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The effect of Isoprinosine treatment on persistent infection of Balb/c mice infected with murine gammaherpesvirus 68

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Summary. – We demonstrated the positive effect of Isoprinosine treatment on persistent infection of Balb/c mice with murine gammaherpesvirus 68 (MHV-68). Increased number of leukocytes, increased percentage of neutrophils, elevated levels of virus-neutralizing (VN) antibodies, reduced number of atypical lymphocytes and reduced virus titers were detected in the examined organs after a 14-day treatment. The positive effect of Isoprinosine therapy vanished after 120–150 days. After this interval, we demonstrated lower numbers of leukocytes, lower levels of VN antibodies and an increased number of atypical lymphoid monocytes in the Isoprinosine-treated group. Immunological parameters correlated with increased titers of virus in all investigated organs. Evidence of immunostimulation was demonstrated by lower incidence of tumor formation (7.5%) in the group of MHV-infected and Isoprinosine-treated mice in comparison to group without Isoprinosine treatment (17.5%). The presented results showed that Isoprinosine therapy had a positive impact on persistent infection of mice with MHV-68, but this effect was time-limited. The improvement of the investigated parameters lasted for five months only. Our presented results confirmed that each treatment with Isoprinosine should be repeated and must be long-term in some chronic infections.

Keywords: MHV-68; Isoprinosine; immunity; reactivation of latent virus; tumors

Introduction

Isoprinosine (Inosine acedoben dimepranol) is known as a drug with immunomodulatory and antiviral effects, which is registered in 75 countries all over the world and has been used for more than 20 years (Campoli-Richards *et al.,* 1986). Originally introduced in 1974 by Gordon for treatment of geriatric patients, it was later found to have a significant immunostimulating effect. Isoprinosine was also used as a very effective antiviral drug in combating herpes virus infection and is still regarded as the forerunner of acyclovir (Gordon *et al.,* 1974). Immunomodulatory effects of Isoprinosine results in antiviral activity, which can be demonstrated *in vitro* and *in vivo* (Campoli-Richards *et al*., 1986).

In vivo, Isoprinosine is believed to stimulate the production of lymphokines. This hypothesis was confirmed by a number of experiments, which demonstrated the increased amounts of lymphokines (IL-1, IL-2) as wells as an increase of blood mononuclear cells in patients following treatment with Isoprinosine (Nakamura *et al*., 1983*;* Tsang *et al.,* 1985). It was also shown that the antiviral and immunomodulating activity of Isoprinosine increased a number of parameters of either specific or non-specific (cell-mediated) immunity (Petrisková *et al*., 2002). Isoprinosine stimulates the function of blood mononuclear cells (PBMC), which leads to increased response against different mitogens (Jacobsen and Greenspan, 1982; 1983). The antiviral activity of Isoprinosine was confirmed to result in inhibition of replication of DNA and RNA viruses and in improvement of cell-mediated immunity (Simon and Glasky, 1978). An inhibition of replication was demonstrated for herpes simplex virus, cytomegalovirus, adenovirus, po-

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Abbreviations: dpi = days post infection; IS = Isoprinosine; MHV- 68 = murine gammaherpesvirus 68; VNA = virus neutralization assay; EBV = Epstein-Barr virus

