NOTE *Immunology*

Intracellular Reactive Oxygen Species Production by Polymorphonuclear Leukocytes in Bovine Leukemia Virus-Infected Dairy Cows

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ABSTRACT. The present study assesses the oxidative burst activity from polymorphonuclear leukocytes (PMNLs) from bovine leukemia virus (BLV)-infected cows. Fifteen clinically healthy cows were divided into serologically positive cows without any hematological alteration, serologically positive animals with persistent lymphocytosis (PL) and healthy serologically negative cows. The oxidative burst activity from the PMNLs was evaluated by flow cytometry using 2',7'-dichlorofluorescein diacetate as a probe. PMNLs from each cow were incubated with heat-killed *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) to stimulate oxidative burst activity. The results of the present work showed no significant difference in the oxidative burst activity without any stimulus and elicited by *S. aureus*. Conversely, a decrease in the oxidative burst index induced by *E. coli* in PMNLs was observed in BLV-infected cows.

KEY WORDS: deltaretrovirus, neutrophils, oxidative burst.

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Bovine leukemia virus (BLV) is a member of the Retroviridae family, belonging to the genus deltaretrovirus, which is genetically and structurally similar to human T-cell leukemia virus-1 to 4. Although, it has been successfully eradicated in some regions of Europe, BLV is among the most widespread livestock pathogens in many countries, especially in dairy herds [9].

BLV tropism for B cells has been well documented, but other cell populations also bear the provirus, including $CD8^+$ T cells, monocytes and polymorphonuclear leukocytes (PMNLs) [22]. However, the role of these cells in BLV pathogenesis is not completely understood.

PMNLs, which are mainly neutrophils, are the main cells involved in the innate host defense against invading microorganisms by the process known as phagocytosis. During phagocytosis, PMNLs produce reactive oxygen species (ROS) to kill phagocytosed bacteria, a process that relies on the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [15].

Various viruses, analogously to some microorganisms, can affect the regular functions of PMNLs. This phenomenon predisposes the animal to different coinfections or superinfections and increases the severity of the infections [16, 29]. In fact, BLV has been associated with the coinfection of some microorganisms [20, 23, 26, 28, 30]. Therefore, the increase in the incidence of some infections associated with BLV could be the result of some alteration in the innate immune response of these animals.

The role of lymphocytes and monocytes/macrophages in BLV infection has been the subject of many studies in BLV-infected cattle [2, 6, 8, 9, 24, 31, 32], but PMNLs function has been less well investigated [1, 7, 13, 19, 29]. Thus, the aim of the present study was to evaluate the function of PMNLs from naturally BLV-infected cows through the evaluation of the intracellular ROS production, even that elicited by *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*).

One hundred adult Holstein cows from the same herd were sera-tested using an agar gel immunodiffusion test (Tecpar®, Curitiba, Brazil) and enzyme-linked immunosorbent assay (cat. no. 284–5, VMRD, Inc., Pullman, WA, U.S.A.) using the glycoprotein, gp51. From these animals, fifteen clinically healthy animals in the middle of lactation during their second or third parity were selected.

Based on the results of serological testing and their hematological profile, the selected animals were divided uniformly into three groups: healthy animals that were serologically negative in both tests without any hematological alterations [5], aleukemic (AL) animals that were serologically positive in both tests without any hematological alterations [5] and cows with persistent lymphocytosis (PL) that were serologically positive in both tests (Table 1). The

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Table 1. White blood cell differential count of BLV-free (BLV^-) and BLV-infected cattle (BLV^+) without (AL) and with persistent lymphocytosis (PL^+) used in the present study

	Leukocytes ($10^3/\mu l$)	Neutrophils ($10^3/\mu l$)	Lymphocytes ($10^3/\mu l$)	Eosinophils ($10^3/\mu l$)	Monocytes ($10^3/\mu l$)
BLV^+/PL^+	19.70[a)] (17.50–35.70)	4.37[a)] (3.09–5.71)	14.38[a)] (11.70–29.27)	0.79[a)] (0.36–1.40)	0.27[a)] (0.18–0.71)
CV (%)	31.75	21.42	39.04	50.59	62.86
BLV^+/AL	10.80[b)] (9.6–12.0)	2.94[a)] (2.30–4.10)	6.50[b)] (5.78–7.24)	0.76[a)] (0.70–0.96)	0.29[a)] (0.11–0.46)
CV (%)	9.16	24.69	8.39	14.10	46.43
BLV^-	10.80[b)] (9.6–11.7)	2.21[a)] (1.69–4.68)	6.60[b)] (5.70–8.53)	0.96[a)] (0.11–1.48)	0.23[a)] (0.10–0.61)
CV (%)	9.21	46.21	17.89	63.04	84.00

Data are presented as medians and ranges. Different letters between lines indicate $P<0.05$. CV: coefficient of variation. BLV^-: bovine leukemia virus serologically negative animals. BLV^+/AL: bovine leukemia virus serologically positive animals without any hematological alteration. BLV^+/PL^+: bovine leukemia virus serologically positive animals with persistent lymphocytosis.

