PHYSIOLOGICAL REVIEWS
Vol. 76, No. 4, October 1996
Printed in U.S.A.

Endocytosis, Intracellular Transport, and Cytotoxic Action of Shiga Toxin and Ricin

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Sandvig, Kirsten, and Bo van Deurs. Endocytosis, Intracellular Transport, and Cytotoxic Action of Shiga Toxin and Ricin. *Physiol. Rev.* 76: 949–966, 1996.—Protein toxins such as ricin and Shiga toxin with intracellular targets have to be endocytosed and translocated to the cytosol to inhibit the protein synthesis and thereby kill the cell. Ricin is internalized by both clathrin-dependent and -independent endocytic mechanisms, whereas Shiga toxin seems to be taken up exclusively from clathrin-coated pits. After endocytosis, internalized membrane and content are delivered to endosomes, where sorting for further routing in the cell takes place. Toxins that remain membrane bound at low endosomal pH can be recycled to the cell surface or transcytosed in polarized epithelia. A large proportion of internalized toxin is transported to lysosomes for degradation. Most importantly, a fraction of the internalized ricin and Shiga toxin molecules is delivered to the *trans*-Golgi network (TGN). Shiga toxin can, in some very sensitive cells, be transported retrogradely through the Golgi cisterns all the way back to the endoplasmic reticulum (ER), and it is possible that also ricin is transported retrogradely to the ER. In this review, a cell biological overview of these intracellular transport steps is presented, and evidence is provided that the delivery to the TGN and the subsequent retrograde transport to the ER are required for optimal intoxication. Moreover, it is argued that knowledge of this transport is important for targeted drug delivery such as the application of immunotoxins in cancer therapy.

I. INTRODUCTION

Studies on the endocytosis, intracellular transport, and cytotoxic action of bacterial and plant protein toxins with intracellular targets are of interest for a number of reasons. Several of the bacterial toxins are responsible for or are important factors in the development of widespread and serious diseases such as diphtheria (for review, see Ref. 166), cholera (for review, see Refs. 45, 196), and dysentery and hemolytic uremic syndrome (for review, see Refs. 1, 87, 133, 203). Unraveling the mechanism of action of these toxins might help in understanding the diseases and improving treatment. Because of their extreme toxicity, the toxins might even be used to cure disease such as cancer, if directed to specific cell types. For this purpose, toxins or the enzymatically active part of them can be covalently coupled either to antibodies directed against surface antigens or to ligands binding to surface receptors of cells that one wishes to kill (targeted drug delivery) (for review, see Refs. 13, 56, 221). A large number of laboratories have been involved in the development of immunotoxins directed against cancer cells, and promising results have been obtained. To construct active immunotoxins, it is important to know which part of the toxin molecule to include and whether or not the construct has to be targeted to a specific intracellular compartment in the cell. For this purpose, knowledge of the mechanisms involved in intracellular sorting is essential.

Protein toxins can also be used to study translocation of proteins across membranes. Their high toxicity provides the investigator with a very sensitive test system for translocation to the cytosol. Furthermore, the toxins can

even be used as vectors to bring other peptides or proteins into the cytosol (197, 228). This is likely to have wide implications both for basic studies and for treatment of and protection against disease.

The toxins themselves have proven valuable as tools in cell biological research to clarify basic mechanisms of intracellular sorting and transport (for review, see Refs. 179, 181, 216). Ricin (6, 111), which binds to a large number of molecules at the cell surface, has turned out to be useful in studies of membrane traffic. Not only has ricin provided some of the early evidence for different types of endocytosis (179), but as described below, this toxin has also given us valuable information about transport to the Golgi apparatus and regulation of transcytosis in polarized epithelial cells. Transcytosis is for instance responsible for transepithelial transport of immunoglobulins that will protect the organism (127), and the process is essential for uptake of drugs.

Shiga toxin, which is produced by *Shigella dysenteriae*, and Shiga-like toxins produced by *Escherichia coli* or by *Citrobacter freundii* (184) are not only important in connection with disease (for review, see Refs. 1, 87, 133, 203); Shiga toxin was in fact the first glycolipid-binding molecule shown to be internalized from clathrin-coated pits (167). Moreover, it was the first molecule shown to move all the way from the cell surface, through the Golgi apparatus, to the endoplasmic reticulum (ER) and the nuclear membrane, thus demonstrating a new pathway in intracellular transport (163, 174).

II. STRUCTURE AND PHYSICAL PROPERTIES OF PROTEIN TOXINS

A number of protein toxins produced by bacteria or found in plants exert their toxic action on cells by entering the cytosol and inactivating components of the machinery required for normal cell function (6, 39, 40, 85, 86, 111, 133, 136, 166). In many cases, the toxins first bind to cell surface receptors by one moiety of the toxin molecule, then they are endocytosed, and finally an enzymatically active part of the toxin enters the cytosol from an intracellular compartment. Although the toxins are structurally related, the mechanism of internalization varies, and even the intracellular target for the protein toxins differs. Several plant toxins, ricin, abrin, viscumin, volkensin, modeccin, and the bacterial toxin Shiga toxin, inhibit protein synthesis by removal of one single adenine from the 28S RNA of the 60S subunit of the ribosome (39, 40), thereby inhibiting protein synthesis. Two other bacterial toxins, diphtheria toxin and *Pseudomonas* exotoxin A, also inhibit protein synthesis, but by ADP-ribosylation and consequent inactivation of elongation factor 2 (166, 227). In several cell types, the toxins that inhibit protein synthesis induce cell lysis in a manner that is characteristic for

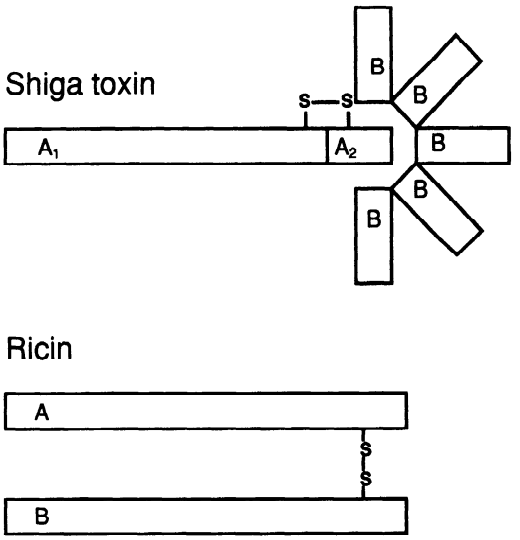

FIG. 1. Schematic models of Shiga toxin and ricin.

programmed cell death or apoptosis (21, 22, 63, 178). There is cell blebbing and DNA degradation, and the typical DNA ladder seen in other apoptotic processes can be found both in Vero and Madin-Darby canine kidney (MDCK) cells (178). Other protein toxins, such as cholera toxin and pertussis toxin, use ADP-ribosylation of other target proteins to exert their effect (for review, see Ref. 34). Recently, the intracellular action of *Clostridium difficile* toxins A and B was clarified. These toxins add glucose to rho and other small GTP-binding proteins of the same class, leading to changes of the cytoskeleton (85, 86).

