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Adjuvant Activity of 6-*O*-Acyl-Muramyldipeptides to Enhance Primary Cellular and Humoral Immune Responses in Guinea Pigs: Adaptability to Various Vehicles and Pyrogenicity

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Thirteen 6-*O*-acyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamines (6-*O*-acyl-MDPs), including four inactive D-isoasparagine and L-isoglutamine analogs, were tested for their pyrogenicity and immunopotentiating activity to stimulate primary humoral and cellular immune responses in guinea pigs to a model protein antigen, ovalbumin, when administered in various vehicles. Among them, derivatives whose muramic acid residue was substituted by α-branched (and β-hydroxylated) higher fatty acids at the carbon-6 position, especially 6-*O*-(2-tetradecylhexadecanoyl)-MDP (B3O-MDP) and, to a lesser extent, 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-MDP (BH48-MDP) and its L-serine analog [BH48-MDP(L-Ser)], were found to exert strong adjuvant activity in both the induction of delayed-type hypersensitivity and the stimulation of circulating precipitating antibody levels when combined with nonirritating vehicles (liposomes, squalene-in-water emulsion, and phosphate-buffered saline). These vehicles did not efficiently support the adjuvant activity of MDP, the parent molecule of the above lipophilic derivatives. Pyrogenicity tests showed that introduction of α-branched higher fatty acid groups but not of straight, long-chain fatty acids at the 6-position of the muramic acid residue resulted in marked decrease of the pyrogenicity inherent to MDP via intravenous administration.

To develop more effective and safer vaccines, much work has been done to identify the antigenic determinants (protective epitopes) responsible for the efficacy of vaccines and to prepare the epitopes identified by chemical synthesis or gene technology (2, 28, 29, 31). One of the problems encountered in this approach is that purified protective epitopes often possess only poor immunogenicity, at least partly because of removal of the components carrying the adjuvant activity of the original vaccines. Thus, the development of new types of vaccines requires studies on safe and potent immunoadjuvants that effectively potentiate the immunogenicity of protective epitopes.

From 1974 to 1975, the active component that could replace the role played by mycobacterial cells in Freund complete adjuvant in stimulation of immune responses was identified by synthesis as *N*-acetylmuramyl-L-alanyl-D-isoglutamine (generally abbreviated MDP), first by Ellouz et al. (7) and subsequently but independently by the group of Kotani et al. (17). Numerous studies have since revealed that MDP is the minimum structural unit responsible for most of the biological activities of bacterial cell walls and that the range of bioactivities of MDP is extensive (1, 5, 32), with the greatest attention being paid to the immunopotentiating activity (adjuvanticity) of MDP.

However, there are problems to be solved before attempting to apply the adjuvanticity of MDP to vaccines for humans. The biggest problem is that MDP requires a water-in-mineral oil (w/o) emulsion as a specified vehicle to exert its full adjuvant capacity, particularly the ability to induce cellular immunity, e.g., delayed-type hypersensitivity. In fact, MDP is quite effective in the induction of delayed-type hypersensitivity to ovalbumin when it is injected in guinea pigs as a w/o emulsion (Freund incomplete-type adjuvant) with test antigens, but has little activity as a saline solution or with other nonirritative vehicles (11), although an aqueous MDP solution has been reported to work as an effective adjuvant to raise serum antibody levels (3, 4, 6). However, w/o emulsions, especially those prepared with mineral oil, induce severe injurious reactions both at the injection site and in the regional lymph nodes (those pertaining to the injection site), making them unsuitable vehicles for practical vaccination. Another problem is that the biological activities of MDP are so multifold that some of the bioactivities are more harmful than beneficial.

