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The feline immunodeficiency virus infected cat as a model of AIDS pathogenesis and antiviral therapy

Hayes, Kathleen Ann, Ph.D.
The Ohio State University, 1993
THE FELINE IMMUNODEFICIENCY VIRUS INFECTED CAT
AS A MODEL OF AIDS PATHOGENESIS
AND ANTIVIRAL THERAPY

Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

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* * * * *

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To my husband Kevin Hayes
and to my mentor Lawrence Mathes
for their love and faith in me.
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1. Hayes KA, Lafrado LJ, Ericson JG, Marr JM, Mathes LE. Prophylactic
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3. Hayes KA, Rojko JL, Tarr MJ, Polas PJ, Olsen RG, Mathes LE. Atypical
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INTRODUCTION

The work encompassed within this dissertation can be divided into two parts: the first evaluates aspects of prophylactic azidothymidine (AZT) treatment on feline immunodeficiency virus (FIV) infection in a cat model for acquired immune deficiency syndrome (AIDS). The second explores the effects of cofactors of FIV infection, namely, intercurrent viral infection and tumor necrosis factor alpha activity, on disease pathogenesis at the level of the infected cell.

Since the discovery of AIDS a decade ago, the presumptive etiological agent, human immunodeficiency virus type 1 (HIV-1), has become arguably the most widely investigated human pathogen. Infection with HIV-1 leads inevitably to immunologic decline and death, as a result of direct infection and killing of immune cells or indirectly by alteration of the immune competence of the host facilitating opportunistic infection by pathogens to which the host would normally be resistant (1-4).

The apparent long latency period (of perhaps ten years or more) should work to the advantage of HIV-1 infected individuals as a lengthy window for treatment. However, recent studies have discovered chronic, low-level viremia
(5) and active virus expression in lymphoid tissue (6, 7) in asymptomatic HIV-1 infected patients. These developments tend to dispel the notion of an eclipse period in which HIV is not readily detectable prior to the onset of ARC and AIDS and lend support to HIV as the etiologic agent for AIDS. Such discoveries may also bring additional pressure to bear on beginning antiviral therapy much earlier than is currently practiced in order to intervene at the site of focal infection where the infectious process is active and unrelenting.

Most therapeutic strategies to combat HIV-1 infection are prophylactic where at primary exposure prevent infection of the host or, more commonly, in HIV-1 infected individuals, to prevent infection of naive cells by infected cells within the host. The current state of chemotherapy is concerned with both direct and indirect effects of virus infection. Drug therapies ideally target a unique part of the life cycle of the pathogen to avoid toxicity to the host. In the case of HIV-1 (or other retroviruses) experimental drugs are directed against 1) virus adsorption to the cell, 2) reverse transcription, 3) posttranslational processing of viral polyproteins and 4) virus budding from the cell membrane (8). Three of the currently prescribed chemotherapeutics for HIV-1 infection focus on intervention at the reverse transcription stage. Zidovudine, a nucleoside analog (also called azidothymidine or AZT), was the first antiviral therapy approved for the treatment of HIV-1 infection (9). In the initial clinical trials, AZT treatment of AIDS patients was associated with a significant increase in survival and decrease in morbidity (10). One mechanism of action
of AZT relies on its ability to be preferentially utilized by the viral reverse transcriptase enzyme leading to termination of the viral DNA transcript (11). Triphosphorylated AZT acting as a competitive substrate inhibitor (with respect to thymidine triphosphate) for the viral reverse transcriptase, represents a second mechanism of antiviral activity (12). AZT is thought only to prevent infection of naive cells, as reverse transcription precedes proviral integration (and hence, permanent infection of the cell) (9, 11-13). At the present time AZT is the standard against which new AIDS therapies are measured. Dideoxycytidine (ddC) and dideoxyinosine (ddl) are nucleoside analogs currently licensed for treatment of AIDS which also affect the reverse transcription step of the virus life cycle (14-16). Both ddC and ddl have shown therapeutic promise especially in combination regimes with AZT (17, 18). Ideally, antiviral therapy would eliminate all infected cells (or instruct the host’s immune system to do so) without harming uninfected cells. Unfortunately, with current antivirals (and cancer therapies as well), this critical specificity rarely exists and the levels of chemotherapy necessary to achieve this goal often contribute to significant toxicity.

Indirect effects of HIV-1 infection include impairment of the normal function of the host immune response (3, 4). T-lymphocytes, in particular, are eventually rendered unable to respond to biologic immune stimulators (ie, cytokines) as a consequence of microbial invasion (19). On the other hand, shifts in the balance of cytokine production due to loss or anergy in a particular
subset of helper T-lymphocytes may be responsible for increased levels of circulating TNF-α (20). Chronic expression of TNF-α is associated with the cachexia seen in AIDS patients (21,22). Another area of therapy is concerned, therefore, with immune modulation. Recent studies point to alterations in the cytokine network as the forbear of frank AIDS disease (20,23). Administration of interferon-α, interferon-γ or interleukin 3 to AIDS patients is an attempt to reestablish immune competence and immune homeostasis (18). Inhibitors of TNF-α (eg., pentoxyffylline, cyclosporin A or glucocorticoids) also show promise of reducing host-mediated pathogenesis (22).

AIDS therapies are also oriented to the treatment of opportunistic infections as indirect effects of HIV-1 infection. Gancyclovir and acyclovir are prescribed for herpes retinitis and cytomegalovirus infections commonly seen in AIDS patients (24,25). Trimethoprim-sulfamethoxasol (26) and rifabutin (26) are currently used for the treatment of Pneumocystis pneumonia and AIDS-related tuberculosis, respectively.

In cats, a common retroviral pathogen, belonging to the subfamily oncovirinae, is the feline leukemia virus (FeLV) (27). Infection is most often associated with immunosuppression and/or leukemogenesis (27,28). The feline immunodeficiency virus (FIV) is in another class of retroviruses belonging to the subfamily lentivirinae (29). FIV infection is associated with the induction of an AIDS-like-syndrome in cats (29,30). Having two distinct retrovirus pathogens in the same host may offer a unique parallel between FeLV/FIV dual infection
in cats and concurrent infection of humans with human T-cell leukemia virus type I (HTLV-I) and HIV-1. Although different in many respects such as genomic complexity and some disease manifestations, both HTLV-I and FeLV are classified as type-C oncovirinae, are lymphocytotropic and are etiologically linked to leukemia induction (31,32). Intercurrent HTLV-I infection has been suggested to potentiate HIV susceptibility and infection (33,34). The FeLV-FIV cat model could provide valuable insight into oncovirus-lentivirus disease interaction within the host.

Another aspect of disease pathogenesis is host initiated. The host immune response, attempting to defend self against a pathogen may unwittingly potentiate the infection leading to pathology in the host. A vital part of immunological communication and surveillance is that of the cytokine network. The host reacts to immunologic insult with the elicitation of a complex cytokine response. In an ideal and prototypical sense, cytokine expression aids the host in fending off the pathogen. On occasion, however, the pathogen is able to use the immune cytokine response to its advantage. TNF-α is an important mediator of inflammation and the acute phase response (22,35,36). Untimely or overproduction of TNF-α is involved in septic shock (37), tissue injury due to inflammation (37,38), and cachexia in chronic disease (22), and may play a deleterious role in rheumatoid arthritis (39) and graft-versus-host disease (40). Importantly, this cytokine can potentiate HIV-1 infection by direct transactivation of the HIV-1 genome (41,42). Further, HIV-1
infection of monocytes and lymphocytes can stimulate the production of TNF-α leading to paracrine/autocrine enhancement of infection (42). Additionally, plasma levels of TNF-α are elevated in many HIV-1 infected patients at various stages of disease (21). Due to significant interspecies crossreactivity, it is possible to study TNF-α production and effects in the feline model using recombinant human TNF and anti-human TNF antibodies (43,44). Through epidemiological studies, an important factor in AIDS pathogenesis is the state of immune activation of the host to which the virus is introduced (45). Most individuals are resistant to infection by a wide variety of pathogens most of the time. However, due to the nature of the virus (i.e., that HIV-1 infects and disables the very cells involved in host defense), what a host is infected with or has been exposed to at the time of HIV-1 exposure may be a determining factor in AIDS pathogenesis.

Importantly, animal models of disease allow us to manipulate circumstances in order to better understand the roles of the many players in the multifactorial process of degeneration that is disease. Further, the discovery and development of means to impede the disease process rely entirely on an understanding of the mechanisms involved.

References


CHAPTER I

PROPHYLACTIC AZT THERAPY PREVENTS EARLY VIREMIA AND LYMPHOCYTE DECLINE BUT NOT PRIMARY INFECTION IN FELINE IMMUNODEFICIENCY VIRUS INOCULATED CATS

Abstract

Prophylactic azidothymidine (AZT) therapy was evaluated in the feline immunodeficiency virus (FIV)-cat model for HIV-1 infection in humans. AZT treatment (30 mg/kg/day via continuous subcutaneous infusion) was initiated 48 hrs prior to virus inoculation and continued for 28 days. Transient plasma antigenemia evident in 6/6 untreated cats at week 2 post inoculation (pi) was absent in the AZT-treated cats although at 10 and 14 weeks pi (6 and 10 weeks after drug treatment), one of the AZT-treated cats had low-level antigenemia. Both CD4 and CD8 lymphocyte numbers were consistently higher in the AZT-treated cats when compared to both the FIV-inoculated-untreated cats and the virus-naive age-matched controls. CD4:CD8 ratios were lower for the AZT-treated cats than either the FIV-inoculated-untreated or virus-naive control cats. The decreased CD4:CD8 ratios were the result of an increase in CD8 lymphocytes in the AZT-treated cats while decreased ratios in the FIV-
inoculated-untreated cats were due to cell loss. Both AZT-treated and untreated cats showed nearly identical FIV-specific antibody responses beginning 2 weeks pi. Polymerase chain reaction (PCR) results from blood lymphocytes showed 6/6 AZT-treated and 6/6 untreated cats were positive for FIV-specific gag sequences. Although primary infection was not prevented, these results suggest prophylactic AZT therapy deterred early systemic spread of infection mediated by viremia and delayed absolute CD4 and CD8 lymphocyte decline.

**Introduction**

Azidothymidine (AZT) was the first FDA-approved drug for the treatment of acquired immune deficiency syndrome (AIDS). The virustatic effect of AZT is the result of interference with the reverse transcription of the viral RNA genome into proviral DNA (1). AZT therapy in AIDS patients reduces morbidity and prolongs life (2,3). Although the value of AZT as an early post-exposure treatment for preventing the primary spread of HIV-1 infection has not been established (4,5), AZT therapy may alter the natural progression of infection.

Two previous studies using the feline and murine leukemia oncornavirus animal models suggested that prophylactic or early post-exposure AZT therapy was effective in preventing retrovirus disease induction (6,7). In the mouse study (6) and our own studies in cats (8), prophylactic AZT therapy did not prevent primary virus infection but was effective in suppressing viremia,
allowing establishment of effective immune responses and preventing disease related to the early dispersion of virus. Studies using animal models of lentiviral infection to assess the efficacy of AZT have shown less dramatic results. Work with lentivirus infections in nonhuman primates indicated that AZT therapy in SIV-inoculated macaques and cynomolgus monkeys did not prevent infection but did reduce or delay the appearance of plasma antigenemia and CD4 lymphocyte decline (9,10). In contrast, Fazely et al. (11) reported no significant difference in survival between untreated and AZT-treated SIV-inoculated rhesus macaques although combination therapy using AZT and alpha interferon reduced plasma levels of p27. However, these authors could not rule out the potential therapeutic benefit of early post-exposure AZT treatment had a larger number of animals been studied (11). A study performed in the scid-hu mouse model showed that whereas virus was suppressed below the level detectable by PCR during the two week AZT treatment period, within two weeks after cessation of drug treatment, the engrafted human tissues were HIV-1 positive (12).

Feline immunodeficiency virus (FIV, 13), as a naturally occurring lentiviral infection of cats, has emerged as an important animal model for HIV-1 infection (14-16). FIV primarily infects lymphocytes leading to immunosuppression in the face of a strong antiviral antibody response (16-18). North et al. (19) have demonstrated similarities between the reverse transcriptase enzyme (RT) of HIV-1 and FIV with respect to template specificity and requirement for
magnesium. In addition, the RT of FIV is sensitive to inhibition by several promising antiviral compounds including AZT (20). *In vitro* inhibitory concentrations of AZT for HIV-1 and FIV are comparable (19) which makes the FIV-cat model particularly well-suited to study chemotherapeutic agents targeted at the RT in an *in vivo* setting. The purpose of this work, based on previous studies of prophylactic treatment in the FeLV-cat and MuLV-mouse models for AIDS, therefore, was to determine if the antiretroviral agent, AZT would prevent primary infection in the FIV-cat model.

**Materials and Methods**

*Animals:*

Eight week old specific pathogen-free cats were obtained from a colony maintained at the Department of Veterinary Pathobiology, The Ohio State University. The cats were randomly selected and assigned to one of three groups: AZT-treated-FIV-inoculated (*n* = 6), FIV-inoculated-untreated (*n* = 6), and uninoculated-age matched controls (*n* = 5). All work was performed in accordance with University Laboratory Animal Care and Use guidelines.

*Virus:*

The Mount Airy, Maryland strain of FIV (FIV$_{MD}$) was used as the source of inoculum. Virus stocks were prepared from clarified cell culture supernatant of FIV$_{MD}$-infected PBL cultures. One thousand tissue culture infectious dose
units-50% (TCID<sub>50</sub>) were administered by intravenous injection to anesthetized cats 48 hours following initiation of drug treatment.

Treatment Protocol:

AZT was administered by continuous subcutaneous infusion as previously described (21). Drug dosage was 30 mg/kg/day based on previous studies of toxicity and pharmacokinetics in the cat as well as efficacy against FeLV (8). The treatment period continued for 28 days with no adjustments in dosage for weight gains during the treatment period.

Sample Collection:

Blood was collected on a weekly basis for complete blood counts (CBC), lymphocyte phenotyping, and serology. Cats were evaluated weekly for weight gain, lymphadenopathy and any signs of illness or drug toxicity.

Serology:

Antiviral antibody responses to FIV<sub>MD</sub> were determined by a live cell immunofluorescence assay (IFA) and by immunoblot analysis. Briefly, the IFA was performed using the chronically FIV<sub>Petaluma</sub>-infected 3201 feline lymphoid cell line, 3201-FIV (22). The cells were incubated with serial two-fold dilutions of feline plasma followed by washing and a second incubation with FITC-tagged goat anti-feline IgG. Endpoint was determined as the highest dilution with ≥
50% positive cells. Virus protein-specific antibody responses were determined by immunoblot assay using disrupted, sucrose gradient-purified FIV \textsubscript{Petaluma} as the source of antigen. Polyacrylamide gel electrophoresis and transfer to nitrocellulose were performed as described elsewhere (23). Antibody reactivity was visualized after incubation with 1:100 dilutions of plasma, followed by a two-step avidin-biotin amplification method: the secondary antibody was a biotinylated staphylococcus Protein A (Zymed, San Francisco, CA) followed by alkaline phosphatase-labelled Extravidin (Sigma, St. Louis, MO). The blot was developed with the BCIP/NBT alkaline-phosphatase kit (Kirkegaard and Perry, Gaithersburg, MD).

