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Bacterial-associated cholera toxin and GM_1 binding are required for transcytosis of classical biotype *Vibrio cholerae* through an *in vitro* M cell model system

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Summary

To elucidate mechanisms involved in M cell uptake and transcytosis of *Vibrio cholerae*, we used an *in vitro* model of human M-like cells in a Caco-2 monolayer. Interspersed among the epithelial monolayer of Caco-2 cells we detect cells that display M-like features with or without prior lymphocyte treatment and we have established key parameters for *V. cholerae* transcytosis in this model. Cholera toxin (CT) mutants lacking the A subunit alone or both the A and B subunits were deficient for transcytosis. We explored this finding further and showed that expression of both subunits is required for binding by whole *V. cholerae* to immobilized CT receptor, the glycosphingolipid GM_1. Confocal microscopy showed CT associated with transcytosing bacteria, and transcytosis was inhibited by pre-incubation with GM_1 before infection. Finally, heat treatment of the bacterial cells caused a loss of binding to GM_1 that was correlated with a significant decrease in uptake and transcytosis by the monolayer. Our data support a model in which the ability of bacteria to interact with GM_1 in a CT-dependent fashion plays a critical role in transcytosis of *V. cholerae* by M cells.

Introduction

Vibrio cholerae, a Gram-negative, human-specific bacterial pathogen, is the aetiological agent of cholera, a severe diarrhoeal disease afflicting large parts of the developing world. The infection is acquired from contaminated food or water and ingested bacteria are able to colonize the small intestine, where they may interact with 'microfold' cells or M cells (Owen *et al*., 1986). M cells are specialized epithelial cells interspersed among enterocytes within the ileum and are responsible for sampling and internalizing antigens from the intestinal lumen and delivering them to underlying antigen-presenting cells (Savidge, 1996; Niedergang and Kraehenbuhl, 2000; Didierlaurent *et al*., 2002). For *Salmonella* and some other pathogens, a hallmark of pathogenicity is uptake by M cells, which initiates a complex series of interactions culminating in infection of cells of the reticuloendothelial system (Jones *et al*., 1994; VanCott *et al*., 2000; Neutra *et al*., 2001). *V. cholerae* is not traditionally considered an invasive pathogen in terms of being able to survive within cells or disseminate to deeper tissue, and its uptake by M cells of the enteric epithelium likely initiates the process leading to a protective immune response.

Disease caused by *V. cholerae* has been extensively studied, and much is understood about the mechanisms of pathogenicity (DiRita, 2001). Less is known about immunity to infection, and in particular the nature of the interaction between *V. cholerae* and M cells that likely represents the earliest step in this process. Although *V. cholerae* is not an invasive microbe, it is found within M cells during experimental infection of rabbit ileal loops (Owen *et al*., 1986), presumably due to the phagocytic nature of M cells. One reason for the paucity of information regarding how M cells take up *V. cholerae* and the role the microbe itself may play in this process is that there are no M cell tissue cell lines available to study the process, and primary M cells are not easily purified from tissue. Consequently, knowledge about the receptors and the molecular mechanisms involved in attachment and trafficking of microorganisms through M cells is limited (Neutra *et al*., 2001).

An *in vitro* model was developed to investigate M cell biology, including uptake and trafficking of microbes (Kerneis *et al*., 1997). In this model as originally published, cells of the polarized human colon carcinoma epithelial line Caco-2 were grown as a monolayer in the filter of a transwell apparatus. After establishment of the monolayer, B cells (primary lymphocytes or a cultured B cell-like line called Raji cells) were added to the basolateral side of the monolayer. Co-culturing of Caco-2 cells with lymphocytes this way induced a re-differentiation of some of the epithelial cells into those with features of M cells, including

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the ability to take up *V. cholerae* added to the lumenal side and transcytose them to the basolateral side (Kerneis *et al*., 1997). Indeed, uptake and transcytosis of this non-invasive pathogen by cells in the Caco-2/lymphocyte co-culture was offered as important evidence for the presence of M cells within the monolayer (Kerneis *et al*., 1997).

The receptors that participate in M cell uptake of *V. cholerae* and the mechanisms for transcytosis through M cells are not well understood. To initiate study of this process, we adapted the *in vitro* model described above. We found that significant transcytosis of *V. cholerae* occurs in Caco-2 monolayers alone, which we show is likely due to the fact that the Caco-2 cell line contains cells with features of M cells even without addition of B lymphocytes. We also discovered that transcytosis is dependent on expression of cholera toxin (CT) by the bacteria, and toxin expression also correlates with the ability of whole *V. cholerae* cells to bind immobilized ganglioside GM_1 in an *in vitro* binding assay. Using confocal microscopy we detected CT on *V. cholerae* cells inside cells from the Caco-2 monolayer. These findings highlight a potentially new role for CT in the association of *V. cholerae* with host cells that leads to immunity against cholera.

Results

Characterization of the in vitro *model of M-like cells*

Caco-2 cells grow in a polarized fashion, forming an epithelial-like monolayer with an organized brush border and tight junctions between adjacent cells (Fig. 1A and B). Similar to what was reported earlier (Kerneis *et al*., 1997), in the presence of Raji cells (a human B lymphocyte cell line) we detected cells with the features of M cells, including a disorganized brush border and, in experiments in which *V. cholerae* were added, the presence of both extra- and intracellular bacteria associated with them (Fig. 1C and D; see below). However, we consistently detected cells lacking an organized brush border within Caco-2 monolayers not co-cultured with Raji cells (Fig. 1E and F). This led us to conclude that some differentiation had occurred in the Caco-2 monolayer even without prior lymphocyte treatment.

To investigate the monolayer more carefully, we used indirect immunofluorescence to identify expression of phenotypic markers of human M cells. Two such markers have recently been described. Monoclonal antibodies against the sialyl-Lewis A antigen recognized that structure on Peyer's patch enterocytes from follicle-associated epithelium but not on adjacent brush border enterocytes (Giannasca *et al*., 1999). In human tissues, Ulex Europaeus lectin 1 (UE-1) recognizes only enterocytes and not M cells (Giannasca *et al*., 1999). More recently, a novel IgA receptor specific for M cells was identified based on the observation that antibodies against IgA stained M cells both from human biopsy samples and from murine Peyer's patches (Mantis *et al*., 2002). As shown by laser confocal microscopy in Fig. 2A, the expression of sialyl-Lewis A antigen (red fluorescence) colocalizes at the surface of some cells in the Caco-2 epithelial monolayer with the receptor for secretory IgA (green fluorescence), and those IgA receptor-positive cells (green fluorescence, Fig. 2B) are negative for the expression of UE-1 (red fluorescence, Fig. 2B). To determine the numbers of cells bearing M cell markers that are present in the Caco-2 monolayers, monolayers cultured alone or in the presence of Raji cells were labelled with a monoclonal antibody directed against sialyl-Lewis A, followed by PE-labelled second antibody, which rendered the positive cells red. Irrespective of whether the monolayer had been exposed to Raji cells or not, approximately 27% of the total events in the flow cytometry analysis were positive for sialyl-Lewis A. In other experiments we observed a slight increase in the numbers of cells bearing M cell markers in response to co-culture with Raji cells and bacteria, but we always observe positive cells in the monolayer without Raji treatment.

We conclude that transcytosis of *V. cholerae* through the monolayer without prior lymphocyte treatment is likely due to uptake by specialized cells (M-like cells) present within the Caco-2 epithelial monolayer. Support for this comes from laser confocal analysis of *V. cholerae* expressing chromosomally encoded green fluorescent protein (GFP); the bacteria are largely found within Caco-2 epithelial cells negative for the UE-1 lectin (Fig. 2C). In this experiment the integrity of the surface of the Caco-2 epithelial monolayer was preserved, allowing simultaneous detection of the UE-1 in the enterocytes surface with trafficking fluorescent bacteria. Depth analysis, in which the fluorescent signal is colour-coded based on its depth in the *z*-axis of the confocal image, was also performed. In this analysis, the fluorescence from the monolayer is colour-coded according to its depth on a continuum from red (shallow) to blue (deep). Figure 2D shows the depth of the signal coming from the UE-1 and Fig. 2E shows the depth coming from the GFP signal of the bacteria. Most of the latter is coming from deep in the monolayer (coded blue in Fig. 2E). In contrast, the few bacteria that are associated with UE-1-positive cells are found higher in the monolayer, as indicated by their green or yellow colour.

