Comparative Efficacy of Two Leading Candidate Ricin Toxin A Subunit Vaccines in Mice

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The two leading ricin vaccine candidates, RVEc and RiVax, are recombinant derivatives of the toxin’s 267-amino-acid enzymatic A chain (RTA). RVEc is truncated at the C terminus (residues 199 to 267) to improve protein thermostability, while RiVax has two point mutations (V76M and Y80A) that eliminate the RNA N-glycosidase activity of RTA, as well as its ability to induce vascular leak syndrome. The two vaccines have never been directly compared in terms of their ability to stimulate RTA-specific antibodies (Abs), toxin-neutralizing activity (TNA), or protective immunity. To address this issue, groups of female BALB/c mice were immunized two or three times with Alhydrogel-adsorbed RiVax or RVEc at a range of doses (0.3 to 20 μg) and then challenged with 10 50% lethal doses (LD50s) of ricin. We found that the vaccines were equally effective at eliciting protective immunity at the doses tested. There were, however, quantitative differences in the antibody responses. RVEc tended to elicit higher levels of ricin-specific RTA IgG and TNA than did RiVax. Pepscan analysis revealed that serum Abs elicited by RVEc were skewed toward a solvent-exposed immunodominant α-helix known to be the target of potent toxin-neutralizing Abs. Finally, immunodepletion experiments suggest that the majority of toxin-neutralizing Abs elicited by RiVax were confined to residues 1 to 198, possibly explaining the equal effectiveness of RVEc as a vaccine.

Ricin, one of the most potent biological toxins known, consists of two subunits, RTA and RTB. RTA is a 267-amino-acid RNA N-glycosidase that selectively and irreversibly inactivates eukaryotic ribosomes (1). RTB is a galactose/N-acetylgalactosamine-specific lectin that facilitates the delivery of RTA into the cytoplasm of eukaryotic cells in general (2). Once in the cytoplasm, it is estimated that a single molecule of RTA can inactivate >1,000 ribosomes per minute.

Despite the history of ricin as an agent of biological warfare and bioterrorism, there is currently no available ricin toxin vaccine (3, 4). As early as the 1940s, the United States military focused on the development of a simple formalin-treated holotoxin vaccine. Although ricin toxoid is highly efficacious in rodents and nonhuman primates, its use in humans was abandoned because of manufacturing problems and safety concerns (5). For those reasons, current efforts are aimed at the development of a recombinant subunit vaccine. While RTB is an obvious candidate, RTB immunization confers only partial protection against ricin challenge (6, 7). In contrast, immunization of mice with RTA or non-toxic derivatives of RTA is sufficient to protect mice against a 10 50% lethal dose (LD50) ricin challenge (8).

There are currently two RTA-based vaccines under development: RiVax and RVEc. RiVax is a recombinant derivative of RTA with two point mutations at residues Y80 and V76. The Y80A mutation abolishes the RNA N-glycosidase activity of the toxin, while the V76M mutation eliminates the ability of RTA to elicit vascular leak syndrome (VLS) (9, 10). RVEc was engineered with the primary objective of increasing the solubility of recombinant RTA and reducing its propensity to self-aggregate in solution (11–13). RVEc lacks the C terminus of RTA (residues 199 to 267) as well as a small hydrophobic loop in the N terminus (residues 34 to 43). Thus, RVEc (often referred to as RTA 1-33/44-198) is only 188 residues in length, compared to the 267 residues of RiVax. When described in terms of the three arbitrary folding domains (FD), RiVax represents all three domains of RTA, while RVEc essentially consists of FD1 and FD2 (14). In mice, RiVax immunization via the intramuscular (i.m.), subcutaneous (s.c.), or intradermal (i.d.) route elicits toxin-specific serum IgG antibodies (Abs) that are sufficient to confer protection against a lethal dose of ricin (8–10, 15–17). Phase I clinical trials have demonstrated that RiVax is safe and immunogenic in healthy human volunteers (18, 19). Similarly, RVEc is effective at eliciting toxin-neutralizing antibodies in mice and rabbits (20–23) and is now in phase I clinical trials.

