Leukemia-like syndrome in Balb/c mice infected with the lymphotropic gamma herpesvirus MHV-Šumava: an analogy to EBV infection*

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Based on our previous observation that primary infection with the murine gamma herpesvirus (MHV) isolate Šumava (MHV-ŠU) undergoes a lymphoproliferative phase resembling to Epstein-Barr virus (EBV) induced infectious mononucleosis (IM), we evaluated white blood cell (WBC) counts at late stages following MHV-ŠU infection. In consequence of intranasal inoculation with MHV-ŠU a leukemia-like syndrome in Balb/c mice developed. The syndrome in question was accompanied with significant splenomegaly; in the peripheral blood leukocytosis (from $8x10^4$ to $5x10^5$ leukocytes/μl) and a high percentage of atypical lymphocytes (60–80%) was found. Presented results are bringing further evidence for lymphoproliferative effect of MHV and point at analogic course of MHV-ŠU and EBV infections.

Key words: murine gamma herpesvirus, MHV-Šumava, BALB/c mice, differential white blood cell count, atypical lymphocytes

MHV-Šumava is one of the eight isolates of MHV-68 (a virus belonging to species *Murid herpesvirus 4*, genus *Rhadinovirus,* subfamily *Gammaherpesvirinae,* family *Herpesviridae* [20]. The MHV-ŠU, similarly as MHV-68, MHV-60, MHV-72, MHV-76, MHV-78, MHV-4556, MHV-5682, are natural pathogens of wild rodents. All the isolates were obtained from organs of free-living rodents *Apodemus flavicollis* and *Clethrionomys glareolus* [1]. These viruses are widespread among populations of free-living rodents, since serological surveys revealed an 12% MHV antibody prevalence [11]. The MHV isolates grew in a variety of cell lines of epithelial or fibroblast origin derived from many mammalian species including man [26]. They were found to establish chronic infection at least in the lungs and spleen of outbred mice when administered by intranasal (i.n.) route [1]. When given i.n. to inbred Balb/c mice, the virus replicated mainly in the lungs and spleen [24] and in adherent mononuclear cells [12]. Inbred mice chronically infected with MHV-68 developed a lymphoproliferative disease (LPD). The frequency of LPDs over a period of 3 years was 9% out of all infected animals, with 50% of them displaying high grade lymphomas [25]. The frequency of LPD following MHV-ŠU inoculation was 14% [15]. The pathogenesis of MHV-ŠU was found similar to that of MHV-68 [16], when causing acute lymphocytosis, splenomegaly and an IM-like syndrome with expansion of $CD8^+$ T cells, NK cells and $CD19^+$ B cells [17]. These findings in suggest that the murine gamma herpesvirus infection may represent an important model for studying the pathogenesis of LPD associated with human gamma herpesviruses such as EBV [28]. It is well known that EBV persists in B cells; in addition to Burkitt's lymphoma, it is associated with lymphoproliferative syndromes such as Hodgkin's disease and certain adult T cell lymphomas, some of which occur during long-

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Abbreviations: BEM – Basal Eagle's Medium, CPE – cytopatic effect, EBV – Epstein-Barr virus, MHV – mouse herpesvirus, PBS – phosphate buffered saline, p.i. – post infection.

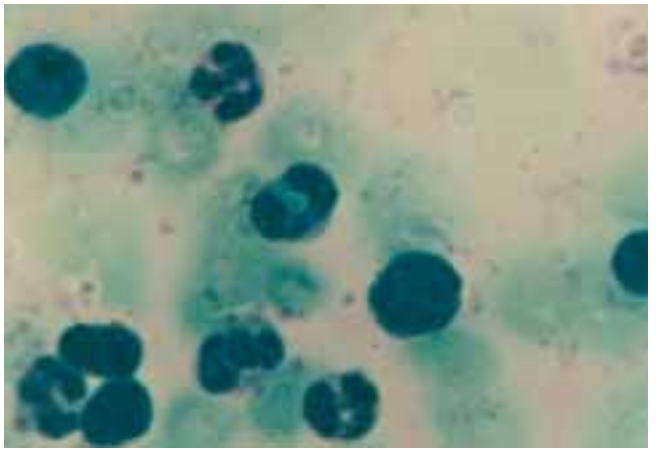

Figure 1. Differential WBC in mouse no. 4 at 665 days after MHV-ŠU infection (compare Tab. 1 A).
The nuclei of atypical lymphocytes are lobulated or kidney shaped. The chromatin, which is in core strands, is irregularly distributed, giving a mottled appearance. Note the variation in size of these atypical lymphocytes, and that the cytoplasm tends to be more basophilic than normal (May-Grünwald – Giemsa-Romanovski stain, magn. x 1000).

