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CELL SURFACE RECEPTORS AND THEIR DYNAMICS ON TOXIN-TREATED MALIGNANT CELLS

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The binding, mobility, and mode of cell entry of the plant toxin ricin (or RCAII) were investigated on susceptible and partially resistant murine cell lines. When susceptible cells (SV40-transformed 3T3 fibroblast cells and BW5147 lymphoma cells) were examined, ricin bound rapidly, induced endocytosis, and entered the cell cytoplasm via broken endocytotic vesicles to inhibit cell protein synthesis, as found previously (1). Addition of lactose within 15 min after initial ricin binding prevented toxicity. After this time lactose addition no longer blocked the inhibition of protein synthesis.

In a partially resistant lymphoma (BW5147/RCA3) that shows only a slight reduction in the total number of ricin-binding sites, ricin bound rapidly to the cell surface, but was endocytosed significantly less at low ricin doses compared to its parental line, indicating a possible difference in cell surface behavior. The exposed surface proteins on the BW5147 parental and BW5147/RCA3 resistant lines were examined by ^{125}I-labeling utilizing lactoperoxidase-catalyzed iodination. The radiolabeled components were solubilized and separated by slab gel electrophoresis in sodium dodecyl sulfate. Autoradiograms of the slab gels indicated that two surface components of approximately 80,000 and 35,000 mol wt were much less exposed or were missing on the resistant line.

INTRODUCTION

Cell surface receptors comprise an important class of membrane molecules that form communication links to the cell's interior, providing the cell with environmental information and instructions to metabolize, depolarize, grow, divide, differentiate, secrete, die, etc. Little is known of the actual mechanisms that receptors use to relay their information, but in many systems processes subsequent to the actual binding of an external ligand are necessary for information to be transmitted (2–4). Thus, the transfer of extracellular information depends upon the structure and properties of the plasma membrane.

Proposals for cell membrane structure have evolved from earlier "sandwich" or "unit" models (5, 6) and globular "subunit" models (7, 8) to "liquid crystalline" or "fluid mosaic" models (9–12). These latter models seem to fit all the available data on membrane structure, and we feel that they represent the basic structure of most plasma

membranes and can explain the variety of phenomena that occur at the cell surface (3, 11). In the fluid mosaic membrane model (12), the globular integral proteins and glycoproteins are intercalated into and stabilized by a fluid lipid bilayer matrix forming a dynamic structure that also has transmembrane structural linkages formed by protein-glycoprotein complexes which traverse the lipid bilayer. Some of these transmembrane structures may be linked at the inner membrane surface to cell cytoskeletal elements such as microtubules, microfilaments, etc. (Fig. 1). Evidence for such linkages comes from experiments on the membrane association of the cytoskeletal system (13, 14) and its role in modulating receptor mobility (15–17), the effects of drugs which disrupt cytoskeletal organization on cell surface receptor dynamics (18–24), and experiments where the distribution and mobility of one class of surface receptors seems to be controlled by cytoskeletal elements that are linked to a second class of receptors (15, 25–27).

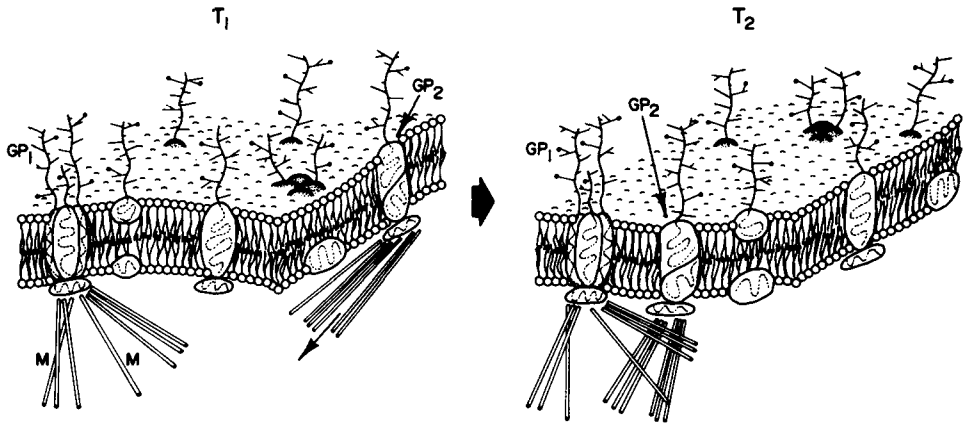

Fig. 1. Modified version of the fluid mosaic membrane model. T_1 and T_2 represent different points in time. Certain hypothetical integral membrane protein and glycoprotein components are free to diffuse laterally in the membrane plane formed by a fluid lipid bilayer matrix, while others, such as the integral glycoprotein complex GP_1, may be impeded by a membrane-associated microfilament-microtubule system (M). Under certain conditions some complexes (GP_2) can be displaced by membrane-associated contractile components in an energy-dependent process (Reproduced by permission from reference 3).

