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Early haematological and pathological abnormalities of pathogen-free cats experimentally infected with feline immunodeficiency virus (FIV)

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Summary — Twelve 8–12-week-old specific-pathogen-free (SPF) cats were inoculated intraperitoneally with feline immunodeficiency virus, or with blood from inoculated cats. Three cats of the same age were used as controls. All animals were sacrificed 10 weeks after inoculation. The inoculated cats seroconverted between the 3rd and 6th weeks after inoculation. For 6 infected cats, a decrease in the CD4/CD8 lymphocyte ratio was observed as early as the 6th to the 10th week after inoculation. Granular lymphocytes and atypical cells with cytoplasmic vacuoles and very irregular nuclei were observed in the blood from the 1st to the 10th weeks after inoculation. The only statistically significant differences were obtained 10 weeks after inoculation. Mean leucocyte and lymphocyte numbers were decreased (8 000 and 3 200 per μ l respectively compared with 14 500 and 7 200 per μ l before inoculation). The mean CD4/CD8 ratio was also decreased (from 2.60 to 1.50). The percentage of lymphocytes in the bone marrow was increased, reaching 34% as a mean for infected cats as opposed to 20% for control animals. The atypical cells found in the blood were not observed in the marrow. The sternal bone marrow did not contain lymphoid follicles, as described for HIV infection. Severe follicular hyperplasia was only found in the lymph nodes, but no viral particles could be observed in them.

cat / feline immunodeficiency virus (FIV) / primary stage / experimental infection / haematology

Résumé — Étude hématologique et histopathologique de l'infection expérimentale par le virus de l'immunodéficience féline (FIV) du chat exempt d'organismes pathogènes spécifiques

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(EOPS). Douze chats EOPS âgés de 8 à 12 sem ont été inoculés par voie intrapéritonéale avec du virus de l'immunodéficience féline ou du sang provenant d'animaux infectés ; 3 chats du même âge ont été conservés comme témoins. Ces animaux ont été sacrifiés 10 sem après l'inoculation. Les chats inoculés sont devenus séropositifs entre la 3^e et la 6^e semaine suivant l'inoculation. Pour 6 chats infectés, nous avons observé une diminution du rapport CD4/CD8, entre les 6^e et 10^e semaines après inoculation. Des lymphocytes granuleux et des cellules atypiques à noyau très irrégulier et des vacuoles cytoplasmiques ont été retrouvés dans le sang de la 1^{re} semaine à la 10^e semaine après inoculation. Les seules anomalies statistiquement significatives ont été observées à la 10^e semaine après inoculation ; les chiffres moyens des leucocytes et des lymphocytes étaient diminués, atteignant respectivement 8 000 et 3 200/µl en comparaison de 14 000 et 7 200/µl avant inoculation ; le rapport CD4/CD8 était également diminué passant de 2,6 à 1,5. Le pourcentage des lymphocytes dans la moelle osseuse était augmenté, à 34% en moyenne pour les animaux inoculés, alors qu'il atteignait 20% chez les chats témoins. Les cellules atypiques du sang n'ont pas été retrouvées au niveau de la moelle osseuse. La moelle osseuse sternbrale ne contenait pas de follicules analogues à ceux décrits dans l'infection par le VIH. En revanche, les nœuds lymphatiques présentaient une hyperplasie folliculaire mais il n'a pas été possible de mettre en évidence des particules virales.

chat / virus de l'immunodéficience féline (FIV) / infection expérimentale / stade précoce de l'infection / hématologie

INTRODUCTION

Feline immunodeficiency virus (FIV), initially called feline T-lymphotropic virus (FTLV), were first isolated in the USA (Pedersen *et al*, 1987), and has a worldwide distribution. FIV, like HIV (the aetiological agent of AIDS) belongs to the sub-family Lentivirinae in the family Retroviridae.

Seroepidemiological studies have shown that the prevalence of infection in France was 13% in healthy cats and 36% in sick animals (Moraillon, 1989).