\textit{Immunophenotypic Analysis:}

Phenotypic analysis of PBL was performed by the Flow Cytometry Laboratory, the Center for Retrovirus Research, the Ohio State University. Monoclonal antibodies to feline pan T, CD4 and CD8 were purchased from Southern Biotechnologies (Birmingham, AL). Results were expressed as proportions of total lymphocytes obtained by CBC evaluation. Graphs are mean values with standard error bars. Shaded areas represent the standard deviations of the mean values obtained from five control cats.
Plasma Antigenemia:

Plasma FIV p26 antigen levels were determined by a commercial ELISA (IDEXX, Westbrook, ME). Plasma levels of p26 were determined by plotting optical density values against a standard curve. The lower level of sensitivity of this assay was $\geq 47$ pg/ml FIV p26 antigen based on repeated measurements.

Polymerase Chain Reaction:

A nested polymerase chain reaction was utilized for detection of FIV provirus levels in peripheral blood cells. Briefly, DNA was isolated from washed blood cells with guanidine HCl and proteinase K treatment (24), quantitated by DNA fluorometry (25), digested with EcoRI restriction enzyme and then used in a series of three PCRs: 1) Large nest product: 500 ng DNA was added to a 100 ul reaction which included the 927/979 primer pair (kindly supplied by Drs. Philip Johnson and Robert Olmsted) which amplifies a 1.3 kb product in the gag region of FIV. 2) Small nest product: a 5 ul aliquot of the "large nest" product was obtained after chloroform-isoamyl alcohol treatment of the large nest product, then added to a new PCR which included the 667/668 primer pair which amplifies a 0.8 kb product within the large nest product. These products were run concurrently with a panel of controls which consisted of serial 1:10 dilutions of 3201-FIV cells (which are 100% infected as shown by IFA) in a background of uninfected 3201 cells for a total of $10^7$ cells per standard. The
dilutions were $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ 3201-FIV cells. The standards were treated in the same manner and at the same time as the samples being run. 3) To control for amplifiable DNA, 500 ng from each sample was also subjected to amplification of a 1.7 kb product from the TNF-α gene. Products were visualized by UV transillumination of an ethidium bromide stained agarose gel and confirmed by Southern blot and autoradiography (26) using an internal end-labelled oligo-probe specific for the 0.8 kb FIV product.

**Determination of Plasma AZT Levels:**

Plasma AZT concentrations were determined by HPLC analysis using a Hewlett-Packard Model HP1090 binary pumping system equipped with a diode-array detector (27). Two hundred ul plasma samples collected from individual animals were filtered through Amicon centrifree micropartition filters (Amicon Division, W.R.Grace and Co., Danvers, MA) to remove plasma proteins. The filtrate was injected onto a Hewlett-Packard 100 x 2.1 Hypersil MDS C8 column with a mobile phase consisting of a mixture of 95% 0.02M potassium phosphate buffer (pH 6.0) and 5% acetonitrile. The flow rate was 1 ml/min and run time was 5.6 min. AZT was detected at 266 nm with peak elution at 3.2 min. External standards were used to prepare a calibration table for concentration determination. The procedure was sensitive to approximately 0.4 ug/ml concentration.
 Statistics:

The Mann-Whitney nonparametric test was used to evaluate differences in lymphocyte subpopulation values and antibody titers between the AZT-treated and untreated groups of cats (Instat v. 1.10, GraphPad). Data are presented as mean +/- standard error of the mean.

Results

Effect of prophylactic AZT therapy on FIV antigenemia:

Six out of 6 FIV-inoculated-untreated cats were transiently positive for FIV antigen in plasma at 2 weeks pi (Figure 1). These cats had plasma levels of 128.5, 411.6, 436.0, 151.0, 59.1 and 267.2 pg/ml, respectively. By 3 weeks pi, FIV antigen was below the level of detection. In addition, at 6 weeks pi 2/6 cats had low levels of antigenemia (70.4 and 74.1 pg/ml) and at week 9, 3/6 (79.8, 47.9, 79.8 pg/ml) and at week 10 2/6 (59.0 and 64.8 pg/ml).

By comparison, 0/6 AZT-treated cats had detectable plasma antigenemia at 2 weeks pi, although one of these cats did show low-level antigenemia at weeks 10 (91.0 pg/ml) and 14 (47.9 pg/ml) pi.

Virus-specific antibody responses:

Antibody responses, as determined by immunofluorescence (Figure 2), were nearly identical and evident by two weeks pi. Immunoblot results from samples at 4 week intervals showed strongest reactivity to major core (p26)
and core precursor (pp52) proteins by 4 weeks pi and immunoreactivity to the transmembrane glycoprotein, gp41, by 12 weeks pi (data not shown).

**CD4 and CD8 lymphocyte phenotypic analysis:**

CD4 lymphocyte numbers of the FIV-inoculated-untreated cats showed an early, age-related rise which peaked at 9 weeks pi (mean 0.79 x 10^6/ml, Figure 3). From 9-15 weeks, CD4 lymphocyte numbers began to decline (mean 0.52 x 10^6/ml at week 16). The FIV-inoculated-AZT-treated cats also had increasing numbers of CD4 lymphocytes which peaked at 8-9 weeks pi (mean 1.59 x 10^6/ml). By 15 weeks pi, the CD4 lymphocytes from the AZT-treated cats had begun to decline only slightly (mean 1.37 x10^6/ml).

CD8 lymphocyte numbers also showed an early age-related increase in the untreated cats which peaked at week 10 pi (mean 0.60 x10^6/ml) followed by a decline (mean 0.33 x10^6/ml at week 15 pi)(Figure 4). For the AZT-treated cats, however, CD8 lymphocytes increased dramatically up through week 12 pi (mean 1.61 x10^6/ml) followed by a decline to 1.28 x10^6/ml by week 15 (11 weeks after cessation of drug treatment). By comparison, the age-matched virus-naive control cats showed steady age-related increases in both CD4 (mean 1.51 x10^6/ml) and CD8 cells (mean 0.79 x10^6/ml) at 23 weeks of age, which corresponds to 15 weeks pi for the inoculates.

CD4/CD8 ratios declined steadily for the AZT-treated and untreated cats throughout the course of the study. Although no statistically significant
differences were observed between ratios in the AZT-treated and untreated, FIV-inoculated groups, the mean ratios were consistently lower for the AZT-treated group (Figure 5). This lack of statistical significance was due to small differences in the actual numbers.

_Evaluation by the polymerase chain reaction (PCR):_

PCR analysis of PBL samples collected at 6 weeks pi for 6 untreated cats and 6 AZT-treated cats showed the 0.8 kb band corresponding to levels of 1/10 to 1/100 infected cells in 5/6 untreated cats and in 3/6 AZT-treated cats (data not shown). By 14 weeks pi, PBL samples from all 12 cats in both the AZT-treated and untreated groups were positive by PCR (Plate IA,B). Amounts of the 1.7 kb product of TNF-α control gene amplification were present and equivalent for all samples (Plate IC,D). The amplified PCR products are FIV-specific as shown by Southern hybridization (Plate II). Samples from 4 age-matched, virus-naive control cats were negative for FIV as shown by the ethidium bromide-stained agarose gel (Plate IA and IB) and by Southern blot (Plate II).

_Plasma AZT levels:_

Mean plasma AZT levels attained during the treatment period were 1.40 μg/ml (5.19 μM), 1.20 μg/ml (4.44 μM), 1.22 μg/ml (4.51 μM) and 0.93 μg/ml (3.44 μM) at days 7, 14, 21 and 28, respectively (Figure 6).
Discussion

AZT therapy is widely accepted as the primary antiviral treatment for HIV-1 infection and has been shown to reduce symptoms and prolong life (2,3). Many stage III and stage IV AIDS patients receiving AZT therapy have reduced plasma viremia levels and some stabilization or increase in lymphocyte numbers (2,3,28,29). The efficacy of early post-exposure AZT therapy has not been well characterized. There are no solid guidelines for treatment of patients that are occupationally exposed. A number of reports have suggested that AZT treatment does not prevent HIV-1 infection even when administered within a short time after primary virus exposure (30,31).

Using the FIV/cat model, the work presented here confirms that even under optimal conditions, AZT treatment does not prevent infection. However, it is apparent that AZT treatment does curtail the early progression of infection. Transient antigenemia, which is likely responsible for the initial systemic dissemination of virus, was evident in 6/6 untreated cats 2 weeks after virus inoculation, while it was absent in 6/6 AZT-treated cats. One of the AZT-treated cats did show detectable antigenemia, but not until 10 and 14 weeks pi, respectively. Suppression of early antigenemia and delayed PCR positivity in the treated cats as compared to the untreated cats, suggest there is a benefit to prophylactic AZT therapy.

A hallmark of both human and feline lentivirus infections is the profound loss of CD4 lymphocytes (32-34). Immunophenotypic analysis of circulating
lymphocyte populations showed a larger and faster increase in CD4 lymphocytes in the AZT-treated cats than in either the untreated or age-matched control cats. Interestingly, the CD8 subpopulation rose even more dramatically than did the CD4 cells leading to apparent low or inverted CD4/CD8 ratios in the AZT-treated cats. It is not clear if this effect is direct result of AZT-induced lymphocyte proliferation or that the AZT-treated cats were better able to mount a stronger anti-FIV cellular immune response than the untreated cats as manifested by increased CD8 cells. It must be considered, however, that these studies were performed with weanling animals and it is normal to observe increases in lymphocyte numbers as the animals mature. Fluxes in lymphocyte values in cats of this age should therefore be taken in the context of the age-matched control values.

The CD4/CD8 ratios were consistently, although not significantly, lower for the AZT-treated cats than either the inoculated-untreated or virus-naive controls which illustrates the importance of evaluating this parameter more critically. In HIV-1 infection, a decline in the CD4/CD8 ratio in early infection is due to an acute reduction in circulating CD4 lymphocytes with CD8 lymphocytes at normal levels or slightly increased (32). The CD4/CD8 ratio inversion in later stage infection (AIDS) is reflective of generalized lymphopenia with loss of CD4 and CD8 cells (32). Because FIV infects both CD4 and CD8 lymphocytes (35), the results suggest that AZT treatment prevented the lymphocyte population stasis and loss seen in the untreated cats, as compared
to the age-matched virus-naive controls, and augmented the lymphocyte numbers overall (data not shown). In FIV infection, it is misleading to present this data solely in the context of a ratio. As shown here, the lower CD4/CD8 ratios of the AZT-treated cats were not due to either CD4 or CD8 loss but rather to a higher rate of increase in CD8 cells with respect to CD4 cells. Meanwhile, the FIV-inoculated-untreated cats showed the characteristic early age-related increases in both CD4 and CD8 cells followed by a steady reduction in the CD4 lymphocytes and CD8 lymphocytes beginning at 9 weeks pi.

Although it is conceivable that an expanded CD8 cell population might contribute to the immunosuppressed state of the HIV-1-infected individual (36), this has not been borne out. It has been reported that CD8 lymphocytes suppress HIV-1 infection in vitro in a target:effector cell dose-dependent manner (37). This suppression is not mediated by direct cytotoxicity but rather an undefined, soluble factor. Further, the suppressive activity of CD8 lymphocytes can be correlated to the stage of HIV-1 infection with CD8 lymphocytes from asymptomatic carriers having the highest activity and those from AIDS patients having the lowest activity (38). Cooper et al. followed early T-lymphocyte population changes at the time of HIV-1 seroconversion (39). There too, it was characteristic for CD8 cells to increase at a faster rate than CD4 cells leading to CD4/CD8 ratio inversions as early as 16 days after the onset of clinical illness (39). Along with increased CD8 levels was an associated decrease in levels of plasma antigenemia. Of 6 patients studied,
only one patient who did not show a decrease in plasma antigenemia progressed to AIDS (39). In late stage HIV-1 infection, AZT treatment in AIDS patients was reported to result in transient elevations in both CD4 and CD8 lymphocytes as well as increased mitogen responsiveness and gamma-interferon production during the first 10-20 weeks of treatment (28). Presently, it is not known if the dramatic CD8 lymphocyte increase seen in the AZT-treated cats in this study was the result of stimulation by AZT treatment itself or FIV-specific induction of proliferation.

In this study, AZT treatment was initiated 48 hours before virus exposure and was limited to 4 weeks. Although declining, absolute lymphocyte counts were still above control levels in the AZT-treated cats out to 15 weeks pi, 11 weeks after cessation of drug therapy. These data suggest that AZT treatment begun prior to the time of virus challenge, protected both CD4 and CD8 lymphocytes from early virus-mediated decline. PCR results showed the 4-week AZT treatment did not prevent infection of circulating lymphocytes, but it was able to delay infection which was likely a result of AZT suppression of antigenemia. The delay in PCR detection of FIV implies AZT treatment at the time of virus exposure minimized early dissemination of virus via the blood to susceptible tissues. The minimization of viral spread shortly after inoculation may have direct impact on the development of disease and length of survival, even if infection is not prevented by AZT treatment.
Plasma levels of AZT in the Fazely et al. nonhuman primate study (mean 42.3 μM and 46.0 μM at weeks 1 and 3 pi) were much higher than those attained in Lundgren's study (mean 22.7 μM) (10,11). In the present study, even lower plasma levels (mean 1.40 μg/ml, 5.19 μM) were attained during the first week of treatment. Drug administration was via continuous infusion in this protocol, as compared to intermittent dosing in the nonhuman primate studies, thereby avoiding problems associated with "peaks and troughs" in therapeutic levels of drug. This may account for the greater efficacy seen in the present experiment.

The quantity of infectious virus in the inoculum also could affect the efficacy of therapy. In this study, cats were inoculated with five-fold less virus (1000 TCID₅₀). The \( \geq 5 \times 10^3 \) tissue culture infectious doses of SIV used as the inoculum in the nonhuman primate studies (10,11) may have exceeded a therapeutic threshold \textit{in vivo} for AZT.

In the optimal setting, the inhibitory constant of AZT-triphosphate effective against purified FIV-RT \textit{in vitro} is in the range of 3.3 \( \pm \) 1.6 nM (19). Similarly, the inhibitory constant of AZT-triphosphate effective against purified HIV-1-RT \textit{in vitro} is in the range of 6.5 \( \pm \) 1.8 nM (19). It is well known that AZT must be phosphorylated to the triphosphate form before it can be effective, however (40). Different cell types vary in their ability to phosphorylate AZT (41). Susceptible cell types \textit{in vivo} have not been elucidated thoroughly in the HIV-1 system and much less so in the FIV system.
It is likely that the failure of prophylactic AZT therapy to prevent infection is due to the susceptibility of a cell type(s) which is unable to utilize AZT.

The FIV cat model of lentiviral infection parallels HIV-1 infection in terms of measurable endpoints of infection, including an early and brief antigenemic episode, CD4/CD8 ratio decline, and loss of lymphocyte and accessory cell function, all in the face of a formidable antibody response. Further, biochemical similarities between FIV and HIV-1 make the FIV model ideal for in vivo evaluation of chemotherapeutic and biologic response-modifying modalities.

