Neisseria meningitidis Lipopolysaccharide Modulates the Specific Humoral Immune Response to Neisserial Porins but Has No Effect on Porin-Induced Upregulation of Costimulatory Ligand B7-2

NAVNEET BHASIN, YU HO, AND LEE M. WETZLER*

Division of Infectious Diseases, Boston University School of Medicine and Evans Biomedical Research Center, Boston, Massachusetts

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In this study we examined the role of LPS in modulating the immune response to meningococcal PorA and PorB. Experiments were designed to evaluate the immunogenicity of PorA and PorB by immunizing mice with live meningococci or with purified PorA and PorB. The effect of LPS on the humoral response to the meningococcal porins was assessed by comparing the antiporin antibody levels in mice immunized with meningococcal strain H44/76 and its LPS-negative mutant. OMCs from either meningococcal strain were able to increase the surface expression of the costimulatory ligand B7-2 on B cells from either murine strain. Consistent with previously reported findings, LPS does not significantly affect the ability of neisserial porins to induce the costimulatory ligand B7-2.

In humans, meningococcal disease is caused by Neisseria meningitidis belonging to a number of capsular polysaccharide serogroups, including A, B, C, Y, and W-135. The presently available vaccine against N. meningitidis is a quadrivalent vaccine composed of capsular polysaccharides from serogroups A, C, Y, and W-135 (18). This preparation does not provide protection against serogroup B strains, which cause approximately one-third of the total cases in the United States and 45 to 80% or more of the cases in Europe (6, 27). Capsular polysaccharide from serogroup B strains mimics the human neuronal cell adhesion molecule (10); hence, the use of group B capsule in a vaccine introduces the risk of autoimmunity. Also, the group B capsular polysaccharide is poorly immunogenic, as indicated by the limited antibody response to the capsule seen in patients’ sera after natural infections by the strain (13). Consequently, a number of groups are currently researching the potential of various surface-exposed outer membrane proteins (OMPs) and lipopolysaccharide (LPS) as vaccine candidates (1, 3, 8, 25). LPS, while being reactogenic in the host, has been explored by Steeghs et al. (29) for its potential as an adjuvant (when present in trace amounts) in vaccine preparations.

The potential of neisserial porins PorA and PorB as vaccine candidates and immune adjuvants has been explored by various investigators. Porins, which constitute about 60% of the bacterial surface proteins (4), have been shown to be immunogenic without the addition of exogenous adjuvants (36, 39). Moreover, neisserial porin preparations are known to augment the humoral response to otherwise poorly immunogenic substances, e.g., peptides (20, 21), and induce T-cell-dependent immune response for normally T-cell-independent antigens, e.g., polysaccharides (9, 11, 12, 22, 35). In addition, neisserial porins have been used as immunologic adjuvants in a number of varied cases, including noncovalent complexes with malarial peptides (20, 21), group A streptococcal M protein (19), influenza virus hemagglutinin (19), and covalent complexes with multiple bacterial capsular polysaccharides (2, 9, 22, 35). In addition to their use as vaccine adjuvants, the use of neisserial porins as specific antinesiserial vaccine candidates has also been investigated (5, 36, 39). Despite these efforts, the effect that LPS may have on the immunogenicity of the major outer membrane porins (PorA and PorB) has never been adequately explored.
and has amounts of OMPs similar to those possessed by the wild-type parent (28). To further define the role of LPS in modulating the immune response to PorA and PorB, the immunizations were done in an LPS-responsive murine strain, C3H/HeOuJ, and an LPS-hyposensitive murine strain, C3H/HeJ (24). The role of meningococcal LPS in modulating the potential immunostimulatory effect of PorB was evaluated by measuring the ability of outer membrane complexes (OMCs) prepared from either the wild-type strain or its LPS- mutant, to induce B7-2 expression in naïve murine B cells in vitro.

MATERIALS AND METHODS

Mice. Two isogenic strains of female mice at 6 weeks of age were used for the study and were obtained from the Jackson Laboratory (Bar Harbor, Maine). These were the LPS-responsive strain C3H/HeOuJ and the LPS-hyposensitive strain C3H/HeJ. The LPS hyposensitiveness of C3H/HeJ mice is attributed to a defect in the cytoplasmic tail of the LPS signal transducer TLR4 (24).

