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Chemical Composition and Biological Functions of *Listeria monocytogenes* Cell Wall Preparations

NICHOLAS W. HETHER,[1] PRISCILLA A. CAMPBELL,[2] LAWRENCE A. BAKER,[3] AND LARRY L. JACKSON,[1]*

Biochemistry Department, Montana State University, Bozeman, Montana 59717[1]; Department of Medicine, National Jewish Hospital and Research Center, Denver, Colorado 80206[2]; and Abbott Laboratories, North Chicago, Illinois 60064[3]

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A crude *Listeria* cell wall fraction, a purified fraction (PF) with demonstrated biological activity, as well as a third fraction of base-hydrolyzed PF (BHPF) were analyzed for chemical composition and activities not previously described. *Listeria* cell wall fraction and PF contained significant quantities of lipid, whereas BHPF was lipid depleted. Fatty acid compositions were typical of gram-positive bacteria. PF and BHPF were depleted in protein. Alanine, glutamic acid, diaminopimelic acid, glucosamine, and muramic acid were found in all fractions, in enhanced concentration in PF and BHPF, and with molar ratios typical of bacterial peptidoglycans. Major neutral sugars were rhamnose, ribose, ribitol, and glucose. The concentrations of rhamnose, ribose, and glucose were increased in BHPF. Differences in chemical composition of the fractions reflected differences in their biological activities: *Listeria* cell wall fraction induced resistance to *Listeria* infection, whereas PF did not. Mitogenic and adjuvant activities were demonstrated for *Listeria* cell wall fraction and PF but were lost in BHPF. BHPF retained the ability to induce macrophage-mediated tumoricidal activity and decrease resistance to *Listeria* infection.

Immunological properties of cell wall preparations from a virulent strain of *Listeria monocytogenes* (serotype 1) have been described. A crude *Listeria* cell wall fraction (LCWF) acts as an adjuvant (6, 7, 28) and B-cell mitogen (8). LCWF induces chemotaxigenesis of neutrophils and activates complement by the alternative pathway (4). LCWF also induces macrophage activation (5) and causes decreased resistance to bacterial infection (3). A purified fraction (PF) was prepared by protease and nuclease digestion of LCWF (4). The loss of protein and nucleic acid in the preparation of PF does not abolish activity observed with LCWF (3–5), implicating the cell wall milieu as the possible agent of activity. LCWF, PF, and a third preparation of base-hydrolyzed PF (BHPF) were examined for chemical composition and for biological properties not previously reported.

MATERIALS AND METHODS

Preparation of LCWF. LCWF was prepared as described previously (24). PF was prepared from LCWF by protease and nuclease digestion of LCWF (3). BHPF was prepared from PF by mild base hydrolysis. PF was dispersed in 0.1 M ethanolic NaOH and allowed to react for 30 min at 20°C. Insoluble material was removed by centrifugation at 23,300 × *g* for 15 min at 4°C. The pellet (BHPF) was washed five times in distilled water, lyophilized, and stored at −20°C until use. The supernatant liquid was examined for lipids.

Lipid analysis. Lipids were extracted from the liquid phase of the mild base hydrolysis of PF. Nonsaponifiable lipids were extracted from the supernatant liquid three times with a hexane-ether mixture (1:1 [vol/vol]). Pooled extracts were dried and weighed. The supernatant liquid was then adjusted to pH 2 with 1.0 N HCl, and the saponifiable lipids were extracted with hexane-ether. Duplicate samples of LCWF were hydrolyzed, respectively, in 4 N NaOH and 4 N HCl to release hydroxy fatty acids (23), and the lipids were extracted in the manner described for PF. Control samples of *Escherichia coli* lipopolysaccharide (Sigma Chemical Co., St. Louis, Mo.) and of methyl-(3-hydroxy)tetradecanoate (Applied Science Laboratories, State College, Pa.) were treated in the same manner as the LCWF samples throughout the experiment.

