Cell Lysis Induced by Ricin D and Ricin E in Various Cell Lines

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Ricin, one of two isolectins from small castor beans showed stronger cytotoxicity than another one, ricin E, based on the inhibition of colony formation and the inhibition of protein synthesis. Both ricin D and ricin E induced cell lysis to different extents in each cell line tested, albeit ricin E was slightly less effective than ricin D. DNA fragmentation, a characteristic feature of apoptosis, was also induced by ricin D and ricin E in Vero cells. Scatchard plot analysis showed that ricin D binds to cells with higher affinity than ricin E, while the number of binding sites per cell was not much different, suggesting that the differences in the cytotoxicity between ricin D and ricin E is mainly due to their differential binding affinity to cells. In Vero cells, the cytolytic activities of ricin D and ricin E were inhibited by brefeldin A (BFA), which is known to affect the Golgi apparatus, but no significant effect of BFA was observed in a BFA-resistant cell line, MDCK cells. These results suggest that the Golgi apparatus may be involved in ricin-induced cell lysis.

Key words: ricin D; ricin E; cytolysis; apoptosis; brefeldin A

Ricin, an extremely toxic lectin present in castor beans (Ricinus communis), is composed of two disulfide-linked subunits. The A chain is an enzyme that inactivates the 60S ribosomal subunit by catalyzing the N-glycosidic cleavage of a specific adenine residue from 28S ribosomal RNA, and the B chain binds to cell surface receptors containing galactose or N-acetylgalactosamine residues.1−4) The intoxication pathway of ricin consists of (i) binding to cell-surface receptors, (ii) receptor-mediated endocytosis and intracellular transport through the vesicular system, (iii) translocation of enzymatically active A chain across the vesicle membrane to the cytosol, and (iv) enzymatic inactivation of ribosomes. In addition to the ability of ricin to inhibit protein synthesis, recent studies have demonstrated that ricin can also lyse target cells and induce DNA fragmentation in a process reminiscent of programmed cell death or apoptosis.5,6) Other protein toxins such as diphtheria toxin and Pseudomonas toxin have also been reported to induce target cells lysis.7,8) Although the mechanism of cell lysis by these protein toxins is still unclear, ricin-induced apoptotic changes have been reported to be prevented by cycloheximide and 3-methyldadenine, a specific inhibitor of autophagy.9) Thus autophagy may be important for ricin-induced cell lysis.

It has been known that there are two isofoms of ricin. The large grain castor beans grown in Thailand contain two types of lectins, ricin D and relatively non-toxic castor bean hemagglutinin (CBH).9,10) The small grain castor beans grown in Japan contain another lectin, ricin E, in addition to ricin D and CBH.11) Although the molecular weight, toxicity to mice, and cytotoxicity to protein of ricin E are almost equal to those of ricin D, the two ricins are different in isoelectric point, affinity for Sepharose 4B, and the growth inhibition toward certain cultured cells.11,12) These differences between ricin D and ricin E have been thought to be due to the differences in the saccharide-binding properties of the B chain, since they have identical A-chain.13) Equilibrium dialysis studies showed that ricin has two galactose-binding sites, a high affinity site (HA-site) and a low affinity site (LA-site), and saccharide-binding ability of the HA-site of ricin E is less than one-half that of ricin D, but no significant difference is observed in their LA-site.14) In this study, we investigated the cytotoxicity of ricin D and ricin E based on the inhibition of colony formation and the inhibition of protein synthesis, and their cytolytic activity in various cell lines. To study the mechanism of ricin-induced cell lysis, we tested the effects of brefeldin A and cycloheximide, which have been reported to affect ricin cytotoxicity.5,15,16) To elucidate the relationship between the differences in B-chain of two ricins and their cytotoxicity, we also examined the cell-surface binding and subsequent internalization of ricin D and ricin E to several cell lines by using FITC-labeled ricins.

Materials and Methods

Materials. Ricin and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chem. Co. (St. Louis, MO). Small castor beans (R. communis) were the generous gift of Dr. T. Hatakeyama and Dr. M. Kimura (Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University). Ricin D and ricin E were isolated from small castor beans as described by Mise et al.11) Brefeldin A (BFA) was obtained from Epicentre Technologies (Madison, WI). [3H]Leucine (60Ci/mmol) was obtained from Du Pont—New England Nuclear Research Products.

