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CONSTRUCTION & CHARACTERIZATION OF ADENOVIRAL VECTORS
EXPRESSING CYTOKINES FOR CANCER IMMUNOTHERAPY

By
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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
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CHAPTER 1. INTRODUCTION

A. The Adenoviruses

1. Characteristics of Adenoviruses

   The human adenoviruses are members of the adenoviridae family, and the genus mastadenovirus, which also includes simian, bovine, equine, porcine, ovine, canine and opossum viruses. There are over 40 different serotypes of human adenoviruses, and they are grouped based on their ability to haemagglutinate rat and rhesus monkey red blood cells, as well as by their ability to induce tumors in rodents. Human adenovirus (Ad) types 2 and 5, which are the most commonly used viruses for vector production, belong to the subclass C group of human adenoviruses, and have been shown to have no, or very low ability to induce tumors in rodents. The Ad 2 and Ad 5 genomes have been sequenced, and this has facilitated their manipulation at the molecular level to generate vector systems which have proven useful in gene therapy protocols. The characteristics of Ads will be discussed below, and for more detail the reader is referred to: "The Adenoviruses" edited by H.S. Ginsberg (1984) and "Adenoviridae: The viruses and their replication" by T. Shenk, in Fields Virology (3rd edition, 1996).

2. Virion Structure

   The Ad double stranded DNA genome is contained within a non-enveloped icosahedral protein capsid (Figure 1-1). The capsid is composed of 252 capsomers (240
Figure 1-1: Structure of the Adenovirus Virion.

A cross-section of the adenovirus virion is graphically represented based on the known associations of the polypeptide components and DNA. The virion is composed of the outer protein capsid, and the inner protein/DNA core. It should be noted that real sections of the icosahedron are unlikely to contain all the virion components, as diagrammed here. Modified from Shenk, “Adenoviridae: The viruses and Their Replication”, In Fields Virology, 3rd Edition (1996).
hexons and 12 pentons) which together form an icosahedron of 20 triangular surfaces and 12 vertices with a diameter of ~140 nm. The hexon capsomers are formed by the non-covalent association of three identical viral polypeptides, and nine of these capsomers associate to form each quasi-triangular face of the icosahedron. The penton capsomers form the vertices of the icosahedron, and are produced by the non-covalent association of 5 penton polypeptides, which are in turn non-covalently associated with the fiber protein. The fiber protein is composed of three monomers of the fiber polypeptide that associate with the penton capsomer through their amino termini. The carboxy termini of the fiber trimer extend outwards from the capsid and end in a knob-like structure. It is the knob portion of the fiber molecule that is involved in the initial attachment of the virion to the host cell surface during infection. In addition to these three major virion capsid proteins, hexon, penton and fiber, there are at least three other viral encoded proteins which associate with the hexon capsomers: viral polypeptides VI, VIII, and IX. Polypeptides VI and VIII are thought to act as scaffolding proteins that aid in the assembly of the virion capsid, while protein IX is thought to be necessary for the packaging of full-sized viral genomes and for the heat stability of the virion. Virions lacking protein IX have been shown to only package approximately 97% of wild-type genome size in a stable virion (Ghosh-Choudhury et al., 1987).

The viral DNA is contained within the capsid as a DNA:protein complex called the viral core. The core consists of an approximately 36 kb double stranded linear DNA genome (the size of the genome varies according to serotype), that is associated with four viral proteins: polypeptides V, VII, μ and the terminal protein (TP). Polypeptides V and VII
associate non-covalently with the viral DNA, and impart a nucleosome-like structure to the viral genome. In addition, polypeptide V can associate with the penton capsomer, and may assist in the positioning of the viral DNA during packaging. The function and location of the μ protein within the virus core is currently unknown. The TP is covalently attached to each 5' end of the virus genome, and is involved in priming viral DNA replication.

3. Viral Infection

Infection of mammalian cells by Ad involves two separate phases: attachment of the virion to the cell surface, and internalization. Attachment occurs as a result of the binding of the fiber protein to a cellular receptor which, considering the broad range of cell types which can be infected by Ads, must be expressed in many different cell types (Philipson et al., 1968; Defer et al., 1990). Recently, a putative receptor for both Ad2 and Ad5 was identified (Bergelson et al., 1997). The Coxsackie-adenovirus receptor (CAR) is a 46 kDa transmembrane protein with an extracellular domain that possesses two immunoglobulin-like domains. The importance of these domains, and the biological function of CAR have yet to be determined.

Following viral adsorption, the Ad virion is internalized as a result of the attachment of the penton capsomers to α, integrins on the cell surface, and subsequent receptor-mediated endocytosis (Wickham et al., 1993). Once in the cytoplasm, the endosomal vesicles are ruptured through a poorly understood process that is mediated by the shedding of the penton base, and the virion is subsequently released into the cytoplasm (Seth et al., 1984; Curiel et al., 1991). The virion is then transported through the cytoplasm to the nucleus by
the host cell microtubules which have been shown to associate with the hexon proteins (Luftig et al., 1975; Dales et al., 1973). Upon reaching the nuclear pore, the viral DNA enters the nucleus, leaving many of the virion proteins within the cytoplasm (Philipson et al., 1968). Viral DNA replication and virion assembly both occur in the nucleus of infected cells.

4. Viral Genome Structure and Replication

The virus replication cycle is divided into an early and a late phase, with the late phase initiating concomitant with viral DNA replication. Similarly, viral transcription is divided into early and late transcription units based on when they are synthesized during the replication cycle. There are four early regions in the virus genome (E1, E2, E3 and E4), and eight late regions (L1, L2, L3, L4, L5, pIX, pIVa2, and the virus associated (VA) RNAs I and II).

The only factors required in cis for Ad DNA replication are the inverted terminal repeats (ITRs) located at each terminus of the Ad genome. The ITRs are the recognition and binding sites for the Ad DNA polymerase (Hay et al., 1985). DNA replication is initiated at each 5' end of the molecule via a protein priming step involving the covalent linkage of a dCMP moiety to the Ad pTP. Replication then proceeds by a semi-conservative continuous mechanism. The non-template strand is displaced as the replication fork continues down the length of the molecule, and this displaced strand can then form panhandle structures, via hybridization of the ITRs, that allows for subsequent protein priming and replication of this strand (Daniell 1976, Lippe & Graham 1989). Following replication of the viral genome, protein capsids are assembled, the viral DNA is packaged, and the infectious virions are released by cell lysis.
5. Transcriptional Units of the Ad Genome.

*Early Genes*

**E1.** The first transcription unit to be expressed following viral infection is the E1 region, which can be divided into two groups of transcripts: the E1α and the E1β regions. E1α is considered an immediate early gene and is the first viral gene transcribed following infection. This region encodes six polypeptides, two of which (13S and 12S) are required for transactivation of other viral genes and the stimulation of host cellular DNA synthesis. E1β encodes 3 polypeptides, 19 kDa, 20 kDa, and 55 kDa proteins. The 19 kDa protein is required along with E1α for stable transformation of cells, and can block p53-mediated transcriptional repression (Shen & Shenk, 1994). The 55 kDa E1β protein interacts with cellular proteins to prevent E1α induced apoptosis, and along with the E4 34 kDa protein, functions to mediate the early to late switch in viral gene expression, and shut-off of host cell protein synthesis. Deletion of the E1 region generates replication-defective viruses, which have been used extensively as vectors for expression of foreign genes in mammalian cells (reviewed by Hitt et al., 1997).

**E2.** There are two transcription units within the E2 region (E2α and E2β), and unlike most of the other viral gene regions, these transcripts use the left (bottom) strand of the viral genome as a template (see Figure 1-2). E2α encodes the 72 kDa DNA binding protein, which is required during viral replication, and contributes to the processivity of the viral DNA polymerase. The E2β region encodes two polypeptides: the precursor TP (pTP) and the viral DNA polymerase. Both these transcription units are absolutely required for viral replication.
Figure 1-2: Transcriptional Map of Adenovirus Type 5.

The major Ad promoted transcription units are indicated by brackets, with the early mRNAs designated by E, and the late mRNAs by L. Arrows represent individual mRNA species for each transcriptional unit. Most of the late mRNAs initiate at the major late promoter (map unit 16.3) and contain the tripartite leader sequence (derived from sequences between map units 16.3 and 27), and within each of these transcription units, the mRNAs share a common polyadenylation signal. Those transcription units diagrammed above the genome are derived from the right (r) strand template, and those diagrammed below the genome are derived from the left (l) strand template. Modified from Shenk, "Adenoviridae: The viruses and Their Replication", In Fields Virology, 3rd Edition (1996).
E3. The E3 transcription unit encodes at least 8 polypeptides, and is not required for virus replication in tissue culture. Recent studies have determined that E3 encodes at least three proteins that are essential for host immune modulation in vivo. The 19 kDa protein binds to major histocompatibility (MHC) class I molecules and prevents their presentation in association with viral antigens on the surface of infected cells, resulting in the inability to stimulate effective cytotoxic T lymphocyte (CTL) responses against virally infected cells (reviewed by Wold and Gooding 1989). The 14 kDa E3 protein is involved in inhibiting tumor necrosis factor α (TNFα)-mediated lysis of infected cells. The 10.4 kDa E3 protein has been shown to bind to the epidermal growth factor receptor; however, the significance of this binding remains unclear.

E4. Little is known about the E4 transcription unit. At least seven open reading frames (ORFs) exist in this region; however, all of these, with the exception of ORF 6, can be removed without affecting viral replication in tissue culture (Bridge and Ketner 1989). The 34 kDa ORF 6 polypeptide binds to the 55 kDa E1b protein, which functions to shut off host protein synthesis (Sarnow et al., 1984; Babbiss et al., 1985; Pilder et al., 1986; Cutt et al., 1987). E4 proteins have recently been shown to also be involved in the induction of apoptosis of infected cells (Marcellus et al., 1996).

Late Genes

The Ad late transcription units, by definition, are expressed following the onset of viral DNA replication. The majority of late viral genes are transcribed from the major late promoter (MLP) which is active to a low level during the early phase of viral replication.
Transcripts which are made from the MLP in the early stages are not full length transcripts, and thus the only late protein which can be made at this time is the L1 protein. Following the initiation of viral DNA replication however, the level of transcription initiating from the MLP drastically increases, and full length transcripts are made which give rise to all the other late viral genes through differential splicing. In addition to L1, pIX and pIVa2 are expressed to low levels before DNA replication, and like L1 their transcription levels drastically increase following DNA replication. These transcripts are thus considered intermediate early or late genes. In the late stages of viral replication, host protein synthesis is shut off, and virtually all the mRNA found in polyribosomes in infected cells is of viral origin (Raskas et al., 1970; Lucas et al., 1972; Beltz et al., 1979). As mentioned, the majority of the late mRNA transcripts initiate at the MLP, and are generated through differential splicing. There are five families of late genes controlled by the MLP, and the transcripts within each region share a common polyadenylation site and 5' tripartite leader sequence.

**L1.** L1 encodes three transcripts that give rise to proteins of molecular weight 52, 55, and 66 kDa. The functions of the 52 kDa and the 55 kDa proteins are unknown; however, the 66 kDa protein is the virion polypeptide IIIa. Polypeptide IIIa is a minor structural component of the virion capsid that appears to associate with the penton vertices, and may also associate with the polypeptides V and VII in the virus core. Polypeptide IIIa is thought to impart stability to the virion structure, and viruses with mutations in polypeptide IIIa fail to assemble virions properly (Devaux et al., 1982).

**L2.** The L2 region also encodes three mRNA transcripts, and gives rise to proteins
of 85, 20, and 48.5 kDa. All three of these proteins are components of the capsid and represent the penton capsomer, polypeptide VII, and polypeptide V, respectively. As mentioned, polypeptides VII and V are non-covalently linked to the viral DNA molecule and in addition to imparting a nucleosome structure, may also serve to position the DNA within the capsid.

L3. The L3 region encodes three mRNA transcripts which are translated into the polypeptide VI precursor (27 kDa), the hexon protein (polypeptide II), and an additional protein of molecular weight 23 kDa. The precursor of polypeptide VI associates with the hexon and, during virion assembly, becomes cleaved into its mature form. The 23 kDa protein is the viral protease which is responsible for the cleavage of the precursor viral peptides into their mature forms.

L4. The L4 region encodes at least three proteins of molecular weights, 100, 33, and 26 kDa. The 100 kDa protein may act as a scaffold to allow the association of the hexon polypeptides into trimers. The function of the 33 kDa protein is, as yet, unknown. The 26 kDa protein is the precursor to polypeptide VIII, which is hexon associated in the virion, and becomes cleaved into its mature form during virion assembly.

L5. The L5 gene region encodes two mRNA species; however, both of these mRNA transcripts are translated into the 62 kDa fiber protein (polypeptide IV). As mentioned previously, fiber is a trimer of polypeptide IV in its mature form in the virion.

pIX. pIX shares a common polyadenylation signal with the E1b proteins, and encodes a polypeptide of 17 kDa. The pIX transcription unit represents the only Ad gene which is not
processed and remains unspliced. pIX was initially classified as a late viral protein because it is a virion structural protein; however, pIX is expressed in the absence of viral DNA replication and is therefore now considered an intermediate early gene. Although pIX is expressed at early times post-infection, its transcription drastically increases at late times post-infection. pIX is required for the packaging of larger Ad genomes, and to maintain the heat stability of the virion (Ghosh-Choudhury et al., 1987).

pIVa2. Like pIX, pIVa2 is expressed early after infection and is therefore considered an intermediate early gene; however, pIVa2 synthesis is drastically increased at late times post-infection. The function of the 51 kDa pIVa2 gene product remains unclear; however, it is thought to act as a scaffolding protein during virion assembly, and may play an additional role in packaging of the viral DNA. pIVa2 does not remain associated with the mature virion.

Viral Associated (VA) RNAs. The VA RNAs differ from all other Ad transcription units in that they are transcribed by RNA polymerase III rather than RNA polymerase II. There are two RNA species produced, VA-RNAI and VA-RNAII, which are both approximately 160 nucleotides long, and both form similar secondary structures that resemble cellular tRNAs. The function of the VA-RNAs remains unclear, although, it is hypothesized that they bind specific splice junctions within the late Ad transcripts. There is also evidence that VA-RNAI may control the rate of translation of late viral proteins, and mediate the selective translation of viral proteins instead of cellular proteins. This preferential translation of viral RNA may be due to the binding of VA-RNAI to the 68 kDa cellular protein kinase R, preventing its autophosphorylation, which in turn prevents activation of elongation factor
2α, resulting in an inhibition of host protein synthesis (Kitajewski et al., 1986; O'Malley et al., 1989).

B. The Use of Adenovirus Vectors for Foreign Gene Expression

1. Construction of First Generation Recombinant Adenoviral Vectors

Although many novel strategies for the generation of recombinant Ad vectors have arisen in recent years, the simplest, and most widely used, is the construction of so called first generation Ad vectors. Typically, first generation Ad vectors are deleted for both the E1 and the E3 regions of the Ad genome. The E3 region is dispensable for viral replication in tissue culture, however, it encodes proteins that are involved in host immune evasion, and thus E3 expression may be desirable for optimum transgene expression in vivo. The E1 region is necessary for viral replication, and its removal from the vector backbone generates a vector that cannot replicate. E1-deleted vectors can be propagated in 293 cells (Graham et al., 1977), which contain nucleotides 1 to 4344 of the wild-type Ad 5 genome, and can provide the E1 proteins in trans (Louis et al., 1997). Foreign DNA sequences can be inserted in either the E1 or the E3 region of first generation Ad vectors.

There are two commonly used methods for generating recombinant genomes containing foreign DNA inserts: A) restriction of the Ad genomic DNA and ligation to a complementing plasmid containing the foreign DNA in vitro or recombination with the complementing plasmid in vivo, or B) homologous recombination in 293 cells between a circular Ad genome and the unrestricted plasmid containing the foreign DNA insert. The
studies performed in this thesis were all based on the use of the two plasmid system developed
by Graham and coworkers (McGrory et al., 1988; Bett et al., 1994), which is dependent on
homologous recombination in 293 cells.

2. The Two Plasmid System for Construction of First Generation Recombinant Ad Vectors

During viral replication, the Ad genome can undergo spontaneous circularization at
low frequency (Ruben et al., 1983). This feature has been exploited to generate circular viral
genomes which contain bacterial origins of replication, and can thus be propagated as
plasmids in *E. coli* (Graham, 1984a; Ghosh-Choudhury et al., 1986; McGrory et al., 1988;
Bett et al., 1994). These plasmids have been modified to prevent them from generating
recombinant virus in 293 cells unless they have undergone homologous recombination with
a "shuttle" plasmid. One such Ad 5 genomic plasmid, pJM17, contains an insertion of ~4.3
kb of bacterial plasmid DNA (McGrory et al., 1988), resulting in a genome size which
exceeds the upper packaging constraints of the virus. Another non-infectious Ad 5 genomic
plasmid is pBHG10. This plasmid contains the Ad 5 sequences from base pairs (bp) 19 to
188, 1339 to 28,133, and 30,818 to 35,934 (Bett et al., 1994). Removal of the Ad genomic
sequences from bp 189 to 1338 results in the deletion of the packaging signal, which is the
only viral factor required in cis for encapsidation of viral DNA, and thus, pBHG10 cannot be
packaged into infectious genomes.

The genomic plasmids are used in conjunction with "shuttle plasmids" to generate
recombinant Ad vectors. All shuttle plasmids constructed in these studies possessed Ad 5
genomic sequences from bp 22 to 341, and from bp 3525 to 5790. The shuttle plasmids also
possessed heterologous promoters and polyadenylation signals, with multiple restriction enzyme cloning sites between these two elements, flanked by the sequences homologous to Ad5 (see Appendix I). The foreign gene of interest is cloned into the multiple cloning site of the shuttle plasmid, and the shuttle plasmid is cotransfected with an Ad genomic plasmid into 293 cells. It has been previously shown that the viral DNA can undergo homologous recombination with as little as a 1 kb stretch of homology (Berkner & Sharp, 1983). The shuttle plasmids have over 2 kb of overlap with the Ad genomic plasmids, and thus efficient recombination is possible. Homologous recombination between pJM17 and the shuttle plasmid results in a genome size which can now be packaged into Ad capsids. Similarly, recombination between pBHGI0 and the shuttle plasmid, results in the acquisition of the packaging signal and subsequent encapsidation. Both of these events result in the foreign gene of interest replacing the Ad E1 sequences in the vector genome.

3. Advantages of Ad Vectors for Use in Gene Therapy

Ad vectors possess many features which make them ideally suited for use as a vehicle of gene delivery. First, deletion of the E1 sequences from the Ad genome renders the vector unable to replicate, thus in vivo administration of first generation Ad vectors does not lead to a productive infection of the vector in the host. First generation Ad vectors have a relatively large cloning capacity, and can accommodate 8 kb of foreign DNA (Bett et al., 1994). Unlike other gene therapy vectors, such as retroviruses, Ad vectors can infect a wide variety of different cell types, and have the ability to infect and mediate transgene expression in both replicating and non-replicating cells. Propagation of E1-deleted Ad vectors in 293
cells allows the efficient production of very high titered viral stocks which can be easily purified. A further advantage of Ad vectors for some purposes is that since the transgene is produced in mammalian cells, it can undergo the proper post-translational modifications, unlike recombinant proteins produced in *E. coli*. In addition, Ad vectors have been shown to produce extremely high levels of transgene encoded protein, with reports of Ad vectors producing up to 90 mg of protein from 1 L of infected 293 cells, or approximately 10-20% of the total cellular protein (Massie *et al.*, 1995).

Ads have been shown to be relatively safe *in vivo*. Live Ad viruses were used as enteric vaccines in military recruits to prevent the development of acute respiratory disease (ARD). It was found that there was a greater than 80% reduction in the incidence of ARD in vaccinated individuals, and no detrimental effects were noted following its administration, thus indicating the safety and efficacy of adenovirus administration *in vivo* (Edmondson *et al.*, 1966; Smith *et al.*, 1970, Top *et al.*, 1971). Although Ad has been shown to induce oncogenic transformation in rodent cells, no Ads have been shown to be associated with any known human malignancies or with any naturally occurring malignancy in other species (reviewed by Graham, 1984b).

Due to the recent interest in Ad for the use in gene therapy, Ad vectors have now been extensively characterized, and have been shown to mediate transgene expression in a variety of gene therapy target tissues including: airway epithelia, skeletal and cardiac muscle, endothelial cells, neuronal cells, hepatocytes, and stromal cells (recently reviewed by Hitt *et al.*, 1997). One disadvantage to the use of Ad vectors in these studies was the induction of
potent cellular and humoral anti-viral immune responses which limited transgene expression (Yang et al., 1994). The induction of this immune response is due, in part, to the low level expression of viral genes from E1-deleted vectors, and this expression is sufficient to induce a host immune response against the Ad proteins and the transduced cell (Engelhardt et al., 1993; Yang et al., 1994). Furthermore, the Ad virion has been shown to induce potent anti-viral responses (McCoy et al., 1995). Another factor which may limit transgene expression, is the induction of an immune response against the transgene (Tripathy S. et al., 1996). Taken together, these immune responses not only limit transgene expression following vector administration, but also reduce the effectiveness of vector readministration (Dai et al., 1995; Gilgenkrantz et al., 1995; Yang Y. et al., 1995). The induction of these immune responses contributes to the transient nature of transgene expression from Ad vectors which limits their use for some therapies. However, for certain applications, such as the immunotherapy of cancer, these properties are not necessarily a disadvantage, and may in fact be useful for the induction of anti-tumor responses. Cell-mediated immunity against virally-infected tumor cells may lead to concomitant induction of anti-tumor immunity. Furthermore, for immunotherapy, persistent over-expression of host molecules, such as cytokines, could be detrimental to the host, and lead to adverse side-effects or development of auto-immune disorders. Thus the transient nature of Ad vector expression may be ideally suited for immunotherapy of cancer as well as for other therapies that may require only transient transgene expression.
4. Factors Influencing Transgene Expression from First Generation Ad Vectors

One of the most important factors which determine whether Ad vectors will mediate efficient transgene expression, is the ability of the virus to infect the target cell type. Ad vectors attach to the cell surface via binding of the fiber protein to the putative CAR receptor (Bergelson et al., 1997), and then mediate internalization through binding of the penton proteins to αv integrins (Wickham et al., 1993). Therefore, the level of expression of either CAR, or the αv integrins on the cell surface will determine the efficiency of Ad vector infection. Some cells, such as monocytes or T-lymphocytes, are known to be poorly infected by Ad vectors, and transgene expression is only detected following infection at high multiplicities of infection (moi's); however, following transfection of these cells with expression plasmids for either αvβ3 or αvβ4 integrins, Ad-mediated gene delivery was greatly enhanced (Huang et al., 1995). Other factors which influence efficiency of Ad transduction of target cells include the duration of incubation with virus, and the depth of virus inoculum during infection [i.e. smaller volumes of higher concentrated virus transduce more efficiently than lower concentrated, larger volumes] (Johnson et al., 1996).

Another factor which may influence transgene expression from Ad vectors, is the viral sequences flanking the expression cassette within the vector backbone. When the gene for firefly luciferase was cloned under an SV40 promoter in the E3 region of an Ad vector in either the leftwards or rightwards orientation, differences in the levels of luciferase expression were noted between the two constructs (Mittal et al., 1993). In these constructs the E3 coding sequences, but not the E3 promoter had been deleted, and it was observed that
expression of luciferase was higher when the transcription unit was directed towards the right end of the genome (i.e. parallel to E3 transcription). This was due to initiation of transcription from the Ad5 MLP and the E3 promoter. Similar effects on transgene expression in the E3 region of Ad vectors have been noted by others for expression of the herpes virus glycoprotein B (Johnson et al., 1988) and vesicular stomatitis virus glycoprotein (Schneider et al., 1989). Foreign gene expression cassettes inserted in place of E1 sequences also appear to be affected by the surrounding vector sequences. Xu et al. (1995), observed that transgene expression from the human cytomegalovirus promoter/enhancer or the Ad 2 MLP in a first generation Ad vector, was higher when the expression cassette inserted in E1 was oriented parallel to E1 transcription.

The level of transgene expression is also determined by the elements controlling transcription. Several studies have indicated that different promoter/enhancer elements can vary greatly in their ability to promote transgene expression and in their cell type specificity. For example, Thompson et al. (1993), observed a hierarchy of luciferase expression using various viral promoters in mammary cells transduced with plasmid DNA: Rous sarcoma virus long terminal repeat (LTR) ~ human cytomegalovirus immediate-early (HCMV IE) promoter ~ simian virus 40 (SV40) promoter > moloney murine leukaemia virus LTR > mouse mammary tumor virus LTR. In contrast, Foecking & Hofstetter (1986) observed that expression from the HCMV IE promoter following transduction of cells with plasmid DNA, was significantly higher than either the SV40 or the RSV LTR in six different cell types of human or murine origin, indicating that some of these promoters may have different kinetics
of expression depending on the cell type examined. Transgene expression differences due to
promoter elements have also been shown for Ad vectors. For example, the Ad2 MLP and the
HCMV IE promoter were found to express better than the SV40 and human β-actin
promoters in 293 cells (Xu et al., 1995). It has also been demonstrated that some adenoviral
proteins can have effects on the transcription of some promoters. The Ad E1A proteins can
transactivate expression from the HCMV IE promoter (Gorman et al., 1989; Cockett et al.,
1990; Olive et al., 1990), while E1A represses transcription from the RSV LTR or the SV40
promoter (Borelli et al., 1984; Alwine, 1985; Velcich & Ziff, 1985). A recent study has also
shown that the Ad E4 proteins can increase expression from the HCMV IE promoter
(Armentano et al., 1997; Kaplan et al., 1997). Taken together, these data suggest that there
are many factors which may influence transgene expression in Ad vectors and these factors
must be considered when designing expression cassettes.

5. The Use of Ad Vectors in Cancer Gene Therapy

To date, most cancer gene therapies have used retrovirus vectors to transduce tumor
cells with genes that promote tumor elimination by administration of cytotoxic drugs ("suicide
genes"), induction of apoptosis, or recognition by immune effector cells. Recently, Ad
vectors have become more widely used in cancer gene therapy because of ease of propagation
and administration of Ad compared to retrovirus vectors, and the efficiency of gene delivery
associated with Ad vectors. Since it is difficult to efficiently target the Ad vector to infect
only the desired cell types in vivo, the non-integrating nature of Ad vectors results in transient
expression of the transgene and avoids the potential for permanent transduction of non-tumor
cell types. Furthermore, the anti-viral immune responses which are generated following *in vivo* administration of Ad vectors, may aid in the removal of virally transduced tumor cells. Ad transduced tumor cells would be recognized by Ad specific CTL, resulting in CTL-mediated elimination of the cell. During this process, the generation of specific anti-tumor antigen CTL may also be enhanced.

Studies have been performed using Ad vectors to deliver "suicide genes" into tumor cells. Genes encoding *E. coli* cytosine-deaminase (CD), or herpes simplex virus thymidine kinase (HSV TK), were delivered to target cells, thus rendering the transduced cells sensitive to killing following the administration of 5-fluorocytosine (5FC) or ganciclovir (GCV), respectively. Ad vectors were used to transduce HT29 human colon-carcinoma cells with the CD gene *in vitro*, and following 5FC treatment, the overall cell growth was suppressed in a dose-dependent manner (Hirschowitz *et al.*, 1995). Hirschowitz *et al.* (1995) also demonstrated a suppression of tumor growth *in vivo*; however, despite the prolonged survival of the animals, no tumor regression was observed. Similarly, HSV TK has been delivered to a variety of tumor types using Ad vectors, and has resulted in tumor regression in some models. Chen *et al.* (1994) demonstrated that the delivery of an Ad-HSV TK vector to established C6 glioma tumors in rats resulted in a significant decrease in tumor size following addition of GCV. Ad-HSV TK delivery followed by GCV treatment has also been shown to be efficacious in other tumor models, including; mesothelioma (Smythe *et al.*, 1995), prostate cancer (Hall *et al.*, 1996; Shaker *et al.*, 1996; Eastham *et al.*, 1996), hepatocellular carcinoma (Qian *et al.*, 1995) and squamous cell carcinoma of the head and neck (O'Malley *et al.*, 1995).
Ad vectors have also been used in cancer therapy studies to deliver tumor suppressor genes, such as p53 or cyclin-dependent kinase inhibitors. These genes are involved in cell cycle regulation, and are mutated in a variety of cancers, resulting in an increased proliferative capacity of the tumor cells. Induction of growth suppression, expression of p53-regulated genes, and an increase in apoptosis have been observed following introduction of wild-type p53 by Ad vector transduction in vitro (Bacchetti & Graham, 1993; Liu et al., 1994; Yang C. et al., 1995; Katayose et al., 1995a). Transduction by Ad-p53 was also found to modulate tumorigenicity of C6 gliomas (Yang C. et al., 1995), H226Br lung carcinomas (Fujiwara et al., 1994), and Tu-177 or Tu-138 squamous carcinoma of the head and neck tumor cells (Liu et al., 1994) in vivo. Ad vectors expressing p21^{waf}, a cyclin dependent kinase inhibitor, have been shown to induce cell cycle growth arrest of a variety of tumor cell lines including; human lung carcinoma, breast carcinoma (Katayose et al., 1995b), murine melanoma and renal cell carcinoma (Yang C. et al., 1995), or murine prostate cancer (Eastham et al., 1995). Furthermore, suppression of tumor growth in vivo was demonstrated following Ad-p21^{waf} transduction (Eastham et al., 1995; Yang C. et al., 1995). An Ad vector encoding another cyclin-dependent kinase inhibitor, p16^{INK4}, was found to arrest tumor cell growth in vitro, and modulate tumor growth in vivo, following Ad-p16^{INK4} vector transduction (Jin et al., 1995).

The use of tumor suppressor or suicide genes has been shown to be efficacious; however these types of therapeutic approaches have several disadvantages. These treatments do not usually protect against tumor recurrence nor have an effect on distal untreated tumors. Since the majority of patients do not die from the primary tumor, but rather from the spread
of metastatic disease, methods of therapy which induce protective immunity that can mediate killing of distal tumors and metastases are desirable. Induction of specific anti-tumor immune responses could achieve this result, and thus use of Ad vectors for immunotherapy of cancer is one approach which might be effective in the induction of protective anti-tumor immunity.

C. Immunotherapy of Cancer

Immunotherapy involves the introduction of immunologically active proteins (e.g. cytokines) in an attempt to stimulate, or abrogate, the activity of immune effector cells and achieve resolution of the disease. Current cancer therapies, such as chemotherapy and irradiation, have been relatively unsuccessful in achieving long-term survival of patients suffering from solid tumors. This is mainly a result of the inability to completely eradicate the residual tumor cells or prevent metastatic spread of the disease. Moreover, some subpopulations of tumor cells may become resistant to the chemotherapeutic drugs or irradiation. The delivery of cytokines to induce anti-tumor immune responses could lead to the induction of protective immunity that would abrogate the residual tumor or metastases.

1. Evasion of Immune-Detection by Tumors

Many tumor cells evade detection by the immune system in a manner which is not completely understood; however, some mechanisms of evasion have been elucidated. A decrease in the level of major histocompatibility complex (MHC) class I expressed on the surface of tumor cells compared to normal cells has been observed (Browning & Bodmer, 1992), resulting in the failure to present tumor associated antigens in a context that would
stimulate cytotoxic T cell responses. Tumor cells may also downregulate or lose the expression of specific tumor associated antigens (George & Stevenson, 1989), similarly resulting in the absence of cytotoxic T cell recognition. Recently, it has been demonstrated that the expression of MHC class I or class II molecules or tumor specific antigens by tumor cells (i.e. by transduction of tumor cells with the appropriate cDNA) results in the induction of tumor cell specific CTL activity (Plautz et al., 1993; Leach et al., 1995; Mandelboim et al., 1995; Hock et al., 1996; Zhai et al., 1996) that inhibits tumor growth in vivo.

Another mechanism of tumor immune-evasion is by the induction of T-cell anergy. T-cells can be rendered tolerant to tumor antigens upon recognition of tumor cells lacking the costimulatory molecules such as B7.1 or B7.2 (Chen et al., 1992; Baskar et al., 1993; Harding & Allison, 1993; Townsend & Allison, 1993). It has been shown that the activation of T-cells requires two signals; recognition of antigen in association with MHC molecules by the T-cell receptor (TCR), and costimulation by the binding of CD28 on T-cells to B7.1 or B7.2 on the target cell (recently reviewed by Judge et al., 1996; Van Gool et al., 1996). The binding of the TCR to MHC-antigen complexes results in the induction of signal transduction pathways leading to cell proliferation, and the binding of costimulatory molecules results in the activation of transcription of many cytokines required for T-cell activation and proliferation. Activation through the TCR in the absence of the costimulatory signal, results in a state of anergy whereby the T-cells do not proliferate or gain effector function. Many tumor cells do not express B7 molecules, and thus fail to elicit appropriate anti-tumor CTL responses (Barth et al., 1990; Fearon & Vogelstein, 1990; Bishop, 1991;
Van der Bruggen et al., 1991). Introduction of the gene for B7 into murine melanoma cells was shown to induce specific anti-tumor activity in vivo which prevented the growth of the tumors and protected the animals against a subsequent challenge with parental untransfected tumors (Townsend & Allison, 1993). This anti-tumor activity was a result of the induction of specific CD8+ CTL recognizing the tumor cells, thus demonstrating the ability to counteract the tumor-induced immunosuppression and restore normal immune effector function.

Immune effector cell function may also be impaired by the expression by the tumor cell of immunosuppressive molecules, such as transforming growth factor-β (TGF-β) or interleukin (IL)-10. These cytokines downregulate cell-mediated immunity, and hence tumor cell killing. IL-10 can induce the maturation of T-helper cells into the Th2 lineage profile, which are responsible for humoral immune reactions (reviewed by Mossman & Sad, 1996), and can also decrease the expression of T-cell growth factors such as IL-2 (de Waal Malefyt et al., 1993; Taga et al., 1993). Furthermore, IL-10 has been shown to inhibit the expression of B7.1 and B7.2 which are required as costimulatory signals for efficient activation of T-cell effector function (Ding et al., 1993; Willems et al., 1994). Taken together, the effects of IL-10 would induce a potent immunosuppressive environment within the tumor, and this suppression would need to be overcome to mount an effective anti-tumor immune response. Since the downstream result of most of these suppressive effects is the prevention of IL-2 expression and T-cell proliferation, introduction of IL-2 at the tumor site may counteract the effects of immunosuppression and lead to the induction of potent anti-tumor responses.
2. **Anti-Tumor Activity of Interleukin-2**

Interleukin-2 (IL-2) was originally identified as a T-cell growth factor that was involved in T-lymphocyte proliferation. IL-2 is a 14-17 kDa glycosylated protein that is produced mainly by activated CD4+ T-cells, and also, to a lesser extent, by CD8+ cells. IL-2 can act on T-cells in both an autocrine and a paracrine fashion and, upon binding its receptor on T-cells, can induce its own expression, as well as the expression of other cytokines such as interferon (IFN)-γ and lymphotoxin. The cytotoxic activity of both T-cells and natural killer (NK) cells is increased following IL-2 stimulation, and incubation of NK cells with high concentrations of IL-2 generates lymphokine activated killer (LAK) cells which have non-specific cytotoxic activity on a wide variety of tumor cells *in vitro* (reviewed by Kohler *et al.*, 1989). IL-2 can also act as a B-cell growth factor, and will induce antibody synthesis which could also contribute to anti-tumor immunity.

The anti-tumor activity of IL-2 was discovered through its ability to induce LAK cell activity following incubation of tumor-associated lymphocytes in high concentrations of IL-2 (Yron *et al.*, 1980; Grimm *et al.*, 1982). Initially, LAK cells appeared very promising for cancer therapy due to their ability to selectively lyse freshly isolated tumor tissue, but not normal cells (Grimm *et al.*, 1982; Vujanovic *et al.*, 1988). In preliminary *in vivo* studies, the administration of LAK cells alone, or in combination with recombinant IL-2, resulted in a reduction in metastases in tumor-bearing mice (Mazumder, *et al.*, 1984; Mule *et al.*, 1984; LaFreineire *et al.*, 1985). These initial successes led to the use of LAK cells and IL-2 in clinical trials of a variety of human malignancies. Results in the clinic were less promising
however, only 8 patients of 106 underwent a complete response, and approximately 70% of the treated patients did not respond to the treatment (Rosenberg et al., 1987). These studies also showed that high systemic doses of recombinant IL-2 alone were equally as therapeutic as the LAK plus IL-2 therapy. Rosenberg et al. (1987) also noted that the degree of antitumor responses was dependent on the type of cancer, with renal-cell cancer, malignant melanoma and colorectal cancer being the most responsive to the IL-2 or IL-2 plus LAK cell treatment. The systemic administration of the high doses of recombinant IL-2 was associated with severe side-effects, such as vascular leak syndrome, edema, anemia, fevers and chills, nausea and hypotension (reviewed by Siegel & Puri, 1991). These side effects (associated with systemic administration of IL-2) limited the efficacious dose that could be administered, and thus more effective ways of delivering IL-2 locally were needed.

