INFECTION AND IMMUNITY, Feb. 1977, p. 500-509

Vol. 15, No. 2
Printed in U.S.A.

Purification of a Monocytosis-Producing Activity from *Listeria monocytogenes*

S. B. GALSWORTHY,* S. M. GUROFSKY, AND R. G. E. MURRAY

Department of Bacteriology and Immunology, The University of Western Ontario, London, Ontario, Canada N6A 5C1

Received for publication 19 August 1976

The monocytosis-producing activity (MPA) from *Listeria monocytogenes* is shown to be associated with the cell envelope. Both MPA and immunosuppressive activity were readily extracted with aqueous solvents and separated as two independent activities by gel filtration and ion-exchange chromatography. As little as 10 μg of the resulting MPA-containing fraction caused a ninefold elevation in the level of circulating monocytes. The molecular weight of the fraction was approximately 22,000, and it contained phosphorus and carbohydrate.

Monocytosis is a hallmark of infections with *Listeria monocytogenes* in some animals (28). Comparable degrees of monocytosis can be produced by injection of chloroform-soluble extracts of whole organisms (3, 16, 39, 41) or aqueous extracts of the lipid-depleted residue (42). Crude active material liberated with either polar or nonpolar solvents consistently produces lymphopenia, granulocytosis, and immunosuppression as well as monocytosis after injection into mice (24, 42). In this communication we present evidence that the monocytosis-producing activity (MPA), although extractable, is associated with the cell envelope. Furthermore, gel filtration of extracts produces two fractions that possess MPA and immunosuppressive activity. These two activities can be separated by diethylaminoethyl (DEAE)-cellulose chromatography.

MATERIALS AND METHODS

Strains, media, and cultivation. *L. monocytogenes* strain 42 (serotype 1), originally isolated by Stanley (38), was used as the source of MPA-containing fractions. A 70-g batch of this strain, prepared as described below, was the gift of K. K. Carroll of the Department of Biochemistry, University of Western Ontario. The cells, grown as described by Holmström (20), were killed by heating to 120°C for 30 min, lyophilized, and stored at room temperature for use as starting material for extraction and chromatographic separation of biologically active fractions.

For the preparation of membranes, *L. monocytogenes* strain 42 was obtained in the lyophilized state from the culture collection of the late E. G. D. Murray (University of Western Ontario collection no. 42), revived in 5 ml of the medium of Girard and Murray (16) for 12 h at 30°C, and streaked on blood agar for incubation (37°C for 24 h) to check the purity of the culture. Batch cultures were grown in 2 liters of the medium of Girard and Murray (16) in 6-liter flasks, using an inoculum of 20 ml from a 12-h culture of *L. monocytogenes* in the same medium. The flasks were shaken on a rotating platform (Psychrotherm incubator G26, New Brunswick Scientific Co., New Brunswick, N.J.) at 100 excursions/min at 30°C for 12 h to bring them to the late logarithmic phase of growth. Cells were harvested by batch centrifugation in stainless-steel bottles at 20,000 × *g* (Sorvall RC2B centrifuge, Newtown, Conn.). The supernatant was filtered, concentrated, and prepared for bioassay as described below. Pelleted cells were rinsed twice with cold distilled water.

Preparation of membranes. Protoplasts were formed from the pelleted, rinsed cells of freshly grown strain 42 by incubation with pancreatic lipase followed by incubation with lysozyme as described by Ghosh and Murray (14). Procedures for preparation of protoplasts and membranes are summarized in Fig. 2.

Extraction of MPA from heat-killed *L. monocytogenes* and preparation of cell envelopes. Ten grams of the lyophilized cells was extracted with chloroform-methanol (2:1) and then with 1 M NaCl as described by Tadayon et al. (42). This is summarized in Fig. 1, which shows how the saline-extractable material (SE) was obtained.

The residue (5 g) from the above extractions was mixed with 25 ml of 50-μm glass beads (3M Co., St. Paul, Minn.) and 20 ml of 1 M NaCl in a 50-ml Braun homogenizer bottle and agitated at 4,000 rpm in a Braun homogenizer (model MSK, Bronwill Scientific Corp., Rochester, N.Y.) while being simultaneously cooled with liquid Co_2. After each 2-min agitation period, a sample of the mixture was observed under the phase-contrast microscope. After three agitation periods virtually all cells appeared to be broken, and cell envelopes were collected by centrifugation at 10,000 × *g* for 20 min. Further agitation of cell envelopes prepared in this manner did not

release further protein into the supernatant as determined by the method of Lowry et al. (25). Envelopes were washed four times with distilled water and lyophilized.

Fractionation on Bio-Gel A-1.5m. A column (2.7 by 111 cm) of agarose gel (Bio-Gel A-1.5m, Bio-Rad Corp., Richmond, Calif.) was equilibrated with 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5, at a flow rate of 14 ml/h. SE was dissolved in the same buffer at a concentration of 15 mg/ml, the equivalent of 75 to 110 mg was applied to the column, and material was eluted with the above buffer. Fractions (4 ml) were collected, and the optical density of each fraction at 260 nm (A_{260}) was measured (Hitachi Perkin-Elmer spectrophotometer, model 139). Aliquots of alternate fractions were analyzed for protein and phosphorus as described below. Eluates from subsequent identically run columns were pooled as described in Fig. 3, dialyzed, lyophilized, and stored in a desiccator at room temperature.