We focus in this review on ricin and Shiga toxin, because our knowledge about the intracellular transport of these toxins has increased considerably during the last years (for review, see Ref. 181). Ricin is found in the seeds of *Ricinus communis*, and there are various isoforms with molecular masses of 63–66 kDa (for review, see Refs. 6, 111). The toxin consists of an A chain that has enzymatic activity and a B chain that binds to the cell-surface receptor (Fig. 1). The three-dimensional structure of ricin was solved by X-ray crystallography (154). The B chain contains two domains originating from gene duplication; these domains have 32% homology, and each of them has a binding site for galactose (for review, see Refs. 6, 111). The two chains (A and B) are linked by a disulfide bond. The ricin gene codes for a preprotoxin that has 576 amino acids (preproricin D, GenBank accession no. X52908). The mature form of ricin is formed by removal of the leader sequence (35 amino acids) and by removal of the interchain linker of 12 amino acids. This leaves the A chain with 267 amino acids and the B chain with 262 residues. The toxin is glycosylated; it contains mannose and glucosamine on both chains (137). Most of the carbohydrate is associated with the B chain.

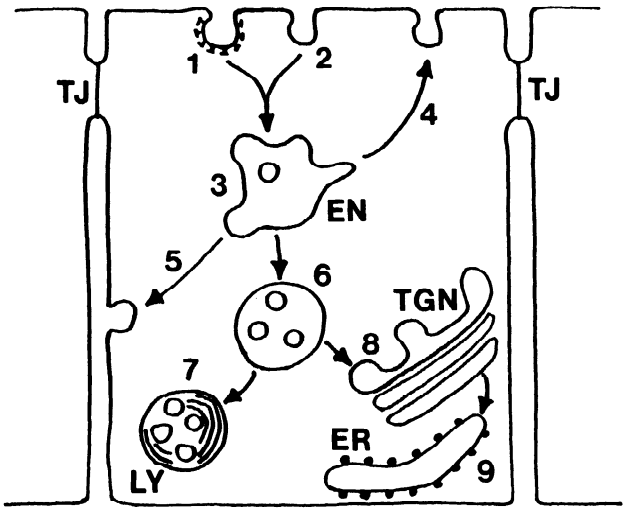

FIG. 2. Schematic model of endocytosis and intracellular membrane traffic in a polarized epithelial cell. For simplicity, only endocytosis and subsequent transport from apical surface are shown. TJ, tight junctions. Endocytosis may be clathrin dependent (*1*) or clathrin independent (*2*) and leads to an early endosome compartment (*3*; EN) that acts as a sorting station. Thus, from this compartment, internalized membrane and content may be recycled to apical surface (*4*), transcytosed to basolateral surface (*5*), delivered via maturing endosomes (*6*) to lysosomes (*7*; LY), or be transported to *trans*-Golgi network (*8*; TGN) and all the way through Golgi cisterns to endoplasmic reticulum (*9*; ER).

Shiga toxin is produced by *Shigella dysenteriae* (for review, see Ref. 133). The binding moiety of this toxin consists of five small B subunits (molecular mass 7,691 Da for one subunit) (Fig. 1). These subunits are noncovalently associated with an A subunit (molecular mass 32,225 Da), which is proteolytically cleaved to an A_1 subunit with enzymatic activity (molecular mass 27 kDa) and an A_2 subunit that is connected with the A_1 subunit by a disulfide bond. The A fragment can be cleaved with trypsin, but as described in section VII, it was recently shown that furin is the cellular enzyme responsible for cleavage and activation in several cell types (52). In cells without furin, the toxin is also processed and activated, but much more slowly and less extensively, possibly by the cytosolic enzyme calpain (51, 52).

III. MECHANISMS OF ENDOCYTOSIS

Protein toxins such as ricin and Shiga toxin have to be endocytosed to exert their toxic effect on cells (165). Because ricin binds to both glycolipids and glycoproteins with terminal galactose, a large number of toxin molecules become bound to the cell surface (3×10^7 molecules per HeLa cell; Ref. 170). Ricin provided some of the first evidence that there is more than one mechanism for endocytosis (128, 168, 216) (Fig. 2). The best characterized mechanism is the clathrin-dependent process responsible for receptor-mediated uptake of transport proteins like low-density lipoprotein and transferrin, growth factors, and hormones (3, 10, 31, 67, 72, 81, 101, 155, 159). Special protein sequences, often including an essential tyrosine residue in the cytosolic tail of the receptors, are required for uptake by this mechanism (220). Recently, it has been shown that also a dileucine motif (67) and the motif K-K/R-F/Y-F/Y (81) can function as endocytosis signal. Such internalization sequences interact with adaptor proteins leading to aggregation of receptors in clathrin-coated pits. As expected, ricin is also bound to molecules internalized by this pathway, and with the use of electron microscopy (EM), ricin-gold or ricin-horseradish peroxidase (HRP) conjugates can be visualized in clathrin-coated pits and vesicles (for review, see Refs. 214, 216). However, ricin is internalized even when the clathrin coats are removed from the cell surface by hypotonic shock followed by potassium depletion (128), and also when the formation of the clathrin-coated vesicles is blocked by acidification of the cytosol (168). Such acidification of the cytosol can be carried out in several ways that basically give the same result: paralysis of clathrin-coated pits (69, 168). In the case of potassium depletion, it can be argued that the decoated or naked pits are still able to pinch off even in the absence of the clathrin coat (30), whereas the latter method (acidification) clearly tells us that there must be a second form of endocytosis responsible for uptake of a large fraction of the endocytosed toxin. Although the relative importance of clathrin-independent endocytosis varies from one cell type to another, several studies have revealed that the clathrin-independent mechanism is responsible for ~50% of fluid-phase endocytosis (38, 143, 168, 169). That cells have such a clathrin-independent mechanism has recently been confirmed by the finding that when clathrin-dependent endocytosis is inhibited by transfection of Cos-7 cells with a mutant of the 100-kDa GTPase dynamin, clathrin-dependent endocytosis is inhibited, whereas fluid-phase uptake still occurs (211). Similarly, also in HeLa cells with a mutant dynamin unable to hydrolyze GTP, fluid-phase endocytosis continues and is reduced only to one-half the original value (31). Furthermore, when HeLa cells were transiently transfected with a mutant dynamin that blocks clathrin-mediated endocytosis, they were still intoxicated with ricin entering by clathrin-independent endocytosis (193).

The term *clathrin-independent endocytosis* may actually cover more than one mechanism (179). In A 431 cells, there is, after stimulation of protein kinase C, increased uptake of ricin by a clathrin-independent mechanism that involves formation of relatively large vesicles (macropinocytosis) (177). However, in HEp-2 cells, clathrin-independent endocytosis is mediated by vesicles with a diameter of ~95 nm (68), significantly smaller than the diameter of clathrin-coated vesicles in the same cells. It is being discussed whether caveolae, small uniformly shaped invaginations with a diameter of ~60 nm and re-

cently shown to be coated with caveolin, can pinch off from the cell surface or not (213). In most cell types there is no evidence for such a process, but it has been shown that a combination of hypertonic medium and treatment with okadaic acid can induce internalization of whole membrane areas that are enriched in caveolae (141). However, clathrin-independent endocytosis can clearly occur even in the absence of caveolae. In lymphocytes, which have no caveolae as defined by morphology as well as by the absence of caveolin (46), interleukin-2 is internalized by a clathrin-independent process (199). Also, ricin is internalized by a clathrin-independent mechanism in Caco-2 cells, which were reported to be without caveolae (100), and, as described below, clathrin-independent endocytosis occurs on the apical side of polarized MDCK cells (38), although this membrane domain does not contain caveolae (van Deurs, unpublished data). The MDCK line is an epithelial cell line that forms polarized cell layers when grown on filters. Therefore, although the sensitivity of cells to ricin varies, ricin can be internalized in several ways, and endocytosis is presumably not the limiting factor for cytotoxicity in most cells.

