bearing hosts and strong protective immunity following a rechallenge with tumour cells (Cayeux et al., 1995).

4.1.6 Interleukin-10

IL-10 was initially designated cytokine synthesis inhibitory factor (CSIF) and was identified as a product of murine T2 clones that suppressed the production of cytokines by T1 clones responding to stimulation by antigen-presenting cells (Fiorentino et al., 1989). In the mouse, IL-10 is produced by T2 cells, activated foetal thymocytes, macrophages, keratinocytes, $LY\text{-}1^+$ ($CD5^+$) and normal B cells (Moore et al., 1993) and exerts its biological functions by down-regulating MHC Class II and costimulatory molecules (like B7) at the surface of monocytes, macrophages, Langerhan cells and dendritic cells, reducing both the ability of these cells to present antigen and to provide costimulatory signals to $CD4^+$ lymphocytes (de Vries 1995). IL-10 has also been shown to inhibit the release of cytokines such as TNF-α, IL-1, and IL-12 by activated macrophages (Moore et al., 1993; Scott 1993; Nabioullin et al., 1994). The IL-10 receptor is a 110kDa receptor protein that has a four α-helix structure characteristic of cytokines binding to hematopoietin receptors, with the receptor most closely resembling the receptor for IFN-γ (Shanafelt et al., 1991; Sato and Miyajima, 1994). The IL-10R binds IL-10 with a high affinity (K_d=70 pM) (Ho et al., 1993).

Interleukin-10 (IL-10) has a wide range of *in vivo* biological activities and is a key regulatory cytokine of immune-mediated inflammation. Murine IL-10 given 12 hours after a recombinant vaccinia virus (rVV) containing the LacZ gene significantly enhances the treatment of mice bearing 3-day-old pulmonary metastases expressing beta-galactosidase.

Although IL-10 did not alter levels of anti-vaccinia antibodies or natural killer cell activity, rVV-primed mice treated with IL-10 had enhanced vaccinia-specific cytotoxic T-lymphocyte activity (Kaufman et al., 1999). Despite IL-10 being a key cytokine in the down-regulation of the T1 cellular immune response, IL-10 also has demonstrated antitumour capabilities in other murine model systems characterised by the generation of tumour-suppressing cytotoxic T cells and in some cases, the establishment of immunological memory (Giovarelli 1995, Gérard 1996). In the B16F1 melanoma model, tumour cells transfected with recombinant IL-10 and injected into mice surprisingly resulted in a loss of tumourigenicity that was proportional to the amount of IL-10 being secreted. This was an unusual finding because of IL-10's strong T2 influence, yet the response was certainly T cell and NK cell mediated as the antitumour response was abolished in nude mice (Gérard 1996).

4.1.7 Interleukin-12

IL-12, formerly termed natural killer cell-stimulatory factor (Kobayashi et al., 1989), or cytotoxic lymphocyte maturation factor (Stern et al., 1990) is a disulfide-linked heterodimeric cytokine composed of a 35kDa light chain (p35) and a 40kDa heavy chain (p40) (Kobayashi et al., 1989; Stern et al., 1990), both being necessary for the induction of biologically active IL-12 (3,4). IL-12 is produced by macrophages in response to infection with various organisms (Hendrzak and Brunda, 1995). IL-12 also has potent biological effects inducing IFN-γ production from NK and T cells (Kobayashi et al., 1989; Chan et al., 1991; Wolf et al., 1991), enhancing cytotoxic T cell function and proliferation of NK and activated T cells (Wong et al., 1988; Gately et al., 1992 and 1994), and promoting T1 cytokine responses (Kennedy et al., 1994). Importantly, IL-12 also conditions naive helper cells to differentiate to the T1 pathway (Hsieh et al., 1993; Seder et al., 1993; Manetti et al., 1993) and stimulates T2 cells to transiently express IFN-γ (Manetti et al., 1994).

Recombinant IL-12 has significant therapeutic effects when administered either locally or systemically, or when delivered by viral vectors or gene transfer. IL-12 exerts its antitumour and antimetastatic effects in multiple murine tumour models including, melanoma (Brunda et al., 1993; Tahara et al., 1994), M5076 reticulum cell sarcoma (Brunda et al., 1993), Renca renal cell adenocarcinoma (Brunda et al., 1993), the MCA207 and MCA105 sarcomas (Tahara et al., 1995, Tahara and Lotze, 1995; Nastala et al., 1994), Meth A sarcoma (Noguchi et al.,

1995), MC-38 adenocarcinoma (Nastala et al., 1994), B cell lymphoma (O'Toole et al., 1993), colon carcinoma (Caruso et al., 1996) and Lewis lung carcinoma (Stern et al., 1994). Although IL-12 possesses the most potent single-cytokine antitumour efficacy, the mechanism by which IL-12 exerts its antitumour activities, was unclear until a study by Tsung et al in 1997. In a tumour regression model induced by IL-12 treatment, the antitumour response was shown to be mediated by a T1 directed process, with macrophages as the effector cells and nitric oxide produced by the activated macrophages as the effector molecule. Associated with the induction of tumour necrosis, activated macrophages expressing high levels of inducible nitric oxide synthetase were found surrounding the tumour. The importance of nitric oxide as the effector molecule was further confirmed by the delay and loss of tumour regression in the presence of a nitric oxide synthetase inhibitor *in vivo* (Tsung et al., 1997). These findings demonstrate that IL-12 plays an essential role in the induction of an effective T1 type cell-mediated immune response against established tumours through the production of nitric oxide from macrophages.

4.1.8 TNF-α

TNF-α is a 17kD cytokine produced by activated macrophages and is predominantly referred to as an inflammatory cytokine that promotes margination of leukocytes at inflammatory sites, cytotoxic effects on tumour cells and exerts little direct effect on T lymphocytes (Beautler and Cerami, 1989; Bemelmans 1996). TNF-α has been shown to have a wide range of biological activities such as cytotoxicity and activation of cellular genes, kinases and transcription factors (Vilcek and Lee 1991). These effects are mediated by two TNF receptors with molecular weights of 55kDa and 75kDa (Tartaglia et al., 1991). Cytotoxicity effects are thought to occur via binding to the 55kDa receptor followed by internalisation and degradation of the ligand in the lysosomes (Larrick and Wright 1990; Fruehauf and Sinha 1992).

In vivo antitumour activity is due to the direct cytotoxic action of TNF on tumour cells and on vascular endothelial cells leading to an increase in procoagulant activity and increased adhesion of neutrophils, monocytes and lymphocytes to endothelial cells (Shimomura et al., 1988). TNF-α has demonstrated antitumour capabilities in murine model systems characterised by the generation of tumour-suppressing cytotoxic T cells and in some cases, the establishment of immunological memory (Blankenstein 1991, Teng 1991, Tepper 1994). Yet

despite impressive antitumour effects in mice, TNF-α trials in humans have been disappointing due to its severe side-effects (Kemeny et al., 1990).

4.1.9 Interferons

Interferons (IFN) are inducible proteins which are important not only in defence against a wide range of viruses but also in the regulation of immune responses in hematopoietic cell development. IFN-α genes encode proteins of 165-172 amino acids in length (Langer and Pestka 1985) while IFN-β genes encode a 183 amino acid protein which is only 30% homologous to IFN-α at the amino acid level. Both IFN-α and IFN-β share a common receptor (Langer and Pestka 1988), and are produced by most cells in response to viral infection or stimulation with natural or synthetic double-stranded RNA. They have potent antiviral activity and at a higher concentration have antitumour effects, especially IFN-α which has been extensively tested in phase I, II and II human clinical trials (Spiegel et al., 1987).

IFN-γ is a multifunctional protein first observed to have antiviral activity in cultures of human leukocyte cultures inoculated with Newcastle disease virus (Wheelock 1966). Produced by both $CD4^+$ and $CD8^+$ lymphocytes and NK cells, IFN-γ is now known to be both an inhibitor of viral replication and regulator of numerous immunological functions. IFN-γ regulates the induction of cytolytic T cells, increases natural killer cell activity, increases the antigen presenting capacities of macrophages and promotes macrophage tumouricidal activity (Hayashi 1985, Weigent 1983, Birmingham 1982, Pace 1983). Due to its role in CTL induction and tumouricidal capabilities, IFN-γ gene transfer has been used to exert its antitumour effects in many mouse tumours models (Matory 1995, Watanabe 1989, Gansbacher 1990).

4.1.10 GM-CSF

Granulocyte macrophage colony stimulating factor (GM-CSF) belongs to a family of colony stimulating factors that regulates the survival, growth and differentiation of hematopoietic progenitor cells. GM-CSF induces the proliferation of macrophages (Metcalf 1985) and enhances T cell immune responses by potently recruiting and activating antigen presenting cells (Grabe 1991, Tazi 1993, Cohen 1994). GM-CSF also activates dendritic antigen-presenting cells derived from epidermis (Langerhans cells), bone marrow, and peripheral

blood cells to present a wide variety of antigens, including tumour-associated antigens, for various immune responses. The development and function of dendritic cells is dependent upon a number of cytokines including GM-CSF. For example, Langerhans cells can present tumour-associated antigens for the induction of substantial *in vivo* anti-tumour immunity but only after activation *in vitro* by GM-CSF (Ozawa et al., 1999). GM-CSF has been shown to produce antitumour responses in several tumour models (Armstrong 1996, Dranoff 1993, Saito 1994) including in a murine breast cancer model, C3HBA, where mice immunised with tumour cells infected with VV-GM-CSF were completely protected against a challenge of parental tumour cells and mice not infected with VV-GM-CSF were not (Peplinski 1996).

4.2 The T1 / T2 paradigm

Functionally different subsets of $CD4^+$ and $CD8^+$ cells have important regulatory and effector roles in immune responses. The subdivision of T helper cells into two classes (Th1 and Th2) was originally described for murine $CD4^+$ T cell cultures (Mosmann et al., 1986; Cherwinski et al., 1987) and later among human T cells (Del Prete et al., 1991). Based on the cytokine synthesis patterns of long term clones, $CD4^+$ T cells were classified into Th1(or T1), which secreted IL-2, IFN-γ and TNF-β and the Th2 (or T2) which secreted, IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and Sad, 1996). Several other cytokines are secreted by both T1 and T2 cells including IL-3, TNF-α and GM-CSF. A third subset of cytokines also exist, these are T cells that express both cytokines patterns and are called Th0 cells (Mosmann and Coffman, 1989). Th0 cells represent a heterogenous population of partially differentiated effector cells comprising multiple discrete subsets that can secrete both T1 and T2 cytokines (Romagnani 1996). A fourth set of cells secreting large amounts of TGF-β have been termed Th3 (Chen et al., 1994). Distinct cytokine-secreting subsets of $CD8^+$ T cells have also been subdivided into subsets similar to their $CD4^+$ counterparts (Salgame, 1991). TC1 cells secrete IL-2 and IFN-γ associated with cellular immune responses and TC2 cells secrete IL-4, IL-5, IL-6, and IL-10 associated with humoral immune responses (Salgame, 1991).

Recently however, studies have emerged demonstrating that a rigid T1/T2 subdivision does not occur for some murine and human T cell clones (Paliard 1988; Kelso 1998). What does remain clear however, are the functions associated with the cytokines. Most T cells functions are mediated by cytokines which are secreted upon their stimulation. The

distinctive cytokine profiles of the two classes are believed to be associated with these distinctive functions. The T1 cells are primarily involved in cytotoxic (CD8) and inflammatory (CD4) responses (Mosmann and Coffman, 1989). T1 cells, through their production of IFN-γ and TNF-β, induce delayed-type hypersensitivity and microbicidal activity in macrophages (cellular immunity) (Yamamura et al., 1991; Tsicopoulos et al., 1992). Some B-cell help can be provided by T1 cells, but at higher T1-cell numbers, this can become suppressive (Coffman et al., 1988; Del Prete et al., 1991a). T2 cells secrete cytokines that help B cells become antibody-producing cells (humoral immunity), particulary IgE responses, and also enhance eosinophil proliferation and function in allergy responses (Mosmann and Coffman, 1989).

The characteristic cytokine products of T1 and T2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype. Therefore IFN-γ and IL-12 selectively inhibits the proliferation of T2 cells (Mosmann and Coffman, 1989; Romagnani 1997), and IL-10 inhibits cytokine synthesis by T1 cells (Fiorentino et al., 1989). This cross regulation may partly explain the strong biases towards T1 or T2 responses seen in some tumour immunotherapy studies with cytokines. The mannan-MUC1 tumour model is an excellent example of how selective immunisation with an antigen can stimulate both the T1 and T2 cytokine pathways simultaneously or independently (Lofthouse et al 1997). However in this model, while the concentration of antigen used is critical to the immune response generated (Pietersz et al., 1998), even with the same amount of antigen, the immune response can be manipulated to produce either a T1 or T2 response depending on the choice of either oxidised or reduced mannan-MUC1 as the immunogen (Apostolopoulos et al., 1995a, 1996). This unique ability of being able to select the desired immune response and specifically induce antitumour cytotoxic T cells has long been a goal of tumour immunotherapy studies. T1 cytokines have been incorporated into many immunotherapy strategies in an attempt to bias the immune response towards a cellular or cytotoxic response. Interestingly, the T2 cytokines IL-4 and IL-10 have also been shown to exhibit antitumour characteristics in some tumour models (Golumbek et al., 1991; Tepper et al., 1989; Giovarelli et al., 1995), therefore widening the choice of application for T1 and T2 cytokines in tumour immunotherapy studies. The following section reviews the use of both T1 and T2 cytokines in antitumour immune responses.

4.3 Cytokine delivery mechanisms

The most straight forward approach to tumour immunotherapy with cytokines involves the systemic delivery of recombinant cytokines to tumour bearing hosts. This method of cytokine delivery has been studied in several tumour models described in more detail below, and has met with some success with some studies advancing into human clinical trials. There are however problems associated with this delivery mechanism which have been overcome using cytokine gene transfection. Preclinical studies have shown that *in vitro* cytokine gene transfection of tumour cells and their use as vaccines result in the enhanced development of antitumour immunity and in some cases can be used to successfully treat pre-existing tumours. Other studies have explored the use of vector delivery systems such as avipox virus, adenovirus and vaccinia virus recombinants to directly transfect tumour cells in situ with cytokine genes as a strategy for enhancing the development of antitumour immunity. All of these mechanisms are described in more detail below.

4.3.1 Recombinant cytokines

Many cytokines can mediate antitumour activity *in vitro* and *in vivo* (Pardoll, 1993). For example, interleukin 2 has been demonstrated to possess antitumour activity through its ability to stimulate the cell-mediated killing activity of CTLs (Fearon et al., 1990), to induce LAK cells (Lotze et al., 1981; Grimm et al., 1982) and to activate tumour-infiltrating lymphocytes (TILs) (Cameron et al., 1990; Lindgren 1993). Systemic delivery of cytokines has been used in animal models and in the clinic and has met with some success. However the short-life of cytokines in serum requires repeated high doses, which for IL-2, induces severe side-effects including vascular leak syndrome, oedema, anaemia, fevers and chills, nausea and hypotension (Siegal and Puri, 1991). To avoid these problems, the local delivery of low-dose cytokines has been investigated in many animal models and has met with antitumour immunity resulting in increased survival and reduced tumour growth without the side effects of the high dosing regimens (Yeung et al., 1992; Tohmatsu et al., 1993). This section reviews some of the many preclinical tumour immunotherapy experiments with recombinant cytokines and looks specifically at regimens which advanced into human clinical trials.

Preclinical cancer gene therapy with recombinant-cytokines

The T1 cytokines IL-2, IFN-γ, IL-12, and TNF-β (lymphotoxin-α) have generally been favoured for use in tumour immunotherapy as their production results in the development of a

cellular immune response and tumour protection in many models (Mosmann 1986, 1989, Cherwinski 1987, Salgame 1991). Of the T1 cytokines studied, IL-2 has been used extensively in murine (Bubenik 1990, Fakhari 1995, Fearon 1990, Mule 1984) and human (Rosenberg 1987) tumour models because it is able to both increase T cell proliferation and recruitment to tumour sites and to induce antitumour immune responses. In a murine colon cancer hepatic metastases model, intrasplenic injection of tumour cells with vaccinia colon oncolysate (VCO) and low-dose interleukin-2 (IL-2) therapy, demonstrated consistently better survival (75% survival) than mice treated with IL-2 alone or controls. *In vitro* assays revealed enhanced NK activity and suggested CTL induction as possible mechanisms responsible for these biologic effects, demonstrating the potent effects of this immunoregulatory cytokine (Barnavon 1988).

Similarly, IL-2 administration enhanced the therapeutic effect of a single immunisation of a recombinant vaccinia virus-carcinoembryonic antigen (rV-CEA) vaccine in an experimental murine model system. A single rV-CEA immunisation of mice bearing palpable CEA-positive tumours resulted in complete tumour regression in approximately 20% of the mice. The addition of a course of low-dose IL-2 resulted in complete tumour regression in 60-70% of the mice. Moreover, the combination of rV-CEA and IL-2 induced systemic immunity, which protected those tumour-free mice from subsequent rechallenge with CEA-expressing tumour cells. The enhanced CEA-specific immune response, coupled with the improved experimental therapeutic outcome following IL-2 administration, suggested that treatment with recombinant IL-2 cytokine may effectively substitute for multiple rV-CEA immunisations (McLaughlin et al., 1996).

IFN-γ has also proven to be an effective anti-tumour cytokine inducing tumour regression and protection, indicated by CTL induction in tumour models including fibrosarcomas (Gansbacher 1990a), mammary adenocarcinomas (Lollini 1993), and lung and colon carcinomas (Esumi 1991, Porgador 1993). More recently, IL-12 has been much favoured as it has shown therapeutic effects in tumour-bearing animals (Nastala 1994, Noguchi 1995, reviewed by Tahara 1995). Owing to the promising results of IL-12 gene therapy, clinical trials are now in progress (Leonard 1997).

Many of the T2 cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann 1986, 1989, Cherwinski 1987, Salgame 1991) known for their involvement in humoral responses, have

also been studied for their effect on tumourigenicity and found to be effective in many tumour models. IL-4, which is commonly known to exert its biological effects on the proliferation, activation and differentiation of B cells has also been shown to play an important role in T cell tumour immunity (Brown 1997), and has been engineered into tumour cells to treat various cancers including renal carcinoma (Golumbek 1991, Tepper 1989). IL-6, a predominant B cell growth factor, also plays a role in tumour protection when engineered into fibrosarcoma or Lewis lung carcinoma cells (Mullen 1992, Porgador 1992). GM-CSF and TNF-α have also demonstrated antitumour immune responses when administered in several tumour models. GM-CSF belongs to a family of cytokines which regulate the growth, survival and differentiation of haematopoietic progenitor cells. When transfected into melanoma (Armstrong 1996, Dranoff 1993), bladder cancer (Saito 1994), and leukeamia (Hsieh 1997) models, GM-CSF induces potent anti-tumour immunity. Although toxic in high doses, TNF-α has also been used in tumour models to show regression of autologous tumours (Blankenstein 1991, Teng 1991).

The antitumour effects of more recently discovered cytokines have also been examined. In a murine tumour model for pulmonary metastases, IL-15 was found to be half as potent as IL-2 in suppressing metastases (Munger et al, 1995) and therapeutically was able to prolong the survival of mice bearing lymphomas after syngeneic bone marrow transplantation (Katsanis et al, 1996). IL-15 has also been shown to induce human killer cell activity from PBMCs against a variety of lung cancer cell lines (Takeuchi et al, 1996). IL-18, a potent inducer of IFN-γ, has also exhibited antitumour effects when recombinant cytokine is delivered *in vivo*. Mice injected with IL-18 on days one, two and three after a challenge with Meth A sarcoma were completely protected against the tumour, and induced cytotoxic activity augmented by $CD4^+$ T cells (Sanchez-Beuno et al., 1996; Micallef et al, 1997).

Human clinical trials with recombinant- cytokines

Recombinant cytokines have been used in clinical trials in cancer patients and also form part of the treatment regime for specific tumours. The findings that leucocytes incubated with IL-2 gave rise to cytotoxic cells capable of killing tumour cells but not normal cells (Grimm et al., 1982) prompted both laboratory investigation and application to human tumour therapy. LAK cells have been shown to be effective in mediating regression of lung and liver metastases in various murine models (Mule et al., 1984). Although IL-2 alone in very large amounts was shown to affect these tumours, the injection of IL-2 with LAK cells proved to be the most

beneficial. Laboratory studies led to clinical trials in which LAK cells were administered with IL-2 in humans (Rosenberg et al., 1985). Patients' leucocytes were incubated *in vitro* with IL-2 and then re-administered with IL-2. Significant tumour regressions were reported although there was also significant toxicity associated with the treatment. The IL-2 gave rise to leaky capillaries and this resulted in fluid retention which caused dose-limiting toxicity. Much of the toxicity probably arose from the induction of other cytokines such as TNF. Of the tumours that regressed, the most significant and long-lasting changes were seen for renal cell carcinoma and melanoma. IL-2 or IL-2 and LAK cells seem to show consistent benefit in 10-20% of patients with these two tumours (Smith et al., 1991).

Other potential agents for tumour therapy are the interferons IFN-α and IFN-γ, TNF and GM-CSF. IFN-γ is immunomodulatory and has been shown to cause macrophage activation in clinical trials (Nathan et al., 1985) but has not shown consistent activity in any malignancies although trials still continue. In contrast, IFN-α has been reported to be effective in treating hairy cell leukaemia (Thompson and Fefer, 1987), Kaposi's sarcomachronic (Volberding et al., 1987), myeloid leukaemia (Talpaz et al., 1987), and myeloma (Castanzi et al., 1985). TNF has also been studied and although extremely toxic when administered systematically, local administration by perfusion of isolated limbs affected by melanoma and in the treatment of ascites from ovarian cancer may be effective (Lejeune et al., 1994). In a more recent clinical trial based on the success of GM-CSF in gene transfer experiments, patients with solid tumour malignant mesothelioma were given continuous intratumoural injections of GM-CSF over 8 weeks. Two patients demonstrated tumour necrosis, one of whom had a partial response (>50% reduction in tumour area) (Robinson et al., 1998).

4.3.2 Transfection into tumours

While the systemic administration of recombinant cytokines have clearly shown to be therapeutic in many tumour models, there are severe side-effects associated with this kind of therapy. As a more effective method for achieving high intratumoural cytokine concentrations, scientists have focused their attention on the direct introduction of exogenous genes into tumours. While the use of this type of immunotherapy in patients may be only limited to solid tumours accessible by surgery, undoubtedly, it provides an effective therapeutic approach for the delivery of localised cytokines to tumours, without the side-effects of systemic delivery.

The transfection of tumour cells with cDNA coding for cytokines will produce those cytokines when the cells are injected into experimental animals. In some cases, such as with tumours producing IL-1, IL-2, IL-4, IL-6, IL-7, IL-12, IFN, TNF, G-CSF or GM-CSF, tumours are subsequently rejected often with the development of tumour specific immunity. Therefore if the same tumour cells, not secreting cytokines, are reimplanted the tumour is rejected and immunological memory established (Doudevani et al., 1992; Fearon et al., 1990; Gansbacher et al., 1990; Ley et al., 1991; Tepper et al., 1989; Porgador et al., 1992; Hock et al., 1991; Nastala et al., 1994; Aoki et al., 1992; Karp et al., 1992; Colombo et al., 1992; Restifo et al., 1992; Watanabe et al., 1989; Dranoff et al., 1993). Cytokines transfected into tumours have produced such antitumour immunity in many tumour models including neuroblastoma (Corrias et al., 1998), fibrosarcoma (Patel et al., 1993), and colorectal carcinoma (Shawler et al., 1995).

While the transfection of cytokine cDNA into tumour models was a popular approach to cytokine delivery in the early 1990's, the advent of viral vector delivery mechanisms meant scientists quickly began focussing their attention to these new delivery systems to increase cytokine concentrations in tumour immunotherapy studies. These experiments are described in detail below.

4.3.3 Viral vectors

Viral vectors have strong potential as vehicles for the targeted delivery of cytokines into tumour cells *in vivo*. Unlike the delivery of systemic recombinant cytokines, viral vectors do not induce severe side effects (although weak anti-viral immune responses can be induced) and they are able to continually express cytokines within or around the tumour. Candidate viruses for cytokine delivery include retroviruses, adenoviruses and poxviruses. Transduction of tumour cells with retroviral vectors expressing cytokines, in particular IL-2, have been shown to reduce the tumourigenicity and metastatic potential of B16 melanoma (Karp et al., 1993), CMS-5 fibrosarcoma (Gansbacher et al., 1990; Bannerji et al., 1994) and the MBT-2 bladder carcinoma (Conner et al., 1993). However, certain problems associated with the use of retroviral vectors (e.g low titres and stability) have resulted in many scientists focussing on the use of avipox, adenovirus and vaccinia viruses as vectors for cytokine delivery. The use of each of these viruses in tumour immunotherapy studies is described in more detail below.

a) Avipox

Despite their use as delivery vectors for antigens such as HIV (Kent et al., 2000) and CEA (Marshall et al 1999), there are no reports of avipox vector being used to deliver cytokines in tumour immunotherapy studies. In a Listeria model comparing the use of vaccinia and fowl pox delivery vectors, vaccinia virus was found to be more efficient at delivering IFN-gamma-mediated protection than the fowlpox virus, which was unable to proliferate in mammalian cells (Cheers et al., 1999).

There are however studies which have utilised the avipox vectors to deliver IL-6, IL-10 and IFN-γ to various *in vivo* models. Recombinant fowlpox virus expressing influenza virus hemagglutinin and interleukin-6 augmented both primary systemic and mucosal antibody responses whereas the expression of interferon-γ, markedly inhibited antibody responses without affecting the generation of cell-mediated immunity (Leong et al., 1994). Similarly, the expression of the gene for IL-6 in recombinant fowlpox vectors markedly increased IgA reactivity to co-expressed heterologous antigen in the lungs of mice inoculated intranasally with the viruses (Ramsay et al., 1994). Recombinant fowl pox viruses co-expressing chicken type I interferon and/or hemagglutinin-neuraminidase and fusion proteins of Newcastle disease virus (NVD), produce less antibodies against NDV in comparison with vaccinated chickens (Karaca et al., 1998). In another NVD model in turkeys, fowlpox vectors delivering chicken type I interferon (IFN) or type II IFN, protect against a challenge with wild-type NVD virus (Rautenschlein et al., 1999). Complex recombinant fowlpoxvirus vaccines expressing both HIV-1 antigens and human interferon-gamma can facilitate the induction of cellular immunity against HIV-1 through the enhancement of cellular immune responses to the Gag antigen on HIV (Kent et al., 2000).

b) Adenovirus

Adenoviral vectors have also been used to deliver recombinant cytokines to tumour-bearing hosts as another major immunotherapeutic strategy to combat cancers. One of the earliest reports of cytokine gene transfer into tumour cells using the replication-defective adenovirus vector used the murine mastocytoma cell line P815, and evaluated the effect of interleukin-2 (IL-2) gene transfer on its tumourigenic capacity (Haddada et al. 1993). The study showed that P815 cells infected with recombinant adenovirus secreted significant amounts of

biologically active IL-2, and furthermore when injected into syngeneic DBA/2 mice, the tumourigenic phenotype is lost in up to 80% of the animals. Moreover, protected animals developed a long-lasting state of immunisation against the P815 tumour cells and their splenocytes were able to transfer the immunity to syngeneic naive recipients (Haddada et al. 1993).

Adenoviral vectors are very attractive for clinical use as they have the ability to produce large quantities of purified virus with relative ease. Moreover, for cancer immunotherapy studies, adenovirus vectors have many other features including a deletion of the early region 1 genes rendering the virus replication deficient, the ability to carry up to 8kb of foreign DNA (Bett et al., 1994), the ability to infect a wide variety of replicating and nonreplicating cell types, and most importantly, adenoviral DNA does not integrate into the infected cell genome and thus expression of the foreign gene product (or cytokine) is typically transient. For cytokine gene therapy, this means that the introduction of an adenovirus containing a cytokine gene will not result in chronic stimulation of the immune system. The use of adenoviruses to deliver cytokines in tumour immunotherapy studies have been extensively investigated and are described in more detail below.

Preclinical cancer gene therapy with adenovirus- cytokines

Adenoviral-mediated gene transfer of IL-2 has also proven to be very successful at inducing tumour reactive immune responses, tumour regression and indeed immunity in many other *in vivo* tumour models. These include hepatocellular carcinoma (Huang et al., 1996; Bui et al., 1997), medullary thyroid carcinoma (Zhang et al 1998 and 1998a), head and neck carcinoma (O'Malley et al., 1997), murine mastocytoma (Cordier et al., 1995) and murine breast cancer (Addison et al., 1995). Similarly, adenoviral vectors used to transduce freshly isolated human neuroblastoma cells with IL-2 do so at a far greater efficiency than retroviral vectors, and when cultured with patient lymphocytes, increase the proportion of T cells and generate MHC unrestricted cytotoxic effector cells active against parental (nontransduced) tumour cells (Leimig et al., 1996). Human lung adenocarcinoma cells transduced with IL-2 also result in enhanced cytokine secretion from gene-modified cells for 8 -days after the transfection which was successful at eradicating pre-existing lung tumours with malignant pleural effusions but not pre-existing subcutaneous tumours (Heike et al., 1997).

Other strategies to induce tumour immunity using cytokines and adenoviral vectors include combination gene therapy and adoptive transfer. The efficacy of combination gene therapy using adenovirus vectors expressing human IL-2 and the wild-type human p53 gene was examined in a transgenic mouse mammary adenocarcinoma model (Putzer et al. 1998). Following a single intratumoural injection of mice, tumour regression was observed in 65% of the mice suggesting that cancer treatment strategies involving combined delivery of immunomodulatory and antiproliferative genes may be highly effective (Putzer et al. 1998). In an adoptive immunotherapy study, murine tumour-specific CTL were transduced using an adenovirus expressing IL-2 to successfully reduce tumour metastasis and produce longer survival from intracerebral tumour death (Nakamura et al., 1994).

Other adenoviral transduced cytokines have also been used to inhibit tumour growth and induce immunity in cancer immunotherapy studies. IL-4 has demonstrated therapeutic value in a rat glioma model (Wei et al., 1998) and the administration of adenoviral vectors expressing IL-6 induce $CD8^+$ human CTLs specific for autologous human tumour cells and inhibits the growth and metastasis of autologous human cancers in a SCID mouse model (Tanaka et al., 1997). Intratumoural administration of adenoviral IL-7 gene modified dendritic cells augments specific antitumour immunity and achieves tumour eradication in two murine lung cancer models (Miller et al., 2000).

IL-12 has been used extensively in adenoviral gene transfer studies to induce the regression of colorectal carcinomas (Caruso et al., 1996; Mazzolini et al., 1999), to induce NK cell activity in the treatment of a melanoma lung metastases (Hirschowitz and Crystal 1999) and to eradicate murine bladder carcinoma (Chen et al., 1997), murine adenocarcinomas and murine fibrosarcomas (Gambotto et al. 1999). Adenoviral gene transfer of IL-12 has also been used in combination with the costimulatory molecule B7-1 (Putzer et al., 1997) and IL-2 (Addison et al., 1998) to facilitate tumour regression.

The most recently described cytokine, IL-18, has also been used in adenoviral gene transfer immunotherapy studies. Retroviral and adenoviral vectors encoding murine IL-18 were injected directly into an established MCA205 murine fibrosarcoma and completely eradicated the tumours in all animals with concomitant induction of protective systemic immunity. Co-administration of systemic IL-12 provided synergistic antitumour effects when combined with peritumoural injections of IL-18 without apparent side-effects. The anti-tumour effects

observed were mediated by NK cells and interestingly, IL-18 reduced the numbers of $CD8^+$ cells found within the tumour (Osaki et al., 1999).

The anti-tumour activities of interferon-gamma (IFN-γ) have been well described (Matory 1995, Watanabe 1989, Gansbacher 1990). Adenoviral gene transfer of IFN-γ in several tumour models has also induced potent antitumour immune responses *in vivo*. Treatment of a human breast cancer cell line (MDA-MB-435) in nude mice with a recombinant adenovirus containing human IFN resulted in tumour regression in 100% of the animals (Zhang et al., 1996). Interestingly however, control mice immunised with only wild-type adenovirus also partially rejected the tumours indicating that viral oncolysis occurred whereby tumour cells were selectively lysed by the replication-competent virus and the enhanced effect of IFN expression. Adenoviral gene transfer of IFN-γ has also shown therapeutic effects in an adult T cell leukaemia model (Xu et al., 1996) and IFN-β has demonstrated antitumour activity when transferred into a murine fibrosarcoma model (Lu et al., 1999).

Irradiated tumour cells genetically modified to secrete granulocyte/macrophage-colony-stimulating factor (GM-CSF) are potent stimulators of systemic antitumour immunity. Adenoviral transfer of GM-CSF effects the local and systemic expansion of haematopoietic cells and in particular, dendritic cells, a powerful antigen-presenting cell (Burger et al., 2000). The introduction of GM-CSF cDNA into dendritic cells for use in tumour immunotherapy studies results in the release of abundant quantities of GM-CSF, a greater expression of MHC class II molecules and B7. Most importantly, GM-CSF gene-transferred epidermal cells present tumour-associated antigens and induces anti-tumour immunity, demonstrating that GM-CSF gene-transferred DC-cells are useful in tumour vaccination strategies (Ozawa et al., 1999). The use of GM-CSF gene-transferred DC cells has been taken one step further to produce a new DC-based vaccine for melanoma (Cao et al., 1999). A hybrid vaccine has been produced between the fusion of GM-CSF gene-modified DCs and B16 melanoma cells. The hybrid vaccine exhibits a higher expression of B7 when fused, elicits a specific CTL response, protects immunised mice from B16 tumour challenge, reduces pulmonary metastases and extends the survival of B16 tumour-bearing mice (Cao et al., 1999). This data indicates that GM-CSF gene-modified DCs may lead to the generation of hybrid vaccines with potentially increased therapeutic efficacy and may be an attractive strategy for future cancer immunotherapy.

Despite the successful adenoviral-mediated gene transfer of many cytokines in numerous *in vivo* tumour models, there are limitations to the use of this cytokine transfer system to develop cancer vaccines. Initially, it was hypothesised that the use of replication-deficient adenovirus for human gene therapy may be limited by host anti-vector immune responses that result in transient recombinant protein expression and blocking of gene transfer when rechallenged, however recent clinical trials indicate that this may not be a problem (Gahery-Segard et al 1997). While delayed growth and rejection of some tumours can be achieved with cumulative viral doses in two or three injections (as previously discussed), lower viral doses are ineffective at inducing tumour regression, and higher viral doses result in animal death due to toxicity (Toloza et al 1996; Marr et al., 1998). Similarly, the amount of recombinant protein expressed can effect the tumourigenic capabilities of the vaccine as evident in an oral cancer model using adenoviral IL-2 gene therapy, where intratumoural levels of IL-2 was a major factor limiting tumour regression (O'Malley et al. 1999).

Human clinical trials with adenovirus-cytokines

To date there have been two cancer immunotherapy human clinical trials studying the effect of adenoviral vectors in humans. The first trial investigated the immune responses to an adenoviral vector and to the beta-galactosidase protein in four patients with lung cancer and found that recombinant adenovirus injected directly into the tumour is a highly efficient vector for immunising patients against the transgene protein. (Gahery-Segard et al., 1997). The second trial investigated the delivery of IL-2 in patients with metastatic breast cancer and melanoma and confirmed the safety of adenoviral vectors for use in gene delivery to humans and demonstrated successful transgene expression even in the face of pre-existing immunity to adenovirus (Stewart et al., 1999).

Gahery-Segard et al examined the cytotoxic and humoral immune responses to an adenoviral vector and to the beta-galactosidase protein in four patients with lung cancer given a single intratumour injection of 10^9 plaque-forming units of recombinant adenovirus. The results showed that a high level of neutralising anti-adenovirus antibodies were detected in only one patient, and two other patients developed anti-adenovirus CTL responses. All patients developed potent CD4 type 1 helper T cell (Th1) responses to adenoviral particles which increased gradually over time after injection. Anti-beta-galactosidase IgG was observed in all patients except patient 1, and consistent with anti-beta-gal antibody production, all patients except patient 1 developed dose-dependent Th1 responses to soluble beta-galactosidase which increased over time. Strong beta-galactosidase-specific cytotoxic T lymphocyte responses

were detected in patients 2, 3, and 4. These results clearly showed that despite the intensity of anti-adenovirus responses, transgene protein expression was sufficient to induce strong and prolonged immunity in three patients (Gahery-Segard et al., 1997).

A recent phase 1 clinical trial investigated the toxicity and immune responses produced in 23 patients, with subcutaneous deposits of melanoma or breast cancer injected directly with an adenovirus encoding IL-2. Local inflammation was observed at the site of injection in 60% of patients, but side-effects were otherwise minor. Incomplete local tumour regression occurred at the site of injection in 24% of patients, but no conventional clinical responses were seen. Circulating CD4 and CD8 counts fell significantly 24 h after injection. Post-injection biopsies demonstrated tumour necrosis and lymphocytic infiltration with the predominant tumour-infiltrating cells both CD3- and CD8-positive. While IL-2 was detectable by ELISA in tumour biopsies at 48 h, no protein was detectable in injected tumours after seven days and no circulating IL-2 was detectable at any time-point. Anti-adenovirus and neutralising antibody titres were elevated one month after injection in all patients (Stewart et al., 1999).

However, the recent death of an 18-year old man, Jesse Gelsinger, who was a patient in a clinical trial at the hospital of the University of Pennsylvania, has caused great concern over the use of adenovirus vectors in humans. Gelsinger was in the highest-dose group of a clinical trial receiving escalating doses of an adenovirus carrying a gene to restore ornithine transcarbamylase (OTC)-an enzyme that, when missing, renders people unable to break down dietary protein. On the second day of the trial, Gelsinger inexplicably fell ill and lost the ability to clot blood, while toxic ammonia built up in his liver. By the third day he developed respiratory problems and on day four, died after physicians were unable to rescue him from brain and kidney failure (Smaglik 1999). The death of this man, apparently caused by the adenovirus, raises the question of safety as the number one issue in the development of all recombinant cancer therapies.

c) Vaccinia virus

In the early 1990's, the use of vaccinia virus as a successful delivery vector for proteins was extended to include the delivery of biologically active cytokines *in vivo*. Recombinant VVs were constructed that secreted IL-2, IL-4 and IL-5 from infected cells during replication. It was observed that the secreted IL-2 enhanced the hosts antiviral responses mediated by NK cells and a population with γδ T cell characteristics, allowing athymic nude mice to resolve

the viral infection (Ramshaw et al., 1992). Similarly, IL-5 and IL-6 both selectively stimulated the production of different immunoglobulin isotopes during mucosal and systemic antiviral antibody responses (Ramshaw et al., 1992). Taken together these results demonstrated that VV, which had previously been shown to faithfully process and transport foreign proteins (Moss and Flexner 1987), can indeed be used as a successful vector for the delivery of biologically active cytokines.

Preclinical cancer gene therapy with VV-cytokines

Vaccinia virus was first used in the mid 1990's in cancer gene therapy as a vector for delivering recombinant cytokines to tumour cells *in vivo*. Several studies have demonstrated that vaccinia viruses expressing recombinant cytokines can infect tumour cells and secrete biologically active protein that is capable of exerting its effect on surrounding effector cells (Bash 1993, Acres et al 1994, Whitman et al 1994). Acres et al (1994) showed that several days after an intravenous inoculation of tumour-bearing mice with various VV-cytokine recombinants, tumour cells are well infected with vaccinia particles while organs such as the liver and spleen show minimal levels of virus infection. Injection of tumour bearing mice with VV transfected with either IL-2 or IL-6 results in the secretion of detectable cytokine levels in the sera of immunised mice (Acres et al 1994) and specifically for IL-2, is secreted from infected tumour cells (Whitman et al., 1994) and can indeed result in tumour regression (Bash 1993).

In tumour immunotherapy studies with VV-cytokine recombinants, VVIL-6 has been shown to significantly reduced the growth rate of tumours in nude mice and in some cases led to the complete rejection of the tumour, while VVIL-2 exhibited similar effects in tumour-bearing DBA mice (Acres et al 1994). In a similar study, A/J mice were protected against a challenge of 1 x 10^3 neuroblastoma (C1300) cells after receiving three weekly immunisations of C1300 tumour cells transfected with recombinant VV expressing human IL-2. Even after surviving more than 75 days after the initial challenge, mice were rechallenged with a greater number of tumour cells and remained alive (Qin and Chatterjee, 1996). Both of these studies demonstrate that the delivery of recombinant cytokine to the site of tumourigenesis can produce antitumour responses.

To study the immunotherapeutic potential of interleukin-4 (IL-4) delivered *in vivo* via a recombinant VV, a thymidine kinase-negative (TK-) vaccinia virus that expressed the murine

IL-4 gene (VVIL-4) was constructed. A single s.c. inoculation with VVIL-4 delayed the development of NCTC 2472 tumours, but when VVIL-4 was inoculated s.c. weekly for 8 weeks, tumour development was completely prevented in 93% of mice. Similarly, the development of M-3 melanoma tumours was also prevented by weekly s.c. inoculations of VVIL-4. Weekly virus treatment did not prevent NCTC 2472 tumour development in athymic nu/nu mice, suggesting that mature T cells are required for expression of VVIL-4 induced antitumour activity (Elkins et al 1994). In a study investigating the role of IL-4 in anti-viral mediated cellular immunity, VVIL-4 infected spleens produced a remarkably lower number of antiviral cytotoxic T cell precursors compared to the number produced by VV infected spleens (Sharma 1996), demonstrating IL-4 is directly involved in the suppression of cell-mediated immune responses *in vivo*.

The efficacy of a recombinant VV-cytokine vaccine also been established in a melanoma model of tumour metastasis. C57BL/6 mice with established B16-F10 melanoma and lung metastasis treated with a VV expressing murine GM-CSF (rvv-mGM-CSF) vaccine that produced a B16 tumour cell specific immune response, survived longer, were free of palpable tumours and had smaller mean tumour volumes compared to control mice (Qin and Chatterje 1996a). However, when the vaccine was prepared by infecting irradiated tumour cells with rVV-GM-CSF, tumour development and lung metastases could be inhibited. The vaccine was also effective when rVV-GM-CSF was directly injected into the tumour (McLaughlin et al 1997, Chatterje et al 1999). Interestingly in this melanoma metastatic model VVIL-2, which was successful at inducing tumour protection in other murine models, was ineffective (Qin and Chatterje 1996a). This data suggests that using VV-cytokine in metastatic tumour immunotherapy also has potential use in cancer treatment, especially for patients with easily accessible tumours.

Cancer gene therapy with replication-deficient recombinant vaccinia viruses (for increased safety) expressing cytokines has also been investigated in several studies. A noncytopathic recombinant VV was constructed encoding murine IL-12 and used to infect MCA 101 fibrosarcoma cells and PAN 02 pancreatic tumour cells. As seen in earlier studies with other VV strains, the recombinant virus produced large amounts of biologically active cytokine (up to 793ng/10^6 cells/24 hr), however it continued to replicate with no cytopathic effects. Mice injected with VVIL-12 and subsequently challenged with either tumour, exhibit delayed tumour establishment and significant tumour inhibition growth compared to control mice

(Meko et al., 1996). In a similar study, another replication-deficient recombinant VV, NYVAC, was developed by deleting 18 open reading frames in the vaccinia virus genome. Recombinant NYVAC encoding the murine T cell costimulatory gene B7.1 (CD 80) (NYVAC-B7.1) and the murine interleukin-2 gene (NYVAC-IL-2) were studied in a syngeneic murine colon adenocarcinoma model. Mice immunised with either NYVAC-B7.1 or NYVAC-IL-2 alone, showed little if any decrease in tumour burden compared to control mice. However when mice were immunised with both NYVAC-B7.1 and NYVAC-IL-2 before being challenged with 10^8 naive CC-36 tumour cells, they were protected against tumour development (Sivanandham et al., 1998).

To date, tumour immunotherapy research using vaccinia virus to delivery biologically active cytokines to tumours *in vivo* have been extensively studied. Table 4 below summarises vaccinia virus cytokine gene therapy studies for cancer immunotherapy from the past decade.

Table 4: A Summary of the tumour immunotherapy studies using VV-cytokines

Cytokine	VV-cytokine Tumour Immunotherapy Studies
IL-1β	Peplinski et al 1995, Peplinski et al 1996
IL-2	Bash 1993, Acres et al 1994, Sivanandham et al 1994, Tanaka et al 1994, Whitman et al 1994, Bronte et al 1995, Peplinski et al 1996, Qin and Chatterjee 1996, Robinson et al 1998, Sivanandham et al 1998,
IL-4	Elkins et al 1994
IL-6	Acres et al 1994,
IL-12	Meko et al 1995, Meko et al 1996, Rao et al 1996,
IFN-α	Tanaka et al 1994
IFN-γ	Bronte et al 1995, Peplinski et al 1996
G-CSF	Bronte et al 1995
GM-CSF	Peplinski et al 1996, Qin and Chatterjee 1996a, Ju et al 1996, McLaughlin et al 1997, Chatterje et al 1999
TNF- α	Bronte et al 1995, Peplinski et al 1996

Human clinical trials with VV- cytokines

Despite the successful pre-clinical antitumour effects of VV-cytokine recombinants the clinical application of VV as a potential vaccine for cancer patients with internal cancers (such as malignant mesothelioma) has met with little success (Robinson et al., 1998), however external skin cancers (melanomas) have shown considerable promise (Lattime et al 1996, Mastrangelo et al 1999).

In 1996, cytokine-encoding recombinant vaccinia virus was injected in a trial in melanoma patients with accessible lesions. This study found that VV productively infects human melanoma cells following intratumoural injection, the in situ transfections are highly efficient, and therapy with increasing doses of virus is safe with only minor side effects (Lattime et al 1996). It was therefore concluded that recombinant VV vectors are suitable and safe for *in situ* transfection in patients with melanoma. Following on from this work was a later study which investigated melanoma patient response rates after being injected with recombinant VV-GM-CSF (Mastrangelo et al 1999).

In this trial, seven immunocompetent, revaccinated patients with surgically incurable cutaneous melanoma underwent treatment of dermal and/or subcutaneous metastases with intratumoural injections of escalating doses of a vaccinia/GM-CSF recombinant virus for six weeks. The two patients with the largest tumour burdens failed to respond even at treatment sites. Three patients had mixed responses, with regression of treated and untreated dermal metastases and progression of disease elsewhere. One patient had a partial response, with regression of injected and uninjected regional dermal metastases. When the residual melanoma was excised, the patient was rendered disease free. Only one patient with dermal metastases confined to the scalp achieved a complete remission (Mastrangelo et al 1999). Therefore, administration of a GM-CSF recombinant vaccinia virus is safe, effective and can induce tumour regression.

4.4 The future of tumour immunotherapy with cytokines

The incorporation of cytokines into potential cancer vaccines holds great promise for future tumour immunotherapy studies. Cytokines have proven to be useful at modifying biological responses in the treatment of various cancers. They can successfully be used to manipulate immune responses and direct uncommitted T cells to the cellular pathway and stimulate the growth and migration of many immune effector cells including antigen presenting cells and cytotoxic T cells.

Cytokines often act locally, at the sites of inflammation or at high regional concentrations during interactions between lymphocytes, but diffuse rapidly such that the lymphokine is undetectable systemically. With the availability of purified recombinant cytokines, clinical protocols involving the injection of cytokines has become feasible, although expensive. In therapy, large doses must be administered systemically to produce the desired effect, and this can results in serious unwanted side effects (Balkwill 1989; Rosenberg et al., 1987; Siegal and Puri 1991).

The use of recombinant cytokine delivery vectors such as adenovirus and vaccinia virus are also an attractive option for tumour immunotherapy as cytokines can be delivered in high doses to rapidly dividing tumour cells to exert their biological effects. Similarly, the transfection of recombinant cytokines into solid tumours also has the advantage of being able to produce high concentrations of cytokines at the site of the tumour. The clinical applicability of these observations is however, restricted to surgically accessible tumours and to tumours from which cell lines can be established. Furthermore the re-injection of tumour cells, even if transfected with cytokines, may not always be desirable.

However the ability to manipulate and stimulate the immune response in such a way as to induce anti-tumour immunity is very attractive. Despite some of the problems associated with their use, cytokines remain potent mediators of anti-tumour effects *in vivo*.

Section 5: Conclusion

It has been over 200 years since Edward Jenner first used the term 'vaccine' to name the prophylactic preparation of cowpox viruses used to condition the human defensive system to recognise and fight smallpox. A hundred years later William Coley, the first tumour immunologist, used preparations derived from streptococcal cultures to treat human tumours. As we enter the new millennium, scientists still continue to research ways and devise new strategies to activate immunological mechanisms to combat cancer. While initial immunotherapy studies focussed on inducing tumour immune responses to whole tumour cells (both autologous and allogenic), tumour cell lysates, defined antigens and anti-idiotypic antibodies; second generation tumour vaccines now use new technologies to develop genetically modified cellular and recombinant vaccines.

With an increasing understanding of the requirements for the development of an anti-tumour immune response, immunotherapeutic strategies predominantly focus on identifying novel tumour antigens and providing mechanistic requirements. These include enhancing or providing tumour or accessory antigen expression, localising treatment with immune-active adjuvants, and systematically administering cytokine-based 'immune help'. New strategies continue to emerge every decade and with the advent of recombinant delivery systems, DNA based vaccines and a better understanding of the immune system, the future for tumour immunotherapy has never looked brighter.

This thesis focuses on three aspects of tumour immunotherapy; antigen delivery, immune system manipulation and the administration of cytokine-based 'immune help'. The studies contained within investigate the use of various cytokines to boost and manipulate the immune response generated to the MUC1 cancer antigen – coupled the carrier, oxidised mannan. It is envisaged that combination therapies such as those being studied, may hold the secret to protecting against tumour growth *in vivo*, and perhaps, a combination of immunotherapy and classical cancer treatments such as surgery, chemotherapy and/or radiotherapy may be required to effectively treat established tumours. What does remain conclusive however, is only through clinical trials will scientists be able to develop a greater understanding of the human immune response to cancer and manipulate this response to ultimately protect and or treat malignant carcinomas.

Chapter 2:

Common materials and methods

Table of contents

Introduction

This chapter describes materials and methods frequently used in this thesis. Each method is described in detail and, where appropriate, results included. This chapter is divided into seven major sections describing the production of mannan - MUC1 fusion protein, cellular assays, flow cytometry, the production of recombinant cytokines from vaccinia virus, characterisation of tissue samples, cell lines, and the characterisation of MUC1 transgenic mice.

Any methods not listed in this chapter and variations to methods described herein, are located in the materials and methods section of the corresponding chapter.

1. Production of mannan-MUC1 fusion protein (MFP)

1.1: Production of soluble GST-MUC1 fusion protein (FP)

The 309 base pair cDNA clone, pDF9.3, (Siddiqui et al., 1988) containing 5 x 20 amino acid repeat regions from the variable number of tandem repeat (VNTR) extracellular region of human MUC1, was previously cloned into the pGEX-3X plasmid vector and transfected into DH5α ampicilin resistant *Escherichia coli* (Siddiqui et al., 1988) (Figure 2.1). *E. coli* were grown overnight at 37°C in Luria broth containing ampicilin (Sigma, St. Louis, U.S.A). The culture was diluted 1:25 with fresh medium and grown for 1 h at 37°C before adding 0.1M IPTG to induce fusion protein production from bacteria (Smith and Johnson, 1988). After a further 3 h incubation at 37°C, cultures were centrifuged at 2,500 x g for 15 min at 4°C. Supernatants were discarded and pellets resuspended in 5ml phosphate buffered saline (PBS). Cells were lysed for 3 x 30 sec with a probe sonicator, mixed with 1% Triton X-100 (BDH Chemicals, Dorset, England), and centrifuged at 10,000 x g for 15 min at 4°C. The soluble GST-MUC1 fusion protein [C-PAHGVTSAPDTRPAPGSTAP x 5 - GST] was obtained from the supernatant and stored at -20°C (Apostolopoulos et al., 1993).

1.1.1: Purification of soluble GST-MUC1 FP

Fusion protein was purified by rotating a mixture of soluble FP with a 50% suspension of glutathione-agarose beads (Sigma, St.Louis, U.S.A) at a 5:1 (fusion protein:beads), for 1 h at room temperature. After absorption, beads were collected for centrifugation at 800 x g for 5 min, and washed 3 times in PBS. Fusion protein was eluted by competition with free glutathione (Sigma, St. Louis, U.S.A) with 1.5 bead volume of 50mM Tris HCl (pH 8.0) containing 5mM reduced glutathione for 1 h on a rotating wheel. The first elution of pure fusion protein was collected in the supernatant. This process was repeated twice to obtain further purified product (Figure 2.2). The eluted protein was dialysed at 4°C against PBS and stored at -20°C.

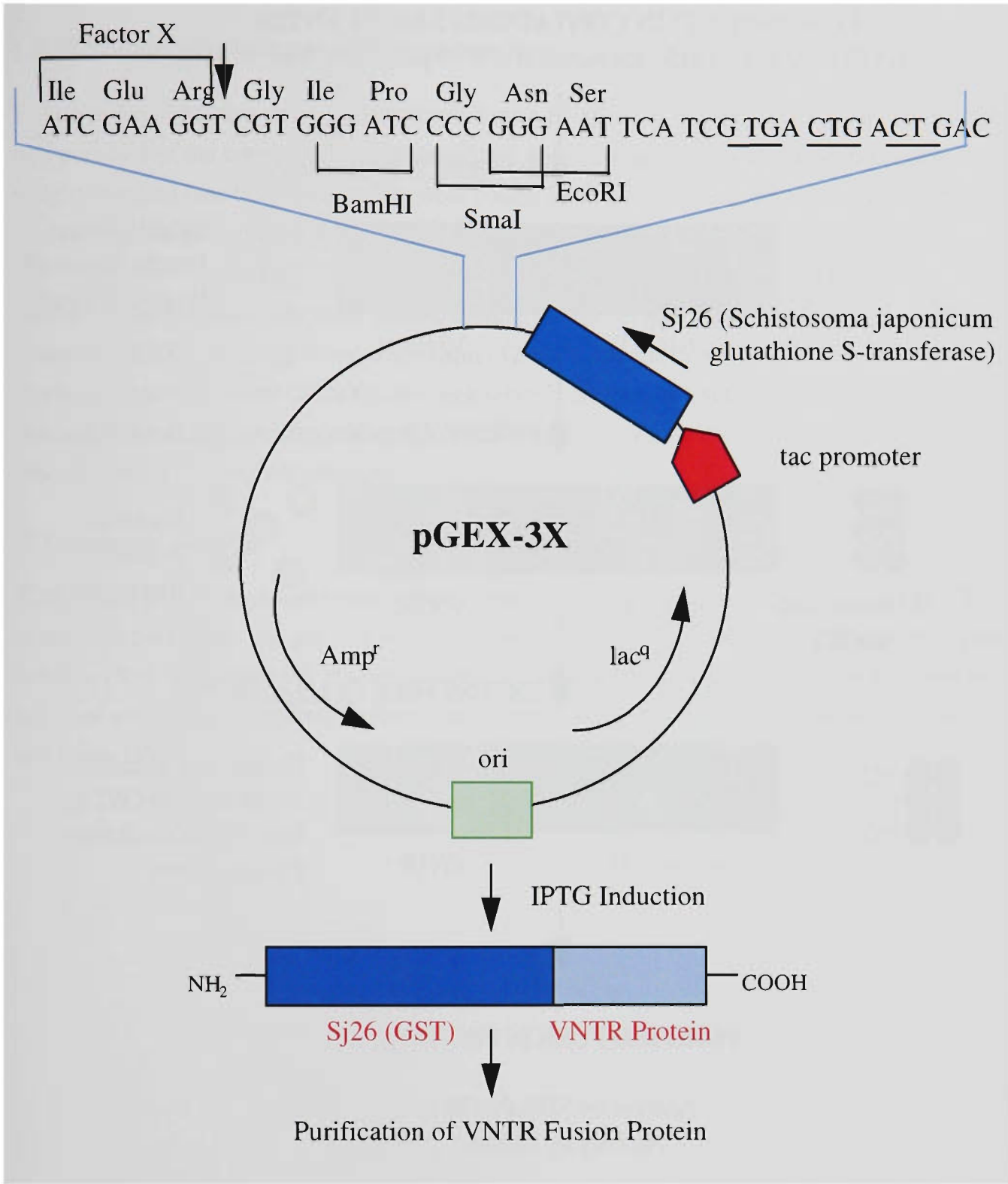


Figure 2.1: Structure of the gene fusion vector pGEX-3X

The tac promoter is shown upstream of the Sj26 cDNA, which encodes the entire GST polypeptide, followed by a polylinker region and a stop codon in each reading frame. Nucleotide sequence at the C-terminus of the Sj26 cDNA are shown. The cleavage sites for BamHI, SmaI and *Eco*RI and the stop codons in all three reading frames, are underlined. An additional sequence encoding the Factor Xa protease cleavage site is recognised. (Figure based on Apostolopoulos, "Immunotherapy of Cancer using Mucin (MUC1) Antigens" PhD thesis, p91, 1995)

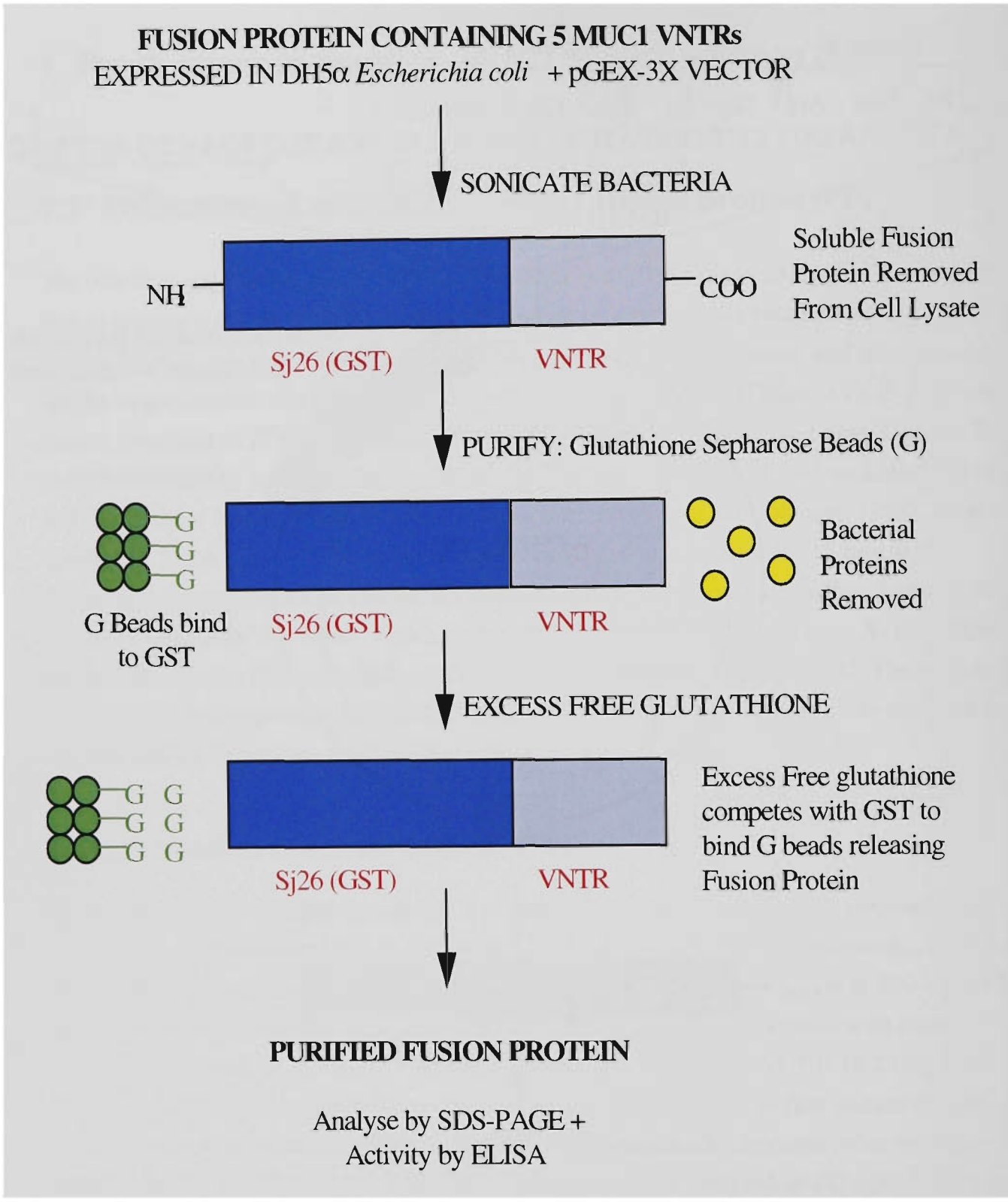


Figure 2.2: Purification of Soluble Fusion Protein

The gene encoding 5 MUC1 VNTRs was amplified and inserted into the pGEX vector. Fusion Proteins were expressed in *Escherichia coli* and sonicated to release protein. Bacterial proteins were removed by centrifugation and fusion protein isolated using Glutathione Sepharose beads and eluted upon addition of excess reduced free glutathione.

1.1.2: Polyacrylamide gel electrophoresis

Fusion protein samples were electrophoresed on a 12.5% SDS-PAGE gel to ensure the protein was pure and of the correct molecular weight by direct comparison with a low molecular weight standard (see below). Samples were boiled for 5 min with non-reducing SDS buffer, loaded onto the gel, and run for 3-4 h at 150 volts to ensure complete protein separation.

Molecular weight standards used: Rabbit muscle phosphorylase B (97,400), Bovine serum albumin (66,200), Hen egg white ovalbumin (42,699), Bovine carbonic anhydrase (31,000), Soybean trypsin inhibitor (21,500), Hen egg white lysozyme (14,400). The concentration of fusion protein was determined using UV spectrophotometry (wavelength = 280 nm; absorbance 1.43 = 1mg/ml solution).

(i) Coomassie staining

SDS-PAGE gels were stained with 0.2% Coomassie blue for 30 min and destained with 7% acetic acid overnight. The gel was washed 3 times with distilled water (DW), soaked in drying solution (30% methanol, 5% glycerol and 65% double DW) for 1 h, and air dried between two layers of cellophane (Bio-Rad, Sydney, Australia) overnight at room temperature (Michaels and Ford, 1991).

1.2: Conjugation of mannan to MUC1 FP

1.2.1: Oxidised mannan-MUC1 fusion protein (ox MFP)

Mannan, obtained from the cell walls of *Saccharomyces cerevisiae* (Sigma, St. Louis, U.S.A), was oxidised and coupled to MUC1-FP according to Apostolopoulos et al. (1995). Mannan, at 14 mg/ml in 0.1M phosphate buffer (pH 6.0), was oxidised to a poly-aldehyde with 0.1M sodium periodate (100µl) for 1 h at 4^{0}C. Ethandiol (10µl) was added to the oxidised mannan and the mixture incubated for 30 min at 4^{0}C before being passed down a PD-10 column (Sephadex G-25 column, Pharmacia Biotech, Uppsala, Sweden) equilibrated with pH 9.0 bicarbonate buffer and non-specific binding blocked with 0.5% BSA. Oxidised mannan, which eluted in the void volume, was mixed with 900µg MUC1-FP overnight at room temperature (in the dark) resulting in Schiff base and aldehyde formation and the coupling of oxidised mannan to MUC1-FP (oxidised MFP). (See Figure 2.3; Apostolopoulos et al., 1996a)

1.2.2: Reduced mannan-MUC1 fusion protein (red MFP)

Reduced MFP was produced by reacting oxidised MFP with 1 mg/ml sodium borohydride for 3 h at room temperature to reduce the Schiff bases and aldehydes to amines and alcohols. (See Figure 2.3; Apostolopoulos et al., 1996a)

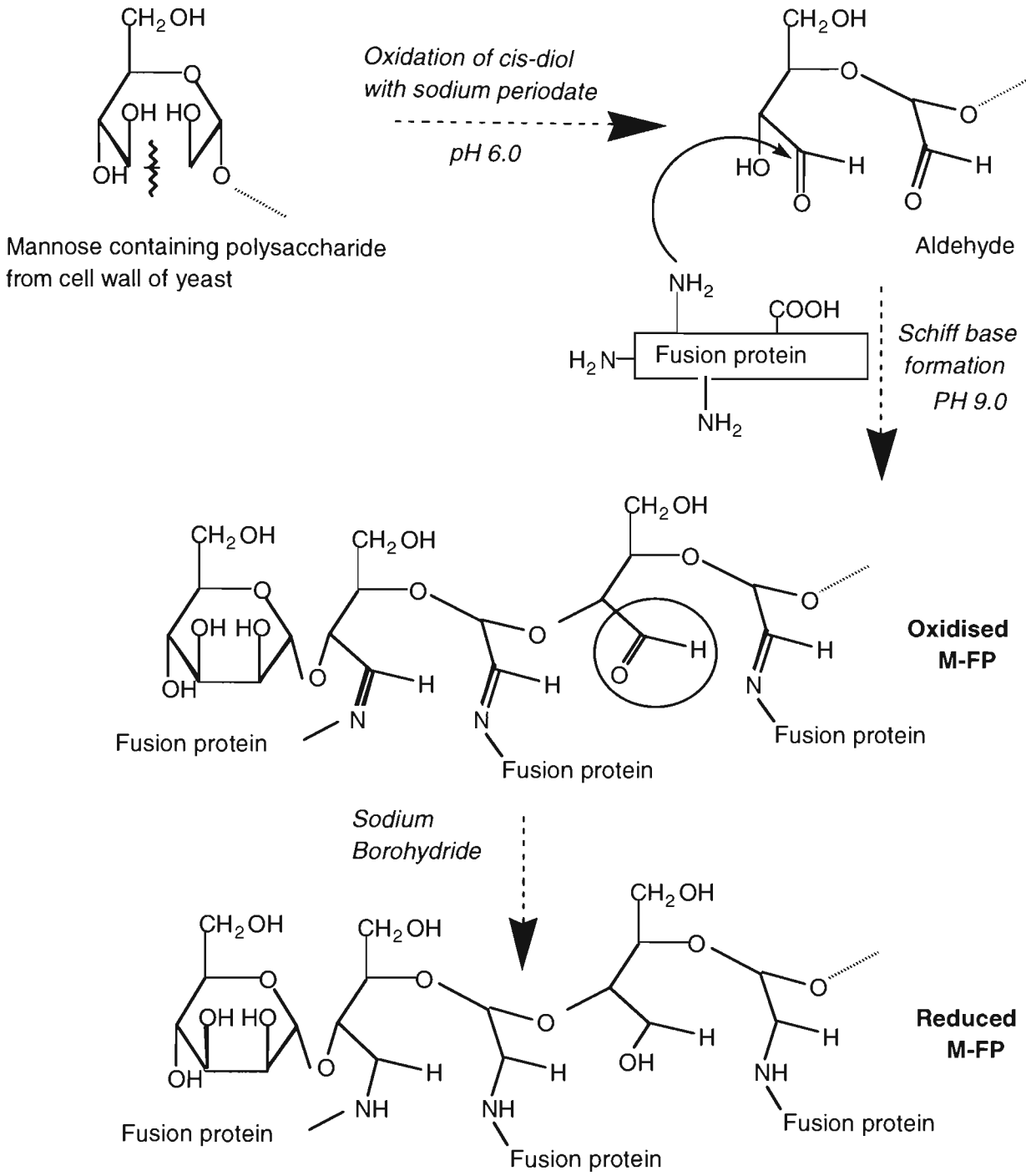


Figure 2.3: Chemical conjugation of MUC1 fusion protein to mannan

Production of oxidised MFP and reduced MFP (Apostolopoulos et al. 1996a).

2. Cellular assays

2.1: Direct binding enzyme linked immunosorbent assay

To determine the MUC1 activity of soluble GST-MUC1 FP, a direct binding enzyme linked immunosorbent assay (ELISA) was performed using the MUC1 antibody BC2. MUC1-FP and the MUC1 VNTR peptide, Cp13-32: C-PAHGVTSAPDTRPAPGSTAP were coated on a 96 well round-bottom microtitre plate (50 µl/well) at 10 µg/ml in a 0.05 M carbonate buffer pH 9.6 for 2 h at 37^{0}C. Unbound antigen was removed by washing the plates 3 times in MPBS/0.05% Tween 20 and non-specific binding blocked with 2% BSA for 1 h at 37^{0}C. The plates were again washed 3 times in MPBS/Tween before adding serially diluted (1:2) antibody (50 µl) in PBS and incubated for 2 h at room temperature. Excess antibody was removed by thorough washing with PBS/Tween and 50 µl of sheep anti-mouse immunoglobulin-horseradish peroxidase linked to conjugate (Amersham, Buckinghamshire, U.K.) diluted 1:500 (MPBS) added to the plate for 1 h at room temperature. After thorough washing in PBS/Tween to remove excess conjugate, the assay was developed with 50 µl, 0.03% ABTS enzyme [2,2'-azino-di(3-ethylbenzthiazoline sulphonate)] (Amersham, Buckinghamshire, U.K.), 0.02% H_2O_2 (100 Volume, Ajax Chemical, N.S.W, Australia) in 0.1 M citrate buffer, pH 4.0 and incubated at room temperature until a colour change occurred. Absorbance was read in an EL 312e Microplate Bio-Kinetics reader at 405 nm.

