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Enhancement by muramyldipeptides of the activities of early-type inducers of interferon

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Summary

A synthetic muramyldipeptide (MDP) and two analogues, B30-MDP and MDP-Lys(L18), augmented serum interferon (IFN) production in mice by the inducers lipopolysaccharide (LPS) and polyinosinic acid : polycytidylic acid (poly I : C), and also augmented immune IFN production induced by purified protein derivative (PPD) in mycobacteria-sensitized mice. These compounds were most effective when administered to mice one day before the interferon inducer. By contrast, IFN production in mice by either oral tilorone or virus infection was not enhanced with these compounds. Since LPS and poly I : C are well known as early-type IFN inducers, and tilorone and virus infection are late-type inducers, we presume that MDP and its analogues are able to augment only early-type IFN production. This enhancing effect may be mediated by macrophage activation.

In vivo antiviral activity of MDP and its analogues was further tested in mice infected with vaccinia virus (VV) using early-type inducers. When mice previously treated with MDP or its analogues were stimulated for IFN production with a low dose of LPS, protective activity against VV infection was markedly enhanced.

muramyldipeptide; interferon; antiviral activity; augmentation

Introduction

Since Freund [13] discovered the adjuvant activity of mycobacteria for immune

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responses, muramyldipeptide (MDP), a minimum structural unit of mycobacterial cell-wall materials, has been extensively investigated and found to possess activities for both humoral antibody formation and delayed-type hypersensitivity [1,4]. In addition, efforts have been made to establish the relationships between the structures of MDP analogues and their activities with regard to antitumor activity and resistance to bacterial infection [16,20]. In a previous paper [14], we demonstrated evidence that MDP and two lipophilic derivatives, B30-MDP and MDP-Lys(L18), possess protecting activity against virus infection in mice due to macrophage activation. However, these compounds by themselves showed no IFN-inducing activity in mice [14]. De Clercq et al. [9] reported that IFN responses of mice to bacterial endotoxins or poly I:C were enhanced by the use of Freund's complete adjuvant. Since MDP is a minimum active component of mycobacterial cell-wall materials, we investigated whether MDP and its analogues could enhance IFN production by known inducers, hence increasing protection against virus infection in mice.

In the present paper, we report that serum IFN production was significantly augmented by MDP and its analogues, when administered one day before the interferon inducer. Similarly, immune IFN production in mycobacteria-sensitized mice was also enhanced by pre-treatment with these compounds one day before PPD administration. The mechanism of the enhancing effects of MDP and its derivatives on IFN induction will be discussed in connection with activation of the immune system. Cooperation of these compounds with IFN inducers in antiviral protection was also demonstrated in mice infected with vaccinia virus.

Materials and Methods

Animals

Female BALB/c mice, aged 8–10 weeks, and ddY mice, aged 4–5 or 8–10 weeks, were obtained from Shizuoka Cooperative for Experimental Animals (Hamamatsu, Japan). These mice were kept for 1 week in a specific pathogen-free environment before use.

Test compounds

N-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP), 6-*O*-(2-tetradecyl-hexadecanoyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (B30-MDP), and N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-N^{ε}-stearoyl-L-lysine (MDP-Lys(L18)) were supplied by the Research Institute of Daiichi Seiyaku Co. Ltd., Tokyo, Japan. MDP and MDP-Lys(L18) are soluble in a sterile phosphate-buffered saline (PBS), but B30-MDP is not. The latter was thus used as a suspension in sterile PBS. Poly I:C was purchased from Yamasa Shoyu Co. Ltd., Japan, LPS from Difco Lab., U.S.A., BCG and PPD from Nihon BCG Co. Ltd., Japan. These compounds were dissolved in sterile PBS before use. Carrageenan was purchased from Sigma Chemical Co., U.S.A.

Cells and viruses

The cells were cultured in Eagle's minimal essential medium (EMEM) supplement-

ed with 3 mM glutamine, 0.07% bicarbonate, and either 5% fetal calf serum (FCS) for HEL cells or 10% FCS for L-929 cells. Vaccinia virus (Lister strain) and vesicular stomatitis virus (New Jersey strain) were propagated in HEL cells and L-929 cells, respectively.

Assay for interferon

The assay for mouse IFN was carried out in L-929 cells infected with VSV [19]. IFN titers are expressed as reciprocals of the highest dilution of test samples that reduced virus cytopathic effect (CPE) by 50%. Plastic microplates with 96 wells were used for in vitro assay of IFN. Four wells were used for each test sample diluted in 3-fold series. All experiments were carried out in duplo.