liovirus, poxvirus, influenza A and B, rhinovirus, echovirus, rabies virus, encephalomyocarditis virus and eastern equine encephalitis virus (Muldoon *et al*., 1972; Ginsberg *et al.,* 1973; Gordon *et al*., 1974; Hernández-Jáuregui *et al*., 1980; Ohnishi *et al*., 1983). Antiviral activity was also demonstrated against monkey rotavirus, where the level of inhibition was dependent on the concentration of Isoprinosine (Linhares *et al.,* 1989). Effective inhibition of replication of Newcastle disease virus was achieved by combining Isoprinosine and immunomodulator KLP-602 (Malaczewska and Rotkiewicz, 2005). Immunomodulatory and antiviral effects of Isoprinosine have been tested *in vivo* in experimental animals infected with DNA and RNA viruses, and with various doses of medicated Isoprinosine. The recommended daily dose is 50 mg of Isoprinosine per kg. Positive clinical improvements have been observed with infection by herpes simplex virus, subacute sclerosing panencephalitis, genital warts, flu, in herpes zoster virus infection, hepatitis B, as well as in homosexual men with persistent generalized lymphadenopathy. Statistically important therapeutic results were reported in the treatment of tropical measles, but not in measles. A variety of studies deal with the therapeutic effects of Isoprinosine in infectious diseases and autoimmune diseases (Campoli-Richards *et al*., 1986; Sadowska-Wróblewska *et al*., 1988). The treatment of patients infected with human immunodeficiency virus maintains the standard levels of $CD4^+$ and $CD8^+$ T-lymphocytes, and increases the amount of leukocytes (De Simone *et al*., 1989). Studies have also shown a positive effect of Isoprinosine in the treatment of various skin diseases that are caused by infectious viruses (herpes simplex, herpes zoster and human papillomavirus-induced genital warts) (Matusiak and Szepietowski, 2010). Therapeutic efficacy of Isoprinosine was confirmed especially in papillomavirus infections. The treatment of genital warts represents a safe alternative of systematic therapy (Mohanty and Scott, 1986; Georgala *et al*., 2006). Isoprinosine can also be used in patients with varicella-zoster (VZ) infection and has been tested in patients with acute hepatitis B. Under the influence of this drug, normalization of biochemical markers and liver functions occurred, leading to overall recovery of patients (Karimov, 2004).

However, Isoprinosine affects the whole immune system and has also been tested in tumor diseases. In patients with primary tumors (lung cancer, mammary adenocarcinoma, melanoma), all reduced parameters returned to normal after application of Isoprinosine (Tsang *et al*., 1983).

Isoprinosine was also tested with respect to Epstein-Barr virus (EBV) that causes lymphoproliferative disorders. The study was focused on the ability of Isoprinosine to increase lymphocyte response against EBV antigens and EBV-transformed lymphocytes (Sundar *et al.,* 1985).

Isoprinosine is also known under the trade name Imunovir. The generic name of the molecule is inosine acedoben dimepranol or methisoprinol, also referred to as inosine pranobex or inosiplex (Hrubiško, 2009). All recent reports demostrated that Isoprinosine has immunomodulation, antiviral and antiproliferative effect (Campoli-Richards *et al*., 1986; Petrova *et al*., 2010; Samuel, 2011).

MHV-68, a murine virus, serves as a model to study the pathogenesis (immunology and oncogenesis) of EBV in experimental animals (Blaškovič *et al*., 1980; Mistríková *et al*., 2000; Čipková-Jarčušková *et al*., 2013). It allows us to monitor the progress of virus distribution in different stages, i.e. in acute and chronic infection (Stevenson and Doherty, 1998). MHV-68, namely its genetic and biological properties, is very similar to EBV, both possessing the characteristics of human gammaherpesviruses (Sunil-Chandra, 1992). In view of the fact that infections with gammaherpesviruses are frequent in the population and Isoprinosine is often used as an immunomodulatory agent, we decided to test the effect of Isoprinosine treatment in Balb/c mice infected with the MHV-68.

Epstein-Barr virus is a human gammaherpesvirus that induces persistent infection and is associated with several lymphoproliferative disorders, including infectious mononucleosis (IM). IM is accompanied by abnormalities of the immune system, including a temporary reduction in the activity of the immune system. Due to this observation we assume that Isoprinosine may be very useful for therapy of immunosupression following gammaherpesvirus infection.

The aim of this work was to investigate the immunomodulatory effect of Isoprinosine treatment of chronic infection of Balb/c mice focused on the following parameters:

- to compare the number of leukocytes and percentage of neutrophils in the blood of infected mice either treated or not treated with Isoprinosine
- to compare differential blood cell count (DBC) at different stages of infection in both animal groups
- to determine the virus-neutralizing antibody titer in both groups
- to determine the presence of the virus in different organs of infected and drug-treated mice
- to confirm the efficacy of Isoprinosine and/or characterize its role in persistent infection and in reactivation of latent virus as well as on lymphoproliferative processes related to the development of virus-related lymphomas.

