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Adjuvant Activity of Purified Peptidoglycan of *Listeria monocytogenes* in Mice and Guinea Pigs

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The immunological properties of peptidoglycan (L-PG) purified from the cell wall skeleton (L-CWS) of *Listeria monocytogenes* strain EGD were investigated and compared with the properties of L-CWS. L-PG consisted of alanine, glutamic acid, α,ε-diaminopimelic acid, muramic acid, and glucosamine. L-PG showed potent adjuvant activities for circulating antibody formation and development of delayed-type hypersensitivity to bacterial α-amylase in vivo and for the primary immune response to sheep erythrocytes in vitro, as well as L-CWS. Both L-PG and L-CWS enhanced the generation of cell-mediated cytotoxicity in allogeneic mice and activated thioglycolate-elicited peritoneal macrophages and macrophage cell line RAW 264 to kill tumor target cells in vitro. We also found that L-PG acted on normal spleen cells as a mitogen. Both L-PG and L-CWS had tumor (Meth A)-suppressive and -regressive activities in syngeneic mice. Our results suggest that the L-PG moiety retains the adjuvant and antitumor activities of L-CWS.

Previously, we have reported that the cell wall skeletons of various bacteria, such as *Mycobacterium bovis* BCG, *Nocardia asteroides* 131, *Nocardia rubra*, *Corynebacterium diphtheriae* PW8, and *Propionibacterium acnes* C7, show immunoadjuvant activity for circulating antibody formation, induction of cell-mediated immunity, and antitumor activity in experimental animals (3, 4, 6, 31, 32). We have also shown that the peptidoglycan moieties of *M. bovis* BCG, *N. asteroides* 131, and *C. diphtheriae* PW8 prepared by acid treatment from the corresponding cell wall skeletons retain adjuvant activity (3). Several investigators (1, 2, 17, 22) clearly showed that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is the minimum adjuvant-active subunit of these bacterial cell wall skeletons and peptidoglycans. The results obtained from extensive studies have suggested that the immunological properties of MDP are partially different from those of cell wall skeletons or peptidoglycan moieties. The most distinctive differences was identified in the case of the antitumor activities of these adjuvants. The cell wall skeletons of *M. bovis* BCG, *Nocardia*, and *P. acnes* were shown to have potent antitumor activities in transplantable tumor systems in syngeneic mice and guinea pigs; however, MDP showed no antitumor activity in these tumor systems (7). These results may be considered to be due to the physicochemical properties of these adjuvants. The bacterial cell wall skeletons and peptidoglycans are non-metabolizable and water-insoluble polymers; on the other hand, MDP is a low-molecular-weight, water-soluble glycopeptide.

Recently, we have been interested in the biochemical and immunological properties of the cellular components, especially the cell surface components, of *Listeria monocytogenes*, which is known to be one of the facultative intracellular bacteria, in comparison with the properties of the cellular components of tubercle bacilli, which are typical intracellular bacteria. More recently, we have described the detailed chemical structure of the peptidoglycan moiety (L-PG) which was prepared from the purified cell wall skeleton (L-CWS) of *L. monocytogenes* strain EGD (20a). The L-PG was easily prepared by mild acid treatment of L-CWS. The results of chemical analyses of L-PG and L-CWS suggested that L-PG could be prepared from L-CWS without serious damage to its chemical nature. This allowed us to study the biological properties of L-PG, and in the present paper we describe the adjuvant activities of L-PG and compare them with the activities of L-CWS, heat-killed cells of *L. monocytogenes*, and MDP.

MATERIALS AND METHODS

L-PG, L-CWS, and other adjuvants. *L. monocytogenes* strain EGD was cultured on Trypto-soya broth (Nissui Seiyaku Co., Ltd., Tokyo, Japan) and grown

TABLE 1. Chemical compositions of L-CWS and L-PG[a]

Compound	Lipid (%)	Total carbohydrate (%)	Neutral sugars (molar ratios)			Amino acids and amino sugars (μmol/mg)						Phosphorus (μmol/mg)
			Rhamnose	Ribose	Glucose	D-Glutamic acid	Total alanine	L-Alanine	*meso*-Diaminopimelic acid	Muramic acid	Glucosamine	
L-CWS	<1	20.0	8.7	1.3	1.0	0.435	0.552	0.390	0.426	0.403	1.145	1.156
L-PG		<0.1				1.000	1.247	0.949	0.955	0.923	0.921	0.103

[a] L-CWS and L-PG were *N*-acetylated before acid hydrolysis because a non-*N*-substituted glucosamine residue is very resistant to acid hydrolysis.

in a controlled environment incubator shaker (New Brunswick Scientific Co., Edison, N.J.) at 37°C for 3 days with constant shaking (120 rpm). The cells were heat killed at 100°C for 30 min in an autoclave, collected by centrifugation at 3,000 × *g* for 20 min, and then washed three times with water. Purified L-CWS was prepared from these heat-killed *L. monocytogenes* strain EGD cells by a method similar to one described previously (4). Briefly, the heat-killed *L. monocytogenes* cells (killed *Listeria* cells) were suspended in cold water and disrupted with a Dynomill (type KDL) at 3,000 rpm for 10 to 15 min at 5 to 10°C by using 0.1-mm glass beads. After the unbroken cells and debris were removed by centrifugation, the crude cell wall fraction was collected by centifugation at 20,000 × *g* for 60 min. The fraction obtained in this way was treated repeatedly with DNase, pronase, trypsin, and α-chymotrypsin and then washed several times with water. The enzyme-treated cell wall fraction was extracted with diethylether-ethanol (1:1) and chloroform-methanol (2:1). The residue prepared in this way was referred to as L-CWS. The L-CWS was treated with 5% trichloroacetic acid at 37°C for 48 h. After the residue was washed three times with water, it was extracted with diethylether-ethanol (1:1). The resulting residue was referred to as L-PG. The chemical compositions of L-CWS and L-PG are summarized in Table 1. The cell wall skeleton of *N. rubra* was prepared by a similar method, as described previously (4). MDP was chemically synthesized in our laboratory. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5, prepared by phenol-water extraction, was purchased from Difco Laboratories, Detroit, Mich.

