Pathways Involved in Fluid Phase and Adsorptive Endocytosis in Neuroblastoma

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ABSTRACT

The endocytosis of ricin, horseradish peroxidase (HRP), and a conjugate of ricin-HRP by monolayer cultures of murine neuroblastoma was studied using morphological and biochemical techniques.

The binding of $^{125}$I-ricin and $^{125}$I-ricin-HRP to cells at 4°C, as a function of ligand concentration, was a saturable process. The apparent affinity constants, determined at equilibrium, were $2.8 \times 10^6$ M$^{-1}$ for ricin and $1 \times 10^8$ M$^{-1}$ for ricin-HRP. The number of binding sites per cell was $8 \times 10^7$ and $3 \times 10^7$ for the lectin and the conjugate, respectively. The binding of $^{125}$I-ricin to monolayers was not proportional to cell density. We found reduced binding at higher cell concentrations, suggesting a decrease in the accessibility of the ligand for the receptor site or fewer sites with increasing cell population.

Neuroblastoma cells have an acid-phosphatase-positive network of cisternae and vesicles near the Golgi apparatus (GERL). Ricin-HRP undergoes endocytosis in vesicles and cisternae corresponding to GERL, and in residual bodies (dense bodies). The cellular uptake of ricin-HRP was 100-200 times greater than free HRP and there was no stimulation of fluid phase endocytosis by ricin. When monolayers were exposed to concentrations of native HRP 100-fold that of the conjugate, cellular uptake of peroxidase was comparable, but HRP was localized only in residual bodies and never in elements of GERL. These results support the conclusion that GERL is involved in the adsorptive endocytosis of ricin-HRP, while residual bodies are involved in the bulk uptake of HRP.

In addition, the binding, uptake, and possible recycling of $^{125}$I-subunit B (the binding subunit) of ricin and of $^{125}$I-ricin was examined by quantitative electron microscope autoradiography. Both ricin and its binding subunit displayed similar autoradiographic grain distributions at 4°C, and there was no evidence of their breakdown or recycling to the plasma membrane during endocytosis for 2 h.

Various ligands that bind on the plasma membranes of plasma cells, cultured neurons, and neuroblastoma cells undergo endocytosis into cisternae and vesicles of the Golgi apparatus or GERL$^1$ (Golgi apparatus-endoplasmic reticulum-lysosome) (1, 9, 10, 20, 21, 27). To gain further insight into the process of adsorptive endocytosis in neurons, we studied the binding and uptake of ricin, horseradish peroxidase (HRP), and a conjugate of ricin with HRP (ricin-HRP), by cultured murine neuroblastoma. Murine neuroblastoma cells have certain morphologic and electric properties of sympathetic neurons which render them a suitable, if not an ideal, substitute for cultured neurons; furthermore, quantitative studies are feasible in cultured murine neuroblastoma, whereas, in cultured neurons, limitations of cell numbers and heterogeneity of cell types preclude combined morphologic and quantitative studies (26).

Our results indicate that the adsorptive endocytosis of ricin-HRP by neuroblastoma cells is different both quantitatively and qualitatively from the endocytosis of free HRP (35). Ricin-HRP underwent endocytosis predominantly in GERL, while

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$^1$ The term GERL is used in this paper to indicate an acid phosphatase-positive system of vesicles and cisternae at the trans-aspect of the Golgi apparatus, without implying whether or not it is a separate organelle or a component of the Golgi apparatus (5, 16, 27).
free HRP was found only in residual bodies (27). The internalized lectin or its binding subunit did not undergo detectable breakdown for up to 24 h after their endocytosis (34). By quantitative ultrastructural autoradiography, recycling of internalized ricin or its binding subunit back into the plasma membrane was not demonstrated (33, 34).

MATERIALS AND METHODS

Ricin 60 (RCA 60), lens culinaris lectin, and peanut agglutinin were purchased from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.) and from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). HRP (type VI) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and lactoperoxidase from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, Calif.). Carrier-free 125I-Na was obtained from New England Nuclear (Boston, Mass.). All other reagents were of the highest grade available. Viokase was purchased from Grand Island Biological Co. (GIBCO, Grand Island, N.Y.).

Preparation of 125I-Ricin

Iodination of ricin was performed using lactoperoxidase according to the method of Marchall (25). 1 mcg of carrier-free 125I was added to 1 mg of ricin (ESF, mol = 12.5) previously dialyzed against phosphate-buffered saline (PBS). Lactoperoxidase (10 µg) and 10 µl of 4 mM hydrogen peroxide were added and the mixture was incubated at room temperature for 30 min with gentle shaking. The iodinated ricin was separated from free 125I by a G-25 (fine) Sephadex column (0.9 x 30 cm). After further dialysis against PBS, the specific activity of the radioactively labeled ricin was 3.7 x 10^5 cpm/µg and polyacrylamide gel electrophoresis in 0.1% SDS-8 M urea showed a single peak corresponding to a mol wt of ~60,000. The labeled ricin was stored at 4°C and used within 2 wk.

