Adjuvant Activities of Quinonyl-*N*-Acetyl Muramyl Dipeptides in Mice and Guinea Pigs

IKUO SAIKI,^{1*} YOSHIRO TANIO,² MIKIO YAMAWAKI,² MASANAO UEMIYA,² SHIGERU KOBAYASHI,² TSUNEHICO FUKUDA,² HIDEFUMI YUKIMASA,² YUICHI YAMAMURA,² AND ICHIRO AZUMA¹

Institute of Immunological Science, Hokkaido University, Kita-ku, Sapporo 060,¹ and Third Department of Internal Medicine, Osaka University Medical School, Fukushima-ku, Osaka 553,² Japan

The adjuvant and tumor-suppressive activities of the quinonyl [2,3-dimethoxy-5-methyl-6-(9'-carboxynonyl)-1,4-benzoquinone (QS-10)] derivatives of *N*-acetyl muramyl dipeptides were examined. *N*-Acetyl muramyl-L-valyl-D-isoglutamine (MurNAc-L-Val-D-isoGln), QS-10-MurNAc-L-Val-D-isoGln, and their methyl esters were shown to have potent adjuvant activity on the induction of delayed-type hypersensitivity to monoazobenzenearsonate-*N*-acetyl-L-tyrosine in guinea pigs and on the primary immune response against sheep erythrocytes *in vitro*; however, only QS-10-MurNAc-L-Val-D-isoGln methyl ester, i.e., QS-10-MurNAc-L-Val-D-Glu(OCH₃)NH₂ (quinonyl-MDP-66), was shown to be an active adjuvant for the induction of allogeneic killer T cells in mice and the suppression of tumor growth in syngeneic mice when it was administered as a suspension in phosphate-buffered saline. The effectiveness of the chain length of the quinonyl moiety in quinonyl-MDP-66 and the replacement of the L-valine residue with L-serine or L-threonine were also examined in comparison with the adjuvant and tumor-suppressive activities of quinonyl-MDP-66.

Synthetic *N*-acetyl muramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln) was demonstrated to be the minimal structure of bacterial peptidoglycans required for adjuvant activity on immune responses (1, 2, 13, 19).

Previously, MurNAc-L-Ala-D-isoGln was shown to be an active adjuvant for the induction of delayed-type hypersensitivity to monoazobenzenearsonate-*N*-acetyl-L-tyrosine (ABA-*N*-acetyl-L-tyrosine) in guinea pigs and for circulating antibody formation to bacterial α -amylase; however, it was inactive as an adjuvant for cell-mediated cytotoxicity in allogeneic mice *in vivo* and for suppression of tumor growth in syngeneic mice (5, 8).

Recently, we examined the effectiveness on the adjuvant activity of the replacement of the L-alanine residue by L-serine, L-valine, or glycine in MurNAc-L-Ala-D-isoGln and its 6-*O*-“mycoloyl” derivatives (6-*O*-mycoloyl, 6-*O*-nocardomycoloyl, and 6-*O*-corynomycoloyl) (6, 8, 21, 25). It was shown that 6-*O*-mycoloyl-MurNAc-L-Ala-D-isoGln, 6-*O*-“mycoloyl”-MurNAc-L-Ser-D-isoGln, and 6-*O*-“mycoloyl”-MurNAc-L-Val-D-isoGln were less effective on the humoral immune response or on the function of carrier-primed helper T cells, but active as adjuvants for cell-mediated cytotoxicity in allogeneic mice and for induction of delayed-type hypersensitivity to ABA-*N*-acetyl-L-tyrosine in guinea pigs. In contrast, MurNAc-L-Ala-D-isoGln, MurNAc-

L-Ser-D-isoGln, and MurNAc-L-Val-D-isoGln were inactive as adjuvants for cell-mediated cytotoxicity in allogeneic mice, but were active for the humoral immune response and for the induction of delayed-type hypersensitivity to ABA-*N*-acetyl-L-tyrosine in guinea pigs.

Consequently, we have concluded that the introduction of the “mycolic acid” residue as the lipophilic group into *N*-acetyl muramyl dipeptides modified qualitatively the adjuvant activity on the immune response, predominantly on the cellular immune response (6, 26).

