Routing of Internalized Ricin and Ricin Conjugates to the Golgi Complex

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Abstract. Receptor-mediated endocytosis and intracellular routing of native ricin, and of ricin conjugated to colloidal gold (Ri-Au) and to horseradish peroxidase (Ri-HRP), have been studied in cultured MCF-7 and Vero cells by electron microscopical techniques including serial section analysis. Both native ricin, as demonstrated by immunoperoxidase cytochemistry, and the ricin conjugates were internalized via a common coated pit–coated vesicle pathway to reach vacuolar and tubulo-vesicular portions of the endosomal system. In addition, native ricin and a purified monovalent fraction of Ri-HRP reached distinct Golgi cisterns, whereas Ri-Au and polyvalent Ri-HRP did not. The results delineate intracellular routing of native ricin and compare it with the routing of different ricin conjugates. Moreover, our study shows that conjugates of a particular ligand (ricin) and various probes (e.g., gold and peroxidase), may be handled differently by cells. Sorting apparently takes place in the endosomal system, allowing some but not other molecules to reach Golgi elements. This sorting seems to depend on the valency of the ricin conjugate.

R ICIN, a representative of a group of highly toxic plant and bacterial proteins (31, 32, 40), consists of two polypeptide chains (each of about 30 kD) linked by a disulfide bond. The B-chain is a lectin that binds to cell surface receptors (galactose-terminating glycoproteins and glycolipids). The A-chain is an enzyme which, following translocation into the cytosol, interferes with protein synthesis (31, 32). Ricin has recently been paid considerable attention because of its application in the preparation of immunotoxins, where monoclonal antibodies are used to direct the toxin or its enzymatically active A-chain towards specific target cells (4, 47, 52).

In order to act, ricin A-chain must gain access to its intracellular target, the ribosomal 60S subunit. As deduced from biochemical studies, the first step in this entry process is receptor-mediated endocytosis (RME)[1] of ricin (31, 32, 36, 38–40). Internalization of ricin has also been elucidated ultrastructurally by using ricin conjugated to markers such as ferritin (Ri-Fe) (15, 28, 29), horseradish peroxidase (Ri-HRP) (13), and colloidal gold (Ri-Au) (51), and in the form of a ricin-immunotoxin adsorbed to colloidal gold (6). However, these studies are contradictory as regards the intracellular routes followed by the conjugates. Moreover, it is important to stress that the route followed by a ligand conjugate does not necessarily reflect the route followed by the native ligand (51). Using Ri-HRP, Gonatas et al. (13) observed labeling of Golgi elements, an observation which could not be confirmed with Ri-Fe (15) or Ri-Au (51). Recent studies by Gonatas et al. (14) have revealed that also wheat germ agglutinin (WGA) conjugated with HRP appeared in Golgi elements, whereas WGA-Fe did not.

By using Ri-Au, Ri-HRP in both monovalent (Ri-HRPmv) and polyvalent (Ri-HRPpv) forms, native ricin/anti-ricin immunocytochemistry, and analysis of serial sections, we have addressed the following key questions in relation to RME of ricin and ricin conjugates. (*a*) Are there differences in the intracellular routing to Golgi elements of ricin conjugated with different probes—particularly HRP and Au—which cannot be explained simply by the enzymatic action and thereby higher sensitivity of the HRP conjugate? If so, (*b*) what is the intracellular route(s) followed by native (unconjugated) ricin? (*c*) What is the explanation of the discrepancies in the results obtained with the different conjugates?

Materials and Methods

Cell Cultures

The established human breast cancer cell line MCF-7 (43) was obtained from the Fibiger Institute (Copenhagen, Denmark). Vero cells (African green monkey kidney cells) were obtained from the National Institute of Public Health (Oslo, Norway). The cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (MCF-7) and minimal essential medium (Vero cells) supplemented with 5% and 10% fetal calf serum, respectively. Experiments were always performed with cultures which have had their growth medium changed the day before. The cells were washed in ice cold 20 mM Na-phosphate,
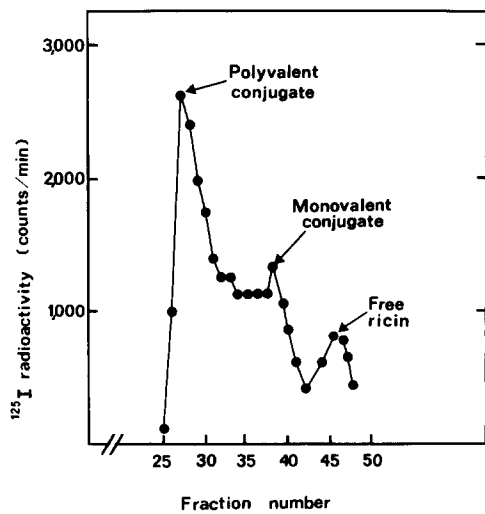

Figure 1. Separation of Ri-HRPmv from Ri-HRPpv. Ri-HRP was prepared as described in Materials and Methods and then polyvalent and monovalent conjugates were separated by gel filtration through a Sephacryl S-200 column. SDS PAGE showed that fraction 28

pH 7.4, 140 mM NaCl, or in Hepes buffer, before addition of ricin and ricin conjugates.

Experiments with Ri-Au

The 5-nm colloidal gold particles and the ricin conjugate were prepared as previously reported (51). Dilutions of 1:10 or 1:20 of the Ri-Au stock solution were added to pre-washed cells in 2 ml of Hepes or phosphate buffer at 0–4°C. After 1–4 h, the cells were washed carefully, and further incubated at 37°C for various periods of time (5 min to 6 h) before fixation. In some experiments cells were incubated at 0–4°C with Ri-Au in the presence of 0.1 M lactose or 1 mg/ml ricin, or lactose washing was performed after binding of the Ri-Au to the cells, before fixation.

Preparation of Ri-HRP

Ricin was coupled covalently to HRP (Sigma type VI) with SPDP (N-succinimidyl-3-[2-pyridylthio]propionate), essentially as described by Carlsson et al. (5). Unlabeled ricin was mixed with a trace of ^{125}I-ricin and treated with a 10-M excess of SPDP while HRP was treated with a 20-M excess of SPDP. Unreacted SPDP was removed by gel filtration on Sephadex G-25. The HRP treated with