Analytical thin-layer chromatography (TLC) was done on silica plates (Absorbosil 2; Applied Science Laboratories) developed in hexane-diethyl ether-acetic acid (85:15:1 [vol/vol]; solvent 1) and, to detect hydroxy fatty acids (20), in hexane-diethyl ether (1:1 [vol/vol]; solvent 2). Visualization was done by spraying with sulfuric acid-potassium dichromate followed by charring. To separate lipid fractions, preparative TLC was done on analytical plates (developed in solvent 1) after spraying with 0.5% rhodamine 6 G in

ethanol. Argentation TLC was done to examine fatty acid fractions for unsaturated fatty acids. Lipid standards (Applied Science Laboratories) were run concurrently on all plates. Lipid samples and fatty acid fractions were methylated either by the method of Schlenk and Gellerman (27) or by coinjection into a gas-liquid chromatograph with Meth-Prep I (Applied Science Laboratories) (18). Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC). Before GLC analysis, the respective samples and controls to be examined for hydroxy fatty acids were split, and one portion of each was treated to form the trimethylsilyl ether derivatives (15). Chromatograms of trimethylsilyated portions of the respective controls and samples were observed for diminution of peak areas, for peaks with altered retention times, and for the appearance of new peaks relative to those of untreated portions.

GLC was done on a 10-m fused silica capillary column of SE-30 programmed from 150 to 230°C at 5°C/min after a 1.0-min initial hold and with a flow rate of 5 ml/min. Iso- and anteiso-branched-chain fatty acid methyl ester isomers were resolved on a 50-m stainless steel capillary column of Dexil 300 programmed from 110 to 250°C at 8°C/min. Quantitation was by electronic integration, and peak identification was done by comparison to retention times of standards. Normal-chain, branched-chain, unsaturated, and hydroxy fatty acid standards were obtained from Applied Science Laboratories.

Amino acid and amino sugar analyses. Amino acid and amino sugar analyses were performed on a Beckman model 120C amino acid analyzer by using a modification of the method of Spackman et al. (29). Long column runs were programmed with a temperature change from 30 to 55°C after 15 min to resolve muramic acid and glutamic acid (26). Amino acids were released by hydrolysis of respective samples in 6.0 N HCl for 18 to 24 h at 100°C. Amino sugars were released by hydrolysis in 4 N HCl for 4 h at 100°C. Hydrolysates for amino sugars were neutralized with an equal volume of 4 N NaOH and diluted to 1.0 ml with sample buffer to avoid destruction of muramic acid, which may occur during the drying step employed for analysis of amino acids (11). The internal standard was 2-amino-*n*-butyric acid (Sigma). Commercial amino acid standards (Pierce Chemical Co., Rockford, Ill.) as well as standards of diaminopimelic acid, muramic acid, and glucosamine (Sigma) were prepared with the internal standard to determine relative molar responses. Total amino sugars were also estimated colorimetrically (11).

Neutral sugar and phosphate analyses. Neutral sugars were analyzed by GLC as the alditol acetates after hydrolysis of the respective cell wall fractions in trifluoroacetic acid (1, 12). Inositol (Sigma) was the internal standard. In selected samples, reduction with sodium borohydride was omitted to determine the quantities of alditols present. Standards containing ribose, 2-deoxyribose, ribitol, glycerol, rhamnose, glucose, galactose, mannose, α-mannoheptitol, D-glucoheptose, 2-keto-3-deoxyoctonate, and glucosamine (Sigma) were prepared with the internal standard to determine relative molar responses. Total sugar estimates were done by the micro phenol-sulfuric acid method (19), and phosphate was estimated by the method of Ames (2). GLC analysis was done on a glass column (2 m by 2 mm inner diameter) of 3% ECNSS-M on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories) programmed from 140 to 210°C at 3°C/min. Isothermal runs were done at 180 and 195°C. The carrier gas flow rate was 30 ml/min. Sugars were also analyzed on the SE-30 fused silica capillary column previously described. Temperature was programmed from 140 to 250°C at 5°C/min.

Mitogenesis. To assay for the ability of various *Listeria* fractions to serve as mitogens, spleens were removed from 8- to 12-week-old (C57BL/6 × BDA/2) F_1 (BDF_1) normal mice, teased into single-cell suspensions, washed, and counted. Then 2×10^5 cells per 0.1 ml per well were cultured in quadruplicate in microtiter wells, and mitogens were added to different groups as indicated. The medium was RPMI 1640 plus 1% fetal calf serum plus 50 μg of gentamicin per ml plus 5×10^{-5} M 2-mercaptoethanol. Cells were incubated in 8% oxygen, 12% CO_2, and 80% N_2 at 37°C for 48 h and then were pulsed with 0.01 ml of [^{125}I]iodouracildeoxyriboside (100,000 cpm) and harvested 20 to 24 h later. Cells were collected on glass fiber filters, and uptake of isotope was determined by counting on a gamma counter.

