5-2003

Using Immune Modulators to Ameliorate Aleutian Disease in Mink

Kie Hoon Jung
Utah State University

USING IMMUNE MODULATORS TO AMELIORATE ALEUTIAN DISEASE IN MINK

by

Kie Hoon Jung

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

Edmund D. Brodie, Jr., Ph.D. Major Professor	Dale L. Barnard, Ph.D. Committee Member
Robert W. Sidwell, Ph.D. Committee Member	Joseph K.K. Li, Ph.D. Committee Member
Daryll B. DeWald, Ph.D. Committee Member	Thomas L. Kent, Ph.D. Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2003

ABSTRACT

Using Immune Modulators to Ameliorate Aleutian Disease in Mink

by

Kie Hoon Jung, Doctor of Philosophy

Utah State University, 2003

Major Professor: Dr. Edmund D. Brodie, Jr.
Department: Biology

Aleutian disease of mink (AD) is an immunopathological disorder induced by persistent infection with AD virus. Many breeds of mink seem to have a high susceptibility to AD. Interestingly, the primary feature of AD in susceptible adult mink is a chronic, fatal immune complex-mediated glomerulonephritis comparable to that seen in human diseases such as systemic lupus erythematosus. Because of its comparative aspects to human diseases and the substantial economic loss to the fur industries in the world, classical AD and the nature of the virus has been studied extensively for three decades. Currently no therapies are available and vaccination has been a failure; the latter exacerbated the disease.

The hypothesis tested in this research was that the treatment of AD-infected mink with TGF-β_1 and dehydroepiandrosterone (DHEA) inhibited or ameliorated the disease. The hypothesis was tested by evaluating the effects of TGF-β_1 and epiandrostene-like compounds on the immune response of AD-infected animals on the normal physiological parameters of animals and by evaluating the effects of both compounds on virus loads.

The TGF-β_1 study was designed to evaluate three different doses (200, 100 and 1 ng/kg) of recombinant human TGF-β_1 and one dose (500 ng/kg) of TGF-β_1 inhibitor at three different times (treated TGF-β_1 2 weeks pre-, 8 hours post-, and 2 weeks post-virus inoculation) of administration relative to virus exposure. Animals were administered drug or placebo intravenously via the inguinal vein injection and animals were held to up to 9 months.

For the epiandrosterone study, five mink received 16 ng/kg HE2300 once per week, 17 animals received 16 ng/kg HE2300 three times per week, and five animals were injected with 16 ng/kg HE2500 three times per week. In addition, 17 animals received vehicle three times per week. All treatments were injected subcutaneously into the nape of the neck of the mink. Animals were held to up to 8 months.

Pathological analysis of various physical, physiological and immunological parameters in surviving mink treated with either immunomodulator (treated with TGF-β_1 at 100 ng/kg and treated with HE2300 at 16 ng/kg three times per week) revealed that these parameters were near the levels measured for normal uninfected mink. In contrast, in untreated infected animals the measured virus pathology, and physiological and immunological parameters were significantly different from the untreated control animals. In a comparison of survival times, treated animals with either immunomodulator survived almost twice as long as untreated animals. A polymerase chain reaction (PCR) assay of lymph node tissue confirmed that AD-infected animals treated with either immunomodulator had significantly lower levels of virus DNA than untreated infected animals.

These findings support the hypothesis that TGF-β_1 and DHEA and its analogs may afford mink protection from chronic lethal AD.

(171 pages)

ACKNOWLEDGMENTS

I would especially like to thank my dissertation advisor, Dr. Dale L. Barnard, and major professor, Dr. Edmund D. Brodie, Jr., for their guidance and for teaching me invaluable lessons of life. I will always remember their example as hard-working and dedicated researchers who were willing to share ideas and teach the principles behind the experiments that were being conducted.

I would like to thank the other members of my committee, Dr. Robert W. Sidwell, Dr. Joseph K.K. Li, Dr. Daryll B. DeWald, and Dr. John Stark, for sharing their expertise and giving me suggestions and advice during this research project. I would like to acknowledge the help and support of Scott Winslow, Darin Chase, Valerie Hubbard, and Jason Roth.

Special thanks to my wife, Jung-Ae Choi, and my two daughters, Rebekah and Michelle Jung, as well as to my mother and my father, who passed away from lung cancer several years ago, my father- and mother-in-law and family, Ruth and Dr. Author Moss, and Dr. Kiyo Kamikawa, for their encouragement and interest in my work, and thanks to God.

Kie Hoon Jung

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Context

Aleutian disease (AD) is an immunopathological disorder of mink induced by persistent infection with AD virus (ADV), a naturally occurring infection caused by a virus from the Parvoviridae family (Porter et al., 1980). The chronic infection often leads to immune complex disease and is marked by a progressive renal failure. It is characterized by a hypergammaglobulinemia, a large fraction of which consists of antibody complexed with non-neutralizable virus. Pathological findings include mesangial proliferative glomerulonephritis and severe interstitial nephritis comparable to that seen in human diseases such as systemic lupus erythematosus (Bloom et al., 1994). AD often leads to tremendous weight loss and premature death, both of which severely impact the profitability of mink farming

Currently no therapies are available and vaccination has been a failure; it exacerbated the disease (Bloom et al., 1994). For producers who have AD in their mink herds, their only recourse is to pelt all animals or to take a chance that the disease will not afflict a significant portion of their animals. Therefore, a modality needs to be developed that could boost the susceptible animals' immune responses in an appropriate manner to allow mink to survive AD. Such a treatment would allow the mink industry to remain a profitable endeavor.

Extensive studies suggest that there is a genetic predisposition to AD. Some mink breeds, like those designated as sapphires or having dark coat color, are much more

susceptible to chronic lethal AD than other breeds such as the wild type strains (Bloom et al., 1994). In addition, many immunological studies have suggested that an abnormal immune response in susceptible animals may cause the pathology of AD (reviewed by Aasted and Leslie, 1991; Mori et al., 1991; Kanno et al., 1992; Bloom et al., 1994).

There are two kinds of immune responses based on the cytokine profile expressed during immune response. Th1 type cells are involved in inflammatory responses, release of interferon gamma (IFN-γ), interleukin-6 (IL-6), and tumor necrosis factors (TNF-α, β), activation of macrophages, induction of $CD8^+$ cytolylic T-cells and clearance of viral and intracellular infection. Th2 cells are involved in the anti-inflammatory responses and in stimulating antibody production by B-cells. In addition, Th2 type cells do not promote tissue damage as do Th1 cells, the damage by these latter cells being mediated by release of IL-3, 4, 5, 6 and 10. A Th1 predominance leads to the suppression of Th1 cytokines such as IL-12 and IFN-γ.

From early studies of AD (Hahn and Hahn, 1983; Bloom et al., 1994), it is apparent that the immunological response to AD is overwhelming humoral (hypergammmaglobulinemia) and mediated by Th2 type cytokines, especially IL-6. Perhaps not coincidentally, human diseases (e.g., Castleman's disease) that are characterized by hypergammaglobulinemia are also associated with high levels of IL-6 production. Based on these findings, AD may be a virus-induced cytokine disorder; a syndrome of chronic, inappropriate production of IL-6 and perhaps other Th2-type cytokines (Bloom et al., 1994). Perhaps, if one could modulate the immune response in AD-infected animals to a largely cell-mediated response with Th1-type cytokines instead of the typical Th2 cytokine response, the severity of AD might be diminished or perhaps

even prevented. A pertinent observation for the treatment of AD in mink is that an attenuated vaccine only enhances AD (a Th2 response), leading to an especially extreme B cell hyperplasia, while immunosuppressive therapy has been shown to slightly ameliorate the severity of disease, e.g., preventing AD lesions (Cheema et al., 1972). One of the compounds chosen, levamisole, does not directly modulate the B cell function of producing antibody (Amery and Hörig, 1984). The other compound, isoprinosine, actually enhances B cell antibody production in the presence of pokeweed mitogen (Tsang et al., 1984). Thisa result is not a desirable effect in AD, where over-production of antibody leads to fatal disease.

There is considerable evidence for an immunomodulatory role for a cytokine, transforming growth factor beta (TGF-β). It both potentiates and inhibits various functions of the immune system in its regulatory role, depending on when and where it is expressed. Therefore, the regulatory functions ascribed to TGF-β sometimes appear to be in opposition. However, its potent effects on immune cells in vitro are reflected in the various effects it has in a variety of animal model systems, strongly suggesting an important role in immune disorders such as autoimmune disease (Derynck and Choy, 1998).

A finding relevant to the potential immunotherapy of AD is that exogenous administration of TGF-β in vivo markedly depresses inflammatory and immunological responses. For example, TGF-β delays the onset of experimentally-induced arthritis in mice, a disorder involving immune complexes (Brandes et al., 1991). In addition, TGF-β also regulates expression of primary antigen presenting cells, which activate T-lymphocyte responses (Steinman, 1991) that are responsible for correct processing of

virus antigen. Thus, it is conceivable that TGF-β could elicit a Th1 response adequate enough to ameliorate AD by inducing appropriate processing of AD antigen. Finally, TGF-β_1 seems to potently inhibit cell proliferation and is able to induce apoptosis (programmed cell death) in B cells (Gordon et al., 1994) in addition to reducing the secretion of immunoglobulin from activated B cells (Stavnezer, 1996). These mechanisms suggest that TGF-β_1 may be able to down-regulate what appears to be a B cell hyper-response such as is seen in mink with AD.

Another set of compounds that appear to have useful immunomodulatory properties is dehydroepiandrosterone (DHEA) and its analogs. DHEA has been shown to have immunomodulating effects in several experimental infections involving viruses and protozoans (Rasmussen et al., 1991; Mulder et al., 1992; Uozumi et al., 1996; Loria and Padgett, 1998). In HIV infections, 16a-bromoepiandrosterone (a-epi-Br), an analog of DHEA, may have shifted cytokine production to a Th1 response and increased circulating activated immune effecter cells (Freilich et al., 2000). This activity appeared not to be androgenic, since the compound only bound weakly to the androgen receptor. Thus, DHEA and its analogs and TGF-β_1 may be able to promote Th1 cytokine-type responses that could afford protection for mink from chronic lethal AD.

Therefore, we attempted to develop several approaches for treating experimental AD using TGF-β_1 and epiandrosterone analogs. We tested the hypothesis that treatment of AD-infected mink with TGF-β_1 or epiandrosterone-like compounds would ameliorate the development of the AD as shown by enhanced survival time of the animals.

Literature Review

This review considers several aspects of a Aleutian disease (AD). After an overview of current information about the structure and function of the AD virus (ADV) genome, the major clinical forms of AD are discussed. This is followed by summary of recent pathogenesis studies providing potential insight into the genesis of the immune disorder.

Aleutian Disease of Mink

AD is an immunopathological disorder of mink induced by persistent infection with ADV (Porter et al., 1980). It is a naturally occurring virus infection caused by a virus from the family *Parvoviridae*. The virus can be detected in saliva, urine, and feces and the virus can persist for long periods in the environment. During the virus life cycle, certain obligate intermediates of ADV replication are produced. These include replicative forms of ADV DNA, viral messenger RNA (mRNA), various proteins, progeny genomes, and infectious progeny virus (Bloom et al., 1994). A major goal of ADV research is to relate virus infection to the pathogenesis of the clinical syndromes.

AD is a persistent viral infection of mink. In the early 1940's a spontaneous mutation resulting in a dark blue coat color occurred in ranch-raised mink in Oregon and was designated the Aleutian mink coat color (Alexandersen, 1986); this mutation is homozygous recessive for the Aleutian gene *a*. In the early 1950's, as the Aleutian mink were continuously in- bred, a chronic wasting disease was noticed in many of them. It was mainly characterized by weight loss but on occasion some mink would have abnormal hemorrhaging from the mucous membranes. A syndrome involving the

kidneys, the liver and lymphoid tissue of *aa* mink was reported, and was referred to as AD (Hartsough and Gorham, 1956). It first seemed that only Aleutian mink were affected; however, it has been subsequently shown that all genotypes of mink are susceptible to the disease, but mink homozygous for a gene develop more severe disease with a more rapid course. Mortality from AD can be as high as 100% in Aleutian mink. The disease is not as severe in other breeds. Wild-type mink known as demi (brown), are least affected, with about a 6% mortality. It appears that a small percentage of non-Aleutian mink may either recover or live for years with AD (Porter et al., 1980).

Clinically, AD is characterized by gradual loss of weight, anemia, and uremia ulceration of oral mucosa and in rare occasions, nervous signs. The gross lesions include emaciation (Fig. 1.1), hepatomegaly with small pinpoint pale foci scattered through the parenchyma, generalized lymphadenopathy with the spleen and lymph nodes two to four times their normal size, nephritis characterized by enlarged kidneys with widespread petechiae early in the course and by shrunken pale kidneys with cortical cysts during the later stages. Histologically, there is widespread proliferation and infiltration of plasma cells in practically all organs (Obel, 1959; Henson et al., 1992). These cells are mostly of the preivascular type early in the disease. The plasmacytosis leads to a marked hypergamma-globulinemia, which may exceed 50% of the total serum proteins. The glomeruli in affected kidneys are relatively a vascularized and contain a large amount of slightly granular, eosinophilic material. Mononuclear cells proliferate in the liver. In the early stages of the disease the all infiltrate is a mixed population of lymphocytes, histiocytes, and immature plasma cells. Later, mature and immature plasma cells dominate. In addition, there is a progressive renal disease characterized by mesangial

proliferative glomerulonephritis and severe interstitial nephritis comparable to that seen in human diseases such as systemic lupus erythematosus (Bloom et al., 1994). Additional symptoms include decreased fertility, abortion, acute pneumonia in kits, and chronic, persistent infections, all of which severely impact the profitability of mink farming (Porter et al., 1980).

ADV Genome Structure, Protein, and Replication

The organization of the 4,748-nucleotide ADV DNA genome (Bloom et al., 1990) resembles other autonomous parvoviruses in that there are major right and left open reading frames, and palindromic structures at both 3' and 5' termini (Bloom et al., 1990). The nucleic acid consists of a 4.8 kilo base single-stranded DNA (Mayer et al., 1983). The size of AD virus particles ranges from 22 to 25 nm. Each 22 to 25 nm particle contains only a single strand of DNA, the minus sense strand in > 90% of virions (Bloom et al., 1993). The virus is non-enveloped and has cubic symmetry and contains 32 capsomeres. The density of the complete virus in cesium chloride (CsCl) is 1.39 to 1.42 g/cm^3 (Berns and Labow, 1987).

Permissive replication of the non-pathogenic strain of virus, ADV-G (ADV-Utah 1 by Gorham) in Crandell feline kidney (CRFK) cells resembles that of other parvoviruses (Bloom et al., 1990). After adsorption and uncoating, the single-stranded genome is converted into a variety of double-stranded replicative forms, from which the single-stranded genomes are derived. The number of DNA molecules in an infected cell is large, $\approx 10^5$ copies of both duplex replicative forms and single-stranded virion molecules per infected cell (Bloom et al., 1985; Alexandersen and Bloom, 1987).

During the infectious cycle, four viral mRNAs (R1, R2, R2′, and RX) are synthesized from a promoter at map unit 4 (P4) and one mRNA (R3) from another promoter at map unit 36 (P36) (Alexandersen et al., 1988a). R1, R2, and R3 terminate with a polyadenylation signal located at map unit 92, whereas R2′ and RX utilize a termination signal at map unit 53. Cells permissively infected with ADV contain $> 10^5$ copies of viral mRNA (Alexandersen et al., 1988b), and all the RNA species are complementary to single-stranded virion DNA.

The 2.8-kb R3 mRNA codes for the two capsid proteins, VP-1 (P85) and VP-2 (P75), which arise by alternate initiation on the same mRNA (Alexandersen et al., 1988a; Christensen et al., 1993). VP-2 coding sequences are fully contained within the large right open reading frame, whereas VP-1 contains additional sequences joined to the amino end of VP-2 (Bloom et al., 1982; Alexandersen et al., 1988a; Christensen et al., 1993).

The mRNAs specified by P4 encode at least two nonstructural (NS) proteins (Bloom et al., 1982; Alexandersen et al., 1988a; Christensen et al., 1993), analogous to those found for minute virus of mice (MVM) and other parvoviruses. The ADV NS proteins are derived by complex, differential mRNA splicing of the major left open reading frame and smaller open reading frames in the mid-genomic region (Alexandersen et al., 1988a). All of the ADV NS proteins share a common amino terminal portion of 59 residues, but there are unique regions for each NS protein as well.

The large 4.3-kb mRNA, which contains almost the entire left open reading frame, codes for the 71-kDA (Bloom et al., 1983) major nonstructural protein 1 (NS1), a protein with pleiotropic function (Bloom et al., 1983; Christensen et al., 1993).

Parvoviral NS1 is required for viral genomic replication and excision of single-stranded virion DNA from double-stranded replicative form DNA (Bloom et al., 1983; Rhode, 1985, 1989; Christensen et al., 1993). In addition, NS1 functions as a transcriptional regulator by trans-activating the P36 promoter and thus controlling the level of capsid protein mRNA (Ahn et al., 1989; Doerig et al., 1990; Hanson and Rhode 1962). NS1 can also regulate its own transcription and may be involved in influencing the level of cytopathology induced by the virus (Labow et al., 1986; Berns and Labow, 1987; Ozawa et al., 1988; Legrand et al., 1993).

Both the 2.8-kb R2 and 0.85-kb R2′ mRNAs code for a smaller nonstructural protein 2 (NS2), ~13.4 kDa in size (Cotmore and Tattersall, 1986; Alexandersen et al., 1988a). The difference between R2 and R2′ is that R2′ terminates at a polyadenylation site at map unit 53, whereas R2 continues with a long 3' noncoding region that ends at the map unit 92 polyadenylation signal (Alexandersen et al., 1988b). The function of NS2 is not well understood, but it is required for efficient replication in a cell-type specific fashion (Brandenburger et al., 1990; Brownstein et al., 1992). It should be noted that the ADV R2 transcript also contains all the required coding sequences necessary for production of the capsid proteins, and in fact, a recombinant baculovirus expressing this mRNA direct will synthesis of small amounts of capsid proteins in addition to NS2 (Christensen et al., 1993).

The 1.1-kb RX mRNA may specify an additional nonstructural protein, tentatively designated as NS3 (Alexandersen et al., 1988b). A baculovirus containing this gene directs expression of an appropriate sized protein in insect cells (Christensen et al., 1993). NS3 has not been found for the other autonomous parvoviruses; however,

MVM has several different isoforms of NS2. Thus, if NS3 is a real viral gene product, it may subserve the function of NS2 isoforms. Preliminary sequence analysis of additional strains of ADV suggests that the requisite splicing junction for RX may be lacking from the RNA R1 in some in vivo derived ADV strains.

At the peak of replication, viral antigen is detected in cells associated with the distribution of macrophages in lymphoid organs and Kuffer's cells liver, and the location of viral antigen is cytoplasmic, suggesting that the virus is sequestered in phagocytic cells (Porter et al., 1969). At later times in the infection, virus levels decrease significantly, but complete viral clearance is infrequently observed (Larsen and Porter, 1975). Because ADV occurs in infectious immune complexes and is probably sequestered in phagocytic cells, the simple identification of virus, virion proteins, and DNA is not adequate proof that actual replication is occurring in a particular site. Therefore, it is necessary to use reagents that mark obligate intermediates of active viral gene expression (e.g., replicative from DNA, mRNA, and nonstructural proteins; Alexandersen et al., 1988a).

Virion DNA is widespread in lymphoid and phagocytic organs. It is apparently a sequestered in cells of the reticuloendothelial system, including follicular dendritic cells (FDC) and macrophages (Kanno et al., 1992). This suggests that some virus might be bound as immune complexes to the FDC (Mori et al., 1991). Replicative from DNA and mRNA, the two obligate intermediates of de novo virus replication, are present at greatly reduced or restricted levels as compared to permissively infected mink kit lungs or cell culture (Alexandersen et al., 1988a). Nevertheless, evidence suggests that a small percentage of macrophages support viral replication and gene expression (Mori et al., 1991) and that FDC and lymphoblasts may also be targets. Importantly, the number of

cells sequestering virion DNA is at least 10 times greater than the number containing ADV RNA, suggesting that the number of cells containing ADV support an active infection (Alexandersen et al., 1988a).

At later times after infection, when the immune disorder is evident, the amount of virus is further reduced (Alexandersen et al., 1988a). Virion DNA remains detectable in the sequestered pattern observed at earlier times, but only in cells in lymphoid tissues that appear to be macrophages (Mori et al., 1991). Furthermore, throughout the course of disease, other organs such as nucleated blood cells, bone marrow, liver, kidney, and spleen also contain viral sequences (Alexandersen et al., 1988a).

AD of Newborn Mink

Less than 2 weeks after neonatal infection, mink kits can develop an acute interstitial pneumonia (Alexanderson, 1986). Within a few days, they succumb to a fatal respiratory distress syndrome characterized by parenchymal hemorrhage, extensive atelectasis, and hyaline membrane formation. With highly virulent strains, incidence and mortality are >90%. In contrast, the incidence of disease is between 50-70% and mortality is 30-50% with low-virulence strains. Survivors develop typical lesions of the classical chronic adult form of AD regardless of the infecting strain (Alexanderson et al., 1994).

In AD of newborn mink, there is no question of where the virus is replicating. The disease in kits is characterized by ADV infection of type II alveolar cells, with capsid and nonstructural proteins readily demonstrable in the infected cells. Approximately 10% of the type II cells are actually infected in these animals (Alexanderson et al., 1987).

Infection of these cells, which produce pulmonary surfactant, presumably leads to a functional surfactant deficiency and to the fatal severe respiratory distress. Although the main target in kits is the type II alveolar cell, virus replication occurs in cells of other tissues, such as in the lymph node, spleen, kidney (Alexanderson et al., 1987; Alexanderson, 1990). *In situ* and Southern blot hybridization studies indicate that the levels of replicative intermediates in the infected alveolar type II cells are very high, on a par with those found in cell culture ($\approx 10^5$ copies per infection cell; Alexanderson et al., 1987). This apparently unique susceptibility of the type II cells in the newborn period may be due to several factors. First, during the immediate postnatal period, type II pneumocytes are rapidly dividing and rapidly dividing cells are, in general, more susceptible to parvoviruses. In addition, newborn mink kits are not able to mount as rapid an immune response as adults (Alexanderson, 1986; Alexanderson et al., 1994).

Pathogenesis of Aleutian Mink Disease

Most pathogenesis studies have involved experimentally induced infections in which uninfected mink are exposed to spleen homogenates from other infected mink, either intraperitoneally or intranasally. Under these conditions, not only are viral particles introduced, but also antibodies that may enhance uptake of the virus into cells and other inflammatory mediators are also inoculated with virus. It has been assumed that the virus is naturally transmitted via a mucosal route where it penetrates the epithelium of the gastrointestinal or respiratory systems. The incubation period in natural infections appears to be longer than that in experimentally-induced infections, indicating possible fundamental differences in the pathogenesis of disease between natural and

experimentally-induced infection. In experimentally infected mink, a three to ten day interval may exist between appearance of viral DNA in blood leukocytes, as determined by the polymerase chain reaction (PCR), and appearance of antibody. When mink are first infected with the virus, there are large amounts (10^7-10^8) of virus particles in the blood before antibody appears. With the appearance of antibody, the virus titer in the blood decreases. After a few weeks, the virus either disappears or can be detected in only small quantities in the blood. In demi mink, there is a good linear relationship between the amount of virus in blood after 6 months of infection and lesions. In dark mink, there is not a linear relationship because smaller amounts of virus in blood can be associated with severe lesions.

A simplified scheme for a grand unified theory of AD pathogenesis might be portrayed as follows: An initial round of AD virus infection occurs in antigen presenting cells, perhaps in macrophages, follicular dendritic cells (FDC), or an as yet in an unrecognized primary target cell (such as renal tubular cells), providing an initial burst of virus that stimulates the immune system to produce anti-ADV antibody. Further ADV antigen stimulation leads to preferential induction of Th2 helper T-cell types, and the creation of a Th2 cytokine-laden milieu in lymphoid follicles. The FDCs present ADV antigens to B cells, programming them for differentiation into anti-ADV antibody plasma cells. In a subpopulation of macrophages, a restricted, antibody-dependent infection (ADI) leads to the production of the cytokine, IL-6. IL-6 mediates a polyclonal stimulation of the programmed B cells and synthesis of large amounts of antiviral antibody. The stimulated B cells also release IL-6 and stimulate other B cells to follow the same path. In the face of the persistent infection, the antiviral antibody reacts with

virus, creating a chronic load of virus-containing immune complexes. This sustains a repeating cycle of virus loading onto FDC and macrophages, programming of anti-ADV B-cells and production of IL-6. The self-reinforcing circuit that results impairs normal regulatory mechanisms, leading to hypergammaglobulinemia and plasmacytosis. Deposition of immune complexes in glomeruli and arteries results in immune complex disease. Virus also infects cells in the renal tubular epithelium, and the infected cells become a target for T cells and other lymphocytes primed to react against infected cells, leading to interstitial nephritis.

Transforming Growth Factor-β (TGF-β)

The initial identification of transforming growth factor (TGF-β), was based on its ability to reversibly induce phenotypic transformation of elect fibroblast cell lines (Derynck, 1992). Although transforming growth factor (TGF-β) was originally named for its ability to reversibly cause a phenotypic transformation of rat fibroblasts (Moses et al., 1981), numerous bioassays describing cartilage inducing factor (Seyedin et al., 1985), glioblastoma immunosuppressive factor, myoblast differentiation inhibition factor (Florini et al., 1986), and epithelial growth inhibitor (Holley et al., 1980) were found to be measuring the same TGF-β protein.

The initial detection of TGF-β activity in a transformation assay suggested a role for TGF-β in malignant transformation and tumor development. It is now well established that the activities of TGF-β are by no means restricted to tumor cells, but that TGF-β exerts a multiplicity of biologic activities on most cells, both normal and transformed, and regulates many cell physiologic processes. The biologic response to

TGF-β is complex and depends not only on the cell type but on the physiologic conditions. Furthermore, TGF-β also plays an important role in the control of the immune response and wound healing and in the development of various tissue and organs.

Genomic Organization of the TGF-β_1 Gene

Multiple species of TGF-β which are encoded by different genes have been identified. TGF-β's are disulfide-linked polypeptide dimers of molecular mass 25 kDa. Each mature chain is 112 amino acids in length and is derived from a larger precursor. In mammalian genomes, at least three genes encode TGF-β precursors (TGF-β_1, β_2, and β_3). TGF-β_1, β_2, and β_3 are each coded by a single gene located on separate chromosomes, in humans on 19q13.1, 1q41, and 14q23 (Fujii et al., 1986; Barton et al., 1988).

The human TGF-β_1 precursor is encoded by seven exons. The similarity of their seven exon structures and the high degree of conservation of their exon/ intron borders indicate that these genes are derived from a common ancestor (Derynck et al., 1987). TGF-βs have unique 5′ long untranslated regions (UTR). For TGF-β_1, there is a region of 841 untranslated base pairs and 50 untranslated base pairs in 7 exon. In contrast to TGF-β_2 and β_3 promoters, β_1 does not have a traditional "TATA" sequence. Instead, just upstream of the first transcriptional start site, the TGF-β_1 promoter has a very GC-rich region with several Sp1 binding sites.

The TGF-β_1 precursor is 390 amino acid. All the potential N-linked glycosylation sites lie outside the bioactive domain. The TGF-β_1 precursor contains an amino-terminal hydrophobic signal sequence (aa 1-23) for translocation to the endoplasmic reticulum to

undergo exocytosis. The pro-region (aa 24-278) guides the correct folding and disulfide bonding of the bioactive domain during TGF-β synthesis (Gray and Mason, 1990). The bioactive domain (aa 279-390) is the carboxyl-terminus of the precursor, preceded by a sequence of four basic amino acids, which form the cleavage site. Intracellular proteolytic cleavage probably occurs by furin peptidase after dimerization of the precursors (Dubois et al., 1995). Active TGF-β is usually a homodimer but naturally occurring TGF-β_1, β_2 and β_3 heterodimers can also be found.

The amino acid sequences of all three mature TGF-β isoforms are strictly conserved; there is 98-100% identity between chicken and human. The evolutionary pressure to retain such a degree of conservation suggests that the functions of each isoform are critical. The degree of homology of the bioactive domain between the three human isoforms ranges from 70-80%, with much greater sequence divergence in the precursor region.

Structure of TGF-β

Since the original construction of the cDNA clone of TGF-β, extensive protein characterization and cDNA analysis have revealed a large group of structurally related secreted factors. In addition to being multifunctional, TGF-β is a member of a large family of factors with widely diverse activities. The TGF-β superfamily was first recognized in the late 1980s (Sporn et al., 1987). Since then, the superfamily has grown to more than 25 members, with some of the molecules being grouped into four distinct subfamilies owing to highly related structures (Roberts and Sporn, 1990). Each precursor contains an N-terminal signal peptide, a long prosegment, also called the "latency-

associated polypeptide" (LAP) and a 112-amino-acid C-terminal polypeptide that constitutes the mature TGF-β monomer. This monomer is cleaved from the remaining precursor segment following a tetrabasic peptide.

The active form of TGF-β is a hydrophobic, disulfide-linked dimer of the C-terminal segment of the pre-pro-TGF-β (Derynck et al., 1985; Cheifetz et al., 1987). The three TGF-β species, with a sequence identity of 70-80% (Derynck et al., 1988), are thus generated as homodimers of two identical C-terminal polypeptides, although heterodimers consisting of TGF-β_1 and TGF-β_2 have also been isolated (Cheifetz et al., 1987). The biologic relevance of the heterodimeric species is unclear, especially as they presumably correspond to only minor species. The 112-amino-acid mature TGF-β polypeptide includes nine conserved cysteines, eight of which are paired. All cysteines form intra-chain disulfide bonds, with the exception of one intermolecular cysteine bridge responsible for dimerization. Three-dimensional structure analysis has revealed an extended butterfly-like structure of the TGF-β_2 homodimer, rather than the compact globular structure frequently seen in highly stable and temperature and protease resistant growth factors (Daopin et al., 1992). The sequence between the fifth and sixth cysteine, which has the most sequence divergence among the different TGF-β isoforms and the other members of the TGF-β superfamily, is exposed at the surface of this molecule and plays an important role in the determination of the specificity of ligand-receptor recognition.

The prosegments of the three TGF-β precursors show a sequence identity of only 25-35%. Nevertheless, some structural elements are strongly conserved, suggesting

critical roles either in the function or in the folding of this prosegment. Among these conserved features are three cysteine residues and two N-glycosylation sites. Two of the three cysteines are involved in intermolecular disulfide bridge formation (Miyazono et al., 1988; Derynck et al., 1998). Finally, a hydrophobic stretch of amino acids closely following the signal peptide is conserved as well and may be involved in the folding of the precursor segment (Lopez et al., 1992).

Biochemical characterization has shown that TGF-β is normally secreted as a protein complex, consisting of the mature TGF-β homodimer and two prosegments, which interact non-covalently with the mature dimer. The participation of mature TGF-β in this complex prevents it from interacting with the TGF-β receptors, thus rendering this complex inactive or latent. Based on antibody recognition researches, it is likely that this interaction of TGF-β with the much larger prosegments leaves little of the mature TGF-β exposed at the surface of this complex (Wakefield et al., 1988).

The expression of the three TGF-β isoforms is differentially regulated. Thus, different inducers or exogenous agents have distinct effects on the secretion of individual TGF-β species. This differential regulation of expression is largely due to a characteristic and independent pattern of transcriptional regulation of the three TGF-β species, which is in turn regulated by distinct control elements in the promoters of the genes (Roberts et al., 1991). In addition, all three TGF-β isoforms have long 5′ untranslated sequences which most likely play an isoform specific role in the regulation of translation efficiency (Derynck et al., 1985; Kim et al., 1992).

Receptors and the Cytokine Network

Low numbers of high-affinity receptors for TGF-β are found on most normal resting and transformed mammalian cells. The two major TGF-β receptors, types I and II (TGFβRI, 53 kDa, and TGFβRII, 70-80 kDa), are both serine/ threonine kinases used by many of the mitogens, whose action TGF-β opposes (Derynck, 1994; Massague et al., 1994). Each has a small extracellular region, a single transmembrane domain, and an intracytoplamic serine/ theronine kinase domain. The large numbers of cysteines in the extracellular domain with a cluster of three just upstream of the transmembrane are characteristic of all signaling receptors of the TGF-β superfamily. Almost all cells, regardless of their origin, express TGF-β receptors and bind TGF-β with high affinity. Signaling has been shown to require the formation of a trimeric complex consisting of both TGF-β receptors I and II. The type III receptor, a transmembrane proteoglycan, has a short, highly conserved cytoplasmic domain without an apparent signaling motif (Lopez-Casillas et al., 1993). Other binding proteins for TGF-β include α_2–macroglobulin, fibronectin and β-amyloid precursor.

The cloned type I receptor has a high-affinity binding for TGF-β_1 and TGF-β_3 but not TGF-β_2 (Lin et al., 1992). The kinase domain of TGFβRII is flanked by a spacer between the domain and the transmembrane region and a carboxy-terminal tail. The kinase domains of the associated type II receptors of the superfamily show less than 40% amino acid sequence identity, suggesting that this divergence might be involved in conferring some signaling specificity.

In addition, type I receptors have a much shorter carboxyl-terminal tail and the extracellular domain has a distinct clustering of five cysteines. The type II receptors can bind ligand directly from the medium, while type I receptors cannot (Massague et al., 1994). Type I receptors recognize ligand that is bound to type II receptors, with which they form a complex, probably a heterotetramer. If distinct heterodimers have different functional specificities, this could provide another way of expanding the regulatory flexibility of TGF-β superfamily.

The role of TGF-β in immune surveillance and immunotherapy must be conceptualized in the context of its role as a cytokine, participating in the cytokine network. This integrated network includes contribution and immunity. The outcome of an immune response is the result of the balance between synergy and antagonism of several cytokines, and dysregulation at any level can contribute to disease pathogenesis. Like other cytokines, TGF-β is highly potent, acting on cell surface receptors at femtomolar concentrations (10^{-15}M). Since different cell types possess receptors for the same cytokine, TGF-β like other individual cytokines, may have multiple effects on the growth, differentiation and function of many cell types.

TGF-β in the Immune System

There is considerable evidence for an immunomodulatory role of TGF-β and its function as a potent differentiation modulating and immunosuppressive agent (Letterio and Roberts, 1998). In culture most cells of the immune system, such as lymphocytes, monocytes and dendritic cells, synthesize TGF-β that is almost exclusively TGF-β_1. The TGF-β released by these cells is mostly not active, suggesting that activation of the latent

complex is required before TGF-β can exert its function. The target cells are not only the different leukocytye cell types, but also various other cell types in the immediate tissue environment, such as endothelial cells or tumor cells, indicative of autocrine and paracrine actions. Once activated, TGF-β exerts a variety of activities on proliferation, differentiation and function of various cell types of the immune system.

TGF-β often inhibits cell proliferation, as has been shown for T lymphocytes, B lymphocytes, thymocytes, natural killer (NK) cells, large granular lymphocytes, and lymphocyte activated killer (LAK) cells (Rook et al., 1986; Kehrl et al., 1986a; Wahl et al., 1988). Furthermore, the response may depend strongly on the state of differentiation of the cell and on the context of cytokines to which the cells are exposed. Accordingly, these different types of immune cells all have TGF-β receptors.

One of the cell populations, which is strongly regulated by TGF-β are the T lymphocytes. Activated T cells secrete TGF-β and exogenous TGF-β inhibits interleukin-2-dependent proliferation (Kehrl et al., 1986b). In addition, TGF-β also inhibits the secretion of a variety of cytokines and in this way inhibits the effector function of the activated cells. Thus, TGF-β inhibits the effects and/ or the production of interferon-γ (IFN-γ), tumor necrosis factor (TNF) -α and -β and interleukins-1, -2, -3, and expression of interleukin-2 receptor (Ohta et al., 1987; Letterio and Roberts, 1998). The endogenous synthesis of the TGF-β and the increased cytokine production in TGF-β_1-deficient mice strongly suggests that cytokine production is under autocrine control of TGF-β (Shull et al., 1992). The inhibition of cytokine production and the anti-proliferative effect are presumably the major effector functions in TGF-β-induced

immunosuppression, especially as it relates to the cytolytic activities of the immune system. Accordingly, TGF-β has been shown to be a strong inhibitor of the generation and cytolytic activity of cytotoxic T cells, NK cells and LAK cells, and to suppress the natural and lymphokine-activated killing by large granular lymphocytes (Roberts and Sporn, 1990).

Other evidence for the strong immunosuppressive effect of TGF-β on lymphocytes comes from the observation that TGF-β downregulates interferon-γ-induced major histocompatibility complex (MHC) class II antigen expression by both lymphoid and non-lymphoid cells (Czarniecki et al., 1988). In contrast to the inhibitory effects of TGF-β on T lymphocytes, TGF-β also enhances the growth of immature lymphocytes and inhibits T-cell apoptosis, thus allowing expansion of the effector population (Rich et al., 1996).

Finally, TGF-β may play an important regulatory role in differentiation of T cells. TGF-β can induce the expression of CD8 and can synergize with TNF-α to favor the development of cells expressing CD8. Conversely, TGF-β inhibits later stages of differentiation and thus inhibits the generation of $CD4^+$/ $CD8^+$ cells (Suda and Zlotnik, 1992; Takahama et al., 1994). Whereas TGF-β was initially shown to strongly promote the generation of Th1 cells (Schmitt et al., 1994), a subset of helper T cells with a characteristic cytokine secretion pattern, TGF-β has recently been more clearly implicated as a positive regulator of differentiation of Th2 cells and a suppressor of differentiation of Th1 cells (Schmitt et al., 1994).

TGF-β also plays an important role in the function of B cells. The expression of TGF-β is regulated and is likely to depend on the differentiation state of these cells. For example, activation of B cells with *Staphylococcus aureus* Cowan mitogen strongly increases TGF-β_1 secretion without a major effect on mRNA levels, white lipopolysaccharide (LPS) stimulates the activation of TGF-β, which is required for LPS-induced immunoglobulin G (IgG) secretion by B cells (Kehrl et al., 1986a; Snapper et al., 1993). Furthermore, activation of the B cells by binding of an anti-IgM antibody to cell surface IgM also induces the secretion of active TGF-β_1 (Warner et al., 1992). The stimulation of TGF-β expression by B cells most likely plays an important autocrine role in B cell function, as TGF-β has a potent ability to inhibit cell (Gordon et al., 1994). TGF-β also regulates B-cell differentiation and immunoglobulin expression. Indeed, TGF-β suppresses the expression by activated B lymphocytes of membrane immunoglobulin and decreases secretion of immunoglobulins of different classes (Kehrl et al., 1986b). However, antibodies to TGF-β decrease the secretion of various immunoglobulin classes without affecting IgM secretion, suggesting that low levels of TGF-β may regulate and be required for immunoglobulin secretion (Snapper et al., 1993). Furthermore, the presence of TGF-β promotes isotype switching of immunoglobulins, likely associated with differential transcriptional regulation (Coffman et al., 1989).

TGF-β also plays an important role in the physiology and functional regulation of monocytes and macrophages. As with cells of many other hematopoietic cell lineages, monocytes and macrophages secrete TGF-β_1 in a highly regulated fashion. In addition,

the activation of the latent TGF-β complex is also highly regulated. For example, treatment of monocytes with LPS or macrophages with concanavalin A results in a drastically increased activation of TGF-β without changes in mRNA levels (Grotendorst et al., 1989). In addition, TGF-β_1 synthesis by monocytes is strongly induced by TGF-β itself (Wahl et al., 1989). Thus, TGF-β can induce or inhibit cell proliferation depending on the physiologic context (Fan et al.,1992). Low concentrations of TGF-β induce a chemotactic response, which may be important for the recruitment of these cells at sites of inflammation, in activating cells to secrete a variety of cytokines which may play a role in the inflammatory response and often associated angiogenesis, and to increase their expression of adhesion receptors (Wahl et al., 1993). The important role of TGF-β is further illustrated by the inflammatory response to subcutaneous injection of TGF-β, which elicits a sequence of events, including inflammatory cell recruitment, fibroblast accumulation and vascular growth, which is similar to the normal inflammatory response to injury (Roberts et al., 1986). It is thus conceivable that the release of TGF-β by activated degranulating platelets results in an influx of monocytes, which accompanies the initiation of the inflammation, and of fibroblasts, and may explain the appearance of fibroblasts at the site of injury (Postlethwaite et al., 1987). Following recruitment of monocytes, the TGF-β at the site of inflammation then presumably activates monocytes to induce secretion of growth factors and inflammatory mediators, that are likely to amplify further the inflammatory response and to initiate the repair processes at the site of injury.

