



University of Zurich  
Zurich Open Repository and Archive

Winterthurerstr. 190  
CH-8057 Zurich  
<http://www.zora.unizh.ch>

---

*Year: 1998*

---

## The role of in vitro-induced lymphocyte apoptosis in feline immunodeficiency virus infection: correlation with different markers of disease progression

Holznagel, E; Hofmann-Lehmann, R; Leutenegger, C M; Allenspach, K; Huettner, S; Forster, U; Niederer, E; Joller, H; Willett, B J; Hummel, U; Rossi, G L; Schüpbach, J; Lutz, H

Holznagel, E; Hofmann-Lehmann, R; Leutenegger, C M; Allenspach, K; Huettner, S; Forster, U; Niederer, E; Joller, H; Willett, B J; Hummel, U; Rossi, G L; Schüpbach, J; Lutz, H. The role of in vitro-induced lymphocyte apoptosis in feline immunodeficiency virus infection: correlation with different markers of disease progression. *J. Virol.* 1998, 72(11):9025-33.

Postprint available at:  
<http://www.zora.unizh.ch>

Posted at the Zurich Open Repository and Archive, University of Zurich.  
<http://www.zora.unizh.ch>

Originally published at:  
*J. Virol.* 1998, 72(11):9025-33

# The role of in vitro-induced lymphocyte apoptosis in feline immunodeficiency virus infection: correlation with different markers of disease progression

## Abstract

Human immunodeficiency virus infection is characterized by a progressive decline in the number of peripheral blood CD4(+) T lymphocytes, which finally leads to AIDS. This T-cell decline correlates with the degree of in vitro-induced lymphocyte apoptosis. However, such a correlation has not yet been described in feline AIDS, caused by feline immunodeficiency virus (FIV) infection. We therefore investigated the intensity of in vitro-induced apoptosis in peripheral blood lymphocytes from cats experimentally infected with a Swiss isolate of FIV for 1 year and for 6 years and from a number of long-term FIV-infected cats which were coinfecting with feline leukemia virus. Purified peripheral blood lymphocytes were either cultured overnight under nonstimulating conditions or stimulated with phytohemagglutinin and interleukin-2 for 60 h. Under stimulating conditions, the isolates from the infected cats showed significantly higher relative counts of apoptotic cells than did those from noninfected controls (1-year-infected cats,  $P = 0.01$ ; 6-year-infected cats,  $P = 0.006$ ). The frequency of in vitro-induced apoptosis was inversely correlated with the CD4(+) cell count ( $P = 0.002$ ), bright CD8(+) cell count ( $P = 0.009$ ), and CD4/CD8 ratio ( $P = 0.01$ ) and directly correlated with the percentage of bright major histocompatibility complex class II-positive peripheral blood lymphocytes ( $P = 0.004$ ). However, we found no correlation between in vitro-induced apoptosis and the viral load in serum samples. Coinfection with feline leukemia virus enhanced the degree of in vitro-induced apoptosis compared with that in FIV monoinfected cats. We concluded that the degree of in vitro-induced apoptosis was closely related to FIV-mediated T-cell depletion and lymphocyte activation and could be used as an additional marker for disease progression in FIV infection.

## The Role of In Vitro-Induced Lymphocyte Apoptosis in Feline Immunodeficiency Virus Infection: Correlation with Different Markers of Disease Progression

EDGAR HOLZNAGEL,<sup>1,2</sup> REGINA HOFMANN-LEHMANN,<sup>1</sup> CHRISTIAN M. LEUTENEGGER,<sup>1</sup>  
KARIN ALLENSPACH,<sup>1</sup> SILKE HUETTNER,<sup>1</sup> URSULA FORSTER,<sup>2</sup> EVA NIEDERER,<sup>3</sup>  
HELEN JOLLER,<sup>4</sup> BRIAN J. WILLETT,<sup>5</sup> URS HUMMEL,<sup>6</sup> GIOVANNI L. ROSSI,<sup>2</sup>  
JÖRG SCHÜPBACH,<sup>6</sup> AND HANS LUTZ<sup>1\*</sup>

*Clinical Laboratory, Department of Internal Veterinary Medicine,<sup>1</sup> and Swiss National Center for Retroviruses,<sup>6</sup> University of Zurich, Institute of Biomedical Engineering, Swiss Institute of Technology,<sup>3</sup> and Laboratory of Clinical Immunology, Department of Internal Medicine, University Hospital,<sup>4</sup> Zurich, and Division of Experimental Pathology, Institute of Animal Pathology, University of Bern, Bern,<sup>2</sup> Switzerland, and Department of Veterinary Pathology, University of Glasgow, Glasgow, United Kingdom<sup>5</sup>*

Received 28 May 1998/Accepted 13 July 1998

**Human immunodeficiency virus infection is characterized by a progressive decline in the number of peripheral blood CD4<sup>+</sup> T lymphocytes, which finally leads to AIDS. This T-cell decline correlates with the degree of in vitro-induced lymphocyte apoptosis. However, such a correlation has not yet been described in feline AIDS, caused by feline immunodeficiency virus (FIV) infection. We therefore investigated the intensity of in vitro-induced apoptosis in peripheral blood lymphocytes from cats experimentally infected with a Swiss isolate of FIV for 1 year and for 6 years and from a number of long-term FIV-infected cats which were coinfecting with feline leukemia virus. Purified peripheral blood lymphocytes were either cultured overnight under nonstimulating conditions or stimulated with phytohemagglutinin and interleukin-2 for 60 h. Under stimulating conditions, the isolates from the infected cats showed significantly higher relative counts of apoptotic cells than did those from noninfected controls (1-year-infected cats,  $P = 0.01$ ; 6-year-infected cats,  $P = 0.006$ ). The frequency of in vitro-induced apoptosis was inversely correlated with the CD4<sup>+</sup> cell count ( $P = 0.002$ ), bright CD8<sup>+</sup> cell count ( $P = 0.009$ ), and CD4/CD8 ratio ( $P = 0.01$ ) and directly correlated with the percentage of bright major histocompatibility complex class II-positive peripheral blood lymphocytes ( $P = 0.004$ ). However, we found no correlation between in vitro-induced apoptosis and the viral load in serum samples. Coinfection with feline leukemia virus enhanced the degree of in vitro-induced apoptosis compared with that in FIV mono-infected cats. We concluded that the degree of in vitro-induced apoptosis was closely related to FIV-mediated T-cell depletion and lymphocyte activation and could be used as an additional marker for disease progression in FIV infection.**

Feline immunodeficiency virus (FIV) infection is a naturally occurring infection, and disease progression in infected cats is associated with a decline in the number of CD4<sup>+</sup> cells (2, 6, 22, 23, 36), a loss of bright CD8<sup>+</sup> cells in the advanced stage of the disease (22), an increased number of activated T cells (39, 41), and a changed cytokine production, i.e., decreased production of interleukin-2 (IL-2) and concomitantly increased production of tumor necrosis factor alpha (TNF- $\alpha$ ) (25, 26). The increased production of TNF- $\alpha$  has been reported to induce apoptosis in chronically FIV-infected cells (38). Apoptosis, a controlled mode of cell death (34), plays an important role in the regulation of immune responses (5, 14). As described for FIV (23), the hallmark of human immunodeficiency virus (HIV)-induced disease is the loss of T-helper cells (31, 43). Theoretically, cell loss can be caused by decreased production of cells, increased destruction, or a combination of the two mechanisms. Findings of an early infection of thymocytes followed by pathologic changes in the thymus support the model of decreased T-helper cell production triggered by HIV (13)

and FIV (52). The destruction model is supported by findings of an increased number of peripheral blood T cells undergoing apoptosis upon HIV (20, 32) and FIV (11, 21, 33) infection. However, increased CD4<sup>+</sup>-T-cell turnover may not be the main cause of the observed T-helper cell decline in HIV-1 infection, as reviewed by others (44, 51). In addition, the degree of HIV-induced apoptosis correlates with the T-helper cell decline and disease progression (19, 40). However, such a relationship has not yet been described for FIV. It has been reported that cross-linking of CD4 molecules by HIV gp160 triggers apoptosis in noninfected CD4<sup>+</sup> T cells (1). Investigation of this aspect in the feline system is especially interesting since FIV does not use the feline homologue of CD4 (50).

The aim of the present study was to compare the degree of in vitro-induced lymphocyte apoptosis in FIV-infected cats with normal and decreased T-helper cell counts. We used two different culture conditions to trigger apoptosis in vitro: cells were either cultured overnight under nonstimulating conditions and in the absence of growth factors or cultured for 60 h in the presence of phytohemagglutinin, IL-2, and fetal calf serum. We additionally examined cats which were coinfecting with the feline leukemia virus (FeLV). This coinfection is known to accelerate the progression toward feline AIDS (23) by an unknown mechanism (8).