Animals and tumors. Six- to eight-week-old, female, inbred C57BL/6J, BALB/c, and DBA/2 mice and Hartley strain guinea pigs weighing 350 to 400 g were purchased from Shizuoka Agricultural Co-operatives for Experimental Animals, Hamamatsu, Japan. These animals were given food (Nihon Nosan Kogyo Co., Ltd., Yokohama, Japan) and water freely. Mastocytoma P815-X2 (derived from DBA/2 mice) and Meth A fibrosarcoma (derived from BALB/c mice) were serially passed in ascitic form in syngeneic mice. Cell line FBL-3, Friend virus-induced leukemia cells of C57BL/6 mouse origin (19), and macrophage cell line RAW 264 (an Abelson leukemia virus-transformed line of BALB/c mouse origin) (26) were kindly supplied by M. Ito (Toyama Medical and Pharmaceutical University, Sugitani, Toyama, Japan) and were maintained in in vitro cultures.

Antigens and mitogens. Crystalline bacterial α-amylase (BαA; EC 3.3.1.1; Seikagaku Kogyo Co., Ltd., Tokyo, Japan) from *Bacillus subtilis* was obtained commercially. Sheep erythrocytes (SRBC) preserved in Alsever solution were purchased from Nippon Bio-Test Laboratories Inc., Tokyo, Japan, and were washed three times with sterilized phosphate-buffered saline (PBS) before use. Concanavalin A (ConA; batch 4000; Pharmacia Fine Chemicals, Uppsala, Sweden) and LPS were used as T- and B-cell mitogens in this study.

Medium solution. Hanks balanced salt solution and RPMI 1640 medium were obtained from Nissui Seiyaku Co., Ltd., and were supplemented with 100 U of penicillin G per ml and 100 μg of streptomycin per ml. Fetal bovine serum (lot R781615) was purchased from GIBCO Laboratories, Grand Island, N.Y., and was inactivated by heating at 56°C for 30 min before use.

Determination of immune response to BαA. Each Hartley strain guinea pig was immunized in its four footpads with a total of 300 μg of BαA with or without killed *Listeria* cells, L-CWS, and L-PG in Freund incomplete adjuvant (a mixture of liquid paraffin and Aracel A [85:15]). After 4 weeks, the guinea pigs were bled by puncturing the retroorbital venous plexus with fine capillary glass pipettes. Anti-BαA antibody in sera was measured by the enzymatic method described by Okada et al. (24). At 24 h after bleeding, skin tests were performed with 100, 50, or 10 μg of BαA, and skin reactions were measured 4, 24, and 48 h after intradermal injection of the test antigen.

In vitro culture system and PFC assay. Using the Marbrook technique (23), 10^7 normal BALB/c spleen cells and 4×10^6 SRBC suspended in 1 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum were cultured with or without killed *Listeria* cells, L-CWS, and L-PG. Triplicate cultures were harvested on day 4 and were assayed for plaque-forming cells (PFC) by the method of Jerne and Nordin (20), using the modifications described previously (5).

Allogeneic cell-mediated cytotoxicity. C57BL/6 inbred mice ($H\text{-}2^b$) were immunized intraperitoneally with 1.5×10^5 viable mastocytoma P815-X2 ($H\text{-}2^d$) cells in the presence or absence of a suspension of killed *Listeria* cells, L-CWS, and L-PG in PBS. On day 14 after immunization, a cytotoxicity assay was carried out by using the spleen cells of immunized mice and ^{51}Cr-labeled mastocytoma P815-X2 cells at a ratio of effector cells to target cells of 100:1, using the method of Brunner et al. (10), with some modifications. The culture fluids were harvested after 20 h, and the radioactivity in the supernatant fluids was measured with a gamma scintillation counter. Target cell

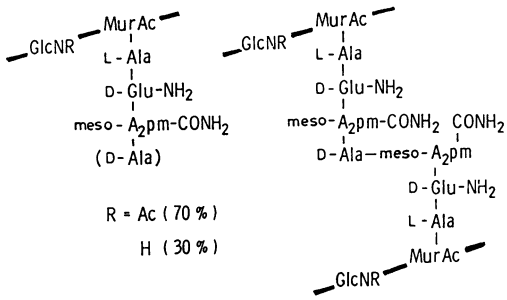

FIG. 1. Proposed structure of L-PG. Abbreviations: GlcN, glucosamine; MurAc, *N*-acetylmuramic acid; A_2pm, Diaminopimelic acid; Ac, acetyl.

lysis was expressed as the percentage of specific target cell lysis, calculated as follows: [(release with effector cells − spontaneous release)/(maximum release − spontaneous release)] × 100. Maximum chromium release was measured at complete cell lysis after target cells were frozen and thawed three times.

