

ADENOVIRUS VECTORS FOR CYTOKINE GENE EXPRESSION

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ABSTRACT

Cytokines are polypeptide hormones that act nonenzymatically to regulate host cell functions. These glycoproteins make up a fourth class of soluble intercellular signalling molecules that also include neurotransmitters, endocrine hormones and autacoids and are believed to play a central role in tissue remodelling in inflammation, infection, and wound repair. Numerous studies have now implicated cytokines to be of critical importance in host defense, and a more complete understanding of their molecular function is essential. What is also evident is that the majority of biological functions assigned to cytokines have been characterized by in vitro systems.

In vivo confirmation of these reported biological functions is required and has been attempted. To date, this has been difficult to attain with the available animal models. While studies in transgenic mice have revealed a number of biological activities, they probably do not reflect normal physiological responses, since tissues chronically exposed to a cytokine throughout development may undergo alterations in its effector phenotype. Administration of recombinant protein is also problematic as repeated injections with large doses of purified recombinant protein are usually required to maintain physiologic concentrations due to the short half life of most cytokines in the

circulation.

To overcome these problems, we have developed an alternative approach to investigate cytokine function in vivo. This approach, which we have defined as a "pseudo transgenic" animal model, uses recombinant adenovirus vectors containing cytokine genes to deliver and **transiently** overexpress cytokines in vivo in a tissue-directed manner to normal adult animals. Using this vector approach, cytokine expression can be targeted to a tissue in a way that may mimic more normal physiologic responses. In this study, recombinant adenovirus type 5 vectors capable of expressing the murine cytokines interleukin-5 (IL-5), interleukin-6 (IL-6), and RANTES were constructed to investigate the in vivo effects of these cytokines on immune and inflammatory responses.

The first vector constructed, Ad5E3mIL6, contained the murine IL-6 gene incorporated into the E3 region of the viral genome and was used to characterize the capacity of recombinant adenovirus vectors for cytokine expression. This vector was very efficient for cytokine expression both in vitro and in vivo. In addition, using an adenovirus vector containing luciferase as a reporter gene, we demonstrated that expression could be targeted in a highly tissue-specific manner dependent upon the route of administration.

Since IL-6 was reported to be the major mediator of the acute phase

response and intraperitoneal administration of adenovirus vector primarily targeted cytokine expression to the liver and spleen in Balb/c mice, our initial investigation involved intraperitoneal injection of the Ad5E3mIL6 vector into Balb/c mice. This study confirmed in vivo biological roles for IL-6 as a major mediator of the acute phase response and as a B and T cell proliferation factor.

We then analyzed the effects of expression of IL-5 and IL-6, alone and in combination, on humoral immune responses in the mucosal tissue of the lung. Both cytokines, produced by T helper type 2 lymphocytes, are critical to the development and differentiation of B lymphocytes and in particular to IgA antibody production in the mucosa-associated lymphoid tissue (MALT) and bronchus-associated lymphoid tissue (BALT). These studies, using Ad5E3mIL5 (a vector expressing murine IL-5 in the E3 region of the virus) in conjunction with Ad5E3mIL6, provided in vivo support for the roles of IL-5 and IL-6 in inducing lung mucosal immune responses. Co-administration of these two vectors in C57Bl/6 mice synergistically induced up to 8-fold increases in antigen-specific IgA antibody production in the lung.

In addition, we studied the in vivo effects of RANTES, a molecule reported to be chemotactic for monocytes, on lung inflammatory responses. A vector, Ad5E3mRANTES, was constructed which contained the murine RANTES cDNA in the E3 region of the virus. This vector, when intratracheally instilled into Sprague Dawley rats, targeted expression to the mucosal tissue

and induced the recruitment of monocytes to the lung within 24 hours. These effects were transient and this expression did not result in detectable lasting pathologic changes to the organ. These in vivo findings on RANTES function are consistent with its proposed function as a potent monocyte chemotactic factor.

Finally, we applied this newly developed adenoviral technology to immune modulation in an animal model of breast cancer. Mammary tumor cells from transgenic mice expressing the polyoma middle T antigen under the control of the mouse mammary tumor virus promoter were transplanted into syngeneic mice to establish subcutaneous tumors. These tumors were directly injected with control virus or Ad5E1mIL6A+ vector, a replication-deficient vector containing the murine IL-6 gene in the E1 region of the Ad5 genome. We found that localized vector-derived expression of IL-6 could attenuate tumor growth.

In conclusion, the results of this thesis study demonstrate the practical use for recombinant adenovirus vectors to aid in the investigation of cytokine function in vivo. This demonstration that vector-derived expression of cytokine is high and can be targeted to specific tissues suggests their use as potential therapeutic agents for the modulation of immune responses in the treatment of cancer and other diseases.

ACKNOWLEDGEMENTS

I dedicate this thesis to my mother and the loving memory of my father.

PREFACE

This dissertation consists of seven chapters. Chapter 1 is the introduction and reviews basic concepts and pertinent background to the thesis, as well as the objectives of the study. Chapters 2 - 6 represent the core of the thesis and comprise 5 manuscripts, one of which has been published and four which are at various stages of review at the time of writing this thesis. Chapter 7 is a summary of the major findings and their relevance to the body of scientific knowledge.

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LIST OF ABBREVIATIONS

Ad5	human adenovirus type 5
BAL	bronchoalveolar lavage
C-C	cysteine-cysteine
CD	cluster designation
cDNA	complementary deoxyribonucleic acid
CNTF	ciliary neurotrophic factor
CTL	cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
E1	early region 1
E2	early region 2
E3	early region 3
E4	early region 4
GM-CSF	granulocyte macrophage colony-stimulating factor
IFN- γ	interferon gamma
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin-1

IL-2	interleukin-2
IL-3	interleukin-3
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-11	interleukin-11
IL-12	interleukin-12
IL-13	interleukin-13
kb	kilobase
kDa	kilodalton
LIF	leukemia inhibitory factor
mRNA	messenger ribonucleic acid
OM	oncostatin M
PBS	phosphate buffered saline
RANTES	regulated on activation, normal T cell expressed and secreted
TGF β	transforming growth factor beta
Th1	Type 1 T helper lymphocyte
TNF α	tumor necrosis factor alpha
Th2	Type 2 T helper lymphocyte

Chapter One

INTRODUCTION

1.1. CYTOKINES

1.1.1 Overview of Cytokine Function

Cytokines are a diverse group of low molecular weight glycoproteins (usually <20 kDa) that act as powerful mediators of a broad spectrum of cellular functions. Cytokines generally act in a paracrine or autocrine fashion and are predominantly released in response to host stress to restore homeostasis (Oppenheim et al., 1994). This expression is believed to play a central role in tissue remodelling during inflammation, infection, and wound repair (Nathan and Sporn, 1991). In addition, cytokines have been shown to be intimately involved in the regulation and development of hematopoiesis (Williams et al., 1994; Hirano, 1992; Tominaga et al, 1992).

Cytokines to share several common characteristics regarding their biological function. These molecules are pleiotropic in nature in that one cytokine may act on many cell targets. Redundancy in their function has also been noticed and may reflect in part the use by many cytokines of common (public) receptor elements (Falus, 1995). All cytokines are extremely potent, acting at picomolar concentrations by binding to specific high affinity cell surface receptors, initiating intracellular transcription of RNA which encode proteins that trigger effector cell function (Arai et al., 1990). These functions

include the induction of other cytokines, often resulting in cytokine networks or cascades which act in concert to modify immune and inflammatory response (Balkwill et al., 1989). Because cytokine-mediated tissue damage can occur, cytokine expression is highly regulated, often by other networks of cytokines consisting of anti-inflammatory and immunosuppressive cytokines (Abbas et al., 1991; Oswald et al., 1992). In many cases, cytokine expression is down-regulated by an autocrine mechanism. In addition cytokine expression is often transient due to short periods of transcription and an associated short half-life of cytokine mRNA. AU-rich sequences in 3' untranslated regions, which have been suggested to be involved in mRNA turnover, have been found in many cytokines (Balkwil, 1991).

The realization that cytokine networks are intimately involved in the regulation of host defense, immune regulation, and hematopoiesis has led to our study which has been designed to elucidate the functions of the individual components of these cytokine cascades. The observation that distinct cytokine profiles are associated with either T helper 1 (Th1) or T helper 2 (Th2) lymphocytes has implicated a process whereby T cells may orchestrate these cytokine cascades differentially in response to stimulus (Romagniani, 1991; Anderson et al., 1994). Using this insight as a basic premise for study, we constructed individual recombinant adenovirus type 5 (Ad5) vectors expressing IL-5, IL-6, and RANTES to investigate the effects of these cytokines on in vivo

inflammatory, systemic and mucosal immune responses by tissue-directed vector expression in mice (Takatsu et al., 1994; Borden et al., 1994; Schall et al., 1990). Recombinant adenovirus vectors expressing various genes, including reporter gene constructs, had already been demonstrated to express the transgene protein in a variety of cells and organ tissues and therefore served as excellent vehicles for the in vivo targeted and transient expression of cytokine (Rosenfeld et al., 1991; Mittal et al, 1993; Xing et al., 1994; Bramson et al., 1995).

1.1.2 Interleukin-6

IL-6 is a low molecular weight polypeptide hormone exhibiting pleiotropic activity in the regulation of hematopoiesis, immune and acute phase responses (Wong and Clark, 1988; Sehgal et al., 1989; Akira et al., 1990; Kishimoto et al 1992; Kishimoto et al, 1995). Human and mouse IL-6 proteins purified to homogeneity from a variety of cellular sources show multiple forms with apparent molecular weights ranging in size from 20 to 30 kD (May et al., 1988a; Gross et al., 1989; Schiel et al., 1990). The mouse, rat and human genes code for mature proteins of 183 to 185 amino acids, depending on the site of cleavage of the signal peptides. The heterogeneity of protein size can be accounted for largely by post-translational modifications such as N- and O-linked glycosylation (May et al., 1988a), phosphorylation, and possible

myristylation (May et al, 1988b). The rat and mouse proteins do not possess N-linked glycosylation sites but do have a few putative O-linked sites, although the purified native mouse molecule is shown to have no carbohydrate attached (Fuller et al., 1989).

The human, mouse and rat genes, as well as the porcine cDNA, have been cloned and sequenced (Yasukawa et al., 1987; Tanabe et al., 1988; Northemann et al., 1989; Richards et al., 1991). All genes contain a high degree of conserved intron/exon boundary structure and are made up of 4 introns and 5 exons (Northemann et al., 1989). IL-6 is a single copy gene which has been mapped to chromosome 7p21 in humans (Bowcock et al., 1988) and chromosome 5 in the mouse (Mock et al., 1989).

IL-6 can be produced by a variety of cells including fibroblasts (Weissenbach et al., 1980), endothelial cells (Corbel et al., 1984; Sironi et al, 1989), myoblasts (Bartoccioni et al., 1994), keratinocytes (Baumann et al., 1984; Derocq et al., 1994), mesangial cells (Ikeda et al., 1992), monocyte/macrophages (Aarden et al., 1987), T cell lines (Hirano et al., 1985) and a number of tumor cell lines (Hirano et al., 1986; Kawano et al, 1988; Tosato et al, 1990; Miles et al, 1990). The most potent stimulator of IL-6 protein expression appears to be the endotoxin lipopolysaccharide (LPS) in monocyte/macrophages, while stromal cells are optimally stimulated by the macrophage-derived proteins interleukin-1 (IL-1) and tumor necrosis factor-alpha

(TNF α) (Arai et al., 1990).

IL-6 has been shown to possess B cell stimulatory activity, inducing final maturation of B lymphocytes into high level antibody producing plasma cells (Hirano et al., 1986). It also acts as both a human and a murine plasmacytoma/hybridoma growth factor (Nordan et al., 1986; Van Damme et al., 1987; Klein et al., 1990). IL-6 stimulates the growth and differentiation of T lymphocytes (Takai et al., 1988) and supports the proliferation of hemopoietic stem cells in synergy with IL-3 (Ikebuchi et al., 1987; Okano et al., 1989). In IL-6 knockout mice, decreases in granulocyte-monocytic, megakaryocytic, and erythroid lineage progenitors were evident and resulted in slow recovery from hematopoietic ablation (Bernard et al., 1994).

As one of its primary roles in homeostasis, IL-6 can elicit hepatic acute phase responses and induce the synthesis and secretion of a series of acute phase plasma proteins by hepatocytes (Gauldie et al., 1987); however, studies of IL-6 knockout mice revealed that there is some overlap with other cytokines in this function, since an IL-6 independent acute phase response could be elicited, depending on the agent of induction (Kopf et al., 1994).

IL-6 may also contribute to the pathology associated with atherosclerosis. It participates in the growth of vascular smooth muscle cells and has recently been shown to have inhibitory effects on vascular smooth muscle contraction (Ikeda et al., 1991; Ohkawa et al., 1994).

IL-6 has also been shown to act on the nervous system. It induces the secretion of nerve growth factor from brain astrocytes and stimulates the release of adrenocorticotrophic hormone by the anterior pituitary through the induction of corticotropin-releasing factor from the hypothalamus (Kishimoto, 1989; Naitoh et al., 1988). IL-6 is detected in a model of central nervous system viral infection, supporting a possible *in vivo* regulatory function for IL-6 on neural tissue (Frei et al., 1989).

The actions of IL-6 are mediated by specific receptors expressed on both lymphoid and nonlymphoid cells (Yamasaki et al., 1988). The IL-6 receptor is composed of two chains, a ligand-specific chain, gp80, and a common signal transducer molecule, gp130 (Yawata et al., 1993). IL-6 belongs to a family of cytokines, that includes leukemia-inhibitory factor (LIF), oncostatin M (OM), interleukin-11 (IL-11), and ciliary neurotrophic factor (CNTF) that act through the gp130 subunit required for signal transduction (Taga et al., 1989; Kishimoto et al., 1995). These IL-6 related proteins (LIF, OM, and IL-11) have been shown to induce the acute phase response (Baumann et al., 1989; Baumann et al., 1992; Richards et al., 1992). These other related IL-6 proteins may account for the acute phase response seen in IL-6 knockout mice after LPS challenge (Kopf et al., 1994). The IL-6 receptor binds IL-6 in both low and high affinity complexes. The IL-6 high affinity complex is a hexamer consisting of two molecules each of IL-6, gp80, and gp130 (Ward et al., 1994).

Dimerization of the gp130 subunits causes activation of the janus associated kinase-signal transducer and activator of transcription (JAK-STAT) as well as the mitogen activated protein kinase (MAPK) cytoplasmic signal transduction pathways leading to the activation of IL-6 specific nuclear factors (including nuclear factor NF-IL6 which binds to IL-6 responsive elements on acute phase genes (Murakami et al., 1993; Narazaki et al., 1994; Stahl et al., 1995; Darnell et al., 1994; Akira et al., 1992; Zhong et al., 1994).

1.1.3 Interleukin-5

Interleukin-5 (IL-5) is a pleiotropic cytokine that was first described as T cell replacing factor (TRF) due to its ability to replace T lymphocytes in the induction of in vitro B cell antibody responses to sheep red blood cells (Dutton et al., 1971; Schimpi et al., 1972). Like IL-6, it is involved in the differentiation of B lymphocytes (Takatsu et al., 1980). In mice, IL-5 has been shown to induce the production of polyclonal IgM, IgA, and IgG1, albeit by different mechanisms. Stimulation of B cells by antigen plus IL-5 stimulation causes induction of μ -chain mRNA, leading to increased IgM production (Webb et al., 1991). IL-5 acts in concert with transforming growth factor beta (TGF β) to induce the production of IgA (Sonoda et al., 1989; Coffman et al., 1989). IL-5 does not induce class switching from IgM to IgA but rather acts as a maturation factor for the development of IgA-committed B cells into IgA

secreting cells (Kim et al., 1990; Matsumoto et al., 1989; Sonoda et al., 1992). IL-5 also acts in combination with IL-4 to enhance the production of IgE and IgG1 in murine B cells (Purkerson et al., 1992).

While the exact role of IL-5 in human B cell growth and differentiation remains controversial, IL-5 has been demonstrated to affect the growth and differentiation of many cell types (Clutterbuck et al., 1987). IL-5 promotes the growth of dextran sulphate-stimulated normal B cells and of the murine chronic leukemic B cell line, BCL1, and acts as a growth and differentiation factor for Ly-1⁺ B cells (Swain et al., 1982; Harada et al., 1985; Tominaga et al., 1989). It acts as a growth and chemotactic factor for eosinophils (Yamaguchi et al., 1988; Wang et al., 1989) and can promote differentiation of bone marrow precursors into mature eosinophils (Campbell et al., 1987). It also has been shown to affect the growth and differentiation of human basophils (Denburg, 1992).

In the human, IL-5 exists as a 112 amino acid mature protein, possessing a signal peptide of 22 residues (Yokota et al., 1987) and two sites for N-linked glycosylation (Azuma et al., 1986). Mature mouse IL-5 also consists of 112 amino acids with a signal peptide of 21 amino acids, and it has 3 potential sites for N-linked glycosylation (Kinashi et al., 1986); however, carbohydrate modification does not affect its biological activities (Tominaga et al., 1990). In its native state, IL-5 protein exists as a dimer. Under non-reducing

conditions, human and mouse proteins migrate with apparent molecular weights of 45 and 50 kD, respectively (Takatsu et al., 1988). The IL-5 subunits are cross-linked in an antiparallel arrangement through two of the three conserved cysteine amino acid residues (Minamitake et al., 1990). This homodimeric arrangement is critical for function, as mutation of these cysteine residues causes loss of biological activity (Takahashi et al., 1990; McKenzie et al., 1991).

The mouse and human IL-5 genes have been cloned. Both were found to be single copy genes composed of 4 exons and 3 introns (Campbell et al., 1987; Tanabe et al., 1987; Campbell et al., 1988). The gene has been mapped to chromosome 5 in humans (LeBeau et al., 1989) and chromosome 11 in the mouse (Lee et al., 1989).

The IL-5 receptor complex consists of two distinct polypeptide subunits, designated alpha and beta, with molecular weights of 60 kDa (IL-5R α) and 130 kDa (β chain) (Miyajima et al., 1993; Tavernier et al., 1991). The β chain is shared with the receptors for both granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) (Lopez et al., 1991). IL-5 binds to the IL-5 receptor α chain alone but will not bind to the β chain by itself (Devos et al., 1995). The cytoplasmic portions of both α and β chains are required for signal transduction, suggesting that heterodimerization must occur for signal transduction (Takaki et al., 1993; Sakamaki et al., 1992). Like the IL-6

receptor, IL-5 receptor binding activates both JAK-STAT and MAPK signal transduction pathways, but requires STAT 1 protein for eosinophil activation (Alam et al., 1995; Pazdrak et al., 1995).

1.1.4 RANTES

RANTES is a member of the β subfamily of chemokines which has been shown to be chemotactic for monocytes, eosinophils, basophils and a memory cell subset of CD4+ T lymphocytes (Zhang et al., 1994). In addition, it has recently been shown to be chemotactic for mast cells (Taub et al., 1995). The protein was first described in 1988 as a novel T cell gene recovered from a T lymphocyte library subtracted against B lymphocyte mRNA and designated RANTES an acronym for Regulated on Activation, Normal T Expressed and Secreted. RANTES cDNA encodes a predicted 91 amino acid protein with a molecular weight of 10 kDa and contains no sites for N-linked glycosylation (Schall et al., 1988). In vitro expression of recombinant human RANTES reveals a non-glycosylated form of the mature protein with a molecular weight of 8 kDa consistent with cleavage of signal peptide sequences producing a mature 68 amino acid protein (Schall et al., 1990). The protein forms dimers at physiologic pH and this conformation is important for biological function (Skelton et al., 1995).

The gene for RANTES has been localized to human chromosome 17

(q11.2-q12), closely linked to other members of the β chemokine gene family (Donlon et al., 1990). The RANTES gene is organized into 3 exons and 2 introns; the first and second introns are highly conserved amongst the members of this family (Nelson et al., 1993).

RANTES can be expressed by a number of cell types. These include T cells (Shall et al., 1988) and activated synovial fibroblasts (Rathanaswami et al., 1993), monocytes (Schall et al., 1991a), platelets (Kameyoshi et al., 1992), renal tubular epithelial cells and mesangial cells (Heeger et al., 1992), and rhabdomyosarcoma and osteosarcoma cells (Schall, 1991b).

In vitro analysis has indicated that RANTES expression is modulated by various cytokines and inflammatory factors. RANTES expression was stimulated by IL-1 and TNF α in synovial fibroblasts (Rathanaswami et al., 1993). In addition, TNF α and interferon gamma (INF γ) act in a synergistic manner to increase RANTES expression in human bronchial epithelial cells (Stellato et al., 1995). This expression was inhibited by glucocorticoids. Negative regulation of RANTES expression was also found in endothelial cells treated with interleukin-4 (IL-4) or interleukin-13 (IL-13) (Marfaing-Koka et al., 1995).

In addition to its chemotactic activities, RANTES has been reported to induce the release of eosinophil cationic protein from eosinophils, as well as histamine from basophils (Alam et al., 1993; Kuna et al., 1992); however,

Zeck-Kapp et al. (1995) have demonstrated that eosinophil release of ECP requires signals in addition to RANTES. In that study, RANTES caused intracellular mobilization and activation of ECP granules but did not trigger exocytosis.

The RANTES chemokine receptor is related to the G-protein-coupled receptors and consists of seven membrane-spanning segments (Ahuja et al., 1994). Three types of β chemokine receptors appear to exist on monocytes, but only one of these appears to bind RANTES. There also is evidence for more than one class of β chemokine receptor capable of binding RANTES on both basophils and eosinophils (Ahuja et al., 1994). The RANTES receptor on human monocytes binds with high affinity but has also been shown to bind two additional cysteine-cysteine (C-C) chemokines, monocyte chemotactic peptide 1 (MCP-1) and macrophage inflammatory protein-1-alpha (MIP-1 α) albeit with lower affinity (Wang et al., 1993). Receptor binding is associated with a rapid and transient increase in cytosolic free calcium ion (Ca²⁺) concentration which affects chemotaxis, interleukin-2 (IL-2) expression, proliferation and cytokine release in T cells (Bacon et al., 1995).

1.2 ASPECTS OF CYTOKINE FUNCTION ON HOST DEFENSE

1.2.1 Inflammation

Inflammation is the host response to infection or injury. It is elicited in response to a variety of agents including cold, heat, trauma, and microbial infection. The primary function of inflammation is to restore homeostasis in the host by eliminating or inactivating foreign agents and to set the stage for tissue repair. The key cellular mediators of this response are phagocytes (neutrophils, monocytes, and macrophages) whose recruitment from the blood into sites of insult is controlled by an array of cytokine mediators including chemokines (Beekhuizen et al., 1993; Lukacs et al., 1995). These phagocytic cells are the effector cells of innate resistance and represent the first line of defense in the host against infections or foreign pathogens.

1.2.2 Acute Phase Response

An additional protective aspect of inflammation is the induction of the acute phase response (Baumann and Gauldie, 1994). This response is characterized by a dramatic change in plasma concentrations of a defined group of polypeptide molecules, known collectively as the acute phase proteins, which mediate a number of functions involved in host defense. Acute phase proteins are predominantly produced in the liver by hepatocytes and are

released into the bloodstream where they influence inflammatory and tissue repair processes. For example, fibrinogen, a strongly induced acute phase protein, is involved in blood clotting and wound healing (Koj, 1985). Haptoglobin, hemopexin, and ceruloplasmin have been shown to have antioxidant properties while α -1 protease inhibitor, α -1 antichymotrypsin, and C-1 inhibitor can protect against tissue damage by controlling protease activity (Kushner, 1993). While a number of cytokines have been shown to be able to induce this response, it is clear the major mediator of the hepatic acute phase response is IL-6 (Gauldie et al., 1987; Richards et al., 1992). However, it must be recognized that the pattern of acute phase protein production by hepatocytes is complex and can be differentially regulated by different combinations of cytokines (Makiewicz et al., 1991; Zuraw et al., 1990). In addition, glucocorticoids are important co-factors in this response and can synergistically increase the production of many acute phase proteins (Baumann et al., 1987).

1.2.3 Adaptive Immune Responses

In addition to innate or natural defense mechanisms, adaptive immune responses are also elicited in the protection of the host. These responses are mediated by lymphocytes and induce specific immune responses against foreign microbes or substances. Specific immune responses can be classified into two

types based on the effector components of the response. Humoral immunity is mediated by antibodies (serum proteins which recognize specific antigen epitopes on a given pathogen or noxious substance). Cell-mediated immune responses are controlled by T lymphocytes and play a critical role in the regulation of both humoral and cell-mediated immune response (Abbas et al., 1994).

T cells control these immune responses by producing several cytokines and have been classified into two distinct subpopulations based on their cytokine profile (Street et al., 1991). Th1 cells produce primarily IL-2 and IFN- γ , but not IL-4 or IL-5, and are associated with classic delayed-type hypersensitivity reactions, whereas Th2 cells produce primarily IL-4 and IL-5, but not IL-2 or IFN γ , and mediate humoral immune responses (Street et al., 1991).

1.2.3.1 Humoral Immune Response

Humoral immune responses are dependent on interactions between B and T lymphocytes. Soluble cytokines produced by T cells are thought to act on B cells to influence their differentiation as well as their isotype specificity. Several models have been used to study the effects of cytokines on B cells, and a restricted number of cytokines have been demonstrated to play critical roles in the humoral immune response. IL-4 acts early in B cell responses and

was designated as B cell stimulatory factor 1 (BSF1) since it could cause the proliferation of resting B cells as well as inducing major histocompatibility complex (MHC) class II antigens (Noelle, et al., 1984). IL-4 may also play a central role in the regulation of Ig isotype. Treatment of LPS-stimulated murine B cells with IL-4 induced the secretion of IgG1 and IgE antibodies and inhibited the secretion of IgM, IgG2B and IgG3 antibodies (Snapper et al., 1988). In addition, IL-4 caused class switching to IgE and IgG4 secretion in human B cells (Gascan et al., 1991).

IL-5 may be a differentiation factor for IgA-committed B cells (Schoenbeck et al., 1989). It enhances IgA-specific responses since treatment of LPS-stimulated B cells with IL-5 induced secretion of IgA antibody without increasing the synthesis of IgG or IgM (Coffman et al., 1987). The demonstration that vaccinia virus-derived IL-5 expression increased IgA anti-hemagglutinin titers in vivo after targeted expression of IL-5 and antigen to the mucosal tissue of the lung supports an important role for this cytokine in mucosal immune response (Ramsay et al., 1993).

Interactions between IL-2, IL-4 and IL-5 have profound effects on isotype regulation. For example, while IL-4 was shown to down-regulate IgM responses, this effect could be overcome by the addition of IL-5 (McHeyzer-Williams, 1989). Treatment of LPS-activated B cells with IL-4 and IL-5 in combination increased IgA production 2- to 3-fold (Murray et al., 1987).

In addition, IL-2 induced growth and differentiation of Staphylococcus aureus Cowen strain I (SAC)-activated B cells appears to be mediated by autocrine production of IL-6 (Xia et al., 1989). IL-6, on the other hand, has been shown to enhance IgG, IgM and IgA production. In synergy with IL-1, IL-6 increased IgM and IgG production of T cell-depleted Peyer's patch B cells. In the same model system, IL-6 enhanced the production of IgA induced by IL-5 (Kunimoto et al., 1989). From these studies, an understanding of cytokine effects on B cell differentiation and isotype selection is emerging which implicates interactions between IL-4, IL-5 and IL-6 as being critical in isotype-specific responses.

1.2.3.2 Cytotoxic T Cell Cytokine Regulation

The function of cell-mediated immunity is closely related to the cascade of cytokines produced upon activation of the host defense responses. One of the most powerful effector arms of the cell-mediated immune response consists of cytotoxic T lymphocytes (CTL) (Maryanski et al., 1986). The induction of CTL from precursor T cells requires the presence of cytokine signals in conjunction with T cell receptor recognition of antigen (Kern et al., 1981). Multiple cytokine signals are needed to generate a full CTL response. CTL development from isolated spleen lymphocytes has been shown to require both IL-2 and IFN γ (Maraskovsky et al., 1989). Interleukin-12 (IL-12) is also a

potent mediator of CTL function. IL-12, in combination with IL-2, synergistically enhances the generation of CTL in response to antigen stimulation (irradiated melanoma cells) and it independently augments CTL activity in anti-CD3-stimulated human CD8+ T cells (Wong et al., 1988; Gately et al., 1992).

Cytokines produced by Th2 cells also influence CTL function and differentiation. IL-4 promotes the development of CTL from mature thymocytes, but CD8+ T cells expressing this cytokine exhibit greatly diminished CTL activity in vitro (Collins et al., 1988; Erard et al., 1993). IL-6, a cytokine which can be produced by Th2 lymphocytes, promotes the proliferation of both thymocytes and peripheral T cells (Lotz et al., 1988). IL-6 is also an important mediator for the optimal induction of CTL (Rogers et al., 1991). In mixed lymphocyte/tumor cell cultures measuring CTL response to P815 mastocytoma tumor cells, IL-6 failed to costimulate proliferation or cytotoxicity in naive CD8+ cells. The combination of the costimulatory molecule B7-1 and IL-12 and IL-6 was sufficient to induce CTL activity, but only IL-6 could induce proliferation (Gajewski et al., 1995). IL-6 did not induce cytotoxic activity of dengue virus-specific human CD8+ CTL cell clones, suggesting that IL-6 may function primarily as a differentiation factor for CTL (Livingston et al., 1995). Many of the T cell-enhancing effects of IL-6 may occur in synergy with IL-1 (Mizutani et al. 1989) and appear to be mediated

through IL-2 by induction and enhancement of IL-2 receptor expression on T cells (Noma et al., 1987).

In addition to its effects on CTL, IL-6 also promotes the differentiation and proliferation of natural killer (NK) cells, (a cell type involved in innate immune response with a broader specificity for killing than antigen specific CTL) (Takai et al., 1988; Okada et al., 1988) and NK activity is enhanced by IL-6 (Lugar et al., 1989).

1.2.4 Mucosal Immunity

Lymphoid tissues associated with mucosal membranes contain more immune cells than any other tissue in the body. For example, it has been determined that there are 10^{10} immune cells, both T and B lymphocytes, in each meter of small bowel (Brandtzaeg et al., 1976). The mucosal B cells are primarily involved in producing IgA antibodies (Brandtzaeg et al., 1984). These polymeric IgA molecules combine with a secretory component present on the surface of mucosal epithelial cells; this secretory IgA is then transported to luminal secretions (Brandtzaeg et al., 1985). Mucosal immune responses are dominated by IgA at membrane sites, presumably to protect the tissue by aggregating and neutralizing luminal antigens (Mestecky, 1987). Mucosal immunity is complex and immune regulation depends upon interactions between antigen presenting cells, such as macrophages and dendritic cells and

T and B cells found throughout the mucosa-associated lymphoid tissue (MALT). These interactions take place both within the epithelium and in subepithelial tissue (Brandtzaeg et al., 1995). The primary mucosal immune response occurs in the MALT, where antigen responses are mediated through the T and B cell interactions. The MALT includes Peyer's patches, gut associated lymphoid tissue (GALT), and bronchus-associated lymphoid tissue (BALT), as well as other lymphoid tissues (Brandtzaeg et al., 1995). Mucosal T cell factors have been implicated in preferentially supporting IgA secretion from Peyer's Patch (PP) B cells (McGhee et al., 1989), the PP being the gastrointestinal equivalent of lymph nodes. Some of these factors have been identified and include IL-5 and IL-6. Both of these cytokines have been shown to regulate the terminal differentiation of IgA-committed B cells to IgA-secreting plasma cells (Beagley et al., 1990). This key role for IL-6 in mucosal IgA production was confirmed by studies in IL-6 'knockout' mice (Ramsay et al., 1994). In this study, mutant mice were observed to have greatly reduced numbers of IgA-producing cells in their mucosae and were grossly deficient in antibody responses after mucosal challenge. IgA responses in the lungs of these animals were completely restored with a recombinant vaccinia virus engineered to express IL-6. In a similar fashion, a recombinant vaccinia virus expressing IL-5 induced IgA antibody production 4-fold in the lungs of normal mice, indicating that IL-5 can selectively enhance IgA production in vivo (Ramsay et al., 1993).

