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Adjuvant Activity of Synthetic 6-*O*-"Mycoloyl"-*N*-Acetylmuramyl-L-Alanyl-D-Isoglutamine and Related Compounds

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Adjuvant and antitumor activities of synthetic 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamine were examined. All the synthetic 6-*O*-corynomycoloyl-, 6-*O*-nocardomycoloyl-, and 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine were active as adjuvants for cell-mediated immune responses. However, 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was less active as an adjuvant on circulating antibody formation. It was shown that pyrogenic activity of *N*-acetylmuramyldipeptide was reduced by 6-*O*-acylation with mycolic acid, but not with nocardomycolic or corynomycolic acid. Tumor-suppression activity was observed by the synthetic 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine by using transplantable tumor in syngenic mice.

It has been reported that synthetic *N*-acetylmuramyldipeptide, i.e., *N*-acetylmuramyl-L-alanyl-D-isoglutamine, is the minimum adjuvant-active structure in peptidoglycan of bacterial cell walls (7, 11). The results of an immunological study on *N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine indicated that these synthetic *N*-acetylmuramyldipeptides were adjuvant-active for the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine and for the circulating antibody formation to bacterial α-amylase (BαA). However, they were inactive as adjuvants on cell-mediated cytotoxicity in allogenic mice (4). It also has been shown that *N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine show no antitumor activity on transplantable tumor systems in syngenic mice (4, 9).

More recently, we synthesized 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, which is more lipophilic than *N*-acetylmuramyl-L-alanyl-D-isoglutamine or 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (13), and compared its adjuvant activity with that of the *Mycobacterium bovis* BCG-cell wall skeleton, *N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (17). The results obtained in the previous study indicated that 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was active as an adjuvant on cellular immune response such as induction of cell-mediated cytotoxicity to mastocytoma P815-X2 in allogenic mice, but less effective on the humoral immune response or on the function of helper T cells (17). From the above-mentioned data we concluded that the mycolic acid moiety introduced into the *N*-acetylmuramyldipeptide molecule modifies qualitatively the adjuvant activity on immune responses, predominantly on the cellular immune response.

In this study, we compared the adjuvant and antitumor activities of three kinds of "mycoloyl" derivatives of *N*-acetylmuramyldipeptides (Fig. 1), namely, 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, 6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, and 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, particularly in reference to their chain lengths of mycolic acid residues.

MATERIALS AND METHODS

Synthetic peptides and other adjuvants. Syntheses and properties of *N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine were reported in previous papers (13, 14). The chemical syntheses of 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (6-*O*-nocardomycoloyl-*N*-acetylmuramyldipeptide) and 6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (6-*O*-corynomycoloyl-*N*-acetylmuramyldipeptide) were carried out by a method similar to that described for 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide (13), and the details will be published soon elsewhere. The nocardomycolic and

corynomycolic acids used for the esterification of *N*-acetylmuramic acid residue were isolated from the cells of *Nocardia asteroides* strain 131 and *Corynebacterium diphtheriae* strain PW8, respectively (2). The cell wall skeletons of *M. bovis* BCG (3) and *N. rubra* (5) were prepared by methods described previously. The average molecular formulas of "mycolic acid" analogs are described in Table 1.

Medium solution. Eagle minimal essential medium containing 100 U of penicillin and 100 μg of streptomycin per ml was obtained from the Research Foundation of Microbial Diseases of Osaka University. Medium RPMI-1640 for tissue culture was obtained from Nissui Seiyaku Co., Tokyo. Fetal calf serum (lot no. 4055952) was purchased from Flow Laboratories (Rockville, Md.) and was inactivated by heating at 57°C for 30 min before use.

Animals. Six- to eight-week-old female and male mice of DBA/2, C57BL/6J, SWM/Ms, and (C57BL/6J × DBA/2)F_1 (BDF_1) and female Hartley strain guinea pigs weighing 350 to 450 g were obtained from Shizuoka Jikken-Dobutsu Nokyo (Shizuoka) and Ohmura Jikken Dobutsu Co., Ltd. (Zama.). The animals were given food (from Oriental Yeast Industries Ltd., Osaka) and water freely.