Preparation of Ricin-Subunit B, and Iodination

Ricin B-chain was isolated as described (29). 2 mcg carrier-free 125I was added to 2 mg of ricin B-chain and then lactoperoxidase and H2O2 were added as above. The incubation at room temperature lasted for 45 min. The material was then applied to a 5-ml column of acetic-acid treated Sepharose 4B equilibrated with PBS (29). After extensive washing of the column with PBS, the labeled ricin B-chain was eluted with 0.1 M lactate in PBS. Finally lactate was removed by dialysis. The specific activity obtained was 396 cpm/µg.

Conjugation of Ricin to HRP

The iodinated or free ricin was coupled to HRP by the two-step method of Avrameas and Ternynck (2) and Gonatas and Avrameas (8). In a typical experiment, 30 µg of HRP was dissolved in glutaraldehyde (1.25% solution in 0.1 M potassium phosphate, pH 6.8) and left at 25°C for 16 h. The reaction mixture was applied to a Sephadex G-25 fine column (0.9 x 65 cm) and developed with 0.15 M NaCl. The brown band containing “activated” HRP was collected and combined with 2 mg ricin. In one experiment incubation of activated HRP with ricin was carried on in the presence of 0.2 M lactose. The pH was adjusted to 9.0 with 1 M sodium bicarbonate buffer and after 24 h at 4°C any remaining aldehyde groups were blocked with 1 M lysine, pH 7.4. After an additional 4 h at 4°C the solution was concentrated, dialyzed against 0.1 M phosphate buffer adjusted to pH 5.0 with citric acid. The reaction was stopped by adding 0.1 ml of 6 N HCl. The optical absorbance of the conjugate was measured at 492 nm and the amount of HRP was calculated from a standard curve of HRP diluted in 0.1% BSA. Final concentrations of SDS >0.001% produced turbidity on acidification, but identical results were obtained after centrifugation in a microfuge.

Polyacrylamide Gel Electrophoresis

Monolayers of neuroblastoma cells were rinsed free of medium with ice-cold Earle's balanced salt solution (Microbiological Associates, Walkersville, Md.) buffered with HEPES (Microbiological Associates) (Earle's-HEPES) and incubated for 2 h at 4°C in a total volume of 3 ml Earle's-HEPES containing one of the following: (a) ricin-HRP, (b) ricin, (c) Earle's-HEPES. The lectin concentration was 25 µg/ml. The cells were then washed five times with cold Earle's-HEPES. The flasks previously exposed to buffer or ricin were incubated with 3 ml of a solution of HRP (1 mg/ml) in Earle's-HEPES, while the flasks containing ricin-HRP were incubated with 3 ml of Earle's-HEPES. Endocytosis of HRP or the conjugate was allowed to proceed for 1 h at 37°C. The cultures were washed an additional six times and solubilized in 0.1% SDS aided by brief sonication. A sample was removed for measurement of HRP activity, and protein was assayed by the method of Lowry using BSA as a standard (24). A modification of the o-phenylenediamine (OPD) assay as described by Wolters et al. was used to estimate the amount of peroxidase taken up by the monolayer (37). Substrate solution was prepared immediately before use by adding 0.1 ml of 3% (vol/vol) H2O2 to 100 ml of a solution containing 0.005% (wt/vol) OPD and 0.0025% (wt/vol) urea in 0.1 M phosphate buffer adjusted to pH 5.0 with citric acid. The reaction was initiated by the addition of 0.8 ml of substrate solution to 0.1 ml of the sample (0.2-2 µg of HRP). After incubation for 15 min at room temperature in the dark, the reaction was stopped by adding 0.1 ml of 6 N HCl. The optical absorbance was read at 492 nm and the amount of HRP was calculated from a standard curve of HRP diluted in 0.1% BSA. Final concentrations of SDS >0.001% produced turbidity on acidification, but identical results were obtained after centrifugation in a microfuge.

Cell Culture

Monolayers of the neuro-2A cell line (CCL 131) obtained from the American Type Culture Collection (Rockville, Md.) were grown in Falcon culture bottles (Falcon Labware, Div. Becton, Dickinson & Co.), according to described methods (14). We used Dulbecco's modified Eagle's medium (GIBCO) containing 10% alpha MoM fibroblastic newborn calf serum and glutamine at a final concentration of 2 mM. For electron microscopy, cells were grown on plastic strips (Aclar 33 C, 5 mil, Allied Chemical Corp., Specialty Chemicals Div., Morrisstown, N. J.) (21). Between 75 and 85 x 10^4 cells/ml from a monolayer culture were added to bottles containing one 5 x 10 cm strip of Aclar. Strips were used 48 h later, when cells had become confluent.