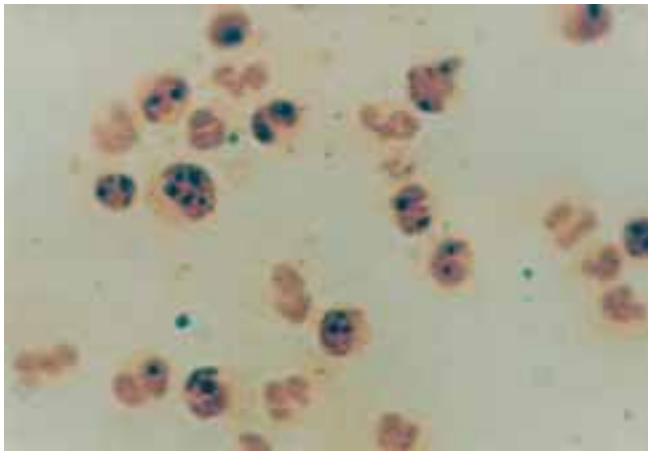

Figure 2. Silver-stained AgNORs in the peripheral blood leukocytes of MHV-ŠU infected mouse.
The staining was performed at day 730 p.i. The AgNORs are clearly visible within the nuclei. Mean number of AgNOR dots per nucleus was 5.68 (magnification 1000x).

term immunosuppression. EBV infection may induce also epithelial tumors such as nasopharyngeal carcinoma, gastric carcinoma and oral hairy leukoplakia, which appears in longterm immuno-suppressed patients. In such patients EBV may cause even leiomyosarcoma or tumors of smooth muscle origin [3].

Respiratory challenge with murine gamma herpesvirus leads to productive virus replication in lungs and to a persistent or latent infection in B-lymphocytes, epithelial cells and macrophages [21, 23]. MHV-68 was first used for studying of gamma herpesvirus pathogenesis in conventional mice [19]. The MHV-68 induced splenomegaly and increased number

Figure 3. Detection of viral antigen in splenocytes of a mouse which developed the leukemia-like syndrome by indirect IF.

of activated atypical $CD8^+$T cells in the circulation were described in Balb/c mice [14, 27] as well as in nude mice [18]. The shape of atypical murine leukocytes was highly similar to those observed in patients with IM [14]. Therefore, MHV infection in mice has become an experimental model for IM [2, 4, 5, 7]. Here we describe a leukemia-like syndrome associated with MHV-ŠU infection.

Material and methods

Virus. The MHV-Šumava stock was prepared in Vero cells propagated in Eagle's Basal Medium (BEM) supplemented with 7% inactivated bovine serum, glutamine (300 mg/l) and antibiotics (100 units of penicilin and 100 μg of streptomycine per ml). Virus was harvested at 72 or 96 hrs post infection (p.i.). Cells were disrupted and the virus was released by homogenization and sonication. The viral suspension obtained was clarified by low-speed centrifugation. The supernatant was stored at –70 °C. Infectious virus was assayed by titration in Vero cells. Virus titre was up to 10^7 $TCID_{50}$/ml.

Animals. Balb/c mice were obtained from the breed of the Institute of Virology, Slovak Academy of Sciences, Bratislava. Fifty female 4–6 week-old Balb/c mice were inoculated intranasally (i.n.) with $2x10^5$ $TCID_{50}$ of MHV-ŠU in 20 μl per mouse under light ether anaesthesia. Fifty non-infected mice served as controls. Mice were killed at different intervals p.i. and blood was drawn from *sinus orbitalis*. Spleens were removed and used for preparation of cell suspensions for detection of viral antigen by immunofluorescence test.

Blood samples taken at intervals shown in Tables 1 and 2 were mixed with a drop of heparin diluted 1:100 (to a final concentration of 2–4 U/ml).

Staining of white blood cells (WBC). Smears were made

immediately after blood collection, fixed by air drying and stained with the May-Grünwald solution for 10 min and the Giemsa-Romanovski solution for 15 min. Leukocyte were counted after 10 min staining with Türk solution. The differential counts were expressed as rate for each kind of WBC. Spleens were removed under sterile conditions, minced and gently dispersed to prepare a cell suspension. The cells were repeatedly washed in phosphate buffered saline, filtered through nylon wool in a small syringe and counted similarly as the WBC.

