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IMMUNOADJUVANT ACTIVITIES OF SYNTHETIC 6-O-ACYL-N-ACETYLMURAMYL-L-ALANYL-D-ISOGLUTAMINE WITH SPECIAL REFERENCE TO THE EFFECT OF ITS ADMINISTRATION WITH LIPOSOMES

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SUMMARY Addition of a lauroyl, stearoyl or docosanoyl group to the primary hydroxy group at the C-6 position of N-acetylmuramyl-L-alanyl-D-isoglutamine gave lipophilic derivatives that had definite adjuvancies in induction of delayed-type hypersensitivity and enhancement of antibody production against a test protein antigen, ovalbumin, when administered to guinea pigs as liposomes, that is without mineral oil. When administered as mineral oil-in-water emulsion, including Ribi-type emulsions, rather than as water-in-mineral oil emulsions, N-acetylmuramyl-L-alanyl-D-isoglutamine and its 6-O-acyl derivatives showed only weak immunoadjuvancies.

INTRODUCTION

The immunoadjuvant activities of bacterial cell walls or their components and especially the activities to induce cell-mediated immune responses are greatest when the materials are injected into test animals as water-in-mineral oil emulsions with antigens (cf., review by Kotani, 1976). But this method of administration of antigens and adjuvants leads to severe tissue reactions at the site of the sensitizing injection and swelling of the regional lymph nodes, making it impracticable to utilize the ad-

juvancies of cell wall components, of either natural or synthetic origin in humans.

In studies on the immunoadjuvancies of synthetic derivatives of N-acetylmuramyl-L-alanyl-D-isoglutamine, the minimum structure required for the adjuvant activity of bacterial cell walls, we found that some 6-O-acyl derivatives of the muramyl dipeptide showed definite adjuvancy to induce delayed-type hypersensitivity to ovalbumin in guinea pigs without the aid of the highly irritative

water-in-mineral oil emulsion. This paper describes the adjuvant activities of several synthetic 6-O-acyl-N-acetylmuramyl-L-alanyl-D-isoglutamines when given in various vehicles with ovalbumin to guinea pigs.

MATERIALS AND METHODS

1. *Synthesis of* **6-O-acyl-N-acetylmuramyl-L-alanyl-D-isoglutamine** *(Fig. 1)*

6 - O - Acyl - N -acetylmuramyl - L - alanyl-D-isoglutamines (**Ia–f**) were prepared by monoacylation at C-6 position of l-α-O-benzyl-N-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester (**III**) and then hydrogenolytic deprotection.

The starting compound (**III**) was obtained by heating 1-α-O-benzyl-4,6-O-benzylidene-N-acetyl-muramyl-L-alanyl-D-isoglutamine benzyl ester (**II**) in 60% acetic acid at 100 C for 30 min (Kusumoto

et al., 1976). Selective acylation of the primary hydroxy group at C-6 position of **III** was accomplished by treatment with excess of the corresponding acyl chloride in a mixture of pyridine and tetrahydrofuran at 0 C (or 12–15 C for the stearoyl and docosanoyl derivatives). Acetylation with acetic anhydride in pyridine at 0 C afforded the 6-O-monoacetyl derivative (**IVf**), whereas the 4,6-di-O-acetyl derivative (**IVg**) was obtained by treatment with the same reagent at 45 C. The acylation products (**IVa–g**) were then hydrogenolyzed in acetic acid in the presence of palladium black catalyst to afford the 6-O-acyl or 4,6-di-O-acetyl-N-acetyl-muramyl dipeptides (**Ia–g**), which were isolated as somewhat hygroscopic solids by lyophilization. Some of their physical constants are summarized in Table 1. The details of the synthesis will be published elsewhere (Kusumoto, Okada and Shiba, in press).