It has also been reported that related compounds of ubiquinone, such as QS-10 [2,3-dimethoxy-5-methyl-6-(9'-carboxynonyl)-1,4-benzoquinone], QS-3 [2,3-dimethoxy-5-methyl-6-(2'-carboxyethyl)-1,4-benzoquinone], Q acid-I [2,3-dimethoxy-5-methyl-6-(5'-carboxy-3'-methyl-2'-phenyl)-1,4-benzoquinone], and Q acid-II [2,3-dimethoxy-5-methyl-6-(3'-carboxybutyl)-1,4-benzoquinone], have an additional effect on the humoral immune response besides their various biological activities, such as effects on the lysosomal membrane, the heart cyclic adenosine 3',5'-monophosphate phosphodiesterase, and the mitochondrial electron transport system (3, 15, 22). More recently, we determined the adjuvant activity of the compounds in which the L-alanine residue in MurNAc-L-Ala-D-isoGln was replaced by various kinds of amino acids (4). The results obtained indicated that the L-valine,

L-threonine, and L-serine analogs of *N*-acetyl muramyl dipeptide have potent adjuvant activity on the induction of delayed-type hypersensitivity to ABA-*N*-acetyl-L-tyrosine in guinea pigs.

In a previous communication (9), MurNAc-L-Val-D-isoGln, its methyl ester MurNAc-L-Val-D-Glu(OCH₃)NH₂, and their QS-10 derivatives were shown to be active for the development of delayed-type hypersensitivity in guinea pigs. However, only quinonyl-MDP-66 (Fig. 1) showed adjuvant activity for the induction of killer T cells in allogeneic cell-mediated cytotoxicity and potent suppressive activity of tumor growth in syngeneic mice. In this study, we will describe the details of the adjuvant and tumor-suppressive activities of quinonyl-MDP-66 and its related compounds.

MATERIALS AND METHODS

Adjuvants. The chemical syntheses of synthetic adjuvants used in this study were performed by the procedures reported by Kobayashi et al. (18). The *Nocardia rubra* cell wall skeleton was prepared by the method described previously (7). The *N. rubra* cell wall skeleton and the quinonyl derivatives of *N*-acetyl muramyl dipeptide methyl esters are insoluble in water, and these adjuvants were used as suspensions in phosphate-buffered saline (PBS) in most experiments. Other adjuvants, *N*-acetyl muramyl dipeptides, and their quinonyl derivatives are soluble in water and were used as solutions in water.

Medium solution. Eagle minimal essential medium and RPMI 1640 medium were obtained from Flow Laboratories, Inc., McLean, Va. They were supplemented with 100 U of penicillin G and 100 µg of streptomycin per ml. Fetal bovine serum (lot R781615) was purchased from GIBCO Laboratories, Grand Island, N.Y., and inactivated by heating at 56°C for 30 min before use.

Animals. Six- to eight-week-old, female inbred mice of C57BL/6J, DBA/2, BALB/c, and Hartley strain guinea pigs, weighing 350 to 400 g, were purchased from Shizuoka Experimental Animal Cooperative, Hamamatsu, Japan. The animals were given food (Nihon Nosan Kogyo Co. Ltd., Yokohama, Japan) and water freely.

Antigens. Crystalline bacterial α-amylase prepared from *Bacillus subtilis* was purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. ABA-*N*-acetyl-L-tyrosine and ABA-bacterial α-amylase were prepared by the method described previously (23). Sheep erythrocytes (SRBC) preserved in Alsever solution were obtained from Nippon Bio-Test Laboratories Inc., Tokyo, Japan, and were washed three times with PBS before use.

Tumors. Mastocytoma P815-X2 of DBA/2 mice and Meth-A fibrosarcoma of BALB/c mice were serially passed in the ascitic form in syngeneic mice.

Determination of adjuvant activity on induction of delayed-type hypersensitivity. Guinea pigs were immunized into four footpads with a total of 50 µg of ABA-*N*-acetyl-L-tyrosine with or without various

FIG. 1. Chemical structure of quinonyl-MDP-66.

adjuvants in Freund incomplete adjuvant. After 2 weeks, skin tests were carried out with 100 µg of ABA-bacterial α-amylase and the reactions (induration) were measured at 24 and 48 h after intradermal injection of the test antigen.

Determination of antibody response in vitro and in vivo. The in vitro culture technique described by Marbrook (20) was used. Briefly, 2×10^7 spleen cells of normal C57BL/6J mice and 4×10^6 SRBC were suspended in 1 ml of RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum in the presence or absence of 10 and 100 µg of adjuvants and cultured for 4 days. Plaque-forming cells against SRBC were assayed by the method of Jerne and Nordin (16) with some modifications as described previously (5).