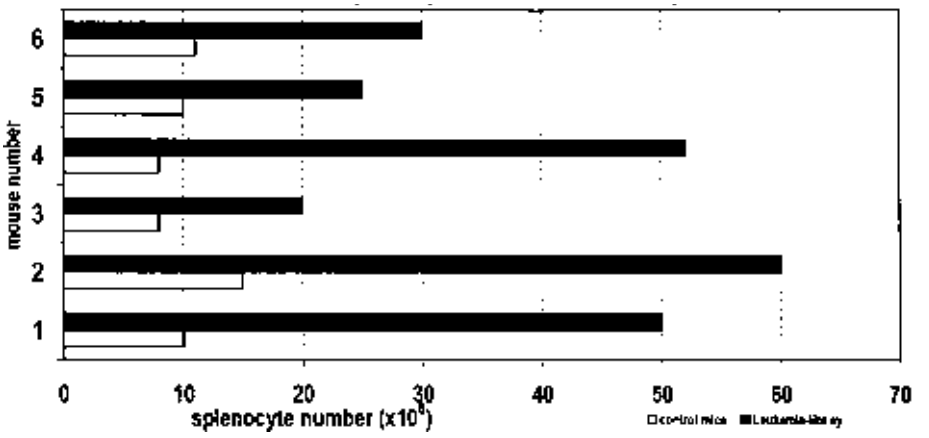

Figure 4. The numbers of splenocytes in mice non-infected (control) and infected with leukemia-like syndrome.

Immunofluorescence (IF) test. The presence of the MHV-antigen in organs of infected mice was determined by indirect IF test and the percentage of positive cells was calculated. Suspensions of cells (100 μl) obtained from lymphatic tissues of infected mice were stained with a monoclonal antibody (MAb) prepared against MHV-ŠU (MAb/MHV-ŠU, [6]) diluted 1:100–1:500 and with a goat anti-mouse IgG (H+L) conjugated with rhodamine (Immunotech, Slovak Republic).

Estimation of argyrophilic (AgNOR) proteins. Slides with air dried leukocyte smears were fixed in cooled (4 ˚C) methanol for 5 min and rinsed with deionized water. The slides were then immersed into a freshly made mixture of one volume of 2% gelatine in 1% formic acid and two volumes of 50% silver nitrate and incubated at room temperature for 30 min in the dark. Slides were then washed with distilled water and dipped in 5% sodium thiosulfate to remove the non-specific silver background. The number of AgNOR dots was assessed by direct examination under a light microscope. At least 300 cells per each sample were examined using a 100x oil immersion lens. The mean number of the dots per cell nucleus was calculated.

Statistical analysis. Statistical significance of differences was estimated using the Student's t-test. Differences with $p<0.05$ were considered significant.

Results and discussion

Fifty female 4–6 week-old Balb/c mice were inoculated by i.n. route under light anaesthesia with $2x10^5$ $TCID_{50}$ /20 μl of MHV-ŠU, while another fifty non-infected animals served as controls. Six mice showed splenomegaly i.e. it was seen in 12% out of a total of 50 infected Balb/c mice kept under observation for two years. Differential WBC counts revealed that these animals had developed a leukemia-like syndrome with extensive leukocytosis ranging from 80.000–500.000 per μl blood. The mice in question had as many as 13–80% atypical leukocytes in their blood. The atypical lymphocytes (Fig. 1) could be classified as lymphoblasts, myeloblasts and promyelocytes (Tab. 1A). In contrast, when infected mice were taken at random for WBC examination at the same late p.i. intervals, no high leukocyte counts were found, though a proportion of these animals showed a low rate of atypical lymphocytes (Tab. 1B). No leukocytosis was found in control mice; in the control group WBC counts revealed no atypical lymphocytes, though their leukocyte numbers ranged from 4,900 up to a relatively higher values of 15,000/μl (Tab. 2). The enumeration of AgNOR dots for demonstration of argyrophilic proteins in the leukemia-like syndrome was chosen since this method reflects the proliferative activity of atypical leukocytes. The AgNOR proteins were detected as clearly visible black dots within the cell nucleus (Fig. 2). In atypical leukocytes of mice who developed the leukemia-like syndrome during persistent infection with MHV-ŠU, a significantly increased number of AgNOR dots could be detected (Tab. 3). As mentioned above, the mice with leukemia-like syndrome had enlarged spleen. The splenocyte counts showed $20–60x10^6$ cells per spleen in comparison with non-infected ones which had $8–15x10^6$ splenocytes per spleen (Fig. 4), the difference being 2.5–4 fold. The splenocytes were occasionally positive for the MHV-ŠU antigen, when stained with a monoclonal virus-specific antibody confirming persistent infection (Fig. 3).

