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Augmentation of immune responses by a muramyl dipeptide analog, MDP-Lys(L18)

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Abstract

The effects of N^2-(N-acetyl-muramyl-L-alanyl-D-isoglutamyl)-N^6-stearoyl-L-lysine (MDP-Lys(L18)), a muramyl dipeptide (MDP) analog, on the immune responses in mice were studied. MDP-Lys(L18) augmented the mitogenic responses of splenic lymphocytes to phytohemagglutinin (PHA) and lipopolysaccharide (LPS) at 0.1–10 μg/ml, and antibody formation to sheep red blood cell (SRBC) in normal and immunosuppressed mice, and to dinitrophenyl (DNP)-Ficoll. In addition, MDP-Lys(L18) potentiated polyclonal B cell activation both *in vivo* and *in vitro*. It was also found that MDP-Lys(L18) augmented the cellular immune responses, such as mixed lymphocyte reaction (MLR) and delayed type hypersensitivity (DTH). These effects of MDP-Lys(L18) were more potent than those of MDP. These findings may be attributed to the interleukin 1 (IL-1)-inducing activity of MDP-Lys(L18).

Introduction

It is well known that N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide, MDP) is the minimum adjuvant-active unit in the molecular structure of bacterial cell wall peptidoglycan [1, 2]. Since Chedid et al. [3, 4] found that MDP enhanced non-specific immunity to *K. pneumoniae* infection, a variety of MDP analogs have been synthesized in the search for more effective potentiators against bacterial infection [5]. Among these MDP analogs N^2-(N-acetyl-muramyl-L-alanyl-D-isoglutamyl)-N^6-stearoyl-L-lysine (MDP-Lys(L18)) was reported to show potent activity in protecting mice against *E. coli* infection [6]. Since MDP had been shown to potentiate various immune responses [7–10], we considered it of interest to investigate the effects of MDP-Lys(L18) on the immune responses in mice, and found that MDP-Lys(L18) is a potent immunopotentiator.

Materials and methods

Mice

In all experiments except for MLR, C57Bl/6×DBA/2F_1 (BDF_1) female mice were used. C3H/He and DBA/2 female mice were employed for MLR. These mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Shizuoka, Japan) at 6 weeks of age and conditioned in our facility for 1 or 2 weeks prior to use. Five mice per group were used for *in vivo* experiments.

MDP and MDP-Lys(L18)

MDP and MDP-Lys(L18), synthesized in this institute, were dissolved in PBS and used for the experiments after being passed through Millex-HA (Japan Millipore Ltd., Tokyo, Japan). In experiments *in vivo*, drugs were injected subcutaneously 24 hr before sensitization.

Spleen cell suspension

A spleen single cell suspension was aseptically prepared by teasing spleens in RPMI 1640 (GIBCO, Grand Island, NY, USA) and washing twice with the same medium. The spleen cell number was determined by a Coulter Counter (Coulter Electronics Inc, Hialeah, FL, USA).

Mitogenic response to lectins

Spleen cells (4×10^5 cells) suspended in RPMI 1640 containing 5% heat-inactivated fetal calf serum (Flow Laboratories, Stanmore, Australia) were incubated in microwells (Falcon 3042, Oxnard, CA, USA) with phytohemagglutinin (PHA, 50 μg/ml, E.Y. Laboratories, San Mateo, CA, USA), concanavalin A (ConA, 5 μg/ml, Sigma Chemical Co., St. Louis, MO, USA) or lipopolysaccharide (LPS, 50 μg/ml, DIFCO, Detroit, MI, USA, *E. coli* 055 : B5) in the presence or absence of MDP or MDP-Lys(L18) at 37 °C for 66 hr. Incorporation of ^{3}H-thymidine (0.5 μCi/well, New England Nuclear, Boston, MA, USA) into DNA for the last 18 hr of incubation was determined by a liquid scintillation counter (Aloka, Tokyo, Japan) after the harvesting of the cells by a microharvester (Bellco, Vineland, NJ, USA). The results were expressed as a stimulation index, which was the ratio of ^{3}H-thymidine incorporation in the presence to that in the absence of MDP or MDP-Lys(L18).

