Antiviral Research, 5 (1985) 207–215
Elsevier

AVR 00166

Enhancement of non-specific resistance to viral infection by muramyldipeptide and its analogs

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(Received 17 July 1984; accepted 22 October 1984)

Summary

Antiviral activity of muramyldipeptide (MDP) and its lipophilic derivatives, B30-MDP and MDP-Lys(L18), was investigated in mice infected with vaccinia virus (VV) and herpes simplex virus type 2 (HSV-2). Mice administered these compounds subcutaneously or orally were protected against VV in tail lesion tests and against HSV-2 in skin lesion tests, respectively. Since in vitro antiviral activity was not demonstrated with these compounds in cultured mammalian cells infected with either VV or HSV-2, host-mediated defense mechanisms may play a role in the activity of the compounds. As for serum interferon (IFN) induction, MDP and its analogs showed no activity in mice, suggesting that IFN does not participate in the antiviral mechanisms against VV and HSV-2.

An extrinsic antiviral activity was demonstrated when peritoneal macrophages from the mice administered these compounds were cocultivated with VV-infected 3T3 cells. The results indicate that macrophage activation by MDP and its analogs plays a role in the defense mechanisms against viral infection. This activity was not virus-specific.

We also demonstrate that the introduction of lipophilic residue(s) into MDP enhances the antiviral activity of mice against VV and HSV-2.

muramyldipeptide; vaccinia virus; herpes simplex virus; macrophages; non-specific resistance

Introduction

Muramyldipeptide (MDP), an active component in Freund's complete adjuvant [7], is known to be a minimal structural unit of peptidoglycan of bacterial cell walls.

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Research has been conducted by many investigators to synthesize MDP derivatives with varying lipophilic groups in the structure, and the biological properties tested for immune responses and antitumor activity. As biological response modifiers (BRM), MDP and its derivatives showed not only adjuvant activity for delayed-type hypersensitivity (DTH) and humoral antibody formation [1,4], but also induction of non-specific tumor immunity [2], enhancement of host resistance against bacterial infection [5], augmentation of cell-mediated cytotoxicity [10], and mitogenic activity [18]. However, undesirable biological properties such as pyrogenicity [6] and arthritogenicity [13] have been reported by other investigators. Concerning antiviral activity of MDP, few reports are available; Brehmer et al. [3] demonstrated that MDP does not protect mice against influenza virus.

We carried out experiments to examine whether MDP and its lipophilic derivatives, B30-MDP and MDP-Lys(L18), protect mice against viral infection caused by vaccinia virus (VV) and herpes simplex virus type 2 (HSV-2). This paper describes the antiviral activity and the mechanisms of the above compounds in mice.

Materials and Methods

Test compounds

MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine), B30-MDP [6-*O*-(2-tetradecylhexadecanoyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine], and MDP-Lys(L18) [N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyl-L-lysine] were provided from The Research Institute of Daiichi Seiyaku Co., Ltd., Tokyo, Japan. MDP and MDP-Lys(L18) were dissolved in a sterile phosphate-buffered saline (PBS), and B30-MDP suspended in sterile PBS for experimental use.

Cells and viruses

Vaccinia virus (Lister strain) and herpes simplex virus type 2 (HSV-2) (UW strain) were grown in BHK-21 and HEp-2 cells, respectively. Virus titers of the preparations were $10^{6.5}$ for vaccinia virus (VV) and $10^{7.5}$ $TCID_{50}$/ml for HSV-2. Influenza virus ($A/PR/8/34/H_0N_1$) was prepared in allantoic fluid of infected fertile hens' eggs. The titer of influenza virus (InfV) preparations was $10^{7.5}$ $TCID_{50}$/ml when assayed in SK-K cells, a swine kidney cell line sensitive to InfV [19]. Vesicular stomatitis virus (New Jersey strain) was propagated in L-929 cells. The virus preparations were stored in a deep freezer at -80°C until use. BALB/3T3 clone A31 cells were used for the assay of extrinsic antiviral activity of peritoneal macrophages against VV. The cells were cultivated in Eagle's minimum essential medium (EMEM) supplemented with 3 mM glutamine, 0.07% bicarbonate and either 10% fetal calf serum or 5% calf serum, depending on the cell-lines used.

