Flow cytometric determination of the expression of gp 51 protein of bovine leukaemia virus in experimentally infected sheep

Maria Szczotka

Department of Biochemistry, National Veterinary Research Institute, 24-100 Pulawy, Poland
szczotka@piwet.pulawy.pl

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Abstract

The study was performed on lambs experimentally infected with bovine leukaemia virus (BLV). The presence of BLV antibodies in sera of infected animals was detected by agar gel immunodiffusion test and ELISA. Proviral DNA was detected by PCR and nested PCR. Dual-colour flow cytometry analysis was performed with the use of specific monoclonal antibodies against lymphocyte CD markers and gp51 viral envelope protein, followed by incubation with fluorescent-labelled secondary antibodies conjugated with FITC or PE. Gp51 viral envelope protein was detected in tumours caused by BLV infection. The BLV infection resulted in depletion of CD4+ lymphocytes, increase in CD8+ lymphocytes, and decrease in CD4+ to CD8+ ratio in infected sheep. Proliferation of IgM+ CD19+ cells was also detected. These cells had an immature character without tendency to differentiate, and their vitality was prolonged. Flow cytometry enabled detection of gp51 expression in sheep blood lymphocytes at the early stages of the infection, before detection of serum antibodies using ELISA.

Keywords: bovine leukosis, gp51 glycoprotein, lymphocytes, flow cytometry.

Introduction

Bovine leukaemia virus (BLV) belongs to the family Retroviridae, genus Deltaretrovirus and is closely related by genomic organisation and disease progression to human T-cell leukaemia viruses (HTLV-1 and HTLV-2) and simian T-cell leukaemia virus. Retroviruses are a family of RNA viruses that use reverse transcription in the replication (15). They have been classified as simpler or more complex on the basis of genetic organisation and replication. The population of these viruses indicates a great genetic variability (3, 5, 19). This retroviral genetic variation is the composite of three variables: the mutation rate per replication cycle, the number of replication cycles, and the selective advantage or disadvantage possessed by variants of the viruses (4, 11, 24, 25, 27). Retroviral envelope (Env) proteins play multiple functions that are critical for viral replication and pathogenicity. They serve as principle targets for humoral and cellular responses (1, 13). They are synthesised as glycosylated polyproteins that are proteolytically processed by host enzymes into surface and transmembrane subunits during their passage through the Golgi apparatus, and then they are selectively incorporated into budding virions (27). The env protein of bovine leukaemia virus is synthesised as a 72-kDa precursor that is cleaved to yield a 51-kDa surface protein (gp51) and a 30-kDa transmembrane protein (gp30). Cellular tropism of the virus is determined by gp51 protein, whereas gp30 is responsible for anchoring the complex into the membrane, and mediates virus-cell fusion (6, 22).

BLV infection remains subclinical in the majority of cattle, but about one third of infected animals develop persistent lymphocytosis (PL). About 1% to 5% of infected animals develop lymphosarcoma, with or without prior PL (12, 14). The primary cellular target of BLV is the B-lymphocyte (15, 16). The susceptibility of other cells to BLV infection is less clear. Monocytes were first implicated as potential carriers of BLV in sheep on the basis of cell morphology and in situ hybridisation. Some authors reported that BLV was present in 5% to 40% of purified adherence monocytes, but not in T-cells or granulocytes from BLV infected cattle with or without PL (23, 27). T-cell susceptibility to BLV infection was evidenced when immunoaffinity depletion of B-cells and monocytes from peripheral blood or positive
selection of T-cells with immunomagnetic beads were performed (28). Under experimental conditions, sheep can be easily infected with BLV. This species is more sensitive to infection than cattle and some sheep develop B-cell leukaemia or lymphoma after shorter latency period and at higher frequencies than cattle (6, 7, 10).

The aim of the study was to analyse the presence of gp51 in blood lymphocytes and cells isolated from organs with limfoproliferative changes in lambs experimentally infected with BLV.

Material and Methods

Experimental infection. Ten Kent breed sheep, at age of five months, were intramuscularly inoculated with peripheral blood leukocytes isolated from a BLV-infected cow. Infection status of the cow was confirmed by PCR, AGID, and ELISA. Each animal was inoculated with a dose of 1 × 10⁶ cells. Four animals served as the negative control group.

