Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells

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Enterotoxigenic *Escherichia coli* (EPEC) is a prevalent cause of traveler's diarrhea and infant mortality in third-world countries. Heat-labile enterotoxin (LT) is secreted from EPEC via vesicles composed of outer membrane and periplasm. We investigated the role of EPEC vesicles in pathogenesis by analyzing vesicle association and entry into eukaryotic cells. Fluorescently labeled vesicles from LT-producing and LT-nonproducing strains were compared in their ability to bind adrenal and intestinal epithelial cells. EPEC-derived vesicles, but not control nonpathogen-derived vesicles, associated with cells in a time-, temperature-, and receptor-dependent manner. Vesicles were visualized on the cell surface at 4°C and detected intracellularly at 37°C. EPEC vesicle endocytosis depended on cholesterol-rich lipid rafts. Entering vesicles partially colocalized with caveolin, and the internalized vesicles accumulated in a nonacidified compartment. We conclude that EPEC vesicles serve as specifically targeted transport vehicles that mediate entry of active enterotoxin and other bacterial envelope components into host cells. These data demonstrate a role in virulence for EPEC vesicles.

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Introduction

Enterotoxigenic *Escherichia coli* (EPEC) is a leading cause of childhood and traveler's diarrhea (Levine, 1987; Hyams *et al.*, 1991; Gyles, 1992; Smith, 1992). Diarrheal disease is the third leading cause of death by infectious diseases in the world (WHO, 1997), and an estimated 800 000 childhood deaths per year have been attributed to EPEC (Gaastra and Svennerholm, 1996). Half of EPEC strains isolated from

children with diarrheal disease were positive for the production of heat-labile enterotoxin (LT; Qadri *et al.*, 2000). LT is an AB₅ toxin consisting of a catalytically active LT_A subunit and a pentameric ring of LT_B subunits responsible for binding and internalization. The LT_B subunit binds monosialoganglioside (G_{M1}) and (with a lower affinity) other receptors with terminal galactose units (Fukuta *et al.*, 1988). Subsequent to internalization, LT is transported via the endoplasmic reticulum (ER) to the cytosol. The ADP-ribosylation of the G_{ss} subunit in the adenylate cyclase pathway by LT_A leads to an increase in cAMP (Spangler, 1992; Verlinde *et al.*, 1994; Cieplak *et al.*, 1995) and a net efflux of electrolytes and water into the lumen of the proximal small intestine, known clinically as watery diarrhea.

LT is 80% homologous to cholera toxin (CT) produced by *Vibrio cholerae* and is similar in both structure and function (Dallas and Falkow, 1980; Gyles, 1992; Lencer *et al.*, 1999). Both LT and CT bind G_{M1}, which is enriched in eukaryotic membrane lipid rafts found in microdomains that are also enriched in cholesterol and glycosphingolipids. After binding G_{M1}, CT is internalized via a non-clathrin-dependent endocytic pathway that leads to delivery to the Golgi complex (Orlandi and Fishman, 1998; Lencer *et al.*, 1999; Nichols *et al.*, 2001). Recent studies have shown that cell-associated LT fractionates with detergent-resistant membranes in light-density fractions, a property characteristic of lipid rafts (Shimizu *et al.*, 2003).

Prior investigations of the activity of LT on host cells used purified soluble LT prepared from *E. coli* cell extracts (Schnitzer *et al.*, 1996; Orlandi and Fishman, 1998; Wolf *et al.*, 1998), which is unlikely to be a physiologically relevant form of LT. LT is not detected in the soluble fraction of EPEC culture supernatants, but instead is found associated with bacterial outer membrane vesicles (Gankema *et al.*, 1980; Wai *et al.*, 1995; Horstman and Kuehn, 2000). Until recently, LT was thought to be a periplasmic protein complex that EPEC was incapable of secreting. We and others determined that the general secretory pathway does transport LT out of the periplasm (Horstman and Kuehn, 2002; Tauschek *et al.*, 2002), but that LT remains membrane-associated due to its affinity to the Kdo core of lipopolysaccharide (LPS) (Horstman *et al.*, 2004). Greater than 95% of the catalytically active LT secreted via the general secretory pathway is associated with vesicles, in the vesicle lumen and bound externally via LPS (Horstman and Kuehn, 2002).

Several lines of data support an active role for vesicles in bacterial pathogenesis. Vesicles are ubiquitously shed by Gram-negative bacteria and have been proposed to be vehicles for virulence factor delivery to host cells (Kadurugamuwa and Beveridge, 1997; Beveridge, 1999; Wai *et al.*, 2003). Bacterial vesicles consist of outer membrane lipids and a subset of outer membrane proteins and soluble periplasmic components (Beveridge, 1999; Horstman and Kuehn, 2000; Wai *et al.*, 2003; Kesty and Kuehn, 2004). Several investigations have demonstrated that budded

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portions of outer membrane material are shed *in vivo*: vesicles produced by *Helicobacter pylori* were found in human gastric epithelium biopsies (Fiocca *et al*, 1999; Heczko *et al*, 2000), and outer membrane protein–LPS complexes have been found in the serum of septic patients and rats (Brandtzaeg *et al*, 1992; Hellman *et al*, 2000). Vesicles from pathogenic strains such as *Pseudomonas aeruginosa*, *H. pylori*, *Actinobacillus actinomycescomitans*, and pathogenic *E. coli* contain active virulence factors, such as proteases, proinflammatory proteins, and toxins (Kadurugamuwa and Beveridge, 1995, 1997; Kolling and Matthews, 1999; Keenan and Allardyce, 2000; Keenan *et al*, 2000; Kato *et al*, 2002; Wai *et al*, 2003). However, the molecular mechanism of virulence factor delivery via vesicles has been unclear.

