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Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles

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INTRODUCTION

Outer membrane (OM) vesicles are a naturally secreted product of Gram-negative bacteria. Vesicles form when a portion of the outer membrane with periplasmic content is selectively "blebbed" off to form round vesicles (11, 94, 95). The production of OM vesicles has been observed for a wide variety of gram-negative bacteria in all stages of growth as well as in a variety of growth environments, such as infected tissues. The production of vesicles was further demonstrated to be linked to the bacterial stress response (97). Vesiculation levels increase during periods of bacterial stress, such as what might be experienced during the colonization of host tissues.

Analyses of OM vesicle components have demonstrated that vesicles contain a wide variety of virulence factors (see references in Table 1). These virulence factors include protein adhesins, toxins, and enzymes as well as nonprotein antigens such as lipopolysaccharide (LPS). Purified vesicles have displayed the ability to act as a delivery system for virulence factors by interacting with both prokaryotic and eukaryotic cells. Furthermore, vesicles are laden with pathogen-associated molecular patterns (PAMPs) and other OM components that can impact the course of infection and host responses to infection. This review will focus on examining the roles that OM vesicles and their specific components play as virulence factors and their ability to interact with and trigger responses from target cells. The diverse abilities of OM vesicles to modulate immune responses, deliver toxins and other virulence factors to host cells, and aid in biofilm formation all attest to the importance that these secreted elements can have in bacterial pathogenesis.

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OUTER MEMBRANE VESICLE FORMATION BY PATHOGENS

An ever-growing number of pathogens have been documented to produce and secrete natural OM vesicles (Table 1). Morphological and biochemical evidence for infected host tissues and fluids supports the idea that the production of vesicles by pathogens occurs during infection and, in fact, may be induced during infection.

What Are OM Vesicles?

OM vesicles are closed spheroid particles of a heterogeneous size (~10 to 300 nm in diameter) released from Gram-negative bacteria during all phases of growth (11, 94, 95). Electron microscopy (EM) studies reveal that OM vesicles are formed from OM bulges and the subsequent fission of vesicles containing electron-dense material (11, 18, 48,

TABLE 1. Virulence factors and activities associated with native OM vesicles[b]

Species	Vesicle-associated virulence factor(s) (reference[s])	Virulence-associated activity (reference[s])[a]
Actinobacillus actinomycetemcomitans	Leukotoxin, GroEL (46, 73)	Bone-resorbing activity, chicken embryo lethality, cytotoxic (22, 73, 105)
Actinobacillus pleuropneumoniae	Apx toxin, proteases (102)	Proteolytic (102)
Bacteroides fragilis	Hemagglutinin, alkaline phosphatase, esterase lipase, acid phosphatase, phosphohydrolase, α- and β-galactosidases, α-glucosidase, glucosaminidase, β-glucuronidase (109)	Hemagglutinating and enzymatic activities (109)
Bacteroides succinogenes	Cellulase, xylanase (35)	Aryl-β-glucosidase, aryl-β-xylosidase, endoglucanase, xylanase activities (35)
Bordetella pertussis	AC-Hly, FHA, pertussis toxin (Ptx) (62)	ND
Borrelia burgdorferi	OspA, OspB, OspD (26, 123)	HUVEC adherence (123)
Brucella melitensis	Omp25, Omp31 (42)	ND
Burkholderia cepacia	PLC-N, lipase, PSCP, 40-kDa protease (2)	Enzyme activities (2)
EHEC O111:H–	ClyA (136)	Pore forming (136)
ETEC	LT (61, 137, 138)	Enterotoxic and vacuolating activities (61, 78)
ExPEC	Alpha-hemolysin, CDT, iron and hemin binding OMPs (6, 10)	Hemolytic, causing detachment of cells from monolayer (6)
STEC O157:H7	Shiga toxin 2 (81, 143)	Cytotoxic (143)
UPEC	CNF1 (83)	Cytotoxic (83)
Helicobacter pylori	VacA, Lewis antigen LPS (34, 63, 75)	Vacuolating activity (75), cytotoxic, stimulating proliferation, IL-8 secretion (66, 76)
Legionella pneumophila	Mip (lpg0791), IcmK/IcmX, flagellin, phospholipase C, LaiE/LaiF, phospholipase, chitinase, acid phosphatases, Hsp60, proteases, diphosphohydrolase (40)	Inhibition of phagolysosome fusion, proteolytic and lipase activity (33, 40)
Moraxella catarrhalis	UspA1/UspA2 (127)	Binds C3 complement in serum (127)
Neisseria meningitidis	PorA, NlpB, NarE (putative) (93, 134)	TNF-α, IL-6, activation of tissue factor (procoaggulant), profibrinolytic and antifibrinolytic factors (12, 119)
Photorhabdus luminescens	Toxin AB, GroEL (49)	Insecticidal (79)
Porphyromonas (*Bacteroides*) *gingivalis*	Arg- and Lys-gingipain cysteine proteinases (27, 48, 72)	Cleavage and loss of CD14 from macrophage, cleavage of IgG, C3, IgM (47)
Pseudomonas aeruginosa	Phospholipase C, hemolysin, alkaline phosphatase, Cif, PQS, quinolines, protease, β-lactamase (20, 21, 70, 89, 91, 92, 119)	Decrease of apical CFTR expression, *in vitro* enzyme activities, bactericidal quinolines, IL-8 stimulation (8, 20, 21, 70, 91, 92)
Salmonella enterica serovar Typhimurium	Protective antigens (9)	ND
Shigella dysenteriae serotype 1	Shiga toxin 1 (30)	Toxicity (30)
Shigella flexneri	IpaB, IpaC, IpaD (68)	Invasion (68)
Treponema denticola	Dentilysin, adhesins, proteases (19, 116)	Chymotryptic activity, disruption of tight junctions (19, 116)
Vibrio anguillarum	Metalloprotease, hemolysin, phospholipase (58)	Protease, metalloprotease, hemolytic activities (58)
Vibrio cholerae	RTX toxin (14)	Cell rounding, depolymerizing actin (14)
Xanthomonas campestris	Cellulase, β-glucosidase, xylosidase, avirulence proteins, type 3 secretion system proteins (124)	ND
Xenorhabdus nematophilus	Bacteriocin, fimbrial adhesin, pore-forming protein, chitinase (79)	Chitinase activity, insecticidal (79)

[a] In addition to proinflammatory properties of endotoxin and porins (130, 131).
[b] Abbreviations not defined in the text: AC-Hly, adenylate cyclase-hemolysin; FHA, filamentous hemagglutinin; CDT cytolethal distending toxin; OMP, outer membrane protein; PLC-N, phospholipase, nonhemolytic; UPEC, uropathogenic *E. coli*; CNF1, cytotoxic necrotizing factor 1; ND, not determined.

70, 81, 88, 94, 109). In general, OM vesicles reflect the OM composition, containing LPS, glycerophospholipids, and OM proteins as well as enclosed periplasmic components (43, 56, 61, 70, 95) (Fig. 1). Importantly, OM vesicles are not a product of cell death since they contain newly synthesized proteins and are produced without concomitant bacterial lysis (96, 99, 144). Several OM vesicle proteomes have been evaluated recently, and all were determined to be enriched in envelope components, although some cytosolic and inner membrane proteins were also present in these preparations (10, 40, 84, 86, 87, 124, 141).