The assay for investigating the enhancing activity on IFN production was conducted as follows. Mice were given MDP or its analogues one day before the interferon inducer and then stimulated by poly I:C, LPS, tilorone or influenza virus. For experiments on immune IFN production, mice were sensitized with 1 mg of mycobacteria 2 weeks before intravenous injection of 50 μg PPD per mouse. Mice were treated with 100 μg MDP or its analogues one day before injection of PPD. Mouse sera were collected at 2, 6, and 12 h, and IFN titers assayed according to the method described above.

Protection test against viral infection

Cooperative effects of MDP and IFN-inducers were also determined in VV-infected mice by the tail-lesion test [6]. Briefly, ddY mice of 4–5 weeks were treated subcutaneously with 100 μg MDP one day before i.v. LPS injection. After injection, mice were inoculated intravenously with $10^{4.8}$ $TCID_{50}$ of VV. The number of lesions appearing on the tail were counted on the 7th day post-infection after staining with 1% fluorescein/0.5% methylene blue solution [3].

Statistical analysis of results

The average numbers of VV tail lesions were evaluated statistically by the Mann–Whitney U-test.

Results

Inability of MDP and its analogues to induce IFN in mice

To investigate whether MDP and its analogues possess IFN-inducing activity, each compound was administered subcutaneously to BALB/c mice at a dose of 100 μg per mouse, and sera were collected at various time intervals. IFN titers of the collected sera were determined in L-929 cells infected with VSV. None of these compounds was found to induce IFN in mice (data not shown), even when mice were previously sensitized with MDP and/or its analogues. Experiments carried out with cultured spleen cells demonstrated that these compounds were not able to induce IFN in an in vitro system.

Augmenting effects of MDP and its analogues on IFN production using LPS or poly I:C as the inducer

When mice were treated with MDP or its analogues one day before injection of the inducer (either LPS or poly I:C), IFN titers of sera collected at 2 h were enhanced as compared to the IFN titers obtained in mice injected with the inducers only. The results in Table 1 show that serum IFN titers reached their peak at 2 h and then decreased in mice administered either 25 or 100 μg LPS. When MDP or its analogues were administered to mice prior to the interferon inducer, serum IFN titers were increased 3-fold or more as compared to mice which had not been pretreated with MDP or its analogues. The same was true for mice treated with these compounds one day before receiving poly I:C as inducer at a dose of either 1 or 10 μg per mouse (Table 2).

Augmenting effects of MDP and its analogues on immune IFN production in mice

To investigate whether MDP or its analogues could also enhance immune IFN production, mice were first sensitized by intravenous inoculation with live mycobacteria, and treated with MDP or its analogues one day before PPD stimulation for IFN production. As shown in Table 3, immune IFN production was enhanced from 94–281 to 842 U/0.1 ml, as compared to untreated mice at 2 and 6 h after PPD injection. MDP could not substitute for the live mycobacteria in sensitization or PPD in stimulation of the mice, although it is a structural component of the mycobacterial cell-wall skeleton.

Non-enhancement by MDP and its analogues of IFN induction with oral tilorone and virus infection

We investigated the effect of MDP and its analogues on tilorone-induced IFN production in mice. De Clercq and Merigan [8] reported that lymph nodes, thymus and serum IFN titers in mice reached maximum at 16 h after oral tilorone administration. Serum IFN titers in response to oral tilorone were not augmented when mice

TABLE 1

Enhancement of LPS-induced IFN production by pretreatment with MDP or its analogues

Treatment		Serum IFN titer (U/0.1 ml)	
Day −1[a]	Day 0[b] (μg/mouse)	2 h	6 h
-	LPS 100	94	<6
-	LPS 25	54	<6
MDP	LPS 100	281	<6
MDP	LPS 25	162	<6
MDP-Lys(L18)	LPS 100	841	<6
MDP-Lys(L18)	LPS 25	281	<6
B30-MDP	LPS 100	281	<6
B30-MDP	LPS 25	281	<6

[a] BALB/c mice were injected s.c. with 100 μg MDP or its analogues one day prior to injection of the inducer.

[b] After pretreatment with MDP or its analogues, mice were injected i.v. with the indicated LPS doses.

TABLE 2

Enhancement of poly I : C-induced IFN production in mice by pretreatment with MDP or its analogues

Treatment		Serum IFN titer (U/0.1 ml)	
Day -1[a]	Day 0[b] (μg/mouse)	2 h	6 h
-	Poly I : C 10	2525	281
-	Poly I : C 1	94	< 6
MDP	Poly I : C 10	7575	281
MDP	Poly I : C 1	281	< 6
MDP-Lys(L18)	Poly I : C 10	7575	281
MDP-Lys(L18)	Poly I : C 1	281	< 6
B30-MDP	Poly I : C 10	7575	281
B30-MDP	Poly I : C 1	281	< 6

[a] BALB/c mice were injected s.c. with 100 μg MDP or its analogues one day prior to injection of the inducer.