IL-2 can also induce the cytotoxic activity of tumor infiltrating lymphocytes (TILs) (Cameron et al., 1990; Lindgren, et al., 1993). TILs could be isolated from resected tumors, and expanded in vitro following incubation with high concentrations of IL-2 (Yron et al., 1980). TILs are predominantly CD3+, CD8+ T-lymphocytes, and demonstrate greater cytolytic activity in vitro and in vivo, compared to LAK cells (Rosenberg et al., 1986; Beldegrun et al., 1989). However, when TILs were administered to patients in combination with recombinant IL-2, only a 10-30% response rate was observed, which was similar to that noted for the LAK cell plus recombinant IL-2 therapy (Kradin et al., 1989). Furthermore, TILs were thought to retain their ability to migrate and localize to the tumor site, however, studies have failed to show preferential localization of TILs at tumor sites, and most were
found in the liver and lungs of injected animals (Wong et al., 1991). These results led to the investigation of gene transduction of lymphocytes or of tumor cells to induce anti-tumor effector mechanisms.

As a result of the high degree of toxicity following the systemic delivery of recombinant IL-2, attention turned to local delivery of IL-2 at the site of the tumor. Injection of low doses of IL-2 into the tumor resulted in increased survival and a delay of tumor growth in animal models, without the IL-2 associated toxicity (Yeung, et al., 1992; Tohmatsu et al., 1993). Unfortunately, the short half-life of recombinant IL-2 in the serum requires the administration of multiple doses. In order to produce a more continual supply of IL-2, gene therapy approaches were used to introduce the gene for IL-2 into the tumor cells using retroviral vectors. Transduction of tumor cells with the gene for IL-2 resulted in a reduction of tumorigenicity in vivo, compared to non-transduced parental tumor lines in a wide variety of cancer types including; B16 melanoma (Karp et al., 1993), CMS-5 fibrosarcoma (Gansbacher et al., 1990; Bannerji et al., 1994), and MBT-2 bladder carcinoma (Conner et al., 1993). Although the use of retivoiral vectors to transduce tumor cells with IL-2 was efficacious, most of these studies utilized ex vivo approaches to modify the tumor cells. Since Ad are more efficient at transducing cells, the use of Ad vectors would allow for the in vivo administration of IL-2 expressing vectors by direct intra-tumoral injection.

During the course of the studies presented in this thesis, Ad vectors were used to deliver the gene for IL-2 to tumor cells and this treatment resulted in a modulation of tumorigenicity compared to non-transduced tumor cells following injection in vivo (Haddada
et al., 1993; Addison et al., 1995). More importantly, injection of Ad-IL-2 into established tumors mediated their regression, and protected animals from subsequent challenges of fresh tumor cells (Addison et al., 1995; Cordier et al., 1995; Toloza et al., 1996). These results demonstrate the efficacy which may be achieved using Ad vectors expressing cytokines in cancer immunotherapy, and will be discussed in more detail in Chapter 4.

3. Anti-Tumor Activity of Interleukin-4

Another cytokine which has been shown to possess potent anti-tumor activity in vivo is interleukin-4 (IL-4). IL-4 was originally identified as an approximately 20 kDa protein produced by helper T-cells that stimulated the proliferation of resting B-cells. IL-4 stimulates B-cells to produce antibody, and can induce antibody isotype switching to produce IgE. It is also an autocrine growth factor for Th2 helper cells and, together with IL-10, may promote Th2 differentiation of T-cells. The activation of various immune effector cells such as TILs (Kawakami et al., 1988), CTLs and macrophages (Mossman et al., 1986), has also been attributed to IL-4, and these may contribute, in part, to its anti-tumor activity.

The majority of studies using IL-4 as an anti-tumor agent involved the ex vivo transduction and subsequent implantation of IL-4-expressing tumor cells in a variety of animal models. When tumor cells were transfected with a retrovirus vector expressing IL-4, the tumorigenicity of these cells in vivo was decreased in a dose-dependent manner (reviewed by Tepper, 1994). In vivo injection of IL-4-expressing tumor cells resulted in the recruitment and activation of eosinophils around the tumor (Tepper et al., 1989; Tepper et al., 1992). Eosinophils are believed to be directly responsible for the anti-tumor activity induced by IL-4,
as IL-4-expressing tumor cells did not establish themselves in either nu/nu, bg/bg, bg/nu/xid, scid, nor w/w" mice, indicating an anti-tumor mechanism which is not dependent on T-cells, B-cells, NK cells, nor mast cells (Tepper, 1994). Although the initial studies using IL-4-transduced tumor cells suggest a major role for eosinophils, Golumbek et al. (1991) demonstrated that the presence of CD8+ T-cells was required for the development of a protective anti-tumor immunity.

The systemic delivery of recombinant IL-4 has been used as an anti-tumor therapy in clinical trials; however, as for recombinant IL-2, the administration of high doses of IL-4 has been associated with the induction of toxic side-effects, and a lack of anti-tumor efficacy (Atkins, et al., 1992; Prendiville et al., 1993). These results clearly demonstrate the necessity for administering cytokines locally in an attempt to increase efficacy and reduce toxicity.

4. Anti-tumor Activity of Interleukin-12

Interleukin-12 was initially discovered as a T-cell maturation factor that synergized with IL-2 to induce CTL and LAK cell activity (Wong et al., 1988; Stern et al., 1990), and as a factor that augmented NK cell-mediated cytotoxicity (Kobayashi et al., 1989). It is a unique cytokine, in that it is a heterodimer made up of a 40 kDa and a 35 kDa subunit linked by a disulphide bond. The disulphide-linked heterodimer is required for the biological activity of IL-12. IL-12 is produced mainly by B-cells, and cells of the monocyte/macrophage lineage, and in addition to increasing the cytotoxic activity of immune effector cells, IL-12 can induce the production of IFN-γ by NK cells (Chan et al., 1991; Perussia et al., 1992), T-cells (Bertagnolli et al., 1992), and TILs (Andrews et al., 1993). IL-12 also induces an
upregulation of expression of various cell surface molecules including: the IL-2 receptor on both NK and T-cells; the IL-12, IL-4 and TNF-α (p75) receptors on NK cells; and the adhesion molecules CD2, CD11a and CD54 on NK cells (reviewed by Brunda, 1994). The upregulation of these cell surface molecules would aid in enhancing the cytolytic and proliferative capacities of NK and T-cells. Additionally, IL-12 plays a major role in the generation of a Th1-type cell from naive T-cells, and will induce the proliferation of, and production of IFN-γ, by Th1 cell clones (Germann et al., 1993).

The anti-tumor activity of IL-12 has been well documented and administration of IL-12 either systemically or locally at the tumor site has been shown to inhibit the in vivo growth of murine melanoma, renal cell carcinoma, colon carcinoma, sarcoma, B-cell lymphoma, and Lewis lung carcinoma (reviewed by Brunda, 1994). The anti-tumor effects of intraperitoneal IL-12 administration were also observed against experimentally induced metastases. Anti-tumor activity following IL-12 administration was found to be far more potent than that induced by IL-2 or IFN-α when compared in murine melanoma and renal cell carcinoma models (Brunda et al., 1993a). The mechanism for IL-12-mediated tumor killing appears to be partially T-cell dependent, since its activity was substantially reduced in nude mice (T-cell deficient), and following depletion of CD8+ (but not CD4+) T-cells (Brunda et al., 1993b).

Administration of IL-12 also leads to an increase in the expression of IFN-γ, which is important for the anti-tumor activity mediated by IL-12, since neutralizing antibodies to IFN-γ abrogate these effects (Nastala et al., 1994). The use of IL-12 in vivo can be associated with reversible dose-dependent side effects such as, anemia, leucopenia, thrombocytopenia,
and splenomegaly (Gately et al., 1994; Sarmiento, et al., 1994; Tare et al., 1995). A phase II clinical trial was briefly suspended due to toxicity noted with IL-12 systemic administration; however, the problems appear to have been overcome and the trial has resumed (Cohen, 1995). These problems suggest that local delivery of IL-12 might reduce the toxicity associated with systemic administration, and to this end several gene therapy vectors have been constructed and were shown to be efficacious in inducing anti-tumor effects in murine models (Zitvogel et al., 1994; Meko et al., 1995; Bramson et al., 1996a).

An Ad vector (AdmIL-12.1) which expressed both subunits of IL-12 induced secretion of biologically active protein from infected cells (Bramson et al., 1996b), and mediated regression of established mammary adenocarcinoma tumors in 75% of treated animals following intra-tumoral injection of the Ad vector (Bramson et al., 1996a). Of these responding animals, 1/3 remained tumor free while the others succumbed to regrowth at the primary tumor site. These data suggest that although efficacious, administration of IL-12 is associated with tumor recurrence, and to prevent recurrence or metastatic spread of disease, IL-12 administration may be more effective when used in conjunction with other anti-tumor therapies.

5. Induction of Th1 Versus Th2 Profiles in Anti-tumor Activities

It is now clear that there are two different populations of CD4+ T-helper cells, Th1 and Th2. These cells are divided on the basis of their profiles of cytokine secretion; Th1 cells secrete IFN-γ, IL-2 and lymphotoxin (LT), while Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (reviewed by Mossman & Sad, 1996). Th1 and Th2 cells are associated with
different effector arms of the immune system: Th1 cells are involved in cell-mediated immunity and inflammatory responses, while Th2 cells are involved in the induction of humoral responses and mediate antibody production by B-cells, and proliferation and activation of eosinophils. Each T-helper cell type appears to regulate the activity and effector functions of the other. For example, the production of IFN-γ by Th1 cells inhibits the proliferation of Th2 cells (Mossman & Coffman, 1989), and the production of IL-10 by Th2 cells inhibits the production of cytokines by Th1 cells (Fiorentino et al., 1989). The induction of a Th1 or a Th2 phenotype is thought to occur as a result of stimulation of an uncommitted precursor cell by various cytokines; IFN-γ, IL-12 and TGF-β enhance the development of Th1 cells, while IL-4 promotes a Th2 phenotype. There is now evidence that CD8+ cells may also be grouped into similar profiles, Tc1 and Tc2, and that the differentiation of cells into each phenotype is induced by the same cytokines as for the CD4+ cells. The regulation of the type 1 T-cells by the type-2 T-cells and vice versa, may be critically important in the downregulation of strong immune responses.

To induce effective tumor killing in vivo, a Th1/Tc1 lymphocyte profile is desirable. Thus mechanisms which target the promotion of these cells to the type-1 profile should be examined for tumor therapy. Tumor cells themselves appear to modulate the immune response to render it ineffective to mediate tumor killing by preventing T-cells from differentiating into a type-1 phenotype through the production of molecules such as IL-10 (Sulitzeanu, 1993). Therefore, the expression of IL-12 or IFN-γ in tumor cells should counteract this effect, and promote type-1 cell mediated immune responses. Indeed, this has
been shown to occur in various animal models of tumorigenesis (Brunda et al., 1993b; Gately et al., 1993; Nastala et al., 1994).

6. Breast Cancer and the Polyoma Middle T Antigen Model of Mammary Adenocarcinoma

Breast cancer will affect 1 in every 9 women, and it was estimated that approximately 183,000 new cases and 46,000 deaths from breast cancer would occur in the United States in 1995 (Tripathy D. & Henderson, 1996). Although new techniques for early detection, and the identification of hereditary risk factors for the development of breast cancer are now routinely used, there is still no efficacious treatment for breast cancer that will prevent the recurrence and metastatic spread of the disease in the majority of patients. In fact, the mortality rate of patients with metastatic disease is close to 100%, with a mean survival of only 2 to 4 years (Tripathy D. & Henderson, 1996).

Metastases of primary tumors is likely a very complex event which requires multiple processes, such as, activation of proteases to allow tumor cells to invade basement membranes, induction of neovascularization by the tumor cells at the site of metastases, and expression or alteration of cell surface molecules that have been found to correlate with metastatic potential. Overexpression of some oncogenes, such as Neu/ErbB-2 and Int-2, has been found to correlate with the metastatic potential of tumors, and an inverse correlation between their overexpression and mean survival time has been observed (Slamon et al., 1987; Lidereau et al., 1988).

In an attempt to examine the role that oncogene overexpression plays in mammary adenocarcinoma, transgenic mouse models have been constructed which express such
oncogenes under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR), which limits transgene expression to mainly the mammary tissue. Overexpression of the neu-protooncogene in the mammary tissue of transgenic mice resulted in the development of focal mammary tumors within approximately 4 months of birth (Guy et al., 1992a). Moreover, many of these animals developed pulmonary metastases, indicating that oncogene over-expression may confer enhanced metastatic potential to the tumor cell.

To study the efficacy of Ad vectors encoding cytokine genes in the immunotherapy of cancer, we used a transgenic mouse model of mammary adenocarcinoma based on the over-expression of the polyoma virus middle T antigen (PyMidT) oncogene (Guy et al., 1992b). These animals develop adenocarcinoma of all mammary epithelium by 8-10 weeks of age, and like the tumors which arose in the neu-transgenic mice, the PyMidT induced tumors have the potential to metastasize to the lungs. These properties make the PyMidT tumors an excellent model to examine the effect of Ad-cytokine vector delivery both locally and at distal sites. We found that these tumors could be explanted and, upon injection in syngeneic mice, formed tumors in 100% of injected animals by 21 days post-injection (Chapters 4 and 5).
This thesis presents data on the use of Ad vectors expressing IL-2, IL-4 and IL-12, alone and in combination, for the immunotherapy of cancer. In Chapter 3 data are presented that examine factors which influence the level of transgene expression from Ad vectors, such as orientation of the transgene expression cassette within the Ad vector backbone, and the choice of promoter elements controlling transgene transcription. I constructed Ad vectors expressing IL-2 and IL-4 and characterized these vectors for their ability to produce biologically active protein in infected cells, and to modulate tumorigenicity (Chapter 4 and 5). Further experiments were performed to determine the mechanism of tumor killing following these treatments by examining the induction of tumor-specific CTLs (Chapter 4 & 6). The use of combinations of Ad vectors expressing different cytokines (i.e. IL-2, IL-4, and IL-12) was also examined in order to determine if more efficacious approaches could be defined (Chapter 6). Furthermore, I tested the ability of the Ad-cytokine vectors to induce anti-tumor immune responses that had the potential to recognize and mediate regression of distal untreated tumors (Chapter 6). Also included in this work are descriptions of bacterial plasmids (Appendix I), and Ad vectors which have been constructed (Appendix II), strategies for construction of "stuffer viruses" (Appendix III), and construction and characterization of tumor-antigen specific CTL target cell lines (Appendix IV).

All plasmid and viral constructs described in this thesis were constructed by me with the following exceptions: pMH4, pMH5, pJM17, pBH10G10, Addl70-3, AdmIL-12.1, and AdDK1. Unless otherwise stated in the text, all in vitro assays for protein detection and
quantitation were performed by me. All the animal experiments testing the ability of the Ad-cytokine vectors to modulate tumorigenicity were performed by me with the exception of data that compared the efficacy of administration of AdCAIL-2 in combination with AdmIL-12.1 in animals bearing single tumors (performed by Dr. Jonathan Bramson). The majority of the CTL assay data presented within this thesis was generated by me with some assistance from Dr. Jonathan Bramson.
CHAPTER 2. MATERIALS AND METHODS

A. Recombinant DNA Techniques

1. Bacterial Cultures

The bacterial host used for plasmid propagation was the *Escherichia coli* strain DH5α (supE44, hsdR17, recA1, gyrA96, thi-1, relA1). Bacteria were grown on solid phase on Luria-Bertani broth agar (LBA) or in liquid phase in Luria-Bertani broth (LB) or Terrific Broth (TB) supplemented with the appropriate antibiotic (80 μg/ml ampicillin or 50 μg/ml kanamycin sulphate).

2. Bacterial Transformation

Bacterial cells were transformed by calcium chloride transformation or electroporation. Calcium chloride competent cells were prepared by inoculation of 1 L of LB with 1/100 volume of an overnight bacterial culture, followed by incubation at 37°C with shaking until an OD₅₉₀ of approximately 0.375. The culture was poured into pre-chilled 50-ml sterile polypropylene tubes and incubated on ice for 10 min. Cells were centrifuged for 10 min at 2000 xg at 4°C, the cell pellet resuspended in 10 ml of ice-cold CaCl₂ solution (60 mM CaCl₂, 10 mM PIPES pH 7, 15% glycerol, filter sterilized), and centrifuged for 5 min at 1400 xg at 4°C. The cell pellet was again resuspended in 10 ml of ice-cold CaCl₂ solution and incubated on ice for 30 min. The cells were recentrifuged as in the previous step and the
pellet resuspended in 2 ml of the CaCl₂ solution. Cells were aliquoted (220 μl per tube) and frozen at -70°C until use. For transformation, 100 μl of competent cells was added to varying quantities of ligated plasmid DNA, and incubated on ice for 10 min. Cells were heat shocked by incubation at 42°C for 2 min, 1 ml of LB was added, and the cultures were incubated at 37°C with shaking for a minimum of 1 hour.

To prepare bacterial cells for electroporation, 10 ml of an overnight bacterial culture was used to inoculate 1 L of LB, and the culture was incubated at 37°C with shaking until an OD₆₀₀ of 0.5-1.0. The culture was incubated on ice for 30 min, and centrifuged at 2500 xg for 10 min. The cells were washed sequentially in 500 ml and 20 ml of ice cold 10% glycerol, and resuspended in 2-3 ml of 10% glycerol (final concentration of approximately 3x10¹⁰ cells/ml). Aliquots (50-100μl) in microfuge tubes were frozen in a dry ice bath, and stored at -70°C. For electroporation of plasmid DNA, 50 μl of cells were mixed with 1-2 μl of ligated DNA (~5 ng), incubated on ice for at least 1 min, and electroporated in a cold 0.2 cm electroporation cuvette at 25 μF, 2.25 kV or 1.6 kV, and 200 Ω (BioRad Electroporator) to generate a time constant of 4.5-5 msec. Immediately following electroporation, the cells were diluted to 1 ml with LB and incubated at 37°C, with shaking for at least 1 hr.

Following either calcium chloride transformation or electroporation, appropriate dilutions of the transformed cells were plated on LBA containing the appropriate antibiotic.

3. Small Scale DNA Preparation

Antibiotic resistant colonies were inoculated into 2 ml of TB containing the appropriate antibiotic, and incubated at 37°C overnight with shaking. Approximately 1.5 ml of the culture
was aliquoted into microfuge tubes and centrifuged at 16,000 xg for 1 min. The cell pellet was resuspended in 200 μl of lysis buffer (50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA), and incubated at room temperature (RT) for 5 min. Four hundred μl of freshly prepared alkaline-SDS buffer (0.2 N NaOH, 1% SDS) was added, the sample was mixed by inversion, and incubated on ice for 5 min. Three hundred μl of 7.5 M ammonium acetate solution (pH 7.8) was added, the solution mixed by inversion, and the tube incubated on ice for 10 min. The cell debris was pelleted by centrifugation in a microfuge for 5 min at 16,000 xg, and the supernatant was transferred to a fresh tube containing 450 μl of isopropanol (0.6 volumes). The tube was mixed and incubated at RT for 15 min and the DNA pelleted by centrifugation for 10 min at 16,000 xg. The DNA pellet was washed with 70% ethanol, dried at RT for ~15 min, and resuspended in 75 μl of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). TER buffer (TE plus 10μg/ml Rnase, 25 μl per tube) was added, and the samples were incubated at 65°C for 20 min.

4. Large Scale Cesium-Chloride Purified Plasmid Preparation

For large scale preparation of plasmid DNA, 1 L of TB (plus appropriate antibiotics) was inoculated with 5 ml of a 6-8 hr old bacterial culture, and incubated overnight with shaking at 37°C. Cells were harvested by centrifugation at 6000 xg for 10 min at 4°C. The cell pellet was resuspended in 40 ml of lysozyme buffer (lysis buffer supplemented with 5 mg/ml lysozyme), and incubated at RT for 20 min. Alkaline-SDS buffer (80 ml) was added, the solution mixed by swirling, and incubated for 10 min on ice. Forty ml of 5 M potassium acetate solution (3 M potassium and 5 M acetate made by addition of 60 ml 5 M potassium
acetate and 11.5 ml glacial acetic acid to 28.5 ml ddH₂O) was added, mixed by swirling and incubated on ice for an additional 20 min. Distilled H₂O was added (10 ml) and the solution centrifuged at 6000 xg for 10 min at 4°C. The resulting supernatant was filtered through cheesecloth, 100 ml of isopropanol was added, and the solution was incubated at RT for 30 min. The precipitated DNA was pelleted by centrifugation at 6000 xg for 10 min at RT, and the DNA pellet resuspended in 7 ml of 0.1x SSC (15 mM NaCl, 1.5 mM sodium citrate). Cesium chloride (1.2 g per ml of solution) was added, and the solution was incubated on ice for 30 min. Following centrifugation at 6000 xg at 4°C for 20 min, the supernatant was transferred to a Beckman 13 ml quickseal tube, 400 μl of 6mg/ml ethidium bromide was added, and the cesium mixture was overlayed with light paraffin oil. The tube was subsequently heat sealed, mixed by inversion, and the DNA centrifuged to equilibrium in a Beckman Vti65.1 rotor at 55,000 rpm at 15°C for 16-18 hours. Plasmid bands were removed from the cesium chloride gradients using a syringe and an 18 gauge needle, and the ethidium bromide was extracted with equal volumes of cesium chloride saturated isopropanol. Three volumes of TE was added and the DNA was precipitated by addition of 8 volumes of 96% ethanol. The DNA was pelleted by centrifugation at 6000 xg at 4°C for 15 min, the pellet was washed with 70% ethanol, dried, and resuspended in 0.1x SSC. Nucleases were inactivated by incubation at 65°C for 10-20 min. DNA concentrations were determined from the absorbance at 260/280 nm using the equation: [DNA] = 50 μg/ml x Abs₂₆₀ x dilution factor; or by fluorometric determination in a Hoeffer TKO fluorometer and comparison to known quantities of DNA.
5. Enzymatic Manipulations of DNA

Restriction and Repair of DNA

Restriction enzyme digestion of plasmid DNA was performed according to the manufacturer's protocol. For the repair of 3' recessed DNA ends, plasmid DNA was diluted to a concentration of approximately 50-100 ng/μl, 4 μl of a solution containing 2.5 mM of each dNTP and 1 unit of Klenow enzyme per μg of DNA was added, and the reaction was incubated at 37°C for 30 min. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM and the enzyme was inactivated by incubation at 75°C for 10 min. Reactions to repair 5' overhanging DNA ends were performed as described above, however, 1 unit of T4 DNA polymerase was used in place of Klenow enzyme.

Purification of Restricted DNA Fragments

Restricted DNA fragments were recovered from either low melting temperature or regular agarose gels by excision of the desired fragment from the gel with a clean scalpel, and the DNA was isolated from the gel using the Wizard PCR DNA isolation kit (Promega) according to the manufacturer's protocol. For small DNA fragments (less than 250 bp), samples were separated on a polyacrylamide gel, and the DNA was isolated using the crush and soak technique (Maniatis et al., 1989).

DNA Sequencing

DNA sequences were determined using the Sequenase Kit (USB) according to the manufacturer's protocol or, alternatively, by the automated sequencer in the MOBIX central facility, McMaster University.
Ligation ofRestricted DNA Fragments

All ligationsof bacteriophage T4 DNA ligase, and reactions were incubated overnight at 14°C, or at RT for 1-3 hr. Ligation reactions were terminated by incubation at 65°C for 10 min.

B. Northern Blot Analysis

Total RNA was isolated from virus- or mock-infected cells using the RNeasy kit (Qiagen) according to the manufacturer's protocol, and the concentration of RNA was calculated from the absorbance at 260 nm using the following equation: 40 μg/ml x Abs_{260} x dilution factor. Equal quantities of total RNA were separated on formaldehyde agarose gels (Maniatis et al., 1989) until the bromophenol blue had run 3/4 of the length of the gel. RNA was transferred to positively charged nylon membranes (Boehringer Mannheim or Amersham) by capillary transfer (Maniatis et al., 1989). In short, the gel was incubated in 10x SSC for 45 min, and placed on Whatman #3 filter paper that was saturated with 10x SSC and whose ends were submerged in a tray of 10x SSC. A positively charged nylon membrane cut to the size of the gel was placed on top of the agarose gel, followed by 5 sheets of Whatman #3 filter paper moistened in 10x SSC. A 2 cm stack of dry Whatman #3 filter paper was placed on top of this, followed by a 6 cm stack of paper towels. A glass plate was used to cover that stack of towels, and a bottle filled with 100 ml of liquid was placed on top as a weight. Following overnight transfer, the assembly was dismantled, the membrane was dried at RT and UV crosslinked at 150 mJoules. Detection of specific transcripts was performed using
either DNA probes derived from random prime labelling of purified plasmid DNA fragments using the DIG high prime labelling and detection kit (Boehringer Mannheim) or a commercially available RNA probe against human β-actin (Boehringer Mannheim). The DIG high prime labelling system is based on the incorporation of digoxigenin-11-UTP (DIG) into the DNA or RNA probes, followed by the chemiluminescent detection of the DIG-labelled molecule using an alkaline phosphatase-conjugated DIG specific antibody. The luminescent reaction was detected by autoradiography using XAR-5 film (Kodak).

C. Mammalian Cell Culture

All tissue culture cell lines were grown at 37°C in a 5% CO₂ atmosphere. All media reagents were obtained from Gibco, unless otherwise indicated, and culture medium was supplemented with 100 U/ml of penicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine, and 25 μg/ml fungizone (Squibb Canada). Cells were seeded into new dishes following two washes with PBS and treatment with trypsin-EDTA (Gibco), except for 293 cells which were passaged by washing confluent monolayers twice with sterile citric saline (130 mM KCl, 15 mM sodium citrate). The cell lines used in these studies, and the medium in which they were cultured, are listed in Table 2-1. All cells were grown in 10% fetal bovine serum, except for 293N3S cells which were cultured in 10% horse serum.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Culture Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>293</td>
<td>human embryonic kidney cells transformed with the adenoviral type 5 E1 region</td>
<td>MEM</td>
</tr>
<tr>
<td>MRC5</td>
<td>human fibroblast strain (ATCC CCL 171)</td>
<td>$\alpha$-MEM</td>
</tr>
<tr>
<td>A549</td>
<td>human lung carcinoma (ATCC CCL 185)</td>
<td>$\alpha$-MEM</td>
</tr>
<tr>
<td>983M</td>
<td>human metastatic melanoma (Herlyn et al., 1985)</td>
<td>RPMI</td>
</tr>
<tr>
<td>793</td>
<td>human radial phase melanoma (gift of N. Lassam)</td>
<td>RPMI</td>
</tr>
<tr>
<td>MeWo</td>
<td>human metastatic melanoma (Herlyn et al., 1985)</td>
<td>RPMI</td>
</tr>
<tr>
<td>Wm35</td>
<td>human vertical phase melanoma (gift of N. Lassam)</td>
<td>RPMI</td>
</tr>
<tr>
<td>MCF7</td>
<td>human mammary adenocarcinoma (ATCC HTB 22)</td>
<td>DMEM</td>
</tr>
<tr>
<td>B16BL6</td>
<td>murine melanoma (Fidler, 1975)</td>
<td>MEM + NEAA + Sodium pyruvate</td>
</tr>
<tr>
<td>MT1A2</td>
<td>murine PyMidT induced mammary adenocarcinoma isolated by C. Addison from explanted tumors of the PyMidT transgenic strain #634 (Guy et al., 1992b)</td>
<td>MEM</td>
</tr>
<tr>
<td>SV329</td>
<td>SV40 T antigen transformed FVB/N mouse kidney cells (constructed by C. Addison)</td>
<td>$\alpha$-MEM</td>
</tr>
<tr>
<td>PT0516</td>
<td>murine fibroblast cells from FVB/N kidneys (isolated by C. Addison)</td>
<td>$\alpha$-MEM</td>
</tr>
<tr>
<td>Cell Line</td>
<td>Description</td>
<td>Medium</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>516MT3</td>
<td>PT0516 cells cotransfected with neo resistance plasmid and polyoma middle T antigen-expressing plasmid (constructed by C. Addison)</td>
<td>$\alpha$-MEM + 400ng/ml G418</td>
</tr>
<tr>
<td>MDCK</td>
<td>canines kidney cells (ATCC CCL 34)</td>
<td>$\alpha$-MEM</td>
</tr>
<tr>
<td>RF</td>
<td>rat fibroblasts cells from explanted lung (isolated by Z. Zhang)</td>
<td>$\alpha$-MEM</td>
</tr>
</tbody>
</table>
D. Construction and Propagation of Recombinant Adenovirus

1. Generation of Recombinant Adenoviruses by Cotransfection of 293 Cells

All recombinant viruses constructed in this thesis are first generation adenoviral (Ad) vectors, that have been rendered replication defective by deletion of the E1 genes from the adenoviral genome. Recombinant viral vectors were generated by homologous recombination in 293 cells of two plasmids as has been previously described (McGrory et al., 1988; Bett et al., 1994). A schematic for the construction of Ad vectors is shown in Figure 2-1. In short, the gene of interest was cloned into a "shuttle" plasmid under the regulation of an exogenous promoter and the simian virus 40 (SV40) polyadenylation (An) sequences. The promoters used in these studies include: the human CMV IE (-299 to +72 relative to transcriptional start site), or the murine CMV IE (-1491 to +36 "long", or -491 to +36 "short"). Descriptions of all plasmids constructed in these studies can be found in Appendix I. All shuttle plasmids contain the sequences from the Ad type 5 genome from nucleotides 22 to 341 (which contains the Ad packaging signal required for genome encapsidation) and from 3525 to 5790, and do not include the E1 sequences required for transactivation of viral promoters and for virus replication. The shuttle plasmids, containing the gene of interest inserted in the E1 region, were cotransfected in 293 cells along with a second plasmid containing complementary Ad sequences. pJM17 is a 40 kb plasmid that contains the entire adenoviral genome, ITR's joined in a head to tail manner, and a 4.3kb insert (containing a bacterial ampicillin resistance gene and origin of replication) in the E1 region. Upon
Figure 2-1: Rescue of Foreign DNA Sequences into First Generation Ad Vectors Using the Two Plasmid System.

The strategy for rescuing recombinant Ad vectors is outlined. 293 cells are cotransfected with a circular Ad genome plasmid (pJM17 or pBHG10) and a "shuttle plasmid" that is deleted for the Ad E1 sequences. The foreign DNA sequences (black bar) are flanked by exogenous regulatory sequences (usually viral promoters and polyadenylation signals), and the left end adenoviral sequences. Homologous recombination between these two plasmids, within a 2-kb sequence common to both, results in the generation of an adenoviral genome containing a foreign DNA insert in the left end of the Ad vector genome.
linearization and replication in 293 cells, this plasmid is too large to package into viral capsids (McGrory et al., 1988). In addition, the E3 region of pJM17 is inactivated and therefore does not synthesize any of the E3 viral proteins, with the exception of the 19 kDa protein. Cotransfection and subsequent homologous recombination with the shuttle plasmids results in a viral genome lacking E1 sequences and a bacterial origin of replication and ampicillin resistance gene, but contains the gene of interest, that can now be packaged into virions. The plasmid pBHG10 also contains the entire adenovirus genome; however, in this plasmid, the E3 sequences and the Ad packaging signal are deleted (Bett et al., 1994). The absence of the packaging signal results in viral genomes that cannot be packaged into viral capsids. However, cotransfection and subsequent homologous recombination with the shuttle plasmid in 293 cells generates genomes which possess the packaging signal (present in the shuttle plasmid) and can therefore be efficiently packaged into virions. Recombination between the shuttle vector and pBHG10 results in a viral vector that is deleted for both E1 and E3 sequences, and contains the gene of interest in the E1 region. Cotransfections were performed in 60 mm dishes containing 70-80% confluent, low passage 293 cells (passages 27 to 38) using calcium phosphate transfection (Graham & Van der Eb, 1973). For each 60 mm dish, 10 µg of salmon sperm DNA was added to 0.5 ml sterile 1x HeBS (21 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 137 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄·2H₂O, 5.6 mM glucose, adjusted to pH 7.1), and the DNA was sheared by vortexing for 1 min. The mixture was then aliquoted to polystyrene tubes, the shuttle plasmid DNA and either pBHG10 or pJM17 were added, and the samples were mixed by shaking.
gently. Typically the two plasmids were mixed in varying ratios, such as 5 µg:5 µg, 5 µg:10 µg, or 10 µg:10 µg per dish. Following this, 25 µl of 2.5 M CaCl₂ was added per 0.5 ml of DNA solution, and the samples were again mixed by gentle shaking. The solutions were incubated at RT for 30 min, and 0.5 ml of precipitate was added to each 60 mm dish of 293 cells. Dishes were incubated with the DNA precipitate for 4-5 hr, or overnight at 37°C, in 5% CO₂. After incubation, the medium was removed, and cells were overlayed with 10 ml of a 1:1 mixture of overlay medium (2x MEM supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 50 µg/ml fungizone, 600 µg/ml L-glutamine, 0.2% yeast extract, and 10% heat inactivated horse serum) to 1% agarose. Once the overlay had solidified, dishes were incubated at 37°C, until plaques developed or for a maximum of 21 days. Typically, visible plaques were observed 10-16 days post-cotransfection. All first generation recombinant adenoviral vectors that have been rescued are listed in Appendix II.

2. Screening Recombinant Viral Plaques

An agar plug containing the viral plaque was removed from the dish using a sterile Pasteur pipette, and the picked plaque was then stored at -70°C in 0.5-1 ml of sterile PBS (137 mM NaCl, 3 mM KCl, 4 mM Na₂HPO₄, 1.5 mM KH₂PO₄) containing 10% glycerol. In order to identify positive plaque isolates, 0.2 ml of the plaque isolate was used to infect a 60 mm dish of 293 cells. To infect 293 cell monolayers, medium was removed from the dish, virus inoculum was added to the surface of the cells, and the dish was incubated at 37°C for 30 min. Following incubation, medium containing 5% horse serum was replaced and the cells were incubated at 37°C. When complete cytopathic effects (CPE) was observed, (ie. 90-
100% of cells had rounded up and were no longer attached to the dish), the cells were allowed to settle for approximately 15-30 min at RT, and the supernatant was removed, added to glycerol (10% final concentration), and stored at -70°C. The remaining cells on the dish were digested in 0.5 ml of pronase-SDS (500 µg/ml pronase in 10 mM Tris pH 7.4, 10 mM EDTA, 0.5% SDS) at 37°C for a minimum of 4 hr. The cell extract was transferred to a sterile microfuge tube, the DNA was extracted with one volume of buffer-saturated phenol, and precipitated by addition of 1/10 volume of 3 M sodium acetate and 2 volumes of cold 96% ethanol. The solution was mixed by inversion, and DNA was pelleted by centrifugation at 16,000 xg in a microfuge for 15 min. The DNA pellets were washed twice with 96% ethanol, dried at room temperature, and resuspended in 50 µl of 0.1x SSC. The structure of the viral DNA was analysed by restriction enzyme digestion, and isolates with the predicted digestion pattern were plaque purified and used in subsequent experiments.

3. Plaque Purification of Recombinant Virus

Positive vector isolates were plaque purified prior to expansion to high titer stocks and use in further experiments. Serial dilutions of the original plaque agar plug were made in PBS supplemented with 0.68 mM CaCl₂ and 0.5 mM MgCl₂ (PBS²⁺), and 0.2 ml of each dilution was used to infect a 60 mm dish of 293 cells as described above. Following viral adsorption, the monolayers were overlayed with 10 ml of a 1:1 ratio of overlay medium to 1% agarose, and upon solidification of the overlay, dishes were incubated at 37°C until visible plaques could be seen. Well isolated plaques were removed as agar plugs, and screened as described in the above section. The supernatant that was saved following plaque screening
was used in subsequent infections to generate high titer viral stocks.

4. Propagation of Virus

Medium was removed from dishes of 293 cell monolayers and virus inoculum (plaque plugs, screening supernatant or high titer crude stocks) was added to the surface of the monolayer: 0.2 ml for 60 mm dishes, and 2 ml for 150 mm dishes. Cells were incubated at 37°C for 30 min to allow adsorption of the virus, and MEM plus 5% heat inactivated horse serum was added. After complete CPE, the infected cells were treated with pronase-SDS as described above, or were harvested for preparation of a high titer crude stock as follows: the cells were scraped into the medium, the cell debris (containing the majority of the virus which remains cell associated) was centrifuged at 6000 xg for 15 min, and the cell pellet was resuspended in PBS + 10% glycerol. 293N3S cells were used for the preparation of cesium chloride gradient purified viral stocks. One to three litres of 293N3S spinner culture was centrifuged at 2300 xg for 30 min, half of the conditioned medium was saved, and the cell pellets were resuspended in 50 to 100 ml of fresh supplemented Joklik's medium with 5% horse serum. High titer crude viral preparations or infected cell supernatant was added to the 293N3S cells at a multiplicity of infection (moi) of 1 to 5. Cells were incubated at 37°C, with stirring, for 1.5 hours, at which time the infected cells were transferred to the spinner flask along with the reserved conditioned medium and an equal volume of fresh medium. Cells were monitored for the progression of infection by inclusion body staining. When 80-100% of cells contained inclusion bodies, the infected cells were harvested by centrifugation at 2300 xg for 30 min. The supernatant was discarded, the cell pellet was resuspended in a small
volume of the remaining medium, transferred to a 50 ml polypropylene tube and centrifuged at 600 xg. The cell pellet was resuspended in 15-20 ml of 0.1 M Tris-HCl pH 8 and stored at -70°C until cesium chloride gradient banding.