In addition to naturally shed vesicles, proteoliposomes can be artificially generated by sonication or detergent treatment of bacteria or the bacterial OM. These proteoliposomes are often referred to as bacterial vesicles. In some cases, it was claimed that brief treatments of detergent or sonication simply release preformed OM vesicles. However, in most cases, these treatments have been proven to differ in composition and ac-

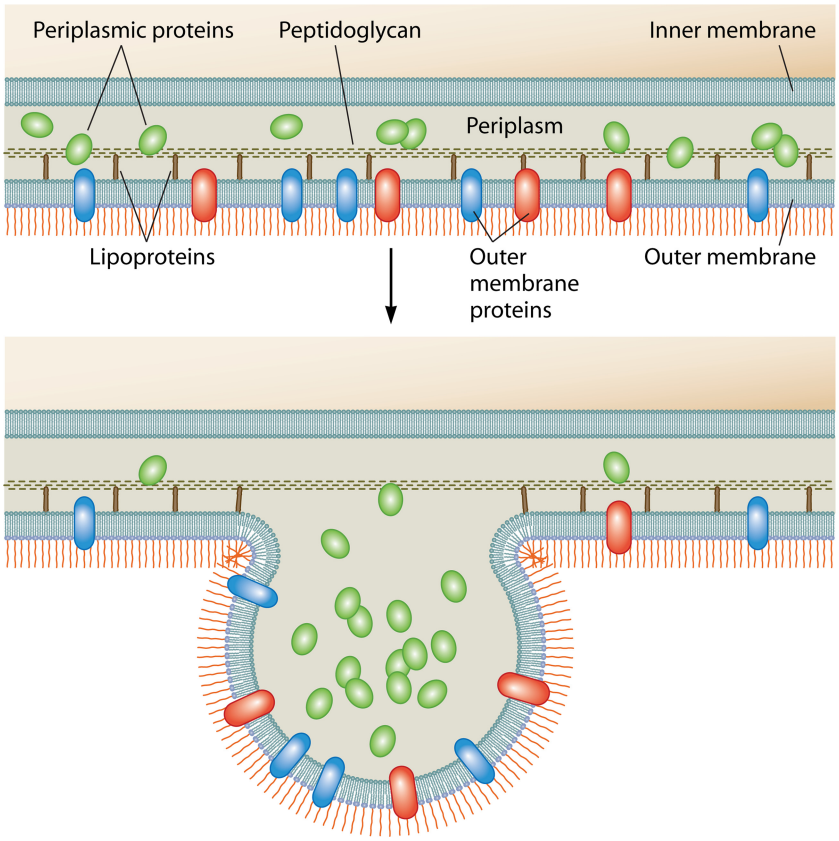

FIG. 1. Model of OM vesicle production. Shown is the budding of the Gram-negative bacterial envelope. Released OM vesicles contain periplasmic material and OM proteins and lipids, including PAMPs and other virulence factors, as described in the text. Although details of the mechanism remain unclear, budding is thought to occur in places where lipoprotein links between the OM and the peptidoglycan are broken or missing.

tivity from naturally produced OM vesicles (12, 23). We have focused this review of the literature on naturally produced OM vesicles.

Natural OM Vesicles

All Gram-negative bacteria investigated to date naturally release OM vesicles. Calculations of native OM vesicle production show that the vesicles represent a significant fraction of cellular material. For instance, vesicles produced by typical laboratory cultures of growing and dividing *Pseudomonas aeruginosa* and *Escherichia coli* cells account for ~1% of the OM material in the culture (8, 43, 139). In contrast, *Neisseria meningitidis* produces abundant numbers of vesicles, constituting 8 to 12% of radiolabeled protein and endotoxin in log-phase cultures (24). Not only are OM vesicles produced by free-living cells, they are also abundant in naturally occurring biofilms (120). In addition, intracellular pathogens such as *Legionella pneumophila*, *Salmonella* spp., and *Francisella* spp. produce OM vesicles in both intraphagosomal and extraphagosomal compartments (3, 33, 44, 45). In the case of *Flavobacterium*, vesicles are made late in the growth phase (82). The mechanism of vesicle production is complex (95), and progress in this field will be updated in detail in a separate review (A. Kulp and M. J. Kuehn, unpublished data).

Modulation of Vesiculation *In Situ*

Rates of OM vesicle production are not uniform, even for a particular strain, as production has long been seen to be influenced by environmental factors and by sources of cellular stress (74, 103, 129). In studies of both nonpathogenic and pathogenic species, vesiculation was found to be upregulated by conditions that activate the σ^E envelope stress response (97). In fact, vesiculation appears to be critical to surviving stress. When vesiculation mutants of *E. coli* were challenged with lethal envelope stressors, the vesicle-underproducing mutant succumbed, but the overproducing mutants survived better than the wild type (97). Considering the harsh antimicrobial environments encountered in a host during infection, the capacity to modulate vesicle production is likely critical for pathogens.

EM evidence has shown that vesiculation can be induced by exposure to host components and tissue. In a model that simulates meat spoilage, *Pseudomonas fragi* was inoculated into pig muscle (29). Unlike counterparts grown in a protein-free medium, the surfaces of bacteria that contacted the meat were covered with vesicles. These vesicles are thought to contain proteolytic enzymes that disrupt myofibrils seen in the bacterial spoilage of meat. In addition, using a mouse model of enterotoxigenic *E. coli* (ETEC) infection, vesicles were highly

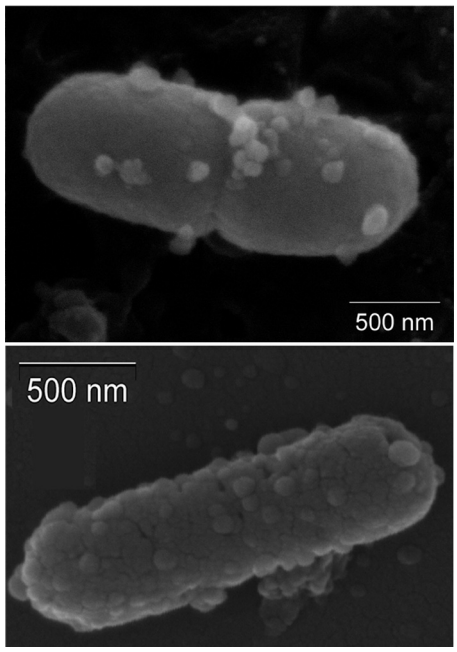

FIG. 2. Enterotoxigenic *E. coli* recovered postinfection. Shown are data from scanning electron microscopy of a representative ETEC strain recovered from mouse small intestines 2 h after intragastric inoculation. (Courtesy of Amanda McBroom.)

evident on ETEC cells recovered from the intestine 2 h after intragastric inoculation (Fig. 2).