\* Corresponding author. Mailing address: Clinical Laboratory, Department of Internal Veterinary Medicine, University of Zurich, Winterthurer Str. 260, CH-8057 Zurich, Switzerland. Phone: 41-1-6358312. Fax: 41-1-6358906. E-mail: hanslutz@vetklinik.unizh.ch.

## MATERIALS AND METHODS

**Animals.** To determine the effect of short-term and long-term FIV infection on the degree of lymphocyte apoptosis, specific-pathogen-free cats (Novartis AG, Basle, Switzerland) of two age groups were used. Twenty-two 17-month-old cats were used to study the degree of in vitro-induced apoptosis in 1-year-infected cats (group F1;  $n = 12$ ) and in noninfected and nonimmunized age- and sex-matched controls (group C1;  $n = 10$ ). The cats in group F1 had been experimentally infected for the purpose of the present experiment with a Swiss isolate of FIV (Zurich-2 strain [FIV Z2]) under conditions described previously (30). The second age group consisted of 19 6.5-year-old cats, which had been used in a FeLV vaccine study 5 years before the present experiment. In that FeLV vaccine study, we had investigated the degree to which cats with a preexisting FIV infection could be vaccinated against FeLV infection with a recombinant FeLV envelope preparation (27). From that study, 19 cats were selected for inclusion in the present experiment and assigned to one of the following three groups. Group F2 consisted of eight cats which had been experimentally infected with FIV Z2 alone at the age of 6 months and which had been used as non-FeLV-vaccinated controls (27). Group C2 consisted of eight cats which had been vaccinated with a recombinant FeLV envelope vaccine and challenged with FeLV subtype A. These cats were immune to FeLV as judged by the absence of FeLV antigenemia and viremia during the 5-year observation period. They were included as FIV- and FeLV-negative cats in the present study. Group FF2 consisted of three cats which had been experimentally infected with FIV and which had served as non-FeLV-vaccinated FeLV challenge controls.

**Cell preparation and culture conditions.** Peripheral blood lymphocytes (PBLs) were isolated from heparinized blood by centrifugation on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient as previously described (24), washed, and resuspended ( $10^6$  cells per ml of medium; lymphocyte purity,  $\geq 90\%$ ) in buffered RPMI 1640 medium supplemented with 2 mM L-glutamate, 10% (vol/vol) heat-inactivated fetal calf serum, 100 IU of penicillin G, and 100  $\mu$ g of streptomycin per ml of medium (all reagents from Gibco Life Technologies, Basle, Switzerland); this medium was designed basal medium (BM). BM was supplemented with 0.2% (vol/vol) phytohemagglutinin (PHA-M; Gibco catalogue no. 10576-015, lot no. 10P2145) and IL-2 (100 U of recombinant human IL-2 per ml of medium; kindly provided by M. H. Schreier, Sandoz, Basle, Switzerland) to stimulate cells. Zinc sulfate (Sigma, Buchs, Switzerland) was added to the culture medium to inhibit apoptosis as previously described (24). Cell viabilities were assessed by the trypan blue exclusion test (24). Cyto-centrifuged and stained cell preparations were examined for lymphocyte purity by light microscopy. Human peripheral blood mononuclear cells were purified by a standard method from heparinized blood samples collected from healthy human donors (24).

**Flow-cytometric analysis of PBL subsets.** A fluorescein isothiocyanate (FITC)-conjugated murine monoclonal antibody (MAb) against anti-feline major histocompatibility complex (MHC) class II antigen (clone vpg 3) and FITC- or phycoerythrin (PE)-conjugated moabs against feline CD4, CD5, and CD8 antigens (Southern Biotechnology, Birmingham, Ala.), diluted as recommended by the manufacturer were used to stain subsets in 100  $\mu$ l of platelet-depleted whole blood as previously described (23) or in 100  $\mu$ l of medium containing  $0.5 \times 10^6$  gradient-purified PBLs. Stained PBLs were processed by a routine whole-blood lysis technique (Coulter Q-Prep Workstation solutions). Single- and dual-color analyses were performed with an EPICS Profile analyzer. A total of  $10^4$  events were collected in the lymphocyte gate, which was set according to light-scattering properties ( $90^\circ$  log side scatter or  $90^\circ$  side scatter versus forward scatter). Multiparametric data analysis was performed with Lysis II software (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). Data from the EPICS Profile analyzer were made compatible for Lysis II software with ProFCS software (Verity Software, Topsham, Maine). The fluorescence intensities in histograms and contour plots were displayed on a logarithmic ( $\log_{10}$ ) scale. The absolute counts of lymphocyte subsets were calculated by multiplying the absolute lymphocyte number (determined by routine hematological procedures) by the relative subset count (expressed as the percentage of PBLs determined by flow cytometry). The lymphocyte bitmap comprised  $\geq 95\%$  peripheral blood lymphocytes, determined by the sum of CD5<sup>+</sup> and CD21<sup>+</sup> cells, as determined in a separate experiment (data not shown). As previously described, dim CD8<sup>+</sup> cells were gated in relation to the fluorescence signals of bright CD8<sup>+</sup> cells and isotype-matched control antibody of FIV negative cats (29).

**Detection and quantitation of apoptosis.** (i) Internucleosomal DNA fragmentation was detected by agarose gel electrophoresis as previously described (24). Briefly, 10  $\mu$ g of phenol-extracted cellular DNA was transferred to a 1% (wt/vol) agarose gel and electrophoresed, and DNA was visualized by ethidium bromide staining. A murine tumor-specific cytotoxic T-lymphocyte cell line (CTL-2) that undergoes apoptosis upon IL-2 or IL-4-withdrawal served as a positive control. (ii) Electron microscopic (EM) analysis was used to detect typical morphological changes in apoptotic cells. Briefly, cell pellets were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 2% osmium tetroxide (pH 7.4)–3% potassium cyanoferrate II solution, and incubated in phosphate buffer with 1% uranyl acetate. Samples were embedded in Spurr's low-viscosity medium, and thin sections were cut from the postfixed blocks and stained for contrast with lead citrate for subsequent electron microscopic analysis. (iii) The percentage of apoptotic cells in cultures was determined by flow cytometry. The apoptotic cell

population was flow cytometrically identified by changed light-scattering signals and a reduced DNA content (subdiploid cells) upon ethanol fixation and propidium iodide (PI) staining (24). Briefly, cell suspensions were stained with PI, which stoichiometrically binds to nucleic acids. Analysis of the red fluorescence intensity (linear scale) was then used as a parameter of the relative DNA content. We used area and peak fluorescence processing of the PI signal to discriminate doublet cells, i.e., clumping cells, and debris.

**Detection and quantitation of FIV p24 and TNF- $\alpha$ .** FIV p24 (MAbs 43-1B9 and 43-1E2, kindly provided by Niels C. Pedersen, Davis, Calif.) and TNF- $\alpha$  (Endogen, Boston, Mass.) were quantified by sandwich enzyme-linked immunosorbent assays in culture supernatants of PBLs stimulated for 60 h as previously described (9, 28). For p24 quantitation, supernatants of an FIV-positive lymphocyte culture, defined as a 100% standard, and of FIV-negative culture were used. The detection limit of the TNF- $\alpha$  assay was 10 pg/ml.

**Quantitation of the viral burden in sera by the product-enhanced reverse transcriptase assay.** The viral burden in sera from the infected animals was determined indirectly by quantitation of reverse transcriptase activity levels (product-enhanced reverse transcriptase assay), as described for HIV-1 (12), on an ABI PRISM 7700 sequence detection system (Perkin-Elmer, Applied Biosystems Division, Rotkreuz, Switzerland).

**Statistical analysis.** All the data in the text are presented as the mean  $\pm$  standard deviation for an experiment. The flow-cytometric parameters of the groups were analyzed for significant differences ( $P < 0.05$ ) by the Kruskal-Wallis *H*-test. Linear regression of the form  $y = a + bx$  were calculated, including the Spearman coefficient of correlation ( $r$ ). As described elsewhere (23), in all figures displaying box plots, the boxes extend from the 25th to the 75th percentile, with a horizontal line at the median, and the whiskers extend down to the smallest and up to the largest value.

## RESULTS

**Clinical outcome.** No severe clinical signs were seen during the observation period. Some cats, especially but not exclusively the FIV- and the FIV-plus FeLV-infected animals, showed mild stomatitis and gingivitis.

