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ORIGINAL ARTICLE

Association of TNF-α gene promoter region polymorphisms in bovine leukemia virus (BLV)-infected cattle with different proviral loads

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Abstract Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine involved in the immune response against viral and other infections. Its expression levels are affected by a polymorphism in the promoter region of the gene. Bovine leukemia virus is a retrovirus that infects cattle and develops two different infection profiles in the host. One profile is characterized by a high number of proviral copies integrated into the host genome and a strong immune response against the virus, while the most relevant property of the other profile is that the number of copies integrated into the host genome is almost undetectable and the immune response is very weak. We selected a population of cattle sufficiently large for statistical analysis and classified them according to whether they had a high or low proviral load (HPL or LPL). Polymorphisms in the promoter region were identified by PCR-RFLP. The results indicated that, in the HPL group, the three possible genotypes were normally distributed and that, in the LPL group, there was a significant association between the proviral load and a low frequency of the G/G genotype at position -824.

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Introduction

Bovine leukemia virus (BLV) is a retrovirus that belongs to the family *Deltaretrovirus* and is the causative agent of enzootic bovine leucosis. It is distributed all over the world and mainly infects domestic cattle, with major incidence within dairy herds due to management conditions [13]. Many efforts have been made to eradicate this virus from the herds, and some countries, mostly in the EU, have completely eradicated the infection [1, 5, 27]. Once the animal becomes infected with BLV, the provirus integrates into the host genome, and the host remains infected for life. The infection is associated with a chronic benign polyclonal expansion of $CD5^+$ B cells or persistent lymphocytosis (PL) in 30-40 % of the animals. However, only 1 to 5 % of these animals develop malignant lymphoma five years or more after infection. The other 60-70 % of infected animals (non-PL) remain subclinically infected, with no clinical or hematological symptoms. Many authors consider the state of lymphocytosis a preleukemic state [10].

This $CD5^+$ B cell expansion allows the classification of the infected animals as PL or non-PL. Animals with an increase in white cell count of three standard deviations above the median and with similar counts for a period of not less than two months can be considered to have PL [21]. Over the last decade, major studies involving infected animals have allowed a more thorough classification [2, 11, 15, 16]. Within the PL group, all animals carry a high proviral load and develop a strong immune response against the major antigenic proteins of the viral particle, p24 and gp51, and these are called the high proviral load (HPL) group. In the non-PL group, 40 % of the animals develop a humoral response comparable to that of the HPL group. The remaining animals, with a frequently

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undetectable proviral load and a weak immune response against the same antigenic proteins, can be grouped into another category, usually designated as the low proviral load (LPL) group. There are several genetic factors that are thought to influence the development of each infection profile, but none of them is absolutely conclusive, and this leads us to believe that the resistance to viral dissemination is associated with an undefined genetic profile [16, 24]. Many attemps have been made to develop an efficient vaccine. Nevertheless, none of them seems to be fully effective for inducing an immune response strong enough to protect the host against infection [11].

It is known that tumor necrosis factor alpha (TNF-α) plays an important role in protecting the organism against several viral infections. It is a potent cytokine that plays a crucial role in the immune response against infections, exerting its action by inducing the expression of adhesion molecules and other cytokines, cell proliferation, and apoptosis [14]. It is produced by many cell types, including monocytes/macrophages, T lymphocytes, neutrophils and NK cells. Its association with disease progression has been well established in several acute and chronic infections and autoimmune diseases in humans [3, 6, 19]. Overexpression of TNF-α in sheep experimentally infected with BLV might contribute to the progression of bovine leukosis in animals developing persistent lymphocytosis or B-cell lymphosarcoma [17, 18]. It has been demonstrated in cattle that the level of expression of TNF-α can be related to the progression into BLV-induced lymphoma and that this level of expression can be related to genetic polymorphisms in the promoter region. Specifically, a point mutation at position -824 is associated with differences in the expression of TNF-α in concanavalin A–stimulated peripheral blood leucocytes (PBLs) in normal cattle, and the proviral load is significantly increased in cattle homozygous for the -824 G/G allele [20]. With all this in mind, we can speculate that animals belonging to the LPL group do not carry the G/G genotype, or it should be present at a low frequency. In this work, we study the TNF-α polymorphism in the promoter region in two different groups of animals classified as LPL or HPL in order to investigate the association of the G/G genotype with the proviral load.