For the determination of antibody response in vivo, four female C57BL/6J mice were sensitized intraperitoneally with SRBC (10^8) suspended in PBS with or without adjuvants. After 2 weeks, blood was obtained by micropipette from the retro-orbital vein. A hemagglutinin test against SRBC was performed by a microtitration method.

Cell-mediated cytotoxicity test. C57BL/6J inbred mice ($H-2^d$) were immunized intraperitoneally with viable cells of mastocytoma P815-X2 ($H-2^d$) in the presence or absence of a suspension of various adjuvants in PBS. On day 11 after immunization, a cell-mediated cytotoxicity assay was carried out, using the spleen cells of immunized mice and ^{51}Cr -labeled mastocytoma P815-X2 cells by the method of Brunner et al. (10) with some modifications. The ratio of spleen cells (effector cells) to ^{51}Cr -labeled mastocytoma P815-X2 cells (target cells) was 100:1. Target cell lysis was expressed as the percentage of specific target cell lysis by the following formula: percentage of specific target cell lysis = [(release with effector cells - control release)/(maximum release - control release)] $\times$ 100. Maximum chromium release was measured at complete cell lysis when target cells were frozen and thawed three times.

Assay for footpad reaction. Four female C57BL/6J mice were sensitized intraperitoneally with SRBC (10^8) suspended in PBS with or without adjuvants. After 4 days, an eliciting dose of 10^8 SRBC in a volume of 25 µl was injected into one hind footpad. The degree of swelling was measured at 24 and 48 h after challenge. The footpad reaction was expressed as the difference in thickness between one foot injected with SRBC and the other injected with PBS.

Suppression test of transplantable tumor in syngeneic mice. A mixture of tumor cells (Meth-A) and adjuvants suspended in PBS was inoculated intradermally into the flanks of BALB/c mice. Tumor sizes at the inoculated site and survival rates were examined every week. The results shown in the tables were obtained at 4 weeks after tumor inoculation.

RESULTS

Adjuvant activity of quinonyl derivatives of *N*-acetyl muramyl dipeptides. Table 1 shows the effects of synthetic adjuvants such as MurNAc-L-Val-D-isoGln, its methyl ester MurNAc-L-Val-D-Glu(OCH₃)NH₂, and their quinonyl derivatives on the primary immune response against SRBC in vitro. All of the synthetic adjuvants showed adjuvant activity on the formation of 19S plaque-forming cells to SRBC after 4 days of culture in vitro. In particular, QS-10-MurNAc-L-Val-D-isoGln (group 3b) showed remarkable adjuvant activity at a 10-μg dose. The effect of the chain length of the quinonyl moiety in quinonyl-MDP-66 on the adjuvant and tumor-suppressive activities is shown in Table 2. The short-chain quinonyl (QS-3) derivative of MurNAc-L-Val-D-Glu(OCH₃)NH₂ [QS-3-MurNAc-L-Val-D-Glu(OCH₃)NH₂ (group 1)], as well as quinonyl-MDP-66, was shown to have potent adjuvant activity on the induction of delayed-type hypersensitivity in guinea pigs. However, this derivative is not effective for the suppression of syngeneic tumors in this experimental system. The adjuvant and tumor-suppressive activities of the compounds in which

the L-valine residue in quinonyl-MDP-66 was replaced with L-serine or L-threonine were determined in comparison with those of quinonyl-MDP-66 (Table 2). Both the L-serine and the L-threonine analogs of quinonyl-MDP-66 (groups 3 and 4) were also shown to be active as adjuvants for the induction of delayed-type hypersensitivity in guinea pigs; however, the L-serine analog was less active for the suppression of Meth-A in BALB/c mice when administered as a suspension in PBS. Table 3 shows the effect of the compounds in which MurNAc-L-Val-D-isoGln or MurNAc-L-Val-D-Glu(OCH₃)NH₂ was combined with the quinonyl moiety (QS-10) via glycine or leucine as a spacer on the adjuvant and tumor-suppressive activities. Three kinds of compounds in which MurNAc-L-Val-D-Glu(OCH₃)NH₂ was combined with QS-10 (groups 4 through 6) had both potent adjuvant activity on the generation of allogeneic cell-mediated cytotoxicity and suppressive activity of tumor growth, as compared with other compounds (groups 1 through 3). The adjuvant and tumor-suppressive activities of the compounds in which the γ-carboxylic acid of D-isoglutamine in QS-10-MurNAc-L-Val-D-isoGln was esterified with aliphatic alcohol (methanol, ethanol, isopropanol, hexanol, or methoxyethanol) are shown in Table 4. All synthetic compounds had not only adjuvant activity on the induction of delayed-type hypersensitivity in guinea pigs but also suppressive activity of tumor growth in syngeneic mice. However, the compound which esterified with methoxyethanol (group 5) was