Previously, we demonstrated that naturally shed ETEC vesicles induced a dose-dependent morphological change in Y1 cells that was inhibited by the addition of G_{M1} (Horstman and Kuehn, 2000). Here, we investigate how LT directs ETEC vesicle association with host cells and the consequent internalization of vesicle components. The results elucidate a novel mechanism for bacterial virulence factor transmission into host cells.

Results

ETEC vesicles associate with host cells via the toxin receptor

To investigate how toxic bacterial vesicles interact with Y1 cells, purified vesicles from ETEC and a nontoxic *E. coli* strain, HB101, were labeled with fluorescein isothiocyanate (FITC). FITC vesicles were incubated with Y1 adrenal cells, which become round in response to incubation with soluble toxin or toxic vesicles (Donta *et al*, 1974; Horstman and Kuehn, 2000). FITC labeling did not substantially reduce vesicle toxicity (0–20% reduced activity). The intensity of fluorescence associated with Y1 cells increased as the incubation time with the FITC-ETEC vesicles increased (2–8 h) and paralleled the increase in cell rounding (Figure 1A–C, see toxicity scores in lower right of panels). After an 8 h incubation, the rounded Y1 cells that had been incubated with FITC-ETEC vesicles contained intensely fluorescent ‘punctate’ areas (Figure 1C–E). In contrast, cells incubated with FITC-HB101 vesicles were not rounded and contained a low level of dispersed fluorescence (Figure 1F). These results show that the toxicity of ETEC vesicles correlates with the association of the vesicles with the cells.

To determine whether the observed fluorescence of Y1 cells incubated with FITC-ETEC vesicles depended on the interaction between the vesicle-associated LT and G_{M1}, the eukaryotic cell receptor for LT, we performed competition experiments. Fluorescence of Y1 cells was significantly reduced for incubations with FITC-ETEC vesicles pretreated with G_{M1} (Figure 1G), suggesting that LT located on the surface of vesicles directed binding of vesicles to Y1 cells. The low level of fluorescence with G_{M1}-treated vesicles was similar to the fluorescence observed when cells were incubated with FITC-HB101 vesicles (Figure 1F). The addition of 100-fold excess unlabeled nontoxic HB101 vesicles did not alter the fluorescence pattern observed with FITC-ETEC vesicle binding, indicating that the nontoxic vesicles did not compete with the LT-containing fluorescent vesicles. In con-

trast, a 100-fold excess of unlabeled ETEC vesicles decreased the amount of FITC-ETEC vesicles associated with Y1 cells (data not shown). These data suggest that ETEC vesicles interact specifically with host cells via the LT–receptor interaction resulting in localized high concentrations of cell-associated fluorescence.

HT29 human intestinal epithelial cells exemplify a cell type that pathogenic *E. coli* strains may encounter *in vivo*. A quantitative assay was developed based on the linear relationship between FITC-vesicle fluorescence and vesicle protein concentration to assess objectively FITC-vesicle association with HT29 cells. The amount of ETEC vesicles associated with HT29 cells increased over a 24 h time course (Figure 2A). ETEC vesicle association dropped by 52% when vesicles were preincubated with G_{M1} prior to an 8 h incubation with HT29 cells, a level similar to the low association observed with nontoxic HB101 vesicles (Figure 2A). Soluble LT causes vacuole formation in HT29 cells (Charantia *et al*, 1992; Spangler, 1992), and we observed that after an 8 h incubation, ETEC vesicles also caused vacuolization of HT29 cells (compare Figure 3A and B) that was inhibitable with G_{M1} (data not shown). Another indicator of LT toxicity is an increase in cellular cAMP. ETEC vesicles increased the cAMP level in HT29 cells and this increase was reduced by preincubation with G_{M1}, similar to the effects seen with CT and G_{M1}-treated CT (Figure 2B).

ETEC vesicle association with cells may depend on factors located on the surface of the vesicles other than LT. To isolate the role of LT in vesicle binding to cells, we used vesicles purified from MC4100 Δhns/GSP/LT (LT+), a genetically modified nonpathogenic *E. coli* strain previously shown to export and surface-localize plasmid-encoded LT, as well as an isogenic strain, MC4100 Δhns/GSP (LT–), that does not express LT (Horstman and Kuehn, 2002). Similar to HT29 cells incubated with FITC-ETEC vesicles, bright punctate staining was seen in HT29 cells incubated with the vesicles purified from the LT+ strain (Figure 4A), and this staining was dramatically reduced with G_{M1} pretreatment (Figure 4B). We observed 60% less cell-associated fluorescence in incubations using LT– vesicles compared with LT+ vesicles (Figure 4C and D). These results are consistent with the clear reduction in cell-associated fluorescence when nontoxic FITC-vesicles are incubated with Y1 or HT29 cells and when LT on the vesicles is ‘blocked’ by preincubating ETEC vesicles with G_{M1} (Figures 1F, G, and 2A). We conclude that LT on ETEC vesicles is critical for both epithelial cell binding and toxicity.