CT mutants do not transcytose

To analyse the molecular basis for recognition and uptake of *V. cholerae* in the monolayer, we assessed the capability of well-described mutant strains to be transcytosed in

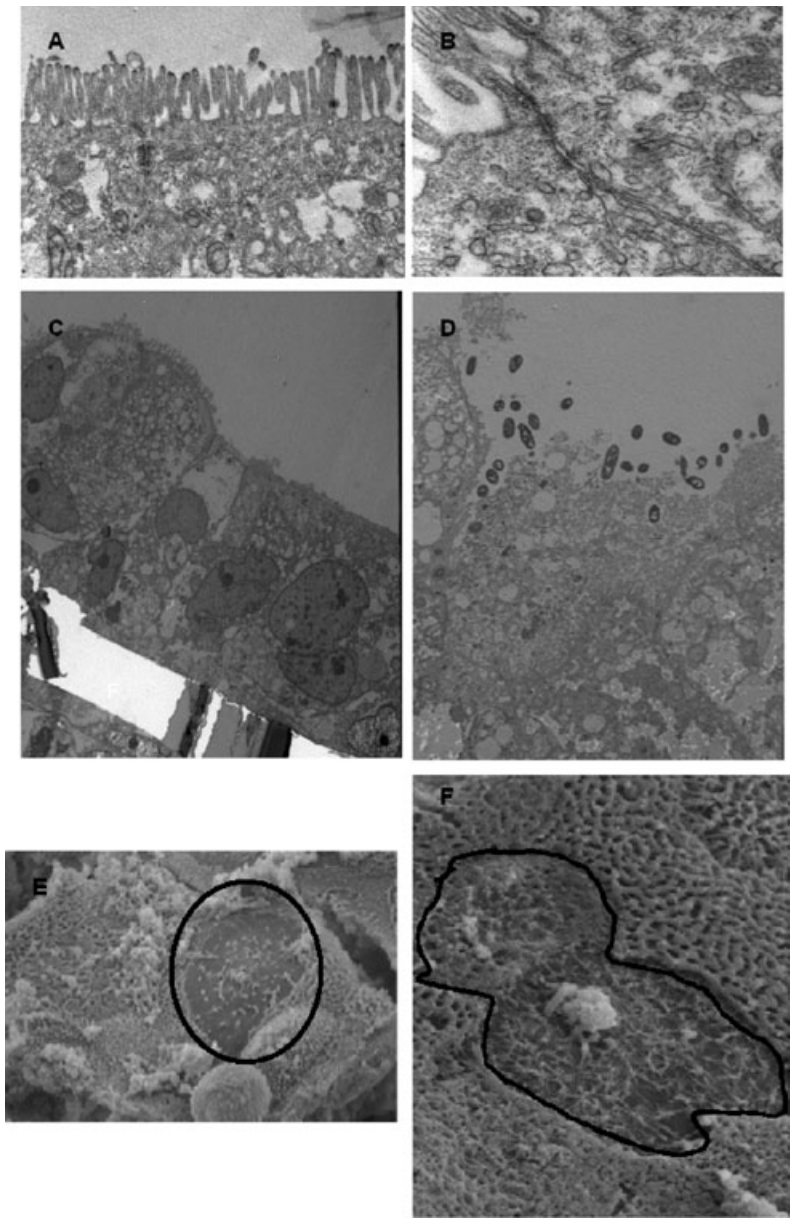

Fig. 1. Transmission and scanning electronic micrographs of epithelial monolayers of Caco-2 cells displaying M-like features.
A. Transmission electron microscopy (TEM) showing a monolayer of Caco-2 cells following 21 days of culture over the transwell device (13 500× magnification).
B. TEM showing tight junctions established between adjacent Caco-2 cells (46 000× magnification).
C. TEM of the monolayer following incubation with Raji cells (1100× magnification).
D. TEM of a cell with extra- and intracellular *V. cholerae* classical O935 strain after 30 m of infection (2600× magnification).
E. Scanning electron microscopy (SEM) showing an epithelial monolayer of Caco-2 cells following 21 days of culture in the transwell device and treated with Raji cells (4600× magnification). Encircled area highlights region lacking the brush border morphology, as expected of M cells.
F. SEM in the area from a monolayer of Caco-2 cells without prior Raji cell treatment; area delimited in black marks a similar flattened, brush-borderless morphology as that in (E) (5000× magnification).

comparison with wild type. Among these were two CT-deficient mutants, O395NT (*ctxAB*$^-$) and O395N1 (Δ*ctxA*) (Mekalanos *et al*., 1983). The O395NT mutant was reported as being less adherent and exhibiting reduced uptake by intestinal Peyer's patch cells in experimentally infected rabbit ileal loops (Owen *et al*., 1988), which compelled us to examine the role of CT in recognition and transcytosis of *V. cholerae* in the Caco-2 model.

By comparison with the parental strain O395, each mutant was transcytosed with greatly reduced efficiency; 2 h after introducing wild-type O395 into the lumenal well, 27% of the bacteria collected from the total transwell apparatus were in the basolateral well, as opposed to much less than 1% for either mutant strain (Table 1).

We consistently observe a drop in the transepithelial electrical resistance (TER) of monolayers cultured with CT-producing (wild-type) strain O395 (Table 1). Others studying CT effects on the T84 polarized cell line reported a similar drop in TER and attributed it to ion channel activation, and not to a disruption in the tight junctions *per se* (Lencer *et al*., 1992; Fullner *et al*., 2001). We do not observe significant decrease in TER of Caco-2 monolayers by adding purified CT (Table 2), so this reported effect might be cell-line specific.

We also considered that zonula occludens toxin (Zot), encoded upstream of *ctxAB* in the genome of toxigenic *V. cholerae*, might be playing a role in the drop of TER when wild-type cells are used for transcytosis. Zot alters cellular junctions in epithelial cells. We speculated that perhaps in constructing the *ctx* mutants used in this study,

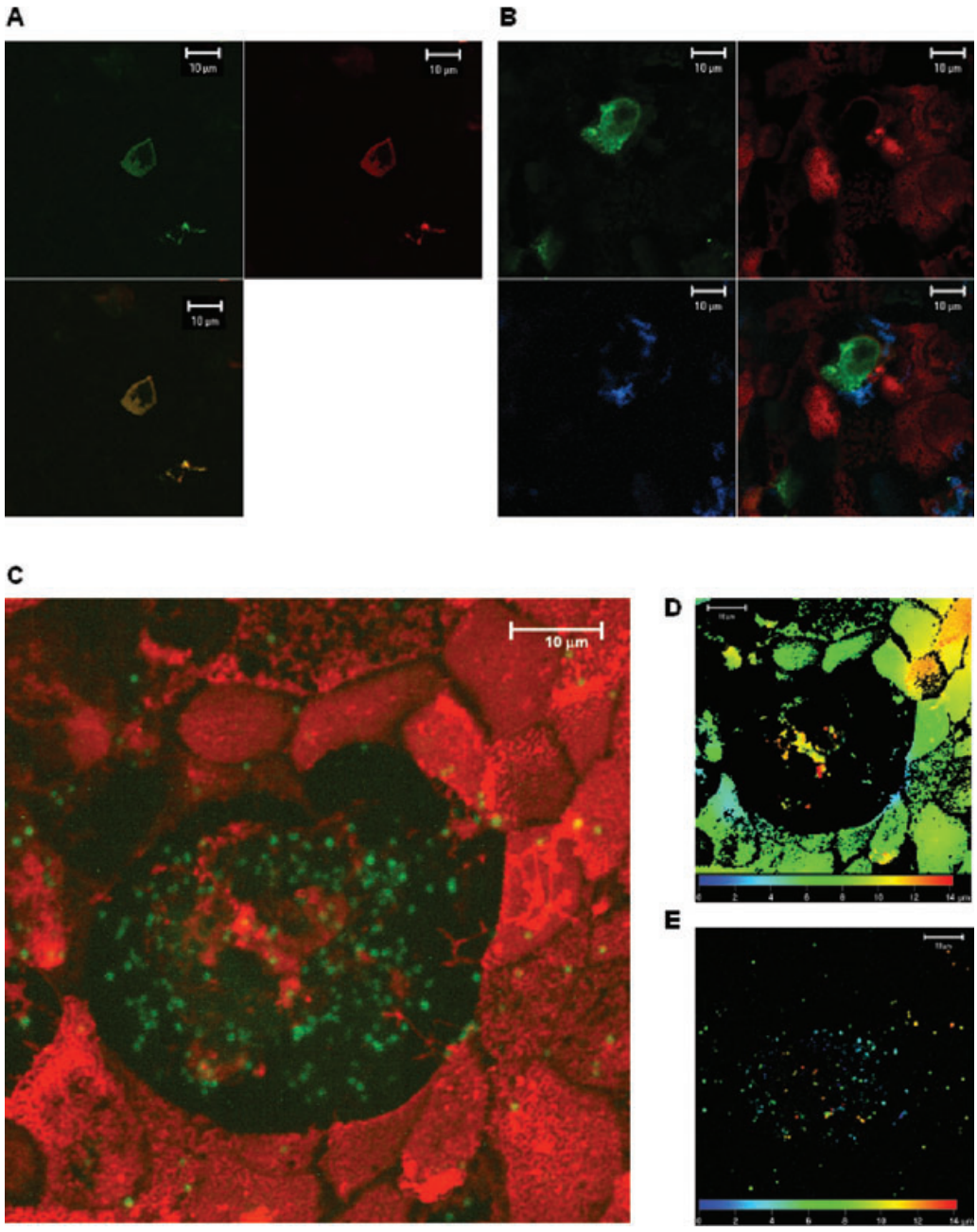

Fig. 2. Expression of M cell phenotypic markers and traffic of *V. cholerae* through cells in the Caco-2 epithelial monolayer.
A and B. *x*–*y* views at the plane of the surface in the Caco-2 monolayer. In (A) upper quadrants show a cell positive for the receptor for the secretory IgA (left, green fluorescence), that also expresses the sialyl-Lewis A antigen (right, red fluorescence). In the lower quadrant is showed the merge of the two fluorescences and the colocalization of the positive staining signals. Then in (B) is shown an *x*–*y* view of the epithelial monolayer of Caco-2 cells in the plane of the surface upper quadrants shown left the green fluorescent cell positive for the receptor of sIgA, the right panel with red fluorescent cells that are recognized by UE-1 lectin, the lower quadrants are left for the blue fluorescent channel detecting *V. cholerae* attached to the epithelial surface and in the right panel the merge of the three fluorescent colours detected.
C–E. In (C) the projection of the *z*-scanning sections through the unfixed and non-permeabilized epithelial monolayer of Caco-2 cells through which fluorescent *V. cholerae* (chromosomally expressing GFP) are trafficking. Also shown is the respective depth analysis for red (UE-1) fluorescence in (D) and green (bacteria) fluorescence in (E). It is evident that bacteria are trafficking through UE-1-negative cells.

Table 1. Transcytosis of CT mutants of *V. cholerae* through M-like cells.

