EFFECT OF IMMUNOSUPPRESSION ON BALB/C MICE INFECTED WITH MURINE HERPESVIRUS

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Summary. – Balb/c mice were infected with murine herpesvirus (MHV-72) and subjected to immunosuppression (IS) with the antibiotic FK506 either during the acute or chronic phase of infection. Attempts to detect virus in various organs of immunosuppressed and non-immunosuppressed mice at different time interval were made. In the mice immunosuppressed on days 3-23 p.i. of the acute phase of infection virus was detected in various tested organs and tissues at 2.0 times higher rate than those of control mice. At later intervals of the acute phase of infection (56-84 days p.i.) virus was still recovered in bone marrow and lymph nodes peritoneal cells of immunosuppressed mice, but not in those of control animals. In the chronically infected mice immunosuppressed on days 290-320 p.i., the virus was detected in lungs, thymus, bone marrow, spleen and peritoneal cells at 3.5 times higher rate than in those of control mice.

Key words: murine herpesvirus; Balb/c mice; immunosuppression; acute infection; chronic infection

Introduction

MHV strains 68 and 72 were isolated from organs of freely living rodents *Apodemus flavicollis* and *Clethrionomys glareolus* (Blaškovič *et al.*, 1980; Mistríková and Blaškovič, 1985). Both the MHV strains grew in a variety of cell lines of epithelial or fibroblast origin, derived from many mammalian species including man (Svobodová *et al.*, 1982). Studies on MHV distribution in infected newborn and adult outbred laboratory mice showed its presence in lungs, kidneys, spleen, and liver with occasional haematogenic spread to nervous system (Blaškovič *et al.*, 1983; Rajčáni *et al.*, 1985). In addition, MHV-68 was found to establish chronic infection at least in the lungs and spleen of outbred mice when administered by intranasal (i.n.) route (Rajčáni *et al.*, 1985). When given i.n. to inbred Balb/c mice, the virus replicated mainly in the lungs and spleen (Sunil-Chandra *et al.*, 1992a). Later on it persisted in the lungs and lymphatic system, especially in B lymphocytes (Sunil-Chandra *et al.*, 1992b) and in adherent mononuclear cells (Mistríková *et al.*, 1994). The results of genetic analysis of MHV-68 DNA showed that MHV belongs the subfamily of *Gammaherpesvirinae* similarly to Epstein-Barr virus (EBV) and herpesvirus saimiri (Efsthatiou *et al.*, 1990). Most recently, mice infected with MHV-68 were found to develop a lymphoproliferative disease (lymphomas and lymphoblastomas) at a rate of approximately 10% (Nash *et al.*, 1994). Thus, lymphotropic properties of MHV rise the possibility of using the chronic MHV infection in mice as a model for elucidation of the possible role of gammaherpesviruses in lymphoproliferative disorders arising in man due to chronic EBV infections. The lymphoproliferative disorders in organ transplant patients may be related to the immunosuppression which reactivates latent EBV and cytomegalovirus (CMV) (Nalesnik *et al.*, 1991; Yoshizava *et al.*, 1993). Here we describe the effect of immunosuppression with FK506, a novel compound from *Streptomyces* (Kino *et al.*, 1987), on Balb/c acutely and chronically infected with MHV-72.

Abbreviations: BEM = Eagle's Basal Medium; CMV = cytomegalovirus; CPE = cytopathic effect; EBV = Epstein-Barr virus; i.n. = intranasal(ly); IS = immunosuppression; MHV = murine herpesvirus; PBS = phosphate buffered saline; p.i. = post inoculation; sc = subcutaneous(ly)

Materials and Methods

Animals. Balb/c mice originating from the breed Velaz (Prague, Czech Republic) were propagated at the Institute of Virology, Bratislava under standard conditions. When 6-week-old, the animals were inoculated with 2 x 10^5 $TCID_{50}$ of MHV-72 in 0.02 ml by i.n. route.

Virus. MHV-72 was grown in Vero cells in the presence of Eagle's Basal Medium (BEM) supplemented with 7% calf serum, 0.3% glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). To prepare a stock virus, the infected cell suspension was sonicated, clarified by low-speed centrifugation and the supernatant was stored in 1 ml aliquots at -70°C. The stock virus titer was 1 x 10^7 $TCID_{50}$/1 ml.

Immunosuppression (IS). Compound FK506 was a kind gift from Dr. M.A. Nalesnik, Pittsburgh Medical Center, Pittsburgh, PA, USA. The lyophylized substance was dissolved in phosphate buffered saline (PBS). Freshly prepared solutions were inoculated subcutaneously (sc) to virus-infected and uninfected Balb/c mice daily with exception of weekend days in the course of 4-6 weeks. The administered dose was 2 mg/kg/day as recommended by Yamamoto *et al.* (1990).

