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Adjuvant Activity of Mycobacterial Fractions: Adjuvant Activity of Synthetic *N*-Acetylmuramyl-dipeptide and the Related Compounds

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Immunological activity of synthetic cell wall peptidoglycan subunits was examined in guinea pigs and mice. It was concluded that the minimal adjuvant-active subunit of cell wall peptidoglycan for the induction of delayed-type hypersensitivity to monoazobenzenearsonate-*N*-acetyl-L-tyrosine and for circulating-antibody formation to bacterial α-amylase and the thymus-independent antigen DNP-Ficoll was *N*-acetylmuramyldipeptide, MurNAc-L-Ala-D-isoGln. *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide showed no adjuvant activity in the generation of cell-mediated cytotoxic effector cells in the spleens of C57Bl/6J mice after in vivo immunization with the allogeneic antigen mastocytoma P815-X2 cells, but *N*-acetylmuramyldipeptide showed adjuvant activity after in vitro sensitization of C57Bl/6J mouse spleen cells to the alloantigen mitomycin C-treated DBA/2 mouse spleen cells. It was also shown that 6-*O*-stearoylation of *N*-acetylmuramylpeptide could not potentiate the adjuvant activity of *N*-acetylmuramyldipeptide. Mitogenic and antitumor activities were not observed in either *N*-acetylmuramyldipeptide or 6-*O*-stearoyl-*N*-acetylmuramyldipeptide in mouse systems.

Previously, it was well established that a "mycolic acid-arabinogalactan-peptidoglycan (mucopeptide)" complex is the principle structure of the cell wall of *Mycobacterium* as well as of *Nocardia* and *Corynebacterium*. We have also reported that the mycobacterial cell wall is the adjuvant-active fraction, having the ability to induce delayed-type hypersensitivity and enhance circulating-antibody formation in the immune response, and that the peptidoglycan moiety is the adjuvant-active unit in the chemical structure of the cell walls of mycobacteria, nocardia, and corynebacteria (2, 3, 28). More recently, we clearly demonstrated that the cell wall of *Mycobacterium bovis* BCG (BCG-CWS) acts on thymus-derived lymphocytes (T cells) and bone marrow-derived lymphocytes (B cells) as adjuvant (2, 27-28) and as mitogen (I. Azuma, T. Taniyama, K. Sugimura, and Y. Yamamura, Jpn. J. Microbiol., in press). It was also shown that BCG-CWS suppressed tumor growth when tumor cells and BCG-CWS that were treated as an oil-in-water emulsion were mixed and injected intradermally in syngeneic mice and that they induced specific and systemic tumor immunity in mice (7; Y. Yamamura, I. Azuma, T. Taniyama, K. Sugimura, F. Hirao, R. Tokuzen, M. Okabe, W. Nakahara, K. Yasumoto, and M. Ohto, Ann. N. Y. Acad. Sci., in press). The recent data suggest that the cell walls not only of *Mycobacterium* but also of other bacteria such as *Nocardia*, *Corynebacterium*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Escherichia*, *Neisseria*, *Moraxella*, *Proteus*, and anaerobic coryneforms are active as adjuvant for the induction of delayed-type hypersensitivity and circulating antibody in guinea pigs (2, 6, 10-12, 14, 22). Recently, it was shown that *N*-acetylmuramyldipeptide, MurNAc-L-Ala-D-isoGln, is the simplest adjuvant-active unit of these cell wall peptidoglycans for the induction of both circulating antibody and delayed-type hypersensitivity in guinea pigs (1, 5, 13, 16, 17). More recently, 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (Fig. 1) was prepared by the acylation of the primary hydroxy group of muramic acid of *N*-acetylmuramyldipeptide to examine the effect of introducing the hydrophobic group (stearoyl residue) into hydrophilic adjuvant (*N*-acetylmuramyldipeptide) on its immunological activity. The present paper deals with the adjuvant activity of synthetic peptides, such as tetrapeptide, *N*-acetylmuramyldipeptide, and 6-*O*-ste-

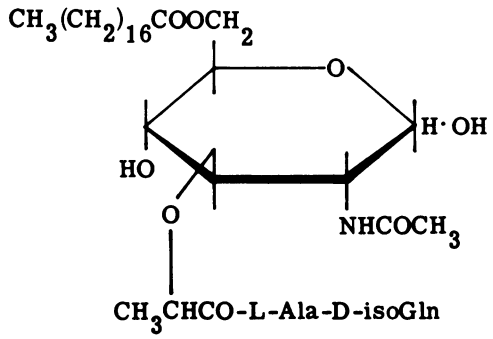

FIG. 1. *Structural formula of 6-O-stearoyl-N-acetylmuramyldipeptide*

aroyl-*N*-acetylmuramyldipeptide, in the induction of delayed hypersensitivity to monoazobenzenearsonate-*N*-acetyl-L-tyrosine (ABA-*N*-acetyltyrosine) in guinea pigs and in the production of circulating antibody to bacterial α-amylase (BαA) in mice and to the T-independent antigen DNP-Ficoll. We also report the adjuvant activity of these synthetic compounds on the generation of allogeneic cell-mediated cytotoxic effector cells in mice. The mitogenic activity of these synthetic peptides in the spleen cells of mice is examined.