The primary phase of infection was identified after animal inoculation, and is characterized by fever, leukopenia due to neutropenia, and sometimes by anemia. These symptoms persist for 4–9 weeks (Pedersen *et al*, 1987; Yamamoto *et al*, 1988, 1989; Shelton *et al*, 1989, 1990a). Similar observations were made for HIV infection in man (Spivak *et al*, 1984; Murphy *et al*, 1987; Zon *et al*, 1987; Oksenhendler, 1989; Vainchenker *et al*, 1989).

A generalized lymphadenopathy similar to the human AIDS-related complex (ARC)

occurs during the initial stage of FIV infection and persists from 2 to 9 months after inoculation (Yamamoto *et al*, 1988). We recently reported that the CD8 cells were increased in number in the follicles of infected animals but the distribution of CD4 lymphocytes resembled that observed in control lymph nodes (Parodi *et al*, 1994).

The purpose of this work was to determine the early haematological changes and histological alterations of lymph nodes in cats experimentally infected with FIV.

MATERIALS AND METHODS

Animals and experimental design

Fifteen 8–12-week-old specific pathogen free (SPF) domestic short-hair cats, both male and female, were studied. These animals were obtained from IFFA Credo (Les Oncins, France) and Charles River SA (Saint-Aubin-les-Elbeuf, France).

A total of 12 cats were inoculated, as 2 series of 6 animals (A and B); 3 others were used as controls. In each series 1 cat received intraperi-

toneal inoculations of 2 ml supernatant (reverse transcriptase activity: 50 000 cpm/ml from an FIV culture of a Zurich isolate obtained from H Lutz (Morikawa *et al*, 1991)). At 6-week intervals a further cat received a 0.7 ml intraperitoneal infection of heparinised blood from the preceding animal (A1 → A2 → A3 → A4 → A5 → A6) and (B1 → B2 → B3 → B4 → B5 → B6).

Jugular venous blood was collected in EDTA and heparin just prior to inoculation, and 3 or 4, 6 and 10 weeks after inoculation. Complete clinical examination was performed before each sampling. Each sample underwent a complete blood count, a CD4-CD8 lymphocyte differential count and serological tests.

Haematological tests

Erythrocyte, leukocyte and direct platelet counts, haemoglobin concentration and haematocrits were determined with an ABX counter (MINOS ST). A leukocyte differential count was determined for each sample after staining with the May-Grünwald-Giemsa stain.

Serological tests

A serological examination was performed weekly by using a commercial ELISA kit (Petchek, Idexx Portland, USA), with entire virus (Petaluma strain) (Pedersen *et al*, 1987) as antigen. After incubation of the serum sample, bound antibodies were detected with an anti-cat IgG serum conjugated with peroxidase.

Measurement of CD4⁺ and CD8⁺ lymphocyte subsets by flow cytometry

The CD4⁺ and CD8⁺ lymphocytes were measured according to Pedersen *et al* (1990). Briefly 600 000 to 1 000 000 lymphocytes, isolated on ficoll hypaque (Pharmacia), were placed in each of a 96-well sterile plate (Titertek, Flow, France). The primary antibody used was either an anti-CD4 or anti-CD8 mouse monoclonal antibody (Ackley *et al*, 1989; Klotz *et al*, 1986) (Immunochemicals, Clinisciences, France) and the secondary antibody was a goat anti-mouse IgG labelled

with fluorescein isothiocyanate (GAM/FITC, Nordic-Tebu SA, France) used at a 1:50 dilution. The cells were washed extensively in Hanks' solution and fluorescence analysis was carried out with a Profile II flow cytometer (Coultronics, France) at 488 nm excitation with a laser power at 15 mW. A 525-nm bandpass filter was used for FITC fluorescence. Scatter gates were set to minimize analysis of debris. Fluorescence signals were processed by using a log amplifier with a 4-log-scale representation. Results were expressed as mean fluorescence intensity (MFI) converted to a linear value. The linearity of the fluorescence measurement was checked using calibrated fluorescent beads.

