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Protection of Mice from Ricin by Oral Administration of Ricinus communis Agglutinin

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Background: Ricin is a potent plant toxin known to cause cell death by inhibiting protein synthesis. The currently used vaccine is prepared mainly from biohazardous toxin. Thus, this research studied an alternative protein, *Ricinus communis* agglutinin (RCA), which has the same structure as ricin but much lower toxicity, and demonstrated that RCA provided sufficient protection against ricin in a murine model. **Method:** The serum antibody levels were determined using ELISA. The ricin-neutralized antibody, protection against ricin and cross-protection against abrin were assayed under direct challenge of lethal-dose ricin. **Results:** After four cycles of immunization, mice evoked high levels of serum antibodies and survived lethal doses of ricin. Immunized mice also elicited partial cross-protection against the challenges of abrin. The serum antibody titer of RCA-immunized mice was almost the same as that of ricin toxoid-immunized mice. Serum antibody titers induced by i.p. route immunization with either RCA or ricin toxoid were slightly higher than those induced by oral route with RCA, but there is no difference between RCA (i.p.) and ricin toxoid (i.p.) or RCA (i.p.) and RCA (oral). **Conclusions:** Serum from the immunized animals that had received RCA either i.p. or orally showed completely neutralized ricin activity. This is the first study demonstrating the potential use of RCA as an oral vaccine candidate for ricin protection.
Key words: Ricinus communis agglutinin (RCA), oral vaccine, ricin

INTRODUCTION

Ricin and Ricinus *communis* agglutinin (RCA) are homologous lectins that can be isolated from the castor bean plant *Ricinus communis*.[1,2] Ricin (M.W. approx. 65,000), which contains an A chain and a B chain linked by a disulfide bond, is extremely toxic to eukaryotic cells while RCA (M.W. approx. 130,000), which is composed of two A chains and two B chains, is relatively non-toxic. Ricin kills eukaryotic cells by inhibiting protein synthesis. The B chain binds specifically to galactosyl residues on the cell surface and appears to trigger the endocytic uptake of ricin molecules.[3] The A chain is separated from the B chain in the cytoplasm of the cell and inactivates enzymatically the 60S ribosomal subunit, thus disrupting protein synthesis of the cell.[4] The toxin has been extensively studied in both medical and basic research since its discovery in the 1880s and is one of the most toxic compounds known, with a mouse intravenous LD_{50} of 2.7 μg/kg.[5] The potential application of the toxin to cancer therapy, and its use in many laboratories throughout the world as a useful tool in biomedical studies have revealed potential health problems.[6,7] The toxin is also considered a potential threat to selected populations as a potential agent of warfare or terrorist attack.[8,9] Therefore, protection against possible exposure would be desirable.

Previous investigators have shown that mice can be protected by passive immunization with ricin toxoid,[10,11] and ricin intoxication is prevented if antibody is given soon enough after toxin administration.[12] Although offering complete protection against ricin challenge, ricin toxoid must be carefully prepared because it is extremely toxic to human.

This study used RCA, a natural analogue of ricin with weak toxicity, as a vaccine to immunize mice and compare it with ricin toxoid in vaccination and challenge study. When administrated orally, RCA was found to evoke systemic antibody production and immunity against native toxin.

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MATERIALS AND METHODS

Materials

Abrin was purified from the beans of *Abrus precatorius* using the method of Hegde.[13] Sepharose 6B and Sephacryl S-200 columns were obtained from GE Pharmacia (Uppsala, Sweden). Alkaline phosphatase-labeled goat anti-mouse IgG antibody and p-nitrophenol phosphate were obtained from Sigma (St. Louis, MO, U.S.A.). Protein sequence was aligned by Basic Local Alignment Search Tool (BLAST®, National Center for Biotechnology Information, U.S.A.).