1.3 RECOMBINANT VIRAL VECTORS

1.3.1 Gene Therapy and Recombinant Viral Vectors

Manipulation of viral genomes has now become relatively easy to accomplish with the available techniques of molecular biology (Maniatis et al., 1982). Foreign genes can now be inserted into viral genomes to create recombinant viral vectors capable of expressing the gene upon infection in the host. The initial applications of recombinant viral vectors to treat cancer and correct genetic defects used retroviral vectors (Miller, 1992; Rosenberg, 1992).

This technology has now been used to study the function of biologically active molecules in vivo. Recombinant viral vectors which express cytokine have been constructed and used in vivo. Expression of IL-6 by a retrovirus in hematopoietic cells produced a condition in experimental animals resembling Castleman's disease, an autoimmune condition characterized by anemia, hypoalbuminemia, hypergammaglobulinemia and marked splenomegaly, amongst other effects (Brandt et al., 1990). This finding suggested an important role for IL-6 in antibody production. Transformation of IL-5 dependent cells with a retrovirus expressing IL-5 caused the cells to become tumorigenic in mice (Blankenstein et al., 1990).

More recently, recombinant vaccinia virus vectors (VV) have been used to express cytokines in vivo. Initial studies involving these vectors

demonstrated immune effects which resulted in enhanced clearance of viral vectors expressing IL-2 or TNF α (Karupiah et al., 1990; Sambhi et al., 1991). These responses appeared to be localized to tissue sites of vector expression, since co-infection of VV containing IL-2 with VV wild type resulted in only VV-IL2 being cleared in an enhanced way (Dr. A. Ramsey, personal communication). No aberrant pathology was noticed in VV-treated animals. This phenomena was relatively surprising given the known proinflammatory effects of TNF α and may reflect the localized pattern of cytokine expression resulting from these vectors.

Vaccinia virus vectors expressing IL-5 and IL-6 were also tested for their effects on immune response. The vector-derived expression of IL-5 or IL-6 modified the mucosal immune responses to hemmagglutinin glycoprotein which was coexpressed in the same vector as the cytokine (Ramsay et al, 1993 and 1994). These responses demonstrated roles for IL-5 and IL-6 in enhancing IgA mucosal antibody, however these changes may only reflect the cumulative effects of localized interactions between antigen-specific lymphocytes and cytokine.

While both retrovirus and vaccinia virus vectors provided useful insight into cytokine function, each appeared to have an inherent limitations in investigating normal physiologic cytokine effects in vivo. Retroviral vectors require the reintroduction of transformed cells into the animal and they were

found to cause pathologic changes in treated animals. Vaccinia virus vectors appear limited in their capacity for cytokine expression and are quickly cleared because of their lytic activity, making cytokine expression relatively transient. To overcome these obstacles, we began an alternative strategy to investigate cytokine function by developing recombinant adenoviruses capable of expressing cytokines in vivo in a targeted manner.

1.3.2 Recombinant Adenovirus Vectors

At the initiation of this thesis study, the use of recombinant adenovirus type 5 (Ad5) vectors for cytokine delivery had not yet been reported; however, a generation of recombinant Ad5 vectors expressing viral antigens from genes inserted into the E3 region of the viral genome had already been used to elicit protective immune responses in a number of animal models (Morin et al., 1987; Dewar et al., 1989; McDermott et al., 1989; Prevec et al., 1989; Prevec et al., 1990). Recombinant Ad5 vectors expressing genes coding for antigens from rabies, herpes and vesicular stomatitis viruses protected animals from lethal challenge of the respective wild type virus. The current adenovirus technology was developed to allow for the construction of replication-deficient vectors with the capacity to carry foreign genes of up to 8 kb into either the E1 or the E3 regions of the viral genome (Bett et al., 1994).

Examples of this generation of recombinant Ad5 vectors have proven to

be efficient for the delivery of foreign genes in vivo. Expression of vector derived-genes has been demonstrated in airway epithelial cells, hepatocytes, endothelial cells, skeletal muscle cells, and brain ependymal cells and neurons, as well as other cell types (Rosenfeld et al., 1991 and 1992; Xing et al., 1994; Morsy et al., 1993; Lemarchand et al., 1992; Stratford-Perricaudet et al., 1992; Bajocchi et al., 1993; Davidson et al., 1993; Le Gal La Salle et al., 1993; Bramson et al., 1995). Because of the natural tropism of adenoviruses for the respiratory tract, adenovirus gene therapy clinical protocols have been developed in order to correct the pulmonary manifestations of cystic fibrosis by delivering a properly functioning cystic fibrosis transmembrane conductance regulator gene to airway epithelial cells (Crystal et al., 1993). Preliminary results from these trials have demonstrated the capability of these recombinant adenovirus vectors to express protein in the lung.

Due to its wide range of cellular targets and the fact that adenoviruses likely do not integrate into the host cell genome, adenoviral vectors are currently being tested as gene transfer vehicles for the treatment of cancer. P815 mastocytoma cells which were transduced to express IL-2 by an adenovirus vector lost their tumorigenic phenotype in 80% of injected syngeneic animals (Haddada et al., 1993). Intratumoral injection of a similar IL-2 vector was shown to cause the resolution of a subcutaneous tumor in a murine breast cancer model (Addison et al., 1995). In each of these instances,

animals with demonstrable IL-2 responses developed immunity and rejected a subsequent challenge with tumor cells, indicating that adenoviral vectors may be useful in the treatment of metastatic forms of cancer.

1.3.2.1 Adenovirus Structure

Adenoviruses are nonenveloped viruses that have been identified in several mammalian hosts, the first of which was isolated by Rowe et al. (1953). The virion is an icosahedral structure and contains at least nine different structural proteins (Horne et al., 1959). The structural proteins of adenovirus have been labelled II to IX on the basis of their molecular weight pattern on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Maizel et al., 1968). Polypeptides II, III, and IV (hexon, penton base, and fiber, respectively) form the major components of the outer capsid of the virion (Everitt et al., 1975). The viral DNA is associated with polypeptides V and VII and form a nuclear core of 12 spherical subunits in contact with the vertices of the icosahedral outer capsid (Brown et al., 1975). Polypeptides VI and VIII are associated with hexon protein and VI is also found to have a high affinity for DNA (Russell et al., 1982). Polypeptide IX is also associated with hexon and is involved in the stabilization of the capsid (Colby et al., 1981).

1.3.2.2 Adenovirus Life Cycle

Adenoviruses infect cells via binding of fiber protein to specific receptors on the cell surface (Svensson, 1985). The adenovirus genome is composed of double-stranded linear DNA of approximately 36 kb (Boulanger et al., 1991). Viral genes are transcribed in the nucleus of the cell by host nuclear factors and are expressed in two broad phases, the "early" genes being expressed at early stages of infection and the "late" genes being expressed following viral DNA replication (Flint, 1986). The first genes to be expressed are the "immediate early" genes in the E1A transcription unit. The two major proteins encoded in E1A, a 243 and a 289 amino acid protein, are translated from alternatively spliced mRNAs (Moran et al., 1987). E1A acts to transactivate expression from other early regions (E1B, E2, E3 and E4) shortly after the expression of E1A protein (Nevins et al., 1979; Spector et al., 1978). Late genes are primarily transcribed after viral DNA replication off the major late promoter and predominately encode the adenoviral structural proteins (Flint, 1986).

1.3.2.3 Early Region Gene Functions

In addition to its transactivation function, E1A has been shown to effect transformation and immortalization processes in cells and cause the induction of DNA synthesis in quiescent cells, functions that may be mediated through its interactions with retinoblastoma protein (Moran et al., 1987; Quinlan et al., 1988; Whyte et al., 1989). Region E2 encodes proteins that are essential for

viral genome replication and include the DNA-binding protein, DNA polymerase, and the terminal protein that primes the initiation of viral DNA replication (Tamanoi, 1986). The E4 region encodes proteins whose functions are required for efficient DNA replication, late gene expression, and host cell shutoff (Halbert et al., 1985). These early region genes, E1, E2 and E4 function in a coordinated fashion to prepare the cell for efficient viral DNA replication, transcription of RNA, and expression of protein.

The E3 region genes, on the other hand, are not required for viral replication but appear to be involved in regulating host cellular functions to enhance the survival of the virus in a host (Kelley et al., 1973; Wold et al., 1989). E3 encodes nine genes, two of which counteract different branches of the host anti-viral defense. The 19 kDa E3-encoded protein has been demonstrated to retain MHC class I molecules within the endoplasmic reticulum of infected cells to protect against CTL lysis (Paabo et al., 1987), while a 14.7 kDa protein was demonstrated to protect infected cells from tumor necrosis factor cytolysis (Gooding et al., 1988).

1.3.2.4 Adenovirus Vector Construction

The adenovirus type 5 recombinant vectors are the most commonly used system for gene expression by adenoviruses. Three regions of the Ad5 viral genome can accommodate insertions or deletions of DNA in the construction of

a helper-independent virus. These regions are located in E1, E3, and a short region between E4 and the end of the genome (Graham and Prevec, 1991). The E3 region is nonessential for viral replication and can accommodate deletions of up to approximately 3 kb (Bett et al., 1994). Extension of this deletion is not possible due to the presence of essential virion structural genes, protein VIII and fiber, flanking this region. An additional deletion of 3 kb in the E1 region can be made. Insertions in the E1 region cannot interfere with the inverted terminal repeat (ITR) or the packaging signal of the viral genome (Bett et al., 1994). Due to a packaging capacity of approximately 105% of the wild type Ad5 genome, combination of both E1 and E3 should allow for gene inserts of up to 8.3 kb to be incorporated into vector. A vector system incorporating these deletion modifications in E1 and E3 demonstrated the efficient expression from the E1 region of a 7.8 kb insert comprised of two genes (Bett et al., 1994).

Construction of a prototypical vector uses methodology based on manipulation of bacterial plasmids containing circular forms of the Ad5 genome. One plasmid is modified to incorporate exogenous genetic information into either the E1 or E3 region of the Ad5 genome and is used in cotransfections of 293 cells with a plasmid containing additional complementary Ad5 sequences to the first plasmid (Graham and Prevec, 1991). A homologous recombination event between these two plasmids allows the exogenous gene sequence to be

rescued into a recombinant vector (Graham and Prevec, 1991). Recombinant adenovirus vectors with deletions in the E1 region must be rescued on 293 cells (a human embryonic kidney cell line transformed with Ad5 DNA which can provide E1 protein in trans) (Graham et al., 1977). All forms of recombinant adenoviral vectors (E1 or E3 gene insert constructs) are propagated in 293 cells and these vectors can be produced in titers of up to 10^{12} plaque forming units/ml.

1.4 PROJECT OUTLINE AND OBJECTIVES

The study of local and systemic effects of cytokines in vivo has proven more difficult than investigation of these effects in vitro due to limitations of the available experimental model systems in the manner in which the cytokines can be expressed in animals. Administration of recombinant cytokine protein probably does not reflect normal physiologic effects due to the rapid distribution of these small polypeptides away from instillation sites and to the short half-life of the recombinant proteins in the circulation. Moreover, the normal physiologic response of an infected or traumatized tissue likely involves slow sustained release of the mediator. Transgenic expression of cytokines gives rise to difficulties in interpretation of data, as chronic stimulation and prolonged in utero and neonatal exposure by cytokine may alter normal tissue effector functions in the adult.

Recently, several recombinant viral vectors have been described which express foreign genes upon replication in infected cells. In particular, vaccinia virus has been used to express cytokines in vivo; however, the lytic nature of this virus and the damage caused by the infection also confuses the interpretation of the results. Retroviral vectors are inefficient at infecting non-replicating cells and are not useful in delivering cytokine genes in a direct manner. Previous studies involving modified recombinant adenoviral vectors demonstrated expression of foreign antigens in vivo with a minimum of tissue injury.

A great deal of expertise in the use of adenovirus expression systems was in place at McMaster prior to the initiation of this thesis study. We used this accrued knowledge to generate recombinant adenoviral vectors expressing cytokines to investigate the in vivo effects of cytokines on host immune and inflammatory responses. The rationale is that the adenovirus vectors will deliver the cytokines to local tissue in a sustained fashion over a sufficient length of time that subsequent immune and tissue pathologic reactions will closely mimic those seen in normal physiologic responses. The impact of local delivery and expression of cytokine was assessed by monitoring immune and inflammatory responses against adenovirus antigens and changes in the infected tissues. Unlike the transgenic mice or animals receiving exogenously administered recombinant cytokine protein, animal models using recombinant

adenovirus vectors for the delivery of cytokine are powerful tools in the investigation of cytokine biological function in vivo. Adenovirus vectors can be used to target the expression of cytokine to local tissues (adenoviruses are naturally tropic for respiratory epithelium) and cytokine effects may be determined in the context of tissue response.

In this study, we used recombinant Ad5 vectors expressing the cytokines IL-5, IL-6 and RANTES to determine the in vivo effects of these cytokines on inflammatory, mucosal and systemic immune responses. These cytokines have been implicated as playing critical roles in host defense responses.

Specifically, we investigated the in vivo effects of IL-6 expression on the acute phase response in mice after intraperitoneal injection of the Ad5E3mIL6 vector. In this study, we addressed the tissue specificity and the capacity of adenovirus vectors to express cytokine in vivo. In addition, the production of an IL-6 dependent acute phase protein, haptoglobin, was examined.

We used the recombinant Ad5 vectors expressing IL-5 and IL-6 to target the expression of these biologically potent molecules to mucosal tissues to investigate their respective roles in mucosal immune responses. We sought to determine whether local expression of immunomodulatory cytokines at the same time as an antigen exposure enhanced the immune response and whether IL-5 or IL-6 expressed by Ad5 recombinants, alone or in combination, mediated antibody, cellular or local mucosal immune responses.

We also administered the Ad5E3mRANTES vector intratracheally and monitored the effects of RANTES expression on changes in chemotaxis in the lung and the effects on lung physiology.

Finally, we tested this cytokine-adenovirus vector technology in its application for the treatment of cancer. We used the replication deficient vector Ad5E1mIL6A⁺ to target the expression of IL-6 to tumor cells. Several tumor regimens were attempted and the effects on tumor growth and immunity were monitored.

Chapter two

The Use of Recombinant Adenoviruses to Investigate the Effects of Cytokine Function In Vivo.

The following article, entitled "Construction of recombinant human type 5 adenoviruses expressing rodent IL-6 genes: an approach to investigate in vivo cytokine function", is published in the *Journal of Immunology*. 151:5145-5153, 1993. This paper describes the first use of recombinant adenovirus vector for cytokine gene expression and characterizes the effects of IL-6 expression on acute phase response and anatomical changes in mice after intraperitoneal administration.

The work presented in this study was performed by the author of the thesis. Dr. S. Mittal provided assistance in performing luciferase assays and lab technicians helped with cytokine bioassays. This research paper was written by the author of the thesis. Supervision was provided by Drs. J. Gauldie, F.L. Graham and C.D. Richards resulting in the multiple authorship of this paper.

Construction of Recombinant Human Type 5 Adenoviruses Expressing Rodent IL-6 Genes

An Approach to Investigate in Vivo Cytokine Function¹

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ABSTRACT. The majority of biologic functions assigned to cytokines have been characterized by in vitro assay systems which may not necessarily reflect cytokine roles in vivo. Recently, recombinant virus approaches have allowed tissue-specific expression of foreign gene products in experimental animal models. We have constructed recombinant human type 5 adenoviruses, deficient in the E3 region of the genome, with incorporated rodent IL-6 cDNA that express significant levels of biologically active IL-6 on infection both in vitro and in vivo. After i.p. injection, the liver, spleen, and peritoneum appear to be primary sites of expression, whereas the lung and bronchus are the main sites of expression after intratracheal instillation. Injection i.p. of BALB/c mice with the murine rIL-6 virus causes an increase in serum levels of bioactive IL-6 for up to 6 days post-infection, whereas similar changes are not seen in animals infected with control viruses. Coincident with enhanced plasma levels of IL-6, we detect raised serum levels of hepatic-derived acute phase proteins. Associated with the expression of IL-6 in the liver and spleen, at 7 days we note a fourfold splenomegaly with expansion of B and T cell compartments, as well as the presence of lymphoid aggregates in the liver. These morphologic changes had resolved by 16 days. Our findings demonstrate that recombinant human type 5 adenoviruses expressing cDNA for various cytokines could be used as a transient pseudo-transgenic animal model to investigate the biologic function of cytokines in vivo. *Journal of Immunology*, 1993, 151: 5145.

In vitro experiments designed to test the biologic function of cytokines have led to the characterization of a number of bioactivities which are often contradictory. It is becoming increasingly clear that the actions of a cytokine must now also take into account the extracellular

environment in which the cell receives the cytokine signal. Extracellular matrix proteins have been demonstrated to induce as well as modify the production of cytokines in a number of cell types. Conversely, cytokines have been demonstrated to alter the production of extracellular matrix proteins by stromal cells and the composition of matrix structure, indicating that an intricate regulatory network that controls immune and inflammatory responses exists within the context of a tissue (1, 2). We have designed a unique recombinant human type 5 adenovirus (Ad5) that contains cDNA for the mouse cytokine IL-6 inserted into a relevant E3-depleted site in the altered virus and that produces bioactive IL-6 upon infection (Ad5mIL-6). Due to the natural tropism and high expression capacity of adenovirus, these recombinant viruses can be used to target the expression of cytokine to local tissue sites in transient

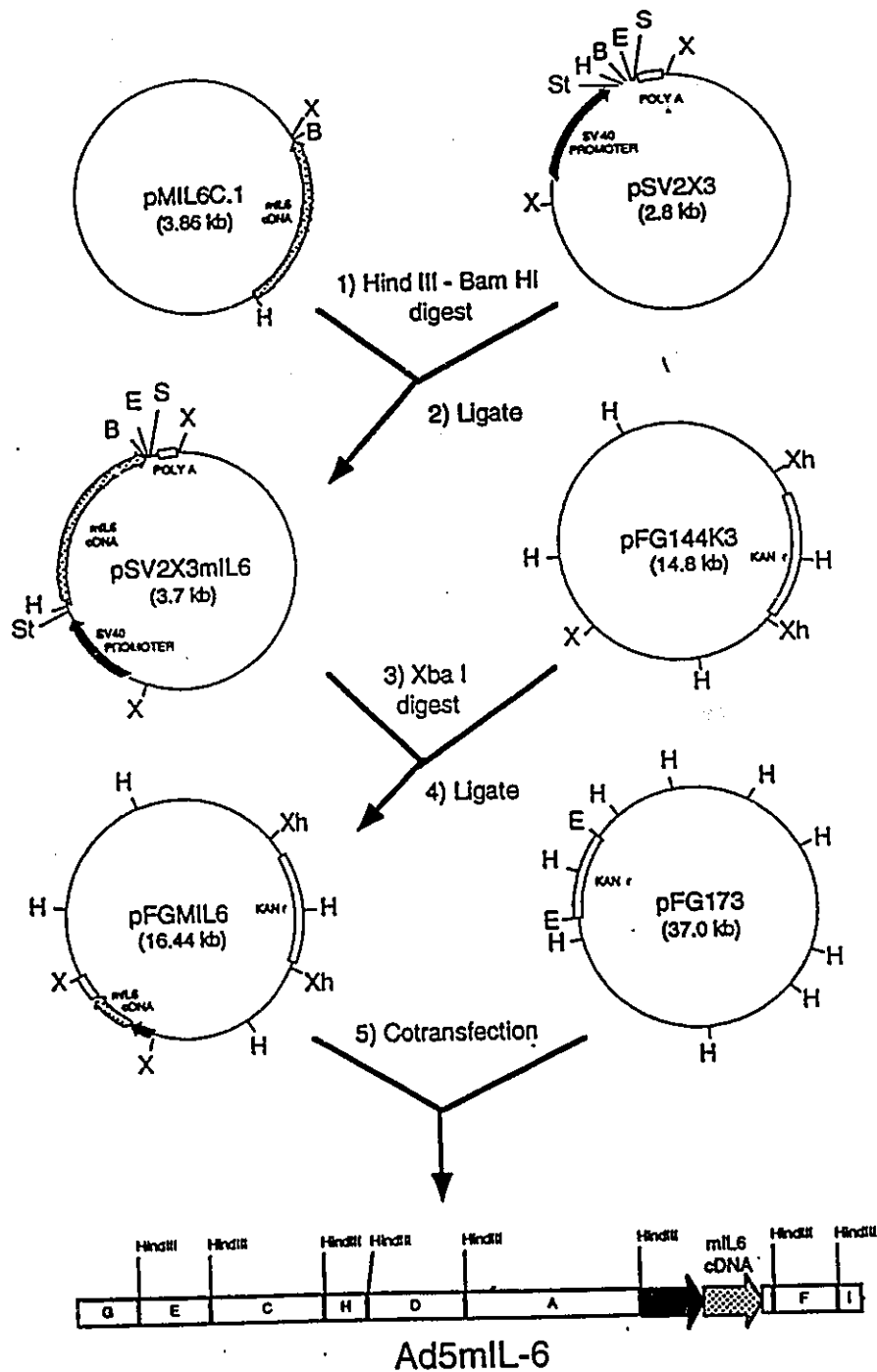
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FIGURE 1. Construction strategy of rAd5 vectors expressing mouse IL-6 cDNA. Numbers 1-5 refer to the steps as outlined in *Materials and Methods*. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sma*I; St, *Stu*I; X, *Xba*I; Xh, *Xho*I.



fashion and at levels that may be physiologically relevant. This use of rAd5 vectors provides a novel *in vivo* model in which to investigate cellular cytokine responses in the context of tissue structure.

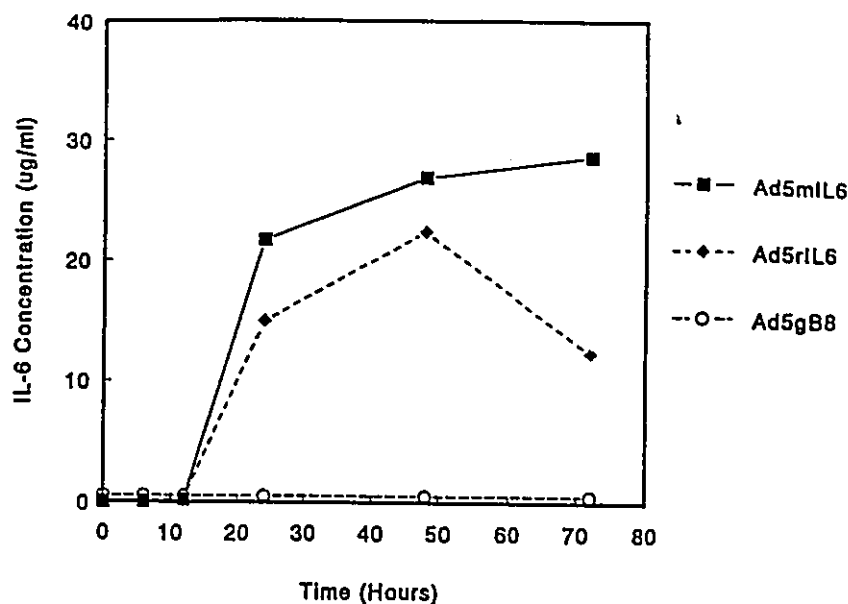
Materials and Methods

Adenovirus vector

Steps described below refer to outlines on Figure 1. 1: To construct the recombinant type 5 adenoviruses, a 945-bp

fragment containing the coding region of murine IL-6 cDNA was isolated from plasmid pMIL-6C.1 (3) by digestion with *Bam*HI and *Hind*III restriction enzymes. A 910-bp fragment containing the coding region of rat IL-6 cDNA was recovered by a *Bam*HI and *Eco*RI restriction enzyme digest of plasmid pRIL-6C.94 (4). 2: These cDNA fragments were then ligated into the multicloning site of the plasmid pSV2X3. This plasmid, a derivative of pSV2neo containing a cassette with the promoter and poly(A) addition signal sequences of SV40, was used to insert the IL-6

FIGURE 2. 5×10^6 HeLa cells were infected with 5×10^7 pfu of recombinant virus. Supernatants were tested for IL-6 bioactivity in a B9 hybridoma growth factor assay at various times. Representative data are shown for one set of cultures. No IL-6 activity was detected for cells alone, or cells infected with wild type Ad5 virus.



cDNA downstream of the SV40 promoter in an orientation that would allow transcription of the IL-6 message (5). 3: The resulting plasmid (pSV2X3mIL-6), identified and characterized by restriction mapping, contained the respective species IL-6 cDNA in the proper orientation and was digested with *Xba*I. A 1682-bp fragment containing the hybrid SV40/IL-6 gene was purified. 4: The fragment was ligated into the *Xba*I site of pFG144K3. The plasmid pFG144K3 contains the rightward 30% of the Ad5 genome, with a single *Xba*I site located in the E3 region obtained by deletion of the Ad5 *Xba* "D" fragment (mu 78.5 to 84.3) contained within the E3 region of the virus and sequences from the left end of the genome, but lacking Ad5 DNA from mu 16 to 70. 5: pFG144K3 containing the derived inserts in the E3 parallel or rightward orientation (pFGmIL-6) was used to rescue the hybrid IL-6/SV40 DNA into the Ad5 genome by cotransfection of 293 cells along with a second plasmid, pFG173, that contains all of the Ad5 genome except for a lethal deletion extending rightward from E3 into the sequences encoding fiber. The use of 293 cells allows efficient homologous recombination to occur and generates an infectious replication-competent virus carrying the insert of mIL-6 in Ad5mIL-6. Background viral plaques are eliminated by use of this cotransfection technique as the individual plasmids, each carrying a lethal deletion, are unable to produce infectious virus unless they recombine. We have used these approaches previously to construct recombinant vaccines (6).

A similar approach with the rat cDNA for IL-6 leads to Ad5rIL-6, an infectious recombinant adenovirus that expresses rat IL-6. The viruses Ad5mIL-6 and Ad5rIL-6 were recovered from the cotransfections and were plaque purified as previously described (6) an additional two times before being used in subsequent studies. For purposes of

simplicity only the Ad5mIL-6 construct is shown in Figure 1. Ad5gB8, used as a control, is a similarly constructed rAd5 virus that expresses glycoprotein B of herpes virus as previously described (7). Ad5Luc3 was constructed in a similar fashion to express firefly luciferase as described (8).

In vitro infection

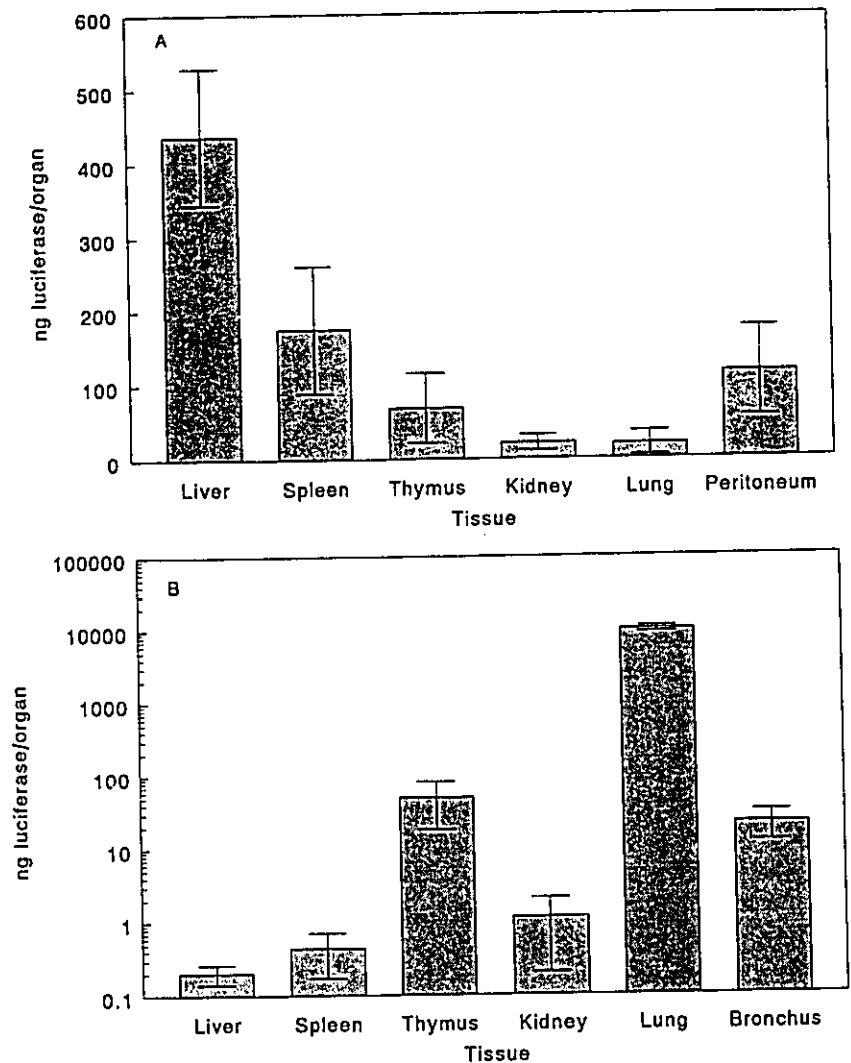
HeLa (5×10^6) cells in a 60-mm tissue culture dish were infected with Ad5mIL-6, Ad5rIL-6, Ad5gB8 or wild-type virus at 10 pfu/cell³ for 45 min at 37°C. Then 5 ml of fresh DMEM containing 5% calf serum was added to the infected cells and 100- μ l aliquots of supernatant were collected at 6, 12, 24, 48, and 72 h. Live virus was inactivated by UV irradiation for 10 min and IL-6 bioactivity was then determined for each sample by using the B9 hybridoma cell growth assay method with minor modifications (9). B9 cellular growth was determined by using the monotetrazolium colorimetric assay as described (10). The assay was standardized by using purified human and rat rIL-6 protein.

In vivo infection and distribution

A recombinant adenovirus expressing luciferase was used to determine the tissue distribution of expression in rats. 1×10^9 of Ad5Luc3 virus was administered to male Sprague-Dawley rats (Charles River Canada, St.-Constant, Quebec) (180–250g) by injection into the peritoneal cavity or by i.t. instillation. Infections were then allowed to proceed for 24 h before the animals were killed. Whole organs or tissues were removed and placed in 2, 5, or 10 ml of

³ Abbreviations used in this paper: pfu, plaque-forming unit; i.t., intratracheal.

