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 2.8×10^6 M^{-1} for ricin and 1×10^6 M^{-1} for ricin-HRP. The number of binding sites per cell was 8×10^7 and 3×10^7 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

[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).

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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 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 ^{125}I-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 ^{125}I-Ricin

Iodination of ricin was performed using lactoperoxidase according to the method of Marchalonis (25). 1 mCi of carrier-free ^{125}I was added to 1 mg of ricin ($E^{1\%}_{280\,nm}$ = 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 ^{125}I by a G-25 (fine) Sephadex column (0.9 × 30 cm). After further dialysis against PBS, the specific activity of the radioactive lectin was 3–7 × 10^6 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 mCi carrier-free ^{125}I was added to 0.4 mg ricin B-chain and then lactoperoxidase and H_2O_2 were added as above. The incubation at room temperature lasted for 45 min. The material was then applied to a 5-ml column of 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 lactose in PBS. Finally lactose was removed by dialysis. The specific activity obtained was 396 cpm/ng.

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 mg 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 × 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 in an Amicon G-50 filtration cone. (Amicon Corp., Scientific Sys. Div., Lexington, Mass.). The ricin-HRP conjugate was separated from unreacted HRP and ricin by a Sephadex G-200 column (1.6 × 93 cm), equilibrated with PBS, and monitored by absorbance at 403 nm and gamma radioactivity. The fractions containing the lectin conjugates were concentrated, dialyzed against PBS, and stored at 4°C. Polyacrylamide gel electrophoresis confirmed the absence of free ricin and native HRP. With this method, a conjugate of ricin-HRP having a 1:3 molar ratio was prepared.

Binding Assay

Cells grown as monolayers in 25-cm^2 Falcon flasks (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) were washed five times with Earle's-HEPES at room temperature and two times with ice-cold Earle's-HEPES and incubated in 3 ml of buffer solution containing 1% bovine serum albumin (BSA) and ricin or the lectin conjugate at 4°C for the desired length of time. Because cell number had a significant effect on the binding data, only those flasks where cell density did not vary >10% were chosen for binding studies with increasing concentrations of lectin. The lectin concentration ranged from ~25–850 pmol/ml. After incubation, the cells were washed five more times with 10 ml of cold buffer and dissolved in 2–3 ml of 1 N NaOH with the aid of sonication. Aliquots were taken for radioactivity and protein measurement from which cell number was derived. The amount of ricin specifically bound to the cells was obtained by subtracting the amount bound in the presence of 0.2 M lactose. Nonspecific binding of ^{125}I-ricin was <3% of the total binding at all ricin concentrations used in the experiments; the percentage of inhibition of binding of ^{125}I-ricin-HRP by 0.2 M lactose was 27–29. There was no binding of ^{125}I-ricin-HRP or of ^{125}I-ricin on bottles incubated with the culture medium alone.

The number of binding sites per cell and the apparent Ka values were calculated by plotting the data according to Steck and Wallach (36).

Endocytosis and Measurement of Peroxidase

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) H_2O_2 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 ng 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 H_2SO_4. 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.

Polyacrylamide Gel Electrophoresis

Monolayer cultures were incubated for 2 h at 37°C with ^{125}I-ricin, or ^{125}I-subunit B of ricin, washed with Earle's-HEPES, and incubated for 1 min with Earle's-HEPES containing 0.23% Viokase (VioBin Corp., Monticello, Ill.). They were then washed three times with Earle's-HEPES to remove any residual Viokase and detached by gentle shaking. The cells were pelleted by centrifugation at 300 *g* for 10 min and solubilized in 0.063 M Tris buffer, pH 6.7, containing 8 M urea by boiling for 2 min. The samples were loaded in a 10% discontinuous slab gel according to Gonatas et al. (11) and Laemmli (22); after fixation and staining, radioactivity in 1.2-mm slices was determined.

Cell Culture

Monolayer cultures 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% agammaglobulinemic 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., Morristown, N. J.) (21). Between 75 and 85 × 10^3 cells/ml from a monolayer culture were added to bottles containing one 5 × 1.0 cm strip of Aclar. Strips were used 48 h later, when cells had become confluent.

Light and Electron Microscopy and Cytochemistry 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.) (13). Neuroblastoma 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% $CaCl_2$. Cells were fixed for

30 min at room temperature, washed with Earle's-HEPES, and stained with DAB (13). They were postfixed in 1% OsO_4 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 were examined at 100 kV in a Siemens Elmiskop 1A. Thin sections were examined at 80 kV.

Cytochemistry for Acid Phosphatase

Fixation of strips with cells was carried out for 30 min at 22°C in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer containing 0.025% $CaCl_2$. Strips were washed at 4°C in 0.1 M cacodylate buffer containing 5% sucrose and incubated for 2 h at room temperature and for 30 min at 37°C in a medium containing disodium salt of cytidylic acid as substrate, according to Novikoff and Novikoff (27).