Purification of ricin and RCA

Ricin and RCA were purified from unshelled castor beans using the method of Lin and Liu with minor modification.[14] Through acid extraction and 60% ammonium sulfate fractionation, the soluble protein of the dialyzed ammonium sulfate fraction obtained from 200 g of beans was applied to a 5×24 cm Sepharose 6B column equilibrated at 25°C in PBS. After washing the column with 1 to 1.5 liters of buffer, a fraction containing both ricin and RCA was eluted with 200 ml of PBS containing 0.1 mM galactose. The fraction was concentrated to 5 ml and applied to a 2.5×60 cm Sephacryl S-200 column equilibrated at 25°C in PBS. The column was eluted with the same buffer and toxin fractions were collected. The purity of purified ricin and RCA were analyzed by SDS-PAGE under 8% polyacrylamide gel and non-reducing condition.

Immunization and sample collection

Eight-week-old female mice (25 g) were immunized intraperitoneally (i.p.) or orally. For i.p. injection, each animal received 2 μg of protein in 0.1 ml of PBS. For oral administration, each animal was fed 4 μg of protein in 0.2 ml of PBS administered through an intragastric feeding needle. The mice were immunized on day 0, and boosters were given on days 14, 28, and 42. Samples of serum from identically immunized mice were collected and pooled on days 21, 35, and 49. For collection of serum, the mice were bled through heparinized capillary tubes at the retro-orbital plexus under isofiurane anesthesia.

Titration of sera by enzyme-linked immunosorbent assay (ELISA)

Sera were titrated for ricin-specific antibody with an ELISA by first adsorbing 0.1 μg ricin onto each well of a 96-well microplate. After incubating at 4°C overnight, wells were then blocked for 4 h with PBS containing 0.1% bovine serum albumin and 0.1% casein. Serial dilutions of sera were added and the plates were incubated at 37°C for 2 h. Phosphatase-labeled goat anti-mouse IgG antibody was added and the plates were incubated at 37°C for 2 h. The plates were developed at 25°C for 10 min with p-nitrophenol phosphate in carbonate buffer, pH 9.8, and the absorbance (A) was read at 405 nm in an ELISA reader. The titer was defined as the reciprocal of the highest dilution having an A_{405} > 0.2 after corrections for background. Statistical significance was calculated by two-tailed t-test.

Serum neutralization assay

Ricin (5.2 μg) was incubated with 10 μl of preimmune or immune serum at 37°C for 1 h. Subsequently, the incubation mixture was diluted with 80 μl of PBS-BSA and injected intraperitoneally. The mice were monitored for 12 days to assess the residual toxicity of various mixtures.

Challenge of immunized mice with ricin and abrin

At 3 months after the third booster, immunized mice were challenged i.p. with 50 μg of ricin per animal. The survival of challenged animals was monitored for 12 days. Immunized mice were also challenged with 50 μg of abrin and the survival rate was monitored.

RESULTS

Purity of RCA and ricin

To avoid extreme toxicity of ricin, the RCA was highly purified by galactose affinity and gel filtration chromatography. The last molecular sieve chromatogram is shown in Fig. 1. The first peak containing RCA and the second peak containing ricin were analyzed by SDS-PAGE. The RCA lane showed two bands with M.W. 16,500 and 14,500, which represented two isoforms of RCA (Fig. 1 insert).[2] There were no cross contamination of ricin and RCA.

Serum antibody response in mice immunized with RCA and ricin toxoid

Mice were immunized i.p. 2 μg with RCA, orally 4 μg with RCA, i.p. 2 μg with ricin toxoid, and PBS-BSA as control to assess the ability of these proteins to evoke a serum immune response. All the animals were given booster doses on days 14, 28, and 43. Samples of sera were collected on days 21, 35, and 49 after immunization and analyzed by ELISA for antibody reactivity to