**Lymphocyte subsets.** The CD4/CD8 ratio was significantly lower in the long-term FIV-infected group (F2) than in the age- and sex-matched controls (C2), whereas the analysis of 1-year-infected cats (F1) showed only a trend (Fig. 1). One-year-infected animals had only slightly decreased CD4<sup>+</sup> counts and slightly increased CD8<sup>+</sup> counts (Fig. 1). These changes did not reach significant levels. The long-term FIV- and FeLV-coinfected group (FF2) showed the lowest ratio of all the investigated groups (Fig. 1). In long-term virus carriers, lower CD4/CD8 ratios were caused predominantly by significantly decreased CD4<sup>+</sup> lymphocyte counts (Fig. 1), whereas the total number of CD8<sup>+</sup> cells remained constant (Fig. 1). However, a number of long-term-infected cats had a reduced number of cells showing bright CD8<sup>+</sup> fluorescence (bright CD8<sup>+</sup> cells) (Fig. 2 and 3). This loss was accompanied by the occurrence of CD8<sup>+</sup> cells showing lower CD8<sup>+</sup> fluorescence intensity (dim CD8<sup>+</sup> cells) (Fig. 2). Both bright and dim CD8<sup>+</sup> lymphocytes coexpressed CD5 antigen (Fig. 2). In long-term-infected cats, the percentage of bright MHC class II-positive lymphocytes (Fig. 4) was significantly higher than in both healthy controls ( $P = 0.008$ ) and 1-year-infected animals ( $P = 0.001$ ) (Fig. 5). Again, the highest values were found in the three coinfecting animals (Fig. 5). However, there was also an age-dependent difference between the two control groups (C1 and C2;  $P = 0.04$  [Fig. 5]).

**Detection and degree of apoptosis.** Apoptosis was not found in freshly obtained PBL samples by agarose gel electrophoresis or by EM, whereas overnight incubation in BM triggered apoptosis as revealed by agarose gel electrophoresis and EM in samples from all groups, including infected and noninfected cats (data not shown). Flow-cytometric DNA cell cycle analyses revealed the appearance of a discrete cell population below  $G_0/G_1$  (subdiploid) only in cultured samples (Fig. 6 through 8). This subdiploid cell population could be dose-dependently inhibited by addition of zinc sulfate to the culture medium or by lowering the incubation temperature (Fig. 7) as previously

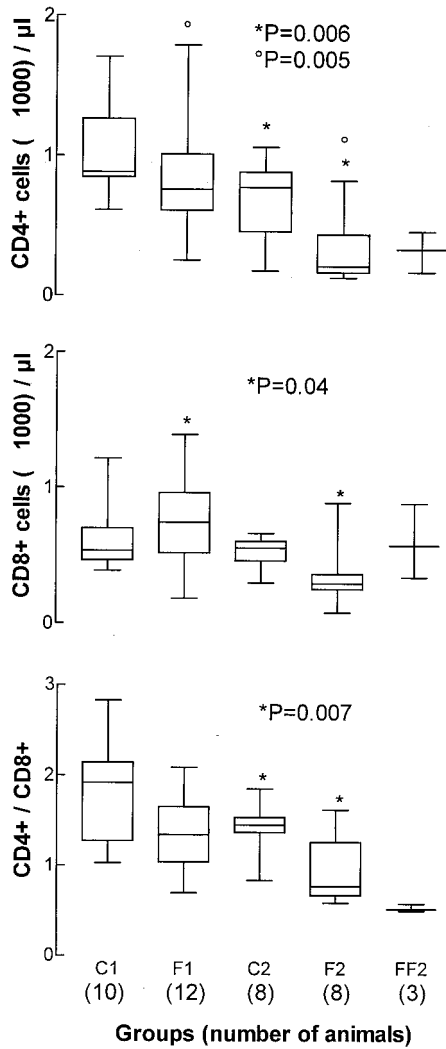


FIG. 1. Absolute numbers of peripheral blood T-cell subsets and CD4/CD8 ratios in short- and long-term retrovirus-infected cats. Box plots of short-term (F1) and long-term FIV-monoinfected (F2), long-term FIV- and FeLV-coinfected (FF2), and healthy noninfected age- and sex-matched control (C1 and C2) cats are shown. The numbers in parentheses are the numbers of animals per group. Note that there is also a decrease in absolute CD4<sup>+</sup> counts during aging (C1 versus C2).

described (24). A time course study was performed to evaluate the rate of apoptosis as a function of culture duration (Fig. 7C). A rapid increase in apoptosis was detected in all samples, and the rate peaked at around 24 h. However, human lymphocytes from healthy donors were more resistant to the induction of apoptosis (Fig. 7C). A significantly higher degree of apoptosis was found in 1-year-infected cats than in age- and sex-matched controls under these nonstimulating conditions (F1, 23.8% ± 4.8% [n = 12]; C1, 17.9% ± 5.7% [n = 16]; P = 0.004), whereas in long-term-infected cats this difference was only slightly higher than in age- and sex-matched controls (F2, 19.6% ± 5.7% [n = 9]; C2, 14.9% ± 5.6% [n = 6]; P = 0.07). When PBLs were stimulated for 60 h with PHA and IL-2, the degree of apoptosis was significantly higher in 1- and 6-year-infected cats than in age- and sex-matched controls (Fig. 9). Again, the highest values were found in the three coinfecting animals (Fig. 9).

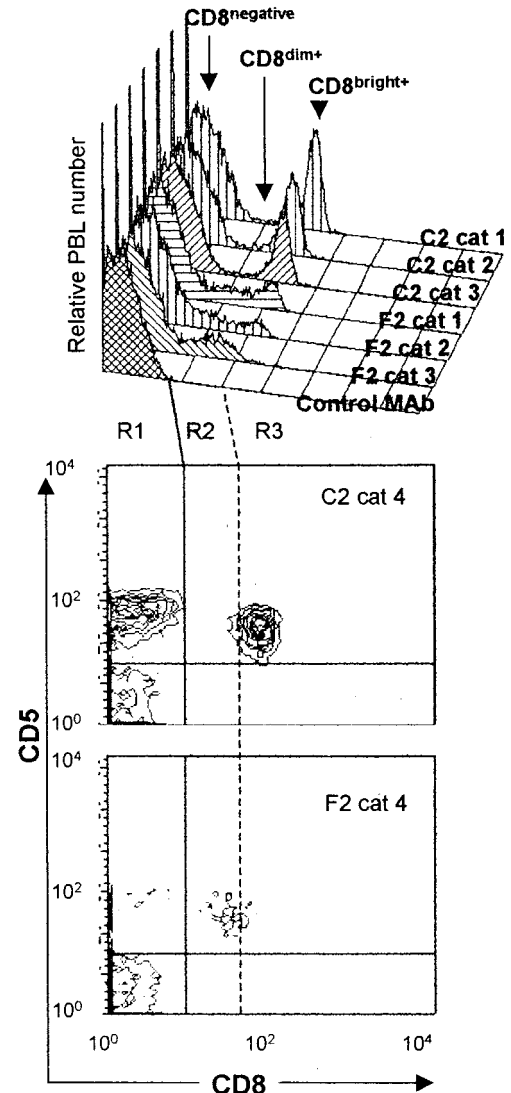


FIG. 2. Peripheral blood CD8<sup>+</sup> T-cell populations characterized by different CD8 antigen densities. The upper diagram is a CD8 histogram overlay and shows representative fluorescence intensities corresponding to CD8 antigen densities in PBLs from three long-term FIV-infected cats (F2) and three age- and sex-matched healthy control cats (C2). The middle and lower diagrams show representative contour plots of CD8-FITC- and CD5-PE-labeled PBLs in a healthy control and a long-term FIV-infected cat, respectively. Note that the lower contour plot comprises only 1,500 analyzed events, due to the lymphopenic status of the cat, compared to 5,000 analyzed events in the histograms and the middle contour plots, respectively. Two subsets of CD8<sup>+</sup> PBLs are found in infected cats (CD8<sup>dim+</sup> and CD8<sup>bright+</sup> cells), and the appearance of CD8<sup>dim+</sup> cells is accompanied by a loss of CD8<sup>bright+</sup> cells (upper diagram). To exclude that CD8<sup>dim+</sup> cells were natural killer (NK) cells, we used a feline pan-T-cell marker (CD5) to characterize CD8<sup>+</sup> cells. The middle and lower diagrams show representative examples of dual-color analyses. The coexpression of CD5 in CD8<sup>bright+</sup> and CD8<sup>dim+</sup> cells confirmed their T-cell origin. Abbreviations: R1, analysis region of CD8<sup>negative</sup> PBLs as determined by an isotype-matched control monoclonal antibody; R2, analysis region of dim CD8<sup>dim+</sup> PBLs; R3, analysis region of CD8<sup>bright+</sup> PBLs. The dotted line represents the threshold between R2 and R3; for definition, see the text.