Macrophage-mediated cytotoxicity. As a source of macrophages, peritoneal exudate cells were harvested sterilely from C57BL/6 mice which had received intraperitoneally 1 ml of a 3% thioglycolate solution (Difco Laboratories) 4 or 5 days previously. The cells were seeded into a hemacytometer (Bürker-Türk type; Erma Optical Works, Tokyo, Japan) and were incubated in a humidified culture dish for 2 h at 37°C to avoid drying. The glass plate of the hemacytometer was washed intensively with Hanks balanced salt solution to remove nonadherent cells, and then the remaining adherent cells (i.e., glass-adherent cells) on the hemacytometer were counted. Using this method of calculation, we adjusted the peritoneal exudate cells to a concentration of 5×10^6 adherent cells per ml in RPMI 1640 medium supplemented with 10% fetal bovine serum. More than 98% of the adherent cells, which constituted 70 to 80% of the elicited peritoneal exudate cells, displayed morphological characteristics of macrophages.

The adjusted cells were plated at a concentration of 5×10^5 cells per well into a micro tissue culture plate (type 3040; Micro Test II; Falcon Plastics, Oxnard, Calif.) and cultured for 2 h at 37°C. After the nonadherent cells were removed by intensive washing, killed *Listeria* cells, L-CWS, and L-PG suspended in culture medium (0.1 ml) were added to each well, and the preparations were cultured for 24 h at 37°C. After additional washing, 0.2 ml of a solution containing 10^4 ^{51}Cr-labeled FBL-3 target cells was added to each well, and incubation was continued for additional 20 h. In another experiment with macrophage cell lines, RAW 264 cells and ^{51}Cr-labeled FBL-3 target cells were cultured in the presence of L-CWS and L-PG for 20 h. After centrifugation of the plate, 0.1 ml of supernatant fluid was removed from each well and measured with a gamma counter. Target cell lysis was expressed as described above.

Determination of mitogenic activity. A total of 4×10^5 normal spleen cells from BALB/c mice suspended in 150 μl of RPMI 1640 medium containing 10% fetal bovine serum were cultured with or without 50 μl of killed *Listeria* cells, L-CWS, and L-PG in micro tissue culture plates for 72 h at 37°C, and then 0.25 μCi of [^{3}H]thymidine (Radiochemical Centre, Amersham, England) was added 24 h before the end of the culture. The cells were harvested on a glass filter by using a multiple cell harvester. The incorporation of [^{3}H]thymidine was measured with a liquid scintillation counter. In another experiment, 5×10^7 spleen cells were treated with either a 1:48 dilution of rabbit anti-mouse brain-associated θ serum prepared by the method of Golub (18) or a 1:20 dilution of rabbit anti-mouse immunoglobulin serum prepared in our laboratory in the presence of a 1:3 dilution of agarose-absorbed guinea pig complement (14) at 37°C for 40 min. Furthermore, θ-positive T cells and immunoglobulin-bearing B cells were detected in these cell populations by using the immunofluorescence technique described previously (30).

Tumor suppression and regression test. A single-cell suspension of 2×10^5 Meth A fibrosarcoma cells in Hanks balanced salt solution was mixed with L-CWS or L-PG suspended in PBS and then injected intradermally into syngeneic BALB/c mice for the suppression test. After 4 weeks cured mice were rechallenged with 2×10^5 of same tumor cells. For the regression test, BALB/c mice were injected intradermally with 2×10^5 Meth A fibrosarcoma cells, and L-CWS or L-PG diluted in PBS was injected intralesionally on different days after tumor cell inoculation. After 6 weeks cured mice were rechallenged with the 2×10^5 of same tumor cells. In both experiments, the tumor sizes at the inoculated sites and the survival rates were examined every week.

RESULTS

Chemical compositions of L-CWS and L-PG. As shown in Table 1, L-CWS was mainly composed of polysaccharide and peptidoglycan components. The polysaccharide contained rhamnose and glucosamine as major constituents and small amounts of ribose and glucose. L-PG, which was obtained by treating L-CWS with 5% trichloroacetic acid as described above, consisted of alanine, glutamic acid, *meso*-diaminopimelic acid, muramic acid, and glucosamine. Based on the results of the composition study and a determination of the N- and C-terminal amino acids, in addition to a characterization of fragments obtained by enzymatic treatment and partial acid hydrolysis of L-PG, which will be described in detail elsewhere (20a), the possible structure of L-PG is illustrated in Fig. 1. We suggest that L-PG contains a cross-linkage between *meso*-diaminopimelic acid and D-alanine, which belongs to the $A_{1\gamma}$ type according to the classification scheme of Schleifer and Kandler (28). It should be noted that L-PG contains glucosamine residues with free amino groups (about 30%), which are responsible for the resistance of this compound to lysozyme.