FITC labeling of ricin. Fluorescein isothiocyanate (FITC)-labeled ricin D (F-ricin D) and ricin E (F-ricin E) were prepared by essentially the same method as described previously.17) In brief, 2 mg of FITC was added to the 1 ml of 0.5 M sodium bicarbonate buffer, pH 8.3, containing 10 mg of ricin and 0.1 M lactose. After stirring for 4 h at 4°C, the reaction mixture was applied to a column (1.5×10 cm) of Sephadex G-25 previously equilibrated with phosphate-buffered saline (PBS), followed by dialysis against PBS. Both F-ricin D and F-ricin E had original hemagglutinating activity toward human erythrocytes (O-type).

Cell culture. Vero (African green monkey kidney), MDCK (Madin-Darby Canine kidney), XC (Rat sarcoma), CHO (Chinese hamster—ovary),

Abbreviations: BFA, brefeldin A; CBH, castor bean hemagglutinin; FBS, fetal bovine serum; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; F-ricin, FITC-labeled ricin; HA-site, a high affinity saccharide-binding site; LA-site, a low affinity saccharide-binding site; MEM, minimal essential medium; PBS, phosphate-buffered saline; LD50, 50% colony formation inhibiting dose; ID50, 50% protein synthesis inhibiting dose.
HeLa (Human epithelial carcinoma), and PtK₁ (Potoroo rat kangaroo kidney) cells were cultured in z-minimal essential medium (z-MEM) supplemented with 10% fetal bovine serum (FBS), 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml), and streptomycin (100 μg/ml), as described.¹⁹

*Measurement of cytotoxicity of ricin.* The cytotoxicity (LD₅₀) of ricin was measured by the inhibition of colony formation as described previously, with a slight modification.¹⁹ In brief, 100–150 cells per well in 24-well plates were cultured with varying concentrations of ricin, in the growth medium for 5–8 days. The number of colonies formed was counted after staining with 1% methylene blue in 50% methanol. Clusters of 40 or more cells were considered as colonies. From the dose-response curves, the 50% colony formation inhibiting dose was estimated as the 50% lethal dose (LD₅₀).

*Measurement of protein synthesis inhibition.* Cells were inoculated at a density of 1 x 10⁶ cells/well for Vero, HeLa, CHO, and XC cells, or 2 x 10⁵ cells/well for MDCK and PtK₁ cells, in 0.5 ml of medium using 48-well plates. One day later, cells were incubated with varying concentrations of ricin in z-MEM containing 10% FBS for 3 h at 37°C. After removal of the medium, cells were incubated with 50 μCl/ml of [³H]leucine for 45 min at 37°C in leucine-free medium. The incorporation of [³H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was measured as described previously.¹⁹

*Measurement of cell lysis.* Ricin-induced cell lysis was assessed using the MTT tetrazolium cytotoxicity assay.²¹ In brief, 1–2 x 10⁴ cells per well in a 96-well plate were cultured with varying concentrations of ricin in z-MEM containing 35 μg bovine serum albumin (BSA) for 24 h, and then incubated with MTT for 4 h. After aspiration and washing once with PBS, dimethylsulfoxide (DMSO) was added to dissolve the MTT formazan reaction product and the optical density was measured using multwell scanning spectrophotometer at 570 nm wavelength.

*DNA fragmentation assay.* Cells in 6-well plates (1 x 10⁶ cells/well) were incubated for 24 h in the presence or the absence of the indicated concentrations of ricin. The cells were washed once with PBS and lysed in 1 ml lysis buffer (0.2 M NaCl, 10 mM EDTA, 20 mM Tris, 1% sodium dodecyl sulfate, pH 8.0) as described previously.²² The lysate was incubated with 250 μg/ml of protease K for 10–15 h at 37°C. The solution was then extracted with phenol/chloroform and precipitated with 2.5 volume of ethanol at −83°C, and the precipitate was dried by evaporative centrifugation. The DNA was then dissolved in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0 containing 100 μg/ml RNase A) and incubated for 4 h at 37°C. The DNA of each sample was then analyzed by electrophoresis on 2.0% agarose gels containing 50 μg/ml ethidium bromide and using TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA, pH 8.0).