2.2: T cell assays

2.2.1: Cytotoxic T cell assay

Immunised mice were sacrificed 7-10 days following their final injection, and spleen cells collected in 4% foetal calf serum/PBS and treated with warmed 0.83% NH_4Cl (Ajax Chemicals, N.S.W, Australia) for 10 min at 37°C to lyse red blood cells. Target cell lines were radiolabelled with 100 µCi of $Na_2{}^{51}Cr0_4$ (Amersham, Buckinghamshire, U.S.A) for 1 h at 37°C and washed to remove free radioactivity. Cells were resuspended in RPMIM culture media [Roswell Park Memorial Institute Medium 1640, (Flow Laboratories, N.S.W, Australia) supplemented with 10% foetal calf serum (Flow Laboratories, N.S.W, Australia), 2 mM glutamine, $5x10^{-5}$ mM 2-mercaptoethanol (2ME; Sigma Chemicals, St. Louis, U.S.A) 100 U/ml penicillin (Commonwealth Serum Laboratories (CSL), Victoria, Australia), 100 µg/ml streptomycin (CSL, Victoria, Australia) and 0.02 M HEPES buffer (CSL, Victoria, Australia)] and combined with spleen cells at various effector to target ratios in a 96 well round bottom microtitre plate. Plates were briefly centrifuged at 1000 x g to bring cells into contact, and incubated for 4 h at 37°C. The spontaneous release of ^{51}Cr from the labelled cells was determined by incubating target cells in RPMIM media and the maximum release was determined by incubation with 10% SDS (sodium dodecyl sulphate) (BDH Chemicals, Dorset, England). After incubation, plates were centrifuged at 2000 x g and 100 µl supernatant collected and transferred to 96 well flat Optiplates (Disposable Products, S.A, Australia) containing 100 µl of Microscint 40 (Packard, Meriden, U.S.A) for analysis on a Microplate Scintillation Counter (Packard, U.S.A). The specific percentage lysis of target cells was determined by [(experimental - spontaneous) cpm / (maximum - spontaneous) cpm] x 100%.

2.2.2: Limiting dilution cytotoxic T cell precursor assay

Immunised mice were sacrificed and their spleen cells collected in 4% foetal calf serum/phosphate buffered saline (PBS) and treated with warmed 0.83% NH_4Cl (Ajax Chemicals, N.S.W, Australia) for 10 min at 37°C to lyse red blood cells. After washing, cells were collected and added, in replicates of 32, to 96 well microtitre plates with cell numbers varying from 1 x 10^3 to 1.28 x 10^5 cells/well; stimulator spleen cells from naive mice were irradiated using 3,000 rads from a Caesium source (Gammacell 1000 elite irradiator: Nordion International Inc.) to inhibit their proliferation. Stimulator cells (5 x 10^5 cells/well), 5 µM MUC1 peptide Cp13-32 and 10 U/ml of recombinant human IL-2 were added to all effector wells. Controls containing 5 x 10^5 cells/well stimulators and either 10 U/ml IL-2 and 5 µM peptide Cp13-32, or $5x10^5$ cells/well stimulators and 1.28 x 10^5 cells/well effectors were also

set up in replicates of 32 wells. Plates were incubated for 7 days at 37^{0}C 10% CO_2. After incubation, 100 µl supernatant from all wells was discarded and 1 x 10^4 ^{51}Cr labelled target cells added. Cultures were incubated for a further 4 h before transferring 100 µl of supernatant to 96 well flat Optiplates (Disposable Products, S.A, Australia) containing 100 µl of Microscint 40 (Packard, Meriden, U.S.A) for analysis on a microplate scintillation counter (Packard, Meriden, U.S.A). Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release three standard deviations above the mean release from 10^4 effector cells cultured alone, or 10^4 effector cells and 5 x 10^5 stimulators together or stimulators and peptide and rIL-2 together. A linear relationship existed between the dose of effector cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative well (Taswell 1981; Lefkovits and Wladmann 1984).

3. Flow cytometry

Cell surface marker expression from various cells were analysed by the FACScan (Beckton Dickenson, California, U.S.A). Cells (2 – 5 x 10^5 cells/ml) prepared for flow cytometry were incubated with 180 µg/sample heat aggregated gamma globulin (HAG) for 30 min at 4^{0}C to block Fc Receptors, washed three times with PBS and incubated with various antibodies at 4^{0}C for 45 min After washing three times in PBS to remove excess unbound antibody, cells were incubated for 45 min at 4^{0}C with FITC-conjugated (Fab')2 immunoglobulin (Amersham, Buckinghamshire, U.K) (1/50 dilution). Cells were washed thoroughly with PBS and transferred to FACScan tubes for analysis.

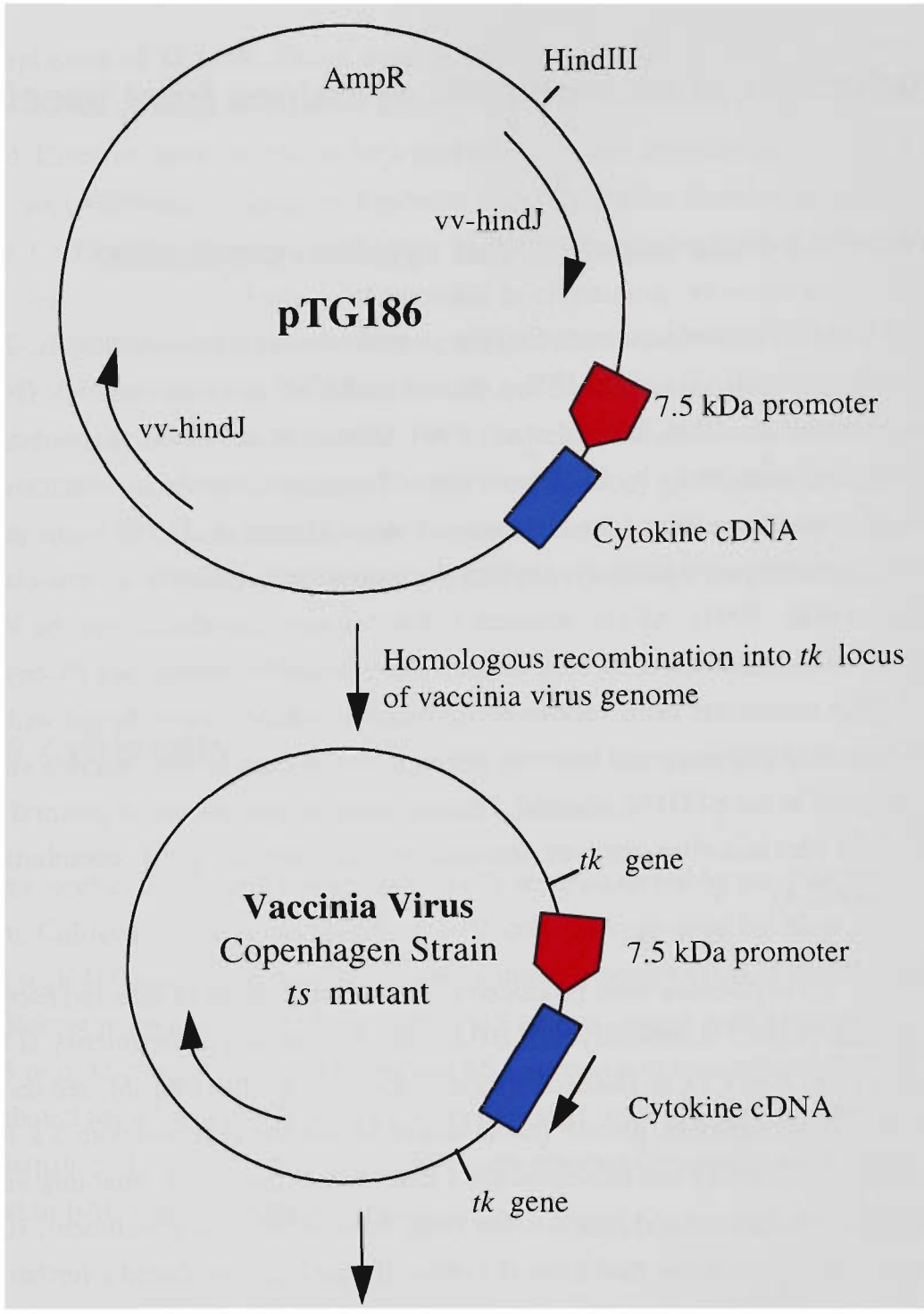
4. Production of recombinant cytokines from vaccinia virus

4.1: Vaccinia virus infection and cytokine production

Vaccinia virus (VV) constructs encoding the recombinant cytokines human IL-2, murine IL-4, murine IL-6, murine IL-7, murine IFN-γ, murine GM-CSF and wild-type VV (Nakagawa et al., 1991, Acres et al., 1994, Balloul et al., 1994, Wen Ju et al., 1997), were obtained and tested for biological activity by Dr Bruce Acres (Transgène, Strasbourg, France). The VV strains used were *ts* mutants of the Copenhagen strain (Kieny et al., 1984) and possessed a thymidine kinase negative phenotype which decreases viral virulence *in vivo* (Buller et al., 1985; Taylor et al., 1991). All recombinant cytokines were introduced into the VV genome by homologous recombination according to previously described techniques (Kieny et al., 1984). Briefly, cDNA constructs from various recombinant cytokines were aligned with an early 7.5 kDa VV promoter sequence and inserted within a cloned copy of the vaccinia virus thymidine kinase *(tk)* gene in the pTG186 plasmid. Homologous recombination of plasmid DNA into the *tk* locus of the vaccinia virus genome, resulted in virus harbouring the recombinant cytokine cDNA (Figure 2.4).

Recombinant VV-cytokines were produced from the supernatent of infected baby hamster kidney-21 cells (BHK). A monolayer of BHK cells were grown to confluency at the time of infection at 37^{0}C 10% CO_2 in Dulbeco's modified eagles media (DMEM, see section 6.1) (10% FCS). Once confluent, growth media was removed and replaced with 5 x 10^6 pfu virus in 5ml DMEM (1% FCS) and incubated for 1 h at room temperature, enabling virus particles to come into close contact and attach to the BHK cells. After viral attachment, 10 ml DMEM (10% FCS) was added to the flasks (ie. 0.1 pfu/cell) and cells incubated a further 48 h until infected cells became detached. Recombinant cytokine was harvested from the supernatant of infected cells, UV treated to inactivate virus particles, and tested for recombinant protein.

To determine recombinant cytokine activity and concentration, both biological assays and cytokine ELISAs were performed in conjunction with Dr Bruce Acres (Transgène, Strasbourg, France) after the generation of each VV-cytokine product and for the production of VV-IL-2, VV-IL-4 and VV-IL-12 in the studies contained herein. Results from all of these experiments concluded that the ELISA assays detected biologically active cytokine in all circumstances and was used for quantifying cytokine production in this thesis (refer to section 4.4).



Purification of recombinant Vaccinia Virus-cytokine

Figure 2.4: Production of recombinant vaccinia virus containing cytokine genes

Recombinant cytokine cDNA was aligned with an early 7.5 kDa promoter and inserted into the HindIII fragment of the VV genome (containing a complete *tk* gene creating a TK-deficient phenotype) in the pTG186 plasmid. Homologous recombination of vaccinia virus and plasmid DNA generates recombinant VV.

4.2: Virus concentration for *in vivo* immunisations

VV encoding recombinant cytokines were semi-purified for *in vivo* applications in mice (Figure 2.5). Monolayers of BHK cells infected with VV (as described above) were harvested 48 h after infection and centrifuged at 1000 x g for 10 min and the pellets, containing virus to be purified, resuspended in 5 ml/flask RSB buffer (10 mM Tris pH 7.6-8.0, 1 mM $MgCl_2$, 1 mM KCl, in 1 L distilled H_2O). Cells were sonicated for approximately 30 sec on ice using a probe sonicator to release viral particles and centrifuged 1000 x g for 10 min to remove cellular debris. Supernatant containing virus was collected, the pellet resuspended in RSB buffer (2 ml/flask) and further sonicated to collect any remaining virus. After centrifugation, the supernatants containing vaccinia virus were pooled and under-layered onto 10 ml of pre-prepared 36% sucrose (autoclaved and cooled to room temperature) in SW28 polyallomer centrifuge tubes (Beckman, California, U.S.A). The sucrose/virus mixture was ultra-centrifuged (Sorvall Ultracentrifuge, DuPont, Delaware, U.S.A) for 2 h at 14,000 rpm, 4^0C. After ultra-centrifugation, the pellet containing virus was resuspended in 2-5 ml/flask 10 mM Tris (pH 8.0) and sonicated using the probe sonicator on ice for approximately 30 sec to obtain semi-purified virus for *in vivo* use.

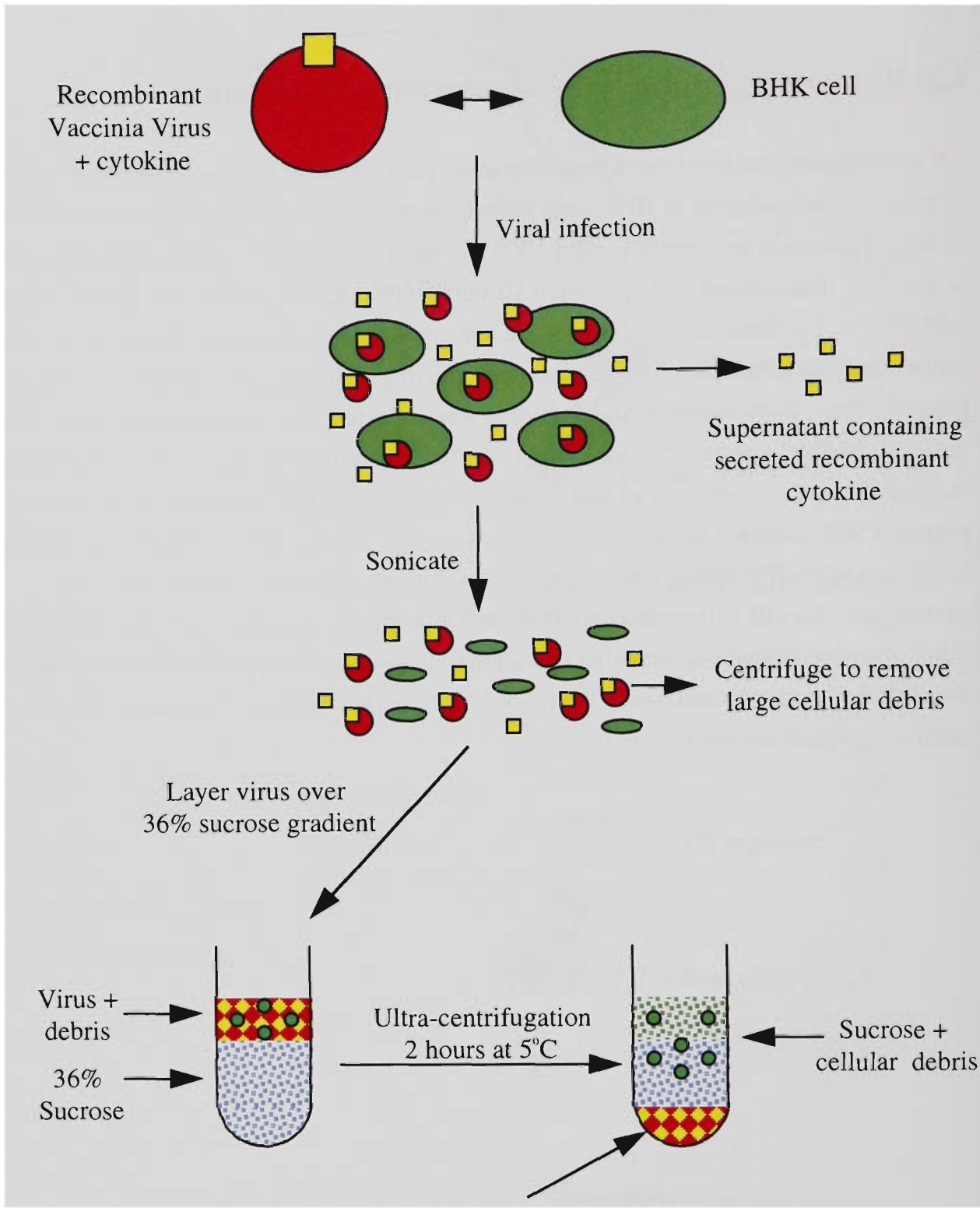


Figure 2.5: Production of semi-purified recombinant Vaccinia virus expressing cytokines

VV-cytokine infect BHK cells and non-purified recombinant cytokine collected from the supernatant 48hrs post infection. Infected cells are sonicated to release virus and semi-purified by ultra-centrifugation over a 36% sucrose gradient.

4.3: Determination of viral titres by a plaque forming assay

Viral titres were determined by plaque forming assays in which monolayers of confluent BHK cells prepared in 6 well Multiwell tissue culture plates (Becton Dickinson, Franklin Lake, U.S.A) were infected with dilutions ($1 \times 10^5 - 1 \times 10^9$) of vaccinia virus for 1 h at room temperature, and incubated in growth medium at 37°C 10% CO_2. Twenty-four h later, growth media was removed, cells stained with aniline blue for 30 min, dried, and microscopic plaques quantified. The semi-purified virus titres are summarised in Table 3.1.

Table 3.1: Summary of Semi-purified VV-Cytokine titres and cytokine concentrations

VV-Cytokine	Virus Titres[1] (pfu/ml)	Cytokine Conc.[2] (µg/ml)
VV	7.0×10^9	N/A
VV IL-2	2.3×10^{10}	4.0
VV IL-4	1.9×10^{10}	-
VV IL-5	-	0.105
VV IL-6	4.0×10^{10}	22.0
VV IL-7	4.7×10^{10}	-
VV IL-12	4.0×10^{10}	0.012
VV IFN-γ	1.6×10^{10}	3.35
VV GM-CSF	6.3×10^{10}	1.8

1. Virus titres from semi-purified stocks used *in vivo*.
2. Cytokines were quantified using commercial Cytokine ELISA kits (Pharmingen, San Diego, U.S.A)
3. N/A: Not Applicable
4. -: No test performed

4.4: Sandwich ELISA for detection and quantitation of cytokines

Cytokines were detected and quantified using commercially available sandwich ELISA kits (Pharmingen, San Diego, U.S.A) (Figure 2.6). Optimised concentrations of capture and detection antibodies for each cytokine ELISA was determined by setting up a 'chequer board' scenario whereby both coating antibodies and detection antibodies were titrated between 0.5 – 4 µg/ml and optimised conditions established (Table 3.2). Purified anti-cytokine capture antibodies were diluted according to Table 3.1. in 0.1 M Na_2HPO_4 pH 9.0 (HCl) and 50 µl added to the wells of a protein enhanced binding ELISA plate (Nunc Maxisorb; Nunc, Denmark), sealed in a humidified container, and incubated overnight at 4^0C. After washing several times in 0.5% PBS/Tween-20 non-specific binding was blocked by adding 200 µl 2% PBS/BSA and incubating at room temperature for 30 min. Recombinant cytokine standards at 20 ng/ml (100 µl) were diluted in PBS and cytokine samples added to wells, sealed in a humidified container, and incubated at 4^0C overnight. ELISA plates were washed 10-15 times in PBS/Tween and 100 µl PBS diluted biotinylated anti-cytokine detection antibody added to each well and incubated at room temperature for 1 h (see Table 3.2).

Streptavidin-HRP conjugate diluted 1/500 in PBS (100 µl) was added to thoroughly washed wells and incubated for 30 min at room temperature. Cytokine detection and quantitation was determined by washing plates 10-15 times with PBS / Tween and adding 50 µl of developing reagent [1 – 2 mg TMB substrate (Tetramethyl-benzidine dihydrochloride hydrate 97%) (Aldrich, Australia) dissolved in 1 ml 0.1 M citric acid, 7 ml DW, 2 ml 0.5 M sodium acetate and 5 µl H_2O_2] and incubated in the dark at room temperature for colour development. The reaction was stopped by the addition of 0.18 M Sulphuric acid after the optimal colour intensity was reached and the absorbance read at 450 nm on a plate reader (Figure 2.7).

Table 3.2: Optimised Cytokine Sandwich ELISA Conditions

Cytokine	Anti-Cytokine Capture Antibody µg/ml	Biotinylated Anti-Cytokine Detection Antibody (µg/ml)	Cytokine Standard Biol. Activity (ng/ml)
IL-2	2	1	0.1-10.0[1,2]
IL-4	2	1	0.05-5.0[1,3]
IL-5	4	2	0.015-2.0[1]
IL-10	4	1	0.01-20.0[1]
IL-12[4]	4	1	0.015-2.0[1]
IFN-γ	4	1	0.03-4.0[1]
GM-CSF[5]	N/A[6]	N/A	0.001-0.25
TNF-α	1	1	0.05-20.0[1,3]

Recombinant cytokine standards purchased from:

1. Pharmingen (San Diego, U.S.A)
2. Genzyme (Cambridge, U.S.A)
3. R&D Systems (U.S.A)
4. IL-12 sandwich ELISA detects only p35 subunit from the p70 heterodimer. ie. only biologically active IL-12.
5. GM-CSF commercial ELISA kit from Endogen (Cambridge, U.S.A)
6. N/A: Not Applicable

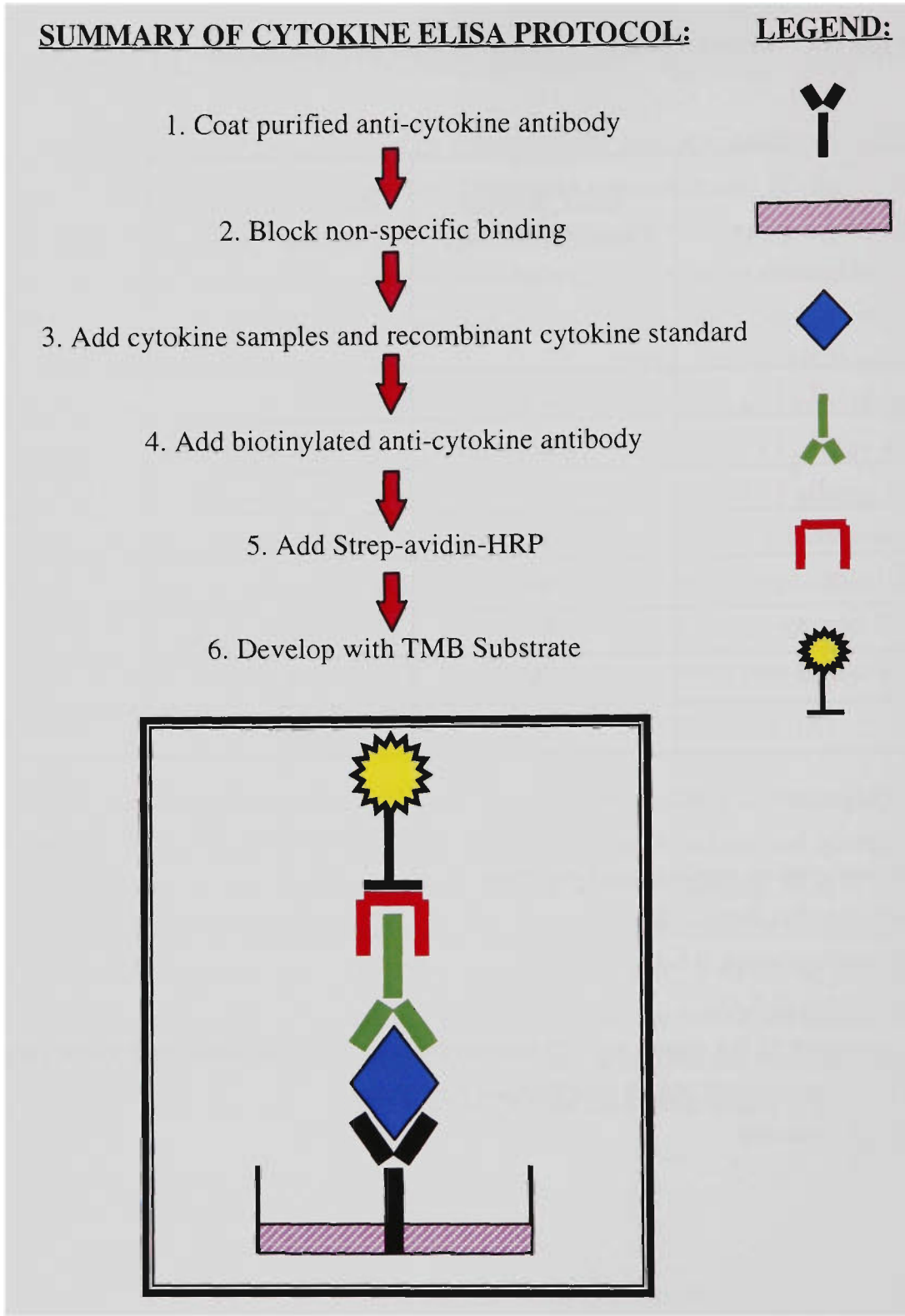


Figure 2.6: Schematic representation of sandwich cytokine ELISA protocol

Purified anti-cytokine antibodies were coated at pre-determined concentrations. Non-specific binding of cytokine was blocked with 2% BSA / PBS and both cytokine samples and recombinant standards incubated overnight at 4^{0}C. Cytokines were detected by reacting TMB substrate with strep-avidin-HRP bound to biotinylated anti-cytokine antibodies. VV-cytokine sample concentrations were calculated from cytokine standard curves.

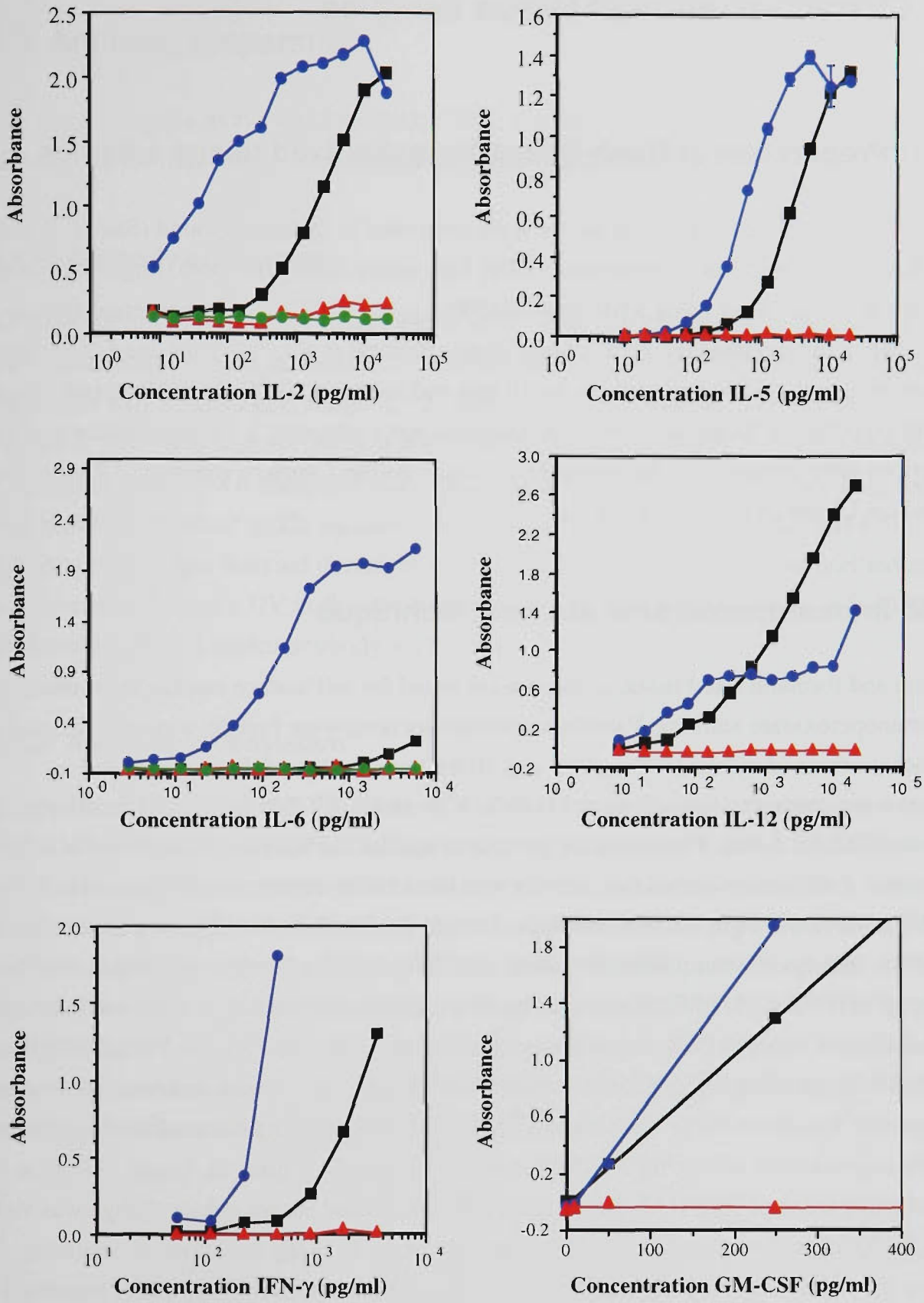


Figure 3.7: VV-cytokine concentrations as determined by a sandwich ELISA

VV- cytokine titres (■), recombinant cytokine standards (●), and VV alone (▲). VV-cytokine controls (●).

5. Characterisation of tissue samples

5.1: Preparation of fresh frozen/formalin fixed tissue samples

Fresh tissue samples to be fresh frozen were immersed in OCT compound (Sakura, California, U.S.A) and snap frozen in isopentane (BDH Laboratory Supplies, Poole, England). Samples were cut 5-6 μm thick using a Microm HM500 cryostat (MICROM Laborgerate, Strässe, Germany) and mounted on silane coated slides (Rentrop et al., 1986), air dried overnight at room temperature, fixed in acetone for 10 min and stored at - 20^{0}C. Formalin fixed samples were prepared by fixing fresh tissue in specimen jars containing 3.7% formeldehyde. Paraffin sections were prepared by Anatomical Pathology, Austin Campus, Austin and Repatriation Medical Centre (Victoria, Australia).

5.2: Immunoperoxidase staining technique

Fresh and formalin fixed tissue sections were tested for cell surface marker expression using immunoperoxidase staining. Paraffin embedded sections were first de-waxed in decreasing concentrations of xylene (100%, 90% and 70%) 2 min each, and then re-hydrated in decreasing concentrations of ethanol (100%, 90% and 70%) 2 min each and finally placed in mouse PBS for 5 min. The following procedure applies for both fresh and formalin fixed sections. Endogenous peroxidase activity was blocked for 40 min at room temperature by 0.5% of 30% w/v H_2O_2 (BDH Chemicals, Dorset, England). Sections were washed three times in PBS, laid out in a humidified container, and incubated for 60 min with 100μl of antibody diluted in 0.5% BSA/DME at room temperature. Excess antibody was removed and slides washed three times in PBS. Secondary antibodies were diluted 1/50 in 0.5% BSA/DME and added to tissue samples for 45 min in a humidified container at room temperature. Excess conjugate was removed by washing in PBS and antibody binding detected after incubating slide sections with 1.5 mg/ml 3-3 diaminobenzidine (DAB, Sigma, St. Louis, U.S.A) in PBS containing 0.3% of 30% H_2O_2 for 5 min or until an optimal brown colour change was visible. Excess DAB was removed and slides washed extensively in tap water before mounting. Slides were stained by covering the sections in eosin for 30 sec, washed in tap water, immersed in haematoxylin for 2 min and washed again in tap water before soaking in Scott's tap water (0.02 M $KHCO_3$, 0.08 M $MgSO_4$) for 1 min. Slides were dehydrated by immersion in increasing concentrations of absolute ethanol (70%, 90%, and 100%) for 2 min each and then increasing concentrations of xylene (70%, 90%, and 100%). Coverslips were prepared with xylene mountant reagent and slides mounted for examination under the microscope.

5.3: Antibody preparation

The anti-$H2^d$ IgG2a monoclonal antibody, 34.1.2s, (Ozato et al., 1982) was purified and biotinylated for immunoperoxidase staining performed in chapter 5.

5.3.1: Antibody purification

Anti-$H2^d$ ascites to be purified was centrifuged at 15,000 x g for 10 min to remove any particulate matter and the supernatant containing antibody, diluted 1:4 in 0.14 M phosphate buffer (pH 8.0). A peristaltic pump (0.5 ml/min) was used to load the diluted ascites onto a Protein A Sepharose column (Protein-A-Sepharose CL4B, Pharmacia, Uppsala, Sweden) equilibrated with pH 8.0 phosphate buffer and excess antibody removed by washing for 30 min with PBS. Purified IgG2a antibody was eluted with 0.1 M citrate buffer (pH 4.5), fractions pooled, and dialysed overnight at 4^0C in PBS. The concentration of purified antibody was determined using a UV visible spectrophotometer (Beckman, California, U.S.A) whereby absorbance 1.43 = 1 mg/ml antibody at 280 nm.

5.3.2: Antibody biotinylation

Biotinylation of purified anti-$H2^d$ antibody was achieved by dialysing the antibody overnight in 0.1 M Borate buffer pH 8.8 (0.2 M Boric acid and 0.2 M Borax solution in distilled water) at 4^0C. The antibody was mixed with biotin ester (Calbiochem, California, U.S.A) [10mg N-hydroxysuccinimide biotin in 1 ml anhydrous dimethylsulphoxide (Ajax Chemicals, N.S.W, Australia)] at a ratio of 1 mg purified antibody: 100 µg biotin ester, and incubated at room temperature for 4 h. Excess active esters were blocked by adding 8 µl of 1 M NH_4Cl per 100 µg of biotin ester for 5 min at room temperature and dialysing overnight at 4^0C in PBS. The concentration of biotinylated antibody was determined using a UV visible spectrophotometer whereby absorbance 1.43 = 1 mg/ml antibody at 280 nm.

6. Cell lines and MUC1 tumour models

6.1: BHK-2I

Mammalian baby hamster kidney cells obtained from Dr. B. Acres (Transgène, Strasbourg, France). BHK cells grow in DMEM supplemented with 10% foetal calf serum (Flow Laboratories, N.S.W, Australia), 2 mM glutamine, 100 U/ml penicillin (CSL, Victoria, Australia), 100 mg/ml streptomycin (CSL, Victoria, Australia).

6.2: MCF-7

MCF-7 is a human mammary breast cancer cell line which expresses cell surface MUC1 and HLA-2 major histocompatibility complexes (Gendler et al., 1987). MCF-7 tumours were cultured in RPMIM media.

6.3: P815-MUC1

P815-MUC1 and P815-Tm2 are DBA/2 P815 mastocytoma cell lines transfected with the cDNA of the transmembrane form of human MUC1 and was provided by Dr B Acres (Acres et al., 1993). Both P815-MUC1 (Tm211) (high MUC1 surface expression) and P815-Tm2 (low MUC1 surface expression) are cultured in RPMIM growth media. MUC1 expression is selected for every 14-20 days with 1.25 mg/ml G418 Sulfate (Gibco BRL, New York, U.S.A) and MUC1 expression tested for serologically.

6.4: RMA-MUC1

RMA-MUC1 is a C57BL/6 RMA T cell thymoma cell line transfected with the human MUC1 gene (Graham et al., 1995). RMA-MUC1 expresses cell surface MUC1 and was cultured in RPMIM growth media. MUC1 expression was selected for every 25-30 days with 1.1 mg/ml Hygromycin B (Boehringer Mannheim, Indianapolis, U.S.A) and expression tested for serologically.

6.5: DA3-MUC1

DA3-MUC1 is a metastatic BALB/c DA3 mammary cell line transfected with the cDNA of the transmembrane form of human MUC1 and was provided by Dr N Smorodinsky (Tel Aviv University, Tel Aviv, Israel; Baruch et al., 1997). DA3-MUC1 cells expressing surface MUC1 were cultured in DMEM growth media supplemented with x1 OPI [x100 stock = 3.3 g oxaloacetic acid, 1.24 g pyruvic acid and 5000 units of Actrapid (human insulin)]. MUC1 expression was selected for every 14-20 days with 1.25 mg/ml G418 Sulfate and expression tested for serologically.

7. Characterisation of MUC1 transgenic mice

MUC1 transgenic (Tg) mice expressing a 40kB human MUC1 gene product were developed by Acres et al. (1998) in CBA x C57BL/6 F_1 mice. The expression of MUC1 on tissues from the MUC1 Tg mice was determined by immuno-histology with the anti-MUC1 antibody, BCP-8 (Xing et al., 1992); and was evident in lung bronchioles, β-islets of the pancreas, kidney tubules and strongest expression in the stomach (Acres et al., 1998). MUC1 Tg mice were supplied by Dr B Acres (Transgene, France) and crossed onto a DBA/2 background at the Austin Research Institute (Victoria, Australia) for immunotherapy studies.

7.1: Production of MUC-1 cDNA probe

7.1.1: MUC1 "midi" DNA preparation from *Escherichia coli*

E. coli transformed with pGEX-3X plasmids containing 5 x 20 amino acid VNTR regions of the MUC1 cDNA clone, PDF9.3 (Siddiqui et al., 1988) were grown overnight at 37°C with agitation, in Luria broth supplemented with 100 µg/ml Ampicillin (Sigma, St. Louis, U.S.A). Cultures were centrifuged for 10 min at 2,500 rpm at 4°C and the pellets resuspended in 2 ml of Solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0). To the preparation, 4 ml of Solution II (0.2 M NaOH, 1% SDS) was added and inverted 3-4 times and 3 ml of Solution III (5 M potassium acetate, 11.5 ml glacial acetic acid) added before transferring the DNA preparation to 1.5 ml eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuging at 13,000 rpm at room temperature for 5 min. The pellets were collected and 4.5 ml isopropanol (BDH Chemicals, Victoria, Australia) added and incubated for 30 min at 4°C. Following centrifugation at 13,000 rpm for 4 min at 4°C, all supernatants were discarded and 1 ml 75% cold ethanol added. DNA preparations were centrifuged at 13,000 rpm for 4 min at 4°C, supernatant discarded, and ethanol washes repeated twice. DNA was completely air dried and 4 ml TE buffer (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0) containing 400 µg of pancreatic RNAse added and incubated for 15 min in a 37°C water bath to degrade all RNA. A phenol-chloroform extraction was performed whereby a 50% phenol/chloroform mixture was added to the DNA preparation, vortexed, and centrifuged at room temperature for 5 min at 2,000 rpm. The supernatant was removed and replaced with an equal volume of chloroform, vortexed and centrifuged at room temperature for 5 min at 2,000 rpm. To the pellet, 10 ml 100% ethanol containing 3 M sodium acetate was mixed and incubated for 1 h at -20°C. The DNA preparation was ultra-centrifuged in oakridge tubes using the SS34 rotor for 10 min at 10,000 rpm at 4°C. The pellet was washed x 2 with 70% ethanol and then 100%

ethanol in eppendorf tubes and the resulting DNA preparation resuspended in 250-500 µl TE buffer and stored at -70^{0}C. The concentration of DNA was determined using the UV visible spectrophotometer whereby absorbance of 1.0 = 50 µg DNA at 260 nm.

7.1.2: Agarose gel electrophoresis

A 1% agarose gel was prepared from 1% DNA grade agarose (Sigma, St. Louis, U.S.A), 1% TBE buffer (0.9 M Trizma base, 0.9 M Boric Acid and 0.5 M EDTA pH 8.0) in double DW and 1 µl of Ethidium bromide (Sigma, St. Louis, U.S.A) to test the quality and molecular weight of the plasmid DNA preparation. DNA samples consisting of 10 µg of the plasmid DNA prep, TE buffer and loading dye were electrophoresed on the gel with a lambda Hindi III standard (New England Biolabs, Hertfordshire, England) at 100 volts for 30 min. The gel was scanned using the Eagle Eye II Scanner (Stratagene) (Figure 2.8). The MUC1 plasmid DNA prep was of the correct size, 2.3 kB.

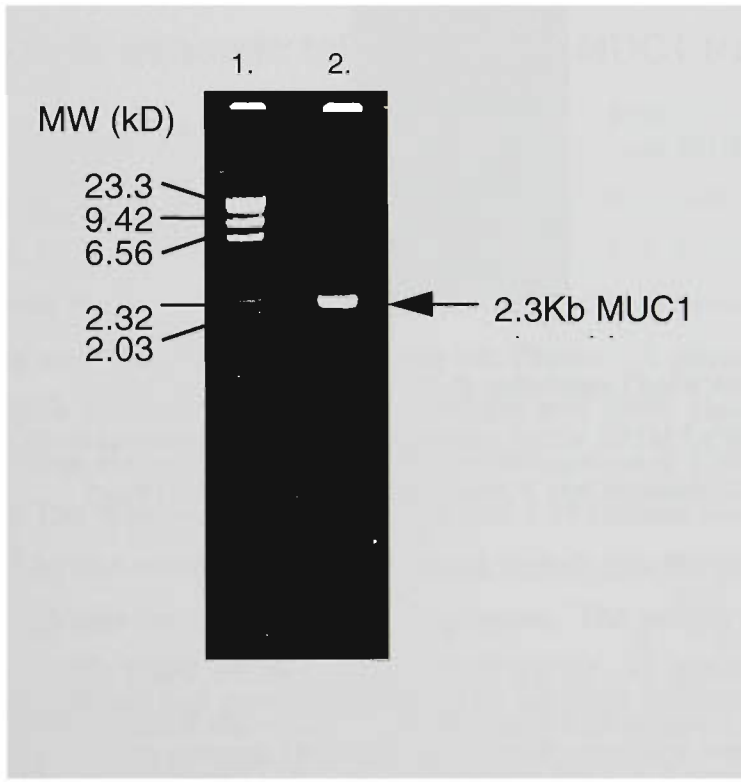


Figure 2.8: MUC1 cDNA Plasmid PUC18

Gel electrophoresis of MUC1 DNA plasmid preparation. Lane 1: Lamda HINDI III standard; lane 2: MUC1 cDNA plasmid PUC18 (10 µg).

7.1.3: Restriction digests and purification of MUC1 DNA probe

MUC1 cDNA DF9.3 is inserted into the *Eco*RI sites of the PUC18 vector (Siddiqui et al., 1988). To obtain purified cDNA as a probe for screening genomic DNA from MUC1 Tg mice, a restriction digest using the *Eco*RI enzyme was performed. MUC1 cDNA was digested with *Eco*RI (1 unit enzyme = 1 μg cDNA) for 2 h in a 37°C water bath. The digestion was checked on a 1% agarose gel and DF9.3 cDNA observed at the correct molecular weight of 0.34 kB (Figure 2.9).

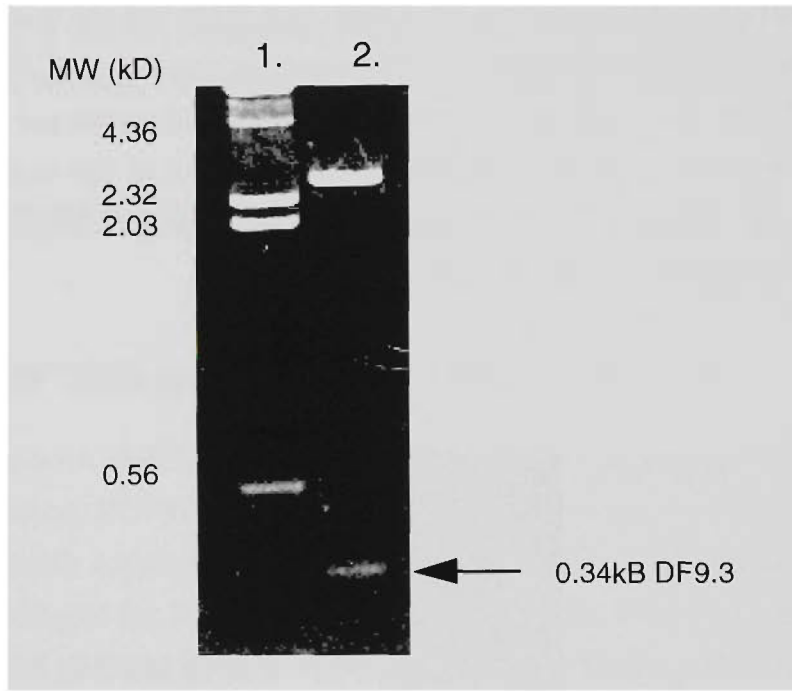


Figure 2.9: MUC1 cDNA EcoRI restriction digest

Gel electrophoresis of DF9.3 MUC1 cDNA removed from the PUC18 plasmid by ECORI restriction enzyme. Lane 1: Lamda HINDI III standard; lane 2: EcoRI MUC1 cDNA digest (10 μg)

MUC1 cDNA was purified from the PUC18 vector using low melting agarose gel purification. A 1% low temperature melting (L.T.M) agarose gel was prepared with 1% L.T.M agarose (NuSeive, FMC Bioproducts, Rocklands, U.S.A), TBE buffer and ethidium bromide. Digested DNA was electrophoresed at 60 volts at room temperature and the DF9.3 cDNA cut from the gel using a sterile blade under UV light from the Eagle Eye Scanner (Stratagene). The gel containing cDNA, was placed in an eppendorf tube and melted at 65°C on a single dry block heater (Ratek Instruments, Victoria, Australia). A 1% agarose gel was used to demonstrate purified MUC1 cDNA (0.34 kB).

7.1.4: Labelling MUC1 DF9.3 cDNA probe with ^{32}P

Purified MUC1 cDNA DF9.3 was labelled with radioactive ^{32}P using the Megaprime DNA labelling System kit, PRN 1604 (Amersham, Buckinghamshire, U.K). A fixed volume of 33 µl containing between 2.5 and 25 ng of DF9.3 cDNA, 5 µl Primers NIF541 and double DW was boiled for 5 min and centrifuged at 13,000 rpm at 4^{0}C and then added to 10 µl labelling buffer, 5 µl ^{32}P dCTP (Amersham, Buckinghamshire, U.K) and 2 µl enzyme for 1 h in a 37^{0}C water bath. After 0.2 M EDTA (BDH Chemicals, Victoria, Australia) was added to the mixture, free ^{32}P was separated from the labelled cDNA by passing the mixture through a column containing G50 Sepharose beads by centrifugation for 1 min at 1,000 rpm. The fraction collected after centrifugation contained ^{32}P labelled MUC1 cDNA which was tested for labelling efficacy by the β-counter (Liquid scintillation analyser; Packard, Canberra, Australia).

7.2: Production of genomic tail DNA from MUC1 transgenic mice

Genomic DNA was prepared from a 1-1.5 cm length of tail from MUC1 Tg mice aged 3 - 5 weeks. Tails were incubated overnight at 55^{0}C in a hybridisation oven (Hybaid Micro-4) with 1 mg of Proteinase K (Boehringer Mannheim, Indianapolis, U.S.A) and lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 10 mM EDTA, 1% NP-40, 1 mM protease inhibitor) in a Vacutainer gelplug tube (Becton Dickinson, Meylan, France). A phenol-chloroform extraction was performed: 100% phenol, 50% phenol/chloroform and 100% chloroform was added to the preparation in three steps with vortexing and centrifugation at 2,000 rpm for 10 min in between each step. The supernatant was removed and 3 M sodium acetate pH 6.0 added and vortexed, followed by the addition of 100% ethanol, mixed into the preparation and centrifuged at 13,000 rpm for 5 min at room temperature. The pellets of DNA were briefly air dried, washed again with 100% ethanol and air dried for 30 - 45 min. Genomic DNA was resuspended in double distilled water and sheened using a 26 gauge 1 ml syringe and stored at 4^{0}C. The concentration of genomic DNA prepared from MUC1 Tg mice was determined by the UV visible spectrophotometer whereby absorbance 1.0 = 50 µg DNA at 260 nm.

7.3: Hybridisation of ^{32}P radiolabelled probe to genomic DNA

The ^{32}P -MUC1 cDNA probe DF9.3 was used to screen for positive MUC1 Tg mice by hybridisation. Genomic DNA (10 µg), SSC buffer (3 M sodium chloride, 0.3 M sodium citrate pH 7.0) and dye were mixed together, transferred to a 96-well dot blot apparatus and absorbed

onto 0.45 µm Hybond N^+ Nylon membrane (Amersham, Buckinghamshire, U.K) under suction. The nylon membrane was removed and incubated for 5 min at room temperature in denaturising solution (0.5 M NaOH and 1.5 M NaCl) and 5 min at room temperature in neutralising solution (1 M Tris pH 8.0 and 1 M NaCl) and air dried. The nylon membrane was loosely folded into two nylon mesh strips and incubated in a hybridisation oven (Hybaid Micro-4) containing prehybridisation solution (0.5% skim milk powder, 0.1 mg/ml Herring sperm DNA, 5 x SSC, 5% SDS and 0.2 M phosphate) overnight at 42°C. The ^{32}P -MUC1 cDNA probe was boiled for 5 min, ice quenched for 5 min and added to the prehybridisation solution containing the nylon membrane for 2-3 h in the hybridisation oven at 42°C to allow hybridisation of the cDNA probe to the genomic DNA. The nylon membrane was then washed for 2-3 h with 2 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS to remove excess ^{32}P. After washing, the nylon membrane was exposed to Kodak Scientific Imaging film (Integrated Sciences, Vic., Australia) for 24 – 72 h at -70°C and developed using a Storm 840 Phosphoimager (Molecular Dynamics, Vic., Australia) (Figure 2.10).

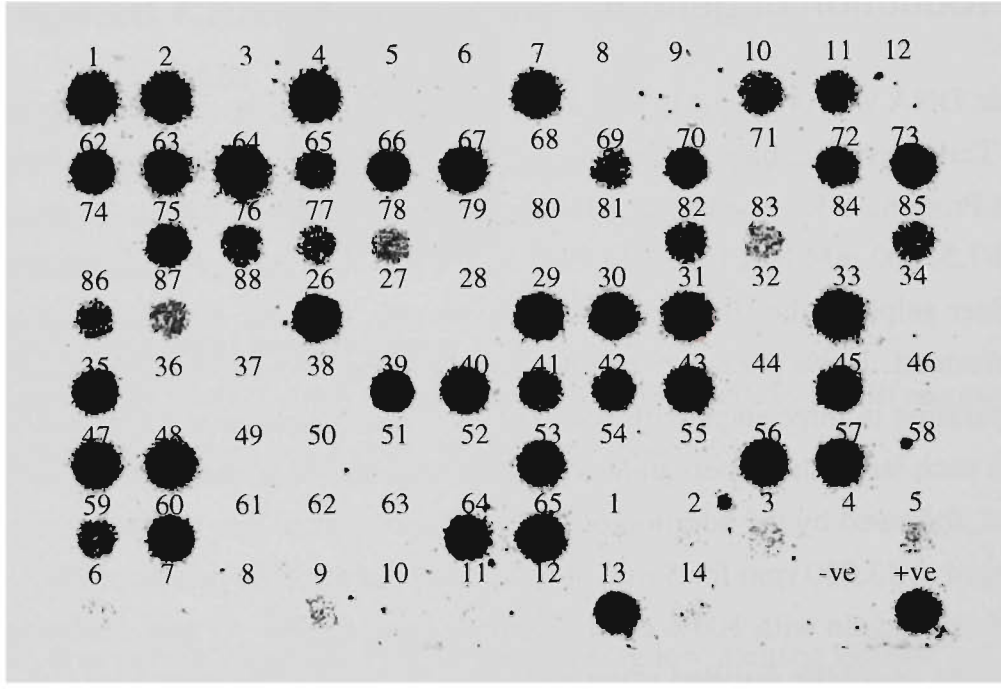


Figure 2.10: Hybridisation of MUC1 probe to genomic DNA from MUC1 transgenic mice

Each number represents a genomic DNA preparation from a MUC1 transgenic mouse. Negative (-ve) control and positive control DNA samples are from wild-type DBA/2 and MUC1 transgenic mice.

Chapter 3:

CD4 and CD8 cytokine profiles for mannan-MUC1 immunotherapy

Catherine J Lees, Vasso Apostolopoulos, and Ian F.C McKenzie. Cytokine production from murine CD4 and CD8 cells after mannan-MUC1 immunisation. Journal of Interferon and Cytokine Research, 19:1373-1379, 1999.

Summary

Immunotherapy with oxidised mannan-MUC1 fusion protein (MFP) leads to a T1 immune response characterised by the generation of CTLs, few antibodies, secretion of IL-2, IL-12 and IFN-γ and tumour protection. Immunotherapy with reduced MFP or FP alone leads to a T2 immune response characterised by the generation of MUC1 antibodies, few CTLs, IL-4 secretion and no tumour protection. In these studies, cytokine production from T cells was measured in cultures containing whole spleens. This study reports the cytokine secretion patterns from spleen cells separated into $CD4^+$ and $CD8^+$ T cells obtained from mice immunised with either oxidised MFP, reduced MFP or FP, or from the simultaneous administration of oxidised MFP and FP.

Immunisation with oxidised MFP led to the secretion of T1 cytokines from $CD8^+$ T cells (IL-2, IFN-γ and TNF-α) and from $CD4^+$ T cells (IL-2 and IFN-γ). IL-12 production, presumably from activated macrophages, was observed in $CD8^+$ but not $CD4^+$ cultures. Immunisation with either reduced MFP or FP led to the secretion of predominantly T2 cytokines from $CD4^+$ T cells (IL-4 and IL-10) and IL-2 production in both $CD4^+$ and $CD8^+$ T cell cultures. The simultaneous immunisation of both oxidised MFP and FP led to the production of both T1 and T2 cytokines from $CD8^+$ T cells (IL-2, IFN-γ and TNF-α) and $CD4^+$ T cells (IL-2, IFN-γ, IL-4, and IL-10) and IL-12 production in $CD8^+$ cultures that is, both types of immune responses could occur together. The results demonstrate that the cellular immune response observed in oxidised MFP-immunised mice is dependent on the T1 cytokine profile secreted by $CD8^+$ T cells, and the simultaneous production of both T1 and T2 cytokines is not cross-inhibitory.

3.1 Introduction

The original TH1/TH2 paradigm described that murine $CD4^+$ T cells produce two types of cytokines, Th1 (T1): IL-2, IFN-γ, and TNF-β, and Th2 (T2): IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann 1986, 1996; Coffman 1991). Murine $CD8^+$ T cells have now also been subdivided into two subsets, Tc1 and Tc2, following the T1 and T2 cytokines produced (Salgame 1991). Recently, however, studies have emerged demonstrating that a rigid T1/T2 subdivision does not occur for some murine and human T cell clones (Paliard 1988; Kelso 1998). What does remain clear are the functions associated with the cytokines in which T1 cells are primarily involved in cellular cytotoxic and DTH immune responses, and T2 cells in antibody and allergic reactions. For the immunotherapy of solid tumours, it is likely that successful therapy will require the *in vivo* production of the T1 cytokines, IL-2, IFN-γ, IL-12 and TNF, either from or associated with activated $CD8^+$ cells and a $CD8^+$ CTL response, presumably with $CD4^+$ help. Production of the T2 cytokines, IL-4, IL-5, and IL-10, associated with antibody production, appears to have little effect on solid tumours (Boon 1993).

MUC1 based cancer immunotherapy with mannan and MUC1 can induce either T1 (CTL) or T2 (antibody) immune responses depending on the chemical conjugation of mannan MUC1 fusion protein (MFP) (Apostolopoulos 1995, 1996). In mice, tumour protection is associated with mannan conjugated to MUC1 FP in the oxidised form (oxidised MFP), and is characterised by the production of CTL and T1 cytokines (IL-2, IL-12, IFN-γ) from whole spleen cell cultures (Apostolopoulos 1995, 1996). By contrast, MUC1 FP alone or mannan conjugated to MUC1 FP and subsequently reduced (reduced MFP) does not protect against $MUC1^+$ tumours, the immune response being characterised by a weak CTL response, MUC1 antibodies and the secretion of the T2 cytokine IL-4 (Apostolopoulos 1995). Recently, it was also demonstrated that the simultaneous immunisation of oxidised MFP and FP resulted in immune responses whereby both cellular and antibody mediated responses were induced together (Lofthouse 1997), rather than the two acting antagonistically. This is perhaps of more importance in infectious disease than in immune responses to tumours (Jankovic 1999). The role that cytokines play in these immune responses has not been examined, other than from whole spleen cultures, and it is not known if a T1/T2 bias exists between the cytokines produced by either $CD4^+$ or $CD8^+$ T cells. To further characterise the T1 and T2 cytokine profiles associated with the MFP immune responses, $CD4^+$ and $CD8^+$ T cell cultures were established after immunisation with oxidised MFP, reduced MFP, MUC1 FP, or the simultaneous administration of ox MFP and FP, and the production of IL-2, IL-4, IL-10, IL-12, IFN-γ and TNF-α cytokines was measured.

3.2 Materials and methods

MUC1 antigens

MUC1 fusion protein (five repeats from the VNTR region of the MUC1 protein core) was produced in a pGEX-3X bacterial expression system (Siddiqui 1988). Purified MUC1 FP was conjugated to oxidised mannan (Sigma, St Louis, U.S.A) (Apostolopoulos 1995) using sodium periodate in pH 9.0 bicarbonate buffer (refer to chapter 2, section 1). Reduced MFP was produced by reacting oxidised MFP with sodium borohydride (to reduce the Schiff bases and aldehydes to amines and alcohols) (refer to chapter 2, section 1) (Apostolopoulos 1993, 1995). The peptide Cp13-32 [(C) PAHGVTSAPDTRPAPGSTAP] contains two MUC1 VNTR regions following N-terminal dimerisation and was synthesised (>95% pure) with a peptide synthesiser (Applied Biosystems, Foster City, U.S.A) (Hodges 1975, Kent 1985). Peptide Cp13-32 was used to stimulate spleen cells *in vitro* from MUC1 immunised mice.

Mice and immunisations

Female BALB/c mice aged 6-10 weeks were immunised intraperitoneally with 5µg of either oxidised MFP, reduced MFP, MUC1 FP or, oxidised MFP and FP given on opposite sides. A control, pH 9.0 bicarbonate buffer was also given. Mice were given three 200 µl immunisations on days 0, 7 and 14 and sacrificed on day 21. Spleen cells from three immunised mice per group were pooled, and the results represent the average cytokine production from three experiments, unless otherwise indicated.

Enzyme linked immunosorbent assay

Serum from immunised mice were collected 4 - 6 days after the third injection and tested for MUC1 antibody levels by ELISA (refer to chapter 2, section 2.1). Serum samples were serially diluted (1:2) and added to 96 well round-bottom microtitre plates pre-coated with 10 µg Cp13-32. Non-specific binding was blocked with 2% BSA/PBS. MUC1 antibody levels were detected with sheep-anti mouse HRP (Amersham, Buckinghamshire, U.K) and developed with 0.03% 2,2' – azino – di (3 - ethylbenzthiazoline sulphonate) (Amersham), 0.02% H_2O_2 (100 volumes; Ajax Chemicals) in 0.1 M citrate buffer (pH 4.0). Absorbances were read at 405 nm.

Cytotoxic T cell precursor assay

A limiting dilution cytotoxic T cell precursor assay was performed 14 - 21 days after immunisation with MFP (refer to chapter 2, section 2.2.2). Spleen cells from immunised mice were collected and added in 32 replicates to 96 well microtitre plates varying from 1 x 10^3 to 1.28 x 10^5 cells / well. Stimulator spleen cells (5 x 10^5 cells / well) from (BALB/c x DBA)F_1 naive mice were irradiated and added to all effector wells with 5 μM MUC1 peptide Cp13-32 and 10 U/ml of rh IL-2. Controls containing stimulators and either IL-2 and peptide Cp13-32, or stimulators and effectors were included. Plates were incubated for 7 days at 37^{0}C, 10% CO_2. After incubation, 1 x 10^4 ^{51}Cr-labelled P815-MUC1, or P815 target cells were added. Cultures were incubated for a further 4 h and analysed on a microplate scintillation counter (Packard, U.S.A). The number of CTL precursors present was determined as a frequency of the number of cells not lysed on a logarithmic scale and the number of cells/well on a linear scale.

CD4 and CD8 T cell depletions

Spleen cells from immunised mice were collected and pooled in RPMI, treated with 0.83% NH_4Cl to remove red blood cells, washed and resuspended in complete RPMI medium supplemented with 10% foetal calf serum (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Sigma), 0.02 M HEPES (Commonwealth Serum Laboratories [CSL], Victoria, Australia), 100 U/ml of penicillin and 100 U/ml streptomycin (CSL). $CD4^+$ T cells were depleted from spleen preparations (producing a $CD8^+$ T cell enriched population) by incubating 5 x 10^7 washed CD4 (L3T4) Dynabeads (Dynal, Victoria, Australia) per 5 x 10^7 effector spleen cells in 5ml RPMI medium for 45 min and rotating at 4^{0}C until rosette formation was evident. $CD4^+$ T cell-Dynabead complexes were removed from the spleen cell population with a magnetic particle concentrator, MPC-1 (Dynal), for 2 min, and the supernatant containing the remaining spleen cells collected. This method was used to remove $CD8^+$ T cells from spleen preparations (producing a $CD4^+$ T cell enriched population) using CD8 (Lyt-2) Dynabeads.

Flow cytometry and antibodies

Lymphocyte populations were examined by flow cytometry (refer to Chapter 2, section 3). Fluorescein (FITC)-labelled or phycoerythrin (PE)-labelled anti-mouse CD3 (Pharmingen, San Diego, U.S.A), PE-labelled anti-mouse CD4 (Pharmingen), and PE-labelled anti-mouse CD8 (Pharmingen) monoclonal antibodies were used for analysis. Spleen cells (2 – 5 x 10^5 cells/ml) were prepared for flow cytometry by incubation with 180 μg/ml heat-aggregated gamma globulin (HAG) for 30 min at 4^{0}C to block non-specific Fc receptor binding, washed

three times with 0.5% bovine serum albumin (BSA)/phosphate buffer, and incubated for 45 min at 4°C with 1 μg/ml of antibody and 0.5% BSA/phosphate buffer. Background levels were determined using isotype controls, and spleen cells were incubated with 0.5% BSA/phosphate buffer and no antibody. After washing three times in phosphate buffer to remove excess antibody, the cells were transferred to FACScan tubes for two colour fluorescence analysis. Results were expressed as the mean ± standard deviation (SD) of the data based on several experiments.

T cell cultures

Spleen cells obtained from immunised mice (oxidised MFP, reduced MFP, FP, oxidised MFP + FP or pH 9.0 buffer) were mixed with synthetic peptide Cp13-32 (90 μg/ml) and irradiated naive BALB/c spleen cells ($2x10^6$) as antigen presenting stimulator cells in culture. Each culture contained $2x10^6$ cells from immunised mice with either (1) whole spleen cells (containing both $CD4^+$ and $CD8^+$cells), (2) $CD4^+$cells ($CD8^+$ depleted), or (3) $CD8^+$ cells ($CD4^+$ depleted). Control cultures were (1) effectors and stimulator cells without depletion and (2) synthetic peptide Cp13-32 and stimulator cells. Cells were cultured in 24-well flat-bottomed (Costar Corp, Cambridge, MA) in complete RPMI culture medium for 72 h, and supernatants were collected and tested for cytokine production by ELISA.

Cytokine production from T cell cultures

Cytokine assays were performed by ELISA (Pharmingen) to detect IL-2, IL-4, IL-10, IL-12, TNF-α and IFN-γ (refer to Chapter 2, section 4.4). All ELISA assays were set up in duplicate, and supernatants were tested from three independent experiments. Diluted purified anticytokine capture antibodies (1-4μg/ml) were coated onto protein binding ELISA plates (Nunc, Roskilde, Denmark) in 50 μl 0.1M Na_2HPO_4 binding solution, pH 9.0, overnight at 4°C. Plates were washed three times in 0.05% Tween-20/phosphate buffer before blocking in 200 μl 2% BSA/phosphate buffer for 30 min at ambient temperature. After washing, 100 μl sample supernatants and recombinant cytokine proteins were serially diluted and incubated overnight at 4°C. Plates were thoroughly washed and dried and 100 μl of 1-2 μg/ml anti-cytokine detection antibody added and incubated for 1 h at ambient temperature. After washing, 100 μl streptavidin-HRP conjugate (Amersham, Buckinghamshire, U.K) (1:500 dilution) was added to the plates and incubated for 30 min at ambient temperature before detecting cytokine by adding 100 μl of substrate (0.03% 2,2-azino-di-3-ethlybenzthiazoline sulphonate/0.02% H_2O_2). Absorbance was measured at 450 nm on an ELISA plate reader and cytokine concentrations determined from the standard curve. The linear region of each cytokine ELISA standard curve varied between 20-200 pg/ml and 2000-3000 pg/ml. The results were statistically analysed by analysis of variance (ANOVA). There was little

statistical difference ($p > 0.05$) in the amounts of cytokines produced between culture replicates. Similarly in most cases where virtually no cytokines were produced, the difference between the secreted and non-secreted cultures was statistically significant ($p < 0.05$). The one exception was with TNF-α (Figure 3.4F).

3.3 Results

Immune responses in mice

To ensure effective MUC1 immunisation prior to determining cytokine profiles *in vitro*, CTLp frequencies and MUC1 antibody levels were measured in mice given three injections of either oxidised MFP, reduced MFP, FP, oxidised MFP + FP or pH 9.0 bicarbonate buffer. The CTLp frequencies produced from the immunised mice (oxidised MFP - 1/12000, reduced MFP - 1/89000, FP - 1/42000 oxidised MFP and FP - 1/19000) were in accordance with previous published data (Apostolopoulos 1996) (Figure 3.1).

Similarly, MUC1 antibody titres produced from immunised mice (weak titres in mice immunised with ox MFP and pH 9.0 buffer, moderate titres in mice immunised with oxidised MFP + FP and reduced MFP and high titres in mice immunised with FP alone) were in accordance with previous published results (Lofthouse 1997) (Figure 3.2). The CTLp and antibody results confirmed the immune responses produced from the MUC1 immunised mice were representative of previous studies and that the spleen cells from these mice could therefore be confidently used for cytokine analysis.

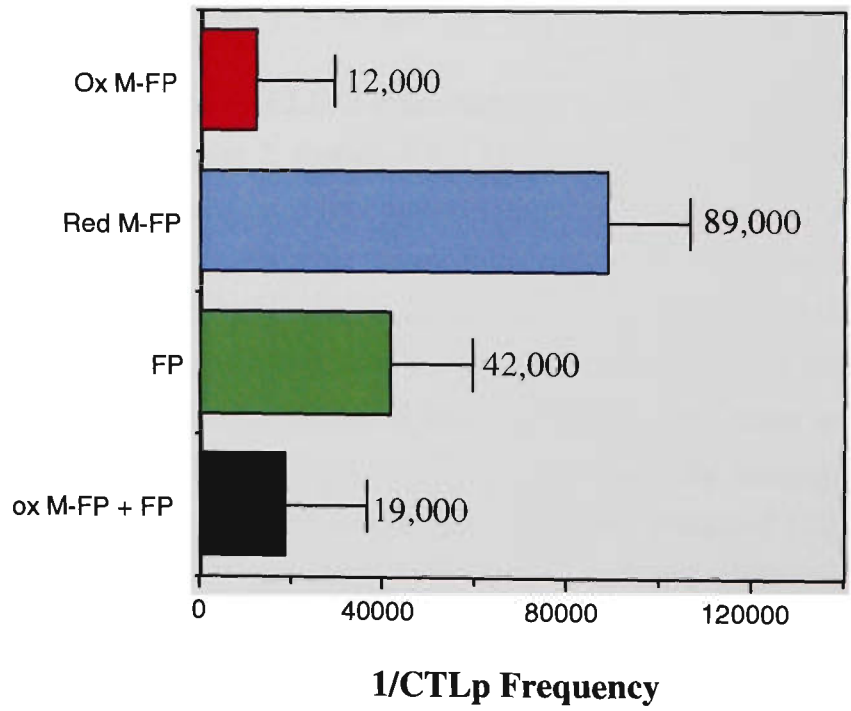


Figure 3.1: MUC1 CTLp immune responses in immunised mice. MUC1 specific CTLp frequencies in BALB/c mice injected once with either 5 μg oxidised (ox) MFP, 5 μg reduced (red) MFP, 5 μg FP or 2.5 μg oxidised MFP + 2.5 μg FP intraperitoneally. The data represents the average CTLp frequency from three determinations and the standard error of the data. CTLp frequencies in mice injected with pH 9.0 bicarbonate buffer < $1/10^6$.

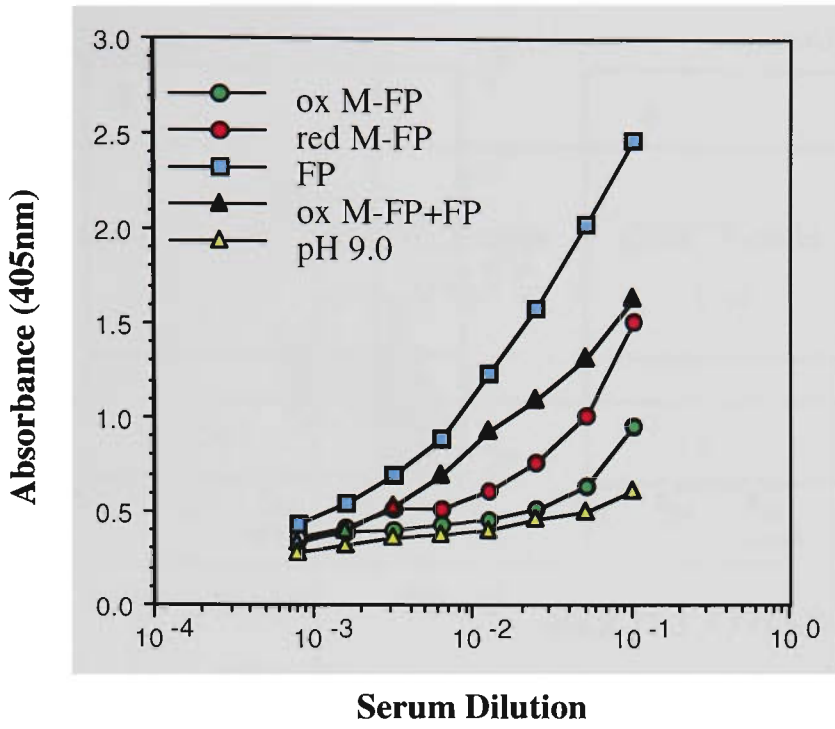


Figure 3.2: MUC1 antibody titres from the sera of BALB/c mice (x3) given three I.P injections with either pH 9.0 bicarbonate buffer, 5 μg oxidised MFP, 5 μg reduced MFP, 5 μg FP or 2.5 μg oxidised MFP + 2.5 μg FP, on days 0, 7 and 14, and eyebled 4-6 days after the final injection. MUC1 serum antibody levels were determined by a direct binding ELISA coated with 10 μg/ml Cp13-32. Serum samples were diluted 1:10 in phosphate buffered saline and serially diluted 1:2. Absorbances read at 405 nm.