TABLE 1. *Physical properties of O-acyl-N-acetylmuramyl dipeptides*

O-Acyl-N-acetylmuramyl dipeptide		Mp (C)	$[\alpha]_D$ (c 0.5, H_2O)	Rf value[a]
6-O-docosanoyl	(**Ia**)	153–155	+37.0°	0.49
6-O-stearoyl	(**Ib**)	133–136	+29.3°	0.35
6-O-lauroyl	(**Ic**)	107–110	+35.6°	0.29
6-O-octanoyl	(**Id**)	66– 72	+32.6°	0.22
6-O-butyryl	(**Ie**)	105–109	+35.6°	0.18
6-O-acetyl	(**If**)	132–134	+38.6°	0.14
4, 6-di-O-acetyl	(**Ig**)	111–116	+31.2°	0.17

[a] Silica gel G, solvent: $CHCl_3$-CH_3OH-CH_3CO_2H (100: 20: 1, v/v)

FIGURE 1 Synthesis of 6-O-acyl-N-acetylmuramyl-L-alanyl-D-isoglutamine. Bzl: $C_6H_5CH_2$. a: R^1=docosanoyl, R^2=H; b: R^1=stearoyl, R^2=H; c: R^1=lauroyl, R^2=H; d: R^1=octanoyl, R^2=H; e: R^1=butyryl, R^2=H; f: R^1=acetyl, R^2=H; g: R^1=R^2=acetyl

2. Preparation of various vehicles for administration of 6-O-acyl-N-acetylmuramyl dipeptides and ovalbumin to guinea pigs

Liposomes: These were prepared in the following way, on the basis of the description of Inoue (1974). Lecithin (DL-α-phosphatidyl choline, dipalmitoyl, Grade I approx. 99%, Sigma Chemical Co., St. Louis, Mo., U.S.A.) 10 μmoles (7.3 mg) and 10 μmoles (3.9 mg) cholesterol (Grade 99+%, Sigma) were dissolved in about 5 ml of chloroform in a 10-ml round-bottomed flask. The solvent was distilled off by evaporation in a rotary vacuum evaporator at below 30 C, to leave a thin film of lecithin and cholesterol on the inside wall of the flask. A solution (2 ml) containing ovalbumin (crystalline, Grade V, Sigma) and test synthetic adjuvants (varying doses) in 1/75 M phosphate buffered saline (pH 7.0) at about 55 C was added little by little to the flask also at 55 C, with gentle heating. The contents of the flask were then submitted to gentle mixing with a THERMO-MIXER (Model TM-100, Tokyo Thermonics Co, Tokyo) to give a liposome suspension containing the test antigen and adjuvant. In some experiments with 6-O-stearoyl or 6-O-docosanoyl-N-acetylmuramyl dipeptide, the test adjuvant was incorporated into a lipid film instead of phosphate buffered saline. When diacetylphosphate (Sigma), phosphatidic acid (from egg lecithin, Grade approx. 70%, Sigma) or stearylamine (Wako Pure Chemical Industries, Osaka) was used as additive, 1.0 μmole aliquots of these materials were dissolved in chloroform with 10 μmoles each of lecithin and cholesterol.

In some experiments, lipids extracted from erythrocytes of guinea pigs or sheep by the method of Bligh and Dyer (1959), and generously supplied by Dr. T. Miyama, Department of Bacteriology, Nara Medical University, were employed for preparation of liposomes. Amounts of 11 mg and 12 mg of these lipids were used to prepare 2 ml of the respective liposome suspensions.

Mineral oil-in-water suspension: Two methods were used to prepare mineral oil-in-water emulsions; one was the method of Mayer et al. (1974) which was essentially that of Ribi et al. (1971), except that test 6-O-acyl derivatives of the N-acetylmuramyl dipeptide were added to a mixture of chloroform and methanol (94:6, v/v) and the solvent was evaporated off under a stream of nitrogen gas. This modification was used to ensure contact of test adjuvants with the mineral oil added later, as re-

commended by Granger et al. (1975). The other type of mineral oil-in-water emulsion was prepared by mixing mineral oil, a mixture of Tween 80 (Wako) and Span 80 (Wako) (7:3, w/w) and 1/75 M phosphate buffered saline, pH 7.0, in a ratio of 4:1:5 (v/v) by sonication.

Water-in-mineral oil emulsion: This was prepared as described in a previous paper (Kotani et al., 1975).

In preparation of the oil-in-water and water-in-oil emulsions described above, Drakeol 6VR (Penreco., Los Angeles, Cal., U.S.A.) was used as a light mineral oil. For the Ribi type oil-in-water emulsion, test adjuvants were added to Drakeol as described, but for other emulsions, adjuvants were added to the phosphate buffered saline with ovalbumin.

