A MOLECULAR AND BIOLOGICAL STUDY OF THE ROLE OF B7-1 AND B7-2 ANTIGENS IN THE IMMUNOSTIMULATORY PROPERTIES OF MYELOID LEUKAEMIC CELLS.

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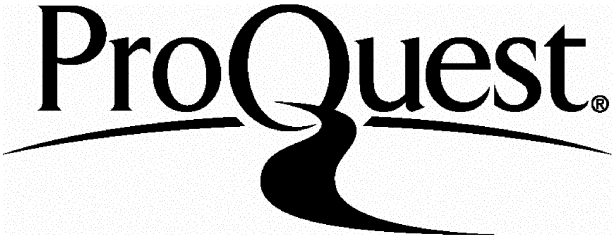

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ABSTRACT

T lymphocytes require two signals for their optimal activation and subsequent proliferation. One of these signals is provided by antigen presented in the relevant HLA groove binding to a reciprocal T cell receptor and the second by a costimulatory molecule interacting with its ligand on the T cell. If antigen is presented to the T cell in the absence of a costimulatory signal the T cell enters into a state of unresponsiveness, termed anergy. The lack of expression of co-stimulatory molecules by tumour cells has been suggested to be a reason why the immune response fails to eradicate the tumour. The major co-stimulatory molecules are B7-1 (CD80) and B7-2 (CD86) which bind to CD28 on the T cell and induction of either of these molecules on tumour cells has resulted in the development of effective anti-tumour immunity in syngeneic murine models.

Phenotyping studies of AML demonstrated B7-1 was infrequently expressed, whereas greater than 50% of the samples analysed expressed B7-2. The duration of first remission was longer in those patients in whom a higher percentage of blasts expressed B7-2 than those that expressed less, suggesting that, in these patients, an anti-leukaemic immune response may be accounting for the longer duration of remission. B7-2+ve AML blasts were better able to stimulate allogeneic T cells to proliferate and produce interleukin-2 (IL-2) when compared to B7-2-ve AML blasts. This suggests that the B7-2 was functional and that expression of B7-2 by the AML blasts may induce an anti-leukaemic immune response, if a suitable immunogenic peptide was expressed.

Transfectants expressing B7-1 or B7-2 were generated in the "293" cell line to compare their co-stimulatory ability. The transfectants were used in a costimulation assay to stimulate allogeneic T cells to show the molecules were functional. In a cytotoxicity assay greatest killing was seen with the B7-1 293 cells as stimulators, followed by the B7-2 293 cells and finally the control 293 cells, suggesting B7-1 is the preferred co-stimulatory molecule for tumour vaccination strategies.

To overcome lack of costimulation by the AML blasts leading to anergy in potential tumour reactive T cells, AML cells were transduced with recombinant AAV containing an expression cassette for B7-1 or B7-2. Compared to control AML blasts, the B7-1 or B7-2 expressing AML blasts induced greater proliferation in allogeneic T cells. This suggests that the strategy of inducing expression of costimulatory molecules on AML cells may be effective in the induction of an anti-leukaemic immune response. It is hoped that in the future these modified leukaemic cells will be used to generate autologous anti-leukaemic T cells ex vivo, which may, after expansion be returned to the patient as specific anti-leukaemic cellular therapy.

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1. INTRODUCTION

1.1 HISTORY OF IMMUNOTHERAPY OF CANCER

The concept of immunotherapy of cancer dates back to the 16th century when it was first noted that patients with some concurrent infections had a coincident reduction in their malignancy (Starnes, 1992). The pioneer of the application of immunotherapy in the treatment of cancer was the New York surgeon William Coley, whose work celebrated its 100th anniversary in 1993. He reported tumour regressions in patients injected with pyogenic bacterial extracts which he postulated to stimulate the immune system in a non specific manner, resulting in an enhanced immune response against the tumour. Using this treatment he achieved a cure rate of up to 10% in patients with inoperable sarcoma (Starnes, 1992).

Modifications of this approach are still in use with the application of bacillus Calmette-Guerin (BCG) in the treatment of superficial bladder carcinoma where it was found that inoculation into the tumour of BCG lead to improved survival (Morales et al., 1976). The mechanism of action is unknown, but an inflammatory response was seen in the bladder epithelium, with upregulation of MHC class II expression and recruitment of CD4+ve T cells into the bladder wall (Bohle et al., 1990). That the immune system was involved was demonstrated in a mouse bladder tumour model where BCG inoculation elicited a CD4+ve T cell response in draining lymph nodes that reacted with BCG antigens presented by MHC class II molecules on epithelial tumour cells (Lattime et al., 1992). This suggests that the tumour cells may express BCG

peptides in the context of MHC class II, leading to the generation of a T cell response which is lytic to tumour cells.

BCG has also been used as an adjuvent in the treatment of patients with malignant melanoma with some success. Remissions have been obtained in some patients injected intradermally with autologous tumour cells together with BCG (Berd et al., 1990). In some cases the tumour cells were haptenised with dinitrophenyl to improve their immunogenicity, with response rates of up to 50% (Murphy et al., 1993). With the isolation of cytotoxic T lymphocyte (CTL) clones from patients immunised with melanoma whole cell vaccine, with activity against melanoma cell lines, it became apparent that a cell mediated immune response was responsible for the clinical responses (Hayashi et al., 1993). Furthermore it also suggested that the tumour cells evaded immune recognition at the outset in order to have escaped immune mediated destruction.

Studies have been performed in which patients with acute myeloid leukaemia, in addition to standard chemotherapeutic regimes, have been vaccinated subcutaneously with a mixture of allogeneic myeloid leukaemia cells and BCG. It was hoped that an immunological response would be elicited against a common antigen specifically expressed on the AML blasts. In one study a significant increase in survival was seen in patients receiving vaccination with BCG and AML blasts in addition to chemotherapy, as opposed to chemotherapy alone. However, prolongation in survival was mainly due to increased survival time after relapse, as opposed to freedom from disease (Powles, 1974). Other studies found no benefit with this strategy of tumour vaccination with a combination of allogeneic AML blasts and BCG (Russell et al., 1976).

More recently, with regard to AML, has been the use of interleukin 2 post autologous bone marrow transplantation, which appeared to significantly decrease the risk of relapse when compared to standard chemotherapy (Benyunes et al., 1993). There have also been documented cases of induction and maintenance of remission in patients with relapsed AML with treatment with IL-2 alone, the mechanism of which was thought to be *in vivo* generation of lymphokine-activated killer cells and subsequent lysis of the malignant cells (Foa et al., 1990). A major drawback to IL-2 therapy has been treatment related mortality related to the development of capillary leak syndrome (Hamon et al., 1993) and in the context of standard chemotherapy, a small study of nine patients suggested no benefit with IL-2 therapy, with relapse occurring in six of the patients (Macdonald et al., 1990).

1.2 THE IMMUNE SYSTEM

1.2.1 Cell-mediated Immunity

T lymphocytes recognise antigens generated within cells, which may be derived from intracellular peptides, such as viruses, or extracellular proteins which are internalised from the extracellular fluid. T cells are broadly divided into two groups, the CD4+ve T cells which recognise peptides presented in the context of MHC class II and CD8+ve T cells which recognise peptides presented in the context of MHC class I molecules.

MHC class I molecules are expressed on most cells and are composed of one α chain non covalently linked to a smaller chain, β_2-microglobulin, presenting peptides originating from the cytosol. The cytosolic proteins are degraded into peptides in the cytosol by a protease complex consisting of 28

subunits, called the proteosome. The resulting peptides are transported into the lumen of the endoplasmic reticulum by two ATP-binding cassette proteins associated with the endoplasmic reticulum termed Transporters associated with Antigen Processing-1 and -2 (TAP-1 and -2). In the endoplasmic reticulum the peptides bind to partially folded MHC class I molecules which are then released from a membrane bound protein, calnexin. The α_1 and α_2 domains of the α-chain pair to form a cleft that is the site of peptide binding. The peptide:MHC complexes are then transported to the cell surface. Individual peptides bind to MHC class I molecules at two or three specific amino acids along the peptide sequence, which are termed the anchor residues. Changing any anchor residue prevents binding to the MHC binding cleft, but because of folding of the peptide in the groove the anchor residues are not necessarily at the same position in all peptides. This enables any given MHC class I molecule to bind a wide variety of different peptides.

MHC class II molecules are principally expressed on lymphoid and myeloid cells and are composed of one α and one β chain delivering peptides from the vesicular compartment of the cell derived from the extracellular fluid. MHC class II molecules are also assembled in the endoplasmic reticulum, where they are bound to a protein called the invariant chain (Ii), the CLIP fragment of which prevents binding to intracellular peptides. The MHC:Ii complexes are then transported through the Golgi apparatus to acidified endosomes containing engulfed extracellular proteins. In the endosomes the acidified environment activates endosomal proteases which cleave Ii and remove the CLIP segment from the class II binding site with the help of HLA DM. The exposed class II binding site can then interact with a ligand, which is

followed by transport of the complex to the cell surface for recognition by T cells. The site of peptide binding on MHC class II molecules is formed by pairing of the α_1 domain with the β_1 domain to form a cleft in a manner analogous to MHC class I molecules. In contrast to MHC class I molecules, peptides bound in the MHC class II cleft do not contain anchor residues, lying in an extended conformation along the groove, the end of the clefts being open. Peptides presented by MHC class I molecules tend to consist of 8-10 amino acids, whereas peptides presented by MHC class II tend to consist of at least 13 amino acids.

Specificity of a T cell response against peptides presented in the context of MHC class I or II, is provided by the T cell receptor, which is composed of one α- and one β-chain. The extracellular portion of each molecule consists of a constant domain and a variable domain which is involved in peptide recognition and confers the specificity of the T cell response. The variable domain of the α-chain is produced by recombination of variable (V) and joining (J) gene segments, to produce a functional exon that, after splicing to the constant (C) gene segment, is translated to yield the TCR α-chain. The TCR β-chain is produced by recombination of variable (V), diversity (D) and joining (J) gene segments, which are then spliced to the C gene segment. After transcription the TCR β-chain pairs with the α-chain to yield the α:β heterodimer. The variable domain of the T cell receptor consists of three loops termed CDR1, CDR2 and CDR3, the diversity being concentrated in the CDR3 loop, which binds to the peptide presented in the appropriate MHC groove. In addition to T cells expressing a TCR composed of an α-chain and β-chain are T cells expressing a γ-chain and δ-chain. These T cells are termed γ:δ T cells and

express the γ:δ TCR due to absence of a silencer protein which acts as a transcriptional repressor of the γ-chain genes.

T cells are stimulated by peptide presented by MHC class I or II and once activated are able to kill cells expressing the specific peptide by two main mechanisms. The first of these is termed apoptosis, which is a normal process the body uses to maintain homeostasis. Apoptosis is characterised by the degradation of the DNA into 200 bp fragments through activation of endonucleases, characterised initially by the preservation of the cell membrane. T cells induce apoptosis in the target cell by the binding of fas (CD95) on the target cell by fas ligand expressed on both CD4 and CD8 T cells. As well as inducing target cells to undergo programmed cell death, CD8+ve T cells can induce necrosis. Necrosis is induced by the release of secretory granules by the T cell on recognition of the antigen on the surface of the target cell. The granules contain two main classes of protein, the first perforin and the second granzymes. On release from granules perforins form polymers which aggregate to form cylindrical structures which insert into the target cell lipid bilayer disrupting the integrity of the cell membrane and allowing entry of granzymes which result in nuclear degradation similar to that seen with apoptosis.

1.2.2 Antigen Presenting Cells

Antigen presenting cells (APC) are cells that specialise in presenting antigen via MHC class I or II to naive T cells. Cells that are capable of antigen presentation include macrophages and B cells. In addition to B cells and macrophages are dendritic cells which are located throughout the body and

whose sole function is antigen presentation. They are classified into interstitial DCs in the heart, kidney, gut and lungs, Langerhans cells in the skin and mucous membranes, interdigitating DCs in the thymic medulla and secondary lymphoid tissue and in the blood are termed veiled cells. All the above DCs specialise in presenting antigen to T cells, whereas follicular DCs found in the primary and secondary B-cell follicles retain antigen for long periods of time and are thought to be implicated in immune memory and maintaining humoral immune responses.

DCs are characterised by their characteristic branched morphology, the expression of $CD1_a$, high levels of MHC class II and the adhesion molecules LFA-3, ICAM-1 and ICAM-3 which bind to CD2 and LFA-1 on the T cell. DCs have low phagocytic activity, contain intracytoplasmic structures termed Birbeck granules, which are specifically found on Langerhans cells and are potent inducers of proliferation of naive allogeneic T cells. DCs also express CD40 which on binding to CD40 ligand (CD40L) upregulates expression of the co-stimulatory molecules B7-1 and B7-2. B7-1 and B7-2 bind to a receptor on the T cell, CD28, which provides a second necessary signal, in addition to an antigen specific signal through the T cell receptor, required for T cell activation (Linsley et al., 1991; Azuma et al., 1993b). The two signal model of T cell activation will be discussed in section 1.3.

1.3 TWO SIGNAL MODEL OF T CELL ACTIVATION

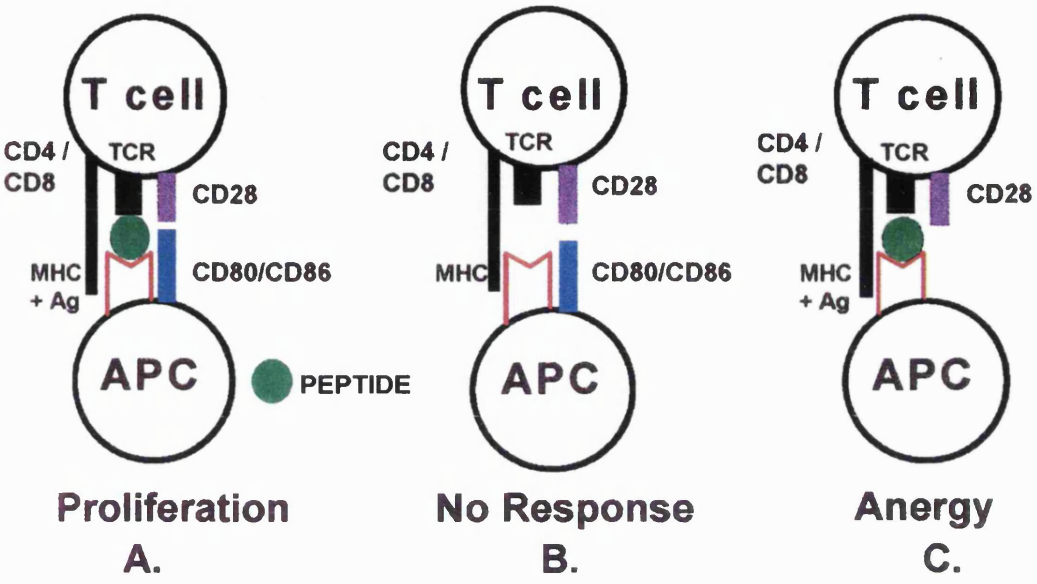
That T lymphocytes required two signals for activation was proposed in 1970. It was suggested that lymphocyte activation required a second signal in addition to an antigen specific signal, whereas an antigen specific signal alone would result in paralysis (anergy) of that population of T cells (Bretscher and Cohn, 1970). The first signal is provided by peptides bound to the MHC molecules binding to the T cell receptor(Schwartz, 1985) and for optimal activation and proliferation a second concurrent costimulatory signal is required. The best studied co-stimulatory molecules are B7-1 and B7-2 which bind to CD28 or CTLA-4 on the T cell. B7-1 and B7-2 are described in sections 1.5 and 1.6 respectively, CD28 in section 1.7 and CTLA-4 in section 1.8. In the absence of a costimulatory signal the T cell enters an unresponsive state known as clonal anergy (Schwartz, 1990). The two signal model of T cell activation is illustrated in figure 1.

1.3.1 Identification of Co-stimulatory Molecules

Localisation of costimulatory molecules to antigen presenting cells (APC) was shown in 1988 when it was noted that stimulation of IL-2 producing T cell clones with chemically modified APC's plus antigen resulted in a state of anergy. Anergy was defined as a failure of T cells to respond to subsequent stimulation with unaltered APC and antigen. However addition of allogeneic spleen cells to the assay resulted in T cell proliferation. Subsequent fractionation of the spleen cells showed that this activity was restricted to B cells or macrophages and was termed co-stimulatory activity (Jenkins et al.,

1988). Further studies demonstrated the role of the CD28 surface antigen on T cells in mediating a costimulatory signal, by the finding that signalling through CD28 together with stimulation through the TCR/CD3 complex enhanced the production of IL-2, γ-interferon, TNF-α and GM-CSF by the T cells (Thompson et al., 1989). The ligand for CD28 was subsequently determined by the transfection of the cDNA for CD28 into Chinese Hamster Ovary (CHO) cells. The transfected cells were shown to bind to human B cell lines and activated B cells. This could be inhibited by an anti-B7-1 mAb (Linsley et al., 1990). The ability of B7-1 to provide a costimulatory signal to T cells, which were stimulated through the T cell receptor with anti-CD3 mAb, was demonstrated using CHO cells expressing B7-1 or immobilised anti-B7-1 mAb (Linsley et al., 1991a).

Figure 1 Two signal model of T cell activation.



Legend to figure 1:

A.- Concurrent signalling through the T cell receptor and CD28 results in optimal T cell activation.

B.- Signalling through CD28 in the absence of an antigen specific signal through the T cell receptor has no effect on the T cell.

C.- Signalling through the T cell receptor without a co-stimulatory signal induces anergy in potentially responsive T cells.

1.3.2 Lack of Costimulation Induces Anergy

Confirmation of the role of CD28 in the prevention of T cell anergy, defined as being unable to respond to specific antigen under optimal activation conditions, was determined using the following murine model. A murine T cell clone (L1) exposed to both specific antigen and spleen cells, as a source of co-stimulatory molecules, proliferated on reexposure to the specific antigen (λ phage protein). However if the T cell clone was initially exposed to specific antigen and chemically fixed spleen cells, preventing delivery of a co-stimulatory signal, proliferation was vastly decreased. Addition of anti-CD28 mAb to the T cell clone and fixed spleen cells in the initial stimulation, resulted in restored proliferation on restimulation. This demonstrated that signalling through CD28 prevented the induction of anergy in T cells (Harding et al., 1992). Further evidence of the role of the CD28/B7-1 interaction in preventing the induction of anergy was demonstrated in an *in vitro* cell model. Peptide treated cells, either B7-1+ve or B7-1-ve, were used to stimulate T cells and then after 48hrs the T cells were restimulated with peptide treated B7-1+ve or B7-1-ve cells. T cells initially exposed to B7-1-ve cells were unable to respond to secondary proliferation with either the B7-1+ve or B7-1-ve cells, whereas the T cells initially stimulated with B7-1+ve cells proliferated on rechallenge with either the B7-1+ve or B7-1- cells as stimulators. The role of B7-1 in the primary stimulation and prevention of anergy was confirmed by the addition of CTLA4-Ig to the initial stimulation with B7-1+ve cells which induced a state of anergy in the T cell population (Gimmi et al., 1993). CTLA4-Ig consists of the extracellular domain of CTLA-4 fused to the hinge region of the immunoglobulin

heavy chain. CTLA4-Ig blocks costimulation as CTLA-4 has a high affinity for B7-1 and B7-2 (section 1.8).

The intracellular mechanisms of anergy have also been studied. TCR engagement by specific peptide activates protein tyrosine kinases leading to phosphorylation of protein substrates (June et al., 1990). Phosphorylation of the ζ-chain of the TCR complex, by the *src* family of tyrosine kinases, *lck* and *fyn*, results in binding of the ζ-associated tyrosine kinase ZAP-70 (Irving and Weiss, 1991; Chan et al., 1992). After binding of ZAP-70 to the ζ-chain, it is tyrosine phosphorylated leading to downstream signal transduction via protein kinase C (PKC) (Chan et al., 1992). Superantigens bind to the Vβ region of the TCR inducing T cell proliferation and in mice intrathymic exposure results in deletion of thymocytes expressing that particular Vβ variable region. *In vivo* exposure of mice to the superantigen staphylococcal enterotoxin B (SEB) results in anergy of SEB-reactive T cells. T cells anergised to SEB failed to produce IL-2 on restimulation with SEB, but produced IL-2 on exposure to PMA and calcium ionophore, indicating that the defect in T cell signalling was at a level proximal to PKC. It was found that TCR signalling in the anergic cells did not induce tyrosine phosphorylation of the TCR ζ-chain and there was impaired association of ζ-chain and ZAP-70 (Migita et al., 1995). Association of ζ-chain and ZAP-70 results in tyrosine phosphorylation of ZAP-70 which leads to phosphorylation of other cellular substrates (Chan et al., 1995). The defect in anergic T cells may therefore involve abnormalities of the *src* family of tyrosine kinases which tyrosine phosphorylate the ζ-chain (Iwashima et al., 1994).

1.3.3 Role of CD28 in Activation of Specific T cell subsets

T cells are a heterogeneous population and the role of CD28 in the costimulation of the various subsets has been studied. The role of costimulation in the proliferation of T_H1 clones was demonstrated using established murine T cell clones (Williams and Unanue, 1990). Culture of the T_H1 clones with immobilised anti-CD3 mAb resulted in failure to proliferate and unresponsiveness to subsequent exposure to specific peptide. Addition of splenic accessory cells resulted in proliferation of the T_H1 clones on exposure to anti-CD3 mAb and it was demonstrated that for a successful costimulatory signal to be delivered the splenic cells had to be in physical contact with the T cells.

Signalling through CD28 is also required by T_H2 T cells. T_H2 cells concurrently stimulated through the T cell receptor and CD28 upregulate production of IL-1 which increases their responsiveness to IL-4 resulting in increased proliferation (McArthur and Raulet, 1993).

The role of CD28 in the generation of cytotoxic T cells was demonstrated by the ability of human peripheral T cells to lyse P815 cells (murine mastocytoma) expressing human B7-1 in the presence of anti-CD3 mAb, which was inhibited by the addition of either anti-CD28 or anti-B7-1 mAb (Azuma et al., 1993a). It was also demonstrated that this cytotoxicity could be mediated by both CD4 and CD8 T cells. T cells can also be subdivided into naive cells, which have not previously responded to a peptide and memory T cells which have previously responded to antigen. Naive T cells express a surface antigen CD45RA and memory T cells express CD45RO (Beverley, 1990). Further analysis of the T cells mediating the cytotoxity against the B7-1+ve P815

targets demonstrated that these were CD45RO+ve and depletion of T cells expressing either HLA-DR or CD25 demonstrated that the cytotoxicity was not mediated by pre-existing activated T cells. Selection of CD45RA+ve (naive) and CD45RO+ve T cells demonstrated the CD28/B7 pathway was important in the costimulation of both naive and memory T cells (Azuma et al., 1993a). A similar study analysed the role of CD28-B7 interactions in the induction of CD8 cytotoxic T cells, demonstrating that B7-1 transfected P815 cells resulted in selective proliferation of CD8+ve T cells with potent cytotoxic activity against parental P815 cells, which was not seen with B7-1-ve P815 cells as stimulators. CD8+ve T cells from the B7-1+ve P815 cultures were then tested for cytotoxicity against both B7-1+ve P815 and parental P815 cells, which was found to be equivalent, demonstrating that although B7-1 is essential for induction of a cytotoxic T cell response, it is not required in the effector phase of the cytotoxic T cell response (Harding and Allison, 1993). This is of relevance to the strategy of creating tumour vaccines by inducing expression of B7-1 on the malignant cell, in that the altered cells stimulate tumour reactive cytotoxic T cells which should then be able to kill unmodified tumour cells. In the above experiment, addition of anti-IL-2 during the induction phase inhibited the generation of CTL to B7-1+ve P815 demonstrating that costimulation resulted in the secretion of IL-2 by the CD8+ve T cells which allowed for proliferation of the antigen specific CTL.

The TCR on most T cells is composed of one α- and one β-chain and are termed α:β T cells. During their development, α:β T cells express a protein which inhibits transcription of a gene coding for a protein called γ-chain. In the absence of the repressor protein the T cells express the γ-chain and produce a

TCR composed of one γ-chain and one δ-chain, γ:δ T cells. One of the differences between γ:δ T cells and α:β T cells is that γ:δ T cells do not express either CD4 or CD8 (Bluestone et al., 1991). The role of costimulation in the activation of γ:δ T cells was demonstrated by the absence of proliferation in the absence of signalling through CD28, the first signal being provided either by alloantigen or cross-linking of the TCR with mAb (Sperling et al., 1993).

1.4 IMMUNOSURVEILLANCE AND ESCAPE FROM IMMUNE RECOGNITION

Evidence that the immune system was involved in the control of certain malignancies was implicated by the increased frequency seen in chronically immunosuppressed patients, either due to disease or immunosuppressive medication. In patients receiving immunosuppressive drugs after organ transplantation an increase in non-Hodgkin's lymphoma (NHL), carcinoma of the skin, lips, vulva and perineum, Kaposi's sarcoma and kidney have been reported (Penn, 1994). In patients infected with HIV type 1 an increase in NHL, Kaposi's sarcoma (KS) and in children, cervical carcinomas has been reported (Mueller and Pizzo, 1996). However, the development of these malignancies may not only be related to immunodeficiency, many oncogenic viruses are detected in the tumour, for example EBV in NHL and in KS Kaposi's sarcoma-associated herpesvirus (HHV8) (Tossing, 1996). That the immune system is important in preventing the tumours from occurring can be demonstrated by the success of infusing EBV specific cytotoxic T cells in inducing remission in patients developing NHL after allogeneic BMT(Smith et al., 1996). There have also been reports of spontaneous remissions of malignant diseases, which may

be immune mediated (Beguin et al., 1985). A variety of mechanisms have been suggested as to how tumour cells escape immune mediated destruction in patients with a normal immune system.

1.4.1 Role of MHC Class I

Failure to express major histocompatibility complex (MHC) class I molecules has been proposed as a reason some tumour cells evade the immune system (Tanaka et al., 1988). Loss off MHC class I expression by tumours may occur through a variety of mechanisms, including mutations in the gene encoding β_2-microglobulin, preventing the generation of MHC class I molecules in the endoplasmic reticulum (Wang et al., 1993), the loss of peptide transporter genes (Franksson et al., 1993) or altered binding of regulatory factors to MHC class I enhancer sequences (Blanchet et al., 1992). To assess the role of MHC class I expression in the immunogenicity of tumours Restifo et al. transfected a sarcoma cell line with the γ-interferon (γ-IFN) gene resulting in the upregulation of MHC class I expression and the induction of tumour infiltrating lymphocytes, which were therapeutic in a murine model of metastatic disease (Restifo et al., 1992).

1.4.2 Immunosuppression Mediated by Tumour Cells

Some tumour cells can cause selective depletion or suppression of T cell populations, resulting in escape from immune responses. In mice, binding of superantigens to particular Vβ regions results in apoptosis of T cells expressing that particular Vβ variable region. Mice inoculated with C4 HAN preneoplastic mammary lesions were found to be depleted of a subset of T lymphocytes

which expressed the TCR Vβ2 phenotype. This suggests that the benign C4 HAN tumours express a Vβ2-restricted superantigen which induces the deletion of this subset of T cells (Wei et al., 1992). Tumour cells may also suppress T cell responses by the secretion of humoral substances. Fibrosarcoma tumours in mice were found to maintain the ability to present antigen, but a progressive loss in CD4 T cell function emerged which was thought to be mediated by TGF-β (Zou et al., 1992). In a further murine sarcoma model, tumours of more than 30 days development led to a progressive decline in CD8 T cell function in the host. This effect was shown to be conferred by an unidentified cytokine present in the tumour cell culture supernatants (Loeffler et al., 1992). Some tumour cells secrete transforming growth factor (TGF-β) which inhibits both production of γ-interferon (γ-IFN) and proliferation of T cells (Lagadec et al., 1996). Interleukin-10 (IL-10) is another cytokine produced by some tumour cells which suppresses the secretion of γ-IFN by T_H1 T cells and may inhibit the generation of a cytotoxic T cell response (Sulitzeanu, 1993).

Downregulation of the ζ-chain in the T cell receptor in patients with malignancy has also been noted, which prevents the production of IL-2 and subsequent proliferation of potentially tumour reactive T cells. Interestingly this defect can be overcome by concurrent stimulation through CD28, which returned a normal pattern of response to the T cells (Renner et al., 1996). This observation is of particular importance to the strategy of inducing expression of B7-1 or B7-2 on malignant cells, which would presumably overcome this signalling defect in the T cell population. However, a murine model of leukaemia, in addition to demonstrating downregulation of the ζ-chain, demonstrated a suppressor factor secreted by the leukaemic cells which had an

inhibitory effect on naive T cells (Hirst et al., 1997). This suggests that more than one mechanism may be implicated in lack of immune recognition of tumour cells *in vivo.*

1.4.3 Lack of Costimulation resulting in Escape from Immunosurveilance

Another mechanism whereby tumour cells may evade immune recognition and therefore destruction, is their inability to provide an adequate costimulatory signal, which is necessary for optimal T cell activation. Costimulation of T cells is described in section 1.3. The first costimulatory molecule to be identified was B7-1 (CD80) (Freedman et al., 1987), which binds to CD28, which is innately expressed on most T cells (Aruffo and Seed, 1987). Using a poorly immunogenic murine melanoma cell line K1735 which did not express B7-1, it was demonstrated that induction of B7-1 expression led to rejection of the tumour and protection against subsequent exposure to the parental tumour (Chen et al., 1992). This strategy will be discussed in more detail in section 1.13.

1.5 CLONING OF B7-1

In 1987 the first member of the B7 family was identified by a group examining B cell activation antigens (Freedman et al., 1987). BALB/c mice were immunised with syngeneic B cells that had been preactivated with anti-immunoglobulin coupled to polyacrylamide beads which cross-linked surface immunoglobulin. Monoclonal antibody (mAb) producing cell lines were then produced by somatic cell hybridisation and one clone was found to be highly reactive with the immunising cells, as well as several B cell lines. This mAb was subsequently termed anti-B7. The anti-B7 mAb was used to identify cell types that expressed the B7 antigen, which was found to be mainly expressed on a subpopulation of B cells. B7 antigen levels increased after *in vitro* activation with anti-immunoglobulin beads. Analysis of tumour cells by the same group demonstrated expression of B7 on B cell chronic leukaemias, including chronic lymphocytic leukaemia (CLL), prolymphocytic leukaemia (PLL) and hairy cell leukaemia (HCL), with some expression by Hodgkin's and non Hodgkin lymphoma cells of B cell origin. No B7 was detected on any malignancy of T cell origin or on the blasts from patients with either acute lymphocytic leukaemia (ALL) or acute myeloid leukaemia (AML). The B7 protein was characterised by immunoprecipitation from the Burkitt`s lymphoma cell line, Raji, demonstrating a single chain cell surface protein with a molecular weight of approximately 60,000 Da.

In 1989 the gene coding for B7 was identified by the same group (Freeman et al., 1989). A cDNA library was made from Raji cells and pools of

clones transfected into COS cells, which are derived from monkey kidney cells. B7 expressing cells were identified by immunoprecipitation with anti-B7 mAb. The episomal DNA was recovered by a process called Hirt extract and transformed into E. coli. Plasmid DNA was prepared from individual colonies and found to contain a cDNA insert of between 1.4 and 1.6 kb. Transfection of these clones resulted in B7 expression in fresh COS cells. Immunoprecipitation from the B7 transfected COS cells using the anti-B7 mAb revealed a broad protein band of 44 to 54 kDa. The B7 cDNA was found to consist of 1491 base pairs with an open reading frame extending from nucleotides 318/320 to 1181. The polypeptide was predicted to be composed of 262 amino acids consisting of a 216 amino acid extracellular domain, a 27 amino acid transmembrane region and a 19 amino acid cytoplasmic domain. There were eight potential N-linked glycosylation sites (Asn-X-Ser/Thr), all located in the extracellular region of the molecule. When compared with databases of previously characterised proteins, homology was found with members of the immunoglobulin superfamily, in particular with two immunoglobulin like domains in the extracellular region between residues 1 and 112 and 113 and 210. The first immunoglobulin domain had the characteristics of a V set domain and the second with that of a C1 set domain.

1.6 IDENTIFICATION OF A SECOND LIGAND FOR CD28

After B7 was characterised it became apparent that more than one ligand for CD28 existed, as anti-B7 only partially inhibited responding lymphocytes. B7 was subsequently renamed B7-1 and the new ligand B7-2. In mixed lymphocyte cultures preincubation of both responder and irradiated stimulator cells from MHC mismatched normal human donors with CTLA4-Ig inhibited primary proliferative responses by 50-85%. In contrast preincubation with the anti-B7-1 monoclonal antibody BB1 inhibited the MLR by only 30% (Tan et al., 1993) , suggesting the existence of more than one ligand for CD28. In addition, monoclonal antibodies thought to bind to the same B7 antigen did not stain target cells or tissues equivalently, an example being keratinocytes which stained with the monoclonal antibody BB1 but not with the anti-B7 antibody (Nickoloff et al., 1993) . Finally, in B7 knockout mice, mixed lymphocyte reactions were reduced to only 30% of normal controls and activated B cells from the mice retained the ability to bind CTLA4-Ig, providing strong evidence for the existence of additional members of the B7 family of molecules (Freeman et al., 1993a).

The gene coding for B7-2 was cloned by both Freeman and Azuma in 1993 (Freeman et al., 1993b; Azuma et al., 1993b). Azuma's group generated a monoclonal antibody (IT2) by immunising BALB/c mice with cells from a B-lymphoblastoid cell line (JY B-LCL) and subsequently fusing the splenocytes with P3U1 myeloma cells. Supernatants were screened using the human NK-like leukaemia cell line YT2C2 which preferentially kills cell lines expressing B7

(Azuma et al., 1992). Anti-CD28 antibody alone completely inhibited, whereas anti-B7 antibody only partially inhibited, YT2C2 cytotoxicity against JY cells. Addition of the supernatant containing IT2 together with saturating amounts of anti-B7 antibody totally inhibited lysis of JY B-LCL cells by YT2C2 demonstrating blockade of the second ligand for CD28. In order to demonstrate that IT2 was not binding B7, murine P815 mastocytoma cells were transfected with human B7, which IT2 but not anti-B7 failed to bind to. In addition IT2 inhibited binding of CTLA4-Ig to JY cells but did not affect binding of CTLA4-Ig to B7 transfected P815 cells and precoating of JY cells with both anti-B7-1 and IT2 antibodies completely inhibited binding of CTLA4-Ig. This confirmed the existence of a second ligand for CD28, which was recognised by the mAb IT2 (Azuma et al., 1993b).

1.6.1 Cloning of B7-2

Azuma's group cloned the cDNA coding for B7-2 transfecting COS7 cells with a cDNA library derived from JY B-LCL and screening the transfectants with both IT2 and CTLA4-Ig. Positive cells were isolated and the plasmid recovered by Hirt extract which was then transformed into *E.Coli.* Sequencing of the cDNA demonstrated that it coded for a 323 amino acid consisting of a N-terminal signal peptide, a 223 amino acid extracellular domain containing eight sites for N-linked glycosylation, a 23 amino acid transmembrane domain and a 61 amino acid cytoplasmic domain containing three potential sites for protein kinase C dependant phosphorylation. Surprisingly, considering that they shared the same receptors, CD28 and CTLA-4, B7-1 and B7-2 have limited amino acid homology but in common with B7-1 the extracellular domain of B7-2

consisted of a variable and C2, immunoglobulin like, domain. Immunoprecipitation demonstrated a single glycoprotein of 70K whereas anti-B7-1 precipitated a smaller glycoprotein of 55-60K.

Freeman's group cloned the cDNA coding for B7-2 from an activated B cell cDNA library and transfected COS cells with the construct (Freeman et al., 1993b). The expressed B7-2 protein bound CTLA4-Ig, but not the control immunoglobulin or anti-B7-1 monoclonal antibody, demonstrating that B7-1 was distinct from B7-2. The cDNA contained an open reading frame of 987 nucleotides coding for a 220 amino acid extracellular region, a 23 amino acid transmembrane domain and a 60 amino acid intracellular portion in contrast to Azuma's clone in which the extracellular domain was predicted to consist of 220 amino acids and the intracellular portion 61 amino acids. COS cells transfected with either B7-1 or B7-2 resulted in T cell proliferation and IL-2 production, with either phorbal 12-myristate 13-acetate (PMA) or anti-CD3 monoclonal antibody (mAb) providing the first signal. Proliferation induced by B7-1 was inhibited by anti-B7-1 mAb and confirming that B7-2 signalled through the CD28 pathway, blocking anti-CD28 mAb was shown to inhibit proliferation mediated by B7-2. They postulated that B7-2 provides the initial costimulatory signal, as it is present on resting monocytes and early on activated B cells and that B7-1 is important in driving T cell clonal expansion as expression occurs 24 hours after activation of monocytes and B cells (Freeman et al., 1989).

Expression of B7-2 was determined on a variety of normal cells and cell lines by flow cytometric analysis and found to be highly expressed on human B-LCL and the Burkitt's B lymphoma cell lines Daudi and Raji. Resting peripheral blood B cells expressed low levels of both B7-1 and B7-2 , but activation with

lipopolysaccharide, pokeweed mitogen or by crosslinking CD40 induced high levels of expression of both B7-1 and B7-2. Similarly resting T cells were negative for both molecules but after 10 days culture with anti-CD3 both were induced. However, in fresh resting monocytes only low amounts of B7-1 were expressed compared to significant expression of B7-2, which could be further augmented by 24 hours incubation with γ-interferon (Azuma et al., 1993b).

1.7 FUNCTION OF CD28

CD28 was cloned by constructing a plasmid cDNA library from a human T cell tumour line. The libraries were introduced into COS cells where cells expressing surface CD28 were isolated using a monoclonal antibody. The plasmid DNA was recovered from the positive cells using a Hirt extract and the plasmid transformed into Escherichia coli. The cDNA was found to code for a mature protein of 202 amino acids and immunoprecipitation from transfected COS cells demonstrated that CD28 is a 42-kDa homodimer (Aruffo and Seed, 1987). CD28 is constitutively expressed on virtually all murine T lymphocytes, on 95% of human peripheral blood CD4+ve T cells and on approximately 50% of human CD8+ve T cells (Aruffo and Seed, 1987). Ligation of CD28 together with signalling through the T cell receptor results in upregulation of the α,β and γ chains of the IL-2 receptor (Cerdan et al., 1992; Cerdan et al., 1995; Freeman et al., 1995). IL-2 mRNA transcription is increased (Lindstein et al., 1989) resulting in increased IL-2 secretion and subsequent T cell proliferation (Gimmi et al., 1991; Linsley et al., 1991a). Transcription of CTLA-4 mRNA is also increased (Freeman et al., 1992b) and upregulation of CD40 ligand occurs (de Boer et al., 1993).

1.7.1 Intracellular Signalling After CD28 Ligation

The intracytoplasmic domain of CD28 consists of a 41 amino acid peptide which contains 4 tyrosines. The precise mechanism whereby ligation of CD28 results in upregulation of the IL-2 gene is unknown, but a variety of kinases have been associated with CD28. CD28 has been shown to be associated with the kinase p72-ITK/EMT which is rapidly phosphorylated on cross-linking of CD28 (August et al., 1994). This leads to tyrosine phosphorylation of the cytoplasmic tail of CD28 and recruitment of phosphoinositide-3 (PI-3) kinase (Nunes et al., 1993; Pages et al., 1994). In addition the *lck* kinase belonging to the *src* family of kinases has been shown to phosphorylate CD28 upon ligation and PI-3 kinase and grb2/SOS binding is dependant on CD28 phosphorylation by *lck* (Raab et al., 1995). However *lck* does not appear to be obligatory in the downstream signalling of CD28 as stimulation of an *lck* deficient cell line with PMA and ionomycin resulted in similar IL-2 secretion to the wild type cell line (Stein et al., 1994). Similarly site directed mutagenesis of PI-3 kinase has demonstrated that PI-3 kinase binding to CD28 is also not an absolute requirement for IL-2 production (Lu et al., 1995).

Studies suggest that tyrosine phosphorylation of CD28, as a consequence of cross-linking of CD28, results in binding of grb2 to the pYMNM motif in the cytoplasmic tail and this binds with SOS to form a CD28/grb2/SOS complex (Schneider et al., 1995). However when CD28 ligation was mediated by B7-1 or B7-2, grb2 did not complex with CD28, but resulted in tyrosine phosphorylation of p62, an adapter protein not involved in the TCR pathway (Richard et al., 1995; Nunes et al., 1996). How these changes mediated

expression of cytokine remains unclear, although it would seem that signalling through CD28 inhibits the degradation of mRNA of several cytokines (Lindstein et al., 1989; Cerdan et al., 1992; Holter et al., 1992; de Boer et al., 1993).

CD28 ligation has also been shown to result in the phosphorylation of *Jun* via activation of Jun kinase. Phosphorylated *Jun* is a component of the AP-1 transcription factor which binds to several target sequences in the 5' IL-2 enhancer region (Su et al., 1994). The MAP kinases ERK 1 and ERK 2 are fully activated by TCR ligation alone, whereas the MAP kinases involved in phosphorylation of the Jnk activation domains JNK 1 and JNK 2 require signalling through both the TCR and CD28 suggesting that this is the point where integration of the two signals results in optimal T cell activation (Su et al., 1994).

1.8 CTLA-4

1.8.1 Identification of CTLA-4

The gene coding for CTLA-4 was originally cloned in 1987 in a study examining genes involved in cytotoxic T cell function (Brunet et al., 1987). It was initially identified as a member of the immunoglobulin superfamily and was found to have a 75% nucleotide sequence homology with CD28, with the greatest degree being in the juxtamembrane and cytoplasmic regions (Dariavach et al., 1988). This high level of homology raised the question as to whether the molecules were functionally related. In 1991 it was demonstrated that CTLA-4 was a second receptor for B7-1 (Linsley et al., 1991b). To analyse the binding characteristics of CTLA-4 a fusion gene consisting of the extracellular domain of CTLA-4 and the Immunoglobulin Cγ1 domain was constructed. Incubation of the purified CTLA4-Ig with the B7-1+ve lymphoblastoid cell line PM LCL and CHO cells transfected with B7-1 revealed strong binding to both. Comparison between the ability of a corresponding CD28Ig and CTLA4-Ig to bind membrane bound B7-1, immunoprecipitate solubilised B7 and inhibit T cell proliferation in an MLR revealed CTLA4-Ig to be more effective in all cases. In direct comparison of the ability to bind soluble ^{125}I-labelled B7Ig, CTLA4-Ig had a twentyfold greater affinity than CD28Ig indicating that it was probably the high affinity receptor for B7-1.

1.8.2 Function of CTLA-4

The discovery that CTLA-4 was a second receptor for B7-1 raised the question as to its function. Two hypotheses were proposed, the first being that expression of CTLA-4 defines a subset of T cells distinct from CD28+ve T cells, or that CTLA-4 might deliver a negative signal to T lymphocytes. This question was addressed in 1992 by Freeman et al. (Freeman et al., 1992b). He analysed expression of CTLA-4 and CD28 on resting and activated T cells, T cell clones and T cell tumour cell lines by analysis of mRNA as there was no anti-CTLA-4 antibody available at that time. CD28 mRNA was expressed in all T cell populations and was moderately increased 24 hours after activation with either PHA or PMA. In contrast CTLA-4 mRNA was undetectable in resting T cells but was strongly induced 24 hours after mitogenic activation of T cells. Analysis of murine and human activated T cell clones demonstrated coexpression of CTLA-4 and CD28 mRNA confirming that both B7-1 receptors were coexpressed in the same cell, thus demonstrating that the two receptors do not identify reciprocal subsets.

With the development of anti-CTLA-4 monoclonal antibodies the role of CTLA-4 in T cell activation became more apparent. It was initially found that anti-CTLA-4 alone was unable to provide a costimulatory signal to T cells *in vitro* alone, but did augment the effect of anti-CD28 when used in combination. This led to the hypothesis that CTLA-4 synergised with CD28 to prolong IL-2 production and T cell proliferation (Linsley et al., 1992). Analysis of temporal expression of CTLA-4 demonstrated that it was not expressed on resting T cells, but present on activated T cells maximally at 48 hours in both CD4+ve and CD8+ve subsets at a level no more than 1/30th that of CD28. Despite the

lower level of expression of CTLA-4 as compared to CD28, CTLA-4 was responsible for the majority of binding of B7-1 demonstrating its higher affinity. In combination, anti-CTLA-4 and anti-CD28, blocked T cell proliferation in a primary mixed lymphocyte culture (MLC). When immobilised anti-T cell receptor monoclonal antibody (mAb) was used to activate T cells, anti-CTLA-4 mAb was less efficient at costimulating proliferation as compared to anti-CD28 mAb. When anti-CD28 and anti-CTLA-4 were used together they were synergistic in their ability to augment anti-TCR induced proliferation (Linsley et al., 1992). These results appeared to confirm the hypothesis that CTLA-4 and CD28 acted together to augment T cell proliferation and IL-2 production.

1.8.3 CTLA-4 Delivers an Inhibitory Signal to T cells

Another possible interpretation of the above results was that the enhancement of T cell proliferation when anti-CTLA-4 and anti-CD28 were used together was that the anti-CTLA-4 was purely acting to block an inhibitory signal. This hypothesis was supported by Krummel and Allison in 1995 (Krummel and Allison, 1995). To determine the functional consequences of CTLA-4 engagement, soluble anti-CTLA-4 or anti-CD28 were added to T cells exposed to immobilised anti-CD3 as described above. Similar results were seen with no increased proliferation with anti-CTLA-4, a marked increase with anti-CD28 and a synergistic effect with the two antibodies used together. When the antibodies were cross-linked with anti-hamster immunoglobulin differing results were seen. Cross-linking CD3, CD28 or CTLA-4 alone had no effect on proliferation, cross-linking of CD3 and CD28 resulted in potent costimulation as expected and cross-linking of CD3 and CTLA-4 had no effect. When CTLA-4

was co-cross-linked with CD28 and CD3 a 5-to 10-fold reduction in proliferation was seen together with a reduction in IL-2 production. These results appeared to demonstrate that CTLA-4 delivers a negative signal to inhibit T cell responses following signalling through the T cell receptor.

The role CTLA-4 plays *in vivo* was identified following the generation of CTLA-4 deficient mice that were homozygous for the mutation. CTLA-4 deficiency resulted in a lethal phenotype with death occurring at three to four weeks of age. At autopsy massive splenomegally and lymphadenopathy was found, due mainly to infiltration by CD3+ve T cells. Further analysis showed there was an increase in the activation markers CD69, CD25 and CD44 on the T cells and there was infiltration of the myocardium and pancreas by T cells. The severe phenotype seen in the CTLA-4 deficient mice suggested a critical role for CTLA-4 in down-regulating T cell activation and maintaining immunological homeostasis since the majority of T cells in these mice expressed activation markers (Tivol et al., 1995; Waterhouse et al., 1995). Thus the outcome of T cell activation seemed to depend on a balance between CD28-mediated T cell activation and CTLA-4-mediated downregulation.

The evidence that CTLA-4 delivers a negative signal to T cells is not conclusive. In CD28 deficient mice, T cells proliferate in response to costimulation provided by B7-1, which can be blocked by anti-CTLA-4 mAb, indicating that CTLA-4 delivers a positive signal (Wu et al., 1997). *In vivo* T cell mediated rejection of autologous and syngeneic tumours was maintained in these mice (Wen et al., 1997). These results were further supported by the finding that a mutant B7-1 molecule, which did not bind to CD28 while retaining binding to CTLA-4, was able to costimulate T cell proliferation which was also

blocked with anti-CTLA-4 mAb (Wu et al., 1997). It has also been demonstrated that a monovalent ligand can result in receptor dimerisation (Blechman et al., 1995), indicating that Fab fragments of anti-CTLA-4 mAbs may not necessarily inhibit signalling through CTLA-4.

The lymphocyte infiltration seen in many organs of CTLA-4 deficient mice has been proposed to be due to generation of autoreactive T cells due to a defect in immune tolerance (Liu, 1997). This is supported by the finding that in CTLA-4 deficient mice using two transgenic T cell receptor models, lymphoproliferation is decreased in the absence of an antigen specific signal (Waterhouse et al., 1997). Thymic selection is thought to be governed by the strength of interaction between the T cell and the thymic cell (Vukmanovic, 1996), a high avidity leading to negative selection and a low avidity to positive selection. As CTLA-4 is the high affinity receptor, deficiency may lead to transformation of negative into positive selection resulting in the generation of self-reactive T cells. With regard to induction of B7-1 or B7-2 expression on tumour cells, it is important that the function of CTLA-4 is fully understood, as it may be that blocking CTLA-4 interactions would augment an anti-tumour response if it was indeed a negative regulator of T cells. However if the converse is true, blocking CTLA-4 interactions would have a detrimental effect on induction of an anti-tumour immune response. *In vivo* administration of blocking anti-CTLA-4 mAb to mice exposed to B7-1+ve tumour cells, resulted in tumour rejection and protection against secondary exposure to the tumour (Leach et al., 1996). Only a proportion of mice exposed to the B7-1+ve tumour cells rejected the tumour. These results suggest that blockade of CTLA-4 potentiates anti-tumour immunity.

1.9 TH1/TH2 AND COSTIMULATION

The necessity for two CD28 ligands with limited sequence homology and relatively low conservation between species was initially unexplained (Azuma et al., 1993b; Freeman et al., 1989; Freeman et al., 1993b; Bajorath et al., 1994; Freeman et al., 1991), but subsequent evidence has pointed to distinct functions between these two proteins in directing the immune response. That their functions may differ was initially supported by the differential expression of B7-1 and B7-2 on B cells , T cells, monocytes, macrophages and dendritic cells (Hathcock et al., 1994; Azuma et al., 1993b). In addition it was shown that B7-1 has a higher affinity for both CD28 and CTLA-4 than B7-2 and that B7-2 dissociates quicker from human CTLA4-Ig than B7-1, which correlated with human CTLA4-Ig being less effective at inhibiting a B7-2 costimulated T cell response than a B7-1 driven response (Linsley et al., 1994).

1.9.1 Differential Function of B7-1 and B7-2 in Responding T cells

Differential function of B7-1 and B7-2 in T cell costimulation has been suggested by both *in vitro* and *in vivo* experiments. It was noted that although costimulation with B7-1 was required for the generation of a mixed lymphocyte response directed against alloantigens, this was only partially inhibited by anti-B7-1 antibodies. The role of B7-2 in generating an alloantigenic response was therefore examined. Fresh non adherent peripheral blood lymphocytes were incubated with irradiated allogeneic peripheral blood mononuclear cells for seven days. Addition of IT2 (an anti-B7-2 mAb) completely inhibited the proliferative response comparable to that seen with anti-CD28 antibody,

compared to only partial inhibition with anti-B7-1 antibody. It was postulated that this difference may be due to B7-2 which is highly expressed on the monocytes initiating the allogeneic response by binding to CD28 expressed on the T cells (Azuma et al., 1993).

In a murine experimental allergic encephalitis (EAE) model, treatment of proteolipid protein immunised mice with anti-B7-1 mAb was associated with less severe disease and T cell clones grown from these mice produced large amounts of IL-4, a T_H2 profile cytokine. In contrast treatment of these mice with anti-B7-2 mAb enhanced the severity of the disease and T cell clones from these mice produced γ-interferon (γ-IFN), a T_H1 cytokine (Kuchroo et al., 1995). That B7-1 and B7-2 have a role in the generation of a Th1 or a Th2 response was further supported by *in vitro* data. Repetitive stimulation of CD45RA+ve T cells with NIH 3T3 cells transfected with B7-1 or B7-2 resulted in moderate levels of IL-4 production with the B7-2 transfectants and only low levels of IL-4 with the B7-1 transfectants. With both the B7-1 and B7-2 transfectants high levels of IL-2 were produced (Freeman et al., 1995). However a number of other studies have shown that B7-1 and B7-2 can provide costimulatory signals for both Th1 and Th2 lymphokine production (Lanier et al., 1995; Levine et al., 1995). That B7-2 may be more involved in the generation of a primary immune response was suggested by the fact that B7-1 knockout mice had relatively normal Th1 and Th2 responses compared to B7-2 knockout mice which were severely immunocompromised (Lenschow et al., 1996). In addition, in an allogeneic islet cell transplant model, anti-B7-2 but not anti-B7-1 mAbs prevented graft rejection (Lenschow et al., 1995b). As graft rejection is thought to be mediated by a T_H1 immune response treatment it might have been

expected that treatment with anti-B7-1 mAb would have been more effective at inhibiting graft rejection.

Further evidence that B7-1 and B7-2 differ in their function is provided by studies using NOD mice which are characterised by the development of an autoimmune insulitis followed by diabetes. Female mice, which are more frequently affected than males, treated with either CTLA4-Ig or anti-B7-2 mAb at the onset of insulitis did not develop diabetes although the severity of the insulitis is not affected. Treatment of female mice with anti-B7-1 mAb alone or in combination with anti-B7-2 mAb surprisingly results in a more severe insulitis with rapid progression to diabetes (Lenschow et al., 1995a). An interesting observation was made in a murine relapsing-EAE model where it was noted that treatment of affected mice, after the resolution of the initial phase of the disease, with anti B7-1 mAb increased the rate of onset, frequency and severity of relapse. When treatment was initiated with non cross-linking F(ab) fragments of the anti-B7-1 mAb clinical relapses were blocked, raising the possibility that the exacerbations were due to direct signalling through B7-1 on antigen presenting cells or activated T cells (Miller et al., 1995).

1.9.2 Intracellular Signalling Following Ligation of CD28 with B7-1 or B7-2

How B7-1 or B7-2 might mediate different effects on ligation of a common receptor is still unknown. The MYPPPY motif in the complementarity-determining region 3 (CDR3) of the variable like domain in CD28 and CTLA-4 is a key recognition site for both B7-1 and B7-2 (Peach et al., 1994). In a hCTLA4-Ig containing a single amino acid mutation in the MYPPPY region binding of B7-1 was only reduced compared to complete inhibition of binding

with B7-2, thus demonstrating that the precise interaction sites of the two proteins may be distinct (Peach et al., 1994). In addition, it was recently demonstrated that five single amino acid mutations in the MYPPPY region of CTLA-4 completely inhibited binding of B7-2 with only partial inhibition of B7-1 (Morton et al., 1996). This raises the possibility that B7-1 and B7-2 are differentially recognised by their receptors and different downstream pathways are activated resulting in different functional outcomes. This theory was supported by the finding that, although cross-linking of CD28 by anti-CD28 mAb or B7-1 transfectants induced phosphorylation of p95 and stimulated IL-2 secretion, only anti-CD28 mAb induced tyrosine phosphorylation of p36/SOS/grb2 complex, p21 ras activation, Raf kinase hyperphosphorylation and MAP kinase activation (Nunes et al., 1994). This supported the hypothesis that B7-2 may activate pathways not activated by B7-1. However when the above experiments were performed with B7-2 transfectants, activation of the Ras pathway was not seen as with the B7-1 transfectants (Nunes et al., 1996). However it is possible that B7-1 or B7-2 CD28 mediated pathways may differ at other points in the signalling pathway.

1.10 B7-3

B7-1, recognised by anti-B7 and anti-BB-1 mAb, was classified as CD80 in 1994 (Engel and Tedder, 1994). However in 1993 a study suggested that anti-B7 and anti-BB-1 may recognise different molecules, when it was noted that keratinocytes, psorriatic keratinocytes and thymus epithelial cells bound to anti-BB1 but not anti-B7 mAb (Nickoloff et al., 1993). A further study demonstrated that T cells, in psorriatic lesions, bound to anti-B7-1 but not anti-BB-1 mAb (Nickoloff et al., 1994). In addition the BB-1 gene was mapped to chromosome 12 (Katz et al., 1985), whereas the B7-1 gene was localised to chromosome 3 (Freeman et al., 1992a; Selvakumar et al., 1992). To differentiate B7-1 from BB-1, activated B cells were sorted into B7-1+ve and B7-1-ve fractions. As might be expected the B7-1+ve cells were able to costimulate T cells stimulated with anti-CD3 mAb, but anti-B7-1 only inhibited proliferation by 50%, compared to anti-BB-1 which inhibited proliferation by 90%. Furthermore the B7-1-ve activated B cells were able to costimulate T cells receiving concurrent stimulation through the T cell receptor, which was completely inhibited by anti-BB-1 and unaffected by anti-B7-1 mAb. This was interpreted as confirmation of the existence of a third ligand for CD28 which was termed B7-3 (Boussiotis et al., 1993). As no antibody to B7-2 was available, distinction from B7-2 was made indirectly, as it provides the main co-stimulatory signal 24 hours after B cell activation, as opposed to B7-1 and B7-3 which appeared to be expressed 48 to 72 hours after activation. However the gene for B7-3 has yet to be cloned so the existence of B7-3 has yet to be confirmed. Indeed, in the original paper describing the cloning of B7-1, both

anti-B7-1 and anti-BB-1 mAb were able to immunoprecipitate a 44-54 kDa protein from COS cells transfected with one of the B7-1 clones, suggesting that they were identifying the same protein (Freeman et al., 1989).

1.11 OTHER COSTIMULATORY MOLECULES

As well as the B7 family of molecules, other molecules expressed on the surface of APCs have been shown to costimulate T cell activation. They are also members of the immunoglobulin superfamily and are intercellular adhesion molecule-1 (ICAM-1) (Van Seventer et al., 1990), leucocyte function-associated antigen-3 (LFA-3) (Bierer et al., 1988) and vascular cell adhesion molecule-1 (VCAM-1) (Damle and Aruffo, 1991). They function as costimulatory molecules by binding to their counterreceptor on the T cell. ICAM-1 binds to the CD11a/CD18 complex (Springer, 1990), LFA-3 binds to the CD2 molecule (Springer, 1990) and VCAM-1 interacts predominantly with the CD49d/CD29 integrin (Damle and Aruffo, 1991). Fusion chimeras of ICAM-1, LFA-3 and VCAM-1 were able to stimulate resting CD4 T cells to proliferate when stimulated through the T cell receptor with immobilised mAb to CD3 (Damle et al., 1992), demonstrating their individual costimulatory abilities. The role of ICAM-1 in generating an *in vivo* anti-tumour response was investigated using tumourigenic cells lines that either constitutively expressed ICAM-1 or lacked the molecule. Transfection of all cell lines with B7-1 resulted in rejection of the tumour cells that also expressed ICAM-1, whereas the tumours that lacked ICAM-1 were not rejected despite the expression of B7-1 (Cavallo et al., 1995). Thus it would seem that B7-1 and ICAM-1 synergise in inducing a T cell mediated immune response in the cell lines studied.

A further molecule shown to have costimulatory function is CD70, found on activated B and T cells. It binds to CD27, present on resting T cells, which is a member of the TNF-receptor family. Ligation of CD70 with CD27 provides a second stimulus resulting in T cell proliferation and induction of TNF-α, but lower IL-2 secretion than that induced by B7-1 ligation with CD28 (Hintzen et al., 1995).

CD40 ligand is an early activation antigen expressed on CD4 T cells whose expression peaks within 6 hours of T cell activation. It binds to CD40 on B cells and promotes B cell activation, differentiation and Ig class switching (Lederman et al., 1992). In addition B cells are induced to express B7-1, which increases their ability to induce proliferation in allogeneic T cells (Yellin et al., 1994).

Heat stable antigen is a cell adhesion molecule which is capable of providing a costimulatory signal to T cells (Liu et al., 1992) and is expressed on B cells, activated T cells, monocytes, granulocytes, Langerhans cells and thymocytes. Transfection of cDNA for heat stable antigen into a murine melanoma cell line was able to induce proliferation and generation of cytotoxic T cells in syngeneic mice (Wang et al., 1995) demonstrating that the costimulatory signal was able to assist in the generation of an anti-tumour immune response.

Finally 4-1BB is a glycoprotein belonging to the tumour necrosis factor receptor superfamily (Smith et al., 1994), expressed on primed CD4+ve and CD8+ve T cells. 4-1BB ligand is expressed on antigen presenting cells and signalling through 4-1BB results in T cell proliferation on concurrent stimulation through the T cell receptor (Hurtado et al., 1995). A role for 4-1BB in tumour

immunity has been suggested, anti-4-1BB mAb given to mice with established tumours leads to regression mediated by both CD4+ve and CD8+ve T cells (Melero et al., 1997).

