Characterization of neutrophil extracellular traps in cats naturally infected with feline leukemia virus

Amanda B. Wardini,1 Anderson B. Guimarães-Costa,2 Michelle T. C. Nascimento,2 Natalia R. Nadaes,1 Maria G. M. Danelli,1 Carlos Mazur,1 Claudia F. Benjamim,3 Elvira M. Saraiva2† and Lucia H. Pinto-da-Silva1†

1Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Seropédica, RJ, Brazil
2Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil
3Departamento de Farmacologia Básica e Clínica, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brazil

INTRODUCTION

Neutrophils are among the first cells involved in the defence against pathogens such as bacteria, fungi and protozoa. These cells utilize mechanisms such as phagocytosis, degranulation and oxidative burst to efficiently kill and eliminate micro-organisms (Nathan, 2006). Recently, a new microbicidal mechanism named NETosis was described in human, bovine and fish neutrophils, as well as in chicken heterophils. In this mechanism, neutrophils die after releasing ‘neutrophil extracellular traps’ (NETs), threads composed of DNA, histones and granular proteins that not only ensnare microorganisms but also provide a high local concentration of antimicrobial molecules (Brinkmann et al., 2004; Lippolis et al., 2006; Urban et al., 2006; Guimarães-Costa et al., 2009). The release of NETs is dependent on reactive oxygen species (ROS) production and can be stimulated by bacteria, fungi, protozoa and some soluble molecules (e.g. interleukin-8, lipopolysaccharide, β-glucan and phorbol esters) (Brinkmann et al., 2004; Lippolis et al., 2006; Urban et al., 2006; Fuchs et al., 2007; Chuammitri et al., 2009; Guimarães-Costa et al., 2009).

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus found worldwide in domestic cats and some small, related felids (Hoover & Mullins, 1991; Leutenegger et al., 1999). The release of NETs is dependent on reactive oxygen species (ROS) production and can be stimulated by bacteria, fungi, protozoa and some soluble molecules (e.g. interleukin-8, lipopolysaccharide, β-glucan and phorbol esters) (Brinkmann et al., 2004; Lippolis et al., 2006; Urban et al., 2006; Fuchs et al., 2007; Chuammitri et al., 2009; Guimarães-Costa et al., 2009).

Feline leukemia virus (FeLV) is a naturally occurring gammaretrovirus found worldwide in domestic cats and some small, related felids (Hoover & Mullins, 1991; Leutenegger et al., 1999). FeLV is a horizontally transmitted virus that can lead to fatal neoplasia, degenerative diseases of the haematopoietic system and immunodeficiency (Dunham & Graham, 2008). There is interest in this
Cat infection with FeLV has two possible outcomes: approximately 60% of cats recover from a mild, transient infection with a high titre of neutralizing antibodies, whilst about 30% fail to develop an effective humoral immune response and become persistently viraemic, leading to immunosuppression and susceptibility to opportunistic infections (Hardy, 1982; Rojko & Kociba, 1991; Dunham & Graham, 2008). Dysfunction of cellular immunity by FeLV infection, however, is not restricted to T or B cells (Cockerell & Hoover, 1977; Tompkins et al., 1989), as the loss of neutrophil function has also been reported as a consequence of FeLV infection. Moreover, neutrophils from FeLV-infected cats suffer reduced ROS production, phagocytosis and chemotaxis (Lafrado & Olsen, 1986; Lewis et al., 1986; Kiehl et al., 1987; Lafrado et al., 1987).

The aim of this study was to analyse NETs from healthy (FeLV−) and FeLV naturally infected (FeLV+) symptomatic and asymptomatic cats. We have demonstrated for the first time that cat neutrophils release pathogen-induced NETs and that, most significantly, NET induction is associated with the health status of FeLV-infected cats.