Ion-exchange chromatography of SE. A 20-mg sample of SE was dissolved in 2 ml of distilled water and adjusted to pH 2 by dropwise addition of 0.05 N HCl. The solution was applied to a column (1 by 10 cm) of AG 50W-X2, hydrogen form (Bio-Rad Laboratories, Richmond, Calif.), and the column was washed with distilled water until no more A_{260}-absorbing material could be detected in the eluate. Cationic material that had adsorbed to the column was obtained by eluting in a similar manner with 6 N HCl. The material eluted with distilled water was freeze-dried, redissolved in 2 ml of distilled water, adjusted to pH 10 by the addition of 0.05 N NaOH, and applied to a column (1 by 10 cm) of AG 1-X8, acetate form (Bio-Rad Laboratories, Richmond, Calif.). Material that was nonanionic under these conditions was eluted with distilled water. Anionic material was eluted with 2 N sodium acetate. Cationic, neutral, and anionic materials were dialyzed for 8 h at 4°C against four changes of distilled water and lyophilized.

Fractionation on DEAE-cellulose. A column (2.7 by 45 cm) of DEAE-cellulose (J. T. Baker Chemical Co., Phillipsburg, N.J.) was equilibrated with 0.01 M phosphate buffer, pH 7.0. Material (88 mg) corresponding to fraction C from the Bio-Gel column was applied in 5.9 ml of the same buffer. After elution with 100 ml of the same buffer, a 400-ml linear gradient from 0 to 1 M NaCl in the same buffer was passed through the column, and 5-ml fractions were collected. Then 400 ml of a second gradient from 1 to 2 M NaCl was established, and fractions were collected as before. Finally 200 ml of 2 M NaCl in the same buffer was passed through the column, and 5-ml fractions were collected. The A_{260} of each fraction was determined, and fractions were pooled as described in Fig. 4, dialyzed at 4°C against four changes of distilled water, and lyophilized.

Analytical methods. The methods of Lowry et al. (25) and Dryer et al. (11) were used for protein and phosphorus estimations, respectively. For carbohydrate analysis, 5-mg samples in 2 ml of 2 N HCl were sealed in vacuo and heated to 110°C for 2 h. Acid was removed from samples by evaporation, and samples were dissolved in distilled water for analysis by the orcinol method (36, 37). Alternatively, aliquots of fractions were analyzed for carbohydrate by the phenol-sulfuric acid method (12).

Preparation of samples of bioassay. (i) Samples derived from live cells. The lipase digestion mixture was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.), dialyzed, frozen, and lyophilized. Washed plasma membranes and soluble cytoplasm preparations were frozen and lyophilized. The lysozyme digestion mixture was freed of sucrose and other low-molecular-weight components by ultrafiltration through a UM-05 filter, which excludes compounds with a molecular weight of 500 at 70 lb/in^2 in a pressure cell (Amicon model 402, Amicon Corp., Lexington, Mass.). The 400-ml mixture was reduced to 30 ml, the pressure was released, 350 ml of distilled water was added, and the process was repeated twice. The resulting concentrate was passed through a 0.45-μm membrane filter (Millipore Corp.), and the filtrate was frozen and lyophilized. All lyophilized samples were reconstituted in sterile water at a concentration of 5 mg/ml, placed in sterile screw-top containers, and heated in a water bath at 70°C for 30 min. An aliquot of each sample was plated onto blood agar and incubated 48 h at 37°C to check sterility before bioassays were performed. Pasteurized samples were stored at −20°C.

(ii) Samples derived from heat-killed cells. Lyophilized material was dissolved in 0.15 M NaCl or distilled water at a concentration of 5 mg/ml, filter sterilized, dispensed aseptically in sterile universal containers, and stored at −20°C.

Assay of MPA. MPA activity was determined by a modification of the mouse assay described by Tadayon et al. (41). Samples (0.2 ml) at an appropriate concentration were injected intraperitoneally into a group of 18- to 20-g female Swiss albino mice. Control animals were injected with the same volume of isotonic saline. Samples of tail vein blood were obtained immediately before injection and at 12, 36, 60, and 84 h after injection. Blood was diluted 1:20 in Turk solution and mixed, and the number of leukocytes was determined in a Neubauer hemocytometer (A. O. Baker). Smears were fixed in Carnoy solution and stained with Giemsa stain. Differential leukocyte counts (at least 500 cells per sample) were made to ascertain the absolute levels of blood monocytes. MPA is expressed as the sum of the number of monocytes per cubic millimeter at the three peak periods of the response. The level of circulating monocytes in saline-injected control mice ranged from 0 to 2% and seldom exceeded 0.5%. The average MPA activity for control animals was calculated to be between 30 and 100; values between 200 and 700 were considered to represent low MPA activity; values greater than 700 were considered to represent high activity. The response to MPA has been shown to vary with age, sex, and species of test animals (41). In the experiments reported in this communication, with these variables controlled, there was still variation in the response of individual animals. To discriminate between varied responses of successive batches of mice and real differences between

activities of various fractions, a "standard" SE preparation was made by dissolving 250 mg of SE in 50 ml of 0.15 M NaCl. Aliquots (2 ml) were stored in tightly stoppered Universal containers at −20°C. One group of assay animals in all experiments was injected with 0.2 ml of the standard SE preparation, and another was injected with 0.2 ml of 0.15 M NaCl. Despite considerable variation between responses of individual animals, the activities of different preparations relative to the standard remained consistent in different experiments.

Plaque-forming assay. The immunosuppressive effect of crude and fractionated SE was tested by injecting groups of mice with 1 mg of each fraction 24 h before injection of the antigen, 0.1 ml of a 10% suspension of sheep erythrocytes. Control animals were injected with 0.2 ml of phosphate-buffered saline 24 h before administration of sheep erythrocytes. Five days after injection of antigen, mice were killed by cervical dislocation, and spleens were removed and assayed for plaque-forming cells by the method of Cunningham and Szenberg (10).