1.12 TUMOUR SPECIFIC ANTIGENS

The concept of cancer immunotherapy relies on the premise that tumour antigens exist and that they can be presented to the T cell receptor, providing the first signal required for T cell activation. One of the first indications of the existence of tumour antigens was in a non immunogenic murine thymic leukaemia, which on mutation led to a variant that was rejected by syngeneic mice and partially protected against exposure to original tumour. *In vitro* generation of CTL's revealed the response was directed against an antigen present on the original tumour, indicating that a putative tumour antigen was present and that the deficiency was in the induction of the immune response (Van Pel and Boon, 1982). In 1985 it was demonstrated that intracellular protein was processed into small peptides and presented on the cell surface by MHC class I (Townsend et al., 1985). This led to the hypothesis that peptides derived from upregulated self proteins or mutant self proteins could be displayed on the surface of tumour cells allowing the immune system to recognise them. The generation of tumour-specific cytotoxic T lymphocytes (CTLs) *in vitro* from the peripheral blood or draining lymph nodes of patients with malignancy was strong evidence that tumour specific antigens were present and were capable of stimulating an immune response (Chen and Hersey, 1992).

1.12.1 Identification of Tumour Antigens

Three strategies have been used to identify antigens presented to tumour specific T cells. The first involves the cloning of genomic fragments from the tumour into a cosmid library (Lurquin et al., 1989). These are transfected into MHC matched cells and CTL clones are generated. The gene in the cosmid can then be sequenced.

A second approach involves the elution of tumour specific peptides from the tumour MHC class I with acid (Slingluff, Jr. et al., 1993). The peptides are then fractionated by passing over a high performance liquid chromatography (HPLC) column and are then added to a target cell not recognised by the tumour specific CTLs but expressing the same MHC class I molecules. Fractions can then be identified that sensitise the target cells to the tumour specific CTLs.

A third method targets proteins known to be overexpressed or mutated in tumour cells. Peptides with high affinity binding to MHC class I are selected and loaded onto professional APC's which are used to stimulate CTL's which are then tested for their ability to lyse original tumour cells (Celis et al., 1994).

1.12.2 Embryonic Derived Tumour Antigens

The first group of tumour antigens to be identified were the MAGE, BAGE and GAGE proteins which are reactivated embryonic gene products (Van den Eynde et al., 1995; van der Bruggen et al., 1991; Boel et al., 1995; De Plaen et al., 1994). The first to be characterised was encoded by a gene called MAGE-1, which was found to be expressed on 37% of all melanomas and some other tumours including lung and bladder carcinomas or sarcoma (van der Bruggen et al., 1991). Another antigen was found to be coded for by a gene called BAGE which, like the MAGE genes, is not expressed in normal adult tissues, except testis (Boel et al., 1995). The final family of genes, termed GAGE, were found to code for an antigenic peptide expressed on melanomas, sarcomas, non-small cell lung carcinomas, head and neck tumours and bladder tumours (Van den Eynde et al., 1995).

1.12.3 Lineage Restricted Tumour Antigens

CTL's generated using human melanoma cell lines were also, in some cases, able to recognise common antigens expressed on all cells of melanocyte lineage. The first of these common antigens was found to be derived from the enzyme tyrosinase (Brichard et al., 1993). Several tyrosinase peptides have been identified, being presented by HLA-A2 (Brichard et al., 1993), HLA-A24 (Robbins et al., 1994), HLA-B44 (Brichard et al., 1996) and HLA-DR4 (Topalian et al., 1994). Therefore tyrosinase derived epitopes can be presented by both MHC class I and II, which may be more effective in tumour vaccination strategies due to recruitment of both helper and cytotoxic T cells.

Other common melanoma antigens were also found to be derived from melanocytic proteins. Pmel17 and gp100 were two similar proteins that were found to produce five distinct peptides recognised by HLA-A2 restricted tumour infiltrating lymphocytes (TILs)(Bakker et al., 1994; Kwon et al., 1991; Kawakami et al., 1994a; Kawakami et al., 1995; Cox et al., 1994). Other differentiation antigens identified include $gp75^{TRP1}$ (Wang et al., 1995) and Mart-1 (Kawakami et al., 1994b). In contrast to the MAGE, GAGE and BAGE genes, CTL's can be readily detected in most patients against peptides from the genes encoding melanoma differentiation antigens (Brichard et al., 1996) (Sensi et al., 1995). However, many of these patients have progressive disease which implies that CTL against these targets are not effective. That CTL against melanoma differentiation antigens might have a therapeutic role is supported by the association of vitiligo with spontaneous regression and improved survival (Bystryn et al., 1987).

Of particular interest, with regard to myeloid leukaemic cells, are the granulocytic proteins, proteinase 3 and myeloperoxidase, which are expressed in both some normal haematopoietic cells and myeloid leukaemic cells (Dengler et al., 1995). In Wegener's granulomatosis the anti-neutrophil cytoplasmic antibodies are directed against both proteinase 3 and myeloperoxidase and T cells from these patients proliferate on exposure to the proteins demonstrating that these proteins can be presented by HLA molecules and be recognised subsequently by T cells (Brouwer et al., 1994). A nine amino acid peptide (PR-1) derived from proteinase 3 was found to bind to HLA-A2 and a T cell line was generated after immunisation with the peptide, which demonstrated specific cytotoxicity against a cell line loaded with PR-1 and myeloid leukaemic cells,

the degree of cytotoxicity being dependant on the expression of proteinase 3 within the leukaemic cells (Molldrem et al., 1996). Again this demonstrated that a protein expressed within normal cells can act as a tumour antigen.

1.12.4 Tumour Antigens Arising from Genetic Mutation

Many tumours posses point mutations or translocations involving oncogenes which result in changes to the amino acids encoded. These mutated gene products are potential tumour specific antigens. An example of a mutated gene product, found in over 95% of patients with chronic myeloid leukaemia, is the product of the t(9;22)(q34;q11) translocation. This leads to the formation of a fusion gene termed bcr/abl, coding for a protein of 210 kDaltons which has abnormal tyrosine kinase activity (Shtivelman et al., 1985). Both the BCR and ABL proteins are expressed in normal cells and would not be appropriate targets for an anti-leukaemic immune response, but the joining segment of the bcr-abl fusion gene codes for a unique peptide which would be a potential target for an anti-leukaemic T cell response, specific for the malignant clone. Mice vaccinated with synthetic peptide corresponding to the joining region of the BCR-ABL fusion protein generated a synthetic peptide specific class II restricted T cell response that was mediated by CD4+ve T cells (Chen et al., 1992), demonstrating that the fusion gene represents a potential target for an anti-leukaemia specific immune response. In humans the BCR-ABL junctional region is also able to elicit a T cell response. Immunisation of human lymphocytes with a 17 amino acid peptide encompassing the joining region of the BCR-ABL peptide resulted in the successful generation of T cell lines which were HLA DR4 restricted and only recognised cells encoding the

bcr-abl translocation (Bosch et al., 1996). This demonstrates that the BCR-ABL junctional region can be processed and presented as a tumour specific peptide by leukaemia cells expressing the relevant MHC class II molecules. In addition, two 9 amino acid peptides encompassing the BCR-ABL protein junctional region were found to bind to HLA-B8. Stimulated cytotoxic T cells were able to specifically lyse autologous lymphoblastoid cells and a class I deficient cell line transfected with HLA-B8, after being pulsed with either of the two 9 amino acid peptides (Dermine et al., 1995). Preliminary data suggested that these T cell clones were able to recognise CML cells, again supporting the role of this fusion protein as a tumour specific antigen.

In acute promyelocytic leukaemia a 15:17 chromosomal translocation is found in up to 90% of cases (Rowley et al., 1977). The result of this translocation is the generation of a unique fusion gene product called the PML/RARα fusion protein, generated by a translocation of the rarα gene on chromosome 17 to the pml gene on chromosome 15 (de The et al., 1990). As with the bcr-abl translocation a unique junctional sequence is created by the translocation which creates a possible tumour specific peptide. A 25 amino acid peptide encompassing the fusion region of the PML/RARα hybrid protein was found to be able to be presented in a HLA-DR restricted pattern by APC's, which were able to be recognised by autologous CD4+ve T cells. In addition one of the T cell clones demonstrated specific cytotoxicity against autologous lymphoblastoid cell lines pulsed with the fusion peptide. This demonstrated that the junctional region of the PML/RARα can also be processed and presented by leukaemia cells expressing the relevant MHC class II molecule and that the

junctional region may act as a potential tumour specific antigen (Gambacorti-Passerini et al., 1993).

Point mutations within genes may also lead to single amino acid substitutions which may act as potential tumour specific antigens. An example of this is the mutated ras proto-oncogene found in pancreatic carcinoma which results in the expression of oncogenic $p21^{ras}$ protein. Synthetic peptides encompassing the amino acid substitution in $p21^{ras}$ were able to bind to murine MHC class I and immunisation of lymphocytes with the peptides led to the generation of cytotoxic T cells specific for the $p21^{ras}$ protein, demonstrating its ability to act as a tumour specific antigen (Peace et al., 1994). Similarly the product of a mutated tumour suppressor gene p53, present in colon and breast cancers, was found to be recognised by both CD4+ve and CD8+ve T cells demonstrating its possible role as a tumour specific antigen (Noguchi et al., 1994).

1.12.5 Tumour Antigens Encoded by Viral Genes

A further class of putative tumour specific antigens is found in those tumours that appear to be virally mediated. An example is found in human cervical cancer which is associated with papilloma virus infection in over 90% of cases. CTL directed against the HPV type 16 oncogenes E6 or E7 were able to protect against development of tumour by HPV-16 transformed cells (Feltkamp et al., 1993). Epstein-Barr virus infection is widespread and is kept latent in healthy individuals by EBV specific CTL. EBV nuclear antigen derived peptide epitopes, recognised in association with several MHC molecules, have been identified and it has been shown that EBV induced Burkitt's lymphoma

cells can evade recognition by down-regulating expression of EBV antigens encoding these epitopes, which was independent of downregulation of the adhesion molecules LFA-1, LFA-3 and ICAM-1 (Khanna et al., 1993).

1.12.6 Idiotypes as Tumour Antigens

Further potential candidates as tumour specific antigens are found in tumours expressing surface immunoglobulin or the T cell receptor, such as lymphomas and myeloma. Immunoglobulins are composed of both heavy and light chains which possess at their termini specific variable regions which form unique antigen recognition sites. These variable regions contain determinants that can themselves be recognised as antigens or idiotypes. As malignancies are clonal in origin, any expressed surface immunoglobulin or T cell receptor will possess the same idiotypic determinant which may serve as a unique potential tumour specific antigen. The induction of anti-idiotype specific immunity by immunisation with tumour derived Ig has been demonstrated in myeloma (Freedman et al., 1976), B cell lymphomas (Sugai et al., 1974) and B cell leukaemias (Stevenson and Gordon, 1983).

1.13 INDUCTION OF EXPRESSION OF CO-STIMULATORY MOLECULES ON TUMOURIGENIC CELLS

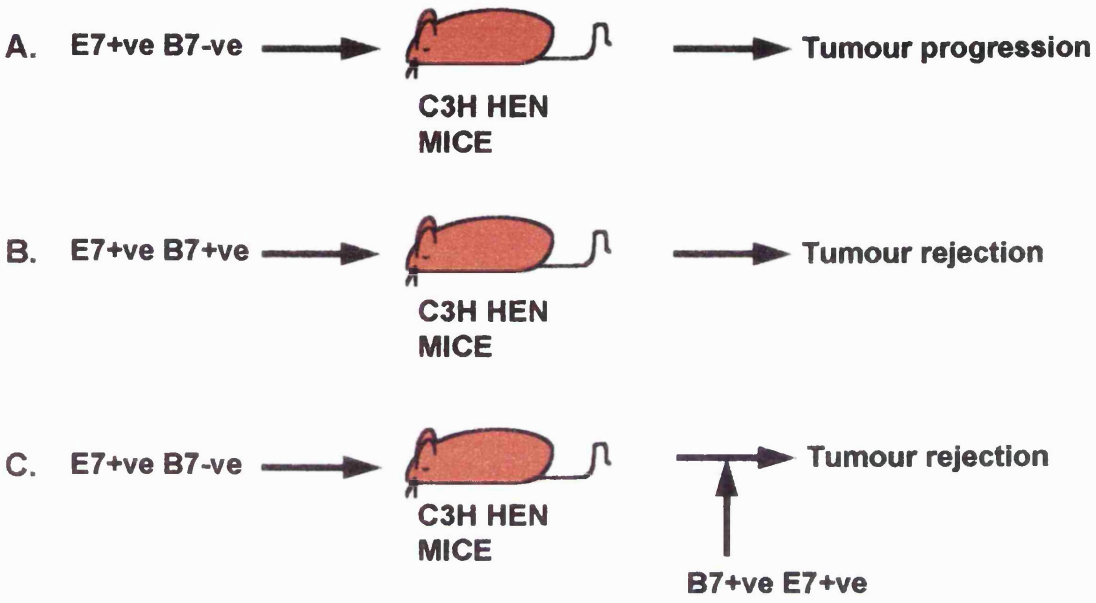
1.13.1 B7-1 Expression on Tumourigenic Cells

As mentioned in section 1.4.3 the poor immunogenicity of many tumours may be attributed to the lack of expression of a costimulatory molecule resulting in the induction of anergy to potential tumour antigens (Bretscher and Cohn, 1970). Demonstration that the induction of a costimulatory molecule could lead to immune recognition of the tumour and induction of an effective CTL response was first demonstrated in a murine melanoma model (Chen et al., 1992). The poorly immunogenic murine melanoma K1735-M2 cell line was transfected with the E7 gene of human papilloma virus 16 (HPV-16). This line was then termed E7C3. Stable transfectants, expressing B7-1, were generated in the E7C3 cell line with an expression plasmid vector and expression confirmed by flow cytometric analysis. Subcutaneous inoculation of both the parental K1735-M2 and E7C3 cell lines were tumourigenic in syngeneic immunocompetant mice, whereas inoculation of E7C3B7-1+ve cells resulted in tumour regression in all mice studied. In contrast inoculation of B7-1 expressing tumour cells in nude mice resulted in progressive tumour growth suggesting tumour rejection was immune mediated. This was confirmed by preincubating B7-1+ve cells with CTLA4-Ig prior to administration to the mice, which abrogated tumour rejection confirming that this was mediated by B7-1. Depletion of T cell subsets in the murine recipients revealed that it was the CD8+ve T cells which were responsible for tumour rejection. Of particular

interest was the finding that simultaneous inoculation of B7-1+ve tumour cells, in one flank of the mouse, with B7-1-ve tumour cells on the other flank resulted in complete rejection of both B7-1+ve and B7-1-ve tumour cells demonstrating induction of a systemic immune response (Figure 2). Indeed, in mice previously inoculated with B7-1-ve tumour cells with established micrometastases in the lungs, injection of B7-1+ve cells prolonged survival in all mice tested and 40% of mice were completely tumour free on histological analysis. This is of particular relevance as candidates for immunotherapy would be expected to have residual disease prior to return of any altered cells.

A separate group performed a similar series of experiments with the K1735 tumourigenic cell line, not modified to express the viral rejection antigen E7. Thus, any specific immune response would have to be directed against peptides innately presented by the tumour cells, as would be the case with naturally occurring malignancies expressing both MHC class I and class II molecules. To assess the role of costimulation in this setting, parental tumour was inoculated subcutaneously into syngeneic mice who received repeated intraperitoneal injections of anti-CD28 mAb, to provide a co-stimulatory signal. Although this did not result in complete tumour rejection it slowed growth of the tumour in the mice. As with Chen's experiments K1735 cells expressing B7-1 were rejected by the syngeneic mice which were protected for up to 90 days against exposure to parental tumour cells. *In vivo* depletion of CD4 and CD8 T cell subsets again demonstrated that the cells mediating the rejection of the tumour were contained within the CD8+ve T cell population (Townsend and Allison, 1993).

Figure 2 B7-1 Expression on Tumourigenic Cells Induces Rejection and Confers Protection



Legend to Figure 2:

A.- Inoculation of syngeneic mice with E7+veB7-ve tumourigenic cells leads to tumour progression.

B.- Inoculation of syngeneic mice with E7+veB7+ve cells leads to rejection of the cells.

C.- Mice with established E7+veB7-ve tumours on inoculation with E7+veB7+ve cells reject both the established tumours and the E7+veB7+ve cells.

1.13.2 Expression of B7-2 on Tumour Cells Results in Tumour Rejection

As well as expression of B7-1 by tumourigenic cells, the effect of expression of B7-2 has been assessed. In one study recombinant vaccinia virus was used, as the vector, to induce expression of either B7-1 or B7-2 in MC38 murine adenocarcinoma cells (Hodge et al., 1994). Syngeneic mice inoculated with MC38 cells expressing B7-1 or B7-2 remained tumour free, whereas control MC38 cells all resulted in progressive tumour growth which was slightly delayed as compared to the wild type tumour. Rechallenge of mice with wild type tumour, injected 40 days before with B7-1+ve or B7-2+ve tumour, resulted in delayed tumour formation and decreased growth rate as compared with injection of wild type tumour into unprimed mice. No difference was seen in tumour growth between the mice vaccinated with B7-1+ve or B7-2+ve tumour cells. A subsequent study using the murine mastocytoma cell line, P815, as the target found expression of B7-2 resulted in inhibition of tumour growth and protection against subsequent exposure to wild type tumour which was mediated by CD8+ve T cells (Yang et al., 1995). *In vitro* CTL generated from vaccinated mice were class I restricted and were specific against P815 cells failing to lyse syngeneic cell lines and an NK sensitive cell line. Comparison with B7-1 expressing P815 cells from the same group demonstrated slightly higher *in vitro* cytotoxicity from primed mice, but the B7-1 expression as measured by FACS analysis was slightly higher making it difficult to compare the degree of immunity generated. However *in vivo* there were no differences seen between mice primed with either B7-1+ve or B7-2+ve cells. Indeed

transfection of B7-2 into the non immunogenic cell line MCA102 failed to elicit protective immunity even with coexpression of B7-1.

1.13.3 Immunogenicity of the Tumour Cells is Important in Generating CTLs

Subsequent work with tumourigenic cell lines demonstrated that the immunogenicity of the tumour was critical to the development of a cytotoxic T cell response. Immunogenic tumours are those which are rejected after transplantation into syngeneic animals which have been previously exposed to irradiated tumour cells, indicating that the tumour cells express specific antigens. In this study, four immunogenic (mastocytoma P815, melanoma E6B2, lymphoma RMA and EL4) and four non immunogenic (sarcomas MCA101, MCA102 and Ag104 and melanoma B16) tumourigenic cell lines were studied (Chen et al., 1994a). The immunogenicity of the tumour cells was determined by the ability of irradiated tumour cells, inoculated subcutaneously in syngeneic mice, to protect against subsequent challenge of the minimal tumourigenic dose of tumour cells required for outgrowth in 100% of exposed mice. With the RMA and E6B2 cell lines a single injection conferred protection, whereas multiple injections were required with the EL4 and P815 cell lines. The four non immunogenic tumours were sarcomas MCA101, MCA102, Ag104 and melanoma B16. All 8 cell lines were induced to express B7-1. The four immunogenic B7-1+ve tumour cell lines were rejected by syngeneic mice, whereas tumour growth was progressive with the four B7-1+ve non immunogenic tumours. All B7-1-ve control tumours grew progressively. Exposure of the mice to B7-1+ve immunogenic tumour cells conferred protection to subsequent exposure with wild type tumour, which was not seen

with any of the non immunogenic tumours studied and in the case of the EL4 lymphoma, injection into mice 4 days previously exposed to wild type tumour, resulted in 60% of mice being cured. Harvested T cells from the spleens of inoculated mice stimulated further with wild type tumour led to an increase in *in vitro* CTL activity in those mice which were initially inoculated with B7-1+ve EL4 or P815 cells compared to B7-1-ve cells. In contrast no CTL response was seen with either B7-1+ve or -ve non immunogenic tumours. Chen et al. postulated that the failure of B7-1 transduced non immunogenic tumours to elicit a T cell response may be related to failure of a first signal which could be due to either lack of a target antigen or failure of presentation of a target antigen. That the defect is due to failure of antigen presentation rather than lack of tumour antigen is supported by the finding that three of the non immunogenic cell lines fail to express MHC class I and transduction of the B16 cell line with murine MHC class I resulted in failure of tumour growth when injected into syngeneic mice and conferred protection to subsequent exposure to wild type tumour (Tanaka et al., 1988).

1.13.4 Expression of B7-1 or B7-2 on Murine AML Leads to Tumour Rejection

Many studies examining the effect of B7-1 or B7-2 expression in tumourigenic cell lines used solid tumour models. The first demonstration of B7-1 expression on murine acute myeloid leukaemic cells was with a non immunogenic AML cell line, which was rapidly fatal in syngeneic mice. Inoculation of mice with B7-1+ve expressing AML cells conferred protection against subsequent exposure to B7-1-ve AML cells for up to five months. In addition mice with established leukaemia survived longer if exposed to

repeated doses of B7-1+ve AML cells, with some mice surviving "long term". After exposure to B7-1+ve AML cells, the mice had demonstrable leukaemia at day 7, which became undetectable at day 14, suggesting immunological rejection of the leukaemia. Depletion of CD4+ve and CD8+ve T cells demonstrated that it was the CD8+ve T cells which were mediating the rejection of the tumour (Matulonis et al., 1995).

Direct comparison between the effect of B7-1 or B7-2 expression was subsequently performed using the same murine myeloid leukaemic cell line. Mice injected with wild type leukaemic cells all rapidly developed leukaemia, whereas 100% of mice exposed to B7-1+ve leukaemic cells and 60% of mice exposed to B7-2+ve cells survived long term (Matulonis et al., 1996). As only 60% of mice survived after exposure to B7-2+ve leukaemia cells, this suggested that these cells were less efficient at stimulating an effective immune response. *In vivo* depletion of CD8+ve T cells from mice resulted in the rapid development of leukaemia after injection with wild type tumour and also B7-1+ve and B7-2+ve leukaemic cells. In contrast *in vivo* depletion of CD4+ve cells resulted in development of leukaemia in only those mice receiving wild type or B7-2+ve leukaemic cells, with the B7-1+ve cells still being rejected. This suggested that only CD8+ve T cell were involved in the immune response against the B7-1+ve leukaemic cells and both CD4+ve and CD8+ve T cells were involved in the immune response against the B7-2+ve leukaemic cells. Further differences between B7-1 and B7-2 in this setting were found when the capacity of the transduced leukaemic cells to protect against subsequent exposure to wild type tumour was examined. Mice exposed to live B7-1+ve leukaemic cells were protected in 100% of cases, whereas only 20-60% of mice

survived after vaccination with the B7-2+ve leukaemic cells when injected, three months later, with wild type leukaemic cells. In addition, there were differences between the ability of B7-1+ve or B7-2+ve leukaemic cells to eradicate existing leukaemia. Mice with existing wild type leukaemia exposed to multiple injections of B7-1+ve cells survived greater than 4 months in 60% of cases, whereas no survivors were seen with mice receiving B7-2+ve leukaemic cells with pre-existing leukaemia. This data suggests that B7-1 and B7-2 possess functional differences and that B7-1 transduction might be a more effective strategy in the generation of an effective anti-tumour immune response (Matulonis et al., 1996).

In the previous two studies a murine AML cell line, which had been transformed with the p210 BCR/ABL oncogene was used, which may have provided a potential tumour specific antigen (Chen et al., 1992). To determine whether B7-1 expression on primary AML would also lead to immunological rejection, a radiation induced leukaemia was used in mice. In addition, the cells used to vaccinate the mice were irradiated, which in some studies has decreased the ability to induce an anti-tumour response (Chen et al., 1994b; Matulonis et al., 1995). Similar results were obtained, with rejection of B7-1+ve AML cells and protection against B7-1-ve AML cells for up to 6 months, which was mediated by CD8+ve T cells. In addition a single dose of B7-1+ve AML cells lead to immune rejection of pre-existing early leukaemia (Dunussi-Joannopoulos et al., 1996). In the above experiment the nature of the tumour antigen is not known, as is the case with human AML, but the CD8 T cell response suggests that it is presented in the context of MHC class I molecules. In addition the normal haemopoiesis observed in surviving animals, suggest

that an immune response against epitopes expressed on both AML cells and normal haemopoietic progenitors is not occurring, which is encouraging given this potential complication in using a tumour modification strategy. The same group also observed that mice only rejected pre-existing AML when vaccinated with irradiated B7-1+ve AML cells within 1 week of the inoculation with wild type AML. If vaccination with B7-1+ve AML cells occurred 2 weeks after inoculation with wild type tumour, almost no long term survivors were seen. One possibility is that anergy had developed in AML reactive T cells, on exposure to the tumour antigen in the absence of costimulation (Harding et al., 1992). However, it was demonstrated that the week 2 vaccinated mice developed stronger CTL activity than the week 1 vaccinated mice. When mice received early chemotherapy followed by week 2 vaccination with B7-1+ve AML cells, 100% of mice were cured, whereas late chemotherapy and week 2 vaccination cured only 20% of mice. Taken together these results suggest that it is tumour bulk that is the limiting factor in immune mediated killing of the AML cells and induction of anergy does not seem to be occurring (Dunussi-Joannopoulos et al., 1997).

Using a different strategy, a further group used a myeloid leukaemic cell line to generate cytotoxic T lymphocytes with anti-leukaemic specific cytotoxicity. They found that expression of B7-1 was required to generate leukaemia reactive CTL's and expansion occurred on restimulation with B7-1 or B7-2 expressing AML cells. CTL infusions into mice after inoculation with a lethal dose of wild type AML resulted in 100% survival and protected mice against rechallenge for up to 3 months (Boyer et al., 1997). Therefore B7-1+ve AML cells can be used *in vivo* as tumour vaccines, or *in vitro* to generate CTL's

with anti-leukaemic specific activity, which can be subsequently returned and successfully protect against leukaemic relapse.

1.14 AML AS A TARGET FOR IMMUNOTHERAPY

AML is a malignancy of myeloid haematopoietic progenitors that without treatment results in complete bone marrow failure within a few weeks. It is classified on morphological grounds, shown fully in table 1. With current chemotherapeutic regimes up to 60% of patients can be cured, but of those that relapse the chance of cure with chemotherapy alone is low. The best chance of cure in patients relapsing with AML is afforded by allogeneic bone marrow transplantation, which is mainly attributed to an immune anti-leukaemic response, Graft versus Leukaemia (GvL) (Horowitz et al., 1990). However, only 25% of patients requiring a bone marrow transplant have a suitably matched HLA-identical donor, thus there is a large body of patients which could benefit from possible immunotherapeutic strategies.

That AML cells are capable of eliciting an anti-leukaemic immune response was first recognised in the setting of allogeneic bone marrow transplantation (BMT), by decreased relapse risk compared to standard chemotherapy (Horowitz et al., 1990) and increased relapse in those patients receiving a syngeneic bone marrow transplant (Gale et al., 1994). This anti-leukaemic immune response was termed Graft versus Leukaemia (GvL). That donor lymphocyte infusions were able to reinduce remission in patients relapsing after allogeneic BMT was further evidence of the ability of the immune system to generate an effective anti-leukaemic immune response (Kolb et al.,

1990). Associated with the GvL effect is graft-versus-host disease (GvHD), which results characteristically in immune mediated damage to the skin, liver, gastrointestinal tract and bone marrow, occurring more frequently in unrelated donor or HLA mismatched marrow transplants (Vogelsang and Hess, 1994). GvL and GvHD are closely linked, in that those patients developing GvHD have a decreased risk of relapse (Weiden et al., 1979), although decreased relapse is also seen in those patients without GvHD demonstrating that GvL is present in the absence of clinically detectable GvHD (Horowitz et al., 1990). That T cells are involved in mediating GvL is suggested by the increase in relapse associated with depletion of the marrow graft of T cells (Marmont et al., 1991). The lymphocyte subsets involved in GvL have not been fully characterised, but both CD4+ve and CD8+ve T cells have been implicated in GvL in murine and human systems (OKunewick et al., 1992; Truitt and Atasoylu, 1991; Jiang and Barrett, 1995; Jiang et al., 1991), although in man depletion of CD4 or CD8 T cell subsets suggests that CD4 cells contribute more to GvL (Jiang et al., 1991). Natural killer (NK) cells have also been implicated in the GvL effect, the relapse risk in a murine model correlating with the susceptibility of the leukaemic cells to NK cell mediated cytotoxicity and depletion of NK cells increasing the relapse risk in one of the leukaemic models (Glass et al., 1996). In patients with AML post autologous BMT, NK cells have been shown to mediate anti-leukaemic specific cytotoxicity (Lowdell et al., 1997). In addition delayed NK cell recovery was found to be associated with an increased relapse risk in patients undergoing allogeneic BMT for CML, suggesting these cells are involved in GvL (Jiang et al., 1997). Interleukin-2 infusions have been given following autologous bone marrow transplantation in an attempt to induce autologous

anti-leukaemic activity with reduction in the expected relapse risk, but toxicity was significant (Benyunes et al., 1993).

One of the potential group of target antigens in GvL are the minor histocompatability antigens (mHa), which are allelic peptides expressed on all cells expressing MHC molecules (Goulmy et al., 1991). These peptides are MHC restricted. Two such mHa are HA-1 and HA-2, which are HLA A2.1 restricted and are expressed on MHC class I positive cells in 69% and 95% of the HLA-A2.1 positive population respectively. HA-4 and HA-5 are also restricted to HLA A2.1 and are expressed in 16% and 18% of the same population respectively (van Els et al., 1992). It has been demonstrated with the mHag HA-1, that the HA-1 negative allelic phenotype is due to a single amino acid difference in the peptide, due to two nucleotide substitutions, from the HA-1 positive phenotype. Family screening revealed that HA-1 negative members of the family only possessed the HA-1 negative DNA sequences, whereas the HA-1 positive family members possessed both positive and negative DNA sequences (Joke et al., 1998). As the negative phenotype for HA-1 is not recognised by HLA A2.1 T cells, only bone marrow transplantation from a homozygous negative donor into a heterozygous or homozygous HA-1 recipient would be associated with GvHD and GvL (~~Joke~~ den Haan et al., 1998). Minor histocompatibility antigens which may be of particular interest with regard to GvL are the mHa HA-1 and HA-2, which are restricted to cells of haematopoietic and lymphoid origin and are expressed on leukaemic cells (van der Harst et al., 1994). The HA-2 peptide has been identified as a member of the non filament forming class I myosin family, which are involved in cell locomotion and organelle transport (den Haan et al., 1995).

Table 1 Morphological Classification of AML

M0	undifferentiated myeloblastic
M1	myeloblastic without maturation
M2	myeloblastic with maturation
M2-Baso	M2 with basophil-blasts
M3	hypergranular promyelocytic
M4	myelomonocytic, with both granulocytic and monocytic differentiation
M4-Eo	M4 with bone marrow eosinophilia
M5	monocytic; monoblastic (M5a) and promonocytic-monocytic (M5b)
M6	erythroleukaemia
M7	megakaryoblastic

Minor histocompatibility antigens are of relevance as a target for GvL, but not in an autologous anti-leukaemic immune response. As described in section 1.12.4, a variety of genetic abnormalities have been found in AML which result in the generation of tumour specific peptides. In AML some proteins are also overexpressed which may act as an immunogenic target (section 1.12.3).

In human pre-B ALL, lack of expression of B7-1 or B7-2 have been shown to prevent allogeneic T cells from proliferating in mixed lymphocyte reactions and furthermore prevent proliferation with the presence of signalling through CD28 demonstrating the induction of anergy in potentially reactive T cells. Cells innately expressing B7-2 or induced to express B7-2 were capable of eliciting an allogeneic T cell response, indicating that lack of B7-1 or B7-2 expression may be involved in preventing an anti-leukaemic immune response and induction of B7-2 expression on the pre-B ALL cells may be able to induce an anti-leukaemic immune response (Cardoso et al., 1996). Murine models of AML, using tumourigenic cell lines or radiation induced leukaemia have demonstrated that induction of B7-1 expression on the AML cells has lead to immune rejection, mediated by T cells, which also confers protection to subsequent exposure to B7-1-ve AML cells (Matulonis et al., 1995; Matulonis et al., 1996; Dunussi-Joannopoulos et al., 1996; Boyer et al., 1997). This demonstrates that in the murine setting, induction of B7-1 expression enables the AML cells to act as professional antigen presenting cells, presenting a tumour specific antigen and delivering a co-stimulatory signal. In addition AML blasts express both MHC class I and II as well as several adhesion molecules important in the induction of a an immune response and thus many of the

factors important in the generation of an immune response are innately expressed by AML cells (Reuss-Borst et al., 1995).

1.15 SUMMARY

The aim of this study is to induce expression of the co-stimulatory molecules B7-1 or B7-2 on myeloid leukaemia cells, to provide a co-stimulatory signal to potentially leukaemia reactive T lymphocytes. The modified leukaemia cells will function as professional antigen presenting cells and will enable the induction of an effective anti-leukaemic immune response leading to immune mediated destruction of residual disease in patients after chemotherapy.

In chapter 3 I have analysed inate expression of B7-1 and B7-2 on myeloid leukaemic cells and found B7-1 to be only rarely expressed. In contrast B7-2 was frequently expressed and in those patients with high expression I found that the duration of first remission was longer, suggesting that an immune mediated response might be responsible for this observation. The B7-2 was demonstrated to be functional in an allogeneic setting and induced responding T cells to proliferate and produce IL-2. All leukaemic cells expressed both HLA class I and II and therefore should be able to present peptides including leukaemic specific antigens and expressed the adhesion molecules LFA-1 and ICAM-1 important in the induction of an immune response. That first remission was prolonged in those patients whose leukaemia innately expressed B7-2 suggests that a strategy of inducing AML blasts to express B7-1 or B7-2 might be effective.

In chapters 5 and 6, using stable transfectants, I have confirmed the co-stimulatory function of the expressed B7-1 and B7-2 and analysed their role in

the generation of CTL's. Stimulator cells expressing B7-1 resulted in the greatest cytotoxicity, followed by B7-2 expressing stimulator cells and finally control cells. Some studies have suggested that B7-1 and B7-2 may play a role in the development of a T_H1 or T_H2 immune response, although analysis of the cytokines produced by the responding T cells in the above assay did not demonstrate a significant difference in the amount of IL-4 or γ-interferon produced.

In chapter 7 I describe the production of recombinant AAV containing B7-1 or B7-2 expression cassettes, which were used to transduce myeloid leukaemia blasts. This vector was chosen for the ability of the virus to infect slowly or non dividing cells. After infection with recombinant AAV, up to 15% of AML blasts expressed either B7-1 or B7-2, although the results were inconsistent. The B7-1 or B7-2 expressing AML blasts were able to induce increased proliferation of allogeneic T cells, compared to control AML blasts. Future work will include analysing the ability of these altered AML blasts to induce CTL's *in vitro* and improving the consistency of the transduction efficiency with recombinant AAV into AML blasts. It is hoped that this strategy will be used in clinical trials, either involving *ex vivo* generation of anti-leukaemic CTL's which can be returned to patients after chemotherapy, or returning the altered cells directly to patients in an attempt to induce an anti-leukaemic response *in vivo*.

2.1 MATERIALS AND METHODS (CELL BASED)

2.1.1 AML Cell Preparations

Leukaemic cells were obtained from either the bone marrow or peripheral blood of patients with acute myeloid leukaemia. 10mls of Ficoll-hypaque (Lymphoprep^{TM}), warmed to room temperature, was added to a 30ml universal container and 10mls of peripheral blood or bone marrow was carefully layered on top, avoiding any mixing of the blood and lymphoprep. The sample was centrifuged at 400 x g for 20 minutes at room temperature. After centrifugation the mononuclear cells were harvested from the sample/lymphoprep interface and diluted in an equal volume of HBSS. The cells were washed once in HBSS (Life Technologies) and pelleted by centrifugation at 250 x g for 5 minutes. Cells were then either immunophenotyped immediately or stored in liquid nitrogen for later analysis. Cells to be frozen were resuspended in RPMI (Gibco) to a cell density of $2x10^7$/ml and diluted with an equal volume of fetal calf serum containing 20%DMSO. The cells were frozen, initially in the vapour phase of liquid nitrogen and after 24 hours transferred to liquid nitrogen for long term storage. To decrease the possibility of contamination from normal mononuclear cells only samples with a blast population of greater than 95% were analysed and the cells were also labelled with anti-CD3$_{PerCP}$ and analysed by flow cytometric analysis to ensure that samples used were not contaminated significantly with T cells.

2.1.2 Separation of Peripheral Blood Mononuclear Cells (PBMC) From Blood

Healthy human volunteers were used in all cases. 10-20 millilitres (mls) of peripheral blood was taken into a 30mls universal container (Sterilin), containing preservative free heparin (CP Pharmaceuticals Ltd.) at a final concentration of 10 IU/ml, from the volunteer on the day the cells were to be used. The mononuclear cells were separated as described in section 2.1.1. The mononuclear cells were pelleted by centrifugation at 250 x g, the supernatant discarded and the cells resuspended in 10mls of HBSS. The sample was recentrifuged at 250 x g, the supernatant discarded and the cells resuspended in 10 mls RPMI 1640 medium with Glutamax-I (GibcoBRL) supplemented with penicillin (50 IU/ml), streptomycin (50μg/ml) and 10% human AB serum (Sigma) at the appropriate cell concentration for the assay (termed complete medium).

2.1.2.1 Enrichment of T cells From PBMC

To enrich the mononuclear cells of T cells, the sample was depleted of monocytes, B cells and NK cells by magnetic separation after labelling with the appropriate mAbs and magnetic microbeads. The PBMC, resuspended in RPMI 1640 medium from above, were incubated at 37°C and 5% CO_2 in a 90mm cell culture plate (Nunc™) for 1 hour. After 1 hour the non adherent cell population were harvested, leaving the adherent monocytes. The cells were pelleted by centrifugation at 250 x g for 5 minutes in a 30ml universal container, resuspended in 10mls of HBSS and recentrifuged at 250 x g for 5 minutes. The supernatant was discarded and the cells resuspended in the residual HBSS.

20μl of anti-CD14 (Becton Dickinson - clone MϕP9), 20μl of anti-CD19 (Becton Dickinson - clone SJ25C1) and 20μl of anti-CD56 (Becton Dickinson - clone NCAM16.2) were added to the sample per $2x10^7$ cells. The cells were incubated at room temperature for 15 minutes, after which the cells were resuspended in 10mls of HBSS and centrifuged at 250 x g for 5 minutes. The supernatant was discarded and the cells resuspended in the residual HBSS. The cells were then incubated with 20μl of goat anti-mouse IgG conjugated to FITC (Becton Dickinson - polyclonal) for 15 minutes at room temperature in the dark. The cells were resuspended in 10mls of MACS buffer (Phosphate Buffered Saline (PBS), 5mM Ethylenediaminetetraacetic acid (EDTA) and 0.5% bovine serum albumin (Sigma)) and centrifuged at 250 x g for 5 minutes at room temperature. The supernatant was decanted, the cells resuspended in the residual fluid and 40μl of anti-FITC microbeads (Miltenyi Biotec) were added. The sample was incubated on ice for 15 minutes and the volume made up to 10mls with MACS buffer. During the final 15 minute incubation period the separation column was prepared. The separation column used was an AS negative selection column (Miltenyi Biotec), which was placed in the MACS separator and the flow resistor used was a 25 gauge needle. The column was primed with MACS buffer from below and the column rinsed with 10mls of MACS buffer added from the top. The magnetically stained cell suspension was applied to the top of the column, the cell suspension was allowed to pass through and the cells collected in a sterile 30ml universal container. The column was rinsed with a further 5mls of MACS buffer and this fraction was also collected. The collected cell fraction, containing the enriched T cells, was centrifuged at 250 x g for 5 minutes and the cells resuspended in supplemented

RPMI 1640 medium as before. The cell concentration was calculated with a haemocytometer (Neubauer/ARH) and the cell concentration adjusted to $2x10^6$ cells/ml. An aliquot of the T cells was taken and split into two fractions. The cells were washed in HBSS and to one aliquot was added 10µl of anti-CD3 conjugated to PerCP (Becton Dickinson - clone SK7), which was incubated at room temperature in the dark for 15 minutes. The cells were washed once and resuspended in FACSFlow prior to flow cytometric analysis as described in section 2.1.3, using the unlabelled T cells as negative control cells.

2.1.3 Flow Cytometric Analysis

$0.5x10^6$ cells to be analysed were transferred into 12 x 75mm polystyrene tubes (Falcon Plastics). 2mls HBSS was added to each tube which were centrifuged at 200 x g (MSE Mistral 2000) for 5 minutes. The supernatant was discarded from each tube and the cells resuspended in the residual fluid. A further 2 mls of HBSS was added to each tube and recentrifuged at 200 x g. The supernatants were discarded and the cell pellet resuspended in the residual HBSS in the bottom of the tubes. To each tube was then added the appropriate monoclonal antibody directly conjugated to a fluorochrome and the cells and antibody were mixed by briefly vortexing the tubes. The cells were incubated at room temperature in the dark for 15 minutes. 2mls of HBSS was added to each tube and both were centrifuged at 200 x g for 5 minutes. The supernatant was again discarded and the cells were resuspended in 1ml of FACSFlow (Becton Dickinson). The cells were then analysed by flow cytometric analysis (FACScan with Lysis II software - Becton Dickinson). The forward scatter (FSC), FL1 and FL2 signals were standardised prior to each

analysis against a commercial bead preparation (Calibrite - Becton Dickinson). 10,000 viable cells as determined by light scatter, were analysed from each sample.

2.1.4 Mixed Lymphocyte Reaction (MLR)

T cells were enriched from the peripheral blood of healthy volunteers, as described in sections 2.1.2 and 2.1.2.1. After counting, using a haematocytometer, the cells were resuspended in supplemented RPMI 1640 at a concentration of $2x10^6$ cells/ml. Stimulator cells were washed and resuspended in supplemented RPMI 1640 at a concentration of $1x10^6$ cells/ml. All stimulator cells were then irradiated with 4000 rads in a cell irradiator (Nordion/Gammacell 2000) to inhibit proliferation during the assay. To assess background proliferation of donor T cells, 100μl of T cells was added to 100μl of medium. To assess background proliferation of stimulator cells, 100μl of each of the stimulators was added to 100μl of medium. 100μl of T cells were added to 100μl of irradiated stimulator cells. All the above combinations were performed in replicates of 6 and cultured for 5 days at 37°C and 5% CO_2. For the last 18 hours of the culture 1 μCi (37 kBq) of [methyl-^{3}H]-thymidine (Amersham) was added to each well in the assay. The cells were then harvested onto glass fibre filters (Helis Bio Ltd.) using a cell harvester (Dynatech Multimash 2000). The filters were dried and then added to a 20ml scintillation vial (Canberra Packard). 1ml of scintillation fluid (Canberra Packard - Filter count) was added to each vial, before measurement of β-emission in a scintillation counter (Wallac 1209 Rackbeta).

2.1.5 ELISA Assays

Appropriate reference concentrations of the cytokine were produced from a stock solution by serial dilution with sample diluent. 100μl of each standard and 100μl of each sample to be analysed were added to each test well of the microtitre plate (Genzyme). The plate was sealed, incubated at 37°C for 60 minutes and washed 5 times in wash reagent (proprietary formulation). After the last washing step the plate was blotted on paper towels to remove residual fluid and then 100μl of anti-cytokine biotinylated antibody was added to each well of the assay. The plate was resealed and incubated at 37°C for 60 minutes, after which it was washed a further 5 times, with wash reagent. 100μl of streptavidin reagent conjugated to horseradish peroxidase was added to each test well and incubated at 37°C for 15 minutes. The wells were washed a further 5 times with wash reagent and 100μl of working substrate solution was added to each test well. The plate was incubated at room temperature for 10 minutes and then 100μl of stop solution (1M Sulphuric acid) was added to each test well. The absorbance in each test well was read at 450nm using a spectrophotometer (Anthos Reader 2001). A standard curve was generated from the reference concentrations of the cytokine and the concentration of cytokine in the test samples was calculated from the standard curve. Statistical analysis of the amount of cytokine produced was performed using the Student's paired T test.

2.1.6 Transfection

Transfections were performed using cationic lipid, which relies on the binding of the positively charged lipid headgroup to the negatively charged

phosphate groups of the DNA to form lipid DNA complexes (Felgner et al., 1987). Prior to transfection of eukaryotic cells with plasmid DNA, fresh plasmid DNA was prepared using the Qiagen QIAprep plasmid miniprep column as described in section 2.2.3 and cleaned as described in section 2.2.3.4. The concentration of the DNA was calculated by measuring the absorbance at 260nm (A_{260}) using a UV visible spectrophotometer (Cam Spec M302).

Target cells used were 293 cells (ECACC No:85120602), which are derived from human embryonal kidney cells transformed with sheared human adenovirus type 5 DNA. The 293 cells were seeded at a concentration of $1x10^5$ cells/ml in a Nunclon 6 well plate, with a total of 2 mls in each well, in Dulbecco's Modified Essential Medium (DMEM) (Gibco), supplemented with 10% Foetal Calf Serum (FCS) (GibcoBRL), penicillin (GibcoBRL) at a concentration of 50IU/ml and streptomycin (GibcoBRL) at a concentration of 50μg/ml. The cells were cultured at 37°C and 5% carbon dioxide (CO_2) until they reach 40-60% confluency. The desired amount of plasmid DNA to be transfected was placed in a 17 x 120mm conical polystyrene sterile tube (Falcon) and to this 100μl of serum free medium was added (Opti-MEM GibcoBRL). In a separate 12x75 mm sterile tubes was aliquoted the cationic lipid (Lipofectin (1:1 ratio N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA) and dioleoyl phosphotidylethanolamine (DOPE) GibcoBRL), which was at a concentration of 1mg/ml, to which was added 100μl of serum free medium. The plasmid DNA and lipofectin solutions were mixed gently and incubated at room temperature for 15 minutes, after which 800μl of serum free medium was added to each tube, making the total volume 1ml. Meanwhile the cells to be transfected were washed by adding once to each well 2mls of serum

free medium. This was aspirated from each well and to each well was added the DNA/lipofectin mixture ensuring the cell monolayer was completely covered. The cells were incubated for 5 hours at 37°C and 5% carbon dioxide (CO_2), after which the DNA:lipofectin mixture was carefully aspirated and replaced with DMEM supplemented as before. Incubation was then continued for 48 hours before harvesting of the cells to assess expression of the gene of interest.

2.1.6.1 Detection of β-Galactosidase Activity in Transfected Cells

To assay the cells for β-galactosidase activity the medium was carefully aspirated from the cell monolayer from each transfected well. The cells were then washed twice with PBS buffer (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH adjusted to 7.1) (Mg^{2+} and Ca^{2+} free) taking care not to dislodge any of the cells. The residue of the PBS was aspirated using a pipette tip. 400µl of 1x Reporter Lysis Buffer (Promega) was added to the cells so that the cell monolayer was completely covered. This was incubated at room temperature for 15 mins, gently rocking the cells once midway through this period. The cells were then carefully scraped off from each well and transferred to separate 1.5ml microcentrifuge tubes (Eppendorf). Each tube was vortexed for 15 seconds and centrifuged at 10,000 x g in a benchtop microcentrifuge for 2 minutes. After this the supernatants containing β-galactosidase activity were transferred to fresh microcentrifuge tubes.

25µl of each cell extract was added to a flat bottomed 96 well plate (Nunc), including the negative control and was diluted with 25µl of 1x Reporter Lysis Buffer (Promega). In a separate 96 well plate 50µl of Assay 2x Buffer (120mM Na_2HPO_4, 80mM NaH_2PO_4, 2mM $MgCl_2$, 100mM β-mercaptoethanol

and 1.33mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG)) was added to the appropriate number of wells. To each of these wells was added 50μl of each cell extract, the samples mixed by pipetting up and down and the plate was covered and incubated at 37°C for 30 minutes. The reaction was then stopped by the addition of 150μl of 1M Sodium carbonate. The absorbance of the samples was read at 405nm using a plate reader (Anthos reader 2001).

2.1.6.2 Generation of Stable Transfectants

293 cells were cultured in a 96mm sterile petri dish (Nunc) in supplemented DMEM as described in 2.1.6 until they had reached 60% confluency. The plasmid was transfected into the 293 cells as described in 2.1.6, using lipofectin. 5 hours post transfection, the lipofectin:DNA complexes were removed and replaced with fresh medium. After incubation overnight at 37°C with 5%CO_2, the medium was replaced with fresh supplemented DMEM containing G418 sulphate at an active concentration of 500μg/ml (Canaani and Berg, 1982). Culture was continued, with death of non resistant cells between 3 and 7 days post exposure to G418. During selection, the medium was replaced every day and dead cells removed. When the cells had reached confluency, they were harvested by incubation with 5mls of trypsin-EDTA (0.5g trypsin and 0.2g EDTA in 1000mls PBS), after aspiration of the medium and washing the cell monolayer once with HBSS. The cells were then analysed by flow cytometry for expression of the gene of interest, as described in section 2.1.3.

2.1.6.3 Sorting of 293 B7-1 and B7-2 293 Cells into Uniformly Expressing Populations

Stable transfectants expressing either B7-1 or B7-2 were harvested by trypsinisation. $1x10^6$ cells from each population were washed and labelled with either anti-B7-1 FITC (Pharmingen - clone BB1) or anti-B7-2 FITC (Pharmingen - clone FUN-1) respectively. After labelling and washing the cells were resupended in 1ml of FACSFlow. The cells were sorted on a FACS Vantage, the cells separated into two populations, the highest surface expressors of B7-1 and B7-2 and the low or negative expressors of B7-1 or B7-2. The high expressors of B7-1 or B7-2 were washed once in 1ml of HBSS, centrifuged at 200 x g, supernatant discarded and the cell pellet resupended in 2mls of DMEM, supplemented with 10% FCS, penicillin and streptomycin and G418 sulphate at 500μg/ml. The B7-1 and B7-2 high expressors were each inoculated into one well of a 6 well plate and cultured at 37°C and 5%CO_2 until the cells had reached confluency. An aliquot of each of the two samples was harvested and labelled with either 10μl anti-B7-1 PE or 10μl anti-B7-2 FITC accordingly, as described in section 2.1.3, prior to flow cytometric analysis.

2.1.7 Costimulation Assay

The sorted 293 cells expressing B7-1 or B7-2 and the 293 cells stably transfected with pBK-CMV *lac-ve* were harvested, washed twice in HBSS and resuspended in supplemented RPMI 1640 at a concentration of $2x10^5$ cells/ml. The 293 cells were then irradiated with 125 Gray in a cell irradiator (Nordion - Gammacell 3000 Elan). After irradiation $1x10^4$ cells were added to a flat bottomed well in a 96 well plate. Six replicates of each of the B7-1 and B7-2

expressing 293 cells as well as the negatively expressing 293 cells were used. $1x10^5$ enriched T cells were added to each of the wells to a total volume of 100μl. To assess intrinsic proliferation $1x10^5$ T cells were added to 6 wells with no 293 cells and $1x10^4$ irradiated 293 cells were added to 6 wells with no T cells, both controls also being made up to a total volume of 100μl with 50μl of fresh medium. PMA was added to all wells in the assay to a final concentration of 2.5ng/ml. To determine if proliferation was due to costimulation with either B7-1 or B7-2, $1x10^5$ B7-1 and B7-2 expressing 293 cells were preincubated for 15 minutes with 1μg of anti-B7-1 (Pharmingen - clone BB1) or 1μg of anti-B7-2 (Pharmingen - clone IT2.2) which was screened for the absence of endotoxin and low in azide. The cells were washed and resuspended in 500μl of RPMI 1640 with Glutamax-I supplemented with penicillin, streptomycin and 10% human AB serum at a concentration of $2x10^5$ cells/ml. $1x10^4$ of the antibody coated B7-1 or B7-2 expressing 293 cells were then added to a well in the 96 well plate, again 6 replicates of each being performed. $1x10^5$ T cells were added to each of these wells and PMA was added to a final concentration of 2.5ng/ml to each of these wells also. The cells were incubated for 72 hours at 37°C in 5% CO_2 and for the last 18 hours of the assay each well was pulsed with 1 μCi (37 kBq) of [methyl-^{3}H]-thymidine (Company). Incorporation of tritiated thymidine, as a measure of proliferation, was assessed as described in section 2.1.4.

2.1.8 Cytotoxicity Assays

PBMC were obtained from the peripheral blood of healthy volunteers as described in section 2.1.2 and enriched for T cells by depletion of monocytes, B

cells and NK cells as described in section 2.1.2.1. The T cells were resuspended at $1x10^6$ cells/ml in supplemented RPMI 1640. The stimulator cells were 293 cells stably transfected with either *lac-ve*, pBK-CMV B7-1 or pBK-CMV B7-2, the B7-1 and B7-2 expressing 293 cells having been sorted to obtain a uniformly highly expressing population (section 2.1.6.3). The stimulator cells were harvested by incubating the cells with trypsin-EDTA solution (GibcoBRL) until they became non-adherent and were washed twice in HBSS. After washing they were counted and resuspended in RPMI 1640 with Glutamax-I supplemented with penicillin (50IU/ml), streptomycin (50μg/ml) and 10% human AB serum (Sigma) at a concentration of $0.5x10^6$ cells/ml. All of the stimulator cells were then irradiated to 125 Gray in a cell irradiator (Gammacel 3000 Elan/Nordion International). After irradiation 100μl of each of the control 293, 293B7-1 and 293B7-2 cells were mixed with 100μl of the T cell preparation in separate wells of a sterile 96 round bottomed plate (Nunclon™). To control for inate cytotoxic activity of the T cells, 100μl of the T cells were cultured with 100μl of medium for the duration of the assay. All variables were performed in replicates of 8. The cells were cultured at 37°C and 5%CO_2 for 10 days. After 5 days all the wells were supplemented with interleukin 2 (R&D Systems) at a final concentration of 5ng/ml. On the 10^{th} day fresh parental 293 cells were harvested as described above, washed in HBSS twice and resuspended in 10mls of RPMI 1640 with Glutamax-I supplemented with penicillin, streptomycin and 10% human AB serum. The cells were pelleted by centrifugation at 200 x g for 5 minutes and the cells resupended in the residual medium after the supernatant was decanted. 50μCi of ^{51}Chromium (Cr) (Amersham) was added to the 293 target cells, which were then incubated at 37°C and 5%CO_2 for 2

hours. The target cells were then washed twice in 10mls of RPMI 1640 medium, before being resuspended in supplemented RPMI 1640 (Sigma) at a concentration of $0.1x10^6$cells/ml. The T cells incubated with the irradiated stimulator cells were washed twice in 10mls of HBSS before being resuspended in RPMI 1640 with Glutamax-I supplemented with penicillin, streptomycin and 10% human AB serum at a concentration of $1x10^6$ cells/ml. In a round bottomed 96 well plate (Nunclon™) 100μl of each of the three T cell preparations were incubated with 100μl of the labelled 293 target cells. As a spontaneous release control 100μl of the target cells were added to 100μl of RPMI 1640 with Glutamax-I supplemented with penicillin, streptomycin and 10% human AB serum. As a total release control 100μl of Triton X (BDH) was added to 100μl of target cells, which results in lysis of 100% of the cells on visualisation by microscopy. 100μl of the unstimulated T cells were added to 100μl of the labelled target cells to control for innate cytoxic activity against the 293 cells. All of the effector phase of the assay was performed in replicates of at least 6. The cells were cultured for 4 hours at 37°C and 5%CO_2. The cells were then pelleted by centrifugation at 200 x g for 5 minutes and 50μl of the supernatant was carefully aspirated from each well and transferred to a fresh 96-well sample plate (Wallac). 200μl of Optiphase Hi-Safe scintillation fluid was added to each well and the plate covered and shaken for 15 minutes on a plate shaker. After shaking the plates were loaded into a β-counter to assess the ^{51}Cr released into the supernatant (MicroBeta - Wallac).

2.1.9 Detection of the Intracellular Cytokines IL-4 and γ-IFN

Detection of the intracellular cytokines IL-4 and γ-IFN has been shown to be a useful technique for the analysis of T cells of either T_H1 or T_H2 cytokine profiles (Picker et al., 1995). This technique was therefore applied to the T cells after the stimulation phase of the cytotoxicity assay. After 9 days incubation of the T cells with the 293 transfectants as described in section 2.1.8, monensin (Sigma) was added to the well at a final concentration of 3μM, together with phorbal 12-myristate 13-acetate (PMA) (Sigma) at 25 ng/ml and calcium ionophore (Sigma) at 1μg/ml. Monensin interferes with intracellular transport mechanisms and causes an accumulation of cytokine within the Golgi apparatus of the cell. PMA and calcium ionophore result in accessory cell independent T cell activation, upregulating cytokine production. After 18 hours incubation at 37^0C and 5% CO_2, the cells were harvested and washed twice in HBSS. The cells were counted and divided into aliquots of $0.5x10^6$ cells, which were labelled with 10μl anti-CD3 PerCP (Becton Dickinson - clone SK7) as described in section 2.1.3. After 15 minutes incubation in the dark, the cells were washed in 2 mls of wash buffer (PBS, 0.5% BSA and 0.1% sodium azide). After washing the T cells were resuspended in 500μl of FACS™ permeabilising solution (Becton Dickinson - proprietory formulation containing 0.1% saponin) and incubated for 10 minutes at room temperature in the dark. The cells were washed with a further 2 mls of wash buffer and then labelled with 20μl of anti-γIFN-FITC and anti-IL-4 PE (Becton Dickinson). As a negative control, an aliquot of T cell was also incubated with isotype control antibodies (20μl gamma 2a FITC and gamma 1 PE (Becton Dickinson)). The cells were incubated at

room temperature in the dark for 30 minutes and then washed in 2mls of wash buffer. After washing the cells were resuspended in 500μl PBS + 1% paraformaldehyde, prior to flow cytometric analysis as described in section 2.1.3.

2.1.10 Production of Recombinant AAV B7-2

Production of recombinant AAV requires a two plasmid system, with the *rep* and *cap* gene functions being supplied in trans by the AAV helper plasmid and the vector plasmid containing the expression cassette between the two ITR's. 293 cells were grown to 80% confluency in 92mm tissue culture plates (Nunclon) in DMEM supplemented with 10% FCS. 3.5μg of AAV B7-2 and 10.5μg of helper plasmid (3:1 ratio of helper to vector plasmid) were transfected into the 293 cells using cationic lipid as described in section 2.1.6. The 293 cells were washed twice with HBSS and the DNA lipofectin mixture was layered onto the cells, after the total volume had been made up to 2mls to ensure the cells were adequately covered. The cells were then incubated for 5 hours at 37°C and 5% CO_2, after which the serum free medium was replaced with fresh DMEM containing 10% FCS and adenovirus type 5 at a multiplicity of infection (MOI) of 5. Incubation was then continued until full cytopathic effect of the adenovirus was seen on the 293 cells, typically after 48 to 72 hours. The infected cells were scraped off and collected together with the supernatant. Trypsin was added to a final concentration of 0.02% and sodium deoxycholate at a final concentration of 0.5%, which digests and precipitates free capsid protein respectively while maintaining the titre of complete virions [Anderson et al submitted]. The sample was then incubated at 37°C for 2 hours and then

subjected to three cycles of freeze-thawing to release any virus remaining within the cells. Following this the sample was incubated at 56°C for 1 hour to inactivate the heat-labile adenovirus. The sample was spun at 2100 x g for 10 minutes to pellet the cellular debris and the supernatant containing the recombinant AAV was decanted into a separate 50ml tube (Falcon).

2.1.10.1 Purification of Recombinant AAV Using Cellufine Affinity Column

Affinity column chromatography has been shown to be an effective technique for large scale purification of recombinant AAV (Tamayose et al., 1996). Sulfonated cellulose (Cellulofine) was packed into the column (Pharmacia Biotech XK50) and equilibrated with PBS (pH 7.8). The supernatant containing recombinant AAV was then applied to the column, which was then washed with PBS (pH 7.8) until the absorbance of the eluate at 280nm had returned to the baseline level seen before the supernatant had been applied. The AAV bound to the cellulofine was eluted with 10mM phosphate buffer containing 1.0M NaCl. Fractions of 3ml aliquots were collected and the aliquots containing eluted AAV were identified by the increase in absorbance detected at 280nm. These fractions were then pooled and applied to a Centriprep 50 concentrator, which was repeatedly spun at 1500 x g until the total volume had been reduced to approximately 2.5mls. The concentrated viral preparation was then applied to a sephadex desalting column (PD-10 column sephadex G-25 Pharmacia Biotech), equilibrated with 25mls of DMEM and then eluted from the column with 3.5mls of DMEM. The desalted viral preparation was then applied to a centricon 30 concentrator, which was spun at 5000 x g for 30 minutes. The concentrated virus was

recovered by inverting the filter which was spun at 5000 x g for 5 minutes to collect a final volume of typically 500μl. Prior to infecting cells with the viral preparation the sample was passed through a 0.2μm filter (Sartorius - Centrisart C4) and spun at 10,000 x g in a benchtop microcentrifuge. The viral concentrate was then either analysed immediately, used to infect target cells or stored at -70°C.