Antigens and mitogens. Sheep erythrocytes (SRBC) preserved in Alsever solution were obtained from the Research Foundation of Microbial Diseases of Osaka University and washed with saline solution before use. Crystalline BαA prepared from *Bacillus subtilis* was purchased from Seikagaku Kogyo Co., Tokyo. *m*-[4(4'-Arsonophenylazo)-phenyl]-*N*-acetyl-L-tyrosine (ABA-*N*-acetyltyrosine) and ABA-BαA were prepared by the method described previously (16). *m*-[4(4'-Arsonophenylazo)-phenyl]-*N*-(*N*-2,4-dinitrophenyl-ε-aminocaproyl)-L-tyrosine [DNP-ε-Acp-(ABA)-Tyr] was prepared by coupling diazotized arsanilic acid with DNP-ε-Acp-Tyr (1). Concanavalin A (batch no. 4000, Pharmacia Fine Chemicals, Uppsala, Sweden) and lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 (Difco Laboratories Inc., Detroit, Mich.) were used as T- and B-cell mitogens.

Tumors. Mastocytoma P815-X2 cells chemically induced in DBA/2 mice were kindly supplied by C. Henney (Department of Medicine, Johns Hopkins University, Baltimore, Md.) and were serially passed in ascites fluid through adult DBA/2 female mice. Fibrosarcoma induced in SWM/Ms mice by 3-methylcholanthrene was kindly supplied by T. Tanaka, National Cancer Center, Tokyo, and tumor cells were maintained serially by subcutaneous transplantation in SWM/Ms female mice. The ninth generation of this tumor was used in this experiment.

Preparation of oil-attached 6-*O*-mycoloyl-*N*-acetylmuramyldipeptides. A 5-mg portion of mineral oil (Drakeol 6VR, Pennsylvania Refining Co., Butler, Pa.) was added to 4 mg of 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide placed in a 5-ml tissue grinder equipped with a Teflon pestle. The mixture was ground to a smooth paste by rotation at 800 rpm. After addition of 1 ml of phosphate-buffered saline (PBS) containing 0.2% Tween 80, grinding was continued for 2 to 3 min to obtain a uniform suspension of small oil droplets containing 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide at a concentration of 4 mg/ml. The oil-attached 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide was sterilized by heating at 60°C for 30 min.

Determination of adjuvant activity on the induction of delayed-type hypersensitivity and the helper function of carrier-primed T-cells. Guinea pigs received a primary immunization into four footpads of a total of 50 μg per animal of ABA-*N*-acetyl-L-tyrosine in the presence or absence of various adjuvants in Freund incomplete adjuvant (FIA). Two weeks later, skin tests were carried out with 100 μg of ABA-BαA, and the reactions were measured at 24 and 48 h after intradermal injection of test antigen. After 16 days, the guinea pigs received a secondary immunization subcutaneously and intraperitoneally of 1.0 mg of DNP-ε-Acp-(ABA)-Tyr in Freund complete adjuvant emulsion (200 μg of BCG-cell wall skeleton per 0.5 ml of FIA per guinea pig). Each guinea pig was bled 7, 14, and 21 days after immunization with DNP-ε-Acp-(ABA)-Tyr. Antibody determinations were performed as described below.

Measurement of anti-DNP antibody. Anti-DNP antibody in sera was determined by the method of Katz et al. (10). To 50 μl of antiserum (undiluted and serially diluted in 20% normal homologous serum), 50 μl of [^{3}H]DNP-ε-aminocaproic acid ([^{3}H]DNP-ε-Acp) (1×10^{-8} M) was added. After standing at 4°C for 1 h, 0.1 ml of saturated ammonium sulfate solution was

TABLE 1. *Average molecular weights and formulas of "mycolic acid" analogs*

"Mycolic acid"	Source of defatted cells of:	Avg molecular wt[a]	% Elemental analysis[b] C	% Elemental analysis[b] H	Avg molecular formula	Molecular wt	Chain length of α-branches[c] (R')
Mycolic acid	*M. tuberculosis* Aoyama B	1,180	81.45	13.55	$C_{80}H_{158}O_{3.5}$	1,176	$C_{24}H_{49}$
Nocardomycolic acid	*N. asteroides* 131	766	79.84	12.76	$C_{51}H_{97}O_{3.6}$	768	$C_{10}H_{21}C_{14}H_{29}$
Corynomycolic acid	*C. diphtheriae* PW8	483	76.97	12.38	$C_{31}H_{59}O_{3.3}$	485	$C_{10}H_{21}C_{14}H_{29}$