Light and Electron Microscopy and Cytchemistry for HRP

For these experiments cells grown on Aclar strips were used. For light microscope studies, cells were fixed, stained with toluidine blue, and strips were mounted on glass slides with Permount (Fisher Scientific, Fair Lawn, N. J.). For the cytochemical demonstration of HRP, the method of Graham and Karnovsky was used with diaminobenzidine tetrahydrochloride as substrate (DAB, Sigma Chemical Co.). Neuroublastoma cells plated onto Aclar Strips were washed three times with Earle's-HEPES. Cells were incubated at 4°C (ricin-HRP), or at 37°C (HRP) for various time intervals. For studies of endocytosis of ricin-HRP, cells were incubated at 4°C with ricin-HRP, washed with Earle's-HEPES, and incubated in a medium free of HRP or ricin-HRP at 37°C. The fixative for electron microscopy consisted of 2.5% glutaraldehyde, 1% formaldehyde in 0.2 M sodium cacodylate buffer, pH 7.35, containing 0.02% CaCl2. Cells were fixed for
30 min at room temperature, washed with Earle's-HEPES, and stained with DAB (13). They were postfixed in 1% OsO₄ and 1.5% potassium ferrocyanide in distilled water. The strips were then processed for electron microscopy as described previously (9, 10, 20, 21). Sections 0.5-1 μm thick, stained only with DAB distilled water. The strips were then processed for electron microscopy as described before (11). Quantitative analysis was done according to established methods of Salpeter et al. (11, 31); a half distance of 1,000 Å was used (7, 11).

**RESULTS**

The binding of ricin-HRP to neuroblastoma cells at 4°C as a function of time reaches a plateau as shown in Fig. 1. As 2 h was the time required for maximal binding at the lowest lectin concentration, it was used throughout the binding experiments.

The binding of ricin to monolayers was not proportional to cell density, although a sufficiently high concentration of lectin was present in the medium. When monolayer cells at four different concentrations were incubated at 4°C in a 200 pmol/ml ricin solution, the binding per cell decreased with increasing cell number (Fig. 2). The same phenomenon was observed at a lower lectin concentration, but the values did not differ in the same ratios. Sandvig reported similar findings with abrin binding to Hela cells in monolayer cultures (32). These results could not be attributed to nonspecific binding, as the presence of 1% BSA virtually eliminated nonspecific binding, and control flasks without cells, carried through the entire procedure, did not bind any radioactivity. Figs. 3 and 4 are Steck and Wallack plots of the data obtained when neuroblastoma cells were incubated with increasing concentrations of ricin and ricin-HRP at 4°C. Both cases were saturable and gave a straight line suggestive of one class of noninteracting receptor sites.

The number of binding sites per cell is equal to the inverse of the slope and the number of binding sites per cell. For ricin, the value was $2.8 \times 10^6$ M⁻¹ and for the conjugate, $1 \times 10^6$ M⁻¹.

**Morphologic Studies**

**LIGHT MICROSCOPY:** The cytochemical reaction product for HRP was not visible when cells were incubated with the conjugate in the presence of 0.2 M lactose or 100-fold unlabeled ricin. Peanut and lens culinaris lectins did not inhibit the stain.

**ELECTRON MICROSCOPY:** A continuous peripheral staining of oxidized DAB was observed on the external surfaces of the plasma membranes of cells incubated at 4°C with ricin-HRP (ricin: 25 μg/ml, conjugated HRP: 46 μg/ml) for 2 h. To study the progress of adsorbive endocytosis of ricin-HRP, cells were incubated with ricin-HRP for 2 h at 4°C, washed, and then incubated at 37°C in a medium without ricin-HRP or HRP for 15 and 30 min, and 1, 2, 3, and 6 h. They were subsequently fixed and stained for HRP. After 15 min at 37°C, there was no detectable redistribution of surface stain for HRP in the form of patches or caps, or any intracytoplasmic stain. Small invaginations of the plasma membrane containing oxidized DAB-osmium black precipitates, hence to be referred as oxidized DAB, seen also at 4°C, probably represent the beginning...
Figure 3: Steck-Wallach plot of 125I-ricin binding by neuroblastoma monolayers. Cells (2 x 10^8) were incubated at 4°C for 120 min with increasing concentrations of lectin in a final volume of 3 ml. After incubation, the cells were washed, dissolved in 1 N NaOH, and measured for radioactivity and protein, from which cell numbers were derived. The free lectin concentration was obtained by subtracting the amount bound from the total added. Lactose inhibited 97% binding of ricin at concentrations used. Cell number was expressed per liter, and lectin concentration was expressed in molality. K is the affinity constant and n is the number of receptor binding sites according to the equation (32):

\[
\frac{\text{cells}}{\text{bound ricin}} = \frac{1}{K \cdot n} + \frac{1}{\text{free ricin} \cdot n}
\]

Often clusters of acid phosphatase-positive vesicles were proximal to one or both edges of the unstained cisternae of the Golgi apparatus (Fig. 7). The resemblance of the vesicular and cisternal staining obtained with the DAB and acid phosphatase stains led us to conclude that ricin-HRP undergoes endocytosis in GERL as well as in other vesicles (compare Fig. 5 with Figs. 6 and 7).