Effects of L-CWS and L-PG on the immune response to BαA. As Table 2 shows, guinea pigs immunized with BαA and MDP showed potent

TABLE 2. Effects of L-CWS and L-PG on the immune response to BαA in guinea pigs

Fraction	Dose (μg)	Skin reaction (mm) with 50 μg of BαA at:[a]			Anti-BαA titer (U)[b]
		4 h	24 h	48 h	
Killed *Listeria* cells	100	10.4 ± 0.5	15.4 ± 0.4	11.0 ± 0.4	976 ± 66 (6.6)
	10	8.3 ± 0.5	15.4 ± 0.6	7.3 ± 1.8	555 ± 48 (3.8)
L-CWS	100	9.9 ± 0.5	16.4 ± 0.6	10.5 ± 0.6	1278 ± 241 (8.7)
	10	9.8 ± 0.5	12.5 ± 1.6	6.0 ± 2.3	703 ± 61 (4.8)
L-PG	100	8.3 ± 0.4	15.1 ± 1.2	9.4 ± 0.5	746 ± 73 (5.1)
	10	9.2 ± 0.7	12.2 ± 0.6	4.7 ± 1.5	652 ± 111 (4.4)
MDP	100	10.3 ± 0.2	14.6 ± 0.5	12.3 ± 0.4	930 ± 50 (6.3)
	10	8.5 ± 0.5	15.2 ± 1.7	11.5 ± 1.2	967 ± 155 (6.6)
Control (BαA + FIA)[c]		9.0 ± 0.4	10.5 ± 1.4	0	147 ± 28 (1.0)

[a] The data are expressed as mean diameter ± standard error of the skin reaction for four guinea pigs.
[b] The data are expressed as mean units ± standard error. The numbers in parentheses are stimulation indexes, as calculated with the control.
[c] FIA, Freund incomplete adjuvant.

Arthus type and delayed-type skin reactions after intradermal injection of 50 μg of BαA. Killed *Listeria* cells, L-CWS, and L-PG also had adjuvant activity on the induction of delayed-type hypersensitivity to BαA in guinea pigs; at 48 h this activity was similar to or less than that of MDP. In contrast, only Arthus type skin reactions were observed in guinea pigs immunized with BαA in Freund incomplete adjuvant (control group). We also found that killed *Listeria* cells, L-CWS, L-PG, and MDP enhanced the production of anti-BαA antibody in sera compared with the control. In particular, L-CWS was highly effective as an adjuvant at a dose of 100 μg.

Effects of L-CWS and L-PG on the primary immune response to SRBC in vitro. As Table 3 shows, L-CWS and L-PG, as well as MDP and LPS, showed adjuvant activity on the formation of 19S PFC in response to SRBC after 4 days of culture. In addition, L-CWS and L-PG also increased the background PFC response of normal spleen cells cultured in the absence of SRBC, but L-CWS and L-PG were less active than LPS. However, killed *Listeria* cells were less active for both the formation of 19S PFC and background PFC responses compared with L-CWS and L-PG. In both cases, there was no difference in the number of viable cells recovered after 4 days of culture (about 70%).

TABLE 3. Effects of L-CWS and L-PG on the primary immune response to SRBC in vitro

Fraction	Dose (μg)	No. of 19S PFC (×10⁶)[a]	
		+SRBC	−SRBC
Killed *Listeria* cells	100	67 ± 9 (0.7)[b]	13 ± 1 (1.3)
	10	120 ± 15 (1.3)	
L-CWS	100	222 ± 18 (2.4)	42 ± 4 (4.2)
	10	119 ± 10 (1.3)	
L-PG	100	214 ± 37 (2.3)	52 ± 13 (5.2)
	10	432 ± 77 (4.6)	
MDP	100	315 ± 66 (3.4)	55 ± 11 (5.2)
	10	261 ± 10 (2.8)	
LPS	100	322 ± 67 (3.4)	126 ± 4 (12.6)
Control		94 ± 23 (1.0)	10 ± 3 (1.0)

[a] The data are expressed as arithmetic means of triplicate cultures ± standard errors.
[b] The numbers in parentheses are the ratios which were calculated with the respective controls.

Effects of L-CWS and L-PG on the induction of allogeneic cell-mediated cytotoxicity in mice. Table 4 shows that killed *Listeria* cells, L-CWS, and L-PG enhanced the generation of cell-mediated cytotoxicity in allogeneic C57BL/6 mice; this enhancement was similar to that of *N. rubra* cell wall skeleton which was demonstrated to have potent adjuvant activity previously (3, 6, 31, 32).

Cytolytic activities of peritoneal macrophages and a macrophage cell line activated by L-CWS and L-PG in vitro. As Table 5 shows, in two experiments killed *Listeria* cells, L-CWS, and L-PG were capable of activating the thioglycolate-elicited peritoneal macrophages of C57BL/6 mice to be cytotoxic for ^{51}Cr-labeled FBL-3 tumor cells compared with the control; however, these preparations were not as active as LPS, which is well known as a macrophage activator. Also, MDP could slightly activate peritoneal macrophages to kill tumor target cells at a ratio of effector cells to target cells of 50:1. In the case of the macrophage cell line, L-CWS and L-PG could similarly activate and become cytotoxic against ^{51}Cr-labeled FBL-3 tumor cells (Table 6).

