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Ricinus Communis Toxin-Mediated Inhibition of Protein Synthesis in Cell-Free Extracts of a Toxin-Resistant Variant Mouse Lymphoma Cell Line

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Ricinus communis agglutinin II (RCA_{II}, ricin, toxin) at low concentrations inhibits protein synthesis in cell-free extracts, but not in intact cells, of an RCA_{II}-resistant mouse lymphoma variant cell line. The concentration dependence of the inhibition by RCA_{II} was the same in cell-free extracts of both RCA_{II}-resistant variant and RCA_{II}-sensitive parental cells, while intact parental cells are 250 times more sensitive to RCA_{II} toxicity. The onset of RCA_{II} inhibition of cell-free protein synthesis was extremely rapid in both cases, being complete in a few minutes. Under these conditions RCA_{II} inhibits protein synthesis in intact RCA_{II}-sensitive parental cells, but maximal inhibition requires several hours to occur. These results support our previous electron microscopic observations that the variant cells are defective in the uptake of RCA_{II} by endocytosis at low toxin concentrations.

Key words: mutant RCA_{II}, ricin, toxin, protein synthesis, variant cell line

INTRODUCTION

RCA_{II} (ricin, toxin), a toxic lectin isolated from Ricinus communis (castor bean) (1, 2) kills human and mouse fibroblasts in vitro by a) binding to the cell surface, b) entering cells by endocytosis, and c) irreversibly inhibiting cytoplasmic protein synthesis (3–5). Several mouse (6, 7) and hamster (8–14) cell variants resistant to killing by RCA_{II} or other lectins have been isolated, aiding in the study of the mechanisms of toxin-mediated cell killing. We have selected and isolated an RCA_{II}-resistant variant (BW5147 Ric^{R}·3) of the mouse lymphoma cell line BW5147 that binds 60–70% as much RCA_{II} as does the parental cell line, but is up to 250 times more resistant to cell killing by RCA_{II} (7). Electron microscopic observation of cells labeled with ferritin-conjugated RCA_{II} showed that the variant resistant cells fail to endocytose the toxin at low RCA_{II} concentrations (15). The present report demonstrates that RCA_{II} at low concentrations inhibits protein synthesis in intact cells only of the parental line, and only after a delay of over 1 hr. However, RCA_{II} swiftly and equally inhibits protein synthesis in cell-free extracts of both resistant and sensitive lines. These results confirm that the RCA_{II}-resistant variant cells are defective in the uptake of the toxin.

METHODS

Materials

The BW5147 cell line is a spontaneous AKR/J mouse lymphoma that has been adapted to tissue culture (16, 17). A thioguanine-resistant subclone, BW5147·G·1, was used in these experiments. The RCA_{II}-resistant variant, BW5147 Ric^R·3, was isolated by repeated growth of cells in toxic levels of RCA_{II} as described elsewhere (7). 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 as described in Nicolson et al. (2). Radioisotopes were obtained from New England Nuclear.

Whole-Cell Protein Synthesis

Cells were suspended at 1×10^6/ml in DMEM plus 10% serum and separated into control and experimental samples. RCA_{II} (0.8 μg/ml) was added to the experimental cells at zero time, and duplicate 1.0 ml aliquots of cells dispensed to centrifuge tubes. The tubes were kept at 37°C in a tissue culture incubator, with occasional swirling. At desired times the tubes were swirled to suspend the cells, 50 μl portions were removed for cell counting, and 10 μCi of ^{3}H-amino acid mixture was added to each tube. After 1 hr at 37°C the cells were pelleted by centrifugation, dissolved in 1 N NaOH containing 1% casein hydrolysate, and incubated 10 min at 37°C to hydrolyze aminoacyl-t-RNA. Protein was precipitated with 5 ml of cold 10% TCA and aged 1 hr at 4°C, and the precipitate was collected on Whatman GF/C filters. Each filter was washed with cold 5% TCA and with 95% ethanol and dried, and the radioactivity was 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.

