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Antiviral Research 71 (2006) 64–68
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

Evaluation of methyl inosine monophosphate (MIMP) and peramivir activities in a murine model of lethal influenza A virus infection

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Received 31 October 2005; accepted 15 February 2006

Abstract

An inbred murine model (BALB/c) was utilized to assess the protective effect of the immunomodulator methyl inosine 5′-monophosphate (MIMP) against infection with influenza A/PR/8/34 (H1N1) virus. Contrary to the data reported for outbred mice (NMRI) infected with the aerosolized virus (Masihi, Hadden, 2003. J. Int. Immunopharmacol. 3, 1205–1215), there were no improvements in the outcomes of infection in the inbred animals treated with MIMP intranasally 1 day before the challenge and/or orally after the challenge for 5 days (up to 10 mg/kg/day). Nevertheless, complete protection against lethality was afforded by the treatment with the neuraminidase inhibitor peramivir given once daily for 5 days after the challenge (10 mg/kg/day). We speculate that the rapid progression of the disease in inbred mice caused by the intranasal challenge may render the MIMP-treatment ineffective. Our results emphasize the need for careful consideration of murine strains and routes of virus challenge in the design of experiments utilizing lethal influenza virus infection.

Keywords: Antiviral; Immunomodulator; Animal model; Neuraminidase inhibitor

Influenza virus is one of the major causes of morbidity and mortality from respiratory infections (Sullivan, 1996). The genetic variability of influenza viruses necessitates a continuous update of vaccine formulations and a search for new antivirals. In addition to amantadine and rimantadine that target the ion channel formed by the M2 protein of influenza A viruses, two inhibitors of the viral neuraminidase (NA) zanamivir and oseltamivir have been approved to manage influenza virus infections in humans (reviewed by Gubareva et al., 2000). However, emergence of influenza viruses resistant to the drugs of both classes has been reported (Belshe et al., 1988; Hayden, 1996), including influenza A viruses that exhibited dual resistance (Weinstock et al., 2003; Ison et al., 2006). Moreover, the recent introductions of avian influenza A viruses of novel antigenic subtypes (H5, H7 and H9) into humans (Webby and Webster, 2003; Li et al., 2004), including H5N1 viruses resistant to amantadine and rimantadine (Hayden et al., 2005), emphasize the need for new therapeutics with alternative modes of action. Immunomodulators that enhance the host's ability to combat various infections have been considered as such alternatives (Dickneite et al., 1991; Kobayashi et al., 1999; Utsunomiya et al., 1997). Among those, the synthetic purine methyl inosine 5′-monophosphate (MIMP) was shown to stimulate T-lymphocyte differentiation and function in vitro and in vivo (Hadden et al., 1995; Signorelli and Hadden, 2003; Sosa et al., 1993; U.S. Patent No. 5614504). Importantly, the pre-treatment of mice with MIMP was reported to improve the outcome of the lethal infections caused by intracellular bacteria (*Listeria monocytogenes* and *Salmonella typhimurium*) and viruses (Friend leukemia virus and influenza A virus) (reviewed by Signorelli and Hadden, 2003). Moreover, a single intranasal administration of MIMP to outbred mice 1 day before or 1 h after the challenge with aerosolized influenza A virus conferred protection against lethality (Masihi and Hadden, 2002). The use of inbred mice allows detailed assessment of the host immune response, and therefore in the present study, we investigated the anti-influenza efficacy of MIMP in inbred mice BALB/c

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doi:10.1016/j.antiviral.2006.02.006

(H-2^d) (Hilltop Lab Animals, Inc., Scottdale, PA), which are the most used murine strain in influenza virus research (Sidwell and Smee, 2000; Sidwell et al., 2001a; Webby et al., 2003).