The interaction of surface receptors with cytoskeletal systems seems to be required for processes such as endocytosis, the removal of surface components by internalization. Endocytosis appears to be an important means by which cells can modulate their surfaces by removal of specific components when external ligands are bound at their surfaces. Although quite complex, surface modulation by endocytosis seems to occur as follows: (a) an external multivalent ligand is bound to a specific cell surface receptor; (b) ligand binding initiates receptor clustering due to the lateral mobility of the ligand-receptor complex; (c) the clustered ligand-receptor complexes somehow signal the cell cytoskeletal system to contract at the inner membrane surface in regions of ligand-receptor complexes, causing this area to be pulled into the cell, forming a membrane invagination; (d) the membrane invagination pinches off and reseals, leaving the ligand-receptor complexes sealed

inside endocytotic vesicles; and (e) the endocytotic vesicles are transported to specific cell regions for storage, breakdown, or fusion with lysozomal vesicles.

Certain plant and bacterial toxins must enter cells before exerting their toxic effects. In at least one case (ricin or RCAII from Ricinus communis [28, 29]), the toxin molecules must bind to cell surface receptors and be endocytosed before killing occurs by inhibition of protein synthesis (1, 30). Ricin is more toxic toward certain virus-transformed cell lines than to their normal counterparts, although these cell types contain equivalent numbers of surface receptors for ricin. To investigate the nature of the functional receptors for toxin binding and cell entry, hamster and mouse cell variants have been derived that are highly resistant to killing by ricin (31, 32). One of these ricin-resistant lines which was selected by direct toxicity still contains most of its binding sites for ricin, but two parental surface proteins which may serve as ricin receptors are no longer exposed on the cell surface. This ricin-resistant murine lymphoma line fails to endocytose or endocytoses less ricin under conditions where the ricin-sensitive parental line transports the toxin and is subsequently killed.

MATERIALS AND METHODS

Cells

Murine BALB/c 3T3 and SV40-transformed 3T3 lines were obtained from Dr. S. Aaronson (National Institutes of Health). The cell lines were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) containing 10% calf serum as described (1). The BW5147·G·1 line arose as a spontaneous lymphoma in the AKR strain mouse and was originally obtained from the Jackson Laboratories, Bar Harbor, Me., and adapted to tissue culture (33). Cells were grown in DMEM containing 10% horse serum (34). The parental line BW5147·G·1 is sensitive to low ricin concentrations (0.1 μg/ml). Ricin-resistant variants were selected in vitro by repeated exposure to increasing concentrations of ricin. One resistant clone (BW5147·G·1/RCA3) which grows in the presence of 1 μg/ml, but is inhibited by 4 μg/ml ricin, was chosen for further study. The immunological and surface properties of this and other toxin-resistant variants will be discussed elsewhere (35).

Surface Labeling Catalyzed by Lactoperoxidase

The lymphoma cells were harvested by centrifugation, rinsed three times with growth medium (lacking serum), once with sodium phosphate-buffered saline (PBS), and suspended at a concentration of 2×10^7 cells/ml in PBS. Cell viability was $> 95\%$ as determined by dye exclusion. Na ^{125}I (New England Nuclear) was added to the cell suspension at a concentration of 10–20 μCi/ml and lactoperoxidase (Calbiochem) at 2 U/ml. The labeling reaction was run at room temperature with additions of 5 μl 0.03% H_2O_2 per milliliter of cell suspension at 0, 1, 2, and 3 min. An additional 2 U/ml of lactoperoxidase were added at 2 min. The reaction was terminated at 4 min by addition of >20 vol of chilled DMEM containing 10% horse serum, and the cells were washed three times with this medium. Cells were suspended at a concentration of 2×10^7/ml in chilled 0.5% Nonidet P-40 (NP-40; Particle Data Laboratories) in PBS and incubated for 0.5 hr in an ice bath with occasional swirling. Nuclei were removed by slow-speed centrifugation at 5°C, and

the NP-40 supernatant was completely solubilized by heating in 2% sodium dodecyl sulfate (SDS) containing 0.1% 2-mercaptoethanol. SDS-polyacrylamide gel electrophoresis was run essentially by the method of Laemmli (36), and autoradiography was performed with NS54T medical X-ray film (Eastman Kodak).