T cell populations from spleen cultures

Flow cytometry was used to determine the composition of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in spleen cell cultures after Dynabead depletions (Table 3.1, Figure 3.3). Non-depleted cultures consisted of ~47% (± 5%) $CD3^+$ T cells, of which 28% (± 4%) were $CD4^+$ and 19% (± 7%) $CD8^+$ cells (Figures 4.3 A-C). The remaining 53% (± 8%) of cells consisted of B cells, macrophages and other haematopoietic cells. $CD8^+$ T cell enriched cultures ($CD4^+$ depleted) consisted of ~60% (± 7%) $CD3^+/CD8^+$ cells and <5% $CD3^+/CD4^+$ cells (Figures 4.3 D-F). $CD4^+$ T cell enriched cultures ($CD8^+$ depleted) consisted of ~70% (± 7%) $CD3^+/CD4^+$ cells, <5% $CD3^+/CD8^+$ cells (Figures 4.3 G-I).

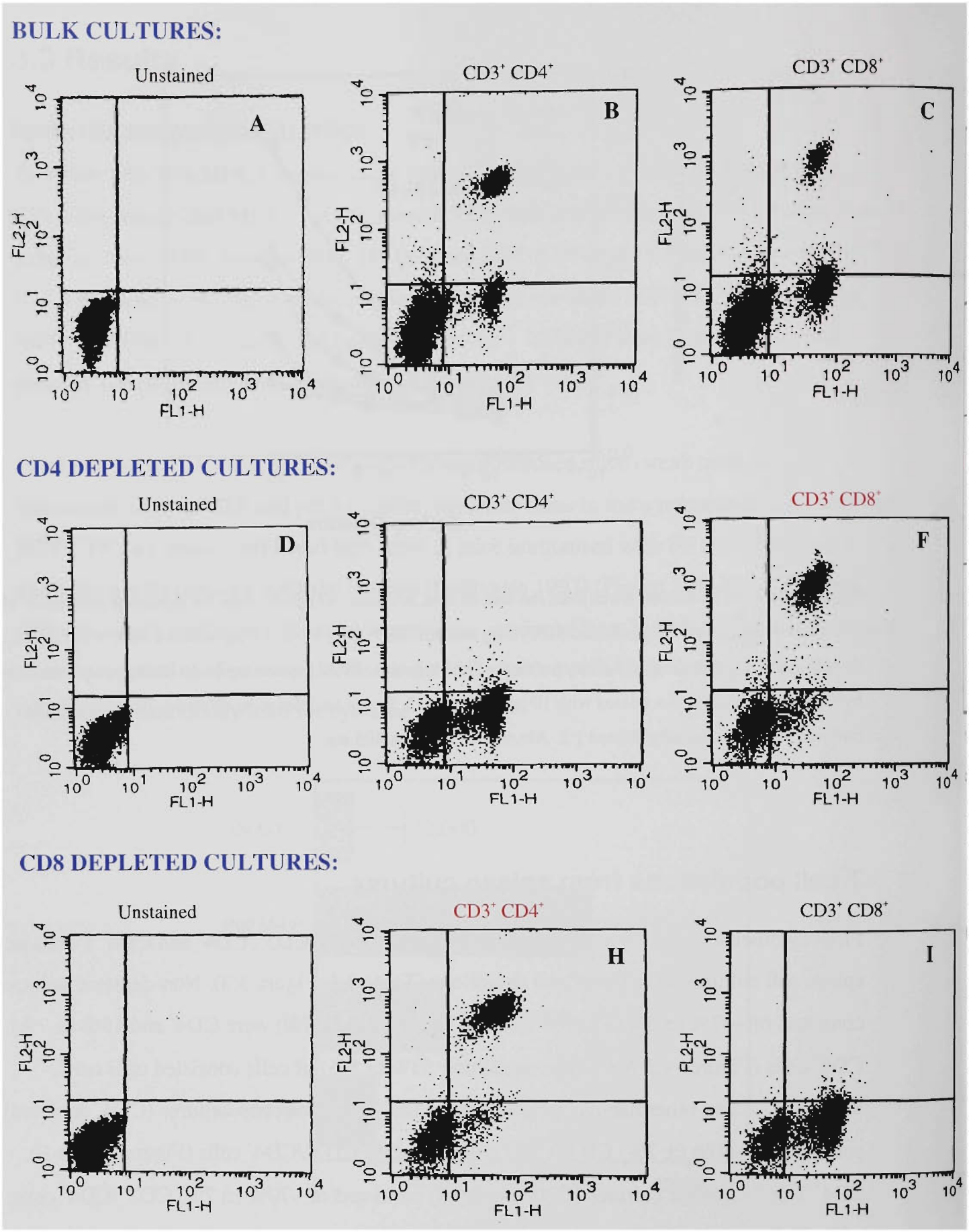


Figure 3.3: Flow cytometry of $CD4^+$ and $CD8^+$ T cell populations from either oxidised MFP, reduced MFP, FP or oxidised MFP + FP immunised mice. FACS profiles representative of T cell populations from all immunised mice.

Table 3.1: $CD3^+$, $CD4^+$, and $CD8^+$ T cell numbers present in depleted cultures determined by flow cytometry

Culture composition	$CD3^+$ T cells (%)	$CD4^+$ T cells (%)	$CD8^+$ T cells (%)	Other Cells[a] (%)
Non-depleted	47 ± 5	28 ± 4	19 ± 7	53 ± 8
CD4 ($CD8^+$ Depleted)	70 ± 7	70 ± 9	< 5	28 ± 10
CD8 ($CD4^+$ Depleted)	60 ± 7	< 5	60 ± 5	37 ± 11

[a] Other cells constitute undepleted B cells, macrophages and other haematopoietic cells. All controls of unstained cells <5%. FACS profiles represent T cell populations from two experiments.

Cytokine production in T cell cultures from MFP immunised mice

Spleen cell cultures from MFP immunised mice were either not depleted ($CD4^+$ + $CD8^+$) or depleted to produce $CD4^-$ ($CD8^+$ enriched) or $CD8^-$ ($CD4^+$ enriched) cultures. T cell cytokine profiles from either:

1) oxidised MFP
2) reduced MFP or FP, or
3) oxidised MFP + FP immunised mice, were compared (Table 3.2 and Figure 3.4).

There were few or no cytokines produced in the absence of peptide (0 - 120 pg/ml). The mean ± SD (pg/ml) is shown in Figure 3.2; only the means are noted in the text.

Table 3.2: Cytokine profiles from $CD8^+$ T cells and $CD4^+$ T cells from MFP immunised mice determined by ELISA.

	Oxidised MFP		Reduced MFP		FP		Oxidised MFP + FP		pH 9.0 buffer	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
IL-2	+	+	+	+	+	+	+	+	-	-
IL-4	-	-	+	-	+	-	+	-	-	-
IL-10	-	-	+	-	+	-	+	-	-	-
IL-12	-	+	-	-	-	-	+	+	-	-
IFN-γ	+	+	-	-	-	-	+	+	-	-
TNF-α	-	+	-	-	-	-	-	+	-	-

[a] +, cytokine present above detection threshold limit (values >50-100 pg/ml);
- , cytokine not present above detection threshold limit (values <50-100 pg/ml). Spleen cells from three immunised BALB/c mice per group were pooled, and the results are the average cytokine production from three experiments.

IL-2 (Figure 3.4A): IL-2 was secreted in similar amounts after immunisation with oxidised MFP in all cell populations; $CD4^+$/ $CD8^+$ (1005 pg/ml), $CD4^+$ (790 pg/ml), and $CD8^+$ (785 pg/ml) cells. When reduced MFP, FP, or oxidised MFP + FP were used, similar amounts of IL-2 were secreted by $CD4^+$/ $CD8^+$, $CD4^+$ or $CD8^+$ (976 - 1105 pg/ml). Thus, both $CD4^+$ and $CD8^+$ cells secrete IL-2 after all modes of immunisation.

IL-4 (Figure 3.4B): IL-4 was not made after stimulation with oxidised MFP (< 30 pg/ml) in any cell population but was found in similar amounts after immunisation with either reduced MFP, FP, or oxidised MFP + FP (presumably from the FP immunisation). In the cultures where IL-4 was produced, it was clear that only $CD4^+$ cells made IL-4 (874 – 1000 pg/ml), as the $CD8^+$ population did not (< 112 - 162 pg/ml) and IL-4 was found in the $CD4^+$/$CD8^+$ cultures (800 - 1010 pg/ml), presumably due to the $CD4^+$ cells.

IL-10 (Figure 3.4C): IL-10 was not produced by mice immunised with oxidised MFP (< 300 pg/ml) but was found after the other immunisations (reduced MFP, FP and both oxidised MFP and FP together produced similar amounts of IL-10 (~2800 pg/ml). Only $CD4^+$ cells produced IL-10, and in contrast to the IL-4 results (Figure 3.4B), in the presence of $CD8^+$ cells (ie. the $CD4^+$/ $CD8^+$ cultures), IL-10 was not produced, that is, $CD8^+$ cells inhibited IL-10 production by $CD4^+$ cells.

IL-12 (Figure 3.4D): IL-12, a macrophage derived cytokine, was produced only in cultures derived from mice receiving oxidised MFP and predominantly by $CD8^+$ cells (700 pg/ml after oxidised MFP; 1000 pg/ml after oxidised MFP + FP). However, smaller amounts (380 pg/ml) were detected in the $CD4^+$ (oxidised MFP) cultures. No IL-12 was detected in mixed $CD4^+$ and $CD8^+$ cultures. As in the IL-10 study, the $CD4^+$ cells inhibited $CD8^+$ cells from producing IL-12.

IFN-γ(Figure 3.4E): IFN-γ was produced only from mice immunised with oxidised MFP (1000 pg/ml) and was made by both $CD4^+$ (780 pg/ml) and $CD8^+$ (700 pg/ml) cells in similar amounts (ie. present in both of these and in $CD4^+$ + $CD8^+$ cells).

TNF-α(Figure 3.4F): TNF-α was produced only after oxidised MFP immunisation and only in the presence of $CD8^+$ cells (i.e. 990 pg/ml in both $CD8^+$ cultures and $CD4^+$/ $CD8^+$ cells).

Thus after immunisation with (1) oxidised MFP, the T1 cytokines IL-2, IFN-γ, TNF-α, and IL-12 were produced predominantly by, or in the presence of, $CD8^+$ cells; (2) reduced MFP, T2 cytokines IL-2, IL-4, and IL-10 were produced, particularly by $CD4^+$ cells; (3) oxidised MFP and FP give both types of response.

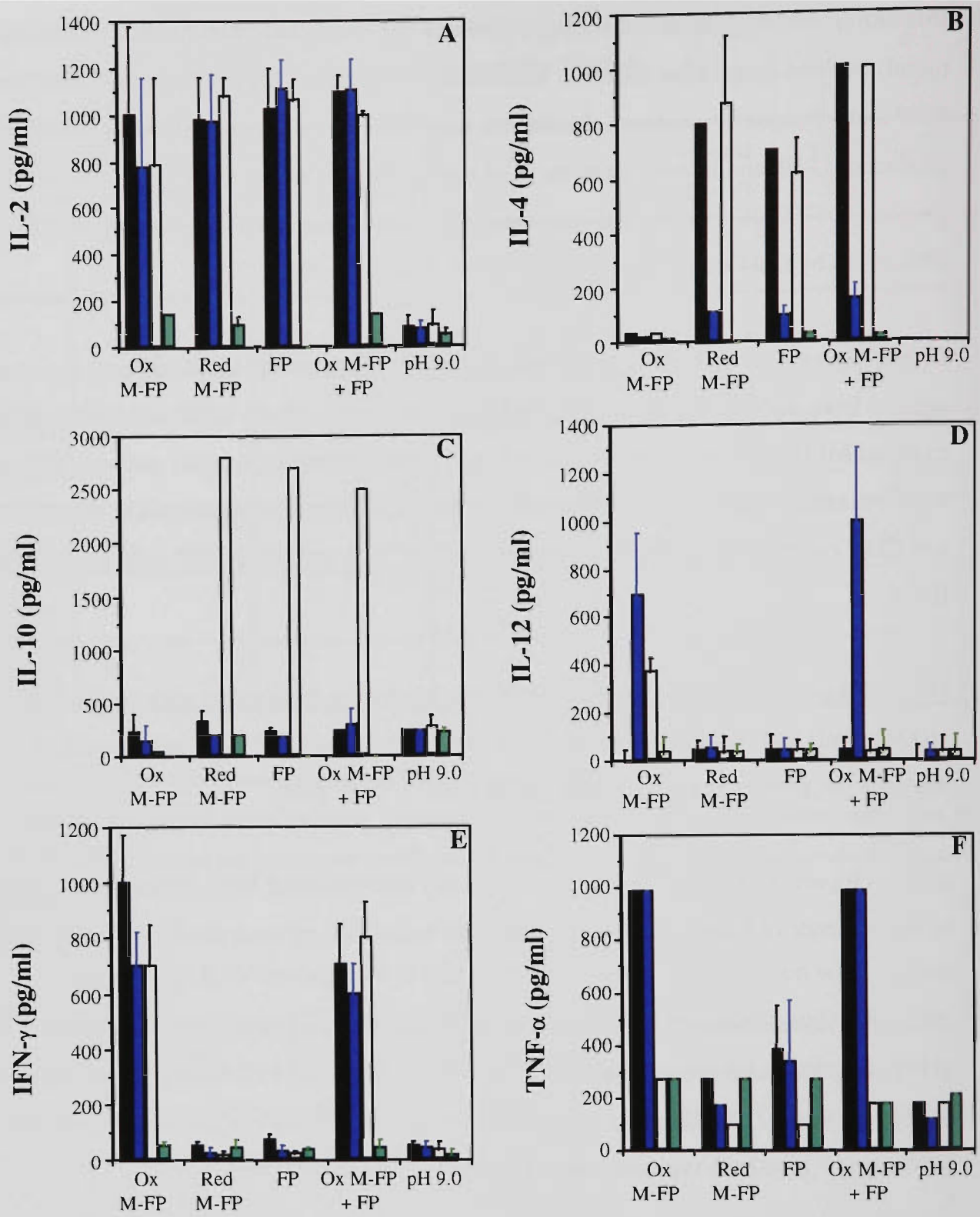


Figure 3.4: ELISA assay showing cytokine production (mean ± SD) after 72 h from depleted BALB/c spleen cultures. $CD4^+/CD8^+$ T cell (■), $CD4^-$ ($CD8^+$) (■), and $CD8^-$ ($CD4^+$) ( ) cultures stimulated with 20 ng/ml Cp13-32 peptide, from mice immunised with either oxidised (ox) MFP, reduced (red) MFP, FP, and oxidised MFP + FP. Mice injected with phosphate buffer and cultures incubated only with phosphate buffer and no peptide (■) were used as controls. ELISA graphs representing (A) IL-2, (B) IL-4, (C) IL-10, (D) IL-12, (E) IFN-γ, and (F) TNF-α production from *in vitro* cultures. Spleen cells from three immunised mice per group were pooled, and the results are the mean (± SD) cytokine production from independent experiments.

3.4 Discussion

Immunisation of mice with oxidised or reduced MFP generates anti-MUC1 immune responses, and the cytokine profiles produced by $CD4^+$ or $CD8^+$ cells in culture was examined (Apostolopoulos et al., 1995,1996).

In oxidised MFP immunised mice, both $CD4^+$ and $CD8^+$ T cells produced T1 cytokines, IL-2 and IFN-γ, but only in the $CD8^+$ T cell cultures were TNF-α and IL-12 produced (IL-12 coming from macrophages). In reduced MFP or FP-immunised mice, IL-2 was also produced by both $CD4^+$ and $CD8^+$ T cells, and the T2 cytokines IL-4 and IL-10 were produced by $CD4^+$ T cells. The cytokine response to oxidised MFP was clearly T1, with $CD8^+$ T cells producing IL-2, IFN-γ, and TNF-α and these cultures producing IL-12. It was of interest that although the *in vivo* response to oxidised mannan-MUC1 is predominantly $CD8^+$ dependent, *in vitro* both $CD4^+$ and $CD8^+$ cells made IL-2 and IFN-γ, and only $CD8^+$ cells made substantial amounts of TNF-α and IL-12 (Table 3.2). As noted previously, the early secretion of IL-2 from activated T cells leads to their differentiation into $CD4^+$ and $CD8^+$ effector T cells secreting both T1 and T2 or Tc1 and Tc2 cytokines (Mosmann et al.,1996). Also, the amounts of some cytokines (eg. IFN-γ and IL-2) produced by individual cultures of $CD4^+$ and $CD8^+$ splenocytes did not equal the sum of the cytokine secreted in $CD4^+$/ $CD8^+$ bulk cultures. This effect is presumably a result of maximum cytokine secretion in these cultures, which subsequently exceeds the linear region of the ELISA standard curve, and therefore, ELISA sensitivity.

It has previously been reported that immunisations with both oxidised MFP and FP induced both cell-mediated immunity and antibody immune responses simultaneously (Lofthouse et al., 1997). In this study, cytokine profiles obtained from the T lymphocytes of mice immunised with oxidised MFP and FP also show the production of both T1 and T2 cytokines. $CD8^+$ T cell cultures produced IL-2, IFN-γ and TNF-α, and $CD4^+$ T cell cultures produced IL-2, IL-4, IL-10 and IFN-γ demonstrating that both subsets of T cells are independently activated by stimulation with MUC1 antigen. In this environment, IL-4/IL-10 and IFN-γ did not induce antagonistic T1 and T2 cytokine pathways as previously reported (Powrie et al., 1993, Li et al., 1994, Sad et al., 1995). In a similar *in vivo* study, both $CD4^+$ and $CD8^+$ T cells were shown to be induced concurrently during tumour growth, and both subsets were functionally efficacious in tumour-bearing hosts (Arca et al., 1997), again demonstrating a

direct role for collaboration between both subsets of activated T cells in tumour immunotherapy studies.

An interesting finding was the production of IL-12 in $CD8^+$ T cell cultures from mice immunised with oxidised MFP or with the combination of oxidised MFP and FP. As IL-12 was detected only in the supernatants of oxidised MFP immunised mice (both alone and in combination with FP) and not in reduced MFP or FP cultures, IL-12 production in this system is $CD8^+$ dependent and presumably produced by activated macrophages (Apostolopoulos, submitted, 1999) or dendritic cells (De Saint-Vis et al., 1998) following oxidised MFP immunisations. Unlike with IL-12 production, TNF-α production occurred in both $CD8^+$ T cells immunised with oxidised MFP and $CD8^+$ / $CD4^+$ cultures demonstrating that the presence of $CD4^+$ cells does not inhibit TNF-α production when in combination, whereas with IL-12 it did. This interaction of cytokines is of interest and further demonstrates the relationship of T1 and T2 responses.

Thus, *in vitro*, in the presence of $CD4^+$ cells (presumably via IL-4 or IL-10) lower amounts of both TNF-α and IL-12 are produced. Similarly, in $CD4^+$ cells the production of IL-10 was decreased in the presence of $CD8^+$ cells, although IL-4 production was not decreased. Thus, the T1 cytokines present (particularly IL-2, IFN-γ, TNF-α and IL-12) could decrease IL-10 and served to decrease TNF-α and IL-12 but not IFN-γ or IL-2. However, these *in vitro* results did not translate into differences *in vivo* where the simultaneous administration of antigen gave a T1 response (oxidised MFP) and a T2 response (reduced MFP or fusion protein) (Lofthouse et al., 1997). The finding could be explained in that although the VNTR of MUC1 is the most immunogenic region, presumably different epitopes are presented by class I and class II molecules (class II epitopes have not yet been mapped), leading to separate induction of $CD8^+$ and $CD4^+$ immune cells. If these occurred in different microenvironments, that is, different anatomical sites in lymph nodes and spleen, the cytokines acting locally may not be sufficiently close to inhibit other immune responses.

Thus, immunisation with oxidised MFP led to a T1 cytokine immune response where $CD8^+$ T cells produce IL-2, IFN-γ, TNF-α, and IL-12 in $CD8^+$ cultures, presumably by macrophages and no IL-4 or IL-10, and $CD4^+$ T cells produce IL-2, and IFN-γ. Immunisation with either reduced MFP or FP led to a T2 cytokine immune response where $CD4^+$ T cells produce IL-2, IL-4 and IL-10 and no IL-12, IFN-γ and TNF-α and $CD8^+$ T cells produce only IL-2.

Immunisation with both oxidised MFP and FP in combination led to a combined T1/T2 cytokine immune response where $CD8^+$ T cells produce IL-2, IFN-γ, TNF-α and IL-12 production in $CD8^+$ cultures, and $CD4^+$ T cells produce IL-2, IL-4, IL-10 and IFN-γ. Thus, although the response can be predominantly T1 or T2, both CD4 and CD8 cells contribute to the response.

Chapter 4:

The effect of T1 and T2 cytokines on the cytotoxic T cell response to mannan-MUC1

Catherine J. Lees, Vasso Apostolopoulos, Bruce Acres, Chin-Swee Ong, Violeta Popovski, Ian FC. McKenzie. The effect of T1 and T2 cytokines on the cytotoxic T cell response to mannan-MUC1. Cancer Immunology Immunotherapy. 48:644-652, 2000.

Summary

MUC1 is a mucin over-expressed in breast cancer and a proposed target for immunotherapy. By immunising mice with MUC1 conjugated to mannan (MFP), $CD8^+$ MHC class I restricted CTLs of high CTL precursor (CTLp) frequency (1/8,000) and with significant tumour protection, can be induced. The effect of various cytokines (IL-2, IL-4, IL-6, IL-7, IFN-γ, and GM-CSF) on the MUC1 CTL immune response was investigated by, a) measuring the frequencies of CTLp in mice immunised with vaccinia virus constructs containing recombinant cytokines and MFP, or b) by immunising cytokine or cytokine receptor knockout (-/-) mice with MFP. Vaccinia virus (VV) constructs containing recombinant cytokines were used either individually or in combination *in vivo* with MFP immunisation. MFP immunisations combined with VV-IL-2, VV-IL-7, and VV-GM-CSF and combinations of VV-IFN-γ + VV-IL-2, VV-IFN-γ + VV-IL-4 or VV-GMCSF + VV-IL-7, increased CTLp frequencies up to threefold (1/17,666: MFP + VV-GMCSF + VV-IL-7) compared to MFP (1/77,500) alone. By contrast, MFP combined with VV-IL-4 decreased the CTLp frequency threefold and VV-IL-6 and VV-IFN-γ had no effect. Studies in cytokine and cytokine receptor gene knockout (-/-) mice demonstrated that IL-2 -/- and IL-7 receptor -/- produce the same CTLp response to MFP as do control mice, whereas responses in the IL-6 -/-, IL-10 -/- and IFN-γ -/- mice were marginally improved and responses to MFP in IL-4 -/- and TNF-receptor 2 -/- mice were weaker. In spite of the increase in CTLp frequency, this was not reflected in an *in vivo* tumour model. Tumour challenges using $MUC1^+$P815 cells demonstrated that the addition of cytokines had little additive effect on the already effective tumour-regression capabilities of MFP alone.

4.1 Introduction

Oxidised mannan, coupled to MUC1 fusion protein (MFP), has previously been shown to successfully induce MUC1 specific tumour immunity in mice, generating a high CTLp frequency, $CD8^+$ MHC-restricted CTLs, low antibody titres and significant tumour protection (Apostolopoulos et al., 1995, 1996). The cytokines produced by spleen cells from mice immunised with oxidised MFP demonstrated a distinct T1-type profile consistent with the immune response generated, with IL-2 and IFN-γ production. However, mice immunised with reduced MFP produce a low CTLp frequency, high antibody titres, little tumour protection and IL-4 production, i.e. T2-type immunity (Apostolopoulos et al., 1996a). As tumour protection to MUC1 is associated with $CD8^+$ CTL and T1-type cytokine production in mice immunised with MFP (Pietersz et al., 1998), it was of interest to determine the effect additional T1 or T2 cytokines had on the MUC1 immune response.

Cytokines have been used extensively to manipulate immune responses in animal models and as a result, many have progressed into clinical trials and use in humans eg. IL-2 in metastatic renal cell carcinoma and melanoma (Danheiser et al., 1997). Many of these studies have focused on the use of recombinant cytokines, administered either locally or intratumourally by repeated low-dose injections including IL-1, IL-2, IL-4 and IFN-γ (Musiani et al., 1997), or on cytokine gene therapies whereby murine tumours have been transfected to secrete cytokines *in vivo* including IL-2, IL-4, IL-6, IL-7, IL-12, GM-CSF, TNF, IFN-γ etc. (Colombo et al., 1994, Tepper et al., 1994, Dranoff et al., 1995). In most of these examples the amount of cytokine delivered to the tumour determines both the intensity of tumour rejection and immune response; and it is of interest that high levels of cytokines can lead to rapid tumour rejection, with little protection from subsequent challenges, whereas low doses induce slower rejection rates of tumour but also induce memory which protects from further challenges (Cavallo et al., 1992).

The T1-type cytokines IL-2, IFN-γ, IL-12, and TNF-β (lymphotoxin-α) (Mosmann et al., 1986, 1989, Cherwinski et al., 1987, Salgame et al., 1991) have generally been favoured for use in tumour immunotherapy as their production results in the development of a cellular immune response and tumour protection in many models. Of the T1 cytokines

studied, IL-2 has been used extensively in murine (Bubenik et al., 1990, Fakhari et al., 1995, Fearon et al., 1990, Mule et al., 1984) and human (Rosenberg et al., 1987) tumour models because it is able both to increase T cell proliferation and recruitment to tumour sites and to induce anti-tumour immune responses. IFN-γ has also proven to be an effective anti-tumour cytokine inducing tumour regression and protection, indicated by CTL induction in tumour models including fibrosarcomas (Gansbacher et al., 1990a), mammary adenocarcinomas (Lollini et al., 1993), and lung and colon carcinomas (Esumi et al., 1991, Porgador et al., 1993). More recently, IL-12 has been much favoured as it has shown therapeutic effects in tumour-bearing animals (Nastala et al., 1994, Noguchi et al., 1995, reviewed by Tahara et al., 1995). Owing to the promising results of IL-12 gene therapy, clinical trials are now in progress (Leonard et al., 1997).

Many of the T2-type cytokines IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Mosmann et al., 1986, 1989, Cherwinski et al., 1987, Salgame et al., 1991) known for their involvement in humoral responses, have also been studied for their effect on tumourigenicity and found to be effective in many tumour models. IL-4, which is commonly known to exert its biological effects on the proliferation, activation and differentiation of B cells has also shown to play an important role in T cell tumour immunity (Brown et al., 1997), and has been engineered into tumour cells to treat various cancers including renal carcinoma (Golumbek 1991, Tepper et al., 1989). IL-6, a predominant B cell growth factor, also plays a role in tumour protection when engineered into fibrosarcoma or Lewis lung carcinoma cells (Mullen et al., 1992, Porgador et al., 1992). GM-CSF and TNF-α have also demonstrated anti-tumour immune responses when administered in several tumour models. GM-CSF belongs to a family of cytokines that regulate the growth, survival and differentiation of haematopoietic progenitor cells. When transfected into melanoma (Armstrong et al., 1996, Dranoff et al., 1993), bladder cancer (Saito et al., 1994), and leukeamia (Hsieh et al., 1997) models, GM-CSF induces potent anti-tumour immunity. Although toxic in high doses, TNF-α has also been used in tumour models to show regression of autologous tumours (Blankenstein et al., 1991, Teng et al., 1991).

Immunotherapy with the target MUC1 antigen and recombinant cytokines has previously been studied by Acres et al. (1993) and Balloul et al. (1994). These groups constructed vaccinia viruses co-expressing the MUC1 antigen and various cytokines: IL-2, IL-4, IL-5,

IL-6, IL-7, on the basis of their previously described anti-tumour activities. Mice immunised with VV-MUC1 were protected against tumour challenge, however no evidence of MUC1 CTL activity was found after immunisation (Acres et al., 1993). Based on these findings, we used IL-2, IL-4, IL-6, IL-7, IFN-γ, and GM-CSF in a MUC1 model to examine the effect of these cytokines on CTLp frequency and tumour protection. The role of various cytokines in the initiation of the immune response against MFP was further examined in cytokine gene knockout mice, and in a MUC1 DBA/2 *in vivo* immunotherapy tumour model.

4.2 Materials and methods

MUC1 antigens

MUC1 FP was produced in a bacterial expression system (pGEX-3X) containing 5 VNTR regions of MUC1, and conjugated to oxidised mannan (Apostolopoulos et al., 1993, 1995) (refer to Chapter 2, section 1). The MUC1 peptide Cp13-32 was synthesised using an Applied Biosystems Model 430A biosynthesiser.

Cytokine and cytokine receptor knockout mice

Cytokine and cytokine receptor knockout mice produced by homologous recombination on a C57BL/6 background, were generously supplied by the following: IL-2 -/- (R.M. Zinkernagel, Institute of Experimental Immunology, Switzerland: Kundig et al., 1993), IL-4 -/- (Institute for Genetics, University of Cologne, Germany: Kuhn 1991), IL-6 -/- (R.M. Zinkernagel; Kopf et al., 1994), IL-7 receptor (IL-7R) -/- (Jackson Laboratory, Maine, U.S.A: Peschon et al., 1994), IL-10 -/- (Jackson Laboratory: Kuhn 1993), IFN-γ receptor (IFN-γR) -/- (R.M. Zinkernagel: Huang et al., 1993), and TNF-R_2 -/- (Department of Cell Genetics, Genetech, California: Erickson et al., 1994).

Immunisations

BALB/c, DBA/2, (BALB/c x C57BL/6) F_1, (BALB/c x DBA) F_1 mice were bred at the Austin Research Institute (Victoria, Australia). Female mice aged 6 - 10 weeks were immunised intraperitoneally with MFP (containing 5 μg of FP) either once, or weekly for three weeks. Mice injected with cytokines received 1 x 10^7 pfu/ml (total) vaccinia virus containing the cytokine intraperitoneally either alone, or mixed with MFP (5 μg). Both cytokine and cytokine receptor -/- mice were immunised intraperitoneally with MFP conjugate either once or weekly for 3 weeks.

Vaccinia virus-cytokines

Vaccinia virus (VV) constructs encoding the recombinant cytokines human IL-2, murine IL-4, murine IL-6, murine IL-7, murine IFN-γ, murine GM-CSF and wild-type VV were obtained and tested for biological activity by Dr Bruce Acres (Transgène, Strasbourg, France). Vaccinia virus cytokine constructs were produced (refer to Chapter 2, section 4) from infected baby hamster kidney cells (BHK), sonicated to release virus particles, and purified over a 36% sucrose gradient by ultra centrifugation to obtain virus for immunisations as described (Acres et al., 1994). Virus titres were determined by plaque forming assays in which 5×10^6 BHK cells in 6 well plates were incubated for 24 h at 37°C 10% CO_2 with serial dilutions ($1 \times 10^5 - 1 \times 10^9$) of vaccinia virus and stained with aniline blue to count plaque formation. To determine recombinant cytokine activity and concentration, both biological assays and cytokine ELISAs (commercially available kits: Pharmingen U.S.A; Genzyme U.S.A; R&D Systems U.S.A; Endogen U.S.A), were performed in conjunction with Dr Bruce Acres (Transgène, Strasbourg, France) after the generation of each VV-cytokine product.

Tumours

Two DBA/2 P815 mastocytoma cell lines transfected with the human MUC1 gene were used to study the *in vivo* effects of MFP immunisations in combination with cytokines. P815-Tm211 (high MUC1 expression) was used as a target in CTLp assays, and the immunogenic clone P815-Tm2 (low MUC1 expression) (Acres et al., 1993) was used to challenge mice. DBA/2 mice (10 per group) were injected subcutaneously with 1×10^6 P815-Tm2 tumour cells until tumours of 0.6 ± 0.1 cm^2 were established (day 8). Mice were then given one intraperitoneal injection of MFP + VV-cytokine, MFP, VV or cytokine alone. Tumour sizes were measured every 2 - 3 days using electronic callipers.

Cytotoxic T cell precursor assays

A limiting dilution cytotoxic T cell precursor assay was performed 14 - 21 days after immunisation with MFP (refer to Chapter 2, section 2.2.2). Briefly, spleen cells from immunised mice were collected and added varying from 1×10^3 to 1.28×10^5 cells/well. Stimulator spleen cells (5×10^5 cells/well) from (BALB/c x DBA)F_1 or (BALB/c x

BL/6)F_1 naive mice were irradiated and added to all effector wells containing 5 μM MUC1 peptide Cp13-32 and 10 U/ml of rhIL-2. Controls containing stimulators and either, IL-2 and peptide Cp13-32, or stimulators and effectors were included. After incubation, 1 x 10^4 ^{51}Cr labelled P815-MUC1, P815, RMA-MUC1 or RMA target cells were added. Cultures were incubated for a further 4 h and analysed on a microplate scintillation counter (Packard USA). The number of CTL precursors present was determined as a frequency of the number of cells not lysed on a logarithmic scale and the number of cells/well on a linear scale. CTLp assays were repeated a minimum of three times on each VV-cytokine and at least twice on cytokine knockout mice due to the availability of mice.

4.3 Results

Production and characterisation of VV-cytokines

Recombinant VV-cytokines were produced and tested for viral titres and cytokine concentration by ELISA and summarised in Table 4.1 (refer to Chapter 2, section 4.4). The concentration of cytokine produced from 1 x 10^7 pfu of vaccinia virus was determined by a plaque forming assay whereby the concentration of cytokines ranged from > 20 ng/ml to < 1 μg/ml in BHK cell culture supernatants, at confluency (4 x 10^6 cells), after 24 h (data not shown).

The effect of cytokines on MFP immune responses *in vivo*

VV-cytokines were used either alone or in various combinations with MFP to determine the effect of each cytokine on the cellular immune response, and measured by a CTLp assay (Figure 4.1). Firstly, mice injected with either VV-cytokines + MFP or MFP alone could generate a MUC1 specific CTLp response as the CTLp frequency in mice immunised with VV-alone and VV-cytokines without MFP was < 1 / 10^6. Secondly, in keeping with previous observations, the T1 cytokines IL-2, IFN-γ and GM-CSF were beneficial to the CTLp response whereas the T2 cytokine IL-4, was antagonistic. The following was observed:

a) Increases in CTLp responses were seen after one injection of MFP (CTLp frequency of 1/77,500) combined with VV-IL-2 (1/49,000; p = 0.013), VV-IL-7 (1/42,000; p = 0.0014) or VV-GM-CSF (1/53,000; p = 0.0278) (Figure 4.1).

b) No difference was noted in CTLp responses to MUC1 after the addition of VV-IFN-γ (1/73,500; p = 0.359) or VV-IL-6 (1/92,000; p = 0.213) (Figure 4.1).

c) A decrease in CTLp frequency was evident after VV-IL-4 was injected with MFP (1/223,500; p = 0.0039). CTLp frequencies against MUC1 were strongest with MFP and VV-cytokine combinations of VV-IFN-γ+VV-IL-2 (1/33,500; p = 0.0019), VV-IFN-γ+VV-IL-4 (1/39,000; p = 0.0068), and the best response seen with VV-GM-CSF+VV-IL-

7 (1/17,600 p = 0.00004) (Figure 4.1). It was of interest that when used alone, VV-IFN-γ had no effect on the CTLp frequency and VV-IL-4 decreased CTLp responses, however, when used together, VV-IFN-γ+VV-IL-4 increased the CTLp frequency.

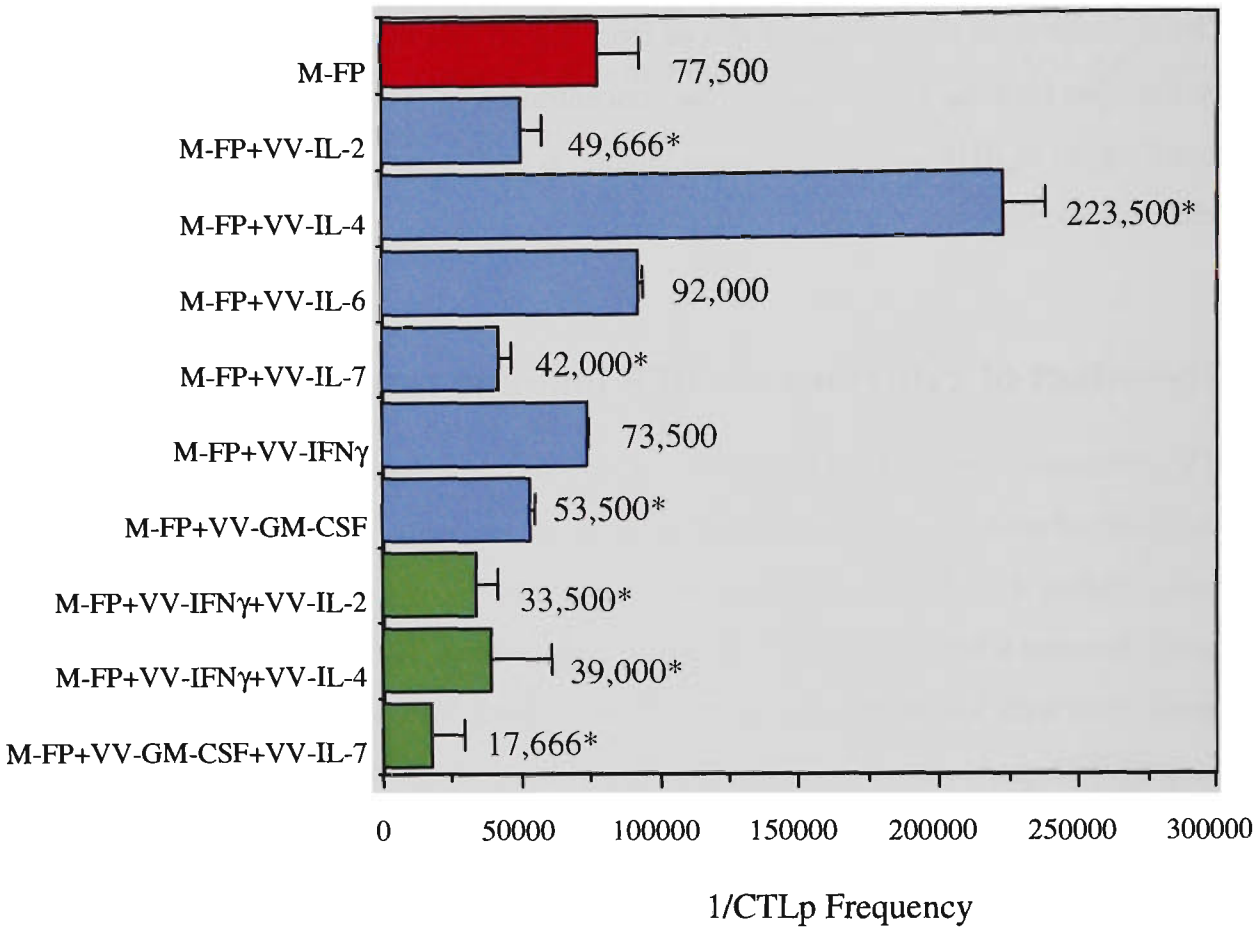


Figure 4.1: MUC1 specific CTLp frequencies in BALB/c mice injected once with 5 μg MFP with or without various VV-cytokines (1 x 10^7 pfu / mouse total) intraperitoneally. Vaccinia virus and VV-cytokine without MFP had CTLp frequencies of < $1/10^6$. The data represents the average CTLp frequency from three determinations. * Frequencies statistically different to MFP, as determined by a student's T-test.

MFP *in vivo* CTLp responses in cytokine - gene and cytokine - receptor knockout mice

The effect of cytokines on the immune response to MUC1 was further examined using cytokine and cytokine receptor gene knockout mice (-/-). IL-2, IL-7R, IL-10, IFN-γ, and TNF-R_2 -/- mice were injected once, and IL-4 and IL-6 -/- mice were injected three times with MFP, and the MUC1 specific CTLp frequencies determined and compared with those found in C57BL/6 control mice (Table 4.1).

a) There was no effect on the MUC1 CTLp frequency after a single injection of MFP in IL-2 -/- mice (1/72,900), IL-7R -/- mice (1/78,000), TNF-R_2 -/- mice (1/69,000), or IFN-γ -/- mice (1/66,000) compared to MFP in wild-type mice (1/72,000) (Table 4.1). No significant difference after three injections of MFP in IL-4 -/- mice (1/9,500) as noted compared to control mice (1/7,000).

b) Stronger CTLp responses were evident after three injections in IL-6 -/- (1/2,500) and one injection in IL-10 -/- (1/60,000) mice compared to their respective controls (1/7,000 and 1/72,000), indicating an inhibitory role for these cytokines in the implementation of a MUC1 immune response *in vivo* (Table 4.1). The MUC1 negative target (RMA) was not killed in any of the CTLp assays (CTLp $< 1/10^6$). Thus, the cytokine -/- mice results provide further evidence that certain cytokines can influence the immune response generated to MUC1.

Table 4.1: MUC1 CTLp frequencies in cytokine gene knockout mice

Gene Knockout Mice	Number of M-FP Injections	TARGETS	
		RMA-MUC1	RMA
IL-2	1	1/72,900	$<1/10^6$
IL-4	3	1/9,500	$<1/10^6$
IL-6	3	1/2,500	$<1/10^6$
IL-7R	1	1/78,000	$<1/10^6$
IL-10	1	1/60,000	$<1/10^6$
IFN-γ	1	1/66,000	$<1/10^6$
TNF-R2	1	1/69,000	1/980,000
Wild-type (control)	1	1/72,000	1/350,000
Wild-type (control)	3	1/7,000	$<1/10^6$

CTLp frequencies from cytokine -/- mice immunised intraperitoneally with 5μg MFP, either once (IL-2 -/-, IL-7R -/-, IL-10 -/-, IFN-γ-/-, TNF-R_2 -/-) or three times (IL-4 -/-, IL-6 -/-). Control mice were wildtype C57BL/6.

Cytokines + MFP in the Tm2 tumour model

Three groups of MFP and various VV-cytokine combinations were studied in the DBA/2 P815-Tm2 tumour model, on the basis of the significant increases observed in the CTLp frequencies seen in Figure 4.1. VV-IFN-γ + VV-IL-2, VV-IFN-γ + VV-IL-4 and VV-GM-CSF + VV-IL-7 were used to inject DBA/2 mice with established P815-Tm2 tumours, both alone and together with MFP, and their tumour growth was measured over 30 days (Figure 4.2). Mice injected only with cytokine combinations and no MFP had no effect on tumour growth, which was similar to that of control mice; in mice injected with MFP and cytokine combinations, a complete regression of established tumours occurred after 30 days. No significant advantages in the anti-tumour effects produced by MFP were evident after the incorporation of cytokines into the MFP immunisations in this tumour model. A different *in vivo* model for MUC1 tumour immunotherapy needs to be established to determine whether cytokines enhance the anti-tumour responses to MFP.

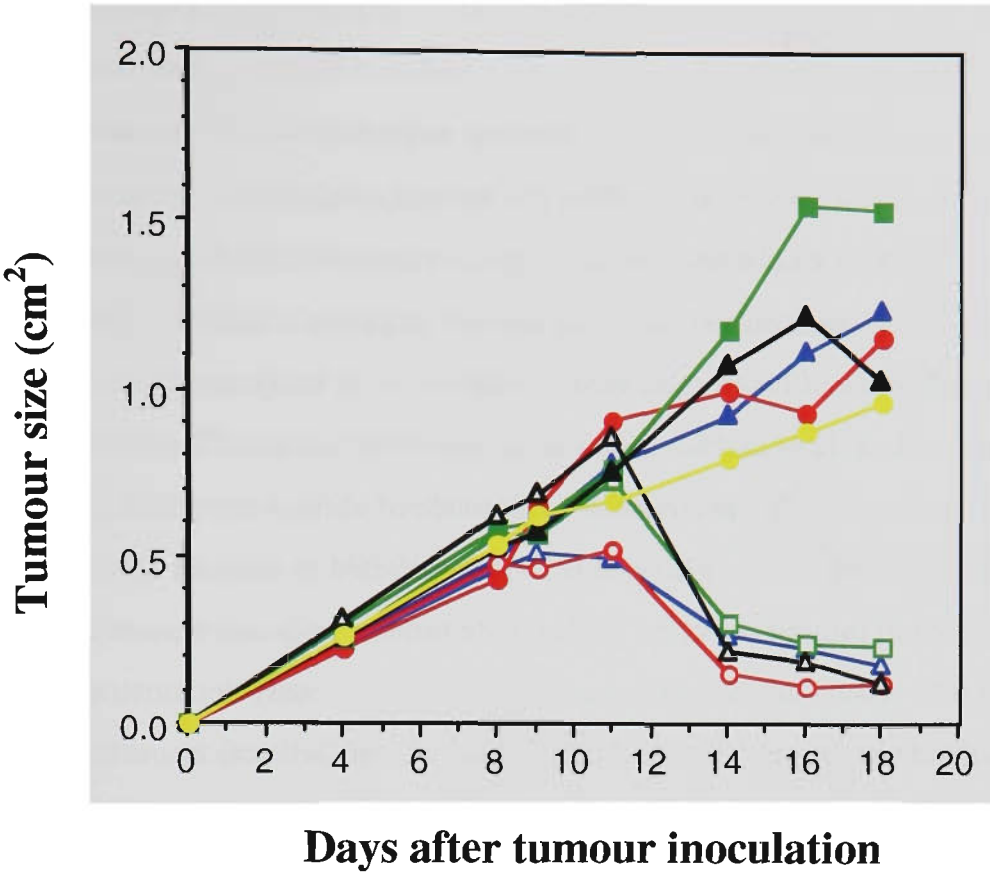


Figure 4.2: Subcutaneous tumour growth in DBA/2 mice with established 8 day Tm2 tumours (1 x 10^6 cells/mouse). Mice were immunised on day 8 with VV-cytokines (1 x 10^7 pfu/mouse total) with or without 5μg MFP. The graph represents MFP (△) MFP + VV-IFN-γ + VV-IL-4 (△), VV-IFN-γ + VV-IL-4 (▲), MFP + VV-IFN-γ + VV-IL-2 (○), VV-IFN-γ + VV-IL-2 (●),MFP + VV-GM-CSF + VV-IL-7 ( ), VV-GM-CSF + VV-IL-7 (■), VV (▲) and pH 9.0 buffer (●). All tumours in rejecting mice disappeared after 30 days. Student T tests were performed to show a significant difference between mice immunised with MFP and VV-cytokines compared to VV-cytokines alone.

4.4 Discussion

The induction of anti-tumour cellular immune responses require tumour specific antigens to be processed and presented by either the tumour, professional antigen presenting cells (eg. B cells, dendritic cells and macrophages), or both, in association with MHC class I and costimulatory molecules to activate naive T cells into tumour specific cytotoxic cells, under the influence of locally secreted cytokines. Early in the immune response, cytokines such as IL-1, IL-2, IL-4 and GM-CSF act to recruit APCs and $CD4^+$ T cells to the site of antigenic challenge. After presentation, a cascade of various cytokines including IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, TNF and IFN-γ are secreted to activate $CD8^+$ T cells and other cells involved in tumour rejection to eliminate tumour cells and ensure T cell memory is established to protect against recurrences. We had previously demonstrated that MUC1, when coupled to mannan, induced $CD8^+$ CTLs, tumour protection and memory in MUC1 tumour models (Apostolopoulos et al., 1995). To determine if the anti-tumour immune response generated against MFP could be increased by the addition of cytokines; and identify the role various cytokines play in the immune response generated against MFP *in vivo*, cytokines were incorporated into MFP immunisations and both CTLp and tumour protection studies in a MUC1 model were examined together with CTLp responses to MFP in cytokine knockout mice studied.

Vaccinia viruses encoding the recombinant cytokines, human IL-2, murine IL-4 , murine IL-6, murine IL-7 , murine IFN-γ, murine GM-CSF and wild-type VV (Nakagawa et al., 1991, Acres et al., 1994, Balloul et al., 1994, Wen Ju et al., 1997), were combined with MFP, and the influence of each individual cytokine on the MUC1 cellular immune response measured by CTLp frequency. IL-2, IL-7 and GM-CSF increased the MUC1 CTLp frequency, additional IL-4 decreased the CTLp frequency whilst IL-6 and IFN-γ had no effect. Whether the addition of these cytokines acts directly on enhancing recruitment or proliferation of CTLs, or indirectly acting on other cells involved in the MUC1 immune response, is not clear and will be discussed below.

IL-2, a T cell growth factor, acts to increase T cell proliferation and recruitment at sites of antigen recognition early in the immune response. Tumour immunotherapy studies with IL-2 have demonstrated additional IL-2 is beneficial to tumour rejection and the

establishment of memory. Numerous IL-2 transfection studies into non-immunogenic tumour models show that continual low dose secretion of IL-2 provides sufficient cytotoxicity necessary to reduce the tumourigenicity and metastatic potential of numerous tumour models including melanomas (Karp et al., 1993), fibrosarcomas (Gansbacher et al., 1990), bladder carcinomas (Conner et al., 1993) and neuroblastomas (Corrias et al., 1998). Indeed many clinical trials such as those with melanoma, include IL-2 as it improves tumour immune responses *in vivo* (Stidham et al., 1996). In the MUC1-MFP model system, additional IL-2 also improved the immune response by producing a two-fold increase in MUC1 specific CTLp frequencies and additional IL-2 could be a beneficial cytokine for increasing MUC1 cytotoxic T cells and would be advantageous to include in MUC1 tumour therapy.

The IL-2 -/- mice showed that IL-2 was not required for the initiation of a MUC1 immune response nor did its absence hinder the generation of cytotoxic T cells, as no difference in CTLp frequencies in MFP immunised wild-type or IL-2 -/- mice were observed. Phenotypic studies in IL-2 deficient mice show these mice are normal in regard to thymocyte and peripheral T-cell subset composition (Schorle et al., 1991), however they are susceptible to autoimmunity at a very early age due to a breakdown in regulatory functions leading to uncontrolled proliferation and activation of lymphocytes which results in haemolytic anaemia and colitis (Sadlack et al., 1994, Klebb et al., 1996). Immune responses generated against tumour antigens in IL-2 deficient mice have not been well documented (as these mice do not survive well) (Sadlack et al., 1993), however the results from a study investigating antiviral immune responses show that virus specific CTL induction is only moderately reduced in IL-2 -/- mice compared to wild-type mice perhaps attributed to cytokine compensation (Bachmann et al., 1995). As the absence of IL-2 did not change the MUC1 specific CTLp frequency in IL-2 deficient mice, it is possible that the loss of functional IL-2 was compensated by other cytokines, in particular, IL-15, which has similar biological activities to IL-2 (Grabstein et al., 1994). Hence this evidence suggests that although additional IL-2 produced from vaccinia virus is beneficial to the MFP immune response as it increases the production of cytolytic T cell precursors, IL-2 may not play a significant role in the initiation of the immune response against MFP as its absence in the knockout mice does not effect the CTLp frequency.

Many studies indicate that IL-4, a T2 cytokine, gene transfer into tumour cells confers a certain extent of protection from subsequent lethal challenges of tumour, however this protection is generally attributed to nonspecific effector cells and not always $CD8^+$ CTLs required for complete tumour rejection (Tepper et al., 1989, 1992, Li et al., 1990, Hock et al., 1993, Pericle et al., 1994). In the MFP model, IL-4 significantly decreased the MUC1 CTLp frequency when added to MFP injections. This was not surprising as it has been shown that IL-4 inhibits the development of cytotoxic T1 cells whilst inducing the proliferation of T2 cells (Erard et al., 1993; Brown et al., 1997). In a study investigating the role of IL-4 in anti-viral mediated cellular immunity, VVIL-4 infected spleens produced a remarkably lower number of antiviral cytotoxic T cell precursors compared to the number produced by VV infected spleens (Sharma et al., 1996), demonstrating IL-4 is directly involved in the suppression of cell-mediated immune responses *in vivo*. Apostolopoulos and colleagues (1995) demonstrated that during the immune response to MFP, cytotoxic T cell production was directly associated with IFN-γ and no detectable IL-4 secretion; and when IL-4 production was evident, it was associated with no cytotoxic T cell development. Therefore, when IL-4 was included in MFP injections, the T1 immune response originally produced switched to a T2 immune response due to the presence of IL-4; hence resulting in a down regulation in the number of CTLps produced in response to MFP.

MFP injections into IL-4 deficient mice showed no effect on the number of CTL precursors produced against MUC1 in -/- mice compared to control mice producing IL-4. This result is in keeping with observations made when studying the immune response to contact hypersensitivity in IL-4 -/- mice (Weigmann et al., 1997). Results from this study show the disease progressed in two phases, the first of which was dependent on T1/Tc1 cells and was unaffected by the absence of IL-4, whilst the second phase of the condition was T2/Tc2 dependent and was totally undeveloped in mice lacking IL-4. Similarly, Kuhn and colleagues (1991) reported IL-4 deficient mice to have normal T and B cell development, but strongly reduced serum levels of IgG1 and IgE. These results demonstrate that T1/Tc1 immune responses progress independent from the presence of IL-4 and suggests that the T1 immune response generated against MUC1 is also IL-4 independent, however when introduced in excess, hinders the production of CTLps in the immune response against a MUC1 antigen.

IL-6 has many biological functions including acting as a differentiation factor for B cells, a growth factor for B cell hybridomas and a role in the activation of T cells (Van Snick et al., 1990). IL-6 plays an active role in stimulating the proliferation of mature $CD8^+$ T cells and cytolytic T cell responses (Lotz et al., 1988) and for these reasons has shown potent anti-tumour activity when transfected into several tumour models (Mullen et al., 1992, Porgador et al., 1992, Tanaka et al., 1997, Mullen et al., 1996). In MFP injections however, additional IL-6 had no effect on the CTLp response generated against MUC1. As the above studies demonstrated IL-6 to be an active stimulator of cytotoxic T cell development, it would appear that in the MUC1 immune response induced by MFP, significant levels of IL-6 may already be induced. Further studies examining the cytokines secreted from T cells of mice immunised with MFP are in progress and will determine which are produced upon MFP stimulation. IL-6 deficient mice generated a stronger CTLp response to MFP compared to MFP in control mice. The increase in CTL precursors observed in the absence of IL-6 indicate IL-6 is an inhibitory factor in the early stages of MUC1 CTL precursor development. Studies from IL-6 deficient mice show that in a *candida albicans* model, IL-6 -/- mice have impaired neutrophil and $CD4^+$ T cell development (Romani et al., 1996). Based on this study, the presence of neutrophils and $CD4^+$ T cells early in the MUC1 immune response, may hinder the generation of MUC1 CTL precursors, however the presence of IL-6, and therefore neutrophils and $CD4^+$ T cells at a later time, has no effect on the T1 immune response generated to MFP.

IL-7 is a growth factor for early B and T cell precursors (Morrissey et al., 1989). Additional IL-7 included in MFP injections increased the CTLp frequency to MUC1 two-fold. Numerous *in vivo* studies have also shown the therapeutic effects IL-7 exerts by increasing $CD8^+$ T cell responses (Lynch et al., 1994, Komschiles et al., 1994). Studies examining VVIL-7, have also shown enhanced T cell proliferation during viral infection whereby both $CD4^+$ and $CD8^+$ T lymphocytes were increased 2-3 fold, and both antigen-specific and antigen-non-specific immune activity characterised by the production of anti-viral CTL, natural killer cells and lymphokine activated killer cells was enhanced (Leong et al., 1997). From these studies it was not surprising that IL-7 increased the immune response to MFP by generating a stronger MUC1 cytotoxic T cell precursor response. MUC1 CTLp responses generated by an MFP injection in IL-7 receptor alpha-chain deficient mice (IL-7R -/-), were not effected by the absence of IL-7. Phenotypic studies in

IL-7R -/- mice show severely depleted lymphocyte populations where γδ T cells are absent and αβ T and B cell numbers are reduced, NK populations are normal, and thymocyte development is arrested at the double negative stage, although it can be restored upon the introduction of a T cell receptor (Maki et al., 1996; Crompton et al., 1997). In the MFP model, these results suggest that cytotoxic T cell precursors are either generated in an IL-7 independent manner or MFP provides the antigenic stimulation required to activate the small amount of αβ T cells present to generate into MUC1 specific $CD8^+$ T cell precursors.

Neither VV-IL-10 or VV-TNF-α recombinants were available to determine the effects of these cytokines on the immune response generated to MUC1 when included in MFP immunisations. Both cytokines have however demonstrated anti-tumour capabilities in other murine model systems characterised by the generation of tumour-suppressing cytotoxic T cells and in some cases, the establishment of immunological memory (TNF-α: Blankenstein et al., 1991, Teng et al., 1991, Tepper et al., 1994: IL-10: Giovarelli et al., 1995, Gérard et al., 1996). To investigate the role IL-10 and TNF have on the generation of MUC1 CTL precursors, MFP was injected into both IL-10 deficient and TNF receptor deficient mice. There was an increase in the CTLp frequency observed in IL-10 -/- mice demonstrating IL-10 has an inhibitory effect on CTLp induction in the early stages of the MUC1 immune response. This observation is true of IL-10 which has been described as indirectly preventing antigen-specific T cell activation by down-regulating the antigen presentation capabilities of monocytes, macrophages, Langerhan cells and dendritic cells (de Vries et al., 1995). Studies investigating MFP CTLp responses in TNF-R_2 deficient mice showed no effect on the number of CTL precursors produced demonstrating CTLp induction in MFP stimulated mice is TNF-α independent. This finding is in keeping with a report from Erickson and colleagues (1994) which described TNF-R_2 -/- mice to have normal T-cell development and activity, but an increased resistance to TNF-induced death. The unaffected T cell response in TNF-R_2 -/- mice is not unusual as TNF-α is predominantly referred to as an inflammatory cytokine that promotes margination of leukocytes at inflammatory sites and cytotoxic effects on tumour cells and exerts little direct effect on T lymphocytes (Bemelmans et al., 1996).

IFN-γ is a T1 cytokine which regulates the induction of cytolytic T cells, increases natural killer cell activity, increases the antigen presenting capacities of macrophages and promotes macrophage tumouricidal activity (Hayashi et al., 1985, Weigent et al., 1983, Birmingham et al., 1982, Pace et al., 1983). Due to its role in CTL induction and tumouricidal capabilities, IFN-γ gene transfer has been used to exert its anti-tumour effects in many mouse tumour models (Matory et al., 1995, Watanabe et al., 1989, Gansbacher et al., 1990). In the MFP immune response, IFN-γ is secreted in quantities sufficient enough to generate a strong MUC1 CTLp response (Apostolopoulos et al., 1995). It was therefore not surprising that additional IFN-γ included in MFP injections, had no effect on the levels of MUC1 CTLps generated even though it is a cytokine renowned for its ability to increase CTL production. MFP injections into IFN-γ deficient mice showed no effect on the number of cytotoxic T cell precursors produced against MUC1 in -/- mice compared to control mice producing IFN-γ. Studies in IFN-γ -/- mice report the development of a normal immune system, however, the mice have defective natural resistance to viral infections despite normal cytotoxic and T helper cell responses (Huang et al., 1993). As the CTLp response to MFP was not effected by the absence of IFN-γ, this suggests that IFN-γ is not directly involved in the early stages of MUC1 CTL induction, but instead, exerts its biological effects later on in the immune response, as indicated by the significant levels of IFN-γ induced after MFP immunisations, by acting as a growth factor for CTL precursors.

GM-CSF belongs to a family of colony stimulating factors that regulates the survival, growth and differentiation of haematopoietic progenitor cells. GM-CSF induces the proliferation of macrophages (Metcalf et al., 1985) and enhances T cell immune responses by potently recruiting and activating antigen presenting cells (Grabe et al., 1991, Tazi et al., 1993, Cohen et al., 1994). GM-CSF has been shown to produce anti-tumour responses in several tumour models (Armstrong et al., 1996, Dranoff et al., 1993, Saito et al., 1994) including in a murine breast cancer model, C3HBA, where mice immunised with tumour cells infected with VV-GM-CSF were completely protected against a challenge of parental tumour cells and mice not infected with VVGM-CSF were not (Peplinski et al., 1996). In the immune response generated against MUC1, GM-CSF increased the number of CTL precursors when included in MFP injections. This result, plus the observation that GM-CSF increased the number of macrophages at the site of MFP injection, (Apostolopoulos,

manuscript in preparation) suggests that GM-CSF acts to increase the number of antigen-presenting macrophages to the area of MFP injection enhancing the MUC1 T cell immune response.

Apart from the advantages single cytokines attribute to tumour immunotherapy, the use of multiple cytokine therapies is rapidly being explored due to both the additive and synergistic effects of cytokine combinations in an *in vivo* situation. Combinations of cytokines included in MFP immunisations (IFN-γ+IL-2, IFN-γ+IL-4, and GM-CSF+IL-7) were successful at enhancing the MUC1 CTLp response, and all were either synergistic or additive in their effect. IFN-γ+IL-2 acted synergistically to significantly increase MUC1 CTLps by stimulating T1-type cells. Others have shown that IFN-γ and IL-2 in combination can prolong the survival of mice with melanoma and induce both natural killer/lymphokine activated killer cells and anti-melanoma specific CTLs in a murine melanoma model (Kim et al., 1995). IFN-γ+IL-4 acted synergistically to increase the number of MUC1 CTLps to the same extent seen with IFN-γ+IL-2, although alone IFN-γ and IL-4 act quite differently. The synergistic effect seen when IFN-γ and IL-4 are combined could be the result of an increase in macrophage activation as previously shown by Crawford and colleagues (1987) and Belosevic and colleagues (1988), therefore increasing the antigen presenting capacities for MUC1 antigen to be processed. The combination of GM-CSF and IL-7 produced an additive effect when compared to the reaction of each cytokine alone. Both cytokines were able to increase the MUC1 CTLp frequency individually, however the combination of the two enhanced this response dramatically. An increase in macrophage availability and hence antigen presenting capabilities, and a growth factor for activated T cells, may explain the synergistic effects seen when GM-CSF and IL-7 and used in combination with MFP to induce MUC1 CTL precursors.

The CTLp responses observed in the MFP and cytokine immunisations show a clear advantage for using combinations rather than individual cytokines in the MUC1 model for those cytokines tested. To examine this *in vivo*, the combinations of IFN-γ+IL-2, IFN-γ+IL-4 and GM-CSF+IL-7 were used in conjunction with MFP to immunise mice against an established MUC1 tumour, P815-Tm2. No significant difference in tumour growth was noted between MFP and MFP with cytokine combinations. However, the use of cytokines

without MFP clearly had no effect on tumour growth demonstrating that the cytokines do not exert their effects in a non-specific manner on cells of the immune system and act only on antigen specific effector cells. No advantage was obtained in the MUC1 P815-Tm2 model by including cytokines into the MFP immunisations, as alone MFP could successfully eradicate the established tumour. To therefore show a requirement for additional cytokines in MUC1 tumour therapy, a new MUC1 tumour model needs to be established whereby MFP immunisations alone are not effective at abolishing tumours.

Chapter 5:

Characterisation of a metastatic mammary adenocarcinoma model: DA3-MUC1

Catherine J Lees, Vasso Apostolopoulos, Chin-Swee Ong Nechama Smorodinsky, and Ian F.C. McKenzie. Characterisation of a metastatic murine adenocarcinoma model: DA3 MUC1. J. Immunotherapy, submitted (2000).

Summary

MFP immunotherapy was investigated in an aggressive MUC1$^+$ metastatic tumour, DA3-MUC1 because, unlike other MUC1$^+$ tumour models, DA3-MUC1 is not spontaneously rejected in mice making it an alternative model for immunotherapy studies. Further, MUC1$^+$ DA3 cells are restricted to lysis by anti-MUC1 CTLs. An absence of MUC1 induced anti-tumour immunity in DA3-MUC1 was attributed to an absence of MHC class I molecules on the tumour cell surface. Both *in vitro* and *in vivo* analyses of subcutaneous tumours and lung metastases demonstrated that DA3-MUC1 tumour cells have a low expression (< 6%) of MHC class I which can be upregulated (>90%) following culturing with IFN-γ. Results from FACS analyses and immunoperoxidase staining indicated that the up-regulation of class I could be maintained for up to seven days *in vivo* after removal of the cytokine, without effecting the expression levels of MUC1 antigen. Interestingly, the presence of induced MHC class I molecules led to MUC1$^+$ specific CTL from MFP immunised mice to lyse DA3-MUC1 targets *in vitro* however *in vivo* this did not produce anti-tumour immunity. These results highlight the importance of MHC class I molecules in the induction of anti-tumour immunity and the MFP immune response.

5.1 Introduction

Anti-tumour immunity and tumour eradication are induced by cell-mediated immune responses (Greenberg, 1991; Melief, 1992). The activation of tumour-specific $CD8^+$ T lymphocytes and their subsequent differentiation into cytolytic cells is dependent on two signals from the antigen-presenting cell. One signal is provided through the interaction of the antigenic peptide (from the tumour) and the MHC complex that engages the T cell receptor. The other is a costimulatory signal, efficiently provided by B7 binding to CD28 or CTLA-4 on the T cell (Leung and Linsley, 1994). However, malignant cells have evolved mechanisms enabling them to successfully evade the immune system, which in many cases directly affects this two signal process (Hellström and Hellström, 1991). These mechanisms include inadequate expression of costimulatory molecules, Fas ligand, or adhesion molecules on cancer cells, antigen processing defects, the secretion of inhibitory peptides or molecules into the tumour microenvironment or absent or poorly expressed MHC molecules on the tumour cell surface (Nawrocki and Mackiewicz, 1999).

The failure of tumours to adequately process and present antigens to T cells is in many cases, directly attributed to a reduced expression of MHC class I molecules on the tumour cell surface (reviewed in Garrido et al, 1993). In many tumour models however, this can be rectified with the transfection of the MHC class I gene (Hui et al., 1984; Tanaka et al., 1984; Wallich et al., 1985). Another approach is to transfect cytokine cDNA, in particular IFN-γ, into tumours as it directly causes an up-regulation of cell surface MHC class I expression (Watanabe et al., 1989, Gansbacher et al., 1990a).

This study characterises the DA3-MUC1 metastatic tumour following the failure of mannan-MUC1 (MFP) immunisations to induce a protective immune response in this new $MUC1^+$ model. It was demonstrated that this lack of immunogenicity was due to an absence of MHC class I expression on the tumour cell surface, which could be upregulated by IFN-γ, but not sustained long enough *in vivo* to cause tumour eradication.

5.2 Materials and methods

Mice and immunisations

A MUC1-GST fusion protein containing 5 VNTR regions from the extracellular protein core of MUC1 (Apostolopoulos et al 1993), was produced in a bacterial expression system (pGEX-3X) and conjugated to oxidised mannan to form MFP as described previously (Apostolopoulos et al 1995) (see Chapter 2, section 1). BALB/c mice aged 6-10 weeks were given three intraperitoneal immunisations (on days 0, 7 and 14) with either MFP (containing 5μg of MUC1 fusion protein) or a control pH 9.0 phosphate buffer. BALB/c mice immunised with mannan coupled to oxidised GST (M-GST) were included as controls in the lung metastases study (see below).

Cell lines

DA3-MUC1 is a metastatic BALB/c DA3 mammary cell line transfected with the cDNA of the transmembrane form of human MUC1 and was provided by Dr. Nechama Smorodinsky (Tel Aviv University, Tel Aviv, Israel; Baruch et al., 1997) (see Chapter 2, section 6.5). P815-MUC-1, a DBA/2 P815 mastocytoma cell line transfected with the cDNA of the transmembrane form of human MUC1 (Acres et al., 1993) was cultured in RPMI (see Chapter 2, section 6.3) and MUC1 expression selected for every 14 - 20 days with 1.25 mg/ml G418-sulfate (Gibco BRL, U.S.A).

Flow cytometry

The expression of various DA3-MUC1 cell surface molecules was measured by flow cytometry (see Chapter 2, section 3).

The following monoclonal antibodies were used:

a) MUC1 (BC2: supernatant) (Xing et al., 1989),

b) MHC class 1 $H2^d$ (34.1.2s, 1 / 1000 dilution of ascites fluid) (Ozato et al., 1982),

c) MHC class II I-A8 (1 / 500 dilution of ascites fluid),

d) B7.1 (4 µg) (Pharmingen, San Diego, USA),

e) ICAM-2 (1 µg) (Pharmingen),

f) CD28 (4 µg) (Pharmingen),

g) LFA-2 (1 µg) (Pharmingen) and

h) CTLA-4 (1 µg) (Pharmingen).

DA3-MUC1 tumour cells were prepared for FACS analysis by either a) culturing in growth medium, b) culturing with 20 ng/ml VV-IFN-γ (see Chapter 2, section 13) for 72 h, or c) culturing with 20 ng/ml IFN-γ for 72 h and then removing IFN-γ for subsequent culturing. In preparation for flow cytometry, tumour cells (2 – 5 x 10^5 cells/ml) were incubated with the specified antibodies for 45 min at 4 ^{0}C, washed with phosphate buffer and incubated with either FITC-conjugated sheep (Fab')2 anti-mouse, anti-rat or anti-hamster immunoglobulin, (Amersham, UK) (1 / 50 dilution) for a further 45 min at 4 ^{0}C. Cells were washed and analysed by flow cytometry.

Immunoperoxidase staining of DA3-MUC1 tumour cells

Cell surface expression of MUC1 and MHC class I proteins on DA3-MUC1 tumour cells *in vivo* were analysed by immunoperoxidase staining (see Chapter 2, section 5). DA3-MUC1 tumour cells were either,

a) injected subcutaneously into BALB/c mice and grown for >30 days to establish lung metastasis. Mice were culled and samples taken from both the subcutaneous tumour site and from lung metastasis; or

b) cultured with 20 ng/ml IFN-γ for 72 h and injected subcutaneously into BALB/c mice. Mice were culled and samples taken from the subcutaneous tumour site on days 4 and 7 and from lung metastasis >30 days after injection.