However, in engineering RVEc for stability purposes, it was unclear what impact eliminating virtually one-third of RTA would have on the ability of the recombinant antigen to stimulate toxin-neutralizing activity (TNA) and protective immunity (12, 13). On the one hand, if residues T34 to P43 or A199 to F267 are important in eliciting TNA, then RVEc would be expected to be less effective than RiVax at eliciting protective immunity. Alternatively, we have postulated that RVEc may be slightly more effective than RiVax because residues T34 to P43 and A199 to F267 contain numerous epitopes recognized by nonneutralizing monoclonal antibodies (MAbs) (14). We speculated that elimination of these nonneutralizing B cell epitopes could actually “focus” the Ab response to targets elsewhere on the protein. In this study, we have directly compared the immunogenicity and relative efficacy of RiVax and RVEc.
fied from 100-liter scale fermentation, and stored in stabilizing buffer of RiVax protein manufactured by Cambrex (Baltimore, MD), puri-

His, 10 mM histidine, 144 mM NaCl (pH 6.0); Suc, 20 mM Na succinate, 100 mM NaCl, 0.10% Tween 20 (pH 6.5). The vaccines were adsorbed to Alhydrogel at a final concentration of 0.85 mg/ml or 0.7 mg/ml Al equiv-

were made from RVax lot 190-100L-FF-090105; WC 121211 was made from RVax lot KU Gao1, obtained from the University of Kansas (KU); and WC 004 and WC 032112 were made from RVEc lots ER-004 and 032112 from USAMRIID. WC, Wadsworth Center.

MATERIALS AND METHODS

Chemicals, biological reagents, and cell lines. Ricin was purchased from Vector Laboratories (Burlingame, CA) and dialedyzed against phosphate-buffered saline (PBS) at 4°C in 10,000-molecular-weight (MW) cutoff Slide-A-Lyzer dialysis cassettes (Pierce, Rockford, IL) prior to use in cyto
toxicity and mouse studies. The sources and dates of manufacture (DoM) of all vaccines used in this study are listed in Table 1. Lot PBR-0047-001 (obtained from Soligenix, Inc.) is a batch manufactured as an engineering run of Alhydrogel-adsorbed RiVax, representing a run of 350

The N-terminal biotinylated A11 peptide (YFFHPDNQEDAEAITHLF; NeoBioLab, Cambridge, MA) was attached to flow channel 2 (Fc2) of a streptavidin (SA)-coated chip (GE Healthcare). Flow channel 1 (Fc1) served as an uncoated-surface control. Both Fc1 and Fc2 were blocked with free biotin. The peptide was bound to a ligand density of 1,055 res-
served as an uncoated-surface control. Both Fc1 and Fc2 were blocked

Ricin neutralization assays. Toxin-neutralizing activity (TNA) was based on the reciprocal dilution of serum required to protect 50% (50% effective concentration [EC_{50}] of the Vero cells in the well of a 96-well plate from the effects of ricin (10 ng/ml). Vero cell cytotoxicity assays were
done as described previously (14, 26). RTA peptide array consisted of 2918-mers, each overlapping by 9 amino acids (see Table S1 in the supplemental material). The peptides were synthe-
sized unbond in 96 individual tubes, in a 96-well plate format, and were provided at 3 mg per peptide (>75% purity) (NeoBioLab, Cambridge, MA). The peptides were solubilized in dimethyl sulfoxide (DMSO), and aliquots were stored at −20°C.

ELISA and RTA peptide arrays. RTA-specific immunoglobulin G (IgG) reciprocal endpoint (RET) and geometric mean titers (GMT) were determined by enzyme-linked immunosorbent assay (ELISA) (26). Affini-

Ricin and RVEc in mice at a range of doses and after two or three immunizations.

Mouse studies. Female BALB/c mice, approximately 8 to 12 weeks of age, were purchased from Taconic Labs (Hudson, NY). Animals were housed under conventional specific-pathogen-free conditions and were treated in compliance with the Wadsworth Center’s Institutional Animal Care and Use Committee guidelines and approved protocol. Both vacci
cines were adsorbed to an aluminum hydroxide gel (Alhydrogel; E.M. Sergeant, Clifton, NJ) prior to immunization (11). RiVax and RVEc were adsorbed to Alhydrogel under conditions previously shown to give >95% adsorption (11, 24). Specifically, RiVax and RVEc were incubated with Alhydrogel at a final concentration of 0.85 mg/ml or 0.7 mg/ml Al equiv-

cine concentration in 1 mg/ml and then evaluated in duplicate for TNA in a Vero cell cytotoxicity assay.