Quantitative Binding of [^{125}I] Ricin

Ricin was radioiodinated by the ^{125}I-monochloride method of McFarlane (37) in 0.2 M D-galactose. The lectin was dialyzed and diluted to a specific activity of 30–60 X 10^{10} cpm/g for labeling experiments. No detectable loss of lectin-agglutinating activity occurred during ^{125}I-labeling. Cell labeling procedures followed the procedures of Nicolson and Lacorbiere (38) and Nicolson et al. (1).

Cell Protein Synthesis

Petri dishes containing confluently grown SV3T3 or 3T3 cells were washed twice with DMEM minus leucine. DMEM plus [^{3}H] leucine (3 μCi/ml) was added and the dishes were incubated at 37°C in a CO_2 incubator for 1 hr. After the [^{3}H] leucine labeling the cells were washed three times with phosphate-buffered saline and dissolved in 1 M NaOH. Protein was precipitated in excess cold 10% trichloroacetic acid. After 1 hr at 4°C the contents of each dish were filtered through Whatman GF/C filters, and the filters were washed twice with 5% trichloroacetic acid and once with 95% ethanol and then dried prior to counting in a Beckman Model LS-200 scintillation counter.

Ultrastructural Localization of Toxin Receptors

Ferritin-conjugated ricin (Fer-ricin) was synthesized in buffer containing 0.1 M D-galactose according to Nicolson and Singer (39, 40) or Kishida et al. (41). The ferritin conjugates were purified by affinity chromatography (40). Immediately before use the conjugate solutions were dialyzed into phosphate-buffered balanced salt solution (300 mosM).

Cells were labeled with Fer-ricin at 0°C for 10 min. Aliquots of the labeled cells were removed and rapidly washed and fixed in 2% buffered glutaraldehyde as described (1). The remainder of the cells were washed once by centrifugation at 4°C and then incubated at 37°C for 30 min. At that time cold glutaraldehyde was added to the cell suspensions and fixation continued for 1 hr at 4°C. Glutaraldehyde-fixed samples were washed twice at 4°C and postfixed in 1% phosphate-buffered osmium tetroxide. The fixed cell samples were dehydrated in ethanol-propylene oxide and embedded in Epon 812. Thin sections were stained with 2% uranyl acetate and observed in a Hitachi model HU-12 electron microscope at 75 kV.

RESULTS AND DISCUSSION

Mechanism of Ricin Entry and Killing of SV3T3 Cells

Addition of ricin (1–10 μg/ml) to cultures of SV3T3 resulted in growth inhibition, but these same ricin concentrations allowed 3T3 cell growth to proceed (1). More SV3T3 cells were killed initially compared to 3T3 cells, and few SV3T3 cells were able to grow

out after 6 days in medium containing ricin. In contrast to its effects on SV3T3 cells, ricin inhibited growth rate but did not stop 3T3 cell growth when used at concentrations (0.1–1 μg/ml) that were toxic to SV3T3 cells. The differential killing of SV3T3 compared to 3T3 cells was not due to a difference in the number of surface ricin receptors. Quantitative labeling of confluently grown cells with saturating concentrations (100 μg/ml) of [^{125}I] ricin resulted in a similar number of bound lectin molecules (approximately 10^7) on these cells (1). The kinetics of [^{125}I] ricin labeling indicated that receptor saturation occurs rapidly (<10 min), even at 4°C. However, when inhibition of cell protein synthesis was monitored after addition of ricin, a lag time of approximately 60 min was required before significant inhibition of cell protein synthesis occurred (1). For at least some of this lag time (after ricin binding to the cell surface) the toxin molecules were accessible to removal by the addition of inhibitory saccharides. When excess lactose was added at various times up to 15 min after a ricin pulse for 10 min, the disaccharide was effective in preventing the inhibition of protein synthesis. Addition of lactose 30 min after ricin did not prevent the subsequent shutdown of protein synthesis (Fig. 2). This indicated that time-dependent event(s) subsequent to ricin cell binding are important in eventual cell killing. If ricin was added in a 10-min pulse to cells at 0°C and the cells were washed and maintained at that temperature for 30 min, lactose was effective in preventing the subsequent inhibition of protein synthesis. Cell-free protein synthesis systems were sensitive to ricin and were not susceptible to lactose reversal of the ricin effects. The time course of ricin inhibition of cell-free protein synthesis indicated that the toxin shuts down translation within 1–3 min at 0.1 μg/ml (1).