[a] Determined by acid titration.
[b] Mean values of two individual analyses.
[c] R—CH(OH)—CH(R')—CO—

added. The mixture was kept at 4°C for 30 min and then centrifuged at 3,000 rpm for 20 min. A 50-μl portion of each supernatant fluid was added to 4 ml of a scintillation solvent, and radioactivity was measured by a liquid scintillation counter. Control samples contained normal homologous serum diluted adequately with PBS (0.01 M, pH 7.2). The binding of [^{3}H]DNP-ε-Acp by normal nonimmune guinea pig sera was usually less than 5%. Binding curves were constructed with selected mouse and guinea pig anti-DNP sera that had been analyzed by the quantitative precipitation technique. By using these curves, the percentages of binding of all other antisera in each experiment were converted into amounts of anti-DNP antibody in micrograms per milliliter.

Tissue culture. The in vitro culture technique described by Marbrook (15) was used. Plaque-forming cells against SRBC were assayed by the method of Jerne and Nordin (8).

Cell-mediated cytotoxicity test. Inbred C57BL/6J mice (H-2^b) were immunized intraperitoneally with viable cells of mastocytoma P815-X2 (H-2^d) with or without 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine suspended in PBS. On day 11 after immunization, the cell-mediated cytotoxicity assay was carried out by using the spleen cells of immunized mice and ^{51}Cr-labeled mastocytoma P815-X2 cells by the method of Brunner et al. (6) 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 according to the following formula: percentage of specific target cell lysis = [release of specific target cell lysis/(maximal release − control release)] × 100. Maximal chromium release was measured at complete cell lysis where target cells alone were frozen and thawed twice.

Determination of pyrogenic activity. Pyrogenic activity of synthetic *N*-acetylmuramyldipeptide and its acylated derivatives was determined by the method of *The Pharmacopeia of Japan*, 9th ed. (15a). Briefly, the test specimen was dissolved or suspended in pyrogen-free physiological saline at an appropriate concentration. A 10-ml volume of the solution or suspension containing the required dose was injected intravenously into rabbits weighing 1 to 2.7 kg, and rectal temperatures were measured continuously with an automatic body temperature-recording device. When at least two out of three rabbits tested showed individual rises in temperature of 0.6°C or more above their control temperatures, the test specimen was considered pyrogenic. When none of the three rabbits showed such a rise in temperature, the test material was regarded as nonpyrogenic. On the other hand, when one out of the three rabbits showed a temperature rise of 0.6°C or more, or when the sum of the temperature rises exceeded 1.4°C, even if neither one was over 0.6°C, the test materials were judged weakly or doubtfully pyrogenic.

Determination of mitogenic activity. Cells (4×10^5) suspended in 150 μl of RPMI-1640 containing fetal calf serum were cultured in the presence or absence of various concentrations of mitogens in a microtissue culture plate (Falcon 3040, MicroTest II, Falcon Plastics, Oxnard, Calif.) for 72 h at 37°C in a CO_2 incubator. [^{3}H]thymidine (0.5 μCi) (The Radiochemical Centre, Amersham, England) was added 24 h before the end of the culture. Cells were harvested on a glass filter by a Dynatech Automash cell harvester (Dynatech, England). The incorporation of [^{3}H]thymidine was measured by a liquid scintillation counter. All experiments were carried out in triplicate cultures.

RESULTS

Adjuvant activity of 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides on delayed-type hypersensitivity to ABA-*N*-acetyltyrosine. Hartley strain guinea pigs were immunized with FIA containing 50 μg of ABA-*N*-acetyltyrosine dissolved in PBS with or without synthetic *N*-acetylmuramyldipeptides or 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides as a water-in-oil emulsion. Two weeks later, skin tests with ABA-BαA were done. *N*-acetylmuramyl-L-alanyl-D-isoglutamine, 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamines, and BCG-cell wall skeleton showed the potent adjuvant activity for the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs (Table 2).

Adjuvant activity on helper T-cells in guinea pigs. After skin testing with ABA-BαA, guinea pigs were immunized with 1 mg of DNP-ε-Acp-(ABA)-Tyr and 200 μg of BCG-cell wall skeleton in FIA. Anti-DNP antibody in sera was determined at 14 and 21 days after secondary immunization. As shown in Table 2, *N*-acetylmuramyl-L-alanyl-D-isoglutamine (group 1a) and 6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (group 2a) as well as BCG-cell wall skeleton (group 5) showed adjuvant activity on the helper function of carrier-primed T-cells. On the other hand, 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide (group 4) at the 50-μg dose level was not adjuvant active compared with control 2. 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine stimulated helper function at the 40-μg, but not at the 400-μg, dose level.