Adjuvant activity. To determine whether these fractions could act as immunological adjuvants, their ability to enhance an in vitro immune response by normal mouse spleen cells to the antigen sheep erythrocytes was determined as described previously (6). Briefly, a single-cell suspension of normal mouse spleen cells was prepared, and then cells were cultured at 1×10^7 to 2×10^7 cells per ml in RPMI 1640 plus 50 μg of gentamicin per ml plus 2 mM glutamine plus 5% fetal calf serum. Antigen (2×10^7 sheep erythrocytes) was added to the dishes, which were incubated with

TABLE 1. Fatty acid composition of *Listeria* fractions[a]

Fatty acid	% of total fatty acids	
	LCWF	PF
12:0	Tr[b]	Tr
13:0	Tr	NF[c]
14:iso	2	3
14:0	3	3
15:iso	2	1
15:anteiso	36	32
15:0	1	2
16:iso	6	7
16:anteiso	Tr	Tr
16:0	17	18
17:iso	1	1
17:anteiso	24	24
17:0	2	2
18:1	1	2
18:iso	Tr	Tr
18:0	6	5
19:anteiso	Tr	Tr
19:0	Tr	Tr
20:iso	Tr	Tr
20:0	Tr	Tr
21:anteiso	Tr	Tr

[a] Averages of triplicate determinations.
[b] Trace was considered to be >0.1% but <0.5%.
[c] NF, Not found.

TABLE 2. Amino acid composition of *Listeria* fractions[a]

Amino acid	Composition (μmol/mg)[b]		
	LCWF	PF	BHPF
Alanine	0.48	0.44	0.95
Glutamic acid	0.46	0.31	0.63
Diaminopimelic acid	0.16	0.25	0.64
Valine	0.14	Tr[c]	Tr
Glycine	0.32	0.03	Tr
Isoleucine	0.13	Tr	Tr
Leucine	0.21	Tr	Tr
Proline	0.10	Tr	Tr
Threonine	0.19	Tr	Tr
Serine	0.17	Tr	Tr
Cysteine	Tr	NF[d]	NF
Aspartic acid	0.30	0.03	0.04
Methionine	Tr	NF	NF
Phenylalanine	0.11	Tr	Tr
Histidine	0.07	NF	NF
Tyrosine	0.09	Tr	Tr
Lysine	0.27	0.03	0.04
Arginine	0.12	NF	NF

[a] Averages of triplicate determinations.
[b] The alanine/diaminopimelic acid ratio for each fraction was: LCWF, 3.0; PF, 1.8; and BHPF, 1.5. The glutamic acid/diaminopimelic acid ratio for each fraction was: LCWF, 2.9; PF, 1.2; and BHPF, 1.0.
[c] Trace was considered to be >0.005 μmol/mg but <0.01 μmol/mg.
[d] NF, Not found.

rocking in the gas mixture described above at 37°C for 4 days. Cultures were fed daily, and after 4 days, three dishes per group were harvested and assayed for hemolytic antibody-forming cells as described earlier (6).

Increased resistance. To determine whether *Listeria* fractions could induce increased resistance, normal 8- to 10-week-old BDF_1 mice were injected intravenously with 50 μg of the *Listeria* fraction and were boosted 2 and 4 weeks later intraperitoneally with 100 μg of the same material. Four weeks later they were challenged intraperitoneally with five 50% lethal doses of *Listeria*, and the number of survivors was counted 2 weeks later.

Decreased resistance. To test whether *Listeria* fractions could decrease resistance as described previously (3), normal mice were injected intraperitoneally with the fraction to be tested and were challenged 2 h later intraperitoneally with different amounts of viable *Listeria*. The ability to decrease resistance was identified as a decrease in the number of survivors upon challenge.