Blood samples were collected from the jugular vein, first in two week intervals starting from the beginning of the experiment up to three months, then once a month, up to 26 months postinfection.

Cell preparation. For the haematological examination and fluorescence-activated cell sorting (FACS) analysis, blood was collected to the tubes containing EDTA-K2 as anticoagulant. Total white blood cells count, lymphocyte count, and Schilling formula were determined. At the end of the experiment, the animals were bled and tissue samples from the spleen, lymph nodes, heart, kidneys, liver, and muscles were collected. The BLV infection was monitored by AGID and ELISA methods using commercial TestLine and Merial kits.

PCR and nested PCR. The proviral DNA of BLV was detected by PCR according to the method of Klintevall and Bellagi-Pordany (24).

Cell cultures. To enable expression of bovine leukaemia gp51 antigen, blood cells and cells isolated from the spleen, lymph nodes, heart, kidneys, liver, and muscles of BLV-positive and BLV-negative cows were cultured in short-term cell cultures. Blood samples and cells isolated from tumours and organs with typical lesions were layered on the Histopaque gradient 1.077 and centrifuged (30 min, at 600 × g, at 4°C). The cells from interphase after three washings were counted and suspended at the concentration of 1 × 10⁶ cells/mL in RPMI-1640 medium, supplemented with 10% of heat inactivated foetal calf serum, fungizone, and penicillin/streptomycin. Concanavalin A (Con A) was added to the medium at the concentration of 5 μg/mL. The cells were cultured in plastic Petri dishes for 48 h at 37°C, in 5% CO₂. After incubation, cell pellets were tested by flow cytometry and immunofluorescence.

Flow cytometry analysis. Lymphocyte subpopulations were determined using dual staining method comprising a panel of monoclonal antibodies (MAbs) (VMRD Inc. Pullman, USA) and fluorescent-labelled secondary antibodies conjugated with FITC or PE (Medac, Germany). Monoclonal mouse anti-bovine antibodies detecting bovine CD2, CD4, CD8, CD19, pre-B cells, and gp51 of BLV were used. Briefly, for FACS analysis, lymphocytes (50 μL) were incubated for 30 min at room temperature with 1 μL of primary MAb, washed, and then incubated in the darkness with goat anti-mouse (IgG+IgM) FITC-labelled secondary Ab. After washing, the cells were coupled with the second MAbs and incubated as previously. Afterwards, washing was performed, then goat anti-mouse (IgG+IgM) PE-labelled secondary Ab was added and cells were again incubated. Then, red blood cells were lysed with FACS Lysing Buffer (Becton Dickinson). Leukocytes were washed, fixed in the buffer containing formaldehyde, and analysed in FACS Calibur flow cytometer (Becton-Dickinson) with argon source of excitation. Percentages of B- and T-cells were determined by Simulset and PC Lysis Software.

Immunofluorescence (IF). Smears of cultured lymphocytes after air drying were fixed in cold acetone (10 min, 4°C). Monoclonal mouse gp51 antibody (VMRD Inc., Pullman), diluted 1:100, was dropped on the slides and incubated for 30 min. at 4°C in humid atmosphere, in the darkness. After washing, the slides were covered with FITC-labelled goat anti - murine immunoglobulins (H+L) diluted 1:300, and incubated as mentioned above. Then, the slides were washed, buffered glycerol was added, and immunofluorescence of the cells was analysed under UV microscope (Olympus).

Statistical evaluation. The data was analysed by Student’s t-test and the level of significance was set at P < 0.05 in all cases.