[b] After pretreatment with MDP or its analogues, mice were injected i.v. with the indicated doses of poly I : C.

TABLE 3

Enhancement of immune interferon production in mycobacteria-sensitized mice by pretreatment with MDP or its analogues

Sensitization[a]	Treatment[b]		Serum IFN titer (U/0.1 ml)		
Day -14	Day -1	Day 0	2 h	6 h	12 h
Mycobacteria	-	PPD	94	281	< 6
Mycobacteria	MDP	PPD	842	842	< 6
Mycobacteria	MDP-Lys(L18)	PPD	842	842	< 6
Mycobacteria	B30-MDP	PPD	842	842	< 6
Mycobacteria	-	MDP	< 6	< 6	< 6
MDP	-	PPD	< 6	< 6	< 6
-	MDP	PPD	< 6	< 6	< 6

[a] Female ddY mice were sensitized i.v. with 1 mg mycobacteria.

[b] The mycobacteria-sensitized mice received 100 μg MDP or its analogues i.v. one day before PPD as the inducer.

were pretreated with MDP or its analogues (data not shown). Similar results were found regarding the late-type serum IFN titers following influenza and vaccinia virus infection. We therefore conclude that MDP and its analogues could not enhance late-type IFN production in mice.

Characteristics of the enhancement of LPS-induced IFN production by MDP

To study the mechanism of the enhancement of LPS-induced IFN production by MDP, we determined the optimum time for pretreatment by MDP. As shown in Fig. 1,

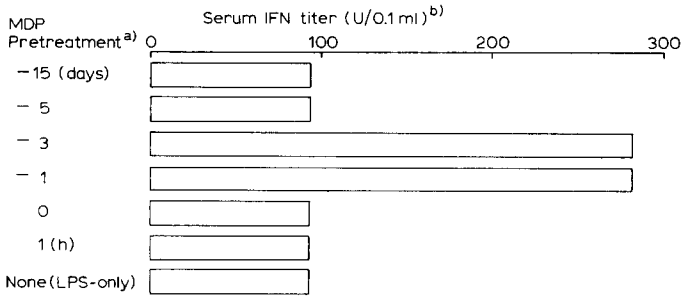

Fig. 1. Efficiency of MDP pretreatment in enhancing interferon production by LPS. (a) After pretreatment with 100 μg MDP, mice were i.v. injected with the inducer LPS (100 μg). (b) Serum specimens for IFN assay were collected 2 h later, pooled and assayed for IFN.

augmentation of IFN production by MDP occurred only if MDP was administered between 1 and 3 days before LPS. If memory cells of the immune systems would take part in this process, the enhancement might occur if MDP was given between 5 and 15 days before induction. From our results it can be concluded that the reticuloendothelial system, especially macrophages, plays an important role in this enhancement process. Therefore, experiments were carried out to investigate whether carrageenan, a specific macrophage inhibitor, prevented the enhancing activity of MDP on IFN production in mice. When mice were given 200 mg/kg carrageenan via the intraperitoneal route one day before MDP administration, LPS-induced IFN production was not enhanced, as can be seen from Table 4 which indicates that macrophages may indeed contribute to the enhanced IFN production in response to LPS.

Enhancement of in vivo antiviral activity of LPS by pretreatment of mice with MDP

Experiments were conducted with MDP and its analogues to examine their protective effect in mice against virus infection. Female ddY mice were inoculated intravenously with $10^{4.8}$ $TCID_{50}$ VV via the tail vein. On the 5th day after inoculation, discrete dermal vesicles appeared on the tail, reached their maximal number on the 7th day, and began to heal after the 8th day post-infection. Intravenous administration of LPS at low doses decreased the appearance of tail lesions in VV-infected mice. When mice were pretreated with 100 μg of MDP, the antiviral activity of LPS was enhanced by 63.3% at 1 μg LPS and by 84.5% at 10 μg LPS, respectively. The results summarized in Table 5 indicate that a low and non-toxic dose of LPS can protect mice effectively against virus infection in cooperation with MDP and its analogues. Serum IFN titers of these mice were increased in parallel with the enhancement of antiviral protection.

Discussion

The adjuvant activity of MDP and its derivatives on antibody formation and DTH reaction in mice has been substantiated by many investigators [1,4]. As described in this paper, MDP and its lipophilic analogues, B30-MDP and MDP-Lys(L18), showed

TABLE 4

Effect of carrageenan, a macrophage inhibitor, on enhancement of LPD-induced IFN production by MDP

Treatment[a]			Serum IFN titer[b] (U/0.1 ml)
Day -2	Day -1	Day 0	
-	-	LPS (100 μg/mouse i.v.)	94
-	MDP (100 μg/mouse s.c.)	LPS	281
Carrageenan (200mg/kg i.p.)	-	-	<6
	-	LPS	94
	MDP	LPS	94
(50mg/kg i.p.)	-	-	<6
	-	LPS	94
	MDP	LPS	281

[a] Female BALB/c mice were injected i.p. with the indicated doses of carrageenan 2 days prior to the inducer s.c. with 100 μg MDP one day prior to the inducer. The inducer LPS was administered intravenously.