References


Figure 1. The effect of prophylactic AZT treatment on FIV-antigenemia as determined by antigen capture ELISA. Weekly mean (+/- S.D.) plasma p26 antigen levels (pg/ml) were determined by extrapolation from a linear regression analysis of the standard curve (not shown). The mean plasma levels at week 2 pi for the FIV-inoculated, untreated cats (n = 6) (●) were significantly different from those of the AZT-treated cats (n = 6) (○) (p = .0022).
Figure 1

**PLASMA p26 (pg/ml)**

- **AZT TREATED**
- **UNTREATED**

AZT TREATMENT vs. WEEKS
Figure 2. Antiviral antibody responses as measured by live cell immunofluorescence assay for the FIV-inoculated, untreated (n = 6) (●) and AZT-treated cats (n = 6) (○). The results are presented as geometric mean (+/- S.D.) titers.
Figure 2

GEOMETRIC MEAN TITER

- UNTREATED
- AZT TREATED

AZT TREATMENT WEEKS

Figure 2
Figure 3. Mean (+/− S.E.) CD4 lymphocyte population changes in the FIV-inoculated, untreated (n = 6) (●) and AZT-treated (n = 6) (○) cats. Shaded area represents the variances of mean values obtained from control cats (n = 5). Differences between the FIV-inoculated, untreated and AZT-treated cats were statistically significant at pi weeks: 7 (p = .0087), 9 (p = .0087), 11 (p = .0087), 12 (p = .0043), and 15 (p = .0317).
Figure 3

CD4 LYMPHOCYTES $\times 10^6 / \text{mL}$

- UNTREATED
- AZT TREATED

AZT TREATMENT

WEEKS
Figure 4. Mean (+/-) CD8 lymphocyte population changes in the FIV-inoculated, untreated cats (n = 6) (●) and AZT-treated cats (n = 6) (○). Shaded area represents the variances of mean values obtained from control cats (n = 5). Differences between the FIV-inoculated, untreated and AZT-treated cats were statistically significant at pi weeks: 7 (p = .0087), 10 (p = .0260), 11 (p = .0087), 12 (p = .0022), and 15 (p = .0079).
Figure 4
Figure 5. Mean (+/- S.E.) CD4/CD8 ratios for the FIV-inoculated, untreated (n = 6) (●) and AZT-treated cats (n = 6) (○). Shaded area represents the variances of the mean values obtained from control cats (n = 5). Differences between the FIV-inoculated, untreated and the AZT-treated cats were not statistically significant at any time point.
Figure 6. Mean (+/− S.D.) plasma AZT levels attained during the treatment period were 1.40 µg/ml (5.19 µM), 1.20 µg/ml (4.44 µM), 1.22 µg/ml (4.51 µM) and 0.93 µg/ml (3.44 µM) at days 7, 14, 21 and 28, respectively.
Figure 6

AZT (μg/ml)

0.0  0.5  1.0  1.5  2.0

WEEKS

1  2  3  4

Figure 6
Plate I.

A. & B. Ethidium bromide-stained agarose gel showing PCR-amplified 803 bp FIV product (14 week pi samples). A. Lanes: 1, molecular weight marker; 2, 4787 untreated; 3, 4788 untreated; 4, 4789 untreated; 5, 4796 untreated; 6, 4797 untreated; 8, 4798 untreated; 8, 4790 AZT-treated; 9, 4791 AZT-treated; 10, 4793 AZT-treated; 11, 4794 AZT-treated; 12, 4795 AZT-treated; 13, 4804 AZT-treated. B. Lanes: 1, molecular weights; 2, reaction mixture without DNA; 3-6, four virus-naive, age-matched control cats; 7-11, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ FIV-positive cells in a background of uninfected cells for a total of $10^7$ cells per sample, respectively.

C. & D. Ethidium bromide-stained agarose gel showing PCR-amplified 1.7 kbp TNF-α product (14 week pi samples). C. Lanes 1, molecular weight marker; 2, 4787 untreated; 3, 4788 untreated; 4, 4789 untreated; 5, 4796 untreated; 6, 4797 untreated; 8, 4798 untreated; 8, 4790 AZT-treated; 9, 4791 AZT-treated; 10, 4793 AZT-treated; 11, 4794 AZT-treated; 12, 4795 AZT-treated; 13, 4804 AZT-treated. D. Lane 1, molecular weight marker; lane 2, reaction mix without DNA; lanes 3-6 four virus-naive, age-matched control cats; lanes 7-11, $10^2$, $10^3$, $10^4$, $10^5$ and $10^6$ FIV-positive cells in a background of uninfected cells for a total of $10^7$ cells per sample.
Plate II. Autoradiograph of a Southern blot showing four age-matched, virus-naïve controls (lanes 1-4), six FIV-inoculated, untreated cats (cats 4787, 4788, 4789, 4796, 4797, 4798) (lanes 5-10), and six FIV-inoculated, AZT-treated cats (4790, 4791, 4793, 4794, 4795, 4804) (lanes 11-16).
CHAPTER II
LONG TERM EFFECTS OF AZIDOTHYMIDINE TREATMENT OF CATS WITH ACUTE OR CHRONIC FELINE IMMUNODEFICIENCY VIRUS INFECTION

Abstract

As a sequel to the work presented in Chapter 1 which evaluated the first 16 weeks of prophylactic efficacy of 2',-3'-azidothymidine (AZT) in the FIV cat model, Chapter 2 extended the observation period to 1 year post-infection (pi) and 11 months post-treatment. In addition, AZT therapy was evaluated in chronically infected cats.

Although both AZT-treated and untreated FIV-inoculated cats remained FIV-DNA polymerase chain reaction (PCR) positive throughout the 1 year observation period, the CD4 and CD8 lymphocyte populations remained at higher levels in the AZT-treated cats at least 48 weeks after cessation of AZT treatment. The CD8 values of the treated cats were within the normal range of the age-matched uninfected control cats to at least 52 weeks pi. Anti-FIV antibody responses as measured by live cell immunofluorescence were comparable between the treated and untreated cats during the experimental
period. Antigenemia remained below the level of detection at all time points tested after the initial 16 week evaluation period.

Results of histological examination of lymphoid tissues were within the normal limits of the age-matched control cats. Low level antigen was detected in thymus or lymph node homogenates from 3 of the 11 cats tested. PCR results showed provirus in blood, lymph node, bone marrow, spleen thymus and brain samples from inoculated cats but not naive controls.

To evaluate AZT treatment in chronically infected cats, seven cats at ≥ 10 months pi were treated with 30 mg/kg/day AZT administered by subcutaneous injection every eight hours for a 4 week period. This treatment regimen had no effect on the following parameters evaluated over a 6 month period: CD4/CD8 lymphocyte numbers, FIV-DNA PCR positivity, antigenemia.

Introduction

Antiviral drug efficacy for the treatment of human immunodeficiency virus infection (HIV-1) in vivo has been measured by a variety of parameters: immunological function, cell numbers, plasma antigen and viral RNA levels, provirus levels, morbidity and mortality (1-8).

AZT has been the premier antiviral therapy employed in early post-exposure prophylaxis as well as in treatment of chronic HIV-1 infection (9). Phase I clinical trials of AZT in AIDS and ARC patients indicated improvement
in parameters such as increased numbers of circulating CD4+ lymphocytes, loss of skin test anergy and increased patient survival (1,4,8,10).

*In vitro* studies have shown that AZT does not affect antigen and reverse transcriptase levels in chronically infected U937 (11), U1 or ACH-2 cell lines (12). Although ineffective against virus already integrated into the genome of an infected cell or against intracellular viral DNA, in the dynamics of an *in vivo* system, AZT may prevent infection of newly generated cells by acting in concert with endogenous antiviral factors (such as interferons) (13). Further, the extent of virus transmission between cells in the infected host via cell fusion is not known. AZT therapy has been suggested to reduce the ability of uninfected CD4+ lymphocytes to take part in the fusion process (14). Ideally, AZT therapy could help restrict viral infection of new cells until normal or disease-induced cell turnover renders the infected host virus free.

There are several limits to the hypothesis that AZT treatment is an effective prophylactic therapy in chronic infection. First is the differential ability of cells to triphosphorylate the compound to its effective form (15). Second is the necessity to reach all virus-susceptible cells with the compound. Third are the limitations of maintaining consistent and high enough levels of the active drug in all susceptible cells for a long enough duration to prevent reverse transcription. Consequently, the ideal circumstance is not likely attainable. Yet, based on the results of clinical trials which suggest AZT treatment as
beneficial in AIDS patients (1,4,6,8,10), evaluation of AZT therapy in ARC and asymptomatic patients seems warranted.

There has been a series of animal model studies using AZT alone or in combination with other agents (interferon-α, 3′-fluorothymidine), to assess prevention of lentivirus and oncovirus (feline leukemia virus, simian immunodeficiency virus and murine leukemia virus) infection (16-22). However, none has evaluated the long term effects of AZT therapy.

To determine whether prophylactic AZT therapy would have long term effects on FIV pathogenesis, the first part of this chapter extends the results of the prophylactic AZT treatment regimen described in chapter 1. The purpose of the second part of this chapter was to determine if AZT treatment of chronically FIV-infected cats would retard disease progression.

Materials and Methods

**Animals:**

For the acute infection treatment regimen, eight week old specific pathogen-free cats were obtained from a colony maintained at the Department of Veterinary Pathobiology, The Ohio State University. The cats were randomly selected and assigned to one of three groups: AZT-treated-FIV-inoculated (n = 6), FIV-inoculated-untreated (n = 6), and uninoculated-agematched controls (n = 5). All work was performed in accordance with University Laboratory Animal Care and Use guidelines.
Cats (n = 7) used in the therapeutic AZT treatment regimen were inoculated at eight weeks of age and shown by serology and PCR to have been chronically infected for 10 to 18 months.

**Virus:**

The Mount Airy, Maryland strain of FIV (FIV\textsubscript{MD}) was used as the source of inoculum. Virus stocks were prepared from clarified cell culture supernatant of FIV\textsubscript{MD} -infected PBL cultures. One thousand tissue culture infectious dose units-50% (TCID\textsubscript{50}) were administered by intravenous injection to anesthetized cats at eight weeks of age and 48 hours following initiation of drug treatment in the acute infection treatment regimen. For the chronic infection treatment regimen, cats were inoculated at eight weeks of age and monitored for 1-2 years.

**Treatment Protocol:**

For the acute treatment regimen, AZT was administered by continuous subcutaneous infusion as previously described (23). Drug dosage was 30 mg/kg/day based on previous studies of toxicity and pharmacokinetics in the cat as well as efficacy against FeLV (16). The treatment period continued for 28 days with no adjustments in dosage for weight gains during the treatment period. For the chronic infection treatment regimen, administration was by
intermittent subcutaneous injection at eight-hour intervals. Drug dosage was 30 mg/kg/day and treatment period continued for 28 days.

**Sample Collection:**

Blood was collected on a weekly basis for complete blood counts (CBC), lymphocyte phenotyping, and serology. Cats were evaluated weekly for weight gain, lymphadenopathy and any signs of illness or drug toxicity. Complete post mortem evaluations were performed on animals at the termination of the study. Tissues were collected for routine histopathology and for nucleic acid and protein isolation.

**Serology:**

Antiviral antibody responses to FIV\textsubscript{MD} were determined by a live cell immunofluorescence assay (IFA). Briefly, the IFA was performed using the chronically FIV\textsubscript{Petaluma}-infected 3201 feline lymphoid cell line, 3201-FIV (24). The cells were incubated with serial two-fold dilutions of feline plasma followed by washing and a second incubation with FITC-tagged goat anti-feline IgG. Endpoint was determined as the highest dilution with \( \geq 50\% \) positive cells.

**Immunophenotypic Analysis:**

Phenotypic analysis of PBL was performed by the Flow Cytometry Laboratory, the Center for Retrovirus Research, the Ohio State University.
Monoclonal antibodies to feline pan T, CD4 and CD8 were purchased from Southern Biotechnologies (Birmingham, AL). Results were expressed as proportions of total lymphocytes obtained by CBC evaluation.

Plasma and Tissue Antigenemia:

Plasma FIV p26 antigen levels were analyzed by a commercial ELISA (IDEXX, Westbrook, ME). Plasma levels of p26 were determined by plotting optical density values against a standard curve. The lower level of sensitivity of this assay was $\geq 47$ pg/ml FIV p26 antigen based on repeated measurements. Tissues were snap frozen in liquid nitrogen at necropsy and were homogenized in an extraction buffer (0.05M NaCl, 0.5% NP-40, 0.02M Tris-HCl, 0.5% Na desoxycholate and 10% aprotinin) by a Polytron homogenizer while frozen. Centrifuge-clarified homogenates were tested for p26 by ELISA.

Polymerase Chain Reaction:

A "hot start" nested polymerase chain reaction (PCR) technique was used to amplify FIV-gag specific DNA sequences from peripheral blood, lymph node, thymus, spleen, bone marrow and brain samples. Briefly, DNA was isolated from washed blood cells and frozen homogenized tissues with guanidine HCl and proteinase K treatment (25), quantitated by DNA fluorometry (26), and digested with EcoRI restriction enzyme. Five-hundred ng samples of
purified DNA was amplified with the 927,979 primer pair in tubes in which primers, magnesium, and deoxynucleotides were separated from Taq polymerase, buffer and DNA by a wax bead. Following initial amplification, 10 ul of the amplified product was used as a template in a second PCR using the 667/668 primer pair. The control panel was a series of 1:10 dilutions of FIV-positive DNA (100 ng to 0.1 pg) in a background of FIV-negative DNA for a total of 500 ng/sample. Amplified products were visualized by UV transillumination of an ethidium bromide stained agarose gel.

Post mortem Evaluation:

Complete necropsies were performed following euthanasia. Tissues collected for routine histopathology were: liver, kidney, spleen, mesenteric lymph node, thymus, adrenal glands, thyroids, heart, lungs, stomach, intestines, bone marrow, urinary bladder, brain, skin, and ovary/testes. Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, section to 6 micron thickness and stained with hematoxylin and eosin. Lymph node, thymus, bone marrow, spleen and brain sections were snap frozen in liquid nitrogen and stored at -85 C. For homogenization, tissues were kept in liquid nitrogen, quickly sectioned and homogenized in either DNA or protein extraction buffers.
Statistics:

The Mann-Whitney nonparametric test was used to evaluate differences in lymphocyte subpopulation values and antibody and antigen titers between the AZT-treated and untreated groups of cats (Instat v. 1.10, GraphPad). Data are presented as mean +/- standard error of the mean.

Results

Clinical Signs:

As presented in Table 1, during the course of the experiment, many cats in both the AZT-treated and untreated groups demonstrated one or more of the following clinical signs: ocular opacity, cachexia, cystitis, seborrhea, alopecia and lymphadenopathy.

Lymphocyte Phenotypic Analysis:

The numbers of CD4 lymphocytes increased at a faster rate and to a higher level in the AZT-treated cats as compared to the untreated, FIV-inoculates (Figure 7). The values then stabilized for both groups from 16 weeks pi to approximately 32 weeks pi. Meanwhile CD4 numbers for the age-matched controls steadily increased to 32 weeks pi (40 weeks of age). While CD4 lymphocyte numbers were generally within the normal range (FIV inoculates) or above (AZT-treated) for the first 16 weeks of the study, these populations experienced growth stasis while virus-naive animals showed
continued age-related increases in CD4 lymphocytes. The static period was
followed by a mild decline after 40 weeks pi. Statistically significant
differences between AZT-treated and untreated-FIV inoculates were noted at
weeks 7 (p = .0087), 10 (p = .041), 11 (p = .0087), 12 (p = .0043), 15
(p = .0318) and 48 (p = .0318) as determined by Mann-Whitney U test.