2.1.11 AML Cell Culture

All AML samples had been stored in liquid N_2, in 10% dimethyl sulfoxide, 40% FCS and 50% RPMI 1640 medium. Vials were retrieved prior to transduction and defrosted rapidly in a water bath at 37°C. Blasts were then washed twice in HBSS, prior to resuspension at $5x10^6$ cells/ml in Iscove's Modified Dulbecco's Medium (IMDM) containing penicillin (50iu/ml), streptomycin (50μg/ml) and gentamycin (100μg/ml) and supplemented with 10% FCS, 10% Horse serum, 20ng/ml stem cell factor (SCF)(R+D), 10ng/ml IL-3 (R+D) and 10ng/ml granulocyte monocyte colony stimulating factor (GM-CSF)(R+D).

2.1.11.1 Infection of AML Blasts with Recombinant AAV

100μl ($5x10^6$ cells) of the leukaemic blasts were pipetted into round bottomed wells of a 96 well plate. The plate was then centrifuged at 200 x g for 5 minutes to pellet the cells and the medium was aspirated from the wells leaving the cell pellet. The concentrated recombinant AAV was then added to each well to be infected at the required multiplicity of infection (MOI) and the cells were resuspended by gently pipetting up and down. The cells were then

incubated for 60 minutes at 37°C, after which 150μl of IMDM containing penicillin (50iu/ml), streptomycin (50μg/ml) and gentamycin (100μg/ml) and supplemented with 10% FCS, 10% Horse serum, 20ng/ml stem cell factor (SCF)(R+D), 10ng/ml IL-3 (R+D) and 10ng/ml granulocyte monocyte colony stimulating factor (GM-CSF)(R+D) was added to each well. Analysis of the cells for expression of the transgene was typically performed 72 hours after the infections.

2.2 MATERIALS AND METHODS (DNA BASED)

2.2.1 DNA Sequencing

All sequencing reactions were carried out in 0.5ml plastic centrifuge tubes (Eppendorf). 4μl of pUC 18 B7-2 (1μg DNA) was added to 3μl of distilled water, 5μl of plasmid denaturing reagent (10mM Tris-HCl, pH 7.5, 1mM EDTA, 50% Glycerol, 50% Ethylene Glycol) and 1μl of forward primer (2.0pmol/μl 5'-GTTTTCCCAGTCACGACGTTGTA-3'), mixed thoroughly and heated to 95°C for 5 minutes to denature the DNA. The mixture was chilled in an ice water bath and 2μl of plasmid reaction buffer (400mM Tris-HCl, pH 7.5, 100mM $MgCl_2$, 250mM NaCl) was added. The mixture of template, buffer and primer was then incubated at 37°C for 10 minutes to allow annealing of the primer to the template and then the mixture was chilled in ice. To label the DNA strands 1μl DTT(0.1M), 2μl labelling mix (1.5μM 2'-Deoxyguanosine 5'-triphosphate (dGTP), 1.5μM 2'-Deoxycytidine 5'-triphosphate (dCTP), 1.5μM 2'-Deoxythymidine 5'-triphosphate (dTTP), 0.5μl (5μCi) [^{35}S] 2'-Deoxyadenosine 5'-triphosphate (dATP) and 2μl sequenase polymerase (13 units/μl in 20mM

KPO_4, pH 7.4, 1mM DTT, 0.1mM EDTA, 50% Glycerol diluted 1:8 with ice cold Enzyme Dilution Buffer) was added to the ice cold annealed DNA mixture. This was mixed and incubated at room temperature for 8 minutes.

Prior to termination of the generation of the DNA strands 4 0.5ml microcentrifuge tubes were labelled G, A, T and C respectively. Into the tube labelled G was added 2.5µl of the ddGTP Termination mix (80µM 7-deaza-GTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddGTP, 50mM NaCl) and similarly into the A,T and C tubes was added 2.5µl of the ddATP (80µM 7-deaza-GTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddATP, 50mM NaCl), ddTTP (80µM 7-deaza-GTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddTTP, 50mM NaCl) and ddCTP (80µM 7-deaza-GTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 8µM ddCTP, 50mM NaCl) termination mixes respectively. These termination tubes were then prewarmed to 37°C and on completion of the labelling reaction 3.5µl was added to each of the termination tubes, mixed and reincubated at 37°C for 5 minutes. 4µl of stop solution (95% formamide, 20mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) was added to each of the termination reactions, mixed thoroughly and stored on ice until ready to load onto the sequencing gel.

2.2.1.1 Preparation of Acrylamide Gel and Running of Sequencing Reactions

The sequencing gel (8% acrylamide) was made by dissolving 75g urea in 67.5mls of distilled water. 6mls of 20x glycerol tolerant gel buffer (216g Tris base (Tris[hydroxymethyl]aminomethane), 72g Taurine (2-Aminoethanesulfonic acid), 4g NaEDTA.$2H_2O$, made up to 1ltr volume with distilled H_2O) and 30mls of a 40% solution of acrylamide was added to this. 1.5mls of 10% ammonium

persulphate was added and prior to pouring of the gel was added 30μl of TEMED (N,N,N',N' tetramethylethylenediamine). Prior to this the glass plates, graduated spacers and comb were thoroughly cleaned and then rinsed with distilled H_2O. The inner surface of the smaller glass plate was coated with a film of sigmacote® (Sigma) and left to dry. The glass plates were then taped together with insulating tape to ensure a watertight seal, with the sigmacoted surface of the smaller plate facing inwards and the graduated spacers at each margin of the glass plates. The acrylamide solution was the poured between the glass plates immediately after addition of the TEMED (Sigma), taking care to remove any air bubbles and the flat side of the shark's tooth comb was inserted into the top of the plates when the solution had reached the top of the plates. The comb was the clamped in position using bulldog binder clips. The gel was then left until it had set and then the insulating tape was removed and the gel loaded onto a Model S2 Sequencing Gel Electrophoresis Apparatus (Bethesda Research Laboratories, Life Technologies Incorporated) with the smaller glass plate facing inwards. The top reservoir and the bottom reservoir of the gel tank was then filled with 1x Glycerol Tolerant Gel Buffer so that the lip of the smaller glass plate was covered. The comb was then reversed to provide discrete wells for loading of the sequencing reactions and each well was flushed with the gel buffer prior to loading of the sequencing reactions. 4μl of each of the 4 sequencing reactions was loaded into each well, after being heated to 75°C for 2 minutes and run at 1400 volts for 3 hours. A careful note of the order of loading of the sequencing reactions was taken. The gel was removed from between the glass plates by blotting it onto Whatman 3MM paper, which was then laid on a further two pieces of the 3MM paper before

being covered in Saran Wrap (Dow Chemical Company). The 3MM paper and the Saran Wrap was then trimmed so that they were only slightly larger than the gel. The gel was then dried under vacuum for three hours in a Biorad Model 483 gel dryer. Once the gel was dry the gel was developed using Kodak Scientific Imaging Film (35x43cms) placed over the dry gel in a exposure cassette for 24 hours at room temperature.

2.2.1.2 Generation of Nested Deletions

4μg of pUC 18/B7-2 was digested with the restriction enzymes *Sal* I and *Sph* I (section 2.2.3.1). The mixture was then incubated at 37°C for 3 hours to ensure full digestion of the DNA with both restriction enzymes. Prior to digestion with Exonuclease III (Exo III), the DNA was cleaned with a Qiagen clean up column as described in section 2.2.3.4., except that the DNA was eluted from the column with 45μl H_2O. The cleaned digested DNA was topped up to 45μl with distilled H_2O if required, transferred to a 0.5ml microcentrifuge tube (Eppendorf) and 5μl of Exo III 10x buffer (660mM Tris-HCl, pH 8.0, 6.6mM $MgCl_2$) was added. The mixture was then warmed to 34°C and 7.5μl of S1 mix (172μl distilled H_2O, 27μl S1 7.4x Buffer (0.3M potassium acetate pH 4.6, 2.5M NaCl, 10mM $ZnSO_4$, 50% glycerol), 60 units S1 nuclease) was added separately to 12 wells of a 96 well round bottomed plate (Nunclon) and then placed on ice. 500 units of Exo III was added to the digested pUCB7-2, incubated at 34°C and after 30 seconds a 2.5μl aliquot was removed and put in well 1 of the S1 mix ensuring the sample was well mixed. This process was repeated at 15 second intervals until 12 samples had been aliquoted. The samples were taken off ice and incubated at room temperature for 30 minutes.

After this time 1μl of S1 stop buffer (0.3M Tris base, 0.05M EDTA) was added to each of the samples, which were then heated at 70°C for 10 minutes to inactivate the enzymes. To determine the extent of digestion 2μl aliquots were removed to run on a 1% agarose gel. To prepare the gel, 1g of agarose (Gibco) was weighed and added to 125mls of 0.5x TBE (5x 54g Tris base, 27.5g Boric acid, 20mls 0.5M EDTA (pH 8.0). The mixture was heated in a microwave oven until the agarose had completely dissolved. 5μl of ethidium bromide (10mg/ml) was added to the melted agarose and the mixture poured into the gel plate, sealed with watertight tape, with a 30 well comb in place. The agarose was allowed to cool until completely solidified and the tape and comb were carefully removed. The solidified gel in its tray was inserted into the gel tank and 0.5x TBE was poured in until the gel was submerged. Each of the aliquots was added to 2μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and 6μl of distilled H_2O, before being loaded into a well in the gel. The DNA was then run at 5 volts/cm until the DNA had run far enough into the gel, as indicated by the dyes in the loading buffer. The digested DNA was visualised using a UV light box and the gel photographed (Polaroid 667). The remainder of the samples were then transferred to a 37°C incubator and 1μl of Klenow mix (30μl Klenow buffer(20mM Tris-HCl, pH 8.0, 100mM $MgCl_2$), 5 units Klenow DNA polymerase) was added to each sample, incubated for 3 minutes at 37°C, before the addition of 1μl of dNTP mix (0.125mM each of dATP,dCTP,dGTP and dTTP) to each well. The DNA was then incubated for a further 5 minutes at 37°C. At room temperature 40μl of ligase mix (790μl deionised H_2O, 100μl ligase 10x buffer(500mM Tris-HCl, pH 7.6, 100mM $MgCl_2$, 10mM ATP), 100μl

50% PEG, 10μl 100mM DTT, 5 units T4 DNA polymerase) was added to each well, mixed and incubated at room temperature for 1 hour. 10μl of each ligation product were then transformed into E. coli (strain TG-1) competent cells.

2.2.2 Preparation of Competent Cells

Competent cells were produced using the Hanrahan technique. 5mls of LB medium ((950mls deionised H_2O, 10g Bacto-tryptone (GibcoBRL), 5g Bacto-yeast extract (GibcoBRL), 10g NaCl (BDH), sterilised by autoclaving (15-18 p.s.i and 121-124°C for 15mins), supplemented with 50μl 1M $MgCl_2$ and 50μl 20% glucose was inoculated with a fresh scraping of E.coli bacteria (strain TG-1) from the stock agarose plate. This was cultured overnight at 37°C and the following day 50mls of LB medium supplemented with 500μl of 1M $MgCl_2$ and 500μl of 20% glucose was inoculated with 500μl of the overnight bacterial culture. This was then cultured at 37°C for between 90 and 120 minutes until the optical density of the bacterial culture was between 0.5 to 0.55 when measured on a spectrophotometer (CamSpec M302) at a wavelength of 600nm. The bacterial culture was transferred to a 50ml centrifuge tube (Falcon) and then cooled on ice for 10 minutes before centrifugation at 350 x g for 10 minutes. The medium was decanted and the bacterial pellet resuspended in 16.66mls of ice cold TFB medium (10mM 2[N-morpholino]ethanesufonic acid, 45mM $MnCl_2.4H_2O$, 10mM $CaCl_2.2H_2O$, 100mM KCl, 3mM Hexamminecobalt chloride) and incubated on ice for 15 minutes. The bacteria were spun at 300 x g for 10 mins, the supernatant discarded and the bacteria resuspended in 4.12mls of ice cold TFB medium. 144μl DnD (1.53g dithiothreitol, 9ml DMSO, 100μl 1M potassium acetate pH

7.5, H_2O to 10 mls) was added to the tube and gently agitated to ensure adequate mixing. Incubation was continued for 10 minutes on ice before the addition of a further 144μl DnD. The bacteria were then incubated on ice for a further 15 minutes before being transformed with the DNA.

2.2.2.1 Transformation of Competent Cells

10μl of the ligation product was added to 200μl of the competent cells in a 15ml polystyrene centrifuge tube (Falcon), thoroughly mixed and incubated on ice for 30 minutes. The tubes were then heated at 42°C for 45 seconds and immediately put in ice for a further 2 minutes. 800μl of prewarmed LB medium supplemented with 0.2% glucose and 0.01M $MgCl_2$ was then added. The bacteria were then transferred to the shaker at 37°C for 1 hour. 200μl of each culture was then plated out in a sterile hood on LB agar plates containing the appropriate antibiotics for selection (ampicillin 50μg/ml, tetracycline 25μg/ml or kanamycin 50μg/ml). LB agar is 950 mls deionised H_2O, 10g Bacto-tryptone, 5g Bacto-yeast extract, 10g NaCl, dissolved and the pH adjusted to 7.0 with 5M NaOH, 15g Bacto-agar and autoclaved as described above. The antibiotic was added prior to pouring into 9.6cm diameter culture plates in a sterile hood and allowing the agar to solidify at room temperature. The bacteria were spread evenly over the LB agar using a sterile plastic loop (Nunc) and the plates allowed to dry, before incubation at 37°C overnight. The following day the plates were removed, colonies picked from the plates and inoculated into 5ml of LB medium containing the appropriate antibiotic for selection. The bacteria were cultured overnight and the plasmids extracted using the Qiagen plasmid miniprep columns, described below.

2.2.3 Extraction of Plasmid DNA

After the overnight culture the bacteria were pelleted by centrifugation at 3500rpm for 10 minutes. Excess medium was carefully pipetted off and the pellet resuspended in 250μl of resuspension buffer P1(100μg/ml RNase A, 50mM Tris/HCl, 10mM EDTA, pH 8.0). This was then transferred to a 1.5ml microcentrifuge tube (Eppendorf) and 250μl of buffer P2 (200mM NaOH, 1%SDS) was added, which denatures cellular proteins and chromosomal DNA. The suspension was gently inverted to avoid shearing of chromosomal DNA and incubated for 5 minutes at room temperature. 350μl of chilled buffer N3 (3.0M $KC_2H_3O_2$, pH 5.5) was added to the mixture, which results in precipitation of the denatured proteins, chromosomal DNA, cellular debris and SDS, while allowing the shorter plasmid DNA to stay in solution. The samples were then centrifuged at 10,000 x g in a benchtop microcentrifuge for 10 minutes to pellet the precipitate along the side of the tube. The supernatant was aspirated and applied to a QIAprep-spin column. The column was centrifuged for 1 minute at 10,000 x g and the flowthrough fraction was discarded. In the column is an anion-exchange resin containing positively charged Diethylaminoethanol groups which bind to the negatively charged phosphate groups of the DNA backbone. Binding and elution of the DNA is determined by the pH and salt concentration of the buffers used. The column was washed with 500μl of wash buffer PB (proprietary formulation), which was then centrifuged at 10,000 x g in the benchtop microcentrifuge. Again the flowthrough fraction was discarded and 750μl of buffer PE (10mM Tris/HCl pH 8.0, 80% Ethanol) was added to wash the column which was centrifuged at 10,000 x g in the microcentrifuge.

The flowthrough fraction was discarded and the column was recentrifuged at 10,000 x g for a further 1 minute to remove any residual wash buffer. The QIAprep-spin column was then placed in a fresh 1.5ml microcentrifuge tube (Eppendorf) and the DNA was eluted by the addition of 100μl of distilled H_2O to the column ensuring the resin was completely covered. The column was spun at 10,000 x g for 1 minute and the plasmid DNA collected in the 1.5ml microcentrifuge tube.

2.2.3.1 Restriction Digestions

Approximately 1μg of each of the plasmid DNA was incubated with 2μl of the appropriate 10x restriction enzyme buffer, 10 IU of the desired restriction enzyme, bovine serum albumin (BSA) to a final concentration of 100μg/ml if required and sterile H_2O to a final volume of 20μl. The mixture was incubated for 2 hours at 37°C. To visualise the digested DNA 2μl of each digest was added to 2μl of DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and 6μl H_2O and loaded onto a 0.8% agarose gel (section 2.2.1.2). The gel was run at 5 volts/cm in 0.5x TBE and the DNA visualised using a UV light box.

1x concentration of restriction enzyme buffers used:

Promega Buffer B (pH 7.5, 6mM Tris-HCl, 6mM $MgCl_2$, 50mM NaCl, 1mM DTT): (*Nhe* I / *Spe* I)

Promega Buffer D (pH 7.9, 6mM Tris-HCl, 6mM $MgCl_2$, 150mM NaCl, 1mM DTT): (*Sal* I)

Promega Buffer G (pH 8.2, 50mM Tris-HCl, 5mM $MgCl_2$): (*Mlu* NI)

Promega buffer H (pH 7.5, 90mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl): (*Pst* I)

NEB buffer 2 (pH 7.9, 10 mM Tris-HCl, 10mM $MgCl_2$, 50 mM NaCl, 1mM DTT): (*Hind* III / *Xba* I + BSA / *Xho* I + BSA / *Eco* RV + BSA)

NEB buffer 3 (pH 7.9, 50mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT): (*Bgl* II / *Not* I + BSA / *Dra* III + BSA)

NEB buffer 4 (pH 7.9, 20mM Tris-acetate, 10mM Magnesium acetate, 50mM potassium acetate, 1mM DTT): (*Sna* BI + BSA / *Sma* I)

*Bam*HI buffer (NEB(pH 7.9, 150mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol) supplemented with BSA 100μg/ml)

Nsi I buffer (NEB(pH 8.4, 10mM Tris-HCl, 10mM $MgCl_2$, 100mM NaCl, 1mM DTT))

Sal I buffer (NEB(pH 7.9, 150mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol) supplemented with BSA 100μg/ml)

Boehringer Buffer H (pH 8.0, 10mM Tris-HCl, 5mM $MgCl_2$, 100mM NaCl, 1mM 2-Mercaptoethanol): (*Pvu* I)

2.2.3.2 Extraction of DNA From 0.8% Agarose Gel

After restriction digestion and prior to ligation, DNA fragments may require separation which was achieved by running the DNA on an agarose gel. The DNA was visualised using a long wavelength UV lamp to minimise damage to the digested DNA and the desired DNA band was cut out, minimising the surrounding area of agarose and transferred to a 1.5ml microcentrifuge tube. Each gel fragment was weighed and the DNA extracted from the gel using the Qiagen Qiaquick gel extraction kit. To the sample was added 100μl Buffer QX1 (proprietory formulation - solubilises the agarose) per 100mg agarose and 10μl of QIAEX II (proprietory formulation - DNA absorbs to silica particles) and the

samples mixed by pipeting up and down (Vogelstein and Gillespie, 1979). The sample was then incubated at 50°C for 10 mins, gently agitating the tubes 2-3 times during this period to aid mixing of the solution. The sample was centrifuged at 10,000 x g in a microcentrifuge for 1 minute and the supernatant removed with a pipette. The sample was resuspended in 500µl Buffer QX1 and recentrifuged at 10,000 x g in a microcentrifuge and the supernatant again removed with a pipette. 500µl of Buffer PE was added to the tube, the pellet resuspended and sample recentrifuged at 10,000 x g. This step with Buffer PE was repeated one more time as described above and after careful removal of the supernatant the pellet of QIAEX II bound to the DNA fragments was allowed to dry for 15 minutes at room temperature. To elute the DNA 20µl of H_2O was added to the sample and the pellet resuspended by briefly vortexing. The sample was again centrifuged at 10,000 x g for 1 minute and the supernatant containing the eluted DNA was transferred to fresh 1.5ml microcentrifuge tubes.

2.2.3.3 Ligation

Ligation of vector and insert DNA fragments was performed using T4 DNA ligase, which catalyses the joining of two DNA strands between the 5'-phosphate and 3'-hydroxyl groups of adjacent nucleotides in either a blunt-ended or cohesive-end configuration. The vector and the insert DNA at a ratio of 1:5 were added to the ligation mix consisting of polyethylene glycol at a final concentration of 5%, 50mM Tris-HCl (pH 7.5), 10mM $MgCl_2$, 10mM dithiothreitol, 1mM adenosine triphosphate (ATP), 25µg/ml bovine serum albumin and 100 units of T4 DNA ligase (NEB). As an indicator of the ability of the linearised vector to religate an aliquot of the linear vector was incubated

with the ligation mix and to assess the presence of uncut vector contaminating the linearised sample of vector plasmid an aliquot of the linearised vector was incubated in the absence of the ligase mix. All the above mixtures were then incubated at 16°C overnight and then 25µl of each of the three mixes were transformed into competent cells (see 2.2.2.1), which were then selected on LB agarose plates containing the appropriate antibiotics.

2.2.3.4 Cleaning of Plasmid DNA Using Qiagen Clean up Column

Plasmid DNA was purified prior to restriction digestion, ligation or transfection to remove substances that may interfere with these processes. The purification process is based on binding of the plasmid DNA to a silica membrane, which is enhanced at a high salt concentration (Vogelstein and Gillespie, 1979). To the plasmid DNA was added 10 times the volume of the DNA sample to be cleaned of buffer PN (proprietory formulation - enhances binding of DNA to silica gel due to high concentration of chaotropic salt). The DNA mix was then loaded onto a clean up column, containing the silica membrane, which was placed in a 2ml microcentrifuge tube provided. The sample was spun at 5000 x g in a benchtop microcentrifuge for 1 minute at room temperature. The flow through fraction was discarded and 750µl of wash buffer PE was added to the column, which was then respun at 5000 x g in the microcentrifuge for 1 minute at room temperature. The eluate was again discarded and the column was respun at 10,000 x g for 1 minute in the microcentrifuge to remove any residual Buffer PE. The DNA was then eluted by pippetting 50µl of distilled H_2O, taking care to cover the entire membrane and allowing the column to stand for 1 minute at room temperature. The

column was placed in a fresh 1.5ml microcentrifuge tube (Eppendorf) and both were spun at 10,000 x g for 1 minute at room temperature to elute the DNA into the microcentrifuge tube.

2.2.4 Extraction of Genomic DNA from Eukaryotic Cells

Genomic DNA was recovered from eukaryotic cells using the Nuclitips™ DNA extraction system (Amersham Life Science). 293 cells were grown in a 90mm tissue culture plate and washed twice in PBS. 0.5ml of PBS was added to the cell monolayer, after aspiration of the residual wash solution and the cells were harvested by scraping them off using a cell scraper (Nunc). The cells were transferred to a 1.5ml microcentrifuge tube and centrifuged at 10,000 x g for 2 minutes in a benchtop microcentrifuge. The supernatant was removed and the cells resuspended in 0.5mls of PBS. The cells were lysed by the addition of 0.5mls of lysis buffer 1 (10mM Tris pH 7.5, 10mM NaCl, 3mM $MgCl_2$ and 1% N-lauryl sarcosine), mixed by inversion and incubated at room temperature (18°C) for 20 minutes. A Nuclitip was placed on a 1000µl pipette (Gilson) and 800µl of the lysed cell solution was drawn up into the tip and then expelled back into the 1.5ml microcentrifuge tube, to capture the nuclei on the membrane. This process was repeated a further 4 times and then this process was repeated 5 times in PBS, each time drawing up 800µl of PBS into the tip, to wash away cellular debris leaving the nuclei adherent to the membrane in the tip. The PBS was expelled from the tip after the final washing step and the tip was placed in a fresh 1.5ml microcentrifuge tube. The upper half of the tip was snapped off and discarded, the lower half containing the membrane remaining in the microcentrifuge tube. 40µl of lysis buffer 2 (20mM Tris pH 8.0, 1mM

EDTA, 0.2% SDS, 0.2mg/ml RNAse A, 2U/ml RNAse T_1 and 1mg/ml proteinase K) was added to the inside of the Nuclitip to lyse the nuclei, releasing the DNA and digesting the protein (de Yebra and Oliva, 1993). The sample was then incubated at 55°C for 30 minutes and the DNA eluted by centrifugation at 10,000 x g for 2 minutes in a microcentrifuge. The nuclitip was removed from the microcentrifuge tube, discarded and the DNA sample was incubated at 80°C for 10 minutes to neutralise the proteinase K. Prior to PCR analysis of the DNA, neutralising reagent (proprietory formulation) was added to the PCR reaction at a final concentration of 1%. This was to prevent inhibition of Taq DNA polymerase in the PCR reaction due to the presence of SDS in the DNA sample.

2.2.5 Polymerase Chain Reaction (PCR)

5μl of the sample to be analysed was added to 1μl of forward primer (0.5μmol/μl), 1μl of reverse primer (0.5μmol/μl), 3μl of dNTP's (2mM dATP, dTTP, dCTP and dGGT), 1.5μl of $MgCl_2$ (1.5mM), 3μl of 10x Taq DNA polymerase buffer(500mM Kcl, 100mM Tris-HCl (pH 9.0 at 25°C) and 1.0% Triton®X-100), 0.1μl of Taq DNA polymerase (5u/μl) and 15.4μl of sterile H_2O (Saiki et al., 1988). The sample was thoroughly mixed and overlaid with 1 drop of mineral oil (Sigma) to prevent evaporation of the sample during the procedure. The sample was then subjected to 30 cycles of denaturation at 94°C for 60 seconds, annealing at 52°C for 60 seconds and extension at 72°C for 90 seconds, when using primers specific for the CMV promoter. As a negative control, DNA not containing primer binding sites was used and as a positive control DNA known to contain the primer binding sites was used. To

ensure no cross contamination sterile H_2O was also subjected to PCR. After the required cycles of PCR, 5μl of each PCR product was taken, added to 2μl of DNA loading buffer and run on a 2% agarose gel.

2.2.6 Western Blot Analysis

Precast polyacrylamide gels consisting of 10% Tris-Glycine resolving gel and 4% stacking gel (Biorad) were loaded into an electrophoresis tank (Mini-Protean II cell). The top and bottom reservoirs were then filled with x1 reservoir buffer (3.03g Tris, 14.4g glycine, 1g Sodium dodecyl sulfate (SDS) in 1000mls H_2O) and each of the wells was carefully flushed to remove any unpolymerised acrylamide. 16.66μl of sample to be analysed was added to 3.34μl 6x sample buffer (7mls 0.5M Tris pH 6.8, 3mls 100% glycerol, 1.0g SDS, 0.6ml 2-mercaptoethanol (2-ME), 1.2mg bromophenol blue and H_2O to 10mls total volume), which was then loaded into a well using a Hamilton microliter syringe. In the wells either side of the samples to be analysed were added high molecular weight markers (Amersham) to determine the molecular weight of the proteins detected on the gel. 5μl of high molecular weight markers (14,300-200,000 kDa) being added to 3.34μl 6x sample buffer and 11.66μl of H_2O before being loaded into a vacant well. Prior to loading, all samples were heated for 5 minutes at 100°C for 5 minutes and then kept on ice until loaded into the wells. Once loaded the gel was run at 60mA until the bromophenol blue reached the bottom of the resolving gel.

As the gel was nearing the end of its run a piece of nitro-cellulose filter (Hybond ECL- Amersham) and 18 pieces of Whatman 3MM paper were cut to the size of the gel. The glass plates holding the gel were prised apart and the

gel floated off the remaining glass plate by submerging them in blotting buffer (Tris 5.81g, glycine 2.93g, 10%SDS 3.75mls, methanol 200mls and made up to 1000mls with H_2O) 9 pieces of 3MM paper were placed on the anode of the electro transfer plate (LKB electrophoresis unit) and soaked in blotting buffer, taking care to remove any air bubbles. On this was placed the nitrocellulose filter, the gel and then a further 9 pieces of 3MM paper also soaked in blotting buffer. The upper electrode (cathode) was then placed over the layers of 3MM paper, nitrocellulose filter and gel and a current of 320mA was passed across these for 1 hour. After disconnecting the apparatus, the nitrocellulose filter was carefully removed and transfer of the rainbow markers confirmed successful electrotransfer of the proteins from the gel to the filter. The filter was then placed in 100mls of blocking buffer pH 7.8 (10x TBS 50mls, Marvel 25g, FCS 10mls, made up to 500mls with H_2O) and shaken gently for 1 hour at room temperature. The filter was then transferred to a heat sealable plastic bag and to this was added 1μg of a murine anti-VP1/VP2/VP3 monoclonal antibody (Progen clone B1), added to 5mls of blocking buffer. Taking care to remove any air bubbles present, the bag was sealed and incubated for 18 hours at 4°C with gentle agitation on a platform shaker. The filter was removed and placed in 100mls of blocking buffer and shaken for 15 minutes at room temperature on a platform shaker. The filter was removed and washing in 200mls of blocking buffer was repeated for a total of four washes. The filter was removed after the final wash and placed in a fresh heat sealable plastic bag, to which was added 20μl (20μg) goat anti-mouse antibody conjugated to horseradish peroxidase (Dako) in 5mls of blocking buffer. The bag was heat sealed and incubated on a platform shaker for 2 hours at room temperature. The filter was removed and

washed for 10mins in 100mls of blocking buffer with 0.01% Tween. The filter was removed and excess blocking buffer was removed and over this was poured 2mls of ECL A added to ECL B (Amersham -Life Sciences), ensuring the filter was adequately covered. After 1 minute the excess ECL was removed and the filter was wrapped in cling film and exposed to a X-Ray film (Fuji RX) for between 1 and 5 minutes. If background was seen on the film, the filter was washed for a further 5 - 10 minutes in blocking buffer with 0.01% Tween and the filter then reexposed after incubation with ECL.

2.2.7 Dot Blot Hybridisation

To quantitate the number of AAV genomes obtained in the rAAV preparations, dot blot hybridisation was performed using a primer specific for the expression cassette (Chiorini et al., 1995). Hybond-N nylon membrane (Amersham-Life Science) was prewetted in 6x SSC (52.6g NaCl, 26.5g sodium citrate, made up to 1 litre with H_2O, pH 7.0) and then inserted in the dot blot apparatus (Bio-Rad). The reference control DNA (pUC AAV poly A B7-2) was diluted in 10x SSC to the following concentrations 10, 1, 0.1 and 0.01μg/ml. 20μl of the rAAV preparation was incubated with 10units DNase I (Sigma) and 10mM $MgCl_2$ at 37°C for 1 hour, to digest residual unpackaged DNA derived from the transfections. Prior to loading, the virus and plasmid samples were heated at 100°C for 5 minutes and then incubated on ice until loading. 2μl of virus and reference plasmid samples were then loaded onto the nylon membrane and sucked through under negative pressure. The nylon filter was then removed from the dot blot apparatus and dried by incubation for 2 hours at

80°C. After drying both sides of the filter were UV irradiated (Stratagene UV Stratalinker).

The nylon filter was added to prehybridisation buffer (24 mls 20x SSC (175.3g NaCl, 88.2g sodium citrate, H_2O to 1 litre, pH 7.0), 8 mls 50x Denharts (5g Ficoll (Type 400, Pharmacia), 5g polyvinylpyrrolidone (Sigma), 5g BSA, H_2O to 500mls), 4mls 10% sodium dodecyl sulfate (SDS), 80μl (10ng/ml) salmon sperm DNA), prewarmed to 65°C. Prehybridisation was for 4 hours at 65°C. The prehybridisation buffer was then discarded and replaced with hybridisation buffer (9 mls 20x SSC, 3mls 50x Denharts, 1.5mls 10% SDS, 300μl EDTA 0.5M, 30μl salmon sperm DNA (10ng/ml), 16.2ml H_2O) to which was added the radiolabelled probe. Hybridisation was for 18 hours at 65°C. DNA used for the probe was the PCR product using primers specific for the CMV promoter (see section 4.6.8) and the probe was made using the rediprime DNA labelling system (Amersham Life Science). The DNA was diluted in H_2O to a concentration of 25ng/ml and then heated to 100°C for 5 minutes in a 1.5ml microcentrifuge tube. The tube was then spun at 10,000 x g for 1 minute in a microcentrifuge. The denatured DNA was then added to the labelling mix (proprietory formulation) and mixed, followed by centrifugation at 10,000 x g for 1 minute. 50μCi of [^{32}P] dCTP (Amersham) was then added to the microcentrifuge tube which was then spun at 10,000 x g for 1 minute, followed by incubation at 37°C for 10 minutes. To remove unincorporated [^{32}P] dCTP, the probe was spun at 200 x g for 5 minutes through a G-50 Sephadex column. Prior to addition of the probe to the filter, the labelled probe was heated to 95°C for 5 minutes and chilled on ice. After hybridisation the filter was washed twice in 2x SSC and 0.1% SDS at 37°C for 10 minutes, followed by two washes in 2x

SSC and 0.1% SDS at 65°C for 10 minutes. After washing the filter was wrapped in Saran wrap, placed next to X-Ray film (Fuji) and exposed for 3 hours at -70°C. The exposed film was then developed.

2.2.7.1 Calculation of Titre of rAAV From Dot Blot

Density of the signals of the reference plasmid samples and rAAV were measured by densitomotry (Molecular Dynamics Personal Densitometer) and a standard curve generated. The amount of DNA in the rAAV preparation was then calculated from the standard curve. The number of genomes in the rAAV preparation was then calculated using the following formula:

5685 (Molecular weight (MW) reference plasmid) x 660g (MW nucleotide) = 6.023×10^{23} (Avagadro's constant)

3.75×10^{6}g = 6.023×10^{23} molecules

1g = 1.606×10^{17} molecules

1ng = 1.606×10^{8} molecules

The amount of DNA in the rAAV generated from the standard curve needs to be multiplied by a factor of 2 as the AAV DNA is single-stranded and the reference plasmid double-stranded. The number of genomes in 2µl of rAAV can then be calculated from the number of molecules in 1ng of the reference plasmid. This figure is then multiplied by 500 to give the number of genomes per ml in the rAAV preparation.

3.1 PHENOTYPING OF AML SAMPLES

3.1.1 Introduction

The strategy of overcoming anergy to tumour cells by inducing expression of a co-stimulatory molecule shows promise as demonstrated in numerous murine models using tumourigenic cell lines as described in section 1.13. That AML can be successfully targeted by the immune system is seen by the established GvL effect seen after allogeneic bone marrow transplantation (BMT) (Weiden et al., 1979), the ability of DLI to reinduce remission in patients whose disease has relapsed after allogeneic BMT (Kolb et al., 1990; Popat et al., 1995) and documented cases of spontaneous remission, which are possibly immune mediated (Dore et al., 1976). These observations do not necessarily indicate the presence of a specific anti-leukaemic immune response, but may reflect an immune response directed against myeloid restricted antigens. In addition a number of studies have demonstrated that the induction of expression of B7-1 in murine AML can lead to an effective immune response which is able to eradicate existing unmodified tumour cells (Matulonis et al., 1995; Matulonis et al., 1996; Boyer et al., 1997; Dunussi-Joannopoulos et al., 1996).

To determine whether this strategy might be effective in human AML, it first needed to be determined whether AML cells innately expressed either of the B7 family of co-stimulatory molecules. A previous study has demonstrated that B7-1 is only rarely expressed (1.9%), but that B7-2 is more frequently expressed (28%) on AML cells (Hirano et al., 1996). In addition HLA class I and class II expression needed to be determined as putative tumour antigens

would be presented in the context of MHC class I or class II. Those tumour antigens presented by MHC class II stimulate a CD4 T cell response, leading to production of greater amounts of IL-2 and maintain the immune response over a longer time period (Kern et al., 1986; Fearon et al., 1990). Finally, I wanted to determine expression of the adhesion molecules LFA-1 and ICAM-1 on the myeloid leukaemia cells. Both LFA-1 and ICAM-1 have been shown to play an important role in T cell activation (Schmits et al., 1996; Sligh, Jr. et al., 1993), in particular LFA-1 has been found to enhance the contact between T cells and APC's and facilitate cytotoxic T lymphocyte - target cell interactions (Davignon et al., 1981). A study comparing LFA-1 and CD28 in T cell activation demonstrated that the LFA-1/ICAM-1 interaction decreased the amount of antigen required for T cell activation, facilitating the triggering of TCR's by promoting adhesion of the T cells to the APC's. In contrast signalling through CD28 reduced the number of TCR's needing to be triggered for T cell activation and only CD28 and not LFA-1 prevented the induction of T cell responsiveness after signalling through the T cell receptor (Bachmann et al., 1997). That signalling through CD28 reduces the number of TCR's needing to be triggered for T cell activation may be of particular importance to the strategy of inducing B7-1 or B7-2 expression on AML cells, where potential tumour antigens may be present in low abundance. Class I, class II and ICAM-1 expression have also been described in human AML (Hirano et al., 1996). 100% of AML samples expressed class I and between 69% and 100% express class II, the lower percentage being found in AML M1 and the higher percentage in AML M0, M5 and M6. ICAM-1 expression ranged between 0% and 43%, 0% in FAB types

M0, M3, M5 and M6 and 43% in M2. Samples were only considered positive if more than 20% of blasts in each sample expressed the antigen.

Density of surface expression of costimulatory molecules may be important in determining whether a costimulatory signal is delivered, as increased expression is associated with increased costimulation (Bluestone, 1995). To compare intensity of expression of B7-1 and B7-2, I also analysed surface expression of both molecules on activated B cells and monocytes which are known to be able to deliver a costimulatory signal (Hathcock et al., 1994) and induce T cell proliferation in conjunction with an appropriate antigen specific signal. As a negative control expression of B7-1 and B7-2 was also determined on resting T cells which are known not to express either molecule (Azuma et al., 1993b).

3.1.2 Materials and Methods

3.1.2.1 Immunophenotyping

AML blasts were isolated and stored as described in section 2.1.1 and were analysed either directly after harvesting or after rapid defrosting at 37°C from cryopreservation in the vapour phase of liquid nitrogen. Individual samples of $0.5x10^6$ cells were labelled with the following monoclonal antibodies (mAb) and analysed by flow cytometric analysis as described in section 2.1.3. 10μl antiB7-2-FITC (Pharmingen - clone FUN-1), 10μl antiB7-1-PE (Becton Dickinson - clone L307.4) and 10μl antiCD3-PerCP (Becton Dickinson - clone SK7). Four further separate aliquots of the cells to be analysed were each incubated with one of the following monoclonal antibodies for 15 minutes at room temperature, 10μl antiHLA-A/B/C-FITC (Pharmingen -clone G46-2.6),

10μl antiHLA-DR-PE (Becton Dickinson - clone L243), 10μl antiLFA-1α-FITC (Becton Dickinson - clone G25.2) or 10μl antiICAM-1-PE (Becton Dickinson - clone LB-2). Isotype control antibodies are sometimes used to control for non specific binding to the cell population being analysed, but are themselves not ideal controls, as the amount of fluorochrome bound to control antibody would be unlikely to be the same as that on the test antibody. With this in mind it was decided to use unlabelled cells as the negative control. To avoid contamination from non leukaemic cells, when the samples were analysed by flow cytometry, data was acquired by gating around the population of blast cells. By calculating the percentage of CD3 positive cells in the analysed cell population, the purity of the AML sample could be assessed with regard to contaminating T cells.

Expression of B7-1, B7-2, MHC class I and II, LFA- and ICAM-1 was also determined on resting T lymphocytes, resting monocytes and activated B lymphocytes from the peripheral blood of five normal donors. T cells were analysed by 3 colour labelling incubating the mononuclear cells with 10μl anti-CD3-PerCP (Becton Dickinson - clone SK7) and either 10μl anti-B7-2-FITC (Pharmingen - clone FUN-1) and 10μl anti-B7-1-PE (Becton Dickinson - clone L307.4) or 10μl antiHLA-A/B/C-FITC (Pharmingen -clone G46-2.6) and 10μl antiHLA-DR-PE (Becton Dickinson - clone L243), or 10μl antiLFA-1α-FITC (Becton Dickinson - clone G25.2) and 10μl antiICAM-1-PE (Becton Dickinson - clone LB-2). FL3 PMT voltage was set after standardisation of FL1 and FL2 to the bead preparation. The intensity of FL3 output was not formally standardised against a bead preparation as this parameter was used solely for identification of specific cell subsets rather than precise measurements of fluorescence intensity. The monocyte population was determined by gating

and the expression on T lymphocytes by analysis of the CD3+ve population. B lymphocytes were analysed after 24 hours incubation with pokeweed mitogen (Sigma) at a concentration of 5μg/ml. The cells were washed and then incubated with 10μl anti-CD19-FITC (Becton Dickinson - clone 4G7) and either 10μl anti-B7-1-PE (Becton Dickinson - clone L307.4) or 10μl anti-B7-2-PE (Pharmingen - clone IT2.2), or 10μl antiICAM-1-PE (Becton Dickinson - clone LB-2) or 10μl antiHLA-DR-PE (Becton Dickinson - clone L243) for 15 minutes at room temperature. To analyse LFA-1 expression on B cells, the lymphocytes activated with pokeweed mitogen were incubated with 10μl anti-CD19-PE (Becton Dickinson - clone 4G7) and 10μl antiLFA-1α-FITC (Becton Dickinson - clone G25.2) for 15 minutes at room temperature. The cells were then analysed by flow cytometry as described in section 2.1.3.

3.1.2.2 Analysis of Flow Data

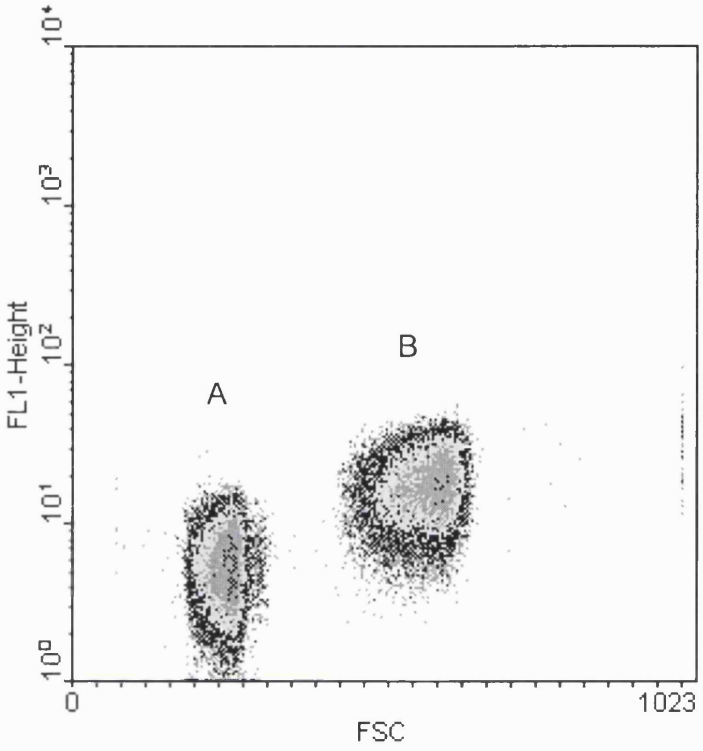
Flow histograms were constructed for each leukaemic sample for B7-1 and B7-2 expression. The percentage of positive cells was calculated from each sample as compared to the negative control cells and the median channel fluorescence was calculated from the positive cell population.

The median channel fluorescence signal is a correlate of the amount of antibody binding to each cell. Myeloid leukaemia cells were usually larger than normal lymphocytes, are often heterogeneous in size and thus to compensate for size differences between the control cells and the leukaemic blasts the median channel fluorescence was divided by the median channel forward scatter (FSC), which is an indicator of cell size. The resulting fluorescence/FSC ratio (FFR) gives an indication of density of B7-1 and B7-2 expression on cells.

The need to compensate for differences in size was demonstrated by analysing two populations of non-fluorescent polycarbonate beads (Park Scientific, Cambridge, UK) after excitation by 488nm light (FACScan, Becton Dickinson, UK). These beads are of the same polycarbonate, but bead A has a diameter of 6μm, while bead B has a diameter of 13μm. Analysis of the FL1 signal (515-535nm) emitted by the two populations of beads demonstrates an apparent increase in fluorescence simply due to the greater amount of non-specific light scatter by the larger particles as shown in figure 3.

Percentage expression of HLA-A/B/C, HLA-DR, LFA-1 and ICAM-1 was also determined from the samples by comparison with unlabelled cells. Median channel fluorescence was calculated in the positive cell population and compared with the median channel fluorescence in the unlabelled cell population. Median channel fluorescence was again divided by the median channel forward scatter to give the FFR ratio approximating to the density of surface expression of the molecules being analysed.

Figure 3 Fluorescence of 3μm and 6.5μm beads in FL1



Legend to Figure 3:
Fluorescence of 6μm (A) and 13μm (B) beads in FL1, demonstrating increased median channel fluorescence seen with the larger beads. Median channel fluorescence of the 6μm beads was 180 and of the 13μm beads 307.

3.1.3 Results

39 samples were analysed from patients with AML. Median age was 51 years (range 14-90). Diagnosis is summarised in table 2.

3.1.3.1 Surface B7-1, B7-2, HLA A/B/C, HLA-DR, LFA-1 and ICAM-1 Expression on Monocytes, T cells and Activated B cells

The highest level of B7-1 expression was detected in activated B lymphocytes with an FFR of 0.83 (sd 0.035). A similar level of B7-1 expression was detected in monocytes with an FFR of 0.79 (sd 0.036), the two were not statistically different ($p>0.1$- paired Student's T test). As expected the level of expression of B7-1 on T lymphocytes was low with an FFR of 0.4 (sd 0.014) which was significantly lower ($p<0.001$) than that of the other two cell types. A similar pattern of expression was seen with B7-2 as with B7-1. The FFR for B7-2 on T lymphocytes was 0.42 (sd 0.017) and was significantly lower (p,0.001)than that on monocytes (FFR - 1.02 (sd 0.017)), or on activated B lymphocytes (FFR - 1.16 (sd 0.017))($p=0.001$). The results are summarised in table 3.

The FFR of HLA-A/B/C expression on monocytes, B cells and T cells was 1.3, 1.81 and 1.65 respectively and the FFR of HLA-DR on monocytes, B cells and T cells was 1.00, 1.66 and 0.75 respectively. The FFR of LFA-1 expression on monocytes, B cells and T cells was 0.97, 1.03 and 1.30 respectively and the FFR of ICAM-1 on monocytes, B cells and T cells was 0.82, 1.04 and 0.68 respectively. These results are summarised in Table 3.

Table 2 FAB Type of AML at Diagnosis

Diagnosis	Number of patients
AML secondary to myelodysplasia	6
AML secondary to myeloproliferative disorder	4
AML M1	2
AML M2	2
AML M3	2
AML M4	14
AML M4Eo	1
AML M5	6
AML M6	2

Table 2:
Subtypes of AML samples analysed for expression of B7-1, B7-2, MHC class I and II, LFA-1 and ICAM-1.

Table 3 Expression of B7-1, B7-2, HLA-A/B/C, HLA-DR, LFA-1 and ICAM-1 on T Cells, Monocytes and Activated B Cells

	Monocytes (sd)	B Lymphocytes (sd)	T Lymphocytes (sd)
Median channel fluorescence B7-1	421 (15.9)	457 (11.3)	165 (5.3)
Median channel forward scatter	532 (16.3)	549 (13.0)	413 (2.7)
FFR B7-1	0.79 (0.036)	0.83 (0.035)	0.4 (0.014)
Percentage cells B7-1+ve	83	61	0
Median channel fluorescence B7-2	553 (8.3)	603 (19.7)	175 (7.4)
Median channel forward scatter	544 (14.0)	519 (18.0)	412 (2.1)
FFR B7-2	1.02 (0.017)	1.16 (0.017)	0.42 (0.017)
Percentage cells B7-2+ve	97	81	2
Median channel fluorescence HLA-A/B/C	925 (33.6)	939 (70.0)	857 (56.8)
Median channel forward scatter	709 (67.0)	518 (62.1)	480 (45.2)
FFR HLA-A/B/C	1.3	1.81	1.78
Percentage cells HLA-A/B/C+ve	100	100	100
Median channel fluorescence HLA-DR	727 (135.8)	825 (142.3)	389 (133.6)
Median channel forward scatter	724 (68.9)	498 (72.3)	465 (93.1)
FFR HLA-DR	1.00	1.66	0.84
Percentage cells HLA-DR+ve	99.5	97.5	15
Median channel fluorescence LFA-1	701 (55.5)	498 (89.0)	628 (73.9)
Median channel forward scatter	719 (66.4)	482 (75.4)	463 (56.8)
FFR LFA-1	0.97	1.03	1.36
Percentage cells LFA-1+ve	95	94.5	99.5
Median channel fluorescence ICAM-1	580 (76.5)	501 (100.6)	354 (85.2)
Median channel forward scatter	709 (65.6)	483 (125.9)	451 (67.9)
FFR ICAM-1	0.82	1.04	0.78
Percentage cells ICAM-1+ve	96.5	96	81.5

Table 3:
Expression of B7-1, B7-2, HLA-A/B/C, HLA-DR, LFA-1 and ICAM-1 on T cells, monocytes and activated B cells. Results are expressed as median channel fluorescence, FFR and percentage of cells expressing the relevant antigen.

3.1.3.2 Surface B7-1 and B7-2 Expression on Myeloid Leukaemia Cells

Of the 39 samples analysed only 5 were positive for B7-1 expression, with between 5 and 27.5% of cells expressing the protein (table 4). In the six positive samples the mean FFR of the positive cells within the samples was 0.63 (sd 0.107) which, while significantly greater than that on resting T cells ($p=0.006$), was significantly lower than that on monocytes ($p=0.007$) or activated B cells ($p=0.005$) (figure 4).

In contrast to B7-1, 31 out of the 39 AML samples contained B7-2+ve cells (table 4). The percentage of cells which were positive in these populations ranged from 5.5 to 94.9%. In the positive samples the mean FFR of the positive cells was 0.723 (sd 0.151), which was significantly lower than that of monocytes or activated B cells ($p<0.001$) and higher than that seen in the T cells ($p<0.001$) (figure 4). However, in the samples with the highest expression, the FFR was approaching that seen on monocytes and activated B lymphocytes.

The percentage of cells CD3+ve in the samples analysed ranged from 0 to 6.3%, with a mean of 2.1% (sd 2.13). This indicates that the samples were not heavily contaminated with T cells.

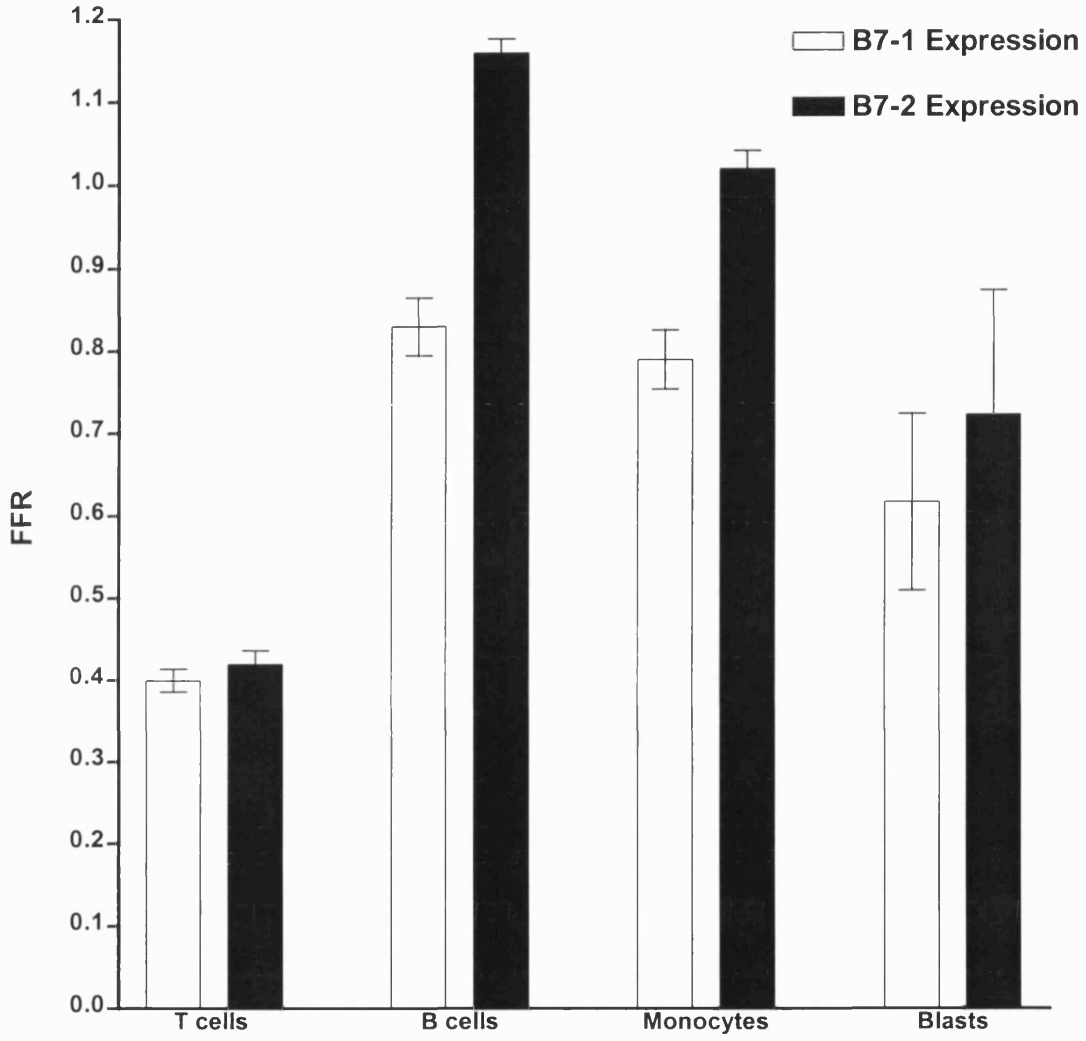
Table 4 B7-1 and B7-2 Expression on AML Blasts

	% cells B7-1+ve	Median channel fluorescence (MCF) B7-1	Median channel FSc.	FFR B7-1	% cells B7-2+ve	MCF B7-2	FFR B7-2
Patient 1	13	354	442	0.80	14.5	433.5	0.98
Patient 2	0	NA	657	NA	0	NA	NA
Patient 3	0	NA	489	NA	0	NA	NA
Patient 4	0	NA	644	NA	20.5	576	0.89
Patient 5	0	NA	699	NA	20.5	337	0.48
Patient 6	0	NA	720	NA	25.5	437	0.61
Patient 7	0	NA	NA	NA	50.5	505	0.76
Patient 8	20	383	661	0.58	80	422	0.64
Patient 9	0	NA	648	NA	11.5	314	0.48
Patient 10	0	NA	570	NA	46	432	0.76
Patient 11	0	NA	693	NA	5.5	457	0.66
Patient 12	0	NA	656	NA	35	336	0.51
Patient 13	0	NA	722	NA	7	383	0.53
Patient 14	0	NA	617	NA	85	492	0.80
Patient 15	0	NA	565	NA	44.5	408	0.72
Patient 16	0	NA	553	NA	0	NA	NA
Patient 17	0	NA	518	NA	35.5	430	0.83
Patient 18	0	NA	432	NA	5	333	0.77
Patient 19	0	NA	630	NA	16.5	429	0.68
Patient 20	4	404	563	0.72	21.5	416	0.74
Patient 21	0	NA	456	NA	26.5	419	0.92
Patient 22	0	NA	669	NA	48	507	0.76
Patient 23	0	NA	679	NA	19.5	484	0.71
Patient 24	0	NA	533	NA	33	305	0.57
Patient 25	27.5	329	655	0.50	30.5	635	0.97
Patient 26	0	NA	645	NA	10	524	0.81
Patient 27	0	NA	506	NA	0	NA	NA
Patient 28	0	NA	545	NA	16	524	0.96
Patient 29	0	NA	708	NA	49	357	0.50
Patient 30	0	NA	694	NA	0	NA	NA
Patient 31	0	NA	703	NA	78.5	463	0.66
Patient 32	0	NA	659	NA	0	NA	NA
Patient 33	0	NA	629	NA	0	NA	NA
Patient 34	0	NA	675	NA	0	NA	NA
Patient 35	15.9	307	560	0.55	88.5	558	1.00
Patient 36	0	NA	635	NA	24	376	0.59
Patient 37	0	NA	562	NA	80.5	344	0.61
Patient 38	0	NA	616	NA	94.9	456	0.74
Patient 39	0	NA	600	NA	8.5	364	0.61

Table 4:

Expression of both B7-1 and B7-2 on AML blasts, expressed as percentage cells positive, median channel fluorescence and FFR. Where no cells were positive the median channel fluorescence and FFR were not calculated (not applicable-NA).

Figure 4 Comparison of B7-1 and B7-2 Expression on AML cells, T cells, B cells and Monocytes



Legend to Figure 4:
Comparison of B7-1 and B7-2 expression on T cells, B cells, monocytes and AML blasts. Expression is measured as the FFR which gives an indication of density of expression of the surface proteins.

3.1.3.3 HLA-A/B/C and HLA-DR Expression on Myeloid Leukaemic Blasts

23 samples were analysed for expression of HLA-A/B/C and HLA-DR. 90 to 100% of cells in all samples expressed HLA-A/B/C (mean -99.3% sd -2.14), with the majority of samples having 100% of cells positive, with a mean of median channel fluorescence of 714 (sd 97.0) and a mean FFR of 1.32 (sd 0.25). In contrast the percentage of cells expressing HLA-DR in each sample ranged from 15 to 100% (mean -88.7% sd -19.6), with a mean median channel fluorescence of 774 (sd 153.7)and a mean FFR of 1.33 (sd 0.32) in the positive cell population. The sample with only 15% HLA-DR expression was a solitary finding with 16 of the 23 samples having greater than 90% of the cells expressing. The results are summarised in table 5.

3.1.3.4 LFA-1 and ICAM-1 Expression on Myeloid Leukaemic Blasts

22 samples were analysed for expression of LFA-1 and 23 samples for expression of ICAM-1. The percentage of cells in each sample expressing LFA-1 ranged from 10 to 100% (mean -71% sd -26.9) and the percentage of cells expressing ICAM-1 in each sample ranged from 43 to 99% (mean -81.8 sd -16.7). The mean FFR of the cells expressing LFA-1 was 0.855 (sd 0.22) and the FFR of the cells expressing ICAM-1 was 0.835 (sd 0.19). The results are summarised in table 5.

