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RiVax, a recombinant ricin subunit vaccine, protects mice against ricin delivered by gavage or aerosol

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Abstract

Ricin is a plant toxin that is a CDC level B biothreat. Our recombinant ricin A chain vaccine (RiVax), which contains mutations in both known toxic sites, has no residual toxicity at doses at least 800 times the immunogenic dose. RiVax without adjuvant given intramuscularly (i.m.) protected mice against intraperitoneally administered ricin. Furthermore the vaccine without alum was safe and immunogenic in human volunteers. Here we describe the development of gavage and aerosol ricin challenge models in mice and demonstrate that i.m. vaccination protects mice against ricin delivered by either route. Also RiVax protects against aerosol-induced lung damage as determined by histology and lung function tests.

Keywords

vaccine; ricin; bioterrorism; RiVax

Introduction

Ricin toxin is a 64 kDa protein produced by castor beans (*Ricinus communis*) [reviewed in ^{1, 2}]. The holotoxin consists of two polypeptide chains (A and B) joined by a disulfide bond. The A chain (RTA) is a ribosome inactivating protein (RIP) that inhibits protein synthesis in mammalian cells. The B chain (RTB) is a lectin, which binds to galactose residues on the surface of cells. Once internalized by a cell, RTA translocates into the cytosol where it enzymatically inactivates 60S ribosomes. A single molecule of RTA in the cytoplasm of a cell completely inhibits protein synthesis. The reported estimated lethal dose of ricin in humans is 1-25 µg/kg when inhaled or injected, and 20 mg/kg when ingested [^{2,3}]. Because of its wide availability and extraordinary toxicity, ricin represents a potential agent for use in bioterrorism and is therefore classified by the Centers for Disease Control, Atlanta GA (CDC) as a level B biothreat. Ricin intoxication can be prevented in experimental animals by vaccination with toxoid [⁴] or deglycosylated ricin A chain (dgRTA) (Leonard Smith, USAMRIID, personal communication), or by passive immunization with anti-ricin antibodies [^{4,5}]. However the

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toxoid is considered to be too toxic for routine use in humans and the dgRTA is difficult and expensive to produce, and since it retains both active sites it could induce toxic side effects in humans [6]. Passive immunization with anti-ricin antibodies is only effective if the ricin dose is relatively low and the antibody is administered within a few hours following exposure [4, 7-8].

In order to avoid these limitations, we developed a recombinant RTA vaccine, named RiVax, into which we have incorporated two point mutations, Y80A and V76M, to remove both of its known toxicities, i.e. ribotoxicity and vascular leak-inducing ability [9-10]. This vaccine, in the absence of adjuvant, is non-toxic and immunogenic in mice, rabbits and humans [9-11]. Mice immunized three times at four week intervals with at least 1.0 µg of vaccine were consistently protected against an intraperitoneal (i.p.) ricin challenge of 10 LD₅₀s. Immune sera from all three species contained ricin-neutralizing antibodies, which could passively protect non-vaccinated mice from a lethal i.p. challenge with ricin.

In the present study, we determined whether i.m. vaccinations could protect mice against either intragastric gavage or inhaled aerosolized ricin. Generally speaking, mucosal protection is accomplished by secretory IgA (sIgA) at the mucosal site, which is most commonly induced by administering vaccines orally or intranasally [12]. sIgA traps antigens such that they cannot breach epithelial barriers in the mucosa. In this regard, it should be noted that the mechanism by which ricin kills following oral or aerosolized challenge is not understood. Ricin causes local tissue damage but because it is able to disrupt the endothelium and cause massive edema, it may also enter the bloodstream where it could be neutralized by IgG antibodies in the blood. In addition, since small amounts of certain IgG subclasses from the blood can enter mucosal sites and bind to proteins, very small amounts of toxin might be effectively neutralized in the mucosa.

In this report we describe the two challenge models that we have developed to determine the LD₅₀ of ricin administered by two different routes, and then demonstrate that i.m. vaccination with RiVax protects mice against 10 LD₅₀s of ricin administered by gavage or aerosol. Additionally we describe the degree of tissue damage induced in the lung by aerosolized ricin as determined both histologically and by lung function tests. We also report that this damage is inversely related to the vaccine dose used to protect the mice. In summary, vaccination by the i.m. route prevents ricin-induced death and tissue damage when the toxin is delivered directly into the stomach by gavage or into the lung by an aerosolizer.