Materials and methods

Animals

A total of 274 Holstein (Holando Argentino) cows were selected for the study. The animals came from twelve high-BLV-prevalence dairy herds from different and remote geographical areas in Argentina. The smallest dairy farm analyzed had 200 milking cows, and the largest one had approximately 1,500 milking cows. In all of them, without exception, the seroprevalence was higher than 80 %, as tested by ELISA108 [12]. We analyzed pedigree data at least four generations back in order to avoid any blood relationship among animals.

BLV infection and proviral load determination

Every cow from the selected herds was bled, tested by ELISA108 [12], and separated according to whether they were positive or negative for BLV infection. Briefly, 3 ml of heparinized blood (5 U/ml) was obtained by jugular vein puncture. PBLs were obtained by mixing the "buffy coat" with 11 ml of a cold solution containing 150 mM NH_4Cl, 8 mM Na_2CO_3 and 6 mM EDTA (pH 7) for 1 min to completely lyse red blood cells. The white cell pellet or "buffy coat" obtained after centrifugation at 1,000*g* for 7 min at 4 °C was resuspended in 1 ml of PBS solution, transferred to a 1.5-ml tube and centrifuged at 10,000*g* for 2 min. Genomic DNA was isolated by using the standard protocol of the Illustra blood genomic kit (GE Healthcare) and quantified by measuring the optical density at 260 nm with a Nanodrop2000 (Thermo Scientific). Animals were classified as HPL or LPL by a semiquantitative PCR technique, either by conventional PCR, amplifying a fragment of the *pol* gene [15], or by real-time PCR in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems), also amplifying a fragment of the *pol* gene (Forletti *et al.* submitted). The classification of animals by conventional PCR was confirmed by real-time PCR. In all cases, when the number of integrated copies of provirus was less than 100/μg of DNA from peripheral blood of the host genome, the animal was classified as belonging to the LPL group. In the same way, when the number of integrated copies of the provirus was greater than 10,000, in the same conditions as the other group, animals were assigned to the HPL group.

TNF-α promoter region genotyping

The bovine TNF-α promoter region sequence was downloaded from the GenBank database (accession no. Z14137) and analyzed using the RestrictionMapper software. As a result, the simultaneous digestion with two restriction enzymes, *SacI* and *AluI,* allowed the three possible genotypes to be distinguished. From each animal, a fragment of the promoter region of 639 nucleotides upstream of the first coding codon was amplified by conventional PCR and digested with the two above-mentioned enzymes. The specific primers used were TNFAF (forward) 5′CTGGAGAAGTGGGGGTCA3′ and TNFAR (reverse) 5′ATAAAGCCCCTCCCATTTCTAA3′ [20]. The following reaction mix was used: 250 ng of template DNA, 10x Taq buffer without Mg^+ (Invitrogen), 0.5 mM dNTPs, 0.25

μM each primer, and 2 mM $MgCl_2$ in a final volume of 50 μl. The amplification program consisted of a denaturation step (5 min at 94 °C), 27 cycles (95 °C for 1 min, 62 °C for 1 min, and 72 °C/1 min) and a final elongation step (72 °C for 10 min). The amplified fragments were separated by electrophoresis in an agarose gel, purified following the instruction from the PureLink Quick Gel Extraction Kit (Invitrogen), and sequenced in an automatic sequencer (ABI377 Genetic Analyzer, Applied Biosystems) with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.

Two restriction enzymes were used. *Sac*I recognizes the sequence 5′GAGCT↓ G3′, and *Alu*I recognizes the sequence 5′AG↓ CT3′. Aliquots (10 μl) of amplified TNF-α promoter region were digested separately with *Sac*I or *Alu*I according to the manufacturer's instructions. Each digested product was mixed with 2 μl of 5x loading buffer and resolved by electrophoresis in a 1 % agarose gel. The RFLP pattern was resolved by staining with Sybr Safe dye according to the manufacturer's instructions, visualized in a blue light transilluminator and photographed.

Statistical analysis

In order to estimate the distribution of the genotypes in a population with the premise that this variable is multinomial with three types (homozygous A/A or G/G and heterozygous A/G), it must be assumed that there is an equal proportion of each of the three types. An equation of multinomial distributions was applied to estimate the size of the sample to be analyzed [7]. Under this premise, with a confidence level of 0.95 and an error of 0.1, the minimum size of the sample was determined to be 127 individuals of each category (HPL or LPL).

The association of A/A, G/A, and G/G genotypes with the proviral load (high or low) was estimated by the chi^2 test and quantified by means of the odds ratio (OR). This analysis was performed with the PROC FREQ program, SAS 9.3 (2011).

