Activity of Alveolar, Peritoneal Macrophages and Blood Phagocytes during Experimental Infection of BALB/c Mice with MHV-Šumava

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Abstract

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MHV-Šumava (MHV-Š) represents murine gammaherpesvirus. Experimental intranasal infection of BALB/c mice with MHV-Š caused leukocytosis and increased number of alveolar macrophages. Atypical blastic leukocytes were observed. The number of alveolar macrophages correlated with the amount of MHV-Š in lungs. Macrophages have been identified as the major reservoir of latent virus in peritoneal exudate cells (PECs). Using indirect immunofluorescence method we have found the number of CD14 positive cells correlating with the number of MHV-Š antigen positive PECs MHV-Š infected leukocytes were found to have significantly reduced phagocytic activity. As evaluated by quantification of digested *Candida albicans* we observed slightly increased phagocytic index of infected leukocytes compared to controls. Taken together, we proved a decrease in ability of these cells to phagocyte due to MHV-Š infection.

Murine gammaherpesvirus, MHV-Šumava, infection, latency, PECs, macrophages, phagocytic activity

The murine herpesvirus isolate Šumava (MHV-Š) is an oncogenic gammaherpesvirus which serves as a model for the study of pathogenesis and immunology of human gammaherpesviruses (Mistríková et al. 2002). MHV-Š was isolated from the lungs of seropositive *Apodemus flavicollis* (Mistríková and Blaškovič 1985) and represents one of the eight isolates of mouse gammaherpesviruses. Isolates MHV-60, 68, 72, 76 and 78 are widespread in population of free living rodents (Blaškovič et al. 1980) as well as those isolated later such MHV-4556, MHV-5682 (Kožuch et al. 1993). All isolates represent one group of antigenically identical viruses (Svobodová et al. 1982), however, MHV-Š differs from other MHV isolates in minimal 3 polypeptides (Reichel et al. 1991). When administered intranasally the MHV-68 virus replicates in the lungs. Spread then occurs in the B-cell compartment resulting in splenomegaly. The virus then becomes latent in B-cells and persists. The long-term infection is associated with the development of lymphoproliferative disease (Sunil-Chandra et al. 1994). Particular studies of peripheral blood adherent mononuclear cells (AMCs) infected with MHV-72 *in vivo* and *in vitro* confirmed their important role for dissemination and latency of the virus in organism. It was found that the peripheral blood AMCs from non-infected mice were permisive to MHV-72 infection. AMCs play an important role in latency and reactivation of latent virus. Important are findings of increased virus neutralization antibodies in correlation with activation of latent virus (Mistríková et al. 1994). The aim of this work was to confirm our previous observation (Mistríková et al. 1994) about importance of peritoneal macrophages in chronic phase of infection on the model of BALB/c mice experimentally infected with MHV-72. Our main aim was to (i) quantify alveolar

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macrophages in broncho-alveolar area in acute MHV-Š infection and so prove the correlation with the amount of virus in the lungs, (ii) detect viral antigen in infected cells with specific monoclonal antibodies using the method of indirect immunofluorescence and monitored the kinetics of Ag+ cells in PECs and spleen after MHV-Š infection, (iii) examine phagocytic efficiency of leukocytes after infection with MHV-Š as a new model for EBV.

Materials and Methods

Mice
    Female BALB/c mice were obtained from the Institute of Virology of the Slovak Academy of Sciences and infected at 4-6 weeks of age.

Virus
    MHV-Š was isolated by (Mistríková and Blaškovič 1985). Virus working stocks were prepared by infection of VERO cells.

Infection of mice
    Female (4 - 6 weeks old) BALB/c mice were inoculated with 2×10^5 PFU MHV-Š per mouse. Twenty μl of the virus was administered intranasally (i.n.) to mice under light ether anaesthesia.

Sampling
    Mice were killed at different times p.i. by cervical dislocation. The blood, alveolar macrophages, spleen and peritoneal macrophages were removed and used for preparation of cell suspensions for detection of viral antigen.