Animals

Female ddY, BALB/c and CDF_1 (BALB/c × DBA/2) mice, 4–8 weeks of age, were obtained from Shizuoka Cooperative for Experimental Animals (Hamamatsu, Japan). Female C57BL/6 hairless mice were bred and provided by The Research Institute of Daiichi Seiyaku Co., Ltd., Tokyo, Japan.

In vitro antiviral activity and cell toxicity tests of the compounds

The tests were carried out as follows. The cells (either MRC-5, a human diploid cell-line of embryonic lung cells, or SK-K, a swine kidney cell-line) were seeded into a 24-well microplate (TERUMO Co., Ltd., Japan) and cultivated at 37°C in a CO_2 incubator. For antiviral tests, $10^{1.7} \times TCID_{50}$ of test viruses were inoculated into monolayer cells, and then serial one-fourth diluents of the compounds were added to cell cultures. After cultivation for 3 days at 37°C, cytopathic effects (CPE) were observed under the reversed microscope. MIC_{50} of test compounds was then calculated according to the formula of Kärber [12] by a method of Fiala et al. [8]. Direct cell toxicity of the compounds was evaluated simultaneously by cultivating cells in maintenance medium containing various concentrations of the compounds. MTC_{50} (minimum toxic concentration for 50% CPE) was then calculated in the same way as described above. The maintenance medium for cell cultivation consisted of EMEM supplemented with 1% calf serum, 3 mM glutamine and 0.07% bicarbonate.

Protection tests against viral infection in mice

For in vivo antiviral activity against VV, 7–10 BALB/c mice in a group were inoculated intravenously with 0.1 ml of $10^{6.0}$ $TCID_{50}$/ml of VV preparations at a site of about 3 cm from the tail base. Test compounds diluted to various concentrations were administered subcutaneously to mice at various time intervals as indicated in the Tables. Experiments by oral administration were also performed 1 day before virus infection. The number of lesions on the tail were counted at the 7th day of infection after staining with 1% fluorescein–0.5% methylene blue solution [11].

Antiviral activity against herpes simplex virus was assayed by skin lesion test. Test compounds were applied to the mice subcutaneously at a concentration of 100 μg/mouse. After administration of the test compounds, C57BL/6 hairless mice were inoculated with HSV-2 at an area of scarified skin as follows. An area 2.5×2.5 cm^2 on the back skin was scarified 5 times with a bound-needle for smallpox vaccination and then rubbed with a cotton swab saturated with 0.05 ml of 10^6 $TCID_{50}$/ml HSV-2 inoculum [11]. Protective effects were evaluated by observing local skin reactions such as vesicle formation, thickening and ulceration, and systemic reactions such as paralysis and mortality.

Interferon-inducing ability of the compounds in mice

Each experimental group consisted of three female BALB/c mice, 8 weeks of age. Test compounds were administered at a dose of 100 μg/mouse. Mice were bled for sera at various time intervals after administration of the test compounds, and serum interferon (IFN) titers were measured in L-929 cells infected with vesicular stomatitis virus (VSV) [17]. IFN titers were expressed as the reciprocal of the highest serum dilution which reduced VSV-specific CPE on L-929 cells by 50%.

Extrinsic antiviral activity of macrophages

Macrophages were prepared from peritoneal exudate cells (PEC) of female CDF_1 mice which were irritated with 100 μg of the compounds via the intraperitoneal route. PEC were harvested 1 day after inoculation, and cell numbers were adjusted to between 4×10^4 and 4×10^5 per suspension. BALB/3T3 cells were grown in a 24-well microplate with EMEM containing 8% fetal calf serum, infected with an appropriate amount of VV that produced between 30 and 60 plaques per well. The PEC suspension was added to VV-infected 3T3 cells in the wells and cultivated for 2 h at 37°C in a CO_2 incubator. The cell layers were then washed twice with Hank's–PBS to remove non-adherent cells and overlayed with EMEM containing 1% calf serum and 1% Noble agar. The extrinsic antiviral activity was determined after 3 days' cultivation in a CO_2 incubator by counting plaques stained with 0.03% neutral red solution. The rates of plaque reduction were calculated compared to the plaque numbers of VV-infected 3T3 cells without PEC suspension.

