

Female cats have lower rates of apoptosis in peripheral blood lymphocytes than male cats: Correlation with estradiol-17 β , but not with progesterone blood levels

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Abstract

During earlier study, we quantified by flow cytometry the rate of apoptotic feline lymphocytes after overnight culture. We found evidence that the sex of the animals influences the rate of apoptosis, intact females showed lower rates of apoptosis in lymphocytes cultured overnight than castrated male cats. This observation was also confirmed for cats that were previously experimentally infected with the feline immunodeficiency virus (FIV). In an attempt to find an explanation for these sexually determined differences, plasma estradiol-17 β and progesterone levels were measured by radio-immuno assay in the blood of these cats. The hormone levels were analyzed with respect to the rate of lymphocyte apoptosis. As expected, castrated males had lower blood levels of estradiol and progesterone than females. However, no overall correlation was found between hormone blood levels and rate of apoptosis under non-stimulating conditions. Interestingly, the rate of apoptosis found in lymphocytes collected from females and stimulated overnight in phytohaemagglutinin-containing medium, showed a strong negative correlation with the estradiol levels in the blood of these cats. To our knowledge, this is the first confirmation that estradiol in physiological concentrations may protect peripheral lymphocytes from apoptosis after stimulation. No correlation was found in male cats. In conclusion, these observations broaden the list of sexually determined differences of the immune system, sex and sex hormones predispose males and females for certain immune responses and dysfunctions. The present observations have to be taken into account when designing or interpreting experiments on apoptosis and, for example, evaluating the influence of a preexisting FIV infection on the rate of apoptosis. It would be highly advisable to include only spayed cats in studies on the immune system so as to minimize the influence of sex hormones. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Peripheral blood lymphocytes; Cats; Sex hormones; Estradiol; Progesterone

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1. Introduction

Apoptosis is an active mode of cell death characterized by morphological and biochemical features that allow its differentiation from necrosis (Wyllie, 1994). Apoptosis is involved in the development and regulation of the immune system (Cohen, 1993). Immature T cells undergo clonal deletion when they encounter (self) antigen (Smith et al., 1989). Moreover, spontaneous apoptosis occurs in mature T cells when cultured *in vitro* (Perandones et al., 1993). Recently, one of us (EH) has established a flow cytometrical method to quantify apoptosis in feline peripheral blood lymphocytes (Holznagel et al., 1996). We were able to demonstrate that mature lymphocytes of healthy cats undergo apoptosis when cultured overnight (Holznagel et al., 1996). During a further experiment where we determined the influence of an *in vivo* immune activation on the immune system in cats (Hofmann-Lehmann et al., 1998), we found evidence that sex of the animals influences the rate of apoptosis: females showed lower rates of lymphocyte apoptosis than male cats. The immune system has been shown to be broadly affected by sex: females also have higher levels of immunoglobulins, also after immunizations, and they have a markedly more pronounced cellular immune response than males (Butterworth et al., 1967; London and Drew, 1977; Ahmed et al., 1985). Recent studies suggested a central role of female sex hormones in the immune response and in immune-mediated diseases (Stoeger et al., 1988; Shuurs and Verheul, 1990; Da Silva and Hall, 1992; Cutolo et al., 1995). Physiological levels of estrogens stimulate the immune response (Grossman, 1985; Stoeger et al., 1988). Estrogens act dose-dependently via specific receptors that now have been demonstrated on lymphocytes (Cohen et al., 1983; Stimson, 1988). Our goal was to determine whether physiological blood levels of sex hormones influence the rate of lymphocyte apoptosis. Therefore, we measured sex hormone blood levels of the above-mentioned cats and related them to the rate of lymphocyte apoptosis occurring after overnight culture.