3. Immunological methods

Groups of 5 female guinea pigs (200–300 g) were used for assays. Corneal and skin tests were done as described previously (Kotani et al., 1975), except that the corneal response was graded from the mean
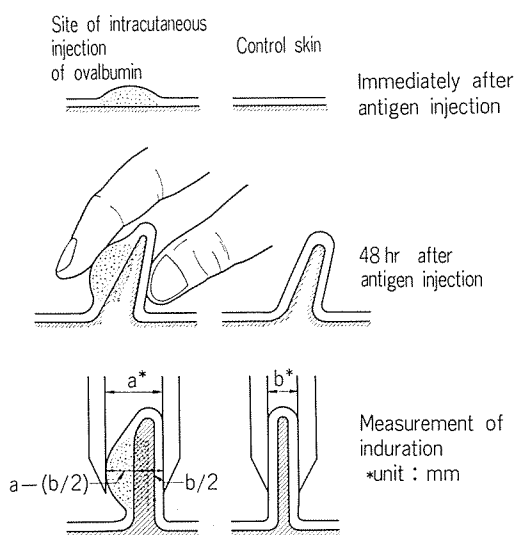

Site of intracutaneous injection of ovalbumin / Control skin

Immediately after antigen injection

48 hr after antigen injection

Measurement of induration *unit : mm

a^* b^*

$a-(b/2)$ $b/2$

FIGURE 2 Measurement of the extent of induration at the site of intracutaneous injection of ovalbumin solution (100 μg/0.1 ml). The thickness of test and control skins was measured with a vernier calliper as illustrated. The extent of induration was calculated from the formula, $(a-b/2)/(b/2) = 2(a/b) - 1$.

score calculated on the basis of the stronger of the two reactions exhibited by each animal 2 and 3 weeks after the immunization and the skin reaction was evaluated by measuring the extent of induration, as illustrated in Fig. 2. At the time of the second corneal test, the reactions at the sites of the sensitizing injections of ovalbumin and adjuvants in various vehicles and those in the regional lymph nodes were examined.