Toxic vesicles are internalized

We investigated the fate of ETEC vesicles by examining whether the emergence of punctate fluorescence was temperature dependent, a hallmark of cellular internalization (Anderson *et al*, 1992; Pelkmans *et al*, 2001). Incubation of Y1 cells with FITC-ETEC vesicles at 4°C resulted in diffuse, peripheral fluorescence (Figure 1H). We also detected 60% less cell-associated fluorescence with HT29 cells incubated with vesicles at 4°C compared with incubations performed at 37°C (Figure 2A). Because vesicle association with Y1 and epithelial cells was distinctly temperature dependent, it was possible that vesicles accumulated intracellularly at 37°C.

We probed the cellular localization of FITC-labeled vesicles using variations in temperature and cell media pH. Because

Figure 1 Interaction of ETEC vesicles with Y1 adrenal cells depends on the LT receptor G_{M1} and is temperature sensitive. FITC-ETEC vesicles were incubated with Y1 cells at 37°C for 2 h, (A), 4 h (B), or 8 h (C) and visualized by phase contrast (left panel) and confocal microscopy (right panel). Toxicity scores (see Materials and methods) are indicated in the left panels. (D, E) Magnified confocal microscopy of isolated Y1 cells following incubation with FITC-ETEC vesicles for 8 h. Confocal microscopy of Y1 cells incubated for 8 h with FITC-HB101 vesicles at 37°C (F), FITC-ETEC vesicles pretreated with G_{M1} and incubated for 8 h at 37°C (G), or FITC-ETEC vesicles incubated for 8 h at 4°C (H). Size bars apply to all panels in each section (A–C, 20 μ m; D, E, 5 μ m; F–H, 20 μ m).

FITC is pH sensitive (Pelkmans *et al*, 2001), the fluorescence due to external cell surface-bound vesicles would be susceptible to changes in media pH, whereas the fluorescence of internalized vesicles would not depend on media pH. To investigate whether cell-associated fluorescence was internal, we performed a temperature pulse-chase experiment and

subsequently compared the amount of cell-associated fluorescence in pH 4 and pH 8 media. HT29 cells were incubated with FITC-ETEC vesicles for 2 h at 4°C, washed, and then 'chased' at 37°C for either 4 or 24 h. The treated cells were then incubated briefly in pH 4 or pH 8 media and the amount of cell-associated fluorescence was measured. In cell-free

Figure 2 ETEC vesicle association with HT29 colorectal, epithelial cells is G_{M1}-, time-, and temperature-dependent and causes an increase in cAMP. (A) Cell-associated fluorescence was quantitated for HT29 cells incubated with FITC-labeled vesicles. Left, time course for FITC-ETEC vesicle incubations. Right, FITC-HB101 vesicles incubated for 8 h at 37°C; FITC-ETEC vesicles preincubated with G_{M1} for 8 h at 37°C; or FITC-ETEC vesicles incubated for 8 h at 4°C, as indicated. Data are represented as the percent of cell-associated fluorescence relative to the 8 h incubations with untreated FITC-ETEC vesicles $\pm$ s.e.m. Significant difference was determined using Student's *t*-test. *Treatment is significantly different from 8 h treatment with FITC-ETEC vesicles (*P*-value < 0.001), *n* > 9. (B) Concentration of cAMP (pmol/well) produced by HT29 cells incubated for 4 h with CT, CT preincubated with G_{M1}, ETEC vesicles, or ETEC vesicles preincubated with G_{M1}. *n* = 3.

incubations, 20% of the fluorescence of FITC-vesicles was resistant to incubation in pH 4 media compared with pH 8 media (data not shown). Approximately 60% of the cell-associated fluorescence of HT29 cells incubated with vesicles at 4°C was resistant to acidic (pH 4) conditions (Figure 5A, 0 h chase), suggesting that even at 4°C, the cells afforded some protection to acid quenching of the fluorescence. Acid-resistant fluorescence increased significantly to nearly 100% when vesicles had been incubated with the cells for 4 or 24 h at 37°C (Figure 5A, 4 and 24 h chase).

To examine the cellular localization of ETEC vesicles further, wheat germ agglutinin (WGA) was utilized as a eukaryotic cell surface marker. Following incubations of HT29 cells with FITC-ETEC vesicles for 8 h at 37 or 4°C, the plasma membrane was labeled with WGA. As demonstrated by confocal microscopy, vesicles incubated with cells at 4°C colocalized with WGA on the exterior of the cell (Figure 5B). For incubations at 37°C, few FITC-ETEC vesicles colocalized with WGA, and distinct green punctate and red membrane staining was observed (Figure 5C). These data support the

Figure 3 ETEC vesicle association with HT29 cells causes vacuolization. Electron microscopy of thin sections of HT29 cells (A) and HT29 cells incubated with 10 μ g ETEC vesicles for 8 h (B). Crescent-shaped vacuoles (CV) were apparent in both vesicle-treated and untreated cells. Other subcellular structures include microvilli (MV), toxin-induced vacuoles (Vac), and tight junction desmosomes (TJ). Ex designates the extracellular space. Ves designates possible extracellular ETEC vesicles. Possible intracellular vesicles are visible in and around vacuoles. Size bar indicates 5 μ m.

hypothesis that ETEC vesicles are internalized by host cells and accumulate in an intracellular compartment distinct from the plasma membrane.