The question has been raised whether endocytosis of ricin by a clathrin-independent mechanism occurs only in nonpolarized cells or whether such a process occurs in more complicated systems like polarized epithelia. Recently, evidence was obtained for internalization of ricin by clathrin-independent endocytosis on both the apical and the basolateral pole of MDCK cells (38). Both when the cytosol was acidified to block the pinching-off of clathrin-coated pits and when the cells were treated with hypotonic shock and potassium depletion to remove clathrin-coated pits, ricin endocytosis continued on both sides of the epithelial cells. The data suggest that the proportion of the two pathways may differ on the two poles. Interestingly, clathrin-independent endocytosis in these cells seems to be under selective regulation at the apical side, which lacks caveolae. Both activation of protein kinases C (74) and A (38) as well as treatment with the drug mastoparan (38), which is known to activate heterotrimeric G proteins, increase the apical endocytosis of ricin as well as of the fluid-phase marker horseradish peroxidase by a clathrin-independent process. Also, ricin endocytosis in another polarized cell line, Caco-2, is increased apically after stimulation of protein kinase C (74), demonstrating that ricin uptake can depend on whether the cell has been stimulated by, for instance, growth factors or hormones, and also emphasizing that clathrin-independent endocytosis can be under regulation highly specific for one domain in a polarized cell. These data suggest that clathrin-independent endocytosis can be regulated independently of the uptake mediated by clathrin-dependent endocytosis. Also, mastoparan, which seems to increase the apical clathrin-independent endocytosis, actually inhibits the clathrin-mediated process in both permeabilized (19) and intact cells (38).

Another important question is whether clathrin-independent endocytosis is a phenomenon only associated with established cell lines. Recent investigations reveal that this is not the case. In *Xenopus* oocytes, clathrin-independent endocytosis occurs, and it seems to be regulated by protein kinase C and RhoA (183). Moreover, rapid membrane retrieval after exocytosis in adrenal chromaffin cells (5) and in pituitary melanotrophs (206) takes place by clathrin-independent endocytosis.

The physiological significance of clathrin-independent endocytosis has not been completely clarified. However, some roles for the clathrin-independent uptake have been reported. As mentioned above, clathrin-independent endocytosis is involved in retrieval of membrane after induced exocytosis in pituitary cells (206) and in adrenal chromaffin cells (5), and one could speculate that this would also be the case in, for example, thyrocytes and secretory epithelial cells of exocrine salivary glands. Moreover, clathrin-independent endocytosis is involved in actin-dependent uptake of desmosomal plaques after loss of cell-to-cell contact (33, 75). It is conceivable that clathrin-independent endocytosis is important for uptake of membrane molecules for repair or degradation; clearly, the composition of membrane proteins is changed during differentiation of cells. Recently, HeLa cells transfected with the dynamin mutant that blocks clathrin-dependent endocytosis were shown to upregulate the clathrin-independent process, until the total endocytic uptake reached the original level (31). Probably, the fact that clathrin-independent endocytosis seems to be upregulated by factors other than clathrin-dependent endocytosis and perhaps can be more readily changed than the clathrin-dependent uptake could somehow be of advantage to the cells.

Shiga toxin binds to a more limited number of receptors at the cell surface than ricin ($\sim 10^6$ receptors/cell in sensitive HeLa cells; Ref. 37). The functional receptor is the neutral glycolipid Gb3 (28, 82, 107, 114), which is not even present on all cell types (37, 112, 114, 173). It is present on, for instance, kidney endothelial cells of children, a physiological property giving rise to kidney failure in some of the children infected with bacteria producing Shiga toxin (or Shiga-like toxin). Receptor synthesis is under regulation by tumor necrosis factor (TNF), interleukins, and butyric acid, and receptor regulation may be important during development of disease (83, 114). The finding that B cells express Gb3 has been suggested to be important for the lack of antibody response in humans exposed to Shiga toxin (114, 115). The interaction of Shiga toxin with Gb3 is not only dependent on the carbohydrates; the lipid part of the receptor is important for binding (90, 114), and possibly also for the ability to intoxicate the cells (174).

The endocytosis mechanism used by Shiga toxin to enter the cell turned out to be clathrin dependent (167, 173). This was somewhat surprising, considering that

Shiga toxin binds to a glycolipid, and in fact, Shiga toxin was the first lipid-binding ligand shown to be endocytosed by this mechanism. Two other lipid-binding toxins, cholera toxin and tetanus toxin, have been claimed to be internalized by clathrin-independent mechanisms (124, 208). It seems reasonable to suggest that Shiga toxin or the toxin-receptor complex interacts with a protein that aggregates in clathrin-coated pits. Recently, a lipid-anchored prion protein was found to enter by clathrin-dependent endocytosis (191), and in this case, it was found that the NH_2-terminal end of the protein is required for the endocytic uptake by this mechanism (192). Shiga toxin seems to be evenly bound all over the cell surface at low temperature. However, after a short incubation at 37°C, the toxin aggregates in clathrin-coated pits and is endocytosed (167, 173). This has been demonstrated by EM, where the toxin was visualized by using antitoxin antibodies followed by protein G-gold. Biochemical studies support the idea that clathrin-dependent endocytosis is required for intoxication of cells with Shiga toxin. When the cytosol is acidified to block clathrin-dependent endocytosis, the toxin is not endocytosed and the cells are protected (167). Likewise, when the clathrin coats are removed by potassium depletion, the cells are not intoxicated (180). Furthermore, cell surface-bound toxin rapidly becomes inaccessible to antitoxin (167), a finding which is in agreement with the rapid uptake mediated by clathrin-dependent endocytosis.

IV. SORTING IN ENDOSOMES

The preendosomal vesicles formed by clathrin-independent endocytosis in HEp-2 cells fuse with endosomes containing transferrin receptors taken in by the clathrin-dependent pathway (70), implying that at least in this cell line the fate of endocytosed ricin is the same, whether it is taken in by one pathway or the other. The endosome acts as a major sorting station on the endocytic pathway (Fig. 2). Thus there are several possible routes that can be followed by ricin and Shiga toxin delivered to this compartment. A large fraction of endocytosed ricin is recycled to the cell surface, and, in fact, ricin was the first endocytosed ligand shown to recycle (164). To what extent Shiga toxin is recycled is not known. The cell surface seems to be cleared of the toxin (except for membrane regions enriched in caveolae; unpublished data) in experiments where the toxin was prebound (167, 173), suggesting that there may be limited or no recycling. Both toxins can be transported across epithelial cell layers by transcytosis, and this process is likely to be important for intoxication in vivo (173, 212). In the case of ricin, it has been shown that the toxin is active even after transcytosis (212), and this is probably the case also for Shiga toxin, since both toxin molecules are extremely stable and quite resistant to proteolytic degradation (135, 137) and since Shiga toxin can cause a systemic disease after infection with bacteria that produce the toxin (1, 87, 88, 114). Even though a large fraction of both toxins is transported to lysosomes (167, 173, 217), the degradation rate is quite low both for ricin (164) and for Shiga toxin (unpublished results). As described below, a relatively small fraction of both ricin (120, 146, 172, 217) and Shiga toxin (161, 173) is transported to the Golgi apparatus (Fig. 2), and this transport step seems to be essential for intoxication.