Strains	(Basolateral cfu/basolateral + lumenal cfu) × 100 at[a]			Transepithelial resistance ($\Omega \times cm^2$)	
	15 min	60 min	120 min	Initial	Final
O395	0.06 ± 0.05 ($n = 8$)[b]	2.82 ± 1.76 ($n = 8$)	27.36 ± 8.50 ($n = 5$)	299.25 ± 28.11 ($n = 8$)	196.25 ± 27.24 ($n = 8$)
O395N1 (*ctxA*)	0.09 ± 0.04 ($n = 7$)	0.17 ± 0.09 ($n = 7$)	0.25 ± 0.25[c] ($n = 5$)	315.00 ± 38.40 ($n = 6$)	381.50 ± 52.25 ($n = 6$)
O395NT (*ctxAB*)	0.08 ± 0.06 ($n = 9$)	0.38 ± 0.18 ($n = 9$)	0.02 ± 0.01[c] ($n = 5$)	321.13 ± 30.59 ($n = 8$)	389.00 ± 37.77 ($n = 8$)
O395NT revertant (O395NT::*ctxAB*)	0.01 ± 0.004 ($n = 6$)	0.19 ± 0.03 ($n = 6$)	27.23 ± 3.78 ($n = 6$)	524.50 ± 10.60 ($n = 6$)	132.50 ± 4.11 ($n = 6$)
O395-cells[d]	44.50 ± 6.90 ($n = 7$)	68.47 ± 6.33 ($n = 7$)	68.64 ± 3.37 ($n = 7$)	[122.23 ± 3.07 ($n = 13$)][e]	[134.69 ± 3.50 ($n = 13$)][e]

a. The epithelial monolayer of Caco-2 cells was infected adding 2–6 × 10^7 *V. cholerae* per well (approximately 80 bacteria per cell) by the lumenal side of the transwell and the number of colony-forming units (cfu) determined at the times indicated. Data are the average ± SEM.
b. The number of transwells analyzed is in parentheses.
c. $P < 0.05$ Student's *t*-test.
d. Only the transwell apparatus without Caco-2 cells.
e. Basal value of transepithelial resistance of the membrane without cells already subtracted from the above resistance measurements.

the *zot* gene might have been disrupted in some way. Zot activity would then be diminished in strains lacking CT (O395N1 and O395NT), and if Zot is responsible for drop of TER, that would explain why the CT mutant strains do not alter TER while the wild type does.

To test this, we used the classical strain CVD101, which is similar to O395N1 ($CtxA^-CtxB^+$) but is known to have Zot activity (Fasano *et al*., 1991). If Zot activity were responsible for disrupting tight junctions and allowing *V. cholerae* to transit between cells, we would expect that CVD101 would be readily transcytosed in the monolayer system. Transcytosis of CVD101 was very low [0.007 ± 0.002 ($n = 6$) after 2 h] and the TER did not drop (from initial 267.2 ± 9.9 to final 273.0 ± 13.1), suggesting that Zot is not responsible for the TER effect in the Caco-2 monolayer and providing further evidence that cells lacking one of the CT subunits [CT subunit A (CTA) in this case] are only very weakly proficient in their ability to undergo transcytosis. Other *V. cholerae* proteins that might have an effect on tight junction integrity, such as the haemagglutinin/protease or the RTX toxin (Wu *et al*., 2000; Fullner *et al*., 2001), are not expressed by classical strains such as O395, so their role in the TER drop we observe is ruled out *a priori*.

To make a more direct assessment of whether the drop in TER is accompanied by significant damage to the junctions that would be sufficient to allow paracellular movement of the bacteria across the monolayer, we performed confocal fluorescence microscopy using an antibody that recognizes the tight junction protein ZO-1 (also termed TJP-1). We reasoned that if junctions were being damaged by the bacteria, that would be reflected in loss of staining with antibodies to ZO-1. Such an effect has been observed with enteropathogenic *Escherichia coli* (EPEC), which does disrupt barrier function in part by damaging tight junctions (Dean and Kenny, 2004). Figure 3 shows representative confocal images of uninfected and infected (for 2 h) Caco-2 epithelial monolayer cells treated as described in *Experimental procedures*. In the right panel of the figure, bacteria were also detected using anti-*Vibrio* antibody and a PE-conjugated second antibody. DAPI was used to stain the Caco-2 cell nuclei. In both panels,

Table 2. Effect of exogenously added CT in the transit of *V. cholerae* through the Caco-2 epithelial monolayer.

Condition	Final transcytosis (basolateral cfu/basolateral + lumenal cfu) × 100	Transepithelial resistance ($\Omega \times cm^2$)	
		Initial	Final
Caco-2 O395	4.522 ± 1.178	229.7 ± 10.0	148.6 ± 13.6 ($n = 9$)[a]
(Caco-2 + CT) O395[b]	2.233 ± 0.707*	256.6 ± 9.8	184.9 ± 16.4 ($n = 8$)
Caco-2 O395NT	0.173 ± 0.070	380.6 ± 5.1	382.3 ± 12.6 ($n = 9$)
(Caco-2 + CT) O395NT[c]	0.138 ± 0.030	374.8 ± 8.7	370.2 ± 12.7 ($n = 9$)
Caco-2 (O395NT + CT)[d]	0.017 ± 0.002	393.8 ± 15.9	416.8 ± 3.1 ($n = 4$)

a. Number or transwells used (average of at least two experiments ± SEM).
b. CT 60 nM was added to the epithelial monolayer 15 min before infection with the wild-type O395 strain and remained during the transcytosis assay.
c. CT 46.7 nM was added to the epithelial monolayer 5 min before infection with O395NT strain.
d. O395NT was incubated for 10 min at 37°C with CT (46.7 nM) and then used to infect the Caco-2 cells keeping CT during the transcytosis assay at 9.3 nM.
*$P < 0.05$, regard Caco-2 O395 Student's *t*-test.

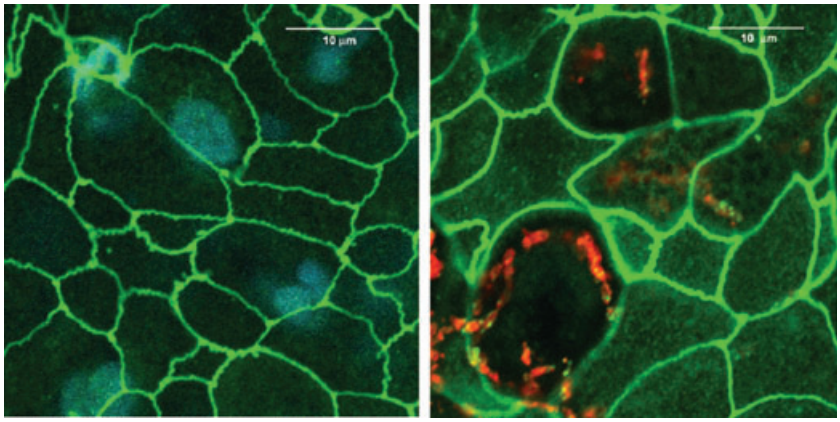

Fig. 3. Tight junctions integrity and transcellular traffic of *V. cholerae* through Caco-2 cells. Representative images from the laser confocal microscopy analysis of the epithelial monolayer of Caco-2 cells that show the integrity of the tight junctions detecting ZO-1 protein in green and *V. cholerae* (green plus red) trafficking transcellularly through the cells. After 2 h the epithelial monolayer was fixed and permeabilized as explained in *Experimental procedures* and treated with the antibodies. In the left panel no bacteria were added and in the right panel the cells were infected with *V. cholerae* O395 strain chromosomally expressing GFP that was immunodetected using anti-*V. cholerae* mouse monoclonal antibody and a second anti-mouse antibody PE (red) conjugated. ZO-1 was immunostained with anti-ZO-1 rabbit polyclonal antibody and a second anti-rabbit Alexa 488- (green) conjugated antibody. The final transepithelial resistance measurements were: 254 $\Omega \times cm^2$ and 190 $\Omega \times cm^2$ for non-infected versus infected Caco-2 cells respectively.

ZO-1 staining is observed throughout the focal plane and junctions surrounding cells where bacteria are localized are undamaged (Fig. 3). These results are in distinct contrast to what was recently reported for EPEC, in which staining for another junction protein (occludin) was poorly detectable in polarized Caco-2 monolayers after incubation with the bacteria (Dean and Kenny, 2004). In addition, we note that monolayer TER consistently remained at levels sufficiently high (well over 100 $\Omega \times cm^2$) to indicate that junctions are intact (Canil *et al.*, 1993; Heppner *et al.*, 2001). Paracellular movement would presumably disrupt tight junctions and reduce the monolayer TER more dramatically, which we did not observe even after 4 h with the wild-type O395 strain.

Another way we assessed the barrier function of the Caco-2 monolayer was to monitor transit of the paracellular marker lucifer yellow after incubation with the bacteria. For this experiment, 200 µg of lucifer yellow was added to the apical side of the monolayer at the end of the transcytosis assay and the amount of the dye that came through to the basolateral side after 10 min in the presence and absence of *V. cholerae* was monitored fluorometrically. About 0.3% of added lucifer yellow crossed the monolayer when infected with wild-type strain O395, similar to what was found in the basolateral well when dye was added apically to uninfected monolayers (0.2%). In contrast, when monolayers were treated with 0.1% SDS before addition of lucifer yellow, 1% of the dye was found in the basolateral well.

Our results are consistent with there being minimal, if any, damage to the cell–cell junctions in the Caco-2 monolayer that would allow for significant bacterial movement through a paracellular pathway. We thus conclude that the observed movement of *V. cholerae* through the monolayer is predominantly transcellular and not paracellular.