Antibiotic treatment has been demonstrated to influence several aspects of vesiculation. The level of Shiga toxin-associated OM vesicle production by *Shigella dysenteriae* increased with mitomycin C treatment but not upon treatment with other tested antibiotics (30). Mitomycin C induces toxin production (135), and it is tempting to consider that one consequence of increased toxin production is hypervesiculation; however, this has not been proven. Gentamicin treatment also increased the vesiculation of *P. aeruginosa*; however, when the composition of these vesicles was inspected, they were not identical to native OM vesicles, as they also contained inner membrane and cytoplasmic material (70). Nevertheless, it is important to consider the tendency for particular antibacterials to elicit the release of proinflammatory and toxic materials.

A careful biochemical analysis by Fernandez-Moreira et al. highlighted how naturally produced vesicles can mediate the OM surface remodeling that is critical for virulence (33). Their data demonstrated significant differences in the composition, LPS, and activity of OM vesicles shed by *Legionella* during developmentally distinct periods of growth. This work supports a model by which transmissive *Legionella pneumophila* sheds OM vesicles into the phagosome not only to inhibit fusion with lysosomes but also to promote the remodeling of the bacterial surface to the intracellular replicative form (33).

Vesiculation Observed during Infection

Examination of animal and human biopsy specimens, tissues, and host fluids provides evidence that bacterial vesicles are formed during infection. EM has provided the most direct observation of vesicle production by pathogens in host tissues. Some of the oldest evidence of bacterial OM vesicles comes from EM of gingival plaque, which demonstrates abundant membrane vesicles interspersed between Gram-negative and Gram-positive bacteria (50). This material, termed biofilms, has been the subject of much further study and is discussed in more detail below.

Several biopsy studies have identified OM vesicles within human tissues. EM examination of *Helicobacter pylori*-infected human biopsy specimens revealed that bacterially derived vesicles contact intestinal epithelial cells (34, 51, 63, 75). Hynes et al. further noted that these vesicles could adsorb antibodies in sera from infected patients (63). EM examination revealed a highly vesiculating *Neisseria meningitidis* serogroup B strain associated with a fatal septic infection in a 20-year-old patient. The abundant "blebs" were thought to be the contributor to the high endotoxin level of 1,700 endotoxin units/ml (equivalent to the activity of 170 ng/ml pure *E. coli* LPS) that led to this fatal septicemia (101). Additionally, OM vesicles were observed in the cerebrospinal fluid of an infant infected with *N. meningitidis* and in the sera of three patients with lethal meningococcal endotoxemia (16, 125).

In several cases, microscopic examination of infected tissues revealed that OM vesicles are produced (or at least are more apparent) near host cells. A nasal sample from a child with *Moraxella catarrhalis* sinusitis was examined by transmission EM, and it was determined that, when in close association with leukocytes, *M. catarrhalis* secretes OM vesicles. Immunogold labeling of the C3 complement binding factor UspA1/UspA2 showed that these proteins were located near or on OM vesicles in the biopsy specimen (127). In another study, a *Salmonella* strain isolated from a human food-poisoning infection was inoculated into the ileum of chickens. A subsequent EM analysis determined that a majority of the *Salmonella* cells proximal to the ileal epithelial cells could be seen producing OM vesicles (142).

OM vesicles have also been observed at sites more disseminated from the direct site of bacterial colonization. OM vesicle antigens were found in urine, blood, and several organs of mice, dogs, and humans infected with *Borrelia burgdorferi* (26). Vesicles were observed on the surfaces of these spirochetes recovered from infected ticks and from infected mouse urinary bladder, spleen, liver, heart, and brain tissues, indicating that these vesicles are formed by *B. burgdorferi in vivo*.

Even without direct observation by EM, OM vesicles have been implicated in the pathogenic process. In a series of studies of *E. coli* infection, LPS and OM protein-containing bacterial fragments were isolated from the serum of septic rats (52–54). *E. coli* cells grown in culture with serum were found to shed OM material with the same composition as the material shed *in vivo* (55). Several factors indicate that these OM components are actually OM vesicles. They are composed of LPS, OM proteins, and lipoproteins; are larger than 0.1 μm in size; and are stable (55).

These studies leave no doubt that OM vesicles or blebs are found surrounding and attached to the surface of pathogenic Gram-negative organisms during infection. Subsequent studies have been done to examine what roles they may play in this niche and to address whether the vesicles are instruments of virulence and inflammation. It remains to be determined

whether OM vesicles contribute to the benefit of the bacterium (as a mediator of toxin transfer or inflammatory damage), to the benefit of the host (as a mobile, potent indicator of infection), or to both.

ROLES OF OM VESICLES IN INTERBACTERIAL INTERACTIONS

The interaction of bacteria with cocolonizing and commensal bacteria can be hostile or neighborly. As discussed below, bacteria destroy coinfectors to prevent competition for limited nutrients or to provide nutrients for themselves. In other cases, vesicles facilitate bacterial communities where interspecies interactions are not only tolerated but are promoted in order to combat otherwise lethal environmental conditions. In yet other cases, OM vesicles from one species have further been observed to aid in the survival of an entire mixed bacterial infective population by actively destroying host defenses.

Vesiculating bacteria may have a survival advantage in mixed-population infections by their capacity to eliminate competing bacterial strains. As fuel for competition, OM vesicles package periplasmic peptidoglycan hydrolases (70, 88). Their activity in OM vesicles has been attributed to the killing of cocultured Gram-negative as well as Gram-positive bacteria (89). Thus, OM vesicle production could be an advantage for growth in a mixed bacterial population where nutrition is limiting. Further, β-lactamase was found to be packaged into *P. aeruginosa* vesicles produced by strains that expressed β-lactamase (20). OM vesicles containing β-lactamase can protect the vesicle-producing strain from a cocolonizing, β-lactam-producing species that could otherwise eliminate them. Additionally, some OM vesicles have been found to contain scavenging proteases (29, 129), xylanase, and cellulase (35), which can aid in nutrient acquisition and thereby provide a survival advantage.

Vesicles can act as bridging factors in biofilms that produce an environment that is resistant to antibiotics and antibacterials. Vesicles from oral bacteria, in particular, promote biofilm formation and colonization (48, 71, 72, 94). Vesicles are abundant in natural biofilms, and OM vesicle surface-bound DNA appears to be an electrostatic, bridging component that is central to membrane vesicle-bacterium-biofilm matrix interactions (120, 121). *Porphyromonas gingivalis* vesicles mediate the coaggregation of the periodontopathogen *Tannerella forsythia*, and vesicles mediate the interaction/aggregation of staphylococci and *Prevotella intermedia* (65, 71, 72). By participating in quorum sensing, OM vesicles can also aid survival by contributing to communication within mixed populations of pathogens. *P. aeruginosa* OM vesicles contain quorum-sensing molecules, and vesicle production is also stimulated by the addition of the *Pseudomonas* quinolone-signaling molecule, PQS (92).

Not only can OM vesicles provide interbacterial glue to generate a nearly impervious multicellular structure, they can also promote the growth of a cocolonizing pathogen. For instance, in a mixed population of bacteria, secreted vesicles that titrate an antimicrobial agent or degrade β-lactams (described above) could significantly aid in the survival of any neighboring bacteria. Furthermore, OM vesicles released by one strain could cause increased inflammation, resulting in the exposure of host extracellular matrix proteins and the upregulation of epithelial cell surface receptors that are beneficial to colonization by another strain. *Moraxella catarrhalis* is frequently found in mixed infections with pathogens such as *Haemophilus influenzae*. As such, the ability of *M. catarrhalis* OM vesicles to bind complement and allow *H. influenzae* survival is a relationship that might also benefit *M. catarrhalis* (127). Thus, it was proposed that proinflammatory vesicle-mediated changes in host tissue can pave the way for the adherence and survival of cocolonizing pathogens.