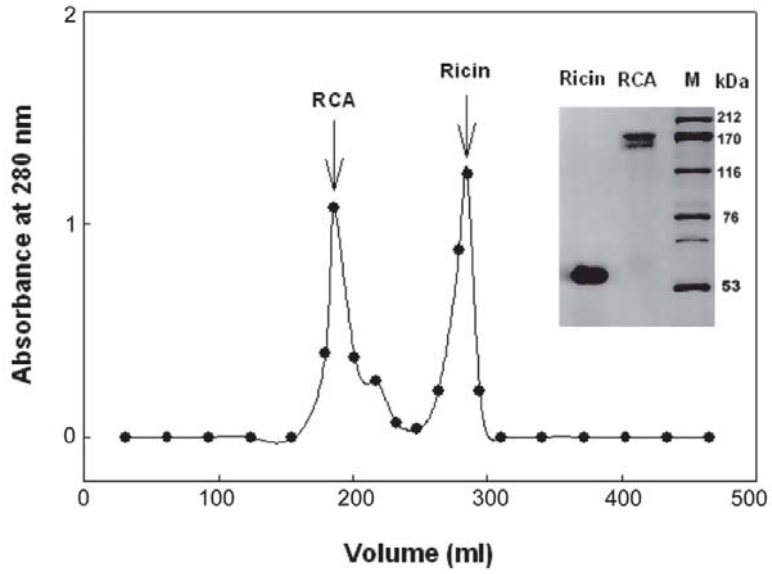

Fig. 1 Purification of Ricin and RCA
The galactose-eluted fraction from the Sepharose-6B column was applied to a Sephacryl S-200 column and eluted with PBS.
Insert: SDS-PAGE of purified ricin and ricin hemagglutinin. The samples were run under 8% polyacrylamide gel and non-reducing condition. Each lane contains 2 μg of protein.

Table 1 Summary of significant serum antibody titers induced by i.p. route immunization with either RCA or ricin toxoid
The antibody titers were slightly higher than those induced by oral route with RCA but there is no apparent statistical difference between RCA (i.p.) and ricin toxoid (i.p.) or RCA (i.p.) and RCA (oral).

Immunization	ELISA titer[a] Group Mean ± SD	p[b]
RCA (i.p.)	10400±2055 (n = 10)	0.21
Ricin toxoid (i.p.)	11520±1576 (n = 10)	0.15
RCA (oral)	9600±2491 (n = 7)	

[a]Serum was bled from each mouse individually. Titer is the reciprocal of the highest dilution having an A405 > 0.2 after corrections for background.
[b]Difference in titer value between ricin toxoid (i.p.) / RCA (i.p.) group and ricin toxoid (i.p.) / RCA (oral) were tested with two-tailed Student's t-test, the P values were 0.21 and 0.15, respectively and showed no significant difference (α = 0.05).

ricin. Sera collected after the third boost (day 49) induced high titer anti-ricin antibodies. The mean antibody titers at day 49 and standard error of each group were given in Table. 1. The average serum antibody titer of RCA i.p. immunized mice was almost the same as that of ricin toxoid i.p. immunized mice. Serum antibody titers induced by i.p. route immunization with either RCA or ricin toxoid were slightly higher than those induced by oral route with RCA but there is no apparent statistical difference between RCA (i.p.) and ricin toxoid (i.p.) or RCA (i.p.) and RCA (oral) (Table. 1).

Neutralizing activity of serum from immunized mice

Experiments were performed to assess the ability of various serum samples to neutralize ricin (Fig. 2 insert). The following four different sources of serum were tested: (1) PBS-BSA immune serum (control), (2) serum from animals that received ricin toxoid i.p., (3) serum from animals that received RCA i.p., and (4) serum from animals that received RCA orally. The toxicity of ricin incubated with non-immune serum was equivalent to that of toxin incubated in physiological saline. The toxicity of ricin incubated with serum from animals that had received ricin toxoid showed completely neutralized ricin activity. Serum from animals that had received RCA either i.p. or orally also showed completely neutralized ricin activity (100% inhibition; Fig. 2 insert).

Protection of mice against challenge with ricin

Data in the preceding section indicate that mice immunized with ricin toxoid should show resistance against toxin. At 3 months after the final boost, the experimental mice from which serum was obtained and tested earlier in the serum neutralization assay (see above) were challenged i.p. with ricin (50 μg per animal). As predicted, animals that received RCA either i.p. or orally had protection against challenge with ricin (Fig. 2).

