Lipopolysaccharide 3-Deoxy-α-manno-octulosonic Acid (Kdo) Core Determines Bacterial Association of Secreted Toxins*

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Pathogenic bacteria produce toxins that cause the symptoms of the diseases associated with infection. Several of these, including shiga toxin, pertussis toxin, cholera toxin (CT), and heat-labile enterotoxin (LT), belong to a family related by structural homology. These AB$_2$ toxins consist of a catalytic A subunit (e.g. LTA, CTA) and a pentamer of receptor-binding B subunits (e.g. LTB, CTB) (1, 2). In addition to structural homology, the enterotoxigenic Escherichia coli (ETEC) toxin, LT, shares 80% nucleotide sequence identity with the Vibrio cholerae toxin, CT (3, 4). The ring-shaped B pentamers of both LT and CT mediate binding to the host cell receptor, G$_{MT}$ (5–7). LTB is more promiscuous than CTB in that it can also bind other receptors containing a terminal galactose (6). After binding, the receptor/toxin complex is internalized by the host cell and the A subunit undergoes retrograde trafficking to the cytosol via the endoplasmic reticulum (ER) (6, 8, 9). Upon entry into the cytosol, the catalytic subunit constitutively activates adenylyl cyclase, resulting in water and electrolyte efflux from the host cells (10).

Despite the equivalent activity CT and LT exhibit in cell culture, disease caused by ETEC is much less severe than that caused by V. cholerae (11). Several factors, including the toxins themselves, are likely to contribute to the severity of these diseases. Volunteers treated with 25 µg of CT lost an average of 20 liters of fluid, compared with only a 6 liter loss caused by the same amount of LT (12, 13). Hypotheses regarding the processing and host cell trafficking of the toxins have been proposed to explain this difference in toxicity. For example, the C terminus of CTA has a prototypical KDEL ER retrograde trafficking signal sequence whereas LTA has an EDEL sequence; however, this difference has not been shown to affect the intracellular trafficking of the toxins inside the host (14). Further, CTA secreted from V. cholerae is activated by a hemagglutinin/protease encoded by V. cholerae resulting in two polypeptides, A1 and A2, that remain linked by a disulfide bond until the toxin reaches the ER (15–17). LTA is nicked in vitro, but not when expressed and secreted by V. cholerae (16, 18). However, inefficient LTA processing is probably not a relevant factor since this result may be explained by an insufficient level of soluble hemagglutinin produced by the mutant used in those studies (19), and recently several serine proteases in the endocytic pathway have been demonstrated to proteolytically activate LTA (20). Finally, structural studies have shown that a difference in the A2 linker of CT and LT causes a difference in stability: the A2 portion of CT confers a greater stability, and thus a greater ability to survive intact within the host (14). This difference in stability, as well as host cell receptor binding differences, may explain some of the differing effects of purified LT and CT on human volunteers described above.

Other hypotheses to explain the difference in the severity of ETEC and V. cholerae infections focus on differences in the bacteria themselves. Certainly, many factors including colonization, persistence, adhesins, and other toxins, in addition to the processing, secretion, and presentation (e.g. soluble versus insoluble) of CT and LT are likely to play an enormous role in determining the severity of the diseases caused by ETEC and V. cholerae. Both CT and LT are secreted by the general secretory pathway (GSP) across the outer membrane (21–23), however, our group has shown that, in contrast to the secretion of soluble CT, LT remains associated with the cell surface and is transported to the host cell in association with outer membrane vesicles (21, 24). The association between LT and the bacterial surface was determined to be mediated by an interaction between LTB and LPS (21).