Table 5 HLA-A/B/C, HLA-DR, LFA-1 and ICAM-1 Expression on AML Blasts

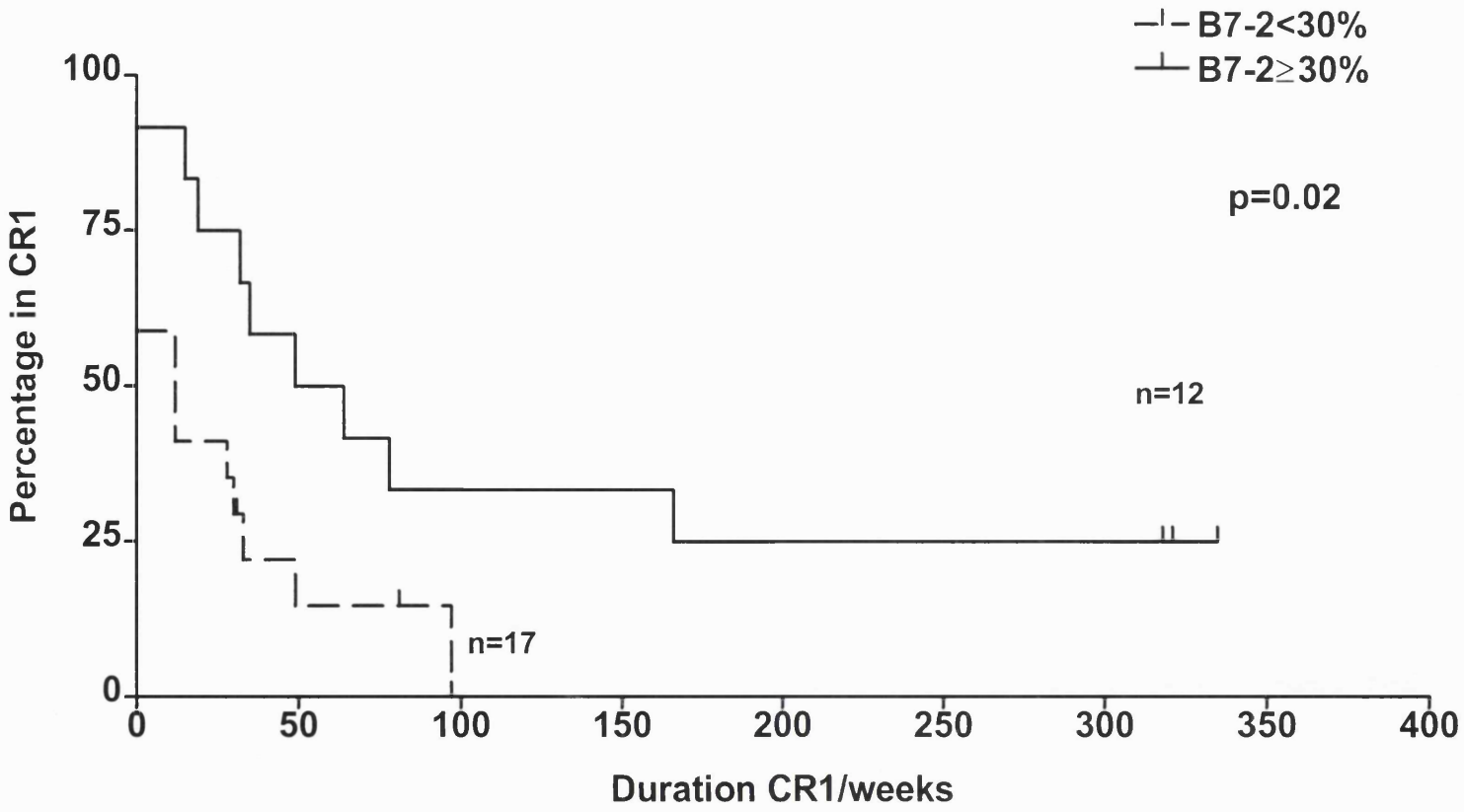
	% HLA-A/B/C +ve	FFR HLA-A/B/C	% HLA-DR+ve	FFR HLA-DR	% LFA-1+ve	FFR LFA-1	% ICAM-1 +ve	FFR ICAM-1
Patient 1	100	1.64	78.5	1.89	72.5	1.17	68	1.33
Patient 2	100	0.96	100	1.05	100	0.73	90.5	0.64
Patient 3	98	1.25	98.5	1.88	80	0.97	95	1.18
Patient 4	100	1.31	100	1.59	96	0.89	97	0.96
Patient 5	100	0.99	100	1.15	100	0.60	43	0.56
Patient 6	100	1.00	100	1.14	55	0.56	99	0.77
Patient 7	100	1.18	98.5	1.37	91	0.78	89.5	0.75
Patient 8	100	1.14	85	1.00	37	0.65	78	0.68
Patient 9	100	1.02	15	0.69	10	0.50	48.5	0.54
Patient 10	100	1.43	74.5	1.07	53	0.88	49	0.76
Patient 11	90	1.22	67.5	1.43	70	0.88	66	0.76
Patient 12	100	1.23	97	1.08	71	0.64	68	0.61
Patient 13	100	1.02	98	1.19	ND	ND	87	0.56
Patient 14	100	1.40	100	1.42	99.5	1.11	92	0.84
Patient 15	100	1.39	95	1.20	89.5	1.05	99	0.96
Patient 16	100	1.42	99	1.25	96	0.86	96	0.88
Patient 17	96.5	1.53	100	1.92	81.5	0.83	77	0.91
Patient 18	100	2.02	99	1.91	99	1.23	86	0.92
Patient 19	100	1.33	98	1.28	45	0.75	96	0.89
Patient 20	100	1.46	57	1.16	57	0.80	91	0.93
Patient 21	99	1.68	84	1.34	15.5	1.34	80	1.06
Patient 24	100	1.30	44.5	0.59	67	0.64	78.5	0.66
Patient 38	100	1.25	98.5	1.16	96	0.94	93	0.84
Patient 39	100	1.49	97	1.34	51	0.65	93	0.87

Table 5:
Expression of HLA-A/B/C, HLA-DR, LFA-1 and ICAM-1 on AML blasts, expressed as percentage of cells positive and FFR. One sample was not analysed for expression of LFA-1 and this has been marked ND.

3.1.3.5 Duration of First Remission in Patients Depending on Expression of B7-2 on AML Blasts

Of the 39 patients whose AML was phenotyped for expression of B7-1 and B7-2, follow up was not available in 5 (patients 10, 16, 17, 22 and 30). In three patients the AML samples were obtained from patients not presenting with AML (patients 11, 20 and 34). Of the remaining 31 patients, two were not treated with chemotherapy and were excluded from the analysis (patients 28 and 36). In the remaining 29 patients, the date of presentation and date of relapse was noted, as well as defined risk factors including white cell count at presentation, age and cytogenetic abnormalities. Relapse was defined as greater than 5% blasts found in the bone marrow, of similar morphology and phenotype to the presentation blasts. In those patients who had not relapsed the duration of first remission (CR1) was calculated based on the date of analysis. The patients were divided into those where less than 30% of the AML blasts expressed B7-2 (17 patients) and those where 30% or more of the AML blasts expressed B7-2 (12 patients). Survival curves were then generated for the two groups using the method of Kaplan and Meier. These results are presented in figure 5.

Figure 5 Duration of First Remission in Patients Depending on B7-2 Expression on the AML Blasts



Legend to Figure 5:
Survival curves of patients with either less than 30% of AML blasts expressing B7-2, or 30% or more of the AML blasts expressing B7-2.

3.1.4 Discussion

Tumour cells are thought to evade the immune response via a variety of mechanisms. These include the inability of tumour cells to provide an adequate costimulatory signal, which is a prerequisite for optimal T cell activation and production of IL-2 (Bretscher and Cohn, 1970). Induction of expression of a costimulatory molecule on tumourigenic cell lines has been demonstrated to induce an immune response which leads to rejection of the tumour and eradication of established micrometastases (Chen et al., 1992; Townsend and Allison, 1993; Hodge et al., 1994; Yang et al., 1995). Another mechanism is loss of MHC class I molecules on the tumour cell, preventing presentation of putative tumour specific antigens (Tanaka et al., 1988). Induction of MHC class II molecule expression on some tumours has also resulted in the generation of an effective immune response (Baskar et al., 1995). Finally, some tumour cells have been demonstrated to secrete humoral factors which suppress a T cell mediated immune response, affecting both CD4 (Zou et al., 1992) and CD8 (Loeffler et al., 1992) T cell subsets.

The results show that B7-1 is infrequently expressed on myeloid blasts and when present only a minority of cells express the protein and at levels less than that seen on activated B cells and monocytes which are known to be able to deliver a costimulatory signal.

B7-2 in contrast was expressed in 30 of the 39 samples and in 7 samples over 50% of cells expressed B7-2. In the 30 samples where B7-2 expression was detected, this was at a level intermediate between T cells and monocytes, but in the four samples with the highest expression the FFR was

approaching that seen on monocytes. In pre-B ALL the expression of B7-2 required to deliver a costimulatory signal and prevent the induction of anergy was low (Cardoso et al., 1996). If this is the case in myeloid leukaemic cells, then in the majority of cases an effective T cell response might be generated. This raised the question as to whether the AML cells are able to deliver a costimulatory signal and stimulate an anti-leukaemic immune response. If this is the case then it might be expected that patients should generate an autologous T cell response against potential leukaemic antigens, in the absence of other factors suppressing the immune response.

All the samples analysed expressed MHC class I molecules at a similar density to that found on normal haematological cells, so loss of class I expression would not appear to be a mechanism whereby myeloid leukaemia cells prevent induction of an anti-leukaemic immune response, although allelic specific expression was not tested. In addition these cells will be able to present cytosolic peptides to T cells, so any potential tumour antigens will be effectively presented to suitable T cells, which in the presence of a costimulatory signal will result in proliferation of responding T cells. T cells responding to peptides presented by MHC class I molecules are generally CD8+ve T cells. CD8+ve T cells are to a certain extent dependant on sufficient help from tumour activated T cells and that optimal immunological memory is generated if both CD4+ve and CD8+ve T cell are stimulated (Kern et al., 1986; Fearon et al., 1990). In the majority of samples analysed a high percentage of blasts also expressed HLA-DR at a similar or higher density to monocytes. MHC Class II expression is required for induction of a CD4 T cell response, which recognises secreted proteins. In addition coexpression of both B7-1 and

MHC class II by a sarcoma cell line was found to be the most effective combination for inducing rejection of the tumour, which was mediated by both CD4+ve and CD8+ve T cells (Baskar et al., 1995). Therefore coexpression of both class I and class II may induce a mixed CD4 and CD8 T cell response. CD8 cells are low producers of IL-2 and protection against rechallenge of tumour tends to lapse after about 90 days (Townsend and Allison, 1993; Townsend et al., 1994). However CD4 T cells are potent producers of IL-2, after appropriate activation (Deeths and Mescher, 1997) and protection against reexposure to tumour cells is longer lived than with CD8 T cells (Cavallo et al., 1992). Although the CD4 T cells recognise different antigens to those recognised by the CD8 T cells, they could act as helpers for a CD8 cytotoxic T cell response and maybe prolong the response. It should be advantageous therefore to be able to activate both CD4 and CD8 reactive T cells (Chen et al., 1993). In contrast, a study comparing the effectiveness of B7-1 mediated tumour rejection in a variety of tumourigenic cell lines did not improve with coexpression of MHC class II, protection against rechallenge with parental tumour of greater than 90 days was seen in a thymoma model which was MHC class II negative and was mediated by CD8+ve T cells alone (Townsend et al., 1994).

ICAM-1(CD54) is an adhesion molecule which binds to LFA-1 (CD11a) on T cells, which acts not just as an adhesion molecule, but also provides a costimulatory signal to T cells (Van Seventer et al., 1990). Coexpression of B7-1 and ICAM-1 on tumourigenic cells has been shown to be beneficial in inducing an effective immune response against the malignant cells (Cavallo et al., 1995). All the samples analysed expressed ICAM-1, most at a level

comparable to that on T lymphocytes. The percentage of cells expressing ICAM-1 in each sample ranged from 40 to 99%. Similarly, all samples expressed the adhesion molecule LFA-1, which binds to ICAM-1 present on a broad range of different cells. However, density of expression in most samples was less than that seen on monocytes, which expressed less than B cells and T cells. Adhesion is required in both the induction (Schmits et al., 1996; Sligh, Jr. et al., 1993) and effector phases (Davignon et al., 1981) of the immune response and thus expression of LFA-1 would be beneficial in a strategy designed to induce an anti-leukaemic immune response. That inate expression of MHC class I and II, ICAM-1 and LFA-1 is seen on AML blasts means many of the factors required for T cell activation are present, indeed in many of the samples B7-2 expression was found at a level which might be expected to deliver a co-stimulatory signal. Costimulation through CD28 has been demonstrated to reduce the number of T cell receptors needed to interact with the relevant peptide presented in the MHC groove (Bachmann et al., 1997). This is relevant to T cell recognition of tumour antigens which may be expressed at a low density. Indeed B7-1 expression on a tumour cell line has been shown to increase the number of epitopes which can be recognised by potential tumour reactive T cells (Johnston et al., 1996). With this in mind I decided to look at the duration of CR1 in the patients, to see if there was any benefit in B7-2 expression. Prolongation of CR1 in the patients whose AML cells expressed B7-2 might suggest the presence of an anti-leukaemic immune response *in vivo*.

The data suggests that CR1 is prolonged in those patients with B7-2 expressed on 30% or more of their AML blasts, compared to those patients

whose AML blasts expressed B7-2 at a lower percentage. This suggests that, if the B7-2 is functional, that costimulation is playing a role in preventing relapse of the AML. It may be that in these patients with higher B7-2 expression on their blasts, an autologous anti-leukaemic immune response is being generated. Although an immune response may be responsible for this, many other factors affect duration of first remission, including age, sex, white cell count at presentation, susceptibility to chemotherapeutic agents, cytogenetic abnormalities in the leukaemic clone and whether the patient underwent allogeneic bone marrow transplantation. All the preceding risk factors were similar between the two groups of patients, except for three patients in the higher B7-2 expression group who underwent allogeneic BMT, as opposed to none in the lower B7-2 expression group. Of the three patients undergoing allogeneic BMT, two are long term survivors and one died 15 weeks after the procedure. Removing these three patients renders the prolonged survival not statistically significant ($p=0.07$).

An explanation as to why high B7-2 expression on the AML cells does not result in a more marked effect on freedom from disease is that B7-2 appears to promote a T_H2 type immune response (Kuchroo et al., 1995; Freeman et al., 1995). A humoral immune response might be expected to be less effective than a cell mediated immune response in effectively eradicating residual myeloid leukaemic cells, which could result in relapse at a later date. Expression of B7-1 on myeloid leukaemic cells may induce a cell mediated immune response which may be more effective at eradicating residual leukaemic cells and thus preventing relapse. Indeed a study comparing the effectiveness of B7-1 or B7-2 expression on AML cells in a murine model,

showed that vaccination with the B7-1 expressing AML cells was more effective than the B7-2 AML cells. Although immunophenotyping detects expression of the two co-stimulatory molecules, it does not determine whether the expressing cells are able to deliver a co-stimulatory signal. With this in mind, I assessed the ability of the different AML samples to stimulate allogeneic T cells to proliferate and produce IL-2, indicative of the delivery of a co-stimulatory signal.

3.1.5 Co-stimulatory Ability of AML Blasts

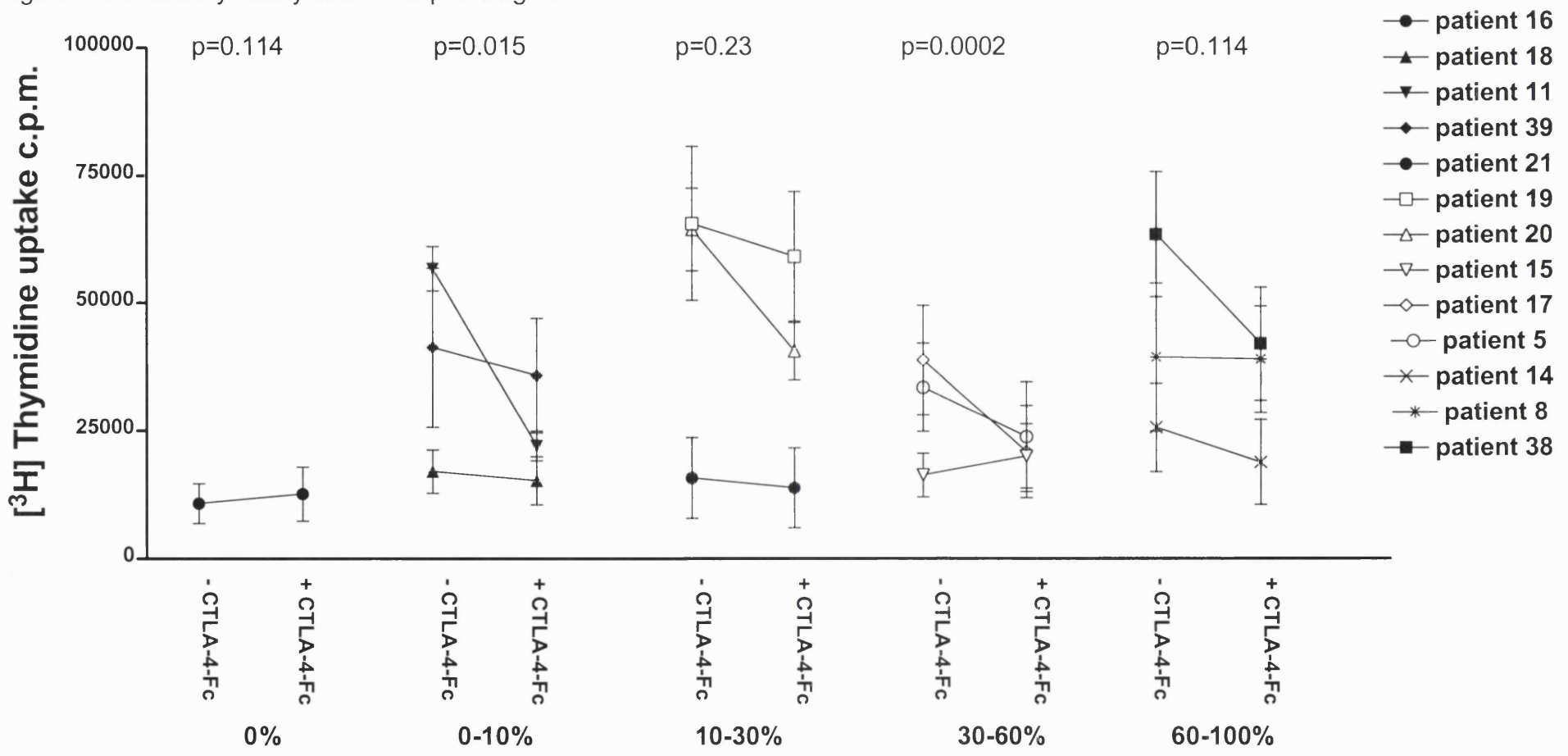
AML blasts were assessed for their ability to stimulate allogeneic T cells to proliferate in a 5 day MLR as described in section 2.1.4. Provision of a co-stimulatory signal has been shown to be a requirement for efficient generation of an allogeneic T cell response (Azuma et al., 1993b). All experiments were performed in duplicate with 2 different donors of normal T cells and all conditions were performed in replicates of 6. To assess the role of CD28/B7 interaction in proliferation of responding T cells CTLA-4/Fc (a fusion protein of the extracellular domain of CTLA-4 and the Fc region of human IgG (R&D Systems)) was added to the combination of T cells and AML blasts at a final concentration of 10μg/ml. This control was also performed in replicates of six with both T cell donors. As well as the B7 family of molecules, a variety of other molecules including ICAM-1, have been shown to deliver a co-stimulatory signal to T cells resulting in proliferation. However this tends not to be sustained and induction of IL-2 in the responding T cells is low (Damle et al., 1992). A feature of costimulation through CD28 is upregulation of IL-2 production (Freeman et al., 1995; Cerdan et al., 1992; Cerdan et al., 1995) and therefore the supernatants from the MLR's was assessed for IL-2 production

after 96 hours, as described in section 2.1.5. AML samples were divided into five groups, depending the percentage of AML blasts expressing B7-2, being 0%, 0-10%, 10-30%, 30-60% and >60%.

3.1.5.1 Results

There were three AML samples in each of the five groups, except for the 0% expression group, where there was only patient's sample available. Mean proliferation of unstimulated T cells was 3505 c.p.m. (sd 2911) and mean proliferation of irradiated AML cells was 268 c.p.m. (sd 144). Mean proliferation of the allogeneic T cells with the blasts negative for B7-2 as stimulators was 10750 c.p.m. (sd 3885) and in the presence of CTLA-4/Fc 12629 c.p.m. (sd 5303) (p=0.114). With 0-10% expression of B7-2 mean proliferation of T cells was 37581 c.p.m. (sd 18991) and in the presence of CTLA-4/Fc 24372 c.p.m. (sd 11192) (p=0.0147). With 10-30% expression of B7-2 mean proliferation of T cells was 47190 c.p.m. (sd 26071) and in the presence of CTLA-4/Fc 37885 c.p.m. (sd 20778) (p=0.23). With 30-60% expression of B7-2 mean proliferation of T cells was 30765 c.p.m. (sd 12800) and in the presence of CTLA-4/Fc 21530 c.p.m. (sd 9063) (p=0.0002). Finally with greater than 60% expression of B7-2 mean proliferation of T cells was 37838 c.p.m. (sd 18963) and in the presence of CTLA-4/Fc 30201 c.p.m. (sd 14557) (p=0.114). These results obtained with each patients' sample are shown in figure 6.

Figure 6 Stimulatory Ability of B7-2 expressing AML blasts

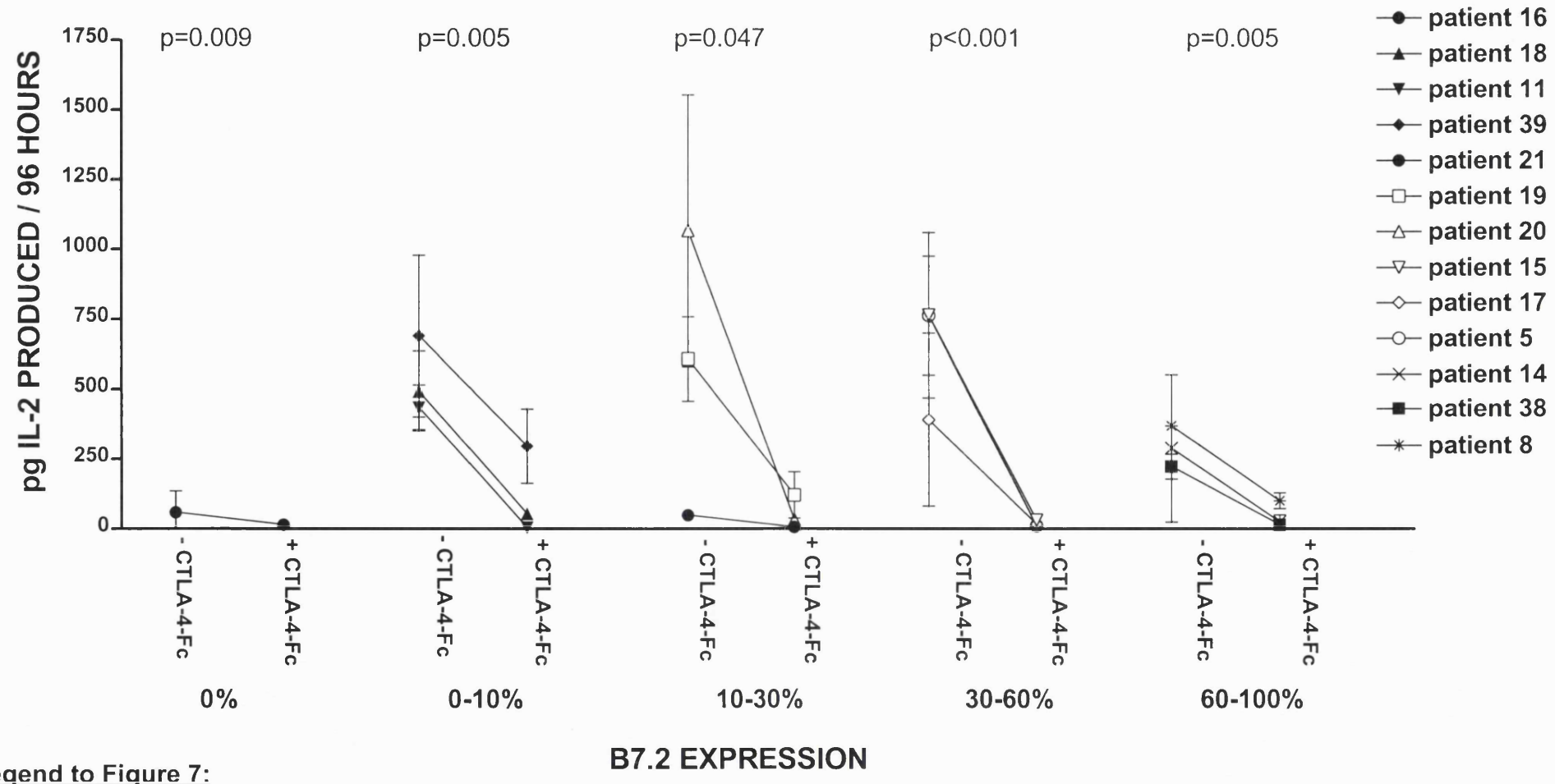


Legend to Figure 6:

Ability of AML blasts innately expressing B7-2 to stimulate proliferation of allogenic T cells, with and without CTLA-4/Fc at 10µg/ml. Results are expressed as mean counts per minute (c.p.m.) as an indicator of incorporation of [^{3}H]-thymidine, together with the standard deviation. Patient identification corresponds to that used for the immunophenotyping.

Mean production of IL-2 from unstimulated T cells was 6.5 pg/ml (sd 4.3) and from irradiated AML blasts 3.2 pg/ml (sd 5.2). Mean production of IL-2 from the allogeneic T cells stimulated with blasts negative for B7-2 was 60.0 pg/ml (sd 17.2) and in the presence of CTLA-4/Fc 15.5 pg/ml (sd 5.0) ($p=0.009$). With 0-10% expression of B7-2 mean production of IL-2 from the T cells was 540.3 pg/ml (sd 220.1) and in the presence of CTLA-4/Fc 118.7 pg/ml (sd 149.4) ($p=0.005$). With 10-30% expression of B7-2 mean production of IL-2 from the T cells was 575.0 pg/ml (sd 510.8) and in the presence of CTLA-4/Fc 53.6 pg/ml (sd 68.4) ($p=0.047$). With 30-60% expression of B7-2 mean production of IL-2 from the T cells was 588.1 pg/ml (sd 343.6) and in the presence of CTLA-4/Fc 19.9 pg/ml (sd 14.0) ($p<0.001$). Finally with greater than 60% expression of B7-2 mean production of IL-2 from the T cells was 292.7 pg/ml (sd 195.4) and in the presence of CTLA-4/Fc 41.7 pg/ml (sd 41.2) ($p=0.005$). These results are shown for each individual sample in figure 7.

Figure 7 Ability of B7-2 Expressing AML Blasts to Induce IL-2 Production from Allogenic T cells



Legend to Figure 7:

Ability of AML blasts innately expressing B7-2 to stimulate production of IL-2 from allogenic T cells, in the presence and absence of CTLA-4/Fc at 10µg/ml. Results are expressed as mean production of IL-2 in pg/ml per 96 hours, together with the standard deviation. Patient identification corresponds to that used for the immunophenotyping.

3.1.5.2 Discussion

AML blasts negative by flow cytometric analysis for B7-2 and B7-1 stimulated only a modest increase in proliferation of allogeneic T cells which was not inhibited by the addition of CTLA-4/Fc. Production of IL-2 was also modestly stimulated and was largely inhibited by the addition of CTLA-4/Fc. That CTLA-4/Fc inhibited IL-2 production from the T cells incubated with the B7-2-ve and B7-1-ve blasts indicates either that there was expression of either co-stimulatory molecule below the level of detection of flow cytometric analysis, or that there were cells in the T cell preparation, such as B cells or monocytes that were capable of providing bystander 3rd party costimulation, despite the T cell enrichment steps (Reiser et al., 1992). Unfortunately there were no other AML samples negative for B7-2 expression available, to see whether the results obtained with patient 16 would be repeated. All the AML samples expressing B7-2 stimulated T cell proliferation and in the 10 of the 12 cases addition of CTLA-4/Fc partially inhibited T cell proliferation, although this was only statistically significant in the 0-10% and 30-60% B7-2 expression groups, indicating that there were other molecules expressed on the AML blasts capable of providing a second co-stimulatory signal. Indeed as shown in section 3.1.3.4 all AML samples expressed ICAM-1 and there are several other co-stimulatory molecules that may also be expressed but were not analysed on the blasts. The results are more clear when analysing IL-2 production, which is upregulated more when the co-stimulatory signal is through CD28 as compared to other co-stimulatory molecules (Damle et al., 1992). IL-2 production was significantly increased in all the B7-2 expressing samples, except for patient 21

and was inhibited by the addition of CTLA-4/Fc, demonstrating that the expressed B7-2 was functional. With patient 21, in whom 26.5% of the leukaemic blasts expressed B7-2, at an intensity close to that on monocytes, proliferation of allogeneic T cells was lower than many of the other samples and IL-2 production was markedly low. Interestingly only 15.5% of this patients' AML cells expressed LFA-1. HLA-A/B/C, HLA-DR and ICAM-1 were expressed at comparable levels to the other samples, indicating that in this case it may have been failure of adhesion of the AML blasts to the allogeneic T cells which prevented an adequate co-stimulatory signal from being delivered. Also of interest is that AML cells, with less than 10% of cells expressing B7-2 were able to deliver a co-stimulatory signal capable of inducing T cell proliferation and IL-2 production of similar levels to samples where greater than 60% of AML cells expressed B7-2. This is of relevance to the strategy of inducing expression of B7-1 or B7-2 on AML blasts, where transduction efficiencies of viral vectors is generally low.

That the B7-2 expressing AML cells were able to costimulate IL-2 production and CR1 appears to be prolonged with higher inate B7-2 expression on the AML blasts, suggests that the strategy of inducing expression of B7-1 or B7-2 is worthwhile. In addition, studies in mice seem to suggest that B7-1 would be the preferred co-stimulatory molecule to induce an effective anti-leukaemic immune response (Matulonis et al., 1996) and therefore survival might be expected to be improved with expression of B7-1 as opposed to B7-2.

4.1 SEQUENCING OF B7-2

4.1.1 Introduction

4 clones of B7-2 were supplied, in the plasmid pUC 18, which were produced by reverse transcriptase PCR from the cell line Daudi (ECACC No: 89120702). The cell line, which innately expresses B7-2, was derived from a patient with Burkitt's lymphoma. The method involved extracting RNA from the cells, which means that only transcriptionally active genes could be cloned. A complementary DNA strand to the mRNA was produced by incubating with reverse transcriptase and a primer specific for B7-2. The mRNA was then removed by hydrolysis, primers specific for B7-2 and Taq polymerase were added and the DNA amplified by PCR. A limitation to this technique was the accuracy of Taq polymerase in producing a complementary DNA strand. The fidelity of the PCR reaction depends upon the error rates of three steps. The first step is the ability of Taq polymerase to distinguish the incoming dNTP substrates and incorporate the correct nucleotide. The second step is the ability of the polymerase to continue extension on a correctly paired primer-template as opposed to extending a primer-template containing a terminal mismatch. The final step is the ability of the polymerase to have 3' to 5' exonuclease activity, which selectively removes misincorporated nucleotides replacing them with the correct nucleotide. Taq polymerase does not have 3' to 5' exonuclease activity and produces single-base substitution errors at a rate of 1 in 9000 nucleotides (Tindall and Kunkel, 1988). The cDNA for B7-2 is 1180 base pairs and thus there was a chance of a mutation in the open reading frame (ORF) which may have affected the amino acid structure of the molecule.

A change in the amino acid structure of B7-2 might have altered the ability of the molecule to deliver a co-stimulatory signal and thus one of the clones had to be sequenced before cloning of the cDNA into an expression plasmid.

4.1.1.1 DNA Sequencing

The method of sequencing which was chosen was the chain-termination DNA sequencing method (Sanger et al., 1977). This method involves the synthesis of a complementary strand of DNA using a single-stranded DNA template. The site of initiation of DNA synthesis by the DNA polymerase was determined by the binding of an oligonucleotide primer to the template. Termination occurs with incorporation of a chain terminating nucleotide analogue which does not support further DNA elongation. The chain terminating nucleotide analogues are the 2',3'-dideoxynucleoside 5'-triphosphates (ddNTPs). These lack the 3'-OH group necessary for DNA chain elongation. When mixtures of dNTPs and one of the four ddNTPs are used, polymerisation will be terminated in a fraction of the population at each site where that particular ddNTP could be incorporated. When four separate reactions, each with a different ddNTP, are undertaken the entire sequence of the template DNA can be determined. Incorporation of a radioactively labelled nucleotide into the reaction allows visualisation of the labelled chains of various lengths by autoradiography after separation by high-resolution electrophoresis.

Limitations to this technique include resolution of autoradiography which used high energy ^{32}Phosphorus, resulting in diffuse bands on the film. This problem has been largely overcome with the use of lower energy ^{35}Sulphur which improves the resolution of the bands greatly (Biggin et al., 1983).

Another limitation is the quality of the DNA polymerisation which ideally would result in equal numbers of chains terminated at each correct nucleotide and no false terminations at incorrect nucleotides. This would result in uniform band intensity and absence of background bands which is seen more with methods using the large fragment of *E. coli* DNA polymerase I (Klenow enzyme) or AMV reverse transcriptase, than with the method employed which utilises Sequenase T7 DNA polymerase (Tabor and Richardson, 1987). DNA synthesis is split into two steps the first being the labelling step in which the primer is extended using limiting concentrations of the deoxyribonucleoside triphosphates (dNTPs), including radioactively labelled (^{35}S) dATP. This step continues until incorporation of labelled nucleotide into DNA chains, which are distributed randomly in length from several nucleotides to hundreds of nucleotides, is virtually complete. In the second step, the concentration of all the dNTPs is increased and a dideoxynucleoside triphosphate is added. Progressive DNA synthesis occurs until all growing chains are terminated by a dideoxyribonucleotide. During this step the chains are extended by only a few dozen nucleotides on average.

A further limitation is incomplete denaturation of the DNA strands during electrophoresis, seen in particular with sequences containing dGTP and dCTP residues. This can result in interruption of normal migration of the DNA strands which results in bands being compressed or further apart than normal. The incorporation of nucleotide analogues of dGTP, which form weaker secondary structures, has helped eliminate these gel artefacts (Mizusawa et al., 1986).

4.1.1.2 Nested Deletions

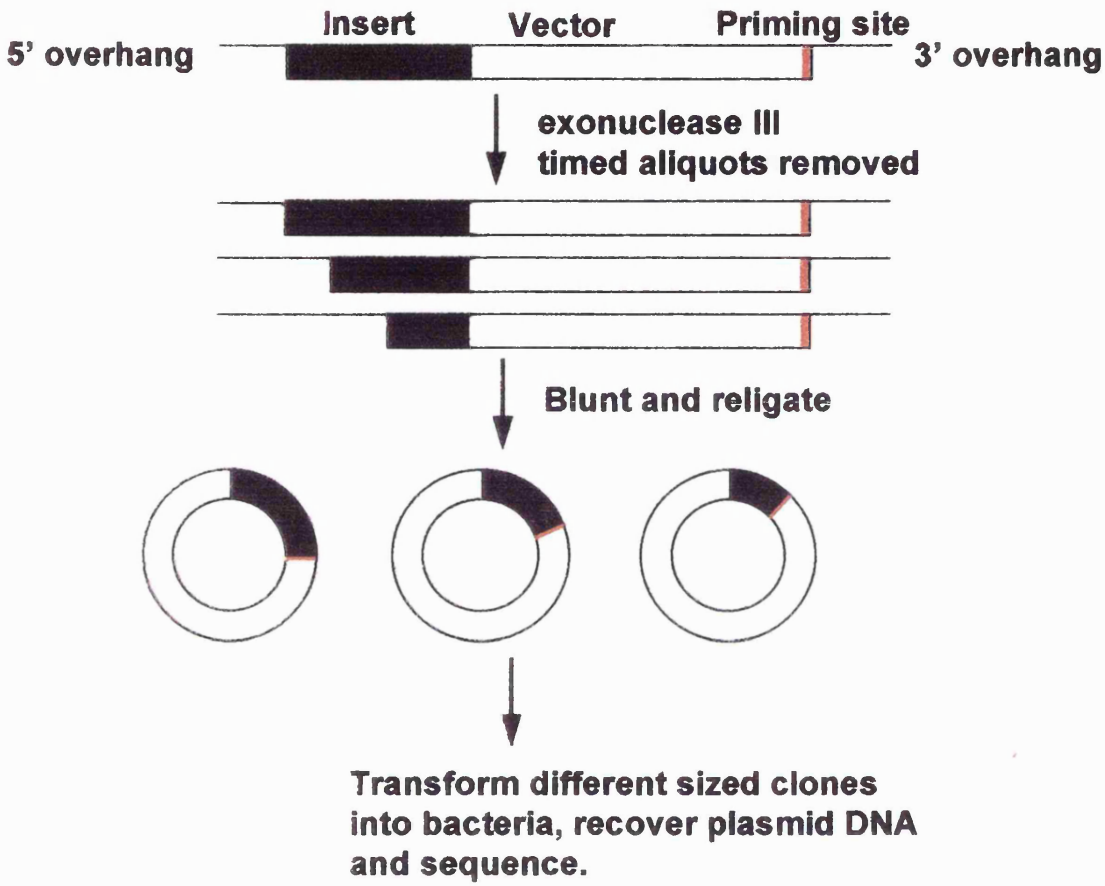
The use of forward and reverse primers allows approximately 500 bases to be sequenced. Sequencing of DNA fragments larger than 500 bases in length, as is the case with the cDNA for B7-2, involves a different strategy. One of these involves the generation of a set of nested deletions in the target DNA, which effectively moves the site of binding of the primer closer to the sequence of interest. This system is based on the ability of the enzyme Exo III to specifically digest insert DNA from a 5' protruding restriction site (Henikoff, 1984), whereas 3' overhang restriction sites are protected (Putney et al., 1981). DNA cloned into the multiple cloning site of an appropriate vector will in most cases allow the choice of two restriction enzymes which cut between the primer binding site and the DNA to be sequenced. The use of a restriction enzyme which cuts closest to the primer binding site must leave a 3' overhang, which is resistant to digestion by Exo III. The enzyme that cuts closest to the DNA to be sequenced must leave a 5' overhang which is susceptible to digestion by Exo III. Digestion of the DNA with the two appropriate restriction enzymes, followed by digestion with Exo III will result progressive digestion of the DNA from the 5' overhang which results in progressive digestion of the DNA to be sequenced. Timed removal of aliquots of the reaction into tubes containing S1 nuclease removes remaining single strand DNA tails and the presence of zinc cations and low pH inhibits further digestion by Exo III. This results in differing sized fragments depending on the time the DNA was exposed to Exo III, those being exposed the longest bringing the 3' end of the DNA to be sequenced closer to the primer binding site. After heating the samples to inactivate the S1 nuclease, the ends of the DNA are blunt-ended using Klenow DNA polymerase

and religated to circularise the deletion containing vectors. The ligation product from each timepoint is then used to transform competent cells and each one will yield a number of subclones containing deletions extending further into the original insert. These subclones can then be screened to select candidate clones which will enable the entire DNA insert to be sequenced. This technique is shown diagramatically in figure 8.

4.1.2 Materials and Methods

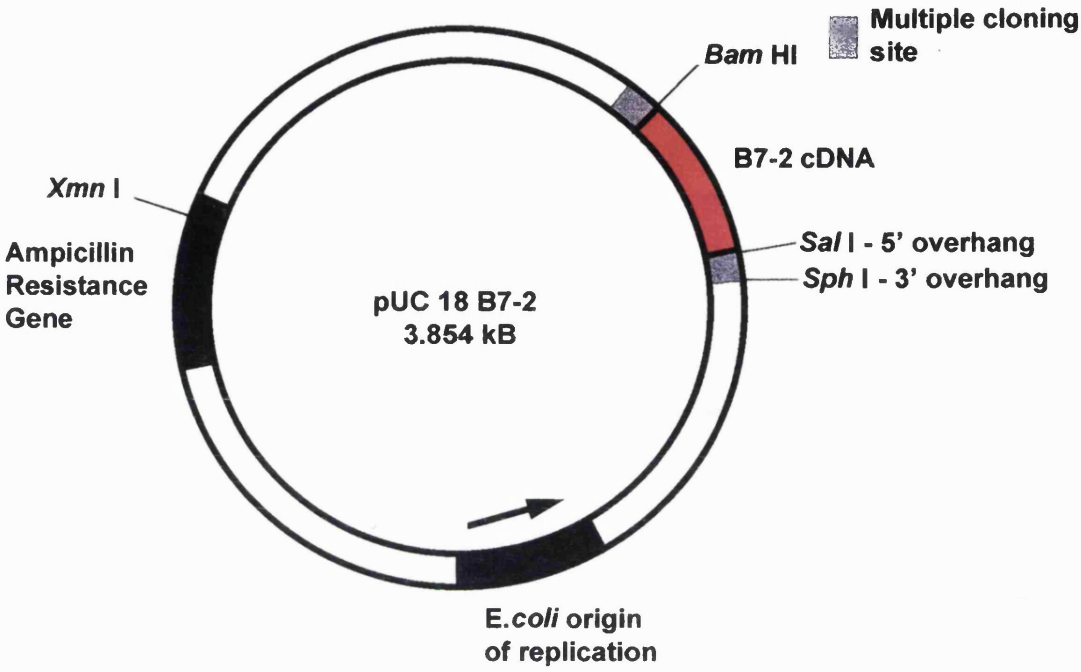
The clone chosen was pUC18 B7-2 clone A, which was sequenced using forward and reverse primers as described in sections 2.2.1 and 2.2.1.1. To sequence the entire cDNA, nested deletions of clone A were generated as described in section 2.2.1.2. To determine the likely clones for sequencing the entire open reading frame for B7-2 the subclones were linearised and visualised by running on a 0.8% agarose gel. As described in section 2.2.3.1, each of the subclones was digested with the restriction enzyme *XMn* I, which cuts pUC18/B7-2 once outside the multiple cloning site (Figure 9). To see how much of the gene had been removed, cl.6 was also linearised with the restriction enzyme *Xmn* I. An aliquot of each digest was then visualised after running on a 0.8% agarose gel.

Figure 8 Schematic diagram of Erase-A-Base



Legend to Figure 8:
Exonuclease III digests DNA from susceptible 5' overhang. Removal of aliquots at timed intervals creates subclones with gradually increasing amounts of 5' DNA digested from the insert, which can then be sequenced using the single primer binding site.

Figure 9 Restriction map of pUC 18 B7-2



Legend to Figure 9:
Restriction enzyme map of the plasmid pUC 18 B7-2, demonstrating sites used in generation and screening of nested deletions.

4.1.3 Results

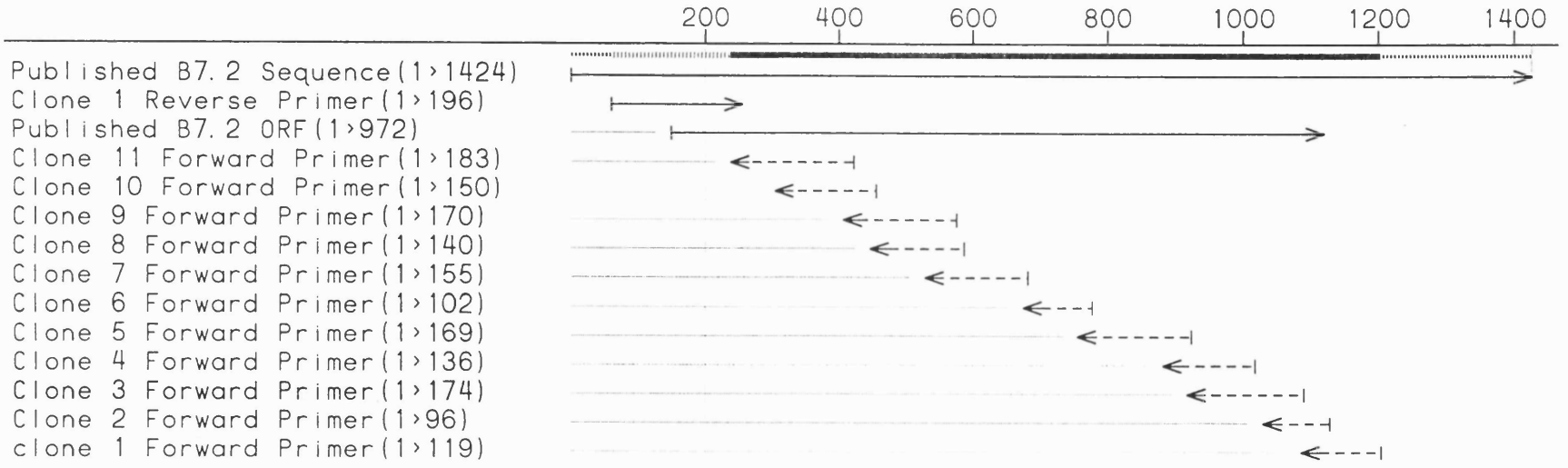
4.1.3.1 Generation of Nested Deletions

To sequence the entire open reading frame for B7-2 a total of 29 nested deletions of pUC B7-2 were generated by digestion with Exo III. They were linearised, together with parental pUCB7-2, by digestion with the restriction enzyme *Xmn* I and visualised on an agarose gel, to demonstrate the graded digestion of the B7-2 cDNA with Exo III. By comparing the size of the Exo III digested clones with the parental pUC B7-2 the likely candidates for sequencing were determined. Of the nested deletions generated and sequenced, 10 were required to cover the open reading frame for B7-2 and these were termed clones 2 to 11. The original undigested clone A was termed clone 1 (Fig 10).

4.1.3.2 Sequencing of B7-2

The cDNA coding for B7-2 in clone A was sequenced using the forward and reverse primers on the original clone (clone 1). Sequencing of 10 nested deletions (clones 2 to 11), using the forward primer, enabled the entire cDNA of B7-2 in pUC18 B7-2 clone A to be determined. The sequence was read using the DNA* SEQ.EASY system, which uses the software Lasergene Navigator and the sequence was compared with the sequence published by Azuma in 1993 (Azuma et al., 1993b). There were two mutations found at positions 429 and 585 of the open reading frame both of which were silent. The two mutations left the amino acids encoded unchanged, Asn and Thr respectively, involving a substitution of cytosine for thymidine (Figs 11 and 12).

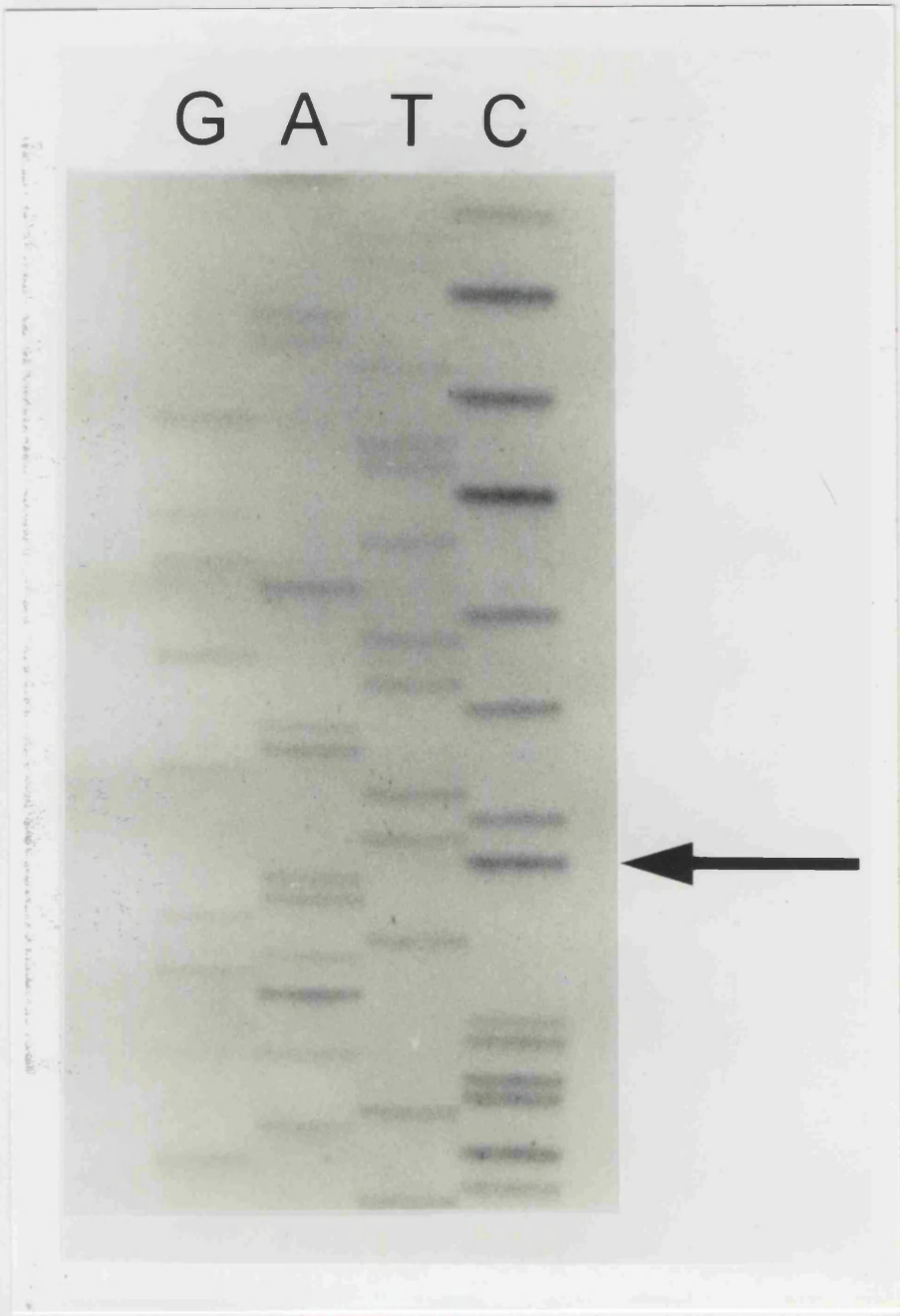
Figure 10 Contig map of B7-2 with 10 nested deletions



Legend to Figure 10:

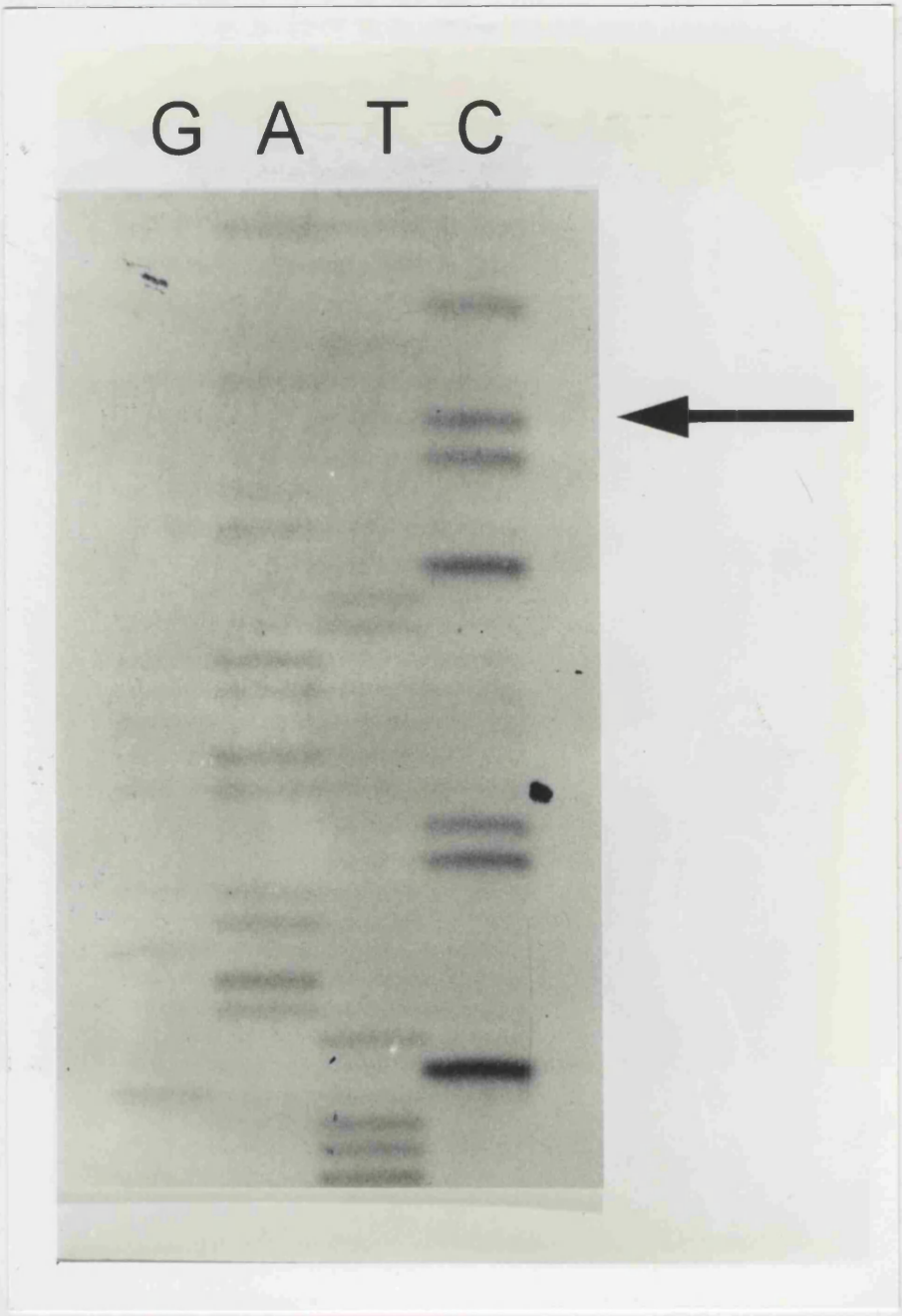
Contig map of clone 1 and the 10 nested deletions (clones 2 to 11) covering the ORF of B7-2, compared against the published sequence of B7-2 including the ORF.

Figure 11 Mutation 1 in Clone A



Legend to Figure 11:
The order of loading of the sequencing gel was G (guanosine), A (adenosine), T (thymidine) and C (cytidine). The sequence is read from the bottom and reads GAA**C**TC, the arrow points to the mutation where thymidine is substituted with cytidine, which is highlighted in bold.

Figure 12 Mutation 2 in Clone A



Legend to Figure 12:
The order of loading of the sequencing gel was G (guanosine), A (adenosine), T (thymidine) and C (cytidine). The sequence is read from the bottom and reads CAAC**C**A, the arrow points to the mutation which involves a substitution of cytidine for thymidine, which is highlighted in bold.

4.1.4 Discussion

Sequencing of the cDNA for B7-2 cloned by reverse transcriptase PCR, highlighted two mutations, when compared with the published sequence (Azuma et al., 1993b). Fortunately both mutations were silent. Thus I proved that one of the clones, clone A, contained the entire open reading frame of B7-2 and should code for the correct amino acid sequence of B7-2. Clone A was then chosen for all further experiments.

4.2 CONSTRUCTION OF EXPRESSION PLASMID

4.2.1 Introduction

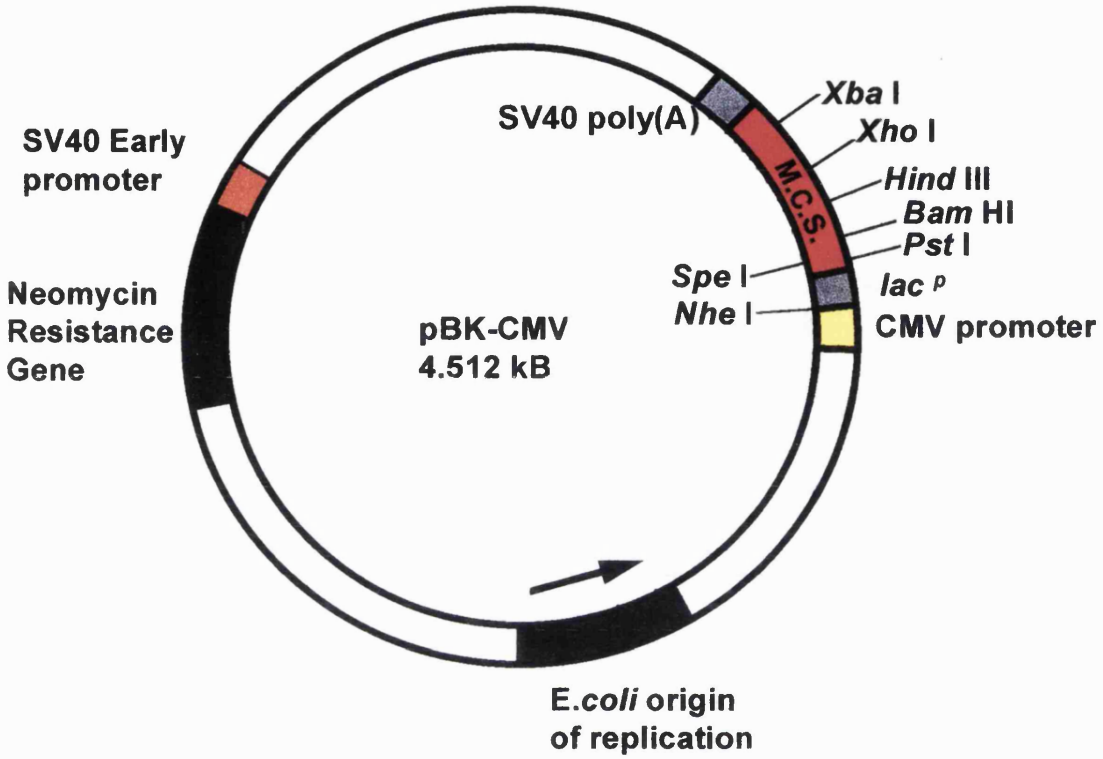
After confirmation that clone A contained the complete ORF for B7-2, I needed to determine whether the cDNA for B7-2 could be expressed as a transmembrane protein on the surface of eukaryotic cells. The vector chosen for subcloning of the B7-2 cDNA from clone A was the phagemid pBK-CMV (Stratagene). This vector allows expression in both prokaryotic and eukaryotic systems. In eukaryotic cells, expression is driven by the cytomegalovirus (CMV) immediate early promoter, which is a strong heterologous promoter and in prokaryotic cells expression is driven by the *lac* promoter. 3' to this is a polylinker cloning site and polyA tail derived from the SV40 virus. The vector also contains the neomycin phosphotransferase gene, which allows for selection with kanamycin in prokaryotic cells or geneticin in eukaryotic cells. It has been demonstrated that two promoters within the same vector may downregulate one of the promoters by a process called promoter interference (Proudfoot, 1986; Emerman and Temin, 1984). To prevent the possibility of promoter interference occurring in pBK-CMV, the *lac* promoter was removed before any cloning of DNA into the expression plasmid was performed.

4.2.2 Removal of *lac* Promoter

Parental pBK-CMV was provided in E.coli (strain TG-1) and grown overnight in 5mls LB medium containing 50μg/ml kanamycin. Plasmid DNA was extracted as previously described in section 2.2.3. and digested with *Nhe* I

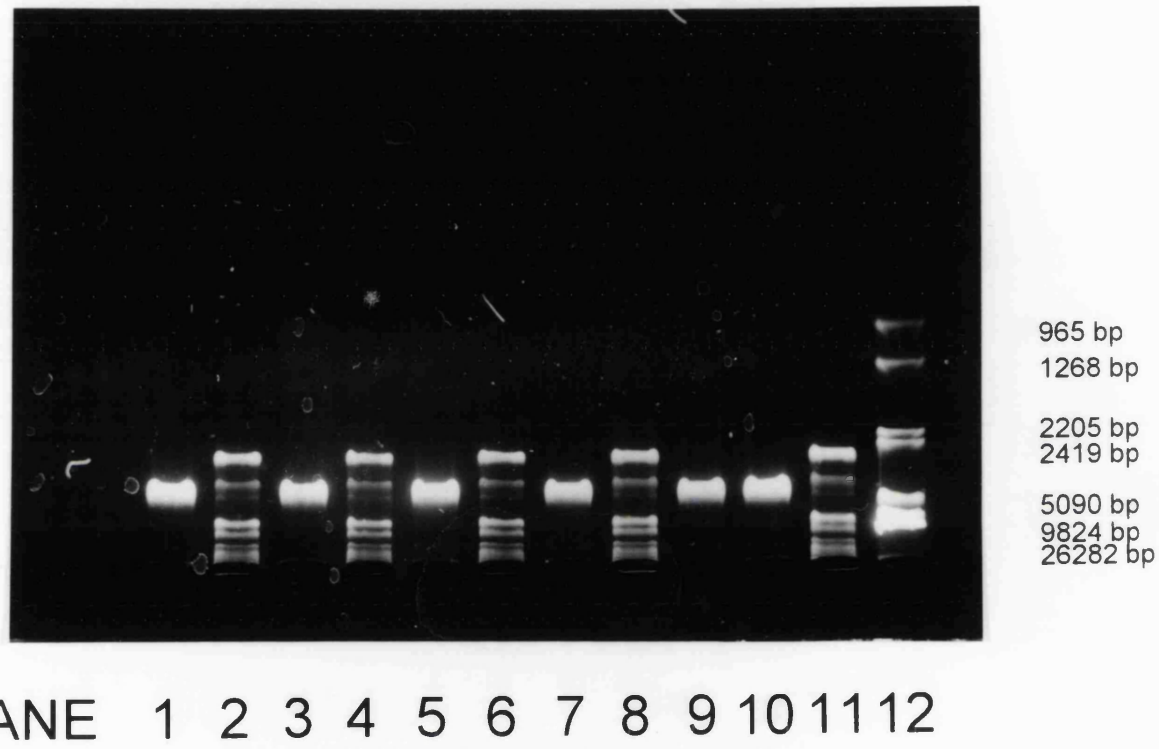
(Promega) and *Spe* I (Promega), as described in section 2.2.3.1. The digested DNA was cleaned using a Qiagen clean up column as described in section 2.2.3.4. Digestion with both restriction enzymes removes the prokaryotic promoter and since both restriction enzymes result in complementary overhanging ends after digestion no further manipulation was required prior to ligation. The digested DNA was religated as described in section 2.2.3.3. 20μl of the ligation product was used to transform 200μl competent cells (TG-1), prepared as described in section 2.2.2. The transformed cells were selected on LB agarose plates containing 50μg/ml kanamycin. The plates were incubated overnight at 37°C. 4 colonies were picked and expanded in LB medium containing 50μg/ml kanamycin at 37°C overnight. The plasmid was then extracted as described in section 2.2.3. Successful removal of the *lac* promoter was determined by digesting the four clones with *Pst* I (Promega) as described in section 2.2.3.1. There is a single *Pst* I site in the parental plasmid which is removed with the removal of the *lac* promoter. *Pst* I digestion of the parental plasmid ~~was~~ assured the enzyme was functional. Each of the four subclones was also digested with the restriction enzyme *Hind* III (NEB), since this enzyme cuts once in the multiple cloning site and remains in the construct after removal of the *lac* promoter (see figure 13). All four clones appeared to have lost the *lac* promoter (see figure 14).