Vesicles can also enable the exchange of bacterial products. Biochemical analyses revealed that DNase-resistant DNA and a periplasmic antibiotic could be transferred between bacteria by OM vesicles, suggesting a fusion event (25, 67). In EM studies where nonhydrolytic OM vesicles were used, *P. aeruginosa* and *Shigella flexneri* vesicles were found to confer stably integrated LPS onto the surfaces of other Gram-negative bacteria (69). These results are particularly intriguing considering that they show a potential role for vesicles in natural transformation and the acquisition of drug resistance in infections.

VESICLE-ASSOCIATED VIRULENCE FACTORS

Extracellular products of pathogens are often associated with acute infection and are essential for maximal virulence. As secreted extracellular entities, OM vesicles can actively modify the bacterial environment. OM vesicles have been found to be capable of delivering active toxins and other virulence-associated bacterial proteins to host cells. In some cases, toxins are enriched in OM vesicles, suggesting that their entry into vesicles is somehow regulated.

Vesicle-Associated Toxins

Toxic materials are associated with native OM vesicles from a variety of Gram-negative pathogens. In many cases, vesicle-associated toxins have indeed been found to deliver active toxins to host cells (Table 1). Enterotoxigenic *E. coli* (ETEC) produces heat-labile enterotoxin (LT) that associates with LPS in the particulate fraction of the cell culture supernatant (43, 61, 137, 138). Purified ETEC vesicles specifically bind, enter, and deliver active toxin into epithelial and Y1 adrenal cells (61, 78). OM vesicles from *Actinobacillus actinomycetemcomitans* exhibit cytotoxicity (22, 73, 105), and *Xenorhabdus nematophilus* and *Photorhabdus luminescens* OM vesicles were cytotoxic for insect larvae as well as in tissue culture (79). Kolling and Matthews demonstrated, by immunoblotting, that Shiga toxin is present inside OM vesicles produced by O157:H7 cells (81), and this was corroborated for many strains of Shiga toxin-producing *E. coli* (STEC) under both aerobic and anaerobic conditions (143). NarE is a putatively vesicle-associated protein of *Neisseria meningitidis* vesicles that exhibits ADP-ribosyltransferase and NAD-glycohydrolase activities typically associated with toxicity (93). Toxic vesicles produced by extraintestinal *E. coli* (ExPEC) include a hemolysin; an RTX toxin, which becomes surface bound; as well as cytolethal distending toxin, which has a lipid binding domain to bind the OM (6, 10).

In some cases, vesicle-associated toxins have been observed directly in biopsy specimens of infected tissues. For instance, OM vesicles containing the vacuolating cytotoxin VacA were

observed in *H. pylori*-colonized human gastric epithelium biopsy specimens and were similar in appearance and composition to *H. pylori* vesicles made *in vitro* (34). The VacA-related toxicity of *H. pylori* vesicles has been studied in some detail. OM vesicle-associated VacA causes vacuolization in HEP-2 cells (75), although the relative contribution of vesicle-associated VacA and free-soluble VacA is debated (114). In contrast, Galka et al. found that extracellular Mip was found exclusively in the OM vesicle fraction of *Legionella pneumophila* cultures (40).

H. pylori vesicles with either full-length VacA or a natural carboxyl-terminal truncate of VacA (from a VacA$^-$ phenotype strain) induce cytochrome *c*-independent apoptosis in gastric epithelial cells (5). The truncated VacA contains the active domain but not the type I secretion system signal; thus, the results with the truncate were surprising because this was classified as a "nonsecreted" version of VacA. It was proposed that the OM vesicles mediate the toxicity of the truncate by enabling its presentation to host cells. This type of datum suggests that care should be taken when bacteria are assigned as having a toxin-deficient phenotype—they could be secreting toxins via vesicles.

Investigations of several pathogens demonstrate directly or support that toxins are often localized to the surface of the vesicles. Extensive studies have detailed how LT is secreted, becomes associated with LPS, and eventually is shed via OM vesicles, which have LT tightly bound to LPS on their surface as well as soluble periplasmic LT in their lumen (59–61, 98) (Fig. 3). Although Kolling and Matthews demonstrated that Shiga toxin (Shiga toxin 1 and/or 2) is protease resistant in OM vesicles produced by O157:H7 (81), the ability of LPS to bind and neutralize Shiga toxin 2 (41) suggests that at least some of the Shiga toxin may be found on the OM vesicle surface in a protease-resistant form. Immunogold EM was used to detect a high-molecular-mass protease on the surface of *Actinobacillus pleuropneumoniae* vesicles, which also package Apx toxin (102). The vacuolating toxin VacA was also seen to be closely associated with the membrane of *H. pylori* vesicles (75). VacA appears to be in a mature, monomeric form when associated with vesicles, as it is when it is associated with cells (64, 114). Studies of *Campylobacter coli*, *C. fetus*, and *C. jejuni* OM vesicles showed that although present in both whole cells and OM vesicles, some proteins were not exposed to labeling in whole cells but were labeled in OM vesicles (90). This suggests that either the topology changed (which is considered unlikely) or the proteins internal in whole cells are externally associated with OM vesicles.

Toxins associated with the OM vesicle surface are typically very protease resistant, although they are demonstrably on the exterior surface of vesicles. Leukotoxin detected on the surface of *A. actinomycetemcomitans* cells is found in a tight association with the membrane of its vesicles (73, 106). Similarly, the cytotoxins found to be associated with *X. nematophilus* OM vesicles were not strippable from OM vesicles except with sodium dodecyl sulfate, which destroyed its activity as well (79). LT and alpha-hemolysin (HlyA) were also tightly bound to the membrane of ETEC and ExPEC vesicles, respectively (6, 61). Whether the ability of toxins to tightly associate with the vesicle surface is due to the high degree of curvature of the OM vesicle or to their interaction with a specific component of the vesicles can be tested experimentally.

Nontoxin Virulence Factors in OM Vesicles

OM vesicles are a mechanism by which bacteria can secrete many periplasmic and membrane components associated with the virulence of the bacterium. Often, OM components of vesicles include adhesins that allow vesicles to interact with host cells (Table 1) (discussed in more detail below). In addition, OM vesicles can include proteases and signaling molecules. For example, *Pseudomonas* OM vesicles include virulence-associated enzymes, antimicrobial quinolines, and quorum-sensing molecules (70, 89, 91, 92, 113). Also, the *P. aeruginosa* CiF protein is preferentially secreted into vesicles, and CiF-containing vesicles decrease levels of apical lung epithelial expression of the cystic fibrosis transmembrane conductance regulator protein (CFTR), the central protein in the genetic disease of cystic fibrosis (15, 91).