The outer membrane of Gram-negative bacteria is unique in

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‡ The abbreviations used are: CT, cholera toxin; LT, heat-labile entero-toxin; ETEC, enterotoxigenic E. coli; LPS, lipopolysaccharide; Kdo, 3-deoxy-α-manno-octulosonic acid; ER, endoplasmic reticulum.
its asymmetry. The inner leaflet is composed of the same phospholipids as are found in the cytoplasmic membrane: phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (25). The predominant outer leaflet lipid component is lipopolysaccharide (LPS), an amphiphilic molecule made up of lipid A (an acylated disaccharide of glucosamine), core sugars (including Kdo [3-deoxy-D-manno-octulosonic acid]) specific to each genus, and O-antigen sugars that define serotype (reviewed in Refs. 26 and 27). Under standard laboratory conditions, the minimum essential LPS structure in E. coli is Kdo2-lipid A. Two Kdo sugars are added to lipid A by a bifunctional E. coli Kdo transferase (28). The core structures of V. cholerae and H. influenzae LPS differ from E. coli in that they consist of Kdo-phosphate-lipid A, which are synthesized by a monofunctional Kdo transferase and a Kdo kinase (29, 33). Studies have shown that E. coli can survive with a single Kdo when its Kdo-transferase mutant expressing the bifunctional E. coli Kdo transferase mutant expressing the monofunctional H. influenzae Kdo transferase, KanR, CmR, ts (33) E. coli Kdo-transferase mutant expressing the monofunctional H. influenzae Kdo transferase, CmR, ts (28)

### EXPERIMENTAL PROCEDURES

**Strains and Media**—Bacterial strains and plasmids used in this investigation are listed in Table I. Strains were grown in LB (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0), and maintained on LB agar. Transformations were performed using a modified CaCl2 protocol (34). Antibiotics were added as required at the following concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 50 μg/ml; chloramphenicol (Cm), 35 μg/ml; isothiogalactosyranoside (IPTG, 1.0 mM) was used to induce expression of LT and CT constructs. Unless specified, reagents were purchased from Fisher.

**LT-Bacterial Surface Binding Assay**—Similar to the previously described assay (21), bacteria were grown to mid-log phase, 1.0 ml was pelleted, washed twice in cold HEPES (50 mM, pH 6.8), and resuspended in 0.5 ml of HEPES. LT (0.5 μg, 60 pmol) (List Biologicals) was preincubated (25 °C, 30 min.) with HEPES or with a 10-fold molar excess of sonicated E. coli LPS (O55, Ra, Re, Rd, and Re), E. coli lipid A, Kdo, or V. cholerae LPS (Sigma) in HEPES. Washed bacteria (50 μl) were pelleted, washed, and resuspended, and 20 μl applied to 15% SDS-PAGE. A standard immunoblotting protocol was performed (34) using an LT cross-reactive anti-CT antibody, anti-rabbit-horseradish peroxidase (Sigma), and the ECL detection reagents (Amersham Biosciences). NIH-Image was used for quantitative densitometry of the immunoblots.

**BODIPY-GM1 Labeling**—Labeling was performed as described (21).

### RESULTS

**LT Binds to the Core Sugars of LPS**—In previous work we have determined that after secretion across the outer membrane, LT binds to LPS on the surface of ETEC. In addition, the addition of soluble, purified LT to an LT-deficient ETEC strain E9034P leads to LT binding and this interaction can be blocked with full-length purified LSps. However, G41 was incapable of blocking the interaction between LT and the bacteria. These data demonstrated that LT has the capability to bind to LPS at a binding site distinct from the GM1 binding site (21). We were interested in determining the minimum LPS components that are required for LT binding and, in particular, whether LT binds the lipid or sugar moieties. As published previously, purified full-length O55 LPS and Ra LPS, which lacked O-antigen, blocked the binding of LT to the surface of ETEC ((21) and Table II). In fact, Ra LPS blocked binding more effectively than did O55, indicating that LT binding does not require O-antigen. The lipid core of LPS, lipid A, was not able to inhibit LT binding. We tested shorter truncated forms of LPS and found that Ra LPS, which lacks most of its outer core sugars, did block binding (Table II). LPS derived from mutants expressing fewer core sugars (Rd and Re) were unable to inhibit binding significantly. These data indicate that LT binds to LPS independent of O-antigen and complete outer core sugars.

