CD4+ and CD8+ T lymphocyte counts and ratio in cats with chronic gingivostomatitis and naturally infected with feline immunodeficiency virus: a preliminary study

Razão e contagem de linfócitos T CD4+ e linfócitos T CD8+ em gatos com gengivoestomatite crônica e infectados naturalmente pelo vírus da imunodeficiência felina: estudo preliminar

Kátia Haipek[1] , Alexandre Gonçalves Teixeira Daniel[2] , Kilder Dantas Filgueira[3]* ,
Fábio Parra Sellera[3] , Ronaldo Gomes Gargano[3] , José Roberto Kfoury Júnior[4] ,
Lilian de Jesus Oliveira[5] , Janaína Munuera Cunha[6] , Archivaldo Reche-Júnior[1,3]

ABSTRACT: This study aimed to evaluate the CD4+ and CD8+ T lymphocytes counts and CD4+: CD8+ ratio in a colony of cats with chronic gingivostomatitis (CGS). We used forty domestic short-haired cats inhabiting the same colony. Ten cats with CGS were immunodeficiency virus-positive (group IV), and ten with CGS were immunodeficiency virus-negative (group III). As a control, twenty cats without CGS were used: ten cats were immunodeficiency virus-positive (group II) and ten cats were immunodeficiency virus-negative (group I). We employed flow cytometry to count CD4+ and CD8+ T lymphocytes. In cats infected with the immunodeficiency virus, the presence of CD4+ lymphocytes were lower both for animals with and without CGS. Conversely, not immunodeficiency virus-infected cats with CGS had a higher amount of CD4+ when compared to seronegative animals without CGS. The counts of CD8+ T lymphocytes showed no significant difference among cats with CGS, whether infected with immunodeficiency virus or not. The CD4+: CD8+ ratio was only different for group III, which was higher than any other group. No difference was observed for total lymphocyte number and CD8+ among groups. By contrast, mean CD4+ levels were different, with cats from groups III and IV showing higher levels than those from groups I and II. The flow cytometry could be a useful tool for the diagnosis and prognosis of cats with CGS infected by the immunodeficiency virus.

KEYWORDS: Oral inflammation, Gingivitis, Feline immune suppression, Immune response.

RESUMO: Este estudo teve como objetivo avaliar a contagem e a razão de linfócitos T CD4+ e CD8+ em uma colônia de gatos com gengivoestomatite crônica (CGS). Foram analisados quarenta gatos domésticos que habitavam a mesma colônia. Dez gatos com CGS foram positivos para o vírus da imunodeficiência (grupo IV), e dez com CGS foram negativos para o vírus da imunodeficiência (grupo III). Como controle, vinte gatos sem CGS foram usados: dez gatos foram positivos para o vírus da imunodeficiência (grupo II) e dez gatos foram negativos para o vírus da imunodeficiência (grupo I). Empregou-se a citometria de fluxo para contagem de linfócitos T CD4+ e CD8+. Nos gatos infectados pelo vírus da imunodeficiência, a presença de linfócitos CD4+ foi menor tanto para os animais com e sem CGS. Por outro lado, gatos não infectados e com CGS apresentaram maior quantidade de linfócitos CD4+ quando comparados a animais soronegativos sem CGS. A contagem de linfócito T CD8+ não mostrou diferença significativa entre gatos com CGS, infectados ou não com o vírus da imunodeficiência. A razão CD4+:CD8+ foi diferente apenas para o grupo III, que foi maior do que qualquer outro grupo. Não foi observada diferença para o número total de linfócitos e CD8+ entre os grupos. Em contraste, os níveis médios de CD4+ foram diferentes, com os gatos dos grupos III e IV apresentando níveis mais elevados do que os dos grupos I e II. A citometria de fluxo pode ser uma ferramenta útil para o diagnóstico e prognóstico de gatos com CGS infectados pelo vírus da imunodeficiência.

PALAVRAS-CHAVE: Inflamação oral; Gengivite; Imunossupressão felina; Resposta imune.

