Biochemical Studies on Oral Toxicity of Ricin. V.[1] The Role of Lectin Activity in the Intestinal Absorption of Ricin

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In order to investigate a possible role of lectin activity of ricin in its absorption from the small intestine, we prepared two ricin derivatives. BMH-ricin, prepared by crosslinking A and B chains of ricin with 1,6-bismaleimidohexane, was nearly non-toxic but the lectin activity was unaltered. And, NBS-ricin, prepared by the oxidation of tryptophanyl residues of ricin with *N*-bromosuccinimide, was not only non-toxic but also non-lectinic.

After the oral administration of ricin derivatives to rats, their interaction with the digestive tract and absorption into the circulatory systems have been compared with those of ricin, immunochemically and histologically. It was shown by immunostaining that ricin and BMH-ricin could bind to the intestinal mucosa, whereas NBS-ricin could not. No appreciable damage in the small intestine from rats treated with either BMH-ricin or NBS-ricin has been observed, in contrast to ricin treatment where severe impairment of the small intestinal tissues resulted after 5 h. Immunoreactive ricin in the liver has been determined with the ricin enzyme immunoassay (EIA). When compared at 48 h after oral administration, NBS-ricin was not detected, whereas BMH-ricin was found to be 38 μg/liver and ricin 100 μg/liver.

From these results, it was inferred that the lectin activity of ricin plays an important role in the absorption of ricin from the small intestine and that the absorption of ricin protein was enhanced by its high toxicity.

Keywords ricin; lectin; oral toxicity; intestinal absorption; rat small intestine; chemical modification; ricin EIA; histoimmunochemical staining; 1,6-bismaleimidohexane; *N*-bromosuccinimide

Introduction

Ricin, a toxin from the castor bean seed (*R. communis* L.), is a glycoprotein comprising two polypeptide chains, A and B, joined by a disulfide bond. Its toxic action against eukaryotic cells as well as many types of the transformed cells has been well documented.[2,3] Ricin had been discovered as an agglutinin which can interact with the sugar moiety of glycoproteins or glycolipids on the cell surface and thus can agglutinate cells if it binds at more than two binding sites per cell. The toxic action of ricin, on the other hand, is explained in that the B chain of ricin is first bound to the cell surface receptors followed by the internalization of the whole molecule into the cytosol and the A chain released inhibits enzymatically the protein synthesis, resulting in the cell death.[4]

One of the remarkable characteristics of ricin protein is that ricin exerts its toxic action in animals by oral administration regardless of its protein nature. Recently, considerable attention has been payed to the physiological roles of certain proteins ingested as foodstuffs in the digestive organs.[5,6] We have previously reported that ricin given *p.o.* to rats caused severe damage to the small intestine resulting in the impairment of absorption of the nutrients.[7–9] It has, however, been unclear whether or not the death of the rats was caused by the active ricin absorbed from the small intestine. Then, we could determine the immunoreactive ricin in blood, lymph, and liver of the orally-intoxicated rats by sensitive enzyme immunoassay (EIA) and gel filtration followed by identification with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and the immunobinding method.[1] When ricin (10 mg/kg rat) was orally given a dose equivalent to 1/3 LD_{50}, about 75% of the ricin was found in the stomach and small intestine within 2 h, and most of it was transferred to the large intestine after 24 h. It was also demonstrated by *in vitro* toxicity test of immunoreactive ricin in the blood and lymph obtained from the intoxicated rats that part of the ricin was absorbed from the small intestine into the tissues and organs *via* the circulatory systems. Ricin, after absorption, was detected mainly in the liver and was predominantly in the form of intact ricin, although the possible distribution of an undetectable amount of ricin in other organs cannot be eliminated.[1] It still remained unsolved whether this transfer of ricin from the small intestine to the circulating system occurred as a secondary effect due to destruction in the absorptive villi of the small intestine or if it was a normal process essential for the normal digestion and absorption of food proteins. In the present study, therefore, we intended to clarify the effects of the two distinctive properties of ricin, *i.e.*, lectin activity and toxicity, on the absorption process of ricin in the rat small intestine. For this purpose, two chemically modified ricins, one 1,6-bismaleimidohexane (BMH)-ricin, which is non-toxic but lectinic, and the other, *N*-bromosuccinimide (NBS)-ricin, which is not only non-toxic but also non-lectinic, have been prepared, and the interaction with the intestinal mucosa and absorption of these derivatives after *p.o.* administration into rats have been compared with those of intact ricin, immunochemically and histologically.