Blood samples
    Were taken from *sinus orbitalis* at different times p.i. for preparing of serum and for examination of leukocytes. Obtained sera were inactivated at 56 °C for 30 min and titrated.

Staining of blood leukocytes
    Blood smears were made immediately after blood collection. After fixation by air drying they were stained with May-Grünwald solution for 10 min and Giemsa Romanowski solution for 15 min. Number of leukocytes was determined after 10 min of staining with Türk solution.

Differential white blood cell count
    Blood picture consisted of calculation of percentage of each kind of white blood cells.

Immunofluorescence (IF) test
    The presence of the virus antigen and surface antigen in cells from organs of infected mice was determined by indirect immunofluorescent assay.

Monoclonal antibodies (MoAbs)
    Mouse MoAbs prepared against MHV-Š was diluted 1 : 100-500 and vizualized with goat anti mouse IgG (H + L) conjugated with rhodamine (Immunotech, Slovak Republic). Rat MoAbs to mouse CD14 monocytes were purchased from Pharmingen (USA) and diluted 1 : 50. A goat anti rat IgG (H + L) conjugated with fluoresceinisothiocyanate (Sigma) was used as the secondary antibody. All the antibodies were diluted in bloking solution before use. Mouse MoAbs reacted with denatured viral antigen in Western blot and showed specificity for 50-55 000 protein. This MoAb exhibited virus-neutralizing potency indicating that the 50-55 000 viral antigen might be relevant for the infectivity of MHV (Matušková et al. 2003).

Virus neutralization test (VNT)
    was performed with 2-fold dilutions of sera from infected mice to which 1000 ID of appropriate virus was added. The virus-serum mixtures were incubated at 37 °C for 90 min and then inoculated into 24 - 48-hr-old VERO cell cultures according to the growth requirements of the respective virus. The titre was calculated when 1000 ID of virus caused a complete CPE in infected cells used as controls.

Determination of phagocytic efficiency
    The phagocytic efficiency was examined via co-incubation of leukocytes altogether with inactivated *Candida albicans* at the ratio of 1 : 5. The same volumes of *C. albicans*, heparinized blood, autologous serum and Hank's solution were mixed together and incubated for 30 min at 37 °C at continual mixing to provide precise opsonization. The smears were then stained with Wright's solution and neutral water and determined the phagocytic activity (FA) and phagocytic index (FI). FA was calculated as percentage of phagocytes out of 100 examined able to phagocyte *C. albicans*. FI was calculated as average number of *C. albicans* being phagocyted by one phagocytic cell.

Statistical analysis
    Statistical significance of differences was assessed using Student's *t*-test.

Results

Since the MHV-Š infection was lethal for newborn suckling mice (Mistríková et al. 2002), we decided to infect 4 - 6 week-old mice. In our experiment 50 female BALB/c mice were used, 25 served as controls and 25 were inoculated with MHV-Š with 2×10^5 PFU per mouse. Twenty µl of virus was administered intranasally (i.n.) to mice under light ether anaesthesia. Infected mice did not show apparent symptoms of illness. However, they showed an increase in the number of leukocytes and appearance of atypical leukocytes in peripheral blood. In the first part of our work presented here, mice infected with MHV-Š were sacrificed during 27 days of acute infection and 90 and 240 days of chronic infection. Using monoclonal antibodies against MHV-Š via immunofluorescent method we detected viral antigen in immunocompetent cells from the samples from lymphatic organs. Table 1 shows that in acute infection the highest number of cells positive on viral antigen were

Table 1. Detection of viral antigen MHV-Š in lymphatic organs of infected BALB/c mice