In contrast to these proinflammatory activities, TGF-β also inhibits a variety of activities of macrophages, which result in a suppression of macrophage function and are likely to contribute to a resolution of the inflammatory response. This drastic change in cell response may be causally related to decreased expression of cell surface TGF-β receptors as cells mature. Thus, TGF-β can inhibit the production of cytokines, including interferon-γ and increase the expression of the interleukin-1 receptor antagonist by macrophages (Fargeas et al., 1992; Bauvois et al., 1996). Finally, TGF-β also strongly inhibits the expression of nitric oxide synthetase and in this way is likely to downregulate the antimicrobial and tumoricidal response (Bogdan and Nathan, 1993). The pro-inflammatory activity of TGF-β on monocytes and the subsequent deactivating activity on macrophages clearly illustrate the highly coordinated role of TGF-β in the complex response to injury and its modulatory role at the site of repair and inflammation. Furthermore, TGF-β also inhibits differentiation of cytotoxic $CD8^+$ T cells, which are instrumental in eliminating infected cells (Barral et al., 1995).

Endogenous TGF-β may also plays a regulatory role in limiting immune function *in vivo* and in some pathologic dysfunctions of the immune system. Some new insights into the role of TGF-β in immune suppression and the mechanism of TGF-β presentation have been revealed through studies of MRL/1pr mice, which develop systemic autoimmune disease resembling human systemic lupus erythematosus, probably resulting in part from defective apoptosis (Steinberg, 1994). These mice have high levels of plasma TGF-β_1 bound to IgG antibodies. The complex potently suppresses $CD8^+$ cytotoxic T-lymphocyte responses in the presence of macrophages (Caver et al., 1996;

Stach and Rowley, 1993). Binding to IgG could restrict or target the activity of TGF-β to antigenic sites where it could play an important role in suppression of autoimmune disease (Stach and Rowley, 1993). Furthermore, the IgG-bound TGF-β complexes could increase the susceptibility to bacterial infection and increase defects in polymorphonuclear leukocyte function (Caver et al., 1996). Finally, TGF-β bound to IgG results in a considerably higher (10-500-fold) activity than free TGF-β, suggesting that this recently discovered form of delivery of TGF-β results in an efficient targeting to specific receptors (Caver et al., 1996).

Perhaps mice in which the TGF-β_1 gene is functionally inactivated by gene targeting provide the best illustration of the endogenous role of TGF-β_1 in normal immune function and infiltration. Shortly after birth, these mice develop a multifocal mixed inflammatory disease with rapid and massive infiltration of lymphocytes and neutrophils in many tissues, and subsequently die (Shull et al., 1992). Thus, TGF-β_1 deficiency results in a severe pathology with dysfunction of the immune and inflammatory systems, which is accompanied by increased production of several cytokine mediators of inflammation, such as interferon-γ, TNF-α and macrophage inflammatory protein-1α (MIP-1α) (Shull et al., 1992). These mice also show enhanced expression of MHC class I and II antigens, circulating antibodies to nuclear antigens, and pathogenic glomerular IgG deposits, thus resembling human autoimmune disease (Letterio and Roberts, 1998). The enhanced expression of MHC class II antigen in TGF-β_1 knockout mice is consistent with the ability of TGF-β to suppress its expression (Czarniecki et al., 1988).

In summary, there is ample evidence that TGF-β is major determinant of the immune and inflammatory response. Among the TGF-β isoforms, TGF-β_1 is probably the major effector molecule in most contexts, and TGF-β_2 and TGF-β_3 may exert their major normal effects outside the immune system.

References

Aasted, B., Leslie, R.G.Q., 1991. Virus-specific B-lymphocytes are probably the primary targets for Aleutian disease virus. Vet. Immunol. Immunopath. 28, 127-141.

Ahn, J.K., Gavin, B.J., Kumar, G., Ward, D.C., 1989. Transcriptional analysis of minute virus of mice P4 promoter mutants. J. Virol. 63, 5425-5439.

Alexandersen, S., 1986. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. Vet. Pathol. 23, 579-588.

Alexandersen, S., 1990. Pathogenesis of disease cause by Aleutian mink disease parvovirus. Acta. Pathol. Microbial. Immunol. Stand. 98, l-32.

Alexandersen, S., Bloom, M. E., 1987. Studies on the sequential development of acute interstitial pneumonia caused by Aleutian disease virus in mink kits. J. Virol. 61, 81-88.

Alexandersen, S., Bloom, M.E., Perryman, S., 1988a. Detailed transcription map of Aleutian mink disease parvovirus. J. Virol. 62, 3684-3694.

Alexandersen, S., Bloom, M.E., Wolfinbarger, J., 1988b. Evidence of restricted viral replication in adult mink infected with Aleutian disease of mink parvovirus. J. Virol. 61, 1495-1507.

Alexandersen, S., Larsen, S., Aasted, B., Uttenthal A., Bloom, M.E., Hansen, M., 1994. Acute interstitial pneumonia in mink kits inoculated with defined isolates of Aleutian mink disease parvovirus. Vet. Pathol. 31, 216-228.

Amery, W.K., Hörig, C., 1984. Levamisole. In: Fenichel, R.L., Chirigos, M.A. (Eds.), Immune modulation agents and their mechanisms. Marcel Dekker, Inc., New York, pp. 383-408.

Barral, A., Teixeira, M., Reis, P., Vinhas, V., Costa, J., Lessa, H., Bittencourt, A.L., Reed, S., Carvalho, E.M., Barr-al-Netto, M., 1995. Transforming growth factor-p in human cutaneous leishmaniasis. Am. J. Pathol. 147, 947-954.

Barton, D.E., Foellmer, B. E., Du, J., Tamm, J., Derynck, R., Franke, U., 1988. Chromosomal locations of TGF- βs 2 and 3 in the mouse and human. Oncogene Res. 3, 3230-3231.

Bauvois, B., Van Weyenbergh, J., Rouillard, D., Wietzerbin, J., 1996. TGF-beta 1-stimulated adhesion of human mononuclear phagocytes to fibronectin and laminin is abolished by IFN-gamma: dependence on alpha 5 beta 1 and beta 2 integrins. Exp. Cell Res. 222, 209-217.

Berns, K.I., Labow, M.A., 1987. Parvovirus gene regulation. J. Gen. Virol. 68, 601-614.

Bloom, M.E., Alexandersen, S., Garon, CF., Mori, S., Wei, W., Perryman, S., Wolfinbarger, J.B., 1990. Nucleotide sequence of the 5′-terminal palindrome of Aleutian mink disease parvovirus and construction of an infectious molecular clone. J. Virol. 64, 3551-3556.

Bloom, M.E., Berry B. D., Wei, W., Perryman S., Wolfinbarger J.B., 1993. Characterization of chimeric full-length molecular clones of Aleutian mink disease parvovirus (ADV): identification of a determinant governing replication of ADV in cell culture. J. Virol. 67, 5976-5988.

Bloom, M, E., Kanno, H., Mori, S., Wolfinbarger, J.B., 1994. Aleutian mink disease: puzzles and paradigms. Infect. Agents Dis. 3, 279-301.

Bloom M. E., Mayer, L.W., Garon, C. F., 1983. Characterization of the Aleutian disease virus genome and its intracellular forms. J. Virol. 45, 977-984.

Bloom, M, E., Race R.E., Asted, B., Wolfinbarger, J.B., 1982. Identification of a nonviron protein of Aleutian disease virus: mink with Aleutian disease have antibody to both virion and nonviron proteins. J. Virol. 43, 608-616.

Bloom, M, E., Race R.E., Asted, B., Wolfinbarger, J.B., 1985. Analysis of Aleutian disease virus infection in *vitro* and *in vivo*: demonstration of Aleutian disease virus DNA in tissues of infected mink. J. Virol. 55, 696-703.

Bogdan, C., Nathan, C., 1993. Modlation of macrophage function by transforming growth β, interleukin-4, and interleukin-10. Ann. N.Y. Acad. Sci. 685, 713-739.

Brandenburger, A., Legendre, D., Avalosse., Rommelaere, J., 1990. NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virol. 171, 576-584.

Brandes, M.E., Mat, U., Ohura, K., Wahl, S.M., 1991. Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta. J. Immunol. 147, 1600-1606.

Brownstein, D.G., Smith, A.L., Johnson, E.A., Pintel, D.J., Naeger, L.K., Tattersall, P., 1992. The pathogenesis of infection with minute virus of mice depends on expression of the small nonstructural protein NS2 and on the genotype of the allotropic determinants VP-1 and VP-2. J. Virol. 66, 3118-3124.

Caver, T.E. O'Sullivan, F.X., Gold, L.I., Gresham, H.D., 1996. Intracellular demonstration of active TGF-β_1 in B cells and plasma cells of autoimmune mice. IgG bound TGF- β_1 suppresses neutrophil function and host defense against *Staphylococcus aureus* infection. J. Clin. Invest. 98, 2496-2506.

Cheema, A., Henson, J.B., Gorham, J.R., 1972. Aleutian disease of mink: prevention of lesions by immunosuppression. Am. J. Pathol. 66, 534-556.

Cheifetz, S., Weatherbee, J.A., Tsang, M.L.S., Andersen, J.K., Mole, J.E., Lucas, R., Massaguè, J., 1987. The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. Cell 48, 409-415.

Christensen, J., Storgaard, T., Bloch, B., Alexandersen, S., Aasted B., 1993. Expression of Aleutian mink disease parvovirus proteins in a baculovirus vector system. J. Virol. 67, 229-238.

Coffman, R.L., Lebman, D.A., Shrader, B., 1989. Transforming growth factor β specifically enhances IgA production by lipopolysac-charide-stimulated murine B lymphocytes. J. Exp. Med. 170, 1039.

Cotmore, S.F., Tattersall, P., 1986. Organization of non-structural genes of the autonomous parvovirus minute virus of mice. J. Virol. 58, 724-732.

Czarniecki, C.W., Chiu, H.H., Wong, G.H.W., McCabe, S.M., Palladino, M., 1988. Transforming growth factor-β_1 modulates the expression of class II histocompatibility antigens on human cells. J. Immunol. 140, 4217-4223.

Daopin, S., Piez, K.A., Ogawa, Y., Davies, D.R., 1992. Crystal structure of transforming growth factor-β_2: an unusual fold for the superfamily. Science 257, 369-372.

Derynck, R., 1992. The physiology of transforming growth factor-α. Adv. Cancer Res. 58, 27-52.

Derynck, R., 1994. TGF-β-receptor-mediated signaling. TIBS 19, 548-553.

Derynck, R., Choy, L., 1998. Transforming growth factor-β and its receptor. In: Thomsen, A. (Ed.), The cytokine handbook. Academic Press, Inc., San Diego, CA, pp. 593-636.

Derynck, R., Jarrett, J.A., Chen, E.Y., Eaton, D.H., Bell, J.R., Assoian, R.K., Roberts, A.B., Sporn, M.B., Goeddel, D.V., 1985. Human transforming growth factor-β cDNA sequence and expression in tumor cell lines. Nature 316, 701-705.

Derynck, R., Lindquist, P.B., Lee, A., 1998. A new type of transforming growth factor-β, TGF-β_3. EMBO J. 7, 3737-3743.

Derynck, R., Rhee, L., Chen, E.Y., Van Tiburg, A., 1987. Intron-exon structure of human transforming growth factor-p precursor gene. Nucleic Acids Res. 15, 3188-3189.

Doerig, C., Hirt, B., Antonietti, J.P., Beard, P., 1990. Nonstructural protein of parvoviruses B19 and minute virus of mice controls transcription. J. Virol. 64, 387-396.

Dubois, C.M., Laprise, M.H., Blanchette, F., Gentry, L.E., Leduc, R., 1995. Processing of transforming growth factor beta 1 precursor by human furin cinvertase. J. Biol. Chem. 270, 10618-10624.

Fan, K., Ruan, Q., Sensenbrenner, L., Chen, B., 1992. Transforming growth factor-β_1 bifunctionally regulates murine macrophage proliferation. Blood 79, 1679-1685.

Fargeas, C., Wu, C.Y., Nakajima, T., Cox, D., Nutman, T., Delespesse, G., 1992. Differential effect of transforming growth factor-β on the synthesis of Thl- and Th2- like lymphocytes by human T lymphocytes. Eur. J. Immunol. 22, 2173-2176.

Florini, J., Roberts, A., Eaton, D., Falen, S., Flanders, K., Sporn, M., 1986. Transforming growth factor-β, a very potent inhibitor of myoblast differentiation, identical to differentiation inhibitor secreted by Buffalo rat cells. J. Biol. Chem. 261, 16509-16513.

Freilich, D., Ferris, S., Wallace, M., Leach, L., Kallen, A., Frincke, J., Ahlem, C., Hacker, M., Nelson, D., Hebert, J., 2000. 16-Alpha-bromoepiandrosterone, a dehydroepiandrosterone (DHEA) analogue, inhibits Plasmodium falciparum and Plasmodium berghei growth. Am. J. Trop. Med. Hyg. 63, 280-283.

Fujii, D., Brissendem, J., Derynck, R., Franke, U., 1986. Transforming growth factor-β maps human chromosome 19 long arm and mouse chromosome 7. Somat. Cell Mol. Genet. 12, 281-288.

Gordon, J., Katrira, A., Holder, M., MacDonald, I., Pound, J., 1994. Central role of CD40 and its ligand in B lymphocyte responses to T-dependent antigens. Cell Mol. Biol. 40, 1-13.

Gray, A.M., Mason, A.J., 1990. Requirement for acticin A and transforming growth factor-β_1 pro-regions in homodimer assembly. Science 247, 1328-1330.

Grotendorst, G.R., Smale, G., Pencev, D., 1989. Production of transforming growth factor-β by human peripheral blood monocytes and neutrophils. J. Cell Physiol. 140, 396-402.

Hahn, E.C., Hahn, P.S., 1983. Autoimmunity in Aleutian disease: contribution of antiviral and anti-DNA antibody to hypergammaglobulinemia. Infect. Immun. 41, 494-500.

Hanson, N.D., Rhode, S.L. III., 1962. Parvovirus NS 1 stimulates P4 expression by interaction with the terminal repeats and through DNA amplification. J. Virol. 65, 4325-4333.

Hartosough, G.R., Gorham, J.R., 1956. Aleutian disease in mink. Natl. Fur News. 28, 10-11.

Henson, J.B., Gorham, J.R., Leader, R.W., Wagner, B.M., 1992. Experimental Hypergammaglobulinemia in mink. J. Exp. Med. 116, 357-364.

Holley, R.W., Bohlen, P., Fava, H., Baldwin, J.H., Kleeman, G., Armour., 1980. Purification of kidney epithelial cell growth inhibitors. Proc. Natl. Acad. Sci. U.S.A. 77, 5989-5992.

Kanno, H., Wolfinbarger, J.B., Bloom, M.E., 1992. Identification of Aleutian mink disease parvovirus transcripts in macrophages of infected adult mink. J. Virol. 66, 5305-5312.

Kehrl, J.H., Roberts, A.B., Wakefield, L.M., Jakowlew, S., Sporn, M.B., Fauci, A.S., 1986a. Transforming growth factor-β is important immunomodulating protein for human B lymphocytes. J. Immunol. 137, 3855-3860.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, Derynck, R., Sporn, M.B., Facui, A.S., 1986b. The production of TGF-β by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. 163, 1037-1050.

Kim, S. J., Park, K., Koeller, D., Kim, K.Y., Wakefield, L.M., Sporn, M.B., Roberts, A.B., 1992. Post-transcriptional regulation of the human transforming growth factor-β_1 gene. J. Biol. Chem. 267, 13702-13707.

Labow, M.A., Hermonat, P.L., Berns, K.I., 1986. Positive and negative autoregulation of the adeno-assocated virus type 2 genome. J. Virol. 60, 251-258.

Larsen, A.E., Porter, D.D., 1975. Pathogenesis of Aleutian disease of mink: identification of non-persistent infections. Infect. Immun. 11, 92-94.

Legrand, C., Rommelaere, J., Caillet-Fauquet, P., 1993. MVM(p) Ns-2 protein expression is required with NS-1 for maximal cytotoxicity in human transformed cells. Virology 195, 149-155.

Letterio, J.J., Roberts, A.B., 1998. Regulation of immune response by TGF-beta 1. Annu. Rev. Immunol. 16, 137-161

Lin, H.Y., Wang, X.F., Ng-Eatron, E., Weinger, R., Lodish, H., 1992. Expression cloning of the TGF-β type II receptor, a functional transmemberane serine/ threonine kinase. Cell 76, 797-805.

Lopez, A.R., Cook, J., Deininger, P.L., Derynck, R., 1992. Dominant negative mutants of transforming growth factor-β_1 inhibit the secretion of different transforming growth factor-β isoforms. Mol. Cell Biol. 12, 1674-1679.

Lopez-Casillas, F., Wrana, J.L., Massague, J., 1993. Betaglycan presents ligand to the TGF-β signaling receptor. Cell 73, 1435-1444.

Loria, R.M., Padgett, D.A., 1998. Control of the immune response by DHEA and its metabolites. Rinsho. Byori. 46, 505-517.

Massague, J., Attisano, L., Wrana, J., 1994. TGF-β family and its composite receptors. Trends Cell Biol. 4, 172-178.

Mayer, L.W., Aasted, B., Garon, CF., Bloom, M.E., 1983. Molecular cloning of the Aleutian disease virus genome: expression of Aleutian disease virus antigens by a recombinant plasmid. J. Virol. 48, 573-579.

Miyazono, IX., Hellman, U., Wernstedt, C., Heldin, C.H., 1988. Latent high molecular weight complex of transforming growth factor-β_1 purification from human plates and structural characterization. J. Biol. Chem. 263, 6407-6415.

Mori, S., Wolfinbarger, J.B., Miyazawa, M., Bloom, M.E., 1991. Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages. J. Virol. 65, 952-956.

Moses, H.L., Branum, E.L., Proper, J.A., Robinson, R.A., 1981. Transforming growth factor production by chemically transformed cells. Cancer Res. 41, 2842-2848.

Mulder, J.W., Frissen, P.H., Krijnen, P., Ender, T.E., de Wolf, F., Goudsmit, J., Masterson, J.G., Lange, J.M., 1992. Dehydroepiandrosterone as predictor for progression to AIDS in asymptomatic human immunodeficiency virus-infected men. J. Infect. Dis. 165, 413-418.

Obel, A.L. 1959. Studieson a disease in mink with systemic proliferation of the plasma cells. Am. J. Vet. Res. 20, 384-393.

Ohta, M., Greenberger, J.S., Anklesaria, P., Bassols, A., Massague, J., 1987. Two forms of transforming growth factor-β distinguished by hematopoietic progenitor cells. Nature 329, 539-541.

Ozawa, K., Ayub, J., Kajigaya, S., Shimada, T., Young, N., 1988. The gene encoding the nonstructural protein of B 19 (human) parvovirus may be lethal in transfected cells. J. Virol. 62, 2884-2889.

Porter, D.D., Larsen, A.E., Poter, H.G., 1969. The pathogenesis of Aleutian disease of mink. I. In vivo viral replication and the host antibody response to viral antigen. J. Exp. Med. 130, 575-589.

Porter, D.D., Larsen, A.E., Poter, H.G., 1980. Aleutian disease of mink. Adv. Immunol. 29, 261-288.

Postlethwaite, A.E., Keski-Oja, J., Moses, H.L., Kang, A.H., 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor-β. J. Exp. Med. 165, 251-256.

Rasmussen, K.R., Martin, E.G., Arrowood, M.J., Healey, M.C., 1991. Effects of dexamethasone and dehydroepiandrosterone in immunosuppressed rats infected with Cryptosporidium parvum. J. Protozool. 38, 157-159.

Rhode, S .L. III., 1985. Trans-activation of parvovirus P_{38} promoter by the 76K noncapsid protein. J. Virol. 55, 886-889.

Rhode, S.L. III., 1989. Both excision and replication of cloned autonomous parvovirus DNA require the NS-l (rep) protein. J. Virol. 63, 4249-4256.

Rich, S., Van Nood, N., Less, H.M., 1996. Role of a 5pl integrin in TGF-β_1 costimulated CD8+ T cell growth and apoptosis. J. Immunol. 157, 2916-2923.

Roberts, A.B., Kim, S.J., Noma, T., Glick, A.B., Lafyatis, R., Lechleider, R., Jakowlew, S.B., Geiser, A., O'Reilly, M.A., Danielpour, D., Sporn, M.B., 1991. Multiple forms of TGF-β_1: distinct promoters and differential expression. Ciba Found. Symp. 157, 7-15.

Roberts, A.B., Sporn, M.B., 1990. Transforming growth factor-βs. In: Spon, M.B., Robert, A.B. (Eds.), Peptide growth factors and their receptors. Springer, Heidelberg, pp. 421-472.

Roberts, A.B., Sporn, M.B. Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H., Fauci, A.S., 1986. Transforming growth factor β: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro.* Proc. Natl. Acad. Sci. U.S.A. 83, 4167-4171.

Rook, A.H., Kehrl, J.H., Wakefield, L.M., Sporn, M.B., Burlington, D.B., Lane, H.C., Fauci, A.S., 1986. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J. Immunol. 136, 3916-3920.

Schmitt, E., Hoehn, P., Huels, C., Goedert, S., Palm, N., Rude, E., Germann, T., 1994. T helper type 1 development of naive CD4+ cells requires the coordinate action of interleukin-12 and interferon-y and is inhibited by transforming growth factor β. Eur. J. Immunol. 24, 793-798.

Seyedin, S., Thomas, T., Thompson, A., Rosen, D., Piez, K., 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc. Natl. Acad. Sci. U.S.A. 82, 2267-2271.

Shull, M.M., Ormsby, I., Kier, A.B., Pawlowski, S., Diebold, R.J., Yin, M., Allen, R., Sidman, C., Proetzel, G., Calvin, D., Annunziata, N., Doetschman, T., 1992. Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease. Nature 359, 693-699.

Snapper, C.M., Waegell, W., Beernink, H., Dash, J.R., 1993. Transforming growth factor-β_1 is required for secretion of IgG of all subclasses by LPS-activated murine B cells *in vitro*. J. Immunol. 151, 4625-4636.

Sporn, M., Roberts, A. Wakefield, L., de Crombrugghe, B., 1987. Some recent advances in the chemistry and biology of transforming growth factor- β. J. Cell Biol. 105, 1039-1045.

Stach, R.M., Rowley, D.A., 1993. A first or dominant immunization. II. Induced immunoglobulin carries transforming growth factor β and suppresses cytolytic T cell responses to unrelated alloantigens. J. Exp. Med. 178, 841-852.

Stavnezer, J., 1996. Transforming growth factor-β. In: Snapper, C.M. (Ed.), Cytokine regulation of humoral immunity: basic and clinical aspects. John Wiley, New York, pp. 289-324.

Steinberg, A.D., 1994. MRL-lpr/lpr disease: theoriesmeet Fas. Semin. Immunol. 6, 55-69.

Steinman, L., 1991. Prospects for immunotherapy directed to the T cell receptor in human autoimmune disease. Ann. N.Y. Acad. Sci. 636, 147-153.

Suda, T., Zlotnik, A., 1992. *In vitro* induction of CD8 expression on thymic pre-T cells. II. characterization of CD3-CD4-CD8 cells generated *in vitro* by culturing $CD25^+$, $CD3^-$, $CD4^-$ and $CD8^-$ thymocytes with T cell growth factor-p and tumor necrosis factor-α. J. Immunol. 149, 71-76.

Takahama, Y., Letterio, J.J., Suzuki, H., Farr, A.G., Singer, A., 1994. Early progression of thymocytes along the CD4/ CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor β. J. Exp. Med. 179, 1495-1506.

Tsang, P.H., Tangnavarad, K., Solomon, S., Bekesi, J.G., 1984. Modulation of T- and B-lymphocyte functions by isoprinosine in homosexual subjects with prodromata and in patients with acquired immune deficiency syndrome (AIDS). J. Clin. Immunol. 4, 469-478.

Uozumi, K., Uematsu, T., Otsuka, M., Nakano, S., Takatsuka, Y., Iwahashi, M., Hanada, S., Arima, T., 1996. Serum dehydroepiandrosterone and DHEA-sulfate in patients with adult T-cell leukemia and human T-lymphotropic virus type I carriers. Am. J. Hematol. 53, 165-168.

Wahl, S.M., Hunt, D. A., Wong, H. L., Dougherty, S., McCartney-Francis, N., Wahl, L.M., Ellingsworth, L., Schmidt, J.A., Hall, G., Roberts, A.B., 1988.

Transforming growth factor-β is a potent immunosuppressive agent that inhibits IL-1-dependent lymphocyte proliferation. J. Immunol. 140, 3026-3032.

Wahl, S.M., McCartney-Francis, N.M., Mergenhagen, S.E., 1989. Inflammatory and immunomodulatory roles of TGF-β. Immunol. Today 10, 258-261.

Wahl, S.M., Allen, J.B., Weeks, B.S., Wong, H.L., Klotman, P.E., 1993. Transforming growth factor β enhances integrin expression and type IV collagenase secretion in human monocytes. Proc. Natl. Acad. Sci. U.S.A. 90, 4577-4581.

Wakefield, L.M., Smith, D.M., Flanders, K.C., Sporn, M.B., 1988. Latent Transforming growth factor-β from human platelets. A high molecular weight complex containing precursor sequences. J. Biol. Chem. 263, 7646-7654.

Warner, G.N., Ludlow, J.W., Nelson, D.A., Gaur, A., Scott, D.W., 1992. Anti-immunoglobulin treatment of murine B cell lymphomas induces active transforming growth factor β independent. Cell Growth Differ. 3, 175-181.

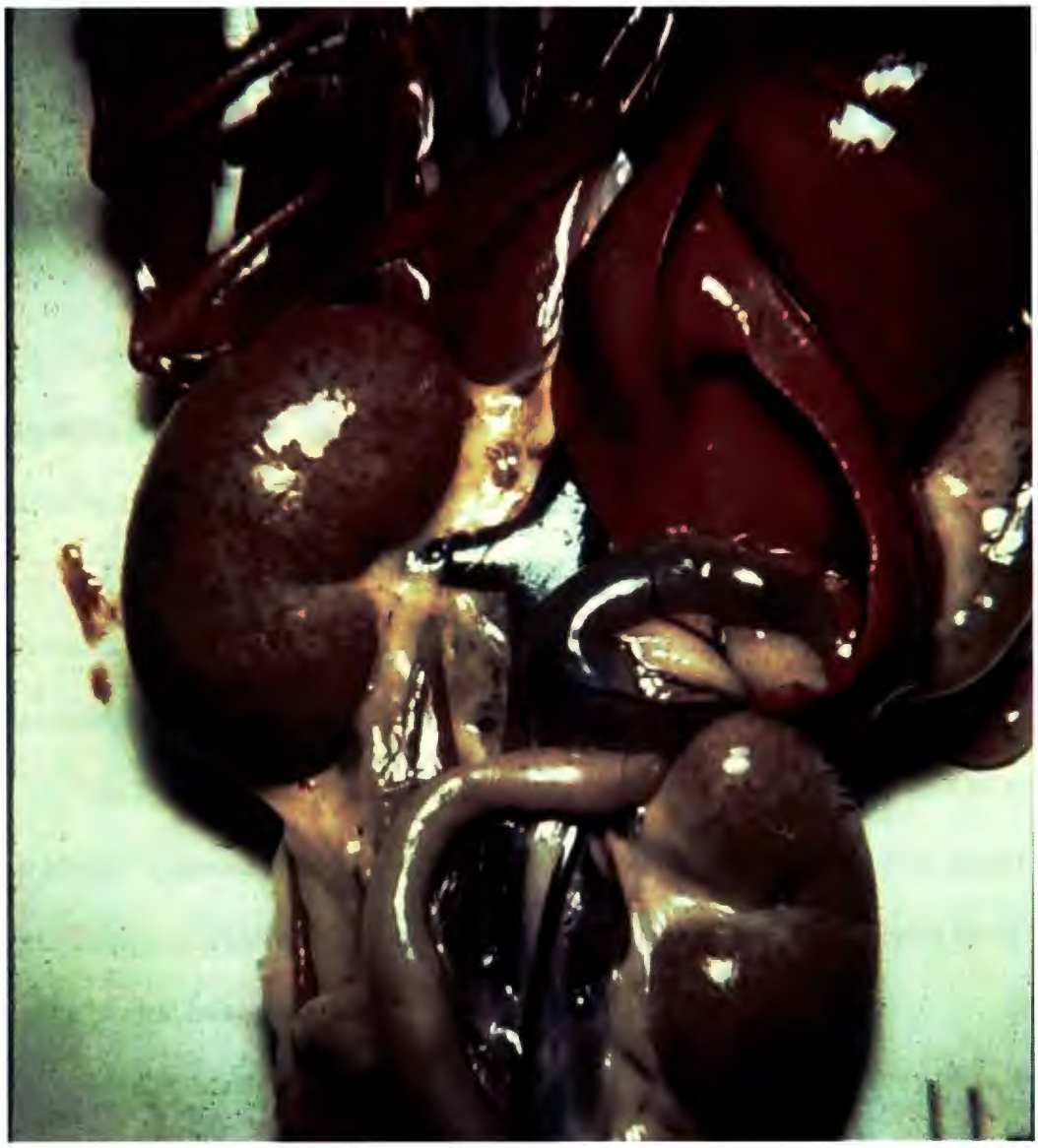


Fig. 1.1. An illustration of the postmortem lesions of AD on mink. The spleen, liver, and lymph are enlarged, and kidney have also enlarged, pale, yellow, and mottled.

CHAPTER 2

AMELIORATING ALEUTIAN DISEASE IN MINK BY TREATMENT WITH HUMAN rTGF-β_1

Abstract

Aleutian disease of mink (AD) is an immunopathological disorder of mink induced by persistent infection with AD virus. It is a naturally occurring viral infection caused by a parvovirus.

AD is serious problem in mink throughout the world. A major outbreak occurred in Summit and Morgan counties in Utah in 1994. This outbreak is still continuing and has resulted in some mink farms deciding to stop raising mink.

The hypothesis to be tested in this research was that TGF-β_1 treatment of mink will inhibit or ameliorate AD in mink. The study was conducted using four groups of mink. Each group included twenty-five mink and were treated at a different times relative to viral infection.

At nine months, all the treated animals had low anti-ADV antibody titers, blood-urea-nitrogen (BUN), interferon-gamma (IFN-γ) and circulating-immune-complex (CIC) levels which were well within normal parameters. However, the non-treated, infected animals all died within four months after infection. These animals had high antibody titers and high BUN, IFN-γ and CIC levels, suggesting that their kidneys were damaged by immune complex deposition, a hallmark of AD. Comparison of survival times between treated and non-treated animals demonstrated that treated animals survived

almost twice as long as non-treated animals. Also, polymerase chain reaction (PCR) assay of lymph node tissue confirmed that non-treated animals had significant levels of virus whereas treated animals had none.

These findings support the hypothesis that TGF-β_1 supplementation does ameliorate the severity of AD in mink.

Introduction

Aleutian disease (AD) is an immunopathological disorder of mink induced by persistent infection with Aleutian disease virus of mink (ADV) (Porter et al., 1980), a naturally occurring infection caused by a virus from the *Parvoviridae* family. The term "Aleutian disease" was coined because the recessive Aleutian genotype (gray coat color) of mink has a predilection for AD. Not only is this breed of mink characterized by extreme susceptibility to lethal, chronic AD, it also has a genetic defect called Chediak-Higashi syndrome in which the lysosomes of the mink cells are very large and have some functional abnormalities, resulting in decreased lysosomal activity and/ or fusion of lysosomes with phagosomes in leukocytes, impairing phagocytosis (Padgett et al., 1967; Ward et al., 2000).

The course and type of disease in ADV-infected mink is dependent on the age of the mink. In newborn kits, an acute AD presents fatal interstitial pneumonitis (Alexandersen, 1986). In adult mink, AD often progresses to a chronic, lethal infection characterized by proliferative glomerulonephritis and severe interstitial nephritis due to deposition of immune complexes consisting of antibody bound to ADV or to auto antigens (Porter et al., 1980). The adult mink show hair loss, poor quality fur, severe

emaciation (Fig. 2.1) and a diminished capacity to reproduce when infected with AD. The chronic infection also leads to immune complex disease. The mortality and morbidity of AD can reach up to 100%. These clinical signs are culminated by an early death in both adults and kits. Once the virus is present, complete herd infection is often imminent (Bloom, 1984).

Attempts to immunize with attenuated or killed virus have failed; they only exacerbated the severity of the disease (Bloom et al., 1994). However, immunotherapy using immunosuppressive drugs such as levamisole and Isoprinosine, resulted in some slight amelioration of disease pathogenesis (Cheema et al., 1972).

When ADV is first presented in to the mink immune system, both T helper 1 (Th1) and T helper 2 (Th2) type of immune responses are activated. As the infection becomes chronic, a shift from the Th1 to a dominant Th2 response is seen. The Th2 predominance leads to the suppression of Th1 type cytokines such as interleukin 12 (IL-12) and interferon-γ (IFN-γ) (Bloom et al., 1994). This is critical due to the role these two cytokines play in the differentiation of T-cells and Th1 responses. Thus, it would be interesting to determine if the immune response of mink exposed to ADV could be modulated to a cell-mediated response characterized by a Th1 cytokine pattern.

A cytokine of special interest for this research is transforming growth factor beta (TGF-β), which can modulate Th1 and Th2 immune responses. Two families of "transforming growth factors" have been identified as TGF-α and β. These are distinct peptides with very different spectra of biological activity. TGF-α is closely related to epidermal growth factor (EGF), whereas the family of TGF-β proteins not only plays an important role in cell growth control and neoplasm development but also in inflammation

and immune regulation. TGF-β proteins are multifunctional dimeric cytokines originally isolated from tumor cells, with molecular weights of 25kD and exerting pleiotropic effects on immune function.

Among the important actions of TGF-β proteins are the recruitment and activation of mononuclear cells, promotion of wound healing, fibrosis, angiogenesis, potent immunosuppressive action on numerous functions of T lymphocytes (Roberts and Sporn, 1990) and modulation of cytokine production (Chantry et al., 1989). Inactivation of TGF-β inhibits early hematopoietic growth and differentiation, alters B cell immunoglobulin (Ig) production and class switching (Coffman et al., 1989). Inactivation of TGF-β inhibits mast cell, thymocyte, B cell and T lymphocyte proliferation.

In vitro studies have provided evidence that, in addition to inhibiting T lymphocyte proliferation, TGF-β_1 inhibits the generation of cytotoxic T lymphocyte (CTL) activity, induction of IL-2-induced lymphokine-activated killer cell activity, generation of natural killer (NK) cell activity from murine NK cell bone marrow precursors (Migliorati et al., 1989), and IFN-γ augmentation of NK cell activity mediated by human peripheral blood leukocytes. It is particularly plentiful in platelets, but it is also present in substantial quantities in mesenchymal cells, where it plays an important role in the remodeling of connective tissue that occurs during inflammation, bone re-absorption, and wound healing.

TGF-β_1 produced by macrophages, T and B cells and other cells generally inhibits development and proliferation of cells in culture (Derynck and Choy, 1998). IL-2 or IL-5

(Th2-type cytokines) may modify this inhibition while at the same time enhancing the production of IgA.

TGF-β_1 may play an important regulatory role in differentiation of T cells. TGF-β_1 has been found to induce the expression of $CD8^+$ on thymocytes suggesting that it has a differentiative function in addition to its anti-proliferative activity. For example, TGF-β_1 can induce expression of $CD8^+$ protein and can act synergistically with TNF-α to favor development of cells expressing $CD8^+$. Conversely, TGF-β_1 inhibits later stages of differentiation and thus inhibits the generation of $CD4^+$ and $CD8^+$ cells (Suda and Ziotnik, 1992). Whereas TGF-β_1 was initially shown to strongly promote the generation of Th1 cells, a subset of helper T cells with a characteristic cytokine secretion pattern (Swain et al., 1991), it also has more recently been more implicated as a positive regulator of differentiation of Th2 cells and a suppressor of differentiation of Th1 cells (Schmitt et al., 1994). Consequently, the microenvironment in which TGF-β_1 interacts with varying concentrations of a mixture of other cytokines may determine the ultimate behavior of this multifunctional growth factor.

Since TGF-β_1 seems to modulate both Th1 and Th2 responses, we wanted to investigate the role of this cytokine in ameliorating AD. The hypothesis to be tested in this research was that TGF-β_1 treatment would inhibit or ameliorate AD in mink. The hypothesis was tested by evaluating the effects of TGF-β_1 on the immune response of AD-infected animals, on the pathological features of disease, on certain physiological factors detectable in serum and by evaluating its effects on viral load.

Materials and Methods

Virus

The Utah I strain of ADV was obtained from Marshall Bloom (NIH, NIAID, Rocky Mountain Laboratories, Hamilton, MT). All animals exposed to virus were injected with virus intraperitoneally and received 5 X 10^5 fluorescent focusing units of ADV of mink. This inoculum was used to induce detectable disease within 2 to 5 months after virus exposure.

Cells

The macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were routinely passaged with in Dulbecco's modification of minimal essential medium (GIBCO Invitrogen Corporation, Grand Island, NY) containing 4000 mg/L glucose. The medium was supplemented with 4 mM L-glutamine (GIBCO Invitrogen Corporation), 0.1 mM sodium pyruvate (GIBCO Invitrogen Corporation), 0.15% sodium bicarbonate and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT).

Animals

Unmated or non-parous one-year-old female mink weighing 600-1000 g were used. These mink were obtained from the Windy Peaks ranch facility (Franklin, ID). This facility has been free of AD for about 6 years as determined by serological testing and pathology results. Serum from each animal was tested by counter-immune

electrophoresis (CEP) for the presence of antibody to AD. All animals received at the animal research facility were negative for AD by CEP testing.

Mink were fed once daily ad libitum diet (approximately 100 g) with a fish-based diet formulated for optimal growth, pelt quality and seasonal variations in nutritional needs by the Mink Farmer's Cooperative of Utah (Fig. A.1).

Blood for analysis was obtained by toe clip, and when mink were sacrificed, they were euthanized by carbon monoxide inhalation in a sealed plexiglass chamber in an aerated room (Fig. A.2).

Animals were held to up to 9 months. Signs of disease were measured monthly by analyzing serum samples using antibody, blood urea nitrogen, interferon gamma, and circulating immune complex assays. Confirmation of disease was measured by histopathological examination of kidney, liver, and spleen, and by detection of viral DNA in the mesenteric lymph nodes. In addition, animals were weighed monthly to determine weight loss or gain, weight loss being a symptom of AD.

Study Design

The study was designed to evaluate three different doses (200, 100 and 1 ng/kg) of recombinant human TGF-β_1 (rhTGF-β_1) and one dose (500 ng/kg) of TGF-β_1 inhibitor (latency associated peptide [rhLAPTGF-β_1] R&D Systems, Minneapolis, MN) at one of three different times of administration relative to virus exposure (Table A.1). Group 1 received TGF-β_1 two weeks prior to virus exposure, group 2 received treatment eight h post-virus exposure and group 3 received TGF-β_1 2 weeks after virus exposure. Twenty-five animals were assigned to each administration group, and within that group, five

animals were designated to receive one of three doses of rhTGF-β_1, rhTGF-β_1 inhibitor, or 0.1% BSA in PSS (placebo). A separate group (group 4) of five animals received TGF-β_1 at 200 ng/kg 2 weeks prior to virus exposure, again 8 h after virus exposure and then 2 weeks after virus exposure (Fig. A.3).