Quantitative Autoradiography

The quantitative autoradiographic methods of Salpeter were used (7, 11, 31). Silver to gold sections of cells incubated with ^{125}I-ricin or ^{125}I-ricin-B (the binding subunit of ricin) were mounted on slides covered with 1% collodion in amyl acetate. The slides were coated with carbon and then covered with a monolayer of Ilford-L4 emulsion using a dipping machine (V. Avarloid, Toronto, Ontario) (11). Sections were developed 4–5 wk later with gold latensification (5 min), Elon ascorbic (4 min) and fixed (1 min) in nonhardening fixer (7). Sections were stained with uranyl acetate and lead citrate before or after coating with emulsion 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.

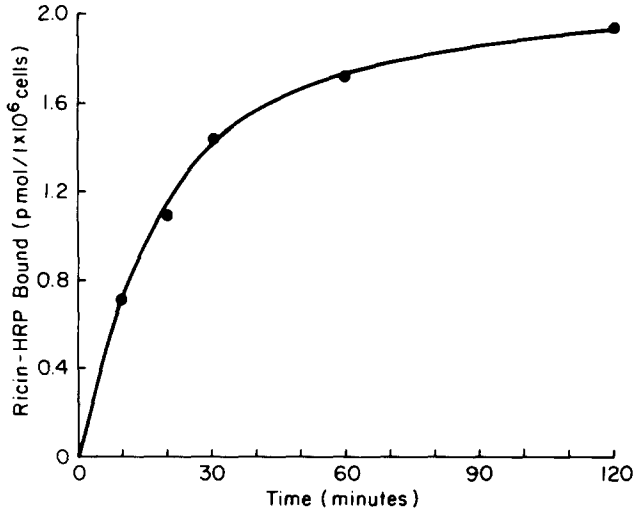

FIGURE 1 Neuroblastoma monolayers were incubated for 30, 60, 90, and 120 min at 4°C with 10 pmol/ml of ^{125}I-ricin, conjugated with HRP. The amount of ^{125}I-ricin-HRP bound by cells shows saturation as a function of time of incubation.

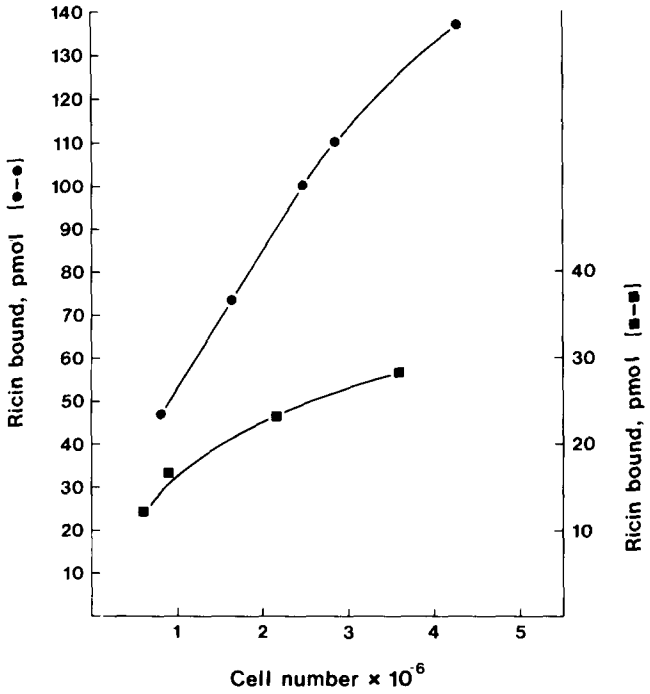

FIGURE 2 Binding of ^{125}I-ricin to monolayer neuroblastoma cells as a function of cell density. The experiment was performed at 4°C for 120 min. (●) Lectin concentration was 212 pmol/ml in a final volume of 3 ml. (■) 50 pmol/ml. Each point represents the average value of duplicate samples. The variation was <15%.

The number of binding sites per cell is equal to the inverse of the intercept with the ordinate and is 8×10^7 and 3×10^7 per ricin and ricin-HRP, respectively.