Numbers of CD8 lymphocytes increased dramatically for the AZT-treated
cats during the AZT-treatment period and were stable to 32 weeks pi (Figure
8). These values remained within the control range from 13 weeks pi through
the remainder of the experiment. Initially, the FIV inoculates also showed a
CD8 lymphocyte population rise, although to a lesser extent and for a shorter
time than those of the AZT-treated cats. A 31 week period of growth stasis
was apparent from weeks 9-40 pi. Statistically significant differences between
the AZT-treated and untreated inoculates were observed at weeks 7
(p = .0022), 10, (p = .0260), 11 (p = .0043), 12 (p = .0022), 15 (p = 0079), 24
(p = .0318) and 48 (p = .0159) as determined by the Mann-Whitney U test.

The CD4/CD8 ratios of the AZT-treated cats were consistently lower
than those of the untreated FIV-inoculates throughout the experimental period
due to the higher numbers of CD8 lymphocytes in the AZT-treated cats (Figure
9). Statistically significant differences between AZT-treated and untreated FIV-
inoculates were observed at weeks 12 (p = .0152) and 52 (p = .0159).
**Anti-FIV Antibody Titers:**

Anti-FIV antibody titers as measured by immunofluorescence assay were detectable by week 2 pi and rose quickly to levels of approximately 1:32,000 by 12 weeks pi (Figure 10). There were no statistical differences between the two groups at any time point as measured by Mann-Whitney U test.

**FIV Antigen in Plasma and Tissues:**

All plasma samples beyond the initial 16-week study period tested by FIV ELISA were below the level of detection. Acid pretreatment of samples did not improve detectability (27).

Of the protein samples isolated from spleen, lymph node, thymus, bone marrow and brain sections from 8 FIV-infected and 3 control cats, only 3 samples had detectable FIV antigen: cat 4671 thymus (86.4 pg/ml), cat 4790 thymus (121.7 pg/ml) and cat 4804 lymph node (63.5 pg/ml) (Table 2). Protein values were 3.28 mg/ml, 1.42 mg/ml and 3.32 mg/ml, respectively as determined by BioRad Protein Assay (Bio Rad, Hercules, CA). Proteins tested from the various samples ranged from 0.962 mg/ml to 14.48 mg/ml.

**Polymerase Chain Reaction:**

PCR results from peripheral blood samples during the chronic infection AZT-treatment period (week 3) and at 6 months after treatment are shown in Plate III. The integrity of the EcoRI-digested DNA and equivalence in loading
amounts are shown by ethidium bromide-stained agarose gel (Plate IV). The amounts of amplified product appeared equivalent between the two time points and no differences were noted between the AZT-treated and untreated cats.

DNA was extracted from snap-frozen lymph node, thymus, spleen, bone marrow and brain samples collected at necropsy from both treated and untreated cats. FIV-gag sequences were amplified from the lymph nodes of 6/7 animals, from the spleens of 7/8 animals, from the thymuses of 8/8 animals, from the bone marrow of 3/3 animals and from the brain of 5/8 animals (plate V A, V B, Table 2). The level of detection of this PCR assay was 1 pg FIV-positive DNA in a background of 500 ng FIV-negative DNA.

Histopathology:

Tissues sampled at necropsy were sectioned and stained with hematoxylin and eosin for histopathologic examination. Results from the lymphoid tissues examined showed no differences between AZT-treated or untreated, FIV-infected animals (between 1-2 years pi) and age-matched control animals.

AZT Treatment of Chronically Infected Cats:

Seven FIV-positive cats between 10 and 18 months post inoculation were treated with AZT by intermittent subcutaneous infusion for a 28-day period. The effects of AZT treatment in these chronically-infected cats were
evaluated by changes in proportions of CD4 and CD8 lymphocyte populations, plasma antigenemia, anti-FIV antibody response, peripheral blood cell DNA PCR, DNA PCR of tissues (described above) and clinical signs. No measurable changes were observed in any of the treated animals in any of the stated parameters during the course of treatment or 5 months after treatment ended.

Discussion

Using the FIV/cat as a model for HIV-1 infection, even under optimal circumstances, prophylactic AZT treatment did not prevent infection. However, early AZT treatment prevented detectable systemic spread of virus (via the blood) delaying the initial infection and preventing lymphocyte population growth stasis due to FIV-infection. These effects continued throughout the one year observation period. Recent reports used an ultrasensitive polymerase chain reaction technique and have described significant amounts of HIV-1 RNA in the plasma of infected patients even during the "asymptomatic" phase of infection (28). In this study, three of six of the AZT-treated cats were PCR positive for FIV DNA in peripheral blood cells by 6 weeks pi as compared to 5/6 untreated challenge controls. Thus, AZT treatment was able to slow, but not prevent, the initial spread of virus and dramatically reduce the amount of circulating virus to below the level of detection of the ELISA test. Antigenemia
remained below the level of assay detection for both the treated and untreated cats at all time points tested beyond the initial 16 week period.

The effects on CD4 and CD8 lymphocyte populations were seen early after inoculation. The cats used in this experiment were inoculated at 8 weeks of age to parallel work our laboratory had done in the FeLV model where the infectious window is < 10 weeks of age. The CD4 lymphocytes increased in an age-related manner similar to the age-matched controls in the AZT treated cats for the first 8 weeks after FIV inoculation. In contrast, the untreated FIV-inoculated cats failed to undergo the age-related increase in CD4 numbers in this early phase post inoculation which translated into maintenance of CD4 lymphocyte numbers below those of the age-matched controls. Beyond the first 16 weeks pi, CD4 and CD8 numbers remained stable at their respective levels for the remainder of the experiment.

In the AZT-treated cats, CD8 lymphocyte numbers expanded during the AZT treatment period to levels above those of the age-matched controls. Whether or not this effect was related directly to drug stimulation or indirectly, through drug suppression of virus replication to a level which allowed immune cell expansion in response to the virus, was not determined. The CD8 values for the AZT treated cats remained within the normal range out to at least 48 weeks after treatment. The results suggest that FIV slows or stops the normal age-related rise in T lymphocytes and that AZT treatment early post inoculation prevents this FIV effect.
In contrast, a recent report described a decrease in absolute CD8 numbers in the second month of AZT treatment of HIV-1 subjects who were started on therapy close to the time of primary exposure, while placebo-treated controls experienced CD8 lymphocytosis (29). The implication was that AZT treatment early post infection (i.e., within 10 days of presentation with acute symptoms) depressed a normal host response to HIV-1 infection. The FIV challenge controls in this study did not undergo primary CD8 lymphocytosis in response to FIV infection but this might be explained by the age of the animals and the stage of lymphocyte distribution at the time of virus inoculation. Experimental cats in these studies experienced an age-related increase in circulating CD4 and CD8 lymphocytes which did not stabilize until approximately 40 weeks of age. Further, timing of drug treatment initiation and dose is likely critical in the antiviral response. In a feline leukemia virus/cat model for HIV-1 infection, AZT treatment initiated within the first 4 days of virus challenge afforded complete protection while treatment initiated 1 week post exposure elicited a delay in infection as measured by plasma viremia (Mathes, personal communication). Further, a bolus dose of AZT administered within the first 8 hours of FeLV inoculation abrogated the protective effect of the drug and potentiated the infection (Mathes, personal communication).

In this study, AZT treatment administered to chronically infected cats had no effect on CD4/CD8 lymphocyte numbers, antigenemia, anti-FIV antibody response and peripheral blood PCR positivity. While phase I clinical trials in
AIDS and ARC patients showed clinical benefit, extending treatment to the asymptomatic HIV-1 infected population has become controversial (30). Initial studies suggested a delay in progression to AIDS in asymptomatic patients initiating AZT therapy. Early results from the Concorde trial suggest, however, that there is no long term clinical benefit and that the problems of acquired AZT resistance, drug toxicity and cost make AZT treatment of asymptomatic individuals prohibitive (31).

It is conceivable that AZT-resistant strains of FIV arose in the chronically infected cats before any apparent effects of AZT on the parameters measured were detectable. Drug resistance acquired during the treatment period was not evaluated in this study. However, Remington et al. (32) have shown that in vitro, the emergence of AZT resistant FIV can be documented in as few as three serial passages in tissue culture (in vitro). The clear differences relating to effects of AZT treatment at the time of virus inoculation would suggest that if AZT-resistance was a factor, it occurred later in the course of treatment period if at all. That there might be more of a propensity to develop drug resistance in chronic infection as opposed to acute infection is also a possibility.

The expression of viral protein in select tissues, as measured by ELISA, was low and only detectable in 3 cats (all of which were in the AZT-treatment group). It is possible, if not probable, that there was significant virus antigen expression (especially the lymphoid tissue) below the threshold detectable by
this technique (33,34). By DNA PCR, most cats, irrespective of AZT treatment, carried detectable amounts of FIV provirus in lymph node, spleen, thymus, bone marrow and brain in addition to peripheral blood. The level of virus activity (i.e., messenger RNA) was not determined.

Histologically, lymph node, spleen, bone marrow and thymus sections of the infected cats (both treated and untreated) were within the range of the age-matched controls. The lack of histological change correlates with the lack of demonstrable disease and with the status of these animals as "asymptomatic carriers". Rideout et al. reported a lack of FIV-specific lentiviral induced change in lymph node sections from cats with naturally acquired FIV infection in all stages of disease (35). It is true that many of these animals experienced periodic bouts of illness, but most of the clinical signs observed regressed with or without supportive care or simply were not obvious histologically. Many of the infected animals had low levels of T-lymphocytes for much of the experimental period and perhaps if not housed under specific pathogen-free conditions would have progressed to frank AIDS-like disease within the time period examined. Otherwise, like HIV-1 infection and typical FIV infection in the field, the cat must be infected for several years before immune deterioration lends itself to the development of AIDS. This work suggests a long term benefit to early AZT therapy, for instance in cases of occupational exposure or when time of exposure is known, if administered within the right time frame.
and at the right dose. The benefit of AZT administration in cats which do not exhibit frank disease remains questionable.

References


Table 1.  Clinical Signs of FIV Infection in AZT-Treated and Untreated, FIV inoculated Cats.

<table>
<thead>
<tr>
<th>Clinical Signs</th>
<th>FIV n = 4</th>
<th>AZT-Treated n = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>ocular opacity</td>
<td>1/4</td>
<td>4/7</td>
</tr>
<tr>
<td>conjunctivitis</td>
<td>ND</td>
<td>2/7</td>
</tr>
<tr>
<td>alopecia</td>
<td>ND</td>
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<tr>
<td>seborrhea</td>
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<tr>
<td>lymphadenopathy</td>
<td>2/4</td>
<td>2/7</td>
</tr>
<tr>
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<td>1/4</td>
<td>ND</td>
</tr>
<tr>
<td>cachexia</td>
<td>3/4</td>
<td>2/7</td>
</tr>
</tbody>
</table>

ND: nothing documented
Table 2. Polymerase chain reaction and viral antigen results from tissues of AZT-treated and untreated, FIV inoculated cats.

<table>
<thead>
<tr>
<th>Animal</th>
<th>FIV Status*</th>
<th>Tissue</th>
<th>DNA**</th>
<th>Protein***</th>
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<tbody>
<tr>
<td>4805(cont.)</td>
<td>-</td>
<td>lymph node</td>
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<td>-</td>
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<tr>
<td>4806(cont.)</td>
<td>-</td>
<td>lymph node</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4807(cont.)</td>
<td>-</td>
<td>lymph node</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4671(AZT)</td>
<td>+</td>
<td>lymph node</td>
<td>-</td>
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<td></td>
<td></td>
<td>thymus</td>
<td>+</td>
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<td>spleen</td>
<td>+</td>
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<td></td>
<td>brain</td>
<td>+</td>
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<td></td>
<td></td>
<td>bone marrow</td>
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<td>-</td>
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<td>thymus</td>
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<td>brain</td>
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<td>brain</td>
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<td>4796(FIV)</td>
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<td>bone marrow</td>
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<td>lymph node</td>
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*FIV inoculation positive/negative
**FIV-gag sequences amplified
***protein level determined by FIV p26 ELISA
AZT: AZT treated, prophylactic treatment experiment
Cont.: untreated, uninoculated control
Figure 7. Long term effects of prophylactic AZT therapy on CD4 lymphocyte numbers. Mean (+/- S.E.) values for the AZT-treated (n=6) (○ =), FIV-inoculated, untreated cats (n=6) (●). Significant differences between AZT-treated and untreated cats were noted at pi below 7 (p = .0087), 10(p = .041), 11(p = .0087), 12(p = .0043), 15(p = .0318) and 48(p = .0318) as determined by Mann-Whitney U Test. Shaded area denotes the variances of mean values obtained from control cats (n=5).
Figure 7

CD4 LYMPHOCYTES x10^6 /m

- • UNTREATED
- ○ AZT TREATED

AZT TREATMENT

WEEKS

Figure 7
Figure 8. Long term effects of prophylactic AZT therapy on CD8 lymphocytes numbers. Mean (+/- S.E.) values for the AZT-treated (n = 6) (○), FIV-inoculated, untreated cats (n = 6) (●). Significant differences between AZT-treated and untreated cats were noted at pi weeks 7 (p = .0022), 10 (p = .0260), 11 (p = .0043), 12 (p = .0022), 15 (p = .0079), 24 (p = .0318) and 48 (p = .0159) as determined by Mann-Whitney U Test. Shaded area denotes the variances of mean values obtained from control cats (n = 5).
Figure 8
Figure 9. Long term effects of prophylactic AZT therapy on CD4/CD8 ratios. Mean (+/- S.E.) values for the AZT-treated (n = 6) (○), FIV-inoculated, untreated cats (n = 6) (●). Significant differences between AZT-treated and untreated cats were noted at pi weeks 12(p = .0152) and 52(p = .0159) as determined by Mann-Whitney U Test. Shaded area denotes the variances of mean values obtained from control cats (n = 5).
Figure 9

CD4/CD8 RATIOS

AZT TREATMENT

WEEKS

UNTREATED

AZT TREATED

Figure 9
Figure 10. Long term effects of prophylactic AZT therapy on anti-FIV antibody response. Mean (+/- S.D.) values of the AZT-treated (n = 6) (○), and FIV-inoculated, untreated cats (n = 6) (●). There were no significant differences between treated and untreated cats at any time point as determined by Mann-Whitney U Test.
Figure 10

Browser treatment weeks.
Plate III. Polymerase chain reaction results from peripheral blood cell DNA--AZT Treatment in chronic infection experiment. (TOP) Lane 1, molecular weight marker; 2, reaction mix, no DNA; 3, 4807-negative control cat; 4, 4650 (treatment 3); 5, 4658 (tr. wk. 3); 6, 4659 (tr. wk. 3); 7, 4671 (untreated); 8, 4672 (unt.); 9, 4787 (tr. wk. 3); 10, 4790 (unt.); 11, 4791 (unt.); 12, 4795 (unt.); 13, 4796 (tr. wk. 3); 14, 4797 (tr. wk. 3); 15, 4798 (tr. wk. 3). (BOTTOM) Lane 1, molecular weight marker; 2, 4804, (tr. wk. 3); 3, 10ng FIV positive DNA in 500ng background negative DNA; 4, 4650 (5 mo. post treatment); 5, 4667 (unt.); 6, 4659 (5 mo. post tr.); 7, 4671 (unt.); 8, 4672 (unt.); 9, 4787 (5 mo. post tr.); 10, 4790 (unt.); 11, 4796 (5 mo. post tr.); 12, 4797 (5 mo. post tr.); 4798 (5 mo. post tr.); 4804 (unt.).
PLATE III

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Plate IV. EcoRI enzyme digested DNA from tissue samples. (TOP) Lane 1, molecular weight markers (λ HindIII digested) (MW); 2, 4790 bone marrow (BM); 3, 4797 lymph node (LN); 4, 4796 spleen (SP); 5, 4796 LN; 6, 4790 thymus (TH); 7, 4790 brain (BR); 8, 4671 BM; 9, 4791 BR; 10, 4658 TH; 11, 4671 TH; 12, 4804 BM; 13, 4797 SP; 14, 4658 TH; 15, 4805 SP; 16, 4671 SP; 17, 4797 BR; 18, 4791 TH; 19, 4795 SP; 20, 4790 SP. (BOTTOM) Lane 1, MW; 2, 4671 BR; 3, 4807 LN; 4, 4791 BR; 5, 4796 BR; 6, 4804 LN; 7, 4791 LN; 8, 4796 TH; 9, 4805 LN; 10, 4671 LN; 11, 4790 LN; 12, 4806 LN; 13, 4658 BR; 14, 4804 SP; 15, 4658 LN; 16, 4791 SP; 17, 4795 BR; 18, 4797 TH. Cats 4671, 4790, 4791, 4795, and 4804 were AZT-treated. Cats 4658, 4796, and 4797 were FIV-inoculated, untreated. Cats 4805, 4806 and 4807 were uninoculated, untreated controls.
Plate V.