Cholera toxin expression confers GM_1 binding to whole V. cholerae *cells*

The O395N1 mutant, which lacks only the enzymatic CTA subunit, was not transcytosed. This led us to investigate the hypothesis that toxin enzymatic activity on the cell monolayer was leading to uptake and transcytosis of the bacteria. We ruled this possibility out by determining that addition of purified CT to the monolayer did not enhance transcytosis of the mutant bacteria irrespective of whether the toxin was added first to the monolayer before addition of the bacteria, or mixed with the bacteria before inoculation of the monolayer (Table 2). Exogenously added CT holotoxin slightly but consistently inhibited transcytosis of the wild-type O395 strain (Table 2). We therefore focused on another aspect of CT function – ganglioside binding – as a potential explanation for the role of toxin in transcytosis.

Ganglioside GM_1 binding is implicated in uptake and trafficking of some viruses (Tsai *et al.*, 2003). Because this glycosphingolipid is the CT receptor as well, we hypothesized that perhaps a similar mechanism is at work in uptake of CT-producing *V. cholerae* by M cells in the monolayer. This hypothesis would imply that some fraction of secreted toxin remains associated with the bacterial cell. To our knowledge, bacterial surface-associated CT has not been reported in this or any other *V. cholerae* strain before. The mutant strain O395N1 was originally

reported to secrete wild-type levels of CT subunit B (CTB) into the supernatant (Mekalanos *et al.*, 1983), which we also observed, and was especially abundant when the mutant strain was incubated in cell culture media compared with the wild-type O395 strain (data not shown).

To test whether CT expression could direct binding of whole bacteria to GM_1, we adapted the GM_1-ELISA, widely used to quantify secreted CT. Whole *V. cholerae* strains were added to GM_1-coated wells of a 96-well plate. Bound wild-type *V. cholerae* was detectable after treatment with anti-*V. cholerae* antibody (Fig. 4A). In contrast, strains O395NT (*ctxAB*$^-$) and O395N1 (Δ*ctxA*), and EK407, which is deleted for the *ctxAB* gene activator *toxR*, bound the GM_1-coated wells with significantly lower efficiency in this assay (Fig. 4A). Probing the wells with antibody against CT (Fig. 4B) allowed us to assess the relative levels of CT that remain associated with GM_1-coated wells to which bacteria had been added. Toxin associated with wild-type O395 was saturating in this assay across a range of twofold dilutions of the cells. In contrast, toxin associated with strains O395NT and EK407 was undetectable even when cells were undiluted. A derivative of O395NT in which the *ctxAB* deletion allele was replaced with the wild-type genes by homologous recombination (strain O395NT::*ctxAB*) exhibited restored GM_1 binding, CT production and ability to be transcytosed through the Caco-2/M cell monolayer (Fig. 4A and B, and Table 1). Strain O395N1 displayed an intermediate phenotype, manifest as poor binding to the ganglioside but high levels of toxin attached.

Inhibition of transcytosis by exogenously added GM_1 or monoclonal antibody against CTB

Our results suggest that *V. cholerae* can bind GM_1 in a CT-dependent fashion, and that this binding plays a role in recognition, uptake or transcytosis of *V. cholerae* in the Caco-2 monolayer. If the CT effect on these processes involves binding to GM_1 in the monolayer, then we would predict that addition of either exogenous GM_1 or anti-CTB antibodies to the bacteria before inoculating the monolayer would block interaction of the bacteria with the monolayer.

We tested this prediction by pre-incubating 10^8 *V. cholerae* O395 with 1 mg ml^{-1} GM_1 before infection of the monolayer. The number of bacteria recovered in the lower well was significantly reduced by this treatment, compared with that seen when untreated bacteria were used (Fig. 5A). Furthermore, inhibition of *V. cholerae* transcytosis by GM_1 was not observed if the GM_1 was pre-mixed with CTB before it was added to the bacteria (Fig. 5A). That exogenous gangliosides reduce the amount of uptake and transcytosis, and that this effect can be reversed if the gangliosides are mixed with CTB ahead of time, suggest that bacterial interaction with GM_1 is a feature of uptake and/or transcytosis mechanism.

We also tested a mouse monoclonal antibody against CTB. When this antibody was present during the infection assay transcytosis of strain O395 through M-like cells was inhibited (Fig. 5B). These results support the hypothesis that recognition of *V. cholerae* by cells in the monolayer occurs through interaction between bacterial-associated CT and cell-associated ganglioside GM_1.

Association of V. cholerae *and CT during interaction with cells in the Caco-2/M cell monolayer*

We next sought to determine whether CT is produced by *V. cholerae* when bacteria are associated with the cells in the tissue culture system described above. To detect CT associated with bacteria during tissue culture infection, we allowed infection with GFP-labelled *V. cholerae* to proceed

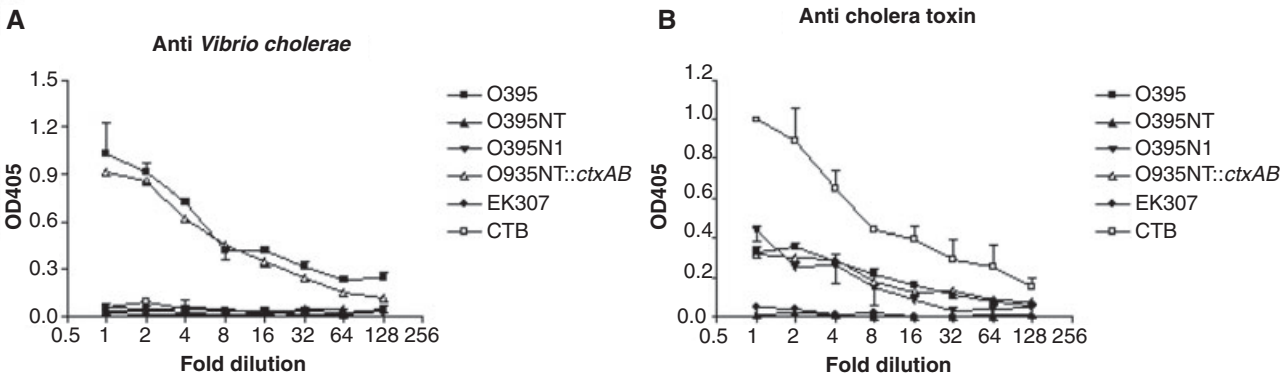

Fig. 4. *In vitro* interaction of *V. cholerae* with GM_1. To evaluate the ability of *V. cholerae* to interact with GM_1 we used GM_1 attached to inert ELISA wells. See *Experimental procedures* for details on the ELISA. The ELISA was developed with anti-*V. cholerae* antibody (A) or with anti-CT antibody (B). Bacterial strains used were *V. cholerae* classical O395, O395NT (O395*ctxAB*$^-$), O395N1 (O395Δ*ctxA*), O395NT::*ctxAB* (O395NT in which the *ctx* locus was re-introduced by homologous recombination) and EK307 (O395Δ*toxR*). Pure commercial CTB at (500 ng ml^{-1} before the first dilution) was used as a positive control.

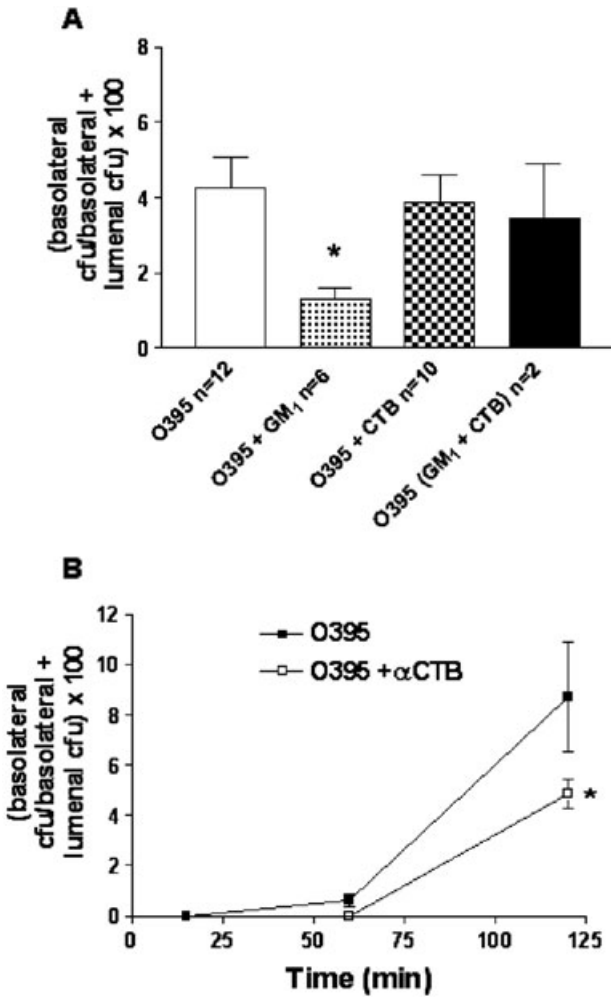

Fig. 5. Effect of exogenously added GM_1 or an antibody against CTB on the transcytosis of *V. cholerae* through Caco-2 cells. Shown in (A) and (B) is transcytosis of wild-type *V. cholerae* strain O395 through the epithelial monolayer of Caco-2 cells measured after 2 h.
A. The monolayer of Caco-2 cells was treated with exogenously added CTB; for GM_1, the ganglioside was pre-mixed with the bacteria before infection of the monolayer. When CTB and GM_1 were tested for their combined effects, they were pre-mixed and then added to the bacteria. $^*P < 0.05$ (Student's *t*-test).
B. The inhibitory effect in the transcytosis of *V. cholerae* O395 wild-type strain through M-like cells by the monoclonal antibody against CTB is shown. $^*P < 0.05$ Mann–Whitney analysis.

for 30 min, followed by addition of anti-CT antibody. Using laser confocal microscopy we detected focal production of the toxin in association with GFP-expressing *V. cholerae* (Fig. 6A); in this case it is possible to show fluorescence concomitantly with differential interference contrast (DIC), contrary to analysis of the monolayer in the transwell where the opaque membrane material does not allow capturing this type of image. By analysing *z*-sections through the monolayer of cells growing in the transwell apparatus, we detected intracellular *V. cholerae*, often with associated CT, by their being in the same plane as the nuclei (stained blue with DAPI in Fig. 6B). The staining profile of a section taken through the monolayer (Fig. 6C) shows that peaks of red fluorescence (CT) are typically associated with peaks of green fluorescence (bacteria), leading us to conclude that CT remains in the proximity of the bacteria as they traffic through the cells.