The apparent association constant is derived from the product of the inverse of the slope and the number of binding sites per cell. For ricin, the value was 2.8×10^6 M^{-1} and for the conjugate, 1×10^6 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 adsorptive 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 begin-

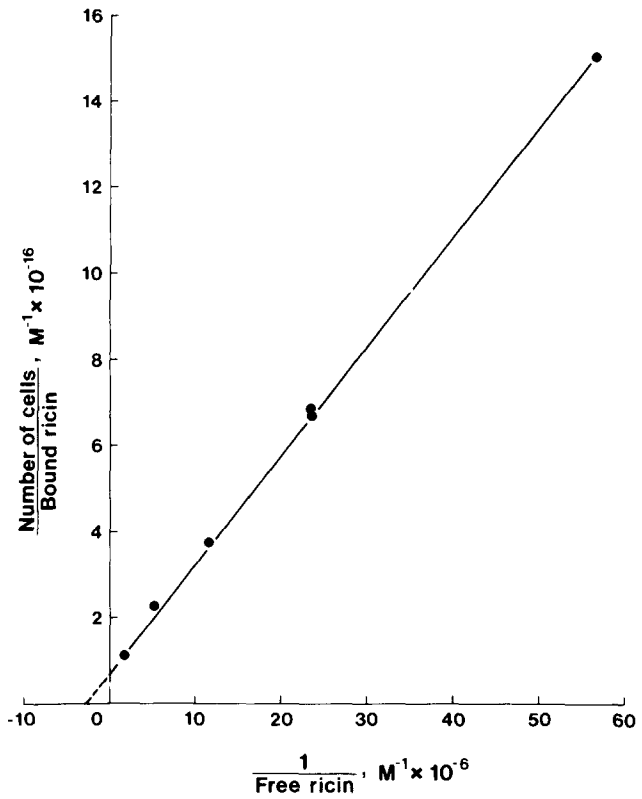

FIGURE 3 Steck-Wallach plot of ^{125}I-ricin binding to neuroblastoma monolayers. Cells (2×10^6) were incubated at 4°C for 120 min with increasing concentrations of lectin in a final volume of 3 ml. After incubation, the cells were washed, dissolved in 1 N NaOH, and measured for radioactivity and protein, from which cell numbers were derived. The free lectin concentration was obtained by substracting 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 molarity. K is the affinity constant and n is the number of receptor binding sites according to the equation (32):

$$\frac{\text{cells}}{\text{bound ricin}} = \frac{1}{K \cdot n} \cdot \frac{1}{\text{free ricin}} + \frac{1}{n}.$$

ning of endocytosis of ricin-HRP. Some of these vesicles were "coated" but the majority of them lacked a distinct coat of "bristles" at their intracytoplasmic aspects. After 30 min at 37°C, scattered intracytoplasmic vesicles measuring 20–200 nm in diameter contained oxidized DAB. After 1 h of endocytosis of ricin-HRP, the positive vesicles were clustered near the trans-aspect of the Golgi apparatus (Fig. 5). In addition, one cisterna next to the compact cisternae of the Golgi apparatus was often stained with DAB (Fig. 5). Neither the compact lamellae of the Golgi apparatus, nor their dilated lateral terminals contained oxidized DAB. After 2–6 h at 37°C, the clustering of the vesicular and cisternal staining near the Golgi apparatus became more prominent. Oxidized DAB was also observed in dense or residual bodies 2–3 h after endocytosis. The surface stain of DAB disappeared after cells were incubated at 37°C for 1 or 2 h. Incubations at 37°C for longer periods, did not result in the reappearance of the surface stain or in the appearance of intracytoplasmic HRP stain which was not associated with vesicles or cisternae (28). Neuroblastoma cells, stained for acid phosphatase, contained reaction product in vesicles and in one cisterna near the unstained cisternae of the Golgi apparatus, as well as in lysosomes (Figs. 6 and 7). 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

