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Mechanism of Resistance to Ricin Toxin in Selected Mouse Lymphoma Cell Lines

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The role of impaired toxin uptake in conferring cellular resistance to the plant toxin RCA_{II} (ricin) has been examined using a murine BW5147 lymphoma line and a toxin-resistant variant (BW5147Ric^R.3) selected by repeated exposure to RCA_{II}. The toxin-resistant variant is 250 times more resistant to RCA_{II} in long-term growth experiments and 1,000 times more resistant in short-term protein synthesis assays. Experiments with ferritin-conjugated ^{125}I-labeled RCA_{II} (ferritin-^{125}I-RCA_{II}) indicated that toxin binding to sensitive and resistant cells is similar at low toxin concentrations where maximum differential cytotoxicity occurs but that major differences exist with respect to toxin uptake. In sensitive cells toxin is internalized via endocytosis, and as seen previously in other systems subsequent rupture of some of the toxin-containing endocytotic vesicles releases toxin into the cytoplasm, where it inhibits protein synthesis. The process of toxin transfer to the cytoplasm is presumed to account for the one-hour lag before toxin-induced inhibition of protein synthesis can be detected. Endocytotic uptake of toxin is impaired in resistant BW5147Ric^R.3 cells, and they are unaffected by toxin concentrations that inhibit protein synthesis and kill sensitive parental cells. Killing of resistant cells at low toxin concentrations was accomplished by encapsulating RCA_{II} into lipid vesicles capable of fusing with the plasma membrane. Direct introduction of toxin into resistant cells using lipid vesicles as carriers produced rapid inhibition (< 15 min) of protein synthesis and eliminated the lag in toxin action seen in sensitive cells exposed to free toxin. These findings are discussed in relation to the mechanism of toxin action and proposals that toxin activity requires structural modification of the toxin molecule at the cell surface before transport into the cell.

Key words: endocytosis, lipid vesicles, RCA_{II}, ricin, toxin, protein synthesis, variant cell line

The cytotoxicity of the plant protein ricin (RCA_{II}) [1–3] is thought to involve initial binding of toxin at the cell surface and subsequent entry into the cell by endocytosis [4–6] and final transfer to the cytoplasm to produce inhibition of protein synthesis [6–8]. In order to study this sequence of events in more detail, several investigators have selected somatic cell mutants that exhibit resistance to the direct cytotoxic

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effects of ricin or other lectins [9–14]. Many of these toxin- and lectin-resistant variants have lost the cell surface receptors involved in binding specific toxins or lectins [1, 15, 16], but other resistant variants that bind substantial quantities of toxin or lectin molecules have been identified in several laboratories [10, 13, 14, 16].

We have selected a murine BW5147 lymphoma variant line by stepwise selection against a series of increasing ricin (RCA_{II}) concentrations. The variant line, called BW5147Ric^R.3, is approximately 250 times more resistant to RCA_{II} than the wild-type BW5147 line in cytotoxic assays [14] and 1,000 times more resistant in cell protein synthesis inhibition assays. Interestingly, this resistant line still possesses a large fraction of cell surface receptors for RCA_{II} and can bind similar amounts of toxin compared to wild-type cells [14, 17]. Studies on RCA_{II}-mediated inhibition of protein synthesis in cell-free systems also indicate that the ribosomes of this variant are equally as sensitive to toxin inactivation as those of wild-type sensitive cells [18]. The similarity of toxin binding and ribosomal susceptibility in sensitive and resistant cells has focused attention on the possibility that the resistant phenotype is determined by failure to internalize toxin and/or transfer it to the cytoplasm. Support for this possibility has come from studies on the kinetics of toxin-mediated inhibition of protein synthesis and ultrastructural studies of toxin uptake. Inhibition of protein synthesis by RCA_{II} in sensitive BW5147 cells is not immediate and only occurs after a delay of 1 h [17, 18]. Ultrastructural analysis of the uptake of ferritin-conjugated RCA_{II} suggests that this delay corresponds to the time required for the internalization of toxin by endocytosis and subsequent escape of toxin molecules from endocytotic vesicles into the cytoplasm [17]. However, in toxin-resistant BW5147Ric^R.3 cells exposed to similar low toxin concentrations, endocytotic uptake of toxin is impaired [17]. In this paper we present further evidence to support the view that the resistance of BW5147Ric^R.3 cells to low toxin concentrations is due to impaired toxin entry. By fusing lipid vesicles containing encapsulated toxin with the plasma membrane of resistant cells to introduce toxin directly into the cytosol we have been able to render BW5147Ric^R.3 cells sensitive to low toxin concentrations. The lag time normally seen in the inhibition of protein synthesis by toxin in intact cells is eliminated under these conditions. The rapid inhibition of protein synthesis (< 15 min) produced by vesicle-delivered toxin presumably reflects that toxin introduced directly into the cytoplasm can gain access to ribosome "targets" more quickly than free toxin, which must bind to the cell surface receptors, be internalized by endocytosis and finally escape from endocytotic vesicles into the cytoplasm.