MATERIALS AND METHODS

Synthetic peptides and adjuvant. The syntheses and properties of the peptides and *N*-acetylmuramylpeptides used in this study were described previously (18). 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (6-*O*-stearoyl-*N*-acetylmuramyldipeptide) was prepared by coupling 1-α-*O*-benzyl-6-*O*-stearoyl-*N*-acetylmuramic acid with L-alanyl-D-isoglutamine benzyl ester, using the 1-succinimidyl ester method followed by hydrogenolytic deprotection with palladium black catalyst. D-Lactyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (D-lactyltetrapeptide) and D-lactyl-L-alanyl-D-isoglutamine (D-lactyldipeptide) were prepared by coupling *O*-benzyl-D-lactic acid with N^{α}-(L-alanyl-D-isoglutaminyl)-N^{ϵ}-benzyloxycarbonyl-L-lysyl-D-alanine benzyl ester and L-alanyl-D-isoglutamine benzyl ester, respectively, using ethyl chloroformate followed by hydrogenolytic deprotection. Details of the synthesis and chemical properties of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide, D-lactyltetrapeptide, and D-lactyldipeptide will be published elsewhere. The cell wall of *M. bovis* BCG (BCG-CWS) was prepared as described previously (4).

Antigens. Sheep erythrocytes (SRBC) preserved in Alsever solution were obtained from the Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan, and washed with saline solution before use. Crystalline BαA prepared from *Bacillus subtilis* was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. ABA-*N*-acetyltyrosine and ABA-BαA were prepared by the method of Tabachnik and Sobotka (26). Ficoll 400 (lot no. 6849, molecular weight 4×10^5) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and DNP_{26}-Ficoll was prepared by the method of Sharon et al. (25).

Mitogens. Phytohemagglutinin (Bacto-PHA-P, Difco Laboratories, Detroit, Mich.), sodium dextran sulfate (DS-500, molecular weight 5×10^5; Pharmacia Fine Chemicals, Uppsala, Sweden), and concanavalin A (batch no. 4000, Pharmacia Fine Chemicals, Uppsala) were obtained commercially and used as authentic T- and B-cell mitogens.

Tumor. Mouse mastocytoma P815-X2 cells, which originally were chemically induced in DBA/2 mice, were kindly provided by C. Henny, Department of Medicine, Johns Hopkins University, Baltimore, Md., and were serially passed in ascites fluid through adult (C57Bl/6J × DBA/2)F_1 female mice.

Medium solution. Minimum essential Eagle medium (MEM) containing 100 U of penicillin and 100 μg of streptomycin per ml was obtained from the Research Foundation for Microbial Diseases of Osaka University. RPMI 1640 for tissue culture was obtained from Nissui Seiyaku Co. Ltd., Tokyo. Fetal calf serum (lot no. 4055722) was purchased from Flow Laboratories, Rockville, Md., and was inactivated by heating at 56 C for 30 min before use.

Animals. The mice, 6- to 8-week-old (C57Bl/6J × DBA/2)F_1 and C57Bl/6J females, and female Hartley strain guinea pigs, weighing 350 to 450 g, were used. These animals were obtained from Shizuoka Jikken-Dobutsu Nokyo, Shizuoka. The animals were given food (from Oriental Yeast Industries Ltd., Osaka) and water ad libitum.

Preparation of oil-treated 6-*O*-stearoyl-*N*-acetylmuramyldipeptide. 6-*O*-stearoyl-*N*-acetylmuramyldipeptide was placed in a 5-ml tissue grinder. One drop of mineral oil (Drakeol 6VR, Pennsylvania Refining Co., Butler, Pa.) was added, using a syringe with a 26-gauge injection needle, to 4 mg of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide placed in a 5-ml tissue grinder equipped with a Teflon pestle, and the mixture was ground to a smooth paste by rotation at 800 rpm. Then 4 ml of saline solution containing 0.2% Tween 80, which had been kept for 30 min at 60 C, was added to the tube, and grinding was continued for 2 to 3 min to obtain a uniform suspension of small oil droplets containing 6-*O*-stearoyl-*N*-acetylmuramyldipeptide at a concentration of 1 mg/ml. Oil-treated 6-*O*-stearoyl-*N*-acetylmuramyldipeptide was sterilized by heating at 60 C for 30 min.

Determination of antibacterial α-amylase antibody titer in sera. This was carried out by the method of Okada et al. (23).

Tissue culture. The in vitro culture technique described by Marbrook (19) was used. Briefly, 1 ml of cell suspension containing 2×10^7 nucleated cells in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum plus 100 μg of streptomycin and 100 U of penicillin per ml in a glass tube (15 by 80 mm) covered with dialysis membrane at the top was placed in a bottle (30 by 70 mm) and incubated at 37 C in a humidifed CO_2 incubator with a gas flow of 10% CO_2 and 90% air. After 4 days of incubation, cells were harvested and a hemolytic plaque assay was done. Plaque-forming cells (PFC) against SRBC were assayed as described previously (15, 28). The 19*S* PFC were developed by incubation for 1 h at 37 C and then treated with guinea pig complement

diluted 1:10 in barbital buffer (pH 7.3 to 7.4) for 30 min at 37 C.