FIGURE 3. Expression of Ad5Luc3 in the tissues of Sprague-Dawley rats. *A*, after i.p. infection on a linear ordinate; *B*, after intratracheal instillation on a logarithmic ordinate. Data points are expressed as the mean \pm SE measurement of luciferase activity recovered from individual tissue samples. $n = 3$ for each data point.



a solution of 100 mM potassium phosphate containing 1 mM PMSF and 10 μ g/ml aprotinin. Tissues were homogenized and sonicated. Cellular debris was removed by centrifugation from each tissue sample and 50 μ l of this supernatant was then assayed for luciferase activity by using a luminometer (Lumat LB 9501) in 350 μ l of assay reagent (11). Total luciferase expression per organ or tissue was determined against a 1 mg/ml standard solution of luciferase enzyme supplied by Sigma (St. Louis, MO).

In vivo expression of IL-6

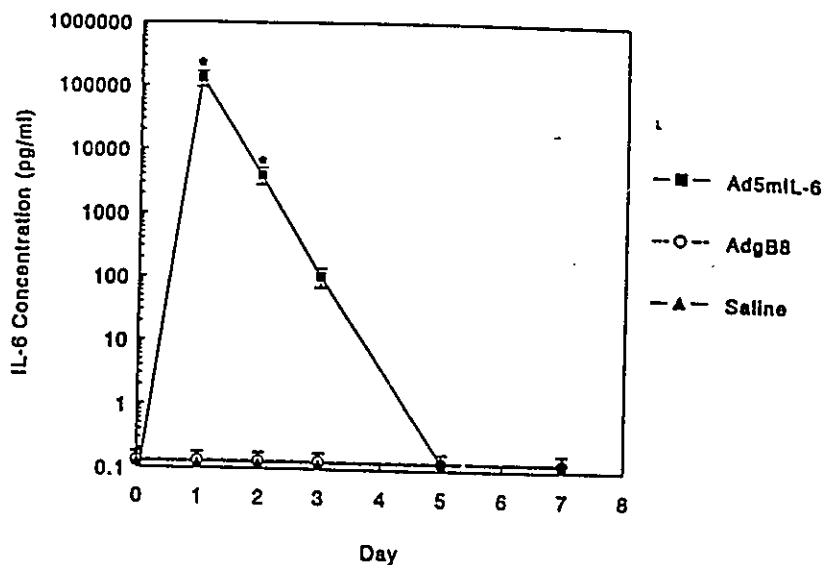
Six- to 8-wk-old BALB/c female mice (four animals per group) were injected i.p. with either Ad5mIL-6 or Ad5gB8 at 2×10^8 pfu of virus per animal. Individual blood samples were collected by retro-orbital bleed from each group on days 0, 1, 2, 3, 5, and 7. Serum samples were then analyzed for IL-6 biologic activity in the B9 hybridoma growth assay (9). Rocket immunoelectrophoresis for acute phase proteins as previously described (12) was performed on sera collected from infected BALB/c mice. For the analysis of

haptoglobin levels, 200 μ l of haptoglobin antisera (Atlantic Antibodies, Stillwater, MN) was added to 12 ml of a 1% agarose gel in barbital buffer. For α_1 -acid glycoprotein levels, 200 μ l of a monospecific rabbit anti-mouse α_1 -acid glycoprotein antiserum (generously provided by Dr. H. Baumann, Roswell Park, NY) was used. Seven microliters of 150-fold diluted serum samples were loaded per well and the gel was run overnight at 60 V. The gels were then dried and stained with Coomassie blue. After destaining, peak heights were measured and protein amounts were determined against a standard curve that was generated from a pool of serum obtained from mice undergoing LPS-mediated inflammation and quantified against purified protein standards. All statistical analysis was performed using STATPAK 4.1 programs.

Results and Discussion

Infectious virus was used in vitro to infect 293 and HeLa cells. Bioactive IL-6 was detectable in the supernatant at

FIGURE 4. Determination of serum levels of IL-6 in rAd5 infected animals. Data points are expressed as the mean \pm SE measurement of bioactive IL-6 as measured by the B9 hybridoma growth factor assay. $n = 4$ for each data point. The levels for AdgB8 were not different from those seen in saline-treated animals. * $p < 0.01$ for values compared to control virus or saline by Student's paired *t*-test.



approximately 12 h post-infection and increased to very high levels (20–30 $\mu\text{g/ml}$) up to 48 or 72 h post-infection (Fig. 2 shows representative data for HeLa cell infection). The IL-6 bioactivity in the B9 assay was neutralized by a rabbit antiserum raised against purified recombinant rat IL-6. This antiserum neutralized mouse and rat IL-6 from natural and recombinant sources in both B9 hybridoma growth and hepatocyte stimulation assays (12). In addition, the antiserum precipitated ^{35}S -methionine-labeled proteins in the 23 to 30-kDa range, consistent with the molecular masses reported for IL-6 (data not shown). These multiple bands may reflect differences in glycosylation as reported for human cell-derived IL-6 (13, 14).

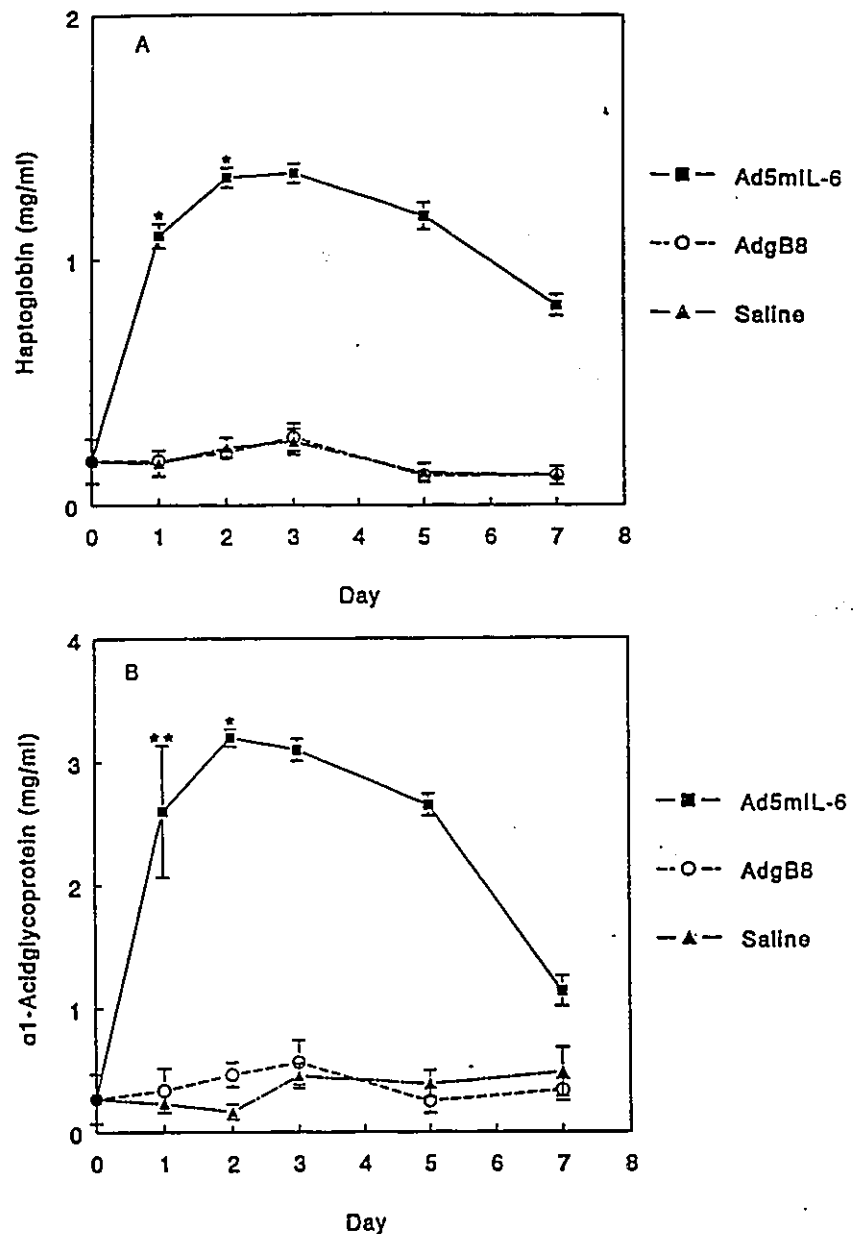
Infection *in vivo* by i.p. injection of 1×10^9 pfu of Ad5Luc3 virus into rats resulted in the greatest levels of luciferase expression being recovered from the liver (53% of the total activity) at 24 h post-inoculation, with lesser amounts expressed in the spleen and the peritoneal lining of the cavity (Fig. 3A). In addition, the outer peritoneal edges of the tissues contained higher amounts of recoverable luciferase activity than did the center of the organ (data not shown). Small but significant amounts (<12% of total activity) were expressed in the kidney, thymus, and lung. This tissue distribution is similar to that we have reported for the same virus in mouse by i.p. injection (8). Infection of rats by i.t. instillation (1×10^9 pfu Ad5Luc3) resulted in expression of luciferase almost exclusively (>98%) in the lung and bronchus with a small amount in the thymus (Fig. 3B: note that the recoverable luciferase activity is expressed in logarithmic manner) indicating there are preferential sites of virus infection and localization after different routes of administration, with i.p. injection targeting the liver, spleen, and peritoneum, and i.t. instillation targeting the lung and airways. It has previously been shown that IL-6 is the major regulator of the hepatic acute phase response through *in vitro* and *in vivo* challenges with ex-

ogenous recombinant cytokine (15–17). Injection i.p. of 2×10^8 pfu Ad5mIL-6 into the mouse resulted in a time-dependent increase in circulating levels of IL-6 bioactivity. The serum levels were raised as early as 12 h and were at a maximum by 24 h. Enhanced serum levels were present up to 4 days after infection (Fig. 4) and in some experiments, serum IL-6 activity was detected up to day 6. Infection with control virus, either wild-type Ad5 or one expressing an unrelated Ag (Ad5gB8), did not elicit a detectable serum IL-6 response. As was the case for the product expressed *in vitro*, the IL-6 bioactivity in the serum was neutralized in a B9 hybridoma growth assay by the rabbit anti-IL-6 antibody. The levels of circulating IL-6 found in Ad5mIL-6-infected mouse sera were comparable to those seen after a s.c. injection of turpentine, known to elicit a marked acute phase response.

Coincident with the detection of increased levels of IL-6 in the circulation, we detected significantly raised serum levels of several hepatic-derived acute phase proteins including haptoglobin and α_1 -acid glycoprotein (Fig. 5A and B). The levels of induction of the acute phase proteins were similar to those seen in an experimental inflammatory model, such as the response to turpentine (18). As the levels of IL-6 in the circulation subsided, so did the levels of serum acute phase proteins. No acute phase protein response was seen in infections with either wild-type or Ad5gB8 viruses with values falling within the expected normal range for the animals (day 0 values). Thus the IL-6 that is produced during infection with the rAd5mIL-6 virus expresses a biologic role by initiating the hepatic acute phase response.

The use of an E3-deleted Ad5 mutant leaves the recombinant virus intact in the E1 region and therefore functionally competent for replication, particularly in human cells. In mouse and rat cells *in vitro*, replication is detected only at very low levels because the virus is only semi-permissive

FIGURE 5. Serum levels of acute phase proteins in rAd5-infected animals. Data points are expressed as the mean \pm SE measurement of haptoglobin (A) and α_1 -acidglycoprotein (B) as measured by immunoelectrophoresis. $n = 4$ for each data point. Normal ranges for untreated animals are shown on the ordinate at time = 0. * $p < 0.001$, ** $p < 0.02$ for values compared to control virus or saline by Student's paired t -test.



in the rodent species so that a low level of replication *in vivo* in the rodent is possible. Although the level of expression of the cytokines encoded in the recombinant viruses is significant, it is still not clear whether viral replication *per se* occurs. The kinetics of the *in vitro* expression for Ad5mIL-6 (Fig. 2) coincides with Ad5Luc3 virus luciferase expression shown previously (8) and implies that the IL-6 expression is primarily derived from the activity of the adenovirus major-late promoter, although some early expression may be derived from the SV40 or E3 promoter, all of which are located upstream of the IL-6 cDNA sequences in our rAd5 viruses. The decrease in serum IL-6 seen over 4–5 days indicates that the limited yields of infectious virus after first round replication and cell lysis in the mouse is insufficient to reinfect adjacent cells and, thus, the high

level of expression seen immediately after first inoculation begins to decrease. A similar decrease in expression was previously reported for the luciferase virus construct (8).

At day 7, we detected a fourfold splenomegaly with evidence of lymphoid expansion of germinal centers and of trabecular areas of the spleen (Fig. 6A) similar to results reported for systemic administration of rIL-6 over 3 days (19). Preliminary data indicate a significant increase in the antibody response against adenovirus Ag (8) in the Ad5mIL-6 infected animals at this time (data not shown). Also at day 7, many lymphoid aggregates were noted in the liver of Ad5mIL-6-treated mice (Fig. 6B). No such changes were seen in mice receiving saline or an injection of an equal number or 10-fold greater pfu of control Ad5gB8 virus. At 16 days, spleen size and germinal center mor-

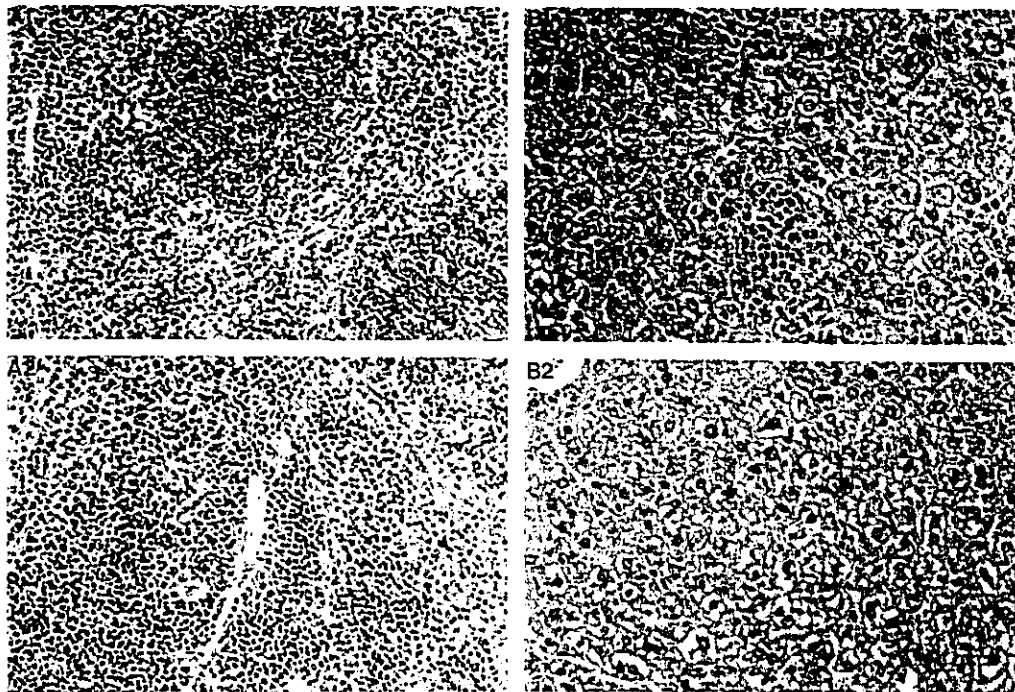


FIGURE 6. Morphology of spleen and liver at 7 days after infection with (1) Ad5mIL-6 or (2) control virus. A, spleen $\times 300$; B, liver $\times 400$. Tissues were fixed in 4% paraformaldehyde and sections stained with hematoxylin and eosin. Spleen germinal centers were greatly expanded in IL-6-treated mouse.

phology had returned to normal, indicating the transient nature of the splenomegaly. Similarly, the lymphoid aggregates in the liver were no longer apparent at 16 days. No other abnormalities in hepatic morphology were seen, such as those described by Hawley et al. (20) where the use of a retrovirus vector to introduce IL-6 into bone-marrow cells followed by reconstitution of an X-irradiated syngeneic host caused multinucleated giant hepatocyte formation in the liver.

Our previous experience with chronic (greater than 10 days) injections of rat rIL-6 into rats indicated that the liver continued to respond to exogenous administration of IL-6 by inducing the synthesis of acute phase proteins. In the current model, the method of hepatic stimulation by the adenovirus-encoded IL-6 is, at this point, speculative. Given that the adenovirus expression of incorporated genes occurs within the liver and the peritoneal lining of the organ as well as other tissues, the data are consistent with the hepatocyte either responding to IL-6 generated at other tissue sites and arriving at the liver through the circulation (exocrine) or responding to IL-6 generated within the liver in a paracrine or perhaps autocrine (21) fashion or a combination of pathways depending on the cell type(s) infected with the virus. Examination of the liver by *in situ* hybridization techniques may provide information regarding these possible mechanisms.

Eukaryotic viruses, including retrovirus and vaccinia virus, have been increasingly used as expression vectors for

gene transfer and as experimental recombinant viral vaccines (20, 22–26). The current use of homologous cDNA in the respective species (mouse IL-6 into mouse) eliminates the concern of immune responses against foreign cytokines, which exists in a number of other *in vivo* studies. rAd5 vectors deficient in E3, E1, or both may provide unique applications distinct from vaccinia and retroviral vectors due to differences in their tissue localization, pattern of expression in non-replicating cells, and mode by which they can be administered (5, 8, 21, 27–30). The recent occurrences of expression of human α_1 -antitrypsin and human cystic fibrosis transmembrane-conductance-regulator genes in the lungs of cotton rat (31, 32) and our findings reported here on expression of rAd5 vectors in the lung of Sprague-Dawley rat demonstrate the potential usefulness of this system for tissue-localized gene expression, especially at mucosal sites. The fact that biologically active mediator molecules or cytokines can be transiently overexpressed in a normal tissue through directed infection with pathophysiologic outcomes shows that this system could be applied to numerous other molecules. The transient but prolonged nature of overexpression of bioactive mediators may be more representative of tissue responses to infection or trauma, than to exogenous injection of factors or constant expression as in transgenic systems. Moreover, it may be possible to use a similar system to deliver anti-sense sequences for transient cytokine “knock-out” experiments. Finally, recombinant adenovirus/cytokine constructs may

prove useful as infectious vectors to induce high cytokine production in tumor cells for autologous vaccine use, as has recently been described for retroviral vectors (33).

Acknowledgments

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Chapter three

Generation of a Polyclonal Antiserum Against Cytokine Expressed by a Recombinant Adenovirus Vector.

The following article, entitled "Vector derived expression of recombinant rat interleukin-6", is accepted for publication in *Protein Expression and Purification*, 1996. This paper describes the novel expression vector, plasmid pRIL6.992, which expresses rat IL-6 as a fusion protein with histidine hexapeptide at its N-terminus. It also demonstrates for the first time the use of a recombinant adenovirus to generate a polyclonal antiserum against vector expressed cytokine after immunization in rabbits.

The work presented in this study was performed by the author of the thesis with help from lab technicians with cytokine purification and bioassays. Dr. W. Northemann contributed the expression plasmid pRIL6.992 used in this study. This paper was written by the author of the thesis. Drs. J. Gauldie and W. Northemann provided supervision to this study resulting in multiple authorship.

VECTOR DERIVED EXPRESSION OF RECOMBINANT RAT INTERLEUKIN-6

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RUNNING TITLE

Expression of recombinant rat interleukin-6 hexahistidine fusion protein

ABSTRACT

Rat interleukin-6 cDNA (IL-6), coding for an important inflammation and immune regulatory polypeptide cytokine, was cloned into the novel expression vector pH6EX3 which directs the synthesis of inserted genes as a fusion protein with histidine hexapeptide (HH). The resultant vector (pRIL6.992) was shown to produce significant amounts of recombinant rat IL-6 fusion protein with HH at its N-terminus in various strains of *E. coli*. The expression of the HH-IL-6 fusion protein was demonstrated to be under the control of the tac-promoter and could be induced by IPTG. This protein was isolated from bacterial inclusion bodies and purified to homogeneity in a one-step procedure by affinity chromatography using a nickel chelating column. The HH-IL-6 fusion protein isolated in this manner was biologically active as determined by hepatocyte stimulation and B9 hybridoma growth assays. Further, this activity was neutralized with a polyclonal anti-serum raised against rat IL-6 protein generated in a novel fashion from rabbits infected with a recombinant human type 5 adenovirus vector expressing rat IL-6 protein (Ad5E3rIL6). The recombinant HH-IL-6 protein was then used to boost Ad5E3rIL6 immunized rabbits. This resulting antiserum was shown to neutralize recombinant and natural rat and murine IL-6 bioactivity *in vitro* and was useful in Western blot analysis and immunohistochemistry of rat IL-6.

KEYWORDS

Interleukin-6, expression, recombinant protein, recombinant adenovirus, rat, protein purification, prokaryotic system

INTRODUCTION

The initiation of the human genome project has spurred on the rapid advances in sequencing technology and has led to the dramatic increase in the number of genes available for basic research study (1,2). These changes have brought to the forefront limitations in the technologies to keep pace and characterize the biological functions of the now numerous gene sequences generated. Detailed studies on the function and regulation of these novel genes will require the availability of antibody reagents and sufficient quantities of highly pure recombinant protein.

In this report we describe the expression and purification of recombinant rat IL-6 in Esherichia coli as a hexahistidine fusion protein using a novel highly efficient expression vector system pH6EX3. The use of this vector system allows high-level expression and single step purification of recombinant protein by affinity chromatography using a chelating matrix charged with Ni²⁺ ions. In addition, we demonstrate the use of the recombinant adenovirus vector Ad5E3rIL6 to express IL-6 and to directly generate polyclonal anti-sera against rIL-6 protein after vector administration in rabbits. This use of recombinant adenovirus vectors may be appropriate for generating polyclonal antisera to other novel genes since it does not require purification of the unknown protein and only involves a single cloning step of the gene into an adenovirus rescue vector. Incorporation of these strategies may facilitate in the more rapid

characterization and functional analysis of unknown proteins.

MATERIALS AND METHODS

Plasmids and cDNA

Plasmid pRIL-6.96, an expression vector containing the rat IL-6 cDNA which produces recombinant rat IL-6 as a fusion protein with glutathione S-transferase, was used to isolate the rat IL-6 cDNA for the construction of pRIL6.992 (3). As described, plasmid pRIL-6.96 was constructed using the plasmid pRIL6.94 containing the full length cDNA clone of rat IL-6 (4) and further modified to contain only a 791-bp fragment encoding the reading frame of rat IL-6.

Expression vector pH6EX3 was constructed from pGEX-2T (5) and possesses the ampicillin-resistant gene, the modified *E. coli* lac repressor gene and transcriptional termination sequences. The lac promoter controls the expression of a fusion protein with histidine hexapeptide at its N-terminus separated from a multicloning site by a site-specific sequence for thrombin cleavage as described (6).

Expression of HH-IL-6 Fusion Protein by *E. coli* and Time Course

Various *E. coli* strains transformed with IL-6 plasmid (pRIL-6.992) were cultured in LB-medium containing ampicillin overnight, diluted 10-fold with fresh pre-warmed LB-medium, and incubated for 90 min at 37°C prior to the

induction with 1.0 mM IPTG for 8 hours. Aliquots of 100 μ l were collected and analyzed by PAGE. For the time course determination, the highest HH-IL-6 protein expressing *E. coli* strain K5254 was used. Samples were prepared and analyzed as previously described (6,7).

Purification of the HH-IL-6 Fusion Protein

5 ml of an overnight culture of IL-6 plasmid (pRIL-6.992) transformed K5254 were inoculated into 50 ml of LB-medium containing 150 μ g/ml of ampicillin and were incubated for 1.5 hrs at 37°C. Cells were then induced with 1 mM IPTG for 8 hrs and pelleted by centrifugation at RT for 10 min at 4000 rpm. Cells were resuspended in 10 ml of PBS. For lysis the cells incubated in the presence of 1 mg/ml lysozyme (Sigma, St. Louis/MO) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) for 20 min at 0°C followed by treatment with 1% Triton X-100 for 10 min at 0°C. After sonication (2 x 20 sec) the cell homogenate was separated by centrifugation (8,000 x g) for 5 min at 4°C. The insoluble fraction containing the recombinant IL-6 protein was dissolved in 6 ml of sample buffer (50 mM Tris-acetate pH 7.2, 6M guanidine.HCl and 0.5 M NaCl) for 1 hr at 0°C. The sample was sonicated an additional 2 x 2 sec to further solubilize this fraction. To remove remaining insoluble protein, the fraction was centrifuged again at (8000 x g) for 20 min at 0°C. Supernatants were then loaded onto 2 ml of a Nickel-Sepharose

Column. To prepare the column, 2 ml of Chelating Sepharose FF (Pharmacia) was loaded into 5 ml disposable syringe. Sepharose was then washed twice with deionized water (dH₂O). The column was then loaded with 10 mg/2 ml of a NiCl₂.6H₂O solution. Finally, the column was washed with 5 ml of sample buffer. Protein was eluted from the column in 5 ml aliquots of 0.1 M Tris-acetate ranging in pH from 7.2 to 4.0. The sample eluting at pH 4.0 to 4.5 was dialyzed against PBS and stored at -70 °C.

IL-6 Bioassays

IL-6 biological activity of the isolated recombinant HH-IL-6 fusion protein was determined in the B9 hybridoma growth assay and in a hepatocyte stimulation assay as previously described (8).

Generation of Polyclonal Neutralizing Sera using Recombinant Adenovirus Vector

Ad5E3rIL-6 is a recombinant human type 5 adenovirus incorporating the rat IL-6 cDNA as described previously (8). In short, the Ad5E3rIL6 recombinant vector contains the coding sequences of rIL-6 cDNA inserted into the E3 region of the adenovirus genome in an orientation in which the majority of transcription is driven off the major late promoter. This vector can express significant levels of rat IL6 both *in vitro* and *in vivo*. New Zealand white rabbits

were given intraperitoneal injections with 1×10^9 pfu of Ad5E3rIL-6 virus. After 3 weeks, animals were tested and boosted at 1 month intervals with 50 μ g purified recombinant IL-6 in saline. Sera were collected on a weekly basis subsequent to the initial immunization with vector and after boosting with recombinant HH-IL-6 fusion protein. These serum samples were tested in both the B9 hybridoma growth factor and hepatocyte stimulation assays for the ability to neutralize a recombinant rat IL-6 standard. To neutralize the HH-IL-6 fusion protein bioactivity in the B9 assay, various concentrations of fusion protein were diluted in 50 μ l of the complete B9 growth media and incubated with a 1/50 dilution of the polyclonal rabbit anti-rat IL-6 sera for 1 hr at 37 °C. Samples were then run in the B9 assay and biological activities were determined.

Western Blot Analysis of the HH-IL-6 Fusion Protein

The inclusion body fraction of the bacterial cell lysate was separated by 10% PAGE and electrophoretically transferred onto nitrocellulose filters (Amersham, Buckinghamshire/England) using the trans-blot semi-dry electrophoretic transfer cell (Bio-Rad, Richmond/CA). The filter was incubated with a 250-fold dilution of the rabbit anti-rat IL-6 recombinant adenovirus vector generated sera. The bound antibodies were visualized with anti-rabbit immunoglobulins conjugated with alkaline phosphatase (Promega, Madison/WI)

followed by the color reaction using NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) as substrates (Promega).

RESULTS

Construction of the expression fusion vector pRIL-6.992

To construct the plasmid pRIL-6.992, a 791 bp Bam H1/EcoR1 digest cDNA fragment coding for rat IL-6 (3) was ligated into the multicloning site of pH6EX3 as described (6). The plasmid pH6EX3 contains the tac promoter with the translation start site (ATG, position 288) ten bases downstream of the ribosome binding site. The expression of the cloned rat IL-6 cDNA is then controlled by the tac promoter which can be induced by IPTG (Figure 1). This tac promoter is normally repressed by the lac repressor which is directed by the lac repressor gene contained in pH6EX3. Therefore, this expression system can be used in all available *E. coli* strains independently of endogenous established lac repressor genes (6). The correct orientation and the restored open reading frame of the fusion construct between the HH and rIL-6 were confirmed by sequencing the cloning junctions.

Expression of the HH-Rat IL-6 Protein

To confirm the expression of IL-6 by pRIL-6.992 induced with IPTG, five *E. coli* strains LE392, CAG440, CAG456, GE196 and K5254 were transformed and induced with 1 mM IPTG for 8 hrs with pRIL-6.992 or pH6EX3 as a control (Figure 2). All five strains transformed with pRIL-6.992 produced a protein

band with an apparent molecular weight of 25 kD. This protein was confirmed to be rat IL-6 by western blot (Figure 3). Two of the strains (GE196 and LE392) produced a similar molecular weight protein species in the pH6EX3 control, however, these proteins did not react with the rat IL-6 antibody in the western blot and must be due to the presence of an unidentified 25 kD protein in the extract. The observed molecular mass of the protein band was in agreement with the predicted amino acid sequence of the mature rat IL-6 which lacks potential N-glycosylation sites (4). The highest yield of recombinant protein was obtained in the *E. coli* strain K5254 and the kinetics of expression of IL-6 was determined for this strain (Figure 4). Expression could be detected as early as 2 hrs and reached a maximum by 10 hrs. Under optimal conditions the recombinant protein constituted up to 20% of total cellular proteins.

Purification of the HH-IL-6 Fusion Protein

The *E. coli* strain K5254 cell culture transformed with pRIL-6.992 was induced with 1 mM IPTG for 8 hrs and harvested. Cells were lysed by treatment with lysozyme and sonication and the cell homogenate separated into soluble and insoluble fractions (Figure 5, lane 1,2,3). As shown in lane 3, the rat HH-IL-6 fusion protein remained entirely in the insoluble cell fraction representing the inclusion bodies. This insoluble fraction was dissolved in 6 M guanidine.HCl and loaded onto a chelating Sepharose column charged with Ni²⁺

ions and washed with pH-step gradients from pH 7.2 to 4.0 (Figure 5). The rat HH-IL-6 fusion protein eluted in a highly purified form in the pH 4.0-4.5 fraction (Figure 6).

RECOMBINANT RAT IL-6 BIOACTIVITY AND NEUTRALIZATION

The various recovered pH gradient samples were tested for bioactivity in the IL-6 dependent B9 cell growth assay. IL-6 bioactivities were measured using mouse recombinant IL-6 protein as a standard (Table 1). While the solubilized inclusion body and pH 5.0 fraction samples contained high levels of rat HH-IL-6 fusion protein, the majority of the recombinant HH-IL-6 protein was found to elute at pH 4.0. This pH 4.0 fraction appeared to contain HH-IL-6 fusion protein in its purest form based on the Western blot analysis. The purified recombinant rat protein was shown to cause a dose-dependent stimulation of rat H35 hepatoma cells to express and secrete cysteine proteinase inhibitor and α_1 -acid glycoprotein, two acute phase proteins known to be stimulated by IL-6 (9). Interfering or inhibitory substances were removed from the crude supernatant yielding higher specific activities in the purified material. Thus, the column purified rat HH-IL-6 fusion protein retained bioactivity and yielded recoveries ranging from 0.8 to 2.5 mg of protein per 50 ml of bacterial culture in the pH 4.0 eluted fraction. This represented a yield of approximately 4% of IL-6 protein from 600 mg of total protein loaded onto the Ni²⁺ chelating

column.