*Light microscopy.* For light microscopical studies, MDCK and CHO cells were grown on glass cover slips and incubated with ricin for 24 h at 37°C. Ricin-treated and untreated control cultures were examined and photographed directly in phase contrast with microscope (Carl Zeiss Axiophot).

*Binding and internalization of FITC-labeled ricin.* The cells were cultured as described above, and cell monolayers were incubated with the indicated concentration of FITC-labeled ricin at 37°C for 2 h in z-MEM containing 10% FBS. After removal of the medium by aspiration, cells were washed three times with ice-cold PBS. Washed cells were solubilized in 1 ml of 20 mM Tris-HCl buffer, pH 8.5, containing 0.2% sodium dodecyl sulfate. The fluorescence intensity of the solubilized cell solution was measured with a fluorescence spectrophotometer (Hitachi Model 650-40) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. In this condition no quenching of fluorescence due to binding to cells was observed. The amount of cell-associated fluorescent ricin (F-ricin D or F-ricin E) was measured from the relationship between the concentration and fluorescent intensity of each FITC-labeled ricin. To measure the internalized ricin, the cells were incubated with F-ricin at 37°C for 2 h. After removal of the medium, cells were incubated with 0.1 μl lactose in PBS for 10 min at 37°C, followed by washing three times with the same solution, and cell-associated fluorescent intensity was measured. In these binding experiments, the amounts of F-ricin bound to cells in the presence of 0.1 mM lactose were subtracted from each value as the nonspecific binding.

The nonspecific bindings of F-ricin D and F-ricin E were less than 5% of the total binding. The number of apparent binding sites per cell and the apparent association constants were calculated from Scatchard plot analysis.²²

**Results**

The cytotoxicities of ricin D and ricin E in various cell lines

The cytotoxicities of ricin D and ricin E were measured by their inhibition of colony formation in Vero and CHO cells. Adherent cells were incubated with varying concentrations of ricin in growth medium for 5–8 days. The numbers of colonies formed were counted after staining with 1% methylene blue. The dose-response curves of ricin D and ricin E showed that ricin D had a stronger toxicity than ricin E in both cell lines, while the sensitivity of Vero cells

![Fig. 1. Dose-Response Curves of the Cytotoxicity of Ricin D (O, □) and Ricin E (●, ▪) in Vero (○, ●) and CHO (□, ▪) Cells.](image-url)

The cytotoxicity of ricin D and ricin E was measured by the inhibition of colony formation (A) or by the inhibition of [³H]leucine incorporation (B, A). After adherent cells (100–150 cells/well) were cultured with varying concentrations of ricin D or ricin E in the growth medium for 2 days, the numbers of colonies formed were counted as described in Materials and Methods. (B): Adherent cells (10⁶ cells/well) were incubated with varying concentrations of ricin D or ricin E for 3 h at 37°C, and labeled with [³H]leucine (0.5 μCi/ml) for 45 min in leucine-free medium for the measurement of protein synthesis.

**Table 1. The Cytotoxicity of Ricin D and Ricin E in Various Cell Lines**

<table>
<thead>
<tr>
<th></th>
<th>LD₅₀ (ng/ml)</th>
<th>ID₅₀ (ng/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ricin D</td>
<td>Ricin E</td>
</tr>
<tr>
<td>Vero</td>
<td>0.12</td>
<td>0.94</td>
</tr>
<tr>
<td>CHO</td>
<td>17.00</td>
<td>180.00</td>
</tr>
<tr>
<td>XC</td>
<td>0.56</td>
<td>23.00</td>
</tr>
<tr>
<td>HeLa</td>
<td>0.05</td>
<td>0.46</td>
</tr>
<tr>
<td>MDCK</td>
<td>0.10</td>
<td>0.88</td>
</tr>
<tr>
<td>PtK₁</td>
<td>0.10</td>
<td>0.83</td>
</tr>
</tbody>
</table>

All measurements were done in duplicate. The differences between values in duplicate experiments were within 10%.

