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Effect of Quinonyl-*N*-Acetyl Muramyl Dipeptide on Immune Responses in Tumor-Bearing Mice

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The efficacy of 6-*O*-QS-10-*N*-acetyl muramyl-L-valyl-D-isoglutamine methyl ester (quinonyl-MDP-66) for restoring impaired immune status was examined in mice bearing Lewis lung carcinoma. Quinonyl-MDP-66 suspended in phosphate-buffered saline was shown to restore the depressed allogeneic cell-mediated cytotoxicity of spleen cells from mice with Lewis lung carcinoma when the chemical was injected twice intraperitoneally, intravenously, or intratumorally. However, primary tumor size and the numbers of lung metastases were not affected when quinonyl-MDP-66 was administered under the present experimental conditions. Intraperitoneal injection of quinonyl-MDP-66 in mice with Lewis lung carcinoma enhanced host resistance to *Listeria monocytogenes* infection.

N-Acetyl muramyl-L-alanyl-D-isoglutamine [MurNAc-L-Ala-D-Glu(OH)-NH_2, or MDP] has been demonstrated to be a minimal structure of bacterial peptidoglycans required for adjuvant activity on immune responses (1, 2, 9, 19).

Previously we have synthesized various derivatives of MDP and examined their adjuvant and antitumor activities in mice and guinea pigs (5). It has been shown that methyl 2-{2-acetamido-2-deoxy-6-*O*-[10-(2,3-dimethoxy-5-methyl-1,4-benzoquinon-6-yl)-decanoyl]-D-glucopyranos-3-*O*-yl}-D-propionyl-L-valyl-D-isoglutaminate [6-*O*-QS-10-MurNAc-L-Val-D-Glu(OCH_3)-NH_2, or quinonyl-MDP-66], a QS-10 ester of MurNAc-L-Val-D-Glu(OCH_3)-NH_2, has potent adjuvant activity on both humoral and cell-mediated immune responses, such as antibody formation to sheep erythrocytes in vivo and in vitro, delayed-type hypersensitivity to monoazobenzenearsonate-*N*-acetyl-L-tyrosine in guinea pigs, allogeneic cell-mediated cytotoxicity against P815 mastocytoma cells, and tumor-suppressive activity in mice and guinea pigs (6, 18, 23, 24).

In the present paper, we investigate the restorative effect of quinonyl-MDP-66 on the impaired immune responses and the lowered resistance to bacterial infection in mice bearing syngeneic transplantable tumors.

MATERIALS AND METHODS

Adjuvant. Quinonyl-MDP-66 was synthesized by the procedure described previously (18) and was used as a suspension in phosphate-buffered saline (PBS) in this study.

Animals. Female inbred C57BL/6 and DBA/2 mice 8 to 10 weeks old were purchased from the Shizuoka Agricultural Cooperative for Experimental Animals, Hamamatsu, Japan. The animals were given food (Nihon Nosan Kogyo Co., Ltd., Yokohama, Japan) and water freely.

Tumors. Mastocytoma P815-X2 cells (P815) from DBA/2 mice were serially passed in the ascitic form through syngeneic mice. Lewis lung carcinoma (3LL) from C57BL/6 mice, a malignant metastasizing tumor, was kindly supplied by T. Kataoka (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan) and was maintained by biweekly subcutaneous transplantation in C57BL/6 mice. For the preparation of 3LL cell suspension, the solid 3LL tumor was removed, trimmed off the necrotic regions, and minced in PBS. Each cell suspension was obtained by mildly stirring the minced tissue in a mixture of 0.5% pronase (Kaken Kagaku Co., Ltd., Tokyo, Japan) and 0.07% collagenase (Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) in Veronal buffer (pH 7.5) for about 2 h at 37°C. After the reaction was terminated with RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y., lot R781615), the cells were washed and suspended in cold Hanks balanced salt solution (Nissui Seiyaku Co., Ltd., Tokyo). The viability of the cells determined by trypan blue dye exclusion was always >95%.