TABLE 1. Adjuvant activity of MurNAc-L-Val-D-isoGln derivatives on primary immune response to SRBC in vitro

Group	Adjuvant	Dose (μg)	Activity ^a
1a	OH MurAc-L-Val-D-Glu-NH ₂	100	1,733 ± 393 (5.3) ^b
b	MurAc-L-Val-D-Glu-NH ₂	10	848 ± 155 (2.6)
2a	OCH ₃ MurNAc-L-Val-D-Glu-NH ₂	100	4,100 ± 675 (12.5)
b	MurNAc-L-Val-D-Glu-NH ₂	10	3,706 ± 429 (11.3)
3a	OH QS-10-MurAc-L-Val-D-Glu-NH ₂	100	3,100 ± 278 (9.5)
b	QS-10-MurAc-L-Val-D-Glu-NH ₂	10	7,613 ± 1,034 (23.2)
4a	OCH ₃ QS-10-MurNAc-L-Val-D-Glu-NH ₂ (quinonyl-MDP-66)	100	3,473 ± 197 (10.6)
b	QS-10-MurNAc-L-Val-D-Glu-NH ₂ (quinonyl-MDP-66)	10	2,900 ± 440 (8.8)
5	Control		328 ± 35 (1.0)

^a Each value represents the arithmetic (mean 19S plaque-forming cells per culture) of triplicate cultures ± standard error of the mean.

^b Numbers given within parentheses are stimulation ratios.

TABLE 2. *Adjuvant and tumor-suppressive activities of QS-10-N-acetyl muramyl dipeptides*

Group	Adjuvant	Dose (μ g)	Skin reaction with ABA-bacterial α -amylase at 24 h (mm $\pm$ SE) ^a	Suppression of Meth-A growth in BALB/c mice
1	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-3-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	22.6 $\pm$ 0.7	0/10 ^b
2	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \text{ (quinonyl-MDP-66)} \end{array}$	100	24.4 $\pm$ 1.2	10/10
3	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Ser-D-Glu-NH}_2 \end{array}$	100	23.8 $\pm$ 0.6	3/10
4	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Thr-D-Glu-NH}_2 \end{array}$	100	21.1 $\pm$ 0.8	6/10
5	Control		0	0/10

^a Values show the degree of delayed hypersensitivity. SE, Standard error.^b Values represent the ratio of the number of tumor-free mice to the number of mice tested.TABLE 3. *Adjuvant and tumor-suppressive activities of QS-10-N-acetyl muramyl dipeptides*

Group	Adjuvant	Dose (μ g)	Specific target cell lysis (% $\pm$ SE) ^a	Suppression of Meth-A growth in BALB/c mice	
				Expt 1	Expt 2
1	$\begin{array}{c} \text{OH} \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	24.9 $\pm$ 5.5	0/10 ^b	ND ^c
2	$\begin{array}{c} \text{OH} \\ \\ \text{QS-10-Gly-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	31.8 $\pm$ 7.5	1/10	ND
3	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-Leu-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	10.1 $\pm$ 4.6	0/10	ND
4	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	84.2 $\pm$ 5.7	8/10	6/10
5	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-Gly-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	73.8 $\pm$ 8.3	ND	8/10
6	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-Leu-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	91.6 $\pm$ 4.0	4/10	5/10
7	Control		26.9 $\pm$ 6.2	0/10	0/10

^a Values show the degree of cytotoxicity. SE, Standard error.^b See Table 2, footnote b.^c ND, Not done.