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to the two ricins was more than 100-fold higher than that of CHO cells (Fig. 1A). To examine the inhibition of protein synthesis by ricin, cells were incubated with varying concentrations of ricin for 3 h. After removal of the medium, the cells were labeled with [3H]leucine in leucine-free medium, and the amount of [3H]leucine incorporation into proteins was measured. As shown in Fig. 1B, the cytotoxicity of ricin D was stronger than that of ricin E in CHO cells similar to the inhibition of colony formation. However, no significant difference in the ability of the inhibition of protein synthesis between ricin D and ricin E was observed in Vero cells. To further characterize the toxic effects of ricin D and ricin E, we examined the cytotoxicities of two ricins in other cell lines based on both the inhibition of colony formation (LD50) and the inhibition of protein synthesis (ID50). The LD50 and ID50 of each ricin estimated from the dose–response curves in each cell line are summarized in Table I. In all cell lines tested, both the LD50 and ID50 values of ricin E tended to be higher than those of ricin D, indicating the stronger cytotoxicity of ricin D than ricin E, while the differences in ID50 of these ricins were to a lesser extent than differences in LD50 especially in Vero and PTK1 cells. Ricin obtained from Sigma showed similar ID50 and LD50 to ricin D rather than to those of ricin E in these cell lines (data not shown).

**The cell lysis induced by ricin D and ricin E in various cell lines**

Since the protein toxins including ricin and diphtheria toxin have shown to induce lysis of several cell lines in a process characteristic for programmed cell death or apoptosis,5–8 we examined the cytolytic activities of ricin D and ricin E in various cell lines. After 24 h of incubation with varying concentrations of ricin D or ricin E, cell lysis of each cell line was assessed by the MTT assay. As shown in Fig. 2, both ricins induced cell lysis with varying efficiency among the cell lines tested, albeit ricin E was slightly less effective than ricin D. Microscopic examination of cultured cells exposed to ricin has shown morphological changes typical of apoptosis with condensation of chromatin and cell blebbing.5 In MDCK and CHO cells, ricin D caused membrane blebbing similar to characteristic morphological changes typical for apoptosis and many cells were detached from the plates after the treatment with ricin D, especially in relatively sensitive MDCK cells (Fig. 3). Similar morphological changes were also induced by ricin E (data not shown). These morphological changes induced by ricin D and ricin E were dose-dependent and correlated with the results of MTT assay in terms of the different susceptibility of each cell line against ricin-induced cell lysis. These results suggest that the MTT assay may reflect ricin-induced cell lysis. Fragmentation of cellular DNA is also the most typical feature of apoptosis. To further confirm that ricin induces cell lysis by triggering apoptosis, we did gel electrophoresis of DNA isolated from Vero cells incubated with ricin D or ricin E. Both ricins induced DNA fragmentation of Vero cells and DNA of Vero cells incubated with 10 ng/ml of ricin D or ricin E for 24 h showed a ladder-like pattern of fragmented DNA (Fig. 4). Thus, these results suggest that ricin D and ricin E induce cell lysis by a mechanism similar to programmed cell death.

**The binding and internalization of ricin D and ricin E**

To examine whether the different cytotoxicity of ricin D and ricin E is due to a differential binding ability and subsequent internalization, we examined the binding and internalization of F-ricin D and F-ricin E in various cell lines. Table II shows that the amounts of bound and internalized ricin D and ricin E in each cell line at 37°C after 2 h of incubation. In all cell lines tested, the amounts of bound and internalized F-ricin D were higher than those of F-ricin E. The differential binding ability between ricin D and ricin E could be accounted for either by a difference in the number of binding sites on the cell surface or by a difference in the affinity for the binding sites. To discriminate between these possibilities, we measured the number of binding sites and apparent association constant (Kd) of ricin D and ricin E in Vero and CHO cells. Vero and CHO cells were incubated for 2 h with various concentrations of

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**Table II. The Binding and Internalization of F-ricin D and F-ricin E in Various Cell Lines**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Bound (ng/10⁶ cells)</th>
<th>Internalized (ng/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ricin D</td>
<td>Ricin E</td>
</tr>
<tr>
<td>Vero</td>
<td>11.20±0.26</td>
<td>5.13±0.09</td>
</tr>
<tr>
<td>CHO</td>
<td>4.33±0.28</td>
<td>1.25±0.05</td>
</tr>
<tr>
<td>XC</td>
<td>3.13±0.14</td>
<td>1.02±0.06</td>
</tr>
<tr>
<td>HeLa</td>
<td>14.60±1.03</td>
<td>7.19±1.16</td>
</tr>
<tr>
<td>MDCK</td>
<td>3.48±0.32</td>
<td>1.42±0.06</td>
</tr>
<tr>
<td>PTK1</td>
<td>4.02±0.00</td>
<td>2.01±0.00</td>
</tr>
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</table>