A. Polymerase chain reaction results from tissue samples DNA. (TOP) Lane 1, molecular weights; 2, reaction mix, no DNA; 3, 4805 spleen (SP); 4, 4805 lymph node (LN); 6, 4806 LN; 6, 4807 LN; 7, 4658 LN; 8, 4658 thymus (TH); 9, 4658 SP; 10, 4658 brain (BR); 11, 4671 LN; 12, 4671 BR; 13, 4671 bone marrow (BM); 14, 4671 SP; 15, 4671 TH. (BOTTOM) Lane 1, molecular weights; 2, 4790 TH; 3, 4790 SP; 4, 4790 BR; 5, 4790 LN; 6, 4790 BM; 7, 4791 LN; 8, 4791 TH; 9, 4791 SP; 10, 4791 BR; 11, 4795 BR; 12, 4795 LN; 13, 4795 SP; 14, 4795 TH; 15, 4796 LN.

B. Polymerase chain reaction results from tissue sample DNA, continued. (TOP) Lane 1, molecular weights; 2, 4796 TH; 3, 4796 SP; 4, 4796 BR; 5, 4797 BR; 6, 4797 LN; 7, 4797 TH; 8, 4797 SP; 9, 4804 BM; 10, 4804 BR; 11, 4804 LN; 12, 4804 TH; 13, 4804 SP; 14, $10^3$ FIV-positive DNA; 15, $10^2$ pg. (Bottom) Lane 1, molecular weights; 2, 10pg FIV-positive DNA; 3, 1pg; 4, 0.1pg; 5, 0.01pg. Cats 4671, 4790, 4791, 4795, and 4804 were AZT-treated. Cats 4658, 4796, and 4797 were FIV-inoculated, untreated. Cats 4805, 4806 and 4807 were uninoculated, untreated controls.
PLATE V

A

B
CHAPTER III
FELINE LEUKEMIA VIRUS AND FELINE IMMUNODEFICIENCY VIRUS
COINFECTION IN THE CAT AS A MODEL OF COFACTOR INFLUENCE ON
RETROVIRAL DISEASE PATHOGENESIS

Abstract

Coinfection with feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) was evaluated for effects on \emph{in vivo} retroviral disease pathogenesis. Cats were infected with FeLV at eight weeks of age and with FIV nine weeks later. Three groups of cats were held as age-matched controls: FeLV-infected, FIV-infected, and virus-naive cats. The following parameters were measured: FeLV and/or FIV antigenemia, anti-FeLV and/or FIV antibody titers, lymphocyte numbers and phenotypes, PCR conversion for FIV, and clinical signs. Virologic parameters showed delayed FIV antigenemia in the dually-infected cats with respect to the FIV-inoculation controls while detection of PCR positive blood cells occurred at approximately three weeks pi for cats in both the FeLV-FIV group and the FIV group. FeLV antigenemia was slightly lower in the dually-infected cats than in the FeLV inoculation controls. Immunoblot reactivity against FIV was delayed in the dually-infected cats but was greater than in the
FIV-infected cats. Anti-FeLV reactivity as measured by immunoblot, however, showed earlier and stronger reactivity in the dually-infected cats than in the FeLV-infected cats. The results suggest that in dual FeLV/FIV infection does not potentiate FIV infection and further, appeared to retard the progression of FIV infection.

**Introduction**

Human immunodeficiency virus (HIV-1) infection is associated with the development of acquired immunodeficiency syndrome (AIDS) (1-3). In the pathogenesis of AIDS there is a variable and often protracted (median 10 years) latency period during which the patient is essentially symptom-free (4,5). The variable response to HIV-1 infection suggests the involvement of cofactors in the development of disease. It is not known what factors definitively lead to disease progression and specifically, the profound CD4 T-lymphocyte depletion which is the hallmark of AIDS (6).

Given the protracted time period between exposure to HIV-1 and clinically apparent disease, many infected people carry the virus for years with no apparent ill-effects. It would seem plausible that the host initially establishes a stable relationship with the virus until the equilibrium is altered. In light of this, there has been an ongoing search for potential cofactors which may shift the balance toward frank disease. There are a number of possible modes of cofactor interaction with HIV-1 and conceivably, no one mode may
be all-inclusive. In general, interactions may be direct (physical association at
the intracellular or molecular level) or indirect (modulation of the host immune
response, cytokine generation). While certain intercurrent microbial infections
have been considered as possible cofactors because they are often encountered
in HIV-1 infected patients (7-11) establishing cause and effect relationships is
often nebulous.

Epidemiologically, disease associations have been made between HIV-1
and: HIV-2 (12), human T-lymphotropic virus type 1 (HTLV-I, 8,13), hepatitis
B (14), Mycobacterium sp. (15), cytomegalovirus (16), and Epstein-Barr virus
(17), Mycoplasma sp.(9), and pneumocystis sp.(18). Immune activation or
immune suppression due to any of these infections may directly or indirectly
potentiate susceptibility to HIV-1 infection and disease. Dual infection with
HTLV-I and HIV-1 has been associated with accelerated progression to AIDS
(8,19).

Studies at the molecular level have shown direct transactivation of HIV-
1 transcription by cytomegalovirus (20), Epstein-Barr virus (21), human
herpesvirus-6 (22), herpes simplex-1 (23), adenovirus (24) and human T-cell
leukemia virus-1 tax gene (25). Gene products from these viruses bind directly
to tat or NF-kB-like sequences in the HIV-1 LTR modulating viral mRNA
transcription (20-25). Interactions at the molecular level or at the level of the
host immune response could render the host incapable of maintaining a latent
HIV-1 infection.
The factors involved in disease progression are many and complex. Sero-epidemiology and *in vitro* experiments have uncovered possible contributors to the pathogenesis of HIV-1 infection (see above) yet it would be valuable to study the role of cofactors in lentiviral infection prospectively and *in vivo*. Animal models of retroviral infection are a logical and indispensable vehicle in which to investigate cofactor contributions to lentiviral infection and disease. The use of animal models which have evaluated cofactor phenomena to date have been limited (26,27). The cat system is unique in that it harbors two naturally occurring retroviruses, the feline leukemia virus (FeLV) and the recently described feline immunodeficiency virus (FIV).

Classified as a type C oncovirus, FeLV has been studied extensively since its discovery in 1964 (28). Classically, FeLV has been associated with leukemogenesis although it is identified with a much broader spectrum of disease in the cat (29). More commonly recognized as FeLV infections are syndromes related to immunosuppression (29,30).

An important feature of FeLV is its presence as an endogenous provirus in the cat (31). This allows for recombination between endogenous FeLV and exogenous FeLV sequences sometime resulting in the generation of viral variants with increased pathogenicity (32,33). Overbaugh et al. have characterized a particularly immunosuppressive isolate of FeLV (FeLV-FAIDS) which causes T-lymphocyte cytopathicity, profound immune deterioration, opportunistic infection and death (34).
Another significant characteristic of FeLV infection is that in most cats the infection exists in a state of latency until it is eventually extinguished by normal cell turnover or clonal deletion, or is activated by stress or intercurrent infection (35). Recently, it was determined that there also may be a state of "quasi latency" in which viral replication is active but sequestered to the lymphoid tissue compartment and remains undetectable by standard clinical diagnostic methods (36,37). This is an important parallel to the HIV-1 story in that there is now evidence to suggest a similar condition exists in the period between the acute phase of infection and the eventual development of AIDS (38,39). Previously, the asymptomatic period was thought to be a state of clinical latency (4).

Infection by FeLV is efficiently amplified in cell types which undergo rapid turnover such as epithelial cells and bone marrow cells (40). While cellular infection generally is thought to be nonlytic, normal cellular functions are often altered. It was determined that a virion envelope protein, p15E, could be directly responsible for the immunosuppression observed in FeLV viremic cats by interfering with T-lymphocyte function via inhibition of IL-2 secretion and downmodulation of IL-2 receptors (41,42). Further, FeLV infection interferes with neutrophil respiratory burst as measured by the chemiluminescence response (43,44).

The route of FeLV transmission is thought to be either through grooming and/or biting as FeLV is secreted in saliva and is found at high titer in the blood
Whether inoculation occurs predominantly via exposure of the oral mucosa to infected blood or saliva or by transfer of blood or saliva to an open wound has not been definitively determined. Clinically, most frequently noted in infected cats are diseases associated with immunodeficiency and cytopenia. Upper respiratory tract infections, gingivitis/stomatitis, uveitis, infectious peritonitis, panleukopenia, toxoplasmosis, and anemia are common (29). FeLV-infected cats in our laboratory setting occasionally succumb to a feline infectious peritonitis-like disease. Cases of uveitis/conjunctivitis, and cystitis and, infrequently, lymphoma induction and spontaneous abortion have been noted. Interestingly, risk factors and clinical syndromes associated with FeLV infection are the same or similar for those of infection with another feline retrovirus, feline immunodeficiency virus (FIV).

A lentivirus, FIV is also indigenous to cats and associated with feline AIDS (46,47). The virus preferentially infects T-lymphocytes (both CD4 and CD8) leading to their eventual depletion but also infects B lymphocytes and macrophages (46,48,49). Further, FIV infection is associated with decreased natural killer cell activity and neutrophil function (50).

More cell-associated than FeLV, FIV is not thought to be spread via grooming behavior but rather by fighting/bloodborne exposure (46,47,51). Infected cats typically carry the virus for an extended period of time before presentation with overt clinical signs compatible with immunodeficiency (47). FIV disease manifestations in the field are similar enough to those of FeLV that
FIV infection must be determined by the presence of specific antibody (29). In our laboratory setting within a two year time period, inoculated cats most frequently show lymphadenopathy and failure to thrive and, occasionally, uveitis/conjunctivitis and cystitis.

Given that FeLV and FIV infections occur with some frequency in the natural cat population and occasionally infect animals simultaneously (some reports estimate the prevalence of dual infection at 10-15%)(51,52), it is important to ask if infection with FeLV potentiates FIV infection (or vice versa) and if resulting conclusions from evaluation of this model are extrapolatable to the human condition.

Pedersen et al. described accelerated development of an AIDS-like disease in cats experimentally infected with FeLV followed by inoculation with FIV (27). Potentiation of immunodeficiency and disease in FeLV/FIV infections may occur on several levels. First, each virus is known to induce immunosuppression by several mechanisms (29): 1) direct infection and cytopathicity of immune cells, 2) direct infection and loss of immune cell function (without cell death) 3) disruption or alteration of normal cytokine and intercellular communicative processes. Whether immunosuppression due to one agent sets the stage for infection and disease with the other is not known. Secondly, these two viruses may interact at the molecular level within the same cell. While FeLV has a relatively simple (and typical for oncoviruses) genome (29) and codes for no known transactivating protein, within its LTR are at least
three major nuclear protein binding sites and several enhancer sequences (52,53). Phillips et al. have demonstrated a putative transactivation element in the genome of FIV which encodes a singly sliced message (54). Limited \textit{in vitro} work by Sparger using FeLV and FIV chloramphenicol acetyltransferase (Cat)-virus LTR constructs shed doubt on the ability of FIV to directly transactivate the FeLV LTR (55). However, it is conceivable that viral gene transactivation (of either virus) could take place by less direct mechanisms such as induction of intracellular DNA-binding proteins which could upregulate viral message transcription (56).

The purpose of the work presented in this chapter was to evaluate whether FeLV infection would potentiate FIV infection in dually-infected cats in order to gain insight into role of dual retroviral infection in disease pathogenesis.

\textbf{Materials and Methods}

\textit{Animals:}

Eight week old specific pathogen-free cats were obtained from a colony maintained at the Department of Veterinary Pathobiology, Ohio State University. The cats were randomly selected and assigned to one of four groups: FeLV inoculation controls (n = 6), FIV inoculation controls (n = 5), FeLV-FIV-inoculates (n = 8), and age-matched uninoculated controls (n = 5). All work was performed in accordance with University Laboratory Animal Care and Use
Committee and by DHEW publication No. NIH 74-23, Guide for the Care and Use of Laboratory Animals.

Virus Inocula and Inoculation Schedule:

a. FeLV: Feline leukemia virus was originally obtained as a tumor homogenate from a cat infected with the Rickard strain of FeLV and serially passaged in vivo as infectious plasma. This laboratory-derived isolate was determined by infection interference to be FeLV subgroup A. Eight-week old anesthetized cats were inoculated intravenously with 0.1 ml of a plasma pool containing 900 focus-forming units/ml. A 100% incidence of infection of weanling animals with this preparation is routinely attained (Mathes, personal communication).

b. FIV: The virus isolate utilized in this study was the FIV Mount Airy; Maryland (MD) strain isolated by Johnson and Olmsted. 1000 TCID₆₀ units were administered by intravenous injection to anesthetized cats. In this case, the animals were 15-16 weeks of age at the time of inoculation.