	Acute infection				Chronic infection	
	2 d.p.i.	7 d.p.i.	14 d.p.i.	27 d.p.i.	90 d.p.i.	240 d.p.i.
Peripheral blood	+	++	+	+	−	−
Alveolar macrophages	++	+++	+++	+	−	−
Spleen	−	+	+	++	+	−
PECs	−	−	−	+	+	++
VNT	1 : 16	1 : 64	1 : 256	1 :128/256	1 : 64/128	1 : 128

Viral antigen was detected using MoAb against MHV-· via immunofluorescent test

−	0%	+	2 – 4%
±	1%	++	5 – 10%
		+++	50%

present in alveolar exudate peaking in period from day 7 to day 14 p.i. with positivity more than 50%. In the same intervals as harvesting the samples for the IF test, using virus neutralization test (VNT) we examined sera. The antibodies against virus reached a maximum of 1 : 256 on day 14 p.i. During the acute infection, the number of leukocytes increased, peaking with 14 700 on day 14 p.i. Atypical leukocytes were observed in differential white blood cell counts, reaching a maximum of 14% on day 9 p.i. (Fig. 4).
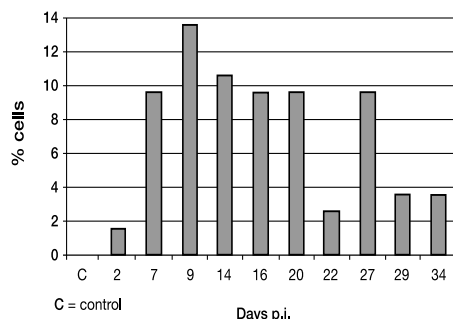
Fig. 4. Appearance of atypical forms of leukocytes in peripheral blood of BALB/c mice infected with MHV-Š
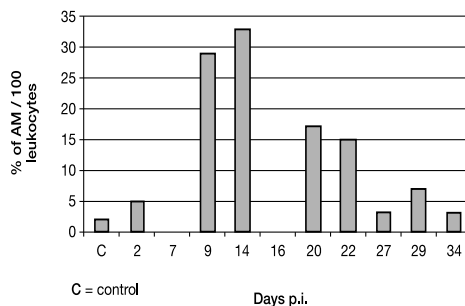
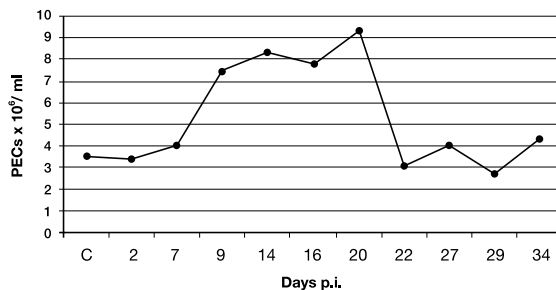Fig. 5. The amount of alveolar macrophages (AM) of BALB/c mice infected with MHV-Š

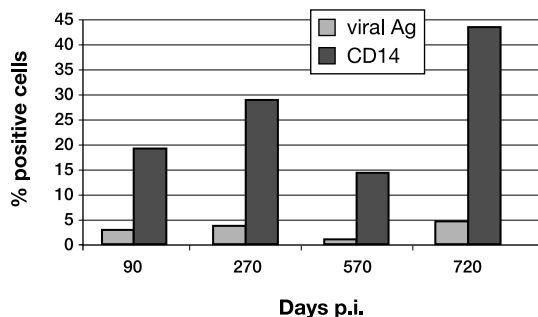Fig. 6. The amount of PECs obtained from BALB/c mice infected with MHV-Š in relation to day p.i.

