Vaccine 33 (2015) 7299–7306

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Safety and immunogenicity of ricin vaccine, RV*Ec*^{TM}, in a Phase 1 clinical trial

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ARTICLE INFO

Article history:
Received 26 March 2015
Received in revised form 24 July 2015
Accepted 24 October 2015
Available online 3 November 2015

Keywords:
Ricin vaccine
Recombinant vaccine
Clinical trial
Dose
Safety
Immune response

ABSTRACT

Ricin is a potent toxin and potential bioterrorism weapon for which no specific licensed countermeasures are available. We report the safety and immunogenicity of the ricin vaccine RVEc^{TM} in a Phase 1 ($N = 30$) multiple-dose, open-label, non-placebo-controlled, dose-escalating (20, 50, and 100 μg), single-center study. Each subject in the 20- and 50-μg dose groups ($n = 10$ for each group) received three injections at 4-week intervals and was observed carefully for untoward effects of the vaccine; blood was drawn at predetermined intervals after each dose for up to 1 year. RVEc^{TM} was safe and well tolerated at the 20- and 50-μg doses. The most common adverse events were pain at the injection site and headache. Of the 10 subjects who received a single 100-μg dose, two developed elevated creatine phosphokinase levels, which resolved without sequelae. No additional doses were administered to subjects in the 100-μg group. Immunogenicity of the vaccine was evaluated by measuring antibody response using the well standardized enzyme-linked immunosorbent assay (ELISA) and toxin neutralization assay (TNA). Of the subjects in the 20- and 50-μg dose groups, 100% achieved ELISA anti-ricin IgG titers of 1:500 to 1:121,500 and 50% produced neutralizing anti-ricin antibodies measurable by TNA. Four subjects in the 50-μg group received a single booster dose of RVEc^{TM} 20–21 months after the initial dose. The single booster was safe and well tolerated, resulting in no serious adverse events, and significantly enhanced immunogenicity of the vaccine in human subjects. Each booster recipient developed a robust anamnestic response with ELISA anti-ricin IgG titers of 1:13,500 to 1:121,500 and neutralizing antibody titers of 1:400 to 1:3200. Future studies will attempt to optimize dose, scheduling, and route of administration. This study is registered at clinicaltrials.gov (NCT01317667 and NCT01846104).

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1. Introduction

Ricin is a potent protein synthesis inhibitor and a potential biological weapon [1–4]. Currently, no medical countermeasures for ricin intoxication are licensed by the U.S. Food and Drug Administration (FDA).

The toxic consequences of ricin are attributed to the ricin toxin A-chain (RTA), a 32-kDa *N*-glycosidase. The B-chain (RTB), a 34-kDa galactose- or *N*-acetylgalactosamine-binding lectin, binds the toxin to host cell surface receptors. Following endocytosis, the disulfide bond linking the RTA and RTB is reduced in the endoplasmic reticulum, leaving free RTA and RTB. In the cytosol, the RTA attacks ribosomes, inhibiting protein synthesis [5–7]. The rapid rate at which ricin is taken up into host cells [1], coupled with the toxin's enzymatic efficiency [8], leaves a short window for treatment of ricin intoxication. Vaccination is therefore the preferred approach to biological defense preparedness with respect to ricin.

Ricin vaccine candidates, such as a ricin toxoid [9,10] and a native subunit deglycosylated RTA vaccine [11,12], showed promise in animal models but had a number of shortcomings, such as potential reversion to a toxic form, a potential to cause vascular leak syndrome (VLS), protein instability, and the lack of a reproducible and well-characterized manufacturing process [13–15]. Recombinant DNA technology has been employed to address the shortcomings noted above. For example, researchers at the University of Texas produced a recombinant RTA (residues 1–267) vaccine candidate, RiVax, with two amino acid substitutions: a Y80A mutation to reduce ribotoxicity and a V76M mutation to

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http://dx.doi.org/10.1016/j.vaccine.2015.10.094
0264-410X/Published by Elsevier Ltd.

mitigate potential VLS [16]. A dose-escalating Phase 1 clinical study demonstrated the safety and immunogenicity of RiVax [17,18].

