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Mitogenic Effects of Purified Outer Membrane Proteins from *Pseudomonas aeruginosa*

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Three major outer membrane proteins from *Pseudomonas aeruginosa* PAO1 were purified and tested for their ability to stimulate resting murine lymphocytes to proliferate. It was demonstrated that picomole amounts of all three proteins were mitogenic for both intact and T-lymphocyte-depleted populations of spleen cells from C3H/HeJ mice. In contrast, they had no activity against either mature or immature thymocytes. Since the strain of mice used is unable to respond to lipopolysaccharide, we conclude that the three proteins are B-cell mitogens.

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Pseudomonas aeruginosa is an opportunistic pathogen which has become of increasing medical importance in recent years. With the exception of lipopolysaccharide (LPS), few studies have looked at the involvement of cell surface components in the pathogenesis of this organism. The recent demonstration that outer membrane proteins, like LPS, are excreted during normal cell growth (23) suggests that they could potentially interact with host cells. We have attempted to probe such interactions by measuring the ability of purified outer membrane proteins to stimulate mitogenically lymphocytes from C3H/HeJ mice which do not respond to LPS (29).

Many substances of bacterial and plant origin have been identified which can stimulate murine lymphocytes to proliferate. In particular, in recent years several components of the outer membranes of enteric bacteria were shown to be specific B-cell mitogens (2, 3, 11, 17, 23, 28, 29, 30). Recently, one of us reported the separation of outer and inner membranes of *P. aeruginosa* PAO1 (15) and the basic methodology for fractionation of the major outer membrane proteins of this strain (14). In this paper we demonstrate that three purified major outer membrane proteins of *P. aeruginosa* PAO1, proteins F, H, and I (see references 13 and 20 for nomenclature), are specific B-lymphocyte mitogens. Furthermore, protein F retains its mitogenicity under conditions known to cause partial denaturation of this protein.

MATERIALS AND METHODS

Bacterial strain. *P. aeruginosa* PAO1 was used. Cells were grown and the outer membrane fraction was obtained as previously described (15).

Purification of *P. aeruginosa* outer membrane proteins. The technique used to purify protein H, protein I (lipoprotein), and the F form of porin was similar to that previously described (15), with the following modifications: (i) the combined outer membrane protein was solubilized in Triton X-100 and ethylenediaminetetraacetic acid at a concentration of 20 mg of protein per ml, and (ii) the column buffer contained 2% rather than 0.1% Triton X-100. These modifications prevented the contamination of the lipoprotein with the major outer membrane protein G. The F* form of porin (which migrates more slowly in sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis than F) was obtained by phenol extraction as previously described (15). The phenol extraction also removed contaminating LPS from the preparation.

To remove the Triton X-100 detergent, the protein in each fraction was precipitated by addition of NaCl to 0.5 M and two volumes of ethanol. After overnight incubation at −20°C, the protein was pelleted by centrifugation (2,000 × *g*, 10 min), washed twice with ethanol, and resuspended in 0.3 ml of water. After a second cycle of ethanol precipitation, the pellet was washed twice with ethanol and once with sterile balanced salt solution (18). The pellet was resuspended in balanced salt solution to an appropriate concentration for biological testing. All protein suspensions were sonicated for 1 to 2 min before addition to cell cultures.

Preparation of purified LPS. LPS from *Salmonella typhimurium* LT2M1, a deep rough mutant, was purified by the method of Galanos et al. (9). LPS from *P. aeruginosa* PAO1 was prepared as previously described (15), except that the LPS suspension in 0.01 mM $MgSO_4$ was additionally treated with the following enzymes: pronase (0.2 mg/ml), pancreatic deoxyribonuclease (0.2 mg/ml), and pancreatic ribonuclease (0.2 mg/ml) for 24 h at 6°C. The enzyme-treated LPS was then centrifuged and resuspended in deionized water. These additional enzyme treatments resulted in LPS that was protein- and nucleic acid-free as determined by the lack of absorbance at 260 and 280 nm.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide slab gel electrophoresis was carried out with 17.5% acrylamide in the presence of 0.07 M

NaCl by the method of Anderson et al. (1) as modified by DeMartini and Inouye (8).