Materials and Methods

Ricin was prepared from Chinese castor bean seeds according to the method described by Hara *et al.*[10] Its individual chains have been prepared as described before.[11] Reagents were obtained from the following sources: BMH from Pierce (Rockford, IL 61105, U.S.A.); NBS and *o*-nitrophenyl-galactopyranoside (ONPG) from Nacalai Tesque (Kyoto, Japan); β-galactosidase (*E. coli*) from Boehringer Mannheim (Tokyo, Japan); and ABC reagent (Vectastain), which is Avidin: Biotinylated enzyme complex kit from Funakoshi (Tokyo, Japan). Polyclonal anti-polyethylene glycol-modified (PEG_2)-ricin and anti-BMH-ricin antisera were raised and ricin EIA was performed as described previously.[1]

Preparation of BMH-Ricin Ricin (48 mg) in 6 ml of 0.1 M Tris–HCl buffer (pH 8.0) containing 5 mM ethylenediaminetetraacetic acid (EDTA) was reduced by incubation with 60 μl of 2-mercaptoethanol (2-ME) for 2 h at room temp. After reduction, the reaction mixture was filtered to remove 2-ME with a Sephadex G-25 column (2.5 × 40 cm) equilibrated with 50 mM sodium phosphate buffer (pH 5.5) containing 5 mM EDTA.

To the reduced ricin solution whose pH was adjusted to 6.5 with 0.2 M Na_2HPO_4, 100 times molar BMH in methanolic solution was added dropwise and the mixture was incubated to form BHM-ricin for 2 h at room temp. Excess reagents were removed by dialysis against 5 mM Tris–HCl buffer (pH 8.5). To remove the unreacted ricin, the dialyzed solution above was applied onto a column of Sepharose 4B (1.5 × 33 cm) after reducing with 1% (v/v) 2-ME for 2 h at room temp. Since BMH-ricin and B chain from the unreacted ricin were adsorbed to this column, any traces of free A chain were eliminated by washing with 5 mM Tris–HCl buffer, pH 8.5, containing 0.1% 2-ME. Adsorbed fractions were eluted with 0.25 M galactose in the same buffer and were finally fractionated on a DE 52 column (1.5 × 20 cm) which had been equilibrated with 5 mM Tris–HCl buffer, pH 8.5, containing 0.1% 2-ME and 0.25 M galactose. Proteins were eluted stepwise with the increasing sodium chloride concentration in the same buffer, pH 8.5. The fraction corresponding to BMH-ricin was eluted with 5 mM Tris–HCl buffer, pH 8.5, containing 20 mM sodium chloride, 0.1% 2-ME and 0.25 M galactose, whereas the fraction containing B-chain was eluted with 0.25 M sodium chloride in the same solution.

Preparation of NBS-Ricin Oxidation of ricin with NBS was carried out according to the method described by Taira *et al.*[12] NBS-ricin was purified using a Sepharose 4B column (1.5 × 10 cm) which was equilibrated with 100 mM acetate buffer, pH 4.5, and only the break-through fraction that was assumed not to have lectin activity was collected. In NBS-ricin, prepared by the treatment of ricin with NBS at pH 4.5 and at room temp. for 10 min and purified, it was found by spectroscopic determination that about 4 residues of tryptophanyl residues were oxidized.