Macrophage-mediated tumor cell cytotoxicity. To determine whether these fractions could activate macrophages, their ability to stimulate macrophages to lyse tumor target cells was tested as described earlier (5). Briefly, normal mice were injected with thioglycollate medium, and peritoneal exudate cells were harvested 5 days later. Cells (3×10^5) were seeded in 0.1 ml in microtiter plates, and nonadherent cells were removed 2 and 24 h later, at which time macrophages were activated with the fraction to be tested. After a further 20 to 24 h of incubation, ^{51}Cr-labeled P-815 mastocytoma cells were added, and the amount of label released was determined after an additional 20- to 24-h incubation. Data are expressed as specific release, calculated as:

$$\text{specific release} = \frac{\text{(mean experimental cpm} - \text{mean control cpm)}}{\text{(mean maximal release cpm} - \text{mean control cpm)}}$$

TABLE 3. Amino sugar composition of *Listeria* fractions[a]

Amino sugar	Composition (μmol/mg)[b]		
	LCWF	PF	BHPF
Glucosamine	0.28	0.34	0.56
Muramic acid	0.05	0.10	0.34

[a] Averages of triplicate determinations.
[b] The glucosamine/muramic acid ratio for each fraction was: LCWF, 5.6; PF, 3.4; BHPF, 1.6.

RESULTS

Biochemical analysis of *Listeria* fractions. (i) Lipid content. The lipid contents of LCWF, PF, and BHPF were 20, 25, and <5%, respectively. The major lipid components derived from base hydrolysis of LCWF and PF were always free fatty acids (greater than 90%). Two other saponifiable fractions and five nonsaponifiable fractions were observed on TLC but not further characterized. Base hydrolysis of LCWF and PF released fatty acids of similar composition (Table 1). The major fatty acids were 15:anteiso, 17:anteiso, 16:iso, palmitic, and stearic acids. These five fatty acids represented greater than 80% of the fatty acid composition in both cases.

TABLE 4. Neutral sugar composition of *Listeria* fractions[a]

Neutral sugar	% of total neutral sugars		
	LCWF	PF	BHPF
Rhamnose	29	59	65
Glucose	5	13	15
Ribose	28	9	15
Ribitol	29	16	2
Galactose	4	2	1
Mannose	2	Tr[b]	Tr
Deoxyribose	2	Tr	Tr

[a] Averages of triplicate determinations.
[b] Trace was considered to be >0.1% but <0.5%.

A small quantity of 18:1 fatty acid was found to be present and was the only unsaturated fatty acid detected. There was insufficient 18:1 fatty acid to locate the position of the double bond. Trace quantities of 3-hydroxytetradecanoic acid (β-hydroxymyristic acid) were detected in lipid samples obtained from LCWF. No other hydroxy fatty acids were detected.

(ii) Amino acid analysis. In LCWF, amino acids composed 40% of the mass, and all of the common amino acids plus diaminopimelic acid were present (Table 2). The amino acid contents of PF and BHPF were 15 and 30%, respectively. The amino acid compositions of PF and BHPF were similar to each other but distinct from that of LCWF. The major amino acids present in PF and BHPF were alanine, glutamic acid, and diaminopimelic acid, which together made up over 80% of the amino acid composition of both cell wall preparations. The molar ratios of alanine to diaminopimelic acid and glutamic acid to diaminopimelic acid were similar for PF and BHPF but distinctly different for LCWF.

(iii) Amino sugar analysis. The total amino sugar contents of LCWF, PF, and BHPF were 6, 9, and 19%, respectively. The only amino sugars detected by amino acid analysis were glucosamine and muramic acid. Glucosamine was the only amino sugar found by GLC. The molar ratio of glucosamine to muramic acid decreased considerably from LCWF to PF to BHPF (Table 3).

(iv) Neutral sugar analysis and phosphate content. LCWF was 29% neutral sugars by weight, and PF and BHPF were, respectively, 44 and 36% neutral sugars. GLC of neutral sugar fractions from LCWF, PF, and BHPF showed the major components to be rhamnose, ribose, ribitol, and glucose, with lesser amounts of galactose, mannose, and deoxyribose (Table 4). Glycerol was detected in all cell wall preparations but not quantified. Glycerol and ribitol were determined by omitting the reduction step before preparation of alditol acetates for GLC. The relative concentrations of rhamnose and glucose were enhanced in PF over LCWF and in BHPF over PF. Ribose remained a major component of BHPF. No monosaccharides with molecular weights higher than those of hexoses were detected. LCWF and PF contained 5 and 4% phosphate, respectively, whereas BHPF was 2%