Analysis of binding versus transcytosis

To determine which process – attachment or trafficking – is more affected in the O395NT mutant, we measured the number of cell-associated bacteria and the number of gentamicin-resistant (intracellular or trafficking) bacteria for both the wild-type strain O395 and O395NT strains, as described in *Experimental procedures*. After 2 h the ratio between numbers of O935 versus O395NT intracellular bacteria was dramatically greater (138-fold) than the ratio of cell-associated bacteria of each strain (12-fold less; Table 3). This result suggests that although CT expression is important for both association with and subsequent trafficking through the Caco-2 monolayer the more critical effect is on trafficking. Overall, our results suggest that CT expression by *V. cholerae* plays an important role in the recognition and trafficking of the bacteria in the monolayer.

Transcytosis of V. cholerae *requires bacterial viability*

When tested in experimentally infected rabbit ileal loops (Owen *et al.*, 1986), live and dead (heat-killed) *V. cholerae*

Table 3. Analysis of binding versus transcytosis of *V. cholerae* wild-type and CT mutant strains.

	Number of bacteria	
Strain	Cell associated	Intracellular
O395	$2.88 \pm 0.63 \times 10^7$	$1.44 \pm 0.77 \times 10^6$
O395NT	$2.38 \pm 1.44 \times 10^6$ ($P < 0.05$)	$1.04 \pm 0.80 \times 10^4$ ($P < 0.001$)
Ratio O395/O395NT	12.11	138.46

The number of bacteria was determined after a 2 h assay with the epithelial monolayer of Caco-2 cells. The cells were infected with a multiplicity of infection (moi) of approximately 80 bacteria per cell (total 2×10^7 bacteria by the lumenal side). The number of cell-associated bacteria was measured after three washes of the monolayer followed by detergent lysis and the number of intracellular bacteria was determined similarly but after an incubation of 15 min with gentamicin. This is the average of two different experiments ± SEM. The statistic analysis was accomplished by the Student's *t*-test.

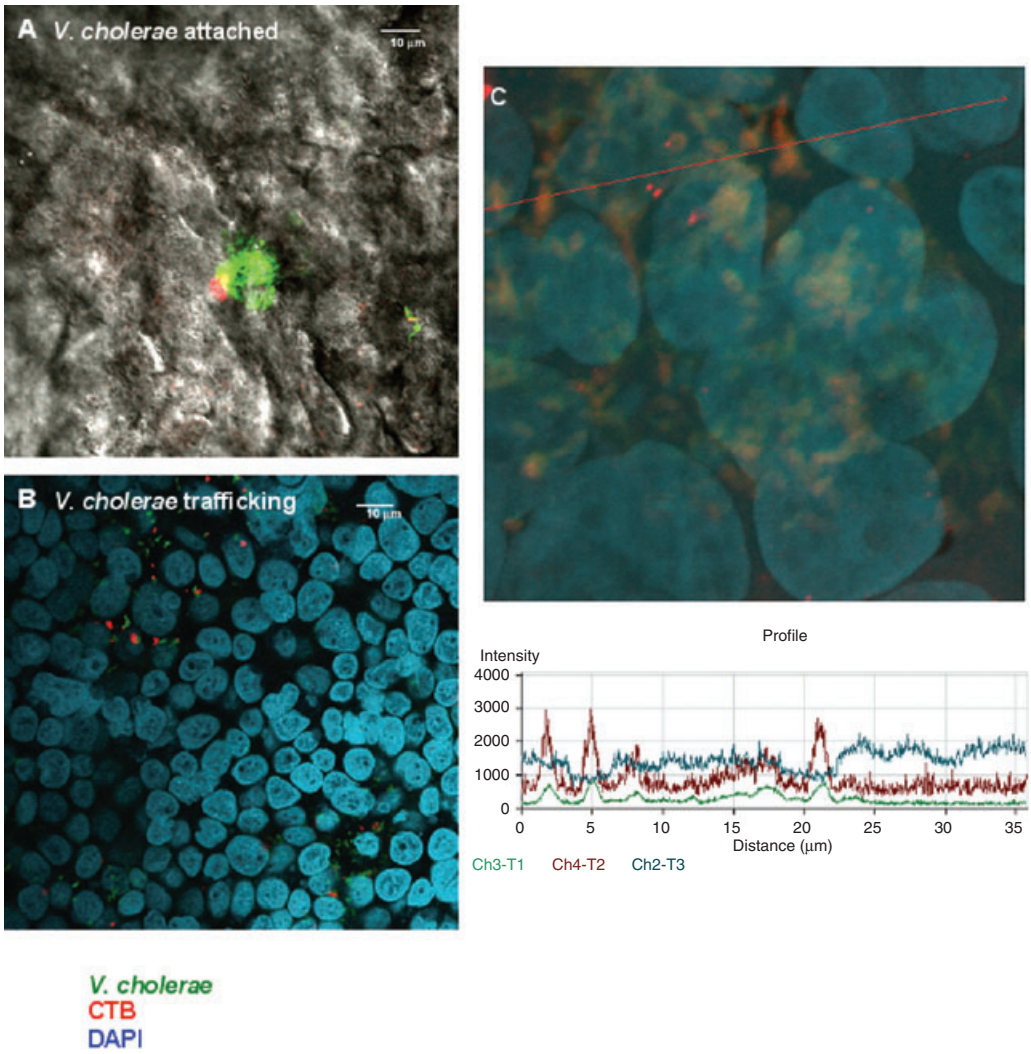

Fig. 6. Expression of CT during surface attachment and transcytosis through Caco-2 cells. Images in (A), (B) and (C) were taken with the laser confocal microscope and *x–y* views are shown for each. (A) shows *V. cholerae* strain O395/pGFPmut3.1 (see *Experimental procedures*) on the surface of a Caco 2 cell monolayer growing confluently over a coverglass. CT was detected using rabbit anti-CT antibody followed by TRITC-labelled anti-rabbit antibody. The background image of the monolayer is shown in differential interference contrast (DIC). For the image in (B), the monolayer was fixed and permeabilized to detect intracellular *V. cholerae* (green fluorescence) in conjunction with CT (red fluorescence) and nuclei in blue (DAPI). The fluorescence signals for bacteria (green) and for CT (red) remain closely associated as demonstrated by the fluorescence profile through the traced red line shown in (C).

cells appeared to be taken up with different efficiencies by M cells. One of two hypotheses may account for this observation: first, perhaps only live cells can make their way through the lumen to the host cell surface with the requisite efficiency to enable sampling by the M cells. Second, perhaps heat-killed *V. cholerae* cells lack a factor required for recognition by M cells. To investigate the latter hypothesis, we analysed transcytosis of heat-killed *V. cholerae* through the Caco-2 monolayer. Our standard method for determining transcytosis depends on plating bacteria from the lower (basolateral) well of the transwell apparatus, which obviously is not possible with heat-killed bacteria. We used two different approaches to monitor dead bacteria on both sides of the transwell in this experiment. One was based on detection of *V. cholerae* DNA in the upper and lower wells with PCR after inoculating either live or heat-killed bacteria. We reasoned that if bacteria are able to transcytose, we would be able to amplify *Vibrio*-specific sequences from the lower well after the standard transcytosis assay. The second approach for detecting transcytosis of heat-killed cells was to use fluorescently labelled bacteria to monitor the level of fluorescent particles in the basolateral well of the transwell apparatus after the transcytosis assay.

The results of the two experiments are shown in Fig. 7A and B. Two hours after live bacteria were added to the