Externally applied antibodies are unable to access internalized antigens and therefore can also be used as probes to detect internalization. Anti-fluorescein antibodies quenched 73% of the FITC-ETEC vesicle fluorescence in cell-free incubations (Figure 5D, left two lanes). Similarly, 80% of the fluorescence was quenched by anti-fluorescein when vesicles were coincubated with cells for 8 h at 4°C, demonstrating the

continued accessibility of the external probe to the FITC-ETEC vesicles at cold temperature (Figure 5D, middle two lanes). However, when vesicles were incubated with cells at 37°C, greater than 80% of the fluorescence remained unquenched after 8 h (Figure 4D, right two lanes). This result indicates that a majority of the cell-associated FITC vesicles were intracellular.

For the localization experiments described thus far, it was possible, albeit unlikely, that the FITC-labeled material associated with cells was only LT that had dissociated from

Figure 4 LT mediates the interaction of *E. coli* vesicles with HT29 cells. Confocal microscopy of HT29 cells incubated at 37°C for 8 h with MC4100 Δhns/GSP/LT (LT+) FITC-vesicles (A), G_{M1}-pretreated LT+ FITC-vesicles (B), or FITC-MC4100 Δhns/GSP (LT-) FITC-vesicles (C). Size bars indicate 5 μm. (D) Cell-associated fluorescence was quantitated for HT29 cells incubated with FITC-MC4100 Δhns/GSP/LT vesicles (LT+) and FITC-MC4100 Δhns/GSP vesicles (LT-) for 8 h at 37°C. Data are represented as the percent cell-associated fluorescence relative to the incubations with FITC-MC4100 Δhns/GSP/LT vesicles ± s.e.m. Significant difference was determined using Student's *t*-test. *Treatment is significantly different (*P*-value < 0.001), *n* > 6.

Figure 5 ETEC vesicles are internalized by HT29 cells. (A) HT29 cells were incubated with FITC-ETEC vesicles at 4°C ('pulse') and fluorescence was measured in buffer at pH 4 and pH 8 after incubations for 0, 4, or 24 h at 37°C ('chase'). The percent acid-resistant fluorescence represents the ratio of fluorescence at pH 4 as compared to the fluorescence at pH 8 ± s.e.m. Significant difference was determined using unpaired Student's *t*-test. *Acid-resistant fluorescence after the 4 and 24 h chase is significantly different from acid-resistant fluorescence after the pulse (0 h) (*P* < 0.003), *n* > 5. HT29 cells were incubated with FITC-ETEC vesicles for 8 h at 4°C (B) or 37°C (C) before incubation with WGA at 4°C to label the exterior of cells. Yellow indicates colocalization of vesicles (green) with WGA (red) at the cell surface. Size bars indicate 20 μm in the upper panels and 5 μm in the lower panels. (D) First panel: 11 ng of FITC-ETEC vesicles (equivalent to the amount of vesicles bound to HT29 cells after an 8 h incubation) was incubated without (-) and with (+) anti-fluorescein antibody (αFL) for 1 h in the absence of cells (-cells). HT29 cells incubated with FITC-ETEC vesicles for 8 h at 4°C (middle panel) and 37°C (last panel) before incubation without (-) and with (+) anti-fluorescein antibody for 1 h. Data are represented as the percent fluorescence relative to incubations without antibody for each treatment ± s.e.m. Significant difference was determined using Student's *t*-test. *Treatment is significantly different from treatment without quenching (*P* < 0.001), *n* > 6.

vesicles, and that we were detecting only the trafficking of LT. To examine whether cells internalized components of ETEC vesicles other than LT, we used an antibody raised to a non-LT-producing *E. coli* strain and probed the localization of vesicle components with a rhodamine-labeled secondary antibody and confocal microscopy. Consistent with our results demonstrating vesicle internalization after an 8 h incubation, the brightest FITC-labeled spots that were predicted to be in the interior of the cells were not accessible to the externally applied, rhodamine-labeled anti-*E. coli* antibody and thus appeared green in the merged images (Figure 6A and B). Colocalization of rhodamine with some of the FITC dots appeared yellow and was detected primarily on the cell periphery (Figure 6A, arrows) demonstrating that vesicle antigens other than LT were also bound to the cell surface. By contrast, if the cells were permeabilized with 1% Triton X-100 prior to antibody labeling, all FITC-labeled spots colocalized with rhodamine, both externally and internally (Figure 6C). The presence of *E. coli* antigens inside permeabilized cells demonstrates that vesicle components in addition to the toxin were internalized.