Materials and Methods

Vaccine, ricin and antibodies

The construction, production and purification of RiVax have been described [9, 10]. Briefly, the gene for the enzymatically active wild type RTA, kindly provided by Dr. J. Michael Lord, Department of Biology Sciences, University of Warwick, Coventry, U.K. [13, 14], was genetically altered to eliminate both its cytotoxic activity (Y80A) and its ability to induce vascular leak (V76M). This construct was then inserted into pET28a (Novagen, Madison, WI), which relies on kanamycin rather than ampicillin for selection. Overnight cultures of *E. coli* strain BL21 (DE3) (Novagen) freshly transformed with this plasmid in Terrific Broth [15] containing 30 µg/mL kanamycin grown with agitation at 37°C were used to inoculate the same media in two 5 L fermentors (New Brunswick Scientific, Edmon, N.J.). Cultures were grown at 37°C with aeration to an OD₆₀₀ of 0.6 to 0.8, then at 30°C to an OD₆₀₀ of 1.0. Cultures were induced with 1.0 mM isopropyl-B-D-thiogalactopyranoside (IPTG) and grown overnight. Cells were harvested, resuspended in 2-3 mL phosphate-buffered saline (PBS) (50 mM phosphate buffered saline, pH 7.0) per gram of cell paste, and lysed by sonication. Cell debris was removed by centrifugation at 25,000 g for 30 min and supernatants were filtered (0.45

μm). This lysate was dialyzed overnight at 4°C against 10 mM phosphate buffer pH 6.5 and chromatographed on CM-Sepharose FF (Pharmacia, Piscataway, NJ) equilibrated with the same buffer. After washing, a 100 to 175 mM NaCl gradient was used for elution. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the RiVax peak. The fractions containing this peak were pooled, dialyzed against PBS pH 7.5 buffer, and the preparation was chromatographed over an Acticlean column (Sterogene Bioseparations, Carlsbad, CA) to remove endotoxin (this and all subsequent steps were carried out using water and reagents that were endotoxin-low). The collected fractions containing purified RiVax (flow through) were pooled and the amount of endotoxin was measured using a Limulus Amebocyte Lysate (LAL) assay (Associates of Cape Cod, East Falmouth, MA) as per the manufacturer's instructions. The protein concentration of the final product was usually between 0.5 and 1.0 mg/mL and the endotoxin levels were 0.5 to 10 EU/mg. This preparation was formulated at 0.2 mg/mL in PBS with 0.1 M sucrose (USP, JTBaker, Phillipsburg, NJ) and 0.04% polysorbate (Tween) 80 (USP, JTBaker), pH 7.4, then vialled, plugged, sealed, labeled, and frozen at -80°C. Periodically stability analysis of this material was performed [10]. Alternately, it was formulated in 10 mM Histidine-HCl, 144 mM NaCl, pH 6.0, 50% v/v glycerol, stored at -20°C, which has been determined to be an ideal formulation for stability (Dr. Russ Middaugh, personal communication). This material was dialyzed overnight into 10 mM Histidine-HCl, 144 mM NaCl, pH 6.0 before use. Both formulations protect equally well in a mouse i.p. challenge assay (data not shown). Ricin was prepared in house as described [16]. Antibodies specific for the vaccine were purified from pooled immune serum using ricin-conjugated CL-B4 Sepharose affinity chromatography in the presence of 0.1 M galactose. Radioimmunoassay (RIA) and SDS-PAGE were used to verify the purity and integrity of the antibody.

Radioimmunoassay (RIA) of anti-RiVax antibody

96 well plates (Costar, Corning, NY) were coated overnight at 4°C with 100 μL wild type (wt) RTA, or RiVax in 20 μg/mL in PBS. After washing and blocking the plates with 5% fetal calf serum (FCS), 100 μL of mouse anti-RiVax antibody at concentrations ranging from 1 to 1000 ng/mL (standard curve) or 100 μL of appropriate dilutions of the sera from vaccinated or control mice were added in triplicate. After overnight incubation at 4°C the plates were washed and [¹²⁵I]-labeled affinity purified rabbit anti-mouse Ig (10⁵ cpm/100 μL) was added. After a 2 h incubation at room temperature, the plates were washed again and the radioactivity in the wells was counted in a gamma-counter (Pharmacia).

Determination of the LD₅₀ of ricin delivered by i.p. injection

The LD₅₀ dose was determined as described [9]. For all i.p. challenges with ricin, mice were injected with ricin in a volume of 100 μL PBS, monitored and weighed daily for 14 days, and euthanized by CO₂ asphyxiation followed by spinal dislocation when moribund or following 25% weight loss.

