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Immunopotentiating Activities of Cell Walls, Peptidoglycans, and Teichoic Acids from Two Strains of *Listeria monocytogenes*

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Crude cell wall (CCW), enzyme purified wall (PF), peptidoglycans (PEP), and teichoic acid fractions from two strains of *Listeria monocytogenes* (EGD and a human isolate) were analyzed by electron microscopy, chemical analyses, and various immunological assays. Electron microscopy and chemical analyses revealed that both strains were quite similar. The CCW, PF, and PEP fractions were mitogenic for mouse lymphocytes, could activate macrophages in vivo but not in vitro, and stimulated nonspecific immunity in vivo to *Candida albicans*. The PF and PEP fractions but not the CCW induced migration inhibition factor production. The CCW and PEP fractions were tested and found to elevate natural killer activity in vivo. Teichoic acid fractions had no activity in the above assays. Since most of the immunological activities were retained in the PEP fraction, we examined this fraction to determine the kinetics of the mitogenic response and the nature of the responding cell population. The peak mitogenic response occurred on day 2 over a 6-day period. This response was not enriched in T-cell-enriched populations but followed the pattern of LPS in B-cell-enriched populations.

The enhancement of immune responsiveness, immunopotentiation, may be a desirable therapeutic approach in those clinical situations where immune responses appear to be suppressed (e.g., cancer patients, burn victims, chronic disease states). Many bacterial products have been tested for use as immunopotentiators or immunomodulators. The BCG vaccine, *Propionibacterium acnes* ("*Corynebacterium parvum*"), endotoxin, and muramyl dipeptide are noteworthy examples. Some have shown promise but only in limited situations; others may be inadequate because of toxicity problems. In the absence of a universal immunomodulator, one alternative is the development of a complete arsenal of immunomodulators.

Cell wall components from *Listeria monocytogenes* should be considered as potential immunomodulators, since they have been found to increase resistance to Listeria infection (16), act mitogenically (6, 14), act as an adjuvant (3, 4, 17), fix complement and be chemotaxigenic (1), stimulate nonspecific immunity to *Candida albicans* and several tumors (11, 14), stimulate macrophages to become tumoricidal (10, 11), and elevate natural killer (NK) activity (14). This study reports the isolation of four different cell wall factions from two commonly used strains of *L. monocytogenes* and their comparison via electron microscopy, chemical analyses, and various immunological assays.

MATERIALS AND METHODS

Animals. Six- to ten-week-old C3H/He mice, raised in departmental facilities, were used in all experiments.

Mitogens. *Escherichia coli* O127:B8 lipopolysaccharide (LPS) -phenol extract and concanavalin A (ConA) grade IV were obtained from Sigma Chemical Company, St. Louis, Mo. Phytohemagglutinin M (PHA) was obtained from Difco Laboratories, Detroit, Mich.

Tissue culture medium. RPMI 1640, containing L-glutamine and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), was obtained from Biofluids, Inc., Rockville, Md. The pH of the cold medium was adjusted to 7.8 and supplemented with 50 μg of gentamicin sulfate (Sigma) per ml. Where specified, the medium was supplemented with fetal calf serum (FCS).

Preparation of cell wall fractions. The EGD strain (rabbit isolate, serotype 1/2a) was originally obtained from George Spitalny, Trudeau Institute, Saranac Lake, N.Y., and the ISC strain (human isolate, serotype 1) was obtained from George B. Olson, University of Arizona, Tucson, Ariz. These strains differ in growth rate and colony morphology. Both strains were grown for 24 h at 37°C in tryptic soy broth (Difco). Crude cell walls (CCW) were prepared after sonication and differential centrifugation as previously described (14). The purified fraction (PF) was prepared from the CCW by a modification of the method of Baker et al. (1), by treatment with trypsin (Sigma, type I), DNase II, and RNase A (Sigma, types VI and I-A, respectively) for 2 h at 37°C, then centrifugation (15,000 × *g*, 15 min, 4°C). The pellets were washed four times by suspension in sterile deionized water and were centrifuged as described above. Next the pellets were suspended in 400 ml of 0.01 M Tris buffer (pH 7.6) containing 0.005 M $CaCl_2$, 0.01 M sodium azide, and 100 mg of protease (Sigma, type XXI) and incubated for 2 h at 37°C. This mixture was centrifuged and washed four times (as above) in sterile deionized water and then lyophilized. The peptidoglycan (PEP) and the teichoic acid (TEICH) fractions were prepared by the method of Rasanen et al. (15) and lyophilized.