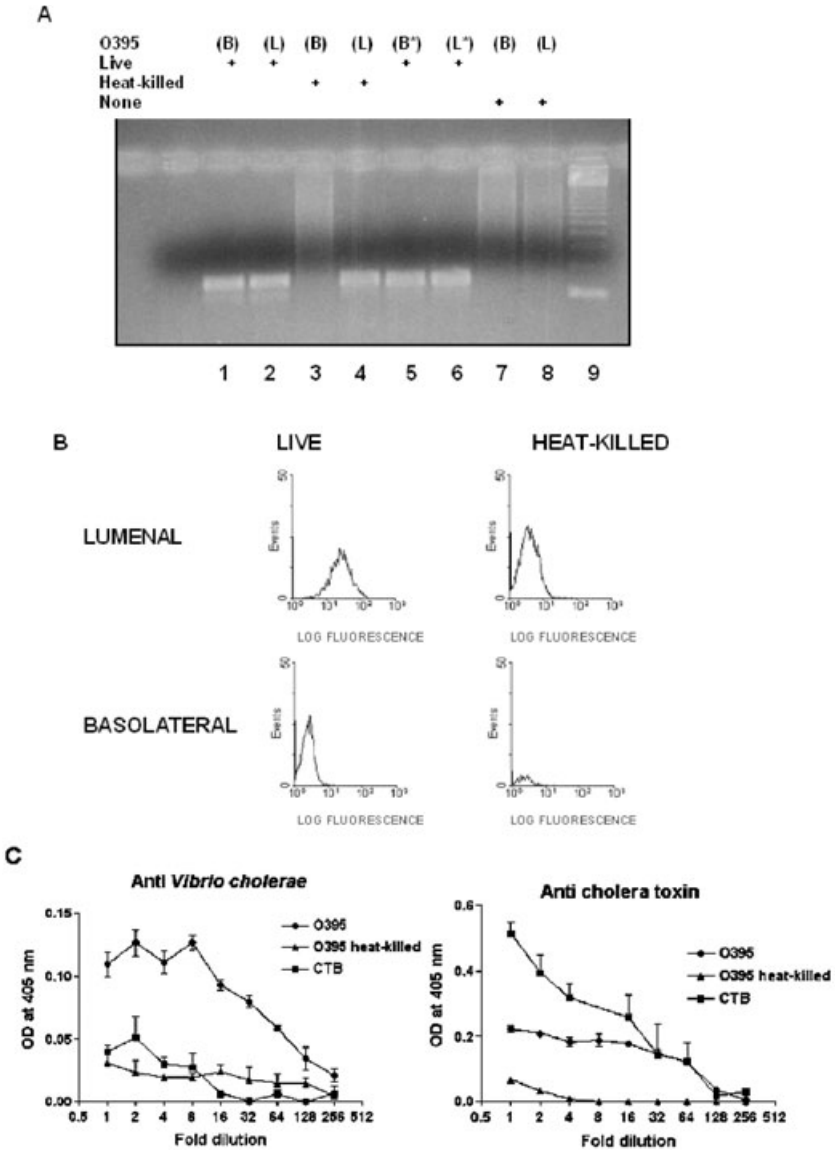

Fig. 7. Transcytosis of heat-killed *V. cholerae* through Caco-2 cells. Two methods were used to assess transcytosis of heat-killed bacteria, as described in the text.
A. Results of PCR to identify a 192 kb product from *V. cholerae* DNA isolated from lumenal (L) or basolateral (B) wells of the transwell apparatus after 2 h infection with live or heat-killed bacteria. Lines marked with asterisks represent experiments in which Caco-2 cells were not co-cultured with Raji cells; all other lines represent experiments in which Caco-2/Raji co-cultures were used. The DNA ladder on the right (line 9) is the 123 Ladder Plus from Invitrogen Life Science.
B. Flow cytometry analysis of medium from each side of the transwell apparatus following a 2 h transcytosis assay with fluorescently labelled heat-killed or live *V. cholerae*, as noted.
C. GM_1-ELISA using wild-type O395 or heat-killed O395. Bacteria were detected using an antibody against *V. cholerae* or with an antibody against CT (as in legend to Fig. 4) to determine the effect of heat treatment CT-dependent binding of GM_1 by whole *V. cholerae*.

apical surface of the monolayer, extracts were prepared from upper and lower wells of the transwell and subjected to PCR using *V. cholerae*-specific primers. Products of the predicted size were obtained in each extract, reflecting bacteria present in both upper and lower wells (Fig. 7A, lanes 1 and 2). In contrast, the predicted product was not obtained from extracts prepared from the lower well 2 h after heat-killed bacteria were added to the upper well (Fig. 7A, lane 3), although products were obtained from extracts of the upper well (Fig. 7A, lane 4). The smear of products from the lower well when heat-killed cells were added (Fig. 7A, lane 3) was similar to what we observed when no bacteria were added at all (Fig. 7A, lanes 7 and 8). In this experiment, we used both monolayers alone (Fig. 7A, lanes 5 and 6) and monolayers that were treated with Raji cells (Fig. 7A, lanes 1, 2–4, 7 and 8) and there was a difference in the percentage of cells transcytosed, about sevenfold, although the gel showing the PCR reaction does not reflect this. This result highlights the sensitivity of the PCR assay as well as the point noted above that co-culture with Raji cells is not a strict requirement for transcytosis of *V. cholerae* through the Caco-2 monolayer. Electron microscopy confirmed that heat-killed bacteria were structurally intact (data not shown).

In the fluorescence-based assay for testing the transcytosis of heat-killed bacteria, bacteria were labelled with the membrane dye PKH-2. The stained bacteria were then split into two aliquots, one of which was subjected to heat treatment (60°C for 15 min). The labelled live and heat-killed cells were washed in Dulbecco's minimal essential media (DMEM) and inoculated into the upper well of a transwell apparatus in which a confluent Caco-2 cell

monolayer had been cultured. Samples were collected from the upper and lower wells after 2 h, and the total number of fluorescent particles from each well was then determined by flow cytometry.

Figure 7B shows the events detected by gating for the bacteria-sized particles in supernatants from the transwell apparatus when infected with live or heat-killed bacteria. When live bacteria were used, both wells contained detectable fluorescent events. In contrast, when heat-killed bacteria were used, fluorescent events were detectable only in supernatants of the upper well. This result supports the observation from the PCR-based assay that heat-killed bacteria are unable to be efficiently recognized by cells in the monolayer responsible for the transcytosis of *V. cholerae*.

We conclude that heat-labile determinants expressed by *V. cholerae* are important for the transcytosis process. Because of the apparent connection between CT-dependent GM_1 binding and transcytosis, we tested whether heat-treated microbes could bind to GM_1 in the whole cell binding assay described above. Heat-killed bacteria were significantly less able to bind to GM_1 compared with wild-type strain O395 (Fig. 7C, left), and CT associated with the heat-killed microbes was nearly undetectable (Fig. 7C, right). We conclude that heat killing may either destroy CT or remove it from its association with the bacteria, thereby eliminating an important determinant of M cell recognition.

Discussion

We used an *in vitro* model to study transcytosis of a non-invasive pathogen, *V. cholerae*, through a monolayer consisting of polarized intestinal epithelial cells. Cells bearing markers associated with human intestinal M cells are present in the monolayer irrespective of addition of lymphocytes, and we are able to detect bacteria within such cells. We thus conclude that transcytosis is a consequence of bacterial uptake and trafficking by M-like cells present in the Caco-2 monolayer.

That confluent growing Caco-2 cells show features of M cells is consistent with the fact that Caco-2 cells are able to display a combination of colonocyte and enterocyte phenotypes, which is likely attributable to crypt-derived cells (Engle *et al*., 1998). Crypt cells in the rabbit gut were shown to contain precursors for both M cells and enterocytes as different cell lineages (Lelouard *et al*., 2001). Indeed, there is some controversy about the origins and elements involved in the process by which M cells differentiate from within brush border epithelia, but contact between epithelial cells and lymphocytes is apparently not a prerequisite for M cells development (Nicoletti, 2000). This *in vitro* model system could be useful for dissecting the mechanisms involved in M cell development. Furthermore, M cell development has recently been described outside the Peyer's patch environment in the mouse villous epithelium as well (Jang *et al*., 2004).

Recognition of a microbial cell through a lipid raft-associated molecule like GM_1 is a widespread mechanism in host/pathogen biology (Shin and Abraham, 2001; Harris *et al*., 2002). Many microbes, including viruses, bacteria and parasites, interact with eukaryotic cells through lipid raft structures (Simons and Ehehalt, 2002). In addition, gangliosides are receptors for murine polyoma virus and SV40 (Tsai *et al*., 2003) as well as for *Pseudomonas aeuroginosa* pili binding and internalization into epithelial cells (Comolli *et al*., 1999) and entry of non-opsonized *Brucella suis* into murine macrophages (Naroeni and Porte, 2002). Gangliosides are also able to recognize and participate in the attachment of certain bacteria to oropharyngeal epithelial cells, i.e. *Haemophilus influenzae* (recognizing GM_1, GM_2 and GM_3) and *Moraxella catarrhalis* (recognizing GM_2, asialo-GM_1 and asialo-GM_2) (van Alphen *et al*., 1991; Ahmed *et al*., 2002).

A number of well-characterized bacterial toxins can bind to eukaryotic cells through lipid raft-associated molecules. In addition to the CT, this is the case for the heat-labile toxin LTI (Wimer-Mackin *et al*., 2001), shiga toxin (Sandvig *et al*., 1989; Kovbasnjuk *et al*., 2001), anthrax toxin (Abrami *et al*., 2003a), vacuolating toxin VacA (Schraw *et al*., 2002), lysteriolysin O (Coconnier *et al*., 2000), tetanus toxin (Herreros *et al*., 2001; Shimada *et al*., 2002), aerolysin (Abrami *et al*., 2003b) and even LPS (Triantafilou *et al*., 2002). Given the results presented here, it is worth exploring the possibility that microbial toxins that bind gangliosides may play a role in microbial invasion, colonization and/or interaction with M cells.

Cholera toxin is well understood to be a secreted protein, but our results suggest that at least some of the protein remains sufficiently cell-associated to confer on the bacteria the ability to bind GM_1. The mechanism by which some fraction of the CT remains associated with the bacteria is unclear. LT secreted via the general secretory pathway in *E. coli* is able to associate with the bacterial surface through the lipolysaccharide, and most of the LT in the supernatants of bacterial cultures is associated with vesicles made of outer membrane (Horstman and Kuehn, 2002). Perhaps a similar mechanism operates in *V. cholerae* to keep some of the CT associated with whole cells. If CT confers upon whole *V. cholerae* the ability to bind GM_1 in a way that ultimately leads to transcytosis, it is not clear why the CTB^+A^- strain O395N1 strain binds poorly. This strain produces and secretes high levels of CTB pentamers, so perhaps the mechanism by which toxin remains cell-associated is more efficient when both CTA and CTB are expressed. Previous findings demonstrated a role of CT in the colonization of rabbit intestine, where either O395N1 or O395NT (the same strains

used in our studies) colonized rabbit intestines at levels up to two orders of magnitude lower than wild-type strain O395. In this case, however, simply adding purified holotoxin to the mutant inoculum could enhance colonization by O395NT (Pierce *et al*., 1985).

