Biochemical Studies on Oral Toxicity of Ricin. IV. A Fate of Orally Administered Ricin in Rats

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(Received September 17, 1991)

After oral administration of ricin in rats, its distribution in the gastrointestinal tract, body fluids and principal organs was determined by an enzyme immunoassay, and the immunoreactive ricin detected was identified by gel filtration followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, protein blotting and the immunobinding method. When ricin D (10 mg/kg rat) was given orally to a rat, which dose is equivalent to $1/3$ LD₅₀, 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 an *in vitro* toxicity test of immunoreactive ricin in the blood and lymph obtained from the intoxicated rats that a part of the ricin was absorbed from the small intestine into the tissues and organs *via* the circulatory systems (lymphatic and blood vessels) as the active ricin. The participation of the blood vessels was greater in the absorption of ricin from the gastrointestinal tract than that of the lymphatic system. Ricin, after absorption, was detected in liver and spleen and ricin found in the liver was predominantly in the form of intact ricin, although an undetectable amount of ricin in other organs cannot be eliminated. These results infer that a small fraction of orally-given ricin was transferred to the circulating system and was responsible for rat's death as in the case of i.p. administration.

Keywords — ricin; toxic lectin; oral toxicity; ricin enzyme immunoassay; intestinal absorption; immunoreactive ricin

Introduction

Ricin D, a toxic lectin from castor bean seed, consists of two different polypeptide chains, A and B. The biological function of the former catalytically inhibits cellular protein synthesis by cleavage of adenine 4324 from the 28S ribonucleic acid (RNA) in the 60S ribosomal subunit, whereas the latter induces internalization of the A chain as a complex with the galactose-containing receptors on the cell surface, resulting in strong cytotoxicity of the ricin.^{1,2)}

Numerous attempts which utilize the very strong cytotoxic property of ricin or ricin A chain as anti-cancer agents have been reported. An example is an "immunotoxin" which contains a tumor cell-specific antibody equipped with ricin A chain as a magic bullet to the tumor or transformed cells.^{3,4)} Despite a wide use of this hybrid protein for drugs, there have been few studies on how the hybrid or ricin is metabolized and/or what happens to the ricin moiety *in*

vivo.⁵⁻⁹⁾

In the previous papers, we have reported that ricin administered orally caused rats death after a long lag time, usually more than 20 h, with severe diarrhea. Microscopic examination of the dead rats revealed atrophy of the villus, elongation of crypt, degeneration of epithelium, decrease in goblet cells, fusion of intervillus epithelia, infiltration of neutrophils and eosinophils, and dissociation between epithelium and lamina propria had occurred in the jejunum with the ricin intoxication.¹⁰⁻¹²⁾ It was also found that the epithelial cells isolated from the rat small intestine were sensitive toward ricin *in vitro*.¹³⁾ However, the death of rats cannot be explained solely with the gastrointestinal damages described above.

In addition, nutritional roles of plant lectins have recently been re-evaluated, and certain lectins could have deleterious effects on the intestinal villi. Absorption of macromolecules such as lectin proteins through intestinal mucosa as an intact form has been discussed in relation to their

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nutritional values.¹⁴⁻¹⁷⁾

Under these circumstances, it is very important to know how ricin protein behaves in the gastrointestinal tract after oral administration, how much it may be transferred to the circulatory systems, and be accumulated in the tissues *in vivo*. In the present study, the effort has been devoted to develop a sensitive enzyme-linked immunoabsorbent method for the detection of a trace amount of ricin in the rat after oral administration. Several lines of evidence clearly showed that ricin was absorbed from the intestine into the circulatory system (*via* both lymphatic and blood vessels) in an almost intact form, and was primarily accumulated in liver, although a possible distribution of undetectable amount of ricin in other organs cannot be excluded.