We attempted to overcome these problems by using 6-*O*-acyl-MDPs that possess various acyl groups at the carbon-6 position of the muramic acid residue and that are more lipophilic than the parent molecule. We found that 6-*O*-lauroyl-, 6-*O*-octadecanoyl-, and 6-*O*-docosanoyl-MDPs showed definite adjuvant activities to potentiate cellular as well as humoral immune responses to ovalbumin when administered with liposomes to guinea pigs (11). These 6-*O*-acyl-MDPs, however, had only weak adjuvanticity when given to guinea pigs either as a saline solution or as a Ribi-type oil-in-water emulsion, which is far less irritative than a Freund-type w/o emulsion (33, 34). Therefore, we focused our attention on 6-*O*-acyl-MDPs whose acyl groups are longer-chain fatty acids, including either α-branched or α-branched and β-hydroxylated ones, to develop MDP derivatives that exert high adjuvanticity when injected with protein antigens in nonirritative vehicles and have less deleterious effects than MDP.

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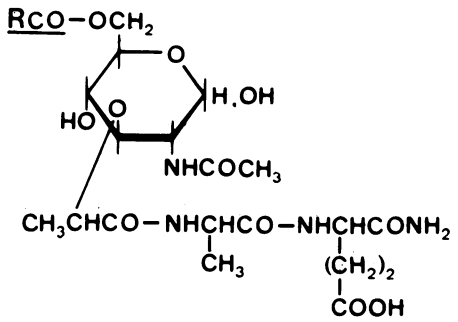

FIG. 1. Chemical structure common to test 6-*O*-acyl-MDPs. RCO–, Acyl group.

MATERIALS AND METHODS

Synthetic 6-*O*-acyl derivatives of MDP. The chemical structure common to all the test compounds is illustrated in Fig. 1, and a list of the structures and chemical names of the acyl portions and the abbreviation of test 6-*O*-acyl-MDP derivatives are presented in Table 1. They were synthesized as described previously (18–22, 26, 27).

Assay for immunoadjuvant activities. Groups of five female albino guinea pigs of a closed colony, weighing about 300 g (purchased from Nihon Rabbit Inc., Osaka, Japan), were immunized by footpad injection (intraperitoneal injection in one experiment) of an appropriate amount of test adjuvant and an indicated dose of crystalline ovalbumin (grade V; Sigma Chemical Co., St. Louis, Mo.) incorporated in the various vehicles described below. The test doses of adjuvants and ovalbumin were selected on the basis of our findings in the preceding study (11).

At 2 and 3 weeks after immunization, a corneal test was done to evaluate the induction of delayed-type hypersensitivity to a test antigen, as described previously (13). Circulating antiovalbumin precipitating antibody levels of serum specimens, which were taken 29 days after immunization, were estimated by the quantitative precipitin method (30).

Vehicles used for the administration of test adjuvants with antigen. The following vehicles were examined for their ability to support the adjuvanticity of test 6-*O*-acyl-MDPs.

Vehicle i. Freund type w/o emulsion as a reference vehicle was prepared by the conventional method, using 1/75 M phosphate-buffered saline (PBS; pH 7.0), Drakeol 6VR (Penreco, Los Angeles, Calif.) and Arlacel A (mannide monooleate; Atlas Chemical Industries Inc., Wilmington, Del.) at a ratio of 5:4:1 (vol/vol/vol) (9).

Vehicle ii. Oil-in-water emulsion was prepared by the method of Ribi et al. (25) with minor modifications. Briefly, 0.1 ml of squalene (a metabolizable shark liver oil, purity 98 to 100%; Sigma) was added to 10 mg of ovalbumin and an appropriate amount of test adjuvant, which were placed in a 5-ml tissue homogenizer equipped with a Teflon pestle. The mixture was ground to a smooth paste by rotation at 800 rpm for several minutes. After addition of 1.9 ml of PBS containing 0.2% Tween 80 (Wako Pure Chemical Industries Ltd., Osaka), grinding was continued for several minutes to obtain a uniform emulsion.