LPS dispersed in aqueous solution may not possess the same conformation as native LPS presented on the surface of a bacterium. Although purified O55, Ra, and Re LPS were able to block LT from binding to the surface of an ETEC cell expressing

### Table I

| Strains/plasmid  | Genotype or alias | Relevant characteristics | Ref. |
|------------------|-------------------|--------------------------|------|
| **Strains**      |                   |                          |      |
| E9034P           |                   | ETEC cured of virulence plasmid (LT-deficient) | (54) |
| E. coli CTB      |                   | E. coli K-12 with hns mutation, expressing GSP and CTB, KanR, CmR, AmpR | (55) |
| E. coli LTB      |                   | E. coli K-12 with hns mutation, expressing GSP and LTB, KanR, CmR, AmpR | (56) |
| K-12             |                   | E. coli GCSC #5158, StrR | (57) |
| Rd               |                   | E. coli Rough mutant, rfa1, CGSC #5158, StrR | (58) |
| Re               |                   | E. coli Deep rough mutant, rfa1, rfa31, CGSC #5156, StrR | (59) |
| Vibrio/LTB       |                   | Vibrio expressing LT, RifR, AmpR | (60) |
| Vibrio/LTB/KdtAa |                   | Vibrio expressing LTB and bifunctional E. coli KdtA, RifR, AmpR, CmR | (61) |
| CJB26            |                   | E. coli kdtA::kan recA−/pKdtAa | (62) |
| Kdo1             |                   | E. coli kdtA::kan recA−/pKdtAa | (63) |
| Kdo2             |                   | E. coli kdoA-transferase mutant expressing the bifunctional E. coli KdtA, KanR, CmR, ts | (64) |
| Kdo1P            |                   | E. coli Kdo-transferase mutant expressing the monofunctional H. influenzae KdtAa, KanR, CmR, ts | (65) |
| Plasmids         |                   | gspAB and gspC-B, CmR | (66) |
| GSP              | pCHAP4278          | IPTG-inducible LTB | (67) |
| LTB              | pMMB68            | IPTG-inducible CTB | (68) |
| CTB              | pLMP1             | Bifunctional E. coli Kdo transferase, CmR, ts | (69) |
| KdtAa            | pJSC2             | Monofunctional H. influenzae Kdo transferase, CmR, ts | (70) |

* This work.

K. White and C. Raetz, unpublished data.

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**TABLE I**

| Strains and plasmids | Strains and Media | Relevant characteristics | Ref. |
|----------------------|-------------------|--------------------------|------|
| **Strains**          |                   |                          |      |
| E9034P               |                   | E. coli K-12 with hns mutation, expressing GSP and CTB, KanR, CmR, AmpR | (55) |
| E. coli CTB          |                   | E. coli K-12 with hns mutation, expressing GSP and LTB, KanR, CmR, AmpR | (56) |
| K-12                 |                   | E. coli GCSC #5158, StrR | (57) |
| Rd                   |                   | E. coli Rough mutant, rfa1, CGSC #5158, StrR | (58) |
| Re                   |                   | E. coli Deep rough mutant, rfa1, rfa31, CGSC #5156, StrR | (59) |
| Vibrio/LTB           |                   | Vibrio expressing LT, RifR, AmpR | (60) |
| Vibrio/LTB/KdtAa     |                   | Vibrio expressing LTB and bifunctional E. coli KdtA, RifR, AmpR, CmR | (61) |
| CJB26                |                   | E. coli kdtA::kan recA−/pKdtAa | (62) |
| Kdo1                 |                   | E. coli kdtA::kan recA−/pKdtAa | (63) |
| Kdo2                 |                   | E. coli kdoA-transferase mutant expressing the bifunctional E. coli KdtA, KanR, CmR, ts | (64) |
| Kdo1P                |                   | E. coli Kdo-transferase mutant expressing the monofunctional H. influenzae KdtAa, KanR, CmR, ts | (65) |
| Plasmids             |                   | gspAB and gspC-B, CmR | (66) |
| GSP                  | pCHAP4278          | IPTG-inducible LTB | (67) |
| LTB                  | pMMB68            | IPTG-inducible CTB | (68) |
| CTB                  | pLMP1             | Bifunctional E. coli Kdo transferase, CmR, ts | (69) |
| KdtAa                | pJSC2             | Monofunctional H. influenzae Kdo transferase, CmR, ts | (70) |
LPS with O-antigen, LT may not be able to bind to the surface of a K-12 strain (lacking O-antigen) or an LPS mutant (lacking core sugars). We therefore wanted to compare the ability of LT to bind bacteria expressing wild type and mutant forms of LPS. LT bound to the surface of K-12, as well as isogenic Rd and Re mutant strains, demonstrating that LT binding to LPS is, in fact, independent of all core sugars but Kdo (Fig. 1A). These results were unexpected, given that purified Rd and Re LPS were unable to block LT from binding to wild type cells (Table II). The fact that this binding was inhibited with wild type O55 LPS (Fig. 1A), suggested that LT may have a higher affinity for LPS with sugars and a lower affinity for LPS with only core sugars. If this is the case, we predicted that, unlike the previous results with wild type LPS expressing bacteria (Table II), purified Re LPS would be able to block the binding of LT to Re mutant bacteria. Indeed, LT binding to the Re mutant was inhibited by purified Re LPS (Fig. 1B). Additionally, we found that preincubation with pure Kdo reduced LT binding to the Re mutant by 74 ± 8% (n = 3) (Fig. 1C), however Kdo did not inhibit LT binding to E9034P (data not shown). Together, these data suggest that LT binding to LPS relies minimal on the Kdo core however LT has a higher affinity for LPS with full-length core sugars.

