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Enhancement of Macrophage-Mediated Tumor Cell Killing by Bacterial Outer Membrane Proteins (Porins)

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Various microbial products are known to influence the function of mouse peritoneal macrophages. Lipopolysaccharide (LPS) and certain lipid A-associated proteins are known to enhance the tumoricidal effects of macrophages. The purpose of this study was to determine whether porins (outer membrane proteins) of *Salmonella typhimurium* G30/C21 would influence the activity of macrophages from lipid A-responsive and -unresponsive mice. Porins, extracted by a combined sodium dodecyl sulfate-EDTA method from cell walls, were free of LPS as determined by *Limulus* amebocyte lysate assay and appeared as a band at approximately 36,000 molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In tumor cell killing assays done under LPS-free conditions, the porins in doses of 1 to 10 ng/ml enhanced the tumoricidal effect of macrophages from bacillus Calmette-Guérin-infected C_3H/HeN or C_3H/HeJ mice. Protein-free LPS enhanced the tumoricidal activity of macrophages from bacillus Calmette-Guérin-infected C_3H/HeN but not C_3H/HeJ mice. The tumoricidal-enhancing activity of protein-free LPS was blocked by the lipid A-binding antibiotic polymyxin B sulfate, but the effects of porins were not altered by the polymyxin B sulfate. These results suggest that porins, proteins known to alter membrane function, may alter macrophage function by interaction with macrophage membranes.

Mouse peritoneal macrophages, after appropriate in vivo or in vitro manipulation, can selectively kill tumorigenic target cells (9, 18, 20, 25). Components of gram-negative bacteria have been shown to enhance the tumor cytotoxic effects of macrophages. Endotoxin (lipopolysaccharide [LPS]) (9, 17, 18, 20, 25) and a protein associated with LPS (5) can render normal or partially "activated" macrophages tumoricidal. Although protein-free LPS does not affect macrophages from lipid A-unresponsive C_3H/HeJ mice (19, 25), the lipid A-associated protein enhances the activity of macrophages from lipid A-responsive and -unresponsive mice (5).

The precise identity of this lipid A-associated protein or endotoxin protein (8, 12) is not known, but in other systems porins, protein II*, and lipoprotein are thought to be parts of the endotoxin proteins (6). The purpose of this study was to determine whether outer membrane proteins (specifically, porins [4, 10, 15]) of gram-negative bacteria were capable of enhancing the tumoricidal effects of macrophages. It is known that porins remain associated with LPS during butanol or trichloroacetic acid extraction procedures like those used by workers studying the effects of various LPS preparations on macrophages (5). Also, porins remain associated with peptidoglycan in the presence of Mg^{2+}, whereas other components may be extracted with sodium dodecyl sulfate (SDS) (16, 21). We have used this differential extraction as a means of preparing porins for our studies of biological activities of porins. Our results show that LPS-free porins isolated from *Salmonella typhimurium* G30/C21 enhance tumor cell killing by macrophages from lipid A-responsive or -unresponsive mice, and function in ways comparable to those described before for lipid A-associated protein (5).

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MATERIALS AND METHODS

Mice. C_3H/HeN and C_3H/HeJ female mice (H-2^k), aged 12 to 18 weeks, were obtained from Charles

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TABLE 1. *Limulus* amebocyte lysate clotting assay[a]

Amt of LPS or porin present (ng/ml)	LPS	Porin
0	−	−
0.1	−	−
0.5	+	−
1.0	+	−
10.0	+	−
100.0	+	−
1,000.0	+	−

[a] +, Solid clot present after 60 min at 37°C; −, no clot present after 60 min at 37°C.

River, Wilmington, Mass., and Jackson Laboratories, Bar Harbor, Maine. Infection with bacillus Calmette-Guérin (BCG) was established by injecting 10^6 CFU of Trudeau BCG (TMC no. 1926) intraperitoneally. The mice were used 2 to 4 weeks after infection.