Vehicle iii. Single lamellar liposomes, which incorporate both ovalbumin and a test adjuvant, were prepared, using DL-α-phosphatidylcholine, dipalmitoyl (PC; grade 1, purity approx. 99%, synthetic; Sigma), and cholesterol (CL; grade 99+%, the standard for chromatography; Sigma) (11). The dose of these ingredients per animal was 1 μmol (730 μg for PC and 390 μg for CL).

An immunogen containing an indicated amount of ovalbumin and a test adjuvant, dissolved or emulsified in the above vehicles and PBS, were injected into the left hind footpad unless otherwise stated.

TABLE 1. Chemical structure and abbreviation of synthetic 6-*O*-acyl-MDPs

Chemical structure [name] of acyl moiety	Structure of MDP moiety	Abbreviation
$CH_3(CH_2)_{16}CO$- [6-*O*-octadecanoyl-]	MurNAc-L-Ala-D-isoGln	L18-MDP
$CH_3(CH_2)_{16}CO$- [6-*O*-octadecanoyl-]	MurNAc-L-Ala-L-isoGln	L18-MDP(L-isoGln)
$CH_3(CH_2)_{18}CO$- [6-*O*-eicosanoyl-]	MurNAc-L-Ala-D-isoGln	L20-MDP
$CH_3(CH_2)_{22}CO$- [6-*O*-tetracosanoyl-]	MurNAc-L-Ala-D-isoGln	L24-MDP
$CH_3(CH_2)_{28}CO$- [6-*O*-triacontanoyl-]	MurNAc-L-Ala-D-isoGln	L30-MDP
$CH_3(CH_2)_{13}$ > $CH_3(CH_2)_{13}$ CHCO- [6-*O*-(2-tetradecylhexadecanoyl)-]	MurNAc-L-Ala-D-isoGln	B30-MDP
$CH_3(CH_2)_{13}$ > $CH_3(CH_2)_{13}$ CHCO- [6-*O*-(2-tetradecylhexadecanoyl)-]	MurNAc-L-Ala-D-isoAsn	B30-MDP(D-isoAsn)
$CH_3(CH_2)_{14}CH(OH)$ > $CH_3(CH_2)_{13}$ CHCO- [6-*O*-(3-hydroxy-2-tetradecyloctadecanoyl)-]	MurNAc-L-Ala-D-isoGln	BH32-MDP
$CH_3(CH_2)_{21}$ > $CH_3(CH_2)_{21}$ CHCO- [6-*O*-(2-dococyltetracosanoyl)-]	MurNAc-L-Ala-D-isoGln	B46-MDP
$CH_3(CH_2)_{22}CH(OH)$ > $CH_3(CH_2)_{21}$ CHCO- [6-*O*-(3-hydroxy-2-dococylhexacosanoyl)-]	MurNAc-L-Ala-D-isoGln	BH48-MDP
$CH_3(CH_2)_{22}CH(OH)$ > $CH_3(CH_2)_{21}$ CHCO- [6-*O*-(3-hydroxy-2-dococylhexacosanoyl)-]	MurNAc-L-Ser-D-isoGln	BH48-MDP(L-Ser)
$CH_3(CH_2)_{22}CH(OH)$ > $CH_3(CH_2)_{21}$ CHCO- [6-*O*-(3-hydroxy-2-dococylhexacosanoyl)-]	MurNAc-L-Ala-L-isoGln	BH48-MDP(L-isoGln)
$CH_3(CH_2)_{22}CH(OH)$ > $CH_3(CH_2)_{21}$ CHCO- [6-*O*-(3-hydroxy-2-dococylhexacosanoyl)-]	MurNAc-L-Ala-D-isoAsn	BH48-MDP(D-isoAsn)

Pyrogenicity. Pyrogenicity was tested essentially as described in Japanese Pharmacopoeia X. The test specimen was dissolved or suspended in pyrogen-free physiological saline for injection. The solution or suspension was injected intravenously or intracerebroventricularly to Japanese domestic white rabbits (weight, 2.0 to 2.7 kg). Rectal temperature was measured continuously with an automatic rectal temperature recording device (model EP-670; Iio Electorical Equipment Co., Tokyo, Japan).