Electron microscopy. The cell wall fractions were negatively stained by placing a drop of diluted wall material on a carbon-coated grid with one drop of 3% uranyl acetate. After 1 min, the excess liquid was removed, leaving a thin film which dried before examination with a Philips EM 300.

Chemical analyses. The Bradford assay (2) was used to measure protein, and carbohydrate (glucose equivalents) was measured by the phenol-sulfuric acid reaction (7). Both values were expressed as a percentage of the dry weight.

Lymphocyte stimulation (mitogenicity). Spleens were aseptically removed from normal mice and pressed through a sterile wire screen into 5 ml of cold tissue culture medium. The suspension was centrifuged (200 × *g*, 10 min), and then the cell pellet was suspended in 2 ml of cold sterile lysing

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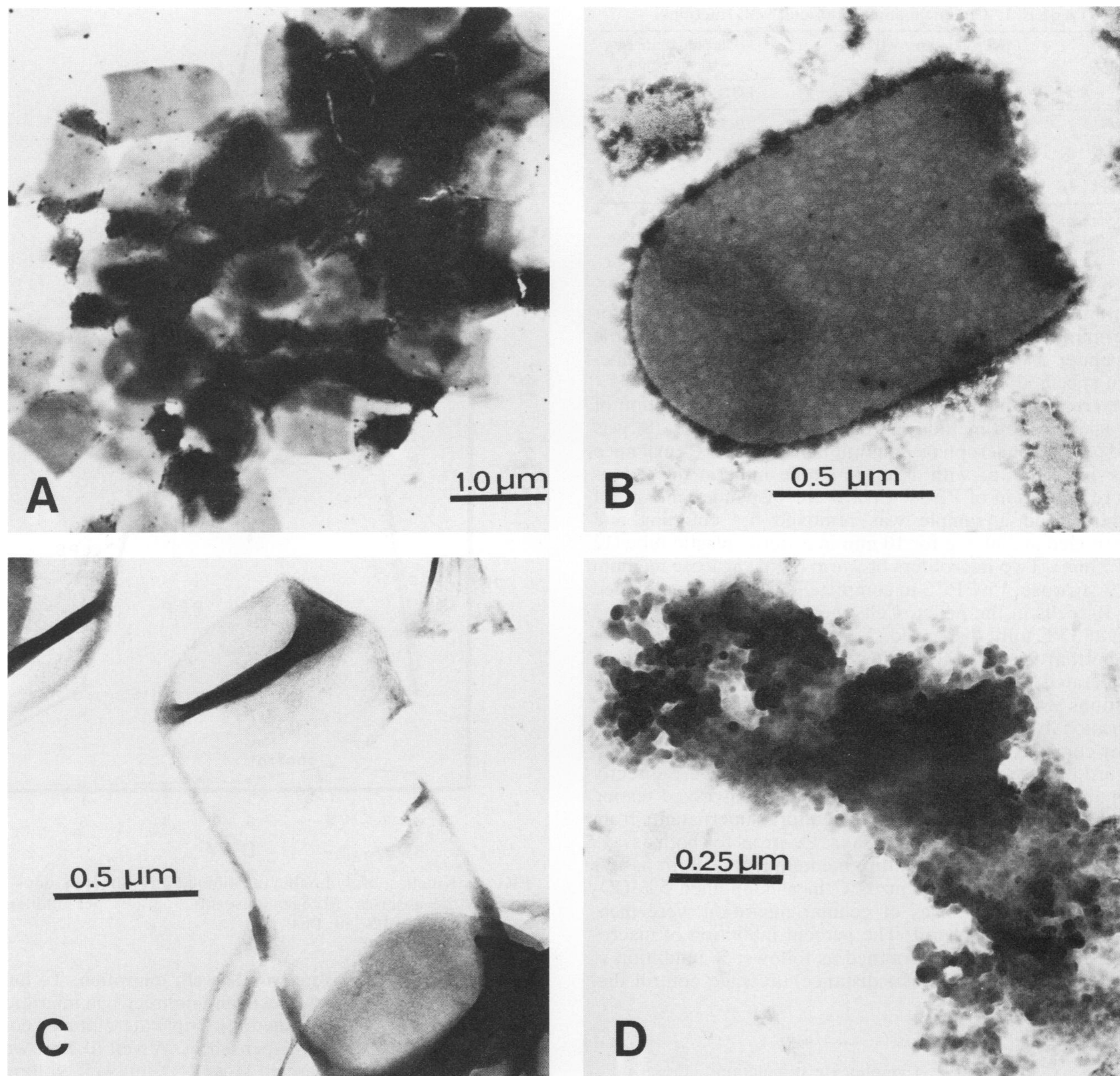