[1]Veterinary Hospital 4*cats* - Av. Pacaembu, 1311, Perdizes, 01234-001, São Paulo-SP, Brazil.
[2]*Gattos* - Clinic Specializing in Feline Medicine and *Cat Expert* Platform, São Paulo, Brazil
[3]University of São Paulo - School of Veterinary Medicine and Animal Science - Department of Animal Reproduction – Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária, 05508-270, São Paulo-SP, Brazil.
[4]Department of Surgery, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, Brazil
[5]Department of Basic Sciences, School of Animal Science and Food Engineering, University of São Paulo, Pirassununga, São Paulo, Brazil
[6]São Paulo City Hall, Municipal Health Department, São Paulo, Brazil
*Corresponding author: kilder@usp.br

INTRODUCTION

Among the retroviruses capable of producing infection and disease in cats, the feline immunodeficiency virus (FIV), from the Lentivirus subfamily, has received special attention in recent years (TANIWAKI; FIGUEIREDO; ARAUJO, 2013). FIV may have gained popularity because of its worldwide distribution and ability to cause a syndrome like the human acquired immunodeficiency syndrome (SIDA), which is caused by another Lentivirus, the human immunodeficiency virus (HIV) (RECHE-JÚNIOR, HAGIWAR; LUCAS, 1997; MILLER et al., 2018).

In cats, chronic gingivostomatitis (CGS) is one of the most common consequences of FIV infection (MILLER et al., 2018; RAVI et al., 2018). Studies have demonstrated such a relationship by correlating FIV–infected cats with CGS in 15 to 80% of animals (DANIEL; HAIPEK; RECHE-JÚNIOR, 2006; ROLIM et al., 2017). Although some studies have suggested that severe immunological dysfunction caused by FIV may be one important factor for CGS development (ROLIM et al., 2017), others have not found any correlation between them (SOUSA-FILHO et al., 2018). Additionally, cats showing acute CGS may be FIV–negative (HARLEY; GRUFFYDD-JONES; DAY, 2011).

Flow cytometry has been widely used in human medicine as well as in other animal species, including cats (CHABANNE et al., 2000). Flow cytometry allows a better understanding of the physiopathology of many diseases, especially those that affects in the immune system (CHABANNE et al., 2000). In feline medicine, flow cytometry has been proposed to applied CD4$^+$ and CD8$^+$ cell counts in FIV-infected animals (WILKERSON, 2012). Quantification of CD4$^+$ and CD8$^+$ lymphocytes is an indirect method to detect FIV (WILKERSON, 2012). The dysfunction of T cells may lead to the progression of FIV and also favor the appearance of other comorbidities such as CGS (CHABANNE et al., 2000; HOSIE et al., 2009).

The stages of FIV infection are based on clinical criteria (HOSIE et al., 2009), with CGS extremely common. During the FIV disease development, animals may undergo a gradual decrease in CD4$^+$ and increase in CD8$^+$, leading to an inversion of the CD4$^+$: CD8$^+$ T lymphocyte ratio, increasing susceptibility to opportunistic infections (TANIWAKI; FIGUEIREDO; ARAUJO, 2013). At the final stage, FIV patients acquire immune deficiency syndrome, with accentuated depletion of CD4$^+$ and CD8$^+$ T lymphocytes (BYRNE et al., 2000), which progresses to diseases that are chronic in nature. The causes of CD4$^+$ T lymphocyte losses may result from decreases in production by the thymus and bone marrow, virus-induced lysis of infected cells, and induction of apoptosis (HOLM; GABUZDA, 2005). CD4$^+$ T lymphocytes act as helper cells, which play a role in immune function and facilitate the development of cell-mediated and humoral immunity. On the other hand, CD8$^+$ T lymphocytes also

called cytotoxically or suppressors, can identify and eliminate cells infected by viruses, such as FIV (HOSIE et. Al., 2009). In the first days after FIV infection, the virus replicates in CD4$^+$ T lymphocytes. CD8$^+$ cytotoxic T cells are detected in circulating blood within one-week post-infection (HOSIE et. Al., 2009).