Mitogenic activities of L-CWS and L-PG. Normal BALB/c spleen cells were cultured with different doses (1, 10, and 100 μg/ml) of killed *Listeria* cells, L-CWS, and L-PG. As Table 7

TABLE 4. Effects of L-CWS and L-PG on the induction of allogeneic cell-mediated cytotoxicity in mice

Mice immunized with:	Dose (μg)	^{51}Cr release (cpm)	
		Expt 1	Expt 2
Mastocytoma P815 (1.5×10^5 cells)			
+killed *Listeria* cells	100	3,628 ± 62 (89.9)[a]	11,896 ± 678 (83.2)
	10	3,686 ± 48 (91.9)	
+L-CWS	100	3,308 ± 122 (78.7)	11,238 ± 818 (76.6)
	10	3,350 ± 122 (80.2)	
+L-PG	100	3,676 ± 52 (91.6)	12,822 ± 472 (92.5)
	10	2,594 ± 384 (54.0)	
+*N. rubra* cell wall skeleton	100	3,660 ± 62 (91.0)	13,200 ± 174 (96.3)
	10	2,508 ± 160 (51.0)	
Mastocytoma P815 alone (1.5×10^5 cells)		1,410 ± 122 (12.9)	5,702 ± 636 (21.0)
Mastocytoma P815 alone (4×10^7 cells)		3,384 ± 100 (81.4)	13,082 ± 150 (95.1)

[a] The results are expressed as mean ± standard error. The numbers in parentheses are cytotoxicities, expressed as percentages of specific target cell lysis. The spontaneous release was 1,040 ± 34 cpm (experiment 1) or 3,610 ± 12 cpm (experiment 2), and the maximum release was 3,919 ± 48 cpm (experiment 1) or 13,567 ± 54 cpm (experiment 2).

shows, killed *Listeria* cells, L-CWS, and L-PG showed remarkable mitogenic activity on normal spleen cells from BALB/c mice. In particular, L-CWS and L-PG at concentrations of 100 μg/ml acted as mitogens as well as LPS at a concentration of 100 μg/ml. In another experiment, both L-CWS and LPS could act as mitogens on spleen cells treated with rabbit anti-mouse brain-associated θ serum supplemented with complement; however, ConA could not act as a mitogen. In contrast, L-CWS and LPS could not act as mitogens on spleen cells treated with rabbit anti-mouse immunoglobulin serum in the presence of complement, but ConA could act as a mitogen.

Antitumor activities of L-CWS and L-PG. As Table 8 shows, L-CWS and L-PG suspended in PBS had tumor-suppressive activities at varying doses (100, 25, and 5 μg), and tumor-suppressed mice showed resistance to reinoculated tumor cells. Table 9 shows that repeated intralesional injection of L-CWS or L-PG was more effective for regression of a Meth A fibrosarcoma than a single injection on day 2 or 5. Similar to the suppression test, tumor-regressed mice rejected reinoculated tumor cells.

TABLE 5. Effects of L-CWS and L-PG on the cytolytic activity of thioglycolate-elicited peritoneal macrophages in vitro

Fraction	Dose (μg)	Cytotoxicity (cpm)	
		Expt 1	Expt 2
Killed *Listeria* cells	100	1,350 ± 9 (18.5)[a]	1,618 ± 32 (39.4)
	10	1,150 ± 36 (9.8)	1,210 ± 50 (11.3)
L-CWS	100	1,312 ± 4 (16.9)	1,410 ± 34 (25.1)
	10	1,192 ± 4 (11.7)	1,220 ± 46 (12.0)
L-PG	100	1,758 ± 32 (36.3)	1,400 ± 62 (24.4)
	10	1,138 ± 26 (9.3)	1,316 ± 46 (18.6)
MDP	100	1,076 ± 78 (6.6)	1,170 ± 54 (8.5)
	10	1,066 ± 36 (6.2)	ND[b]
LPS	25	1,980 ± 34 (46.0)	1,728 ± 24 (47.0)
	10	1,652 ± 74 (31.7)	1,722 ± 40 (46.6)
Control		934 ± 32 (0.4)	1,134 ± 88 (6.1)

[a] The results are expressed as mean ± standard error. The numbers in parentheses are cytotoxicities, expressed as percentages of specific target cell lysis. The maximum release was 3,221 ± 36 cpm (experiment 1) or 2,498 ± 34 cpm (experiment 2), and the spontaneous release was 924 ± 19 cpm (experiment 1) or 1,046 ± 32 cpm (experiment 2).

[b] ND, Not done.

DISCUSSION

Our results indicate that both L-CWS and the purified L-PG moiety of the gram-positive, intracellular bacterium *L. monocytogenes* strain EGD have immunological and antitumor activi-

TABLE 6. Effects of L-CWS and L-PG on cytolytic activity of macrophage cell line RAW 264 in vitro

Fraction	Dose (μg)	Cytotoxicity (cpm)
L-CWS	100	776 ± 42 (45.6)[a]
	10	676 ± 22 (29.8)
L-PG	100	858 ± 16 (58.8)
	10	758 ± 10 (42.8)
LPS	10	842 ± 14 (56.2)
	1	784 ± 22 (47.0)
Control		486 ± 12 (−0.5)

[a] The results are expressed as mean ± standard error. The numbers in parentheses are cytotoxicities, expressed as percentages of specific target cell lysis. The maximum release was 1,117 ± 22 cpm, and the spontaneous release was 489 ± 6 cpm.