Studies on CT biogenesis (and that of the closely related *E. coli* toxin LT) have focused on mechanisms of periplasmic protein assembly and extracellular secretion (Hardy *et al*., 1988; Sandkvist, 2001) under the assumption that there is no other compartment in the bacterial cell where CT is found. Our results may call that assumption into question. A similar finding was reported concerning the VacA toxin expressed by *Helicobacter pylori*. Some of the VacA toxin produced (most is secreted into the culture supernatant) was recently shown to remain bacterially associated within specific domains on the cell surface (Ilver *et al*., 2004). This fraction of toxin appears to be delivered directly into the host cells upon bacterial contact with them.

Vibrio cholerae CTA mutants in human beings are immunogenic and have been developed for use as live attenuated vaccine strains (Killeen and DiRita, 2001). So even though $CTA^{-}CTB^{+}$ *V. cholerae* was poorly transcytosed compared with wild type in our *in vitro* culture model, we conclude either that the low level of measurable transcytosis is nevertheless sufficient to stimulate an immune response or that other factors can stimulate M cell recognition and uptake of CTA mutant *V. cholerae in vivo*. For example, dendritic cells that may directly sample the lumenal environment might be important for the immune response induced against the mutant bacteria *in vivo* (Rimoldi and Rescigno, 2005).

Our results show that CT expression in the O395 classical biotype is more critical for the internalization process (Table 3) than for simply attachment *per se*. A similar observation was made in the study of polyoma virus internalization by C6 glioma cells, which lack complex gangliosides expression. Addition of GD_{1a} stimulated polyoma virus uptake and internalization without enhancing viral attachment to the cells (Gilbert and Benjamin, 2004). Gangliosides have also been implicated in internalization and axonal retro transport of encapsulated pneumococcus *in vivo* in mice from the nasal cavity to the brain (van Ginkel *et al*., 2003).

An alternative hypothesis to explain our results without the participation of a specialized M-like cell is that bacterial-associated CT damages the Caco-2 epithelial monolayer, thereby allowing penetration of bacteria. However, we observe very little transit of the paracellular marker lucifer yellow after *V. cholerae* transcytosis, compared with what we observe in the absence of any bacteria and ZO-1 fluorescent staining confirmed the integrity of tight junctions (Fig. 3). Furthermore, the TER values remain well above what is thought to represent the value of monolayers with tight junction damage (Canil *et al*., 1993; Heppner *et al*., 2001).

A hallmark in *V. cholerae* pathogenesis is the pathophysiological effect of secreted CT, which is also widely recognized as a powerful mucosal adjuvant (VanCott *et al*., 2000; Lencer, 2001; Ericksson and Holmgren, 2002). CTB conjugated with colloidal gold can attach to and enter M cells in the rabbit small intestine, probably through recognition of its enterocyte receptor, the glycosphingolipid GM_1 (Frey *et al*., 1996). CTB binding to GM_1 also has been used extensively to characterize and isolate lipid rafts in cells derived from different tissues (Parton, 1994; Blank *et al*., 2002). CT is endocytosed by different cell types by both clathrin-dependent and clathrin-independent mechanisms as well as through raft microdomains at the plasma membranes (Orlandi and Fishman, 1998; Torgersen *et al*., 2001). The trafficking mechanism of CT inside the enterocyte-like T84 cell line has been clearly elucidated (Lencer, 2001).

The exact mechanisms involved in the adjuvant properties of the holotoxin CT or just CTB remains elusive (Lycke, 1997). In mice CTB has been shown to regulate the dendritic cell response (Ericksson *et al*., 2003) and, once orally administrated, holotoxin CT is able to recruit dendritic cells towards Peyer's patches (Shreedhar *et al*., 2003), which may be important for stimulating the immune response. A study using liposomes as antigen carrier for the induction of mucosal immune response demonstrated that the CTB adjuvant effect is accomplished successfully when the CTB molecule is exposed in the surface of the liposome, but not when CTB is immersed along with the antigen inside the liposome (Harokopakis *et al*., 1998). This observation suggests a role for CTB in the initial attachment and recognition of the cells that will induce the immune response, such as dendritic cells or M cells (Zhou and Neutra, 2002) and supports our findings with CT in *V. cholerae*.

Interaction between bacteria and enterocytes *in vivo* is likely to occur less readily than interaction between bacteria and M cells. In the small intestine, absorptive enterocytes are covered by a mucus layer of up to 500 nm thick, which in fact does not allow gold-CTB particles to approach (Frey *et al*., 1996). But the mucus layer that covers M cells is thinner, allowing microorganisms, antigens and CT to interact more easily with these cells. As for the trafficking mechanisms in the different cell types that may use lipid rafts – which would probably be the case for a particle that initially interacts with GM_1 – the role of lipid raft-mediated trafficking in enterocytes and M cells may be different. In enterocytes, rafts serve for acquisition and trafficking of cholesterol (Field *et al*., 1998; Hansen *et al*., 2001) whereas in M cells they may be involved in internalizing antigens for further processing.

Our findings may have implications for development of vaccines delivered by mucosal routes. In the case of anti-cholera vaccines, dead bacterial strains are only able to induce limited protection (Killeen and DiRita, 2001). It appears from our work that heat-inactivated *V. cholerae* cells are less readily taken up by M cells, which is consistent with earlier studies using rabbit intestinal ligated loops (Owen *et al*., 1986). Poor immunizing capacity of heat-inactivated strains is not restricted to anti-cholera vaccines *per se*. An attenuated *V. cholerae* strain used as a vector to deliver a model antigen, tetanus toxoid (TT) induced a potent anti-TT response when delivered by the intranasal route but failed to do so when it was heat-inactivated before inoculation (Chen *et al*., 1998). The authors speculated that the ability of the strain to interact with M cells of the respiratory mucosa had been compromised by heat inactivation (Chen *et al*., 1998).

Experimental procedures

Bacteria, cell lines, culture conditions and reagents

The bacterial strains used in this work were *V. cholerae* O395 classical strain, *V. cholerae* O395 GFP-expressing strain transformed with the pGFPmut3.1 plasmid from Clontech. *V. cholerae* O395NT has a deletion/insertion mutation in which the *ctxAB* locus is replaced with a kanamycin resistance gene; O395N1 has a deletion of the *ctxA* gene (Mekalanos *et al*., 1983). These mutant strains were kindly provided by Dr John Mekalanos. EK307 is *V. cholerae* O395 classical strain with a deletion of the *toxR* locus (Krukonis *et al*., 2000) and CVD101 is also a derivative of *V. cholerae* O395 classical strain with a *ctxA* deletion, but which is known to secrete Zot (Fasano *et al*., 1991). The O395NT revertant strain was generated by homologous recombination between the O395NT strain chromosomal DNA and the suicide plasmid pKAS32 containing the *ctx* locus cloned into the BglII–EcoRI sites of the plasmid. The *ctx* locus was cloned by PCR amplification from chromosomal DNA of wild-type strain O395 using the following primers: GCGCGAATTCGAAATGATGCAAC TATCGACTC and CGCGCCTAGGGCTTGCGAATACAAGGT GAG, which contained the EcoRI and BamH1 restriction sites respectively. The selection of the recombinants, in which the mutant allele of O395NT was replaced with the wild-type gene, was performed by screening kanamycin-sensitive strains following a step of growth enrichment over GM_1-ELISA-coated wells. *V. cholerae* O395 chromosomally expressing GFP was obtained after mutagenesis with the miniTn5gfp transposon contained in the vector pAG408 (Suarez *et al*., 1997), kindly provided by Dr Carlos Guzman. The SM10 lamda *pir* strain transformed with pAG408 was conjugated with wild-type O395 *V. cholerae*. The exconjugants were isolated in gentamicin and streptomycin media and then those bright fluorescent positive colonies in DMEM agar-containing media (1:1 complete DMEM in water containing 1.5 g agar per litre) were tested for their ability to transit through Caco-2 epithelial monolayer. Bacterial strains were kept at –80°C in Luria broth containing 20% glycerol and were cultured in Luria broth at 37°C. Eukaryotic cell lines used in this work were Caco-2 human enterocyte-like cells line HTB-37 from ATCC and Raji human B-lymphocytes CCL-86 from ATCC. The Caco-2 cell line was cultured in 75 cc cell culture flasks changing media three times a week and new passages were prepared once a week following trypsin treatment. Cells were seeded at 1×10^5 cells ml^{-1} in 10 ml of fresh Dulbecco's modified essential media (complete DMEM) containing MEM non-essential amino acids 0.1 mM, glutamine 2 mM, high glucose, Hepes buffer 25 mM and fetal bovine serum 10%, from Gibco at 37°C with 5% CO_2. The Raji cell line was cultured in RPMI 1640 (plus MEM non-essential amino acids 0.1 mM, Hepes buffer 25 mM, glutamine 2 mM and fetal bovine serum 10%, Gibco) at 37°C with 5% CO_2, and the culture was diluted 10 times with fresh media twice a week.

Purified CTB, CT, polyclonal rabbit anti-CT, anti-rabbit IgG-TRITC, -FITC or -alkaline phosphatase conjugated, anti-human IgA-FITC, anti-mice IgG-TRITC were from Sigma Aldrich, and anti-mice IgG-PE was from BD Biosciences Pharmingen. Polyclonal rabbit antibody raised against *V. cholerae* was from LEE Laboratories, GM_1 was from Matreya LLC, and mice monoclonal anti-*V. cholerae* or anti-CTB were from USBiological. Alexa 350-conjugated polyclonal antibody and DAPI were from Molecular Probes. UE-1-rhodamine-conjugated lectin was from Vector Laboratories.