Materials and Methods

Materials — An electrophoretically homogeneous ricin D and castor bean hemagglutinin (CBH) preparations were obtained from the seeds of castor bean (*Ricinus communis* L., small grains imported from China) according to the method described before.¹⁰⁾ We purchased ¹²⁵I-Na (74 MBq) and donkey anti-rabbit immunoglobulin G (IgG) ¹²⁵I-labeled whole antibody (370 kBq/ μ g) from Amersham Co., Ltd., and *N*-(γ -maleimidobutyryloxy)succinimide (GMBS) from Dojindo, and β -galactosidase (*E. coli*) from Boehringer Mannheim, respectively. ¹²⁵I-Ricin (1.8×10^4 Bq/ μ g) was prepared by the chloramine-T method according to Oda *et al.*¹⁸⁾ A specific antibody to ricin was obtained by the repeated injection of polyethylene glycol-modified ricin (PEG₂-ricin: a ricin toxoid) into rabbits. Modification of ricin with triazine-activated polyethylene glycol to obtain an antigen with weakened toxicity was carried out according to the method described by Kamisaki *et al.*¹⁹⁾ IgG fraction was prepared from the serum by ammonium sulfate fractionation and diethylaminoethyl (DEAE)-cellulose chromatography. Ricin was also labeled with β -galactosidase by the cross-linking agent, GMBS, according to Kitagawa's method.²⁰⁾ Sodium dodecyl sulfate polyacrylamide gel electropho-

resis (SDS-PAGE) was performed according to the method of Laemmli using a 10% polyacrylamide gel under either reducing or non-reducing condition as described previously.²¹⁾ Male albino rats of the Wistar strain (body weight, 300–350 g) and male ddY mice (18–20 g) were purchased from the local animal supplier. Animals were fed with a commercial chow *ad libitum* until the initiation of experiments. Thereafter, they were fasted for 36 h but allowed water *ad libitum*.

Preparation of the Biological Samples from the Intoxicated Rats — Ricin emulsified with olive oil (10 mg/kg; equivalent to 1/3 LD₅₀) was orally administered to rats. Blood was taken from the inferior vena cava, and the lymph from the mesenteric lymphatic duct was collected, respectively, at 1 h interval according to the method described by Bollman *et al.*²²⁾ Organs were removed from the intoxicated rats after perfusion from the inferior vena cava with 500 ml of 50 mM phosphate buffered saline (PBS) and homogenized with a 4 fold volume of 50 mM PBS containing 0.1 M galactose, 0.01% Tween 20 and 0.1% NaN₃. From the normal rats given orally the saline, blood, lymph, and the homogenates of organs were obtained as described above and employed as the blank samples. The blood, lymph and homogenates were centrifuged at 15 000 rpm for 15 min and the supernatant solutions were stored at –80 °C until use.

Enzyme Immunoassay (EIA) of Ricin — Ricin contained in the body fluids and organs was determined by solid-phase EIA. The following buffer solutions were employed for ricin EIA: buffer A, 20 mM phosphate buffer, pH 7.0, containing 100 mM NaCl, 1 mM MgCl₂, 0.1% NaN₃, and 0.1% bovine serum albumin (BSA); buffer B, 50 mM phosphate buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% NaN₃, and 0.1% BSA; and buffer C, 10 mM Tris-HCl buffer, pH 8.5, containing 10 mM NaCl and 0.1% NaN₃. Polystyrene plates (Nunc-Immuno Plate, MaxiSorp) were coated with the buffer C containing rabbit anti-PEG₂-ricin IgG (1 μ g/ml), then covered with Parafilm and kept at 4 °C overnight. The plates were washed 3 times with buffer B. To

block the plates, 200 μ l of the buffer B was added to the wells and the plates were kept at 37 °C for 2 h. Then the plates were washed and stored at 4 °C until use. For assay, each sample or standard solution diluted appropriately with buffer A (100 μ l) and β -galactosidase-labeled ricin (100 μ l), diluted with buffer A to 3 mU/well, were mixed and incubated at room temperature for 3 h. Plates were washed 3 times with buffer A containing 0.05% Tween 20 and 200 μ l of 0.1% *o*-nitrophenyl- β -galactopyranoside (ONPG) solution was added to each well of the plate. The plate was incubated at 37 °C for 2 h and the resulting color intensities of each well were measured at 415 nm with a microplate reader. The present EIA allowed detection of ricin in the biological samples at levels as low as 0.5 pM (3 pg—10 ng/well).