Statistical analysis and software. Statistical analysis was carried out with GraphPad Prism 5 (GraphPad Software, San Diego, CA). The significance level threshold was set at an α value of 0.05.
1 week following the second and third immunizations. Two weeks
RTA-specific Ab titers and TNA were determined in sera collected
(Table 2). A sham group of animals received Alhydrogel only.

RESULTS AND DISCUSSION

Means (geometric mean titers).
ND, not detected.

Reciprocal RTA-specific Ab endpoint titer.
TNA, toxin-neutralizing activity in sera, which is defined as the reciprocal dilution of sera required to protect 50% of cells from ricin (10 ng/ml).
Aluminum was adsorbed to the vaccines prior to the 1st vaccination, and adsorbed vaccines were subsequently stored at 4°C until the consecutive vaccinations.

Comparative immunogenicity of RiVax and RVEc across three doses

| Dose (µg) | Mouse no. | 2nd immunization | 3rd immunization | Survival |
|-----------|-----------|-------------------|-------------------|----------|
| 10        | 1         | 64,000            | 64,000            | +        |
| 10        | 2         | 128,000           | 192,000           | +        |
| 10        | 3         | 128,000           | 256,000           | +        |
| 10        | 4         | 128,000           | 192,000           | +        |
| 10        | 5         | 32,000            | 256,000           | +        |
| 10        | 6         | 64,000            | 128,000           | +        |
| 10        | 7         | 64,000            | 128,000           | +        |
| 10        | 8         | 64,000            | 192,000           | +        |
| 10        | 9         | 64,000            | 128,000           | +        |
| 10        | 10        | 64,000            | 192,000           | +        |
| 3         | 1         | 51,200            | 128,000           | +        |
| 3         | 2         | 51,200            | 128,000           | +        |
| 3         | 3         | 25,600            | 128,000           | +        |
| 3         | 4         | 51,200            | 128,000           | +        |
| 3         | 5         | 51,200            | 128,000           | +        |
| 3         | 6         | 25,600            | 128,000           | +        |
| 3         | 7         | 25,600            | 128,000           | +        |
| 3         | 8         | 102,400           | 128,000           | +        |
| 3         | 9         | 102,400           | 128,000           | +        |
| 3         | 10        | 25,600            | 128,000           | +        |
| 1         | 1         | 12,800            | 32,000            | +        |
| 1         | 2         | 12,800            | 64,000            | +        |
| 1         | 3         | 25,600            | 64,000            | +        |
| 1         | 4         | 6,400             | 51,200            | +        |
| 1         | 5         | 25,600            | 128,000           | +        |
| 1         | 6         | 25,600            | 128,000           | +        |
| 1         | 7         | 12,800            | 64,000            | +        |
| 1         | 8         | 12,800            | 64,000            | +        |
| 1         | 9         | 51,200            | 128,000           | +        |
| 1         | 10        | 12,800            | 102,400           | +        |

a Aluminum was adsorbed to the vaccines prior to the 1st vaccination, and adsorbed vaccines were subsequently stored at 4°C until the consecutive vaccinations.
b Reciprocal RTA-specific Ab endpoint titer.
c TNA, toxin-neutralizing activity in sera, which is defined as the reciprocal dilution of sera required to protect 50% of cells from ricin (10 ng/ml).
d ND, not detected.
e Means (geometric mean titers).

RESULTS AND DISCUSSION

RTA-specific serum Ab titers, TNA, and protection against ricin challenge elicited by RiVax and RVEc immunizations. The goal of this study was to compare, in a mouse model, the immunogenicity and efficacy of two leading candidate RTA subunit vaccines, RiVax and RVEc. A series of pilot studies in BALB/c mice suggested that at high doses, RiVax and RVEc were more or less equally effective at eliciting protective immunity to ricin (J. M. O’Hara and N. J. Mantis, unpublished data). We therefore sought to compare the vaccines across a range of doses and after two or three immunizations.

In the first part of this study, groups of BALB/c mice were vaccinated s.c. three times at monthly intervals with 1 µg, 3 µg, or 10 µg of RiVax or RVEc adsorbed to aluminum salts (Alhydrogel) (Table 2). A sham group of animals received Alhydrogel only. RTA-specific Ab titers and TNA were determined in sera collected 1 week following the second and third immunizations. Two weeks after the third immunization, the mice were challenged with 10 LD50s of ricin administered by i.p. injection.