Histopathology

All cats were sacrificed 10 weeks after inoculation.

A bone marrow puncture of the iliac crest was performed under general anaesthesia just before death. The marrow collected was then smeared on several slides, dried and stained with May-Grünwald-Giemsa stain. Samples were also obtained from the prescapular, submaxillary and popliteal lymph nodes for transmission electron microscopy.

For electron microscopy studies, lymph nodes were cut into 2-mm-thick cubes and fixed in Karnovsky's liquid (Karnovsky, 1985) at 4°C. After postfixation in 1% osmium tetroxide, samples were embedded in epoxy resin. Semithin sections of the embedded specimens were first prepared in order to locate the germinal centers; the sections were stained with toluidine blue. Ultrathin sections, cut with an ultramicrotome, were then stained from the selected regions, mounted on 200 mesh grids, stained with uranyl acetate and lead citrate, and then examined by electron microscopy.

For histological studies, prescapular, submaxillary, popliteal, iliac and mesenteric lymph nodes, were fixed in Bouin's fluid or frozen in liquid nitrogen. A piece of sternebra was fixed in 10% formalin and decalcified for 24 in 25% formic acid. All fixed samples were finally embedded in paraffin and cut into 4-µm-thick slices and stained with Hematoxylin-Eosin-Safran (HES) and Giemsa (Lennert, 1978).

Staining of the CD4⁺ and CD8⁺ lymphocytes was done on frozen specimens, sectioned in a

cryostat at 4 μm and stained by avidin biotin peroxidase (Peroxidase Universal Kit, Immunotech SA, France) using the same mouse anti-feline CD4⁺ and CD8⁺ (Immunochemicals, Clinisciences, France) monoclonal antibodies as previously described.

Statistical analysis

Using Student's *t* test on paired series (Schwartz, 1983), we compared mean numbers observed at weeks 0 and 10 for leukocytes, erythrocytes, thrombocytes, polynuclear neutrophils and lymphocytes for all 12 cats, and the CD4/CD8 ratio for 6 cats.

RESULTS

Clinical signs

Clinical examinations were carried out the day of inoculation and subsequently on the 3rd, 4th, 6th, and 10 th weeks after inoculation.

Lymph node enlargement (prescapular, popliteal, submaxillary) appeared between the 4th and the 6th weeks for the first 3 cats in each series of inoculations and persisted until death. One cat (B2) also developed tonsillitis, conjunctivitis and an ulcer of the hard palate. The other cats did not develop enlarged lymph nodes. No diarrhoea, fever or behavioural changes were observed.

Serological alterations

Seroconversion was demonstrated in all experimentally inoculated cats. FIV specific antibodies appeared between the 3rd and 6th weeks after inoculation (table I), and all animals remained positive in all subsequent tests.

Table I. Clinical data observed and seroconversion in inoculated cats.

Cat	Superficial lymph adenopathy (weeks pi)	Seroconversion (weeks pi)
A1	+ (6)	3
B1	+ (6)	4
A2	+ (4)	6
B2	+ (4)	6
A3	mild (4)	4
B3	mild (4)	6
A4	No	6
B4	No	6
A5	No	6
B5	No	6
A6	No	6
B6	No	6

pi = post-infection.

Haematological changes

The complete blood counts on weeks 0, 3 or 4, 6 and 10 showed changes in the numbers of leucocytes, erythrocytes and platelets. A significant decrease was only observed in mean leucocyte counts ($p = 0.05$) and lymphocytes ($p = 0.02$) at week 10 (table II).

Leucocyte changes

Neutropenia developed in 4 cats. In one of them (A2) it developed at 10 weeks. In 3 others (A4, A5 and B5) it developed earlier, at 4 or 6 weeks and was transitory, being absent at the subsequent examination, 2 or 4 weeks later. The neutropenia (322, 2 300 and 1 974/mm³) was occasionally associated with leucopenia (1 400, 5 000 and 4 200/mm³).