Lectins (mitogens). Twice-crystallized concanavalin A in saturated NaCl was purchased from Miles-Yeda, Ltd. (Kankakee, Ill.). Phytohemagglutinin was purchased from Burroughs-Wellcome Laboratories (Greenville, N.C.). Lyophilized, salt-free peanut agglutinin was purchased from Vector Laboratories (Burlingame, Calif.).

Mice. C3H/HeJ mice were bred in the mouse colony at the Department of Microbiology and Immunology, University of California, Berkeley. Mice of the same sex and age were used in individual experiments. Eight- to twelve-week-old mice were used in most experiments. Six-week-old female C3H/HeJ mice were the source of thymocytes.

T-cell depletion and preparation of mature and immature thymocytes. T-cell depletion of spleen cells by rabbit anti-mouse brain sera and guinea pig complement was performed by the method of Golub (10, 27). Mature and immature thymocytes were separated by the peanut agglutinin method described by Reisner et al. (6, 26).

Measurement of mitogenic responses. Spleen cells, or thymocytes, or both were cultured in 200-μl volumes at a concentration of 4×10^5 cells per well in Microtest II plates (Falcon, Oxnard, Calif.). Culture medium was RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, sodium pyruvate, L-glutamine, nonessential amino acids, penicillin, and streptomycin. It was used without serum. Mitogens and purified outer membrane proteins in the doses described were added at the time cultures were established. Cultures were pulsed at 48 h with 1 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) and harvested 18 to 24 h later with a multiple sample harvester (Otto Hiller Corp., Madison, Wis.). Radioactivity incorporated into acid-precipitable material was measured by scintillation counting in 3 ml of Omnifluor (New England Nuclear). Data are expressed as mean counts per minute of triplicate cultures of stimulated cells minus those of cells cultured without mitogens (background).

RESULTS

Isolation and characterization of the outer membrane proteins. Triton X-100–ethylenediaminetetraacetic acid-soluble proteins were fractionated on a diethylaminoethyl-Sephacel column by eluting with a salt gradient (Fig. 1). As observed previously (15), two major peaks were seen: one which eluted just after the void volume, and one which was eluted with approximately 0.2 M NaCl. After two cycles of ethanol precipitation, the individual fractions were analyzed by SDS-polyacrylamide slab gel electrophoresis, and three fractions were chosen for further study. Fractions 21, 27, and 127 were substantially purified preparations of protein H, protein I (lipoprotein), and the F form of porin, respectively (see Fig. 2).

In other studies (data not shown), protein H had the same mobility during electrophoresis as the previously described 17K major band of *P. aeruginosa* PAO1 (14, 15, 20). Furthermore, it was recently reported to contain lipid and therefore is a new lipoprotein (19, 22). Protein I was identified as the originally described *P. aeruginosa* lipoprotein by comparison with *P. aeruginosa* outer membranes, by its slightly lower mobility relative to *Escherichia coli* lipoprotein (Braun lipoprotein), and by its identical mobility to free-form lipoprotein of *P. aeruginosa* purified by another method (21). We have looked extensively for a bound-form lipoprotein in *P.*