Circulating anti-ovalbumin precipitin levels were estimated by reversed-type single radial immunodiffusion (Vaerman et al., 1969; Nariuchi et al., 1970). Amounts of 60–600 μg nitrogen of antibody/ml could be measured accurately using agar
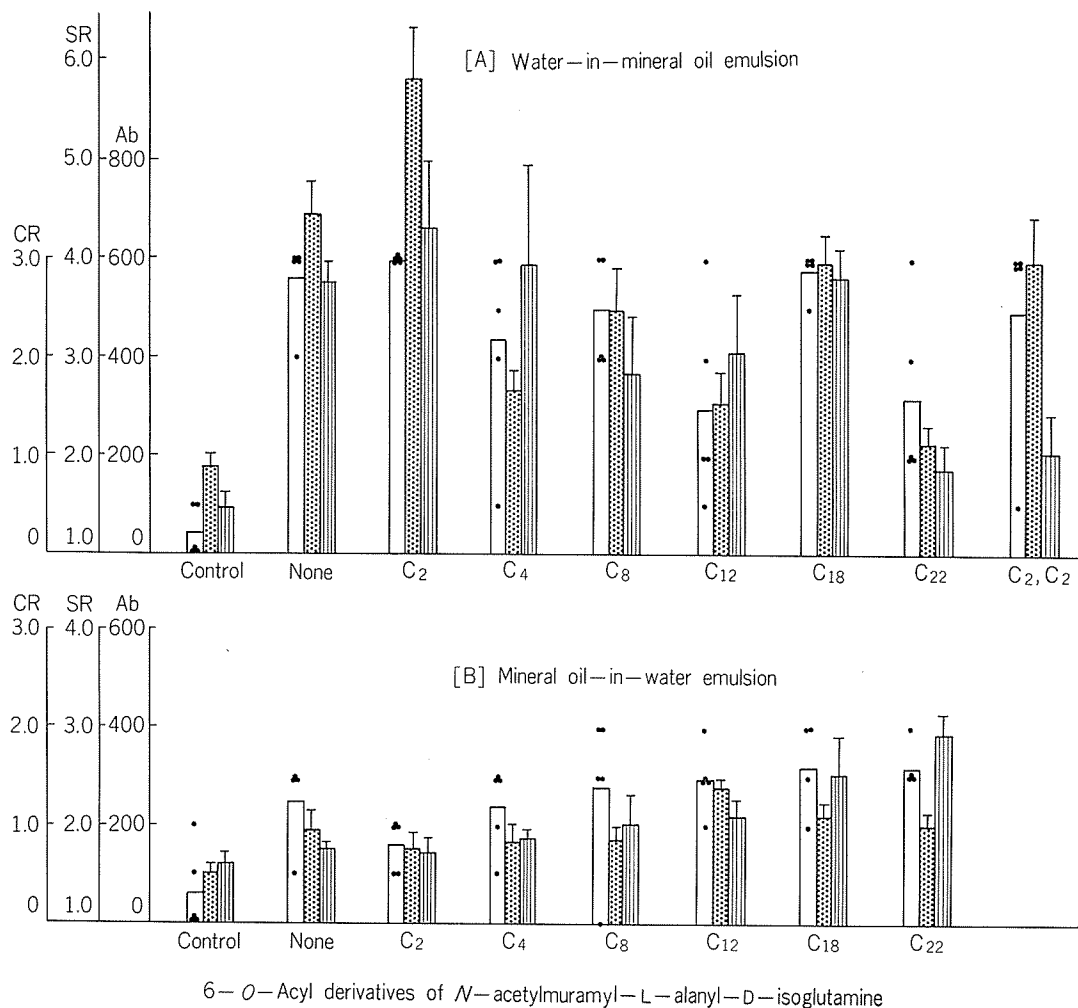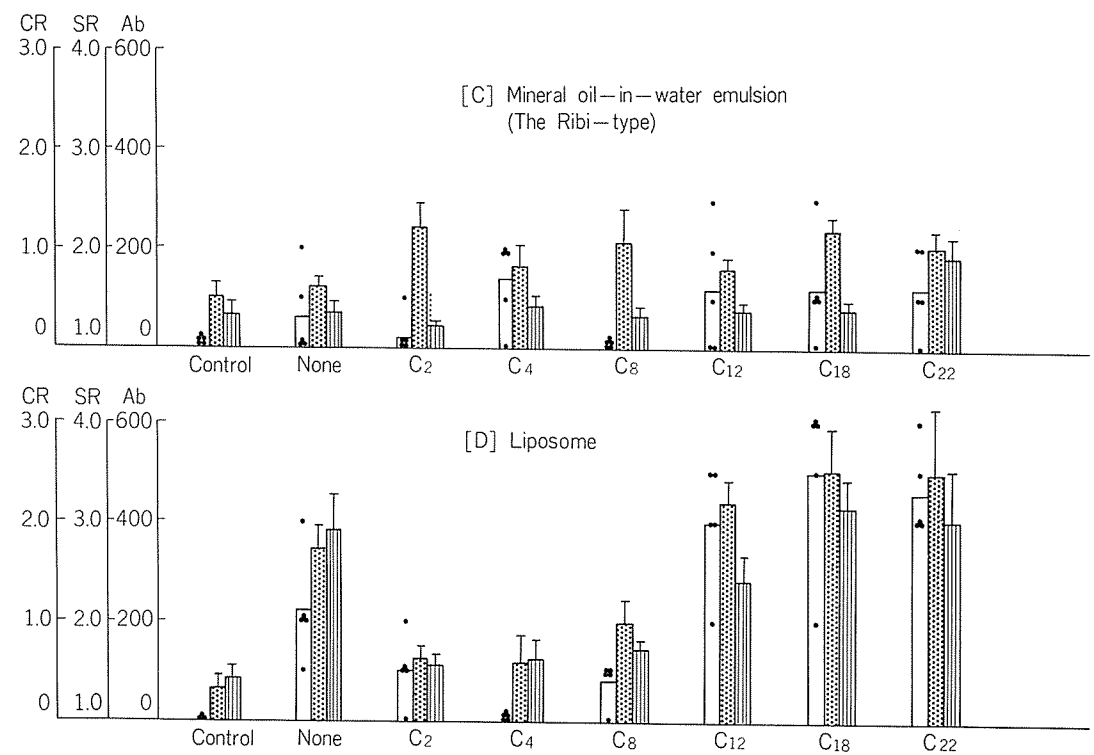

FIGURE 3. Immunoadjuvancies of 6-O-acyl derivatives of N-acetylmuramyl-L-alanyl-D-isoglutamine administered to guinea pigs in various vehicles with ovalbumin. CR, ☐ corneal response (48 hr reading), SR, ▨ skin reaction (induration, 48 hr reading), Ab, ▤ serum antibody level (μg antibody nitrogen/ml serum), ⟩––┤ +1 standard error and • the corneal response of an individual animal. None: N-acetylmuramyl-L-alanyl-D-isoglutamine, C_2: 6-O-acetyl, C_4: 6-O-butyryl, C_8: 6-O-octanoyl, C_{12}: 6-O-lauroyl, C_{18}: 6-O-stearoyl, C_{22}: 6-O-docosanoyl, C_2, C_2: 4,6-di-O-acetyl derivatives of the above N-acetylmuramyl dipeptide.