MATERIALS AND METHODS

Cells and Materials

The BW5147 cell line is a spontaneous AKR/J murine lymphoma and was obtained originally from Dr P. Ralph. The toxin-resistant BW5147Ric^R.3 variant cell line was selected by direct RCA_{II} cytotoxicity as previously described [10]. Both cell lines were grown as suspension cultures in Dulbecco's modified Eagle's medium (DMEM) with 2 mM additional glutamine and 10% horse serum. RCA_{II} was purified from Hale hybrid castor beans (Baker Castor Oil Company) by isolation, ammonium sulfate precipitation, and affinity chromatography as described previously [1, 2]. Radioisotopes were obtained from New England Nuclear, osmium tetroxide and glutaraldehyde from Polysciences, and other chemicals from Calbiochem.

Protein Synthesis

Cells were suspended at (1–2) × 10^6/ml in DMEM plus 10% horse serum. RCA_{II} or vesicle-encapsulated RCA_{II} (see below) were added to duplicate samples at 37°C in a tissue culture incubator with occasional swirling. The cells were suspended and 50 μl aliquots were removed for cell counting and 10 μCi of ^{3}H-amino acids were added to each tube. At various times the cells were pelleted by centrifugation, dissolved in 1 N NaOH containing 1% casein hydrolysate, and incubated for 10 min at 37°C to hydrolyze aminoacyl-tRNA. Protein was then precipitated with 5 volumes of cold 10% trichloroacetic acid and the precipitates left at 4°C for 1 h. Precipitates were collected on Whatman GF/C filters which were washed with cold 5% trichloroacetic acid and with 95% ethanol, dried and their radioactivity determined by scintillation counting with 5 ml Aquasol (New England Nuclear). Cells dissolved with sodium hydroxide before addition of ^{3}H-amino acids served as a blank.

Ferritin-Toxin Conjugates

RCA_{II} was radioiodinated with iodine^{125} as described previously [2]. ^{125}I-RCA_{II} was conjugated to ferritin (immunodiagnostics) and then affinity-purified according to Nicolson, Smith, and Hyman [17]. The ferritin-^{125}I-RCA_{II} obtained was essentially indistinguishable from unconjugated ^{125}I-RCA_{II} in biological assays (growth inhibition) and quantitative cell-binding studies [17].

Cells were labeled with ferritin-^{125}I-RCA_{II} by the following procedures [17]. BW5147 or BW5147Ric^R.3 cells were incubated for 10 min at 22°C with the label in DMEM plus 0.1% bovine serum albumin (BSA). The cells were then washed twice in DMEM plus 0.1% BSA and either fixed in phosphate-buffered 1% glutaraldehyde for 5 min at 22°C or incubated in DMEM plus 5% horse serum for an additional 60 min at 37°C. At the end of the incubation, cells were fixed in phosphate-buffered glutaraldehyde for 5 min at 22°C and washed in 0.1 M cacodylate buffer prior to postfixation in 1% osmium tetroxide in 0.1 M cacodylate for 1 h at 22°C. The cells were then centrifuged onto soft agar and the hardened pellets treated as tissue blocks. The blocks were processed for embedding in Spurr resin (Electron Microscopy Sciences). Thin sections were examined unstained on a Hitachi Model HU-12 (Perkin Elmer) at 75 kV.