M cell generation and transcytosis assays

The epithelial monolayer of Caco-2 cells was obtained by growing cells to confluence in the transwell system (Costar, 3 μm pore diameter, 1 cc growth area). Typically this was for 17 days in complete DMEM plus high glucose at 37°C with 5% CO_2. The medium was changed three times a week. When Raji cells were used, they were added to the basolateral side by submerging the inserts in a dish containing 10×10^6 Raji cells in 10 ml of complete DMEM overnight. On the next day (day 18) inserts were returned to the plates in complete DMEM containing 1×10^5 Raji cells by the basolateral well of the transwell device. Infection with bacteria was performed at day 21 or day 22. To infect the monolayer of M-like cells, *V. cholerae* were grown to mid-log phase in Luria Broth at 37°C and the infection was accomplished using previously DMEM-washed bacteria. A bacterial suspension of 100 μl containing approximately 1×10^7 viable bacteria was used to infect the Caco-2 epithelial monolayer from the lumenal side. The lumenal and basolateral media were sampled over time and serially diluted to estimate the number of colony-forming units. Transcytosis was quantified as follows: (number of bacteria basolateral × 100)/(number of bacteria basolateral + number of bacteria lumenal).

The effect of GM_1 was determined by resuspending bacteria in DMEM containing 1 mg ml^{-1} GM_1 or 1 mg ml^{-1} GM_1 and 0.5 mg ml^{-1} CTB and then infecting the epithelial monolayer of Caco-2 cells as described above. Numbers of cell-associated bacteria were determined after three washes with Dulbecco's PBS (DPBS; Gibco) and lysis of the monolayer with 0.5% sodium deoxycholate in PBS, followed by plating on Luria agar plates. The number of intracellular bacteria was similarly detected but after 15 min of treatment with gentamicin 100 μg ml^{-1}. The inhibitory effect of mouse monoclonal antibody against CTB was determined by adding the antibody along with the bacteria in a 1:10 final dilution to the epithelial monolayer by the lumenal side.

Transepithelial electrical resistance was routinely measured at the beginning and end of each experiment using the epithelial voltohmmeter EVOM (World Precision Instruments, FL). To

establish the integrity of the tight junctions, 0.5 ml of lucifer yellow die (400 μg ml^{-1}) in DPBS was added at the end of the infection by the lumenal side of the DPBS-washed monolayer and incubated during 10 min at 37°C. Then the supernatants were collected and the fluorescence was measured in a SPF-500 spectrofuoremeter SLM AMINCO model using *V. cholerae* (1×10^7) in DMEM as the blank and 488 nm excitation and 530 nm emission. Sialyl-Lewis A antigen expression by the epithelial monolayer of Caco-2 cells was detected using a mouse monoclonal antibody (clone KM231, Kamiya Biomedical Company 1:50) followed by PE-conjugated anti-mouse IgG from Becton Dickinson Biosciences Pharmingen (1:4); cells were then treated with trypsin for 5 min and fixed in cytofix buffer (Pharmingen). Fluorescent cells were analysed by flow cytometry in a Coulter EPIXCS XL cytometer.

Transmission electron microscopy and scanning electron microscopy

For transmission electron microscopy (TEM), cell monolayers grown on transwell membranes were rinsed with 0.1 M Sorensen's phosphate buffer (200 mM mono/di-basic sodium phosphate, pH 7.4) and fixed for 1 h in 2.5% glutaraldehyde in the same buffer. Following a buffer rinse, they were post-fixed for 30 min in 1% osmium tetroxide in the same buffer. They were rinsed in double-distilled water and then block-stained for 30 min in aqueous 3% uranyl acetate. Membranes were dehydrated in a graded series of different ethanol concentrations, infiltrated and embedded in Epon, and polymerized. Ultrathin sections were post-stained with uranyl acetate and lead citrate and viewed on a Philips CM100 at 60 kV. For scanning electron microscopy (SEM), cell monolayers grown on transwell membranes were rinsed in 0.1 M Sorensen's buffer and fixed for 1 h in 2.5% glutaraldehyde in the same buffer. Following a buffer rinse they were post-fixed for 30 min in 1% osmium tetraoxide in buffer, dehydrated by a graded series of increasingly higher concentrations of ethanol, transferred to hexamethyldisilazane and air-dried. Dried membranes were sputter coated with gold and viewed on an Amray 1000 SEM at 10 kV.

Transcytosis of heat-killed bacteria detected by PCR or flow cytometry

The transcytosis assay was developed as described previously above but using heat-killed O395 *V. cholerae* (15 min 60°C). To detect transcytosis of heat-killed bacteria, PCR and flow cytometric assays were developed. For the PCR-based assay, DNA was purified from the media over the lumenal and basolateral sides using a QIAamp DNA mini kit (Quiagen). *V. cholerae* DNA was detected following 100 cycles of standard PCR using primers against the *rfb* locus of *V. cholerae* (Hoshino *et al.*, 1998). For the flow cytometry assay *V. cholerae* classical O395 outer membranes were stained with the PKH-2 kit from Sigma Aldrich following the instructions provided in the manufacturer's manual, and samples were analysed in a Coulter EPICS XL Cytometer.

GM_1-ELISA with whole bacteria

The standard GM_1-ELISA method to measure the amount of CT in solution was modified using whole *V. cholerae*. Bacteria growing to mid log phase in DMEM were centrifuged (7 min at 10 000 *g*) and washed once with complete DMEM, centrifuged again and suspended in complete DMEM in a concentration of $1–5 \times 10^7$ viable bacteria per 100 μl. Each well of the GM_1-ELISA-coated well plate (prepared as describe in Svennerholm and Holmgren, 1978) was incubated with 100 μl of bacteria and serial dilutions were prepared in DMEM for 2 h at 37°C in the cell culture incubator with 5% CO_2. Following a brief wash with PBS containing 0.05% Tween 20, the plate was incubated for 2 h with PBS/Tween containing 0.2% BSA at 4°C. Each well was then incubated overnight at 4°C with 100 μl of rabbit anti-CT or rabbit anti-*V. cholerae* antibody diluted 1:50 or 1:10 in PBS/Tween/BSA respectively. This was followed by three washes of 5 min each with PBS/Tween. Wells were then incubated for 2 h at 4°C with 100 μl of anti-rabbit antibody conjugated with alkaline phosphatase, diluted 1:5000 in PBS/Tween/BSA. Each well was washed three times again with PBS/Tween and the enzymatic alkaline phosphatase activity was determined using the substrate p-nitro-phenyl-phosphate 0.4% in buffer Tris 10 mM pH 8.0. The optical density at 405 nm was determined in an ELISA reader after 30 min and 1.5 h for anti-CT or anti-*V. cholerae* reactions respectively. For a negative control, wells without bacteria but with complete DMEM were analysed, and for a positive control wells were incubated with dilutions of pure CTB (starting with 500 ng ml^{-1}) instead of bacteria. For GM_1-ELISA using heat-killed *V. cholerae* O395, the bacteria were resuspended in DMEM after heat killing for 15 min at 60°C.

Laser confocal microscopy

To detect M cell markers in the Caco-2 monolayer, cells were incubated for 30 min at 37°C with either secretory IgA (Sigma, 0.5 mg ml^{-1}) or a monoclonal antibody against sialyl-Lewis A (1:10). Monolayers were then fixed in 1% formaldehyde for 5 min. Following three washes in DPBS, cells were treated with the anti-human IgA-FITC antibody (1:4) and the anti-mouse IgG-PE antibody (1:4), incubated for 1 h and washed three times in DPBS. The epithelial monolayer was prepared for the laser confocal microscopy as described below. In a similar experiment 100 μl of the fluorescent UE-1 was added to the lumenal side of the epithelial monolayer in the final antibody incubation. To detect production of CT during bacterial association with M-like cells, Caco-2 cells growing to confluency over sterile coverglass (Laboratory-TeckII, Nalge Nunc International) were infected as described above with GFP-expressing *V. cholerae* strain O395 transformed with pGFPmut3.1 plasmid from Clontech. After 30 min, anti-CT antibody (1:4) was added to the cells, followed by another 30 min incubation. After replacing the media carefully, the anti-rabbit TRITC conjugated was added (1:4) in DMEM and incubated for 30 min at 37°C in the cell culture incubator. Subsequently, the epithelial monolayer of Caco-2 cells was washed carefully twice with PBS and fixed in paraformaldehyde 2.5% for 1 h.

To detect production of CT by intracellular bacteria, the epithelial monolayer of Caco-2 cells was infected with *V. cholerae* strains for 1 h. Cells were fixed in 1% formaldehyde in PBS for 5 min followed by permeabilization for 20 min with 0.2% Triton X-100 in PBS. Mouse monoclonal antibody anti-CT (1:50) together with a rabbit polyclonal antibody against *V. cholerae* was added and incubated at room temperature for 1 h. Following two washes with DPBS, the epithelial monolayer was incubated with the anti-

rabbit and anti-mouse antibody conjugated with TRITC or FITC (1:50) containing DAPI (500 nM) overnight at 4°C. Subsequently, the samples were washed twice with DPBS and the membrane containing the epithelial cells was cut out from the transwell with a scalpel and mounted between a microscope slide and a coverslip with Pro-Long (Molecular Probes). Samples were observed using the PlanApo objective 63x with Zeiss LSM 510 using the Argon, HeNe and Enterprise lasers and the LS10 META software. The fluorescent staining of ZO-1 was accomplished after 2 h where the epithelial monolayer of Caco-2 cells was fixed with formaldehyde 1% in DPBS for 15 min, washed three times with DPBS and permeabilized with saponin 0.1% in DPBS for 15 min. First antibodies were added (1:4 in DPBS containing saponin 0.1%) mouse monoclonal anti-*V. cholerae* (USBiological) and rabbit polyclonal against ZO-1 (mid) from Zymed. The epithelial monolayer was incubated at 37°C for 1 h. After three washes with DPBS the cells were incubated overnight with the second antibodies (1:2 in DPBS containing saponin 0.1%). Next day the epithelial monolayer was washed three times in DPBS and treated for 2 h with DAPI 500 nM in DPBS. Then, the epithelial monolayer was washed and prepared as described for the laser confocal analysis.

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