RESULTS AND DISCUSSION

Neutrophil phagocytosis

Neutrophils are among the first leukocytes to reach the site of infection and play important roles in immune responses to infection. Previous studies have demonstrated that FeLV infection affects neutrophil function and their response to different stimuli (Kiehl et al., 1987; Dezzutti et al., 1989, 1990; Hoffmann-Jagielska et al., 2005). Thus, neutrophils from FeLV+ experimentally infected and clinically ill cats show a reduced chemotactic response in vitro in comparison with neutrophils from FeLV− cats (Kiehl et al., 1987). Using flow cytometry, Hoffmann-Jagielska et al. (2005) demonstrated that neutrophils from naturally infected animals, although clinically asymptomatic, presented an impaired phagocytic capacity compared with neutrophils from FeLV− animals. In their study, although the mean number of bacteria phagocytosed was similar, differences in the percentage of cells able to phagocyte Escherichia coli were observed. In our work, the phagocytic capacity of purified neutrophils from FeLV−, FeLV+ asymptomatic and FeLV+ symptomatic cats was evaluated in vitro using Leishmania promastigotes. Our results showed that the neutrophils obtained from these different groups were able to bind and phagocytose promastigote forms of Leishmania regardless of their FeLV infection status. No difference was observed in the number of parasites phagocytosed per neutrophil (FeLV− = 1.107 ± 0.06; FeLV+ asymptomatic 1.104 ± 0.07; FeLV+ symptomatic = 1.146 ± 0.05), in the percentage of infected neutrophils (FeLV− = 48 ± 13.5%; FeLV+ asymptomatic = 49.5 ± 11.3%, FeLV+ symptomatic = 47 ± 13%) or in the infectivity index (FeLV− = 26.07 ± 2.65; FeLV+ asymptomatic = 27.02 ± 2.69; FeLV+ symptomatic = 27.62 ± 2.86) among the studied groups, indicating that the recognition and uptake mechanisms involved in Leishmania ingestion by neutrophils are not affected by FeLV infection. These apparently contradictory results could be explained by methodological differences and by the specific micro-organisms used to evaluate neutrophil phagocytic properties, or by the particles utilized, given that Leishmania can be recognized by lipophosphoglycan and glycoprotein 63 (gp63) surface molecules (Van Strijp et al., 1993).

Characterization of feline NETs

The NETosis process has an important microbicidal function, as demonstrated in human neutrophils during infection with bacteria, fungi and Leishmania (Brinkmann et al., 2004; Urban et al., 2006; Guimarães-Costa et al., 2009), as well as in bovine neutrophils during mastitis infection (Lippolis et al., 2006). Generation of oxygen radicals has been associated with NET release (Fuchs et al., 2007). Thus, exogenous H2O2 generated from NADPH oxidase induced NET release in mammals and release of heterophil extracellular traps in chickens (Fuchs et al., 2007; Chumamitri et al., 2009). FeLV infection inhibits neutrophil activation by inhibition of protein kinase C and a decrease in ROS production (Lafrado & Olsen, 1986; Lewis et al., 1986; Lafrado et al., 1987; Dezzutti et al., 1989, 1990; Hoffmann-Jagielska et al., 2005).

We therefore initially characterized the capacity of healthy cat neutrophils to release NETs following incubation with Leishmania promastigotes. A classical NET fibrous structure protruding extracellularly from neutrophils enwrapping a number of parasites was observed by optical microscopy, indicating the ability of cat neutrophils to release NETs following Leishmania stimulus (Fig. 1a, b). As histone is a known NET-associated component, we stained NETs with 4,6-diamidino-2-phenylindole (DAPI) and anti-H2A histone antibody (Fig. 1c, d). We found promastigotes ensnared by meshes labelled by these two markers (Fig. 1c, d). Secondary control antibody did not label the parasites (Fig. 1h) or the neutrophils (data not shown). To demonstrate the DNA content of these meshes further, DNase was added to the promastigote–cat neutrophil interaction. It was seen that digested NETs disappeared after enzymic treatment, confirming the presence of DNA in NETs released by feline neutrophils (Fig. 2).

NET release and myeloperoxidase (MPO) activity

Previous studies have shown that FeLV infection impairs neutrophil microbicidal functions and that affected cats are more susceptible to opportunistic infections (Lafrado & Olsen, 1986; Lewis et al., 1986). Decreased activation of
neutrophils from FeLV experimentally infected animals during phagocytosis of serum-opsonized particles has been described previously (Lewis et al., 1986). Likewise, neutrophils from FeLV animals were not activated by opsonized particles with serum obtained from FeLV+ cats (Lewis et al., 1986). Dezzutti et al. (1989, 1990) demonstrated that neutrophils from experimentally viraemic cats presented reduced protein kinase C activation when stimulated by phorbol myristate acetate (PMA). Similarly, neutrophils from healthy cats exposed to inactivated FeLV or purified p15E FeLV protein resulted in a diminished response to Ca\(^{2+}\) ionophore (Lafrado et al., 1987).

Moreover, a reduction in the oxidative activity of neutrophils from FeLV naturally infected asymptomatic animals was observed after stimulation with \textit{E. coli} and/or PMA (Hoffmann-Jagielska et al., 2005).

