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Effects of Ricin, a Protein Toxin, on Glucose Absorption by Rat Small Intestine. (Biochemical Studies on Oral Toxicity of Ricin. II¹⁾)

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The effects of ricin, a proteinous toxin from castor bean seeds, on glucose absorption by rat small intestine have been examined by the everted sac method. Glucose absorption was affected by ricin poisoning at 1 h after oral administration, and the inhibition reached the maximum at 5 h, whereas very slight impairment of glucose absorption was observed at 5 h after intraperitoneal injection of ricin. The dose required for 50% impairment of glucose absorption was 10 mg ricin/kg body weight of rat when determined at 5 h after oral administration. This inhibition of glucose absorption was found only when ricin or ricin B-chain had been in contact with the mucosal membrane of the small intestine of the normal rats. The inhibition was prevented by the presence of a galactose-containing sugar, lactose. The effect of ricin on glucose absorption under physiological conditions was analyzed *in situ*, and the increase in blood glucose level was inhibited in ricin-intoxicated rats.

These results suggest that ricin, especially its B-chain, interacts primarily with the intestinal mucosa and inhibits sugar absorption of the rat small intestine. It was also inferred that ricin B-chain is cytotoxic to the epithelial absorptive cells of the small intestine, but that impairment of sugar absorption by the small intestine alone is not the direct cause of death of animals following oral administration of ricin.

Keywords—ricin; phytotoxin; oral toxicity; glucose absorption; everted sac method; small intestine

In recent years, several proteins interacting with the carbohydrate moieties of cell surface glycoproteins and/or glycolipids have been isolated.^{2,3)} One of them, ricin, a highly toxic glycoprotein that occurs in the seeds of *Ricinus communis* L. (Euphorbeaceae), has been purified and studied extensively and the mechanism of its action is now understood at the molecular level.^{4,5)} Ricin is composed of subunit B (MW 32000) which binds the toxin to galactose-containing receptors of the plasma membrane, and subunit A (MW 30000) which blocks protein synthesis by modification of the 60S-ribosomal subunit.⁴⁾ The two subunits are linked by a disulfide bridge.⁶⁾

In a previous paper, we have shown that ricin exerted its toxic action after oral administration even though it is a proteinous substance which would be degraded in the stomach and intestines. It was also inferred that ricin administered *p.o.* acted primarily on the intestinal mucosa and impaired sugar absorption of the small intestine.¹⁾ The aim of the present work was to establish whether the impairment of sugar absorption is a characteristics of the oral administration of ricin or not, and if so, which subunit of ricin is responsible for this inhibition and further whether this would occur *in situ*. The results presented here seem to

suggest that the early inhibition is characteristic to oral administration and that the subunit B of the ricin molecule is mainly responsible for this impairment, although the possibility still remains that subunit A may cause later impairment.

Experimental

Ricin was prepared according to Hara *et al.*⁷⁾ Subunits of ricin were prepared by reducing the single disulfide bond with 2-mercaptoethanol in the presence of 0.5 M lactose at 25 °C overnight, followed by DEAE- and CM-cellulose column chromatographies.⁸⁾ Each isolated subunit was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis⁹⁾ and was stored as a precipitate in saturated ammonium sulfate solution. Male albino rats of Wistar strain (body weight 180–270 g) were used. Animals were fed with a commercial chow *ad libitum* before the initiation of the experiments. Thereafter they were fasted for 24 h but allowed water *ad libitum*.

Everted sacs from the jejunal portion of the rat small intestine were prepared according to Wilson and Wiseman.¹⁰⁾ After the freed intestine had been everted over a long stainless steel probe, segments of jejunum were filled with approximately 0.5 ml of the fluid medium (the serosal fluid, SF; Krebs–Ringer bicarbonate solution, pH 7.4, KRBS) and the end was tied off to form an individual sac (2–4 cm long and 0.5–1.0 cm diameter). Sacs were incubated singly in 30 ml Erlenmeyer flasks containing additional medium (the mucosal fluid, MF). After being gassed with 5% CO₂–95% O₂, the sacs were incubated at 37 °C. D-Glucose absorbed was determined colorimetrically by the *o*-toluidine-boric acid method as described previously¹¹⁾ and the results are expressed as µg D-glucose absorbed per 100 mg tissue per h.