Encapsulation of Toxin Within Lipid Vesicles and Incubation With Cells

Unilamellar vesicles were prepared from chromatographically pure bovine brain phosphatidylserine (Applied Science Labs) as described previously [19]. Phosphatidylserine (10 μmoles/ml) was suspended in Ca^{2+}- and Mg^{2+}-free PBS by vortex mixing at room temperature and then sonicated in a closed tube under nitrogen for 1 h at 25°C in a bath-type sonicator (Model T-80-IRS; Laboratory Supplies Co). After sonication, Ca^{2+} (100 mM $CaCl_2$) was added and the mixture was incubated at 31°C for 1 h, after which the resulting precipitate was pelleted by centrifugation at 3,000 g for 10 min. The wet pellet was then mixed with 1 ml of Ca^{2+}-Mg^{2+}-free DMEM containing a known concentration of ^{125}I-RCA_{II}. The lipid pellet was suspended in this solution by vortex mixing for 10 min at room temperature, after which 100 ml ethylendiaminetetraacetic acid (EDTA) (0.1 M) and 50 μl 0.1 NaOH (0.1 M) were added, followed by incubation at 37°C for 10 min. The vesicles formed by this procedure were then pelleted by centrifugation at 45,000 g for 30 min at 20°C. Control vesicles without encapsulated toxin (referred to as "empty" vesicles) were produced by the same method in Ca^{2+}-Mg^{2+}-

free DMEM without toxin. Aliquots of the pellet and the supernatant were taken for radioactivity measurements to determine the amount of ^{125}I-RCA$_{II}$ encapsulated in the vesicles. For the preparations used in the present experiments, 6–12% of the original ^{125}I-RCA$_{II}$ was captured within vesicles. The vesicle pellet was suspended in DMEM containing 0.5 mM $CaCl_2$ without serum and added to cell cultures to give a final toxin concentration of 2 μg/ml. Vesicles containing encapsulated toxin were devoid of cell agglutination activity indicating that lectin molecules with exposed reactive sites were not bound to the outer surface of the vesicles. The rate of leakage of ^{125}I-toxin from vesicles was approximately 2% per h at 31°C. However, to avoid any possible biological effects from toxin leaking from vesicles, cells were incubated with toxin-containing vesicles in the presence of 50 mM lactose to neutralize any released toxin molecules. However, in control experiments in which cells were incubated with empty vesicles together with free toxin, lactose was omitted.

RESULTS

The biochemical and biological activities of ferritin-^{125}I-RCA$_{II}$ preparations were examined by quantitative measurements of binding to cells and inhibition of cell growth and protein synthesis. In each case ferritin-^{125}I-RCA$_{II}$ was compared with RCA$_{II}$ and ^{125}I-RCA$_{II}$ with respect to its ability to interact with BW5147 and BW5147Ric^R.3 cells and affect cell protein synthesis. Ferritin-^{125}I-RCA$_{II}$ and RCA$_{II}$ had equivalent effects on the growth characteristics of parental or resistant lines both when present continuously in the cell culture for 24–72 h and when present for 10 min and then removed from the cell cultures. These effects were completely abrogated by inclusion of lactose or D-galactose in the medium [6, 17]. At low toxin concentrations, binding of ferritin-^{125}I-RCA$_{II}$ was similar to, but generally slightly lower than, that of ^{125}I-RCA$_{II}$, but there was essentially no difference in the quantitative binding of the two labels to BW5147 or BW5147Ric^R.3 cells during 10-min incubations (Table I). When the kinetics of inhibition

TABLE I. Binding of Ferritin-^{125}I-RCA$_{II}$ to BW5147 and BW5147Ric^R.3 Cells

Cell line	Concentration ferritin-^{125}I-RCA$_{II}$[a]	Lactose (100 mM)	CPM bound per 10^6 cells[b]
BW5147	1.0	–	1,800 ± 200
	3.0	–	5,100 ± 400
	7.0	–	10,600 ± 500
	10	–	16,500 ± 900
	10	+	1,100 ± 400
BW5147Ric^R.3	1.0	–	1,700 ± 200
	3.0	–	5,600 ± 400
	7.0	–	11,100 ± 500
	10	–	17,300 ± 800
	10	+	1,500 ± 300

[a] μg/ml of ^{125}I-RCA$_{II}$ in ferritin conjugate.

[b] 2×10^6 cells were labeled with ferritin-^{125}I-RCA$_{II}$ in DMEM + 1% BSA for 10 min at 22°C and washed twice with PBS + 0.1% BSA.

of cell protein synthesis by RCA_{II} or ferritin-^{125}I-RCA_{II} at 3 μg/ml toxin (pulsed for 10 min at 22°C and subsequently incubated at 37°C) were examined in parental BW5147 and resistant BW5147Ric^R.3 cells, only the sensitive BW5147 cells were affected (Fig 1). Ferritin-^{125}I-RCA_{II} or RCA_{II} produced a marked inhibition (> 80%) of cell protein synthesis after an initial lag of approximately 45–60 min (Fig 1). Higher toxin concentrations reduced slightly, but did not abolish, this initial lag in inhibition of cell protein synthesis.