Sera generated after recombinant adenovirus vector (Ad5E3rIL6) immunization of New Zealand rabbits was preliminarily tested for its ability to neutralize IL-6 bioactivity. This early anti-sera was able to neutralize natural rat IL-6 bioactivity in the B9 hybridoma growth assay at low dilutions (data not shown). These rabbits were then boosted with recombinant HH-IL-6 protein and the sera collected was used in subsequent analyses. Recombinant rat and mouse and natural IL-6 activity was neutralized with polyclonal anti-sera raised against rat IL-6 with 10 μ l of antibody able to neutralize 10 ng of recombinant rat IL-6 in the B9 assay.

Discussion

Previously, we have demonstrated the use of the pH6EX3 expression system to express antigenic epitopes for the screening of autoimmune disorders (6). This vector containing a multicloning region possessing eight restriction sites allows the insertion of cDNA sequences in all three possible protein reading frames fused to the sequence coding for the histidine-hexapeptide through a thrombin-sensitive sequence. This fusion with the histidine-hexapeptide allows for easy purification of protein retained in bacterial inclusion bodies by retention on Ni²⁺-Sepharose columns and elution by a pH gradient in the presence of denaturing buffer if necessary. Here we demonstrate the ability

of this system to produce significant amounts of highly purified bioactive recombinant rat IL-6 to aid in the investigation of the function of this cytokine. The availability of large quantities of purified rat IL-6 protein will complement rat animal models and eliminate the possibility of immune response against foreign IL-6 from a non-homologous species when used *in vivo*.

This system should be suitable for the expression of other bioactive molecules including the increasing number of protein's of unknown function whose gene sequences have been determined. Even in the absence of a biofunctional assay, the abundant production of recombinant protein from the pH6EX3 vector system should be suitable for the generation of anti-sera. These anti-sera may be useful in helping to determine the unknown proteins biological functions or at least provide information on the cellular localization assuming proper refolding of the protein occurs.

In addition to the pH6EX3 system, we demonstrate the ability to generate a neutralizing antibody against a foreign protein without the need to purify it first. Using a recombinant adenovirus containing the rat IL-6 cDNA sequence (Ad5E3rIL6), it was possible to generate a neutralizing antisera against rat IL-6 protein. This technology could be used to raise antisera against proteins which are difficult to recover and/or generate antibodies against.

In conclusion, the combination of the pH6EX3 expression system and recombinant adenovirus technology provide easy and rapid methods for

producing recombinant protein and neutralizing antisera for a number of biofunctional molecules, thus providing the necessary tools for the characterization of their function.

Abbreviations

His, histidine; HH-IL-6, fusion protein of histidine-hexapeptide and interleukin 6; IPTG, isopropyl- β D-thiogalactoside; PBS, phosphate-buffered saline

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Figure Legends

Figure 1 Structure of the rat IL-6 expression vector pRIL6.992. The pRIL6.992 vector contains the ampicillin-resistant gene (amp^r), the modified *E. coli* lac repressor gene ($lacI^q$), and the transcriptional termination sequence (rrnBT). The tac promoter (P^{tac}) controls the expression of the rat IL-6 fusion protein with a histidine hexapeptide (H_1-H_6) at its N-terminus. The rat IL-6 cDNA sequences are inserted into the multicloning region between the Bam HI and Eco RI restriction sites. The multicloning site is flanked by three stop codons located in all three reading frames. Sequences coding for a site-specific thrombin cleavage domain are located adjacent to the histidine hexapeptide.

Figure 2 Expression of HH-IL-6 fusion protein by *E. coli* CAG440 cells were transformed with pH6EX3 (lane 1) and pRIL6.992 (lane 2), CAG456 cells with pH6EX3 (lane 3) and pRIL6.992 (lane 4), GE196 cells with pH6EX3 (lane 5) and pRIL6.992 (lane 6), K5256 cells with pH6EX3 (lane 7) and pRIL6.992 (lane 8), LE392 cells with pH6EX3 (lane 9) and pRIL6.992 (lane 10), respectively. 100 μ l total cell lysate was analyzed in SDS-PAGE after induction with 1 mM IPTG for 8 h.

Figure 3 Western blot analysis of HH-IL-6 fusion protein. CAG440 cells were transformed with pH6EX3 (lane 1) and pRIL6.992 (lane 2), CAG456 cells with pH6EX3 (lane 3) and pRIL6.992 (lane 4), GE196 cells with pH6EX3 (lane 5) and pRIL6.992 (lane 6), K5256 cells with pH6EX3 (lane 7) and pRIL6.992 (lane 8), LE392 cells with pH6EX3 (lane 9) and pRIL6.992 (lane 10), respectively. 100 μ l total cell lysate was separated in SDS-PAGE after induction with 1 mM IPTG for 8 h, transferred to nitrocellulose membrane and analyzed by Western blotting using rabbit anti-rat IL-6 antiserum.

Figure 4 Expression kinetic of HH-IL-6 fusion protein. K5254 cells transformed with pRIL6.992 were induced with 1 mM IPTG, lysed with lysozyme and Triton X-100 followed by sonication. Total E. coli cell lysate were analyzed after 0h (lane 1), 2h (lane 2), 4h (lane 3), 6h (lane 4), 8h (lane 5), 10h (lane 6), 12 h (lane 7), and 24 h (lane 8) induction.

Figure 5 Expression of HH-IL-6 fusion protein. K5254 cells transformed with pRIL6.992 were induced with 1 mM IPTG for 8h, lysed with lysozyme and Triton X-100 followed by sonication. Total E. coli cell lysate (lane 1), soluble protein fraction (lane 2), and insoluble cell fraction (inclusion bodies) (lane 3) are shown.

Figure 6 Purification of HH-IL-6 fusion protein. K5254 cells transformed with pRIL6.992 were induced with 1 mM IPTG for 8h, lysed with lysozyme and Triton X-100 followed by sonication and loaded onto Ni²⁺ chelating column and pH gradient eluted fractions collected. Solubilized inclusion body (lane 1), pH 7.2 (lane 2), pH 6.5 (lane 3), pH 6.0 (lane 4), pH 5.5 (lane 5), pH 5.0 (lane 6), and pH 4.0 (lane 7), respectively. 100 μ l of eluted fraction was separated in SDS-PAGE.

Table 1

IL-6 Bioactivity as Measured in the B9 Hybridoma Growth Assay

Column Fraction	rat IL-6 (units)
Inclusion Body	15.2×10^8
pH 7.2	2.9×10^7
pH 6.5	0.2×10^8
pH 6.0	0.0
pH 5.5	0.0
pH 5.0	12.8×10^8
pH 4.0	18.3×10^8

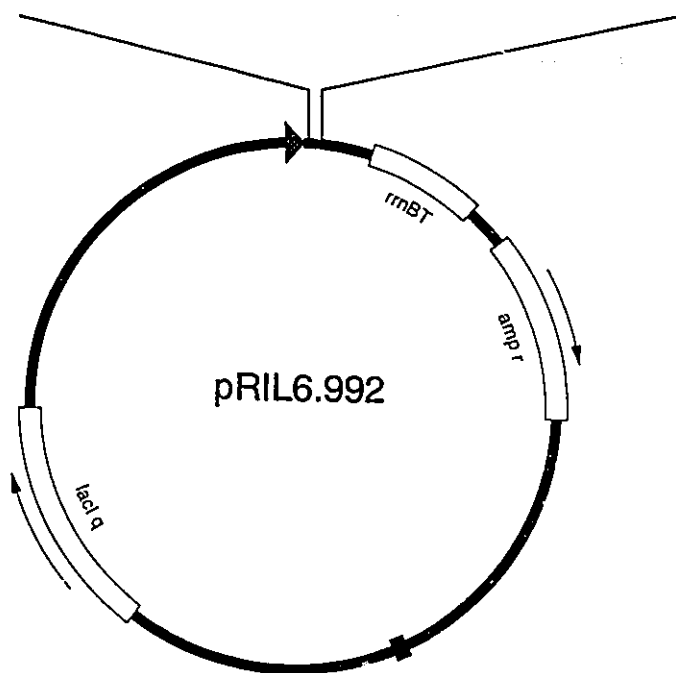
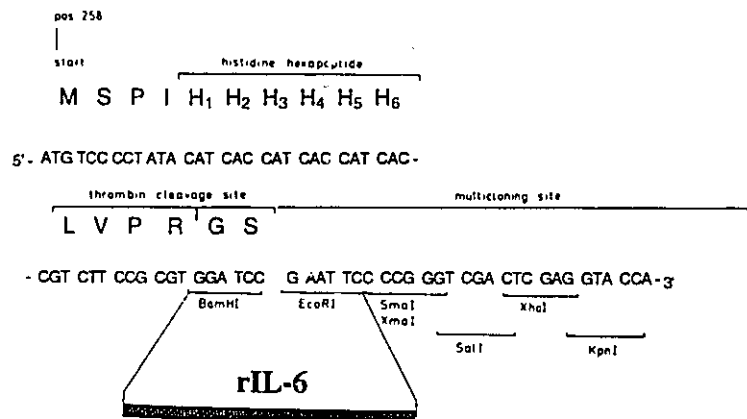


Figure 1

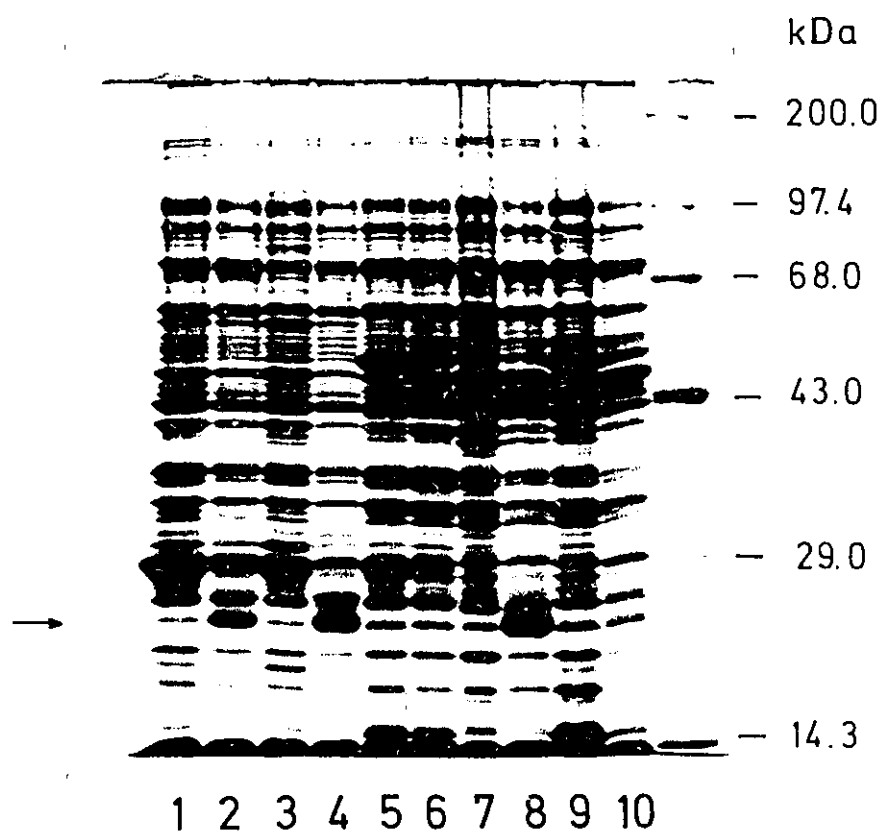


Figure 2

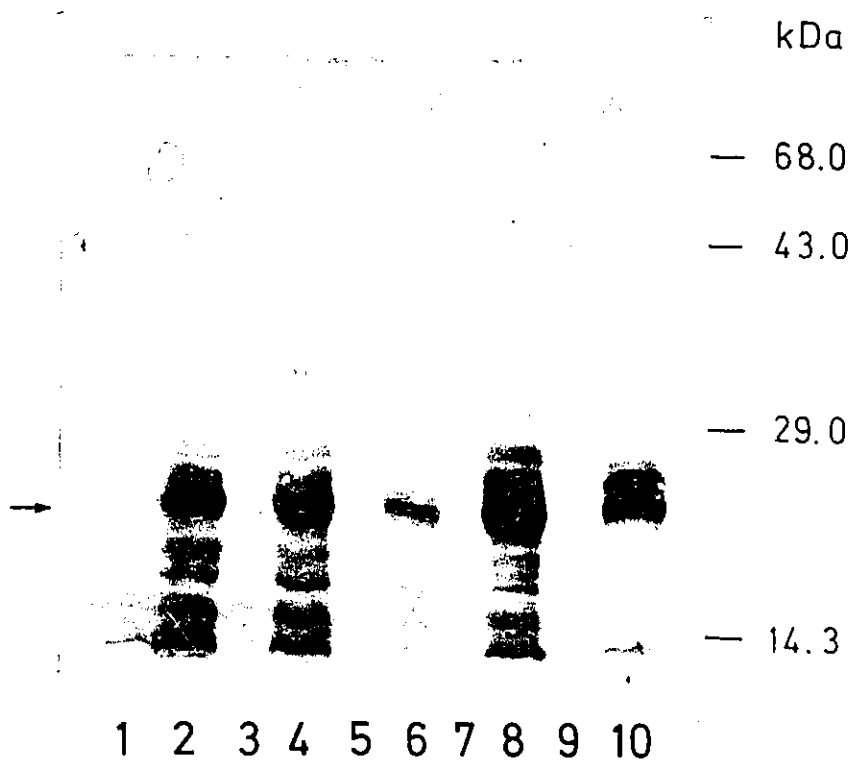


Figure 3

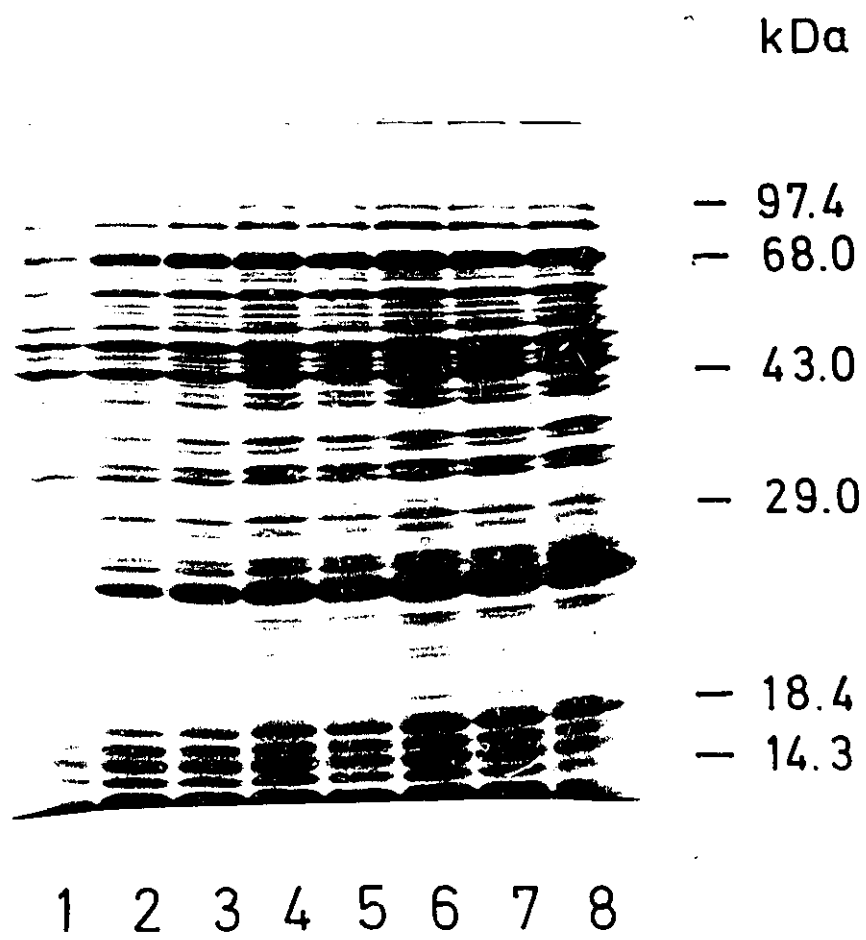


Figure 4

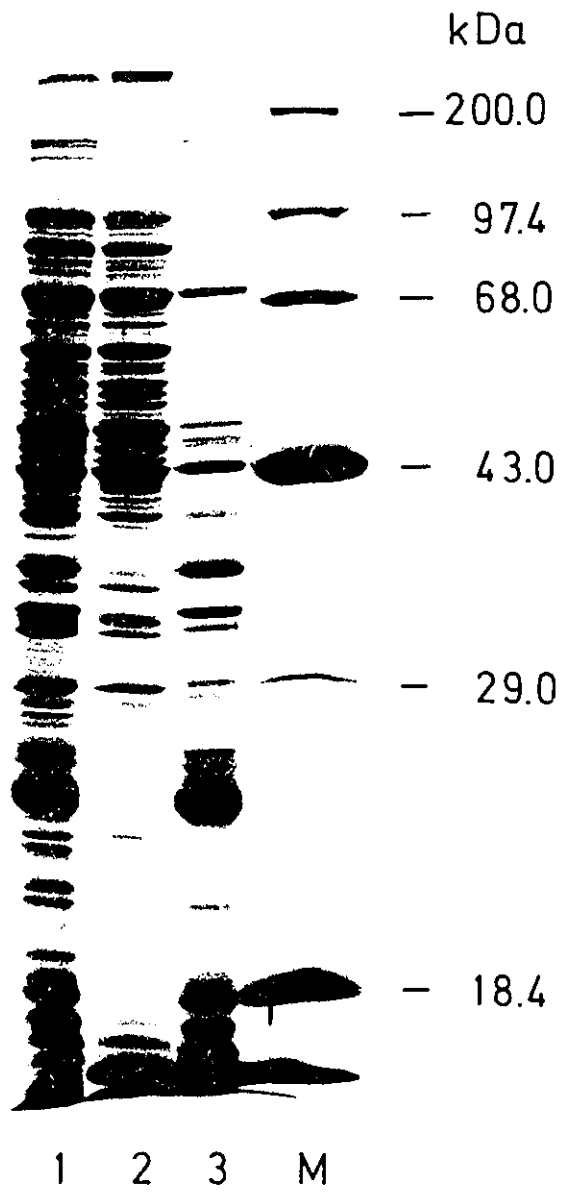


Figure 5

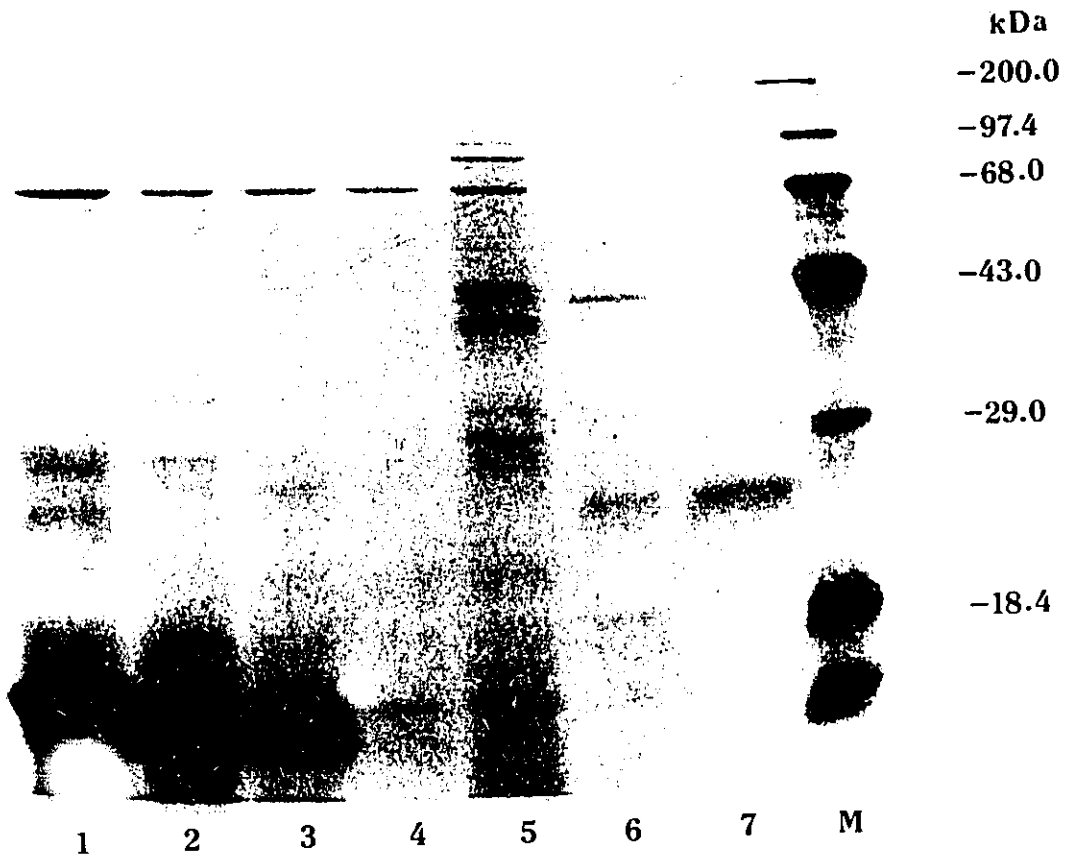


Figure 6

Chapter four

Modification of Lung Mucosal Immune Responses by Cytokines Expressed from Recombinant Adenovirus Vectors.

The following article, entitled "Recombinant human adenoviruses expressing interleukin 5 and 6 synergistically enhance mucosal IgA responses in the lung", is submitted for publication in the Journal of Immunology, 1995. This paper addresses the effects of expression by the cytokines IL-5 and IL-6 on lung mucosal IgA and IgG antibody production. Most of the work included in this manuscript was performed, and the paper was written, by the author of the thesis. Elispot analyses were performed with the help of W.S. Gallichan. Dr. A. Ramsay provided the plasmid containing the mL-5 cDNA used to construct the Ad5E3mIL5 recombinant adenovirus vector. Drs. J. Gauldie, F.L. Graham, C.D. Richards and K.L. Rosenthal provided supervision over this study and resulted in the multiple authorship of this paper.

**Recombinant Human Adenoviruses Expressing Interleukin 5 and 6
Synergistically Enhance Mucosal IgA Responses in the Lung.**

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Running title:

Recombinant adenoviruses expressing IL-5 and IL-6 enhance mucosal IgA production.

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Abstract

In this study, we have examined the in vivo effects of interleukin 5 (IL-5) and 6 (IL-6) overexpression on systemic and mucosal immune responses using recombinant human type 5 adenoviruses (Ad) capable of expressing these cytokines upon infection. A recombinant adenovirus Ad5E3mIL5 was constructed which contained the murine IL-5 gene within the E3 region of the adenovirus genome and was demonstrated to express high levels of IL-5 protein both in vitro and in vivo. Intranasal inoculation of C57Bl/6 mice with the Ad5E3mIL5 vector expressing murine IL-5 and Ad5E3mIL6 vector expressing murine IL-6 (previously described) markedly increased specific anti-Ad IgA antibody recovered in lung lavage fluid at both days 7 and 14 post inoculation. Individually, interleukin 5 or 6 expression increased IgA titres threefold at day 14 compared to those elicited by control virus. In addition, the simultaneous expression of both cytokines by co-inoculation appeared to alter the kinetics of the mucosal IgA response since titres at day 14 were rising and increased the response up to 7.8 times that of control virus. This represented a synergistic increase in the response to interleukin 5 or 6 expression alone. The co-expression effect on IgA synthesis was not due to increases in the number of specific resident lung tissue lymphocytes as determined by Elispot analysis for antigen specific reactivity. Mucosal anti-Ad IgG responses were primarily affected by

interleukin 6 expression with a 4.6 fold increase in titre being recovered in the BAL of Ad5E3mIL6 treated mice. Co-expression produced an intermediate response compared to that of IL-5 or IL-6 alone. Systemic immune responses were affected by IL-6 expression as 2-fold increases in serum IgG anti-Ad titres after a secondary challenge with wild type Ad virus were found. Our results demonstrate a relevant role for interleukin 5 and 6 in the development of mucosal immune responses in vivo and suggest that the incorporation of either interleukin 5 and/or 6 into recombinant adenovirus vectors may be a useful tool in the development of mucosal vaccines.

Introduction

Antibodies of the IgA isotype are commonly thought to be critical in the protection of tissues of the mucosa (1). In fact, there are now many examples where resistance to infection has been correlated to the titre of organism-specific secretory IgA antibody (2-4). With regards to mucosal immune responses, cytokines expressed by type 2 T helper lymphocytes (TH2 cells) have been implicated as being important in the development of isotype specific antibody responses (5). In vitro studies have shown that the TH2 cytokines, interleukin 5 and 6, can both enhance IgA production (6-9).

In order to test the relevance of IL-5 and IL-6 expression on mucosal immune responses in vivo, we have constructed independent recombinant adenovirus vectors capable of expressing these cytokines upon infection. We and others have previously shown that intranasal or intratracheal administration of recombinant Ad vectors leads to a highly compartmentalized expression of recombinant protein, such as IL-6, within the lung and bronchus of treated animals (10-13). In addition, recombinant Ad vectors have been used to induce systemic and mucosal immune responses to a variety of viral antigens (14-16). Here we report, the in vivo interactive effects of IL-5 and/or IL-6 expression on the mucosal immune response by monitoring the specific IgA and IgG reactivity generated against

Ad antigen after treatment with recombinant Ad vectors expressing the cytokines of interest. Our results demonstrate that IL-5 and IL-6 act synergistically to enhance local mucosal IgA responses whereas, IL-6 primarily enhances mucosal IgG antibody production.

Materials and Methods

Animals

Inbred, 6 to 8 week old female C57Bl/6 mice obtained from Charles River, St. Constant, Canada were used in this study.

Adenovirus vectors

Construction of the Ad5E3mIL5 vector involved cloning a 600 bp Eco RI/Xma I IL-5 cDNA digest fragment isolated from plasmid pEDFM-16 (17) containing the mIL-5 coding sequences into the multicloning site of plasmid pSV2X3. The resultant plasmid pSV2X3mIL5 has the murine IL-5 cDNA sequences in an orientation which allows for the transcription of mIL-5 message off the SV40 promoter provided by the pSV2X3 vector. To rescue the mIL-5 cDNA sequences into the E3 region of human type 5 adenovirus genome, the Xba I digest fragment of pSV2X3mIL5 containing the SV40/mIL-5 cassette was ligated into Xba I site of plasmid pFG144K3. Plasmid pFGmIL-5 was rescued from this ligation and contained the SV40/mIL-5 cDNA sequences in the proper configuration to allow for the rescue of infectious replication-competent virus. Cotransfection of plasmids

pFGmIL-5 with pFG173 in 293 cells produced recombinant viruses which, when characterized by Hind III digest and southern blot, were shown to contain the mIL5 cDNA sequence. One virus was picked and then plaque purified as previously described and designated as the Ad5E3mIL5 vector (A schematic diagram for the similarly constructed Ad5E3mIL6 vector is provided in reference 10). The control vector Ad5LacZA1, contains the *E. coli*. β -galactosidase cDNA inserted into the E3 region of the adenovirus genome and Ad5E3⁻ is an adenovirus vector containing an E3 region deletion (18,19).

IL-5 and IL-6 Protein Quantification

IL-5 quantification was performed using an Endogen murine IL-5 ELISA kit supplied by Cedarlane Labs, Hornby, Ont., Canada. In brief, for in vitro analysis, serial dilutions of supernatant from 1×10^6 293 cells infected at 10 multiplicity of infection (m.o.i.) dose were tested for IL-5 levels. For in vivo analysis, mice were given intraperitoneal injection with 2×10^8 pfu of recombinant adenovirus and sera were collected from blood samples obtained by retro-orbital bleeding. Sera were serially diluted and analyzed in the IL-5 ELISA assay. IL-6 levels were determined using the murine B9 hybridoma growth assay as previously described (10).

Intraperitoneal and Intranasal Immunization

For intraperitoneal immunization mice (4 animals per group) were

injected with 2×10^8 pfu of recombinant Ad vector in $300 \mu\text{l}$ of PBS. As a secondary challenge, mice were injected IP with 2×10^8 of wild type Ad virus. Sera were collected weekly post-immunization by retroorbital bleed and anti-Ad5 Elisa analysis was performed. For intranasal immunization, mice were instilled with various combinations of recombinant adenovirus vectors. Five treatment groups containing four animals each were established. Animals were given two $25 \mu\text{l}$ intranasal instillations of recombinant vectors to deliver a total of 3×10^8 pfu of vector in $50 \mu\text{l}$ of PBS per immunized animal. To control for antigen dosage, 1.5×10^8 pfu of Ad5E3⁻ vector were given in various combinations with 1.5×10^8 pfu of the vector to be tested. The treatment groups were as follows: Group 1, PBS medium control; Group 2, Ad5E3⁻ + Ad5E3⁻ vectors; Group 3, Ad5E3mIL5 + Ad5E3⁻ vectors; Group 4, Ad5E3mIL6 + Ad5E3⁻ vectors; Group 5, Ad5E3mIL5 + Ad5E3mIL6 vectors. All statistical analysis was performed using STATPAK 4.1 programs.

Bronchoalveolar lavage and Anti-adenovirus ELISA Assay

Bronchoalveolar lavage (BAL) was performed by inserting a 0.58mm polyethylene tube attached to a 1 ml syringe through a 27 gauge needle into the trachea of recombinant adenovirus treated mice. 1 ml of PBS was injected and re-collected and then stored at -20°C . To quantitate mucosal IgA and IgG anti-adenovirus antibody titres, ELISA assays were performed

on collected BAL samples. Round-bottomed 96-well Nunclon microtitre plates (Roskilde, Denmark) were precoated for 2 hours at 37°C with 5 μ g/well with wild type adenovirus infected Hela cell protein extract in PBS. Wells were washed with PBS containing 0.05 % Tween 20 (PBS-Tw) and incubated with PBS containing 0.1 % BSA (PBS-BSA) for 30 min at 37°C. BAL collected at day 7 or 14 were diluted in PBS-BSA and incubated in the wells for 1 hr at 37°C. The wells were then washed with PBS-Tw and incubated for 30 min at 37°C with biotin-conjugated goat anti-mouse IgA or IgG diluted 1:10000 in PBS-BSA. The wells were washed with PBS-Tw and incubated with Extravidin-peroxidase conjugate diluted 1:2000 for 15 min at 37°C. Tetramethylbenzidine (TMB) substrate was then added according to manufacturers instruction and incubated 20 min at RT. The optical density at 450 nm was measured. Immunoreagents were obtained from Sigma Chemical Co., St. Louis, MO.

ELISPOT ANALYSIS

Lungs were obtained and pooled together from 4 intranasal recombinant vector and PBS control treated mice. Lung lymphocytes were isolated by mincing tissue for 90 min at 37°C in 2 ml of PBS per lung supplemented with collagenase (Boehringer-Mannheim, Tutzing, F.R.G.) at 2 mg/lung, Dispase II (Boehringer-Mannheim, Tutzing, F.R.G.) at 1.2 units/ml

and deoxyribonuclease type II (Calbiochem, La Jolla, CA) at 5 units/ml. Cell suspensions were passed through sterile cotton gauze and purified by Ficoll-Paque density gradient separation.