Experimental design in tumor-bearing mice. From the results obtained by preliminary experiments (Table 1), the following experimental systems were designed. C57BL/6 mice were inoculated subcutaneously with 5×10^5 viable 3LL cells (0.05 ml) in the right flank, and 7 days later they were immunized intraperitoneally (i.p.) with 4×10^5 to 6×10^5 P815 cells. At this time, the primary tumor was 7 to 12 mm in diameter. Cell-mediated cytotoxicity against P815 cells was determined 18 days after tumor inoculation. In separate experiments, 3LL-bearing mice were injected intrave-

TABLE 1. Kinetics of cell-mediated cytotoxicity and pulmonary metastases in 3LL-bearing C57BL/6 mice

Group	No. of mice	Time of inoculation with 3LL (day)[a]	Wt of primary tumor ± SE (g) on day 11	Lysis (%)	P[b]	Lung nodules per mouse	
						Individual	Median
1	3			78.5 ± 8.3			
2	5	−1	0.31 ± 0.10	69.5 ± 13.8	NS[c]	7, 6, 5, 5, 0	5
3	6	−6	2.13 ± 0.30	12.1 ± 6.5	<0.01	41, 38, 31, 16, 10, 5	23.5
4	8	−13	5.66 ± 0.43	5.2 ± 1.9	<0.01	>100, >100, >100, >100, >100, >100, >100, 74	>100

[a] Mice were inoculated with 5×10^5 3LL cells on the days indicated and then were immunized on day 0 with 6×10^5 P815 cells.

[b] Determined by Student's *t* test. Groups 2, 3, and 4 were compared with group 1.

[c] NS, Not significant.

nously (i.v.) with various amounts of viable *Listeria monocytogenes* 10 or 15 days after tumor inoculation. The viable units of *L. monocytogenes* in the spleens were measured 3 or 8 days after infection. In both experiments, four or five C57BL/6 mice in each group were used, and quinonyl-MDP-66 was injected i.p., i.v., or intratumorally (i.t.) twice at 2 and 5 days after tumor inoculation. No antigenic cross-reactivity between 3LL and P815 cells was confirmed by the assay of cell-mediated cytotoxicity (data not shown).

Cell-mediated cytotoxicity. The cell-mediated cytotoxicity assay was carried out by the method of Brunner et al. (7) with some modifications. Briefly, ^{51}Cr-labeled P815 cells (target cells, 10^5 per ml) and immune spleen cells (effector cells, 10^7 per ml) in RPMI-1640 medium with 10% fetal bovine serum were incubated for 18 to 20 h at 37°C. At the end of incubation, the radioactivities in the culture supernatants were measured with a gamma counter. The percentage of target cell lysis was [(release with effector − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum release was determined by freezing and thawing ^{51}Cr-labeled P815 cells three times.

Pulmonary metastases of 3LL in C57BL/6 mice. Lung metastases were counted by the method of Wexler (30) with a dissecting microscope. They were revealed as white spots on lungs infused with India ink. No attempt was made to count beyond a total of 100.

Protection against *L. monocytogenes* infection. *L. monocytogenes* strain EGD was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), harvested while still in log phase (about 5×10^8 cells), dispersed in vials in 2-ml lots, frozen, and stored at −70°C until used. For each experiment, a vial was thawed and diluted appropriately in saline for i.v. inoculation. The infectious inoculum was 4×10^2 to 6×10^2 bacteria in a volume of 0.2 ml of saline. Bacterial growth in the spleen was followed 3 or 8 days after infection by plating 10-fold serial dilutions of whole spleen homogenates on brain heart infusion agar. Colonies were counted after 24 h of incubation at 37°C.

RESULTS

In preliminary experiments, the kinetics of cell-mediated cytotoxicity against P815 cells in 3LL-bearing C57BL/6 mice were examined. C57BL/6 mice were inoculated subcutaneously with 5×10^5 viable 3LL cells and were immunized i.p. with 6×10^5 P815 cells at various days after inoculation with 3LL. At 11 days after immunization, pulmonary metastases and cell-mediated cytotoxicity against P815 cells were determined. Cell-mediated cytotoxicity in control mice (group 1) reached about 80% lysis (Table 1). In contrast, cell-mediated cytotoxicity in the mice receiving 3LL cells decreased markedly with the tumor-bearing period of the hosts. Similarly, the number of lung nodules increased as tumor growth progressed. Moreover, since 3LL-bearing mice die in about 25 days in these experimental systems, we determined the cell-mediated cytotoxicity and pulmonary metastases 18 days after tumor inoculation (described above).