Statistics

The statistical significance of differences in average lesion numbers was revaluated using the Mann–Whitney U-test, because there was a considerable variation in distribution of lesion counts of the individual mouse.

Results

In vitro antiviral activity of MDP and its analogs

A preliminary experiment was carried out to determine the minimum toxic concentration of the compounds on MRC-5 and SK-K cells. The former cells were used as hosts for VV and HSV-2, and the latter as hosts for InfV in subsequent experiments. The toxic dose of MDP against two cell lines was more than 2000 μg/ml, while the toxic doses of the MDP analogs were between 1/15 and 1/60 that of MDP. As a result, MDP had no antiviral activity against VV, HSV-2 and InfV, while the other two compounds, B30-MDP and MDP-Lys(L18), appeared to be active at concentrations near the toxic ranges (data not shown). These results indicate that none of the compounds possesses in vitro antiviral activity.

In vivo antivaccinial activity in mice

When BALB/c mice were inoculated with $10^{5.0}$ $TCID_{50}$ of VV intravenously via the tail vein, discrete dermal vesicles appeared on the tail at day 5, reaching maximum size at day 7, and then began to heal after day 8 of infection. The dermal lesions had completely healed between 10 and 15 days after inoculation. Subcutaneous administration of MDP and its analogs to infected mice prevented tail lesion formation as shown in Table 1. A dose–effect relationship was apparent with MDP between 20 and 500 μg/mouse, but not apparent with the other compounds. No antiviral activity was seen at a dose lower than 1 μg/mouse. Antiviral activity was also demonstrated in VV-infected mice by oral administration of these compounds 1 day before infection (Table 2). Less protective effects were seen if administration of the compounds was delayed until 3 days post-infection.

TABLE 1

Effect of different doses of MDP and its analogs on tail lesion formation by vaccinia virus in BALB/c mice

Doses[a] (μg/mouse)	Numbers of lesions, mean ± S.D.[b] (% inhibition)		
	MDP	B30-MDP	MDP-Lys(L18)
20	21.4 ± 1.3 (29)	13.4 ± 4.9 (50)	17.9 ± 5.6 (47)
100	17.6 ± 5.9 (42)	14.1 ± 9.3 (47)	17.4 ± 4.3 (49)
500	14.8 ± 3.1 (51)	12.0 ± 9.5 (55)	17.2 ± 1.7 (50)
Virus control	30.1 ± 4.1	26.6 ± 5.8	34.0 ± 6.1

[a] The compounds were administered subcutaneously to BALB/c mice 1 day before VV infection via the tail vein.
[b] $P<0.05$, Mann–Whitney U test.

TABLE 2

Effect of different doses of MDP and its analogs administered orally to BALB/c mice infected with vaccinia virus

Compounds[a]	Doses (mg/mouse)	Numbers of lesions[b] (mean ± S.D.)	Inhibition (%)
MDP	1.0	14.1 ± 6.9	38
	3.0	13.4 ± 5.8	41
	6.0	15.0 ± 5.1	34
B30-MDP	1.0	12.0 ± 4.4	47
	3.0	7.8 ± 3.4	66
	6.0	8.3 ± 4.5	64
MDP-Lys(L18)	0.5	9.3 ± 3.7	59
	1.0	9.8 ± 3.0	57
Virus control	-	22.8 ± 6.5	-

[a] The compounds were administered orally to mice 1 day before VV infection via the tail vein.
[b] $P<0.05$, Mann-Whitney U test.