2. Animals, materials and methods

2.1. Study design and cats

A total of 22 SPF cats, 20 months of age, were observed over a period of 5 months (Hofmann-Lehmann et al., 1998). Ten cats (group 1) were FIV-negative, 12 cats (group 2) were experimentally infected with FIV Zurich 2 (Morikawa et al., 1991) and were FIV-positive since 12 months (Hofmann-Lehmann et al., 1998). However, these cats were clinically healthy and did not have reduced CD4⁺ lymphocyte counts. Group 1 consisted of eight males and two females; group 2 included four males and eight females. Male animals were castrated before the start of the experiment. The cats were housed in a special facility of the Department of Internal Veterinary Medicine, University of Zurich. They were fed canned and dry cat food (Whiskas and Brekkies, Effems AG, Zug, Switzerland). All cats were bled regularly over the whole observation period ($n=9$). After several weeks, half of the cats were immunized with a recombinant FeLV vaccine as part of a different study (Hofmann-Lehmann et al., 1998). Blood samples of immunized cats

were excluded from the present analysis. Blood samples were examined for lymphocyte subsets, rate of apoptosis and progesterone and estradiol-17 β blood levels.

2.2. Hematology

Hematology parameters were evaluated in EDTA blood with a Contraves AL 820 autolyzer (Winkler et al., 1995). Differential blood counts were performed manually on 2×100 leukocytes.

2.3. Lymphocyte subsets

Feline lymphocyte subsets were determined flow cytometrically (Hofmann-Lehmann et al., 1997). Briefly, whole blood samples free from platelets were indirectly stained with murine MAbs directed against feline CD4 (Ackley et al., 1990), CD8 (Klotz and Cooper, 1986) or MHCII (Holznagel et al., 1996), and detected with FITC-conjugated F(ab)₂ fragment goat anti-mouse IgG (Jackson Laboratories, represented by Milan Analytica AG, La Roche, Switzerland).

2.4. Cell preparation and culture condition

Lymphocytes were purified from heparinized blood samples as described previously (Holznagel et al., 1996; Hofmann-Lehmann et al., 1998). Lymphocyte purity was determined by differential counts. Cells were cultured in round bottom tubes (Falcon 2059, Inotech, Basle, Switzerland) either in the medium alone (sodium bicarbonate and HEPES-buffered RPMI-1640, 10% FCS, L-glutamine, (Gibco Life Technologies, Basle, Switzerland) (Holznagel et al., 1996), or in medium supplemented with 0.2% phytohaemagglutinin (PHA-M, Gibco, cat. no. 10576-015, lot no. 10P2145). Cell viability was evaluated with the trypan blue exclusion test (Sigma, Buchs, Switzerland).

2.5. Detection and quantification of apoptotic cell by flow cytometry

Detection of apoptotic cells was performed by a DNA staining protocol using propidium iodide (PI, Sigma, Buchs, Switzerland) as described previously for human (McCloskey et al., 1994) and feline lymphocytes (Holznagel et al., 1996). Apoptotic cells were identifiable by their reduced DNA content (subdiploid cells) and changed light scattering properties. The DNA content was determined in the cells that were fixed in alcohol for 1 h at 4°C, washed in PBS, treated with RNase and stained with PI (Holznagel et al., 1996). Fluorescence was assessed with an EPICS Profile Analyzer (EPICS Division Coulter Immunology, Hialeah, FL, USA). Area and peak of the PI fluorescence were used to discriminate doublet cells and debris (Zamai et al., 1993; Holznagel et al., 1996). Multimetric data analysis was performed by PC Lysis I software (Becton Dickinson Immunocytometry Systems, San Jose, CA). This method was evaluated extensively for feline lymphocytes and compared to other methods in an earlier study (Holznagel et al., 1996).

2.6. Blood levels of estradiol-17 β and progesterone

Estradiol-17 β and Progesterone were measured in EDTA plasma samples by radio-immuno assay (RIA; Estradiol-¹²⁵I-kit ER-150, Sorin Diagnostics, Saniggio, Italy; Doebeli, 1980).

2.7. Statistics

Data obtained from the different groups were displayed or calculated by the box plot method (McNeil, 1977) using the Astute, Statistics Add-In for Microsoft Excel (version 1.51, DDU Software, University of Leeds, Leeds, UK). In all figures displaying a box plot, the boxes extend from 25% to 75%. A horizontal line represents the median, and the whiskers extend down to the smallest and up to the largest value. Parameters were analyzed for significant differences by the Kruskal Wallis and the Wilcoxon rank sum test in the SAS System for Windows 6.10 (SAS Institute, Cary, NC). Differences were considered significant if $p < 0.05$. Linear regressions of the form $y = a + bx$ were calculated with intercept and slope as well as Pearson's rank correlation coefficients using Astute.

