Outer Membrane Vesicles Induce Immune Responses to Virulence Proteins and Protect against Colonization by Enterotoxigenic Escherichia coli

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Enterotoxigenic Escherichia coli (ETEC) strains are a heterogeneous group of pathogens that produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. Collectively, these pathogens are responsible for hundreds of thousands of deaths annually in developing countries, particularly in children under the age of 5 years. The heterogeneity of previously investigated molecular targets and the lack of complete sustained protection afforded by antitoxin immunity have impeded progress to date toward a broadly protective vaccine. Many pathogens, including ETEC, have the capacity to form outer membrane vesicles (OMV), which often contain one or more virulence proteins. Prompted by recent studies that identified several immunogenic virulence proteins in outer membrane vesicles of ETEC, we sought to examine the immunogenicity and protective efficacy of these structures in a murine model of infection. Here we demonstrate that immunization with OMV impairs ETEC colonization of the small intestine and stimulates antibodies that recognize the heat-labile toxin and two additional putative virulence proteins, the EtpA adhesin and CexE. Similar to earlier studies with EtpA, vaccination with LT alone also inhibited intestinal colonization. Together, these findings suggest that OMV could be exploited to deliver protective antigens relevant to development of ETEC vaccines.

Enterotoxigenic Escherichia coli (ETEC) strains are a diverse group of diarrheal pathogens that share the ability to colonize the small intestine, where they produce heat-labile (LT) and/or heat-stable (ST) enterotoxins. These organisms are a leading cause of diarrhea in developing countries, where they are responsible for an estimated 300,000 to 500,000 deaths per year, mostly in young children (33).

Unfortunately, there is no broadly protective vaccine available to prevent these infections (5). Although plasmid-encoded colonization factors (CFs) have been a major focus of vaccine development efforts for ETEC to date, the underlying plasticity of E. coli genomes (25) and the antigenic heterogeneity of CFs (5) have impeded vaccine strategies based solely on these antigens. Additional approaches that incorporate highly conserved molecular targets are therefore needed to focus future endeavors toward the design of effective vaccines.

Recent immunoproteomic studies (27) identified a number of known and putative ETEC virulence factors associated with outer membrane vesicles (OMV), small spherical “blebs” released from the surfaces of E. coli and other Gram-negative bacteria (20). Given the established association of LT with OMV (16) and the potential utility of vesicle-based vaccines (4, 14, 31), we elected to examine the immunogenicity and protective efficacy of ETEC-derived OMV in an animal model of ETEC infection.

MATERIALS AND METHODS

Bacterial strains and plasmids. A complete list of bacterial strains and recombinant expression plasmids used in these experiments is included in Table 1.

Preparation of outer membrane vesicles. Vesicles were prepared from culture supernatants of either strain H10407 or jfl412, a previously described flagellin-negative derivative of H10407 (27, 30). Briefly, cultures of H10407 and jfl412 were grown overnight in Luria broth (without antibiotics and supplemented with kanamycin at 25 μg/ml, respectively). Cultures were centrifuged at 5,000 × g for 10 min to pellet bacteria, and supernatants were clarified through a 0.45-μm vacuum filter (Millipore). The filtrate was then centrifuged at 100,000 × g to pellet vesicles. The resulting pellet was then resuspended in 500 μl of phosphate-buffered saline (PBS). These OMV preparations were used to immunize mice. To obtain OMV in isolation from smaller membrane fragments, additional purification was carried out using density gradient centrifugation (Optiprep) as previously described (16).

Preparation of recombinant proteins. Recombinant EtpA was prepared as previously described (11). Briefly, cultures of E. coli Top10 carrying plasmids pJJ1017 and pJJ1030 grown in Luria broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) were induced with 0.02% arabinose, and the supernatant proteins were concentrated by ultrafiltration through a 100,000-MWCO filter (Millipore). Polyhistidine-tagged EtpA was then purified by metal affinity chromatography. CexE-His6 was prepared by metal affinity chromatography as previously noted (24). Briefly, strain BL21(DE3)/pET22b(+) containing the plasmid pET22b(+) containing the plasmid pGPM1034 (24) was cultured aerobically in LB Miller medium supplemented with 0.2% (wt/vol) glucose and 50 μg/ml kanamycin. Following induction with IPTG (isopropyl-β-D-thiogalactopyranoside), cells were harvested, suspended in IMAC buffer (30 mM Tris-Cl [pH 7.4], 200 mM KCl, 20 mM imidazole), and lysed, and the soluble fraction was recovered following centrifugation. Nickel-Sepharose column chromatography was then used to prepare CexE-His6, over a linear imidazole gradient (20 to 250 mM). CexE-His6 was further purified and then exchanged into PBS buffer (pH 7.4) by size exclusion chromatography (Superdex 200 10/300 GL column; GE Healthcare). Heat-labile toxin was obtained from List Biological Laboratories, Campbell, CA.