Other procedures. The statistical significance of the difference between each test result and the respective control results was examined by Student's *t* test for most of the assays.

RESULTS

Immunoadjuvant activities on ovalbumin of 6-*O*-acylmuramyl dipeptides administered with various vehicles. (i) Use of

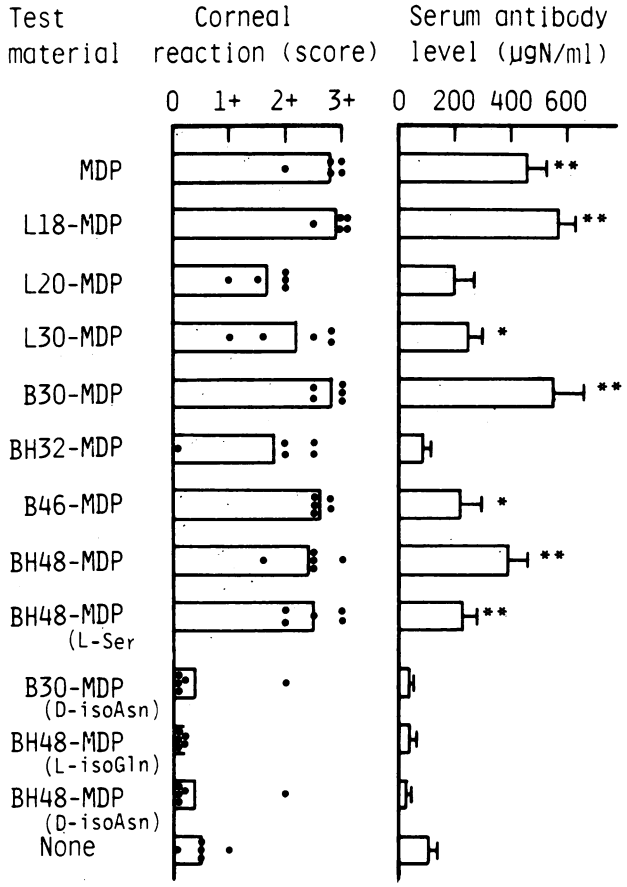

FIG. 2. Immunoadjuvant activities of 6-*O*-acyl-MDPs in Freund-type w/o emulsion to stimulate the primary immune response (humoral and cellular) in guinea pigs. Groups of five female albino guinea pigs were immunized by footpad injection of 1 mg of ovalbumin with 202 nmol (equivalent to 100 μg of MDP) of 6-*O*-acyl-MDPs. Induction of delayed-type hypersensitivity was evaluated in terms of positive corneal reaction on week 3. Stimulation of antibody production was estimated by determination of antiovalbumin precipitin levels in serum specimens drawn 29 days after immunization. For the corneal reaction, each dot represents the score (arbitrarily graded, with a maximum of 3+) for an individual animal, and each column shows the mean score. The antibody level in serum was expressed as micrograms of antibody nitrogen per milliliter of serum (mean ± standard error [SE]). Significant difference from control value: *, $P < 0.05$; **, $P < 0.01$.