Bacteria. N. meningitidis serogroup B strain H44/76 (B:15:P1.7,16), originally isolated from a patient with meningococcal bacteremia and meningitis from Norway, was used in the study. Its recently isolated LPS-negative isogenic mutant strain was a gift from Peter van der Ley, National Institute of Public Health and the Environment, Bilthoven, The Netherlands (28). This LPS-negative mutant strain is totally devoid of LPS and has amounts of OMPs similar to those held by the wild-type parent (28). The bacteria were grown in gonococcal liquid medium (23) at 37°C with shaking to an optical density at 600 nm of 0.1 to 0.2. The bacteria were then washed with phosphate-buffered saline (PBS) once and were resuspended in PBS at concentrations of 2 × 10^7 to 2 × 10^8 cells per ml.

Porins and LPS. PorA and PorB were purified (37) from H44/76 mutant strains H44/76ΔAα (15) and H44/76ΔA4 (14), respectively. These mutant strains allowed purification of the desired porins without contamination from other OMPs. The porins were purified by detergent extraction and column chromatography as described previously (37). Polyacrylamide gel electrophoresis and silver staining (30) (data not shown) demonstrated negligible contamination by other proteins and LPS. Purified LPS from strain H44/76 was kindly provided by Michael Apicella (University of Iowa Medical Center, Iowa City, Iowa).

Immunization of mice. Mice (n = 4) were immunized subcutaneously with 2 × 10^6 or 2 × 10^7 bacteria of either the wild-type strain H44/76 or its LPS-negative isogenic mutant in 100 µl of PBS. Control mice were given 100 µl of PBS. The mice were immunized at days 0 and 21, and sera were obtained every 7 days, ending on day 42. In a separate experiment, following the same immunization regimen, mice were given 10 µg of purified PorA or PorB formed into proteosomes (36).

ELISA. The concentrations of anti-PorA- and anti-PorB-specific immunoglobulin G (IgG) present in the immune and nonimmune murine sera were determined by quantitative enzyme-linked immunosorbent assay (ELISA) as described previously (36). Briefly, flat-bottomed 96-well plates were coated overnight at room temperature with 100 µl of a 2-µg/ml solution of purified PorA or PorB in carbonate-bicarbonate buffer (pH 9.6). IgG concentrations were measured for each individual murine serum. The ELISA plate contents were developed by using the chromogenic substrate for alkaline phosphatase (Sigma Diagnostics; St. Louis, Mo.). The concentrations of porin-specific IgG antibodies in serum samples were calculated by extrapolation from standard IgG log-log-transformed titration curves run in parallel. Anti-LPS IgG titers were determined by performing an ELISA similar to that described above, except that the plates were coated with 100 µl of a 10-µg/ml solution of purified LPS in sodium barbital buffer (pH 4.6).

Preparation of OMCs. N. meningitidis cultures were grown to an optical density at 600 nm of 0.8. The cells were suspended in PBS, and OMCs were prepared according to the method described by van der Ley et al. (31). Briefly, meningococci were inactivated at 56°C for 30 min and were lysed by resuspending in 10 mM Tris (pH 8.0) followed by sonication. Cell debris was removed by centrifuging at 10,000 × g for 30 min. OMCs were obtained from the supernatant by subsequent centrifugation at 50,000 × g. The OMCs were then treated with 1% sodium laurylsarcosinate to remove cytoplasmic membrane proteins bound to the OMCs. The protein content of the OMCs was determined by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co.) as per the manufacturer’s protocol. Bovine serum albumin was used as a standard.