Characterization of Ricin Derivatives Each ricin derivative after purification was analyzed by SDS–PAGE.[13] Toxicity toward animals was determined as the half lethal dose at 48 h (LD_{50}) of the derivatives injected intraperitoneally to mice (ddY strain, 20—25 g).[14] Hemagglutinating activity was measured as lectin activity of the derivatives employing rabbit erythrocyte as described previously.[14] The activity was expressed as the minimum amount of protein required to agglutinate.

Preparation of Tissue Sections[15] Ricin or derivatives (2 mg) were orally given to Wistar male rats (200 g). Animals were sacrified at 0.5, 1 and 5 h, and organs were removed and fixed in 10% formalin. After the organs were sectioned, they were stained with hematoxylin and eosin (HE staining) for histological observation.

Immunohistochemical Staining Deparafinized and hydrated tissue sections were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate the endogeneous peroxidase activity. After washing completely with phosphate buffered saline, the sections were treated with normal swine serum for 20 min followed by incubation with diluted anti-ricin antiserum (anti-BMH-ricin) overnight at 4 °C. They were then immersed in the biotinylated anti-rabbit immunoglobulin G (IgG) solution for 30 min at room temp. The resulting sections were next incubated with ABC reagent for 30 min. Finally, bound peroxidase activity was visualized with 0.1% diaminobenzidine in Tris–HCl buffer (pH 7.2), containing 0.02% H_2O_2 and the sections were observed microscopically under counterstaining with methyl green.

Determination of Immunoreactive Ricin in the Liver of Intoxicated Rats After ricin, BMH- or NBS-ricin (each 10 mg/kg) was orally administered to rats, the livers were taken out after 5—72 h and homogenized with 10 mM sodium phosphate buffered saline containing 100 mM galactose and 0.1% Tween 20. After centrifugation, the supernatant solution was used for the determination of immunoreactive ricin by the ricin EIA system as described previously.[1] Briefly, 100 μl of samples, diluted appropriately with the buffer, and 100 μl of β-galactosidase-labeled ricin, 3 mU, were mixted in wells of a microtiter plate which had been coated with rabbit anti-PEG_2-ricin IgG. After incubation at room temp for 3 h, plates were washed 3 times with the buffer containing Tween 20. Color intensities developed by the incubation with ONPG were determined at 415 nm with a microplate reader. This EIA allows the detection of ricin in the biological samples at levels as low as 0.5 pM (3 pg—10 ng/well).

Results

Characterization of Chemically Modified Ricins BMH-ricin and NBS-ricin were found homogeneous and free from the intact ricin as judged by SDS–PAGE (Fig. 1). Comparisons of some properties of ricin derivatives with those of ricin are shown in Table I. BMH-ricin, in which the interchain disulfide bond became uncleavable, (Fig. 1, lane 2), exhibited the similar lectin activity based on its hemagglutinating activity to ricin, whereas its toxicity was reduced to less than 10% of ricin. Lectin activity and toxicity of NBS-ricin were reduced to less than 25% and 10% of those of ricin, respectively. In the ricin EIA, rabbit

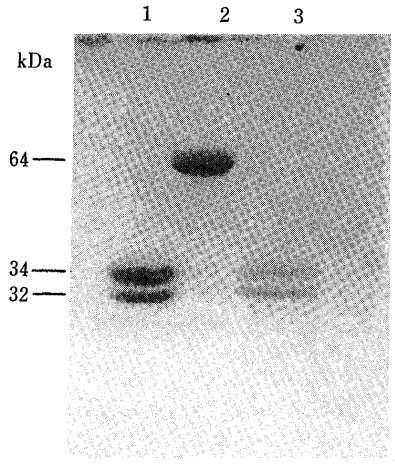

Fig. 1. Analysis of Ricin Derivatives by SDS–PAGE

In order to confirm purity of the derivatives used throughout this study, SDS–PAGE was performed with 11% gel in the presence of 2-ME. Lane 1, ricin; 2, BMH-ricin; 3, NBS-ricin.