V. TOXIN TRANSPORT TO THE *TRANS*-GOLGI NETWORK AND IMPORTANCE FOR INTOXICATION

The first demonstration that internalized protein can be transported to the Golgi apparatus was based on ultrastructural localization of cholera toxin conjugated to HRP (84, 126). Later, it was similarly shown that ricin and Shiga toxin conjugated to HRP were delivered to the Golgi complex (59, 167, 172, 173, 176, 218) (Fig. 3). Normally, HRP alone is never delivered in detectable amounts to the Golgi complex; however, two interesting exceptions to the rule have been reported (119, 138).

Today we do not find it surprising that internalized protein molecules at least to some extent will be sorted from the endocytic pathway to the Golgi complex, provided that they remain membrane associated at low pH. Ricin and Shiga toxin in fact bind very efficiently to the membrane even at the low endosomal pH; at pH 5, ~75% of ricin molecules are still bound compared with binding at pH 7 (170, 217; Sandvig, unpublished data). Resialylation of membrane molecules has been reported, a process which most likely involves recycling through the *trans*-most portion of the Golgi apparatus (195, 222). Furthermore, glycoprotein recycling to the galactosyltransferase-containing compartment of the Golgi apparatus has been demonstrated (76). Moreover, the role of the cation-independent mannose-6-phosphate receptor (M6PR) in delivering lysosomal enzymes with a mannose-6-phosphate tag from the *trans*-Golgi network (TGN) to late or mature endosomes is well established (58). After delivery and pH-induced release of the cargo to endosomes, the unoccupied M6PRs are recycled in vesicles back to the TGN (58). Even a lipid-linked protein, Thy-1, is transported from the cell surface and to the Golgi apparatus, although at a slower rate than transmembrane proteins (61). Moreover, it is now established that some molecules such as furin (12, 182, 223) and TGN38 (11, 79, 229) have specific TGN sorting signals. These transport systems thus provide a pathway for opportunistic molecules bound to the endosome membrane. Also, glycolipids that bind ricin may be transported from endosomes to the Golgi apparatus, and clearly, the lipid-binding ligands Shiga toxin (see sect. III) and cholera toxin (42, 43, 45) are transported to the Golgi

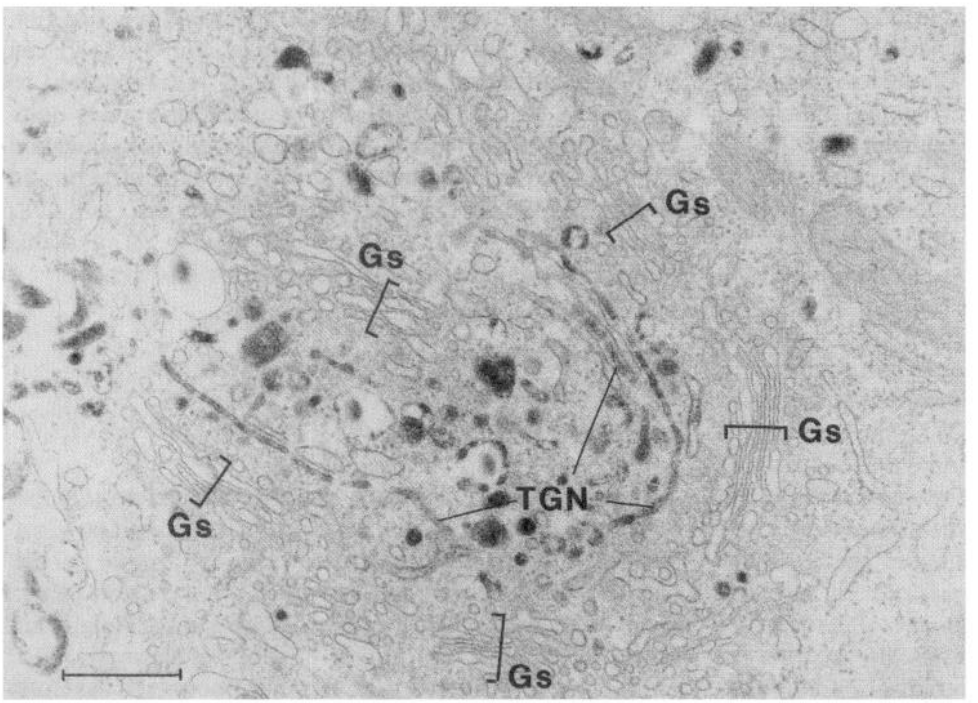

FIG. 3. Electron microscopic picture of a T47D human breast carcinoma cell that has internalized a monovalent ricin-horseradish peroxidase conjugate for 1 h. Conjugate is seen in extensive *trans*-Golgi network (TGN), while surrounding Golgi stacks (Gs) are not labeled. Bar, 0.5 μm.

apparatus in spite of the fact that they do not bind directly to proteins with sorting sequences. It has been found that some membrane molecules like TGN38 and furin require low endosomal pH for sorting from endosomes to the Golgi apparatus (23). However, ricin transport to the Golgi apparatus is not inhibited by agents that neutralize the low endosomal pH (see below). In fact, transport from the basolateral surface to the Golgi apparatus in polarized MDCK cells is even increased after neutralization of the endosomal/lysosomal pH by addition of monensin, and interestingly, also the toxic effect is increased (120). That there is not a similar increase in the fraction of ricin transported to the Golgi after apical toxin addition could be explained by a differential effect of monensin on the rate of toxin transport to a common compartment from where ricin is sorted to the Golgi apparatus (perhaps a late endosomal compartment), but the finding also raises the question as to whether ricin is transported to the Golgi apparatus from different compartments after endocytosis from the two sides. Perhaps ricin can be transported to the Golgi apparatus from an earlier station on the endocytic pathway than the late or mature endosome. Interesting in this connection is the finding by Simpson et al. (193) that overexpression of a rab9 mutant that blocks transport of M6PR from late endosomes to the Golgi (110) apparently does not protect against ricin, suggesting that ricin transport to the Golgi might occur at an earlier step. Alternatively, the rab9 is essential for transport of receptors like M6PR but not for membrane flow in general.

Early studies with transport of internalized ricin-HRP to the Golgi complex demonstrated that polyvalent HRP conjugates (several ricin molecules per conjugate) as well as ricin-colloidal gold conjugates, which are also polyvalent, are delivered directly from endosomes to lysosomes (218). Only monovalent ricin-HRP conjugates could be traced to the Golgi apparatus (Fig. 3). That the HRP molecule in the monovalent conjugate in itself was not responsible for the sorting to the Golgi apparatus was confirmed by using preembedding immunoperoxidase detection of internalized unconjugated ricin. However, even though serial section analysis convincingly demonstrated a consistent ricin-labeling of only one side of the Golgi stack, it could not unequivocally be concluded that this side represented the TGN. Moreover, the use of peroxidase conjugates did not make it possible to draw any conclusions with respect to the amount of internalized ricin that reached the Golgi complex. In a subsequent study, immunogold labeling was used to colocalize internalized native ricin with the G protein of a temperature-sensitive mutant of vesicular stomatitis virus (VSV) (217). With the cells incubated at the nonpermissive temperature, the VSV G protein could be accumulated in the TGN and thus served as a marker of this compartment. In this way, it was demonstrated that ~5% of the total amount of ricin internalized after 1 h was present in the Golgi apparatus. Of this amount, ~75% was localized to the TGN. It remains an open question whether the remaining 25% was transported directly from endosomes to the Golgi cisterns or whether they arrived at the cisterns from the TGN, either by simple backflow in the Golgi stack or by an active sorting process. We tend to favor the latter possibility that all toxin molecules are first transported to the TGN and then fur-

ther sorted to the cisterns (see below and sect. VI). The amount of ricin transported to the Golgi apparatus was later confirmed by using cell fractionation (120, 146, 172), and other studies revealed that a similar amount of internalized Shiga toxin reaches the TGN (161, 173). The delivery of toxin to the TGN is a temperature-sensitive process. Thus, at 18–20°C, internalized ricin is present in endosomes, but not in the Golgi apparatus (nor in lysosomes) (176, 215), and importantly, the cells are protected against the toxin (167, 215) (see below).