In order to identify and better localize vesicle components inside and outside cells, thin sections of HT29 cells incubated without and with ETEC vesicles were examined and compared (Figure 3A and B, and Supplementary figure). Structures were visible that appeared to be cell-associated and internalized vesicles. Immunogold electron microscopy was used to confirm the extracellular and intracellular location of cell-associated vesicles. Immunogold staining of both external vesicles and internal vesicle components was detected following an 8 h incubation using either anti-*E. coli* (Figure 7A–D and F) or anti-LPS antibodies (Figure 7E). Since these antibodies do not recognize the toxin, these results support the findings that outer membrane components, including but not limited to LT, are internalized. We note that internalized vesicle antigens were often seen in association with internal membrane structures and at the edges of the large, toxin-induced vacuoles (Figure 7D and F, arrows). Together, these results show that LT mediates toxic vesicle binding to host cells and subsequent internalization, resulting in long-lived accumulation of vesicle components within the cells.

Vesicle endocytosis occurs via lipid rafts

Bacteria and bacterial products are internalized by host cells via endocytic pathways including lipid rafts and clathrin-coated pits. Soluble LT and CT bind G_{M1} , a receptor present in lipid rafts (Parton, 1994; Orlandi and Fishman, 1998; Lencer *et al*, 1999; Nichols *et al*, 2001; Nichols, 2003). Since re-incubation with G_{M1} reduced ETEC vesicle association with host cells (Figures 1G and 2A), we wanted to determine whether ETEC vesicles enter cells via lipid rafts. We used inhibitors of endocytosis and examined fluorescent vesicle uptake. Filipin, an inhibitor of cholesterol-rich lipid raft formation (Orlandi and Fishman, 1998; Lencer *et al*, 1999), inhibited soluble rhodamine-CT internalization but not rhodamine-transferrin internalization (data not shown). Filipin dramatically reduced the association of FITC-ETEC vesicles with HT29 cells (compare Figure 8A and B). In contrast, chlorpromazine, an inhibitor of clathrin-dependent endocytosis, had no effect on FITC-ETEC vesicle association (Figure 8C) but significantly reduced the uptake of transferrin

Figure 6 ETEC vesicle components other than LT are bound to and within HT29 cells. Confocal microscopy of Y1 cells incubated for 8 h at 37°C with 1 μ g FITC-ETEC vesicles (green), fixed, and treated with anti-*E. coli* antibody detected using rhodamine-labeled anti-rabbit antibody (red). (A, B) Intact cells exhibit distinct internalized FITC vesicles (green) and FITC vesicles on the cell surface colabeled with rhodamine antibody (arrowheads, yellow). (C) Cells permeabilized with detergent prior to antibody labeling demonstrate rhodamine and FITC colocalization (yellow) of all cell-associated vesicles. Size bars represent 10 μ m (A) and 20 μ m (B, C).

(data not shown). In untreated cells, FITC-ETEC fluorescence was seen throughout the cell (Figure 8D), while in filipin-treated cells, fluorescent vesicle aggregates remained localized at the cell surface (Figure 8E). These data suggest that ETEC vesicle components enter cells via cholesterol-rich lipid rafts.

Since FITC fluorescence is sensitive to a reduction in pH, we used HT29 cell-associated fluorescence to determine if

Figure 7 Thin-section electron microscopy of ETEC vesicle binding and internalization by HT29 cells. Immunogold electron microscopy of thin sections of HT29 cells incubated for 8 h at 37°C with ETEC vesicles, treated with anti-*E. coli* antibody (A–D, F) or anti-LPS antibody (E), and detected with gold-labeled anti-rabbit antibody. Gold-labeled vesicles were detected bound to the exterior (Ex) of cells (arrowheads) and in internal compartments (arrows), often near vacuoles (Vac). (A, inset) Magnification of bound vesicle (large arrowhead in A). Size bars represent 100 nm.

vesicle components enter an acidified compartment. We calculated that the total amount of vesicle fluorescence in the incubation at the initial time point was accounted for by adding cell-associated and unbound fluorescence after an

incubation for 8 h at 37°C. In 4°C/37°C pulse–chase experiments, cell-associated fluorescence did not decrease during an 8 h chase (data not shown). This strongly indicates that FITC-labeled ETEC vesicles are trafficked into stable,

nonacidified intracellular compartments, consistent with the intracellular destination of other products that have been endocytosed by lipid rafts (van der Goot and Harder, 2001).

To characterize further the endocytic pathway used by ETEC vesicles, we examined whether vesicle-associated fluorescence colocalized with caveolin or clathrin by microscopy. Rhodamine-ETEC vesicles were incubated with HT29 cells before immunostaining with FITC for caveolin or

clathrin. The punctate rhodamine staining of the vesicles was frequently colocalized with the punctate stain of the FITC-caveolin and appeared yellow in the merged images (Figure 9A, arrows). In contrast, rhodamine-labeled vesicles and FITC-labeled anti-clathrin rarely colocalized (Figure 9B).

Figure 8 Association of ETEC vesicles with HT29 cells is filipin sensitive and chlorpromazine insensitive. Confocal microscopy of untreated (A), filipin-pretreated (B), or chlorpromazine-pretreated (C) HT29 cells incubated with FITC-ETEC vesicles for 24 h at 37°C. Z-section of a single HT29 cell incubated with FITC-ETEC vesicles without (D) and with (E) filipin. Size bars indicate 20 μm (A–C) and 2 μm (D, E).