Table II. Leukocytes and lymphocyte counts in cats after inoculation with feline immunodeficiency virus.

Cat	Leukocytes ($\times 10^3/\mu\text{l}$)					Lymphocytes ($\times 10^3/\mu\text{l}$)						
	W0	W4	W6	W10	W0	W4	W6	W10	W0	W4	W6	W10
A1	9.6	22.6	8.5	5.5	3.1	3.2	3.1	1.3				
B1	4.9	22.1	17.3	10.4	0.9	3.9	4.5	3.2				
A2	23.7	7.3	12.2	7.7	17.1	1.3	1.6	4.6				
B2	23.7	12.1	6.7	6.3	8.5	3.5	3.3	2.0				
A3	18.3	17.3	19.7	8.93	6.8	9.7	5.5	4.0				
B3	9.8	9.9	22.5	12.1	4.8	2.9	10.8	4.3				
A4	20.8	15.8	5.5	4.2	14.6	5.7	1.6	1.2				
B4	23.8	1.4	1.9	8.9	16.7	0.9	0.7	4.0				
A5	7.9	-	5	6.5	2.7	-	2.1	1.2				
B5	15.5	-	4.2	8.1	5.7	-	1.9	3.9				
Mean \pm SD	14.6 \pm 7.21	13.6 \pm 7.33	11.1 \pm 6.77	8.1 \pm 2.25	7.24 \pm 5.74	3.90 \pm 2.77	3.90 \pm 3.02	3.19 \pm 1.44				

W0, W4, W6 and W10: weeks after inoculation; normal reference values (Jain, 1986): leucocytes = 5 500–19 500/mm³, lymphocytes = 1 500–7 000/mm³.

One of the cats with transitory neutropenia (A5) also developed lymphopenia at 10 weeks after inoculation. Lymphopenia was seen in another cat (A1) at 10 weeks and as a transitory feature at 4 weeks after inoculation in cat B2. In cat A4 lymphopenia also developed after 4 weeks and persisted until death.

Erythrocyte abnormalities

One case (cat A4) of regenerative normochromic normocytic anaemia was observed in the 10th week after inoculation.

Platelet abnormalities

Cat B4 developed transitory thrombocytopenia at the 4th week. Cats A4, A5, A6 and B6 developed thrombocytopenia at the 6th week and 10th week.

Abnormal cells

Smears from the 1st week to the 10th week after inoculation revealed the presence of various abnormal lymphoid cells involving atypical lymphoid cells, lymphoblasts, lymphocytes with basophilic cytoplasm and granular lymphocytes (fig 1). The atypical lymphoid cells (fig 1a, b) were large (15–18 µm) with an irregular and sometimes notched nucleus. The not very dense chromatin was very heterogeneous. A juxtannuclear archoplasm was usually observed. The cytoplasm was basophilic and often vacuolated. The lymphoblasts were constituted of large cells (12–18 µm) with a basophilic cytoplasm and smooth chromatin, characteristics of relatively immature lymphocytes. Lymphocytes with basophilic cytoplasm were also found as small mature lymphocytes (10–12 µm) with a basophilic cytoplasm. Granular lymphocytes (fig 1c) were mature cells on average 10–12 µm with a round or sometimes slightly notched nucleus. The cytoplasm contained a few azurophilic granules.

There was some difficulty in detecting the different lymphocytic population in cats, mainly due to the heavy contamination by platelets. Nevertheless, CD4 and CD8 lymphocyte subpopulations could be distinguished (table III). Results were first expressed as ratios: for the 4 cats tested at week 6, the CD4/CD8 ratio was significantly ($p = 0.05$) decreased reaching a mean of

Measurement of CD4 and CD8 lymphocyte subsets

Table III. CD4/CD8 lymphocyte ratio and absolute CD4 numbers.