Cells were plated in 48-well plates at a cell density of 1×10⁵ cells/well and incubated overnight. F-ricin D (5 μg/ml) or F-ricin E (5 μg/ml) was added, and the amounts of cell-bound and internalized ricin at 37°C after 2 h incubation were measured as described in Materials and Methods. The amount of bound or internalized ricin is expressed in ng/10⁶ cells. The values are the averages of duplicate determinations.
F-ricins, and the amount of bound F-ricins to cells was measured. Scatchard plots of these binding data (Fig. 5) gave straight lines under the conditions used, which indicates that the binding sites have a single affinity. The results are summarized in Table III. The apparent association constants of F-ricin D for both Vero and CHO cells were greater than those of F-ricin E, while the numbers of binding sites of these ricins were not much different. These results indicate that the higher binding ability of ricin D than ricin E is due to higher affinity to cell surface receptors rather than to difference in the number of ricin-binding sites.

Competitive inhibition of the binding of F-ricin D and F-ricin E to Vero cells by unlabeled ricins

The abilities of ricin D and ricin E to inhibit the binding of F-ricin D or F-ricin E to Vero cells were examined. As shown in Fig. 6, ricin E was 5-fold less effective at inhibiting the binding of FITC-ricin D than ricin D (binding reduced to 50% of control by 25 μg/ml ricin D and 122 μg/ml ricin E), while ricin D and ricin E were similarly efficient in competing with the binding of F-ricin E. These results are consistent with the fact that ricin D has higher affinity to cell surface receptors than that of ricin E (Table III).
Table III. Association Constant ($K_a$) and Number of Binding Sites of Ricin D and Ricin E Estimated from Scatchard Plot Analysis

<table>
<thead>
<tr>
<th>Cells</th>
<th>$K_a$ ($\times 10^{-9}$ M$^{-1}$)</th>
<th>No. of binding sites/cell ($\times 10^{-5}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ricin D</td>
<td>Ricin E</td>
</tr>
<tr>
<td>Vero</td>
<td>1.84</td>
<td>0.99</td>
</tr>
<tr>
<td>CHO</td>
<td>2.18</td>
<td>1.61</td>
</tr>
</tbody>
</table>

Fig. 4. Agarose Gel Electrophoresis of DNA of Vero Cells Treated with Ricin D or Ricin E.
Cells grown in 6-well plates were incubated with varying concentrations of ricin D or ricin E for 24 h at 37°C. DNA was extracted and analyzed on 2% agarose gel. Cells treated with ricin D at 0.1 ng/ml (lane 3), 1 ng/ml (lane 4), or 10 ng/ml (lane 5). Cells treated with ricin E at 0.1 ng/ml (lane 6), 1 ng/ml (lane 7), or 10 ng/ml (lane 8). DNA extracted from untreated cells was run in lane 2. Molecular weight standards (kb), lane 1.

Fig. 5. Equilibrium Binding and Scatchard Plot Analysis for the Binding of F-Ricin D (○, △) and F-Ricin E (●, ▲) to Vero (○, ●) and CHO (△, ▲) Cells.
Cells were incubated with various concentrations of F-ricin D or F-ricin E for 2 h at 37°C. The amounts of cell-bound F-ricin were determined as described in Table II. The insets show the saturation curves for the binding of F-ricin to cells. The data were plotted by the method of Scatchard. 3,21

Effects of cycloheximide and BFA on ricin-induced inhibition of protein synthesis and cell lysis.
To characterize the process involved in ricin-induced cell lysis, we tested the effects of cycloheximide, which is known to affect apoptosis in a number of systems, 5,23-24 on ricin-induced cell lysis. As shown in Fig. 7A, C, 10 μg/ml of cycloheximide protected Vero and MDCK cells against

Fig. 6. Competitive Inhibitory Effect of Unlabeled Ricin D (A) and Ricin E (B) on the Binding of F-Ricin D (○) or F-Ricin E (●) to Vero Cells.
Cell monolayers in 48-well plates were incubated in α-MEM containing 10% FBS with 2 μg/ml of F-ricin D or F-ricin E in the presence or absence of indicated concentrations of unlabeled ricin for 2 h at 37°C. The amounts of cell-bound F-ricin were measured as described under Materials and Methods. Competition curves were plotted as a percentage of the amount of each F-ricin bound without unlabeled ricin. Each point represents an average of duplicate measurements.