Animals were administered drug or placebo intravenously via the inguinal vein and virus was injected intraperitoneally using a 1:500 dilution of clarified spleen homogenate (approximately 1 X 10^5 fluorescent focus units). This inoculum was used to induce detectable disease or death within 2 to 5 months after virus exposure (Fig. A.4).

Counter-immune Electrophoresis (CEP)

The CEP tests (Aasted and Cohn, 1982) were performed according to the manufacturer's (United Vaccines, Inc. Madison, WI) instructions. Eight "units" of antigen per 10 µl were added to the appropriate wells and 10 µl of serum dilutions as well a positive control serum were added to wells in apposition to the antigen containing wells. Electrophoresis (55 volts for 60 min) was done using Tri-Barbital buffer (pH 8.6, Pall Gelman Sciences, Ann Arbor, MI) as the wick buffer and 0.9% agarose (FMC corporation) as the matrix for the precipitation reaction (Fig. A.5).

To visualize precipitin lines, the agarose-coated plate was illuminated bilaterally from below and viewed against a dark background. When results were obscured by hemolysis, the precipitin lines became more clearly visible by soaking the plates 15 min in 2% saline solution. Antibody titers were expressed as the last dilution of serum at which a precipitin line formed.

Blood Urea Nitrogen (BUN)

Sigma Diagnostics Urea Nitrogen kit (Sigma, St. Louis, MO) was used for the quantitative, colorimetric determination of BUN in serum at 540 nm (Crocker, 1967).

To prepare serum samples for analysis, the samples were treated with 3% cold trichloroacetic acid (Sigma) for 5 min at refrigerator temperature. The supernatant fluid used for the analysis was collected by centrifugation at 1500 X g for 10 min. Positive controls from the kit were used to generate a standard curve for calculating concentrations of nitrogen (mg/dl) detected in each sample.

Interferon Gamma (IFN-γ) Assay

Interferon-γ-stimulated macrophages produce large amounts of nitric oxide (NO) by cytosolic, NADPH-dependent inducible NO synthase (iNOS) (Kim and Son, 1996). Once cells have been stimulated by interferon-γ, NO concentration can be determined by the amount of nitrite (NO_2) accumulation in cell culture medium. Therefore, a nitric oxide production bioassay using the macrophage cell line RAW 264.7 was done to detect interferon-γ mink sera (Kim and Son, 1996).

RAW 264.7 cells were seeded in 96-well plates flat-bottomed (Corning Costar, NY) at a concentration of 1×10^5 cells/well. The were incubated 4-6 h and stimulated with mink serum samples or with 25 U/ml mouse interferon-γ. All samples were diluted using a two-fold geometric dilution series. A row of wells was set aside for mink sera from normal, untreated mink to serve as a negative control. After 24 h of stimulation at 37°C, the medium was collected from each well for measurement of nitrite accumulation.

Nitrite production was determined by adding 100 µl of Griess Reagent (1 part 0.1% naphthylethyleniamine dihydrochloride to 1 part 1% sulfanilamide in 2% H_3P0_4) to 100 µl of supernatant fluid from stimulated RAW cells in a 96 well plate and mixing well. The absorbance was measured at 540 nm using a microplate reader. Mouse interferon-γ concentration were plotted versus absorbance values to generate a standard curve from which NO concentration for each sample were extrapolated.

Circulating Immune Complexes (CIC) Assay

The BÜHLMANN CIC-C1Q ELA Kit (ALPCO, Windham, NH) was used to quantitative determine the amount of circulating immune complexes (CIC) detectable in mink serum. CIC usually activate complement when formed in the host and normally are cleared by phagocytosis. The component of the complement system that binds these complexes is Clq. For this assay Clq was used to capture any immune complexes found in serum samples. CIC binding to Clq was detected using an alkaline phosphatase conjugated Protein A, which binds to the Fc region of IgG, the predominate antibody associated with immune complexes (Wehler et al., 1981; Endo et al., 1983).

Dilutions of mink sera (beginning at 1:50) kit standards and controls were incubated with human Clq pre-adsorbed onto microtiter wells for 1 h at room temperature. The kit protocol for detection of CIC was then followed. Absorbance at 405 nm was read. CIC concentrations detected in various mink sera were calculated from standard curves generated using the positive controls provided in the kit.

TGF- β_1 ELISA Development Assay

Detection of TGF-β_1 in serum samples was done using a DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN) and following the instructions of the kit.

Briefly, capture antibody was absorbed onto wells of a 96-well microplate and incubated overnight at room temperature. After washing, blocking buffer was added to each well and the plate incubated at room temperature for 1 h. Following three buffer rinses, diluted serum samples, standards in reagent diluent, and serum diluent, were added to appropriate wells in duplicate, and incubated 2 h at room temperature. Following the incubation and rinsing, detection antibody was added to each well and incubated two hours at room temperature. Following three rinses, streptavidin-horseradish peroxidase conjugate (HRP) was added to each well, the plate sealed and incubated for 20 min at room temperature. After appropriate rinsing, substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was put into each well and incubated for 20 min at room temperature. After stopping the reactions with stop solution (2N H_2SO_4), absorbance was read using a microplate reader set to 450 nm and 540 nm.

Mean absorbance for the standards plotted versus concentration of the standards to generate a standard curve and mean concentrations of TGF-β_1 in each sample were derived from the standard curves.

Detection of Viral DNA

Viral DNA was detected by polymerase chain reaction (PCR) amplification of viral DNA extracted from mesenteric lymph nodes of mink with subsquent visualization of the DNA gel electrophoresis.

Approximately 0.25 g of mesenteric lymph node tissue was digested with proteinase K (Sigma). The DNA was then suspended in 500 µl of digestion buffer (0.01 M Tris HC1, 0.001 M EDTA, pH 7.2 TE buffer), phenol-chloroform extracted, and ethanol precipitated using standard procedures. The sample DNA was stored at -80°C until amplified by PCR.

Ten microliters of hydrated DNA (0.5-1.0 µg) was used for each PCR reaction. The reaction mix consisted of the following: 0.25 µM concentration of each primer, 0.2 mM deoxynucleotide triphosphates, 50 mM KC1, l0 mM Tri HC1 (pH 8.8), 15 mM $MgCl_2$, 0.2% Triton X-100, 2.5 units of *Taq* DNA polymerase (total volume of 100 µl). Primers were selected from the 5' end of the viral genome in a highly conserved non-translated region (Bloom et al., 1988). Primer sequences were as follows, with nucleotide positions given in parentheses: upstream primer–GATGAGCAGAGGAGAC TGCA (224-243): downstream primer–AGTAACCTAAGCAACAGTGA (583- 602). The expected fragment size was 379 base pairs, representing nucleotides 224-602. For each set of reactions, positive and negative controls were used (Jackson et al., 1996). DNA for the positive controls consisted of 10 ng of the gel-purified amplified fragment. Negative controls consisted of approximately 1 µg of DNA isolated from blood cells of uninfected mink. Each cycle consisted of 1 min each at 95°C, 55°C, and 72°C. A total of thirty cycles was done, using a thermal cycler (Singblock TED, Ericomp, Inc., San

Diego, CA). After amplification, samples were separated on a 1.0% agarose gel in TAE buffer. Bands were visualized with ultraviolet light and photographed using Polaroid film.

Histopathology Examinations

Upon the death of each mink, either from AD or when sacrified the kidneys and liverwere removed and tissues from each were sectioned, fixed and then stained with hematoxylin and eosin. Typical pathological findings for AD included thickened mesangium in the glomeruli with homogenous eosinophilic material (membranous glomerulopathy), detection of multifocal infiltrates of plasma cells and lymphocytes in the interstitium of the kidney cortex (interstitial nephritis) and also in the medulla, multiple convoluted tubules containing eosinophilic material or granular casts, occasional mineralization basement membranes, and portal areas of the liver with varying numbers of infiltrated plasma cells and lymphocytes (hepatitis) randomly dispersed among the hepatocellular cords. Samples were coded and sent to a board certified pathologist (Utah State Veterinary Diagnostic Laboratory, Logan, UT), who examined the samples for the above-defined pathology; abnormalities were scored as mild (+), moderate (++), and severe (+++). Samples with the pathology described above were considered positive for AD.

Statistical Analysis

Statistical significances were determined by analysis of variance and Student's t-test where appropriate. Standard deviations (SD) were determined for all mean values.

Results

Survival Time

Infected, placebo treated animals all died of AD before the termination of the experiment (by 5 months into the experiment), with mean survival time of 149 ± 8 days. Mink treated with 1 ng/kg of TGF-β_1 had survival times comparable to the infected, placebo control group as did mink treated with TGF-β_1 inhibitor, regardless of the treatment regimen. Animals treated with TGF-β_1 at 100 and 200 ng/kg had significantly ($p \leq 0.05$) prolonged mean survival times, again regardless of the treatment regimen. The average survival time for the four treatments groups at these dosages ranged from 200 ± 33 days to 248 ± 17 days (Fig. 2.2). Survival increased of 10% to 60% ($p \leq 0.05$) were seen in the TGF-β_1-treated groups.

Body Weight

Mink were weighed prior to any treatment and that weight was designated "pre-exposure weight." The post-virus exposure weights represent the average weight of animals in each treatment group just prior to death or sacrifice. Mink which were untreated and not exposed to virus had significant weight increases relative to the infected, untreated (placebo) groups (Fig. 2.3, $p < 0.01$) and suffered from severe emaciation (Fig. 2.1).

All TGF-β_1-treated animals lost some weight compared to pre-exposure body weights (Fig. 2.3). However, those mink treated with TGF-β_1 inhibitor placebo group suffered tremendous weight losses, consistent with typical AD pathology (Fig. 2.1).

Specific ADV Antibody Levels

AD may be manifested at certain stages by exhibiting elevated serum gammaglobulin levels. High titers of antibody (>512) are often associated with progressive chronic AD (Bloom et al., 1994). Therefore, serum was analyzed for ADV-specific antibody as a marker for progression to chronic disease.

Animals treated with TGF-β_1 at 100 and 200 ng/kg had significantly lower anti-ADV antibody titers than did the placebo-treated mink or animals treated with TGF-β_1 inhibitor ($p \leq 0.05$). The lowest average antibody titer occurred in the TGF-β_1 group 2 in which treatment was given 8 h post virus inoculation (Fig. 2.4). The antibody titers for the placebo groups and the TGF-β_1 inhibitor-treated groups exceeded the threshold (>512) commonly accepted as the point at which mink are likely progress to chronic AD often culminating in death (Bloom et al., 1994).

Comparing the mean average CEP titers between the groups of TGF-β_1-treated mink showed that those treated with TGF-β_1 at l00 ng/kg in group 2 and those animals treated with TGF-β_1 at 200 ng/kg in group 4 had significantly decreased mean CEP titers 218 ($p < 0.001$) and 256 ($p < 0.01$), respectively, compared to controls group.

Serum Levels of Urea Nitrogen

Detectable levels of urea or other nitrogenous compounds in serum are sometimes indicative of damage to the kidneys, probably at the level of readsorption through the tubules. The BUN test used in this study provides results which often correlate with chronic renal failure due to distal tubule transport malfunction.

Blood levels of nitrogenous compounds for all groups of mink were higher after

virus exposure when compared to the pre-virus exposure, pretreatment levels (Fig. 2.5). However, animals receiving treatment with TGF-β_1 at 100 or 200 ng/kg had, in general, significantly lower BUN levels after virus exposure compared to those levels in mink not treated with TGF-β_1 or treated with TGF-β_1-inhibitor ($p < 0.05$, Fig. 2.5).

Serum Gamma Interferon (IFN-γ) Activity

Levels of interferon-γ, a Th1 type of cytokine, were assessed as a reflection of the effects of TGF-β_1 treatment on Th1 type responses using the method whereby mouse macrophage cells are stimulated in vitro with a serum sample putatively containing interferon-γ and looking for a proportionate production of nitric oxide (Kim and Son, 1996).

Serum interferon-γ levels were higher in all groups after virus exposure and/or treatment than in sera taken from mink prior to those events (Fig. 2.6). Levels of interferon-γ were 1.2- to 3-fold lower in animals treated with TGF-β_1 at 100 or 200 ng/kg than the levels in the placebo groups or in the TGF-β_1 inhibitor mink. The levels in the mink treated with TGF-β_1 at 1 ng/kg seemed to be midway between the levels detected in the other treatment groups described above.

Circulating Immune Complex (CIC)Ttiters

Circulating immune complexes (CIC) are formed by the interaction of antibodies with immunogenic antigens. Immune complexes with only a slight excess of antibody or antigen are soluble and activate complement and high levels of CIC are sometimes symptomatic of various autoimmune and other CIC-related diseases.

Since one of the hallmarks of AD pathology is the destruction of kidney tissue due to deposition of CIC in the kidney and subsequent antibody-mediated destruction of the basement membrane of kidney tubules, assays were done to determine CIC levels in mink using a commercially available kit.

CIC levels were significantly lower in mink treated with TGF-β_1 at 100 and 200 ng/kg than those animals receiving TGF-β_1 inhibitor or placebo-treated mink (Table 2.1, $p \leq 0.05$). Animals treated with TGF-β_1 at 1 ng/kg seemed to have CIC levels midway between the groups discussed above. However, the CIC levels for the placebo-treated mink and mink treated with TGF-β_1 at 1 ng/kg, and the TGF-β_1 inhibitor were dramatically increased in comparison to levels detected in those same mink prior to virus exposure and/or treatment, suggesting chronic exposure to some agent(s) like ADV.

Serum Levels of TGF-β_1

The effect of treatment with exogenous TGF-β_1 on serum levels of TGF-β_1 in treated mink, is shown in Fig. 2.7. In general, TGF-β_1 levels seemed to slightly increase in animals treated with TGF-β_1 at 100 or 200 ng/kg compared to baseline levels. For mink treated with TGF-β_1, the levels seemed to remain the same throughout the duration of the experiment. In the placebo groups and the groups of mink treated with TGF-β_1 inhibitor the levels of TGF-β_1 appeared to drop compared to the levels found inthose groups prior to treatment and/ or exposure to virus (Fig. 2.7).

Virus DNA Levels

To determine if virus was present in treated, infected animals, mesenteric lymph nodes of mink (Bloom et al., 1994) were harvested and assayed for viral DNA by PCR amplification and gel analysis from samples taken as soon after death as possible or immediately after sacrifice.

Viral DNA was not detected in animals treated with TGF-β_1 at 100 or 200 ng/kg with the exception of one animal treated with 200 ng/kg in group 3 (Table 2.2). For the other groups of mink, viral DNA was present in the majority of animals. In mink treated with 1 ng/kg TGF-β_1, viral DNA could be detected in 60% of the animals. For the other groups, viral DNA was detected in four or five mink in each treatment regimen (Fig. B.1).

Histopathological Examinations

Typical pathological findings for AD included thickened mesangium in the glomeruli with homogenous eosinophilic material (membranous glomerulopathy), detection of multifocal infiltrates of plasma cells and lymphocytes in the interstitium of kidney cortex (interstitial nephritis) and also in the medulla, multiple convoluted tubules containing eosinophilic material or granular casts, occasional mineralization basement membranes, portal areas of the liver with varying numbers of infiltrated plasma cells and lymphocytes (hepatitis) randomly dispersed among the hepatocellular cords.

With few exceptions, mink treated with TGF-β_1 at 100 or 200 ng/kg tended not to have visible lesions in the kidneys and liver attributable to AD (Table 2.3). Most mink treated at the lowest dose of TGF-β_1 did exhibit mild to severe lesions characteristic of

AD, with one animal from each treatment regimen not showing any lesions typical of AD.

All animals in the placebo groups or in the TGF-β_1-treated groups were positive for AD lesions in the kidneys and the liver; most mink being scored as having moderate to severe pathology.

Discussion

Aleutian disease virus (ADV) causes a number of interesting disease syndromes in mink. These syndromes include decreased fertility, abortion, acute pneumonia in kits, and chronic and persistent infections, all severely impacting the profitability of mink farming in Utah and throughout the world. Interestingly, the main feature of AD in susceptible adult mink is chronic, but fatal immune complex-mediated glomerulonephritis comparable to that seen in human disease such as systemic lupus erythematosus (Bloom, 1984). Because of its comparative aspects to human diseases and the substantial economic loss to the fur industries in the world, classical Aleutian disease and the nature of the virus has been studied extensively for 3 decades (Alexandersen, 1990).

Recently, several studies have been done to understand how the virus disturbs host immune function (Aasted and Leslie, 1991). From these studies, it has become apparent that the initial target for ADV replication is probably the macrophage found in the medullary sinuses of the lymph nodes of infected mink (Kanno et al., 1992). Secondary targets are B lymphocytes, circulating macrophages and follicular dendritic cells. The phenotype of Aleutian disease is characterized by extreme

hypergammaglobulinemia caused by the production of non-neutralizing anti-ADV antibodies (Alexandersen, 1990) and polyclonal antibody production, including anti-DNA antibodies (Hahn and Hahn, 1983). Subsequently, plasmacytosis (Porter et al., 1980), arthritis (Porter et al., 1973) as well as immune complex-mediated glomerulonephritis develops. These features suggest that some step in B cell development and/ or maturation is over-stimulated by ADV infection (Kanno et al., 1992).

Several theories have been advanced to account for the hypergammaglobulinemia described above (Bloom et al., 1994). Among them are i) cytokines are produced in response to ADV infection of target cell macrophages that enhance B cell proliferation and maturation; ii) viral components (e.g., structural proteins) might directly stimulate B cell development and clonal expansion; iii) abnormal antigen presentation by infected macrophages might disturb subsequent B cell development and proliferation.

In support of the first hypothesis are the findings of Isaka et al. (1993). The cytokine of special interest for this research is TGF-β, which consists of a group of multifunctional dimeric cytokines. Originally isolated from tumor cells, with molecular weights of 25 kD, TGF-βs exhibit pleiotropic effects. Among the important actions of TGF-β proteins are the recruitment and activation of mononuclear cells, promotion of wound healing, fibrosis and angiogenesis, and a potent immunosuppressive action on numerous functions of T lymphocytes. There is now considerable evidence to support an important role for TGF-β in both the regulation of extracellular matrix accumulation and the pathogenesis of glomerulosclerosis. Recently, Isaka et al. (1993) used an *in vivo* renal transfection technique (injection of liposome-bound TGF-β cDNA into the rat renal

artery), which resulted in over-expression of TGF-β_1 in glomeruli and induced marked extracellular matrix accumulation. These and other findings support a direct role for this cytokine in the pathogenesis of glomerulosclerosis. Since TGF-β_1 is a disparate modulator of cell recruitment, proliferation and extracellular matrix phenotype for mesenchymal and non-mesenchymal cells, we wanted to investigate the role of this cytokine in the potential amelioration of the immunopathology in the kidneys and liver of animals with AD.

From the data of the current experiments, it is apparent that treated animals (TGF-β_1 at 100 and 200 ng/kg) had low antibody titers and BUN, IFN-γ and CIC levels, most lower than those levels detected in placebo-treated mink (see summary Table 2.4). The infected placebo- and TGF-β_1 inhibitor-treated animals all died by four months. These animals had high antibody titers and high BUN, IFN-γ and CIC values and pathology attributable to AD, suggesting that the kidneys were damaged by immune complex deposition, a hallmark of AD. In a comparison of survival times between infected TGF-β_1-treated and placebo- and TGF-β_1 inhibitor-treated animals, TGF-β_1-treated animals receiving 100 or 200 ng/kg TGF-β_1 survived almost twice as long as untreated animals or inhibitor-treated mink. Also, PCR assay of lymph node tissue confirmed that infected placebo- and TGF-β_1 inhibitor-treated animals had significant levels of virus whereas animals treated with at 100 or 200 ng/kg TGF-β_1 had none. These findings support the hypothesis that TGF-β_1 treatment does ameliorate the severity of AD in mink.

The effect of TGF-β_1 treatment on just the animals surviving the 9-month experiment duration was assessed using the previously parameters described. Mink

treated with TGF-β_1 at 200 ng/kg in all groups (group 1, 2, 3, and 4) lost significantly less weight than the control group mink ($p < 0.01$), although all surviving animals did lose some weight throughout the experiment (Table 2.4). Serum levels of BUN, IFN-γ and CIC were significantly decreased from what was detected in placebo-treated mink ($p < 0.01$, Table 2.4). Eleven animals (11/16) in the 200 ng/kg group and 9/13 in the 100 ng/kg treated-group had no pathology attributable to AD. No virus was detected in almost all of the surviving animals. The antibody titer specific for ADV was also significantly lower than the titer in mink from the control group ($p \leq 0.01$) and typical for animals exposed to disease that are long-term survivors. These results lend credence to the conclusion that the various treatment regimens with TGF-β_1 at 100 or 200 ng/kg provide a protective effect against chronic, lethal AD, even when administered after virus exposure. However, dosing at 1 ng/kg was not very effective.

The data in this study support the idea that a dosing regimen of TGF-β_1 at 100 ng/kg significantly increases the survival time of mink after exposure to ADV. Nevertheless, a small number of mink still succumbed to the chronic effects of ADV exposure. All parameters measured indicated that using the TGF-β_1 dose of 100 ng/kg injected 8 h after virus injection (group 2 dosing regimen) was not effective in delaying time to death, but that the severity of the disease was lessened within the time period studied in that particular treatment group (Table 2.4). All parameters measured in a subset of the TGF-β_1 at 100 ng/kg treatment group treated with which survived were comparable to those measured in the infected control group. However, in the survivor group, mild to moderate lesions were still found despite treatment, and weight gains were less, lending credence to the supposition that treatment may only delay the time to death

and appearance of severe lesions, but not permanently alter the course of a chronic AD infection. Similar results were seen with the TGF-β_1 at 200 ng/kg treatment regimen. Time to death was extended substantially and the measured parameters in surviving mink were similar to the TGF-β_1 at l00 ng/kg group, but again pathology attributable to AD was still detected in the kidneys and liver of a small group of treated animals (Table 2.4). Interestingly, a parameter that seemed predictive of a negative outcome was the presence of high levels of interferon-γ, a Th1 cytokine. Interferon-γ has also been shown to correlate with pathogenesis in another immune complex-mediated disease, systemic lupus erythematosus (Kuroiwa and Lee, 1998). In knock-out mice, interferon-γ apparently was an important cytokine in amplifying the local immune responses of lupus induced nephritis. Infiltrating mononuclear cells exerted their effects on resident renal cells through secretion of soluble factors and apparent promotion of cell-to-cell contact. This scenario may not be the case with AD because in AD large numbers of lymphocytes are found at the lesion and not macrophages/monocytes. Instead, the high interferon-γ levels could activate localized cytotoxic T cells by stimulating expression of MHC class I antigen cells on putative target cells (De Maeyer and De Maeyer-Guignard, 1998), for example, cells of the basement membrane coated with immune complexes. The latter scenario may correlate with our findings that infiltrating lymphocytes are often at the site of lesions associated with AD.

Whereas high levels of interferon appeared to be a predictor of a negative outcome, high levels of TGF-β_1 bode well as a harbinger of survival for AD-infected mink (Table 2.4). The 100 and 200 ng/kg doses that seemed to ameliorate the disease in infected mink were also characterized by high serum levels of TGF-β_1, seemingly

supporting our hypothesis. This is further strengthened by the fact that mink treated with an inhibitor of TGF-β_1 had much lower levels of TGF-β_1 in their sera and had disease equal in severity, if not greater severity, than the untreated, control animals.

From the data shown in this study, it is readily apparent that 100 ng/kg or a 200 ng/kg dosages of TGF-β_1 were most effective in treating ADV-infected mink; the TGF-β_1 at 1 ng/kg regimen failed to protect most mink from lethal chronic AD. The experimental design did not allow the determination of whether the compounds ameliorated the severity of the pathogenesis permanently, whether the lesion damage was reversible or irreversible or if the compounds "cured" mink of the disease. However, the lack of detection of viral DNA and the reasonably low levels of specific ADV antibody and circulating immune complexes all suggest that the virus was cleared, thus eliminating a continual stimulator of what appears to be type III hyper- sensitivity response. This would support the notion that at the very least, TGF-β_1 at 200 and 100 ng/kg dosages can temporarily halt or significantly slow down the progression of the disease.

Avenues of future research may include more practical ways to administer TGF-β_1 to the mink, such as by vaccination in a similar way to how the vaccination for distemper is given. Perhaps it would be possible to combine TGF-β_1 with one of the vaccines that is regularly given to mink and administer both at the same time in one injection thus being less labor-intensive for the farmer, or perhaps evaluated orally as part of the diet to see if it could be effectively administered as a supplement in the feed.

References

Aasted, B., Cohn, A., 1982. Inhibition of precipitation in counter current electrophoresis. a sensitive method for detection of mink antibodies to Aleutian aisease virus. Acta. Path. Macrobiol. Immunol. Stand., Section C 90, 15-19.

Aasted, B., Leslie, R.G.Q., 1991. Virus-specific B-lymphocytes are probably the primary targets for Aleutian disease virus. Vet. Immunol. Immunopathol. 28, 127-141.

Alexandersen, S., 1986. Acute interstitial pneumonia in mink kits: experimental reproduction of the disease. Vet. Pathol. 23, 579-588.

Alexandersen, S., 1990. Pathogensis of disease cause by Aleutian mink disease parvovirus. Acta. Pathol. Microbial. Immunol. Stand. 98, 1-32.

Bloom, M.E., 1984. Parvovirus infections: features reminiscent of AIDS. Ann. N.Y. Acad. Sci. 437, 110-120.

Bloom, M.E., Alexandersen S., Perryman S., Lechner, D., Wolfinbarger J.B., 1988. Nucleotide sequence and genomic organization of Aleutian mink disease parvovirus (ADV): sequence comparisons between a nonpathogenic and pathogenic strain of ADV. J. Virol. 62, 2903-2915.

Bloom, M, E., Kanno, H., Mori, S., Wolfinbarger, J.B., 1994. Aleutian mink disease: puzzles and paradigms. Infectious Agents and Disease 3, 279-301.

Chantry, D., Turner, M., Abney, E., Feldmann, M., 1989. Modulation of cytokine production by transforming growth factor-β. J. Immunol. 142, 4295.

Cheema, A., Henson, J.B., Gorham, J.R., 1972. Aleutian disease of mink: prevention of disease by immunosuppression. Am. J. Pathol. 66, 543-551.

Coffman, R.L., Lebman, D.A., Shrader, B., 1989. Transforming growth factor β specifically enhances IgA production by lipopolysac-charide-stimulated murine B lymphocytes. J. Exp. Med. 170, 1039.

Crocker, C.L., 1967. Rapid determination of urea nitrogen in serum or plasma without deproteinization. Am. J. Med. Technol. 33, 361.

De Maeyer, E., De Mayer-Guignard, J., 1998. Type I interferons. Int. Rev. Immunol. 17, 53-73.

Derynck, R., Choy, L., 1998. Transforming Growth Factor-β and its Receptors. In: Thomson, A. (Ed.), The cytokine handbook, 3rd ed. Academic Press, San Diego, CA, pp. 491-516.

Endo, Y., Yamamoto, I., Ohtaki, S., Yoshitake, S., Ishikawa, E., 1983. An enzyme-linked immunoassay for the measurement of circulating immune complexes. Ann. Clin. Biochem. 20, 163-168.

Hahn, E.C., Hahn, P.S., 1983. Autoimmunity in Aleutian disease: contribution of antiviral and anti-DNA antibody to hypergammaglobulinemia. Infect. Immune. 41, 494-500.

Isaka, Y., Fujiwara, Y., Ueda, N., Kaneda, Y., Kamada, T., Imai, E., 1993. Glomerulosclerosis induced by *in vivo* transforming growth factor-β or platelet-derived growth factor gene into the rat kidney. J. Clin. Invest. 92, 2597-2601.

Jackson, M.K., Ellis, L.C., Morrey, J.D., Li Z.Z., Barnard, D.L., 1996. Progression of Aleutian disease in natural and experimentally induced infections of mink. Nor. J. Agr. Sci. 57, 1753-1758.

Kanno, H., Wolfinbarger, J.B., Bloom, M.E., 1992. Identification of Aleutian mink disease parvovirus transcripts in macrophages of infected adult mink. J. Virol. 66, 5305-5312.

Kim, Y. M., Son, K., 1996. A nitric oxide production bioassay for interferon-γ. J. Immunol. Methods 198, 203-209.

Kuroiwa, T., Lee, E.G., 1998. Cellular interactions in the pathogenesis of lupus nephritis: the role of T cells and macrophages in the amplification of the inflammatory process in the kidney. Lupus 7, 597-603.

Migliorati, G., Cannarile, L., Herberman, R.B., Riccardi, C., 1989. Effect of various cytokines and growth factors on the interleukin-2-dependent in vitro differentiation of natural killer cells from bone marrow. Natl. Cell Growth Regul. 8, 48.

Padgett, G.A., Reiquam, C.W., Gorham, J.R., Heson, J.B., O'Mary C.C., 1967. Comparative studies of the Chediak-Higashi syndrome. Am. J. Pathol. 51, 553-571.

Porter, D.D., Larsen, A.E., Poter H.G., 1973. The pathogenesis of Aleutian disease of mink. III. Immune complex arthritis. Am. J. Pathol. 71, 331-344.

Porter, D.D., Larsen, A.E., Poter H.G., 1980. Aleutian disease of mink. Adv. Immunol. 29, 261-288.

Roberts, A.B., Sporn, M.B., 1990. Transforming Growth Factor-βs. In: Spon, M.B. and Roberts, A.B. (Eds.), Peptide growth factors and their receptors. Springer, Heidelberg, pp. 421-472.

Schmitt, E., Hoehn, P., Huels, C., Goedert, S., Palm, N., Rude, E., Germann, T.T., 1994. Helper type 1 development of naive CD4+ cells requires the coordinate action of interleukin-12 and interferon-γ and is inhibited by transforming growth factor β. Eur. J. Immunol. 24, 793-798.

Suda, T., Zlotnik, A., 1992. *In vitro* induction of CD8 expression on thymic pre-T cells. II. characterization of $CD3^-$, $CD4^-$ and CD8 cells generated *in vitro* by culturing $CD25^+$, $CD3^-$, $CD4^-$ and $CD8^-$ thymocytes with T cell growth factor-β and tumor necrosis factor-α. J. Immunol. 149, 71-76.

Swain, S.L., Huston, G., Tonkonogy, S., Weinberg, A.D., 1991. Transforming growth factors-β and IL-4 cause helper T cell precursors to develop into distinct effector helper cells that differ in lymphokine secretion pattern and cells surface phenotype. J. Immunol. 147, 2991-3000.

Ward, D.K., Griffiths, G. M., Stinchombe, J.C., Kaplan, J., 2000. Analysis of the lysosomal storage disease Chediak-Higashi syndrome. Traffic 1, 816-822.

Wehler, C., Andrews, J.M., Bing, D.H., 1981. The use of solid phase Clq and horseradish peroxidase-conjugated goat anti-IgG for the detection of immune complexes in human serum. Molec. Immunol. 18, 157-16.

Table 2.1
Effect of intravenous TGF-β_1 treatment on circulating immune complexes (CIC) in mink with Aleutian disease.

	Mean Circulating-Immune-Complex (μg E/ml ± SD)							
	Group 1 (treated 2 weeks pre-virus inoculation)		Group 2 (treated 8 h post-virus inoculation)		Group 3 (treated 2 weeks post-virus inoculation		Group 4 (3 treatments)[a]	
Treatment	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure[b]
TGF-β_1 200 ng/kg	2.95 ± 0.90	8.25 ± 0.95*	3.12 ± 1.19	7.98 ± 1.59*	2.92 ± 1.37	9.09 ± 3.00*/$^+$	3.16 ± 0.99	4.16 ± 0.79*
TGF-β_1 100 ng/kg	2.81 ± 0.60	4.41 ± 0.74*	2.95 ± 0.56	3.51 ± 0.35*	2.81 ± 0.89	5.22 ± 1.85*		ND[c]
TGF-β1 1 ng/kg	2.76 ± 0.73	15.49 ± 1.97$^+$	2.98 ± 1.16	16.36 ± 2.27*/$^+$	2.69 ± 0.98	17.15 ± 2.88*/$^+$		ND[c]
TGF-β_1-Inhibitor	2.68 ± 1.24	36.46 ± 9.02$^+$	2.85 ± 1.22	34.52 ± 7.52$^+$	3.06 ± 1.72	30.79 ± 3.99$^+$		ND[c]
Placebo	2.98 ± 0.77	28.44 ± 6.15$^+$	3.11 ± 1.58	32.85 ± 5.95$^+$	2.76 ± 1.12	35.84 ± 4.36$^+$	2.95 ± 1.16	32.38 ± 5.49$^+$

[a]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
[b]Represents data from the last serum sample taken prior to death or sacrifice of the animal.
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.
$^+p \leq 0.05$ compared to pre- vs. post-virus exposure.

Table 2.2
Detection of ADV DNA in mink treated intravenously with TGF-β_1.

Group No.	Treatment	Presence of viral DNA ADV+	Presence of viral DNA ADV−	Percentage of mink positive for viral DNA[a]
Group 1 (treated 2 weeks pre-virus inoculation)	TGF-β_1 200 ng/kg	0	5*	0
	TGF-β_1 100 ng/kg	0	5*	0
	TGF-β_1 1 ng/kg	3	2	60
	TGF-β_1-Inhibitor	4	1	80
	Placebo	4	1	80
Group 2 (treated 8 h post-virus inoculation)	TGF-β_1 200 ng/kg	0	5**	0
	TGF-β_1 100 ng/kg	0	5**	0
	TGF-β_1 1 ng/kg	3	2	60
	TGF-β_1-Inhibitor	5	0	100
	Placebo	5	0	100
Group 3 (treated 2 weeks post-virus inoculation)	TGF-β_1 200 ng/kg	1	4*	20
	TGF-β_1 100 ng/kg	0	5**	0
	TGF-β_1 1 ng/kg	3	2	60
	TGF-β_1-Inhibitor	4	1	80
	Placebo	5	0	100
Group 4[b] (3 treatments)	TGF-β_1 200 ng/kg	0	5	0

[a]Lymph nodes of animals were harvested and evaluated for the presence of viral DNA upon death or at the determination of the experiment.
[b]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation
*$p \leq 0.05$ compared to placebo-treated controls.
**$p \leq 0.01$ compared to placebo-treated controls.

Table 2.3
Summary of histopathological findings of AD-infected mink treated with TGF-β_1.

		Histopathological Examination				
		Pathology Severity Scores				Percentage of animals AD+ by Pathology
Group No.	Treatment	0	+	++	+++	%
Group 1 (treated 2 weeks pre-virus inoculation)	TGF-β_1 200 ng/kg[b]	3	1	0	0	25
	TGF-β_1 100 ng/kg	3	1	1	0	40
	TGF-β_1 1 ng/kg	1	1	2	1	60
	TGF-β_1-Inhibitor[c]	0	1	2	0	100
	Placebo[b]	0	2	2	0	100
Group 2 (treated 8 h post-virus inoculation)	TGF-β_1 200 ng/kg	3	1	1	0	40
	TGF-β_1 100 ng/kg[b]	3	1	0	0	25
	TGF-β_1 1 ng/kg[b]	1	1	2	0	75
	TGF-β_1-Inhibitor	0	0	3	2	100
	Placebo	0	1	3	1	100
Group 3 (treated 2 weeks post-virus inoculation)	TGF-β_1 200 ng/kg[c]	2	0	1	0	33
	TGF-β_1 100 ng/kg[b]	3	0	1	0	25
	TGF-β_1 1 ng/kg[b]	1	1	2	0	75
	TGF-β_1-Inhibitor	0	0	3	2	100
	Placebo	0	1	4	0	100
Group 4[a] (3 treatments)	TGF-β_1 200 ng/kg[b]	3	0	1	0	25

[a]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
[b]One animal from this group (n=5) died from bacterial infection or other causes not attributable to AD pathology.
[c]Two animals from this group (n=5) died from bacterial infection or other causes not attributable to AD pathology.

Table 2.4

Summary of results from 8 months treatment of Aleutian mink disease with TGF-β_1.[a]

Group	Treatment	Mean survival time		% AD (+)		Reciprocal	Average (± SD)				
		(day ± SD)	Alive/total	Viral DNA	Patholoy	CEP Titer	Weight (g)	BUN (nm)	IFN-γ (μM)	CIC (nm)	TGF-β(ng/ml)
Group 1	200 ng/kg	207 ± 21*	3/5*	0	25	358 ± 64**	828 ± 87*	17.9 ± 4.9*	18.1 ± 3.3*	8.3 ± 1.0*	0.95 ± 0.07*
(treated	100 ng/kg	221 ±26*	3/5*	0	40	307 ± 57**	820 ± 114*	14.1 ± 3.5*	12.0 ± 1.7*	4.4 ± 0.7*	0.91 ± 0.06*
2 weeks	1 ng/kg	170 ± 31	1/5	60	60	614 ± 150*	730 ± 120*	31.3 ± 7.0*	25.0 ± 4.2	15.5 ± 2.0	0.77 ± 0.02
pre-virus	Inhibitor[c]	116 ± 9**	0/5	80	100	1126 ± 280	340 ± 22	84.1 ± 17.4	40.9 ± 7.0	36.5 ± 9.0	0.55 ± 0.07*
inoculation)	Placebo[d]	149 ± 8	0/5	80	100	1024 ± 0	339 ± 24	63.8 ± 12.7	31.6 ± 2.8	28.4 ± 6.2	0.73 ± 0.05
Group 3	200 ng/kg	204 ± 9*	3/5*	0	40	410 ± 64**	808 ± 65*	23.1 ± 5.2*	15.5 ± 5.6*	8.0 ± 1.6*	0.88 ± 0.11*
(treated	100 ng/kg	247 ± 17*	3/5*	0	25	218 ± 43**	883 ± 94*	15.3 ± 3.4*	17.6 ± 4.9*	3.5 ± 0.4*	0.88 ± 0.07*
8 h	1 ng/kg	170 ± 38	1/5	60	75	563 ±140**	765 ± 145	33.2 ± 11.8	25.0 ± 5.3*	16.4 ± 2.3*	0.77 ± 0.03
post-virus	Inhibitor	123 ± 12	0/5	100	100	1024 ± 0	373 ± 22*	73.2 ± 18.3	39.8 ± 6.2	34.5 ± 7.5	0.67 ± 0.03
inoculation)	Placebo	120 ± 25	0/5	100	100	922 ± 115	470 ± 60	56.6 ± 9.2	37.3 ± 4.2	32.9 ± 6.0	0.73 ± 0.04
Group 3	200 ng/kg	200 ± 33*	2/5	20	33	307 ± 57**	788 ± 103*	22.8 ± 4.5*	16.6 ± 2.4*	9.1 ± 3.0*	0.84 ± 0.11*
(treated	100 ng/kg	223 ± 28**	3/5*	0	25	327 ± 88**	853 ± 100*	15.1 ± 7.0*	16.9 ± 3.6*	5.2 ± 1.9*	0.84 ± 0.06*
2 weeks	1 ng/kg	179 ± 31	1/5	60	75	666 ± 128*	655 ± 53*	29.2 ± 8.2*	23.9 ± 8.8	17.2 ± 2.9*	0.77 ± 0.03
post-virus	Inhibitor	135 ± 26	0/5	80	100	922 ± 115	380 ± 37	61.8 ± 15.7	50.4 ± 11.0	30.8 ± 4.0	0.62 ± 0.11*
inoculation)	Placebo	129 ± 24	0/5	100	100	1126 ± 280	365 ± 39	67.6 ± 5.0	42.0 ± 11.3	35.8 ± 4.4	0.74 ± 0.02
Group 4[b]	200 ng/kg	248 ± 31**	3/5*	0	25	256 ± 29	969 ± 103	10.4 ± 2.7*	16.0 ± 4.8*	4.2 ± 0.8*	1.07 ± 0.26*

[a]Values are averages of last measurable parameters prior to death or sacrifice.

[b]3 times treatment: TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.

[c]Latency associated peptide, recombinant human (LAPrhTGF-β_1 at 500 ng/kg). [d]Control; PSS 0.1% BSA

*$P \leq 0.05$ and **$P \leq 0.01$ compared to placebo-treated controls.