Polyclonal antisera. Antisera used in these studies were prepared as described previously (24, 30) by preabsorption against AAEC191A, an afimbriate E. coli K-12 mutant, and with an E. coli lysozyme column (Pierce) to remove cross-reactive antibodies. Polyclonal rabbit antisera raised against the A and B subunits of LT were supplied by John Clements of Tulane University.
TABLE 1. Bacterial strains and recombinant plasmids used in this study

| Strain or plasmid | Description | Reference or source |
|------------------|-------------|-------------------|
| H10407           | Wild-type ETEC strain; serotype O78:H11; CFA/I LT+ STh+ STp+ | 9                  |
| jf1412           | fliC* mutant of H10407; nonmotile; Km* | 30                 |
| Top10            | F* merA Δ(mrr-hsdRMS-mcrBC) 680lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL Strr endA1 nupG Invitrogen |
| j876            | ΔlacZYA derivative of H10407 | 8                 |
| BL21(DE3)       | F* ompT hsdSde(rk - mK -) dcm gal (DE3) |                |

Plasmids

- pJL017  
  - etpBA cloned into pBAD/Myc-HisA, with etpA in frame with Myc and His, coding regions 28
- pLJ030  
  - etpC gene cloned into pACYC184, Cm* 30
- pGPM1034  
  - cexE gene cloned into pET33b in frame with His* coding region 24

Transmission electron microscopy. Five microliters of each vesicle suspension, prepared as described above, was spotted on a nickel grid and incubated at room temperature for 30 min. The grid was then quickly blotted and fixed with a freshly prepared solution of 2% formaldehyde-0.5% glutaraldehyde for 15 min. Grids were then washed once with PBS before negative staining with 2% phosphotungstic acid.

Immunization with OMV and challenge with ETEC. All experimental procedures involving animals were reviewed and approved by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. Animals were housed, cared for, and used in compliance with the Guide for the Care and Use of Laboratory Animals (23a) in an AAALAC International-accredited program. CD-1 mice (n = 10) were immunized intranasally with OMV protein suspensions containing 20 µg of total protein in a maximum volume of 20 µl on days 0, 14, and 28. Control mice (n = 10) received by gavage. Approximately 24 h after administration of bacteria, small intestines were harvested and the colonizing ETEC were recovered by plating saponin lysates onto Luria agar plates containing kanamycin (25 µg/ml).

Immunoblotting was performed on proteins transferred to nitrocellulose. Recombinant proteins were diluted to a final concentration of 4 µg/ml in 0.1 M NaHCO3 buffer, pH 8.6, and used to coat enzyme-linked immunosorbent assay (ELISA) plates overnight at 4°C. These were washed with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and blocked for 1 h at 37°C with 1% bovine serum albumin (BSA) in TBS-T (Blocker; Pierce). Dilutions of mouse antisera were prepared in TBS-T containing 1% BSA. To determine fecal antibody concentrations, 5 or 6 fecal pellets were collected from each mouse, housed briefly in an individual enclosure. Pellets were then resuspended in 1.5 ml of extraction buffer containing Tris (10 mM), NaCl (100 mM), Tween 20 (0.05%), and sodium azide (5 mM), pH 7.4. Fecal extracts were then incubated overnight in ELISA plates containing target antigens at 4°C, followed by incubation for 1 h at 37°C. For sera containing primary antibody, incubations were performed for 1 h at 37°C. Plates were washed with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (anti-IgA, -IgM, and -IgG) at a final concentration of 1:5,000. Testing for fecal IgA was performed with HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz) at a final concentration of 1:5,000. After 1 h at 37°C, plates were washed and developed with TMB peroxidase substrate (3,3′,5,5′-tetramethylbenzidine). Absorbance measurements were obtained at a wavelength of 620 nm, with data acquired at 1-min intervals, to allow kinetic measurement of absorbance as previously described (30, 32). Absorbance values were expressed as Vmax values, in milliliters/minute.