In vitro plaque forming cell (PFC) assay

The splenic lymphocytes were obtained by Ficoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden)-Conray (Daiichi Seiyaku Co., Ltd., Tokyo, Japan) density gradient centrifugation at $400 \times g$ for 20 min. The lymphocytes (2×10^6 cells) were incubated with sheep red blood cell (SRBC, 1×10^6 cells) in RPMI 1640 containing 5% heat inactivated fetal calf serum, 50 μ*M* 2-mercaptoethanol, 20 μ*M* pyruvic acid, 4 m*M* L-glutamine and 10 m*M* N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) in the presence or absence of MDP-Lys(L18) in a final volume of 0.4 ml for 5 days. PFC assay was carried out according to Cunningham and Szenberg [11].

In vivo PFC assay

Mice were given MDP or MDP-Lys(L18) by a subcutaneous injection 24 hr before sensitization with SRBC (1×10^7 cells/mouse, i.v.). In some experiments, cortisone acetate (Merck Banyu Co., Tokyo, Japan, 2.5 mg/mouse, i.p.) was given to mice 48 hr before SRBC sensitization (5×10^8 cells/mouse, i.v.) for induction of immunosuppressed conditions. The PFC assay was performed by the method of Cunningham and Szenberg [11] 4 days after SRBC sensitization.

Anti-dinitrophenyl (DNP) PFC assay

Mice were intraperitoneally given 10 μg of DNP_{74}-Ficoll, synthesized according to Inman [12], and 4 days later anti-DNP PFC was measured using DNP_{74}-bovine serum albumin (BSA) coupled SRBC as indicator cells.

Polyclonal B cell activation assay

Polyclonal B cell activation was assessed by SRBC PFC assay in a similar way to those used for the *in vitro* and *in vivo* PFC assays described above without pre-sensitization with SRBC.

Mixed lymphocyte reaction (MLR)

DBA/2 spleen cells suspended in RPMI 1640 were irradiated (700R) by an X-ray machine (Hitachi, Model MBR-1505R, Hitachi Medico, Tokyo, Japan) and adjusted to 4×10^6 cells/ml in RPMI 1640 containing 5% heat-inactivated fetal calf serum. C3H/He spleen cells, used as the responder cells, were also adjusted to 4×10^6 *cells/ml in the same medium. 50* μl of each cell suspension was distributed in microwells (Falcon 3042), then 100 μl of MDP-Lys(L18) solution was added and incubated for 4 days in 5% CO_2 at 37 °C. Each culture was then labeled with 0.5 μCi of ^{3}H-thymidine for the last 18 hr for determination of DNA synthesis. At the end of the culture period, the cells were harvested and the ^{3}H-thymidine uptake was measured by a liquid scintillation counter. The results were expressed as a stimulation index, the ratio of ^{3}H-thymidine uptake in the presence to that in the absence of MDP or MDP-Lys(L18).

Delayed type hypersensitivity (DTH) assay

DTH was induced in mice by injection of SRBC (1×10^6 cells) into the tail vein and a subcutaneous injection of SRBC (1×10^8 cells) in the left hind footpad was given 4 days later. The extent of the DTH response was assessed 24 hr later and was expressed as the difference in the thickness of the footpad between the left and right hind feet. The thickness of the footpad was measured by a dial thickness gauge (Peacock, Tokyo, Japan).

Interleukin 1 (IL-1) assay

$P388D_1$ cells (1×10^6), a gift from National Institute of Health, Japan, were incubated in 1 ml of RPMI 1640 in the presence or absence of MDP or MDP-Lys(L18) and culture fluid was obtained by centrifugation. The culture fluid was dialyzed against RPMI 1640 ad then passed through Millex-GV. The IL-1 activity of the culture fluid was assessed according to Mizel et al. [13]. Briefly, the culture fluid was added into 0.1 ml of thymocyte suspension (1.5×10^7/ml) from C3H/HeJ mice and culture was initiated by the addition of 50 µl of 0.1% PHA-P (DIFCO). 0.5 µCi of ^{3}H-thymidine was added 66 hr later, and ^{3}H-thymidine uptake for additional 6 hr was measured as described above.