plates containing 5 μg ovalbumin/ml, where the antigen in the agar and the antiserum (3–6 μl) in the circular wells were allowed to react at room temperature for 48 hr in a moist chamber. With some representative serum specimens, parallel determinations were made of the antibody contents by the reversed-type single radial immunodiffusion and the quantitative precipitin method described previously (Kotani et al., 1975), with fairly good agreement between the values obtained.

RESULTS

Figure 3 summarizes the results of assays of

oil emulsions [A], mineral oil-in-water emulsions [B and C], or in liposomes [D]. The dosages of test adjuvants per animal was adjusted to be equimolar to 100 μg of N-acetylmuramyl-L-alanyl-D-isoglutamine in Experiments [A], [B] and [C], and to 200 μg of 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine in Experiment [D]. The enhancement of immune responses was evaluated in terms of the corneal and skin reactions to ovalbumin, regarding delayed-type hypersensitivity and the serum anti-ovalbumin antibody levels as indices of humoral immunity.

the immunoadjuvancies of 6-O-acyl (acetyl, butyryl, octanoyl, lauroyl, stearoyl and docosanoly)-N-acetylmuramyl-L-alanyl-D-isoglutamines and a 4, 6-di-O-acetyl derivative to stimulate cell-mediated immune response and antibody production against ovalbumin when administered to guinea pigs as water-in-mineral

The following conclusions can be drawn from the findings presented in Fig. 3. 1) When a water-in-mineral oil emulsion was used as a vehicle for the administration of ovalbumin and a test adjuvant, all the 6-O-acyl-N-acetylmuramyl-L-alanyl-D-isoglutamines exhibited definite immunoadjuvancies

although the replacement of the primary hydroxy group of C-6 of N-acetylmuramyl-L-alanyl-D-isoglutamine by a butyryl, lauroyl or docosanoyl group caused some decrease in the adjuvancies of the molecule and monoacetylation at C-6 position of the muramyl dipeptide resulted in some increase in the adjuvancies (a 4,6-di-O-acetyl-N-acetylmuramyl dipeptide seemed to be less active than the 6-O-acetyl derivative) [A]. 2) All test adjuvants except the 6-O-docosanoyl derivative showed weaker adjuvancies when administered as mineral oil-in-water emulsions than when given as water-in-mineral oil emulsions (the reverse was true for the 6-O-docosanoyl derivative), the Ribi-type emulsion being especially ineffective as a vehicle for administration of the N-acetylmuramyl dipeptides and the test acyl derivatives [B and C]. 3) When test adjuvants were given in liposomes consisting of lecithin and cholesterol, 6-O-lauroyl, 6-O-stearoyl and 6-O-docosanoyl-N-acetylmuramyl-L-alanyl-D-isoglutamines had definitely adjuvant activities in

both induction of delayed-type hypersensitivity and stimulation of serum antibody levels, whereas the other 6-O-acyl-N-acetylmuramyl dipeptides had hardly any activity [D].

The effect of the composition of the liposomes used for administration of 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine on the immunoadjuvancies were then studied. Figure 4 shows that the effectiveness of liposomes as a vehicle for 6-O-stearoyl derivatives was not increased significantly by addition of dicetylphosphate, phosphatidic acid or stearylamine. An interesting finding in Fig. 4 is that a lipid fraction isolated from the ghosts of guinea pig erythrocytes seemed more effective than synthetic lipids in preparation of liposomes as a vehicle for 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine. A similar lipid fraction from the membranes of sheep erythrocytes was ineffective.