In one experiment, neuroblastoma cells which had been placed at 37°C for 2 h after the initial incubation with ricin-HRP at 4°C, were fixed and stained for acid phosphatase. We were not able to detect any difference in the stain of lysosomes or GERL elements between these cells and control cells which had not been exposed to ricin-HRP, but otherwise treated identically.

In three separate experiments designed to examine whether ricin "stimulates" fluid phase endocytosis, cells were incubated with 27 μg/ml of ricin for 1 h at 4°C, washed, and then incubated for 2 h at 37°C in a medium containing free HRP at a concentration of 1 mg/ml. The cells were then fixed and stained with DAB or assayed for HRP (Table I). These cells contained only a rare DAB-positive body. The amount of

Figure 4: Steck-Wallach plot of 125I-ricin-HRP binding by neuroblastoma cells. The cells (3 x 10^8) were incubated in a final volume of 2 ml under the conditions described in Fig. 3. Lactose inhibited 29% of binding of 125I-ricin-HRP.
FIGURE 5  Neuroblastoma incubated for 2 h at 4°C with a conjugate of ricin-HRP in which 25 μg/ml of ricin and 46 μg/ml of conjugated HRP were present. Cells were washed and incubated for 1 h at 37°C in Earle's-HEPES, before fixation and staining for HRP with DAB. Vesicular and cisternal stain of HRP is prominent. G, unstained cisternae of the Golgi apparatus. Bar in this and subsequent electron micrographs represents 1 μm.

FIGURE 6  Acid phosphatase stain. Arrows: area seen under higher magnification in Fig. 7.
intracytoplasmic HRP in these cells was comparable to the amount found in cells not previously treated with ricin (Table 1). These morphologic and quantitative experiments strongly suggest that the intense intracellular stain of ricin-HRP (Fig. 5), is not caused by a mere acceleration of an endocytic mechanism that is common to both adsorptive and fluid phase endocytosis. Finally, to confirm the above hypothesis, we examined in the electron microscope neuroblastoma cells that internalized HRP in amounts comparable to those obtained by the endocytosis of ricin-HRP. Cells were incubated for 2 h at 37°C in a medium containing HRP (5 mg/ml); at the end of this period a portion of cells was stained for DAB and processed for electron microscopy (Fig. 8), while the rest were used for determination of HRP activity. These cells had taken in 71 ng HRP/1 × 10⁶ cells. In a parallel experiment, cells from sister cultures were incubated with ricin-HRP for 1 h at 4°C, washed, and then placed for 2 h in a medium free of HRP or ricin-HRP. The concentration of ricin and HRP in the conjugate was 25 and 46 µg/ml, respectively. All quantitative assays were done in duplicate. Cells originally incubated with ricin-HRP had taken in 131 ng HRP/1 × 10⁶ cells. In cells incubated with ricin-HRP, the HRP was observed in GERL and occasionally in residual bodies; in cells incubated with free HRP, the DAB stain was seen only in residual bodies and in small vesicles close to them but not in elongated cisternae or vesicles of GERL (Fig. 8). Our findings suggest that ricin-HRP undergoes endocytosis into vesicles, vesicles and cisternae of GERL, and residual bodies. In view of these results, the internalization of ricin-HRP is qualitatively and quantitatively different from that of native HRP.

**Recycling of the Ligand**

In a recent report, Sandvig et al. showed that ¹²⁵I-ricin ingested by Hela cells was subsequently released in the medium intact and in acid-precipitable form (33). This study by Sandvig et al., as well as recent electron microscope studies by Farquhar,

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**TABLE 1**

| Exp 1 | Exp 2 | Exp 3 | Exp 4 | Exp 5 |
|-------|-------|-------|-------|-------|
| Buffer | 33 | 33 | 23 | 23 | 30 | 30 | 26 | 26 | 31 | 31 |
| Ricin | 16 | 16 | — | — | — | — | 49 | 49 | 23 | 23 |
| Ricin-HRP | 110 | 4,238 | 70 | 2,692 | 278 | 6,048 | 172 | 3,738 | 105 | 3,181 |