Investigators at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) employed computational and structure-guided methods and protein engineering technologies to redesign the RTA into a protein structure that is more stable and soluble than its parent RTA and unable to inhibit protein synthesis. Amino acid C-terminal residues 199–267 in the RTA, which constitute the hydrophobic interfacial surface interacting with the RTB and contributing to the ribosomal inactivating activity, were genetically removed, generating RTA1–198. A protein loop composed of residues 34–43 in the RTA1–198, which was predicted to unfavorably increase the overall solvent accessibility of the protein, was also removed to create the recombinant rRTA1–33/44–198 (RV*Ec*^{TM}) as a lead vaccine candidate [13]. These structural changes to the RTA did not alter a neutralizing epitope previously identified in residues 95–110 [19,20]. In fact, Mantis and colleagues reported that the regions deleted in RV*Ec*^{TM} (residues 199–267 and 34–43) corresponded to RTA regions that they identified as targets for non-neutralizing epitopes and did not contribute to protective immunity [21].

RV*Ec*^{TM} elicited protective immunity in rodents [13,22], New Zealand white rabbits [23], and African green monkeys (L. Smith, unpublished results). In cell binding studies and in an *in vitro* vascular leak assay, the vaccine did not bind to human umbilical vein endothelial cells (HUVECs) or disrupt monolayer integrity of cultured HUVECs at concentrations up to 9 μM, demonstrating that the vaccine lacked cytotoxicity toward primary human endothelial cells [24]. The ricin vaccine was further evaluated in a rabbit reticulocyte cell-free translation assay system [25]; at concentrations of up to 4 μM, no protein inhibition was detected, whereas the half maximal inhibitory concentration of control RTA in the assay was 2.3×10^{-11} M.

A scaled-up manufacturing process for the ricin vaccine, expressed and purified from *Escherichia coli*, was developed, along with associated analytical and biological assays to characterize both the manufacturing process and the ricin vaccine bulk drug substance (BDS) [26]. The vaccine was manufactured in compliance with current Good Manufacturing Practices (cGMP). In a current Good Laboratory Practices preclinical toxicity study conducted in New Zealand white rabbits, no treatment-related or toxicologically significant effects were observed with a dose of up to 200 μg vaccine on study days 1, 29, 57, and 85 [27].

Here, we describe a Phase 1 escalating, multiple-dose study in which we evaluated the safety and immunogenicity of RV*Ec*^{TM} in healthy adults [28]. We hypothesized that RV*Ec*^{TM} would (a) display an acceptable safety profile and (b) elicit enzyme-linked immunosorbent assay (ELISA) IgG titers ≥1:500 and anti-ricin toxin neutralizing antibody titers ≥1:50 in vaccinees.

2. Methods

2.1. RVEc^{TM} vaccine candidate

Recombinant RTA 1–33/44–198 vaccine BDS was manufactured under cGMP at the Biological Process Development Facility at the University of Nebraska (Lincoln, NE). The BDS was formulated into the final drug product (RV*Ec*^{TM}) and fill/finished at the Walter Reed Army Institute of Research Pilot Bioproduction Facility (Silver Spring, MD). Each 3-mL, single-use vial of RV*Ec*^{TM} (lot 1545) contained the following components (1 mL total volume): 0.2 mg rRTA 1–33/44–198 protein, 20 mM sodium succinate, 100 mM NaCl, 0.2% Alhydrogel, and 0.03% Tween-20. Each 5-mL, single-use vial of diluent (lot 1544) contained the following components (2 mL total volume): 20 mM sodium succinate, 100 mM NaCl, and 0.2% Alhydrogel. Both RV*Ec*^{TM} and diluent were stored at 5 °C (2–8 °C).