Figure 13 Restriction enzyme map of pBK-CMV



Legend to Figure 13:
Restriction enzyme map of pBK-CMV, highlighting the restriction sites used in removing the prokaryotic promoter and subcloning of the cDNA's for β-galactosidase and B7-2. The multiple cloning site (M.C.S.) is highlighted in red and the prokaryotic promoter is labelled *lac* ^{P}.

Figure 14 Pst I /HinD III digest of pBK-CMV *lac*-ve



Legend to Figure 14:
All 4 clones are linearised with *Hind* III (lanes 1, 3, 5 and 7), but not with *Pst* I (lanes 2, 4, 6 and 8) which remains uncut as compared with the uncut control (lane 11). Parental pBK-CMV is cut with both restriction enzymes (lanes 9 and 10), demonstrating both enzymes are functioning and the size marker (lane 12) is an *Mlu* I digest of λ, giving fragments of 26282, 9824, 5090, 2419, 2205, 1268, 965 and 458 base pairs. Due to the limitations of the gel, the smallest DNA fragment of the size marker cannot be seen.

4.2.3 Cloning of β-Galactosidase into pBK-CMV *lac*-ve

4.2.3.1 Introduction

After successful removal of *lac* ^{p} from pBK-CMV, the expression plasmid, now termed pBK-CMV *lac*-ve, was ready for subcloning the cDNA for B7-2. It remained to be determined whether the plasmid would express a functional protein. If surface expression of B7-2 was not found on cells transfected with a B7-2 expression plasmid, it could be due to a defect in the plasmid rather than with the cDNA for B7-2. To control for this variable a marker gene, *Lac*Z, was cloned into the multiple cloning site of pBK-CMV *lac*-ve. Cells expressing β-galactosidase, the *Lac*Z gene product, stain blue on exposure to the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). As opposed to *in situ* staining of cells with X-Gal, β-galactosidase activity of cell extracts can be assessed by incubation with o-nitrophenyl-β-D-galactopyranoside (ONPG). Not only does this confirm whether the plasmid can express a gene in eukaryotic cells, but can be used to assess the efficiency of transfer of the plasmid into the cells. Therefore, prior to subcloning of the B7-2 cDNA, the *Lac*Z gene was subcloned into pBK-CMV *lac*-ve.

4.2.3.2 Materials and Methods

The β-galactosidase cDNA used was derived from the vector pSV-β-Galactosidase. pBK-CMV *lac*-ve was digested simultaneously with the restriction enzymes *Xba* I (NEB) and *Hind* III (NEB)(see section 2.2.3.1). pSV-β-Galactosidase (Promega) was also digested with the restriction enzymes *Xba* I (NEB) and *Hind* III (NEB)(see figure 15). The β-galactosidase cDNA released

was ligated into the digested pBK-CMV *lac*-ve plasmid. To a fresh 1.5ml microcentrifuge tube was added 15μl of digested pSV-β-Galactosidase which had been cleaned using a Qiagen column (see 2.2.3.4), 5μl of digested pBK-CMV *lac*-ve similarly purified, which were then ligated as described in section 2.2.3.3 using the appropriate no insert and no ligase controls. 200μl aliquots of competent cells (see 2.2.2) were transformed with 25μl of the ligation product from each of the three samples (see 2.2.2.1). The transformed bacteria were then plated out on LB agar plates containing 50μg/ml kanomycin and cultured overnight at 37°C. 6 colonies were picked from the plate containing ligated pBK-CMV and β-galactosidase and grown in LB medium containing 50μg/ml kanamycin. The plasmids were prepared as described in section 2.2.3. The six subclones were each digested simultaneously with the restriction enzymes *Xba* I (NEB) and *Hind* III (NEB) to determine if the correct clones had been achieved. An aliquot from each of the restriction digestions was visualised on a 0.8% agarose gel.

4.2.3.3 Results of Subcloning β-galactosidase into pBK-CMV *lac*-ve

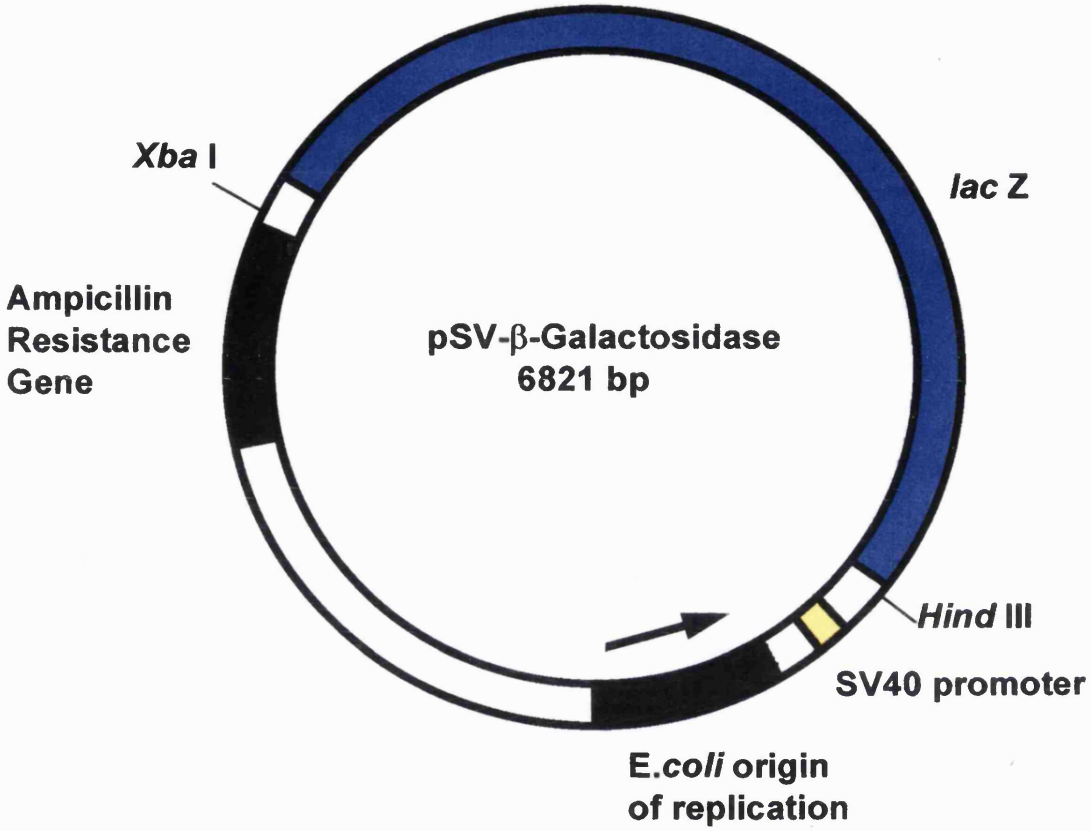
6 subclones were analysed by digesting with the restriction enzymes *Xba* I and *Hind* III. All 6 clones released 2 fragments, both between 5090 bp and 2419 bp as compared to the size markers. The parental plasmid pSV-β-gal on digestion with the same restriction enzymes released two fragments of between 5090 and 2419 bp, the larger fragment corresponding in size to the smaller fragment released from the 6 putative β-galactosidase clones. Furthermore, pBK-CMV *lac*-ve incubated with *Xba* I and *Hind* III resulted in a

single band of between 5090 and 2419bp, which corresponded to the larger band seen in all the 6 subclones (figure 16).

4.2.3.4 Discussion

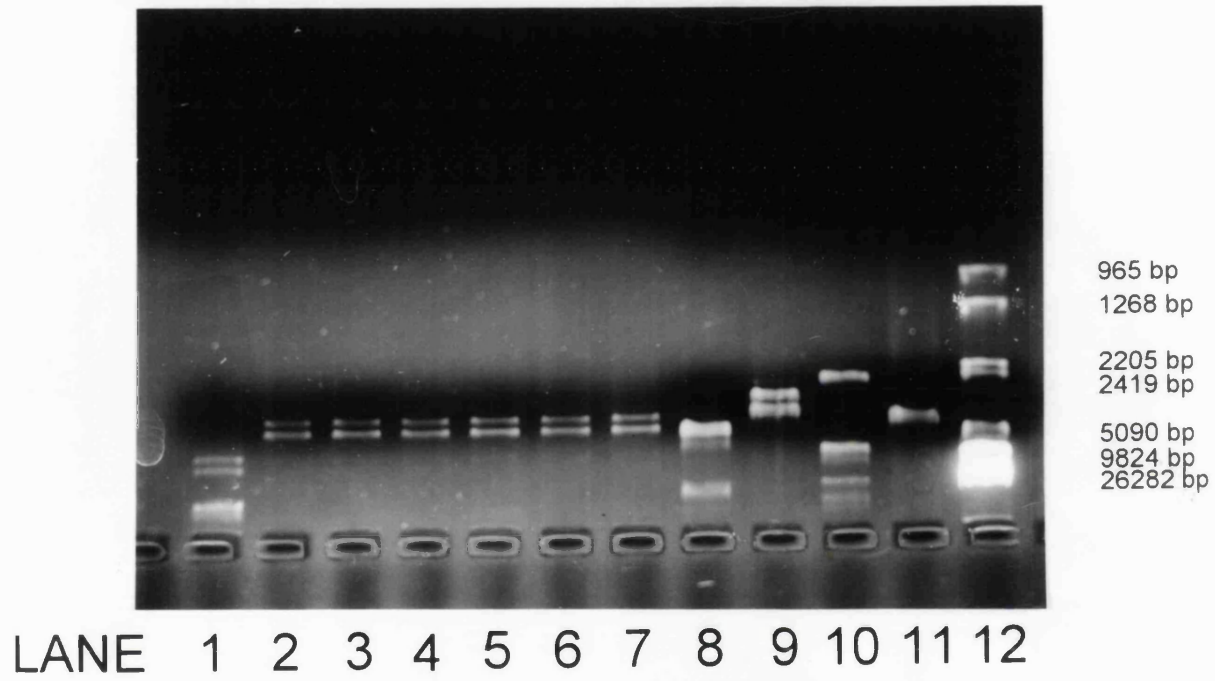
The vector pSV-β-galactosidase on digestion with *Xba* I and *Hind* III would be predicted to yield two fragments of 3743 and 3078bp, the larger fragment corresponding to the *lac* Z gene (figure 15). The larger fragment in lane 8 corresponds in size to the smaller fragment released from the 6 putative pBK-CMV *lac*-ve β-galactosidase clones (lanes 2-7), indicating that all six clones contain the *lac*Z gene. Furthermore digestion of pBK-CMV *lac*-ve with *Xba* I and *Hind* III yields a single visible fragment of 4299bp, which corresponds in size to the larger fragment seen in the six digested clones. This suggests successful cloning of the *lac*Z gene into the vector pBK-CMV *lac*-ve. The pBK-CMV *lac*-ve β-galactosidase clones were then assessed for β-galactosidase activity in eukaryotic cells after transfection and then used to optimise this technique prior to transfection with pBK-CMV *lac*-ve containing the B7-2 cDNA.

Figure 15 Restriction map of pSV-β-Galactosidase



Legend to Figure 15:
Restriction enzyme map of pSV-β-Galactosidase demonstrating the restriction sites used in releasing the *lac* Z cDNA from the vector.

Figure 16 Xba I/ HinD III digest of pBK-CMV *lac*-ve β Gal



Legend to Figure 16:
The six putative pBK-CMV *lac-ve* β-galactosidase clones (lanes 2-7) digested with *Xba* I and *Hind* III. Lane 1, uncut control of clone1. Lane 8, uncut control of pSV-β-galactosidase and lane 9 contains pSV-β-galactosidase digested with *Xba* I and *Hind* III. Lane 10 contains an uncut control of pBK-CMV *lac*-ve and lane 11 pBK-CMV *lac*-ve digested with *Xba* I and *Hind* III. Lane 12, *Mlu* I digest of λ giving fragments of 26282, 9824, 5090, 2419, 2205, 1268, 965 and 458bp, the smallest fragment not being visible on this gel.

4.3 TRANSFECTION OF 293 CELLS

4.3.1 Optimisation of Transfection with pBKCMV *lac*-ve β-Gal Construct

The use of cationic lipids to transfer DNA into eukaryotic cells has been used for several years, first being reported in 1987 (Felgner et al., 1987). The technique relies upon the positively charged headgroup of the lipid associating with the negatively charged phosphate groups on the DNA. However for the successful transfer of the DNA into the cell the DNA/lipid complex must still retain a net positive charge to attract it to the negatively charged cell surface. Thus the ratio of DNA to lipid is crucial to optimise transfection of the DNA. Other variables also affect transfection efficiency, in particular the quality of the plasmid DNA, which should be free of protein, RNA and chemical contamination. The optimal transfection time also varies depending on the plasmid and target cell type, with cells losing viability under prolonged transfection conditions since the use of serum free medium during the transfection period, while increasing transfection efficiency, also decreases the time the cells tolerate transfection conditions.

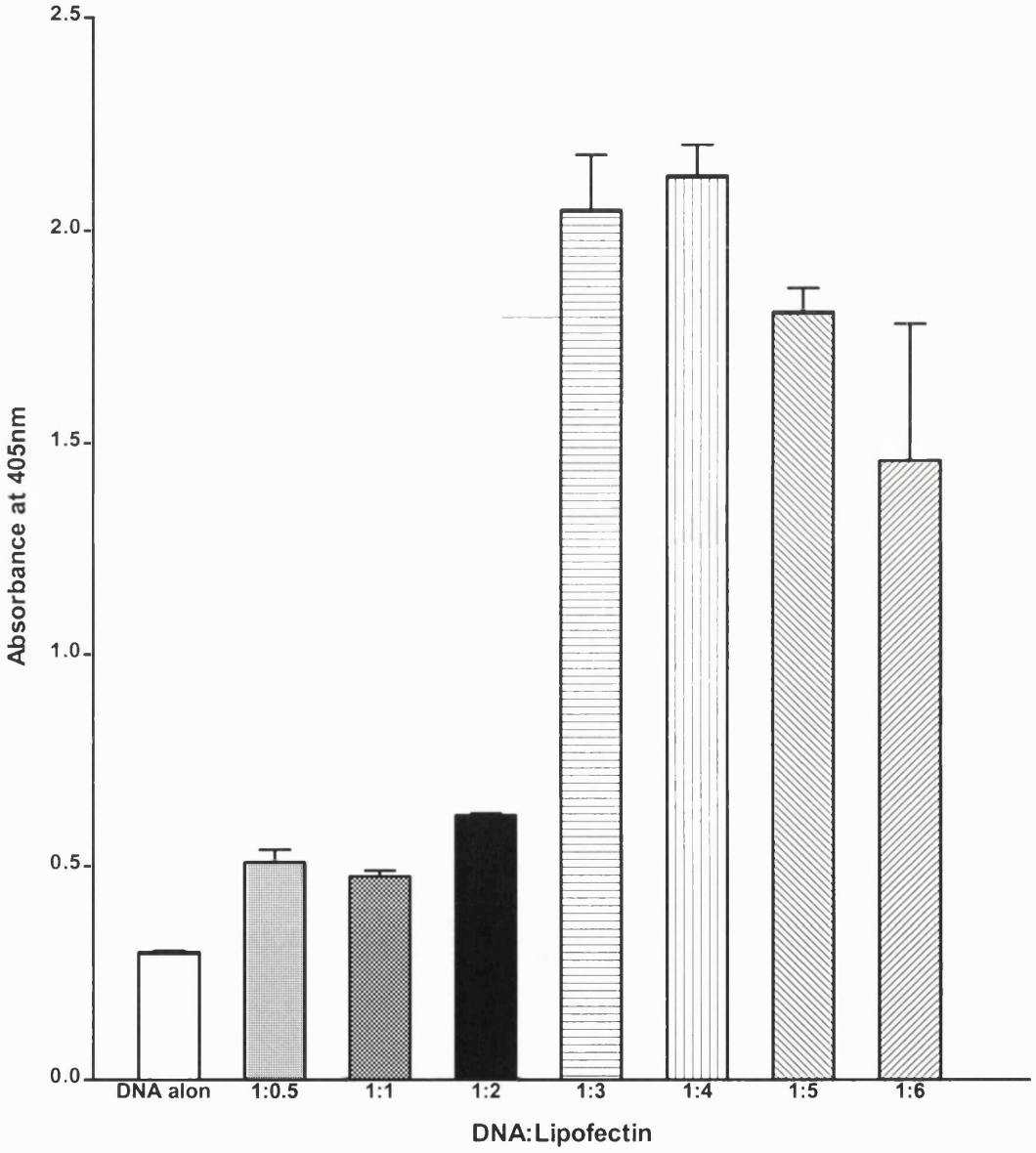
4.3.1.1 Optimisation of Transfection Varying the Lipofectin:DNA Ratio

To determine the optimal ratio of DNA to cationic lipid (lipofectin), fresh pBK-CMV *lac*-ve β-galactosidase plasmid (clone 1) were transfected into 293 cells (ECACC No:85120602), which are derived from human embryonal kidney cells transformed with sheared human adenovirus type 5 DNA. Four 6 well

plates (Nunclon), were seeded at a concentration of $1x10^5$ 293cells/ml and cultured until they had reached 40-60% confluency (see 2.1.6). At this time 1.0μg (1.3μl) aliquots of the plasmid pBKCMV *lac*-ve β-gal were placed in 24 separate 17 x 120mm conical polystyrene sterile tubes and in separate 12x75 mm sterile tubes was aliquoted the cationic lipid (Lipofectin) at a concentration of 1mg/ml. Triplicate tubes containing 0.5μl, 1μl, 2μl, 3μl, 4μl, 5μl and 6μl of lipofectin were prepared. The plasmid DNA and lipofectin solutions were mixed, incubated and transfected into the cells in individual wells as described in section 2.1.6. As a negative control 1μg of pBK-CMV *lac*-ve β-gal was added to 1ml of serum free medium and layered onto the cells in the absence of lipofectin. The ratios of lipofectin to DNA tested were 0.5, 1, 2, 3, 4, 5 or 6. After transfection incubation was then continued for 48 hours before harvesting of the cells to assess β-galactosidase activity, as described in section 2.1.6.1.

From the graphical representation it may be determined that the maximum β-galactosidase activity was seen in the 293 cells transfected with a 1:4 ratio of plasmid:lipofectin (see figure 17).

Figure 17 β-galactosidase Activity in 293 cells following transfection with pBKCMV *lac*-ve β-gal Varying the Lipofectin:DNA ratio.



Legend to Figure 17:

β-galactosidase activity in 293 cells, transfected with 1μg pBK-CMV *lac-ve* β-galactosidase and varying the amount of lipofectin used so the DNA to lipofectin ratio is as shown. As a negative control the plasmid was used without lipofectin and this is labelled "DNA alone". The results are expressed as a mean, together with the standard deviation.

4.3.1.2 Optimisation of Transfection in 293 Cells Varying the Amount of DNA and Maintaining the Lipofectin:DNA Ratio

Next, the optimal amount of DNA to be transfected into 293 cells, in 6 well plates, needed to be determined at a lipid:DNA ratio of 4:1.

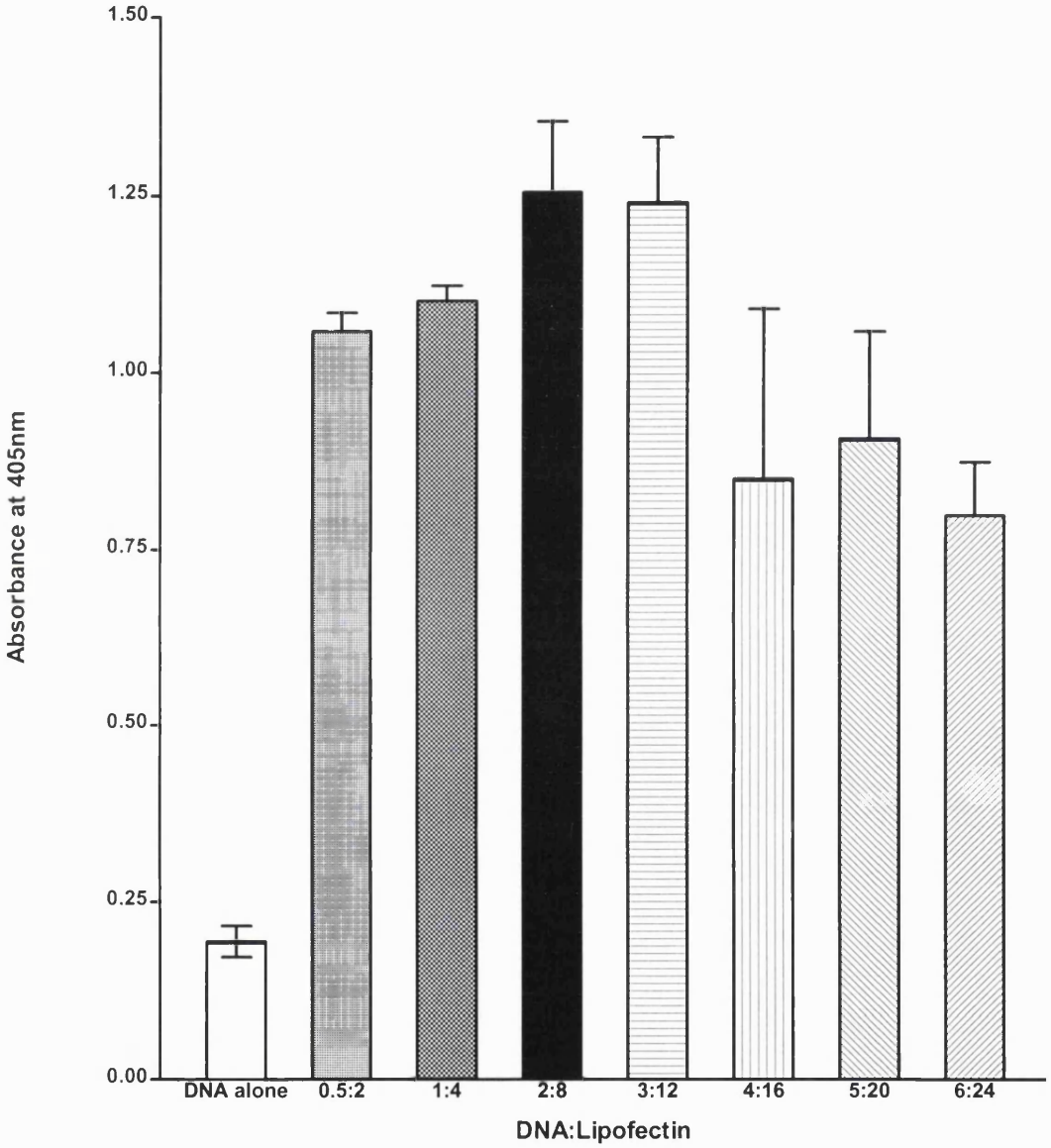
293 cells were seeded into 4 six well plates and cultured at 37°C and 5% CO_2 until approximately 60% confluent. Transfections were performed as described in section 2.1.6, all test samples being performed in triplicate in separate 17 x 120mm conical polystyrene sterile tube (Falcon). As a control 0.5μg of pBK CMV *lac*-ve β-gal was used without the addition of lipofectin. 0.5, 1, 2, 3, 4, 5 and 6μg of pBK CMV *lac*-ve β-gal in 100μl of serum free medium were added to 2, 4, 8, 12, 16, 20 and 24μl lipofectin respectively, also diluted in 100μl of serum free medium. After transfection the cells were cultured for 48 hours at 37°C and 5% CO_2, after which the cells were assayed for β-galactosidase activity as described in section 2.1.6.1.

For transfection of 293 cells in a six well plate, the optimal amount of plasmid DNA was determined to be 2μg with 8μl of lipofectin (see figure 18).

4.3.1.3 Discussion

Transfection of 293 cells with clone 1 pBK-CMV *lac*-ve β-galactosidase revealed that the plasmid was expressing *lacZ* in eukaryotic cells. In addition, by varying the conditions the optimal ratio of DNA:lipofectin was found to be 1:4. When transfecting into 6 well plates, with a surface area of 9.6 cm^2 and the 293 cells at 40-60% confluency, the optimal amount of DNA for transfection was found to be 2μg. This data was only valid for 293 cells and would have to be repeated for each different eukaryotic cell line to be transfected.

Figure 18 Transfection of 293 cells with pBK CMV *lac*-ve β-gal varying the amount of DNA Transfected



Legend to Figure 18:

β-galactosidase activity in 293 cells, transfected with the DNA to lipofectin ratio fixed at 1:4. The amount of plasmid DNA used is varied, together with the appropriate volume of lipofectin. As a negative control the plasmid was used without lipofectin and this is labelled "DNA alone". The results are expressed as a mean of triplicate samples, together with the standard deviation.

4.4 SUBCLONING OF B7-2 INTO pBK-CMV *lac*-ve

4.4.1 Introduction

The ability of pBK-CMV *lac-ve* β-gal to express *lac Z* in 293 cells, demonstrated the ability of the CMV promoter in the vector to express a gene cloned into the multiple cloning site. The next step was to clone the B7-2 cDNA from pUC18 B7-2 into pBK-CMV *lac*-ve to see whether transfection of this construct into 293 cells would result in surface expression of B7-2.

4.4.2 Materials and Methods

pBK-CMV *lac-ve* was digested with the restriction enzymes *Bam*HI (NEB) and *Xho* I (NEB) (see section 2.2.3.1). Simultaneously pUCB7-2 (clone A) was digested with the restriction enzymes *Bam*HI (NEB) and *Sal* I (NEB) (section 2.2.3.1) to release the B7-2 cDNA (Fig 9). The DNA was visualised on a 0.8% agarose gel using a long wavelength UV lamp to minimise damage to the digested DNA. Complete digestion of pBK-CMV *lac-ve* resulted in visualisation of a single fragment of 4314 base pairs (bp), the other expected fragment being only 24bp and beyond the limitations of visualisation on the gel. Complete digestion of pUCB7-2 resulted in two fragments, the larger consisting of 2674 bp and the second of 1180 bp, which contained the cDNA for B7-2. The single visible fragment of pBKCMV *lac*-ve and the smaller fragment from the digested pUCB7-2 were gel purified (see section 2.2.3.2).

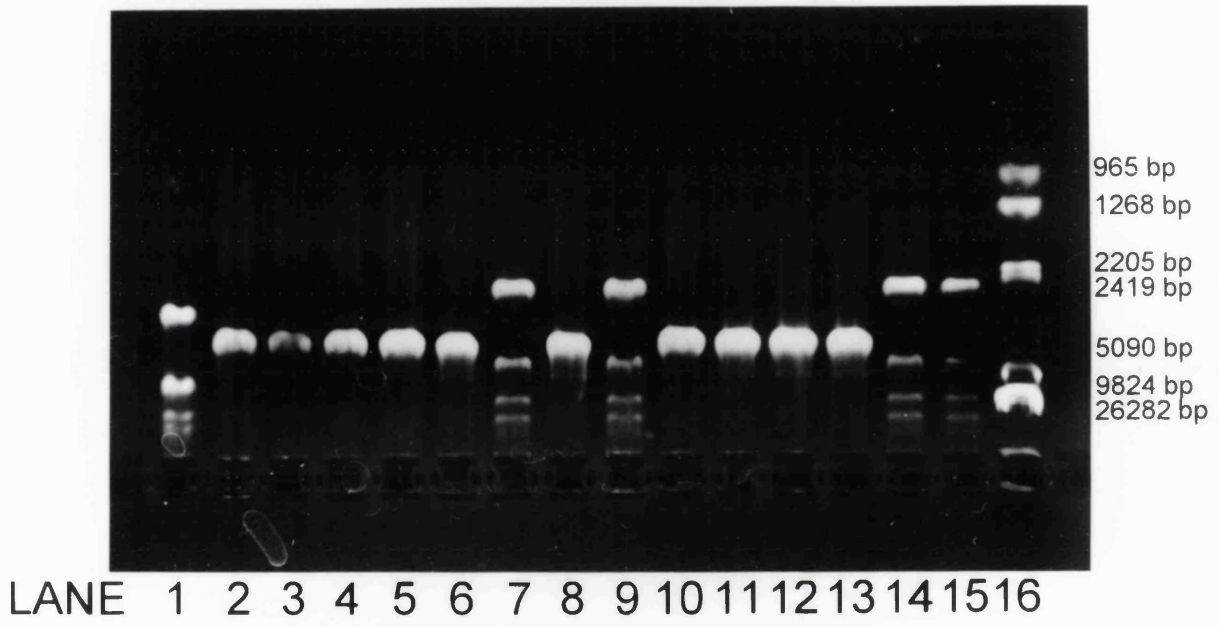
The ligation was performed with an insert:vector ratio of 5:1 as described in section 2.2.3.3. 25μl of each ligation mix were transformed into competent

cells (TG-1) (see sections 2.2.2 and 2.2.2.1) and the cells selected on LB agar containing 50μg/ml kanamycin. 12 kanamycin resistant colonies were picked and expanded overnight in 5 mls of LB medium containing kanamycin (50μg/ml). The plasmid DNA was extracted and digested with *Pst* I(Promega) (see 2.2.3.1).

4.4.3 Results of Subcloning B7-2 into pBK-CMV *lac*-ve

Clones 1,2,3,4,5,7,9,10,11 and 12 were linearised after *Pst* I digestion (see figure 19). pBK-CMV *lac*-ve does not contain a *Pst* I site and the cDNA for B7-2 contains one *Pst* I site (see figure 36). The expected size of pBK-CMV *lac-ve* containing the B7-2 insert is 5494 bp, which is approximately the size of the linearised bands as compared to the size markers. The *Pst* I linearised clones were the expected size, suggesting that clones 1,2,3,4,5,7,9,10,11 and 12 contained the DNA coding for B7-2.

Figure 19 *Pst* I digest of pBK-CMV *lac*-ve B7-2



Legend to Figure 19:
Putative pBK-CMV *lac*-ve B7-2 clones 1-12 digested with *Pst* I (lanes 2-13). Uncut control pBK-CMV *lac*-ve B7-2 clone 1 (lane 1). *Pst* I digest of pBK-CMV *lac*-ve (lane 14). Uncut control pBK-CMV *lac*-ve (lane 15). *Mlu* I digest of λ, giving fragments of DNA of 26282, 9824, 5090, 2419, 2205, 1268, 965 and 458 b.p., the smallest fragment being beyond the resolution of the gel.

4.5 TRANSFECTION OF 293 CELLS WITH pBKCMV *lac*-ve B7-2

4.5.1 Introduction

As demonstrated in section 4.4.3 the cDNA coding for B7-2 was successfully cloned into the expression vector pBK-CMV *lac*-ve. It needed to be determined whether the cDNA for B7-2 could be successfully transcribed, translated and expressed in eukaryotic cells. 293 cells were chosen for the following reasons. Firstly, they are human in origin and express MHC class I molecules, which would make them suitable for use later in assays examining the co-stimulatory ability of the expressed B7-2 to stimulate allogeneic T cells (section 6.1). Secondly, they do not innately express either of the co-stimulatory molecules B7-1 or B7-2 and only express low levels of the adhesion molecules LFA-1 and ICAM-1 which have some co-stimulatory ability. Lastly, they are easy to maintain in culture and are readily transfected using cationic lipids.

4.5.2 Materials and Methods

293 cells were seeded in 6 well tissue culture plates (Nunclon). When the cells had reached 60% confluency they were washed once in serum free medium (OPTIMEM). Once washed, the cells in one of the wells were transfected with 2μg of pBK-CMV *lac*-ve B7-2 clone 1 (section 2.1.6). As a negative control, duplicate wells of 293 cells were transfected with 2μg of pBK-CMV *lac*-ve. The cells were then incubated at 37°C and 5% CO_2 for 5 hours. After this time the mixture of DNA, lipofectin and OPTI-MEM was carefully

aspirated from the wells and replaced with DMEM supplemented with 10% FCS and penicillin (50IU/ml) and streptomycin (50μg/ml). Incubation was continued at 37°C and 5% CO_2 for a further 72 hours.

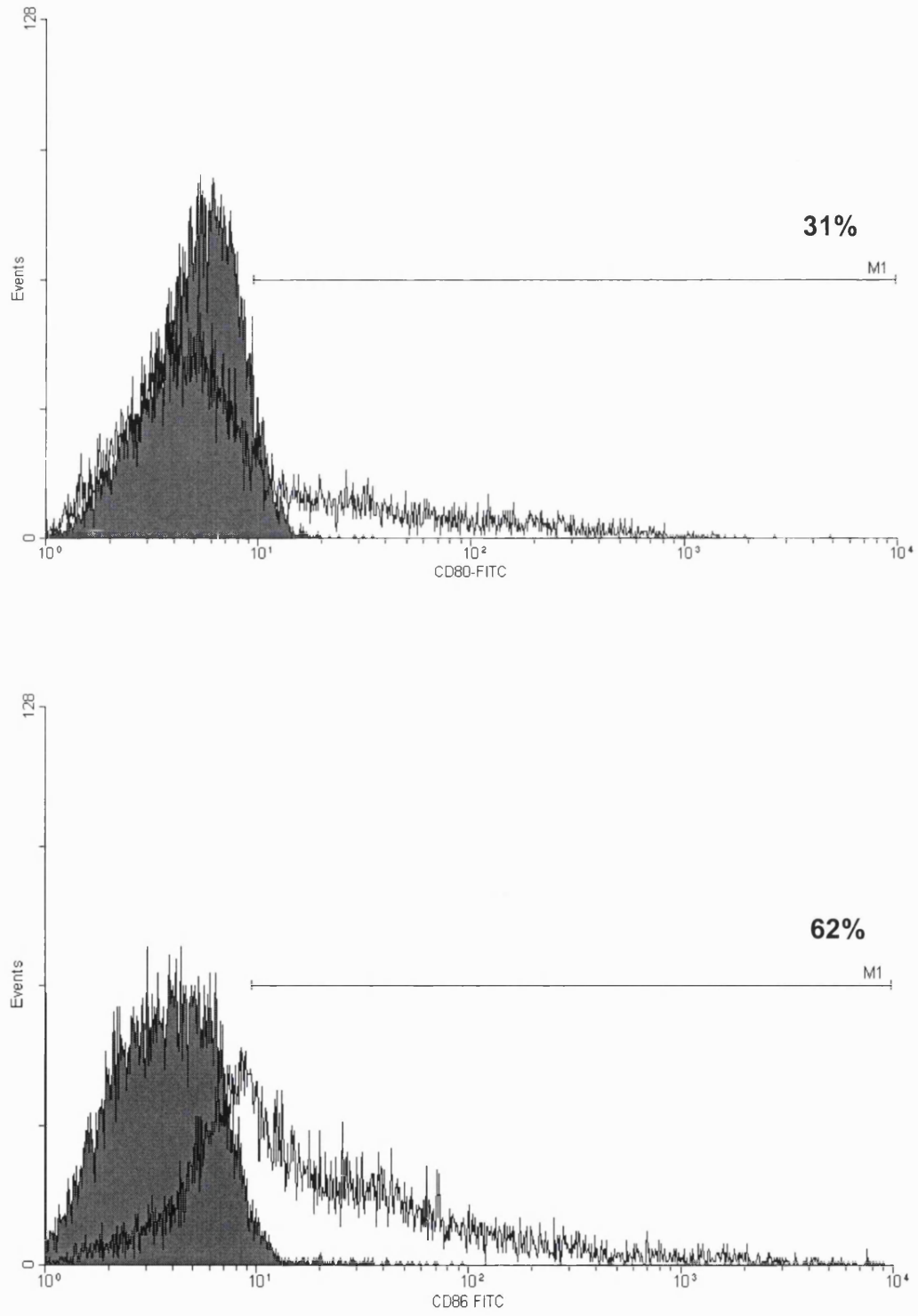
4.5.2.1 Flow Cytometric Analysis of Transfected Cells

Cells were washed with 3 mls of Hanks' Balanced Salt Solution without calcium and magnesium (HBSS) (Gibco BRL) and then incubated with 2mls of trypsin EDTA (GibcoBRL(0.5g Trypsin, 2.0g EDTA in 1 ltr of PBS) at 37°C until the cells had gone into suspension. The cells transfected with either pBK-CMV *lac*-ve or pBK-CMV *lac*-ve B7-2 were washed prior to labelling with monoclonal antibody as described in section 2.1.3. To each tube was added 10μl of anti-B7-2 antibody ((1mg/ml) Pharmingen-clone2331(FUN-1)) conjugated to fluoresceine isothiocyanate (FITC). The cells were incubated with the antibody for 15 minutes at room temperature, washed and analysed by flow cytometry as described in section 2.1.3.

4.5.3 Results of Transfection of 293 cells with pBK-CMV lac-ve B7-2

The 293 cells transfected with pBK-CMV *lac-ve* were found to be negative for expression of B7-2, as compared to unlabelled 293 cells. By comparison 62% of 293 cells transfected with pBKCMV B7-2 clone 1 expressed surface B7-2 after 72 hours, as shown in figure 20.

Figure 20 B7-1 or B7-2 expression on 293 cells 72 hours post transfection with pBK-CMV *lac*-ve B7-1 or pBK-CMV *lac*-ve B7-2



Legend to Figure 20:

Surface expression of B7-1 (upper histogram) or B7-2 (lower histogram) on 293 cells 72 hours post transfection with cationic lipid with the construct pBKCMV lac-ve B7-2 clone 1 (open histogram) or pBK-CMV *lac*-ve B7-1. The cells transfected with pBK-CMV *lac*-ve are shown as the shaded histogram.

4.5.4 Discussion

Transfection of pBK-CMV *lac*-ve B7-2 clone 1 resulted in surface expression of B7-2 on the 293 cells. Comparison of the unlabelled 293 cells and the cells transfected with pBK-CMV *lac*-ve revealed no change in median channel fluorescence, demonstrating absence of non specific binding of the monoclonal antibody to the 293 cells. That B7-2 was expressed on the surface of the transfected cells did not determine whether the expressed protein would be capable of providing a co-stimulatory signal.

4.5.5 Transfection of 293 cells with pBK-CMV *lac*-ve B7-1

The other member of the B7 family of co-stimulatory molecules, excluding B7-3 which has yet to be cloned, is B7-1. To compare the co-stimulatory ability of B7-1 and B7-2, I was supplied with the cDNA for B7-1, which had been sequenced and cloned into pBK-CMV *lac*-ve. To determine whether *lac*-ve B7-1 would result in surface B7-1 expression, this construct was also transfected into 293 cells.

293 cells were transfected with 2μg pBK-CMV *lac*-ve B7-1 as described in section 2.1.6. Cells were harvested 72 hours after transfection and labelled with 10μl of anti-B7-1 mAb conjugated to phycoerythrin (PE) (Becton Dickinson - clone L307.4). As a negative control 293 cells transfected with 2μg pBK-CMV *lac-ve* were either labelled with the anti-B7-1 mAb, or were unlabelled to exclude non specific binding of the antibody to the 293 cells. B7-1 expression was determined by flow cytometric analysis as described in section 2.1.3.

Compared to the negative control cells, 31% of cells transfected with pBK-CMV B7-1 expressed surface B7-1 72 hours post transfection in the experiment presented. This result is shown in figure 20.

4.5.6 Discussion

Expression of B7-1, in the 293 cells transfected with pBK-CMV *lac*-ve B7-1, demonstrated this construct was able to induce surface expression of B7-1 in eukaryotic cells. In this particular experiment, only 31% of cells expressed B7-1 72 hours post transfection, compared to 62% of cells expressing B7-2 after transfection with pBK-CMV *lac*-ve B7-2. Although the same amount of DNA and lipid were used, contaminating endotoxin in the DNA preparation adversely affects transfection efficiency and may explain the decreased expression seen. In addition the confluency of the cells may affect transfection efficiency. As the cells become more confluent, they divide more slowly which decreases transfection efficiency. In other experiments, expression of B7-1 was equivalent to B7-2, demonstrating that the decreased expression was not due to an inherent problem with the plasmid.

Transfection leads only to transient expression of the gene, which peaks at 48 to 72 hours, declining rapidly thereafter. To compare the function of B7-1 and B7-2, I needed cells with stable surface expression of the molecules. The pBK-CMV *lac*-ve plasmid contains a selectable marker, the neomycin resistance gene (figure 13). In a minority of cells transfected with plasmid DNA, the DNA may integrate into the genomic DNA which results in persistent expression of the gene. Culturing the transfected cells in a selectable marker,

allows these stable transfectants to selectively grow, resulting in a population of transfected cells which constantly express the gene.

4.6 GENERATION OF STABLE TRANSFECTANTS

4.6.1 Introduction

Expression of B7-1 and B7-2 on the surface of the transfected 293 cells, as shown by flow cytometric analysis, demonstrated that the expression cassette was functional and the translated protein was expressed as a transmembrane protein. In the case of B7-2, two substitutions were identified in the cDNA and although these did not affect the amino acid structure, the ability of the expressed protein to deliver a costimulatory signal needed to be determined. In addition, to compare the ability of B7-1 and B7-2 in delivering a costimulatory signal and identifying any difference in the generation of cytotoxic T cells (CTL's) with the two molecules, cell lines stably expressing either B7-1 or B7-2 needed to be constructed. Both pBK-CMV *lac*-ve B7-1 and pBK-CMV *lac*-ve B7-2 contain the neomycin phosphotransferase gene. Geneticin (G418 sulphate) is an aminoglycoside related to gentamycin which is toxic to both prokaryotic and eukaryotic cells (Canaani and Berg, 1982). Culturing of the transfected cells in medium containing geneticin results in death of cells not expressing the neomycin phosphotransferase gene, while positively selecting those cells which contain an integrated copy of the plasmid.

Quantitative differences in gene expression occur in stable transfectants, depending on the number of copies of the plasmid in each cell. This gives a variable level of expression of the protein when looking at a population of cells. To compare the costimulatory function of B7-1 and B7-2, it was necessary to generate stable transfectants that expressed either B7-1 or B7-2 at similar

levels, with each cell having similar surface expression of the protein. To do this stable transfectants were sorted using a cell sorter, with the cells being selected on the basis of surface expression of B7-1 or B7-2.

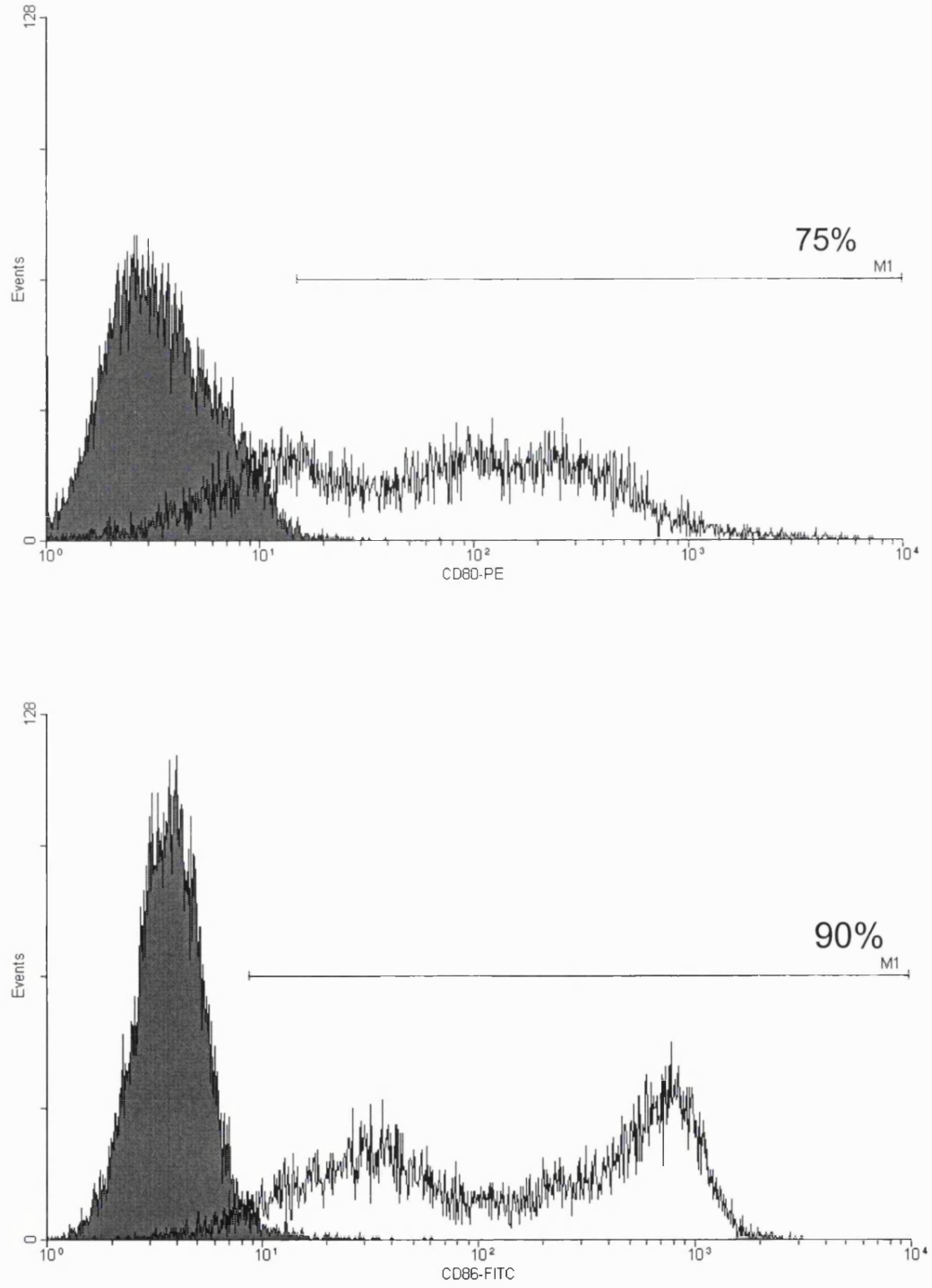
4.6.2 Materials and Methods

293 cells were cultured in a 96mm sterile petri dish (Nunc), in DMEM supplemented with 10% FCS, penicillin and streptomycin as described in 2.3.1 until they had reached 60% confluency. 13.8μg of either pBK-CMV B7-1 or pBK-CMV B7-2 were transfected into 293 cells as described in 2.1.6, using 55μl of lipofectin. This keeps to the optimised conditions determined for lipofection of 293 cells. The cells were then cultured in G418 as described in section 2.1.6.2. After 10 days selection the cells were analysed for expression of B7-1 or B7-2 by flow cytometric analysis as described in section 2.1.3. Negative controls consisted of 293 cells transfected with pBK-CMV *lac*-ve, selected for G418 resistance and labelled with the same amount of the appropriate mAb as the test cells.

4.6.3 B7-1 and B7-2 Expression on Stable Transfectants

75% of the pBK-CMV *lac*-ve B7-1 transfectants expressed B7-1 (see figure 21) and 90% of the 293 cells transfected with pBK-CMV *lac*-ve B7-2 expressed B7-2 (see figure 21). The median channel fluorescence of the B7-1 transfectants was 466 (sd 183) and median channel fluorescence of the control 293 cells was 132 (sd 64). The median channel fluorescence of the B7-2 transfectants 569 (sd 182) and median channel fluorescence of the control 293 cells was 147 (sd 42).

Figure 21 B7-1 or B7-2 expression on 293 cells after selection in G418



Legend to Figure 21:
B7-1 (upper histogram) or B7-2 (lower histogram) expression on 293 cells transfected with pBK-CMV *lac*-ve B7-1 or pBK-CMV *lac*-ve B7-2 and selected in G418 (open histogram). Negative control cells, transfected with pBK-CMV *lac*-ve are presented as the shaded histogram.

4.6.4 Discussion

Selection of transfected 293 cells in G418 resulted in generation of stably transfected 293 cells expressing either B7-1 or B7-2. However, there was a great variation in the intensity of expression of both B7-1 and B7-2 in the transfected cell population, there being both high expressing and low expressing subpopulations. Level of expression of B7-1 or B7-2 on the 293 cells may affect the ability of the cells to deliver a co-stimulatory signal and cells innately expressing the molecules tend to have a high level of expression of the molecules (Engel et al., 1994; Azuma et al., 1993b). Various methods exist to produce cell populations expressing uniform amounts of cell surface proteins. The first of these involves the cloning of individual colonies of cells, after selection in G418, using cloning cylinders. The individual colonies can then be screened by flow cytometric analysis, to select those which are high expressors. An alternative method is the use of a cell sorter. This enables specific populations of cells to be selected, allowing discrimination between the low and high expressing cells.

4.6.5 Sorting of B7-1+ve and B7-2+ve 293 Cells into Uniformly Expressing Populations

Generation of a uniformly expressing B7-1 or B7-2 expressing population of cells was achieved with a FACS Vantage cell sorter, as described in section 2.1.6.3. The cells were sorted into two populations, one with high surface expression of B7-1 or B7-2 and the other with low or negative expression of B7-1 or B7-2. The highly expressing cells were cultured until enough cells had expanded to be analysed. 0.5 x 10^6 cells from each of the two samples were

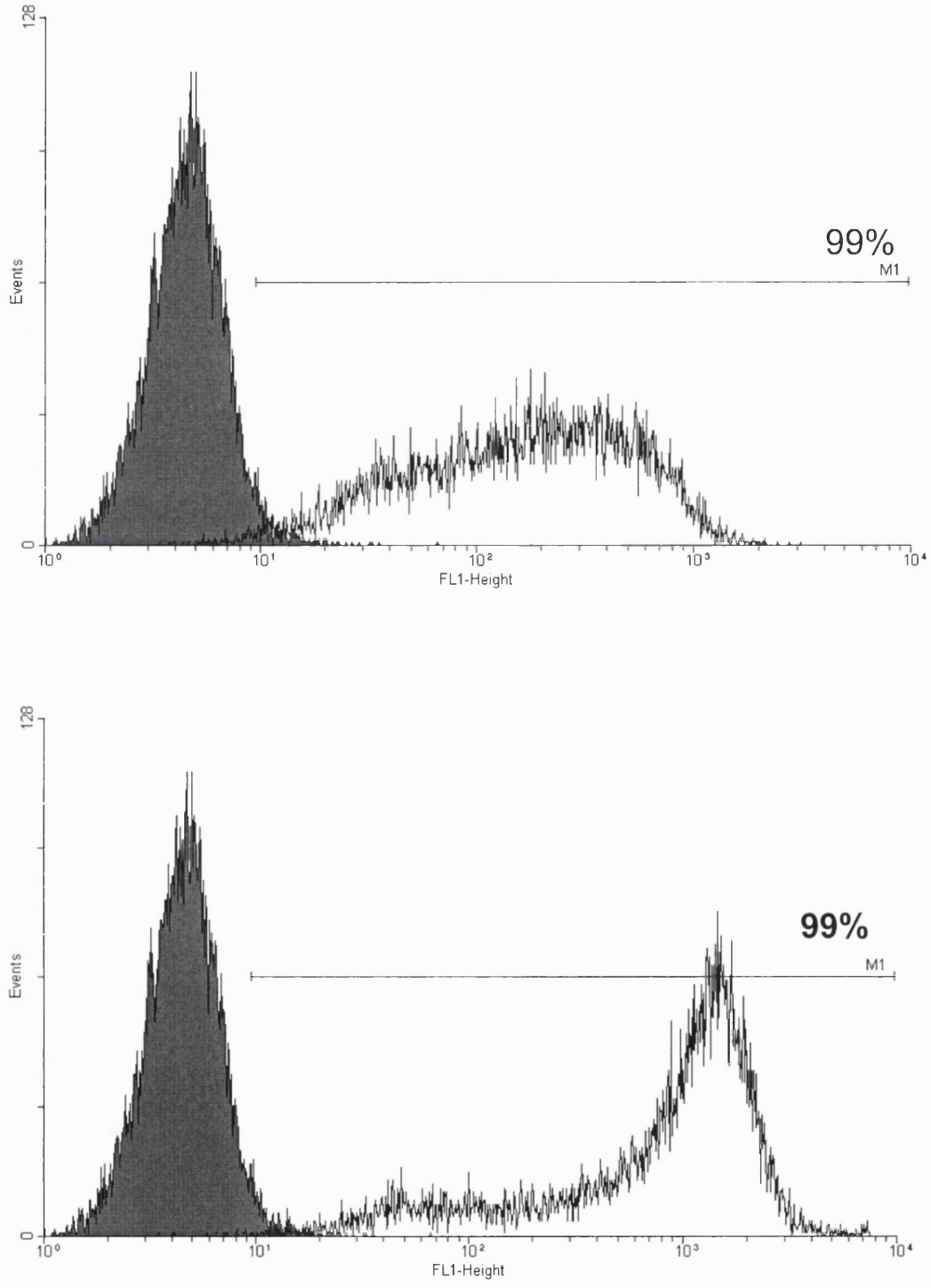
harvested and labelled with either 10μl anti-B7-1 PE (Becton Dickinson - clone L307.4) or 10μl anti-B7-2 FITC (Pharmingen - clone FUN-1) accordingly and analysed by flow cytometry as described in section 2.1.3. 293 cells stably transfected with pBK-CMV *lac*-ve and labelled with the appropriate mAb were again used as negative control cells.

4.6.6 Expression of B7-1 and B7-2 on Sorted 293 Stable Transfectants

293 stable transfectants, sorted on the basis of high expression of surface B7-1 or B7-2, were reanalysed by flow cytometric analysis, after the sorted cells had been expanded. Compared to negative control 293 cells transfected with pBK-CMV *lac-ve*, 99% of sorted 293 cells were positive for B7-1 expression (Figure 22). The median channel fluorescence, which correlates to the amount of antibody bound to each cell, was 573 (sd 131) in the B7-1 sorted transfectants, compared to 169 (sd 45) in the negative control cells. The median channel forward scatter of the 293 cells was 764 (sd 80) and dividing the median channel fluorescence by the median channel forward scatter gave an FFR of 0.75.

99% of sorted B7-2 expressing cells were positive, compared to the negative control cells (Figure 22). The median channel fluorescence of the sorted B7-2 expressing 293 cells was 776 (sd 139), compared to the median channel fluorescence of the negative control cells which was 165 (sd 48). Median channel forward scatter of the 293 cells was 796 (sd 78), so the FFR of the sorted B7-2 expressing cells was 0.97.

Figure 22 B7-1 or B7-2 expression on sorted 293 cells



Legend to Figure 22:
B7-1 (upper histogram) or B7-2 (lower histogram) expression on 293 cells transfected with pBK-CMV *lac*-ve B7-1 or pBK-CMV *lac*-ve B7-2 (open histogram), in contrast to control 293 cells transfected with pBK-CMV *lac*-ve (shaded histogram).

4.6.7 Discussion

Sorting of the B7-1 and B7-2 expressing 293 cells resulted in the generation of a more uniform expressing population of cells. Comparison of figures 21 and 22 (upper histograms) demonstrated that the higher B7-1 expressing population had been selected. This was reflected in the median channel fluorescence which was 466 (sd 183) in the cells pre sorting and 573 (sd 131)in the cells post sorting. The FFR in the B7-1 sorted 293 cells was 0.75, which is at a level intermediate between that seen on T cells and monocytes. As with B7-1, the population of B7-2 expressing cells was more uniform post sorting, which can be seen by comparing figures 21 and 22 (lower histograms). The median channel fluorescence of the B7-2 expressing 293 cells was 569 (sd 182) before sorting and 776 (sd 139) after sorting. The FFR of the B7-2 expressing 293 cells post sorting was 0.97, which is at level approaching that seen on monocytes. Whether the B7-1 and B7-2 expressing 293 cells were capable of delivering a co-stimulatory signal needed to be determined. As well as expressing B7-1 or B7-2, the stable transfectants also contained at least one copy of the plasmid pBK-CMV *lac*-ve, which could act as a possible immunogenic target in any functional assays. As a negative control, in the phenotyping and also in any functional assays 293 cells, stably transfected with the plasmid pBK-CMV *lac-ve* were used. These cells were resistant to G418 and thus presumably carried a copy of the plasmid.

4.6.8. Determination of Whether Stable Transfectants with pBK-CMV *lac*-ve Contain a Copy of the Plasmid

To determine that stable transfectants with pBK-CMV *lac-ve* contained a copy of the plasmid, genomic DNA was recovered from these cells using the Nuclitips^{TM} DNA extraction system (Amersham Life Science) as described in section 2.2.4. The CMV promoter was used as a marker for presence of the plasmid. PCR amplification of this marker from recovered genomic DNA was attempted using the protocol described in section 2.2.5. The annealing temperature used was 51^{o}C and 30 cycles of amplification were performed. The forward primer was 5'-ATTACGGGGTCATTAGTTCA-3' and the reverse primer was 5'-AATGGGGCGGAGTTGTTACG-3'. As a negative control, genomic DNA was extracted from untransfected 293 cells and was subjected to PCR amplification using the identical conditions. The PCR positive control sample was the pBK-CMV *lac*-ve plasmid. Sterile H_2O was the PCR -ve control. 5μl of each PCR product was analysed on a 2% agarose gel. A PCR product of 497bp was expected.

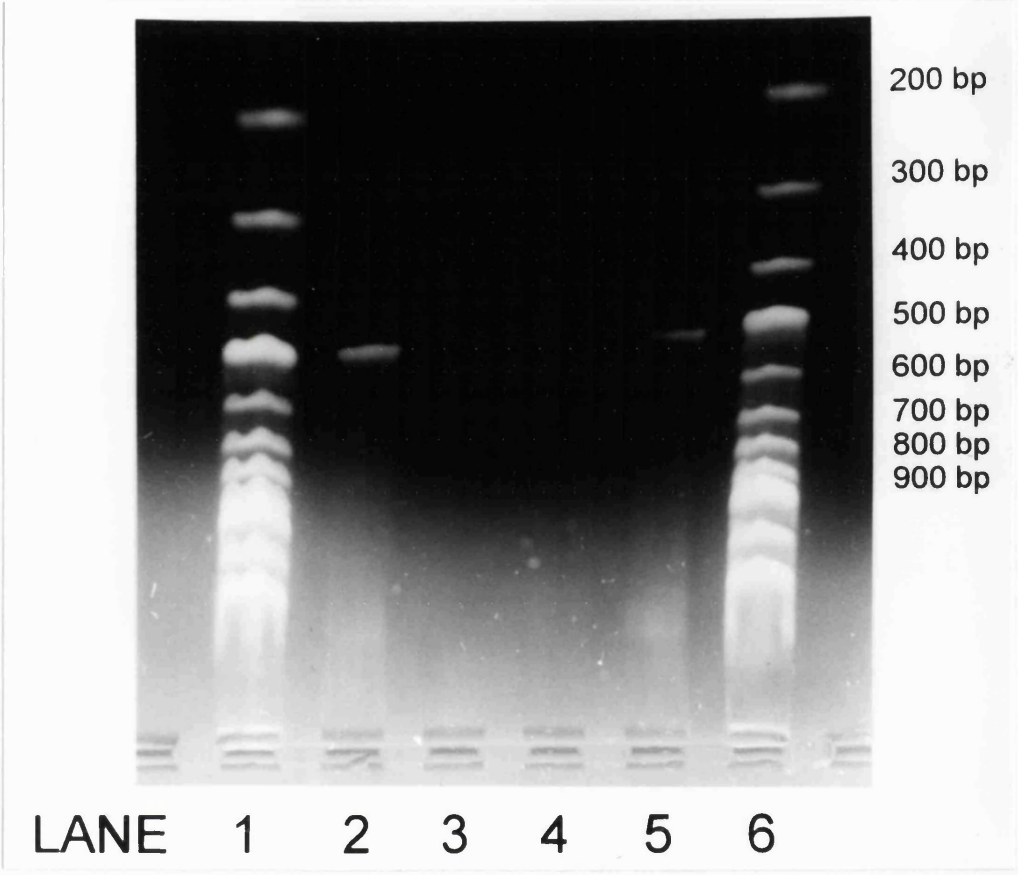
4.6.8.1 Results

PCR of the extracted genomic DNA from the 293 cells stably transfected with pBK-CMV *lac-ve*, using CMV primers produced a PCR product of approximately 497bp as expected. The +ve and -ve controls all produced the expected products (Figure 23).

4.6.8.2 Discussion

PCR of the genomic DNA extracted from the 293 cells stably transfected with pBK-CMV *lac-ve*, demonstrated that at least some of the cells contained a copy of the CMV promoter. Together with the resistance of these cells to G418 it can be inferred that these cells contained at least one stably integrated copy of the plasmid and would provide a suitable control for the B7-1 or B7-2 expressing 293 cells in functional assays.