Results

Proviral load distribution

Animals were classified as HPL or LPL using a semi-quantitative PCR technique. Animals that in two consecutive samples over a period of three months or more, showed a proviral load that was intermediate (IPL) between HPL and LPL were not included in our study, Fig. 1. Only two animals were discarded for this reason. We believe, after many years of research in this field, that

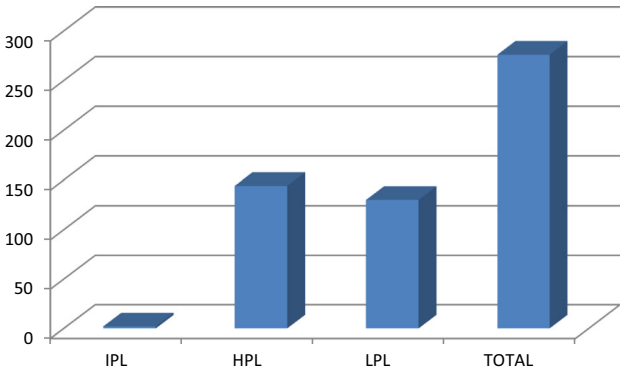

Fig. 1 Distribution of the sampled animals into each category of proviral load (IPL, intermediate proviral load; HPL, high proviral load; LPL, low proviral load)

the classification of animals in an intermediate proviral load category does not reflect the true situation. Animals become infected, and they develop a high or low proviral load at least three months after infection is resolved. In some special cases, it takes more time for the host to acquire the proviral load that will characterize it throughout its life. This situation can be avoided by bleeding the animals every three months for one year. After that period of time, it will be possible to classify the animals as belonging to either the HPL or the LPL group.

Polymorphism in the TNF-α promoter region

PCR-RFLP was conducted using the restriction enzymes *SacI* and *AluI* (Fig. 2). Most of the PCR products showed the pattern associated with genotype G/A (639, 222 and 417 bp with *SacI* and 514, 292, 222 bp, and minor fragments with *Alu I*).

The Hardy-Weinberg equilibrium was verified in this population. The frequency of the A allele was 0.54, while the frequency of the G allele was 0.46. With these data, the expected number of individuals can be estimated and compared with the observations. The obtained value ($\chi^2 = 0.26$, $p < 0.05$) indicates that the distribution of alleles is under Hardy-Weinberg equilibrium for this locus.

The genotype frequencies in both HPL and LPL are shown in Table 1. The G/G-harboring animals, compared to the other two genotypes (A/A and G/A), have a 3.22-times greater chance of belonging to the HPL group than the animals with the genotype A/A (OR: 3.225; IC 95 % Li: 1.5349 – Ls: 6.7759). In the same way, if the comparison is made with the G/A-harboring animals, the homozygous G/G animals have a 3.76-times greater chance of having a high proviral load than the animals bearing the G/A genotype for the same locus (OR: 3.726; IC 95 % Li: 1.8655 – Ls: 7.4421).