In order to determine the location of toxin molecules during the lag period, cells were examined ultrastructurally after a 10-min pulse with ferritin-^{125}I-RCA_{II} at 22°C and subsequent incubation at 37°C. When BW5147 and BW5147Ric^R.3 cells were labeled with ferritin-^{125}I-RCA_{II} (3 μg/ml toxin) for 10 min and then fixed with glutaraldehyde, approximately equal numbers of ferritin-toxin molecules bound specifically to either cell type (Table I). On these cells the ferritin-^{125}I-RCA_{II} molecules were found at the cell surfaces in a more or less dispersed distribution, and inclusion of lactose in the labeling medium prevented binding of label to the cells (Table I). When sensitive BW5147 cells were pulsed for 10 min at 22°C with ferritin-^{125}I-RCA_{II} (1–3 μg/ml toxin) and then washed and incubated for 60 min at 37°C before fixation, the ferritin-toxin molecules did not remain at the cell periphery, and many ferritin-toxin molecules were transported into the cell by endocytosis (Fig 2). Eventually some of the endocytotic vesicles apparently break down, allowing the toxin access to the cytoplasm [6]. In contrast, in BW5147Ric^R.3 cells most of the ferritin-^{125}I-RCA_{II} molecules remained at the cell surface after the 60-min pulse-incubation, and internalization via endocytosis was not observed (Fig 3). However, at higher toxin concentrations where both the parental and resistant variant cells are sensitive to toxin effects, RCA_{II}-mediated endocytosis occurs in both cell types [17].

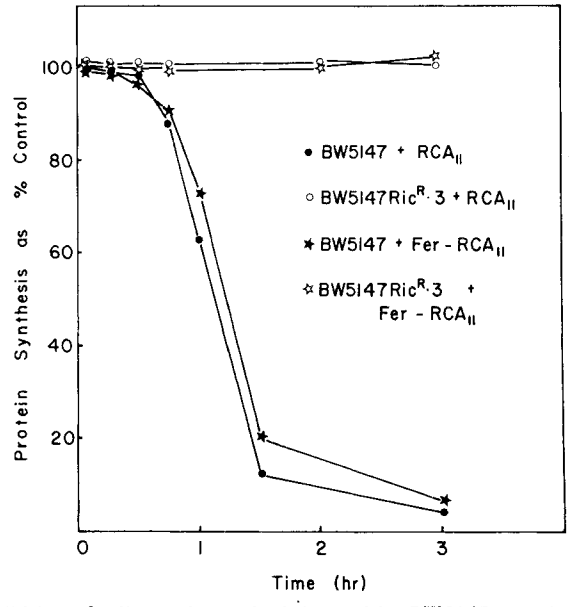

Fig 1. Kinetics of inhibition of cell protein synthesis in sensitive BW5147 or resistant BW5147Ric^R.3 cells by 3 μg/ml RCA_{II} or ferritin-^{125}I-RCA_{II} (3 μg/ml RCA_{II}).