[b] Serum IFN titers were determined 2 h after LPS administration.

TABLE 5

In vivo antiviral activity of LPS increased by pretreatment of mice with MDP

Treatment		No. of lesions (mean±S.D.)	Serum IFN titer[c] (U/0.1 ml)
Day -2[a]	Day -1[b]		
MDP(100μg)	-	21.2±8.1	<6
-	LPS 0.1(μg)	13.0±4.6	18
MDP	LPS 0.1	10.4±5.7(20.0%)NS	31
-	LPS 1.0	9.8±5.2	31
MDP	LPS 1.0	3.6±3.1(63.3%)*	54
-	LPS 10	5.8±4.6	31
MDP	LPS 10	0.9±1.1(84.5%)*	94
-	LPS 100	3.6±3.1	94
Virus control		33.2±9.4	-

The values in parentheses are % increase of inhibition by LPS in the presence of 100 μg MDP, as compared with administration of LPS alone. The formula for calculation is as follows: % increase = 100 × (LPS - (LPS + MDP))/LPS. NS: not significant, * $P<0.05$ (Mann-Whitney U-test).

[a] Female ddY mice were injected subcutaneously with 100 μg MDP two days prior to virus challenge.

[b] Mice were injected intravenously with the indicated doses of LPS one day before virus challenge. The challenge dose of vaccinia virus was $10^{4.8}$ $TCID_{50}$ intravenously.

[c] Serum specimens were collected 2 h later, pooled and assayed for IFN in the VSV-L cell assay system.

no IFN-inducing activity by themselves in either in vitro or in vivo experiments. In murine models, these compounds are not antigenic and do not exert mitogen-like activities. However, MDP and its analogues increased the 'early-type' serum IFN production, i.e. IFN induced by LPS or poly I : C, but not the 'late-type' IFN induced by the oral inducer tilorone or virus infection. Release of 'early-type' IFN is usually observed from macrophages 1 and 2 h after treatment with the inducer, while B cells and other leukocytes release a 'late-type' IFN some 16 and 18 h later [15]. When cell cultures are stimulated by an 'early-inducer' such as poly I : C, they produce IFN between 2 and 4 h, while, with virus infection, IFN does not appear until several hours later [12,17]. Thus, different mechanisms are probably triggered by different inducers. It has been postulated that differences in the lag period between addition of an inducer and appearance of IFN depend on the synthesis of the triggering molecule within the cells [7,12]. This may also explain why poly I : C induces an 'early-type' IFN in vivo whereas virus-induced IFN appears much later [10]. One possible explanation is that the short lag IFN production is stimulated by activation of macrophages. In fact, when mice were treated with carrageenan one day before MDP treatment, LPS-induced IFN production was not enhanced at all (Table 4).

Involvement of macrophages as accessory cells has been clearly demonstrated in immune IFN production by IFN-producing T cells [11]. In a previous paper [14], we demonstrated that MDP and its analogues augment the host defense mechanisms against viral infection through activation of macrophages. These activated macrophages acquire extrinsic antiviral activity and kill virus-infected cells before these cells can release virus into the extracellular environment.

In the light of our results, a desirable inducer should have both interferon-inducing activity and adjuvant activity. MDP and its analogues possess only adjuvant activity in that they augment 'early-type' IFN production. We are currently investigating how MDP works and how macrophages contribute to their enhancing effect on IFN production. De Clercq et al. [9] demonstrated that mice treated with complete Freund's adjuvant and injected with either endotoxin or poly I : C 2 days later became more resistant to VSV infection than mice injected with the inducer alone. They suggested that Freund's complete adjuvant acts on those cells of the reticuloendothelial system that are involved in the processing of the inducer. Since MDP is a minimum active structural component of mycobacterial cell-walls, our results may define the chemical entity responsible for the action of Freund's complete adjuvant.

Clinical studies with both natural and recombinant IFNs have been initiated in patients with cancer or viral diseases [2,18]. Great expectations are vested in the efficacy of IFN against malignant and viral diseases. In this perspective, new therapeutic modalities should be explored, i.e. by using IFN in concert with other therapeutic agents. Also worth considering is the combined use of IFN inducers with adjuvant substances which enhance IFN-inducer activity. Among the many natural and synthetic IFN inducers that have been discovered, some might be useful for therapeutic purposes [5]. However, most of these inducers are fairly toxic. Less toxic inducers that are effective at low doses in cooperation with appropriate adjuvants like MDP, would seem a lead that should be pursued further.

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