RESULTS

MPA activity of fractions derived from heat-killed cells. The preparation of the lipid fraction, SE, residue, and envelopes from heat-killed cells is summarized in Fig. 1. MPA was demonstrated in heat-killed cells, lipid fractions, SE, residue, and envelopes; the results (Table 1) were comparable to those of Tadayon et al. (42) for lipids and SE. The deviation between individual determinations made it impossible to ascertain differences between activities of different fractions, with the exception of the lipid fraction, which was considerably less active than the others. Since MPA could be extracted from the residue with saline, we sought to determine whether the removal of cytoplasmic material from the residue would result in the complete solubilization of MPA. Cell envelopes salvaged from the residue as described in Materials and Methods still exhibited MPA. Although further studies were done to localize MPA as described below, the SE fraction was used as the starting material for further purification steps because it was likely to involve separation from a reduced range of molecular species.

TABLE 1. *MPA of fractions from lyophilized, heat-killed L. monocytogenes*

Fraction[a]	Dose (mg)	No. of expts	MPA[b]
Lyophilized, heat-killed cells	5	1	1,010 ± 140
	2	3	739 ± 163
	1.5	1	839 ± 110
	0.5	1	788 ± 116
Lipid	2	2	296 ± 118
SE	5	1	1,538 ± 287
	2	6	990 ± 263
	1	4	683 ± 147
	0.5	4	496 ± 122
Residue	2	2	819 ± 230
Cell envelope	2	1	1,024 ± 100
	1	3	698 ± 180
Saline controls		9	26 ± 14

[a] Fractions were prepared and assayed as described in the text and Fig. 1.

[b] MPA activity is expressed as the sum of monocytes per cubic millimeter of tail vein blood on the peak 3 days of response. Three mice were used in each experiment. Results express the arithmetic mean of values from all animals ± standard error of the mean.

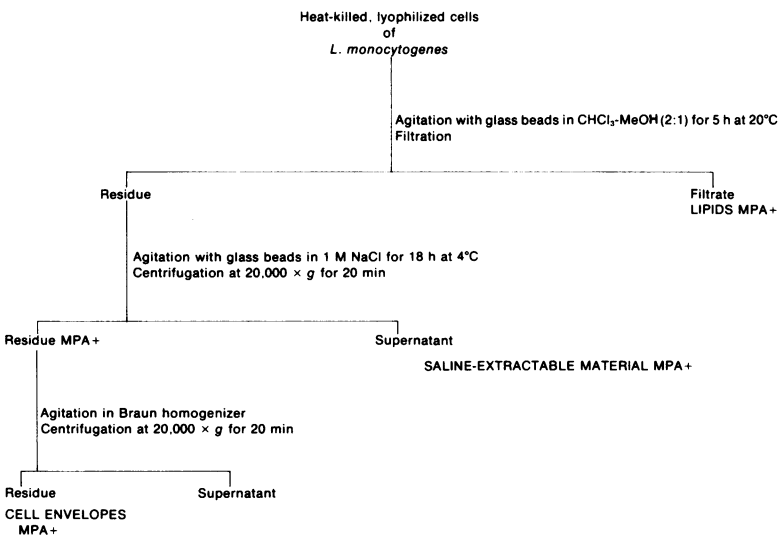

FIG. 1. *Extraction of MPA from L. monocytogenes. "MPA+" indicates presence of MPA.*

Intracellular localization of MPA. Because considerable MPA appeared to be associated with the cell envelope (Table 1), protoplasts of the organisms were prepared according to the method of Ghosh and Murray (14, 15) for use as a source of plasma membrane (15) and other fractions (Fig. 2). The MPA of each fraction is shown in Table 2. More MPA was associated with the plasma membrane than with any other cell fraction.

Fractionation of SE on Bio-Gel A-1.5m. Filtration of crude SE, which possessed both MPA and immunosuppressive activity (Table 3), on a Bio-Gel A-1.5m column yielded material with A_{260}, protein, and phosphorus content as shown in Fig. 3. Fractions A through E were pooled as shown. Materials for assay corresponding to fractions A, D, and E were obtained by pooling the appropriate fractions from three identical columns. Phosphorus, protein, and reducing-sugar content of each fraction were measured, and the MPA and immunosuppressive activity of a 1-mg dose of each fraction were determined (Table 3). Fraction A, richest in immunosuppressive activity and composed primarily of protein, retained significant MPA. Although this fraction represented a well-isolated peak, recovery was too small to make further purifi-

TABLE 2. *MPA of subcellular fractions of L. monocytogenes*

Fraction[a]	Dose (mg)	No. of expts	MPA[b]
SE	4.0	1	708 ± 104
	2.0	2	996 ± 242
	0.5	1	208 ± 50
Culture filtrate	1.0	1	136 ± 17
	0.5	1	33 ± 30
Lipase-solubilized wall	2.0	1	112 ± 36
Lysozyme-solubilized wall	4.0	1	317 ± 73
	2.0	1	255 ± 42
	1.0	1	202 ± 108
Cytoplasm	4.0	1	663 ± 46
	2.0	2	233 ± 117
	1.0	2	305 ± 105
Plasma membrane	4.0	1	865 ± 92
	2.0	2	616 ± 142
	1.0	2	475 ± 58
	0.5	2	612 ± 107
Saline controls		5	75 ± 20

[a] Fractions except SE were prepared as described in Fig. 2; SE was prepared as described in Fig. 1 and text.

[b] Expressed as described in Table 1. Three mice were used for each experiment.