Identification of Ricin in the Biological Samples — After 125 I-labeled ricin (1×10^7 cpm) was given orally to rats, the blood was collected at 90 min. The plasma was subjected to gel filtration through a Sephadex G-75 column (2.5×40 cm), the column was then eluted with

50 mM phosphate buffer, pH 7.4, containing 0.1% BSA and 0.1% NaN₃, and the radioactivity in each fraction was determined. A peak fraction corresponding to ricin (64 kDa) was collected, concentrated *in vacuo* and subjected to SDS-PAGE. The resulting gel was visualized by autoradiography. Alternatively, the lymphatic solution was collected for 3 h from the orally intoxicated rats (10 mg/kg), and the liver was removed at 48 h after ricin administration. The lymph and the supernatant solution prepared from the liver homogenate as described above were separately concentrated and applied on the Sephadex column, and were eluted with 0.1% NH₄HCO₃ solution, pH 7.4. The content of the immunoreactive ricin in each fraction was determined by ricin EIA. Peak fractions were combined, concentrated and subjected to SDS-PAGE. The separated proteins in the gel were detected by immunobinding analysis using anti-PEG₂-ricin antiserum after being transferred onto a Durapore filter (Millipore). This was followed by reaction with donkey anti-rabbit IgG 125 I-labeled whole antibody, washing and auto-

Fig. 1. *In Vivo* Digestion of Ricin in Rats

Ricin (10 mg/kg) emulsified with olive oil was orally administered in rats. Immunoreactive ricin in stomach, small intestine, large intestine and feces was determined at various time intervals by EIA. The results were expressed by percent ratio of the remaining ricin to the given dose, and represented as mean of 2 experiments.

radiography. Recoveries of ricin from the biological samples were determined separately and found to be between 85% (blood) and 75% (liver homogenate).

Additionally, the plasma and lymph of intoxicated rats collected at the indicated times were tested for toxicity. Each 250 μ l of the fluids was i.p. injected to mice and the death of mice was observed after 24, 48, and 72 h.

Results and Discussion

We have reported in the previous papers that ricin administered orally in rats resulted in the severe damage to the epithelial cells of small intestine suggesting that this harmful effect on the

gastrointestinal tract could be one of the possible causes of oral toxicity of ricin.¹⁰⁻¹²⁾ Recently, a number of studies have appeared on the absorption of several kinds of proteins from the intestine to the circulating fluids.^{14-17, 23, 24)} Thus, trace amounts of proteins after oral administration have been clearly shown to be absorbed from the intestine into blood or lymph. It was also shown that ricin given intravenously in rats was distributed in liver, spleen, lymph nodes, mesenterium as well as in the gastrointestinal tract with congestion, edema and necrosis.²⁵⁾ The above observations suggest that ricin administered orally might be absorbed into the circulating system and would give rise to deleterious effects on organs other than the intestine. In the present study, we have examined the fate of orally-given ricin in the rats, *i.e.*, absorption and distribution of immunoreactive ricin for the elucidation of the mechanism of oral toxicity of ricin.

Distribution of Orally-Given Ricin in the Rat

The amount of ricin in the digestive system and feces of rats was determined by EIA after ricin (10 mg/kg) was orally administered. As shown in Fig. 1, about 40% of ricin was found to translocate from the stomach to the large intestine within 12 h and remained there for a rather long time (—72 h) though it gradually decreased. It was also found that about 20% of the ricin given was excreted in the feces for 72 h and that the total recovery of ricin became to about 45% at 72 h. The results suggest that the transfer of ricin into the circulating system had occurred and the decrease in the total immunoreactive ricin in the gut shows the degradation of ricin into small peptides which are insensitive to the present assay.