5. Inclusion Body Staining

At various times post-infection, a 3 ml sample of infected 293N3S spinner cells was removed and centrifuged at 600 xg for 5 min. The cell pellet was resuspended in 0.5 ml of 1% sodium citrate, and incubated at RT for 10 min. Carnoy's fixative (3:1 methanol:glacial acetic acid, 0.5 ml per sample) was added, and cells were incubated at RT for another 10 min. An additional 2 ml of Carnoy's fixative was added, and the cells were centrifuged for 5 min at 600 xg. The cell pellet was resuspended in 5 drops of Carnoy's fixative, and 1 drop of this solution was placed onto a microscope slide and allowed to air dry. The inclusion bodies were then stained with 1-2 drops of orcein stain (2% orcein in 50% glacial acetic acid). Inclusion bodies appeared as refractile densely-staining nuclear structures.

6. Cesium Chloride Gradient Banding of Virus

The frozen infected cell suspension (15-20 ml) was thawed, and 1/10 volume of 5% sodium deoxycholate was added. The solution was incubated at RT for 30 min with repeated mixing by inversion every 10 min. The viscosity of the solution was reduced by the addition of 1/100 volume of 2 M MgCl₂ and 1/200 volume of DNase I solution (100 mg pancreatic DNase I in 10 ml of 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, in 50% glycerol). The solution was mixed by inversion and incubated at 37°C for 1.5 hr, with repeated mixing every 15 min. A cesium chloride-saturated TE
solution was added (1.8 ml per 3.1 ml of viral suspension), giving a final density of 1.35 g/ml. The solution was mixed and transferred to a 13 ml Beckman ultraclear quickseal tube, and centrifuged at 35,000 rpm in a Beckman 50Ti rotor at 4°C for 16-20 hr. The virus band, visible as a whitish band in the upper 1/3 of the tube, was collected by puncturing a hole in the top and bottom of the tube and allowing the virus to drop into a fresh tube. The virus was transferred to a 4.5 ml Beckman ultraclear tube and centrifuged at 35,000 rpm in an SW50.1 rotor at 4°C for 16-20 hours. The viral band was collected by dropping, as described above, and the virus was dialysed twice against 500 ml of 0.01 M Tris-HCl pH 8, or against PBS supplemented with 10% glycerol for at least 4 hr per dialysis medium change. Glycerol was added (to all virus stocks) to a final concentration of 10% before being stored at -70°C.

7. Titration of Viral Stocks

All adenovirus vectors were titered on 293 cells by plaque assay and expressed as plaque forming units per ml (pfu/ml). Virus stocks were serially diluted in PBS²⁺, and 0.2 ml of each dilution was used to infect 60 mm dishes of 293 cells. Following infection, the cells were overlayed with a 1:1 ratio of supplemented 2x medium to 1% agarose, as previously described. Plaques were counted at 7 days post-infection and again at 10 days post-infection, and the titer was calculated as follows: average number of plaques x dilution factor x 5 = pfu/ml.
E. Detection of Protein from Infected Cells

1. β-galactosidase assays

Cell monolayers infected with Ad vectors expressing the lacZ gene were assayed for β-galactosidase (β-gal) activity as follows. Cells were scraped into the medium, transferred to a 15 ml polypropylene tube, pelleted by centrifugation at 2000 xg for 5 min, and the pellet stored at -70°C until the time of assay. Prior to the β-gal assay, the cell pellet was thawed, resuspended in 200 μl of either reporter lysis buffer (Promega) or PMSF/NP40 lysis buffer [250 mM Tris-HCl pH 7.8, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.5% nonidet P 40 (NP40)], vortexed, centrifuged for 20 min at 2000 xg at 4°C, and the supernatant was transferred to a fresh microfuge tube. For assay of β-gal activity, 40 μl of sample was combined with 350 μl of mercaptoethanol solution (10 mM KCl, 1 mM MgSO₄, 100 mM sodium phosphate pH 7.5, 50 mM 2-mercaptoethanol). The samples were then pre-incubated at 37°C for 5 min, 132 μl of ONPG solution [4 g/L o-nitrophenol-β-D-galactopyranoside (ONPG) in 100 mM sodium phosphate pH 7.5] was added, and the reaction was incubated at 37°C for 1 hr. The reaction was terminated by the addition of 172 μl of 1 M Na₂CO₃. The absorbance at 420 nm was measured and the quantity of β-gal present in each sample was determined by interpolation of a standard curve generated by known quantities of β-gal protein.

2. Detection of β-gal in Paraffin Embedded Sections

The distribution of injected Ad vector in tumors in vivo was analysed by
immunohistochemistry of paraffin embedded tumor sections. Tumors were injected with 5x10^8 pfu of Ad vectors expressing the lacZ gene, and at various times post-injection, the tumors were removed, fixed in neutral buffered formalin for 24 hours, and then washed in increasing concentrations of ethanol (from 50% to 70%, for two 30 min washes at each concentration). Tumor samples were cut longitudinally with a razor blade, placed in a tissue cassette, and paraffin embedded in the Histology Department of McMaster University Hospital. The embedded tissue was sectioned into 3 μm thick sections using a microtome and, following air drying overnight, the sections were fixed onto Aptex coated slides by incubation at 60°C for 45 min. The tissue section was de-paraffinized by three consecutive 15 min incubations in xylene, and three 1 min washes in 100% ethanol. Slides were incubated in 1.5% hydrogen peroxide and 0.03 N HCl in methanol for a minimum of 30 min, and rehydrated by successive incubations in 100%, 95%, and 70% ethanol for a minimum of 1 min each. Slides were then rinsed in tap water (the metals found in tap water aid in the staining of tissue in subsequent steps) for 5 min, distilled water for 1 min, and incubated in TBS (0.05 M Tris pH 7.6, 0.85% NaCl) for 15 min. Excess liquid was removed from the slides with a kimwipe, the tissue was circled with a PAP pen (Dako), and 2 drops of normal swine serum (Dako X901 diluted 1:100 in TBS) was added to the tissue. The slides were incubated in a humid chamber for 15 min, the normal swine serum was removed, 1-2 drops of the β-gal antibody was added (Oncogene Science anti-β-gal antibody Ab-1, diluted to a concentration of 0.4 μg/ml in Dimension D-2030 antibody dilution fluid), and the slides were incubated overnight at RT in a humid chamber. Next day, the slides were washed 3x 5 min by flooding
the slides with TBS, the excess liquid was removed with a kimwipe and the tissue was overlayed with 1-2 drops of biotinylated rabbit anti-mouse antibody (Dako E464 diluted 1:300 in Dimension D-2030 antibody dilution fluid). The slides were incubated in the secondary antibody for 45 min at RT in a humid chamber, and washed as described above. After the excess liquid was removed, 1-2 drops of horse radish peroxidase (HRP) conjugated to strepavidin (Dako P397, diluted 1:600 in Dimension D-2030 antibody dilution fluid) was added, and the slides were incubated at RT in a humid chamber for 45 min. The slides were washed, rinsed in TBS, and incubated in DAB solution (0.02% 3'3' diaminobenzidine tetrahydrochloride (DAB) in TBS) for 2 min to equilibrate. Hydrogen peroxide was then added to the DAB solution to a final concentration of 0.03%, and the slides were incubated for a further 15 min, or until a sufficient colour had developed in the tissue sample. The slides were then washed three times in distilled water, and incubated in 2% copper sulphate solution for 5 min. The slides were rinsed in distilled water, counterstained with haematoxylin, dehydrated, and mounted under a coverslip in permount resin.

3. Detection of Luciferase Reporter Gene Expression

Cell monolayers infected with Ad vectors expressing the gene for firefly luciferase were assayed for luciferase activity as follows. At various times post-infection, the cells were washed three times with PBS and the cells were overlayed with 0.5 ml per dish of 0.1 M potassium phosphate pH 7.8 and 1 mM dithiothreitol (DTT). The cells were scraped into the solution, the mixture was transferred to a microfuge tube and the cells pelleted by centrifugation at 16,000 xg for 2 minutes at 4°C. The supernatant was discarded, and the
pellet was resuspended in 100 μl of 0.1 M potassium phosphate pH 7.8 with 1 mM DTT, briefly vortexed, and the samples were freeze-thawed three times to disrupt the cells. Cellular debris was pelleted by centrifugation at 16,000 xg for 5 min at 4°C, and the supernatant was reserved for subsequent assay.

Luciferase was also detected *in vivo* in tumor bearing mice that were injected intratumorally with 1x10⁸ pfu of Ad vectors expressing luciferase. Forty-eight hr post-injection, the tumors were removed, frozen in liquid nitrogen and stored at -70°C. Approximately 0.5-1 ml of 0.1 M potassium phosphate pH 7.8 supplemented with 35 μg/ml PMSF and 9.5 μg/ml aprotinin was added to each tumor, and the tissue was homogenized on ice with a Tekmarr tissuemizer. Samples were then sonicated for 3x 5 sec and the lysates were centrifuged for 15 min at 2500 xg to pellet cellular debris. The supernatants were removed and luciferase assays were performed on the appropriate dilutions.

Luciferase assays were performed by combining 0.2 ml of assay solution [25 mM glycyl glycine pH 7.8, 15 mM MgSO₄, 5 mM adenosine triphosphate (ATP)] with 20 μl of the appropriate dilution of the sample in a luminometer tube (Starstedt). Within the luminometer, 100 μl of luciferin solution (50 μg/ml luciferin in 3 mM potassium phosphate, 0.03 mM DTT, 23 mM glycyl glycine, pH 7.8) is automatically added to each sample, and the light emission resulting from the luciferase enzyme reaction is detected by the luminometer. The quantity of luciferase protein was determined by interpolation of a standard curve derived from known amounts of purified luciferase protein (Boehringer Mannheim).
4. Western Blot Analysis

Western blot analysis was used to detect specific proteins from infected cells. Cell monolayers in 60 mm dishes were infected at an moi of 10 pfu/cell, and at various times post-infection, the monolayers were placed on ice, washed with PBS, and cells lysed by addition of either 500 μl of RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 35 μg/ml PMSF, and 9.5 μg/ml aprotinin) or 500 μl of TNE lysis buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 2.5 mM EDTA containing 35 μg/ml PMSF and 9.5 μg/ml aprotinin). The cell lysates and debris were scraped into clean microfuge tubes, incubated on ice for 30 min, and centrifuged at 16,000 xg at 4°C for 30 min to pellet cellular DNA and debris. Supernatants, containing cellular proteins, were removed and stored at -70°C.

Aliquots of the crude cell extracts (5-30 μl) were added to 1 volume of 2x polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.2% bromophenol blue), and the samples were boiled in a water bath for 5 min. The samples were separated on a 10-15% PAGE gel overnight at 5-10 mAmps. Following electrophoresis, the gel was soaked in transfer buffer (20 mM Tris-HCl and 150 mM glycine pH 8, with 1/5 volume methanol) for 15 min at RT. The transfer sandwich was then assembled as follows: Scotch brite pad, 2 pieces of 3M Whatmann, PAGE gel, Immobilon-P membrane (Millipore), 2 pieces of 3M Whatmann paper, and a Scotch brite pad. The sandwich was placed in a Biorad transfer apparatus filled with transfer buffer, and the proteins were transferred to the nylon membrane at 4°C overnight at
30 volts. Next day, the nylon membrane was removed and incubated in blocking solution [3-5% powdered milk in PBS or PBS plus 0.5% Tween-20 (PBST)] for 2 hr at RT. The membrane was then incubated in 20-40 ml of the primary antibody solution (antibody against protein of interest in blocking solution) in a heat-sealed bag for 2 hr at RT. The membrane was washed 3 times for 10 min, in 200-500 ml of PBST to remove unbound primary antibody. The membrane was then incubated with the secondary antibody solution (a species-specific antibody conjugated to HRP that recognizes the primary antibody, in blocking solution) in a heat-sealed bag at RT for 1 hr. The membrane was washed 3 times for 10 min with 200-500 ml of PBST and the membrane was then subjected to chemiluminescent detection [using the ECL detection system (Amersham)] according to the manufacturer's protocol. Following exposure to the ECL solution, the membrane was placed between acetate sheets and exposed to Kodak XAR-5 film for varying lengths of time (between 30 seconds to 1 hour) until significant signal was detected. For western blot analysis of interleukin-2 (IL-2), the mouse anti-human-IL-2 monoclonal antibody 881B1 (Chiron Corporation) was used as a primary antibody at a 1:2000 dilution, and a sheep anti-mouse HRP conjugated antibody (Boehringer Mannheim) was used as a secondary antibody at a 1:5000 dilution. For detection of polyoma middle T antigen, tissue culture supernatant from the rat anti-middle T hybridoma cell line C1 [gift of Beverly Griffin] was used as a primary antibody at a 1:100 dilution, and a rabbit anti-rat HRP conjugated monoclonal antibody (DAKO Inc.) was used as a secondary antibody at a 1:2000 dilution.
5. ELISA Assays for Detection of Cytokines

To detect human IL-2, ELISA kits for human IL-2 from R&D Systems or the hIL-2 Biotrak kit from Amersham were used according to the manufacturer's protocol. The quantity of IL-2 present was determined by interpolation of a standard curve produced by known amounts of human IL-2 using the Deltasoft II computer program (MacIntosh). Murine IL-4 was detected by ELISA using the mIL-4 Biotrak ELISA kit from Amersham or the mIL-4 ELISA kit from Cedarlane laboratories according to the manufacturer's protocol. The quantity of murine IL-4 present in each sample was determined by interpolation of a standard curve generated from known amounts of murine IL-4 using the Deltasoft program.

Interferon-\(\gamma\) (IFN-\(\gamma\)) was detected by ELISA using a polyclonal antiserum against murine IFN-\(\gamma\) (gift of Steve Kunkel) as both the capture and detection antibody. 96-well flat bottom maxisorb microtiter plates (Nunc) were coated with the antibody by addition of 50 \(\mu\)l per well of coating buffer (120 mM NaCl, 50 mM H\(_2\)BO\(_3\), 0.016 N NaOH) containing 10 \(\mu\)g/ml anti-IFN-\(\gamma\) polyclonal antiserum. The plates were incubated overnight at 4\(^\circ\)C, and the next day, were washed 3 times with 200 \(\mu\)l per well of wash buffer (PBS, 0.05% Tween-20), and dried by blotting on paper towels. The wells were blocked by the addition of 200 \(\mu\)l of blocking solution [PBS with 2% bovine serum albumin (BSA)], for 2 hr at 37\(^\circ\)C. The plates were washed 3 times with wash buffer, and 50 \(\mu\)l of sample (diluted in 2% FBS in wash buffer) was added to each well. After a 1 hr incubation at 37\(^\circ\)C, the plates were washed four times, 50 \(\mu\)l of a 4 \(\mu\)g/ml biotinylated IFN-\(\gamma\) polyclonal antibody solution was added to each well, and plates were incubated at 37\(^\circ\)C for 45 min. The plates were washed four times, and
100 μl of a 1:3000 dilution of HRP conjugated to avidin (BioRad) in 2% FBS in wash buffer was added to each well. The plates were then incubated at 37°C for 30 min, and washed three times. Finally, 100 μl of the peroxidase substrate solution [four orthophenylenediamine dichloride (OPD) tablets in 12 ml citrate/phosphate buffer (24 mM citric acid, 0.05 M Na₂HPO₄), 5 μl H₂O₂] was added to each well, and the plates were incubated in the dark for 5 min at RT. The reaction was terminated by the addition of 50 μl of 3 M sulphuric acid to each well, and the absorbance at 490 nm was measured. The concentration of IFN-γ present in each sample was determined by comparison to a standard curve generated by known amounts of IFN-γ and interpolation using the Deltasoft program.

F. Polyoma Middle T Antigen Model of Mammary Adenocarcinoma

1. Preparation of Polyoma Middle T Antigen-Derived Tumor Cells from Transgenic Animals

Transgenic animals (FVB/N background) that express the polyoma middle T antigen (PyMid T) under control of the murine mammary tumor virus (MMTV) long terminal repeat (LTR) were generated in Dr. William J. Muller’s laboratory (strain #634), and have been previously described (Guy et al., 1992b). These animals develop adenocarcinoma of all mammary epithelium by 8–10 weeks of age. For the preparation of tumor cells in tissue culture, tumor-bearing mice were sacrificed, the mammary tumors were removed, minced, and placed in collagenase/displace (CD) solution (0.25 mg/ml collagenase A, 0.25 mg/ml dispase in PBS). The minced tissue was transferred to a sterile bottle containing approximately 50 ml of CD, and the mixture was stirred at 37°C for 1 hr. Following
incubation, the undigested tissue was allowed to settle, the supernatant was transferred to a 50 ml polypropylene tube and the cells were pelleted at 5°C at 300 xg for 10 min. The cell pellet was resuspended in 20 ml of MEM supplemented with 10% FBS, and recentrifuged. Finally, the pellet was resuspended in complete MEM, and approximately 1x10^7 cells were seeded into 150 mm dishes and incubated overnight at 37°C in 5% CO₂. Once the cells had attached to the dishes, the monolayers were washed twice with 5 ml of PBS and fresh complete MEM medium was replaced.

2. *In Vitro* Infection of Explanted Tumor Cells

Dishes of explanted tumor cells were infected with Ad vectors expressing cytokines or control Ad vectors at an moi of 100 pfu/cell, or were mock infected by addition of PBS, as described previously. If two viruses were used to coinfect tumor cells, an moi of 50 pfu/cell was used for each virus giving a total of 100 pfu/cell. Following infection, medium was replaced, and the infected tumor cells were incubated at 37°C for a minimum of 18 hr. The cells were harvested by trypsinization, 2% FBS was added, and the cells were counted in a haemocytometer. Cells were then pelleted at 300 xg at 5°C for 5 min, and resuspended in PBS to a final concentration of 5x10^6 cells/ml. Six to eight week old syngeneic FVB/N mice were anesthetized with isoflurane and injected subcutaneously (sc) with 200 µl of mock or infected cell suspension. Animals were closely monitored for the onset of tumor development.
3. Intra-tumoral Injection of Established Tumors with Ad Vectors

Tumor cells which had been explanted from transgenic animals were harvested as described above, and 200 µl of the cell suspension (1x10⁶ cells total) was injected sc in the hind flank of anaesthetized six to eight week old syngeneic FVB/N female mice. Fifteen to twenty days later, when tumors were palpable, the tumors were injected intra-tumorally with 5x10⁸ pfu of cesium chloride gradient banded Ad vector expressing cytokines or control Ad vectors diluted to a final volume of 50-100 µl in PBS, or were injected with 50-100 µl of PBS alone. Immediately prior to injection, the tumors were measured in three dimensions (length, width, and depth) with calipers, and the tumors were measured every 4-7 days post-viral injection to monitor tumor size. Tumor volumes were calculated assuming a prolate spheroid using the following equation: \( \pi/24 \times [\text{length} \times (\text{width}+\text{depth})^3] \). All animals which had undergone tumor regression, following intra-tumoral injection of Ad vectors, were challenged by injection of freshly isolated tumor cells on the opposite flank to the primary tumor.

To examine the effect of intra-tumoral injection of Ad vectors on regression of an un.injected tumor on the opposite flank (contralateral experiments), animals were injected in each hind flank with 200 µl of tumor cell suspension as described above. Approximately twenty days later, these animals were injected with Ad vectors in the right hind flank tumor only, and the size of both tumors was monitored.
G. Cytotoxic T Lymphocyte Assays

1. Generation of Polyoma Middle T Antigen Expressing Cell Lines

To generate a cell line which expressed polyoma middle T that could be used as a target line in cytotoxic T lymphocyte (CTL) assays, 18 kidneys from 5-8 day old FVB/N mice were removed and placed in a tissue culture dish containing PBS supplemented with 200 U/ml penicillin and 200 μg/ml streptomycin (PBS-PS). The sheath surrounding each kidney was removed, and the kidneys were washed twice with fresh PBS-PS, and transferred to a sterile 50 ml polypropylene tube. The kidneys were minced with scissors until the kidney pieces were less than 1 mm in size, and the material was transferred into a sterile 100 ml bottle containing 20 ml of a 2x trypsin-EDTA solution. The 50 ml tube was washed with an additional 20 ml of 2x trypsin-EDTA to ensure all the kidney material was removed, and this wash was added to the 100 ml bottle. The kidney tissue was digested in the 2x trypsin-EDTA solution at RT for 20 min with stirring, to dissociate the kidney cells. The supernatant containing the kidney cells was transferred to a bottle containing 20 ml of cold FBS to inactivate the trypsin, and this mixture was kept on ice during subsequent incubations. The remaining tissue was digested twice with an additional 30 ml of 2x trypsin-EDTA, as described above. The supernatant was removed as described previously and the process was repeated. Following trypsinization, the cells were transferred to 50 ml polypropylene tubes and centrifuged at 600 xg for 10 min. The cell pellets were resuspended in 100 ml warm α-MEM supplemented with 10% FBS, and incubated at 37°C for 20 min. The cells were then
poured through sterile cheesecloth into a 500 ml sterile beaker, and the cheesecloth was rinsed with medium, yielding a final volume of approximately 300 ml. A 5 ml aliquot of this cell suspension was plated into each 60 mm tissue culture dish and incubated at 37°C for 2-3 days, or until the kidney cells had grown to 70-80% confluency. The kidney cells were then transfected using calcium phosphate transfection procedure described below.

2. The Bubbling HeBS Method of Calcium Phosphate Transfection for Mammalian Cells

Calcium phosphate precipitation of plasmid DNA for transfection of mammalian cells was performed essentially as previously described with some modifications (Graham & Van der Eb, 1973; Wigler et al., 1979). Briefly, 0.25 ml of 2x HeBS (42 mM HEPES, 274 mM NaCl, 10 mM KCl, 1 mM Na₂HPO₄2H₂O, 11.2 mM glucose, adjusted to pH 7.1) was aliquoted to a 15 ml polystyrene tube, and air was bubbled into the solution using a sterile Pasteur pipette. Plasmid DNA (1-5μg/dish) in 0.25 ml of the CaCl₂ solution (0.125 mM CaCl₂, 10 mM Tris-HCl, 1 mM EDTA, 20 μg/ml salmon sperm DNA) was added dropwise to the bubbling 2x HeBS forming a precipitate. The solutions were incubated at RT for 30 min, and 0.5 ml was added to each 60 mm dish of cells to be transfected. The cells were incubated with the precipitate overnight, and the medium was replaced the next morning. Transfected cells were incubated for 3 days, and then changed to medium containing 400 μg/ml G418 for selection. G418 resistant clones were expanded and tested for the expression of polyoma middle T Ag by western blot analysis.

3. Coculturing of Isolated Splenocytes

To detect the presence of CTL cells which specifically recognized the PyMid T
protein, spleens from animals which had undergone tumor regression were used in CTL assays. Spleens were mashed with a 3 ml syringe barrel through a sterile wire mesh in PBS supplemented with 2% FBS, the cells were collected in a 15 ml polypropylene tube, and pelleted at 300 xg for 5 min. The pellets were resuspended in 5 ml of cold 0.83% NH₄Cl, (to lyse red blood cells), incubated for 5 min at RT, and repelleted. Pellets were then resuspended in 10 ml of PBS supplemented with 2% FBS, and live cells were counted in a haemocytometer using trypan blue exclusion. Cells were repelleted and resuspended in RPMI medium supplemented with 10% FBS, 20 mM HEPES, and 100 μM 2-mercaptoethanol.

Polyoma Middle T antigen-recognizing splenocytes were stimulated by coculture with 516MT3 cells [from an FVB/N background which express polyoma middle T antigen under control of the MMLV LTR (see Appendix IV)]. The 516MT3 cells were harvested by trypsinization, counted in a haemocytometer, and centrifuged at 300 xg for 5 min. The pellets were resuspended in RPMI medium to a final cell concentration of 10⁶ cells/ml. These cells were then irradiated with 5000 rad of γ-irradiation. For coculturing, 120 μl of the 516MT3 stimulator cell suspension was aliquoted into each well of a 12-well dish (1.2x10⁵ stimulator cells per well), and 1.2x10⁷ splenocytes were added to each well, creating an effector to stimulator ratio of 100:1. Supplemented RPMI medium was added to achieve a final volume of 4 ml per well. Cocultures were incubated for 5 days at 37°C in 5% CO₂, and 2 ml of fresh supplemented RPMI medium was used to replace some of the spent medium 2-3 days later.
4. **CTL assays**

Dishes of 516MT3 cells, used as targets in the CTL assay, were harvested by trypsinization, FBS was added to a final concentration of 2%, and the cells were pelleted at 300 xg for 5 min. The pellets were resuspended in fresh RPMI medium and the number of cells were counted. The appropriate number of target cells (5x10^5 cells are needed for each 96-well microtiter plate) were placed into a 15 ml polypropylene tube and centrifuged at 300 xg for 5 min. The pellet was resuspended directly in 50-100 μCi of ^{51}Cr for each 5x10^5 cells used (depending on the age of the ^{51}Cr), and incubated at 37°C for 1.5 hr. After incubation, the labelled cells were washed in 10 ml of PBS and pelleted at 300 xg for 5 min. The supernatant was removed, the pellet was resuspended in 10 ml of PBS, and the cells were again centrifuged at 300 xg for 5 min. This washing step was repeated for a total of 4 times. The cells were then resuspended in 10 ml of RPMI, and incubated at RT for 15 min. This incubation allows for spontaneous ^{51}Cr release, and reduces background levels in the CTL assay. The cells were pelleted, and resuspended in RPMI medium to a final concentration of 1x10^5 cells/ml.

The cocultured lymphocytes were harvested by vigourous pipetting to resuspend the cells, and pelleted by centrifugation at 300 xg for 5 min. Pellets were resuspended in 1/3 of the initial volume in RPMI and the number of viable cells was determined by trypan blue exclusion. The lymphocytes and the ^{51}Cr-labelled target cells (5000 cells per well) were combined in a V-bottomed 96-well microtiter plate at an effector to target ratio of 90:1, 30:1, 10:1 and 3.3:1. Minimum and maximum lysis were determined by the addition of 5000
labelled target cells to supplemented RPMI medium alone, or to 1 N HCl respectively. The 96-well microtiter plates were incubated at 37°C in 5% CO₂ for 6 hours. Following incubation, 80 μl of supernatant was removed from each well, and the ⁵¹Cr released from the lysed target cells was detected in a γ-irradiation counter. All samples were assayed in duplicate. The resulting radioactive counts were normalized to percent lysis compared to the minimum and maximum lysis readings.

5. Cytokine Profiles of Activated Splenocytes

Spleens were removed from animals which had undergone tumor regression as described above, and the splenocytes were cocultured with 516MT3 stimulator cells as described previously. Following the 5 day coculture, the splenocytes were incubated at an effector to stimulator ratio of 30:1 in 96-well V-bottomed microtiter plates at 37°C for a further 24 hours. Supernatants were removed and frozen at -20°C until the time of assay. Supernatants were assayed for the presence of murine IL-4 or for the presence of interferon-γ (IFN-γ) using ELISA, as previously described.
CHAPTER 3: EVALUATION OF PROMOTERS AND OTHER FACTORS AFFECTING TRANSGENE EXPRESSION IN ADENOVIRAL VECTORS

A. Introduction

Ad has shown great promise as a vector for delivery of DNA in gene therapy. First generation Ad vectors have been shown to direct the expression of transgenes in a variety of both replicating and non-replicating cell types (recently reviewed by Hitt et al., 1997). As Ad vectors become an increasingly popular vector of choice in gene therapy protocols, it is important to determine what might influence transgene expression. This chapter presents results from experiments that were designed to examine the effect of various factors on transgene expression by adenoviral vectors, including: choice of promoter to drive transgene expression, orientation of the expression cassettes within the vector backbone, and the effect of the presence of replication competent adenoviruses (RCA) on expression from non-replicating vectors. Some of these data have been previously published (Addison et al., 1997, "Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression in adenoviral vectors", J. Gen. Virol. 78: 1653-1661).

The development of vectors that function efficiently both in animal models and in humans is critically important for their use in gene therapy. For transduction of mammalian cells, the majority of gene transfer experiments to date have used viral vectors encoding
transgenes under the control of promoter elements derived from viruses. One of the most frequently used promoters in these expression cassettes is the human cytomegalovirus (HCMV) immediate early (IE) gene. The HCMV enhancer/promoter directs high levels of transgene expression in a wide variety of cell types (Boshart et al., 1985; Schmidt et al., 1990). The activity of this promoter depends on a series of 17, 18, 19 and 21 base pair (bp) imperfect repeats (Boshart et al., 1985; Ghazal et al., 1988; Fickenscher et al., 1989; Hunninghake et al., 1989), some of which bind transcription factors of the NF-kappa B, cAMP responsive binding protein (CREB), and the nuclear factor-1 families (Henninghausen & Fleckenstein, 1986; Fickenscher et al., 1989; Sambucetti et al., 1989; Stamminger et al., 1990; Niller & Henninghausen, 1991). These repeats occur within the enhancer region which has been localized to -524 to -118 relative to the transcriptional start site of the immediate early gene (Boshart et al., 1985). Approximately 300 bp of this enhancer sequence (-458 to -118) were sufficient for the direction of high level expression in an enhancer trap assay (Boshart et al., 1985). This fragment of the HCMV IE enhancer retained the ability to induce expression in a broad host range of cell types including human, mouse and frog cells, although the absolute levels of expression varied considerably between species. We and others have used a fragment of the HCMV IE promoter for transgene expression in Ad vectors, and have shown that this element provides high levels of expression both in vitro and in vivo (Wilkinson & Akrigg, 1992; Addison et al., 1995a,b; Hitt et al., 1995; Bramson et al., 1996a,b). To further examine the ability of the HCMV IE promoter to drive expression of foreign genes in Ad vectors, viral vectors with lacZ or luciferase reporter genes under the
control of the HCMV IE promoter element, from -299 to +72 relative to the transcriptional start site, were constructed. These Ad vectors were used to determine whether orientation of the expression cassette within the viral vector had an effect on gene expression, and to examine variations in expression associated with different cell types in vitro. During the course of these studies, it was observed that both human and murine cells infected with Ad vectors encoding either the interleukin-2 (IL-2) or murine interleukin-4 (IL-4) cDNA under control of the HCMV IE promoter could produce biologically active protein, but the levels produced in murine cells were 10-50 fold lower than those produced in human cells (Chapters 4 & 5). To determine whether the higher level of expression in human cells was due to a species preference of the promoter, or due to a greater efficiency of infection of human cells by the human Ad 5-based vectors, the murine CMV (MCMV) IE promoter (Dorsch-Hasler et al., 1985) was cloned into these vectors and the expression levels were compared to those achieved by the HCMV IE promoter. The MCMV IE promoter contains imperfect repeat elements similar to the HCMV promoter, and is believed to bind some of the same transcription factors (Dorsch-Hasler et al., 1985).

First generation Ad vectors are rendered replication defective by deletion of the E1 sequences from the vector backbone. Since E1 genes are absolutely required for viral replication, E1- defective vectors must be grown in 293 cells (Graham et al., 1977), which provide the E1a and E1b genes in trans and thus allow propagation of the E1- deleted vectors. In addition to the E1 region, the 293 cell line also contains E1-flanking sequences that are homologous to sequences retained in the Ad vector backbone. Therefore,
homologous recombination between the vector and the adenoviral sequences in 293 cells can occur, resulting in the reacquisition of the E1 sequences by the vector and the generation of replication competent adenoviruses (RCA). RCA possess all viral proteins necessary for autonomous replication and could, in theory, provide helper functions in trans that might result in the concomitant replication of E1-deleted vectors. Amplification of an Ad vector in this manner would result in an increase in the number of copies of the transgene present within a cell, and could result in increased expression levels. Experiments were thus designed to examine the effect of contaminating RCA on transgene expression from first generation Ad vectors by "spiking" Ad vector-infected cell cultures with wild-type replicating Ad 5 virus. This chapter discusses results of experiments that determined the effects of promoter choice and orientation of expression cassettes on transgene expression by Ad vectors along with the effect of RCA contamination on transgene expression.

B. Results

1. Effect of Orientation on Gene Expression.

The lacZ gene was cloned into a series of shuttle plasmids containing either the HCMV IE promoter (-299 to +72) or one of two forms of the MCMV IE promoter (-1336 to +36 or -491 to +36) (Figure 3-1A) and the resulting plasmids were used to generate a series of recombinant Ad vectors (Figure 3-1B). Initially, we compared expression of lacZ under the control of the HCMV IE promoter inserted in the right to left (i.e. anti-parallel to the direction of E1 transcription, AdCA12lacZ), versus left to right orientation (i.e. parallel
Figure 3-1: (A) Shuttle Plasmids for Transgene Insertion.

The coding sequences for β-galactosidase were cloned into the shuttle plasmids pCA4, pCA14, pMH4 or pMH5, and the resulting plasmids pCA12, pCA17, pCA36, and pCA35 respectively, were used to generate the viruses used in this study. All four plasmids contain Ad sequences from m.u. 0 to 1 (nucleotides 22 to 341) and from 9.8 to 16.1 (nucleotides 3525 to 5790), with a polycloning site and the SV40 polyadenylation signal inserted between the two segments of the Ad left end genomic sequences. In addition, pCA4 and pCA14 carry the HCMV IE promoter from -299 to +72 relative to the transcriptional start. pMH4 and pMH5 are derivatives of pCA14 in which two different fragments of the MCMV IE promoter from -491 to +36 (pMH4) or from -1336 to +36 (pMH5) replace the HCMV IE promoter. Expression of lacZ is directed rightwards (parallel to E1) in pCA17, pCA36 and pCA35, while it is directed leftwards (anti-parallel to E1) in pCA12. Similar constructs containing the luciferase cDNA in place of the lacZ sequences were also constructed, generating pCA18 and pDK1 (rightwards directed) or pCA15 (leftwards directed).
(B) Recombinant Ad Vectors.

Each of the plasmids described in (A) was cotransfected with either pJM17 (McGrory et al., 1988) or pBHGI0 (Bett et al., 1994) to generate replication deficient adenoviral vectors. The viruses used in this study all possess the E. coli lacZ gene under control of the HCMV IE (AdCA12lacZ, or AdCA17lacZ), a 1.4 kb MCMV IE (AdCA35lacZ) or a 0.5 kb MCMV IE (AdCA36lacZ) promoter. The vectors AdCA15luc, AdCA18luc, and AdDK1 resemble AdCA12lacZ, AdCA17lacZ, and AdCA36lacZ respectively, except the cDNA for firefly luciferase replaces the lacZ gene within the transgene expression cassettes. Solid arrows represent the inverted terminal repeats of the adenoviral genome, the shaded arrows represent the HCMV IE or the MCMV IE promoters, the solid bar represents the SV40 polyadenylation signal, and the shaded bar represents the lacZ sequence or the firefly luciferase cDNA.
B

\[ \Delta E1 \]

- AdCA12lacZ (HCMV IE)
- AdCA17lacZ (HCMV IE)
- AdCA36lacZ (0.5kb MCMV IE)
- AdCA35lacZ (1.5kb MCMV IE)

- AdCA15luc (HCMV IE)
- AdCA18luc (HCMV IE)
- AdDK1 (0.5kb MCMV IE)
to the direction of E1 transcription, AdCA17lacZ) in a recombinant Ad vector. Human MRC5 fibroblasts were infected at an moi of 10, harvested at 24 hour intervals, and assayed for β-gal activity. AdCA17lacZ, produced 7-fold higher levels of β-gal than did AdCA12lacZ (Table 3-1). Similar virus constructs encoding either the luciferase reporter gene or the human IL-2 cDNA, also showed increased expression when the cassette was inserted in the rightwards orientation (Table 3-1). This orientation dependent difference was also noted in a variety of other human and murine cell types (data not shown).

2. HCMV IE Driven Expression in Various Cell Types

AdCA17lacZ was chosen for further analysis to determine the level of lacZ expression in different cell types. Several murine and human cell lines were infected at an moi of 10, and the cells were harvested at 24 hour intervals for β-gal assays. Up to 100 μg of β-gal per 10^6 cells was detected following infection of human cells with AdCA12lacZ (Figure 3-2). Murine cell lines produced approximately 10 to 100 fold lower levels of β-gal than human cells and similar differences were observed with other reporter genes (data not shown). Nonetheless, several hundred nanograms of β-gal were produced per 10^6 infected murine cells by day 3 post infection, from the HCMV IE construct.