contained only polyvalent Ri-HRP, while fraction 38 contained monovalent Ri-HRP and fraction 45 contained free ricin.
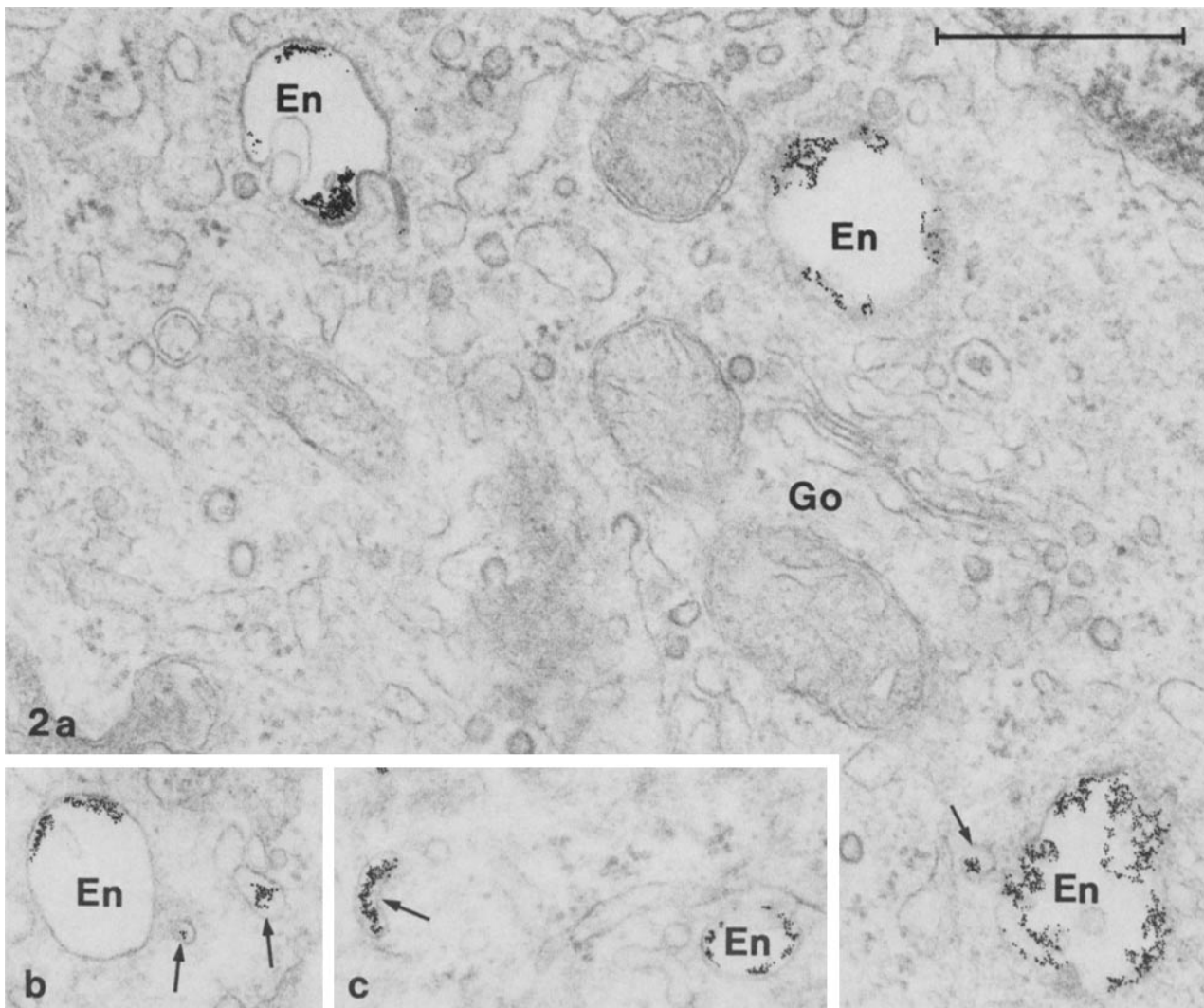

Figure 2. MCF-7 cells incubated with Ri-Au at 37°C for 30 min. The micrographs show the vacuolar portion (*En*) and the tubulo-vesicular portion (arrows) of the endosomal system, both labeled with Ri-Au. Note that the Golgi complex (*Go*) in *a* is unlabeled. Section thickness, 40 nm. Bar, 0.5 μm.

SPDP was reduced with 50 mM dithiothreitol for 20 min at room temperature, run through a Sephadex G-25 column, and then reacted with the SPDP-treated ricin overnight at room temperature. The conjugate was then dialyzed in PBS at 4°C. PAGE showed that conjugates in a 1:1 ratio as well as higher molecular weight conjugates were present in the preparation. These conjugates were used in the experiments with Ri-HRP.

To separate polyvalent (with more than one ricin molecule) and monovalent conjugates, Ri-HRP was run through a Sephacryl S-200 column equilibrated with 0.14 M NaCl, 20 mM Na-phosphate, pH 7.4, and 0.1 M lactose. As shown in Fig. 1, the conjugates were well separated according to their molecular weight. Subsequent SDS PAGE showed that fraction 28 contained only polyvalent Ri-HRP, fraction 38 contained monovalent Ri-HRP, while fraction 45 contained free ricin. In the further experiments, fraction 28 and fraction 38 were used as Ri-HRPpv and Ri-HRPmv, respectively.

Experiments with Ri-HRP, Ri-HRPpv, and Ri-HRPmv

Cells were incubated with Ri-HRP (2-10 µg/ml) in Hepes buffer for 60 min at 0-4°C followed either by direct transfer in this solution to 37°C or by washing and further incubation for 5-60 min at 37°C in Hepes buffer without Ri-HRP. Thereafter the cells were fixed.

Cells were incubated with Ri-HRPpv or Ri-HRPmv (2 µg/ml) in Hepes buffer for 60 min at 0-4°C. In some experiments they were then washed with buffer and fixed. In other experiments, cells were incubated with the conjugate in the presence of 0.1 M lactose, or they were washed with 0.1 M lactose in the buffer after binding of the conjugate to the cell surface, before fixation. In some experiments, cells pre-labeled at 0-4°C were further incubated at 37°C for 5-120 min, either directly or following a wash with buffer, before fixation. Finally,

in some experiments cells were not pre-labeled at 4°C, but incubated directly at 37°C.

Experiments with Native HRP and Cationized Ferritin (CF)

HRP (Sigma type II or VI) in PBS or Hepes buffer was added to cells at 4°C at a final concentration of 1 mg/ml. CF (Miles Laboratories, Inc., Elkhart, IN) in PBS was added to cultures (only MCF-7) at 4°C at a final concentration of 0.1 mg/ml. After 60 min at 0-4°C, the cells were washed and incubated further for 30-120 min at 37°C before a final wash and fixation.

Anti-ricin Immunocytochemical Experiments

A ricin stock solution (1 µg/ml) was diluted in Hepes buffer (1-10 µl stock solution per ml Hepes) and added at 0-4°C to pre-washed cells. After 60 min, the cells were washed with buffer and incubated for 5-60 min at 37°C, either directly or after washing, followed by two rinses with buffer.