LT and CT Bind the Surface of E. coli but not Vibrio—Unlike E. coli strains of Vibrio expressing CT, LT, and LTB secrete these products solubly, suggesting little to no association with the Vibrio membrane. Thus, we wanted to investigate the interaction of the toxins with Vibrio LPS and the surface of Vibrio using our in vitro assays. We observed that purified V. cholerae LPS was unable to block the binding of LT to ETEC. In case the native context of the LPS was critical to binding, we wanted to examine whether LT was able to bind to the surface of a Vibrio strain. We previously developed a quantitative fluorescence assay to demonstrate that expressed and secreted LT bound to the surface of ETEC (21). Using this assay with Vibrio expressing LTB, we found little BODIPY-GM1 binding (Fig. 2A). Immunoblotting confirmed that LTB was expressed and secreted by this strain (data not shown). Thus, although LT binds to E. coli LPS, it does not bind to Vibrio LPS.

Because CT and LT are structurally almost identical, we wondered whether CT would bind LPS. Interestingly, similar to LT, purified CT bound to the surface of ETEC and this association was blocked by soluble O55 LPS (Fig. 2B). Likewise, CTB expressed in a secretion-competent E. coli strain, which has a mutation in hns and overexpresses the GSP (21), was detected by BODIPY-GM1 binding, similar to LTB (Fig. 2A). Based on these data, we propose that CT and LT are secreted solubly from Vibrio because they cannot bind to Vibrio LPS, and that CT and LT remain associated with E. coli because they can bind E. coli LPS. These results further indicate that differences in the conformation or shape of E. coli and Vibrio LPS are critical to LT and CT binding.
This work describes the nature of the association between LT and the surface of the outer membrane of ETEC. Our prior work determined that LT binds LPS on the surface of E. coli independent of the O-antigen (21). LT also bound purified Ra LPS, which lacks O-antigen, and Rc LPS, which lacks outer core sugars, however, LT did not bind lipid A. Therefore, LT appeared to bind specifically the core sugars of LPS.

To examine the LT binding site on LPS, the ability of LT to bind to the surface LPS biosynthesis mutant strains expressing different truncations of LPS was tested. We were surprised to find that LT bound to rough (Rd) and deep rough (Re) E. coli. This result apparently contradicted the previous data that showed that purified Rd and Re LPS were unable to inhibit LT binding the surface of E. coli expressing full O-antigen. Therefore, we considered the possibility that LT may have a lower affinity for truncated LPS compared with longer LPS. The ability of purified Re LPS to block LT binding to Re E. coli proved that LT binds to E. coli Kdo2-lipid A but has a higher affinity for less truncated forms of LPS.