TABLE 7. Mitogenic activities of L-CWS and L-PG in normal BALB/c spleen cells

Mitogen added	Dose (μg)	Incorporation of [^{3}H]thymidine into spleen cells (cpm)[a]			
		Expt 1 (normal)	Expt 2		
			Normal	Anti-BAθ + complement	Anti-MIg + complement
Killed *Listeria* cells	100	11,613 ± 183 (2.8)[b]			
	10	15,019 ± 1,041 (3.6)	ND[c]	ND	ND
	1	10,320 ± 495 (2.5)			
L-CWS	100	46,660 ± 213 (11.2)	10,226 ± 539 (9.7)	7,449 ± 621 (11.1)	3,437 ± 770 (1.9)
	10	22,062 ± 1,093 (5.3)	2,339 ± 251 (2.2)	1,785 ± 397 (2.7)	1,613 ± 166 (0.9)
	1	11,292 ± 493 (2.7)	1,079 ± 290 (1.0)	1,336 ± 297 (2.0)	1,711 ± 394 (1.0)
L-PG	100	34,285 ± 944 (8.2)			
	10	12,455 ± 398 (3.0)	ND	ND	ND
	1	9,994 ± 178 (2.4)			
LPS	100	35,342 ± 318 (8.5)	9,815 ± 642 (9.3)	8,008 ± 429 (11.9)	4,805 ± 187 (2.8)
ConA	1	82,048 ± 4,029 (17.6)	28,500 ± 1,623 (27.1)	1,514 ± 47 (2.3)	95,933 ± 2,169 (55.0)
Medium alone		4,174 ± 148 (1.0)	1,053 ± 121 (1.0)	673 ± 81 (1.0)	1,745 ± 282 (1.0)

[a] The data are expressed as arithmetic means of triplicate cultures ± standard errors. Anti-BAθ, Rabbit anti-mouse brain-associated θ serum; anti-MIg, rabbit anti-mouse immunoglobulin serum.
[b] The numbers in parentheses are stimulation indexes, which were calculated with the respective controls.
[c] ND, Not done.

ties in mice and guinea pigs. Other workers have reported that a crude cell wall fraction (11–13, 15, 27), monocytosis-producing extracts (29), a heat-labile water-soluble extract (21), and a culture filtrate (25) of *L. monocytogenes* enhance murine immune responses in vivo and in vitro. However, the biochemical properties of these cellular fractions used in the immunological studies were not demonstrated. Also, the biological activities of purified peptidoglycan have been little studied. As Table 1 shows, L-CWS was composed of polysaccharide and peptidoglycan components. L-PG was easily obtained by mild acid treatment of L-CWS. Table 2 shows that L-PG and L-CWS have adjuvant activities on both the formation of circulating antibody and the development of delayed-type hypersensitivity to BαA in guinea pigs; these activities are similar to or slightly lower than the activity of MDP. L-CWS and L-PG also increased both 19S PFC and background PFC responses to SRBC in vitro when they were added with antigens at the same time (Table 3). Previously, Campbell et al. (12, 15) reported that a crude cell wall-containing fraction of *L. monocytogenes* enhanced humoral immune responses to erythrocyte antigens in vivo and in vitro and mitogenic responses in mice and also could act directly on murine B cells independent of any T-cell influences. As Table 7 shows, both L-CWS and LPS could act as mitogens on spleen cells treated with rabbit anti-mouse brain-associated θ serum in the presence of complement; however, ConA could not act as a mitogen. In contrast, L-CWS and LPS could not act as mitogens on spleen cells treated with anti-mouse immunoglobulin serum in the presence of complement, but ConA could act as a mitogen. Wexler and Oppenheim (34) indicated that an endotoxin-like component could be isolated from a phenol-water extract of *L. monocytogenes* and that this component had potent biological activities, such as pyrogenicity, endotoxin activity, and the cold agglutinin syndrome. However, although data were not shown, no contamination of endotoxin was detected at a concentration of 10^{-1} μg of L-CWS or L-PG per ml, as determined by the *Limulus* assay. From the results described above, we concluded that L-CWS was a B-cell adjuvant or a B-cell mitogen and that the effects of L-CWS and L-PG were not due to a small amount of contamination with LPS from gram-positive bacteria. This conclusion was supported by the finding of Campbell et al. (15) that the *Listeria* cell wall fraction stimulated the B cells

TABLE 8. Suppression of growth of Meth A fibrosarcoma with L-CWS and L-PG in BALB/c mice

Fraction	Dose (μg)	No. of tumor-free mice/no. of mice tested[a]	No. of tumor-free mice after rechallenge/no. tested[b]
L-CWS	100	10/10	5/10
	25	8/10	3/8
	5	5/10	3/5
L-PG	100	10/10	3/10
	25	9/10	9/9
	5	9/10	7/9
PBS		0/10	0/10

[a] Results 4 weeks after injection of the mixture.
[b] Results 2 weeks after rechallenge of tumor-suppressed and tumor-bearing (control) mice.

TABLE 9. Regression of Meth A fibrosarcoma with L-CWS and L-PG in BALB/c mice

Fraction	Dose (μg)	No. of tumor-free mice/no. of mice tested[a]	No. of tumor-free mice after rechallenge/no. tested[b]
L-CWS	300 × 4 (on days 2, 5, 8, 15)	10/10	9/10
	100 × 4 (on days 2, 5, 8, 15)	10/10	8/10
	100 (on day 2)	9/10	9/9
	100 (on day 5)	6/10	6/6
L-PG	300 × 4 (on days 2, 5, 8, 15)	10/10	7/10
	100 × 4 (on days 2, 5, 8, 15)	10/10	10/10
	100 (on day 2)	9/10	9/9
	100 (on day 5)	5/10	5/5
PBS		0/10	0/10

[a] Results 6 weeks after injection of the mixture.
[b] Results 2 weeks after rechallenge of tumor-suppressed and tumor-bearing (control) mice.

of C3H/HeJ mice, which generally do not respond to LPS.