Effects of quinonyl-MDP-66 on cell-mediated cytotoxicity in tumor-bearing mice. Impaired cell-mediated cytotoxicity was restored to normal levels when 3LL-bearing mice were treated i.p., i.v., and i.t. with quinonyl-MDP-66 (Table 2). However, no statistically significant differences in primary tumor size and the number of lung nodules among groups 2, 3, 4, and 5 were observed by the Mann-Whitney *U* test.

Effects of quinonyl-MDP-66 on bacterial growth in 3LL-bearing mice. The effects of quinonyl-MDP-66 on the nonimmune macrophage-mediated bacterial resistance on day 3 after infection (experiment 1) and on the T cell-mediated immune resistance on day 8 (experiment 2) were examined (Table 3). The viable units of *L. monocytogenes* in the spleens of 3LL-bearing mice (group 2) were markedly decreased compared with those in the spleens of normal mice on day 3 after infection (Table 3, experiment 1). Moreover, in 3LL-bearing mice, quinonyl-MDP-66 further reduced the viable units in the spleens when it was injected i.p., i.v., or i.t. By contrast, the viable units of *L. monocytogenes* in the spleens of 3LL-bearing

TABLE 2. Effect of quinonyl-MDP-66 on depressed cell-mediated cytotoxicity and pulmonary metastases in 3LL-bearing mice

Group[a]	Treatment (days 2 and 5)	Wt of primary tumor ± SE (g) on day 18	Lysis ± SE (%)	Lung nodules per mouse	
				Individual	Median
1			69.3 ± 2.2		
2	PBS, i.p.	2.01 ± 0.05	3.1 ± 4.6	29, 34, 40, 61	37
3	Quinonyl-MDP-66 (400 μg twice), i.p.	1.65 ± 0.34	67.0 ± 1.5	32, 33, 33, 37, 52	33
4	Quinonyl-MDP-66 (400 μg twice), i.v.	1.73 ± 0.22	53.2 ± 6.3	17, 37, 51, 58, >100	51
5	Quinonyl-MDP-66 (400 μg twice), i.t.	1.89 ± 0.36	61.1 ± 2.7	9, 21, 31, 42, 70	31

[a] C57BL/6 mice were used. All, except those in group 1, were inoculated with 3LL cells on day 0; all mice were inoculated with 4×10^5 P815 cells on day 7.

mice were remarkably increased compared with those in the spleens of normal mice on day 8 after infection (Table 3, experiment 2). Treatment i.p. with quinonyl-MDP-66 restored the viable units of *L. monocytogenes* to a normal level. However, the lowered bactericidal activity of 3LL-bearing mice could not be improved by i.v. or i.t. injection of quinonyl-MDP-66.

DISCUSSION

Our previous reports have indicated that quinonyl-MDP-66 suspended in PBS has potent adjuvant activity in mice and guinea pigs and that it has suppressive activity against tumors (Meth A fibrosarcoma) in syngeneic mice when injected together with tumor cells (6, 23, 24). We also found that quinonyl-MDP-66 treated with oil (squalane) has more potent regressive activity against the growth of tumors (line 10 hepatocarcinoma) in strain 2 guinea pigs and inhibits metastases into the regional lymph nodes when injected two or four times i.t. (23). These results suggest that quinonyl-MDP-66, a new synthetic MDP derivative, may be useful as an immunotherapeutic agent.

It is generally accepted that a tumor-bearing host shows immunological hyporesponsiveness, indicating in particular the presence of suppressor cells in various organs (3, 4, 12, 13, 17, 27). Moreover, some experimental data suggest that the immune status of the host is related to the growth of local and disseminated tumor cells and to host resistance to bacterial infections (8, 20, 22, 28, 29). Relieving immunosuppression in a tumor-bearing host might provide a means for restoring both immune defenses to bacterial infection and the ability to inhibit primary and local metastasizing tumor growth. Therefore, we attempted to examine the effect of quinonyl-MDP-66 suspended in PBS on the impaired immune response and lowered resistance to bacterial infection in tumor (3LL)-bearing mice.

