PATHOLOGICAL FEATURES OF EXPERIMENTAL BOVINE LEUKAEMIA VIRAL (BLV) INFECTION IN RATS AND RABBITS

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Abstract

Rabbits and rats were inoculated with material derived from FLK cells producing permanently bovine leukaemia virus (BLV). The viral presence in the inoculum was proved by transmission electron microscopy, immunofluorescence, immunogold labelling demonstrating viral Tax protein, and PCR analysis. About 30% of the infected animals sustained BLV seropositivity during the experiment, and demonstrated symptoms of lympholeukaemia – clinical manifestation of an immunosuppressive condition, increased number of lymphocytes and lymphoblasts, and preneoplastic lymphoid cell accumulations in the liver, lungs, kidneys, and lymph nodes. BLV DNA, detected by PCR in diseased animals, indicates the role of BLV as an aetiological factor of lympholeukaemia, developed in these animals after BLV infection. The alterations in rats were more pronounced than those in rabbits. The results prove that these two species of laboratory animals, especially rats, are suitable models for the in vivo studies of leukaemogenesis caused by BLV/HTLV infections.

Key words: rats, rabbits, bovine leukaemia virus, pathology.

The enzootic bovine leukosis (EBL) caused by the RNA bovine leukaemia virus (BLV) is widespread throughout the world due to the easy transmission of the infection both in the vertical and horizontal way. Economic losses are significant but the fight against the disease is limited to organisational and preventive measures, since all developed countries have adopted radical methods and the diseased animals are sacrificed. The interest in this disease is prompted because of the genetic relatedness of the BLV to a large group of retroviruses responsible for leukaemia in humans, such as HTLV-I, HTLV-II, and HIV (1, 14). The genomes of BLV and HTLV I and II show over 50% similarity in their nucleotide sequences (9).

It has been established that BLV affects other species as well. Sheep and goats are strongly sensitive. Pigs, rabbits, rats, cats, dogs, and some primates are also susceptible to BLV infection (7). Human cells of neural origin have also been proved to be sensitive to the infection (2).

The successful study of the EBL as a model of some human leukaemias depends to a great extent on the possibility of modelling the disease in experimental animals and creating easily manipulated animal models with a shorter incubation period. However, the attempts of reproducing the ailment in small laboratory animals have been unsuccessful so far. In most cases, BLV antibodies were produced without manifestation of visible pathological processes (4). Restricted pathological changes, resembling the lesions occurring in the human in the course of acquired immune deficiency syndrome (AIDS), have been reported by Altanerova et al. (3) and Todorova et al. (15) in rabbits infected with BLV.

The aim of the present investigation was to obtain additional clinical and immunological data and to describe histopathological characteristics of the disease in rats and rabbits infected with BLV.

Material and Methods

Infection material. FLK cells from a culture permanently producing high amounts of BLV, kindly provided by Prof. Altaner from the Slovakian Academy of Sciences, were maintained under standard conditions in Dulbecco’s Modified Eagle Medium (DMEM) and tested for viral activity by immunofluorescence, immunogold labelling, transmission electron microscopy (TEM), and PCR analyses.

Immunofluorescence. The production of viral Tax protein was visualised by indirect immunofluorescence in FLK-BLV cells. The cells were fixed in cold methanol-acetone (3:7) for 5 min at –20°C
both sexes, at the age of 1-2 months, with a weight of

- DNA Purification Kit (Fermentas/Thermo Fisher Scientific) according to the manufacturer’s instructions. The BLV pol and tax regions of BLV were amplified using BLV pol and tax primers, respectively (Fig. 2). No BLV DNA was detected in PBMCs of non-infected animals. 

For immunogold labelling, FLK-BLV cells were fixed in 2% paraformaldehyde, dehydrated in a series of increased concentrations of ethanol, and embedded in antigen activity preserving low temperature embedding resin Lowikryl K4M (Serva) at room temperature. The immune complexes were detected using anti-mouse immunoglobulin conjugated to colloidal gold particles (10 nm in diameter) (Sigma). After washing, the grids were stained with uranyl acetate. Controls were carried out in the same way, except that the incubation with antibodies was replaced by incubation with normal serum. The samples were observed under electron microscope.

**Electron microscopy.** The monolayer culture from FLK-BLV cell line was fixed in 2.5% glutaraldehyde in Petri dishes for 1 h, scraped, centrifuged, post-fixed in osmium tetroxide, and Epon embedded. Ultrathin sections were stained with uranyl acetate and observed under a JEOL 1200 EX electron microscope.

For immunogold labelling, FLK-BLV cells were fixed in 2% paraformaldehyde, dehydrated in a series of increased concentrations of ethanol, and embedded in antigen activity preserving low temperature embedding resin Lowikryl K4M (Serva) (8). Ultrathin sections, mounted on golden grids, were incubated with Tax-protein mAb for 30 min at room temperature. The immune complexes were detected using anti-mouse immunoglobulin conjugated to colloidal gold particles (10 nm in diameter) (Sigma). After washing, the grids were stained with uranyl acetate. Controls were carried out in the same way, except that the incubation with antibodies was replaced by incubation with normal serum. The samples were observed under electron microscope.