RESULTS

Outer membrane vesicles contain multiple ETEC virulence proteins. Encouraged by earlier studies in our laboratory demonstrating that several known or putative ETEC virulence proteins are associated with outer membrane vesicles (27) and by the apparent ability of OMV immunization to protect against other enteric pathogens (4, 31), we prepared ETEC-derived OMV for use in mouse immunization (Fig. 1a). We found, however, that OMV prepared from wild-type (wt) ETEC strain H10407 were heavily contaminated with flagellar fragments that persisted even after attempted gradient centrifugation. As a result, flagellin (FliC), the major molecular structural subunit of flagella, appeared as the predominant protein in these OMV preparations (Fig. 1a, inset). Because we had previously shown that flagellin itself is a

FIG. 1. OMV preparations contain ETEC virulence proteins. (a) OMV preparation. The transmission electron microscopy image (magnification, ×100,000) shows OMV prepared from the flagellin deletion strain jf1412. The inset shows a one-dimensional SDS-PAGE gel image of vesicle preparations from wt ETEC strain H10407 and the ΔfliC strain jf1412, showing the predominant flagellin band in the wt preparation (arrow). Five micrometers of protein was loaded in each lane. (b) Immunoblots of OMV preparations obtained using antisera specific for EtpA, LT-A, LT-B, and CexE. Negative-control (−) blots obtained using preimmune rabbit sera are shown at the left of each group.
protective antigen in the murine model of ETEC infection (29) and we wished to examine the effect of OMV vaccination in isolation, vesicles for these studies were prepared from jf1412, a flagellin-negative mutant of H10407 (Table 1). As predicted from earlier immunoproteomic experiments (27), these flagellin-free OMV contained several potential virulence proteins, including LT, EtpA, and CexE, as demonstrated by immunoblotting using specific antisera (Fig. 1b).

Immunization with OMV elicits immune responses to multiple proteins. Following vaccination with OMV, mice mounted immune responses to multiple proteins (Fig. 2a). To evaluate immune responses to proteins shown here and in earlier proteomic studies to be vesicle associated, we used the respective purified recombinant proteins LT, EtpA, and CexE in immunoblotting and ELISA studies. These studies revealed that OMV vaccination resulted in significant serum (Fig. 2b) and fecal (Fig. 2c) antibody responses to each of these antigens, confirming results of previous immunoproteomic studies conducted with convalescent-phase antisera from naturally infected patients and animals experimentally challenged with ETEC H10407 (27).

Ideally, an ETEC vaccine destined primarily for developing countries would be inexpensive to produce and manufacture. Therefore, we chose to examine responses to vaccination with OMV preparations produced in a fashion similar to that recently described for Vibrio cholerae (31) rather than with more-refined OMV preparations requiring additional purification steps (16). Although LT is known to be associated largely with OMV, we carried out additional gradient purification steps to isolate highly purified OMV fractions (Fig. 3a) and to verify the association of EtpA and CexE with OMV. We identified the putative virulence proteins EtpA and CexE in these purified OMV fractions by using specific antisera (Fig. 3b), suggesting that these immunogenic proteins, like LT, are associated at least in part with OMV.

Protective efficacy of OMV vaccination in a murine model of ETEC infection. Mice immunized with OMV were subsequently challenged with ETEC to assess the potential role of these structures in preventing ETEC infection. Compared to mice immunized with the vehicle alone (PBS), OMV-immunized mice had significantly fewer ($P = 0.002$) colonizing organisms in the small intestine (Fig. 4). Collectively, the studies included here suggest that vaccination with outer membrane-derived or associated antigens in the form of vesicles would be a feasible option to protect against ETEC infections.

Vaccination with LT protects against intestinal colonization with ETEC. Heat-labile toxin has previously been shown to promote epithelial cell adhesion (17) and intestinal colonization (1, 3) by ETEC. Given the association of LT with OMV and the protective effect of these vesicles, we examined whether immunization with LT alone would afford protection against colonization. As anticipated, intranasal immunization with LT yielded robust antibody responses in both serum (Fig. 5a) and stool (Fig. 5b and c). In addition, mice vaccinated with LT were significantly protected against subsequent intestinal colonization compared to control mice (Fig. 5d). These results are similar to those previously demonstrated for EtpA (28, 29),
another antigen which is associated at least in part with vesicles (27). Together, these data suggest that OMV contain at least two protective antigens and that these structures potentially offer an effective strategy for immunization against ETEC infections.