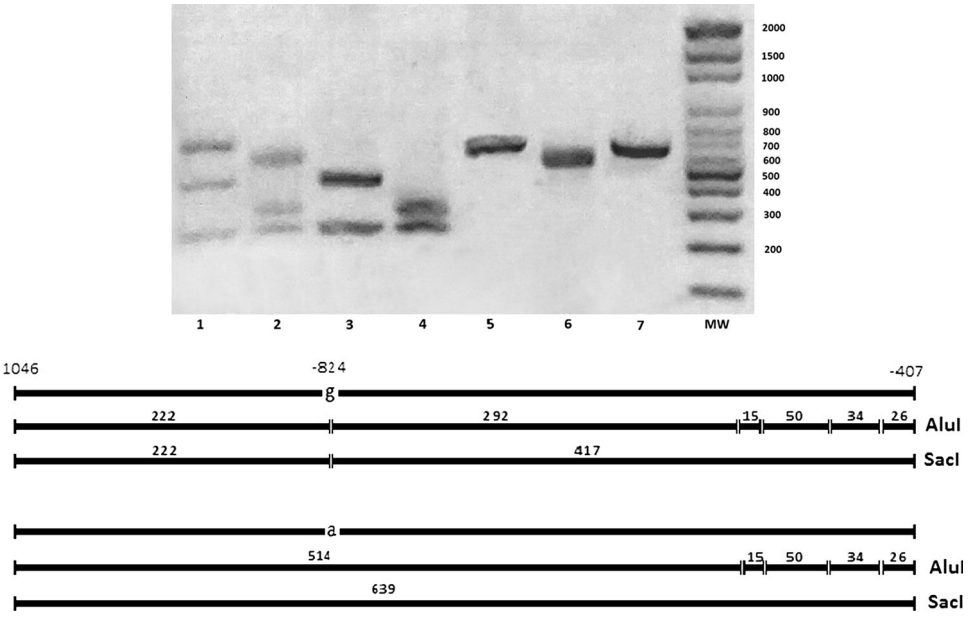

Fig. 2 PCR-RFLP of the TNF-α promoter region. Upper panel: lanes 1 and 2, heterozygous G/A digested with *SacI* and *AluI*; lanes 3 and 4, homozygous G/G digested with *SacI* and *AluI*; lanes 5 and 6, homozygous A/A digested with Sac*I* and Alu*I;* lane 7, non-digested control. Lower panel: schematic diagram of the expected fragment sizes after digestion with either *SacI* or *AluI* when G or A is present at position -824

Table 1 Genotype frequencies in HPL and LPL infection profiles. A statistically significant association was found between the proviral load and the genotype ($Chi^2 = 0.0005$)

Genotype	Proviral load		
	HPL	LPL	Total
A/A	40	42	82
G/A	61	74	135
G/G	43	14	57
Total	144	130	274

Discussion

BLV infection is highly prevalent in dairy herds in many countries and leads to major economic losses in cattle production and exportation. Only a few countries have developed a successful policy to completely eradicate the virus from their herds [1, 5, 27]. Considering this fact, it is necessary to identify the molecular genetic markers of susceptibility or resistance to this infectious disease for effective breeding [31].

Thorough studies over the past decade have allowed us to identify an interesting group among the infected animals that had a different humoral response against the infection. This group was classified as belonging to a low proviral profile, and its most conspicuous properties were the weak humoral response against the two main antigenic proteins of the virus, p24 and gp51, and a very low, mostly undetectable, proviral load by conventional PCR [15]. An important feature lies in the fact that the animals in this group can be used as a way of slowing down viral dissemination in an infected herd. BLV transmission occurs by the transfer of infected lymphocytes from one animal to another by practices that enhance fluid exchange, such as sharing contaminated needles, dehorning, tattooing, transrectal palpation, and, to a minor extent, by milk, colostrum, semen, and hematophagous insects [13, 22]. Previous work from our group has shown that infection can spread within a herd in a relatively short period of time [8, 9]. Several efforts have been made to define a genetic profile that could be associated with a low proviral load. Some polymorphisms of the *BoLA* region are present in the majority of LPL animals. However, they are not enough to justify the existence of these particular animals in every herd. Most animals belonging to this group carry the described resistance alleles in a homozygous or heterozygous manner, but there are still some animals that, despite belonging to the LPL group, do not carry any specific allele in this locus. Moreover, there are some animals in the HPL group that also carry alleles described as conferring resistance to BLV infection [15].

While significant research has been conducted to explore the role of viral determinants in the transformation process, the participation of host-related mechanisms has been poorly addressed. Published results in human cancer support the hypothesis that epigenetic events, which were initially identified as a causative mechanism of virus silencing, are also major players in host gene regulation [23]. In BLV infection, the RNA viral genome is retrotranscribed into double-stranded DNA, which is integrated into the host genome and organized into chromatin. This chromatin environment is likely to be a key parameter for the control of viral gene expression because transcriptional activation by cellular or viral trans-acting factors is

dependent on chromatin accessibility. BLV expression is then assumed to be controlled by chromatin structure [29]. The bovine cellular response against BLV has not been well characterized yet. After infection, both humoral and cell-mediated responses are induced, and they play an important role in protecting the host against the infection.

Many studies have suggested that cellular immunity against BLV antigens mainly contributes to the suppression of BLV replication by Tax protein inactivation, thereby delaying disease progression. If the modulation from type 1 to type 2 immunity is caused by BLV infection, the extended viral propagation at the initial phase of the infection could prompt the subclinical progression from the non-PL to the PL stage as well as the outcome of BLV-induced pathogenesis in the late stage. Therefore, the host immune responses immediately after BLV infection should be elucidated.

TNF-α is a functional pro-inflammatory cytokine with a role in modulation of acute inflammation and host innate immunity. Its association with disease progression has been observed in several acute and chronic infections and autoimmune diseases in humans [3, 4, 6]. In BLV infection, TNF-α expression is upregulated in the first stages of infection, resulting in the elimination of infected cells. As infection progresses, an imbalance in the expression of the receptors of the cytokine finally ends with the resolution of the infection.