**PCR analysis.** PCR was used to detect BLV in peripheral blood mononuclear cells (PBMCs) of animals inoculated with FLK cells. PBMCs were isolated by a Ficoll-Paque (Pharmacia) gradient centrifugation. DNA was extracted from PBMCs using GeneJET™ Genomic DNA Purification Kit (Fermentas/Thermo Fisher Scientific) according to the manufacturer’s instructions. Five microlitres of PBMCs DNA were used for each PCR. Primers to the pol and tax regions of BLV were used for amplification. The primer sequences were as follows: GAA CGC CTC CAG GCC CTT CAA GA (BLV pol primer - sense), CAT TGG AGG TCT CCT AAG ACC (BLV pol primer - antisense), AACGACAAAAATTTTCTTGTTC (BLV tax primer - sense), and CGGGGCGGTGGCGGCCGCCTAG (BLV tax primer - antisense). The BLV pol and tax primers amplify 709-bp and 1008-bp PCR products, respectively. The final 50 µl of the PCR mixture contained: 5 µl sample, 25 µl PCR Master Mix (Fermentas/Thermo Fisher Scientific), 3 mM MgCl₂, and 20 pmol of each primer. Amplifications were performed with the following cycling profile: 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C for 30 cycles, and 10 min at 72 °C. BLV producing FLK cell line and PBMC from non-inoculated animals were used as positive and negative controls, respectively. The PCR products were analysed on a 2% agarose gel stained with ethidium bromide and visualised by UV-transillumination.

**Laboratory animals.** Thirty-two Wistar rats of both sexes, at the age of 1-2 months, with a weight of about 50 g, and 18 New Zealand White rabbits as well as six California rabbits of both sexes, aged 1-2 months, weighing 300-400 g, were used in the experiments. The animals were infected twice at 10-d interval. The rats were inoculated intraperitoneally (i.p.) with 2.6 x 10⁶ FLK-BLV cells. The rabbits received i.p. 5.1 x 10⁸ FLK-BLV cells. All animals were kept under standard conditions in the Institute’s animal facilities according to the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (10).

The clinical condition was checked daily and the diseased animals were euthanised. All experimentally infected animals that survived, as well as six rabbits and 10 rats used as controls, were sacrificed under narcosis at the end of the second year after infection.

**Haematology.** Blood samples from all animals were collected every 4 months starting 2 weeks after the last inoculation. Leukocytes were counted using the Bürker camera. Blood smears were also prepared and stained with May–Grünwald–Giemsa method.

**Serology.** All blood serum samples were tested for gp51 and p24 antibodies, using the single radial immunodiffusion method originally described by Manchini et al. (12).

**Pathomorphology.** Autopsies were performed on all dead or sacrificed experimental and control animals. Samples from the liver, spleen, lymph nodes, kidneys, and lungs were collected from all autopsied animals. All samples were fixed in 10% buffered formalin, dehydrated in graded ethanol, and paraffin embedded. Tissue sections were cut at 5 µm, deparaffinised in xylene, hydrated, and stained with haematoxylin and eosin (H&E) for microscopic observation.

**Results**

**Inoculum.** TEM revealed numerous C-type virus particles in the intercellular spaces of the FLK-BLV culture (Fig. 1a). BLV protein production was proved by immunofluorescence (Fig. 1b) and immunogold labelling (Fig. 1c).

PCR was applied to detect DNA extracted from PBMCs, obtained from rats and rabbits with symptoms of lympholeukaemia, which developed after inoculation with cells of BLV producing FLK cell line. The diseased animals were positive for BLV DNA. PCR products of 709-bp and 1008-bp were amplified using BLV pol and tax primers, respectively (Fig. 2). No BLV DNA was detected in PBMCs of non-infected animals.

**Clinical findings.** During the experiment and especially after the first year, 60% of the rats and no more than 25% of the rabbits developed disorders such as: weight loss, alopecia, pruritus, erythema, conjunctivitis, rhinitis, and pneumonia (Fig. 3).

In the second year, by the end of the experiment, most of the treated rats were already dead. All control animals were in good health and no mortality was observed.
Fig. 1. BLV viral particles in intercellular space (a), perinuclear pearl-like accumulation of BLV Tax protein in cytoplasmic vacuoles (CV) (b), and Tax immunogold labelling (c).

Fig. 2. BLV DNA detection by PCR in PBMCs of diseased rats: amplification of 709 bp fragment of pol gene (line 2) and 1008 bp fragment of tax (line 3). PBMC DNA of non-infected rats amplified by BLV pol (line 4) and tax (line 5) primers was used as negative control. BLV producing FLK cell line was used as positive control – amplification with BLV pol and tax primers (lines 6 and 7). Line 1 - molecular weight marker (100 bp).