OM vesicle-associated proteins include active proteases that can degrade host cells. Vesicles from *Treponema denticola*, a periodontopathogen, have active proteases that confer host cell-damaging characteristics similar to those of the whole bacterium (19, 116, 119). In addition, *Neisseria meningitidis* culture supernatant endotoxic filtrates have procoagulant and fibrinolytic factors for human monocytes (119).

OM vesicles can directly bind, titrate, and even destroy host bactericidal factors. Indeed, *Neisseria* vesicles can bind and remove cell-targeted bactericidal factors in serum (110). *H. pylori* expresses Lewis antigens on its OM LPS, and this "molecular mimicry" contributes to *H. pylori* virulence. Since OM vesicles produced by *H. pylori* contain those Lewis antigens on their LPS, it was proposed that these vesicles could contribute to the chronic stimulation of the host immune system (63). These vesicles can absorb anti-Lewis antigen autoantibodies from the serum, and it was suggested that they may play a role in the putative autoimmune aspects of *H. pylori* pathogenesis. *M. catarrhalis* OM vesicles carry UspA1 and UspA2, which bind to C3 in the complement cascade (127). As a result, *M. catarrhalis* vesicles inhibit the bactericidal effects of complement in normal human sera that are also directed against nontypeable strains of *H. influenzae*. Thus, vesicles from one species can also contribute indirectly to the pathogenicity of another by binding and depleting complement in their immediate environment.

Additionally, OM vesicles can influence critical bacterial cell-host cell associations in a variety of tissues. *P. gingivalis* vesicles enhance the attachment and invasion of *T. forsythia* in periodontal epithelial cells (65). Likewise, the addition of adherent-invasive *E. coli* (AIEC) vesicles to intestinal epithelial cells significantly increased the internalization of a vesicle-underproducing mutant of AIEC (115). The vesicle components that contribute to bacterial entry have yet to be determined.

Enrichment of Vesicle-Associated Virulence Factors

Particular OM vesicle components may be enriched compared to their abundance in the originating bacterial cell. While vesicle OM protein profiles are typically very similar to

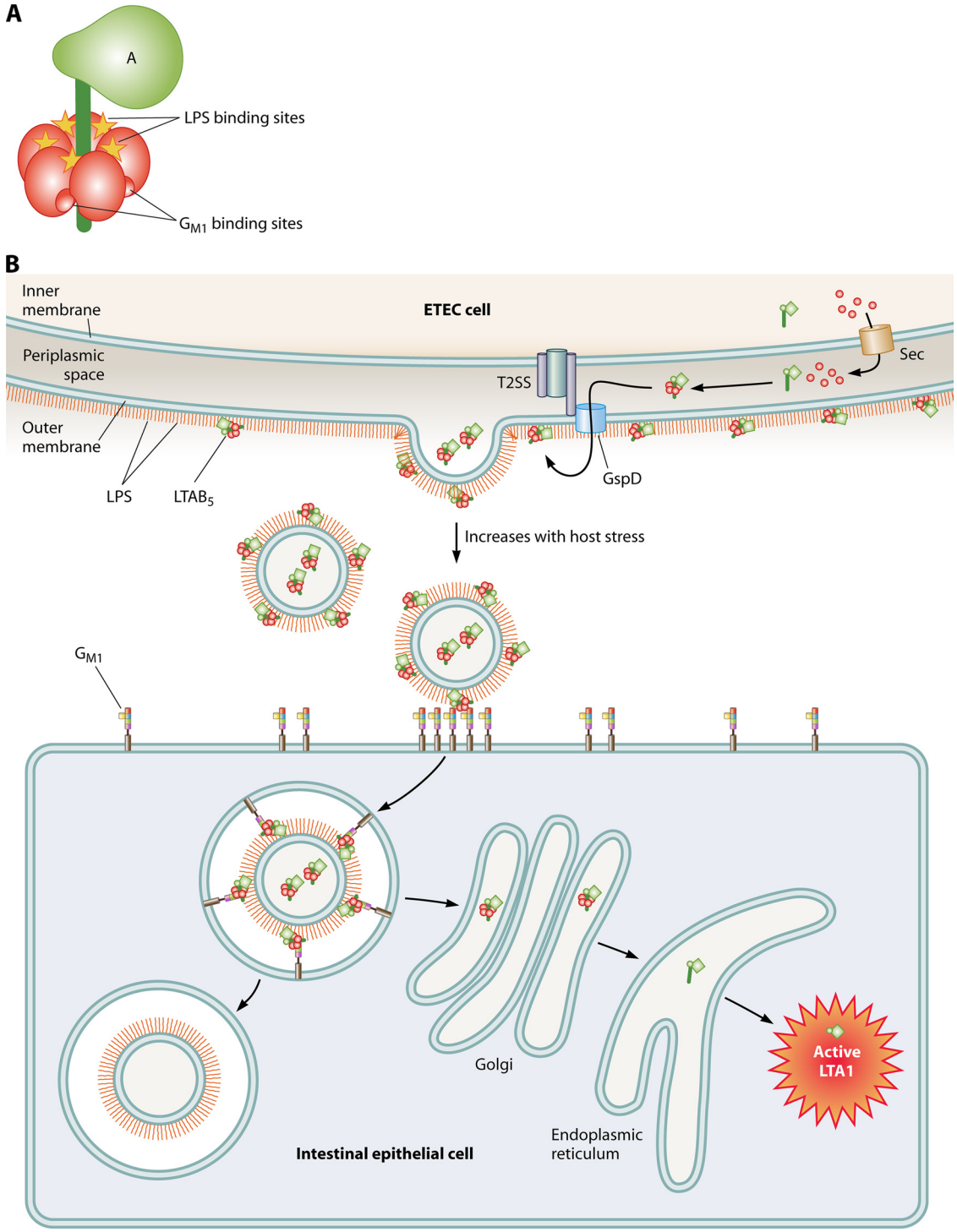

FIG. 3. Models of binding, secretion, and vesicle-mediated LT transport into host cells. (A) LT consists of an LTA subunit in a complex with 5 LTB subunits ($LTAB_5$). LPS and G_{M1} both bind in interfaces between subunits of the $LTAB_5$ complex, but the binding sites are distinct from each other. (B) LT is secreted through the inner membrane by the Sec machinery, folds into the periplasm, is secreted through the outer membrane via GspD of the type 2 secretory system, and binds to LPS on the cell surface via LTB_5. Consequently, vesicles released from the cell have LT on their surface that can act as a tether between G_{M1} on the host cell and LPS on the vesicle. Vesicles are internalized by G_{M1}- and cholesterol-rich microdomains (lipid rafts). Vesicle-associated LT traffics through the Golgi apparatus and ER and is retrograde translocated into the cytosol, where the A1 fragment of LTA is catalytically active. The remaining vesicle material is maintained intracellularly in a nonacidified compartment.

those of the OM, they are not identical. Enrichment and depletion of the OM and periplasmic cargo were found when the contents of vesicles and bacterial OM fractions were compared (95). Most relevant to this discussion is the enrichment of virulence factors in OM vesicles. Proteins identified as being enriched in vesicles include LT of ETEC and the *P. aeruginosa* aminopeptidase, both of which increase the association of vesicles with cultured epithelial cells (7, 78). Leukotoxin and leukotoxic activities are 4- to 5-fold enriched in OM vesicles compared with OM preparations for some strains of *A. actinomycetemcomitans* (73). Similarly, a subfraction of the vesicles secreted by ExPEC cells expressing HlyA is enriched in that virulence factor (6). In contrast, CagA, an *H. pylori* virulence factor, is notable for its absence from VacA-containing OM vesicles from Cag pathogenicity island-positive strains (66, 75).

