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Antibody-Producing Cell Responses to an Isolated Outer Membrane Protein and to Complexes of This Antigen with Lipopolysaccharide or with Vesicles of Phospholipids from *Proteus mirabilis*

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Antibody-producing cell responses of mice to a protein isolated from the outer membrane of *Proteus mirabilis* were typical of the responses to a thymus-dependent antigen. The immunoglobulin G antibody-producing cell responses to the protein were increased after administration of the antigen complexed with either lipopolysaccharide or with vesicles of phospholipids extracted from *P. mirabilis*. The protein in turn significantly increased the immune response to lipopolysaccharide and also converted this response from predominantly immunoglobulin M to predominantly immunoglobulin G.

A few major characteristic proteins are present in relatively large amounts in the outer membrane of gram-negative bacteria (21). In this regard, they represent likely candidates for target antigens involved in specific immunological reactions of a host infected with these organisms. Although several reports in the literature have indicated that some of these proteins have defined mitogenic activity for B cells (2, 3, 5, 12, 18) and that immune responses to cell surface proteins may play a role in protection against experimental infections with gram-negative bacteria (6, 15, 19, 27), the actual characteristics of the immune responses to outer membrane proteins have not been studied extensively.

In the native outer membrane structure, proteins are complexed with other major membrane components, such as lipopolysaccharide (LPS) and phospholipids. What contributions these components make to the immune responses directed against outer membrane proteins is also a question that has not been dealt with sufficiently.

In the present investigation we have studied the quantitative and the qualitative antibody-producing cell responses of mice to a major protein (M_r 39,000) isolated from the outer membrane of *Proteus mirabilis*. We have also compared the responses to the isolated M_r 39,000 protein with the responses to complexes of this antigen with LPS or with vesicles of phospholipids extracted from *P. mirabilis*.

We chose the M_r 39,000 protein as a model outer membrane protein antigen in this investigation for several reasons. After isolation and purification, the lyophilized protein is readily soluble by brief sonication in aqueous solvents and reacts strongly with antibodies directed against *P. mirabilis* cell walls, i.e., antibodies to the protein in its native form (4). Furthermore, this protein is undoubtedly important for the structure and function of the outer membrane. It forms pores that allow the penetration of hydrophilic molecules through reconstituted model membrane vesicles of protein and phospholipids from *P. mirabilis* and can protect model membranes from disruption by detergents (22).

MATERIALS AND METHODS

Bacterial strain. The bacterial strain used for this investigation was *P. mirabilis* 19 from this laboratory (H. H. Martin). It was cultivated as described previously (17).

Isolation of protein. The M_r 39,000 protein was isolated from purified cell walls of *P. mirabilis* 19 essentially according to a method described previously (21, 22) except that an additional gel filtration step was carried out to remove traces of LPS detectable by gas-liquid chromatography of fatty acids (9). Briefly, 10 g (wet weight) of purified cell walls (22) was extracted with 1% sodium deoxycholate in 200 ml of a buffer consisting of 0.2 M NaCl, 1 mM ethylenediaminetetraacetate, and 10 mM tris(hydroxymethyl)-aminomethane hydrochloride, pH 8.0, at 37°C for 45 min (21). Insoluble material was sedimented by centrifugation at 100,000 × *g* for 1 h in an ultracentrifuge. The supernatant was concentrated by negative-pressure dialysis against the same buffer and filtered on a column (2.5 by 100 cm) of Sephadex G-200 in the same buffer as above except that it contained 0.25%, instead of 1%, sodium deoxycholate. Fractions containing the M_r 39,000 protein peak, which was well separated from other outer membrane proteins (22), were pooled, concentrated, and subjected to a second gel filtration

as above. The product obtained after the final gel filtration was concentrated as above against the column buffer and dialyzed extensively against 5 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 8.0, and finally against double-distilled water to remove all traces of detergent (21, 22).

Extraction of LPS and phospholipids. LPS I of *P. mirabilis* 19 was extracted from whole cells with phenol-water and purified according to Gmeiner (8). All samples were electrodialyzed (7). Phospholipids were extracted from whole cells of *P. mirabilis* 19 as previously described (9) and contained approximately 80% phosphatidylethanolamine, 12% phosphatidylglycerol, 4% diphosphatidylglycerol, 1% lysophospholipids, and 2% "neutral lipids" (9).

Analytical methods. Protein content was determined by a modification of the Lowry method (16). Amounts of phospholipids and LPS were determined by gas-liquid chromatography of fatty acids (9). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins was carried out as previously described (22).