The experiments were performed according to the protocol approved by the Animal Care and Use Committee (ACUC) and conducted at the Center for Comparative Medicine at the University of Virginia accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). In a murine model of influenza virus infection, animals are typically infected either by exposure to virus aerosol in a closed chamber or by direct instillation of virus into nares while animals are under light ether anesthesia (Hayden, 1986; Johansson and Kilbourne, 1991; Schulman and Kilbourne, 1963; Sidwell and Smee, 2000). Aerosol challenge may provide a viral exposure more representative of natural transmission in humans, however it is a time-consuming procedure and in experiments employing several experimental groups that must be kept separated, the challenge by aerosol is less convenient compared to the intranasal instillation. Moreover, it has been reported that intranasal instillation was more effective than infection by aerosol in inducing protection against a subsequent infection (Johansson and Kilbourne, 1991). Therefore, in our studies, we utilized the intranasal route for infection essentially as described (Gubareva et al., 1998) in contrast to the aerosol route employed by Masihi and Hadden (2002). The mouse-adapted influenza virus A/Puerto Rico/8/34 (H1N1) (A/PR/8) was used for the challenge (a kind gift of Dr. Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN). The virus stock was prepared in Madin-Darby canine kidney (MDCK) cells, aliquoted and stored at −70 °C. To accurately evaluate the optimal challenge dose, the stock virus (2.5×10^8 $TCID_{50}$/ml) was serially 10-fold diluted in the phosphate-buffered saline (PBS) and each virus preparation was instilled intranasally in eight animals (100 μl per animal). To determine the mouse ID_{50} (MID_{50}), four animals per group were sacrificed on day 3 post challenge, and the pulmonary viral titers were assessed in MDCK cells as described (Gubareva et al., 1998). The remaining animals were monitored until day 14 post infection in order to determine the mouse lethal dose 50 (MLD_{50}) (Hierholzer and Killington, 1996). The 1 MLD_{50} was equal to 100 MID_{50} or 500 $TCID_{50}$. In studies of antiviral agents, mice are typically infected with relatively low virus inocula to avoid obscuring potential drug effects (Hayden, 1986). Therefore, in all subsequent experiments, the virus challenge dose was 800 $TCID_{50}$, which produced approximately 85% lethality in the placebo-treated animals.

The synthetic compound MIMP (lot # 923–0015) was supplied by ImmunoRx/IRX Therapeutics, Inc. (Farmingdale, NY) in a form of a white powder. Based on the report by Masihi and Hadden (2002), MIMP intranasally delivered in 1% squalane-PBS emulsion (Allison, 1999) was anticipated to produce the best protective effect against influenza virus-induced lethality in mice. Therefore, the compound was prepared freshly either in sterile PBS or in 1% squalane-PBS emulsion and stored at 4 °C during 1 week. One day before the challenge, mice received MIMP at dose 10 mg/kg (200 μg/mouse) via the intranasal route while under light anesthesia (Table 1, group A). The other groups of mice (B–F) received MIMP diluted in 1% squalane-PBS emulsion via intranasal route. Two lower doses of MIMP were tested, namely 3 mg/kg/day (70 μg/mouse) (groups C and E) and 0.3 mg/kg/day (7 μg/mouse) (groups D and F). The animals in the groups E and F received the additional oral treatment with MIMP in PBS at dose 3 and 0.3 mg/kg/day twice daily for 5 days starting 1 h following the challenge. The control animals received 100 μl of 1% sqaulane-PBS emulsion intranasally 1 day before the challenge and 100 μl PBS orally twice daily for 5 days (group G). The survival in the MIMP-treated animals ranged from 0 to 13% and the mean day to death (MDD) ranged from 7.3 to 8.0 days (Table 1, groups A–F). The outcomes of the treatment at the highest dose of MIMP (groups A and B) were no different from the results of the lower dose groups (groups C–F) and were not improved compared to the control (group G) (Table 1). On average, 8% of the animals that received MIMP

Table 1
Effect of administration of MIMP and peramivir on the influenza A virus infection in inbred mice