2.2. Study design

The protocol for this study (FY09-03) was approved by the USAMRIID Human Use Committee (HUC). Healthy, nonsmoker, ricin vaccine–naïve 18- to 50-year-old male and nonpregnant female volunteers were recruited from the military and civilian populations in and around Fort Detrick, Maryland. A synopsis of the study was presented to eligible volunteers; each individual still interested signed an informed consent document. Each enrolled subject was assigned to one of three groups; all subjects received RV*Ec*^{TM} as an intramuscular (IM) injection in the deltoid muscle. Group 1 received 20 μg IM × 3 doses, Group 2 received 50 μg IM × 3 doses, and Group 3 was scheduled to receive 100 μg IM × 3 doses but received only a single dose due to toxicity.

The primary endpoints for measuring safety were the frequency, nature, severity, and causality of adverse events (AEs). These endpoints were evaluated on a per-dose basis and overall on each vaccination day; days 1, 3, 7, 14, and 28 after each dose; and at 6 months after the first dose. Subjects in Groups 1 and 2 were additionally evaluated at 9 and 12 months after the first dose. The endpoints used to measure immunogenicity of the RV*Ec*^{TM} vaccine were total anti-ricin IgG antibody titers, as determined by ELISA, and anti-ricin toxin neutralizing antibody titers, as determined by colorimetric toxin neutralization assay (TNA), at all specified time points. Time points for subjects in Groups 1 and 2 included study days 7, 14, 28, 35, 42, 56, 63, 70, and 84 and months 6 and 9; subjects with detectable titers at month 9 were additionally evaluated at month 12. Time points for subjects in Group 3 included study days 7, 14, 28, 56, 84, and 112 and month 6.

To ensure the safety of subjects, doses were administered in a staggered fashion within and between groups. Specifically, three subjects in Group 1 (20 μg) received dose 1 and were followed for at least 14 days. When safety criteria (triggers for halting dose escalation or for halting the administration of consecutive doses within a dose level) were not observed during the 14-day interval, the remaining seven subjects in Group 1 received dose 1. Doses 2 and 3 were administered 4 weeks and 8 weeks, respectively, after administration of dose 1. When safety criteria were not observed for at least 14 days after 9 of 10 individuals in Group 1 received dose 2, the first three subjects in Group 2 (50 μg) received dose 1. Dose administration and escalation continued in this fashion through dose 3 for Groups 1 and 2 and through dose 1 for Group 3 (100 μg).

A separate protocol (FY12-16) for a single booster dose administered to a subset of subjects in this study was also approved by the HUC. Subjects in Group 2 (50 μg) were invited to participate in the booster study. Of the 10 eligible subjects, four (two males and two females) were available and consented to participate in the booster study. All booster recipients were white, non-Hispanic, and aged 28–49.

2.3. Ricin-specific IgG antibody by ELISA

We used a standardized ELISA to analyze serum specimens for total anti-ricin IgG endpoint titers. Briefly, polyvinyl chloride microtiter plates were coated with ricin toxin at 2.5 μg/mL in phosphate-buffered saline (100 μL per well) and allowed to adhere overnight at 4 °C. Plates were washed three times with ELISA wash buffer and blocked with assay buffer (phosphate-buffered saline with 5% skim milk and 0.5% Tween-20, pH 7.4) for 40–120 min at 37 °C. Unknown serum was diluted in assay buffer to produce seven 3-fold serial dilutions ranging from 1:500 to 1:364,500. A positive control (PC; pooled positive monkey serum, 1:4000 in assay buffer), two negative controls (NC; pooled non-vaccinated

human serum, NC1, 1:500 and NC2, 1:1500, diluted in assay buffer), and a buffer control (BC; as baseline blank) were included in each plate. After three washes, unknown samples and controls were added to the plates (100 μL per well) in triplicate. Plates were incubated for 60 min at 37 °C and washed. The secondary antibody, affinity-purified peroxidase-conjugated goat anti-human IgG (H + L) at 0.1 μg/mL, was added (100 μL per well), and the plates were incubated for 60 min at 37 °C and washed. ABTS peroxidase substrate was added (100 μL per well) to develop the color. Optical densities (OD) were measured at a wavelength of 405 nm. The IgG endpoint titer of a test sample was reported as the dilution factor of the highest triplicate dilution that had a mean OD value ≥0.1. Response was defined as an ELISA titer ≥1:500; samples with titers <1:500 were determined to be nonresponders. For statistical purposes, ELISA titers <1:500 were set to 353.6 (500/$\sqrt{2}$).