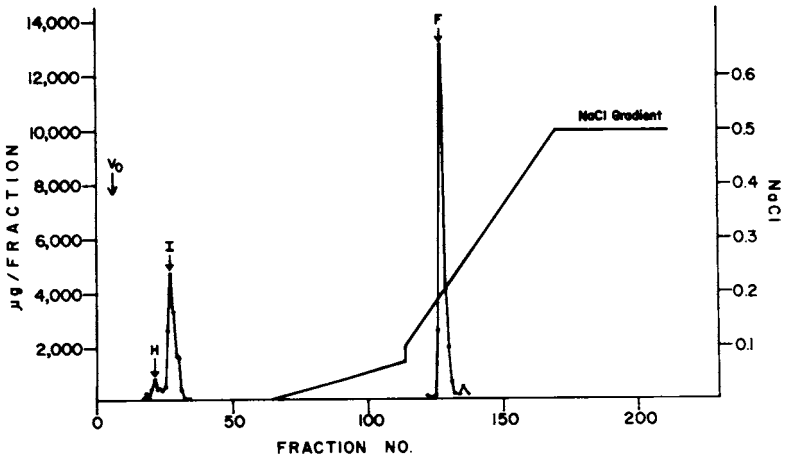

FIG. 1. *Diethylaminoethyl-Sephacel chromatographic separation of Triton X-100–ethylenediaminetetraacetic acid-soluble outer membrane proteins of Pseudomonas aeruginosa. Outer membrane proteins solubilized in Triton X-100–ethylenediaminetetraacetic acid were applied to a diethylaminoethyl-Sephacel column and eluted with a NaCl gradient as indicated. The running positions of purified proteins H, I (lipoprotein), and F (porin) are indicated. The protein concentration in each fraction was assayed by the procedure of Sandermann and Strominger (26a).*

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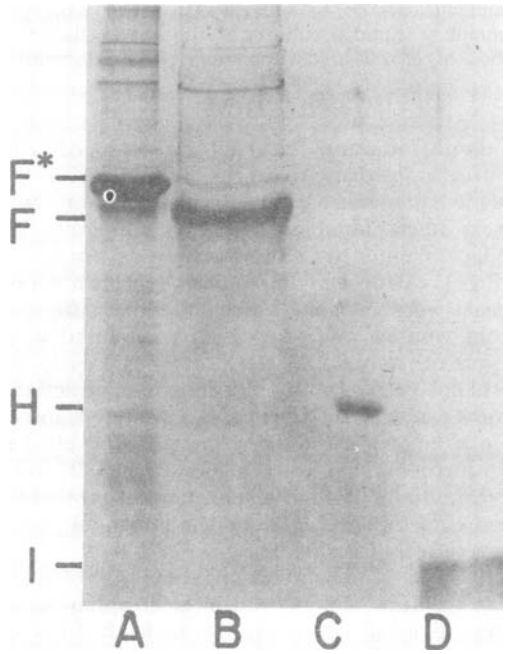

FIG. 2. *SDS-polyacrylamide gel electrophoretograms of purified P. aeruginosa outer membrane proteins. (A) phenol-extracted protein F (in the F* form); (B) (fraction 127, diethylaminoethyl-Sephacel chromatograph, Fig. 1), porin protein F; (C) (fraction 21, diethylaminoethyl-Sephacel chromatograph, Fig. 1), protein H; (D) (fraction 27, diethylaminoethyl-Sephacel chromatograph, Fig. 1), protein I (lipoprotein). The positions of the protein bands were determined by comparison with standard proteins (not shown).*

aeruginosa PAO1 and have found none. We therefore conclude that the material we are working with is the free-form lipoprotein. Even in heavily overloaded gels (15 to 20 μg of protein per gel), we were unable to detect cross-contamination of the protein H and protein I preparations with any of the other major outer membrane proteins including porin (protein F and F*) (Fig. 2; compare gels C and D with gels A and B).

The porin preparations used, although slightly contaminated (<5%) with minor protein bands, contained no detectable amounts of either protein H or protein I, even when very large amounts of protein (15 to 20 μg) were applied to the gels (Fig. 2A and B). The porin preparations were identified by the following criteria: (i) one of the preparations (F form) has been shown to have in vitro porin activity (14). Since ethanol precipitation irreversibly destroys in vitro porin activity (14), we were unable to show such activity for the porin in fraction 127 after ethanol precipitation. (ii) Phenol treatment converted the F form of porin into the F* form (Fig. 2A); and (iii) the F and F* bands comigrated on SDS-polyacrylamide gels with the equivalent bands of *P. aeruginosa* whole outer membranes (data not shown). No other *P. aeruginosa* outer membrane proteins run in an equivalent position (13).