Immunization of mice. For immunization of mice, 1.25 mg of lyophilized M_r 39,000 protein was mixed with 1.0 ml of buffer [0.1 M NaCl–0.02 M tris(hydroxymethyl)aminomethane hydrochloride, pH 7.3] alone, with 2.5 mg of LPS in 1.0 ml of buffer, or with 37.5 μmol of dried phospholipids suspended in 1.0 ml of buffer. Also, 2.5 mg of LPS alone was suspended in 1.0 ml of buffer. All four samples were sonicated for 4 min in an ice bath under a stream of nitrogen by using a Branson Model S-125 apparatus (Branson Instruments Co., Danbury, Conn.) at power level 1 with the microtip. The solutions were diluted to give the correct concentrations (see below), and 0.2-ml amounts were injected intraperitoneally into white, female, specific-pathogen-free NMRI mice weighing 22 to 25 g (Wiga, Sulzfeld, West Germany). A primary injection was given on day 0, and a secondary injection containing the same amount of material was given on day 14.

Assay of antibody-producing cells. The immunoglobulin M (IgM) and the IgG antibody-producing cell responses were measured in the hemolytic plaque test, using a modification of the microscope slide assay (20) as previously described (25). Briefly, 2.0 ml of a 10% suspension of sheep erythrocytes (SRBC) (Deutsche bioMerieux, Nürtingen, West Germany) sensitized with antigen (see below) was added to 2.0 ml of a 1:2 dilution of lyophilized guinea pig serum (Serva, Heidelberg, West Germany) reconstituted in Hanks balanced salt solution (20). Then 0.1 ml of this mixture was added to 0.5 ml of 0.6% agarose (Serva) in Hanks balanced salt solution in a water bath at 42°C. Immediately thereafter, 0.1 ml of a dilution of spleen cells in Hanks balanced salt solution from two mice immunized with a given antigen was added, and the mixture was spread on a microscope slide. Slides were incubated for 1.5 to 2 h in a moist chamber at 37°C. For each spleen cell dilution, three parallel slides were prepared. For the measurement of indirect plaques, 20 μl of a dilution of rabbit anti-mouse immunoglobulin prepared as previously described (25) was added to the test system directly before the addition of spleen cells. A dilution of serum was used which gave an optimal number of indirect plaques with the least amount of inhibition of IgM plaques. The small amount of inhibition observed (approximately 7 to 10%) did not affect the results significantly, even in the case of possible earlier detection of IgG antibody-producing cells.

Responses to the M_r 39,000 protein were measured against the protein coupled to sheep erythrocytes by a modification of the chromium chloride method (11), using aged $CrCl_3$ solution (10). Briefly, 600 μg of protein, solubilized by sonication in 2.0 ml of 0.9% NaCl, plus 1.8 ml of an aged 0.0133% $CrCl_3$ solution in 0.9% NaCl were added simultaneously with stirring to 0.4 ml of a 50% solution of SRBC in 0.9% NaCl for 15 min at room temperature. This mixture was incubated for 15 min at 37°C and subsequently washed in phosphate-buffered saline (10). The SRBC were suspended in Hanks balanced salt solution (20) for use in the plaque test. This amount of protein coupled to SRBC gave optimal numbers of plaques in our system. The M_r 39,000 protein coupled to tannin-treated SRBC (23) gave similar results, but twice as much protein was needed to produce the same optimal numbers of plaques. Responses to *P. mirabilis* 19 LPS were measured against LPS coupled to SRBC as previously described (25).

RESULTS AND DISCUSSION

The responses of mice to various amounts (12.5, 20, 40, and 100 μg) of the M_r 39,000 protein alone are presented in Table 1. The numbers of IgM and IgG antibody-producing cells were measured on days 19 and 20 (days 5 and 6 after the second injection). The results indicate that 40 μg of the protein induced an optimal immune response. The responses to the protein at all doses tested after the second stimulus were mainly of the IgG type.

TABLE 1. *Immune responses of mice to various amounts of M_r 39,000 protein isolated from the outer membrane of P. mirabilis*[a]

Protein injected (μg)	Antibody type	Response (PFC/10^6 spleen cells)[b] on day:	
		19	20
12.5	IgM	39	29
	IgG	237	190
20	IgM	76	56
	IgG	402	325
40	IgM	100	60
	IgG	1,180	714
100	IgM	83	52
	IgG	891	454

[a] Mice were given a primary injection on day 0 and a secondary injection with the same amount of protein on day 14.

[b] Values represent geometric means of responses measured in two separate experiments.

A comparison of the IgM and the IgG antibody-producing cell responses of mice to (i) 12.5 μg of M_r 39,000 protein alone, (ii) 12.5 μg of M_r 39,000 protein plus 300 μg of phospholipid vesicles, (iii) 12.5 μg of M_r 39,000 protein plus 25 μg of LPS, or (iv) 25 μg of LPS alone is presented in Table 2. In all cases, a primary injection was given on day 0 and a secondary injection containing the same amount of material was given on day 14. To see possible effects of membrane components on the immune responses to the M_r 39,000 protein, we purposely chose 12.5 μg of the antigen for this experiment because this amount gave a minimal response (Table 1). The amount of LPS (25 μg) was chosen for the same reason (25). Also, 300 μg of phospholipids was used because this amount produced the greatest effects in another system tested previously with LPS as antigen (25).