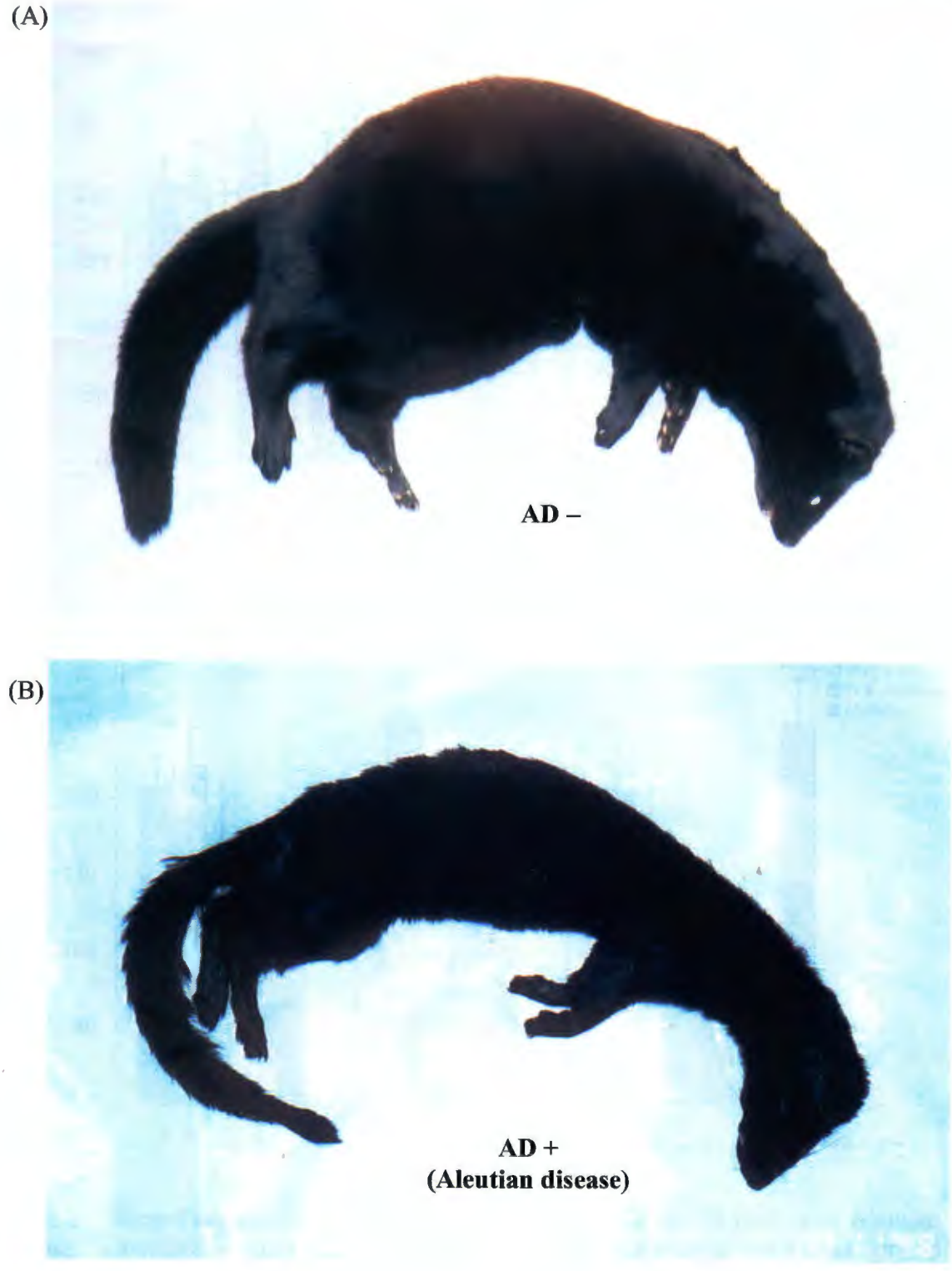


Fig. 2.1. An illustration of the visible effects of AD on mink.

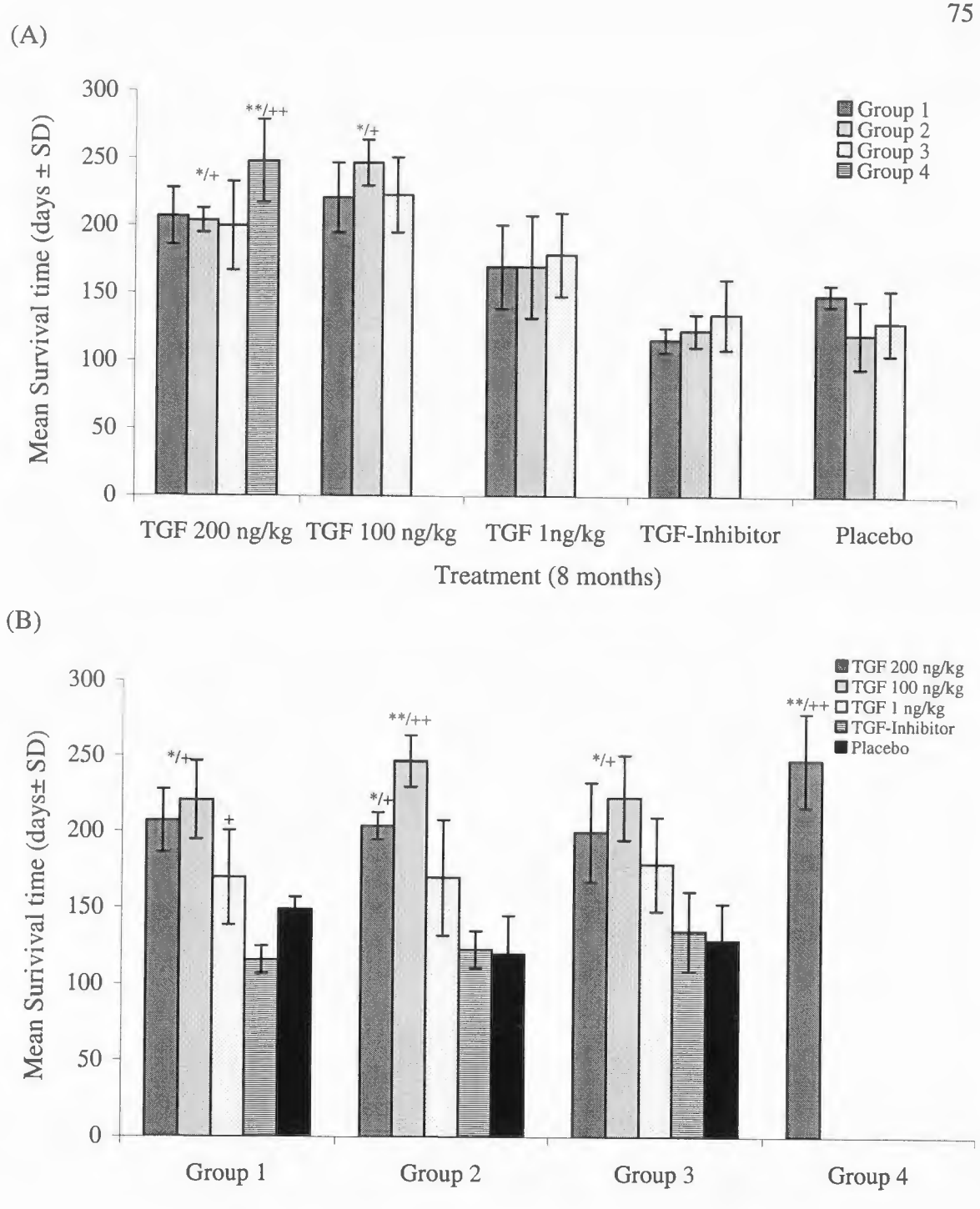
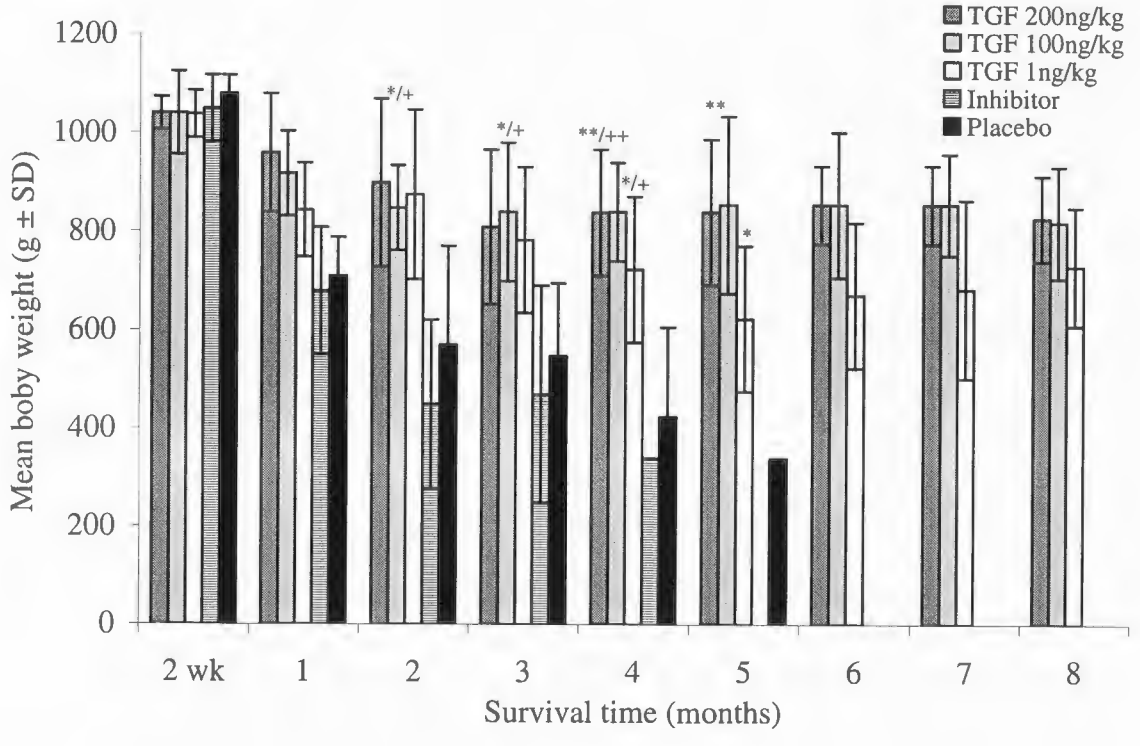


Fig. 2.2. Effect of intravenous TGF-β_1 treatment on survival time of mink with Aleutian disease. Animals dying prior to day 266 days. TGF-β_1 was administered 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

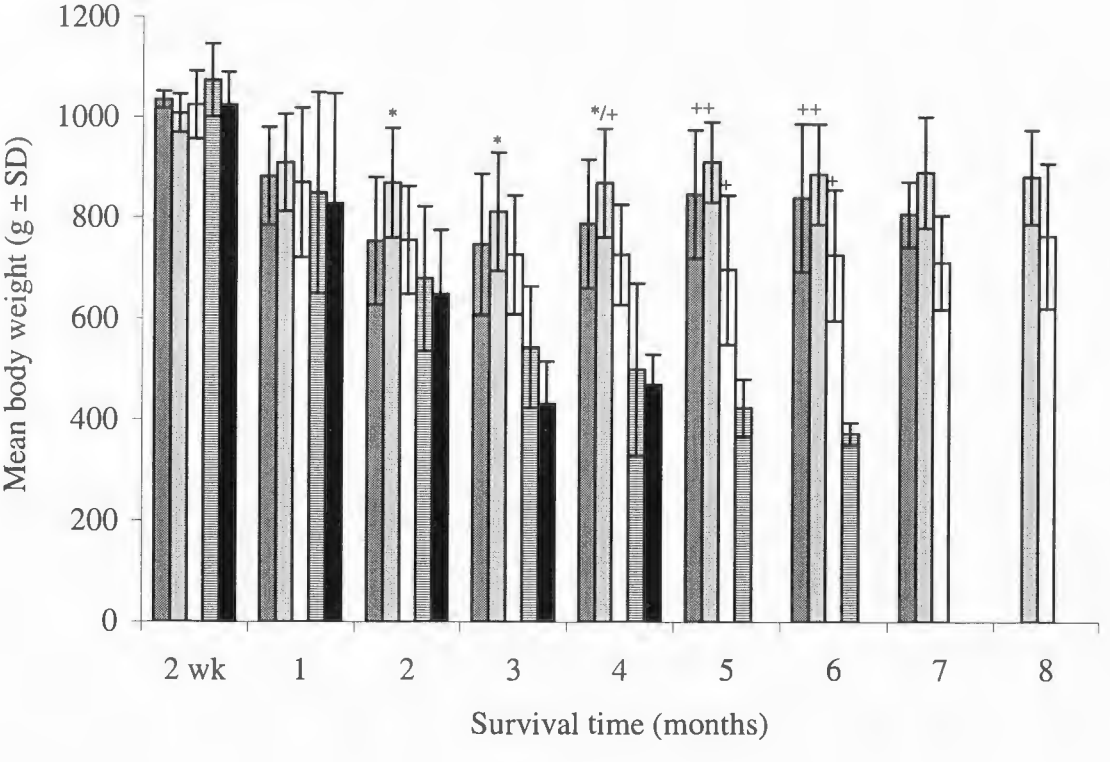
*$p < 0.05$ and **$p < 0.01$ compared to placebo group.

$^{+}p < 0.05$ and $^{++}p < 0.01$ compared to inhibitor group.

(A)


TGF 200ng/kg
TGF 100ng/kg
TGF 1ng/kg
Inhibitor
Placebo
Mean boby weight (g ± SD)
1200
1000
800
600
400
200
0
*/+
*/+
**/++
*/+
**
*
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(B)


Mean body weight (g ± SD)
1200
1000
800
600
400
200
0
*
*
*/+
++
++
+
+
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(C)

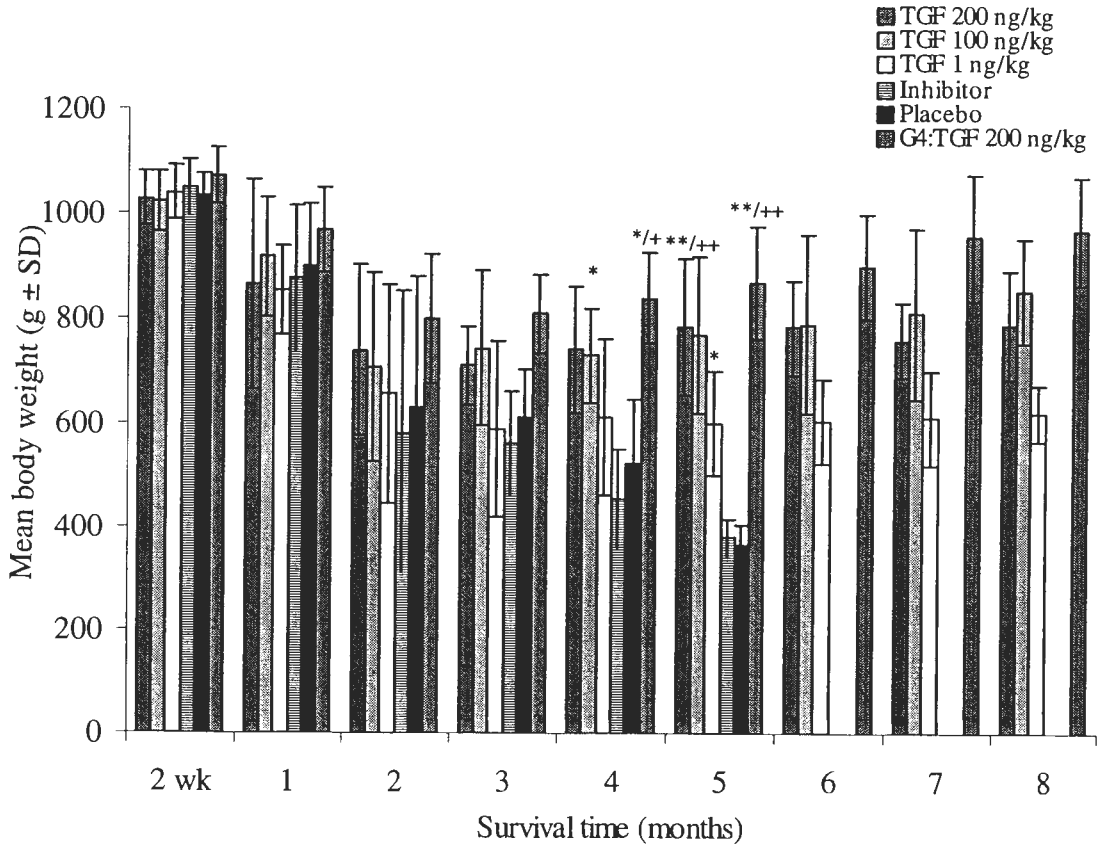


Fig. 2.3. Effect of intravenous TGF-β_1 treatment on body weights of mink with Aleutian disease. Group 1: treated with TGF-β_1 injected 2 weeks pre-virus inoculation (A), Group 2: TGF-β_1 injected 8 h post-virus inoculation (B), and Group 3: TGF-β_1 injected 2 weeks post-virus inoculation and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation) (D). Animals treated with recombinant human TGF-β_1 (rhTGF-β_1) at 200, 100, and 1 ng/kg. Inhibitor: recombinant human latency associated peptide TGF-β_1 (rhLAPTGF-β_1) at 500 ng/kg. Placebo: 0.1 % BSA in PSS. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

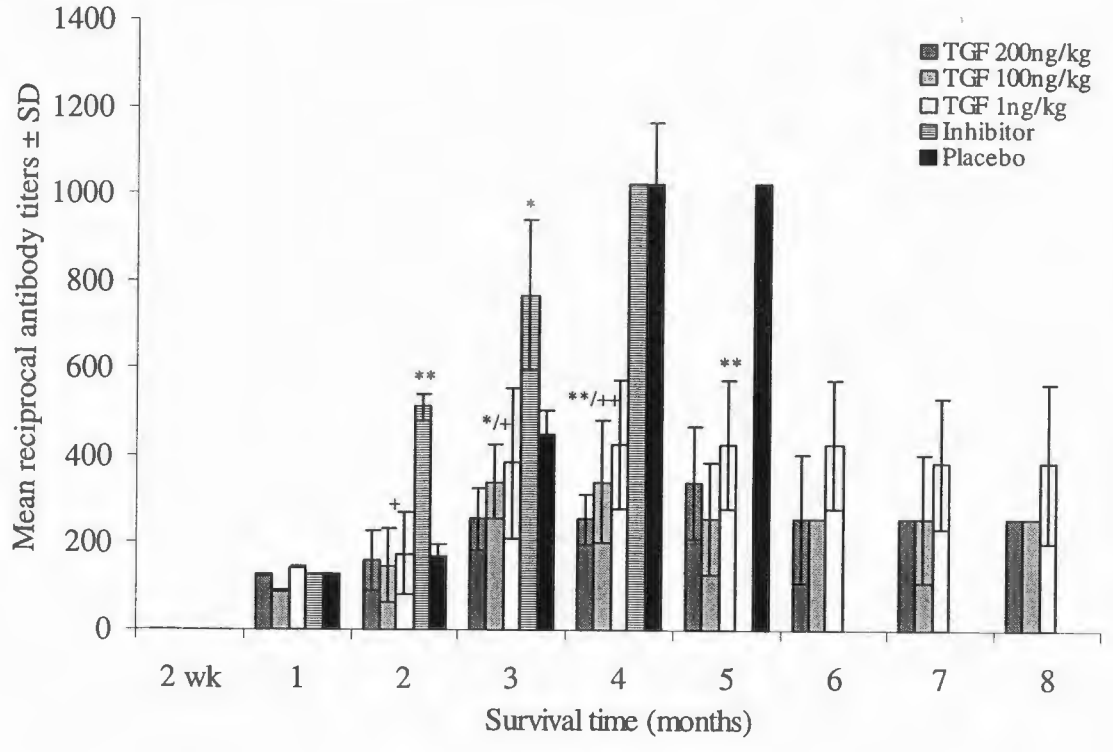
*$p < 0.05$ compared to placebo group.

**$p < 0.01$ compared to placebo group.

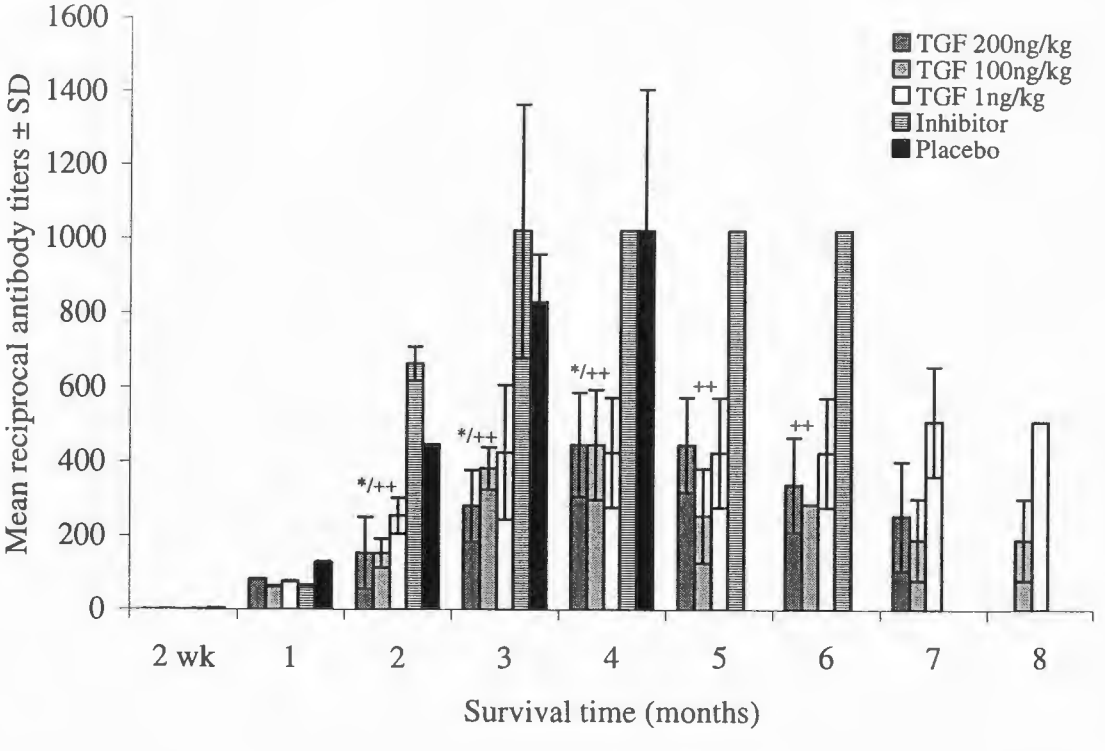
$^{+}p < 0.05$ compared to inhibitor group.

$^{++}p < 0.01$ compared to inhibitor group.

(A)


Mean reciprocal antibody titers ± SD
1400
1200
1000
800
600
400
200
0
TGF 200ng/kg
TGF 100ng/kg
TGF 1ng/kg
Inhibitor
Placebo
+
**
*/+
*
**/++
**
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(B)


Mean reciprocal antibody titers ± SD
1600
1400
1200
1000
800
600
400
200
0
TGF 200ng/kg
TGF 100ng/kg
TGF 1ng/kg
Inhibitor
Placebo
*/++
*/++
*/++
++
++
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(C)

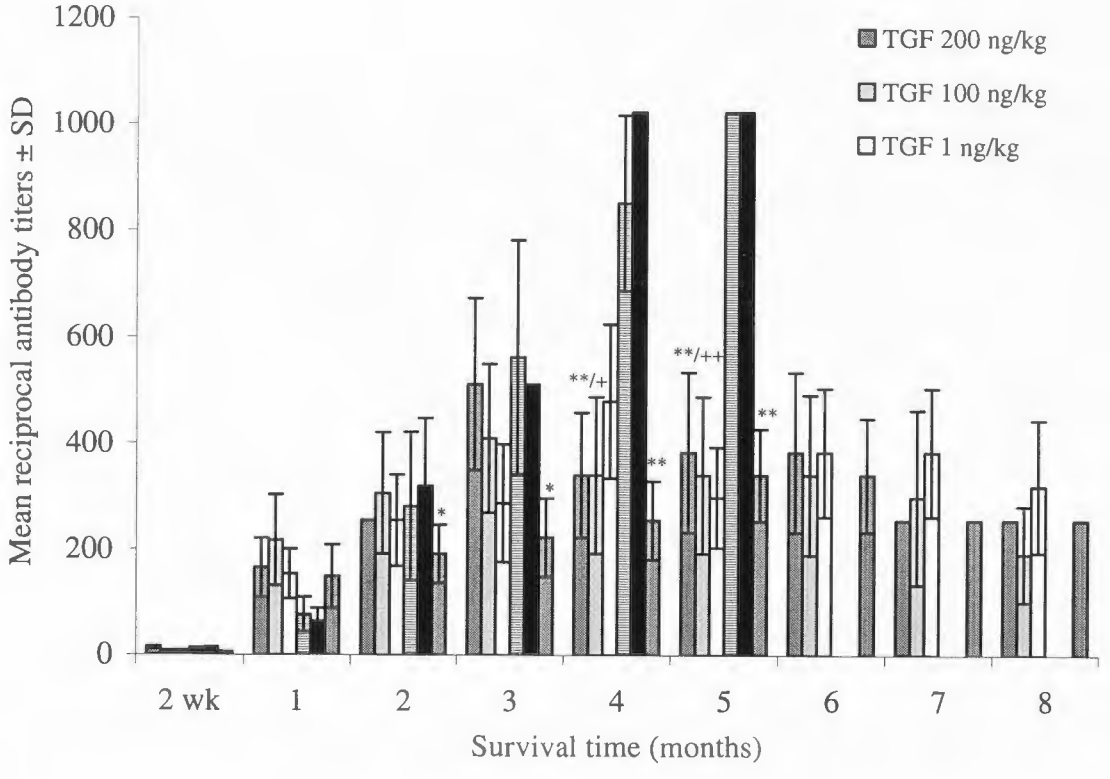


Fig. 2.4. Effect of TGF-β_1 treatment on the anti-ADV antibody titers in mink. Group 1: treated with TGF-β_1 injected 2 weeks pre-virus inoculation (A), Group 2: TGF-β_1 injected 8 h post-virus inoculation (B), and Group 3: TGF-β_1 injected 2 weeks post-virus inoculation and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation) (C). Animals treated with recombinant human TGF-β_1 (rhTGF-β_1) at 200, 100, and 1 ng/kg. Inhibitor: recombinant human latency associated peptide TGF-β_1 (rhLAPTGF-β_1) at 500 ng/kg. Placebo: 0.1 % BSA in PSS. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

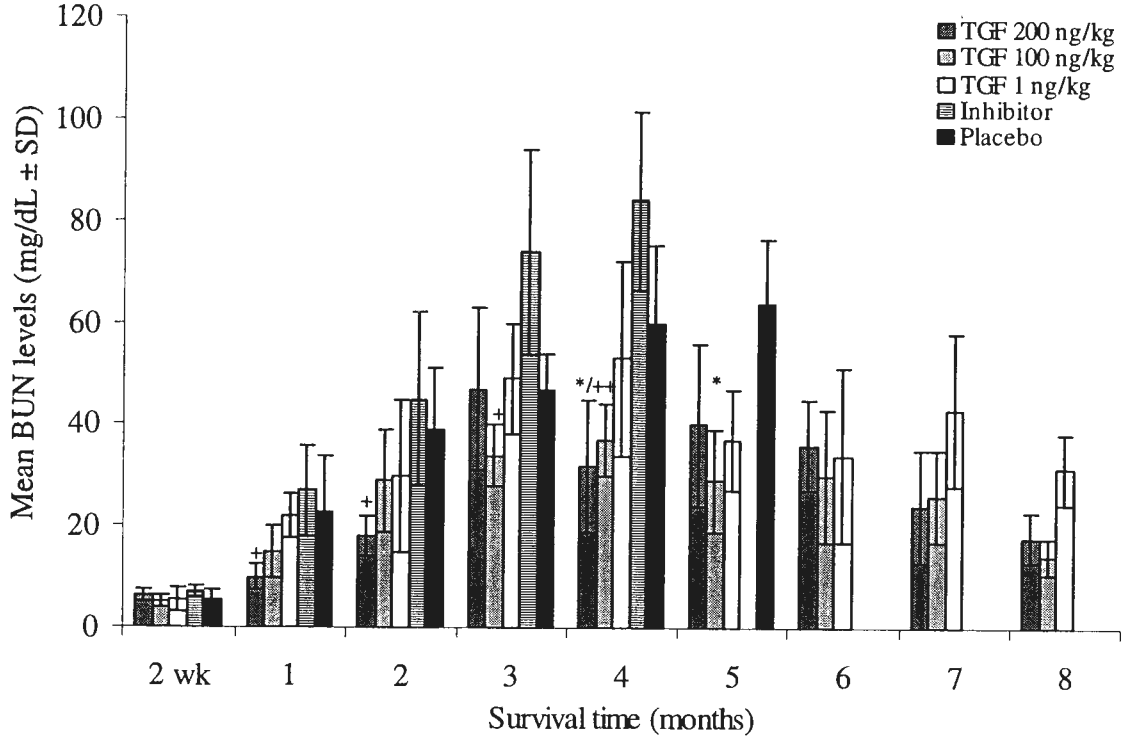
*$p < 0.05$ compared to placebo group.

**$p < 0.01$ compared to placebo group.

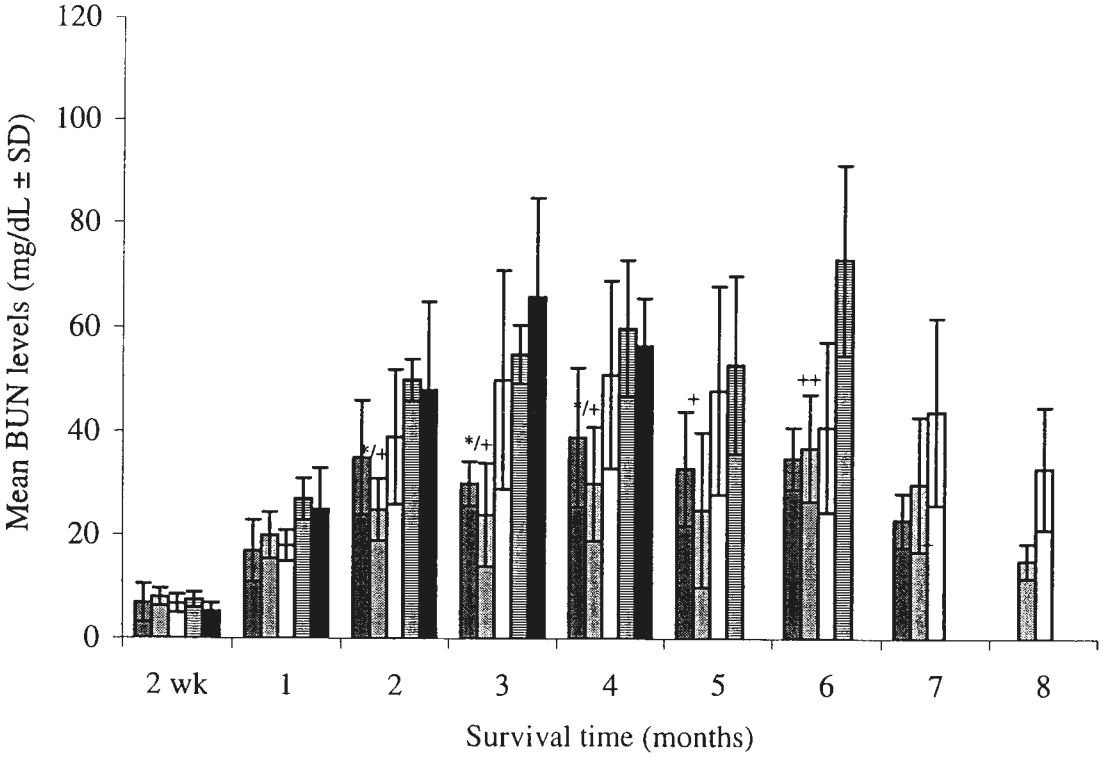
$^{+}p < 0.05$ compared to inhibitor group.

$^{++}p < 0.01$ compared to inhibitor group

(A)


TGF 200 ng/kg
TGF 100 ng/kg
TGF 1 ng/kg
Inhibitor
Placebo
Mean BUN levels (mg/dL ± SD)
120
100
80
60
40
20
0
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(B)


Mean BUN levels (mg/dL ± SD)
120
100
80
60
40
20
0
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(C)

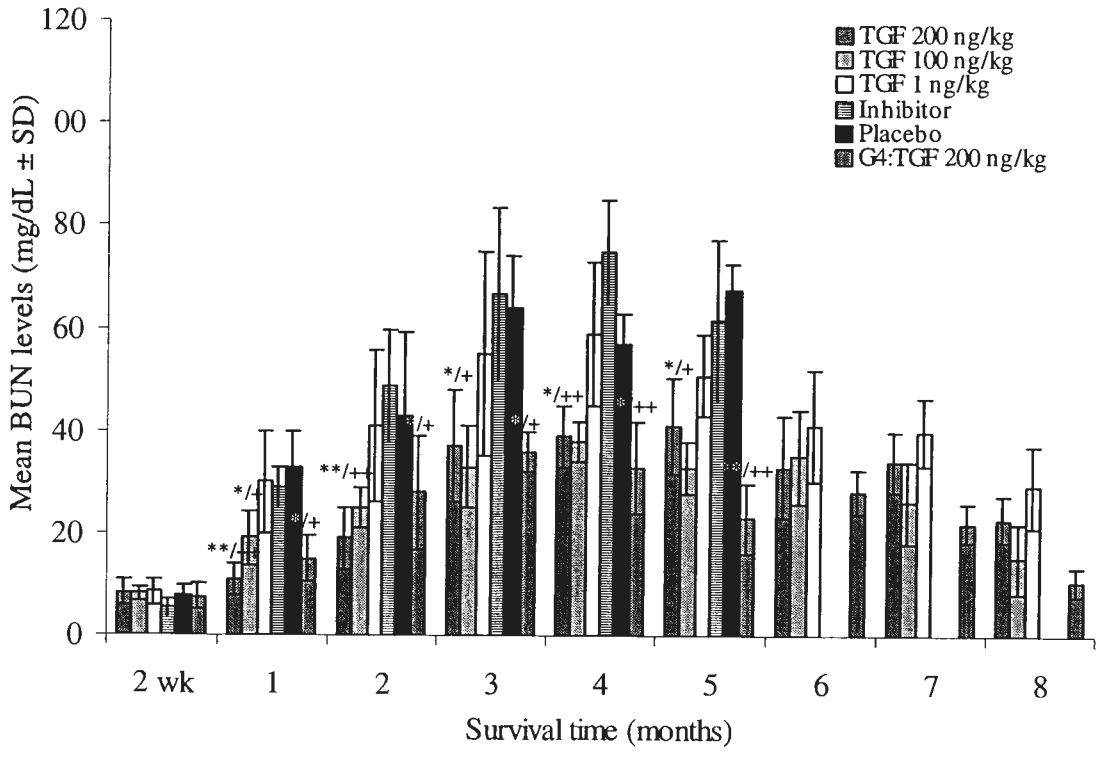


Fig. 2.5. Effect of intravenous TGF-β_1 treatment on blood urea nitrogen (BUN) levels in mink with Aleutian disease. Group 1: treated with TGF-β_1 injected 2 weeks pre-virus inoculation (A), Group 2: TGF-β_1 injected 8 h post-virus inoculation (B), and Group 3: TGF-β_1 injected 2 weeks post-virus inoculation and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation) (C). Animals treated with recombinant human TGF-β_1 (rhTGF-β_1) at 200, 100, and 1 ng/kg. Inhibitor: recombinant human latency associated peptide TGF-β_1 (rhLAPTGF-β_1) at 500 ng/kg. Placebo: 0.1 % BSA in PSS. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

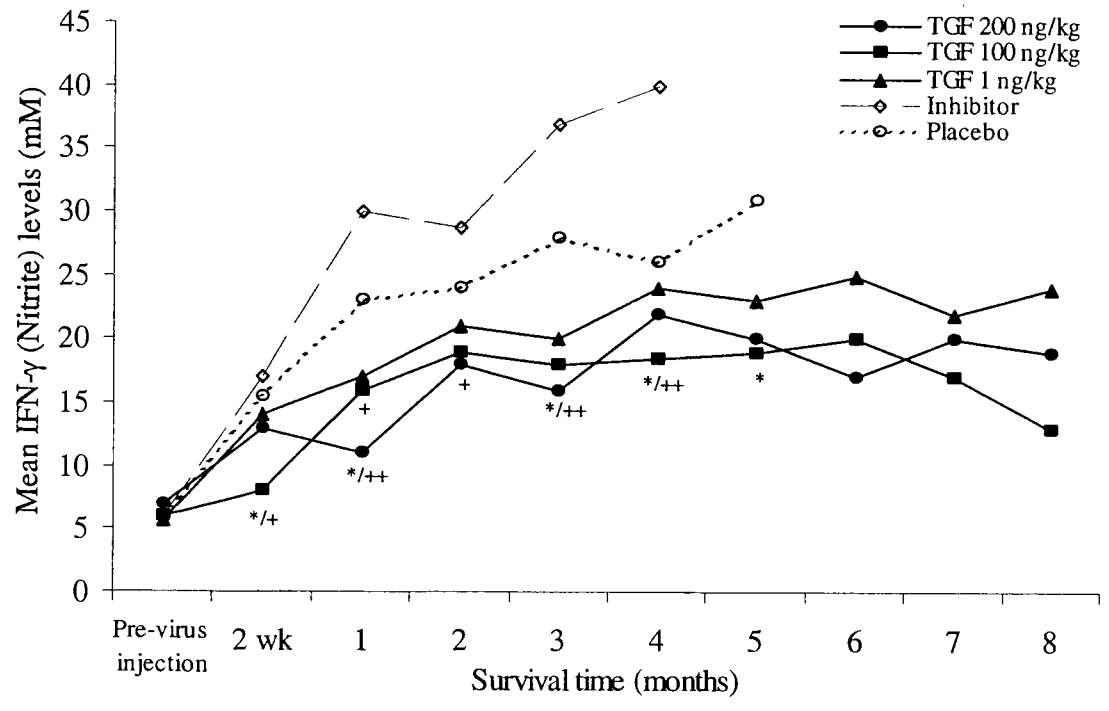
*$p < 0.05$ compared to placebo group.

**$p < 0.01$ compared to placebo group.

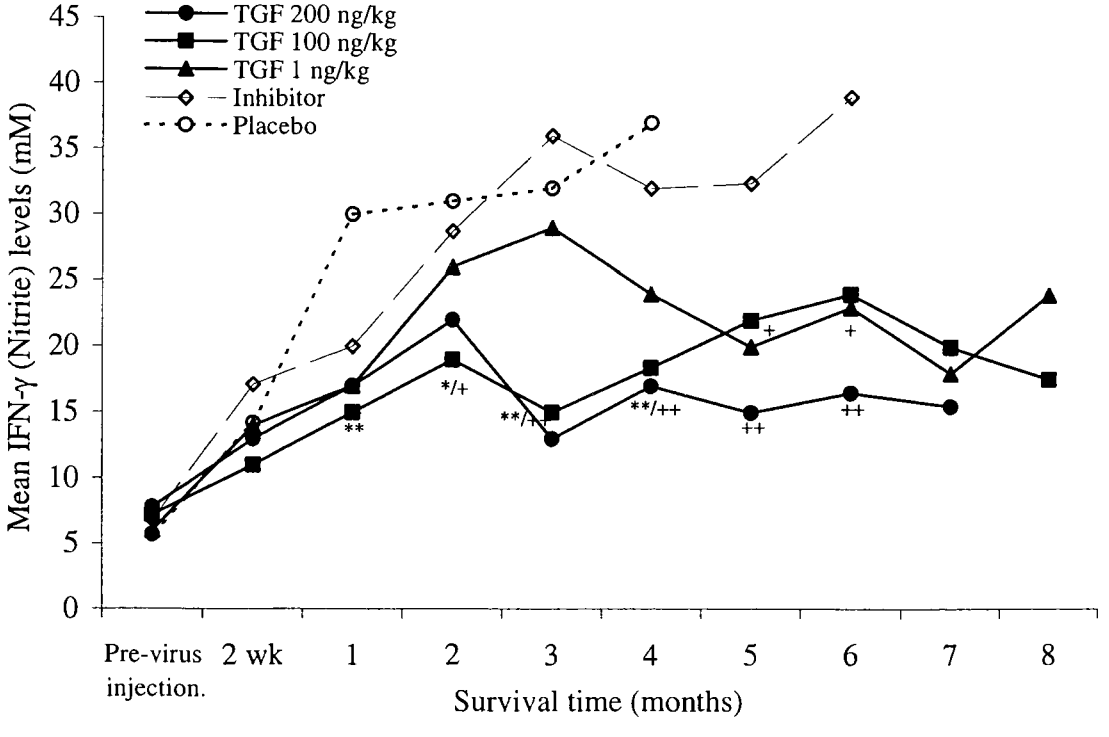
$^{+}p < 0.05$ compared to inhibitor group.