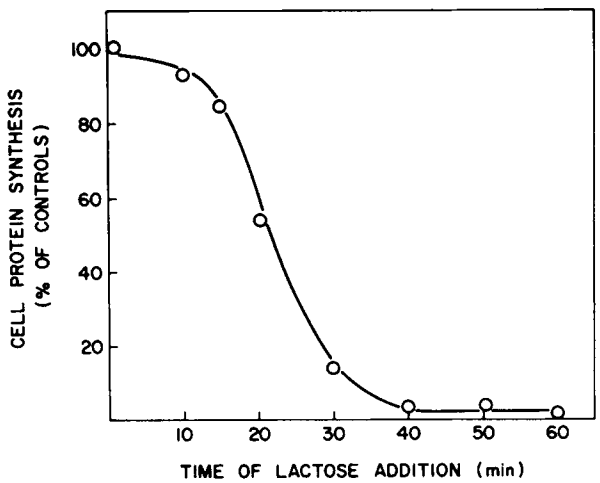

Fig. 2. Effect of lactose addition after ricin binding on the inhibition of cell protein synthesis. SV3T3 cells were pulsed in situ with 1 μg/ml recin for 10 min at 4°C and washed with cold DMEM. The cells were then incubated at 37°C, lactose (final 50 mM in DMEM) was added at various times, and the level of protein synthesis was measured at 1 hr by incorporation of [^{3}H] leucine into acid-precipitable protein (see Methods for details).

These results suggested that ricin enters susceptible cells to directly inhibit protein synthesis (1, 29, 42, 43). To examine this possibility we utilized an electron-dense derivative of ricin, ferritin-conjugated ricin (Fer-ricin), which is biologically active in this system (43). Fer-ricin bound rapidly to SV3T3 cell surfaces (<5 min), but its binding could be prevented, or reversed, if lactose was present during or added immediately after the labeling incubation. If lactose was not added during the experiment, eventually the cell surface-bound Fer-ricin induced clustering and endocytosis of some of its receptors (1). 60 min after a pulse labeling with Fer-ricin numerous endocytotic vesicles containing Fer-ricin were seen inside cells, and some of the Fer-ricin appeared to have been released into the cytoplasm (Fig. 3). The appearance of Fer-ricin in the cytoplasm paralleled the time course for inhibition of cell (but not cell-free) protein synthesis. By 60–90 min after Fer-ricin labeling, a large proportion of the lectin was transported inside cells, but a significant proportion remained at the cell surface. This suggests that there are different classes of surface toxin receptors, and some toxin-receptor complexes probably remain cell surface associated and do not stimulate endocytosis at low ricin concentrations. Similar results have been observed for certain concanavalin A receptors on murine fibroblasts (17). Thus, there exists a possibility that some receptor-ligand complexes can form on cell surfaces without stimulation of endocytosis. This class of receptors may fail to "communicate" with the cell cytoplasm for a variety of reasons, but a simple explanation may be that only certain surface receptors are transmembrane linked to structures within the cytoplasm (microfilaments?) which are responsible for surface modulation via endocytosis.