Results

Neither clinical, nor haematological signs indicating bovine leukosis were found in seronegative animals (negative control group). The presence of proviral DNA in infected sheep was confirmed by PCR. The first seropositive animals were found by ELISA two weeks postinfection, but the level of specific antibodies was rather low, with a tendency to increase with time. Significantly high level of BLV antibodies was observed in animals with clinical signs of the disease, with the highest titres 22-24 months postinfection (Fig. 1). In four animals, lymphoma developed with high lymphocytosis and tumorous changes in internal organs. Persistent lymphocytosis (PL) was observed in four sheep, with the level of lymphocytes ranging from 68 000 to 716 000 cells/mL. The lymphocyte percentages were elevated up to 100% in two sheep and clinical symptoms were developed in these sheep (Fig. 2). The differences in leukocyte and lymphocyte counts in experimental and healthy animals were statistically significant (P < 0.05).
Proviral DNA of BLV was detected in blood lymphocytes and cells isolated from tumours in internal organs by PCR and nested PCR (Figs 3, 4).

Multiple lymphatic tumours were found in the internal organs during necropsy. The organs were enlarged and soft, with subcapsular haemorrhages (spleen, liver, kidneys) on the section surface. These changes were present in peripheral and internal lymph nodes, spleen, kidneys, heart, ribs, and stomach (Figs 5-9).
Fig. 5. Tumour of mesenteric lymph node

Fig. 6. Enlarged spleen with subcapsular haemorrhages

Fig. 7. Section of tumourous iliac lymph node

Fig. 8. Multiple tumours in abdominal cavity.

Fig. 9. The lymphatic tumours on mesentherium

Fig. 10. Expression of BLV gp51 in blood lymphocytes and cells isolated from internal organs of sheep No. 4 with lymphoma
The results of flow cytometry analysis of gp51 expression in blood lymphocytes and cells isolated from internal organs with tumourous changes are presented in Fig. 10. The highest percentage of cells with gp51 expression was detected in lymph nodes (60%–80%), kidneys (70%), liver (65%), and spleen (60%). The gp51 expression was detected in about 45% of heart muscle cells, in 33% of blood lymphocytes, and in 31% cells of preascapular muscle. The presence of gp51 glycoprotein was confirmed by immunofluorescence assay, in which cells expressing the BLV-gp51 protein were visualised by green fluorescence. The positive IF reaction was detected in blood lymphocytes and tumours of internal organs (Figs 15-17).

The expression of gp51 in short-term culture of lymphocytes was first observed 14 days postinfection, but the percentage of these cells was low. Later these values increased and the maximal level was found from six up to 10 months after experimental inoculation. Then, the amount of gp51 positive cells elevated and in sheep 9 the expression was present on 60% of the cells. In this period, some sheep developed lymphoma with clinical symptoms and high lymphocytosis. These animals were bled in terminal stadium of the disease. At this time, the percentage of blood lymphocytes with gp51 expression rapidly decreased; in some cases the values were from 3% to 21%, but the highest percentage of these cells was found in internal and lymphatic organs. In some animals, from 16 to 19 months postinfection, the expression of gp51 further increased, but decreased afterwards. Twenty-four months postinfection, a decrease in gp51 expression was detected in sheep No 2, 3, 4, and 7. In sheep 4 and 7, a very low level of gp51 expression in the blood lymphocytes was observed prior death (Figs 11, 12).

Fig. 13 presents the changes of gp51 expression in blood lymphocytes during the experiment and in cells isolated from internal organs after animal bleeding. The differences are remarkable: a very low level of gp51 expression in blood lymphocytes and much higher expression in lymph nodes and internal organ cells were found. This was probably caused by transmission of BLV from the blood to the organs in developed stage of the disease. Fig. 14 shows the percentage of blood lymphocytes expressing CD19 marker. The levels of these cells are similar to those of the cells with gp51 expression: similar values, increases, and decreases, indicating that the expression of gp51 was mainly in the B lymphocytes.

Flow cytometry analysis revealed that in animals with PL, about 90% of lymphocytes expressed antigen CD19, which is a characteristic marker of B-type lymphocytes (Fig. 14).

In sheep with lymphoma, the cells isolated from blood and internal organs had CD19+ marker and high percentage of these cells was IgM+CD19+. These cells had characteristics of immature B cells. In the tumour of internal iliac lymph node, 100% of cells were IgM+CD19+, while in mesenteric lymph node 78% of cells were immature B cells. In animals with developed leukaemia/lymphoma, the CD4/CD8 ratio was very low; only in blood lymphocytes these values reached 1.05, but in cells of internal organs they ranged from 0 to 1.0 (Table 1). In contrast, in healthy animals, the CD4+/CD8+ ratio is 2:1. These results indicate that BLV infection caused depletion of CD4+ lymphocytes. This phenomenon is similar to that in HIV infection in humans (36).