The variation in the capacity to produce cytokines in different individuals has been attributed to the existence of polymorphisms within the regulatory regions or signal sequences of the cytokine genes. In HTLV infections, a relationship between the susceptibility to disease progression after infection and TNF gene polymorphism has been reported. Individuals carrying a polymorphism at position -857 T, associated with an increase in TNF-α expression, are more susceptible to developing HTLV-associated myelopathy [25] and ATL (adult T-cell lymphoma) [30] than healthy HTLV carriers. Other polymorphisms have been studied, and controversy exists regarding the relationship between the expression of TNF-α, the proviral load, and susceptibility to disease. Indeed, in asymptomatic carriers, no association was found between the proviral load and genetic polymorphisms in cytokine promoters, including TNF-α [26]. In the particular case of bovine TNF-α, Konnai *et al.* [20] reported that the frequency of the -824 G allele, which has been associated with low transcription activity of the promoter/predicted enhancer region of the gene, was higher in individuals with BLV-induced lymphoma than in asymptomatic carrier individuals. They also observed a tendency toward an increased BLV provirus load in cattle with the TNF-α -824G/G homozygote compared to the TNF-α -824 A/A homozygote or TNF-α -824A/G. These data could suggest that this observed polymorphism in the promoter region of the bovine TNF-α gene may at least in part contribute to the progression of lymphoma in BLV infection. Many authors have suggested that an increase in TNF-α may promote the growth of lymphoid cells. In the case of HTLV, a high level of expression of TNF-α can be associated with the development of HAM/TSP, an inflammatory disease, but not with the development of ATL [28] . In cattle, the worst scenario after BLV infection, the development of lymphoma, is associated with a polymorphism that may diminish TNF-α expression.

In this work, we have analyzed 274 unrelated dairy cows that were classified on the basis of their proviral load into two groups, HPL and LPL. Polymorphisms in the above-mentioned region of the TNF-α promoter were investigated, and we found that the genotype G/G was present in only 14 animals of the LPL group (14/57). This is a clear indicator that the absence of this polymorphism can be associated with the low proviral load profile. It has been reported previously that the genotype G/G at position -824 of the promoter region of TNF-α can be associated with low transcriptional activity of the promoter region, and hence lower expression of TNF-α [20]. As this cytokine is intimately related to the immune response against viral dissemination, stimulating the elimination of infected lymphocytes, we speculate that the presence of this allelic combination favors viral dissemination in the infected animals. The other allelic polymorphisms, *i.e.*, G/A and A/A were homogeneously distributed in the population despite belonging to the LPL and HPL groups. This analysis leads to the conclusion that the three possible genotypes are normally distributed in the HPL group, and that there is a significant association between the proviral load and the low frequency of the G/G genotype among the animals of the LPL group. This means that an animal carrying the G/G genotype at position -824 of the promoter region of TNF-α most likely belongs to the HPL group. This single nucleotide change cannot be responsible for the progression to any of the different stages of the infection. There are some other polymorphisms in the *BoLA* region that can be associated with the resistance to viral dissemination [16]. If we analyze bovine chromosome 23, the *BoLA* genes and TNF genes are only 2.3 cM apart, both located in the q22 arm. These data indicate that the probability of recombination of both genes is less than 0.2 %, a clear indication that these genes are in linkage disequilibrium. Our hypothesis is that there are several polymorphisms in different genes that contribute to the development of a resistance profile in cattle.

The humoral and cellular responses of bovines immediately after BLV infection are a very delicate imbalance between the expression/repression of cytokines, stimulation/inhibition of viral Tax, and many other factors that

have been described previously. In the specific case of TNF-α, which is closely involved in the development of the infection regardless the genetics of the host, more-thorough studies are being performed to understand the role of the imbalances in the expression of its receptors in the regulation of the activity of this cytokine. TNF-α receptor type 1 is pro-apoptotic and hence can help to eliminate BLV-infected lymphocytes. On the other hand, receptor type 2 not only lacks the death domain but can also stimulate cell proliferation. Furthermore, when the TNF-α concentration is low, receptor type 2 is able to recruit and transfer the molecule to receptor type 1. Further studies are being conducted to understand the role of the imbalances in TNF-α concentrations and the expression of both receptors in animals belonging either to the LPL or HPL group.

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