In the above-mentioned studies, a correlation between delivery of toxin molecules to the TGN/Golgi cisterns and cytotoxicity was indicated. In addition, the list of arguments that can be presented in favor of a role of toxin delivery to the TGN for toxicity is long.

1) Drugs that inhibit various steps on the biosynthetic pathway, e.g., the protein synthesis (cycloheximide), *N*-linked glycosylation in the ER (tunicamycin) or mannosidase activity in the Golgi apparatus (swainsonine), sensitize cells to ricin and Shiga toxin (176). One possible explanation for these results is that toxins entering the Golgi apparatus might compete with newly synthesized molecules for transport or processing.

2) Low concentrations of the ionophore monensin, which have no pH-neutralizing effect in endosomes/lysosomes (117) but influence Golgi structure and function (205), sensitize cells to ricin (165, 176) and, interestingly, also to a number of immunotoxins (20, 148), suggesting that Golgi transport is important also for the toxic action of these molecules. Moreover, as mentioned above, the low pH of late or mature endosomes and lysosomes is not required for ricin toxicity. Ammonium chloride (10 mM) and high concentrations of monensin (2–10 μM) which neutralize pH in organelles on the endocytic pathway do not protect against, but rather sensitize cells to ricin (120, 165, 171). Also, bafilomycin, which is a specific inhibitor of the vacuolar proton ATPase, does not protect against ricin (Sandvig, unpublished data).

3) In cells incubated with ricin and Shiga toxin at 18–20°C, the delivery of toxin to the Golgi apparatus is blocked, and also the toxic effect is strongly reduced (167, 176, 215). At this low temperature, also transport of M6PRs and asialotransferrin receptors to the TGN is prevented (167, 176, 215). In contrast, diphtheria toxin that becomes translocated from endosomes clearly intoxicates cells at 18–20°C (116, 175).

4) A hybridoma cell line producing monoclonal antibodies against ricin was found to be resistant to ricin (234). This is most readily explained by assuming that internalized ricin meets the antibody before ricin A chain translocation can take place, i.e., in the TGN.

5) Studies with brefeldin A (BFA) have shown that there is a correlation between an intact Golgi apparatus and the sensitivity of cells to ricin and Shiga toxin (134, 172, 233). In cells where the organization of the Golgi apparatus is sensitive to BFA, this drug protects against intoxication. Brefeldin A is a fungal drug known to have many effects on cells (25, 32, 35, 36, 49, 50, 80, 105, 108, 109, 113, 122, 132, 139, 144, 146, 150, 156, 189, 190, 202, 207, 210, 225, 230, 232, 235). One very remarkable effect is that it disrupts the Golgi stack in many cell types; membrane and content are subsequently transported in a retrograde fashion all the way back to the ER (35, 50, 108, 109, 122). However, many studies report that the TGN remains structurally intact, although the preferential perinuclear position is lost (25, 35, 109, 172). In some cell lines, BFA apparently has no effect on Golgi structure, e.g., in MDCK and PtK2 cells (80, 98, 99, 172), and BFA treatment of MDCK cells does not protect against ricin (172). It should be noted that, also in these cells, BFA affects endosome morphology. However, in numerous cell lines like HEp-2, T47D, Vero, and MCF-7, a dramatic transformation of the Golgi stack into characteristic tubulovesicular structures and a subsequent retrograde transport of these structures to the ER occur upon BFA exposure. In these cells, BFA protects against ricin (172). Interestingly, after BFA treatment of these cells, the TGN appeared structurally discrete and, as revealed by EM, labeled by ricin-HRP conjugates (172). With the assumption that retrograde transport of ricin and Shiga toxin to the ER is necessary for A-chain translocation to occur, a straightforward interpretation of the data is that when BFA causes retrograde transport of the Golgi stack, but not of the TGN, to the ER, then internalized toxin will end up in the TGN without any chance of further transport backward through the Golgi cisterns to the ER; accordingly, the cells are protected. In agreement with the idea that toxin translocation depends on transport to the Golgi and possibly also on retrograde transport to the ER is the finding by Wu et al. (231) that an immunotoxin that acts after addition of retinoic acid no longer intoxicates the cells when BFA is also added. It should, however, be noted that results that do not support this model have been reported. For instance, there is no protection against ricin in a temperature-sensitive mutant cell line where the Golgi apparatus is disrupted at the nonpermissive temperature (7). However, it could not be ruled out that some intact Golgi cisterns were still present. Also, Hudson and Grillo (78) reported that the effect of some immunotoxins was increased instead of decreased by BFA, suggesting that some immunotoxins can enter from a different compartment than others. When cells are incubated with high concentrations of ricin A or diphtheria A chain, some toxic effect can be seen even on intact cells (Sandvig, unpublished data), an effect which may be due to leakage of protein into the cytosol from another compartment than the one used by toxins containing both the A and the B chains. In agreement with this hypothesis is the finding that the galactose binding sites of ricin seem to be important for efficient intoxication (111, 131).

VI. RETROGRADE TRANSPORT TO THE ENDOPLASMIC RETICULUM

The concept of retrograde transport (Fig. 2) raises two important questions: *1*) Is internalized toxin actually transported to the ER, and if so, what is the mechanism of this retrograde transport? *2*) Is retrograde transport of toxin to the ER necessary for optimal toxicity? In answering these questions, much attention has been paid to the KDEL (-Lys-Asp-Glu-Leu-) sequence, and similar sequences that function as ER retention signals present on luminal proteins (129) and on transmembrane proteins with the COOH-terminal luminal (204). The sequence is recognized by the KDEL receptor, which seems to be present throughout the Golgi stack and even in the TGN (62, 121), and the receptor is involved in sorting of proteins to the ER. Consequently, if an internalized protein which for some reason reaches the TGN/Golgi stack is provided with a KDEL or with a similar sequence recognized by the KDEL receptor, it will be carried retrogradely to the ER. There is evidence that this system could be of importance for the sorting and cytotoxicity of some protein toxins, but the existing data are far from unambiguous. *Pseudomonas* exotoxin A has a KDEL-like sequence (REDLK), and removal of this sequence significantly reduces toxicity (24, 186). Similarly, cholera toxin has a KDEL sequence in its A chain (RDEL in *E. coli* heat-labile toxin) (for review, see Refs. 194, 196), and A-chain translocation to the cytosol is inhibited by BFA (106, 140), suggesting that retrograde transport also of this toxin may be required for translocation to the cytosol. However, mutation of the RDEL sequence of LT1 did not change the ability of the toxin to increase the level of cAMP in cells (27), suggesting that it is not of importance for translocation of the A fragment to the cytosol. This does not imply that cholera toxin is not transported in a retrograde manner through the Golgi apparatus. It should be noted that even cholera B chain, which has no KDEL sequence, is transported to all the Golgi cisterns in A 431 cells (174). Also, ricin (102), the related plant toxin modeccin (145), and Shiga toxin (96, 187, 198) do not have KDEL or KDEL-like sequences. In spite of a lack of the KDEL sequence, retrograde transport of Shiga toxin to the ER has been shown to occur (163, 174) (see below). Although ricin is highly toxic even without a KDEL sequence, insertion of a KDEL sequence in ricin increases the cytotoxicity in some cell types, whereas there is no difference in others (200, 224). It turns out that the cytotoxicity of ricin with KDEL correlates with the distribution of the KDEL receptor in the Golgi (200). Ricin has so far not been demonstrated in the ER. Recently, Simpson et al. (193) suggested that recycling between the ER and the Golgi complex is required for ricin and Shiga-like toxin 1 cytotoxicity. They have overexpressed GTPase mutants of Rab1, Sar1, and Arf1 and have shown that the cells expressing these proteins are protected against the toxins. The authors suggest that a recycling receptor is required for retrograde transport of toxin, but they also discuss other possible explanations for the protection observed, and the study does not prove that ricin has to enter the ER to intoxicate cells. However, the experiments clearly suggest that the Golgi is essential for intoxication.