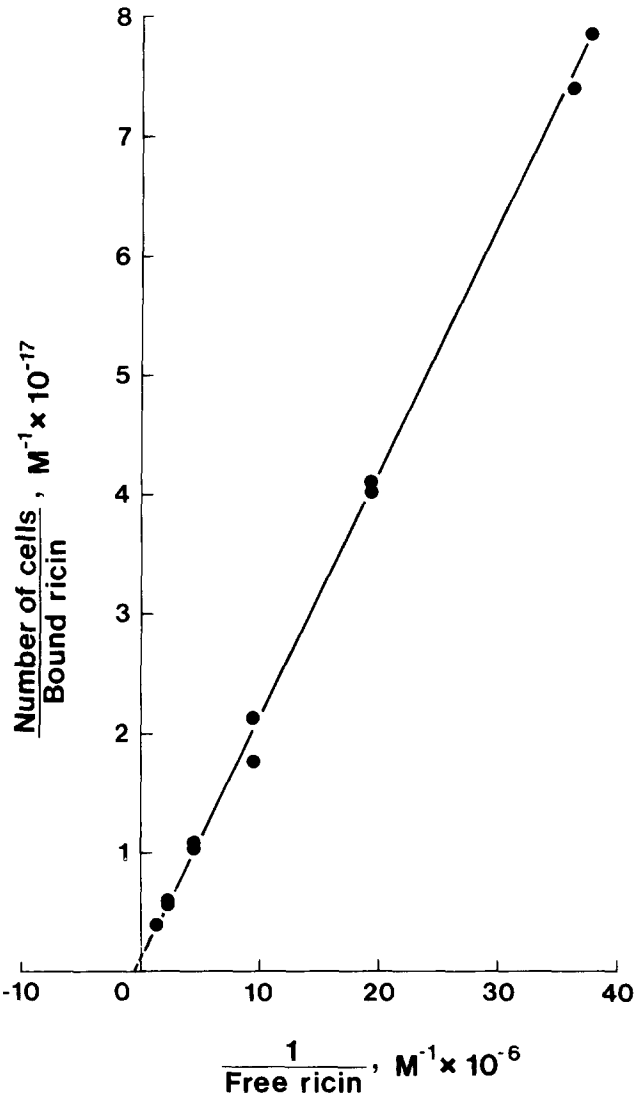

FIGURE 4 Steck-Wallach plot of ^{125}I-ricin-HRP binding by neuroblastoma cells. The cells (3×10^5) were incubated in a final volume of 2 ml under the conditions described in Fig. 3. Lactose inhibited 29% of binding of ^{125}I-ricin-HRP.

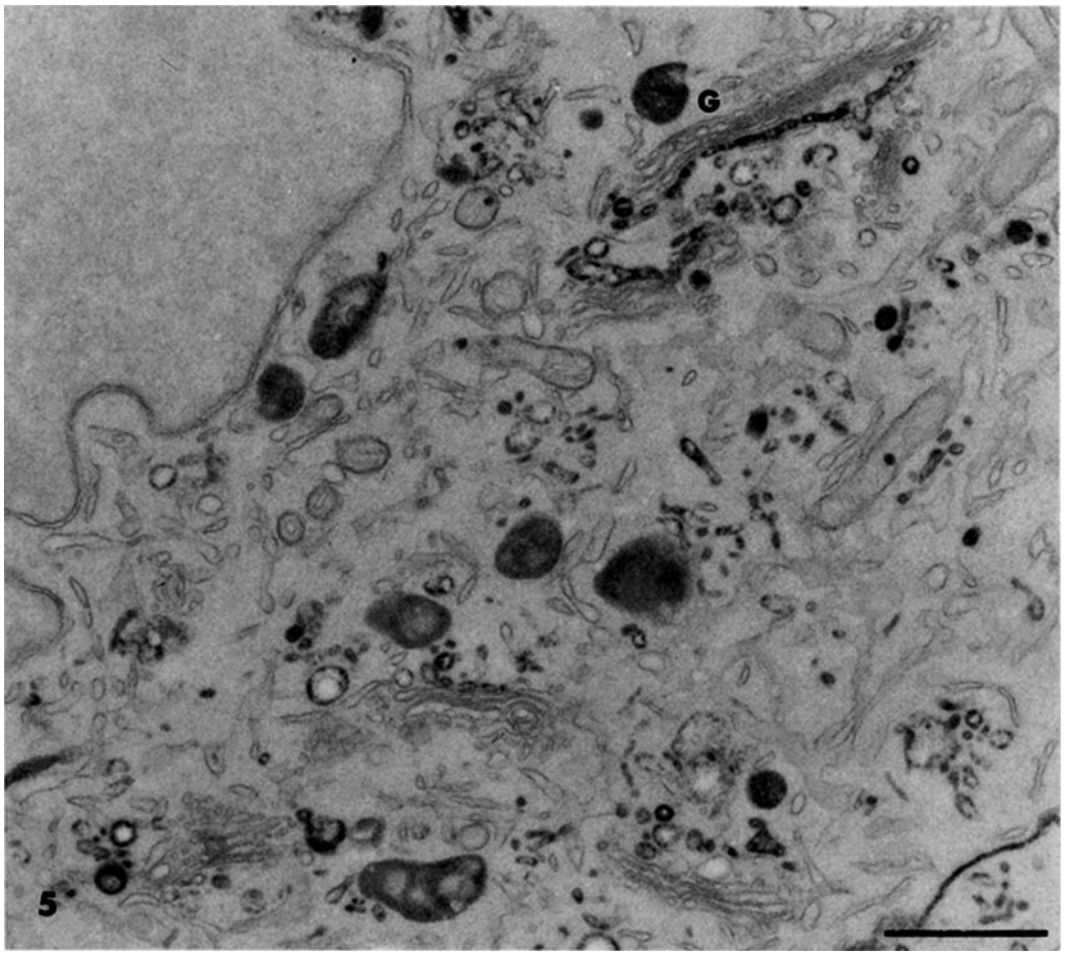

FIGURE 5 Neuroblastoma incubated for 2 h at 4°C with a conjugate of ricin-HRP in which 25 μg/ml of ricin and 46 μg/ml of conjugated HRP were present. Cells were washed and incubated for 1 h at 37°C in Earle's-HEPES, before fixation and staining for HRP with DAB. Vesicular and cisternal stain of HRP is prominent. *G*, unstained cisternae of the Golgi apparatus. Bar in this and subsequent electron micrographs represents 1 μm.