Ricin-Resistant Lymphoma Variants

Cell lines that show increased resistance to the toxic effects of ricin have been isolated principally by two methods: direct cytotoxic selection and immunoselection with ricin-antiricin plus complement. Gottlieb et al. (31) and Hughes (44) have used direct cytotoxic selection to obtain variants which grow in the presence of toxic concentrations of ricin. Alternatively, Hyman et al. (32) treated cells with low concentrations of ricin followed by antiricin and complement to select variants that were subsequently shown to have greater resistance to direct ricin killing. By these methods two general classes of ricin-resistant variants have been obtained: those showing a dramatic reduction in ricin-binding sites (31), and those showing almost normal levels of ricin-binding sites (32). This latter class of variants is of particular value in determining the mechanism of ricin cell entry and killing, because of the possibility discussed above that only a fraction of the total toxin-binding sites may serve as functional receptors involved in endocytosis and cell killing. We have been engaged in selecting and characterizing several ricin-resistant variants that show reductions of ~30–50% in the number of ricin receptors. Although the details of these selections and extensive characterizations of the variants obtained will be presented elsewhere (35), preliminary evidence suggests that two surface-exposed (glyco) proteins on one of the variants isolated by direct cytotoxicity from the murine lymphoma line BW5147·G·1 could be important for ricin cell entry at low toxin concentrations.

When BW5147 lymphomas and ricin-resistant variant (BW5147/RCA3) cells are labeled by lactoperoxidase-catalyzed ^{125}I iodination procedures under controlled conditions (45), only outer surface plasma membrane protein tyrosine residues are labeled.

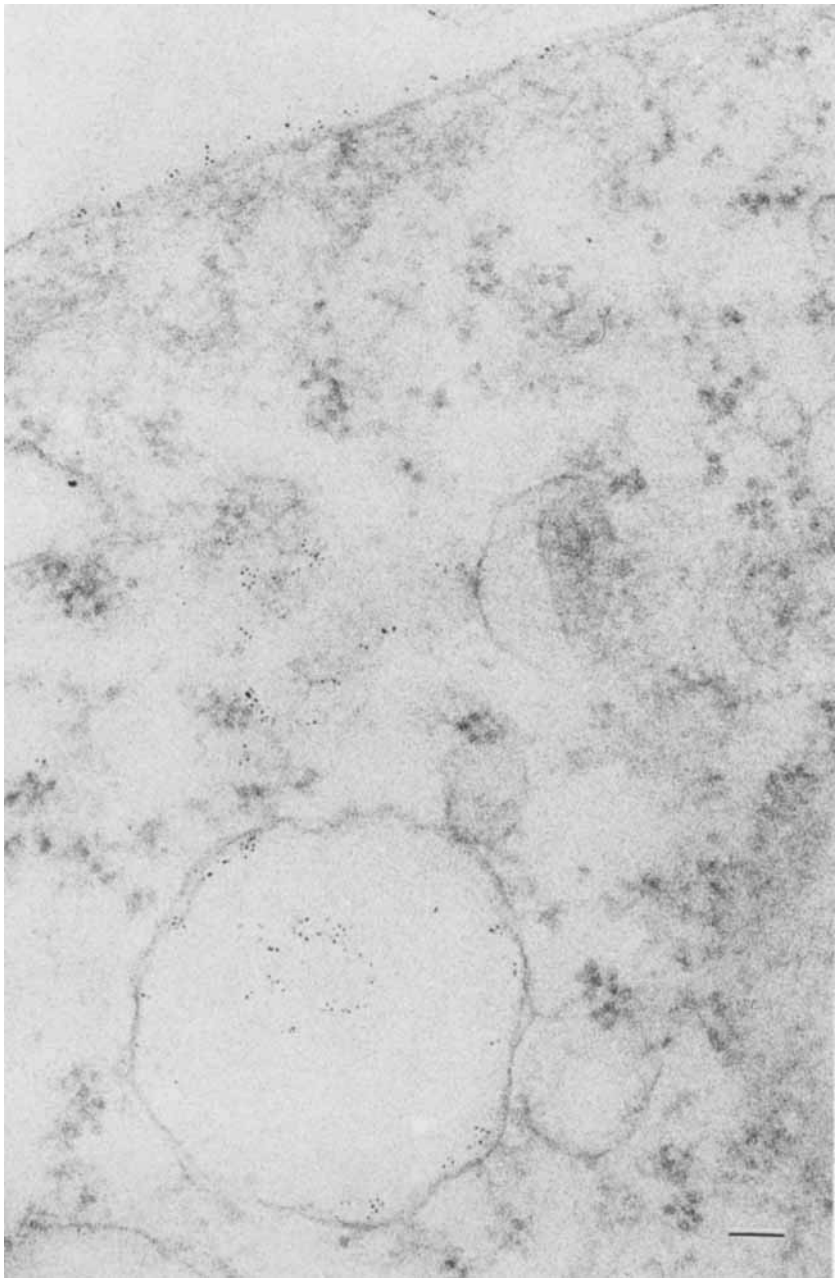

Fig. 3. Cell entry of Fer-ricin. SV3T3 cells were pulsed with Fer-ricin for 10 min at 4°C, and the cells were washed and resuspended in fresh DMEM at 37°C for 60–90 min. Extensive endocytosis has occurred, and some of the endocytotic vesicles appear to have broken, releasing their contents into the cell cytoplasm. Bar equals 0.1 μm, × 75,600. (Reproduced by permission from reference 1).