Sample Collection:

Blood was collected on a weekly basis for complete blood counts (CBC), lymphocyte phenotyping, DNA isolation and serology. Cats were evaluated weekly for weight gain, lymphadenopathy and any signs of illness.
Serology:

Antibody responses were determined by 2 methods:

a. *Live cell immunofluorescence (IFA)* of plasma antibody against chronically infected 3201-FIV target cells (58) for FIV and FL-74 cells for FeLV (59). Briefly, serial 1:2 dilutions of test plasma were made in 96-well microtiter plates. Approximately $10^6$ 3201/FIV or FL-74 cells were added to each well. Following incubation and wash steps, the cells were incubated with the appropriate dilution of fluoresceinated anti-cat IgG. Reactivity was evaluated using a Zeiss Axioplan UV light microscope. The titration endpoint was the highest dilution of 50% positivity.

b. *Immunoblot analysis* of plasma reactivity to specific viral proteins used 3201-FIV and FL-74 cell lysates as sources of antigen. The lysates were prepared by cold lysis in a phosphate-buffered solution containing 1% NP-40, 1 mM EGTA and 0.1 mM phenyl-methyl-sulfonyl fluoride (as a protease inhibitor). Lysates were clarified by centrifugation, the supernatant was diluted in a reducing buffer and boiled for 5 minutes. Three hundred fifty micrograms of protein was loaded per gel. After electrophoresis through a 12% polyacrylamide gel and electrotransfer of the viral proteins to nitrocellulose, the strips were incubated with 1:25 dilutions of plasma. The secondary antibody was a 1:1000 dilution of biotinylated staphylococcus Protein A (Zymed, San Francisco, CA) prepared in phosphate-buffered saline/1% tween 20, followed by incubation with alkaline phosphatase-labelled Extravidin (Sigma, St. Louis,
MO) diluted to 1:5000 in PBS/tween. The blot was developed with the BCIP/NBT alkaline-phosphatase kit (Kirkegaard and Perry, Gaithersburg, MD). Molecular weight standards (Rainbow Markers, Amersham,) were plotted on a log scale and used to extrapolate molecular weights of the reactive bands of the test samples.

**Immunophenotypic Analysis:**

Fresh leukocytes were prepared by whole blood hypotonic lysis, counted for viability and dispensed into wells of a microtiter plate. Mouse anti-feline Pan T, anti-feline CD4 and anti-feline CD8 (Southern Biotechnologies, Birmingham, AL) diluted at 1:100 in 1% bovine serum albumin/PBS solution were used to label peripheral blood cells. Secondary antibody consisted of phycoerythrin-tagged goat anti-mouse IgG Fab\textsubscript{2} fragment (Sigma, St. Louis, MO). Mouse IgG\textsubscript{1} kappa chain served as an isotypic immunoglobulin control. After labelling and fixation in 1% paraformaldehyde, the samples were analyzed by an EPICS 753 flow cytometer (Coulter, Hialeah, FL). Percent positives were determined by the Immuno program of the E.A.S.Y. 88 sample analysis package (Coulter Electronics, Hialeah, FL). Results, expressed as numbers of CD4 and CD8 lymphocytes, were obtained by multiplying percent positive populations from immunostain with CBC lymphocyte values.
Plasma Antigenemia and Cell Culture Antigen:

FIV p26 and FeLV p27 levels were determined by commercial ELISA (IDEXX, Westbrook, ME and Synbiotics, San Diego CA, respectively) consisting of an antibody sandwich format with the second antibody directly labelled with horseradish peroxidase. In the case of FeLV, results are expressed as optical density values. For FIV, picograms/ml plasma (or culture supernatant or cell lysate) values were obtained by extrapolation against a standard curve. The level of detection of the FIV antigen ELISA was repeatedly determined to be \( \geq 42 \) pg/ml.

Polymerase Chain Reaction:

A "hot start" nested polymerase chain reaction (PCR) technique, was used to amplify FIV-\textit{gag} specific sequences from 500 ng DNA isolated from peripheral blood. Briefly, DNA was isolated from washed blood cells with quanidine HCl and proteinase K treatment (60), quantitated by DNA fluorometry (61), digested with EcoRI restriction enzyme. Five-hundred ng samples of purified DNA was amplified with the 927,979 primer pair in tubes in which primers, magnesium, and deoxynucleotides were separated from Taq polymerase, buffer and DNA by a wax bead. The control panel consisted of serial 1:10 dilutions of FIV-positive DNA (100 ng to 0.1 pg) in a background of FIV-negative DNA. Following initial amplification, 10ul of the amplified product was used as a template in a second PCR using the 667/668 primer pair.
Amplified products were visualized by UV transillumination of an ethidium bromide stained agarose gel.

Statistics:

Differences in lymphocyte phenotype results, antiviral antibody responses and antigenemia levels between groups were determined by Mann-Whitney nonparametric test (Instat, Graph Pad). Results were expressed as mean and standard error of the mean.

Results

Clinical Signs:

Clinical signs documented during the experimental period are presented in Table 3. There is a trend toward increased prevalence of disease-associated signs in the FeLV and FeLV-FIV groups when compared to the FIV-only group. However, due to the small numbers of documented clinical signs no further correlation could be made.

Total lymphocytes:

Lymphocyte numbers were within the control range for the FeLV, FIV and FeLV-FIV groups out to 26 weeks post FeLV inoculation and 17 weeks post FIV inoculation with the exception of week 2 post FIV inoculation when both the FIV and FeLV-FIV groups dropped beneath the control range (Figure
From 22 to 32 weeks of the study, lymphocyte numbers in the dually infected cats were consistently less than the control range but appeared stable.

**CD4 lymphocytes:**

The numbers of CD4 lymphocytes were not significantly different between the FeLV, FIV and FeLV-FIV groups at any time post inoculation (Figure 12). During the first 19-21 weeks of FeLV infection, the values for the FeLV-inoculates were within the control range. The FIV-inoculates and the FeLV-FIV inoculates CD4 numbers dropped below the normal range within 2 weeks of FIV inoculation. Following an initial recovery, between weeks 5 and 8 after FIV inoculation, CD4 numbers for the FIV inoculates steadily declined out to the 29 week time point. The dually infected cat CD4 values remained at the level reached by week 2 post FIV inoculation and did not undergo any perceived increase during the sampling period.

**CD8 lymphocytes:**

CD8 lymphocyte numbers were within the control range for the FeLV group out to 22 weeks pi (Figure 13). Values for the FIV group but not the FeLV-FIV group, dropped below the control range at weeks 2 and 3 post FIV inoculation. CD8 levels for the FeLV and FeLV-FIV groups remained static for the remaining time points while those for the FIV group recovered from the initial drop at weeks 2-3 pi, increased slightly out to 11 weeks pi then began
a steady decline for the remainder of the study period. Values between the FIV, FeLV and FeLV-FIV groups were not statistically different at any time point.

**CD4/CD8 ratios:**

CD4/CD8 ratios for the FeLV group remained within the control range out to 23 weeks pi (Figure 14). Ratios for the FIV and FeLV-FIV groups were similar and clustered below the control range beginning 5 weeks post FIV challenge.

**FeLV antigenemia:**

Plasma FeLV antigenemia was slightly but not significantly lower in the FeLV-FIV group except at weeks 2 and 4 post FIV inoculation (Figure 15).

**FIV antigenemia:**

Plasma FIV antigenemia was delayed in appearance by 1 week and was lower (peak mean 100 pg/ml) in the FeLV-FIV group compared to the FIV group (peak mean >200 pg/ml) (Figure 16). However, FIV antigenemia was cleared less rapidly by the FeLV-FIV group (duration 3 weeks) than the FIV group (duration 1.5 weeks). Following the initial antigenemic phase, plasma p26 was below the level of detection for the remainder of the experimental period.
**Anti-FIV antibody responses:**

Anti-FIV antibody responses measured by live cell immunofluorescence assay was detectable one week earlier in the FeLV-FIV group than in the FIV group (Figure 17). By immunoblot assay, dually infected cats showed reactivity to FIV \textit{gag} precursor, pp52, \textit{env} transmembrane gp41 and major \textit{gag}, p26 beginning 4-6 weeks post inoculation (Table 4, representative immunoblots Plates VI and VII). In comparison, FIV-inoculates showed the same reactivity detectable beginning 2 weeks pi.

**Anti-FeLV antibody responses:**

Anti-FeLV antibody titers as measured by live cell immunofluorescence assay was similar between the FeLV and FeLV-FIV groups during the experimental period reaching a plateau at 5 weeks post FeLV inoculation (Figure 18). By immunoblot assay, dually-infected cats showed the strongest reactivity against FeLV proteins: FeLV-infected control reactivity was detectable after 18-20 weeks pi if at all (Table 5, representative immunoblots Plates VI and VIII). Reactivity was detected against \textit{gag}-precursor, pp52, integrase p46 and infrequently, major core, p27.
**Polymerase chain reaction results:**

Nested PCR results for FIV provirus in peripheral blood samples for the FIV and FeLV-FIV groups are shown in Table 6. In the FeLV-FIV group 4/5 cats were PCR positive by week 3 post FIV inoculation and 4/5 cats in the FIV group were detectable at week 3 pi.

**Discussion**

For retrovirus-infected cats, disease progression may take place over several years for some animals while others succumb much more rapidly. It is not known what triggers the final descent into immunodeficiency disease and eventual death. Possible factors include genetic makeup of the animal, age, immune status at the time of infection, intercurrent infection and/or viral strain. For humans, genetic traits, the degree of lymphocyte activation and immunocompetent state at the time of HIV-1 exposure are all considered as contributing factors in the progression to disease (62).

The results of the current study suggest that FeLV preinfection resulted in a delay in the progression of FIV infection. FIV plasma antigenemia was detectable between weeks 1.5 and 3 pi with a peak mean of >200 pg/ml for the FIV infected cats while for the dually infected cats, antigenemia was detectable between weeks 2 and 4 with a peak mean of 100 pg/ml. FIV antigenemia was undetectable in both groups for the remainder of the experimental period. The level of FeLV antigenemia dropped slightly in the
dually infected cats within one week of FIV challenge (10 weeks post FeLV challenge) while FeLV-infected cats showed a decline in plasma antigenemia to the level of the FeLV-FIV group beginning at week 12 pi. Thereafter, FeLV antigenemia was at a comparable level for the two groups.

Total lymphocytes were within the control range for the FeLV-inoculates, the FIV-inoculates and the dually inoculated cats to 25 weeks post FeLV inoculation and 16 weeks post FIV inoculation with the exception of a brief decline in the FIV and FeLV-FIV groups at week 2 post FIV inoculation and a trend toward decline in the FIV and FeLV-FIV groups at 19 and 18 weeks post FIV inoculation, respectively.

CD4 lymphocyte numbers were dramatically reduced in the FIV inoculates between weeks 2-5 pi. A comparable decrease was not noted in the FeLV inoculated cats and was slight in the FeLV-FIV cats. Thereafter, CD4 lymphocyte numbers were generally highest in the FeLV group and lowest in the dually infected animals and these values remained relatively stable throughout the observation period.

CD8 lymphocyte numbers also showed a brief drop between 2-4 weeks pi in the FIV group but not the FeLV or FeLV-FIV groups. Cats in the FeLV-only group maintained CD8 levels within the control range to 22 weeks pi followed by a decline to below the control range by week 24 pi. For the FIV-only group, aside from the initial transient decline, CD8 numbers were within the control range to 14 weeks pi while the dually-infected cats fell below the control range
by week 10 pi. The results for the dually infected cats were compatible with lack of age-related increase. The CD8 values initially rose in both the FeLV and FIV groups then began to decline when the animals reached 21 and 12 weeks pi, respectively.

CD4/CD8 ratios were maintained at the low end or below the control range for the FeLV-FIV cats starting at week 4 post FIV inoculation, for the FIV group starting at week 5 post FIV inoculation and for the FeLV group at week 15 post FeLV inoculation. No true, sustained ratio inversion was noted for any of the groups at any time point. This is in contrast to the reported inversion of CD4/CD8 ratios in 4/5 dually infected cats but not in any FIV or FeLV infected cats in the Pedersen study (27).

The effect of FeLV infection on lymphocyte population fluxes has not been reported previously. FeLV infection appeared to have an effect similar to that of FIV infection on CD4 and CD8 lymphocyte populations. In contrast to the age-related increase noted in the control cats, FeLV-infected cats showed a lack of lymphocyte population expansion in the circulation during the time period examined. In addition, the FeLV-infected cats that later were inoculated with FIV did not show the short-term drop in circulating CD8 lymphocytes that was noted in the FIV-inoculates between 1 and 4 weeks post FIV-inoculation. The CD4 lymphocytes from the FeLV-FIV cats showed less of a decrease at that same time point than the FIV group. One could speculate that immune suppression mediated by FeLV infection could make lymphocytes less able to
respond to FIV (i.e., proliferate due to exposure to antigen) and therefore are less susceptible to FIV infection. It is also possible that the signals normally received by lymphocytes to home to a lymph node in order to be exposed to antigen are not operable (perhaps due to a defect in the cytokine network or antigen presentation) (11).

The anti-FeLV antibody response as measured by immunofluorescence (FOCMA test) (58) showed no difference between FeLV and FeLV-FIV cats at any time point. However, by immunoblot, the dually-infected cats had measurable responses to gag precursor, endonuclease and major core proteins which was evident at least 4 weeks earlier than in the FeLV cats. Generally, the FeLV-infected cats did not show detectable reactivity by immunoblot until at least 24 weeks pi. Immunofluorescence assay for the anti-FIV antibody response was very similar between the FeLV-FIV and FIV groups. Reactivity to individual FIV proteins measures by immunoblot was evident in the FIV group by approximately 2 weeks pi and by 4 weeks pi in the dually infected cats. Both the FeLV-FIV and FIV groups had antibodies against FIV gag precursor, envelope transmembrane and major core proteins. Reactivity to major envelope glycoproteins of either FeLV or FIV was not detectable by immunoblot analysis.

Concurrent infection with HTLV-I and HIV-1 is associated with a more rapid AIDS-related decline (8,13). A primary cellular target of infection with both viruses is the CD4-bearing T lymphocyte (63-66). Within the same cell the tax protein of HTLV-I, a known transactivator of interleukin 2 and
interleukin 2 receptor genes, also is a capable transactivator of the tat gene of HIV-1 (25). Increased HIV-1 replication may be due to increased T-cell activation or to upregulated HIV-1 gene transcription leading to loss of the infected cell. Unlike HTLV-I, FeLV does not code for any known transactivator while FIV is suggested to include a putative transactivating element in its genome (54,67). The results reported here suggest that prior FeLV infection actually retards FIV pathogenesis. The nature of FeLV infection and pathogenesis would purport that FeLV-infected lymphocytes (and other cell types) would be less active and able to function appropriately in response to infection with a second pathogen. Both FeLV-infected lymphocytes and FIV-infected lymphocytes lose mitogen responsiveness and therefore, are probably not good targets for secondary retroviral infection (37,38,46).

Pedersen et al. (27) reported expanded FIV infection in cats dually infected with FeLV and FIV. It is conceivable that FIV infection enhances FeLV infection as well. Clinical manifestations of infection with either FeLV or FIV are difficult to separate. In that report, the criteria used to determine enhancement of FIV infection was the increased detection of FIV-DNA in tissues of the dually infected cats. Nothing was stated concerning the level of activity of FIV (ie, mRNA levels) nor was FIV antigenemia measured. Further, the authors cited a lack of increase in FeLV plasma antigenemia as the basis for the observation that FeLV infection was not enhanced. Meanwhile, the prevalence of FeLV in tissues was not determined.
There are currently two schools of thought surrounding the progression to AIDS in humans based largely on epidemiology and in vitro work (19-25,62). The cofactor hypothesis purports the necessity of immune activation or immune suppression due to chemicals (drugs, hormones, cytokines) or microbial infection for disease progression (68). These agents may act directly on the viral genome (transactivation) or amplify infection by stimulating the proliferation of virus-susceptible cells. The second hypothesis asserts that the outgrowth of a particularly virulent viral genotype is responsible for developing full-blown AIDS (68). A thorough evaluation of this issue is central to understanding the pathogenesis of retroviral disease. Importantly, the feline retrovirus models can provide valuable insight into particular pathogenic mechanisms.