Transfer of Ricin to the Circulatory Systems

The transfer of orally-given ricin from the gastrointestinal tract to the circulating systems, was determined with the lymph and the plasma collected at 1 h intervals by EIA. In Fig. 2(A), ricin appeared in the lymph after 1 h, reached at the maximum at 2 h and later remained under 0.1 μ g/ml until 6 h, while ricin appeared more slowly in the plasma reaching to its maximal level after 4 h and remained constant thereafter. Based on the amount of ricin absorbed into the plasma and

Fig. 2. (A) Concentration of Ricin and CBH in Plasma and Lymph and (B) Transfer of Ricin in Plasma and Lymph

(A) Ricin (10 mg/kg) or CBH (10 mg/kg) was administered in rats as olive oil suspension. The amount of lectins in plasma and lymph was determined by EIA. The results were represented as mean $\pm$ S.E. of 5 experiments.

(B) Ricin absorbed into the plasma and lymph was calculated by the area under the curves of ricin concentration described above.

●, ricin in plasma; ▲, ricin in lymph; ○, CBH in plasma; △, CBH in lymph.

lymph, the apparent total amount of ricin was estimated by the areas under the curves and the results are shown in Fig. 2(B). It was shown that $8.0 \mu\text{g}$ ricin or 0.27% of the dose was transferred to the blood and $0.7 \mu\text{g}$ ricin or 0.02% to the lymph after 6 h, indicating that ricin transfer to the blood was greater than that to the lymph.

According to the results of Sekine *et al.*,¹²⁾ the gastrointestinal damages began to be significant at 2 h after oral administration of ricin with the same dose as ours (10 mg/kg) and became more thereafter. We have reported in *in vitro* experiments that ricin contained in the outer incubation medium was transferred to the inner solution of the everted sacs prepared from the jejunum in rats.¹¹⁾ Further, Yoshikawa *et al.*, employing human fibroblast interferon which is thought to be non-toxic, showed that interferon (20 kDa) was never transferred to the blood after injection of emulsified interferon into the intestine.²³⁾ When CBH (120 kDa),²⁶⁾ a homologous protein of castor bean with ricin, was given orally as a less toxic control,¹³⁾ immunoreactive CBH was scarcely detected in the plasma for 6 h, while it appeared in the lymph after 2 h although the amount of absorbed CBH was as a half as that of ricin (Fig. 2(A)). These results seem to suggest that the transfer of ricin into the blood as

found above was due to destructive damages of the intestine by the deleterious effects of ricin, whereas CBH was less because of its incapability of absorption from the intestine due to its weaker toxicity. It is, however, unclear whether the lectin activity of ricin, another significant biological property, plays any role in the absorption of ricin from intestine to the circulating system, and further study will be necessary.

On the other hand, with regard to the transfer to the lymph, those of ricin and CBH both reached the maxima at 2 h and later decreased. Yoshikawa *et al.* showed that the transfer of interferon into the lymph reached maximum within 1–3 h after intestinal administration.²³⁾ Further, Warshaw *et al.* reported that by the oral administration of BSA (68 kDa) and horseradish peroxidase (30 kDa) their transfer to lymph became significant after 1 h.^{27,28)} Irrespective to the nature of proteins and forms of injections (olive oil emulsion and saline solution), the protein transfer from the intestine to lymph is thought to be rather fast, giving its maximum concentration within a short time (1–3 h). Comparing these reports with ours, it was concluded that a part of ricin given orally was transferred to the blood by a different mechanism from that to the lymph.

Distribution of Ricin in the Organs

Distribution of orally-given ricin in the various organs of rats was investigated by the EIA determination of immunoreactive ricin in the homogenates of the organs such as liver, kidney, pancreas, spleen, lung, heart and brain taken at various times between 0–72 h. Ricin was detected in liver first at 6 h, its amount increased gradually with time and reached its maximum at 48 h ($95 \pm 20 \mu\text{g}/\text{organ}$) and decreased thereafter (72 h). In spleen, ricin appeared after 3 h, reached to its maximum level ($0.1 \pm 0.025 \mu\text{g}/\text{organ}$) at 6 h and kept its level till 72 h. Ricin was, however, undetectable by this EIA in pancreas, kidney, lung, heart and brain between 6 h and 72 h.