Adjuvant activity on the generation of cell-mediated cytotoxic effector cells in allogenic mice. Mice of the C57BL/6J strain were immunized intraperitoneally with mastocytoma P815-X2 cells with or without 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamine suspended in PBS. Eleven days after the immunization, cell-mediated cytotoxicity of mouse spleen cells was determined by the method of Brunner et al. (6) by using ^{51}Cr-labeled mastocytoma P815-X2 cells as target cells. These 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamines enhanced the generation of effector cells in allogenic mouse spleen cells (Table 3). 6-*O*-nocardomycoloyl-*N*-acetylmura-

TABLE 2. *Adjuvant activity of 6-O-mycoloyl-N-acetylmuramyldipeptide and related compounds on the induction of delayed-type hypersensitivity to ABA-N-acetyltyrosine and on the helper function of carrier-primed T-cells in guinea pigs*

Group no.	Adjuvant added	Dose (μg)	Primary antigen (ABA-N-acetyltyrosine) (50 μg)	Skin reaction with ABA-BαA (100 μg) at 24 h[a] (mm ± SE)	Secondary antigen [DNP-ε-Acp-(ABA)-Tyr] (1 mg)	Anti-DNP antibody (μg/ml) after second immunization[b]	
						Week 2	Week 3
1a	MurNAc-L-Ala-D-isoGln	100	+	19.7 ± 1.0	+	119.6 ± 46.6	196.5 ± 69.6
b		10	+	12.5 ± 1.5	+	44.8 ± 7.5	78.4 ± 12.7
2a	6-O-corynomycoloyl-	300	+	22.0 ± 0.6	+	269.5 ± 24.1	275.8 ± 49.2
b	MurNAc-L-Ala-D-isoGln	30	+	21.2 ± 1.9	+	170.3 ± 44.2	186.2 ± 69.9
3a	6-O-nocardomycoloyl-	400	+	23.0 ± 1.5	+	61.2 ± 12.7	62.6 ± 80.0
b	MurNAc-L-Ala-D-isoGln	40	+	24.5 ± 0.7	+	149.5 ± 30.9	155.4 ± 23.4
4	6-O-mycoloyl-MurNAc-L-Ala-D-isoGln	50	+	14.1 ± 1.1	+	27.0 ± 8.0	49.9 ± 11.5
5	BCG-cell wall skeleton	100	+	24.7 ± 1.2	+	114.8 ± 35.9	133.5 ± 27.1
6	Control 1		+	0	+	29.2 ± 8.4	34.5 ± 7.4
7	Control 2		−	0	+	21.0 ± 3.9	104 ± 29.1

[a] Hartley guinea pigs were immunized in four footpads with 50 μg of ABA-N-acetyltyrosine in FIA with synthetic compounds or BCG-cell wall skeleton. Control groups were immunized with ABA-N-acetyltyrosine alone in FIA (control 1) or FIA alone (control 2). After 2 weeks, skin tests were performed with 100 μg of ABA-BαA, and skin reactions were measured 24 h after intradermal injection of test antigen.

[b] On day 16 after primary immunization with ABA-N-acetyltyrosine, guinea pigs were immunized both subcutaneously and intraperitoneally with 0.5 ml of FIA containing 1.0 mg of DNP-ε-Acp-(ABA)-Tyr and 200 μg of BCG-cell wall skeleton.

TABLE 3. *Adjuvant activity of synthetic 6-O-mycoloyl-N-acetylmuramyldipeptides on the induction of cell-mediated cytotoxicity in allogenic mice*[a]

Group	Mice immunized with:	Dose (μg)	Specific target cell lysis (% ± SE)
	Mastocytoma P815-X2 cells (1×10^4) and:		
1a	6-O-corynomycoloyl-MurNAc-L-Ala-D-isoGln in PBS[b]	100	28.8 ± 14.4
b		10	16.1 ± 5.0
2a	6-O-nocardomycoloyl-MurNAc-L-Ala-D-isoGln in PBS	100	76.9 ± 3.2
b		10	21.0 ± 7.6
3a	6-O-mycoloyl-MurNAc-L-Ala-D-isoGln in PBS	100	35.7 ± 13.6
b		10	47.5 ± 10.9
4	Mastocytoma P815-X2 cells (1×10^4) alone (control 1)		10.3 ± 0.7
5	Mastocytoma P815-X2 cells (3.5×10^7) alone (control 2)		89.7 ± 4.0