All tissue samples were snap frozen in isopentane, cut 5 – 6 µm thick using a Microm HM500 cryostat (MICROM Laborgerate, Strässe, Germany), mounted and fixed on silane coated slides (Rentrop et al., 1986). Endogenous peroxidase activity was blocked for 40 min at room temperature using 0.5% H_2O_2. Immunoperoxidase staining of tissue samples were performed as described previously (Stacker et al., 1985). Briefly, samples were incubated for 45 min at 4^{0}C with biotinylated BC2 (neat) to detect MUC1 expression or biotinylated anti-$H2^d$ (1/1000

dilution) to detect MHC class I expression (see Chapter 2, section 5.3). Excess antibodies were removed by thorough washing and samples incubated with strep-avidin conjugate (Amersham, UK) (1/50 dilution) for a further 45 min at 4 ^{0}C. Antibody binding was detected with 1.5 mg/ml 3-3 diaminobenzidine (DAB, Sigma, St. Louis, USA) in phosphate buffered saline containing 0.5% H_2O_2 for 5 min, slides washed and mounted. The biotinylated anti-$H2^d$ antibody reacted with macrophages, dendritic cells and normal cells in control experiments.

Tumour models

Several tumour models were used to both characterise the DA3-MUC1 tumour *in vivo* and establish the immunogenicity of the tumour following MFP immunisations. These were as follows:

a) BALB/c mice (x 10) were subcutaneously injected with 5 x 10^6 DA3-MUC1 tumour cells and tumour growth measured with electronic callipers every week for 10 weeks to establish a DA3-MUC1 growth curve. Mice were sacrificed >30 days after the tumour challenge and lung metastasis determined by microscopically counting the number of metastasis present in random cross sections of similar sizing from formalin fixed lung samples (Anatomical Pathology Unit, Austin and Repatriation Medical Centre, Vic, Australia).

b) BALB/c mice (x 20 per group) were immunised three times with either MFP (5µg) or M-GST (5µg) and challenged with 5 x 10^6 subcutaneous DA3-MUC1 tumour cells. A minimum of four mice from each group were sacrificed each week for five weeks and the number of metastatic lesions present on each lung determined microscopically.

c) BALB/c mice (x 10 per group) were injected subcutaneously with 5 x 10^6 DA3-MUC1 tumour cells until tumours of ~50 mm^2 were established (Day 17). Mice were then immunised intraperitoneally on days 17, 19 and 21 with 5 µg MFP. Tumour sizes were measured every 2 - 3 days for 30 days using electronic callipers.

d) BALB/c mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 µg MFP or pH 9.0 buffer, and challenged subcutaneously on day 21 with 3 x 10^6 DA3-MUC1 tumour cells. Prior to challenge, the tumour cells were cultured with 20 ng/ml

VV-IFNγ supernatant (UV inactivated) for 72 h to increase cell surface MHC class I expression. Tumour growth was measured every 2 - 3 days for two weeks using electronic callipers.

e) BALB/c mice (x 7 per group) were immunised intraperitoneally three times with either 5 μg MFP or pH 9.0 buffer. On day 21, mice were challenged subcutaneously with varying low doses (1×10^5, 5×10^5 or 1×10^6) of DA3-MUC1 tumour cells previously cultured with 20 ng/ml IFNγ for 72 h, and the tumour growth measured with electronic callipers.

Cytotoxic T cell ^{51}Cr release assay

BALB/c mice immunised (x 3) with MFP (5μg) were culled and their spleen cells collected and treated with 0.83% NH_4Cl. Two-fold serial dilutions of effector spleen cells from the immunised mice were plated into a 96 well plate beginning at a concentration of 1×10^6 cells per well in duplicate. 1×10^4 ^{51}Cr labelled DA3-MUC1 cells cultured with IFN-γ (20 ng/ml) for 72 h, DA3-MUC1, P815-MUC1 or P815 target cells were added to the effectors. The spontaneous release of ^{51}Cr from the labelled cells was determined by incubating target cells in RPMIM media and the maximum release was determined by incubation with 10% SDS (BDH Chemicals, Dorset, England) (see Chapter 2, section 2.2.1). Cultures were incubated for 4 h before transferring 100 μl of supernatant to 96 well flat Optiplates (Disposable Products, Australia) containing 100 μl of Microscint 40 (Packard, USA) for analysis on the microplate scintillation counter (Packard USA). The specific percentage lysis of target cells was determined by; [(experimental-spontaneous) cpm / (maximum-spontaneous) cpm] x 100%.

5.3 Results

Immunotherapy of DA3-MUC1 with MFP

To examine the anti-tumour effects of MFP immunisations on the DA3-MUC1 tumour *in vivo*, two immunotherapy models were examined.

In the first model, BALB/c mice with an established DA3-MUC1 tumour (~50 mm^2) were immunised three times (days 17, 19 and 21) with either MFP or a control pH 9.0 buffer and tumour growth and lung metastases measured for 30 days. Unlike other tumour models (Apostolopoulos et al., 1996b) in the DA3-MUC1 model, there was no difference in tumour growth (Figure 5.1a) or the number of lung metastases (as determine by lung weight) (data not shown) in mice immunised with either MFP or the control pH 9.0. Therefore, therapy with MFP is not effective at treating established DA3-MUC1 tumours.

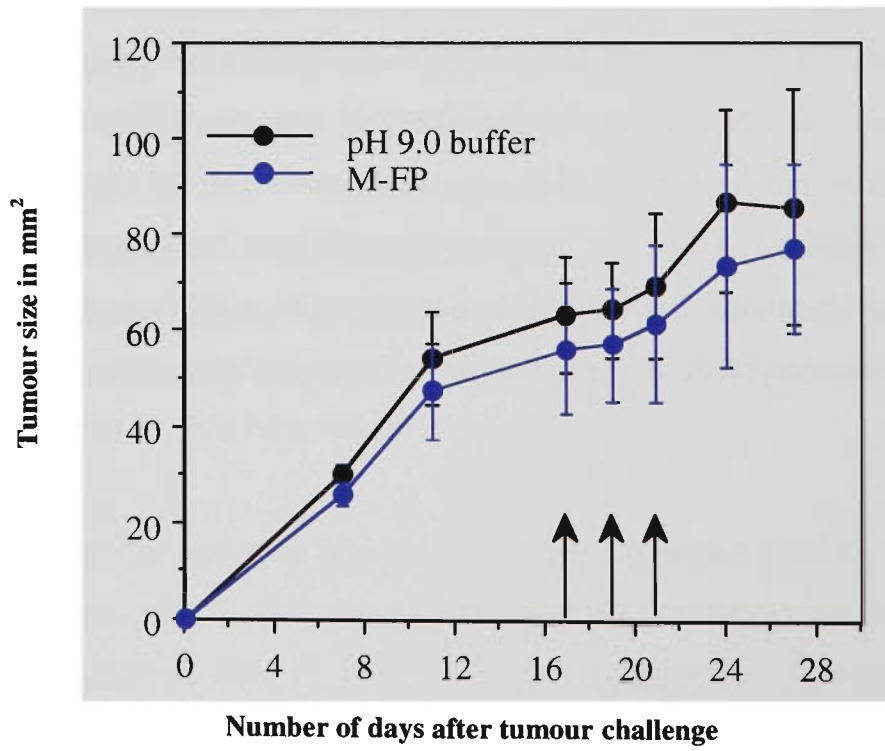


Figure 5.1a: Subcutaneous DA3-MUC1 (5 x 10^6 cells/mouse) tumour growth in BALB/c mice with an established 17 day tumour. Mice were immunised intraperitoneally on days 17, 19 and 21 (↑) with 5 μg MFP or pH 9.0 buffer and tumour growth measured.

In the second model, BALB/c mice were immunised three times with either MFP or a control preparation, M-GST, and challenged with 5 x 10^6 DA3-MUC1 tumour cells subcutaneously. Metastatic lung nodules from 4 - 6 mice per week were examined microscopically for five weeks (Figure 5.1b). Immunisation with MFP did not protect mice challenged with DA3-MUC1 from developing lung metastases nor decrease the number of lung metastases per lung compared to immunised control mice.

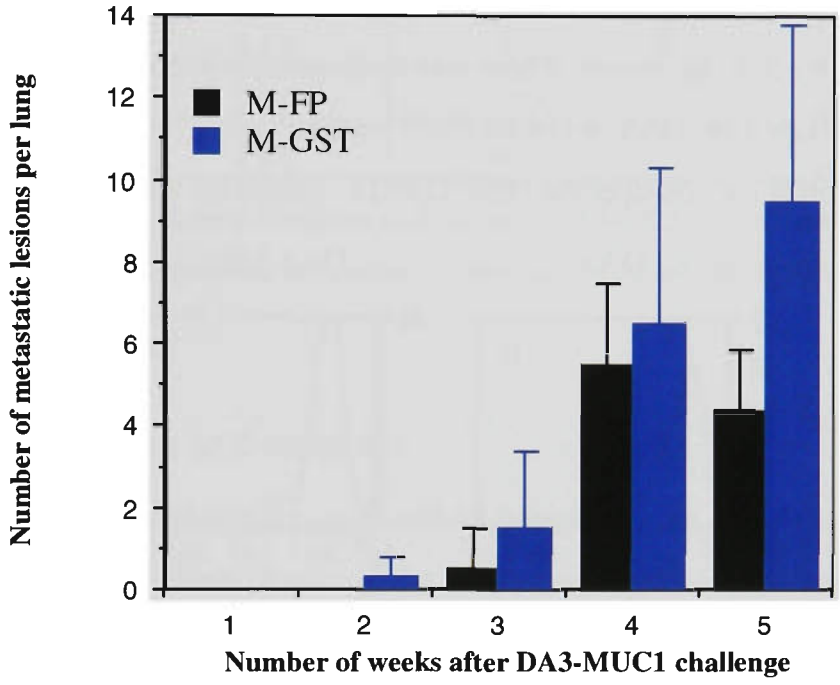


Figure 5.1b: Lung metastases in BALB/c mice immunised with either MFP or a control M-GST three times (days 0, 7 and 14), then challenged with DA3-MUC1 (5 x 10^6 cells/mouse). Four to five mice were culled each week for five weeks and microscopic lung metastasis counted.

From these studies, it was concluded that immunisation with MFP could not induce tumour protection in mice challenged with DA3-MUC1 tumour cells, nor could it offer protection against an established DA3-MUC1 tumour. These results were in contrast to findings in all other MUC1$^+$ tumour models investigated to date, where MFP has been able to successfully induce anti-tumour immunity and tumour protection *in vivo*. It was hypothesised that the DA3-MUC1 tumour is not immunogenic because of a decrease in either costimulatory or MHC molecules on the surface of the tumour cell. To investigate these hypotheses, the DA3-MUC1 tumour was characterised for cell surface molecule expression *in vitro* and *in vivo*.

Serological characterisation of the tumours

a. *In vitro* characterisation of DA3-MUC1

The DA3-MUC1 metastatic cell line was examined for the expression of human MUC1, MHC class I and other cell surface antigens by flow cytometry (Table 5.1 and Figure 5.2). MUC1 is highly expressed on the surface (> 85%) of DA3-MUC1 cells compared to < 2% on non-transfected parental DA3 cells (Figure 5.2ii and v). In contrast, MHC class I expression was considerably decreased in both DA3-MUC1 cells (6%) and non transfected DA3 cells (<3%) (Figures 5.2i, iii, iv, vi). There was no detectable MHC class II, B7.1, ICAM-2, CD28, LFA-2 or CTLA-4 on DA3 or DA3-MUC1 tumour cells (Table 5.1). Phosphate buffer was used as a control for non-specific (Fab')2 FITC-conjugate binding (Figure 5.2i, and iv) .

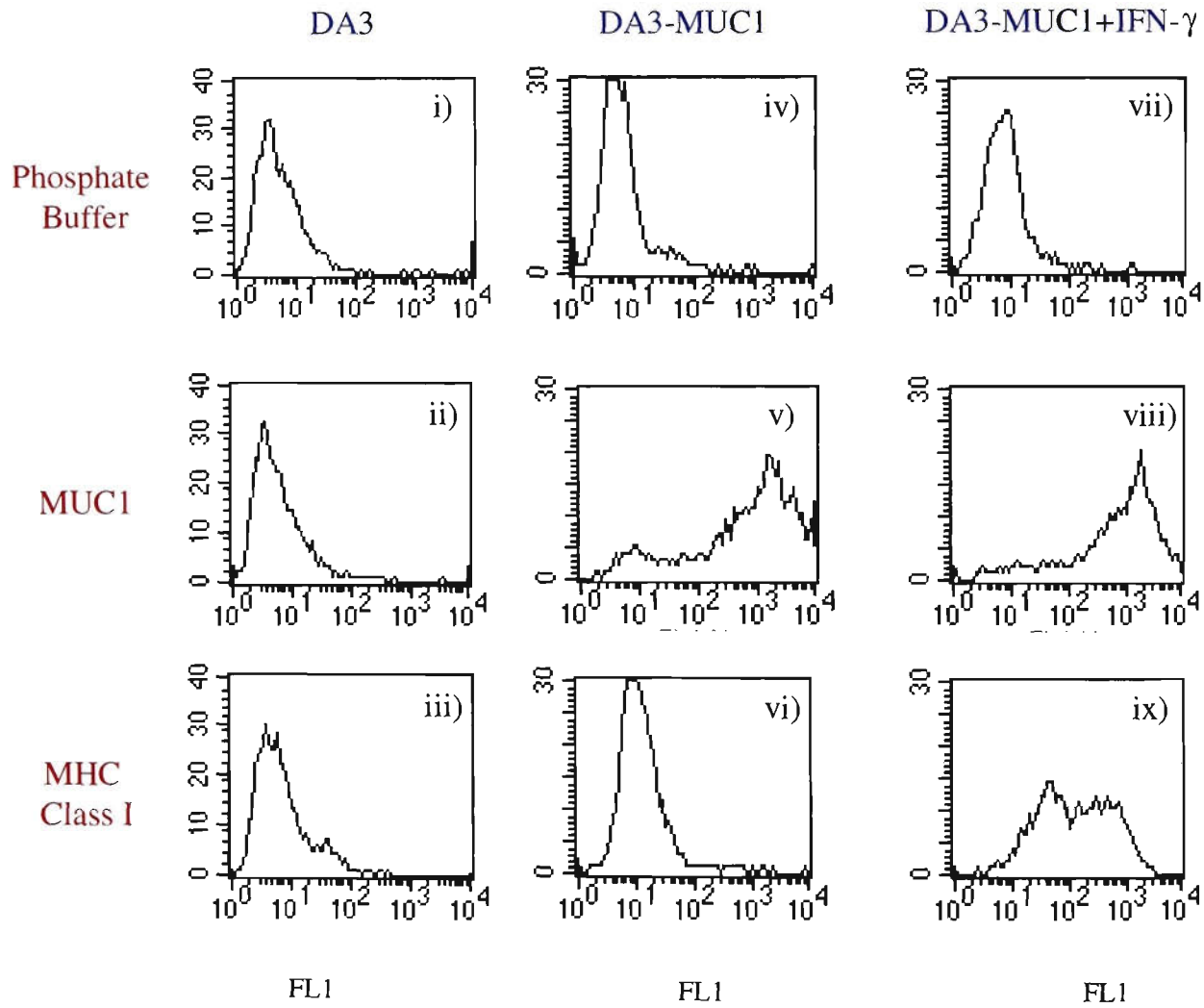


Figure 5.2: *In vitro* expression of cell surface MUC1 and MHC class I on DA3-MUC1 cells cultured with or without 20 ng/ml IFN-γ for 72 h. The non transfected parental cell line, DA3 was used as a control. Phosphate buffer represents negative control binding of FITC-conjugated sheep $(Fab')_2$ anti-mouse (1/50) to the tumour cell lines.

Cell Surface Markers	DA3 (%)	DA3-MUC1 (%)	DA3-MUC1+IFN-γ (%)
negative control	1.94	3.12	2.71
MUC1	3.36	87.73	93.6
MHC class I	2.98	5.67	76.85
MHC class II	3.81	3.72	2.01
B7.1	1.49	3.03	1.67
ICAM 2	3.70	4.42	2.75
CD28	4.13	5.22	4.03
LFA-2	2.27	3.09	2.12
CTLA-4	4.81	6.09	3.70

Table 5.1: *In Vitro* expression of cell surface markers on DA3 and DA3-MUC1 cells cultured with or without 20 ng/ml IFN-γ for 72 h. Values represent the percentage of cells positive for each antibody determined by flow cytometry.

b) *In vivo* characterisation of DA3-MUC1

To characterise the DA3-MUC1 tumour *in vivo*, BALB/c mice were challenged with 5 x 10^6 metastatic cells and tumour growth monitored for 10 weeks (Figure 5.3). Mice had palpable subcutaneous tumours after 2 - 3 weeks and were culled after 10 weeks and their subcutaneous tumours, and lungs removed for tumour analysis.

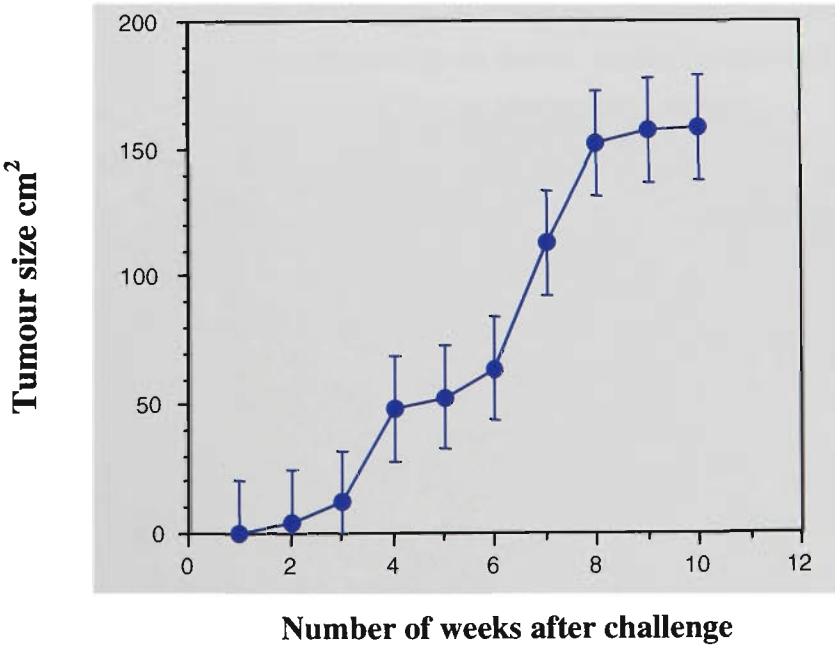
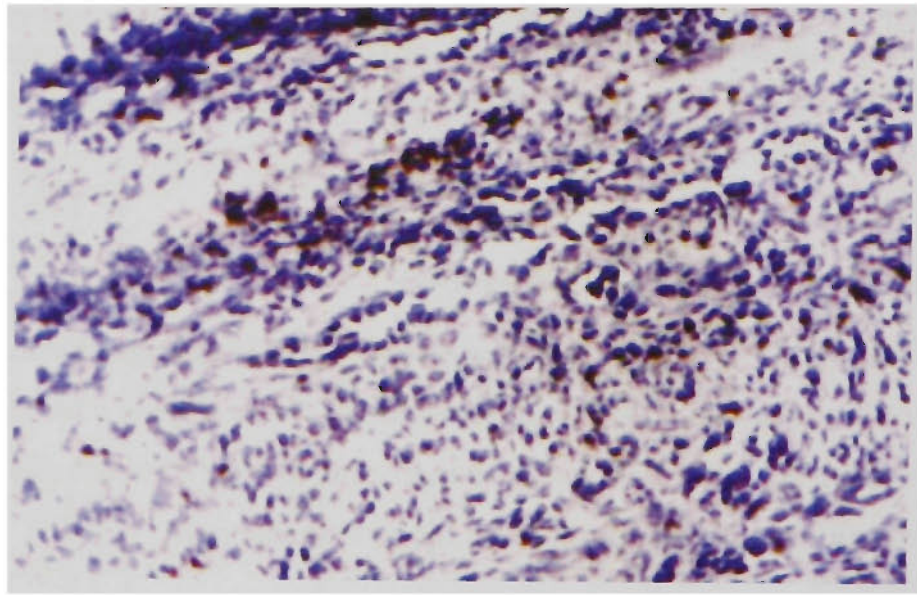
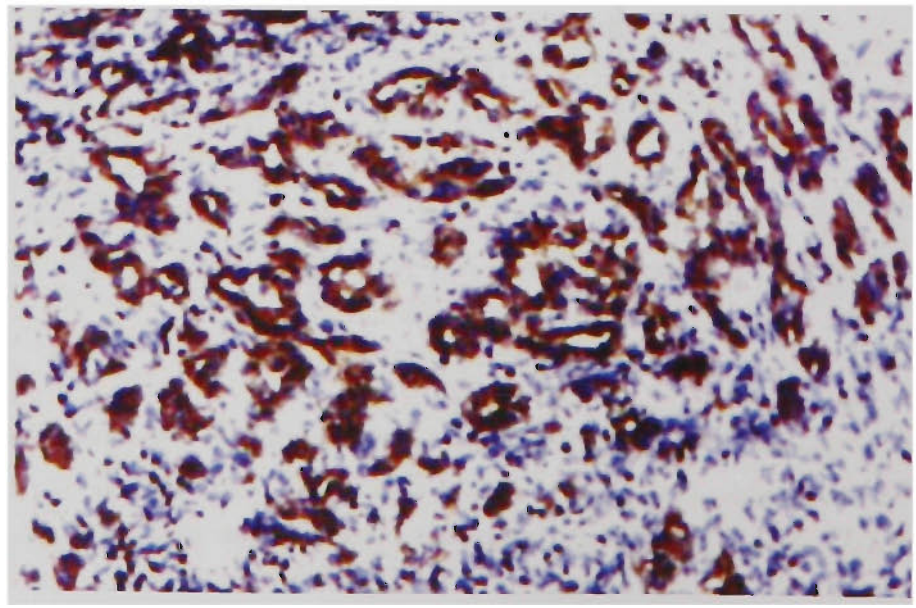


Figure 5.3: Tumour growth in BALB/c mice challenged with 5 x 10^6 DA3-MUC1 cells

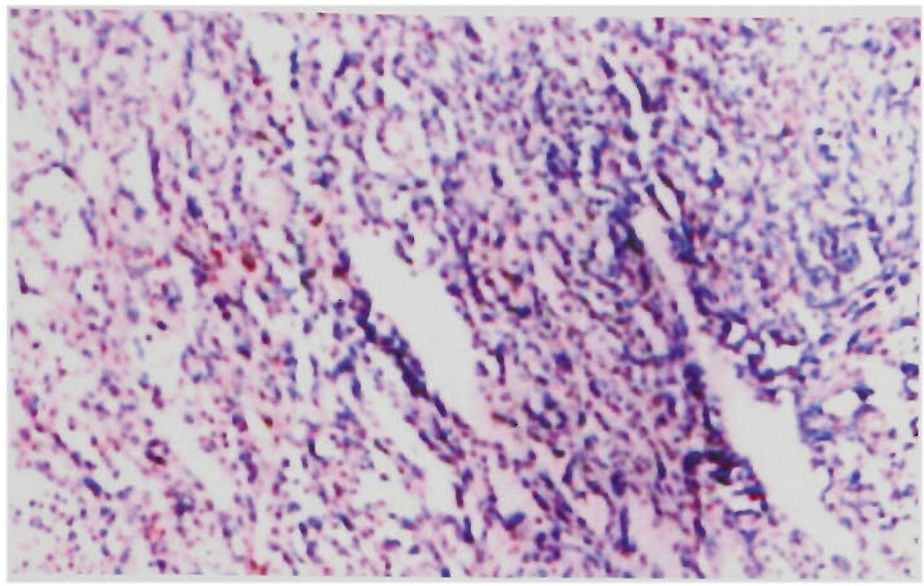
As expected, immunoperoxidase staining for MUC1 and MHC class I expression on subcutaneous established DA3-MUC1 tumours found similar findings to earlier *in vitro* studies. DA3-MUC1 tumour cells express high levels of MUC1 (75-100% of cells) (Figures 5.4a and b) and very little, if any, MHC class I (0-15% of cells) (Figures 5.4a and c) on their cell surface. Lung metastases (macroscopic and microscopic) were observed 30-35 days after a subcutaneous injection of 5 x 10^6 DA3-MUC1 cells, however there was no evidence of metastases to the liver (data not shown). Immunoperoxidase staining for MUC1 and MHC class I expression on lung metastases showed MUC1 expression on ~50% of tumour cells in the lung but no evidence of MHC class I expression (Figures 5.4d and f).



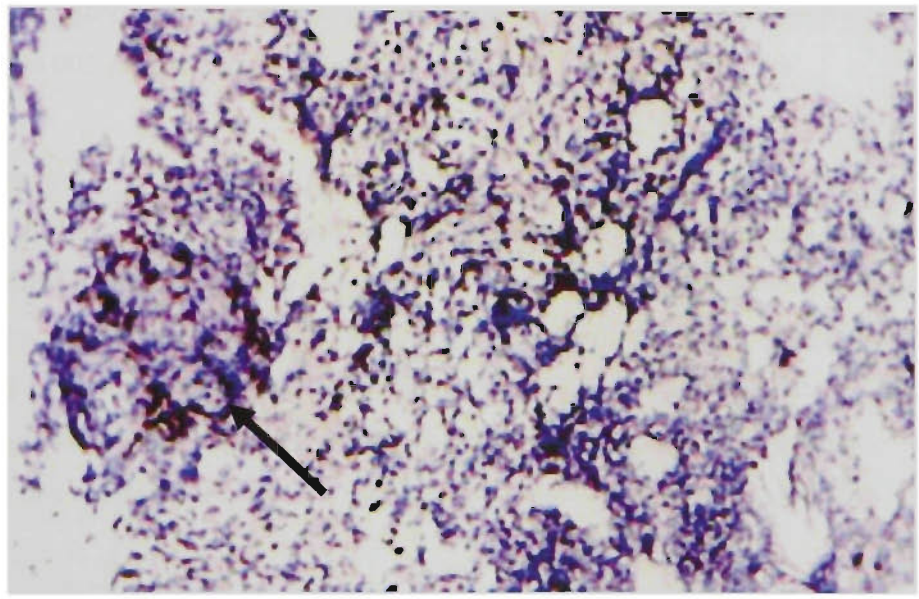
a) DA3-MUC1 S.C tumour, negative control (x 200 mag)



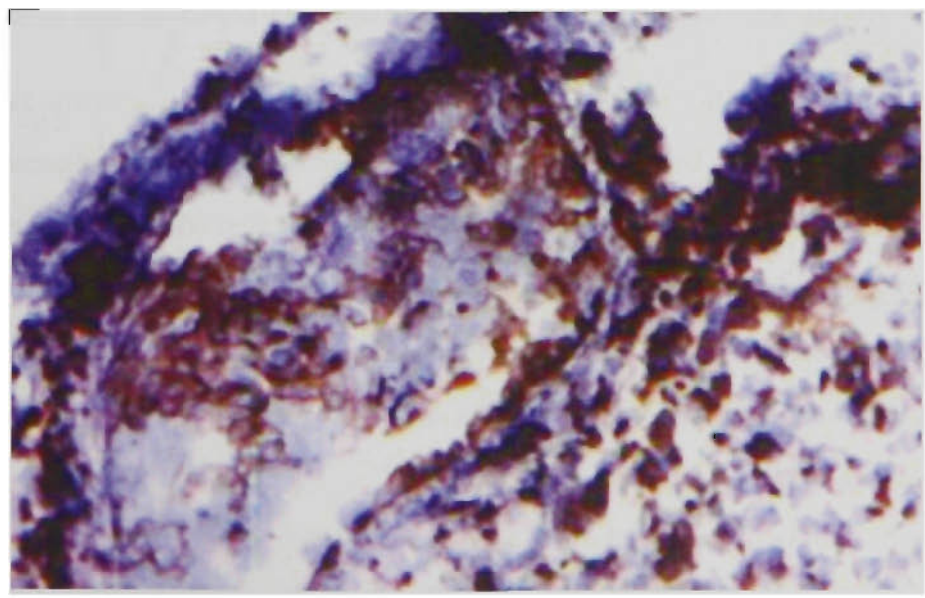
b) DA3-MUC1 S.C tumour, MUC1 expression (x 200 mag)



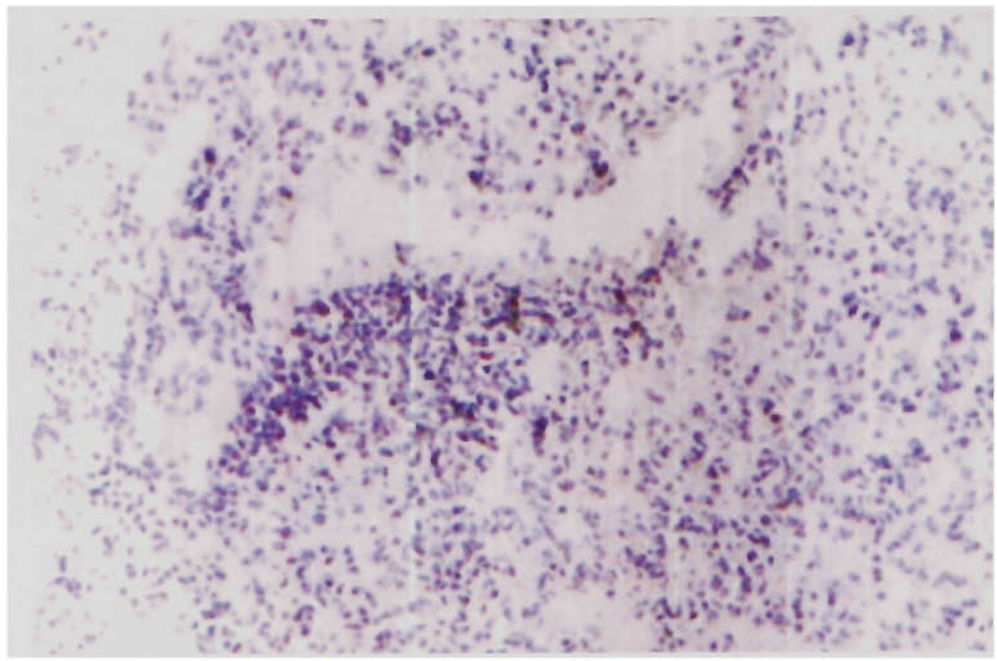
c) DA3-MUC1 S.C tumour, MHC Class I expression (x 200 mag)



d) DA3-MUC1 lung metastasis (as indicated by the arrow), negative control (x 200 mag)



e) DA3-MUC1 lung metastasis, MUC1 expression (x 200 mag)



f) DA3-MUC1 lung metastasis, MHC class I expression (x 200 mag)

Figure 5.4: MUC1 and class I ($H2^d$) surface expression on DA3-MUC1 tumour cells determined by immunoperoxidase staining. DA3-MUC1 tumour cells from the site of a BALB/c subcutaneous tumour were tested by MUC1 expression using biotinylated-BC2 (4b) and MHC class I expression using biotinylated-34.1.2s (4c). DA3-MUC1 lung metastases were tested for MUC1 expression using biotinylated-BC2 (4e) and MHC class I expression using biotinylated-34.1.2s (4f). Non-specific binding of $(Fab')_2$ conjugate was blocked with 10% BSA in DME and control samples were incubated with 10% BSA in DME (4a and 4d).

Elevation of MHC class I expression on DA3-MUC1 with IFN-γ

From earlier *in vitro* and *in vivo* findings, it was evident that one of the factors hindering the immunogenicity of the DA3-MUC1 tumour was a decrease in the expression of MHC class I cell surface molecules. Numerous studies have shown that MHC class I expression, and therefore tumour immunogencity, can be increased by culturing the tumour with recombinant IFN-γ. To investigate if MHC class I expression could be up-regulated on DA3-MUC1 cells, tumour cells were cultured with 20 ng/ml VV-IFN-γ for 72 h and MHC class I expression investigated using flow cytometry.

DA3-MUC1 expression of MHC class I molecules on the tumour surface could be greatly increased *in vitro* by culturing the cells with IFN-γ (Table 5.1 and Figure 5.2vii, viii and ix). Prior to *in vitro* culturing with IFN-γ, only 6% of DA3-MUC1 cells expressed MHC class I on their cell surface (Figure 5.2vi), however after culturing the cells with IFN-γ for 72 h, 77% of DA3-MUC1 tumour cells expressed MHC class I (Figure 5.2ix) and MUC1 expression still remained high (Figure 5.2v, vii).

To determine the length of time MHC class I expression remained elevated on DA3-MUC1, tumour cells were cultured with IFN-γ for 72 h, removed, and then examined daily for MHC class I expression (Figure 5.5). DA3-MUC1 MHC class I expression peaked at 72 h (~90%) (as expected with IFN-γ in the cultures) and remained elevated (>70%) for three days after the removal of the cytokine, after which time the expression dropped constantly to plateau at ~55% by day 10.

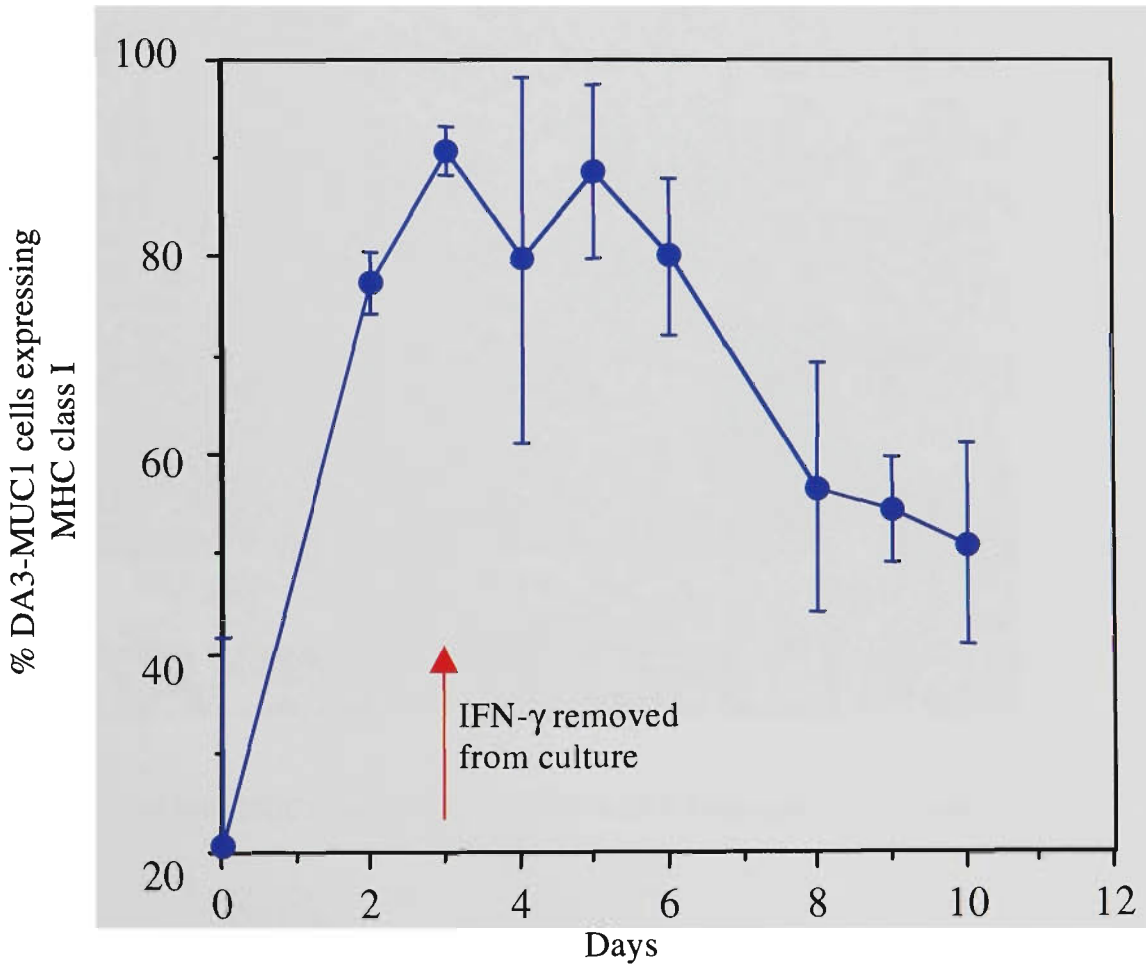
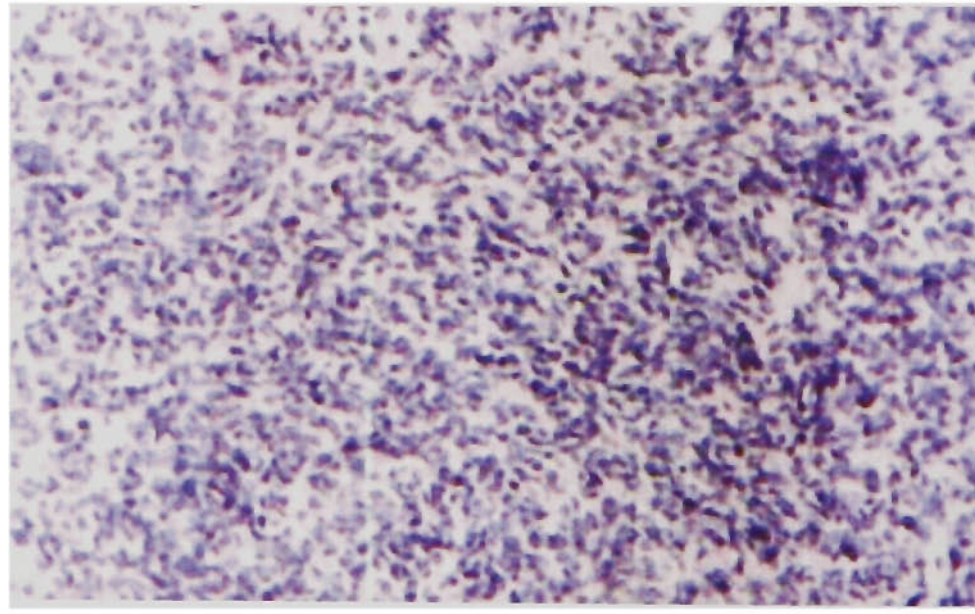
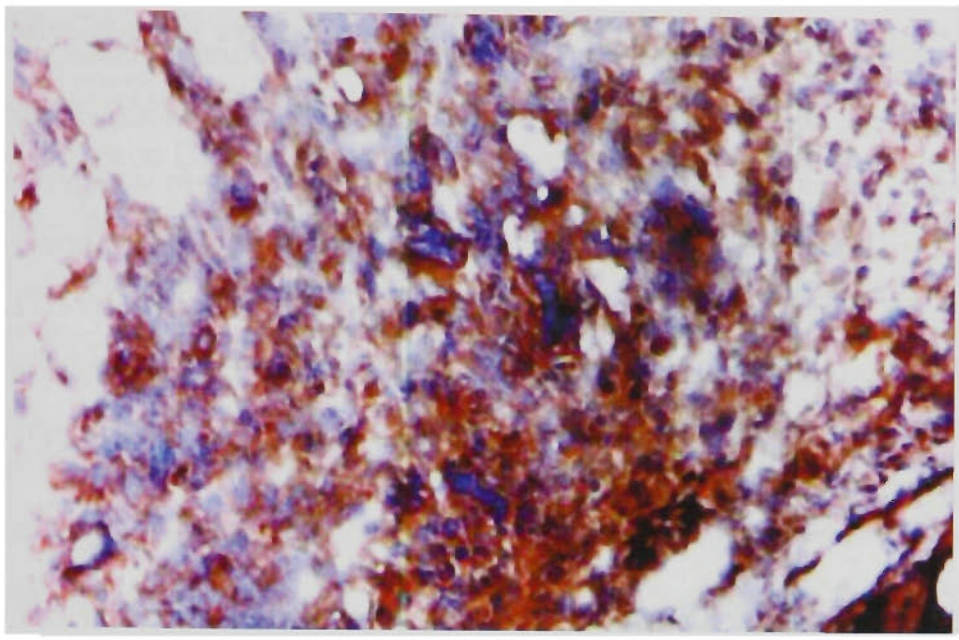


Figure 5.5: FACS analysis determining the length of time class I expression remained elevated on DA3-MUC1 cells cultured with IFN-γ *in vitro*. Tumour cells were cultured for 72 h with 20 ng/ml IFN-γ to increase MHC class I expression, the cytokine removed from culture and class I expression measured daily by FACS analysis.

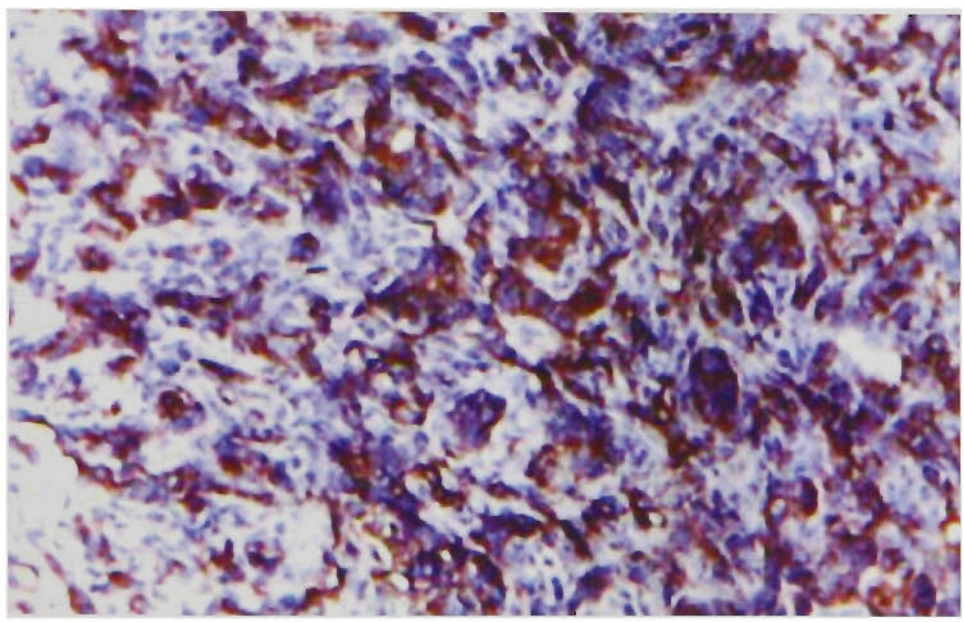
To ensure the elevated class I levels observed *in vitro* could be sustained *in vivo,* DA3-MUC1 cells were cultured with IFN-γ and injected subcutaneously into BALB/c mice. Mice were examined on days 4 and 7 for MHC class I expression by immunoperoxidase staining (Figure 5.6). DA3-MUC1 cells cultured with IFN-γ expressed high levels of MHC class I molecules on 75% of tumour cells removed from the subcutaneous site on day 4 (data not shown), with 50% of tumour cells still remaining positive on day 7 (Figure 5.6a and 5.6b). *In vivo* expression of MUC1 on the subcutaneous tumour was not altered after culturing with IFN-γ (Figure 5.6c). Thus, culturing DA3-MUC1 cells with IFN-γ increases the expression of MHC class I molecules on the cell surface for at least seven days after removal of the cytokine both *in vivo* and *in vitro.*



a) DA3-MUC1 pre-cultured with IFN-γ, negative control (x 200 mag)



b) DA3-MUC1 pre-cultured with IFN-γ, MHC class I expression (x 200 mag)



c) DA3-MUC1 pre-cultured with IFN-γ, MUCI expression (x 200 mag)

Figure 5.6: Immunoperoxidase staining of DA3-MUC1 cells pre-cultured with IFN-γ and grown *in vivo*. Mice were sacrificed every three days for 10 days, tumours removed, formalin fixed and stained for class I expression using anti-$H2^d$ ascites (b) and MUC1 expression using biotinylated BC2 (c). Phosphate buffer represents negative control binding of FITC-conjugated sheep $(Fab')_2$ anti-mouse (1/50) to the tumour (a).

Cytotoxic T cell assay on DA3-MUC1 targets

From the results so far, it would appear that the DA3-MUC1 tumour is not immunogenic because it has a decreased expression of MHC class I molecules on its cell surface. However, culturing the tumour with IFN-γ increases MHC expression both *in vitro* and *in vivo* for at least seven days after the cytokine is removed. What remains to be seen however, is if the increase in MHC class I molecules is enough to stimulate MUC1 specific CTL. To investigate this hypothesis further, a CTL assay was used to determine if BALB/c mice immunised with MFP could indeed recognise and kill DA3-MUC1 tumour cells expressing increased MHC class I molecules (Figure 5.7).

MUC1$^+$ CTL isolated from the spleen cells of mice immunised with MFP, were indeed able to kill DA3-MUC1 tumour cells cultured with IFN-γ at the same effector:target ratio as the positive control, P815-MUC1 cells i.e. at 6:1. Interestingly, without culturing with IFN-γ, MUC1 CTL were still detected, however the response was much weaker i.e. 40:1. There was no lysis of non-transfected P815 negative control cells. Therefore, *in vitro* MUC1 T cell cytotoxicity to DA3-MUC1 tumours increases substantially with elevated MHC class I expression.

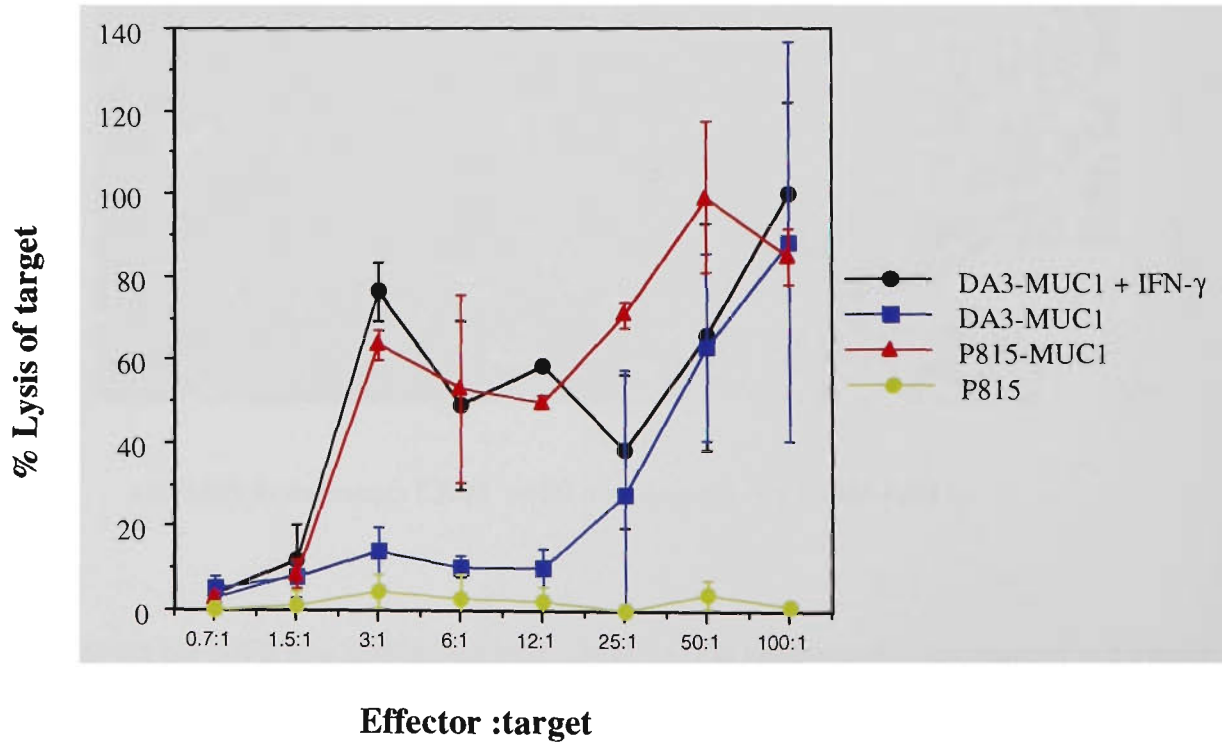


Figure 5.7: CTL assay of spleen cells from effector BALB/c mice immunised with 5 μg MFP, on ^{51}Cr-labelled DA3-MUC1 cultured with IFN-γ for 72 h, DA3-MUC1, P815-MUC1 and P815 tumour cell targets.

Immunotherapy of DA3-MUC1 expressing elevated MHC class I

As IFN-γ treated tumours express elevated levels of MHC class I, and *in vitro*, MFP can stimulate $MUC1^+$ CTL capable of killing DA3-MUC1 target cells, the antitumour effects of MFP immunisation on DA3-MUC1 tumours expressing MHC class I was investigated in two *in vivo* experiments. In both studies, mice were immunised three times with either MFP or a control pH 9.0 phosphate buffer and challenged with DA3-MUC1 tumour cells pre-cultured for 72 h with IFN-γ to increase MHC class I expression.

In the first study (Figure 5.8), BALB/c mice were challenged with 5×10^6 DA3-MUC1 cells with elevated MHC class I expression. Interestingly, a small reduction in tumour growth, which correlated with an increase in MHC class I expression (Figures 5.5 and 5.6), was evident between days 2 and 5 in both MFP and control pH 9.0 immunised mice. A decrease in tumour burden was evident in mice immunised with MFP compared to control mice, on day 3, suggesting that elevated levels (90-95%) of MHC class I expression on DA3-MUC1 tumour cells may increase their susceptibility to CTL lysis. However, no differences in tumour size were observed between MFP and control mice on any other days, and from day 6 onwards, DA3-MUC1 tumours continued to grow steadily which corresponded to a steady drop in surface MHC class I levels (Figure 5.5) as the positive tumour cells lost MHC class I expression.

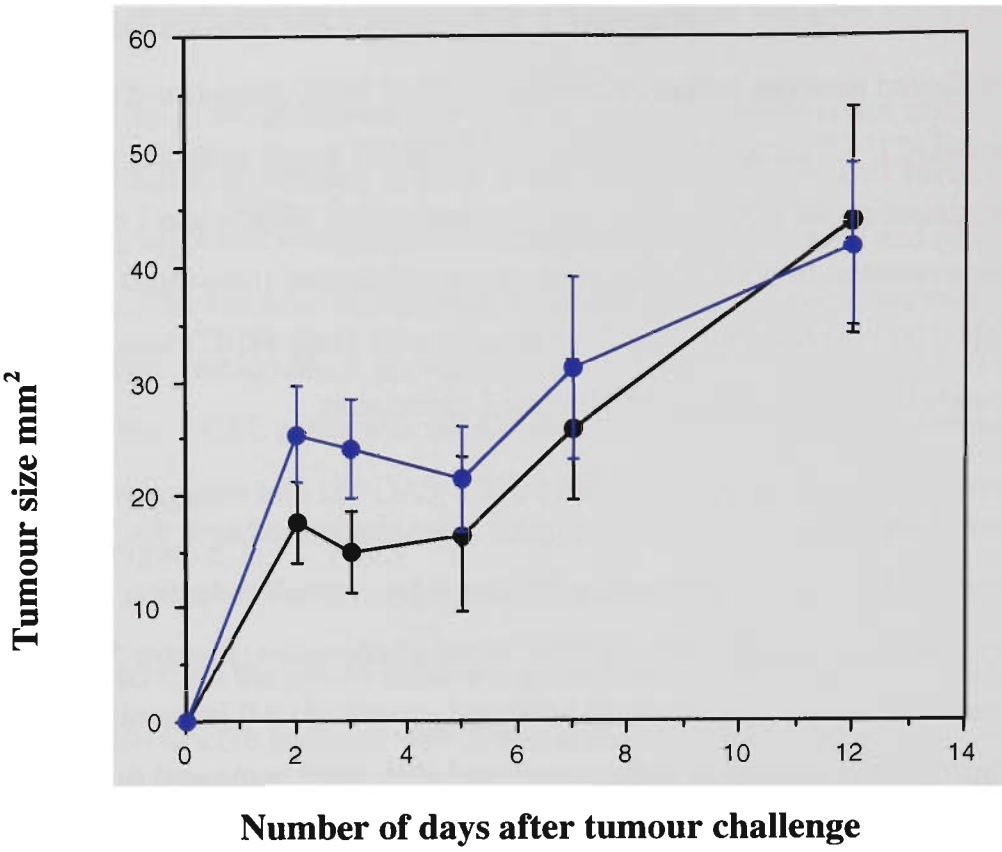


Figure 5.8: Subcutaneous tumour growth of DA3-MUC1 cultured with IFN-γ in BALB/c mice. Mice (x 10 per group) were immunised intraperitoneally on days 0, 7 and 14 with either 5 μg MFP (●) or pH 9.0 buffer (●) and challenged with tumours with 3 x 10^6 DA3-MUC1 tumour cells previously cultured with 20 ng/ml VV-IFN-γ supernatant (UV inactivated) for 72 h. Mice were culled after 30 days to determine lung metastasis.

In the second study (Figure 5.9), BALB/c mice were immunised with MFP and challenged with various doses of DA3-MUC1 tumour. Mice were given three immunisations with either MFP or a control pH9.0 buffer and were challenged with low doses (1 x 10^6, 5 x 10^5, 1 x 10^5) of DA3-MUC1 tumour cells cultured with IFN-γ to elevate surface MHC class I expression. Despite elevated MHC class I expression and the generation of $MUC1^+$ CTL able to recognise and kill DA3-MUC1 tumour cells *in vitro*, there were no differences in tumour burden between mice immunised with either MFP or pH 9.0 buffer, at any of the three doses of tumour examined. Thus elevated class I expression could not be sustained *in vivo* to induce anti-tumour CTL responses.

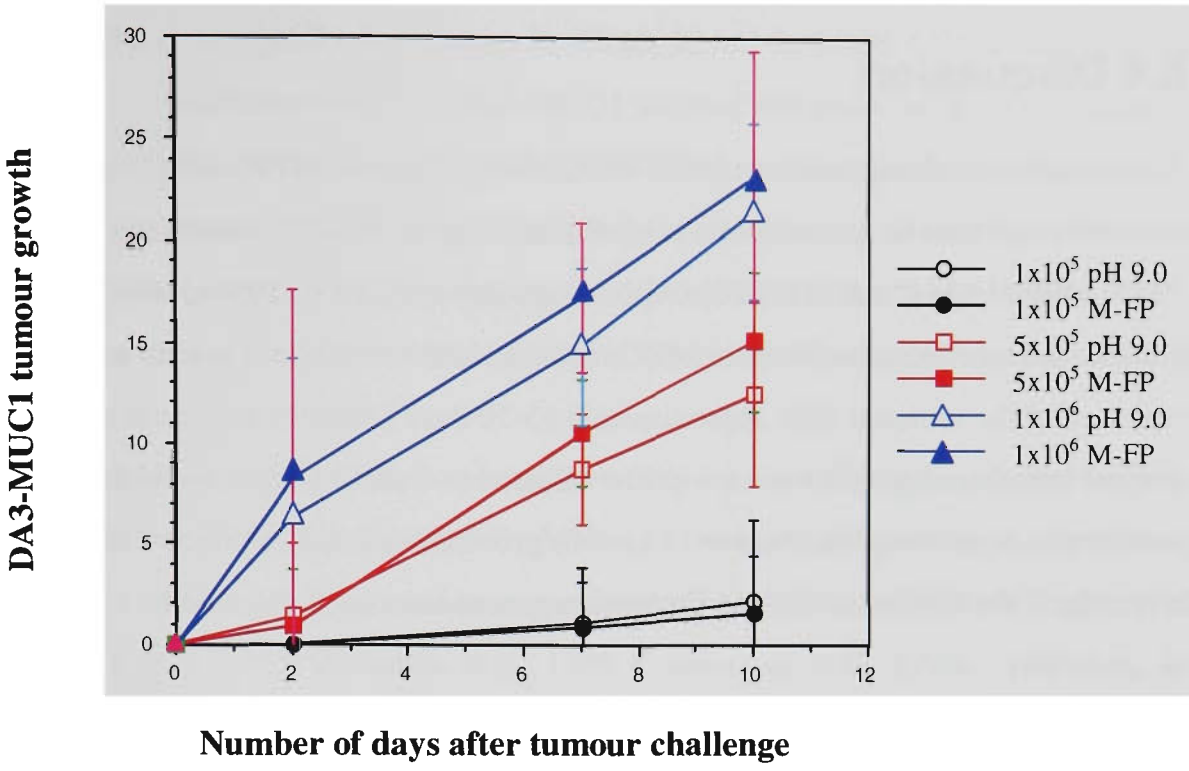


Figure 5.9: Dose-response tumour challenge of BALB/c mice immunised with 5 μg MFP or pH 9.0 buffer and challenged subcutaneously with either 1 x 10^5, 5 x 10^5 or 1 x 10^6 DA3-MUC1 tumour cells cultured with IFN-γ for 72 h to increase surface class I expression on the tumour.

5.4 Discussion

Tumour immunotherapy with mannan MUC1 fusion protein (MFP) induces $CD8^+$ cellular immunity and tumour protection in several immunogenic $MUC1^+$ tumour models ($MUC1^+$ P815, $MUC1^+$ RMA, $MUC1^+$ 3T3) (Apostolopoulos et al, 1995, 1996a, 1996b). In these models, the transfection of human MUC1 into the tumour cell lines results in the spontaneous rejection of the tumours after approximately 15-20 days (Apostolopoulos et al, 1994). Yet despite this, there has still been a window of between 0 and 11 days in which to observe either accelerated rejection or an absence of tumour growth in immunised mice – the basic model with which the MFP anti-tumour immune responses have been described.

In this study, the aggressive $MUC1^+$ metastatic tumour, DA3-MUC1, was investigated as a model to study MFP immunotherapy as it is not spontaneously rejected in mice and therefore a better indicator of anti-tumour immunity. However, when previously all other MUC1 tumour models have been immunogenic, a lack of tumour protection in the DA3-MUC1 model, resulted in an investigation of the expression of various cell surface markers required to induce cell mediated immunity. Both *in vitro* and *in vivo* studies confirmed that in contrast to other $MUC1^+$ tumour models, DA3-MUC1 has a low expression of cell-surface MHC class I molecules that can be upregulated with IFN-γ, however it directly reduces the immunogenicity of this tumour *in vivo*.

Initial immunotherapy studies demonstrated that mice immunised with MFP and then challenged with DA3-MUC1 tumours were not protected from tumour growth. Similarly in a therapy experiment, three injections with MFP was also unsuccessful at decreasing the tumour burden in mice with established DA3-MUC1 tumours. These findings were unlike other studies with MFP, whereby mice immunised with MFP were totally protected against a challenge of $MUC1^+$ 3T3 tumours (Apostolopoulos et al, 1995a) and the induction of a $CD8^+$ cellular immune response caused the regression of 15 day-old $MUC1^+$ P815 established tumours in DBA/2 mice (Apostolopoulos et al, 1996).

In vitro and *in vivo* characterisation of DA3-MUC1 showed the tumour was weakly immunogenic because even though high surface levels of the MUC1 antigen were expressed (>85%), there was a low level of all other cell surface molecules needed for T cell activation

including MHC class I (<6%), MHC class II, CD80, ICAM-2, CD28, LFA-2 and CTLA-4. Similarly, *in vivo* characterisation of DA3-MUC1 induced metastatic lung nodules again demonstrated MUC1 expression to be present on 50% of metastatic cells but there was no MHC class I expression. The absence, or relatively low expression, of these molecules on the tumour cell surface causes anergy in any activated T cells and is an effective mechanism many tumours have evolved to evade the immune system (Nawrocki and Mackiewicz, 1999).

However, tumour immunogenicity can be successfully increased by up-regulating the expression of these molecules (particularly MHC and costimulatory molecules) by either gene transfection or culturing with cytokines – specifically IFN-γ (Hui et al., 1984; Tanaka et al., 1984; Wallich et al., 1985; Watanabe et al., 1989, Gansbacher et al., 1990a). Therefore, to increase the expression of MHC class I on DA3-MUC1 cells, the tumour was cultured with IFN-γ. Culturing DA3-MUC1 with IFN-γ increased surface expression of MHC class I from <16% to >90% after 72 h. The class I expression remained elevated for several days before declining to 50% one week after the cytokine was removed from culture. *In vivo* investigations of MHC class I expression on DA3-MUC1 after IFN-γ culturing, revealed a similar pattern whereby levels previously not detected in a subcutaneous tumour, were elevated to 50-75% of cells expressing MHC class I four days later, and still present on day 7. Interestingly, culturing DA3-MUC1 with IFN-γ did not increase the surface expression of MHC class II, CD80, ICAM-2, CD28, LFA-2 or CTLA-4 as had been previously reported in other tumour models (Sgagias et al., 1996; Gastl et al, 1996).

Following the up-regulation of MHC class I on the surface of DA3-MUC1 tumours, MUC1 specific CTL isolated from the spleen cells of MFP immunised mice could only kill DA3-MUC1 tumour cells cultured with IFN-γ. This result was considerably higher than untreated tumour cells, demonstrating that DA3-MUC1 immunogenicity is increased in the presence of MHC class I, and can be killed by MUC1 restricted CTL *in vitro*. This study again confirms previous findings by Apostolopoulos et al (1995b) demonstrating there are no non-restricted MUC1 CTLs. However, the generation of MUC1 specific CTL able to kill DA3-MUC1 tumour cells cultured with IFN-γ, did not extend to the *in vivo* scenario as MFP was not able to induce tumour protection in mice challenged with various doses of DA3-MUC1 tumour.

The lack of an effective anti-tumour response in DA3-MUC1 tumours is, in part, a result of the down-regulation in MHC class I expression which can be overcome by culturing the tumour with IFN-γ. As culturing with this cytokine only temporarily increases MHC expression; evidently not enough to induce anti-tumour immunity following MFP immunisation, it is suggested that future studies in this model would focus on the transfection of the IFN-γ gene into tumour cells. Alternatively, the decrease in DA3-MUC1 immunogenicity may also be a result of tumour-reactive T cells receiving inadequate costimulation through the absence of the costimulatory molecules B7-1 and B7-2. This again can be over come through transfection with these molecules (Chen et al., 1992) and is also suggested for future immunotherapy studies with MFP.

Chapter 6:

A role for IL-5 in the induction of cytotoxic T lymphocytes *in vivo*

Vasso Apostolopoulos, Ian FC McKenzie, Catherine Lees, Klaus I Matthaei, Ian G Young, A role for IL-5 in the induction of cytotoxic T lymphocytes *in vivo*. Eur. J. Immunol. 30:1733-1739, 2000.

Summary

IL-5 is generally regarded as a T2 cytokine involved in eosinophil maturation and function and in B cell growth and antibody production, but without any well-established effects on T cells. Early reports suggested that IL-5 could stimulate the production of cytotoxic T lymphocytes (CTL) *in vitro*, but no evidence has been obtained to date for such a role in studies with IL-5 deficient (IL-5 -/-) mice. This study demonstrates that when oxidised mannan MUC1 (MFP) is used as an antigen in mice, IL-5 is required for the optimal generation of the CTL response. IL-5 was as effective as IL-2 for the *in vitro* induction of CTL from spleen cells *in vitro,* and both $CD4^+$ and $CD8^+$ T cells from MFP immunised animals could be shown to secrete IL-5 in culture. In IL-5 -/- mice, the CTLp frequency was greatly diminished resulting in the inability to reject $MUC1^+$ tumours.

The CTL, CTLp and tumour challenge experiments in this chapter were performed by Dr Vasso Apostolopoulos. The cytokine profile studies of IL-5 and the CTL and tumour protection studies, clearly demonstrate IL-5 is produced by functional T cells, especially the Tc1 type, after MFP immunisation and is required for an optimal CTL response to this antigen.

6.1 Introduction

It is generally accepted that $CD4^+$ and $CD8^+$ T cells secrete both T1 (IL-2, IFN-γ and TNF-α) and T2 (IL-4, IL-5, IL-6, IL-10 and IL-13) cytokines. IL-5 has been considered to be a typical T2 cytokine as it acts as a differentiation factor and regulator for eosinophils and is involved in B cell growth and antibody production (IgA, IgM and IgE). There are many reviews on the role of IL-5 as a T2 cytokine, concentrating on both *in vitro* and *in vivo* studies (Coffman et al., 1991; Sanderson 1992; Mosmann et al., 1996). In particular, there has also been much progress in defining the biological role of IL-5 *in vivo*. For example, mice transgenic for IL-5 under a metallothionein promoter have an increased number of $CD5^+$ (B-1) B cells, develop eosinophilia and high levels of autoantibodies (Tominaga et al., 1991). However, mice transgenic for IL-5 under the control of the lymphocyte specific CD2 promoter showed no effects on B cells or antibody levels, although they developed eosinophilia (Dent et al., 1990). Further studies on the biological role of IL-5 in B cell development and on eosinophils were stimulated by the development of IL-5 gene knockout mice (IL-5 -/-). Such mice lacking IL-5 did not develop eosinophilia when infected with the helminth *Mesocestoides corti* and had a conventional development of B (B-2) cells, cytotoxic T cells (CTL), T cell dependent antibody responses and NK cell activity (Kopf et al., 1996). The number of $CD5^+$ (B-1) B cells however, was reduced in 2-week old mice but returned to normal by 6-8 weeks (Kopf et al., 1996).

More recent studies with the IL-5 -/- mice indicate a continuing reduction in B-1 lymphocytes and in mucosal IgA production (Bao et al., 1998). Similar findings were obtained with mice deficient in the IL-5-α receptor (Yoshida et al., 1996). Thus the studies to date with transgenic animals support a key role for IL-5 in the regulation of eosinophilia and in involvement in B-1 cell development and in antibody production. Several early reports (Takatsu et al 1987; Ramos 1989) showed that IL-5 could stimulate CTL production *in vitro*, but no evidence for such a role *in vivo* in relation to anti-viral CTL responses has come from studies with IL-5 -/- mice. Clearly it is necessary to obtain data *in vivo* since *in vitro* activities are generally not accurate predictors of the biological role of cytokines.

In the present work we have used IL-5 -/- mice to demonstrate that IL-5 plays a significant role in the CTL response to the modified tumour antigen oxidised mannan MUC1 (MFP) and have shown that in the absence of IL-5, the CTL response is insufficient to enable $MUC1^+$ tumour rejection. These findings indicate that an IL-5 dependent mechanism exists for the generation of CTL and suggests a broader role for IL-5 than previously envisaged.

6.2 Materials and Methods

Antigens, tumours and immunisation of mice

Human MUC1 was produced as a GST-fusion protein which contained five VNTR regions and consisted of 105 amino acids (FP), and was conjugated to mannan using oxidising conditions (oxidised-mannan-MUC1; MFP) (Apostolopoulos et al 1993; 1995; 1996) (refer Chapter 1, section 1). For reduced MFP the Schiff bases and aldehydes of the oxidised MFP complex were reduced to amines and alcohols with 1mg sodium borohydride overnight at room temperature. Synthetic MUC1 20mer peptides of sequence (PDTRPAPGSTAPPAHGVTSA) were synthesised using an ABI peptide sequencer.

IL-5-/- mice (C57BL/6 background) (Kopf et al., 1996) were generated and bred at The John Curtin School of Medical Research. B6.IL-5-/- mice (Kopf et al., 1996) or the parental C57BL/6 strain were immunised intraperitoneally with oxidised or reduced MFP conjugate (containing 5 μg of FP) either once, or weekly for 3 weeks. It has previously been demonstrated that the optimal route of immunisation is i.p (although i.v., i.d., and i.m. immunisation generated similar responses), and 5 μg was shown to be optimal in generating CTL responses (Pietersz et al., 1998). The number of injections was also important for CTL generation, three injections were found to be optimal, more or fewer injections generated weaker CTLp frequencies (Pietersz et al., 1998).

RMA-MUC1 cells (Graham et al., 1995) were obtained from Dr J. Burchell, (ICRF, London, GB). Groups of seven mice immunised with oxidised MFP, were challenged 7 days after the final immunisation with a 0.2 ml subcutaneous injection of 5 x 10^6 RMA-MUC1 tumour cells in PBS and subsequent tumour growth measured with dial gauge calipers (Schnelltaster, H. C. Kroplin, Hessen, Germany) and the size of the tumours were expressed as the product of the two perpendicular parameters.

CTL assay

Spleen cells from mice immunised with MFP (either once or three times i.p.) were obtained 7 days after the third immunisation, washed in PBS containing 2% foetal calf serum and 2 x 10^6 cells/ml seeded in 24 well tissue culture plates (refer to Chapter 2, section 2.2.1). Non-immune spleen stimulator cells (as antigen presenting cells) were irradiated with 3,000 rad (Gammacell 1000 elite irradiator; Nordion International) and 2 x 10^6 cells/ml (stimulators) were added to the effector cells. 90 μg/ml of the 20 mer MUC1 peptide (PDTRPAPGSTAPPAHGVTSA) and 10U/ml of recombinant IL-2 or IL-5 (Pharmingen, CA, USA) were added. After 1 week of restimulation, effector cells were harvested and mixed at various effector:target with ^{51}Cr-labeled RMA-MUC1 cells (Apostolopoulos et al., 1993; 1995; 1996). RMA (non-MUC1) tumour targets were not lysed in any experiments.

CTLp frequency analysis

Mice immunised once or three times with oxidised MFP were sacrificed 2 weeks after the final injection. For each cell suspension in which CTLp frequencies were determined, a minimum of 32 replicates for each of at least six effector cell doses (ranging from 1 x 10^3-5 x 10^5 spleen cells per well) were cultured in U-bottomed microtitre trays, with 5x10^5 C57BL/6 spleen cells irradiated using 3,000 rad (stimulator cells; antigen presenting cells), in modified Eagle's medium supplemented with 10% foetal calf serum, 90 μg/ml synthetic MUC1 peptide (C- PAHGVTSAPDTRPAPGSTAP) and 10 U/ml recombinant human IL-2 (refer to Chapter 2, section 2.2.2). Seven days later each microculture was assayed for cytotoxicity by replacing 100 μl of culture medium with 100 μl target cell suspension containing 10^4 ^{51}Cr-labelled RMA-MUC1 or RMA tumour target cells. Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release three standard deviations above the mean release from 10^4 effector cells cultured alone, or 10^4 effector cells and 5 x 10^5 stimulators together or stimulators and peptide and rIL-2 together. A linear relationship existed between the dose of effector cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative well (Taswell 1981; Lefkovits and Wladmann 1984). Each experiment was performed three to four times.