Koga reported after i.p. injection of ^{131}I -ricin D into rats that radioactivity was found in pancreas, liver and small intestine after 5 h,⁵⁾ and Godal *et al.* demonstrated by i.v. injection of ^{125}I -ricin into mice and whole-body autoradiog-

Fig. 3. Ricin Concentration in Plasma after Different Routes of Administration

Ricin dissolved in PBS (0.3 mg/kg) was administered intravenously, injection *via* portal vein, and lymphatic injection. Plasma was obtained at indicated times was analyzed by EIA for the determination of immunoreactive ricin. The results were represented as mean $\pm$ S.E. of 3 experiments. Routes of administration: $\bullet$ — $\bullet$, intravenous injection (i. v.); $\blacktriangle$ — $\blacktriangle$, injection *via* portal vein (p.v.); $\blacksquare$ — $\blacksquare$, injection in the mesenteric lymphatic (lym.).

raphy that the strong accumulation of radioactivity was observed in liver and spleen, moderate activity in kidney after a short period (30 min—3 h), but no radioactivity in the whole body at 24 h.²⁵⁾ However, it was obvious as above that immunoreactive ricin was undetectable in kidney in the case of oral administration. The present results probably indicate greater contribution of the ricin transfer from the blood stream to the organs than that from the lymph. Ricin absorbed from the gastrointestinal tract into the blood will

be primarily accumulated in the liver and later be metabolized. The detection of smaller amount of ricin in spleen might be due to the presence of ricin receptors or unknown factors which remain to be clarified.

Effect of the Route of Administration on the Distribution of Ricin in Rats

In order to know whether the transfer and distribution described in the previous section was dependent on the route of administration or not, ricin (0.3 mg/kg rat) was separately administered

Fig. 4. (A) Distribution of Ricin in Liver and Spleen and (B) Concentration Ratio of Ricin in Organs and Plasma
 (A) Ricin-PBS solution (0.3 mg/kg) was given to rats as described in the legend of Fig. 3. Liver and spleen were removed at indicated times and the concentration of ricin was determined by EIA as described in the text. The results were represented as mean $\pm$ 3 experiments. ●—●, i.v.; ▲—▲, p.v.; ■---■, lym.
 (B) Concentration ratio was obtained by dividing the ricin concentration of liver and spleen at 6 h after administration by that of plasma. The ratio obtained by oral administration were also included.

Fig. 5. Characterization of Immunoreactive Ricin in the Biological Samples (A) Gel Filtration of Plasma from Ricin-Intoxicated Rats, (B) Gel Filtration of Lymph and (C) Gel Filtration of Liver Extract on Sephadex G-75

(A) Plasma prepared from the blood collected at 90 min after *p.o.* administration of ^{125}I -ricin (1×10^7 cpm) was subjected to gel filtration through a Sephadex G-75 column (2.5×40 cm). Radioactivity in each fraction was determined and the peak 1 was further analyzed by SDS-PAGE as shown in the insert.

(B) The lymph collected for 3 h since *p.o.* administration of ricin (10 mg/kg) emulsified with olive oil was analyzed by gel filtration through a Sephadex G-75 column (2.5×40 cm). Immunoreactive ricin in fractions was determined by EIA. The insert represents an immunoblot of SDS-PAGE of the peak fraction.

(C) The extract was prepared from liver removed at 48 h after *p.o.* administration of ricin (10 mg/kg), and applied on to the same Sephadex G-75 column as (B). Immunoreactive ricin in each fraction was determined by EIA. The insert represents an immunoblot of SDS-PAGE of the peak fraction.

by the different routes, intravenous injection (i.v.), injection *via* portal vein (p.v.) and lymphatic injection. The concentration of ricin in plasma and in the organs (liver and spleen) were determined with EIA. When ricin was administered either intravenously or by injection *via* the portal vein, the plasma ricin disappeared rapidly within 2 h to the constant level (1 $\mu\text{g}/\text{ml}$), while by the lymphatic route later appearance of ricin into the plasma was observed (Fig. 3). After 2 h, however, the plasma ricin concentration remained constant (approximately 1 $\mu\text{g}/\text{ml}$) until 6 h regardless of the routes.