2.4. Neutralizing anti-ricin antibodies by TNA

We used a standardized colorimetric TNA to analyze serum specimens for neutralizing anti-ricin antibody endpoint titers. Briefly, each test plate included a PC (positive monkey serum, 1:100 dilution), an NC (pooled non-vaccinated human serum, 1:50 dilution), toxin control (TC; ricin working solution, 8.0 ng/mL), cell control (CC; without toxin), medium control (MC; Dulbecco's minimum essential medium, incomplete), and seven 2-fold serial dilutions of four test human serum samples. All PC, NC, and sample dilutions were run in triplicate (50 μL per well), whereas CC, TC, and MC were run in duplicate. Ricin toxin working solution (50 μL) was added to each well except the CC and MC wells. The plates were incubated at 37 °C for 30 min. During incubation, an EL4 cell suspension was prepared at a concentration of 1.05×10^6 per mL. After incubation, 50 μL of cell suspension was added to each well except the MC wells. The plates were incubated at 37 °C overnight. On the following day, CellTiter 96® AQueous One Solution (30 μL) was added to each well of the plates. The plates were incubated at 37 °C for 4 h. After the incubation, each plate was read at a wavelength of 490 nm. The mean OD of the MC was used as the baseline (blank) of the assay. The results were reported as the human neutralizing anti-ricin antibody endpoint titer, which was the dilution factor of the highest dilution of a test sample that retained a positive reading [29]. Response was defined as a TNA titer ≥1:50. Samples with TNA titers <1:50 were classified as nonresponders. For statistical calculation purposes, TNA titers <1:50 were set to 35.4 (50/$\sqrt{2}$).

2.5. Statistical methods

All significance tests performed were two-tailed. Differences in age were tested by Kruskal–Wallis one-way analyses of variance. Differences in sex, race, and ethnicity were tested by Fisher's exact tests. Confidence intervals of response rates for both immunogenicity and safety were calculated using exact binomial methods. Comparisons of response rates were performed using Fisher's exact tests. No correction for multiple comparisons was used to adjust comparisons of overall immunogenicity response rates between groups. Comparisons of titer values between late and per-protocol groups were performed using Wilcoxon rank-sum tests. All vaccinated subjects were included in the analysis of data. No interim analyses were performed. All analyses were performed using SAS version 9.3.

3. Results

3.1. General

Thirty subjects were enrolled (10 subjects per group) and received at least one dose of vaccine. All 10 subjects in Group 1 (20-μg dose) and all 10 subjects in Group 2 (50-μg dose) received all three doses of vaccine; each of the 10 subjects in Group 3 (100-μg dose) received only one dose before being withdrawn due to AEs.

The majority of subjects were male (67%) and white (97%). The groups did not differ significantly in demographic characteristics (Table 1).

3.2. Adverse events

3.2.1. Systemic adverse events

Pulse oximetry, which was assessed each time vital signs were taken, measured 96–100% throughout the trial for all subjects. Peripheral edema was not observed in any subject throughout the trial. Eighty percent of subjects complained of having at least one systemic AE deemed possibly, probably, or definitely related to vaccine administration; these events were generally mild. The most common systemic AE was headache (experienced by 33% of subjects). Table 2 shows the number of subjects in each group who experienced the most commonly occurring systemic AEs.

3.2.2. Local adverse events

Seventeen subjects (57%) complained of having at least one related local AE; 16 subjects (53%) reported pain at the injection site, as shown in Table 2. All local reactions were mild. Most notably, no instances of erythema, induration, itchiness, or edema were noted.

3.2.3. Clinical laboratory values

Two subjects in Group 3 (100-μg dose) had a grade 4 elevation in creatine phosphokinase (CPK).