Media and cell lines. Supplemented Dulbecco's modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) was formulated with LPS-free water as described before (24, 25). Only media which tested negatively in *Limulus* amebocyte lysate assay were used. LPS-free adult bovine serum was obtained from Sterile Systems, Logan, Utah. The tumorigenic fibrosarcoma 3T12 cell line ($H\text{-}2^d$) was used as the target cell in the tumor cell killing assays.

Tumor cell killing assays. This assay was performed as described before (25) in assays in Dulbecco's modified Eagle medium with 10% adult bovine serum. Resident peritoneal cells are very resistant to activating agents (25, 26), so mice received 1 ml of 10% peptone intraperitoneally 3 days before cell harvest. Peritoneal cells (4×10^5) in 0.1 ml of Dulbecco's modified Eagle medium were added to chambers of microtiter plates (Falcon no. 3040), and after adherence for 1 hour at 37°C, nonadherent cells were removed by washing twice with phosphate-buffered saline. The resultant monolayers (ca. 1.2×10^5 per chamber) had more than 90% macrophages as assessed by their phagocytic ability and their presence of nonspecific esterase (26). 3T12 target cells were labeled with tritiated thymidine before the assay. The effector to target ratio was approximately 12 to 1. Supernatants were harvested after 3 days of coculture with the macrophages. Spontaneous release from the target cells did not exceed 20% over the 3-day period. The percent of tumor cell killing was calculated as the percent tritiated thymidine release by the formula $[(Sx-Sc)/total] \times 100$, where Sx is counts per minute in the supernatant of the experimental well, Sc is counts per minute in the supernatant of the control well (tumor cell alone wells), and total is the total counts per minute in the tumor cell alone well determined by lysing with 0.2% SDS (25). The cultures were monitored visually with an inverted phase microscope, and the tumoricidal effect as determined by the tritiated thymidine release assay reflected the cytotoxicity as determined visually (25, 26). Experiments were done at least three times with each of three separate preparations of porins.

Porin preparation. The porins were extracted from *S. typhimurium* cell walls by using a procedure modified from those described by Nakae (cf. 16) and Schnaitman (21). Re mutant *S. typhimurium* G30/C21 cell walls were prepared as previously described by Takayama et al. (22), except for the omission of the trypsin treatment. The lyophilized cell wall pellet was washed twice with water at 22°C and extracted thrice with 2% SDS-0.5 mM $MgSO_4$ at 60°C. Insoluble peptidoglycan complexes were recovered by centrifugation at 30,000 × *g* for 30 to 90 min at 22°C. The insoluble Mg^{2+}-porins-peptidoglycan complex was then extracted five times with 2% SDS-50 mM EDTA at 60°C to solubilize and separate the porins from the insoluble peptidoglycan. The soluble porins were then repeatedly precipitated in 0.5 M NaCl with 50% ethanol-water to remove SDS (3), and the porins pellet was suspended in acetone and subsequently dried. For use in experiments, the porins were dissolved in water at 70°C and sonicated in a bath sonifier. SDS-polyacrylamide gel electrophoresis was done as described by Lugtenberg et al. (11) by a modified Laemmli system. Proteins were prepared by boiling for 5 min in 62.5 mM Tris-hydrochloride (pH 6.8) with 2% SDS, 10% glycerol, 0.001% bromophenol blue, and 2% 2-mercaptoethanol.

Testing for LPS. The presence of LPS was determined by the *Limulus* amebocyte lysate assay (24, 25) with Westphal-extracted *Escherichia coli* 0111:B12 LPS as standard. The *Limulus* amebocyte lysate (PanMed, Chicago, Ill.) was capable of detecting >0.5 to 1.0 ng of LPS per ml.

Materials. Polymyxin B sulfate and *E. coli* 0111:B12 LPS were from Sigma Chemical Co., St. Louis, Mo. Protein-free LPS (phenol extraction) from *E. coli* K235 was given to us by David Morrison, Emory University, Atlanta, Ga.