The minimal LT binding site on Re LPS was defined with further experiments. LT binding to Re LPS was significantly inhibited by preincubation with Kdo, implicating Kdo as the minimal portion of LPS required for LT binding. Purified lipid A did not inhibit LT binding to ETEC, suggesting that lipid A did not contain the LT binding site. However, it was possible that the affinity for purified lipid A was too low to compete with full length LPS in its native conformation. The fact that the Re-truncated LPS is the minimal requirement for E. coli viability prevented us from testing this directly. However, if lipid A is sufficient for LT binding, then every Gram-negative bacterial surface would be expected to bind LT, and we found that this was not the case. LT did not bind Vibrio. Thus toxin binding depends on the Kdo core of LPS. This result also suggested that LT discriminated between Kdo2-lipid A and Kdo1-phosphate-lipid A. Indeed, LT could not bind E. coli expressing phosphorylated Kdo-lipid A, whereas LT bound to E. coli expressing Kdo-lipid A. Additionally, LT could bind better to Vibrio expressing the E. coli Kdo-transferase which results in Kdo2-lipid A. Therefore, we conclude that LT binding to LPS depends on the unphosphorylated Kdo core.

Both LT and CT expressed in E. coli bind to the surface of E. coli. This indicates that LPS binding is not a characteristic unique to LT and cannot be explained by the 20% difference in amino acid sequence between LT and CT. Although the toxins LT and CT are almost identical, the diseases caused by ETEC and V. cholerae are very dissimilar. Whereas cholera is a disease of pandemic proportions, diarrhea caused by ETEC is much less severe. This difference was proposed to be due to the mode of toxin delivery: CT was secreted extracellularly and purportedly LT remained periplasmic. Work from our group and others, however, has disproven that theory since extracellular LT secretion can occur in E. coli by the GSP (21–23). Based on the results in this paper, we propose that a factor contributing to the difference between the diseases is the fate of the secreted toxin. LT and CT do not associate with the surface of Vibrio, nor does V. cholerae LPS block binding of LT to E. coli LPS. Therefore, perhaps the difference in LPS structure, rather than in the toxins of ETEC and V. cholerae significantly contributes to the difference in disease. LT and CT secreted by Vibrio is liberated into the medium because it cannot bind to Vibrio LPS which contains Kdo-phosphate (29). LT and CT secreted by ETEC remain associated with the surface of ETEC because E. coli LPS contains a non-phosphorylated Kdo core (35). Thus, the difference between the extracellular localization of these two similar toxins is most likely due to the differing
LPS core structures of *E. coli* and *V. cholerae* and this difference in the affinity of LPS for the toxins could significantly contribute to the difference in activity and spread of the toxin in the host.

As a consequence of the LT-LPS interaction, one would predict LT to remain associated with the outer surface of ETEC and never be released into the extracellular milieu. However, ETEC, similar to all Gram-negative bacteria, produce outer membrane vesicles (21, 36, 37). We have shown that ETEC vesicles harbor LT both in the lumen and bound to their surface (21, 24). The secretion of LT on the surface of vesicles effectively decreases the number of functional toxin units secreted when compared with the release of soluble CT by *V. cholerae*, thereby potentially decreasing the number of host cells affected by the toxin. In addition, LT and CT have been shown to inhibit cytokine release (38, 39). Thus the association of LT with vesicles also affects the host immune response.

Because all Gram-negative bacteria produce vesicles, we do not believe that ETEC evolved vesiculation exclusively for the transport of LT. Nor do we suggest that the core of *E. coli* LPS evolved for the sole purpose of binding to LT. It is far more likely that LT, acquired by *E. coli* from *V. cholerae* via horizontal gene transfer (40), bound *E. coli* LPS by chance and therefore was retained on the cell and vesicle surface. Regardless of the manner by which ETEC came to transport LT via vesicles, vesicles are the final step in LT secretion and LT transported on vesicles is toxic. Therefore vesicles should be considered a specific secretion mechanism for virulence factors.

Several recent observations in other Gram-negative bacteria have revealed that host cell delivery of toxin bound to the surface of outer membrane vesicles is a strategy not unique to ETEC. Kolling et al. (41) demonstrated that shiga toxin is present in vesicles produced by O157:H7. Vesicle-associated toxin was partially protease sensitive, which we interpret as demonstrating surface association of some of the toxin. Leukotoxin has been detected on the surface of *Actinobacillus actinomycetemcomitans* cells (42), and that toxin has been found in association with the membrane of the vesicles produced by that organism (43). Immunogold electron microscopy was used to detect a protease on the surface of Apx-containing *Actinobacillus pleuropneumoniae* vesicles (44) and *Helicobacter pylori* vacuolating toxin, VacA, closely associated with the membrane of *H. pylori* vesicles (45). These vesicles all deliver physiologically active toxin to host cells.