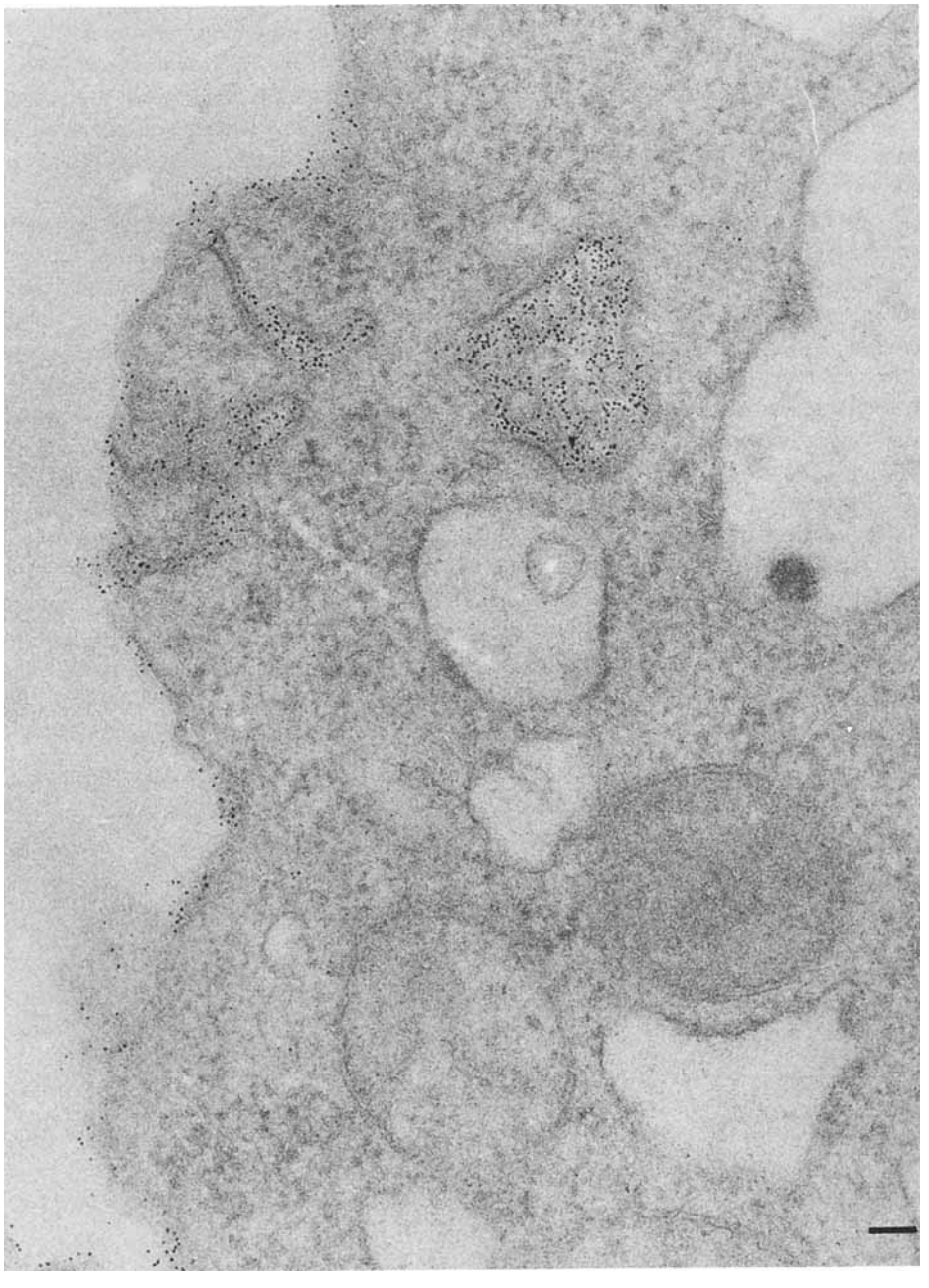

Fig 2. Sensitive BW5147 cells labeled with ferritin-^{125}I-RCA$_{II}$ (3 μg/ml RCA$_{II}$) for 10 min at 22°C, washed, and incubated for 60 min at 37°C prior to fixation. Note extensive internalization of ferritin-toxin molecules; bar equals 0.1 μm (× 65,000).

The necessity of toxin transport into cells can be bypassed using newly developed techniques employing lipid vesicles that are able to fuse with the plasma membrane releasing material(s) encapsulated within the vesicles directly into the cytoplasm [20, 21]. Direct introduction of RCA$_{II}$ into cells using lipid vesicles as carriers should thus circum-