Group	Mice age (weeks)	Treatment				Survived/total	Survival (%)	MDD[c]
		Compound	Dose (mg/kg/day)	Intranasal route, 1 day before challenge[a]	Oral route, 1 h after challenge, ×5 days[b]			
A	4–6	MIMP	10	+	None	1/8	13	7.3
B	4–6	MIMP	10	+	None	1/8	13	7.3
C	4–6	MIMP	3	+	None	0/8	0	8.0
D	4–6	MIMP	0.3	+	None	1/8	13	8.0
E	4–6	MIMP	3	+	b.i.d.	1/8	13	8.0
F	4–6	MIMP	0.3	+	b.i.d.	0/8	0	8.0
G	4–6	PBS	0	+	b.i.d	4/24	17	7.8
H	4–6	Peramivir	10	None	q.d.[d]	12/12[e]	100	>14
I	30–32	MIMP[a]	10	None	b.i.d.	8/15	53	7.0
J	30–32	MIMP	10	+	None	6/14	43	8.5
K	30–32	PBS	0	+	b.i.d.	5/10	50	8.4

[a] MIMP used for intranasal instillation was diluted either in PBS (group A) or in 1% squalane-PBS emulsion (groups B–F and I).
[b] MIMP or PBS was given twice daily (b.i.d.) for 5 days starting 1 h after the virus inoculation.
[c] MDD (mean day to death) for animals that died or were humanely euthanasia during 14 days after the virus inoculation.
[d] Peramivir was given once a day (q.d.) for 5 days starting 1 h after the virus inoculation.
[e] $p < 0.05$, protection in peramivir-treated group compared to PBS-treated animals.

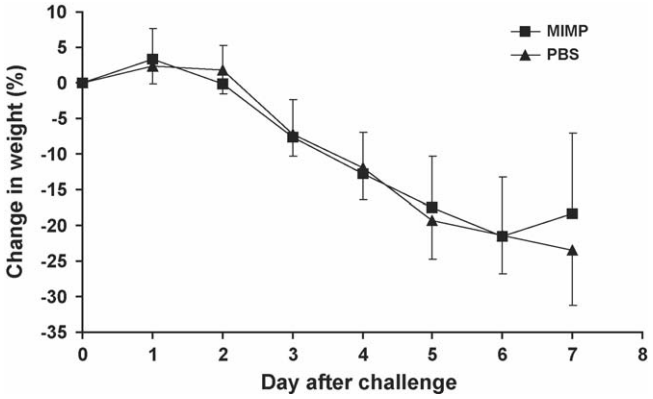

Fig. 1. Changes in the mean body weight of influenza virus-infected mice (4–6-week-old). MIMP diluted in either PBS or in 1% squalane-PBS emulsion was administered intranasally at dose 10 mg/kg (100 μl) 1 day before the virus inoculation (■); PBS was in administered intranasally as a control (▲).

survived the infection (4/48) versus 17% of animals survived in the control (4/24). The change in the body weight serves as a sensitive indicator of the progression of viral pneumonia in mice (Sidwell and Smee, 2000). Nevertheless, we did not detect any delay or amelioration of the weight loss in the MIMP-treated groups compared to the control (Fig. 1). As a positive control, an additional group of animals (Table 1, group H) was treated in parallel with the neuraminidase inhibitor peramivir (BCX-1812, RWJ-270201) kindly provided by BioCryst, Inc. (Babu et al., 2000). Peramivir diluted in PBS was administered orally at dose 10 mg/kg/day (200 μg/mouse in 100 μl of PBS) once daily for 5 days starting 1 h after the challenge. The peramivir treatment was accompanied by the complete protection of mice against lethality. Moreover, based on the decelerated loss of the body weight, the beneficial effect of the treatment was noticeable as early as on day 3 post challenge (Fig. 2). This outcome exceeded the results of the previous studies (Sidwell et al., 2001b) where peramivir was only moderately effective (25% increase in the survival rate) in protecting young BALB/c mice infected with the