3. Examination of Promoter Specificity

The consistently low expression observed in murine cells compared to human cells could be due to species preferences associated with the HCMV IE promoter or to inefficient infection of rodent cells by a human adenovirus. To determine if the difference in expression observed in cells of different species was due, at least in part, to reduced promoter activity,
Table 3-1: Effect of Cassette Orientation on Relative Gene Expression in Infected Human MRC5 Fibroblast Cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Direction of*</th>
<th>Reporter Expression†</th>
<th>Fold Increase‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transcription</td>
<td>(µg protein /10⁶ cells)</td>
<td></td>
</tr>
<tr>
<td>AdCA17lacZ</td>
<td>R</td>
<td>21.4</td>
<td>6.5</td>
</tr>
<tr>
<td>AdCA12lacZ</td>
<td>L</td>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>AdCA18luc</td>
<td>R</td>
<td>0.13</td>
<td>2.4</td>
</tr>
<tr>
<td>AdCA15luc</td>
<td>L</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>AdCAIL-2P</td>
<td>R</td>
<td>0.58</td>
<td>7.4</td>
</tr>
<tr>
<td>AdCAIL-2AP</td>
<td>L</td>
<td>0.08</td>
<td></td>
</tr>
</tbody>
</table>

* R=rightwards and L=leftwards with respect to the Ad genome
† Cells were infected, harvested and assayed for β-gal, luciferase or IL-2 activity as described in materials and methods. Expression levels shown are those measured at 48 hours post-infection.
‡ The fold increase is the increase in the quantity of protein produced by cells infected with viruses driving expression rightwards relative to leftwards.
Figure 3-2: Expression of the lacZ Reporter Gene Under Control of the HCMV IE Promoter in Murine and Human Cells.

Cells were infected at an moi of 10 pfu/cell and pellets were harvested daily and assayed for β-gal activity as described in materials and methods. The murine cell lines used were P815 mastocytoma, and B16BL6 melanoma, and the human cells used were 983M melanoma and the MRC5 fibroblast strain.
two viruses were constructed which expressed $lacZ$ under the control of the MCMV IE promoter in a rightwards orientation. One vector contained a larger MCMV IE enhancer/promoter region (from -1336 to +36, AdCA35lacZ) and the other contained a smaller fragment (from -491 to +36, AdCA36lacZ). The promoter activity of these two recombinant viruses was compared to that of AdCA17lacZ. A variety of cell types from species which might be used as models for gene therapy studies, including human, murine, rat, canine and monkey cells, were infected with the above viruses, and the level of $lacZ$ expression was determined. In human MRC5 cells, both MCMV IE promoters produced levels of $\beta$-gal protein about 2-fold greater than observed for the HCMV IE promoter over a period of 4 days (Figure 3-3A). In contrast, in human 983M melanoma cells, the MCMV IE promoters were significantly more effective than HCMV IE in driving expression of $\beta$-gal, resulting in 15-30 fold higher levels (Figure 3-3B). In two murine cell lines, MT1A2 and SV329, both MCMV IE promoters induced $\beta$-gal expression at least 6-fold and 22-fold more efficiently than the HCMV IE promoter (Figure 3-3C and 3-3D). The 1.4 kb MCMV IE promoter expressed 2-4 fold greater amounts of $\beta$-gal than the HCMV IE promoter in canine MDCK cells (Figure 3-3E) and primary canine osteosarcoma cells (data not shown), and 4-5 fold better in RF rat lung fibroblasts (Figure 3-3F). In addition, the 1.4 kb MCMV IE promoter expressed 4-5 fold higher than the HCMV IE promoter in two monkey cell lines, LLC-MK$_2$ (ATCC CCL 7) and BS-C-1 (ATCC CCL 26) (Bett, Addison and Graham, unpublished data). The increase in expression driven by MCMV IE compared to HCMV IE was also noted for the Ad vectors containing the luciferase reporter gene (data not shown).
Figure 3-3: β-galactosidase Expression in Various Cell Lines Infected with Recombinant Ads Containing HCMV IE or MCMV IE Promoter-driven Expression Cassettes.

All cells were infected at an moi of 10 pfu/cell, harvested daily and assayed for β-gal expression as described in Materials and Methods. The cell lines used were MRC5 human fibroblasts (panel A), human 983M melanoma cells (panel B), MT1A2 murine mammary adenocarcinoma cells (panel C), SV329 SV40 transformed murine epithelial cells (panel D), MDCK canine epithelial cells (panel E), and rat lung fibroblasts (panel F). The viruses are represented as follows: AdCA17lacZ (HCMV IE) - open squares, AdCA35lacZ (1.4 kb MCMV IE) - closed squares, AdCA36lacZ (0.5 kb MCMV IE) - closed circles. The expression cassettes in all three viruses are in the rightwards orientation. It should be noted that the graphs in panels D, E and F are on different scales than those in panels A, B, and C.
No consistent differences were observed between the expression of lacZ driven by the 1.4 kb and that driven by the 0.5 kb MCMV IE promoter in any of the cell lines tested, indicating that the smaller fragment was sufficient to obtain high levels of expression. In summary, we found that in all cell types examined, transgene expression driven by either the small or large MCMV IE promoter was as good as, and frequently several fold better than, that of the HCMV IE promoter in vitro.

4. Analysis of HCMV IE and MCMV IE Derived Transcripts

Higher β-gal levels in cells infected with MCMV IE promoted compared to HCMV IE promoted vectors could be caused by increased levels of transcription or increased efficiency of translation. To distinguish between these two possibilities, northern analysis was performed to determine the quantity of lacZ transcript present. Human MRC5 or murine SV329 cells were infected with either the HCMV IE (AdCA17lacZ) or the MCMV IE (AdCA35lacZ and AdCA36lacZ) promoter constructs. Two bands at approximately 3.3 kb and 3.8 kb were visible upon hybridization with a lacZ probe (Figure 3-4). The 3.3 kb transcript was the predominant transcript, and is likely the result of transcriptional termination at the SV40 An site in the expression cassette. Because the vector backbone retains the E1b polyadenylation signals downstream of the expression cassette, termination at this second An site would give rise to a fragment of 3.8 kb; however, this appears to occur at a reduced frequency compared to termination at the SV40 polyadenylation site as indicated by the reduced intensity of the 3.8 kb band (Figure 3-4). In both human and mouse cells, the MCMV IE promoter (either large or small fragment) directed a higher level of transcription.
Figure 3-4: Northern Blot Analysis of \( \text{lacZ} \) Transcripts Produced by Infected Human or Murine Cells.

MRC5 human fibroblasts or SV329 murine epithelial cells were either mock infected or infected at an moi of 10 pfu/cell with either AdCA17lacZ (HCMV IE), AdCA35lacZ (1.4 kb MCMV IE), or AdCA36lacZ (0.5 kb MCMV IE). Cells were harvested 48 hours post-infection and RNA isolated as described in materials and methods. Equal amounts of total RNA were subjected to formaldehyde agarose gel electrophoresis, and were transferred to positively charged nylon membranes by capillary transfer. Specific \( \text{lacZ} \) and \( \beta \)-actin transcripts were visualized using the chemiluminescent DIG detection system (Boehringer Mannheim Inc.) as described in the materials and methods, and subsequent exposure to Kodak XAR-5 film.
than the HCMV IE promoter, in agreement with the differences observed in protein levels. Because the genes were all cloned in a similar manner [i.e. identical Kozak sequences (Kozak, 1991) were present at the translational start site, and identical polyadenylation signals 3' of the coding sequences], differences in protein levels are most likely due to differences in the rate of transcription or stability of the transcripts rather than differences in the efficiency of translation.

5. *In vivo* Expression of Luciferase and β-gal

For comparison of *in vivo* expression levels from the HCMV IE and MCMV IE promoters, we injected 1x10^8 pfu of AdCA18lac (HCMV IE) or AdDK1 (0.5 kb MCMV IE) intra-tumorally in animals bearing subcutaneous (sc) PyMidT induced tumors. At 48 hours post-injection, tumors were removed, snap frozen in liquid nitrogen, and lysates were prepared. Approximately 7-fold more luciferase was detected in animals injected with AdDK1 compared to AdCA18lac (9.3 ± 3.8 x10^6 RLU/tumor compared to 1.4 ± 0.7 x10^6 RLU/tumor, respectively). *Intra-tumoral* injection of vectors expressing β-gal under control of the HCMV IE promoter (AdCA17lacZ) or the 1.4 kb MCMV IE promoter (AdCA35lacZ) was also performed, and β-gal was detected by immunostaining of paraffin embedded tissue sections. The number of positively stained cells, and the intensity of staining, was significantly greater in the MCMV IE vector compared to the HCMV IE vector injected tumor (data not shown). These data are in agreement with previous results obtained *in vitro* (see MT1A2 cell line in Figure 3-3D), and suggest that the MCMV IE promoter may direct increased levels of expression *in vivo* as well as *in vitro*. 
6. **Comparison of a Larger Fragment of the HCMV IE Promoter to the MCMV IE Promoters**

The fragment of the HCMV IE promoter from -299 to +72 was selected for these studies since it had been shown to induce high levels of transgene expression in human cells, and its reduced size allowed for the insertion of larger genes into the Ad vector backbones. Compared to the full length HCMV IE enhancer, however, this fragment has fewer repeats of the DNA sequence motifs required for transcription factor binding. Therefore, we examined the ability of a 0.8 kb HCMV IE promoter fragment (-760 to +54) in an Ad vector to drive transgene expression in murine cells. Following infection of SV329 murine cells with an Ad vector expressing *lacZ* under control of this larger HCMV IE promoter (AdCA38lacZ), we detected a 3-fold increase in the amount of β-gal protein when compared to the small HCMV IE promoted vector (AdCA17lacZ). This level of expression, however, was still 4 fold-lower than that obtained following infection of cells with the Ad vector expressing *lacZ* under control of the 0.5 kb MCMV IE promoter (AdCA36lacZ). Similar trends were observed in other cell types including the canine MDCK cells, murine MT1A2 cells, and 983M human melanoma cells. We found that the only cell type in which the large HCMV IE promoted construct expressed higher than the small MCMV IE promoted construct was in the human MRC5 fibroblast cell strain (2-fold higher). These data indicate that although the use of a 0.8 kb fragment of the HCMV IE promoter can result in higher levels of expression compared to the 0.4 kb HCMV IE promoter fragment, the MCMV IE promoter still outperformed the HCMV IE promoter in cells of murine origin.
7. Effect of RCA on Transgene Expression from First Generation Ad Vectors

To determine if the presence of contaminating RCA would affect transgene expression from an E1-deleted, first generation Ad vector, an experiment was performed whereby cells were infected with a constant amount of vector and an increasing quantity of wild-type adenovirus, and the level of transgene expression was monitored. A549 cells, which are non-permissive for replication of E1-deleted vectors but permissive for wild-type virus, were infected at an moi of 10 with AdCAIL-2 (approximately $10^7$ pfu per 60 mm dish), and coinfectected with either 0, 10, 100, 1000, or 10000 pfu of wild-type Ad 5. Aliquots of the supernatant were removed at 3 and 5 days post-infection, and assayed for IL-2 expression by ELISA. The addition of small amounts (10 to 1000 pfu) of wild-type replicating virus did not appear to have a significant effect on vector-derived IL-2 expression (Figure 3-5). The presence of RCA appeared to have an effect on transgene expression when the ratio of RCA to vector was high (1:1000). At 5 days post-infection, cells infected with the highest amount of wild-type virus showed a 2-fold increase in IL-2 expression compared to cells infected with AdCAIL-2 vector alone. Although a 2-fold increase may not seem significant, it does suggest that, at high RCA to vector ratios, the presence of RCA may provide helper functions and allow for the replication of E1-deleted vectors.
Figure 3-5: Effect of RCA on Transgene Expression From First Generation Ad Vectors.

A549 cells were infected with AdCAIL-2 at an moi of 10 and were then coinfected with either 0, 10, 100, 1000, or 10000 pfu of wild-type Ad 5 virus (as an RCA). At various times post-infection, supernatants were removed and assayed by ELISA for the presence of IL-2. All samples were assayed in duplicate and the graph is a representation of the amount of IL-2 detected at each time point for each sample as follows: AdCAIL-2 plus 0 pfu Ad 5 - white box; AdCAIL-2 plus 10 pfu Ad 5 - light grey box; AdCAIL-2 plus 100 pfu Ad 5 - medium grey box; AdCAIL-2 plus 1000 pfu Ad 5 - dark grey box; AdCAIL-2 plus 10000 pfu Ad 5 - black box.
C. Discussion

This chapter examined factors that influence transgene expression from first generation Ad vectors. It was observed that transgene expression was affected by the orientation of the expression cassette relative to sequences in the Ad vector backbone: expression cassettes directed rightward (i.e. parallel to the direction of E1 transcription) expressed higher levels of protein (Table 3-1). Xu et al. (1995) noted the same orientation dependence using E1 replacement Ad vectors expressing rotavirus antigen VP7sc regulated by the 0.4 kb HCMV IE promoter or the Ad 2 major late promoter. This effect may be due, in part, to the presence of the E1a enhancer which remains in the vector backbone. It is conceivable that in the rightwards oriented recombinants, transcription factors binding to the E1a enhancer may act synergistically with those binding the HCMV IE enhancer sequences.

The cell type and species preferences observed may reflect a property of HCMV itself. HCMV has a relatively narrow host range for productive infection and does not replicate efficiently in transformed human cells, being restricted mostly to human diploid fibroblasts (Stamminger et al., 1990; Stinski, 1990). Recent studies on the ability of HCMV and MCMV to infect and replicate in cells of various species have shown that MCMV has a much wider host cell range than HCMV and can replicate in murine, simian and human cells (LaFemina et al., 1988). The block in HCMV replication in non-human cells correlates with reduced levels of transcription of the major IE gene (Nelson & Groudine, 1986). It is possible that expression driven by the HCMV IE promoter within an Ad vector is subject to the same host restriction found for HCMV replication. Recent studies using the HCMV IE promoter for
transgene expression in transgenic mice, [including one which used an HCMV IE promoter fragment similar to the one used in these studies i.e., -302 to +72 (Kothary et al., 1991)], indicated that the HCMV IE promoter expressed in a tissue and cell specific pattern (Kothary et al., 1991; Baskar et al., 1996a,b), and the majority of expression occurred in tissues that are naturally infected by HCMV (Baskar et al., 1996b). This finding suggests a role for tissue-specific factors that bind the viral promoter elements and contribute to the variation in the levels of transgene expression.

To examine the possibility of species preferences of the CMV IE promoters in Ad vectors, Ad vectors containing lacZ or luciferase under the control of either the MCMV or HCMV IE promoter were constructed. The activity of the MCMV IE promoter was consistently higher than the HCMV IE promoter in murine cells and equal to or higher than the HCMV IE promoter in all other cell types examined (Figure 3-3), and this increase was also observed at the transcriptional level (Figure 3-4). It was also observed that in vivo the MCMV IE promoter directs higher levels of transgene expression than the HCMV IE promoter. This observation has also been made by others using the AdCA17lacZ and AdCA35lacZ vectors, where expression from the MCMV IE promoter following intraocular vector injection was higher and persisted longer in rat intraocular tissue (K. Csaky, personal communication).

These results indicate that Ad vectors containing the MCMV IE promoter express much more efficiently in rodent cells than do vectors containing the HCMV IE promoter, although the absolute levels of transgene product detected in murine cells were still slightly
lower than those produced in human cells. It is possible that Ad vectors do not infect murine cells as efficiently as human cells due to the lack of Ad 5-specific cell receptors; however, within each cell line, the different Ad vectors would infect with equal efficiency (since they all contain identical structural proteins), and thus differences in promoter activity seen in infected cells are not due to differences in vector infection.

To construct vectors which will be efficacious as vehicles for gene delivery, it is important to choose a system which results in a high degree of cell transduction and appropriate levels of foreign gene expression. Ad vectors can infect replicating and non-replicating cells yielding a very high degree of cell transduction. One of the drawbacks of Ad vectors for gene therapy is the anti-viral immune response associated with their administration in vivo (Yang et al., 1994). This anti-viral immunity limits the duration of expression and effectiveness of the transgene following a second administration of the vector. If the level of transgene expression is low, it may require the administration of greater amounts of vector in order to attain therapeutic levels of transgene product, and may result in stronger anti-viral immune responses, further limiting transgene expression. By maximizing expression levels, e.g. through the use of strong promoters, one can minimize the amount of vector required to achieve high levels of the transgene, possibly prolonging transgene expression and allowing vector readministration. We and others have found that the HCMV IE promoter can direct expression of very high levels of transgene, but as reported here, these levels are reduced in rodent compared to human cells. The HCMV IE promoter also appears to be more subject to species and cell type specificity than the MCMV IE promoter. These data suggest that the
MCMV IE promoter results in expression levels which rival that of the HCMV IE promoter in human cells, and outperforms it in cells of murine and rat origin.

Finally, it was determined that coinfection of cells with an E1-deleted, first generation Ad vector and wild-type Ad 5 virus (i.e. RCA), did not significantly affect expression of the transgene encoded by the E1-deleted vector. This result indicates that small quantities of contaminating RCA in viral preparations should not affect experimental results obtained using first generation Ad vectors; however, attempts should be made to prevent RCA contamination, or maintain it at a minimal level since high levels of RCA contamination could have potential effects on transgene expression and might alter the effective administered "dose".
CHAPTER 4. INTRA-TUMORAL INJECTION OF AN ADENOVIRUS

EXpressing interleukin-2 induces regression and immunity in a murine breast cancer model

A. Introduction

The use of Ad vectors in gene therapy is well documented, and Ad vectors have been shown to be efficient at delivering foreign genes to a wide variety of cell types (recently reviewed by Hitt et al., 1997). This high transduction efficiency makes Ad vectors very attractive for the delivery of therapeutic genes for the treatment of diseases such as cancer. The cytokine interleukin-2 (IL-2), has been previously shown to induce anti-tumor effects by its ability to stimulate the activity of host cytotoxic T lymphocytes (CTL), lymphokine activated killer (LAK) cells, and tumor infiltrating lymphocytes (TIL) (Lotze et al., 1981; Grimm et al., 1982; Fearon et al., 1990; Cameron et al., 1990; Lindgren et al., 1993). This chapter presents results using a first generation Ad vector that expresses the cDNA for human IL-2 under the control of the HCMV IE promoter and SV40 An signals for the immunotherapy of cancer. Included in this chapter, are data that have been previously published (Addison et al., 1995, "Intratumoral injection of an adenovirus expressing interleukin-2 induces regression and immunity in a murine breast cancer model", Proc. Natl. Acad. Sci. USA, 92: 8522-8526), which discusses the level of IL-2 expression following
infection of various tumor and normal cell types \textit{in vitro}, and the ability of an Ad vector expressing IL-2 to induce regression of established tumors \textit{in vivo}. This chapter also includes unpublished results demonstrating the kinetics of IL-2 expression following infection of cells \textit{in vitro}, immunostaining of tumor sections to determine the pattern of vector transduction \textit{in vivo}, the duration of IL-2 expression \textit{in vivo} following intra-tumoral injection, and data demonstrating a correlation between the presence of tumor antigen-specific CTL cells and tumor regression.

B. Results


This paper demonstrates that a first generation Ad vector which expresses the cDNA for IL-2 under the HCMV IE viral promoter (AdCAIL-2, Figure 4-1) could be used to efficiently infect a variety of cell types and induce the expression of microgram quantities of biologically active IL-2 protein (Figure 4-2). AdCAIL-2 was shown to modulate tumor growth \textit{in vivo} following \textit{in vitro} transduction of mouse mammary tumor cells with the AdCAIL-2 vector, and subsequent introduction of the infected cells into recipient mice. There was a delay of at least 3-5 weeks in the onset of tumor formation in animals that had received AdCAIL-2 transduced cells compared to those which had received control- or mock-infected tumor cells (Figure 4-3). More importantly, when tumor-bearing animals were injected intra-tumorally with \(5 \times 10^8\) pfu of the AdCAIL-2 vector, 54\% of treated animals underwent complete regression of the tumor, and these animals remained tumor free for
greater than 18 months (Table 4-1). Injection of a control E1 deleted Ad vector, Addl70-3 (constructed by Andrew Bett), had a slight effect on tumor growth compared to animals injected with PBS; however, no tumor regression was observed in control animals, as was noted following injection of AdCAIL-2 (Figure 4-4). Animals that had been "cured" of tumors were protected from a subsequent challenge of freshly isolated tumor cells on the opposite flank, indicating that a protective form of anti-tumor immunity had been induced. These results demonstrate that intra-tumoral injection of an Ad vector expressing IL-2 is effective at inducing protective anti-tumor immune responses.
Intratumoral injection of an adeno-virus expressing interleukin 2 induces regression and immunity in a murine breast cancer model

(cancer therapy/expression vectors)

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ABSTRACT Rodent tumor cells engineered to secrete cytokines such as interleukin 2 (IL-2) or IL-4 are rejected by syngeneic recipients due to an enhanced antitumor host immune response. An adeno-virus vector (AdCAIL-2) containing the human IL-2 gene has been constructed and shown to direct secretion of high levels of human IL-2 in infected tumor cells. AdCAIL-2 induces regression of tumors in a transgenic mouse model of mammary adenocarcinoma following intratumoral injection. Elimination of existing tumors in this way results in immunity against a second challenge with tumor cells. These findings suggest that adeno-virus vectors expressing cytokines may form the basis for highly effective immunotherapies of human cancers.

Many tumor cells express various peptide antigens on their cell surface in association with major histocompatibility class I molecules (MHC I), and these antigens may allow immune effector cells to distinguish tumor from normal tissue (reviewed in ref. 1). Many of these tumor antigens have been isolated and shown to be recognized by human cytotoxic T lymphocyte (CTL) cell lines (2, 3) or tumor-infiltrating lymphocytes (TILs) (4, 5). In addition, synthetic peptide epitopes displayed on the surface of tumor cells in association with MHC I molecules result in specific antitumor CTL activity (6). Nevertheless, tumor cells known to express potentially antigenic peptides manage to evade host immunosurveillance and proliferate in vivo (2, 3, 7). TILs are usually present in tumor biopsies but are unresponsive to the tumor antigens and fail to mediate cytotoxic activity (8). It has been suggested that in some instances tumor cells may lack secondary costimulatory signals required to induce expression by immune effector cells of cytokines necessary for activation and proliferation of CTLs (9, 10). This can result in anergy and thus failure of the T cells to respond to the antigen in a subsequent exposure. Indeed, it has been shown that CTL-mediated rejection of the tumor could be induced by transfection of melanoma tumor cells with DNA encoding the B7 costimulatory molecule (11). In the absence of costimulatory signals, it may be possible to bypass this requirement by providing exogenous cytokines, the downstream effect of costimulation, and overcome or prevent anergy of the immune effector cells.

Many cytokines can mediate antitumor activity in vitro and in vivo (summarized in ref. 12). Interleukin 2 (IL-2) has been demonstrated to possess antitumor activity through its ability to stimulate the cell-mediated killing activity of CTLs (13), to induce lymphokine-activated killer cells (14, 15), and to activate TILs (16, 17). Systemic delivery of recombinant IL-2 has been used in animal models and in the clinic and has met with some success (reviewed in ref. 18). However, the short half-life of IL-2 in serum requires repeated high doses, resulting in severe side effects including vascular leak syndrome, edema, anemia, fever and chills, nausea, and hypotension (reviewed in ref. 19). To avoid these problems, local delivery of low doses of IL-2 has been investigated in animal models and was found to mediate antitumor activity, resulting in increased survival and reduced tumor growth without the side effects associated with high dosing regimens (20, 21). As a more effective method of achieving high intratumoral concentrations of IL-2, attention has turned to the use of gene delivery systems to express IL-2 continuously within or around the tumor. Transduction of tumor cells with retrovirus vectors expressing the IL-2 gene has been shown to reduce the tumorigenicity and metastatic potential of B16 melanomas (22), CMS-3 fibrosarcoma (22, 24), and the MBT-2 bladder carcinoma (25). However, certain problems associated with the use of retroviral vectors (e.g., low titers, stability) have led us to examine adeno-virus (Ad) vectors for immunotherapy of cancer.

The activity of adeno-viruses (Ads) as gene therapy vectors is currently being intensively investigated (26-30). The ability to produce large quantities of purified virus with relative ease makes this system very attractive for clinical use. Moreover, for immunotherapy of cancer, Ad vectors have many advantages over other commonly used systems. Deletion of the early region 1 (E1) genes renders the virus replication deficient and combining E1 deletions with deletion of the nonessential E3 region results in Ad vectors with a capacity of up to 8 kb of foreign DNA (31). The vectors can infect a wide variety of replicating and nonreplicating cell types and efficiently express genes linked to appropriate promoters. More important, Ad DNA does not normally integrate into the infected cell genome, and thus expression of the foreign gene product is typically transient. When used to deliver cytokines in immunotherapy, an Ad-based vector would therefore not result in chronic stimulation of the immune system as might occur following administration of vectors capable of integration. We have constructed an E1/E3-deleted recombinant Ad vector that expresses human IL-2 (hIL-2) under control of the human cytomegalovirus immediate early promoter (HCMV IE) and the simian virus 40 poly(A) signals (SV40 poly(A) An). This vector was found to express high levels of hIL-2, modulate tumorigenicity, and induce regression of existing tumors leading to protective immunity in a transgenic mouse model of mammary adenocarcinoma.

MATERIALS AND METHODS

Construction of Recombinant Plasmids and Viruses. Plasmids were constructed according to standard protocols (32). The DNA for hIL-2 was inserted between Xho I and EcoRI

Abbreviations: IL, interleukin; Ad, adeno-virus; h, human; CMV, cytomegalovirus; SV40, simian virus 40; pfu, plaque-forming units; PyMu, polyoma middle T antigen; CTL, cytotoxic T lymphocyte; TIL, tumor-infiltrating lymphocyte.
sites in the polylinker region of the shuttle plasmid pCA14 (33) to create pCAIL-2P. After purification by alkaline lysis (34) and cesium chloride density gradient banding, pCAIL-2P was cotransfected into 293 cells (35) with pBHG10 DNA (31) to generate AdCAIL-2 (see Fig. 1).

Cells and Viruses. Cell lines used include the following: MRC5, human fibroblast strain (ATCC CCL 171); 793, vertical phase human melanoma (36); MeWo, metastatic human melanoma (36); Wn35, radial phase human melanoma (37); MCF7, human breast carcinoma (ATCC HTB 22); FVBMT, primary murine polyoma middle T antigen (Py MrD1)-induced mammary adenocarcinoma explanted from transgenic mice (this paper); B16H1L, murine melanoma (38); 293, adenoviral E1 transformed human embryonic kidney cells (35), and 293N3S, 293-derived spinner cells (39). All cell culture media and supplements was obtained from Gibco. All viruses were grown and titered in 293 cells except for cesum chlorided banded stocks of virus, which were grown in 293N3S spinner cultures.

Detection of IL-2 Expression. Cells were infected at a multiplicity of infection of 10 plaque-forming units (pfu) per cell and at various times postinfection aliquots of infected cell supernatants were removed, quick frozen in a dry-ice/ethanol bath, and stored at −70°C for detection of IL-2. Levels of secreted hIL-2 were quantitated using the Quantikine ELISA kit (R & D Systems).

Preparation of Tumor Cells from Transgenic Animals. The transgenic mice used as a model for mammary adenocarcinoma in this study [strain M1P (40)] possess the PyMrD1 expressed under the control of the mouse mammary tumor virus long terminal repeat. These mice develop spontaneous adenocarcinomas of all mammary epithelium by 8–10 weeks of age.

Tumor-bearing transgenic mice were sacrificed, minced, and incubated at 37°C with gentle stirring in collagenase/dispase solution (25 mg of collagenase and 250 mg of dispase (both from Boehringer Mannheim) in 100 ml of phosphate-buffered saline (PBS)). Large clumps of cells were allowed to settle and the supernatant was centrifuged at 1500 rpm for 2 min at 4°C in a Beckman GPR centrifuge to pellet the cells. The pellets were resuspended in minimal essential medium (supplemented with penicillin/streptomycin and l-glutamine) and 10% fetal bovine serum, and cells were plated at 10^3 per 150-mm tissue culture dish.

After an overnight incubation to allow the cells to adhere, the tumor cell cultures were rinsed twice with PBS, fresh medium was added, and the cells were incubated for a further 24 hr.

In Vitro Infection of Tumor Cells. Cells were rinsed twice with PBS and incubated with cesum chloride banded viral stocks (41) of either AdCA1L-2 or Add70P3 [an E1-deleted control virus (31)] at 100 pfu per cell or were mock infected. Following viral adsorption at 37°C for 30 min, the growth medium was replaced and the cells were incubated 20–24 hr and then harvested by trypsinization, centrifuged, and resuspended in PBS at 10^6 cells per 200 µl. Syngeneic PVB/N mice (Tacoma Farms) were anesthetized with sodium pentobarbital (Abbott) and injected s.c. in the right hind flank with 200 µl of tumor cell suspension. Mice were then monitored visually and by palpation for time of tumor onset.

Intratumoral Injections of Ad Virus. Tumor cell cultures were prepared as described above but were not infected with virus. After incubation for 48 hr, the cells were harvested and 10^6 cells were injected s.c. into syngeneic mice. After 2 days, when palpable tumors had developed in all animals, the mice were injected intratumorally with 5 × 10^6 pfu of AdCA1L-2 or Add70P3 in 100 µl of PBS or with PBS alone. Tumors were measured using callipers prior to injection of virus and at weekly intervals thereafter. Tumor size was estimated by determining the longest diameter and average width and calculating the volume assuming a prolate spheroid.

RESULTS AND DISCUSSION

Kinetis of IL-2 Expression in Cells Infected with AdCAIL-2. The structure of AdCAIL-2 is illustrated in Fig. 1. The vector has deletions of E1 and E3 sequences and a substitution of E1 by an expression cassette containing the HCMV IE promoter, the hIL-2 cDNA, and the SV40 An signals. Cells infected with AdCAIL-2 produced two polypeptides of 15 kDa and 17 kDa, recognized by hIL-2 specific monoclonal antibodies. Western blots of infected cell extracts and culture supernatants. IL-2 produced by infected cells was found to be biologically active as demonstrated by proliferation of the IL-2-dependent cell line CTLL-2 in bioassays (data not shown). IL-2 production following AdCAIL-2 infection of a variety of different cells was quantified by ELISA (Fig. 2). Most human cell lines were found to produce and secrete 1–2 ng of IL-2 per 10^5 infected cells over a period of 3–4 days, whereas expression by murine cells was 50–100-fold lower. The differences in expression between murine and human cell lines are likely due at least in part to the relatively lower activity of the HCMV IE promoter in murine cells compared to human cells (C.L.A. and F.L.G., unpublished). The levels of IL-2 produced, in the ng range, should nonetheless be sufficient for biological activity in treated animals.

In Vivo Modulation of Tumorigenicity. To determine whether expression of IL-2 by cells transduced with AdCAIL-2 could promote antitumor activity in vivo we chose to study a transgenic mouse model of mammary adenocarcinoma. Breast cancer affects one in nine women in North America and is the leading cause of death in nonsmoking women (42). Although primary tumors can usually be surgically removed, the a-
come of current therapies is often unsuccessful in preventing metastases (43) and 50% of women developing primary malignancy will eventually die from metastatic disease (44). A majority of breast cancers have been shown to overexpress various oncogenes, such as c-neu (erbB-2) (reviewed in ref. 45), and these molecules may be potential tumor antigens by which immune effector cells can distinguish tumor tissue from normal tissue. Breast cancer is therefore a prime target for treatment by immunotherapy whereby immune responses triggered against the tumor may result in the establishment of memory immune effector cells that recognize and destroy tumor cells and may prevent metastatic disease.

The transgenic mice possessing the PyMuMT under the control of the mouse mammary tumor virus long terminal repeat develop adenocarcinoma of all mammary epithelium by 8–10 weeks of age. These tumors resemble the scirrhous carcinomas found in human breast cancers (45), are heterogeneous, and will metastasize to the lungs. Cells from primary tumors can be explanted from the transgenic mice and treated with a collagenase/dispase solution to generate single cell suspensions that can be maintained in culture. Because the tumor cells express PyMuMT they should be relatively immunogenic and, consistent with this, delivery of two consecutive doses of irradiated tumor cells i.p. could induce protection against lung metastases when live tumor cells were subsequently delivered by tail vein injection (T.B. and T.G., unpublished). Although PyMuMT should be a potential target for recognition by specific CTLs the cells are not rejected following injection of 10^5 tumor cells s.c. into immunosuppressed syngeneic animals, and palpable solid tumors develop in 100% of injected animals by 15–20 days postinjection. The tumors continue to grow and have never been observed to regress spontaneously. This system therefore provides a good model of mammary carcinogenesis in humans and an excellent model in which to test the effect of cytokine expression on the immune response to immunogenic tumors that evade antitumor responses.

In initial studies on the effect of AdCAIL-2 on tumorigenicity of PyMuMT tumor cells, the cells were explanted and infected in vitro with either AdCAIL-2 or Add70-3 [an E1-deleted control virus containing no insert (31)] at 100 pfu per cell or were mock infected. After an additional 24 hr, by which time AdCAIL-2-infected cells had begun to express IL-2 (Fig. 2), syngeneic animals were injected s.c. with 10^6 tumor cells and monitored for tumor development (Fig. 3). There was no significant difference in tumor onset in animals injected with mock-infected versus Add70-3-infected cells, but a delay of 3–5 weeks was seen for animals receiving AdCAIL-2-infected cells. However, 100% of animals in all groups eventually developed tumor(s). Viral infection of tumor cells (with either Add70-3 or AdCAIL-2) did not inhibit cell growth or proliferation in vitro, as determined by [H]thymidine incorporation assays (data not shown), suggesting that the delay in tumor onset was not a result of cell killing due to viral infection or IL-2 production but was most likely due to IL-2-induced immune responses that were able to delay, but not prevent, tumor growth.

![Figure 3](image-url)  
**Fig. 3.** Transduction of PyMuMT tumor cells with AdCAIL-2 results in a delay in tumor onset. Tumor-bearing transgenic mice were sacrificed and the tumors were explanted and grown in tissue culture dishes overnight. Cells were then infected with either AdCAIL-2 or Add70-3 at 100 pfu per cell or were mock infected. Syngeneic FVB/N mice were injected s.c. on the right hind flank with 10^6 tumor cells. Mice were monitored for the onset of tumor formation and the percentage of mice remaining tumor free is shown as a function of time post injection. The graph represents the pooled data from two independent experiments. •, Uninfected, n = 16; ◦, Add70-3 infected, n = 9; ○, AdCAIL-2 infected, n = 19.

**Fig. 4.** Tumor regression following intratumoral injection of AdCAIL-2. Tumor cells from transgenic mice were explanted and cultured for 24 hr, at which time 10^6 tumor cells were injected s.c. into syngeneic mice. Twenty-one days postinfection of tumor cells, when palpable tumors had developed in all animals, the mice were injected intratumorally with 5 x 10^6 pfu of AdCAIL-2 or Add70-3 or with PBS alone. (A) Tumors were measured prior to injection of the virus and then weekly (see text). •, PBS, n = 4; ◦, Add70-3, n = 8; ○, AdCAIL-2, n = 8. Tumor volume for the single AdCAIL-2–infected animal that failed to respond is indicated by the dashed line. (B) Fraction of mice surviving following treatment with PBS or virus. Symbols are as in A. One animal infected intratumorally with AdCAIL-2 died of unknown causes 6 days postinfection and is not included in the data set.
PBS controls, possibly a result of anti-adenoviral immune responses generated against virus-infected cells (46). However, all Ad70B-3 injected tumors continued to enlarge until the mice became moribund (Fig. 4B). In contrast, eight of nine tumors injected with AdCAIL-2 underwent complete regression, and by 3–4 weeks postinjection neither visible nor palpable tumors could be detected. Mice in which complete tumor regression occurred remained tumor free for 12 weeks postvirus injection, at which time they were used in a challenge experiment (see below). In the one mouse in which tumor regression failed to occur (dashed line in Fig. 4A), the kinetics of tumor growth was not significantly different from that of the PBS control. This could reflect a failure to deliver virus efficiently into the tumor of this mouse or a subsequent leak of virus out of the site of injection.

Tumor-bearing animals that were injected with the same dose of virus s.c. in the flank opposite to that of the tumor failed to show any tumor regression or growth delay (data not shown), suggesting that IL-2 must be delivered intratumorally to mediate the effective immune responses. These results support the hypothesis that TILs within the tumor mass become activated and subsequently kill the tumor cells mediating regression. Histological sections of tumors 7 days postinjection with AdCAIL-2 showed a marked increase in lymphocytes present within the tumor compared to sections from control tumors, suggesting that IL-2 induces proliferation and accumulation of lymphocytes in the tumor (data not shown).