Since the amino acid sequences of ricin and abrin showed 50% identities (BLAST® sequence alignment), it was hypothesized that mice acquire cross-protective ability against abrin through ricinus antigen immunization. Therefore, the protection assay for abrin was also examined. After an abrin (50 μg) challenge, 30% of ricin toxoid-vaccinated mice (n = 10), 20% of i.p. RCA-vaccinated mice (n = 10) and 14% of orally RCA-vaccinated mice (n = 7) survived. Ricin toxoid-vaccinated mice had longer life span after abrin challenge (Fig. 3). Although

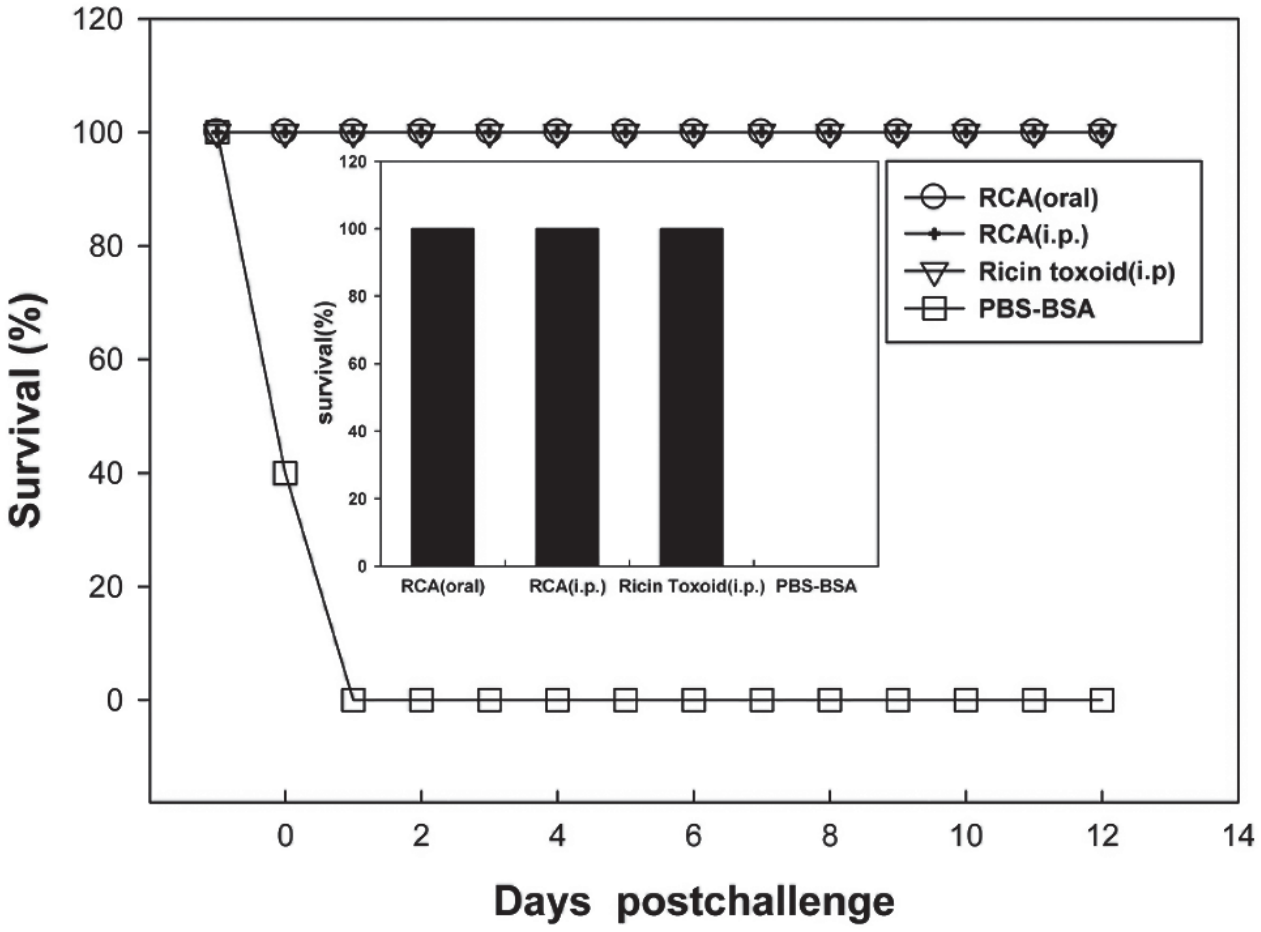

Fig. 2 Protection of mice immunized with RCA or ricin toxoid
Serum neutralization assay (insert). Ricin (5.12 μg) was incubated with serum from mice immunized with RCA i.p., ricin toxoid i.p., RCA oral or PBS-BSA. Toxin mixtures were injected i.p. into nonimmunized mice (four animals/group), and percent survival was determined at day 12. Direct challenges on immunized animals. Ricin (50 μg) was injected i.p. into mice immunized with RCA i.p. (+, n = 10), ricin toxoid i.p. (▽, n = 10), RCA oral (○, n=7) or PBS-BSA (□, n=10). The percent survival was monitored for 12 days.

the protection against abrin was less efficient than that against ricin, the development of a multivalent hemagglutinin vaccine for ricin and abrin remains a possibility.

DISCUSSION

RCA and ricin are homologous in structure and function. The amino acid sequences of ricin and RCA have 89% identities (BLAST® sequence alignment). Moreover, both show immunological partial identity. However, RCA is 2000 times less toxic than ricin to mice upon intraperitoneal injection.[15] The reason for the much lower toxicity of RCA compared with ricin is unknown. Owing to immunological and structural similarity between RCA and ricin, RCA is regarded as a natural mutant of ricin with weak toxicity. In this study, after immunization with native RCA, mice were found to have smooth fur and their level of activity and food consumption showed no decrease.

The development of oral vaccines that evoke systemic immunity is one of the major challenges facing investigators interested in the control of infectious diseases and various forms of poisoning.[16] This task is made challenging by the fact that most potential antigens do not survive transit in the gastrointestinal system and are not efficiently transported from the lumen of the gut into the general circulation. However, ricin may be an exception.