All sham-immunized mice succumbed to ricin intoxication and expired within 48 h (data not shown). All RiVax- and RVEc-vaccinated mice survived the ricin challenge, indicating that at the doses tested, there was no qualitative difference between the two vaccines with respect to their ability to elicit protective immunity (Table 2). There were, however, differences at the serologic level. After the second immunization, sera from mice immunized with RVEc displayed significantly higher RTA-specific IgG titers than did the RiVax-immunized mice (Table 2; Fig. 1); this difference was no longer apparent after the third immunization, except in the high-dose group. Interestingly, however, after the third immunization, the RVEc-immunized mice had consistently higher serum TNA (2- to 4-fold) than did the RiVax-immunized mice (Table 2; Fig. 1), suggesting that the truncated derivative of RTA is slightly...
more effective than full-length RTA at eliciting ricin-neutralizing antibodies.

We next set out to determine whether the vaccines were qualitatively or quantitatively different when administered to mice at an even lower dose and after only two immunizations. Groups of BALB/c mice were vaccinated s.c. two times at monthly intervals with 0.3 μg of Alhydrogel-adsorbed RiVax or RV Ec adsorbed to Alhydrogel. Two weeks after the second immunization, mice were challenged with 10 LD₅₀s of ricin, as described above. As shown in Table S2 in the supplemental material, all of the mice immunized with the 0.3 μg of RiVax or RV Ec survived the ricin challenge, underscoring that there is no qualitative difference between the two vaccines at the doses tested in this study. However, RV Ec-vaccinated mice again produced higher RTA-specific serum IgG titers than the RiVax-vaccinated mice (Fig. 1D).

Following this low-dose immunization, neither the RiVax- nor the RV Ec-immunized animals had detectable TNA in their sera, despite the fact that the animals were immune to the ricin challenge. This phenomenon (i.e., immunity in the absence of detectable serum-neutralizing antibodies) was observed previously and is likely due to the relative insensitivity of the Vero cell-based cytotoxicity assay used to measure TNA (27). We cannot, however, formally rule out the possibility that alternative (innate or adaptive) mechanisms of ricin toxin neutralization exist in vivo that are not reflected in standard in vitro Vero cell-based TNA assays (e.g., Fc-mediated clearance). Another apparent discrepancy that needs to be pointed out is the fact that mice immunized twice with 0.3 μg of Alhydrogel-adsorbed RiVax or RV Ec had higher serum RTA-specific Ab titers than mice immunized twice with 1 μg of the “same” vaccines (compare Table 2 versus Table S2 in the supplemental material). The disparity between these two different studies is likely due to differences in vaccine lots, as well as the time interval between the adsorption of the proteins to Alhydrogel and immunization. (28, 29). For example, Wagner and colleagues recently reported that mice immunized with freshly prepared formulations of anthrax-protective antigen (PA) developed significantly higher toxin-neutralizing antibody titers than mice immunized with the stored preparations (30).

**Pepsan analysis of serum antibodies from RiVax- and RV Ec-immunized mice.** Using pepscan analysis, we recently described six immunodominant (ID) regions (I to VI) on RTA (14). Regions II and IV are postulated to be the target of toxin-neutralizing Abs, whereas regions I, V, and VI are proposed to be targets of nonneutralizing Abs (14). To determine whether the two RTA-based vaccines elicited different pepscan profiles, sera from RV Ec- and RiVax-immunized mice (n = 6 mice/group) were subjected to an RTA 18-mer peptide array (Fig. 2). As described previously, sera from RiVax-immunized mice reacted with immunodominant regions I (residues V28 to S63; peptides A04 to A06), II (residues V82 to F117; peptides A10 to A12), III (A118 to Y153; peptides B02 to B04), IV (T163 to L207; peptides B07 to B10), V (residues E208 to I252; peptides B12 to C03), and VI (residues A253 to F267; peptide C05). In contrast, the RV Ec sera reacted almost exclusively with peptide A11 (residues Y91 to F108), which corresponds to an immunodominant neutralizing linear epitope on RTA (26, 31, 32).

This observation prompted us to examine more closely A11 peptide reactivity in sera from the previous RV Ec dose range immunization study (Table 2). We found that RV Ec immune sera consistently had enhanced reactivity with the A11 peptide compared to that of RiVax immune sera (Fig. 3). Although there was no correlation between A11 peptide reactivity and TNA, as determined by linear regression analysis (data not shown), these data nonetheless reveal differences in recognition of immunodomin-
nant epitopes between sera from RiVax- and RVEc-immunized mice. These differences may be due to subtle conformation differences between RVEc and RiVax that make residues Y91 to F108 more (or less) prone to B cell reactivity (33,34).