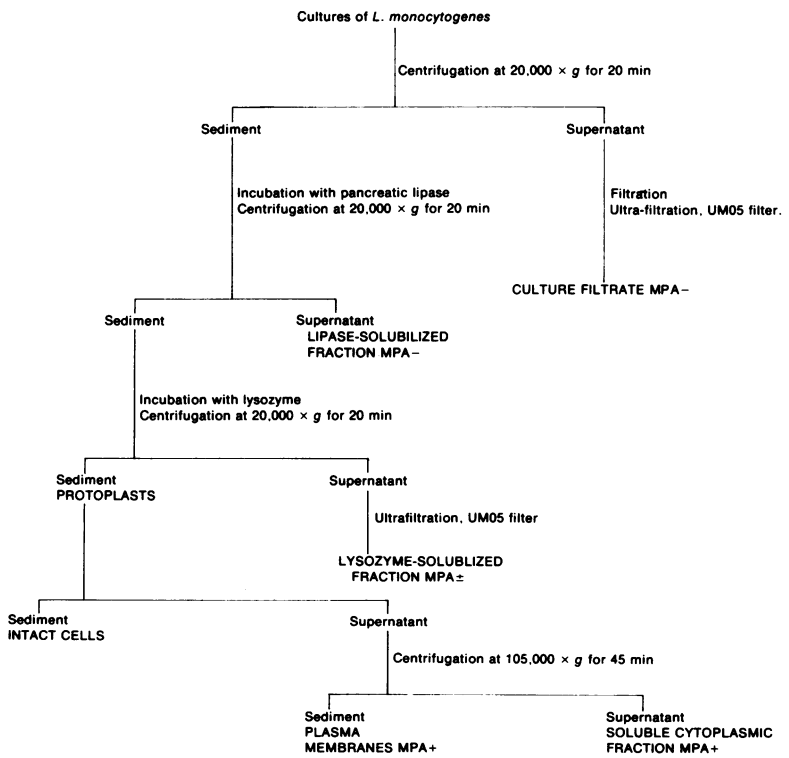

FIG. 2. *Preparation of plasma membranes from L. monocytogenes. "+" and "−" indicate presence and absence, respectively, of MPA.*

TABLE 3. *MPA and immunosuppressive activities and chemical composition of fractions of saline extract separable by gel filtration*

Fraction[a]	Recovery (mg)	MPA[b]	No. of plaque-forming cells/spleen[c]	Phosphorus[d] (% of dry wt)	Protein[e] (μg/mg [dry wt])	Reducing sugar[f] (μmol/mg dry wt])
SE	106 (applied to column)	747 ± 209	63,500 ± 13,500	2.3	180	100
Bio-Gel						
A	6.7	583 ± 114	8,625 ± 2,625	0.54	360	64
B	27.5	292 ± 274	33,750 ± 4,687	1.35	332	84
C	36.0	1,489 ± 275	52,375 ± 18,187	2.50	100	156
D	3.5	294 ± 249	179,500 ± 37,500	0.66	255	78
E	1.0	519 ± 204	332,062 ± 43,000	0.78	175	38
Saline control		60 ± 20	92,000 ± 39,937			

[a] Gel filtration of SE was performed as described in the text. Fractions pooled as described in Fig. 3 were used for bioassays and analyses.
[b] MPA expressed as described in Table 1. Doses of 1 mg of each fraction were used for each assay.
[c] Determined by the method of Cunningham and Szenberg (10) as described in the text.
[d] Determined by the method of Dryer et al. (11).
[e] Determined by the method of Lowry et al. (25).
[f] Determined by the phenol-sulfuric acid method (12).

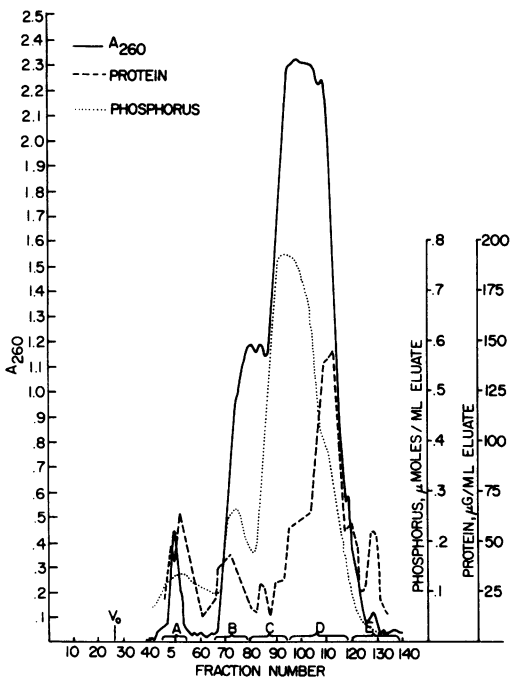

FIG. 3. *Gel filtration of SE on Bio-Gel A-1.5m. SE was prepared and subjected to gel filtration as described in the text. Aliquots of alternate fractions were analyzed for protein and phosphorus as described in the text. Values are expressed as micromoles per milliliter of eluate. Fractions A–E were pooled as indicated.*

cation steps feasible at this point. Fraction B was immunosuppressive but low in MPA. Fraction C, with the greatest MPA, retained some immunosuppressive activity and was relatively enriched in phosphorus and carbohydrate but depleted in protein. Fractions D and E, comprising a very small proportion of recovered material, appeared to promote an enhanced response to antigen. Since the dose effect of MPA was not linear, no attempt was made to compare activities of marginally active fractions. The enhanced response of fraction C (Table 3) becomes even more apparent when smaller doses are compared with SE (see Table 6).