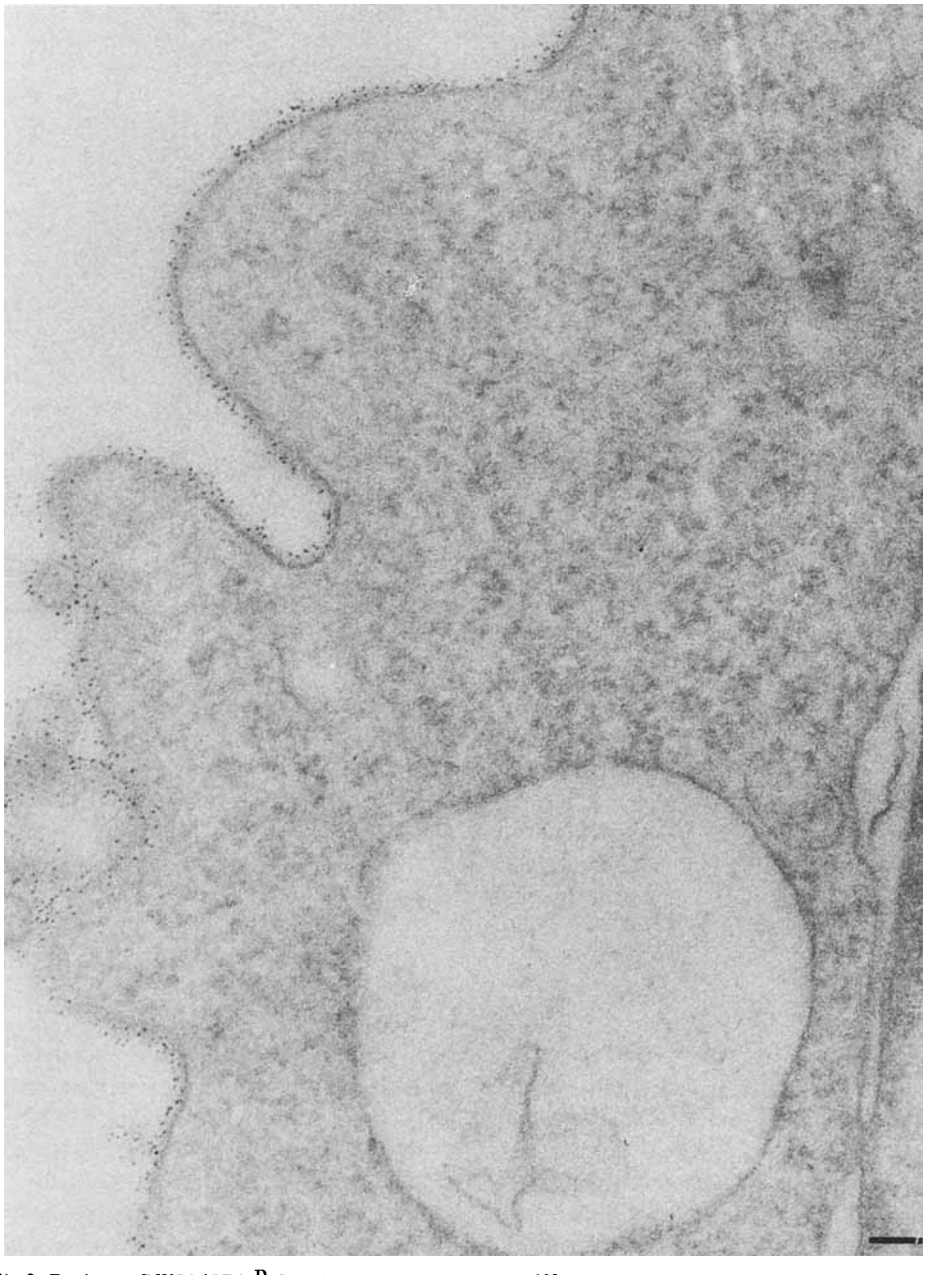

Fig 3. Resistant BW5147Ric^{R}.3 cells labeled with ferritin-^{125}I-RCA$_{II}$. Details as in Figure 2.

vent the normal mode of toxin transport and decrease the lag time for inhibition of protein synthesis.

Incubation of parental BW5147 cells with vesicles containing encapsulated RCA$_{II}$ (2 μg/ml) produced rapid inhibition of cellular protein synthesis (Fig 4). Inhibition of protein synthesis under these conditions was significantly faster than that seen in repli-

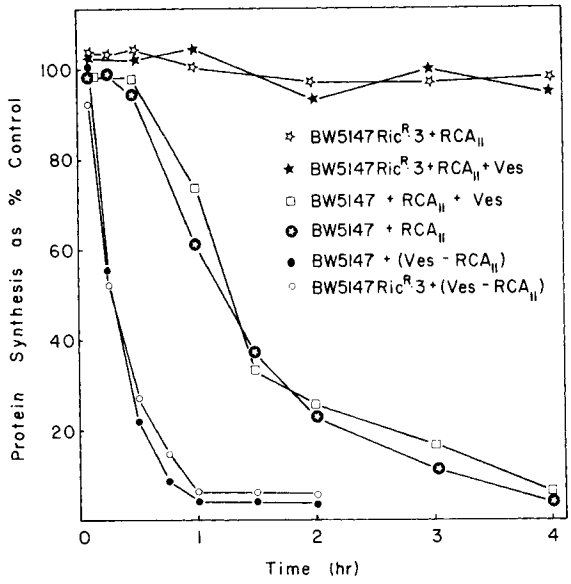

Fig 4. Kinetics of inhibition of cell protein synthesis in sensitive BW5147 or resistant BW5147Ric^R.3 cells by RCA_{II} (2 μg/ml), lipid vesicle-encapsulated RCA_{II} (2 μg/ml) (Ves-RCA_{II}) or empty lipid vesicles together with free RCA_{II} (2 μg/ml) (RCA_{II}+Ves).

cate cell populations exposed to the same concentration of free toxin (Fig 4) or a combination of empty vesicles together with free toxin (Fig 4), or empty vesicles alone (not shown). RCA_{II} encapsulated within lipid vesicles also caused rapid inhibition of protein synthesis in resistant BW5147Ric^R.3 cells, but similar concentrations of free toxin, either alone or added together with empty lipid vesicles, had no effect (Fig 4). Possible effects due to leakage of encapsulated RCA_{II} out of vesicles were excluded by adding excess lactose to the incubation medium during incubation of cells with vesicles.