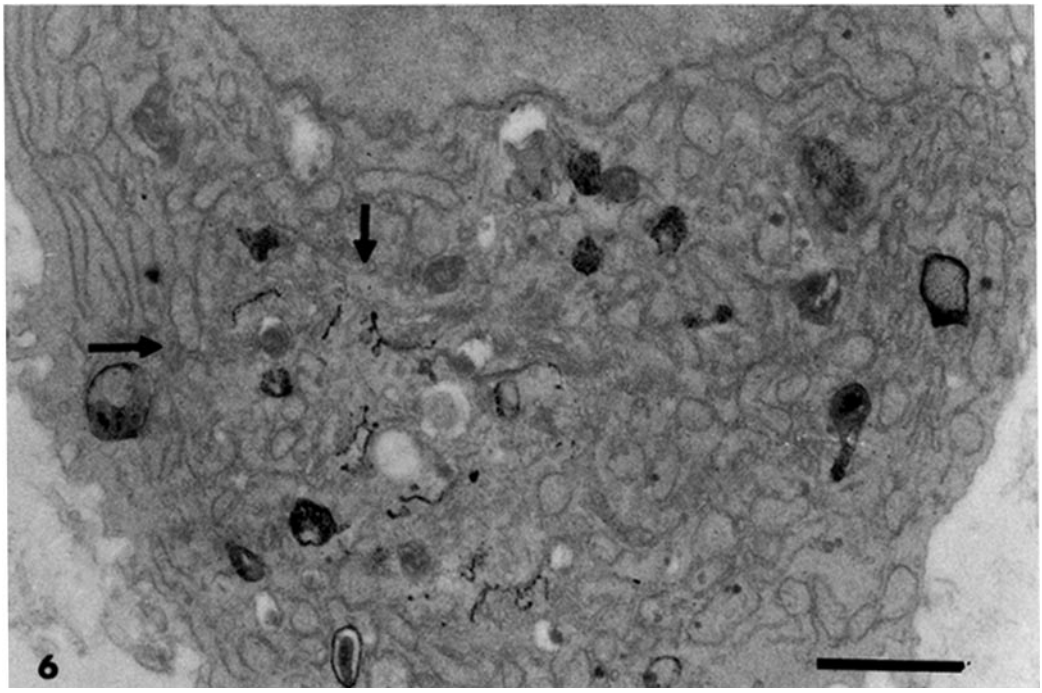

FIGURE 6 Acid phosphatase stain. Arrows: area seen under higher magnification in Fig. 7.

intracytoplasmic HRP in these cells was comparable to the amount found in cells not previously treated with ricin (Table I). 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 × 10^6 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 × 10^6 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 ^{125}I-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,