Distribution of ricin in liver and spleen were compared by the different routes of administration. As shown in Fig. 4(A), about 20% of the dose was found by injection *via* portal vein in the liver after 3 h whereas a less (about 15%) by either intravenous or lymphatic injection. In the spleen, lymphatic administration resulted in the highest ricin concentration after 3 h although any route could not yield as high a concentration as that in liver. These results show that ricin is primarily distributed in the liver by injection *via* the portal vein and in the spleen by the lymphatic route. Figure 4(B) represents organ/plasma concentration ratios calculated from the data given above. In the spleen, the ratios obtained from any route were found to be less than 1.0, while in the liver higher ratios (5–8) were obtained in all cases. From the definition, when this ratio is higher than 1.0, one can assume that ricin in the plasma was eventually transferred to an organ, *i.e.* accumulation. It was, therefore, considered that orally-given ricin tends to accumulate in the liver mainly *via* the portal vein. Since a larger amount of ricin was transferred to blood than that to lymph (Fig. 2(B)), ricin found in the liver was mainly derived from the blood stream.

Characterization of “Immunoreactive” Ricin in the Biological Samples

In order to know whether ricin hitherto determined with EIA is the intact ricin or not, the biological samples except from the plasma prepared from the ricin-intoxicated rats were successively analyzed by gel filtration, SDS-PAGE, and an immunobinding method. Plasma was prepared from the blood collected at 90 min after oral administration of ^{125}I -ricin and analyzed

with gel-filtration and SDS-PAGE followed by autoradiography. As shown in Fig. 5(A), two radioactive peaks, one being eluted at 75 ml where the authentic ricin ($M_r = 64 \text{ kDa}$) is eluted, and the other being eluted at 178 ml where free ^{125}I is eluted were found. Analysis by SDS-PAGE as shown in the insert revealed that the first peak contained a protein with the closely similar molecular weight as ricin, whereas no high molecular weight compounds were detected in the second peak (data not shown). The recovery of the first peak in radioactivity was only 26%, whereas it was 70% for the second peak. Samples obtained at 3, 4 and 5 h, respectively, after *p.o.* administration of ^{125}I -ricin gave apparently one peak at 178 ml with the recovery of more than 90% (data not shown). It was considered that ^{125}I was easily released during the circulation from the labeled ricin when it was given orally. Figures 5(B and C) represent the results of the lymph after 3 h and the liver homogenate after 48 h, respectively. In both cases, a single immunoreactive peak was detected at the same position (75 ml) as ricin itself. Analysis of the peak fractions with SDS-PAGE followed by the immunobinding method and autoradiography revealed that both peaks corresponded with the authentic ricin and subunits under the non-reducing and reducing conditions, respectively.

In addition, intravenous injection of either the lymph obtained after 2 h or the plasma collected at 4 h after oral administration of ricin caused the death of all mice used. The injected immunoreactive ricin in the plasma and lymph were about 0.1 $\mu\text{g}/\text{dose}$ which is equivalent to the reported lethal dose of ricin (Fig. 2(A)). The results suggested that toxic immunoreactive ricin has been transferred into both the blood and lymphatic streams from the small intestine.

It was thus concluded that the immunoreactive ricin found in blood, lymph and liver were a closely similar one in its molecular size and toxicity without any presence of degraded forms, which, if present, are undetectable by the present method.

In conclusion, ricin, a macromolecular and toxic lectin, was absorbed after *p.o.* administration from the small intestine differently into the

blood and lymph, followed by the transfer to tissues in rats, the highest fraction being present in liver, about one tenth of 1% of that in spleen and undetectable in kidney, pancreas, heart, lung and brain, respectively. It was also demonstrated that ricin was found to be closely similar to the intact ricin even after the transfer.

These results seem to indicate for the mechanism of oral toxicity that the death of rats was caused by a mechanism similar to that of i.v. or i.p. injection of ricin. However, there still remains a possibility of the presence of an undetectable amount of active ricin in other organs, which would give rise to a lethal effect. More detailed biochemical and pathological study will be necessary before we understand the oral toxicity of ricin.

Acknowledgement This work was supported in part by a grant from the Ministry of Education, Science and Culture, Japan. The authors are grateful to Professor Junzo Nakamura, School of Pharmaceutical Sciences, Nagasaki University, for earnest advice and discussion during the course of this study.

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