The permeabilization and immunoperoxidase protocol was based on studies by Ohtsuki et al. (30), Tougard et al. (46), and Louvard et al. (21). All steps were carried out at room temperature. Rinsed cells were fixed for 30 min in hypotonic phosphate buffer (10 mM Na-phosphate, pH 7.4, 2 mM MgCl$_2$, 0.08 M NaCl) containing 2% formaldehyde. Thereafter the cells were washed for 5 min with hypotonic buffer, and incubated for 30 min with hypotonic buffer containing 0.05% (wt/vol) saponin. The cells were then incubated for 60 min with rabbit anti-ricin serum (36) in hypotonic buffer plus saponin. The amount of anti-ricin antibody used was based on experiments where the ability of the antibody to block the toxicity of ricin at a concentration of 1 µg/µl was measured. After three 5-min washings with hypotonic buffer plus saponin, the
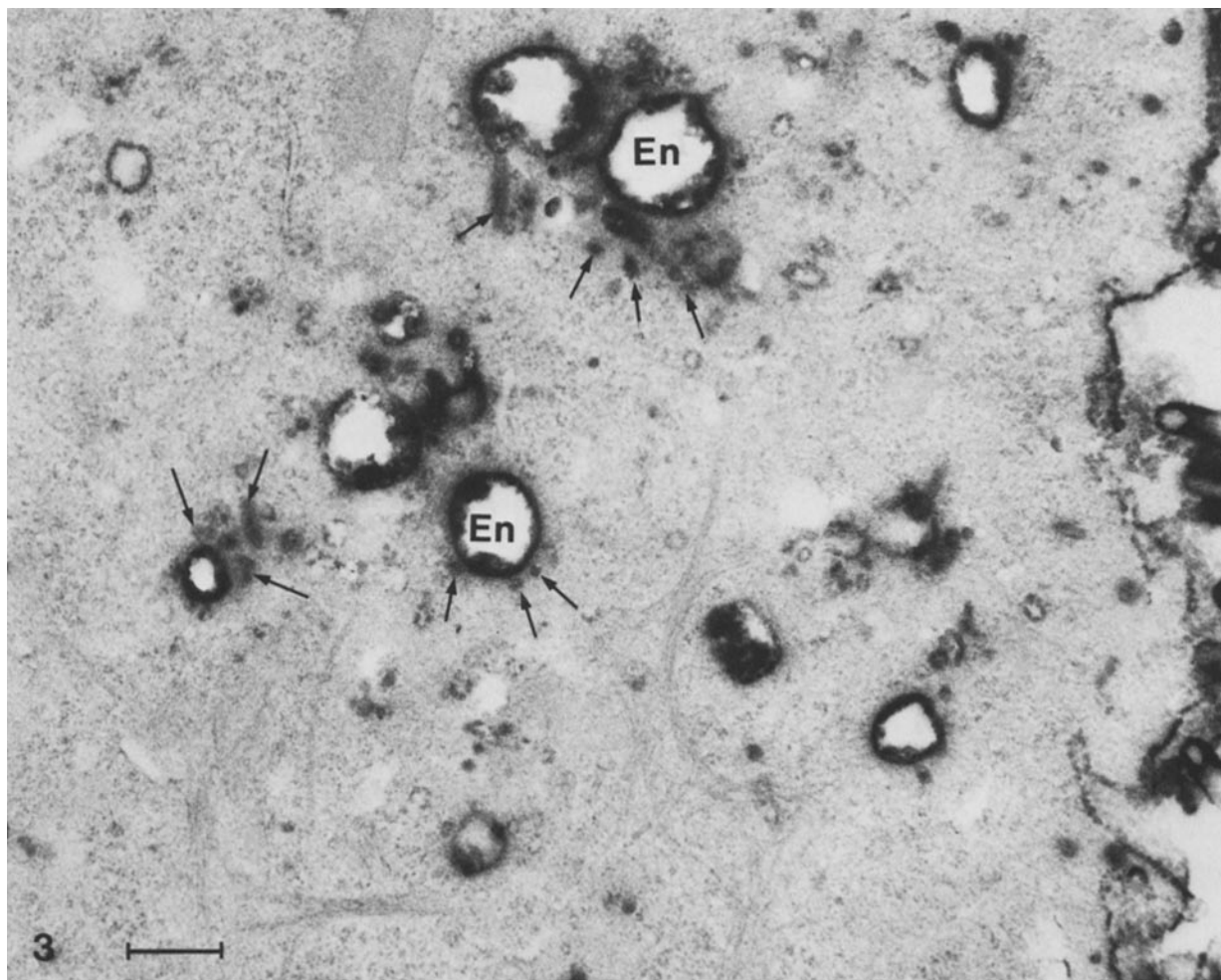

Figure 3. MCF-7 cell incubated with Ri-HRP at 37°C for 30 min. This thick (~200-nm) section clearly shows how the Ri-HRP–labeled vacuolar portion (*En*) of the endosomal system is surrounded by labeled elements of the tubulo-vesicular portion (arrows). In this section, no Golgi complexes are visible. Bar, 0.5 µm.

cells were incubated for 60 min with peroxidase-conjugated swine anti-rabbit antibody (1:20) (DAKO, Denmark) in hypotonic buffer plus saponin. After three 5-min washings with hypotonic buffer plus saponin, fixative was added. In some controls, a similar incubation procedure was followed, but without the anti-ricin antibody. In other controls, cells were not incubated with ricin, but thereafter permebilized and incubated with both antibodies.

Measurement of Cytotoxicity of Ricin, Ri-HRPpv, and Ri-HRPmv

Cells were seeded into disposable 24-well trays at a density of $0.5-1 \times 10^5$ cells/well the day before the experiment, and kept in minimal essential medium with 10% fetal calf serum overnight. The cells were then transferred to medium containing 20 mM Hepes instead of bicarbonate and increasing amounts of ricin or conjugate were added. After incubation for different periods of time, the cells were incubated for 15 min with [^{3}H]leucine in Hepes medium containing no unlabeled leucine. Subsequently, the medium was removed and the cells were incubated with 5% (wt/vol) trichloroacetic acid for 10 min. After a second incubation with trichloroacetic acid, the cells were dissolved in 0.1 M KOH (200 μl/well) and the samples were transferred to counting vials. 1 ml of Opti-fluor 1 (Hewlett-Packard Co., Palo Alto, CA) was added and the radioactivity was measured. The results are expressed as percent of the incorporation in control cells without toxin.

Electron Microscopy

After all experiments for electron microscopy, cells were fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature.

In the peroxidase experiments, cells were thereafter washed carefully with PBS and incubated for 30 min at room temperature with 2 ml PBS containing 1 mg diaminobenzidine and 2 μl of a 15% H_2O_2 solution per flask.

Cells were scraped off the flasks and centrifuged in buffer for 25 min at 1,600 g. Pellets were treated with 2% OsO_4 in cacodylate buffer, pH 7.2-7.4, for 1 h at 4°C, and thereafter with 1% uranyl acetate in distilled water for 1 h at room temperature, followed by ethanol dehydration and embedding in Epon. 20-200-nm-thick sections were cut with a diamond knife and collected on Formvar-coated grids (single slot grids for serial sections). They were photographed in a JEOL 100 CX electron microscope operated at 40-80 kV without further contrasting.

Results

The two cell lines used in this study were quite different in morphological appearance. While MCF-7 cells show a typical epithelial appearance in monolayer culture, with marked polarization, junctional complexes including desmosomes, and distinct bundles of 10-nm filaments, Vero cells appeared rather fibroblastic. Moreover, while the vacuolar apparatus was very well developed in MCF-7 cells, it was in general less prominent in Vero cells. By using both cell lines, however, we feel more sure that our main findings are not only valid for one particular, established cell line.