Cat	W0		W6		W10	
	CD4/CD8	CD4/µl	CD4/CD8	CD4/µl	CD4/CD8	CD4/µl
A1	3.19	705	ND	ND	2.6	172
B1	2.1	235	ND	ND	1.47	806
B3	2.31	1 250	1.96	3 132	0.62	653
B5	2.73	1 457	1.23	430	0.62	543
A6	2.62	1 593	0.85	1 042	0.45	519
B6	2.87	1 240	1.49	2 143	1.02	1 336
Mean ± SD	2.64 ± 0.39	1 080 ± 512.7	1.38 ± 0.47	1 687 ± 1 196	1.13 ± 0.81	672 ± 387

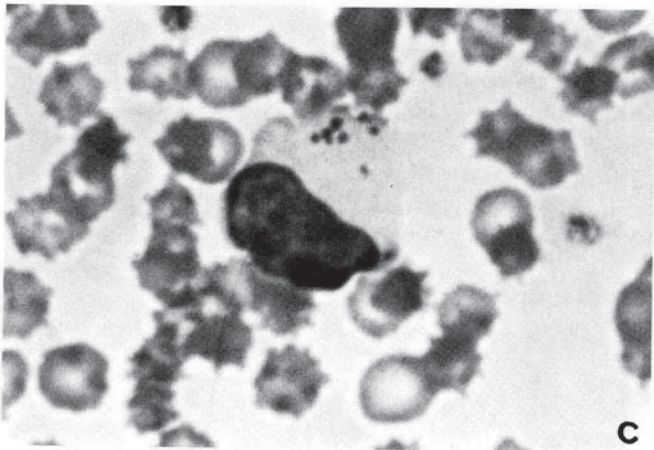
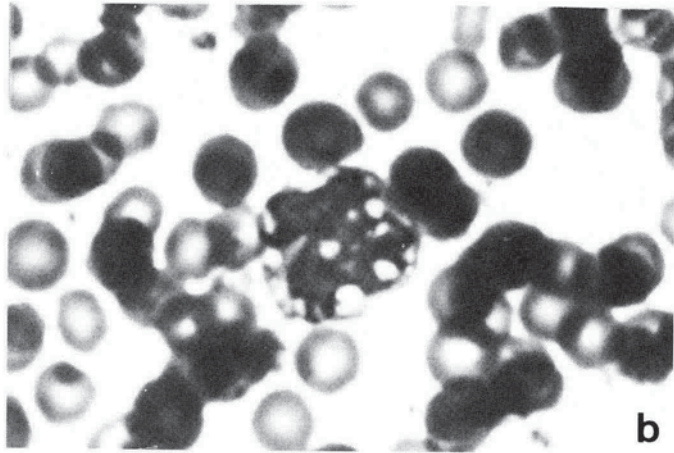
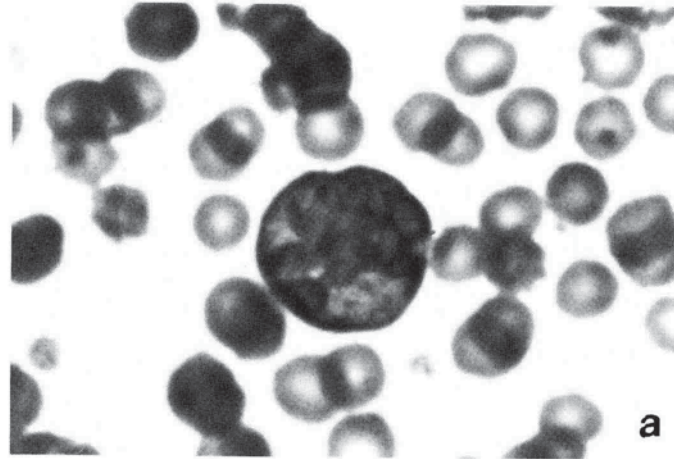


Fig 1. May–Grünwald–Giemsa-stained films of peripheral blood showing (a,b) atypical lymphoid cells with an irregular and sometimes notched nucleus, and (c) granular lymphocyte with cytoplasm containing a few azurophilic granules (original magnification x 4 000).