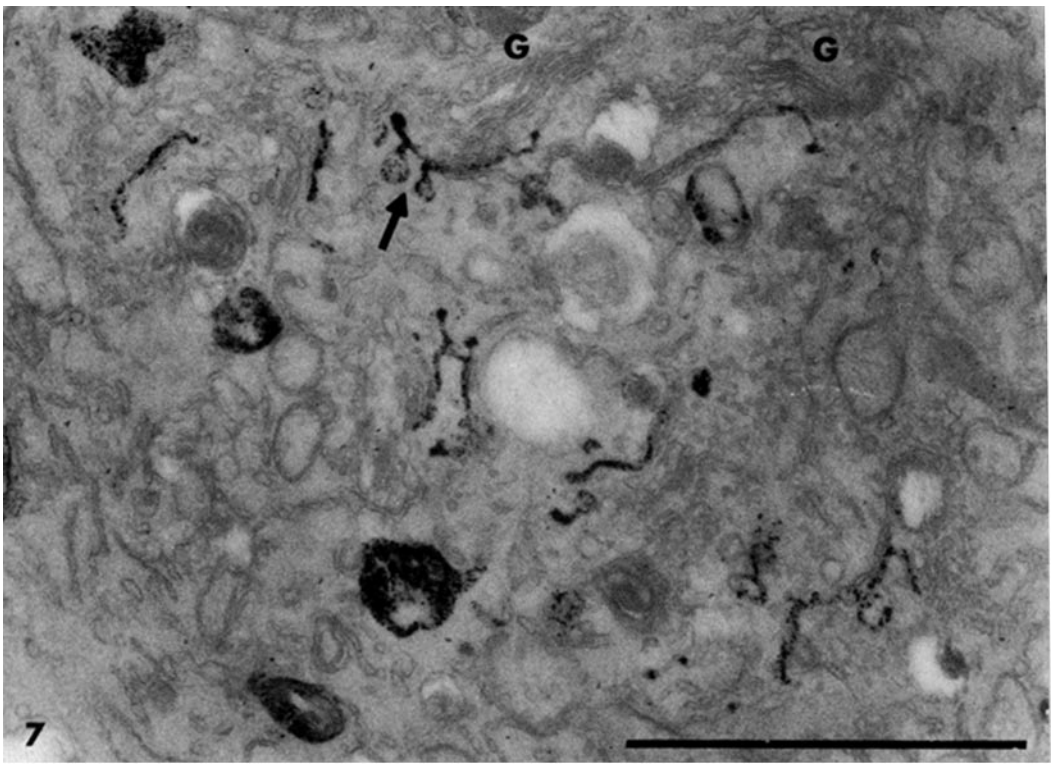

FIGURE 7 Enlargement from Fig. 6. G, unstained cisterna of Golgi apparatus: note positive cisternae of GERL at trans-aspect of the Golgi apparatus. Arrow: vesicular acid phosphatase stain in apparent continuity with positive cisterna, suggestive of two primary lysosomes, "budding" off from GERL.

TABLE I

Effect of Ricin on Subsequent Uptake of HRP by Neuroblastoma

	exp 1		exp 2		exp 3		exp 4		exp 5	
	ng HRP/ mg cell pr.	ng HRP/mg cell pr./mg inc. HRP	ng HRP/ ng cell pr.	ng HRP/mg cell pr./mg inc. HRP	ng HRP/ mg cell pr.	ng HRP/mg cell pr./mg inc. HRP	ng HRP/ mg cell pr.	ng HRP/mg cell pr./mg inc. HRP	ng HRP/ mg cell pr.	ng HRP/mg cell pr./mg inc. HRP
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*).

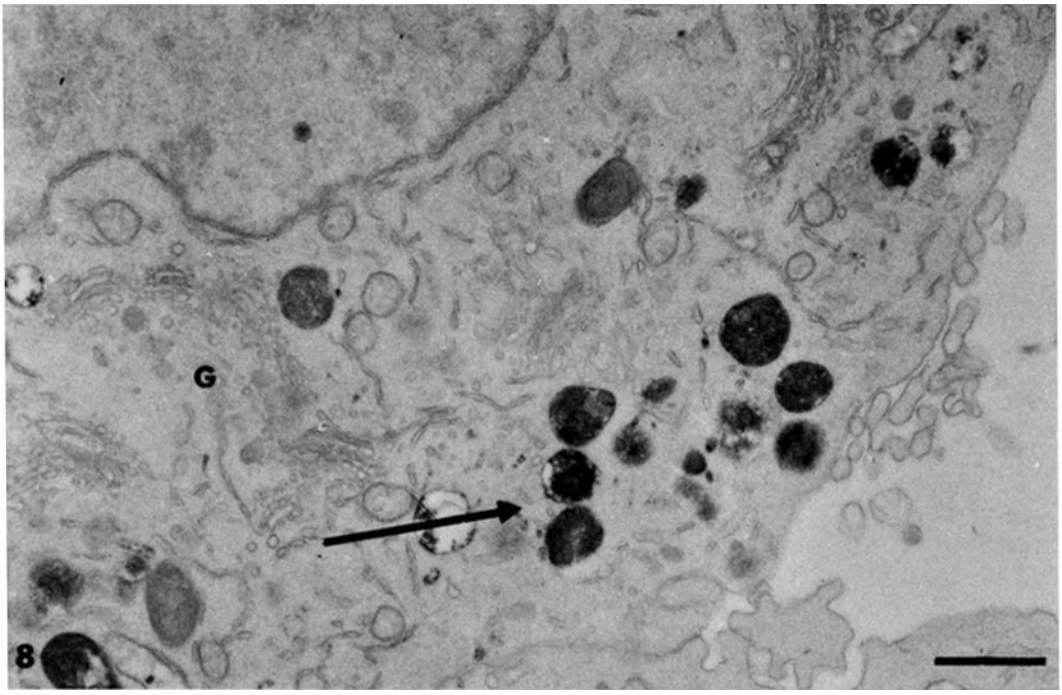

FIGURE 8 Neuroblastoma cells incubated with free HRP, 5 mg/ml, for 2 h at 37°C. *G*, unstained cisternae of the Golgi apparatus. Arrow: peroxidase-positive residual (dense) bodies.