Fig. 7. Number of CD14 and virus Ag positive cells in relation to days p.i. in chronic in fection

Intranasal inoculation with MHV-Š led to an initial lung infection with peak titres of virus in lungs, which were accompanied with increased number of alveolar macrophages in alveolar exudate (Fig. 5). Alveolar monocytes and macrophages are crucial for the virus, since they become the main target and the place of viral replication after the first contact with its host. Further, we investigated the number of cells in peritoneal exudate. We found that the number of PECs kept an increased level from day 9 to day 20 p.i. (Fig. 6). PECs consisted of heterogenic cell population such as lymphocytes, monocytes and macrophages (Plate V, Fig. 1). Detecting CD14+ cells via IF test we proved that macrophages represented the major part of PECs. Using MAbs against MHV-Š we detected the presence of viral antigen in these cells (Plate V, Fig. 2). Our intention was to find relationship between the number of CD14 positive PECs and number of cells positive on viral Ag. We focused on chronic infection from day 90 up to 720 p.i. As Fig. 7 shows the number of CD14+ cells peaked on day 720 as well as number Ag+ cells suggesting viral reactivation. As well known, the spleen harbours virus in a latent form in B lymphocytes, however the major reservoir of the latent virus are macrophages in peritoneal cells. Using IF test we compared the presence of viral antigen in splenocytes and PECs during the late phase of chronic infection in order to confirm meaning of PECs, macrophages particularly, as major reservoir even in infection of MHV-Š. The samples were taken 150, 240 and 570 days post infection.

In peripheral blood, we examined the efficiency of leukocytes to phagocyte during acute infection and chronic infection, particularly. Using co-incubation with *Candida albicans* we determined phagocytic activity (FA) and phagocytic index (FI) non-infected controls as well as infected groups in acute and chronic stage of infection. The average values of FA and FI are shown in Table 2. In control group FA = 78% and FI = 5.1. During acute phase FA = 41%,

Table 2. Phagocytic efficiency of leukocytes obtained from non-infected controls and infected BALB/c mice

Mice (No.)	FA	FI
non-infected controls (5)	78% ± 0.15	5.1 ± 0.78
acute infection (3)	41% ± 0.01	5.35 ± 0.4
chronic infection (8)	58.8% ± 0.18	5.3 ± 0.14

FA = phagocytic activity
FI = phagocytic index

which indicate significant decrease of phagocytic activity comparing to controls. Slightly increased value was detected in FI = 5.35. In chronic phase of infection we monitored phagocytic efficiency FA = 58.8% and FI = 5.3.

Discussion

Pathogenetic studies of MHV demonstrate that after acute infection the virus causes chronic infection and is capable of reactivation forming tumors. Components of host immune system are actively involved in all phases of MHV infection and play a key role in latency and following reactivation. Although little is known about the mechanism of MHV-68 transmission, the early stages of infection after intranasal inoculation and the ensuing antiviral host immune response have striking similarities with EBV. After intranasal infection the virus establishes a lytic infection. It is rapidly cleared by the host immune response with CD8+T cells playing a prominent, but not exclusive role. Latency is established largely in splenic B-cells, although other cell type can harbour latent virus (Blackman et al. 2000). This was proved by Flaňo et al. (2000), who showed that latent MHV-68 is harboured in three types of cells in spleen after intranasal infection, namely, B-cells, macrophages and dendritic cells. Our results show that the infection with MHV-Š was accompanied by major changes in number as well as in morfology of leukocytes. Compared to isolates MHV-72 (Mistríková et al. 1994) and MHV-78, (Mrmusová - Šupolíková et al. 2003) the profile and the percentage of Ag+ cells is similar, particularly with MHV-78. In previous experiments we found, that during 180 - 240 days p.i. MHV-72 persisted in the highest titre in peritoneal macrophages. VNT antibodies showed an increase in the same interval as reactivation of latent virus (Mistríková et al. 1994). Mistríková et al. (1994) and Weck et al. (1999) demonstrated that macrophages (F4/80+) in PECs harbour latent MHV-72, MHV-68 and are the major reservoir, comparing to B-cell-reservoir, where the frequency of CD19+ B-cells carrying latent virus was 10-fold lower. This fact puts B-cells into position of the second reservoir of latency. Little is known about the interaction of EBV with human monocytes. Savard et al. (2000) demonstrated that EBV infects and replicates in human monocytes, a process accompanied by the supression of phagocytosis by these cells. The results indicate that the virus adsorbs and penetrates into monocytes without being phagocytosed. EBV-infected monocytes are significantly impaired in their ability to phagocyte. Impairment of the phagocytosis machinery is expected to be advantageous for the viral outcome (Savard et al. 2000). Taken together, it can be postulated that targeting monocytes/macrophages may represent an evolutionary advantage for ensuring propagation and persistance of EBV and other herpesviruses within the host (Blasig et al. 1997; Kondo et al. 1991; Larsson et al. 1998; Maciejewski et al. 1993). Shimakage et al. (1999) in his study provides additional indications that monocytes/macrophages may serve as reservoirs of EBV infection. EBV-infected monocytes showed reduced capacity to phagocyte. The mechanisms by which EBV affects phagocytosis remain to be elucidated (Savard et al. 2000). Results demonstrated in our study show similarities with those of other authors, who proved suppressive effect of human