Co-cultivation method. Autopsy was made on different days p.i. or after the onset of IS as specified in Results. 10% suspensions of tissue homogenates in serum-free BEM were prepared and pooled from lungs, livers and kidneys of 2 mice. The suspensions were frozen and thawn, sonicated and clarified by low-speed centrifugation (2000 x g for 10 mins). Concentrated and diluted (10^{-1}) supernatants were used for infection of Vero cell monolayers in 24-well microtiter plates (NUNC). CPE was read after 3-5 days of incubation at 37°C in 4% CO_2 atmosphere. Thymus, lymph nodes (mainly parabronchial, thoracal and cervical), spleen and bone marrow were mechanically disrupted in PBS under sterile conditions. The cells were repeatedly washed with PBS, filtrated through a cotoon wool, centrifuged at 800 x g for 5 mins and co-cultured with Vero cells as described above.

Explantation method. Tissue fragments were prepared from lungs, kidneys, spleen and liver, pooled from 2 mice, minced under sterile conditions and cultured in 24-well microtiter plates at 37°C for 10 days in 4% CO_2 atmosphere in medium RPMI-1640 supplemented with 10% foetal calf serum and antibiotics. The medium was changed for fresh one on days 4 and 7 of cultivation. By day 10, the medium was removed and the fragments of the given tissue were homogenized described above. A typical positive sample shed virus since day 7 into the medium and the corresponding homogenate was positive at dilutions $\geq 10^{-1}$. The sample was taken for positive also if the explanted tissue homogenate yielded CPE by day 5 p.i. (at least at dilution 10^{-1}), though the corresponding medium samples remained negative. Peritoneal adherent cells and peripheral blood cells suspensions were prepared as described previously (Mistríková *et al.*, 1994). Samples of media and of homogenates of explanted fragments from both the co-cultivation and explantation assays were used for virus titration in Vero cells by standard procedures.

Results

Effect of IS applied during the acute phase of infection

The virus-infected mice were immunosuppressed with antibiotic FK 506 on days 3-23 p.i. and sacrificed on various days p.i. The non-immunosuppressed mice served as controls. Various organs were tested for virus presence by co-cultivation and explantation procedures. Table 1 shows that the virus recovery rate in all organs as a whole in immunosuppressed mice was about 2.0 times higher than that in control animals.

Table 1. Effect of IS applied during the acute phase of MHV-72 infection of Balb/c mice

A. Immunosuppressed group	Virus recovery rate on days p.i.					
Organ (tissue)	7	14	28	56	84	7-54
Thymus (Co)	–	–	+	–	–	1/5
Lymph nodes (Co)	–	–	–	–	+	1/5
Bone marrow (Co)	+	–	+	–	+	3/5
Peritoneal cells	+	–	+	–	+	3/5
Peripheral blood	–	–	–	–	–	0/5
Spleen (Co/E)	+	+/+	–/–	+/+	+/+	4/5
Lungs (Co/E)	+	+/+	–/–	+/+	+/+	4/5
Kidney (Co/E)	+	–/–	–/–	–/–	–/+	2/5
Liver (Co/E)	ND	–/–	–/–	–/–	–/–	0/5
Total						18/45=40%
B. Control group						
Thymus (Co)	–	ND	–	–	–	0/4
Lymph nodes (Co)	–	ND	–	–	–	0/4
Bone marrow (Co)	–	ND	–	–	–	0/4
Peritoneal cells	–	ND	+	–	–	1/4
Peripheral blood	–	ND	–	–	–	0/4
Spleen (Co/E)	+/+	ND	+/+	–/+	–/–	3/4
Lungs (Co/E)	+/+	ND	–/–	–/+	–/+	3/4
Kidney (Co/E)	–/–	ND	–/–	–/–	–/–	0/4
Liver (Co/E)	–/–	ND	–/–	–/–	–/–	0/4
Total						7/36=19.4%

Mice were infected with MHV-72 and one half of them was subjected to IS on days 3-23 p.i. On various days p.i. mice were sacrificed and their organs were tested for virus presence by explantation (Ex) and co-cultivation (Co) procedures. Another half of infected mice served as control. ND = not done.