Cell-mediated cytotoxicity test. (i) Effector cells. Inbred C57Bl/6J mice (H-2^b) were immunized intraperitoneally with viable mastocytoma P815-X2(H-2^d) cells with or without the oil-treated preparation, or with a phosphate-buffered saline (PBS) solution of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide or oil droplets. At day 11 after immunization, spleens were removed aseptically and minced with tweezers and then passed through a 100- to 200-mesh stainless-steel sieve. After being washed two times, spleen cells were resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum and adjusted to 1×10^7 viable cells/ml.

(ii) Target cells. Viable mastocytoma P815-X2 tumor cells, harvested from the peritoneal cavity of (C57Bl/6J × DBA/2)F_1 mice, were washed two times with MEM, resuspended in MEM, and incubated with 100 μCi of [^{51}Cr]chromate (Nippon Isotope Kyokai, Tokyo) for 30 min at 37 C. After being washed three times with RPMI 1640 containing 10% fetal calf serum, ^{51}Cr-labeled mastocytoma P815-X2 cells were adjusted to 1×10^5 viable cells/ml.

(iii) Cell-mediated cytotoxicity assay. Assay was carried out by the method of Brunner et al. (9) with slight modifications. One milliliter of a ^{51}Cr-labeled target cell suspension (1×10^5/ml) mixed with an equal volume of effector cell suspension (1×10^7) was incubated at 37 C for 20 h in a humidified CO_2 incubator. All assays were set up in duplicate or triplicate. The ratio of effector cells to target cells was 100:1. As a control, target cells, either mixed with nonimmunized spleen cells or alone, were incubated for the same period. At the end of the incubation period, the cells were centrifuged at $1,000 \times g$ for 10 min. One milliliter of supernatant was carefully removed and counted in a well-type gamma radiation counter. Target cell lysis was expressed as percentage of specific target cell lysis according to the following formula:

$$\% \text{ of specific target cell lysis} = \frac{\text{release with effector cells} - \text{control release}}{\text{maximal release} - \text{control release}} \times 100$$

Maximal chromium release was measured at the time of complete cell lysis, when target cells alone were frozen and thawed two times.

Determination of adjuvant activity on antihapten response to the T-independent antigen DNP-Ficoll in vivo and in vitro. C57Bl/6J mice were immunized intraperitoneally with a PBS solution of DNP_{26}-Ficoll (100 μg) and synthetic muramylpeptides (100 μg). One week later, anti-trinitrophenyl (TNP) PFC in spleen cells were assayed. Anti-TNP antibody in sera was determined by passive hemagglutination with TNP SRBC by using microtiter plates (Cooke Engineering Co., Alexandria, Va.). Details of the in vivo and in vitro assay of antihapten antibody were described previously (28). In the in vitro system, spleen cells (2×10^7) were cultured with 10 ng of DNP_{26}-Ficoll and synthetic muramylpeptides for 4 days by Marbrook's method (19), and anti-TNP PFC were determined.

Determination of mitogenic activity in mouse spleen cells. Spleen cells (2×10^6) of C57Bl/6J mice suspended in 1 ml of RPMI 1640 containing 10% fetal calf serum were incubated with synthetic peptide dissolved in PBS or with mitogens for 48 h at 37 C in a humidifed CO_2 incubator, and 0.5 μCi of [^{3}H]thymidine (The Radiochemical Centre, Amersham, England) was added from 24 h until the end of culture. The spleen cells were collected by centrifugation and washed with PBS and 5% trichloroacetic acid. After dissolution of the cells by the addition of NCS tissue solubilizer (Amersham/Searle, Arlington, Ill.), 10 ml of toluene scintillator {5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1,000 ml of toluene} was added, and then the incorporation of [^{3}H]thymidine was counted in a liquid scintillation counter (Nuclear Chicago Mark II).

RESULTS

Adjuvant activity of synthetic peptides on the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs. Hartley strain guinea pigs were immunized with 50 μg of ABA-*N*-acetyltyrosine dissolved in PBS with or without 100 μg of synthetic peptides dissolved in PBS in Freund incomplete adjuvant as a water-in-oil emulsion. Two weeks later, a skin test with ABA-BαA was done. *N*-acetylmuramyltetrapeptide and *N*-acetylmuramyldipeptide showed adjuvant activity on the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs (Table 1). It has been observed that D-lactyltetrapeptide (or D-lactyldipeptide) is liberated when *N*-acetylmuramyltetrapeptide was kept at alkaline conditions (higher than pH 11.0) (Kusumoto et al., unpublished data). In this experiment, the adjuvant activity of chemically synthesized D-lactyltetrapeptide and D-lactyldipeptide on the induction of delayed-type hypersensitivity to ABA-*N*-acetyltyrosine in guinea pigs was also examined. Both D-lactyltetrapeptide and D-lactyldipeptide were inactive as adjuvant in this system even at a dose of 500 μg (Table 1). To confirm this result, the adjuvant activity of tetrapeptide, *N*-acetylmuramyldipeptide, and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide was examined at various doses in guinea pigs. *N*-acetylmuramyldipeptide showed potent adjuvant activity on the induction of delayed hypersensitivity to ABA-*N*-acetyltyrosine at doses of 1, 10, and 100 μg per guinea pig (Table 2). On the other hand, 6-*O*-stearoyl-*N*-acetylmuramyldipeptide showed clear adjuvant activity only at a dose of 100 μg. The delayed-type skin reaction to ABA-*N*-acetyltyrosine was not clear in guinea pigs when 10 or 1 μg of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide was used as adjuvant in this study. Tetrapeptide did not have

adjuvant activity, as was shown previously (4).