herpes viruses on phagocytic activity. Decreased phagocytic efficiency caused by herpes viruses allows intensive replication and establishment of different forms of infection typical for them. Our results presented here confirm the important role of peritoneal macrophages in chronic infection of MHV-Šumava and point out eminent effect of the infection on phagocytic efficiency of macrophages. Similar results obtained in EBV studies confirm suitability of murine herpesvirus as an animal model.

Aktivita alveolárnych, peritoneálnych makrofágov a krvných fagocytov v experimentálnej infekcii BALB/c myši vírusom MHV-Šumava

MHV-Šumava predstavuje myšací gamaherpesvírus. Experimentálne sme infikovali inbrédnu líniu BALB/c myší intranazálne, čo u nich vyvolalo leukocytózu, zvýšenie počtu alveolárnych makrofágov a prítomnosť atypických leukocytov v periférnej krvi. V pľúcach sme zistili koreláciu medzi počtom alveolárnych makrofágov a množstvom MHV-Š. V bunkách peritoneálneho výplachu (PECs) boli makrofágy identifikované ako hlavný rezervoár latentného vírusu. S použitím špecifických monoklonálnych protilátok voči MHV-Š sme v PECs pomocou imunofluorescenčnej metódy detegovali vírusový antigén a pozorovali koreláciu medzi bunkami pozitívnymi na marker CD14 a bunkami pozitívnymi na antigén MHV-Š. Leukocyty infikované MHV-Š vykazovali signifikantne zníženú fagocytárnu aktivitu a kvantifikácia pohltených *Candida albicans* v porovnaní s kontrolnými vzorkami ukázala mierne zvýšenú hodnotu fagocytárneho indexu.

Acknowledgement

Note

The authors claim that all procedures using animals were performed in accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes from 1986.

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Plate V
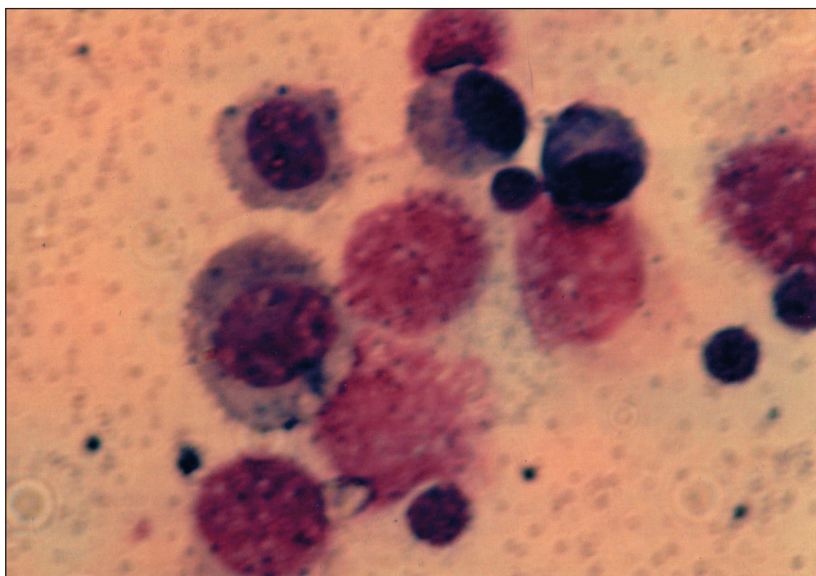Spiššáková I. . et al.: The Role of Alveolar ... pp. 353-359