Determination of the LD₅₀ of ricin delivered by intragastric gavage

Several different variations of this protocol were tried (see Results and Discussion) and the following has become our 'standard' IACUC-approved procedure. Swiss Webster mice, 6-8 weeks of age, (Taconic, Germantown, NY) were moved to a clean cage without food 20 h prior to the intragastric gavage challenge. Mice were single-hand restrained and a 1.25 mm diameter gavage needle was gently inserted directly into their stomachs. The mice were dosed with a volume corresponding to 1% of their body mass (10 μL/g). Food was withheld for an additional 4 hours following the challenge. Mice were monitored and weighed daily for 14 days and, when moribund or following 25% weight loss, euthanized by CO₂ asphyxiation followed by

spinal dislocation. The LD₅₀ was calculated graphically from the curves generated by survival vs. challenge dose.

Determination of the LD₅₀ of ricin delivered by aerosol

Mice were exposed to ricin in a nose-only exposure chamber (InTox, Moriarty, NM) with a total system airflow rate of rate of 10 L min⁻¹, precisely measured using a DryCal air flow meter (Bios, Butler, NJ). A small-particle aerosol with a median particle diameter of 2 µm was generated using a Lovelace nebulizer (InTox) in conjunction with a particle dryer. Respiratory minute volumes (total volume of air inhaled per minute) were estimated by using the formula, $\text{Log}_{10} (V) (\text{mL min}^{-1}) = -0.899 + 1.725 (\text{Log}_{10} \text{ body weight}) (\text{g})$, which is based on animal weight [17]. The estimated inhaled and retained dose of ricin was then determined from the minute volume, the calculated ricin concentration per L air, published particle retention rates, and exposure time. The exposed mice were observed daily for 14 days. Lung function in the mice was measured using a Buxco 12 chamber whole body plethysmograph (Wilmington, NC). This technique has been used to assess airway obstruction and, following methacholine treatment, hyperresponsiveness in rodents [18 and references therein].

Histopathology

To determine whether there was ricin-related tissue damage in the mice challenged with ricin, representative animals were euthanized at various time points post-challenge for histopathological evaluation. At least one time point for harvesting tissues from surviving vaccinated mice was well beyond the time of death of the non-vaccinated mice, which typically survived for 24-72 hours, depending upon the challenge route. Tissues were harvested following systemic paraformaldehyde perfusion under anesthesia; major organs (liver, lungs, heart, and kidneys) were harvested from challenged mice; the lungs were inflated with paraformaldehyde following removal from the chest cavity. In addition, sections of the gastrointestinal (GI) tract (stomach, duodenum, jejunum, ileum, and proximal colon) were harvested from ricin-gavaged mice. Tissues were stained with hematoxylin and eosin, coded and evaluated by a pathologist.

Vaccination and ricin challenge

Groups of 5 Swiss Webster mice, 5-6 weeks old, were injected i.m three times, once every four weeks, with various doses of vaccine (0.1 to 10 µg) in 50 µL formulation buffer. As a control, two mice were injected i.m with formulation buffer only. One to two weeks after the last injection, all mice were challenged with 10 LD₅₀s of ricin using one of the challenge protocols described above. The weights and deaths of animals were recorded daily for 14 days, a period of time sufficient for almost all vaccinated animals to regain their initial weight. Blood was collected from each mouse one to two days prior to the ricin challenge to determine the serum concentration of anti-RTA antibody.

Passive Protection of Mice Against Ricin Challenge by Gavage

Anti-vaccine antibodies were affinity purified from pooled sera collected from the vaccinated mice and then tested for their ability to protect mice against ricin intoxication. Briefly, groups of two mice fasted for 12 hours were injected i.v. with 50 µg/g immune or normal (non-immune) IgG. This dose was chosen to achieve serum levels >100 µg/mL, which we determined from our active vaccination studies would be protective against ricin administered i.p. Three hours later, blood was collected to determine levels of anti-RiVax antibodies. Five hours later (20 hours fast time total and 8 hours after the injection) the mice were gavaged with 10 LD₅₀s of ricin as described above. Food was returned 4 hours later. Mice were monitored twice daily for the first three days and daily thereafter and euthanized when they had difficulty breathing or moving, usually within 24-48 h for mice that had not been vaccinated.