TABLE 5. Mitogenic activity of *Listeria* fractions[a]

Mitogen	Amt added (μg per well)	Expt 1		Expt 2	
		Mean (cpm) ± SE	S.I.[b]	Mean (cpm) ± SE	S.I.
None		46 ± 4	1.0	790 ± 28	1.0
LCWF	50	ND[c]	ND	4,724 ± 183	6.0
	5	ND	ND	4,961 ± 158	6.3
	5	ND	ND	4,514 ± 117	5.7
	1	ND	ND	3,779 ± 191	4.8
PF	50	5,181 ± 474	112.0	6,551 ± 459	8.3
	10	2,733 ± 151	59.1	5,807 ± 115	7.3
	5	1,610 ± 101	34.8	3,491 ± 240	4.4
	1	409 ± 30	8.8	2,519 ± 39	3.2
BHPF	50	110 ± 4	2.4	891 ± 58	1.1
	5	89 ± 10	1.9	776 ± 59	1.0
	1	111 ± 3	2.4	871 ± 138	1.1
Lipopolysaccharide	1	4,013 ± 98	86.8	7,553 ± 240	9.6
Concanavalin A	0.1	30,396 ± 4612	657.2	ND	ND

[a] Normal mouse spleen cells (2×10^5 per well) were stimulated in the presence of mitogen as indicated. After 48 h they were pulsed with [^{125}I]iodouracildeoxyriboside and counts per minute were determined 20 h later. Data are expressed as mean of quadruplicate cultures.
[b] S.I. (stimulation index) = mean experimental cpm/mean control cpm.
[c] ND, Not done.

TABLE 6. Adjuvant activity of *Listeria* fractions[a]

Listeria fraction	Amt (μg per dish)	Plaque-forming cell responses	
		Per 10^6 spleen cells	Per dish
None		59	635
PF	100	322	4,257
PF	50	229	3,054
PF	10	215	2,259
BHPF	100	45	676
BHPF	50	34	438
BHPF	10	20	235

[a] Normal mouse spleen cells (10^7) were cultured for 3 days with 2×10^8 sheep erythrocytes plus *Listeria* fractions as indicated. Plaque-forming cell responses were determined on a pooled sample of three dishes per group.

phosphate. Greater than 95% of the phosphate detected was present as organic phosphate.

Biological activities of *Listeria* fractions. Since previous experiments (8) showed that LCWF is a mitogen, we wished to determine whether components removed when preparing the derivatives PF or BHPF resulted in loss of mitogenic activity. A representative experiment shown in Table 5 indicated that PF, like LCWF, was a good mitogen, whereas BHPF lost its mitogenic activity. These experiments would suggest that lipids are required for the mitogenic activity of these *Listeria* cell wall fractions.

To test whether PF and BHPF could enhance immune responses, their ability to increase plaque-forming cell responses by spleen cells in vitro was tested. Table 6 shows that PF was an immunological adjuvant for normal spleen cells, whereas BHPF was not. In addition, other experiments (data not shown) showed that PF could enhance in vivo immune responses to sheep erythrocytes. These experiments, again, suggest that the active components for immunological adjuvant effects may also contain lipids.

Previous experiments reported that LCWF, administered either with lipopolysaccharide as an immunopotentiating agent or administered in multiple injections alone, could induce resistance to *Listeria* (24). Table 7 shows that PF lost the ability to induce resistance to *Listeria* in vivo. BHPF was not tested since it is a derivative of PF.

TABLE 7. Induction of resistance by LCWF but not PF[a]

Immunogen	No. of survivors/ no. injected
Saline (0.1, 0.5, and 0.5 ml)	2/10
LCWF (50, 100, and 100 μg)	8/10
PF (50, 100, and 100 μg)	3/10

[a] Mice were injected intravenously on day 0 and intraperitoneally on days 15 and 28 with the amounts indicated, respectively, in parentheses. On day 56, they were challenged with 1.2×10^6 *Listeria* intraperitoneally (5 50% lethal doses). Survivors were determined on day 71.