In these experiments, cells were incubated for 1 h at 4°C in Earle's-HEPES (buffer), Earle's-HEPES containing 25 µg/ml ricin (ricin), or in Earle's-HEPES containing a conjugate of ricin with HRP (ricin-HRP); subsequently, cells were washed at 4°C with Earle's-HEPES and incubated for 1 h at 37°C in Earle's-HEPES containing HRP, (buffer and ricin), or in Earle's-HEPES (ricin-HRP). Cells were then washed and content of HRP determined. Uptake of HRP is expressed per mg of cell protein (mg cell pr.) and per mg of incubated HRP (mg inc. HRP).
Herzog and Farquhar, and Linthicum et al., are consistent with the hypothesis that ligands, and presumably, their plasma membrane receptors, might be recycled from the interior of the cell back into the plasma membrane (6, 18, 23). For this reason, we performed a quantitative electron microscope autoradiographic study, using $^{125}$I-ricin and $^{125}$I-labeled subunit B (binding subunit) of ricin. Cells grown on Aclar strips were labeled at 4°C for 2 h with $^{125}$I-ricin (380 pmol/ml) or $^{125}$I-ricin subunit B (300 pmol/ml). After several washes, a portion of cells was fixed and the rest were incubated for 1 h at 37°C. Subsequently, cells were washed with Earle's-HEPES containing either 0.1 M lactose or 0.1 M methylmannoside. A portion of cells washed with each sugar was fixed with 4% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.35, plus 0.02% CaC$_{12}$. All cells were processed for quantitative ultrastructural autoradiography (Figs. 11-13). Both $^{125}$I-ricin and $^{125}$I-ricin B were intact in samples of cells in which the ligands had undergone endocytosis for 2 h at 37°C (Figs. 9 and 10).

In parallel studies we determined the amounts of $^{125}$I-ricin found in the medium during its endocytosis. Aliquots of the medium were obtained at 15, 30, and 60 min after the commencement of the incubation at 37°C, and radioactivity counts were performed. Data from three different experiments indicate that $\sim 85\%$ of $^{125}$I-ricin, bound on cells at 4°C, is shed in the medium in the first 30 min of incubation at 37°C. Polyacrylamide gel electrophoresis of the medium has shown that the radioactivity is associated with intact ricin.

Quantitative autoradiographic studies consisting of grain density distribution analysis showed that there was no detectable return of $^{125}$I-ricin from the interior of the cell to the plasma membrane during a 1–2 period at 37°C. (Fig. 12C and C$_1$ and Fig. 13, last two columns).

**DISCUSSION**

Ricin (RCA 60) inhibits protein synthesis of cultured mammalian cells by inactivating the 60S ribosomal subunits (32–34). We have demonstrated that ricin and ricin-HRP undergo retrograde transport from the axonal terminals of the rat submandibular gland into the superior cervical ganglion and produce toxic effects consisting of depletion of the neuronal rough endoplasmic reticulum and aggregation of ribosomes into large basophilic bodies (17). The toxicity of ricin was reduced by its conjugation to HRP.

When ricin is added to cell culture medium a lag time of 20–30 min is noted before inhibition of protein synthesis can be detected (32–34); this lag time of 30 min coincides with the earliest time required for the intracytoplasmic visualization of ricin-HRP. The mechanism by which ricin crosses the plasma or other cell membranes to enter the cytoplasm is unknown. Ricin may cross the plasma membrane either at the site of its initial binding or at the membranes of the endocytic vacuoles and cisternae of GERL, which may be more favorable sites for the translocation of the toxic lectin from the membrane into the cytosol.

Neuroblastoma cells have a large number of saturable binding sites for ricin-HRP of neuroblastoma cells, human fibroblasts contain only 40,000–100,000 binding sites for epidermal growth factor (EGF) and a maximum of 20,000–50,000 receptors to low-density lipoproteins (LDL) (3, 4, 15). Unlike the slow uptake of ricin-HRP into GERL, $^{125}$I-EGF, EGF-ferritin, and LDL ferritin undergo endocytosis within minutes, via coated pits.
Polyacrylamide Gel Electrophoresis of cells that had taken in $^{125}$I-ricin for 2 h. Note that radioactivity is found in a single peak corresponding to ricin. PA: alkaline phosphatase, GDH: glutamic dehydrogenase, Ricin: the two subunits of ricin, Oval: ovalbumin.

into multivesicular bodies and lysosomes of cultured human carcinoma cells (EGF) and fibroblasts (LDL). Thus, the diffuse adsorptive endocytosis of a ligand such as ricin which has widespread specificities and numerous binding sites is quantitatively and qualitatively different from the receptor-mediated endocytosis of EGF, LDL, and other ligands with limited specificities and limited number of receptors (receptor-mediated or concentrated adsorptive endocytosis).

Recently, Yokoyama et al., using a conjugate of HRP with ricinus communis agglutinin (RCA-120), have stained the cisternae of the Golgi apparatus, phagocytic vacuoles, and multivesicular bodies of fixed cells in several mouse tissues including cerebellum and adrenal medulla (38); 0.2 M lactose added to RCA-120 HRP diminished the HRP stain. This observation suggests that certain cytomembranes, including those of the Golgi apparatus, are particularly rich in moieties with terminal D-galactose.

The binding of $^{125}$I-ricin to monolayers of neuroblastoma cells is not proportional to cell number (Fig. 2). Similar observations were made with abrin (32). These data suggest that cells at higher densities have fewer lectin receptors or that the ligand is less accessible to cell surfaces of confluent cells.