Results from the experiment of Fig. 4 and two additional experiments are presented in Table 1. Although complete tumor regression was observed only in a minority of the animals treated in experiments 2 and 3, a pronounced delay in tumor growth was observed in most animals (87% for pooled data from all three experiments), and 54% of AdCAIL-2-treated tumors completely regressed. In animals showing a partial response, tumor development was significantly delayed and survival was increased for up to 6 weeks longer than for untreated animals or control virus-injected animals. Factors that might contribute to the variability in the response to AdCAIL-2 injection are the efficiency of delivery of the transducing virus to the tumor and its retention and dissemination within the tumor mass. In addition, the tumor cells are derived from different individual transgenic mice for each experiment and the variation noted between experiments may be due to differences in immune susceptibility of the tumor cells obtained from different animals.

Table 1. Tumor response following intratumoral injection of AdCAIL-2

<table>
<thead>
<tr>
<th>Response</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Total*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None†</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>3/24 (13)</td>
</tr>
<tr>
<td>Partial‡</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>8/24 (33)</td>
</tr>
<tr>
<td>Complete§</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>13/24 (54)</td>
</tr>
</tbody>
</table>

*Percentage in parentheses.
†Tumor growth not significantly different from that of animals treated with control Ad70B-3.
‡Tumor growth significantly delayed and animals survived 3–4 wk longer than controls.
§Complete regression of tumors and no recurrence.

(five in each experiment) developed tumors between days 15 and 21, whereas the experimental mice showed no sign of tumor development and are currently tumor free at 32 (eight mice) and 42 weeks (three mice) postchallenge in two independent experiments. Thus, mice in which tumors had regressed following AdCAIL-2 injection developed long-term immunity against a subsequent tumor challenge. This immunity is likely to be tumor specific, as it has been previously shown that animals that have been injected with IL-2-transduced tumor cells are protected from challenge with parental tumor cells but not from unrelated tumors (13, 23, 25).

In view of the fact that AdCAIL-2 transduction of tumor cells prior to injection resulted only in a delay in tumor development, the potency of AdCAIL-2 when directly injected into existing tumors is surprising. It would appear that the time and mode of delivery of AdCAIL-2 may be critically important in mediating tumor regression. This may reflect a requirement for immune effector cells to be recruited to the tumor site, primed, and activated, by which time the immune response may be insufficient or too late to completely abolish tumor growth. In contrast, when animals are tumor bearing at the time the virus has passed for recruitment and priming of immune effector cells within the tumor prior to cytokine treatment, and thus intratumoral expression of IL-2 at this point may be much more effective, resulting in activation of effector cells and potent antitumor activity. Our results suggest that in the intratumoral injection experiments, a resident effector cell population is present but does not respond to the tumor load and mediate regression. The delivery of IL-2 into the tumor results in the activation of this cell population and induces the potent antitumor responses seen. That not all tumors regressed when treated in this way may be a reflection of the use of primary tumor cells, which, like tumors in a clinical setting, may vary in their susceptibility to therapy. It is important to note that mice injected s.c. with PyMID T tumor cells always develop tumors at the site of injection and that the tumors have the potential to metastasize and never regress spontaneously. Thus ability to obtain regression of a majority of tumors by AdCAIL-2 administration is highly significant.

Pearson et al. (13) demonstrated that tumor cells secreting IL-2 could induce an antitumor activity that was dependent on CD8+ T cells and that this treatment could induce protective immunity. However, they found that this protection was short lived and that 50% of animals challenged 4 weeks after immunization developed tumors. In contrast, our findings that "cured" mice were protected against a challenge 3 months after primary tumor regression strongly suggest that these mice had developed a long-lasting immunity to the PyMID T tumor cells. Unlike cancer therapies that use cytokine genes or drugs to mediate tumor cell killing (47–50), this form of immunotherapy may generate immune responses that would lead to protection from distal secondary tumor development or metastases. Development of this form of immunity is extremely important for the treatment of cancers such as breast cancer and melanoma, where the patient usually succumbs to the metastatic spread of the disease. Recently, Hudda et al. (20) and Cordier et al. (50) have also reported a therapeutic effect of an Ad vector carrying the murine IL-2 gene in the P815 mastocytoma model. Our studies provide further evidence that the delivery of Ad vectors carrying cytokine genes can induce highly effective antitumor responses and long-lasting immunity in a primary tumor model and may therefore be useful in establishing protective immunity in patients, thus preventing the metastatic spread of the cancer.

We thank Laura Levy for technical assistance and Silva Bacchetti for critical comments on the manuscript. C.L.A. and T.B. are, respectively, recipients of a Steve Fonyo Studentship from the National Cancer Institute of Canada (NCIC) and a studentship from the
2. Kinetics of IL-2 Expression Following Transduction with AdCAIL-2

To examine the kinetics of IL-2 expression from AdCAIL-2, monolayers of 293 cells in 60 mm dishes were infected with AdCAIL-2 at an moi of 10, and at various times post-infection the culture medium was removed, and the cells were lysed in RIPA buffer. Twenty-five μl of cell lysate from each time point was applied to a SDS-polyacrylamide gel, separated by electrophoresis, and IL-2 protein detected by western blot. IL-2 protein was first observed in infected cell lysates at 8 hr post-infection; however, significant quantities of the protein did not accumulate until 20-24 hr post-infection (Figure 4-5). Thus, the kinetics of transgene expression resembled that of late viral proteins during Ad replication. Based on this result, tumor cells were incubated for a minimum of 16 hr following transduction to ensure IL-2 was expressed at high levels before cells were implanted into recipient animals.

3. Pattern of Transduction Following Intra-tumoral Injection of Ad Vectors

The distribution of the vector following intra-tumoral injection of established tumors was examined using immunohistochemical detection of the vector-encoded protein. Tumors were injected with 5x10⁸ pfu of AdCA35lacZ, which encodes the lacZ gene under the control of the long MCMV IE promoter and SV40 An sequences (see Chapter 3 and Appendix II). At various times post-injection, tumors were removed, fixed in neutral buffered formalin, and immunostained for β-gal. The β-gal specific antibody used in this procedure was detected by a secondary antibody conjugated to horse-radish peroxidase which, in the presence of the DAB substrate, converts the chromogenic molecule, and thus a brown colour is produced in
Figure 4-5: Kinetics of IL-2 Expression Following Infection of 293 Cells with AdCAII-2.

293 cells were infected at an moi of 10 with the vector AdCAII-2, and at various times post-infection, cell monolayers were overlayed with 0.5 ml of RIPA buffer and crude cell lysates were separated by SDS-PAGE. Proteins were transferred to a nylon membrane and human IL-2 protein was detected by western blot analysis. The IL-2 primary antibody detected two bands that migrated at molecular weights of approximately 15 kDa and 17 kDa, a pattern which has been previously reported for IL-2 (Abbas et al., 1991).
cells that contain β-gal (Figures 4-6 and 4-7). Intra-tumoral injection of the vector resulted in transduction of cells throughout the tumor mass, and was not restricted to cells along the needle track nor the outer layer of the tumor (Figure 4-6); however, infection was not evenly distributed within the tumor. Typically, infected cells were found in groups of 3-10 cells surrounded by non-infected cells (Figure 4-6, Figure 4-7A). β-gal expression appeared maximal at 3 days post-injection (the first time point examined), and rapidly decreased over time. By day 11, only a few positive cells were detected, and by day 16 virtually all cells in the tumor were negative for β-gal (Figure 4-7, panels A-D). As β-gal protein is fairly stable (the half-life of β-gal in vitro is 96 hr, Andrew Bett, unpublished data), it is unclear whether the protein detected at day 11 is newly synthesized, or represents protein synthesized at an earlier time.

4. Duration and Level of IL-2 Expression from AdCAIL-2 in Tumors

The expression level of IL-2 within tumors following intra-tumoral injection of AdCAIL-2 was next determined. Tumors were injected with 5x10⁸ pfu of AdCAIL-2 or Adl70-3, and at various times post-injection, the tumors were removed, frozen in liquid N₂ and stored at -70°C until the time of assay. Crude tumor protein lysates were prepared, and the amount of IL-2 was quantitated by ELISA. Since the vector encodes the cDNA for human IL-2, an ELISA which specifically detects the human IL-2 protein was used; therefore, the protein detected by ELISA is strictly vector-derived and is not a result of cross-reaction with the endogenously produced murine IL-2. The expression of IL-2 within the tumor paralleled that previously noted for β-gal (Figure 4-7). IL-2 expression was maximal at 1 day
Figure 4-6: Pattern of Vector Transduction Following Intra-tumoral Injection.

Syngeneic FVB/N mice were injected sc with $10^6$ PyMidT tumor cells, and 21 days later when palpable tumors had developed, mice were injected intra-tumorally with $5 \times 10^8$ pfu of AdCA35lacZ. Three days post-injection the tumor was removed, fixed in neutral buffered formalin, and paraffin embedded. The embedded tumor was sectioned on a microtome (3 µm thickness) and β-gal protein was detected by immunohistochemistry as described in the materials and methods. β-gal positive cells appear dark brown. The injection track can be seen through the centre of the field of view as an area of stromal and fibrotic tissue. Cells staining positive for β-gal are seen throughout the tumor mass. The magnification is 100x in a light microscope.
Figure 4-7: Kinetics of Expression of β-gal From an Ad Vector *in Vivo*.

Established PyMidT tumors in syngeneic FVB/N mice were injected intra-tumorally with $5 \times 10^8$ pfu of AdCA35lacZ. At various times post-injection, tumors were removed, fixed in neutral buffered formalin, and paraffin embedded. Tumor tissue was sectioned (3 μm thickness) using a microtome, and β-gal positive cells detected by immunohistochemistry. Positive cells appear dark brown. The panels represent tumors removed at the following time points: (A) 3; (B) 6; (C) 11; and (D) 16 days post-injection, respectively. The magnification is 400x under a light microscope.
post-injection, and steadily decreased over time until it was undetectable at 15 days post-injection (Figure 4-8, closed symbols). As expected, tumors that had been injected with Add170-3 had no detectable levels of human IL-2 at any time examined (Figure 4-8, open symbols). These data indicate that β-gal or IL-2 transgene expression from first generation Ad vectors in immunocompetent animals is transient, lasting only ~7-10 days.

5. Toxicity of AdCAIL-2 In Vivo

In the initial experiments describing the anti-tumoral response following intra-tumoral injection of AdCAIL-2 (presented in Addison et al., 1995, "Intratumoral injection of an adenovirus expressing interleukin-2 induces regression and immunity in a murine breast cancer model", Proc. Natl. Acad. Sci. USA, 92: 8522-8526) there was little toxicity associated with this treatment, although one animal which had died of "unknown causes" (Figure 4-4), likely succumbed to toxic side effects due to AdCAIL-2 administration. When additional experiments were performed it was observed that injection of high-doses of AdCAIL-2 into tumor-bearing mice led to toxicity and morbidity in a significant proportion of the treated animals. When doses of 1x10⁵ pfu or 5x10⁵ pfu were delivered intra-tumorally, 100% of the animals became moribund. Fatal toxicity was induced by AdCAIL-2 at a dose of 5x10⁸ pfu in approximately 38% of the animals (based on eight separate experiments). AdCAIL-2-induced toxicity was reduced by injecting lower doses of vector; however, when 1x10⁶ pfu of AdCAIL-2 was injected intra-tumorally in two separate experiments, only one animal had a significant tumor growth delay, and no animals underwent complete tumor regression. Thus, the effective dose of AdCAIL-2 which resulted in tumor regression (i.e.
Figure 4-8: Duration of Transgene Expression from AdCAIL-2 In Vivo.

PyMidT tumor cells (10⁶) were injected sc into syngeneic FVB/N mice, and 21 days later, the animals were injected intra-tumorally with 5x10⁸ pfu of AdCAIL-2 or Addl70-3. At various times post-injection, tumors were removed, frozen in liquid nitrogen, and stored at -70°C. Tumor protein lysates were prepared and analyzed for the presence of IL-2 by ELISA. Two animals were examined at each time point, for each treatment, and are represented as small symbols while the means are represented by the larger symbols. AdCAIL-2 - closed squares; Addl70-3 - open circles.
5x10^8 pfu), was also associated with a high degree of toxicity.

Toxicity, leading to morbidity, appeared eight to ten days following intra-tumoral injection of AdCAIL-2, when animals became extremely unresponsive with a "ruffled" appearance. Post-mortems were performed on these animals, and tissues from all major organs were fixed in neutral buffered formalin, paraffin embedded, sectioned, stained with haematoxylin and eosin, and analyzed by Dr. Jacek Kwiecien (Veterinary Pathologist, Central Animal Facility, McMaster University). The animals had no apparent lesions in the brain, lung, colon, small intestine, heart or kidney; however, severe lesions were observed in the spleen and liver of euthanized animals. Spleens were atrophied in the perivascular cuffs with a marked necrosis of lymphocytes. In the liver, there was acute severe hepatitis, with many infiltrating lymphocytes and macrophages, as well as a large number of necrotic leucocytes and hepatocytes. This morphology is consistent with hepatocellular necrosis caused by Ad infection of the liver, and is most likely due to "leaking" of the AdCAIL-2 vector from the tumor, the site of injection, into the liver. Following intra-tumoral injection of an Ad vector expressing luciferase, analysis of liver lysates indicated that a significant amount of vector was found in liver tissue (Jonathan Bramson, unpublished data). Thus, toxicity associated with AdCAIL-2 injection is likely a result of the infection of hepatocytes, and subsequent expression of vector-derived IL-2 in the liver cells. Expression of IL-2 in hepatocytes could lead to the recruitment and activation of lymphocytes in the liver, and ultimately lead to the destruction of virally transduced hepatocytes. These data indicate that, although AdCAIL-2 was able to cause the regression of established tumors, the toxicity associated with AdCAIL-2
treatment suggests that other therapeutic approaches need to be examined.

6. Tumor Regression Mediated by AdCAIL-2 is Associated with the Induction of Anti-Tumor CTL Activity

We wished to determine if the mechanism of AdCAIL-2-mediated tumor regression was due to the recruitment and activation of tumor antigen-specific CTL. To examine this hypothesis, we used a $^{51}$Cr release assay based on the recognition of a polyoma middle T Ag expressing target cell line (see Appendix IV) by reactive lymphocytes. Spleens were removed from responding or non-responding animals, and cocultured with irradiated 516MT3 PyMidT-expressing cells for 5 days. Following this incubation, a CTL $^{51}$Cr release assay was performed, and the specific lysis observed on 516MT3 and PT0516 control target cells was compared. Lymphocytes from animals which had undergone tumor regression, had a high level of middle T Ag specific killing (Figure 4-9 panel A), while animals which had not exhibited any tumor regression had weak or no detectable levels of CTL killing (Figure 4-9, panel B). The specific killing on 516MT3 cells, (but not the killing noted on PT0516 cells, which is an indication of non-tumor antigen-specific killing) was inhibited by the addition of antibodies against CD3, suggesting that the killing of the $^{51}$Cr-labelled PyMidT-expressing target cells was mediated by T-lymphocytes (Figure 4-10). There were no middle T specific CTL cells detected in naive or Addl70-3 injected animals (Figure 4-10). Thus, AdCAIL-2-mediated tumor regression is due to the recruitment and activation of tumor antigen-specific CTLs.
Figure 4-9: Detection of CTLs Following AdCAIL-2 Mediated Tumor Regression.

Spleens from animals that had been intra-tumorally injected with $5 \times 10^8$ pfu of AdCAIL-2 were removed and the isolated lymphocytes were restimulated by coculture with irradiated 516MT3 PyMidT-expressing cells for 5 days. The lymphocytes were used in a $^{51}$Cr release CTL assay that used PyMidT-expressing cells (516MT3 - closed squares) or a parental cell line that does not express PyMidT (PT0516 - open circles) as CTL targets. The percent specific lysis was measured in a gamma counter, and plotted against the effector to target ratio for tumor free (panel A) and tumor bearing (panel B) animals.
Figure 4-10: Specific Lysis in CTL Assays is Inhibited by Anti-CD3 Antibodies.

Spleens from naive animals, or animals that had been intra-tumorally injected with 5x10^8 pfu of AdCAIL-2 or Addl70-3, were removed and the isolated lymphocytes were maintained in coculture with irradiated 516MT3 PyMidT-expressing cells for 5 days. The lymphocytes were used in a ^{51}Cr release CTL assay that used PyMidT-expressing cells (516MT3 - closed symbols) or a parental cell line that does not express PyMidT (PT0516 - open symbols) as CTL targets. The graph represents the specific lysis detected from each target cell line in the absence (squares) or the presence (circles) of antibody against the CD3 T-cell receptor molecule. Panel A - tumor-free animal injected with AdCAIL-2; panel B - tumor-bearing animal injected with Addl70-3; panel C - naive non-tumor-bearing animal.
C. Summary

An Ad vector expressing human IL-2 was constructed (Figure 4-1) and resulted in the expression of high levels of biologically active human IL-2 in infected human (1-5 μg/10⁶ cells) and murine cells (10-100 ng/10⁶ cells), [Figure 4-2]. In vitro infection of polyoma middle T antigen-induced mammary tumor cells by AdCAIL-2 resulted in a delay in the onset of tumor formation following sc injection of the transduced cells; however, 100% of animals eventually developed tumors (Figure 4-3). When tumor bearing animals were injected intra-tumorally with an Ad vector expressing lacZ, the pattern of transduction indicated that the virus spread throughout the tumor, suggesting that this was an effective mode of delivery (Figure 4-6). Expression of transgenes from intra-tumorally injected Ad vectors (either AdCAIL-2 or AdCA35lacZ) was transient, with expression limited to approximately 7-10 days post-injection (Figures 4-7 and 4-8). Intra-tumoral injection of AdCAIL-2 resulted in complete regression of established tumors in 54% of animals (Table 4-1), and these animals were protected from a subsequent tumor challenge. Tumor regression was associated with the presence of PyMidT-specific CTL activity (Figure 4-9), which was T cell-mediated, as demonstrated by the ability of the addition of anti-CD3 antibody to inhibit target cell lysis (Figure 4-10). This treatment led to some toxicity that, in the future, could be overcome by the use of lower doses of IL-2 in combination with other therapeutic cytokines. These data demonstrate the efficacy of an Ad vector expressing the therapeutic cytokine IL-2 to modulate tumorigenicity and induce regression of established tumors in vivo.
CHAPTER 5. AN ADENOVIRAL VECTOR EXPRESSING INTERLEUKIN-4
MODULATES TUMORIGENICITY AND INDUCES REGRESSION IN A
MURINE BREAST CANCER MODEL

A. Introduction

Many cytokines have been used for the immunotherapy of cancer, and one of these, interleukin-4 (IL-4), has been previously shown to possess anti-tumor activity through its ability to direct the accumulation and activation of eosinophils (Tepper, 1994). IL-4 is also an autocrine and paracrine activator of CTLs, TILs and macrophages (Kawakami et al., 1988; Mossman et al., 1986). This chapter describes studies involving the construction and characterization of an Ad vector that expresses the cDNA for murine IL-4 under the control of the HCMV IE promoter and SV40 Ag sequences. It includes data that has been previously published (Addison et al., 1995, "An adenoviral vector expressing interleukin-4 modulates tumorigenicity and induces regression in a murine breast cancer model". Int. J. Oncol. 7:1253-1260), which discusses the level of IL-4 expression following infection of various cells in vitro, and the ability of the Ad vector expressing IL-4 (AdCAIL-4) to modulate tumorigenicity in vivo. This chapter also includes additional unpublished data demonstrating the duration of expression and toxicity of IL-4 following in vivo injection of AdCAIL-4.
B. Results


An Ad vector expressing murine IL-4 was constructed (outlined in Figure 5-1), and was shown by ELISA and IL-4-specific bioassay to direct the expression and secretion of microgram quantities of biologically active IL-4 from infected cells (Figure 5-2, panel A and B, respectively). Subcutaneous injection into syngeneic mice of tumor cells that had been transduced *in vitro* with AdCAIL-4 demonstrated the ability of IL-4 to modulate tumorigenicity *in vivo*. A significant delay in the onset of tumor formation was observed (greater than 13 weeks compared to control animals), with approximately 61% of animals remaining tumor free for greater than 17 months (Figure 5-3). Furthermore, intra-tumoral injection of AdCAIL-4 into established tumors resulted in significant delays in tumor growth compared to control animals, and approximately 50% of animals underwent complete tumor regression (Figure 5-5). Animals that had undergone tumor regression were also protected from a subsequent challenge of freshly isolated tumor cells injected on the opposite flank from the initial tumor. *In vivo* injection of AdCAIL-4 into established tumors, or sc injection of tumor cells expressing IL-4, resulted in the infiltration of large numbers of eosinophils compared to Addl70-3 treated tumors (Figure 5-4). Eosinophils have been indicated as the effector cell responsible for tumor regression associated with IL-4 treatment, and therefore our observations are consistent with other published data (Tepper et al., 1989; Tepper et al., 1992). The mechanism of tumor-killing by eosinophils is currently unclear, however, eosinophils are not known to possess immunological "memory". The fact that in our studies,
animals that had undergone tumor regression were also protected from subsequent tumor challenge indicates that immunological memory has been induced, and suggests a role for T-lymphocytes in IL-4-mediated tumor cell killing. These results demonstrate that an Ad vector expressing IL-4 can modulate tumorigenicity and induce regression of established tumors in vivo, and that tumor regression is associated with the induction of protective anti-tumor immunity.
An adenoviral vector expressing Interleukin-4 modulates tumorigenicity and induces regression in a murine breast cancer model

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Contributed by F.L. Graham, September 26, 1995

Abstract. Anti-tumor activity of a recombinant adenovirus expressing murine IL-4 (AdCAIL-4) was investigated in a murine model of mammary adenocarcinoma. Primary tumor cells derived from mammary adenocarcinomas induced in transgenic mice by the middle T antigen gene of polyomavirus were infected with AdCAIL-4 and injected into syngeneic non-tumor bearing recipients. Expression of IL-4 by AdCAIL-4 transduced tumor cells significantly prolonged survival of all animals and prevented tumor development in 61% of recipient mice. When tumor bearing animals were injected intra-tumorally with AdCAIL-4, all animals survived at least 8 to 10 weeks longer than controls, and 50% of treated animals underwent complete tumor regression. Both in vitro and in vivo treatment with AdCAIL-4 resulted in infiltration by eosinophils in and around the tumor site. Animals which had undergone complete tumor regression were protected from a second challenge suggesting that immunotherapy with Ad vectors expressing cytokines may protect from metastatic disease.

Introduction

Breast cancer affects 1 in 9 women in North America (1), and even though primary tumors can be surgically removed with some success, over 50% of affected women will die from metastatic spread of the disease. Breast cancer is therefore a primary candidate for treatment by immunotherapy where the induction of an effective immune response might mediate the regression of the primary tumor, establish protective immunity against tumor cells and block the development of metastatic disease. Most tumors, including those of the breast, possess antigens which are potentially recognizable by the immune system (2) but still manage to evade cytotoxic responses which might otherwise lead to their destruction. It is possible to boost the immune response and obtain anti-tumor activity by delivering exogenous cytokines, many of which have been shown to be capable of stimulating anti-tumor responses in vivo. Interleukin-4 (IL-4) is a pleotropic cytokine, produced mainly by T lymphocytes, which has many effects on various arms of the immune system including the ability to enhance antibody production by B cells (3), and to activate tumor infiltrating lymphocytes (TILs) (4), cytotoxic T lymphocytes (CTLs) and macrophages (5). Its anti-tumor activity however, appears to be mediated by its ability to direct the accumulation and activation of eosinophils (6).

The systemic delivery of IL-4 has been used in clinical trials for the immunotherapy of cancer. However, due to a lack of effectiveness together with toxic side effects, the trials have been discontinued (7,8). Toxicity may in part be due to the requirement for high systemic doses which may be necessary to compensate for the short half life of IL-4 in serum. To develop therapeutic approaches that may circumvent this problem, we are exploring the use of replication defective adenovirus (Ad) vectors for local delivery of IL-4. Ad vectors (9) are well suited for the immunotherapy of cancer since expression of the therapeutic gene from replication defective vectors is transient in nature and will not lead to persistent activation of the immune system. When deletions of the early region 1 (E1) and 3 (E3) genes are combined, Ad vectors can incorporate up to 8 kb of foreign DNA, a capacity that will permit the cloning of cDNAs for any of the known cytokines. Many studies have shown that Ad vectors infect many cells near the injection site (10,11) and therefore local and high level expression of the gene of interest is readily achieved.

Using the murine breast cancer model described herein, we have previously shown that intra-tumoral injection of a recombinant Ad vector expressing interleukin-2 (IL-2) can cause regression of primary tumors and induce protection against secondary tumor challenges (12). We wished to determine whether IL-4 expressed by an Ad vector could similarly induce anti-tumor activity. To this end, we have constructed a recombinant Ad vector that expresses the murine IL-4 (mIL-4) gene under control of the human cytomegalovirus immediate early (HCMV IE) promoter and found it able to direct the production of high levels of biologically active IL-4 by infected cells. In addition, we

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have tested the ability of this IL-4-expressing vector to mediate anti-tumor responses in a murine model of mammary adenocarcinoma (13).

Materials and methods

Construction of recombinant plasmids and viruses. A HindIII/EcoRI fragment containing the cDNA for mIL-4 was removed from the plasmid p309.9 (gift of Dr R. Tepper) (14) and cloned into the polylinker region of pCA13 (15), to generate pCAIL-4P (Fig. 1A). This placed the gene under the control of the HCMV IE promoter (-299 to +72 relative to the transcriptional start site) and simian virus 40 (SV40) polyadenylation signals (SV40 polyA). pCA13 is a shuttle plasmid that contains Ad5 sequences from nuc 0 to 1 and 9.8 to 16.1 surrounding the HCMV IE - SV40 polyA expression cassette. To obtain an Ad vector expressing IL-4, pCAIL-4P DNA was cotransfected with pBH10 (16) into 293 cells (17) according to standard protocols (18). Homologous recombination between these two plasmids yielded a genome which could be packaged into virions. Viral plaques were analyzed for the presence of the mIL-4 gene by restriction enzyme digestion (18), and a recombinant virus having the expected DNA structure was identified, plaque purified, and designated AdCAIL-4 (Fig. 1B).

Cell culture and virus propagation. All cell culture media were obtained from Gibco laboratories (Grand Island, NY). Cell lines used include: MRC5, human fibroblast strain (ATCC CCL 171); 793, vertical phase human melanoma (19); McWo, metastatic human melanoma (19); Win35, radial phase human melanoma (20); MCF7, human breast carcinoma (ATCC HTB 22); 293, adenoviral E1 transformed human embryonic kidney cells (17); 293N3S, 293 derived
cells adapted for growth in suspension (21); B16BL6, murine melanoma (22); LT-1, J558L, plasmacytoma derived cell line which produces IL-4 (gift of Dr R. Tepper) (23); CT-4S, an IL-4 dependent cell line (gift of Dr R. Tepper) (24). FVB/BMT cells were prepared by explanting tumor tissue from tumor bearing PyMID T transgenic mice (strains MT6634) as described previously (12). All virus stocks were grown in 293 cells, except that for large scale virus production 293N3S cells were used. Virus were titrated, grown and purified according to standard protocols (18).

Detection of mIL-4 expression. Cells were infected with AdCAIL-4 at a multiplicity of infection (m.o.i) of 10 plaque forming units (pfu) per cell, incubated in 5 ml of growth medium, and at various times post-infection, 0.5 ml of medium was removed, quickly frozen, and stored at -70°C. Expression levels of mIL-4 were quantified using an mIL-4 ELISA kit (Cedarlane laboratories, Hornby, Ont., Canada) according to the manufacturer's protocol. In addition all samples were tested for biological activity by assaying proliferation of the IL-4 dependent CT-4S cell line (24). The various supernatants from infected cells were added to the growth medium of the CT-4S cells in the absence of exogenously added IL-4. Proliferation of the CT-4S cells in response to IL-4 was measured using the MTT assay described by Mossman (25), and the units of IL-4 present in each supernatant was determined by interpolation of a standard curve derived from the proliferation of CT-4S cells provided with known amounts of IL-4.

In vitro infection of tumor cells. Cultured FVB/BMT cells derived from explanted mammary tumors of polyoma middle T antigen (PyMID T) transgenic mice (12,13) were rinsed twice with PBS and infected with cesium-chloride purified stocks of AdCAIL-4 or AdFl70-3 [an E1 deleted control adenovirus (16)] at an m.o.i of 100 pfu/cell, or were mock infected with PBS. After adsorption at 37°C for 30 minutes, growth medium was replaced, and the cells were incubated for a further 20-24 hours. At this time, cells were harvested by trypsinization, and resuspended at a concentration of 10⁶ cells per 200 μl in PBS. Syngeneic FVB/N mice (Taconic Farms, Germantown, NY) were anaesthetized with isofluorane (Abbott laboratories, Montreal, Que., Canada), injected subcutaneously (s.c.) in the right hind flank with 200 μl of the tumor cell suspension, and monitored for tumor formation.

Intra-tumoral injections of AdCAIL-4. FVB/N mice were injected s.c. with 200 μl of PyMID T tumor cells at a concentration of 10⁶ cells per 200 μl. Upon development of palpable tumors (approximately twenty-one days post tumor cell injection), the mice were injected intra-tumorally (i.t.) with 5x10⁶ pfu of AdCAIL-4 or AdFl70-3 in 100 μl PBS, or with PBS alone. Tumors were measured using calipers prior to i.t. injection of virus, and then at weekly intervals. The longest diameter and average width of tumors were used to calculate tumor volumes assuming a prolate spheroid.

Challenge experiments. Mice which had undergone primary tumor regression were challenged 12 weeks post virus infection. Animals were given 10⁴ tumor cells s.c. on the opposite flank and monitored for the onset of tumor formation. Age-matched controls were similarly injected to ensure the tumor preparation had the potential to form tumors.

Histological examination. All tissues were fixed in 10% neutral formalin for 24 hours at room temperature, then washed and stored in 70% ethanol. Samples were paraffin-embedded, sectioned and stained with haematoxylin and eosin (H&E).

Results

Construction of and characterization of AdCAIL-4. The shuttle plasmid pCAIL-4P was generated by insertion of the cDNA for mIL-4 into the plasmid pCAIL-13 (15) which contains the HCMV IE promoter and the SV40 polyadenylation signals (Fig. 1A). Because this plasmid lacks the E1 sequences necessary for viral replication, recombinant viruses derived from it are replication deficient in cells other than 293S. The recombinant adenovirus AdCAIL-4 (Fig. 1B) was generated by cotransfection of 293 cells with the shuttle plasmid pCAIL-4P and pBH/G1 (16) and homologous recombination between overlapping sequences. Candidate plaques were screened by restriction digest analysis and recombinants with the predicted DNA structure were plaque-purified and grown to high titre for subsequent studies.

To determine if AdCAIL-4 produced intact IL-4 protein, radiolabelled infected cell lysates were immunoprecipitated with a specific monoclonal antibody against mIL-4. A product of approximately 17 kDa was present in immunoprecipitations from a variety of lysates of AdCAIL-4 infected cells, but not from AdFl70-3 or from mock infected cells (data not shown). Supernatants from infected cells were also collected and used in bioassays based on the proliferation of the IL-4 dependent cell line CT-4S. All supernatants from AdCAIL-4 infected cell cultures stimulated proliferation of CT-4S cells, indicating that the virus could produce bioactive protein in a wide variety of infected human and murine cell types (Fig. 2A). The amount of IL-4 produced in infected cell supernatants was also quantitated by ELISA (Fig. 2B). Human cells, including fibroblasts and melanoma lines (open symbols), were found to express 2-8 μg of mIL-4 per 10⁶ infected cells. Murine cells (closed symbols) consistently expressed mIL-4 at approximately 10-fold lower levels than infected human cells. This difference in expression levels between murine and human cells has been observed with several vectors analogous to AdCAIL-4 (Addison et al., unpublished data) and probably reflects cell dependent differences in the activity of the HCMV IE promoter and in the efficiency of infection. However, the levels of expression of IL-4 in murine tissue were nonetheless relatively high (approximately 30 ng/10⁶ cells/day) and appear to be sufficient to mediate biological effects in a murine model.

AdCAIL-4 transduction of tumor cells affects tumorigenicity. Initially, we examined the ability of AdCAIL-4 to modify the tumorigenicity of FVB/BMT cells by infecting explanted cells in vitro, and injecting them s.c. into syngeneic mice. There
was no significant difference in the onset of tumor formation in PBS treated or Ad170-3 infected control cells, and all animals had developed palpable tumors by 25 days post-injection (Fig. 3, open symbols). In contrast, all animals that had been injected with cells transduced by AdCAIL-4 showed a pronounced delay in tumor onset with animals remaining tumor free until 13 weeks post-injection of tumor cells. More strikingly, the majority of the animals (61%) injected with AdCAIL-4 transduced tumor cells remained completely tumor free for up to 68 weeks.

In a separate experiment, tissue surrounding the injection site in animals that had received virally infected tumor cells was removed 7 days post-injection and analyzed histologically. In mice injected with Ad170-3 treated tumor cells development of solid tumors was observed (Fig. 4A). In contrast, in animals injected with the AdCAIL-4 infected tumor cells, no definite tumor formation was apparent although some tumor cells could be seen (Fig. 4B). There was a significant infiltration of eosinophils at the site of AdCAIL-4 transduced cell injection compared to the Ad170-3 infected tumor cells (Fig. 4A and B), an observation consistent with the anti-tumor effects being mediated by IL-4 expressing cells as demonstrated by Topper et al in a different tumor model (14,26). The actual mechanism of anti-tumor activity shown here, however, has yet to be determined.

*Direct injection of AdCAIL-4 into existing tumors can mediate tumor regression.* Following the initial success in modulating tumorigenicity by *ex vivo* treatment of tumor cells, we tested the anti-tumor activity of AdCAIL-4 upon injection of the vector into pre-existing tumors. Syngeneic mice were given $10^6$ FVBMT cells s.c., and twenty-one days later, when palpable tumors (approximately 10-13 mm$^3$), had
developed in all mice, the tumors were directly injected with 5x10^6 pfu of either AdCAIL-4, or Addl70-3, or with PBS alone. Tumors treated with PBS (Fig. 5A, open circles) or Addl70-3 (Fig. 5B, open squares) continued to grow until the mice became moribund. Treatment with Addl70-3 appeared to induce a growth delay compared to PBS injections, as has been noted previously (12), possibly because of immune responses against viral products (27). However, infection by Addl70-3 has never caused tumor regression in any mice. In contrast, after direct intra-tumoral injection with AdCAIL-4, all animals showed significant delays in tumor growth (Fig. 5A, closed symbols), and 50% of animals underwent complete tumor regression (Fig. 5A, closed squares), then remained tumor free for the duration of the experiment (90 days post virus injection). The remaining 50% of AdCAIL-4 injected mice (Fig. 5A, closed circles), showed a dramatic delay in tumor growth, with a significant prolongation of survival lasting up to 8 weeks post-injection (Fig. 5B), but eventually became moribund. In a separate experiment, similarly treated tumors were excised 36 days post virus injection and histological sections were stained with haematoxylin and eosin. Extensive eosinophil infiltration was seen in AdCAIL-4 treated tumors compared to control tumors and the tumor structure had been almost completely destroyed (Fig. 4C and D). Two animals had to be sacrificed after intra-tumoral injection of AdCAIL-4, when they developed severe abdominal ascites. The direct cause of this was unclear and factors contributing to this are currently under investigation.

'Cured' mice are protected from subsequent tumor challenge. In the case of breast cancer, 50% of successfully treated patients will eventually succumb to metastatic spread of the disease without recurrence of the primary tumor (1).
Therefore, it was important to determine if animals in which primary tumor regression had occurred were protected from a subsequent tumor challenge. Sixteen weeks post injection of AdCAIL-4 intra-tumorally, mice in which no primary tumor was detectable were challenged with a second tumor dose by delivery of 10⁶ tumor cells s.c. in the opposite flank. All animals were protected from the development of subsequent tumors, and remain tumor free at the present time (25 weeks after the second challenge). This suggests that the initial treatment of existing tumors by direct injection of AdCAIL-4 had the ability to generate a long-term memory response and to provide protection from subsequent tumor development at distal sites.

Discussion

IL-4 is produced mainly by activated T-lymphocytes and has a wide variety of effects on different immune cell populations. It can enhance the cytotoxic activity of CTLs, LAK cells, and TILs, which in turn may increase the anti-tumor activity of these effector cells (4,5). It can also induce the expression of MHC class II molecules by macrophages (5), which could enhance their ability to present antigen to T-cells and lead to induction of protective immunity. At the tumor site, IL-4 induces massive infiltration of eosinophils able to mediate tumor cytotoxicity in nu/nu, bg/bg, bg/nutid, scid, and w/wv+ mice. This suggests a mechanism which is not T-cell, B-cell, NK cell or mast cell dependent, and supports a role for eosinophils as the principal effector cell. However the mechanism of eosinophil-mediated tumor killing remains unclear (6). IL-4 has been shown to mediate anti-tumor activity in a wide variety of murine and human tumors in tumor transplantation assays (6), and such activity is clearly host and dose dependent, with the tumor clones producing the highest levels of IL-4 being the ones which are more readily killed. The requirement for high doses of IL-4 for effective anti-tumor activity, and the toxicity known to be associated with high concentrations, makes local delivery of IL-4 to tumors a more attractive means of immunotherapy. Ad vectors are well suited for delivery of the cytokine gene and for production of IL-4 in a local manner, and the choice of the appropriate promoters can result in high levels of protein production. Ad vectors can be used to transduce tumor cells in vivo with excellent efficiency, and because they do not replicate or efficiently integrate their genome, the expression of the transduced gene is transient. Thus persistent activation of the immune response, that could occur following use of retroviral vectors which integrate into the chromosomes of cells in or around the tumor, would be avoided with the use of Ad vectors to deliver the gene of interest.