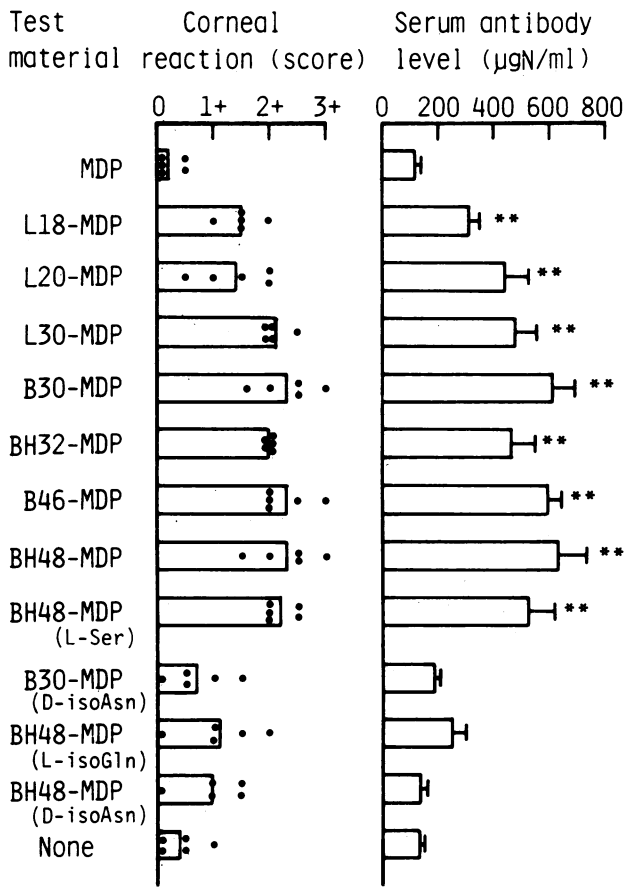

FIG. 3. Adjuvant activities of 6-*O*-acyl-MDPs in Ribi-type squalene-in-water emulsion. See the legend to Fig. 2 for the dose size of test adjuvants and ovalbumin and other details.

Freund-type w/o emulsion as a vehicle. Figure 2 summarizes the assay results of a control experiment in which ovalbumin with test 6-*O*-acyl-MDPs was injected as a w/o emulsion. The introduction of some acyl groups, such as L20-, L30- and BH32- groups, caused a decrease in the adjuvanticity of the molecule, particularly the stimulation of antibody formation. Analogs of B30- and BH48-MDPs [B30-MDP(D-isoAsn) and BH48-MDP(L-isoGln)], in which the D-isoglutamine residue of the MDP portion was substituted with D-isoasparagine and L-isoglutamine, respectively, were completely inactive.

(ii) Use of Ribi-type squalene-in-water emulsion. While the parent molecule, MDP, had almost no adjuvanticity, all the test 6-*O*-acyl-MDPs except the inactive analogs B30-MDP(D-isoAsn), BH48-MDP(L-isoGln), and BH48-MDP(D-isoAsn) markedly potentiated both cellular and humoral primary immune responses to ovalbumin (Fig. 3). Thus, a sharp difference was found between MDP and 6-*O*-acyl derivatives concerning their compatibility with this type of vehicle. The ability of 6-*O*-acyl-MDPs incorporated in squalene-in-water emulsion to stimulate serum antibody production was more or less greater than that of the corresponding compound incorporated in other vehicles, including w/o emulsion, although larger doses of test adjuvants were used in combination with liposomes and PBS as vehicles.

(iii) Liposomes as an administration vehicle. MDP had little ability to induce delayed-type hypersensitivity, although it