Subject 0049, male, Group 3. The subject's first and only dose was received on 17 April 2012. CPK started to rise on post-vaccination day 1 (18 April 2012). Rigorous oral hydration was instituted. CPK peaked on day 4 (21 April 2012) at 3960 U/L and returned to normal by day 10 (27 April 2012). Dose 2 was cancelled. The subject's kidney function remained normal.

Subject 0053, male, Group 3. The subject's first and only dose was received on 20 March 2012. CPK was 463 U/L on post-vaccination day 27 (16 April 2012) and 1,220 U/L on day 28 (17 April 2012). CPK peaked at 30,718 U/L on day 31 (20 April 2012) and returned to normal by day 56 (14 May 2012). Dose 2 was cancelled. The subject was hospitalized for intravenous hydration and observation; thus, this event qualified as a serious AE (SAE), as described below.

3.2.4. Serious adverse events

Two SAEs occurred during the course of this study.

Subject 0023, female, Group 1. Onset: 112 days after dose 3. Duration: 53 days. Diagnosis: shoulder injury related to vaccine administration (SIRVA). Treatment: outpatient surgery. This individual, who had a previous injury in the right shoulder that was not disclosed until after dose 3, had joint pain in that shoulder after dose 1. The subject took ibuprofen for 1 week and the pain ceased. The subject had no pain in the left shoulder after dose 2. The subject had severe persistent joint pain in the right shoulder after dose 3 (given in the right shoulder). This individual received cortisone/lidocaine injections in that shoulder three times and continued to have shoulder pain. The subject then had surgery to remove the injured tissue of the shoulder. The joint pain was determined by the study principal investigator to be a probable SIRVA.

Subject 0053, male, Group 3. Onset: 31 days after dose 1 (subject's only dose). Diagnosis: rhabdomyolysis. This 28-year-old male was enrolled in the study on 17 February 2012. He received his only dose of RV*Ec*™ (100 μg) on 20 March 2012. He reported no complaints. The subject was scheduled to receive his second dose on 17 April 2012. He returned early on 16 April 2012, and CPK was

Table 1
Demographic characteristics of subjects.

		Group 1, 20 μg (*N* = 10)	Group 2, 50 μg (*N* = 10)	Group 3, 100 μg (*N* = 10)	All subjects (*N* = 30)
Gender	Male	7 (70%)	6 (60%)	7 (70%)	20 (67%)
	Female	3 (30%)	4 (40%)	3 (30%)	10 (33%)
Age	Median	33.5	34.0	31.0	32.5
	Min–max	(25–47)	(25–49)	(23–45)	(23–49)
Race	Caucasian/white	10 (100%)	10 (100%)	9 (90%)	29 (97%)
	African-American/black	0	0	1 (10%)	1 (3%)
Ethnicity	Hispanic/latino	1 (10%)	1 (10%)	0	2 (7%)
	Not Hispanic/latino	9 (90%)	9 (90%)	10 (100%)	28 (93%)

Table 2
Number and percentage of subjects experiencing vaccine-related adverse events.

	Group 1, 20 μg (*N* = 10)	Group 2, 50 μg (*N* = 10)	Group 3, 100 μg (*N* = 10)	All subjects (*N* = 30)
Systemic AEs				
Any systemic AE	7 (70%)	10 (100%)	7 (70%)	24 (80%)
Headache	4 (40%)	5 (50%)	1 (10%)	10 (33%)
Hypernatremia	2 (20%)	2 (20%)	3 (30%)	7 (23%)
Nausea	1 (10%)	2 (20%)	2 (20%)	5 (17%)
CPK increase	0	2 (20%)	2 (20%)	4 (13%)
Hypophosphatemia	1 (10%)	3 (30%)	0	4 (13%)
Hematuria	2 (20%)	1 (10%)	0	3 (10%)
Hyperkalemia	1 (10%)	0	2 (20%)	3 (10%)
Local AEs				
Any local AE	7 (70%)	8 (80%)	2 (20%)	17 (57%)
Injection site pain	7 (70%)	8 (80%)	1 (10%)	16 (53%)
Injection site ecchymosis	1 (10%)	0	0	1 (3%)
Injection site papule	1 (10%)	0	0	1 (3%)
Arthralgia	1 (10%)	0	0	1 (3%)
Joint range of motion decreased	0	0	1 (10%)	1 (3%)
Muscle twitching	0	1 (10%)	0	1 (3%)
Lymphadenopathy	1 (10%)	0	0	1 (3%)
Axillary pain	0	0	1 (10%)	1 (3%)
Pain in extremity	0	0	1 (10%)	1 (3%)