Table I. Properties of BMH-Ricin and NBS-Ricin[a]

	BMH-ricin	NBS-ricin	Ricin
Binding ability to Sepharose 4B[b]	(+)	(−)	(+)
Hemagglutinating activity[c]	5.8 (100%)	>23.2 (25%>)	5.8 (100%)
Immunological cross-reactivity[d]	10 ng	10 ng	10 ng
Toxicity (i.p.)[e]	>0.18 (10%>)	>0.18 (10%>)	0.018 (100%)

a) BMH- and NBS-ricins are ricin preparations modified with BMH and NBS, respectively, as described in the text. *b*) A protein with binding ability to Sepharose 4B column is ususally considered as lectinic, and (−) expressed no binding. *c*) Lectin activity of proteins is also represented as hemagglutinating activity, which is expressed as the minimum concentration necessary to agglutinate rabbit erythrocytes (μg protein/ml). *d*) This was calculated from Fig. 2 as the amount of protein in the well at $B/B_0 = 50\%$ in ricin EIA. *e*) A half lethal dose (i.p.) at 48 h (μg/g).

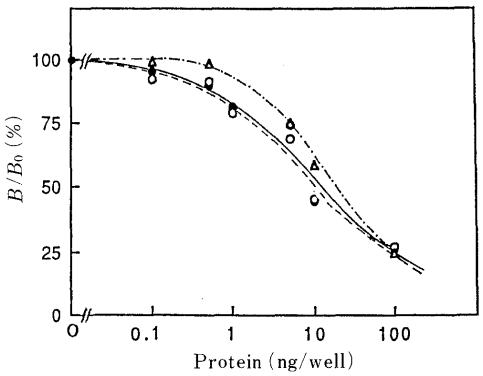

Fig. 2. Immunological Cross-Reactivity of Ricin Derivatives in Ricin EIA

Ricin, BMH- and NBS-ricins contained in the liver homogenates were determined by solid-phase EIA as described previously.[9] For details of the method, see the text. Values are the mean of 3 determinations. ●—●, ricin; ○---○, BMH-ricin; △—·—△, NBS-ricin.

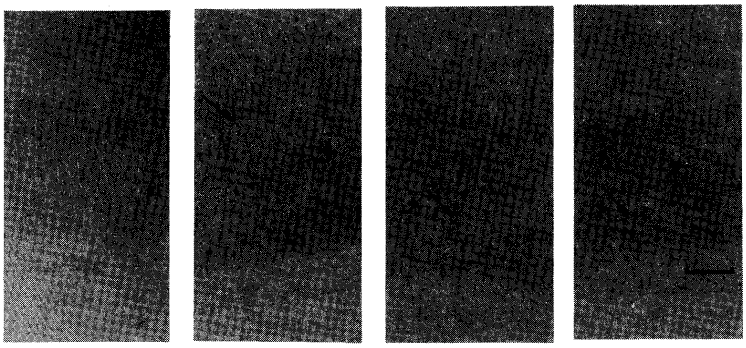

Fig. 3. Immunostaining of Small Intestine after Oral Administration of Ricin

Rats given ricin (2 mg), *p.o.*, were killed after 0.5, 1 and 5 h, respectively. The small intestinal segments were removed and fixed in 10% formalin. Thin sections were stained for ricin by ABC stain. Scale bar, 100 μm. Arrows indicate positive grains.