**Relationship between apoptosis and different markers of disease progression.** The relationship between the percentage of apoptotic cells after 60 h of stimulation and disease markers was examined by linear regression. In infected animals (n = 21), the analysis demonstrated significant inverse correlations with immunologic markers: cats with the highest rate of apo-

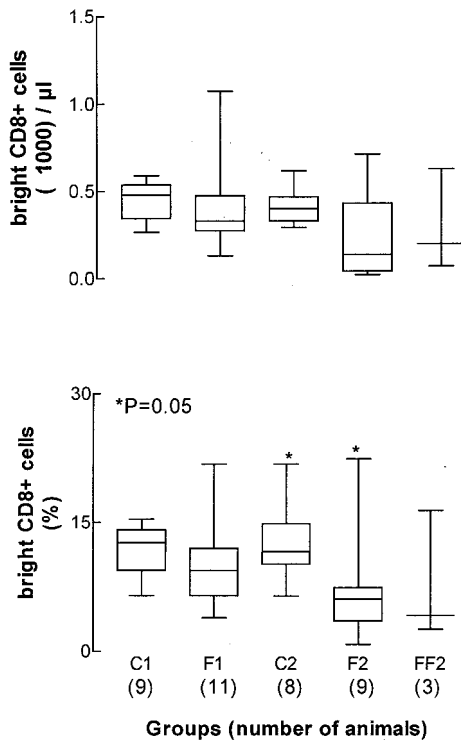


FIG. 3. Absolute numbers and percentages of peripheral blood CD8<sup>bright+</sup> T cells in short- and long-term retrovirus-infected cats. The percentage of bright-positive cells gives the proportion of CD8<sup>+</sup> lymphocytes with bright fluorescence of all lymphocytes. The absolute number of bright CD8<sup>+</sup> cells represent the bright CD8<sup>+</sup> counts per microliter of whole blood. The numbers in parentheses are the numbers of animals per group. It is evident that cats with long-term FIV infection (F2) and FIV and FeLV coinfection (FF2) have significantly lower relative bright CD8<sup>+</sup> counts than do noninfected age- and sex-matched controls (C1 and C2).

ptosis had the lowest absolute (Fig. 10) and relative numbers of CD4<sup>+</sup> cells ( $y = -1.26 \times + 62.3$  [ $r = -0.71$ ;  $P = 0.0004$ ]), the lowest CD4/CD8 ratios ( $y = -14.3 \times + 55.7$  [ $r = -0.51$ ;  $P = 0.01$ ]), and the lowest absolute (Fig. 10) and relative numbers of bright CD8<sup>+</sup> cells ( $y = -1.24 \times + 48.3$  [ $r = -0.57$ ;  $P = 0.006$ ]), whereas analysis of the total CD8<sup>+</sup>-cell count showed only a trend in the percentage of CD8<sup>+</sup> cells ( $y = -0.80 \times + 53.0$  [ $r = -0.37$ ;  $P = 0.08$ ]) and the CD8<sup>+</sup> cell count per microliter of blood ( $y = -0.01 \times + 49.7$  [ $r = -0.42$ ;  $P = 0.05$ ]). The percentage of bright MHC class II-positive PBLs directly correlated with the occurrence of apoptosis ( $y = 0.27 \times + 25.4$  [ $r = 0.67$ ;  $P = 0.001$ ]). In contrast to infected animals, the degree of apoptosis found in cultures from noninfected controls ( $n = 20$ ) did not correlate with any of the above-mentioned immunologic markers. In contrast to the observed relationship between apoptosis and immunologic markers, no correlation existed between apoptosis and viral load in serum samples as determined by FIV reverse transcriptase activity ( $r = 0.33$ ;  $P = 0.18$ ).

**FIV p24 and TNF- $\alpha$  concentrations in 60-hour-culture supernatants.** PBL culture supernatants from FIV-infected cats were FIV p24 antigen negative, and only a basal TNF- $\alpha$  secretion was detected following 60 h of PHA-plus-IL-2-stimulation in cell cultures from FIV-infected cats ( $9.8 \pm 6$  pg per ml of medium [ $n = 5$ ]) and noninfected controls ( $10.4 \pm 7$  pg per ml of medium [ $n = 5$ ]). These results do not differ significantly.

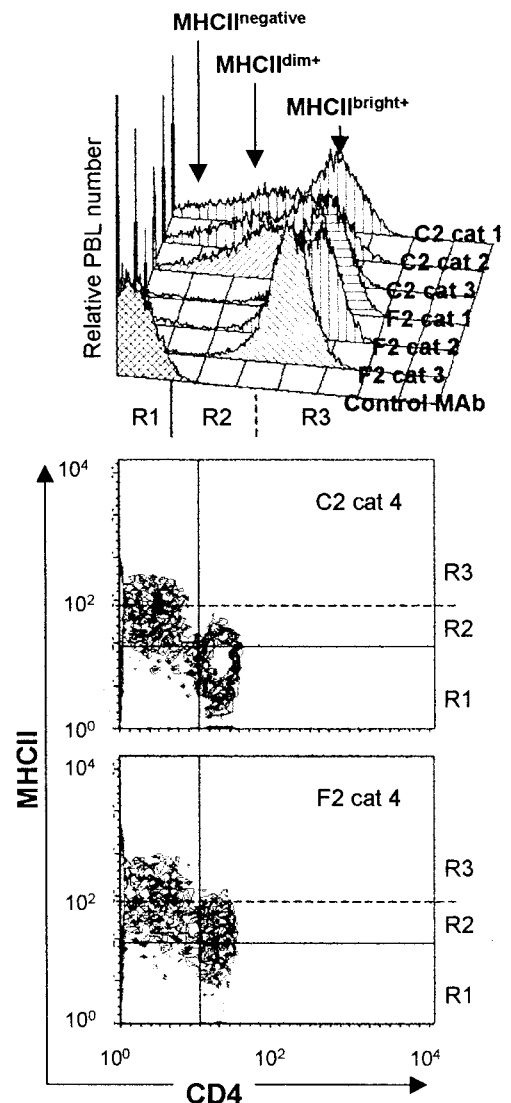


FIG. 4. Increased MHC class II antigen densities in PBLs of long-term FIV-infected cats. The upper diagram is an MHC class II antigen histogram overlay and shows representative fluorescence intensities of MHC class II antigen-expressing PBLs in three long-term FIV-infected cats (F2) and three age- and sex-matched healthy controls (C2). The middle and lower diagrams show representative examples of the MHC class II antigen expression (PE-labeled) on peripheral blood CD4<sup>+</sup> T cells (FITC-labeled) of a healthy control cat (middle diagram) and a long-term FIV-infected cat (lower diagram) displayed by contour plots. In healthy cats, three PBL populations exist in peripheral blood: MHC class II<sup>negative</sup>, MHC class II<sup>dim+</sup>, and MHC class II<sup>bright+</sup> PBLs (upper diagram). In long-term FIV-infected cats, virtually all PBLs express MHC class II, the majority of them with a high antigen density (upper diagram). FIV infection triggered an increase of MHC class II expression on T-helper cells (lower diagram), which contributed to the overall increase in the number of MHC class II-positive PBLs in peripheral blood samples. These observations indicate that the immune system is activated in persistently retrovirus-infected cats. Abbreviations: R1, analysis region of MHCII<sup>negative</sup> PBLs as determined by an isotype-matched control MAb; R2, analysis region of MHCII<sup>dim+</sup> PBLs; R3, analysis region of MHCII<sup>bright+</sup> PBLs. The dotted line represents the threshold between R2 and R3. Note that the scale of the contour lines is displayed in 1% steps, leading to a plateau on the top of the middle diagram.

## DISCUSSION

For some time it has been known that FIV infection leads to the loss of T helper cells (2) and that coinfection with FeLV enhances this effect (8). The FIV-mediated loss of CD4<sup>+</sup> cells

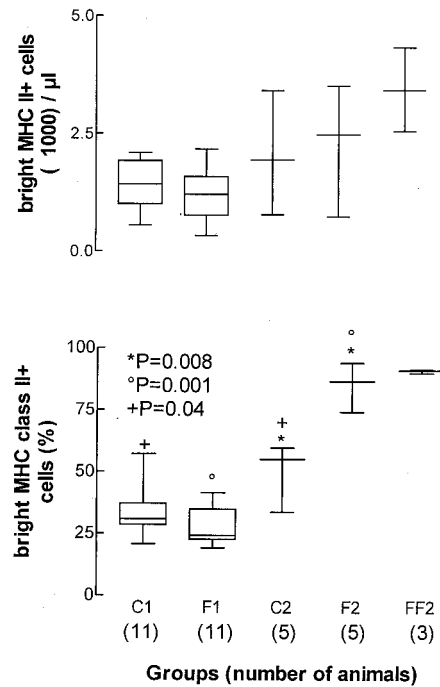


FIG. 5. Absolute numbers and percentages of peripheral blood MHC II<sup>bright+</sup> lymphocytes in short- and long-term retrovirus-infected cats. The percentage of bright-positive cells gives the proportion of MHC class II<sup>bright+</sup> lymphocytes of all lymphocytes. The absolute number represent the MHC class II<sup>bright+</sup> cell counts per microliter of whole blood. The numbers in brackets are the numbers of animals per group. The designation of the groups corresponds to that in Fig. 1. It becomes clear that in long-term FIV-infected cats the relative numbers of bright MHC II-positive cells is significantly increased. However, this increase is not seen at the same level of significance in the absolute counts.

and the concomitantly decreased CD4/CD8 ratio are immunologic markers for disease progression in the feline AIDS model (23). In the present study, we have investigated the possible role of in vitro-induced lymphocyte apoptosis at different stages of experimental FIV infection. In search of possible immunosuppressive mechanisms, we were interested to know whether the extent of in vitro-induced apoptosis could be used as a marker for disease progression in the feline AIDS model and whether coinfection with FeLV leads to enhanced apoptosis.