Ricin binds to cell surfaces through receptors containing terminal D-galactose, and the binding of the lectin is inhibited by D-galactose or lactose (29). In a recent publication it was reported that the binding of ricin covalently linked to a carbohydrate was not inhibited by lactose (39). Similarly, we have shown that while the binding of $^{125}$I-ricin was inhibited by lactose, the binding of $^{125}$I-ricin covalently linked with HRP was reduced but not entirely inhibited by this sugar. However, the peroxidase stain was abolished in cells incubated with $^{125}$I-ricin-HRP in the presence of 0.2 M lactose, or with 100-fold concentrations of ricin. The apparent discrepancy of the effect of 0.2 M lactose on $^{125}$I-ricin-HRP binding, studied by radioactivity counting (inhibition of binding 27–29%) and peroxidase stain (complete inhibition), is probably caused by the higher sensitivity of radioactivity counting over the optical detection of peroxidase stain.

The increased uptake of HRP bound to ricin, in comparison to that of free HRP, is consistent with known quantitative differences between bulk and adsorptive endocytosis (Table I) (12, 35). Furthermore, ricin did not stimulate the uptake of free HRP (Table I). Thus, ricin does not have an "enhancing" effect on the mechanisms underlying bulk endocytosis, i.e., membrane invagination, and pinocytic vesicle formation and transport.

HRP is covalently linked to ricin using glutaraldehyde, and the detection of the HRP stain in GERL strongly suggests that ricin is also present in this area. Unpublished ultrastructural autoradiographic and cytochemical studies with $^{125}$I-ricin-HRP, and the lack of any degradation of $^{125}$I-ricin support the conclusion that intact ricin undergoes endocytosis in GERL. However, it remains to be determined whether ricin-HRP in GERL is associated with its "receptor," with certain domains of the plasma membrane, or with the entire plasma membrane which may have undergone endocytosis with ricin-HRP. Previous combined quantitative ultrastructural autoradiographic and cytochemical studies of iodinated plasma membranes of lymphocytes during segregation and internalization of surface immunoglobulins induced by anti-immunoglobulin antibodies strongly suggest that only certain domains of the plasma membrane undergo endocytosis (11).

The following observations are consistent with the hypothesis that separate endocytic pathways are involved in the uptake of presumed ligand-receptor complexes (ricin-HRP) and soluble enzyme (HRP): (a) GERL is the primary recipient of ricin-HRP, (b) free HRP is taken up only in dense or residual bodies,
FIGURE 11 Quantitative autoradiography: Bars represent grain density distribution in neuroblastoma cells incubated with $^{125}$I-ricin (A), 380 pmol/ml; or $^{125}$I-subunit B or ricin (B), 300 pmol/ml, for 2 h at 4°C before fixation. Solid vertical line at 0 represents the plasma membrane. in: Inside cell; out: outside cell. Solid line represents theoretical grain scatter from $^{125}$I of Fertuck and Salpeter (7, 11). Actual grain distribution from $^{125}$I-ricin or $^{125}$I-subunit B of ricin is consistent with labeling of plasma membrane. Abcissa: half distances. (HD) Grains from 6 HD and over were computed together. Ordinate: grains per 1 µm². Values at 0-2 HD were normalized to 1.

and (c) unlabeled ricin does not induce any significant increase of the uptake of free HRP by neuroblastoma cell (Table I). Furthermore, while ricin-HRP is found in GERL during the first 2 h of endocytosis and later in dense bodies, HRP is found only in dense bodies. This observation suggests that there is a unidirectional flow of ligand, and presumably of membrane-associated receptors, from GERL to lysosomes; the continuity of cisternae of GERL with lysosomes has been noted in various cells (27).

Recent studies have implicated GERL in adsorptive endocytosis and secretion (5, 16, 27). The isolation from cultured fibroblasts of a fraction enriched in GERL elements with lysosomal enzyme activities suggests that the plasma membrane undergoes endocytosis in GERL for degradation (19, 30).

The hypothesis of recycling of biological membranes has been formulated from several morphologic and morphometric studies (reviewed in reference 35). Recent evidence by Farquhar (6) and Herzog and Farquhar (18) supports the concept that several elements of the Golgi apparatus, i.e., the stacks of the Golgi cisternae including both cisternal elements and dilated rims, the secretory granules, and vesicles at the cis side of the Golgi apparatus play a significant role in the retrieval of plasma membranes for possible membrane reutilization. The findings of Linthicum et al. on the endocytosis and exocytosis of phytohemagglutinin cell surface receptors are also consistent with the view that plasma membrane receptors to ligands undergo recycling (23). However, the above studies, as well as our own observations based on the visualization of ligands, which do not bind covalently to their receptors, are suggestive but not conclusive of the intracellular traffic of receptors and membranes. For example, it is quite conceivable that during endocytosis, the ligand dissociates from its plasma membrane receptor and follows a different pathway than its receptor.