FIG. 1. (A) CCW fraction obtained after sonication. (B) CCW at higher magnification. The cellular debris attached along the edge of the broken cell is visible. (C) PF obtained after enzymatic treatment. (D) PEP fraction. The wall skeletons are disrupted, leaving only aggregates of material.

buffer (0.87% NH_4Cl) for 5 min. A 10-ml portion of cold medium was then added, and the cells were centrifuged as before, washed three times in cold medium, and suspended to 4×10^6 cells per ml in medium containing 10% FCS. Cells were dispensed in 0.1-ml volumes (4×10^5 cells) into the wells of flat-bottom 96-well Micro Test II plates (Falcon; Becton Dickinson Labware, Oxnard, Calif.). Triplicate or quadruplicate cultures then received 0.1-ml volumes of either medium alone or medium containing various doses of *Listeria* cell wall fractions, ConA, PHA, or LPS. Cultures were incubated at 37°C for the appropriate times in a humidified atmosphere of 5% CO_2 in air. Six to eight hours before harvesting, 0.05 ml of tritiated thymidine (1 µCi; specific activity, 20 Ci/mmol) was added. Cultures were harvested onto fiber glass filters with a semiautomatic sample harvester (Brandel), and the amount of incorporated radioactivity was measured by liquid scintillation spectrometry. A stimulation index (S.I.) was determined by dividing the average counts per minute of the experimental wells by the average counts per minute of control wells (cells in medium alone). In some experiments spleen cells were enriched for T or B cells after passage through nylon wool columns (9). The nylon wool nonadherent T-cell-enriched and the adherent B-cell-enriched populations were then cultured as described above.

Collection of peritoneal exudative cells (PECs). PECs were harvested by intraperitoneal (i.p.) injection and withdrawal of 10 ml of cold Hanks balanced salt solution and pooled

TABLE 1. Chemical analysis of cell wall fractions

Fraction	% Protein (dry wt)[a] for strain		% Carbohydrate (dry wt)[b] for strain	
	EGD	ISC	EGD	ISC
CCW	13.2	13.3	9.4	7.9
PF	3.0	3.6	13.2	14.5
PEP	13.1	16.3	2.9	2.3
TEICH	2.2	2.9	24.4	19.7

[a] Average from two separate assays.
[b] Glucose equivalents; average from two separate assays.

from mice in each group. A sample was removed for determining total, viable, and differential cell counts, and the remainder was washed and suspended in appropriate medium specified by the assay being performed.

Macrophage migration inhibition. A slight modification of the agarose-drop method of Harington and Stastny (8) was used to assay macrophage migration inhibition. Normal mice were injected i.p. with 1 ml of sterile mineral oil 3 days before collection of PECs. Cells were suspended in 1 ml of medium, and a sample was removed for counting and centrifuged at 200 × *g* for 10 min in a sterile plastic tube (12 by 75 mm). Two microliters of warm (37°C) agarose medium (0.2% agarose, 15% FCS in complete RPMI 1640) was added per 10^6 cells in the pellet. Cells were gently suspended and held at 37°C until dispensed (2-μl droplets) into the wells of flat-bottom 96-well Micro Test II plates. The plates were refrigerated for 5 min to gel the droplets. The cell wall fractions were tested for their ability to inhibit macrophage migration by both direct and indirect assays. The direct assay consisted of mixing cell wall fractions or mitogens with culture medium and placing 0.2 ml of this mixture directly over the agarose drop. The indirect assay involved testing 0.2-ml volumes of filter-sterilized culture supernatants from the lymphocyte stimulation assays. Controls for both assays were 0.2-ml volumes of culture medium alone. Both assays were incubated for 24 h at 37°C in a humidified 5% CO_2 incubator. The distances of cellular migration were measured with an ocular grid. The percent inhibition of macrophage migration was determined as follows: % inhibition = [1 − (average experimental distance)/(average control distance)] × 100.