Adjuvant activity of synthetic peptides on the induction of the circulating antibody to BαA in mice. Saline solutions of the synthetic peptides were mixed with BαA dissolved in saline and emulsified in a water-in-oil form in Freund incomplete adjuvant. Each 0.2 ml of the emulsion was injected intraperitoneally into (C57Bl/6J × DBA/2)F_1 mice and BαA in sera was determined by the method of Okada et al. (23). All the synthetic compounds tested in this study suppressed circulating-antibody formation to BαA in sera (Table 3); however, potent adjuvant activity was observed when a mixture of PBS solution of synthetic *N*-acetylmuramyldipeptide or 6-*O*-stearoyl-*N*-acetylmuramyldipeptide and saline solution of BαA was emulsified in a water-in-oil form in Freund incomplete adjuvant and injected intraperitoneally into mice. Tetrapeptide was inactive as adjuvant for the induction of circulating antibody to BαA in mice (Fig. 2). Maximal antibody formation was

TABLE 1. *Adjuvant activity of synthetic peptides on the induction of delayed-type hypersensitivity to ABA-N-actyl-L-tyrosine in guinea pigs*[a]

Synthetic peptides tested	Dose (μg)	Skin reaction with ABA-BαA at 48 h	
		No. positive/total	Avg sizes (mm)
N-acetylmuramyltetrapeptide (MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala)	100	6/6	18 by 20
N-Acetylmuramyldipeptide (MurNAc-L-Ala-D-isoGln)	100	5/5	16 by 16
N-Acetylmuramyl-L-alanine (MurNAc-L-Ala)	100	0/4	0
Tetrapeptide (L-Ala-D-isoGln-L-Lys-D-Ala)	100	0/12	0
Tripeptide (L-Ala-D-isoGln-L-Lys)	100	0/6	0
Dipeptide (L-Ala-D-isoGln)	100	0/2	0
D-Lactyltetrapeptide (D-CH_3CHOHCO-L-Ala-D-isoGln-L-Lys-D-Ala)	500	0/5	0
	50	0/5	0
D-Lactyldipeptide (D-CH_3CHOHCO-L-Ala-D-isoGln)	500	0/5	0
	50	0/5	0
Control		0/4	0

[a] Hartley guinea pigs were immunized in each footpad with 50 μg of ABA-*N*-acetyltyrosine in Freund incomplete adjuvant with synthetic peptides. The control group was immunized with 50 μg of ABA-*N*-acetyltyrosine alone in Freund incomplete adjuvant. Two weeks later, a skin test with 100 and 10 μg of ABA-BαA was carried out, and the skin reaction was measured at 24 and 48 h after the intradermal injection of test antigen. The table indicates the results with 100 μg of ABA-BαA at 48 h.

TABLE 2. *Adjuvant activity of synthetic peptides on the induction of delayed-type hypersensitivity to ABA-N-acetyltyrosine in guinea pigs*[a]

Synthetic peptide	Dose (μg)	No. of animals	Skin reaction (mm ± SE)[b] to ABA-BαA at:	
			24 h	48 h
Tetrapeptide	100	4	(4.3 ± 2.7)[c]	0
	10	4	(4.3 ± 1.4)	0
	1	4	(6.0 ± 0.7)	0
N-Acetylmuramyldipeptide	100	3	22.0 ± 0.7	17.5 ± 0.3
	10	3	21.0 ± 0.9	20.0 ± 1.1
	1	3	18.5 ± 0.8	13.5 ± 1.3
6-*O*-stearoyl-*N*-acetylmuramyldipeptide	100	3	17.2 ± 1.2	15.2 ± 1.3
	10	4	(11.4 ± 0.5)	(6.1 ± 1.7)
	1	6	(11.6 ± 2.6)	(8.1 ± 2.1)
Control		4	(6.7 ± 1.5)	0

[a] Hartley guinea pigs were immunized in each footpad with ABA-*N*-acetyltyrosine (50 μg) in Freund incomplete adjuvant with synthetic peptides. The control group was immunized with ABA-*N*-acetyltyrosine alone in Freund incomplete adjuvant as a water-in-oil emulsion. Two weeks later, a skin test with ABA-BαA (100 μg) was carried out, and the reaction was measured at 24 and 48 h after the intradermal injection of test antigen.

[b] SE, Standard error.

[c] Numbers in parentheses indicate the size of erythema.

observed at 30 days after immunization with 100 μg of synthetic *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide. Synthetic peptides were then dissolved in PBS and used in the following experiments.

Adjuvant activity of synthetic *N*-acetylmuramyldipeptides on the primary immune response to SRBC in vitro. Adjuvant activity of synthetic *N*-acetylmuramyldipeptides was examined by the method of Marbrook (19) in vitro, using a culture from a mixture of mouse spleen cells and SRBC with or without synthetic peptides. Both *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide showed marked adjuvant activity on the formation of 19*S* PFC to SRBC by 4 days of culture.

