DETERMINATION OF LYMPHOCYTE SUBSETS AND PCNA ACTIVITY IN SHEEP EXPERIMENTALLY INFECTED WITH BOVINE LEUKEMIA VIRUS (BLV)

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Lymphocyte phenotypes were determined in sheep experimentally infected with bovine leukemia virus (BLV). BLV infection was detected by serological tests AGID and ELISA. Flow cytometry analysis with the use of specific monoclonal antibodies for CD markers and conjugates labeled with FITC or PE was performed. Proliferating cell nuclear antigen (PCNA) was estimated in lymphocytes of infected and healthy sheep by flow cytometry. Activity of acid hydrolases ANAE and AP in blood lymphocytes was also determined. The results demonstrated much higher level of PCNA in leukemic lymphocytes than in healthy ones. These values were dependent on the stage of the disease. The BLV infection caused depletion of CD4 lymphocytes in infected sheep.

Key words: sheep, bovine leukemia virus, lymphocyte CD markers, monoclonal antibodies, flow cytometry.

Bovine leukemia virus (BLV) belongs to the family of oncogenic retroviruses which includes human T cell leukemia virus (HTLV) I and II and simian T cell leukemia virus I and II. These retroviruses share a common genomic organization (16) and are associated with non-neoplastic lymphocyte disorder, lymphoid neoplasia and/or progressive myelopathies (23). BLV infects B lymphocytes and induces a persistent lymphocytosis (PL) of B lymphocytes in cattle (2, 4, 11, 13, 20), but other viruses in this group have a tropism to T lymphocytes (13, 23). Animals with PL are at a greater risk of developing BLV-induced lymphosarcoma, but it remains unclear, whether this form is a pre-neoplastic state or a lympho-proliferative response to the presence of the virus (11). Due to the difficulty in detecting BLV proteins or BLV mRNA in freshly isolated lymphocytes or tissues (1), it has been proposed, that BLV is functionally latent in vivo, especially in infected cells (13, 17). A characteristic feature of BLV-
HTLV-induced disease is spontaneous lymphocyte proliferation of cultured peripheral blood mononuclear cells (19, 23). To investigate the possible role of virus expression on lymphocyte proliferation, proliferating cell nuclear antigen (PCNA) or cycline C in ovine lymphocytes from experimentally BLV infected and non infected animals was estimated. Proliferating cell nuclear antigen, a major nuclear protein, has been shown to be associated with human leukemia and malignancies (7, 23).

PCNA is a 36 kD nuclear protein associated with cell cycle and DNA synthesis, and is expressed in the nuclei of proliferating cells during S-phase (7, 15). PCNA functions as a co-factor for DNA polymerase delta in S phase and also during DNA synthesis associated with DNA damage repair mechanisms. The PCNA molecule has a half-life longer than 20 h, and therefore may be detected in non-cycling cells, e.g. those in G0 phase (14, 15).

Material and Methods

Experimental infection. Ten sheep at age of 5 months were intramuscularly inoculated with peripheral blood leukocytes isolated from a BLV-infected cow. Infection status of this donor cow was confirmed by PCR, AGID and ELISA tests. Each animal was inoculated with a dose of $1 \times 10^6$ cells. Four animals at the same age served as the control group.

Blood samples from sheep were taken from jugular vein at 2 week intervals at the beginning of the experiment, then at 1 month up to 26 months of the experiment. For the haematological examination and FACS analysis blood was taken to the tubes containing EDTA-K2 as anticoagulant. Total blood white cells count, lymphocytes and Schilling formula were determined. At the end of the experiment animals were bled and tissue samples from inner organs were taken. BLV infection was monitored by AGID and ELISA methods using commercial TestLine and Rhône-Merieux kits.

Flow cytometry analysis. Lymphocyte subpopulations were analysed using dual staining method and a panel of monoclonal antibodies (Mabs) produced by VMRD Inc. Pullman USA. Monoclonal antibodies against CD2, CD4, CD8, CD19 and pre-B cells were used. Mabs anti-PCNA were produced by Novocastra Laboratories Ltd. Briefly, for FACS analysis, blood samples (50 μl) were incubated with Mabs, washed and then incubated with FITC conjugate. After washing cells were coupled with the second Mabs and incubated as previously. Next washing was performed, then PE labeled conjugate (Medac Germany) was added and cells were again incubated. Then, red blood cells were lysed in FACS Lysing Buffer (Becton Dickinson) and leukocytes were analysed in FACs Calibur flow cytometer (Becton Dickinson) with argon source of excitation. B- and T-cells were determined by Simulset and PC Lysis Software.