TABLE 2. Lymphocyte stimulation

Test material[a]	Avg cpm ± SE	S.I.[b]
Control medium	490 ± 52	1.0
ConA	18,321 ± 760	37.4
LPS	7,803 ± 272	15.9
Strain EGD		
CCW	4,903 ± 734	10.0
PF	5,441 ± 166	11.1
PEP	5,439 ± 150	11.1
TEICH	955 ± 128	1.9
Strain ISC		
CCW	4,176 ± 1,010	8.5
PF	4,162 ± 187	8.5
PEP	5,664 ± 131	11.6
TEICH	836 ± 118	1.8

[a] All materials were tested in quadruplicate at a final concentration of 50 μg/ml except ConA (3 μg/ml).
[b] Values less than 2.0 were not significant; all other values were significantly different from controls at $P < 0.01$.

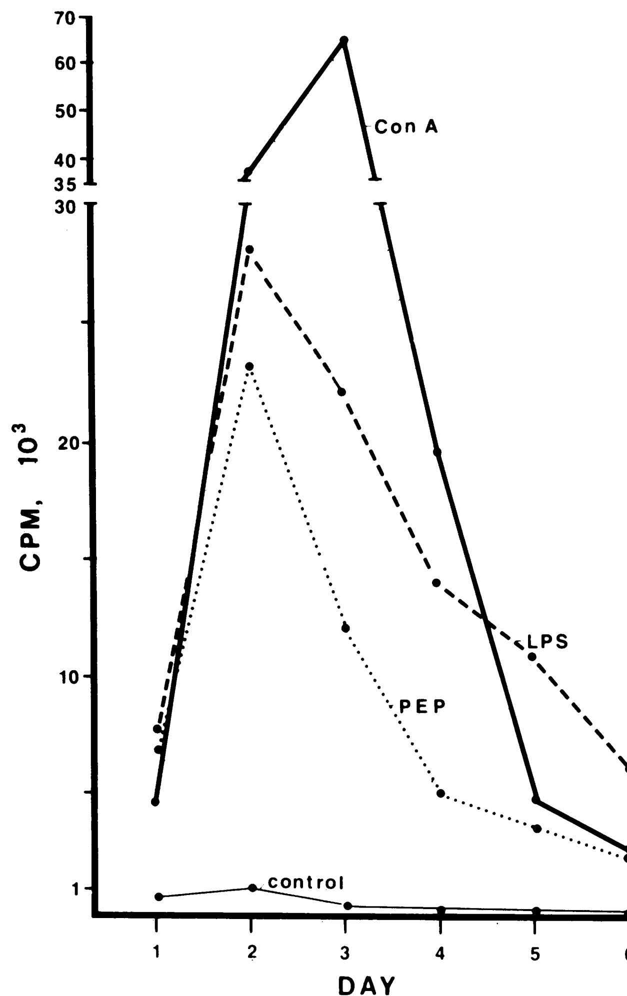

FIG. 2. Kinetics of lymphocyte stimulation. Time in days is shown on the abscissa. Mitogen dose (micrograms per milliliter) used: ConA, 1.5; LPS, 50; PEP, 50.

Micro (96 well) versus macro (24 well) migration. To help distinguish between direct effects and indirect (via migration inhibition factor [MIF] production), triplicate cultures containing 2-μl droplets were dispensed in 96-well (0.2-ml well volume) plates and in larger 24-well (1-ml well volume) plates for comparison. The CCW and PEP fractions from both strains were tested at a concentration of 100 μg/ml. Control wells contained droplets plus medium alone. Migration distances were measured, as described above, after 24 h at 37°C.

TABLE 3. Lymphocyte stimulation with nylon-wood separated cells

Treatment, mitogen dose (μg/ml)	Avg cpm ± SD[a] for the following cell types:		
	Unseparated	T-cell enriched	B-cell enriched
Control	847 ± 23	393 ± 15	1,631 ± 535
PHA, 50	9,935 ± 214	26,765 ± 875	787 ± 20
ConA, 1.5	57,565 ± 1,273	53,194 ± 2,363	10,190 ± 188
LPS, 50	22,817 ± 607	19,987 ± 854	22,007 ± 1,087
PEP, 50	12,696 ± 355	9,498 ± 281	20,312 ± 1,034

[a] From two separate experiments.