Results

The intragastric gavage challenge route

Our efforts to define an LD₅₀ of ricin by intragastric gavage were complicated by two factors. First, we based our initial experiments on the reported oral LD₅₀ of 20-30 mg/kg [19,2]. We found that doses in the range of 5 to 40 mg/kg in groups of 2 mice were not consistently lethal, so we initially believed that this value was essentially correct. However, in trying to test doses in this range in enough mice to validate the LD₅₀, we found that even doses at the low end of this range were lethal far greater than 50% of the time. Furthermore, while some mice from the same dose group would either die within 24-48 hours, with the associated weight loss and GI tract tissue damage, others would show no signs of toxicity (data not shown). After eliminating the possibility that this was a result of technical variation in the gavage procedure itself, we reasoned that the different amounts of food in the stomach might have an impact on ricin toxicity. Hence, the individual habits of the mice, specifically the variations in daytime eating, suggest that even within a group of mice, there was a wide range in the amount of food in their GI tracts at the time of challenge. To circumvent this variability, we fasted the mice for 20 hours prior to ricin challenge and 4 hours after challenge, to ensure good uptake of the ricin. This had the added benefit of having each experiment start at approximately the same time in the day/night cycle (to fit into a work day) thereby improving consistency between experiments. We also adjusted our ricin challenge dose from a uniform 100 µL to a volume corresponding to ~1% v/w body mass (i.e. 10 µL/g, typically 300 - 500 µL for 30 to 50 g mice) to ensure that we had adequate stomach filling to trigger gastric motility. We found that this regimen yielded much more uniform results within dose groups than did the shorter fasting times and that the LD₅₀ of ricin administered with fasting was consistently 10 µg/kg (Table 1). This value was over three logs lower than the reported dose of 20 mg/kg [19], and the same as our LD₅₀ dose of ricin by i.p. injection [9]. With our newly established fasting/challenge regimen, we challenged groups of three mice with 10 µg, 100 µg, 1 mg, or 10 mg ricin per kg, (corresponding to 1, 10, 100, or 1,000 times our LD₅₀) and examined the duodenum for tissue damage 18 hours post-challenge. As shown in Figure 1, at 0 and 10 µg/kg there were no detectable changes in the gut. In mice challenged with 100 µg/kg, intestinal villi were slightly shortened and scattered rare apoptotic cells were identified in the lamina propria. Rare sloughed necrotic epithelial cells were also seen in the gut lumen. At 1 mg/kg, villi were further blunted and crypt epithelial cells were hyperplastic; mitotic figures were common in these cells. In addition, necrosis was common in crypt epithelial cells. Apoptotic and necrotic cells were also observed in the edematous lamina propria and sloughed necrotic epithelial cells were identified in the lumen. At 10 mg/kg villi were dramatically blunted and there was extensive necrosis and apoptosis in the cells of the lamina propria; necrosis of crypt epithelial cells was common and numerous necrotic epithelial cells accumulated in the gut lumen.

The aerosol challenge route

In a series of eight experiments, a total of 40 mice were exposed to a range of doses of aerosolized ricin, from approximately 1 to 16 µg/kg, as described in the Methods section. Mice were monitored and weighed daily for 14 days. Mice that appeared moribund or who had lost >25% of their pre-challenge body weight were sacrificed. Most mice died or were euthanized within 2-6 days of exposure. The data are shown in Figure 2. There was very little overlap between the lethal and 'safe' doses, and the estimated LD₅₀ of ricin in mice was approximately 4 µg/kg, in agreement with the published value of 3-5 µg/kg [9]. Histopathological analysis of the lungs (including the trachea and alveoli) from the mice showed necrosis of alveolar cells, indicating that the aerosol particles were small enough (1-2 µm) to penetrate to the alveolar region of the lung [20] (data not shown).

Serum titers following vaccination

All the mice in this study were vaccinated using the same vaccine formulation, doses and dosing schedule. The titers, presented in Figure 3, showed a clear dose response relationship. While we anticipate inducing higher levels of serum antibody and better protection with larger vaccine doses and the addition of an adjuvant, the objective of this study was to determine whether our previously characterized dose regimen would also protect mice against ricin delivered into the stomach or lung so no attempt was made to optimize the vaccination regimen. We also used our previously reported i.p. challenge route for comparison [9].

Ricin challenge by i.p. injection of vaccinated mice

In two separate experiments, groups of 5 mice were vaccinated with various doses of vaccine, or excipient, and challenged with 10 LD₅₀s by i.p. injection, as described in Methods. As shown in Figure 4, 100% of mice from the three highest vaccine dose groups, 10 µg, 3.3 µg or 1.0 µg, were protected and, on average, maintained at least 90% of their pre-challenge weights. In contrast, only 50% of the mice that received 0.33 µg per dose survived and lost 15% of their body weight. Although all the mice receiving the lowest dose of the vaccine eventually died, they did survive marginally but statistically longer than the control mice ($P < 0.05$ by Logrank test). Histopathologic evaluation of major organ systems revealed mild acute alterations. There was diffuse interstitial pneumonitis in the lung. Sub capsular hepatocytes were necrotic and neutrophils were margined in sinusoids throughout the sections. There were no lesions in the kidney but the adrenal medulla and zona fascicularis contained necrotic cells. Apoptosis of apical epithelia cells in the small intestine were increased. Given the lack of extensive tissue damage that would explain the cause of death, we speculate that death might be due to a lethal drop in their blood glucose levels, as reported by Pincus *et al* [21]. In that study, lethal doses of ricin resulted in a significant and probably ultimately lethal drop (down to less than 50 mg/dL) in blood glucose levels within 16-24 h.