Cell-Free Protein Synthesis

S30 cell-free extracts were prepared from both BW5147 and BW5147 Ric^R·3 cells essentially as described by Matthews and Korner (18) except that the preincubation and gel filtration steps were omitted. Approximately 10^8 cells were used in each case, which made about 0.3 ml S30. Such nonpreincubated S30s are active in protein synthesis utilizing endogenous mRNAs. Sixty μl reactions containing 45 μl of S30 were incubated at 32°C, containing the following final concentrations: 5mM $MgCl_2$, 100 mM KCl, 30 mM Tris-Cl pH 7.5, 1 mM ATP, 0.1 mM GTP, 10 mM creatine phosphate, 50 μg/ml creatine phosphokinase, 1mM dithiothreitol, 50 mM each of 19 unlabeled amino acids (omitting methionine), 100 μCi/ml ^{35}S- methionine (273 Ci/mmole) and the desired concentrations of RCA_{II}. Samples (12 μl) were withdrawn for estimation of alkali-stable acid-precipitable radioactivity as described in Nicolson et al. (5).

RESULTS

Protein Synthesis by Intact Cells

Whole cells were incubated with or without RCA_{II} (0.8 μg/ml), ^{3}H-amino acids were added, and protein synthesis was measured over the next hour. The results are shown in Fig. 1. RCA_{II} did not inhibit protein synthesis in the RCA_{II}-resistant BW5147 Ric^R·3 cells. After a 1 hr incubation with RCA_{II}, protein synthesis in sensitive BW5147 cells during the next hr (still with RCA_{II}) was inhibited by 32%, with complete inhibition

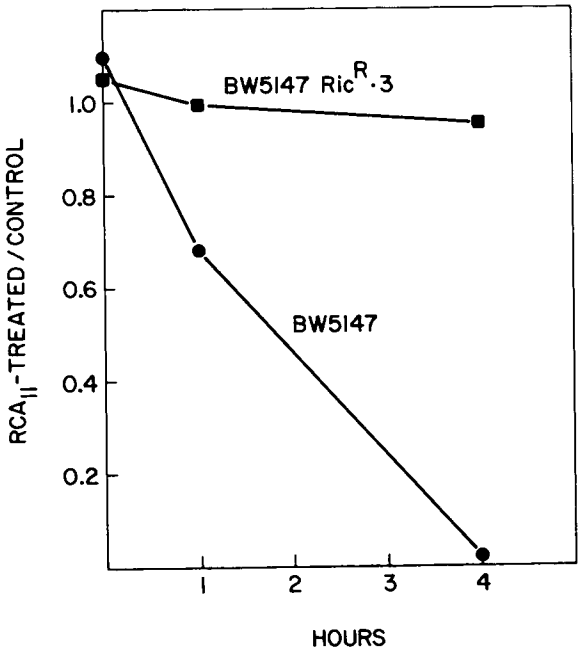

Fig. 1. Inhibition of whole-cell protein synthesis by RCA_{II}. The ratios of protein synthesis by cells incubated with and without RCA_{II} (0.8 μg/ml) are plotted against the length of the incubation. Control values at zero time were 21,699 cpm for BW5147 and 15,961 cpm for BW5147 Ric^R·3.

obtained after 4 hr. Both cell lines retained > 98% viability throughout this 4 hr incubation with RCA_{II}, as judged by trypan blue exclusion. Continuation of the incubation for 24 hr gave ∿ 1% viability for BW5147 cells and ∿ 70% for BW5147 Ric^R·3.

Protein Synthesis by Cell-Free Extracts

RCA_{II} rapidly inhibits protein synthesis in S30 cell-free extracts of both BW5147 and BW5147 Ric^R·3 cells. The time course and concentration dependence of this inhibition are the same for both parental and variant cells. RCA_{II} caused 20% inhibition at 1 μg/ml and 50–70% inhibition at 10 μg/ml in only 5 min (not shown). The 15 min data (Fig. 2) show approximately 20% inhibition of both BW5147 and BW5147 Ric^R·3 cell-free protein synthesis by 0.1 μg/ml RCA_{II}, 60–70% inhibition by 1.0 μg/ml, and 85% by 10 μg/ml.