TABLE 4. Inhibition of macrophage migration by cell wall fraction direct assay

Test material and dose (μg/ml)	% Inhibition[a] ± SD for strain:	
	EGD	ISC
CCW		
100	0 ± 13[b]	17 ± 6
10	0 ± 15[b]	14 ± 9
PF		
100	52 ± 9[c]	53 ± 9[c]
10	45 ± 9[c]	49 ± 8[c]
PEP		
100	64 ± 2[c]	63 ± 13[c]
10	56 ± 5[c]	54 ± 12[c]
TEICH		
100	14 ± 14	36 ± 17
10	14 ± 9	21 ± 20

[a] Formula in text. Results are the average of three separate exeriments with each fraction tested in triplicate. Control mitogens ConA (3 μg/ml) and PHA (100 μg/ml) caused 43 and 64% inhibition, respectively.
[b] No inhibition, occasional enhanced migration.
[c] Values are statistically significantly different from controls ($P < 0.01$).

Macrophage activation. The ability of these cell wall fractions to activate macrophages was determined by measuring the uptake of [^{14}C]glucosamine (20) after direct cultivation of wall fractions with normal macrophages in vitro or after injection of wall fractions into mice (in vivo activation) and subsequent assay of the macrophages in vitro. For the direct assay, oil-induced PECs were harvested as described above, and suspended to 1×10^6 cells per ml in culture medium with 15% FCS. A 1-ml portion of this suspension was added to each well of a 24-well flat-bottom plate, and cells were allowed to adhere for 2 h at 37°C in a 5% CO_2 incubator. The wells were washed three times with Hanks balanced salt solution to remove nonadherent cells and then filled with 1 ml of fresh culture medium (15% FCS) with or without test mitogens or cell wall fractions. Plates were incubated for 72 h at 37°C in a 5% CO_2 incubator. During the last 8 h, 0.25 μCi of D-[1-^{14}C]glucosamine (specific activity, 58 mCi/mmol) was added to each well (50-μl volume). The wells were then washed three times with warm Hanks balanced salt solution and incubated with 0.5 ml of 4% sodium lauryl sulfate at 60°C for 30 min. Samples (0.2 ml) were placed in 5 ml of scintillation fluid and counted. The in vivo activation procedure involved injecting normal mice with 500-μg doses of a cell wall fraction or 100 μg of ConA or LPS 3 days before collection of PECs. Cell suspensions were adjusted to contain 1×10^6 macrophages per ml, then plated as described above.

TABLE 5. Micro (96 well) versus macro (24 well) method for assaying macrophage migration inhibition

Test material (μg/ml)	% Inhibition[a] ± SD for method	
	96 Well	24 Well
Control medium	0 ± 4	0 ± 11
ConA (3)	62 ± 3	52 ± 3
EGD		
CCW (100)	0 ± 4	0 ± 12
PEP (100)	57 ± 2	16 ± 5
ISC		
CCW (100)	7 ± 3	0 ± 11
PEP (100)	52 ± 3	13 ± 4

[a] Formula in text. Results are the averages from 12 separate measurements with each test material.

TABLE 6. Macrophage activation in vivo

Test material[a]	Avg glucosamine uptake (cpm) ± SD (S.I.)
Control medium	176 ± 12 (1.0)
ConA	200 ± 10 (1.1)
Strain EGD	
CCW	465 ± 34 (2.6)
PF	221 ± 19 (1.2)
PEP	395 ± 13 (2.2)
TEICH	98 ± 12 (0.6)[b]
Strain ISC	
CCW	382 ± 28 (2.2)
PF	327 ± 18 (1.8)
PEP	371 ± 24 (2.1)
TEICH	163 ± 7 (0.9)[b]

[a] All fractions and mitogens injected i.p. at a dose of 500 μg into normal mice.
[b] Not significant. All other values for S.I. were significantly different from controls at $P < 0.05$.

In vivo protection against *C. albicans*. To measure induction of nonspecific immunity, groups of mice were pretreated by i.p. injection of cell wall fractions or 100 μg of ConA, then challenged 4 days later with *C. albicans* as described previously (14).

NK activity. The ability of the wall fractions to elevate NK cell activity was measured by using a 4-h chromium release assay described previously (14) with YAC-1 cells as targets. Cell wall fractions were injected i.p. into normal mice, and 1 and 4 days later their PECs were collected and incubated with labeled target cells at a ratio of 25 effector cells to 1 target cell.

Statistical analysis. The chi-square ($2 \times k$, contingency) method (5) was used for analysis of the in vivo protection studies, and the Student t test was used for determining the levels of significance of differences between two means.