The presence of BLV gp51 was detected by IF assay in blood lymphocytes and tumour cells. Very strong immunofluorescence signal of gp51 was found in blood lymphocytes of animals with high lymphocytosis and in proliferating cells of internal organs (Figs 15-17).

### Table 1. CD markers on the blood lymphocytes and internal organ cells of sheep with lymphoma

<table>
<thead>
<tr>
<th></th>
<th>CD19+</th>
<th>IgM+CD19+</th>
<th>CD2+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>53</td>
<td>30</td>
<td>15</td>
<td>42</td>
<td>40</td>
<td>1.05</td>
</tr>
<tr>
<td>Liver</td>
<td>74</td>
<td>25</td>
<td>8</td>
<td>18</td>
<td>65</td>
<td>0.27</td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>92</td>
<td>78</td>
<td>0</td>
<td>2</td>
<td>82</td>
<td>0.02</td>
</tr>
<tr>
<td>Internal iliac lymph node</td>
<td>96</td>
<td>96</td>
<td>0.5</td>
<td>6</td>
<td>83</td>
<td>0.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>60</td>
<td>48</td>
<td>26</td>
<td>16</td>
<td>64</td>
<td>0.25</td>
</tr>
<tr>
<td>Heart</td>
<td>14</td>
<td>50</td>
<td>2</td>
<td>7</td>
<td>55</td>
<td>0.127</td>
</tr>
<tr>
<td>Spleen</td>
<td>70</td>
<td>40</td>
<td>11</td>
<td>12</td>
<td>72</td>
<td>0.17</td>
</tr>
<tr>
<td>Muscle</td>
<td>46</td>
<td>28</td>
<td>20</td>
<td>0</td>
<td>29</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 11. Expression of BLV-gp51 in blood lymphocytes in short-term culture (% cells)

Fig. 12. Expression of BLV-gp51 in blood lymphocytes of three sheep with lymphoma

Fig. 13. Expression of BLV-gp51 in blood lymphocytes and tumours of lymphatic organs of sheep No 4 with lymphoma

Fig. 14. Percentage of CD19+ lymphocytes in the blood of BLV infected sheep
The infection of cows with BLV causes the expression of gp51, env glycoprotein, and p24 major structural protein of BLV, B-cell lymphocytosis, and development of lymphosarcoma. The natural disease in cattle is characterised by a low tumour incidence, whereas, experimental infection of sheep leads to symptoms typical for leukosis in the majority of infected animals. Tax-BLV, the major oncoprotein, initiates a cascade of events leading toward malignancy, although the basis of transformation is not fully understood (19). The BLV gp51 protein has the highest degree of antigenicity.

The mechanisms of BLV infection and gp51 expression in experimentally infected sheep were investigated in the present study. The BLV env gene contains a variable region with the sequences coding four epitopes involved in antigen–antibody reactions and virus–host relations. Genetic variation analyses and comparison of gp51 sequences of different isolates have been described by several authors (9, 22). Such studies are important for diagnostic and vaccine construction purposes, as well as for pathogenetic and epidemiological studies (23).

In the study, the proliferation of B-lymphocytes expressing CD19 marker was induced in sheep infected with BLV. In some animals, B lymphomas with great percentage of cells having the IgM+CD19+ phenotype were developed as was reported earlier (34). The results of the experiment are in agreement with the results obtained by other authors (12, 15, 16).

Surface IgM positive B-lymphocytes are predominant virus host cells in the blood of BLV infected sheep and cows. They are predominantly MHC+CD5+CD11b+, but provirus was also found in CD5-CD11b- B cells. Very low levels of provirus were detected in monocytes, CD8+ T lymphocytes, and granulocytes (28, 29). Cell types other than B-lymphocytes may play important but yet unidentified roles in development of BLV infection in vivo. The association between B-cell phenotype and BLV tropism might occur. It is possible that cells bearing IgM and CD5 markers are the main target cells for BLV infection (23, 25).