Recently, EM studies revealed that HRP conjugates of Shiga toxin and its B subunit can be transported all the way back to the ER and the nuclear envelope (which is continuous with the ER) (163, 174) (Fig. 4). Incubation of A 431 cells with butyric acid induced retrograde transport, which correlated with a marked sensitization of A 431 cells to the toxin (163). The A 431 cells had to be incubated with butyric acid for 48 h to induce maximal sensitization, and the sensitization was dependent on protein synthesis during this period. Butyric acid also upregulated toxin binding to A 431 cells, but not to a level that can explain the sensitization. Interestingly, transport to the TGN was also increased (161, 163, 174). Butyric acid is able to change gene transcription in a variety of cell lines and is often used to increase transcription from vectors that have been transfected into cells (9, 44, 53, 142, 147, 153). Actually, butyric acid treatment is not unphysiological; in the intestine the concentration of butyric acid has been reported to be ~20 mM (71). This might certainly affect the epithelial cells in the intestine, and interestingly, butyric acid has been reported to increase the toxin sensitivity and the binding of Shiga toxin also to the intestinal carcinoma cell lines Caco-2 and HT-29 (83). Furthermore, increased intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP) (incubation with the membrane permeant 8-bromo-cAMP or with cholera toxin which increases the level of cAMP) induce retrograde transport of Shiga toxin to the ER and sensitization to the toxin (174). Also, on endothelial cells, the number of receptors can be increased by various factors like TNF and interleukins (88, 114), but it is still not known whether retrograde transport is also induced by these factors. Furthermore, in T47D cells that are normally sensitive to Shiga toxin (without exposure to butyric acid or cAMP), such a retrograde transport has been demonstrated (51). These findings suggest that the retrograde transport of the toxin can be regulated by physiological signals. Also Shiga toxin B fragment alone can be transported retrogradely to the ER, showing that the A fragment is not required for this process (89, 174). However, it is not yet clear whether the Shiga toxin receptor Gb3 is transported retrogradely even without the small B subunit bound.

It should be stressed that the HRP technique often used to follow retrograde transport at the EM level is unfortunately not a quantitative one and that immunogold detection of protein toxins in the ER would have been preferable. However, presumably only a very small amount of ricin reaches the ER, far below the level of

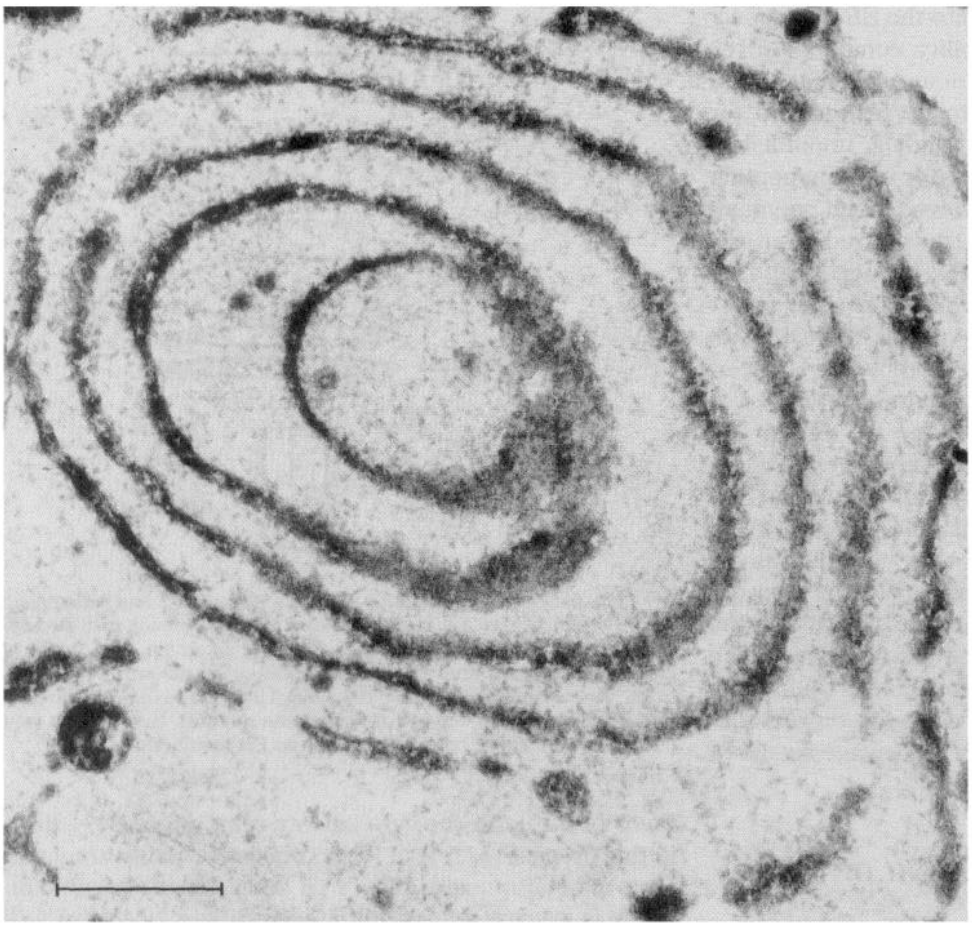

FIG. 4. Electron microscopic picture of an A 431 human epidermoid cancer cell that was first treated for 48 h with 2 mM butyric acid and then incubated with a Shiga toxin B-HRP conjugate for 1 h. Toxin conjugate is distinct in concentrically arranged ER cisterns. Bar, 0.5 μm.

immunogold detection. It has, for instance, not been possible so far to detect the ricin-KDEL in the ER by immunogold labeling on ultracryosections, but the amount of ricin sorted from the TGN to the ER may, even with a KDEL sequence, still be too low to allow detection (J. M. Lord and B. van Deurs, unpublished data). In contrast, immunogold detection of Shiga B chain in the ER has been reported (89). The peroxidase technique, on the other hand, is enzymatic and thus very sensitive. Clearly, however, the amount of detectable reaction product generated by Shiga-HRP varies from cell to cell, from ER cistern to cistern, and even within one and the same cistern (174). At the moment, therefore, quantifications of the retrograde traffic of toxins with and without KDEL sequences or in cells exposed to various signals are severely hampered.