TABLE 7. In vivo protection against *C. albicans*

Treatment group[a]	% Survival[b] (no. of surviving mice/total no. of mice)	Significance (P)[c]
Control	10 (2/20)	
ConA	40 (8/20)	<0.05
ISC		
CCW	33 (6/18)	NS
PF	40 (8/20)	<0.05
PEP	40 (8/20)	<0.05
TEICH	25 (4/16)	NS
EGD		
CCW	67 (12/18)	<0.005
PF	70 (14/20)	<0.005
PEP	80 (16/20)	<0.002
TEICH	29 (4/14)	NS

[a] Mice were injected i.p. with 100 μg of test material or saline as a control 4 days before challenge with 5×10^8 (~3 50% lethal doses) of *C. albicans*.
[b] Determined 7 days after challenge.
[c] Determined by chi-square analysis. NS, Not significant.

RESULTS

Electron microscopic and chemical analyses. Typical electron microscopic views of three of the wall fractions are shown in Fig. 1. The soluble teichoic fractions were not examined. We found no obvious differences between fractions from each strain. The CCW obtained after sonication is shown in Fig. 1A; Fig. 1B shows a higher magnification. The contaminating cellular debris attached to the CCW can be seen. The enzymatically digested PF fraction, which appears much cleaner, with only broken wall skeletons visible, is depicted in Fig. 1C. The PEP fraction is shown in Fig. 1D; it is apparent that the integrity of the walls was disrupted, leaving aggregates of material. The results of brief analyses of protein and carbohydrate contents of the fractions from each strain are summarized in Table 1. There appeared to be no major differences between the strains. However, our measurements of protein and carbohydrate only account for about 20% of the dry weight. The remaining components must be materials not detected by these assays, such as lipids, other carbohydrates (e.g., hexosamines), and other compounds.

Lymphocyte stimulation. Fractions from both strains, when analyzed for their ability to stimulate mouse splenic lymphocytes, yielded similar results (representative experiment shown in Table 2). The CCW, PF, and PEP fractions all showed significant and approximately equal levels of stimulation (S.I. values approximately 9 to 11 times above control), whereas the TEICH fractions yielded no significant stimulation. All fractions were also tested at a concentration of 5 μg/ml (data not shown) and produced S.I. values that were approximately half of those observed at 50 μg/ml.

Later experiments examined the kinetics of this response by using the strain EGD peptidoglycan fraction alone (Fig. 2). The peak stimulation occurred on day 2 with counts approximately 25 times higher than controls. The LPS response also peaked on day 2, then dropped steadily. In contrast, the T-cell mitogen ConA yielded counts-per-minute values that reached a high peak on day 3 approximately 200 times higher than controls. The results of testing this EGD-PEP fraction on T-cell-enriched and B-cell-enriched populations of spleen cells from nylon wool columns are shown in Table 3. In the T-cell-enriched population, only the PHA response was dramatically increased over values obtained with unseparated cells, whereas the LPS and PEP responses remained about the same or were slightly lower. In the B-cell-enriched population, the responses to ConA and PHA were significantly reduced; the LPS response remained about the same, and the PEP response was greater than the values in the unseparated cells. This may indicate that LPS and PEP stimulate different subsets of B cells.

TABLE 8. In vivo protection by EGD-PEP against *C. albicans*

Treatment group (dose [μg])[a]	% Survival[b] (no. of surviving mice/ total no. of mice)
Control	33 (4/12)
ConA (100)	100 (13/13)
EGD-PEP (10)	92 (12/13)
EGD-PEP (50)	100 (13/13)
EGD-PEP (100)	100 (13/13)
EGD-PEP (500)	100 (13/13)

[a] I.p. injections in 0.5 ml of saline 4 days before challenge with 3×10^8 *C. albicans*.

[b] Determined 7 days after challenge. All values were significantly different from control ($P = 0.001$).

TABLE 9. Stimulation of NK activity

Treatment group (dose [μg])[a]	% Lysis[b] at day after i.p. injection	
	1	4
Control	3 (1.0)	3 (1.0)
ConA (100)		23 (7.7)
C. parvum (300)		53 (17.7)
ISC		
CCW (500)	25 (8.2)	13 (4.3)
PEP (500)	18 (6.0)	9 (3.0)
EGD		
CCW (500)	26 (8.7)	12 (4.0)
PEP (500)	18 (6.0)	14 (4.7)

[a] i.p. injections in 0.5 ml of saline. Controls were injected with 0.5 ml of saline.