**DISCUSSION**

The development of enteric vaccines, including those designed to prevent ETEC infections, faces a number of important challenges that must be overcome. The ideal ETEC vaccine would elicit broad sustained mucosal immunity and would be inexpensive to manufacture, without the need for complicated and expensive technology, in order to facilitate production of vaccines in developing countries. Until very recently, most ETEC vaccine development efforts have focused on a limited number of antigens, namely, the heat-labile toxin (LT) and plasmid-encoded colonization factors (CFs). Unfortunately, the lack of complete protection afforded by LT and the expanding heterogeneity among CF antigens have hampered the development of a broadly protective vaccine. This has prompted the investigation of additional candidate ETEC antigens.

Recent immunoproteomic studies revealed that the immune response to ETEC is quite complicated and involves the recognition of many proteins, including secreted antigens, and outer membrane structures (27). Interestingly, these studies identified a number of antigens associated with outer membrane vesicles, including LT, EtpA, and CexE. Because we recently demonstrated that novel protein antigens such as EtpA could potentially be exploited for development of an ETEC vaccine (28, 29), the present study was undertaken to investigate whether vaccination with OMV would induce immune responses to these proteins and achieve some degree of protection in a murine model of ETEC infection.

These efforts were also prompted by prior experiences with OMV-based vaccines for *Neisseria meningitidis*, as wild-type OMV preparations have been used extensively to control epidemics caused by serogroup B infections (10, 14). Moreover, recent animal experiments suggested that OMV preparations could likewise be useful in preventing infections caused by *V. cholerae* (31) and other enteric pathogens.

Here we focused specifically on three OMV-associated antigens, namely, LT, for which there is considerable evidence for its association with vesicles (15, 18, 19, 20), as well as EtpA and CexE, identified in prior immunoproteomic studies of wild-type ETEC H10407 vesicles (27). While both EtpA and CexE were previously identified as secreted proteins of ETEC, these studies seem to indicate that at least some portion of these proteins may remain associated with one or more elements of the outer membrane.

The studies included here were performed with OMV obtained from a flagellin-negative mutant of ETEC H10407, for several reasons. First, we found that even after gradient separation of OMV into distinct fractions, we could not separate OMV from abundant flagellar fragments that copurified in these fractions. Flagellin itself is highly protective against ETEC in animal models (29), and therefore the use of a flagellin-negative background permitted us to examine the relative contributions of other antigens. Likewise, because EtpA also binds to conserved regions of flagellin monomers, purification...
of flagellin-free OMV permitted the identification of EtpA specifically associated with OMV.

While OMV are capable of delivering associated LT to epithelial cells (18), the role of either EtpA or CexE in this process has not yet been explored. Nevertheless, the OMV association of both the EtpA adhesin and CexE, a plasmid-encoded putative virulence protein of unknown function, should prompt additional study of the involvement of these antigens in toxin delivery.

Emerging evidence suggests that bacteria have distinct mechanisms for sorting virulence proteins specifically to OMV (12). Packaging of virulence factors, including LT, into vesicles is well described and has been demonstrated more recently in Gram-positive organisms, including Bacillus anthracis, in which vesicles contain multiple virulence factors (26).

The finding of potential ETEC virulence factors in addition to LT in association with OMV suggests that these structures could serve as a suitable platform for delivery of multiple protective antigens. In addition, the possibility of engineering ETEC OMV to deliver heterologous molecules (6, 22) that may not normally be vesicle associated could be an attractive option in the development of a broadly protective vaccine that incorporates multiple target antigens. Such a strategy is currently being examined by combining recombinant MmB proteins with Neisseria OMV (10). Likewise, it is conceivable that strains could be engineered to express mutant forms of LT incapable of inducing diarrhea or LT-ST fusions that could potentially induce neutralizing antibody against both toxins (21).

While our studies suggest that ETEC-derived OMV could induce responses to multiple antigens, including known and putative virulence proteins, significant efforts will be required to explore their true utility in preventing infections by these remarkably heterogeneous pathogens. The immunizations were performed in the absence of additional adjuvants, relying simply on the inherent immunogenicity of these structures that include significant amounts of LT, a potent mucosacudant. The intranasal route of immunization chosen here is not optimally suited for protection against ETEC, for a number of reasons. First, it is likely that other routes of vaccine administration would more effectively engender small intestinal immune responses that are most relevant to preventing ETEC infection (13). In addition, the association of intranasal LT administration with facial palsy (23) would likely preclude administration by this route (7), and it is likely that oral administration in some form would significantly enhance intestinal immune responses (2, 13). Nonetheless, these preliminary experiments are intended as a first step to suggest that an OMV-based strategy could be exploited as an avenue toward an inexpensive, broadly protective ETEC vaccine.

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