DISCUSSION

Ricin (RCA_{II}) must enter cells to inhibit cell protein synthesis. This toxin binds rapidly to a number of susceptible cells [23] through cell surface oligosaccharides containing terminal D-gal or D-galNAc residues [2, 4, 6, 22, 23]. Toxin binding can be blocked or reversed by D-gal or lactose [1, 2, 4–6, 14, 17, 24]. Once bound, the toxin can be removed by excess competitive saccharide without subsequent cellular cytotoxicity, but only if the toxin molecules are removed within minutes after initial interaction with cell surface receptors. Although toxin binds rapidly to susceptible cells, a lag time of 30–60 min occurs before the onset of inhibition of protein synthesis [4, 6]. This lag time corresponds closely with the time required for toxin-stimulated endocytosis and subsequent rupture of some of the endocytotic vesicles with release of toxin molecules into the cytoplasm [5, 6]. Cells which do not undergo endocytosis at significant rates are not easily inhibited by ricin, even though the toxin binds quickly to cell surfaces. For example, Refsnes et al [4] found that protein synthesis in reticulocytes was not inhibited by ricin for at least four hours after binding to the cell surface.

Compared to cell entry, more is known about the action of ricin and similar toxins on cytoplasmic protein synthetic processes. The toxin catalytically and irreversibly

inactivates ribosomes involved in ribosome-dependent protein synthesis [8, 25–27]. The 60S ribosomal subunit is the target for the toxin [28, 29], which somehow interferes with the GTPase site on the ribosomes. The binding site for EF-2 on ribosomes appears to be identical to or cross-reacts with the target for the toxin, since prebound EF-2 protects ribosomes from inactivation by toxin, and toxin-treated ribosomes show a decreased ability to bind EF-2 [25, 30].

In the present study we have investigated the sequence of events that lead to inhibition of protein synthesis and the kinetics of inactivation of protein synthesis using a toxin-sensitive lymphoma line and a selected variant line that is much more resistant to killing by the toxin. The RCA_{II} toxin-sensitive BW5147 lymphoma line and its toxin-resistant variant were compared for RCA_{II} binding, transport, and cytotoxicity using a biologically active ferritin-^{125}I-RCA_{II} probe [17]. The details concerning this probe and its interaction with sensitive and resistant lymphoma cell lines are presented in detail elsewhere [17]. We found that although similar amounts of ferritin-^{125}I-RCA_{II} were bound to parental and BW5147Ric^R.3 cells at low toxin concentrations (3 μg/ml) where differences in susceptibility to cytotoxicity were manifest, significant internalization of ferritin-toxin molecules by endocytosis occurred only in the sensitive parental cells. In contrast, at high toxin concentrations (10 μg/ml RCA_{II}), where both parental and Ric^R.3 cells are sensitive to the toxin, ferritin-^{125}I-RCA_{II} molecules were bound and transported into both cell lines [17]. This difference in toxin transport at low toxin concentrations was not due to a generalized defect in cellular endocytosis, because both cell lines are capable of transporting low concentrations of other materials such as ferritin-Con A (3–10 μg/ml) into cells by endocytosis [17]. The time required for significant endocytosis of ferritin-^{125}I-RCA_{II} and possible escape of ferritin-toxin molecules after breakdown of some of the endocytotic vesicles could explain the 30–60 min lag time required before the onset of inhibition of protein synthesis. Support for this interpretation is also provided by the present result showing that direct transfer of RCA_{II} into the cytoplasm using lipid vesicles as carriers eliminates the normal lag time in toxin-sensitive BW5147 cells and renders resistant BW5147Ric^R.3 cells equally sensitive to the toxin. Presumably the lipid vesicles fuse with the plasma membrane [21, 31, 32], releasing the encapsulated toxin directly into the cytoplasm, where it can gain immediate access to the ribosome "target" (Fig 5). Direct access of vesicle-delivered toxin to the protein synthetic machinery in the cytoplasm is also suggested by the finding that the kinetics of inhibition of protein synthesis by vesicle-derived toxin in both sensitive and resistant cells closely resembles that obtained in studies with cell-free preparations from these cells in which toxin is incubated directly with ribosomes [18]. The present results showing that *intact* toxin molecules encapsulated in lipid vesicles cause rapid and marked inhibition of protein synthesis are also difficult to reconcile with proposals that toxin activity requires separation of cell surface-bound toxin into its constituent subunits before transport into the cell [33].