Thus, we decided to quantify spontaneous (unstimulated) and \textit{Leishmania}-stimulated NET release into supernatants from the neutrophils in the cat groups under analysis. NETs released by neutrophils obtained from FeLV− and FeLV+ asymptomatic cats increased around nine- and threefold after parasite stimulation in relation to unstimulated neutrophils, respectively (Fig. 3a, b). In contrast, neutrophils from FeLV+ symptomatic cats were not able to significantly increase NET release upon parasite stimulation (Fig. 3c). The same inability to induce NETs was observed in neutrophils from PMA-stimulated symptomatic cats (data not shown). Taken together, these

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**Fig. 1.** Characterization of feline NETs. (a–e) Cat neutrophils were stimulated with promastigotes (cell:parasite ratio of 1:5) at 34 °C and 5% CO\(_2\) for 2 h. Cells were fixed with paraformaldehyde and stained with Diff-Quick (a, b), DAPI (5 ng ml\(^{-1}\)) (c) or anti-histone H2A (diluted 1:800) (d). In (c), an overlay of differential interference contrast (DIC) and DAPI fluorescence shows a parasite (arrowhead) ensnared by the NET (arrow). (e) Merged image of DAPI staining (blue) and anti-histone (red) fluorescence showing pink staining resulting from the overlay of images. (f–h) DIC of paraformaldehyde-fixed \textit{Leishmania} promastigotes (f), DAPI staining (5 ng ml\(^{-1}\)) of the promastigote nucleus and kinetoplast (g) and anti-histone labelling (h). N, Neutrophils. Bars, 20 \(\mu\)m (a–e); 4 \(\mu\)m (f–h).

**Fig. 2.** DNase eliminates feline NET formation. Cat neutrophils were stimulated with promastigotes (cell:parasite ratio of 1:5) at 34 °C and 5% CO\(_2\) for 2 h. Cells were fixed with paraformaldehyde and stained in the absence (a, b) or presence (c, d) of DNase (50 IU ml\(^{-1}\)) for 2 h and then incubated with DAPI (5 ng ml\(^{-1}\)). (a, b) DIC (a) and fluorescent image (b) of \textit{Leishmania} entrapped by NETs (arrowheads) stained by DAPI (arrow). (c, d) Phagocytosis of \textit{Leishmania} (arrowhead) and a degraded NET (arrow) following enzymatic treatment with DNase shown by DIC (c) and fluorescence (d). N, Neutrophils. Bars, 20 \(\mu\)m.

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findings suggest that FeLV infection could lead to an exacerbated neutrophil activation, making these cells unresponsive to new stimuli. Interestingly, when NETs released from unstimulated neutrophils were compared, it was observed that neutrophils from FeLV\(^+\) symptomatic animals produced 15 and 2.3 times more NETs than unstimulated neutrophils from FeLV\(^-\) and FeLV\(^+\) asymptomatic cats, respectively (Fig. 3d).

Several granular proteins, such as elastase, MPO, cathepsin G, lactoferrin and gelatinase, are released in association with NETs (Brinkmann \textit{et al.}, 2004). MPO is an important enzyme involved in oxidative stress and in the inflammatory process itself (Loria \textit{et al.}, 2008). Thus, to characterize further the NET release from neutrophils, MPO activity was quantified in the supernatants of unstimulated cells in the different cat groups being studied. The increase in MPO activity was similar to the increase in DNA concentration measured in the unstimulated neutrophils tested (Fig. 4). Furthermore, MPO activity increased in unstimulated neutrophils from FeLV\(^+\) symptomatic animals compared with healthy ones, implying spontaneous neutrophil activation during progressive FeLV infection. Altered neutrophil functions have been detected in other viral infections, particularly with human immunodeficiency virus type 1 and human T-lymphotropic virus type 1 (Guerreiro \textit{et al.}, 2005; Salmen \textit{et al.}, 2007). An increase in NET release and MPO activity in unstimulated neutrophils from FeLV\(^+\) symptomatic cats was also observed, suggesting that progressive viral infections may induce chronic neutrophil activation.

In this report, along with the finding that NET induction may be modulated by a viral infection, NET release by feline neutrophils is described for the first time. Overall, our results indicate that the NET mechanism appears to be exhausted in FeLV\(^+\) animals, preventing a neutrophil response to other stimuli. We believe that this feature could be considered a marker of disease progression and an important neutrophil trait in FeLV infection, similar to what has been described recently for sepsis and septic arthritis (Margraf \textit{et al.}, 2008; Lögters \textit{et al.}, 2009).

**METHODS**

**Animals.** The experimental group consisted of male cats ranging from 1 to 7 years old, which were evaluated with regard to their haemogram, body mass and temperature, dehydration status, appetite, faeces, mucosal cavities, lymph nodes and the presence of tumour mass. None of the cats presented any laboratory or clinical
signs of concurrent infection, including haemotropic mycoplasmas (Bobade & Nash, 1987; Boujon et al., 1991). All animals included in this study were negative for feline immunodeficiency virus (FIV), as detected by immunoblotting for FIV recombinant p24 protein. The animals were separated into three groups (n=8 per group): (i) clinically healthy cats, negative for FeLV; (ii) FeLV naturally infected asymptomatic cats; and (iii) FeLV naturally infected, clinically ill (symptomatic) cats. The FeLV+ animals were selected using an indirect immunofluorescence kit (VWRD) on blood smears. This work was approved by the Bioethical Committee on Animal Research of the Federal Rural University of Rio de Janeiro (UFRRJ), process #013837.