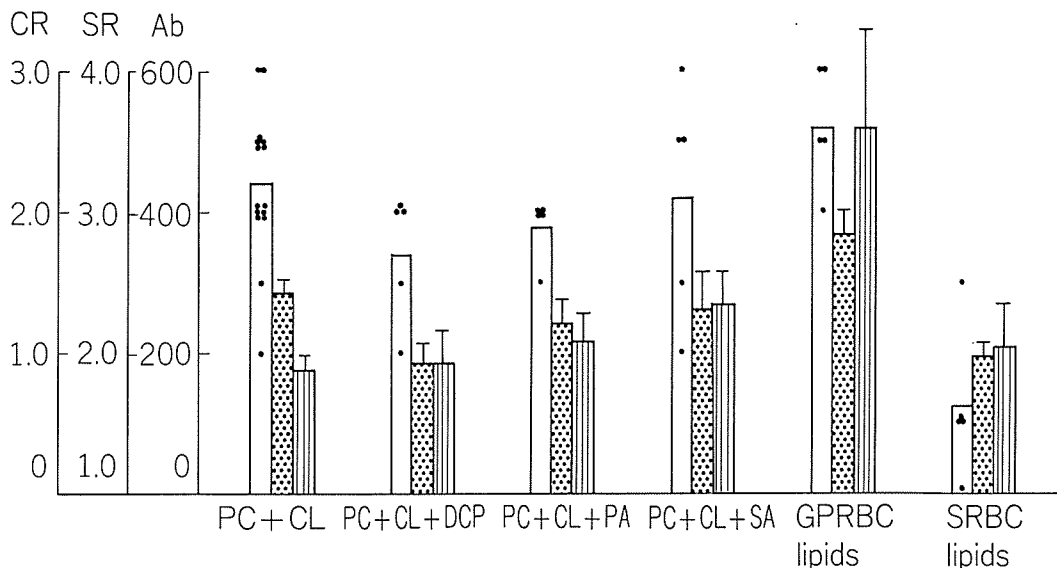Table 2 shows the tissue responses at the site of sensitizing injection (the foot pad) and

FIGURE 4 The effects of the composition of liposomes as a vehicle on the immunoadjuvancies of 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine. Amounts of 200 μg and 1 mg of ovalbumin per guinea pigs, respectively, were used. PC: DL-α-phosphatidyl choline, dipalmitoyl, CL: cholesterol, DCP: dicetylphosphate, PA, phosphatidic acid, SA: stearylamine, GPRBC: guinea pig red blood cells, SRBC: sheep red blood cells. For other details see Fig. 3.

TABLE 2. *Reactions at the site of sensitizing injection and in the regional lymph nodes of guinea pigs after injection of test adjuvants and ovalbumin in various vehicles into the foot pads*

Acyl group at C-6 of MurNAc-L-Ala-D-isoGln	w/o emulsion		o/w emulsion		o/w emulsion (Ribi type)		liposomes (PC+CL)[c]	
	[A][a]	[B][a]	[A]	[B]	[A]	[B]	[A]	[B]
None	2.3	1.7 (0.5–3.0)	1.4	0.8 (0 –1.5)	1.3	0	1.1	0
Acetyl	2.7	1.6 (1.0–2.0)	1.5	0.4 (0 –1.0)	1.0	0.4 (0 –0.5)	1.0	0.1 (0 –0.5)
Butyryl	2.0	0.9 (0.5–1.5)	1.4	0.5 (0.5–1.0)	1.1	0.1 (0 –0.5)	1.0	0
Octanoyl	2.1	1.4 (1.0–2.0)	1.6	0.7 (0 –1.5)	1.2	0	1.1	0.1 (0 –0.5)
Lauroyl	1.9	1.0 (0.5–1.5)	1.7	0.2 (0 –0.5)	1.2	0	1.3	0.9 (0.5–1.5)
Stearoyl	2.2	1.5 (1.0–2.0)	1.4	0.4 (0 –0.5)	1.2	0.7 (0.5–1.0)	1.2	0.9 (0.5–1.5)
Docosanoyl	2.1	0.7 (0 –1.0)	1.9	0.1 (0 –0.5)	1.2	0.4 (0 –0.5)	1.2	0.1 (0 –0.5)
Control[b]	1.5	1.1 (0.5–1.5)	1.6	0.1 (0 –0.5)	1.1	0.1 (0 –0.5)	1.0	0

[a] [A]: Ratio of the cross section of the foot after injection of ovalbumin and a test adjuvant in various vehicles to that of the control foot.
 [B]: Swelling of the regional lymph nodes. Mean value (range). The swelling was arbitrarily graded from 0 for no swelling to 3.0 for marked swelling.
[b] A control containing ovalbumin, but no adjuvants.
[c] PC: DL-α-phosphatidyl choline, dipalmitoyl, CL: cholesterol.

in the regional lymph nodes in the guinea pigs whose corneal and skin responses and serum antibody levels were presented in Fig. 3. Examinations for the tissue responses were carried out 3 weeks after the sensitization. In all cases, injection of ovalbumin and test adjuvants as water-in-mineral oil emulsions caused the strong reactions at the sites of immunogen injection and regional lymph nodes, but their injection in liposomes or as mineral oil-in-water emulsions induced only slight reactions at the site of injection and in the regional lymph nodes.