In cats with CGS, high CD8$^+$ levels are predominant in cases of viral etiology, while CD4$^+$ cells predominate in bacterial-induced diseases (HARLEY; GRUFFYDD-JONES; DAY, 2011; LEE; VERSTRAETE; ARZI, 2020). The CD4$^+$/CD8$^+$ ratio may decrease in cats diagnosed with CGS, resulting from normal circulating levels of CD4$^+$ and increased levels of CD8$^+$ (ARZI et al., 2016; ARZI et al., 2017). Unfortunately, CGS is a severe disease that remains without a successful outcome, with nearly 30% of feline patients not responding to treatment (JENNINGS et al., 2015). On exceptional occasions, it may lead to euthanasia (RAVI et al., 2018). In summary, the cats at more advanced stages of FIV are usually immune suppressed, they would typically present lower lymphocyte levels and not respond to a secondary infection (HARTMANN; WOODING; BERGMANN, 2015). This study aimed to quantify CD4$^+$ and CD8$^+$ T lymphocytes and determine the CD4$^+$: CD8$^+$ ratio in cats with concurrent infection of FIV and CGS.

MATERIALS AND METHODS

Institutional approval

This study was approved by the Animal Care and Ethics Committee of the University of Sao Paulo - Brazil (under the number: 739/2005), and the cat owners signed written informed consents before enrolment in the study.

Animals

We studied 40 cats from the same colony of various sex, breeds, and ages. The age range comprised between one and five years old.

All animals were subjected to a serological enzyme-linked immunosorbent assay (ELISA) to assess whether they were infected with FIV and/or feline leukemia (FeLV). Oral cavity inspection was also performed to evaluate gingival mucosa lesions and classify the degree of CGS (ranging from 1 to 4, namely: 1- mild; 2- moderate; 3- severe; 4- extremely severe) (PIGNONE; MENDICELLI, 2020). All cats with gingivitis were classified as stages 3 or 4. Additionally, based on clinical criteria, CGS cats were also at stage IV of FIV disease, while non-CGS cats were included in stage II of FIV infection, which ranges from I to V (I- initial-acute; II- asymptomatic-latency; III- symptomatic-months to years; IV- SIDA-related complex; and V- terminal stage) (HOSIE et al., 2009; HARTMANN; WOODING; BERGMANN, 2015).

We separated the cats into four groups:
- Group I: Composed of 10 individuals. Cats without CGS and FIV-negative.
- Group II: Composed of 10 individuals. Cats without CGS and FIV-positive.
- Group III: Composed of 10 individuals. Cats with CGS and FIV-negative.
- Group IV: Composed of 10 individuals. Cats with CGS and FIV-positive.

Laboratory tests

Leukogram

We obtained blood samples (5 mL) from animals by venipuncture of the jugular or cephalic veins. These samples were stored in sterile tubes with anticoagulant (EDTA). Total leukocytes were counted using an automated system (Serono®; System 9020X). Moreover, differential leukocyte counts were made from freshly prepared blood smears stained by the Rosenfeld method (VALENCIANO; COWELL, 2020).

Serological Testing

We employed serological testing by the Enzyme-Linked Immunosorbent Assay method to detect anti–FIV antibodies and FeLV antigens in the cats (SNAP® FIV/FeLV Combo Test, IDEXX Laboratories – Westbrook/Maine/United States of America).

We also performed tests using whole blood samples following the manufacturer instructions.

Phenotyping of peripheral CD4+ and CD8+ T lymphocyte subpopulations by the flow cytometry technique

T lymphocyte (CD4+ and CD8+) subpopulations were quantified in the peripheral blood for each of the 40 cats studied by flow cytometry (BYRNE et al., 2000). To this end, we collected 5 mL of blood from each cat into tubes containing EDTA. These samples were maintained at room temperature and processed within 4 h.

For the cytometry technique, 200 µL of whole blood were incubated for 15 min with lysing solution. Each sample was washed with 5% PBS and centrifuged for 8 min at 1800 rpm (BYRNE et al., 2000). The pellet was resuspended and incubated for 35 min with 25 µL of CD4+ (FITC) and CD8+ (RPE) specific monoclonal antibodies, which were previously diluted in a buffer solution. After incubation, we washed tubes twice with 200 µL of FACS buffer solution for 5 min at 1600 rpm (BYRNE et al., 2000).

Statistical Analysis

Based on Shapiro-Wilk, our data were subjected to a parametrical analysis. We also used ANOVA to evaluate the influence of both stimuli upon different experimental groups, and the Bonferroni test was applied as *post hoc*. The statistical analyses were carried out using the PRISMA software. Differences were statistically significant when $P < 0.05$, with results being expressed as mean ± SD.