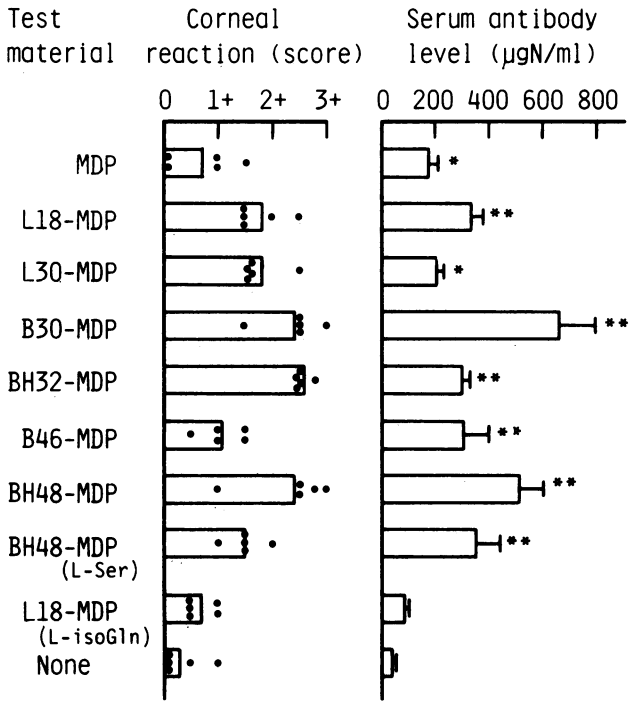

FIG. 4. Adjuvant activities of 6-*O*-acyl-MDPs in liposomes. The dose was 265 nmol (equivalent to 200 μg of L18-MDP) of test compound and 1 mg of ovalbumin per guinea pig. See the legend to Fig. 2 for details.

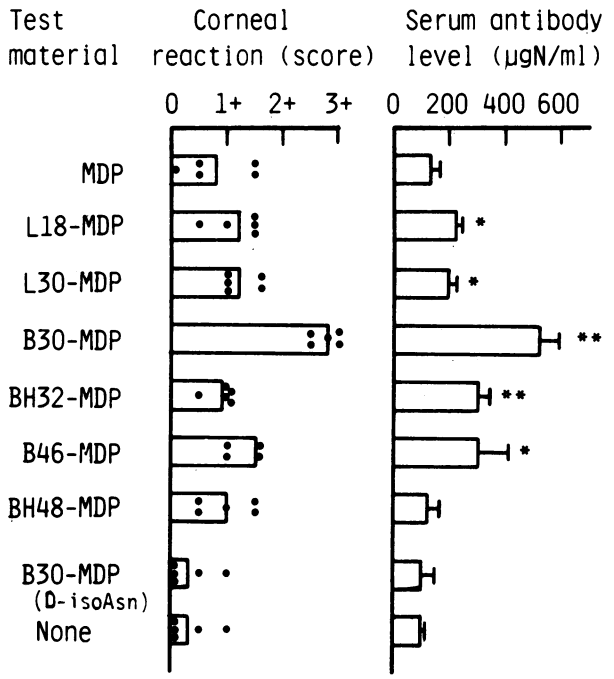

FIG. 5. Adjuvant activities of 6-*O*-acyl-MDPs in PBS. The dose was 525 nmol (equivalent to 400 μg of L18-MDP) of test compound and 2 mg of ovalbumin per guinea pig. See the legend to Fig. 2 for details.

significantly increased antibody production. In contrast, the test 6-*O*-acyl-MDPs, especially B30-MDP and BH48-MDP, effectively induced delayed-type hypersensitivity and increased serum precipitin levels (Fig. 4).

(iv) PBS as a vehicle. Among the adjuvants tested, only B30-MDP was satisfactorily effective in stimulating both cellular and humoral immune responses of guinea pigs against ovalbumin (Fig. 5). Significant adjuvanticity was noted also with B46-MDP and BH32-MDP. BH48-MDP and B30-MDP(D-isoAsn) were hardly active and inactive, respectively.

Figure 6 shows the assay results of some of the test 6-*O*-acyl-MDPs, which were either dissolved or suspended in PBS with ovalbumin and injected intraperitoneally into guinea pigs. B30-MDP in PBS showed strong adjuvanticity in both induction of delayed-type hypersensitivity and stimulation of serum antibody production by this administration route. BH48-MDP was also significantly active.

Pyrogenicity of test 6-*O*-acyl-MDPs. Figure 7 shows the mean maximum elevation in rectal temperature after intravenous injection of three dose levels of MDP and various 6-*O*-acyl-MDPs in the rabbit. At a dose of 10 μg/kg, L18-, L20-, and L24-MDP caused significant rise (over 0.6°C) in temperature, whereas 1 mg of B30- or BH48-MDP (lipophilic MDP derivatives with α-branched acyl groups of 30 or more carbon atoms) per kg did not. However, L30-MDP, possessing an acyl group consisting of 30 carbon atoms like B30-MDP, but of straight-chain, caused a distinct rise in rectal temperature, although the degree of pyrogenicity was lower than that of the MDP derivatives possessing a shorter, straight-chain acyl group. BH48-MDP in squalene-in-water emulsion did not cause any significant febrile response by intravenous or subcutaneous injection at a dose of 40 or 100 μg/kg, respectively (data not shown).