Figure 23 PCR of genomic DNA from pBK-CMV *lac-ve* 293 transfectants



Legend to Figure 23:
Lanes 1 and six contain the 100bp DNA ladders. Lane 2 contains the PCR product of the genomic DNA from the 293 cells stably transfected with pBK-CMV *lac*-ve. Lane 3 contains the PCR product from the genomic DNA from untransfected 293 cells. Lane 4 contains the PCR product from the H_2O control and lane 5 contains the PCR product from the plasmid pBK-CMV *lac-ve* (positive control).

5.1 COSTIMULATORY ABILITY OF 293 STABLE TRANSFECTANTS

5.1.1 Introduction

Engagement of the T-cell receptor complex (TCR) by antigen presented by MHC molecules (signal 1) is not sufficient to induce T cell activation (Schwartz, 1990). A second costimulatory signal, is required to induce T cell proliferation and IL-2 production. One of the main costimulatory signals is provided by ligation of CD28 on the T cell, by its ligands B7-1 (Gimmi et al., 1991) or B7-2 (Freeman et al., 1993b). Phorbal 12-myristate 13-acetate is a phorbal ester which is a potent activator of T cells, which acts by binding and subsequent activation of protein kinase C (PKC) (Kraft and Anderson, 1983). PKC inhibitors block T cell activation and PKC activators induce expression of the IL-2 receptor and production of IL-2 (Isakov and Altman, 1987), suggesting the role of PKC in T cell activation. Activation of PKC also results in phosphorylation of the lymphocyte surface molecules CD3, CD4, CD8 and lymphocyte function-associated antigen-1 (LFA-1) (Chatila and Geha, 1988). Suboptimal activation of T cells with PMA followed by crosslinking of CD28 with anti-CD28 mAb results in enhanced T cell proliferation and IL-2 production (June et al., 1987) and this assay has been used to demonstrate the costimulatory action of ligation of B7-1 (Gimmi et al., 1991) or B7-2 (Freeman et al., 1993b) with CD28 on the T cell.

As described in section 3.1.3.1 expression of B7-1 and B7-2 was demonstrated on several mononuclear cells, including resting monocytes and activated B cells, but not on resting T cells (Lenschow et al., 1996). Addition of

accessory cells expressing a costimulatory molecule is able to provide a costimulatory signal, resulting in T cell proliferation in the responding lymphocytes (Reiser et al., 1992). Therefore, in the costimulation assays using the B7-1 or B7-2 expressing 293 cells, enriched T cell populations were used to decrease third party costimulation, which might mask costimulation provided by the 293 transfectants.

5.1.2 Materials and Methods

5.1.2.1 Determination of Dose of Irradiation to Inhibit Growth of 293 Cells

The transfected 293 cells were to be used as stimulators in a proliferation assay. Uptake of [^{3}H]-thymidine was used as a marker of proliferation of responding T cells. To keep the number of stimulator cells constant in the assays and minimise uptake of [^{3}H]-thymidine, the 293 cells were subjected to a dose of irradiation sufficient to arrest cell division. To determine the optimal dose of radiation, 293 cells were harvested, washed in HBSS twice and resuspended in RPMI 1640 with Glutamax-I supplemented with penicillin (50IU/ml), streptomycin (50μg/ml) and 10% human AB serum at a concentration of $2x10^5$ cells/ml. The 293 cells were then split into 7 equal fractions. Each individual fraction was then irradiated with 0, 25, 50, 75, 100, 125 and 150 Grays (Gy) respectively in a cell irradiator (Nordion / Gammacell 3000 Elan). $2x10^4$ cells from each of the 7 fractions was added to a flat bottomed well of a 96 well plate (Nunclon) in triplicate and cultured for 72 hours at 37°C in 5% CO_2. For the last 18 hours of the culture 1 μCi (37 kBq) of [methyl-^{3}H]-thymidine (Amersham) was added to each well in the assay and proliferation was assessed as described in section 2.1.4.

5.1.2.2 Determination of Submitogenic Dose of PMA

PBMC were obtained from healthy human volunteers and separated from the peripheral blood as described in section 2.1.2. The mononuclear cell population was then depleted of B lymphocytes, natural killer cells and monocytes to enrich the T cell population as described in section 2.1.2.1. After enrichment the T cells were resuspended in RPMI 1640 with Glutamax-I supplemented with 10% human AB serum, penicillin (50IU/ml) and streptomycin (50μg/ml) at a concentration of $1x10^6$ cells/ml. The T cells were split into 7 aliquots and PMA was added to each aliquot at a final concentration of 0, 2.5, 5.0, 7.5, 10 and 12.5 ng/ml. 100μl from each aliquot was added to a flat bottomed 96 well plate (Nunc- Life Technologies) in replicates of 5. The cells were cultured for 72 hours at 37°C and 5% CO_2 and were pulsed with 1 μCi (37 kBq) of [methyl-^{3}H]-thymidine (Amersham) for the last 18 hours of the assay. Proliferation was assessed by incorporation of [methyl-^{3}H]-thymidine, as described in section 2.1.4.

5.1.2.3 Costimulation Assay

Costimulation assays were set up as described in section 2.1.7, using control 293 cells, B7-1 or B7-2 expressing 293 cells as stimulator cells after irradiation with 125 Grays. Peripheral blood mononuclear cells were obtained from the blood of 4 normal volunteers as described in section 2.1.2 and enriched for T cells as described in section 2.1.2.1. An aliquot of T cells was assessed for T cell purity prior to setting up the assay by labelling with anti-CD3 PerCP (peridinin chlorophyll protein) (Becton Dickinson - clone SK7) and analysing by flow cytometry (see 2.1.3). Six replicates were performed for each

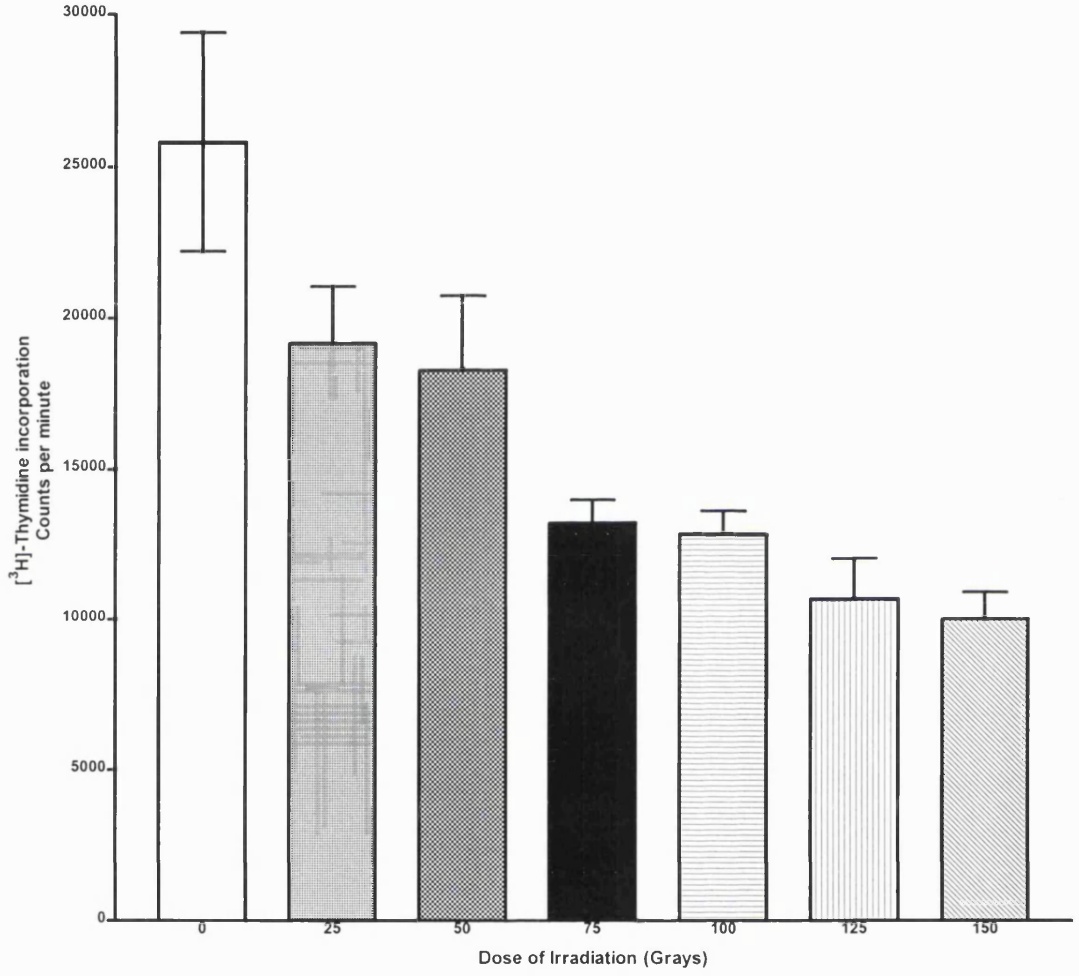
variable in the assay. The results presented were from 8 different experiments, using a total of 4 different donors for the T cells.

5.1.3 Results

5.1.3.1 Dose of Irradiation to Inhibit 293 cells Proliferating

293 cells were resuspended at $2x10^5$ cells/ml in supplemented DMEM and split into seven aliquots. The cells were then irradiated, receiving between 0 and 150 Grays and then cultured in a flat bottomed 96 well plate. After 48 hours culture cell proliferation was assessed, by [^{3}H]-thymidine incorporation over 18 hours, which was measured as a mean of counts per minute (cpm) after a counting period of 150 seconds. A sequential decrease in cell proliferation was seen with increasing doses of radiation up to 75 Grays, after which there was a plateau in the inhibition up to the maximal dose of radiation 150 Grays (see Figure 24). Even with the maximal dose of irradiation there was still a higher uptake of [^{3}H]-thymidine as compared to unstimulated T cells (see section 3.1.5.1). This presumably reflects non specific uptake of [^{3}H]-thymidine by the cells, as on microscopic evaluation all the cells were non adherent and no viable cells were seen with prolonged culture up to 7 days. In the costimulation assays 293 cells received 125 Grays.

Figure 24 Cell proliferation vs increased dose of irradiation with 293 cells



Legend to Figure 24:

Proliferation as assessed by [^{3}H]-thymidine incorporation by 293 cells after exposure to increasing doses of γ-irradiation. After 75 Grays exposure there was a plateau in inhibition of proliferation.

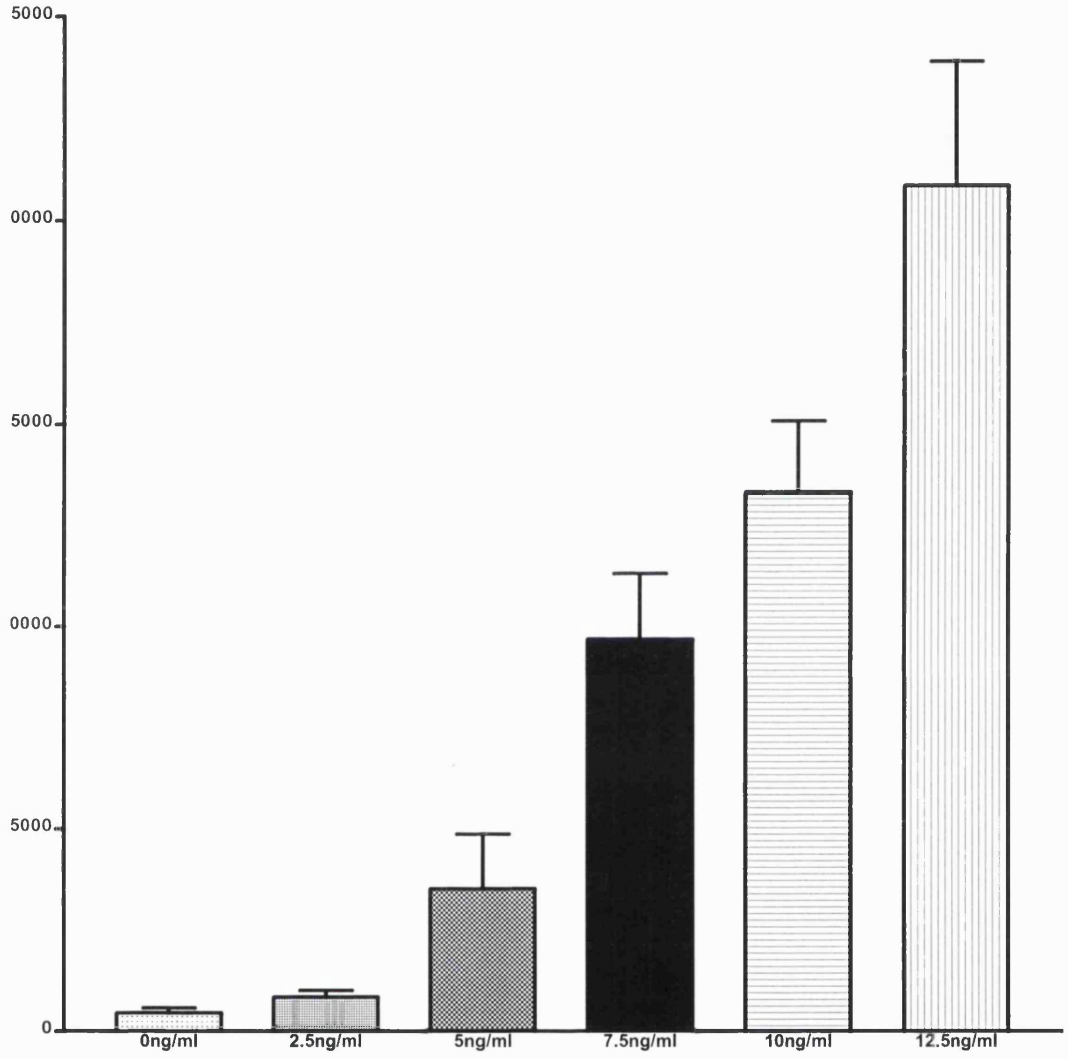
5.1.3.2 Determination of Submitogenic dose of PMA

Mean proliferation of T cells cultured in the absence of PMA was 446 c.p.m. (sd 125.4). Mean proliferation of T cells cultured in 2.5ng/ml PMA was 842 c.p.m. (sd 165). Mean proliferation of T cells cultured in 5ng/ml PMA was 3513.7 c.p.m. (sd 1353). Mean proliferation of T cells cultured in 7.5 ng/ml PMA was 9685 c.p.m. (sd 1635). Mean proliferation of T cells cultured in 10 ng/ml PMA was 13330 c.p.m. (sd 1744) and mean proliferation of T cells cultured in 12.5 ng/ml PMA was 20846 c.p.m. (sd 3044). These results are summarised in figure 25. In the costimulation assays, the dose of PMA the T cells were exposed to was 2.5ng/ml.

5.1.3.3 T cell Purity After Enrichment by Negative Depletion

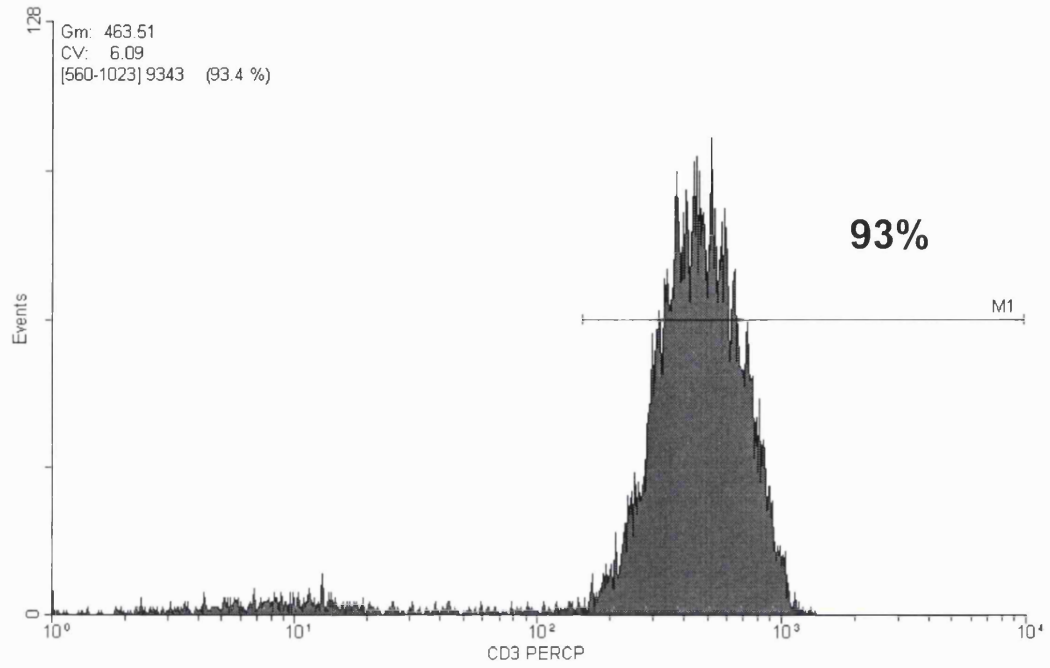
As described in section 5.1.2.3 an aliquot of the enriched T cells was labelled with anti-CD3 conjugated to PerCP, prior to using the cells in functional assays. As a negative control, unlabelled T cells were used from the same preparation. The data was acquired and analysed on a FACScan using Lysis II software. Compared to the negative control cells greater than 85% bound anti-CD3 mAb in all preparations used in the functional assays (see figure 26).

Figure 25 Proliferation of T cells with varying doses of PMA



Legend to Figure 25:
Proliferation of T cells as assessed by [^{3}H]-thymidine incorporation (counts per minute (c.p.m.)) cultured in the presence of varying doses of PMA.

Figure 26 Flow histogram of CD3 expression on sorted T cells

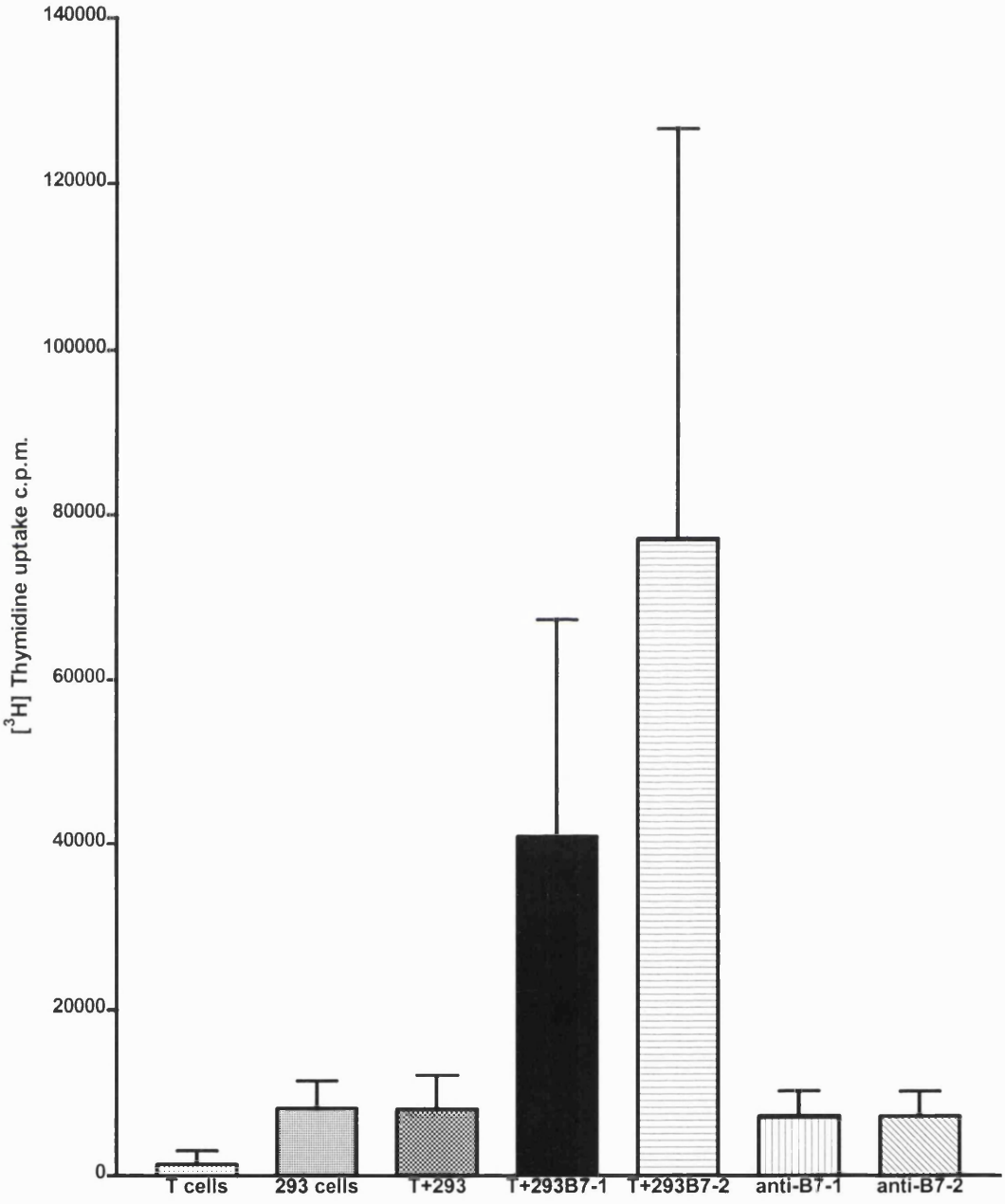


Legend to Figure 26:
Flow cytometric analysis of a typical T cell preparation after depletion of monocytes by plastic adherence and depletion of monocytes, B cells and NK cells by MACS depletion. All cells are analysed, as gating around the lymphocytes would exclude the residual monocyte population. The T cells were labelled with CD3 PerCP and analysed in Fl 3. In this preparation T cell purity was 93%.

5.1.3.4 Co-stimulatory Assays with 293 Transfectants

Results of the co-stimulatory assays are expressed as a mean of eight experiments, using four different normal donors as a source of T cells. Each experiment had six replicates of each variable. Mean proliferation of the T cells alone was 1361 cpm (sd 1673) and of the 293 cells alone after irradiation with 125 Gray was 8089 cpm (sd 3355). Proliferation of T cells with 293 pBK-CMV *lac*-ve stable transfectants as stimulator cells was 8028 cpm (sd 4120). T cell proliferation with the B7-1 293 cells as stimulators was 40906 cpm (sd 26393) and with the B7-2 293 cells as stimulators 77092 cpm (sd 49466). The increase in T cell proliferation induced with the B7-1 and B7-2 expressing 293 cells was statistically significant ($p<0.0001$ with both), as compared to the proliferation induced by the 293 cells stably transfected with pBK-CMV *lac*-ve. Preincubation of 293 B7-1 cells with anti-B7-1 mAb resulted in return of proliferation to baseline levels of 7184 cpm (sd 3034). Similarly preincubation of 293 B7-2 cells with anti-B7-2 mAb inhibited proliferation with a mean of 7169 cpm (sd 3004). These results are summarised in figure 27.

Figure 27 Graph of T cell proliferation with 293 transfectants.



Legend to Figure 27:

Proliferation of T cells as assessed by incorporation of [^{3}H]-thymidine and measured as counts per minute (c.p.m.). B7-1 and B7-2 expressing 293 cells costimulated T cell proliferation, compared to control 293 cells. The T cell proliferation was inhibited by preincubation of the B7-1 or B7-2 expressing 293 cells with anti-B7-1 or anti-B7-2 mAbs respectively.

5.1.4 Discussion

Proliferation was significantly increased in the T cells stimulated with the B7-1 or B7-2 expressing 293 cells ($p<0.0001$ with both), as opposed to the T cells stimulated with the control 293 cells. T cell proliferation was also significantly higher with the B7-2 expressing 293 cells as compared to the B7-1 expressing 293 cells ($p=0.001$), which was a feature seen consistently throughout all the experiments. The reason for this is not determined, but this is not a feature seen with published data using B7-1 or B7-2 transfected cells as stimulators with a submitogenic stimulus through the T cell receptor (Gimmi et al., 1991; Freeman et al., 1993b). However the intensity of expression of B7-2 was higher than that seen with the B7-1 transfectants in the above experiments. The median channel fluorescence of the B7-2 transfectants was 776 (FFR 0.97) compared to the median channel fluorescence of the B7-1 transfectants which was 573 (FFR 0.75). It has been suggested that the level of costimulatory signal may affect the subsequent T cell response. Indeed upregulation of B7-1 and B7-2 on APC's after activation is associated with increased costimulation and T cell proliferation (Bluestone, 1995). Thus it would seem likely that the difference in T cell proliferation was due to the stronger costimulatory signal provided by the B7-2 transfectants, due to the higher level of expression of the molecule on these cells as compared with B7-1. The return of T cell proliferation to levels seen with the control 293 cells, with either blocking monoclonal antibodies to B7-1 or B7-2, demonstrates that the proliferation was due to the specific expression of B7-1 or B7-2 respectively on the 293 cells.

Other groups have used immobilised anti-CD3 mAb as a submitogenic stimulus to T cells in costimulation assays (Harding et al., 1992; Williams and Unanue, 1990; Engel et al., 1994; Gimmi et al., 1991), as this represents a more physiological signal through the T cell receptor as compared to PMA, which directly activates protein kinase C. This was not used in my series of experiments, as I was solely looking at the functional ability of the expressed proteins on the stable transfectants. Both the B7-1 and B7-2 had been cloned from B cell lines by reverse transcriptase PCR and this could have resulted in mutations in the cloned cDNA as discussed in section 4.1.1. Confirmation of the function of B7-1 and B7-2 was required prior to comparison of their co-stimulatory function and construction of the viral vectors containing the B7-1 or B7-2 cDNAs. Another end point of costimulation, particularly with signalling through CD28, is production of IL-2 by responding T cells (Damle et al., 1992). In these series of experiments the supernatants from the costimulation assays were not saved for further analysis. If I were to repeat these experiments, I would look at IL-2 production by the costimulated T cells, as this is an important cytokine in expanding a T cell response and maintaining it over a longer time period, which is an important factor in tumour vaccination strategies (Deeths and Mescher, 1997). However, proliferation as the end point of costimulation, demonstrated the ability of the transfectants to deliver a co-stimulatory signal to the T cells, which was inhibited by blocking with the appropriate mAb.

6.1 CYTOTOXICITY ASSAYS WITH STABLE TRANSFECTANTS

6.1.1 Introduction

In murine models expression of the co-stimulatory molecules B7-1 or B7-2 on tumourigenic cell lines, have been shown to be effective in generating an effective T cell mediated immune response, resulting in eradication of the tumour (Chen et al., 1992; Hodge et al., 1994). In *in vitro* assays, expression of B7-1 or B7-2 on cell lines was effective at generating cytotoxic T lymphocytes (CTL's) (Lanier et al., 1995). Despite the fact that B7-1 and B7-2 have been demonstrated to be effective in generating CTLs, there are several lines of evidence that the two molecules have differing functions. First, they exhibit different temporal expression. B7-2 is expressed innately on resting B cells and upregulated within 6 hours of activation. In contrast B7-1 is absent on unstimulated B cells and upregulated approximately 24 hours after activation with LPS (Hathcock et al., 1994). Second, using blocking antibodies to either B7-1 or B7-2 in a murine model of experimental allergic encephalitis, demonstrates different effects on disease progression. Blocking with anti-B7-1 resulted in increased levels of IL-4, a Th2 cytokine, which was associated with less severe disease progression. In comparison, anti-B7-2 mAb increased the severity of disease and T cells isolated from these mice produced Th1 cytokines (Kuchroo et al., 1995). Finally, repetitive stimulation of naive T cells with transfectants expressing either B7-1 or B7-2 resulted in decreased production of IL-4 from the responding T cells when the B7-1 expressing cells

were the stimulators. When the B7-2 transfectants were stimulating the T cells, increased levels of IL-4 were produced (Freeman et al., 1995).

In tumour vaccination strategies there is evidence that B7-1 or B7-2 expression on the tumour cells is not equally effective in the generation of an anti-tumour immune response. In a murine model of AML, vaccination with AML blasts expressing B7-2 was less effective than B7-1 in eradicating pre-existing disease (Matulonis et al., 1996). If B7-1 expression results in production of Th1 cytokines in responding T cells, then the resulting cytotoxic T cell response may be more effective in eradicating residual disease. T_H1 T cells producing γ-IFN production would activate natural killer (NK) cells, which have been demonstrated to be capable of killing leukaemic cells (Hercend et al., 1986). If B7-2 expression on leukaemic cells induced a Th2 immune response, it would be expected that this would be less effective at removing residual leukaemic cells, which may increase the chances of relapse. However, a Th2 immune response may clear residual leukaemic cells, by antibody dependant cell mediated cytotoxicity (ADCC). As discussed in section 3.1.3.5 the duration of CR1 appeared prolonged in those patients with a higher percentage of AML blasts expressing B7-2. This beneficial effect may have been due to induction of an anti-leukaemic immune response. With this in mind I used the stable transfectants expressing either B7-1 or B7-2, produced as described in section 4.6.5, to stimulate allogeneic T cells and tested these for cytotoxic activity against the parental 293 cells, to see whether the two transfectants were equally effective at generating CTL's. I also collected the supernatants from the stimulation phase of the assay to see whether differential production of Th1 or Th2 cytokines was seen with the two different co-

stimulatory molecules. I also analysed production of γ-IFN and IL-4 from the T cells by flow cytometric analysis, to see whether either B7-1 or B7-2 costimulation would increase the percentage of T cells with either a Th1 or Th2 cytokine profile, as compared to T cells stimulated with the control 293 cells.

6.1.2 Cytotoxicity Assays with PBMC Responders

Initial experiments were performed with PBMC obtained from the peripheral blood of healthy volunteers as described in section 2.1.2. Cytotoxicity assays were then set up as described in section 2.1.8. Two different T cell donors were used in the experiments. All of the effector phase of the assay was performed in at least replicates of 6, with an effector:target ratio of 10:1. Results are expressed as the mean percentage cytotoxicity, from the cumulative results from 4 experiments.

6.1.2.1 Results and Discussion

Percentage specific lysis was calculated using the following formula, the spontaneous release of ^{51}Cr from the labelled target cells always being less than 15% of the total release from the target cells.

$$\% \text{ specific lysis} = \frac{\text{Experimental release(cpm) - Spontaneous release(cpm)}}{\text{Total release (cpm) - Spontaneous release (cpm)}}$$

The mean specific lysis of unstimulated T cells was 1.6%, with a standard deviation (sd) of 0.9. Mean specific lysis of target cells by T cells primed with 293 cells stably transfected with *lac-ve* was 31.3%, with a standard deviation of 10.9. Mean specific lysis with the B7-1 293 cells as stimulator cells was 35.7% with a standard deviation of 9.4 and mean specific lysis with the B7-2 expressing 293 cells as stimulator cells was 27.9% with a standard deviation of

7.6. Using Student's paired T test, there was no significant difference in cytotoxicity against the parental 293 cells depending on whether stimulated with control 293 cells, B7-1 or B7-2 expressing 293 cells.

PBMC preparations contain monocytes and B cells which innately express both B7-1 and B7-2. It has been demonstrated that third party cells expressing B7-1 or B7-2 may provide a co-stimulatory signal to T cells which are responding to an antigen specific signal from a second population of cells (Reiser et al., 1992). This observation could then account for there being no difference in the generation of CTLs between the control 293 cells and the B7-1 or B7-2 expressing 293 cells. With this in mind further cytotoxicity assays were performed using an enriched T cell population, depleted of cells capable of third party costimulation.

6.1.3 Cytotoxicity Assays with T Cell Responders

PBMC were obtained from the peripheral blood of healthy volunteers as described in section 2.1.2 and depleted of monocytes, B cells and NK cells as described in section 2.1.2.1. Cytotoxicity assays were then set up as described in section 2.1.8. Four different T cell donors were used in the experiments. All of the effector phase of the assay was performed in at least replicates of 6, with an effector:target ratio of 10:1. Results are expressed as the mean percentage cytotoxicity, from the cumulative results from 13 experiments.

6.1.3.1 Cytokine Analysis of Stimulated T cells

From the 13 cytotoxicity assays performed using enriched T cells, the supernatants were saved from eight after the 10 day stimulation phase of the assay. Aliquots from each of these were then analysed for the presence of γ-

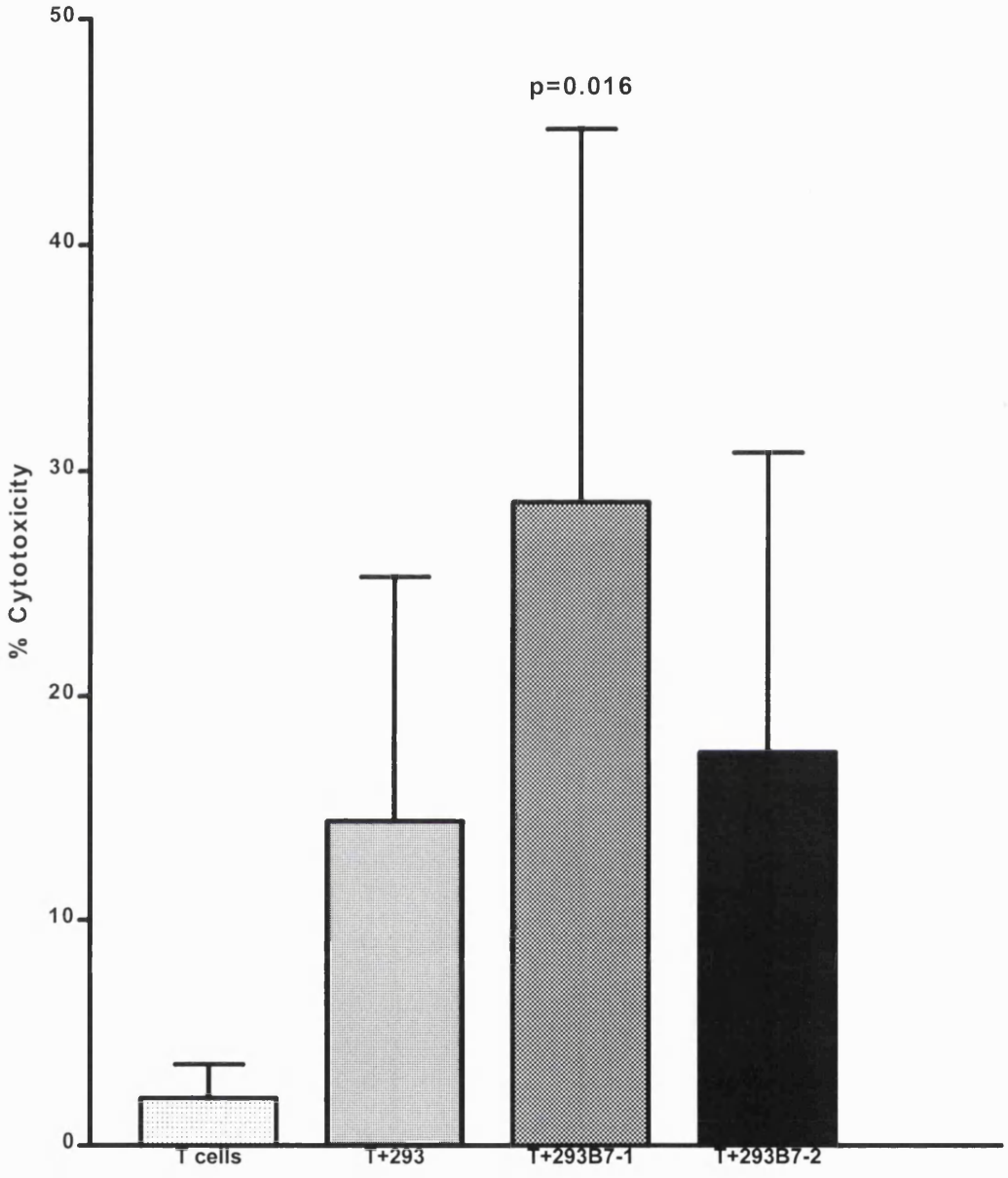
IFN (a T_H1 cytokine) and IL-4 (a T_H2 cytokine), using an ELISA as described in section 2.1.5. Aliquots of the responding T cells from four of the experiments were also analysed for the intracellular presence of the cytokines γ-IFN and IL-4 following the protocol described in section 2.1.9.

6.1.4 Results

6.1.4.1 Cytotoxicity Assays

Percentage specific lysis was calculated as described in section 6.1.2.1. The mean specific lysis of unstimulated T cells was 2.1%, with a standard deviation (sd) of 1.5. Mean specific lysis of target cells by T cells primed with 293 cells stably transfected with *lac-ve* was 14.4%, with a standard deviation of 10.9. Mean specific lysis with the B7-1 293 cells as stimulator cells was 28.6% with a standard deviation of 16.5 and mean specific lysis with the B7-2 expressing 293 cells as stimulator cells was 17.5% with a standard deviation of 13.3. These results are summarised in figure 28. Using Student's paired T test, there was a significant increase in cytotoxicity above background with the unprimed T cells, with stimulation with control 293 cells ($p=0.003$), B7-1 expressing 293 cells ($p=0.0001$) and B7-2 expressing 293 cells ($p=0.0026$). There was a significant increase in cytotoxicity with the B7-1 expressing 293 cells as stimulators, as compared to the control 293 cells ($p=0.016$). There was no significant difference in cytotoxicity between the B7-1 and B7-2 expressing 293 cells as stimulators ($p=0.072$), or between the control 293 cells and B7-2 expressing 293 cells as stimulators ($p=0.517$).

Figure 28 Graph of cytotoxicity assay with 293 transfectants.



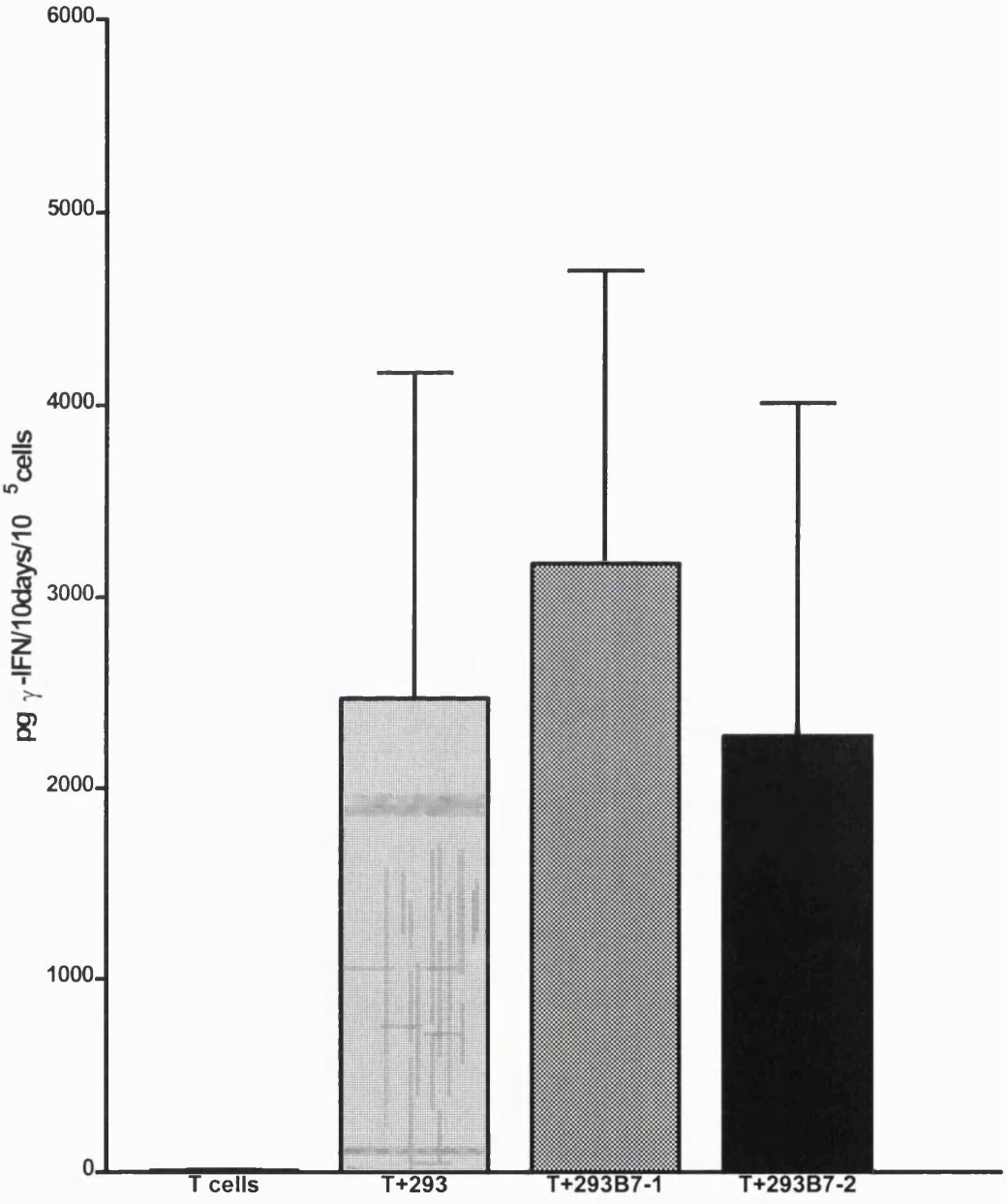
Legend to Figure 28:

Cytotoxicity, expressed as percentage cytotoxicity, against parental 293 target cells, either with unstimulated T cells, T cells stimulated with control 293 cells, or sorted B7-1 or B7-2 expressing 293 cells. Results are expressed as the mean percentage cytotoxicity, together with the standard deviation. There is a significant increase in cytotoxicity with the B7-1 expressing 293 cells as stimulators compared to the control 293 cells (p=0.016).

6.1.4.2 Cytokines Produced by T cells Activated by the 293 Transfectants

Mean amount of γ-IFN produced by unstimulated T cells was 9.3 pg/ml (sd 4.2) and mean amount of IL-4 produced by unstimulated T cells was 3.3 pg/ml (sd 5.3). Mean amount of γ-IFN produced by T cells stimulated with control 293 cells was 2468 pg/ml (sd 1699) and mean amount of IL-4 produced by T cells stimulated with 293 cells was 13.7 pg/ml (sd 16.6). T cells stimulated with B7-1 expressing 293 cells produced a mean of 3174 pg/ml γ-IFN (sd 1519) and a mean of 22.4 pg/ml IL-4 (sd 17.1). T cells stimulated with B7-2 expressing 293 cells produced a mean of 2273 pg/ml γ-IFN (sd 1733) and a mean of 12.3 pg/ml IL-4 (sd 5.3) (see figures 29 and 30). Statistical analysis comparing γ-IFN and IL-4 production from the responding T cells was performed using Student's paired T test. There was no statistically significant difference in the amount of γ-IFN of IL-4 produced by the T cells responding to the control 293 cells, or the B7-1 or B7-2 expressing 293 cells. Compared to the unstimulated T cells there was a statistically significant increase in γ-IFN production from T cells stimulated with control 293 cells ($p=0.018$) and B7-1 ($p=0.002$) or B7-2 ($p=0.028$) expressing 293 cells. In contrast, only the B7-1 expressing 293 cells induced a significant increase in IL-4 production in responding T cells ($p=0.03$), as opposed to unstimulated T cells.

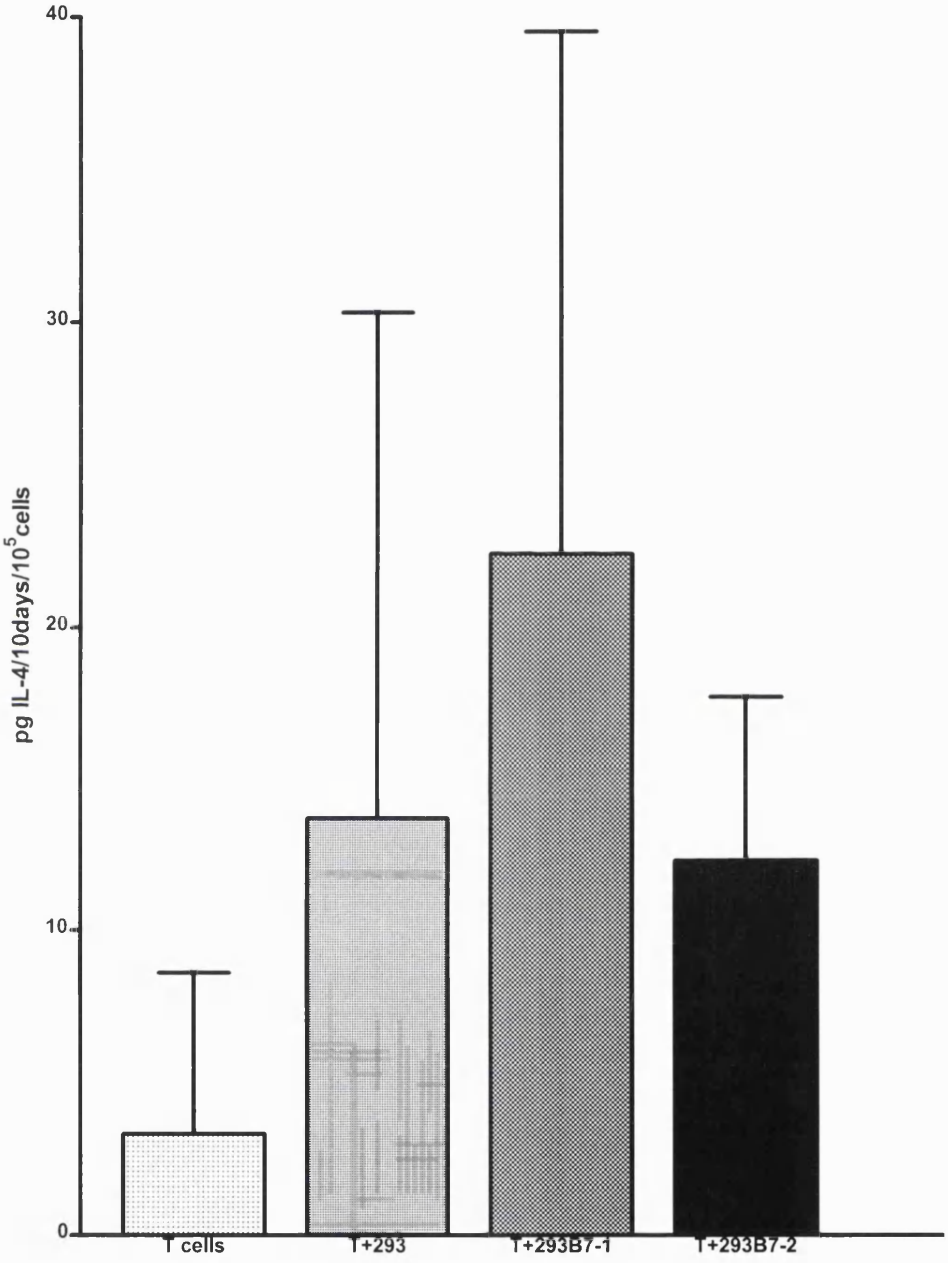
Figure 29 γ-IFN Production from T cells Stimulated with 293 Transfectants



Legend to Figure 29:

γ-IFN production as pg γ-IFN produced per 10 days per 10^5 T cells, from unstimulated T cells, or T cells stimulated with control 293 cells, or 293 cells expressing B7-1 or B7-2.

Figure 30 IL-4 Production from T cell stimulated with 293 Transfectants



Legend to Figure 30:
IL-4 production expressed as pg IL-4 per 10 days per 10^5 T cells from unstimulated T cells, or T cells stimulated with control 293 cells, or 293 cells expressing B7-1 or B7-2.

6.1.4.3 Intracellular γ-IFN and IL-4 in Responding T cells

The percentage of T cells expressing γ-IFN or IL-4 was not significantly different, depending on whether stimulated with control 293 cells, or sorted B7-1 or B7-2 expressing 293 cells. A mean of 14.9% of T cells produced γ-IFN when the stimulator cells were control 293 cells, compared to 13.4% when the stimulator cells expressed B7-1 and 11.1% when the stimulator cells expressed B7-2. With control 293 cells 2.2% of T cells produced IL-4, compared to 1.5% of T cells when B7-1 293 cells were stimulator cells and 2.4% of T cells when B7-2 293 cells were the stimulator cells.

6.1.5 Discussion

No significant differences were seen in the mean specific lysis between T cells stimulated with either control 293 cells, or B7-2 expressing 293 cells ($p=0.517$). However there was significantly increased cytotoxicity with T cells stimulated with B7-1 expressing 293 cells as opposed to control 293 cells ($p=0.016$). Cytotoxicity was also greater with T cells stimulated with B7-1 expressing 293 cells, compared with B7-2 expressing 293 cells, although this was not statistically significant ($p=0.072$). As might be expected, mean specific lysis of labelled target 293 cells was very low when the T cells had been unprimed during the stimulation phase of the assay. This lysis could be attributed to residual natural killer cells in the enriched T cell preparation. One reason why there was not a larger difference in killing with the stimulator cells expressing the co-stimulatory molecules, may be the lack of adhesion molecules expressed on the 293 cells, they lack ICAM-1 and LFA-1 expression. In both the induction of a T cell response (Schmits et al., 1996; Sligh, Jr. et al., 1993) and in the effector phase of a cytotoxic T cell response (Davignon et al., 1981), the first process that needs to occur is binding of the T cell to the stimulator or target cell respectively. In the absence of a suitable adhesion molecule this process will be impaired. To overcome this transfectants could be generated in a cell line that innately expresses an adhesion molecule, for example HeLa cells which express ICAM-1. Another explanation for why there was not higher cytotoxicity with costimulation through B7-1 or B7-2, is that the 293 cells only express class I MHC and do not express MHC class II. Approximately 50% of CD8+ve T cells express CD28 (Aruffo and Seed, 1987)

and CD8 T cells are class I restricted. In the enriched T cell populations obtained from the normal donors the CD4/CD8 ratio varied between 2:1 and 4:1 thus, the majority of T cells in the preparations will not respond to an allogeneic class I signal. Of the CD8 cells present only 50% of these will express the ligand for B7-1 and B7-2 (Aruffo and Seed, 1987). This perhaps could be solved if future cytotoxicity assays were performed with T cells depleted also of CD4+ve T cells, therefore preferentially enriching the CD8 population. Another factor which may have affected the small differences seen in specific cytotoxicity is that the effector target ratio (E:T) was fixed at 10:1. It is often only at higher E:T ratios that differences in cytotoxicity become apparent (Wendtner et al., 1997). Ideally, I would have liked to have performed these cytotoxicity assays using a leukaemic cell line, for example KG1a instead of the 293 cells. These cells are more likely to express a similar array of surface molecules as would be found on myeloid leukaemic blasts and may have given a better indication of the effect of co-stimulatory molecule expression on myeloid leukaemic cells. However, non adherent cell lines like KG1a are difficult to transfect efficiently with cationic lipids, or by electroporation. I used both techniques with KG1a cells, yielding very few positive cells and selection in G418 did not yield a resistant population of cells.

No significant difference was seen in the production of both γ-IFN and IL-4 from the T cells stimulated with control, B7-1 or B7-2 expressing 293 cells. As was expected very little production of γ-IFN or IL-4 was seen with the unstimulated T cells as these cells would not have received either a signal through the T cell receptor or a co-stimulatory signal. There was a trend towards increased production of the T_H1 cytokine γ-IFN, from the T cells

stimulated with the B7-1 expressing 293 cells, when analysing the supernatants. In addition, a large proportion of circulating T cells in peripheral blood have already committed to either the T_H1 or T_H2 phenotype, as demonstrated by the expression of the surface molecule CD45RO. This is perhaps reflected in the intracellular detection of intracellular γ-IFN and IL-4, where no difference was seen in the T cells stimulated with control or B7-1 or B7-2 expressing 293 cells. To demonstrate a possible role of co-stimulatory molecules in the development of T_H1 or T_H2 cells, it might be better to select naive T cells which express the surface antigen CD45RA. These experiments have been performed and do suggest that B7-1 promotes production of a T_H1 response and that B7-2 promotes the development of a T_H2 response with repetitive stimulation with either B7-1 or B7-2 expressing cell lines (Freeman et al., 1995). If B7-1 and B7-2 have equivalent functions, it is surprising that it was the B7-1 rather than B7-2 expressing 293 cells which were better at generating CTL's, as the level of expression of B7-2 was higher than on the B7-1 transfectants. As γ-IFN (a T_H1 cytokine) production was also higher from the T cells stimulated with B7-1 transfectants, it may be that B7-1 does promote a T_H1 response (Kuchroo et al., 1995; Freeman et al., 1995). Similar results were obtained by a separate group who used B7-1 or B7-2 transfectants in the keratinocyte cell line A431, which expressed MHC class II. They found that the B7-1 transfectants consistently induced more proliferation, IL-2 and γ-IFN production from responding T cells (van Dijk et al., 1996). As with my experiments the T cells and stimulator cells were MHC mismatched, with alloantigen providing the signal through the T cell receptor. Another interpretation may be made by using the strength of signal hypothesis

(Lenschow et al., 1996). This hypothesis states that with a low antigen dose, costimulation through CD28 is essential, which is also suggested by experimental data (Bachmann et al., 1997). As B7-2 is innately expressed on APC's, it is proposed that this molecule initiates the T cell response, be it a T_H1 or T_H2 type response. As the immune response progresses, B7-1 is upregulated on APC's and the increased co-stimulatory signal promotes a T_H2 immune response. Blockade of costimulation at this time point, may then skew the T cell response to a T_H1 phenotype. They postulate that this is the reason anti-B7-2 mAb increases disease severity and promotes a T_H1 T cell response in mice with experimental allergic encephalitis (Kuchroo et al., 1995). However, in the same murine model, treatment of mice with anti-B7-1 mAb, again decreasing the strength of the co-stimulatory signal, ameliorated disease severity and promoted a T_H2 T cell response (Kuchroo et al., 1995). The hypothesis also suggests that high antigen dose, together with a strong signal through CD28 may downregulate the immune response. The allogeneic signal provided by the mismatched 293 cells, together with the higher level of expression of B7-2, as compared to B7-1 may have been sufficient to downregulate the immune response, explaining why cytotoxicity was less with the higher B7-2 expressing 293 cells as opposed to the B7-1 expressing 293 cells. This explanation seems unlikely, as the B7-2 transfectants consistently induced higher proliferation of T cells, when all the T cells were potentially responsive, receiving a submitogenic dose of PMA (see section 5.1.3.2). This would suggest that B7-1 costimulation preferentially promotes CTLs when compared to B7-2.

7.1 TRANSDUCTION OF AML BLASTS WITH B7-1 OR B7-2

7.2 INTRODUCTION

The efficient introduction of foreign DNA into target cells is a prerequisite for gene therapy. Recombinant retroviruses have long been used to deliver genes to mammalian cells and over recent years a variety of other viruses have been developed as well as a number of non viral methods of gene transfer. Despite the many vectors available for gene therapy, efficient transduction of primitive haematopoietic cells has proved difficult, due both to inefficient entry of the vector into the target cell and to inhibition of transcription and translation of the gene of interest. No vectors fulfil the ideal requirements of gene transfer which include, ease of construction of the vector, ease of construction, safety, which includes target cell transformation and long term expression of the gene product. Therefore the choice of vector is always a compromise depending on the target cell to be transduced, the desired effect of the gene of interest on the target cell and safety issues, in particular if the transduced cells are to be returned to patients.

7.2.1 Non-Viral Methods of Gene Transfer

Non-viral approaches to gene therapy have been developed because of the inherent limitations of viruses. Retroviruses are only able to be produced to low titre and require cell division for DNA integration. In addition, due to the random nature of their integration, there is the potential for malignant transformation in the target cells. Adenoviruses and Adeno-Associated Virus

(AAV) have the ability to transduce non dividing cells, but AAV in particular has limitations in the amount of DNA that can be packaged and recombinant adenoviruses elicit a potent immune response to viral gene products which negates long term expression of the transgene.

7.2.1.1 Particle Bombardment

One method of non-viral gene transfer involves the coating of plasmid DNA onto gold or tungsten beads 1-3 μm in diameter (Williams et al., 1991). The plasmid coated beads are then fired at the target cells after acceleration either with an electric discharge or gas pulse. This technique relies on penetration of the cell membrane barrier (Cheng et al., 1993). Expression declines rapidly after an initial peak at 72 hours, which limits this approach for most gene therapy applications. This technique has not been applied to AML blasts as targets.

7.2.1.2 DNA Injection

Another non-viral method of transferring DNA to cells involves the direct injection of plasmid DNA into the target tissue, which has been successfully demonstrated using mouse skeletal muscle cells (Wolff et al., 1990). Using direct DNA injection into muscle expression was seen for up to 80 days and by stimulating muscle regeneration levels of expression were improved (Davis et al., 1993). This method has advantages over viral vectors in that it is simple, cheap and there is no constraint on the size of the DNA construct. However it has limitations in that the levels of expression are low and the duration of expression is generally limited. This technique is used on solid tissue as the target and is not relevant to AML blasts.

7.2.1.3 Cationic Lipids

This method of transferring DNA into cells relies on the negative electrical charge of the DNA which is due to the phosphate backbone of the double helix. Complexing of the negatively charged DNA with cationic lipids allows the complex to pass through the negatively charged cell membrane. The first lipids to be used were monocationic such as DOTMA (N[1-(2,3-dioleyloxy)-propyl]-NNN-trimethylammonium chloride) (Felgner et al., 1987). These have been superseded by polycationic lipids such as DOPSA, (2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-NN-dimethyl-1-propanaminiumtrifluoroacetate, which has thirty times the transfection efficiency of the former (Gao and Huang, 1995). As with the other non-viral methods this has the advantages of no DNA size constraints and low immunogenicity. Primary cells are in general resistant to transfection and this is the case with AML blasts. However, transfection is an important part of the process for the production of recombinant Adeno Associated Virus (AAV) which was described in section 2.1.10. I have also used transfection to generate cell lines expressing B7-1 or B7-2 to confirm the co-stimulatory ability of the molecules (sections 4.5.3 and 4.5.5).

7.2.1.4 Receptor Mediated Gene Transfer

In addition to complexing DNA with cationic lipids, conjugation of the DNA with a carrier molecule which is the ligand for a cellular receptor is a way of targeting genes into specific cells. Two carrier proteins are commonly used and include the asialoglycoprotein receptor (Wu et al., 1989) which is hepatocyte specific and transferrin-poly-L-lysine (Wagner et al., 1992) which

binds to the transferrin receptor. This method has advantages in being able to transfer large DNA fragments, it has low immunogenicity and because of the inherent targeting can be given via a peripheral vein rather than selective catheterisation of an organ. Binding of the DNA/receptor complex to the ligand is followed by receptor-mediated endocytosis and the DNA-containing vesicles fuse with cytoplasmic liposomes. Within the resulting acidic endosome the DNA is largely degraded, which is a major limitation to this technique.

To bypass the endosomal pathway the properties of haemagglutinating virus of Japan (HVJ) has been utilised. A hybrid liposome is constructed containing the DNA insert with the HN spike coat glycoprotein of HVJ on the surface of the liposome. The modified liposome attaches to the plasma membrane via the spike coat glycoprotein HN. Membrane fusion occurs releasing the viral contents directly into the host cell cytoplasm, bypassing the endosomal compartment completely (Kaneda et al., 1989b). The DNA remains as an episomal structure, resulting in rapid decline of expression after only 7-8 days (Kaneda et al., 1989a). This technique has not been used with haemopoietic cells as the target cells.

7.2.2 Viral Mediated Gene Transfer

7.2.2.1 Retroviruses

Retroviruses consist of an inner core enclosed in a phospholipid envelope. The inner core consists of the capsid, which contains two copies of positive sense viral mRNA, virally encoded protease, reverse transcriptase and integrase enzymes. The envelope is a spherical phospholipid bilayer covered with oligomeric membrane spike glycoproteins. The viral genome contains

three genes, *gag*, which encodes the structural proteins of the capsid, *pol*, which encodes for reverse transcriptase, protease and integrase and *env*, which codes for the envelope glycoproteins.

The retrovirus attaches to the target cell by binding of viral envelope glycoprotein (Env) to its cell surface receptor. The viral and cellular phospholipid bilayers fuse releasing the viral core into the cytoplasm. The RNA genome is converted to double stranded DNA (Panganiban and Fiore, 1988), is transported to the nucleus and integrates into chromosomal sites, showing a preference for transcriptionally active host integration sites (Mooslehner et al., 1990). After integration, regulatory promoter and enhancer elements in the U3 region of the proviral long terminal repeat (LTR) drive transcription of the viral genome. Full length viral mRNA binds to the Gag polyprotein through its packaging signal sequence (*Psi*) during particle assembly and Env glycoproteins, present in the plasma membrane, are incorporated into the viral progeny as they bud from the cell surface. Viral protease then cleaves the Gag and Gag-Pol polyproteins into their component parts which results in the virions becoming infectious.