Ricin challenge of vaccinated mice by intragastric gavage

In general, i.m. vaccinations induce good serum antibody titers but are not effective in generating robust mucosal immunity. However we tested our vaccine in two mucosal challenge models, intragastric gavage and inhaled aerosol. As shown in Figure 5, in the gavage model we challenged a total of 10 mice per vaccination dose group in two separate experiments, and we found that mice challenged by gavage were protected as well as those challenged by i.p. injection (Figure 4). Protection was vaccine-dose dependent. The mechanism underlying this protection is not clear. Histopathologic studies revealed no specific and consistent tissue damage, either in the GI tract or any of the major organs that would be predictive of death. Lesions were limited to mild interstitial pneumonitis, margination of neutrophils in hepatic sinusoids, and scattered apoptotic apical epithelial cells and apoptotic cells in the lamina propria of the small bowel (data not shown). It is possible that serum IgG migrates into the lumen of the intestine and neutralizes the ricin locally so that damage does not occur [22]. Alternatively, the ricin might migrate into the bloodstream after damaging epithelial cells in the gut or endothelial cells in blood vessels, at which point serum antibodies prevent lethal damage. To show that serum antibodies could indeed protect the gut, naïve mice were injected intravenously with 50 µg/g purified mouse anti-vaccine antibodies (chosen to ensure a protective final serum concentration of at least 100 µg/mL) or with control mouse IgG. Blood was sampled three hours later to measure the titers of the antibodies in the blood to verify that a protective serum level had been reached. After an additional 5 hours, mice were challenged with a 10 LD₅₀s of ricin by oral gavage and monitored for survival. The mice that received anti-RiVax antibodies survived for more than 14 days while those that were given control mouse IgG died within 2 days (data not shown). This experiment suggests that either passively administered anti-ricin

antibody can enter and protect the gut or that the ricin gains access to the blood, where it is neutralized. Distinguishing between these two possibilities is not possible at this time.

Ricin aerosol challenge of vaccinated mice

Groups of mice were vaccinated as described above and were then challenged with 10 LD₅₀s of inhaled ricin aerosol, as described in the Methods. We monitored the mice for survival as well as for lung function by plethysmography. Figure 6 shows the combined survival and weight data from four separate experiments, with 13 to 18 mice in each treatment group. All mice receiving the highest two vaccine doses, 10 and 3.3 µg/dose survived, as did the majority of mice given 1.0 µg/dose. In contrast, few mice in the lowest two dose groups survived and many suffered severe weight loss. By four days after the challenge, all the non-vaccinated mice died or were euthanized because of severe weight loss, which is predictive of death. Higher values for Penh, or 'enhanced pause', are symptomatic of airway resistance or obstruction [18] and as shown in Figure 7A the lung function tests confirmed that the mice receiving higher doses of vaccine suffered less breathing difficulty than the mice in the lower dose groups. After 3 days, these mice also had Penh values that had returned to or near baseline levels more quickly than the survivors of the lower dose groups. By day 10 or 14 most of the Penh values had returned to baseline. However, for the lower dose groups these results are probably skewed since the sicker mice have already died and only the (exceptional) survivors of the low dose groups are included in the later time points, and these might have had less initial lung damage. Since this is likely due to variations in serum anti-RiVax titers, we also plotted peak Penh vs. pre-challenge serum titer for individual mice. As shown in Figure 7B, there is a good correlation between titer, which is a function of vaccine dose, and maintenance of lung function.