Quantitative proteomic techniques are useful tools to determine exactly what is enriched in the OM vesicle population. When investigating the *Xanthomonas campestris* OM vesicle proteome, surprisingly, less than half of the most abundant OM proteins could also be identified in the vesicle fraction (124). Further analysis revealed that nearly half of the proteins associated with the OM vesicle fraction are involved or are putatively involved in virulence, and of the 21 proteins enriched in the OM vesicle fraction, most of them are virulence associated. It was also evident that the composition of the OM vesicle proteins was partially determined by the growth medium. This indicates that in addition to their abundance, OM vesicle proteomes, including their associated virulence factors and toxins, are highly mutable depending on the physiological surroundings.

VESICLE INTERACTIONS WITH HOST CELLS

Protease- and toxin-containing OM vesicles interact with host cells and thereby act as virulence factor delivery vehicles. As discussed below, their interaction can occur via a membrane fusion event or via adhesin-receptor-mediated attachment. In some cases, adherence is followed by vesicle uptake, even by nonphagocytic cell types. Data from diverse pathogens support the theory that vesicle binding contributes to infection by enabling the delivery of toxic bacterial cargo to host cells, often by the internalization of the entire content of the vesicle. Thus, it is not surprising that, in addition to toxicity, the ramifications of such a delivery process include signaling and innate and adaptive immune responses in the host cells.

Adherence of Vesicles to Host Cells

Binding to cultured host cells has been observed for OM vesicles produced by a variety of pathogens. Protease- and toxin-containing OM vesicles from *E. coli*, *Shigella*, *Actinobacillus*, and *Borrelia* strains interact with bacterial as well as mammalian cells (43, 68, 73, 117, 123). Vesicle-associated aminopeptidase increases the ability of *P. aeruginosa* vesicles to associate with both primary and cultured human lung epithelial cells, although whether this is due to a receptor binding or receptor-uncovering property of this enzyme is not yet clear (7).

OM vesicles adhere to host cells not only in tissue culture but also in the complex environment of an infected host tissue. For instance, *H. pylori* OM vesicles were bound to intestinal cells in biopsy specimens from infected patients (51, 75). *Bacteroides fragilis* OM vesicles have hemagglutinating activity, which indicates that they are able to act as adhesive bridging entities between mammalian cells (109).

Host cell adherence tropism depends on bacterial strain-specific factors. Not surprisingly, therefore, vesicles and bacteria can use identical host cell receptors. *B. burgdorferi* vesicles adhere to human umbilical vein endothelial cells (HUVECs) in a manner that competes with whole *Borrelia* cells (140). Tropism in host cell binding was demonstrated for OM vesicles from *A. actinomycetemcomitans* (22) and was specifically engineered for vesicles formed by *E. coli* that expressed the *Yersinia enterocolitica* adhesin/invasin, Ail (77).

Vesicle Entry into Cells

In many cases, after OM vesicles adhere, they can be internalized into host cells. Toxins can act as adhesins for the OM vesicles and allow vesicles to enter using the receptor-mediated endocytic pathway also used by the soluble toxin. Topologically, toxin-mediated vesicle adherence and uptake can occur only if the toxin-vesicle interaction does not interfere with the toxin-receptor interaction. This has been carefully mapped in the case of LT, the toxin/adhesin associated with ETEC vesicles. Although the LT-B subunit forms a pentamer, and thus both LPS and G_{M1} binding sites do not need to be simultaneously occupied to confer vesicle binding, LPS (vesicle) and G_{M1} (host cell) binding sites on LT-B are entirely distinct (60, 98) (Fig. 3A). As a result, ETEC vesicles bind to and enter epithelial cells via lipid raft-mediated endocytosis governed by G_{M1} (78) (Fig. 3B). Once inside the cell, the LT-A subunit is trafficked to the endoplasmic reticulum (ER) and the cytosol, where it catalyzes the ADP-ribosylation of $G_{s\alpha}$, leading to enterotoxicity. The remaining vesicle components are stably maintained in a nonacidified intracellular compartment.

Other examples of toxin-mediated vesicle adherence have been investigated. *H. pylori* VacA is taken up by gastric epithelial cells both as a free toxin and as a vesicle-associated toxin (34, 114). As yet, it is not known if VacA is the vesicle adhesin/entry ligand. Common components of vesicles such as OmpA could also contribute to the host cell entry of vesicles. OmpA mediates invasion for *E. coli* K1, causing neonatal meningitis, by interacting with a surface receptor, Ecgp, on brain microvascular endothelial cells (111, 112). Not all vesicle toxins are required for effective vesicle adhesion. *A. actinomycetemcomitans*-derived OM vesicles, while highly enriched in leukotoxin (73), do not require leukotoxin for rapid vesicle-cell interactions (22).

The destination of some vesicle-associated material is transepithelial. Dentilysin-carrying OM vesicles from *Treponema denticola* can disrupt the epithelial cell monolayer in culture and proceed to penetrate through the monolayer and emerge on the basolateral side, as measured by the appearance of dentilysin activity in that compartment (19).

Host factors that contribute to vesicle uptake by specific host cell types have been identified. For instance, bactericidal/permeability-increasing protein (BPI), but not LPS binding protein (LBP), enhanced the uptake of *N. meningitidis* vesicles

into dendritic cells, and confocal imaging visualized BPI colocalized with internalized OM vesicles (122). These interactions were surprisingly selective, since the same did not occur for macrophages, and BPI did not promote similar interactions of free lipooligosaccharide aggregates with the dendritic cells.

Vesicle Fusion with Cell Membrane

Experimental evidence from several studies supports the hypothesis that certain OM vesicles can fuse with host cell plasma membranes. *Salmonella* cells growing within host cell vacuoles were found to release LPS that could be detected within host epithelial cell vesicle membranes. This process was determined to be dependent on the presence of the O antigen in the LPS structure, as detected by antibodies to the O antigen itself (44). According to another report, *A. actinomycetemcomitans* vesicles conferred a lipid-tracking dye to host cell plasma membranes within 2 min of coincubation (22). Likewise, *L. pneumophila* OM vesicles tracked with a fluorescent tag were observed on the surface of alveolar epithelial cells, suggesting that they are lengthily adherent or have fused (40).

While these biochemical and visual data are compelling, abundant and definitive biophysical evidence of OM vesicle-membrane fusion is lacking. Meanwhile, it is intriguing to consider how the phospholipid bilayer of mammalian cells could possibly accommodate the foreign architecture of LPS, much less bacterial OM protein β-barrel structures (if these are also transferred). The incorporation of such bacterial structures into a eukaryotic membrane could also influence the sensing of the ligands by innate immune receptors and overall host responses.