Contribution of RTA folding domain 3-specific antibodies to TNA. The fact that RVEc, which lacks the so-called folding domain 3 (FD3) of RTA, was as effective as RiVax at eliciting protective immunity suggests that FD3 does not contribute significantly to elicitation of TNA. To address this issue experimentally, a pool of RiVax antisera containing 200 µg/ml of RTA-specific IgG was passed over an RVEc agarose column, as described in Materials and Methods. We then compared TNA associated with pooled antisera, the RVEc agarose column flowthrough fraction (corresponding to FD3 antibodies), and the eluate (corresponding to FD1 and -2 antibodies). The samples were normalized so that each had 50 µg/ml of RTA-specific IgG and then tested in the Vero cell cytotoxicity assay. We found that ~80% of the TNA present in the starting pooled antisera was associated with the eluate, while less than 10% was associated with the flowthrough fraction (Fig. 4). These data reveal that FD3-specific Abs contribute only minimally to the neutralizing anti-RTA Ab response in RiVax immune sera. These data are not surprising considering that, in the context of the ricin holotoxin, FD3 is largely occluded by the RTB.

Conclusion. Although RiVax and RVEc are currently being tested in phase I clinical trials as aluminum-adsorbed vaccines (18–22), our study is the first to compare the two vaccines side-by-side in an animal model. We compared RiVax and RVEc adsorbed to Alhydrogel and administered to mice s.c. in a 2- or 3-dose immunization regimen across a range of doses (0.3 to 20 µg). At the doses tested, we found that the two vaccines were indistinguishable with respect to their ability to elicit protective immunity to an i.p. toxin challenge performed 14 to 21 days after the last immunization. Moreover, the immune responses elicited by the two vaccines were for the most part quantitatively similar, although the RVEc-immunized mice tended to have higher levels

FIG 2 RTA pepscan analysis of sera from RiVax- and RVEc-immunized mice. Individual serum samples (n = 6 per group) from mice immunized three times with 20 µg RiVax or RV Ec were applied to ELISA plates coated with an overlapping 18-mer peptide array spanning the length of RTA. The cumulative reactivities of the individual samples (y axis) are plotted versus each RTA peptide (x axis). The final peak (far right) represents antibody reactivity with RTA. RV Ec antisera displayed little to no reactivity with peptides A04 to A06 or B11 to C05, which correspond to regions of RTA that were deleted in the construction of RVEc. OD450, optical density at 450 nm.

FIG 3 A11 peptide (RTA residues Y91 to F108) reactivities associated with serum antibodies from RiVax- and RVEc-immunized mice. Sera from mice immunized three times with 10 µg, 3 µg, or 1 µg of Alhydrogel-adsorbed RiVax or RV Ec (x axis) were subjected to BIAcore analysis using an A11 peptide-coated chip. Shown on the y axis are relative resonance units (RU). Error bars represent the standard errors of the means (SEM). An unpaired t test with Welch’s correction was used to determine the differences in A11-specific reactivities between sera from RiVax- and RVEc-immunized mice. *, P < 0.05, and **, P < 0.01.

FIG 4 TNA associated with FD3-specific Abs. Shown is the TNA associated with pooled RiVax immune sera (FD1 + FD2 + FD3), the eluate from an RVEc affinity column (FD1 + FD2), or the RVEc affinity column flowthrough (FD3) fraction. The eluate is enriched for FD1- and FD2-specific Abs, whereas the flowthrough is enriched in FD3-specific Abs. The amount of RTA-specific Abs in the pooled sera, eluate, and flowthrough were normalized to 50 µg/ml prior to being tested in the Vero cell cytotoxicity assay. Error bars represent the SEM.
of toxin-specific serum IgG and TNA than did the mice immunized with comparable amounts of RiVax. In addition, antisera from the RVEc-immunized mice were biased toward an immunodominant solvent-exposed α-helix (Y91 to F108) known to elicit toxin-neutralizing antibodies in mice (31, 32). One important caveat to these studies is that they were done with several different lots of research grade reagents and not strict good manufacturing practices (GMP) materials or protocols. While more definitive studies in nonhuman primates (NHP) and/or humans will be required to verify these studies, they were done with several different vaccine potency lots, and the other will probably depend more on extrinsic factors associated with manufacturing, formulation, and/or stability than on vaccine potency per se.

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