Figure 9 ETEC vesicles colocalize with caveolin but not clathrin. Confocal microscopy of HT29 cells incubated with rhodamine-ETEC vesicles (red) for 8 h at 37°C before staining with anti-caveolin (A) or anti-clathrin (B) antibodies detected with FITC-labeled anti-mouse antibody (green). Red vesicles colocalized with green caveolin to give yellow spots (arrowheads) but did not colocalize with clathrin. Size bars indicate 5 μm. (C, D) HT29 cells were incubated with ETEC vesicles for 18 h at 4°C followed by 1 h at 37°C. Vesicles were visualized using anti-*E. coli* antibody and 10 nm gold-conjugated anti-rabbit antibody. Caveolin was detected with anti-caveolin antibody and 5 nm gold-conjugated anti-mouse antibody (arrowheads). No 5 nm gold label for caveolin was detected near bound 10 nm gold-labeled vesicles prior to the 37°C chase (not shown). Ex, extracellular; C, cytoplasm. Size bars represent 100 nm.

Since we demonstrated that vesicles continue to bind and internalize throughout the incubation with cells (Figure 2A), these data, visualized at 8 h, represent the localization of vesicle components and endocytic markers throughout the entire binding and internalization pathway. To investigate vesicle entry, immunogold electron microscopy was performed with 4°C pulse–37°C chased cells using anti-*E. coli* and anti-caveolin antibodies. After a 1 h chase at 37°C, anti-*E. coli* 10 nm gold labeling (large dots) was seen in the vicinity of 5 nm gold/anti-caveolin labeling (small dots indicated with arrowheads), both near the surface and internally (Figure 9C and D). These results agreed with the immunofluorescence results, suggesting that the lipid raft marker, caveolin, is near vesicles at an early stage of vesicle internalization.

Discussion

Despite a great deal of published data regarding the activity of soluble LT derived from the periplasm of *E. coli*, nothing was known regarding the host cell interaction of vesicle-associated LT, which is the secreted form of LT. We describe here that ETEC-derived vesicles are potent vehicles for toxin transmission into host cells, and that LT is the adhesin that catalyzes internalization of ETEC vesicles into host cells. ETEC vesicles associated with eukaryotic cells in a time-, temperature-, and LT-dependent manner, and their toxicity correlated with their internalization into intestinal epithelial cells. A specific potent inhibitor of cholesterol-dependent endocytosis, filipin, was found to diminish vesicle internalization significantly. The toxicity of ETEC vesicles and its inhibition with Brefeldin A (N Kesty, K Mason, and M Kuehn, unpublished data) demonstrate that vesicle-delivered toxin is trafficked via retrograde transport through the Golgi and ER. The other vesicle components (e.g. other luminal vesicle proteins, LPS, outer membrane proteins) appeared to accumulate in nonacidified compartments inaccessible to the extracellular milieu. These data support the model that toxic vesicles bind to a receptor within a lipid raft, lipid raft components such as caveolin are recruited to these sites, the raft-bound vesicle is internalized and retained, and the toxin is trafficked to the Golgi and ER. These results demonstrate for the first time the molecular basis for the intracellular delivery of toxin by native bacterial vesicles.

We were able to distinguish between two potential pathways of vesicle-mediated toxicity. In the first model, LT travels via vesicles, but becomes dissociated before entry into the host cell. In another scenario, LT remains bound to the vesicles upon cell internalization, thereby mediating intracellular delivery of the vesicle together with LT. Using confocal and electron microscopy, we found *E. coli* antigens other than the toxin in intracellular compartments, demonstrating that LT did not dissociate from vesicles prior to binding. Since LT is both luminal and externally bound, and luminal vesicle components are internalized, these data do not distinguish whether ETEC vesicle toxicity is due to the externally bound or lumenally contained LT. We discovered that LT bound to LPS on the bacterial cell surface can simultaneously bind G_{M1} (Horstman and Kuehn, 2002; Horstman *et al*, 2004), further validating the possibility that LT remains bound to the vesicles upon cell internalization.

LT, rather than LPS or other vesicle surface components, performs a specific targeting function that is critical to vesicle–cell association and internalization. G_{M1} addition significantly reduced the association of ETEC vesicles, and vesicles from a laboratory *E. coli* strain expressing secreted LT resulted in cell-associated fluorescence that was bright, punctate, and increased over time, similar to ETEC vesicles. Vesicles produced by strains that do not express toxin bound cells to a lower extent and in a manner that did not inhibit toxin-mediated vesicle binding. These results are consistent with either a model in which the LT/G_{M1} interaction directly triggers vesicle endocytosis or a model in which the LT/G_{M1} binding is followed by secondary contacts mediated by other receptor binding vesicle surface constituents that lead to subsequent endocytosis. Since we observed that cell-associated fluorescence was slightly lower for cells incubated with FITC-LT+ vesicles than with FITC-ETEC vesicles, other properties of ETEC appear to be important for optimal toxic vesicle interactions, perhaps those subsequent to an initial LT/G_{M1} binding event.