TABLE 3. *Effect of synthetic peptides dissolved in saline on the circulating-antibody production to BαA in mice*[a]

Group	Synthetic peptide added (100 μg)	Antibacterial α-amylase antibody titer (U) at:	
		28 days	42 days
1	*N*-Acetylmuramyl-tetrapeptide	3.0	9.6 (0.1)[b]
2	*N*-Acetylmuramyl-dipeptide	7.5	12.0 (0.1)
3	Tetrapeptide	2.0	0
4	Tripeptide	16.6	13.2 (0.2)
5	Dipeptide	3.1	0
6	BCG cell wall	62.2	162.0 (2.6)
7	Control	34.2	62.1 (1.0)

[a] (C57Bl/6J × DBA/2)F_1 mice were immunized intraperitoneally with 100 μg of BαA with synthetic peptides that were dissolved in saline and suspended in Freund incomplete adjuvant as a water-in-oil emulsion. The control group was immunized with BαA dissolved in saline in Freund incomplete adjuvant as a water-in-oil emulsion. Antibacterial α-amylase titer was determined by the method of Okada et al. (23).

[b] Numbers in parentheses are stimulation ratios.

Adjuvant activity of synthetic *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide to the thymus-independent antigen DNP-Ficoll in vivo and in vitro. It has been established that DNP-Ficoll is a thymus- and macrophage-independent antigen (21). To examine the adjuvant effect of these synthetic muramylpeptides to this antigen in vivo, mice were immunized intraperitoneally with 100 μg of DNP_{26}-Ficoll and 1,000, 100, or 20 μg of synthetic muramylpeptides dissolved in PBS. Seven days later, anti-TNP PFC in spleens and anti-TNP antibody in sera of immunized mice were determined. *N*-acetylmuramyldipeptide clearly showed adjuvant activity at 1,000 and 100 μg, and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide showed activity at 1,000, 100, and 20 μg (Table 5). Table 6 shows the effect of synthetic muramylpeptides on the immune response to DNP-Ficoll in vitro. Spleen cells of C57Bl/6J mice were cultured in vitro with DNP_{26}-Ficoll (0.01 μg) and synthetic muramylpeptides for 4 days. Synthetic muramylpeptides, *N*-acetylmuramyldipeptide, and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide were all adjuvant active on

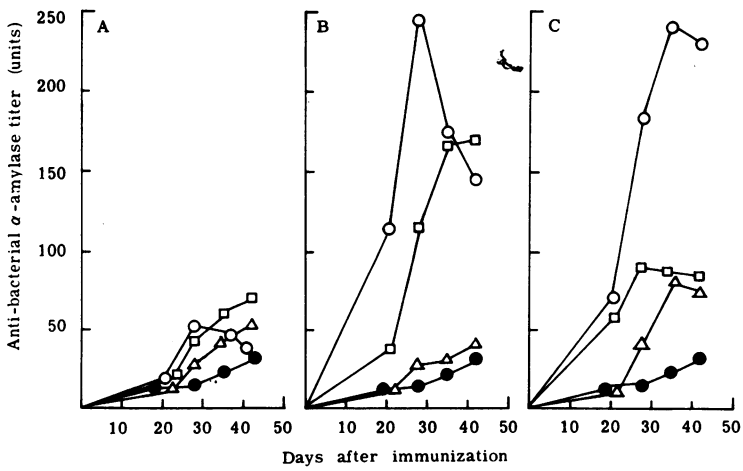

FIG. 2. *Adjuvant activity of synthetic peptides on the production of circulating antibody to bacterial α-amylase. (C57Bl/6J × DBA/2)F_1 mice were immunized intraperitoneally with 100 μg of bacterial α-amylase and 100 (○), 10 (□), or 1 (△) μg of tetrapeptide (A), 6-O-stearoyl-N-acetylmuramyldipeptide (B), or N-acetylmuramyldipeptide (C) in Freund incomplete adjuvant. Control mice (●) were immunized with 100 μg of bacterial α-amylase alone in Freund incomplete adjuvant. Antibacterial α-amylase antibody titer was determined by the method of Okada et al. (23). Each group contained seven mice, and antibody titers were determined by using pooled sera.*

TABLE 4. *Adjuvant activity of synthetic peptides on the primary immune response to SRBC in vitro*[a]

Synthetic peptide	Dose (μg)	19S PFC/culture ± SE	
		Expt 1	Expt 2
N-Acetylmuramyldipeptide	100	2,623 ± 295 (11.9)[b]	440 ± 25 (5.4)
	10	2,775 ± 415 (12.6)	810 ± 36 (6.0)
	1	1,070 ± 75 (4.8)	468 ± 38 (3.4)
6-*O*-stearoyl-*N*-acetylmuramyldipeptide	100	155 ± 15 (0.7)	75 ± 35 (0.5)
	10	2,568 ± 722 (11.7)	1,118 ± 101 (8.2)
	1	3,590 ± 157 (16.2)	1,665 ± 461 (12.3)
Control		220 ± 30 (1.0)	135 ± 19 (1.0)

[a] Normal spleen cells of C57Bl/6J mice (2×10^7 cells/tube) were cultivated for 4 days with SRBC (4×10^6 cells/tube) and synthetic peptides. The control group was incubated with spleen cells and sheep erythrocytes without synthetic muramylpeptides. PFC responses were assayed by the method of Jerne and Nordin (15). Each value represents the arithmetic mean (19*S* PFC/culture) of duplicate cultures ± the standard error (SE) of the mean.