The conclusion to be drawn from the above-mentioned data is that retrograde transport to the ER of protein toxins can take place and that such a process is very likely to be of importance for optimal toxin translocation and subsequent cytotoxicity, at least for Shiga toxin, where there seems to be a correlation between retrograde toxin transport as demonstrated by EM and the intoxication of the cells. The mechanism for the retrograde transport is not known. It is possible that there is a retrograde transport of the lipid receptor and that this process is dependent on the fatty acid tail of the ceramide moiety of the receptor, since lipid analysis of the receptor in butyric acid-treated cells revealed that the sensitive cells in which retrograde transport can be visualized have a longer fatty acid tail than the receptor in control cells (174). That lipid composition can be important for sorting has been demonstrated in the biosynthetic pathway of polarized MDCK cells (219). Furthermore, lipids themselves can undergo retrograde transport in the Golgi complex (73). Also, it cannot be excluded that the B fragment of Shiga toxin is important for retrograde sorting of the toxin; the B fragment might interact with a KDEL-containing protein. Furthermore, the aggregation of lipid molecules (Gb3) induced by the pentamer of B fragments might somehow be important for intracellular routing and retrograde transport.

Why would the ER be a convenient site for translocation of toxins to the cytosol? At this stage, we can only speculate. It is possible that the machinery normally involved in translocation of peptides or proteins across the ER membrane (29, 157, 185) and the components involved in folding of proteins (8, 48, 94, 149) may allow translocation of toxins into the cytosol. Because cells with a defect

in the ability to transport peptides into the ER for presentation by the major histocompatibility complex (MHC) class I-system are not resistant to ricin and Shiga toxin, this mechanism does not appear to be responsible for toxin entry into the cytosol (162; Sandvig, unpublished data). However, other mechanisms for protein/peptide translocation in the ER might be involved. Even polymannose oligosaccharides seem to be transported from the ER to the cytosol for breakdown (125), suggesting that the permeability (either by passive diffusion or by transporters) of the ER membrane to various molecules is larger than that of other membranes. Moreover, it is uncertain to what extent ricin and Shiga toxin have to be unfolded to be translocated, and whether any of the ER chaperones might assist in unfolding. Experiments where an extra disulfide bond was introduced to prevent unfolding of ricin A chain showed that the toxicity of the molecule was reduced, suggesting that unfolding is required for translocation to the cytosol (4). It is also not clear whether reduction of disulfide bonds occurs in the ER or in the cytosol after translocation. If the cytosolic enzyme calpain is involved in cleavage of Shiga toxin without a furin site (see below), then the results suggest that reduction occurs in the cytosol, since cleavage seems to occur more rapidly than reduction.

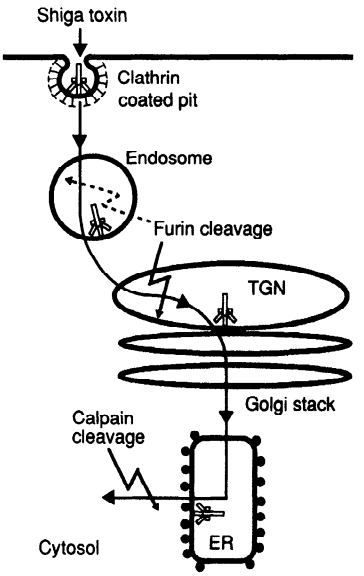

FIG. 5. Schematic model of Shiga toxin transport and processing. Toxin is internalized via clathrin-coated pits and transported through endosomes to TGN. In endosomes or, more likely, in TGN, toxin is cleaved by furin. From TGN, toxin is transported through Golgi stack to ER, where translocation to cytosol takes place. If toxin is not already cleaved by furin, e.g., in case of some toxin mutants, then calpain may cleave molecule after translocation across ER membrane.

VII. INTRACELLULAR PROCESSING OF SHIGA TOXIN

Shiga toxin contains a highly trypsin-sensitive region near the COOH-terminal of the A chain (151), and cleavage has been assumed to be important for the cytotoxic effect (14, 145, 151). The importance of intracellular transport and processing for optimal cytotoxicity has been investigated by using two trypsin-resistant Shiga toxin mutants: a deletion mutant where the -Ser-Arg-Val-Ala-Arg-Met- sequence of the trypsin-sensitive cleavage site was deleted and a substitution mutant where the two arginines in the sequence above were exchanged with histidines (51). These mutants were tested out both in vivo (on intact cells) and in vitro (in a reticulocyte lystate). Although less toxic than the wild-type toxin in short time experiments, the mutant toxins became at least as toxic after longer time (24 h), and EM revealed that both wild-type and mutant toxins were routed through the TGN/Golgi to the ER. Moreover, BFA protected, as expected, against both wild type and mutants. The cells cleaved both wild type and mutants, but the wild-type toxin was, in contrast to the mutants, cleaved in the presence of BFA, suggesting that this cleavage takes place in a station on the retrograde pathway before the Golgi stack, that is, in the TGN or in endosomes. In contrast, the mutants were cleaved more slowly and at a post-Golgi level along the retrograde pathway (ER or cytosol), and evidence was obtained in favor of a role of the cytosolic cysteine protease calpain in this process (51). That trypsin-resistant Shiga toxin mutants can intoxicate cells well after longer incubation times is in agreement with similar studies of mutants of *Escherichia coli* Shiga-like toxin I (15) and of Shiga-like toxin IIv (160). Interestingly, the Arg-X-X-Arg sequence of the highly trypsin-sensitive site in the A chain is the consensus sequence for the membrane-anchored serine endoprotease furin (123), and studies on LoVo cells which do not express the functional form of furin (201) revealed that Shiga toxin was only slowly cleaved in these cells (52). However, in LoVo cells transfected with furin, Shiga A chain was cleaved very efficiently, also in the presence of BFA (52). Untransfected cells were ~20 times more sensitive to nicked than to unnicked toxin. However, the furin-transfected cells were intoxicated equally well with nicked and unnicked Shiga toxin (52). Thus furin can clearly cleave and activate Shiga toxin, but other proteases might be involved as well. Furin-induced toxin cleavage did not take place on the cell surface, but it remains uncertain whether it occurs in endosomes or in the TGN. It is obvious from the literature that furin is mainly present in the TGN (is a TGN resident protein) (12, 123), but as it cycles between the TGN and the cell surface, it is also present in endosomes (12, 95, 123, 209). Furin colocalizes with TGN38 (123), but also this "TGN marker" may be present in endosomes. Therefore, furin may cleave Shiga A chain in both endosomes and the TGN. The current data on transport and processing of Shiga toxin are summarized in a tentative model in Figure 5.

VIII. TARGETED DELIVERY OF PROTEIN TOXINS

Most toxin conjugates made for targeted drug delivery are based on ricin, although other toxins, in particular *Pseudomonas* exotoxin (PE) and diphtheria toxin, are also used (13). Shiga toxin has not so far been used to produce immunotoxins. However, Shiga A chain fused to human CD4 was recently found to selectively kill cells infected with human immunodeficiency virus type 1 (HIV-1), presumably by using HIV-1 glycoprotein gp120 and gp41 as receptors (2). Interestingly, BFA protected against the CD4-Shiga hybrid toxin, suggesting that transport through the Golgi stack is important for the intoxication (see sect. V). Recently, it was also reported that primary ovarian tumors and metastases have increased levels of Gb3, the Shiga toxin receptor (see sect. III), compared with normal ovaries (41). This made the authors suggest that Shiga toxin might be applied directly to treat ovarian cancers. In fact, Shiga toxin has antineoplastic activity against certain skin tumors in humans, and it is also effective in a murine sarcoma tumor model, where it prevents metastasis to the lung (41).