RESULTS

We found no difference in total lymphocyte number and CD8+ among groups. Conversely, mean CD4+ levels were different for all groups. The difference was enhanced if the cat was also FIV-naïve (group III). The lowest CD4+ levels were found in FIV-infected cats without CGS (group II). The CD4+: CD8+ ratio was only different for group III (cats with CGS and FIV-negative), which was higher than the other groups (Table 1).

Hence, the higher CD4+ levels observed in group III, along with undifferentiated CD8+ levels, led to the highest CD4+: CD8+ ratio in this group (Table 1). Furthermore, it should be noted that all animals were FeLV-negative.

DISCUSSION

Our results partially comply with our expectations since FIV-positive cats with CGS (Group IV) had CD4+ lymphocyte counts higher than FIV-positive without CGS (group II). In other words, FIV-infected cats might not be as immune suppressed as previously thought. In agreement with our hypothesis, FIV-naïve cats but with CGS (group III) showed the highest CD4+ levels, while the lowest counts were observed

Table 1. Mean ± Standard deviation of total Lymphocytes, CD4+ and CD8+ Lymphocytes, and CD4+ to CD8+ ratio.

	Non – CGS		CGS	
	FIV negative	**FIV positive**	**FIV negative**	**FIV positive**
	(Group I)	**(Group II)**	**(Group III)**	**(Group IV)**
Lymphocytes	3501.7 ± 1898.4	3514 ± 2048.4	3726.5 ± 1469.1	2843 ± 1087
CD4+	616.1[c] ± 324	190.3[d] ± 70	1144[a] ± 531.9	623.4[b] ± 247.5
CD8+	390.8 ± 200.4	653.6 ± 418.8	616.5 ± 285.3	5474 ± 188.8
CD4+: CD8+ ratio	1.1[b] ± 0.5	0.5[b] ± 0.4	2.3[a] ± 1.1	1.1[b] ± 0.4

Values with different superscript letters indicate statistical significance

in non-CGS cats with FIV (group II). A similar pattern was not observed for CD8$^+$ lymphocyte count, which was undifferentiated among groups. Such a pattern led to a difference in the CD4$^+$: CD8$^+$ ratio only in group III.

Inflammatory diseases of the feline oral cavity are common in clinical practice (ROLIM et al., 2017; LEE; VERSTRAETE; ARZI, 2020). The CGS associated with FIV infection is quite common and is often severe. It also arises from FIV and other immunodeficient diseases such as FeLV, herpesvirus, and/or calicivirus. Other factors may also contribute to the development of CGS and stomatitis, namely diet, oral conformation, owner–performed oral hygiene, specific genetic characteristics (*i.e.,* juvenile CGS of the Abyssinian, Persian, and Maine Coons), and systemic diseases (HOSIE et al., 2009; LEE; VERSTRAETE; ARZI, 2020). It should be noted that the age range used in our study was based on Bellows et al. (2019), since cats can start the CGS process at nine months of age; the maximum limit of five years has become important to avoid geriatric influence on the immune system. Currently, it has been suggested that the immune system dysregulation caused by FIV could contribute to the unbalance of the oral microbiome. Consequently, oral dysbiosis may favor the occurrence of CGS (OLDER et al., 2020).

Analysis of our results showed a marked decrease in circulating CD4$^+$ T lymphocytes in FIV-infected cats without CGS (group II). Nevertheless, when the total amount of circulating lymphocytes was measured, no statistically significant lymphopenia was noted in any of the groups. It is known that FIV-infected cats tend to develop lymphopenia, which becomes accentuated with the progression of the disease. Such cellular depletion is due to a viral induction of the apoptosis mechanism for CD4$^+$ and CD8$^+$ T cells (HOLM; GABUZDA, 2005). Among the biological parameters that are of interest during the clinical monitoring of FIV-carrying cats, the CD4$^+$ T lymphocyte count (in peripheral blood) is particularly important. A gradual decrease in the CD4$^+$ cell count has been observed in the second stage of the disease (CHABANNE et al., 2000).