$^{++}p < 0.01$ compared to inhibitor group

(A)


TGF 200 ng/kg
TGF 100 ng/kg
TGF 1 ng/kg
Inhibitor
Placebo
Mean IFN-γ (Nitrite) levels (mM)
45
40
35
30
25
20
15
10
5
0
*/+
*/++
+
+
*/++
*/++
*
Pre-virus injection
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(B)


TGF 200 ng/kg
TGF 100 ng/kg
TGF 1 ng/kg
Inhibitor
Placebo
Mean IFN-γ (Nitrite) levels (mM)
45
40
35
30
25
20
15
10
5
0
**
*/+
**/++
**/++
++
+
+
++
Pre-virus injection.
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

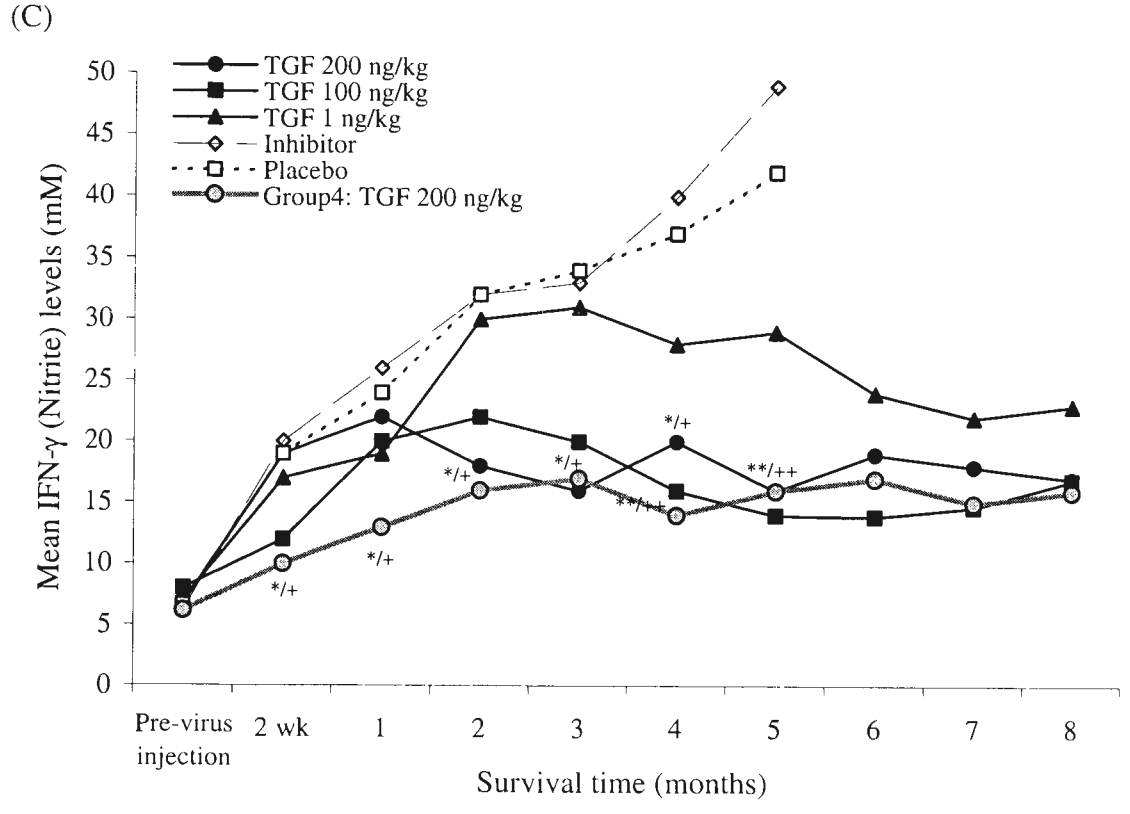


Fig. 2.6. Effect of intravenous TGF-β_1 treatment on serum interferon gamma (IFN-γ) levels in mink with Aleutian disease. Group 1: treated with TGF-β_1 injected 2 weeks pre-virus inoculation (A), Group 2: TGF-β_1 injected 8 h post-virus inoculation (B), and Group 3: TGF-β_1 injected 2 weeks post-virus inoculation and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation) (C). Animals treated with recombinant human TGF-β_1 (rhTGF-β_1) at 200, 100, and 1 ng/kg. Inhibitor: recombinant human latency associated peptide TGF-β_1 (rhLAPTGF-β_1) at 500 ng/kg. Placebo: 0.1 % BSA in PSS. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

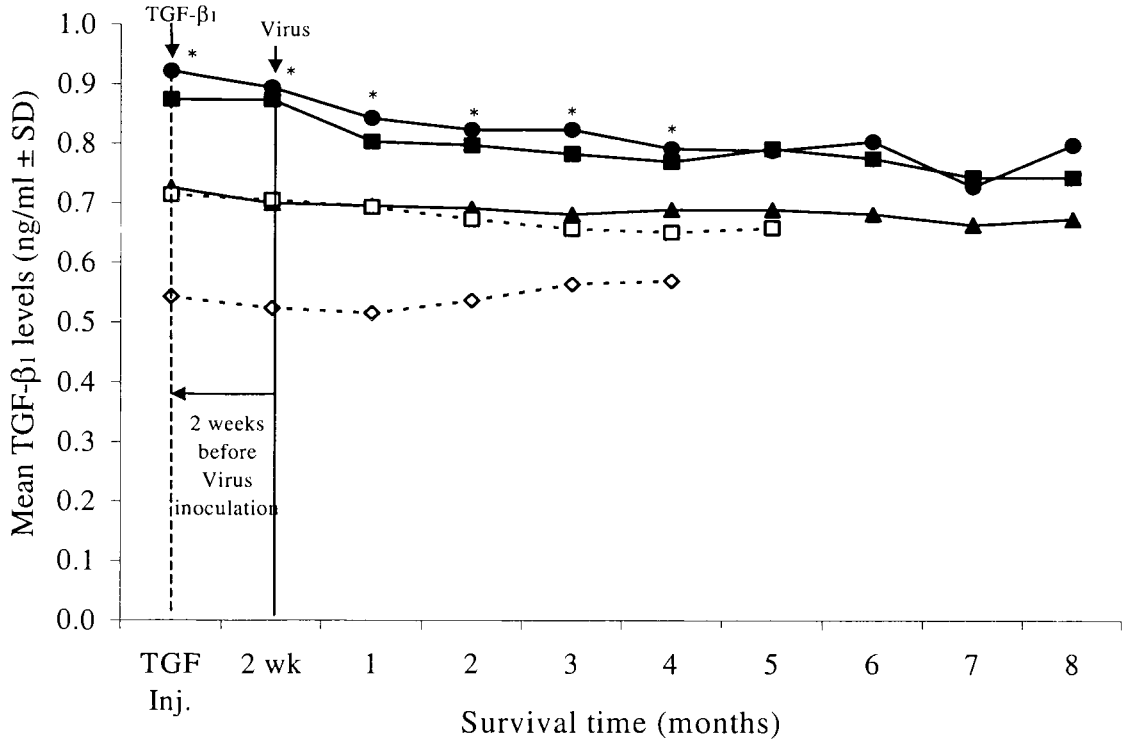
*$p < 0.05$ compared to placebo group.

**$p < 0.01$ compared to placebo group.

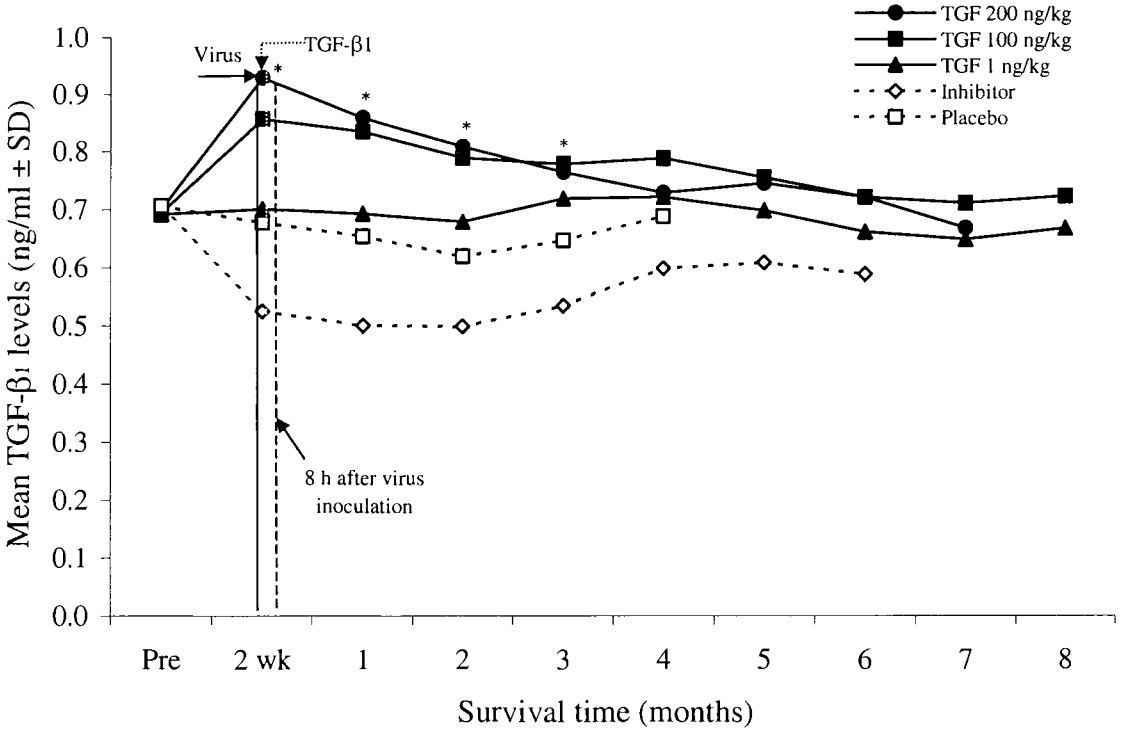
$^{+}p < 0.05$ compared to inhibitor group.

$^{++}p < 0.01$ compared to inhibitor group.

(A)


TGF-β1
Virus
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Mean TGF-β1 levels (ng/ml ± SD)
2 weeks before Virus inoculation
TGF Inj.
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(B)


TGF 200 ng/kg
TGF 100 ng/kg
TGF 1 ng/kg
Inhibitor
Placebo
Virus
TGF-β1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Mean TGF-β1 levels (ng/ml ± SD)
8 h after virus inoculation
Pre
2 wk
1
2
3
4
5
6
7
8
Survival time (months)

(C)

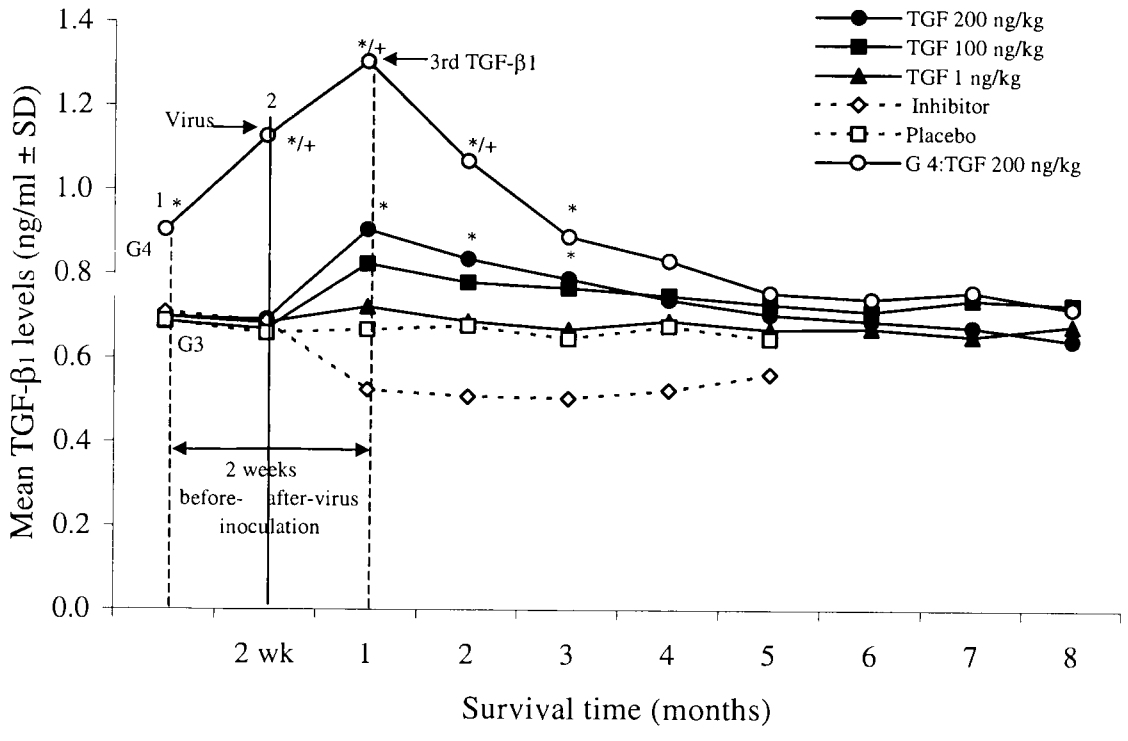


Fig. 2.7. Effect of intravenous TGF-β_1 treatment on serum TGF-β_1 levels in mink. Group 1: treated with TGF-β_1 injected 2 weeks pre-virus inoculation (A), Group 2: TGF-β_1 injected 8 h post-virus inoculation (B), and Group 3: TGF-β_1 injected 2 weeks post-virus inoculation and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation) (C). Animals treated with recombinant human TGF-β_1 (rhTGF-β_1) at 200, 100, and 1 ng/kg. Inhibitor: recombinant human latency associated peptide TGF-β_1 (rhLAPTGF-β_1) at 500 ng/kg. Placebo: 0.1 % BSA in PSS. After 5 months, all animals in TGF-β_1 inhibitor group and the placebo group had died from AD.

*$p < 0.05$ compared to inhibitor group.

+$p < 0.05$ compared to placebo group.

CHAPTER 3

EFFECTS OF HE 2300 AND HE 2500 ON THE OUTCOME OF ALEUTIAN DISEASE IN MINK

Abstract

Aleutian disease of mink (AD) is an immunopathological disorder of mink induced by persistent infection with AD virus. Many breeds of mink seem to have a high a susceptibility to AD.

Interestingly, the primary feature of AD in susceptible adult mink is a chronic, fatal immune complex-mediated glomerulonephritis comparable to that seen in human diseases such as systemic lupus erythematosus. Currently no therapies are available and vaccination has been a failure; the latter exacerbates the disease.

The hypothesis to be tested in this research was that dehydroepiandrosterone-like compounds (DHEA analogs) will inhibit or ameliorate Aleutian disease in mink. The DHEA analog, HE2300 and HE2500, used three times weekly at 13 mg/ml, significantly enhanced mink survival time compared to infected, untreated control mink. The severity of the disease was lessened as determined by histopathological examination. HE2300 (3x/wk) treatment increased survival time and body weight nearly two-fold compared to infected, untreated animals. Untreated animals had high antibody titers and high blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), asparatate aminotransferase (AST), interferon gamma (IFN-γ), and circulating immune complex (CIC) levels, suggesting immune complex deposition, a hallmark of AD. Infected, treated animals showed a dramatic decrease in ADV-specific antibody titers. BUN,

creatinine, ALT, AST, IFN-γ, and CIC levels. This was supported by histopathological examination of the mink. Spleen weights of untreated animals increased by two-fold compared to treated animals and control mink.

Another analog, HE2500, also ameliorated AD in mink when given three times weekly at a dose of 16 ng/kg. Time to death was extended in treated mink and all other parameters measured were similar to the uninfected control animals, although usually mild lesions were present in kidney and liver of surviving mink in the treated group.

These findings support the hypothesis that DHEA analogs may be able to promote an immune responses that affords protection for mink from chronic lethal AD.

Introduction

Aleutian disease virus (ADV) causes a number of interesting and completely different disease syndromes in mink. These syndromes include decreased fertility, abortion, acute pneumonia in kits, and chronic and persistent infections, all severely impacting the profitability of mink farming. Interestingly, the primary feature of Aleutian disease (AD) in susceptible adult mink is a chronic, fatal immune complex-mediated glomerulonephritis comparable to that seen in human diseases such as systemic lupus erythematosus (Bloom, 1984). Currently no therapies are available and vaccination has been a failure; the latter exacerbated the disease (Alexandersen, 1990). For producers who have AD in their mink, their only recourse is to kill the mink early before the coat of the animal becomes less desirable due to AD effects, or when time to harvest the pelts comes to gamble that the disease will not afflict a significant portion of their animals.

Therefore, a need exists to develop a modality that can boost the susceptible animals' immune responses in an appropriate manner to allow them to survive the disease.

Despite intensive studies, the mechanism by which ADV induces a severe disorder of immune function and establishes a chronic, persistent infection remains unclear. It is also not well established whether inbreeding of the animals may be part of the problem. The mink have been so extensively inbred that certain varieties (e.g., sapphires, a.k.a. Aleutian mink) may not be immunocompetent.

Recently, several studies have been done to understand how ADV disturbs host immune function (Aasted and Leslie, 1991; Mori et al., 1991; Kanno et al., 1992). From these studies, it has become apparent that the initial target for ADV replication is probably the macrophage found in the medullary sinuses of the lymph nodes of infected mink (Kanno et al., 1992). Secondary targets are B lymphocytes, circulating macrophages and follicular dendritic cells. The phenotype of AD is characterized by extreme hypergammaglobulinemia caused by the production of non-neutralizing anti-ADV (Alexandersen, 1990) and polyclonal antibody production, including anti-ADV DNA antibodies (Hahn and Hahn, 1983). Subsequently, plasmacytosis (Porter et al., 1980), arteritis (Porter et al., 1973), as well as immune complex-mediated glomerulonephritis, develops. These features suggest that some step in B cell development and/ or maturation is overstimulated by ADV infection (Kanno et al., 1992).

Several theories have been advanced to account for the hypergammaglobulinemia described above (Kanno et al., 1992). Among them are i) cytokines are produced in response to ADV infection of target cell macrophages that enhance B cell proliferation and maturation; ii) viral components (e.g., structural proteins) might directly stimulate B

cell development and clonal expansion; iii) abnormal antigen presentation by infected macrophages might disturb subsequent B cell development and proliferation.

In support of the first hypothesis are the findings of Bloom et al. (1994) that macrophages produce interleukin-6 (IL-6) in antibody-dependent ADV infection. In addition, IL-6 mRNA can be detected in the mesenteric lymph nodes of ADV-infected mink. Perhaps not coincidentally, human diseases that are characterized by hypergammaglobulinemia are also associated with high levels of IL-6 production (e.g., Castleman's disease). Thus, AD may be a virus-induced cytokine disorder; a syndrome of chronic, inappropriate production of IL-6 and perhaps other cytokines (Bloom et al., 1994).

A pertinent observation for the treatment of AD in mink is that an attenuated vaccine only enhances the disease, leading to an especially extreme B cell hyperplasia, while immunosuppressive therapy has been shown to somewhat ameliorate the severity of disease, e.g., preventing Aleutian disease lesions (Cheema, et al., 1972). These therapies have only produced marginal results. One of the compounds chosen, levamisole, does not directly modulate the B cell function of producing of antibody (Amery and Hörig, 1984). The other compound, isoprinosine, actually enhances B cell antibody production in the presence of pokeweed mitogen (Tsang et al., 1984), perhaps a less than desirable effect in AD where over-production of antibody leads to fatal disease.

The β-androstene steroids form a subgroup of steroids that up-regulate immunity, increases resistance against lethal infection and lethal radiation, and mediate a rapid recovery of hematopoietic precursor cells after radiation injury. In vivo, the β-androstenes increase the levels of the Th_1 cytokines such as IL-2, IL-3, IFN-γ; they also

counteract hydrocortisone immune suppression, and function in the maintenance of the Th1/ Th2 balance and immune homeostasis.

There is considerable evidence for an immunomodulatory role for a cytokine, dehydroepiandrosterone (DHEA), β-androstene. It both potentiates and inhibits various functions of the immune system in its regulatory role, depending on when and where it is expressed (Loria, 2002). However, its potent effects on immune cells in vitro are reflected in the various effects it has in various animal model systems, strongly suggesting an important role in immune disorders such as autoimmune disease (Derynck and Choy, 1998). DHEA, one of these β-androstenes, has been shown to have immunomodulating effects in several experimental infections involving viruses and protozoans (Rasmussen et al., 1991; Mulder et al., 1992; Uozumi et al., 1996; Loria and Padgett, 1998). In HIV infections, 17α-bromoepiandrosterone (α-epi-Br), an analog of DHEA, may shift cytokine production to a Th1 response and increase circulating activated immune effector cells (Freilich et al., 2000). This activity appeared not to be androgenic, since the compound only bound weakly to the androgen receptor. In contrast, the 17α-epimer of androstenetriol inhibits proliferation and mediates apoptosis in tumor cells of murine and human origin (Loria, 2002). The antiproliferative functions of 17α-androstenediol are not dependent on either the estrogen or androgen receptors.

Thus, DHEA and its analogs may be able to promote Th1 cytokine-type responses that could afford protection for mink from chronic lethal AD. Therefore, we attempted to develop several approaches for treating experimental AD using epiandrosterone analogs. We tested the hypothesis that treatment of AD-infected mink with TGF-β or

epiandrosterone analogs would ameliorate the development of the AD as shown by an enhanced survival time and lessened other AD parameters in the animals.

Materials and Methods

Animals

Unmated or non-parous 1-year-old female mink weighing 600-1000 g were used. These mink were obtained from the Windy Peaks ranch facility (Franklin, ID). This facility has been free of AD for about 6 years as determined by serological testing and pathology results. All animals were tested for AD by counter-immune-electrophorsis prior to admittance to animal research facility.

Mink were fed a one-time daily ad libitum diet (approximately 100 g) with a fish-based diet formulated for optimal growth, pelt quality, and seasonal variations in nutritional needs by the Mink Farmer's Cooperative of Utah.

Blood for analysis was obtained by toe clip, and when mink were sacrificed, they were euthanized by carbon monoxide inhalation in a sealed plexiglass chamber in an aerated room.

Animals were held to up to 8 months. Signs of disease were measured monthly by analyzing serum samples using antibody, blood urea nitrogen, interferon gamma, and circulating immune complex assays. Confirmation of disease was measured by histopathology examination of kidney, liver, and spleen and by detection of viral DNA in mesenteric lymph nodes. In addition, animals were monitored monthly to determine weight loss or gain, weight loss being a symptom of AD.

Study Design

Five mink received 16 ng/kg of HE2300 (β-androstenetriol (β-AET); 5-androstene-3β-7β-triol; Hollis-Eden pharmaceuticals, San Diego, CA) once per week, 17 animals received 16 ng/kg of HE2300 three times per week and five animals were injected with 16 ng/kg of HE2500 (fluasterone [FLST]; 16α-fluoro-5-androsten-17-one; Hollis-Eden pharmaceuticals, San Diego, CA) three times per week. In addition, 17 animals received vehicle three times per week. All treatments were injected subcutaneously into the nape of the neck of the mink. In addition, six animals only received virus (Table C.1). All animals exposed to virus were injected with virus intraperitoneally and received 5 X 10^5 fluorescent focusing units of ADV of mink (Strain Utah I). Five animals did not receive virus or any treatment. Treatment began 8 h after virus exposure.

Cell

The macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA, USA.). The cells were routinely passaged with in Dulbecco's modification of minimal essential medium (GIBCO Invitrogen Corporation, Grand Island, NY, USA) containing 4000 mg/L glucose. The medium was supplemented with 4 mM L-glutamine (GIBCO Invitrogen Corporation), 0.1 mM sodium pyruvate (GIBCO Invitrogen Corporation), 0.15% sodium bicarbonate and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT).

Counter Current Immunoelectrophoresis

CEP was done according to the manufacturer's instructions using commercially

available reagents (United Vaccines, Madison, WI). Briefly, the samples were run on 80 X 100 mm agarose gels into which wells had been punched. Known antigen plus serum dilutions from the infected animal were placed in wells in apposition to one another. The gels were exposed to electrical current at a constant 40 volts for 1 h. Titers of antibody for each treatment were calculated by taking the inverse of the last dilution at which precipitation was detected using the last available serum prior to death. These titers were then averaged for each treatment group.

Interferon-γ Bioactivity Assay

This assay was used as an indicator of a Th1 cellular response. A nitric oxide production bioassay for interferon-γ was used to assay for interferon-γ in serum samples (Kim and Son, 1996). RAW cells, a murine macrophage cell line (American Type Culture Collection, Manassas, VA), were seeded at 1 X 10^5 cells per well in a 96-well flat-bottomed plate (Corning Costar, NY) and incubated at 37°C for 6 h. The cells were then exposed to a known interferon stimulator or a filtered serum sample for 24 h at 37°C. Nitric oxide produced in response to interferon induction in the cells was detected by the addition of dimethyl-α-naphthylamine, yielding a red color. Absorbances were detected spectrophotometrically at a wavelength of 540 nm. Absorbances were converted to nitrite concentration (μM) using a standard curve for each assay done.

Circulating Immune Complex (CIC) Assay

This serum-based assay was done by following the instructions of a commercially available CIC kit (APLPCO Diagnostics, Windham, NH) to quantitate the amount of CIC found in each mink serum sample.

CICs normally activates complement when they are formed in the host and are normally cleared by phagocytosis. The component of the complement system that binds these complexes is complment-1q (Clq). For this assay Clq was used to capture any immune complexes found in serum samples. CIC binding to Clq was detected using an alkaline phosphatase conjugated Protein A, which binds to the Fc region of IgG, the predominate antibody associated with immune complexes (Wehler et al., 1981).

Dilutions of mink sera (beginning at 1:50 dilution) or standards and controls were incubated with human Clq pre-adsorbed onto microtiter wells for 1 h at room temperature. The detection protocol of the kit was then followed. Absorbance at 405 nm was read. CIC concentrations detected in various mink sera were calculated from standard curves generated using the positive controls provided in the kit.

Blood Chemistry Evaluations

Blood chemistry profiles of sera were determined using a calibrated blood chemistry analyzer at Logan Regional Hospital (Logan, UT). A 15 parameter protocol was used and included detection of Ca^+, Na^+, K^+ and Cl^- levels. Sera were also tested for the serum levels of the following: urea/nitrogen, creatinine, albumin, aspartate and alanine aminotransferases, total bilirubin, alkaline phosphatase, lactate dehydrogenase, glucose, and CO_2.

Polymerase Chain Reaction Detection of Viral DNA

DNA was extracted from mesenteric lymphoid tissue using the DNeasy kit (Qiagen, Valencia, CA). For the PCR reaction the primers used were those described by Jackson et al. (1992). The reactions consisted of the following components: 49.5 μl of

double distilled water. 0.25 μM concentration of each primer, 0.2 mM of all four dexyribonucleotide triphosphates, 50 mM KCL, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.2% Triton X-100, 2.5 units Taq DNA polymerase (Promega) and dilutions of DNA from the samples. For each gel run, there was a positive control, negative control and reagent control. PCR amplification was carried out using a thermal cycler in the following manner:

Denaturation: 95°C for 1 min

Primer annealing: 55°C for 1 min

Primer extension: 72°C for 2 min

Thirty cycles were performed. After amplification, samples were analyzed on a 1.0% agarose gel in TAE buffer (Ausubel et al., 1987). Bands were visualized with ultraviolet light and photographed using Polaroid film.

Histopathological Examinations.

Upon the death of each mink, either from AD or when sacrified the kidneys and liverwere removed and tissues from each were sectioned, fixed and then stained with hematoxylin and eosin. Typical pathological findings for AD included thickened mesangium in the glomeruli with homogenous eosinophilic material (membranous glomerulopathy), detection of multifocal infiltrates of plasma cells and lymphocytes in the interstitium of the kidney cortex (interstitial nephritis) and also in the medulla, multiple convoluted tubules containing eosinophilic material or granular casts, occasional mineralization basement membranes, and portal areas of the liver with varying numbers of infiltrated plasma cells and lymphocytes (hepatitis) randomly dispersed among the

hepatocellular cords. Samples were coded and sent to a board certified pathologist (Utah State Veterinary Diagnostic Laboratory, Logan, UT), who examined the samples for the above-defined pathology; abnormalities were scored as mild (+), moderate (++), and severe (+++). Samples with the pathology described above were considered positive for AD.

Statistical Analysis

Statistical significances were determined by analysis of variance and Student's t-test where appropriate. Standard deviations (SD) were determined for all mean values.

Log rank test was for distribution of survival equality in the two groups. In other words, log rank test was to test statistical significance of survival environment each infected-treated group compared to infected-untreated. Statistically, F-value and p-value of statistical significance were often reported.

Results

Effects of HE2300 and HE2500 Treatment on Physiological and Physical Parameters of Mink exposed the Aleutian Disease Virus

Survival Time

Mean and median survival times in the normal control group was eight months. All infected animals receiving no treatment presumably died from AD, since they all exhibited pathogical lesions consistent with AD. The mean and median survival times in the infected, untreated group was 3.8 and 4 months, respectively, which was significantly ($p < 0.001$) reduced compared to the 8 months survival time of the normal control group

(Fig. 3.1). Sixteen percent of the animals in the virus-infected group survived 4 months. The null hypothesis that there was no difference in survival experience between the two groups was rejected. The mean and median survival time in the infected vehicle-treated group was 4 months in both cases, again a significantly reduction in survival time compared to control (log rank test, $p < 0.01$). Forty-seven percent in the vehicle group survived to 4 months of age. Only one animal survived 8 months in the vehicle-treated group exposed to virus.

The mean and median survival times in the HE2500 (treated three times per week) group was 6 and 8 months, respectively. Sixty percent of the animals in this group survived to 4 months of age, compared to the vehicle and untreated virus control groups. However, one animal in this group apparently did not die from AD; the lesions were not consistent with ADV pathogenesis. Excluding that animal, the survival rate was 75% and the mean time to death was 7 months that is statistically different from vehicle and virus controls. Mink treated with HE2300 one time per week afforded the mink much less protection against lethal AD. These animals had a mean survival time of 4 months and only one animal (20%) in that group survived the 8 months duration of the experiment. By contrast, the mean and median survival time in HE2300 (treated three times per week) group was 7 and 8 months, respectively. Fifty-two percent in this group survived 4 months, which was statistically significant compared to placebo-treated controls ($p < 0.05$, log rank test).

Weight Gain

Mink were weighed prior to any treatment and that weight was designated "pre-exposure weight." The post-virus exposure weights represent the mean weight of animals in each treatment group just prior to death or sacrifice. The mink which were untreated and not exposed to virus (normal control animals) had significant weight increases relative to the infected untreated animals ($p < 0.01$, Fig. 3.2). Mink that were exposed to virus or virus plus vehicle had significant weight losses when compared to the average pre-exposure weights of the same animals. Animals exposed to virus and then treated with any of the treatment regimens gained weight similar to that of the normal controls and gained a significant amount of weight relative to vehicle-treated virus exposed mink and infected untreated mink ($p < 0.01$).

Histopathological Findings

Histopathogical examination of the dying or sacrificed animals exposed to ADV revealed the following results. Twenty-five percent of the mink treated with HE2500 appeared to have no lesions associated with AD, while the remaining mink did exhibit lesions consistent with AD ranging in severity from mild to severe (Table 3.1). One animal in this group had particularly severe lesions and survived 5 months; the remainder survived the entire 8 months of the experiment (Table 3.2).

All animals treated with HE2300 one time per week had lesions indicative of AD. When treated with same compound three times per week, two animals did not exhibit AD-like lesions, whereas 10 had a severity score of + and five had a severity score of ++.

No lesions specific for AD were detected in two animals treated with vehicle. However, four animals had lesions classified as mild and eight had lesions scored as moderate to severe. As expected, all untreated, virus-exposed mink demonstrated pathology attributable to AD.

Serum Levels of Ions

Maintaining of proper ion levels the serum is extremely important for homeostasis. When these levels are abnormal, they often reflect problems with absorption, usually at the level of the glomerular and tubules of the kidney. Since a hallmark of AD is glomerulonephritis and renal dysfunction, the serum levels of sodium, calcium, potassium, and chloride ions were monitored throughout the duration of the experiment.

Mink that were infected and not treated or treated with placebo showed significantly ($p < 0.05$) increased levels of K^+, Na^+ and Cl^- relative to animals in other treatment groups (Fig. 3.3). These levels of K^+, Na^+ and Cl^- were also elevated when compared to levels in each animal prior to infection. Serum Ca^{+2} levels in the placebo and untreated groups dramatically dropped during the experiment and were significantly different from the other groups of mink. In treated animals, the levels of the ions remained relatively constant throughout the duration of the experiment.

Serum Levels of Liver Enzymes

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes produced abundantly in hepatocytes and their release into the serum indicates a reduction in liver function, although diet (mink diet was rich in fish offal containing amines) may influence these enzyme levels to some extent. In mink treated with HE2500

and the one dose of HE2300 (three times per week), the serum levels of these two enzymes were not significantly different from the normal untreated, uninfected controls (Fig. 3.4). However, the virus-infected mink receiving vehicle or no treatment had much higher ($p < 0.05$) serum levels of the two liver enzymes compared to the drug-treated infected animals.

Serum Levels of Nitrogenous Compounds

Detectable levels of urea, creatinine or other nitrogenous compounds in serum are usually indicative of damage to the kidneys, probably at the level of readsorption through the tubules. In mink treated with HE2300 (three times per week) and HE2500, the levels of urea/nitrogen were similar to the normal uninfected, untreated controls (Fig. 3.5). However, the levels of those metabolites in virus-infected mink receiving vehicle and in the untreated group of mink were significantly higher ($p < 0.05$) than drug-treated and the normal control groups.

High serum creatinine levels are a sign of severe renal impairment, but such levels were not found in the serum at any significant amounts above or below the normal control group for the HE2300 (three times per week) and HE2500 group (Fig. 3.5). However, the levels of creatinine in virus-infected mink receiving vehicle and infected, untreated group were significantly higher ($p < 0.05$) than thedrug-treated and normal control group.

Effects of HE2300 and HE2500 on Viral and Immunological Parameters in Mink Infected with ADV

Virus Levels

Viral DNA was detected in the mesenteric lymph nodes of exposed mink by PCR amplification and gel analysis from samples taken as soon after death as possible or after sacrifice. Twenty percent of the mink treated with HE2500 were found to have viral DNA, 40% treated with HE2300 given one time per week were also positive for viral DNA and 18% of the mink treated with HE2300 three times per week were positive (Table 3.3). In the vehicle control animals, 71% were positive and 83% of the mink exposed to virus only were also found to be positive for viral DNA. Only four mink receiving HE2300 treatment three times per week were positive for viral DNA and this was significantly lower than those infected and treated with placebo ($p = 0.003$).

Specific ADV Antibody Levels

High titers of antibody (>512) are often associated with progressive chronic AD (Bloom et al., 1994). Serum was analyzed for ADV-specific antibody by CEP assay. In mink treated with HE2500, the average titer upon death of the animal was 666, for mink treated with HE2300 one time per week, 339 and for mink receiving HE2300 three times per week, 337 (Fig. 3.6). In contrast, the average titers in vehicle-treated mink and in infected, but untreated mink were significantly higher, 1365 and 1536, respectively ($p < 0.001$). All placebo animals were dead after 5 months, generally due to complications from AD.

Circulating Immune Complexs (CIC) Titers

One of the hallmarks of AD pathology is the destruction of kidney tissue due to deposition of CIC in the kidney and subsequent complement-mediated destruction of the basement membrane of kidney tubules. Mink that were infected with virus and not treated or received vehicle had much greater CIC levels in their serum than the treatment group receiving HE2500 and significantly higher levels than those mink receiving either dose of HE2300 ($p < 0.01$, Fig. 3.7). Virus-infected mink that were not treated also had substantially higher levels of CIC than any of the compound treatment regimens.

Serum Gamma Interferon (IFN-γ) Activity

Mink treated with HE2300 given three times per week had NO levels that were much less than were those detected in vehicle-treated mink or in virus-infected mink receiving no treatment ($p < 0.01$, Fig. 3.8). In addition, the mink treated with HE2500 or HE2300 given one time per week, the NO levels were significantly reduced as well ($p = 0.0345$,and 0.007, respectively). Levels of NO with all drug treatments were very similar to the levels detected in uninfected, untreated mink.

Splenomegaly

Splenomegaly is a reflection of an active infection or any number of abnormalities including hypergammaglobulinemia, liver disorders and AD (Bloom et al., 1994). In mink with AD this is probably due the prodigious production of antibody (sometimes 100 mg/dl) in response to virus infection. In this experiment, mink that were infected with ADV had much larger spleen weights than did control mink or mink treated with HE2500

or HE2300 (Fig. 3.9). In addition, the average spleen weight in untreated mink exposed to virus was significantly heavier than in unexposed, untreated mink ($p < 0.01$).

Discussion

Previous preliminary experiments run by us with HE2300 had indicated that treatment of ADV-infected mink three times per week increased the survival time of those mink (data not shown). The current experiments were done to verify those initial findings, as well as to evaluate a less frequent dosing regimen and to evaluate a new compound, HE2500, for efficacy in this immune complex disease model.

HE2500 3x/wk

The effects of treatment on surviving animals were assessed using the parameters discussed above. Mink treated with HE2500 had a significant reduction in weight gain compared to control mink ($p < 0.001$), although all surviving animals did gain some weight throughout the experiment (Table 3.2). Serum levels of nitrogenous compounds and liver enzymes were elevated, although not significantly different from what was detected in normal (uninfected/ untreated) mink. Only one animal in this group had no pathology attributable to AD, even though virus was not detected in any of the surviving animals (Table 3.4). Relative to the sole surviving animal in the vehicle-treated group, the ADV-specific antibody titers in the HE2500-treated animals were much lower as were the levels of CIC ($p < 0.001$), the latter being no different than those levels detected in the control animals. Interferon-γ activity was also comparable to the untreated, uninfected control animals.

A three times per week dosage regimen of HE2500 at 16 ng/kg dosage was effective in treating ADV-infected mink. The compounds appeared to delay the progression of the disease.

HE2300 1x/wk

One animal in this treatment group survived eight months. This mink did not significantly gain weight compared to the control mink, but unlike the surviving animal in the infected vehicle-treated group, it did not lose weight (Table 3.2). This animal had very definite lesions consistent with AD, although no virus was detected in the mesenteric lymph nodes (Table 3.4). The liver enzyme levels in the serum were quite elevated compared to the uninfected controls, but the serum levels of nitrogenous compounds were only slightly elevated compared to controls (Table 3.2). The antibody titer specific for ADV was much lower than that titer in the surviving mink from the vehicle-treated group and typical for animals exposed to disease that are long-term survivors (Table 3.4). The level of circulating immune complexes was commensurate with the low ADV-specific antibody titer and equivalent to that found in the control animals. Interferon-γ activity was also similar to that detected in the uninfected control animals. Despite the favorable physiological and immunological outcomes, this mink did sustain moderate damage to the kidney and liver, which consisted of patchy membranous glomerulopathy, some multifocal interstitial nephritis, and hepatitis with infiltration of a few lymphocytes and plasma cells in the portal areas of the liver.

A one time per week regimen of HE2300 at 16 ng/kg dosage failed to protect most mink from lethal chronic AD.