Prevention of RCA_{II} Endocytosis

RCA_{II}-sensitive BW5147 cells were incubated for 2 hr with or without RCA_{II} (0.5 μg/ml). RCA_{II} remaining free in solution or bound to cell surfaces was removed by rinsing the cells with the hapten inhibitor *D*-galactose, and cells were incubated overnight in growth medium. As Table I shows, cells that had been incubated with RCA_{II} at 37° had taken up enough of the toxin in 2 hr for 56% of the cells to be killed. Chilling the cells during RCA_{II} exposure – which reduces endocytosis of the lectin but not binding – largely protected the cells against killing by RCA_{II}. The presence of *D*-galactose – which inhibits cellular binding by the lectin – during the RCA_{II} incubation also largely protected the cells against killing.

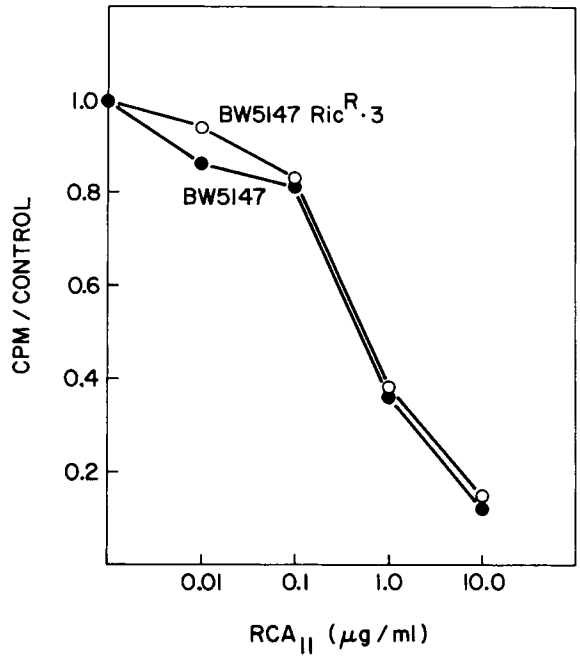

Fig. 2. Inhibition of cell-free protein synthesis by RCA_{II}. Protein synthesis in the presence of RCA_{II} as a fraction of the control without RCA_{II} is plotted against RCA_{II} concentration. Values shown are 15 min. Control values were 83,511 cpm/mg protein for BW5147 and 48,797 for BW5147 Ric^{R}·3.

TABLE I. Killing of BW5147 Parental Cells by RCA_{II}*

Incubation	Live cells/ml × 10^{-6}		With/without RCA_{II}
	Control	With RCA_{II}	
37°C	6.27	2.74	0.44
4°C	6.07	5.31	0.87
37°C, 0.1 M D-gal	6.50	5.35	0.82

*Cells were incubated as shown in DMEM with 10% horse serum for 2 hr with and without 0.5 μg/ml RCA_{II}. D-galactose was added to 0.1 M, and the cells were chilled, centrifuged, rinsed twice with DMEM with 10% horse serum and 0.1 M D-galactose, and resuspended in 3 ml of this medium. After incubation overnight at 37°, viable cells (those excluding trypan blue) were counted.

DISCUSSION

We and others have demonstrated that RCA_{II} probably kills cells by irreversibly inhibiting protein synthesis after entering cells by endocytosis (3–5). Some virally transformed cells are more sensitive to RCA_{II} toxicity than are the corresponding untransformed cells, although the amount of RCA_{II} binding to the two cell types is quite similar (5). In this case the greater sensitivity of transformed cells was thought to be due to greater endocytosis of RCA_{II} into these cells at low toxin concentrations, at which the toxin remains bound to the surfaces of untransformed cells. Several laboratories have selected somatic cell

mutants that are more resistant to the toxicity of RCA_{II} or other lectins (see below). The toxin resistance of some of these variants seems to be due to large decreases in toxin binding compared to parental cells. Other variants do not have deficiencies in toxin binding, however, and their mechanisms of resistance have not been demonstrated.