Materials and Methods

Cells, viruses and mice. Vero cells and NIH 3T3 cells were maintained in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 9% of heat-inactivated fetal bovine serum (FBS), glutamine, penicilline and streptomycine. Cell cultures were grown in a 5% CO_2 humidified atmosphere at 37°C. MHV- 68 stock (isolated from *Myodes glareolus*) was prepared by virus propagation on NIH 3T3 cells, while titers of infectious viruses were determined

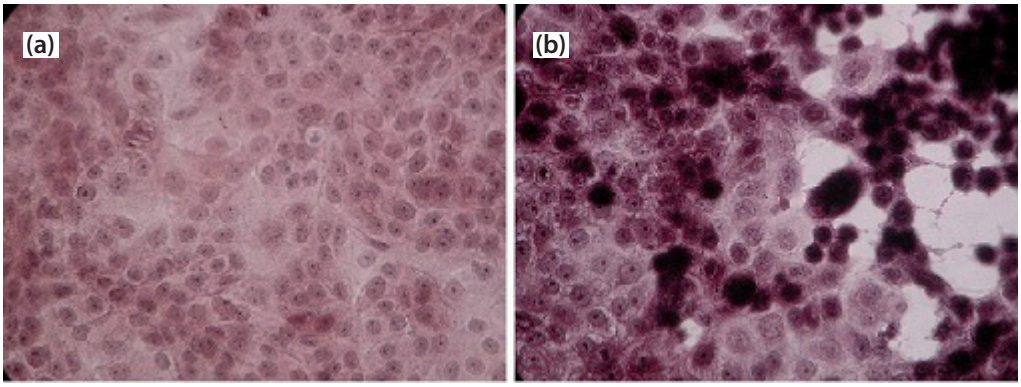

Fig. 1

Infection of Vero cells with MHV-68 from mouse tissues
(a) Control non-infected Vero cells; **(b)** Vero cells infected with suspension from organs of Balb/c mice infected with MHV-68.

by plaque assay on Vero cells. Six-weeks old female Balb/c mice were supplied by the Faculty of Veterinary Medicine, Brno, Czech Republic.

Coumpound. Isoprinosine (4-acetamidobenzoic acid;9-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3H-purin-6-one;1-(dimethylamino)propan-2-ol) is used in the treatment of a variety of viral infection. For peroral aplication, 500 mg tablet (Ewofarma) was diluted in distilled water.

Infection. Six-week-old female Balb/c mice were intranasally infected with $2x10^4$ PFU of MHV-68 per mouse in total inoculum volume of 20 µl, under light anesthesia. 100 mice were used in our experiment, 70 mice were infected with MHV-68, and 35 of 70 infected mice received 14 doses of Isoprinosine (2.5 mg/mouse/day), administered perorally by catheter. Control group of 30 mice received intranasally 20 µl of phosphate buffered saline (PBS).

Blood sample analysis. Blood for serum preparation and leukocytes examination was taken from *sinus orbitalis*. Heparinized blood was used to determine the total leukocyte count and differential blood cell count (DBC count). The total leukocyte count was determined after staining with Türck´s solution. In order to determine DBC count, air dried smears were stained for 10 min with May-Grünwald solution and then for 15 min with Giemsa-Romanowski solution. The stain solution was removed by rinsing with tap water and the smears were examined microscopically using a 100x magnification.

Virus-neutralization assay was performed with 2-fold dilutions of heat-inactivated sera from infected mice. For neutralization, 1000 PFU of appropriate virus was added. The virus-serum mixtures were incubated at 37°C for 30 min and then inoculated on Vero cell cultures according to the growth requirements of the respective virus. The last dilution of serum that was able to prevent virus infection in cell culture was considered as the titer of virus-neutralization antibodies.