TABLE 4. *MPA of SE fractionated by ion-exchange chromatography*

Fraction[a]	MPA[b]
SE	630 ± 59
Cationic	388 ± 122
Neutral	265 ± 113
Anionic	856 ± 104
Saline control	134 ± 55

[a] Fractions were obtained by chromatography of SE on AG 50W-X8 (hydrogen form) and AG 1-X8 (acetate form).
[b] MPA activity of 1-mg samples of each fraction was determined as described in the text and is expressed as the arithmetic mean of assays on three mice ± standard error of the mean.

Ion-exchange chromatography of SE. To explore the feasibility of ion-exchange chromatography as a means of purifying MPA, 20 mg of crude SE was subjected to a sequence of ion-exchange procedures as described in Materials and Methods, which resulted in cationic, neutral, and anionic fractions. When these were assayed in the usual manner, the anionic fraction was found to possess most of the MPA (Table 4).

DEAE-cellulose chromatography of frac-

tion C. Fraction C, subjected to chromatography on a DEAE-cellulose column as described in Materials and Methods, gave an elution pattern as shown in Fig. 4. Eluates were pooled as indicated in Fig. 4 for dialysis and freeze-drying preparatory to analysis for MPA and immunosuppressive activity, protein, phosphorus, and reducing sugars (Table 5). Fractions C-2 and C-3, eluted with lower concentrations of NaCl, had high immunosuppressive activity and either no or moderate MPA. Fraction C-4, eluted at higher concentrations of NaCl, was not appreciably immunosuppressive and contained very high MPA activity. Phosphorus, protein, and reducing-sugar analyses revealed that C-4 was nearly devoid of protein and had a decreased phosphorus and carbohydrate content compared with the crude SE and partially purified C fraction. The elution pattern of the C-4 fraction run on a Bio-Gel A-1.5m column (Fig. 5) revealed a single, symmetrical peak, indicating that the two-step chromatographic procedure eliminated a considerable proportion of the inactive material in the SE.

Estimation of molecular weight of C-4 by gel filtration. Marker molecules of known purity were filtered through the Bio-Gel A-1.5m column, and the molecular weight of C-4 (Fig. 6) approximated 22,000 by the method of Andrew (2).

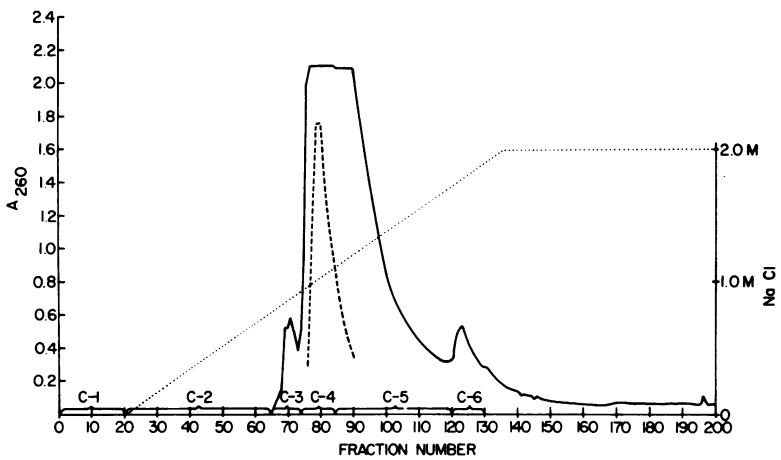

FIG. 4. *DEAE-cellulose chromatography of MPA. Material obtained in fraction C of the Bio-Gel A-1.5m column described in Fig. 3 was chromatographed on DEAE-cellulose as described in the text. The A_{260} values of each fraction are indicated by the solid line. The values of 1:10 dilutions of certain fractions are indicated by the dashed line. The molarity of NaCl is indicated by the dotted line. Fractions C-1 through C-6 were pooled as indicated.*

TABLE 5. *Biological activities and chemical composition of fractions derived from fraction C (in Table 3) by DEAE-cellulose chromatography*

Fraction[a]	Recovery (mg)	MPA[b]	No. of plaque-forming cells/spleen[c]	Phosphorus[d] (μ/mg [dry wt])	Protein[e] (μ/mg [dry wt])	Reducing sugar[f] (μmol/mg)
SE		800 ± 195	110,000 ± 5,000	2.30	180	100
C	88.0 (applied)	1,264 ± 225	64,300 ± 1,100	2.50	100	156
C-1	1.0	313 ± 150	ND	0.75	50	94
C-2	1.8	470 ± 200	48,000[g] ± 100	0.82	218	92
C-3	20.2	753 ± 214	48,250 ± 250	1.21	56	98
C-4	25.5	1,643 ± 218	196,500 ± 3,500	1.22	10	84
C-5	8.5	1,331 ± 254	ND	2.32	0	92
Saline control		75 ± 30	196,000 ± 1,000			

[a] Fractions were prepared by DEAE-cellulose chromatography of fraction C as described in the text and Fig. 4.

[b] Expressed as the arithmetic mean of assays on two groups of three mice ± standard error of the mean.

[c] Measured by the method of Cunningham and Szenberg (10). Values represent the arithmetic mean of assays on three animals ± standard error of the mean. ND, not done.

[d-f] See Table 3.

[g] 100 μg of this fraction was injected for assay of plaque-forming cells.