References


Table 3. Clinical signs of FeLV-FIV, FIV and FeLV infection.

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* Feline infectious peritonitis-like syndrome.
** Dermatitis (1), rectal inflammation (1).
ND nothing documented
Table 4. FIV Immunoblot Results

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FF: FeLV-FIV infected
FIV: FIV-infected
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FF: FeLV-FIV infected
FeLV: FeLV-infected
Table 6. Polymerase chain reaction results for FIV DNA in peripheral blood cell samples from FeLV-FIV and FIV infected cats.

FIV DNA PCR Results

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ND = Not Done  
FF = FeLV-FIV coinfected  
FIV = FIV infected only
Figure 11. Total lymphocyte numbers for the FeLV-FIV dual infection study. ■ FeLV (n = 6), ● FIV (n = 5), ▲ FeLV-FIV (n = 8). Shaded area denotes the variances of mean values obtained from control cats (n = 5). There was no significant difference between the FeLV, FIV and FeLV-FIV groups at any time point as measured by Mann-Whitney U test.
Figure 11

*MEAN +/- S.E.
Figure 12. CD4 lymphocyte numbers for the FeLV-FIV dual infection study. ■ FeLV (n = 6), ● FIV (n = 5), ▲ FeLV-FIV (n = 8). Shaded area denotes the variances of mean values obtained from control cats (n = 5). There was no significant difference between the FeLV, FIV and FeLV-FIV groups at any time point as measured by Mann-Whitney U test.
CD4 LYMPHOCYTES X 10^6/ml

*Mean +/- S.E.

Figure 12

- FeLV
- FeLV-FIV
- FIV
Figure 13. CD8 lymphocyte numbers for the FeLV-FIV dual infection study. ■ FeLV (n = 6), ● FIV (n = 5), ▲ FeLV-FIV (n = 8). Shaded area denotes the variances of mean values obtained from control cats (n = 5). At pi week 11, the FeLV-FIV values were significantly higher than the FIV values (p = .0062) as measured by Mann-Whitney U test.
Figure 13

CD8 LYMPHOCYTES $\times 10^6$/ml

*MEAN +/- S.E.

WEEKS

FeLV
FeLV-FIV
FIV

Figure 13
Figure 14. CD4/CD8 ratios for the FeLV-FIV dual infection study. ■ FeLV (n = 6), ● FIV, ▲ FeLV-FIV (n = 5). At pi week 11, the FIV values were significantly higher than the FeLV-FIV values (p = .0295) as measured by Mann-Whitney U test.
CD4/CD8 RATIO

FeLV
FeLV-FIV
FIV

6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
WEEKS

*MEAN +/- S.E.

Figure 14
Figure 15. Effect of FeLV-FIV dual infection on FeLV antigenemia. ▲ FeLV-FIV (n = 8), ■ FeLV (n = 6). There were no significant differences between the two groups at any time point as measured by Mann-Whitney U test.
Figure 15

ELISA OPTICAL DENSITY

* MEAN +/- S.E.
Figure 16. Effect of FeLV-FIV dual infection on FIV antigenemia. ▲ FeLV-FIV (n = 8), ○ FIV (n = 5). The FIV values were significantly higher at pi weeks 0.5 (p = .0006) and 1 (p = .0019). The FeLV-FIV values were significantly higher at pi week 2.5 (p = .0058) as measured by Mann-Whitney U test.
Figure 16
Figure 17. Effect of FeLV-FIV dual infection on FIV antibody response. ▲ FeLV-FIV (n = 8), ● FIV (n = 5). There were no significant differences between the two groups at any time point as measured by Mann-Whitney U test.
Figure 17

**MEAN +/- S.E.**
Figure 18. Effect of FeLV-FIV dual infection on FeLV antibody (FOCMA) response. ▲ FeLV-FIV (n = 8), ■ FeLV (n = 6). There were no significant differences between the two groups at any time point as measured by Mann-Whitney U test.
Figure 18

GEOMETRIC MEAN TITER

WEEKS

FIV
FeLV
Plate VI. Representative immunoblot for FeLV-FIV dual infection.
PLATE VI

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Plate VII. Representative immunoblot for FIV infection.
PLATE VII

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Plate VIII. Representative immunoblot for FeLV infection.
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PLATE VIII

FeLV 4884

pp52
CHAPTER IV
EVALUATION OF THE INVOLVEMENT OF TUMOR NECROSIS FACTOR
ALPHA IN THE PATHOGENESIS OF FELINE IMMUNODEFICIENCY VIRUS
INFECTION

Abstract

Tumor necrosis factor alpha (TNF) was evaluated for its role in the pathogenesis of FIV infection. A series of cell lines (3201, 3201/FIV, FL-74, FL/FIV, Molt4, Molt4/FIV and HL-60) were treated with doses of TNF (100 and 10 ng/ml) to assess effects on cell growth and FeLV or FIV antigen production. Only 3201, 3201/FIV and HL-60 cells demonstrated cytotoxicity by TNF. The other cell lines were unaffected. FeLV or FIV antigen production by the infected cell lines was unchanged. Neutralizing anti-TNF antibody was able to abolish TNF cytotoxicity in a dose-dependent manner. These same cell lines were tested for ability to consume TNF from the culture medium as a crude way to determine the presence of receptors. The HL-60, U937, 3201 and 3201/FIV cell lines consumed most of the TNF in the culture supernatants by 24 hours. Molt4, Molt4/FIV, FL-74 and FL/FIV showed a general lack of TNF consumption.
In primary mononuclear cell cultures from both FIV-infected and control animals, TNF was induced by phorbol ester and calcium ionophore treatment. Low but detectable levels of FIV antigen expression were also noted in 3/12 samples of PBMC from FIV infected cats by this treatment. The effect of TNF on FIV antigen induction in primary mononuclear cell cultures was negative.

Introduction

Cytokines are essential intermediaries of cellular communication. Tumor necrosis factor-alpha (TNF) is a cytokine primarily derived from monocyte/macrophage cells which serves a fundamental role in inflammation and the acute phase response (1-6). Although initially noted as a soluble factor involved in tumor necrosis and regression (7), TNF is now known to elicit a wide array of biological effects including increased expression of endothelial adhesion and MHC class I/II molecules, prostaglandin E$_2$ production by synovial cells and fibroblasts, increased fibroblast proliferation, induction of bone resorption by osteoclasts, IL-1 induction by macrophages, and suppression of lipoprotein lipase which mediates the cachexia of chronic disease (8).

Importantly, TNF has been associated with antiviral activity demonstrable by cytolysis of virally-infected cells and as such likely is important in the host defense against viral infection (9-12). Cytotoxicity may be directly mediated by TNF or indirectly by increased recognition of virally-infected cells by cytotoxic T-cells in response to upregulation of MHC class I expression and viral
protein expression (13-17). Virus-infected cells may be selectively susceptible (compared to their uninfected counterparts) to direct TNF cytotoxicity if the viral infection interferes with the production of protective proteins by the cell (18,19). Resistance of normal cells and some tumor and virus-infected cell types has been correlated with the ability to synthesize superoxide dismutases and heat shock proteins of the HSP28 and HSP70 families (18-20). Resistance can be abrogated by interference with de novo protein synthesis by such agents as cycloheximide and actinomycin D (21). A second mode of TNF antiviral activity is via stimulation of interleukin 6 (IL-6) (22) which, in turn upregulates cell surface receptors for TNF (22). TNF may also act in concert with gamma interferon to damage some virus-infected cells leading to their eventual destruction, and to protect uninfected cells from infection (23).

Receptors for TNF are present on most somatic cell types and cells lines with the exception of resting lymphocytes, erythrocytes and Epstein-Barr virus-transformed B-cells (24-26). Lymphotoxin, also called a TNF-beta product of T-lymphocytes utilizes the same receptors (27). There are two types of TNF receptors, 55-60 kD and 75-80 kD in size, which are coexpressed on the cell surface but differ in relative amounts of expression between cell types (28,29). The importance of this is that binding of TNF to one receptor type or the other (in addition to binding affinity) governs, to some extent, the effect of TNF (cytotoxicity, cytostasis or proliferation) on the cell (27-29). The pleiotropic
effects of TNF, therefore, are dictated by differential activation of intracellular signalling pathways (30).

Upon receptor binding, a cascade effect in intracellular signalling takes place: TNF-bound receptors stimulate G protein phosphorylation which, in turn, signals phospholipase A₂ to hydrolize membrane phospholipids releasing arachidonic acid and diacylglycerol (31). Arachidonic acid release from membrane phospholipids likely contributes to cytotoxicity by promoting production of oxygen radicals leading to oxidative damage if the cell does not have normal hydroxyl radical scavenging capability (20). Diacylglycerol stimulates protein kinase C to phosphorylate a number of intracellular moieties including the IkB-NFkB complex (14). Phosphorylation of IkB releases NFkB which is a DNA-binding protein that binds to a regulatory element (kB) associated predominantly with the immunoglobulin kappa light chain gene (32). The kB transcriptional enhancer element is found in regulatory regions of many genes, including those for many cytokines and inducible heat shock proteins (32). In addition, viruses such as cytomegalovirus (33), SV-40 (34), HIV-1 and 2 (34,35), SIV (34,35), HTLV-1 (36) and FIV (37) also have one or more copies of the kB element included within their genomes. It is reasonable to consider that TNF may also activate transcription of these viruses in infected cells via activation of NFkB.

*In vitro*, TNF has been demonstrated to selectively kill HIV-1 infected T-cell lines (38,39). However, HIV-1 replication was enhanced in these cell lines.
From this two questions were asked: 1) are HIV-1 infected cells stimulated to produce TNF and 2) is cell killing due to direct TNF cytotoxicity or to TNF-enhancement of virus expression? Peripheral blood mononuclear cell cultures isolated from HIV-1 infected patients and infected cell lines have shown increased production of HIV-1 in response to TNF exposure (38,39). Further, elevated serum levels of TNF have been reported in AIDS patients (40). Interestingly, plasma antigenemia was increased upon therapeutic administration of TNF to AIDS and ARC patients (41).

The involvement of TNF in host defense and potentiation of disease presents itself as a double-edged sword. In HIV-1 infection, the primary immune cells, infected T-lymphocytes, may be killed by either the virus infection directly or by the immune response against the virus-infected cell. On the other hand, infected macrophages, involved in initiating the immune response (via antigen presentation) may be activated in an autocrine manner to amplify the infection while at the same time being immune to the cytolytic effects of either the virus or TNF.

There are many unanswered questions about the role of TNF in retrovirus pathogenesis. The purpose of this work was to explore the role of TNF in the FIV infection model and to test the hypothesis that TNF-α selectively kills FIV-infected cells in vitro and results in increased FIV-antigen expression as reported for HIV-1 infected cells in vitro (39-40).
Materials and Methods

TNF:

Recombinant human tumor necrosis factor alpha (TNF-α, Genzyme Corp., Cambridge, MA) was diluted to 1 μg/ml stock concentration in tissue culture medium containing 10% fetal calf serum. For the virus induction and cytotoxicity experiments, TNF was used at 100, 10, and 0 ng/ml. For TNF absorption studies, 100 and 50 pg/ml was used.

Anti-human TNF antibody:

Rabbit polyclonal antibody to human TNF (Genzyme, Cambridge, MA) preincubated at 1000, 2000, or 4000 neutralizing units with 100 ng/ml TNF for 30 min. at 37°C.

TNF ELISA:

The Predicta Human TNF ELISA kit was purchased from Genzyme (Cambridge, MA). The assay utilized a solid phase anti-TNF monoclonal antibody, a biotinylated secondary antibody and peroxidase-labelled streptavidin. Samples were run concurrently with a standard curve. The amount of TNF in samples was determined by extrapolation against the standard curve. The level of assay sensitivity was 12 pg/ml.
**Cell Proliferation/Viability:**

Cell growth was assessed by the colorimetric MTT assay (Chemicon, Temecula CA). MTT, a tetrazolium bromide salt that when cleaved by living cells yields an insoluble purple formazan product. The formazan crystals were then dissolved in an SDS/formamide solubilizing buffer overnight. Optical density was determined at 580 nm. Enumeration and viability measurements also were performed by trypan blue exclusion/cell counts by hemacytometer.

**Viral Antigen Measurement:**

Antigen for FeLV and FIV were measured by ELISA (Synbiotics, San Diego, CA and IDEXX, Portland, ME, respectively). Results for FeLV were presented as optical density while FIV results were presented as pg/ml of p26 based on extrapolation from a standard curve. The level of sensitivity of the FIV ELISA was 46 pg p26/ml.

**TNF Cytotoxicity Assay:**

Cells (see cell lines, described below) in log phase growth were seeded into 24-well plates (Costar, Cambridge, MA) at a concentration of $2 \times 10^5$ cells. TNF was added to achieve concentrations of 100, 10, and 0 ng/ml. Samples were collected at 24 hour intervals for cell counts/viability, MTT, and antigen production.
In a separate experiment, PBMC were isolated from both FIV-infected and control animals and evaluated for TNF toxicity and TNF-induction of FIV antigen expression.

**TNF Absorption Assay:**

As a crude method to measure the presence of TNF receptors on the cell lines, consumption from tissue culture medium of exogenously added TNF was analyzed. Cells in log phase growth were seeded at a density of $1 \times 10^5$ cells/well in 24-well plates. TNF was added at concentrations of 100, 50 and 0 pg/ml. Wells of TNF without cells were also included as stability controls. Samples of culture supernatants were collected at 0, 8 and 24 hours post exposure and tested for the amount of TNF remaining in the culture supernatant by TNF ELISA.

**TNF Induction Experiment:**

The ability to stimulate peripheral blood mononuclear cells (PBMC) to produce TNF was assessed (42). PBMC were isolated from freshly collected blood samples from both FIV-infected and control animals by standard ficoll separation. Mononuclear cells (lymphocytes and monocytes) were enumerated and seeded in 24-well plates at a density of $10^6$/ml. Duplicate wells were treated with phorbol ester and calcium ionophore (phorbol myristate acetate + A23187) at concentrations of 100ng/ml and 0.5 ug/ml, respectively, or were
untreated. Samples were collected for antigen and TNF production at 24 hour intervals.

**Cell Lines:**

1) **3201 CT:** feline lymphoma, exogenous FeLV negative, expresses endogenous FeLV and RD-114.

2) **3201 FIV:** 3201 cell line chronically infected with the Petaluma strain of FIV (43). The culture has consistently shown 100% virus expression by immunofluorescence assay. Produces ng/ml amounts of FIV in culture supernatants.

3) **FL-74:** feline lymphoma cell line, chronically infected with KT strain of FeLV.