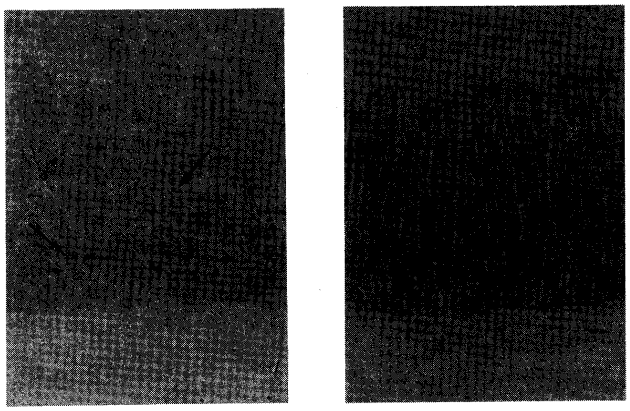

Fig. 4. Immunostaining of Small Intestine after Oral Administration of Ricin Derivatives

Rats given BMH-ricin and NBS-ricin (each 10 mg/kg), separately, *p.o.*, were killed after 5 h. The small intestinal segments were cut out, fixed and stained as in Fig. 1. Arrows indicate positive grains. Scale bar, 100 μm.

anti-PEG_2-ricin antiserum and β-galctosidase-labeled ricin have been employed. It was revealed that ricin, BMH-ricin, and NBS-ricin were equally recognized by PEG_2-ricin antibody (Fig. 2). It is, therefore, concluded to use this ricin EIA for the dtermination of the absorbed ricin derivatives as well as ricin from the small intestine to the liver.

Interaction of Ricin and Its Derivatives with Small Intestine after Oral Administration to Rats As shown in Fig. 3, orally-given ricin was found as positive staining particles immunohistochemically on the surface of the absorptive villi of the small intestine after 30 min. After 1 h, staining particles were detected at the deeper position of the villi, and after 5 h, at the roots of the villi. These results indicate the translocation of ricin across the villi and absorption from the intestinal mucosa. Since the damage caused by ricin in the digestive organs, especially in the small intestine became apparent even after 1 h reaching its highest degree at 5 h and lasting for more than 10 h,[15] 5 h was chosen for comparison in the cases of ricin derivatives. In the case of BMH-ricin, which possesses lectin activity but no toxicity, the positive staining particles were present on the surface and the interior part of the villi after 5 h of administration (Fig. 4, left), which was similar to that of ricin after 1 h (Fig. 3). This result suggests the slower absorption of BMH-ricin could occur due to its non-toxicity. NBS-ricin, without lectin activity, did not give positive staining (Fig. 4, right).

Histological Changes Severe atrophy of the mucosa and villi, and elongation of the crypt of the small intestine were observed even after 5 h of oral administration of ricin (Fig. 5). Figure 5 also shows that the individual chains of ricin (A or B chain) did not cause histological changes of the small intestine although a minor desquamation of the absorptive cells was observed by the B chain. After the administration of either BMH-ricin or NBS-ricin, both of which are non-toxic, no histological changes were observed (Fig. 6). These results confirmed the previous results[7,15] that only ricin in which its A and B chain are covalently bonded could give rise to deleterious effects on the intestinal mucosa and villi of rat.

The Role of Lectin Activity in the Intestinal Absorption of Ricin In order to investigate the role of lectin activity in the absorption of ricin from the small intestine, the amount of immunoreactive ricin in the liver was determined (Fig. 7). When NBS-ricin was given orally, the concentration of immunoreactive ricin in the liver after 48 h was lower than that which can be detected by this EIA system (less than 5 μg/liver). On the other hand, in the case of the oral administration of BMH-ricin, the immunoreactive ricin was detected after 5 h and gradually increased reaching to the highest (38 μg/liver) at 48 h and then decreased at 72 h. Similarly, when the intact ricin was orally given, the amount of immunoreactive ricin increased gradually with the time reaching to the maximum (100 μg/liver) at 48 h and decreased at 72 h. It should be stressed that the amount of immunoreactive ricin in the liver obtained from the intact ricin-intoxicated rat was about 2.5 times higher than that of BMH-ricin.

From these results, it was inferred that the lectin activity of ricin plays an important role in the absorption of ricin from the small intestine to the liver *via* the circulating systems and that the absorption was possibly enhanced by the high toxicity of ricin.