Mitogenicity of *P. aeruginosa* protein H, protein I, and porin. Spleen cells from C3H/HeJ mice were cultured in the presence of varying doses of known mitogens (LPS, concanavalin A, and phytohemagglutinin) and the purified proteins H and I (Fig. 3). Both proteins H and I are strongly mitogenic. The LPS controls were negative, demonstrating that our strain of mice has retained the inherited defect in response to LPS (30) and extending this observation to *P. aeruginosa* LPS. Proteins H and I were also mitogenic for the spleen cell subpopulation remaining after depletion of T lymphocytes, as shown in Table 1. The effectiveness of T-cell removal is indicated by the failure of these cells to respond to the plant mitogens concanavalin

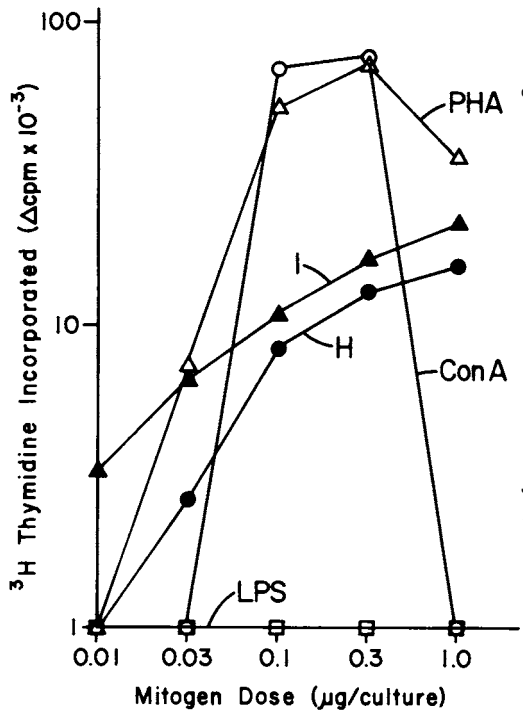

FIG. 3. *Mitogenic effect of outer membrane proteins from P. aeruginosa. Spleen cells were cultured and the proliferative responses measured as described (see text). Triplicate cultures contained the indicated amounts (micrograms per culture) of protein I (lipoprotein), protein H, phytohemagglutinin (PHA), concanavalin A (Con A), and LPS of P. aeruginosa and S. typhimurium LT2M1. Results are the mean counts of triplicate cultures of each experimental group minus that of unstimulated (background) cultures (3,300 cpm/culture).*

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A and phytohemagglutinin.

Similar results were obtained with the F and F* forms of porin as shown in Fig. 4 and 5. None

TABLE 1. *Mitogenic effects of P. aeruginosa outer membrane proteins on unseparated and T-depleted spleen cells of C3H/HeJ mice*[a]

Mitogen	Dose (μg/ml)	Whole spleen cells (Δcpm)	T-depleted cells (Δcpm)
Protein H	1.0	16,990	16,420
	0.3	15,310	15,670
	0.1	7,860	7,490
	0.03	1,310	2,440
Protein I	1.0	16,520	27,690
	0.3	16,330	22,240
	0.1	10,730	14,410
	0.03	5,390	6,950
PHA	1.0	22,360	140
	0.3	66,910	960
ConA	0.1	49,840	10
	0.03	<0	210

[a] T-depleted spleen cells were prepared as described (see text). Cells were cultured and pulsed as described in the text. Data (Δ cpm) are the mean of triplicate experimental cultures minus the mean of triplicate unstimulated (background) cultures. The background cpm of the whole spleen cells was 3,740 cpm and that of the T-depleted cells was 4,280 cpm. C3H/HeJ cells of either whole or T-depleted cells did not respond to LPS stimulation over a wide dose range tested (3 to 0.003 μg, data not shown). The LPSs were from *S. typhimurium* LT2M1 and *P. aeruginosa*. PHA, Phytohemagglutinin; ConA, concanavalin A.

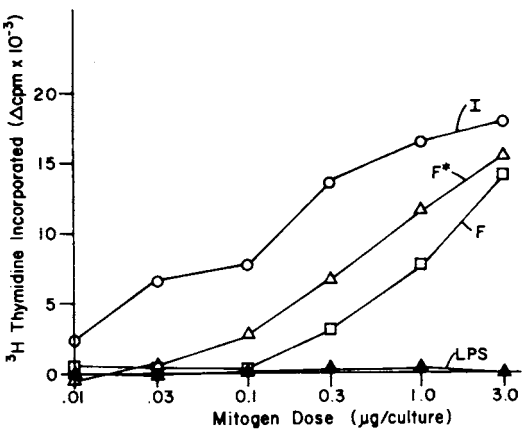

FIG. 4. *Mitogenic effect of P. aeruginosa porin on whole spleen cells. Normal spleen cells from C3H/HeJ mice were cultured, and the proliferative responses were measured as described (see text). The spleen cells were cultured with the indicated doses (micrograms per culture) of porin (F), LPS-depleted, phenol-extracted porin (F*), protein I, and LPS from S. typhimurium LT-2M1. The data are Δ cpm (mean counts of triplicate cultures of each experimental group minus that of background cultures, 1,500 cpm/culture).*