The number of cells producing IgM antibody to the M_r 39,000 protein after immunization with the protein alone reached a maximum on day 4 after the first injection. IgG-producing cells were first detected on day 6, and the number of these cells increased slowly up to day 14, the time of secondary stimulation. The second injection had no apparent effect on either the strength or the kinetics of the IgM responses to this protein. In contrast, the number of IgG antibody-producing cells was greatly increased after the second injection and reached a peak on day 19 (day 5 after the second stimulus). These responses to the M_r 39,000 protein were typical of responses to a thymus-dependent antigen (24). Only a very small number of IgM or IgG antibody-producing cells measured against LPS-SRBC after immunization with the isolated protein alone were detected during the course of immunization. However, since the numbers of these cells were slightly higher than those that could be attributed to a nonspecific response to LPS (25), this might indicate that the protein preparation was still contaminated with amounts of LPS not detectable by gas chromatographic analyses of fatty acids. It should be noted that the M_r 39,000 protein and *P. mirabilis* 19 LPS do not cross-react serologically (4).

Administration of the same amount of M_r 39,000 protein incorporated into 300 μg of bacterial membrane phospholipid vesicles caused an increase in the IgG antibody-producing cell responses which was particularly evident after the second injection (from 237 plaque-forming cells [PFC] per 10^6 spleen cells to 761 PFC/10^6 spleen cells on day 19). The significance of this increase can best be judged by examination of

TABLE 2. *Immune responses of mice to M_r 39,000 protein, phospholipid vesicles, and LPS from P. mirabilis*[a]

Immunogen	Measured against	Antibody type	Response (PFC/10^6 spleen cells)[b] on day:									
			0	3	4	5	6	14	17	18	19	20
M_r 39,000 protein	M_r 39,000 protein-SRBC	IgM	0.05	19	68	56	36	4	21	42	39	29
		IgG	0	0	0	0	1	10	80	140	237	190
	LPS-SRBC	IgM	0.26	1	6	1	0	1	ND[c]	9	13	10
		IgG	0	0	0	0	0	2	ND	12	30	15
M_r 39,000 protein-phospholipids	M_r 39,000 protein-SRBC	IgM	0.13	17	69	51	40	10	38	79	62	39
		IgG	0	0	0	1	2	23	151	433	761	353
M_r 39,000 protein-LPS	M_r 39,000 protein-SRBC	IgM	0.07	58	188	130	75	37	140	310	190	132
		IgG	0	0	0	5	4	37	500	1,100	2,200	1,210
	LPS-SRBC	IgM	0.04	34	65	41	29	6	51	154	79	65
		IgG	0	0	0	1	2	24	350	632	945	390
LPS	LPS-SRBC	IgM	0.15	ND	20	ND	ND	4	ND	31	11	8
		IgG	0	ND	0	ND	ND	0	ND	9	11	8

[a] Responses to (i) 12.5 μg of M_r 39,000 protein, (ii) 12.5 μg of M_r 39,000 protein–300 μg of phospholipid vesicles, (iii) 12.5 μg of M_r 39,000 protein–25 μg of LPS, or (iv) 25 μg of LPS were measured against either M_r 39,000 protein-SRBC or LPS-SRBC. Mice received a primary injection on day 0 and a secondary injection with the same amount of material on day 14.

[b] Values represent the geometric means of PFC measurements in two separate experiments.

[c] ND, Not determined.

the actual values obtained in two separate experiments. For immunization with the protein antigen alone, the numbers of IgG antibody-producing cells on day 19 in two experiments were 254 and 221 PFC/10^6 spleen cells, whereas the numbers of these cells after immunization with the protein incorporated into phospholipid vesicles were 840 and 690 PFC/10^6 spleen cells. Thus, phospholipids induced approximately a threefold increase in the IgG responses to the M_r 39,000 protein in both experiments.

The adjuvant effect of certain defined phospholipid species has been clearly demonstrated for such antigens as diphtheria toxoid (1), serum albumin (13, 28), bovine gamma globulin (28), and lipid A (26). Also, we have recently shown that bacterial membrane phospholipids in their native composition increased the immune responses to *P. mirabilis* LPS (25).

The adjuvant properties of LPS have been well documented in the literature. Accordingly, 25 μg of *P. mirabilis* LPS, which induced only a minimal response in mice (Table 2), had a profound effect on the strength of the responses to 12.5 μg of M_r 39,000 protein when administered together with the protein. Once again, the increase in the IgG response after the second injection was most pronounced.