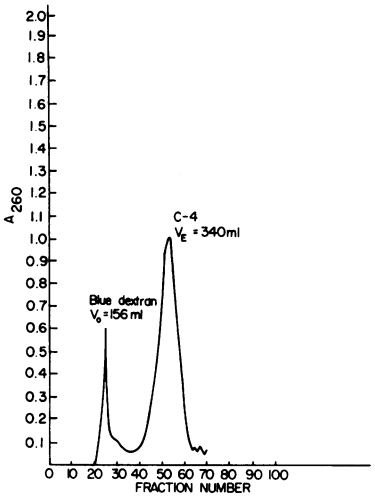

FIG. 5. *Bio-Gel chromatography of C-4. One milliliter of a solution containing 5 mg of blue dextran and 8 mg of fraction C-4 was filtered through the Bio-Gel A-1.5m column as described in Materials and Methods. All fractions were examined for A_{260}, and V_e and V_o were calculated.*

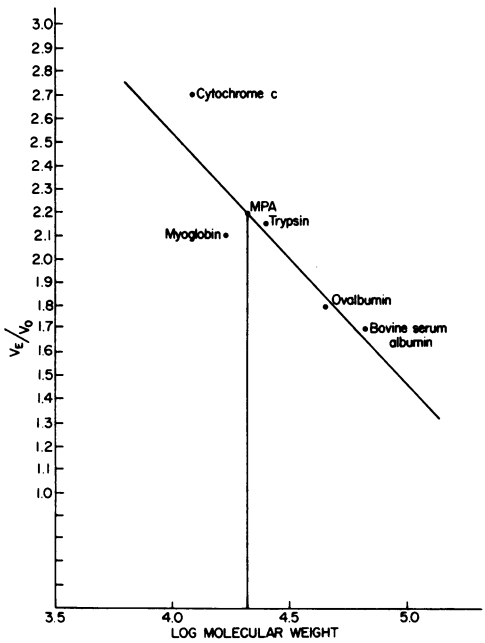

FIG. 6. *Estimation of molecular weight of purified MPA. Samples (10 mg) of cytochrome c, myoglobin, trypsin, ovalbumin, bovine serum albumin, and fraction C-4 were filtered through a Bio-Gel A-1.5m column as described in the text.*

Threshold dose of crude and purified MPA. The degree of enrichment in MPA of fraction C-4 was shown by an experiment designed to determine the threshold dose required to induce monocytosis. Definite amounts of these fractions (Table 6) were injected into mice. The values in Table 6 represent the average of two or more such experiments. Although SE exhibited high activity at 300-μg (dry weight) or higher doses, both C and C-4 exhibited high activity at 30-μg doses, and C-4 retained high activity at a 10-μg dose.

DISCUSSION

The data indicate that MPA from *L. monocytogenes* is associated with the cell envelope, particularly the plasma membrane, from which it can be only partially extracted with chloroform-methanol or saline. So far, attempts to solubilize all the activity using noncovalent bond-breaking reagents (ethylenediaminetetraacetic acid, LiCl, urea) have been only partially successful (S. B. Galsworthy, unpublished data). It appears that, although material of high activity is readily extractable, considerable activity remains an integral part of the cell envelope. MPA is not destroyed during dissolution of the cell wall by lysozyme when protoplasts are formed, but either remains attached to or becomes adsorbed to the cell membrane. MPA appearing in the soluble cytoplasm fraction could have been released from the plasma membrane fraction during the washing of the membranes in a manner analogous to the removal of the SE fraction from the heat-killed cells. Some MPA could be extracted from membranes with chloroform-methanol and considerably more with 1 M NaCl (S. B. Galsworthy,

TABLE 6. *Monocytosis in response to varied doses of crude and purified MPA preparations*

Fraction[a]	Dose (μg)	No. of expts	MPA[b]
SE	1,000	4	1,158 ± 255
	300	2	872 ± 63
	100	4	480 ± 85
	30	2	362 ± 97
	10	2	353 ± 65
C	1,000	4	1,279 ± 205
	300	2	904 ± 117
	100	4	914 ± 130
	30	2	772 ± 105
	10	2	328 ± 89
C-4	1,000	4	1,656 ± 236
	300	2	959 ± 150
	100	4	1,276 ± 165
	30	2	954 ± 89
	10	2	729 ± 43
Saline controls		6	75 ± 20

[a] Fractions were prepared as described in text.
[b] MPA was determined as described in the text and is expressed as the arithmetic mean of values from all animals (three in each experiment) ± standard error of the mean.

unpublished data). These results do not distinguish between the possibility that MPA is an integral part of the membrane and the possibility that MPA is a cell wall component which, when liberated by the dissolution of peptidoglycan, preferentially adsorbs to the membranes. The isolation and characterization of MPA has been hampered in the past by co-fractionation of activities. Crude lipid extracts (16, 39, 41, 42), phospholipids (42), ninhydrin-positive material in phospholipid extracts (42), and water-soluble material separated from the lipid extracts (42) all have been shown to contain MPA. These results are consistent with the hypothesis that MPA is a molecule with both hydrophilic and lipophilic properties. The nature of the active material cannot yet be deduced, but the fraction contains carbohydrate and phosphorus, with the proportions somewhat altered from those in the crude extract. It does not seem likely that it is a protein.

Kim et al. (24) found that the crude SE of delipidated cells markedly suppresses the antibody response of mice to sheep erythrocytes, provided that the extract is administered before antigen. Our similarly prepared extract possessed immunosuppressive activity as well as MPA. In contrast, Girard and Murray (16) showed that MPA-containing lipid extracts of *L. monocytogenes* enhanced antibody responses to several antigens concurrently with induction of monocytosis. Our chromatographic separations have produced evidence for both immunosuppressive and immune-enhancing materials in the crude extract (Table 3). The MPA alone is clearly not immunosuppressive (Table 4).

L. monocytogenes produces several substances of considerable biological interest in addition to MPA. Both the original lipid extracts and the saline extracts of lipid-depleted cells are able to induce lymphopenia and granulocytosis. The latter may be due to contamination of the crude extract with the polysaccharide described by Stanley (39).