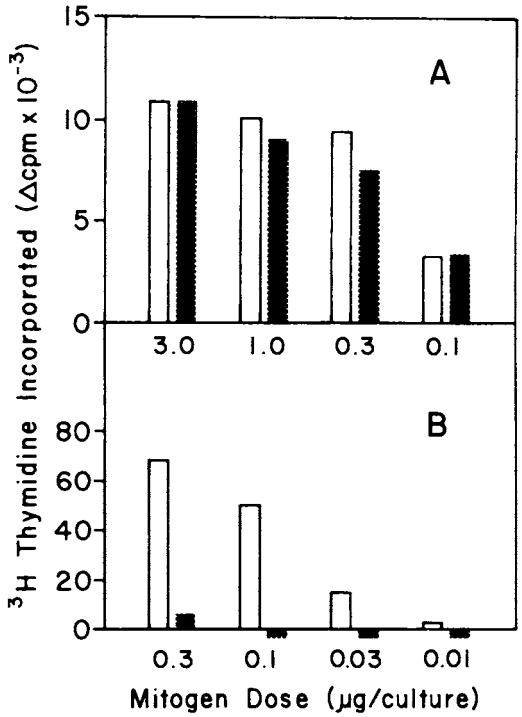

FIG. 5. *Failure of T-cell depletion to affect the mitogenicity of the F* form of porin for C3H/HeJ spleen cells. The mitogenic effects of porin protein F* (A) and phytohemagglutinin (B) on normal spleen cells (⊓) and spleen cells treated with rabbit anti-mouse brain serum to deplete T-cell populations (▨) were tested as described (see text). The data are Δ cpm (mean counts of triplicate cultures of each experimental group minus that of background cultures, 2,620 cpm/culture). T-cell depletion also abolished the mitogenic effect of concanavalin A but had no effect on stimulation by protein I (lipoprotein). LPS from P. aeruginosa and S. typhimurium LT2M1 had no effect on either cell population.*

of the outer membrane proteins was mitogenic for unseparated thymocytes or for either the mature or immature subpopulations of thymocytes (Table 2). In these experiments the mature subpopulation of thymocytes responded to phytohemagglutinin, confirming the effectiveness of the separation procedure (26). The data from Fig. 3 and 4 are replotted in Fig. 6, showing proliferation in response to molar concentrations of the outer membrane proteins. When compared in this manner, the three proteins exhibit similar dose effects.

DISCUSSION

The data reported in this paper indicate that the three major outer membrane proteins of *P. aeruginosa* are specific mitogens for murine lymphocytes. The mitogenic effects were ex-

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TABLE 2. *Mitogenic effect of P. aeruginosa outer membrane molecules on thymocytes of C3H/HeJ mice*[a]

Mitogen	Dose (μg/ml)	Unseparated thymocytes (Δcpm)	Mature thymocytes (Δcpm)	Immature thymocytes (Δcpm)
Porin (F)	3.0	80	<BG	<BG
	1.0	<BG	<BG	<BG
	0.3	<BG	<BG	<BG
Protein H	1.0	190	<BG	<BG
	0.3	<BG	<BG	<BG
	0.1	<BG	<BG	<BG
Protein I	3.0	<BG	570	50
	1.0	50	190	<BG
	0.3	<BG	<BG	<BG
PHA	1.0	4,820	83,480	190
	0.3	4,690	54,890	1,690
ConA	0.1	10,000	70,360	5,920