Statistics

Data were expressed as mean ± S.E.; *in vitro* studies were carried out in triplicate. Comparison between means was performed by *t*-test. The significance level for the experiments was expressed as *: $p < 0.05$, **: $p < 0.01$.

Results

Effects of MDP and MDP-Lys(L18) on the mitogenic response to lectins

First, experiments were carried out to examine the effects of MDP and MDP-Lys(L18) on the mitogenic responses of murine splenic lymphocytes stimulated with PHA, ConA and LPS. As shown in Figure 1, MDP and MDP-Lys(L18) augmented the mitogenic responses of the lymphocytes to PHA and LPS, but not to ConA. MDP-Lys(L18) augmented the responses to PHA at concentrations above 0.1 µg/ml and those to

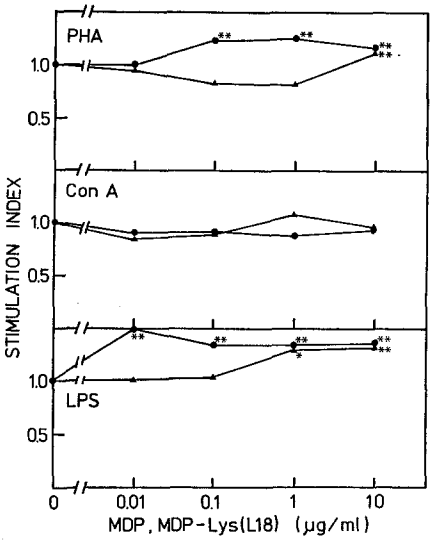

Figure 1
Effects of MDP and MDP-Lys(18) on the mitogenic response. Spleen cells from BDF_1 mice were cultured with PHA, ConA or LPS in the presence of MDP (▲) or MDP-Lys(18) (●) for 66 hr. Each culture was pulsed with ^{3}H-thymidine for the last 18 hr. The results were expressed as a stimulation index as indicated in Materials and Methods. Control responses of spleen cells to PHA, ConA and LPS were 4461 ± 156 cpm, 142061 ± 9313 cpm and 25820 ± 196 cpm respectively. $n = 3$.

LPS at 0.01 µg/ml or more. MDP-Lys(L18) was more effective than MDP. In addition, MDP-Lys(L18) also enhanced, to a slight but significant extent, DNA synthesis of the lymphocytes in the absence of any mitogens (data not shown).

Effects of MDP and MDP-Lys(L18) on SRBC PFC response

The ability of MDP-Lys(L18) to augment IgM antibody response was compared with that of MDP both *in vivo* and *in vitro*. MDP-Lys(L18) increased the number of PFC at doses of 10 and 100 µg/mouse, while PFC response was only slightly augmented by treatment with MDP at 100 µg/mouse (Figure 2). Also, in cortisone-induced immunosuppressed mice, MDP-Lys(L18) augmented the number of PFC in a dose-dependent manner (Figure 3). These effects of MDP-Lys(L18) *in vivo* were observed *in vitro* as well (Figure 4). The maximum effect on *in vitro* PFC response was observed at 1 µg/ml of MDP-Lys(L18).

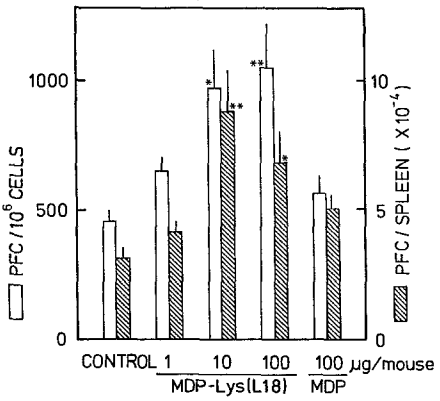

Figure 2
Effects of MDP and MDP-Lys(18) on SRBC-PFC. BDF_1 mice were treated subcutaneously with MDP or MDP-Lys(18) 24 hr before sensitization with SRBC. Four days after sensitization, plaque-forming cells were detected. Bars represent S.E. $n = 5$.