Antibody-secreting cells (ASC) were determined in recombinant adenovirus treated lungs by ELISPOT analysis (20). 96-well nitrocellulose-bottom Millititre HA plates (Millipore, Bedford, MA) were used. Wells were coated with adenovirus antigen at 5 μ g/well using wild type adenovirus infected Hela cell protein extract and incubated overnight at 4°C and then washed 3 times with PBS. The wells were treated with PBS-BSA for 1 hr at 37°C to block non-specific binding sites on the nitrocellulose membrane. Lymphoid cell isolates were serially diluted and incubated in the wells overnight in a 37°C 5% CO₂ incubator. Wells were washed 3 times with PBS to remove cells and then incubated with biotin-conjugated goat anti-mouse IgA or IgG diluted 1:1000 for 3 hrs at 4°C. Wells were rinsed 3 times with PBS-Tw and incubated with Extravidin-peroxidase conjugate for 45 min at 37°C. 3-amino- α -ethyl-carbozole substrate was added and incubated for 5 min to develop stain after which spots were counted.

Results

Expression of mL-5 by Ad5E3mIL5

Hind III restriction enzyme digest and southern blot analysis were performed and confirmed the correct orientation of the murine IL-5 cDNA sequence in the E3 region of the recombinant vector Ad5E3mIL5 (data not shown). Subsequent in vitro analysis using Ad5E3mIL5 vector showed high levels of mIL-5 protein produced in the supernatant of 293 cells post-infection (Fig. 1). IL-5 protein was detectable at approximately 12 hrs and continued to increase over a 48 hr period reaching greater than 20 $\mu\text{g/ml}/10^6$ cells. Control infection with Ad5E3 or Ad5E3mIL6 vector and untreated cells produced no detectable mIL-5 protein.

In Vivo Expression of mIL5 and mIL6

IP administration of the Ad5E3mIL5 vector to C57Bl/6 mice raised expression of mIL-5 detected in serum over a 5 day period (Fig. 2A). Peak protein expression for mIL5 from the Ad5E3mIL5 vector was found 24 hours post-infection reaching levels greater than 30 ng/ml in the serum. Similarly as in vitro, no IL-5 expression was detected in sera taken from control vector treated animals.

As shown in Figure 2B, expression of mIL6 protein from the Ad5E3mIL6 vector in C57Bl/6 mice followed a similar timecourse as that for mIL5 expression from Ad5E3mIL5. Maximum expression was seen at 24 hours (> 30 ng/ml). IL-6 levels dropped more rapidly in the serum compared to the IL-5 expression profile. However, as was the case for IL-5.

IL-6 expression was still detectable on day 5 at picogram levels. The use of homologous cDNA cytokine genes (mIL-5 and mIL-6) avoided concerns of host immunity altering cytokine function.

Effects of Cytokine Expression on Humoral Immune Response

The effects of cytokine expression on the humoral immune response was monitored in animals receiving Ad vector after intraperitoneal injection by performing anti-Ad antigen specific antibody Elisa. Primary humoral anti-Ad IgG responses were found but no differences were noted between vectors expressing cytokine and those of control. As seen in Figure 3A, serum anti-Ad antibody response from Ad5E3mIL6 and Ad5LacZA1 treated animals were almost identical over a 10 week time course. In a separate experiment, treatment with IL5 vector was shown to produce a similar IgG immune profile over a 3 week time course as that of the control vectors (Data not shown). IgG titres in treated animals rose to peak levels by 14 days and remained elevated over the tested time course. No specific anti-Ad IgA antibody was detected in any serum samples collected from vector treated animals (data not shown).

To test for effects on secondary immune responses, Ad vector treated animals were given an additional challenge with wild type Ad virus at day 70 (Figure 3B). In contrast to primary IgG responses, differences in humoral immune responses were evident after secondary challenge. Animals

receiving IL6 vector were found to have a statistically significant 2-fold enhanced serum levels of anti-Ad IgG antibody compared to those of control vector after rechallenge. Anti-Ad IgG antibody titres reached up to 160,000 in IL6 vector treated mice. Animals receiving PBS medium control produced IgG anti-Ad antibody responses consistent with those obtained from primary vector challenged animals.

Effects of Cytokine Expression on Mucosal Immune Responses in the Lung

We next determined the effects for cytokine vector expression on lung mucosal immune responses (Fig 4). Anti-Ad IgA antibody titres recovered in the BAL fluid after intranasal administration from IL5 or IL6 vector treated mice were increased approximately 3-fold over a 14 day time course versus control vector (Fig. 4A). IgA mucosal antibody responses appeared to peak at day 7 for the IL5 and IL6 vectors when each cytokine was expressed individually and in the control vector. However, the simultaneous expression of mIL5 and mIL6 by co-inoculation with the vectors appeared to shift the kinetics of this response. Antibody titres recovered in the BAL samples at day 7 were on the rise and resulted by day 14 in a synergistic enhancement of anti-Ad IgA responses with titres up to 7.8 times those of control vector and approximately 2.7 fold higher than either of IL5 or IL6 vector treatment alone. These anti-Ad immune responses between IL-5 vector and/or IL-6 vector and control treatment were statistically significant.

Mucosal IgG anti-Ad antibody responses were most affected by mL6 expression from Ad5E3mL6 (Fig. 4B). Expression of mL6 resulted in marked increase in the titre of anti-Ad IgG antibody recovered in the BAL both at days 7 and 14 ($p < 0.01$). No increase in IgG response was evident at day 7 for IL5 vector over control vector expression. However, there was a slight increase in anti-Ad IgG noticed at day 14 for IL5 vector but this response was not as dramatic as that elicited by IL6 expression. Co-inoculation led to an intermediate response compared to that of IL5 and IL6 vector expression alone.

Synergy of mL5 and mL6 Co-expression Is Not Due to Increased Antibody Secreting Cells in the Lung

Lymphocytes recovered from the lung tissue of animals intranasally treated with recombinant Ad vectors were assayed for Ad antigen specific antibody secreting cells/ 10^6 cells (ASC) by Elispot analysis (Table 1). As expected, increases in specific IgA anti-Ad ASC were found in all animals receiving recombinant Ad vectors intranasally. Expression of mL5 resulted in an approximately 2 fold increase in anti-Ad IgA ASC number at day 7 compared to that of control vector but this response dropped significantly by day 14 to under 5 ASC cells. No significant increases in anti-Ad IgG ASC responses were found for IL5 or control vector at either days 7 or 14. However, striking enhancement in both anti-Ad IgA and IgG ASC numbers

were found after mL6 expression. Values for IL6 vector anti-Ad IgA ASC reached 174 at day 7 and continued to rise up to 270 ASC by day 14. Similarly, IL-6 expression appeared to exclusively cause increases in anti-Ad IgG ASC. By day 14, anti-Ad IgG ASC were 610 in Ad5E3mIL6 treated mice compared to background levels detectable control vector treated mice. Co-expression of mL5 and mL6 did not further enhance the number of either anti-Ad IgA or IgG ASC in treated lungs above that of mL6 expression alone.

Increases were also found in total numbers of IgA and IgG ASC measured at day 14 in intranasally recombinant Ad vector treated mice (Table 1). IL-5 derived expression from IL5 vector treatment resulted in a 2.4 fold increase (1700 versus 710 ASC) in total IgA ASC while IL-6 (3120 ASC) caused up to a 4.4 fold increase. IgG total ASC (as was the case for specific anti-Ad IgG response) were most profoundly affected by IL6 vector derived IL-6 expression. However, IL5 vector treatment did cause increases in total IgG ASC versus both PBS and control vector (660 ASC versus 330 ASC for PBS). IgG ASC total number in IL6 treated mice reached 4480 (an increase of 13.5 fold compared to PBS controls). Control vector treatment did not increase either anti-ad IgA or IgG ASC compared to PBS and appeared to cause a slight reduction in total IgG ASC number.

Histological Examination of Ad Vector Treated Lungs

Histological analysis of treated lungs at day 7 revealed a markedly different histopathology for Ad5E3mIL5, Ad5E3mIL6, and coinoculated animals (Figure 5). IL6 vector treatment at day 7 resulted in the accumulation of a large number of mononuclear cells (Fig. 5A). In contrast, animals cotreated with IL5 and IL6 vectors (Fig. 5B) had a marked reduction in this mononuclear cell accumulation. Ad5E3mIL5 (Fig. 5C) and Ad5E3- (Fig. 5D) vectors produced little to no mononuclear cell accumulation after intranasal administration and lungs appeared to have a normal physiologic appearance.

Discussion

Results from various studies using in vitro analysis have suggested important roles for the TH2 cytokines interleukin 5 and 6 in the development of mucosal immune responses (5-9). Recombinant IL-5 treatment was shown to induce IgA synthesis in Peyer's patch B cells (6). This IL-5 induced IgA synthesis could be synergistically enhanced in combination with recombinant interleukin 6 (9). Together these findings indicate the importance of IL-5 and IL-6 expression in supporting the maximal terminal differentiation of IgA precursor lymphocytes. However, it was important that the biological function of these cytokines be confirmed in vivo. Here we have reported the in vivo effects of IL5 and IL6 enhanced expression on systemic and mucosal immune responses by using recombinant adenovirus vectors to target tissue directed cytokine expression.

These TH2 cytokine containing vectors were first tested for their ability to express protein both in vitro in 293 cells and in vivo in C57Bl/6 mice. The Ad5E3mIL5 vector which contains the mIL5 cDNA code inserted into the E3 region of the recombinant Ad5 genome expressed protein in similar manner as that of the Ad5E3mIL6 vector and other recombinant Ad5 E3 region vectors that we have constructed (21).

IL6 had previously been demonstrated to enhance both primary and secondary immune responses to sheep red blood cells (SRBC) resulting in

2-fold increases in primary and 11-fold increases in secondary SRBC antibody reactivity (22). Therefore, we tested the recombinant vectors Ad5E3mIL6 and Ad5E3mIL5 for their ability to modify immune responses to adenovirus antigen. No potentiating effects on the primary systemic humoral immune response to Ad antigens for either mIL6 or mIL5 vector expressed cytokine were found following intraperitoneal administration. However, mIL-6 expression enhanced secondary humoral specific Ad antigen responses as specific anti-Ad IgG titres were raised two fold versus controls. These results are consistent with previous findings and again suggest that IL-6 acts in the development of B cell memory responses. The differences noted in primary immune responses after IL-6 expression between our work and that of the previous study may be accounted for by the nature of the antigen and its presentation. Recombinant Ad vector administration results in a highly efficient presentation of both endogenous and exogenous processed Ad antigen promoting optimal immune responses. Thus, background reactivities will be higher in the Ad vector model (control vector anti-Ad responses were significant). The ability to detect differences in the secondary immune response are probably due to the large increases in memory response elicited by the vector derived cytokine expression. After intranasal administration, we saw a differential tissue immune response in the mucosal compartment to Ad antigen dependent on the expression of

mIL5 and/or mIL6 by the recombinant Ad vectors. Anti-Ad IgA antibody titres were increased approximately 3-fold over controls by vector derived expression of either mIL5 or mIL6 cytokine. In addition, IL5 and IL6 co-expression resulted in synergistic enhancement of anti-Ad IgA reactivity recovered in BAL fluid on day 14. These changes in IgA response to Ad antigen are consistent with the biofunctional properties reported for IL-5 and IL-6 on mucosal lymphocytes. Effects of vector derived cytokine expression on IgG anti-Ad response were most markedly influenced by mIL-6. Co-expression of mIL-5 and mIL-6 resulted in slightly lower levels of IgG antibody to be recovered in the BAL fluid. IL5 cytokine expression caused several changes in the lung mucosal immune response consistent with priming of Th2 responses. Expression of IL5 appeared to preferentially affect IgA responses even in combination with IL-6. That this response could be enhanced by IL6 indicates it may mimic normal Th2 responses of the lung. Together these results suggest that IL-5 expression mediates mucosal immune responses in the lung by both upregulating IgA and by dampening IgG production. Bias in IgA antibody production to foreign antigens has been demonstrated for mucosal tissues of the gut (23). T cell subsets from mucosal tissues have been demonstrated to produce predominately TH2 cytokine profile (5). Our results demonstrate cytokine expression alone can account for these effects.

In addition to increasing anti-Ad IgA antibody responses, IL5 expression reduced the overall number of IgG specific anti-Ad ASC recovered from the lungs of co-treated animals at day 14 and yet was capable of increasing specific anti-Ad IgA ASC when administered alone (day 7). Of interest, co-expression of IL-5 and IL-6 did not increase the specific anti-Ad IgA ASC number above that of IL-6 expression alone. However, co-expression did cause a corresponding increase in specific anti-Ad IgA antibody production. IL-5 has previously been shown to increase IgA antibody production by enhancing B cell differentiation without a corresponding increase in lymphocyte proliferation (8). Our results are consistent with IL-5 functioning as differentiation factor in the mucosal tissue of the lung.

IL-6 vector derived cytokine expression produced the greatest enhancement in both specific anti-Ad and total ASC numbers. IL-6 expression increased IgA specific anti-Ad ASC numbers to 270 by day 14 whereas, IL-5 expression appeared to only transiently increase specific IgA ASC. IL-5 increased IgA specific anti-Ad ASC to 81 by day 7 but were below 5 ASC by day 14. In addition, IL-5 had no effect on the generation of specific anti-Ad IgG ASC whereas, IL-6 expression increased both specific and total IgG ASC count. In addition to these effects on IgG ASC response, IL-6 induced increases in total IgA ASC numbers as well. IL-5 expression

also caused slight increases in total IgA ASC but this increase was not as dramatic as those caused by IL-6 expression alone. IL-6 has been reported to function as a B cell proliferation factor and again our findings are consistent with this reported function (26).

Histological analysis of IL6 vector treated lungs at day 7 revealed a significant accumulation of mononuclear cells which were absent in animals receiving the co-inoculation with IL5 vector. Our previous analysis in Sprague-Dawley rats at day 7 showed a similar mononuclear accumulation. The cellularity of this response was shown to consist primarily of CD3+CD8+ T lymphocytes (11). The ability of IL5 expression to dramatically alter the mononuclear cell accumulation may indicate an additional biological property for IL-5 on mucosal immune responses in addition to the enhancement of IgA. It is possible that IL-5 expression can divert TH1 cell mediated immune responses towards TH2. Determination of the effects on cell mediated immune responses in the mucosa are ongoing.

Recent work using recombinant vaccinia viruses containing TH2 cytokines have addressed similar issues of in vivo regulation of mucosal immune responses. Using this approach, common effects for IL-5 and IL-6 expression in enhancing mucosal immune responses were demonstrated. Here we have reported, in addition to the effects of each cytokine alone, the effects of cytokine co-expression on immune responses. The results

generated were consistent between the two approaches and illustrate the usefulness of recombinant viral vectors as a tool to define in vivo cytokine function (24,25).

In addition, the ability of cytokine containing recombinant Ad vectors to modify mucosal immune responses may be exploited in the development of viral vaccines. Since mucosal tissues are primary sites of invasion for a number of pathogenic viruses, these recombinant vectors may be useful to generate more potent local immune responses. Our recent work using a recombinant adenovirus expressing glycoprotein B of herpes has demonstrated protection against wild type infection up to 52 weeks when administered at mucosal tissue sites (2). The incorporation of TH2 cytokines into these recombinant vectors and in particular, recombinant Ad vectors, may produce more attractive viral vaccines which can induce longer and more protective mucosal immune responses.

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FIGURE LEGENDS**Figure 1.**

1x10⁶ 293 cells were infected at a m.o.i. of 10 with Ad5E3mIL5 vector. Supernatants were tested for IL-5 protein levels in a murine IL-5 specific ELISA assay. Representative data are shown for one set of cultures. IL-5 protein was not detected in cells alone or in Ad5E3- control treated cells.

Figure 2.

Determination of serum levels of IL-5 (A) and IL-6 (B) in rAd5 infected animals. Data points are expressed as the mean \pm SE for IL-5 as determined by ELISA and for IL-6 as determined by B9 hybridoma growth factor assay. n=4 for each data point. *p<0.01 for values compared to control virus or PBS by Student's paired t-test.

Figure 3.

Determination of serum anti-Ad5 IgG antibody levels in rAd5 IP treated animals before (A) and after rechallenge with 2x10⁸ pfu of wild type Ad5 virus (B). All data points are expressed as the mean \pm SE with an n=4. *p<0.01 for values compared to Ad5E3- and PBS control treated

animals by Student's paired t-test.

Figure 4.

Determination of anti-Ad5 IgA (A) and IgG (B) in BAL samples recovered from rAd5 IN treated animals. All data points are expressed as the mean \pm SE with an n=4. Values of $p < 0.01$ were obtained for data comparing anti-Ad5 IgA responses at day 14 with Ad5E3mIL5 + Ad5E3mIL6 to either cytokine alone or Ad5E3- vector control treatment. IgG anti-Ad5 responses were also significant at $p < 0.01$ in Ad5E3mIL6 treated animals at both days 7 and 14.

Figure 5.

Representative microphotographs of lung histopathology in mice treated with Ad5E3mIL6 (A), Ad5E3mIL5 + Ad5E3mIL6 (B), Ad5E3mIL5 (C), and Ad5E3- vectors (D) at day 7 (X400). Tissues were fixed in 4% paraformaldehyde and sections stained with hemotoxylin and eosin. Mononuclear cell deposits were evident throughout the parenchyma of Ad5E3mIL6 treated lungs but were markedly reduced in Ad5E3mIL5 co-treated animals.

Table 1. ASC from lung lymphocytes post intranasal immunization

Immunization	IgA ASC/10 ⁶		IgG ASC/10 ⁶	
	α -Adeno	total	α -Adeno	total
Day 7				
PBS	0	-	0	-
E3' + E3'	38	-	0	-
m1L5 + E3'	81	-	0	-
m1L6 + E3'	174	-	6	-
m1L5 + m1L6	175	-	15	-
Day 14				
PBS	0	740	0	330
E3' + E3'	<5	710	<5	160
m1L5 + E3'	<5	1700	<5	660
m1L6 + E3'	270	3120	610	4480
m1L5 + m1L6	277	2040	360	3520
- not done				

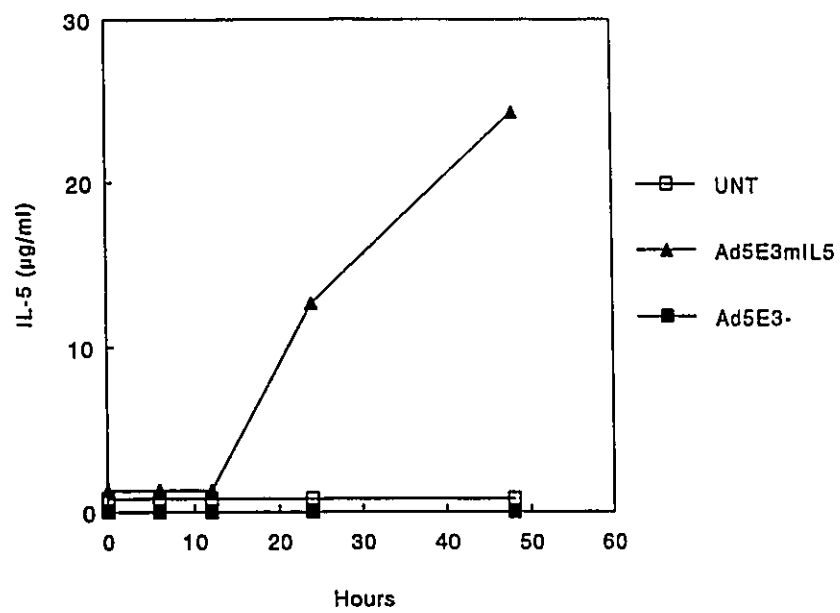


Figure 1

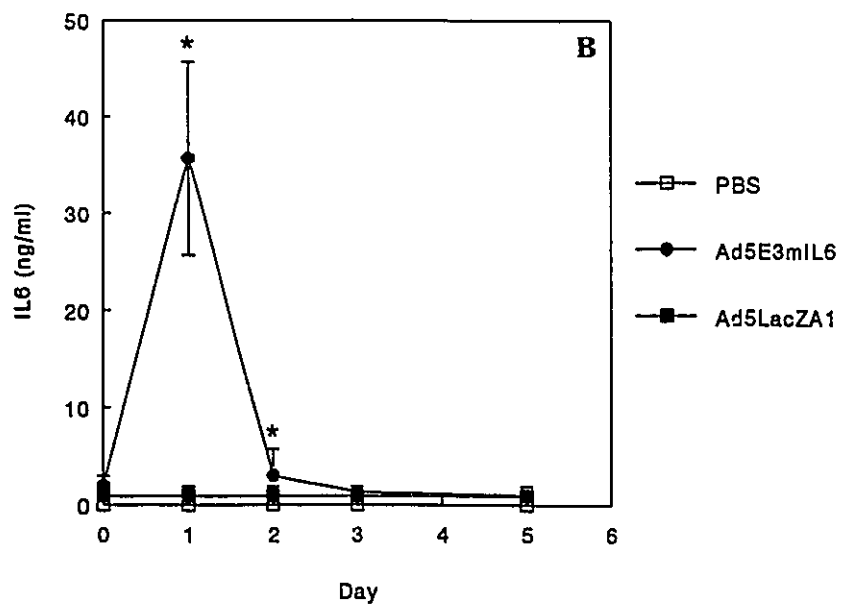
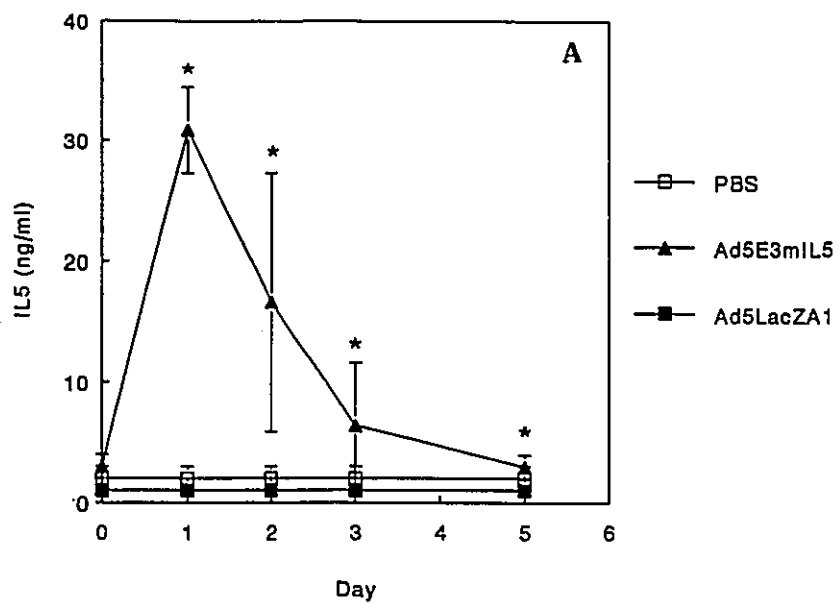


Figure 2

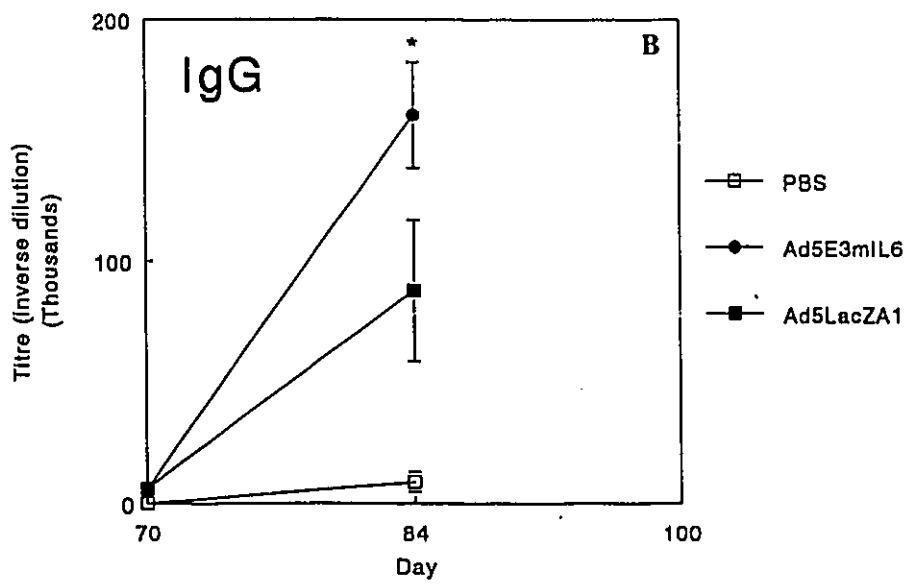
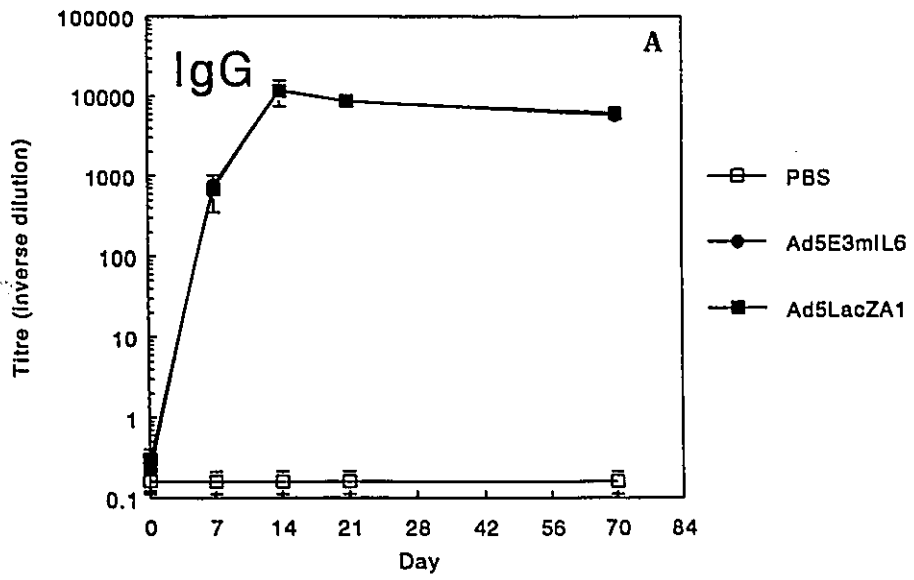


Figure 3

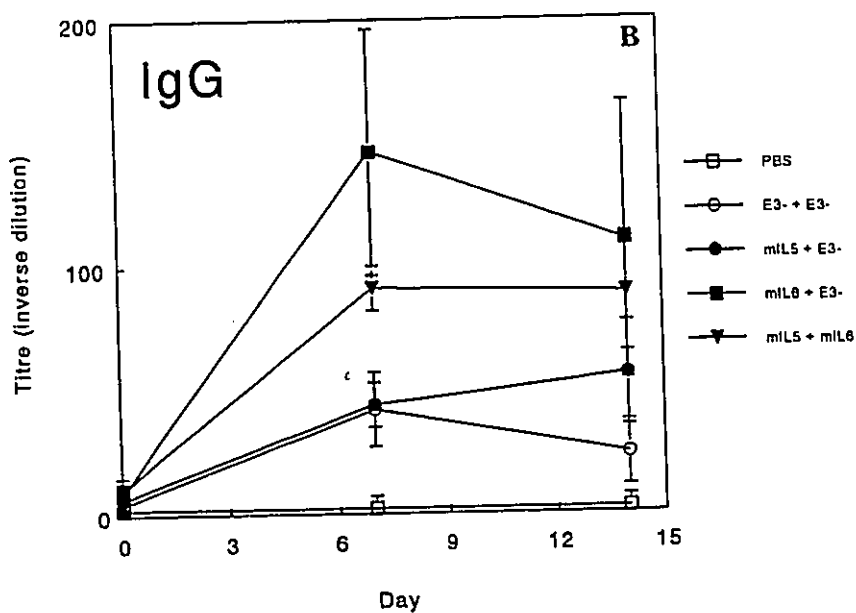
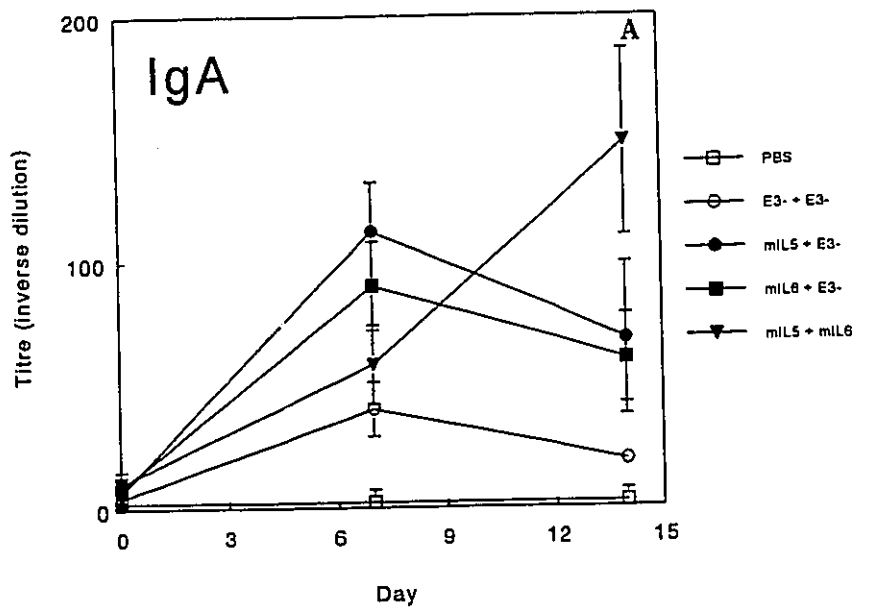


Figure 4

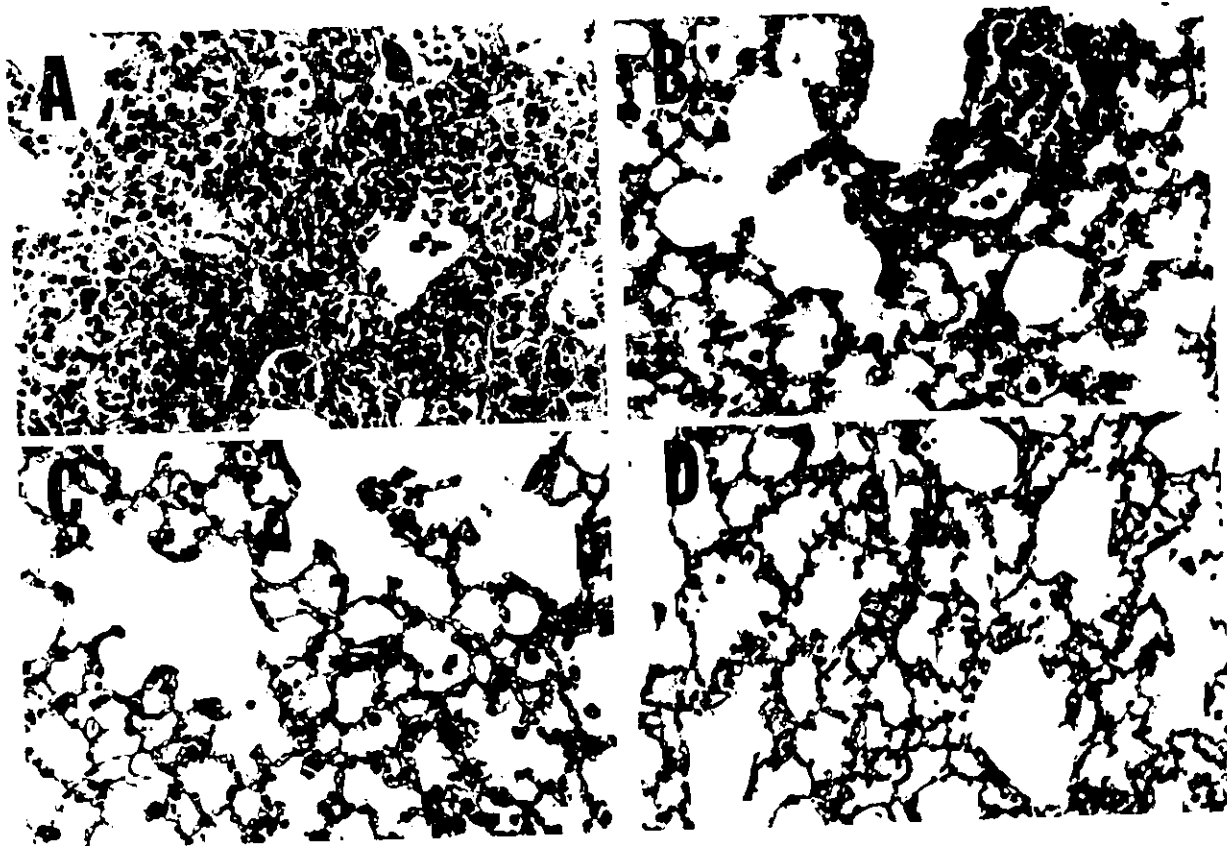


Figure 5

Chapter five

Induction of Monocyte Chemotaxis in the Lung by a Recombinant Adenovirus Expressing RANTES.