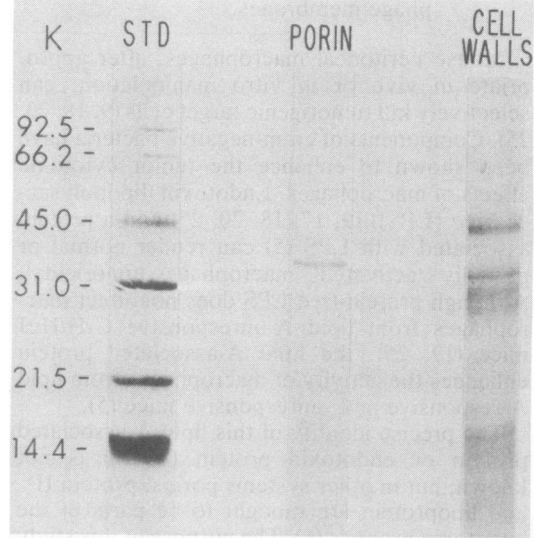

FIG. 1. SDS polyacrylamide gel electrophoresis of *S. typhimurium* G30/C21 cell walls (5 μg of protein) and porins (1 μg of protein). Protein reference standards (STD) (Bio-Rad Laboratories, Richmond, Calif.) include phosphorylase *b* (92,500 dalton), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

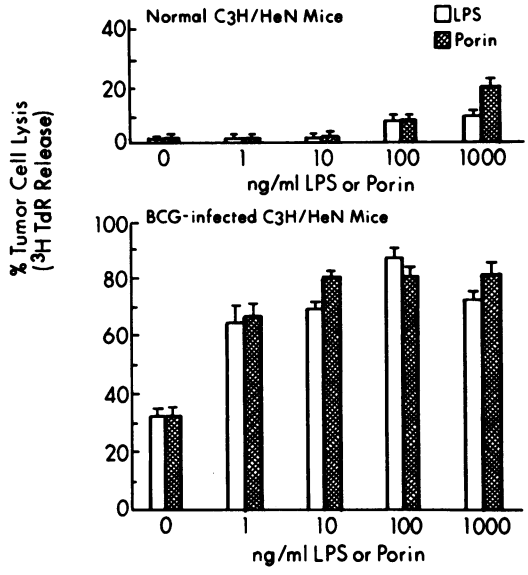

FIG. 2. Tumor cell killing by macrophages from normal or BCG-infected C_3H/HeN mice in assays with either LPS or porins. The bars represent the means plus one standard error of the mean of triplicate samples.

RESULTS

As determined by *Limulus* amebocyte lysate clotting, there was no LPS in the porins preparation (Table 1). Also, the porins did not inhibit the clot-inducing ability of LPS since >1 ng of LPS per ml added to the porins caused clotting of the *Limulus* lysate. Coomassie blue staining of polyacrylamide gel electrophoreses showed one major band at about 36,000 molecular weight and a minor one at 37,000 (Fig. 1). Electrophoresis with higher amounts of protein (e.g., 10 μg per lane) disclosed three or four additional faint bands. The bands at 36,000 and 37,000 molecular weights comprised more than 90% of the protein in the sample.

As noted before (9, 25), macrophages from normal or BCG-infected mice injected with peptone did not kill tumor cells in vitro in the absence of added LPS. However, in cultures supplemented with protein-free LPS, there was enhanced tumor cell killing by the normal and BCG macrophages from C_3H/HeN mice (Fig. 2). The BCG macrophages were much more sensitive to the LPS effects than were the macrophages from peptone-injected normal mice. Macrophages from C_3H/HeJ mice were not rendered tumoricidal by the protein-free LPS. The LPS-free porins were effective in enhancing the tumoricidal effect of macrophages from both C_3H/HeN and C_3H/HeJ mice (Fig. 3).

The antibiotic polymyxin B sulfate binds to the protein-free lipid A and inhibits its effects in various systems including macrophage-mediated tumor cell killing (5, 14, 19, 25). In experiments done here, polymyxin B inhibited the effect of protein-free LPS but did not alter the tumoricidal enhancing activity of porins in C_3H/HeN or C_3H/HeJ mice (Fig. 4). The polymyxin B had no consistent effect on the macrophages in the absence of porins or LPS.