In this report we compare the effects of RCA_{II} on protein synthesis of an RCA_{II}-resistant lymphoma variant and its RCA_{II}-sensitive parental cell line. The lymphoma variant (BW5147 $Ric^R \cdot 3$) is much more resistant to RCA_{II} toxicity than is the parental (BW5147) cell line: parental cells are completely killed after a 1 hr exposure to 0.01 μg/ml RCA_{II} in the absence of serum, while variant cells are only partially killed at 2.5 μg/ml (7). A variant that is resistant to RCA_{II} toxicity could be altered in any of the three steps of toxicity: cell-surface binding, endocytosis, or protein synthesis. The binding of RCA_{II} by the lymphoma variant BW5147 $Ric^R \cdot 3$ is only 30–40% less than that of the parent cell line at RCA_{II} concentrations of 20 μg/ml and above (7), and seems to be equivalent to parental binding at the lower concentrations where differential toxicity is maximal (19). The difference in binding explains the variant cells' resistance to RCA_{II} toxicity only if the relatively few binding sites that are lost are critical for the toxin action, possibly for the endocytosis of bound toxin. Reduced entry of RCA_{II} into the variant cells was shown by electron microscopic observations of cells incubated with ferritin-conjugated RCA_{II} (15, 19). While both parental and variant cells bound the ferritin-RCA_{II} at the cell surface, only the parental, sensitive cells endocytosed substantial toxin within 1 hr at 37°C.

This report supports the proposal that entry of RCA_{II} into the variant cells must be defective, since the cytoplasmic protein synthetic machinery of the variant cells has not mutated to RCA_{II}-resistance. Figure 2 shows that cell-free lysates of both sensitive and resistant cells are equally sensitive to RCA_{II} inhibition of protein synthesis. Since protein synthesis in intact BW5147 $Ric^R \cdot 3$ cells is not affected by RCA_{II} concentrations that strongly inhibit BW5147 cells (Fig. 1), it seems likely that RCA_{II} does not enter the resistant cells at these concentrations. Our electron-microscopic work with ferritin-RCA_{II} (15, 19) argues strongly against the possibilities that RCA_{II} enters the cells but is either stored indefinitely in endocytotic vesicles or is quickly degraded by lysosomal enzymes.

In addition, the kinetics of inhibition of parental cell protein synthesis support the idea that RCA_{II} enters cells by endocytosis. While protein synthesis in cell-free lysates is inhibited by RCA_{II} in a matter of minutes (within 5 min; see Results), this inhibition takes hours in whole cells (Fig. 1). BW5147 cells took up enough RCA_{II} in 2 hr (in DMEM with 10% serum and 0.5 μg/ml RCA_{II}) so that 56% of the cells subsequently died even when RCA_{II} remaining on the cell surface was removed by the hapten inhibitor *D*-galactose (Table I). This killing was substantially decreased by reducing either the binding of the RCA_{II} (*D*-galactose present as hapten inhibitor) or its endocytosis (incubation at 4°C). Preliminary attempts to block the endocytosis of RCA_{II} with colchicine plus cytochalasin B or with dinitrophenol (not shown) have not yet been successful – probably because RCA_{II} is so extremely toxic that the slight residual endocytosis that occurs in the presence of these drugs allows entry of a toxic amount of RCA_{II} into the sensitive cells.

Several laboratories have isolated somatic cell variants resistant to RCA_{II} or other lectins (6–14). Several of these variants lack most ($\geq$ 90%) of the surface binding sites for the selecting lectin and sometimes other lectins, because they lack major glycosyltransferase activities. Many surface glycoproteins on these cells are substantially deficient in glycosylation (9, 10, 20, 21). Other lectin-resistant variants have smaller decreases in lectin binding; mechanisms of resistance and surface changes on most of these variants have not yet been described, with the exception of BW5147 $Ric^R \cdot 3$. Most surface gly-

coproteins of BW5147 $Ric^R \cdot 3$ appear quite similar to those of parental cells, but a major RCA_{II}-binding glycoprotein is changed from a form with apparent molecular weight 80,000 daltons to one of 70,000 (7). We suggest that this glycoprotein may be important for endocytosis of RCA_{II} into parental cells but cannot serve this function effectively in the variant cells.

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