Preliminary experiments reveal that 6-O-stearoyl-N-acetylmuramyl-L-alanyl-D-isoglutamine included in liposomes with ovalbumin enhanced both the humoral and the cellular immune responses to the test antigen, when injected intraperitoneally into guinea pigs. However, the same liposome preparation with the 6-O-stearoyl-derivative had no adjuvant activity when given intravenously.

A preliminary study also showed that the 6-O-docosanoyl-N-acetylmuramyl dipeptide (430 μg dose with 2 mg ovalbumin per guinea

pig) induced a definitely positive corneal response and stimulated precipitin production against ovalbumin when injected intraperitoneally "suspended" in 1/75 M phosphate buffered saline, pH 7.0, with ovalbumin (the 6-O-docosanoyl derivative formed micelles instead of a solution in saline). Under the same experimental conditions, N-acetylmuramyl-L-alanyl-D-isoglutamine was inactive even at the high dose of 5 mg per animal, and 6-O-stearoyl muramyl dipeptide showed very little if any adjuvancy for induction of delayed-type hypersensitivity.

DISCUSSION

This work showed that the replacement of the primary hydroxy group at the C-6 position of N-acetylmuramyl-L-alanyl-D-isoglutamine by a lauroyl, stearoyl or docosanoyl group gave a muramyl dipeptide derivative with adjuvant activities in induction of delayed-type hypersensitivity and enhancement of antibody production against ovalbumin in guinea pigs, without the aid of a water-in-mineral oil emul-

sion, that is to say, when given in liposomes. There are reports that liposomes themselves have adjuvant effects on protein antigens (Allison and Gregoriadis, 1974; Heath et al., 1976); but the possibility that our positive results are due to the adjuvant activity of liposomes themselves is excluded by the fact that the control, liposomes containing ovalbumin but no test compounds, had no adjuvant activity, and that 6-*O*-acetyl, 6-*O*-butyryl or 6-*O*-octanoyl derivatives of the *N*-acetylmuramyl dipeptide had scarcely any adjuvant activity when administered in liposomes.

The intensity of delayed-type hypersensitivity and the extent of elevation of serum antibody levels by 6-*O*-stearoyl (or lauroyl or docosanoyl) -*N*-acetylmuramyl-L-alanyl-D-isoglutamine included in liposomes with ovalbumin were comparable to, or even more than, those induced by the corresponding 6-*O*-acyl derivatives administered as water-in-mineral oil emulsions, but were less than those induced by *N*-acetylmuramyl-L-alanyl-D-isoglutamine given as a water-in-mineral oil emulsion. Attempts to improve the effectiveness of the vehicle for 6-*O*-stearoyl-*N*-acetylmuramyl dipeptide by changing the composition of liposomes have so far been unsuccessful. No information is available about what proportions of the added ovalbumin and test adjuvants were actually included in liposomes in the present study.

The adjuvant activities of 6-*O*-lauroyl, 6-*O*-stearoyl and 6-*O*-docosanoyl derivatives of *N*-acetylmuramyl-L-alanyl-D-isoglutamine when administered with liposomes, unlike derivatives in which the primary hydroxy group on C-6 is unchanged or replaced by only a short saturated fatty acid, can be understood on the basis on their lipophilicity (Table 1).

The administrations of test adjuvants as liposomes or mineral oil-in-water emulsions elicited far less reactions at the site of injection and in the regional lymph nodes than injection of the same adjuvants as water-in-mineral oil emulsions. Although the findings indicate that liposomes and mineral oil-in-water emulsions are by themselves less injurious, it should be pointed out that the reactions at the site of the sensitizing injection and in the regional lymph nodes observed 3 weeks after sensitization could be partly due to the reaction of ovalbumin deposited at these sites and the ensuing state of hypersensitivity.

The immunological adjuvancies and antitumor activities of semisynthetic 6-*O*-mycolyl derivatives of *N*-acetylmuramyl-L-alanyl-D-isoglutamine which were prepared by Kusumoto et al. (1976) have been reported by Yamamura et al. (1976, 1977) in separate papers.

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