We also observed that killed *Listeria* cells, L-CWS, and L-PG could activate elicited peritoneal macrophages to become cytotoxic for FBL-3 tumor target cells in vitro (Table 5). Similarly, Campbell et al. (11) showed that a *Listeria* cell wall fraction could induce peritoneal exudate cells from nude mice to kill tumor cells, suggesting that mature T cells were not required. Since more than 98% of the adherent cells in both experiments were macrophages, as determined by morphological observation and the acid phosphatase staining method, and similar results were obtained from an in vitro cytotoxicity test by using macrophage cell line RAW 264 instead of peritoneal macrophages (Table 6), our results also indicated that both L-PG and L-CWS could directly stimulate elicited peritoneal macrophages and macrophage cell lines. Previously, live or killed *L. monocytogenes*, and other microorganisms, such as *M. bovis* BCG and *Corynebacterium parvum*, have been used for experimental immunotherapy of tumors in mice and guinea pigs (8, 9, 17, 35). Tables 8 and 9 show that L-CWS and L-PG had tumor (Meth A)-suppressive and -regressive activities in syngeneic BALB/c mice when they were administered as a suspension in PBS.

Our data confirmed results on the immunological properties of *Listeria* cells and cell fractions previously published by several workers (11–13, 15, 16, 21, 25, 27, 29, 33). Furthermore, we found that L-PG has adjuvant activities on the induction of delayed-type hypersensitivity, allogeneic cell-mediated cytotoxicity, and in vitro macrophage-mediated cytotoxicity in addition to mitogenicity and that it inhibits tumor growth in syngeneic mice as well as L-CWS (Table 10). Therefore, based on the effects of L-CWS and L-PG on B cells and macrophages, we suggest that the cell-mediated immune response may be associated with the activation of macrophages and B cells by their components. Previously, we have shown that acid-treated cell wall skeletons (peptidoglycan) of *M. bovis* BCG, *N. asteroides*,

TABLE 10. Summary of biological properties of L-CWS, L-PG, and MDP

Biological property	Form of administration[a]	Animals	L-CWS	L-PG	MDP[b]
Adjuvant activity on:					
Delayed-type hypersensitivity to:					
Azobenzenearsonate-*N*-acetyl-L-tyrosine	w/o	Guinea pigs	+	+	+
BαA	w/o	Guinea pigs	+	+	+
Allogeneic cell-mediated cytotoxicity, in vivo	PBS	Mice	+	+	−
Humoral antibody formation with:					
SRBC (in vitro)	PBS	Mice	+	+	+
BαA (in vivo)	w/o	Guinea pigs	+	+	+
Macrophages (in vitro)					
Elicited	PBS	Mice	+	+	
Cell line	PBS	Mice	+	+	
Mitogenic activity (in vitro)	PBS	Mice	+	+	
Antitumor activity (mouse transplantable tumor)	PBS	Mice	+	+	−

[a] w/o, Water-in-oil emulsion; PBS, solution or suspension in PBS.
[b] Present study and reference 7.

and *C. diphtheriae* still retain the adjuvant activities but that their activities are drastically decreased (3) because of the drastic acid treatment (0.1 N HCl, 60°C, 24 h) of these cell wall skeletons. Based on the results described above, we suggest that adjuvant and antitumor activities of L-CWS occur in L-PG having almost intact potency. As Table 10 shows, we conclude that L-PG carries almost all of the biological activities which L-CWS exhibits and which MDP can demonstrate partially. L-PG may also be a useful material to study the relationships between the adjuvant and antitumor activities and the chemical structures and morphological properties of L-CWS, L-PG, and MDP.