BLV-infected cattle were classified with PL when the lymphocyte counts exceeded 10,000/μl and the leukocytes counts exceed 15,000/μl, as established by Thurmond *et al.* [27]. The persistence of lymphocytosis was confirmed within a period of 72 days.

Each animal was then codified and randomized, and further analysis was performed without previous knowledge of the state of the animal from which the sample was drawn. We emphasize that this research was conducted in accordance with the Ethical Principles in Animal Research and approved by the Bioethical Commission of FMVZ-USP (number 1227/2007).

The total leukocytes counts were determined using an automatic cell counter (ABX ABC VET, Horiba ABX Diagnostic®, Montpellier, France). The differential leukocyte count was performed by routine smears.

The intracellular ROS production (oxidative burst activity) and the preparation of propidium iodide (PI)-labeled *E. coli* (O98:H28) and *S. aureus* (ATCC 25923) were performed as described by Hasui *et al*. [10], with some modifications. Briefly, 100 μl of whole blood was incubated at 37ºC for 30 min with 0.3 μM of 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (cat. no. D6883, Sigma Aldrich, St. Louis, MO, U.S.A.). In addition, the oxidative burst activity was measured with 100 μl of heat-killed PI-labeled *E coli* or *S. aureus* to stimulate intracellular ROS production. After centrifugation, the erythrocytes were removed by hypotonic lysis. Lastly, the leukocytes were resuspended in 300 μl of phosphate buffered saline and analyzed using flow cytometry.

A conservative, active gate was created around the PMNLs population, using the cell size (forward scatter) and cell granularity (side scatter) characteristics of the cells, as proposed by Kampen *et al.* [14]. The intracellular 2',7'-dichlorofluorescein (DCF) fluorescence of the PMNLs was determined by flow cytometry using excitation at 488 nm. DCFH-DA is a cell-permeable nonfluorescent probe that becomes highly fluorescent when converted by ROS to DCF, a process that occurs in a dose-dependent manner. The green fluorescence from DCF was detected at 500–530 nm. The quantification of ROS production (oxidative burst) was estimated by the geometric mean fluorescence intensity (MFI) of the DCF green fluorescencecell. The intensity of the intracellular ROS production induced by the phagocytosis of the heat-killed labeled *E. coli* and *S. aureus* was expressed as follows:

$$\text{Oxidative Burst Index} = \frac{\text{MFI of stimulated PMNLs}}{\text{MFI of unstimulated PMNLs}}$$

The percentage of the PMNLs that produced ROS was equal to the number of fluorescent PMNLs divided by the total PMNL count multiplied by 100. At least 20,000 cells were recorded per sample. The data were analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, U.S.A.).

Kolmogorov-Smirnov test was used to check for a Gaussian distribution. If the data had a Gaussian distribution, one-way analysis of variance (ANOVA) was applied, followed by the Tukey-Kramer multiple means comparison test. For nonparametric data, the Kruskal-Wallis test was used, followed by Dunn's test to compare all of the pairs of columns. The oxidative burst index between the BLV-infected cows (AL and PL) was compared by Student's *t*-test for unpaired data. Otherwise, the oxidative burst index between the BLV-infected cows (AL or PL) and the serologically negative animals was analyzed by the Mann-Whitney test. The statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, U.S.A.). The data are presented as the means $\pm$ SD or the medians and ranges. Significance was declared at $P\leq0.05$ unless otherwise indicated.

In the present study, no significant difference was observed in the measurements of the oxidative burst activity without any stimulus in the BLV-infected dairy cows. Indeed, the percentage of PMNLs that produced ROS elicited by *E. coli* and *S. aureus* was not different among the groups (Table 2).

Conversely, a tendency toward a decrease in the oxidative

Table 2. Intracellular reactive oxygen species production by polymorphonuclear leukocytes

	Measurement of ROS production	BLV^-	BLV^+/AL	BLV^+/PL^+	BLV^+	*P* value
Basal	Intensity	711.6 ± 385.1[a)]	630.6 ± 290.1[a)]	665.6 ± 189.2[a)]	648.1 ± 231.7[a)]	0.97
	PMNL ROS^+ (%)	84.1 ± 13.5[a)]	76.0 ± 20.3[a)]	89.1 ± 9.5[a)]	82.6 ± 16.5[a)]	0.67
E*scherichia coli*	Oxidative Burst Index	2.03 ± 0.24[a)]	1.64 ± 0.26[a,b)]*	1.67 ± 0.21[a,b)]*	1.66 ± 0.22[b)]	0.0019
	PMNL ROS^+ (%)	92.7 ± 5.1[a)]	90.6 ± 13.2[a)]	92.5 ± 7.2[a)]	91.6 ± 10.1[a)]	0.97
Staphylococcus aureus	Oxidative Burst Index	2.08 ± 0.93[a)]	1.88 ± 0.58[a)]	1.74 ± 0.42[a)]	1.81 ± 0.48[a)]	0.96
	PMNL ROS^+ (%)	84.8 ± 15.4[a)]	88.7 ± 10.6a[a)]	87.7 ± 10.4[a)]	88.2 ± 9.9[a)]	0.94