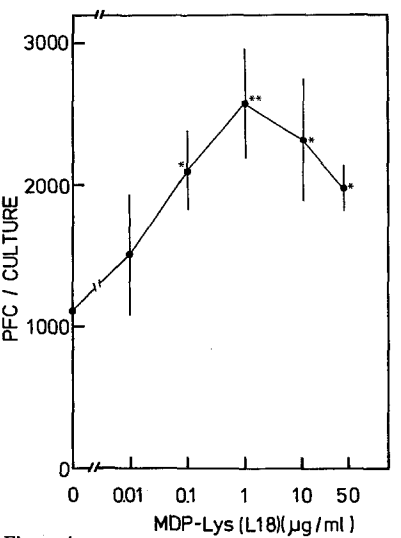

Figure 4
Effect of MDP-Lys(18) on *in vitro* SRBC-PFC. Spleen cells were cultured with SRBC in the presence of MDP-Lys(18). Five days after the culture, plaque-forming cells were detected. Values represent mean ± S.E. $n = 3$.

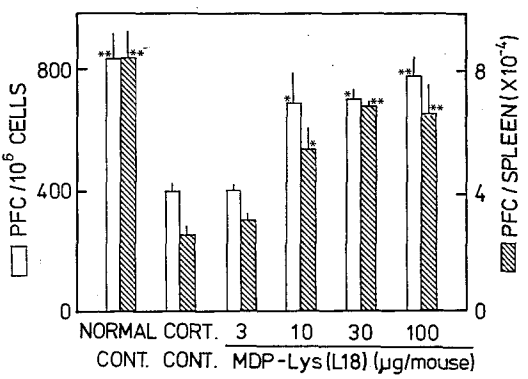

Figure 3
Effect of MDP-Lys(18) on SRBC-PFC in immunosuppressed mice. Mice were treated intraperitoneally with cortisone acetate 48 hr before sensitization with SRBC. Treatment with MDP-Lys(18) and the detection of plaque-forming cells were carried out in the same way as in Figure 2. Bars represent S.E. $n = 5$.

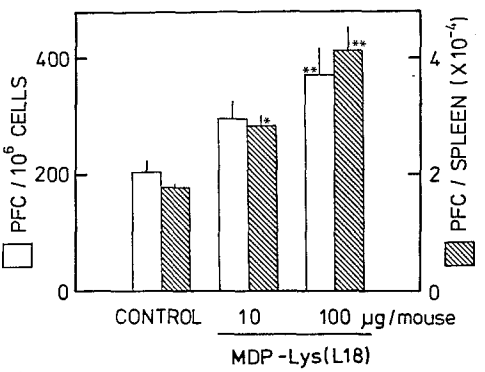

Figure 5
Effect of MDP-Lys(18) on anti-DNP PFC. Mice were treated subcutaneously with MDP-Lys(18) 24 hr before sensitization with DNP-Ficoll. Four days after sensitization, plaque-forming cells to DNP-BSA-coupled SRBC were detected. Bars represent S.E. $n = 5$.

Effect of MDP-Lys(L18) on anti-DNP PFC response

Another experiment concerning antibody responses was carried out to determine whether or not MDP-Lys(L18) augmented the antibody response to one of the T cell independent antigens, DNP-Ficoll. The result shows that the antibody response to DNP-Ficoll, as well as that to SRBC, was enhanced by the treatment with MDP-Lys(L18) (Figure 5).

Effects of MDP and MDP-Lys(L18) on polyclonal B cell activation

Effects of MDP and MDP-Lys(L18) on polyclonal B cell activation were assessed both *in vitro* and *in vivo* using LPS as a positive control. As shown in

Figure 6, MDP-Lys(L18) was found to have more potent activity on polyclonal B cell activation than MDP. MDP-Lys(L18), however, was less active than LPS.