[b] Numbers in parentheses are stimulation ratios.

TABLE 5. *Adjuvant activity of synthetic muramylpeptides on the immune response to DNP-Ficoll in vivo*[a]

Adjuvant added	Dose (μg)	Anti-TNP PFC/spleen[b]	Anti-TNP antibody[c]
N-Acetylmuramyldipeptide	1,000	43,373 ± 2,770 (1.8)[d]	2^4
	100	36,380 ± 1,230 (1.5)	2^4
	20	17,240 ± 1,694 (0.7)	2^3
6-*O*-stearoyl-*N*-acetylmuramyldipeptide	1,000	38,868 ± 9,114 (1.6)	2^4
	100	46,190 ± 5,683 (1.9)	2^4
	20	35,460 ± 4,520 (1.5)	2^4
Control		23,346 ± 2,851 (1.0)	2^3

[a] C57Bl/6J mice were immunized intraperitoneally with 100 μg of DNP_{26}-Ficoll and synthetic muramylpeptides dissolved in PBS. The control group was immunized with DNP_{26}-Ficoll alone. Seven days later, antihapten PFC and antihapten antibody in sera were determined.

[b] Mean of the number of specific anti-TNP PFC in four mice ± standard error.

[c] Dilution titer of passive hemagglutination by using TNP-SRBC in the pooled sera.

[d] Numbers in parentheses are stimulation ratios.

antibody production in the DNP_{26}-Ficoll in vitro system (Table 6).

Adjuvant activity of synthetic peptides on the generation of cell-mediated cytotoxic effector cells in mice. Mice were immunized intraperitoneally with mastocytoma P815-X2 cells combined with an oil-treated preparation, or with a PBS solution of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide, or with oil droplets without adjuvant to mastocytoma cells. Eleven days after immunization, cell-mediated cytotoxicity of mouse spleen cells was determined by using ^{51}Cr-labeled mastocytoma P815-X2 cells as target cells. 6-*O*-stearoyl-*N*-acetylmuramyldipeptide showed no adjuvant activity in either group of mice that were immunized with mastocytoma P815-X2 cells and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide, either treated with oil droplets or dissolved in PBS (Table 7). It was also shown that *N*-acetylmuramyldipeptide dissolved in PBS showed no adjuvant activity in this system. However, the enhancing effect of *N*-acetylmuramyldipeptide dissolved in PBS on in vitro sensitization of C57Bl/6J mouse spleen cells to alloantigen (mitomycin C-treated DBA/2 mouse spleen cells) was observed (Table 8).

Mitogenic activity of synthetic peptides. All the synthetic peptides tested in this study were inactive as mitogen on mouse spleen cells (Table 9).

DISCUSSION

It has been shown that *N*-acetylmuramyldipeptide, MurNAc-L-Ala-D-isoGln, is the minimum adjuvant-active unit of the bacterial cell wall peptidoglycan in the immune system of guinea pigs (Table 1; 1, 6, 17). In this paper, we examined the adjuvant and mitogenic activities of synthetic peptides and 6-*O*-stearoyl-*N*-acetyl-

TABLE 6. *Effect of synthetic muramyldipeptides on the in vitro primary antihapten response to DNP-Ficoll in mice*[a]

Adjuvant added	Dose (μg)	19S PFC/culture ± SE
N-Acetylmuramyldipeptide	100	249 ± 24 (2.6)[b]
	10	358 ± 28 (2.4)
	1	230 ± 41 (2.4)
6-*O*-stearoyl-*N*-acetylmuramyldipeptide	100	147 ± 28 (1.5)
	10	181 ± 31 (1.9)
	1	141 ± 18 (1.5)
Control		96 ± 10 (1.0)

[a] Normal spleen cells (2×10^7) of C57Bl/6J mice were cultured for 4 days with 10 ng of DNP_{25}-Ficoll and synthetic muramyldipeptide. The control group was cultured with spleen cells and DNP_{25}-Ficoll without synthetic muramyldipeptides. Each value represents the arithmetic mean of duplicates and standard error (SE) of the mean.

[b] Numbers in parentheses are stimulation ratios.