3. Results

3.1. Number of lymphocytes and lymphocyte subsets

No differences were found due to the sex of the animals neither in the total number of lymphocytes nor in lymphocyte subsets.

3.2. Apoptosis of peripheral blood lymphocytes

Apoptosis of Percoll purified cells was measured after 20 h of culture in all the samples collected. In addition, apoptosis was measured in cells cultured for 20 h after adding 0.2% PHA in order to determine apoptosis under stimulating conditions. Due to a tremendous number of samples, these measurements were not continued throughout the whole experiment. The lymphocyte purity after Percoll separation was never significantly different between the two groups. However, lymphocyte apoptosis determinations by flow cytometry were accomplished only at a purity of over 80% lymphocytes. Apoptosis data were analyzed with respect to the sex of the animals. Blood samples were examined from a total of 12 male and 10 female cats (up to nine samples per animal). Females showed less apoptotic lymphocytes than male cats ($p = 0.0032$) when the cells were cultured for 20 h (Fig. 1). The gender-related difference persisted when the data were additionally analyzed in relation to FIV status (Fig. 2). The rate of apoptosis did not differ between male and female cats when the lymphocytes were cultured in the presence of PHA.

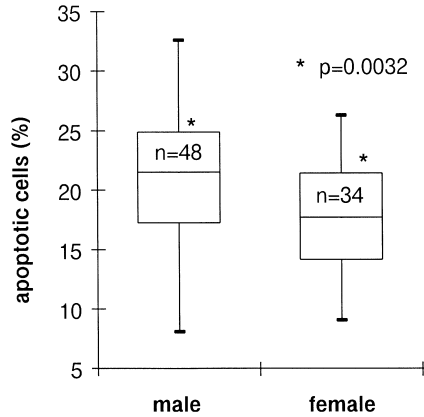


Fig. 1. Box plot of percentage of apoptotic cells determined by flow cytometry as subdiploid cells in peripheral blood lymphocytes: lymphocytes were collected from a total of 12 male and 10 female cats (up to nine blood collections per cat), and were cultured in basal medium for 20 h. Female cats had a significantly lower percentage of apoptotic cells ($*p=0.0032$) than male animals. n =Number of samples analyzed.

3.3. Blood levels of estradiol-17 β and progesterone

Plasma estradiol-17 β and progesterone levels were measured by RIA. Males had significantly lower estradiol levels (5.5–13.6 pg/ml) than females (4.3–32.6 pg/ml, $p=0.007$, Fig. 3). They also had significantly lower progesterone values (0.4–1.7 ng/ml) than the females (0.3–40 ng/ml, $p=0.0004$, Fig. 4). In females, estradiol levels did

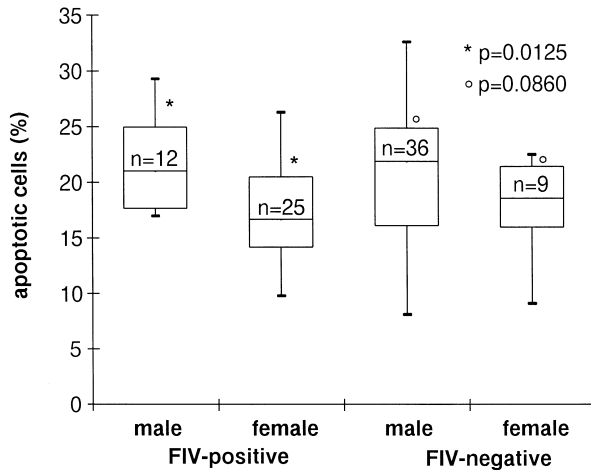


Fig. 2. Box plot of percentage of apoptotic cells after 20 h of cell culture. Females and males were additionally grouped by FIV status. Female FIV-negative cats had a slightly lower percentage of apoptotic cells ($*p=0.0886$). Female FIV-positive cats had a significantly lower percentage of apoptotic cells ($*p=0.0125$). n =Number of samples analyzed.