Another group of mice was vaccinated and challenged by ricin aerosol and then sacrificed at various time points to determine the extent of tissue damage and whether the vaccine protected the lungs of the aerosolized mice. As shown in Figure 8A-C, two days following the challenge, there is a rough correlation between vaccine dose and lung protection, but the mice do suffer some lung damage even at the highest vaccine dose. However by 21 days after challenge there was mild bronchiolar epithelial hyperplasia indicating ongoing repair in the lungs of the mice from the highest vaccine dose groups while those rare mice from the lower vaccine dose groups that survived the challenge (and so were very well protected), still showed significant epithelial hyperplasia, and smooth muscle hyperplasia of the small arteries (Figure 8D-F). In comparison, the damage in the lungs of naïve mice was extensive (Figure 9). Terminal bronchioles were denuded and fibrin lined the bronchiolar lumens and plugged the airways. Extensive necrosis of alveoli resulted in acute fibrinous exudation into the alveolar spaces. Perivascular and peribronchiolar spaces were expanded by extensive protein-rich edema. Multifocal alveolar hemorrhage was evident. The proximal airway epithelium was necrotic. In animals surviving 50 days after ricin challenge, lesions were limited to mild epithelial hyperplasia, pseudopolyps within scattered bronchioles, and mild smooth muscle hyperplasia of small arteries. There was minimal hyperplasia in the bronchial lymphoid tissues and no prominent perivascular inflammation. Multifocal type II pneumocyte hyperplasia was documented in an animal from the low-dose vaccine group (data not shown).

Discussion

We have reported that RiVax administered i.m., protects mice against 10 LD₅₀s of ricin injected i.p. [9]. Rivax had no observed toxic effect on mice at doses that were a minimum of 800 fold higher than the doses necessary to induce neutralizing antibodies and to protect the mice. Furthermore, RiVax administered to human volunteers was both immunogenic and safe and toxicities were those generally observed with any i.m. vaccination. The antibodies from the human volunteers passively protected mice against ricin intoxication [11].

The objective of this study was to determine whether i.m. vaccination with RiVax could protect mice against gavaged or aerosolized ricin. This was important since ricin poisoning is most likely to occur following accidental or intentional contamination of food, water, or air. The major findings to emerge from this study are as follows: 1). A well-defined intragastric gavage model to deliver ricin into the stomach was developed. If the mice were fasted for 20 hours before and 4 hours after the challenge, the LD₅₀ of the ricin was routinely 10 µg/kg, or 2000-fold lower than previously reported for oral exposure [19]. If the mice were not fasted or fasted for shorter periods of time, the results were highly variable with some mice surviving up to 500-fold higher doses of ricin (5 mg/kg). 2). When mice were vaccinated, fasted and challenged with 10 LD₅₀s of ricin by intragastric gavage they survived, and protection was dose-dependent. Non-vaccinated mice died rapidly. 3). A well-defined and reproducible aerosol model was developed and the LD₅₀ of aerosolized ricin was consistent with that reported previously [3]. We also assessed lung function by plethysmography. In the aerosol model, we could calculate the dose of ricin retained in the lung, demonstrate by histology that lung tissue was damaged, and that lung function was compromised. Finally, 4). i.m. vaccination with RiVax reduced ricin-mediated damage to lung tissue in a dose-dependent manner.

With regard to the gavage model, while it is fairly routine to vaccinate mice or humans orally or intranasally with live attenuated microorganisms to induce mucosal immunity, these routes do not effectively induce mucosal immunity against proteins such as toxoids, since most are degraded in the stomach, and those that reach the intestine have the potential to induce tolerance. [12]. In contrast, an i.m. vaccination induces antibody in the blood and some of the IgG from the blood can enter mucosal sites [12,22,23]. If the lethal dose of a toxin is very low, this transudated IgG could be sufficient to neutralize it. Alternatively, ricin delivered by gavage or aerosol might damage the mucosa and gain access to the circulation where it would be neutralized in the blood.

Of note, this study demonstrates that in fasted mice, gavaged ricin is far more toxic and more consistently toxic than previously reported [19]. However, the previously determined LD₅₀ relied on a crude bean extract so that it is difficult to compare published results with those in this study where highly purified ricin was used. In addition, from our study, it appears that toxicity is also related to the amount of food present in the stomach. Hence we would expect a high degree of variability in a human population if ricin were introduced in food or water. Regardless, in order to study the ability of RiVax to protect mice against gavaged ricin, it was important to develop a ricin gavage model that would give consistent LD₅₀s.

Other studies investigating the toxic effects of ricin given orally have since been conducted [24-26], but these relied on the published LD₅₀ of 30 mg/kg in animals that had not been fasted or were fasted for very short intervals before dosing. In one study [24], they harvested tissues for examination after only five hours after ricin was administered, so they would not have known if the lowest dose used (1 mg/kg) was lethal or sublethal. In another study [25], rats were given a 'sublethal' dose of 10 mg/kg (one third of the reported LD₅₀) emulsified in olive oil. They also fasted the mice for 36 h upon the initiation of the experiment but not prior to the delivery of the dose, and were able to follow the mice for 72 h. They went on to report that using gavaged doses of radiolabeled ricin of 10 mg/kg, a total of 8 µg per 300-350 g animal of intact ricin was found in the blood (further evidence that this was not a sublethal dose, since the LD₅₀ of ricin by i.v. injection is reported to be 5 µg/kg), and a further 95 µg/animal in the liver (maximum level over 72 h, so given the high rate of metabolism [3], the total was likely much higher). In our experience, naïve mice fasted both before and after the administration of ricin die within 48 h after receiving a dose of 0.1 mg/kg. In a recent study [26], mice were fasted for one hour both prior to and following the oral administration of 1 to 10 mg/kg doses of ricin. Extensive dose-dependent epithelial damage was reported in the proximal small intestine at doses of ricin exceeding 2.5 mg/kg, and our results are consistent with this