T cell cultures and cytokine production

Spleen cells from immunised mice (Apostolopoulos et al., 1995; 1996; Lofthouse et al., 1997; McKenzie et al., 1998) were collected and pooled in RPMI. B cells were depleted by incubating 5 x 10^7 Pan B (B220) Dynabeads (Dynal, VIC, Australia) in 5ml RPMI rotating for 45 min at 4^0C. Supernatants were collected. $CD4^+$ T cells were removed using 5 x 10^7 CD4 (L3T4) Dynabeads ($CD8^+$ population). $CD8^+$ T cells were removed using 5 x 10^7 CD8 (Lyt-2) Dynabeads ($CD4^+$ population). The separated populations were shown to be rich in $CD4^+$ or $CD8^+$ cells by flow cytometry (Lees et al., 1999). $CD4^+$ T cell enriched cultures [$CD4^+$ ($CD8^+$ depleted)] consisted of ~70% $CD3^+$ / $CD4^+$ cells; <5% $CD3^+$ / $CD8^+$ cells; $CD8^+$ T cell enriched cultures (Lees et al., 1999) (refer to Chapter 2, section 3). $CD8^+$ ($CD4^+$ depleted) consisted of ~60% $CD3^+$ / $CD8^+$ cells, <5% $CD3^+$ / $CD4^+$ cells. Remaining cells in both $CD4^+$ and $CD8^+$ cultures, consisted of B cells, macrophages and other haematopoietic cells.

Cells (2 x 10^6) from mice immunised with oxidised MFP, reduced MFP, or pH 9.0 control buffer were mixed with MUC1 peptide (20 μM) and 2 x 10^6 cells (naive spleen cells, as stimulator cells) irradiated using 3,000 rads. Each culture was separated into either bulk spleen cells, $CD4^+$ cells, $CD8^+$ cells or $CD4^+CD8^+$ cells. Cells were cultured for 72 h and supernatants collected and tested for IL-5 production. Cytokine assays were performed by ELISA using commercial kits (Endogen, USA) as previously described (Lees et al., 1999) (refer to Chapter 2, section 4.4).

6.3 Results

Previous studies have shown that immunisation of mice with oxidised mannan conjugated to MUC1 (MFP), generates MHC-restricted $CD8^+$ CTL that recognise MUC1-transfected cells, $MUC1^+$ tumours and peptide-pulsed target cells (Apostolopoulos et al., 1995; 1996; Lofthouse et al., 1997; McKenzie et al., 1998). In these studies it was demonstrated that by immunising with oxidised MFP, a predominant TI response was generated with secretion of IL-2, IFN-γ, no IL-4, little antibody, strong CTL and tumour rejection (Apostolopoulos et al., 1995; 1996; Lofthouse et al., 1997; McKenzie et al., 1998). We now show that IL-5, a T2 cytokine, is also produced as part of this response and is required for optimal CTL induction and for the development of a CTL response sufficient for $MUC1^+$ tumour rejection.

Effect of IL-5 on *in vitro* stimulated CTL

Spleen cells from C57BL/6 mice immunised with oxidised MFP were restimulated *in vitro* for 7 days in the presence of immunising peptide, with the addition of either IL-2, IL-5 or both together. Effector cells were then mixed at various effector:target with RMA-$MUC1^+$ cells and cell lysis measured. *In vitro* RMA target cells were negative. *In vitro* stimulation in the absence of either IL-2 or IL-5 gave little lysis, but in the presence of IL-2, CTL were effective, giving ~80% lysis at a 6:1. However, when IL-5 was used instead of IL-2, virtually the same CTL profile occurred ie. IL-5 was as effective as IL-2 for the *in vitro* generation and expression of CTL (Figure 6.1). It is of interest that the simultaneous addition of both IL-2 and IL-5 gave no additive effects suggesting that the two cytokines act upon the same pathway. It would be of interest to determine what effects anti-IL-2 antibodies would have if added to the IL-5-supplemented cultures. However, the results clearly suggest that IL-5 in this system is involved in the induction/expression of CTL, an activity similar to that reported earlier (Takatsu et al., 1987), but in this case IL-5 alone is sufficient with no requirement for the presence of IL-2.

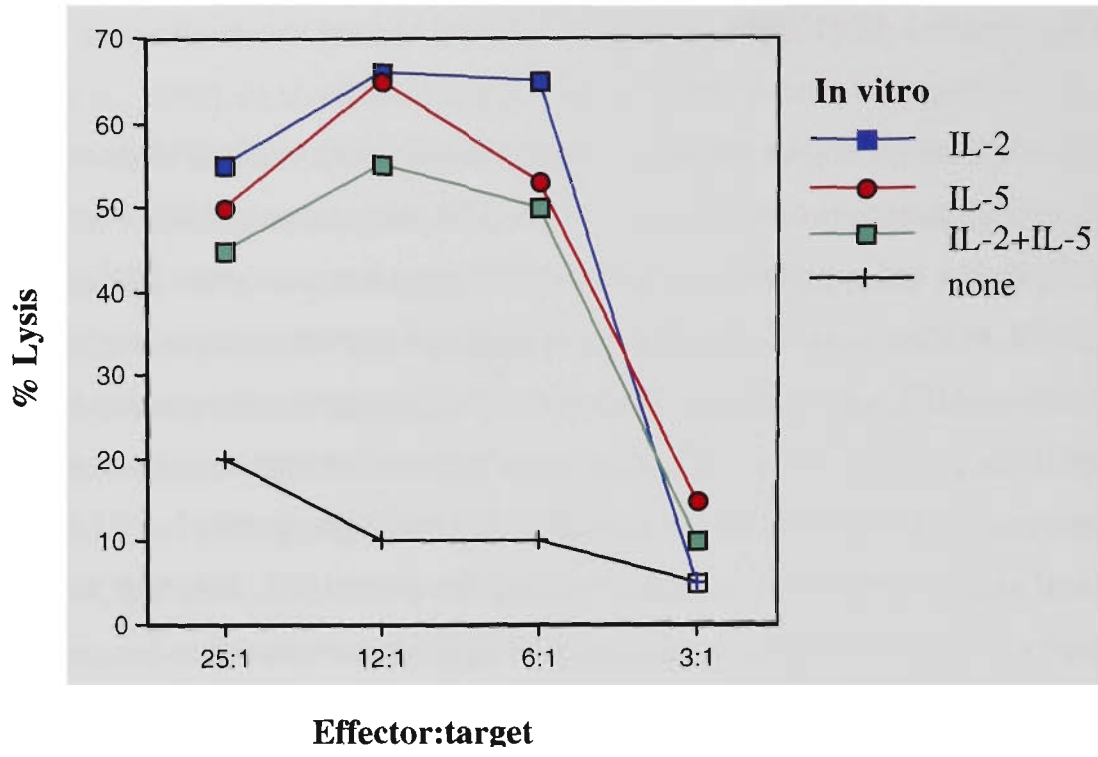


Figure 6.1: CTL assay using ^{51}Cr labeled RMAMUC1 from C57BL/6 mice immunised with oxidised MFP. *In vitro* restimulation with MUC1 peptide in the presence of either IL-2, IL-5, IL-2 + IL-5, or no cytokine. RMA cells were not lysed

CTL precursor frequency in IL-5 -/- mice

To further examine the role of IL-5 in CTL induction, CTLp frequency assays were performed in normal wild-type C57BL/6 and B6.IL-5 -/- mice immunised with oxidised MFP. The results demonstrate (Figure 6.2) that one injection of MFP gave a CTLp frequency 1/70,000 in C57BL/6 mice, however, in IL-5 -/- mice the frequency was reduced to 1/170,000. When 3 injections of MFP were given, there was a frequency of 1/10,000 in C57BL/6 mice and of 1/80,000 in IL-5 -/- mice (Figure 6.2). Clearly, the presence of IL-5 significantly enhances the precursor frequency and with this antigen IL-5 plays an obligatory role in the generation of the CTL response.

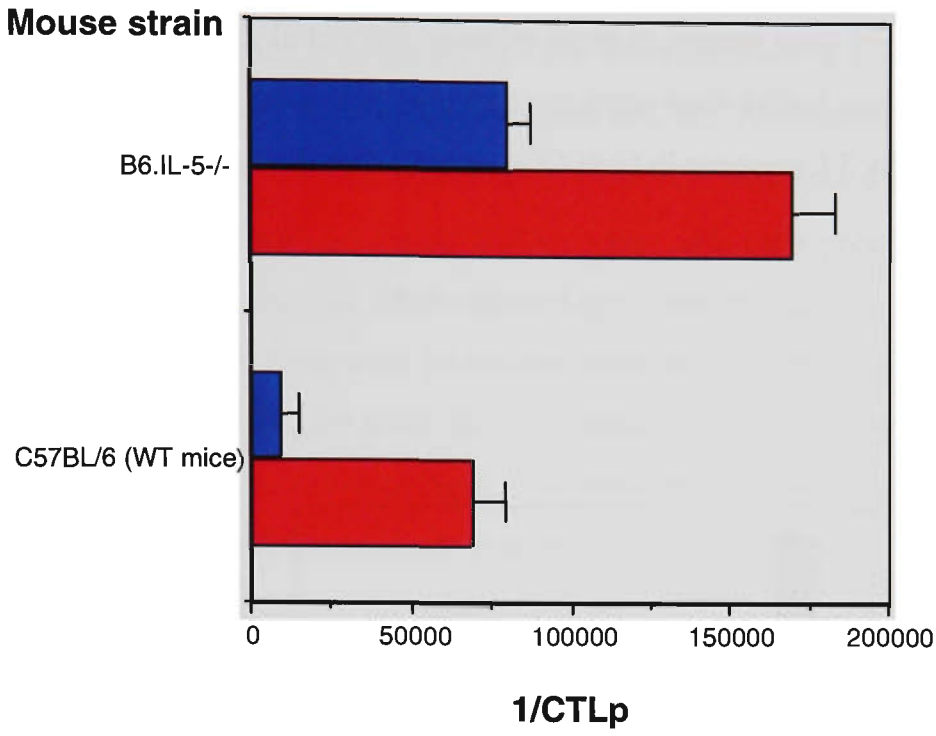


Figure 6.2: CTLp frequencies detected in spleen cultures from C57BL/6 (wild-type) or B6.IL-5 -/- mice, immunised with oxidised MFP, once (■) or three times (■). Target cells were RMA-MUC1$^+$; RMA cells gave a CTLp frequency of >1:10^6. The results are an average of three to four individual mice.

Cytokine production of T cell cultures from mice immunised with MFP

Spleen cell cultures from mice immunised with oxidised MFP were selectively depleted of CD4 or CD8 cells to produce (i) CD8$^+$ (ii) CD4$^+$ (iii) CD4$^+$/CD8$^+$ or (iv) non-depleted bulk cultures, and cytokine profiles were measured after *in vitro* culture with antigen. IL-5 was produced in significant quantities in all the cultures; by bulk cultures (900pg/ml); by CD4$^+$/CD8$^+$cells (1060pg/ml); and by both CD4$^+$ (1080pg/ml) and CD8$^+$ (2000pg/ml) cell cultures (Figure 6.3) ie. both CD4$^+$ and CD8$^+$ T cells produced IL-5. Other cytokines were measured and the T1 cytokines IL-2, TNF-α and IFN-γ were also produced, but the T2 cytokines, IL-4 or IL-10 (Lees et al., 1999) were not detectable. When mice were immunised with reduced MFP (oxidised MFP exposed to sodium borohydride, which reduces Schiff bases to amines and aldehydes to alcohols) which had previously been identified as inducing

T2 responses (Apostolopoulos et al., 1995; 1996; 2000; Lofthouse et al., 1997; McKenzie et al., 1998; Lees et al., 1999), IL-5 was not present (Figure 6.3); and in this case the T2 cytokines IL-4 and IL-10 were present in these cultures (Lees et al., 1999). In control mice immunised with phosphate buffer there were no cytokines produced. Thus, oxidised MFP induced a predominantly T1 response in both $CD4^+$ and $CD8^+$ cells but this also included the production of IL-5.

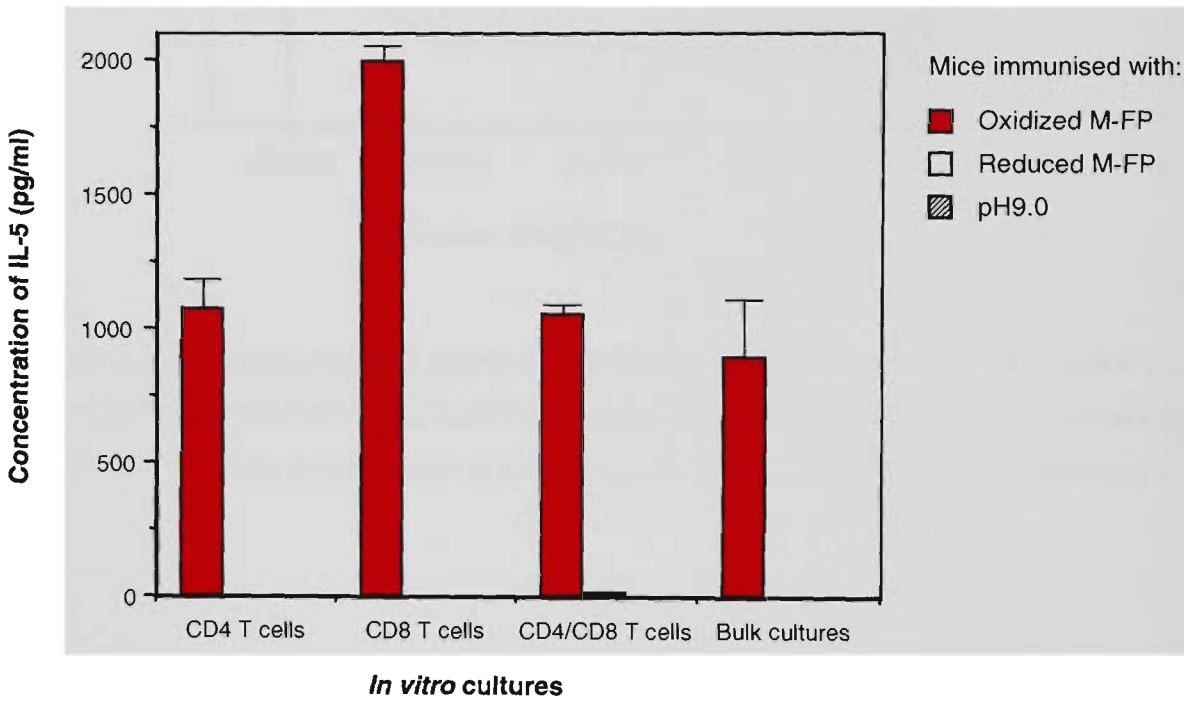


Figure 6.3: ELISA assay for *in vitro* cytokine production after 72 h in cultures of splenic, $CD4^+$ / $CD8^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells stimulated with 20 ng/ml MUC1 peptide, and prepared from mice immunised with either oxidised and reduced MFP or with control phosphate buffer.

Role of IL-5 in the rejection of tumours

MUC1$^+$ tumours in mice are rejected by CD8$^+$ cells and there is a direct correlation between the control of tumour growth and the numbers of CTL (Apostolopoulos et al., 1994; 1995; 1996; Lofthouse et al., 1997; Pietersz et al., 1998). C57BL/6 mice immunised with oxidised MFP and challenged with syngeneic RMA tumour cells grew progressively and were not rejected; in IL-5 -/- mice, the RMA tumours also grew progressively, but more rapidly (Figure 6.4). When C57BL/6 mice were immunised with oxidised MFP and challenged with RMA-MUCI tumours, the tumours grew, but were then rejected, due to the effective CTL-immune response to MUCI. However, in the IL-5 -/- mice the RMA-MUC1 tumours grew progressively and were not rejected (Figure 6.4). Previous studies had indicated that a certain level of CTLp were required for tumour rejection (> 1/20,000), and there was a complete correlation (r^2=0.982) between the CTLp frequency and tumour rejection using various MUC1 formulations (Lofthouse et al., 1997; Pietersz et al., 1998). The CTLp frequency gave a clear and quantitative measure of the rapidity and nature of tumour rejection; thus, CTLp frequency can be used as a readout for *in vivo* efficacy (Pietersz et al., 1998). Since from the studies cited above on CTLp, the immunisation of IL-5 -/- mice with oxidised MFP only gave a frequency of 1/80,000, tumour graft rejection would not be expected. Thus, IL-5 has direct effects on the numbers of CTL and CTLp, and on tumour graft rejection.

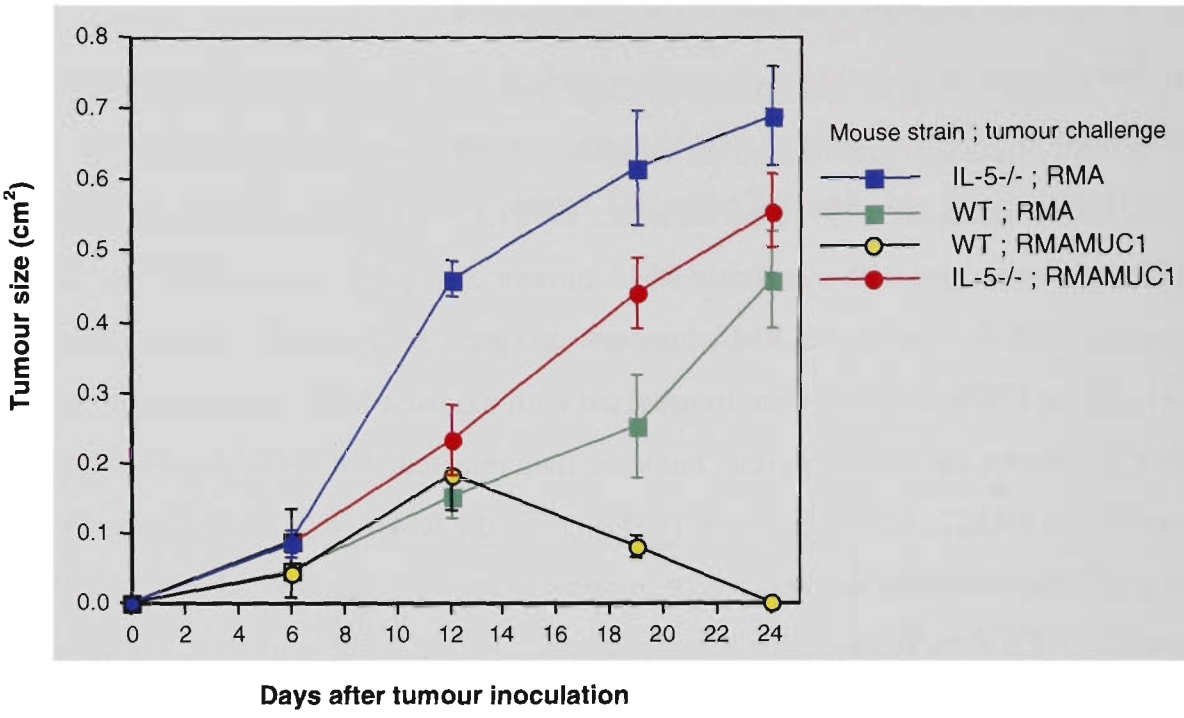


Figure 6.4: Growth of RMA or RMAMUC1$^+$ tumours in IL-5-/- and C57BL/6 (wild-type) mice. Mice were immunised with oxidised MFP and IL-5-/- mice challenged with RMA or RMA-MUC1; wild-type mice challenged with RMA or RMA-MUC1. The mean size of the tumours (the product of two diameters) is shown (vertical axis) with standard deviation (n=7), and days after tumour inoculation (horizontal axis).

6.4 Discussion

Although the involvement of IL-5 in the generation of CTL was suggested by earlier studies *in vitro* (Takatsu et al., 1987; Ramos 1989), this is the first demonstration of a role for IL-5 in a CTL response *in vivo*. These studies indicate that IL-5 appears to be involved in the induction of CTL. Firstly, addition of IL-5 to *in vitro* cultures could effectively replace IL-2 for CTL induction; secondly, IL-5-/- mice had a decreased frequency of CTLp in comparison to C57BL/6 mice and, unlike the parental mice, could not reject $MUC1^+$ tumours. In addition, both $CD4^+$ and $CD8^+$ populations isolated from mice immunised with oxidised MFP could produce IL-5. Thus, IL-5 is required for CTL maturation and is produced *in vitro* by $CD4^+$ and $CD8^+$ T cells derived from mice immunised with oxidised MFP (which is presented by MHC Class I molecules) but not those immunised with reduced MFP. The defect in CTL maturation caused by IL-5 deficiency is unlikely to be due to an indirect effect on T cell cytokine production as previous studies (Kopf et al., 1996) have shown that stimulated T-cells from IL-5 -/- mice produce normal levels of a range of cytokines including IL-2, IL-3, IL-4, IL-10 and IFN-γ.

In these experiments, it appears that IL-2 and IL-5 were equally effective in CTL induction *in vitro* with no additive effects suggests that both cytokines may be stimulating the same pathway. Since it is well established that the effect of IL-2 is directly on the T cells it seems likely that the effect of IL-5 will be analogous to that of IL-2 and not on antigen presentation. Since the experiments with IL-5 -/- mice indicate that IL-5 plays an obligatory role in the CTL response to MFP, it is possible that the effect of IL-2 in this case may be via the induction of IL-5. Of interest in this regard are reports that IL-2 can induce IL-5 in mice (Yamaguchi et al., 1990) and in patients undergoing treatment with recombinant IL-2 (Macdonald et al., 1990).

However the important aspect of the present results is the demonstration of a role for IL-5 in the generation of a CTL response to immunisation with MFP *in vivo*. This is in contrast to a previous study, which showed that anti-viral CTL induction was normal in the IL-5-/- mice (Kopf et al., 1996). This suggests the existence of IL-5-dependent and -independent mechanisms for CTL maturation, which are regulated according to the type of immune response elicited. It is already known that IL-5 is produced in a limited subset of immune

responses, namely those relating to infections with parasitic helminths and to allergies. Possibly a similar limited subset of CTL responses will be IL-5 dependent. IL-5-dependent and -independent mechanisms also appear to regulate the B-1 lymphocyte lineage.

The subdivision of T helper cells into two classes (T1 and T2) was originally described for murine $CD4^+$ T cell cultures. The T1 subclass secreted cytokines IL-2, IFN-γ and TNF-α and the T2 subclass, IL-4, IL-5, IL-6, IL-10 and IL-13 (Coffman et al., 1991; Mosmann et al., 1996). More recently, murine $CD8^+$ T cells have also recently been subdivided into similar subsets, Tc1 and Tc2 (Salagame et al., 1991). The distinctive cytokine profiles of the two classes are believed to be associated with distinctive functions whereby T1 cells are primarily involved in cytotoxic (CD8) and DTH (CD4) responses, and T2 cells with antibody and allergic reactions. In all of these studies it has been noted that IL-5 is mainly produced by $CD4^+$ T2 cells and partly by $CD4^-CD8^-$ cells, but not by $CD8^+$ cells such as in mice infected with *Toxocara canis* (Takamoto et al., 1998). However, the present study shows that IL-5 is produced by both $CD4^+$ and $CD8^+$ T cells from mice immunised with oxidised MFP, an antigen which produces a typical T1 response, and not by mice immunised with reduced MFP which produces a T2 type response.

The results of the present work highlight the fact that, cytokine production in immune responses is not always purely of the T1 or T2 type. In this case, immunisation with oxidised MFP results in IL-5 production but the rest of the response is typically T1. Other Th2 cytokines such as IL-4 and IL-10 were not detected.

A role for IL-5 in the generation of CTL was noted first in 1987 and it was also shown that IL-5 was required to obtain effective CTL function at different stages of T cell differentiation (Takatsu et al., 1987; Ramos 1989). In addition, earlier studies suggested that a non-IL-2 cytokine was required for the generation of CTL (Raulet et al., 1982), possibly this was IL-5. However, more recently interest has centered on the role of IL-5 in regulating B-1 cells and antibody production, in the growth and activity of eosinophils and especially in their trafficking to sites of allergic inflammation in mouse asthma models (Foster et al., 1996). In IL-5 -/- mice, there are basal levels of eosinophils produced that appear to be morphologically and functionally normal. However, in the absence of IL-5 the pronounced eosinophilia characteristic of infections with helminth parasites and of allergic lung disease is not produced

(Kopf et al., 1996). Eosinophil trafficking is also dysfunctional since IL-5 acts as a cofactor with chemokines like eotaxin (Collins et al., 1995). Lack of IL-5 reduces host defence against some intestinal nematodes (Ovington et al., 1998). Most of the effects of IL-5 with respect to parasite infections have been interpreted in terms of effects on eosinophils. However, the demonstrated effects of IL-5 on B-1 cells in previous studies (Kopf et al., 1996; Bao et al., 1998) and on CTL production in the present work means that consideration of other effects of IL-5 may need to be made when interpreting the responses of IL-5 -/- mice.

The present work provides a clear indication of a role for IL-5 in the generation of CTL *in vivo* as part of the immune response to MFP. This observation provides the rationale for examination of possible IL-5 involvement in a range of other CTL responses in future studies. Further studies of the role of IL-5 in the generation of a functional cytotoxic response to tumours may provide opportunities for enhancement of such responses in cancer.

Chapter 7:

Immunotherapy using MFP and IL-12 in MUC1 transgenic models

Catherine J Lees, Vasso Apostolopoulos, Bruce Acres, Ian Ramshaw, Alistair Ramsay, Chin-Swee Ong and Ian FC McKenzie. Immunotherapy with mannan-MUC1 and IL-12 in MUC1 transgenic mice. Vaccine. In Press, 2000.

Summary

Mice immunised with oxidised mannan-MUC1 fusion protein (MFP) produce MHC restricted $CD8^+$ cytotoxic T cells, tumour protection and T1 cytokine production. This study demonstrates that in MUC1 transgenic mice, IL-12 enhances the T1 immune response to MUC1 characterised by enhanced CTL and CTLp responses and tumour protection. The transgenic mouse models used expressed either human MUC1 or both human MUC1 and HLA-A*0201, and examined MUC1 immunity as a prelude to clinical studies in patients with $MUC1^+$ tumours. Significant MUC1 CTLp responses were generated in transgenic mouse models after one immunisation with MFP (1/55,000), however when IL-12 was included with MFP, the responses were enhanced to 1/19,000. Immunisation of MUC1 transgenic mice with MFP and IL-12 also induced tumour protection after challenge with a $MUC1^+$ mastocytoma cell line. The role of IL-12 in the MUC1 immune response was further demonstrated in IL-12 gene knockout mice (-/-) when the CTLp frequency to MUC-1 after one MFP injection was decreased to 1/226,000 compared with 1/77,500 in normal mice. This study demonstrates the enhanced anti-tumour immune responses of MFP and IL-12 administered together in MUC1 transgenic mouse models.

7.1 Introduction

Preclinical tumour immunotherapy studies with MUC1 coupled to oxidised mannan (MFP), have shown MUC1 specific tumour immunity characterised by the production of MHC-restricted MUC1 specific $CD8^+$ cytotoxic T cells, a high CTLp frequency, tumour protection, low antibody levels, and IL-2 and IFN-γ cytokine secretion from spleen cell cultures (Apostolopoulos et al, 1995, 1996). MUC1 transgenic mice provide a more realistic model for MUC1 immunotherapy studies compared to previous studies using human MUC1 in non-transgenic mice. It is easier to generate an immune response to a 'non-self' antigen as has been done previously in non-transgenic mouse models. The human MUC1 transgenic mouse model represents a more realistic model for these studies because MUC1 is a self-antigen, as it is in humans. Therefore the generation of an immune response in this model provides a better indication of the possibilities in the clinic. Already there are indications that in MUC1 transgenic mice, anti-MUC1 immune responses can be induced (Rowse et al., 1998, Gong et al., 1998, and Acres et al., 2000).

MUC1 transgenic mice, bred onto a DBA/2 background, express MUC1 in the lung bronchioles, β-islets of the pancreas, kidney tubules and stomach (Acres et al., 2000). Recent studies show MUC1 transgenic mice to be partially tolerant to human MUC1 as MUC1 antibodies (Rowse et al., 1998), and CTL responses have been induced (Acres et al., 2000; Gong et al, 1998). In another model described herein, HLA-A*0201 x MUC1 (A2-MUC1) transgenic mice were developed by crossing a HLA-A*0201 transgenic mouse expressing the human HLA-A*0201 gene (Vitiello et al., 1991) with the MUC1 transgenic mouse; these mice express both human MUC1 and human HLA-A2 and were used to produce human HLA-A*0201 restricted CTLs specifically against human MUC1.

A number of different cytokines have been used in immunotherapy studies to boost the anti-tumour responses generated against tumour antigens (Forni and Colombo 1994; Musiani et al., 1997). In particular the T1 cytokine IL-12, which promotes T cell and NK cell growth and cell mediated immunity by stimulating IFN-γ production (Brunda et al., 1995; Gately et al 1994; Nastala et al., 1994), has been found to produce significant therapeutic effects when administered either locally or systemically, or by using delivery systems such as viral vectors or gene transfer. IL-12 exerted its anti-tumour and anti-metastatic effects in multiple murine

tumour models including, melanoma (Tahara et al., 1994), the MCA207 and MCA105 sarcomas (Tahara and Lotze, 1995), MC-38 adenocarcinoma (Nastala et al., 1994), B cell lymphoma (O'Toole et al., 1993), colon carcinoma (Caruso et al., 1996) and Lewis lung carcinoma (Stern et al., 1994).

Based on the anti-tumour potential of systemic IL-12, the CTLp frequencies generated in response to MFP compared with MFP + IL-12 in BALB/c, MUC1 and A2-MUC1 transgenic models were investigated. This study demonstrates that IL-12 increased the MUC1 CTLp frequency and anti-tumour effects to mannan-MUC1 in the MUC1 x A2-MUC1 double transgenic mice.

7.2 Materials and methods

Cell lines

The MUC1$^+$/HLA-A*0201 human breast cancer cell line, MCF-7 and DBA/2 P815 mastocytoma cell line transfected with the cDNA of the transmembrane form of human MUC1, MUC1$^+$ P815 (Acres et al., 1993), were cultured in RPMIM growth media and MUC1 expression selection performed using 1.25mg/ml G418-sulfate (Gibco BRL, U.S.A).

MUC1 antigens

MUC1 fusion protein (5 VNTR regions of MUC1) was produced in a pGEX-3X bacterial expression system, purified using excess glutathione and conjugated to oxidised mannan to produce aldehydes and Schiff bases (MFP) (refer to Chapter 2, section 1). The MUC1 peptide Cp13-32 was synthesised using an Applied Biosystems Model 430A biosynthesiser.

Mice and immunisations

BALB/c, DBA/2 MUC1 transgenic and DBA/2 HLA-A*0201 x MUC1 (A2-MUC1) transgenic mice were bred at the Austin Research Institute (Victoria, Australia). Homozygote MUC1 transgenic mice were supplied by Dr B Acres (Transgène, Strasbourg, France) and are described elsewhere (Acres et al., 2000). Briefly, the DPr/pTG8186 plasmid was generated by inserting the 3' half of the MUC1 transmembrane cDNA, plus a 7.2 kB fragment of MUC1 genomic DNA including promoter sequences, into the p-polyIII plasmid. A linear fragment from the p-polyIII plasmid was excised and injected into (B6 x SJL) F_1 eggs and resulting transgenic mice crossed to DBA/2. MUC1 transgenic mice were typed for MUC1 expression using genomic tail DNA and a MUC1 DNA probe (refer to Chapter 2, section 7).

A2-MUC1 transgenic mice were produced by crossing homozygote HLA-A*0201 mice (Vitiello et al., 1991) with MUC1 transgenic mice and typed for genomic MUC1 expression as above. BALB/c IL-12 gene knockout mice (-/-) lacking the p40 monomer were obtained from Dr M Gately, Hoffmann-La Roche, Nutley, U.S.A (Mattner et al., 1996). Mice aged 6-10 weeks were immunised intraperitoneally with 5 μg of MFP either once, or three times on

days 0, 7 and 14. Mice injected with VV-IL-12 received 1 x 10^7 pfu/ml (total virus) intraperitoneally either alone, or mixed with MFP. BALB/c mice injected with murine recombinant IL-12 received 5 daily 1 μg injections of IL-12 per week and a single injection of MFP (5 μg) intraperitoneally.

Vaccinia virus-IL-12

Vaccinia virus (VV) encoding recombinant murine IL-12 (VV-IL-12) produced by Ian Ramshaw (John Curtin School of Medical Research, Canberra, ACT), was used to infect baby hamster kidney (BHK) cells and purified over a 36% sucrose gradient by ultra centrifugation to obtain semi-purified virus for immunisations (Acres et al., 1994) (refer to Chapter 2, section 4). Recombinant IL-12 bioactivity was determined by ELISA (Pharmingen, Australia) and VV- IL-12 titres determined by plaque forming assays in which 5 x 10^6 BHK cells in 6 well plates were incubated for 24 h at 37°C 10% CO_2 with serial dilutions (1 x 10^5 – 1 x 10^9) of vaccinia virus and stained with aniline blue to count plaques.

ELISA for detection and quantitation of VV- IL-12

Recombinant IL-12 was quantified using an ELISA (Pharmingen, San Diego, U.S.A) (refer to Chapter 2, section 4.4). Purified anti-IL-12 capture antibody (4 μg/ml) was coated onto a protein enhanced binding ELISA plate (Nunc Maxisorb; Nunc, Denmark) overnight at 4°C and non-specific binding blocked with 2% PBS/BSA. Samples of VV-IL-12, control VV and VV + irrelevant cytokine (VV-IL-6) were serially diluted and incubated at 4°C overnight. A recombinant murine IL-12 standard (20 ng/ml) was used as a positive control. IL-12 was detected by further incubation with a biotinylated anti-IL-12 detection antibody (1 μg/ml) then Streptavidin-HRP conjugate diluted 1/500. Cytokine levels were detected and quantified using 1-2 mg TMB substrate (Tetramethyl-benzidine dihydrochloride hydrate 97%) (Aldrich, Australia) dissolved in 0.1 M citric acid and mixed with distilled water containing 0.5 M sodium acetate and 5 μl H_2O_2 in the dark at room temperature. The reaction was stopped by the addition of 0.18 M H_2SO_4 after optimal colour intensity was reached and the absorbance read at 450 nm.

Cytotoxic T cell precursor assays

A limiting dilution CTLp assay was performed 14 - 21 days after immunisation with MFP (refer to Chapter 2, section 2.2.2). Briefly, spleen cells from immunised mice were added in 32 replicates to 96 well microtitre plates varying from 1 x 10^3 to 1.28 x 10^5 cells/well. Stimulator spleen cells from (BALB/c x DBA)F_1 mice were irradiated using 3,000 rads from a Caesium source and added at 5 x 10^5 cells/well stimulators, with 5 μM MUC1 peptide Cp13-32 and 10 U/ml of rhIL-2 to all wells. Controls containing 5 x 10^5 cells/well stimulators and either, 10 U/ml IL-2 and 5 μM peptide Cp13-32, or 5 x 10^5 cells/well stimulators and 1.28 x 10^5 cells/well effectors were set up in replicates of 32 wells and incubated for 7 days at 37^{0}C 10% CO_2. After incubation, 1 x 10^4 ^{51}Cr labelled P815-MUC1 (with P815 as a control), MCF-7 (HLA-A2$^+$, MUC1$^+$) or BT20 (HLA-A2$^-$, MUC1$^+$) target cells were added. Cultures were incubated for a further 4 h and analysed on a microplate scintillation counter (Packard USA). The number of CTL precursors present was determined as a frequency of the number of cells not lysed on a logarithmic scale and the number of cells/well on a linear scale. Specificity assays were performed using P815 and BT20 cells and at times, NK and LAK lysis was checked using K562 target cells. As shown extensively elsewhere, in the systems described, the lysis was MUC1 specific, and could be inhibited by CD8$^+$ antibody (Apostolopoulos et al., 1994, 1995, 1995a, 1997).

Antibody assays by ELISA

Serum from immunised MUC1 transgenic mice were collected 4 - 6 days after a third injection and tested for MUC1 antibody levels by ELISA (refer to Chapter 2, section 2.1). Serially diluted (1:2) serum samples were added to 96 well round-bottom microtitre plates coated with 10 μg/ml Cp13-32 and non-specific binding blocked with 2% BSA/PBS. MUC1 antibody levels were detected with sheep anti-mouse HRP (Amersham, U.K) and developed with 0.03% ABTS enzyme in ABTS buffer containing 0.02% H_2O_2 and absorbance was read at 405 nm.

MUC1 transgenic tumour challenge

Two different protocols for a MUC1$^+$ tumour challenge in MUC1 transgenic mice were performed. In the first experiment, MUC1 transgenic mice (5–6 /group) were immunised

intraperitoneally on days 0, 7 and 14 with either 5 μg of MFP, 5 μg of MFP mixed with 1 x 10^7 pfu/ml VV-IL-12, or buffer as a control. Mice were challenged with 3.5 x 10^6 P815-MUC1 tumour cells given subcutaneously on day 22, and the tumour growth measured. In the second experiment, MUC1 transgenic mice (7–9 /group) were immunised intraperitoneally on days 0, 7 and 14 with either 1 x 10^7 pfu/ml VV-IL-12 or 1 x 10^7 pfu/ml VV, alone, or mixed with 5 μg of MFP. Mice were challenged with 1 x 10^7 P815-MUC1 tumour cells subcutaneously on day 21, and 30 days later the number of mice with palpable tumours was determined.

7.3 Results

VV- IL-12 Production

Recombinant murine IL-12 was produced in the supernatant of VV-IL-12 infected BHK cells at a concentration of 57 ng/ml, determined by ELISA (Figure 7.1). VV alone and VV + irrelevant cytokine (IL-6) samples contained no IL-12. The concentration of IL-12 secreted from $1x10^7$ pfu/ml VV-IL-12 infecting confluent BHK cells (4×10^6) after 24 h, was equivalent to 400 ng/ml, as determined by a plaque forming assay (data not shown).

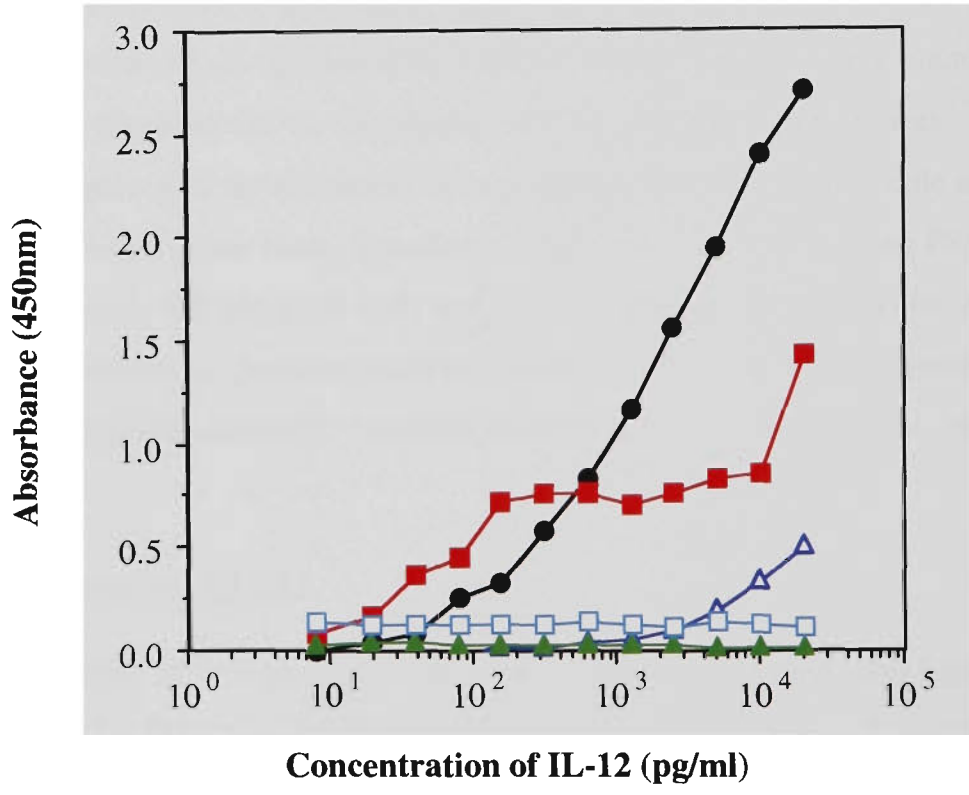


Figure 7.1: The concentration of VV-IL-12 determined by an IL-12 sandwich ELISA. Purified anti-IL-12 and IL-12 biotinylated detection antibodies were used at 4 µg/ml and 1 µg/ml respectively, to detect VV-IL-12 (■). All VV samples were added neat and diluted 1:2. Negative controls consisting of non-biologically active recombinant IL-12 (▲), VV (▲), and VV-IL-6 ( ) were incorporated and murine recombinant IL-12 (20 ng/ml) (●) used as a positive control.

MUC1 antibody levels

The MUC1 antibody levels produced in human MUC1 transgenic mice injected three times with either MFP, MFP + VV-IL-12, MFP + VV, VV-IL-12 or buffer was determined (Figure 7.2A) and the average titre from each group of immunised mice (x5/group) calculated as half the average maximal absorbance at 450 nm. No MUC1 specific antibodies were produced in MUC1 transgenic mice injected with buffer, VV-IL-12 or MFP. Weak antibodies were produced in mice injected with MFP + VV-IL-12 (1/143) and MFP + VV (1/485). The MUC1 antibodies in the A2-MUC1 transgenic mice (Figure 7.2B) were similar to those of MUC1 transgenic mice, in that none were detected after three injections of VV-IL-12, or MFP, and weak responses to MFP + VV-IL-12 (1/292) and MFP + VV (1/317). It was of interest that while MFP alone did not give rise to antibodies, the addition of VV provided additional antigenic stimulus in both sets of transgenic mice which induced antibodies to MUC1. The poor antibody response in mannan MUC1 immunised mice is in accord with previous findings and is due to the T1 nature of the oxidised MFP response in mice (Apostolopoulos et al., 1995).

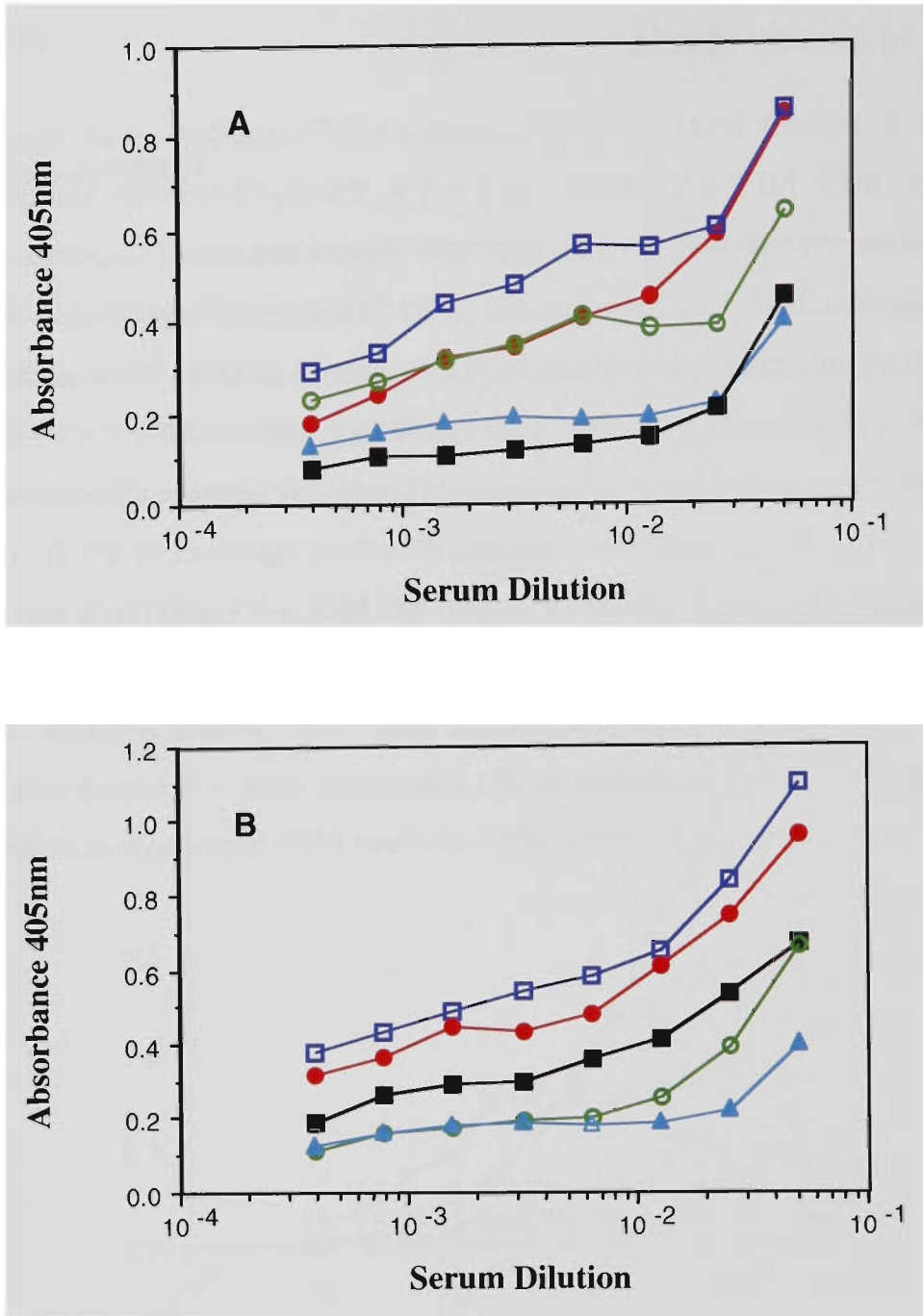


Figure 7.2: MUC1 antibody titres from the sera of: A) MUC1 transgenic and B) A2-MUC1 transgenic mice. Mice were given three IP injections with either pH 9.0 buffer (▲), 5μg MFP (○), 5 μg MFP + VV-IL-12 (●) (1 x 10^7 pfu/ml), 5 μg MFP + VV ( ) (1 x 10^7 pfu/ml), or VV-IL-12 (1 x 10^7 pfu/ml) (■) on days 0, 7 and 14 and bled 4-6 days after a final injection. MUC1 serum antibody levels were determined by a direct binding ELISA coated with 10 μg/ml Cp13-32. Serum samples were diluted 1:10 in phosphate buffered saline and serially diluted 1:2. Average MUC1 sera titres from the transgenic mice were calculated as half the maximum absorbance (405 nm).

MUC1 cytotoxic T cell precursor frequencies

Inbred mice, MUC1 transgenic and A2-MUC1 transgenic mice were immunised with either MFP, or MFP and IL-12, and CTLp frequencies determined.

a) In BALB/c mice (Figure 7.3A) using $MUC1^+$ P815 cells as targets, a single MFP injection generated a MUC1 CTLp frequency of 1/76,000 that significantly increased to 1/19,000 upon addition of recombinant murine IL-12 (five daily injections/week). Mice given only recombinant IL-12 and no MFP had no MUC1 CTLp responses ($<1/10^6$), nor was there lysis of control $MUC1^-$ P815 cells. After three weekly injections of MFP in BALB/c mice, the CTLp frequency of 1/7,500 significantly increased to 1/2,500 with recombinant murine IL-12 (five daily injections/week). Similarly there was no lysis of control $MUC1^-$ P815 cells (CTLp frequency $<1/10^6$ not shown).

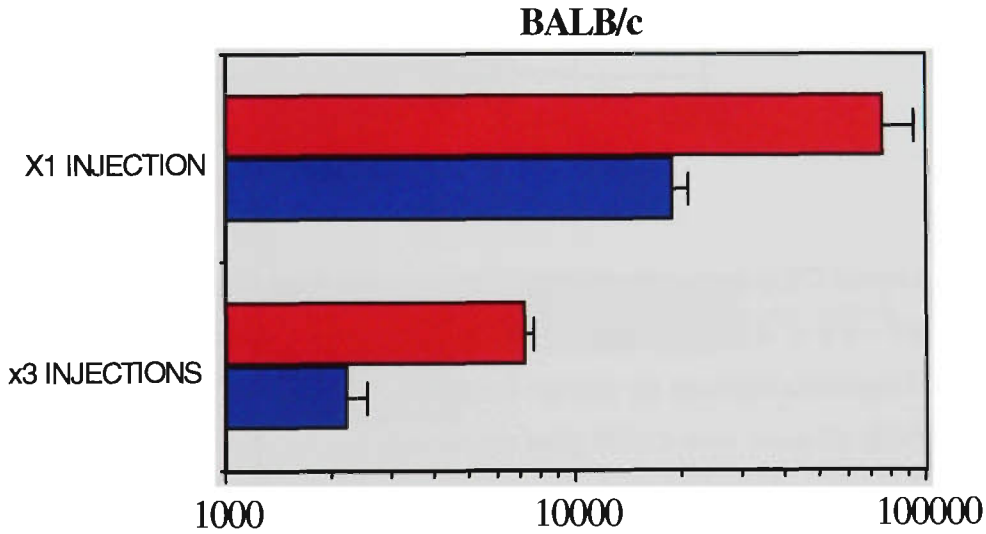


Figure 7.3A: Average CTLp frequencies from BALB/c mice immunised (I.P) with either 5 µg MFP (■), or 5 µg MFP + murine recombinant IL-12 (1 µg/mouse/day IP x 5 days) (■). Results represent the average frequency from 2-3 mice. The CTLp frequencies from mice injected with rIL-12 were < $1/10^6$ (data not shown).

b) In MUC1 transgenic mice (Figure 7.3B) using $MUC1^+$ P815 cells as targets, a single injection of MFP led to a CTLp frequency of 1/420,000 which significantly increased to 1/240,000 with VV-IL-12. A significant improvement in CTLp responses was observed when three injections of MFP were given (1/55,000) which further increased with VV-IL-12 (1/19,000). CTLp were not detected ($<1/10^6$) in mice injected with VV-IL-12 and no MFP, nor was there lysis of control $MUC1^-$ P815 cells.

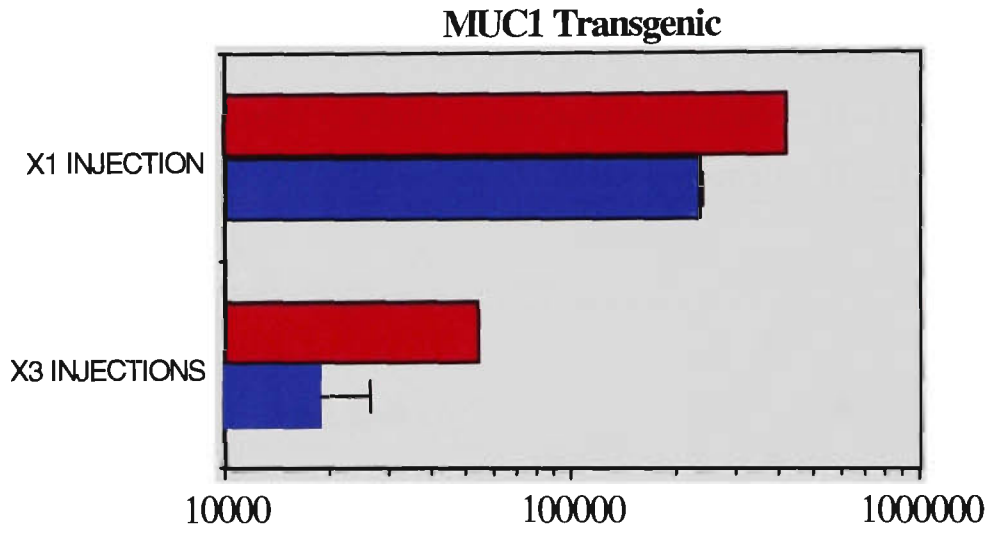


Figure 7.3B: Average CTLp frequencies from MUC1 transgenic mice immunised (IP) with either 5 µg MFP (■), or 5 µg MFP + VV-IL-12 (1 x 10^7 pfu/ml) (■). Results from x 1 injection represent one mouse and the results from x 3 injections represent the average frequency from 2 mice. The CTLp frequencies from mice injected with VV-IL-12 alone were < $1/10^6$ (data not shown).

c) In A2-MUC1 transgenic mice (Figure 7.3C) using MCF-7 target cells, three injections of MFP led to a CTLp frequency of 1/74,250 which significantly increased to 1/35,000 with VV-IL-12. The $MUC1^+$/ $H2^d$ CTLp frequency in A2-MUC1 transgenic mice, using $MUC1^+$ P815 target cells, demonstrated that MFP + VV-IL-12 produced CTLp responses almost four times stronger than MFP alone (1/78,000 compared to 1/280,000). The frequency on $MUC1^-$ P815 ($H2^d$) cells was less than on HLA-A2 MCF-7 cells – possibly due to a decrease in $H2^d$ molecules in A2-MUC1 double transgenic mice. Injections of VV-IL-12 alone were negative ($<1/10^6$) against both target cell lines in A2-MUC1 double transgenic mice. Thus IL-12 substantially increased the CTLp frequencies in both inbred, MUC1 and A2-MUC1 transgenic mice, the findings in the A2-MUC1 mice being in accord with our previous results; there was no lysis of P815 or BT20 ($HLA\text{-}A2^-$, $MUC1^+$) cells (Apostolopoulos et al., 1997).

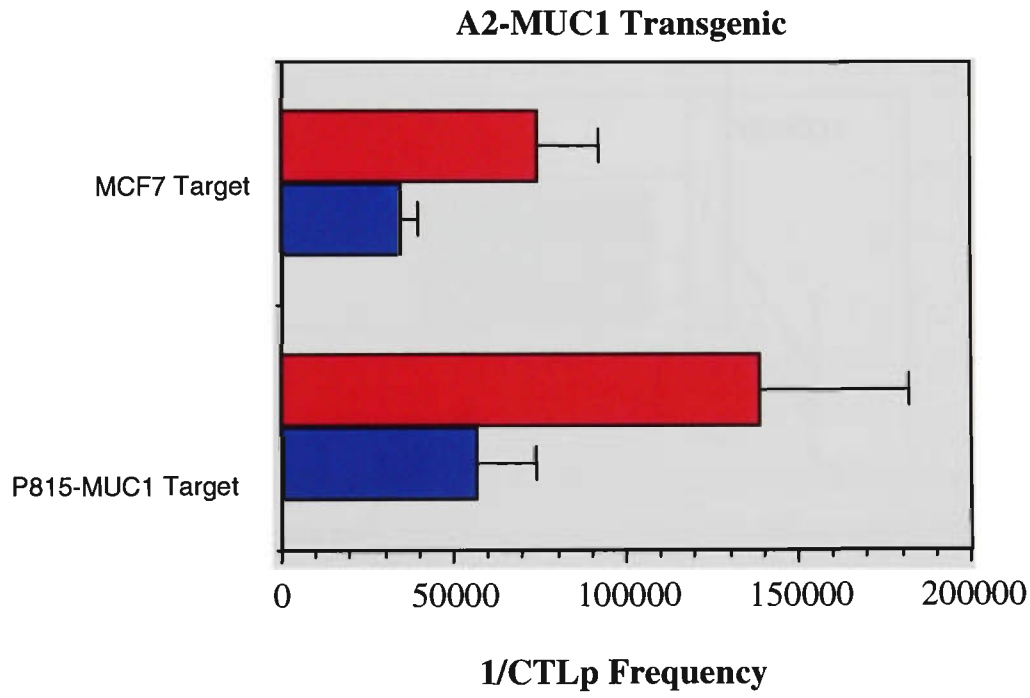


Figure 7.3C: Average CTLp frequencies from A2-MUC1 double transgenic mice immunised (I.P x 3) with either 5 µg MFP (■), or 5 µg MFP + VV-IL-12 (1×10^7 pfu/ml) (■). HLA-2 restricted, MUC1 positive CTL precursors were determined using the MCF-7 target cell line whilst MUC1, $H\text{-}2^d$ CTLp were determined using the $MUC1^+$ P815 target cell line. Average CTLp frequencies for the MCF-7 targets represent an average of four mice and results from P815-MUC1 represent a single mouse. The CTLp frequencies from mice injected with VV-IL-12 alone were $<1/10^6$ on both target cell lines (data not shown).

IL-12 p40 -/- mice

To further examine the effect of IL-12 on the MUC1 cellular immune response, CTLp frequencies from BALB/c IL-12 -/- mice injected with MFP were investigated (Figure 7.4). BALB/c mice generated a CTLp frequency of 1/77,500 after one MFP injection while in IL-12 -/- mice it was decreased to 1/226,000, demonstrating a crucial role for IL-12 in the development of the MUC1 CTL response.

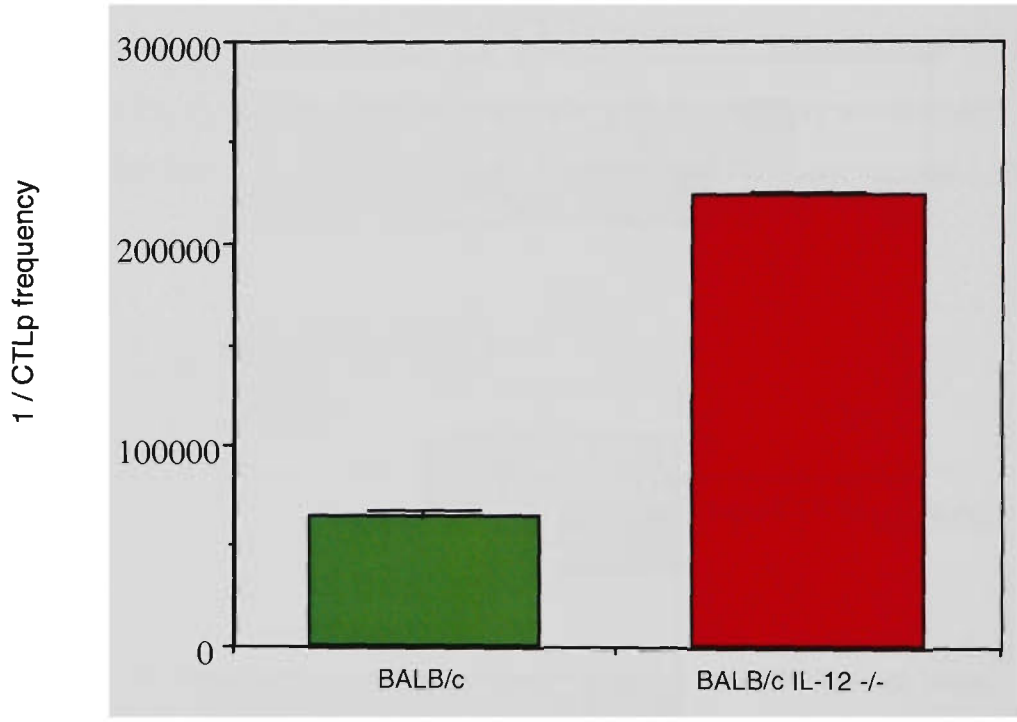


Figure 7.4: Average CTLp frequencies from BALB/c IL-12 -/- and BALB/c wild-type mice injected IP x 1 with 5 μg MFP. Data represents the average CTLp frequency from three mice.

MUC1 transgenic tumour challenge

The ability of MFP + VV-IL-12 to induce anti-tumour immunity was investigated *in vivo* using the MUC1$^+$ P815 tumour in DBA/2 MUC1 transgenic mice. In the first study, MUC1 transgenic mice immunised three times with MFP, MFP + VV-IL-12 or a control buffer, were challenged with 3.5x10^6 MUC1$^+$ P815 tumour cells subcutaneously and the tumour growth measured. Figure 7.5A illustrates the tumour growth (± standard error), and shows that MFP + VV-IL-12 significantly decreased the average tumour size of MUC1$^+$ P815 tumours in MUC1 transgenic mice compared to MFP alone (which incurs a small amount of tumour protection) or controls. These results demonstrate that the cellular immunity induced in MFP + VV-IL-12 immunisations (as demonstrated by CTLp induction) was accompanied by anti-tumour immunity. A proportion of control mice also rejected tumours, ascribed to non-H2 differences between the P815 DBA/2 tumour and the transgenic mice which had been backcrossed (N8) to DBA/2.

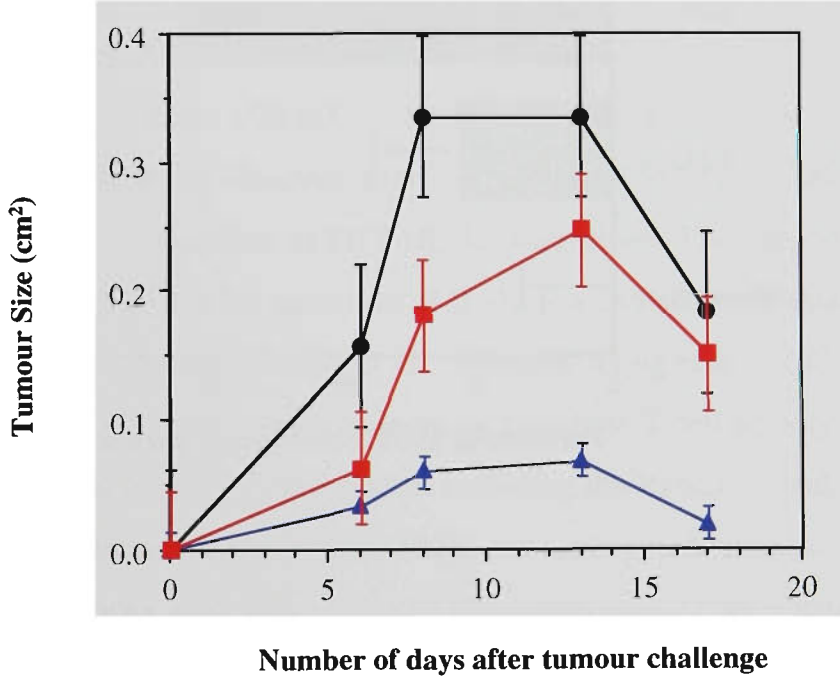


Figure 7.5A: MUC1$^+$ P815 tumour challenge in MUC1 transgenic mice. MUC1 transgenic mice (5– 6 / group) were immunised with either pH 9.0 (●), 5 µg MFP (■), or 5 µg MFP + VV-IL-12 (1 x 10^7 pfu/ml) (▲) on days 0, 7, and 14. Mice were challenged with 3.5 x 10^6 MUC1+ P815 tumour cells (subcutaneously) on day 22 and tumour growth measured.

In the second tumour study, MUC1 transgenic mice immunised three times with either MFP, MFP + VV, MFP + VV-IL-12 or VV- IL-12 were challenged with $1x10^7$ subcutaneous $MUC1^+$ P815 tumour cells, and the number of mice with palpable tumours present after 30 days assessed (Figure 7.5B). Tumours were present in 62.5% of MUC1 transgenic mice immunised with MFP+VV, 37.5% of mice immunised with MFP alone ($p = 0.62$ using a Fisher's Exact Test) and 22.2% of mice immunised with only VV-IL-12 ($p = 0.153$) demonstrating that neither vaccinia virus nor VV-IL-12 can induce statistically significant tumour protection alone. However, mice immunised with MFP + VV-IL-12, were totally protected (0% with tumours) from the P815-MUC1 tumour challenge ($p = 0.025$) compared to control MFP + VV immunised mice. Thus MFP + VV-IL-12 immunisation is able to induce tumour protection in MUC1 transgenic mice.

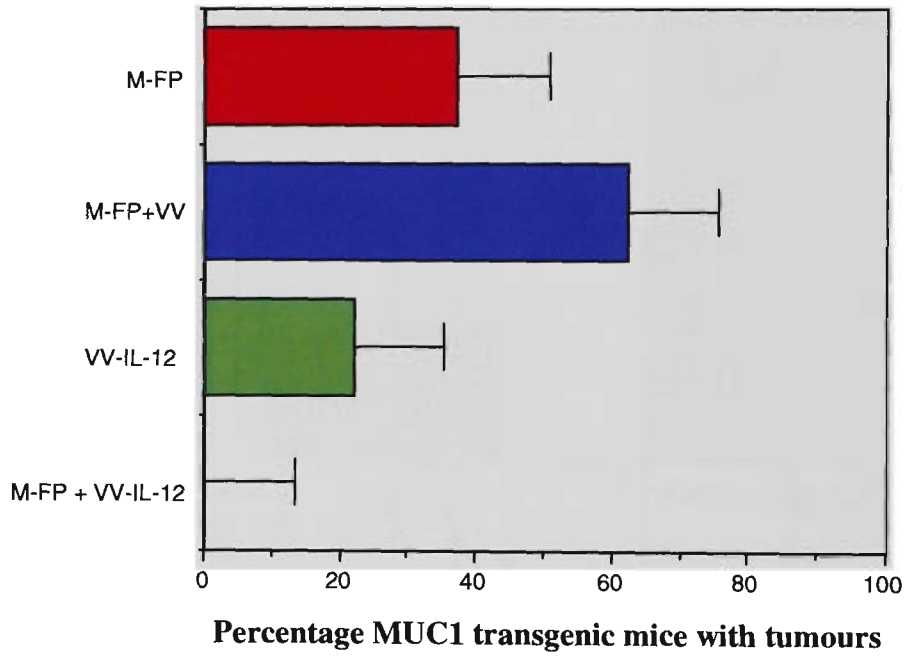


Figure 7.5B: $MUC1^+$ P815 tumour challenge in MUC1 transgenic mice. MUC1 transgenic mice (7–9 / group) immunised intraperitoneally on days 0, 7 and 14 with either 5 μg MFP, 5 μg MFP + VV (1×10^7 pfu/ml), 5 μg MFP + VV-IL-12 (1×10^7 pfu/ml), or VV-IL-12 (1×10^7 pfu/ml). Mice were challenged on day 21 with 1×10^7 $MUC1^+$ P815 tumour cells subcutaneously. The number of mice with palpable tumours 30 days later are represented as a percentage of the total number of mice in each group with a tumour (± SE). A Fisher's Exact Test was used to determine the statistical significance of the data.

7.4 Discussion

The ability to generate an immune response against MFP using the T cell cytotoxic enhancing capabilities of the cytokine IL-12 were investigated in both BALB/c and in human MUC1 transgenic mice. IL-12 is a heterodimeric cytokine produced by macrophages (Hendrzak and Brunda, 1995) and has potent biological effects both *in vitro* and *in vivo* by inducing IFN-γ production from NK and T cells (Kobayashi et al., 1989; Chan et al., 1991; Wolf et al., 1991), enhancing cytotoxic T cell function and proliferation of NK and activated T cells (Wong et al. 1988; Gately et al., 1992 and 1994) and promoting T1 cytokine responses (Kennedy et al., 1994). To determine if the T1 response induced by mannan MUC1 MFP could be increased in inbred BALB/c, DBA/2 MUC1 transgenic and HLA-A*0201 x DBA/2 MUC1 (A2-MUC1) double transgenic mice, IL-12 was incorporated into MFP immunisations and cytotoxic T cell production and tumour protection measured.

IL-12 could increase the CTL responses generated against MUC1 in MUC1 transgenic mice. Initial observations in BALB/c mice demonstrated that after one injection, rIL-12 increased the MUC1 CTLp frequency from 1/76,000 with MFP to 1/19,000 with MFP+IL-12. Furthermore, the CTLp response observed after 3 injections of MFP (1/7,500) was also increased (1/2,500) when 3 injections of MFP+IL-12 were given. The significant increase in cytolytic response observed after the inclusion of IL-12 is a characteristic quality of the cytokine in both mice and humans (Gately et al., 1994 and Wong et al., 1988) in allogeneic responses and IL-12 has also been shown to increase cytotoxic T cell activity mediating anti-tumour activity in several *in vivo* murine models including the Renca (Brunda et al., 1993), and MCA-26 colon carcinoma (Caruso et al., 1996) but not in any transgenic models.

The CTLp frequency in DBA/2 MUC1 transgenic mice was increased from 1/420,000 to 1/240,000 after a single injection of recombinant vaccinia virus secreting IL-12 was included and was further increased (from 1/55,000 to 1/19,000) when 3 injections of MFP+IL-12 were given. The increase in MUC1 CTL production observed in these transgenic mice after MFP+IL-12 injections could also be translated into anti-tumour immunity, as immunisations with MFP+IL-12 significantly decreased the tumour burden of human MUC1 transgenic mice

challenged with MUC1$^+$ P815 tumours, compared to either MFP or IL-12 immunisations alone.

To further examine cytotoxic T cell induction in MUC1 transgenic mice, MFP+IL-12 was injected into mice transgenic for both human MUC1 and HLA-A*0201. Previous studies with MFP have shown that HLA-A*0201/K^b transgenic mice immunised with MFP generate HLA-A*0201 restricted CTLs that recognise MUC1 in association with human HLA-A*0201 (Apostolopoulos et al., 1997). These findings prompted the generation of the HLA-A*0201/K^{b}xMUC1 (A2-MUC1) double transgenic line to provide a model for investigating and enhancing human MUC1 CTL immune responses in mice to MUC1. Immunisation of A2-MUC1 double transgenic mice with MFP+IL-12 resulted in the generation of MUC1 specific HLA-A*0201 CTLps which killed the HLA-A*0201 target human MCF-7 mammary breast cancer line. The inclusion of IL-12 in these injections increased the CTLp frequency from 1/74,250 to 1/35,000; a result similar to that generated in MUC1 transgenic mice. Interestingly, the K^b CTLp response in A2-MUC1 double transgenic mice injected (x3) with MFP+IL-12 (1/78,000) was considerably weaker than the response observed in the MUC1 transgenic mice (1/19,000) following the same injection protocol, albeit stronger than MFP alone (1/280,000) in the A2-MUC1 transgenic mice, suggesting a bias towards HLA-A*0201 CTL production rather than K^b and H-2^d CTL production in A2-MUC1 transgenic mice. These studies may reflect a human immune response to MUC1.