Discussion

Ricin has been known to be one of the most toxic substances when administered intraperitoneally, intravenously or subcutaneously to animals such as rats and mice. In addition, it is lethal, even by oral administration, with

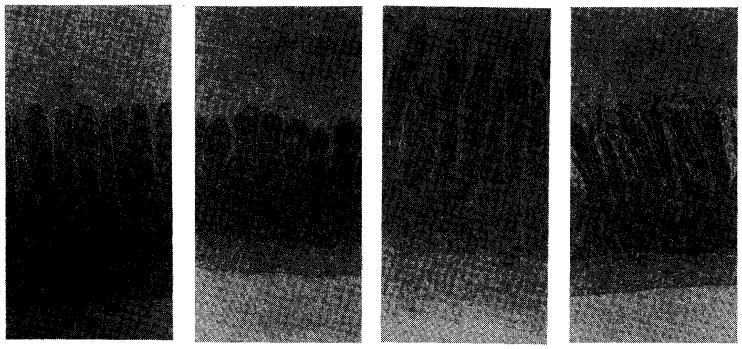

Fig. 5. Histological Changes of Small Intestine after Oral Administration of Ricin and Its Individual Chains

Rats given ricin (10 mg/kg), A- and B-chain (each 15 mg/kg), separately, *p.o.*, were killed after 5 h. Segments were cut out, fixed, sectioned and stained with HE. Note the length of villi. Scale bar, 100 μm.

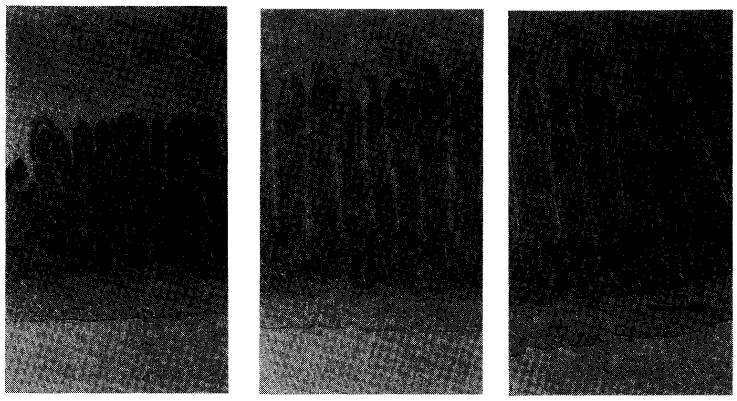

Fig. 6. Histological Changes of Small Intestine after Oral Administration of Ricin and Its Derivatives

Rats given ricin, BMN-ricin and NBS-ricin (each 10 mg/kg), separately, *p.o.*, were killed after 5 h. Segments were fixed, sectioned and stained with HE. Scale bar, 100 μm.

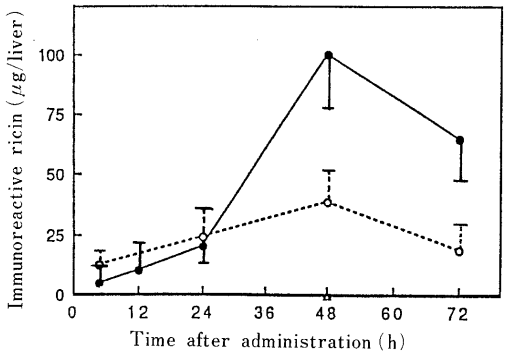

Fig. 7. Determination of Immunoreactive Ricin in the Liver of Intoxicated Rats

Rats given ricin orally, BMH-ricin or NBS-ricin (each 10 mg/kg), separately, were killed after 5—72 h. Livers were removed and homogenized. The amount of immunoreactive ricin in the supernatant solution was determined by ricin EIA as described in Materials and Methods. Values are the mean±S.E. of 3 experiments. ●—●, ricin; ○---○, BMH-ricin; △, NBS-ricin.