Table 2. Effect of IS on the reactivation of MHV-72 in chronically infected Balb/c mice

A. Immunosuppressed group

Virus reactivation rate on days after the onset of IS									
Organ (tissue)	14	21	42	49	65	91	119	147	14-147
Thymus (Co)	–	–	+	–	–	+	–	–	2/8
Lymph nodes (Co)	+	+	+	–	–	–	–	–	3/8
Bone marrow (Co)	+	+	+	+	–	–	–	–	4/8
Peritoneal cells	+	+	+	–	+	–	+	–	5/8
Peripheral blood	–	+	–	–	–	–	–	–	1/8
Spleen (Co/E)	–/–	–/+	–/+	+/–	–/+	–/–	–/–	–/–	4/8
Lungs (Co/E)	–/+	–/+	–/+	–/+	–/+	+/+	–/–	–/–	6/8
Kidney (Co/E)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	0/8
Liver (Co/E)	–/–	–/–	–/–	–/–	–/–	–/–	–/–	–/–	0/8
Total									25/72=34,7%
B. Control group									
Thymus (Co)	ND	–	–	–	–	–	–	–	0/7
Lymph nodes (Co)	ND	+	–	–	–	–	–	–	1/7
Bone marrow (Co)	ND	+	–	+	–	–	–	–	2/7
Peritoneal cells	ND	–	+	–	–	–	–	–	1/7
Peripheral blood	ND	–	–	–	–	–	–	–	0/7
Spleen (Co/E)	ND	–/–	+/+	+/–	–/–	–/–	–/–	–/–	2/7
Lungs (Co/E)	ND	–/–	–/–	–/–	–/–	–/–	–/–	–/–	0/7
Kidney (Co/E)	ND	–/–	–/–	–/–	–/–	–/–	–/–	–/–	0/7
Liver (Co/E)	ND	–/–	–/–	–/–	–/–	–/–	–/–	–/–	0/7
Total									6/63=9.5%

Mice were infected with MHV-72 and one half of them was subjected to IS on days 290-320 p.i. On various days after the onset of IS, mice were sacrified and their organs were tested for virus presence by explantation (Ex) and co-cultivation (Co) procedures. Another half of infected mice served as control. ND = not done.

Effect of IS applied on established chronic infection

The virus-infected mice were immunosuppressed with antibiotic FK506 on days 290-320 p.i. and sacrificed on various days p.i. The non-immunosuppressed mice served as controls. Various organs were tested for virus presence by co-cultivation and explantation procedures. In control mice the virus was recovered in total of 6 out of 63 organ and tissue samples (9.5%). The results of this experiment are shown in Table 2. In contrast, in immunosuppressed mice, the virus was recovered at a 3.5 times higher rate (34.7%). The virus was isolated mainly during the period from 14 to 91 days.

Discussion

The drug FK506 is a macrolidic antibiotic obtained from *Streptomyces tsukubaensis*, which suppresses the T-cell mediated immune response, possibly acting on the IL-2 receptor or on its expression in helper T cells (Honbo *et al.*, 1987). The IS effect of FK506 exceeds that of cyclosporin A about 100 times.

MHV has been recently classified as a gammaherpesvirus because its genes were found colinear with those of EBV (Efstathiou *et al.*, 1990). MHV is similar to EBV also biologically, being able to persist in spleen cells and B lymphocytes of infected mice (Sunil-Chandra *et al.*, 1992b). In addition, MHV can induce lymphomas of mixed B and T cell phenotype in mice (Sunil-Chandra *et al.*, 1994).

In our experiments IS treatment applied to mice with established chronic MHV infection considerably increased the recovery rate of latent virus from thymus, lymph nodes, bone marrow, peritoneal mononuclear adherent cells, spleen and lungs. The distribution of latent MHV found in chronically infected mice in the absence of IS resembled that described earlier (Rajčáni *et al.*, 1985). The frequent reactivation of latent MHV in lungs is in accord with the fact that the virus was originally isolated from the lungs of freely living rodents (Mistríková and Blaškovič, 1985).

Another part of our study is the investigation of the effect of the drug FK506 on chronic MHV-72 infection in Balb/c mice from the viewpoint of possible enhancement of tumor induction.

In these experiments (data not shown) we were unable to confirm the enhancement of lymphoproliferative tumors due to IS with FK506. We observed such tumors as lymphomas, lymphoblastomas and fibrosarcomas without any association to IS. Possibly, this was due to an transient effect of IS, which seemed to be restricted to the 3-month-period since the onset of the drug treatment (the lag period for tumor formation under these experimental conditions ranged from

290 to 720 days). Because the treatment with FK506 had no considerable effect on the outcome of acute MHV infection other than the increased establishment of chronic infection in the lymphatic system and in mononuclear cells, we believe that this effect may lead to an increase of the frequency of lymphoproliferative disease at late post-infection intervals. It is well known that EBV, CMV and HHV-6 frequently reactivate in posttransplantation and immuno-compromised patients (Yoshizava *et al.*, 1993). The pathogenesis of EBV-related lymphoproliferative disease needs elucidation at molecular level (Cohen, 1991; Gulley *et al.*, 1992; Ambinder *et al.*, 1994; Klein *et al.*, 1994). Chronic MHV infection of mice may represent a useful model also regarding the possible development of EBV-related smooth muscle tumors in post-transplantation patients (Lee *et al.*, 1995).

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