Uptake of Ri-Au

RME of the Ri-Au conjugate in Vero cells has been described in detail elsewhere (51), and it is here extended to MCF-7 cells. In brief, at 4°C Ri-Au was bound to evenly distributed cell surface receptors. When the cells were incubated with Ri-
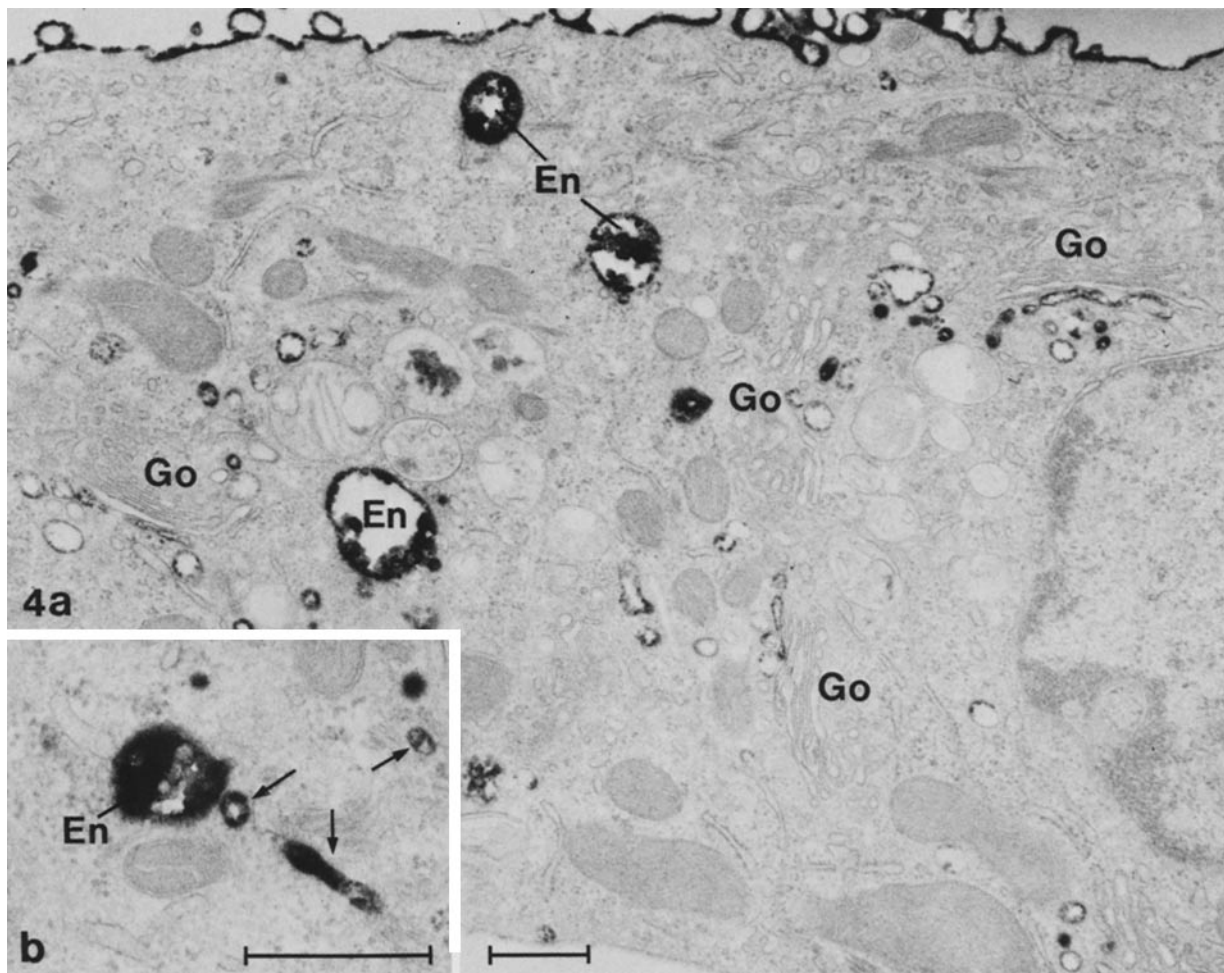

Figure 4. MCF-7 cells incubated as in Fig. 3. (*a*) Distinct labeling is seen of endosomes (*En*) and Golgi complexes (*Go*). (*b*) Ri-HRP labeling of the vacuolar (*En*) and tubulo-vesicular (arrows) portions of the endosomal system. Section thickness, 40-50 nm. Bars, 0.5 μm.

Au at 4°C in the presence of lactose or native ricin, almost no surface labeling was seen. However, after pre-binding of Ri-Au, the conjugate could not be washed off completely with lactose. At 37°C, Ri-Au became internalized—at least in part—via the coated pit pathway to reach the endosomal system. Both the vacuolar and the tubulo-vesicular portions of the endosomal system were labeled within 5–30 min at 37°C (Fig. 2). Typically, the tubulo-vesicular elements were most often seen adjacent to the larger vacuoles, or connected with these (see also reference 51). During these experiments, Ri-Au was seen occasionally in small vesicular and tubular structures close to well-defined Golgi complexes. However, distinct, stacked Golgi cisterns were not labeled (Fig. 2).

To elucidate further the apparent lack of labeling of Golgi cisterns, two approaches were applied: (*a*) quantitation on random, individual sections and (*b*) examination of thick and thin consecutive sections. (*a*) Out of 300 well-defined Golgi complexes examined in 40-nm sections of Vero cells exposed to Ri-Au for 20–60 min, none showed labeling. Similarly, 100 randomly selected Golgi complexes in MCF-7 cells showed no Ri-Au in distinct, stacked Golgi cisterns. (*b*) In very thick (150–200-nm) consecutive sections, Ri-Au was

clearly visible in endosomes (both the vacuolar and tubulo-vesicular portions), whereas Golgi complexes never showed labeling. By using a section thickness of one-fifth to one-tenth of that of the thick sections (i.e., 20–30 nm), and analyzing long series of such sections (10 to 20), a much more detailed image arose. The analysis of thin consecutive sections did not reveal any Ri-Au in stacked Golgi elements. Rather, small vesicular and tubular structures with Ri-Au turned out to be associated with endosomes. We therefore conclude that Ri-Au did not reach the Golgi complex.

Uptake of Ri-HRP

The routes and time sequence for RME of Ri-HRP were comparable to the findings with Ri-Au, although the endosomal system, especially small vesicular and tubular elements, was much more distinctly labeled already within 5–15 min with Ri-HRP than with Ri-Au. The close association of the tubulo-vesicular portion of the endosomal system with the vacuolar portion was in particular obvious after 30 min as revealed in thick (150–200-nm) sections (Fig. 3). However, with the Ri-HRP conjugate, Golgi elements also showed some labeling after 15 min, and they were consistently labeled after
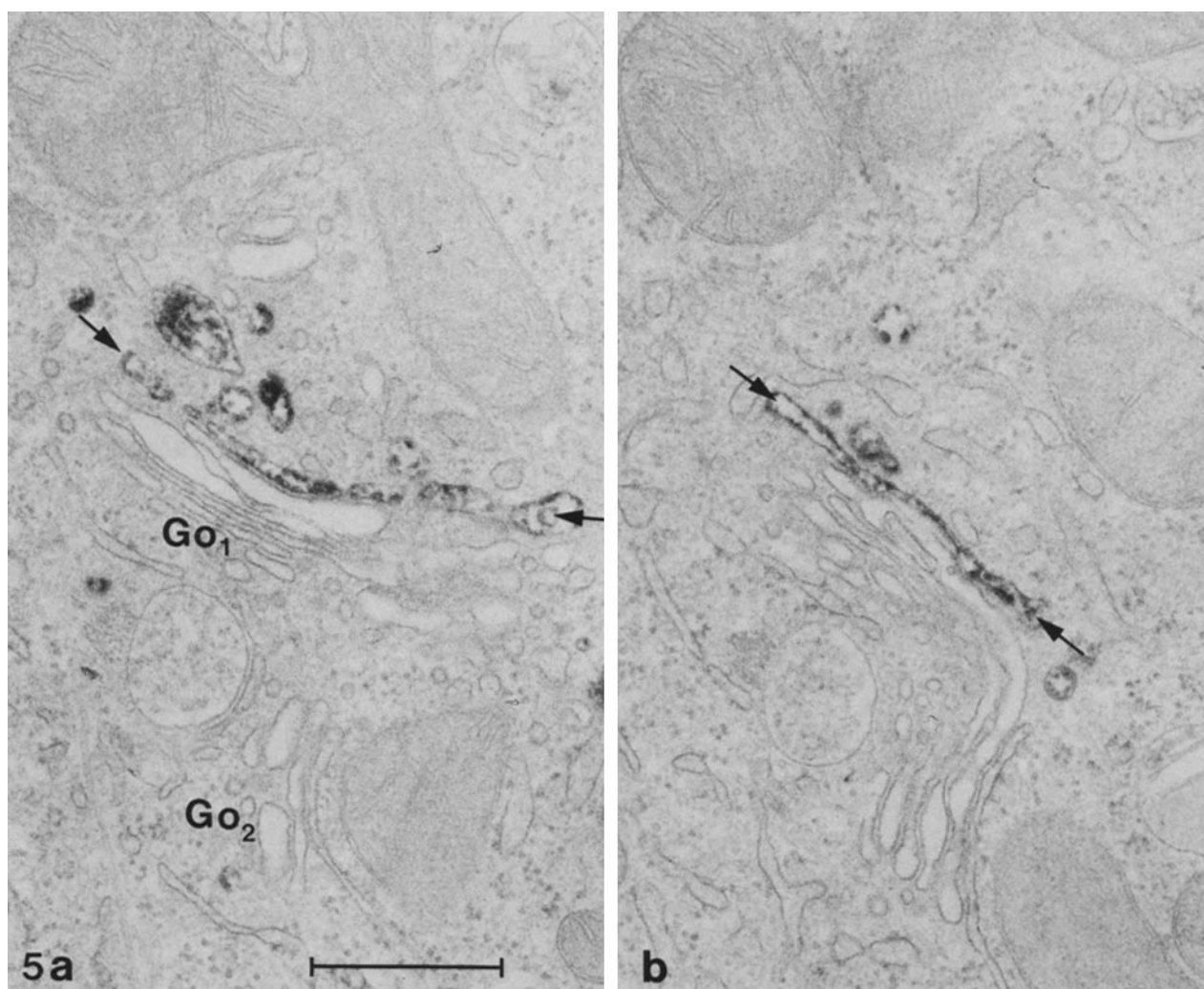
Figure 5. Serial sections of an MCF-7 cell incubated as in Fig. 3. *a* and *b* show sections no. 1 and 8 of the series. In *a* two Golgi profiles are seen (*Go₁* and *Go₂*), which in *b* turn out to be one and the same complex. Ri-HRP is seen in a Golgi cistern (arrows) with a typical *trans* position in *a*, whereas this position is not clear in *b*. Section thickness, 40 nm. Bar, 0.5 µm.