HE2300 3x/wk

The mink in this treatment group also had significant reductions in weight gain (Table 3.2); five of the animals lost weight (10-140 g), although not as severe as the weight loss in the vehicle-treated animal, which lost half its original body weight (-450 g). The remaining animals maintained their weight or gained weight (0-110 g). Only two mink in the HE2300 group did not have demonstrable AD lesions in the kidney and liver, while most remaining mink had moderate lesions in the kidney and liver with one animal having severe lesions. The blood urea/nitrogen serum levels were elevated relative to the normal (uninfected, untreated) controls, although the creatinine levels were similar to the normal controls (Table 3.2). Serum levels of the livers enzymes were elevated in this treatment group, although this was statistically not significant due to the variability in these measurements. Spleen weights were near normal as were the levels of CIC (Table 3.4). ADV specific antibody titers in this group were again typical for animals surviving a potentially lethal exposure to ADV (<512). The large, pronounced level of interferon-γ activity detected in the surviving vehicle-treated mink was not detected in the HE2300-treated mink and was actually no greater than those levels found in the uninfected control group.

HE2300 given three times per week at 16 ng/kg was the most effective means of treating ADV-infected mink. The compounds temporarily halted or slowed down the progression of the disease.

The data in the current study support the idea that a dosing regimen of three times per week with HE2300 significantly increases mink survival time after exposure to ADV. Nevertheless, substantial numbers of mink still succumbed to the chronic effects of ADV

exposure. All parameters measured indicated that HE2300, using the three times per week dosing regimen, was not only effective in delaying time to death, but that the severity of the disease was lessened within the time period studied in that particular treatment group. The parameters measured in a subset of the HE2300-treatment group, surviving mink, were all comparable to those measured in the uninfected control group with two exceptions, the pathology detected and weight gain. In the survivor group, mild to moderate lesions were still found despite treatment and weight gains were less, lending credence to the supposition that treatment may only delay the time to death and appearance of severe lesions, but not permanently alter the course of a chronic AD infection.

Similar results were seen with the HE2500 treatment regimen. Time to death was extended substantially and the measured parameters in surviving mink were similar to the control group, but again pathology attributable to AD was still detected in the kidneys and liver of the treated animals.

Interestingly, a parameter that seemed predictive of a negative outcome was the presence of high levels of interferon-γ, a Th1 cytokine. Interferon-γ has also been shown to correlate with pathogenesis in another immune complex-mediated disease, systemic lupus erythematosus (Kuroiwa and Lee, 1998). In knock out mice, interferon-γ apparently was an important cytokine in amplifying the local immune responses of lupus nephritis. Infiltrating mononuclear cells exerted their effects on resident renal cells through secretion of soluble factors and apparent promotion of cell-to-cell contact. This scenario may not be the case with AD because in AD large numbers of lymphocytes are found at the lesion and not macrophages/monocytes. Instead, the high interferon-γ levels

could activate localized cytotoxic T cells by stimulating expression of MHC class I antigen cells on putative target cells (De Maeyer and De Maeyer-Guignard, 1998), for example, cells of the basement membrane coated with immune complexes. The latter scenario may correlate with our findings that infiltrating lymphocytes are often at the site of lesions associated with AD.

From the data shown in this report, it is readily apparent that a three times per week regimen of either HE compound was most effective in treating ADV-infected mink; the one time per week regimen failed to protect most mink from lethal chronic AD. The experimental design did not allow the determination of whether the compounds ameliorated the severity of the pathogenesis permanently, whether the lesion damage was reversible or irreversible, or if the compounds "cured" mink of the disease. However, the lack of detection of viral DNA and the reasonably low levels of specific ADV antibody and circulating immune complexes all suggest that the virus was cleared, thus eliminating a continual stimulator of what appears to be type III hypersensitivity response. This would support the notion that at the very least, HE compounds can temporarily halt or slow down the progression of the disease.

References

Aasted, B., Leslie, R.G.Q., 1991. Virus-specific B-lymphocytes are probably the primary targets for Aleutian disease virus. Vet. Immunol. Immunopath. 28, 127-141.

Alexandersen S., 1990. Pathogenesis of disease caused by Aleutian mink disease parvovirus. Acta Pathol. Microbiol. Immunol. Scand. 98, 1-32.

Amery, W.K., Hörig, C., 1984. Levamisole. In: Fenichel, R.L. and Chirigos, M.A. (Eds.), Immune modulation agents and their mechanisms. Marcel Dekker, Inc., New York, pp. 383-408.

Ausubel, F.M., Brent, F., Kingston, R.L. (Eds.), 1987. Current protocols in molecular biology. Greene Publishing and Wiley Interscience, New York.

Bloom, M.E., 1984. Parvovirus infections: features reminiscent of AIDS. Ann. N.Y. Acad. Sci. 437, 110-120.

Bloom, M.E., Kanno, H., Mori, S., Wolfinbarger, J.B., 1994. Aleutian mink disease: puzzles and paradigms. Infect. Agents Dis. 3, 279-301.

Cheema, A., Henson, J.B., Gorham, J.R., 1972. Aleutian disease of mink: prevention of lesions by immunosuppression. Am. J. Pathol. 66, 534-556.

De Maeyer, E., De Mayer-Guignard, J., 1998. Type I interferons. Int. Rev. Immunol. 17, 53-73.

Derynck, R., Choy, L., 1998. Transforming Growth Factor-β and its Receptors. In: Thomson, A. (Ed.), The cytokine handbook, 3rd ed. Academic Press, San Diego, CA, pp. 491-516.

Freilich, D., Ferris, S., Wallace, M., Leach, L., Kallen, A., Frincke, J., Ahlem, C., Hacker, M., Nelson, D., Hebert, J., 2000. 16-Alpha-bromoepiandrosterone, a dehydroepiandrosterone (DHEA) analogue, inhibits Plasmodium falciparum and Plasmodium berghei growth. Am. J. Trop. Med. Hyg. 63, 280-283.

Hahn, E.C., Hahn, P.S., 1983. Autoimmunity in Aleutian disease: contribution of antiviral and anti-DNA antibody to hypergammaglobulinemia. Infect. Immun. 41, 494-500.

Jackson, M.K., Ellis, L.C., Morrey, J.D., Barnard, D.L., 1992. Early detection of Aleutian mink disease virus in mink by polymerase chain reaction. Nor. J. Agr. Sci. Supplement 9, 385-387.

Kanno, H., Wolfinbarger, J.B., Bloom, M.B., 1992. Identification of Aleutian mink disease parvovirus transcripts in macrophages of infected adult mink. J. Virol. 66, 5305-5312.

Kim Y.M., Son, K., 1996. A nitric oxide production bioassay for interferon-gamma. J. Immunol. Methods 198, 203-209.

Kuroiwa, T., Lee, E.G., 1998. Cellular interactions in the pathogenesis of lupus nephritis: the role of T cells and macrophages in the amplification of the inflammatory process in the kidney. Lupus 7, 597-603.

Loria, R.M., Padgett, D.A., 1998. Control of the immune response by DHEA and its metabolites. Rinsho. Byori. 46, 505-17.

Loria, R.M., 2002. Immune up-regulation and tumor apoptosis by androstene steroids. Steroids 67, 953-966.

Mori, S., Wolfinbarger, J.B., Miyazawa, M., Bloom, M.B., 1991. Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages. J. Virol. 65, 952-956.

Mulder, J.W., Frissen, P.H., Krijnen, P., Ender,T.E., de Wolf, F., Goudsmit, J., Masterson, J.G., Lange, J.M., 1992. Dehydroepiandrosterone as predictor for progression to AIDS in asymptomatic human immunodeficiency virus-infected men. J. Infect. Dis. 165, 413-418.

Porter, D.D., Larsen, A.E., Porter, H.G., 1973. The pathogenesis of Aleutian of mink. III. Immune complex arteritis. Am. J. Pathol. 71, 331-334.

Porter, D.D., Larsen, A.E., Porter, H.G., 1980. Aleutian disease of mink. Adv. Immunol. 29, 261-286.

Rasmussen, K.R., Martin, E.G., Arrowood, M.J., Healey, M.C., 1991. Effects of dexamethasone and dehydroepiandrosterone in immunosuppressed rats infected with Cryptosporidium parvum. J. Protozool. 38,157-159.

Tsang, P.H., Tangnavarad, K., Solomon, S., Bekesi, J.G., 1984. Modulation of T- and B-lymphocyte functions by isoprinosine in homosexual subjects with prodromata and in patients with acquired immune deficiency syndrome (AIDS). J. Clin. Immunol. 4, 469-78.

Uozumi, K., Uematsu, T., Otsuka, M., Nakano, S., Takatsuka, Y., Iwahashi, M., Hanada, S., Arima, T., 1996. Serum dehydroepiandrosterone and DHEA-sulfate in patients with adult T-cell leukemia and human T-lymphotropic virus type I carriers. Am. J. Hematol. 53, 165-8.

Wehler, C., Andrews, J.M., Bing, D.H., 1981. The use of solid phase Clq and horseradish peroxidase-conjugated goat anti-IgG for the detection of immune complexes in human serum. Molec. Immunol. 18, 157-162.

Table 3.1
Summary of histopathological findings of AD-infected mink subcutaneously treated with HE2300 or HE2500.

	No. of animals displaying Pathology Severity Scores				Percent Animals Positive for AD Pathology
Treatment	0	+	++	+++	+
HE2500[a] 3X/wk*	1	2	0	1	75
HE2300[b] 1X/wk	0	3	2	0	100
HE2300 3X/wk	2	10	5	0	91.5
Vehicle 3X/wk**	2	4	7	1	91.5
Untreated	0	1	5	0	100
Normal control	5	0	0	0	0

[a]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[b]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*One animal from this group died from bacterial infections or other causes not attributable pathology.
**Three animals from this group died from bacterial infections or other causes no attributable AD pathology.
+: mild, ++: modulate and +++: severe

Table 3.2
Summary of selected physical and physiological parameters eveluated in HE2300- or HE2500-treated mink infected with ADV and surviving 8 months.

Treatment Group	Survivors	Average Weight (g ± SD)	Pathology Severity Scores (-/+)	Blood Urea Nitrogen (mg/dl ± SD)	Serum Creatinine (mg/dl ± SD)	Serum Alanine Aminotransferase (U/L ± SD)	Serum Aspartate Aminotransferase (U/L ± SD)
HE2500[a] 3X/wk	3/5	1113 ± 35*	1/2	22.7 ± 17.6	0.4 ± 0.3*	118.1 ± 111.4*	91.7 ± 51.3*
HE2300[b] 1X/wk	1/5	1060 ± 0*	0/1	22 ± 0	0.8 ± 0	243 ± 0*	162 ± 0*
HE2300 3X/wk	9/17	1103 ± 34*	2/7	35.9 ± 33.2*	0.8 ± 0.3	170.3 ± 109.3*	155.7 ± 114.9*
Vehicle 3X/wk	1/17	550 ± 0	0/1[c]	17 ± 0	0.7 ± 0	426 ± 0*	307 ± 0*
Untreated	0/5	0	0/0	0	0	0	0
Normal control	5/5	1360 ± 204	5/0	14.4 ± 4.53	0.7 ± 0.11	42 ± 2.13	42 ± 2.13

[a]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[b]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
[c]Histopathological investigation revealed moderate to severe lesions indicative of AD in kidneys and liver (severity score = ++).
*$p < 0.01$ compared to normal control group.

Table 3.3
Detection of viral DNA in mesenteric lymph nodes of AD-infected mink treated subcutaneously with HE2300 or HE2500.

Treatment Group	Pre-Exposure	Post-Exposure[a]	
	Presence of Viral DNA (Positive/total)	Presence of Viral DNA (Positive/total)	Percent Animals Positive for Viral DNA
HE2500[b] 3X/wk	0/5	1/5	20[+]
HE2300[c] 1X/wk	0/5	2/5	40
HE2300 3X/wk	0/17	4/17	18[+]
Vehicle 3X/wk	0/17	12/17	71*
Untreated	0/6	5/6	83*
Normal control	0/5	0/5	0

[a] Represents the lymph node available prior to death or sacrifice of the animal.
[b] Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[c] β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*$p \leq 0.01$ compared to normal control group.
[+]$p \leq 0.01$ compared to infected, vehicle and untreated groups.

Table 3.4
Summary of selected viral, serological and immunological parameters evaluated in HE2300- or HE2500-treated mink infected with ADV and surviving 8 months.

Treatment Group	Survivors	Presence of viral DNA (–/+)	Average spleen weight (g ± SD)	Average CEP titer (± SD)	Average CIC titer (μg E/ml ± SD)	IFN-γ bioactivity assay average Nitrite serum concentration (μM ± SD)
HE2500[a] 3X/wk	3/5	3/0	2.2 ± 0.8	181 ± 129	5.6 ± 1.5	13.8 ± 3.5
HE2300[b] 1X/wk	1/5	1/0	2.9 ± 0	256 ± 0	2.1 ± 0	7.8 ± 0
HE2300 3X/wk	9/17	9/0	2.4 ± 0.6	213 ± 140	2.6 ± 0.1	11.8 ± 3.7
Vehicle 3X/wk	1/17	0/1	5.8 ± 0*	2048 ± 0*	61.6 + 0*	48.3 ± 0*
Untreated	0/5	0/0	0	0	0	0
Normal control	5/5	5/0	2.1 ± 0.5	0	3.8 ± 2.2	8.6 ± 1.6

[a]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[b]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*$p < 0.01$ compared to normal control group.

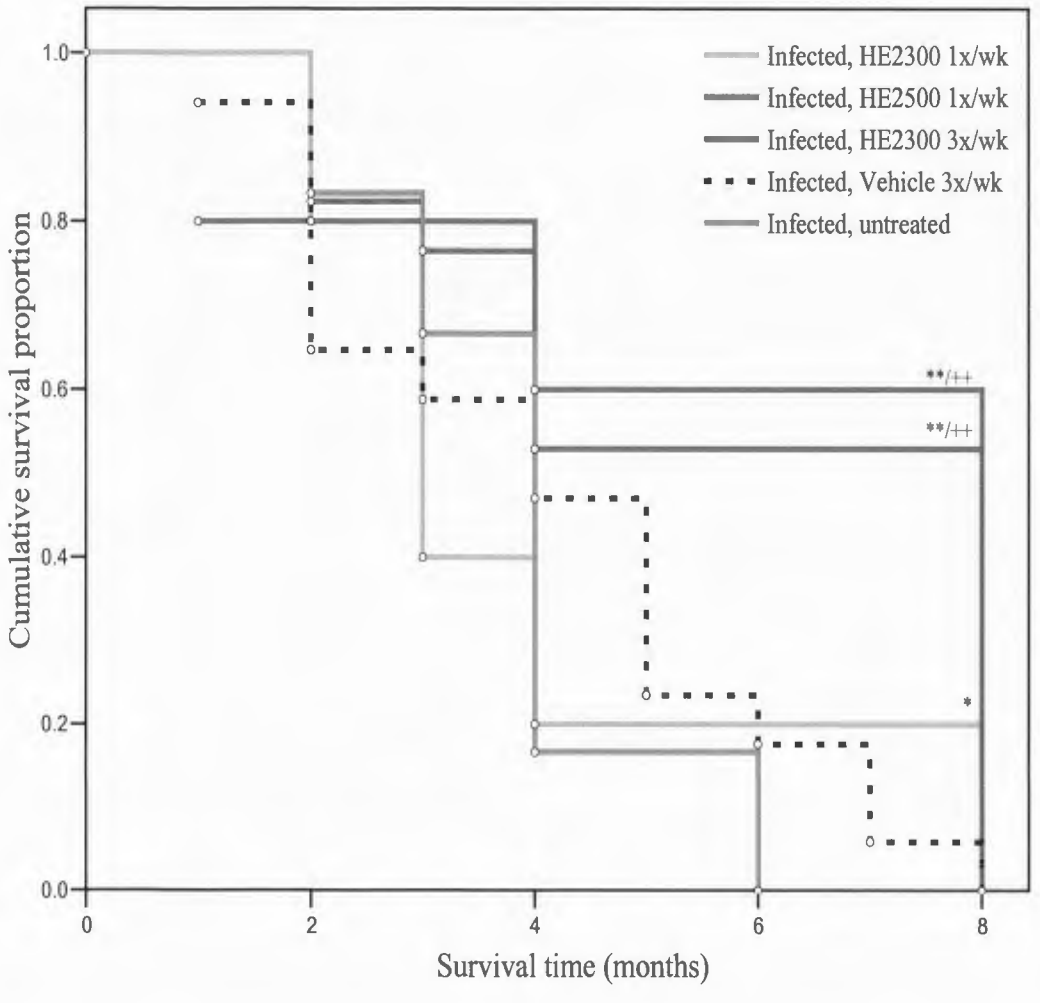


Fig. 3.1. Effects of subcutaneous administration of HE2300 and HE2500 treatment on survival time (Kaplan-Meier analysis) of ADV-infected mink. All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$ compared to infected, no treatment.

**$p < 0.01$ compared to infected, no treatment.

$^{++}p < 0.01$ compared to infected vehicle treated.

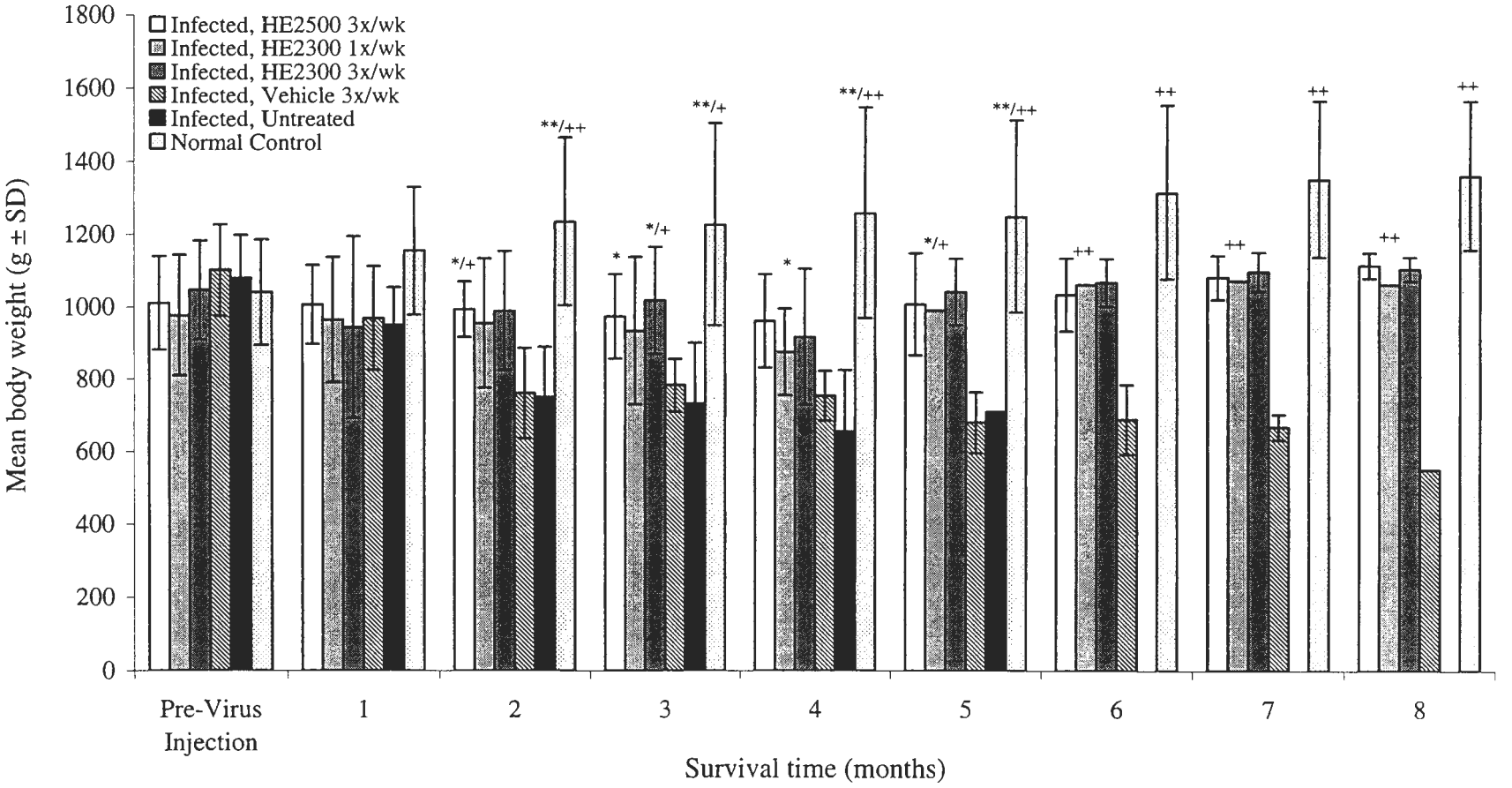
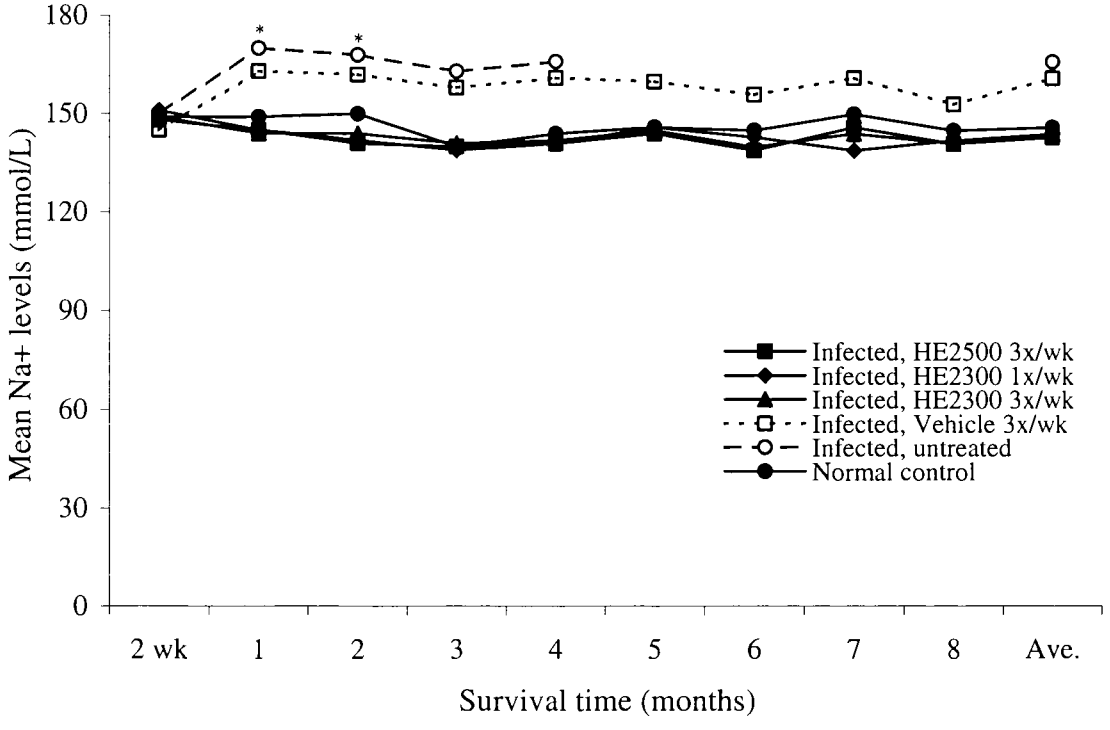


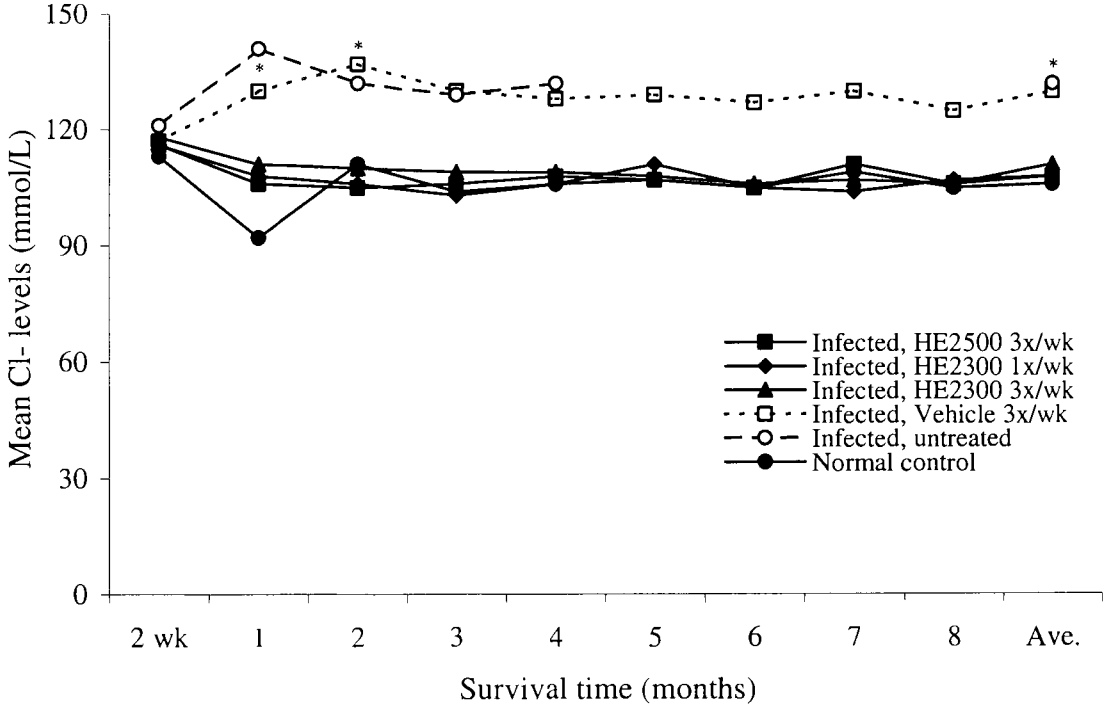
Fig. 3.2. Effect of subcutaneous HE2500 and HE2300 treatment on weight gain of ADV infected mink. All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$, **$p < 0.01$ compared to infected, untreated group; $^{+}p < 0.05$, $^{++}p < 0.01$ compared to infected, vehicle group.

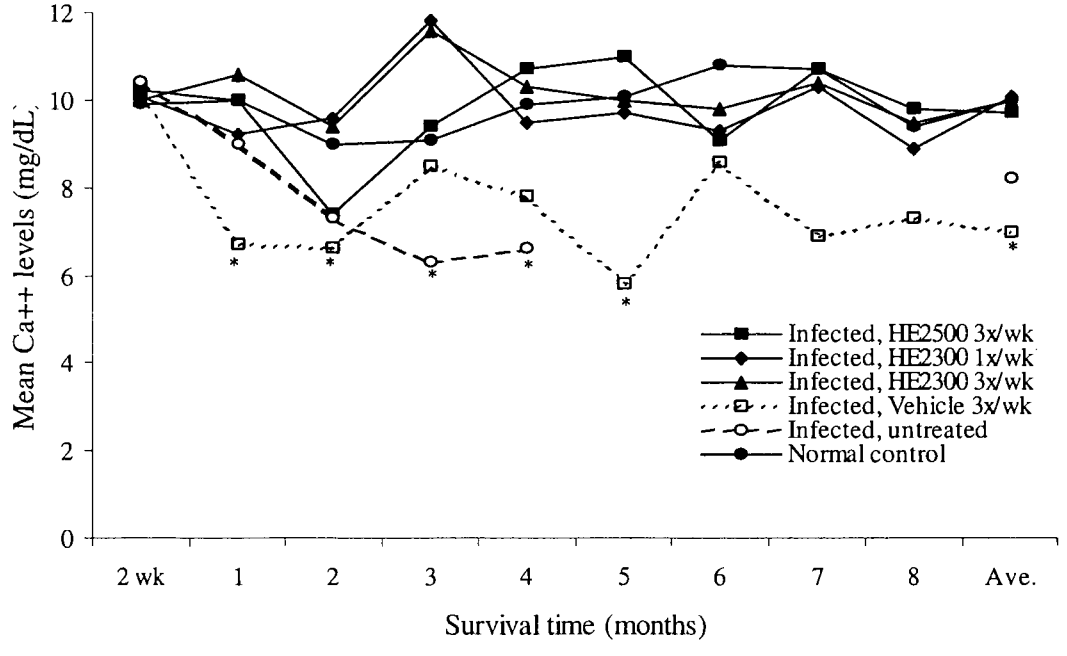
(A)


180
150
120
90
60
30
0
Mean Na+ levels (mmol/L)
2 wk
1
2
3
4
5
6
7
8
Ave.
Survival time (months)
Infected, HE2500 3x/wk
Infected, HE2300 1x/wk
Infected, HE2300 3x/wk
Infected, Vehicle 3x/wk
Infected, untreated
Normal control

(B)


150
120
90
60
30
0
Mean Cl- levels (mmol/L)
2 wk
1
2
3
4
5
6
7
8
Ave.
Survival time (months)
Infected, HE2500 3x/wk
Infected, HE2300 1x/wk
Infected, HE2300 3x/wk
Infected, Vehicle 3x/wk
Infected, untreated
Normal control

(C)



(D)

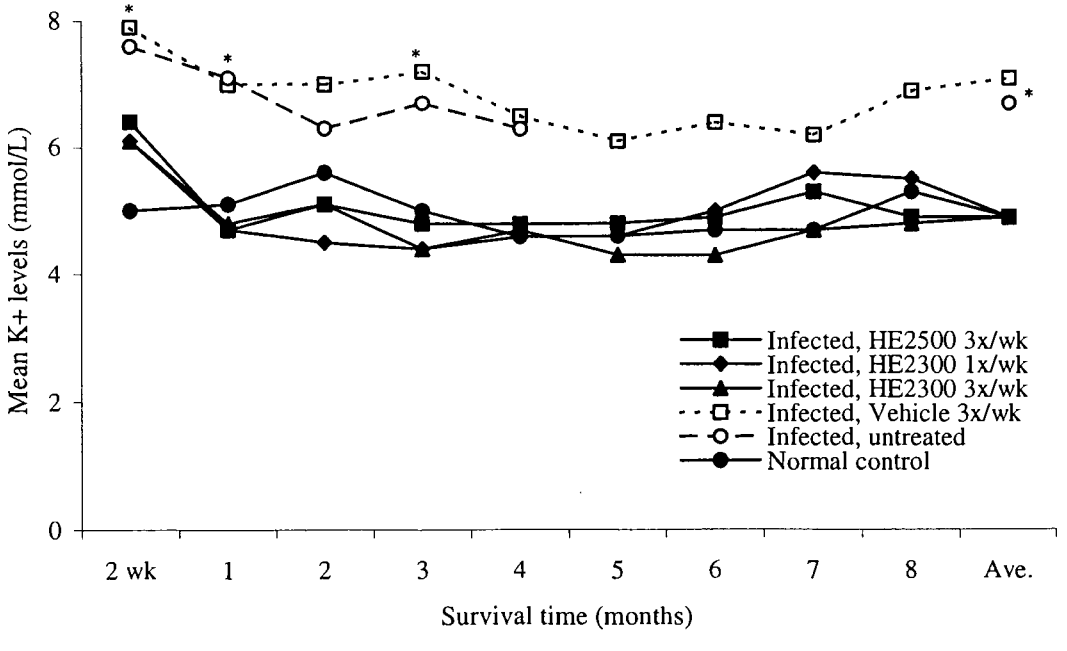
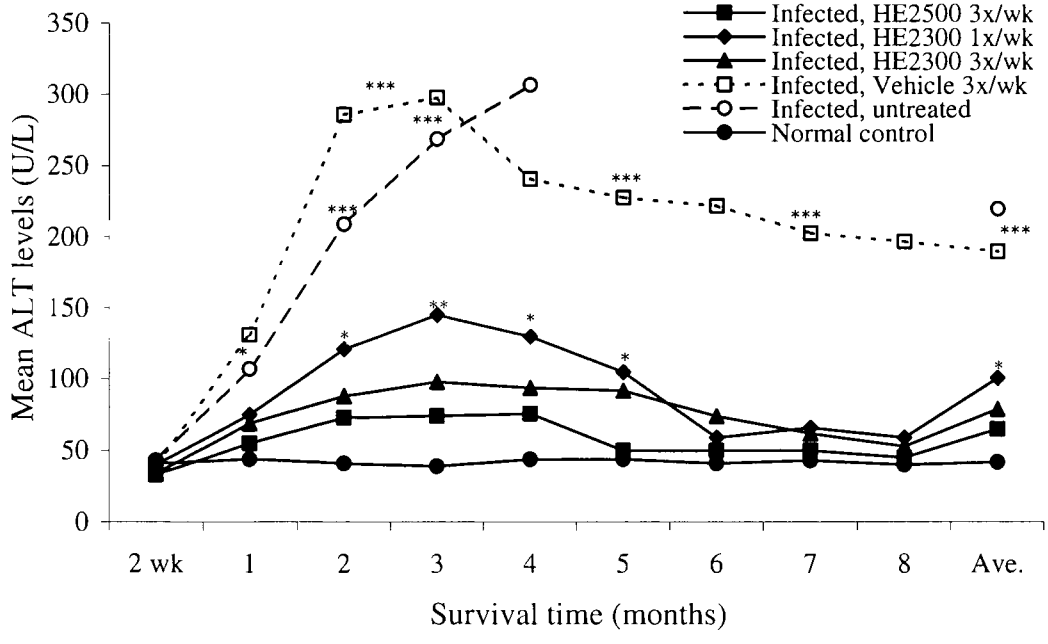


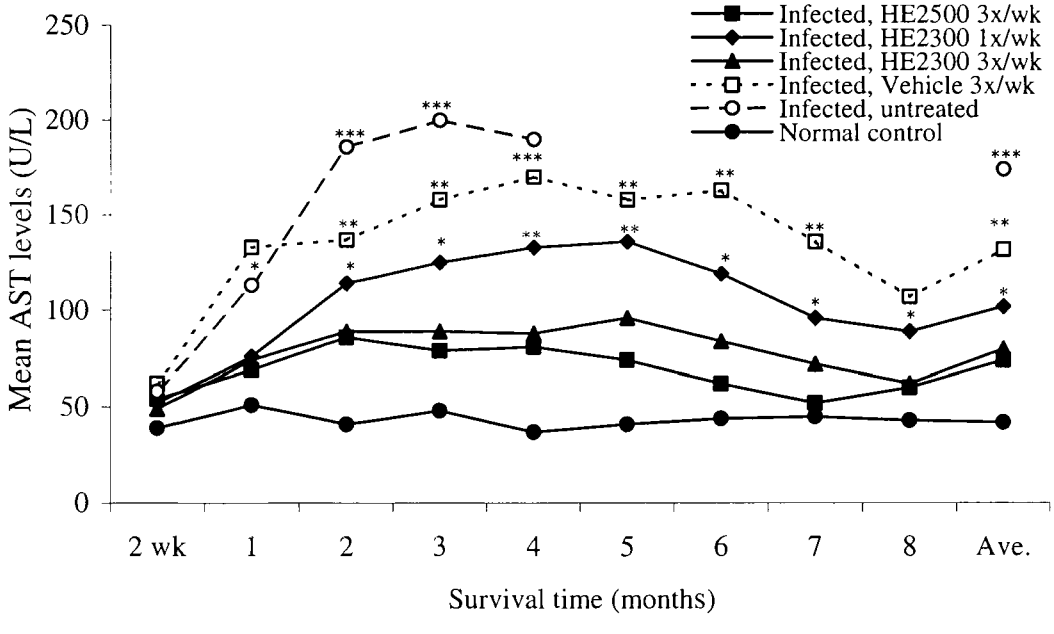
Fig. 3.3. Effects of subcutaneous HE2500 and HE2300 treatment on serum ion levels in ADV-infected mink. Na^+ levels (A), Cl^- levels (B), Ca^{+2} levels (C), and K^+ levels (D). All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$ compared to normal control animals.

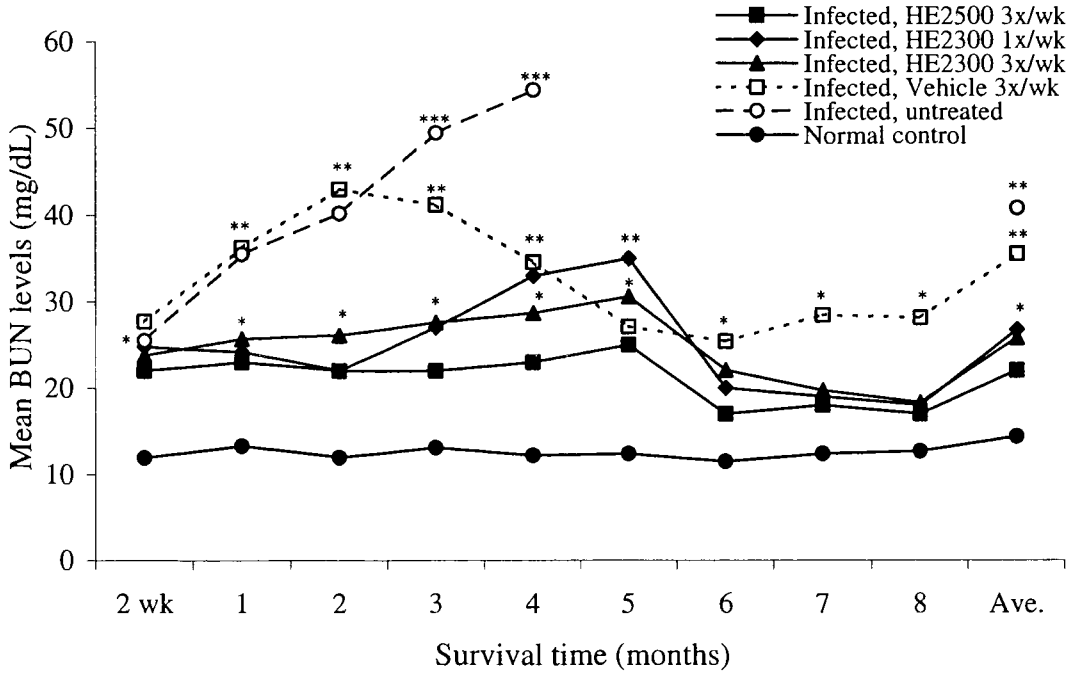
(A)


Infected, HE2500 3x/wk
Infected, HE2300 1x/wk
Infected, HE2300 3x/wk
Infected, Vehicle 3x/wk
Infected, untreated
Normal control
Mean ALT levels (U/L)
350
300
250
200
150
100
50
0
2 wk
1
2
3
4
5
6
7
8
Ave.
Survival time (months)

(B)


Infected, HE2500 3x/wk
Infected, HE2300 1x/wk
Infected, HE2300 3x/wk
Infected, Vehicle 3x/wk
Infected, untreated
Normal control
Mean AST levels (U/L)
250
200
150
100
50
0
2 wk
1
2
3
4
5
6
7
8
Ave.
Survival time (months)

(A)



(B)

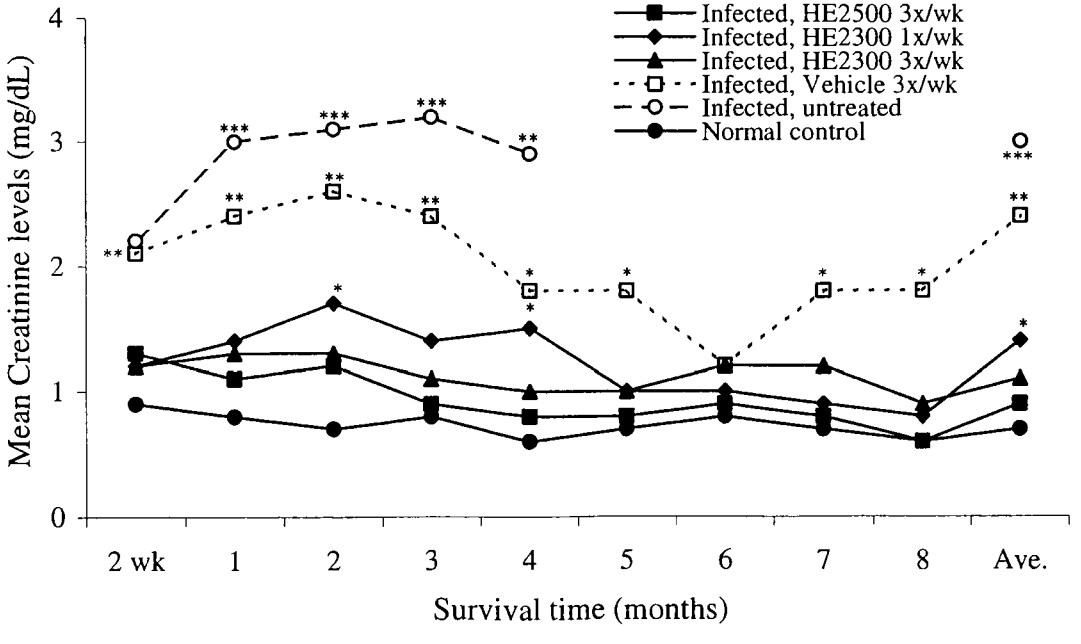


Fig. 3.5. Effects of subcutaneous HE2500 and HE2300 treatment on serum levels of blood urea nitrogen (A) and creatinine (B) in ADV-infected mink. All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to normal control group.