7.2.2.1.1 Recombinant Retroviruses

7.2.2.1.1.1 Retroviral Vector Genome

There are several essential *cis*-acting sequences which must be retained within the retroviral vector genome. First, the packaging signal sequence (*Psi*), which ensures the encapsidation of the vector RNA into the virions, is essential. Second, there are several elements which are required for reverse transcription, including the primer binding site which binds the tRNA primer of

reverse transcription, the terminal repeat sequences that guide the transfer of reverse transcriptase between RNA strands during DNA synthesis and a purine-rich sequence 5' of the 3' LTR that serves as the priming site for synthesis of the second DNA strand. Finally specific sequences near the ends of the LTRs are necessary for integration of the vector DNA into the host cell chromosome in a manner analogous to wild type retrovirus (Panganiban and Temin, 1983). Removal of the protein coding sequences of the retrovirus, *gag*, *pol* and *env* allow incorporation of up to 8 kbp of foreign DNA 3' of the *Psi* and 5' of the 3' LTR. Expression of the inserted gene may be driven off the viral LTR or an internal promoter. Within the packaging constraints of the virus it is possible to insert more than one gene into the vector genome controlled by both viral or non viral promoter sequences. This allows incorporation of an antibiotic resistance marker allowing for selection of successfully transduced cells. Alternatively the incorporation of an Internal Ribosome Entry Site (IRES), derived from picornaviruses, allows cap-independent initiation of translation at internal start codons on polycistronic mRNA. Therefore, inclusion of an IRES sequence between two genes allows for expression of both genes from a single promoter (Boris-Lawrie and Temin, 1993).

7.2.2.1.1.2 Packaging Cell Lines

The purpose of the retroviral packaging cell line is to provide the helper functions deleted from the vector genome, the Gag, Pol and Env proteins. The *gag*, *pol* and *env* genes are normally cloned into one or two helper plasmids which are stably expressed in the packaging cell line and are not packaged into viral particles because they lack the *Psi*. When the recombinant retroviral

genome is transfected into the packaging cell, the viral Gag proteins recognise and package the vector RNA genome into viral particles which are then released into the culture supernatant (Miller, 1990).

7.2.2.1.1.3 Disadvantages of Recombinant Retroviruses

The main disadvantages associated with retroviruses as gene vectors are the possibility of producing replication competent virions and the induction of transformation in the infected target cell. When the vector cDNA is transfected into the packaging cell line all the sequences required to make wild type retrovirus are present. If there is sequence homology between the vector and helper genomes there is a chance they will align and undergo homologous recombination. This risk is increased with the number of vector and helper genomes within each cell and the degree of sequence homology between the vector and helper DNA. To reduce the risk of generating replication competent retrovirus, the plasmid containing the helper functions can be deleted of more than the packaging signal, for example the 3' LTR. This increases the number of recombination events to two in order to generate replication competent retroviruses. To reduce the risk further newer packaging cell lines separate the *gag* and *pol* transcription units from the *env* transcription unit onto different plasmids (Miller, 1990). This prevents recombination producing replication competent retrovirus as the viral genome is split three ways into two helper constructs and the vector construct.

Retroviral vectors are theoretically capable of causing neoplastic transformation in the target cells by insertional mutagenesis (Cornetta, 1992). In animal experiments and human gene therapy trials no cell transformation has

been observed and theoretical models suggest that the risk is low (Moolten and Cupples, 1992). In addition retroviral reverse transcriptase has a high error rate. If the recombinant retrovirus is intended to deliver a tumour suppressor gene, mutation may occur which would result in the generation of a mutant of the tumour suppressor gene which could transform infected cells.

Factors that affect gene transfer include the titre of the recombinant retrovirus, its stability, the target cells and factors that subsequently affect the retroviral integration and expression of the gene. The titre of retroviruses is governed by the rate at which infectious particles are released from the producer cells and their subsequent stability. Under normal conditions most retroviral producer cell lines produce infectious particles at a concentration of 10^6 - 10^7 per ml of tissue culture supernatant. Significant loss of retroviral titre occurs at incubation at 37°C with most having a half life of approximately 8 hours. In addition, a 50% loss of infectivity occurs with each freeze thaw cycle and concentration by centrifugation, which has been attributed to dissociation of $gp70^{su}$ envelope protein from the particle surface. *In vivo* delivery of genes with recombinant retroviruses is affected by rapid inactivation by human complement (Welsh, Jr. et al., 1975). This is thought to be mediated by virally encoded Env protein or the lipid composition of the viral envelope. Quiescence in the target cell impedes entry of the virus into the nucleus and subsequent integration into the host cell genome, which is one of the limiting factors with transduction of haemopoietic stem cells or AML blasts (Dunbar and Emmons, 1994; Hirst et al., 1997).

7.2.2.2 Adenoviral Vectors

Adenoviruses are mild pathogens in man, characteristically infecting the upper respiratory tract, the conjunctiva, gastrointestinal and urinary tracts. Removal of all the viral DNA apart from the packaging signal and inverted terminal repeats allows packaging of up to 27kb of DNA, with the viral proteins being supplied in trans by a helper virus (Parks and Graham, 1997). It has advantages over retroviruses in that it can be produced to high titre, with up to 10^{12} infectious units per ml being feasible. In addition cell division is not required for gene transfer, which is advantageous when non dividing cells are the targets of infection. A wide range of target tissues are susceptible to infection. Using the reporter gene β-galactosidase, successful transduction of both bone marrow mononuclear cells and CD34+ve cells has been achieved with recombinant adenovirus (Watanabe et al., 1996). One of the main disadvantages of adenoviral infection is the existence of pre-existing neutralising antibodies which *in vivo* can lead to rapid elimination of infected target cells, negating long term expression.

Long term expression is not a prerequisite for some cancer strategies, as with the strategy of inducing expression of costimulatory molecules on tumour cells and here the ability of adenovirus to infect non dividing cells is an advantage. However adenovirus is a pathogenic virus and the return of transduced cells may infect the recipient with wild type adenovirus. In patients immunosuppressed after chemotherapy or bone marrow transplantation, this could be significant.

7.2.2.3 Adeno-Associated Virus

7.2.2.3.1 Wild Type Adeno-Associated Virus

Adeno-associated viruses (AAV) belong to the family Parvoviridae and genera Dependovirus (Hoggan et al., 1966). They require coinfection with a helper virus, such as adenovirus, cytomegalovirus or herpes simplex virus, to produce a productive infection (Flotte and Carter, 1995). There are five serotypes in humans (AAV1-5) and are thought to naturally infect cells of the respiratory and gastrointestinal tracts although they have been demonstrated to infect a wide variety of cell types, including haematopoietic stem cells (Anderson et al., 1997). AAV have not been demonstrated to produce symptomatic infections in man and are not known to be tumourigenic (Berns, 1990).

The virus has an icosahedral structure, measuring 18-26nm in diameter. The capsid is composed of three unglycosylated proteins, 90, 72 and 60 kDa in size (Ruffing et al., 1992). Binding to the target cell is receptor mediated and it has been suggested that heparan sulfate proteoglycan may act as the receptor for AAV-2 (Summerford and Samulski, 1998). The AAV-2 genome is a linear single stranded DNA molecule, consisting of 4681 nucleotides (nt) with flanking inverted terminal repeats (ITR) of 145nt each, which form hairpin structures when the DNA is in a single stranded state (Muzyczka, 1992). After entry of virus into the cell the single stranded DNA is converted to double stranded DNA, the 3' hairpin serving as an origin for DNA replication (Flotte and Carter, 1995). The viral DNA, in the absence of helper virus infection, forms head to tail multimers. The ITR's appear to be important for integration, but presence of the Rep proteins is required to direct integration into a specific locus on

chromosome 19 termed 19q13.4 (Samulski et al., 1991) (Yang et al., 1997). The viral genome can be divided into three functional parts. First, the ITR's which are required for encapsidation, replication (Berns, I. and Hauswirth, W. 1984) and integration of the viral DNA into the long arm of chromosome 19 (Samulski et al., 1991). Second, the *rep* gene which codes for four non structural proteins, *rep* 78, 68, 52 and 40. *Rep* 78 and 68 are transcribed with the p5 promoter and *rep* 52 and 40 are transcribed with the p19 promoter, differential splicing of the mRNA accounting for the different size of the proteins (Flotte and Carter, 1995). Rep 68 and 78 bind to the ITR region and are involved in integration into the genomic DNA and are also involved in replication and excision of AAV DNA. Rep 40 and 52 are required for the accumulation of single stranded DNA (Flotte and Carter, 1995). Third, the *cap* gene codes for three structural proteins of 90, 72 and 60kDa termed VP1, VP2 and VP3 respectively (Buller and Rose, 1978). The *cap* gene is driven by the p40 promoter and differential splicing of mRNA accounts for the different sizes of the capsid proteins (Becerra et al., 1988). The precursor RNA of 2.3kB is encoded by the right half of the AAV 2 genome (Jay et al., 1981) and in the presence of adenoviral infection the majority is spliced at nucleotide 2228 (major splice) and a minority at nucleotide 2201 (minor splice) to produce two 2.3kB mRNAs. VP1 transcription is initiated from an AUG codon at nucleotide 2203, only found in the minor splice mRNA, while VP2 and VP3 are expressed from both the minor and major splice mRNAs. Translation of VP3 is initiated from an AUG codon at position 2809 and VP2 is translated 10-fold less efficiently from an ACG initiation codon at nucleotide 2614 (Becerra et al., 1988; Cassinotti et al., 1988). The differential splicing to produce major and

minor splice mRNAs and the decreased translation initiation frequency from the ACG initiation codon results in a ratio of VP1, VP2 and VP3 of 1:1:10 respectively, which is the ratio found in mature AAV particles (Buller and Rose, 1978). In the absence of coinfection with adenovirus, one of the Rep proteins appears to act as a repressor of some promoters (Labow et al., 1987) including the p5 promoter (Tratschin et al., 1986), maintaining the virus in a proviral state. Coinfection with adenovirus relieves transcriptional repression of the AAV genome, resulting in replication of the viral DNA and packaging within the produced capsids. The adenoviral helper functions that are required for productive AAV replication are supplied by the E1A, E1B and E4 polypeptides, the E2A DNA binding protein and the VA RNAs (Kremer and Perricaudet, 1995).

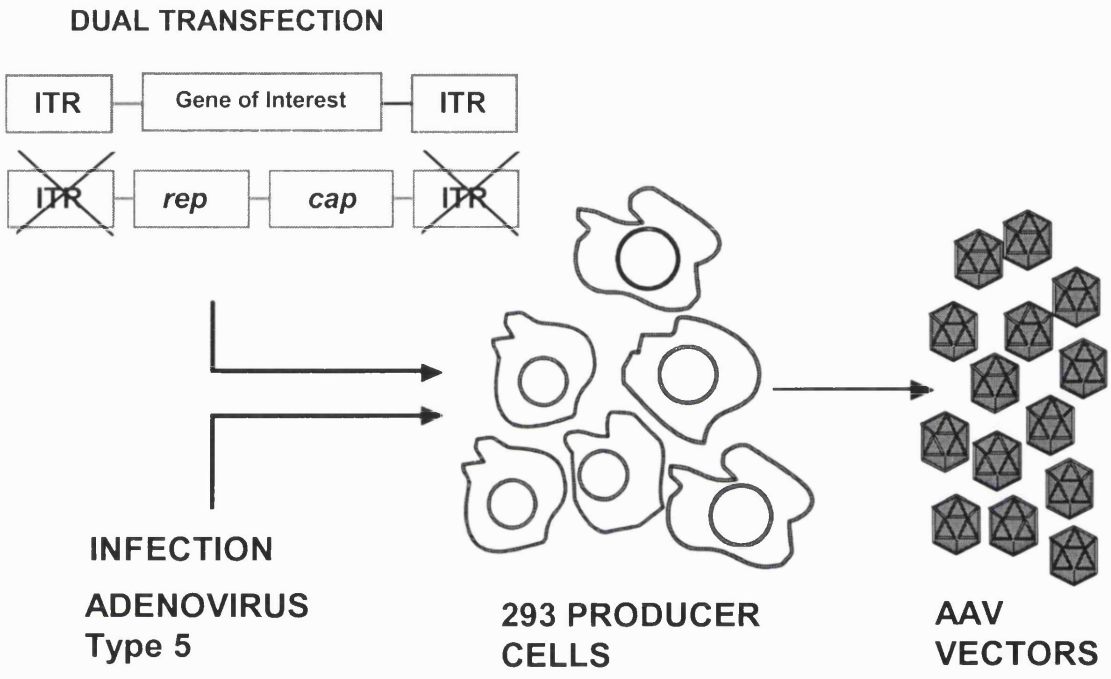
7.2.2.3.2 Recombinant AAV-2

The use of AAV-2 as a vector was first demonstrated with the transduction of the neomycin phosphotransferase gene into human and murine cell lines (Hermonat and Muzyczka, 1984). The neomycin phosphotransferase gene, under the control of the SV40 promoter, was cloned into an AAV mutant deleted of the *cap* gene. Recombinant AAV was produced by transfecting the AAV construct, containing the neomycin phosphotransferase gene, into adenovirally infected cells together with a second plasmid containing the *cap* gene *in trans*. The recombinant virus produced was then used to successfully infect both human and murine cells. A major limitation of this technique was contamination of the recombinant viral stocks with wild type AAV (wtAAV). This problem was addressed by the construction of an AAV plasmid containing only

the 3' and 5' ITRs, between which had been cloned the expression cassette containing the neomycin resistance gene (Samulski et al., 1989). Recombinant AAV was produced by transfection of the AAV plasmid containing the expression cassette, together with a second helper plasmid containing the *rep* and *cap* genes *in trans*. As the two plasmids had no sequence in common, the chance of homologous recombination and production of wtAAV was reduced. This demonstrated that the only *cis*-acting elements required to produce AAV are located in the terminal ITRs, which are each 145nt in length (Samulski et al., 1989). The terminal 125nt of each ITR form palindromic hairpin structures that serve as primers for AAV DNA replication. Each ITR also contains a 20nt sequence, called the D sequence, which is bound by a host cell protein termed the D sequence-binding protein (Wang et al., 1996). Mutational analysis has revealed that it is the proximal 10nt of the D sequence that is necessary for replication and encapsidation of the AAV genome (Wang et al., 1997).

Production of recombinant AAV-2 requires transfection of the producer cells, usually 293 cells, with two plasmids, followed by infection with a helper virus, usually adenovirus type 5. 293 cells (ECACC No:85120602) are derived from human embryonic kidney cells transformed with sheared human adenovirus type 5 DNA. They are used for rAAV production as they are sensitive to adenoviral infection and are readily transfected with plasmid DNA. The packaging plasmid consists of the expression cassette, containing the promoter and gene of interest, flanked by the 145bp ITRs. As described above, the helper plasmid contains the *rep* and *cap* genes, without any flanking ITR sequences. Production of recombinant AAV is shown schematically in figure 31.

Figure 31 Production of recombinant AAV



Legend to Figure 31:

Transfection of 293 cells with both packaging and helper plasmids is followed by infection with a helper virus to enable packaging of recombinant AAV containing the gene of interest. After full cytopathic effect is seen in the producer cells, the supernatant and cells are harvested to produce a crude viral stock, containing both adenovirus and recombinant AAV.

7.2.2.3.2.1 Advantages of AAV Over Other Vectors

AAV-2 has many potential advantages over other vectors used for gene therapy. It has a wide spectrum of target cells and has not been associated with any pathogenic disorders in humans. In addition the prospect of site specific integration with wild type AAV into the long arm of chromosome 19 decreases the possibility of transformation in the target cell, although with rAAV integration occurs at sites other than on chromosome 19 (Ponnazhagan et al., 1997). With recombinant AAV the only viral DNA introduced into the target cell are the two palindromic ITRs. The virus can also be concentrated to a high titre by ultracentrifugation on a CsCl gradient enabling the multiplicity of infection of recombinant virus to target cell to be greatly increased. AAV is also able to infect slowly or non-dividing cells. This was demonstrated with an AAV-2 vector containing the reporter gene *LacZ* which was used to infect human cells either actively proliferating or in a non proliferative state after exposure to DNA synthesis inhibitors. Expression of the reporter gene was equivalent in the non-dividing cells to that in the actively proliferating cells indicating that the mechanisms of viral attachment, uncoating and conversion of the single stranded to double stranded DNA were present in the non-dividing cells (Podsakoff et al., 1994).

Recombinant AAV-2 has been demonstrated to deliver a wide variety of genes to different target cells. With regard to haematological cells, AAV-2 has been used to introduce the γ-globin gene into human erythroleukaemia cells, achieving high-level regulated gene expression (Walsh et al., 1992). The ability of recombinant AAV to transduce haemopoietic progenitor cells was

demonstrated with a vector containing the neomycin resistance gene. CD34+ve cells were transduced, without any prestimulation to induce a higher cell cycle state and expression of the neomycin resistance gene was demonstrated with high efficiency (Zhou et al., 1994). High efficiency transduction of a human lymphoid cell line (LP-1) with recombinant AAV containing the gene for B7-2 has been demonstrated (Chiorini et al., 1995), showing that haematological cells are suitable target cells for this vector. Cystic fibrosis is a disorder characterised by a defect in the cAMP-mediated stimulation of chloride secretion in the airway epithelium. Successful transduction of primary cystic fibrosis nasal polyp cells was achieved with an AAV vector expressing the cystic fibrosis transmembrane conductance regulator. In addition bronchoscopic delivery of this vector to one lobe of the lung of a rabbit resulted in detectable expression six months after inoculation (Flotte et al., 1993). Injection of recombinant AAV, containing either the β-galactosidase gene or human tyrosine hydroxylase gene, into the brain of rats resulted in expression for at least 3 months. In rats with unilateral 6-hydroxydopamine lesions, injection of AAV expressing tyrosine hydroxylase into the lesion resulted in some restoration of tyrosine hydroxylase activity, which was reflected in behavioural recovery in the affected rats (Kaplitt et al., 1994). This strategy may have applications for patients suffering with Parkinson's disease which is characterised by loss of the nigrostriatal pathway. Because of the ability of recombinant AAV to transduce haematological cells, including primitive CD34+ve cells and cells which are non- or slowly dividing and its lack of pathogenicity, AAV was chosen as the vector to deliver the co-stimulatory genes to the leukaemic cells.

7.2.2.3.2.2 Disadvantages of AAV Over Other Vectors

Drawbacks of recombinant AAV are that it is highly labour intensive to produce large amounts of virus. In addition, specific integration into chromosome 19 appears to be a feature of wild type virus alone. Deletion mutants of AAV lacking the *rep* and *cap* genes, as with recombinant AAV, are still capable of integration into the target cell genome, but integration appears to be random (Ponnazhagan et al., 1997). This raises the possibility of target cell transformation due either to activation of a dormant oncogene or repression of a tumour suppressor gene. Although integration can occur with recombinant AAV the transduced DNA seems to exist primarily as an episomal structure and expression of the gene of interest peaks at 48 hours and declines rapidly after this (Muzyczka, 1992). However, the strategy of inducing co-stimulatory molecules on leukaemic cells, does not require long term expression. The packaging capacity of recombinant AAV is approximately 105% of the wild type genome and therefore only cDNAs up to 4.7kB can be incorporated (Muzyczka, 1992).

Certain factors have been found to increase expression of AAV recombinants, such as co-infection with adenovirus or subjecting the cells to ultraviolet irradiation (UV), X-rays, hydroxyurea or heat shock, suggesting that the rate limiting factor in transduction efficiency was not internalisation of the virus. By co-infecting target cells with adenoviral mutants, containing deletions of specific genes, it was determined that the E4*orf*6 gene product was mainly responsible for the increase in expression. Analysis of low molecular weight DNA from transduced cells demonstrated increased conversion of single stranded to transcriptionally active double stranded DNA in target cells

transfected with E4*orf*6 or subjected to UV irradiation. Dose escalation of UV irradiation also increased the conversion of single stranded to double stranded DNA, which was reflected in the expression of the reporter gene packaged in the recombinant AAV (Ferrari et al., 1996). Thus the rate limiting factor for expression of the gene of interest is conversion of the single stranded DNA into double stranded DNA.

Many experiments have used immortalised cell lines as targets for infection with recombinant AAV. Comparison of transduction efficiency in primary epithelial cells to immortalised human epithelial cell lines revealed greater expression of the reporter gene in the immortalised cell lines. This was up to 50 times that seen in the primary human cells and was not related to uptake of the virus which was equivalent in both cell types. This difference between the primary and immortalised cells was reflected in the amount of AAV vector-derived mRNA, indicating that the block occurs prior to translation and after entry of the virion into the cell (Halbert et al., 1995), with the difference appearing to be at the level of transcription.

7.2.2.3.3 Purification and Concentration of Recombinant AAV-2

The crude recombinant AAV-2 produced from the 293 cells contains cellular debris and it is contaminated by the adenovirus required for production of the recombinant virions. Adenovirus can be inactivated by heating the crude preparation at 56^{o}C for 1 hour, which does not significantly decrease the titre of AAV. *In vivo* gene transfer requires the production of pure, high titre recombinant AAV and the classical method involves ultracentrifugation of the crude low titre preparation on a caesium chloride gradient. AAV-2 bands at a

density of 1.42 to 1.45g/ml and the virus can be harvested from this fraction and the caesium chloride removed by dialysis against PBS. The virus can then be used or stored at -70°C. Column chromatography using Cellulofine sulfate has also been utilised for concentrating AAV vectors (Tamayose et al., 1996), an advantage being that a large volume of crude virus can be processed by this method.

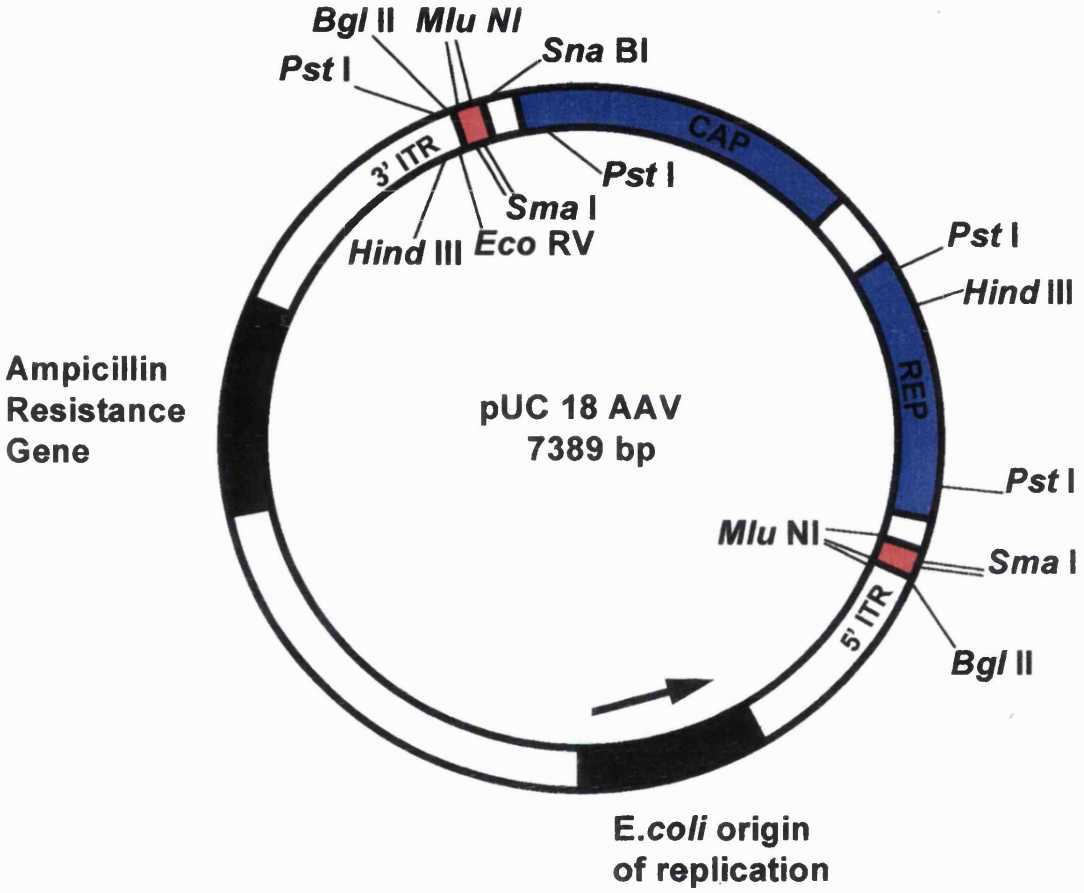
7.3 CLONING OF AAV-2 INTO pUC 18

The cDNA for AAV type 2 was obtained from the ATCC in an unknown plasmid containing the ampicillin resistance gene. The cDNA for AAV was to be released from the vector by digestion with the restriction enzyme *Bgl* II, but on digestion the two fragments were very similar in size. To facilitate removal of the AAV cDNA from the vector the construct was digested with the restriction enzyme *Pvu* I, which has a cutting site within the ampicillin resistance gene. As a positive control pUC18 (containing one restriction site for *Pvu* I also in the ampicillin resistance gene) was also digested with the restriction enzyme *Pvu* I. Each of the DNA constructs were digested once demonstrating that the enzyme was successfully cutting in the ampicillin resistance gene. The *Pvu* I digest of AAV was cleaned using a Qiagen clean up column and was digested with the restriction enzyme *Bgl* II. The digested DNA was then run on a 0.8% agarose gel and the DNA visualised using a long wavelength UV lamp to minimise damage to the DNA. Three bands were seen, the largest band containing the AAV cDNA, was carefully excised from the agarose gel and the DNA extracted as described in section 2.2.3.2.

The AAV cDNA was then subcloned into the vector pUC 18. pUC 18 was digested with the restriction enzyme *Bgl* II and the DNA was cleaned using a Qiagen clean up column. To decrease the risk of the DNA religating, the vector DNA was dephosphorylated. The digested pUC 18 (50μl) was added to 5.7μl NEB buffer 2, 2μl of calf intestinal phosphatase and incubated for 2 hours at 37°C. The dephosphorylated DNA was then cleaned using a Qiagen clean up column. The AAV DNA extracted from the agarose gel was then ligated to

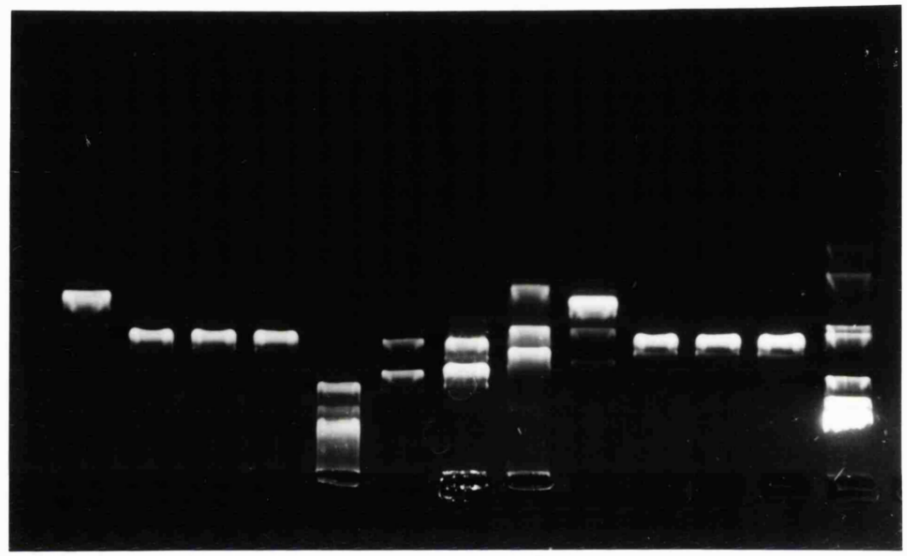
the dephosphorylated pUC 18 at an insert:vector ratio of 5:1, as described in section 2.2.3.3, using the appropriate no insert and no ligase controls. 25μl of each ligation mix were transformed into competent cells (strain SURE) (section 2.2.2.1), prepared as for TG-1 competent cells described in section 2.2.2. SURE bacteria were chosen as they are deficient in the recombinases (b+j). The combination of recB and recJ mutations confers a recombination phenotype to the SURE cells that greatly reduces homologous recombination, which the viral ITRs in the AAV are susceptible to. The transformed competent cells were then selected on LB agar containing ampicillin at a concentration of 50μg/ml. 12 ampicillin resistant colonies were picked and expanded overnight in 5 mls LB medium containing ampicillin (50μg/ml). The plasmid DNA was extracted, digested with *Bgl* II (NEB) and an aliquot of each of the restriction digests was then visualised on a 0.8% agarose gel to see if the cDNA for AAV-2 (4681bp) had been released from pUC 18. To confirm that the insert was the cDNA for AAV each of the 12 clones and pUC 18 were also digested with the restriction enzymes *Hind* III (NEB), or *Pst* I (NEB). Clones containing the AAV insert would expect to yield 2 fragments of 2837 and 4488bp on digestion with *Hind* III and 4 fragments of 460, 1495, 2280 and 3171bp on digestion with *Pst* I. Clones without an AAV insert would yield a single fragment of 2690bp with both restriction enzymes (see figure 32). Of the 12 clones, only clone 5 gave the expected fragments after digestion with *Bgl* II, *Hind* III or *Pst* I. Clones 1, 2, 3, 4, 6, 7, 8, 9, 10,11 and 12 all yielded a single band of 2690bp suggesting that religation of the linearised pUC 18 had occurred in these cases. The restriction fragments produced on digestion with *Bgl* II, *Hind* III and *Pst* I, with clones 1 and 5 are shown in figure 33.

Figure 32 Restriction map of pUC 18 AAV



Legend to Figure 32:
Restriction map of pUC18 AAV, showing the sites used in confirming clones and in the generation of subsequent subclones.

Figure 33 Restriction digest of pUC18 AAV clones with *Bgl* II, *Hind* III and *Pst* I



Legend to Figure 33:
Restriction digest analysis of putative pUC 18 AAV clones 1 and 5. Lane 1 uncut control of clone 1. Lanes 2 to 4 *Bgl* II, *Hind* III and *Pst* I digests of clone1 respectively. Lane 5 uncut control of clone 5. Lanes 6 to 8 *Bgl* II, *Hind* III and *Pst* I digests of clone 5 respectively. Lane 9 uncut control of parental pUC 18 and lanes 10 to 12 contain *Bgl* II, *Hind* III and *Pst* I digests of pUC 18 respectively. Lane 13 λ *Mlu* I size markers (26282, 9824, 5090, 2419, 2205, 1268, 965 and 458 bp).

7.3.1 Production of AAV Helper Plasmid

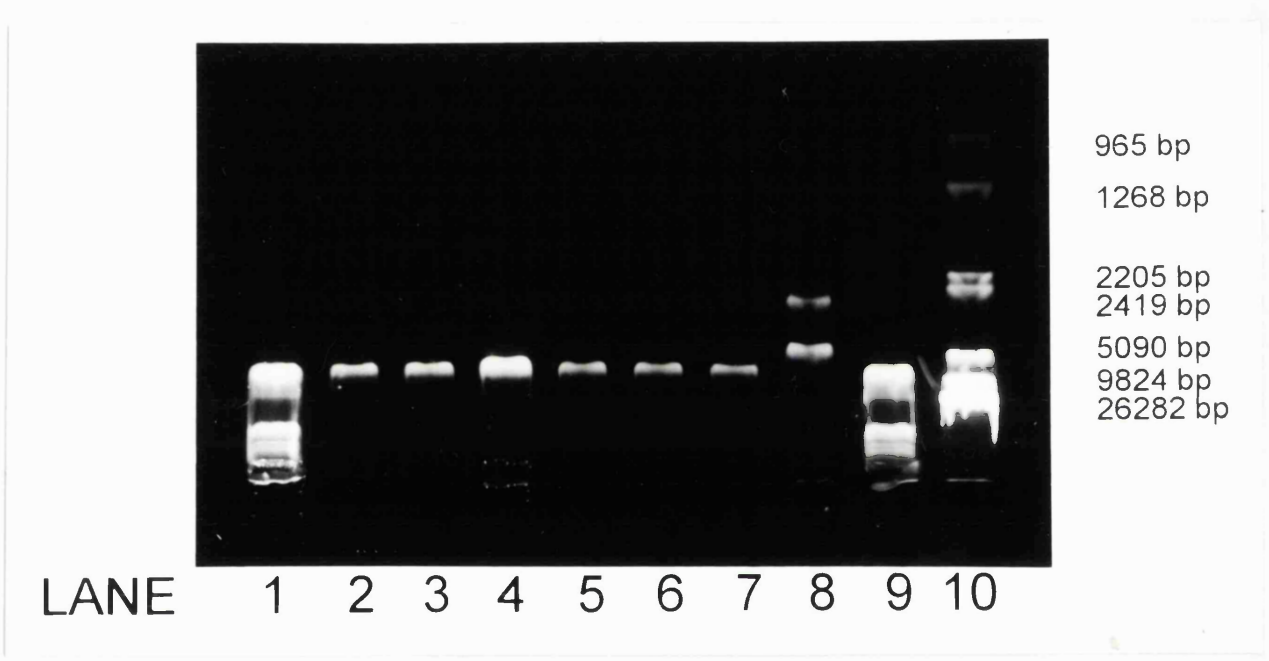
The rAAV helper plasmid supplying the *rep* and *cap* genes *in trans* was produced by removing the two ITRs from the AAV pUC 18 plasmid by serial restriction digestions followed by religation (see figure 32). The 3' ITR was removed by digesting pUC 18 AAV with *Sna* BI (NEB) and subsequently with *Eco* RV (NEB). The linearised DNA was then ligated and transformed into SURE competent cells, which were then selected on LB agarose containing ampicillin at a concentration of 50μg/ml. The following day 6 clones were picked and cultured overnight in LB medium containing ampicillin at 50μg/ml. The plasmid DNA was extracted using the Qiagen miniprep columns (see section 2.2.3). 1μg of each of the 6 clones and the parental pUC 18 AAV was digested with *Bgl* II (NEB). *Bgl* II cuts adjacent to the 5' and 3' ITRs and successful removal of the 3' ITR would result in 1 band of 7389 bp as opposed to 2 bands of 4681 and 2708 bp on digestion (figure 32). The plasmid DNA from the six clones all yielded a single fragment of approximately 7389 bp (Fig 34), demonstrating successful removal of the 3' ITR.

One of the clones (clone 1) with the 3' ITR successfully removed was then digested with *Mlu* NI (Promega) which cuts either side of the 5' ITR. After full digestion the linearised DNA was ligated and then transformed into SURE competent cells. The transformed bacteria were selected on LB agarose containing ampicillin (50μg/ml) and the following day three clones were picked and grown in LB medium containing ampicillin (50μg/ml).

To confirm that the 5' and 3' ITRs had been removed from the helper plasmid the three putative clones were digested with the restriction enzyme

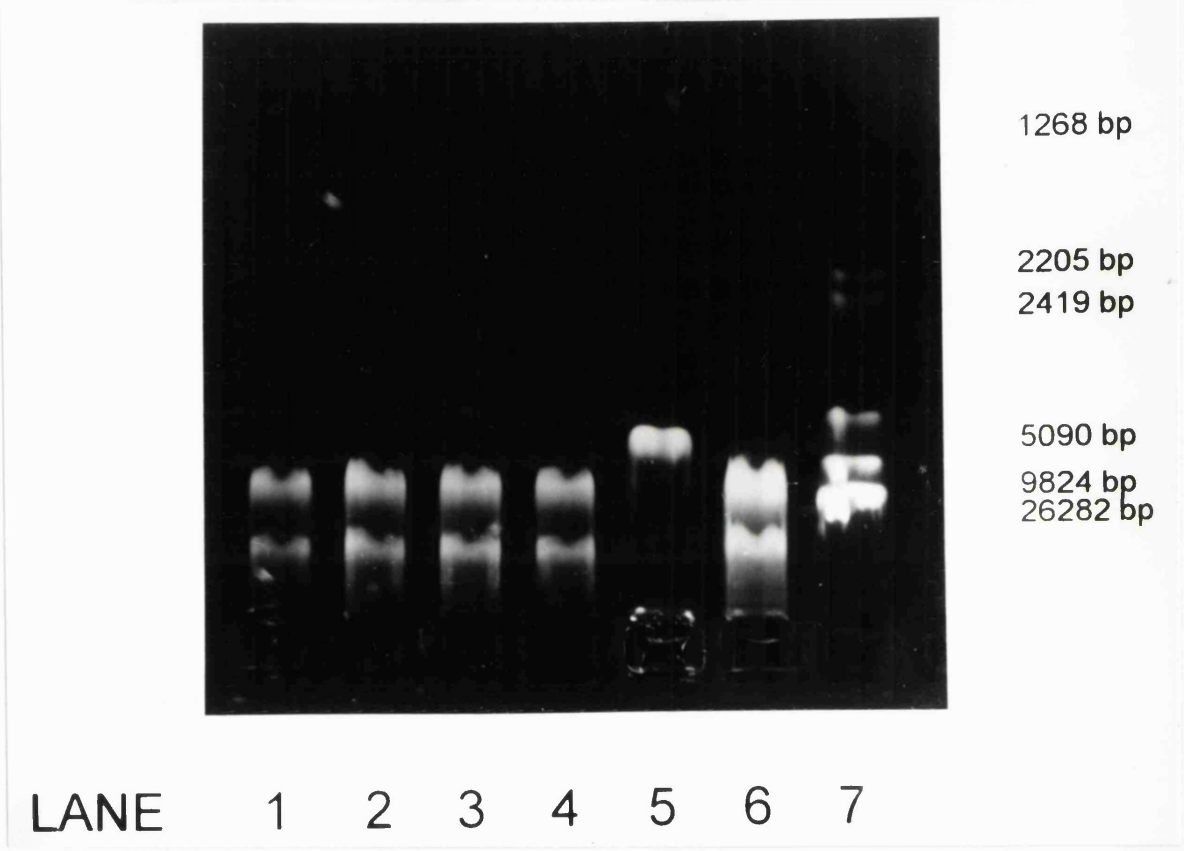
Sma I. This enzyme cuts four times in the parental plasmid, all the digestion points being within the 5' and 3' ITRs (see figure 32). Each of the three helper clones and the parental pUC 18/AAV plasmid were digested with *Sma* I (NEB). An aliquot of each of the restriction digests was visualised on a 0.8% agarose gel. All three clones remained uncut as compared to the uncut controls (fig 35), indicating successful removal of both 3' and 5' ITR's from pUC 18 AAV. As a positive control parental pUC 18 AAV digested with *Sma* I yielded fragments of 2828, 4539, 11 and 11bp, only the two larger fragments being visible within the resolution of a 0.8% agarose gel.

Figure 34 Digest with *Bgl* II demonstrating removal of 3' ITR.



Legend to Figure 34:
Restriction digest with *Bgl* II of 6 putative clones with the 3' ITR removed. Lane 1 uncut control of clone1. Lanes 2 to 7 *Bgl* II digest clones 1 to 6. Lane 8 pUC 18 AAV *Bgl* II digested. Lane 9 an uncut control of pUC 18 AAV and lane 10 λ *Mlu* I DNA digest.

Figure 35 Digest of putative helper plasmids with *Sma* I



Legend to Figure 35:
Restriction digest of 3 putative AAV helper plasmids with *Sma* I . Lane 1 uncut control of clone 1. Lanes 2 to 4 clones 1 to 3 digested with *Sma* I. Lane 5 *Sma* I digest parental pUC 18 AAV. Lane 6 uncut control of parental pUC 18 AAV and lane 7 a λ *Mlu* I DNA digest.

7.3.2 Cloning of B7-2 into AAV

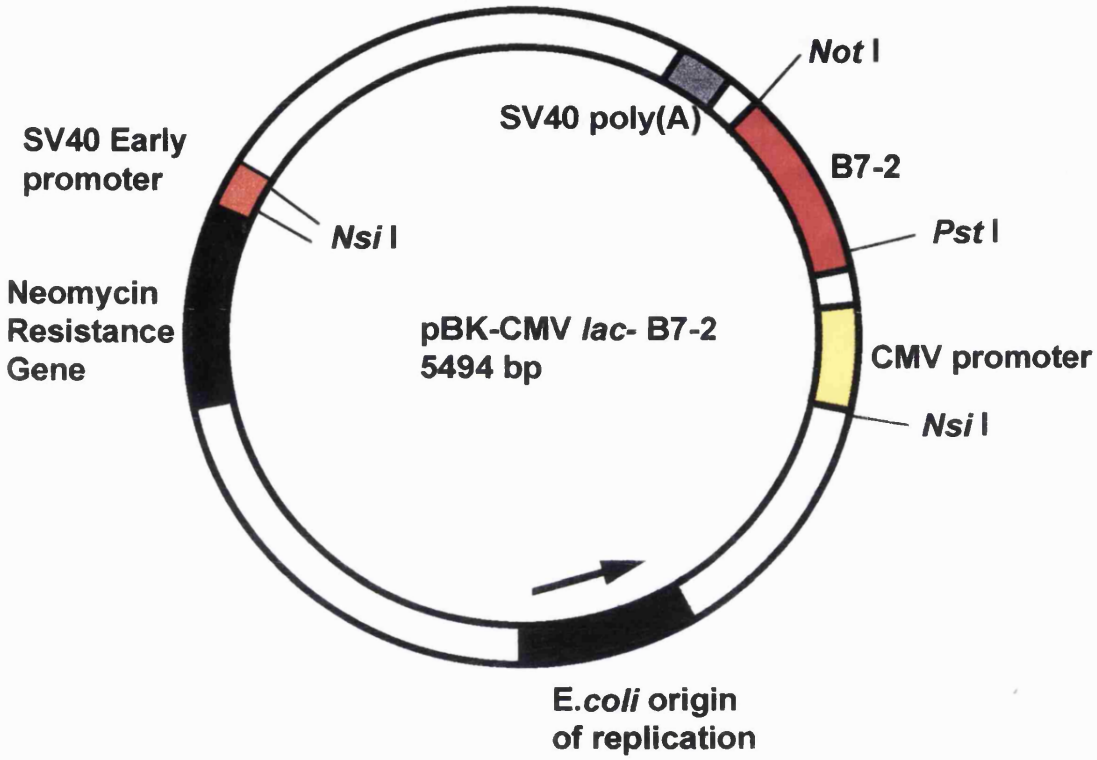
Packaging of the gene of interest within recombinant AAV requires the construction of a second plasmid containing the ITRs which has had the *rep* and *cap* genes removed. Removal of the *rep* and *cap* genes allows the insertion of DNA up to 105% of the wild type genome between the ITRs (Muzyczka, 1992). Thus with recombinant AAV no viral proteins are expressed and the only viral DNA which is returned to the target cell are the ITRs. An AAV pUC 18 construct lacking the *rep* and *cap* genes and into which the SV40 late poly A sequence had been cloned so that only the gene of interest and promoter needed to be subcloned. This construct was termed pUC 18 AAV ITR poly A, into which was cloned the gene of interest, in this case B7-2 and the CMV promoter derived from the plasmid pBK-CMV *lac*-ve B7-2 (see figure 36). pBK-CMV *lac-ve* B7-2 (clone 1) was digested with Nsi I (NEB), using x10 buffer for *Nsi* I (NEB). Two bands were seen, of 2331 and 3115bp, the larger of which contained the CMV promoter and B7-2 cDNA, which was gel purified. The DNA was blunt-ended using T4 DNA polymerase. The blunted DNA was then cut with *Not* I (NEB).

To prepare the vector for ligation with the B7-2 insert, AAV pUC 18 ITR poly A was digested with *Dra* III (NEB) and the single band obtained gel purified. The digested DNA was again blunt-ended and cleaned as before.

The two DNA fragments were ligated and transformed into SURE competent cells. 12 clones were screened for the B7-2 insert by digesting with the restriction enzyme *Xba* I (NEB). *Xba* I cuts once distal to the 3' ITR and once between the B7-2 cDNA and poly A sequence, therefore, those clones

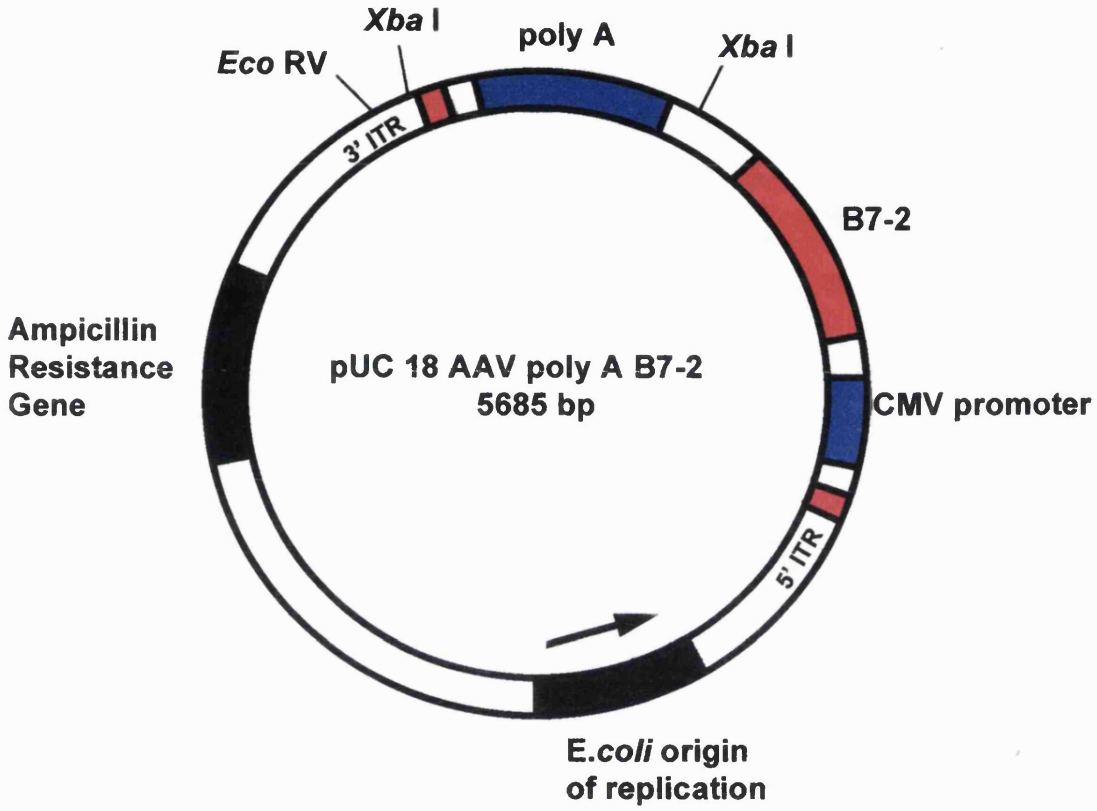
with the B7-2 insert would yield 2 bands of 5200 and 485 bp and those without the insert a single band of 3410 bp (see figure 37). Clones 1, 2, 5, 7, 9 and 10 gave 2 bands of approximately 485 and 5200 bp on digestion with *Xba* I, the smaller band not being visible on the gel (fig 38), suggesting these contained the B7-2 cDNA and CMV promoter.

Figure 36 Restriction map of *lac-ve* B7-2



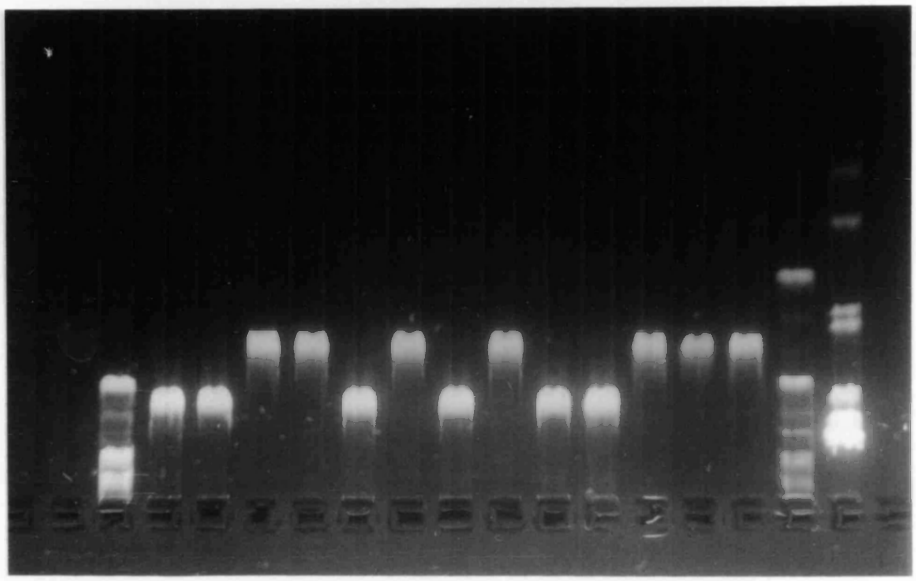
Legend to Figure 36:
Restriction map of pBK-CMV *lac*-ve B7-2 showing sites used in cloning the B7-2 expression cassette into pUC 18 AAV ITR poly A and in confirming successful subcloning of B7-2 into pBK-CMV *lac-ve*.

Figure 37 Restriction map of pUC 18 AAV poly A B7-2



Legend to Figure 37:
Restriction map of pUC 18 AAV poly A B7-2, showing sites used in confirming clones and in further manipulations.

Figure 38 Restriction digest of putative AAV clones with *Xba* I Containing B7-2 Insert.



Legend to Figure 38:

Lane 1 uncut control of clone 1. Lanes 2 to 13 *Xba* I digest clones 1 to 12 respectively. Lane 14 *Xba* I digest pUC 18 AAV poly A and lane 15 uncut control of pUC 18 AAV poly A. Lane 16 λ *Mlu* I size markers (26282, 9824, 5090, 2419, 2205, 1268, 965 and 458 base pairs).

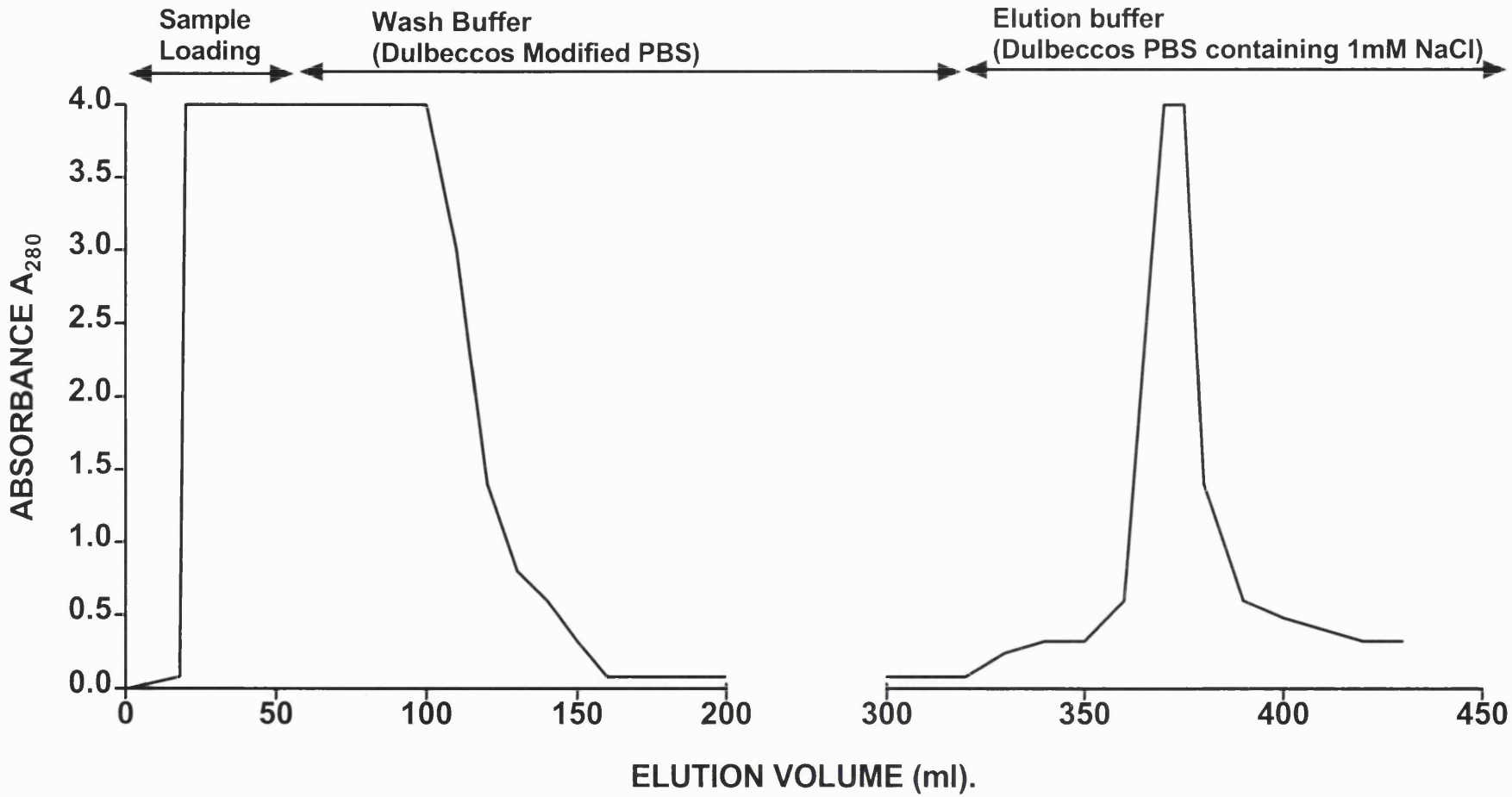
7.4 PRODUCTION OF RECOMBINANT AAV B7-2

293 cells were dual transfected with the helper and AAV vector plasmids, as described in section 2.1.10. Any contaminating adenovirus is heat inactivated at 56°C. To purify the AAV a cellulofine affinity column, which binds AAV, was used as described in section 2.1.10.1. The AAV which bound to the cellulofine column was eluted with 10mM phosphate buffer containing 1.0M NaCl. 3ml aliquots were collected and the fractions containing eluted AAV were identified by the increase in absorbance detected at 280nm (Figure 39). The appropriate fractions were pooled and concentrated as described in section 2.1.10.1. Prior to infecting cells with the viral preparation the sample was filter sterilised through a 0.2μ filter (Sartorius - Centrisart C4).

7.4.1 Detection of Capsid Proteins in Viral Concentrate by Western Blot Analysis

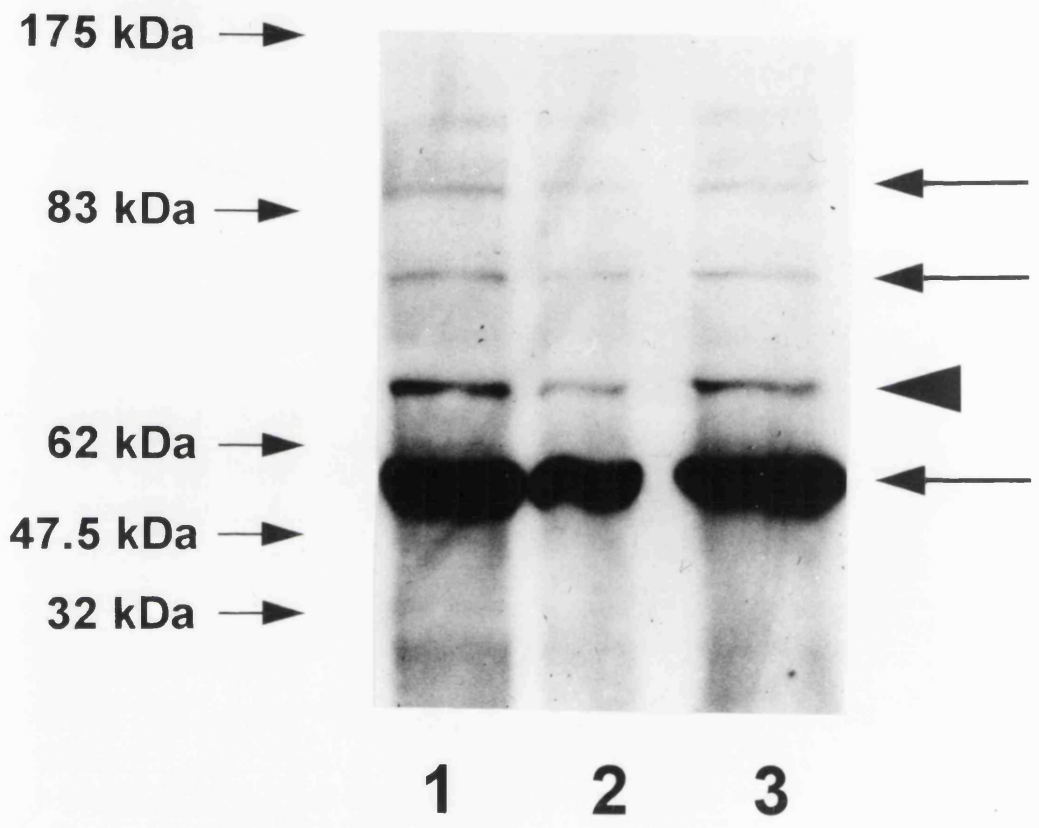
Western blot analysis of the concentrated recombinant AAV preparation was performed as described as in section 2.2.6. Three bands, 69, 72 and 90kDa, were detected in the lanes loaded with rAAV B7-2 or AAV-2. These bands correspond to the VP3, VP2 and VP1 AAV capsid proteins respectively. The strongest band was seen with the 60kDa protein, VP3, which correctly reflects the greater mRNA processing efficiency of the VP3 mRNA species with respect to both VP1 or VP2. Both AAV-2 and rAAV B7-2 showed similar ratios of VP1:VP2:VP3 (Fig 40).

Figure 39 A_{280} nm of Eluate from Cellufine Column.



Legend to Figure 39:
Absorbance at 280 nm of fractions from cellufine affinity column, demonstrating elution of bound protein from the column.

Figure 40 Western blot of AAV-2 and rAAV B7-2



Legend to Figure 40:
Lanes 1 and 3 AAV-2 and lane 2 rAAV B7-2. The accessory band highlighted on the western blot presumably represents a degradation product of the capsid proteins. This was a consistent finding on all samples on which a western blot was performed.

7.4.1.1 Discussion

Efficient production of AAV requires expression of all three capsid proteins, VP1, VP2 and VP3 to be produced in the ratios of 1:1:10 respectively. During a productive AAV infection many empty capsids are also formed. Central to the formation of empty capsids is the production of VP2. Expression of VP1 and VP2, VP2 and VP3, VP2 alone or all three capsid proteins results in empty capsid formation (Ruffing et al., 1992). Production of recombinant AAV is also complicated by the production of empty capsids. Estimates of the ratio of infectious particles to total particles is 1:200 (Chiorini et al., 1995). It has been approximated that wild type AAV yields approximately 10^4 particles per infected cell (Carter, 1992), whereas only 1 recombinant AAV particle is produced per transfected cell (Samulski et al., 1989). Thus it is important with rAAV that the production of the three cap proteins are produced in the same ratio to that seen with wild type AAV to minimise production of empty capsids. This appears to be the case with the recombinant AAV produced by the method described in section 2.1.10.

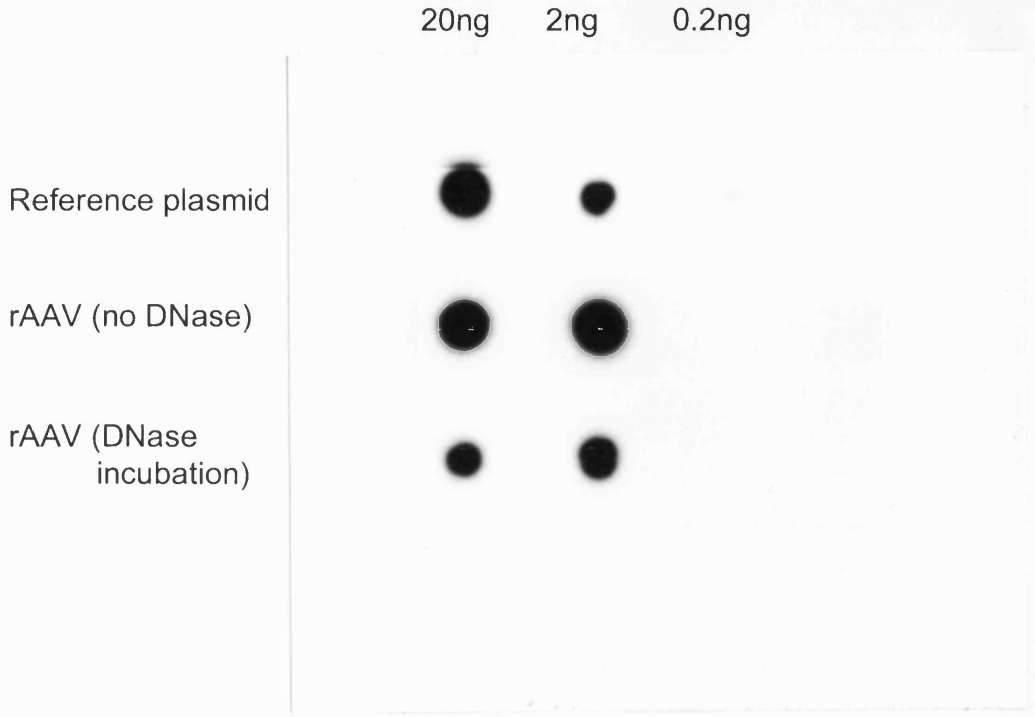
7.4.2 Titration of Recombinant AAV by Dot Blot Hybridisation

Prior to infection of target cells with recombinant virus it is important to determine the titre of AAV in the preparation. Multiplicity of infection (MOI), which is the ratio of virus to target cell, is an important factor in transduction efficiency. The MOI varies depending on the target cells being transduced. To optimise transduction efficiency in different cell types it is essential to know the titre of the virus prior to infection. To take into account empty capsids, the method of titration must assay the concentration of infectious particles. To

detect the number of recombinant AAV genomes, rather than total number of capsids, DNA dot-blot hybridisation was used as described in section 2.2.7.