TABLE 8. Decreased resistance by *Listeria* fractions[a]

Listeria fraction	No. of survivors/no. tested at *Listeria* cell injections of:			
	10^6	10^5	10^4	10^3
None	1/5	5/5	5/5	
PF		0/5	0/5	1/5
BHPF		0/5	0/5	3/5

[a] BDF_1 mice were injected intraperitoneally with saline or 100-μg *Listeria* fractions as indicated and *Listeria* cells intraperitoneally as indicated. The number of survivors was determined 9 days later.

It had previously been shown that both LCWF and PF, when administered as early as 5 days before or along with live *Listeria*, reduced the 50% lethal dose of this organism by as much as 4 orders of magnitude (3). Experiments were then conducted to determine whether the ability of *Listeria* fractions to decrease host resistance to infection with *Listeria* was retained by BHPF. The experiment shown in Table 8 indicates that BHPF was as good as PF at decreasing resistance to *Listeria*. Thus, this material, though depleted of lipids and protein, retained the ability to decrease resistance to *Listeria*. Mechanisms by which this occurs are not yet understood.

Experiments were then conducted to determine whether LCWF and BHPF, as well as PF (5), could activate macrophages to high levels of activity, exemplified by their ability to kill ^{51}Cr-labeled tumor target cells. The experiment shown in Table 9 indicates that LCWF, PF, and BHPF could stimulate macrophages to become tumoricidal. However, BHPF was not as strong an activator as were the other *Listeria* fractions.

The biological and immunological capabilities of the *Listeria* cell wall fractions characterized and described here, as well as those published earlier, are summarized in Table 10.

DISCUSSION

The lipid content of LCWF (20%), PF (25%), and BHPF (<5%) indicates that protease and nuclease treatment of LCWF does not remove much, if any, lipid as the lipid content increases in PF. However, mild base treatment removes nearly all of the lipid in preparation of BHPF. Fatty acid profiles presented here are typical of *L. monocytogenes* (22). Although *L. monocytogenes* is characterized by the presence of five major fatty acids (17:anteiso, 16:iso, 16:0, 18:0, and 14:0), there is variability among strains in

TABLE 9. *Listeria* fraction macrophage-mediated tumoricidal activity[a]

Activator	Mean (cpm) ± SE	% Specific release[b]
Nonidet P-40	5,355 ± 818	100.0
Medium	1,328 ± 87	0.0
LCWF		
10 μg	3,759 ± 139	60.4
1 μg	3,365 ± 86	50.6
PF		
10 μg	4,179 ± 289	70.8
1 μg	3,770 ± 184	60.6
BHPF		
10 μg	2,719 ± 239	34.5
1 μg	1,750 ± 149	10.2

[a] Adherent peritoneal cells were activated for 24 h with *Listeria* fractions as indicated in 0.1 ml cultures in quadruplicate. Then ^{51}Cr-labeled P-815 tumor targets were added, and after an additional 20 h of incubation, the counts per minute released into 0.04 ml of supernatant was determined.

[b] See the equation for specific release in the text.

the relative amounts of these acids (22). Quantitative fatty acid profiles of *L. monocytogenes* appear to be more dependent on growth conditions than on the strain or serotype (13, 31). The origin of the trace (<0.5% of total fatty acids) quantities of 3-hydroxytetradecanoic acid found in LCWF lipid extracts remains to be determined. An endotoxin-like material obtained from serotype 4b cells was found to have high quantities of 3-hydroxytetradecanoic acid (33).

The protein content in LCWF is significant, but protease and nuclease treatment removes most of the protein (3). The amino acid content of PF and BHPF was almost entirely alanine, glutamic acid, and diaminopimelic acid (Table 2). Molar ratios of muramic acid, glucosamine, alanine, and glutamic acid to diaminopimelic acid for BHPF were 0.53, 0.88, 1.5, and 0.98, respectively, which are typical of the ratios found in such examinations of bacterial peptidoglycans (26). The same ratios from an analysis of serotype 4b cells done by others (30) were 0.61, 46.0, 2.2, and 2.3, respectively. The major differences between the peptidoglycans of serotype 1 and serotype 4b cells are in the glucosamine and glutamic acid contents relative to that of diaminopimelic acid. The apparent high glutamic acid content found in the analysis of serotype 4b cells may be the result of failure to resolve muramic acid and glutamic acid, which exit the amino acid analyzer in the same elution volume unless steps are taken to effect the resolution (25). The high glucosamine content of serotype 4b cells may be a serotype-specific phenomenon, although glucosamine did not appear to be a serologically active carbohydrate in an earlier study (32). None of the cell wall preparations described in this study had such a high glucosamine content.