Fig. 1. Heterogenic population in PECs obtained from MHV-Š infected mice
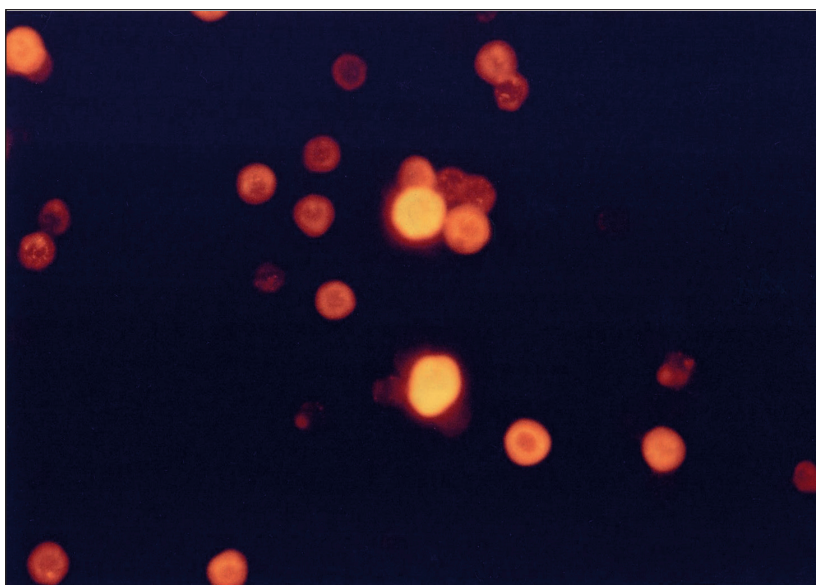

Fig. 2. Detection of viral antigen of MHV-Š
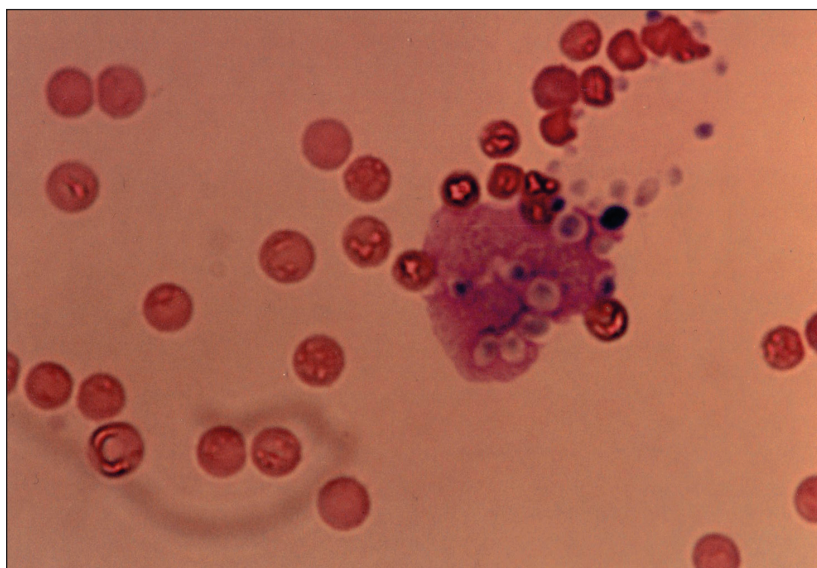
Plate VI

Fig. 3. Blood phagocyte engulfing *Candida albicans* after MHV-Šumava infection