In vitro stimulation of naïve B cells. Naïve B cells were isolated from the spleens of C3H/HeJ and C3H/HeOuJ mice according to the standard protocol (38). Briefly, a single-cell suspension of the spleen tissue was obtained by passing through a fine wire mesh, followed by the lysis of red blood cells (0.15 M Tris-buffered NH₄Cl). T cells were removed by T-cell-specific antibody-mediated complement lysis. Other nonspecific cells were removed by passage through a Sephadex G-10 column followed by a Ficoll gradient separation. The B cells, at a concentration of 5 × 10⁵ cells/ml, were incubated for 24 h at 37°C and 5% CO₂ in the presence of 10 µg of OMCs (quantitated with respect to their protein content)/ml prepared from either strain H44/76 or its LPS- mutant. Negative (incubated with medium alone) and positive (incubated with purified PorA and PorB proteins formed into proteosomes at a concentration of 10 µg/ml) controls were run in parallel. The cells were then probed with rat anti-mouse antibodies and were conjugated with fluorescein isothiocyanate to determine the level of cell surface expression of B7-1 and B7-2 cell surface markers by flow cytometry analysis (7). All the antibody conjugates for flow cytometry analysis were obtained from Caltag Laboratories (Burlingame, Calif.).

RESULTS

Immunogenicity of strain H44/76 and its isogenic LPS-negative mutant. Anti-PorA, anti-PorB, and anti-LPS IgG levels were measured by ELISA in sera from C3H/HeJ and C3H/HeOuJ mice after immunization with meningococcal strain H44/76 or its LPS-negative mutant. The anti-PorB IgG levels were greater in C3H/HeOuJ mice than in C3H/HeJ mice (Table 1). The wild-type strain H44/76 evoked an anti-PorB response about 30 to 40 times higher than that evoked by the LPS-negative mutant strain in the C3H/HeOuJ mice (Table 1). The difference in the anti-PorB response was, however, not as pronounced when the LPS-hyposensitive mice were immu-
nized with either bacterial strain. In addition, as elaborated in Table 1, even though the anti-PorB response was lower in C3H/HeJ mice than in C3H/HeOuJ mice, it was still significant, reaching a maximum concentration of about 6 μg/ml. Furthermore, the LPS-negative bacterial strain evoked a very similar anti-PorB response in the two murine strains. The maximum anti-PorA IgG level attained in mice sera was 2.2 μg/ml (Table 2). Interestingly, a detectable anti-PorA response was obtained only in mice immunized with 10^7 CFU of the LPS-negative mutant strain per dose and was comparable in the two strains of mice.

When intact meningococci were given, PorB was more immunogenic than PorA, as indicated by anti-PorB IgG levels that were higher than anti-PorA IgG levels (Tables 1 and 2). Since the anti-PorB response of mice might be modulated by the presence of LPS on the bacterial surface and by the ability of mice to immunologically respond to LPS, anti-LPS IgG levels were determined. An anti-LPS IgG response was seen only in the LPS-responsive C3H/HeOuJ mice immunized with the higher dose (10^7 CFU/dose) of wild-type strain H44/76 (data not shown). No detectable anti-LPS IgG antibodies were seen in sera from C3H/HeOuJ mice immunized with the LPS-negative bacterial strain or in the sera from the LPS-unresponsive strain of mice immunized with either bacterial strain.

**Immunogenicity of purified PorA and PorB.** As mentioned in the preceding paragraph, both PorA and PorB were found to be immunogenic when the mice were immunized with live cultures of *N. meningitidis*, albeit to different extents. Additionally, variations seen in the IgG response due to modulation by LPS (either at the host level or at the inoculum level) were different for the two proteins. In order to determine if these differences were due to the relative abundance or availability of the two proteins to the immune system or due to their inherently different immunogenicity, mice were coimmunized with purified PorA or PorB formed into proteosomes (36) and the levels of anti-PorA and anti-PorB IgG were determined by ELISA. Anti-PorA IgG levels were comparable to anti-PorB IgG levels in sera from the two strains of mice, C3H/HeJ and C3H/HeOuJ, when they were immunized with purified PorA or PorB (Table 3). However, the anti-PorA and anti-PorB IgG levels in sera from C3H/HeOuJ mice immunized with either porin were about 10-fold higher than in sera from C3H/HeJ mice. These levels were about twofold higher than the maximum IgG concentrations achieved against PorB in C3H/HeOuJ mice immunized with 2 × 10^7 CFU of live cultures of the wild-type bacterial strain per dose. To determine if anti-LPS IgG, which could have been generated against any trace amounts of LPS contaminating the porin preparations, was induced, an ELISA against purified LPS was carried out. No anti-LPS IgG antibodies were detected in sera from either of the two mice strains immunized with either of the purified porins (data not shown).