DISCUSSION

Mouse peritoneal macrophages are known to respond to various environmental signals by acquiring the ability to kill tumor cells in vitro. The process of differentiation of macrophages to the tumoricidal state is not an all or none process but a graded, stepwise path from nontumoricidal resident peritoneal macrophages to macrophages that kill tumor cells (9, 25). In vivo, this activation of macrophages is thought to occur through the action of various lymphokines and possibly other soluble products on the macrophages (9). In vitro systems have enabled investigators to dissect the process of activation and identify agents causing the differentiation. In addition to lymphokines (macrophage-activating factors) (9, 17, 18, 20, 25), products from the cell walls of gram-negative bacteria have been identified as potent activators of macrophages (1, 5, 9, 17, 18, 20, 25). Generally, these bacterial factors act on macrophages which are incom-

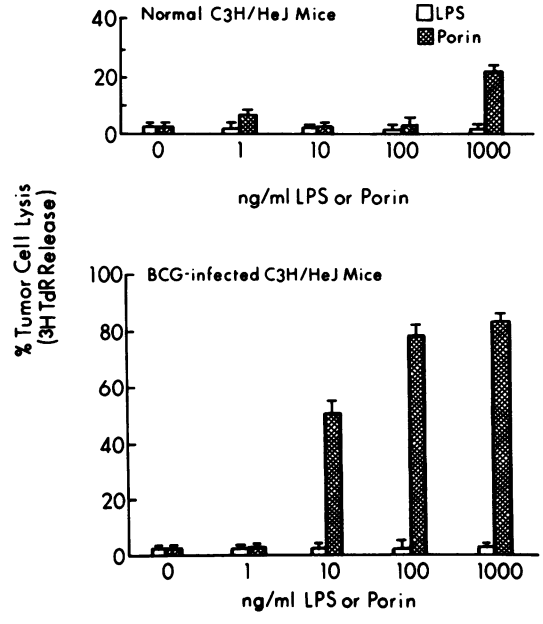

FIG. 3. Tumor cell killing by macrophages from normal or BCG-infected C_3H/HeJ mice in assays with either LPS or porins. The bars represent the means plus one standard error of the mean of triplicate samples.

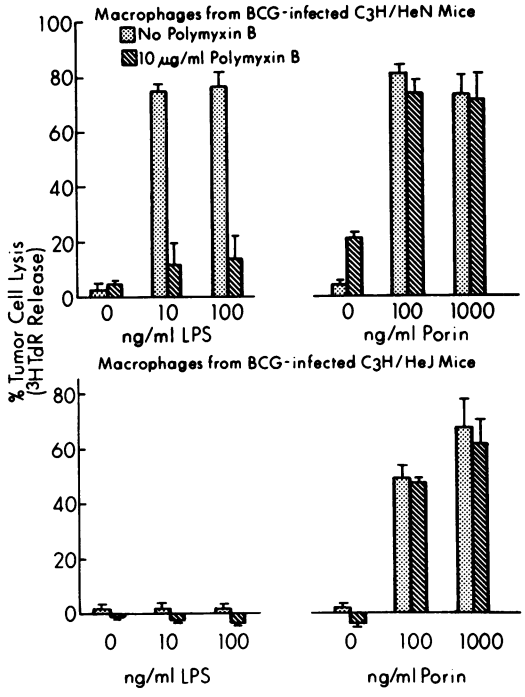

FIG. 4. Tumor cell killing by macrophages from BCG-infected C_3H/HeN or C_3H/HeJ mice in assays with either LPS or porins with or without polymyxin B sulfate. The bars represent the means plus one standard error of the mean of triplicate samples.

pletely activated in vivo ("nontumoricidal activated" [9, 25] or "primed" [17, 20]) or macrophages incompletely activated in vitro by lymphokines (9, 17, 18, 25). The bacterial factors serve as "triggers" for the killing process.

