Cholera toxin internalization and intoxication

We read, with considerable dismay, a recent *Research Article* on cholera toxin (CT) internalization (Torgersen et al., 2001), in which the authors extensively challenged methods, results and conclusions that we had published four years ago (Orlandi and Fishman, 1998). As space limits a point-by-point rebuttal of their comments and critique of the many deficiencies in their study, we encourage readers to evaluate our response by comparing both papers. As a preface to our reply, we state that most aspects of CT intoxication are generally accepted, such as its structure and receptor, its mechanism of retrograde trafficking through the Golgi and ER, its mechanism of activating adenylyl cyclase and its pathophysiological effects on human enterocytes. However, we believe that the crux of the dispute is our differing views on the relationship between CT internalization and intoxication. Whereas most of the cell-surface-bound CT is internalized, only a small percentage is activated on release of the enzymatic A_1 peptide (Kassis et al., 1982; Orlandi and Fishman, 1998). Thus, to understand the mechanism of CT action, one must determine not only the pathway(s) for CT internalization but also whether the uptake leads to intoxication of the cell.

Our paper focused on whether both CT internalization and activation are mediated by caveolae or by detergent-insoluble glycolipid-enriched complexes (DIGs) (also known as lipid rafts) in cells deficient in caveolin and caveolae. Torgersen et al. primarily were interested in showing caveolae-independent endocytosis of CT. Our approach was to compare CT uptake and action in three cells that have no, low or high levels of caveolin and caveolae, and to use the cholesterol modifiers filipin and β-cyclodextrin (βCD) to selectively inhibit caveolae/DIG-mediated endocytosis. Chlorpromazine (CPZ) and diphtheria toxin (DT) served as inhibitor and probe for clathrin-mediated uptake. One of our cell lines, human intestinal CaCo-2, played a major role in their study and appears to be the source of many of their repetitious complaints. By using anti-CT-A_1 antibodies to quantify CT uptake, we found 58% inhibition by filipin. As Torgersen et al. found only 17% inhibition by using a different method, they speculated that our assay may have overestimated CT uptake if the antibodies could not reach CT clustered in the narrow necks connecting caveolae to the cell surface, and if filipin could somehow alter the necks and increase antibody binding. They ignored our second assay in which cells were labeled with rhodamine-conjugated CT-B at 15°C. When warmed at 37°C, there is an extensive redistribution of fluorescence from the plasma membrane to the perinuclear region that is blocked by filipin but not CPZ. Even when Torgersen et al. found filipin to be ineffective on two other cell lines, they failed to show that the filipin was active. Filipin is known to be unstable in solution. This led them to a circular argument: as CT uptake is only slightly inhibited by filipin, it must not be via caveolae/DIGs. Thus when they found that βCD inhibits CT internalization in CaCo-2 cells by 43% (similar to our 39%), they concluded that the uptake is clathrin-dependent based on the weak effect of filipin and cited studies showing that βCD also blocks the latter pathway. Surprisingly, two of the four references cited were not relevant. We found that both βCD- and filipin-treated, but not CPZ-treated, CaCo-2 cells remain sensitive to DT. Others have shown that these agents selectively inhibit caveolae/DIG-mediated, but not clathrin-mediated, endocytosis in a variety of cells (Puri et al., 2001; Wolf et al., 2002).

We also assayed CT activation and activity by A_1 and cAMP formation, respectively. Filipin totally blocks both CT activation and activity in CaCo-2 cells, and βCD inhibits CT activity by 98%. Thus, both filipin and βCD are more effective in inhibiting CT activity than endocytosis. Although filipin- and βCD-treated cells still internalize substantial amounts of the bound CT by other pathways, CT remains inactive. In this regard, filipin blocks CT-B trafficking from plasma membranes to Golgi, but not clathrin-mediated endocytosis of CT-B and transferrin in COS-7 cells (Nichols et al., 2001). We found that filipin also inhibits CT activity in A431 and Jurkat cells that are rich in and lacking caveolin and caveolae, respectively. Thus, the disruption of CT intoxication of cells independent of the presence of caveolin and caveolae led us to conclude that CT internalization and activation are mediated through cholesterol- and glycolipid-rich microdomains rather than a specific morphological structure. Torgersen et al. challenged our thesis by asserting, "DIGs have been proposed to act as the vehicle for CT entry in Jurkat T lymphoma cells (Orlandi and Fishman, 1998), but there are no data indicating how DIGs might be internalized." We refer them to a review (Simons and Ikonen, 1997) and an article on endocytosis of a GPI-anchored protein through DIGs in Jurkat cells (Deckert et al., 1996).