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 of 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 and the remaining cells were incubated at 37°C for one additional hour and then fixed with 4% paraformaldehyde in 0.2 M caccodylate buffer, pH 7.35, plus 0.02% $CaCl_2$. 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 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 ~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. 12*C* 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 (8×10^7), and for ricin-HRP (3×10^7). The large number of binding sites to ricin and ricin-HRP, the high affinity constants (1×10^6 M^{-1}), and the saturability of binding (Figs. 1–4) strongly suggest that at least a proportion of the binding sites represents true plasma membrane "receptors" and not exogenously absorbed glycoproteins, glycolipids, or polysaccharides. The observation that ~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 suggests that ^{125}I-ricin bound by low-affinity receptor sites may not undergo adsorptive endocytosis. This hypothesis requires further investigation.

In contrast to the large number of 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,

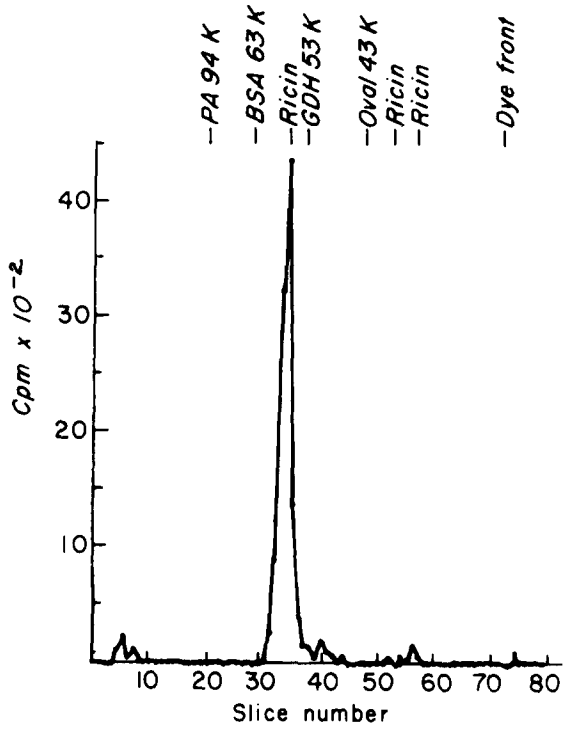

FIGURE 9 Polyacrylamide Gel Electrophoresis of cells that had taken in ^{125}I-ricin for 2 h. Note that radioactivity is found in a single peak corresponding to ricin. *PA:* alkaline phosphatase, *GDH:* glutamic dehydrogenase, *Ricin:* the two subunits of ricin, *Oval:* ovalbumin.

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,

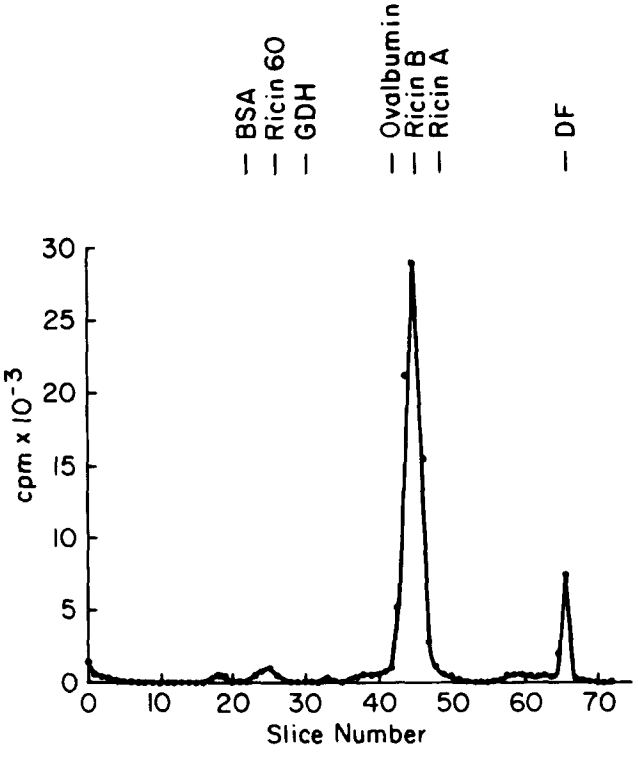

FIGURE 10 Same as in Fig. 9 except that ^{125}I-subunit B of ricin was incubated with cells. Again there is no evidence of breakdown of B subunit of ricin. *DF,* dye front.