observation. However, they did not observe damage with a dose of 1 mg/kg after 18 h while we observed a moderate effect, suggesting that the food might indeed have a protective effect. Since our LD₅₀ dose was 10 µg/kg and our challenge dose was 100 µg/kg, it is not surprising that we are unable to observe significant intestinal damage in our mice. The same authors also reported an interesting correlation between toxicity and an increase in at least one proinflammatory cytokine, but again only at doses of 2.5 mg/kg or greater. Clearly, further studies to determine the effects of food, cytokines, and target cell binding on the lethality of ricin orally administered ricin are needed in order to predict more accurately whether there is a realistic threat to humans of effective large scale delivery of lethal doses of ricin by food or water and exactly what the lethal hit is following exposure to different amounts and different forms (bean mash vs purified) of ricin.

In our gavage model there is little evidence of sufficient tissue damage to explain why the mice died. It has been suggested that hepatocellular and renal lesions are the result of toxin induced vascular damage rather than organ damage *per se* [3], but we rarely observe such lesions. We postulate that death is more likely due to severe hypoglycemia, as reported by Pincus *et al* [20]. With regard to the toxicity of aerosolized ricin, it is not entirely clear how death is caused, though it is likely to be related to a large extent to damage in the lung [3]. However another recent study using doses of 20 µg/100 g of intratracheally delivered ricin (two times their determined LD₅₀ and which they found to be consistently lethal) showed that ricin gained access to the blood and caused inflammatory responses in multiple organ sites [27]. This may explain, at least in part, why our i.m. vaccination protects mice against aerosolized ricin. Our histopathological results from the aerosolized mice also demonstrated that the mice would likely have gone on to die of pulmonary edema and aveolar flooding from vascular leak. We used whole body plethysmography to detect compromised lung function following ricin inhalation, again indicating that this was a major area of tissue damage.

Importantly when RiVax was administered i.m. it conferred protection on mice receiving lethal doses of purified ricin by either gavage or aerosol. The vaccine also protected the mice against a significant amount of lung damage, and at the highest vaccine doses, lung function was largely restored within three days. These results are supported by the histological evaluations carried out at day 2 and again at day 21. In the future we will determine whether larger vaccine doses, or vaccine in adjuvant, can induce more robust and long-lived immunity and further reduce lung damage.

Other ricin vaccines have been considered for human use but have not been developed. These include ricin toxoid [4,26] and dgRTA (Leonard Smith, USAMRIID, personal communication). Both i.m. vaccines protect mice from lethal ricin challenges. However, when aerosolized ricin was delivered to the vaccinated mice they still suffered significant lung damage [7,28]. Intranasal or intratracheal instillation of the toxoid induced good systemic immunity but failed to activate an adequate local response to protect the lungs. The toxoid, and to a lesser extent dgRTA, given as microencapsulated liposome formulations directly into the respiratory tract protected the lung [6,29]. The encapsulated toxoid given orally has also been shown to protect mice against oral ricin challenges [30]. However, both the toxoid and dgRTA (which still retains all of its cytotoxic activity and vascular leak site) have been determined to be too potentially toxic for general use [4].

Two recombinant RTA vaccines are currently being developed, and both have lost their enzymatic activity (but not the vascular leak site). One vaccine was made by inserting a peptide that effectively inactivated its ribotoxic activity [31]. This altered RTA was reassociated with native RTB and, when used to vaccinate rats by the i.m. route, protected them against a challenge with intratracheally instilled ricin. The authors reported no signs of ricin intoxication though they did not present any data concerning lung function or tissue damage. The other

recombinant RTA vaccine contains two large deletions, to eliminate the enzymatic activity as well as to remove a hydrophobic region (normally associated with RTB in the holotoxin) to make the vaccine more stable and soluble [32]. This vaccine administered i.m., with or without adjuvant, protected mice against lethal ricin challenges given either i.p. or by aerosol. However, lung pathology was not reported.