Conversely, the M_r 39,000 protein in this LPS-protein mixture was extremely effective in changing not only the strength but also the type of immune response to LPS (from a predominantly IgM to a predominantly IgG response). We also observed the same change in the type of response to *P. mirabilis* 19 LPS when that antigen was incorporated into phospholipid vesicles (25). Similarly, Hepper et al. (14) reported a switch in mice from a predominantly IgM to an IgG response to *Salmonella enteritidis* LPS when it was contaminated with endotoxin protein (12), which is apparently a mixture of several outer membrane proteins (27). Whether the same mechanisms are involved in the alteration of the response to LPS by phospholipids and proteins will have to be determined.

The values in Table 2 represent the geometric means of PFC measurements taken from two separate experiments. To give a better indication of the significance of differences in the responses to the various immunogens investigated, the geometric mean values ± standard error in two to four separate experiments measured at the height of the primary response (day 4) as well as at the height of the secondary responses (day 18 for IgM and day 19 for IgG) are presented in Table 3.

It has been well documented in the literature that LPS is a polyclonal activator and that for this reason responses to LPS, especially in the early days after immunization, are rather nonspecific. To test the extent of this nonspecific response, we measured the responses to unsensitized SRBC after immunization with LPS alone or with LPS-M_r 39,000 protein mixtures (data not shown). At the height of the primary response to LPS alone, the numbers of PFC measured against unsensitized SRBC were 50 to 60% of the numbers of PFC measured against

TABLE 3. *Geometric means ± standard errors of PFC values obtained after immunization of mice with M_r 39,000 protein, phospholipid vesicles, LPS from P. mirabilis*[a]

Immunogen	Measured against	Antibody type	Response (PFC/10^6 spleen cells)[b] on day:		
			4	18	19
M_r 39,000 protein	M_r 39,000 protein-SRBC	IgM	68 ± 1.00 (2)	43 ± 1.87 (3)	39 ± 4.12 (4)
		IgG	0 (2)	140 ± 20.09 (3)	232 ± 5.57 (4)
M_r 39,000 protein-phospholipids	M_r 39,000 protein-SRBC	IgM	68 ± 10.55 (2)	79 ± 12.82 (3)	64 ± 9.06 (4)
		IgG	0 (2)	433 ± 71.39 (3)	727 ± 95.36 (4)
M_r 39,000 protein-LPS	M_r 39,000 protein-SRBC	IgM	188 ± 21.03 (2)	339 ± 35.08 (3)	171 ± 27.76 (4)
		IgG	0 (2)	1,091 ± 197.01 (3)	2,207 ± 278.70 (4)
	LPS-SRBC	IgM	65 ± 12.59 (2)	154 ± 15.04 (2)	79 ± 10.52 (2)
		IgG	0 (2)	633 ± 55.04 (2)	945 ± 0.71 (2)
LPS	LPS-SRBC	IgM	20 ± 1.00 (2)	31 ± 2.55 (2)	11 ± 1.00 (2)
		IgG	0 (2)	9 ± 0.71 (2)	11 ± 0.71 (2)

[a] Mice were immunized with (i) 12.5 μg of M_r 39,000 protein, (ii) 12.5 μg of M_r 39,000 protein–300 μg of phospholipid vesicles, (iii) 12.5 μg of M_r 39,000 protein–25 μg of LPS, or (iv) 25 μg of LPS, and responses were measured against either M_r 39,000 protein-SRBC or LPS-SRBC. Mice received a primary injection on day 0 and a secondary injection on day 14.

[b] Geometric mean ± standard error of the mean; the number of separate experiments is given in parentheses.

LPS. At the height of the secondary response to LPS alone, the numbers of PFC measured against unsensitized SRBC were 20 to 25% of those measured against LPS. The responses to LPS after immunization with the LPS-M_r 39,000 protein mixture were slightly more specific. In this case, the numbers of PFC measured against unsensitized SRBC were 20 to 40% of the numbers measured against LPS at the height of the primary response and 7 to 15% of those measured at the height of the secondary response. In all cases, PFC measured against unsensitized SRBC after immunization with LPS or with LPS-M_r 39,000 protein mixtures were restricted to the IgM type.

In general, our results demonstrate that complex formation between various membrane components can have pronounced effects on both the quantitative and the qualitative immune responses to antigens in the outer membrane of gram-negative bacteria. Studies are now underway in our laboratory to test whether other outer membrane proteins isolated from *P. mirabilis*, as well as nonmembrane proteins, are as effective as the M_r 39,000 protein in augmenting the immune response and in altering the type of response to LPS and whether this is a characteristic phenomenon for different types of LPS in different strains of mice. We are also investigating possible mechanisms involved in the alteration of the responses to LPS by phospholipids and outer membrane proteins.

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