TABLE 7. *Effect of synthetic 6-O-stearoyl-N-acetylmuramyldipeptide on the induction of cell-mediated cytotoxicity to mastocytoma P815-X2 cells in C57Bl/6J mice*[a]

Mice immunized with:	Specific target cell lysis (%)
Mastocytoma P815-X2 cells (1×10^4)	
+ Oil-treated 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (100 μg)	4.8
+ Oil-treated 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (10 μg)	7.4
+ Oil-treated 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (1 μg)	3.4
+ Oil droplets	5.1
+ 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (100 μg) dissolved in PBS	4.2
+ 6-*O*-stearoyl-*N*-acetylmuramyldipeptide (10 μg) dissolved in PBS	4.9
+ Phosphate-buffered saline (PBS)	4.2
Mastocytoma P815-X2 cells (3×10^7)	89.0

[a] C57Bl/6J mice were immunized intraperitoneally with a mixture of mastocytoma P815-X2 cells and adjuvant or cell alone. Eleven days later, cell-mediated cytotoxicity was determined by the method of Brunner et al. (9) by the incubation of spleen cells obtained from immunized mice and ^{51}Cr-labeled mastocytoma P815-X2 cells (target cells) at a ratio of 100:1 for 20 h.

muramyldipeptide in mice and guinea pigs.

It is evident that *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide are adjuvant active when mixed with antigen in Freund incomplete adjuvant as a water-in-oil emulsion, and the fact that the possible degraded products of *N*-acetylmuramylpeptides, i.e., D-lactyltetrapeptide and D-lactyldipeptide, are inactive as adjuvant suggests that *N*-acetylmuramic acid residue is very important for the adjuvant activity of *N*-acetylmuramyltetrapeptide and *N*-acetylmuramyldipeptide. However, *N*-acetylmuramyldipeptide cannot be associated to oil droplets. The 6-*O*-acyl derivative, 6-*O*-stearoyl-*N*-acetylmuramyldipeptide, being soluble both in water and mineral oil, can be used as adjuvant both as a water-in-oil and an oil-in-water emulsion. Both *N*-acetylmuramyldipeptides and 6-*O*-stearoyl-*N*-acetylmuramylpeptide showed potent adjuvant activity on the induction of delayed-type hypersensitivity to ABA-*N*-acetyl-L-tyrosine in guinea pigs (Table

TABLE 8. *Effect of N-acetylmuramyldipeptide on in vitro sensitization of C57Bl/6J mouse spleen cells (H-2*^{b}*) to alloantigen (H-2*^{d}*)*[a]

Stimulating cells	Adjuvant added (10 μg)	Specific target lysis (%)
MMC-DBA/2 (3×10^4)	*N*-acetylmuramyldipeptide	40.3
MMC-DBA/2 (3×10^4)	None	14.5
MMC-DBA/2 (3×10^5)	*N*-acetylmuramyldipeptide	81.0
MMC-DBA/2 (3×10^5)	None	59.7
MMC-DBA/2 (1.5×10^7)	None	75.4
None	*N*-acetylmuramyldipeptide	2.8

[a] Normal spleen cells (6×10^7) of C57Bl/6J mice were cultured for 6 days with mitomycin C-treated DBA/2 (MMC-DBA/2) mouse spleen cells with or without *N*-acetylmuramyldipeptide dissolved in PBS. After incubation, viable spleen cells were recovered and adjusted to 5×10^6 cells/ml. For the assay of cell-mediated cytotoxicity, spleen cells were incubated with ^{51}Cr-labeled mastocytoma P815-X2 cells (1×10^5 cells/ml) at a ratio of 50:1 for 20 h.

TABLE 9. *Mitogenic activity of synthetic peptides on normal spleen cells of C57Bl/6J mice*[a]

Mitogen added	Dose (μg)	Incorporation of [^{3}H]thymidine (counts/min)	
Tetrapeptide	100	935 ± 88	(0.4)[b]
	10	1,764 ± 238	(0.8)
	1	1,733 ± 33	(0.8)
N-acetylmuramyldipeptide	100	3,359 ± 559	(1.6)
	10	2,834 ± 452	(1.3)
	1	1,583 ± 63	(0.7)
6-*O*-stearoyl-*N*-acetylmuramyldipeptide	100	1,916 ± 425	(0.9)
	10	2,238 ± 236	(1.1)
	1	2,278 ± 79	(1.1)
Control		2,130 ± 134	(1.0)
Phytohemagglutinin	0.1 ml	18,783 ± 234	(8.8)
Sodium dextran sulfate	100	4,835 ± 684	(2.3)
Concanavalin A	10	32,298 ± 1,640	(15.2)

[a] Normal spleen cells (2×10^6) of C57Bl/6J mice were incubated with mitogen or spleen cells alone for 48 h. [^{3}H]thymidine (0.5 μCi) was added from 24 h until the end of culture.

[b] Numbers in parentheses are stimulation ratios.

2). It was also shown that the adjuvant activity of *N*-acetylmuramyldipeptide was not potentiated by 6-*O*-stearoylation. As shown in Table 3, all the synthetic peptides that were dissolved in saline solution and mixed with BαA in Freund incomplete adjuvant as a water-in-oil emulsion showed no adjuvant activity and suppressed circulating-antibody formation in mice. However, *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide, which were dissolved in PBS and mixed with BαA in Freund incomplete adjuvant as a water-in-oil emulsion, clearly showed adjuvant activity on circulating-antibody formation in mice (Fig. 1). Although the discrepancy in these experiments (Table 2 and Fig. 1) is not yet resolved, we suggest that the acidity due to the free carboxylic acid of both synthetic muramylpeptides or to the detergent activity of 6-*O*-stearoyl-*N*-acetylmuramyldipeptide may affect the protein antigen and then influence the immune response in mice. Tetrapeptide showed no adjuvant activity on the production of circulating antibody to BαA in mice (Fig. 1), consistent with the results of Table 1. Both synthetic muramylpeptides were adjuvant active on the production of 19*S* PFC to SRBC in an in vitro culture system (Table 4).