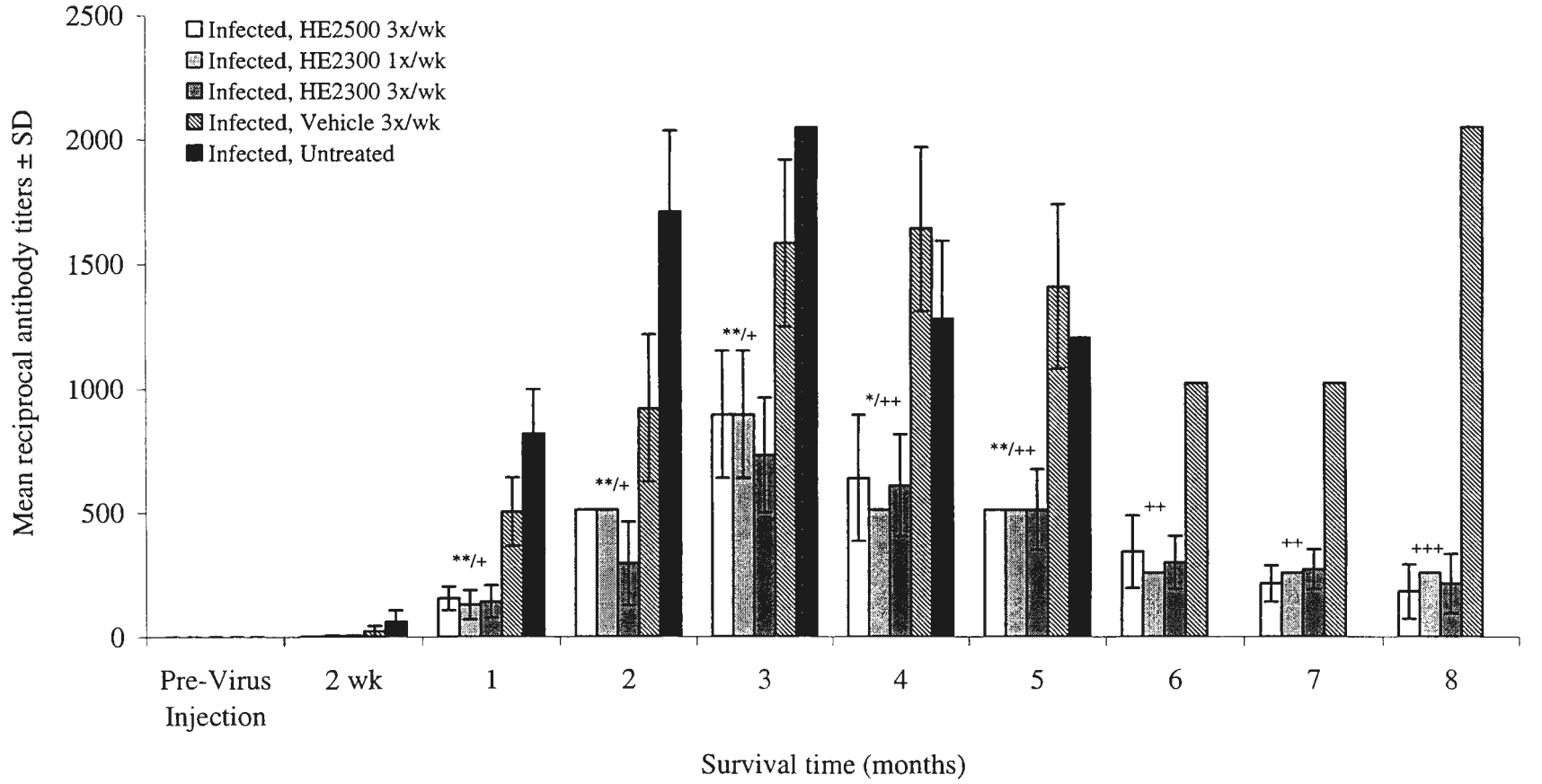


Fig. 3.6. Effect of subcutaneous HE2500 and HE2300 treatment on the anti-ADV antibody titers in mink serum taken after 8 months. All placebo animals were dead after 5 months, generally due to AD.
*$p < 0.05$, **$p < 0.01$ compared to infected, untreated group; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to infected, vehicle group.

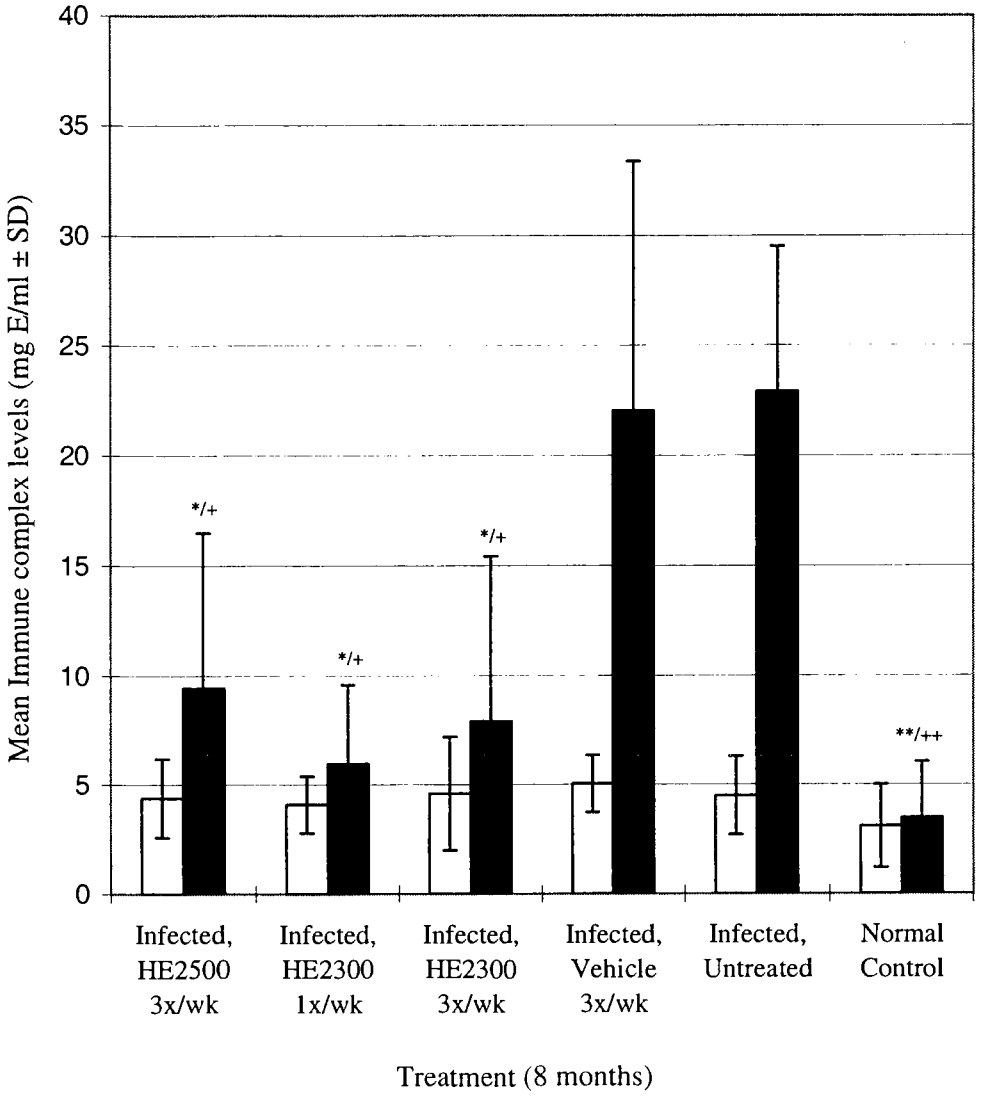


Fig. 3.7. Effects of subcutaneous HE2500 and HE2300 treatment on detectable immune complexes in mink serum taken after 8 months. All placebo animals were dead after 5 months, generally due to AD.

□: Pre-virus injection, ■: Sera from last bleed prior to death or sacrifice
*$p < 0.05$, **$p < 0.01$ compared to infected, untreated group.
$^{+}p < 0.05$, $^{++}p < 0.01$ compared to infected, vehicle group.

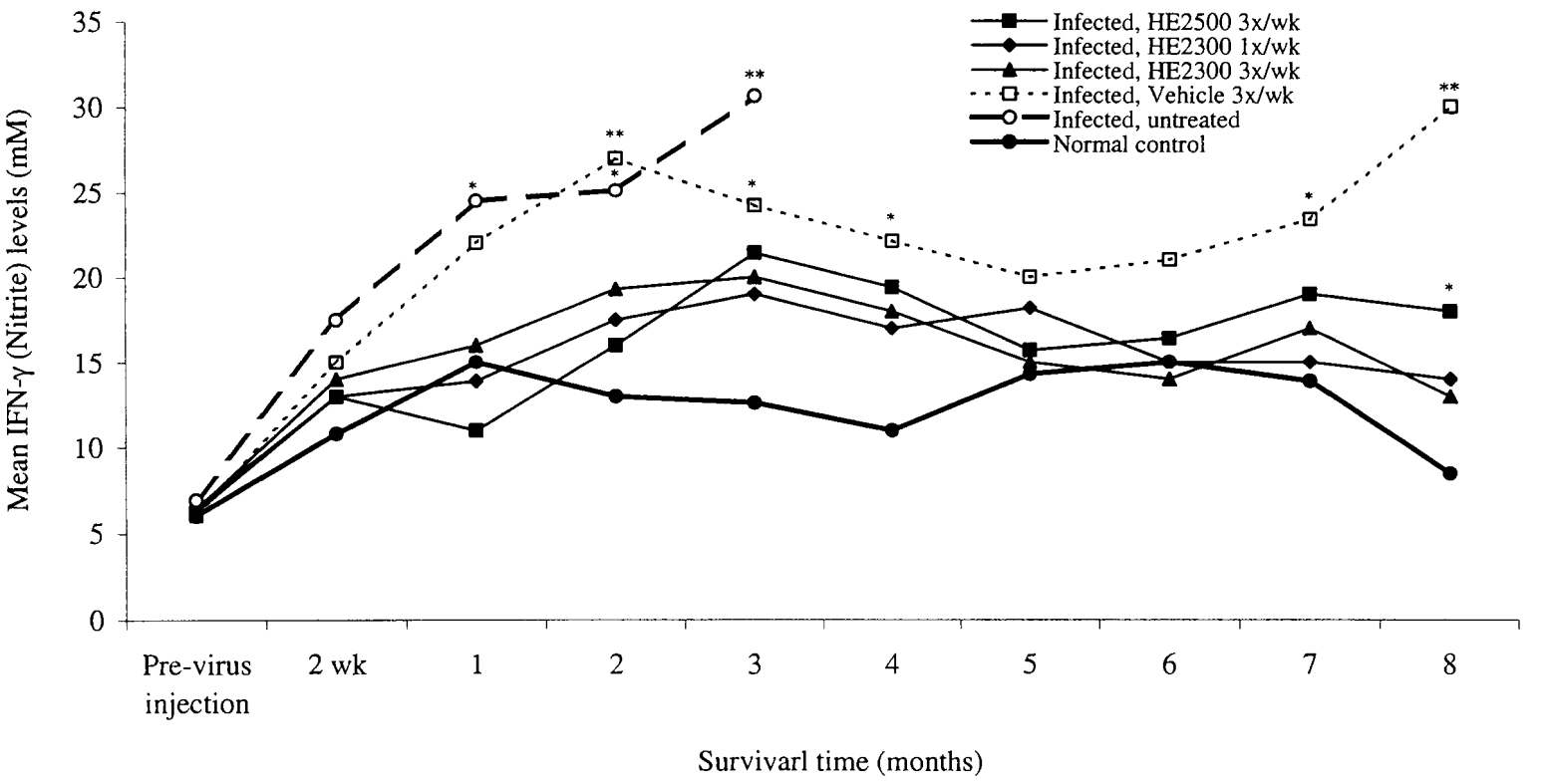


Fig. 3.8. Effects of subcutaneous HE2300 and HE2500 treatment on serum interferon-γ activity of mink exposed to Aleutian disease virus (taken after 8 months). All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$ and **$p < 0.01$ compared to normal control group.

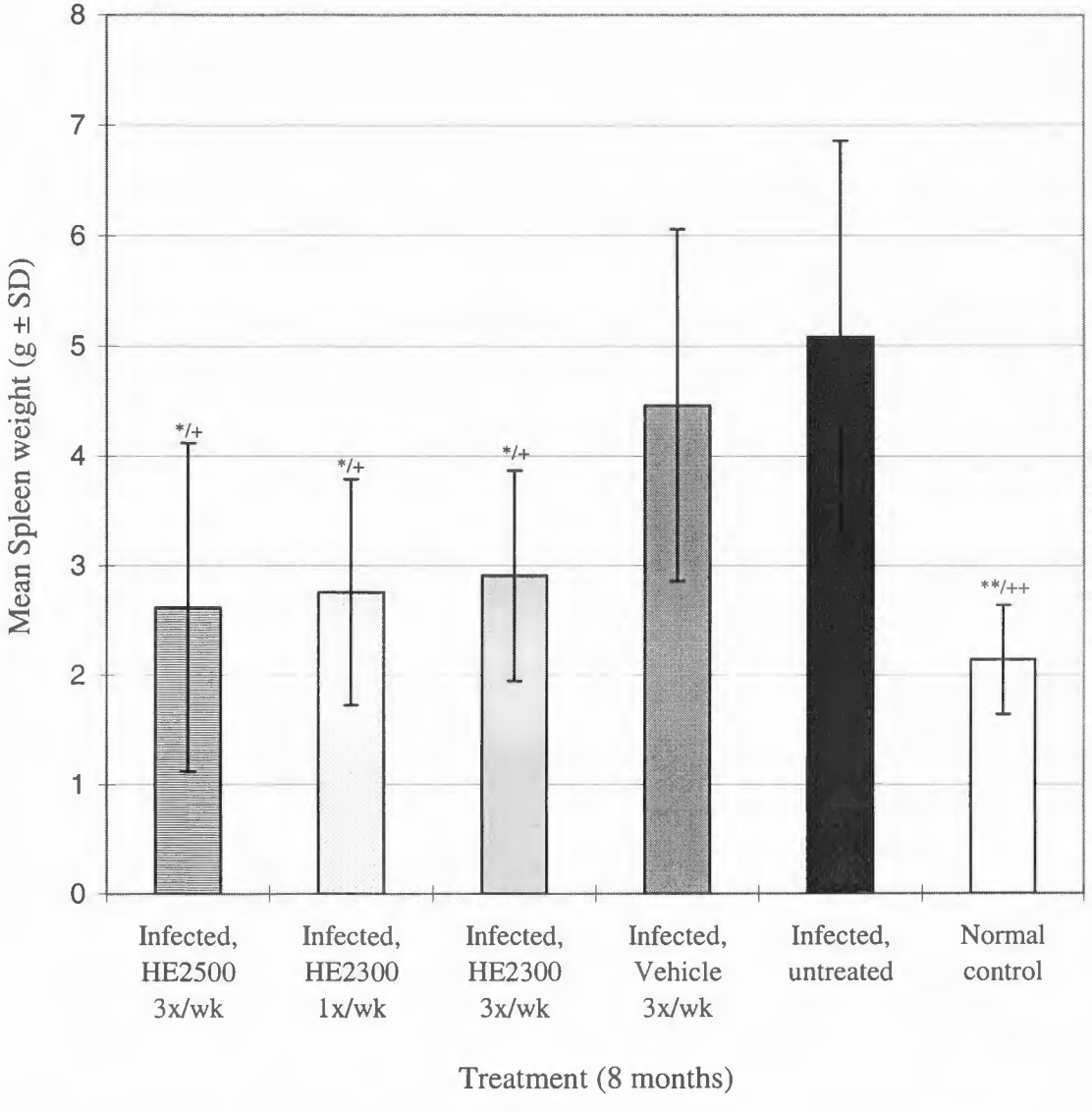


Fig. 3.9. Effects of subcutaneous HE2500 and HE2300 treatment on spleen weight of ADV infected mink. The bars represent the average of the spleen weights of mink at death or sacrifice for each treatment group. All placebo animals were dead after 5 months, generally due to AD.

*$p < 0.05$, **$p < 0.01$ compared to infected, untreated group.

$^{+}p < 0.05$, $^{++}p < 0.01$ compared to infected, vehicle group.

CHAPTER 4

SUMMARY AND CONCLUSIONS

ADV Immunological Response

When ADV is first present in the body, both Th1 and Th2 type of immune responses are activated. As the infection becomes chronic, there is a shift from the Th1 to a Th2. For these studies, the immunological response to AD is overwhelmingly humoral (hypergammaglobulinemia) and mediated by Th2 type cytokines (especially high levels of IL-6). Based on these findings, AD may be a virus-induced cytokine disorder; it is a syndrome of chronic, inappropriate production of IL-6 and perhaps other Th2-type cytokines.

A Th1 immune response is characterized by an inflammatory response with the release of IFN-γ, IL-2, IL-12, TNF-α, β, and activation of macrophages and induction of CD8+ cytolylic T-cells. It is responsible for clearance of viral and intracellular infections.

A Th2 immune response can also mediate an anti-inflammatory responses, but is better characterized by the stimulation of antibody production by B-cells. It does not promote tissue damage as do Th1 cells, which release IL-3, 4, 5, 6, and 10. Th2 predominance leads to the suppression of Th1 cytokines such as IL-12 and IFN-γ.

Thus, if one could modulate the immune response in AD-infected animals to largely cell-mediated response with Th1 type cytokines, the severity of AD might diminished or perhaps even prevented. Previous immunemodulation studies of AD using immunosuppressive therapy has shown slight amelioration of the severity of disease.

Two compounds of interest have been shown to modulate the immune system towards a Th1 response: TGF-β_1 and DHEA; with that in mind we evaluated the effects of exogenous human TGF-β_1 and two DHEA analogs on their ability to diminish the severity of AD mink and enhance survival time in mink infected with AD.

Ameliorating Aleutian Disease in Mink by Treatment with Human rTGF-β_1

Potential Mechanisms of Inhibition

(1) TGF-β_1 may be able to promote Th1 cytokine-type response that could afford protection for mink from chronic lethal AD.

(2) Potent effects on immune cells in vitro are reflected in the various effects TGF-β_1 has in various animal model systems; Inhibition by TGF-β_1 is very important in mediating immune disorders such as autoimmune disease.

(3) A finding pertinent to the potential immunotherapy of AD is that exogenous administration of TGF-β_1 *in vivo* markedly depresses inflammatory and immunological responses (e.g., it delays the onset of experimentally induced arthritis in mice, a disorder involving immune complexe).

(4) TGF-β_1 modulation of Th1 and Th2 immune responses. TGF-β_1 also regulates the primary antigen presenting cell (it is activated T lymphocyte responses that are responsible for correct processing of virus antigen). Thus, it has a regulatory role in the differentiation of T cells (expression of CD8+). It also, it strongly promotes the generation of Th1 cells. Thus, it is conceivable that TGF-β_1 could

elicit a Th1 response adequate enough to ameliorate AD by inducing appropriate processing of AD antigen.

(5) TGF-β_1 inhibition of to B cell proliferation. TGF-β_1 inhibits B cell proliferation and is able to induce apoptosis (programmed cell death) in B cells in addition to reducing the secretion of immunoglobulin from activate B cells. These mechanisms suggest that TGF-β_1 may be able down-regulate a B cell hyper response such as seen in mink with AD.

(6) TGF-β_1 inhibition of antibody class switching. Early hematopietic growth and differentiation alters B cell Ig production and class switching, and inhibits mast cell, thymocyte, B cell and T lymphocyte proliferation

Summary of Results Nine Months (266 days)

after Exposure to ADV

(1) TGF-β_1 treated animals had an increase of nearly two-fold in survival time compared to infected placebo-treated or untreated animals (Table B.1).

(2) TGF-β_1 treated animals had an increase of nearly two-fold in body weight compared to infected untreated animals (Table B.2).

(3) TGF-β_1 treated animals showed a dramatic decrease in ADV-specific antibody titers compared to placebo-treated or untreated infected animals. BUN, IFN-γ, and CIC levels were well within normal parameters (Table B.3, B.4, and B.5), .

(4) TGF-β_1 treated animals had a two-fold increase body weight compared to infected placebo-treated or untreated animals, although they lost weight compared to their initial weight.

Conclusions: *Analysis of the Mink Treated with TGF-β_1*

(1) TGF-β_1 at 100 or 200 ng/kg regimen was most effective in treating ADV-infected mink; both doses appeared to delay the progression of the disease (Table B.6 and B.7).

(2) TGF-β_1 at 1 ng/kg regimen failed to protect most mink from lethal chronic AD.

(3) Treatment with LAPrhTGF-β_1-inhibitor at 500 ng/kg regimen resulted in high physical, physiological and immunological parameter levels in the AD-infected mink; the kidneys were damaged by immune complex deposition, a hallmark of AD.

(4) These findings support the hypothesis that TGF-β_1 treatment of mink by inguinal vein injection can ameliorate AD in mink.

Effects of HE2300 and HE2500 on the Outcome of Aleutian Disease in Mink

Potential Mechanisms of Inhibition

(1) DHEA, an epiandsterone increases the levels of Th1 cytokines, IL-2, IL-3, and IFN-γ and counteracts hydrocortisone-mediated immune suppression that could afford protection for mink from chronic lethal AD.

(2) HE2300 (β-AET), a natural androstene, up-regulates immunity, which increases resistance against lethal infection and lethal radiation, and mediates a rapid recovery of hematopoietic precursor cell after radiation injury

(3) HE 2300 protects the host from lethal infection by DNA or RNA viruses such as herpesvirus type 2, coxsackie virus B4, influenza, and arthropod borne viruses. In

addition, mortality from a lethal challenge of human enteric virus was reduced by 50% when animals were treated with HE2300.

(4) DHEA has been shown to have general immunomodulating effects in experimental infections involving viruses.

(5) DHEA shifts cytokine production to a Th1 response and increases circulating activated immune effects or cells in HIV infections.

Summary of Results Eight Months After Exposure to ADV

(1) HE2500 and 2300 (3x/week) treatment increased survival time and body weight nearly two-fold compared to infected, untreated animals (Table C.2).

(2) HE2500 and 2300 (3x/week) treated animals showed a dramatic decrease in ADV-specific antibody titers, and BUN, creatinine, ALT, AST, IFN-γ, and CIC levels werelowered in treated animals (Table C.3, C.4, and C.5).

(3) In contrast, untreated and placebo-treated animals had high antibody titers and high BUN, creatinine, ALT, AST, IFN-γ, and CIC levels, suggesting immune complex deposition, a hallmark of AD. This was supported by histopathological examination of the kidney and liver tissues of the mink.

(4) Spleen weights of untreated and placebo-treated animals increased by two-fold compared to treated animals and control mink.

(5) PCR assay of lymph node tissue demonstrated that virus DNA could be detected in 83% of the untreated and placebo-treated AD-infected mink, whereas it was only detected in 18% of drug-treated animals (Fig. C.1).

Conclusions: Analysis of The Mink Treated with HE2300 and HE2500

(1) A three times per week dosage regimen of HE2500 and 2300 at 16 ng/kg dosage was the most effective means of treating ADV-infected mink; both compounds appeared to delay the progression of the disease.

(2) A one time per week regimen of HE2300 failed to protect most mink from lethal chronic AD.

(3) The kidneys and liver of vehicle were damaged by immune complex deposition, a hallmark of AD.

(4) These findings tend to support the hypothesis that β-androstene analog treatment of mink by subcutaneous injection can ameliorate Aleutian disease in mink.

Summary of Conclusion for Both Treatments (TGF-β_1 and HE 2300/ 2500)

(1) DHEA (analogs) and TGF-β_1 may be able to promote Th1 cytokine-type response that could afford protection for mink from chronic lethal AD.

(2) TGF-β_1 at 200 and 100 ng/kg treatment temporarily slowed down the progression of the disease.

(3) A three times per week regimen of HE2500 and 2300 was effective in treating ADV-infected mink; both compounds temporarily halted or slowed down the progression of the disease.

(4) These findings support the hypothesis tested in this research that TGF-β_1 treatment of mink by inguinal vein injection and β-androstene analog-treatment of mink by subcutaneously injection can ameliorate Aleutian disease in mink.

APPENDICES

Appendix A. Experimental Methods of Ameliorating Aleutian Disease in Mink by Treatment with Human rTGF-β_1

Table A.1.
Study Design for TGF-β_1 treatment of ADV-infected mink.

Treatment	Treatment Group				Total
	Group 1 Mink injected 2 weeks Pre-virus inoculation	Group 2 Mink injected 8 h Post-virus inoculation	Group 3 Mink injected 2 weeks Post-virus inoculation	Group 4 Mink injected 3 treatments[a]	
TGF-β_1 200 ng/kg	5	5	5	5	**20**
TGF-β_1 100 ng/kg	5	5	5	0	**15**
TGF-β_1 1 ng/kg	5	5	5	0	**15**
TGF-β_1-Inhibitor[b]	5	5	5	0	**15**
Placebo[c]	5	5	5	0	**15**
Total	**25**	**25**	**25**	**5**	**80**

[a]2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
[b]Latency associated peptide, recombinant human (LAPrhTGF-β_1 at 500 ng/kg).
[c]Control; PSS 0.1% BSA.

(A)

(B)

(C)

Fig. A.1. These pictures show mink farm.

(A)

(B)

(C)

Fig. A.2. These pictures depict blood collection method.

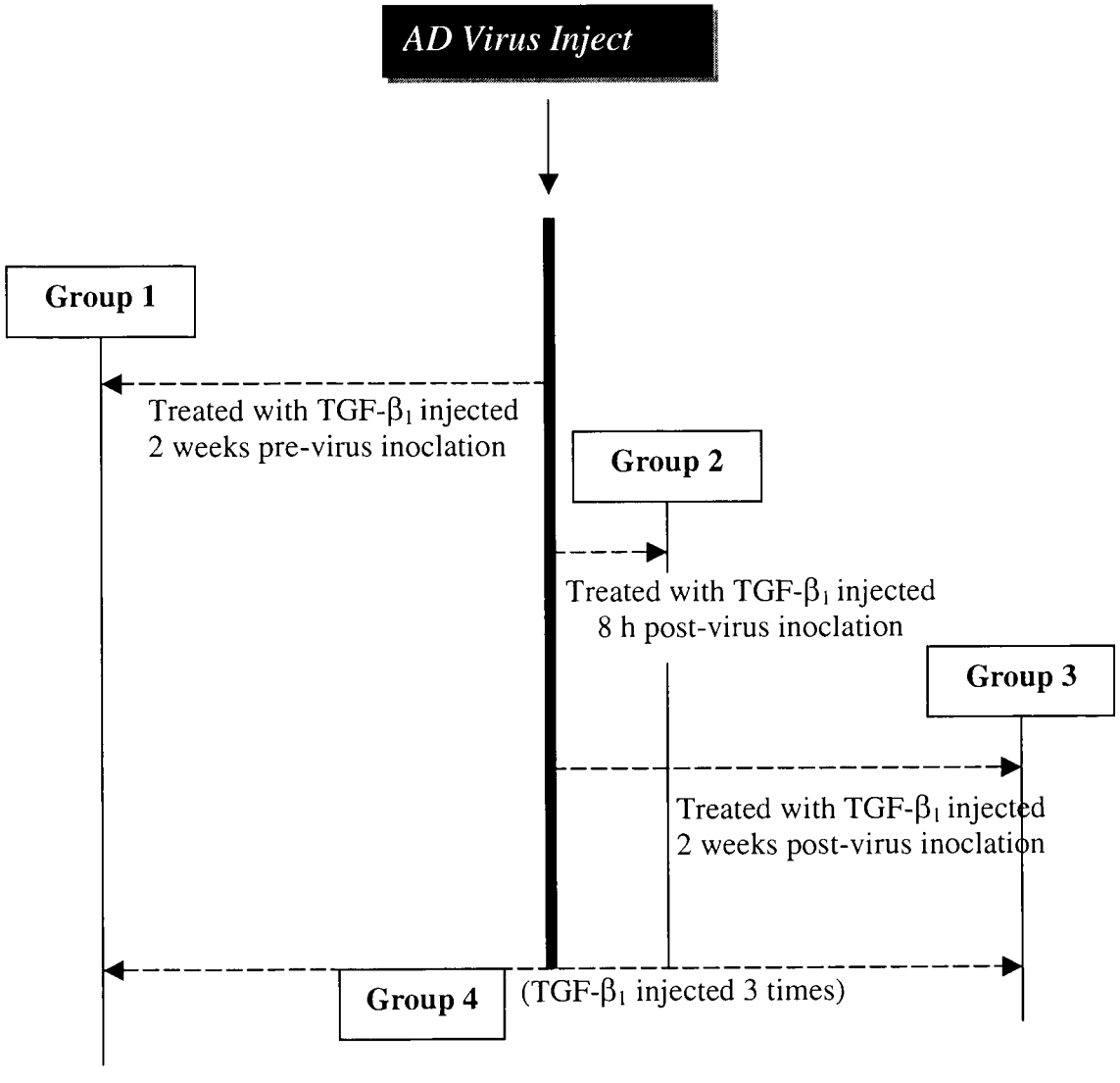


Fig. A.3. TGF-β_1 injection time interval. All animals were injected intraperitoneally (IP) with a 1: 500 dilution of Aleutian disease virus of mink (strain Utah I) in 0.1% PSS and each mink receiving 1ml viral dilution mixture per kilogram body mass.

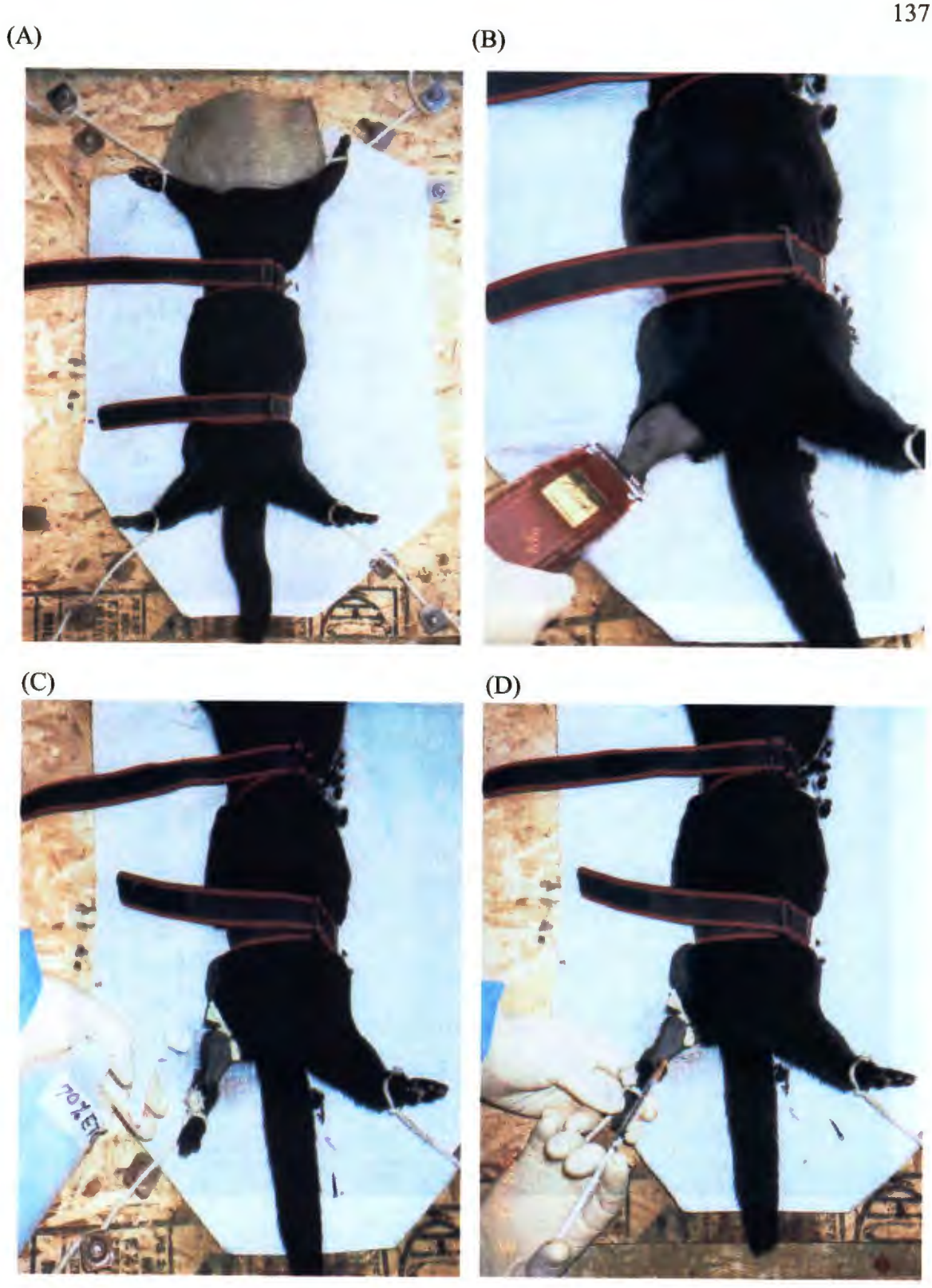
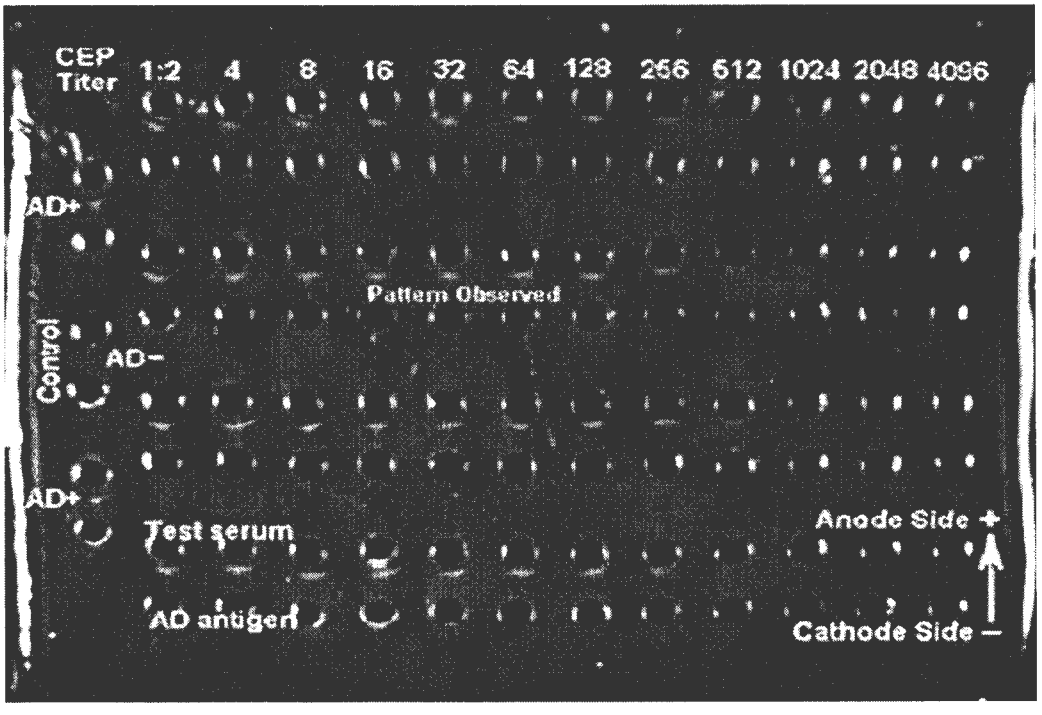


Fig. A.4. These figures show TGF-β_1 injection method.

(A)



(B)

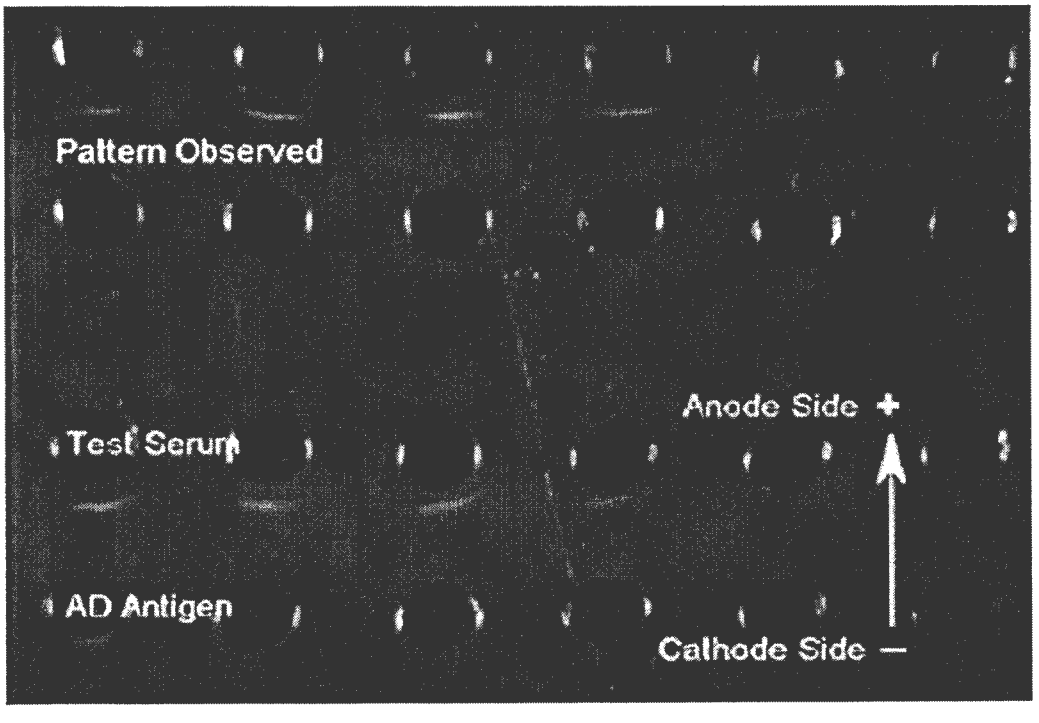


Fig. A.5. These figures depict CEP assay. Runs 1% agarose gel, 55V, 1 hours.

Appendix B. Tables and figures of Ameliorating Aleutian Disease in Mink by Treatment with Human rTGF-β.

Table B.1
Effect of intravenous TGF-β_1 treatment on survival time of mink with Aleutian disease.