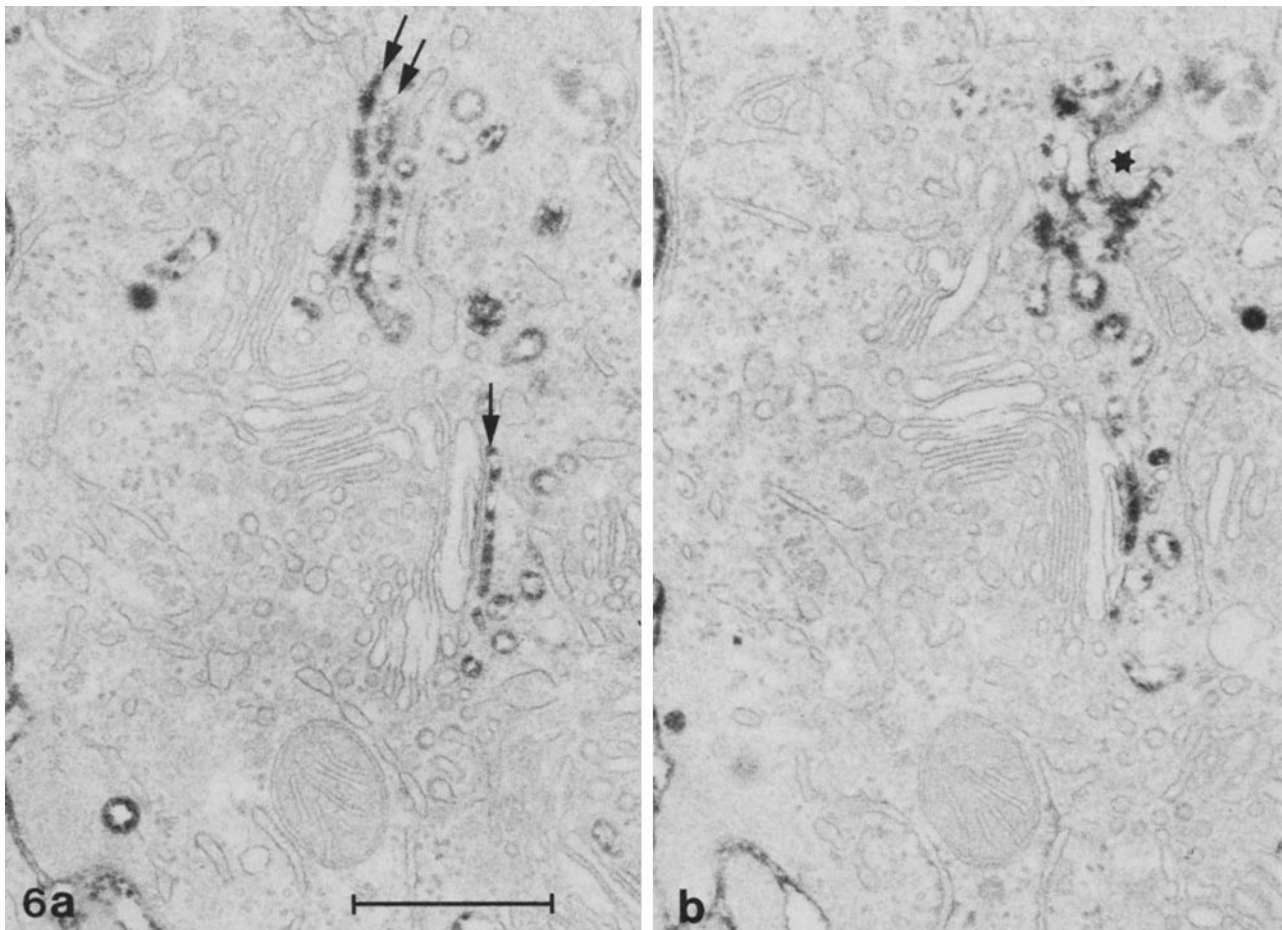van Deurs et al. *Routing of Ricin to the Golgi Complex*

Figure 6. Serial sections of an MCF-7 cell incubated as in Fig. 3. *a* and *b* show sections no. 1 and 6 of the series. The Golgi complex shown has a complicated, wavy structure. Ri-HRP is distinct in some Golgi cisterns (arrows) in *a*. The regular, flattened cisterns in the upper portion of the Golgi complex in *a* appear in *b* as a more complicated system of branched tubules (asterisk). Section thickness, 40 nm. Bar, 0.5 μm.
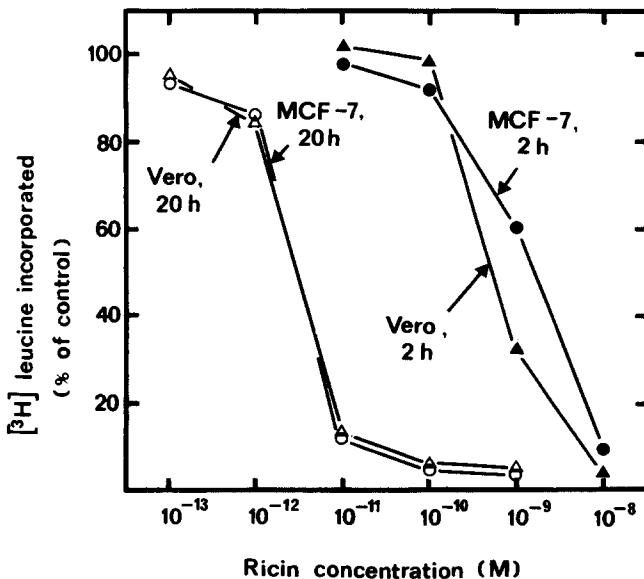