Although the CTLp results from both inbred and MUC1 transgenic mice demonstrate the ability of IL-12 to enhance CTL induction in the MFP immune response, the mechanisms for IL-12 induced immunity is not clear. To further examine the role of IL-12 in the immune response, IL-12 mice deficient in the p40 subunit were generated. IL-12 -/- mice are unable to produce IL-12-dependent IFN-γ production, or to mount T1 and delayed type hypersensitivity (DTH) responses (Lamont and Adorini, 1996). In the MUC1 immune response, the number of cytolytic T cell precursors generated after an injection of MFP decreased from 1/77,500 in control mice to 1/226,000 in IL-12 -/- mice, demonstrating that the cellular immune response produced after MFP injections is partially IL-12 dependent.

Although the cytotoxic T cell precursor results from both IL-12 -/- and MUC1 transgenic mice demonstrate a direct role for IL-12 increasing CTL induction in the MFP immune

response, the exact mechanisms for IL-12 induced immunity remains unclear and varies between the tumour models studied (reviewed by Gately et al., 1994a). In NK-deficient mice, IL-12 has been shown to retain its anti-tumour immunity, suggesting NK cells are not involved in the IL-12 induced immune responses. However, in another tumour model, SCID mice lacking both B and T cells maintain IL-12 anti-tumour activity inferring NK cells are responsible for IL-12 anti-tumour effects. Other roles for T cell involvement in IL-12 mediated anti-tumour effects also occur where IL-12 activity is substantially reduced, although not abrogated in nude mice, suggesting T cells are not directly involved in the anti-tumour response however, depletion of $CD8^+$ T cells in the Renca tumour model significantly reduces IL-12 efficacy. In addition, independent of NK or T cell involvement in the IL-12 anti-tumour immune response, IL-12 has been shown to inhibit angiogenesis induced by human tumours *in vivo* (Majewski et al., 1996) – possibly all of the IL-12 effects occur in the models described herein. Whatever the mechanism, the ability of IL-12 to increase the CTL responses and anti-tumour effects is encouraging and forms the basis of a clinical trial.

Chapter 8:

Conclusions

This chapter reviews the major findings of this thesis and their significance for cancer immunotherapy studies today. After reviewing the preclinical and clinical immunotherapy studies to date, what remains evident is the fact that scientists have devised countless approaches for harnessing the body's immune system to fight cancer – the majority of which are still being tested in clinical trials. Of particular promise for cancers of the breast, pancreas and ovary are studies with the tumour associated antigen, MUC-1. The over-expression of MUC1 in an altered form on these epithelial carcinomas reveals highly immunogenic peptide epitopes which induce humoral immune responses in mice, monkeys and humans. However, the subsequent coupling of a MUC1 fusion protein containing the most immunogenic region of the protein core, to the oxidised carrier mannan, has yielded MHC class I restricted tumour regression and antitumour immunity in several MUC1 tumour models.

Although oxidised MFP induces MUC1 specific cellular immunity in mice, these antitumour responses are not mimicked in either monkeys or humans with $MUC1^+$ tumours. The questions still remains then, can the immune response to MFP be boosted *in vivo* using conventional immunotherapeutic strategies such as the incorporation of cytokines? Therefore the major aim of this thesis was to examine the role of cytokines in the MUC1 immune response to MFP.

In chapter two, the materials and methods commonly used throughout this thesis are described. In particular, the method used to produce soluble GST-MUC1 fusion protein, its purification with glutathione sepharose beads and chemical conjugation to oxidised mannan was adopted from Apostolopoulos et al. (1993, 1995a, 1996). MFP was used as the primary immunogen in all immunotherapy experiments contained within this thesis. Recombinant cytokines were produced and purified from vaccinia viral vectors. Many researchers have used viral vectors to produce protein antigens, and the production of large quantities of biologically active cytokines from VV is not novel. Cytokines were produced following viral infection into BHK cells, purified over a sucrose gradient and quantified using plaque forming assays and ELISAs. The ELISAs used to quantify each cytokine were initially standardised to determine the optimal conditions for use, and also used to characterise the cytokine profiles in chapter 3.

Chapter three investigated the cytokine profile of $CD4^+$ and $CD8^+$ spleen T cells from mice immunised with various forms of the MUC1 tumour antigen (oxidised MFP, reduced MFP and varying combinations). Immunisation with oxidised MFP led to the secretion of T1 cytokines from $CD8^+$ T cells (IL-2, IFN-γ and TNF-α) and from $CD4^+$ T cells (IL-2 and IFN-γ). IL-12 production, presumably from activated macrophages, was observed in $CD8^+$ but not $CD4^+$ cultures. Immunisation with either reduced MFP or FP led to the secretion of predominantly T2 cytokines from $CD4^+$ T cells (IL-4 and IL-10) and IL-2 production in both $CD4^+$ and $CD8^+$ T cell cultures. Interestingly, the simultaneous immunisation of both oxidised MFP and FP led to the production of both T1 and T2 cytokines from $CD8^+$ T cells (IL-2, IFN-γ and TNF-α) and $CD4^+$ T cells (IL-2, IFN-γ, IL-4, and IL-10) and IL-12 production in $CD8^+$ cultures - that is, both types of immune responses could occur together and were not cross-inhibitory. It was concluded from these studies that although the immune response to MFP immunisations could be predominantly T1 or T2, both CD4 and CD8 cells were contributing to the response. Therefore, with the knowledge of the key cytokines active in the antitumour immune response to MUC1, the question remained whether or not additional cytokines could enhance this response further.

Chapter four investigated the effect of various cytokines (IL-2, IL-4, IL-6, IL-7, IFN-γ, and GM-CSF) on the MUC1 CTL immune response based on the findings from chapter 3. An enhanced immune response was measured by an increase in CTLp frequency between mice immunised with MFP alone and MFP plus cytokines. The immune response to MFP was enhanced when it was combined with IL-2, IL-7, or GM-CSF (not conventional T1 cytokines as initially hypothesised) and further enhanced with combinations of IFN-γ + IL-2, IFN-γ + IL-4 and GM-CSF + IL-7. These novel findings demonstrated a clear advantage for using combinations of cytokines rather than individual cytokines in the MUC1 model, and could indeed be used to boost the immune response to tumour antigens in other models. However, in spite of the increases in CTLp frequency, tumour challenges using $MUC1^+$P815 cells demonstrated that the addition of these cytokines had little additive effect on the already effective antitumour response of MFP in the above mentioned model. To therefore show a requirement for additional cytokines in MUC1 tumour therapy, a less immunogenic MUC1 tumour model needed to be tested and experiments carried out in MUC1 transgenic mouse models.

Chapter five characterised an aggressive MUC1$^+$ metastatic tumour, DA3-MUC1 as it is not spontaneously rejected in mice making it an alternative model for MUC1 immunotherapy studies. Results from this study concluded that the DA3-MUC1 tumour does not express MHC class I molecules on the tumour cell surface, however, as shown in many other studies, MHC class I can be upregulated following culturing with IFN-γ. Results indicated that the up-regulation of class I could be maintained for up to seven days *in vivo* after removal of the cytokine, without effecting the expression levels of MUC1 antigen. Interestingly, the presence of induced MHC class I molecules led to MUC1$^+$ specific CTL from MFP immunised mice to lyse DA3-MUC1 targets *in vitro* however *in vivo* this did not produce anti-tumour immunity Further, MUC1$^+$ DA3 cells are restricted to lysis by anti-MUC1 CTLs. These results highlight the importance of MHC class I molecules in the induction of anti-tumour immunity and the MFP immune response. The DA3 tumour model is still being investigated in the laboratory using other therapeutic initiatives ie different immunisation protocols, various forms of other MUC1 antigens (eg. Non-VNTR regions). It would be interesting to see if the enhanced immune responses produced against the MUC1 VNTR using IL-5, IL-12 and IFN-γ could be replicated in this new, more aggressive MUC1 immunotherapy model.

Chapters six and seven both focused on enhancing the MUC1 cellular immune response to MFP using an additional cytokine. Chapter 6 showed that IL-5, a cytokine which to date had not been shown to stimulate the production of CTLs *in vivo*, was indeed required for the optimal generation of the CTL response. This study demonstrated that when oxidised mannan MUC1 (MFP) was used as an antigen in mice, IL-5 was as effective as IL-2 for the *in vitro* induction of CTL, and both CD4$^+$ and CD8$^+$ T cells from MFP immunised animals could be shown to secrete IL-5 in culture. In IL-5 -/- mice, the CTLp frequency was greatly diminished resulting in the inability to reject MUC1$^+$ tumours. These studies provide a clear indication for a role of IL-5 in the generation of CTL *in vivo* as part of the immune response to MFP. This observation provides the rationale for examination of possible IL-5 involvement in a range of other CTL responses in future studies. Further studies may focus on why oxidised MFP may have induced IL-5 in the model studied. In addition, studies investigating the role of IL-5 in the generation of a functional cytotoxic response to tumours may provide opportunities for enhancement of such responses in cancer.

Finally, chapter seven demonstrated in MUC1 transgenic mouse models, MFP plus IL-12 could enhance the antitumour immune response to MUC1 characterised by enhanced CTL, CTLp and tumour protection. The transgenic mouse models used expressed either human MUC1 or both human MUC1 and the human MHC class I gene, HLA-A*0201. Significant MUC1 CTLp responses were generated in transgenic mouse models after one immunisation with MFP and IL-12 compared to MFP alone. Similarly, immunisation of MUC1 transgenic mice with MFP and IL-12 also induced tumour protection after challenge with a $MUC1^+$ mastocytoma cell line. The role of IL-12 in the MUC1 immune response was further demonstrated in IL-12 gene knockout mice (-/-) when the CTLp frequency to MUC-1 after one MFP injection was decreased to 1/226,000 compared with 1/77,500 in normal mice. This study demonstrated the enhanced anti-tumour immune responses of MFP and IL-12 and forms basis for clinical trial in patients with $MUC1^+$ tumours.

As preclinical studies continue to produce promising anti-tumour immune responses *in vivo*, as evident from the studies contained in this thesis using MUC1 and cytokines, for the majority of immunotherapy studies that have advanced into clinical trials they have yet to show the preclinical immune responses anticipated. We still have much to unravel about the tumour immune response in humans. It is anticipated that with the recent completion of the Human Genome Project, cancer immunotherapy will take on a new meaning – one which focuses on the genetic cause of this disease rather than protein to protein interaction. The future for cancer immunotherapy will certainly be enriched with the discovery of new oncogenes and suppressor genes and undoubtedly, DNA vaccine technology will rapidly develop.

However, even with all this new knowledge, one thing that remains inevitable is that only through extensive clinical trials in cancer patients, and indeed, patients with newly diagnosed cancers and 'healthier' immune systems, will scientists learn how to induce, manipulate and measure the anti-tumour immune response in humans and ultimately decrease the tumour burden in humans.

Chapter 9:

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The effect of T1 and T2 cytokines on the cytotoxic T cell response to mannan-MUC1

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MUC1 is a mucin over-expressed in breast cancer and a proposed target for immunotherapy. By immunising mice with MUC1 conjugated to mannan (M-FP), $CD8^+$ MHC-class-I restricted cytotoxic T lymphocytes (CTL), of high CTL precursor (CTLp) frequency (1/8000) and with significant tumour protection, can be induced. The effect of various cytokines [interleukin-2 (IL-2), IL-4, IL-6, IL-7, interferon γ (IFNγ), and granulocyte/macrophage-colony-stimulating factor (GM-CSF)] on the MUC1 CTL immune response was investigated (a) by measuring the frequencies of CTLp in mice immunised with vaccinia virus constructs containing recombinant cytokines and M-FP, or (b) by immunising cytokine- or cytokine-receptor-knockout (−/−) mice with M-FP. Vaccinia virus (VV) constructs containing recombinant cytokines were used either individually or in combination in vivo with M-FP immunisation. M-FP immunisations combined with VV-IL-2, VV-IL-7 and VV-GM-CSF, and combinations of VV-IFNγ + VV-IL-2, VV-IFNγ + VV-IL-4 or VV-GM-CSF + VV-IL-7 increased CTLp frequencies up to threefold (1/17 666: M-FP + VV-GM-CSF + VV-IL-7) compared to M-FP (1/77 500) alone. By contrast, M-FP combined with VV-IL-4 decreased the CTLp frequency threefold whereas VV-IL-6 and VV-IFNγ had no effect. Studies in cytokine- and cytokine-receptor-gene-knockout (−/−) mice demonstrated that mice that are IL-2 −/− and IL-7 receptor −/− produce the same CTLp response to M-FP as do control mice, whereas responses in the IL-6 −/−, IL-10 −/− and IFNγ −/− mice were marginally improved and responses to M-FP in IL-4 −/− and tumour necrosis factor receptor 2 −/− mice were weaker. In spite of the increase in CTLp frequency, this was not reflected in an in vivo tumour model. Tumour challenges using $MUC1^+$ P815 cells, demonstrated that the addition of cytokines had little additive effect on the already effective tumour-regression capabilities of M-FP alone.

MUC1 · Cytokines · Immunotherapy · Mannan · Cytokine-gene-knockout mice

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Introduction

We have previously demonstrated that oxidised mannan, coupled to the mucin MUC1 fusion protein (M-FP), successfully induced MUC1-specific tumour immunity in mice, generating a high cytotoxic T lymphocyte precursor (CTLp) frequency, $CD8^+$ MHC-restricted CTL, low antibody titres and significant tumour protection [4, 5]. The cytokines produced by spleen cells from mice immunised with oxidised M-FP demonstrated a distinct T1-type profile consistent with the immune response generated, with interleukin-2 (IL-2) and interferon γ (IFNγ) production. However, mice immunised with reduced M-FP produce a low CTLp frequency, high antibody titres, little tumour protection and IL-4 production, i.e. T2-type immunity [6]. As tumour protection in response to MUC1 is associated with $CD8^+$ CTL and T1-type cytokine production in mice immunised with M-FP [55], it was of interest to determine the effect additional T1 or T2 cytokines had on the MUC1 immune response.

Cytokines have been used extensively to manipulate immune responses in animal models and, as a result, many have progressed into clinical trials and use in humans e.g. IL-2 in metastatic renal cell carcinoma and melanoma [20]. Many of these studies have focused on the use of recombinant cytokines, administered either locally or intratumorally by repeated low-dose injections including IL-1, IL-2, IL-4 and IFNγ [50], or on cytokine gene therapies whereby murine tumours have been transfected to secrete cytokines in vivo, including

IL-2, IL-4, IL-6, IL-7, IL-12, granulocyte/macrophage-colony-simulating factor (GM-CSF), tumour necrosis factor (TNF), IFNγ etc. [17, 23, 68]. The T1-type cytokines IL-2, IFNγ, IL-12, and TNFβ (lymphotoxin α) [15, 47, 62] have generally been favoured for use in tumour immunotherapy as their production results in the development of a cellular immune response and tumour protection in many models. Of the T1 cytokines studied, IL-2 has been used extensively in murine [14, 26] and human [59] tumour models because it is able both to increase T cell proliferation and recruitment to tumour sites and to induce antitumour immune responses. IFNγ has also proven to be an effective antitumour cytokine, inducing tumour regression and protection, indicated by CTL induction in numerous tumour models including mammary adenocarcinomas [42] and lung and colon carcinomas [25, 57]. More recently, IL-12 has been much favoured as it has shown therapeutic effects in tumour-bearing animals [64]. Owing to the promising results of IL-12 gene therapy, clinical trials are now in progress [39]. Many of the T2-type cytokines (IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 [15, 47, 62]) known for their involvement in humoral responses have also been studied for their effect on tumorigenicity and found to be effective in many tumour models. IL-4, which is commonly known to exert its biological effects on the proliferation, activation and differentiation of B cells, has also shown to play an important role in T cell tumour immunity [13], and has been engineered into tumour cells to treat various cancers including renal carcinoma [67]. IL-6, a predominant B cell growth factor, also plays a role in tumour protection when engineered into fibrosarcoma or Lewis lung carcinoma cells [48, 56]. GM-CSF and TNFα have also demonstrated antitumour immune responses when administered in several tumour models. When transfected into melanoma [22], bladder cancer [61] and leukaemia [31] models, GM-CSF induces potent antitumour immunity. Although toxic in high doses, TNFα has also been used in tumour models to show regression of autologous tumours [12].

Immunotherapy with the target MUC1 antigen and recombinant cytokines has previously been studied by Acres et al. [1] and Balloul et al. [9]. These groups constructed vaccinia viruses co-expressing the MUC1 antigen and various cytokines: IL-2, IL-4, IL-5, IL-6, IL-7, on the basis of their previously described antitumour activities. Mice immunised with vaccinia virus construct VV-MUC1 were protected against tumour challenge; however, no evidence of MUC1 CTL activity was found after immunisation [1]. On the basis of these findings, we used IL-2, IL-4, IL-6, IL-7, IFNγ, and GM-CSF in a MUC-1 model to examine the effect of these cytokines on CTLp frequency and tumour protection. The role of various cytokines in the initiation of the immune response against M-FP was further examined in cytokine-gene-knockout mice, and in a MUC1 DBA/2 in vivo immunotherapy tumour model.

Materials and methods

MUC1 antigens

MUC1 FP was produced in a bacterial expression system (pGEX-3X) containing five VNTR = [Variable number of randem repeat] regions of MUC1 [3] and conjugated to oxidised mannan [4]. The MUC1 peptide Cp13–32 [(C)PAHGVTSAPDTRPAPGSTAP] was synthesised using an Applied Biosystems Model 430A biosynthesiser.

Cytokine- and cytokine-receptor-knockout mice

Cytokine- and cytokine-receptor-knockout mice, produced by homologous recombination on a C57BL/6 background, were generously supplied by the following: IL-2 −/− (R.M. Zinkernagel, Institute of Experimental Immunology, Switzerland [38]), IL-4 −/− (Institute for Genetics, University of Cologne, Germany [36]), IL-6 −/− (R.M. Zinkernagel [35]), IL-7 receptor (IL-7R) −/− (Jackson Laboratory, Maine, USA [54]), IL-10 −/− (Jackson Laboratory [37]), IFNγ receptor (IFNγR) −/− (R.M. Zinkernagel [32]), and TNF receptor 2 (TNF-R_2) −/− (Department of Cell Genetics, Genetech, California [24]).

Immunisation

BALB/c, DBA/2, (BALB/c × C57BL/6) F1, (BALB/c × DBA) F1 mice were bred at the Austin Research Institute (Victoria, Australia). Female mice aged 6–10 weeks were immunised intraperitoneally with M-FP (containing 5 μg of FP) either once, or weekly for 3 weeks. Mice injected with cytokines received 1×10^7 pfu/ml (total) vaccinia virus containing the cytokine intraperitoneally either alone, or mixed with M-FP (5 μg). Both cytokine- and cytokine-receptor −/− mice were immunised intraperitoneally with M-FP conjugate either once or weekly for 3 weeks.

Cytokine-encoding vaccinia virus

Vaccinia virus (VV) constructs encoding the recombinant cytokines human IL-2, murine IL-4, murine IL-6, murine IL-7, murine IFNγ, murine GM-CSF and wild-type VV were obtained and tested for biological activity by Dr. Bruce Acres (Transgene, Strasbourg, France). The constructs were produced from infected baby hamster kidney cells (BHK), sonicated to release virus particles, and purified over a 36% sucrose gradient by ultracentrifugation to obtain virus for immunisations as described [2]. Virus titres were determined by plaque-forming assays in which 5×10^6 BHK cells in 6-well plates were incubated for 24 h 37 °C 10% CO_2 with serial dilutions (1×10^5–1×10^9) of vaccinia virus, stained with aniline blue and counted for plaque formation. To determine recombinant cytokine activity and concentration, both biological assays and cytokine enzyme-linked immunosorbent assays (ELISA) (commercially available kits: Pharmingen USA, Genzyme USA, R&D Systems USA, Endogen USA) were performed in conjunction with Dr. Bruce Acres (Transgene, Strasbourg, France) after the generation of each VV-cytokine product.

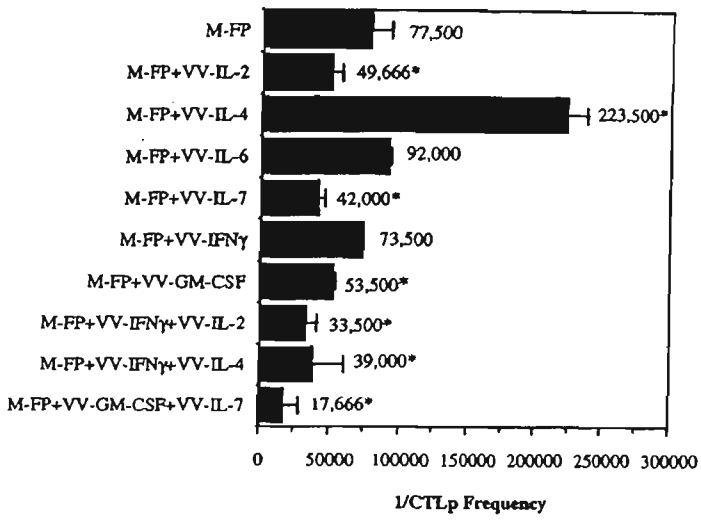
Tumours

Two DBA/2 P815 mastocytoma cell lines transfected with the human *MUC1* gene were used to study the in vivo effects of M-FP immunisations in combination with cytokines. P815-Tm211 (high MUC1 expression) was used as a target in CTLp assays, and the immunogenic clone P815-Tm2 (low MUC1 expression) [1] was used to challenge mice. DBA/2 mice (ten per group) were injected subcutaneously with 1×10^6 P815-Tm2 tumour cells until tumours of 0.6 ± 0.1 cm^2 were established (day 8). Mice were then given one intraperitoneal injection of M-FP + VV-cytokine, M-FP, VV or

cytokine alone. Tumour sizes were measured every 2–3 days using electronic callipers.

Cytotoxic T cell precursor assays

Immunised mice were sacrificed; their spleen cells were collected and added in 32 replicates to 96-well microtitre plates at concentrations varying from 1×10^3 to 1.28×10^5 cells/well. Stimulator spleen cells from (BALB/c × DBA)F_1 or (BALB/c × BL/6)F_1 naive mice were irradiated with 30 Gy from a caesium source [Gammacell 1000 Elite irradiator (Nordion International Inc.)]. Stimulator cells (5×10^5 cells/well), 5 μM MUC1 peptide Cp13–32 and 10 U/ml recombinant human IL-2 were added to all effector wells. Controls containing 5×10^5 cells/well stimulators and either 10 U/ml IL-2 and 5 μM peptide Cp13–32, or 5×10^5 cells/well stimulators and 1.28×10^5 cells/well effectors were set up in replicates of 32 wells. Plates were incubated for 7 days at 37 °C 10% CO_2. After incubation, 100 μl supernatant from all wells was discarded and 1×10^4 ^{51}Cr-labelled P815-MUC1, P815, RMA-MUC1 [30] or RMA target cells were added. Cultures were incubated for a further 4 h before 100 μl supernatant was transferred to 96-well flat Optiplates (Disposable Products, Australia) containing 100 μl of Microscint 40 (Packard, USA) for analysis on a microplate scintillation counter (Packard, USA). The number of CTL precursors present was determined as the frequency of the number of cells not lysed on a logarithmic scale and the number of cells/well on a linear scale [40, 66]. CTLp assays were repeated a minimum of three times on each VV-cytokine and at least twice on cytokine-knockout mice.



MUC1-specific cytotoxic T lymphocyte precursor (*CTLp*) frequencies in BALB/c mice injected once with 5 μg MUC1 conjugated to mannan (*M-FP*) with or without various vaccinia virus (*VV*)-linked cytokines (1×10^7pfu/mouse total) intraperitoneally. Vaccinia virus and VV-cytokine without M-FP had CTLp frequencies lower than $1/10^6$. The data represent the average CTLp frequency from three determinations. * Frequencies statistically different from those obtained with M-FP alone, as determined by Student's *t*-test

Results

The effect of cytokines on M-FP immune responses in vivo

VV-cytokines were used either alone or in various combinations with M-FP to determine the effect of each cytokine on the cellular immune response measured by a CTLp assay (Fig. 1). First, mice injected with either VV-cytokines + M-FP or M-FP alone could generate a MUC1-specific CTLp response as the CTLp frequency in mice immunised with VV alone and with VV-cytokines without M-FP was less than $1/10^6$. Second, in keeping with previous observations, the T1 cytokines IL-2, IFNγ and GM-CSF were beneficial to the CTLp response whilst the T2 cytokine IL-4 was antagonistic. The following was observed:

a. Increases in CTLp responses were seen after one injection of M-FP alone (CTLp frequency 1/77 500) or combined with VV-IL-2 (1/49 000; $P = 0.013$), VV-IL-7 (1/42 000; $P = 0.0014$) or VV-GM-CSF (1/53 000; $P = 0.0278$) (Fig. 1).
b. No difference was noted in CTLp responses to MUC1 after the addition of VV-IFNγ (1/73 500; $P = 0.359$) or VV-IL-6 (1/92 000; $P = 0.213$) (Fig. 1).
c. A decrease in CTLp frequency was evident after VV-IL-4 was injected with M-FP (1/223 500; $P = 0.0039$). CTLp frequencies against MUC1 were strongest with M-FP and the VV-cytokine combinations VV-IFNγ + VV-IL-2 (1/33 500; $P = 0.0019$) and VV-IFNγ + VV-IL-4 (1/39 000; $P = 0.0068$), and the best response was seen with VV-GM-CSF + VV-IL-7 (1/17 600 $P = 0.00004$) (Fig. 1). It was of interest that, when used alone, VV-IFNγ had no effect on the CTLp frequency and VV-IL-4 decreased CTLp responses; however, when used together, VV-IFNγ + VV-IL-4 increased the CTLp frequency.

M-FP in vivo CTLp responses in cytokine-gene- and cytokine-receptor-gene-knockout mice

The effect of cytokines on the immune response to MUC1 was further examined using cytokine- and cytokine-receptor-gene-knockout mice (−/−). IL-2, IL-7R, IL-10, IFNγ, and TNF-R_2 −/− mice were injected once, and IL-4 and IL-6 −/− mice were injected three times with M-FP, and the MUC1-specific CTLp frequencies determined and compared with those found in C57BL/6 control mice (Table 1).

a. There was no effect on the MUC1 CTLp frequency after a single injection of M-FP in IL-2 −/− mice (1/72 900), IL-7R −/− mice (1/78 000), TNF-R_2 −/− mice (1/69 000), or IFNγ −/− mice (1/66 000) compared to M-FP in wild-type mice (1/72 000) (Table 1). No significant differences after three injections of M-FP in IL-4 −/− mice (1/9500) were noted compared to control mice (1/7000).
b. Stronger CTLp responses were evident after three injections in IL-6 −/− (1/2500) and one injection in IL-10 −/− (1/60 000) mice compared to their respective controls (1/7000 and 1/72 000) indicating an inhibitory role for these cytokines in the implementation of a MUC1 immune response in vivo (Table 1). The MUC1-negative target (RMA) was not killed in any of the CTLp assays (CTLp < $1/10^6$). Thus, the results with cytokine-

Cytotoxic T lymphocyte precursor (CTLp) frequencies from cytokine-gene-knockout mice immunised with MUC1 conjugated to mannan (M-FP). CTLp frequencies were determined from cytokine −/− mice immunised intraperitoneally with 5 μg M-FP, either once (IL-2 −/−, IL-7R −/−, IL-10 −/−, IFNγ −/−, TNF-R_2 −/−) or three times (IL-4 −/−, IL-6 −/−). Control mice were wildtype C57BL/6. *IL* interleukin, *TNF* tumour necrosis factor, *R* receptor, *IFN* interferon

Absent gene	Number of M-FP injections	Targets	
		RMA-MUC1	RMA
IL-2	1	1/72 900	$< 1/10^6$
IL-4	3	1/9500	$< 1/10^6$
IL-6	3	1/2500	$< 1/10^6$
IL-7R	1	1/78 000	$< 1/10^6$
IL-10	1	1/60 000	$< 1/10^6$
IFNγ	1	1/66 000	$< 1/10^6$
TNF-R_2	1	1/69 000	1/980 000
Wild type (control)	1	1/72 000	1/350 000
	3	1/7000	$< 1/10^6$

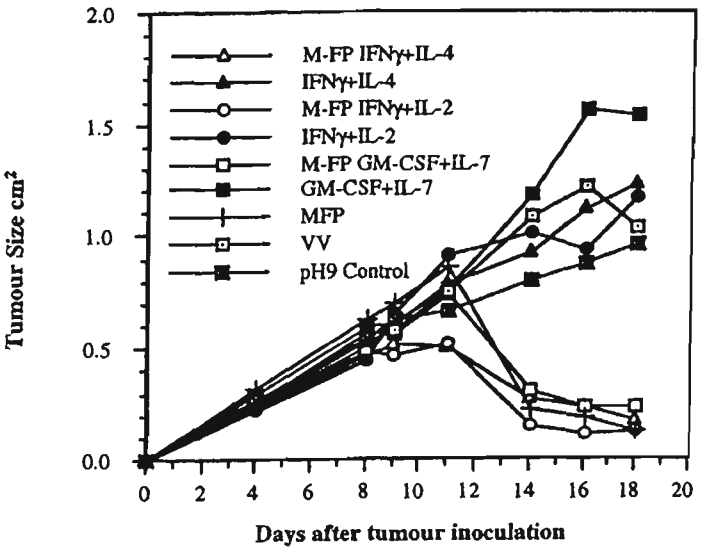
knockout mice results provide further evidence that certain cytokines can influence the immune response generated to MUC1.

Cytokines + M-FP in the Tm2 tumour model

Three groups of M-FP and various VV-cytokine combinations were studied in the DBA/2 P815-Tm2 tumour model, on the basis of the significant increases observed in the CTLp frequencies shown in Fig. 1. VV-IFNγ + VV-IL-2, VV-IFNγ + VV-IL-4 and VV-GM-CSF + VV-IL-7 were used to inject DBA/2 mice with established P815-Tm2 tumours, both alone and together with M-FP, and their tumour growth was measured over 30 days (Fig. 2). Mice injected only with cytokine combinations and no M-FP had no effect on tumour growth, which was similar to that of control mice; in mice injected with M-FP and cytokine combinations, a complete regression of established tumours occurred after 30 days. No significant advantages in the antitumour effects produced by M-FP were evident after the incorporation of cytokines into the M-FP immunisations in this tumour model. A different in vivo model for MUC1 tumour immunotherapy needs to be established to determine whether cytokines enhance the antitumour responses to M-FP.

Discussion

The induction of antitumour cellular immune responses requires tumour-specific antigens to be processed and presented by either the tumour, professional antigen-presenting cells (e.g. B cells, dendritic cells and macrophages) or both, in association with MHC class I and costimulatory molecules to activate naive T cells into tumour-specific cytotoxic cells, under the influence of



Subcutaneous tumour growth in DBA/2 mice with established 8-day Tm2 tumours (1×10^6 cells/mouse). Mice were immunised once on day 8 with VV-cytokines (1×10^7 pfu/mouse total) with or without 5 μg M-FP. All tumours in rejecting mice disappeared after 30 days. Student *t*-tests were performed to show a significant difference between mice immunised with M-FP and VV-cytokines and those receiving VV-cytokines alone

locally secreted cytokines. Cytokines are secreted to activate $CD8^+$ T cells and other cells involved in tumour rejection, to eliminate tumour cells and ensure that T cell memory is established to protect against recurrences. We had previously demonstrated that MUC1, when coupled to mannan, induced $CD8^+$ CTL, tumour protection and memory in MUC1 tumour models [4]. To determine whether the antitumour immune response generated against M-FP could be increased by the addition of cytokines, and to identify the role various cytokines play in the immune response generated against M-FP in vivo, cytokines were incorporated into M-FP immunisations and both CTLp and tumour protection studies in a MUC1 model were examined, together with CTLp responses to M-FP in the cytokine-knockout mice studied.

Vaccinia viruses encoding the recombinant cytokines: human IL-2, murine IL-4, murine IL-6, murine IL-7, murine IFNγ, murine GM-CSF and wild-type VV [2, 9, 51, 70], were combined with M-FP, and the influence of each individual cytokine on the MUC1 cellular immune response measured by determining CTLp frequency. IL-2, IL-7 and GM-CSF increased the MUC1 CTLp frequency, additional IL-4 decreased the CTLp frequency whilst IL-6 and IFNγ had no effect. Whether these cytokines act directly to enhance recruitment or proliferation of CTL, or indirectly, acting on other cells involved in the MUC1 immune response, is not clear and will be discussed below.

Tumour immunotherapy studies with IL-2 have demonstrated that additional IL-2 is beneficial to tumour rejection and the establishment of memory. Numerous studies of IL-2 transfection on non-immunogenic tumour models show that continual low-dose secretion of IL-2 provides sufficient cytotoxicity to

reduce the tumorigenicity and metastatic potential of numerous tumour models including melanomas [33], fibrosarcomas [27] and neuroblastomas [18]. In the MUC1-M-FP model system, additional IL-2 also improved the immune response by producing a twofold increase in MUC1-specific CTLp frequencies; additional IL-2 could also be a beneficial cytokine for increasing MUC1 cytotoxic T cells and it would be advantageous to include it in MUC1 tumour therapy. However, the IL-2 −/− mice showed that IL-2 was not required for the initiation of a MUC1 immune response nor did its absence hinder the generation of cytotoxic T cells, as no difference in CTLp frequencies in M-FP-immunised wild-type or IL-2 −/− mice was observed. Immune responses generated against tumour antigens in IL-2-deficient mice have not been well documented, as these mice do not survive well [60]; however, the results from a study investigating antiviral immune responses show that virus-specific CTL induction is only moderately reduced in IL-2 −/− mice compared to wild-type mice, perhaps because of cytokine compensation [8]. As the absence of IL-2 did not change the MUC1-specific CTLp frequency in IL-2-deficient mice, it is possible that the loss of functional IL-2 was compensated by other cytokines, in particular, IL-15, which has similar biological activities to IL-2 [29]. Hence this evidence suggests that, although additional IL-2 produced from vaccinia virus is beneficial to the M-FP immune response, as it increases the production of cytolytic T cell precursors, IL-2 may not play a significant role in the initiation of the immune response against M-FP as its absence in the knockout mice does not effect the CTLp frequency.

Many studies indicate that transfer of the gene for IL-4, a T2 cytokine, into tumour cells confers a certain degree of tumour protection; however this protection is generally attributed to nonspecific effector cells and not always to $CD8^+$ CTL required for complete tumour rejection [67]. In the M-FP model, IL-4 significantly decreased the MUC1 CTLp frequency when added to M-FP injections. This was not surprising as it has been shown that IL-4 inhibits the development of cytotoxic T1 cells whilst inducing the proliferation of T2 cells [13]. In a study investigating the role of IL-4 in mediating anti-viral cellular immunity, VV-IL-4 demonstrated a direct involvement in the suppression of cell-mediated immune responses in vivo [63]. We have previously demonstrated that, during the immune response to M-FP, cytotoxic T cell production was directly associated with IFNγ and no detectable IL-4 secretion, and when IL-4 production was evident it was associated with no cytotoxic T cell development [4]. Therefore when IL-4 was included in M-FP injections, the T1 immune response originally produced switched to a T2 immune response due to the presence of IL-4, resulting in a down-regulation in the number of CTLp produced in response to M-FP.

M-FP injections into IL-4-deficient mice showed no effect on the number of CTL precursors produced against MUC1 in −/− mice compared to the number in control mice producing IL-4. This result is in keeping with observations made when studying the immune response to contact hypersensitivity in IL-4 −/− mice [69]. Results from this study show that the disease progressed in two phases, the first of which was dependent on Th1/Tc1 cells and was unaffected by the absence of IL-4, whilst the second was Th2/Tc2-dependent and was totally undeveloped in mice lacking IL-4. Similarly, Kuhn et al. [36] reported IL-4-deficient mice to have normal T and B cell development, but strongly reduced serum levels of IgG1 and IgE. These results demonstrate that Th1/Tc1 immune responses progress independently of the presence of IL-4 and suggest that the T1 immune response generated against MUC1 is also IL-4-independent; however, when introduced in excess, it hinders the production of CTLp in the immune response against a MUC1 antigen.

IL-6 plays an active role in stimulating the proliferation of mature $CD8^+$ T cells and cytolytic T cell responses [43] and, for these reasons, has shown potent antitumour activity when transfected into several tumour models [48, 49, 65]. In M-FP injections, however, additional IL-6 had no effect on the CTLp response generated against MUC1. As the above studies demonstrated IL-6 to be an active stimulator of cytotoxic T cell development, it would appear that, in the MUC1 immune response induced by M-FP, significant levels of IL-6 may already be induced. Further studies examining the cytokines secreted from T cells of mice immunised with M-FP are in progress and will determine which are produced upon M-FP stimulation. IL-6-deficient mice generated a stronger CTLp response to M-FP than did control mice. The increase in CTL precursors observed in the absence of IL-6 indicate that IL-6 is an inhibitory factor in the early stages of MUC1 CTL precursor development. Studies of IL-6-deficient mice show that, in a *Candida albicans* model of viral infection, IL-6 −/− mice have impaired neutrophil and $CD4^+$ T cell development [58]. On the basis of this study, the presence of neutrophils and $CD4^+$ T cells early in the MUC1 immune response may hinder the generation of MUC1 CTL precursors; however, the presence of IL-6, and therefore neutrophils and $CD4^+$ T cells at a later time, has no effect on the T1 immune response generated to M-FP.

Additional IL-7 included in M-FP injections increased the CTLp frequency in response to MUC1 twofold. Numerous in vivo studies have also shown the therapeutic effects IL-7 exerts by increasing $CD8^+$ T cell responses [44]. Studies examining VV-IL-7 have also shown enhanced T cell proliferation during viral infection whereby both $CD4^+$ and $CD8^+$ T lymphocytes were increased two- and three-fold and lymphokine-activated killer cells activity was enhanced [41]. In the light of these studies, it was not surprising that IL-7 increased the immune response to M-FP by generating a stronger MUC1 cytotoxic T cell precursor response. MUC1 CTLp responses generated by an M-FP injection in mice deficient in IL-7 receptor α chain (IL-7R −/− mice) were

not affected by the absence of IL-7. Phenotypic studies in IL-7R −/− mice show severely depleted lymphocyte populations where γδ T cells are absent and αβ T and B cell numbers are reduced [45]. In the M-FP model, these results suggest that cytotoxic T cell precursors are either generated in an IL-7-independent manner or that M-FP provides the antigenic stimulation required to activate the small amount of αβ T cells present to generate MUC1-specific $CD8^+$ T cell precursors.

Neither VV-IL-10 or VV-TNFα recombinants were available for the determination of the effects of these cytokines on the immune response generated to MUC1 when included in M-FP immunisations. Both cytokines have, however, demonstrated antitumour capabilities in other murine model systems characterised by the generation of tumour-suppressing cytotoxic T cells and, in some cases, the establishment of immunological memory (TNFα [12, 68], IL-10 [28]). To investigate the role IL-10 and TNF have on the generation of MUC1 CTL precursors, M-FP was injected into both IL-10-deficient and TNF-receptor-deficient mice. There was an increase in the CTLp frequency observed in IL-10 −/− mice, demonstrating that IL-10 has an inhibitory effect on CTLp induction in the early stages of the MUC1 immune response. This observation is true of IL-10, which has been described as indirectly preventing antigen-specific T cell activation by down-regulating antigen presentation [21]. Studies investigating M-FP CTLp responses in TNF-R_2-deficient mice showed no effect on the number of CTL precursors produced, demonstrating that CTLp induction in response to M-FP-stimulated mice is independent of TNFα. This finding is in keeping with a report from Erickson et al. [24], which described TNF-R_2 −/− mice to have normal T cell development and activity, but an increased resistance to TNF-induced death. The unaltered T cell response in TNF-R_2 −/− mice is not unusual, as TNFα is predominantly referred to as an inflammatory cytokine that promotes margination of leukocytes at inflammatory sites and cytotoxic effects on tumour cells, and exerts little direct effect on T lymphocytes [10].

IFNγ is a T1 cytokine that regulates the induction of cytolytic T cells, increases natural killer cell activity, increases the antigen-presenting capacities of macrophages and promotes macrophage tumoricidal activity [11, 52]. In the M-FP immune response, IFNγ is secreted in quantities sufficient to generate a strong MUC1 CTLp response [4]. It was therefore not surprising that additional IFNγ included in M-FP injections had no effect on the levels of MUC1 CTLp generated, even though it is a cytokine renowned for its ability to increase CTL production. M-FP injections into IFNγ-deficient mice showed no effect on the number of cytotoxic T cell precursors produced against MUC1 in −/− mice compared to control mice producing IFNγ. Studies in IFNγ −/− mice report the development of a normal immune system; however, the mice have defective natural resistance to viral infections despite normal cytotoxic and T helper cell responses [32]. As the CTLp response to M-FP was not affected by the absence of IFNγ, this suggests that IFNγ is not directly involved in the early stages of MUC1 CTL induction but, instead, exerts its biological effects later on in the immune response, as indicated by the significant levels of IFNγ induced after M-FP immunisations, by acting as a growth factor for CTL precursors.

GM-CSF induces the proliferation of macrophages [46] and enhances T cell immune responses by potently recruiting and activating antigen-presenting cells [16]. GM-CSF has been shown to produce antitumour responses in several tumour models [7, 22] including a murine breast cancer model, C3HBA, where mice immunised with tumour cells infected with VV-GM-CSF were completely protected against a challenge of parental tumour cells and mice not infected with VV-GM-CSF were not [53]. In the immune response generated against MUC1, GM-CSF increased the number of CTL precursors when included in M-FP injections. This result, plus the observation that GM-CSF increased the number of macrophages at the site of M-FP injection (Apostolopoulos, manuscript in preparation), suggest that GM-CSF acts to increase the number of antigen-presenting macrophages to the area of M-FP injection, enhancing the MUC1 T cell immune response.

Apart from the advantages single cytokines bring to tumour immunotherapy, the use of multiple cytokine therapies is rapidly being explored because of the additive and synergistic effects of cytokine combinations in an in vivo situation. Combinations of cytokines included in M-FP immunisations (IFNγ + IL-2, IFNγ + IL-4, and GM-CSF + IL-7) were successful in enhancing the MUC1 CTLp response, and all were either synergistic or additive in their effect. IFNγ + IL-2 acted synergistically to increase MUC1 CTLp significantly by stimulating T1-type cells. Others have shown that IFNγ and IL-2 in combination can prolong the survival of mice with melanoma and induce both natural killer/lymphokine-activated killer cells and anti-melanoma-specific CTL in a murine melanoma model [34]. IFNγ + IL-4 acted synergistically to increase the number of MUC1 CTLp to the extent seen with IFNγ + IL-2, although alone IFNγ and IL-4 act quite differently. The synergistic effect seen when IFNγ and IL-4 are combined could be the result of an increase in macrophage activation, as previously shown by Crawford et al. [19], which would therefore increase the antigen-presenting capacities for the MUC1 antigen to be processed. The combination of GM-CSF and IL-7 produced an additive effect beyond that of each cytokine alone. Both cytokines were able to increase the MUC1 CTLp frequency individually; however, the combination of the two enhanced this response dramatically. An increase in macrophage availability, and hence antigen-presenting capabilities, and a growth factor for activated T cells, may explain the synergistic effects seen when GM-CSF and IL-7 are used in combination with M-FP to induce MUC1 CTL precursors.

The CTLp responses observed in the M-FP and cytokine immunisations show a clear advantage in using combinations rather than individual cytokines in the MUC1 model for those cytokines tested. To examine this in vivo, the combinations of IFNγ + IL-2, IFNγ + IL-4 and GM-CSF + IL-7 were used in conjunction with M-FP to immunise mice against an established MUC1 tumour, P815-Tm2. No significant difference in tumour growth was noted when M-FP was used alone or in cytokine combinations. However, the use of cytokines without M-FP clearly had no effect on tumour growth, demonstrating that the cytokines do not exert their effects in a non-specific manner on cells of the immune system and act only on antigen-specific effector cells. There was no advantage in the MUC1 P815-Tm2 model when cytokines were included in the M-FP immunisations, as alone M-FP could successfully eradicate the established tumour. Therefore, to show a requirement for additional cytokines in MUC1 tumour therapy, a new MUC1 tumour model needs to be established in which M-FP immunisations alone do not abolish tumours effectively.

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Short Communication

Immunotherapy with mannan-MUC1 and IL-12 in MUC1 transgenic mice

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Abstract

Mice immunised with oxidised mannan-MUC1 fusion protein (M-FP) develop MHC restricted $CD8^+$ cytotoxic T cells. We now demonstrate that in MUC1/HLA-A2 transgenic mice, IL-12 gives enhanced CTL, CTLp and tumor protection. CTLp in MUC1 transgenic mice with M-FP were 1/55,000, and with IL-12, this increased to 1/19,000, with improved tumor protection. Thus, IL-12 is important for effective CTL responses to MUC1 in transgenic mice. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Cytokines; Transgenic; MUC1

1. Introduction

In preclinical studies in mice, human MUC1 coupled to oxidised mannan (M-FP) induced MUC1 immunity with MHC-restricted MUC1 specific $CD8^+$ cytotoxic T cells, a high CTL precursor frequency, significant tumor protection, low antibody levels, and IL-2 and IFN-γ cytokine secretion [1]. MUC1 transgenic mice provide a more realistic model for MUC1 immunotherapy compared to using human MUC1 in mice and recent studies show that MUC1 transgenic mice can be immunised with MUC1 [2, 3]. In another murine model described herein, HLA-A*0201 × MUC1 (A2-MUC1) transgenic mice were developed by crossing HLA-A*0201 transgenic mice [4] with MUC1 transgenic mice; these mice express both human MUC1 and HLA-A2 and were used to produce human HLA-A*0201 restricted CTLs. We now report that IL-12 increased the MUC1 CTLp frequency and anti-tumor effect to mannan-MUC1+ in MUC1 × A2-MUC1 double transgenic mice.

2. Materials and methods

Inbred BALB/c, homozygous DBA/2 MUC1 transgenic and HLA-A*0201 × DBA/2 MUC1 (A2-MUC1) double transgenic [4] mice were used. Mice were immunised IP with 5 μg of MUC-1 fusion protein (5 VNTR repeats of MUC1) conjugated to oxidised mannan (M-FP) [1], either once, or weekly for 3 weeks. Mice injected IP with vaccinia virus (VV) encoding recombinant murine IL-12 (VVIL-12), received 1×10^7 pfu/ml (total virus) either alone or with M-FP. BALB/c mice

Abbreviations: MUC1, human mucin 1; M-FP, mannan-MUC1 fusion protein; A2-MUC1, HLA-A*0201 × MUC1 double transgenic mouse; CTLp, CTL precursor; VV, vaccinia virus.

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sponse leading to rapid increase in post-challenge antibody titres and 100% protection [4]. In case of i.c. challenge, inoculation of a low dose of virus directly into the nervous system may lead to the infection and replication of the virus in central nervous system rather than peripheral sites. This may lead to poor stimulation of pre-existing memory B cells and T helper cells in the DNA vaccinated mice resulting in very little increase in post-challenge antibody titres. However, the fact that DNA vaccinated mice can survive against i.c. challenge even in the absence of a potent B cell response suggests that cellular immune responses may have played a predominant role in the induction of protective immunity against i.c. challenge [3–5,7]. The 100% protection observed in case of the BIKEN vaccine may be due to the presence of high levels of pre-challenge antibody titres that ensure rapid clearance of the virus from the central nervous system. Thus, both cellular and humoral immune responses appear to be involved in protective immunity against JEV. The present study indicates that i.c. JEV challenge model offers a unique opportunity to examine the role of cellular and humoral immune responses in conferring protection against JEV and such studies will aid in the design of better DNA vaccines.

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were injected daily with 1 μg(×5) of murine recombinant IL-12 (rIL-12) and a single injection of M-FP (5 μg) I.P. CTLp assays on spleen cells from immunised mice were described elsewhere [5–8]. The lysis obtained was MUC1 specific, and could be inhibited by $CD8^+$ antibody [9]. Two different protocols for a $MUC1^+$ tumor challenge in DBA/2 MUC1 transgenic mice were performed. In the first, MUC1 transgenic mice (5–6/group) were given 3 weekly IP immunisations (days 0, 7 and 14), with either 5 μg of M-FP, 5 μg of M-FP mixed with 1×10^7 pfu/ml VVIL-12 [10], or buffer as a control. Mice were challenged with 3.5×10^6 P815-MUC1 tumor cells given SC on day 22, and the tumor growth measured. In the second experiment, DBA/2 MUC1 transgenic mice (7–9/group) were given three weekly IP immunisations (days 0, 7 and 14) with either 1×10^7 pfu/ml VV IL-12 or control VV, and/or mixed with 5 μg of M-FP. Mice were challenged with 1×10^7 P815-MUC1 tumor cells SC on day 21, and 30 days later the number of mice with palpable tumors determined. It was not possible to use test tumor growth in the A2-MUC1 double transgenic mice, as there was no tumor available that expressed the genes.

3. Results

3.1. MUC1 cytotoxic T cell precursor frequency

CTLp frequencies were determined in inbred BALB/c mice, MUC1 transgenic and A2-MUC1 double transgenic mice immunised with either M-FP, or M-FP and IL-12.

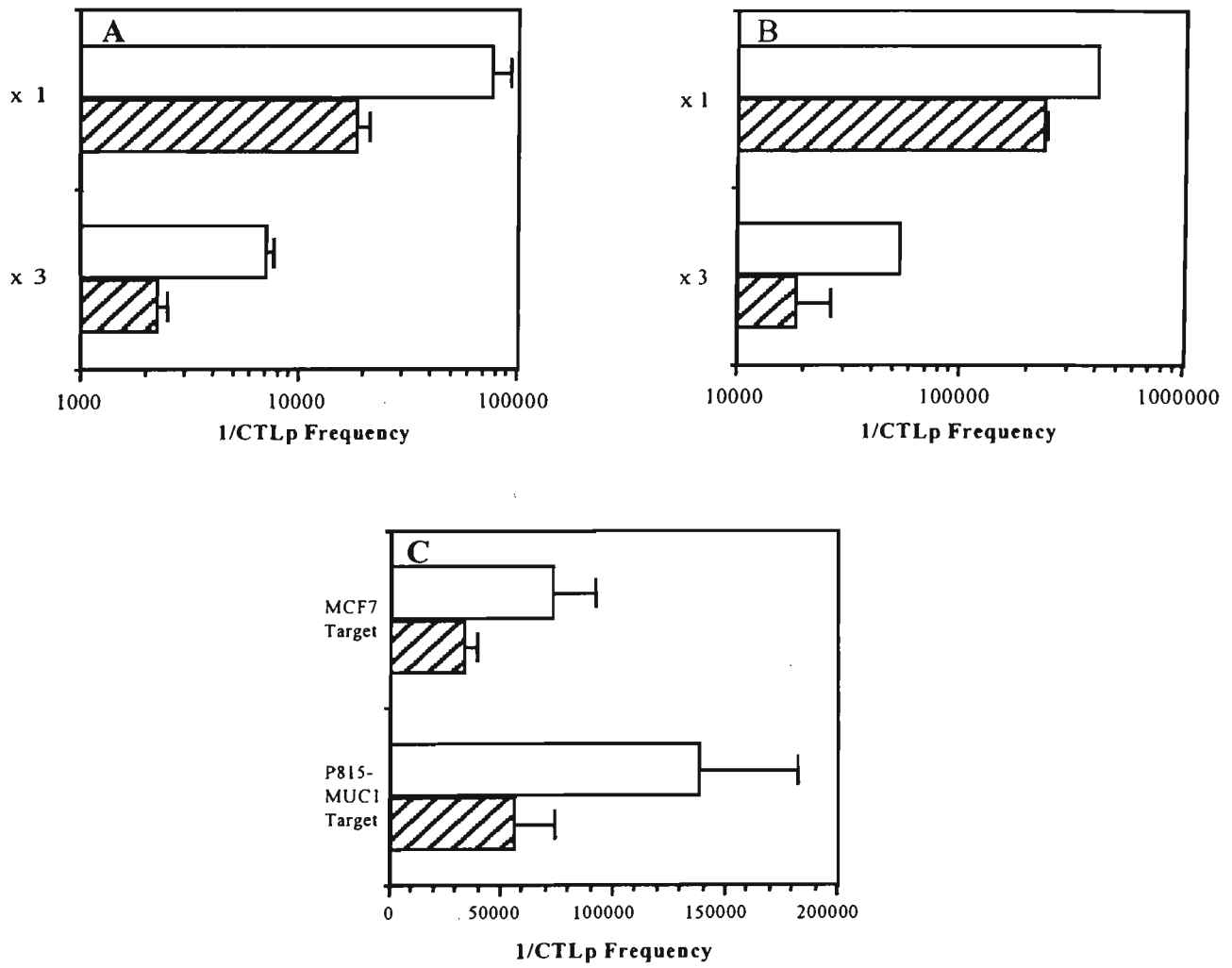

Fig. 1. Average CTLp frequencies from (A) BALB/c mice immunized I.P (×1 or ×3) with either 5 μg M-FP (□) or 5 μg M-FP+murine rIL-12 (■) (1 μg/mouse/day IP × 5 days) (3 mice/group). The CTLp frequencies from mice injected with rIL-12 only were $< 1/10^6$ (not shown); (B) MUC1 transgenic mice immunized I.P (either ×1 or ×3) with either 5 μg M-FP (□) or 5 μg M-FP+VVIL-12 (1×10^7 pfu/ml) (▨). The CTLp frequency for mice injected both ×1 and ×3 with VVIL-12 alone were $< 1/10^6$ (not shown); and (C) A2-MUC1 transgenic mice immunized I.P (×3) with either 5 μg M-FP □ or 5 μg M-FP+VVIL-12 (1×10^7 pfu/ml) ▨. HLA-A2 restricted, MUC1 positive CTL precursors were determined using the MCF-7 target cell line and MUC1, H-2^d CTL precursors were determined using the $MUC1^+$ P815 target cell line. The CTLp frequencies from mice injected with VVIL-12 alone were $< 1/10^6$ on both target cell lines (not shown).

3.1.1. BALB/c mice (Fig. 1A)

Using MUC1$^+$ P815 cells as targets, mice given a single injection of M-FP generated a MUC1 CTLp frequency of 1/76,000 that increased to 1/19,000 upon addition of recombinant murine IL-12 (five daily injections/week). Mice given only recombinant IL-12 and no M-FP had no MUC1 CTLp responses ($<1/10^6$), nor was there lysis of control MUC1$^-$ P815 cells. After three weekly injections of M-FP in BALB/c mice, the CTLp frequency of 1/7500 increased to 1/2500 with recombinant murine IL-12 (five daily injections/week). Similiarily, there was no lysis of control MUC1$^-$ P815 cells (CTLp frequency $<1/10^6$; data not shown).

3.1.2. MUC1 transgenic mice (Fig. 1B)

Using MUC1$^+$ P815 cells as targets, a single injection of M-FP led to a CTLp frequency of 1/420,000 which increased to 1/240,000 with VVIL-12. A significant improvement in CTLp responses was observed when three injections of M-FP were given (1/55,000) which further increased with VVIL-12 (1/19,000). CTLp were not detected ($<1/10^6$) in mice injected with VVIL-12 and no M-FP, nor was there lysis of control MUC1$^-$ P815 cells.

3.1.3. A2-MUC1 double transgenic mice (Fig. 1C)

Using MCF-7 target cells, three injections of M-FP led to a CTLp frequency of 1/74,250 which increased to 1/35,000 with VVIL-12. The MUC1$^+$/H2^d CTLp frequency in A2-MUC1 transgenic mice, using P815-MUC1 target cells, demonstrated that M-FP+VVIL-12 produced CTLp responses almost four times stronger than M-FP alone (1/78,000 compared to 1/280,000). The frequency on MUC1$^-$ P815 (H2^d) cells was less than on HLA-A2 MCF-7 cells — possibly due to a decrease in H2^d molecules in A2-MUC1 double transgenic mice. Injections of VVIL-12 alone were negative ($<1/10^6$) against both target cell lines in A2-MUC1 double transgenic mice. Thus IL-12 substantially increased the CTLp frequencies in both inbred, MUC1 and A2-MUC1 transgenic mice, the findings in the A2-MUC1 transgenic mice being in accord with our previous results; there was no lysis of control P815 or BT20 (HLA-A2$^-$, MUC1$^+$) cells [8,9].

3.2. MUC1 transgenic tumor challenge

The ability of M-FP+VVIL-12 to induce anti-tumor immunity was investigated using the MUC1$^+$ P815 tumor in DBA/2 MUC1 transgenic mice. In the first study, MUC1 transgenic mice immunised ×3 with M-FP, M-FP+VVIL-12 or were challenged with 3.5×10^6 MUC1$^+$ P815 tumor cells SC and the tumor growth measured. Fig. 2a illustrates the tumor growth (±standard error) and shows that M-FP+VV-IL-12 significantly decreased the average tumor size of MUC1$^+$ P815 tumors in MUC1 transgenic mice compared to M-FP alone or controls. These results demonstrate that the cellular immunity induced in M-FP+VVIL-12 immunisations was accompanied by anti-tumor immunity. A proportion of control mice also rejected tumors, ascribed to non-H2 differences between the P815 DBA/2 tumor and the transgenic mice which had been (backcrossed (N8) to DBA/2).

In the second study, MUC1 transgenic mice immunised ×3 with either M-FP, M-FP+VV, M-FP+VVIL-12 or VV IL-12 were challenged with 1×10^7 subcutaneous P815-MUC1 tumor cells, and the number of mice with palpable tumors present after 30 days assessed (Fig. 2b). Tumors were present in 62.5% of MUC1 transgenic mice immunised with M-

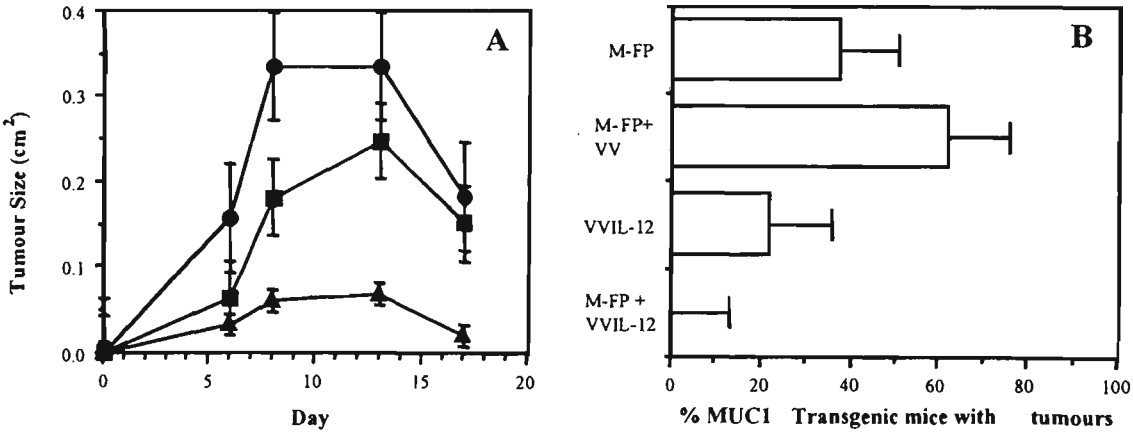

Fig. 2. MUC1 transgenic tumor challenge. (A) MUC1 transgenic mice (5–6/group) were injected with either pH 9.0 buffer (•), 5 μg M-FP (■), or 5 μg M-FP +VVIL-12 (▲) (1×10^7 pfu/ml) on days 0, 7 and 14 and challenged with 3.5×10^6 P815-MUC1 tumor cells on day 22 and tumor growth measured. (B) MUC1 transgenic mice (7–9/group) immunized intraperitoneally on days 0, 7 and 14 with either 5 μg M-FP, 5 μg M-FP+VV (1×10^7 pfu/ml), 5 μg M-FP+VVIL-12 (1×10^7 pfu/ml) or VVIL-12 (1×10^7 pfu/ml) and challenged on day 21 with 1×10^7 P815-MUC1 tumor cells. The number of mice with palpable tumors 30 days later are represented as a percentage of the total number of mice in each group with a tumor (± SE). Fisher's Exact Test was used to determine the statistical significance of the data.

FP+VV, 37.5% of mice immunised with M-FP alone ($p = 0.62$ using Fisher's Exact Test), and 22.2% of mice immunised with only VVIL-12 ($p = 0.153$) demonstrating that neither vaccinia virus or VVIL-12 can induce statistically significant tumour protection alone. However, mice immunised with M-FP+VVIL-12, were totally protected (0% with tumours) from the P815-MUC1 tumor challenge, ($p = 0.025$) compared to control M-FP+VV immunised mice. Thus, M-FP+VVIL-12 immunisation is able to induce tumor protection, in MUC1 transgenic mice.

4. Discussion

IL-12 is a potent enhancer of the T1 cellular immune response as it can enhance cytotoxic T cell function, and induce IFN-γ production [11]. To determine if the T1 response induced by mannan MUC1 M-FP could be increased in inbred BALB/c, DBA/2 MUC1 transgenic and HLA-A*0201 × DBA/2 MUC1 (A2-MUC1) double transgenic mice, IL-12 was incorporated into M-FP immunisations and cytotoxic T cell production and tumor protection measured. IL-12 could increase the CTL responses generated against MUC1 in MUC1 transgenic mice. Initial observations in BALB/c mice demonstrated that after one injection, rIL-12 increased the MUC1 CTLp frequency from 1/76,000 with M-FP to 1/19,000 with M-FP+IL-12. Furthermore, the CTLp response observed after three injections of M-FP (1/7500) was also increased (1/2500) when three injections of M-FP+IL-12 were given. While IL-12 has been shown to increase the cytolytic response in other murine and human models of allogeneic anti-tumor responses [12–14], it has not been used in transgenic models. The CTLp frequency in DBA/2 MUC1 transgenic mice was increased from 1/420,000 to 1/240,000 after a single injection of recombinant vaccinia virus secreting IL-12 was included, and was further increased (from 1/55,000 to 1/19,000) when three injections of M-FP+IL-12 were given. The increase in MUC1 CTL production observed in these transgenic mice after M-FP+IL-12 injections could also be translated into anti-tumor immunity, as immunisations with M-FP+IL-12 significantly decreased the tumor burden of human MUC1 transgenic mice challenged with MUC1$^+$ P815 tumours, compared to either M-FP or IL-12 immunisations alone.

To further examine cytotoxic T cell induction in MUC1 transgenic mice, M-FP+IL-12 was injected into mice transgenic for both human MUC1 and HLA-A*0201. Previous studies with M-FP have shown that HLA-A*0201/K^b transgenic mice immunised with M-FP generate HLA-A*0201 restricted CTLs that recognise MUC1 in association with human HLA-A*0201 [9]. These findings prompted the generation of the HLA-A*0201/K^b × MUC1 (A2-MUC1) double transgenic line to provide a model for investigating and enhancing human MUC1 CTL immune responses in mice to MUC1. Immunisation of A2-MUC1 double transgenic mice with M-FP+IL-12 resulted in the generation of MUC1 specific HLA-A*0201 CTLps which killed the HLA-A*0201 target human MCF-7 mammary breast cancer line, The inclusion of IL-12 in these injections increased the CTLp frequency from 1/74,250 to 1/35,000; a result similar to that generated in MUC1 transgenic mice. Interestingly, the K^b CTLp response in A2-MUC1 double transgenic mice injected (×3) with M-FP+IL-12 (1/78,000) was considerably weaker than the response observed in the MUC1 transgenic mice (1/19,000) following the same injection protocol, albeit stronger than M-FP alone (1/280,000) in the A2-MUC1 transgenic mice, suggesting a bias towards HLA-A*0201 CTL production rather than K^b and H-2^d CTL production in A2-MUC1 transgenic mice. These studies are directly applicable to humans as MCF-7 human tumor cells were lysed in the CTLp studies.

Although the CTLp results from both inbred and MUC1 transgenic mice demonstrate the ability of IL-12 to enhance CTL induction in the M-FP immune response, the mechanisms for IL-12 induced immunity is not clear. We note that IL-12 does not, however, always increase CTLp frequency; and in a recent study [15], the CTLp frequency decreased in patients receiving melanoma peptides plus IL-12, suggesting a loss of CTLp at the tumour site. Whatever the mechanism of IL-12 in MUC1 transgenic mice, the ability of IL-12 to increase the CTL responses and improve the anti-tumor effects is encouraging and forms the basis for clinical trials.

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Cytokine Production from Murine CD4 and CD8 Cells After Mannan-MUC1 Immunization

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ABSTRACT

Immunotherapy with oxidized mannan-MUC1 fusion protein (M-FP) leads to a T1 immune response characterized by the generation of cytotoxic T lymphocytes (CTL), few antibodies, secretion of interleukin-2 (IL-2), IL-12, and interferon-γ and tumor protection. Immunotherapy with reduced M-FP or fusion protein (FP) alone leads to a T2 immune response characterized by the generation of MUC1 antibodies, few CTL, IL-4 secretion, and no tumor protection. In these studies, cytokine production from T cells was measured from cultures containing whole spleens. We now report the cytokine secretion patterns from spleen cells separated into $CD4^+$ and $CD8^+$ T cells obtained from mice immunized with either oxidized M-FP, reduced M-FP or FP, or the simultaneous administration of oxidized M-FP and FP. Immunization with oxidized M-FP led to the secretion of T1 cytokines from $CD8^+$ T cells (IL-2, IFN-γ, and tumor necrosis factor-α [TNF-α]) and from $CD4^+$ T cells (IL-2 and IFN-γ). IL-12 production, presumably from activated macrophages, was observed in $CD8^+$ but not $CD4^+$ cultures. Immunization with either reduced M-FP or FP led to the secretion of predominantly T2 cytokines from $CD4^+$ T cells (IL-4 and IL-10) and IL-2 production in both $CD4^+$ and $CD8^+$ T cell cultures. The simultaneous immunization of both oxidized M-FP and FP led to the production of both T1 and T2 cytokines from $CD8^+$ T cells (IL-2, IFN-γ, and TNF-α) and $CD4^+$ cells (IL-2, IFN-γ, IL-4, and IL-10) and IL-12 production in $CD8^+$ cultures that is, both types of immune responses could occur together. The results demonstrate that the cellular immune response observed in oxidized M-FP-immunized mice is indeed dependent on the T1 cytokine profile secreted by $CD8^+$ T cells, and the simultaneous production of both T1 and T2 cytokines is not cross-inhibitory.

INTRODUCTION

THE ORIGINAL TH1/TH2 PARADIGM described that murine $CD4^+$ T cells produce two types of cytokines, Th1 (T1): interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-β (TNF-β), and Th2 (T2): IL-4, IL-5, IL-6, IL-10, and IL-13.[1–3] Murine $CD8^+$ T cells have now also been subdivided into two subsets, Tc1 and Tc2, following the T1 and T2 cytokines produced.[4] Recently however, studies have emerged demonstrating that a rigid T1/T2 subdivision does not occur for some murine and human T cell clones.[5,6] There are, however, separate functions associated with cytokine production, where T1 cells are primarily involved in cellular cytotoxic and delayed-type hypersensitivity (DTH) immune responses, and T2 cells are involved in antibody and allergic reactions. For the immunotherapy of solid tumors, it is likely that successful therapy will require the *in vivo* production of the T1 cytokines, IL-2, IFN-γ, IL-12, and TNF, either from or associated with activated $CD8^+$ cells and a $CD8^+$ cytotoxic T cell (CTL) response, presumably with $CD4^+$ help. Production of the T2 cytokines, IL-4, IL-5, and IL-10, associated with antibody production, appears to have little effect on solid tumors.[7] It is, therefore, important to document the cytokine profile associated with tumor responses and ensure that the appropriate immune response occurs in vaccine trials where tumor responses may not be as obvious.

Mucin 1 (MUC1) is a membrane-associated glycoprotein (>200 kDa) that has a large extracellular domain stretching far

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(200–500 nm) above the glycocaylx of epithelial cells. MUC1 has the potential to be a target for tumor immunotherapy in the development of vaccines for adenocarcinomas, including that of breast, ovary, and pancreas.[8–10] During cancer, MUC1 expression increases 100-fold, often covering the entire cell surface rather than remaining restricted to the apical surface of secretory cells. New carbohydrate or protein epitopes can appear on the cell surface[8–10] and are immunogenic in both mice and humans, giving both T and B cell responses. [8–10] The protein core of MUC1 contains 40–90 homologous, 20 amino acid repeats (the variable number of tandem repeats [VNTR] region, which is highly immunogenic), with many of the antibodies and T cells reacting with the peptide sequence, APDTR, contained within the VNTR.[8–10]

MUC1-based cancer immunotherapy with mannan and MUC1 can induce either T1 (CTL) or T2 (antibody) immune responses depending on the chemical conjugation of mannan MUC1 fusion protein (M-FP).[8–10] In mice, tumor protection is associated with mannan conjugated to MUC1 FP in the oxidized form (oxidized M-FP), and is characterized by the production of CTL and T1 cytokines (IL-2, IL-12, IFN-γ) from whole spleen cell cultures.[8,9] By contrast, MUC1 FP alone or mannan conjugated to MUC1 FP and subsequently reduced (reduced M-FP) does not protect against MUC1$^+$ tumors, the immune response being characterized by a weak CTL response, MUC1 antibodies, and secretion of the T2 cytokine IL-4.[8] Recently, we also demonstrated that the simultaneous immunization of oxidized M-FP and FP resulted in immune responses whereby both cellular and antibody-mediated responses were induced together[11] rather than the two acting antagonistically. This is perhaps of more importance in infectious disease than in immune responses to tumors.[12] The role that cytokines play in these immune responses has not been examined, other than from whole spleen cultures, and it is not known if a T1/T2 bias exists between the cytokines produced by either CD4$^+$ or CD8$^+$ T cells. To further characterize the T1 and T2 cytokine profiles associated with the M-FP immune responses, CD4$^+$ and CD8$^+$ T cell cultures were established after immunization with oxidized M-FP, reduced M-FP, MUC1 FP, or the simultaneous administration of oxidized M-FP and FP, and the production of IL-2, IL-4, IL-10, IL-12, IFN-γ, and TNF-α cytokines was measured.