Significant changes in the CD4/CD8 ratios, which were mainly due to significantly decreased T-helper-cell counts, indicated a virus-mediated disturbance of the cellular immune system in the long-term-infected animals. FeLV coinfection enhanced these changes, as previously reported (23). We observed a shift from bright CD8<sup>+</sup> to dim CD8<sup>+</sup> cells as previously described (29, 49). Both CD8 subsets coexpressed the feline homologue of CD5, suggesting their T-cell origin. In HIV, a decline in a CD8<sup>+</sup> CD11b<sup>-</sup> subset which correlated with disease progression has been described (10). Unfortunately, neither the function nor the immunophenotype of the feline dim CD8<sup>+</sup> subset is known. Finally, the significantly larger numbers of MHC class II-positive cells indicated both an increased number of feline B cells (17) and an increased T-cell activation (39, 41). In contrast, we found no significant changes among lymphocyte subsets in cats infected for 1 year, although a tendency to decreased CD4/CD8 ratios was also found in these cats.

Flow cytometric DNA analysis revealed that in contrast to human lymphocytes, feline lymphocytes from healthy cats un-

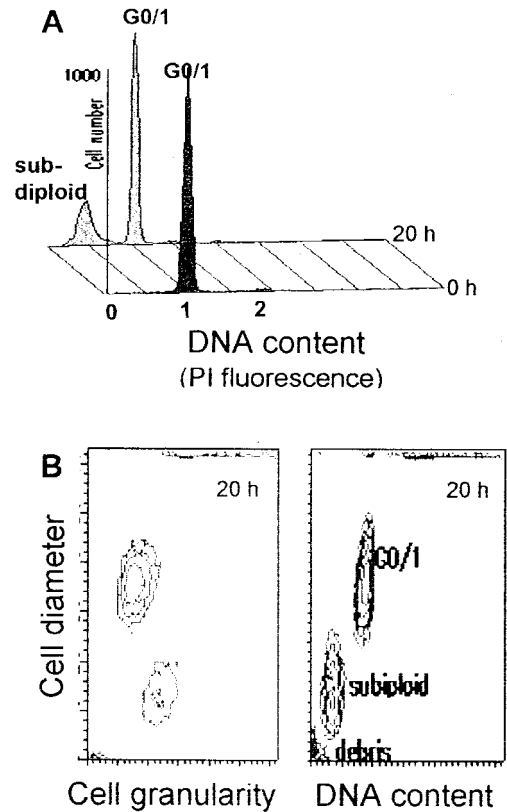


FIG. 6. Quantitation of apoptotic cells by flow-cytometric DNA analysis. (A) Representative DNA analyses of freshly obtained (0-h) and cultured (20-h) PBLs from a short-term FIV-infected cat. (B) Contour plots showing light-scattering properties of the sample cultured for 20 h. For the analysis of the DNA distribution, PBLs were ethanol fixed, RNase treated, washed, and stained with PI. Apoptotic feline PBLs were identified by a reduced (subdiploid) DNA content as described previously (24). Freshly isolated PBLs consisted of a single cell population with a diploid DNA content (1 corresponds to the G<sub>0/1</sub> phase, 2 corresponds to the G<sub>2/M</sub> phase, and the analysis region between 1 and 2 corresponds to the S phase of the cell cycle). Overnight culture triggered the appearance of a subdiploid cell population (analysis region below 1). This hypoploid subset and the cell population in the G<sub>0/1</sub> phase are also shown in the right contour plot in panel B, which combines the forward-scatter signal (cell diameter) with the relative PI fluorescence (DNA content). The corresponding cell populations are also shown in the left contour plot, in which the forward-scatter signals are combined with the side-scatter signals (cell granularity). The data in these two contour plots show that the subdiploid subset consisted of cells showing a reduced cell diameter and an increased granularity and could be easily distinguished from debris. These light-scattering properties are typical of cells passing through the early stages of the apoptotic process (54).

derwent apoptosis to some extent under nonstimulating culture conditions. The cause for this species-specific phenomenon remains unclear, but we speculate that the higher level of in vivo-activated feline PBLs contributed to that phenomenon.

FIV infection caused a significantly higher rate of apoptotic cells in cultures from 1-year- and 6-year-FIV-infected cats than in noninfected age- and sex-matched controls. These findings are in agreement with those of other FIV studies (11, 21, 33). The largest numbers of apoptotic cells were found in FeLV-coinfected cats. No information on the statistics is given for the three coinfecting cats in the experiments in Fig. 1, 3, 5, and 9 since the numbers were too small at the time of the study. However, the animals in this group (originally *n* = 5) have been monitored for more than 5 years and always had the smallest numbers of CD4<sup>+</sup> and bright CD8<sup>+</sup> cells (23). Therefore, it may be speculated that the observed high level of

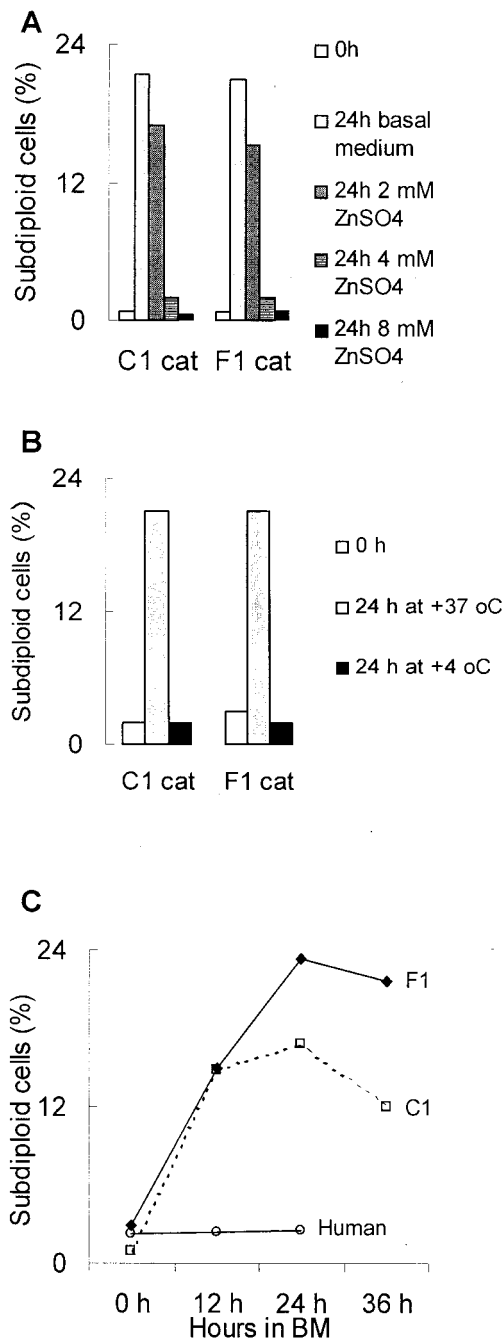


FIG. 7. Effect of zinc sulfate (ZnSO<sub>4</sub>) and of lowered culture temperature (4 versus 37°C) on the induction of subdiploid cells, and time course for the in vitro development of subdiploid cells in human and feline PBLs. (A) ZnSO<sub>4</sub> dose-dependently inhibited the occurrence of the subdiploid cells, as shown in a representative experiment. (B) Complete blocking was achieved when cell aliquots were incubated overnight in basal medium at 4°C. These findings confirm the apoptotic character of the subdiploid cell subset as shown previously (24, 54). (C) In BM, the percentage of subdiploid cells rapidly increased, peaking around 24 h in both short-term FIV-infected and control cats, while at that time no significant increase could be detected in human lymphocytes which were cultured simultaneously and under the same conditions. The representative result of the time course study in panel C reveals the high sensitivity of feline PBLs from young healthy cats to apoptosis in vitro compared to that of human lymphocytes from healthy donors.