Endotoxin (LPS) is one of the bacterial factors that potently serves as a factor to cause differentiation of mouse macrophages to the tumoricidal state (5, 9, 17–20, 25). The LPS acts directly on the macrophages, not on intermediate lymphokine-producing cells (25). The lipid A portion of the LPS is responsible for the action on macrophages, and protein-free LPS does not activate macrophages from mice of the lipid A-unresponsive strain C_3H/HeJ (18, 25). However, if LPS containing lipid A-associated protein is used, macrophages from C_3H/HeJ mice are activated (5). This protein that remains attached to LPS obtained by mild extraction procedures also is mitogenic for B lymphocytes from lipid A-responsive and -unresponsive mouse strains and humans (7, 8, 12, 13). In this study, we have attempted to determine whether porins of the outer membrane of *S. typhimurium* would enhance the tumoricidal effect of macrophages.

The porins of the outer membrane of gram-negative organisms are known to have various biological effects. They were named because they enhance the transport of small molecules across artificial membranes or membranes of bacteria by forming apparent "pores" (4, 10, 15, 16, 23). Porins are apparently the same as the OmpF protein, the matrix protein, or protein Ia (4). In addition to these well-studied functions, the major outer membrane proteins (which contained porins) from *Pseudomonas aeruginosa* PAO1 were found to be mitogenic for splenocytes from C_3H/HeJ mice (3). Also, porins from *S. typhimurium* G30/C21 are mitogenic for human peripheral blood lymphocytes (R. W. Wheat, presented at the 80th Annual Meeting of the American Society for Microbiology, 1980). We propose here that porins of the outer membrane account for the biological activities of endotoxin-associated proteins in enhancing macrophage-mediated tumor cell killing, as well as in causing mitogenesis of lymphocytes described by others (3, 5, 8, 12). In the system studied here, the porins are very potent, with full activity at picomolar to nanomolar concentrations. It is possible that other minor components of the bacterial cell walls contaminating our preparations could have contributed to the findings we noted here, but we feel that this is unlikely.

The mechanisms by which porins alter the function of macrophages is not known. Alteration of the macrophage membrane, possibly induced by a ligand-receptor interaction, may initiate the appropriate change(s). Polyene antibiotics such as amphotericin B and nystatin form channels in cell membranes and enhance the tumoricidal capacity of macrophages in ways comparable to that described here for porins (2). It is possible that mechanistically, these operate in a comparable fashion.

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LITERATURE CITED

1. **Alexander, P., and R. Evans.** 1971. Endotoxin and double-stranded RNA render macrophages cytotoxic. Nature (New Biol.) **232**:76–78.
2. **Chapman, H. A., Jr., and J. B. Hibbs, Jr.** 1978. Modulation of macrophage tumoricidal capability by polyene antibiotics: support for membrane lipid as a regulatory determinant of macrophage function. Proc. Natl. Acad. Sci. **75**:4349–4353.
3. **Chen, Y.-H. U., R. E. Hancock, and R. I. Mishell.** 1980. Mitogenic effects of purified outer membrane proteins from *Pseudomonas aeruginosa*. Infect. Immun. **28**:178–184.
4. **DiRienzo, J. M., K. Nakamura, and M. Inouye.** 1978. The