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LITERATURE CITED

1. **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.
2. **Adam, A., M. Devys, V. Souvannavong, P. Lefrancier, J. Choay, and E. Lederer.** 1976. Correlation of structure and adjuvant activity of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), its derivatives and analogues. Antiadjuvant and competition properties of stereoisomers. Biochem. Biophys. Res. Commun. **72**:229–246.
3. **Azuma, I., F. Kanetsuna, T. Taniyama, Y. Yamamura, M. Hori, and Y. Tanaka.** 1975. Adjuvant activity of mycobacterial fractions. I. Purification and in vivo adjuvant activity of cell wall skeletons of *Mycobacterium bovis* BCG, *Nocarida asteroides* 131 and *Corynebacterium diphtheriae* PW8. Biken J. **18**:1–13.
4. **Azuma, I., K. Sugimura, T. Taniyama, A. A. Aladin, and Y. Yamamura.** 1975. Chemical and immunological studies on the cell walls of *Propionibacterium acnes* strain C7 and *Corynebacterium parvum* ATCC 11829. Jpn. J. Microbiol. **19**:265–275.
5. **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.
6. **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.
7. **Azuma, I., M. Uemiya, I. Saiki, M. Yamawaki, Y. Tanio, S. Kusumoto, T. Shiba, T. Kusama, K. Tobe, H. Ogawa, and Y. Yamamura.** 1979. Synthetic immunoadjuvants—new immunotherapeutic agents, p. 311–330. *In* W. Terry and Y. Yamamura (ed.), Immunobiology and immunotherapy of cancer. Elsevier/North-Holland Publishing Co., New York.
8. **Bast, R. C., B. Zbar, G. B. Mackaness, and H. J. Rapp.** 1975. Antitumor activity of bacterial infection. I. Effect of *Listeria monocytogenes* on growth of a murine fibrosarcoma. J. Natl. Cancer Inst. **54**:749–756.
9. **Bast, R. C., B. Zbar, G. B. Mackaness, and H. J. Rapp.** 1975. Antitumor activity of bacterial infection. II. Effect of *Listeria monocytogenes* on growth of a guinea pig hepatoma. J. Natl. Cancer Inst. **54**:757–761.
10. **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.
11. **Campbell, P. A., J. E. Caldwell, and A. L. Hartman.** 1979. Use of a macrophage cytotoxicity system to show macrophage activation by *Listeria monocytogenes* cell wall fraction. Scand. J. Immunol. **10**:1–9.
12. **Campbell, P. A., G. E. Rodriguez, and C. Schuffler.** 1975. Listeria cell wall fraction: adjuvant activity in vivo and in vitro. Cell. Immunol. **17**:418–422.
13. **Campbell, P. A., C. Schuffler, and G. E. Rodriguez.** 1976. Listeria cell wall fraction: a B cell adjuvant. J. Immunol. **116**:590–594.
14. **Cohen, A., and M. Schlesinger.** 1970. Absorption of guinea pig serum with agar: a method for elimination of its cytotoxicity for murine thymus cells. Transplantation **10**:130–132.
15. **Cohen, J. J., G. E. Rodriguez, P. D. Kind, and P. A. Campbell.** 1975. Listeria cell wall fraction: a B cell mitogen. J. Immunol. **114**:1132–1134.
16. **Dustoor, M. M., A. Fulton, W. Croft, and A. A. Blazkovec.** 1979. Antitumor activity of *Listeria monocytogenes* on a guinea pig fibrosarcoma. Infect. Immun. **23**:54–60.
17. **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.
18. **Golub, E. S.** 1971. Brain-associated θ antigen: reactivity of rabbit anti-mouse brain with mouse lymphoid cells. Cell. Immunol. **2**:353–361.
19. **Igarashi, T., D. Rodrigues, and C. Ting.** 1979. Studies of the mechanisms for the injection of in vivo tumor immunity. IV. Enhancement of the in vitro generation of secondary cell-mediated cytotoxic response by normal peritoneal macrophages and their culture supernatants. J. Immunol. **122**:1519–1527.
20. **Jerne, N. K., and A. Nordin.** 1963. Plaque formation in agar by single antibody-producing cells. Science **140**:405.

20a. **Kamisango, K., I. Saiki, Y. Tanio, H. Okumura, Y. Araki, I. Sekikawa, I. Azuma, and Y. Yamamura.** 1982. Structures and biological activities of peptidoglycans of *Listeria monocytogenes* and *Propionibacterium acnes*. J. Biochem. (Tokyo) **92**:23–33.

21. **Kearns, R. J., and D. J. Hinrichs.** 1978. Heat-labile B-cell mitogen obtained from *Listeria monocytogenes*. Infect. Immun. **22**:676–680.
22. **Kotani, S., Y. Watanabe, F. Kinoshita, T. Shimono, L. 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.
23. **Marbrook, J.** 1967. Primary immune response in cultures of spleen cells. Lancet **ii**:1279–1281.
24. **Okada, Y., K. Onoue, S. Nakashima, and Y. Yamamura.** 1963. Studies on enzyme-antienzyme systems. II. *N*-bromosuccinimide modified bacterial α-amylase. J. Biochem. (Tokyo) **54**:477–483.
25. **Petit, J.-C., and E. R. Unanue.** 1974. Effects of bacterial products on lymphocytes and macrophages: their possible role in natural resistance to *Listeria* infection in mice. J. Immunol. **113**:984–992.
26. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz.** 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell **15**:261–267.
27. **Rodriguez, G. E., J. K. McClatchy, and P. A. Campbell.** 1974. Induction of resistance by *Listeria monocytogenes* cell wall fraction. Infect. Immun. **10**:1163–1169.
28. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan type of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
29. **Stanley, N. F.** 1948. Studies on *Listeria monocytogenes*. I. Isolation of a monocytosis-producing agent (MPA). Aust. J. Exp. Biol. Med. Sci. **27**:123–131.

30. **Sugimura, K., M. Uemiya, I. Saiki, I. Azuma, and Y. Yamamura.** 1979. The adjuvant activity of synthetic N-acetylmuramyl-dipeptides: evidence of initial target cells for the adjuvant activity. Cell. Immunol. **43**:137–149.
31. **Taniyama, T., I. Azuma, and Y. Yamamura.** 1975. Adjuvant activity of mycobacterial fractions. III. Adjuvant effect of cell wall of *Mycobacterium bovis* BCG on cell-mediated cytotoxicity in mice. Jpn. J. Microbiol. **19**:255–264.
32. **Taniyama, T., T. Watanabe, I. Azuma, and Y. Yamamura.** 1974. Adjuvant activity of mycobacterial fractions. II. *In vitro* adjuvant activity of cell walls of Mycobacteria, Nocardia and Corynebacteria. Jpn. J. Microbiol. **18**:415–426.
33. **Ullman, W. W., and J. A. Cameron.** 1969. Immunochemistry of the cell walls of *Listeria monocytogenes*. J. Bacteriol. **98**:486–493.
34. **Wexler, H., and J. D. Oppenheim.** 1979. Isolation, characterization, and biological properties of an endotoxin-like material from the gram-positive organism *Listeria monocytogenes*. Infect. Immun. **23**:845–857.
35. **Youdim, S., M. Moser, and O. Stutman.** 1974. Nonspecific suppression of tumor growth by an immune reaction to *Listeria monocytogenes*. J. Natl. Cancer Inst. **52**:193–198.