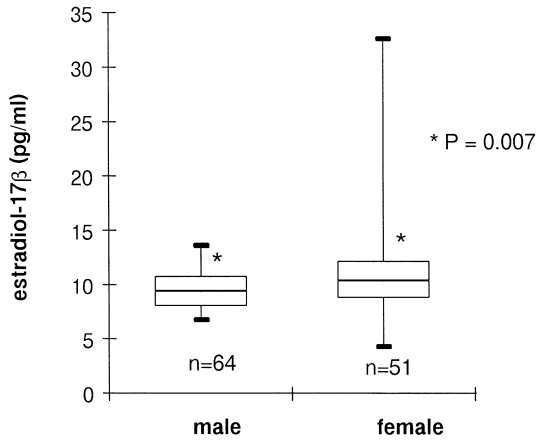


Fig. 3. Plasma estradiol-17β levels were measured by RIA. Males had significantly lower estradiol levels than females (* $p=0.007$). n =Number of samples analyzed.

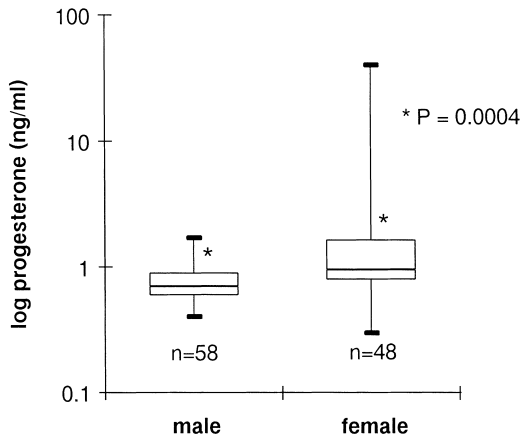


Fig. 4. Plasma progesterone levels were measured by RIA. Males had significantly lower progesterone values than the females (* $p=0.0004$). n =Number of samples analyzed.

significantly correlate with the rate of apoptosis in lymphocytes cultured with PHA ($p=0.0001$, Fig. 5). However, no such correlation was found in lymphocytes of female cats cultured without PHA or in lymphocytes of male animals. Progesterone levels were not correlated in any way with the apoptosis data.

4. Discussion

Apoptosis of feline blood lymphocytes was detected and quantified flow cytometrically. Significant amounts of apoptotic cells were detectable after overnight culture of

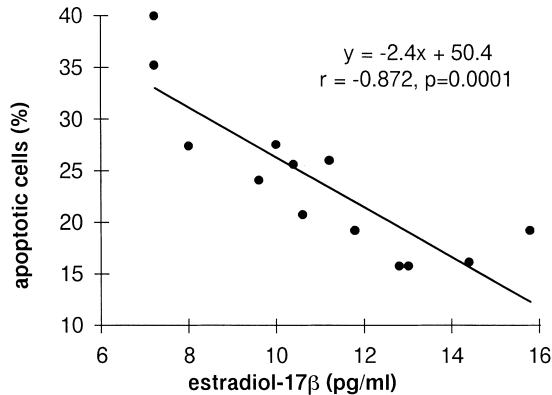


Fig. 5. Significant correlation between the estradiol-17 β blood levels of female cats and the percentage of apoptotic mononuclear cells after 20 h of culture in medium supplemented with PHA.

peripheral blood lymphocytes, but not in uncultured samples. This is similar to the situation in the human system (Gougeon et al., 1996). Interestingly, intact female cats showed a lower rate of apoptotic lymphocytes after overnight culture than castrated males (Fig. 1). This led us to the hypothesis that sex hormones could influence the rate of lymphocyte apoptosis. Steroid hormones cause apoptosis by their presence or absence in various cells and tissues (Thompson, 1994). Estradiol is known to influence apoptosis in several cell-types, for example, osteoclasts (Hughes et al., 1996), erythroid precursors (Blobel and Orkin, 1996) and various cancer cells (Watanabe et al., 1995; Landstrom et al., 1996). Estrogen receptors are also present in lymphocytes and macrophages (Cohen et al., 1983; Stimson, 1988; Cutolo et al., 1993, 1995). Estradiol-17 β and progesterone were determined in the plasma samples of all cats by RIA. As expected, castrated males had lower levels of estradiol (Fig. 3) and progesterone (Fig. 4) than females. Some females showed spiking of estradiol that may indicate the estrous period in these cats (Wildt et al., 1981). The hormone blood levels were put in relation to the rate of apoptosis observed after cell culture. Linear regression and correlation coefficients were calculated. No overall correlation was found between hormone blood levels and rate of apoptosis under non-stimulating conditions when male and female data were included in the analysis. Therefore, it may be speculated that other factors such as other sex hormones or sex-defined differences of the immune system must be responsible for the difference found between female and male animals. The immune system has been shown to be broadly affected by the sex (Butterworth et al., 1967; Ahmed et al., 1985).