Parasite. *Leishmania amazonensis* (WHOM/BR/75/Josefa) promastigotes were maintained at 26°C in Schneider’s insect medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum (Cipiron), 10% human urine and 40 μg gentamicin (Sigma) ml⁻¹. In all assays, promastigotes in the stationary phase of growth (5–6 days) were washed twice in PBS (pH 7.2) with centrifugation at 1300 g for 10 min. Cells were resuspended in RPMI 1640 without serum and their viability was assessed by a 0.01% Trypan blue assay using a haemocytometer.

Neutrophil purification. Freshly drawn peripheral blood was collected from each cat by venipuncture from the cephalic vein into tubes containing heparin (Vacutainer; BD). Feline blood was placed on a discontinuous Ficoll-hyphaque gradient (densities 1.007/1.119, Sigma-Aldrich) and centrifuged at 400 g for 30 min at 22°C. Neutrophils were recovered from the 1.119/1.007 interface, resuspended in PBS and washed at 400 g for 10 min. Cells were resuspended in RPMI 1640 without serum and their viability was evaluated by a 0.01% Trypan blue assay using a haemocytometer.

Neutrophil–parasite interactions. Purified neutrophils (1 × 10⁶ per well) were plated onto 0.001% poly-l-lysine (Sigma-Aldrich)-pretreated coverslips (13 mm²) in 24-well tissue culture plates (Techno Plastic Products). Promastigotes were added to the adhered neutrophils at a parasite : cell ratio of 5 : 1. After 2 h at 34°C in 5% CO₂ free parasites were removed by extensive washing with PBS and the cultures were stained using Diff-Quick solution and mounted in Permount (Fisher Scientific). The association index was obtained by multiplying the percentage of infected neutrophils by the number of parasites per infected neutrophil, by randomly counting at least 200 cells in each of the duplicate coverslips.

Characterization of NETs. Purified neutrophils (1 × 10⁵ per well) were incubated with promastigotes for 2 h, as described above, fixed with 4% paraformaldehyde and stained with Diff-Quick solution. For immunofluorescence, slides were stained with DAPI (5 μg ml⁻¹; Sigma) or anti-histone H2A antibody (diluted 1:150; kindly provided by Dr A. Zychlinsky, Max Planck Institute, Germany), followed by Texas red-labelled anti-mouse (diluted 1:800; Vector Laboratories). Images were taken using an epifluorescence Zeiss Axiosplan microscope.

Quantification of NET released. Neutrophils (5 × 10⁵ per well) were incubated with or without *L. amazonensis* at a promastigote : neutrophil ratio of 5 : 1. After 1 h, restriction enzymes (*Eco*RI and *Hind*III, 20 U ml⁻¹ each; New England BioLabs) were added to the cultures, which were maintained for 1 h at 34°C and 5% CO₂. After centrifugation, NETs were quantified in the culture supernatant using a PicoGreen dsDNA kit (Invitrogen) according to the manufacturer’s instructions. NET DNA concentrations were calculated using herring sperm DNA (Sigma) as a standard.

MPO activity. Supernatants (50 μl) from unstimulated neutrophils were incubated with 25 μl 3,3',5,5'-tetrathymethylbenzidine and 50 μl H₂O₂. The colour change reaction was allowed to proceed for 5 min at 37°C and 100 μl 2 M sulfuric acid was added to stop the reaction.

Different concentrations of lysed neutrophils were used as controls. Absorbance was determined at 405 nm using a microtitre plate spectrophotometer (V-Max; Molecular Devices) with SoftMax PRO 4.0 software.

Statistical analysis. Data are presented as means±SEM and were analysed using Student’s t-test with Kruskall–Wallis or analysis of variance test corrections (GraphPad Prism 3.0).

ACKNOWLEDGEMENTS

We are grateful to Dr Ivan B. M. Sampaio (Federal University of Minas Gerais, Brazil) for helpful statistical analysis, to Dr A. Zychlinsky (Max Planck Institute, Germany) for the anti-H2A antibody, Dr Dumith C. Bou-Habib (Oswaldo Cruz Foundation, Brazil) for critical reading of the manuscript and Judy Greven for editing the text in English. This work was supported by MCT/CNPq/MS-SCTE-DECIT 25/2006 and FAPERJ (170.507/2007; 110.737/2007; 111.584/2008). A. B. W. received a fellowship from CAPES.

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