1.263 as compared with 2.64 before infection. Similarly, the 6 animals tested at week 10 had a CD4/CD8 ratio decreased to 1.50 ($p = 0.01$). When absolute numbers of CD4 lymphocytes were calculated, only one animal (B5) had a marked decreased number at week 6, but 5 of the 6 animals tested at week 10 had a number less than 1 000 CD4 cell/mm³. The only significant variations at the 5% error level were for leukocyte and lymphocyte counts and for the CD4/CD8 ratio.

Bone marrow cytology

Bone marrow from all animals was examined at week 10 and exhibited a high degree of cellularity (table IV).

Myeloblastic and erythroblastic lines were normal, except for cat A4 which was anaemic

at the time of sampling and showed an increase in the erythroblast count. The morphology of these cells was also normal and cytologic tests detected no maturation disorders in these lines.

The myeloid/erythroid ratio was decreased for cats A1, A2, A4, B2, B5, and A6; interestingly these cats had the largest drop in peripheral granulocyte count. The percentage of lymphoid cells in the 2 control cats was 19 and 21 (mean 20). In the inoculated cats values ranged from 19 to 54 (mean 34 ± 3).

This increase in the percentage of lymphocytes was due to an abnormal proliferation of lymphocytes rather than to a reduction of the other cell types. The cytological study of medullary lymphocytes did not reveal atypical lymphocytes as found in the blood. The percentage of lymphoblasts was slightly higher than in the control

Table IV. Bone marrow analysis 10 weeks post-inoculation, normal values.

Cat	Myeloid series (%)	Erythroid series (%)	Lymphoid cells (%)	Monocytes (%)	Myeloid/erythroid
A1	21	33	46	0.6	0.62
B1	37	35	27	0.8	1.05
A2	26	46	27	0.6	0.58
B2	34	43	23	0.4	0.78
A3	45	36	19	0.0	1.27
B3	41	33	24	0.0	1.25
A4	23	51	25	0.3	0.44
B4	51	22	25	0.9	2.28
A5	37	20	42	0.0	1.96
B5	21	25	54	0.2	0.82
A6	21	40	45	0.4	0.65
B6	27	25	48	0.0	1.09
Mean \pm SD	32 ± 3	34 ± 3	34 ± 3	0.4 ± 0.1	1.07 ± 0.15
C1	59	22	19	0.6	2.72
C2	47	32	21	0.0	1.45
Mean	53	27	20	0.3	2.09

C1 = Control 1, C2 = Control 2. Normal reference values (Jain, 1986): myeloid series 29.5–100.1%; erythroid series 12.4–46.9%; lymphoid cells 2.2–15.2%; monocytes 0.2–2.5%; myeloid/erythroid 0.84–1.81%.

cats. The percentage of plasmacytes remained unchanged.

Sternebral histology

Sternebrae of all 12 inoculated cats had the normal histological appearance of growing bone, with no structural abnormality in the bony tissue and no abnormality of ossification. The bone marrow possessed a normal cellularity. The very dense cell population remained polymorphic with a predominance of lymphoid cells and with no nodular or perivascular formation of follicles.

Histological and immunohistochemical changes in lymph nodes

The prominent feature observed in the lymph nodes was a marked follicular hyperplasia frequently associated with irregularities and disappearance of the mantle zone and small lymphocyte infiltration in the centre of the follicles.

Immunohistochemical labelling showed an equal number of CD4 cells in normal and infected cats, whereas CD8 cells in infected animals were larger in number and prominent either scattered or clustered in the follicles.

Electron microscopy examination of at least 2 germinal centres of the prescapular, submaxillary and popliteal lymph nodes was performed and no viral particles were observed in the lymphocytes or dendritic cells of all follicles examined.