Results

The aim of this work was to investigate the effect of Isoprinosine therapy on persistent MHV-68 infection in Balb/c mice. In our experiment were used 100 Balb/c mice, 70 mice were infected with MHV-68, 35 of 70 infected mice received Isoprinosine and another 30 mice served as controls. The

Table 1. Number of leukocytes and percentage of neutrophils during chronic phase of infection with MHV-68 after Isoprinosine treatment in comparison to control (non-infected) mice

	Neutrophils			Leukocytes		
	C	V	V+I	C	V	V+I
60 dpi	4	13	32	5,600	4,350	6,450
90 dpi	5.3	13.5	32.5	7,700	4,400	5,900
120 dpi	6.5	15	30	9,300	8,400	11,500
150 dpi	10.5	15.5	30	8,300	8,900	16,750
180 dpi	12	16	10	9,900	18,000	12,000
210 dpi	8.7	11.5	7.5	11,300	17,100	10,600
240 dpi	10.5	10	9	12,500	11,500	8,400

dpi = days post infection; V = MHV-68; V+I = MHV-68 + Isoprinosine; C = non-infected (control) mice.

Table 2. Percentage of atypical lymphocytes and titer of VN-antibodies during chronic phase of infection with MHV-68 after Isoprinosine treatment in comparison to control (non-infected) mice

	Atypical lymphocytes			Titer of VN antibodies		
	C	V	V+I	C	V	V+I
60 dpi	0	10	1	0	32	64
90 dpi	0	9	0	0	32	64
120 dpi	0	10	1	0	32	64
150 dpi	0	8	0	0	16-32	16-32
180 dpi	0	15	32	0	512	128
210 dpi	0	6	12	0	512	128
240 dpi	0	6	12	0	512	8

dpi = days post infection; V = MHV-68; V+I = MHV-68 + Isoprinosine; C = non-infected (control) mice.

treated group received 2.5 mg/day of Isoprinosine administered orally for 14 days. At regular intervals (60, 90, 120, 150, 180, 210, and 240 days post infection (dpi)), we compared the group of untreated but infected mice with the group of Isoprinosine-treated and MHV-68-infected mice. The monitored parameters were total leukocyte count (percentage of neutrophils, the proportion of atypical lymphocytes) as well as the virus-neutralizing antibody titers (VNT). The purpose of the comparison of these parameters was to confirm the effect of Isoprinosine on the course of persistent infection and its influence on reactivation of persistent infection and on the subsequent lymphoproliferative process (assessed by lymphoma formation). At regular intervals (60, 90, 120, 150, 180, 210, and 240 dpi), the infected mice in both groups (treated and non-treated with Isoprinosine) were sacrificed and their organs were investigated for virus presence using virus titration method (Fig. 1). Blood samples were taken to count leukocyte numbers and to determine differential blood cell count as well as VN antibody titer.

The antiviral and immunomodulatory effect of Isoprinosine has been confirmed in experimental MHV-68 infection. In response to the ongoing virus replication in the body, the level of leukocytes may increase along with the percentage of neutrophils and appearance of atypical lymphoid monocytes. Moreover, VN antibodies were formed simultaneously and their titer increased with the duration of infection.

Table 3. Detection of virus in organs of mice chronically infected with MHV-68 after Isoprinosine treatment

Titration of organs		60 dpi	90 dpi	120 dpi	150 dpi	180 dpi	210 dpi	240 dpi
Peritoneal macrophages	V	+	+	+	–	–	+	–
	V+I	–	–	–	–	–	+	–
Lymphatic nodes	V	–	+	+	–	–	+	–
	V+I	–	–	–	–	–	–	–
Kidneys	V	+	+	+	+	–	–	++
	V+I	–	–	–	+	++	–	++
Heart	V	++	+	+	+++	++	–	+
	V+I	–	–	–	+	+	–	–
Thymus	V	–	–	+	–	–	–	+
	V+I	–	–	–	–	–	–	–
Spleen	V	–	–	+	+	+	+	++
	V+I	–	–	–	+	+	+	++
Bone marrow	V	–	–	–	–	–	+	–
	V+I	–	–	–	–	–	+	–
Liver	V	–	–	–	+	++	+ +	++
	V+I	–	–	–	–	++	++	++
Lungs	V	–	–	+	+	++	+	–
	V+I	–	–	–	+	+	+	–

dpi = days post infection; V = MHV-68; V+I = MHV-68 + Isoprinosin;(+) 10%, (++) 50%,(+++) 75%; CPE (cytophatic effect) on the Vero cells infected with 10% suspension from organs of infected Balb/c mice and treated with Isoprinosine.