MATERIALS AND METHODS

MUC1 antigens

MUC1 FP (five repeats from the VNTR region of the MUC1 protein core) was produced in the pGEX-3X expression system.[13] Purified MUC1 FP was conjugated to oxidized mannan (Sigma, St. Louis, MO)[8] using sodium periodate in pH 9.0 bicarbonate buffer. Reduced M-FP was produced by reacting oxidized M-FP with sodium borohydride (to reduce the Schiff bases and aldehydes to amines and alcohols).[8,14] The peptide Cp13-32 [(C) PAHGVTSAPDTRPAPGSTAP] contains two MUC1 VNTR repeats following N-terminal dimerization and was synthesized (>95% pure) with a peptide synthesizer (Applied Biosystems, Foster City CA).[15,16] Peptide Cp13-32 was used to stimulate spleen cells *in vitro* from MUC1 immunized mice.

Mice and immunizations

Female BALB/c mice aged 6–10 weeks were immunized intraperitoneally with 5 μg of either oxidized M-FP, reduced M-FP, MUC1 FP, or oxidized M-FP and FP given on opposite sides. A control, pH 9.0, bicarbonate buffer was also given. Mice were given three 200 μl immunizations on days 0, 7, and 14 and sacrificed on day 21. Spleen cells from three immunized mice per group were pooled, and the results represent the average cytokine production from three experiments, unless otherwise indicated.

CD4 and CD8 T cell depletions

Spleen cells from immunized mice were collected and pooled in RPMI, treated with 0.83% NH_4Cl to remove red blood cells, washed, and resuspended in complete RPMI medium supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Sydney, Australia), 2 mM glutamine (Sigma), 0.02 M HEPES (Commonwealth Serum Laboratories [CSL], Victoria, Melbourne, Australia), 100 U/ml penicillin, and 100 U/ml streptomycin (CSL). CD4$^+$ T cells were depleted from spleen preparations (resulting in a CD8$^+$ T cell-enriched population) by incubating 5×10^7 washed CD4 (L3T4) Dynabeads (Dynal, Victoria, Melbourne, Australia) per 5×10^7 effector spleen cells in 5 ml RPMI medium for 45 min and rotating at 4°C until rosette formation was evident. CD4$^+$ T cell-Dynabead complexes were removed from the spleen cell population with a magnetic particle concentrator, MPC-1 (Dynal), for 2 min, and the supernatant containing the remaining spleen cells was collected. This method was used to remove CD8$^+$ T cells from spleen preparations (resulting in a CD4$^+$ T cell-enriched population) using CD8 (Lyt-2) Dynabeads.

Flow cytometry and antibodies

Lymphocyte populations were examined by flow cytometry. Fluorescein (FITC)-labeled or phycoerythrin (PE)-labeled antimouse CD3 (PharMingen, San Diego, CA) PE-labeled antimouse CD4 (PharMingen), and PE-labeled antimouse CD8 (PharMingen) monoclonal antibodies (mAb) were used for the analysis. Spleen cells ($2–5 \times 10^5$ cells/ml) were prepared for flow cytometry by incubation with 180 μg/sample heat-aggregated gamma globulin (HAG) for 30 min at 4°C to block nonspecific Fc receptor binding, washed three times with 0.5% bovine serum albumin (BSA)/phosphate buffer, and incubated for 45 min at 4°C with 1 μg/ml antibody and 0.5% BSA/phosphate buffer. Background levels were determined using isotype controls, and spleen cells were incubated with 0.5% BSA/phosphate buffer and no antibody. After washing three times in phosphate buffer to remove excess antibody, the cells were transferred to FACScan tubes for two-color fluorescence analysis. Results were expressed as the mean ± standard deviation (SD) of the data based on several experiments.

T cell cultures

Spleen cells obtained from immunized mice (oxidized M-FP, reduced M-FP, FP, oxidized M-FP + FP, or pH 9.0 buffer) were mixed with synthetic peptide Cp13-32 (90 μg/ml) and irradiated naive BALB/c spleen cells (2×10^6) as antigen-presenting stimulator cells in culture. Each culture contained

TABLE 1. $CD3^+$, $CD4^+$, AND $CD8^+$ T CELL NUMBERS PRESENT IN DEPLETED CULTURES DETERMINED BY FLOW CYTOMETRY

Culture composition	*$CD3^+$ T cells (%)*	*$CD4^+$ T cells (%)*	*$CD8^+$ T cells (%)*	*Other cells[a] (%)*
Nondepleted	47 ± 5[b]	28 ± 4	19 ± 7	53 ± 8
CD4 ($CD8^+$ depleted)	70 ± 7	70 ± 9	<5	28 ± 10
CD8 ($CD4^+$ depleted)	60 ± 7	<5	60 ± 5	37 ± 11

[a]Other cells constitute undepleted B cells, macrophages, and other hematopoietic cells. All controls of unstained cells <5%. FACS profiles represent T cell populations from two experiments.
[b]Mean ± SD.

2×10^6 cells from immunized mice with either (1) whole spleen cells (containing both $CD4^+$ and $CD8^+$ cells), (2) $CD4^+$ cells ($CD8^+$ depleted), or (3) $CD8^+$ cells ($CD4^+$ depleted). Control cultures were (1) effector and stimulator cells without depletion and (2) synthetic peptide Cp13-32 and stimulator cells. Cells were cultured in 24-well flat-bottomed (Costar Corp, Cambridge, MA) in complete RPMI culture medium for 72 h, and supernatants were collected and tested for cytokine production by ELISA.

Cytokine production from cultures

Cytokine assays were perfromed by ELISA (PharMingen) to detect IL-2, IL-4, IL-10, IL-12, TNF-α, and IFN-γ. All ELISA assays were set up in duplicate, and supernatents were tested from three independent experiments. Diluted purified anticytokine capture antibodies (1–4 μg/ml) were coated onto protein binding ELISA plates (Nunc, Roskilde, Denmark) in 50 μl 0.1 M Na_2HPO_4 binding solution, pH 9.0, overnight at 4°C. Plates were washed three times in 0.05% Tween-20/phosphate buffer before blocking in 200 μl 2% BSA/phosphate buffer for 30 min at ambient temperature. After washing, 100 μl sample supernatants and recombinant cytokine proteins were serially diluted and incubated overnight at 4°C. Plates were thoroughly washed and dried on addition of 100 μl of 1–2 μg/ml anticytokine detection antibody and incubated for 1 h at ambient temperature. After washing, 100 μl streptavidin-HRP conjugate (Amersham, Buckinghamshire, UK) (1:500 dilution) was added to the plates, which were incubated for 30 min at ambient temperature before detecting cytokine by adding 100 μl of substrate (0.03% 2,2-azino-di-3-ethylbenzthiazoline sulfonate/0.02% H_2O_2). Absorbance was measured at 450 nm on an ELISA plate reader, and cytokine concentrations were determined from the standard curve. The linear region of each cytokine ELISA standard curve varied between 20–200 pg/ml and 2000–3000 pg/ml. The results were statistically analyzed by analysis of variance (ANOVA). In cultures where cytokines were produced, there was little statistical difference ($p>0.05$) in the amounts in the various cultures, but in most cases where virtually no cytokines were produced, the difference between the secreted and nonsecreted was statistically significant ($p<0.05$). The one exception was with TNFα (Fig. IF).

RESULTS

T cell populations from spleen cultures

Flow cytometry was used to determine the composition of $CD3^+$, $CD4^+$, and $CD8^+$ T cells in spleen cell cultures after Dynabead depletion. Nondepleted cultures consisted of ~47% (± 5%) $CD3^+$ cells, of which 28% (± 4%) were $CD4^+$ and 19% (± 7%) $CD8^+$ cells. The remaining 53% (± 8%) of cells consisted of B cells, macrophages, and other hematopoietic cells. $CD4^+$ T cell-enriched cultures ($CD8^+$ depleted) consisted of ~70% (± 7%) $CD3^+/CD4^+$ cells, <5% $CD3^+/CD8^+$ cells. $CD8^+$ T cell-enriched cultures ($CD4^+$ depleted) consisted of ~60 (± 7%) % $CD3^+/CD8^+$ cells, <5% $CD3^+/CD4^+$ cells (Table 1).

TABLE 2. CYTOKINE PROFILES FROM $CD8^+$ T CELLS AND $CD4^+$ T CELLS FROM M-FP IMMUNIZED MICE DETERMINED BY ELISA

	Oxidized M-FP		*Reduced M-FP*		*FP*		*Oxidized M-FP + FP*		*pH 9.0*	
	CD4	*CD8*	*CD4*	*CD8*	*CD4*	*CD8*	*CD4*	*CD8*	*CD4*	*CD8*
IL-2	+[a]	+	+	+	+	+	+	+	−	−
IL-4	−	−	+	−	+	−	+	−	−	−
IL-10	−	−	+	−	+	−	+	−	−	−
IFN-γ	+	+	−	−	−	−	+	+	−	−
TNF-α	−	+	−	−	−	−	−	+	−	−
IL-12	−	+	−	−	−	−	−	+	−	−

[a] +, cytokine present above detection threshold limit (values >50–100 pg/ml); −, cytokine not present above detection threshold limit (values <50–100 pg/ml). Spleen cells from three immunized BALB/c mice per group were pooled, and the results are the average cytokine production from three experiments.

Cytokine production in T cell cultures from M-FP-immunized mice

Spleen cell cultures from M-FP-immunized mice were either not depleted ($CD4^+ + CD8^+$) or depleted to produce $CD4^-$ ($CD8^+$ enriched) or $CD8^-$ ($CD4^+$ enriched) cultures. T cell cytokine profiles from either (1) oxidized M-FP, (2) reduced M-FP or FP, or (3) oxidized M-FP + FP-immunized mice were compared (Table 2 and Fig. 1). There were few or no cytokines produced in the control, pH 9.0 immunized mice or in cultures in the absence of peptide (0–120 pg/ml). The mean ± SD (pg/ml) is shown in Figure 1; only the means are noted in the text.

IL-2 (Fig. 1A). IL-2 was secreted in similar amounts after immunization with oxidized M-FP in all cell populations; $CD4^+/CD8^+$ (1005 pg/ml), $CD4^+$ (790 pg/ml), and $CD8^+$ (785 pg/ml) cells. When reduced M-FP, FP, or oxidized MFP + FP were used, similar amounts of IL-2 were secreted by $CD4^+/CD8^+$, $CD4^+$, or $CD8^+$ cells (976–1105 pg/ml). Thus, IL-2 is secreted after all modes of immunization and by both $CD4^+$ and $CD8^+$ cells.

IL-4 (Fig. 1B). IL-4 was not made after stimulation with oxidized M-FP (<30 pg/ml) in any cell population but was found in similar amounts after immunization with either reduced M-FP, FP, or oxidized M-FP+FP (presumably from the FP immunization). In the cultures where IL-4 was produced, it was clear that only $CD4^+$ cells made IL-4 (875–1000 pg/ml), as the $CD8^+$ population did not (<112–162 pg/ml) and IL-4 was found in the CD4/CD8 cultures (800–1010 pg/ml), presumably due to the $CD4^+$ cells.

IL-10 (Fig. 1C). IL-10 was not produced by mice immunized with oxidized M-FP (<300 pg/ml) but was found after the other immunizations (reduced M-FP, FP, and both oxidized M-FP and FP together produced similar amounts of IL-10 [~2800 pg/ml]). Only $CD4^+$ cells produced IL-10, and in contrast to the IL-4 results (Fig. 1B), in the presence of $CD8^+$ cells (i.e., the $CD4^+/CD8^+$ cultures), IL-10 was not produced, that is, $CD8^+$ cells inhibited IL-10 production by $CD4^+$ cells.

IL-12 (Fig. 1D). IL-12, a macrophage-derived cytokine, was produced only in cultures derived from mice receiving oxidized M-FP and predominantly by $CD8^+$ cells (700 pg/ml after oxidized M-FP; 1000 pg/ml after oxidized M-FP + FP). However, smaller amounts (380 pg/ml) were detected in the $CD4^+$ (oxidized M-FP) cultures. No IL-12 was detected in mixed $CD4^+$ and $CD8^+$ cultures. As in the IL-10 study, the $CD4^+$ cells inhibited $CD8^+$ cells from producing IL-12.

IFN-γ (Fig. 1E). IFN-γ was produced only from mice immunized with oxidized M-FP (1000 pg/ml) and was made by both $CD4^+$ (780 pg/ml) and $CD8^+$ (700 pg/ml) cells in similar amounts (i.e., present in both of these and in $CD4^+ + CD8^+$ cells).

TNF-α (Fig. 1F). TNF-α was produced only after oxidized M-FP immunization and only in the presence of $CD8^+$ cells (i.e., 990 pg/ml in both $CD8^+$ cultures and $CD4^+/CD8^+$ cells).

Thus, after immunization with (1) oxidized M-FP, the T1 cytokines IL-2, IFN-γ, TNF-α, and IL-12 were produced predominantly by or in the presence of $CD8^+$ cells; (2) reduced M-FP, T2 cytokines IL-2, IL-4, and IL-10 were produced, particularly by $CD4^+$ cells; (3) oxidized M-FP and reduced M-FP give both types of response.

DISCUSSION

Immunization of mice with oxidized or reduced M-FP generates anti-MUC1 immune responses, and we examined the cytokine profiles produced by $CD4^+$ or $CD8^+$ cells in culture.[8,9] In oxidized M-FP-immunized mice, both $CD4^+$ and $CD8^+$ T cells produced T1 cytokines, IL-2 and IFN-γ, but only in the $CD8^+$ T cell cultures were TNF-α and IL-12 produced (IL-12 coming from macrophages). In reduced M-FP or FP-immunized mice, IL-2 was also produced by both $CD4^+$ and $CD8^+$ T cells, and the T2 cytokines IL-4 and IL-10 were produced by $CD4^+$ T cells. The cytokine response to oxidized M-FP was clearly T1, with $CD8^+$ T cells producing IL-2, IFN-γ, and TNF-α and these cultures also producing IL-12. It was of interest that although the *in vivo* response to oxidized mannan-MUC1 is predominantly CD8 dependent, *in vitro* both $CD4^+$ and $CD8^+$ cells made IL-2 and IFN-γ, and only $CD8^+$ cells made substantial amounts of TNF-α and IL-12 (Table 2). As noted previously,[1] the early secretion of IL-2 from activated T cells leads to their differentiation into $CD4^+$ and $CD8^+$ effector T cells secreting both T1 and T2 or Tc1 and Tc2 cytokines. Also, the amounts of some cytokines (e.g., IFN-γ and IL-2) produced by individual cultures of $CD4^+$ and $CD8^+$ splenocytes did not equal the sum of the cytokines secreted in $CD4^+/CD8^+$ bulk cultures. This effect presumably is a result of maximum cytokine secretion in these cultures, which subsequently exceeds the linear region of the ELISA standard curve and, therefore, ELISA sensitivity.

We had reported previously that immunization with both oxidized M-FP and FP induced both cell-mediated immunity and antibody immune responses simultaneously.[11] In this study, cytokine profiles obtained from the T lymphocytes of mice immunized with oxidized M-FP and FP also showed the production of both T1 and T2 cytokines. $CD8^+$ T cells produced IL-2, IFN-γ, and TNF-γ, and, $CD4^+$ T cells produced IL-2, IL-4, IL-10, and IFN-γ, demonstrating that both subsets of T cells are independently activated by stimulation with MUC1 antigen. In this environment, IL-4/IL-10 and IFN-γ did not induce antagonistic T1 and T2 cytokine pathways, as previously reported.[17–19] In a similar *in vivo* study, both $CD4^+$ and $CD8^+$ T cells were shown to be induced concurrently during tumor growth, and both subsets were functionally efficacious in tumor-bearing hosts,[20] again demonstrating a direct role for collaboration between both subsets of activated T cells in tumor immunotherapy studies.

An interesting finding was the production of IL-12 in $CD8^+$ T cell cultures from mice immunized with oxidized M-FP or with the combination of oxidized M-FP and FP. As IL-12 was detected only in the supernatants of oxidized M-FP immunized mice (both alone and in combination with FP) and not in reduced M-FP or FP cultures, IL-12 production in this system is $CD8^+$ dependent and presumably is produced by activated macrophages (Apostolopoulis, submitted, 1999) or dendritic cells[21] following oxidized M-FP immunization. Unlike with IL-12 production, TNF-α production occurred in both $CD8^+$ T

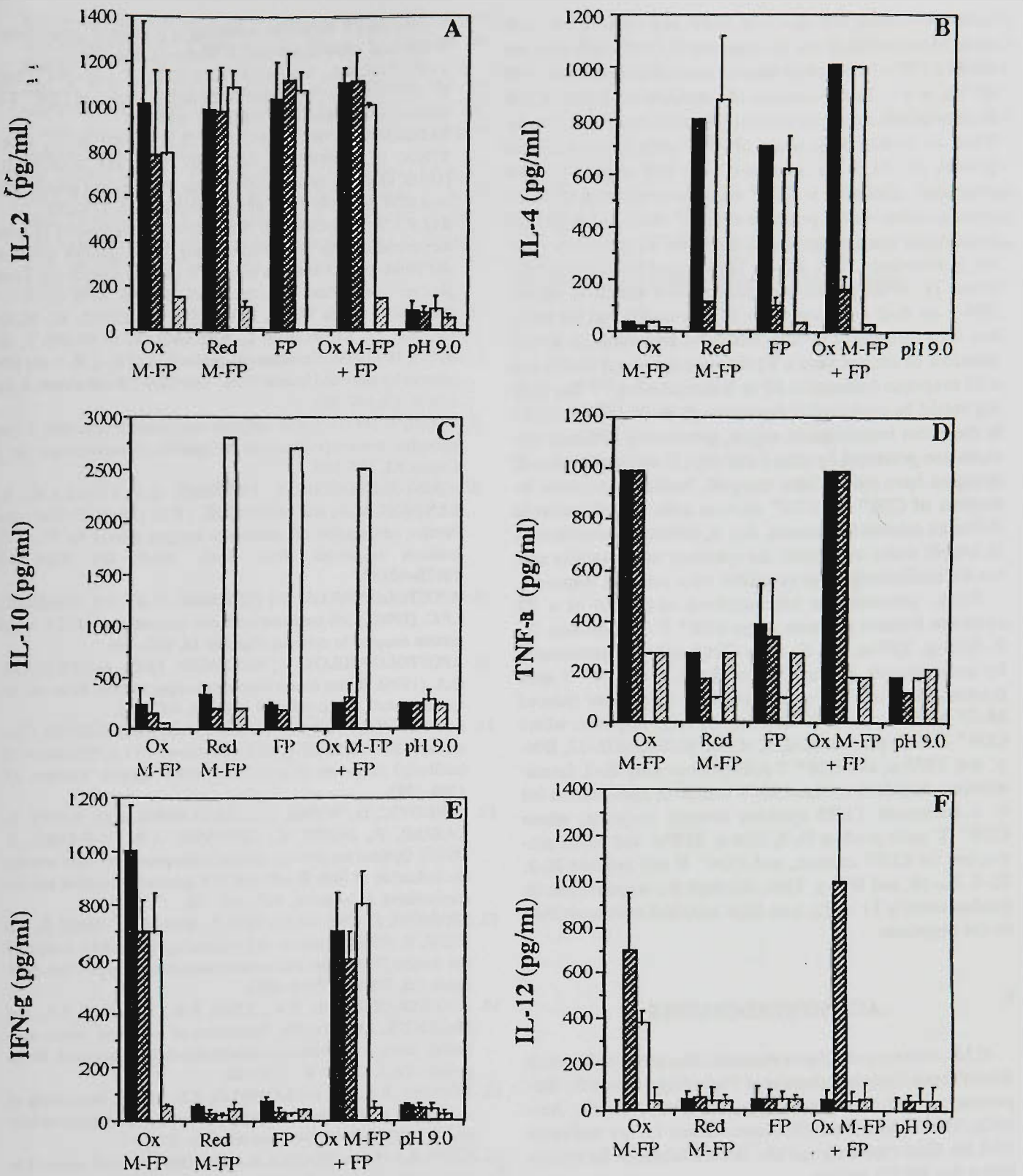

FIG. 1. ELISA assays showing cytokine production (mean ± SD) after 72 h from depleted BALB/c spleen cultures. $CD4^+/CD8^+$ T cell (■), $CD4^-$ T cell ($CD8^+$) (▨), and $CD8^-$ T cell ($CD4^+$) (□) cultures stimulated with 20 ng/ml Cp13-32 peptide, from mice immunized with either oxidized (Ox) M-FP, reduced (Red) M-FP, FP, and oxidized M-FP + FP. Mice injected with phosphate buffer and cultures incubated only with phosphate buffer and no peptide (▨) were used as controls. ELISA graphs representing (**A**) IL-2, (B) IL-4, (C) IL-10, (**D**) IL-12, (**E**) IFN-γ, and (**F**) TNF-α production from *in vitro* cultures. Spleen cells from three immunized mice per group were pooled, and the results are the mean ± SD cytokine production from independent experiments.

cells immunized with oxidized M-FP and $CD8^+/CD4^+$ cultures, demonstrating that the presence of $CD4^+$ cells does not inhibit TNF-α production when in combination, whereas with IL-12, it did. This interaction of cytokines is of interest and further demonstrates the relationship of T1 and T2 responses. Thus, *in vitro* in the presence of $CD4^+$ cells (presumably via IL-4 or IL-10), lower amounts of both TNF-α and IL-12 are produced. Similarly, in $CD4^+$ cells the production of IL-10 was decreased in the presence of $CD8^+$ cells, although IL-4 production was not decreased. Thus, the T1 cytokines present (particularly IL-2, IFN-γ, TFN-α, and IL-12) could decrease IL-10 and served to decrease TFN-α and IL-12 but not IFN-γ or IL-2. However, these *in vitro* results did not translate into differences *in vivo,* where the simultaneous administration of antigen gave a T1 response (oxidized M-FP) and a T2 response (reduced M-FP or fusion protein).[11] The finding could be explained in that although the VNTR of MUC1 is the most immunogenic region, presumably different epitopes are presented by class I and class II molecules (class II epitopes have not yet been mapped), leading to separate induction of $CD8^+$ and $CD4^+$ immune cells. If these occur in different microenvironments, that is, different anatomic sites in lymph nodes and spleen, the cytokines acting locally may not be sufficiently close to inhibit other immune responses.

Thus, immunization with oxidized M-FP led to a T1 cytokine immune response where $CD8^+$ T cells produce IL-2, IFN-γ, TNF-α, and IL-12 in $CD8^+$ cultures, presumably by macrophages, and no IL-4 or IL-10, and $CD4^+$ T cells produce IL-2 and IFN-γ. Immunization with either reduced M-FP or FP led to a T2 cytokine immune response, where $CD4^+$ T cells produce IL-2, IL-4, and IL-10 and IL-12, IFN-γ, and TNF-α, and $CD8^+$ T cells produce only IL-2. Immunization with both oxidized M-FP and FP in combination led to a combined T1/T2 cytokine immune response, where $CD8^+$ T cells produce IL-2, IFN-γ, TNF-α and IL-12 production in $CD8^+$ cultures, and $CD4^+$ T cell produce IL-2, IL-4, IL-10, and IFN-γ. Thus, although the response can be predominantly T1 or T2, both CD4 and CD8 cells contribute to the response.

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A role for IL-5 in the induction of cytotoxic T lymphocytes in vivo

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IL-5 is generally regarded as a Th2 cytokine involved in eosinophil maturation and function and in B cell growth and antibody production, but without any well-established effects on T cells. Early reports suggested that IL-5 could stimulate the production of cytotoxic T lymphocytes (CTL) *in vitro*, but no evidence has been obtained to date for such a role in studies with IL-5-deficient (IL-5$^{-/-}$) mice. Here we demonstrate that when oxidized mannan MUC1 fusion protein (M-FP) is used as an antigen in mice, IL-5 is required for the optimal generation of the CTL response. IL-5 was as effective as IL-2 for the induction of CTL from spleen cells *in vitro* and both CD4$^+$ and CD8$^+$ T cells from M-FP-immunized animals could be shown to secrete IL-5 in culture. In IL-5$^{-/-}$ mice, CTLp frequency was greatly diminished resulting in the inability to reject MUC1$^+$ tumors. Clearly, IL-5 is produced by functional T cells, especially the Tc1 type, after M-FP immunization and is required for an optimal CTL response to this antigen.

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1 Introduction

It is generally accepted that CD4$^+$ and CD8$^+$ T cells secrete both Th1 (IL-2, IFN-γ and TNF-α) and Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13) cytokines. IL-5 has been considered to be a typical Th2 cytokine and to act as a differentiation factor and regulator for eosinophils and to be involved in B cell growth and in antibody production (IgA, IgM and IgE). There are many reviews on the role of IL-5 as a Th2 cytokine, concentrating on both *in vitro* and *in vivo* studies [1–3]. In particular, there has also been much progress in defining the biological role of IL-5 *in vivo*. For example, mice transgenic for IL-5 under a metallothionein promoter have increased number of CD5$^+$ (B-1) B cells, develop eosinophilia and have high levels of autoantibodies [4]. However, mice transgenic for IL-5 under the control of the lymphocyte-specific CD2 promoter showed no effects on B cells or antibody levels, although they developed eosinophilia [5]. Further studies on the biological role of IL-5 in B cell development and on eosinophils were stimulated by the development of IL-5 gene knockout mice (IL-5$^{-/-}$). Such mice lacking IL-5 did not develop eosinophilia when infected with the helminth *Mesocestoides corti* and had a conventional development of B (B-2) cells, cytotoxic T cells (CTL), T cell-dependent antibody responses and NK cell activity [6]. The number of CD5$^+$ (B-1) B cells, however, was reduced in 2-week-old mice, but returned to normal by 6–8 weeks [6]. More recent studies with IL-5$^{-/-}$ mice indicate a continuing reduction in B-1 lymphocytes and in mucosal IgA production [7]. Similar findings were obtained with mice deficient in the IL-5 receptor α [8]. Thus, the studies to date with transgenic animals support a key role for IL-5 in the regulation of eosinophilia and an involvement in B-1 cell development and in antibody production. Several early reports [9, 10] showed that IL-5 could stimulate CTL production *in vitro*, but no evidence for such a role *in vivo* in relation to anti-viral CTL responses has come from studies with IL-5$^{-/-}$ mice. Clearly it is necessary to obtain data *in vivo* since *in vitro* activities are generally not accurate predictors of the biological role of cytokines.

We have been working with MUC1, a large cell surface molecule which contains a 20-amino acid repeat in the extracellular region (the VNTR), which is repeated ~40 times in different alleles [11]. MUC1 is greatly increased in breast cancer and could, therefore, provide a

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Abbreviations: CTL: Cytotoxic T lymphocytes **CTLp:** Cytotoxic T lymphocyte precursor **FP:** MUC1 fusion protein containing 5 VNTR repeats **M-FP:** Mannan coupled to MUC1 FP, either in oxidized or reduced form

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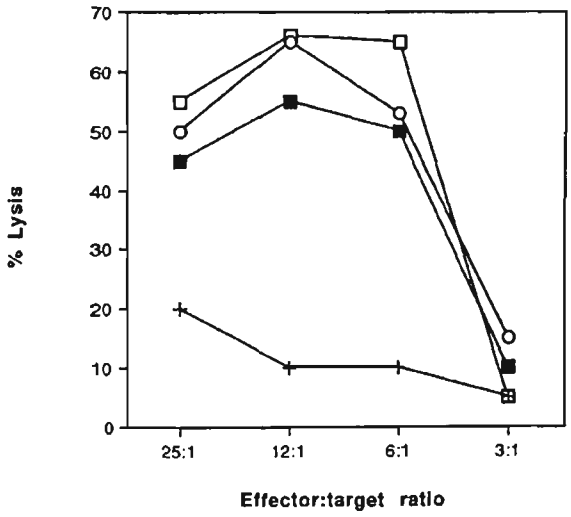

Fig. 1. CTL assay using ^{51}Cr-labeled RMA-MUC1 from C57BL/6 mice immunized with oxidized M-FP. *In vitro* restimulation with MUC1 peptide in the presence of either IL-2 (□), IL-5 (○), IL-2+IL-5 (■) or with no cytokine (+). RMA cells were not lysed.

possible target for CTL [11]. MUC1 is highly immunogenic in mice, both for antibody and for CTL production [12–19]. We have previously demonstrated that conjugation of MUC1 VNTR peptide (or as a 5-VNTR-repeat fusion protein) to oxidized mannan, generates in mice (DBA/2, BALB/c, C57BL/6), high CTL precursor (CTLp) frequency, tumor protection, and low antibody (IgG2a), and predominant Th1 responses generated with secretion of IFN-γ, IL-2 and IL-12 but no IL-4 [13–19]. In contrast, conjugation of MUC1 to reduced mannan, generates in mice low CTLp, no tumor protection, and high antibody (IgG1), and predominant Th2 cytokines with IL-4 and IL-10 production by T cells [13–19]. Both conjugates of mannan-MUC1 target the mannose receptor on antigen-presenting cells [20, 21]; oxidized mannan-MUC1 leading to fast tracking to the MHC class I pathway and reduced mannan-MUC1 predominantly to the MHC class II pathway.

In the present work we have used IL-5$^{-/-}$ mice to demonstrate that IL-5 plays a significant role in the CTL response to the modified tumor antigen oxidized mannan MUC1 (M-FP) and have shown that in the absence of IL-5 the CTL response is insufficient to enable MUC1$^+$ tumor rejection. These findings indicate that an IL-5-dependent mechanism exists for the generation of CTL and suggest a broader role for IL-5 than previously envisaged.

2 Results

2.1 Effect of IL-5 on *in vitro* stimulated CTL

Spleen cells from C57BL/6 mice immunized with oxidized M-FP were restimulated *in vitro* for 7 days in the presence of immunizing peptide, with the addition of either IL-2, IL-5 or both together. Effector cells were then mixed at various E:T ratios with RMA-MUC1$^+$ cells and cell lysis measured. *In vitro* RMA target cells were negative. *In vitro* stimulation in the absence of either IL-2 or IL-5 gave little lysis, but in the presence of IL-2, CTL were effective, giving ~80% lysis at a 6:1 ratio. However, when IL-5 was used instead of IL-2, virtually the same CTL profile occurred, *i.e.* IL-5 was as effective as IL-2 for the *in vitro* generation and expression of CTL (Fig. 1). It is of interest that the simultaneous addition of both IL-2 and IL-5 gave no additive effects, suggesting that the two cytokines act upon the same pathway. It would be of interest to determine what effects anti-IL-2 would have if added to the IL-5-supplemented cultures. However, the results clearly suggest that IL-5 in this system is involved in the induction/expression of CTL, an activity similar to that reported earlier [9], but in this case IL-5 alone is sufficient with no requirement for the presence of IL-2.

2.2 CTLp frequency in IL-5$^{-/-}$ mice

To further examine the role of IL-5 in CTL induction, CTLp frequency assays were performed in normal wild-type C57BL/6 and B6.IL-5$^{-/-}$ mice immunized with oxidized M-FP. The results demonstrate (Fig. 2) that one injection of M-FP gave a CTLp frequency 1/70,000 in C57BL/6 mice; however, in IL-5$^{-/-}$ mice the frequency was reduced to 1/170,000. When three injections of M-FP were given, there was frequency of 1/10,000 in C57BL/6 mice and of 1/80,000 in IL-5$^{-/-}$ mice (Fig. 2). Clearly, the presence of IL-5 significantly enhances the precursor frequency and with this antigen IL-5 plays an obligatory role in the generation of the CTL response.

2.3 Cytokine production of T cell cultures from mice immunized with M-FP

Spleen cell cultures from mice immunized with oxidized M-FP were selectively depleted of CD4 or CD8 cells to produce (i) CD8$^+$ (ii) CD4$^+$ (iii) CD4$^+$/CD8$^+$ or (iv) non-depleted bulk cultures, and cytokine profiles were measured after *in vitro* culture with antigen. IL-5 was produced in significant quantities in all the cultures; by bulk cultures (900 pg/ml); by CD4$^+$/CD8$^+$ cells (1,060 pg/ml); and by both CD4$^+$ (1,080 pg/ml) and CD8$^+$ (2,000 pg/ml) cell cultures (Fig. 3), *i.e.* both CD4$^+$ and CD8$^+$ T cells pro-

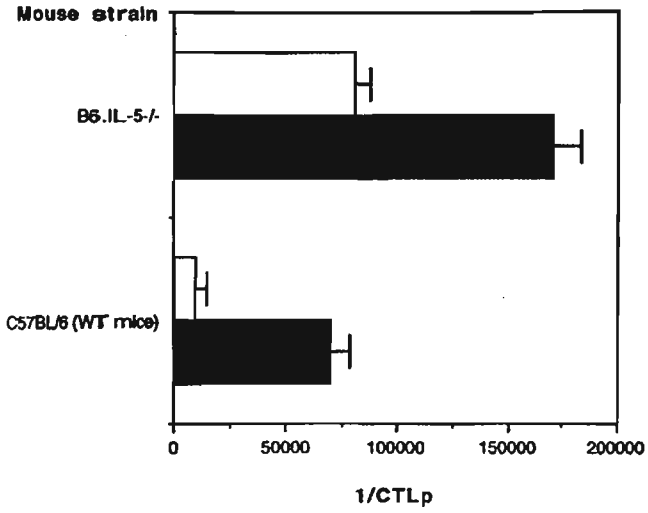

Fig. 2. CTLp frequencies detected in spleen cell cultures from C57BL/6 (wild-type) or B6.IL-5$^{-/-}$ mice, immunized with oxidized M-FP, once (■) or three times (□). Target cells were RMA-MUC1$^+$; RMA cells gave a CTLp frequency of >1:10^6. The results are an average of three to four individual mice.

duced IL-5. Other cytokines were measured and the Th1 cytokines IL-2, TNF-α and IFN-γ were also produced, but the Th2 cytokines, IL-4 or IL-10 [17] were not detectable. When mice were immunized with reduced M-FP (oxidized M-FP exposed to sodium borohydride, which reduces Schiff bases to amines and aldehydes to alcohols), which had previously been identified as inducing

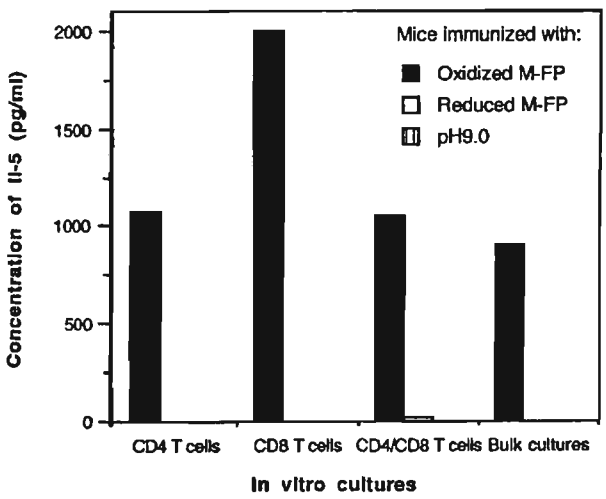

Fig. 3. ELISA assay for *in vitro* cytokine production after 72 h in culture of splenic, CD4$^+$/CD8$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells stimulated with 90 μg/ml MUC1 peptide, and prepared from mice immunized with either oxidized and reduced M-FP or with control phosphate buffer.

Th2 responses [13–17, 20], IL-5 was not present (Fig. 3) and in this case the Th2 cytokines IL-4 and IL-10 were present in these cultures [17]. In control mice immunized with phosphate buffer no cytokines produced. Thus, oxidized M-FP induced a predominantly Th1 response in both CD4$^+$ and CD8$^+$ cells but this also included the production of IL-5.

2.4 Role of IL-5 in the rejection of tumors

MUC1$^+$ tumors in mice are rejected by CD8$^+$ cells and there is a direct correlation between the control of tumor growth and the numbers of CTL [13–15, 18, 19]. C57BL/6 mice immunized with oxidized M-FP and challenged with syngeneic RMA tumor cells grew progressively and were not rejected; in IL-5$^{-/-}$ mice, the RMA tumors also grew progressively, but more rapidly (Fig. 4). When C57BL/6 mice were immunized with oxidized M-FP and challenged with RMA-MUCI tumors, the tumors grew but were then rejected, due to the effective CTL-immune response to MUCI. However, in the IL-5$^{-/-}$ mice the RMA-MUC1 tumors grew progressively and were not rejected (Fig. 4). Previous studies had indicated that a certain level of CTLp were required for tumor rejection (>1/20,000), and there was a complete correlation (r^2=0.982) between the CTLp frequency and tumor rejection using various MUC1 formulations [15, 18]. The CTLp

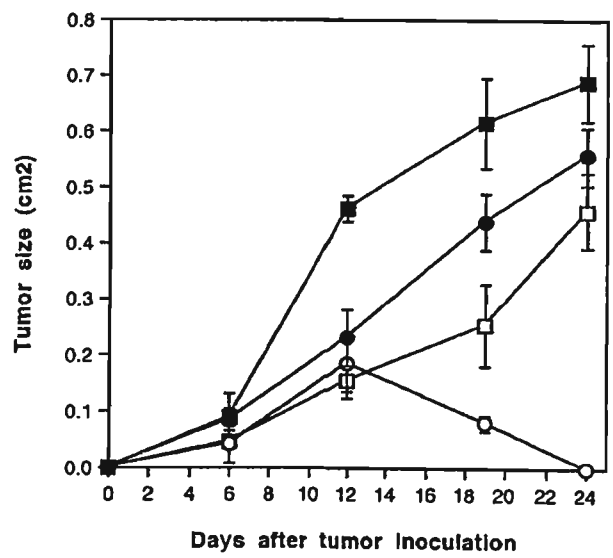

Fig. 4. Growth of RMA or RMA-MUC1$^+$ tumors in IL-5$^{-/-}$ and C57BL/6 (wild -type, WT) mice. Mice were immunized with oxidized M-FP and IL-5$^{-/-}$ mice challenged with RMA (■) or RMA-MUC1 (●); wild-type mice challenged with RMA (□) or RMA-MUC1 (○). The mean size of the tumors (the product of two diameters) is shown (vertical axis) with standard deviation (n=7), and days after tumor inoculation (horizontal axis).

frequency gave a clear and quantitative measure of the rapidity and nature of tumor rejection; thus, CTLp frequency can used as a readout for *in vivo* efficacy [18]. Since from the studies cited above on CTLp, the immunization of IL-5$^{-/-}$ mice with oxidized M-FP only gave a frequency of 1/80,000, tumor graft rejection would not be expected. Thus, IL-5 has direct effects on the numbers of CTL and CTLp, and on tumor graft rejection.

3 Discussion

Although the involvement of IL-5 in the generation of CTL was suggested by earlier studies *in vitro* [9, 10], this is the first demonstration of a role for IL-5 in a CTL response *in vivo*. Our studies indicate that IL-5 is involved in the induction of CTL. Firstly, addition of IL-5 to *in vitro* cultures could effectively replace IL-2 for CTL induction; secondly, IL-5$^{-/-}$ mice had a decreased frequency of CTLp in comparison to C57BL/6 mice and, unlike the parental mice, could not reject MUC1$^+$ tumors. In addition, both CD4$^+$ and CD8$^+$ populations isolated from mice immunized with oxidized M-FP could produce IL-5. Thus, IL-5 is required for CTL maturation and is produced *in vitro* by CD4$^+$ and CD8$^+$ T cells derived from mice immunized with oxidized M-FP (which is presented by MHC class I molecules) but not those immunized with reduced M-FP. The defect in CTL maturation caused by IL-5 deficiency is unlikely to be due to an indirect effect on Th cell cytokine production as previous studies [6] have shown that stimulated T cells from IL-5$^{-/-}$ mice produce normal levels of a range of cytokines including IL-2, IL-3, IL-4, IL-10 and IFN-γ.

The fact that IL-2 and IL-5 were equally effective in CTL induction *in vitro* with no additive effects suggests that both cytokines may be stimulating the same pathway. Since it is well established that the effect of IL-2 is directly on the T cells, it seems likely that the effect of IL-5 will be analogous to that of IL-2 and not on antigen presentation. Since the experiments with IL-5$^{-/-}$ mice indicate that IL-5 plays an obligatory role in the CTL response to M-FP, it is possible that the effect of IL-2 in this case may be via the induction of IL-5. Of interest in this regard are reports that IL-2 can induce IL-5 in mice [22] and in patients undergoing treatment with recombinant IL-2 [23].

However, the important aspect of the present results is the demonstration of a role for IL-5 in the generation of a CTL response to immunization with M-FP *in vivo*. This is in contrast to a previous study which showed that antiviral CTL induction was normal in the IL-5$^{-/-}$ mice [6]. This suggests the existence of IL-5-dependent and -independent mechanisms for CTL maturation, which are regulated according to the type of immune response elicited. It is already known that IL-5 is produced in a limited subset of immune responses, namely those relating to infections with parasitic helminths and to allergies. Possibly a similar limited subset of CTL responses will be IL-5 dependent. IL-5-dependent and -independent mechanisms also appear to regulate the B-1 lymphocyte lineage.

The subdivision of T helper cells into two classes (Th1 and Th2) was originally described for murine CD4$^+$ T cell cultures. The Th1 subclass secreted cytokines IL-2, IFN-γ and TNF-α and the Th2 subclass, IL-4, IL-5, IL-6, IL-10 and IL-13 [1, 3]. More recently, murine CD8$^+$ T cells have also recently been subdivided into similar subsets, Tc1 and Tc2 [24]. The distinctive cytokine profiles of the two classes are believed to be associated with distinctive functions whereby Th1 cells are primarily involved in cytotoxic (CD8) and DTH (CD4) responses, and Th2 cells with antibody and allergic reactions. In all of these studies it has been noted that IL-5 is mainly produced by CD4$^+$ Th2 cells and partly by CD4$^-$CD8$^-$ cells, but not by CD8$^+$ cells such as in mice infected with *Toxocara canis* [25]. However, the present study shows that IL-5 is produced by both CD4$^+$ and CD8$^+$ T cells from mice immunized with oxidized M-FP, an antigen which produces a typical Th1 response, and not by mice immunized with reduced M-FP, which produces a Th2 type response.

The results of the present work highlight the fact that cytokine production in immune responses is not always purely of the Th1 or Th2 type. In this case, immunization with oxidized M-FP results in IL-5 production but the rest of the response is typically Th1. Other Th2 cytokines such as IL-4 and IL-10 were not detected.

A role for IL-5 in the generation of CTL was noted first in 1987 and it was also shown that IL-5 was required to obtain effective CTL function at different stages of T cell differentiation [9, 10]. In addition, earlier studies suggested that a non-IL-2 cytokine was required for the generation of CTL [26], possibly this was IL-5. However, more recently interest has centered on the role of IL-5 in regulating B-1 cells and antibody production, in the growth and activity of eosinophils, and especially in their trafficking to sites of allergic inflammation in mouse asthma models [27]. In IL-5$^{-/-}$ mice, there are basal levels of eosinophils produced which appear to be morphologically and functionally normal. However, in the absence of IL-5 the pronounced eosinophilia characteristic of infections with helminth parasites and of allergic lung disease is not produced [6]. Eosinophil trafficking is also dysfunctional since IL-5 acts as a cofactor with chemokines like eotaxin [28]. Lack of IL-5 reduces host defense against some intestinal nematodes [29]. Most of the

effects of IL-5 with respect to parasite infections have been interpreted in terms of effects on eosinophils. However, the demonstrated effects of IL-5 on B-1 cells in previous studies [6, 7] and on CTL production in the present work means that consideration of other effects of IL-5 may need to be made when interpreting the responses of IL-5$^{-/-}$ mice.

The present work provides a clear indication of a role for IL-5 in the generation of CTL *in vivo* as part of the immune response to M-FP. This observation provides the rationale for examination of possible IL-5 involvement in a range of other CTL responses in future studies. Further studies of the role of IL-5 in the generation of a functional cytotoxic response to tumors may provide opportunities for enhancement of such responses in cancer.

4 Materials and methods

4.1 Antigens, tumors and immunization of mice

Human MUC1 was produced as a GST-fusion protein, which contained five VNTR repeats, and consisted of 105 amino acids (FP), and was conjugated to mannan using oxidizing conditions (oxidized-mannan-MUC1; M-FP) [13, 14, 30]. Briefly, mannan (Sigma, St. Louis, MO) was oxidized to a polyaldehyde by treating 14 mg mannan in 1 ml 0.1 M phosphate buffer pH 6.0 with 100 µl 0.1 M sodium periodate in phosphate buffer for 1 h at 4°C. Following a further 30-min incubation at 4°C with 10 µl ethanediol, the mixture was passed through a PD-10 column (Sephadex G-25 M column, Pharmacia Biotech, Sweden) and the mannan fraction collected; 1 mg MUCI FP was added to the oxidized mannan and reacted overnight at room temperature (M-FP). For reduced M-FP, the Schiff bases and aldehydes of the oxidized M-FP complex were reduced to amines and alcohols with 1 mg sodium borohydride overnight at room temperature. Synthetic MUC1 20-mer peptides of sequence (PDTRPAPGSTAPPAHGVTSA) were synthesized using an ABI peptide sequencer.

IL-5$^{-/-}$ mice (C57BL/6 background) [6] were generated and bred at The John Curtin School of Medical Research. B6.IL-5$^{-/-}$ mice [6] or the parental C57BL/6 strain were immunized i.p. with oxidized or reduced M-FP conjugate (containing 5 g FP) either once, or weekly for 3 weeks. We had previously demonstrated that the optimal route of immunization is i.p. (although i.v., i.d. and i.m. immunization generated similar responses), and 5 µg was shown to be optimal in generating CTL responses [18]. The number of injections was also important for CTL generation, three injections was found to be optimal, more or fewer injections generated weaker CTLp frequencies [18].

RMA-MUC1 cells [31] were obtained from Dr. J. Burchell, (ICRF, London, GB). Groups of seven mice immunized with oxidized M-FP were challenged 7 days after the final immunization with a 0.2-ml subcutaneous injection of 5×10^6 RMA-MUC1 tumor cells in PBS and subsequent tumor growth measured with dial gauge calipers (Schnelltaster, H. C. Kroplin, Hessen, Germany) and the size of the tumors were expressed as the product of the two perpendicular parameters.

4.2 CTL assay

Spleen cells from mice immunized with M-FP (either once or three times i.p.) were obtained 7 days after the third immunization, washed in PBS containing 2% fetal calf serum, and 2×10^6 cells/ml seeded in 24-well tissue culture plates. Nonimmune spleen stimulator cells (as antigen-presenting cells) were irradiated with 3,000 rad (Gammacell 1000 elite irradiator; Nordion International) and 2×10^6 cells/ml (stimulators) were added to the effector cells; 90 µg/ml of the 20-mer MUC1 peptide (PDTRPAPGSTAPPAHGVTSA) and 10 U/ml recombinant IL-2 or IL-5 (PharMingen, CA) were added. After 1 week of restimulation, effector cells were harvested and mixed at various E:T ratios with ^{51}Cr-labeled RMA-MUC1 cells [13–19]. RMA (non-MUC1) tumor targets were not lysed in any experiments.

4.3 CTLp frequency analysis

Mice immunized once or three times with oxidized M-FP were killed 2 weeks after the final injection. For each cell suspension in which CTLp frequencies were determined, a minimum of 32 replicates for each of at least six effector cell doses (ranging from 1×10^3 to 5×10^5 cells/well; cells were taken from the spleen) were cultured in U-bottom microtiter trays, with 5×10^5 C57BL/6 spleen cells irradiated with 3,000 rad (stimulator cells; antigen-presenting cells), in modified Eagle's medium supplemented with 10% fetal calf serum, 90 µg/ml synthetic MUC1 peptide (C-PAHGVTSAPDTRPAPGSTAP) and 10 U/ml recombinant human IL-2. Seven days later, each microculture was assayed for cytotoxicity by replacing 100 µl of culture medium with 100 µl target cell suspension containing 10^4 ^{51}Cr-labeled RMA-MUC1 or RMA tumor target cells. Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release + 3 SD above the mean release from 10^4 effector cells cultured alone, or 10^4 effector cells and 5×10^5 stimulators together or stimulators and peptide and rIL-2 together. A

linear relationship existed between the dose of effector cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative wells [32, 33]. Each experiment was performed three to four times.

4.4 T cell cultures and cytokine production

Spleen cells from immunized mice [13–16] were collected and pooled in RPMI. B cells were depleted by incubating 5×10^7 PanB (B220) Dynabeads (Dynal, Victoria, Australia) in 5 ml RPMI rotating for 45 min at 4 °C. Supernatants were collected. $CD4^+$ T cells were removed using 5×10^7 CD4 (L3T4) Dynabeads ($CD8^+$ population). $CD8^+$ T cells were removed using 5×10^7 CD8 (Lyt-2) Dynabeads ($CD4^+$ population). The separated populations were shown to be rich in $CD4^+$ or $CD8^+$ cells by flow cytometry [17]. $CD4^+$ T cell-enriched cultures [$CD4^+$ ($CD8^+$ depleted)] consisted of ~70% $CD3^+/CD4^+$ cells, <5% $CD3^+/CD8^+$ cells; $CD8^+$ T cell-enriched cultures [$CD8^+$ ($CD4^+$ depleted)] consisted of ~60% $CD3^+/CD8^+$ cells, <5% $CD3^+/CD4^+$ cells [17]. The remaining cells in both $CD4^+$ and $CD8^+$ cultures consisted of B cells, macrophages and other hematopoietic cells.

Cells (2×10^6) from mice immunized with oxidized M-FP, reduced M-FP or pH 9.0 control buffer were mixed with MUC1 peptide (20 μM) and 2×10^6 cells (naive spleen cells, as stimulator cells) irradiated using 3,000 rad. Each culture was separated into either bulk spleen cells, $CD4^+$ cells, $CD8^+$ cells or $CD4^+CD8^+$ cells. Cells were cultured for 72 h and supernatants collected and tested for IL-5 production. Cytokine assays were performed by ELISA using commercial kits (Endogen, USA) as previously described [17].

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Tumour immunotherapy against MUC1 expressing tumours using mannan MUC1

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Introduction

Tumour immunotherapy is now attracting much attention due entirely to the genetic engineering techniques now available, which lead to:- a) the capacity to produce large amounts of synthetic and recombinant materials such as tumour antigens; b) the description and availability of cytokines; c) the identification, using gene cloning techniques of genes encoding tumour antigens; d) the identification of peptides presented by Class I molecules which serve as targets for cytotoxic T cell lymphocytes (CTLs). This is real progress, and we should now be optimistic that a substantial impact in cancer therapy will be made by any or all of these advances. In this light, we are studying Mucin 1 (MUC1) - a ubiquitous mucin, which, in the normal state is found either intracellularly or in the lining of ducts, and in secretions such as breast milk. In the malignant state, MUC1 is found in very large amounts throughout the cell, and on the cell surface, and in which there is abherant glycosylation exposing part of the protein core - which could serve as a target for antibody or non-MHC restricted killer cells [1]. We presume that the massive increase in mucin production by tumour cells should lead to some of the excess MUC1 peptides synthestised and find their way into the Class I MHC groove and be presented to CTLs. Thus, MUC1 should theoretically be a good target for immunotherapy. Two observations lend weight to the concept of targeting MUC1 in immunotherapeutic studies. Firstly, Mucin 1 is highly immunogenic - at least for antibody production in mice, and the amino acids APDTR of the repeat region are the most immunogenic of the whole MUC1 molecule, as monoclonal antibodies made to tumours, mucins such as HMFG or indeed synthetic peptides recognise APDTR [2]. Secondly, Dr O. Finn has found in the lymph nodes of patients with breast cancer can be found CTL precursors which, on stimulation *in vitro*, lead to CTLs which have non-MHC restricted activity; the target of these cells also appears to be the APDTR sequence [3]. On this basis, we and others are determining how is the best way to increase the immune response to MUC1, particularly the VNTR peptides (containing APDTR) and now describe the use of mannan MUC1 conjugates which are immunogenic in mice, monkeys and humans. The results of the studies are summarised in *Table I.*

Murine immune response to MUC1 peptides

As indicated above, synthetic peptides were able to immunise mice for anti-MUC1 antibody production - could they be used to induce cellular immunity and anti-tumour effects? We made a MUC1 synthetic peptide (Cp13-32) containing 20 amino acids of MUC1 (which spontaneously dimerises due to the formation of disulphide bonds, linked this to either KLH, or diphtheria toxoid and immunised mice. As before, antibodies were produced and cellular immunity was demonstrated by $CD4^+$ mediated DTH reactions [4]. Anti-tumour immunity examined by challenging mice with human $MUC1^+$ tumours (induced by transfecting mouse tumours with *MUC1* gene) indicated some anti-tumour effects with protection with low dose of tumour challenge (10^6 cells) [4]. This protection disappeared when 5 times the dose was used. With this immunisation protocol, no CTLs were produced. In this particular model, mice ultimately rejected $MUC1^+$ tumours (in non-immunised mice) and produced weak antibody responses, DTH and CTLs (revealed only after deglycosylation of target antigens) *(Table 1)*. On the basis of the anti-tumour responses (albeit weak) after peptide immunisation, a clinical trial was performed.

Immunogenicity of human MUC1 peptides in humans

A Phase I trial was performed using increasing doses of MUC1 peptides 150-1,000 micrograms in 13 patients, seeking evidence of toxicity, autoimmunity, and immunogenicity. For these studies, diphtheria toxoid was used as a carrier and the only toxicity noted were the severe DTH reactions to the carrier after multiple immunisations. There was no evidence of autoimmunity (which could occur due to anti-MUC1 responses in salivary gland, lung, kidney, pancreas and elsewhere - sites of MUC1 expression). There was weak immunogenicity, with small amounts of antibody produced and T cell proliferation in some of the patients [5]. No anti-tumour responses were noted, although these were not formally sought. Our conclusions were that the peptides were weakly immunogenic, but not sufficiently so to be of any benefit, and we sought to improve the immunogenicity by conjugation to mannan which will form the basis of the rest of this paper.

Murine immune responses to mannan MUC1 (MFP)

After using a number of methods to couple MUC1 to a potential carrier, conjugation to mannan under oxidising conditions by periodate was used and this generated exceedingly good cellular responses *(Table I)* to MUC1 and poor humoral responses [6, 7]. The conjugation conditions to mannan were most important - oxidising conditions leading to cellular responses, reducing conditions leading to humoral responses - indeed, of the same type obtained using MUC1 peptides or a MUC1 fusion protein (produced as a 5 repeat synthetic peptide of 105 amino acids). The strength of the immune response was startling - mice previously susceptible to 5×10^6 tumour cells were now resistant to 5×10^7 cell challenge, little antibody was produced, DTH was again present, but this time, $CD8^+$ CTLs could be induced prior to tumour challenge and with a high frequency ($^1/8{,}000$). Several different tumour models have been used and in all, mannan fusion protein (MFP) given as 3 injections at weekly intervals leads to total resistance to a subsequent tumour challenge (studies with the highly malignant $MUC1^+$ DA3 metastatic tumour are in progress). In addition, the injection of mice carrying $MUC1^+$ tumours led to a rapid reversal of their growth - of importance for patients with cancer.

Table I. Immunogenicity of various MUC1 immunogens in mice[a].

	MUC1 Tumour	MUC1 Peptide Fusion Protein or HMFG	Oxidised MFP	Reduced MFP
Immunogenicity				
Antibody Production	+	+++	±	++
DTH	+++	+++	+++	+++
CTL	+ (after PAGAL)	-	++	-
Tumour Protection	++	±	+++	±
Mediators				
CD4	-	*	-	*
CD8	+	*	+	*
Cytokine secretion after immunisation				
IL-2	*	-	+	-
IL-4	*	+	-	+
IL-12	*	-	+	-
γ-IFN	*	-	+	-
CTL responses				
MHC-restricted		*	Yes	
MHC-non restricted			No	
Responding H-2 haplotypes			b, d, k, s, z	
non-responding H-2 haplotypes			Nil	
HLA responses			HLA-A2 (others not tested)	
Enhancement of responses to mannan MUC1				
Cyclophosphamide			Yes	
Adjuvants				
CFA			No	
IFA			Yes	
Aluminum hydroxide			Yes	
GM-DP			Yes	
M-DP			Yes	
Macrophage ($F4/80^+$, $33D1^-$ presentation by adoptive transfer				
in vivo			Yes	
in vitro			Yes	
Dendritic cell ($F4/80^-$, $33D1^+$) presentation by adoptive transfer				
in vivo			in progress	
in vivo			in progress	
Cytokines				
IL-12			Yes	
IL-7+GM-CSF			Yes	
IL-4+γ-IFN			Yes	

[a] +++, excellent; ++, very good; + good; ± weak; - nil; in progress.
CTL, cytotoxic T lymphocytes; DTH, delayed type hypersensitivity; HMFG, human milk fat globule; MFP, mannan-MUC1fusion protein; PAGAL, phenyl N-acetyl-a-D-galactosamide.

Cytokine profiles after MFP immunisation and MHC restriction

In contrast to the predominant antibody production produced with peptides, fusion protein or reduced MFP, oxidised MFP gave predominant cellular responses and little antibody - suggestive of a T_1 response. This was confirmed in cytokine studies where the former gave rise to IL-4 secretion and little T_1 cytokine, whereas the use of oxidised MFP gave rise to the production of IL-2, γ-IFN, IL-12 and no IL-4 *ie.* a T_1 response [7]. It was intriguing that, by simply altering the oxidising conditions, a response could be switched from T_1 to T_2. MFP induces MHC restricted CTL responses [8]. Although the descriptions of CTLs from Finn indicated these were non-MHC restricted, in all our murine studies only H-2 restricted responses could be induced and indeed, 9 strains of 5 different haplotypes could all be immunised; there were no non-responder strains. By using inbred, congenic, recombinant and mutant mice, the responses are clearly shown to be H-2 restricted and this was confirmed using cells transfected with selected Class I genes and in stabilisation studies using RMA-S cells. There is no doubt the response is MHC restricted and furthermore, non-MHC restricted responses were not seen.

These studies have recently been extended by mapping the epitopes presented by the different Class I molecule (*see Table I*). Of note, now that epitopes did not fit the usual binding rules, but nonetheless, molecular modelling studies clearly demonstrated that MUC1 is firmly bound to Class I molecules, and furthermore, provides the only example wherein peptides in the Class I groove are accessible to anti-MUC1 peptide antibodies (Apostolopoulos *et al.*, MS in preparation).

Presentation of MUC1 by HLA antigens

Using HLA-2 transgenic mice immunised with MFP, it was clearly demonstrated that HLA restricted CTLs could be induced *in vivo* and these could be detected without restimulation *in vitro*. Furthermore, such CTLs could lyse HLA-A2$^+$ MUC1$^+$ MCF7 cells *in vitro*, but not HLA-A2$^-$ MUC1$^+$ BT20 breast cancer cells. The HLA-A2 presented MUC1 epitopes have also been mapped (Apostolopoulos *et al.*, in preparation).

Clinical trials with MFP in patients with adenocarcinoma and in monkeys

Based on the foregoing, 27 patients with adenocarcinoma (predominantly breast or colon) were immunised with MFP. In contrast to the murine studies, most of the patients made high titres of antibody to MUC1 peptide; gave weak DTH responses; T cell proliferation was detected, and CTLs are in the process of being measured. Thus, human MUC1 is highly immunogenic in humans. Similar studies were performed in 4 monkeys and again significant antibody responses were noted - cellular immunity measurements are now in progress. In neither monkeys nor humans, was there evidence of autoimmunity. It appears that MUC1, while being immunogenic, does not incite autoimmune responses.

Enhancement of anti-MUC1 responses

In an endeavour to further increase the immunogenicity of MFP, we used cyclophosphamide, various adjuvants and cytokines *(Table I)* - as shown, a number of the adjuvants can increase

the CTLp frequency as can cyclophosphamide and several of the cytokines, particularly in combination eg. IL-4 and γ-IFN and particularly IL-12. How these could be used in clinical studies is not clear at present.

Mode of action of MFP

At present it is not clear how MFP functions increase immune responses. Clearly mannan is important and could bind to the mannose receptor on macrophages and other cells, however, this is not the whole explanation as oxidised and reduced mannan bind equally well to macrophages and indeed, to the isolated mannose receptor expressed in transfected Cos cells, the reduced form binds better. We consider that macrophages are likely to be the major site of action of MFP rather than in dendritic cells (DC) as MFP does not bind well to DCs or to a DC tumour cell line, whereas it binds to macrophages, but the influence of dendritic cells on macrophages or their interactions are currently under examination. However, what has been shown is that peritoneal exudate cells (containing approximately 90% macrophages, 10% DCs) can be satisfactorily immunised *in vitro*, adoptively transfer immunity to mice and furthermore, one injection of such adoptively transferred cells is equivalent to 3 *in vivo* injections of MFP. We are currently examining the feasibility of sensitising human cells *in vitro* for clinical study.

Conclusion

Our studies have clearly demonstrated that mannan MUC1 (MFP) produced under oxidising conditions is a powerful immunogen for inducing CTL T cell immunity in mice - indeed, the most potent immunogen yet described, giving rise to much greater responses than those found with synthetic MUC1 peptides, whatever carrier is used. However, it should be emphasised that what we are measuring is the murine response to human MUC1, which is merely a model and an indication of what could happen in patients. As noted, when patients were immunised with human MUC1, humoral responses were noted and we are currently determining what degree of cellular immunity was found. However, at this time, other models should be used such as mice transgenic for human MUC1, and we also determining the immunogenicity of mannan conjugated with autologous MUC1 in monkeys (by cloning cynologous monkey MUC1) and also mannan-murine MUC1 in mice; these models are more relevant to the immunisation of patients than cross species immunisation with MUC1. Nonetheless, the results are encouraging and it was appropriate to perform preclinical studies before clinical studies were embarked upon. We are particularly encouraged by preliminary studies wherein MUC1 transgenic mice can be immunised against MUC1, although the CTL precursor frequency (CTLp) is substantially less than that obtained with human MUC1, although with the success in mice immunising macrophages or adoptive transfer - these approaches are likely to be the basis of further clinical trials. At this time the results are encouraging and indicate that the preclinical and clinical studies should be vigorously pursued, simultaneously monitoring immunogenicity, anti-tumour effects and the possibility of auto-immunity - which at this stage does not seem to be a problem.

Abreviations

Cp13-32 peptide, C-PAHGVTSAPDTRPAPGSTAP; CTL, cytotoxic T lymphocytes; DC, dendritic cells; DTH, delayed type hypersensitivity; HMFG, human milk fat globule; PAGAL, phenyl N-acetyl-a-D-galactosamide; MFP, mannan-MUC1 fusion protein; MUC1, Human Mucin 1.

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MOUSE MUCIN 1 (MUC1) DEFINED BY MONOCLONAL ANTIBODIES

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Mucins are highly expressed in many different human cancers and numerous murine monoclonal antibodies (MAbs) to human mucins, particularly Mucin 1 (MUC1), have been produced. However, no such antibodies to murine mucin 1 (muc1) have been described and we now describe 6 different antibodies produced to murine muc1 and to human MUC1 cytoplasmic tail, either by immunising rats, or muc1 o/o mice with synthetic peptides or a fusion protein composed of glutathione-s-transferase (GST) linked to the tandem repeat region of muc1. The antibodies to both the extracellular tandem repeat region and to the cytoplasmic tail were found to react with mucin-containing murine tissues such as breast, stomach, colon, ovary, kidney and pancreas, and the staining patterns were similar to those found in humans. The reagents reacted specifically with muc1 peptides and tissues; however, some cross reactivity with other mucin-derived peptides was noted, particularly those containing the amino acid sequence TSS. Three different epitopes (TSS, TAVLSGTS and LSGTSSP) of the M30, M70 and MFP25 MAbs were detected. Of interest was the finding that some of the antibodies reacted with murine lymphocytes; it was not clear whether these reactions were due to mucin 1 on mouse lymphocytes (MUC1 was considered to be absent from human lymphocyte), or due to cross reaction with a sialic adhesion molecule on lymphocytes. The antibodies should prove valuable reagents when studying differentiation and expression in murine glandular tissues and the ontogeny of mucin-secreting tumours. *Int. J. Cancer* 76:875–883, 1998.
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Human mucin 1 (MUC1) has been extensively described as a tumour-associated antigen since the first description of monoclonal antibodies (MAbs) reactive with the mucin extracellular protein core (Arklie *et al.*, 1981; McKenzie and Xing, 1990). The antibodies identified that MUC1 is overexpressed in cancer tissues (Stacker *et al.*, 1985) and in the serum of patients with epithelial carcinomas of the breast, ovary, lung and pancreas (Arklie *et al.*, 1981; McKenzie and Xing, 1990). Studies showing the presence of MUC1-reactive cytotoxic T cells in the draining lymph nodes of breast and ovarian cancer patients (Barnd *et al.*, 1989) and our own investigations demonstrating the generation of CD8 Class I-restricted cytotoxic T lymphocyte (CTLs) in mice (Apostolopoulos *et al.*, 1994, 1995*a*) have attracted much attention to MUC1 as a potential target for cancer immunotherapy.

The cDNAs for human (*MUC1*) and murine mucin1 (*muc1*) have been isolated, demonstrating that both the human and murine molecules share a basic structure with significant protein homology: there is an extracellular region with 59–62% protein identity, a multiple amino acid repeat region with 34% protein conservation and highly conserved transmembrane (87%) and cytoplasmic (87%) domains (Gendler *et al.*, 1988; Lightenberg *et al.*, 1990; Spicer *et al.*, 1991; Vos *et al.*, 1991; Wreschner *et al.*, 1990). The cloning of the cDNAs has also led to the production of human MUC1 transgenic mice and mice deficient in mouse muc1 (o/o) by homologous recombination (Peat *et al.*, 1992; Spicer *et al.*, 1995). The distribution of human MUC1 in transgenic mice is similar to that seen in human tissues (Peat *et al.*, 1992); further, muc1 o/o mice show no particular phenotype compared with normal littermates, except for a delayed progression of spontaneous mammary tumours, which did not express mouse muc1 (Spicer *et al.*, 1995).

Immunotherapeutic studies of MUC1 have shown that mice can generate $CD8^+$ CTL specific for the tandem repeat region of MUC1 and that these cells are a key part of the immune response that protects mice from challenge with MUC1-expressing tumours (Apostolopoulos *et al.*, 1994, 1995*a*). These findings, although significant, do not represent an ideal model for preclinical studies in humans using human MUC1, as the amino acid differences between the human and murine mucins make human mucin highly immunogenic in mice. To overcome these difficulties, we have established murine models that involve the immunisation of mice with murine muc1. To extend these studies, anti-mouse muc1 antibodies were produced and are described herein. The antibodies were made (a) to the extracellular repeat region using a synthetic peptide (Mp26); (b) to a muc1 fusion protein of tandem repeats (TR) derived from a mouse cDNA clone; and (c) to the cytoplasmic tail region using a human MUC1 peptide (CT-18) that has 87% identity with the murine cytoplasmic tail. The antibodies were used to characterise murine muc1.

MATERIAL AND METHODS

Synthesis of peptides, production of GST-muc1 fusion protein (MFP), human and mouse milk fat globule membranes

Mouse muc1 TR regions consist of 16 degenerate tandem repeats; 5 repeats are each 21 amino acids in length and the remaining 11 repeats are each 20 amino acids long (Spicer *et al.*, 1991; Vos *et al.*, 1991). A series of peptides derived from mouse muc1 TR and human MUC1 were produced for use in these studies (Table I). The first, Mp26, is a part of tandem repeats 2 and 3 and was synthesized using an Applied Biosystems Model 430A automated peptide synthesizer (Foster City, CA) to a purity (by HPLC) of >90%. The hydrophilicity of Mp26 was analysed according to Hopp and Woods (1983). The second peptide, CT18, is the last 17 amino acids of the human MUC1 cytoplasmic tail (15/17 amino acids of which are identical to the mouse muc1 cytoplasmic tail). In addition to these peptides, others were synthesized to test the specificity of the MAbs as described above: (a) 4 peptides corresponding to the variable number of tandem repeats (VNTRs) of human MUC1, 2, 3 and 4 (Cp13–32, MI29, SIB35, M4.22) (Apostolopoulos *et al.*, 1995*b*; Xing *et al.*, 1989*a*, 1992*a*, *b*, 1997) and (b) 6 other peptides derived from the human MUC1 sequences encoding the N-terminal (p31–55, p51–70) and C-terminal (p344–364, p408–423) regions of the VNTR and the cytoplasmic tail (p471–493, p507–526) (the numbers refer to the amino acids in the published sequences) (Wreschner *et al.*, 1990, Xing *et al.*, 1991*b*). Cysteine was added to the N-terminus of these peptides to aid in the production of disulfide bonded dimers; the peptide T4N1 from the N-terminus of mouse CD4 was also synthesized and used as a negative control (Table I).