[b] Average values from three separate experiments in which products were tested in triplicate. Values in parentheses represent the ratio of treatment to control. All ratios >3.0 were significantly different from controls at $P < 0.05$.

Macrophage migration. To further study the effects of *Listeria* cell wall fractions on the immune system, we tested the direct effects of these fractions on macrophage migration in vitro (Table 4). Again both strains yielded comparable results. Surprisingly, the CCW did not induce significant (>25%) macrophage migration inhibition and occasionally caused enhanced migration. The PF and PEP fractions did significantly inhibit migration (45 to 64%). The TEICH fractions were more variable, and their inhibitory effects were not statistically significant. We had assumed that the mitogenicity of CCW, PF, and PEP on lymphocytes would lead to production of MIF, resulting in macrophage migration inhibiton. We did examine culture supernatants from our 3-day lymphocyte cultures but found little MIF activity. Since MIF production can occur earlier than peak mitogenic responses (days 2 to 3), we next compared the migration of macrophages in 96-well (0.2-ml volume) plates with the migration of macrophages in 24-well (1-ml volume) plates (Table 5). The number of cells and the concentration (micrograms per milliliter) of the cell wall fraction were the same in both plates, with only the volume of medium in the wells being different. Therefore, if the inhibition of migration was a direct effect of the fractions on the macrophages, both the 96-well and the 24-well plates should have had the same result. However, if the inhibition of migration was an indirect effect via MIF production, the MIF should have been diluted more in the 24-well (1-ml volume) plates and should have shown less inhibition than in the 96-well plates. The results (Table 5) showed that the cell wall fractions inhibited migration to a greater extent in the 96-well plate (3 to 4 times higher) than in the 24-well plate whose volume was five times greater. This suggested that MIF production occurred and was responsible for this migration inhibition.

Macrophage activation. When the ability of the fractions to activate macrophages in vitro was tested, only the control mitogen ConA was able to cause statistically significant uptake of the radiolabel. LPS and all the *Listeria* fractions did not cause significant uptake. When macrophages were removed from mice previously injected with the cell wall fractions (in vivo activation), ConA and all *Listeria* fractions except the TEICH fractions caused significant uptake of the radiolabel (representative experiment shown in Table 6).

The counts-per-minute values were low but similar to those obtained by Takada et al. (20).

In vivo protection versus *C. albicans*. The fractions were next assayed for their ability to stimulate nonspecific immunity to *C. albicans* (Table 7). All fractions except the ISC-CCW and TEICH were able to increase the number of mice surviving. It is interesting that the *Listeria* fractions were as active or more active than the strong in vitro mitogen ConA. The EGD strain appeared somewhat more effective (29 to 80% survival) than the ISC strain (25 to 40% survival); however, the difference between these two groups was not statistically significant. The EGD-PEP fraction was tested further in a dose-response experiment (Table 8) and found to be very effective in doses as low as 10 μg.

NK activity. Since we previously found that the ISC-CCW enhanced antitumor immunity and NK activitiy in mice (14), we extended these studies to compare the CCW and PEP fractions from both strains in their ability to stimulate NK activity in peritoneal exudates collected 1 and 4 days after i.p. injection (Table 9). The CCW and PEP fractions of both strains stimulated NK activity quite rapidly (day 1 > day 4), as was seen above. This stimulation is especially significant considering the low (25:1) effector-to-target ratio. Again the levels of stimulation were comparable to those achieved by ConA.

DISCUSSION

Cell wall fractions from the two strains appeared to be quite similar when examined by electron microscopy and brief chemical analyses. The protein contents of fractions from each strain were approximately equal (Table 1), with the CCW fractions containing four times more protein than the PF fractions. This agreed with the electron microscopic view that showed that the CCW fraction contained more cellular debris than the protease-treated PF fraction, which provides evidence that most of this debris was proteinaceous. The PEP fractions contained more protein than did the PF fractions, reflecting either the effect of purification or an increased accessibility of the Bradford reagent to peptide side chains. The TEICH fractions should have contained little protein, since they are reported to be primarily polymers of glycerol or ribitol phosphate.