Figure 7. Sensitivity of Vero cells and MCF-7 cells to ricin. Vero cells and MCF-7 cells growing in 24-well disposable trays were incubated with increasing concentrations of ricin for 2 h (closed symbols) and for 20 h (open symbols). Then the medium was removed and protein synthesis was measured during a 15-min interval as

~30 min of incubation (Fig. 4). Thus, based on random individual 40–50-nm sections of MCF-7 cells and Vero cells, about 40% of all Golgi complexes showed distinct Ri-HRP labeling of one or two cisterns. Such labeled cisterns were in some cases located in a typical "*trans*-Golgi" position. However, what in a random section appeared to be an isolated Golgi complex turned out in serial sections to be in connection with other "isolated" Golgi complexes, often in a wavy fashion (Figs. 5 and 6). Such analysis made quantitation on Golgi profiles in individual sections troublesome, and also questioned traditional descriptions of concave and convex Golgi faces, which are often routinely taken for *trans*- and *cis*-faces, respectively (Fig. 5). On occasion, Golgi cisterns showed connection with a more complicated system of branched cisterns and vesicles, but still with a clear Golgi association and thus distinct from the tubulo-vesicular elements of the endosomal system (Fig. 6). Based on these observations, we conclude that internalized Ri-HRP, in contrast to Ri-Au, consistently reached the Golgi complex.

described in Materials and Methods. The results are expressed as percent of the control values (no toxin added). (O and ●) MCF-7 cells; (△ and ▲) Vero cells.

Uptake of Native HRP and CF

For both cell types, native HRP appeared to be internalized without any binding to the cell surface. HRP was, however, seen in coated vesicular profiles close to the cell surface. Within the cells, HRP was found predominantly in endosomal elements and, after 30 min or more, in secondary lysosomes. No labeling of Golgi elements was obtained. CF, on the other hand, was internalized by adsorptive pinocytosis. This marker was found in profiles of coated pits and vesicles, in elements of the endosomal system, and in secondary lysosomes. Moreover, in MCF-7 cells exposed to CF for 30–120 min at 37°C, 8 out of 105 Golgi complexes showed a few CF particles in cisternal Golgi elements (see also references 8, 9, 33).

Uptake of Native Ricin

The toxicity of native ricin to Vero cells and MCF-7 cells is compared in Fig. 7. The two cell types were found to be almost equally sensitive to the toxin, both when the toxic effect (inhibition of protein synthesis) was measured after 2 h and 20 h.

In cells exposed to native ricin for 5 to 60 min at 37°C, and then processed for immunoperoxidase cytochemistry, native ricin was unequivocally localized to the same structures as described for Ri-HRP. Thus, both vacuolar and tubulo-vesicular elements of the endosomal system showed reaction product within 5–15 min. Some reaction product also occurred in Golgi elements after 15 min, and after 30 min Golgi complexes were distinctly labeled (Figs. 8 and 9). In controls, where ricin or the primary antibody was omitted from the experimental protocol, no reaction was obtained.

Uptake of Ri-HRPmv and Ri-HRPpv

To elucidate further the marked difference in intracellular routing of native ricin and Ri-HRP on the one hand and Ri-Au on the other, we used monovalent and polyvalent fractions of the Ri-HRP conjugate (Ri-HRPmv and Ri-HRPpv, respectively). As shown in Fig. 10, Ri-HRPmv was approximately half as toxic as ricin, suggesting that in half of the cases the HRP was bound to the A-chain and thereby inactivated the toxin. Ri-HRPpv was substantially less toxic than ricin (Fig. 10).

While none of the conjugates bound to the cell surface in the presence of 0.1 M lactose, both were bound in the absence of lactose. Pre-bound Ri-HRPmv could be completely removed with lactose, while Ri-HRPpv was only partially removed. Thus in this respect Ri-HRPpv resembled Ri-Au. Also, Ri-HRPpv labeled vacuolar endosomes distinctly, whereas less labeling of tubulo-vesicular elements, and no labeling of Golgi elements, were obtained (Fig. 11). In contrast, Ri-HRPmv, like native ricin, was frequently seen in the tubulo-vesicular portion of the endosomal system and in Golgi elements.