In vitro studies demonstrated that BLV antigen is expressed exclusively in B-lymphocytes. This observation supports the hypothesis that as in the case of other retroviruses, such as HIV, BLV is able to use the regular activation of the immune system for its own replication and transmission. It seems, therefore, that the leukaemia-lymphoma complex in sheep may serve as an experimental model for the investigation of the biological properties of retroviruses (12). Sheep are the only experimental model for tumorigenesis induced by BLV infection (10, 29). In contrast to normal cells, tumor cells proliferate indefinitely and this feature may be a necessary condition for tumourigenesis (30). Inactivation of the p53 and Rb pathways allows the proliferation, but at the expense of escalating chromosomal instability, which is the result of further increases in unprotected chromosome ends. A crisis stage is reached when massive instability prohibits the generation of sufficient viable cells to sustain the proliferation (30).

Tumour incidences are often noted in sheep after experimental infection with BLV. Tumours develop only in small percentage of infected animals (1%-10%), with latency period of 4-8 years. In experimentally

Discussion

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infected sheep, this process developed much earlier and tumours were observed during 1-5 years. In the experiment, changes of proliferating character were present in many internal organs. Tumours were found in lymph nodes, mesentery, liver, spleen, kidneys, ribbes, and uterus. They were infiltrated with B-cell, indicating immature phenotype. Expression of gp51 protein was very high in tumour cells and very low in the blood lymphocytes. Tumours, which developed in internal organs, were the result of virus translocation from the blood to the tissues. The presence of proviral DNA in these cells was detected by nested-PCR, and gp51 expression was confirmed by flow cytometry and immunofluorescence. Tumours develop as an effect of clonal outgrowths of a single BLV-infected cell containing one to several copies of the provirus integrated into host cell DNA (19). Integration sites vary in tumours from different animals, which indicates that the virus does not transform cells by insertional activation of a host gene (32, 36). Tumours may contain full-length provirus, but some harbour incomplete proviruses retaining the 3’ tax/rex region of the genome (2). Tax is necessary for tumourigenesis in sheep. Some data indicates that Tax expression has to be completely suppressed at some stage during tumourigenesis in sheep (27, 12, 18). Tumourigenesis is initiated when the BLV Tax protein alters host cell gene expression (15, 31). Alteration in cellular gene expression appears to render infected cells susceptible to mutations in host genes (19, 32). Last year, a new genotype of BLV was identified and this additional information can be very useful in explication of infection mechanism (4).

Very interesting results were obtained by Llames et al. (20). With the use of monoclonal antibodies and immunofluorescence assay, they investigated cellular distribution of BLV proteins: gp51SU, Pr72env, and Pr66(gag-pro), in live, persistently infected FLK-BLV cells. The results were analysed by electron microscopy. When using a monoclonal antibody against Pr66 (gag-pro), mottled pinpoint fluorescence was seen in the cell surface of polarised cells, but no reaction was observed in cells undergoing mitosis. Positive reaction with monoclonal antibody against Pr72(env) was found only in mitotic cells and cellular fragments. Additionally, in these dividing cells, the envelope (Env) precursor polyprotein was not evenly concentrated, but cellular envelope (Env) precursor polyprotein was not evenly distributed, but concentrated predominantly in only one daughter cell.

Although the development of a tumoural form of bovine leukosis has been described as dependent on host susceptibility (20, 26, 37), currently, there is no evidence whether some genotypes can influence or induce these neoplasias. At the herd level, relative resistance to BLV-dependent B-cell proliferation and lymphocytosis among seropositive cows was associated with bovine lymphocyte antigen (BoLA)-DA7, whereas susceptibility was associated with BoLA-DA12.3 (36). These associations were also confirmed at the family level, where BoLA phenotypes were used as haplotype markers (20, 37).

The flow cytometry method used in the study enabled detection of gp51 expression in sheep blood lymphocytes at the early stages of the BLV infection, before it was possible to detect serum antibodies by ELISA. The study also provided information on the dynamics of BLV infection in sheep and the modulation of immunological system by the virus. Additionally, it evaluated the type of lymphocytes related to lymphocytoses and neoplastic lesions in internal organs.

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