Titre of recombinant AAV genomes by dot-blot hybridisation ranged between 1×10^{11} and 1×10^{12} genomes/ml. These results were consistent with virus produced by the dual transfection method and enabled the MOI to be increased up to 10,000 to 1 with certain target cells. A typical dot blot is shown in figure 41.

Figure 41 Dot-blot hybridisation of serial dilutions of recombinant AAV B7-2 and a reference plasmid preparation



Legend to Figure 41:
Dot blot hybridisation of reference plasmid pUC 18 AAV poly A B7-2 at varying concentrations and two rAAV B7-2 preparations, with and without prior DNase incubation to remove unpackaged DNA. In this sample the approximate amount of DNA in the rAAV preparations after DNase treatment equates to 2ng DNA, which equals 3.2×10^{11} genomes/ml.

7.4.3 Transduction of 293 Cells with Recombinant AAV

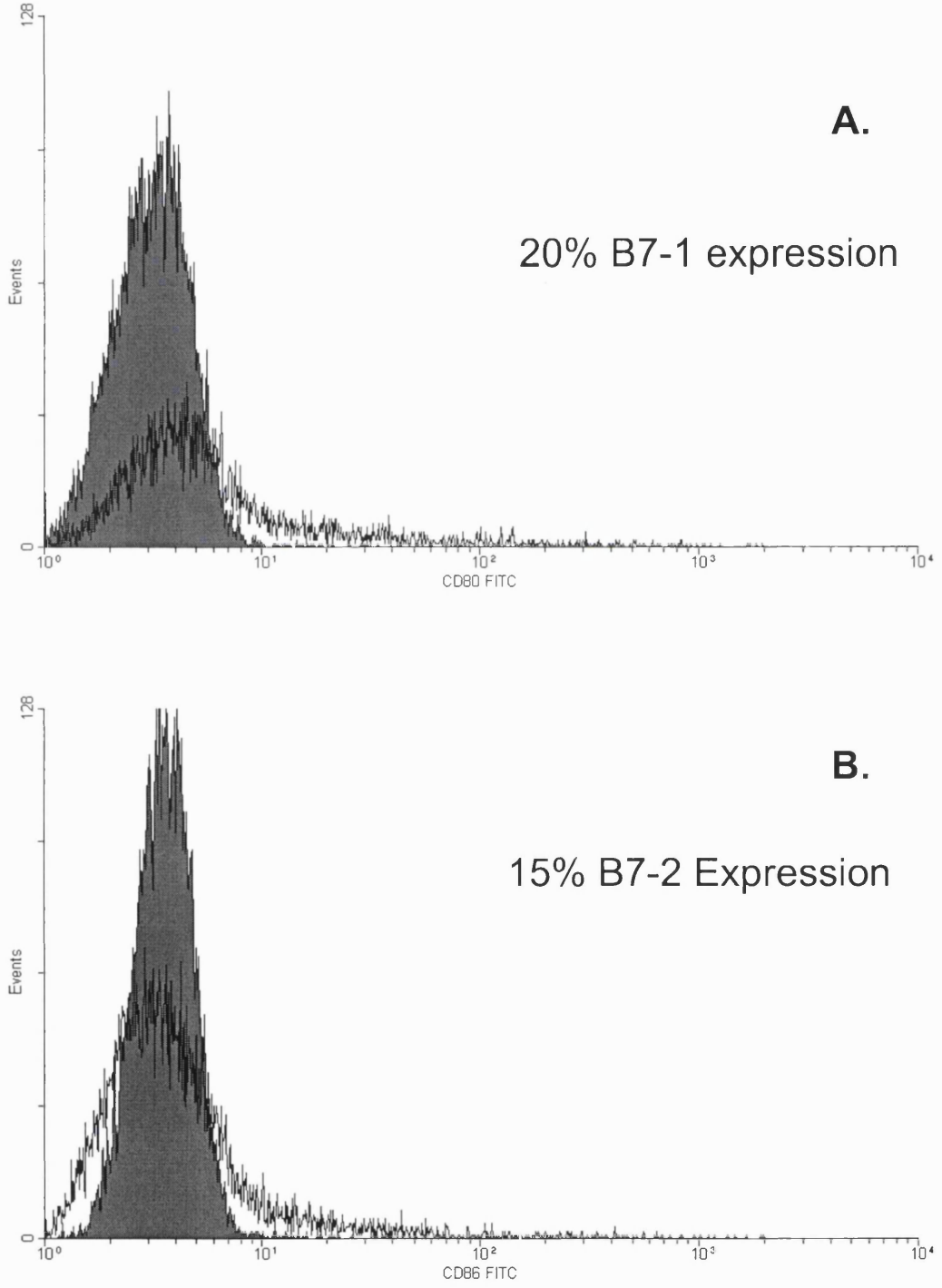
Prior to infecting primary AML blasts with rAAV, it was necessary to determine whether or not the rAAV was capable of inducing expression of the gene of interest on the target cell. With this in mind I initially used 293 cells initially which are known to be permissive to AAV infection (Ferrari et al., 1996).

293 cells were grown in Dulbecco's Modified Essential Medium (DMEM) (Gibco), supplemented with 10% Foetal Calf Serum (FCS) (GibcoBRL), penicillin (GibcoBRL) at a concentration of 50IU/ml and streptomycin (GibcoBRL) at a concentration of 50μg/ml in a 6 well plate. The adherent cells were harvested by incubation with 1:5000 versene (GibcoBRL) which non-enzymatically disadheres the cells, the AAV receptor being trypsin sensitive (Mizukami et al., 1996). The harvested cells were washed twice in HBSS and resuspended in HBSS at $1x10^6$ cells/ml. 200μl aliquots of the 293 cells were transferred to a 96 well plate, which was then centrifuged at 250 x g for 5 minutes to pellet the cells. The supernatant was aspirated and the cells were resuspended in the viral preparation at a MOI of 500:1. Both AAV B7-1 and B7-2 were used and as a negative control 293 cells were infected with AAV-2 at the same MOI. The infected cells were incubated at 37°C and 5%CO_2 for 1 hour and then the infected cells were transferred back to a 6 well plate containing Dulbecco's Modified Essential Medium (DMEM) (Gibco), supplemented with 10% Foetal Calf Serum (FCS) (GibcoBRL), penicillin (GibcoBRL) at 50IU/ml and streptomycin (GibcoBRL) at 50μg/ml. The cells were incubated for a further 48 hours at 37°C, before harvesting using Trypsin/EDTA (GibcoBRL). The cells were then labelled with either 10μl anti-

B7-1 FITC (Pharmingen - clone BB1) or 10μl anti-B7-2 FITC (Pharmingen - clone FUN-1) prior to flow cytometric analysis as described in section 2.1.3. The wild type AAV infected cells were labelled with either the anti-B7-1 FITC or anti-B7-2 FITC antibodies to act as negative controls.

48 hours post-infection approximately 15-20% of 293 cells expressed surface B7-1 or B7-2 as detected by flow cytometric analysis, compared to the control 293 cells infected with wild type AAV. These results are shown in figure 42 and demonstrate that the virus was capable of infecting the 293 cells and the expression cassette was able to drive expression of both B7-1 and B7-2 in the two viral preparations. The transduction efficiency in 293 cells is high compared with other cell lines (Qing et al., 1997) and it might be expected that with an MOI of 500:1 a higher percentage of cells would express B7-1 or B7-2. It has been estimated that titration by dot blot hybridisation overestimates the titre by a factor of 1000 (Samulski et al., 1996). The titres of infectious recombinant AAV in these experiments could therefore be of the order of 1.0 x 10^8/ml, making the effective MOI 0.5 as opposed to 500. The next step was to see whether recombinant AAV was capable of transducing primary human AML cells.

Figure 42 Expression of B7-1 or B7-2 in 293 Cells 48 hours post-infection with rAAV B7-1 or B7-2



Legend to Figure 42:
Surface expression of B7-1 (A) or B7-2 (B) in 293 cells 48 hours post infection with recombinant AAV containing the B7-1 or B7-2 expression cassettes respectively.

7.4.4 Transduction of AML Blasts with Recombinant Virus

AML cells were cultured as described in section 2.1.11. In addition to transducing AML blasts with rAAV B7-2, I also transduced AML blasts with rAAV B7-1. The rAAV B7-1 was also produced by the dual transfection method, purified, concentrated and titred as for the B7-2 virus. Transductions of the AML cells was performed as described in section 2.1.11.1. The AML cells were then infected with recombinant AAV at an MOI of 500, 1000 and 10,000, as determined by dot blot hybridisation, as described in section 2.2.7. As a negative control, target cells were also infected with AAV-2 at the same MOI as the recombinant virus. Cells were analysed for expression of the protein 72 hours post infection by flow cytometric analysis as described in section 2.1.3 after labelling with either 10μl of anti-B7-1 PE (Becton Dickinson - clone L307.4) or 10μl of anti-B7-2 FITC (Pharmingen - clone FUN-1).

72 hours post infection, neither the AML blasts infected with recombinant AAV containing the B7-1 or the B7-2 expression cassette expressed surface B7-1 or B7-2 by flow cytometric analysis. This result was a consistent finding using AML blasts of M1, M2, M4 and M5 FAB types.

7.4.4.1 Discussion

The rate limiting factor for efficient transduction by recombinant AAV is conversion of the single stranded viral genome into transcriptionally active double-stranded DNA. Factors which can increase the rate of conversion of single-stranded to double-stranded DNA include exposure of the infected cells to the adenoviral E4*orf*6 gene product, heat shock, ultraviolet light, hydroxyurea or irradiation (Ferrari et al., 1996; Alexander et al., 1996). More specifically, a

cellular protein has been identified that binds to the single-stranded D sequence within the ITR which has been termed the single-stranded D-sequence binding protein (ssD-BP). This protein is tyrosine phosphorylated and in this state prevents formation of transcriptionally active duplex DNA by inhibiting leading strand synthesis. Cellular tyrosine kinases can be inhibited with genistein resulting in the dephosphorylation of the ssD-BP and increased transgene expression (Qing et al., 1997). In addition the adenovirus E4*orf*6 protein, which also increases transgene expression, resulted in dephosphorylation of the ssD-BP, suggesting a similar mode of action to genistein (Qing et al., 1997). The importance of the ssD-BP in regulating expression from recombinant AAV was shown by the correlation between the transgene expression from recombinant AAV with the dephosphorylated form of the ssD-BP (Qing et al., 1998). Therefore it was next assessed whether irradiation of the AML blasts following infection with recombinant AAV would induce expression of the co-stimulatory molecules B7-1 or B7-2, by overcoming the rate limiting step of second strand synthesis.

7.4.4.2 Transduction of AML Blasts followed by Irradiation

AML blasts were infected with either rAAV B7-1 or B7-2 as described in section 2.1.11.1 using an MOI of 10,000:1. 18 hours post infection both the control cells infected with AAV-2 and the AML cells infected with rAAV were irradiated with 2000 rads. The AML blasts were then analysed for expression of either B7-1 or B7-2 after a further 48 hours by flow cytometric analysis as described in section 2.1.3 after labelling with either 10μl of anti-B7-1 PE

(Becton Dickinson - clone L307.4) or 10μl of anti-B7-2 FITC (Pharmingen - clone FUN-1).

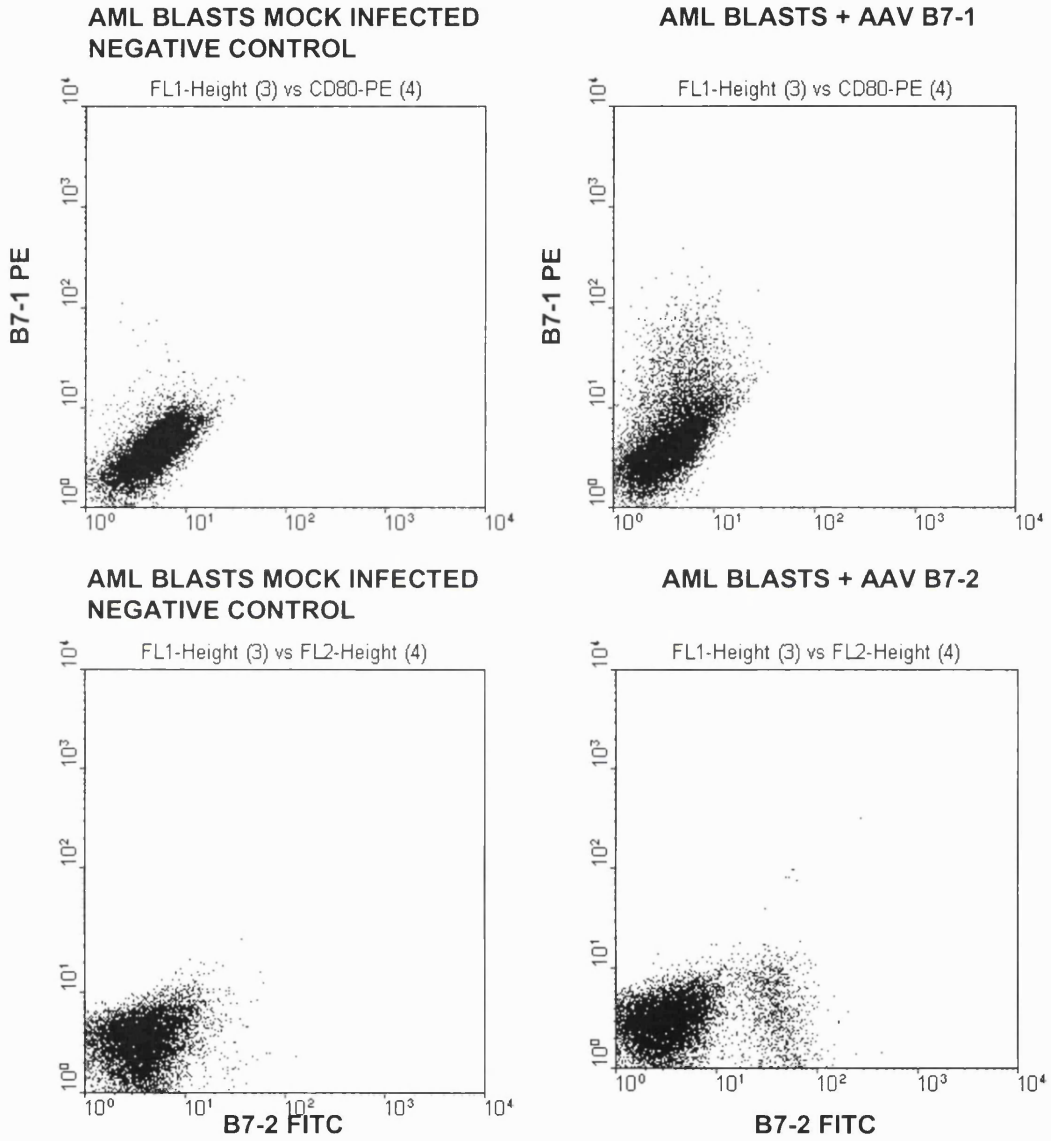
After irradiation of the infected AML cells, up to 15% of cells expressed surface B7-1 or B7-2. The percentage of cells successfully transduced varied and in some samples transduction was unsuccessful despite maintaining the MOI at up to 10000:1. The results are summarised in table 6 and dot histograms of examples of AML cells transduced with either rAAV B7-1 or B7-2 are shown in figure 43.

Table 6 Transduction of AML Blasts with Recombinant AAV

	Percentage Cells Transduced	AAV B7-1/B7-2
AML M4	7%	B7-1
AML M4	4.5%	B7-1
AML M4	4.5%	B7-1
AML M4	6.5%	B7-1
AML M1	13.2%	B7-2
AML M1	10.4%	B7-2
AML M1	8.5%	B7-2
AML M2	0%	B7-2
AML M1	3.5%	B7-2
AML M1	0%	B7-2
AML M4	3%	B7-2
AML M1	5%	B7-1
AML M4	0%	B7-1
AML M4	0%	B7-1
AML M4	15%	B7-1

Table 6:
Transduction of primary AML blasts by FAB type, giving transduction efficiency and whether the recombinant AAV contained the B7-1 or B7-2 expression cassettes.

Figure 43 Expression of B7-1 or B7-2 on AML Blasts 48 hours post infection with rAAV B7-1 or B7-2



Legend to Figure 43:

Surface expression of B7-1 or B7-2 on AML blasts transduced with rAAV B7-1 or B7-2. The FAB type of the AML cells transduced with rAAV B7-1 was M4 and the FAB type of the AML cells transduced with rAAV B7-2 was M2.

7.4.4.3 Discussion

Delivery of cDNA's coding for B7-1 and B7-2 has been demonstrated with AML cells of M1, M4 and M5 FAB types using recombinant AAV as the vector. However, in some samples of M4 and M5 FAB types transduction was unsuccessful despite the same viral preparation successfully transducing other AML blasts. The transduction efficiency with the same AML samples, using different recombinant viral preparations, was also not reproducible. Second strand synthesis is the rate limiting step in transduction efficiency with recombinant AAV (Ferrari et al., 1996) and it may be that this is the step at which expression was inhibited in some AML cells. That this may be the case is indicated by successful transduction occurring only after irradiation of the target cells post infection which increases conversion of single-stranded to double-stranded DNA (Alexander et al., 1996). That different target cells may be less efficiently transduced has been demonstrated to be related to the ratio of a phosphorylated to dephosphorylated cellular protein termed the ssD-BP. In the phosphorylated state the ssD-BP binds to the D region of the viral ITR, inhibiting leading strand synthesis. The higher the ratio of phosphorylated to dephosphorylated ssD-BP the less efficiently the target cells were transduced (Qing et al., 1998). It may be that in AML blasts the ssD-BP is largely phosphorylated and that this ratio varies between different samples. In those samples untransduced with recombinant AAV, the ssD-BP may have been predominantly phosphorylated. Factors which have been shown to increase the ratio of dephosphorylated to phosphorylated ssD-BP include adenoviral infection, in particular expression of the E4*orf*6 protein, treatment with

hydroxyurea or genistein, which is a specific inhibitor of protein tyrosine kinases (Akiyama et al., 1987). It may be that pre-treatment of the AML cells with genistein will increase transduction efficiency with recombinant AAV.

Another factor which might cause low efficiency of transduction is the level of the viral receptor on the target cells. The AAV receptor has only recently been identified as heparan sulfate proteoglycan (Summerford and Samulski, 1998), although it is possible that other molecules may be involved in adsorption of the virus. It may be that the AAV receptor is only expressed at low levels, or not at all in some AML samples. One experiment that could be done to address this issue is to produce radioactively labelled AAV particles. Cells to be labelled are incubated with radioactively-labelled AAV-2, then washed and the β-emission assessed after the addition of scintillation fluid (Qing et al., 1998). Comparison can be made between cells known to be permissive to AAV infection, such as 293 and HeLa cells and cells resistant to AAV infection, such as erythrocytes (Mizukami et al., 1996). If the receptor is absent on some AML samples, then it may be possible to upregulate the receptor, but if this is not possible it would be necessary to use an alternative vector system to transduce these cells.

A factor that may be implicated in transduction with the recombinant AAV is the method of titration of the virus which detects viral genomes. Presence of the viral genome does not equate to an infectious particle. In fact the ratio of genomes to infectious particles has been estimated at 1000:1 (Samulski et al., 1996) and therefore the MOI's in the experiments are probably overestimated by a factor of 1000. The highest MOI used in transducing AML cells may have been as low as 10:1 and an increase in this may produce an increase in

transduction efficiency. The titre of infectious recombinant AAV may have varied between different preparations, despite the titre being of a similar order after estimation by dot blot hybridisation. It may be that in the experiments where transductions were successful in AML cells, that the particular viral preparations contained a higher infectious titre of AAV. In future, a better assessment of infectious titre may be made by infection of 293 cells and analysis of transgene expression after 48 hours. In the AML samples where up to 15% transduction was observed with rAAV B7-1 and B7-2, the transduced cells were tested for their co-stimulatory ability.

7.4.5 Costimulation Assays with Transduced AML Blasts

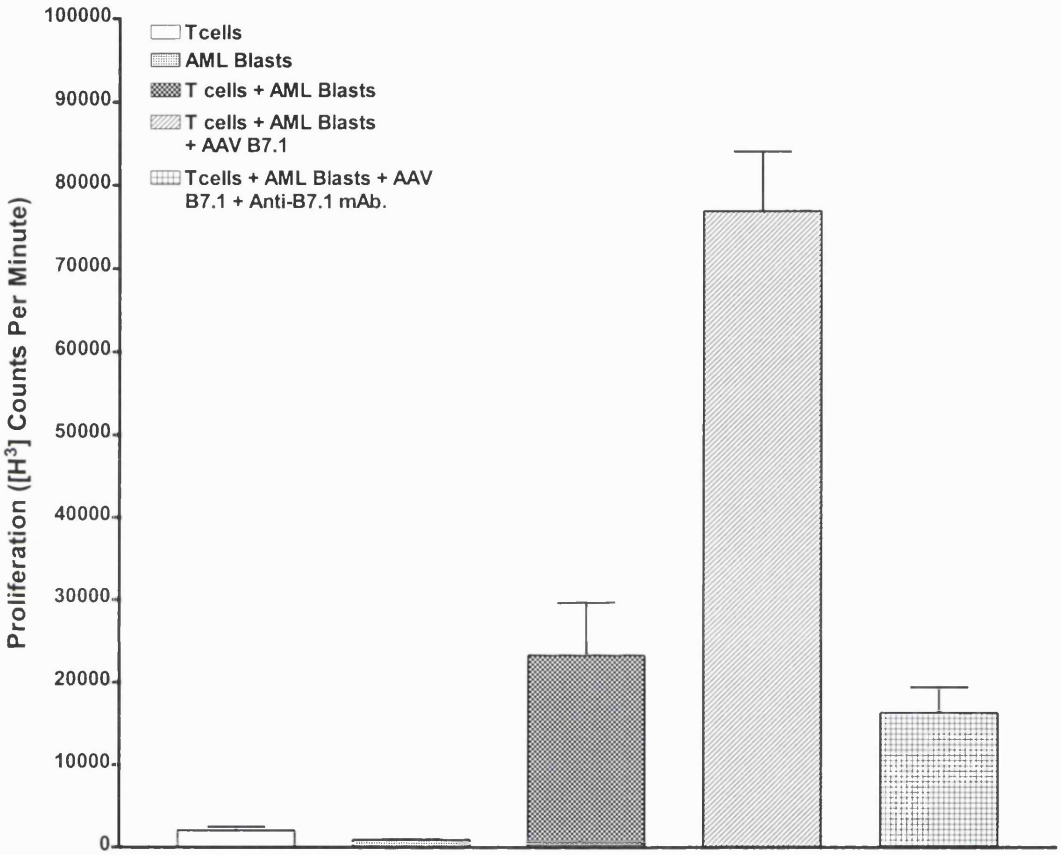
T cells were enriched from the peripheral blood of healthy volunteers, as described in section 2.1.2.1. and the modified MLR was performed as described in section 2.1.4. Since the AML blasts had already been irradiated to 2000 rads, they were not irradiated further. Control AML blasts, infected with AAV-2 at the same MOI as the AML blasts infected with recombinant AAV, were also resuspended at the same concentration of $1x10^6$ cells/ml after washing three times in HBSS. To assess the specific role of the B7-1 or B7-2 induced on the AML blasts after infection with the recombinant virus, an aliquot of each was preincubated with 10μg of either anti-B7-1 (Pharmingen-clone BB1 no azide, low endotoxin) or anti-B7-2 (Serotec-clone IT2.2, no azide, low endotoxin) respectively for 15 minutes, prior to washing and resuspension in 100μl of medium. All variables were performed in triplicate and the cells cultured for 5 days at 37°C and 5% CO_2. For the last 18 hours of the assay each well was pulsed with 1 μCi (37 kBq) of [methyl-^{3}H]-thymidine (Amersham).

Incorporation of tritiated thymidine, as a measure of proliferation, was assessed as described in section 2.1.4. These experiments were repeated twice, using 2 different donors as a source of T cells.

With the AML blasts transduced with rAAV B7-1, with approximately 15% surface B7-1 expression, mean proliferation of the responding T cells was 76920 c.p.m. (sd 7110) and with preincubation with anti-B7-1 proliferation of the responding T cells was 16305 c.p.m. (sd 3067). In comparison proliferation of T cells with control AML blasts as stimulator cells was 23295 c.p.m. (sd 6280). Proliferation of unstimulated T cells was 2064 c.p.m. (sd 435) and background proliferation of irradiated AML blasts was 961 c.p.m. (sd 184). These results are summarised in figure 44.

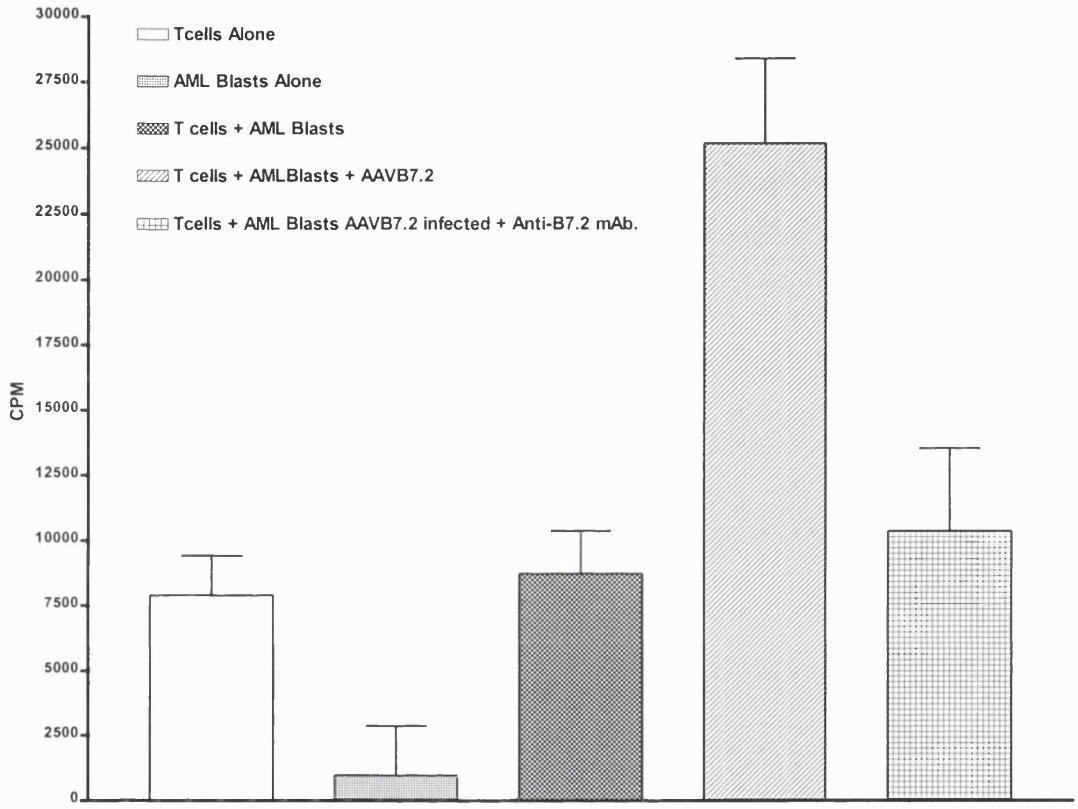
With the AML blasts transduced with AAV B7-2, with approximately 13% of cells expressing surface B7-2, mean proliferation of the responding T cells was 25148 c.p.m. (sd 3218) and with preincubation with anti-B7-2 proliferation of the responding T cells was 10360 c.p.m. (sd 3171). In comparison proliferation of T cells with control AML blasts as stimulator cells was 8718 c.p.m. (sd 1641). Proliferation of unstimulated T cells was 4562 c.p.m. (sd 1521) and background proliferation of irradiated AML blasts was 961 c.p.m. (sd 1924). These results are summarised in figure 45.

Figure 44 Proliferation of Allogeneic T cells in Response to AML blasts Transduced with AAV B7-1



Legend to Figure 44:
Proliferation of allogeneic T cells induced by AML blasts transduced with AAV B7-1 and inhibited by anti-B7-1 mAb.

Figure 45 Proliferation of Allogeneic T cells in Response to AML blasts Transduced with AAV B7-1



Legend to Figure 45:
Proliferation of allogeneic T cells induced by AML blasts transduced with AAV B7-2 and inhibited by anti-B7-2 mAb.

7.4.6 Construction of rAAV Cell Line

A major disadvantage of rAAV over other vectors is the intensity of labour involved in its production. With this in mind a plasmid containing the expression cassette within the ITRs and the *rep* and *cap* genes outside the ITRs was constructed.

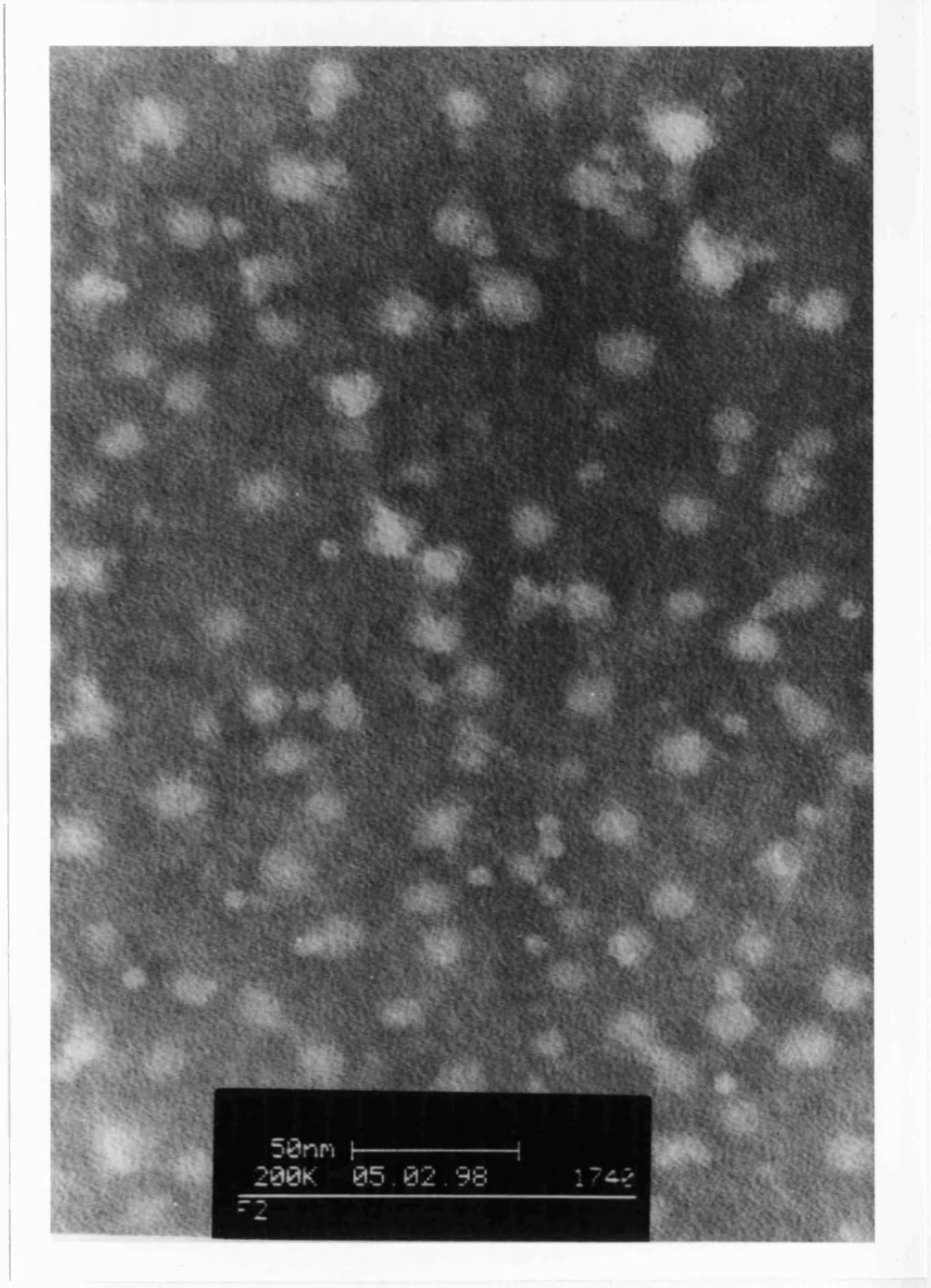
A plasmid containing the *rep* and *cap* genes and neomycin phosphotransferase gene was serially digested with the restriction enzymes *Ase* I and *Apa* LI to yield two bands of approximately 6 and 2.5 kB. The digested DNA was run on a 0.8% agarose gel and the larger band, containing the *rep* and *cap* genes and neomycin phosphotransferase gene, was cut out and gel extracted (section 2.2.3.2). The extracted DNA was then blunted incubating at 12°C for 20 minutes with T4 DNA polymerase (3 units), 0.01mM of each dNTP and T4 polymerase buffer (50 mM NaCl, 10mM Tris-HCl, 10mM $MgCl_2$, 1mM dithiothreitol, pH 7.9). pUC AAV B7-2 poly A was digested with *Eco* RV (Fig 37) and after visualisation on a 0.8% agarose gel was gel extracted. The linearised DNA was then dephosphorylated by incubation with calf intestinal phosphatase (20 units) with NEB buffer 1 at 37°C for 1 hour to inhibit religation of the vector. The vector and insert DNA were then ligated as described in section 2.2.3.3. The ligated DNA was transformed into SURE competent cells and selected on agarose containing kanamycin at 50μg/ml. After selection bacterial colonies were screened by PCR (section 2.2.5) using *cap* specific primers (Forward primer 5' GGGGATTACCTCGGAGAAGCAGTG 3' and reverse primer 5' CATGATCCAGACGGCGGGTGAGT 3'). Annealing temperature used was 64°C and 30 cycles of PCR were performed. Of 23

clones screened, two yielded the expected product of 450bp. One of these clones, clone 14 was transfected into HeLa cells (ECACC No: 85060701) and stable transfectants were selected by culturing in supplemented DMEM containing 500μg/ml G418. HeLa cells were used as 293 cells innately express E1a and E1b which would induce expression of the *rep* gene.

Infection of the transfected HeLa cell with adenovirus resulted in the production of rAAV containing the B7-2 expression cassette. Titration by dot blot demonstrated a similar titre of rAAV was produced by this method and western blot analysis of the viral preparation showed VP1, VP2 and VP3 produced in the same proportions as AAV-2. Electron microscopy of the viral preparation also revealed the presence of virions of expected size of approximately 20nm diameter (see Fig 46).

Infection of 293 cells with the rAAV B7-2 produced with the cell line, using the same MOI as described in section 7.4.3, resulted in similar transduction efficiency as that achieved using rAAV produced by the dual transfection method. The cell line therefore provided an easier method of producing rAAV of similar titre to that obtained using transfection and also decreased the cost of production by negating the requirement of cationic lipid in the procedure. A major drawback of this procedure would be production of AAV-2 due to recombination events in the cell line. AAV-2 has not been detected so far in the rAAV B7-2 produced with the cell line, which have been screened for the *cap* gene with PCR using the conditions described above.

Figure 46 Electron Microscopy of rAAV Preparation



Legend to Figure 46:
Electron microscopy of rAAV B7-2 at x 200,000 magnification, produced from HeLa cells stably transfected with clone 14 and subsequently infected with adenovirus. Expected size of mature AAV particles is 20nm which is approximately the size of the particles seen in comparison with a size marker of 50nm.

7.4.7 Discussion

The increased proliferation of allogeneic T cells with as few as 10 to15% of AML blasts expressing either B7-1 or B7-2 demonstrates that this level of expression is able to deliver a co-stimulatory signal. This supports the data from section 3.1.5.1 in which 5-10% of innately B7-2 expressing AML blasts were able to stimulate allogeneic T cell proliferation and IL-2 production. That a low percentage of cells expressing B7-1 are able to deliver a co-stimulatory signal has been previously shown using a myeloma cell line, where as few as 300 B7-1 positive cells, out of a total of 20,000 cells, were able to induce allogenic T cells to proliferate (Wendtner et al., 1997). Increased proliferation is due to costimulation by B7-1 or B7-2 expressed on the transduced AML blasts as demonstrated by the inhibition of proliferation using anti-B7-1 or anti-B7-2 mAbs respectively. To prevent interference from inate expression of co-stimulatory molecules, the co-stimulatory assays were performed using AML blasts that did not innately express either B7-1 or B7-2. Whether 5 to 15% B7-1 or B7-2 expression is sufficient to costimulate an autologous anti-leukaemic immune response remains to be determined. In this assay stimulation through the T cell receptor is provided by a strong allogeneic signal and as proposed by the strength of signal hypothesis, a weak co-stimulatory signal would be able to induce T cell activation (Lenschow et al., 1996). However, in an autologous setting, the only signal through the T cell receptor would be provided by potential tumour antigens. These may or may not be tumour specific and are likely to be expressed at variable intensity. If the tumour antigen is only expressed at a low intensity, a stronger co-stimulatory signal may be required

to induce T cell activation, necessitating greater transduction efficiency with recombinant AAV. This may not be the case as some potential target antigens, such as proteinase 3, are overexpressed in AML blasts (Dengler et al., 1995). The data suggesting that there was prolongation of CR1 with 30% or more AML blasts innately expressing B7-2, suggests that a higher level of co-stimulatory signal may be beneficial (section 3.1.3.5).

Further studies need to be performed to see whether the AML blasts transduced with B7-1 or B7-2 are more efficient at generating CTL's. These could be performed initially with allogeneic T cells. A problem with AML blasts is that they are difficult to label with ^{51}Cr, so an alternative method of assessing specific cytotoxicity would have to be developed. Some assays use a colorimetric assay which is dependant on release of lactate dehydrogenase (LDH) from the target cells (Weidmann et al., 1995). An alternative method involves labelling of the target cells with a membrane dye and cell death can be assessed by the inability of the target cells to exclude propidium iodide (PI) after labelling with a membrane dye (Lowdell et al., 1997). In addition, *in vitro* studies need to be performed to see whether the transduced AML blasts are capable of stimulating an autologous T cell response and whether the T cells would have specific anti-leukaemic activity. To test whether the transduction efficiency with recombinant AAV is capable of generating an autologous anti-leukaemic response *in vivo* a murine model must also be developed. As the cDNA's for B7-1 and B7-2 are human and the AML targets are human, a human model of AML would have to be developed. Engraftment of both leukaemic and normal haemopoietic progenitors has been demonstrated in SCID mice (Lapidot et al., 1992; Lapidot et al., 1994), which are deficient in T

cell mediated immunity and this would enable the development of a human model of AML in mice. Although engraftment of haematopoietic progenitors has been demonstrated in SCID mice, NOD/SCID mice may prove a better model. In addition to lacking T and B cell function, NOD/SCID mice have low natural killer cell activity, lack haemolytic complement and have defective macrophage function. Consequently engraftment of haematopoietic progenitors is improved in NOD/SCID mice when compared to SCID mice (Hogan et al., 1997).

8.1 GENERAL DISCUSSION

8.1.1 Innate Co-stimulatory Ability of AML Blasts

Since the demonstration that B7-1 expression on tumourigenic cells was required to induce an effective T cell mediated anti-tumour response *in vivo* in a murine model of melanoma (Chen et al., 1992), many studies have demonstrated the effectiveness of this strategy in a variety of murine tumour models, including AML (Chen et al., 1994a; Matulonis et al., 1995; Matulonis et al., 1996). AML would appear to be a suitable candidate disease for immunotherapeutic approaches as the reduced leukaemic recurrence after allogeneic in contrast to autologous BMT is due to an anti-leukaemic immune response termed GvL (Horowitz et al., 1990). The target antigens identified to date, in GvL, are thought to be predominantly minor histocompatibility antigens (Goulmy et al., 1991), although overexpression of myeloid specific proteins or production of fusion proteins due to translocations could act as tumour antigens (Molldrem et al., 1996; Bosch et al., 1996; Gambacorti-Passerini et al., 1993). Phenotyping of 39 AML samples revealed that all samples expressed MHC class I and class II (section 3.1.3.3) and should therefore be capable of presenting such antigens. In addition, all samples expressed the adhesion molecule ICAM-1 (section 3.1.3.4), which is able to deliver a co-stimulatory signal, but does not induce IL-2 production as efficiently as B7-1 or B7-2 (Damle et al., 1992). All samples also expressed the adhesion molecule LFA-1 (section 3.1.3.4), which is important in both induction and effector stages of an immune response (Davignon et al., 1981; Schmits et al., 1996; Sligh, Jr. et al., 1993). As might be expected B7-1 was rarely expressed on AML blasts and

when present, only at an intensity less than that found on cells known to be capable of antigen presentation (section 3.1.3.2). Thus it would seem that inducing B7-1 expression on AML blasts would be an approach that could result in an effective anti-leukaemic response, as the other molecules required are innately expressed. Surprisingly, the majority of AML samples expressed B7-2 and, in some samples, at an intensity seen on monocytes (section 3.1.3.2). In a modified MLR, the B7-2 expressing AML blasts stimulated allogeneic T cells to proliferate, which was only partially blocked with CTLA-4 Fc. This could be explained by ICAM-1 expression on the AML blasts which can costimulate T cell proliferation, but less efficiently IL-2 production (Damle et al., 1992). Assessing IL-2 production from the allogeneic T cells revealed increased production from the B7-2 expressing blasts which was inhibited by CTLA-4 Fc. Thus the B7-2 on the AML blasts was functional in delivering a co-stimulatory signal. As the majority of AML samples expressed B7-2, as well as the other molecules required in the induction of an anti-leukaemic immune response, it may be that some patients were able to induce an autologous anti-leukaemic immune response. Indeed in those patients with 30% or more blasts expressing B7-2, the duration of CR1 was longer than in those patients where less than 30% of AML blasts expressed B7-2 (section 3.1.3.5). This prolongation of CR1 may be due to the presence of an anti-leukaemic immune response, although with the small number of patients it is difficult to control for other factors known to affect prognosis. Age, white cell count at presentation, gender and cytogenetic abnormalities were similar in both groups. Two patients in the lower B7-2 expression group had an autologous BMT, known to improve survival, compared to four patients in the higher B7-2 expression

group. The major discrepancy between the two groups is that three patients in the higher B7-2 expression group had a sibling allogeneic BMT, two of which became long term survivors. None of the patients in the lower B7-2 expression group had a sibling allogeneic BMT. The data still suggests that the strategy of inducing B7-1 or B7-2 expression on AML blasts, or increasing B7-2 expression in those samples innately expressing B7-2 would seem beneficial.

8.1.2 Differential Function of B7-1 and B7-2

Since the discovery of B7-2 there has been debate over whether B7-1 and B7-2 deliver identical co-stimulatory signals, which may have relevance to tumour vaccination strategies using B7-1 or B7-2 expressing tumour cells. Initial studies seemed to suggest that B7-1 and B7-2 delivered identical co-stimulatory signals in terms of T cell proliferation and cytokine production (Lanier et al., 1995). However, since B7-2 is innately expressed on antigen presenting cells and B7-1 is upregulated after induction of an immune response, suggests that the two molecules may have distinct functions (Hathcock et al., 1994; Azuma et al., 1993b). Indeed it has been demonstrated that B7-1 and B7-2 bind with different avidity and to different binding sites of CD28 (Linsley et al., 1994). Subsequent studies have indicated that costimulation through B7-2 may favour development of a T_H2 immune response and B7-1 a T_H1 immune response (Kuchroo et al., 1995; Freeman et al., 1995). In tumour vaccination models, some studies have found no difference in effectiveness between B7-1 or B7-2 expression on the tumour cells (Hodge et al., 1994; Yang et al., 1995), although in other studies B7-1 expression on the tumour cells was more effective than B7-2 in eradicating pre-existing disease (Matulonis et al., 1996). Eradication of existing disease is an important

consideration, as relapse may occur from the small number of cells not killed by chemotherapeutic agents, which may be detected only by molecular techniques (Cross, 1997). With this in mind stable transfectants of 293 cells expressing either B7-1 or B7-2 were generated and used to stimulate allogeneic T cells. Cytotoxicity was assessed against parental 293 cells and was consistently higher with the B7-1 293 cells used as stimulators, followed by B7-2 and finally control 293 cells. There was a significant increase in cytotoxicity with the B7-1 expressing 293 cells as stimulators compared to the control 293 cells, but this was not the case with the B7-2 expressing 293 cells. Analysis of the supernatants revealed higher levels of γ-IFN, a T_H1 cytokine, with the B7-1 293 cells, although this was not significantly different to the control or B7-2 expressing 293 cells. IL-4 production from T cells stimulated with the 293 cells was generally low, but again tended to be higher with the B7-1 293 cells. Similarly detection of intracellular γ-IFN and IL-4 in responding T cells did not reveal any difference between control, B7-1 or B7-2 expressing 293 cells as stimulators. In general these results suggest that B7-1 may be more effective than B7-2 in the generation of CTL's and the induction of γ-IFN. In addition the intensity of B7-2 expression was higher on the 293 cells than B7-1 (section 4.6.6). If the B7-1 expression was of an equivalent level to the B7-2 on the 293 cells, it may be that significant differences would have been seen in production of γ-IFN from the responding T cells and cytotoxicity against parental 293 cells. To demonstrate differences between B7-1 and B7-2 in development of a T_H1 or T_H2 immune response, it may be necessary to use naive T cells as the responders as they have the potential to progress to either a T_H1 or T_H2 phenotype (Freeman et al., 1995). The normal range of CD45RA T cells in the

peripheral blood is up to 50% of the total T cell population (Lowdell et al., 1998). If B7-1 tends to induce a T_H1 and B7-2 a T_H2 immune response, it may be that induction of B7-1 would be more effective than B7-2 expression on AML cells in inducing an effective anti-leukaemic response, which is supported by the results using murine AML (Matulonis et al., 1996).

8.1.3 Recombinant AAV

With the recombinant AAV used in this study, up to 15% expression of either B7-1 or B7-2 has been induced on primary AML blasts. However, transduction efficiency is variable between different samples, which may reflect a variance in the expression of the putative AAV receptor, heparan sulfate proteoglycan (Summerford and Samulski, 1998), or conversion of the single stranded viral genome into transcriptionally active double stranded DNA (Ferrari et al., 1996).

It may be possible to upregulate heparan sulfate proteoglycan on haematological cells. Incubation with heparin or γ-irradiation have been demonstrated to upregulate heparan sulfate proteoglycan on endothelial cells (Nader et al., 1989; Pye et al., 1994). Binding of AAV to the target cells could be assessed by the production of AAV containing [^{35}S] -methionine (Qing et al., 1998). Dot blot hybridisation to determine the titre of the recombinant AAV has been calculated to overestimate the titre by a factor of 1000 (Samulski et al., 1996). The MOI's used in the AML transductions based on viral genomic titre were probably of the order of 10 as opposed to 10,000. An increase in MOI may result in increased transduction efficiency. It may be that in the experiments which resulted in up to 15% transduction of the AML cells

transduced, the MOI of infectious virus was higher than in those experiments which were unsuccessful.

Certain factors, such as irradiation, incubation with hydroxyurea, or exposure to E4*orf*6 protein of the infected cells, have been shown to increase transduction efficiency with recombinant AAV. These work by increasing the rate of conversion of the single stranded DNA to a transcriptionally active DNA duplex (Ferrari et al., 1996). A host cellular protein termed ssD-BP has been found to be involved in the conversion of the AAV genome into double stranded DNA. The ssD-BP binds to the D region of the viral ITR and when phosphorylated at the tyrosine residues inhibits second strand synthesis. The ratio of dephosphorylated to phosphorylated ssD-BP has been shown to be important in transduction efficiency (Qing et al., 1997). Inhibitors of tyrosine kinase, such as genistein, have been shown to increase transduction efficiency with recombinant AAV and E4*orf*6 has also been shown to increase the proportion of dephosphorylated ssD-BP (Qing et al., 1998). Packaging of E4*orf*6 protein within the AAV capsid may be one way of improving transduction efficiency with AML cells, or preincubation of the AML blasts with genistein or an alternative inhibitor of tyrosine kinase.

Transduced AML blasts with as little as 10% of cells expressing B7-1 or B7-2 were able to costimulate allogeneic T cell proliferation. It is not known whether this level of expression of B7-1 or B7-2 on the AML cells is sufficient to induce an autologous anti-leukaemic immune response. It may be that a stronger co-stimulatory signal may be required to induce T cell activation, when the signal through the T cell receptor is provided by a tumour antigen as opposed to an allogeneic signal (Lenschow et al., 1996). The AML samples

innately expressing B7-2 were able to costimulate allogeneic T cell proliferation with as few as 5% of the cells being positive and a higher percentage of cells expressing B7-2 did not increase the proliferation or production of IL-2 from the responding T cells (section 3.1.5.1). This suggests that the transduction efficiency of up to 15% may be sufficient for the infected AML blasts to deliver a co-stimulatory signal and induce an autologous anti-leukaemic immune response *in vivo*. However, the fact that CR1 was longer in those patients with a higher percentage of AML blasts expressing B7-2 suggests that a higher transduction efficiency may be beneficial (section 3.1.3.5). Therefore, it may be worthwhile trying to upregulate the AAV receptor, or increase conversion of single stranded DNA to double stranded DNA by dephosphorylation of the ssD-BP, to increase the transduction efficiency with recombinant AAV.

8.1.4 Complications of Immunotherapy

An advantage of inducing B7-1 or B7-2 expression on tumour cells, is that identification of a potential target antigen is not required. However, this has a disadvantage in that it is possible that an immune response may be generated against a protein expressed on both normal and leukaemic cells. It has been demonstrated in human AML, in some FAB types, that in a system analogous to normal haematopoiesis, not all leukaemic cells are capable of sustaining leukaemic haematopoiesis. It has been found that it is the leukaemic cells expressing CD34 and lacking CD38, the phenotype of stem cells, that are capable of initiating leukaemic haemopoiesis when inoculated into SCID mice (Lapidot et al., 1994). These leukaemic stem cells have been termed leukaemia initiating cells (LIC) and represent the cells that will need to be eradicated to prevent relapse of the leukaemia. These leukaemic stem cells

may share surface peptides with normal stem cells and if an immune response were generated against a shared peptide then the patient may develop aplastic anaemia. That this is possible has been shown by a T cell clone raised against AML, which proliferates and induces cytotoxicity against several different AML samples, but also proliferates on exposure to CD34+ve normal bone marrow cells and inhibits myeloid and erythroid colony formation (Mutis et al., 1997).

To address this possible complication one group introduced a viral peptide into a murine pancreatic tumour model. The tumour remained non immunogenic, but on infection with the virus an anti-tumour response was elicited which resulted in partial regression of the tumour. However, the anti-tumour response was not sustained, despite the presence of CTL's against the viral peptide. Adoptive transfer of T cells from mice exposed to the virus caused further tumour regression. As repetitive immunisations are required, due to the brevity of the anti-tumour response, the risk for induction of autoimmune disease should be low (Speiser et al., 1997).

A similar study used a transgenic mouse that expressed a viral protein in all lymphoid cells, which was highly expressed in an erythroleukaemic cell line (FBL). Adoptive transfer of T cells from non transgenic mice vaccinated with FBL resulted in eradication of tumour cells inoculated into the transgenic mice, with no autoimmune damage to the lymphoid tissues. Although the level of expression of the viral protein was lower than on the FBL cells, these cells were able to stimulate T cells specific for the viral protein, demonstrating that the level of expression on the lymphoid cells was high enough for antigen recognition (Hu et al., 1993). This data is encouraging for tumour vaccines with AML, where overexpression of myeloperoxidase is seen, which may act as a

tumour antigen (Dengler et al., 1995). In a number of murine models of AML, vaccination with B7-1 modified leukaemic cells has been demonstrated to result in a T cell mediated destruction of pre-existing wild type leukaemia (Dunussi-Joannopoulos et al., 1996; Matulonis et al., 1995; Matulonis et al., 1996; Dunussi-Joannopoulos et al., 1997), with prolonged survival of the mice. In one study, mice survived up to 6 months after vaccination with no evidence of residual leukaemia and normal haematological parameters in all cured mice (Dunussi-Joannopoulos et al., 1996). In this study there was therefore no evidence of cross-reactivity against antigens expressed on normal haematopoietic progenitors.

8.1.5 Other Strategies and Future Directions

As well as transducing AML blasts with recombinant AAV, other groups have tried to upregulate expression of B7-1 or B7-2 by a variety of other means. Incubation with γ-IFN has been shown to upregulate expression of B7-1 on AML blasts (Costello et al., 1998). An alternative strategy has utilised innate expression of CD40 on pre-B ALL cells. Incubation of the pre-B ALL cells with cells stably transfected with CD40 ligand, results in upregulation of B7-2 and induction of B7-1 expression, as well as upregulation of MHC class I and II and ICAM-1 and LFA-3 expression (Cardoso et al., 1996). The stimulated pre-B ALL cells were able to stimulate autologous lymphocytes and allowed the generation of leukaemia specific CTL's (Cardoso et al., 1997). Interestingly, CTL's were only able to be generated from T cells derived from the bone marrow and not the peripheral blood of the patients. This raises the question as to whether the peripheral blood T cells had been anergised after exposure to unstimulated pre-B ALL cells lacking B7-1 or B7-2, whereas the

bone marrow may have contained naive T cells. However, the authors did comment that the bone marrow was invariably infiltrated with leukaemic cells, so this may not be a factor. This data does suggest that, at least in pre-B ALL, a target antigen is present and that failure to induce an immune response is partly due to lack of B7-1 or B7-2 expression. A separate approach to allow tumour cells to provide a co-stimulatory signal, is to bind anti-CD28 monoclonal antibody directly to the tumour cell. The antibody can then provide a co-stimulatory signal on binding to CD28, to allow tumour reactive T cells to proliferate (Darling et al., 1997).

Another technique that has been used with chronic myeloid leukaemia (CML) cells, is to culture dendritic cells from CML progenitor cells. The CML cells all contained the 9;22 translocation and would presumably present any tumour antigens efficiently, as they expressed both B7-1, B7-2, MHC class I and II after *in vitro* culture to develop a dendritic cell morphology and phenotype. The CML dendritic cells stimulated autologous proliferation of T cells, which had cytotoxic activity against CML cells, but not against normal bone marrow progenitors (Choudhury et al., 1997). A recent report describes the generation of cells of dendritic cell morphology expressing MHC class I and II, B7-1 and B7-2 after culturing AML blasts with GM-CSF, IL-4 and IL-13 (Strunk and Linkesch, 1997). This seems an attractive strategy which circumvents the inherent inefficiency of current viral vectors. Fusion of tumour cells to dendritic cells has been proposed as a way of allowing tumour cells to efficiently act as antigen presenting cells. This has been shown to be successful *in vivo* in a murine model, with protection on exposure to unmodified tumour cells (Gong et al., 1997). This technique will carry the same potential

complication as B7-1 transduction of tumour cells, in that an autoimmune response against normal cellular proteins may be induced.

Transduction of tumour cells with a variety of cytokines has also been shown to be effective in the generation of a protective immune response. Cytokines used include IL-2, IL-4, IL-6, γ-IFN and GM-CSF (Gansbacher et al., 1990; Tepper et al., 1989; Sun et al., 1992; Watanabe et al., 1989; Dranoff et al., 1993). One possible mechanism for the action of GM-CSF secretion by tumour cells, is that it may promote the differentiation of dendritic cells, which would then be able to present potential tumour antigens to both class I and class II restricted T cells (Dranoff and Mulligan, 1995). In a murine model of acute myeloid leukaemia, blasts engineered to secrete GM-CSF were more effective than B7-1 or B7-2 expressing AML blasts in eradicating disease in mice exposed to the unmodified blasts (Dunussi-Joannopoulos et al., 1998). An important cytokine secreted by antigen presenting cells is interleukin-12, which induces production of γ-IFN from both NK cells and T cells, enhances both NK and T cell-mediated cytotoxicity and promotes development of a T_H1 immune response (Trinchieri, 1994). In addition to promoting a T_H1 immune response, which would be expected to be preferred with tumour vaccination strategies, IL-12 also has the ability to reverse anergised T cells *in vivo* (Grohmann et al., 1997). Therefore it might be expected that a combination of B7-1 expression and secretion of IL-12 by the tumour cells would be more efficient than B7-1 expression alone in inducing anti-tumour immunity. *In vivo*, using murine tumour models, this has been confirmed, the combination of IL-12 and B7-1 being synergistic in the induction of anti-tumour immunity (Coughlin et al., 1995; Zitvogel et al., 1996; Pizzoferrato et al., 1997). We have constructed

a fusion gene coding for the p35 and p40 chains of IL-12, which allows packaging of both the B7-1 and IL-12 genes within AAV. This fusion protein, termed flexi-12 has been demonstrated to possess the same biological properties as native IL-12 and transduction of AML blasts with recombinant AAV containing flexi-12 cDNA under the CMV promoter results in secretion of biologically active flexi-12 from the AML blasts (Anderson et al., 1997).

With the B7-1 or B7-2 expressing AML blasts, there are two broad therapeutic possibilities. The first involves the *in vitro* generation of leukaemia specific CTL's, using the AML blasts as stimulators. The main limitation of this technique is the time taken to generate CTL's. With donor lymphocyte infusions for relapse after BMT, between $1x10^8$ and $1x10^9$ T cells are returned to the patient (Kolb et al., 1990). Successful generation of T cells specific for EBV and CMV have been generated *ex vivo* and returned to patients with lymphoproliferative disorders and CMV disease post BMT (Papadopoulos et al., 1994; Walter et al., 1995), although with a less immunogenic leukaemic antigen this would be expected to be technically more difficult. In mice, *ex vivo* generation of leukaemia specific CTL's have been achieved using B7-1 or B7-2 expressing leukaemic blasts (Cardoso et al., 1997). An advantage of this technique is that cytotoxicity against normal haematological cells can be assessed, to reduce the risk of induction of autoimmune disease. An alternative to *ex vivo* generation of tumour specific CTL's, is the reinfusion of B7-1 or B7-2 expressing modified leukaemic cells to the patient as tumour vaccines. This has been achieved in a variety of murine tumour models. Another problem is that the modified leukaemia cells may have to be irradiated to prevent return of viable cells to the patient. Some studies suggest that

modified tumour cells that have been irradiated do not induce protective immunity (Matulonis et al., 1995; Matulonis et al., 1996), but subsequent studies have shown, including a model of AML, that irradiation or fixing of the tumour cells does not affect the generation of protective immunity (Dunussi-Joannopoulos et al., 1996). Another question to be addressed with the return of transduced AML cells to patients is the route of administration required to achieve the best immune response. A study in which subcutaneous or intravenous administration of B7-1 or B7-2 expressing AML cells, in a murine model, was compared found that protection was conferred only on intravenous administration (Matulonis et al., 1996).

In summary, it is hoped that by upregulation of the AAV receptor on AML cells, or by increasing the ratio of phosphorylated to dephosphorylated ssD-BP in the AML cells, an increase in transduction efficiency with the recombinant AAV will be achieved. Titre of the recombinant AAV will be assessed by infecting 293 cells and looking for expression of the transgene. This will give a more accurate assessment of infectious titre, enabling the MOI required to infect AML blasts to be more accurately quantitated. The transduced AML blasts could then be returned to patients with AML after chemotherapy to minimise the tumour burden. The route of administration will probably be intravenous to induce the anti-leukaemic immune response. First, we will have to prove the efficacy of the system in a murine model using NOD/SCID mice. NOD/SCID mice are able to engraft both normal haematopoietic progenitors and leukaemia initiating cells and would be suitable hosts in which to generate a human model of AML. Prior to the return of modified leukaemia cells to patients, the protocol will require approval by the gene therapy advisory

committee (GTAC). Recombinant AAV will have to produced to clinical grade (GMP) and transductions will have to be performed in suitably approved laboratories.

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