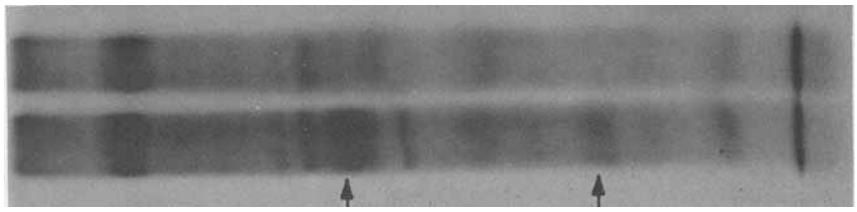

Fig. 4. ^{125} Iodine-labeled surface proteins of parent BW5147 (lower pattern) and ricin-resistant BW5147/RCA3 (upper pattern) lymphomas. The NP-40 supernatants of iodinated cells were solubilized with SDS and electrophoresed in an SDS-polyacrylamide gel slab (3% stacking gel, 7.5% resolving gel) for 5 hr at 12.5 mA. The resolving gel was dried and exposed for 4 days to NS54T film (Eastman Kodak Company). Migration was from left to right, and the sharp band at the extreme right is the dye front marker. Arrows in the lower pattern indicate surface proteins of approximately 80,000 (left arrow) and 35,000 (right arrow) daltons which are more iodinated on the parental line.

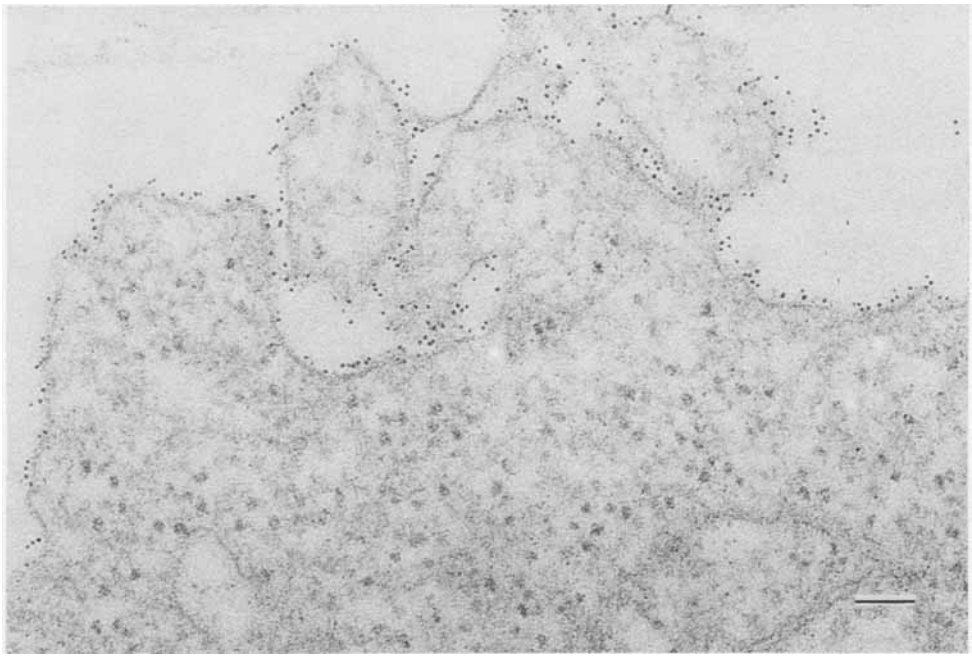

Fig. 5. BW5147 lymphoma labeled at 4°C for 10 min with Fer-ricin. Bar equals 0.1 μm; × 79,000.