Absorption of D-Glucose by the Rat Small Intestine—Exp. I: Rats received ricin *p.o.* (30 mg/kg) or intraperitoneally (0.5 mg/kg), and were killed at 1, 5 and 10 h. Everted sacs were prepared as described above and filled with KRBS. Other groups of rats received subunit A or B (30 mg/kg) *p.o.*, and were killed at 5 h. Everted sacs were prepared as described above. Sacs were incubated singly against 5 ml of 0.5% D-glucose solution for 1 h.

Exp. II: The normal rats were killed and the everted sacs were prepared as above. The everted sacs were filled with KRBS and placed in flasks containing 0.05% ricin, or subunit A or B in KRBS. In some cases, ricin or a subunit was placed on the serosal side. The sacs were preincubated at 37 °C for 40 min then washed with KRBS three times and incubated against 0.5% D-glucose solution at 37 °C for 20 min. In other experiments, the effect of lactose was tested in this system; the preincubation of normal sacs was carried out in the presence of both 0.05% ricin and 0.03% lactose at 37 °C for 40 min.

Exp. III: The fasted rats received ricin (30 mg/kg, *p.o.*) and the intoxicated animals were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) at 4.5 h after administration of ricin. At every 15 min starting from 5 h after ricin administration, blood (0.1 ml) was withdrawn by cannulation from the femoral vein, and blood sugar was determined as described above. After the 3rd blood withdrawal, *i.e.*, at 5.5 h after ricin administration, 1 ml of 50% D-glucose solution was directly introduced into the intestinal loop. Blood samples were collected at 15-min intervals until the death of the animals.

Light Microscopic Examination—The removed organs were fixed in 10% formalin. Segments taken from the small intestinal loops for histological study were opened and fixed in formalin. Thin sections were stained with hematoxylin–eosin and periodic acid–Schiff stain.

Results

As reported previously,¹⁾ rats and mice which received ricin orally died within 36 h. The dying animals showed characteristic severe diarrhea within 5–10 h. The first experiment of the present paper was aimed at testing whether this diarrhea and the impairment of sugar absorption were characteristic of the route of ricin administration, *i.e.* oral or intraperitoneal. As shown in Fig. 1, the inhibition of glucose absorption by the everted sacs from orally-intoxicated rats was significant at as early as 1 h, and 82% inhibition was found compared to that of normal sacs at 5 h, while no significant inhibition was found at 5 h in the rats intoxicated intraperitoneally, and only about 60% was found even after 10 h. No effect on glucose absorption was observed when either subunit of ricin was given *p.o.* [D-glucose absorption: subunit A $109.5 \pm 6.3\%$ and subunit B $102.9 \pm 4.1\%$ of the control]. On light microscopic examination, the intestinal mucosa of ricin-treated (*p.o.*) rats showed remarkable atrophy of the villi, whereas no changes of the intestinal mucosa of intraperitoneally-treated rats were apparent (Fig. 2). From these results, the early inhibition of glucose absorption

Fig. 1. Effect of Ricin Poisoning on Glucose Absorption by Rat Small Intestine

The effect of ricin administered orally or intraperitoneally on glucose absorption by rat small intestine was investigated by the *in vitro* everted sac method. Rats received ricin either orally (30 mg/kg) or intraperitoneally (0.5 mg/kg) and were sacrificed at 0 h (4), 1 h (2), 5 h (4), and 10 h (3). Numbers in parentheses are numbers of rats killed. Everted sacs were prepared as described in the text and placed in 5 ml of the mucosal solution [0.5% D-(+)-glucose in KRB solution, pH 7.4]. All sacs were incubated singly at 37°C for 1 h under gassing with 5% CO₂-95% O₂. Glucose in the serosal solution was determined colorimetrically as described in the text. Each value is the mean $\pm$ S.E.

I. Ricin 5 h

II. Ricin 10 h

Fig. 2. Microphotographs of Small Intestinal Mucosa of Rats Treated with Ricin

Rats given ricin *p.o.* (30 mg/kg) or *i.p.* (0.5 mg/kg) were killed at 5 and 10 h after ricin administration. Segments taken from the small intestinal loops were opened and fixed in 10% formalin. Thin sections were stained with hematoxylin-eosin and periodic acid-Schiff stain. The effect on intestinal mucosa of ricin given by different routes at different times is shown ($\times 100$). I. at 5 h; II. at 10 h.