Curiously, among the FIV-infected cats, an increased number of CD4$^+$ T lymphocytes was noted for animals with CGS (group IV) even though these animals were classified at stage IV of the disease. This fact suggests that, in the event of opportunistic infections, an active immunological response still exists even in the more advanced stages of infection despite the immunodeficiency syndrome (McMICHAEL; ROWLAND-JONES, 2001). Even though cats with CGS and infected with FIV present an increase in CD4$^+$ T lymphocyte counting, it does not mean that these cells are functionally preserved since retrovirus infection could produce qualitative changes in this cell lineage (BYRNE et al., 2000).

Emphasis should be given that cats with both FIV and CGS (group IV) had a CD4$^+$ cell count well below the counts of non–infected cats (group III). Such response seems to be very coherent since retrovirus-infected cats are depleted of CD4$^+$ T lymphocytes (HOLM; GABUZDA, 2005). Animals with feline immunodeficiency show a significantly smaller number of CD4$^+$ T lymphocytes when compared to non–infected animals due to the apoptosis mechanism mentioned earlier (HOLM; GABUZDA, 2005).

However, we observed an increase in the CD8$^+$ T lymphocyte count among FIV- infected cats that did not have severe CGS (group II); however, such an increase did not bear significance. One must consider that in our study the cats with CGS were, based on clinical criteria, at stage IV of the FIV disease, while those without CGS were clinically classified as at stage II of FIV infection. Hence, group IV cats were at a more advanced stage of the disease and must show a decreased level of CD8$^+$ T lymphocyte (OBERT; HOOVER, 2000)

Together, FIV-naïve cats with CGS (group III) had the highest ratios of all groups, which is of great prognostic importance. Therefore, the increase in ratios in this group could be associated with the occurrence of CGS. Our findings are important when considering the treatment of CGS. Classically, glucocorticoids have been used in the CGS treatment, but immunosuppressive effects have limited their use in patients afflicted with diseases that compromise the immune system (HOSIE et al., 2009). Glucocorticoids may interfere with the cellular immune response in a myriad of ways, with the most prominent being a decrease in T lymphocyte blastogenesis (LEE; VERSTRAETE; ARZI, 2020). Therefore, glucocorticoids should be used with caution to treat CGS in FIV-infected cats so that CD4$^+$ and CD8$^+$ T lymphocyte immunological response is not further compromised (BYRNE et al., 2000).

FIV causes immunodeficiency by several mechanisms, mainly because it induces lymphocyte apoptosis and death of other cells. The virus initially infects CD4$^+$ lymphocytes and macrophages, and later CD8$^+$ lymphocytes and B lymphocytes and, when the immunodeficiency syndrome begins, other cell types are also affected, such as dendritic cells, monocytes, microglia, astrocytes and neurons (GUTIÉRREZ et al., 2015). The immunodeficiency syndrome is related to the high level of viral replication and a drastic depletion of CD4$^+$ lymphocytes and consequent greater susceptibility to secondary and opportunistic infections (HARTMANN; WOODING; BERGMANN, 2015). It can also be potentiated in situations of malnutrition, chronic environmental stress, use of immunosuppressive drugs, in addition to endocrinopathies and other chronic diseases (GUTIÉRREZ et al., 2015). Thus, for a better understanding of the results the importance of segregating the animals, with or without CSG, in the different stages of the retrovirus in question was justified. FIV has received considerable attention in recent years because of its worldwide distribution and ability to cause a syndrome like SIDA. Accordingly, FIV-infected cats may be an effective study

model for HIV in developing drugs and vaccines (HOSIE et al., 2009; HARTMANN; WOODING; BERGMANN, 2015). Additional studies are needed to better understand the intersection between FIV and CGS, contributing not only to feline medicine but also to medicine in humans, since animals with FIV can be considered experimental models for HIV patients positives.

CONCLUSIONS

No difference in total lymphocyte number and CD8+ among groups was observed. On the other hand, lower CD4+ levels were detected in FIV-infected cats without CGS. The CD4+:

CD8+ ratio was higher in cats with CGS and FIV-negative. The CD4+: CD8+ ratio was only different for cats with CGS and FIV-negative. The flow cytometry could be a useful tool for the diagnosis and prognosis of cats with CGS infected by the immunodeficiency virus.

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