As shown in Tables 1 and 2, within 60–120 days after treatment with Isoprinosine, MHV-68-infected mice showed increased level of leukocytes, elevated percentage of neutrophils, and increased levels of VN antibodies in comparison to untreated ones, but a decreased level of atypical lymphoid monocytes. In the treated group, the VN antibody titer reached the maximum value at 180 and 210 dpi. At later time points (180–240 dpi), a significant increase of VN antibody levels was observed in the group of infected mice not treated with Isoprinosine. This may indicate reactivation of the virus at the given stage of infection. In contrast, the enhancement of VN antibody levels in Isoprinosine-treated group was insignificant, which may indicate a suppression of virus reactivation elicited by Isoprinosine therapy.

As shown in Table 3, the virus levels in organs of chronically infected mice have decreased during 60–150 days following the Isoprinosine treatment. In these mice, we detected only very low levels of reactivated virus in the tested organs. At these time points, treated mice gained weight when compared with non-treated controls. In the untreated group of infected mice the virus was present in kidneys, heart, peritoneal macrophages and lymphatic nodes at 60–90 dpi. At 120–150 dpi, the virus was demonstrated in almost every organ except for bone marrow and liver. As a result of persistent infection with the MHV-68, infected mice formed tumors.

Evidence of immunostimulation by Isoprinosine therapy was demonstrated by lower incidence of tumor formation in Isoprinosine-treated mice. In this group, we detected lower tumor incidence at 180, 270 and 450 dpi (7.5%), in comparison with the group of MHV-infected mice without Isoprinosine treatment at 180, 210, 300, 310, 320, 450, and 455 dpi (17.5%). A decrease in the number of tumors from 7 to 3 was also observed after immunostimulatory therapy. Because of low number of animals used in the study, these results cannot be considered statistically significant; however, they represent a posssibility that Isoprinosine therapy will be beneficial in the prevention of tumors induced by infection with gammaherpesviruses.

Discussion

Isoprinosine is used in the clinical practice as an immunostimulant with antiviral effect (Gordon *et al.,* 1974). In the state of immunosuppression, Isoprinosine restarts the lymphocyte function, enhances blastogenesis in monocyte population, stimulates cytotoxic T-lymphocytes and NK cells, and enhances the production of IgG, IFN gamma, IL-1 and IL-2 (Hersey *et al.,* 1984). Moreover, it reduces the production of proinflammatory cytokines and stimulates the chemotaxis of neutrophils, monocytes and macrophages (Hadden *et al.*, 1979).

The human gammaherpesvirus EBV establishes lifelong latency in B-lymphocytes in infected individuals, who count more than 90% of human population. It poses a health risk for immunocompromised individuals, since it is associated with several lymphoproliferative diseases and development of malignancies. (Rickinson and Kieff, 2007).

There are currently only few reports documenting the effect of Isoprinosine on gammaherpesviruses. Therefore, studies in a well-established mouse model, analogous to EBV, that is used to study the pathogenesis and immunology in the natural host (Blaškovič *et al*., 1980; Stevenson and Doherty, 1998), can contribute to our understanding of the effects of Isoprinosine on such infection.

The main goal of our study was to analyze the effect of Isoprinosine on the replication of MHV-68 *in vivo*, to quantify the level of blood leukocytes and their subpopulations, the production of antiviral neutralization antibodies and to determine the presence of the virus in different organs during the chronic phase of the infection. Isoprinosine affected selected parameters of the immune response and it reduced the virus levels in the organs of infected mice immediately after premedication, with the effect lasting 120 dpi. Mice were premedicated for 14 days, which is the time recommended for treatment of recurrent infections (Campoli-Richards *et al*., 1986). We did not record any visible symptoms in mice during the acute infection. This is in contrast with the data obtained by Sunil-Chandra *et al*. (1992) who observed symptoms such as ruffled fur, emaciation and severe weakness during the acute phase of infection following intranasal inoculation of Balb/c mice. Later on during infection (90 dpi), Isoprinosine-treated animals showed apparent weight-gain, which was noticeable at dissection as an increased amount of abdominal fat. In contrast to infected but untreated mice, infected and Isoprinosine-treated mice were bigger (data not shown). In addition, no virus was detected by virus titration in any of the analyzed organs of infected and Isoprinosine-treated mice during the chronic phase of infection (60–120 dpi). In animals that were infected but untreated, virus was detected in kidneys, heart and peritoneal macrophages at 90 dpi, and in almost all organs, with the exception of liver and blood marrow, at 120 dpi.