Our quantitative ultrastructural autoradiographic studies of $^{125}$I-ricin or its iodinated binding subunit have not shown recycling of the ligand (Figs. 12 and 13). The fate of the ricin "receptor" during the endocytosis of ricin is unknown, but at least three possibilities should be considered: (a) ricin and its

FIGURE 12 Quantitative autoradiography with $^{125}$I-ricin. Grain density distributions at increments of 2, 4, 6 HD from plasma membrane (solid line). in: Inside cells; out: outside cells. Grains inside cells over 6 HD from the plasma membrane were computed together. A: cells were incubated for 1 h at 4°C with $^{125}$I-ricin, 380 pmol/ml, before fixation (based on 1,463 grains and 5,203 µm² counted). B: initial labeling with $^{125}$I-ricin as in A; subsequently cells were incubated for 1 h at 37°C (1,318 grains, 6,545 µm² counted). C: initial labeling as in A; subsequently cells were incubated at 37°C for 1 h, washed with 0.2 M lactose and fixed (3,335 grains, 16,740 µm² counted). D: same as in C, but washed with 0.2 M methylmannoside (2,617 grains, 14,460 µm² counted).

[125 I] Ric B

FIGURE 13 Same as in Fig. 12 except that $^{125}$I-subunit B was used at a concentration of 300 pmol/ml. GR, number of grains counted. µ², number of square microns counted. A, cells incubated for 1 h at 4°C before fixation. B, after initial labeling with $^{125}$I-subunit B as in A, cells were washed and incubated for 1 h at 37°C before fixation. C, cells were treated as in B but after additional washes with 0.2 M lactose, cells were incubated for one additional hour at 37°C before fixation. D, cells were initially labeled as in A and after washing were incubated for 3 h at 37°C before fixation.
receptor, including closely associated plasma membrane domains, undergo endocytosis into GERL. (b) ricin and its receptor segregate from the plasma membrane and selectively undergo endocytosis in GERL. (c) ricin dissociates from its receptor, either soon after the initiation of endocytosis, or at the site of GERL, and the receptor is either recycled into the plasma membrane or degraded.

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REFERENCES

1. Antoine, J. C., S. Avrames, N. K. Gonatas, A. Stieber, and J. O. Gonatas. 1974. Plasma membrane and internalized immunoglobulin of lymph node cells studied with conjugates of antibody or its Fab fragments with horse-radish peroxidase. J. Cell Biol. 63:12-23.

2. Avrames, A., and T. Ternynck. 1971. Peroxidase labeled antibody and Fab conjugates with enhanced intracellular penetration. Immunochemistry. 8:1175-1179.

3. Brown, S. M., and J. L. Goldstein. 1979. Receptor-mediated endocytosis: Insights from the ligand-protein receptor system. Proc. Natl. Acad. Sci. USA. 76:3330-3337.

4. Carpenter, G., and S. Cohen. 1979. Epidermal Growth Factor. Annu. Rev. Biochem. 48: 193-216.

5. Easner, E., and H. Haines. 1977. Ultrastructural study of GERL in beige mouse alveolar macrophages. J. Cell Biol. 75:381-389.

6. Farquhar, M. G. 1978. Recovery of surface membrane in anterior pituitary cells. Variations in traffic detected with anionic and cationic ferritin. J. Cell Biol. 77:R35-R42.

7. Fertuck, H. C., and M. M. Salpeter. 1976. Quantitation of the junctional and extrasynaptic acetylcholine receptors by electron microscope autoradiography after 125I-bungarotoxin binding at mouse neuromuscular junctions. J. Cell Biol. 79:144-158.

8. Gonatas, N. K., and S. Avrames. 1973. Detection of plasma membrane carbohydrates with lectin peroxidase conjugates. J. Cell Biol. 59:436-443.

9. Gonatas, N. K., S. U. Kim, A. Stieber, D. I. Graham, and S. Avrames. 1975. Internalization of neuronal plasma membrane ricin receptors into the Golgi apparatus. Exp. Cell Res. 94:426-531.

10. Gonatas, N. K., S. U. Kim, A. Stieber, and S. Avrames. 1977. Internalization of lectins in neuronal GERL. J. Cell Biol. 75:11-13.

11. Gonatas, N. K., J. O. Gonatas, A. Stieber, J. C. Antoine, and S. Avrames. 1976. Quantitative ultrastructural autoradiographic studies of iodinated plasma membranes of lymphocytes during segregation and internalization of surface immunoglobulins. J. Cell Biol. 70:477-497.

12. Gonatas, N. K., C. Harper, T. Mitsutani, and J. Gonatas. 1979. Superior sensitivity of conjugates of horseradish peroxidase with wheat germ agglutinin for studies of retrograde axonal transport. J. Histochem. Cytochem. 27:728-739.