Fig. 3. Dose-Response Curve of Inhibitory Effect of Ricin on Glucose Absorption by Rat Small Intestine

At 5 h after rats had received orally various doses of ricin, everted sacs were prepared and glucose absorption was determined as described in the text. The effective dose required for 50% inhibition, $[ED_{50}]$, was about 10 mg/kg. Each value is the mean $\pm$ S.E. of at least three experiments.

through rat small intestine was thought to be specific to oral administration of ricin.

The effect of dose of ricin on the impairment of glucose absorption by the small intestine derived from ricin-intoxicated rats (determined at 5 h after administration) is shown in Fig. 3. The effective dose required for 50% inhibition, ED_{50} , was about 10 mg/kg. No significant inhibition or villous degeneration was found at doses less than 5 mg/kg, and the maximum inhibition was attained with more than 15 mg/kg (Fig. 4, A-H).

It was shown in the previous paper that no inhibition of glucose absorption was observed when everted sacs derived from normal rats were incubated with 0.5% D-glucose in the presence of ricin. Further, after oral administration of subunit A or B, no rats died nor was inhibition observed, as described above. This seems to suggest that an isolated subunit is not resistant to the digestive enzymes. In order to establish which subunit is responsible for the inhibition of glucose absorption described above, a completely *in vitro* method is required. In experiment II, we preincubated the everted sacs derived from normal rats with 0.05% ricin at 37°C for 40 min, and washed the sacs with KRBS. Finally the treated sacs were incubated with 0.5% D-glucose for 20 min and D-glucose transport was determined. Figure 5 shows the *in vitro* effect of ricin on glucose absorption, and reveals that the inhibition occurred only when ricin had been in contact with the mucosal membrane of the rat small intestine, while no inhibition was found when ricin was present in and preincubated with the serosal solution. In the same system, the inhibition of glucose absorption was achieved by ricin B-chain as well as ricin, but not by ricin A-chain, as shown in Fig. 5. It was also clear from Fig. 5 that lactose completely blocked the inhibitory effect of ricin.

In experiment III, the effect of ricin poisoning on glucose absorption by rat small intestine was studied *in situ*. The experimental schedule is shown in Fig. 6. As shown in Fig. 6, when 500 mg of D-glucose was directly introduced into the small intestine of the normal, anesthetized rat, the blood sugar level increased from 120 to 249 mg/dl, *i.e.* a 210% increment after 15 min. On the other hand, the increase in the blood sugar level of the ricin-intoxicated rat was only 45% of the basal level (from 130 to 189 mg/dl after 15 min).

Discussion

Ricin is one of the very few proteinous toxins which elicit toxic action on oral administration. No systematic study has previously been carried out on the oral toxicity or biodegradation of ricin.

When rats were treated with ricin (30 mg/kg, *p.o.*), changes in the villous structure of the intestinal mucosa were seen at 5 h after administration. Moreover, the intoxicated rats suffer-

Fig. 4. Effect of Various Amount of Ricin Administered Orally on Rat Small Intestinal Mucosa

Rats were given various doses of ricin orally and were killed at 5h. Small-intestinal mucosal preparations were obtained and examined by microscopy as described in the text. Atrophy and degeneration of villi were clearly seen at ricin doses of more than 10 mg/kg ($\times 100$). A) normal; B) ricin (1 mg/kg); C) ricin (3 mg/kg); D) ricin (5 mg/kg); E) ricin (10 mg/kg); F) ricin (15 mg/kg); G) ricin (30 mg/kg); H) ricin (60 mg/kg).

Fig. 5. Effect of Ricin and Its Subunits on Glucose Absorption by Rat Small Intestine *in Vitro*

Normal rats were fasted for 24 h then killed and everted sacs were prepared as described above. Sacs were filled with either KRB solution or 0.05% ricin-KRB solution (S.F.: - or +), and incubated in KRB or ricin solution (M.F.: - or +) for 40 min at 37°C. The preincubated sacs were rinsed with fresh cold KRB solution and incubated against 0.5% glucose-KRB solution for 20 min at 37°C. Glucose transported into the serosal solution was determined colorimetrically. In the second experiment, sacs were preincubated with either ricin A-chain or ricin B-chain (each 0.05%) for 40 min at 37°C (M.F.: +). Inhibition of glucose absorption was determined as described above. The final experiment was conducted to determine the effect of lactose on the inhibitory action of ricin on glucose absorption by preincubating normal sacs in ricin-lactose-KRB solution (M.F.: +) and then analyzing glucose transport for 20 min at 37°C. Numbers in the columns are numbers of sacs used. Each value is the mean $\pm$ S.E.