[a] Three or four C57BL/6J mice in each group were immunized intraperitoneally with a mixture of mastocytoma P815-X2 cells and synthetic 6-O-mycoloyl-N-acetylmuramyldipeptides suspended in PBS. After 11 days, cell-mediated cytotoxicity was determined by the method of Brunner et al. (6) by incubating spleen cells that were obtained from each of the immunized mice and ^{51}Cr-labeled mastocytoma cells at a ratio of 100:1 for 20 h. All assays were set up in duplicate. SE, Standard error of the mean.

[b] PBS, 0.01 M (pH 7.2).

myl-L-alanyl-D-isoglutamine at 100 μg showed the highest adjuvant activity in this system.

Adjuvant activity on the primary immune response to SRBC in vitro. N-acetylmuramyl-L-alanyl-D-isoglutamine showed marked adjuvant activity on the formation of 19S-PFC to SRBC after 4 days of culture in vitro (Table 4). However, the adjuvant activity of N-acetylmuramyl-L-alanyl-D-isoglutamine was diminished by the 6-O-acylation of N-acetylmuramyl residue with nocardomycolic acid.

Pyrogenic activity of 6-O-mycoloyl-N-

acetylmuramyldipeptides. The pyrogenic activity of synthetic *N*-acetylmuramyldipeptides and their 6-*O*-acyl-*N*-acetylmuramyldipeptides was determined in rabbits by the method of *The Pharmacopeia of Japan*, 9th ed. (15a). *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its 6-*O*-stearoyl-, 6-*O*-corynomycoloyl-, and 6-*O*-nocardomycoloyl derivatives were definitely pyrogenic (Table 5). However, pyrogenic activity of 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was not observed even at a dose of 875 μg per rabbit.

Mitogenic activity of synthetic peptides. All the synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its 6-*O*-"mycoloyl" derivatives, except 6-*O*-nocardomycoloyl-*N*-acetylmuramyldipeptide, were inactive as mitogen on spleen cells of C57BL/6J, DBA/2, and BALB/c mice (Table 6). Only 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine enhanced slightly the incorporation of [^{3}H]thymidine into spleen cells of C57BL/6J and DBA/2 mice at a 100-μg dose.

TABLE 4. *Adjuvant activity of synthetic N-acetylmuramyldipeptide and its acylated derivatives on primary immune response to SRBC in vitro in mice*[a]

Adjuvant added	Dose (μg)	19S-PFC ± SE (stimulation ratio)
MurNAc-L-Ala-D-isoGln	100	1,577 ± 137 (8.3)
	10	1,707 ± 100 (9.0)
6-*O*-corynomycoloyl-MurNAc-L-Ala-D-isoGln	100	910 ± 107 (4.8)
	10	607 ± 99 (3.2)
6-*O*-nocardomycoloyl-MurNAc-L-Ala-D-isoGln	100	337 ± 11 (1.8)
	10	263 ± 52 (1.4)
Control		190 ± 40 (1.0)

[a] Normal spleen cells (2×10^7) of BDF_1 mice were cultured for 4 days with SRBC (4×10^6) with or without (control) synthetic adjuvants. SE, Standard error of the mean.

Antitumor activity of 6-*O*-mycoloyl-*N*-acetylmuramyldipeptides. The antitumor activity of these synthetic 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamines was examined by using 3-methylcholanthrene-induced fibrosarcoma in SWM/Ms mice. A mixture of tumor cells (1×10^6) and 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (100 μg) suspended in PBS or treated with oil droplets was transplanted intradermally into SWM/Ms mice, and the tumor growth in inoculated sites and survival days of mice were determined. Doses of 100 μg of the oil-attached 6-*O*-corynomycoloyl-*N*-acetylmuramyldipeptide, 6-*O*-nocardomycoloyl-*N*-acetylmuramyldipeptide, and 6-*O*-mycoloyl-*N*-acetylmuramyldipeptide suppressed tumor growth (Table 7). However, all these synthetic 6-*O*-mycoloyl-*N*-acetylmuramyldipeptides suspended in PBS solution were inactive in this tumor system.