Our data demonstrating G_{M1}-dependent internalization, colocalization with caveolin, filipin sensitivity, fluorescence longevity, and insensitivity to external pH provide strong evidence for ETEC vesicle internalization via cholesterol-rich lipid rafts. Lipid raft-mediated binding and internalization appears to be an important entry mechanism for pathogens and toxins that leads to a nondegradative end point (Rosenberger *et al*, 2000; Nichols and Lippincott-Schwartz, 2001; Shin and Abraham, 2001). After 1 h at 37°C, caveolin was found in the vicinity of bound and internalized vesicles. Caveolae, which are flask-shaped plasma membrane domains, are not observed in HT29 cells by electron microscopy, perhaps due to the low levels of caveolin in this cell type (Badizadegan *et al*, 2000; Bender *et al*, 2000; Danielsen and Hansen, 2003; Hansen *et al*, 2003; our unpublished data). Using confocal and immunoelectron microscopy, we did not observe caveolin colocalized with bound vesicles at 4°C (data not shown), thus caveolin may be recruited during vesicle entry. It was previously reported that clustering of G_{M1} by the CT B subunit is sufficient to lead to lipid raft-mediated internalization (Wolf *et al*, 1998). Clustering and recruitment of raft components is also induced by the adherence of other factors (Parton *et al*, 1994; Shin *et al*, 2000; Zobiack *et al*, 2002). Whether caveolin is required for vesicle internalization is not known.

LT-directed vesicle–host cell association may be a paradigm for virulence factor delivery by pathogen-derived vesicles. Shiga toxin, another AB₅ toxin, was demonstrated to be in the protease-protected lumen of vesicles from *E. coli* O157:H7 (Kolling and Matthews, 1999), and, since their data showed that a portion of toxin associated with intact vesicles appeared to be protease sensitive, we propose that it may also localize to the vesicle exterior. In addition, the vacuolating toxin VacA of *H. pylori* is internalized via lipid rafts, and it is associated with vesicles (Fiocca *et al*, 1999; Patel *et al*, 2002; Schraw *et al*, 2002; Ilver *et al*, 2004), suggesting a role for VacA in the adhesion and internalization of *H. pylori* vesicles. However, vesicle–host cell interactions are not limited to toxin-mediated binding and internalization. Vesicles lacking externally localized toxins may interact with eukaryotic cells via other external components, such as outer membrane proteins. OmpA is a major vesicle component for

all strains of *E. coli* we have investigated thus far (Horstman and Kuehn, 2000; our unpublished data), and OmpA has a receptor on human brain endothelial cells and also triggers lipid raft-dependent internalization (Prasadarao, 2002). It is unclear whether toxin mediates the eukaryotic cell interactions of vesicles from *Shigella flexneri*, *Borrelia burgdorferi*, and *P. aeruginosa*, and cytotoxin-containing *E. coli* vesicles interact with eukaryotic cells (Shoberg and Thomas, 1993; Kadurugamuwa and Beveridge, 1998; Beermann *et al*, 2000; Wai *et al*, 2003). Nontoxin-mediated vesicle adhesion and endocytosis may play an equally important role in pathogenesis since these bacterial vesicles could deliver active luminal toxins and endotoxins into the eukaryotic cell.

In summary, we demonstrated LT-dependent ETEC vesicle binding, internalization, and toxicity. We propose that *in vivo*, pathogenic ETEC utilizes vesicles as vehicles to deliver LT and other virulence factors to the host mucosal layer, both locally and away from the site of colonization. Our results suggest that vesicles from bacterial pathogens are a direct conduit for toxin delivery, providing a protective environment for the transmission of luminal proteins and enzymes from the bacterial periplasm into the host cell. The binding specificity of adhesive surface factors on the vesicles dictates which host cells or tissues the vesicles target. Through further study of intracellular events following vesicle internalization, and of the role of bacterial vesicles in immunomodulation, we will begin to clarify virulence attributes of vesicles and the role they play in disease.

Materials and methods

Reagents and cell culture

E. coli strains HB101, ETEC (ATCC 43886), MC4100 Δ hns/GSP (Francetic *et al*, 2000), and MC4100 Δ hns/GSP/LT (Horstman and Kuehn, 2002) were grown in CFA broth (1% casamino acids, 0.15% yeast extract, 0.005% MgSO_4 , 0.005% MnCl_2). Y1 mouse adrenal cells (ATCC CCL-79) were grown in F-12K Kaign's modification media (2.5% bovine calf serum and 12% horse serum; Gibco Life Technologies). Human colorectal HT29 cells (ATCC HTB-38) were maintained in McCoy's 5a media (10% bovine calf serum; Gibco). All cell lines were grown in the presence of penicillin/streptomycin/amphotericin B antibiotic-antimycotic solution (5% CO_2 , 37°C). All reagents were obtained from Sigma unless stated.

Vesicle purification and labeling

E. coli were grown overnight in CFA broth, and vesicles were prepared as described (Horstman and Kuehn, 2000). Vesicles were incubated (1 h, 25°C) with FITC 1:1 (Molecular Probes) or rhodamine B isothiocyanate (1 mg/ml in 50 mM Na_2CO_3 , 100 mM NaCl, pH 9.2), pelleted (52 000 g, 30 min), washed and resuspended in Dulbecco's phosphate-buffered saline (0.2 M NaCl; PBS; Gibco).