The ability to bind both LPS and G *M*₃ enables LT to act as an adhesive, promoting an interaction between the vesicle and host cell, resulting in internalization of the vesicle and all its contents.³ We previously demonstrated that the G *M*₃ and LPS binding sites on LT are distinct (21), and we wondered if a LPS binding site could be elucidated from the solved crystal structures of LT and CT (2, 46–50). Based on examination of the crystal structures of many different lectins (bacterial, mammalian, and plant), a general ligand binding motif emerges. Sugar-binding proteins interact with their receptors at the periphery or surface of the protein, often “snuggled” under an exterior loop. We identified such a region in the crystal structure of CT (and similarly, LT) bound to G *M*₃ pentasaccharide, directly opposite the G *M*₃ binding site on the B-pentamer (Fig. 4A, star). This could be the region that LT/CT bind to *E. coli* LPS. Mutations in this putative LPS binding pocket of LT and CT were found to confer binding to A and B blood group determinants (51). Intriguingly, molecular dynamics simulations conducted in that work defined exactly the same binding pocket for blood group antigens as we have proposed for LPS. As a result of these insights, we propose a model in which LT/CT binds *E. coli* LPS in a manner that allows LT/CT to bind simultaneously to one or more receptors (but not five) on the epithelial cell (Fig. 4B).

When the structure of CT was solved with the G *M*₃ pentasaccharide, Merritt et al. (47) presented a model of how CT (and LT) would interact with the host cell. In this model, the toxin “lands” flat on the surface of the host cell, binding multiple G *M*₃ receptors and filling all of the five binding pockets. Based on this model, however, it is difficult to envision how LT could be bound to LPS on the surface of a vesicle, and still “land” on the surface of an epithelial cell. We propose that the geometry of LT/CT binding to G *M*₃ is more flexible than previously described, in that the toximay bind G *M*₃ at an angle other than 90° and have freedom to rotate and bend. This model does not preclude the possibility that after this “docking event,” the toxin follows the model proposed by Merritt, filling all five of its G *M*₃ binding sites. This may bring the vesicle in closer proximity to the host cell, allowing secondary adhesins in the vesicle membrane to bind their receptors, increasing the intimacy of the interaction between host and vesicle.

To date, no other protein has been demonstrated to interact with LPS in the bacterial membrane in the same manner as LT. The interaction of leukotoxin to the surface of *A. actinomycetemcomitans* appears to be mediated by nucleic acids bound to the bacterial surface (52). Adenylate cyclase toxin binds the surface of *Bordetella pertussis* via filamentous hemagglutinin (53). Because shiga toxin, a member of the AB₅ toxin family, is most likely on the surface of EHEC vesicles, examination of the association between shiga toxin and the outer membrane of EHEC may show that LPS plays a role. In addition, further study of the previously described toxins that are transported via vesicles may yield more proteins that bind to LPS in a lectin-like fashion.

Our studies have disproven the theories that bacterial retention of LT or a lack of the GSP are the reasons for the reduced toxicity of ETEC compared with *V. cholerae*. We have proposed

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² A. Horstman, K. Mason, and M. Kuehn, unpublished data.
³ N. Kesty, K. Mason, and M. Kuehn, submitted manuscript.
that the difference between the diseases caused by *V. cholerae* and ETEC are in part due to the difference in the affinity of their toxins to their LPS. One may question the evolutionary perpetuation of the attenuation of the toxicity of LT bound to their toxins to their LPS. One may question the evolutionary and ETEC are in part due to the difference in the affinity of their toxins to their LPS. One may question the evolutionary perpetuation of the attenuation of the toxicity of LT bound to V. cholerae and E. coli in *Escherichia coli* and *Salmonella* (Neidhardt, F., ed) Vol. 1, pp. 1035–1063, ASM Press, Washington, D. C.

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