Discussion

Several studies have dealt with RME and cellular handling of various bacterial and plant toxins conjugated with ferritin (15, 28, 29), HRP (13, 20), or colloidal gold (27, 51). Moreover,
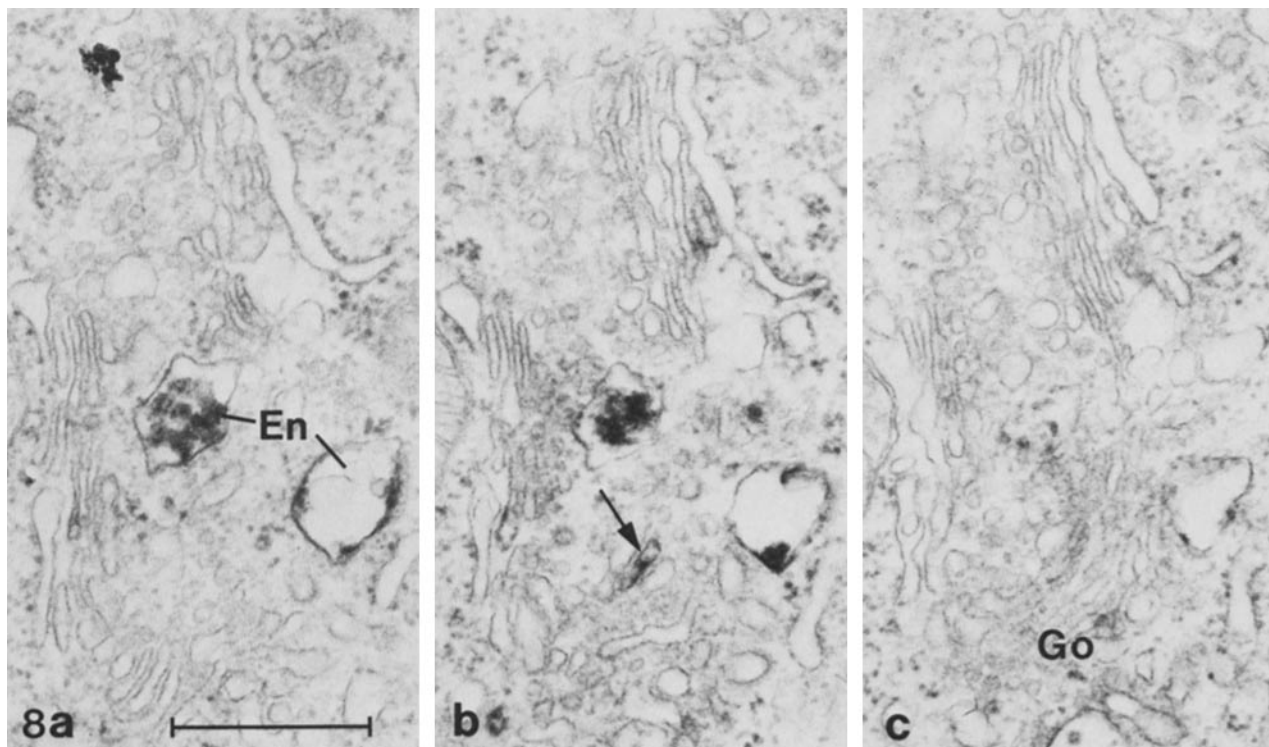
Figure 8. Consecutive ~60-nm sections of an MCF-7 cell incubated with native ricin at 37°C for 15 min before fixation, permeabilization, and immunoperoxidase cytochemistry. Two endosomes (*En*) are labeled. Moreover, a small vesicular or cisternal structure is labeled in *b* (arrow). Although this labeled structure does not appear in *a* or *c*, the last section of the series (*c*) confirms that the labeled structure must have a close relation to the lower Golgi complex (*Go*). Bar, 0.5 μm.
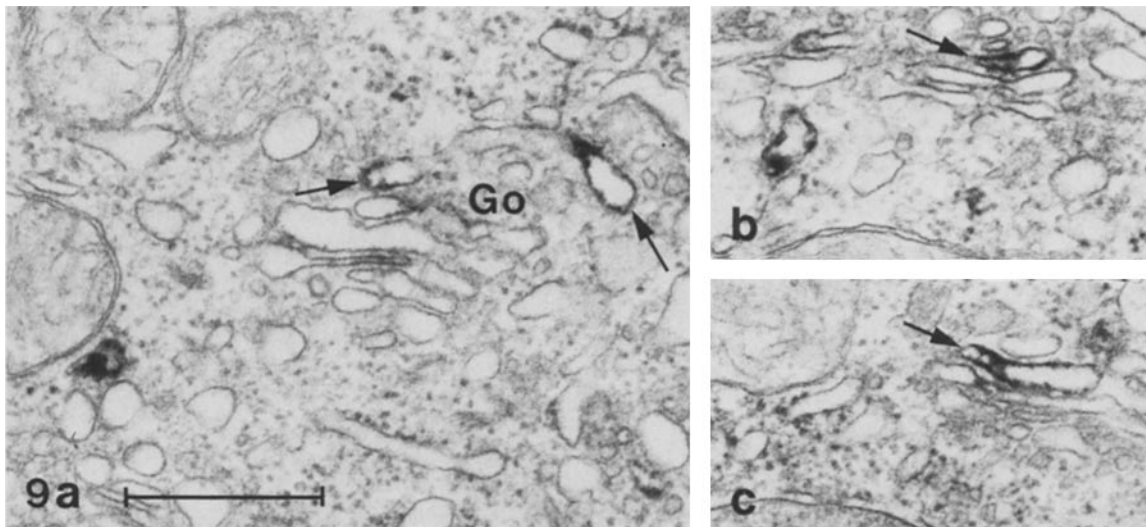
Figure 9. MCF-7 cells incubated with native ricin at 37°C for 30 min before fixation, permeabilization, and immunoperoxidase cytochemistry. Ricin is seen in elements (arrows) of the Golgi complexes (*Go*). Section thickness, 50 nm. Bar, 0.5 μm.
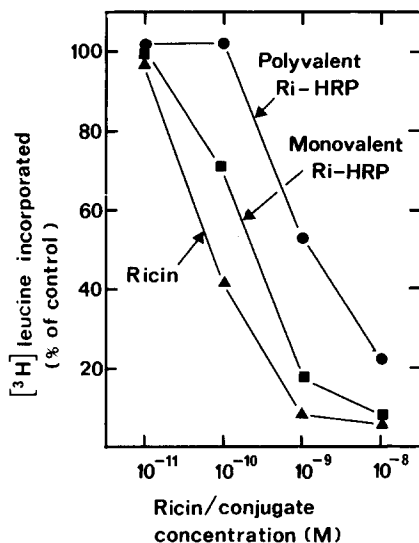

Figure 10. Sensitivity of Vero cells to Ri-HRPmv and Ri-HRPpv. Vero cells growing in 24-well disposable trays were incubated with increasing concentrations of ricin, Ri-HRPmv, and Ri-HRPpv for 3 h. Then the medium was removed and the protein synthesis was measured during 15-min intervals as described in Materials and Methods. The results are expressed as percent of the control value (no toxin or conjugate added). (▲) ricin; (■) Ri-HRPmv; (●) Ri-HRPpv.

FitzGerald et al. (10) showed that Pseudomonas exotoxin A, as detected on the cell surface with antitoxin IgG and ferritin-conjugated anti-IgG, was internalized by RME via coated pits. However, the present study reports the first attempt to determine the intracellular routing of a native (unconjugated) toxin by a combined use of toxin conjugates and immunocytochemistry. The intracellular routing of toxins is of general interest in relation to RME and intracellular routing of other classes of ligands, such as viruses (22, 23) and physiological ligands ligands like low density lipoprotein, asialoglycoproteins, epidermal growth factor, and transferrin (2, 11, 12, 17–19, 24, 44, 53–56). Also, knowledge of the intracellular routing of

native ricin and other toxins is of importance in attempts to design immunotoxins, since after RME the antibody part must deliver the toxin A-chain to a compartment from where translocation can take place (4, 47, 52). This compartment may not be the same for the various toxins (37–41). Finally, the present study throws new light on an obvious problem of many recent studies on RME using ligand conjugates: the same ligand conjugated with different types of probes (e.g., HRP or gold) may be handled differentially by the cell.

The observations of the present and previous (51) studies on RME of ricin and ricin conjugates suggest that they follow a common RME pathway and that the different molecules (conjugates) are separated by an intracellular sorting process. Thus, after binding to cell surface receptors, ricin and ricin conjugate molecules are internalized via coated pits and vesicles (see also references 34 and 50) to reach the vacuolar and tubulo-vesicular portions of the endosomal system. From here, molecules may be delivered to lysosomes. Moreover, some molecules also reach distinct Golgi elements.

The endosomal system is believed to be involved in uncoupling, sorting, and routing of ligands and receptors and in recycling of membrane to the cell surface (11, 12, 16–19, 24, 44, 48, 51, 54–56). Direct morphological evidence for a role of the tubulo-vesicular elements in recycling such as transfer of membrane and content from endosomes to the cell surface has been provided by using time sequence analysis of internalized CF, and double-labeling with HRP and CF (7, 45, 48, 49). Whereas low pH is found in the vacuolar portion of the endosomal sytem, in lysosomes and in *trans*-Golgi elements (1, 3, 42), at least a part of the tubulo-vesicular portion of the endosomal system is non-acidic (42, 56). Since ricin A-chain translocation requires higher pH than, for instance, the diphtheria toxin A-fragment (37, 40, 41), it is conceivable that ricin A-chain translocation takes place in the tubulo-vesicular portion. This notion is supported by our observations that Ri-HRPmv reached this portion of the endosomal system to a higher degree than Ri-HRPpv and was also considerably more toxic than Ri-HRPpv.