Our results are in accordance with those of JANOUTOVÁ et al [8] who had shown that the number of AgNOR dots was significantly higher in less mature than in more mature cells. AgNOR proteins are components of nucleolar organizer regions (NORs) which are segments of DNA with ribosomal genes important for the regulation of protein synthesis in the cell. The number of NORs (rRNA genes) appears to correlate with general metabolic and proliferat-

Table 1 A. Differential WBC counts in MHV-ŠU infected Balb/c mice developing splenomegaly and the leukemia-like syndrome

Day post infection	365	545	575	665	730	730
Mouse number	1	2	3	4	5	6
Leukocytes/μl	80,000	500,000	110,000	500,000	80,000	120,000
Monocytes	0%	40%	11%	20%	1%	6%
Lymphocytes	40%	0%	37%	0%	52%	51%
Segmented neutrophils	0%	0%	4%	0%	30%	20%
Non-segmented neutrophils	0%	0%	0%	0%	3%	4%
Basophils	0%	0%	0%	0%	0%	1%
Eosinophils	0%	0%	0%	0%	1%	1%
Atypical lymphocytes	60%	60%	48%	80%	13%	17%

Table 1 B. Differential WBC counts in MHV-ŠU infected Balb/c mice who did not show splenomegaly

Day post infection	365	545	575	665	730	730
Mouse number	7	8	9	10	11	12
Leukocytes/μl	5,000	8,000	11,400	10,800	7,000	8,800
Monocytes	6%	1%	0%	0%	6%	14%
Lymphocytes	84%	66%	87%	58%	46%	67%
Segmented neutrophils	3%	0%	2%	28%	46%	4%
Non-segmented neutrophils	7%	19%	6%	11%	2%	6%
Basophils	0%	0%	0%	0%	0%	0%
Eosinophils	0%	2%	0%	0%	0%	0%
Atypical lymphocytes	0%	12%	5%	3%	0%	9%

Table 2. Differential WBC count of non-infected Balb/c mice*

Number of mice	1	2	3	4	5	6
Leukocytes/μl	6,000	15,000	9,100	5,000	4,900	7,300
Monocytes	10%	10%	0%	13%	10%	12%
Lymphocytes	88%	73%	78%	54%	76%	72%
Segmented neutrophils	0%	3%	18%	23%	9%	15%
Non-segmented neutrophils	2%	10%	4%	10%	5%	1%
Basophils	0%	3%	0%	0%	0%	0%
Eosinophils	0%	1%	0%	0%	0%	0%
Atypical lymphocytes	0%	0%	0%	0%	0%	0%

*These animals were in the age of their infected counterparts.

ing activities of the cell [9]. As a consequence of persistent infection with MHV-ŠU, the atypical leukocytes in the peripheral blood during leukemia-like syndrome showed a significantly enhanced of AgNOR dots per cell nucleus (Tab. 3). Our results confirmed the analogy between MHV and EBV supporting our hypothesis on analogical biological properties shared by human and mouse herpesviruses (Tab. 4). In conclusion, our results confirmed the usefulness of MHV-ŠU infected mice for studying the lymphoproliferative processes in humans infected with gamma herpesviruses.

This study describes a leukemia-like syndrome in BALB/c mice in consequence of intranasal (i.n.) inoculation with the mouse lymphotropic gamma herpesvirus MHV-ŠU. The results obtained showed that in a proportion of infected mice the number of leukocytes had considerably increased. The increasing numbers of leukocytes as documented by the differential white blood cell counts in infected mice, correlated with the duration of infection. Except of tumors (Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's lymphoma), EBV can induce some forms of leukemia, such as acute lymphoblastic leukemia, subacute granulocytic leukemia and plasmocytic leukemia. The connection between infection with MHV-68 or MHV-72 and the development of tumors had been already proven [13, 25] connection between MHV infection and the occurence of leukemia has not been demonstrated yet. Detection of atypical cells in peripheral blood of Balb/c mice with MHV-72 infection was published [14, 18]. The leukemia-like syndrome (500,000 leukocytes/μl of blood) was but first ob-

Table 3. Number of AgNOR dots in peripheral blood leukocytes of control and MHV-ŠU infected Balb/c mice

Mice	Number of AgNOR dots per nucleus (mean ± SD)
Non-infected controls	2.86 ± 0.42
MHV-ŠU infected (730 days p.i.)	5.68 ± 0.33

Note: AgNOR dots per nucleus (mean ± SD); the difference was significant at $p<0.001$.

Table 4. The analogy between MHV-ŠU and EBV pathogenesis

Sign	MHV-Šumava	EBV
Splenomegaly	+	+
Lymphocytosis/Leukocytosis	+	+
Atypical lymphocytes in the peripheral blood	+	+
Leukemia-like syndrome	+	+
Lymphoproliferative disorders (including tumors)	+	+

served following MHV-ŠU infection, therefore, we decided to examine differential WBC in mice in course of this infection.

The discovery of pathological changes in blood picture (malignant haematopathy) confirm our hypothesis pointing at similar characteristics between EBV and MHV-viruses. How EBV leads to the development of tumors and leukemia in humans cannot be studied in clinical situation. Therefore the MHV-ŠU infected mouse model can serve as a useful model for studying lymphoproliferative disorders. Considering the similarities between virus structure, immunopathology, immune control mechanisms and infectivity in regard to latency, strengthens the importance of the given mouse model for studying the pathogenesis of human gamma herpesviruses.

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