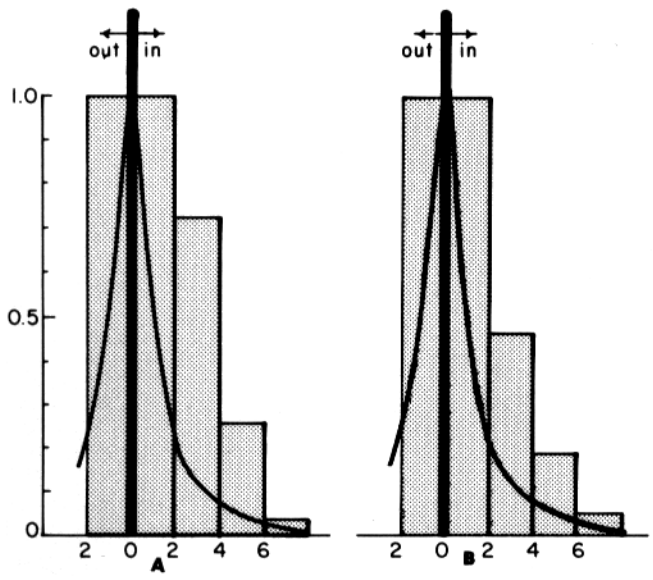

FIGURE 11 Quantitative autoradiography: Bars represent grain density distribution in neuroblastoma cells incubated with ^{125}I-ricin (*A*), 380 pmol/ml; or ^{125}I-subunit B or ricin (B), 300 pmol/ml, for 2 h at 4°C before fixation. Solid vertical line at 0 represents the plasma membrane. *in:* Inside 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 μm^2. 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

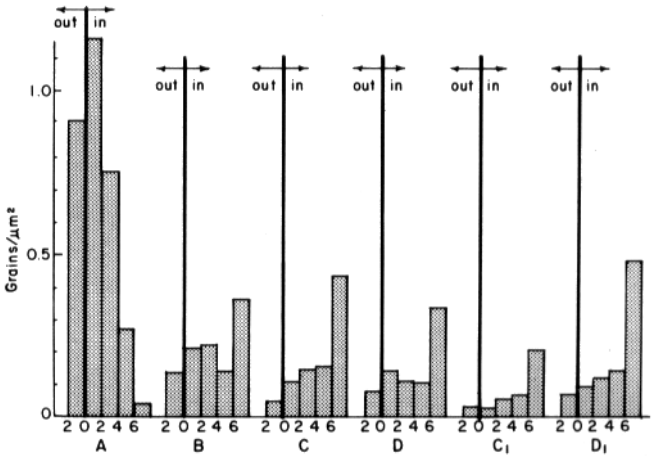

FIGURE 12 Quantitative autoradiography with ^{125}I-ricin. Grain density distributions at increments of 2, 4, 6 HD from plasma membrane (solid line). in: Inside cells, out: outside cells. Grains inside cells over 6 HD from the plasma membrane were computed together. *A*: cells were incubated for 1 h at 4°C with ^{125}I-ricin, 380 pmol/ml, before fixation (based on 1,463 grains and 5,203 μm^2 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 μm^2 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 μm^2 counted). *D*: same as in *C*, but washed with 0.2 M methylmanoside (2,617 grains, 16,151 μm^2 counted). C_1: same as in *C*, but after wash in lactose, cells were incubated for 1 h at 37°C before fixation (2,189 grains, 21,879 μm^2 counted). D_1: same as in *D* but after wash with manoside, cells were incubated for 1 h at 37°C before fixation (2,893 grains, 14,460 μm^2 counted).

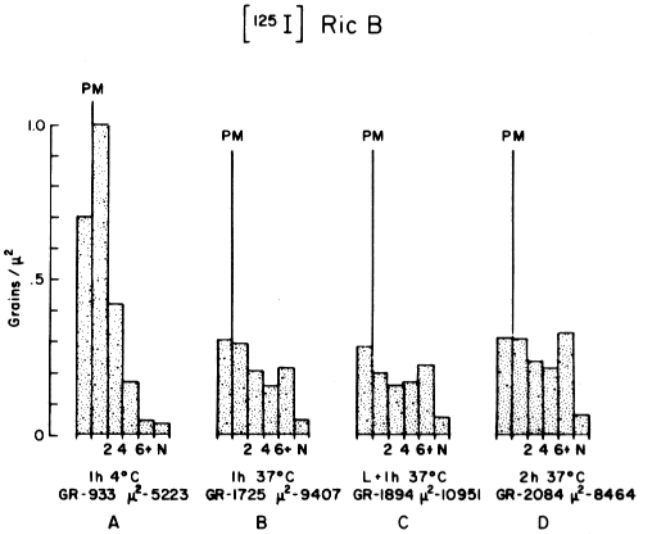

FIGURE 13 Same as in Fig. 12 except that ^{125}I-ricin subunit B was used at a concentration of 300 pmol/ml. *GR*, number of grains counted. μ^2, number of square microns counted. *A*, cells incubated for 1 h at 4°C before fixation. *B*, after initial labeling with ^{125}I-ricin as in *A*, cells were 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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