DISCUSSION

In a previous paper (17), we reported that 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine is a potent adjuvant on the cell-mediated immune response such as induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs and generation of effector cells to mastocytoma P815-X2 in allogenic mice, but not on the circulating antibody formation to SRBC and DNP-Lys-Ficoll. In this experiment, the immunological properties of acyl derivatives of *N*-acetylmuramyldipeptides in which the 6-hydroxy group of muramic acid was esterified with mycolic acid, nocardomycolic acid, or cor-

TABLE 5. *Pyrogenic activity of synthetic N-acetylmuramyldipeptide and its acylated derivatives in rabbits*

Synthetic *N*-acetylmuramyldipeptides	Dose (mg)	Elevation of body temp (°C)				
		Individuals			Total	Pyrogenicity
MurNAc-L-Ala-D-isoGln	0.010	2.45	**0.80**	**0.80**[a]	**2.05**	+
	0.050	**0.85**	**1.00**	**0.85**	**2.70**	+
6-*O*-stearoyl-MurNAc-L-Ala-D-isoGln	0.015	**1.20**	**1.25**	**1.00**	**3.45**	+
	0.075	**1.75**	**0.80**	**1.65**	**4.20**	+
6-*O*-corynomycoloyl-MurNAc-L-Ala-D-isoGln	0.175	0.20	0.20	0.15	0.55	−
	0.875	**0.80**	**0.65**	**0.65**	**2.10**	+
6-*O*-nocardomycoloyl-MurNAc-L-Ala-D-isoGln	0.035	**0.65**	0.30	0.25	**1.20**	±
	0.175	**1.00**	**1.00**	0.30	**2.30**	+
6-*O*-mycoloyl-MurNAc-L-Ala-D-isoGln	0.175	0.10	0.20	0.15	0.45	−
	0.875	0.20	0.35	0.35	0.90	−

[a] Boldface numbers indicate definite pyrogenicity.

TABLE 6. *Mitogenic activity of N-acetylmuramyl-L-alanyl-D-isoglutamine and its acylated derivatives on normal spleen cells of mice*[a]

Mitogen added	Dose (μg/ml)	Stimulation index		
		C57BL/6J	DBA/2	BALB/c
MurNAc-L-Ala-D-isoGln	100	1.38 ± 0.24	1.62 ± 0.46	1.39 ± 0.15
	10	1.36 ± 0.38	1.31 ± 0.30	1.01 ± 0.02
6-*O*-corynomycoloyl-MurNAc-L-Ala-D-isoGln	100	1.63 ± 0.31	1.80 ± 0.28	0.94 ± 0.10
	10	1.32 ± 0.15	1.43 ± 0.29	1.12 ± 0.17
6-*O*-nocardomycoloyl-MurNAc-L-Ala-D-isoGln	100	2.28 ± 0.37	2.61 ± 0.66	1.67 ± 0.35
	10	1.60 ± 0.24	1.87 ± 0.36	1.07 ± 0.14
6-*O*-mycoloyl-MurNAc-L-Ala-D-isoGln	100	1.63 ± 0.24	1.74 ± 0.34	1.54 ± 0.44
	10	1.61 ± 0.38	1.32 ± 0.21	1.13 ± 0.14
BCG-cell wall skeleton	100	4.88 ± 0.82	3.08 ± 0.48	9.14 ± 5.16
	10	2.45 ± 0.53	1.21 ± 0.16	1.64 ± 0.64
Concanavalin A	5	41.72 ± 13.02	18.40 ± 1.20	147.43 ± 42.17
LPS	100	13.24 ± 2.76	5.44 ± 2.15	7.01 ± 1.46
Control		1.0	1.0	1.0

[a] Normal mouse spleen cells (4×10^5) were incubated with mitogen or spleen cell alone for 72 h. [^{3}H]-thymidine (0.5 μCi) was added 24 h before the end of culture. The data indicate the average stimulation ratios of six experiments.

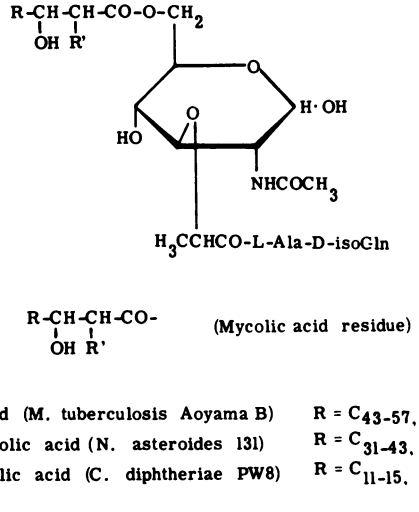

FIG. 1. *Chemical structure of 6-O-mycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine.*

ynomycolic acid were examined particularly with reference to the molecular size of mycolic acids introduced into the muramic acid moiety of *N*-acetylmuramyldipeptides.