When tumor cells were explanted and infected in vitro with AdCAIL-4, the tumorigenicity of FVBMT cells was significantly reduced (Fig. 3). Tumor development was prevented in 61% of animals and these mice remain tumor free to date, 17 months post-injection. Animals which did develop tumors all showed significant delays in tumor onset, with the first animals only developing tumors at least 13 weeks later than control animals. The eventual development of tumors in these animals might be due to an insufficient immune response or to development of resistance to immune mediated killing by a subset of tumor cells. When tumor bearing animals were given intra-tumoral injections of AdCAIL-4, all animals showed significant delays in tumor growth, and more importantly, 50% of these animals underwent complete tumor regression and were protected against a subsequent tumor challenge. Work by Tepper et al (14,26) has shown that a retrovirus expressing IL-4 can
modulate tumorigenicity if cells are transduced prior to injection. However, those studies failed to demonstrate any significant protective immunity against subsequent challenge. In our experiments, we demonstrated that mice which have undergone complete tumor regression were protected from a second challenge with 10^8 tumor cells delivered on the opposite flank. Eosinophils are not known to possess immune memory, and this protection suggests that other immune effector cells are playing a significant role in the anti-tumor activity we observed. Since eosinophils can express MHC class II molecules (28), it seems possible that in addition to mediating the primary regression of the existing tumor, they may be functioning in our experimental system as tumor antigen presenting cells to T-cells which would then provide tumor specific immune memory and protection from subsequent challenge. Data supporting this hypothesis were obtained by Columbuck et al (29) who demonstrated that, although primary tumor regression by IL-4 is most likely eosinophil mediated, the establishment of protective immunity was CD8+ T-cell dependent.

We have previously demonstrated ability of an Ad vector expressing the gene for IL-2 to direct anti-tumor activity in vivo (12), and in the present report we have extended those findings to demonstrate that an Ad vector expressing IL-4 can also be effective. In contrast to the effects of a recombinant Ad vector expressing IL-2 where tumor regression occurred by about 4 weeks post injection of virus (12), animals given the AdCAIL-4 virus did not show complete regression until 8 to 10 weeks post injection of the virus (Fig. 5A). The kinetics of IL-2 and IL-4 action thus differ in the time required to mediate tumor regression, and the immune effector cells present in histological sections were also very different. When IL-4 treated tumors were removed for histological analysis, a massive eosinophil infiltration at the injection site was noted not only surrounding the tumor but also infiltrating into the interior to some degree (Fig. 4B and 4D). There was also a notable absence of lymphocyte infiltration in these sections in contrast to results obtained with IL-2 treatment (12,30). These observations strongly suggest that the anti-tumor responses noted are cytokine specific, and not just a reflection of the inflammation which may occur as a result of the use of Ad as a gene delivery system.

For the treatment of these murine breast tumors with IL-2, the mode and time of delivery of the cytokine could lead to vastly different outcomes (12). We found that all animals developed tumors after a brief delay, when given IL-2 expressing tumor cells, but when existing tumors were directly injected with an IL-2 expressing vector intratumorally, complete tumor regression occurred in 54% of animals. This suggested a possible requirement for the presence of tumor antigen prior to cytokine stimulation which could lead to the recruitment and priming of tumor infiltrating lymphocytes. The delivery of IL-2 at this time would lead to the activation of these cells and to induction of their cytotoxic activity resulting in abrogation of the tumor. In the case of IL-4, the mode and timing of antigen and cytokine delivery does not appear critical. Whether the tumor cells were transduced in vivo with AdCAIL-4 prior to injection, or tumor bearing animals were injected intra-

References


2. Duration of Expression of IL-4 In Vivo Following AdCAIL-4 Injection

The level and duration of expression following intra-tumoral injection of AdCAIL-4 was analyzed. Tumors were injected with 5x10^8 pfu of AdCAIL-4 and, at various times post-injection, tumors were removed, frozen in liquid N\textsubscript{2}, and stored at -70°C. To assay the levels of IL-4 in the tumors, crude protein lysates were prepared and the IL-4 present in supernatants was quantitated by ELISA. The kinetics of IL-4 expression within tumors was found to parallel that observed following intra-tumoral injection of AdCAIL-2 (see Figure 4-7). The level of IL-4 was maximal at 1 day post-injection and then steadily decreased over the time course examined (Figure 5-6). In contrast to the expression of IL-2 following intra-tumoral injection, IL-4 could still be detected in tumors, although at reduced levels, at 15 days post-injection. Since this vector expresses murine IL-4, we were unable to determine whether the levels of IL-4 detected in the tumor were derived solely from the vector, or were are result of endogenously produced protein stimulated by a paracrine mechanism.

3. Toxicity Associated with In Vivo Administration of AdCAIL-4

Addison et al., 1995 reported no toxic side effects following intra-tumoral injection of AdCAIL-4. In subsequent experiments not reported in that study a significant proportion of the treated animals died, and it is now clear that, as with AdCAIL-2, toxic side effects from IL-4 overexpression do occur. This toxicity varied between experiments but, on average, was approximately 49%. Evidence of toxicity appeared 5-10 days post-injection, when animals became unresponsive, "ruffled", and had severe diarrhea. Post-mortems and histological
Figure 5-6: Duration of Expression of IL-4 from AdCAIL-4 In Vivo.

Syngeneic FVB/N mice were injected sc with $10^6$ PyMidT explanted tumor cells, and 21 days later, the animals were injected intra-tumorally with $5 \times 10^8$ pfu of AdCAIL-4 or Adl70-3 as a control vector. At various times post-injection, tumors were removed, frozen in liquid nitrogen, and stored at -70°C. Tumor protein lysates were prepared and analyzed for the quantity of IL-4 present by ELISA. Two animals were examined at each time point for each treatment. Individual animals are represented by small symbols and the mean is represented by the larger symbols. AdCAIL-4 - closed squares; Adl70-3 - open circles.
analyses were performed on these animals by Dr. Jacek Kwiecien (Veterinary Pathologist, Central Animal Facility, McMaster University). There were no apparent lesions in the brain, kidney, lung, or small intestine of affected animals; however, large lesions were found in the liver and spleen. Livers were infiltrated by large numbers of lymphocytes, and many scattered necrotic hepatocytes were identified throughout. The spleen was atrophied with a large accumulation of cells containing segmented nuclei (most likely eosinophils). Additionally, one animal had an infiltration of macrophages in the wall of the right ventricle of the heart, and an increase in cellularity of the femur bone. Both of these observations are consistent with a strong anti-viral immune reaction. The final diagnosis was that the animals were suffering from severe hepatic necrosis.

The toxicity associated with the intra-tumoral injection of AdCAIL-4 was similar to the toxicity observed following injection of AdCAIL-2. These results indicate that the use of lower doses of IL-4 are required to avoid toxicity, however, as with the use of lower doses of IL-2 vector, this treatment may be ineffective in inducing tumor regressions. The use of AdCAIL-4 in combination with other vectors may result in a more efficacious treatment that is associated with less toxicity.

C. Summary

An Ad vector expressing murine IL-4 was constructed (Figure 5-1) and was shown to direct the expression of high levels of biologically active IL-4 (Figure 5-2). Infection in vitro of polyoma middle T Ag-induced mammary adenocarcinoma cells with AdCAIL-4
resulted in a delay in the onset of tumor formation in 40% of animals receiving the transduced cells, and 60% of animals remained tumor free for greater than 17 months (Figure 5-3). Intratumoral injection of AdCAIL-4 into established tumors resulted in tumor regression in 50% of the treated animals, and these animals were subsequently protected from challenge with freshly isolated tumor cells. Treatment of tumors with AdCAIL-4 resulted in the accumulation of eosinophils within the tumor suggesting a role for these effector cells in the anti-tumor response (Figure 5-5). Expression of IL-4 following intra-tumoral injection of AdCAIL-4, was maximal at 24 hours post-injection and then steadily declined over a period of 15 days (Figure 5-6). In vivo administration of AdCAIL-4 was associated with serious toxic side effects, indicating that, although the use of this vector at a dose of 5x10⁶ pfu mediated regression of existing tumors, further studies are required to examine the use of this vector at lower doses for the immunotherapy of cancer, possibly in combination with other therapeutic cytokines.

The mechanism of tumor killing elicited by IL-4 has been demonstrated to be eosinophil-mediated (reviewed by Tepper, 1994). Following injection of AdCAIL-4-infected tumor cells, or intra-tumoral injection of AdCAIL-4, massive infiltration by eosinophils was observed (Figure 5-4), suggesting a role for this cell population in mediating the anti-tumor activity. The fact that animals which had undergone regression of existing tumors were protected from subsequent tumor challenge indicates an additional role for T-lymphocytes in the anti-tumor responses generated. Although no tumor-free animals were examined for the presence of tumor-antigen specific CTLs, a correlation was
noted between tumor volume and CTL activity, with the smaller tumors possessing intermediate levels of CTL activity (data not shown). Animals which had undergone tumor regression following intra-tumoral injection of AdCAIL-4 were also protected from a subsequent challenge of freshly isolated tumor cells. These results support a role for T-cells in the anti-tumor activity mediated by IL-4, and this indeed has also been suggested by others (Golumbek et al., 1991). It is possible that two separate mechanisms of anti-tumor activity are occurring simultaneously, i.e. that eosinophils and T-cells mediate tumor killing. It is also possible that eosinophils act as antigen-presenting cells subsequent to tumor killing, and that they present tumor-specific antigen in the context of MHC class II to T-cells, thus generating specific immune memory responses. In support of this hypothesis, it has been shown that eosinophils express MHC class II on their surface, (Mawhorter et al., 1994) and therefore could act as antigen presenting cells to T-cells.

Regardless of the mechanism involved in tumor regression mediated by IL-4, we have demonstrated that in vitro transduction of tumor cells by AdCAIL-4 or in vivo administration of AdCAIL-4 can modulate tumorigenicity and induce regression of established tumors. The ability to induce a protective immune response in treated animals suggests that this therapeutic approach could be efficacious at mediating regression of distal metastases.
CHAPTER 6. USE OF ADENOVIRAL VECTORS EXPRESSING IL-2, IL-12 OR IL-4 ALONE OR IN COMBINATION TO MODULATE TUMORIGENICITY AND INDUCE REGRESSION OF DISTAL UNTREATED TUMORS

A. Introduction

Previous experiments carried out in the PyM1δ tumor model, demonstrated that intra-tumoral administration of Ad vectors expressing either IL-2 (Chapter 4), or IL-4 (Chapter 5) mediated regression of established tumors in a significant percentage of treated animals; however, these treatments were associated with a high incidence of life-threatening toxicities at the efficacious vector dose. It was hypothesized that using lower doses of two cytokines in combination might result in synergistic action to induce anti-tumor activity, yet would result in a decrease in the incidence of toxicity that had been observed following administration of the high doses used previously (Chapters 4 & 5). Tumor immunotherapy using the combination of IL-2 and IL-4 has been previously shown to induce more potent anti-tumor activity than the use of either cytokine alone (Ohe et al., 1993; Arca et al., 1996; Strome et al., 1996). Pippin et al. (1994) also demonstrated an enhanced anti-tumor response following the administration of systemic IL-2 along with IL-4-transduced irradiated fibroblasts and unmodified tumor cells. Similarly, it has been previously shown that administration of local or systemic IL-2 enhanced the anti-tumor effects induced following in vivo administration of tumor cells transduced to express IL-12 (Pappo et al., 1995). Wigginton et al. (1996a) also observed synergy in mediating tumor regression following
administration of recombinant IL-12 together with IL-2 in a renal cell carcinoma model. Therefore, the use of the combination of two or more cytokines may induce potent anti-tumor responses that are associated with less toxicity in our model. Experiments were thus initiated to determine whether the delivery of Ad vectors expressing IL-2 in combination with vectors expressing IL-12 or IL-4 would result in increased efficacy along with reduced toxicity. This chapter describes the results obtained following transduction of PyMidT tumor cells to express IL-2 and IL-4, and the tumorigenicity of these cells is compared to the tumorigenicity of cells transduced to express IL-2 or IL-4 alone. The ability to mediate tumor regression following intra-tumoral injection of AdCAIIL-2 in combination with AdmIL-12.1 or AdCAIIL-4 in vivo is also compared, and the incidence of toxicity associated with these treatments is discussed.

One of the most important requirements for effective cancer immunotherapy is the ability to prevent the recurrence or metastatic spread of disease. One way to prevent spread of tumors is to induce a systemic protective immune response that can have anti-tumor effects on untreated distal tumor sites. The data presented in Chapters 4 & 5 suggest that a form of protective immunity has been induced in animals which have undergone complete regression of established tumors following intra-tumoral injection of Ad vectors expressing IL-2 or IL-4. Following tumor regression, these animals were protected from a subsequent challenge with freshly isolated tumor cells into the opposite flank to where the primary tumor was located. Furthermore, tumor antigen-specific CTL activity was demonstrated by lymphocytes isolated from the spleens of animals that had undergone tumor regression. These observations
indicated that the immunity which had been generated was systemic and not restricted to the local area of the site of injection. It was thus hypothesized that induction of anti-tumor activity following Ad-cytokine administration might induce immune responses that could mediate regression of distal untreated tumors. This hypothesis was examined in animals bearing tumors on each hind flank, where only one tumor was intra-tumorally injected with an Ad-cytokine vector, and the anti-tumor effect on the distal untreated tumor was monitored. This chapter discusses the results obtained in these contralateral experiments using Ad vectors expressing IL-2, IL-12, or IL-4, and the efficacy of these treatments alone or in combination is compared.

B. Results

1. Ability of AdCAIL-2 and AdCAIL-4 Coinfection to Modulate Tumorigenicity

The Ad vectors used in these experiments have been previously described (see Chapters 4 and 5). Initial animal experiments compared the growth of tumor cells expressing both IL-2 and IL-4 following transduction by Ad vectors, to the growth of tumor cells expressing either cytokine alone. Explanted PyMID tumor cells were infected at an moi of 100 with either AdCAIL-2, AdCAIL-4, or the Addl70-3 control vector, or were infected with a combination of 50 pfu/cell of each of AdCAIL-2 and AdCAIL-4 (total moi of 100). Twenty-four hours later, the infected cells were harvested, and $10^6$ transduced cells were injected sc into mice. Animals that had received tumor cells expressing both IL-2 and IL-4 showed a more pronounced delay in the onset of tumor formation compared to animals that
received cells expressing IL-2 alone; however, these animals tended to develop tumors before animals which had received cells expressing only IL-4 (Figure 6-1). Approximately 38% of animals receiving the cells expressing both IL-2 and IL-4 remained tumor free for greater than 17 months, in contrast to 62% of animals that remained tumor free after receiving tumor cells transduced with AdCAIL-4 alone. These data indicate either that the administration of IL-2 did not act synergistically to increase the anti-tumor activity of IL-4 in a tumor vaccination approach, or that a reduction in the amount of IL-4 expressed by the tumor cells results in a failure to induce appropriate effective anti-tumor responses.

2. Induction of Tumor Regression Following Intra-tumoral Coinjection of AdCAIL-2 and AdCAIL-4

It was next determined whether intra-tumoral injection of both AdCAIL-2 and AdCAIL-4 could induce regression of established tumors more effectively than injection of either vector alone. Tumor-bearing animals were injected with either $5 \times 10^8$ pfu of AdCAIL-2 or AdCAIL-4, or with a mixture of $2.5 \times 10^8$ pfu of each of AdCAIL-2 and AdCAIL-4 together (for a total of $5 \times 10^8$ pfu of viral vector). Injection of the combination of AdCAIL-2 and AdCAIL-4 resulted in complete tumor regression in 83% of treated animals, compared to 33% and 0% when animals were injected with AdCAIL-2 or AdCAIL-4 alone, respectively (Table 6-1). It should be noted that this experiment has not been repeated using a larger number of animals, and thus these data are not statistically significant. Tumor regression occurred by 3-4 weeks post-injection following AdCAIL-2 treatment, and by 5-7 weeks post-injection following treatment with AdCAIL-2 and AdCAIL-4 in combination. Although in
Figure 6-1: Modulation of Tumorigenicity by Coinfection of Tumor Cells with AdCAIL-2 and AdCAIL-4.

Polyoma middle T antigen-induced mammary adenocarcinoma cells were explanted from transgenic animals and infected in vitro at an moi of 100 with either AdCAIL-2, AdCAIL-4, or Addl70-3, or were coinfectected at an moi of 50 with each of AdCAIL-2 and AdCAIL-4. Transduced cells were injected sc into recipient animals and the mice were monitored for the onset of tumor formation. Each point on the graph indicates the percentage of tumor-free animals remaining at various times post-injection. Treatments are represented as follows: Addl70-3 - open circles; AdCAIL-2 - closed squares; AdCAIL-4 - closed circles; AdCAIL-2 plus AdCAIL-4 - open squares.
**Table 6-1: Tumor Regression Induced Following Intra-tumoral Injection of AdCAIL-2 and AdCAIL-4 Alone or in Combination.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Complete</th>
<th>Partial</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCAIL-2 (n=3)</td>
<td>33%</td>
<td>33%</td>
<td>33%</td>
</tr>
<tr>
<td>AdCAIL-4 (n=9)</td>
<td>0%</td>
<td>33%</td>
<td>67%</td>
</tr>
<tr>
<td>AdCAIL-2+AdCAIL-4 (n=6)</td>
<td>83%</td>
<td>17%</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Complete response refers to animals which have undergone total and permanent regression of the injected tumor, partial refers to animals that have undergone partial tumor regression followed by regrowth, or a significant delay in growth, and none refers to animals whose tumors have continued to grow at a rate comparable to controls.*
these particular experiments there were no complete tumor regressions observed following intra-tumoral administration of AdCAIL-4, complete tumor regression was found to occur by 8-10 weeks post-injection of IL-4 vector in previous experiments (Chapter 5). Therefore, each treatment was associated with different kinetics of tumor regression.

In order to determine if T-cells were playing a role in the anti-tumor activity induced following the administration of the AdCAIL-2 and AdCAIL-4 combination, spleens were removed from animals that had undergone complete tumor regression, or from tumor-bearing animals that had failed to undergo tumor regression following Ad-cytokine vector administration, eight weeks following treatment. Lymphocytes were restimulated by coculture with irradiated 516MT3 cells, which express PyMidT (see Appendix IV). Five days later, $^{51}$Cr release CTL assays were performed. The percentage specific lysis was compared to that observed following incubation with the parental cell line, PT0516 (which does not express PyMidT, and is therefore an indicator of non-specific killing by the lymphocytes). Lymphocytes isolated from animals that had undergone complete tumor regression following intra-tumoral injection of the AdCAIL-2 and AdCAIL-4 combination demonstrated high levels of PyMidT-specific killing (Figure 6-2, panels A and B). Even at low effector to target ratios (i.e. 10 to 1) greater than 20% specific lysis was observed. In contrast, animals which failed to undergo tumor regression following administration of the AdCAIL-2 and AdCAIL-4 combination, had no detectable tumor antigen-specific reactive lymphocytes (Figure 6-2, panel C). Therefore, tumor regression following intra-tumoral coinjection of AdCAIL-2 and AdCAIL-4 was associated with the induction of specific anti-tumor antigen-reactive
Figure 6-2: Detection of CTLs Following Intra-tumoral Coinjection of AdCAIL-2 and AdCAIL-4.

Spleens from animals that had been coinjected intra-tumorally with $2.5 \times 10^8$ pfu of AdCAIL-2 and AdCAIL-4 were removed and the isolated lymphocytes were restimulated by coculture with irradiated 516MT3 PyMidT-expressing cells for 5 days. The lymphocytes were used in a $^{51}$Cr release CTL assay using PyMidT-expressing cells (516MT3 - closed squares) or a parental cell line that does not express PyMidT (PT0516 - open circles). The percent specific lysis was measured in a gamma counter, and plotted against the effector to target ratio for tumor free (panels A & B) and tumor bearing animals (panel C).
lymphocytes which can efficiently kill tumor cells. The observation that these cells can be isolated from the spleens of animals that had responded to treatment, suggests that these lymphocytes are circulating throughout the body, and therefore have the potential to react against tumor cells at distal sites.

3. Generation of Tumor Antigen-Specific CTLs Following Treatment with AdCAIL-2 and AdmIL-12.1

Previous work carried out by Dr. Jonathan Bramson demonstrated that intra-tumoral injection of an Ad vector expressing IL-12 could mediate regression in greater than 70% of treated animals; however, only 30% of animals remained tumor free (Bramson et al., 1996b). The remaining animals had recurrence of tumor at the treated site and eventually became moribund. He went on to study whether the administration of Ad vectors expressing IL-2 and IL-12 resulted in a greater incidence of tumor regression compared to treatment with either vector alone. Tumor-bearing animals were injected intra-tumorally with either $5 \times 10^8$ pfu of AdCAIL-2 or AdmIL-12.1, or a combination of $2.5 \times 10^8$ pfu of each of AdCAIL-2 and AdmIL-12.1. It was found that injection of the combination of IL-2 and IL-12-expressing Ad vectors induced regression in 68% of treated animals, while injection of AdCAIL-2 or AdmIL-12.1 alone resulted in complete tumor regression without recurrence in only 45% and 31% of respectively treated animals. All animals which underwent tumor regression were protected from a subsequent challenge of freshly isolated tumor cells on the opposite flank, indicating that protective immunity had been generated. We hypothesized that, as in the case of treatment with AdCAIL-2, the tumor regression following administration of AdmIL-12.1
or the combination of AdCAIL-2 and AdmIL-12.1 was associated with the generation of tumor antigen-specific CTLs that could be isolated from the spleens of responding animals.

Following tumor regression induced by intra-tumoral vector injection, spleens were removed from the animals and the isolated lymphocytes were restimulated by coculture for 5 days with irradiated 516MT3 PyMidT target cells (see Appendix IV). After coculture, the lymphocytes were used in a $^{51}$Cr release assay to detect specific tumor antigen-reactive CTLs. Tumor regression induced by injection of AdCAIL-2, AdmIL-12.1 or a combination of both vectors, was associated with the presence of high levels of tumor antigen-specific lysis that was inhibited by the addition of antibodies against the CD3 molecule on T cells (Figure 6-3, panels A-C). In contrast, tumor-bearing animals that had been injected with Addl70-3 did not have significant levels of tumor antigen-specific CTLs (Figure 6-3, panel D). These data indicate that tumor regression is correlated with the presence of tumor antigen reactive T-lymphocytes in the spleen, and suggest a mechanism which could lead to the induction of anti-tumor activity at distal untreated sites.

For both the single tumor and contralateral tumor experiments, the CTL activity detected in tumor bearing versus tumor free animals for all three treatments was compared (Table 6-2). There was a strong correlation between the degree of specific lysis and the tumor status of the animal. This correlation was most noticeable following treatment of tumors with AdCAIL-2 or with a combination of AdCAIL-2 and AdmIL-12.1. These data support the hypothesis that tumor regression following intra-tumoral injection of AdCAIL-2 or AdmIL-12.1 is mediated by tumor antigen-specific CTLs, and the response correlates with
Figure 6-3. Tumor Regression Induced by Intra-tumoral Injection of AdCAIL-2, AdmIL-12.1 or AdCAIL-2 in Combination with AdmIL-12.1 is Associated with the Presence of Tumor Antigen-Specific CTL.

Spleens were removed from animals that had undergone tumor regression following intra-tumoral injection of $5 \times 10^8$ pfu of either AdCAIL-2 or AdmIL-12.1, or coinjection of $2.5 \times 10^8$ pfu of AdCAIL-2 in combination with AdmIL-12.1, or from a tumor bearing Addl70-3 injected animal. The lymphocytes were isolated and restimulated by coculture with irradiated 516MT3 PyMidT-expressing cells for five days. Following incubation, the lymphocytes were used in a $^{51}$Cr release assay with PyMidT-expressing target cells (516MT3 cells - closed symbols) or a parental cell line that does not express PyMidT (PT0516 cells - open symbols). All samples were assayed in the presence (circles) or the absence (squares) of antibody against the CD3 T-lymphocyte receptor molecule. The treatments are represented as follows: Panel A - AdCAIL-2; panel B - AdmIL-12.1; panel C - AdCAIL-2 plus AdmIL-12.1; panel D - Addl70-3.
Table 6-2. Generation of Tumor Antigen-Specific CTLs Following Intra-tumoral Injection of AdCAIL-2 or AdmIL-12.1.

Specific CTLs Detected

<table>
<thead>
<tr>
<th>Treatment/Tumor Status(^a)</th>
<th>+++(^c)</th>
<th>++(^d)</th>
<th>+(^e)</th>
<th>+/-(^f)</th>
<th>-(^g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCAIL-2/Tumor Free</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdCAIL-2/Tumor Bearing</td>
<td></td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AdmIL-12.1/Tumor Free</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdmIL-12.1/Tumor Bearing</td>
<td></td>
<td>1(^h)</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AdCAIL-2+AdmIL-12.1/Tumor Free</td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdCAIL-2+AdmIL-12.1/Tumor Bearing</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Tumor free refers to animals which have undergone complete tumor regression on both flanks and tumor bearing refers to animals which have measurable tumors on either flank or both flanks.

\(^b\) Effector to target ratio of 30:1.

\(^c\) Specific lysis greater than 70%.

\(^d\) Specific lysis between 40% and 70%.

\(^e\) Specific lysis between 20% and 40%.

\(^f\) Specific lysis between 10% and 20%.

\(^g\) Specific lysis less than 10%.

\(^h\) This animal was from a contralateral experiment and had undergone regression of tumors on both flanks, however, the tumor on the left hind flank had begun to grow back at the time the CTL assay was carried out.
the degree of activation of these lymphocytes.

Following coculture for CTL analysis, the lymphocytes were restimulated with irradiated 516MT3 or PT0516 cells, and 24 hr later, the supernatants were analyzed by ELISA for the presence of IFN-γ. The stimulation index was calculated as the ratio of IFN-γ produced after stimulation with 516MT3 cells compared to PT0516 cells. There was a striking difference in the secretion of IFN-γ by lymphocytes isolated from animals which had undergone tumor regression compared to tumor-bearing animals. Enhanced secretion of IFN-γ was observed for those animals which had been treated with AdCAIL-2 (15.0 ± 10.3 for tumor-free animals compared to 1.4 ± 1.2 for tumor-bearing animals) or the combination of AdCAIL-2 with AdmIL-12.1 (11.0 ± 4.8 for tumor-free animals compared to 1.3 ± 0.9 for tumor-bearing animals); however, the results obtained from animals which had been treated with AdmIL-12.1 alone did not show the same significant increases in IFN-γ expression (5.0 + 4.5 for tumor-free animals compared to 1.3 + 0.9 for tumor-bearing animals). These data suggest that tumor regression is associated with the induction of tumor antigen-reactive CTLs that secrete elevated levels of IFN-γ, and are therefore demonstrating a type 1 lymphocyte profile.

4. The Ability of Intra-tumoral Injection of Ad Vectors Expressing Cytokines to Induce Anti-Tumor Activity at Distal Untreated Sites

Previous experiments suggested that intra-tumoral injection of Ad vectors expressing either IL-2, IL-12 or IL-4, led to tumor regression and the induction of a systemic immune response, as indicated by the ability to induce protection from subsequent tumor challenge or
to isolate tumor-antigen reactive lymphocytes from the spleens of responding animals (this chapter and Chapters 4 & 5; Bramson et al., 1996b). Experiments were initiated to determine the ability of intra-tumoral injection of AdCAIL-2, AdmIL-12.1, AdCAIL-4, or coinjection of AdCAIL-2 and AdCAIL-4, or AdCAIL-2 and AdmIL-12.1, to mediate regression of untreated distal tumors. Syngeneic mice were injected sc on both flanks with $10^6$ PyMidT tumor cells in each. Twenty 20 days post-injection, animals were intra-tumorally injected with $5 \times 10^8$ pfu of AdCAIL-2, AdmIL-12.1, AdCAIL-4 or Addl70-3, or a combination of $2.5 \times 10^8$ pfu of each of AdCAIL-2 and AdmIL-12.1 or AdCAIL-2 and AdCAIL-4, in the right hind flank tumor only. Intra-tumoral injection of AdCAIL-2 or AdmIL-12.1 had a pronounced effect on the growth of the injected tumor compared to the Addl70-3 treated control group, and had a moderate effect on the untreated tumor (Figure 6-4). The coinjection of AdCAIL-2 together with AdmIL-12.1 resulted in more significant anti-tumor responses in both the treated and untreated tumors when compared to treatment with either vector alone or with Addl70-3 (Figure 6-4). The anti-tumor effects observed following injection of AdCAIL-4, or the combination of AdCAIL-2 and AdCAIL-4, were less pronounced in both the injected and uninjected tumors compared to those observed following treatment with AdCAIL-2 or AdmIL-12.1 alone or in combination. Although the anti-tumor responses noted following treatment with AdCAIL-4 alone or in combination with AdCAIL-2 were not as dramatic as those noted for the other treatments, the growth of the treated tumor, but not the untreated tumor, was significantly delayed compared to control injected tumors (Figure 6-4).
Figure 6-4. Regression of Distal Untreated Tumors Following *In Vivo* Injection of Ad Cytokine-expressing Vectors Alone or in Combination.

Animals were injected sc in both hind flanks with 10^6 tumor cells/flank, and twenty days later were injected intra-tumorally in the right hind flank only, with either 5x10^8 pfu of Addl70-3, AdCAIL-2, AdmIL-12.1 or AdCAIL-4, or were coinjected with 2.5x10^8 pfu of each of AdCAIL-2 and AdmIL-12.1, or AdCAIL-2 and AdCAIL-4. Tumor growth was monitored on each flank by caliper measurement and tumor volume was calculated assuming a prolate spheroid. Each panel is a graphic representation of the tumor volume of left hind flank tumors (uninjected tumors - open boxes), and right hind flank tumors (injected tumors - closed boxes) for each treatment at 0 (panel A), 6 (panel B), 12 (panel C) and 19 (panel D) days post-vector injection.
The results of multiple contralateral experiments are summarized in Table 6-3. Following intra-tumoral injection of AdCAIL-2 or AdmIL-12.1, 19% and 38% of the respectively treated animals underwent complete regression of both treated and untreated tumors. In comparison, coinjection of AdCAIL-2 and AdmIL-12.1 resulted in regression of both tumors in 63% of the animals, with an additional 25% undergoing complete regression of the injected tumor, and a partial regression of the untreated tumor. No animals underwent complete regression of both tumors following intra-tumoral injection of AdCAIL-4; however, 1/17 animals had complete regression of the treated tumor, with a partial response in the untreated tumor, and 2/17 animals demonstrated partial responses in both treated and untreated tumors (Table 6-3). Following coinjection of AdCAIL-2 and AdCAIL-4, 13% of animals (2/15) showed complete regression of both tumors, with an additional 33% (5/15) demonstrating complete regression of the treated tumor along with a partial response in the untreated tumor (Table 6-3). These results indicate that the most effective Ad-vector treatment to induce regression of untreated distal tumors is the intra-tumoral injection of AdmIL-12.1 in combination with AdCAIL-2.

5. Toxicity Associated with Intra-tumoral Coinjection of AdCAIL-2 together with AdCAIL-4 or AdmIL-12.1

The toxicity associated with the intra-tumoral injection of 5x10^8 pfu of AdCAIL-2 or AdCAIL-4 has been discussed (see Chapters 4 and 5, respectively). To summarize, between 5-14 days post-vector injection, animals became "ruffled" and non-responsive and had severe hepatic necrosis. Similar toxicities were observed following co-injection of 2.5x10^8 pfu of
Table 6-3: Intra-tumoral Injection of Ad Vectors Expressing Cytokines Can Mediate Regression of Untreated Distal Tumors.

<table>
<thead>
<tr>
<th>Response</th>
<th>Treated</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>IL-2</td>
</tr>
<tr>
<td>Complete</td>
<td>(n=42)</td>
<td>(n=17)</td>
</tr>
<tr>
<td>Complete</td>
<td>Complete</td>
<td>19%</td>
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<td>Complete</td>
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<td>17%</td>
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<td>None</td>
<td>None</td>
<td>21%</td>
</tr>
</tbody>
</table>

*a* Complete refers to animals that have undergone total and permanent regression of tumor, partial refers to animals that have undergone partial tumor regression followed by regrowth, or a significant delay in growth, and none refers to animals whose tumors have continued to grow at a rate comparable to controls.

*b* Animals were intra-tumorally injected in the right hind flank tumor only with 5x10⁶ pfu of vector or 2.5x10⁷ pfu of each vector in the combination treatments.
AdCAIL-2 in combination with $2.5 \times 10^8$ pfu of AdCAIL-4. For the combination treatment, however, only 24% of animals succumbed to the associated toxicities (versus 38% and 49% for AdCAIL-2 and AdCAIL-4, respectively). These results suggest that the use of lower doses of cytokines in combination may aid in reducing toxicity without the loss of efficacy associated with the administration of higher doses of single cytokines. In contrast to the toxicity induced by AdCAIL-2 (Chapter 4), there was no apparent toxicity resulting from the in vivo administration of AdmIL-12.1 at doses as high as $2 \times 10^9$ pfu (Bramson et al., 1996). Toxicity was observed following intra-tumoral injection of the combination of AdCAIL-2 and AdmIL-12.1 ($2.5 \times 10^8$ pfu of each vector); however, the incidence was lower than that previously observed with the AdCAIL-2 treatment alone (only 14% of animals over four independent experiments succumbed to toxic side effects versus 38% when AdCAIL-2 alone was injected). Although the administration of $5 \times 10^8$ pfu of AdmIL-12.1 was not associated with life threatening toxicity, this dose of vector only resulted in complete tumor regression in approximately 1/3 of treated animals (Bramson et al., 1996b). In contrast, treatment with both AdCAIL-2 and AdmIL-12.1 resulted in tumor regression in approximately 89% of animals bearing single tumors (data not shown), and 63% of animals bearing contralateral tumors. The increased efficacy in mediating regression of established tumors in vivo that was observed following the use of AdCAIL-2 in combination with AdmIL-12.1, indicates that this is a very promising therapeutic approach, despite the low level toxicity associated with its use.
C. Discussion

These results demonstrate that following in vitro transduction and subsequent sc injection in mice, a more prolonged delay of tumor onset was observed for tumor cells that were transduced with a combination of AdCAIL-2 and AdCAIL-4, compared to cells transduced by AdCAIL-2 alone. This delay, however, was not as prolonged as that observed for cells transduced with AdCAIL-4 alone (Figure 6-1), and fewer animals remained tumor free in the combination treatment than when IL-4 alone was used. The decreased efficacy observed following the administration of tumor cells expressing both cytokines compared to those expressing IL-4 alone, might simply be due to the dose of IL-4. Tumor cells infected with AdCAIL-4 at an moi of 50 instead of 100 probably produce less IL-4, and as shown by the experiments using either cytokine alone, expression of IL-4, not IL-2, is responsible for the prolonged survival of these animals. The expression of IL-2 in combination with IL-4 in these experiments may have no contribution to tumor modulation, as animals given tumor cells infected with AdCAIL-2 at an moi of 100 had only slight prolongation of survival compared to animals receiving IL-4-transduced tumor cells. To address whether the dose of IL-4 is responsible for the decreased efficacy observed in the combination treatment, the results could be compared to those obtained from tumor cells transduced with the same amount of IL-4 vector (i.e. AdCAIL-4 at an moi of 50 plus Addl70-3 at an moi of 50), and in this case, the combination of IL-2 and IL-4 might prove to be more efficacious than the use of IL-4 alone, as has been reported by others (Ohe et al., 1993; Arca et al., 1996; Strome et al., 1996).
In contrast to the results observed following *in vitro* transduction of tumor cells with the combination of AdCAIL-2 and AdCAIL-4, intra-tumoral injection of the combination treatment resulted in increased efficacy compared to treatment with either vector alone (Table 6-1). Although the number of animals used in the AdCAIL-2 group is small, the response that was observed is similar to that which has been observed previously (on average, approximately 40-50% of animals demonstrate complete regression of the injected tumor), and this response rate is still lower than the response rate observed for the combination treatment in this experiment. The differences observed in the kinetics of tumor regression between AdCAIL-2 treatment alone, or in combination with AdCAIL-4, may be due to the reduced amounts of cytokines that are likely being expressed (i.e. as a result of a lower moi of each virus). The increased ability of the combination treatment, compared to injection of single vectors, to mediate complete tumor regression may be due to a number of reasons. T-cells are usually present in tumor biopsies, but are anergized and rendered tolerant to tumor antigens through a variety of mechanisms (described in Chapter 1). IL-2 and IL-4 are both T-cell growth factors, and thus can induce the proliferation and activation of the T-cells infiltrating the tumor. The burst of cytokine expression from the transduced tumor cells, could overcome T-cell anergy and initiate effective anti-tumor responses. In support of this hypothesis, it has been shown that IL-4 can enhance the CTL activity of antigen-specific T-cell clones (Spits *et al*., 1988), and thus may synergize with IL-2 in activating primed (yet anergized) TILs. Furthermore, Kawakami *et al*. (1993) demonstrated that TILs isolated from human tumors grew better when cultured in the presence of both IL-2 and IL-4, and resulted
in enhanced killing of autologous tumor. Another mechanism by which IL-4 may synergize with IL-2 to enhance anti-tumor immune responses is by the upregulation of the costimulatory molecules required for efficient activation of T-cells. IL-4 can upregulate the expression of the B7 costimulatory molecule on B-cells (Stack et al., 1994), Langerhans cells (Chang et al., 1995), and keratinocytes (Junghans et al., 1996). Tumor cells may have reduced or no expression of costimulatory molecules required for T-cell activation, thus upon encountering tumor antigen, the T-cells become anergized and fail to respond. IL-4-induced expression of B7 on the surface of antigen presenting cells (which may infiltrate the tumor following vector delivery), or on the tumor cells would result in the proper presentation of tumor antigen to specific CTLs and lead to T-cell activation rather than anergy. The observation that tumor regression following treatment with AdCAIL-2 in combination with AdCAIL-4 is associated with the presence of tumor antigen-specific CTLs supports the hypothesis that IL-4 can synergize with IL-2 to induce potent T-cell activation and effective tumor killing.