13. Graham, R. C., Jr., and M. J. Karzowsky. 1966. The early stages of injected horseradish peroxidase in the proximal tubule of mouse kidney. Ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291-303.

14. Graham, D. L., N. K. Gonatas, and F. C. Charalampous. 1974. The undifferentiated and extended forms of C1300 murine neuroblastoma. Am. J. Pathol. 76:285-312.

15. Haigler, H. T., J. A. McKenna, and S. Cohen. 1979. Direct visualization of the binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells A-431. J. Cell Biol. 81:382-395.

16. Harper, C. G., J. O. Gonatas, T. Mitsutani, and N. K. Gonatas. 1980. Retrograde transport of ricin in the autonomic nervous system. Lab. Invest. 42:396-404.

17. Herreg, V., and M. G. Farquhar. 1977. Luminal membrane retrieved after exocytosis reaches most Golgi cisternae in secretory cells. Proc. Natl. Acad. Sci. USA. 74:5073-5077.

18. Holtzman, E., S. Teichberg, S. J. Abrahams, E. Citkowitz, S. M. Cralin, N. Kawai, and E. R. Peterson. 1973. Notes on synaptic vesicles, and synaptic structures, endoplasmic reticulum, lysosomes and peroxisomes in nervous tissue and the adrenal medulla. J. Histochem. 21:349-385.

19. Joseph, K. C., S. U. Kim, A. Stieber, and N. K. Gonatas. 1978. Endocytosis of cholera toxin into neuronal GERL. Proc. Natl. Acad. Sci. USA. 75:2815-2819.

20. Joseph, K. C., A. Stieber, and N. K. Gonatas. 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with GM1 ganglioside. J. Cell Biol. 81:543-554.

21. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). 227:680-657.

22. Linthicum, D. S., E. Elson, J. Mendelsohn, and S. Sells. 1977. Endocytosis and exocytosis of mannose-specific toxin. Proc. Natl. Acad. Sci. USA. 76:927-931.

23. Linthicum, D. S., E. Elson, J. Mendelsohn, and S. Sells. 1977. Detection of plasma membrane carbohydrates with lectin peroxidase conjugates. J. Cell Biol. 70:477-497.

24. Marchalonis, J. J., R. E. Cone, and V. Satter. 1971. Enzymic iodination. A probe for accessible surface proteins of normal and neoplastic lymphocytes. Biochem. J. 124:921-927.

25. McMorris, F. A., F. G. Nelson, and F. E. Ruddle. 1973. Neurosci. Res. Prog. Bull. 11:412-455.

26. McMorris, F. A., F. G. Nelson, and F. E. Ruddle. 1973. Neurosci. Res. Prog. Bull. 11:412-455.

27. Nicolson, G. L., J. R. Smith, and R. Hyman. 1978. Dynamics of toxin and lectin receptors on a lymphoma cell line and its toxin-resistant variant using ferritin-conjugated, 125I-labeled ligand. J. Cell Biol. 78:565-576.

28. Oates, S. 1978. Ricin and ricinus agglutinins, toxin lectins from Castor Bean. Methods Enzymol. 50:330-355.

29. Reines, H. L., A. S. Garvin, M. E. Altietta, and E. F. Neufeld. 1979. Two species of lysosomal organelles in cultured human fibroblasts, Cell. 17:143-153.

30. Salpeter, M. M., and M. Szabo. 1972. Sensitivity in electron microscope autoradiography. I. The effect of radiation dose. J. Histochem. Cytochem. 20:425-454.

31. Sandvig, K. 1978. Cell density effects the binding of the toxic lectin abrin to Hela cells in monolayer cultures. FEBS (Fed. Eur. Biochem. Soc.) Lett. 89:233-236.

32. Sandvig, K., S. Olesen, and A. Pihl. 1974. Kinetics of binding of the toxic lectin abrin to surface receptors of human cells. J. Biol. Chem. 215:3997-3998.

33. Sandvig, K., S. Olesen, and A. Pihl. 1974. Binding, uptake and degradation of the toxic protein abrin and ricin by toxin-resistant cell variants. Eur. J. Biochem. 68:13-21.

34. Silvestrini, C. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. Annu. Rev. Biochem. 46:689-722.

35. Stiek, T. L., and D. F. J. Wallace. 1965. The binding of kidney bean phytohemagglutinin by Ehrlich ascites carcinoma. Biochem. Biophys. Acta. 97:510-527.

36. Wohlers, G., K. Knijpers, K. Kasakii, and A. Schurron. 1976. Solid-phase enzyme-immunosorbent assay for detection of hepatitis B surface antigen. J. Clin. Pathol. 29:873-879.

37. Yokoyama, M., F. Hisiyama, N. Kawai, and H. Hirano. 1980. The staining of golgi membranes with ricin and ricinus agglutinins by electron microscopy. J. Histochem. Cytochem. 28:1:82-86.