Fig. 7. Effects of Cycloheximide (○, ■) and BFA (△, ▲) on the Ricin D (●, ▲)- and Ricin E (○, △, ■)-induced Cell Lysis (A, C) and Inhibition of Protein Synthesis (B, D) in Vero (A, B) and MDCK Cells (C, D).
For the measurement of cell lysis, cells grown in 96-well plates were preincubated in α-MEM containing 35 μg BSA with 10 μg/ml of cycloheximide or 0.1 μg/ml of BFA for 1 h at 37°C, followed by the addition of varying concentrations of ricin D or ricin E. After further incubation with or without these chemicals for 24 h at 37°C, cells were assayed with MTT as described under Materials and Methods. For the inhibition of protein synthesis, adherent cells in 48-well plates were preincubated in α-MEM containing 35 μg BSA with 10 μg/ml of cycloheximide or 0.1 μg/ml of BFA for 1 h at 37°C, followed by the addition of varying concentrations of ricin D or ricin E. After further incubation with or without these chemicals for 3 h at 37°C, incorporation of [3H]leucine into proteins was measured as described in Materials and Methods. ○, ●, control. Each point represents an average of duplicate measurements.
ricin D- and ricin E-mediated lysis. In contrast to these results, ricin D and ricin E had a stronger inhibitory effect on protein synthesis in the presence of cycloheximide especially in Vero cells (Fig. 7B, D). A similar increasing effect of cycloheximide on ricin-induced protein synthesis inhibition has been reported in other cell lines. These results suggest that the cell lysis by ricin is a complex process and is not directly linked to protein synthesis inhibition. BFA, which is known to affect Golgi complex, also protected Vero cells against ricin-induced cell lysis (Fig. 7A). However, no significant protective effect of BFA was observed in naturally BFA-resistant MDCK cells in which the Golgi complex is resistant to BFA and BFA fails to inhibit ricin-induced inhibition of protein synthesis (Fig. 7C, D). Thus, these results suggest that the Golgi apparatus may be involved in the pathway leading to cell lysis by ricin.

**Discussion**

In this study, we have found that ricin D showed 8-40-fold stronger cytotoxicity than ricin E in the inhibition of colony formation assay in the cell lines tested (Table I). The ability of ricin D to inhibit protein synthesis was also 3-14-fold greater than ricin E in CHO, XC, HeLa, and MDCK cells, while no significant difference in this ability of two ricins was observed in Vero and PtK1 cells. Since ricin D and ricin E have identical A-chain, it is probable that the differences in their B-chains account for the differences in the cytotoxicity between two ricins. Analysis of amino acid sequence has shown that the B-chain of ricin E is composed of the N-terminal half of the B-chain of ricin D and the C-terminal half of the B-chain of CBH. Furthermore, equilibrium dialysis analysis has indicated that B-chains of these ricins have two saccharide binding sites with different affinity, and the HA-site of ricin E present on the C-terminal half has a lower affinity than that of ricin D, while there is no significant difference in their LA-sites. In agreement with these findings, the amounts of ricin D bound to cultured cells found to be 2-3-fold greater than those of ricin E in six cell lines tested (Table II). The lower binding ability of ricin E to cells may be due to the lower saccharide binding affinity of the HA-site. Data of Scatchard plot analysis in Vero and CHO cells indicated that the apparent association constants of ricin D to these cell lines were higher than those of ricin E, while their number of binding sites per cell were not much different (Table III). These results suggest that ricin D and ricin E recognize almost the same cell surface carbohydrates with different affinity and ricin D has a higher affinity for these specific carbohydrates than ricin E. This notion is also confirmed by the competition experiments in which the binding of FITC-labeled ricin E to Vero cells was effectively competed for by unlabeled ricin D as well as ricin E, while ricin E was less effective than ricin D in the competition of the binding of FITC-labeled ricin D (Fig. 6).