In vivo antiherpetic activity in mice

C57BL/6 hairless mice were infected intradermally with 5×10^4 $TCID_{50}$ of HSV-2 on scarified back skin. The appearance of the dermal lesions began first with thickening of inoculation sites on the 4th day, changed to localized ulceration on the 5th day, and the hind limbs of the infected mice were paralyzed on the 6th day after infection. Vesicles developed and, as shown in Fig. 1, reached their maximum number on the 3rd day, before they gradually healed. Upon administration of B30-MDP to the infected mice, however, a significant reduction in vesicle numbers was observed at the concentration of 100 μg/mouse (Fig. 1). A lesser effect than that of B30-MDP was observed upon administration of either MDP or MDP-Lys(L18). The compounds were not protective against the systemic symptoms of HSV-2 infection. No anti-influenza

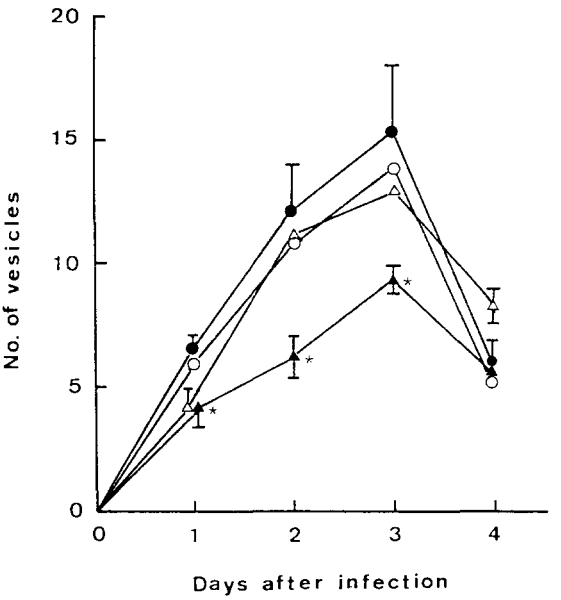

Fig. 1. Effects of MDP and its analogs on vesicle formation in C57BL/6 hairless mice infected with herpes simplex virus type 2 (HSV-2). Mice were injected subcutaneously with 100 μg of the compound, and then inoculated intradermally with 5×10^4 $TCID_{50}$ of HSV-2. ●, Virus control; ○, MDP; △, MDP-Lys(L18); ▲, B30-MDP. *$P<0.05$, Mann–Whitney U test.

activity was demonstrated in mice infected with mouse-adapted InfV (PR 8 strain), according to observations of both mortality and pulmonary consolidation (data not shown).

Effects of the compounds on serum interferon production in mice

The possibility that MDP and its analogs might be able to induce serum IFN in mice was also investigated. Mice administered these compounds subcutaneously were bled after 6, 12, 24 and 48 h. Serum IFN was not detected in the mice, indicating that IFN was not involved in the defense mechanism of antiviral activity by these compounds.

Enhancement of extrinsic antiviral activity of macrophages activated by the compounds

Next, we investigated the role of peritoneal exudate cells (PEC) of CDF_1 mice in the mechanism of antiviral activity of MDP and its analogs. The compounds were administered intraperitoneally to mice, and PEC harvested after 24 h. As shown in Fig. 2, the adherent PEC (as effector cells), actually macrophages, added to VV-infected 3T3 cells (as target cells) prevented virus replication in the cells as compared with resident macrophages. These results explain enhanced extrinsic antiviral activity of macrophages when the mice had been treated with 100 μg of either MDP or B30-MDP. But lesser activity was seen with MDP-Lys(L18) than with the two other compounds. Since they were active against VSV and HSV-2 as well as VV, enhancement of the extrinsic antiviral activity of macrophages by MDP and its analogs was