IMMUNOMODULATORY ACTIVITIES

The composition of OM vesicles makes them significant activators of host innate and acquired immune response pathways. In addition to the potent immunomodulatory molecule LPS, vesicles contain OM porins and other important innate immune-activating ligands. Together, vesicle components appear to act synergistically to modulate the host response in ways that can either stimulate the clearance of the pathogen, enhance the virulence of the infection, or both. In addition, the immunogenic properties of OM vesicles lead to protective mucosal and systemic bactericidal antibody responses that have been exploited for vaccine purposes.

Vesicle-Mediated Activation of Inflammation and Innate Immunity

Vesicles are likely a key factor in effecting an inflammatory response to pathogens. OM vesicles produced by colonizing bacteria encounter and may be taken up by epithelial cells and macrophages to trigger an immediate innate host response. The response of epithelial cells and macrophages to secreted bacterial products is a well-established trigger of the inflammatory cascade.

The ability of OM vesicles to trigger inflammatory responses was thoroughly investigated by Alaniz et al. (1). Their analysis demonstrated that OM vesicles from *Salmonella enterica* serovar Typhimurium are potent stimulators of proinflammatory cytokine secretion and immune cell activation. *Salmonella* OM vesicles activate macrophages and dendritic cells to increase levels of surface major histocompatibility complex class II (MHC-II) expression as well as the production of the proinflammatory mediators tumor necrosis factor alpha (TNF-α) and interleukin-12 (IL-12). OM vesicles also activated $CD4^+$ T cells, indicating that protein antigen components of vesicles were effectively processed and presented by the activated antigen-presenting cells.

A proinflammatory response to OM vesicles was also observed for several other pathogens. Studies of epithelial cell responses found that *H. pylori* OM vesicles potently elicit an IL-8 response (66), as do *P. aeruginosa* vesicles (8). Detergent-generated vesicles from *N. meningitidis*, while not naturally produced, have well-characterized host responses due to their development into a protective vaccine. These manufactured vesicles have been shown to trigger the production of numerous proinflammatory cytokines from neutrophils, including TNF-α, IL-1β, IL-8, macrophage inflammatory protein 1β (MIP-1β), and IP-10. (85) Naturally produced *Neisseria* OM vesicles were further found to stimulate IL-8, RANTES, and IP-10 while activating dendritic cell MHC-II expression (28). Likewise, OM vesicles purified from the fish pathogen *Vibrio anguillarum* stimulated the production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 when inoculated into flounder (58).

It should be considered that stimulation by OM vesicles is more likely to have a pathogen- and site-specific "signature" than is stimulation by individual purified components such as LPS or purified OM proteins. The protein composition of vesicles can vary substantially with the bacterial strain of origin and with the environment (87). Prominent OM proteins such as porins are known to promote proinflammatory activities in a variety of Gram-negative pathogens, including *H. pylori*, *Salmonella*, *Fusobacterium*, and *Yersinia* species (38, 39, 126, 130, 131). Recently, the OM adhesin of *N. meningitidis*, NadA, was shown to be required for the optimal activation of macrophage responses. This adhesin fully activated macrophage cytokine production when presented in the conformation of the OM vesicle membrane rather than as a mixture of purified vesicle components (128). Furthermore, the immunoglobulin responses to the *Neisseria* antigen PorB delivered as a recombinant purified protein, as a virus-like particle, and in outer membrane vesicles were compared. Zhu et al. observed that only the OM vesicle delivery system generated a bactericidal serum response (145).

These data, as well as data from numerous vaccine studies (17, 104), demonstrate that the three-dimensional conformation in which immune ligands are presented can greatly impact the type and potency of the host response generated. Numerous studies have demonstrated that the composition of OM vesicles differs from that of the bacterial OM by the enrichment or exclusion of specific outer membrane proteins and LPS modifications (see above for a discussion of enrichment) (8, 70, 95). Given these differences in composition, it is hypothesized that the responses to these two bacterial membranes would be significantly different. Naturally produced OM vesicles and whole-cell OM preparations have not yet been compared in activation assays. Such experiments would

help elucidate the specific roles that OM vesicles play in diverting host responses to prevent bacterial clearance.

LPS and Other PAMPs

All experimental evidence to date indicates that the innate immune response to OM vesicles results from the combination of vesicle pathogen-associated molecular patterns (PAMPs) and LPS recognized in their natural context. Lipoproteins and OM proteins present in vesicles are biologically active molecules known to activate immune cells and induce leukocyte migration (140). The identity of PAMPs and other components of OM vesicles capable of modulating immune responses can perhaps best be investigated by studying the effects of OM vesicles from genetic mutants lacking specific envelope components.

The single most abundant, and typically considered most potent, immune-stimulating component of OM vesicles is LPS. LPS content can exceed the total protein content of vesicles by ratios as high as 10:1 (our unpublished observations). Numerous studies have characterized how LPS is sensed by the Toll-like receptor 4 (TLR4) complex, triggering a proinflammatory response common to the majority of Gram-negative bacterial infections. High levels of LPS (endotoxin) and TLR4 activation can lead to LPS toxicity and play a role in septic shock (107). Given the high LPS content, all investigations into immune responses to OM vesicles must define the contribution of LPS to the host response. OM vesicles, as LPS delivery vehicles, have the capacity to either enhance bacterial clearance or cause host tissue damage by activating an inflammatory response.

Much of the research into the TLR4-mediated response to LPS has utilized pure, chemically extracted LPS. However, it is more likely that LPS is naturally shed from bacteria in the form of OM vesicles. OM vesicles are heterogeneous proteoliposomes that have a larger dimension than liposomes composed solely of LPS (11), and these properties likely impact the type and potency of the innate immune response. The predicted endotoxic capacity of vesicles has been established by numerous studies, but a more interesting question that has consequently arisen is whether vesicle-associated LPS is equally as endotoxic as cellular LPS.

To address this issue, Munford et al. compared the abilities of phenol-extracted vesicle LPS and whole-cell LPS to bind to high-density lipoproteins (HDLs) (100). Those authors determined that purified and vesicle LPSs were similarly active in binding HDL, while cell-associated LPS was less active. As a result, they concluded that vesicle-associated LPS is the native presentation of LPS that has the highest biological activity. In a similar comparison, endotoxin secreted into the culture supernatant from *N. meningitidis* cells was found to more effectively stimulate monocyte expression of plasminogen activator and the procoagulant factor TF than phenol-extracted, pure LPS (119).

Studies of LPS structure during the course of infection further demonstrated that LPS can be modified in response to environmental stimuli. LPS from *P. aeruginosa* has been well documented to exhibit an altered lipid A acylation pattern when growing in the lungs of cystic fibrosis patients (32). Variations of O-antigen composition have also been shown to vary with environmental conditions, as Sabra et al. were able to induce *P. aeruginosa* to increase B-band LPS production under low O_2 conditions (116a). OM vesicles may also exhibit a distinctive population of LPS molecules, as an analysis of *P. aeruginosa* vesicles revealed vesicles enriched in B-band LPS (70). It is unclear if vesicles aid in the turnover of LPS molecules during colonization; however, the impact of these chemical alterations due to environmental conditions should be factored into the experimental design to determine host responses to these LPS species.