The cell-mediated immune response to alloantigen (P815) was markedly depressed in 3LL-

TABLE 3. Effect of quinonyl-MDP-66 on host resistance against *L. monocytogenes* infection in 3LL-bearing mice

Group[a]	Treatment (days 2 and 5)	Viable units of *L. monocytogenes* in spleen (day 18)[b]	
		Expt. 1 ($\times 10^5$)	Expt. 2 ($\times 10^3$)
1		2,052 ± 108	2.5 ± 0.5
2	PBS, i.p.	714 ± 77	26.3 ± 7.4
3	Quinonyl-MDP-66 (400 μg twice), i.p.	413 ± 124	1.7 ± 1.0
4	Quinonyl-MDP-66 (400 μg twice), i.v.	455 ± 133	17.3 ± 3.2
5	Quinonyl-MDP-66 (400 μg twice), i.t.	316 ± 65	28.3 ± 11.4

[a] C57BL/6 mice were used. All, except those in group 1, were inoculated with 3LL cells on day 0.

[b] Five mice in each group were inoculated i.v. with 4.4×10^2 on day 15 (experiment 1) or 6×10^2 on day 10 (experiment 2) viable cells of *L. monocytogenes*, and viable units of *L. monocytogenes* in the spleen were counted 3 days (experiment 1) or 8 days (experiment 2) after infection. Numbers show means from individual mice ± standard deviation.

bearing mice. However, quinonyl-MDP-66 was shown to restore the depressed cell-mediated cytotoxicity of spleen cells from 3LL-bearing mice when injected twice i.p., i.v., or i.t. (Table 2).

Recently, attention in cancer research has focused on the role of a nonspecific immune mechanism mediated by natural killer cells and macrophages in inhibiting the growth and dissemination of tumors (10, 14–16, 26). In addition, several investigators have reported increased numbers of lung metastases in immunologically impaired animals (8, 20, 28, 29). We have found that quinonyl-MDP-66 was icity against allogeneic and syngeneic tumor cells in vivo and in vitro (I. Saiki, Y. Tokushima, K. Nishimura, Y. Yamamura, and I. Azuma, submitted for publication). However, the data shown in Table 2 indicate that primary tumor size and the number of lung metastases did not decrease significantly when quinonyl-MDP-66 suspended in PBS was injected i.p., i.v., or i.t. More recently, quinonyl-MDP-66 was shown to have potent tumor-regressive activity in a line 10, strain 2 guinea pig system when it was injected intralesionally as a squalene-treated emulsion (23). Fidler et al. and Sone and Fidler have reported that MDP encapsulated within liposomes is more efficient in rendering alveolar macrophages cytotoxic and in inhibiting lung metastases than unencapsulated MDP is (11, 25). These reports suggest that quinonyl-MDP-66 may be expected to be more effective as a cancer immunotherapeutic agent for inhibiting the growth of primary or disseminated tumors if it is administered in an improved adjuvant form or with better timing.

Matsuo et al. and Miyata et al. (21, 22) have shown in detail that nonimmune macrophage-mediated resistance in the host to *L. monocytogenes* is suppressed up to day 4 after tumor inoculation but is enhanced thereafter. On the other hand, T cell-mediated immune resistance remains at the control level up to day 7 but is suppressed thereafter. Similarly in our experiments, nonimmune macrophage-mediated resistance to *L. monocytogenes* was enhanced on day 18 after tumor inoculation (Table 3, experiment 1) and T cell-mediated immune resistance was suppressed (Table 3, experiment 2) compared with results for normal mice. Furthermore, nonimmune macrophage-mediated resistance in 3LL-bearing mice was slightly enhanced when quinonyl-MDP-66 was administered i.p., i.v., or i.t. T cell-mediated immune resistance in 3LL-bearing mice was restored to the normal level when quinonyl-MDP-66 was administered i.p.

From these results, it is suggested that quinonyl-MDP-66 when used as a suspension in PBS provides a means of repairing impaired cell-mediated cytotoxicity and the lowered antibacterial resistance in tumor-bearing mice, in addition to the other immunomodulating activities described previously (6, 23).

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