DISCUSSION

This study reports the clinical and pathological abnormalities observed during early experimental FIV infection of cats.

Clinical symptoms were limited to lymph node enlargement and were only observed in the first 6 cats, suggesting a lessening in the virulence of the FIV strain during the successive passages. Mucosal inflammation was observed in one cat 4 weeks after inoculation. Such lesions are frequently observed in FIV-infected cats (Shelton *et al*, 1989, 1990b). Unlike other reports (Yamamoto *et al*, 1988; Ishida *et al*, 1989) no fever or diarrhoea were noted in infected animals.

The most significant haematological alterations observed were leucopenia and decrease in the CD4/CD8 lymphocyte ratio. These types of abnormalities were described in FIV in both experimentally and naturally infected cats (Sparger *et al*, 1989; Ackley *et al*, 1990; English *et al*, 1990). The leucopenia was linked to the decrease in the number of lymphoid cells per μl . The decrease of the CD4/CD8 ratio was observed in this experiment much earlier (6 weeks) than described by Ackley *et al* (1990). The same alteration of the CD4/CD8 ratio was described in HIV-infected humans (Miedema *et al*, 1990; Pantaleo *et al*, 1993). The absolute number of CD4 lymphocytes was decreased after 6 weeks (1 animal) or 10 weeks (5–6 animals), but no correlation was found with the clinical signs or the gravity of illness. We noted the presence of atypical lymphoid cells, similar to those described in HIV-infected men (Stepper *et al*, 1988). Further definition of these cells could provide some insights into the early events linked to the infection with FIV; however, appropriate reagents are presently missing for their precise characterization in cat. The presence of azurophilic granules in the cytoplasm of some large lymphoid cells recalls the large granular lymphoid cells sometimes observed in humans and considered to display natural killer activities (Reynolds *et al*, 1987). The reduction of lymphoid cells in the peripheral blood is in contrast to a relative increase in bone marrow lymphoid cells. This increase was not associated with the

presence of cytologically abnormal lymphoid cells but was confirmed by the histological appearance of the bone marrow showing a diffuse lymphoid infiltration.

Some cats displayed a clear reduction in the number of circulating neutrophils; interestingly, this reduction seemed to be linked to an insufficient myelopoiesis (decrease of the M/E ratio) without aspects of differentiation blockage, thus excluding an ineffective myelopoiesis. Such an ineffective myelopoiesis (Mandell *et al*, 1992) was characterized by a leftshift of the myeloid series without the signs of dysplasia observed in some cases of HIV infection.

Furthermore no eosinopenia could be detected in our study but the usual low number of circulating eosinophils in normal cats may preclude this observation. In agreement with the study of Mandell *et al*, 1992, only one cat became anaemic and only one transient thrombocytopenia was noticed.

Anemia and thrombocytopenia are observed in natural FIV infection. It has been suggested that this difference is due to factors such as environment, concomitant infection or stage of the disease.

The histological lesions of the lymph nodes are comparable to those described in HIV infection in man and SIV infection in primates (Meyer *et al*, 1985; Diebold *et al*, 1988). In HIV infection in man, viral particles are observed in the cellular prolongations of the dendritic cells during the persistent lymphadenopathy syndrome (LAS) and the acquired immunodeficiency related complex (ARC) syndrome (Le Tourneau *et al*, 1986). In our experiments on cats, no viral particles were found in the dendritic cells, which may be explained by the precocity of the infection (10 weeks after inoculation).

In conclusion, in cats experimentally infected with FIV, we demonstrated a decrease of the CD4/CD8 ratio as early as 6 weeks post-infection and the appearance

of circulating atypical lymphoid cells, which were not observed in the bone-marrow. The only histological alterations were found in the lymph nodes, which exhibited a severe follicular hyperplasia. Such early lesions could provide the basis for the analysis of the mechanisms of early symptoms. They could also help to test new therapeutic approaches for this phase of the disease.

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