TABLE 4. Adjuvant and tumor-suppressive activities of QS-10-*N*-acetyl muramyl dipeptides

Group	Adjuvant	Dose (μ g)	Skin reaction with ABA-bacterial α -amylase at 24 h (mm $\pm$ SE) ^a	Suppression of Meth-A growth in BALB/c mice
1	$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \text{ (quinonyl-MDP-66)} \end{array}$	100	23.8 $\pm$ 0.6	10/10 ^b
2	$\begin{array}{c} \text{OCH}_2\text{CH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	24.1 $\pm$ 0.8	10/10
3	$\begin{array}{c} \text{OCH(CH}_3)_2 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	23.5 $\pm$ 0.8	10/10
4	$\begin{array}{c} \text{OCH}_2(\text{CH}_2)_4\text{CH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	21.6 $\pm$ 0.7	10/10
5	$\begin{array}{c} \text{OCH}_2\text{CH}_2\text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$	100	23.0 $\pm$ 1.2	5/10
6	Control		0	0/10

^a Values show the degree of delayed hypersensitivity. SE, Standard error of the mean.

^b See Table 2, footnote *b*.

less effective for the suppression of tumor growth in comparison with the others (groups 1 through 4).

Furthermore, the adjuvant activity of quinonyl-MDP-66, which showed potent adjuvant and tumor-suppressive activities as described above, was examined in the following experiments.

Adjuvant activity of quinonyl-MDP-66 on footpad reaction and antibody formation in mice. Quinonyl-MDP-66 as a suspension in PBS showed potent adjuvant activity for footpad reaction at a 500- μ g dose, and a 100- μ g dose of *N. rubra* cell wall skeleton also had potent adjuvant and tumor-suppressive activities as reported previously (Table 5). Although the *N. rubra* cell wall skeleton was also active as an adjuvant for footpad reaction at a 20- μ g dose, quinonyl-MDP-66 was less active at 20- or 100- μ g doses. Quinonyl-MDP-66 at a 500- μ g dose, as well as *N. rubra* cell wall skeleton at 20- or 100- μ g doses, was shown to be effective for the humoral immune response. The hemagglutinin titer of quinonyl-MDP-66 was reduced in a dose-dependent manner.

DISCUSSION

Ubiquinone is well known as a fat-soluble vitamin having various biological activities, such as effects on the mitochondrial electron trans-

port system, labilization or stabilization activity of the lysosomal membrane, the production of cyclic adenosine 3',5'-monophosphate phosphodiesterase, and adjuvancy on humoral immune response (15). Imada and his co-workers (15) have reported that the metabolites of ubiquinone and their related compounds such as Q acid-I, Q acid-II, QS-10, and QS-3 have some biological activities which were also observed in ubiquinone homologs.

Previously, it has been clearly shown that related compounds of ubiquinone, as well as other fat-soluble vitamins such as α -tocopherol and phylloquinone (vitamin K₁), were more or as adjuvant active as *N*-acetyl muramyl dipeptide for the circulating antibody formation to bacterial α -amylase in mice (3, 22). The QS-10 used in this study was also shown to have marked adjuvant activity.

In this paper, we examined the adjuvant and tumor-suppressive activities of synthetic quinonyl derivatives of *N*-acetyl muramyl dipeptides and their methyl esters. MurNAc-L-Val-D-isoGln, MurNAc-L-Val-D-Glu(OCH₃)NH₂, and their quinonyl derivatives were shown to be active as adjuvants for the formation of 19S plaque-forming cells to SRBC in vitro (Table 1). As described previously (9), adjuvant activity for the development of delayed-type hypersensitivity in guinea pigs when the adjuvants were administered together with the antigen as a wa-

TABLE 5. Adjuvant activity of quinonyl-MDP-66 on footpad reaction and antibody production to SRBC in mice

Adjuvant	Dose (μ g)	Footpad reaction (1/10 mm $\pm$ SE ^a) at:		Hemagglu- tinin titer (log ₂) $\pm$ SE at 2 weeks
		24 h	48 h	
$\begin{array}{c} \text{OCH}_3 \\ \\ \text{QS-10-MurNAc-L-Val-D-Glu-NH}_2 \end{array}$ (quinonyl-MDP-66)	500	10.7 $\pm$ 0.3	6.0 $\pm$ 0.6	6.0 $\pm$ 0
	100	6.5 $\pm$ 0.9	2.3 $\pm$ 0.3	5.0 $\pm$ 0
	20	6.8 $\pm$ 0.5	2.5 $\pm$ 0.6	4.8 $\pm$ 0.5
<i>N. rubra</i> cell wall skeleton	100	10.7 $\pm$ 1.5	8.7 $\pm$ 1.3	5.3 $\pm$ 0.5
	20	8.5 $\pm$ 1.8	3.5 $\pm$ 0.3	6.0 $\pm$ 0.4
Control		5.0 $\pm$ 0.7	3.0 $\pm$ 0.6	3.8 $\pm$ 0.5

^a SE, Standard error.

ter-in-oil emulsion was shown. However, only quinonyl-MDP-66 was effective for the generation of killer T cells in allogeneic cell-mediated cytotoxicity in C57BL/6J mice and the suppression of tumor growth in syngeneic BALB/c mice (9).