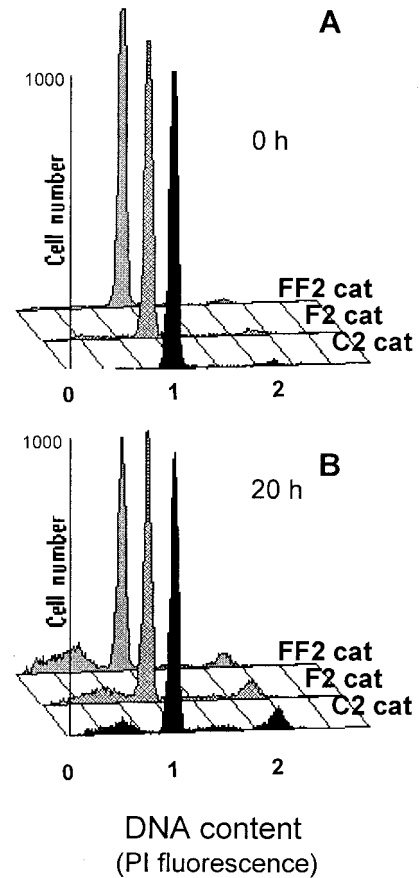


FIG. 8. DNA distribution in infected and noninfected feline lymphocytes before and after culture in BM for 20 h. The DNA distribution curves were determined in lymphocytes collected ex vivo (A) and after 20 h of culture in BM (B) for a noninfected cat (C2), a cat infected with FIV (F2), and a cat coinfecting with FIV plus FeLV (FF2). In the examples shown, subdiploidy was detected in 12% (C2), 17% (F2), and 28% (FF2) of all cells. Note that the PBLs rapidly started dividing under nonstimulating culture conditions (analysis region >1).

apoptotic cells in this group is significant and did not happen by chance. Since FeLV monoinfection can also cause apoptosis in hemolymphatic cells (42), the large number of apoptotic cells in coinfecting cats may be simply the result of the sum of the apoptotic potentials of the two retroviruses contributing to rapid disease progression in coinfecting cats. This hypothesis was not tested because no cats monoinfected for the same duration were available.

We found a strong inverse correlation between the relative and absolute numbers of CD4<sup>+</sup> and bright CD8<sup>+</sup> cells, respectively, and the percentage of apoptosis in PBLs cultured in the presence of PHA and IL-2. A similar relationship was found between the CD4/CD8 ratio and apoptosis. These inverse correlations do not necessarily imply that they are causally related. Nevertheless, it was striking that two well-accepted markers for disease progression, the decrease in CD4<sup>+</sup> counts and CD4/CD8 ratios, and the decline in the number of bright CD8<sup>+</sup> lymphocytes were significantly related to the increased degree of apoptosis. It would have been desirable to observe the FIV-infected cats in a longitudinal study over many months and years. Since our long-term FIV-infected cats were well characterized and represent a source of information that cannot readily be reproduced and since, for ethical reasons, we wanted to obtain as much information as possible, we decided



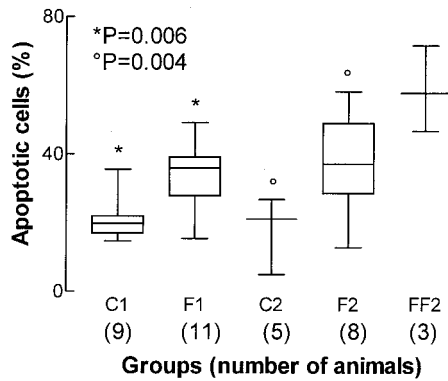


FIG. 9. Percentage of apoptotic cells in stimulated cultures from short- and long-term FIV-infected cats as determined by flow-cytometric cell cycle analysis. Box plots show the rate of apoptosis found in PHA- and IL-2-stimulated cultures after 60 h. The percentage of apoptotic cells refers to all PBLs found in the cell cycle showing a subdiploid DNA content; i.e., all debris, necrotic material, and doublets were excluded as previously described (24). The numbers in parentheses are the numbers of animals per group. The designation of the groups corresponds to that in Fig. 1. It is evident that the rate of apoptosis in FIV-infected animals is significantly higher than in the age- and sex-matched controls under these culture conditions.

to compare short-term and long-term FIV infection. Considering that all the cats originated from the same source and that the same well-defined strain was used for FIV infection, the results obtained in this study mainly reflect differences in the duration of the infection. Since the decline in the number of CD4<sup>+</sup> cells and the reduction of the CD4<sup>+</sup>/CD8<sup>+</sup> ratio are well-documented parameters for disease progression (2, 6, 22, 23, 36) and since we were able to demonstrate a high degree of correlation between apoptotic cells and these two markers, it is quite obvious that in vitro-induced apoptosis can also be considered a marker for disease progression. As shown for HIV-1 (45), there was no correlation between apoptosis and viral load as determined by reverse transcriptase activity in serum samples. We speculate either that low levels of FIV are sufficient to trigger apoptosis or that apoptosis reflects chronic stimulation of the immune system.

The hypothesis that the high degree of apoptosis reflects chronic activation of the immune system is supported by the significant correlation between the percentage of apoptotic cells and bright MHC class II-positive PBLs. The role of MHC class II antigen expression in feline T cells during FIV infection is not yet clear, but in other species MHC class II antigen can

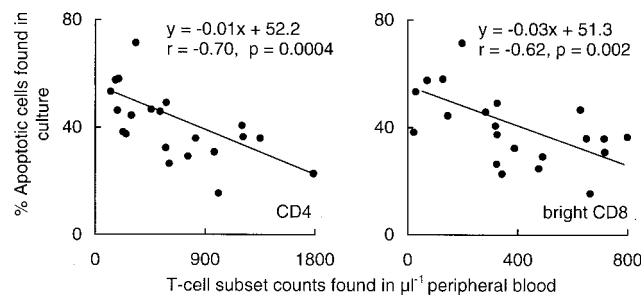


FIG. 10. Relationship between the percentage of in vitro-induced apoptotic cells in PHA- and IL-2-stimulated PBL cultures and immunologic disease markers (CD4<sup>+</sup> T cells, bright CD8<sup>+</sup> T cells) determined in peripheral blood samples of short-term and long-term FIV-infected cats. Each dot represents one cat. It is evident that there is a strong negative correlation between the extent of apoptosis and the absolute counts of CD4<sup>+</sup> and bright CD8<sup>+</sup> T cells, respectively.

promote apoptosis in both T cells (48, 53) and B cells (35). It is interesting that the percentage of MHC class II-positive lymphocytes in cats is significantly higher than that in humans, thereby contributing to the large extent of apoptosis in cultures from noninfected cats, as previously shown (24). The lymphocyte subsets undergoing apoptosis were not determined in the present study, because a clear-cut gating of apoptotic CD4<sup>+</sup> cells and apoptotic CD4<sup>-</sup> cells was not possible due to a decrease in CD4 fluorescence intensity in T-helper cells undergoing apoptosis (unpublished data). However, since the extent of apoptosis reached up to 75%, apoptosis probably was not restricted to CD4<sup>+</sup> or CD8<sup>+</sup> cells alone, because in many cats tested the sum of CD4<sup>+</sup> and CD8<sup>+</sup> cells was lower than 30%. We therefore speculate that apoptosis affected all lymphocyte subsets including B cells (33). This would also explain the relation between the degree of apoptosis and the number of bright MHC class II-positive lymphocytes, since FIV leads to a proliferation of B cells, which physiologically express MHC class II antigen at a high density (17).

The molecular basis of apoptosis is not known. In principle, there are at least two main mechanisms that may initiate apoptosis in vivo as well as in vitro. One mechanism is based on death signals (34) belonging to the TNF superfamily such as TNF- $\alpha$  and CD95 ligand (CD95L) (46), and the other is based on growth factor withdrawal (5). Growth factor withdrawal played a main role when cells were cultured in BM, because preliminary results indicated a reduced rate of apoptosis in the presence of optimal concentrations of autologous plasma together with recombinant IL-2 (unpublished data). Similar results were obtained in HIV studies: lymphocytes from HIV-infected individuals could be rescued from apoptosis by optimal concentrations of IL-2 (3, 4, 15) and even more so by IL-12 (15), possibly by upregulating the expression of the anti-apoptotic *bcl-2* gene (4). In this view, the in vitro induction of apoptosis in lymphocytes from HIV-infected persons may, rather, reflect the death of an increased number of activated cells deprived of growth factors, as previously described (19). This phenomenon also occurs in vivo and downregulates specific immune responses after elimination of an antigen, thereby contributing to T-cell homeostasis (14).