The sensing of LPS by the TLR4 complex requires the extraction of LPS from the membrane by accessory proteins such as CD14, LPS binding protein (LBP), or bactericidal/permeability-increasing protein (BPI). While the mechanism by which these factors remove LPS from the bacterial membrane for presentation to the TLR4 complex is not known, studies have established OM vesicles both as key sources of LPS to activate inflammation and as decoys, binding these soluble factors to impede inflammation.

Vesy et al. found that the LPS in *Salmonella* OM vesicles was extracted by LBP but not by other factors such as CD14. The depletion of LBP from culture supernatants inhibited LPS binding to cell surface TLR4-CD14 complexes (133). In a similar LPS binding study, Schultz et al. investigated the role of BPI in binding *Neisseria* OM vesicle-derived LPS. Those authors observed that BPI binding of LPS was required for the OM vesicle interaction with dendritic cells, leading to CD14-mediated signaling and the expression of proinflammatory cytokines (122).

Vesicle-mediated components other than LPS have also been shown to modulate the sensing of LPS by the host. Vesicles from the dental pathogen *P. gingivalis* have been shown to decrease the level of membrane-bound expression of CD14 on macrophage surfaces, leading to a decreased ability of the macrophages to trigger LPS-stimulated cytokine production. A loss of CD14 is prevalent in cases of chronic periodontitis. Additionally, *P. gingivalis* OM vesicles were shown to bind and actively degrade soluble CD14 with vesicle-derived enzymes. *P. gingivalis* vesicles were also shown to degrade IgG, IgM, and complement factor C3 (47). These studies demonstrate that LPS from OM vesicles both is directly sensed by the innate immune system and directly interacts with host factors to modulate that same response.

Purified LPS and vesicle-bound LPS not only differ in their potencies of stimulating an innate immune response but also may differ in their ability to distribute, and thus be cleared, in host tissues. This is of particular importance for *in vivo* studies that instill pure LPS into animal models. The biophysical properties of vesicles, as heterogeneous, proteinaceous, amphipathic structures, may allow greater movement through tissues. In contrast, pure LPS would likely exhibit more hydrophobic qualities, resulting in more rapid binding to available host membranes. As a result, vesicles could travel deeper into tissues where resident phagocytes are located. These same vesicle properties may also result in vesicles being more readily recognized and cleared by tissue phagocytes. Studies investigating the role of aggregate size in LPS binding by host cells support this hypothesis. Kitchens and Munford found that the size of LPS complexes directly impacts the CD14-dependent internalization of LPS (80). Additionally, Elsbach noted that the inflammatory responses to LPS are directly impacted by the

presence of other membrane components as well as the membrane conformation (31).

OM vesicles have also been shown to contain other PAMPs in addition to LPS. *P. aeruginosa* vesicles were shown to contain both flagellin monomers and CpG DNA (8, 113). Studies utilizing LPS-deficient OM vesicles from *Neisseria* demonstrated the ability of non-LPS PAMPs to enhance immune responses to vesicles (36, 108). However, to date, no studies have demonstrated either flagellin or CpG DNA directly impacting the host responses to intact vesicles containing LPS.

Vaccines, Antigenicity, and Adjuvanticity of OM Vesicles

The capacity of OM vesicles to stimulate an adaptive memory immune response has already been exploited in the development of effective OM vesicle-based acellular vaccines. The most successful use of OM vesicles as a vaccine has been in the development of a vaccine against serogroup B *N. meningitidis*. The development and properties of these serogroup B vaccines have recently been comprehensively reviewed by Holst and colleagues (57). As detailed in that review, vesicle-based vaccines have proven to be the only protective formulation against serogroup B infections, with over 55 million doses administered to date. Several different formulations of the vaccine exist, targeted to strains and antigens specific to a given geographic region. All preparations of *Neisseria* vesicle vaccines stimulate protective mucosal and systemic bactericidal antibody responses, with the antibody response being generated predominantly to the outer membrane porins PorA and PorB (37). Research is currently focused on engineering bacterial strains to produce OM vesicles containing multiple PorA proteins derived from different strains in the hope of developing a global *N. meningitidis* serogroup B vaccine (132).

The concept of developing OM vesicle-based vaccines has also been investigated for cholera infections. Schild et al. used the neonatal mouse model of passive antibody transfer to demonstrate that OM vesicles from *Vibrio cholerae* generate a protective antibody response (118). B-cell responses to OM vesicles from *S.* Typhimurium (1), *B. burgdorferi* (140), and *Flavobacterium* (4) have also been documented, indicating that OM vesicles can easily be used as antigen delivery systems to generate effective antibody responses.

The presence of LPS in OM vesicle-based vaccines has emphasized the ability of LPS to act as a natural adjuvant to the immune system. *Neisseria* is unusual in that it is a Gram-negative pathogen that can tolerate the deletion of the biosynthetic genes for LPS. However, immune responses to LPS-free OM vesicle preparations are poor, and the addition of exogenous PAMP adjuvants was shown to be necessary to generate an optimal host response (28, 36, 108). The most commonly used commercial OM vesicle vaccine against *N. meningitidis* utilizes manufactured vesicles from detergent-treated bacteria, which greatly reduces, but does not eliminate, the endotoxic content of vesicles to prevent toxicity. Data generated by utilizing LPS-deficient OM vesicles as well as the observation that the *Neisseria* adhesin NadA generates an effective immune response only when presented with LPS indicate that the adjuvant activity of LPS may be critical to the engineering of an effective vaccine. Recent investigations into how TLR ligand sensing impacts other phagocyte functions such as phagosomal maturation, the selection of antigens for MHC presentation, and dendritic cell maturation indicate that the immune system may be best adapted to sensing a "mixture" of LPS and other OM bacterial components to generate an effective host response (13).

FUTURE DIRECTIONS

Since every pathogen expresses different virulence characteristics and utilizes these characteristics for distinct purposes, the quest to uncover the roles that OM vesicles play for every pathogen is daunting. Nevertheless, this substantial body of research has generated a great deal of excitement, since it is revealing both common and pathogen-specific roles that OM vesicles can play in bacterial virulence. Future work in this field is likely to take advantage of this knowledge in order to reduce pathogenicity and to exploit the capacities of this complex natural delivery mechanism.

Targeting Vesicles To Reduce Virulence

Disrupting the interactions between host cells and vesicle-associated virulence factors and between cocolonizing bacteria and vesicles is likely to be a good strategy to reduce virulence. Preventing the generation of vesicles by pathogens and reducing the proinflammatory effects of OM vesicles in host tissues are also likely to reduce pathogenicity. As these processes become sufficiently understood, we can proceed to finding therapeutics that can disarm vesicle effects and block vesiculation (if vesiculation is not an essential bacterial function) to make the bacteria more susceptible to host defenses.

Taking Advantage of OM Vesicles

The utilization of OM vesicles as a complex of antigens in their native context with a natural adjuvant has already proven successful for human vaccines. Future efforts will likely result in OM vesicle vaccines engineered to reduce endotoxicity and to include multispecies-specific antigens. Additionally, synthetic proteoliposomes have been developed to deliver drugs to tumors and specific tissues, although off-target effects are a continuing problem. Making use of adherence and entry mechanisms that target native OM vesicles to host cells could improve the specific targeting of engineered therapeutic liposomes.

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