**Immunostimulatory activity of OMCs from strain H44/76 and its isogenic LPS-negative mutant.** PorB from *N. meningitidis* is known to have immunostimulatory activity. PorB can activate B cells and induce the surface expression of the costimulatory B7-2 molecules (38). This effect is related to the porins' adjuvanticity in conjugate vaccines (22). In order to determine if the presence of LPS can modulate this function of PorB, purified splenic naïve B cells were incubated in vitro with OMCs prepared from either of the bacterial strains (wild-type H44/76 and its LPS-negative mutant). The level of expression of B7-2 cell surface molecules was determined in B cells isolated from the LPS-responsive mice strain C3H/HeOuJ. In parallel, B cells prepared from the LPS-hyporesponsive C3H/HeJ mice were also studied. The B cells, after stimulation, were also probed for the presence of another costimulatory cell surface molecule, B7-1. The expression of B7-2 on B cells from

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**Table 2. Anti-PorA response (μg of IgG/ml) generated against wild-type *N. meningitidis* strain H44/76 and its LPS-negative mutant in LPS-responsive C3H/HeOuJ mice and LPS-unresponsive C3H/HeJ mice**

<table>
<thead>
<tr>
<th>Mouse type and day of testing</th>
<th>Results (± SD) for different regimens</th>
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<tbody>
<tr>
<td></td>
<td>PBS W6 W7 M6 M7</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>BDL BDL BDL 0.03 ± 0.03 0.26 ± 0.27</td>
</tr>
<tr>
<td>28</td>
<td>BDL BDL BDL 0.07 ± 0.07 2.24 ± 2.36</td>
</tr>
<tr>
<td>35</td>
<td>BDL BDL BDL 0.06 ± 0.08 0.57 ± 0.51</td>
</tr>
<tr>
<td>42</td>
<td>BDL BDL 0.05 ± 0.04 0.21 ± 0.39 1.71 ± 1.22</td>
</tr>
<tr>
<td>C3H/HeOuJ</td>
<td>BDL BDL BDL 0.23 ± 0.27</td>
</tr>
<tr>
<td>28</td>
<td>BDL BDL BDL 0.07 ± 0.08 0.57 ± 0.51</td>
</tr>
<tr>
<td>35</td>
<td>BDL BDL 0.05 ± 0.04 0.21 ± 0.39 1.71 ± 1.22</td>
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a W6, 2 × 10^6 CFU of wild-type H44/76 per dose; W7, 2 × 10^7 CFU of wild-type H44/76 per dose; M6, 2 × 10^6 CFU of LPS-negative mutant per dose; M7, 2 × 10^7 CFU of LPS-negative mutant per dose; BDL, below detectable limit. For end-group, n = 4. Mice were immunized on days 0 and 21.

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**Table 3. Anti-PorA and anti-PorB responses (in μg of IgG/ml) generated against purified PorA and PorB in LPS-responsive C3H/HeOuJ mice and LPS-unresponsive C3H/HeJ mice**

<table>
<thead>
<tr>
<th>Mouse type and day of testing</th>
<th>Results (± SD) for PBS or porin treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>PBS PorA PorB</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>BDL 11.6 ± 7.27 14.15 ± 13.85</td>
</tr>
<tr>
<td>28</td>
<td>BDL 10.14 ± 6.38 18.07 ± 15.73</td>
</tr>
<tr>
<td>35</td>
<td>BDL 9.9 ± 3.7 17.02 ± 14.79</td>
</tr>
<tr>
<td>42</td>
<td>BDL 7.27 ± 14.79 13.85 ± 13.85</td>
</tr>
<tr>
<td>C3H/HeOuJ</td>
<td>BDL 72.03 ± 49.02 62.67 ± 35.26</td>
</tr>
<tr>
<td>28</td>
<td>BDL 114.21 ± 70.68 196 ± 122.33</td>
</tr>
<tr>
<td>35</td>
<td>BDL 130.62 ± 56.47 109.2 ± 35.86</td>
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</table>