Recently, Mosier et al. (21) and Sharon et al. (25) have shown that DNP-Ficoll is the thymus- and macrophage-independent antigen in vitro. Previously, we reported that the cell walls prepared from cells of *M. bovis* BCG, *Nocardia rubra*, and *Corynebacterium diphtheriae* PW8 acted as adjuvant on the antibody formation to DNP-Ficoll in mice (T. Taniyama et al., unpublished data). Similarly, the present data indicate that *N*-acetylmuramyldipeptide is active as adjuvant on the immune response to DNP-Ficoll in mice in vivo and in vitro. These results suggest that *N*-acetylmuramyldipeptide acts as adjuvant to T-independent antigen.

More recently, we (2, 27) have shown that the cell-mediated cytotoxicity test using the method of Brunner et al. (9) is one of the most useful systems for screening immunotherapeutic agents in experimental animals and humans. 6-*O*-stearoyl-*N*-acetylmuramyldipeptide that was treated with minute oil droplets and suspended in PBS showed no adjuvant activity on the generation of cell-mediated cytotoxic effector cells in the spleen of allogeneic mice in vivo (Table 7). Similarly, *N*-acetylmuramyldipeptide dissolved in PBS was also shown to be inactive as adjuvant for the induction of cell-mediated cytotoxic effector cells by the immunization with mastocytoma P814-X2 cells in vivo. However, *N*-acetylmuramyldipeptide showed adjuvant activity on the in vitro sensitization of C57Bl/6J mouse spleen cells to alloantigen (Table 8). These results suggest that *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide are not effective as adjuvant when injected intraperitoneally. Preliminary experiments indicated that both *N*-acetylmuramyldipeptide and 6-*O*-stearoyl-*N*-acetylmuramyldipeptide were not effective for the suppression of tumor growth of EL4 and melanoma B16 in C57Bl/6J mice by the method described previously (3, 7).

These synthetic muramylpeptides were not mitogenic in the normal spleen cells of C57Bl/6J mice (Table 9). Recently, Rosenstreich et al. (24) reported that the synthetic glycolipid *N*-palmitoyl-D-glucosamine, which was heated and sonicated, was mitogenic for mouse B lymphocytes. In their experiment, it was shown that *N*-palmitoyl-D-glucosamine was mitogenic only after treatment by heating and sonication, and it was also suggested that the physical state, a certain degree of dispersion, of this synthetic glycolipid affected the mitogenic activity. We have reported elsewhere (Azuma et al., in press) that the cell walls, which were water insoluble and polymerized peptidoglycolipid, of mycobacteria, nocardia, corynebacteria, and anaerobic coryneforms were mitogenic in both T and B lymphocytes of mice. However, recent papers (8, 10, 20) have shown that water-soluble adjuvants that consisted of arabinogalactan and mucopeptide (peptidoglycan) were B cell mitogens and acted on macrophages (20). More recently, Damais et al. (11) have reported that peptidoglycans prepared from *Escherichia coli* and *Bacillus megaterium* have potent adjuvant activity on spleen cells of rabbits and of normal or nude mice. They have also shown that the peptidoglycan monomer prepared from *E. coli* is adjuvant active but not mitogenic. These results suggest that both adjuvant and mitogenic activities exhibited in cell walls are clearly dissociated in the low-molecular-weight peptidoglycans and muramyldipeptides, and higher-molecular-weight substances (or the aggregated form of low-molecular-weight substances) may be necessary for mitogenic activity.

As summarized in Table 10, the results reported in this study suggest that *N*-acetylmuramyldipeptide can represent only a part of the immunological activity of the cell walls of mycobacteria, nocardia, and corynebacteria (2–4, 7, 22, 28; T. Taniyama, unpublished data), and they also suggest that mycolic acid and arabinogalactan may modify and potentiate immunological activities such as adjuvant, mito-

TABLE 10. *Comparison of biological activities of synthetic muramyldipeptides with those of BCG cell wall*

Biological activity	BCG cell wall	*N*-acetylmur-amyldipeptide	6-*O*-stearoyl-*N*-acetylmur-amyldipeptide
Adjuvant effect on:			
Circulating-antibody formation to:			
SRBC (in vitro)	++	++	++
BαA (in vivo)	++	++	++
DNP_{26}-Ficoll			
in vivo	++	++	++
in vitro	++	++	++
Cell-mediated immunity to:			
ABA-*N*-acetyltyrosine (in vivo)	++	++	+
Allogeneic cell-mediated cytotoxicity			
in vivo	++	−	−
in vitro	++	+	ND[a]
Mitogenic activity in spleen cells of C57Bl/6J mice (in vitro)	++	−	−
Antitumor activity to transplantable tumors in syngeneic mice (E14, melanoma B16 in C57B1/6J mice	++	−	−

[a] ND, Not done.

genic, and antitumor activities of the peptidoglycan moiety of the cell walls of mycobacteria, nocardia, and corynebacteria.

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