The following article, entitled "Overexpression of RANTES using a recombinant human type 5 adenovirus induces the tissue directed recruitment of monocytes to the lung" is submitted for publication in the *Journal of Immunology*, 1995. This article addresses the function of the chemokine RANTES in the context of a tissue response in vivo.

The work presented in this paper was primarily performed by the author of the thesis. Dr. T.J. Schall provided the plasmid containing the cDNA used in the construction of the Ad5E3mRANTES vector. Dr. K. Bacon carried out the RANTES chemotaxis assays. Dr. Z. Xing aided in the cytological analysis of bronchoalveolar lavage samples and D.J. Torry provided assistance with the immunohistochemical analysis. This paper was written by the author of the thesis, with the supervision of Drs. J. Gauldie, F.L. Graham, and C.D. Richards.

Overexpression of RANTES Using a Recombinant Adenovirus Vector Induces
the Tissue Directed Recruitment of Monocytes to the Lung

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RUNNING TITLE

Adenovirus vector RANTES gene transfer to the lung

FOOTNOTES

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ABSTRACT

RANTES (regulated on activation, normal T cell expressed and secreted) is a member the C-C superfamily of chemokines which is reported to function as a potent chemoattractant for monocytes, eosinophils and a subpopulation of CD4⁺ T cells. Using a recombinant human type 5 adenovirus containing the murine RANTES cDNA (Ad5E3mRANTES) capable of expressing biologically active cytokine upon infection, we initiated a study to characterize the biological functions of RANTES cytokine *in vivo*. Intratracheal administration of Ad5E3mRANTES targeted transient RANTES expression to the bronchial epithelium of the lung in Sprague-Dawley rats. Bronchoalveolar lavage fluids (BAL) collected at 24 hrs. had increased chemotactic activity versus controls as measured in a murine CD4⁺ T cell Boyden chamber microchemotaxis assay. This chemotactic activity was neutralized by a monoclonal antibody raised against purified recombinant murine RANTES protein in a dose dependent manner. In addition, there was a dramatic increase in the number of cells recovered from BAL samples taken from Ad5E3mRANTES treated animals. Most striking were the increases in monocyte and neutrophil numbers with a greater than 50 fold increase in monocytes being found indicating a proinflammatory effect for this cytokine *in vivo*. Histological examination of lung sections revealed greatly increased numbers of mononuclear cells, primarily monocytes, within the lungs of Ad5E3mRANTES treated animals, with

increased extravasation of monocytes around blood vessels, indicating an ongoing process of peripheral blood monocyte recruitment. This study provides another example of the use of recombinant adenovirus as a tissue-specific transient transgenic model to investigate cytokine functions and demonstrates RANTES to be a monocyte chemoattractant *in vivo*.

INTRODUCTION

The resolution of host tissue damage or control of infection is dependent upon the recruitment of various immune and inflammatory cell types to the affected tissue site. However, the composition of this cellular response varies, indicating that chemoattractants specific for distinct cell types are produced at the tissue site and appear to be dependent upon the etiological agent (1). Much of this cellular response is now thought to be controlled by a family of small molecular weight chemotactic cytokines (referred to as chemokines) which can be produced by tissue stromal cells, as well as macrophages, monocytes, and lymphocytes (2-4).

The chemokines are currently divided into two subfamilies designated either alpha or beta dependent upon the positioning of the first two of four conserved cysteines. The chemokine (alpha) subfamily (IL-8 being the best characterized member) possesses an intervening amino acid residue between the first two cysteines (C-X-C) and appears to target primarily neutrophils for chemotaxis. Twelve different C-X-C chemokines have now been identified (5-9). Included in this family are inducible protein (IP-10), growth related oncogene proteins (GRO alpha, beta and gamma) and platelet factor 4 (PF4) proven neutrophil chemoattractant molecules.

Members of the (beta) chemokine subfamily contain a C-C configuration of their first two cysteines and effect a broader range of cell types, however

all beta chemokines appear to have a common chemotactic effect on mononuclear cells. There are seven different C-C family members described to date, which include RANTES, monocyte chemotactic proteins (MCP-1, MCP-2, MCP-3) and the macrophage inflammatory proteins (MIP-1 α , MIP-1 β) (1,2,4). Additional activities for RANTES have been reported and include chemoattractive activity for eosinophils and a subset of CD4+ T cells thought to be involved in memory function (10-12).

There may be an additional novel chemokine family with the recent molecular cloning of lymphotactin (13). This molecule is similar to both alpha and beta chemokines in structure but lacks two of the four conserved cysteines and has chemotactic activity for lymphocytes but not for monocytes or neutrophils.

Since the majority of our knowledge concerning chemokine biologic function has come from *in vitro* experimental analysis, we decided to use a transient gene transfer animal model using a recombinant adenovirus vector to investigate the *in vivo* biological functions of the murine chemokine RANTES. To this end, we designed a recombinant human type 5 adenovirus vector containing and expressing the murine RANTES gene upon infection in an attempt to more precisely define the *in vivo* biological properties of this molecule. We as well as others have shown that intratracheal administration of these rAD5 vectors to rodents leads to highly compartmentalized gene

expression in the lung over a 5 to 10 day period (14-17). In this study, we describe RANTES chemotactic function in the lung of Sprague-Dawley rats after intratracheal instillation of the Ad5E3mRANTES vector. Our findings support the indicated chemotactic function for monocytes assigned for (beta) chemokines and in particular for RANTES.

MATERIALS AND METHODS

Adenovirus vectors

To construct the Ad5E3mRANTES vector, a 335 bp Hind III/Eco RI digest fragment was isolated from plasmid pCR1000 (18) and ligated into the multicloning site of plasmid pSV2X3 (19). The 335 bp fragment contained the entire coding region of the murine RANTES cDNA as well as the signal peptide sequences. The resultant plasmid pSV2X3mRANTES has the murine RANTES cDNA placed between SV40 promoter and polyadenylation signal sequences in an orientation which allows for the transcription of mRANTES mRNA off the SV40 promoter. To rescue the mRANTES cDNA sequences into the E3 region of human type 5 adenovirus genome, a 1073 bp Xba I digest fragment of pSV2X3mRANTES containing the SV40/mRANTES cassette was ligated into Xba I site of plasmid pFG144K3. Plasmid pFGmRANTES was rescued from this ligation and contained the SV40/mRANTES cDNA sequences in the proper configuration to allow for the rescue of infectious replication-competent virus. Co-transfection of plasmids pFGmRANTES with pFG173 in 293 cells produced viral plaques which when characterized by Hind III digest and Southern blot were shown to contain the mRANTES cDNA sequence. This virus was then plaque purified as previously described and designated as the Ad5E3mRANTES vector (For a schematic diagram of the rescue strategy see reference 14). The control recombinant vector, Ad5LacZA1, contains the *E. coli*. β -galactosidase

cDNA inserted into the E3 region of the genome and was constructed in a similar fashion (20).

Restriction mapping and Southern blot

Viral DNA was isolated from Ad5E3mRANTES infected 293 cells as previously described (21). Digestion with Hind III restriction enzyme, electrophoresis and Southern blot analysis were performed by standard procedures (22). The DNA was transferred to Biotrans nylon membrane using 0.4 M NaOH solution by capillary blotting overnight. The membrane was prehybridized and then hybridized with 100 ng of the random primed 335 bp mRANTES cDNA Hind III/ Eco RI fragment at 65 °C overnight. The membrane was then washed 2 times for 15 minutes in a 1X SSC, 0.1% SDS solution and exposed for autoradiography according to standard protocols (23).

Animals and delivery of adenovirus vectors to the lung

Male Sprague-Dawley rats weighing 280 to 340 g (Charles River Laboratories, Ottawa, Canada) were used. Adenovirus vectors were administered by intratracheal instillation at a dose of 2×10^8 plaque-forming units (pfu) with sterile endotoxin-free PBS, pH 7.4, at a final volume of 300 μ l per rat as previously described (15). This vector dosage was used throughout the study since it was previously shown to induce minimal inflammatory responses in the lung over 2-3 days with the Ad5LacZA1 and Ad5E3rIL6 vectors. PBS alone did not affect tissue responses in the lung.

Bronchoalveolar lavage

Animals were sacrificed 24 hours and 7 days after intratracheal administration of the adenovirus vectors. Bronchoalveolar lavage (BAL) was then performed on each treated animal as previously described with minor modifications (24). A total of 30 ml of warm PBS was used for each rat. For each animal an initial 3 ml of PBS was instilled into the lungs and recovered. Five aliquots of the remaining PBS were separately instilled, recovered and pooled. These two BAL samples were then spun at 1000 rpm at 4°C for 8 minutes to pellet cells. Supernatant from the first 3 ml of BAL sample were saved and stored at -20°C for RANTES bioassays. The resulting cell pellets were resuspended and pooled in 5 ml of PBS for cell counting.

Immunostaining of lung tissue sections

Immunoperoxidase staining of recombinant Ad5 vector treated lungs was performed using the mouse anti-monocyte, macrophage monoclonal antibody (clone ED1) (25). Paraffin sections were dewaxed in xylene and passed through an ethanol series. Endogenous peroxidase activity was blocked with a 30 minute incubation in H₂O₂methanol-HCL after which slides were rinsed in 70% ethanol followed by distilled water and finally Tris-buffered saline, pH 7.6 (TBS buffer). Primary antibodies were applied for 1 hr to tissue sections treated with nonimmune serum to block nonspecific binding. A second biotinylated antibody subtracted to eliminate cross reactivity to rat antigens

was then applied and subsequently treated with streptavidin-peroxidase conjugate, incubated with substrate-chromogen mixture, counterstained with hematoxylin, and coverslipped. Reagents were supplied by Zymed, Dimension Lab., Inc. Mississauga, Ont., Canada and used according to manufacturer's instructions.

Chemotaxis assay for determination of RANTES activity in BAL fluid

Chemotaxis was assayed using a modification of the 48-well microchemotaxis assay as described (26). In brief, 25 μ l of BAL fluid was placed to the lower wells of a 48-well microchemotaxis chamber (Neuro Probe Inc, MD, USA), and overlain with a 8 μ m poresize polyvinylpyrrolidone-free (PVPF) polycarbonate membrane. 50 μ l of murine CD4+ Th1 T cell clone HDK-1 cells (kindly provided by Dr. A. O'Garra, DNAX;ref) at a concentration of 2×10^6 /ml was added to the top wells of the chamber and the assay allowed to proceed for 2 hours at 37 C. Following incubation, the filter was removed, the side wiped free of sedimented cells and the cells on the lower surface of the filter were fixed in methanol and stained with Field's stain. Following fixing and staining, the cells on the filter were counted by microscopy and results expressed as cell number per 5 high power fields hpf; (x400). For antibody studies, BAL fluids were pre-incubated with 5 μ g/ml of an anti-mouse RANTES monoclonal antibody for 30 min at 4°C prior to assay BAL fluids were pre-warmed prior to beginning the assay.

RESULTS

Restriction enzyme analysis and Southern blot confirm the structure of Ad5E3mRANTES vector

We have previously used homologous recombination approaches with plasmids to generate recombinant vectors (14). In order to demonstrate the incorporation of the mRANTES cDNA sequences in the Ad5E3mRANTES vector, Hind III restriction enzyme digests were performed on viral DNA samples purified from Ad5E3mRANTES and wild type Ad5. The homologous recombination event resulting in the rescue of Ad5E3mRANTES vector generates an additional Hind III restriction site in the recombinant vector. As shown in Figure 1 the loss of restriction fragment B (the 5665 bp fragment encompassing the E3 region of the wild type genome) and the presence of fragments B' and B* in the Ad5E3mRANTES vector digest verifies recombination in E3 structure (Fig 1B). To confirm that this novel structure of Ad5E3mRANTES contained mRANTES cDNA sequences southern blot analysis was performed with a ³²P-labelled mRANTES cDNA probe. A signal representing the 2040 bp B* fragment of Ad5E3mRANTES can be seen (Fig 1B) indicating that mRANTES cDNA sequences are incorporated into the E3 region of the recombinant genome and are in the correct orientation to allow transcription off the E3 and SV40 promoters as well as the major late promoter of adenovirus.

Kinetics of RANTES expression in 293 Cells

Chemotactic activity for murine CD4+ T cells was monitored up to 24 hours post-infection from supernatant recovered from 293 cells infected with Ad5E3mRANTES (Fig 2). The results show medium recovered from Ad5E3mRANTES infected cells had potent chemotactic activity with levels equivalent to or greater than physiologic concentrations demonstrated for recombinant human RANTES protein in the same assay (10^{-8} M). Activity could be detected as early as 6 hrs. post-infection and reached maximal levels by 12 hrs. This activity could be neutralized by a mAB raised against purified recombinant murine RANTES protein. The inability of the mAB to totally neutralize chemotactic activity reflects high levels of RANTES, as we have previously reported that up to μ g/ml levels of cytokine can be produced by E3 recombinant adenoviruses in 293 cells (14). By contrast supernatants from cells infected with the control recombinant vector Ad5LacZA1 displayed only a random migration pattern throughout the dilution profile.

Detection of mRANTES in the BAL of Ad5E3mRANTES treated rats

Having demonstrated the ability of Ad5E3mRANTES to produce biologically active cytokine *in vitro*, we tested whether RANTES protein would be detected in the BAL samples recovered at 24 hrs from Sprague Dawley rats instilled through the tracheal route (IT) with 2×10^8 pfu of Ad5E3mRANTES or Ad5LacZA1. Chemotactic activity is evident in the BAL of Ad5E3mRANTES

treated rats and appears to be neutralizable in a dose dependent manner with the anti-mRANTES mAB (A dose response curve is shown in Fig 3A). Similar treatment with the Ad5LacZA1 control virus did not produce activity above that of random migration indicating that Ad5 infection itself does not induce endogenous mRANTES protein expression to any significant level (Fig 3B).

Effects of RANTES overexpression on total monocyte and neutrophil number in BAL

To determine if RANTES chemokine expression within the lung effects cellular recruitment, differential cell counts on BAL fluids collected at 24 hrs. were performed. Dramatic differences in cell accumulation were evident for Ad5E3mRANTES treated rats (Fig 4A) with both neutrophil and monocyte increases observed in the BAL samples. Neutrophil numbers approached 12×10^6 /BAL for Ad5E3mRANTES as opposed to 1.2×10^6 /BAL with Ad5LacZA1 treatment. However, the most dramatic effect appeared to be on monocyte recruitment as there was a greater than 50-fold increase in monocytes recovered (Fig 4B) with levels rising to 3×10^6 /BAL with Ad5E3mRANTES treatment. Control virus instillation produced only mild increases in BAL cellularity. The effects produced by Ad5E3mRANTES were transient. Monocyte increases were maintained at 3×10^6 cells up to 7 days but returned to normal BAL levels by day 14 (data not shown).

Effects of RANTES overexpression on lung histopathology

Histological analysis of lungs taken from Ad5E3mRANTES treated animals at 24 hrs revealed marked morphological changes within the tissue. Numerous multi-focal regions of accumulated mononuclear cells are present in the lungs of Ad5mRANTES treated rats as compared to Ad5LacZA1 controls (Figs 5A and 5B). Figures 6A and 6B show higher power resolution of the mononuclear cell accumulation in the Ad5E3mRANTES treated lungs. Figure 6C shows at a similar resolution immunoperoxidase staining of ED1 (specific for rat monocytes and macrophages) positive cells. Numerous positive staining cells representing monocytes are found throughout the parenchyma of Ad5E3mRANTES treated lung tissue. In addition to the localization throughout the parenchyma, there was a marked increase in extravasation of monocytes into lung tissue around blood vessels indicative of active monocyte recruitment (Fig 6D). Immunostaining of control lung showed few monocytes in a diffuse pattern (data not shown). Neutrophils were also evident within the accumulated mononuclear cellular regions and comprised a significant portion of the cellular infiltrate. As evidenced by ED1 immunohistochemistry, the major portion of the accumulated mononuclear cells were monocytes. However, still other cellular subsets may exist within the remaining cellular population. As RANTES has been reported to have chemotactic activity for eosinophils, we performed histological staining with Chrometrope 2R to examine

Ad5E3mRANTES treated lung sections for the presence of eosinophils and did not find any significant presence of eosinophils in lungs taken 24 hrs post IT instillation in these animals (data not shown). In addition, this transient RANTES expression did not produce any evident lasting lung damage. Examination of lung sections at day 14 after instillation showed no evidence of monocyte accumulation or residual tissue damage and no evidence of enhanced matrix deposition using trichrome stains (data not shown).

DISCUSSION

We have previously used the E3 region recombinant adenovirus Ad5E3rIL-6, to investigate the biological function of the cytokine interleukin 6 in the context of lung tissue. In that study, we were able to demonstrate profound effects for IL-6 overexpression on lymphocytic proliferation and infiltration within the lung of treated animals at a period of 7 to 9 days after gene transfer (15). In addition, we demonstrated that intratracheal instillation of recombinant Ad5 vectors leads to a highly compartmentalized expression of cytokine within the epithelial lining and lining fluid of the lung. In this current study, we have constructed a recombinant Ad5 virus containing the mRANTES cDNA within the E3 region of the recombinant genome. This mRANTES vector was shown to express biologically active mRANTES cytokine upon infection of 293 cells. RANTES cytokine expression from the Ad5E3mRANTES vector is

dependent on major late promoter (MLP) activity as the *in vitro* kinetics (peak expression by 12 hrs) is similar to those of our previous vectors, Ad5E3rIL-6 and Ad5Luc3 (E3 region recombinants containing the rat IL-6 and firefly luciferase cDNA, respectively) (14,27).

Intratracheal administration of Ad5E3mRANTES vector resulted in raised concentrations of mRANTES protein produced in the context of lung tissue. BAL samples taken from Ad5E3mRANTES treated animals had increased levels of mRANTES protein as measured in a CD4+ chemotaxis assay with a bell shaped profile, characteristic of *in vitro* chemotaxis assays for chemokines. The overall activity recovered from BAL samples was equivalent to 10^{-8} M recombinant human RANTES protein which can elicit potent chemotactic activity. This value is lower than that obtained from *in vitro* cultures of infected 293 cell supernatants. The differences in expression between these two systems reflects the semipermissive nature of Ad5 replication in rodent cells whereas full replication is seen in 293 cells. In our analysis, we assumed murine RANTES function to be equivalent to that of human RANTES on murine CD4+ T cell chemotaxis.

RANTES is reported to be a potent monocyte chemoattractant and BAL samples taken at 24 hours differed significantly in cellular profile from those infected with control virus. Ad5E3mRANTES treatment appeared to produce a preferential chemotactic effect on monocytes. In addition, histological analysis

of lung tissue sections revealed marked morphologic changes within 24 hrs in Ad5E3mRANTES treated lungs consistent with monocyte chemotactic function. Multifocal mononuclear cell accumulations were evident throughout the lungs of Ad5E3mRANTES treated rats. These cells were shown to be comprised largely of monocytes. No such changes were seen in rats receiving control virus or in a virus vector secreting IL-6 (15).

Recent studies in allergic diseases have begun to focus on the reported eosinophil chemotactic function for RANTES (1,28,29). RANTES overexpression in rat lung tissue did not appear to have an effect on eosinophil accumulation at either 24 hrs or seven days. This finding contrasts previous reports for eosinophil chemotaxis by purified recombinant RANTES protein after injection into canine dermis (30). This difference may reflect variation in inflammatory responses amongst different tissues or species, or may represent the necessity for an eosinophil priming process or enhanced integrin expression in the tissue before accumulation can be seen. Rats used in this study were not previously exposed to parasitic infection as was the case in the canine experiment. Peripheral eosinophil blood counts are low in parasitic naive Sprague Dawley rats. Therefore, localized production of the mRANTES cytokine in the lungs of these animals may not be sufficient to recruit large numbers of eosinophils to the lung. However, animals recovering from parasitic infection would have higher circulating eosinophil levels and these cells may be

actively recruited. Reported increases in chemotactic activity for RANTES on IL-5 primed eosinophils are consistent with this concept (29). We are currently exploring this possibility.

Our findings indicate additional effects on neutrophils as these were found within focal regions of the lung and in the BAL fluid of Ad5E3mRANTES treated rats. Similar observations were made in canines for recombinant RANTES protein injection at local dermal sites. Neutrophil recruitment was shown to be manifest through monocyte mediators released after their recruitment (30). We are not able to discern between effects on neutrophils caused directly by RANTES expression or indirectly through monocyte recruitment as was done in this previous work. However, since RANTES expression produced a more potent effect on monocyte recruitment than on that of neutrophils, it is interesting to speculate that a similar phenomenon is associated with the neutrophil accumulation observed. In a further attempt to discern between the two, we examined both BAL and tissue at the earliest timepoint we could detect RANTES in the BAL (12 hrs), both neutrophils and monocytes were present in similar numbers to the 24 hour timepoint (data not shown).

The early pathologic changes resulting from the transient over-expression of RANTES by the recombinant adenovirus vector did not produce any signs of long term damage in lung structure. Histological examination with trichrome

staining at day 14 showed no evidence of fibrosis or matrix deposition and pulmonary tissue appeared normal. Thus, RANTES overexpression does not appear to induce lung pathology in and of itself. However, it is possible that it is still intimately involved in such processes. Expression of RANTES in an appropriate (or with regards to pathologic outcome, inappropriate) temporal sequence of a cytokine cascade may still significantly contribute to lung tissue destruction evident in pulmonary fibrosis. These temporal considerations are accessible using this adenovirus vector transient expression model and are currently being addressed.

In conclusion, our results show that RANTES is a potent chemotactic factor for monocytes *in vivo* as there were increased numbers of monocytes within the parenchyma of the lungs of rats treated with Ad5E3mRANTES versus controls at 24 hours. These increases correspond to increases in chemotactic activity detected in BAL samples recovered from treated animals. Eosinophils were not detected in Ad5E3mRANTES treated animals and lung tissue sections were negative when they were stained with the eosinophil specific stain chromotrope 2R. The observed changes which occur within 24 hrs are consistent with mRANTES function as a monocyte chemoattractant and as an early wave cytokine.

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FIGURE LEGENDS

Figure 1A Schematic diagram of recombinant adenovirus Ad5E3mRANTES. Hind III restriction map of wild type and recombinant virus indicating the restriction fragments by letter. Black arrow indicates orientation of promoters in relationship to the mRANTES cDNA (hatched box) and includes site of SV40 polyadenylation signal (spotted box).

Figure 1B Hind III restriction map, digest analysis and Southern blot of wild type (WT) and the mRANTES containing recombinant adenovirus type 5, Ad5E3mRANTES (REC). DNA was purified from CsCl₂ banded viruses and analyzed by agarose gel electrophoresis. Molecular weights for the various restriction fragments are labelled according to size in the table A to J with the exception of fragments B' and B* which are unique to the REC vector. The two smallest Hind III restriction fragments I and J indicated in the table are not shown. Southern blot analysis of Hind III restriction digest fragments from wild type (WT) and Ad5E3mRANTES (REC) adenoviruses. Digested viral DNA was run into an agarose gel and transferred onto genescreen nylon membrane by capillary action. Hybridization was performed using a ³²P-labelled

mRANTES cDNA probe which contained the complete protein coding sequence. A signal equivalent to 2040 bp was detected in the REC digest lane and corresponds to the expected size B* fragment of Ad5E3mRANTES which contains the mRANTES gene sequence.

Figure 2 *In vitro* expression kinetics for the Ad5E3mRANTES vector. Confluent monolayers of 293 cells cultured in 60-mm dishes were infected with 10 pfu per cell with Ad5E3mRANTES or Ad5LacZA1 control virus. Supernatants were collected at various times post-infection and analyzed for chemotaxis to HDK-1 cells (a murine CD4+ T cell line). Representative data are shown for one set of cultures at a 1:500 dilution. No chemotactic activity was recovered from cells alone. Supernatants were incubated with 5 μ g/ml concentrations of anti-mRANTES mAb for 30 minutes at 4°C for neutralization experiments. Data points represent the number of cells counted in five microscope objective fields X400. Ad5E3mRANTES (closed circles); Ad5E3mRANTES + mAb (closed triangles); Ad5LacZA1 (open squares).

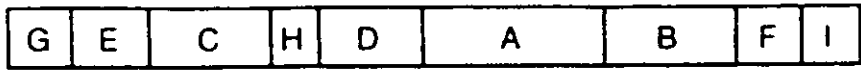
Figure 3 Detection of mRANTES protein in the BAL of Sprague Dawley rats. Rats were intratracheally infected with 2×10^8 pfu of (A) Ad5E3mRANTES or (B) Ad5LacZA1 virus and BAL samples taken 24 hrs post-infection. Samples were analyzed in the microchemotaxis assay using murine HDK-1 line CD4+ T cells. Supernatants were incubated with 5 μ g/ml concentrations of anti-mRANTES mAb for 30 minutes at 4°C for neutralization experiments. Data points are expressed as the mean \pm SD. Viral vectors (closed circles); vector + mAb (closed triangles). $n = 6$ for Ad5E3mRANTES; $n = 4$ for Ad5LacZA1.

Figure 4 (A) Cellular profile of BAL fluids collected at 24 hrs postinfection. Rats were intratracheally instilled with 2×10^8 pfu of Ad5E3mRANTES or Ad5LacZA1 and BAL fluids collected and analyzed for differential cell counts. Neutrophils (PMN), monocytes (MONO), alveolar macrophages (AM), lymphocytes (LC) and eosinophils (EOS). (B) Fold increase in cell number for Ad5E3mRANTES treatment over Ad5LacZA1 control. Results represent mean \pm SD. $n = 6$ for Ad5E3mRANTES; $n = 4$ for Ad5LacZA1.

Figure 5 Morphology of lung at 24 hrs postinfection with (A) Ad5E3mRANTES or (B) Ad5LacZA1. Lung tissues were perfusion fixed at 25 cm with 4% paraformaldehyde overnight. Sections were prepared from paraffin embedded tissues and stained with hemotoxylin and eosin. Numerous multifocal mononuclear cell deposits were evident throughout the parenchyma of Ad5E3mRANTES infected lungs. X100.

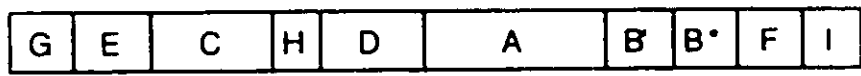
Figure 6 Immunohistochemical analysis of lung histopathology at 24 hrs postinfection in Ad5E3mRANTES infected rats. (A) X400 hemotoxylin/eosin stained Ad5E3mRANTES treated rat lung. (B) X640 resolution. (C) Micrograph of the monocyte/macrophage specific mAb clone ED-1 positive peroxidase stained cells within the parenchyma. X400. (D) Evidence of extravasation of mononuclear cells from blood vessels. Perivascular localization of monocytes was noted throughout the lung. X400.