Ricin intoxication is due to ingestion of castor bean or

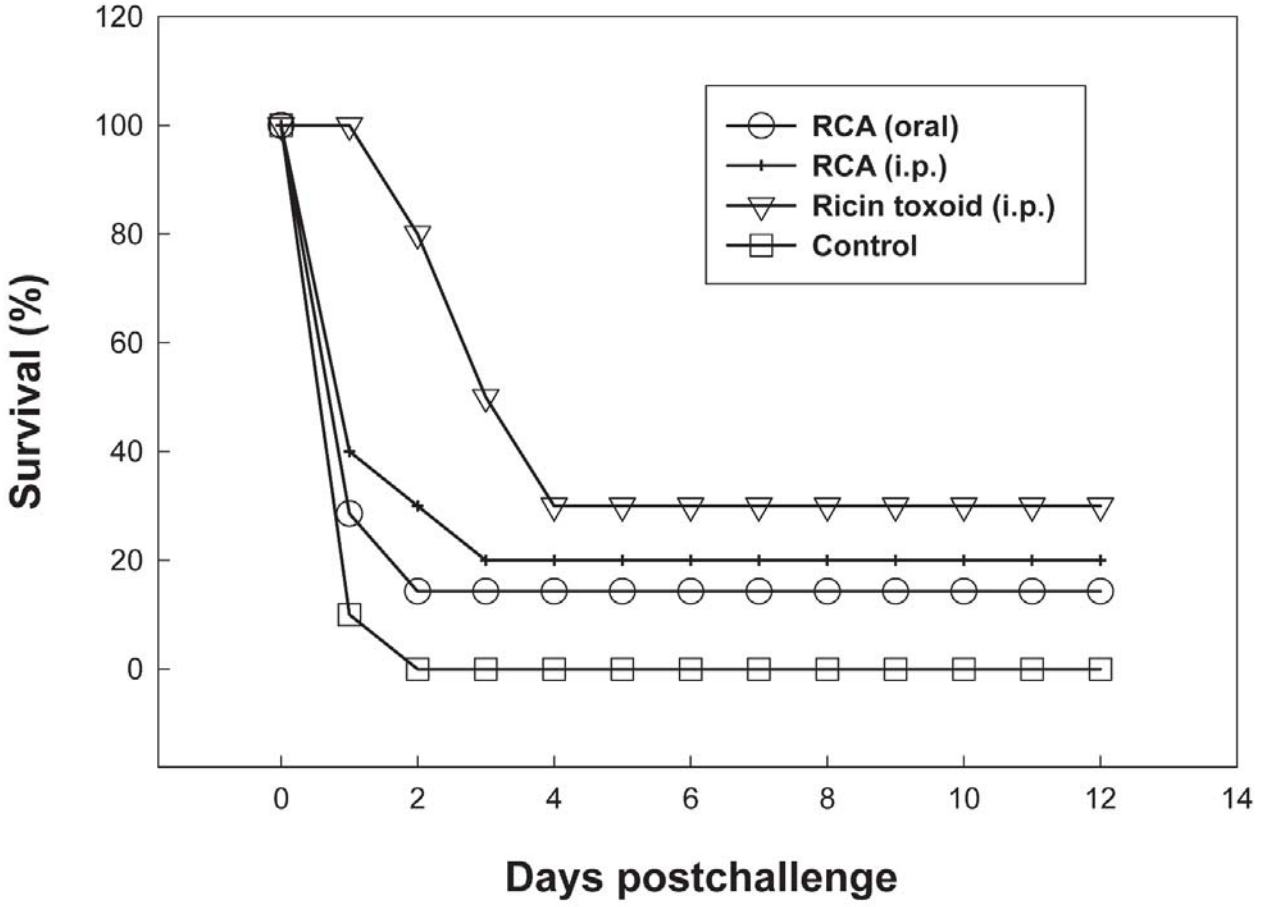

Fig. 3 Cross protection of immunized mice with abrin challenges.
Direct challenge of abrin on immunized animals. Abrin (50 μg) was injected i.p. into mice immunized with RCA i.p. (+, n = 10), ricin toxoid i.p. (▽, n = 10), RCA oral (▽, n = 7) or PBS-BSA (□, n = 10). The percent survival was monitored for 12 days.

food contaminated with ricin. In either case, ricin must escape from the gut into the general circulation, from which it is delivered to its target organ.[17] This suggests that a homologous protein with this ability to reach the general circulation but without the ability to poison cells could be used as an oral vaccine.

Data have shown that RCA escapes from the gut into the general circulation, and this is the site at which antibody production and immunity are induced. This hypothesis is in line with the facts that (1) ricin is known to escape from the gut into the general circulation to produce poisoning effect, and (2) RCA administered i.p. evokes systemic antibodies and induces immunity. However, there is a possibility that RCA can evoke a local response in the gut, which might eventually contribute to the appearance of systemic antibodies and protection and warrants further consideration.[18]

Two types of experiments were performed to estimate the protective effect of the antibodies elicited by i.p. and oral administration of antigens: (1) a serum neutralization test and (2) an in vivo toxicity test. Regardless of the route of administration, serum from animals immunized with RCA inactivated a large dose of ricin (77 LD_{50}). For the in vivo toxicity test, immunization with RCA by either i.p. or oral route produced a dramatic reduction in the potency of a subsequent injection of native toxin (740 LD_{50}). A similar result was obtained when ricin toxoid was given i.p.

In conclusion, the current results provide the first demonstration that RCA can evoke high levels of serum antibodies and protective immunity. The data further show that RCA has the ability to translocate from the gut

into the general circulation and to evoke protective antibodies. Thus, this molecule is an effective oral vaccine against ricin.

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DISCLOSURE

All authors declare that this study has no conflict of interest.

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