Petit and Unanue (33) have described an uncharacterized substance isolated from culture filtrates of *L. monocytogenes* that stimulates proliferation of B-lymphocytes in vivo and in vitro, activates macrophages in vivo, and induces transitory resistance to infection. MPA, in contrast, appears to be envelope associated but can be readily extracted. Separate unpublished evidence from our laboratory shows that MPA increases the labeling index of bone marrow promonocytes labeled with tritiated thymidine in vivo and that the mononuclear phagocytes labeled under these conditions participate in the ensuing monocytosis (S. B. Galsworthy, manuscript in preparation). Other evidence that *L. monocytogenes* secretes substances capable of recruiting lymphocytes and macrophages comes from the experiments of Osebold et al. (32), in which *L. monocytogenes* within chambers implanted in peritoneal cavities of mice appeared to induce macrophages and lymphocytes to migrate to the chamber. A cell wall complex containing protein, lipid, and carbohydrate from *L. monocytogenes* causes a release of the macrophage migration inhibition factor from lymphocytes of animals sensitized by injection of the complex with Freund complete or incomplete adjuvant (23, 27). Another soluble extract of *L. monocytogenes* elicits a delayed hypersensitivity reaction and promotes [^{3}H]thymidine incorporation into lymphocytes in animals recently recovered from infection with *L. monocytogenes* (13). Campbell et al. have demonstrated that a cell wall fraction from *L. monocytogenes* acts as a mitogen for bone marrow-derived cells (5) and as an adjuvant (6, 7).

The roles of thymus-derived lymphocytes (29) and of activated macrophages (26, 30, 31) in resistance to infection by *L. monocytogenes* have been convincingly demonstrated. Recent reports suggest that bone marrow-derived cells are also necessary for resistance (5). Kungshavn (personal communication) has shown that mice devoid of thymus-derived cells effectively combat infection with *L. monocytogenes*.

As is the case for several other bacteria, it is evident that *L. monocytogenes* possesses an array of cell surface components capable of interacting with mammalian cell participants in the immune response in a variety of ways. Immunologically active components of *Streptococcus pyogenes* include an adjuvant peptidoglycan (21), a mitogen for lymphocytes (43), and three immunosuppressants (35). Whole killed cells of *Corynebacterium parvum* depress cell-mediated immunity (9) and can also act as an adjuvant (22). Peptidoglycans and other substances with adjuvant and mitogenic activity have been released from lipid-depleted cell walls of three species of *Nocardia* by lysozyme (4). Material prepared in a similar manner from *Mycobacterium smegmatis* can replace cells or cell walls in Freund's adjuvant (1). Substances with similar properties have been obtained from *Mycobacterium tuberculosis* (19). Both purified protein derivative of *M. tuberculosis* (34) and lipopolysaccharide from *Escherichia coli* (40) are mitogenic for bone marrow-derived lymphocytes.

Extracts from *L. monocytogenes*, however, have the unusual attribute of stimulating monocytosis. The investigations reported in this paper have resulted in the separation of two very useful biological activities from a water-soluble extract of *L. monocytogenes*. MPA, which in

small doses elevates the level of circulating monocytes, will be a useful tool for studying mechanisms of regulating monocyte levels. The immunosuppressive agent does not cause loss of body weight or change in hematocrit values after repeated large (3 mg) doses (24), but causes a marked decrease in numbers of plaque-forming cells after single small (10 μg) doses (24). With a convenient method for separating these activities, it will now be possible to study their individual effects on both humoral and cell-mediated immunological events.

ACKNOWLEDGMENTS

We wish to thank S. K. Singhal and W. Chung for helpful discussions and advice. We are grateful to L. McPhee and A. Levstick for technical assistance and to R. Dishman and J. Verge for preparation of the manuscript.

This investigation was supported by a grant from the Medical Research Council of Canada, MA-5090.