As illustrated in Fig. 8, intracerebroventricular injection of 1 μg of B30- and BH48-MDPs evoked a distinct febrile response in the rabbit. That the fever observed in this pyrogenicity test was not due to contamination by extraneous endotoxins was proved by the observation that B30-MDP(D-isoAsn) and BH48-MDP(L-isoGln or D-isoAsn), the analogs lacking immunoadjuvanticity and other bioactivities (8, 10, 15, 23, 24; S. Kotani, M. Tsujimoto, T. Koga, S. Nagao, A. Tanaka, and S. Kawata, Fed. Proc., in press), did not produce any appreciable signs of pyrogenicity.

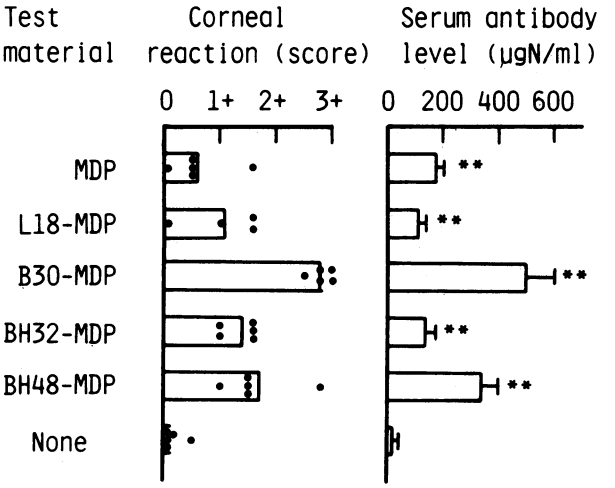

FIG. 6. Adjuvant activities of 6-*O*-acyl-MDPs in PBS administered intraperitoneally. The dose was 525 nmol (equivalent to 400 μg of L18-MDP) of test compound and 2 mg of ovalbumin per guinea pig. See the legend to Fig. 2 for details.

DISCUSSION

Stimulation of the antigen-specific and nonspecific defense mechanisms of humans and animals is the biological activity of MDP that could be useful for medical and veterinary application. Many efforts have been made to minimize possible deleterious effects without accompanying reduction of the useful bioactivities of MDP by chemical modification of the molecule (1, 5, 14, 32). One such attempt is to give MDP lipophilicity by the introduction of acyl groups.

In the present study, synthetic 6-*O*-acyl-MDPs whose physicochemical properties as well as chemical structures were changed by acylation with either straight-chain or α-branched (and β-hydroxylated) fatty acids at the carbon-6 position of the muramic acid residue were examined with respect to their immunoadjuvanticity and pyrogenicity in combination with various vehicles. Some of the test 6-*O*-acyl-MDPs were found to strongly stimulate both cellular and humoral primary immune responses against ovalbumin with vehicles (Ribi-type squalene-in-water emulsion, liposomes, and PBS) that were hardly or only weakly effective in supporting the adjuvanticity of the parent molecule, MDP.

Among the test 6-*O*-acyl-MDPs, B30-MDP and, to a lesser extent, BH48-MDP and BH48-MDP(L-Ser) were superior to the others and found to be promising compounds as a vaccine adjuvant because they can be delivered via a wide range of vehicles, although the time course for a maximum immune response, the dose-response relationships, and the responses to booster immunization were not examined in the present study. This was mainly due to the vast combinations of experimental conditions, which made it impracticable to perform complete comparative studies. Thus, we were compelled to select limited assay conditions, based on the results obtained in our preceding studies in this series (11, 12, 16).