Recent studies have demonstrated that ricin and other protein toxins including diphtheria toxin induce lysis of several cell lines concomitant with DNA fragmentation in a process characteristic of programmed cell death or apoptosis. It is generally considered that apoptosis is an active process and may involve the de novo synthesis or activation of an endogenous Ca2+/Mg2+-dependent endonuclease. Thus, protein synthesis inhibitors such as cycloheximide and actinomycin D can inhibit glucocorticoid-induced apoptosis in rat thymocytes, and radiation-induced apoptosis in lymphocytes. However, this may not be the case for ricin and diphtheria toxin, since they themselves are potent inhibitors of protein synthesis. Moreover, cycloheximide itself has been shown to cause DNA fragmentation typical of apoptosis in macrophages. Therefore, the protein synthesis inhibition is not necessarily required for apoptosis for all cell types, and more than one mechanism may be involved in the apoptotic process. Several compounds or conditions have been reported to modulate protein toxin-induced cell lysis. For instance, cycloheximide inhibits ricin-induced cell lysis but did not affect diphtheria toxin-mediated lysis. In contrast, 3-aminobenzamide, which is reported to inhibit diphtheria toxin-induced lysis of U937 cells, had no protective effect against the ricin-induced lysis in MDCK cells. The phospholipase inhibitors quinacrine and dexamethasone, which are reported to inhibit tumor necrosis factor-induced destruction of L929 cells, did not affect ricin-induced lysis. Although the mechanism by which ricin induces cell lysis is still unclear, the underlying cytolytic mechanism of ricin may be distinct from those of diphtheria toxin or tumor necrosis factor. In agreement with a previous report, cycloheximide was found to inhibit the cell lysis induced by ricin D and ricin E in Vero and MDCK cells (Fig. 7). However, ricin showed a stronger inhibitory effect on protein synthesis in the presence of cycloheximide especially in Vero cells (Fig. 7B), suggesting that the cell lysis induced by ricin does not directly relate to the inhibition of protein synthesis. In addition, it has been shown that macrophage adherence prevents ricin-induced apoptosis but has no effect on the inhibition of protein synthesis by ricin.

Furthermore, it has been reported that some lectins, such as phytohaemagglutinin (PHA), concanavalin A (Con A), wheat germ agglutinin (WGA), and Lentinus edodes (LCA) induce cytology, even though these lectins do not inhibit protein synthesis. Thus, it is interesting to examine whether or not isolated B chains of ricin D and ricin E can induce cell lysis. Such a study may provide insights to our understanding of the mechanism of cell lysis by ricin. Although many details regarding the mechanism of lectin-mediated cytotoxicity remain unknown, a recent study of cytotoxic activity of wheat germ agglutinin (WGA) and Griffonia simplicifolia 1-B4 (GS1B4) lectin suggests that the binding of these lectins to the specific receptors, while essential, is not sufficient for cell lysis and lectin internalization and probably subsequent intracellular mechanisms are involved in the lectin-mediated cytosis, since immobilized lectin failed to trigger cell lysis.

It has been shown that following receptor-mediated endocytosis, ricin molecules are found in the Golgi elements and protein synthesis inhibition by ricin is abolished by BFA, which profoundly affects the structure and functions of the Golgi apparatus without affecting the binding or internalization of ricin. These findings suggest that vesicle trafficking of ricin molecules through the Golgi region is involved in the process leading to the inhibition of protein synthesis by ricin. Since the inhibition of protein synthesis by both ricin D and ricin E was inhibited by BFA, the two ricins basically have same intoxication pathway (Fig. 7). Interestingly, the cell lysis induced by ricin D and
ricin E was also inhibited by BFA in BFA sensitive Vero cells (Fig. 7A), while no protective effect of BFA was observed in BFA-resistant MDCK cells (Fig. 7C, D). Thus, the Golgi region may involve in cytolytic activity of ricin as well as in the inhibition of protein synthesis. During the vesicle trafficking of ricin molecules through the Golgi apparatus, some signals leading to cell lysis may be triggered. We cannot rule out the possibility that other mechanisms such as necrosis might be involved in the ricin-induced cell lysis. However, many investigators have demonstrated that DNA fragmentation is not merely the result of cell death, since killing of target cells by heating, freeze and thawing, or lysis with antibody and complement does not induce DNA degradation. Furthermore, extensive membrane blebbing, another morphological marker for programmed cell death, was observed in ricin-treated cells (Fig. 3). Further studies including examination of cytotoxicity of isolated B chain of ricin are required to elucidate the relationship between the inhibition of protein synthesis and the cell lysis by ricin.

References