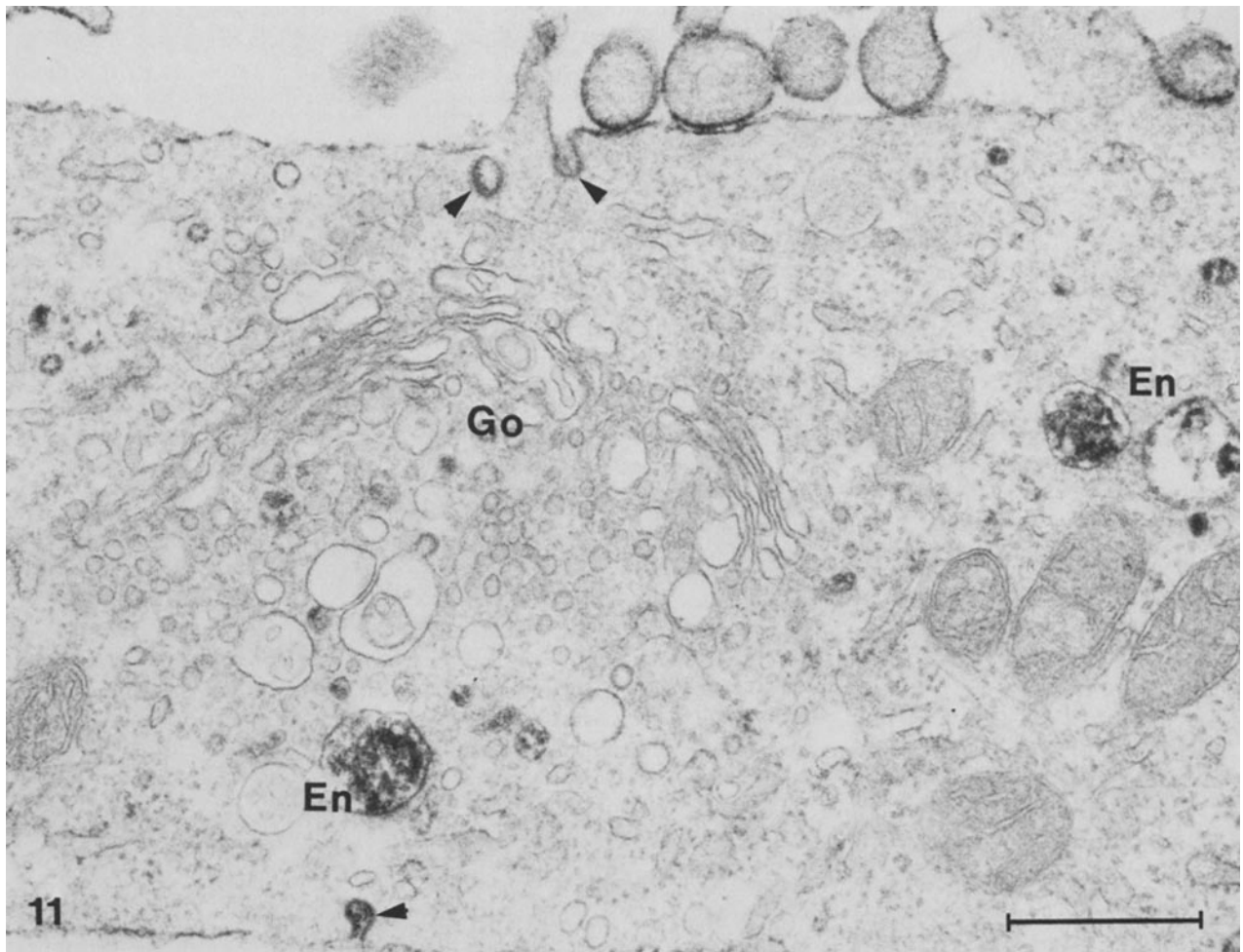The possibility should be considered that the Golgi com-

Figure 11. MCF-7 cell incubated with Ri-HRPpv at 37°C for 60 min. Labeling of the vacuolar portion of the endosomal system (*En*) is distinct, whereas the Golgi complex (*Go*) is unlabeled. Three coated pits with Ri-HRPpv are also seen (arrowheads). Section thickness, 40–50 nm. Bar, 0.5 μm.

plexes are reached by ricin and some ricin conjugates directly from the cell surface, via a pathway bypassing the endosomal system (8, 9, 33). However, the general significance of such a pathway is uncertain. Moreover, we found consistently that the endosomal system became labeled before the Golgi complexes, and therefore assume so far that the tracer molecules reached the Golgi complexes via the endosomal system.

Because of the marked staining of Golgi elements with Ri-HRP conjugates, also reported in sections of pre-fixed cells, it has been suggested that membranes of the Golgi apparatus are particularly rich in moieties with terminal D-galactose (13, 57). While this may very well be true, our observations indicate that this is not the reason why native ricin and Ri-HRPmv reach Golgi elements.

Gonatas et al. (14) recently reported that whereas WGA-Fe was routed only to lysosomes, WGA-HRP was also found in Golgi elements. They discussed several possible explanations of the difference in cellular handling of conjugates made with an enzymatic (HRP) and a non-enzymatic, particulate marker (Fe). They concluded that the differences observed could not be explained simply by the fact that the reaction product of enzymatic probes is diffusible within a certain, membrane-limited compartment, a conclusion we confirm here. They

rather favored the idea that glycoproteins rich in mannosyl residues, such as HRP, are selectively retained in Golgi elements. However, since in the present study (*a*) native ricin was found in Golgi elements, (*b*) native HRP was never found in Golgi elements, (*c*) Ri-HRPpv was not seen in Golgi elements, and (*d*) anti-rabbit IgG antibody-HRP did not label Golgi cisterns in experiments with permeabilized cells when the primary rabbit anti-ricin antibody was omitted, we consider this explanation less likely in our experimental systems.

Thus, neither the ricin molecules nor the probe molecules themselves seem to be the determining factor with respect to the routing of conjugates to Golgi elements. Rather, our present observations suggest that routing and sorting events at the level of the endosomal system are influenced by the valency of the ligand or ligand conjugate. Ri-Au, which is polyvalent (2–4 ricin molecules per gold particle, cf. reference 51), reached vacuolar and tubulo-vesicular elements only. Golgi elements were unlabeled. Native ricin has two galactose binding sites, but since one site binds galactose much more strongly than the other one, ricin can be considered as functionally monovalent (58). It was found that native ricin reached, in addition to the vacuolar and tubulo-vesicular portions of the endosomal system, also Golgi elements. Ri-

HRPpv behaved as Ri-Au, whereas Ri-HRPmv was distributed like native ricin. Moreover, in preliminary studies on MCF-7 cells labeled with a monoclonal antibody against surface proteins and thereafter with a peroxidase-conjugated secondary antibody at 4°C, followed by warming of the cells to 37°C for 30 min, we found labeling of endosomes and lysosomes but not of Golgi complexes (35).

Our considerations about the role of the valency of the conjugate for intracellular routing are supported by previous biochemical data. Thus, Mellman et al. (25) and Mellman and Plutner (26) found that macrophage Fc-receptors tagged by polyvalent IgG were directed to lysosomes only, whereas those tagged by the monovalent Fab fragment of IgG molecules were recycled. Similar conclusions have been drawn by Anderson et al. (2) in studies with polyclonal antibodies and Fab fragments of the antibodies against the low density lipoprotein receptor.

In conclusion, we have shown the following. (a) There are marked differences in the intracellular routing of ricin conjugated with colloidal gold and HRP, respectively, as to whether Golgi elements are involved. This observation is presumably of relevance for other types of ligand conjugates as well. (b) Native ricin is routed to both endosomes and Golgi elements, as documented by ultrastructural immunocytochemistry. (c) The pathways from the endosomal system followed by different conjugates may, in part, be a matter of the valency of the conjugate, a notion which may also be of importance for studies in other cellular systems.

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