The carbohydrate composition of the *Listeria* cell wall fractions (Tables 3 and 4) indicates that associated with typical sugars of peptidoglycan (glucosamine and muramic acid) there is extra glucosamine as well as significant quantities of rhamnose and glucose which increase in content from LCWF to PF to BHPF. Ribose was also a significant component of BHPF and was stable to base hydrolysis. Whether these sugars are covalently attached to the peptidoglycan has yet to be determined, although the significant carbohydrate content of BHPF suggests that many of them are covalently linked. Glucosamine, glucose, and, most importantly, rhamnose are known to be serologically active sugars for *L. monocytogenes* serotype 1 (32). High quantities of rhamnose are also present in serotype 4b cells (30). No monosaccharides with molecular weights higher than those of hexoses or hexosamines were detected in any of the cell wall hydrolysates. Ribitol was found in LCWF and decreased considerably in the preparation of PF and BHPF. Ribitol is a common constituent of teichoic acids, although teichoic acids were not detected in an analysis of serotype 4b cells (30). Ribitol has not been previously recognized in *Listeria* cell walls. The drop in phosphate content from PF (4%) to BHPF (2%) may reflect the loss of membrane material during base hydrolysis. BHPF still contains phosphate apparently resistant to mild base hydrolysis. The nature of the carbohydrate material in the *Listeria* cell wall remains to be determined.

TABLE 10. Biological and immunological properties of *Listeria* fractions

Property	*Listeria* fraction		
	LCWF	PF	BHPF
Mitogen	+[a,b]	+[b]	−[b]
Adjuvant	+[c,d,e]	+[b]	−[b]
Polyclonal B cell activator	+[a]	+[f]	NT[g]
Induces resistance	+[b,h]	+[b]	NT
Decreases resistance	+[i]	+[b,i]	+[b]
Activates complement	+[j]	+[j]	NT
Induces tumoricidal activity	+[b]	+[b,k]	+[b]

[a] See reference 8.
[b] This report.
[c] See reference 7.
[d] See reference 6.
[e] See reference 28.
[f] Unpublished observation.
[g] NT, Not tested.
[h] See reference 24.
[i] See reference 3.
[j] See reference 4.
[k] See reference 5.

Several different laboratories have reported that components and products derived from *L. monocytogenes* are mitogens (8, 14, 21) and immunological adjuvants (6, 7, 21, 27), that they can activate the alternative pathway of complement (4), and that they can induce macrophage-mediated cytotoxicity (5). In addition, *Listeria*-derived materials have been reported to induce T cells to release factors including mitogenic factors, migration inhibition factors, and macrophage-activating factors (9, 10, 16, 17). Careful characterization of the components of *Listeria* responsible for these activities has not previously been conducted.

The cell wall preparations described here are rich in typical bacterial cell wall components. Activity observed with PF may be due to cell wall structures, although the role of lipid cannot be precluded. BHPF appears to be a preparation of peptidoglycan which is depleted of membrane material. BHPF, however, still contains a significant amount of carbohydrate material.

The data described here suggest that mitogenic activity and the ability to function as an immunological adjuvant are properties of crude cell wall derivatives of *Listeria* which are lost when more highly purified preparations of peptidoglycan are prepared. These activities may be dependent on peptides or relatively intact protein. On the other hand, the ability to decrease resistance, which may be due to an effect on the phagocytes responsible for listericidal activity, may not require peptides but may be a property of more purified peptidoglycans (BHPF). Similarly, the ability to induce macrophage-mediated cytotoxicity also appears to be a property of BHPF. Finally, the data presented here could be interpreted to suggest that effects of *Listeria*-derived components on lymphocytes require some intact peptide chains, whereas effects which may be directly on macrophages or other phagocytes may not require *Listeria*-derived peptides but may be due to peptidoglycan or associated carbohydrate.

ADDENDUM IN PROOF

Many of the activities described in this report are confirmed in a recent publication of independent, simultaneous work conducted by others (I. Saiki, K. Kamisango, Y. Tanio, H. Okumura, Y. Yamamura, and I. Azuma, Infect. Immun. **38**:58–66, 1982).

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