Data are presented as means ± SD. (BLV^-=5, BLV^+/AL=5, BLV^+/PL^+=5, BLV^+=10). ROS: reactive oxygen production. Basal: without any stimulus. Different letters among columns indicated $P<0.05$. * $P=0.066$ between BLV^- and BLV^+/AL or BLV^+/PL^+. BLV^-: bovine leukemia virus serologically negative animals. BLV^+/AL: bovine leukemia virus serologically positive animals without any hematological alteration. BLV^+/PL^+: bovine leukemia virus serologically positive animals with persistent lymphocytosis. BLV^+: bovine leukemia virus serologically positive animals.

burst index induced by *E. coli* was found in the AL ($P=0.066$) (n=5) or PL ($P=0.066$) (n=5) BLV-infected animals compared to the non-infected animals (n=5). However, no significant difference was encountered in the oxidative burst index induced by *E. coli* between the BLV-infected animals with different hematological profiles (AL and PL; $P=0.81$). With this in mind, when we compared the oxidative burst elicited by *E. coli* in the BLV-infected cows (n=10) and in the healthy serologically negative animals (n=5), the results were significantly different ($P=0.019$; Table 2). No significant difference in the oxidative burst index elicited by *S. aureus* was observed (Table 2).

The innate immune response senses bacteria by different molecular recognition receptors, such as the Toll-like receptors (TLRs), which are highly expressed on neutrophils, and are the best-characterized family of receptors. These receptors have been implicated as critical mediators that induce inflammatory and host antimicrobial defenses against bacteria via the enhancement of NAPDH oxidase activity and a concomitant high intracellular ROS production [11, 12]. There are different TLRs, and each one is responsible for recognizing key molecules unique to a specific group of pathogens, resulting in different elicitations of the immune response [3]. For instance, TLR2 recognizes a variety of agonists, including peptidoglycan and lipoteichoic acid from Gram-positive bacteria, such as *S. aureus*. In contrast, TLR4 recognizes LPS from enterobacteria, such as *E. coli* [25].

It should be emphasized that the recognition of lipopolysaccharides (LPSs) requires other molecules in addition to TLR4. LPSs binds to the LPS-binding protein (LBP) present in the serum, and this LPS-LBP complex is subsequently recognized by CD14, which is expressed on monocytes/macrophages and neutrophils. Moreover, LPS stimulation is followed by the increased physical proximity between CD14 and TLR4 in the membrane, suggesting that CD14 and TLR4 may interact in LPS signaling [25].

Recent reports showed that TLR4 is targeted by viruses such as HTLV-1 [4]. For instance, HTLV-infected patients have a pronounced immunodeficiency that is associated with frequent opportunistic infections by various pathogens [4, 16, 29], a situation that has also been described in BLV-infected animals [20, 23, 26, 28, 30]. Indeed, BLV has been used as a model to study HTLV-1 pathogenesis [8, 9].

In our study, we observed a reduction in the intracellular ROS index elicited by *E. coli*. Taking into account that the HTLV-1 p30 protein targets the TLR4 signaling pathway, leading to a down-regulation of the TLR4 cell-surface expression [4] and that a protein that closely resembles p30 structurally has also been described in BLV [17, 20], we propose that the reduction in the ROS production observed was induced by an alteration in CD14 and/or the TLR4 signaling pathway in the BLV-infected dairy cows.

Furthermore, it has been demonstrated that p30 suppresses the enzymatic activity of glycogen synthase kinase 3β in HLTV-1 infection. This suppression leads to the induction of IL-10, which suppresses the immune system [4]. In fact, IL-10 expression was elevated in the peripheral blood mononuclear cells in PL and AL BLV-infected cattle after three and twelve weeks of experimental infection, respectively [31]. The serum IL-10 level was also enhanced in HTLV-1-infected subjects [32]. In addition, the LPS-stimulated macrophages expressing p30 exhibit a marked decrease in the release of pro-inflammatory cytokines and an increase in the release of anti-inflammatory cytokines in HTLV-1 infection [4].

In our study, no difference was observed in the oxidative burst activity induced by *S. aureus*. It is well known that there are marked differences between the host innate immune response to *E. coli* and *S. aureus* infections, as has been described by Bannerman *et al.* [3] in intramammary infection. These researchers suggested that the limited cytokine response to *S. aureus* may contribute to the well-known ability of the bacterium to establish a chronic intramammary infection. Additionally, the intramammary inoculation of *E. coli* has been shown to upregulated the expression of TLR4 and TLR2 in the pathogen-inoculated mammary glands and in the mammary lymph nodes. In contrast, *S. aureus* did not significantly regulate the TLRs [18]. Therefore, these facts may explain, at least in part, the non-significant difference observed in the oxidative burst index induced by *S. aureus* in the present study.

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