Fig. 6. Inhibitory Effect of Ricin on Glucose Absorption by Rat Small Intestine *in Situ*

The fasted rats received *p.o.* ricin (30 mg/kg). At 4.5 h, rats were anesthetized with sodium pentobarbital (50 mg/kg, *i.p.*) and the abdomen was opened. At 5 h after ricin administration, 1 ml of 50% glucose solution was introduced directly into the intestinal loop and blood (0.1 ml) was withdrawn every 15 min until the death of the animals. Blood sugar was analyzed as described in the text. Each value is the mean $\pm$ S.E. of two experiments. (○—○ normal; ●—● ricin-intoxicated).

ed from severe diarrhea and died within 36 h. We suggested that ricin administered orally interacted primarily with the small intestine and impaired sugar absorption.¹⁾

In the present study, the inhibition of glucose absorption by everted sacs derived from ricin-intoxicated rats was found to be related to the impairment of the intestinal mucosal membrane. As shown in Figs. 1 and 2, the inhibition and the atrophy were observed as early as 5 h after oral administration, while the intraperitoneal injection of ricin significantly affected both events at 10 h, far later than in the case of oral administration. It was suggested that the early inhibition of glucose absorption was caused by the direct action of ricin on the intestinal mucosa, somehow impairing the epithelial absorptive cells. Since neither the isolated subunit A nor B (30 mg/kg, *p.o.*) killed rats and no inhibition of glucose absorption by the everted sacs derived from subunit-treated rats was observed, it is likely that the isolated individual subunits are digested in the stomach and small intestine. Funatsu *et al.* have also reported that the isolated subunits are not resistant to digestive enzymes.¹¹⁾ The inhibition of glucose absorption after oral administration was dose-dependent, as shown in Figs. 3 and 4.

Next, experiments were carried out to determine whether the inhibition of glucose

transport would occur *in vitro*. In the previous study, we could not find any significant inhibition of glucose transport when the everted sacs from the normal rats were incubated at 37 °C for 1 h in the presence of glucose and ricin. As described in the experimental section, the normal sac was preincubated with 0.05% ricin at 37 °C for 40 min and then the transport of sugar was determined by incubating the ricin-treated sac with glucose for 20 min. In this completely *in vitro* system, the inhibition of glucose absorption was reproduced only when ricin or ricin B-chain had been in contact with the mucosal membrane of the everted sac, as shown in Fig. 5. It was also clear that lactose, a galactose-containing sugar, blocked this inhibitory effect. Lactose or galactose is known to block the binding of ricin/ricin B-chain to the receptors of the cell surface.²⁾ From these results, it was inferred that inhibition of glucose absorption by the small intestine was caused by the binding of ricin or ricin B-chain to the surface cells of the mucosal membrane (epithelial absorptive cells). It may be speculated that ricin binds to the surface receptor of the epithelial absorptive cells but also causes the structural changes of the membrane, which in turn would result in the death of the cells. In this connection, it has already been reported that ricin B-chain causes structural changes of the membrane of human peripheral lymphocytes, disturbing the membrane fluidity.¹²⁾ An *in vitro* study on the interaction of ricin with epithelial absorptive cells of the rat small intestine is being undertaken.

At present, however, it is not certain whether the destruction of the surface cells and the impairment of glucose absorption by the small intestine caused by ricin are directly related to the oral toxicity of ricin. For example, when ricin was administered to rats intraperitoneally, inhibition of sugar absorption by the intoxicated small intestine was observed just before death without significant changes in the mucosal membrane. As reported previously, the amount of ricin absorbed by the small intestine was determined by radioimmunoassay to be 0.006% or 180 ng/rat *in vitro*, and 0.015% or 510 ng/rat *in vivo*. The amount of ricin absorbed could account for the death of the rats if it were transferred to the circulatory system as intact toxin. The nature of the absorbed ricin, however, remains unknown.

In conclusion, inhibition of glucose absorption by the small intestine was caused on the one hand by the direct contact of ricin or ricin B-chain with the mucosal membrane, followed by disturbance of cell membrane structure, although a possible effect of ricin A-chain cannot be excluded. On the other hand, the late inhibition could be explained by the delay of degeneration of absorptive cells, probably resulting from the inhibitory action of ricin on cell growth, especially by interfering with protein synthesis.

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