A

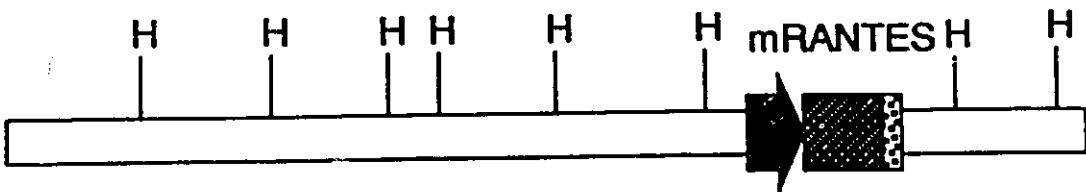


Wild Type

144



Recombinant



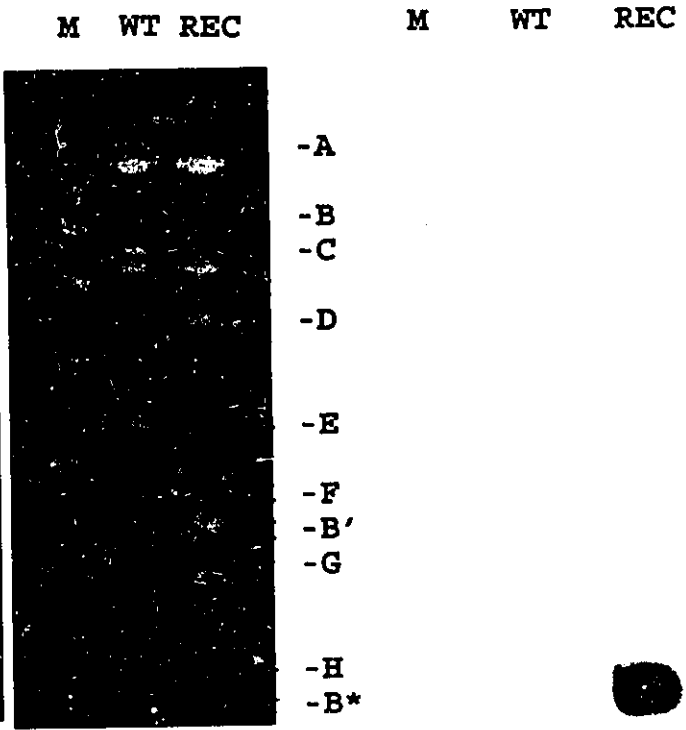
E3 Region

Ad5E3mRANTES

B

RESTRICTION MAP RESTRICTION DIGEST SOUTHERN BLOT

FRAG	WILD TYPE	RECOMBINANT
A	8010	8010
B	5665	lost
C	5324	5324
D	4597	4597
E	3437	3437
F	2937	2937
G	2804	2804
H	2081	2081
I	1005	1005
J	75	75
B'		2828
B*		2040



2040 bp

Figure 1

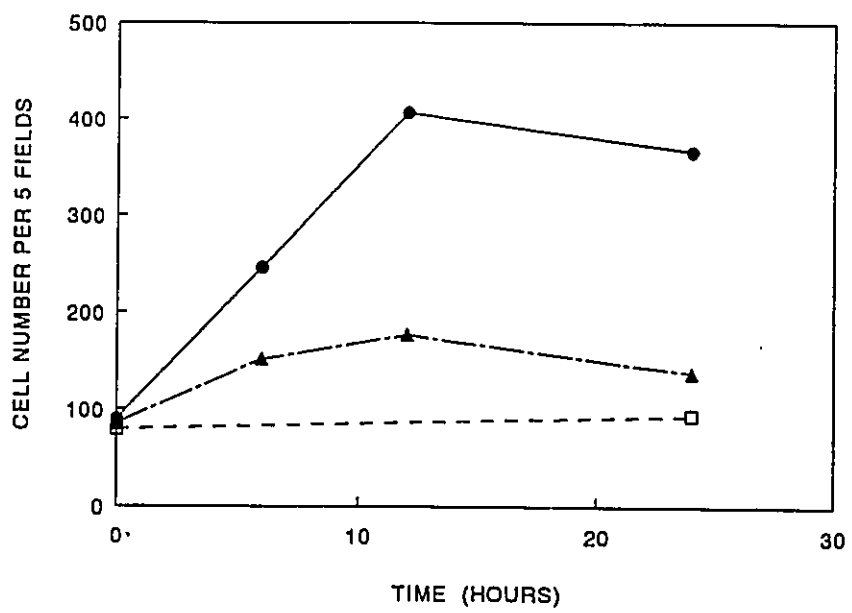


Figure 2

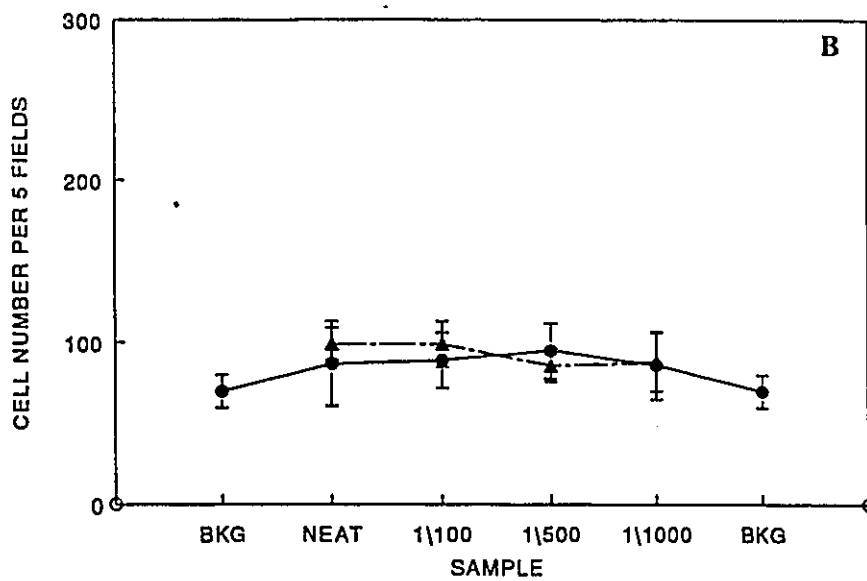
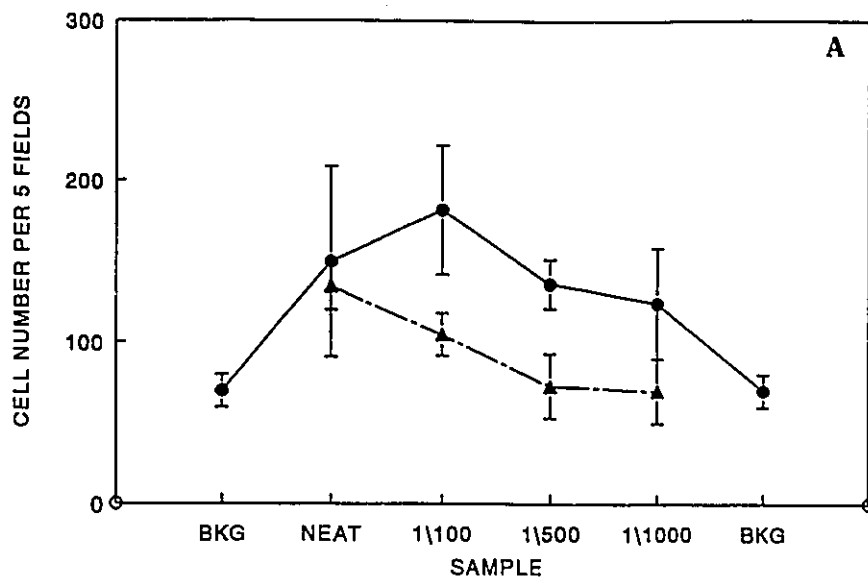


Figure 3

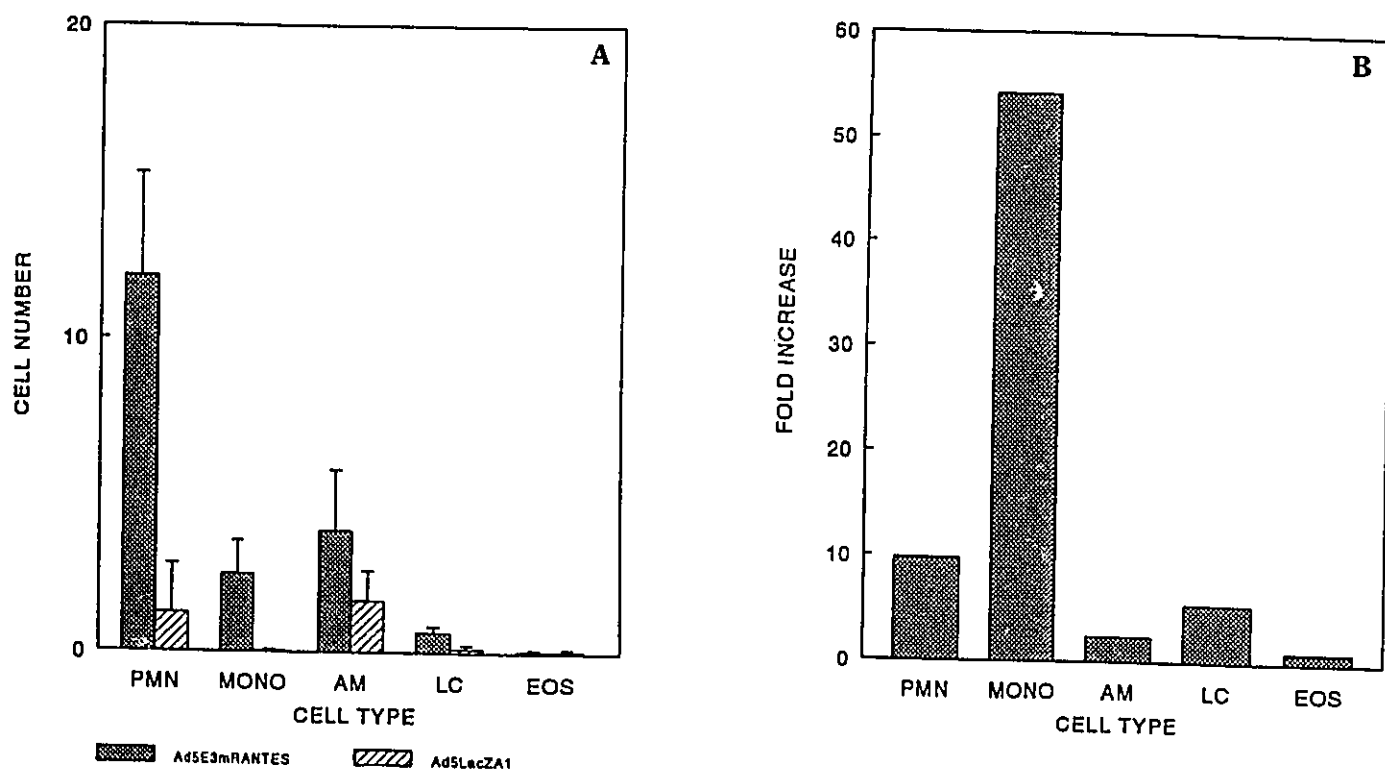


Figure 4

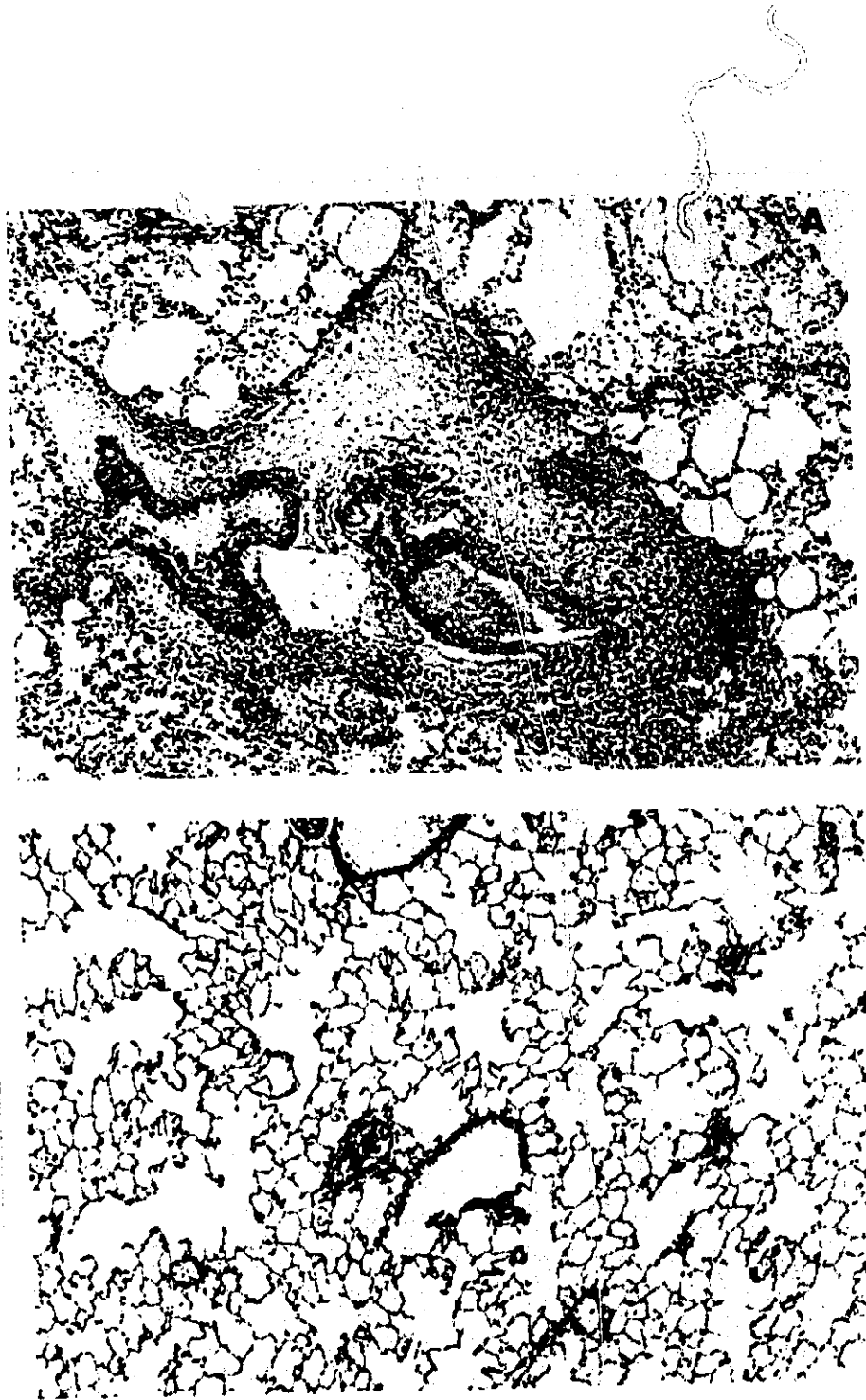


Figure 5

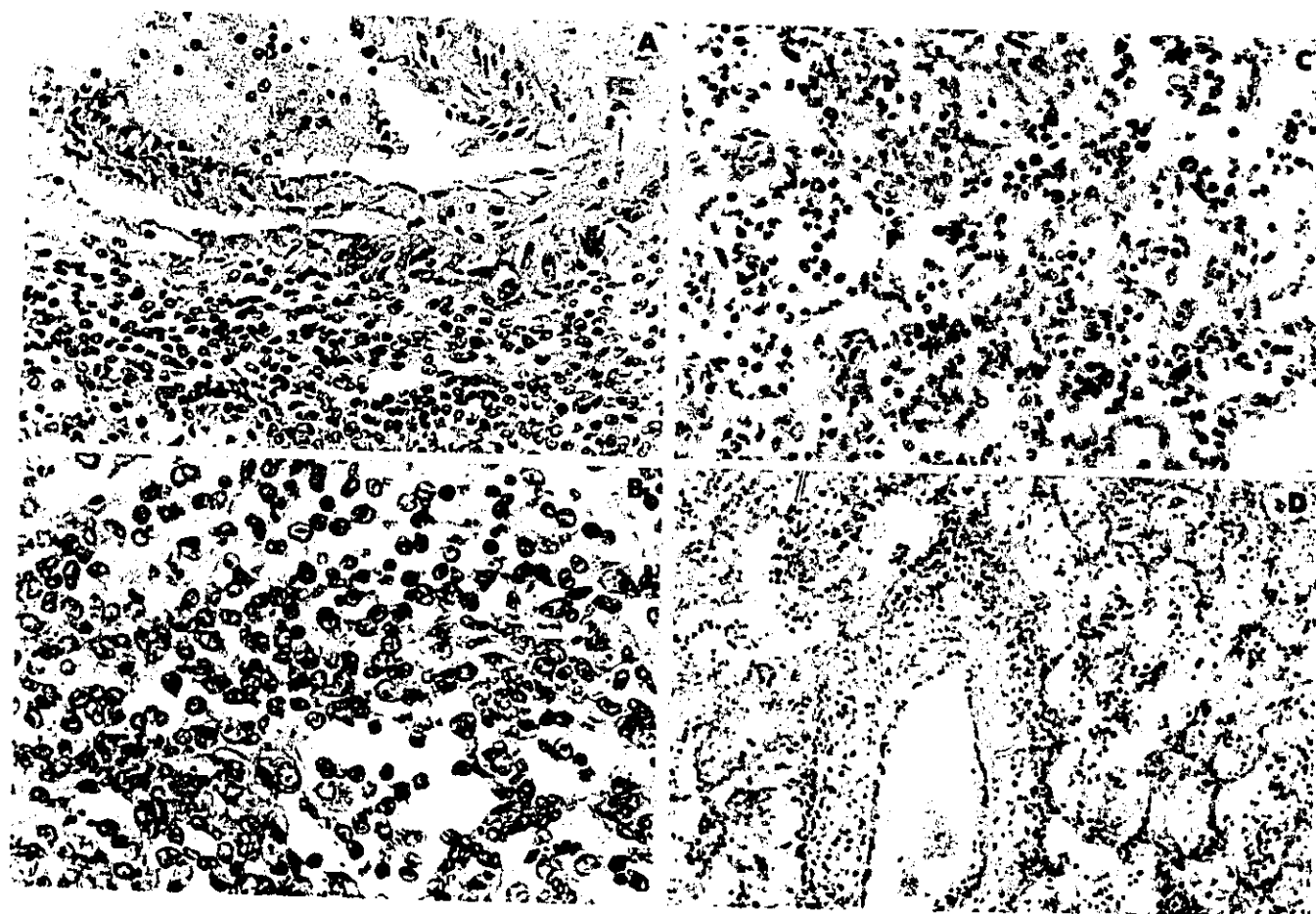


Figure 6

Chapter six

Applications of a Recombinant Adenovirus Vector

Expressing IL-6 in a Murine Breast Cancer Model.

The following article, entitled "Antitumor properties of interleukin-6 expressed from a recombinant adenovirus vector: Intratumoral injection attenuates tumor growth in a murine breast cancer model" is submitted for publication in the *International Journal of Oncology*, 1995. This paper focuses on the effects on tumor growth caused by direct injection of a replication deficient adenovirus vector which expresses IL-6. In addition, tumor cells transduced to express IL-6 by the recombinant adenovirus vector were used as vaccines in animals and immune responses against tumor were monitored.

This study was carried out and written by the author of the thesis. C.L. Addison was involved in the establishment of the breast tumor model in mice. Dr. W.J. Muller provided the transgenic mice used as the source of tumor cells. Drs. J. Gauldie, F.L. Graham, and C.D. Richards provided supervision resulting in the multiple authorship.

**Antitumor Properties of Interleukin 6 Expressed from a Recombinant
Adenovirus Vector: Intratumoral Injection Attenuates Tumor Growth
in a Murine Breast Cancer Model.**

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Running title:

Recombinant adenovirus expressing interleukin 6 attenuates breast cancer tumor growth.

Footnotes:

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Abstract

Cytokines are extracellular signalling glycoproteins which mediate host immune and inflammatory responses and have gained considerable interest in their use as agents in the gene therapy of cancer. Cytokine genes engineered to be expressed in tumor cells have been shown to stimulate in vivo anti-tumor immune responses in a number of rodent models. In this report, we have used a recombinant adenovirus vector (Ad5E1mIL6A⁺) expressing interleukin 6 (IL-6) to test the effects of this cytokine on both tumor growth and metastatic progression in a murine breast cancer model we have established. The Ad5E1mIL6A⁺ vector was shown to direct the synthesis and secretion of high levels of IL-6 in infected tumor cells in vitro. The tumorigenicity of IL-6 vector transduced cells was unaltered compared to that of control virus infected cells. However, direct injection of the IL-6 vector into 17 day old established subcutaneous tumors resulted in the attenuation of tumor growth. Three-fold reductions in tumor mass were evident 28 days later in the IL-6 vector treated animals versus those animals receiving the Add170-3 control vector. A cellular vaccination approach using irradiated breast cancer tumor cells infected with Ad5E1mIL6A⁺ was also used in an attempt to prevent lung metastases. Lungs of animals receiving two doses of tumor cell vaccine prior to tail vein challenge with live tumor cells were found to be tumor free after 30 days versus lungs from untreated animals which showed evidence of heavy tumor

loads. We observed a 20% survival rate in animals with pre-established lung micrometastases after two treatments with IL-6 vector infected attenuated tumor cells used as vaccine. However, this protective response was only slightly greater than that seen in animals treated with irradiated tumor cells alone as a control indicating such protection may be only partly due to the presence of IL-6. Our results show that the localized production of IL-6 has profound effects on breast cancer tumor growth and that humoral adaptive immune responses may contribute to the protection seen against lung metastases in this tumor model of breast cancer.

Introduction

The rate of breast cancer has steadily been increasing since 1950, with a dramatic rise in the 1980's associated with an increase in detection using mammography (1). While early diagnosis and surgical excision can successfully treat this condition, the response of patients with advanced metastatic disease to both surgery and standard chemotherapy is poor and 50% of women presenting with a primary malignancy eventually succumb to complications of metastases (2).

We and others have begun to develop immunological based therapeutic strategies using recombinant adenovirus vectors expressing cytokines. We have used a murine breast cancer model which entails the use of transgenic mice incorporating the polyoma middle T antigen (PMT) expressed under the

control of the mouse mammary tumor virus long terminal repeat (MMTV LTR). These animals spontaneously develop mammary adenocarcinomas by 8 to 10 weeks of age which are heterogeneous in nature and the tumors are used as a source of cells which can be explanted and form tumors in the parental strain syngeneic FVB/N mice (3).

Using this model, we were able to induce tumor immunity and regression by directly injecting subcutaneously formed tumors with an adenovirus vector expressing interleukin 2 (IL-2) (4).

Prior observations of the role of IL-6 in tumorigenesis have produced variable results. IL-6 has been shown to act as a tumor growth factor in variable number of tumor types including multiple myeloma, renal cell carcinoma and kaposi sarcoma's (5-7) and to have potent anti-tumor effects in a number of animal models of cancer (8-11). In addition, IL-6 has been shown to be directly inhibitory to the growth of human breast cancer cell lines (24). In this study, we have evaluated the effects of IL-6 on the pathogenesis of a murine mammary tumor using the recombinant adenovirus vector Ad5E1mIL6A⁺ to target cytokine expression. Our findings indicate an important role for IL-6 in the inhibition of mammary tumor growth and suggest gene therapy using recombinant adenovirus vectors has potential as a novel strategy for the treatment of cancer.

Materials and Methods

Animals

Inbred, female FVB/N mice obtained from Taconic Farms 6 to 8 weeks of age were used in this study.

Adenovirus vectors

Construction of the Ad5E1mIL6A⁺ vector involved cloning a 940 bp Hind III/Xba I digest fragment isolated from plasmid pSV2X3mIL6 containing the mIL-6 coding sequences into the multicloning site of plasmid pHCMVsp13 (12). The resultant plasmid pHCMVsp13.mIL6 has the murine IL-6 cDNA sequences inserted between the human cytomegalovirus immediate early promoter (HCMV) and the SV40 polyadenylation signal. To rescue the mIL-6 cDNA sequences into the E1 region of human type 5 adenovirus genome, cotransfection of plasmids pHCMVsp13.mIL6 and pJM17 was performed using 293 cells. Homologous recombination between these two vectors produced recombinant viruses which when characterized by Hind III digest and southern blot were shown to contain the mIL-6 cDNA sequence. One virus was plaque purified as previously described and designated as the Ad5E1mIL6A⁺ vector (13). The control vector Add170-3 is an adenovirus vector containing an E1 region deletion (14). All vectors were banded on CsCl₂ and purified over a PD-10 desalting and buffer exchange column (Pharmacia, Quebec, Canada).

Preparation of Polyoma Middle T Antigen Tumor Cells and the Establishment of

Experimental Tumors

Tumor bearing polyoma middle T antigen (PMT) transgenic mice were sacrificed and tumors were excised. Single cell suspensions of tumor cells were then prepared by mincing the isolated tumor and incubating the tissue in a collagenase-dispase solution (25 mg collagenase, 250 mg dispase (Boehringer-Mannheim, Tutzig, F.R.G.) in 100 ml of PBS without calcium or magnesium ions) for 30 min. with continuous stirring. The supernatant was collected and cells pelleted by centrifugation at 1500 rpm for 5 minutes. After quantification by hemocytometer, cells were resuspended in Dulbecco's Modified Eagles Medium containing 10% Fetal Calf Serum and 1% Pennicillin-Streptomycin and incubated overnight in T75 tissue culture flasks. Cells were harvested by trypsinization and resuspended in PBS at a concentration of 1×10^6 cells per 200 μ l. Tumors were established in FVB/N parental strain mice by injection of 1×10^6 tumor cells either subcutaneously (SQ) in the right hind flank resulting in detectable tumor growth over 15-20 days or by tail vein injection (IV) resulting in pulmonary metastatic growth over the same time period.

RNase Protection Assay for Polyoma Antigen

RNA was isolated from lungs of FVB/N mice by Chomczynski et al (15) at various days post IV injection of tumor cells by the procedure described . RNase protection assays were performed as described by Melton et al. (16)

with 20 μ g of total cellular RNA per assay using RNA probes made with Bluescript (Stratagene) vector. The riboprotection probe pSP65mT was obtained from J. Hassell, McMaster and contains a 203-bp Hind III/Acc I fragment (nucleotides 165 to 368) of the Polyoma virus middle T antigen cDNA (33).

IL-6 Expression in PMT Tumor Cells

PMT tumor cells were either irradiated with 8000 rad of gamma irradiation provided by a Cs¹³⁷ source or left untreated after infection with either Ad5E1mIL6A⁺ or Add170-3 control vectors. 1×10^6 cells were infected at a multiplicity of infection (moi) of 10 and supernatant samples were collected over a 72 hour period. Secreted IL-6 levels were determined using the murine B9 hybridoma growth assay (17) with modifications as previously described (12). Intratumoral Injection

For intratumoral injection, 1×10^9 pfu of recombinant vector was instilled in a 50 μ l volume of PBS into 14 to 21 day old established right hind flank subcutaneous PMT tumors. Tumor sizes were monitored weekly by caliper measurement and tumor volume was calculated by determining the longest diameter and average width using the equation for the volume of a prolate spheroid.

Cellular Vaccination

Tumor cell vaccines were prepared using PMT tumor cells either

irradiated and infected with 10 moi of Ad5E1mIL6A⁺ or irradiated only as a control. FVB/N mice (4 animals per group) were injected intraperitoneally (IP) with 1×10^6 treated cells. For secondary vaccination, mice were injected IP with the similar additional dose of cells one week after the initial treatment. All statistical analysis was performed using STATPAK 4.1 programs.

Results

Construction of Ad5E1mIL6A⁺ Vector

Figure 1 shows a schematic diagram of the Ad5E1mIL6A⁺ vector construction and rescue strategy. Hind III restriction enzyme digest and southern blot analysis were performed on the rescued recombinant vector Ad5E1mIL6A⁺ in order to confirm the correct orientation of the murine IL-6 cDNA sequence in the recombinant viral genome. A novel 1.4 kb Hind III fragment which hybridized specifically to a radiolabelled mIL-6 cDNA probe was found confirming the correct incorporation of the mIL-6 cDNA sequence into the E1 region of the Ad5E1mIL6A⁺ vector (data not shown). In the resultant vector, IL-6 mRNA transcription is driven off the HCMV promoter in an E1-antiparallel orientation (right to left).

Kinetics of IL-6 Expression in Ad5E1mIL6A⁺ Infected PMT Tumor Cells

To determine whether PMT breast cancer cells were capable of expressing and secreting IL-6 encoded by the recombinant adenovirus vector, cells were infected with the Ad5E1mIL6A⁺ vector at moi of 10 and IL6 levels determined in the B9 hybridoma growth bioassay (Fig. 2). As indicated, untreated and control vector infected tumor cells produced IL-6 at 1.25 ng/10⁶ cells/24 hr levels. In contrast, the Ad5E1mIL6A⁺ vector infected tumor cells produced significant amounts of biologically active IL-6 protein over the 3 day time course with 10 µg/10⁶ cells/24 hr levels being reached. This represented

approximately a 10,000-fold increase in IL-6 expression versus both untreated and control vector treated tumor cells.

The capacity for the IL-6 vector to express cytokine in irradiated tumor cells was also tested. PMT tumor cells were infected with Ad5E1mIL6A⁺ vector and then given an 8000 rad dose of gamma irradiation before being placed into culture. The IL-6 expression profile obtained did not significantly differ from that obtained in primary PMT tumor cells for the IL-6 vector (up to 10 $\mu\text{g}/10^6$ cells/24 hr production). Background expression was slightly increased (between 10 to 25 ng/10⁶ cells/24 hr production) in control irradiated cells although still at magnitudes lower than those obtained by the IL-6 vector treatment.

Tumorigenicity

1x10⁶ tumor cells isolated from PMT transgenic mice were infected with Ad5E1mIL6A⁺ or control vector at a moi of 10 and injected into the right hind flank of syngeneic FVB/N mice and tumor formation was monitored. No effect for vector derived IL-6 expression on inhibiting tumor formation was observed. All animals including those receiving untreated tumor cells developed subcutaneous tumors within a 14 to 17 day period (data not shown).

In Vivo Attenuation of Tumor Growth by Direct Intratumor Injection

To test for the effects of vector derived IL-6 expression on established subcutaneous tumors, tumor cells were isolated from PMT transgenic mice and

1×10^6 cells were injected into the right hind flank of syngeneic FVB/N mice to generate subcutaneous mammary tumors. After 17 days, readily palpable tumors had formed ($\sim 10 \text{ mm}^3$ volume). These experimentally formed tumors were directly injected with the IL-6 vector, control vector or PBS and subsequently monitored for growth (Fig. 3; Tumor volumes are shown as a function of time post-virus injection). Intratumoral injection of the IL-6 vector resulted in the greatest inhibition of tumor growth compared to Add170-3 vector and PBS controls. Tumor volumes measured in IL-6 treated animals were 3 fold smaller than control vector and 5 fold less than PBS treated animals. The differences between IL-6 vector treatment and Add170-3 controls were significant to $p < 0.01$. All tumors regardless of treatment eventually grew in size until the mice became moribund. IL-6 vector tumor injection increased the mean survival time (from 45 to 100 days) of these treated animals by approximately 2-fold compared to animals receiving control vector treatment (data not shown).

Pulmonary Metastatic Model of Breast Cancer

The spontaneous mammary tumors which form in the transgenic PMT strain mice resemble scirrhous carcinomas found in human breast tumors and metastasize to the lung in these animals at around 6 months of age. In order to test the efficacy of recombinant adenovirus vector therapy in the prevention of metastases, we injected isolated PMT tumor cells via the tail vein into

syngeneic FVB/N mice and monitored tumor formation in the lung. As seen in figure 4A, gross morphological analysis revealed multifocal tumor nodules had become evident by day 20 in animals receiving 1×10^6 tumor cells IV. Lung tumors continued to increase in number and became difficult to quantify by day 30. To prove that these tumors were the result of PMT tumor cells and to provide an additional method to quantify tumor load, RNase protection assays were carried out on RNA prepared from the lungs of tumor bearing animals using a polyoma virus middle T antigen RNA probe (Fig. 4B). PMT message signals could be detected and quantified at day 20 in the lungs of tumor bearing mice and continued to increase in intensity to day 30. These results correspond to the tumor load evidenced by morphologic examination and indicate tumor load can be directly correlated and quantified to the PMT RNA protection signal. All tumor bearing animals became moribund and were shown to eventually die from the metastatic disease.

Double Vaccination with Attenuated Cytokine Modified Tumor Cells Prior to Live Tumor Challenge Protects Against Lung Metastases

After demonstrating the ability of PMT tumors to form lung tumors, we next tested whether vaccination with IL-6 vector infected irradiated tumor cells could provide protection against lung metastases. Prior to tumor challenge, FVB/N mice were injected intraperitoneally with either 1×10^6 irradiated PMT tumor cells alone or IL-6 vector treated irradiated cells. Some animals received

an additional treatment one week after the initial vaccination. On day 14, all animals received a tail vein injection of unattenuated PMT tumor cells and the metastatic disease was allowed to progress for 30 days (44 days from the initial treatment). After this period animals were sacrificed and lungs were examined for metastases. The results presented in Table 1 demonstrate that animals receiving irradiated cells infected with IL-6 vector or irradiated cells alone were protected against lung metastases. However, this protection was only elicited in animals receiving two doses of tumor cell vaccine. Approximately 100 tumor nodules per lung formed in all animals receiving a single vaccination and in PBS treated animals indicating that double vaccination was required to generate this protective response. Using this approach, it was not possible to discern a difference in the response of animals receiving the IL-6 vaccine from those receiving irradiated cells alone. Survival analysis were not carried out for this set of experiments (animals were sacrificed for lung gross morphological assessment). However, in a separate experiment, animals receiving double vaccinations with either IL-6 vector modified or unmodified tumor cells did not have lung metastases present at 30 days subsequent to a second challenge of 1×10^6 unattenuated tumor cells (60 days from the initial vaccinations; n = 5).