The use of lipid vesicles as a carrier vehicle for bypassing normal cellular transport mechanisms offers a new and potentially valuable method for studying the biological activity of plant and bacterial toxins. A related approach has also been used recently to study diphtheria toxin activity. Fusion of reconstituted paramyxovirus envelopes [34] containing CRM45 (a mutant form of diphtheria toxin which is nontoxic because it lacks the C-terminal sequence of amino acids needed to bind to the cell surface) with mammalian cells in vitro was found to cause significant cytotoxicity and inhibition of cellular protein synthesis. Since CRM45 has a molecular weight of 45,000 and will yield the intact A subunit (MW 24,000) after proteolysis [35], the finding that the entire CRM45

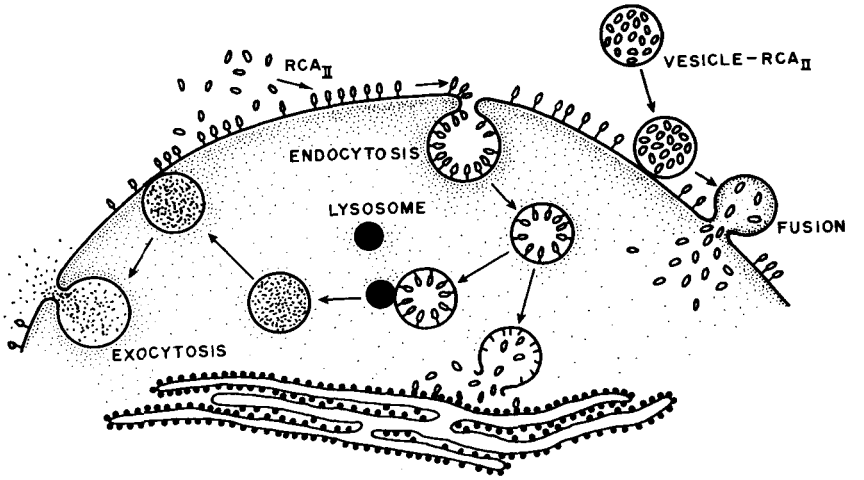

Fig 5. Route of cytoplasmic entry of RCA$_{II}$ into toxin-sensitive cells by RCA$_{II}$ binding, endocytosis, and rupture of some of the endocytotic vesicles. Other endocytotic vesicles containing RCA$_{II}$ may fuse with lysosomes and subsequently excrete degraded toxin. Lipid vesicle-encapsulated RCA$_{II}$ bypasses the normal entry route by fusion of the vesicles with the plasma membrane, releasing the encapsulated toxin directly into the cell cytoplasm.

protein can inhibit protein synthesis when introduced directly into the cytoplasm provides further evidence that splitting of toxins into their subunits at the cell surface is not essential for expression of biological activity in intact cells.

The reason why the present ricin-resistant variants fail to internalize toxin at low concentrations is not known. However, recent studies using lactoperoxidase surface-labeling techniques have shown that two exposed proteins of approximately 80,000 and 35,000 mol wt are present on sensitive wild-type parent cells, but are largely missing or not accessible to iodination on BW5147Ric^R.3 resistant cells [14]. The 80,000 mol wt component has been identified as a binding site for RCA$_{II}$ in experiments in which detergent-solubilized plasma membrane components from BW5147 and BW5147Ric^R.3 cells were purified by affinity chromatography [14]. BW5147Ric^R.3 cells do not possess the 80,000 mol wt glycoprotein but instead appear to have a component of lower molecular weight ($\sim$70,000) which can be precipitated from NP-40 detergent cell extracts by rabbit antisera against BW5147 cells [14]. It was proposed [14] that this 80,000 mol wt glycoprotein-binding site for RCA$_{II}$ might be the productive receptor responsible for toxin transport at low RCA$_{II}$ concentrations and that the altered 70K form in BW5147Ric^R.3 plasma membranes is probably incapable of mediating endocytotic transport at low toxin concentrations. Although direct evidence for this proposal is lacking, the data obtained thus far are at least consistent with the idea that a specialized 80K surface receptor for toxin is responsible for communicating or mediating transmembrane signaling to the cytoplasm [36, 37].

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