Administration of IL-2 together with IL-12 was more effective in mediating regression of distal untreated tumors than any other treatment examined. The enhanced response is likely a result of the ability of IL-2 and IL-12 to synergize to induce potent type-1 T-cell responses, which are more effective in cell-mediated killing. There are a number of mechanisms which may be responsible for the synergistic effects of IL-2 and IL-12 administration in generating anti-tumor activity. Regional draining lymph node lymphocytes from lung cancer patients were found to have increased proliferation, cytotoxicity, and expression of Th1 type cytokines (such as IFN-γ), following incubation with both IL-2 and
IL-12 compared to incubation with either one alone (Hanagiri et al., 1996). The cytotoxic activity of NK cells was also found to be increased following treatment with IL-2 and IL-12 compared to either one alone (Bommenma et al., 1994). Lastly, Wigginton et al. (1996b) have shown that ex vivo incubation of macrophages with IL-12 and IL-2 enhanced their nitric oxide production, and resulted in the ability of the macrophages to decrease tumor growth more effectively than when cultured with either cytokine alone. Each of these mechanisms could contribute to the enhanced anti-tumor activity observed following administration of the combination of IL-12 and IL-2. The CTL data obtained following injection of AdCAIL-2 alone or in combination with AdmIL-12.1, suggest that the CTL which had been generated were of a Th1-type profile, since they had elevated levels of IFN-γ expression but not elevated levels of IL-4 expression (data not shown). The increased levels of IFN-γ production would lead, not only to increased cell-mediated immunity, but also to the induction of an angiostatic environment within the tumor [through upregulation of angiostatic molecules such as IP-10 and MIG (Kaplan et al., 1987; Farber, 1993; Angiolillo et al., 1995; Luster et al., 1995; Strieter et al., 1995)], which would also contribute to tumor regression.

The use of AdCAIL-2 in combination with either AdCAIL-4 or AdmIL-12.1 induced toxicity in fewer treated animals compared to that observed following injection of AdCAIL-2 alone. The reduction in toxicity may be a result of decreased cytokine expression in other organs, rather than a reduction in systemic infection by the administered virus, since equal quantities of virus were used in each treatment. In addition to a reduction in toxicity, the
combination treatments were more efficacious in mediating tumor regression than treatment with the respective vectors alone. Furthermore, intra-tumoral coinjection of AdCAIL-2 and AdmIL-12.1 resulted in regression of distal untreated tumors in a significant percentage of treated animals, suggesting that this therapeutic approach may be extremely effective in the prevention of recurrent or metastatic spread of the disease. Taken together, these data demonstrate that immunotherapy of cancer using combinations of cytokines may be more efficacious, and associated with less toxicity, than treatment with higher doses of single cytokines, and thus warrant further investigation.
CHAPTER 7. DISCUSSION

In the last ten years, there has been a great deal of activity in gene therapy research, leading to the initiation of over 150 human clinical trials, the majority of which are in experimental cancer therapeutics. These approaches have met with some success in the clinic, however, the early results indicate that new, more efficient approaches of gene targeting and delivery, as well as treatments that prevent metastatic spread of cancer are necessary. To this end, we have been developing and characterizing first generation adenoviral vectors as gene delivery systems for use in the immunotherapy of cancer.

A. Factors Influencing Transgene Expression in Adenoviral Vectors

We investigated what factors influenced the expression of transgenes encoded by the viral vector. Initial studies using the HCMV IE promoter to drive transgene expression in Ad vectors indicated that the orientation of the expression cassette within the vector backbone influenced transgene expression. The increase in transgene expression when cassettes were oriented in the rightwards direction (i.e. parallel to E1 transcription), was most likely due to the presence of the E1a enhancer sequences immediately upstream of the HCMV IE promoter, which remained in the vector backbone following deletion of E1 sequences. This effect has been observed by others using the same HCMV IE promoter
fragment to express rotavirus VP7sc antigen (Xu et al., 1995). We also found that the promoter used to drive transgene expression not only influenced the level of expression obtained in infected cells [i.e. the HCMV IE promoter produced higher levels of β-gal protein than did the human β-actin promoter (Bett, 1995)] but, more importantly, also influenced the levels and kinetics of expression in different cell types. The HCMV IE promoter expressed 10-100 fold lower in cells of murine origin compared to human cells (Figure 3-2). It is possible that Ad vectors do not infect murine cells as efficiently as they infect human cells. However, it was demonstrated that the use of the MCMV IE promoter in place of the HCMV IE promoter in our Ad constructs led to an increase in transgene expression in murine cells (Figure 3-3, Table 3-2), thus a reduced infectivity of murine cells by Ad vectors does not entirely explain the reason for the reduction in expression driven by HCMV IE in murine cells. The reasons for the reduced transgene expression levels driven by HCMV IE in infected murine cells are discussed in more detail in Chapter 3.

In vitro, transgene expression following Ad vector infection persisted for at least 7 days, although in some cell types expression levels decreased after 3 days (Figure 3-3). In vivo, transgene expression in tumor tissue peaked between 1 to 3 days post-infection, and then decreased rapidly to undetectable levels by 10 to 15 days post-viral injection (Figure 4-6, and 4-7). The limited duration of transgene expression could be due to not only the infrequency of Ad genome integration into the host cell chromosome, but also to
two additional events: recognition and elimination of virally transduced cells by activated
CTLs, or downregulation of transgene expression through repression of CMV-directed
transcription. It has been shown that, following administration of first generation Ad
vectors in vivo, there are anti-viral immune responses generated as a result of antigen-
presenting cell processing of capsid proteins (McCoy et al., 1995), or of low level
expression of viral proteins in transduced cells resulting in their recognition as virus-
infected cells by the immune system and their subsequent elimination (Yang et al., 1994).
Repression of HCMV IE-mediated transcription was observed in vitro by IFN-γ, while the
expression driven off other eukaryotic promoters, such as that from the MHC I gene,
were activated by the presence of IFN-γ (Harms & Splitter, 1995). This observation is
very important, as one of the first events to occur following viral infection is inflammation
and an increase in the levels of IFN-γ (Abbas et al., 1991). Thus the use of Ad vectors
to deliver transgenes would also increase the endogenous IFN-γ levels and aid in the
repression of the HCMV IE promoter. MCMV replication in infected cells is inhibited by
the expression of interferons (Gribaudo et al., 1993), and this inhibition is due to the
ability of IFN-α to downregulate transcription from the IE promoters (Gribaudo et al.,
1995). Thus, transcription directed by both the HCMV and MCMV IE promoters appear
to be subjected to these same repressional effects and, for some purposes, the use of non-
viral promoters, which are not affected by host immune factors, may be desirable to
promote long term expression.
Another mechanism by which the HCMV IE promoter may be inactivated is through methylation. An HCMV IE promoter/enhancer in transfected cells was subject to sequence-specific methylation, at 5'-CpG, which led to a strong repression of HCMV IE transcriptional activity (Prosch et al., 1996). This repression could not be overcome by the addition of known HCMV IE transcriptional activators, such as TNF-α or phorbol myristate acetate, but could be partially alleviated by the addition of untranscribable highly methylated DNA, suggesting that methyl-CpG specific binding factors prevented transcription. These mechanisms of transcriptional repression may partly explain the limited duration of transgene expression observed in these studies.

B. Effect of RCA Contamination and Approaches to Prevent their Generation

One potential drawback associated with first generation Ad vectors is the ability of the vector to undergo homologous recombination with the Ad sequences encoded within 293 cells. The E1-deleted Ad vectors can only be propagated in 293 cells, as these cells supply *in trans* the E1 proteins required for virus replication. Homologous recombination in 293 cells may result in the reacquisition of the E1 genes, generating an autonomously replicating vector. This could be problematic in a clinical setting, resulting in the dissemination of the virus and induction of viral-associated diseases. During the course of my studies, it became apparent that the first generation vectors that were constructed could indeed undergo homologous recombination and generate replication competent
adenoviruses (RCA) during routine propagation of the viral vector. RCA generation during Ad vector propagation has also been observed by other researchers (Lockmuller et al., 1994; Hehir et al., 1996). As a result, an attempt was made to determine the effect of RCA contamination on transgene expression from first generation Ad vectors. Low level "contamination" of vector stocks with RCA did not appear to have a significant effect on the level of transgene expression from infected cells in vitro; however, at ratios of RCA to vector of 1:1000, a slight increase in transgene expression was noted (Figure 3-5), indicating that at higher concentrations, RCA contamination may alter the effective "dose" of a given transgene. In order to have an effect on transgene expression, the RCA must coinfect the same cell as the vector, thus, significant effects on transgene expression would only occur if the moi of RCA infection is relatively high. It was observed that at an moi of RCA of 0.1, a slight increase in transgene expression occurred (Figure 3-5), thus significant effects on the level of transgene expression would be expected to occur only at an moi of 1 or greater. When aliquots of $2.5 \times 10^9$ pfu of several Ad vector stocks were examined for the frequency of generation of RCA, 66% of samples were contaminated (Hehir et al., 1996). After 12 serial passages of an AdCMVlacZ vector, the stock contained 1% contamination with RCA, which would translate to $10^6$ RCA for every $10^8$ pfu of first generation Ad vector (Lochmuller et al., 1994). Clearly, these results indicate the importance of the prevention of RCA generation during routine vector propagation.

There are many strategies that have been employed to avoid the generation of RCA.
One approach is to modify the packaging cell line and reduce or eliminate the potential of the vector to recombine with the E1 sequences contained in the 293 cell genomic DNA. Fallaux et al. (1996) essentially reconstructed the 293 cell line by transfecting human embryonic retinoblast cells with Ad 5 E1 genomic sequences from nucleotides 79-5789, generating the 911 cell line. It is now known that 293 cells contain wild-type Ad 5 sequences from nucleotides 1 to 4344, and thus although the 911 cells possess a smaller region of overlap with the sequences retained in the first generation vectors on the left end of the genome, the 911 cells have a greater region of overlap than 293 cells on the right end. Hehir et al. (1996) demonstrated that RCA are generated as a result of a double cross-over event and thus the 911 cells still have the potential to generate RCA. The frequency of generation of RCA during vector propagation in 911 cells was not reported; however, subsequent studies have shown that generation of RCA is possible when these cells are used to propagate first generation vectors (J. Rudy & F.L. Graham, personal communication). Another packaging cell line has been generated by transfection of A549 human lung carcinoma cells with Ad 5 sequences from nucleotides 505 to 4034, and these cells do not contain sequences which overlap with those retained in the Ad-vector genome and therefore, theoretically, cannot generate RCA (Imler et al., 1996). These cells expressed the E1-genes for at least 3 months in culture, but the yield of first generation vectors was 10-fold lower than that obtained following propagation in 293 cells (Imler et al., 1996). In order to continue to use 293 cells for vector propagation, other groups have
utilized an Ad 2 vector backbone lacking the protein IX gene, since the protein IX sequences are encoded within the Ad genomic sequences in 293 cells (Hehir et al., 1996). Even though the frequency of RCA generation was reduced, three of thirty stocks were found to contain RCA (Hehir et al., 1996), indicating that other methods of preventing RCA generation are required. We hypothesized that the packaging constraints of the Ad virus could be used to generate a vector that, upon homologous recombination in 293 cells and reacquisition of the E1 sequences, would be too large to package into virions. "Stuffer" sequences (lambda DNA) were inserted into the E3 region of the Ad vector, creating vectors that exceeded 105% of the wild-type Ad genome, a size which had been previously shown to be the upper limit for Ad DNA packaging (Bett et al., 1993). The "stuffer" vectors replicated slightly slower than the same vector without the "stuffer sequence"; however, this vector was stable over 12 serial passages in 293 cells, and RCA could not be detected after assay on A549 cells (Appendix III). These results suggest an alternative strategy to producing RCA-free Ad vector stocks without having to redesign the packaging cells used for these vectors.

C. Polyoma Middle T Induced Murine Model of Mammary Adenocarcinoma

To study the ability to modulate tumorigenicity following Ad-cytokine vector transduction of tumor cells, we chose a transgenic mouse model of mammary
adenocarcinoma. These animals expressed the gene for the polyoma virus middle T antigen (PyMidT) under control of the mouse mammary tumor virus (MMTV) long-terminal repeat (LTR), which resulted in the oncogenic transformation of the normal mammary epithelium (Guy et al., 1992b). The tumors which arose in these animals were explanted and digested into single cell suspensions and, following sc injection in syngeneic animals, would form tumors within 15-21 days post-injection (see Chapter 2). The transgenic animals are likely tolerized to PyMidT, since it should be expressed during thymic maturation and development, and thus be recognized as a self-antigen allowing the mammary tumors to develop without immune recognition in the transgenic animals. It is interesting that, although these tumor cells overexpress PyMidT (which should be recognized by the immune system as a foreign gene product) and thus should be antigenic, 100% of immunocompetent syngeneic animals developed tumors following injection of 10⁶ PyMidT tumor cells. Clearly, other immuno-evasive mechanisms are playing a role in their tumorigenesis. The tumor cells express significant levels of IL-10 (179 ± 13 pg/mg tumor, J. Bramson, personal communication) which would likely induce an immunosuppressive environment via the ability of IL-10 to downregulate a Th1 type immune response and cell mediated immunity (Fiorentino et al., 1989; Matsuda et al., 1994).

Another potential mechanism of immunosuppression utilized by PyMidT tumor cells could be the failure to present tumor antigens in the appropriate context to T-cells
resulting in the subsequent anergization of reactive lymphocytes. Toes et al. (1996) demonstrated that peptide-vaccination with a tumor-specific epitope led to enhanced tumor growth due to the deletion of antigen-specific CTL and induction of antigen-tolerance. It has also been shown that MHC expression on tumor cells can be at a level that is too low to allow recognition of the tumor cells by T-lymphocytes, and following transduction of tumor cells to overexpress MHC class I or class II molecules, anti-tumor immunity was restored (Plautz et al., 1993; Mandelboim et al., 1995; Nabel et al., 1996). In support of this hypothesis, the PyMidT tumor cells were not recognized by allogeneic splenic lymphocytes in a $^{51}$Cr release assay, suggesting that the level of MHC on PyMidT cells was not adequate to promote CTL-mediated killing (J. Bramson, personal communication).

Tolerance to PyMidT tumor cells may also be induced by activation of T-cells in the absence of appropriate costimulatory signals. PyMidT tumor cells express extremely low levels of the B7-1 costimulatory molecule (B. Puetzer, personal communication), which may indeed be contributing to induction of T-cell anergy. Costimulation has been shown to be a requirement for efficient induction of reactive T-lymphocytes (reviewed by Schwartz, 1992; Guinan et al., 1994; Van Gool et al., 1996). Activation of a T-cell by antigen in association with MHC molecules, in the absence of costimulation through binding of either B7-1 or B7-2 to its appropriate ligand on the T-cell, results in the anergization of the T-cell, and its failure to secrete proliferative cytokines and mediate
effective immune responses.

A third mechanism by which these tumor cells may avoid elimination by the immune response is by inducing neovascularization which enables the tumor to grow rapidly and metastasize and thus outcompete the immune system. The PyMidT tumor cells express KC [also known as GRO-α (growth related oncogene); W.J. Muller, personal communication]. KC is a member of the ELR-containing CXC chemokine family, many of which have been shown to induce angiogenesis in rat corneal pocket models (Strieter et al., 1995). Another ELR-CXC chemokine family member, IL-8, is overexpressed in many tumor cells (Smith et al., 1994). Abrogation of IL-8 by addition of neutralizing antibodies resulted in a 40% reduction in A549 tumor size in SCID mice compared to control animals (Arenberg et al., 1996). These results indicate that the ability to induce neovascularization may be a major factor of tumorigenicity in vivo, and may explain the aggressiveness of PyMidT tumor cell growth and their ability to evade elimination by immune-mediated killing.

D. Immunotherapy of Cancer Using Ad Vectors Expressing Cytokine Genes

Effective anti-tumor immune responses may be dependent on the induction of type-1 cell mediated immunity. The production of IL-10 by tumor cells results in the repression of type-1 immune responses, thus delivery of cytokines, or other stimuli, that can initiate
type-1 responses, or counteract the effects of IL-10 may be effective anti-tumor approaches. Indeed, the delivery of IL-12, which can promote the expression of IFN-γ, and induce type-1 T-cell differentiation, has been shown to be extremely efficacious in inducing anti-tumor immune responses \textit{in vivo} (Brunda \textit{et al.}, 1993a,b; Nastala \textit{et al.}, 1994; Zitvogel \textit{et al.}, 1994; Meko \textit{et al.}, 1995; Zou \textit{et al.}, 1995, Bramson \textit{et al.}, 1996b; Tannenbaum \textit{et al.}, 1996). The production of IFN-γ will also result in the suppression of the type-2 responses which are induced by IL-10 (reviewed by Mossman & Sad, 1996). In this manner, the immunosuppression induced by the tumor cells may be overcome. Expression of IFN-γ will also induce the expression of the non-ELR CXC chemokines such as IP-10 and MIG (Kaplan \textit{et al.}, 1987; Farber, 1993), both of which have been shown to induce angiostatic environments (Angiolillo \textit{et al.}, 1995; Luster \textit{et al.}, 1995; Strieter \textit{et al.}, 1995). The over-expression of angiostatic molecules may counteract the effects of the angiogenic molecules expressed by the tumor cells, and remove the growth advantage provided by neovascularization.

Another downstream effect of immunosuppression and induction of T-cell anergy is the failure of T-cells to proliferate and secrete cytokines in response to antigen-recognition. One of the cytokines which is expressed by activated T-cells is IL-2, which can act as an autocrine and paracrine T-cell growth factor. We hypothesized that the immunosuppression induced by the PyMidT tumor cells might be overcome by providing the downstream effector molecules of T-cell activation. We attempted to "kick-start" the
anergized T-cells by delivering strong proliferative signals, such as IL-2 or IL-4. We hoped that delivery of large amounts of IL-2 or IL-4 would bypass the requirement for costimulation, if costimulatory factors were limiting. We found that injection of Ad vectors expressing IL-2 or IL-4 into established tumors resulted in complete tumor regression in a significant proportion of treated animals and, following tumor regression, these animals were protected from a subsequent tumor challenge (Chapters 4 & 5). For IL-2, tumor regression was associated with the presence of antigen-specific CTLs (Figure 4-9), which secreted elevated levels of IFN-γ upon antigen stimulation (Chapter 4), suggesting that a type-1 T-cell response was induced. One outcome of costimulation of T-cells by binding of B7 molecules, is the expression of high affinity IL-2 receptors, and expression of IL-2 by T-cells (Guinan et al., 1994). When excess IL-2 is delivered it is possible that T-cells can become activated through the binding of IL-2 to the low affinity receptor, which is normally expressed on T-cells (Abbas et al., 1991), thus stimulating activation and proliferation in the absence of costimulation. Thus delivery of IL-2 should be effective for tumor cells which lack the expression of appropriate costimulatory molecules, such as B7-1 or B7-2.

Unfortunately, the doses of AdCAIL-2 or AdCAIL-4 that were efficacious in mediating tumor regression, were also associated with life-threatening toxicities. The toxicity that was observed following the in vivo administration of AdCAIL-2 was also noted by others. Toloza et al. (1996) observed that IL-2 treated animals had lesions in the
liver similar to those observed in our experiments. Following injection of $6 \times 10^9$ pfu of an Ad vector expressing murine IL-2, 50% of the animals succumbed to toxicity, while control vector treated animals remained healthy even following delivery of $1.4 \times 10^{11}$ pfu. These results indicate that the toxicity is induced by expression of high levels of IL-2, and not due to anti-vector immunity. By intra-tumoral administration of Ad vectors it was initially hoped that the virus would largely remain locally at the site of injection. However, it is now clear that there is significant dissemination of the inoculum to surrounding organs, particularly the liver. Toloza et al. (1996) estimated that approximately 34% of the administered vector transduced other organs and tissues, indicating that measures must be taken to avoid systemic distribution following Ad vector injection.

One approach which may limit dissemination of the vector, involves the pre-immunization of animals with wild-type Ad, and the subsequent induction of a protective neutralizing anti-viral antibody response (Bramson et al., 1997). This approach resulted in a 100-fold decrease in infection of the liver without severely impairing vector infection and transgene expression at the tumor site (Bramson et al., 1997). Another way to avoid the toxicity associated with Ad-cytokine vector treatment would be to use lower doses of IL-2-vector in combination with other cytokine expressing vectors, such as AdCAIL-4 or AdmIL-12. Intra-tumoral injection with a combination of AdCAIL-2 and AdCAIL-4 was more efficacious in mediating tumor regression than AdCAIL-2 alone, and fewer cases of
life-threatening toxicity were noted (Chapter 6). The increased efficacy noted in these experiments may, at first, seem counter-intuitive, since IL-4 is known to promote Th2-type T-cells, which would result in decreased cell-mediated immunity (Mossman & Sad, 1996). However, IL-4 will stimulate B-cells, which can act as antigen-presenting cells to activate T-cells. IL-4 can also induce the expression of B7 on antigen-presenting cells, which would result in effective costimulation of T-cells and thus synergize with IL-2 to promote T-cell activation.

The combination of IL-2 and IL-12 was also tested for its ability to mediate tumor regression in this model. It was believed that this combination would not only result in reduced toxicity, but also increased efficacy since IL-12 can induce Th1-type T-cell differentiation which, together with IL-2, should lead to induction of potent cell-mediated immune responses. The results obtained in these studies demonstrated that, following injection of Ad vectors expressing IL-2 and IL-12, a more potent anti-tumor response was observed than that following treatment with either cytokine-vector alone. Furthermore, the immune response generated following the IL-2/IL-12 combination treatment could act distally and mediate regression of untreated tumors. Tumor regression was associated with the activation of tumor-specific CTL (Figures 4-9, 6-2 and 6-3) and, following antigen stimulation, the lymphocytes isolated from animals which had undergone tumor regression expressed increased quantities of IFN-γ compared to lymphocytes obtained from control treated or tumor-bearing animals. This data supports the hypothesis that delivery of IL-2,
IL-12, or the combination of both, results in the induction of type-1 cell mediated immune responses that are efficacious in inducing tumor regression. The induction of a strong type-1 immune response resulted in the ability to regress both injected and distal untreated tumors in 63% of animals following intra-tumoral injection with a combination of AdIL-12 and AdIL-2 vectors (Table 6-3). In 2 of 11 animals, tumors recurred after apparent regression following treatment with AdIL-12 alone or in combination with AdIL-2. Since IL-12 induces the expression of IFN-γ, which in turn results in increased expression of angiostatic molecules, it is possible that these tumors regressed as a result, at least in part, of angiostasis. Due to the transient nature of Ad vector transgene expression, this angiostatic environment was lost over time, allowing the remaining tumor cells to grow and establish a tumor at the primary site. Another possibility is that the resulting recurrence of tumors was the result of an antigen-variant subpopulation of tumor cells which may have become resistant to CTL-mediated killing. The latter explanation is unlikely, as recurrent tumors were still sensitive to lysis by PyMidT antigen-specific CTLs (J. Bramson, personal communication).

The increased efficacy of IL-2 in combination with IL-12 may be the result of a number of events. It has been shown that the combination of IL-2 and IL-12 acts synergistically to induce the expression of IFN-γ (Chan et al., 1991, 1992), and thus the levels of IFN-γ may be higher than when IL-2 or IL-12 were used alone. Increased IFN-γ expression, in addition to creating an angiostatic environment, can also lead to an increase
in MHC class I and class II expression on tumor cells (Gerrard et al., 1988), which would render the tumor cells more susceptible to T-cell mediated killing. IL-12 is also required for efficient generation of LAK cells by IL-2, since the presence of neutralizing antibodies to IL-12 inhibits the induction of LAK activity (Gately et al., 1992). Similarly, CTL induction by IL-12 is dependent on the presence of IL-2, and neutralizing antibodies to IL-2 can reduce the IL-12-mediated induction of CTL by 90% (Gately et al., 1992). Furthermore, IL-12 can upregulate the expression of the IL-2 receptor on the surface of NK (Naume et al., 1992; Robertson et al., 1992) and T-cells (Wu et al., 1993). Therefore, the combination of these two cytokines may result in the generation of more potent LAK, CTL and NK cytotoxic activity compared to the use of either one alone.

The ability of Ad vectors delivered intra-tumorally to infect areas other than the tumor, thus delivering doses of cytokines that may be potentially toxic to these tissues, indicates that approaches to reduce toxicity must be taken. Administration of lower doses of cytokines in combination were shown to increase the efficacy of anti-tumor activity and induce less toxicity than using the higher doses of Ad vectors expressing IL-2 or IL-4 alone. Other approaches might include the use of lower doses of cytokines in combination with other mechanisms of inducing anti-tumor activity, such as overexpression of B7 costimulatory molecules. The use of an Ad vector expressing B7-1 and IL-12 in combination resulted in tumor regression in 95% of treated animals at a dose of $1 \times 10^8$ pfu, while intra-tumoral injection of the same dose of vector expressing either molecule alone
resulted in only tumor growth delays (Puetzer et al., 1997), thus indicating the potential of combination treatments. Other approaches which may be successful when used in combination with therapeutic cytokines, are the overexpression of cell cycle arrest or apoptosis-inducing molecules, such as p53 or bax. When used alone, Ad-p53 transduction has been shown to result in reduced proliferative capacity of tumor cells in vitro, and modulation of tumorigenicity in vivo (Fujiwara et al., 1994; Lui et al., 1994; Yang C. et al., 1995). Treatments which combine alternative strategies for modulating tumor growth may prove to be most effective.

E. Conclusions

In conclusion, I have identified factors that may affect transgene expression in Ad vectors, and have outlined some of the considerations which must be taken into account when designing Ad vectors for use in gene therapy. Furthermore, the data presented in Chapters 4 to 6, indicate that Ad vectors expressing cytokines can be efficacious in mediating tumor regression in vivo; however, new approaches to reduce toxicity and increase efficacy are still required. The suggestions outlined in this thesis are currently under investigation and will hopefully lead to the development of more potent therapeutic approaches to cancer which will limit the spread of metastatic disease.
CHAPTER 8. REFERENCES


APPENDIX I. PLASMID CONSTRUCTION

This appendix references all plasmids which have been constructed by me during my PhD training. It should be noted that descriptions of orientation of expression cassettes within plasmids refer to the orientation with respect to the adenoviral genomic sequences flanking these inserts. Some of the "shuttle" plasmids used most frequently are diagrammed in Figure 3-1.

pβACTsp13 - insertion of a double stranded oligonucleotide encoding a multiple cloning site (made by hybridization of oligos AB1531 and AB1532) into a unique SalI site in plasmid pβACTsp1 (Bett, 1995). The oligonucleotide is:

\[ \text{AB1531 5'} TCGACAAGCTTGAATTCGATATCTCTAGACTCGAGGGATCCC 3'} \]
\[ \text{AB1532 3'} GTTCGAACCTTAAGCTATAGCATCTGAGCTCCATGGGAGCT 5'} \]

The order of available restriction enzyme sites in the multiple cloning site is: BamHI, XbaI, XhoI, EcoRV, EcoRI, HindIII and SalI.

pβACTsp14 - created in the same manner as pβACTsp13 except the oligo is inserted in the opposite orientation. The order of the available multiple cloning sites is: SalI, HindIII, EcoRI, EcoRV, XhoI, XbaI, and BamHI.
pSV2XClaI - insertion of a double stranded oligonucleotide encoding a ClaI site and XbaI compatible ends (oligo AB1836 hybridized to itself) into XbaI sites of pSV2X14 (constructed by P. Brinkley). The oligonucleotide is:

\[
\begin{align*}
AB1836 & \quad 5' \quad \text{CTAGCATCGATG} \quad 3' \\
& \quad 3' \quad \text{GTAGCATCGATC} \quad 5'
\end{align*}
\]

ClaI

pSV2PA1 - insertion of a double stranded oligonucleotide containing a BamHI and a SalI site with XhoI compatible ends (made by hybridization of oligos AB1834 and AB1835) into the XhoI site flanking the SV40 polyadenylation signals in pSV2XClal. Insertion of the oligonucleotide destroys the XhoI site. The oligonucleotide is:

\[
\begin{align*}
AB1834 & \quad 5' \quad \text{TCGAAGTCGACGGATCCA} \quad 3' \\
AB1835 & \quad 3' \quad \text{TCAGCTGCCTAGGTAGCT} \quad 5'
\end{align*}
\]

SalI    BamHI

The orientation of the oligonucleotide in this plasmid is SalI, BamHI.

pSV2PA2 - same as pSV2PA1 except the oligonucleotide is in the opposite orientation ie. BamHI, SalI.

pCA1 - insertion of the ClaI/SalI fragment from pSV2PA1 containing the SV40 An fragment into the ClaI/SalI site of pβACTsp14. An Ad shuttle plasmid with the human β-actin promoter (β-act), a multiple cloning site and SV40 polyadenylation (An) signal contained within an E1 deletion, in the leftwards orientation.
pCA2 - insertion of the ClaI/BamHI fragment from pSV2PA2 containing the SV40 An sequences into the ClaI/BamHI sites of pβACTsp13. Same as pCA1 except the multiple cloning site is in the opposite orientation.

pCA3 - insertion of the ClaI/BamHI fragment from pSV2PA2 (containing the SV40 An) into the ClaI/BamHI sites in pHCMVsp13 (constructed by A. Bett). An Ad shuttle plasmid with the human cytomegalovirus immediate early gene (HCMV IE) promoter (from -299 to +72), a multiple cloning site and the SV40 An signals, contained within an E1 deletion in the leftwards orientation.

pCA4 - insertion of the ClaI/SalI fragment from pSV2PA1 (containing the SV40 An sequences) into the ClaI/SalI sites in pHCMVsp14 (constructed by A. Bett). Same as pCA3 except the multiple cloning site is in the opposite orientation.

pCA5 - insertion of a BamHI fragment containing the HisD gene from plasmid pHD1A (gift of R. Mulligan) into the unique BamHI site of pCA4. This plasmid directs expression of the gene for histidinol resistance under control of the HCMV promoter and SV40 An sequences in the leftwards orientation.

pCA6 - insertion of a BamHI fragment containing the HisD gene from plasmid pHD1A (gift of R. Mulligan) into the unique BamHI site of pCA1. This plasmid directs expression of the gene for histidinol resistance under control of the β-act promoter and SV40 An sequences in the leftwards orientation.
pCA1Sfu - insertion of a double stranded oligonucleotide which contains an SfuI site and BglIII overhangs (oligo AB2144 hybridized to itself) into the BglIII site of pCA1. The oligonucleotide is:

\[
\begin{align*}
5' & \text{ GATCTTCGAA } 3' \\
3' & \text{ AAGCTTCTAG } 5'
\end{align*}
\]

SfuI

pCA2Sfu - insertion of the double stranded oligonucleotide (oligo AB2144 as used to create pCA1Sfu) into the BglIII site of pCA2.

pCA7 - ClaI and SfuI digestion of pCA1Sfu and subsequent religation. The resulting plasmid is identical to pCA1 except the \( \beta \)-act promoter, multiple cloning site and SV40 An are directing transcription rightwards.

pCA8 - insertion of a BamHI fragment containing the HisD gene from plasmid pHD1A into the unique BamHI site of pCA7. This plasmid now expresses the gene for histidinol resistance under control of the \( \beta \)-act promoter and SV40 An signals in the rightwards orientation.

pCA9 - digestion of pCA2Sfu with ClaI and SfuI and subsequent religation. This plasmid is identical to pCA7 except the multiple cloning site is in the opposite orientation.

pCA10 - insertion of a BamHI fragment from p548FL (constructed by M. Hitt) which contains the gene for firefly luciferase (luc) into the unique BamHI site of pCA1. Expression of the luc gene is controlled by \( \beta \)-act and SV40 An signals in the leftwards orientation.
pCA11 - insertion of an XbaI/XhoI fragment containing the lacZ gene from pAB16lacZ (constructed by A. Bett) into the XbaI/XhoI sites of pCA2. Expresses lacZ under control of the β-act in the leftwards orientation.
	pCA12 - insertion of an XbaI/XhoI fragment containing the lacZ gene from pAB16lacZ (constructed by A. Bett) into the XbaI/XhoI sites of pCA3. Expresses lacZ under control of HCMV in the leftwards orientation.
	pCA3Δpr - removal of the HCMV promoter in pCA3 following a BgIII digest.
	pCA4Δpr - removal of the HCMV promoter in pCA4 following a BgIII digest.
	pCA3HCMV - insertion of a BgIII/BamHI fragment from pHCMVsp1 (constructed by A. Bett) which contains the HCMV promoter into the BgIII site of pCA3Δpr. Destroys the BgIII site at the 3' end of HCMV leaving a unique BgIII site at the 5' end.
	pCA4HCMV - insertion of a BgIII/BamHI fragment from pHCMVsp1 (constructed by A. Bett) which contains the HCMV promoter into the BgIII site of pCA4Δpr. Destroys the BgIII site at the 3' end of HCMV leaving a unique BgIII site at the 5' end.
	pCA3HSfu - insertion of a double stranded oligonucleotide (oligo AB2144 hybridized to itself as used to create pCA1Sfu) into the unique BgIII site of pCA3HCMV. Insertion of this oligo maintains BgIII sites flanking the SfuI site.
	pCA4HSfu - insertion of a double stranded oligonucleotide (oligo AB2144 hybridized to itself as used to create pCA1Sfu) into the unique BgIII site of pCA4HCMV (identical to pCA3HSfu except multiple cloning site is in opposite orientation).
pCA13 - digestion of pCA3HSfu with ClaI and SfuI and subsequent religation. Identical to pCA3 except the HCMV promoter, multiple cloning site and SV40 polyadenylation signals are in the rightwards orientation. This cloning step resulted in the presence of BglII sites flanking the expression cassette (ie one 5' of the HCMV promoter and one 3' of the SV40 An site).

pCA14 - digestion of pCA4HSfu with ClaI and SfuI and subsequent religation. This plasmid is identical to pCA13 except the multiple cloning site is in the opposite orientation.

pCA15 - insertion of a BamHI fragment from p548FL (constructed by M. Hitt) that contains the firefly luc gene into the unique BamHI site of pCA4HCMV. This plasmid expresses luc under control of HCMV and SV40 An signals in the leftwards orientation.

pCA16 - insertion of a BamHI fragment from p548FL (constructed by M. Hitt) that contains the firefly luc gene into the unique BamHI site of pCA1Sfu. This plasmid now has luc under control of β-act and SV40 An signals and the expression cassette is flanked by ClaI and SfuI. Both of these have compatible ends which are also compatible with BstBI and therefore will allow direct insertion into the BstBI site of the BHG plasmid series (constructed by A. Bett).

pABS.3S - insertion of a double stranded oligonucleotide containing an SfuI and a ScaI site into the pABS.3 shuttle plasmid (constructed by A. Bett). The oligonucleotide is:

\[
\begin{align*}
\text{AB2355} & \quad 5' \quad \text{AATTCTTCGAAAGTACTGAGCT} \quad 3' \\
\text{AB2356} & \quad 3' \quad \text{GAAGCTTTCATGAC} \quad 5' \\
\text{EcoRI} & \quad \text{SfuI} \quad \text{ScaI} \quad \text{SacI}
\end{align*}
\]

compatible end    compatible end
Digestion of pABS.3 with EcoRI and SacI followed by insertion of the hybridized oligonucleotide results in the recreation of the EcoRI and SacI sites with the addition of the SfiI and ScaI sites between them.