Group No.	Treatment	Survivors	Mean Survival Time [a] (days ± SD)
Group 1	TGF-β_1 200 ng/kg	3/5*	207 ± 21*
(treated 2 weeks	TGF-β_1 100 ng/kg	3/5*	221 ± 26*
pre-virus	TGF-β_1 1 ng/kg	1/5	170 ± 31
inoculation)	TGF-β_1-Inhibitor	0/5	116 ± 9**
	Placebo	0/5	149 ± 8
Group 2	TGF-β_1 200 ng/kg	3/5*	204 ± 9*
(treated 8 h	TGF-β_1 100 ng/kg	3/5*	247 ± 17*
post-virus	TGF-β_1 1 ng/kg	1/5	170 ± 38
inoculation)	TGF-β_1-Inhibitor	0/5	123 ± 12
	Placebo	0/5	120 ± 25
Group 3	TGF-β_1 200 ng/kg	2/5	200 ± 33*
(treated 2 weeks	TGF-β_1 100 ng/kg	3/5*	223 ± 28**
post-virus	TGF-β_1 1 ng/kg	1/5	179 ± 31
inoculation)	TGF-β_1-Inhibitor	0/5	135 ± 26
	Placebo	0/5	129 ± 24
Group 4	TGF-β_1 200 ng/kg	3/5*	248 ± 31**
(3 treatments)[b]	Placebo	0/5	133 ± 19

[a]Animals dying prior to day 266 days
[b]TGF-β_1 was administered 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
*$p < 0.05$ compared to placebo-treated controls.
**$p < 0.01$ compared to placebo-treated controls.

Table B.2
Effect of intravenous TGF-β_1 treatment on body weights of mink with Aleutian disease.

	Mean Body Weight (g ± SD)							
	Group 1 (treated 2 weeks pre-virus inoculation)		Group 2 (treated 8 h post-virus inoculation)		Group 3 (treated 2 weeks post-virus inoculation		Group 4 (3 treatments)[a]	
Treatment	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure
TGF-β_1 200 ng/kg	1040 ± 33	828 ± 87*	1035 ± 17	808 ± 65*/$^+$	1028 ± 52	788 ± 103*	1073 ± 53	969 ± 103*
TGF-β_1 100 ng/kg	1040 ± 84	820 ± 114*	1008 ± 38	883 ± 94*	1024 ± 58	853 ± 100*	ND[c]	
TGF-β_1 1 ng/kg	1038 ± 48	730 ± 120*	1025 ± 68	765 ± 145	1040 ± 52	655 ± 53*/$^+$	ND[c]	
TGF-β_1-Inhibitor	1050 ± 67	340 ± 22$^+$	1075 ± 73	373 ± 22*/$^+$	1050 ± 53	380 ± 37$^+$	ND[c]	
Placebo	1080 ± 37	339 ± 24$^+$	1025 ± 66	470 ± 60$^+$	1033 ± 43	365 ± 39$^+$	1046 ± 49	391 ± 41$^+$

[a]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
[b]Represents the last weight available prior to death or sacrifice of the animal.
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.
$^+p \leq 0.01$ compared to pre- vs. post-virus exposure.

Table B.3
Effect of intravenous TGF-β_1 treatment on the anti-ADV antibody titers of mink with Aleutian disease.

Treatment	Mean Reciprocal Antibody Titers[a] ± SD			
	Group 1 (treated 2 weeks pre-virus inoculation)	Group 2 (treated 8 h post-virus inoculation)	Group 3 (treated 2 weeks post-virus inoculation	Group 4 (3 treatments)[b]
TGF-β_1 200 ng/kg	358 ± 64**	410 ± 64**	307 ± 57**	256 ± 29**
TGF-β_1 100 ng/kg	307 ± 57**	218 ± 43**	327 ± 88**	ND[c]
TGF-β_1 1 ng/kg	614 ± 150*	563 ± 140**	666 ± 128*	ND[c]
TGF-β_1-Inhibitor	1126 ± 280	1024 ± 0	922 ± 115	ND[c]
Placebo	1024 ± 0	922 ± 115	1126 ± 280	1024 ± 132

[a]Represents data from the last serum sample taken prior to death or sacrifice of the animal.
[b]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.
**$p \leq 0.01$ compared to placebo-treated controls.

Table B.4
Effect of intravenous TGF-β_1 treatment on blood urea nitrogen (BUN) levels in mink with Aleutian disease.

	Mean BUN levels (mg/dL ± SD)							
	Group 1 (treated 2 weeks pre-virus inoculation)		Group 2 (treated 8 h post-virus inoculation)		Group 3 (treated 2 weeks post-virus inoculation		Group 4 (3 treatments)[a]	
Treatment	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure
TGF-β_1 200 ng/kg	6.21 ± 1.23	17.86 ± 4.90*	6.83 ± 3.68	23.10 ± 5.20*	8.30 ± 2.46	22.77 ± 4.47*	7.57± 2.41	10.42 ± 2.73*
TGF-β_1 100 ng/kg	5.15 ± 1.29	14.14 ± 3.51*	7.98 ± 1.66	15.25 ± 3.40*	8.07 ± 1.23	15.08 ± 6.96*		ND[c]
TGF-β1 1 ng/kg	5.46 ± 2.38	31.32 ± 6.96*/$^+$	6.75 ± 1.76	33.15 ± 11.84	8.54 ± 2.54	29.15 ± 8.15*		ND[c]
TGF-β_1-Inhibitor	7.08 ± 1.23	84.07 ±17.43$^+$	7.53 ± 1.45	73.15 ± 18.3$^+$	5.39 ± 1.69	61.81 ± 15.74$^+$		ND[c]
Placebo	5.48 ± 2.02	63.83 ± 12.68$^+$	5.29 ± 1.64	56.59 ± 9.20$^+$	7.70 ± 1.98	67.55 ± 5.03$^+$	6.16± 1.88	62.66 ± 8.98$^+$

[a]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation
[b]Represents data from the last serum sample taken prior to death or sacrifice of the animal.
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.
$^+p \leq 0.05$ compared to pre- vs. post-virus exposure.

Table B.5
Effect of intravenous TGF-β_1 treatment on serum interferon gamma (IFN-γ) levels in mink with Aleutian disease.

	Mean Interferon-γ levels (μM ± SD)							
	Group 1 (treated 2 weeks pre-virus inoculation)		Group 2 (treated 8 h post-virus inoculation)		Group 3 (treated 2 weeks post-virus inoculation		Group 4 (3 treatments)[a]	
Treatment	Pre-virus Exposure	Post-virus Exposure[b]	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure	Pre-virus Exposure	Post-virus Exposure
TGF-β_1 200 ng/kg	6.36 ± 0.86	18.13 ± 3.31*/$^+$	7.08 ± 1.06	15.48 ± 5.60*	6.76 ± 2.76	16.63 ± 2.42*/$^+$	6.18 ± 1.77	15.96 ± 4.81*
TGF-β_1 100 ng/kg	5.86 ± 1.10	11.97 ± 1.74*/$^+$	7.26 ± 1.71	17.60 ± 4.90*/$^+$	8.01 ± 2.20	16.89 ± 3.60*/$^+$		ND[c]
TGF-β1 1 ng/kg	5.64 ± 0.84	24.99 ± 4.20$^+$	6.25 ± 1.84	25.03 ± 5.32*/$^+$	7.06 ± 2.16	23.90 ± 8.76$^+$		ND[c]
TGF-β_1-Inhibitor	5.41 ± 0.96	40.93 ± 6.99$^+$	6.81 ± 1.85	39.83 ± 6.22$^+$	6.22 ± 1.60	50.41 ± 10.99$^+$		ND[c]
Placebo	6.05 ± 1.51	31.60 ± 2.81$^+$	5.81 ± 1.27	37.29 ± 4.24$^+$	6.65 ± 1.81	42.00 ± 11.26$^+$	6.19 ± 1.53	36.96 ± 6.10$^+$

[a]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation
[b]Represents data from the last serum sample taken prior to death or sacrifice of the animal.
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.
$^+p \leq 0.05$ compared to pre- vs. post-virus exposure.

Table B.6
Effect of intravenous TGF-β_1 treatment on average of serum TGF-β_1 levels in mink.

	Mean TGF-β_1 levels (ng/ml ± SD)[a]							
	Group 1 (treated 2 weeks pre-virus inoculation)		Group 2 (treated 8 h post-virus inoculation)		Group 3 (treated 2 weeks post-virus inoculation		Group 4 (3 treatments)[b]	
Treatment	Baseline levels	Ave.	Baseline levels	Ave.	Baseline levels	Ave.	Baseline levels	Ave.
TGF-β_1 200 ng/kg	1.08	0.95 ± 0.07*	0.78	0.88 ± 0.11*	0.79	0.84 ± 0.11*	1.06	1.07 ± 0.26*
TGF-β_1 100 ng/kg	1.02	0.91 ± 0.06*	0.78	0.88 ± 0.07*	0.77	0.84 ± 0.06*	ND[c]	
TGF-β1 1 ng/kg	0.82	0.77 ± 0.02	0.78	0.77 ± 0.03	0.79	0.77 ± 0.03	ND[c]	
TGF-β_1-Inhibitor	0.58	0.55 ± 0.07*	0.78	0.67 ± 0.03	0.80	0.62 ± 0.11*	ND[c]	
Placebo	0.87	0.73 ± 0.05	0.80	0.73 ± 0.04	0.77	0.74 ± 0.02	1.06	0.73 ± 0.04

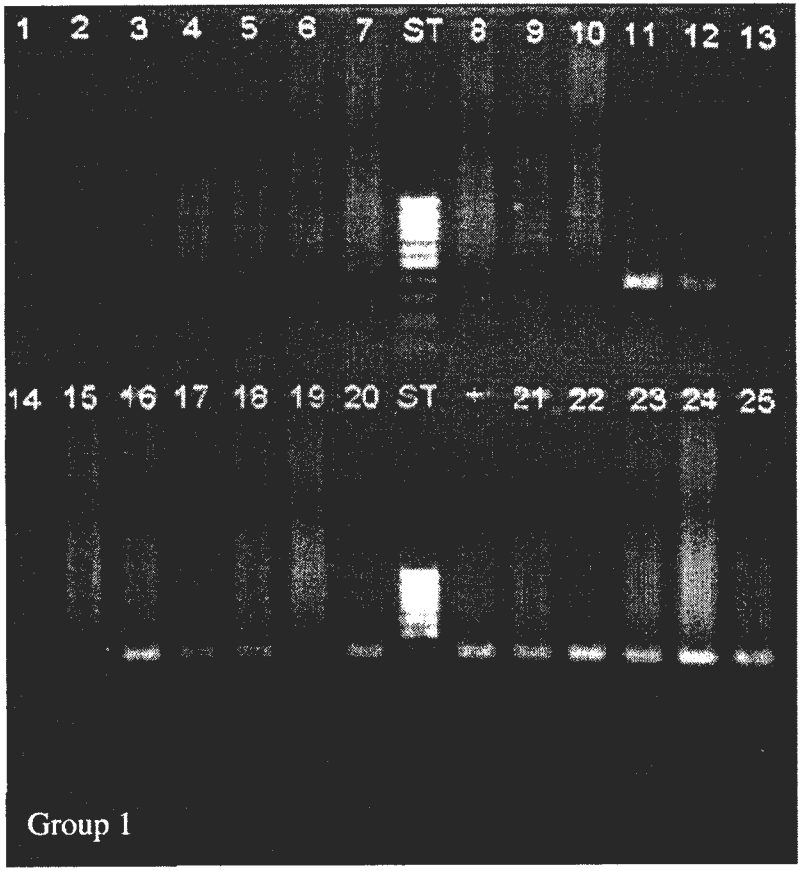
[a]Mean levels after death or at sacrifice.
[b]TGF-β_1 was administered sequentially at 2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
[c]ND = Not Done
*$p \leq 0.05$ compared to placebo-treated controls.

Table B.7
Effect of intravenous TGF-β_1 treatment on serum TGF-β_1 levels in mink.

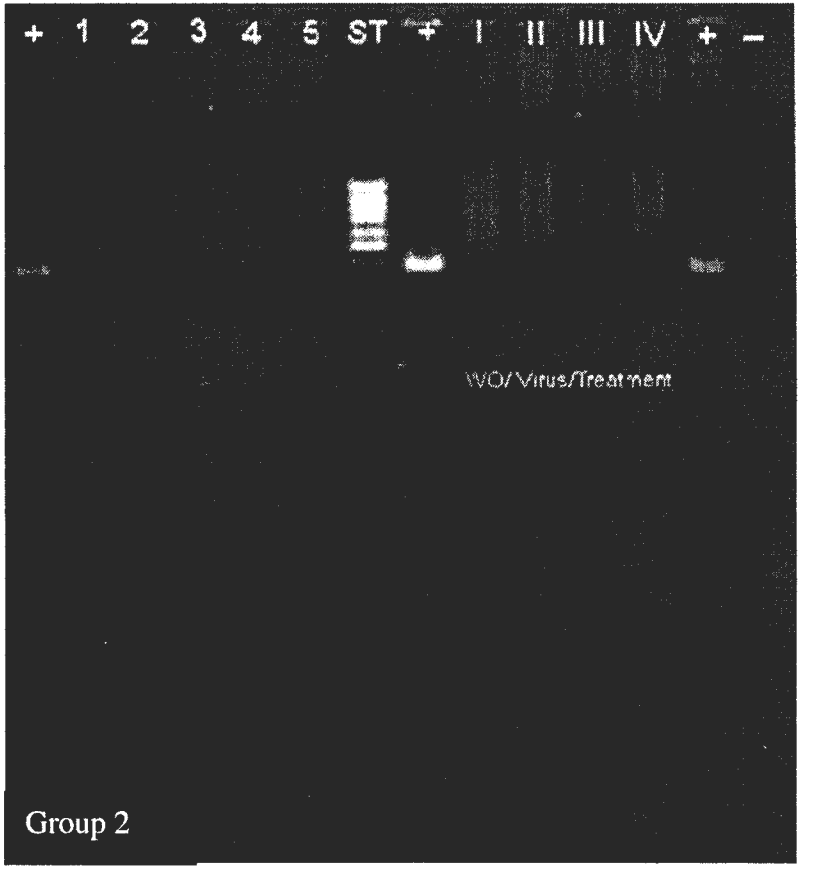
		Mean TGF-β_1 levels (ng/ml ± SD)									
		Pre-virus		Time months virus exposure							
Group No.	Treatment	Exposure	8 h	1	2	3	4	5	6	7	8
Group 1	TGF-β_1 200 ng/kg	1.08±0.16	1.04±0.10	0.98±0.03	0.95±0.08	0.95±0.07	0.91±0.06	0.91±0.10	0.93±0.03	0.83±0.00	0.92±0.00
(2 weeks	TGF-β_1 100 ng/kg	1.02±0.11	1.02±0.11	0.92±0.01	0.92±0.05	0.90±0.04	0.88±0.03	0.91±0.04	0.89±0.09	0.85±0.03	0.85±0.03
pre-virus	TGF-β_1 1 ng/kg	0.82±0.08	0.79±0.08	0.78±0.07	0.78±0.10	0.76±0.08	0.77±0.05	0.77±0.09	0.77±0.04	0.74±0.08	0.76±0.07
inoculation)	TGF-β_1-Inhibitor	0.58±0.05	0.54±0.21	0.55±0.08	0.57±0.10	0.51±0.03	0.52±0.00	–[a]	–	–	–
	Placebo	0.81±0.08	0.70±0.08	0.78±0.08	0.75±0.09	0.73±0.05	0.72±0.04	0.64±0.00	–	–	–
Group 2	TGF-β_1 200 ng/kg	0.79±0.08	1.09±0.07	1.00±0.04	0.93±0.07	0.87±0.10	0.83±0.09	0.85±0.03	0.82±0.06	0.75±0.00	–
(8 h post-	TGF-β_1 100 ng/kg	0.78±0.06	1.00±0.12	0.97±0.10	0.91±0.10	0.89±0.12	0.91±0.09	0.86±0.10	0.78±0.06	0.84±0.02	0.82±0.07
virus	TGF-β_1 1 ng/kg	0.78±0.05	0.79±0.09	0.78±0.07	0.76±0.06	0.81±0.05	0.82±0.05	0.79±0.06	0.74±0.06	0.72±0.03	0.75±0.06
inoculation)	TGF-β_1-Inhibitor	0.78±0.05	0.69±0.09	0.68±0.07	0.66±0.06	0.61±0.05	0.62±0.05	0.69±0.06	0.64±0.06	–	–
	Placebo	0.80±0.09	0.76±0.09	0.73±0.04	0.68±0.02	0.72±0.0 5	0.67±0.00	–	–	–	–
Group 3	TGF-β_1 200 ng/kg	0.79±0.09	0.78±0.09	1.07±0.10	0.97±0.12	0.91±0.11	0.84±0.11	0.79±0.05	0.77±0.07	0.76±0.10	0.72±0.01
(2 weeks	TGF-β_1 100 ng/kg	0.77±0.05	0.75±0.05	0.95±0.13	0.89±0.13	0.88±0.10	0.85±0.01	0.83±0.03	0.81±0.04	0.84±0.06	0.83±0.06
post-virus	TGF-β_1 1 ng/kg	0.79±0.03	0.77±0.03	0.82±0.03	0.77±0.07	0.75±0.02	0.77±0.12	0.75±0.09	0.75±0.03	0.73±0.01	0.76±0.00
inoculation)	TGF-β_1-Inhibitor	0.80±0.05	0.77±0.04	0.56±0.08	0.54±0.10	0.53±0.06	0.56±0.03	0.61±0.03	–	–	–
	Placebo	0.77±0.05	0.74±0.08	0.75±0.06	0.66±0.07	0.62±0.06	0.66±0.08	0.62±0.03	–	–	–
Group 4[b]	TGF-β_1 200 ng/kg	1.06±0.10	1.35±0.08	1.59±0.17	1.27±0.11	1.04±0.05	0.96±0.11	0.86±0.07	0.84±0.01	0.87±0.01	0.81±0.04
3 treatment											

[a]Animal was already dead prior to this sampling time.
[b]2 weeks pre-, 8 h post-, and 2 weeks post-virus inoculation.
The gray region represents the treated (TGF-β_1) and untreated (inhibitor, placebo) injected.

(A)


1 2 3 4 5 6 7 ST 8 9 10 11 12 13
14 15 16 17 18 19 20 ST + 21 22 23 24 25
Group 1

(B)


+ 1 2 3 4 5 ST + I II III IV + –
WO/Virus/Treatment
Group 2

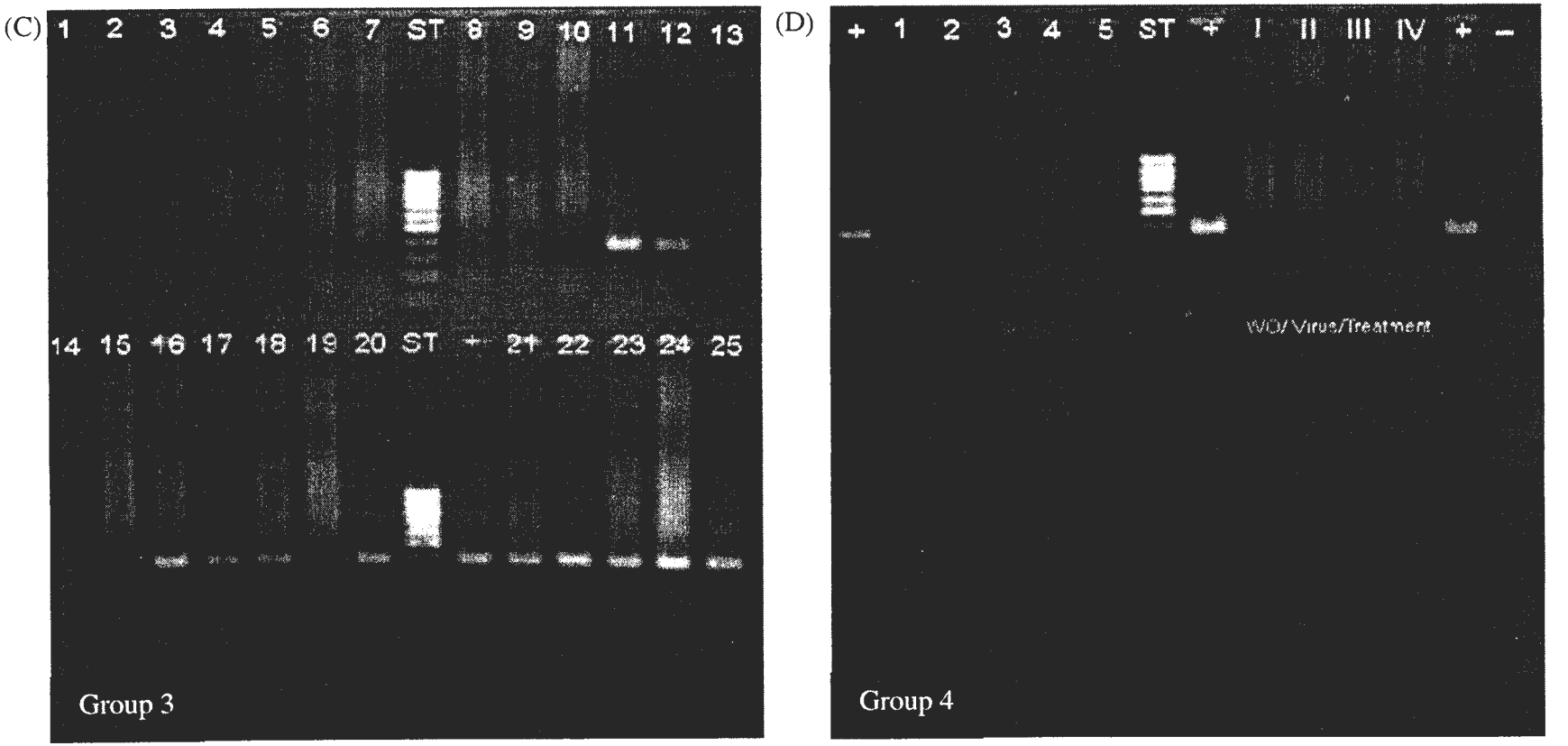


Fig. B.1. Detection of ADV DNA in mink treated with TGF-β_1. The expected fragment size was 379 base pairs, representing nucleotides 224-602. Group 1: treated with TGF-β_1 injected 2 weeks pre- (A), Group 2: 8 hours post- (B), Group 3: 2 weeks after-virus inoculation (C), and Group 4: TGF-β_1 injected 3 times (2 weeks pre-, 8 h post-, and 2 weeks post- virus inoculation) (D). Lane 1-5: TGF-β_1 at 200 ng/kg, 6-10: 100 ng/kg, 11-15: 1 ng/kg, 16-20: rhLAPTGF-β_1 at 500ng/kg (inhibitor) and 21-25: PSS in 0.1% BSA. +: positive control (379 base pairs) , -: negative control and ST: 100kb DNA ladder. PCR assay of lymph node tissue confirmed that untreated mink had significant levels of virus whereas TGF-β_1 treated mink had none.

Appendix C. Tables and figure of Effects of HE2300 and HE2500 on the Outcome of Aleutian Disease in Mink

Table C.1.
Study Design for DHEA treatment of ADV-infected mink.

Treatment	Received Compound	Number of animals
Infected, HE2500[a]	16 ng/kg, 3 times per week received	5
Infected, HE2300[b]	16 ng/kg, one times per week received	5
Infected, HE2300	16 ng/kg, 3 times per week received	17
Infected, Vehicle[c]	16 ng/kg, 3 times per week received	17
Infected, untreated	Virus with untreated	6
Normal Control	No virus, untreated	5
Total		55

[a]Fluasterone (FLST); 16α-fluoro-5-androsten-17-one; synthetic androstene derivative.
[b]β-androstenetriol (β-AET); 5-androstene-3β-7β-triol; natural androsten hormone.

Table C.2.
Effects of subcutaneous HE2500 and HE2300 treatment on survival time of ADV-infected mink.

Treatment Group	Survivors	Survivors (Adjusted[a])	Mean Time to Death (months ± SD)	Mean Time to Death (months ± SD; Adjusted[a])
HE2500[b] 3X/wk	3/5	3/4	5.8 ± 3.2	7.0 ± 2.0
HE2300[c] 1X/wk	1/5*	1/5*	4.0 ± 2.4**	4.0 ± 4.0**
HE2300 3X/wk	9/17	9/17	5.7 ± 2.6	5.7 ± 2.6
Vehicle 3X/wk	1/17*	1/14*	4.0 ± 2.2**	4.5 ± 2.5**
Untreated	0/6*	0/6*	3.8 ± 1.5**	3.8 ± 1.5**
Normal Control	5/5	5/5	8.0 ± 0	8.0 ± 0

[a]Includes only those animals that demonstrated pathology attributable to Aleutian disease.
[b]Fluasterone (FLST); 16α-fluoro-5-androsten-17-one; synthetic androstene derivative
[c] β-androstenetriol (β-AET); 5-androstene-3β-7β-triol; natural androsten hormone.
*$p < 0.001$ compared to normal control animals.
**$p = 0.016$ compared to normal control animals.

Table C.3
Effects of subcutaneous HE2500 and HE2300 treatment on serum ion levels in ADV-infected mink.

Treatment Group	Na^+ (mmol/L ± SD)		Ca^{+2} (mg/dL ± SD)		K^+ (mmol/L± SD)		Cl^- (mmol/L ± SD)	
	Pre-Exposure	After Virus Exposure[a]	Pre-Exposure	After Virus Exposure[a]	Pre-Exposure	After Virus Exposure[a]	Pre-Exposure	After Virus Exposure[a]
HE2500[b] 3X/wk	148 ± 5.6	143 ± 5.1	10.2 ± 0.9	9.7 ± 1.88	6.4 ± 0.5	4.9 ± 0.9	116 ± 6.2	108 ± 5.4
HE2300[c] 1X/wk	151 ± 4.8	144 ± 5.0	10.1 ± 0.9	10.1 ± 1.3	6.1 ± 0.6	4.9 ± 0.8	116 ± 4.8	108 ± 5.5
HE2300 3X/wk	149 ± 4.6	144 ± 3.7	10.0 ± 0.5	10.0 ± 1.0	6.1 ± 0.9	4.9 ± 0.8	118 ± 4.9	111 ± 5.2
Vehicle 3X/wk	145 ± 4.7	161 ± 4.2	10.1 ± 0.8	7.0 ± 1.3*/$^+$	7.9 ± 0.8	7.1 ± 0.9*	117 ± 5.5	130 ± 5.3*
Untreated	150 ± 2.1	166 ± 4.3	10.4 ± 0.4	8.2 ± 2.7	7.6 ± 0.7	6.7 ± 0.8*	121 ± 7.1	132 ± 6.8*
Normal control	149 ± 3.0	146 ± 5.1	9.9 ± 0.9	10.0 ± 0.9	5.0 ± 0.5	4.9 ± 0.3	113 ± 7.3	106 ± 9.6

[a]Represents the last serum available prior to death or sacrifice of the animal.
[b]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[c]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*$p < 0.05$ compared to normal control animals.
$^+p < 0.05$ compared to pre- vs. post-virus exposure.

Table C.4
Effects of subcutaneous HE2500 and HE2300 treatment on serum liver enzyme levels of ADV-infected mink.

Treatment Group	Alanine Aminotransferase(U/L ± SD)		Aspartate Aminotransferase (U/L ± SD)	
	Pre-Exposure	After Virus Exposure[a]	Pre-Exposure	After Virus Exposure[a]
HE2500[b] 3X/wk	40 ± 8.47	65 ± 17.14	54 ± 9.13	74 ± 10.21*
HE2300[c] 1X/wk	33 ± 4.97	101 ± 26.22*/$^{+}$	52 ± 12.24	102 ± 17.26*/$^{+}$
HE2300 3X/wk	34 ± 7.54	79 ± 33.34^{+}	49 ± 36.7	80 ± 19.56*
Vehicle 3X/wk	40 ± 11.13	194 ± 85.73*/$^{+}$	62 ± 38.74	132 ± 68.53*/$^{+}$
Untreated	43 ± 9.63	220 ± 119.4*/$^{+}$	58 ± 36.0	174 ± 50.60*/$^{+}$
Normal control	41 ± 4.44	42 ± 2.13	39 ± 6.02	42 ± 2.13

[a]Represents the last serum available prior to death or sacrifice of the animal.
[b]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[c]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*$p < 0.05$ compared to normal control animals.
$^{+}p < 0.05$ compared to pre- vs. post-virus exposure.

Table C.5
Effects of subcutaneous HE2500 and HE2300 treatment on serum levels of blood urea nitrogen and creatinine in ADV-infected mink.

	Blood Urea Nitrogen (mg/dl ± SD)		Creatinine (mg/dl ± SD)	
Treatment Group	Pre-Exposure	After Virus Exposure[a]	Pre-Exposure	After Virus Exposure[a]
HE2500[b] 3X/wk	22 ± 5.07	22 ± 4.38	1.3 ± 0.2	0.9 ± 0.18
HE2300[c] 1X/wk	24.8 ± 6.50	26.7 ± 4.99*	1.6 ± 1.5	1.4 ± 0.36*
HE2300 3X/wk	23.8 ± 6.02	25.8 ± 5.83	1.2 ± 0.7	1.1 ± 0.29
Vehicle 3X/wk	27.7 ± 10.61	34.5 ± 15.37*	1.4 ± 0.4	2.4 ± 1.01*/$^{+}$
Untreated	25.5 ± 10.68	40.8 ± 12.02*/$^{+}$	0.5 ± 0.5	3.1 ± 1.28*/$^{+}$
Normal control	15.0 ± 9.4	14.4 ± 4.53	0.9 ± 0.2	0.7 ± 0.11

[a]Represents the last serum available prior to death or sacrifice of the animal.
[b]Fluasterone (FLST; 16α-fluoro-5-androsten-17-one; synthetic androstene derivative).
[c]β-androstenetriol (β-AET; 5-androstene-3β-7β-triol; natural androsten hormone).
*$p < 0.05$ compared to normal control animals.
$^{+}p < 0.05$ compared to pre- vs. post-virus inoculation.

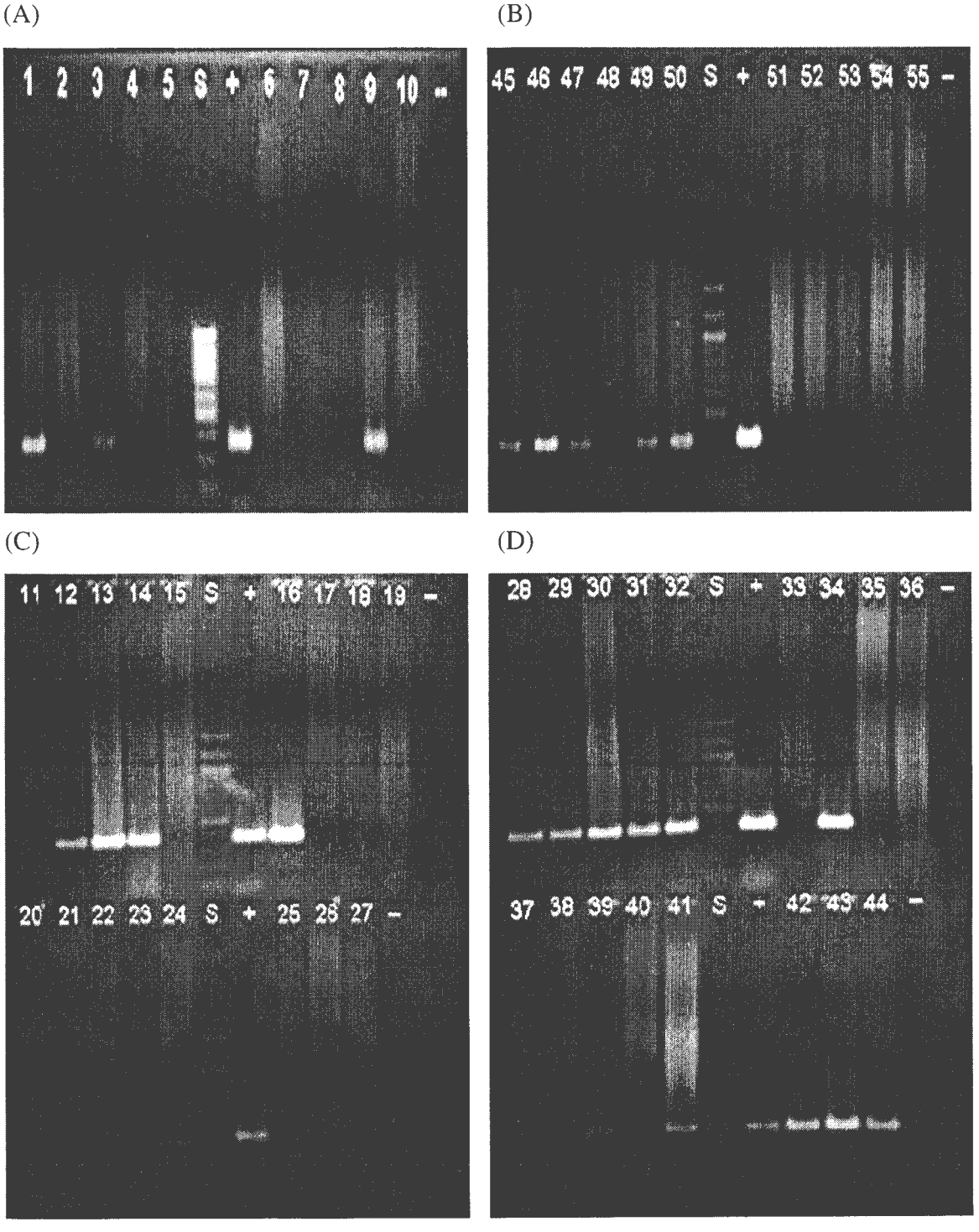


Fig. C.1. Detection of ADV DNA in mink treated with HE2300 and HE2500. (A) Lane 1–5: Infected/HERF 2300 1x/wk, 6–10: Infected/HERF 2500 1x/wk, (B) Lane 45–50: Infected/untreated, 51–55: Normal control, (C) Lane 11–27: Infected/HERF 2300 3/week and (D) Lane 28–44: Infected/vehicle 3x/wk. **S**: DNA ladder 100bp, **+**: AD positive control and **-**: AD negative control.

CURRICULUM VITAE

Kie Hoon Jung

Biology Department
Utah State University
Logan, Utah 84322-5305

phone: (435) 797-1874
email: kiehjung@cc.usu.edu

Education

Doctorate of Philosophy, Biology, **Utah State University** 2003
Major Professor: Dr. Edmund D. Brodie, Jr.
Thesis Director: Dr. Dale L. Barnard
Committee Members: Dr. Robert W. Sidwell,
Dr. Joseph K.K. Li
Dr. Daryll B. DeWald
Dissertation: Using Immune Modulators to Ameliorate Aleutian Disease in Mink.
Mink Farmers Researcher Foundation Grant
Antiviral Research Institute, **Utah State University,** UMC 5600

Master of Science, Biology, **Utah State University** 1997
Major Professor: Dr. Dana K. Vaughan
Committee Members: Dr. Edmund D. Brodie, Jr.
Dr. William Brindley
Dissertation: HNK-1 Immunoreactivity in Site Adult Teleost Retina.
Arbor Research Foundation Grant & NIH
Overall GPA: 3.81/ 4.0

Bachelor of Science, **Kyungpook National University** 1991
Major: Biology, Emphasis: Animal Physiology
Overall GPA: 4.2/ 4.5

Teaching Experience

Dept. of Biology, **Utah State University,** Logan UT 84322
Supervisor: Dr. Dale L. Barnard (435) 797-2696
Immunology 5150 Lab (Teaching Assistant) Spring 2000
Biology 1230 Lab (Teaching Assistant) Fall 1999

Dept. of Biology, **Utah State University,** Logan UT 84322
Supervisor: Dr. Dana K. Vaughan
Physiology Lab 505 (Teaching Assistant) Spring 1996
Biology 135: Brain Behavior (Teaching Assistant) Fall 1995

Training & Work Experience

Safety Training, **Utah State University,** Logan UT 84322

Laboratory and Biological Safety refresher Level II/ III Training	June 2003
Select Agent Awareness Training	June 2003
Biological Safety Level II/ III Training	Apply 2002
Bloodborne pathogens refresher Training	Apply 2002
Laboratory Safety Refresher Training	Apply 2002
Bloodborne pathogens Training	January 2000
Laboratory Safety Initial Training	March 1998
OSHA Laboratory Safety Training	March 1998
Animal Handling Training (Mink)	February 1998

Laboratory Animal Research Center (LARC), **Utah State University**

Occupation Health and Safety Program:

Animal Handling Training (Mouse & Hamster)	September 2003
Control Substance Training	September 2003
Animal Handling Training (Rabbit)	September 2000

Microscopy Control Training, **Utah State University,** Logan UT presented on

Confocal Laser Microscopy (CLM)	1996
Transmission Elector Microscopy (TEM)	1991
Scanning Electron Microscopy (SEM)	1991

Antiviral Research Institute, **Utah State University,** UMC 5600 Present-1998

Antiviral Research Institute, **Utah State University,** UMC 5600

SARS Mouse Model Development for Antiviral Testing Present-Nov. 2003

Research Assistantships, **Utah State University,** Logan UT 84322-5305

Teleost Retina Research: Animal Physiology & Neurobiology
(Arbor Research Foundation & NIH) 1996-1992

Aleutian Mink Disease Research: Virology & Immunology
(Mink Farmer Research Foundation, Antiviral research Institute) 2003-1998

Military Service in the Republic of Korea Marine Corps 1987-1984

Activities

Member of the Antiviral Research Institute, Utah State University	Present-1998
Member of the Hillcrest Elementary School PTA	Present-1998
Member of the Korean Scientist Association in USA	Present-1997
Korean Student Association Advisor	Present-1995
Deacon & Member of the Administration Committee of the Oriental Missionary Church at Logan	Present-1994

Member of the Republic of the Korea Marine Corps in US	Present-1993
Member of the Natural Environment Association in USA	1997-1994
Vice President of the Korea Student Association	1995-1994

Honors & Awards

Teaching Assistant of the Year, International Student & Scholars at USU	2000
Kim Nam Kyun Scholarship, International Student & Scholars at USU	1999
Best Friendship of the Korean Student Association	1995
Full Scholarship, Kyungpook National University	1990-1987
Best Friendship of the Republic of the Korea Marine Corps	1986

Representative Publications / Research /Proposals

Jung, K.H. and Barnard, D.L. Effects of HE 2300 and HE 2500 on the Outcome of Aleutian Disease in Mink	Present-2002
Jung, K.H. and Barnard, D.L. Ameliorating Aleutian Disease in Mink by Treatment with Human rTGF-β_1	Present-2001
Barnard, D.L. and Jung, K.H. The Peplotion Approach to Immunizing Against Aleutian Disease Virus of Mink	2002
Barnard, D.L. and Jung, K.H. Rivavirin (IP injection) Supplementation to Ameliorate Infectious Aleutian Mink Disease in Dark Mink	2001
Barnard, D.L. and Jung, K.H. Using Zinc Supplementation to Ameliorate Infectious Diseases in Dark Mink	2000-1999
Barnard, D.L. and Jung, K.H. The HE-1 (SQ injection) Approach to Immunizing Against Aleutian Disease Virus of Mink	2000-1999
Barnard, D.L. and Jung, K.H. Developing an Immunoadjuvant Vaccine to Prevent Aleutian Disease of Mink	1998
Jung, K. H. HNK-1 Immunoreactivity in Site Adult Teleost Retina M.S. Thesis (Arbor Research Foundation)	1997
Vaughan, D.K., Cole, D.D. and Jung, K. H. Polar Distribution of Neurofilaments in Nonspiking CNS Neuron. (NIH Grant)	1996
Heck, C., Jung, K.H. and Vaughan, D.K. A Rapid Method for Screening Monoclonal Antibodies for Use in Postembedding Electron Microscope Immunolabelling	1996
Vaughan, D.K., Ki, K.S. and Jung, K.H. Expression of the Cell Adhesion-Associated Antigen, HNK-1, in Primary Cultures of retina Neurons	1995
Vaughan, D.K., Jung, K.H., Hoffman, R. and Heck, C. HNK-1 Immuno-Reactivity in Adult Teleost Retina	1994
McManus, C.V., Jung, K.H. and Vaughan, D.K. Postembedding Light Microscope Immunocytochemistry of a Cell Adhesion Molecule-Associated Carbohydrate Antigen in Fish Retina	1993

Presentations

Jung, K.H., Winslow, S. and Barnard, D.L. Ameliorating Aleutian Disease in Mink by Treatment with Human rTGF-β_1, ASM Annual meeting, Post 2003

Jung, K.H. HNK-1 Immunoreactivity in Site Adult Teleost Retina, HNK-1 Immunoreactivity in Site Adult Teleost Retina, Neurobiology Annual Meeting, Post 1996