[a] Cells were cultured and proliferative responses were measured as described in the text. Data are mean counts per minute of triplicate cultures of each experimental group minus the mean counts per minute of the background (BG) (unstimulated) cultures. Background counts per minute of unseparated thymocytes, mature T cells, and immature T cells were 390, 890, and 210 respectively. Mature and immature thymocytes were obtained by agglutination of whole thymocytes with peanut agglutinin as described by Reisner et al. (26). PHA, Phytohemagglutinin; ConA, concanavalin A.

pressed by B lymphocytes as the data show. Thymocytes did not respond to the mitogens. Since the mature thymocytes have similar biological properties as purified T cells, it is unlikely that T lymphocytes respond mitogenically to these proteins. We found that peripheral T cells purified by removal of B cells with immune absorption (panning) (16) are also nonresponsive to these mitogens (Chen and Lucas, unpublished data). Furthermore, it is highly unlikely that contaminating LPS could account for the mitogenic effects since C3H/HeJ mice are genetically incapable of responding to LPS (11, 29, 30), a point that was verified in the present study with LPS from both *S. typhimurium* and *P. aeruginosa*.

Do these mitogenic effects depend on specific recognition by receptors on the B cells, or is perturbation of the B-cell membrane (caused by the hydrophobic nature of the outer membrane proteins) sufficient to initiate the process? Our results suggest that neither hydrophobic interactions nor nonspecific perturbation is involved, since phenol denaturation of porin to the F* form had no inhibitory effect on its ability to stimulate a mitogenic response (Fig. 4), although it destroyed its ability to reconstitute hydrophilic pores in vesicles (R. Hancock, unpublished data). In addition, Triton X-100-extracted membrane proteins from murine red cells did not trigger proliferative responses (Y. U. Chen, unpublished data). Moreover, it is well established that hydrophobicity is not an essential property of murine B-cell mitogens. Dextran sulfate (5) and polymeric peptidoglycans (4, 7) are examples of hydrophilic substances that induce murine B cells to proliferate. In view of the range of molecules which can stimulate murine B cells, it seems likely that specific interactions at the cell surface give rise to the mitogenic responses.

Of the mitogens demonstrated here, proteins H and I, although lipoproteins (19, 21, 22), differ substantially from the Braun lipoprotein of *E. coli*. In particular, protein I, although similar in molecular size and peptidoglycan association (21; Hancock and Carey, manuscript in preparation), has a very different amino acid and lipid composition (21). Since all three lipoproteins are B-cell mitogens, it would be interesting to determine whether the murine structures involved in the interaction with each of these are different. Similarly, the porins of *E. coli* and *P. aeruginosa* have many clearly distinct properties (13, 14), but are both mitogenic.

This paper provides the first demonstration that *P. aeruginosa* outer membrane proteins can interact with mammalian cells. Since outer membrane proteins, like LPS, can be both surface exposed and shed into their environment, their role in the pathogenesis of *P. aeruginosa* deserves further study. One might ask what advantage there is in having B-lymphocyte mitogens in the bacterial outer membrane during infections by *P. aeruginosa*. From the experiments reported here, it would seem that B lymphocytes have evolved surface components which can recognize and interact with foreign

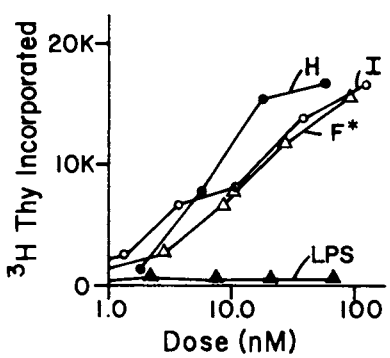

FIG. 6. *The mitogenic data of P. aeruginosa outer membrane molecules from Fig. 3 and 4 are plotted as a function of the molar amounts used.*

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proteins resembling the outer membrane proteins studied in this paper. If these proteins provided essential functions for the bacterial cell, it is unlikely that the bacteria could simply delete them by mutation: the porin protein, for instance, is required for trans-outer membrane permeability (14). It is not clear whether nonspecific stimulation of B lymphocytes provides much advantage to the host since the amount of relevant specific antibody which results may be trivial. Another possibility is that the outer membrane proteins interact with other types of mammalian inflammatory cells (for example, macrophages) and that it is these interactions which provide effective protection for the host.

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