In conclusion, RiVax administered i.m. in the absence of an adjuvant had no detectable side effects and can protect mice from 10 LD₅₀s of ricin administered by injection, aerosol or gavage. Since RiVax is also safe and immunogenic in humans, formulations and dose regimens should be optimized for the most robust and long lasting immunity possible.

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Figure 1. Histopathologic examination of the intestine of the ricin gavaged mice
Sections of duodenum are from naïve mice gavaged with ricin at the indicated dosage per kilogram. Two magnifications of each sample are shown. Bar of measure = 100 μ m.

Figure 2. The LD₅₀ of aerosolized ricin

Mice were exposed to doses of ricin corresponding to ~1 to 16 ng/g body weight by dry aerosol as described in Methods. Mice were weighed and monitored for 14 days. Dot plots of individual mice, arranged as survivors or deaths and/or excessive weight loss within that period plotted by dose. As shown by the horizontal line, the LD₅₀ was estimated to be ~ 4 µg/kg.

Figure 3. Geometric means of titers from mice vaccinated with RiVax
Prechallenge serum titers of individual mice were measured by RIA. Combined results from eight experiments with 3-5 mice per dose group, totaling 33-40 mice per dose group, are shown.

A

B

Figure 4. Vaccination with RiVax followed by 10 LD₅₀s of ricin administered by i.p. injection
 Groups of five mice vaccinated q4w × 3 with doses of: 10 µg (◆), 3.3 µg (◇), 1.0 µg (■), 0.33 µg (□), and 0.1 µg (●) and control (○). **A.** Average weights (as % of pre-challenge) vs. time (days), and **B.** survival curves. n = 2 groups of 5 mice = 10.

A

B

Figure 5. Vaccination with RiVax followed by the administration of 10 LD₅₀s of ricin by intragastric gavage

Groups of five mice vaccinated q4w × 3 with doses of: 10 µg (◆), 3.3 µg (◇), 1.0 µg (■), 0.33 µg (□), and 0.1 µg (●) and control (○). **A.** Average weights (as % of pre-challenge) vs. time (days), and **B.** survival curves. n = 2 groups of 5 mice = 10.

A

B

Figure 6. Vaccination with RiVax followed by the administration of 10 LD₅₀s of ricin by aerosol Groups of five mice vaccinated q4w × 3 with doses of: 10 µg (◆), 3.3 µg (◇), 1.0 µg (■), 0.33 µg (□), and 0.1 µg (●) and control (○). **A.** Average weights (as % of pre-challenge) vs. time (days), and **B.** survival curves. Combined results from four separate experiments with 13-18 mice per dose group in total.

Figure 7. Lung function in mice challenged with 10 LD₅₀s of by aerosolize ricin
 Lung function measured in each mouse on days 0, 1, 2, 3, 5, 7, 10 and 14. **A.** Penh vs time. Doses represented using greyscale bars from 10 µg (black) to 0 µg (white). Student's t-test vs 10 µg dose within each timepoint: * P < 0.01, ** P < 0.001. **B.** Dot plot of Penh vs pre-challenge serum titer for individual mice in each dose group. Groups of mice vaccinated q4w × 3 with doses of: 10 µg (◆), 3.3 µg ◇, 1.0 µg (■), 0.33 µg □, and 0.1 µg (●) and control (○).

Figure 8. Histopathologic alterations in lungs from vaccinated mice at day 2 and day 21 following aerosol exposure to ricin

A. epithelial necrosis and peribronchiolar edema, B. mild epithelial necrosis and perivascular edema, C. no edema or necrosis, D. epithelial hyperplasia with pseudopolyp and alveolar type II pneumocyte hyperplasia, E. arterial smooth muscle hyperplasia, type II pneumocyte hyperplasia and marked perivascular inflammation, F. arterial smooth muscle hyperplasia and mild perivascular inflammation. Bar = 60 μ m

Figure 9. Histopathologic alterations in lungs from unvaccinated control mice at day 2 following aerosol exposure to ricin

A. multifocal alveolar hemorrhage, B. fibrinous exudation and bronchiolar epithelial necrosis, C. fibrinous exudation and hyaline membranes, D. bronchial epithelial necrosis. Bar = 60 μ m

Table 1Determination of the LD₅₀ of ricin delivered by gavage¹

Gavage Dose (µg/kg)	# of Mice Surviving/# of Mice Challenged
500	0/8
100	0/11
10	2/11
3	8/11
1	10/10
0.3	10/10

¹ Following a 20 hour fast, a total of 61 mice were dosed as shown (1% v/w). Food was withheld for an additional 4 hours following the challenge. Mice were monitored daily for 14 days and euthanized when moribund or following a 25% weight loss.