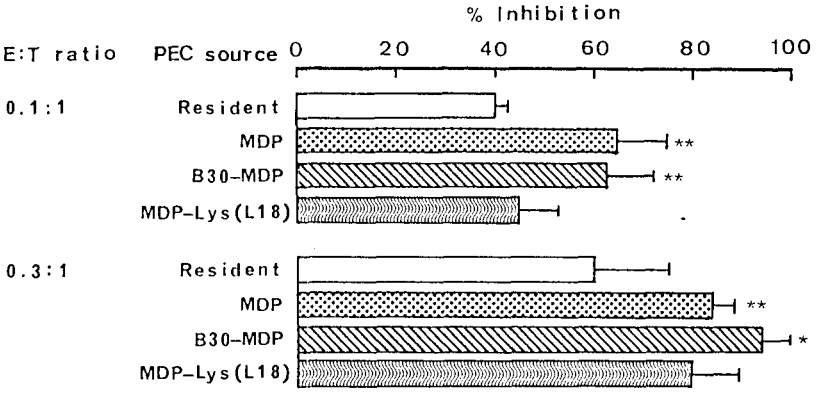

Fig. 2. Enhancement of extrinsic antiviral activity of peritoneal exduate cells (PEC) stimulated by MDP and its analogs. Effector cells were the PEC recovered from CDF_1 mice that received an intraperitoneal injection of 100 μg of the test compound; target cells were vaccinia virus (VV)-infected 3T3 cells. After adsorption of VV on the 3T3 cell monolayers, the cells were washed, and the effector cells added. The cells were then overlaid with 1% agar-containing maintenance medium. $^*P<0.01$, $^{**}P<0.02$, Student's *t*-test.

considered to be nonviral-specific. As a result, it was concluded that macrophage activation may play an important role in the antiviral activity of MDP and its analogs in mice.

Discussion

Our results show that MDP and its two analogs, B30-MDP and MDP-Lys(L18), enhanced non-specific resistance of mice against viral infection. As for in vitro antiviral activity, however, MDP was not effective even at very high concentrations. The two other compounds appeared to be active against the test viruses, but only at concentrations very close to the cytotoxic concentrations.

The in vivo antiviral activity of MDP and its analogs against VV and HSV-2 was prophylactic rather than therapeutic. Therefore, it is believed that the antiviral activity may be revealed through a host-mediated defense mechanism such as immunopotentiation or macrophage activation. As for anti-influenza activity, Brehmer et al. [3] demonstrated that MDP did not prevent InfV infection in mice. We confirmed their results with MDP and its analogs.

Chedid et al. [5] and Matsumoto et al. [15] reported that MDP enhanced non-specific immunity in mice infected with *Klebsiella pneumonia* or *Escherichia coli*. On the other hand, Humphres et al. [9] and Krahenbuhl et al. [14] demonstrated that MDP did not afford any resistance to infection with *Listeria monocytogenesis* or *Toxoplasma gondii* in mice. Thus, the protective effect of MDP against bacterial infection differs from one bacterial species to another. These results can be explained by the fact that protection by MDP against bacterial infection is affected by the mechanisms of bacterial growth, e.g. intracellular or extracellular. Likewise, the antiviral activity of

MDP and its analogs may be influenced by the different factors involved in pathogenesis of the viral disease. The microbicidal activity of MDP-activated macrophages was strong against extracellular but weak against intracellular bacteria. The MDP-activated antiviral activity was strong against localized dermal lesions caused by VV or HSV-2, but weak against the systemic symptoms of the HSV-2 infection such as herpes encephalitis.

According to recent information, it is recognized that macrophages are important effector cells in host-defense mechanisms. Morahan et al. [16] revealed that peritoneal macrophages of mice exhibited extrinsic antiviral activity against HSV in infected cells when the mice had been exposed to HSV. In this paper, we have shown that the extrinsic antiviral activity of mouse macrophages is markedly activated after treatment with MDP or its analogs. Moreover, the augmented antiviral resistance was non-specific since replication of both VV and HSV-2 was inhibited in cultured cells by cocultivation with MDP-activated macrophages. We conclude that macrophage-mediated extrinsic antiviral activity plays an important role in the prevention of VV infection in mice by MDP and its analogs.

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