These results suggest that the lipophilicity which was given to MurNAc-L-Val-D-isoGln by esterifications with QS-10 and methanol played an important role for the adjuvant and tumor-suppressive activities of MurNAc-L-Val-D-isoGln. This consideration was supported by evidence that the compounds esterified with QS-3 instead of QS-10 in quinonyl-MDP-66 were active for the induction of delayed-type hypersensitivity; however, they were inactive for the suppression of tumor growth in syngeneic mice (Table 2, group 1). Although the L-serine and L-threonine analogs of quinonyl-MDP-66, as well as quinonyl-MDP-66 itself, have potent adjuvant activity for the induction of delayed-type hypersensitivity, it is of interest that the L-serine analog was less effective in comparison with quinonyl-MDP-66 for the suppression of tumor growth in syngeneic mice (Table 2, group 3). QS-10-Gly-MurNAc-L-Val-D-Glu(OCH₃)NH₂, QS-10-Leu-MurNAc-L-Val-D-Glu(OCH₃)NH₂ (Table 3, groups 5 and 6), and the compounds in which the γ -carboxylic acid of D-isoglutamine in QS-10-MurNAc-L-Val-D-isoGln was esterified with various aliphatic alcohols (Table 4, groups 1 through 5), as well as quinonyl-MDP-66, showed potent adjuvant and tumor-suppressive activities (Tables 3 and 4). The results obtained above suggest that appropriate physicochemical properties were required for the adjuvant and tumor-suppressive activities of these synthetic adjuvants.

Quinonyl-MDP-66 used in the following experiments was more effective both for footpad reaction to SRBC in mice (Table 5) and for the induction of delayed-type hypersensitivity to

ABA-N-acetyl-L-tyrosine in guinea pigs. It was also shown to be active as an adjuvant for the circulating antibody response to SRBC not only in vitro (Table 1) but also in vivo (Table 5). As reported previously (6, 26), 6-O-mycoloyl-N-acetyl muramyl dipeptides, in which the lipophilic mycolic acid residue was introduced into N-acetyl muramyl dipeptides, have adjuvant activity predominantly on the cellular immune response, such as delayed-type hypersensitivity and allogeneic cell-mediated cytotoxicity. However, quinonyl-MDP-66, in which the lipophilicity was similarly given to the N-acetyl muramyl dipeptide, has potent adjuvant activity not only on the cellular immune response but also on humoral immune response. Quinonyl-MDP-66 suppressed tumor (Meth-A) growth in syngeneic BALB/c mice when it was administered as a suspension in PBS, although the 6-O-mycoloyl derivatives of N-acetyl muramyl dipeptides had suppressive activities when administered as oil-in-water emulsions. Quinonyl-MDP-66, which was treated with squalene and then suspended in 5.6% mannitol containing 0.2% Tween 80 to make an oil-in-water emulsion, also suppressed tumor growth in syngeneic mice (data not shown). In the suppression test described above, the induction of systemic tumor immunity in mice which suppressed tumor growth was demonstrated by the reinoculation of Meth-A cells (10⁵). No direct cytotoxicity of adjuvants to Meth-A cells was observed by the in vitro culture.

Juy and Chedid (17) previously reported that adherent peritoneal exudate cells (macrophages) activated with MurNAc-L-Ala-D-isoGln or other adjuvants in vitro could inhibit the proliferation of mastocytoma target cells. Taniyama and Holden (24) found that MurNAc-L-Ala-D-isoGln augmented the cytolytic activity of activated macrophages obtained from murine sarcoma virus-induced tumors and several macrophage cell

lines. In in vivo and in vitro studies, the effect of the quinonyl derivatives of *N*-acetyl muramyl dipeptides on the function of macrophages is under investigation.