A mouse fusion protein (MFP) containing 550 bp of the mouse tandem repeat region was produced using the bacterial expression vector pGEX2T, into which a mouse cDNA clone, pMUC2TR, was inserted in the correct reading frame and orientation (Smith and Jonson, 1988;

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TABLE I – REACTION OF MAbs WITH SYNTHETIC PEPTIDES TESTED BY ELISA

Peptides	Amino acid sequences	M30 (anti-Mp26) (TSS)[1]		M70 (anti-Mp26) (TAVLSGTS)[1]		MFP25 (anti-MFP) (LSGTSSP)[1]		MFP32 (anti-MFP) (not mapped)[1]		CT91 (anti-cyto-plasmic tail)	CT1: (ant cyto plasm tail
		Direct[2]	Inhibition[3]	Direct[2]	Inhibition[3]	Direct[2]	Inhibition[3]	Direct[2]	Inhibition[3]	Direct[2]	Direc
muc1 (mouse)											
Mp26	CTSSPATRAPEDSTSTAVLSG TSSPA	3+	3+	3+	3+	–	–	–	–	–	–
MFP		3+	3+	3+	2+	3+	3+	3+	3+	–	–
MMFG		+	3+	–	3+	+	2+	–	+	–	–
MUC1 (human)											
C-p13-32	(C)PAHGVTSAPDTRPAPGSTAP	–	–	–	2+	–	–	–	–	–	–
N-terminus to VNTR											
p31–55	TGSGHASSTPGGEKET-SATQRSSVP	–	–	–	–	–	–	–	–	–	–
p51–70	RSSVPSSTEKNAVSMTSSVL	+	3+	–	3+	–	2+	–	+	–	–
C-terminus to VNTR											
p344–364	NSSLEDPSTDYYQELQRDISE	–	–	–	–	–	–	–	–	–	–
p408–423	TQFNQYKTEAASRYNL	–	–	–	–	–	–	–	–	–	–
Cytoplasmic tail of MUC1											
p471–493	AVCQCRRKNYGQLDIFPARDTYH	–	–	–	–	–	–	–	–	–	–
p507–526	(C)YVPPSSTDRSPYEKVSAGNG	–	–	2+	3+	3+	2+	–	–	–	–
CT18	CSSLSYTNPAVVTTSANL	–	–	–	–	–	–	–	–	3+	3+
HFP	(5 VNTR repeats)	3+	3+	2+	3+	–	–	+	–	–	–
HMFG		–	–	–	+	–	2+	–	–	–	–
MUC2											
MI-29	KYPTTTPISTTTMVTPTPTPT-GTQTPTTT	+	+	–	–	–	–	–	–	–	–
MUC3											
SIB-35	CHSTPSFTSSITTTETTSHSTPSF TSSITTTETTS	–	–	–	–	–	–	–	–	–	–
MUC4											
M4.22	CTSSASTGHATPLPVTDTSSAS	–	–	–	–	–	–	–	–	–	–
Controls											
Mouse CD4 T4NI	KTLVLGKEQESAELPCECY	–	–	–	–	–	–	–	–	–	–
GST		–	–	–	–	–	–	–	–	–	–
KLH		–	–	–	–	–	–	–	–	–	–

[1]Epitope of the MAb.–[2]Direct, Direct binding on peptides: OD value: $- < 0.5$; $0.5 \leq + < 1.0$; $1.0 \leq 2 + < 1.5$; $1.5 \leq 3+$.–[3]Inhibition assay, of inhibition: $- < 20$, $20 \leq + < 40$; $40 \leq 2 + < 70$; $70 \leq 3+$.

Spicer *et al.*, 1991). MFP, consisting of glutathione-s-transferase (GST) and 184 amino acids of the mouse muc1 TR region (repeats 7–16) was prepared from transformed *Escherichia coli* DH5α induced with 0.1 mM IPTG and lysed by sonication and 1% Triton X-100 buffer. The MFP was purified from the lysate by binding to a GST-agarose column and elution with 10 mM reduced glutathione (Smith and Jonson, 1988). To test the specificity and cross reaction of the MAbs, human MUC1 fusion proteins (HFP), consisting of five 20 amino acid repeats of VNTR, were also produced using the pGEX vector as described above (Apostolopoulos *et al.*, 1993). GST was prepared using the pGEX2T vector, without any insert, as a negative control. Human and mouse milk fat globules (HMFG, MMFG) were prepared from fresh milk as described elsewhere (Xing *et al.*, 1989*b*). Human milk was obtained from nursing mothers, and mouse milk from lactating mammary glands of nursing mice. Milk samples were centrifuged and the floating cream was collected and homogenized. Crude membranes were prepared by centrifugation at 10,000*g* for 90 min at 4°C. The pellet was resuspended in 0.3 M sucrose, 70 mM KCl, 2 mM $MgCl_2$ and 10 mM Tris-HCl buffer, pH7.4, and stored at −70°C (Jarasch *et al.*, 1977).

Production and testing of MAbs

The peptides Mp26 and CT18 were conjugated to keyhole limpet hemocyanin (KLH) using glutaraldehyde (Xing *et al.*, 1989*a*, 1992*b*); 100 μg Mp26-KLH, CT18-KLH or MFP emulsified in complete Freund's adjuvant were injected i.p. into Lewis female rats and muc1 o/o 129Sv/J mice. After 4 weeks, a second injection of Mp26-KLH, CT18-KLH or MFP was given and the spleen ce were fused with mouse myeloma NS1 cells, 3 days after a thi injection. The hybridomas produced were tested on various pe tides or MFP by an enzyme-linked immunosorbent assay (ELIS (Xing *et al.*, 1989*a*). Briefly, the peptides (20 μg/ml) and MFP μg/ml) were coated in a 96-well polyvinyl chloride plate (Cost Cambridge, MA) in carbonate buffer pH 9.6 for 2 hr at 37°C. T nonspecific binding sites were then blocked with 2% bovine seru albumin (BSA) for 1 hr at 37°C. The tissue culture supernatant w added to the wells and incubated for 2 hr at room temperature; t binding of antibodies detected by sheep [F(ab')$_2$] anti-rat anti-mouse immunoglobulin labelled with horseradish peroxida (Amersham, Aylesbury, UK) and measured after the addition of t substrate (0.03% 2,2-azino-di-3-ethylbenzthiazoline sulphonat 0.02% H_2O_2) on an ELISA reader. To test the specificity of t reactions of the MAbs with peptides, an inhibition assay w performed (Xing *et al.*, 1991*b*). Briefly, MAbs of a consta concentration were incubated for 1 hr with each of the peptid previously serially diluted (1–0.004 mM) and added to the Mp2 (for testing of M30 and M70) or MFP- (for testing of MFP25 an MFP32) coated plates. The inhibition of the MAb binding to th antigen-coated plates was measured by adding anti-rat immun globulin labelled with horseradish peroxidase and measured aft the addition of the substrate (0.03% 2,2-azino-di-3-ethylbenzthiaz line sulphonate/0.02% H_2O_2) on an ELISA reader (Xing *et a*

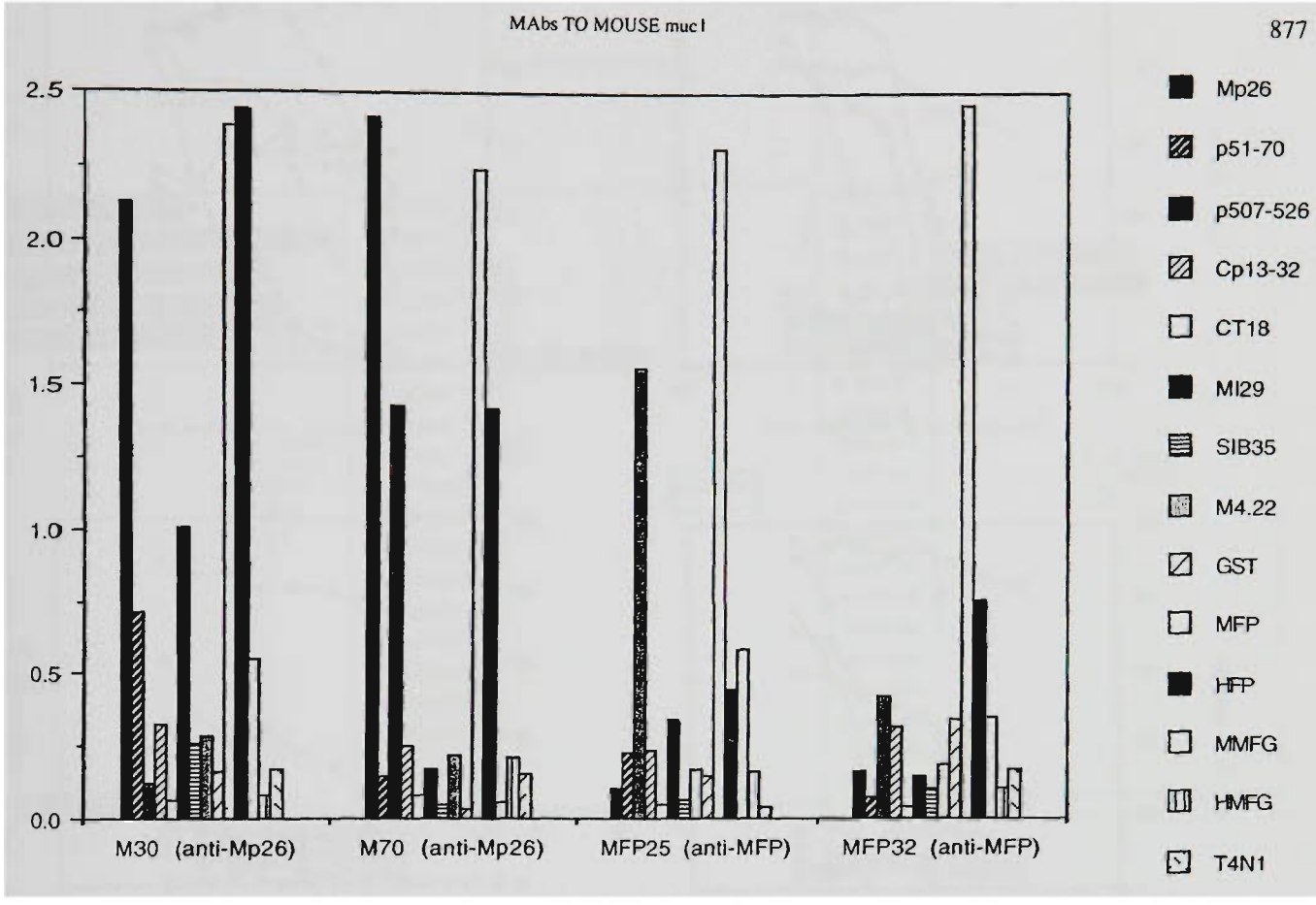

FIGURE 1 – Reactivity of the monoclonal antibodies (MAbs) M30, M70, MFP25 and MFP32 with different antigens by enzyme-linked immunosorbent assay (ELISA) using peptides coated in a plate. The optical density (OD) value at 405 nm is shown on the ordinate.

1992*b*). The percentage of inhibition was calculated as: [1 – (binding of MAb with inhibitor/binding without inhibitor)] × 100%. The subclasses of the MAbs were determined by anti-rat or anti-mouse Ig subclass antibodies (Serotec, Oxford, UK) by Ouchterlony gel diffusion. To map the epitopes of the MAbs to mouse muc1, 8-mer overlapping peptides covering the first 20 amino acids (cysteine not included) of Mp26 were synthesized on pins using a Multipin Peptide Synthesis Kit (Chiron Mimotopes, Clayton, Australia) and tested by ELISA (Geyson *et al.*, 1987).

Immunoperoxidase staining

Both fresh and formalin-fixed mice (from either nursing or normal BALB/c, C57BL/6, DBA/J, 129Sv/J or muc1 o/o 129Sv/J mice) and human tissues were tested for antibody reactivity. A mouse mammary tumour, also tested, was a solid tumour growing in a mouse previously injected with a mouse mammary tumour cell line, DA3 (Fu *et al.*, 1990). The tissues were embedded in O.C.T. Compound (Tissue-Tek, Miles, USA), snap frozen and stored at -20°C. For analysis, tissues were cut 5–6 μm thick, attached to aminoalkylsilane-coated slides (Rentrop *et al.*, 1986), air dried and fixed in cold acetone for 10 min. Endogenous peroxidase activity was blocked for 40 min at room temperature using 0.5% H_2O_2. Fresh human tissues used for testing were obtained from the Department of Pathology, Austin and Repatriation Medical Centre, Australia, within 6 hr after death or operation. Immunoperoxidase staining was performed on these tissues as described elsewhere (Stacker *et al.*; 1985; Xing *et al.*, 1992*b*). F(ab')$_2$ of anti-rat immunoglobulins and an anti-mouse immunoglobulin, linked to horseradish peroxidase (Amersham, Aylesbury, UK) were used to detect rat or mouse MAbs, respectively. Mouse tissues were also fixed using formalin, embedded in paraffin (Stacker *et al.*, 1985). The staining was graded according to the percentage of cells stained: –, <5% ; +, 5–25%; 2+, 25–50% ; 3+, 50–75%; 4+, 75–100%. To demonstrate the specific staining of the MAbs in the tissues, 2 different approaches were used: (a) the MAbs were preincubated with peptides (5 mg/ml) or FP (2 mg/ml) for 1 hr at room temperature before their addition to the tissue sections; and (b) the immunoperoxidase (IP) staining was compared between BALB/c, C57BL/6, DBA/J, normal 129Sv/J and muc1 o/o 129Sv/J mice.

RESULTS

Production of monoclonal antibodies

Mouse muc1 peptide (Mp26) antibodies. Two MAbs M30 (IgM) and M70 (IgG_1) were produced from rats immunised with the muc1 peptide Mp26; both reacted by ELISA with Mp26 and MMFG, but not with the control peptide T4N1 or the carrier protein KLH. These 2 MAbs also reacted with MFP, which contains the amino acids of Mp26, but not with GST, CT18, MUC3 or MUC4 VNTR peptides, indicating that the MAbs reacted specifically with mouse muc1 (Table I, Figs. 1 and 2). The M30 antibody (which detects the TSS epitope, as described below) was found not to react with MUC1 peptides (p31–55, p344–364, p408–423, p471–493, p507–526) but to react with p51–70 (human MUC1 peptide N-terminus to the VNTR), which contains the TSS sequence; on this basis a reaction to human tissues could be predicted (see below). There was also a strong reaction with the HFP consisting of 5 repeats of VNTR, but not with the VNTR peptide Cp13–32; this being likely a conformational epitope formed by folding of the noncontinuous regions of the VNTR. There was also reactivity with the human MUC2 VNTR peptide MI29, which does not contain TSS. Human MUC3 and 4 VNTR peptides, both of which contain TSS, did not react. This finding was not surprising, because although TSS amino acids were present in the immunising peptide, adjacent amino acids SG and PA in the sequence SGTSSPA are clearly important (Xing

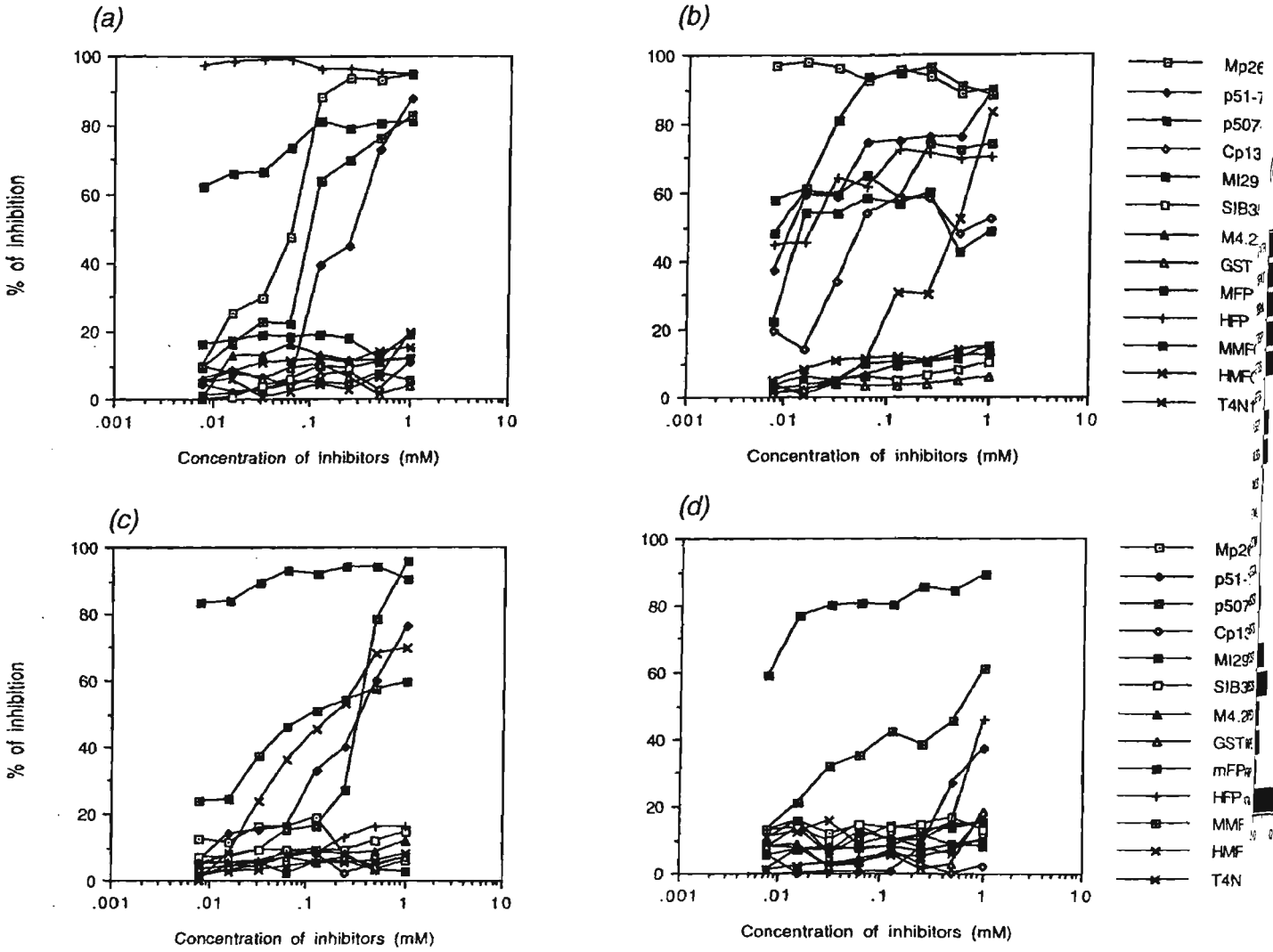

FIGURE 2 – Inhibition assays of the antibodies binding to mouse fusion protein (MFP). The antibodies at 1/200 of tissue culture supernatant ([*a*] and M70 [*b*]) or 1/100 (MFP25 [*c*] and MFP32 [*d*]) were tested in the presence of serial dilutions of inhibitors (abscissa). The percent inhib (see text for calculation) is shown on the ordinate; the concentration of inhibitors is shown on the abscissa.

et al., 1991*a*, *b*). The sequence of the MUC3 VNTR peptide SIB35 is SFTSSIT and the M4.22 (MUC4 VNTR peptide) sequence is TDTSSAS—with different adjacent N- and C-terminal amino acids that differ sufficiently in charge and size to those in Mp26, so that there was no reaction. These findings are good examples of the unpredictability of reactions of antibodies with peptides. The MAb M70 (produced to peptide Mp26 and reacting with TAVLSGTS, see below) reacted with human MUC1, including HMFG, VNTR peptide Cp13–32, N-terminal peptide p51–70 (in an inhibition assay) and a cytoplasmic tail peptide p507–526 (Table I, Figs. 1 and 2); the possible explanation of these reactivities is described above in general terms.

Mouse muc1 tandem repeat fusion protein antibodies. Two MAbs, MFP25 (IgM) and MFP32 (IgM), were produced from rats immunized with MFP. MFP25 (minimum epitope LSGTSSP, see below) reacted with MFP, MMFG, human MUC1 peptides p507–526, p51–70 and also with HMFG in both direct binding and inhibition ELISAs; there was no reaction with the carrier protein GST, other non-VNTR peptides or other mucin VNTR peptides MI29, SIB35 and M4.22. MFP32 (epitope not mapped) had similar reactivities to MFP25, although it reacted weakly with HFP and did not react with the cytoplasmic tail peptide p507–526. Neither MFP25 or MFP32 reacted with Mp26, which is a part of MFP, although the epitope of MFP25 was mapped as LSGTSSP, which is contained in Mp26 (Table I, Figs. 1–3).

Cytoplasmic tail antibodies. Two MAbs, CT91 (rat origin, IgG_1) and CT1.53 (IgG_1, from muc1 o/o 129Sv/J mice) were produced against the human MUC1 cytoplasmic tail peptide CT18. T antibodies were entirely specific for the cytoplasmic tail pe CT18 and did not react with any of the other peptides, nor HMFG or MMFG (Table I).

Reaction of MAbs with peptides and other relevant molecules in inhibition tests

The tests described above were based on "direct" ELISA which the peptide is coated onto a solid support (a plate); other alluded to and described below (epitope detection using pins), one end of the peptide tethered, whilst the other end is free have found (Xing *et al.*, 1991*b*) that testing peptides in sol (liquid phase) is also important, as occasionally different resul obtained (due to the peptide taking a different conformation different conditions). To perform liquid phase inhibition tes peptide is reacted with the antibody in solution and then test solid phase ELISA. For the most part (Table I, Figs. 1 and 2 solid and liquid phase peptide reactions with the antibodies the same. However, some notable exceptions were that (a reaction of M30, M70 and MFP25 with MMFG was we negative by a direct reaction and strong by inhibition, and the was found with MFP32 (Fig. 2); (b) M70 reacted with the h MUC1 peptide in solution but not on the plate (except p507 (Figs. 1 and 2*b*); (c) all antibodies (except CT91 and CT reacted strongly with the human N-terminal peptide p51–70, contains the TSS sequence (for M30 and MFP25) but do contain the M70 TAVLGTS sequence (Fig. 2); and (d) M

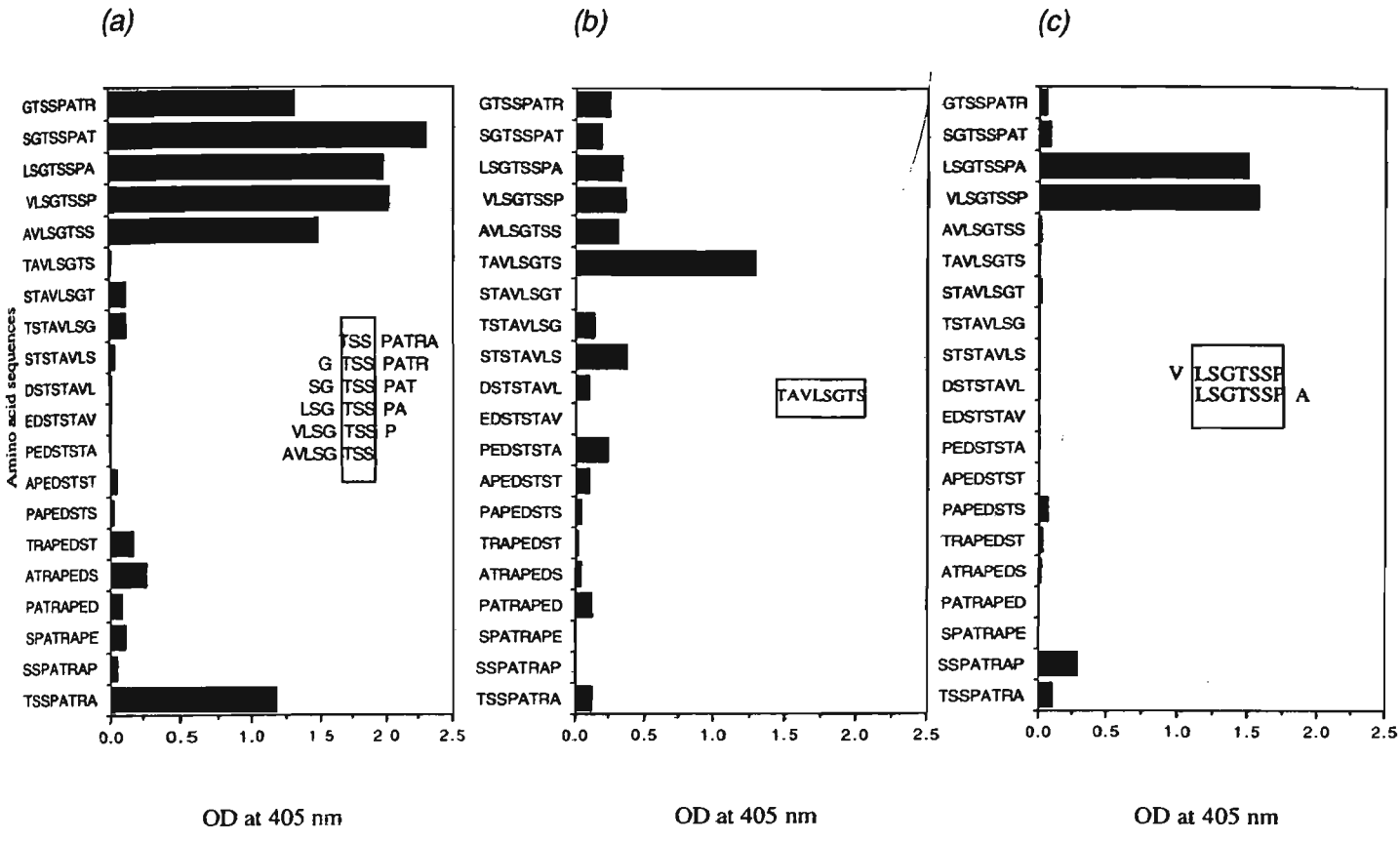

FIGURE 3 – Reactivity of monoclonal antibodies (MAbs) M30 (*a*), M70 (*b*) and MFP25 (*c*) with mouse tandem repeat peptides synthesized on polyethylene pins. The peptide sequences are shown on the ordinate and the OD (at 405 nm) of the enzyme-linked immunosorbent assay (ELISA) on the abscissa. The height of the black bars = OD value. The amino acids common to reactions by MAbs are shown in the boxes.

reacted with HMFG in solution, but not in the plate (Fig. 2*c*). A possible explanation for these findings is given above. However, such "cross reaction" should be kept in mind when using anti-peptide antibodies and different forms of the antigen, for example, tissue sections (solid phase) or serum (liquid phase).

Epitopes of the MAbs

To determine the epitopes of the anti-muc1 antibodies, each antibody (except anticytoplasmic tail MAbs CT91 and CT15.3) were tested on mouse muc1 overlapping peptides, derived from Mp26 (Spicer *et al.*, 1991). The results indicate that the epitopes for the 3 antibodies could be precisely mapped as TSS (M30); TAVLSGTS (M70); and LSGTSSP (MFP25) (Fig. 3). The epitope for MAb MFP32 could not be determined (see below). The sequences of the 3 epitopes detected overlapped, and, in particular, we noted that the epitopes detected by MFP25 and M30, contained the common "TSS" sequence that is also found within the VNTR of MUC3 and 4, as well as in N- and C-terminal regions of human MUC1 amino acids 66–68 (contained in peptide p51–70) and 315–317 (not synthesized in this study) (Table I) (Lightenberg *et al.*, 1990; Wreschner *et al.*, 1990).

Reaction of MAbs with mouse tissues

It is important to demonstrate that MAbs produced against synthetic peptide Mp26 or MFP are reactive not only with the immunogen, but also with native muc1 expressed in the tissues. The M30, M70, MFP25, MFP32 and CT91 MAbs were examined on both fresh and formalin-fixed mouse tissues from 5 strains of mice (BALB/c, DBA/J, C57BL/6, 129Sv/J and muc1 o/o 129Sv/J) and a mouse mammary tumour cell line, DA3, by the immunoperoxidase technique. We found that the staining of the MAbs on formalin-fixed tissues was reduced compared with that observed on the fresh sections, and that the tissue staining patterns from DBA/J, C57BL/6 and 129Sv/J mice were the same as that seen on BALB/c mice, whose results are reported (Table II, Fig. 4). M30 (anti-Mp26) reacted with the apical surface of lining cells of the ducts from resting mammary tissues (Fig. 4*b*) in contrast to negative staining of M70 (Fig. 4*a*) and its staining in lactating breast tissues (Fig. 4*c*); strong staining of the M30 was evident in the cytoplasm of lactating glandular cells (Fig. 4*c* compares the staining of

TABLE II – REACTION OF MAbs WITH FRESH MOUSE TISSUES BY IMMUNOPEROXIDASE STAINING[1,2]

Tissue	M30		M70		MFP25		MFP32		CT91	
	N[3]	muc1 o/o[3]	N	muc1 o/o	N	muc1 o/o	N	muc1 o/o	N	muc1 o/o
Breast										
Rest	+	–	–	–	+	–	+	–	2+	–
Lactating	2+	–	–	–	3+	–	3+	–	4+	–
Lymph node	+	–	–	–	+	–	–	–	–	–
Colon	2+	–	–	–	2+	–	2+	–	–	–
Skin	–	–	–	–	–	–	–	–	–	–
Lung	–	–	–	–	–	–	–	–	2+	–
Spleen	+	–	–	–	+	–	–	–	–	–
Kidney	2+	–	–	–	2+	–	2+	–	2+	–
Submaxillary gland	+	–	–	–	+	–	+	–	+	–
Stomach	3+	–	+	–	2+	–	2+	–	–	–
Liver	–	–	–	–	–	–	–	–	–	–
Pancreas	+	–	–	–	+	–	+	–	–	–
Ovary	2+	–	–	–	2+	–	2+	–	+	–
Fallopian tubes	+	–	–	–	2+	–	2+	–	–	–
Uterus	+	–	+	–	+	–	+	–	3+	–
Heart	–	–	–	–	2+	–	2+	–	–	–
Muscle	3+	–	–	–	+	–	+	–	–	–

[1]Staining was graded according to the percentage of cell stained: <5% (–); 5–25% (+); 25–50% (2+); 50–75% (3+); 75–100% (4+).–[2]CT1.53 was not tested on mouse tissues by immunoperoxidase staining.–[3]N = normal BALB/c mouse; muc1 o/o = mucin gene knock out.

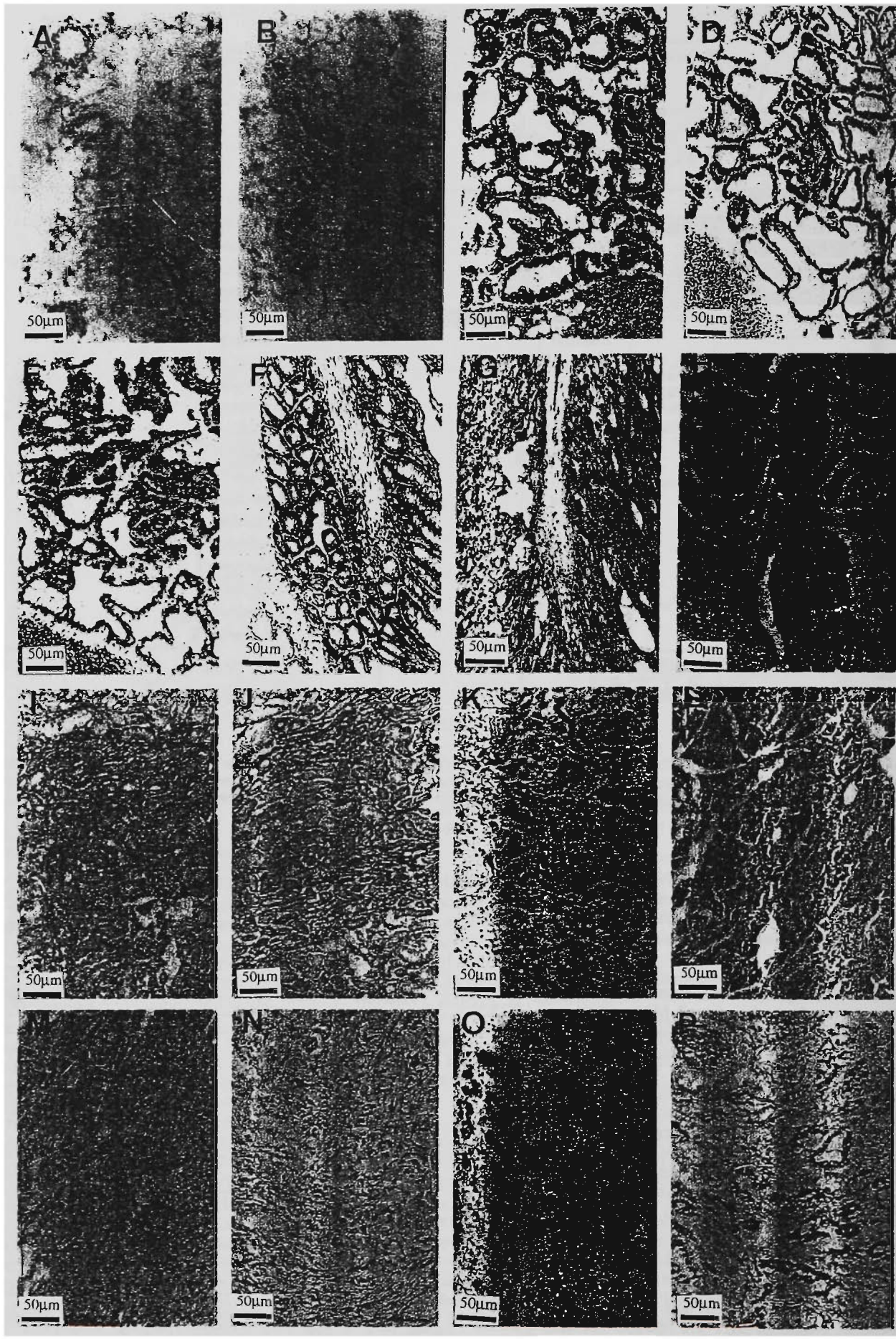

FIGURE 4 – Immunoperoxidase staining of snap frozen tissues by monoclonal antibodies (MAbs) showing M70 (*a*) (nonreactive) and MFP25 (*b*) (reactive) with normal mouse ("resting") breast; M30 (*c*), MFP32 (*d*) and CT91 (*e*) (all positive) reacting with lactating mouse mammary gland; M30 reacting with mouse colon (*f*), stomach (*g*), skeletal muscle (*h*) and kidney (*i*); MFP25 (*j*) and CT91 (*k*) all reacting positively with mouse kidney; M32 (*l*) and CT91 (*m*) reacting with mouse pancreas; M30 (*n*) reacting with mouse mammary tumour cell line DA3 from a BALB/c mouse; CT91 (*o*) reacting with human normal stomach and M30 (*p*) with human breast cancer (both positive).

TABLE III – REACTION OF MAbs WITH MOUSE LACTATING MAMMARY GLAND AFTER BLOCKING WITH Mp26 OR CT18 PEPTIDES OR MFP AND TESTED BY IMMUNOPEROXIDASE STAINING[1,2]

MAbs	Dilution	Inhibitors			
		None	Mp26	MFP	CT18
M30	1/20	2+	–	–	2+
MFP25	1/15	2+	2+	–	2+
MFP32	1/15	2+	2+	–	2+
CT91	1/300	2+	2+	2+	–
CT15.3[1]	1/10	2+	2+	2+	–

[1]Staining of CT1.53 on human breast cancer tissues was inhibited by peptide CT18. M70 was not tested as it was not reactive with most tissues. [2]The staining was graded according to the percentage of cell stained: <5% (–); 5–25% (+); 25–50% (2+); 50–75% (3+); 75–100% (4+).

TABLE IV – REACTION OF MAbs WITH FRESH HUMAN TISSUES BY IMMUNOPEROXIDASE STAINING

Tissue	Antibody reactivity (number of positive/number tested)					
	M30	M70	MFP25	MFP32	CT91	CT1.53
Normal						
Breast	0/2	0/2	0/2	0/2	0/2	0/2
Colon	0/3	0/3	0/3	0/3	0/3	0/2
Stomach	2/3	1/3	2/3	2/3	3/3	NT[1]
Malignant						
Breast	3/15	1/15	5/15	5/15	8/15	9/14
Stomach	0/2	0/2	0/2	0/2	0/2	NT[1]
Lung	3/6	4/6	2/6	2/6	4/6	5/9
Colon	0/3	0/3	0/3	0/3	0/3	3/11

[1]NT, not tested.

MFP32 and CT91 in Figs. 4*d* and 4*e*, see below) and some in the cell surface. Strong reactions were observed with mouse colon (Fig. 4*f*), particularly in the glandular cells of the crypts; M30 also reacted with other epithelial tissues, such as the submaxillary gland (acini), stomach (gastric glands, Fig. 4*g*), heart and skeletal muscle (Fig. 4*h*), kidney (Fig. 4*i* compares the staining of MFP25 and CT91 in Figs. 4*j* and 4*k*, see below for detail), pancreas (acini, Fig. 4*l*, compares the staining of CT91 in Fig. 4*m*), ovary, fallopian tube and the uterus (endometrium and uterine glands of nursing mice) (Table II). These results indicate that the anti-mouse muc1 MAb M30 detects a molecule with distribution similar to human MUC1 from other studies, except that mouse muscles (skeletal and heart) were stained and skin and lung were not stained by M30 (Pemberton *et al.*, 1992; Spicer *et al.*, 1991; Vos *et al.*, 1991; Xing *et al.*, 1989*b*). The M30 also reacted with the mouse mammary tumour cell line DA3, which grew in BALB/c mice (Fig. 4*n*).

MFP25, MFP32 and CT91 had similar reactivities to M30, but some differences were noted in mouse tissues stained by IP (Table II). Firstly, there were 3 types of staining of the mammary glands: (a) cytoplasmic staining by M30 (Fig. 4*c*), MFP25 (similar to M30, figure not shown) and MFP32 (Fig. 4*d*); (b) strong staining of the apical surface of the epithelial cells and the ducts of the mammary glands by CT91 (Fig. 4*e*); and (c) staining of the cytoplasm of lymphocytes adjacent to the mammary glands by M30 (Fig. 4*c*) and MFP25, which was not observed with MFP32 or CT91 (Figs. 4*d* and 4*e*). The staining of lymphocytes was diffuse and mainly in the cytoplasm (Fig. 4*c*), but not obvious on the cell surface; the same staining was also found in the lymphocytes of the red and white pulp of the spleen stained with M30 and MFP25 (not shown). This reaction is important as the anti-lymphocyte reaction of anti-MUC1 was not previously been reported. Secondly, 3 different patterns of kidney staining were observed: (a) staining of both capillary endothelial cells of glomeruli and convoluted tubules by M30 (Fig. 4*i*) and MFP25 (not shown); (b) staining of the capillary endothelial cells of glomeruli but not in convoluted tubules by MFP25 (Fig. 4*j*); and (c) staining of convoluted tubules, but no staining of glomeruli (CT91, Fig. 4*k*). Compared with the staining of the 4 MAbs described above, M70 (anti-Mp26) reacted weakly with mouse stomach and uterus and did not react with other tissues (Table II). CT-1.53 is a murine MAb and was therefore tested on human tissues only (see below).

Specificity of reactions of the MAbs with peptides and tissues

To determine the specificity of the antibody reactions, 2 separate tests were performed by immunoperoxidase staining. In the first, either peptides or the fusion protein was used to block the MAb reaction on mouse lactating mammary gland. The staining of M30 was inhibited by both Mp26 and MFP, whilst the staining of MFP25 and MFP32 was completely inhibited by MFP, but not by peptides Mp26 or CT18 (Table III). We noted that the peptide Mp26 did not inhibit the staining of MFP25 and MFP32, as Mp26 is part of MFP, and may lack the epitope or proper tertiary structure necessary to provide the MAb binding site. Secondly, the MAbs were tested on the muc1 o/o 129Sv/J mice, which do not express mouse muc1 (Spicer *et al.*, 1995). None of the MAbs reacted with muc1 o/o tissues, such as the lactating mammary gland, stomach, kidney and colon, showing that the MAbs were muc1 specific (data not shown).

Reaction of MAbs with human tissues by immunoperoxidase staining

As there are common sequences in mouse muc1 and human MUC1, and the MAbs described above react with some human mucin peptides (Table I, Figs. 1 and 2), a number of fresh human tissues were tested by the immunoperoxidase technique using the MAbs (Table IV, Figs. 4*o* and 4*p*). Firstly, none of the 6 MAbs reacted with normal human breast or colon, although there was a reaction with the glandular cells in the stomach with most of the MAbs (Fig. 4*o*). Unlike normal tissues, breast and lung cancers were reactive in varying proportion (Table IV, Fig. 4*p*); colon and stomach cancers were nonreactive with the 6 MAbs (except anticytoplasmic tail MAb CT1.53, which was reactive with colon cancer) (Table IV). Thus the anti-mouse muc1 antibodies showed some specificity for human cancers and therefore could be useful reagents for human studies.

DISCUSSION

Six different antibodies made to mouse muc1 and human MUC1 cytoplasmic tail using the immunization of rats or muc1 o/o mice with synthetic peptides or fusion protein have been described. Because of the nature of the immunogens (peptide or fusion protein), the antibodies were produced to peptide sequences, and therefore carbohydrates, either in part or *in toto* do not form part of the epitope. Together the antibodies describe the distribution of mucin 1 in the mouse, previously only described using RNA (Vos *et al.*, 1991) or a polyclonal antibody to the human cytoplasmic tail, which cross reacted with mouse mucin 1 (Pemberton *et al.*, 1992). Apart from a few exceptions (muscle and lymph node), our results agree with those described and with what would be expected from the distribution of human MUC1 (Xing *et al.*, 1989*b*). Thus, epithelial tissues particularly lactating breast, submaxillary gland, intestine, ovary and other tissues are reactive, although each of the antibodies, although detecting the same molecule, gave slightly different patterns of reactivity in lactating breast and kidney. A feature also noted was that the anti-mouse MAbs reacted with human MUC1 peptides, with HMFG and, indeed, with cancer tissues. This latter reaction is of interest and, by chance, possibly highly specific reagents may have been produced to detect human cancer mucins—particularly in serum where some of the antibodies reacted with HMFG in solution. These reagents could possibly be useful for human serum MUC1 tests.

One remarkable feature was the finding of mucin 1 on lymphocytes by some of the antibodies, alluded to in the past (Apostolopoulos *et al.*, 1993) but not clearly described. Reactivity of these antibodies with lymphocytes in tissue sections could be seen, and so mucins may be lymphocyte cell surface molecules. Indeed, mucins bear

some resemblance to adhesion molecules such as sialoadhesion, among others, and mucins have been described as adhesion ligand molecules in breast cancer cells (Regimbald *et al.*, 1996; Wesseling *et al.*, 1996). It would be of interest to determine the expression levels of mucin on various subsets of mouse lymphocytes and to confirm the studies by either biochemical analysis of cells or by polymerase chain reaction (PCR) (studies now in progress).

When anti-peptide antibody/peptide interactions are examined it is important to exclude nonspecific reactions, and to do this, a number of other peptides were examined. In some of these peptides, a related TSS sequence was detected by some of the antibodies. One of the peptides (P51–70) of the human MUC1 (N-terminal to VNTR) appeared to react with all of the antibodies except anti-CT antibodies CT91 and CT1.53, and another peptide p507–526 (cytoplasmic tail) reacted with M70 and MFP25. These reactions were likely to be specific and although they were detected or not in reactions with peptides in solid phase, they could be inhibited with the peptides in liquid phase (Table I, Fig. 2). Furthermore in immunoperoxidase staining, the antibody activity could also be inhibited by absorbing with the peptides, but most importantly the reactions with the muc1 o/o mice were entirely negative, providing the best specificity control.

Of interest was that 3 epitopes, TSS (M30), TAVLSGTS (M70) and LSGTSSP (MFP25) all contained the common "TS" sequence. The epitope for MFP32 was not mapped, as it was raised from a immunised with MFP and may react with amino acids no overlapping peptides that were made from repeats 7–8 on the p All epitopes were found in the STAVLSGTSS region of the Th hydrophobic region of the muc1 molecules, rather than in hydrophilic region (Geyson *et al.*, 1987; Welling *et al.*, 19 which is considered to be the most immunogenic. However, as antibodies for the most part were made in rats, the epitopes detec must also be a reflection of the antigenic difference between mo and rat, which at present is not known.

Our results clearly demonstrate that the anti-human cytoplasmic peptide antibodies, CT90 (from rat) and CT1.53 (from 129Sv/J mice), not only react with human tissues but also with mouse tissues that they can be used in both human and mouse mucin studies. Anot interesting finding is that the staining pattern of anticytoplasmic antibodies CT91 and CT1.53, which show apical surface or secreti staining (Figs. 4*e*, 4*k* and 4*m*), indicate that muc1, or at least a portion muc1, may be secreted in an intact form (with an intact cytoplasmic ta rather than a secreted form (without cytoplasmic tail) cleaved fr muc1 outside of the membrane.

We have described anti-mouse muc1 reagents. Six MAbs we produced that reacted with mouse muc1, reagents that should be value in differentiation and oncological studies.

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Oxidised mannan antigen conjugates preferentially stimulate T1 type immune responses

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Abstract

It is desirable to be able to produce either T1 or T2 responses and we have found that, in mice, mannose - coupled antigens stimulated T2 type responses antibodies and CTLs. whereas if oxidized, mannose - coupled antigens stimulated T1 responses little antibody and a potent CTL response. In addition, the cytokine profiles support the T1rT2 differentiation with these immunizations, in that oxidized mannan antigen gives IFNg, IL-2 and IL-12 production, whereas in the absence of oxidization, IL-4 and not the other cytokines is produced. A number of antigens have been examined - particularly Mucin 1 and the delivery method using mannose may be applicable to the other antigens. q 1998 Elsevier Science B.V. All rights reserved.

Keywords: Mannan; Antigen; Immune response

1. Introduction

In designing vaccine strategies, it is apparent that the production of antibodies, using synthetic antigens, is not difficult: thus synthetic peptides and fusion proteins have been used to induce significant antibody responses. However, whether these antibodies are of the appropriate isotype and can function in protection is another matter. For many vaccines, the induction of cellular immunity is considered to be desirable, although it is

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not all that clear whether DTH type mechanisms or cytotoxic T lymphocyte CT production is the appropriate cellular response. We have been trying to induce anti-tum immunity—particularly to breast cancer—and have targeted the Mucin 1 MU antigen as this is increased to 100 times in cancer. MUC1 is modified in cancer, so t certain new protein and carbohydrate epitopes appear Gendler et al., 1989; Papadi itriou et al., 1994. Of importance to the increase in the amount of MUC1, is that so of it should find its way into the cytosol and be presented by MHC Class I molecul Indeed, we have found this to be the case for both murine H-2 and human HL molecules. Our initial attempts to induce immune responses used MUC1 peptides, whi failed to induce adequate protection see below, we then used mannan as a vehicle a satisfactory cellular responses were induced.

2. Mucins, structure and immunology

Mucins are large, heavily glycosylated molecules with a protein core. The gen encoding the protein core of seven different mucins have been cloned MUC1– Apostolopoulos et al., 1996a,b. MUC1 is found in large amounts in many tissu including breast cancer, where there is a 40–100 fold increase in the amount present; has an ubiquitous cellular distribution being found in salivary gland, lung, kidney an pancreas as well as in breast and milk. In cancer, it is found especially on the ce surface, rather than a polar distribution as seen on normal breast cells. There is als defective glycosylation which exposes the protein core which consists of many repea of 20 amino acids. When breast cancer cells are injected into mice to make antibod most antibodies made are to mucins and indeed, to the protein core, and further, to th five amino acids APDTR in the repeat region. Many different antibodies made agains tumours, milk mucins and indeed, to the synthetic APDTR peptide all react similarl with breast cancer Xing et al., 1990. Interestingly, Dr. O. Finn USA found th draining lymph nodes of patients with breast cancer contain T cells, which could b stimulated in vitro with IL-2 and breast cancer cell lines to have cytotoxic activit against breast cancer cell lines. These T cells were unusual in that they were non-MH restricted Barnd et al., 1989. Using this knowledge, our approach has been to us mucins, particularly mucin peptides to develop immunity against breast cancer. W initially used a synthetic peptide in our first clinical trial ARI-1 conducted with th Peter MacCallum Institute, patients received MUC1 peptides with diphtheria toxoid in Phase 1 trial—there was no toxicity important as MUC1 is ubiquitous and autoimmu nity could be a theoretical problem, weak immunity, and no obvious anti-tumou responses Xing et al., 1995. The details of the early and subsequent studies follow.

3. Murine immune responses to MUC1 peptides

As synthetic MUC1 peptides were able to immunise mice for anti-MUC1 antibod production, we asked—could they be used to induce cellular immunity and anti-tumou effects? We made a MUC1 synthetic peptide Cp13–32 containing 20 amino acids o

Table 1
Generation of T1 or T2-type immune responses in mice

Immunogen	CTL	Abs	Cytokines	Immune response	CTLp
MUC1 peptide-KLH	y	qqq	NT	T2	1/150 000
MUC1 peptide-DT	y	qqq	NT	T2	1/150 000
MUC1 fusion protein	y	qqq	NT	T2	1/85 000
MUC1 fusion protein-mannan oxidised	qqq	restricted q	IL-2 IFN-g IL-12	T1	1/8000
MUC1 fusion protein-mannan reduced	y	qq	IL-4	T2	1/85 000

y, Negative; q, weak; qq, strong; qqq, very strong.
NT: not tested.
CTLp: Cytotoxic T lymphocyte precursor frequency after three immunisations.

MUC1 linked to either KLH, or diphtheria toxoid and immunised mice; antibodies were produced and cellular immunity was demonstrated by $CD4^q$ mediated DTH reactions (Apostolopoulos et al., 1996a,b). Anti-tumour immunity, examined by challenging mice with human $MUC1^q$ tumours, indicated some anti-tumour effects, with protection with low dose of tumour challenge (10^6 cells). This protection disappeared when five times the dose was used. With this immunisation protocol, no CTLs were produced and the same was found in patients (Xing et al., 1995).

After using a number of methods to couple MUC1 to a potential carrier, conjugation to mannan under the oxidising conditions of periodate was used to generate exceedingly good cellular responses (Table 1) to MUC1 (Apostolopoulos et al., 1996a,b, 1995a,b). The conjugation conditions to mannan were important—oxidising conditions leading to cellular responses, reducing conditions leading to humoral responses—indeed, of the same type obtained using MUC1 peptides or a MUC1 fusion protein (Table 1). Mice previously susceptible to 5= 10^6 tumour cells were now resistant to 5= 10^7 cell challenge, little antibody was produced, DTH was again present, but this time, $CD8^q$ CTLs could be induced with a high frequency (1/8000).

4. Cytokine profiles after MFP immunisation and MHC restriction

In contrast to the predominant antibody production produced with peptides, fusion protein or reduced MFP, oxidised MFP with cellular responses and little antibody was suggestive of a T1 response. This was confirmed in cytokine studies where the former gave rise to IL-4 secretion and little T1 cytokines, whereas the use of oxidised MFP gave rise to the production of IL-2, g-IFN, IL-12 and no IL-4 a T1 response (Apostolopoulos et al., 1996a,b), i.e. by simply altering the oxidising conditions, a response could be switched from T1 to T2 (Table 1). In addition, it was clearly shown that MFP induced MHC restricted CTL responses (Apostolopoulos et al., 1995a,b).

Nine different mouse strains were readily immunised using oxidised-mannan MUC1 and induced CTLs in a direct assay and a CTLp frequency of ; 1/8000 after three injections. The use of Class I oro mice, H-2 mutants and recombinants clearly

Table 2
Immune responses generated to different antigens in mice using oxidised Mannan

Antigen	CTLp
Listeria peptide	1/17 000
Ovalbumin peptide SIINFEKL	1/15 000
Human papilloma virus E7	
1. Peptide	1/11 000
2. Fusion Protein	1/40 000
Other antigens currently being tested	
Melanoma MAGE-3, gp100, MART-1, Tyrosinase.	
Hepatitis B, C	
Malaria	
CEA	
p53	
Her2/neu	
Influenza	

CTLp: cytotoxic T lymphocyte precursor frequency after three immunisations.

demonstrated the response to be MHC Class I restricted. Furthermore, the MUC1 epitopes binding to K^b, D^b, L^d, D^d and K^k were established.

5. Oxidised mannan—other antigens

The studies described above refer exclusively to the MUC1 peptide from the VNTR. The question that arises is: were the T1/T2 findings restricted to MUC1 or could this be found with other antigens? We have now successfully conjugated other MUC1 peptides, peptides derived from listeria, ovalbumin SIINFEKL, and E7 papilloma peptides and induced satisfactory CTL responses by giving oxidised-mannan antigen immunisations and performing the CTLp frequency assays 14 days later Table 2. A large number of other antigens are currently being examined and we are seeking antigens of veterinary interest to determine if this procedure will work in animals; given the above we have no reason to believe it will not.

6. Mode of action of MFP

At present it is not clear how MFP functions to increase immune responses. Clearly, mannan is important and could bind to the mannose receptor on macrophages and other cells, however, this is not the whole explanation as oxidised and reduced mannan bind to macrophages and to the isolated mannose receptor expressed in transfected COS cells. We consider that macrophages are likely to be the major site of action of MFP as dendritic cells DC. do not bind MFP as macrophages do; the influence of DC or

macrophages on cells or their interactions are currently under examination. However, what we have shown is that peritoneal exudate cells containing approximately 90% macrophages, 5% DC: $F4r80^q$, $33D^y$ cell populations can be satisfactorily immunised in vitro and adoptively transfer immunity to mice and furthermore, one injection of such adoptively transferred cells is equivalent to three in vivo injections of MFP.

7. Increasing the immunogenicity of MFP using T1 or T2 cytokines

As oxidised MFP gives a predominant T1 response, we sought to increase this response using various T1 or T2 stimulators/inhibitory cytokines (Table 3). In these studies, only one injection of oxidised MFP was given compared to three in Tables 1 and 2 above). The oxidised MFP alone gave a frequency of 1/80 000 (Table 3). The addition of IL-2 or IL-12 substantially increased the CTLp frequency—particularly IL-12; whereas IL-4 decreased the frequency. The other cytokines had only a modest effect. These findings have implications for the mode of action of MFP and its clinical use. In other studies, IL-1 has also been found not to alter the CTLp frequency, but to greatly increase antibody production.

In addition, we have found that the simultaneous administration of both oxidised and reduced mannan MUC1—to different sites, both T1 and T2 responses could be induced —findings which have implications for vaccines where *both* antibody and cellular responses are desirable.

Table 3
Cytokines to increase the immunogenicity of MFP[a]

Cytokine	CTLp
	1/80 000
T1-type	
IL-2	1/49 000
INF-g	1/74 000
IL-12	1/19 000
T2-type	
IL-4	1/225 000
IL-10	NT
Other cytokines	
GMCSF	1/54 000
IL-7	1/42 000
In combination	
IFNg+IL-2	1/33 500
IFNg+IL-4	1/39 000[3]
GMCSF+IL-7	1/18 000[4]

[a]NT: not tested; CTLp: Cytotoxic T lymphocyte precursor frequency after one immunisation.
The cytokines were given as recombinant IL-12 cytokines or as part of a vaccinia construct IL-2, g-IFN, IL-4, IL-7, GMCSF given by the intraperitoneal route.

8. Conclusion

The conjugation of different antigens—either as peptides or fusion proteins to mannan under oxidising conditions—appears in mice to preferentially stimulate a T1 type immune response with to production of CTLs and a T1 cytokine profile. By contrast, when the oxidised material is reduced, a T2 cytokine profile occurs with few CTLs and preference for antibody production. From the initial studies with MUC1 peptides we have now extended this to other peptides. It may be this is a preferential mode of inducing T1 responses, but the studies need of be extended to define the optimal modes of and to determine the suitability to such a approach of veterinary practice which is the focus of this meeting. At present clinical studies have been performed in humans; no toxicity was found in Phase 1 studies. It was possible to break tolerance and induce both cellular and humoral anti-MUC1 immune responses in the patients with cancer.

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The immunogenicity of MUC1 peptides and fusion protein

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Abstract

Mucin 1 (MUC1) is highly expressed in breast cancer, has an ubiquitous distribution and, due to altered glycosylation, peptides within the VNTR are exposed. These peptides are the target for anti-MUC1 antibodies, which give a differential reaction on cancer compared with normal tissue. The amino acids, APDTR or adjacent amino acids, are highly immunogenic in mice for antibody production (after immunisation with either breast cancer cells, human milk fat globule (HMFG) or the VNTR peptide). In addition, human studies show that this region of the MUC1 VNTR functions as target epitopes for cytotoxic T cells. We have performed preclinical and clinical studies to examine the immune responses to MUC1 in mice and humans: (a) $MUC1^+$ 3T3 or $P815^+$ 3T3 cells in syngeneic mice are rejected, with the generation of both cytotoxic T lymphocyte (CTL) and DTH responses and a weak antibody response; this type of immunity gives rise to total resistance to re-challenge with high doses of these tumors; (b) immunisation with peptides (VNTR × 2), a fusion protein (VNTR × 5), or HMFG leads to no CTLs, DTH, good antibody production and weak tumour protection (to 10^6 cells, but not 5×10^6 cells) (possibly a TH2 type response); (c) immunisation with mannan-fusion protein (MFP) gives rise to good protection (resistance to 50×10^6 cells), CTL and DTH responses and weak antibody responses (possibly a TH1 type response, similar in magnitude to that obtained after tumor rejection); (d) established tumors can be rapidly rejected by delayed treatment of MFP; (e) the CTL responses are MHC restricted (in contrast to the human studies); (f) APDTR appears not to be the T cell reactive epitope in mice. On the basis of these findings, two clinical trials are in progress: (a) VNTR × 2 (diphtheria toxoid) which gives rise to some T cell proliferation, DTH and antibody responses in some patients and (b) an MFP trial. The ability to alter the immune response towards cellular immunity with mannan or to humoral immunity with peptides, allows the immune response to be selectively manipulated.

Keywords: Vaccine; MUC1 peptides; Fusion protein; Mannan

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1. Introduction

For successful immunotherapy there has to be a suitable target and the immune system has to be stimulated so that an effective antibody response or cellular response (usually cytotoxic lymphocytes) eradicates the tumor. In breast cancer, a number of recent advances have been made wherein a target has been identified and attempts to induce a satisfactory immune response are now being examined. This paper will briefly describe our own studies of immunotherapy of breast cancer using MUC1 peptides and fusion proteins culminating in a clinical trial; no reference will be made to the current work of other laboratories, nor to the use of synthetic carbohydrates for immunisation against breast cancer.

2. Results and discussion

2.1. Human MUC1 molecules as targets for immunotherapy

The structure of mucins have been described in detail elsewhere and a number of different mucins having been identified [1–7]. For the purposes of this study we only consider MUC1 which, in breast cancer, is produced in increased amounts; the cell distribution becomes ubiquitous and altered glycosylation exposes previously hidden peptides. The exposure of these MUC1 peptides provides a satisfactory target for immunotherapy.

When monoclonal antibody technology was established some 15 years ago, a number of murine anti-breast cancer antibodies were produced, many of which appeared to have selective activity on cancers and little or no reaction on normal tissue — most of these appear to act with mucins [8]. With the cloning of MUC1, it was found that many of these antibodies reacted with peptide epitopes in the VNTR of MUC1, indeed with the sequence APDTR. The finding that many antibodies on tissue sections were non-reactive with normal tissue, yet reacted with breast cancer samples, confirmed that peptide epitopes were exposed and using antibodies made to deglycosylated mucins which gave a selective reaction on cancers [9], clearly indicated that the APDTR sequences were more abundant and indeed exposed in breast cancer. These studies also indicated that the APDTR region of the VNTR was immunogenic because it could lead to antibody production and we were able to confirm this by immunising with synthetic peptides which led to the production of antibodies not substantially different from those made by immunising with whole tissue or with mammary mucin (human milk fat globule; HMFG) [10]. Thus, the VNTR, and particularly the APDTR region, was immunogenic in mice particularly for antibody production; it remained to be seen whether cellular immunity could be induced. It should be noted that such findings (extensively described elsewhere) could have been predicted from an analysis of the hydrophilicity plots of the protein structure of the VNTR [11].

2.2. Immunogenicity of MUC1 peptides in mice

It was clear from the foregoing and from the many antibodies that were made that MUC1 is highly immunogenic in mice. While many antibodies react with the carbohydrate coating, e.g. our own 3E1.2 [12] and others, it is apparent that the protein core is highly immunogenic and that many antibodies react with this sequence. Indeed in the First International Workshop on Mucins, all antibodies that reacted with the protein were found to react with the APDTR or related sequence and none were found that reacted outside the VNTR [13]. Using the Geysen method of coupling peptides to pins, it was possible to finitely map the epitopes of the antibodies reacting and all were found to be within the APDTR region with minor variations of amino acids on either side of this. Finally, our own laboratory used synthetic peptides to make second generation antibodies, which gave precisely the same reactivities as those described above, i.e. strong reactions on cancer, virtually no reaction on normal tissue [10]. Thus, the APDTR sequence in the VNTR of MUC1 is highly immunogenic and great progress was therefore made from using crude tumor cells to synthetic peptides containing few amino acids to produce the same antibody response. A word of caution however — protein chemists viewing the sequence of MUC1 from a hydrophilicity viewpoint (which correlates with immunogenicity)

would have predicted that the APDTR sequence would be the most immunogenic. However, the important point is that there is a short immunogenic region in MUC1 which is immunogenic in mice. What is the relevance of this to humans?

2.3. Immunogenicity of MUC1 in humans

The findings of immunogenicity in mice would of itself be of little relevance — everyone knows that foreign proteins can be immunogenic and give rise to either antibodies or cellular responses. The connecting link from these observations to human studies was made by Olivera Finn and colleagues [14,15] who found that in the draining lymph nodes of women with breast cancer are T cells which can be stimulated to react against MUC1 sequences. Finn's work consisted of taking lymph nodes from patients and stimulating them with MUC1 antigen — an interesting feature being the antigen occurring in different cancer cell lines such as T47D and MCF7 and so on. When this was done in the presence of IL-2 (a standard method for making T cell clones), clones were derived which reacted against tumor cells. Of interest was the finding that such reactivity could be blocked by anti-MUC1 antibodies, which demonstrates that there are not only anti-tumor reactive cells in lymph nodes, but that these react to a similar region as found to be immunogenic in mice, i.e. around the APDTR region of VNTR [13]. Studies, particularly in our laboratory, have now gone in several directions: (a) to use the mouse as a model for immunogenicity to MUC1, (b) immunising humans with MUC1 sequences to increase the frequency of the cytotoxic T lymphocytes (CTLs) in the lymph nodes (CTLp-CTL precursors). It is of interest that CTLp have now been found in lymph nodes adjacent to carcinomas of the pancreas, ovary and colon [16,17].

2.4. MUC1 anti-tumor immunity studies in mice

A number of studies have been described which examine the immunogenicity of MUC1 in mice [8–10]; here we will concentrate on our own work. We have made use of two tumors — firstly that obtained from Dr. D. Wreschner and colleagues, consisting of 3T3 cells transfected with a human MUC1 gene which is satisfactorily expressed on the cell surface and a second tumor, P815, which was transfected by Dr. B. Acres and also expresses MUC1. In both of these models, the non-transfected tumors grew unremittingly in the strain of origin (3T3 BALB/c mice, P815 DBA/2 mice) without any obvious evidence of a cellular immune response. However, the transfected lines initially grew and were rejected in times varying from 20–30 days indicating that MUC1 was immunogenic. The mice that rejected the tumor were resistant to subsequent challenge with $MUC1^+$ cells, but had no non-specific immunity to other tumors. Parameters of the rejection were: (a) presence of delayed hypersensitivity (DTH reaction) to MUC1 presented in many different forms — as mucin (HMFG), peptide, fusion protein or as a tumor, (b) cytotoxic T cells, (c) cytotoxic T lymphocyte precursors and (d) little antibody production. Demonstration of CTLs to MUC1 in this model was the first experimental demonstration of anti-MUC1 CTLs in mice. By using antibodies to T cell subsets it was clear that the rejection and CTL cells were $CD8^+$, whereas those mediating DTH were strongly CD4 and weakly $CD8^+$ (the $CD8^+$ DTH cell was itself of some interest, as this has only recently been described in DTH reactions) [18]. Thus, it was reasonable to consider that the rejection response was due to $CD8^+$ cytotoxic cells and the aim should be to induce these with high frequency. Thus, we had a model to study the immunogenicity of MUC1 in mice — there being a 'window' between the host response to MUC1 and the response in an immunised mouse to this [19].

2.5. Immunogenicity of MUC1 peptides in mice

Following the preceding observations, studies were set up immunising mice with synthetic MUC1 peptides — in our case VNTR dimer linked to a carrier — either diphtheria toxoid (DT) or KLH. The 'gold standard' would be early rejection or indeed non-appearance of $MUC1^+$ tumor. In various protocols using synthetic peptides, a fusion protein (to MUC1 containing five VNTR repeats) or HMFG, tumor protection occurred when challenged with a dose of 10^6 cells — this protection had disappeared when a dose 5×10^6 challenging cells were used. It was of interest that

these mice made no CTLs (although a low frequency of CTLp could be detected), a significant DTH reaction occurred and significant antibody responses. We concluded that we were stimulating the wrong 'arm' of the immune response and likely to be generating a TH2 response, with little CTL and significant antibody responses. Other variations in this procedure used immune complexes formed with antigen and antibody and by conjugating the peptide to the surface of sheep red cells. Again, protection occurred, but no total inhibition of tumor growth. Nonetheless, these studies led to a clinical trial (see below). We now have evidence that the response is H-2 restricted in mice, which contrasts with O. Finn's finding in humans where the CTL response is not MHC restricted.

2.6. Murine immune response to mannan-fusion protein

In our quest for a better immunogen that selectively stimulated cellular immunity and gave complete protection (the five reagents used are described above), none was as good as immunising with tumor itself. We then devised a method of conjugating MUC1 (in the form of fusion protein containing five repeats) and the bacterial protein glutathione-*S*-transferase (GST) to mannan which led to some very interesting observations. The conjugation was done under oxidising conditions aiding the formation of Schiff bases; cellular immunity could be generated (see Table 1). The high degree of cellular immunity induced was indicated by protection to a very high dose of tumor cells (5×10^7, which is the highest dose that can be practically administered). This degree of protection was accompanied by the occurrence of significant numbers of CTLps and circulating $CD8^+$ CTLs, a significant DTH reaction, but little antibody, i.e. the major type of immune response we were seeking which was equivalent to or indeed better than the gold standard. At this stage it was not clear how the mannan-fusion protein (MFP) works — it is likely that mannose receptors on macrophages serve to target the MFP to antigen and Schiff bases may lead to more appropriate presentation for a cellular TH1 type of response. At present we are determining where the findings fit into the conventional TH1/TH2 paradigm, but also embarking upon a clinical study. It was of interest to note that only oxidised MFP gave the appropriate cellular responses; when the MFP was reduced it gave the same results as using peptides linked to any other carrier, in that predominantly antibody responses were induced (summarised in Table 1).

2.7. Clinical trial

A clinical trial has been performed in 13 patients using the VNTR dimer (dimerised through disulphide bond) covalently linked to diphtheria tox-

Table 1
Immunogenicity of various MUC1 immunogens in mice[a]

	Tumor protection	CTL	CTLp	DTH	Ab
Tumor	++++	++	++	++	+
Peptide	—	—	—	—	—
Peptide KLH	+ (10^6 cells)	—	+	++	++++
FP GST	+	—	+	++	+++
HMFG	+	—	+	++	+++
Peptide-Ab complex	++	—	+	++	+++
SRBC-peptide	+++	—	NT	++	++
Mannan FP (oxidised)	++++ (5×10^7)	++	+++	++	+
Mannan FP (reduced)	+	—	+	++	+++

[a]++++, excellent; +++, very good; ++, good; +, weak; — , nil; NT, not tested.
CTL, cytotoxic T lymphocytes; CTLp, precursors; DTH, delayed type hypersensitivity; Ab, antibody; KLH, keyhole limpet hemocyanin; FP-GST, fusion protein-glutathione-*S*-transferase; HMFG, human milk fat globule; SRBC, sheep red blood cell.

oid. The point of this study was to examine toxicity and to see if there was any evidence of an immune response to tissues which expressed MUC1 — not only breast, but also lung, salivary gland, pancreas and kidney — and secondly to see if the peptide (which is essentially a self-peptide) was immunogenic in humans. Increasing doses were used starting at 100 μg up to 1 mg of peptide; three injections were given at 14-day intervals and the patients studied. As this was a Phase I study the primary goal was to seek toxicity and immunogenicity; no toxicity was noted and particularly no side-effects or any suggestion of autoimmune disease; the only possible side-effect was the DTH reaction which occurred to repeat administrations of diphtheria toxoid. However, was the material immunogenic? The evidence is that MUC1 peptide was immunogenic in that antibody and T cell proliferative responses occurred with weak skin (DTH) reactivity in several patients. The immunogenicity was not very impressive and at this time we are still attempting to set up the appropriate CTLp assays to perform the studies. These studies, while not demonstrating great immunogenicity, were important as they were the first described wherein patients were injected with MUC1 peptide (although patients in the past have received whole tumor cells which would have certainly included exposed MUC1 peptides). Secondly, some evidence of immunogenicity was shown with no side-effects. On the basis of this, a new trial is now starting with the oxidised mannan-fusion protein and the same observations will be made including the measuring of CTLp to MUC1. We note that other clinical trials are in progress using synthetic carbohydrates [20–22] and peptides with BCG (O. Finn, personal correspondence).

3. Conclusion

New modes of immunotherapy appear every decade or so and as the studies proceed it is clear that the first flourish of excitement dies away and few patients give a prolonged effective immune response — will this be the same with the current immunotherapy? It simply remains to be seen and the clinical trials have to be done. However, with the rapid advance in the production of monoclonal antibodies and in genetic engineering techniques, information accrues very rapidly and now there is a convincing target to which to direct the immunotherapy, i.e. the APDTR exposed sequences. Secondly, there appears to be means at hand to induce both or either humoral or cellular responses at will. The main focus now is trying all possible methods to immunise patients against their own MUC1, but there are many different possibilities which can be examined. Those outlined here are a start, using mannan-fusion protein which selectively stimulates cellular responses. However, in other studies, cytokines are proving of benefit — perhaps the ultimate treatment will be a combination of what is described herein or some other form of induction of cellular immunity aided by the appropriate use of cytokines. What we have now, which is different from the past, is an intimate knowledge of the different steps involved in the immune response and a whole series of reagents which can be used to control the immune system. Hopefully, with these reagents a significant impact on established cancers can be made and then the possibility of immunisation of women against the occurrence of cancer can be considered — at present this remains a dream, but the reality comes closer as clinical trials proceed.

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