Although the introduction of straight-chain fatty acid groups, such as L18-, L20-, L24- and L30-, to the MDP molecule did not decrease the pyrogenicity of the parent MDP molecule, the two test compounds having α-branched

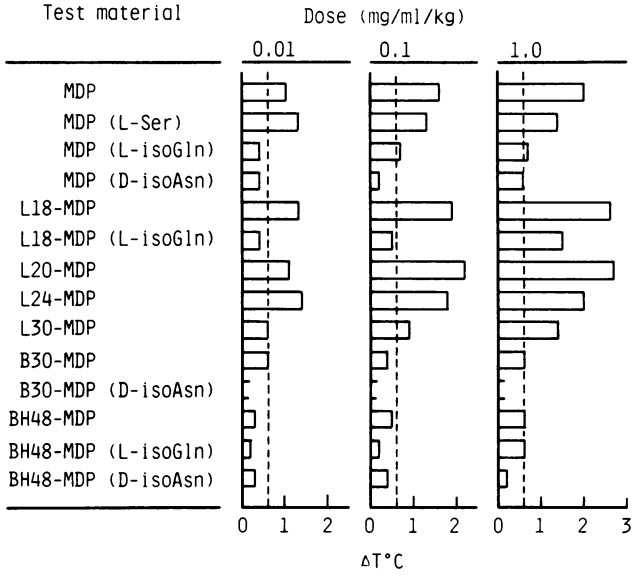

FIG. 7. Pyrogenicity of MDP, 6-*O*-acyl-MDPs, and their analogs in rabbits (intravenous injection). Each column shows the mean maximum rise in rectal temperature in two to six rabbits during a 6-h observation period. Dotted line, Boundary line of significant temperature rise (0.6°C) according to Japanese Pharmacopoeia X.

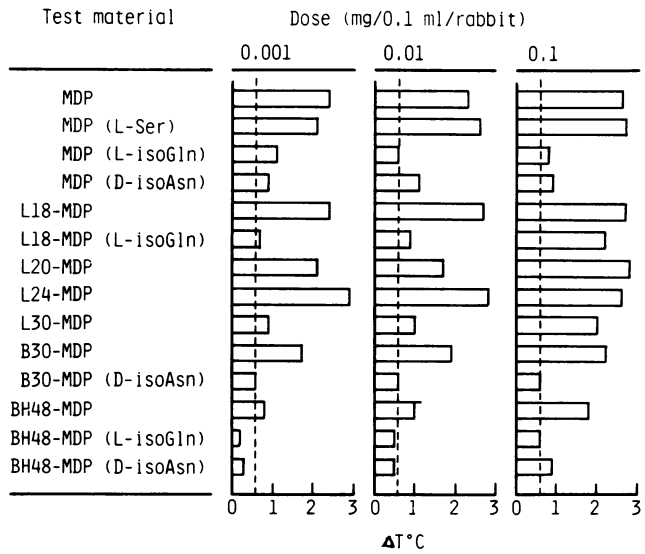

FIG. 8. Pyrogenicity of MDP, 6-*O*-acyl-MDPs, and their analogs in rabbits (intracerebroventricular injection). See the legend to Fig. 7 for details.

higher fatty acids at the C-6 position in the muramic acid residue (B30-MDP and BH48-MDP) were practically nonpyrogenic by intravenous injection. The nonpyrogenicity was not an inherent property of the molecules, because they were highly pyrogenic by administration via intracerebroventricular route under conditions in which B30-MDP(D-isoAsn), BH48-MDP(L-isoGln), and BH48-MDP(D-isoAsn) were found to be nonpyrogenic. The lack of pyrogenicity of B30- and BH48-MDPs after intravenous injection might be explained by the assumption that these compounds cannot pass through the blood-brain barrier, although this has not been proved experimentally.

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