Damais et al. (11, 12) reported previously that MurNAc-L-Ala-D-isoGln acts as a mitogen on mouse spleen cells when cultured for 4 or 5 days in serum-free medium supplemented with 2-mercaptoethanol and shows a different responsiveness in several strains of mice. We observed that MurNAc-L-Val-D-isoGln, MurNAc-L-Val-D-Glu(OCH₃)NH₂, and their quinonyl derivatives can slightly or hardly act as mitogens on spleen cells of C57BL/6J, BALB/c, and DBA/2 mice under the condition of a 3-day culture in RPMI 1640 medium containing 10% fetal bovine serum (data not shown).

It was found that quinonyl-MDP-66, as well as ubiquinone, could restore the reduced nicotinamide adenine dinucleotide oxidase and succinate oxidase activities of pentane- or acetone-treated beef heart mitochondrial preparations (Imada et al., unpublished data). Furthermore, ubiquinone was shown to have protective activity against doxorubicin-induced cardiotoxicity (14, 27). Of interest is the combined effect of the quinonyl derivatives of *N*-acetyl muramyl dipeptides on cancer chemotherapy.

Although the biological significance of the quinonyl (QS-10) group which combined in the 6-*O* position of the muramic acid residue is not yet clear, the biological activities of QS-10, such as its interaction on the cell surface membrane in reference to its lipophilic properties, will play some roles in the development of potent adjuvant and tumor-suppressive activities of quinonyl-MDP-66 as a suspension in PBS.

It is expected that quinonyl-MDP-66, as a new synthetic adjuvant, will have more interesting biological activities besides those of 6-*O*-mycoloyl-*N*-acetyl muramyl dipeptide, as reported previously.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, and from the Ministry of Health and Welfare and also by a Naito research grant.

LITERATURE CITED

- Adam, A., R. Ciorbaru, F. Ellouz, J.-F. Petit, and E. Lederer. 1974. Adjuvant activity of monomeric bacterial cell wall peptidoglycans. *Biochem. Biophys. Res. Commun.* **56**:561-567.
- Adam, A., M. Devys, V. Souvannavong, P. Lefrancier, J. Choay, and E. Lederer. 1976. Correlation of structure and adjuvant activity on *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (MDP), its derivatives and analogues. Antiadjuvant and competition properties of stereoisomers. *Biochem. Biophys. Res. Commun.* **72**:339-346.
- Azuma, I. 1978. Effect of ubiquinones and related compounds on immune responses in mice and guinea pigs. *Vitamins (Kyoto)* **52**:503-507.
- Azuma, I., K. Kamisango, I. Saiki, Y. Tanio, S. Kobayashi, and Y. Yamamura. 1980. Adjuvant activity of *N*-acetyl muramyl dipeptides for the induction of delayed-type hypersensitivity to azobenzenearsonate-*N*-acetyl-L-tyrosine in guinea pigs. *Infect. Immun.* **29**:1193-1196.
- Azuma, I., K. Sugimura, T. Taniyama, M. Yamawaki, Y. Yamamura, S. Kusumoto, S. Okada, and T. Shiba. 1976. Adjuvant activity of mycobacterial fractions: adjuvant activity of synthetic *N*-acetylmuramyl-dipeptide and the related compounds. *Infect. Immun.* **14**:18-27.
- Azuma, I., K. Sugimura, M. Yamawaki, M. Uemiyama, S. Kusumoto, S. Odaka, T. Shiba, and Y. Yamamura. 1978. Adjuvant activity of synthetic 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamine and related compounds. *Infect. Immun.* **20**:600-607.
- Azuma, I., T. Taniyama, M. Yamawaki, K. Sugimura, and Y. Yamamura. 1976. Adjuvant and antitumor activities of *Nocardia* cell-wall skeletons. *Gann* **67**:733-736.
- Azuma, I., M. Uemiyama, I. Saiki, M. Yamawaki, Y. Tanio, S. Kusumoto, T. Shiba, T. Kusama, K. Tobe, H. Ogawa, and Y. Yamamura. 1979. Synthetic adjuvants—new immunotherapeutic agents, p. 311-330. *In* W. D. Terry and Y. Yamamura (ed.), *Immunobiology and immunotherapy of cancer*. Elsevier/North Holland Publishing Co., New York.
- Azuma, I., M. Yamawaki, M. Uemiyama, I. Saiki, Y. Tanio, S. Kobayashi, T. Fukuda, I. Imada, and Y. Yamamura. 1979. Adjuvant and antitumor activities of quinonyl-*N*-acetylmuramyl dipeptides. *Gann* **70**:847-848.
- Brunner, K. T., J. Mauel, H. Rudolf, and B. Chapuis. 1970. Studies of allograft immunity in mice. I. Induction, development and in vitro assay of cellular immunity. *Immunology* **18**:501-515.
- Damais, C., M. Parant, and L. Chedid. 1977. Nonspecific activation of murine spleen cells in vitro by a synthetic immunoadjuvant (*N*-acetylmuramyl-L-alanyl-D-isoglutamine). *Cell. Immunol.* **34**:49-56.
- Damais, C., M. Parant, L. Chedid, P. Lefrancier, and J. Choay. 1978. In vitro spleen cell responsiveness to various analogs of MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine), a synthetic immunoadjuvant, in MDP high-responder mice. *Cell. Immunol.* **35**:173-179.
- Ellouz, F., A. Adam, R. Ciorbaru, and E. Lederer. 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys. Res. Commun.* **59**:1317-1325.
- Ghione, M., and C. Bertazzoli. 1977. Co Q and anthracycline associated cardiomyopathy, p. 183-199. *In* K. Folkers and Y. Yamamura (ed.), *Biomedical and clinical aspects of coenzyme Q*. Elsevier/North Holland Publishing Co., New York.
- Imada, I., M. Watanabe, and H. Morimoto. 1978. Metabolism of ubiquinone, and biological activities of its metabolites and related compounds. *Vitamins (Kyoto)* **52**:493-501.
- Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science* **140**:405.
- Juy, D., and L. Chedid. 1975. Comparison between macrophage activation and enhancement of nonspecific resistance to tumors by mycobacterial immunoadjuvants. *Proc. Natl. Acad. Sci. U.S.A.* **72**:4105-4109.
- Kobayashi, S., T. Fukuda, I. Imada, M. Fujino, I. Azuma, and Y. Yamamura. 1979. Novel quinonyl derivatives of muramyl dipeptide possessing potent an-