Based on the above results, we can conclude that Isoprinosine had a positive effect on suppression of virus replication at 60–120 dpi, e.g. 2–60 days after the end of the treatment. These results correlate with the data published by Campoli-Richards *et al*. (1986).

We next tested the organs for the presence of the virus at later time points of chronic infection, 150–180 dpi. At this time we detected the presence of the virus in kidney, heart, liver and lung in infected animals, thus confirming the hematogenic spread of MHV-68 in the organism. At 210 dpi, the virus was detected in several lymphatic organs – peritoneal macrophages, spleen and in the bone marrow. At this time point, we detected virus also in infected and Isoprinosine-treated mice, which indicates that the therapy by Isoprinosine

does not have a long-term effect on the suppression of latent virus reactivation.

Furthermore, we determined the leukocyte count in the peripheral blood of chronically infected mice. We found that it was affected by Isoprinosine in the timeframe of 60–150 dpi. Animals that were infected and Isoprinosine-treated had a slightly increased leukocyte count, which continuously decreased later after infection (180–240 dpi).

In contrast, infected but untreated animals had a higher leukocyte count, which may be attributed to the reaction of the immune system to the reactivated virus. The reactivation was suppressed in the Isoprinosine-treated animals. Our data are in agreement with the results published by Pfadenhauer and Glasky (1974).

In the course of chronic infection (60–150 dpi), we also detected an increased amount of neutrophils in infected and Isoprinosine-treated animals, with the maximum at 90 dpi, which correlated with the increase of the total leukocyte count in this group of animals.

Following the contact with the virus, the immune system reacts to the infection also by production of antibodies. The titer of virus-neutralizing antibodies increases with the time of infection during the acute infection. These our results are similar to those published by Simas and Efstathiou (1998).

An increased antibody-level during chronic infection may play a role in removing the reactivated virus from infected tissues (Carin *et al.*, 1996). The titer of VN antibodies increased in the course of chronic infection with the time of infection and reached the levels of 16–512. It reached its maximum at 180 and 210 dpi in the group of infected and Isoprinosine-treated mice. The effect of the Isoprinosine treatment on the reactivation suppression subsided at this time, leading to an increase in the antibody level. Our results correlate with the previously published work on positive effect of Isoprinosine on the stimulation of both humoral and cellular immunity, resulting in an increase in CD4+ T cell level in peripheral blood and in the spleen, as well as in the increase of antibody titer (Stenzel *et al.*, 2011).

MHV-68 is related to the EBV and one of their common features is the lymphoproliferation and tumor development in the course of the long-term virus infection. Thus, we set out to examine the lymphoproliferative changes in Balb/c mice infected with MHV-68 in comparison with infected and Isoprinosine-treated mice. Sunil-Chandra and colleagues described lymphoproliferative changes and diseases related to MHV-68 infection in the time span of 165–825 dpi (Sunil-Chandra *et al.*, 1994).

In our hands, tumors developed 180–255 dpi. While 17.5% of infected and untreated mice developed tumors, only 7.5% of infected and Isoprinosine-treated mice developed these. No tumors developed in control group. The presence of viral DNA was confirmed by nested PCR in all animals that developed tumors, thus confirming the association of tumor formation and virus infection.

Based on the presented data, we suggest that treatment with Isoprinosine results in lower level of reactivated virus, which is, during gammaherpesvirus infection, usually associated with increase in the count of atypical blood cells and increase in antibody levels. Despite the lower incidence of tumor formation in the Isoprinosine-treated animals, we do not consider our results statistically significant because of the small group of animals in the study. These results, however, confirm our hypothesis of a potentially positive effect of Isoprinosine on the pathogenesis of oncogenic gammaherpesviruses.

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