pCA17 - insertion of an XbaI/SalI fragment from pAB16lacZ (constructed by A. Bett) that contains the lacZ gene into the XbaI/SalI sites of pCA14. The resulting plasmid expresses lacZ under control of HCMV and SV40 An signals in the rightwards orientation.

pCA18 - insertion of a BamHI fragment from p548FL (constructed by M. Hitt) that contains the firefly luc gene into the unique BamHI of pCA14. The resulting plasmid expresses luc under control of the HCMV promoter and SV40 An signals in the rightwards orientation.

pCA19 - insertion of a BamHI/SalI fragment from pABlacZ21 (constructed by A. Bett) that contains the lacZ gene into the BamHI/SalI sites of pCA13. The resulting plasmid expresses lacZ under control of HCMV and SV40 An signals in the rightwards orientation.

pCAIL-2P - insertion of an XhoI/EcoRI fragment from plasmid pBSIL-2.A2 (gift of R. Ralston, Chiron Corp.) that contains cDNA for human interleukin-2 (IL-2) into the XhoI/EcoRI sites of pCA14. This plasmid expresses IL-2 under control of HCMV and SV40 An signals in the rightwards orientation.

pCAIL-2AP - insertion of an XhoI/EcoRI fragment from plasmid pBSIL-2.A2 (gift of R. Ralston, Chiron Corp.) that contains cDNA IL-2 into the XhoI/EcoRI sites of pCA4. This plasmid expresses IL-2 under control of HCMV and SV40 An signals in the leftwards orientation.
pCAIL-4P - insertion of an EcoRI/HindIII fragment from plasmid p309.9 (gift of R. Tepper) that contains the cDNA for murine interleukin-4 (IL-4) into the EcoRI/HindIII sites in pCA13. Expresses IL-4 under control of HCMV and SV40 An signals in the rightwards orientation.

pCAIL-4AP - insertion of an EcoRI/HindIII fragment from plasmid p309.9 (gift of R. Tepper) that contains the cDNA for mIL-4 into the EcoRI/HindIII sites in pCA3. Expresses IL-4 under control of HCMV and SV40 An signals in the leftwards orientation.

pCAαIL-2 - insertion of an XhoI/EcoRI fragment from plasmid pBSIL-2.A2 (gift of R. Ralston, Chiron Corp.) that contains cDNA IL-2 into the XhoI/EcoRI sites of pCA13. Expresses antisense IL-2 under control of HCMV and SV40 An signals in the rightwards orientation.

pCA20M - BglIII digest of pCA12 to remove the HCMV promoter and the subsequent insertion of the MMTV promoter via a BamHI fragment from plasmid p206 (gift of W. Muller). Expresses lacZ under control of MMTV and SV40 An signals in the leftwards orientation.

pCA21M - insertion of a BamHI fragment from plasmid p206 (gift of W. Muller) into the BamHI site of pCA4Δpr. This plasmid now possesses an expression cassette with the MMTV and SV40 An signals controlling transgene expression in the leftwards orientation.

pCA22M - insertion of a SalI/HindIII fragment from plasmid p206 (gift of W. Muller) into the SalI/HindIII sites of pCA3Δpr. This plasmid now possesses an expression cassette with the MMTV including the H-ras enhancer sequences and SV40 An signals controlling
transgene expression in the leftwards orientation, but the multiple cloning site is in the opposite orientation to that found in pCA21M.

pCA23M - insertion of a SalI/HindIII fragment from plasmid p206 (gift of W. Muller) into the XhoI/HindIII sites of plasmid pCA14dpr (constructed by M. Hitt). This plasmid now possesses an expression cassette with the MMTV and SV40 An signals controlling transgene expression in the rightwards orientation. The multiple cloning site has now been deleted except for the SalI site.

pCA24M - insertion of a double stranded oligonucleotide (hybridization of AB3178 and AB3179) which contains SalI compatible ends into the unique SalI site of pCA23M. The oligonucleotides are:

\[
\begin{align*}
\text{AB3178} & \quad 5' \quad TCGACTCTAGATTGAACTAAGCTCCTCGAGC \quad 3' \\
\text{AB3179} & \quad 3' \quad GAGATCTAAGCTTTCAAATGGTAAGGCTGAGCTCGAGCT \quad 5'
\end{align*}
\]

SalI  XbaI  SfuI  HpaI  EcoRI  XhoI  SalI
compatible end                     compatible end

This results in a plasmid which contains an expression cassette including the MMTV promoter, a multiple cloning site and an SV40 An signal in the rightwards orientation. The order of restriction sites in the multiple cloning site is: HindIII, XhoI, EcoRI, HpaI, SfuI, XbaI, and SalI.

pCA25M - identical to pCA24M except the multiple cloning site is in the opposite orientation i.e: HindIII, SalI, XbaI, SfuI, HpaI, EcoRI, and XhoI.
pCA26ML - insertion of an XbaI/XhoI fragment from plasmid pAB16lacZ (constructed by A. Bett) into the XbaI/XhoI site of plasmid pCA25M. Expresses lacZ under control of the MMTV promoter and SV40 An signals in the rightwards orientation.

pCA27M-2 - insertion of an XhoI/EcoRI fragment from plasmid pBSIL-2.A2 (gift of R. Ralston, Chiron Corp.) into the XhoI/EcoRI sites in pCA24M. Expresses IL-2 under control of the MMTV promoter and SV40 An signals in the rightwards orientation.

pCA28ML - insertion of an XbaI/SalI fragment from pAB16lacZ (constructed by A. Bett) into the XbaI/SalI sites in pCA21M. Expresses lacZ under control of the MMTV promoter (without the H-ras enhancer) and SV40 An signals in the leftwards orientation.

pCA29T2 - insertion of an XhoI/EcoRI fragment from plasmid pBSIL-2.A2 gift of R. Ralston, Chiron Corp.) into the XhoI/EcoRI sites in pMH1 (constructed by M. Hitt). Expresses IL-2 under control of the tyrosinase promoter and SV40 An signals in the rightwards orientation.

pCA30M4 - insertion of a HindIII/EcoRI fragment from p309.9 (gift of R. Tepper) that contains the cDNA for IL-4 into the HindIII/EcoRI sites of pCA24M. Expresses the IL-4 gene under control of MMTV and SV40 An signals in the rightwards orientation.

pCA31ML - insertion of EcoRI/XhoI fragment from pCA11 that contains the lacZ gene into the EcoRI/XhoI sites in pCA22M. Expresses lacZ under control of the MMTV promoter and SV40 An in the leftwards orientation.
pCA32T4 - insertion of a BamHI/XhoI fragment from p309.9 (gift of R. Tepper) that contains the cDNA for murine IL-4 into the BamHI/SalI sites of pMH3 (constructed by M. Hitt). Expresses IL-4 under control of the tyrosinase promoter and SV40 An signals in the rightwards orientation.

pCA33ACMV - created by NotI digestion of pACCMV.PLPASR(+) (gift of J. Gauldie) followed by a klenow reaction to fill in the overhang, and subsequent digestion by EcoRI. This fragment was inserted into the EcoRV/EcoRI sites of pMH3dpr (constructed by M. Hitt). The resulting plasmid contains the HCMV promoter (~450bp), a multiple cloning site and the SV40 An signals in the rightwards orientation.

pCA34AL - insertion of an XbaI/SalI fragment from pAB16lacZ (constructed by A. Bett) into the NheI/SalI sites of pCA33ACMV. Contains the lacZ gene under control of a larger HCMV promoter than the other lacZ constructs used before this and the SV40 An signals in the rightwards orientation.

pCA35 - insertion of an XbaI/SalI fragment from pAB16lacZ (constructed by A. Bett) into the NheI/SalI sites in pMH5 (constructed by M. Hitt). Contains the lacZ gene under control of the 1.5 kb MCMV promoter and SV40 An signals in the rightwards orientation.

pCA36 - insertion of an XbaI/SalI fragment from pAB16lacZ (constructed by A. Bett) into the NheI/SalI sites in pMH4 (constructed by M. Hitt). Contains the lacZ gene under control of the 0.5 kb MCMV promoter and SV40 An signals in the rightwards orientation.

pCAMCIL-2 - insertion of a BamHI/SalI fragment from pCAIL-2P containing the cDNA for hIL-2 into the BamHI/SalI sites of pMH5 (constructed by M. Hitt). Contains the IL-2
cDNA under control of the 1.5 kb MCMV promoter and SV40 An signals in the rightwards orientation.

pCAMcil-4 - insertion of a BamHI/XhoI fragment from p309.9 (gift of R. Tepper) that contains the cDNA for murine IL-4 into the BamHI/Sall sites of pMH5 (constructed by M. Hitt). Contains the IL-4 cDNA under control of the 1.5 kb MCMV promoter and SV40 An signals in the rightwards orientation.

pCA37 - deletion of a BamHI/BglIII fragment from pCA17 containing the HCMV promoter and its replacement by a BamHI fragment from pMA1 (constructed by M. Anton) that contains an ~760 bp HCMV promoter. Contains lacZ under control of the larger HCMV promoter and SV40 An signals in the rightwards orientation. Note: subsequent to construction it was realized that the ATG of the lacZ gene was destroyed following BamHI digestion, therefore this plasmid does not express β-galactosidase.

pCA38 - insertion of a double stranded oligonucleotide into the BamHI site of pCA37 which subsequently reintroduces the lost ATG and an NheI site. The oligonucleotides used are:

restores ATG

\[
\begin{align*}
\text{AB9152} & \quad 5' \quad \text{GATCGCTAGCATG} \quad 3' \\
\text{AB9153} & \quad 3' \quad \text{CGATCGTACCTAG} \quad 5'
\end{align*}
\]

destroys NheI recreates BamHI BamHI

Encodes lacZ under control of the larger HCMV promoter (~760 bp) and SV40 An signals in the rightwards orientation.
pCAMC4IL-2 - insertion of a BamHI/SalI fragment from pCAIL-2P containing the cDNA for hIL-2 into the BamHI/SalI sites of pMH5 (constructed by M. Hitt). Contains the IL-2 cDNA under control of the 0.5 kb MCMV promoter and SV40 An signals in the rightwards orientation.

pCARP1.1 - insertion of a 5.6 kb BamHI fragment of lambda DNA from plasmid pRP1003 (constructed by R. Parks) into unique BamHI site of pUC19.

pCARP2.1 - insertion of a 5.8 kb BsaAI fragment of lambda DNA from plasmid pRP1003 (constructed by R. Parks) into unique SmaI site of pUC19.

pCARP1.abs1 - insertion of a KpnI/SalI fragment from pCARP1.1 that contains the lambda DNA insert into the KpnI/SalI sites of pABS.6 (constructed by A. Bett).

pCARP2.abs1 - insertion of a KpnI/SalI fragment from pCARP2.1 that contains the lambda DNA insert into the KpnI/SalI sites of pABS.6 (constructed by A. Bett).

pBHGI0CARP1K.1 - insertion of a PacI fragment from pCARP1.abs1 which contains the 5.6 kb lambda insert into the unique PacI site of pBHGI0 (constructed by A. Bett), which results in insertion of the lambda sequences along with a kanamycin resistance gene in the E3 deleted region. The lambda sequences in this plasmid are in the parallel orientation to the E3 transcription unit ie rightwards orientation. (Note: this plasmid was created with the assistance of Uma Sankar.)

pBHGI0CARP1K.2 - insertion of a PacI fragment from pCARP1.abs1 which contains the 5.6 kb lambda insert into the unique PacI site of pBHGI0 (constructed by A. Bett), which results in insertion of the lambda sequences along with a kanamycin resistance gene in the E3
deleted region. The lambda sequences in this plasmid are in the anti-parallel orientation to the E3 transcription unit ie leftwards orientation. (Note: this plasmid was created with the assistance of Uma Sankar.)

**pBHG10CARP2K.1** - insertion of a PacI fragment from pCARP2.abs1 which contains the 5.8 kb lambda insert into the unique PacI site of pBHG10 (constructed by A. Bett), which results in insertion of the lambda sequences along with a kanamycin resistance gene in the E3 deleted region. The lambda sequences in this plasmid are in the parallel orientation to the E3 transcription unit ie rightwards orientation. (Note: this plasmid was created with the assistance of Uma Sankar.)

**pBHG10CARP2K.2** - insertion of a PacI fragment from pCARP2.abs1 which contains the 5.8 kb lambda insert into the unique PacI site of pBHG10 (constructed by A. Bett), which results in insertion of the lambda sequences along with a kanamycin resistance gene in the E3 deleted region. The lambda sequences in this plasmid are in the anti-parallel orientation to the E3 transcription unit ie leftwards orientation. (Note: this plasmid was created with the assistance of Uma Sankar.)

**pBHG10CARP1.1** - removal of the kanamycin resistance gene from pBHG10CARP1K.1 by digestion with SwaI and subsequent religation. This plasmid now contains a 5.6 kb lambda insert in the rightwards orientation of the E3 deleted region of pBHG10 and can be used as a "stuffer" plasmid to rescue viral vectors with smaller inserts. (Note: this plasmid was created with the assistance of Uma Sankar.)
pBHGI0CARP1.2 - removal of the kanamycin resistance gene from pBHGI0CARP1K.2 by digestion with SwaI and subsequent religation. This plasmid now contains a 5.6 kb lambda insert in the leftwards orientation of the E3 deleted region of pBHGI0 and can be used as a "stuffer" plasmid to rescue viral vectors with smaller inserts. (Note: this plasmid was created with the assistance of Uma Sankar.)

pBHGI0CARP2.1 - removal of the kanamycin resistance gene from pBHGI0CARP2K.1 by digestion with SwaI and subsequent religation. This plasmid now contains a 5.8 kb lambda insert in the rightwards orientation of the E3 deleted region of pBHGI0 and can be used as a "stuffer" plasmid to rescue viral vectors with smaller inserts. (Note: this plasmid was created with the assistance of Uma Sankar.)

pBHGI0CARP2.2 - removal of the kanamycin resistance gene from pBHGI0CARP2K.2 by digestion with SwaI and subsequent religation. This plasmid now contains a 5.8 kb lambda insert in the leftwards orientation of the E3 deleted region of pBHGI0 and can be used as a "stuffer" plasmid to rescue viral vectors with smaller inserts. (Note: this plasmid was created with the assistance of Uma Sankar.)
APPENDIX II. RECOMBINANT ADENOVIRAL VECTORS

This appendix lists the first generation recombinant adenoviral vectors that were constructed and rescued by me during the course of this PhD thesis work.

Table II-1. Recombinant Adenoviral Vectors.

<table>
<thead>
<tr>
<th>Virus Name</th>
<th>Description</th>
<th>Orientation</th>
<th>Rescue Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdCA5-20</td>
<td>HCMV-HisD</td>
<td>L</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA6-31</td>
<td>β-actin-HisD</td>
<td>L</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA6-32</td>
<td>β-actin-HisD</td>
<td>L</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA8-7</td>
<td>HCMV-HisD</td>
<td>R</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA11-2</td>
<td>β-actin-lacZ</td>
<td>L</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA12-13 or AdCA12lacZ</td>
<td>HCMV-lacZ</td>
<td>L</td>
<td>pJM17</td>
</tr>
<tr>
<td>AdCA15-21</td>
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<td>L</td>
<td>pJM17</td>
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</tr>
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<td>L</td>
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<td>AdCA17-1 or AdCA17lacZ</td>
<td>HCMV-lacZ</td>
<td>R</td>
<td>pJM17</td>
</tr>
<tr>
<td>Virus Name</td>
<td>Description</td>
<td>Orientation</td>
<td>Rescue Plasmid</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------</td>
<td>-------------</td>
<td>---------------</td>
</tr>
<tr>
<td>AdCA18-3 or AdCA18luc</td>
<td>HCMV-luciferase</td>
<td>R</td>
<td>pJM17</td>
</tr>
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<td>pJM17</td>
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<td>AdCAIL-2PB1 or AdCAIL-2</td>
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<td>R</td>
<td>pBHGi0</td>
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<tr>
<td>AdCAIL-4PB1 or AdCAIL-4</td>
<td>HCMV-mIL-4</td>
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<td>pBHGi0</td>
</tr>
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<td>AdCA26MLB2</td>
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</tr>
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<td>R</td>
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<td>MMTV-mIL-4</td>
<td>R</td>
<td>pBHGi0</td>
</tr>
<tr>
<td>AdCA31MLB1</td>
<td>MMTV-lacZ</td>
<td>L</td>
<td>pBHGi0</td>
</tr>
<tr>
<td>Virus Name</td>
<td>Description</td>
<td>Orientation</td>
<td>Rescue Plasmid</td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------</td>
<td>-------------</td>
<td>----------------</td>
</tr>
<tr>
<td>AdCA34-1</td>
<td>0.45kb HCMV-lacZ</td>
<td>R</td>
<td>pBHGI0</td>
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<tr>
<td>AdCA35-1 or AdCA35lacZ</td>
<td>1.5kb MCMV-lacZ</td>
<td>R</td>
<td>pBHGI0</td>
</tr>
<tr>
<td>AdCA36-1 or AdCA36lacZ</td>
<td>0.5kb MCMV-lacZ</td>
<td>R</td>
<td>pBHGI0</td>
</tr>
<tr>
<td>AdCA37-2 (note: missing ATG)</td>
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<tr>
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<td>R</td>
<td>pBHGI0</td>
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<tr>
<td>AdCAMCIL-4</td>
<td>1.5kb MCMV-mIL-4</td>
<td>R</td>
<td>pBHGI0</td>
</tr>
<tr>
<td>AdCAM4IL-2</td>
<td>0.5kb MCMV-hIL-2</td>
<td>R</td>
<td>pBHGI0</td>
</tr>
<tr>
<td>AdCAIL-2lam</td>
<td>HCMV-hIL-2</td>
<td>R</td>
<td>pBHGI0CARP1.2</td>
</tr>
<tr>
<td>AdCAM4IL-2lam</td>
<td>0.5kb MCMV-hIL-2</td>
<td>R</td>
<td>pBHGI0CARP1.2</td>
</tr>
<tr>
<td>AdCA38-1</td>
<td>0.8kb HCMV-lacZ</td>
<td>R</td>
<td>pBHGI0</td>
</tr>
</tbody>
</table>

*The foreign DNA insert in place of the adenovirus E1 sequences in the recombinant vector is indicated as: promoter element controlling transcription - foreign transgene. All transgenes were also under the regulation of the SV40 polyadenylation signal.*

*Orientation refers to the direction of transcription of the foreign gene sequences with respect to the direction of wild-type adenovirus transcription with R indicating rightwards (parallel to E1 transcription), and L indicating leftwards (anti-parallel to E1 transcription).*

*Unless otherwise indicated, the size of the HCMV promoter is 371bp.*
APPENDIX III. CONSTRUCTION AND CHARACTERIZATION OF "STUFFER" VIRUSES

A. Introduction

First generation Ad vectors lack the viral E1 sequences which are necessary for viral replication, therefore use of the 293 cell line, which expresses the E1 proteins in trans, is required for vector propagation. During propagation, these first generation Ad vectors have the potential to undergo homologous recombination with the adenovirus genomic sequences present in the 293 cell line, and can thus generate viral vectors which can replicate autonomously. The presence of replication-competent adenovirus (RCA) might provide helper functions that allow simultaneous vector replication concomitant with RCA replication, and as a result, there is the potential for increases in transgene expression. Experiments were performed that showed that slight increases in transgene expression could be detected when the contamination by RCA was at a ratio of vector:RCA greater than 1000:1 (Chapter 3). Additionally, for the purposes of gene therapy, government regulations require that the RCA contamination is less than 1 per patient dose in vector preparations that are to be used in clinical trials. For these reasons, measures to prevent the generation and propagation of RCA are desirable. Attempts have been made to reconstruct 293 cells, so that homologous recombination with the packaging cell lines is abrogated or minimized (discussed in Chapter 7); however, the cell lines
reported to date do not abrogate the ability to generate RCA, or they result in vector yields significantly lower than that which can be achieved following propagation in 293 cells.

In order to continue to use 293 cells for vector propagation and avoid contamination of first generation Ad vector preparations with RCA, we decided to test the hypothesis that using "stuffer" sequences in these vectors would eliminate the amplification of RCA. The adenovirus capsid has a cloning capacity of 105% of the wild-type genome size (Bett et al., 1993), and genomes larger than this cannot be packaged into infectious virions. Insertion of large amounts of inert DNA into the vector backbone would not prevent the generation of RCA, but would prevent the RCA from being packaged into infectious virions. We have constructed vectors which contain large amounts of lambda DNA inserted into the E3 region of the vector backbone, with the intent that the presence of the large lambda DNA insert would result in a genome that is too large to package into infectious capsids following homologous recombination and acquisition of E1 sequences.

Previously, an Ad vector expressing human interleukin-2 (IL-2), under control of the HCMV IE promoter and SV40 polyadenylation signals, had been constructed in an E1/E3 deleted vector backbone (see Chapter 4). Deletion of both the E1 and E3 sequences from the Ad vector backbone results in the ability to insert approximately 8 kb of foreign DNA and still maintain a packable vector genome. The vector expressing IL-2 (AdCAIL-2) contains only 1.2 kb of inserted foreign DNA and therefore is much smaller than the wild-type Ad 5 genome size (87% of wild-type Ad 5). Upon propagation of this vector,
the viral stocks were frequently found to be contaminated with RCA (19 of 20 stocks expanded from plaque isolates were found to be positive following tests for RCA by growth on A549 cells). It was therefore desirable to construct a vector which would not produce RCA following serial propagation. We determined that insertion of 5.6 kb of lambda DNA into the E3 region of this vector would result in the generation of a vector that was 103% of the wild-type genome and thus could still be packaged, however, upon reacquisition of the E1 sequences (which results in the loss of the 1.2 kb of DNA encoding the IL-2 and promoter sequences and the gain of 3.2 kb of E1 coding sequences), the genome size would be approximately 108% of the wild-type genome and thus could not be packaged into infectious virions.

B. Results

1. Construction of "Stuffer" Viruses Expressing the IL-2 Gene

A 5.6 kb BamHI fragment of lambda DNA (22346 bp to 27972 bp of the lambda genome) was inserted into the E3 deleted region in the rescue plasmid pBHG10 following a series of cloning steps (see Figure III-1A). The lambda stuffer was inserted into two different orientations in pBHG10: parallel (pBHG10CARP1.1) and anti-parallel (pBHG10CARP1.2) to E3 transcription. Following cotransfection of 293 cells, it was found that recombinant virus could only be rescued when pBHG10CARP1.2 was used. This plasmid contains the open reading frames of the lambda sequences in the leftwards
orientation with respect to the viral genome i.e. anti-parallel to E3 transcription. It is possible that in the parallel orientation to E3 transcription, the open reading frames in the lambda sequences somehow interfere with transcription of viral genes or viral DNA replication. The plasmid pBHG10CARP1.2 was used to rescue two viruses expressing IL-2 (Figure III-1B), one under control of the HCMV IE promoter (-299 to +72, AdCAIL-2lam), and one under control of the short MCMV IE promoter (-491 to +36, AdCAM4IL-2lam). These "stuffer" viruses were then compared to the IL-2 vectors lacking lambda sequences for their ability to replicate, their stability, and the degree of contamination by RCA.

2. Growth and Stability of "Stuffer" Viruses

The replication of the four IL-2 expressing viruses was compared by analysis of the "burst size". 293 cells were infected at an moi of 10 pfu/cell, and cells were harvested when complete CPE had occurred. Following freeze-thaw treatment, the titer of each sample was determined by plaque assay on 293 cells as described in Chapter 2. It was found that the viruses containing the lambda DNA "stuffer" sequences yielded 3-4 fold less virus (quantitated in pfu/ml) than those vectors lacking the "stuffer" sequences (titers of 2.3 \times 10^7 pfu/ml, 6.5 \times 10^6 pfu/ml, 2.7 \times 10^7 pfu/ml and 7 \times 10^6 pfu/ml for AdCAIL-2, AdCAIL-2lam, AdCAM4IL-2 and AdCAM4IL-2lam, respectively). This trend was noted for vectors containing either the HCMV IE or the MCMV IE promoter to drive IL-2 transgene expression, indicating that the lambda DNA is most likely responsible for the
Figure III-1: Construction of "Stuffer" Adenoviral Vectors.

(A) Plasmid Construction. A 5.6 kb BamHI fragment of lambda DNA (22346 bp to 27972 bp of the lambda genome) was inserted into the unique BamHI site of pUC19. Positive clones containing inserts of lambda DNA were identified using blue/white screening for β-galactosidase activity upon addition of X-gal. A clone containing the lambda DNA insert was identified and the plasmid was renamed pCARP1. The lambda DNA insert was removed by restriction enzyme digestion with KpnI and SalI, gel purified, and inserted into the unique KpnI and SalI sites in plasmid pABS.6 to generate pCARP1.abs1. The pABS.6 plasmid (constructed by A. Bett), possesses a kanamycin gene and a multiple cloning region flanked by PacI sites and thus allows for inserts to be "shuttled" into the unique PacI site of the pBHG10 rescue plasmid. The PacI fragment from pCARP1.abs1 was cloned into the PacI site of pBHG10 (which contains the gene for ampicillin resistance) and positive clones were identified by resistance to both ampicillin and kanamycin. This resulted in the plasmids pBHG10CARP1K.1 and pBHG10CARP1K.2 which possess the lambda insert in different orientations in an E3 deletion with respect to the adenoviral sequences in pBHG10. The kanamycin gene was then removed from these plasmids by restriction enzyme digestion with Swal (as its retention would result in an IL-2 vector that would also be too large to package) and the resulting plasmids, pBHG10CARP1.1 and pBHG10CARP1.2, were used to rescue an IL-2 expression cassette [contained in plasmid pCAIL-2P, (see Chapter 5 and Appendix I), or in pCAM4IL-2 (see Appendix I)] into a recombinant Ad vector.
A

KpnI/SalI digestion followed by ligation

pCARP1
8312 bp

Pacl digestion followed by ligation

pBHGG10CARP1K.2
41745 bp

Swal digestion

pBHGG10CARP1.2
40487 bp
(B) Recombinant Viral Vectors. Viral vectors were rescued by cotransfection and subsequent homologous recombination in 293 cells of the plasmid containing the IL-2 expression cassette (pCAM4IL-2) along with either pBHGI0 or pBHGI0CARP1.2. The resulting recombinant vectors express IL-2 (light grey box) under control of the short MCMV IE promoter (black arrow) and SV40 An sequences with either an E3 deleted vector backbone (AdCAMC4IL-2), or with a "stuffed" E3 region (AdCAMC4IL-2lam). The lambda sequences used as a stuffer are indicated by a dark grey arrow, and the direction of the arrow indicates the orientation of the lambda open reading frames with respect to the Ad genome backbone. The genome size of the vector AdCAMC4IL-2 is 87% of wild-type Ad 5, while the genome size of the vector AdCAMC4IL-2lam is 104.6% of wild-type Ad 5, and thus acquisition of the E1 sequences by the latter vector results in a genome which is 108% of wild-type Ad 5 and thus cannot be packaged into virions. Similar vectors were constructed using the HCMV IE promoter in place of the MCMV IE promoter (AdCAIL-2 and AdCAIL-2lam).
AdCAMC4IL-2
31334 bp

AdCAMC4IL-2lam
37038 bp
differences in virus yield. It is possible that the open reading frames in the lambda DNA may interfere with viral transcription or replication, or the decreased yields may simply be a result of an increased vector size.

Each of the four vectors were serially propagated to determine if RCA was generated during viral replication. Plaque isolates of each vector were used to infect 293 cell monolayers in 60 mm dishes, and when CPE was complete the supernatants were harvested and the viral DNA structure was examined by restriction enzyme digestion as described in Chapter 2. The supernatants from the first infection were then used to infect another dish of 293 cells, and the process was repeated for multiple serial passages, in order to determine if the viral DNA structure was stable. The AdCAM4IL-2lam virus was found to be stable for greater than 12 serial passages, without the loss of any lambda DNA sequences or acquisition of the E1 sequences. After six serial passages the AdCAM4IL-2lam virus was tested for the presence of contaminating RCA by growth on A549 cells. A549 cells are non-permissive for the growth of virus deleted for the E1 genes, and this method has been deemed one of the most sensitive methods for the detection of RCA and can detect 1 RCA in every $10^4$ virus particles (Lochmuller et al., 1994). No evidence of RCA contamination was found in the AdCAM4IL-2lam stocks, indicating that this vector can be serially propagated and remain RCA-free.

3. Examination of Transgene Expression in "Stuffer" Viruses

The viruses containing the stuffer sequences were found to be stable and did not
result in the generation of RCA, however it was unclear whether the presence of the lambda DNA would affect vector-directed transgene expression. Various murine and human cell types were infected at an moi of 10 pfu/cell, and culture supernatants were removed daily and the amount of IL-2 present quantitated by ELISA. There was less than a 2-fold difference in expression between the vectors in any of the cell lines tested, indicating that the presence of a lambda DNA insert as a stuffer sequence did not have a significant effect on transgene expression (Figure III-2). The experiments described herein indicate that using "stuffer" sequences encoded within the E3 deletion in first generation Ad vectors is an effective way of generating vectors that are stable and do not result in the generation of packagable RCA.

C. Summary

It was shown that the contamination of replication defective vector preparations by RCA can be eliminated by the construction of "stuffer" viruses. The insertion of foreign DNA sequences, in this case, DNA from bacteriophage lambda, into the E3 region of the vector backbone results in genomes of a size which cannot be packaged into virions following homologous recombination and acquisition of the E1 gene sequences. These inserts appear to have only a slight effect on the ability of the virus to replicate in 293 cells, and more importantly, do not impair transgene expression following in vitro infection. The lambda stuffer insert used in these cases was found to be stable over 12
serial passages, and no RCA could be detected in these stocks following examination by restriction enzyme digestion or by replication in A549 cells.
Figure III-2: Effect of "Stuffer" Sequences on IL-2 Expression by Ad Vectors.

Cells were infected at an moi of 10 as described in materials and methods, with either AdCAM4IL-2 or AdCAM4IL-2lam. Aliquots of culture supernatants were removed at days 1, 3, 5, and 7 post-infection, and the amount of IL-2 present in each sample was quantitated by ELISA. All results have been normalized to 10^6 cells. The graphs represent the amount of IL-2 secreted by cells infected with AdCAM4IL-2 (open squares) or with AdCAM4IL-2lam (closed circles), for human MRC5 normal fibroblasts (panel A), human 983M melanoma (panel B), human MCF7 breast carcinoma (panel C), human A549 lung carcinoma (panel D), murine MT1A2 breast carcinoma (panel E) and murine SV329 SV40 transformed kidney (panel F) cells.
A  
B  
C  
D  
E  
F  

Days post-infection

μg IL-2/10^6 cells

μg IL-2/10^6 cells

μg IL-2/10^6 cells

μg IL-2/10^6 cells

μg IL-2/10^6 cells

μg IL-2/10^6 cells

Days post-infection
APPENDIX IV. CONSTRUCTION OF A POLYOMAVIRUS MIDDLE T ANTIGEN-EXPRESSING CTL TARGET CELL LINE

A. Introduction

The tumor model used to study the efficacy of Ad-cytokine vector administration was based on transgenic animals which overexpress the polyomavirus middle T antigen (PyMidT) in an FBV/N genetic background. These animals develop adenocarcinoma of all mammary epithelium by 8-10 weeks of age (Guy et al., 1992b) and the resulting tumors could be explanted and would form tumors in syngeneic immunocompetent mice (Addison et al., 1995a,b; Bramson et al., 1996). In order to study the mechanism of anti-tumor activity in this model system, a CTL target cell line was constructed. We hypothesized that the major tumor antigen in the PyMidT tumors was the PyMidT protein itself. Therefore, cell lines were constructed that overexpressed PyMidT in a syngeneic H-2a murine background.

This appendix describes the construction and characterization of a target cell line for use in cytotoxic T-lymphocyte (CTL) assays to demonstrate tumor antigen specific killing in the polyoma middle T antigen mouse tumor model. Some of the work described herein was carried out by Dr. Jonathan Bramson.
B. Results

1. Construction of PyMidT-Expressing Cell Lines

The FVB/N strain was used to generate the transgenic animals used in this system and since they have an H-2<sup>a</sup> MHC haplotype, it was necessary to generate syngeneic cell lines for targets in the CTL assays. Initially, an attempt was made to transform syngeneic primary cells with polyoma viral sequences. Kidneys were removed from 6 day old baby FVB/N mice, trypsinnized and the cells put into 60 mm culture dishes as described in materials and methods. Approximately 48 hours later, the cells were subjected to calcium phosphate transfection overnight using the plasmid pSVE1-B1a (gift of J. Hassell) which expresses the large, middle and small T antigens of polyomavirus. The medium was changed the next day and the cells were allowed to recover in 10% FBS for 3 additional days. In order to select for polyoma transformed cells, the serum concentration of the medium was reduced to 2%. No polyoma transformed colonies appeared to arise, however after 8-10 weeks, a few fibroblast-like colonies were still alive. These were isolated in cloning cylinders by trysininization and expanded in medium containing 10% FBS. One of these cell lines, PT0516, was used for further experiments to try to generate a CTL cell target.

It was assumed that the major tumor antigen in the mouse model being used was the polyoma middle T Ag itself. Therefore, it was decided that the PT0516 cells would be transfected with a plasmid that expressed PyMidT. The cells were thus cotransfected (using the bubbling calcium phosphate precipitation technique described in Chapter 2), with the following: pSV2neo, which contains a neomycin resistance gene, and pJ4QMT (gift of W.
Muller) which expresses the PyMidT gene under control of the moloney murine leukaemia virus long terminal repeat. Following cotransfection, the medium was changed and three days later medium containing 400μg/ml G418 was added to the cells. Within 2-3 days the majority of cells had died, and a few G418 resistant colonies could be seen. A number of these were isolated by trypsinization using cloning cylinders and expanded. The isolated clones were subsequently tested by Western blot analysis for the expression of PyMidT. All seven G418 resistant clones tested possess a band at approximately 56 kDa which represents PyMidT, while the parental cell line PT0516 does not possess this band (Figure IV-1). A cell line derived from primary tumor cells isolated from the PyMidT transgenic animals, MT1A2, also possesses a band at approximately 56 kDa and an additional band at 58 kDa, which is characteristic of PyMidT expression in transgenic animals (B. Griffin, personal communication), and thus confirms the identity of the bands in the G418 resistant cell lines as PyMidT.

2. Characterization of PyMidT-Expressing Lines as CTL Target Cells

The PyMidT positive cell lines were then tested for their ability to act as target cells in a CTL assay. To generate lymphocytes reactive against PyMidT, syngeneic naive FVB/N mice were injected in the hind footpad with 10⁶ pfu of an E1 deleted Ad vector that expresses PyMidT. Five days post-injection, the popliteal lymph nodes (which drain the footpad area) were removed, and the lymphocytes were isolated and cultured for three days. The potential target lines were labelled with ⁵¹Cr, and a standard ⁵¹Cr release CTL assay was performed as described in the materials and methods. It was found that the cell lines 516MT3 and 516MT4
Figure IV-1: Western Blot Analysis to Detect PyMIDT Expression.

G418 resistant cell lines were harvested, and cell lysates were prepared as described in Chapter 2. Equal volumes of cell lysate were subjected to PAGE, and the gel was transferred to Immobilon-P membranes (Millipore) by electrophoretic transfer. The membrane was exposed to specific monoclonal antibodies for polyoma middle T antigen, and the protein was visualized following incubation with horse-radish peroxidase-conjugated secondary antibody and subsequent chemiluminescent detection (ECL, Amersham). Polyoma Middle T antigen is indicated by the arrow, and runs as a 56-58 kDa protein.
demonstrated the strongest level of antigen-specific CTL killing, while the other lines had weak to no demonstrable killing. The target cell killing observed using the 516MT3 and 516MT4 cell lines as targets was found to be inhibited by anti-CD3 antibodies which indicated that the killing was T-cell mediated. Furthermore, the target cell killing was not inhibited by addition of excess YAC-1 cells which are an extremely sensitive target for killing by NK cells. This data also supports the hypothesis that the killing seen on these target cell lines can be attributed to antigen-specific T-cells. When the parental cell line, PT0516 was used as a target line, no significant T-cell mediated target cell killing was observed, indicating that the cytotoxic activity was PyMidT-specific. Based on these data, the 516MT3 cell line was used as a target line for all subsequent CTL assays.

C. Summary

A cell line that expresses PyMidT was constructed by transfection of murine kidney cells of the FVB/N background. Positive clones were isolated and tested as potential PyMidT-specific CTL targets. The PyMidT-expressing cell line 516MT3 was found to be highly susceptible to PyMidT-specific CTL-mediated lysis, and a standard $^{51}$Cr release assay based on the use of this cell line as a target cell was developed to examine the mechanism of tumor killing following Ad-cytokine-mediated tumor regression.