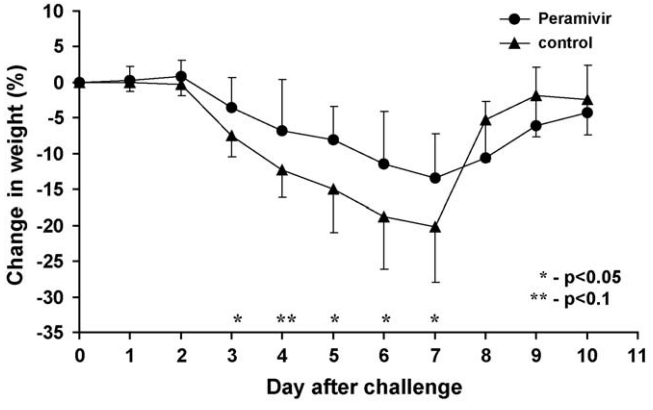

Fig. 2. Changes in the mean body weight of influenza virus-infected mice (4–6-week-old). Either NA inhibitor peramivir at dose 10 mg/kg/day (●) or PBS (▲) were administered orally for 5 days starting 1 h after the virus challenge. Body weights were measured daily and percent of the body weight change was calculated for each animal in the group. Bars indicate standard deviations. $^{*}p < 0.05$ and $^{**}p < 0.1$ Statistical significance compared with the infected PBS-treated mice.

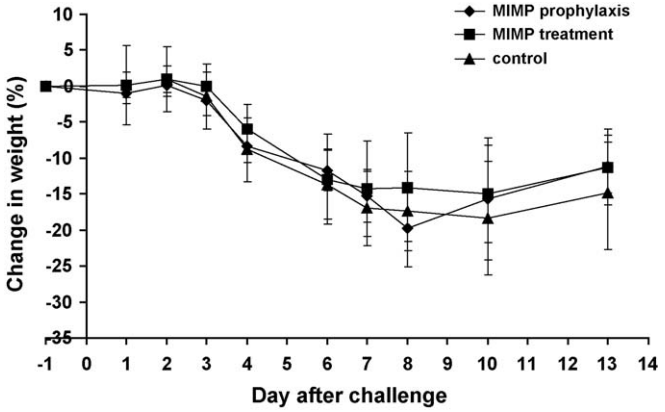

Fig. 3. Changes in the mean body weight of influenza virus-infected mice (30–32-week-old). MIMP diluted in 1% squalane-PBS emulsion was administered intranasally at dose 10 mg/kg 1 day before the virus inoculation (♦); MIMP diluted in PBS at dose 10 mg/kg was administered orally for 5 days starting 1 h after the virus inoculation (■); 1% squalane-PBS emulsion was administered intranasally 1 day before the virus inoculation followed by oral administration of PBS (▲).

mouse-adapted influenza A/PR/8 virus. We attribute the difference in the outcomes of the treatment to the lower challenge dose used in the present study (800 $TCID_{50}$ versus 2000 $TCID_{50}$) and associated difference in the mortality in the placebo-treated animals (83% versus 95%). This highlights how small differences in an animal protocol can have a significant impact on the outcome (Grunert et al., 1965; Sidwell et al., 2001c). In view of the antiviral effect observed with the peramivir treatment, the inability to detect the beneficial effect of MIMP in inbred mice required a further investigation. It is known that influenza virus-induced morbidity and mortality are age-dependent and are decreased in more mature mice (Hayden, 1986). Indeed, the same virus dose that killed ~83% of the younger animals in the present study, caused only 40% mortality in the older mice. Therefore, we next investigated whether the treatment with MIMP of 30–32 weeks-old (26–29 g) female BALB/c mice would produce a more favorable outcome. The mice (group I) received MIMP orally at dose 10 mg/kg/day diluted in PBS twice daily for 5 days starting 1 h after infection. The other group (group J) received 10 mg/kg/day of MIMP diluted in 1% squalane-PBS emulsion via the intranasal route 1 day before the challenge, whereas the control mice (group K) received 1% squalane-PBS emulsion intranasally before the challenge and then orally PBS for 5 days after the challenge. However, the use of the older mice had no apparent effect on the ability of MIMP to improve survival (Table 1). The estimated MDD for the older placebo-treated mice was equal 8.4 days, which is not much different from the value reported for the young outbred mice (8.7 days) (Masihi and Hadden, 2002). Similarly, there was no effect of the MIMP-treatment on the body weight change (Fig. 3). Our results indicate that, despite the decreased mortality, there was no improvement in the outcomes as a result of the MIMP treatment of the older mice.