The carbohydrate contents of fractions from each strain were also approximately equal. The PF fraction contained more carbohydrate than did the CCW, probably because of the removal of nucleic acid and proteinaceous debris; thus, carbohydrate values represent a larger percentage of the dry weight. The low values observed for carbohydrate in the PEP fractions were probably due to the lack of sensitivity of the assay to hexosamines. The TEICH fractions yielded the highest values, presumably reflecting ribitol or glycerol and attached glucose or galactose residues. These carbohydrate values agree with those previously reported (10, 18, 19).

All fractions except TEICH were mitogenic, and when the kinetics of this response was measured by using the EGD-PEP fraction, the peak response occurred on day 2. The counts-per-minute values to EGD-PEP were elevated in B-cell-enriched populations and not in T-cell-enriched populations. Although this technique does not produce pure populations of B or T cells, the levels (Table 2) and patterns (Fig. 2 and Table 3) of stimulation by the *Listeria* fractions are more comparable to the B-cell mitogen, LPS, than the T-cell mitogens ConA and PHA. These results support the designation of EGD-PEP as a B-cell mitogen.

The level of lymphocyte stimulation was not increased in PEP in comparison with that in CCW. This may indicate that the mitogenic component is a predominant constituent of the PEP fraction which in turn represents a major portion of the CCW. The mitogenic activitiy was not lost by the enzymatic treatment, agreeing with the literature (10).

In a similar fashion, injection of the CCW, PF, and PEP fractions in vivo resulted in macrophage activation. However, these fractions were unable to activate macrophages during a short in vitro cultivation. Again, TEICH fractions were inactive. These results paralleled the mitogenic results, with activity remaining through the PEP fraction and with little apparent enrichment in activity.

It was surprising that the CCW fractions, although mitogenic, did not stimulate MIF production and inhibit macrophage migration, whereas the PF and PEP fractions did. This may have been caused by the debris seen to be associated with the walls in this fraction, which might have either interfered with MIF activity or stimulated a competing action like chemokinesis. Chemokinesis is plausible since occasional enhanced migration was observed in individual assays with this CCW fraction.

The most important measure of immunopotentiation was that the CCW, PF, and PEP fractions from both strains could stimulate nonspecific immunity as evidenced by the in vivo protection of mice against *C. albicans*. Furthermore, the *Listeria* fractions appeared to lack the toxicity associated with LPS, in that the mice were not ruffled in appearance as often seen after LPS injection. The EGD-PEP fraction was especially capable of protection (i.e., 10 μg yielded 92% survival). This protection may represent the end result of many different stimulatory events occurring in the animal. Mitogenic stimulation of lymphocytes in vivo could cause production of various lymphokines such as MIF, interferon, chemotactic factor, or others which would recruit or activate other cells. It could be related to macrophage activation, possible enhanced phagocytosis, and fungicidal activity, or even to the elevation of NK cell activity. NK cells have been reported to be fungicidal (13). NK cell activity was shown to be elevated by the CCW and PEP fractions (PF was not tested). This stimulation of NK and antifungal activities are desirable traits for immunopotentiators that might be used in treating cancer patients, who are often troubled with *Candida* infections. The TEICH fractions of both strains were again found to be void of immunopotentiating activity.

Since the majority of immunopotentiating activity found in the cell wall appeared to reside in the PEP fraction, further work should concentrate on isolating the specific portion of the PEP fraction responsible. It may be a small component resembling muramyl dipeptide. Kamisango et al. (11) have chemically described the *Listeria* peptidoglycan as containing *N*-acetylglucosamine and *N*-acetylmuramic acid plus some tripeptide components.

Since there were no significant differences between the two strains (EGD and ISC), either one would be satisfactory for research purposes. Both are virulent strains, which is an important consideration, as avirulent strains are ineffective in stimulating *Listerial* immunity (12). We tested nonpathogenic species of *Listeria* (*Listeria grayi*, *Listeria murrayi*, *Listeria denitrificans*) in NK assays and found them void of NK-stimulating ability, although they were as mitogenic as the *L. monocytogenes* strains.

In summary, we compared cell wall fractions from two different strains of *L. monocytogenes* and found no important differences. A variety of immunological responses were affected by these cell wall fractions, with the activity apparently localized in the peptidoglycans, and most importantly this material conferred in vivo protection, which is the

ultimate function of an immunopotentiator. There is certainly a need for the development of more immunopotentiators which could be added to our arsenal of weapons used in the battle against disease. Peptidoglycans from *L. monocytogenes* show real promise as an immunopotentiator.

ACKNOWLEDGMENTS

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