Fig. 3. Rabbit and rat with alopecia and weight loss.

Fig. 4. WBC count in rats and rabbits during the 24-month experiment.

Fig. 5. Lymphoblasts in the peripheral blood of an experimental rat. May-Grünwald–Giemsa stain.

Fig. 6. Immunodiffusion, rats (gp51 antigen).

Fig. 7. Enlarged prescapular lymph node in a BLV-inoculated rabbit.
Haematology. The dynamic of the white blood cell (WBC) number in infected animals during the experiment was established. During the first eight months, leukocytes decreased even under the reference values (rats – 6-17 x 10^3/µL; rabbits – 7.5-13.5 x 10^3/µL) (11), followed by an increase, more pronounced in rats, with a peak (21 x 10^3/µL) in months 14-18 p.i. (Fig. 4). The elevation of WBC number was due to the appearance mainly of lymphocytes and lymphoblasts in the peripheral blood (Fig. 5). By the end of the experiment, a tendency for a decrease to the reference WBC values was observed in both experimental species. No alterations of WBC number were observed in control animals till the end of the experiment.

Serology. The results from radial immunodiffusion test showed that 34% of the rats and 22% of the rabbits inoculated with BLV-containing material reacted positively 2 weeks p.i.. The gp51 and p24 antibodies persisted till the end of the experiment in almost 90% of the cases (Fig. 6).
**Gross pathology.** Enlarged lymph nodes were sometimes observed in experimental rats and rabbits (Fig. 7). They were of fat consistence and homogeneous cut surface appearance. Alterations in the other viscera were not found.

**Microscopically** perivascular lymphoid infiltrations and hyperplasia of cholangiocytes were observed in the liver of the seropositive animals (Fig. 8). However, no other disturbances of the normal liver architecture were noted.

The histopathological lesions in the lungs were manifested by haemorrhages, laceration of alveoli due to lymphocyte infiltrations, and presence of focal serous or purulent pneumonia (Fig. 9).

Perivascular lymphocyte clusters and haemorrhages in the kidneys were also observed (Fig. 10).

In the spleen the white pulp was spatially reduced, but comparatively rich in cells, and in some cases displayed well-shaped germinal centres. The sinuses were packed with erythrocytes. The lymph nodes showed histiocytosis with many blast cells in mitosis (Fig. 11) and an activation of the vascular endothelium (Fig. 12), rarely with formation of well visible rosette-like structures.

**Discussion**

The data obtained show that treatment of rats and rabbits with BLV-producing cells of the FLK-BLV permanent line leads to infection in approximately 1/3 of the experimental animals. The electron-microscopically observed virus particles, the BLV-protein (Tax) production, and the BLV DNA detection by PCR revealed the infectivity of the FLK - inoculum used. The successful infection of the inoculated laboratory animals was proven by the presence of specific viral antibodies in the serum. These data are in conformity with reports from other authors, who demonstrated the infection with BLV by the detection of antibodies not only in rabbits and rats but also in other experimental animals (6, 7).

In our study, the BLV infection leads to a primary disease with clinical, haematological, and histopathological findings, which were not detected in the controls. Weight loss, alopecia, accompanied by pruritus and erythema, rhinitis, and pneumonia, more pronounced during the second half of the experiment, could be explained by the well known immunosuppressive properties of BLV (13). The clinical indications of upper respiratory disease, exhibited with conjunctival and nasal exudates, severe weight loss, diarrhoea, abscesses, and leg paralysis were observed in other experiments with BLV infected rabbits (5, 16).

The clinical course of the disease, described in our study could be interpreted as AIDS-like symptoms and confirms partially the findings of Altanerova et al. (3). In addition to the immune suppression, carcinogenic action of BLV was observed 16-18 months p.i., manifested by marked lymphocytosis and the appearance of immature PBMCs. Moreover, enlarged lymph nodes, histological lesions in the viscera, such as lymphocyte infiltrations and activation of the reticuloendothelial system, appear to be similar to the alterations induced by the field strain of BLV in cows. BLV DNA detected by PCR in the diseased rats and rabbits indicates the role of BLV as the aetiological factor of lympholeukaemia, which developed in these animals after BLV infection. Other researchers also used PCR with different BLV DNA primers to demonstrate BLV infection in rats and rabbits (5, 6, 16).

The alterations were more pronounced in rats, which, to our opinion, are more suitable as laboratory model of EBL than rabbits. In conformation of this thesis, Altanerova et al. (6) managed to establish a spleen cell line from BLV infected rats, which carry and recover BLV for over 80 in vitro passages. These authors suggested a hypothesis that rats can serve as a reservoir of BLV in the nature.

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**References**