The lack of the beneficial effect of the treatment with MIMP in our study requires analysis of the factors that might contribute to the discrepancy with the previous report (Masihi and Hadden,

2002). The compound MIMP was provided by the same source, Immuno-Rx/IRX Therapeutics, Inc., Farmingdale, NY. The difference in the animal genetic make-up might contribute to the treatment failure, however MIMP immunomodulatory activity and protective effects against bacterial infections (i.e., *L. monocytogenes* and *S. typhimurium*) were previously demonstrated in BALB/c mice (Signorelli and Hadden, 2003). The protective effect could be reduced or even diminished if the challenge was done with a strain of higher virulence. The mouse-adapted influenza A/PR/8 virus was used in both studies, although it was acquired from different sources (Masihi and Hadden, 2002). In our study, the virus challenge was performed while mice being under light ether anesthesia. Because ether can cause irritation of the trachea and bronchi of the animals and is highly flammable, many investigators prefer to use ketamine administered intramuscularly. While, it was reported that ether anesthesia per se does not enhance the severity of influenza in mice (Knight et al., 1983), intranasal administration of sterile fluid to anesthetized animals has been shown to enhance the severity of influenza infection (Gubareva et al., 1998; Johansson and Kilbourne, 1991). Noteworthy, the volumes of the virus inocula used for the intranasal challenge have varied substantially, ranging from 2 to 100 μl per mouse, in different reports (Hori et al., 2001; Johansson and Kilbourne, 1991; Sidwell et al., 2001b; Utsunomiya et al., 1997). Intranasal infection via instillation seems to be more penetrating and its consequences more severe compare to infection caused by aerosolized virus. Yet, the survival of the placebo-treated animals ranged from 4 to 15% after the aerosol challenge (Masihi and Hadden, 2002) and from 13 to 50% after the intranasal challenges (Table 1). Therefore, based on these criteria, the severity of the infection in inbred model was at most only marginally greater than that observed previously in outbred mice. Nevertheless, we found that the oral NA inhibitor was highly protective under our test conditions. It is well recognized that reproducibility of experimental data is enhanced by the use of inbred, specific pathogen-free animals of uniform age, weight and sex (Hayden, 1986). Indeed, outbred NMRI mice challenged by the aerosol route died over a broad period of time, namely from days 5 to 14 (Masihi and Hadden, 2002). In our experiments, the deaths of the inbred mice were recorded within a narrower timeframe, with a majority of the animals dead or humanely euthanized on days 7 and 8 after the virus inoculation. Of note, according to the institutional ACUC requirements, influenza virus-infected mice that exhibited apparent difficulties of breathing and/or lost ≥25% of the initial body weight, should be humanely euthanized; however individual BALB/c mice could recover after ≥25% weight loss caused by influenza infection (Govorkova et al., 2001). The uniformly early death (or euthanasia) of inbred mice caused by the intranasal challenge may diminish the beneficial effect of MIMP and, possibly, other immunomodulators. When young BALB/c mice were challenged with influenza virus by aerosol route, they seem to survive longer (10.5 days) (Utsunomiya et al., 1997) compare to those challenged intranasally in the present study (7.8 days). Therefore, the use of the aerosol route of infection may provide a better approach to assessment of antiviral and therapeutic properties of substances that mediate host responses.

Another concern is the reported variability of the experimental data due to the use of different strains of mice (Sedegah et al., 2004; Toapanta and Ross, 2004; Nahrevanian et al., 2005; Lyons et al., 2005; Gambaryan et al., 2005), which highlights apparent effects of genetic background on host immune response and protection against infection. Given the wide use of murine models to study influenza viral pathogenesis, immunity and antiviral chemotherapy, the results of this study illustrate the uncertainties and potential confounders in making decisions and avoiding pitfalls.

Acknowledgements

This work was supported in part by Immuno-Rx/IRX Therapeutics, Inc. and by a Public Heath Service research grant (AI45782) from the National Institute of Allergy and Infectious Disease (L.V.G.).

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