4) **FL-FIV:** FL-74 cell line chronically coinfected with 3201-FIV-selected FIV. Produces high levels of FeLV and FIV in culture supernatants.

5) **Molt 4 CT:** human lymphoid cell line.

6) **Molt 4 FIV:** Molt 4 cell line which harbors stably integrated FIV provirus (as measured by PCR) but does not express FIV proteins.

7) **HL-60:** human promyelocytic leukemia cell line which is highly inducible for production of TNF by phorbol ester treatment and is highly sensitive to cytotoxicity by TNF treatment.

8) **U937:** human promyelocytic cell line, TNF resistant.
Results:

**TNF Cytotoxicity in Cell Lines:**

In titration studies, TNF treatment resulted in dose dependent cytotoxicity of HL-60, 3201, and 3201/FIV cells measurable by MTT and cell counts within 24-72 hours of treatment (Figures 19-21). FL-74, FL/FIV, Molt 4 and Molt 4/FIV were unaffected even at the highest dose level (Figures 23-26). Cytotoxicity was abolished in 3201/FIV and HL-60 cells by pretreatment of TNF with neutralizing anti-human TNF antibody (Figure 22).

**TNF Absorption from Culture Medium:**

Consumption from cell culture medium of exogenously added TNF was used to measure TNF utilization by cells. Both HL-60 and U937 consumed the 50 pg/ml by 8 hours and 80% of the 100 pg/ml by 24 hours (Figure 27). Both 3201 and 3201/FIV reduced the 50 pg/ml TNF to undetectable by 24 hours and the 100 pg/ml was reduced 50% by 24 hours (Figure 28). FL-74, FL/FIV, Molt 4, Molt 4/FIV showed essentially no change over the 24 hour collection period (Figures 29-30).

**Viral Antigen Production in Cell Cultures Treated with TNF:**

Antigen release from 3201/FIV, FL/FIV and FL-74 into culture supernatants was unchanged (Figure 31-34). However, the level of antigen
release remained very high in the 3201/FIV cultures even as cell death was apparent. TNF treatment failed to reactivate latent FIV in Molt 4/FIV cells.

**TNF Induction in PBMC Cultures Treated with Phorbol Ester:**

Primary mononuclear cells were isolated from fresh heparinized blood samples taken from both uninfected and chronically-FIV infected cats by standard ficoll methodology. Samples were tested for production of TNF and FIV antigen production in response to treatment with phorbol ester/calcium ionophore. As shown in Figure 35, all cultures from both FIV infected cats and uninfected cats were stimulated to produce TNF in response to treatment.

**Effect of TNF Treatment on PBMC from FIV-Infected Cats and Controls:**

TNF treatment of PBMC cultures from FIV-infected cats and controls showed no induction of FIV antigen expression and no effect on growth (data not shown).

**Discussion:**

Tumor necrosis factor, like most cytokines, has many functions and is involved in a wide range of biological processes (1-6,8). As an endogenous factor that selectively kills some types of tumor cells in vivo and in vitro, TNF also elicits cytotoxic effects against many virus-infected cell types (9-12) and
as such, may be an important part of a host's immune response to viral infection.

Seven cell lines were treated with high doses of TNF (100 and 10 ng/ml) and evaluated for effects on cell growth and viral antigen production. The HL-60 promyelocyte cell line, known to be TNF sensitive (44), was very susceptible to TNF-induced cytotoxicity with evidence of cell death as early as 24 hours after treatment. Both 3201 and 3201/FIV were determined to be TNF sensitive, although to a lesser extent than the HL-60 cells. Cell death was not obvious until after 48 hours of treatment. These results correlate well with those of the TNF absorption studies showing faster and more complete consumption of TNF by HL-60 than the 3201 and 3201/FIV cells and that consumption was associated with cytotoxicity. On the contrary, Molt4, Molt4/FIV, FL-74 and FL/FIV were not affected by TNF treatment nor did these cell lines consume exogenous TNF from culture supernatants. Several possible explanations exist for these differences 1) Molt4, Molt 4/FIV, FL-74 and FL/FIV may lack cell surface receptors for TNF, or 2) in response to TNF binding to its receptors, the complex was shed into the supernatant or cleaved by cell surface proteases (45) or, 3) an undetectable amount of TNF bound a few of the receptors which lead to rapid down-regulation (46) of remaining cell surface receptors for TNF utilization and, further, these receptors were not recycled back to the surface.
Protection against the cytotoxic effects of TNF by normal cells may occur at multiple levels: at the cell surface by receptor modulation (down-regulation, shedding, proteolysis of receptor-ligand complex) (45,46), or within the cell by production of superoxide dismutases and specific heat shock proteins (18-20). Viral infections could interfere with the capability of a cell to protect itself by altering membrane phospholipid metabolism (47), by altering normal intracellular signalling pathways, or by interfering with cellular protein synthesis. In this study, the results suggested that the TNF cytotoxicity seen in the 3201 and 3201/FIV cells was independent of FIV infection. Likewise, FL-74 (which are FeLV infected) and FL/FIV (which are both FeLV and FIV infected) cells behaved alike in response to TNF treatment.

With respect to viral infection, TNF has been reported to activate transcription of viral genes (11,39,48-50). Fauci and Folks (39,50) showed TNF induction of HIV-1 protein expression in monocyteid cell lines. TNF treatment of SIV-infected rhesus macaque lung macrophages (51) and SIV-infected Hut-78 cells (52) increased SIV replication.

Induction of HIV-1 protein expression by treatment with phorbol ester has also been shown and has been suggested to occur by activation of the NFkB enhancers present in the HIV-1 LTR (53). Most isolates of HIV-1 include two tandem copies of the kB response element (34,35), FIV was shown to have 8 of 10 bases of a kB enhancer consensus sequence in the 5’ LTR (37,54). In this study, phorbol ester treatment was only able to induce, at best, low level
antigen expression from 3/12 PBMC samples from FIV-infected cats. High dose TNF treatment did not induce FIV antigen expression from either PBMC cultures or the latently infected Molt 4/FIV cell line nor did it enhance antigen production in the chronic producer lines 3201/FIV and FL/FIV. Further, stimulation of PBMC from FIV-infected cats with mitogen and IL-2 (to establish the culture) did not lead to antigen expression. This would suggest that 1) TNF does not induce FIV via NFkB in PBMC of infected cats or that 2) the kB element is not functional in the two isolates of FIV used in this study or 3) that the recombinant human TNF can not stimulate feline kB (due to receptor binding).

Human TNF (and lymphotoxin) binds to two types of receptors, p55-60 (TR60) and p75-80 (TR80) (28,29). The binding of TNF to one or both of these receptors influences the effect TNF has on the cell (27-29). Although TNF is reasonably conserved with significant homology between human, mouse, feline and rabbit (55), TNF receptors are apparently less homologous. It has been demonstrated that in the mouse, both TR60 and TR80 bind human TNF (29). Conversely, in humans, only TR80 binds murine TNF (29). Whether these species differences are valid for the feline system is not known.

Khan et al. (56) implicated a role for TNF in aplastic anemia due to feline leukemia virus infection. It was suggested that bone marrow macrophages exposed to FeLV subgroup C virus were induced to produce TNF which then mediated cytopathic effects against an FeLV-C infected fibroblast cell line (56). Molina et al. (57) showed the induction of TNF from an HIV-1 infected
monocyte cell line due to lipopolysaccharide treatment. In vitro enhancement of TNF production by U937 cells was demonstrated by exposure of the cells to supernatants from HIV-1 infected T-cells (58). Ellis et al. (59) showed induction of TNF-\( \alpha \) from ovine lentivirus infection of ovine pulmonary alveolar macrophages. In this study, TNF was readily inducible in PBMC of both FIV-infected and naive cats by phorbol ester treatment. However, TNF was not induced by acute FIV infection of a PBMC culture used for the purpose of preparing FIV inoculation material.

While it has been shown in vitro that TNF interactions with HIV-1 infected cells may be deleterious to the host from the standpoint of induction of virus replication, and that many HIV-1 infected individuals at later stages of disease have elevating plasma TNF levels, a firm connection between cause and effect remains to be established.

The conclusions from this study suggest that although recombinant human TNF elicited some effect on proliferation in certain, but not all, feline cell lines tested, the lack of apparent effect in primary feline cells may be due to species differences in TNF-receptor binding. A definitive role for TNF in either potentiating or ameliorating FIV infection in this experimental model remains questionable. Several groups have attempted to show elevated plasma TNF levels in FIV infected cats but these reports were conflicting (60,61). Any further connection between TNF and FIV pathogenesis was not demonstrated.
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Figure 19. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of HL-60 as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. Significant differences between treated and untreated at 72 hrs (p = .0286) as determined by Mann-Whitney U Test.
Figure 19

Optical Density, 580 nm

HL-60 Cells

TNF Concentration (ng/ml) & Culture Time

24 hr 48 hr 72 hr
Figure 20. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of 3201 cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. Significant difference between treated and untreated at 72 hrs (p = .0079) as determined by Mann-Whitney U Test.
Optical Density, 580 nm

3201 Cells

24 hr 48 hr 72 hr
TNF Concentration (ng/ml) & Culture Time

Figure 20
Figure 21. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of 3201/FIV cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post treatment. Significant difference between treated and untreated at 72 hrs ($p = .0070$) as determined by Mann-Whitney U Test.
Optical Density, 580 nm

3201/FIV Cells

Optical Density, 580 nm

24 hr 48 hr 72 hr
TNF Concentration (ng/ml) & Culture Time

Figure 21
Figure 22. Effect of TNF treatment (100 ng/ml) on growth of HL-60 and 3201/FIV cells. Inhibition by pretreatment of TNF with 100, 200 or 400 units of anti-human TNF antibody.
Figure 22

Optical Density, 580 nm

Control

100 ug/ml TNF

100 ug/ml TNF + Anti-TNF Ab

Control

100 ug/ml TNF

100 ug/ml TNF + Anti-TNF Ab

HL-60 Cells

320/FLV Cells
Figure 23. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of FL-74 cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference at any time point between treated and untreated.
Figure 23

Optical Density, 580 nm

FL74 Cells

TNF Concentration (ng/ml) & Culture Time

24 hr  48 hr  72 hr

0  10  100  0  10  100  0  10  100
Figure 24. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of FL/FIV cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference at any time point between treated and untreated.
Figure 24

FL74/FIV Cells

Optical Density, 580 nm

<table>
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<tr>
<th></th>
<th>0</th>
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TNF Concentration (ng/ml) & Culture Time

Figure 24
Figure 25. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of Molt4 cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference at any time point between treated and untreated.
Figure 25

MOLT-4 Cells

Optical Density, 580 nm

TNF Concentration (ng/ml) & Culture Time

0 10 100 0 10 100 0 10 100
24 hr 48 hr 72 hr

Figure 25
Figure 26. Effect of TNF treatment (100, 10 and 0 ng/ml) on growth of Molt4/FIV cells as measured by MTT Assay. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference at any time point between treated and untreated.
MOLT-4/FIV Cells

Optical Density, 580 nm

24 hr  48 hr  72 hr

TNF Concentration (ng/ml) & Culture Time

Figure 26
Figure 27. TNF consumption by HL-60 and U937 cells. Samples were collected at 0, 8 and 24 hours.

- ○ = HL-60 treated with 100 pg/ml TNF.
- △ = HL-60 treated with 50 pg/ml TNF.
- □ = HL-60 treated with 0 pg/ml TNF.
- ● = U937 treated with 100 pg/ml TNF.
- ▲ = U937 treated with 50 pg/ml TNF.
- ■ = U937 treated with 0 pg/ml TNF.
Figure 27
Figure 28. TNF consumption by 3201 and 3201/FIV cells. Samples were collected at 0, 8 and 24 hours.

- ☺ = 3201/FIV treated with 100 pg/ml TNF.
- △ = 3201/FIV treated with 50 pg/ml TNF.
- ■ = 3201/FIV treated with 0 pg/ml TNF.
- ● = 3201 treated with 100 pg/ml TNF.
- ▲ = 3201 treated with 50 pg/ml TNF.
- ■ = 3201 treated with 0 pg/ml TNF.
Figure 28: Graph showing the concentration of TNF (pg/ml) over time in different conditions.

- **O 3201FIV-100**
- **△ 3201FIV-50**
- **□ 3201FIV-0**
- **● 3201CT-100**
- **△ 3201CT-50**
- **■ 3201CT-0**
Figure 29. TNF consumption by FL-74 and FL/FIV cells. Samples were collected at 0, 8 and 24 hours.

- ○ = FL/FIV treated with 100 pg/ml TNF.
- △ = FL/FIV treated with 50 pg/ml TNF.
- □ = FL/FIV treated with 0 pg/ml TNF.
- ● = FL-74 treated with 100 pg/ml TNF.
- ▲ = FL-74 treated with 50 pg/ml TNF.
- ■ = FL-74 treated with 0 pg/ml TNF.
Figure 29
Figure 30. TNF consumption by Molt4 and Molt4/FIV cells. Samples were collected at 0, 8 and 24 hours.

- O = Molt4/FIV treated with 100 pg/ml TNF.
- △ = Molt4/FIV treated with 50 pg/ml TNF.
- □ = Molt4/FIV treated with 0 pg/ml TNF.
- ● = Molt4 treated with 100 pg/ml TNF.
- ▲ = Molt4 treated with 50 pg/ml TNF.
- ■ = Molt4 treated with 0 pg/ml TNF.
Figure 30
Figure 31. Effect of TNF treatment (100, 10 and 0 ng/ml) on FIV antigen production by 3201/FIV cells. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference between treated and untreated at any time point.
Figure 31

3201/FIV Cells

Optical Density, 580 nm

<table>
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<th>TNF Concentration (ng/ml) &amp; Culture Time</th>
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<tbody>
<tr>
<td>0 10 100</td>
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<td>0 10 100</td>
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24 hr 48 hr 72 hr
Figure 32. Effect of TNF treatment (100, 10 and 0 ng/ml) on FIV antigen production by FL/FIV cells. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference between treated and untreated at any time point.
Figure 32

Optical Density, 580 nm

FL-74/FIV Cells

24 hr 48 hr 72 hr

TNF Concentration (ng/ml) & Culture Time

Figure 32
Figure 33. Effect of TNF treatment (100, 10 and 0 ng/ml) on FeLV antigen production by FL/FIV cells. Samples were collected at 24, 48 and 72 hours post treatment. No significant difference between treated and untreated at any time point.
Figure 33

Optical Density, 580 nm

FL-74/FIV Cells

24 hr 48 hr 72 hr

TNF Concentration (ng/ml) & Culture Time

Figure 33
Figure 34. Effect of TNF treatment (100, 10 and 0 ng/ml) on FeLV antigen production by FL-74 cells. Samples were collected at 24, 48 and 72 hours post TNF treatment. No significant difference between treated and untreated at any time point.
Optical Density, 580 nm

FL-74 Cells

 Optical Density, 580 nm

0 10 100 0 10 100 0 10 100
24 hr 48 hr 72 hr

TNF Concentration (ng/ml) & Culture Time

Figure 34
Figure 35. Effect of phorbol ester/calcium ionophore treatment on TNF induction in primary blood mononuclear cell cultures from FIV infected and control cats. Samples were collected at 24 hours post PMA/A23187 treatment.
Figure 35
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