However, apoptosis induced under nonstimulating culture conditions did not correlate with the lymphocyte subset changes (data not shown), whereas statistically significant correlations were found when cells were polyclonally stimulated. We therefore suspected that death signals played an additional role in the induction of apoptosis. TNF- $\alpha$  might be one of these death signals, because higher levels of TNF- $\alpha$  were found in cats infected with FIV than in noninfected controls (26, 28). Furthermore, TNF- $\alpha$  was shown to contribute to apoptosis in a persistently FIV-infected cell line (38). Based on our findings that the TNF- $\alpha$  concentration in PBL cultures set up from FIV-infected cats and noninfected controls did not differ and was very low compared with that used in the Japanese study (38), we concluded that TNF- $\alpha$  was not involved significantly in the induction of apoptosis described here. However, the involvement of feline TNF- $\alpha$  in the induction of apoptosis in our culture system cannot be excluded completely. It could be argued that FIV infection may induce expression of TNF receptors, thereby rendering cells more susceptible to TNF- $\alpha$ , a phenomenon which was not measured in the present study. In addition, we did not determine the role of the other receptors of the TNF receptor superfamily and their ligands, which were described as being important in HIV infection, such as the CD95/CD95L system (7, 18, 36, 46, 47).

Finally, since FIV does not utilize the feline homologue of CD4 as a high-affinity receptor (50), it appears unlikely that

FIV gp120 mediated a cross-linking of CD4 antigens which triggered apoptosis in T-helper cells as shown in HIV (1, 16).

We concluded that the degree of lymphocyte apoptosis is an indicator for disease progression in experimental FIV infection. This marker allows a better description of the asymptomatic phase, since it is changed before the T-helper-cell count decreases. We found no evidence that TNF- $\alpha$  and/or the viral burden was involved significantly in the induction of lymphocyte apoptosis. In view of the significant correlation between apoptosis and the number of bright MHC class II-positive lymphocytes, the effect of immune system stimulation and (viral) coinfections on the degree of hyperactivation, CD95/CD95L expression, and lymphocyte apoptosis remains to be determined.

#### ACKNOWLEDGMENTS

This study was supported by grants from the Union Bank of Switzerland on behalf of a customer and from the Swiss National Science Foundation (grant 31-42486.94) and by the European concerted action on FIV vaccination.

The cat food was kindly donated by Effems, Zug, Switzerland. We thank P. Fidler, P. Bruggmann, M. Huder, S. Oswald, E. Rogg, B. Sigrist, and C. Zraggen for excellent technical assistance.

#### REFERENCES

- Accornero, P., M. Radizzani, D. Delia, F. Gerosa, R. Kurrle, and M. P. Colombo. 1997. Differential susceptibility to HIV-GP120-sensitized apoptosis in CD4+ T-cell clones with different T-helper phenotypes: role of CD95/CD95L interactions. *Blood* **89**:558-569.
- Ackley, C. D., J. K. Yamamoto, N. Levy, N. C. Pedersen, and M. D. Cooper. 1990. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J. Virol.* **64**:5652-5655.
- Adachi, Y., N. Oyaizu, S. Than, T. W. McCloskey, and S. Pahwa. 1996. IL-2 rescues in vitro lymphocyte apoptosis in patients with HIV infection: correlation with its ability to block culture-induced down-modulation of Bcl-2. *J. Immunol.* **157**:4184-4193.
- Akbar, A. N., N. J. Borthwick, R. G. Wickremasinghe, P. Panayiotidis, D. Pilling, M. Bofill, S. Krajewski, J. C. Reed, and M. Salmon. 1996. Interleukin-2-receptor common gamma-chain signaling cytokines regulate activated T-cell apoptosis in response to growth factor withdrawal: selective induction of anti-apoptotic (bcl-2, bcl-x<sub>L</sub>) but not pro-apoptotic (bax, bcl-x<sub>s</sub>) gene expression. *Eur. J. Immunol.* **26**:294-299.
- Akbar, A. N., and M. Salmon. 1997. Cellular environments and apoptosis: tissue microenvironments control activated T-cell death. *Immunol. Today* **18**:72-76.
- Barlough, J. E., C. D. Ackley, J. W. George, N. Levy, R. Acevedo, P. F. Moore, B. A. Rideout, M. D. Cooper, and N. C. Pedersen. 1991. Acquired immune dysfunction in cats with experimentally induced feline immunodeficiency virus infection: comparison of short-term and long-term infections. *J. Acquired Immune Defic. Syndr.* **4**:219-227.
- Baumler, C. B., T. Bohler, I. Herr, A. Benner, P. H. Krammer, and K. M. Debatin. 1996. Activation of the CD95 (APO-1/Fas) system in T cells from human immunodeficiency virus type-1-infected children. *Blood* **88**:1741-1746.
- Beebe, A. M., T. G. Faith, E. E. Sparger, M. Torten, N. C. Pedersen, and S. Dandekar. 1994. Evaluation of in vivo and in vitro interactions of feline immunodeficiency virus and feline leukemia virus. *AIDS* **8**:873-878.
- Beebe, A. M., T. G. Gluckstern, J. George, N. C. Pedersen, and S. Dandekar. 1992. Detection of feline immunodeficiency virus infection in bone marrow of cats. *Vet. Immunol. Immunopathol.* **35**:37-49.
- Benito, J. M., J. M. Zabay, J. Gil, M. Bermejo, A. Escudero, E. Sanchez, and E. Fernandez-Cruz. 1997. Quantitative alterations of the functionally distinct subsets of CD4 and CD8 T lymphocytes in asymptomatic HIV infection: changes in the expression of CD45RO, CD45RA, CD11b, CD38, HLA-DR, and CD25 antigens. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **14**:128-135.
- Bishop, S. A., T. J. Gruffydd-Jones, D. A. Harbour, C. R. Stokes. 1993. Programmed cell death (apoptosis) as a mechanism of cell death in peripheral blood mononuclear cells from cats infected with feline immunodeficiency virus (FIV). *Clin. Exp. Immunol.* **93**:65-71.
- Boni, J., H. Pyra, and J. Schuepbach. 1996. Sensitive detection and quantification of particle-associated reverse transcriptase in plasma of HIV-1 infected individuals by the product-enhanced reverse transcriptase (PERT). *J. Med. Virol.* **49**:23-28.
- Bonyhadi, M. L., L. Rabin, S. Salimi, D. A. Brown, J. Kosek, J. M. McCune, and H. Kaneshima. 1993. HIV induces thymus depletion in vivo. *Nature* **363**:728-732.
- Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science* **272**:60-66.
- Clerici, M., A. Sarin, J. A. Berzofsky, A. L. Landay, H. A. Kessler, F. Hashemi, C. W. Hendrix, S. P. Blatt, J. Rusnak, M. J. Dolna, R. L. Coffman, P. A. Henkart, and G. M. Shearer. 1996. Antigen-stimulated apoptotic T-cell death in HIV infection is selective for CD4+ T cells, modulated by cytokines and effected by lymphotoxin. *AIDS* **10**:603-611.
- Corbeil, J., M. Tremblay, and D. D. Richman. 1996. HIV-induced apoptosis requires the CD4 receptor cytoplasmic tail and is accelerated by interaction of CD4 with p56lck. *J. Exp. Med.* **183**:39-48.
- Flynn, J. N., C. A. Cannon, C. E. Lawrence, and O. Jarrett. 1994. Polyclonal B-cell activation in cats infected with feline immunodeficiency virus. *Immunology* **81**:626-630.
- Gehri, R., S. Hahn, M. Rothen, M. Steuerwald, R. Nuesch, and P. Erb. 1996. The Fas receptor in HIV infection: expression on peripheral blood lymphocytes and role in the depletion of T cells. *AIDS* **10**:9-16.
- Gougeon, M. L., H. Lecoecur, A. Dulioust, M. G. Enouf, M. Crouvoisier, C. Goujard, T. Debord, and L. Montagnier. 1996. Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J. Immunol.* **156**:3509-3520.
- Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J. Exp. Med.* **175**:331-340.
- Guiot, A. L., D. Rigal, J. Bernaud, C. Aeberle, M. Soulier, and G. Chappuis. 1993. Demonstration of cell death process by apoptosis in cat lymphocytes infected by FIV (feline immunodeficiency virus). *C. R. Acad. Sci. Ser. III* **316**:1297-1304.
- Hofmann-Lehmann, R., E. Holznagel, A. Aubert, P. Ossent, M. Reinacher, and H. Lutz. 1995. Recombinant FeLV vaccine: long-term protection and effect on course and outcome of FIV infection. *Vet. Immunol. Immunopathol.* **46**:127-137.
- Hofmann-Lehmann, R., E. Holznagel, P. Ossent, and H. Lutz. 1997. Parameters of disease progression in long-term experimental feline retrovirus (feline immunodeficiency virus and feline leukemia virus) infections: hematology, clinical chemistry and lymphocyte subsets. *Clin. Diagn. Lab. Immunol.* **4**:33-42.
- Holznagel, E., R. Hofmann-Lehmann, K. Allenspach, S. Huettner, B. J. Willett, P. Groscurth, E. Niederer, and H. Lutz. 1996. Flow cytometric detection of activation-induced cell death (apoptosis) in peripheral blood lymphocyte subpopulations from healthy cats. *Vet. Immunol. Immunopathol.* **52**:1-14.
- Kraus, L. A., W. G. Bradley, R. W. Engelman, K. M. Brown, R. A. Good, and N. K. Day. 1996. Relationship between tumor necrosis factor alpha and feline immunodeficiency virus expressions. *J. Virol.* **70**:566-569.
- Lawrence, C. E., J. J. Callanan, B. J. Willett, and O. Jarrett. 1995. Cytokine production by cats infected with feline immunodeficiency virus: a longitudinal study. *Immunology* **85**:568-574.
- Lehmann, R., M. Franchini, A. Aubert, C. Wolfensberger, J. Cronier, and H. Lutz. 1991. Vaccination of cats experimentally infected with feline immunodeficiency virus using a recombinant feline leukemia virus vaccine. *J. Am. Vet. Med. Assoc.* **199**:1446-1452.
- Lehmann, R., H. Joller, B. L. Haagmans, and H. Lutz. 1992. Tumor necrosis factor alpha levels in cats experimentally infected with feline immunodeficiency virus: effects of immunization and feline leukemia virus infection. *Vet. Immunol. Immunopathol.* **35**:61-69.
- Lehmann, R., B. von Beust, E. Niederer, M. A. Condrua, W. Fierz, A. Aubert, C. D. Ackley, M. D. Cooper, M. B. Tompkins, and H. Lutz. 1992. Immunization-induced decrease of the CD4+CD8+ ratio in cats experimentally infected with feline immunodeficiency virus. *Vet. Immunol. Immunopathol.* **35**:199-214.
- Lutz, H., R. Hofmann-Lehmann, C. Leutenegger, K. Allenspach, A. M. Cuisinier, J. Cronier, V. Duquesne, and A. Aubert. 1996. Vaccination of cats with recombinant envelope glycoprotein of feline immunodeficiency virus: decreased viral load after challenge infection. *AIDS Res. Hum. Retroviruses* **12**:431-433.
- Margolick, J. B., A. Munoz, A. D. Donnenberg, L. P. Park, N. Galai, J. V. Giorgi, M. R. G. O'Gorman, J. Ferbas, and the Multicenter Cohort Study. 1995. Failure of T-cell homeostasis preceding AIDS in HIV-1 infection. *Nat. Med.* **1**:674-680.
- Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijster, R. P. Keet, and F. Miedema. 1992. Programmed death of T cells in HIV-1 infection. *Science* **257**:217-219.
- Momoi, Y., T. Mizuno, Y. Nishimura, Y. Endo, K. Ohno, T. Watari, R. Goitsuka, H. Tsujimoto, and A. Hasegawa. 1996. Detection of apoptosis induced in peripheral blood lymphocytes from cats infected with feline immunodeficiency virus. *Arch. Virol.* **141**:1651-1659.
- Nagata, S. 1997. Apoptosis by death factor. *Cell* **88**:355-365.
- Newell, M. K., J. Vander Wall, K. S. Beard, and J. H. Freed. 1993. Ligation of major histocompatibility complex class II molecules mediates apoptotic