a BDL, below detectable limit. Mice (n = 4 per group) were immunized on days 0 and 21. For immunizations 10 μg of purified porins (in the form of proteosomes)/ml was used.
C3H/HeJ mice was induced to similar extents by incubation with OMCs prepared from either bacterial strain (Fig. 1A). However, B7-2 on B cells from C3H/HeOuJ mice was induced to slightly higher levels by OMCs from the wild-type strain H44/76 than by OMCs from the LPS\(^-\) mutant strain (Fig. 1A). The levels of B7-1 cell surface marker, however, did not show a significant difference after stimulation with OMCs from the two bacterial strains in B cells isolated from either murine strain (Fig. 1B). These results are consistent with previous data presented after use of purified porins (38), which was repeated as control for this study. The surface expression of B7-2 on B cells from either strain of mice, stimulated with 10 \(\mu\)g of purified PorB protein/ml in the form of proteosomes, was elevated to a level similar to that obtained after stimulation with OMCs prepared from the two bacterial strains. Purified PorA (10 \(\mu\)g/ml) did not induce B7-2 surface expression to the levels induced by PorB in B cells isolated from mice belonging to either of the two murine strains. Levels of expression of B7-1 cell surface marker on B cells isolated from the two murine strains remained unchanged upon stimulation by either PorA or PorB (data not shown).

**DISCUSSION**

The purpose of this study was to examine the immunogenicity of PorA and PorB meningococcal porins in conjunction with LPS in order to evaluate their potential as vaccine candidates and to determine the necessity of LPS for the induction of antiporin immune responses. Both the porins and LPS have been investigated individually as plausible antineisserial vaccine candidates in the past (5, 25, 32, 37, 38). Results from the current study revealed that the presence of LPS in conjunction with PorB on the bacterial cell surface affects the anti-PorB humoral immune response mounted by mice. However, it is important to note that the presence of LPS is not essential for evoking an anti-Por response, as demonstrated by the elevated levels of anti-PorB IgG in mice immunized with the LPS-negative mutant (>3 \(\mu\)g/ml) (Table 2). This is consistent with the initial observation made in *Salmonella enterica* serovar Typhosa regarding the ability of LPS to function as an adjuvant (16). An immunomodulatory role of LPS on the anti-PorA humoral response evoked by OMCs from *N. meningitidis* was observed by Steeghs et al. (29), especially with regard to the...
generation of bactericidal anti-PorA antibodies. However, they did not investigate the anti-PorB response, accounting for their conclusion that LPS is essential for an antiporin humoral response, which we have demonstrated is not necessarily the case.

Interestingly, the anti-PorB response observed in this study is much higher than the anti-PorA response in sera from mice immunized with whole bacteria, which is a pattern similar to that observed in patients with meningococcal disease (14, 15, 17). On the other hand, the anti-PorA and anti-PorB IgG levels obtained in mice immunized with purified porins were very similar. These results could be explained by the presence of higher amounts of PorB than of PorA on the bacterial cell surface (26) and/or by PorB’s possible immunodominance over PorA when the two proteins are present together on intact meningococci. However, when outer membrane vesicle vaccines are used, the immunogenicity of PorA has been shown to be much greater than that of PorB (33, 34), possibly due to an alteration in the structure of PorB during the synthesis of the outer membrane vesicle vaccine, diminishing its immunogenicity.

The augmentation of the antiporin immune response by LPS appears not to be due to an alteration in the stoichiometric presentation of the porins. This was demonstrated by the following findings: (i) anti-PorB IgG levels induced by either the wild-type or LPS-negative meningococcal strain were similar in the LPS hyporesponsive C3H/HeJ mice, and (ii) the anti-PorB wild-type or LPS-negative meningococcal strain were similar in lowFold differences: (i) anti-PorB IgG levels induced by either the wild-type H44/76 strain were 10-fold higher than that of PorB (33, 34), possibly due to an alteration in the stoichiometric presentation of the porins. This was demonstrated by the following findings: (i) anti-PorB IgG levels induced by either the wild-type or LPS-negative meningococcal strain were similar in the LPS hyporesponsive C3H/HeJ mice, and (ii) the anti-PorB wild-type or LPS-negative meningococcal strain were similar in lowFold differences.

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