- titumor activity. *Chem. Pharm. Bull.* **27**:3193-3196.
19. Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, I. Morisaki, T. Shiba, S. Kusumoto, Y. Tarumi, and K. Ikenaka. 1975. Immunoadjuvant activities of synthetic N-acetylmuramylpeptides or amino acids. *Biken J.* **18**:105-111.
20. Marbrook, J. 1967. Primary immune response in cultures of spleen cells. *Lancet* **ii**:1279-1281.
21. Saiki, I., M. Uemiya, T. Kusama, Y. Yamamura, and I. Azuma. 1980. Adjuvant activity of nocardomycoloyl derivatives of N-acetylmuramyl-dipeptide in mice and guinea pigs. *Microbiol. Immunol.* **24**:265-269.
22. Sugimura, K., I. Azuma, Y. Yamamura, I. Imada, M. Watanabe, and H. Morimoto. 1977. Effect of ubiquinone and related compounds on immune response, p. 151-163. *In* K. Folkers and Y. Yamamura (ed.), *Biomedical and clinical aspects of coenzyme Q*. Elsevier/North Holland Publishing Co., New York.
23. Tabachnick, M., and H. Sobotka. 1959. Azoproteins. I. Spectrophotometric studies of amino acid azoderivatives. *J. Biol. Chem.* **238**:1726-1730.
24. Taniyama, T., and H. T. Holden. 1979. Direct augmentation of cytolytic activity of tumor-derived macrophages and macrophage cell lines by muramyl dipeptide. *Cell. Immunol.* **48**:369-374.
25. Uemiya, M., K. Sugimura, T. Kusama, I. Saiki, I. Azuma, and Y. Yamamura. 1979. Adjuvant activity of 6-O-mycoloyl derivatives of N-acetylmuramyl-L-seryl-D-isoglutamine and related compounds in mice and guinea pigs. *Infect. Immun.* **24**:83-89.
26. Yamamura, Y., I. Azuma, K. Sugimura, M. Yamawaki, M. Uemiya, M. Kusumoto, S. Okada, and T. Shiba. 1976. Adjuvant activity of 6-O-mycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine. *Gann* **67**:867-877.
27. Zbinden, G., E. Bachmann, and H. Bolliger. 1977. Study of coenzyme Q in toxicity of adriamycin, p. 219-228. *In* K. Folkers and Y. Yamamura (ed.), *Biomedical and clinical aspects of coenzyme Q*. Elsevier/North Holland Publishing Co., New York.