When ricin is used for the construction of immunotoxins, one has to face the problem that both the A and B chains are glycosylated (see sect. II). This causes binding of intravenously administered ricin immunotoxins to other cell types than the target cells, for instance, to cells in the liver, also in the case of immunotoxins which do not contain the B chain. Moreover, ricin B chain contains the galactose binding sites (see sect. II) normally used to bind the toxin to glycoproteins and glycolipids with terminal galactose on the cell surface; these binding sites will therefore also lead to unspecific binding of ricin immunotoxins. Therefore, much effort has been put into modifying ricin (111). Deglycosylated ricin A chains are widely used in immunotoxins (16, 57). Mostly, however, immunotoxins based only on ricin A are less toxic than those based on the holotoxin. To include the ricin B chain, the galactose binding sites of this chain are therefore blocked, and the modification is referred to as blocked ricin (64–66, 103, 158, 226).

A new and promising approach to improve immunotoxin translocation across membranes (in the Golgi or ER?; cf. sect. VI) and thereby the cytotoxicity has recently been reported. Thus the gene for ricin A chain was fused with DNA encoding for the NH_2-terminal of VSV G protein, leading to a modified ricin that became more readily translocated to the cytosol. This modified ricin was conjugated to transferrin, and the conjugate exhibited 10- to 20-fold higher cell killing efficacy than a conjugate made from native ricin A chain (26).

Another interesting approach would be to try to improve the efficacy of intracellular sorting of immunotoxins to the TGN and ER (see sect. VI). So far, in particular, sorting of immunotoxins with PE to the ER has been of interest. Even though the REDLK sequence of PE is important for the effect of this toxin (see sect. VI), it turned out that replacing this sequence with the KDEL sequence increased the activity. Moreover, with the use of a series of COOH-terminal mutations, it was found that the glutamic acid (E) of the KDEL sequence enhances the cytotoxicity (by improving sorting from the TGN to the ER) compared with the REDL sequence (97). This PE modification has been used for constructing immunotoxins with, for example, transforming growth factor-α (TGF-α), constructions in which it has also been attempted to truncate PE as much as possible (PE37/38KDEL) without losing toxicity (92, 93, 152). A very small but still active chimeric immunotoxin was even constructed with TGF-α and only the domain III of PE (91). However, as discussed in section VI, it is far from clear that addition of, for instance, a KDEL to other immunotoxins would improve the cytotoxicity. Despite obvious improvements in the construction of the next generation immunotoxins and numerous studies with tumor models during the last few years, clinical progress in general is slow. The problem has been formulated by Vitetta et al. (221) "the transfer of basic discoveries to the clinic is a slow, multistep, interdisciplinary process."

From a clinical point of view, the most important progress with immunotoxins has been made for lymphomas and leukemias. For instance, eradication of minimal disease by a combination of conventional chemotherapy and immunotoxin therapy has been reported (54, 55). In this case, two immunotoxins were used: anti-CD19-ricin A and anti-CD22-ricin A. Similarly, an immunotoxin with anti-intracellular adhesion molecule-1 (CD54) and deglycosylated ricin A has been used (77). Also, elimination of residual acute lymphoblastic leukemia cells and non-Hodgkin's lymphoma cells from bone marrow using a blocked ricin immunotoxin has been reported (158). This is of great potential importance for autologous bone marrow transplantation (see also Refs. 64–66, 188). With the use of a mixture of immunotoxins based on PE in combination with chemotherapy, efficient eradication of small cell lung cancer cells from human bone marrow for autologous transplantation has similarly been obtained (130).

Immunotoxin treatment of solid tumors meets several obstacles including problems with diffusion into the tumor and tumor cell heterogeneity. However, successful results have been reported with some brain tumors (human gliomas) in nude mice, when immunotoxins based on monoclonal antibodies to human transferrin receptor plus ricin A chain or human transferrin plus diphtheria toxin were used (104). Promising results have also been obtained with ricin-based immunotoxins directed against the transferrin receptor in cell lines and operative specimens of pediatric brain tumors (118). Also, ricin A-chain

immunotoxins with antidisialoganglioside appear potentially promising in the treatment of human neuroblastoma (60). A summary of ongoing immunotoxin clinical trials in patients with hematologic malignancies and solid tumors was recently published (47).

To circumvent the problem of poor penetration of immunotoxins into solid tumors as well as that of tumor heterogeneity, an interesting approach is vascular targeting. The idea is that the cells of the tumor mass can be killed when the newly formed blood vessels in the tumor are destroyed; the endothelial cells in these vessels are readily accessible from the bloodstream and do not represent a heterogeneous population of cells. In a murine tumor model, the tumor endothelium was induced to express MHC class II (as an experimental tumor marker), and an immunotoxin composed of anti-MHC class II and deglycosylated ricin A chain caused complete thrombosis of the tumor vasculature and subsequent regression of the tumor (16–18). With antibodies selectively directed against markers of proliferating endothelial cells, immunotoxins might be constructed which in combination with conventional chemotherapy should constitute a very promising approach.

IX. DIRECTIONS FOR FUTURE RESEARCH AND PERSPECTIVES

To intoxicate cells efficiently, protein toxins with intracellular targets, such as ricin and Shiga toxin, must first bind to cell surface receptors, then they become endocytosed and sorted to the correct compartment from where translocation of the enzymatic active part of the toxin molecule will take place. Much has been learned about this process during the last few years, but there are still many aspects that are not clear. More has to be learned about the mechanisms of clathrin-dependent and -independent endocytosis of toxins and how these processes are regulated, not least in polarized epithelia. Also, we still have much to learn with respect to the significance of retrograde transport to the ER and the possible role of the KDEL sequence for cytotoxicity. As described above, it is far from evident that the KDEL retrieval system is of importance for cytotoxicity of protein toxins in general, and alternative mechanisms for retrograde transport should be explored. We find that in particular a role of membrane lipid composition appears promising. Also, much more information is required to understand how the toxins are translocated across a membrane and, in particular, what makes the ER membrane, if that is the location for translocation, suited for this purpose.

In parallel with attempts to solve these problems, the potential use of protein toxins to deliver peptides to the cytosol should be further explored. One obvious possibility would be to direct small peptides to MHC class I molecules with respect to antigen presentation on the cell surface.

It seems reasonable to believe that promising results will be obtained with the construction of new generations of more efficient toxin conjugates for targeted drug delivery in cancer therapy. Such immunotoxins are particularly interesting in relation to patients with minimal residual disease. On the basis of detailed knowledge about uptake mechanisms, intracellular sorting, transport, and translocation of the various protein toxins, it should be possible to decide on rational grounds which parts of the toxin molecule are required, and which can be omitted, and whether one can add relevant sorting signals that might be specific for the individual toxin.

Original studies were supported by the Norwegian Research Council for Science and the Humanities, the Norwegian and the Danish Cancer Societies, the Danish Medical Research Council, the Novo Nordisk Foundation, and North Atlantic Treaty Organization Collaborative Research Grant CRG 900517.

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