- cell death in resting B lymphocytes. *Proc. Natl. Acad. Sci. USA* **90**:10459–10463.
36. Noraz, N., J. Gozlan, J. Corbeil, T. Brunner, and S. A. Spector. 1997. HIV-induced apoptosis of activated primary CD4+ T lymphocytes is not mediated by Fas-Fas ligand. *AIDS* **11**:1671–1680.
  37. Novotney, C., R. V. English, J. Housman, M. G. Davidson, M. P. Nasisse, C. R. Jeng, W. C. Davis, and M. B. Tompkins. 1990. Lymphocyte population changes in cats naturally infected with feline immunodeficiency virus. *AIDS* **4**:1213–1218.
  38. Ohno, K., T. Nakano, Y. Matsumoto, T. Wazari, R. Goitsuka, H. Nakayama, H. Tsujimoto, and A. Hasegawa. 1993. Apoptosis induced by tumor necrosis factor in cells chronically infected with feline immunodeficiency virus. *J. Virol.* **67**:2429–2433.
  39. Ohno, K., T. Watari, R. Goitsuka, H. Tsujimoto, and A. Hasegawa. 1992. Altered surface antigen expression on peripheral blood mononuclear cells in cats infected with feline immunodeficiency virus. *J. Vet. Med. Sci.* **54**:517–522.
  40. Pandolfi, F., M. Pierdominici, A. Oliva, G. D'Offizi, I. Mezzaroma, B. Mollicone, A. Giovannetti, L. Rainaldi, I. Quinti, and F. Aiuti. 1995. Apoptosis-related mortality in vitro of mononuclear cells from patients with HIV infection correlates with disease severity and progression. *J. Acquired Immune Defic. Syndr. Hum. Retrovirol.* **9**:450–458.
  41. Rideout, B. A., P. F. Moore, and N. C. Pedersen. 1992. Persistent upregulation of MHC class II antigen expression on T-lymphocytes from cats experimentally infected with feline immunodeficiency virus. *Vet. Immunol. Immunopathol.* **35**:71–81.
  42. Rojko, J. L., J. R. Hartke, C. M. Cheney, A. J. Phipps, and J. C. Neil. 1997. Cytopathic feline leukemia viruses cause apoptosis in hemolymphatic cells, p. 13–43. *In* Y. Kuchino and W. E. G. Mueller (ed.), *Apoptosis—progress in molecular and subcellular biology*. Springer-Verlag KG, Berlin, Germany.
  43. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**:1447–1450.
  44. Rosenberg, Y. J., A. O. Anderson, and R. Pabst. 1998. HIV-induced decline in blood CD4/CD8 ratios: viral killing or altered lymphocyte trafficking? *Immunol. Today* **19**:10–16.
  45. Rothen, M., S. Gratzl, H. H. Hirsch, and C. Moroni. 1997. Apoptosis in HIV-infected individuals is an early marker occurring independently of high viremia. *AIDS Res. Hum. Retroviruses* **13**:771–779.
  46. Sieg, S., Y. Huang, and J. Kaplan. 1997. Viral regulation of CD95 expression and apoptosis in T lymphocytes. *J. Immunol.* **159**:1192–1199.
  47. Silvestris, F., P. Cafforio, M. A. Frassanito, M. Tucci, A. Romito, S. Nagata, and F. Dammacco. 1996. Overexpression of Fas antigen on T cells in advanced HIV-1 infection: differential ligation constantly induces apoptosis. *AIDS* **10**:131–141.
  48. Truman, J. P., M. L. Ericson, C. J. Choqueux-Seebold, D. J. Charron, and N. A. Mooney. 1994. Lymphocyte programmed cell death is mediated via HLA class II DR. *Int. Immunol.* **6**:887–896.
  49. Willett, B. J., M. J. Hosie, J. J. Callanan, J. C. Neil, and O. Jarrett. 1993. Infection with feline immunodeficiency virus is followed by the rapid expansion of a CD8+ lymphocyte subset. *Immunology* **78**:1–6.
  50. Willett, B. J., L. Picard, M. J. Hosie, J. D. Turner, K. Adema, and P. R. Clapham. 1997. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. *J. Virol.* **71**:6407–6415.
  51. Wolthers, K. C., H. Schuitemaker, and F. Miedema. 1998. Rapid CD4+ T-cell turnover in HIV-1 infection: a paradigm revisited. *Immunol. Today* **19**:44–48.
  52. Woo, J. C., G. A. Dean, N. C. Pedersen, and P. F. Moore. 1997. Immunopathologic changes in the thymus during the acute stage of experimentally induced feline immunodeficiency virus infection in juvenile cats. *J. Virol.* **71**:8632–8641.
  53. Yoshino, T., L. Cao, R. Nishiuchi, Y. Matsuo, I. Yamadori, E. Kondo, N. Teramoto, K. Hayashi, K. Takahashi, N. Kamikawaji, and T. Akagi. 1995. Ligation of HLA class II molecules promotes sensitivity to CD95 (Fas antigen, APO-1)-mediated apoptosis. *Eur. J. Immunol.* **25**:2190–2194.
  54. Zamai, L., E. Falcieri, G. Zauli, A. Cataldi, and M. Vitale. 1993. Optimal detection of apoptosis by flow cytometry depends on cell morphology. *Cytometry* **14**:891–897.