LITERATURE CITED

1. **Adam, A., R. Ciorbaru, J. F. Petit, and E. Lederer.** 1972. Isolation and properties of a macromolecular, water-soluble, immuno-adjuvant fraction from the cell wall of *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. U.S.A. 69:851–854.
2. **Andrew, P.** 1964. Estimation of the molecular weights of proteins by Sephadex gel filtration. Biochem. J. 91:222–233.
3. **Beaulieu, M.** 1963. Some factors affecting the yield of the monocytosis-producing agent. Can. J. Microbiol. 9:473–477.
4. **Ciorbaru, R., A. Adam, J. F. Petit, E. Lederer, C. Bona, and L. Chedid.** 1975. Isolation of mitogenic and adjuvant active fractions from various species of *Nocardiae*. Infect. Immun. 11:257–264.
5. **Campbell, P. A., B. L. Martens, H. R. Cooper, and J. L. McClatchy.** 1974. Requirement for bone marrow-derived cells in resistance to *Listeria*. J. Immunol. 112:1407–1414.
6. **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.
7. **Campbell, P. A., C. Schuffler, and G. E. Rodriguez.** 1976. Listeria cell wall fraction: a B-cell adjuvant. J. Immunol. 116:590–594.
8. **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.
9. **Collins, F. M., and M. T. Scott.** 1974. Effect of *Corynebacterium parvum* treatment on the growth of *Salmonella enteritidis* in mice. Infect. Immun. 9:863–869.
10. **Cunningham, A. J., and A. Szenberg.** 1968. Further improvements in the plaque technique for detecting single antibody-forming cells. Immunology 14:599–600.
11. **Dryer, R. L., A. R. Tammes, and J. I. Routh.** 1957. The determination of phosphorus and phosphatase with N-phenyl-p-phenylenediamine. J. Biol. Chem. 225:177–183.
12. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356.
13. **Fulton, A. M., M. M. Dustoor, J. E. Kusinski, and A. A. Blazkovec.** 1975. Blastogenesis as an in vitro correlate of delayed hypersensitivity in guinea pigs infected with *Listeria monocytogenes*. Infect. Immun. 12:647–655.
14. **Ghosh, B. K., and R. G. E. Murray.** 1967. Fine structure of *Listeria monocytogenes* in relation to protoplast formation. J. Bacteriol. 93:411–426.
15. **Ghosh, B. K., and R. G. E. Murray.** 1969. Fractionation and characterization of the plasma and mesosome membrane of *Listeria monocytogenes*. J. Bacteriol. 97:426–440.
16. **Girard, K. F., and E. G. D. Murray.** 1954. The influence of a sustained monocytosis upon the antibody response in rabbits to various antigens. Can. J. Biochem. Physiol. 32:1–13.
17. **Hahn, H.** 1975. Requirement for a bone marrow-derived component in the expression of cell-mediated antibacterial immunity. Infect. Immun. 11:949–954.
18. **Heymer, B., K. H. Schleifer, and B. Bueltmann.** 1975. Migration inhibition of peritoneal macrophages by peptidoglycan. Z. Immunitaetsforsch. Exp. Klin. Immunol. 149:289–294.
19. **Hiu, I.** 1972. Water-soluble and lipid-free fraction from BCG with adjuvant and anti-tumour activity. Nature (London) New Biol. 238:241–242.
20. **Holmström, B.** 1967. Studies on the cultivation of *Listeria monocytogenes* in batch and continuous cultures. Can. J. Microbiol. 13:1551–1559.
21. **Holton, J., and J. H. Schwab.** 1966. Adjuvant properties of bacterial cell wall mucopeptides. J. Immunol. 96:134–138.
22. **Howard, J. G., G. H. Christie, and M. T. Scott.** 1973. Biological effects of the adjuvant *Corynebacterium parvum*. IV. Adjuvant and inhibitory effects on lymphocytes. Cell. Immunol. 7:290–301.
23. **John, C., I. Spratkova, M. Moravkova, F. Patocka, and M. Mara.** 1974. Effect of Listeria-factor E on spleen cell migration in rabbits hypersensitive to *Listeria monocytogenes*. J. Hyg. Epidemiol. Microbiol. Immunol. 18:369–372.
24. **Kim, J. J., N. R. St. C. Sinclair, S. K. Singhal, and K. K. Carroll.** 1976. Immunosuppressive activity of an extract from *Listeria monocytogenes*. Int. Arch. Allergy Appl. Immunol. 50:641–650.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
26. **McGregor, D. D., F. T. Koster, and G. B. Mackaness.** 1971. The mediator of cellular immunity. I. The lifespan and circulation dynamics of the immunologically committed lymphocyte. J. Exp. Med. 133:389–399.
27. **Mara, M., J. Julak, and F. Patocka.** 1974. Isolation and chemical properties of biologically active fractions of the surface of *Listeria monocytogenes*. J. Hyg. Epidemiol. Microbiol. Immunol. 18:359–364.
28. **Murray, E. G. D., R. A. Webb, and M. Swann.** 1926. A disease of rabbits characterized by a large mononuclear leukocytosis caused by a hitherto undescribed Bacillus *Bacterium monocytogenes* (N.SP) J. Pathol. Bacteriol. 29:407–439.
29. **North, R. J.** 1973. Cellular mediators of anti-*Listeria* immunity as an enlarged population of short-lived, replicating T cells. J. Exp. Med. 138:342–355.
30. **North, R. J.** 1974. T-cell dependence of macrophage activation and mobilization during infection with *Mycobacterium tuberculosis*. Infect. Immun. 10:66–71.
31. **North R. J., and G. B. Mackaness.** 1973. Immunological control of macrophage proliferation in vivo. Infect. Immun. 8:68–73.
32. **Osebold, J. W., P. M. Outteridge, L. D. Pearson, and R. A. DiCapua.** 1970. Cellular responses of mice to diffusion chambers. Infect. Immun. 2:127–131.
33. **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.
34. **Rowley, D.** 1955. Stimulation of natural immunity to *E. coli* infection. Lancet i:232.

35. **Schwab, J. H.** 1975. Suppression of the immune response by microorganisms. Bacteriol. Rev. 39:121–143.
36. **Spiro, R. G.** 1966. Analysis of sugars found in glycoproteins. Methods Enzymol. 8:3–26.
37. **Spiro, R. G.** 1966. Characterization of carbohydrate units of glycoproteins. Methods Enzymol. 8:26–52.
38. **Stanley, N. F.** 1948. *Listeria monocytogenes*. A description of a strain of *Listeria monocytogenes* and a report of a case. Med. J. Aust. 2:205–208.
39. **Stanley, N. F.** 1949. Studies on *Listeria monocytogenes*. I. Isolation of a monocytosis-producing agent (MPA). Aust. J. Exp. Biol. Med. Sci. 27:123–131.
40. **Sultzer, B. M., and B. S. Nilsson.** 1972. PPD tuberculin: a B-cell mitogen. Nature (London) New Biol. 240:198–200.
41. **Tadayon, R. A., K. K. Carroll, and R. G. E. Murray.** 1969. Factors affecting the yield and biological activity of lipid extracts of *Listeria monocytogenes*. Can. J. Microbiol. 15:421–428.
42. **Tadayon, R. A., K. K. Carroll, and R. G. E. Murray.** 1970. Purification and properties of biologically active factors in lipid extracts of *Listeria monocytogenes*. Can. J. Microbiol. 16:535–544.
43. **Taranta, A., G. Cuppari, and F. Quagliata.** 1969. Dissociation of hemolytic and lymphocyte-transforming activities of streptolysin S preparations. J. Exp. Med. 129:605–622.