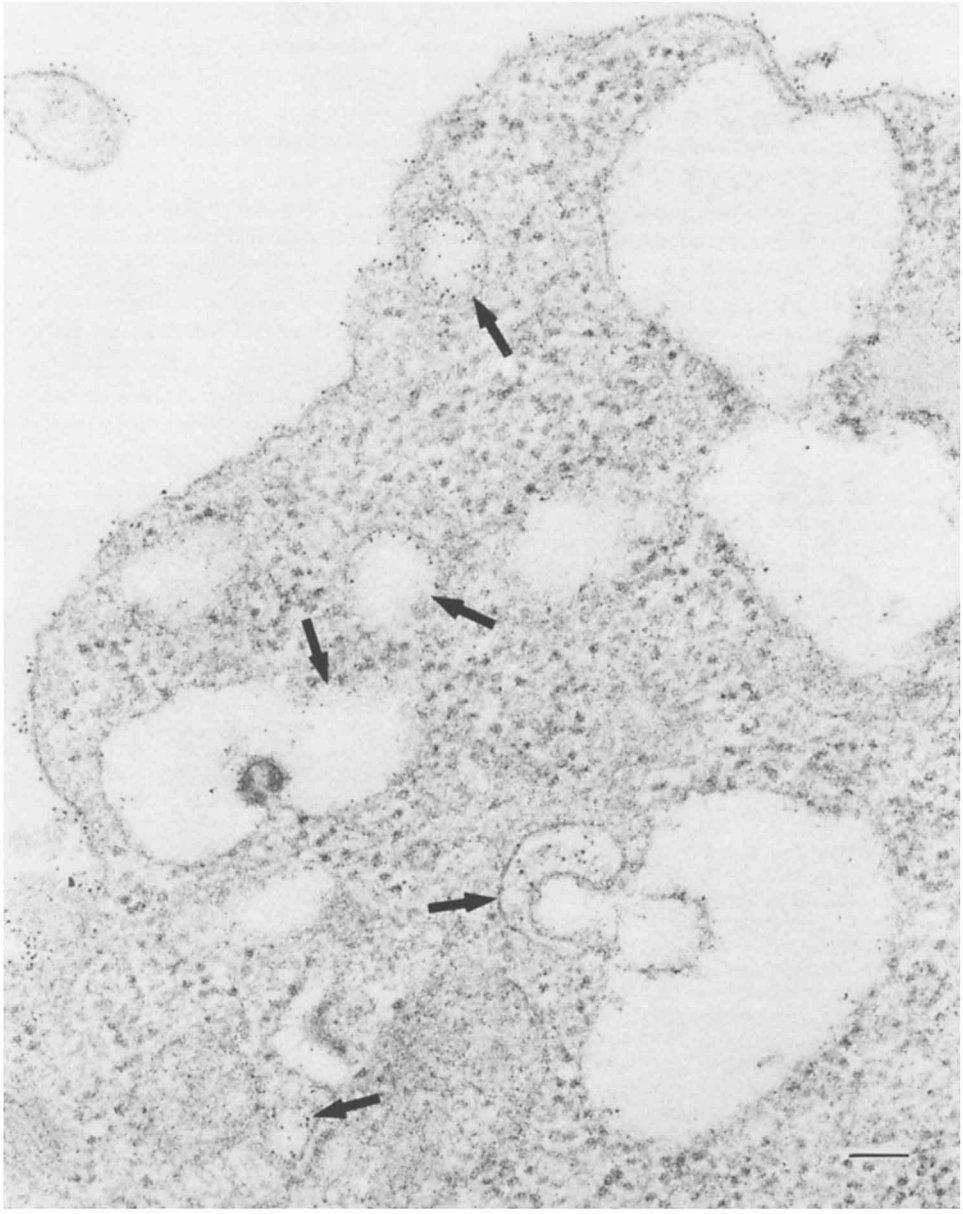

Fig. 6. BW5147 lymphoma labeled at 4°C for 10 min with Fer-ricin and subsequently washed and incubated at 37°C for 30 min. Arrows indicate endocytosed Fer-ricin. Bar equals 0.1 μm; × 79,000.