symptoms such as severe diarrhea and hypothermal effects although it is of a proteinous nature. We have shown in the previous paper that ricin given orally to rats resulted in severe damage of the gastrointestinal mucosa followed by the impairment of glucose absorption.[7] Since only these primary effects of ricin on the digestive gut of rats could not explain the lethality, we assumed that the death of animals could be due to a trace amount of active ricin which might have escaped from the digestion and been absorbed from the small intestine into the circulating systems.[8] We have also demostrated the *in vitro* sensitivity of rat intestinal cell to ricin,[9] and the presence of immunoreactive ricin in the blood, lymph and liver by specific EIA after oral administration of ricin.[1] Since severe gastrointestinal impairment had always been accompanied by the oral administration of ricin, we could not distinguish whether ricin was transferred as a normal process or a secondary one due to the damage of the villus structure of the intestinal mucosa.

The aim of this paper is to clarify why active ricin could be transferred significantly from the small intestine to the blood or lymphatic streams and further to specific organ such as the liver. To this end, we have payed special attention to the two distinctive properties of ricin toxin, lectinic and toxic functions. For this purpose, we have prepared two ricin derivatives, BMH-ricin and NBS-ricin, the former is only lectinic and the latter both non-lectinic and non-toxic. Their interaction with rat small intestine, and the absorption followed by the accumulation of immunoreactive ricin in the liver, after oral administration, have been campared with those of ricin with aids of immunostaining and HE stain and by EIA, respectively.

It was clear that BMH-ricin with decreased toxicity but

full lectin activity can interact with the surface and deeper portions of absorptive villi of the small intestine at 5 h after oral administration (Fig. 4, right), which was similar to that of ricin (Fig. 3, 1 h). It should be mentioned that BMH-ricin (positive stainings in Fig. 4) was found on the surface to the deeper position without severe histological changes of the mucosa, which was in contrast with those of ricin (Fig. 6). Absorption of ricin and BMH-ricin was confirmed by the accumulation of immunoreactive ricin in the liver (Fig. 7). On the other hand, NBS-ricin with neither toxicity nor lectin activity was not detected on the surface of the villi nor in the liver, at the same time no destruction of the villi of the small intestine was observed (Figs. 4, 6 and 7). In addition, it was shown that the A or B chain of ricin alone could not give severe effects histologically on the intestinal villi (Fig. 5). From these results, it was concluded that the damage of the small intestine caused by the oral administration of ricin, which is the most significant symptom of the oral toxicity of ricin, requires both lectin activity (binding ability to the absorptive cells) and toxicity (inhibition of protein synthesis after ricin is internalized into the cells). The second conclusion deduced from these data was that lectin activity plays an important role in the intestinal absorption of ricin and that the change in the permeability of the mucosa had occurred due to its high toxicity, which probably enhanced the absorption of ricin into the circulating system and further to the liver.

Recently, studies on the specific uptake of dietary lectins into the circulating system have appeared. Pusztai *et al.* reported that up to 90% of dietary PHA (kidney bean lectin) can survive gastrointestinal passage in fully reactive form and that by binding to the small intestine, it disrupts the membrane structure of the absorptive epithelium, subsequently being passed from the gut lumen into the circulation.[6] They also reported that non-toxic tomato lectin was really absorbed into the circulation.[16] Further, DE Aizpurua *et al.* described that a protein with lectin activity can specifically bind to various glycolipids and glycoproteins located on the surface of the cells of the intestinal mucosa, and then be transported to the circulation, thereby eliciting a systemic immune response.[17] Our present data together with the results mentioned above clearly support the importance of lectin activity in the intestinal absorption of macromolecular proteins. The possibility exists that such proteins with lectin activity can be designed as carriers in order to deliver drugs, immunotoxins, hormones or antigens more effectively from the intestine into the systemic circulation.[18]

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