Effect of MDP-Lys(L18) on mixed lymphocyte reaction

The results described above show that MDP-Lys(L18) possesses potent adjuvant activity on the humoral immune response. Thus, we then examined the adjuvant activity of MDP-Lys(L18) on the cellular immune response. MLR was performed in the presence or absense of MDP-Lys(L18), using spleen cells of C3H/He mice as responder cells and 700R-irradiated spleen cells of DBA/2 mice. As shown in Figure 7, addition of MDP-Lys(L18) resulted in enhancement of MLR, which indicates that MDP-Lys(L18) augments not only the humoral immune response but also the cellular immune response.

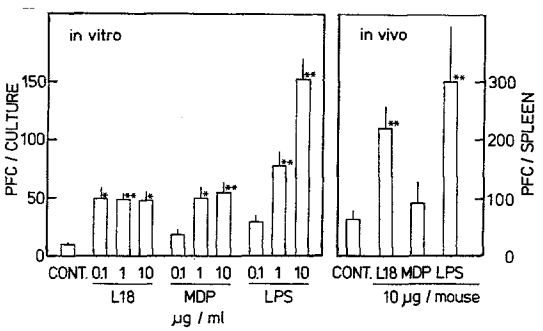

Figure 6
Polyclonal B cell activation by MDP, MDP-Lys(18) and LPS. Polyclonal B cell activation was assessed by the anti-SRBC-PFC response. In *in vitro* experiments, spleen cells were cultured in the presence of MDP, MDP-Lys(18) or LPS. Plaque-forming cells to SRBC were detected after 5 days. $n=3$. In *in vivo* experiment, mice were treated subcutaneously with MDP, MDP-Lys(18) or LPS. $n=5$. Plaque-forming cells to SRBC were detected after 4 days. Bars represent S.E.

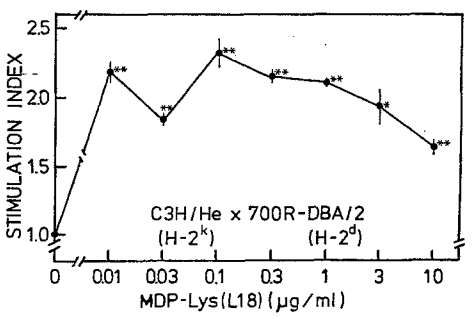

Figure 7
Effect of MDP-Lys(18) on MLR. C3H/HE spleen cells were cultured with X-ray-irradiated DBA/2 spleen cells in the presence or absence of MDP-Lys(18) for 96 hr. Each culture was pulsed with ^{3}H-thymidine for the last 18 hr. The results were expressed as a stimulation index indicated in Materials and Methods. Values represent mean ± S.E. $n=3$. ^{3}H-Thymidine uptake of MLR in the absence of MDP-Lys(18) was 1888 ± 65 cpm.

Effect of MDP and MDP-Lys(L18) on delayed type hypersensitivity to SRBC

In order to examine the effect of MDP-Lys(L18) on an *in vivo* system of the cellular immune response, DTH to SRBC was employed. Figure 8 shows that MDP-Lys(L18) enhanced footpad swelling at 10 μg/mouse, s.c., while MDP at 100 μg/mouse failed to do so.

Effect of MDP and MDP-Lys(L18) on IL-1 induction

Finally, we compared the ability of MDP-Lys(L18) to induce IL-1 with that of MDP. P388D$_1$ cells were employed for this experiment and MDP-Lys(L18) was found to induce IL-1 in a concentration-dependent manner (Figure 9).

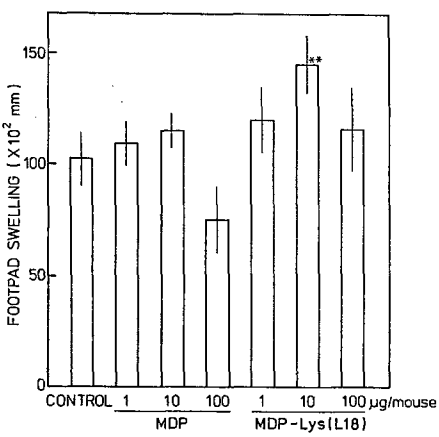

Figure 8
Effects of MDP and MDP-Lys(18) on SRBC-DTH. DTH to SRBC was induced in mice in the way indicated in Materials and Methods. MDP or MDP-Lys(18) was administered subcutaneously 24 hr before SRBC sensitization. The control group was given PBS. The results were expressed as differences between the left and the right hind feet. Values represent mean ± S.E. $n=5$.