Results

First serologically positive results were found after 2 weeks post infection. The level of antibodies increased during the experiment, but very high level of BLV antibodies was observed in animals with clinical signs of the diseases. In some animals lymphoma was developed with high lymphocytosis and tumourous changes in internal organs. No clinical and haematological signs were found in serologically negative animals (control group). Persistent lymphocytosis (PL) was observed in four sheep
We found, using flow cytometry examinations, that in animals with PL about 90% of lymphocytes had antigen CD19+ which is a characteristic marker for B-type lymphocytes (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Blood</th>
<th>Tumour</th>
<th>Spleen</th>
<th>Lymph node</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10 animals)</td>
<td>16</td>
<td>90</td>
<td>51</td>
<td>23</td>
</tr>
<tr>
<td>(10 – 22)</td>
<td>(86-94)</td>
<td>(40-62)</td>
<td>(20-26)</td>
<td></td>
</tr>
<tr>
<td>Control group</td>
<td>2.3</td>
<td>-</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>(4 animals)</td>
<td>(2.0-3.6)</td>
<td>(1.6-2.6)</td>
<td>(2.0-2.6)</td>
<td></td>
</tr>
</tbody>
</table>

Thus, this lymphocytosis was due to B-lymphocytes proliferation with prevalence of immature CD19+IgM+ cells (Table 2). In the spleen, lymph nodes and mesenteric tumours we found that about 100% of cells were B-cell phenotype and in these cells a strong activity of acid phosphatase was found. What is concerning the T-cells – these changes were not so dramatic and comparable with T-cell levels in healthy animals, but depletion of CD4+ cells was observed. In the animals with lymphoma in a terminal stage of the disease we observed that percentages of both CD4+ and CD8+ had similar very low values. Percentage of PCNA positive blood cells was especially elevated in the animals with PL and in lymphoma stage. We observed that in cells isolated from tumours - this percentage was very high and in terminal stages of the disease it reached 90% of the examined cells (Table 1). In the control animals these values were low.

### Table 2

<table>
<thead>
<tr>
<th>Lymphocyte subpopulations</th>
<th>Infected animals</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AL * n = 6</td>
<td>PL * n = 4</td>
</tr>
<tr>
<td>CD2</td>
<td>38 (36-40)</td>
<td>12 (9-15)</td>
</tr>
<tr>
<td>CD19+</td>
<td>35 (29-41)</td>
<td>81 (76-85)</td>
</tr>
<tr>
<td>CD19+ IgM+</td>
<td>26 (22-30)</td>
<td>76 (68-83)</td>
</tr>
<tr>
<td>CD4+</td>
<td>23 (17-26)</td>
<td>3 (2-4)</td>
</tr>
<tr>
<td>CD8+</td>
<td>18 (12-26)</td>
<td>12 (8-15)</td>
</tr>
</tbody>
</table>

*AL - aleukemic form, PL – persistent lymphocytosis
Discussion

In our experiment we observed that in sheep infected with bovine leukemia virus the proliferation of B-lymphocytes with CD19+ markers was induced. In some animals, B lymphomas with great percentage of IgM+CD19+ phenotype were developed what was reported earlier (2, 3, 4). Observations of cell PCNA activity showed that this cycline activity increased in the infected lymphocytes, as compared with the control ones. These findings are in agreement with results obtained in investigations of patients infected with HIV and HTLV and other malignancies (7). Similar results were reported in experiments with canine mammary gland tumours and malignant lymphomas (5, 14, 15).

Retroviral infection caused spontaneous uncontrolled lymphoproliferation of blood cells (4, 8) and lymphocytes expressing BLV p24 during unstimulated culture were spared from apoptosis (2). This suggest that the ability of BLV expression to prevent apoptosis may be connected to its ability to arrest or delay cells in G0/G1 of the cell cycle. A number of in vitro mechanisms used to arrest cells at the G1 checkpoint can prevent cell death (18, 19). For example, G1 cell cycle arrest induced by expression of the cyclin-dependent kinase inhibitor 21 can prevent apoptosis following DNA damage (12, 18). Cell cycle arrested by a retroviral protein has been demonstrated for the human immunodeficiency virus (HIV) Vpr protein. In HTLV-I attention has been focused on the antiapoptotic and cell cycle regulatory properties of the transactivating protein Tax (23). Tax expression has been associated with altered expression of some cyclins and cyclin-dependent kinase inhibitors. In general, HTLV-I Tax induces alterations favouring proliferation and not cell cycle of retroviruses in its association with G0/G1 delay or arrest, but shares similarities with HTLV in its ability to increase arrest (23). Thus, BLV may be the unique among the surviving of virus-expressing cells (18, 19).

Proliferating cell nuclear antigen may play a role in the process of lymphoid transformation as a result of bovine leukemia infection in sheep. This nuclear antigen may have a practical prognostic value in human tumour therapy and in small animal malignancy clinic.

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References