Toxicity assays

The Y1 adrenal cell assay was performed as previously described (Horstman and Kuehn, 2000) using 1 μg FITC vesicles or 1 μg FITC-ETEC vesicles pretreated with 10 μg G_{M1} (30 min, 25°C). Toxin activity scores reflect the percentage of rounded cells: 1 $\leq$ 25%, 2 = 26–50%, 3 = 51–75%, 4 $\geq$ 75%. Cells were observed using a confocal microscope as described below. For cAMP assays, HT29 cells (1×10^4 cells/well) were incubated with 5 μg ETEC vesicles, 5 μg ETEC vesicles preincubated with 50 μg G_{M1} (30 min, 25°C), or 50 ng CT or 50 ng CT preincubated with 50 μg G_{M1} (4 h, 25°C). cAMP concentrations were measured using the Hithunter cAMP Assay (Amersham Pharmacia).

Quantitative fluorescence assays

HT29 cells (8×10^4 cells/well) plated in a 96-well plate overnight (37°C, 5% CO_2) were washed, incubated in serum-free media with FITC vesicles (1 μg /well), washed, solubilized (1% Triton X-100 in

Hanks buffer), and the fluorescence (excitation 485 nm, emission 520 nm) was measured using a FLUOstar Galaxy fluorometer (BMG Labtechnologies). Relative fluorescence units (RFUs) were converted to microgram of vesicle protein using separate standard curves for each strain of vesicles. In G_{M1} experiments, FITC vesicles were preincubated with G_{M1} (10 μg , 30 min, 25°C). To test pH resistance, cells were 'pulsed' with FITC-ETEC vesicles (2–4 μg /well, 2 h, 4°C), washed, and 'chased' in serum-free media (4 or 24 h, 37°C). The media were replaced (Dulbecco's PBS, pH 4 or 8, 30 min) before fluorescence readings. Control cells (0 h) were kept on ice after the pulse in Dulbecco's PBS, pH 4 or 8. For quenching experiments, FITC-ETEC vesicles or washed cells were incubated with anti-fluorescein antibody (1 μg /well, 1 h) prior to solubilization.

Electron microscopy

HT29 cells were plated on thermomax coverslips and incubated for 8 h at 37°C with and without ETEC vesicles. Cells were fixed in 3% glutaraldehyde (Tousimis) and 0.2% tannic acid (Mallinckrodt) in phosphate-free MOPS ringer buffer (1 h, 37°C), rinsed $3 \times$ in phosphate-free MOPS ringer buffer and $2 \times$ in 100 mM KPO_4 (pH 6), fixed in 1% OsO_4 (EM Sciences; in 100 mM KPO_4 , 10 mM MgCl_2 , pH 6, 4°C), rinsed in water, and stained with 2% uranyl acetate. Cells were serially dehydrated in ethanol and embedded in Araldite.

For immunogold electron microscopy, HT29 cells were incubated with ETEC vesicles for 8 h at 37°C or 18 h at 4°C followed by 1 h at 37°C, lightly fixed in 4% paraformaldehyde containing 0.1% glutaraldehyde, dehydrated in a graded series of ethanol, and embedded in LR White resin (EM Sciences). The samples were sectioned, blocked (5% fetal calf serum, PBS), and reacted with rabbit anti-*E. coli* polyclonal antibody, rabbit anti-LPS polyclonal antibody (Cortex Biochem), or mouse anti-caveolin monoclonal antibody, followed by 10 nm goat anti-rabbit immunogold or 5 nm goat anti-mouse immunogold (Amersham Biosciences). Sections were poststained with uranyl acetate and lead citrate and viewed in a Philips EM 301 or 400.

Fluorescent microscopy

Y1 (1.25×10^5) or HT29 (2.0×10^5) cells plated on permanox microwell chamber slides (Nunc) were preincubated (1 h) with serum-free media with or without filipin (1 μg /ml) or chlorpromazine (1 μg /ml) before the addition of FITC or rhodamine vesicles (1 μg). Slides were mounted with glycerol (30 mM; DABCO) or Prolong Antifade (Molecular Probes) and observed using a laser scanning confocal microscope (LSM-410, Carl Zeiss; or TE200, Nikon Eclipse). For WGA studies, nonpermeabilized cells were incubated for 1 h with Texas red WGA (1 μg /ml, 1 h, 4°C; Molecular Probes) following vesicle incubation. For antibody detection, cells preincubated with vesicles were washed, fixed (4% paraformaldehyde), washed, permeabilized (Hanks buffer, 0.1% Triton X-100, 15 min, 25°C), washed, and incubated (20 min, 25°C) with blocking buffer (5% goat serum, 0.1% bovine serum albumin, 0.1% Triton X-100). Cells were incubated (1 h, 25°C) with rabbit anti-*E. coli* polyclonal antibody (2.5 μg /ml; Fitzgerald Industries), mouse anti-caveolin or mouse anti-clathrin antibody (2.5 μg /ml; BD Biosciences) in the presence of 0.1% Triton X-100 followed by an incubation (30 min, 25°C) with rhodamine-labeled anti-rabbit antibody or FITC-labeled anti-mouse antibody (2.5 μg /ml; Jackson ImmunoResearch).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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