outer membrane proteins of gram-negative bacteria: biosynthesis, assembly, and functions. Annu. Rev. Biochem. 47:481–532.
5. **Doe, W. F., S. T. Yang, D. C. Morrison, S. J. Betz, and P. M. Henson.** 1978. Macrophage stimulation by bacterial lipopolysaccharides. II. Evidence for differentiation signals delivered by lipid A and by a protein rich fraction of LPS. J. Exp. Med. **148**:557–568.
6. **Goldman, R. C., D. White, and L. Leive.** 1981. Identification of outer membrane proteins, including known lymphocyte mitogens, as the endotoxin protein of *Escherichia coli* 0111. J. Immunol. **127**:1290–1294.
7. **Goodman, G. W., and B. M. Sultzer.** 1979. Endotoxin protein is a mitogen and polyclonal activator of human B lymphocytes. J. Exp. Med. **149**:713–723.
8. **Goodman, G. W., and B. M. Sultzer.** 1979. Characterization of the chemical and physical properties of a novel B-lymphocyte activator, endotoxin protein. Infect. Immun. **24**:685–696.
9. **Hibbs, J. B., Jr., H. A. Chapman, Jr., and J. B. Weinberg.** 1978. The macrophage as an antineoplastic surveillance cell: biological perspectives. J. Reticuloendothel. Soc. **24**:549–570.
10. **Lugtenberg, B.** 1981. Composition and function of the outer membrane of *Escherichia coli*. Trends Biochem. Sci. **6**:262–266.
11. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alphen.** 1975. Electrophoretic resolution of the major outer membrane proteins of *Escherichia coli* K12 into four bands. FEBS Lett. **58**:254–258.
12. **Morrison, D. C., S. J. Betz, and D. M. Jacobs.** 1976. Isolation of a lipid A bound polypeptide responible for "LPS-initiated" mitogenesis of C_3H/HeJ spleen cells. J. Exp. Med. **144**:840–846.
13. **Morrison, D. C., and B. J. Curry.** 1979. The use of polymyxin B and C_3H/HeJ mouse spleen cells as criteria for endotoxin contamination. J. Immunol. Methods **27**:83–92.
14. **Morrison, D. C., and D. M. Jacobs.** 1976. Binding of Polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry **13**:813–818.
15. **Nakae, T.** 1976. Identification of the outer membrane protein of *E. coli* that produces transmembrane channels in reconstituted vesicle membranes. Biochem. Biophys. Res. Commun. **71**:877–883.
16. **Nakae, T.** 1976. Outer membrane of *Salmonella*. Isolation of protein complex that produces transmembrane channels. J. Biol. Chem. **251**:2176–2178.
17. **Pace, J. L., and S. W. Russell.** 1981. Activation of mouse macrophages for tumor cell killing. I. Quantitative analysis of interactions between lymphokine and lipopolysaccharide. J. Immunol. **126**:1863–1867.
18. **Ruco, L. P., and M. S. Meltzer.** 1978. Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxic activity requires completion of a sequence of short-lived intermediary reactions. J. Immunol. **121**:2035–2042.
19. **Ruco, L. P., M. S. Meltzer, and D. L. Rosenstreich.** 1978. Macrophage activation for tumor cytotoxicity: control of macrophage tumoricidal capacity by the LPS gene. J. Immunol. **121**:543–548.
20. **Russell, S. W., W. F. Doe, and A. J. McIntosh.** 1977. Functional characterization of a stable, noncytolytic stage of macrophage activation in tumors. J. Exp. Med. **146**:1511–1520.
21. **Schnaitman, C. A.** 1973. Outer membrane protein of *Escherichia coli*. I. Effect of preparative conditions on the migration of proteins in polyacrylamide gels. Arch. Biochem. Biophys. **157**:541–547.
22. **Takayama, K., E. Ribi, and J. L. Cantrell.** 1981. Isolation of a nontoxic lipid A fraction containing tumor regression activity. Cancer Res. **41**:2654–2657.
23. **Tokunaga, M., H. Tokunaga, Y. Okajima, and T. Nakae.** 1979. Characterization of porins from the outer membrane of *Salmonella typhimurium*. II. Physical properties of the functional oligomeric aggregates. Eur. J. Biochem. **95**:441–448.
24. **Weinberg, J. B.** 1982. Endotoxin contamination and in vitro monocyte-macrophage function: methods of detecting, detoxifying, and eliminating endotoxin, p. 139–154. *In* D. O. Adams (ed.), Methods for studying mononuclear phagocytes, Academic Press, Inc., New York.
25. **Weinberg, J. B., H. A. Chapman, Jr., and J. B. Hibbs, Jr.** 1978. Characterization of the effects of endotoxin on macrophage tumor cell killing. J. Immunol. **121**:72–80.
26. **Weinberg, J. B., and J. B. Hibbs, Jr.** 1980. In vitro measurement of activated macrophage-mediated cytotoxicity for tumor cells by morphologic observation, p. 345–355. *In* H. B. Herscowitz, H. T. Holden, and J. A. Bellanti (ed.), Manual of macrophage methodology: collection, characterization, and function, Marcel Dekker, New York.