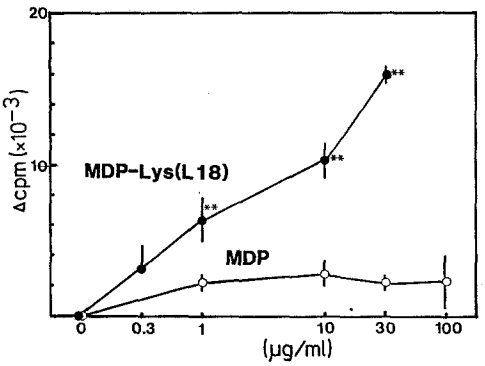

Figure 9
Effects of MDP and MDP-Lys(18) on IL-1 production from P388D_1 cells. P388D_1 cells were cultured with MDP (○) or MDP-Lys(18) (●). Supernatants were obtained after 48-hr incubation and were tested at a 1:4 dilution for their mitogenic effects on PHA-stimulated C3H/HeJ thymocytes. Values represent mean ± S.E. $n=3$.

Discussion

In this paper we examined the effects of MDP-Lys(L18) on the humoral and cellular immune responses in comparison with those of MDP. First of all, augmentation of mitogenic responses to lectins was made clear. MDP-Lys(L18) was shown to augment the mitogenic responses of murine splenic lymphocytes to PHA and LPS but not to ConA. Since PHA and LPS are considered to be mitogens specific to T cells and B cells, respectively, these findings suggest that MDP-Lys(L18) exerts the activity on both T and B cells. This is consistent with the report by Sugimura et al. [14] who have provided evidence that MDP directly stimulated both T and B cells. MDP-Lys(L18), on the other hand, did not augment the mitogenic response to ConA, which has been considered to be specific to suppressor T cells at the concentration we employed [15, 16]. It was suggested that MDP-Lys(L18) did not stimulate suppressor T cells, and it actually inhibited the induction of suppressor T cells to SRBC [17].

These findings led us to further investigation of MDP-Lys(L18) on the immune responses. MDP-Lys(L18) significantly augmented antibody formation against SRBC both *in vivo* and *in vitro*. Antibody formation against DNP-Ficoll, a T cell independent antigen, was also augmented by the treatment with MDP-Lys(L18), which was consistent with the report by Azuma et al. [7]. In addition, MDP-Lys(L18), as well as MDP, as reported by Spector et al. [8], enhanced polyclonal B cell activation. The observation described above suggests that MDP-Lys(L18) augments antibody formation not only against T cell dependent antigens, but also against T cell independent antigens.

MLR and DTH were carried out in order to assess the effect of MDP-Lys(L18) on the cellular immune responses. MLR was markedly enhanced in the presence of MDP-Lys(L18), which might be attributed to the C3H/He mice used in this experiment, because Staruch and Wood [18] have reported that C3H/He is a high responder strain to MDP.

Azuma et al. [7] have compared many MDP analogs with MDP in terms of their ability to potentiate DTH to monoazobenzenarsenate-N-acetyl-L-tyrosine. Our data clearly demonstrated that MDP-Lys(L18) exerted adjuvant activity on DTH more potently than MDP did, showing that MDP-Lys(L18) was more active than MDP not only in the humoral immune responses but also in the cellular immune responses.

Finally, we assessed the ability of MDP-Lys(L18) to induce interleukin-1 (IL-1) using P388D_1 cells, and found that MDP-Lys(L18) stimulated the macrophage cell line P388D_1 to secrete IL-1. Since it is well-known that IL-1 mediates a variety of immune responses [19] and Guenounou et al. [20] reported that immunoadjuvant activity of MDP was mediated by IL-1, the augmentation of the immune responses reported here might be due to enhancement of IL-1 production by MDP-Lys(L18).

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