Torgersen et al. chide us for not investigating the role of dynamin in caveolae-mediated uptake and finally for suggesting that CaCo-2 cells have caveolae based on small amounts of caveolin. The papers that link dynamin with caveolae-mediated uptake were published while ours was in press (Oh et al., 1998; Henley et al., 1998). Although dynamin is now known to be involved in both clathrin- and caveolae-mediated endocytosis, we are not aware of any role in DIG-mediated uptake. Regarding the presence of caveolin and caveolae in CaCo-2 cells, others agree with us (Mayor et al., 1994; Field et al., 1998). Regardless, our major thesis is that of the role of DIGs and not caveolae per se in CT internalization and intoxication. Finally, we are not dogmatic about our conclusions as some cell types may use a different pathway for CT activation. Neurons, although lacking caveolin/caveolae, have DIGs to which CT binds, but the internalization and activation of the toxin is clathrin-mediated (Shogomori and Futerman, 2001).

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Reply

As described in our title, "Internalization of cholera toxin by different endocytic mechanisms", we studied the endocytic pathways by which cholera toxin (CT) can be taken in from the cell surface. We have previously studied CT transport through the Golgi apparatus and to the ER, as well as cAMP production (Sandvig et al., 1996), and we were among the first to demonstrate this transport step for CT. Similarly, as first shown for Shiga toxin (Sandvig et al., 1992), other toxins are transported retrogradely before entry into the cytosol (for a review, see Sandvig and van Deurs, 2002).

However, studies of CT entry are of interest not only because this toxin can increase the level of cAMP in some cells and cause diarrhoea, but also because CT has been commonly used to label GM1 and to study endocytosis from caveolae. In our study (Torgersen et al., 2001), we used three different model systems to modulate uptake by endocytic pathways: (1) Caco cells transfected with caveolin to create caveolae at the cell surface; (2) Hela cells with inducible synthesis of mutant dynamin, which have been reported to inhibit pinching-off of vesicles from both clathrin-coated pits and caveolae; and (3) BHK cells with inducible synthesis of antisense-clathrin heavy chain (cells in which clathrin-dependent endocytosis can be shut off selectively). The data obtained from the three systems reveal that both clathrin-dependent and clathrin- and caveolae-independent mechanisms can lead to endocytosis of CT. This study does not allow us to conclude whether caveolae can be responsible for endocytosis of cholera. Although caveolae (with caveolin) are quite stable structures in some cell types (Thomsen et al., 2002), the toxin itself might affect the stability. To date, no publications have addressed this question. However, our study (Torgersen et al., 2001) clearly shows that different endocytic pathways can be involved. This is in agreement with recent data published by other laboratories (Nichols et al., 2001; Shogomori and Futerman, 2001). We did not make any attempt to answer whether toxin taken in by the various endocytic mechanisms can elicit a biological response (Torgersen et al., 2001). This is of course an important question but was not addressed in our study.

When one investigates the effect of a certain drug (which quite often has more than one effect on cells) or, for instance, the importance of cholesterol (either by adding drugs, removing cholesterol or adding cholesterol) on the action of a toxin, a reduced or increased effect can be caused by an effect on the endocytic uptake, or by an effect on a later step, such as endosome to Golgi transport of the toxin. Along these lines, it has recently been published that transport of CT (Shogomori and Futerman, 2001), ricin (Grimmer et al., 2000) and the Shiga toxin B subunit (Falguieres et al., 2001) from endosomes to the Golgi apparatus are affected by changes in cholesterol. Also, when comparing the effect of a drug on the cytoplasmic action of various toxins, there may not necessarily be a difference because of the different endocytic mechanisms used by the toxins, but a different response could be caused by different intracellular pathways (cholera/diphtheria toxin). Alternatively, the drug could have a direct effect on the target molecule, for instance on the activity of a membrane-associated target such as adenylyl cyclase, which in itself could be regulated by, for instance, cholesterol or drugs affecting cholesterol. Studies with proteoliposomes have even shown that coupling between G_s and adenylyl cyclase can be dependent on the cholesterol:phospolipid ratio (Bai and Youguo, 1998). In order to avoid such complications in our endocytosis studies, we concentrated on the endocytic uptake by directly measuring the internalization from the cell surface (Torgersen et al., 2001).

It should be noted that there are cell-specific differences (as discussed in our article) when it comes to uptake from the cell surface (as well as to intracellular routing of toxins). Thus, whether a toxin is associated with lipid rafts (Falguieres et al., 2001), and to what extent it is transported to the Golgi apparatus, is clearly cell-type dependent and can be dependent on the type of fatty acid in the toxin receptor (Falguieres et al., 2001; Sandvig and van Deurs, 2002; Lingwood, 1999). That endocytosis of CT can occur independently of filipin addition is supported by a previous study (Shogomori and Futerman, 2001). In fact, when clathrin-dependent endocytosis is reduced by antisense-clathrin induction, or when dominant-negative mutant dynamin is induced to

inhibit both clathrin- and caveolae-dependent endocytosis, there is no further decrease in the uptake of CT by extraction of cholesterol with mβCD (M. L. Torgersen, B.v.D. and K.S., unpublished). Thus, in the cells we have studied, CT can be endocytosed even under such conditions.

In conclusion, it is clear that CT can be taken in by various endocytic mechanisms, and that more has to be done to characterize these mechanisms as well as the intracellular transport of CT. To fully characterize the transport of CT, investigation of each step will be necessary.

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