Effects of Double Vaccination on Established Lung Micrometastases

With the demonstration that vaccination prior to tumor challenge

afforded protection against metastases, we analyzed the effects on survival that vaccination would have on animals with established lung micrometastases prior to vaccine treatment (Fig. 5). To analyze these effects, FVB/N mice were given 1×10^6 PMT tumor cells IV and subsequently vaccinated IP with irradiated and cytokine modified tumor cells starting at 1 week post tumor challenge. Some animals received a second dose of vaccine 1 week later (2 weeks after tumor introduction). As shown in figure 5, animals receiving single doses of either IL-6 vector modified or unmodified irradiated tumor cells as vaccines succumbed to metastatic lung disease by 45 days similar to control animals. Again, it appeared that double vaccination provided the best protection against metastatic disease but this response was not nearly as pronounced as that seen with vaccination prior to introduction of viable tumor cells. 2 of 10 animals receiving two doses of IL-6 vector modified irradiated cells versus 1 of 8 animals receiving irradiated tumor cells alone have remained healthy up to 150 days at the time of completion of this study. While IL-6 vector vaccination appeared to induce a slight increase in protective response over cells alone, these differences were not statistically significant. However in double versus single tumor cell vaccination protocols, significant survival advantages were afforded to animals receiving two tumor cell vaccinations in the treatment of pre-established breast cancer metastases ($p < 0.018$).

Discussion

The development of cytokine gene therapy approaches for the treatment of cancer is currently an active area of research (reviewed in ref. 18). In this study, we have used recombinant adenovirus vectors expressing cytokine genes which we feel are best suited for the purpose of transient expression of cytokines and the treatment of cancer (34). One advantage is the ability to express high levels of cytokine in transduced cells. We have been able to demonstrate significant IL-6 expression in IL-6 vector infected tumor cells with or without subsequent irradiation up to $10 \mu\text{g}/10^6$ cells/24 hr levels. These levels of expression are approximately a 100 fold greater than levels obtained by others using retrovirus vectors for cytokine gene expression in transformed tumor cells (18).

In addition, the ability to infect tumor cells and target the expression of cytokine using adenovirus vectors has proven to be a straight forward procedure requiring only a simple injection into the primary tumor in vivo or a short incubation of vector in culture with tumor cells in vitro. Unlike retroviral vectors, adenoviral vectors are capable of transducing genes for expression into non-dividing cells and the vector genomic DNA does not normally integrate into host cell chromosomes but exists episomally. This removes concerns of possible insertional mutagenesis while retrovirus vector approaches require integration for the expression of transduced cytokine gene. Adenovirus vectors

have been shown to infect a wide range of tumor cell types. Recent insights derived from the binding properties of adenovirus may allow vectors to be constructed which express chimeric fiber protein bearing receptor ligand moieties (19-21). This receptor targeting may remove limitations on the capacity for these vectors to infect any cell type, including the myriad of tumor cell types. Our previous demonstration using an IL-2 expressing recombinant adenovirus to elicit potent protective immune responses against breast tumor following direct injection illustrates the effectiveness of this vector for the gene therapy of cancer (4). This observation coupled to the increased expression capacity of Ad vectors make it the ideal system for the transient expression of cytokine in tumor cells and therefore as vectors for tumor immunotherapy.

We have previously shown the ability to modify both systemic and local immune responses using an adenovirus vector with the murine IL-6 gene expressed from the E3 region of the virus (12,22). Since replication competent vectors are unlikely to be suitable agents for the clinical treatment of cancer, we constructed an E1-deleted replication-deficient form of the IL-6 vector. We used this vector to examine the effects of IL-6 expression on breast cancer in a number of modalities. IL-6 has been reported to have direct effects on breast cancer cellular growth (24). However, this was not the case for PMT tumor cells as IL-6 vector modified PMT tumor cell growth in vitro was similar to unmodified (data not shown). IL-6 expression has been reported to affect the

tumorigenicity IL-6 retrovirally transformed melanoma cells injected subcutaneously into mice (11). In that report, the effect of IL-6 appeared to be independent of T cell responses as growth inhibition also occurred in nude mice. These differences noticed between tumor responsiveness to IL-6 in our model versus others highlights the heterogeneous nature of cancer.

While IL-6 expression did not affect the tumorigenicity of IL-6 vector transduced tumor cells, the direct injection of IL-6 vector into established PMT tumor did result in the attenuation of tumor growth (Fig 3.). The inhibitory effects demonstrated for IL-6 on PMT tumor growth may also be the result of non-adaptive immune response, although, it is possible that differences may exist between systemic and local tumor immune responses elicited. Localized cytokine production in the presence of anergic tumor infiltrating lymphocytes (TIL's) may overcome T cell suppression and induce TIL activation (23) as is likely the case in our IL-2 vector treatment (4). We are currently attempting to delineate the mechanism of action of IL-6 on PMT tumor growth. It is possible that the attenuation of tumor growth seen here is mediated through innate immune responses as IL-6 has been shown to enhance Natural Killer cell activity (25). In addition, effects of IL-6 on angiogenesis or shifting proteolytic/inhibitor balances in the local tissue environment should be investigated as each of these responses can effect tumor growth directly (26,27).

Control vector treatment of the subcutaneous tumor also caused a slight inhibition in tumor growth. This inhibition most probably reflects the effects of immune response generated against the Ad vector itself (28). However, it is still possible the slight increase seen in endogenous IL-6 production from the control treated PMT tumor cells (Fig. 2) may contribute to the effects seen on tumor growth.

We also examined the effects of IL-6 using cytokine modified tumor cells as vaccines. Tumor cell vaccinations incorporating the IL-6 vector did not elicit significantly greater protective response than cells alone to pre-established micrometastases (Fig. 5). This finding, while demonstrating that IL-6 was not effective in the enhancement of systemically generated tumor immune responses, was not totally unexpected. A number of tumor immune responses originally attributed to cytokine expression have been found to be similar to those obtained by cellular controls when carefully re-examined (29). The effects seen here by vaccination may only reflect increases in immunogenicity of the tumor cells caused by irradiation. The use of irradiated tumor cells as vaccines has already been demonstrated to induce immune protection against a number of tumors (30).

From this study, it appears that prior vaccination protocols can afford some protection against lung metastases and that double vaccination therapies provided protective advantages versus a single vaccination. The implications

of these findings may have already been revealed in the clinic. The use of human melanoma cell vaccines was shown to increase the delayed type hypersensitivity (DTH) responses in patients after receiving a second vaccination of attenuated tumor cells and provided a survival advantage versus standard therapy (31,32). This multiple vaccination was shown to induce humoral anti-tumor antibody production. An induction of humoral response may account for the protective response seen here for animals receiving double tumor cell vaccination prior to live tumor challenge. Thus, humoral antibodies generated by vaccination may be protective against metastases by clearing potentially invasive tumor cells from the blood.

The mechanisms by which tumors exist at tissue sites despite the presence of tumor infiltrating lymphocytes (TIL) may be many fold. Factors contributing to the ability of tumors to evade host immune responses could include lack or loss of expression of MHC molecules by tumor cells, absence of co-stimulatory signals to induce TIL response (B7 expression), or the production of immunosuppressive molecules from the tumor which inhibit TIL activation. In principle, recombinant adenovirus vectors can be used to provide the appropriate signal(s) to overcome this T cell anergy through the targeted expression of various cytokines to tumor cells at the tissue site as we have previously seen with a vector expressing IL-2 (4). Due to their capacity for inducing expression, adenovirus vectors are potent agents for the transduction

of tumor cells and delivery of cytokine genes.

In this metastatic model of breast cancer, we have shown IL-6 expression to have a pronounced effect on tumor growth when expressed locally. IL-6 by itself does not induce prolonged protection after intratumoral injection as seen with IL-2. Possible combinations of IL-2 and IL-6 or vectors expressing other immune modulating cytokines may prove more efficient at inducing immune protection against metastatic disease. It is important to note that no toxicities were evident in animals receiving either the IL-6 or control vector therapy. Tumor cell vaccinations using IL-6 vector did not appear to enhance protection more than cellular controls alone. However, it does appear from our data and that of others that multiple vaccination approaches are best suited for ex vivo cancer therapies. Thus, adenovirus vectors may serve as safe and reliable agents in gene therapy approaches for the treatment of breast and other forms of cancer.

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FIGURE LEGENDS**Figure 1.**

Rescue Plasmids and Structure of Ad5E1mIL6A⁺. Plasmid pHCMVsp13.mIL-6 was constructed by ligating the mIL-6 cDNA sequences into the Hind III and Xba I sites of the polylinker of pHCMVsp13. Co-transfection of 293 cells with pHCMVsp13.mIL-6 and pJM17 generated the recombinant virus Ad5E1mIL6A⁺. The HCMV IE promoter (black arrow) and the SV 40 polyadenylation signal (speckled box) are shown flanking the mIL-6 cDNA sequence (hatched box).

Figure 2.

Expression Kinetics of Ad5E1mIL6A⁺ Vector. IL-6 levels were determined by B9 hybridoma growth assay in supernatants of Ad5E1mIL6A⁺ infected cells and controls (multiplicity of infection of 10 pfu per cell). Representative data are shown for one set of cultures. Irradiated cells (filled symbols).

Figure 3.

Attenuation of Tumor Growth Following Direct Injection of Ad5E1mIL6A⁺. At 17 days post SC injection of 1×10^6 tumor cells, palpable tumors had formed in the right hind flank of mice. These tumors were injected intratumorally with 1×10^9 pfu of Ad5E1mIL6A⁺, Addl70-3 or with PBS (50 μ l). Tumors were measured weekly following injection. Ad5E1mIL6A⁺, n=10; Addl70-3, n=8; PBS, n=8. $p < 0.01$ for Ad5E1mIL6A⁺ versus Addl70-3 controls.

Figure 4.

Establishment of PMT Lung Metastases by Tail Vein Injection. (A) Mice receiving 1×10^6 PMT tumor cells by tail vein injection were sacrificed and the number of metastatic nodules in the lung were counted. (B) Metastases was also measured indirectly by performing RNase protection assays for PMT antigen mRNA from lungs of mice receiving tail vein injections of tumor cells.

Figure 5.

IL-6 Tumor Cell Vaccine Therapy of Pulmonary PMT Metastases. FVB/N mice were injected IV with 1×10^6 PMT tumor cells. On day 7, animals received 1×10^6 irradiated tumor cells alone or cells transduced with Ad5E1mIL6A+ as a vaccine (sgl). In addition, some mice received an additional dose of tumor cell vaccine on day 14 (dbl) and survival was monitored. Ad5E1mIL6A+, n = 10; Cells alone, n = 8.

Table 1. Effects of Prior Vaccination Using IL-6 Vector Modified
Tumor Cells on Lung Metastases

Treatment (IP)	Number of Animals	Tumor # in Lung
PBS Treatment (Control)	5	~200
Single Vaccination (Irradiated Cells Only)	5	~200
Single Vaccination (Ad5E1mIL6A ⁺ Treated Cells)	5	~200
Double Vaccination (Irradiated Cells Only)	5	0
Double Vaccination (Ad5E1mIL6A ⁺ Treated Cells)	5	0

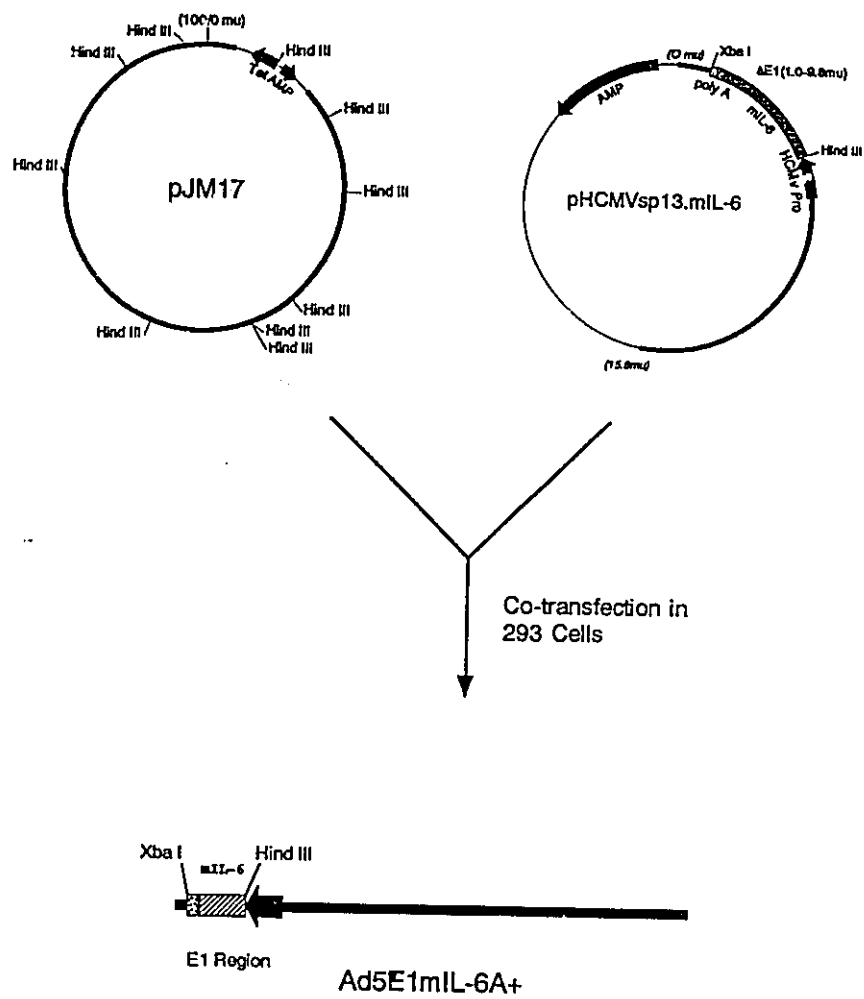


Figure 1

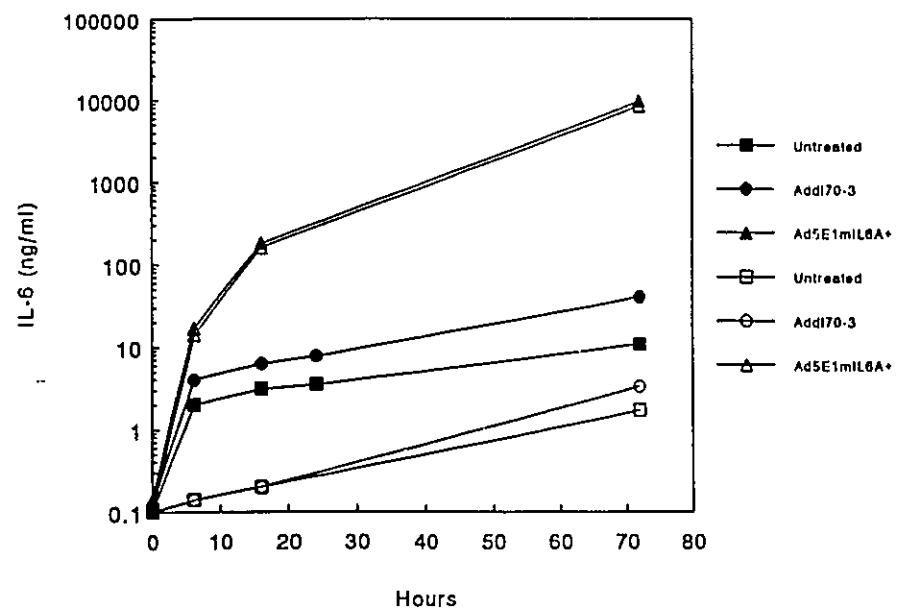


Figure 2

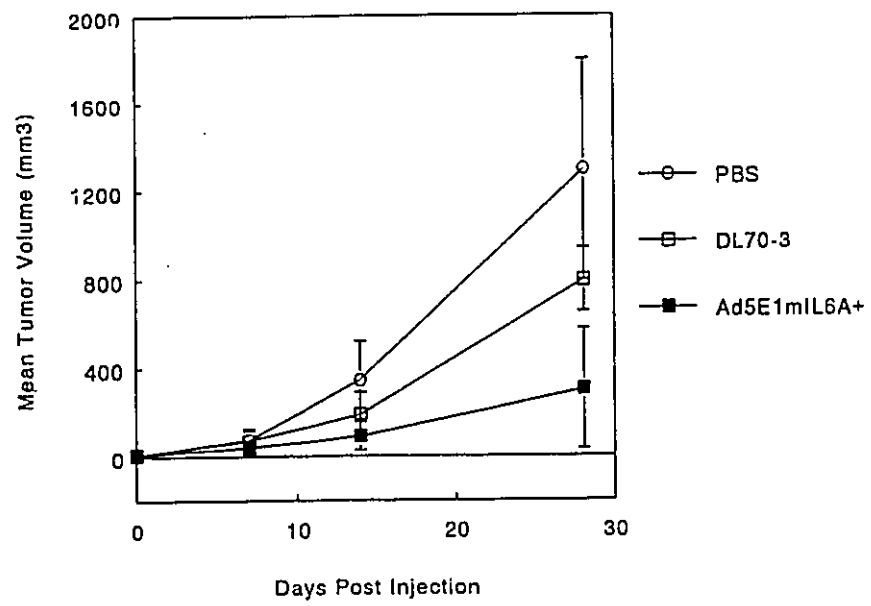


Figure 3

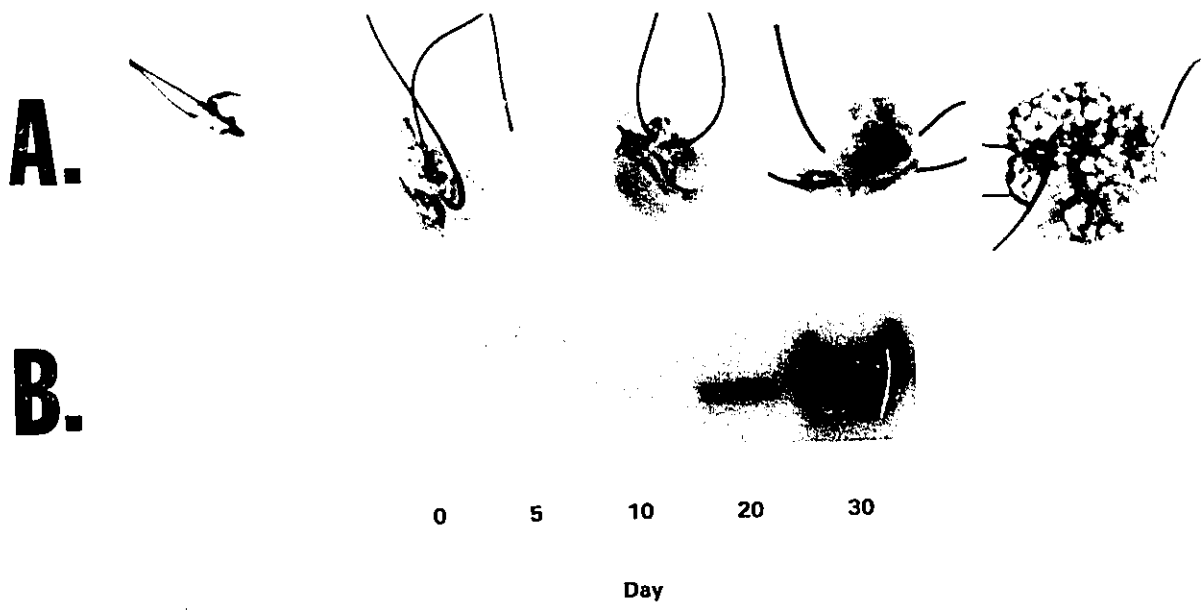


Figure 4

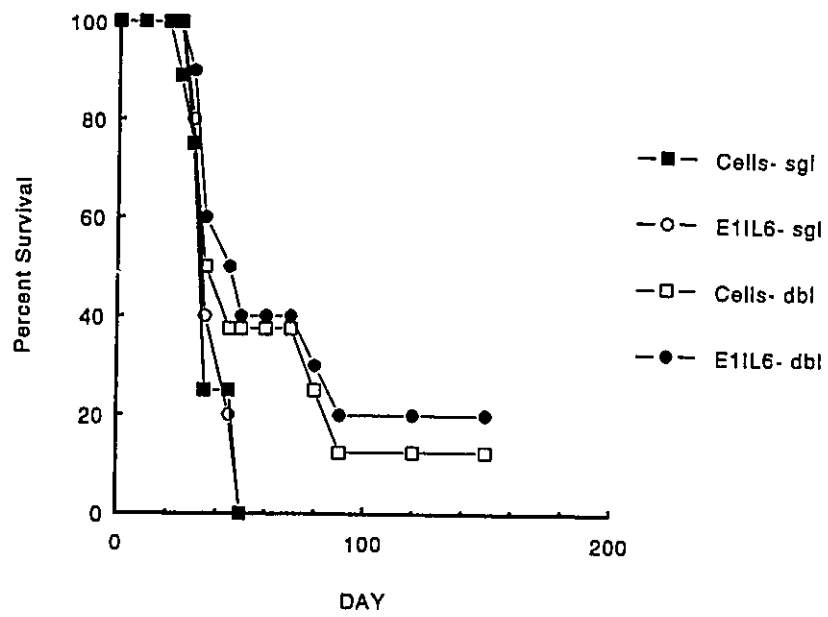


Figure 5

Chapter seven

Summary

It is now well understood that cytokines are powerful mediators involved in the regulation of a wide spectrum of biologic functions in host defense, including immune and inflammatory responses. There have been several ways in which the study of cytokine function has been elucidated, and much is now known about their activities in vitro. Yet, the specific in vivo functions of each cytokine is still not fully understood, particularly as they act not individually but in cascade with evidence of synergy and pleotropic activity.

Techniques in the use of transgenic mice have evolved to aid in the understanding of in vivo cytokine function and insights into these functions are beginning to emerge. Mice transgenic for cytokine genes, or mice whose genes encoding cytokine have been rendered inactive by targeted recombination (knock-out mice), have been used for such investigation. From these studies, some important conclusions can be drawn which have highlighted both the complexity of cytokine regulation and the drawbacks of transgenic mouse models (Taverne, 1993). First, it is clear that biologic effects associated with a particular cytokine are mediated through its induction of, or its synergy with another cytokine as part of a cytokine network. Second, that the inappropriate expression of most cytokines is usually damaging and third, that the site of transgene expression can influence the outcome of cytokine function.

Throughout this thesis, we have described the use of recombinant adenovirus vectors expressing cytokines to aid in the investigation of in vivo

cytokine function. The results from these studies have demonstrated the effectiveness for this use. Using a recombinant virus expressing luciferase as a reporter gene, we were able to define the natural tissue tropism of the vector after various routes of administration in both mice and rats. Intraperitoneal (IP) administration of the vector in mice demonstrated preferential sites for transgene expression (Mittal et al., 1993). Administration of recombinant adenovirus vector by this route resulted in the greatest levels of expression in the liver and spleen.

Since IL-6 was reported to be the major regulator of hepatic acute phase response, we administered the Ad5E3mIL6 vector IP and monitored the effects on acute phase response by measuring serum levels of haptoglobin and α_1 -acid glycoprotein (α_1 AGP). In these mice, serum levels of IL-6 were increased up to 4 days after administration. These IL-6 levels were coincident with dramatically raised serum levels of haptoglobin and α_1 AGP. These results demonstrate that adenovirus vectors can be used to express biofunctional cytokine and most importantly that this expression can be of a relatively short duration. No long lasting pathologic changes were seen in treated animals, although a transient splenomegaly was evidenced in animals receiving the IL-6 vector.

We also produced evidence that vector-derived cytokine expression could be targeted. Intratracheal administration (IT) of recombinant adenovirus vectors resulted in a dramatically different profile in the transgene tissue expression.

In this case, expression was found to be highly localized to the lung. Sprague Dawley rats receiving IT instillation of Ad5Luc3 vector (the luciferase adenovirus expression vector) resulted in expression almost exclusively in the lung. In a collaborative study, we found that vector-derived overexpression of IL-6 in the lungs of rats affected profound morphologic changes resulting in a local lymphocytic hyperplasia in these animals around day 7 (Xing et al., 1994). These changes and expression again were transient in nature and were not of pathologic consequence to the animal. These data demonstrated the ability of adenovirus vectors to express cytokine and affect response in a highly tissue specific manner. In addition, these local effects demonstrated for vector-derived cytokine expression were shown to influence both immune and inflammatory responses. Humoral immune responses of the mucosa tissue in the lung were dramatically enhanced by cytokine expressing adenovirus vectors after IT instillation. In a set of experiments, the expression of IL5, IL-6 or the combination thereof altered the production of immunoglobulin in mucosal secretions. Vector-derived IL-5 expression caused increases in the production of IgA anti-adenovirus antibody. This IgA response could be synergistically enhanced when co-expressed with IL-6. In this model, IL-5 expression appeared to preferentially affect IgA responses whereas, IL-6 expression was shown to affect both IgA and IgG production and acted as an overall proliferation factor for B cells in the lung.

Alterations in inflammatory response in the lung were also demonstrated after intratracheal administration of Ad5E3mRANTES vector. This vector expressing the chemokine RANTES caused a rapid and dramatic 50-fold increase in the number of monocytes recovered in lung BAL fluid. These changes were seen within 24 hours, and corresponded with increases in RANTES protein detected in BAL samples.

Additional differences in tissue tropism were demonstrated with these recombinant adenovirus vectors after intravenous administration and footpad injections in mice (Appendix one and two). From these analyses, it was again seen that preferential sites for tissue expression existed. One mode of delivery in particular may serve as an attractive model to investigate cytokine function. The vector expression profile in mice after footpad injection was bi-polar nature with expression confined to the footpad and popliteal node on the injected side. Therefore, injection of cytokine expressing vector into the footpad on one side should allow the effects of cytokine on immune responses in the ipsilateral popliteal lymph node to be compared to those of the contralateral node. This system would provide an ideal internal control for the comparison of cytokine biofunction.

Even in this early stage of development, our use of the recombinant adenovirus vectors aforementioned, illustrates several advantages for this approach in defining cytokine functions in vivo. Unlike the transgenic mouse

model, cytokine expression derived from recombinant adenovirus vectors is targeted and transient. Animals administered adenovirus vectors for cytokine expression in essence serve as pseudo-transgenic animals. But in this case, the tissue responses of the animal are free of the consequences of cytokine expression during the life from implantation onward. Thus, the use of these vectors in animals may provide a more clear understanding of cytokine function in normal physiologic responses.

In addition to these advantages, innovation of the current adenoviral vector technology is possible and may allow even more highly refined models to be developed for the assessment of cytokine function. One such recombinant adenovirus vector system, incorporating the Cre recombinase of bacteriophage P1 has just been developed, which has important implications to this effect (Anton and Graham, 1995). This system was demonstrated to control transgene expression in a highly regulated manner. The Cre protein catalyzes the precise recombination between two asymmetric 13-bp inverted repeats of loxP and results in the excision of intervening DNA regions. An adenovirus vector encoding Cre was used to initiate expression by excising an extraneous spacer sequence used to block expression of the transgene by separating it from a promoter.

These same principles could also be applied to adenovirus vectors used in the study of in vivo cytokine function. This refinement in adenovirus

technology may increase the capability of this system to target cytokine expression to specific cell types within tissues to more closely mimic true physiologic cytokine responses. This could be accomplished by engineering spacer sequences flanked by loxP sites into promoters controlling cytokine expression to those cells within a tissue. The additional use of tissue specific promoters and modifications of the fibre protein to alter the binding properties in these vectors may further enhance these capacities.

The only real limitation currently in the scope of these vectors use for in vivo investigation is due to the associated immunotoxicity seen after administration of these vectors in vivo (Yang et al., 1995). It should be noted that most of the data have been accumulated from studies using first generation vectors employing strategies requiring stable gene expression. Also, that initially little was done to generate vectors designed to circumvent this problem. However, many modifications in vector design are currently ongoing and have already demonstrated reduction in this immunotoxicity. In the same study of Yang et al., (1995) temperature sensitive mutant E2 region genes have been incorporated which reduce the immunogenicity of these vectors. Similar innovations involving the use of further deletions (e.g. in E4) are being developed at McMaster by Graham et al. Thus, many of the complications of immunotoxicity may soon be removed from the vector which will allow for clearer interperation of cytokine function on host response.

In this initial study using adenovirus vectors, we have used host response against adenovirus antigen as a measure of function. This removes some of the concerns regarding immunotoxicity interfering with the interperation of cytokine function and allowed us to use the E3 region replication competent vectors for study. These E3 vectors are semi-permissive for replication in rodent cells in vitro so that a low level of replication in vivo is possible. However, it is unlikely that replication affected response significantly compared to that of cytokine expression since control vector in every instance demonstrated little alteration of physiologic response.

Since replication competent vectors (E3 region constructs) would not be suitable for clinical applications, we also analyzed the effects of a recombinant adenovirus containing cytokine inserted into the E1 region of the vector. E1 region vectors are replication deficient since they are incapable of producing E1 region gene transcripts which are required for viral synthesis. Using the Ad5E1mIL6A⁺ vector, we were able to demonstrate the efficient expression of cytokine from such vector constructs in vivo. In these E1 region vectors, cytokine expression is dependent on promoter activity in a given tissue.

These E1 region recombinant adenovirus vectors may prove useful for new vaccines designed to enhance protection in mucosal tissues and for the development of new cancer therapies. With regards to the latter, recombinant adenoviruses expressing IL-2 have been shown to affect tumorigenicity and

tumor growth after vector administration (Addison et al., 1995; Haddada et al., 1993). Our demonstration of enhancement of mucosal IgA and IgG antibody responses against adenovirus antigen by IL-5 and IL-6 expression suggests the use of these cytokines as adjuvants in mucosal vaccines. An adenovirus vector expressing herpes glycoprotein B (Ad5gB8) has previously been demonstrated to protect mucosal tissue against wild type infection (Gallichan et al., 1994). Incorporation of these cytokine genes into adenovirus vectors expressing viral antigens, like Ad5gB8, may lead to a generation of vectors capable of inducing long-term mucosal immunity.

In summary, we have begun to develop a novel model for the in vivo characterization of cytokine function using recombinant adenovirus vectors. The use of these vectors has proven useful in elucidating cytokine functions and provides an additional tool to the arsenal of scientific investigation to define these functions more precisely. The future of this technology appears bright both on the forefront of basic research and to the applied clinical sciences. Gene therapy strategies which take advantage of the transient expression capabilities of these vectors are already beginning to be used in clinical trials for cancer therapy.

Appendix One

Expression of Luciferase in the tissues of Sprague-Dawley rats after intravenous injection of the Ad5Luc3 vector. Data points are expressed as the mean \pm SE measurement of luciferase activity recovered from individual tissue samples. n = 3 for each data point.

Appendix Two

Expression of luciferase in the tissues of Sprague-Dawley rats after footpad injection of the Ad5Luc3 vector. Data points are expressed as the mean \pm SE measurement of luciferase activity recovered from the individual tissue samples. n = 3 for each data point.

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