6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine as well as 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine were potent adjuvants for the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs (Table 2). On the helper T-cell function for the antibody response to haptenic group (DNP), 6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, as well as *N*-acetylmuramyl-L-alanyl-D-isoglutamine, was the potent adjuvant compared

TABLE 7. *Suppression of tumor (fibrosarcoma) growth with synthetic 6-O-"mycoloyl"-N-acetylmuramyldipeptides in SWM/Ms male mice*

Synthetic 6-*O*-mycoloyl-*N*-acetylmuramyldipeptides	Dose (μg)	PBS suspension[a]	Oil-attached form[b]
6-*O*-mycoloyl-MurNAc-L-Ala-D-isoGln	100	1/9	7/9
6-*O*-nocardomycoloyl-MurNAc-L-Ala-D-isoGln	100	0/8	5/8
6-*O*-corynomycoloyl-MurNAc-L-Ala-D-isoGln	100	ND	3/8
Control (PBS)		0/8	ND
Control (oil droplets)		ND	0/9

[a] A mixture of fibrosarcoma (1×10^6) and synthetic 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides suspended in PBS was inoculated intradermally into syngenic SWM/Ms female mice. Table shows the results 23 days after inoculation. Number of tumor-free mice per number of mice tested. ND, Not determined.

[b] A mixture of fibrosarcoma (1×10^6) and synthetic 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides attached to oil droplets was inoculated intradermally into syngenic SWM/Ms male mice. Table shows the results 35 days after inoculation. Number of tumor-free mice per number of mice tested. ND, Not determined.

with 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine. These 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamines showed adjuvant activities on the generation of effector cells to mastocytoma P815-X2 in C57BL/6J mice; especially 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine showed the most potent adjuvant activity in this system (Table 3).

We showed that 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was inactive as an adjuvant on the circulating antibody formation to SRBC and DNP-Lys-Ficoll in mice (17). 6-*O*-corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was active as an adjuvant on the 19S-PFC formation, although the activity was diminished by acylation with corynomycolic acid compared with that of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Table 4). In the case of 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, adjuvant activity was less pronounced.

More recently, the correlation between pyrogenicity and adjuvant activity of 14 different *N*-acetylmuramyldipeptides and amino acids was examined by Kotani et al. (12). They concluded that there was an almost perfect correlation between the pyrogenicity and adjuvant activity of *N*-acetylmuramyldipeptide derivatives. The pyrogenic activity of *N*-acetylmuramyl-L-alanyl-D-isoglutamine was observed in rabbits (Table 5), as reported by Kotani et al. 6-*O*-acylation of *N*-acetylmuramyl-L-alanyl-D-isoglutamine with stearic, corynomycolic, and nocardomycolic acids did not diminish the pyrogenicity in rabbits. In contrast, 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine has no pyrogenic activity even at a dose of 875 μg/kg.

From the above results, it may be concluded that pyrogenic activity of *N*-acetylmuramyldipeptide derivatives does not necessarily correlate directly with their adjuvant activities, at least for cell-mediated immunity.

The antitumor activity of 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamines was examined in fibrosarcoma in SWM/Ms mice. All of the 6-*O*-"mycoloyl"-*N*-acetylmuramyl-L-alanyl-D-isoglutamine attached to oil droplets was active for the suppression of fibrosarcoma in SWM/Ms mice (Table 7); however, these 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides suspended in PBS were inactive in this tumor system. 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine and 6-*O*-nocardomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine slightly prolonged survival time of C57BL/6J mice with experimental pleural effusion of fibrosarcoma (T. Yoshimoto et al., unpublished data). Although synthetic 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamines have potent adjuvant activity, especially on cell-mediated immunity, the antitumor activity of these compounds was less than that of the oil-attached cell wall skeleton of *M. bovis* BCG and *N. rubra*. The details of antitumor activity of these 6-*O*-"mycoloyl"-*N*-acetylmuramyldipeptides are well described in another paper (I. Azuma et al., manuscript in preparation). The synthesis of other acyl analogs of *N*-acetylmuramyldipeptide having adjuvant and antitumor activities is in progress.

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