These ^{125}I-labeled surface exposed components can be solubilized in NP-40, separated by electrophoresis with SDS and identified by autoradiography (Fig. 4). The most striking difference between these lines is the reduction or absence of two major iodinatable components of ~80,000 and ~35,000 mol wt in the ricin-resistant variant (Fig. 4). The ~80,000 mol wt component is retained on ricin-sepharose columns, and is probably a receptor for ricin. The smaller component is less efficiently iodinated, and further studies are required to determine whether it is also a ricin receptor. The separation and purification of the BW5147 parental and ricin-resistant variant glycoproteins on ricin-sepharose columns will be discussed elsewhere (35).

An important question remains. What are the functions of the two glycoproteins which are not exposed on the ricin-resistant cell surface? One possibility is that these components function to stimulate transport of the ricin-receptor complexes into the BW5147 cell at low ricin concentrations. We therefore labeled parental and ricin-selected lymphoma cells with Fer-ricin at several conjugate concentrations and followed the ultrastructural fate of the surface-bound toxin molecules. Both parental and resistant line bound Fer-ricin rapidly (<5 min) in a random dispersed distribution across the entire cell surface (Fig. 5). After washing the cells and suspending in fresh media (without serum) at 37°C for 30 min, rapid clustering and endocytosis of Fer-ricin occurred in the parental

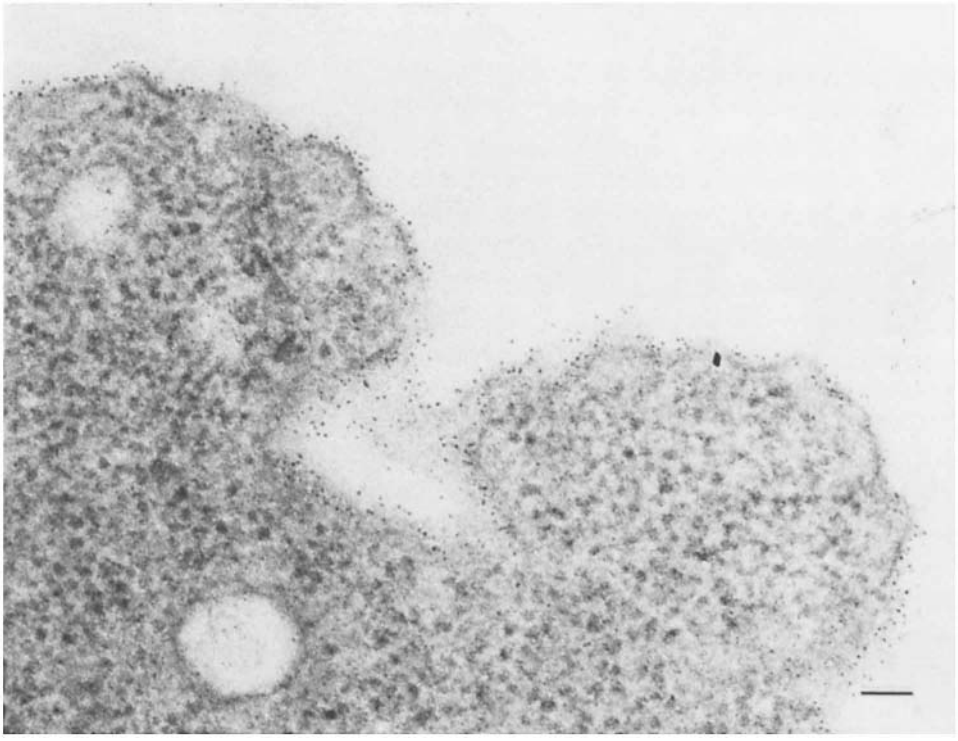

Fig. 7. BW5147/RCA3 ricin-resistant lymphoma variant labeled and incubated as in Fig. 6. Bar equals 0.1 μm; × 79,000.

line at low Fer-ricin concentrations (Fig. 6), but the Fer-ricin remained surface associated on the ricin-resistant variant under similar conditions (Fig. 7). Although preliminary, these results indicate a possible reduction in the uptake of low concentrations of toxin in resistant cells, and this difference may be determined by particular glycoproteins bearing toxin receptors at the cell surface.

The selection and characterization of several different toxin-resistant variants may help in understanding the relationship between cell surface receptors and their ability to transduce information on the state of ligand-receptor occupancy and distribution to the cell cytoplasm. This information is probably critical to the cell in initiating endocytosis resulting in surface modulation. Surface modulation processes utilizing endocytosis may be an important means by which cells transport proteins (46) and may help tumor cells to escape host immunological processes (48–50).

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