The gender-related difference in lymphocyte apoptosis persisted when the data were additionally analyzed in relation to FIV status: male cats always showed higher rates of apoptosis than female cats, independent of the FIV status (Fig. 2). This observation has to be taken into account when designing or interpreting experiments on apoptosis and the influence of a preexisting FIV infection. It is highly advisable to include only spayed cats in studies on the immune system so as to minimize the influence of factors such as sex hormones.

Interestingly, when female samples cultured in the presence of PHA were evaluated by regression analysis, a strong negative relationship was found between the blood estradiol levels and the rate of apoptosis in lymphocytes (Fig. 5). To our knowledge, this is the first confirmation that estradiol in physiological concentrations may protect peripheral lymphocytes from apoptosis after stimulation. We did not see such an influence of estradiol on the lymphocytes of male cats. This may really be a sex-specific difference. More probably, it was created by the statistical method: the very small range of the estradiol levels of the male animals may have led to a 'clouding phenomenon' and thereby to a bad correlation. It is not known how estradiol reduces the susceptibility of lymphocytes to apoptosis after stimulation. However, it may be speculated that the elevated estrogen levels lead to an enhanced expression of nuclear proto-oncogenes in the lymphocytes as described for other tissues and cell lines (Travers and Knowler, 1987; Szijan et al., 1992; Cutolo et al., 1995), and for lymphocytes stimulated to proliferate (Mueller et al., 1984; Reed et al., 1986). Estrogens act dose-dependently via estrogen receptors. Recent studies demonstrated specific receptors on the cells involved in the immune response (Cohen et al., 1983; Stimson, 1988; Cutolo et al., 1993). Binding of the steroid finally results in an activation state of the receptor molecule which in turn binds to specific DNA sequences and changes – as a transcriptional factor – the steady state of messenger RNA of steroid-responsive genes (Landers and Spelsberg, 1992). Thus, the expression of proto-oncogenes and oncosuppressor genes involved in apoptosis may be influenced (Travers and Knowler, 1987; Szijan et al., 1992; Schuchard et al., 1993). In patients suffering from systemic lupus erythematosus (SLE) – preponderance of patients with high estradiol: testosterone balance – an increased expression of *bcl-2* in lymphocytes has been recognized (Aringer et al., 1994; Cutolo et al., 1995). Overexpression of *bcl-2* helps in preventing cell death from apoptosis (Korsmeyer, 1992; Woronicz et al., 1995). Intracellular changes like that may not be evident under non-stimulating conditions. However, after stimulation of the lymphocytes, an apoptosis preventing the effect of estradiol may be evident.

In conclusion, we found higher rates of lymphocyte apoptosis in males than in females, and a protective effect of estradiol in females against apoptosis of lymphocytes after stimulation. These observations broaden the list of sexually determined differences of the immune system by two important points. Sex and sex hormones predispose not only males and females for certain immune responses and dysfunctions, but might also influence many studies elaborating the immune system.

Acknowledgements

This study was supported by a grant from the Union Bank of Switzerland on behalf of a customer, by the Swiss National Science Foundation Grant No. 31-42486.94 and by the European Concerted Action on Feline AIDS. The cat food was kindly donated by Effems, Zug. We are indebted to Dr. K. Allenspach, F. Boretti, P. Bruggmann, P. Fidler, Dr. S. Huettner, Dr. C. Leutenegger, S. Oswald, E. Rogg, D. Wegmann and C. Zraggen for excellent technical assistance and their help with the cats. We kindly thank Dr. M. Doebeli, Hormone Laboratory, Department of Reproduction, Veterinary Faculty,

University of Zurich, for the measurement of estradiol-17 β and progesterone and E. Niederer, Institute of Biomedical Engineering, University of Zurich, and ETH for the help with the flow cytometry.

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