Notes: AE = adverse event, CPK = creatine phosphokinase. AEs included in this table are those deemed possibly, probably, or definitely related to the vaccine. Among systemic AEs, only those experienced by at least three subjects are shown. Local AEs include AEs at the injection site and elsewhere on the injected arm.

463 U/L. Later during the evening of 16 April 2012, he moved and lifted heavy furniture. When he returned for his second dose on 17 April 2012, the CPK test was repeated in accordance with the protocol and was determined to be 1220 U/L. Dose 2 was cancelled. Vigorous water intake was instituted, but he was unable to consume the recommended quantity of water. CPK continued to rise, peaking at 30,718 U/L on 20 April 2012 with fractionation showing CK-MB = 0%, CK-BB = 0%, and CK-MM = 99.9%; serum and urinary myoglobin were positive. The subject was admitted to a military hospital for treatment. During the first night of admission, he complained of headache and nausea and was treated with Zofran (ondansetron) and Phenergan (promethazine). The subject received a total of approximately 7 L of normal saline during the course of hospitalization. He was discharged on 22 April 2012 with instruction to continue oral hydration. CPK was normalized by 14 May 2012.

3.3. Ricin-specific IgG titers by ELISA

Subjects in Groups 1 and 2 received all three doses planned for this clinical trial. The geometric mean titers (GMT) of anti-ricin-specific IgG for the two groups are shown in Fig. 1A. Antibody titers peaked at study day 84 for both Groups 1 and 2, then slowly waned over the next several months. Each subject in Groups 1 and 2 seroconverted by mounting a detectable ELISA titer (Table 3). In Group 3, in which subjects received only a single dose of vaccine, 60% of subjects seroconverted (data not shown).

Five subjects in Group 2 were delayed by 27 days in receiving dose 3 of RV*Ec*^{TM}. A comparison of the mean titer of those who received the third dose on time and those who were delayed is shown in Fig. 1B. The delayed group had higher mean titers that persisted over the course of the study, but the difference was not statistically significant.

3.4. Neutralizing anti-ricin antibody endpoint titer by TNA

Fig. 1C shows that TNA endpoint titers began to rise after dose 3 in Groups 1 and 2, with 50% of subjects achieving a detectable TNA titer (Table 4). In Group 3, only one subject seroconverted (data not shown).

3.5. Correlation between ELISA and TNA titers

A Pearson correlation between $\log_{10}$ ELISA titers and $\log_{10}$ TNA titers, adjusting for time after vaccination and group, yielded an r value = 0.71 ($p < 0.0001$). Considering Groups 1 and 2 separately, $r = 0.69$ ($p < 0.0001$) for Group 1 and $r = 0.73$ ($p < 0.0001$) for Group 2.

3.6. Immune response to a single booster dose of RVEc^{TM} 50 μg

Among the four subjects in Group 2 (50 μg) who received a single booster dose of RV*Ec*^{TM} 20–21 months after the initial dose, all had positive ELISAs in the main study; one subject's peak ELISA was 1:1500 at day 84; each of the other three subjects had a peak ELISA of 1:13,500 at day 70. Two of the booster recipients had positive TNAs in the main study; each had a peak TNA of 1:100 at day 63.

The booster was safe and well tolerated, resulting in no SAEs. All booster recipients developed a robust anamnestic response.

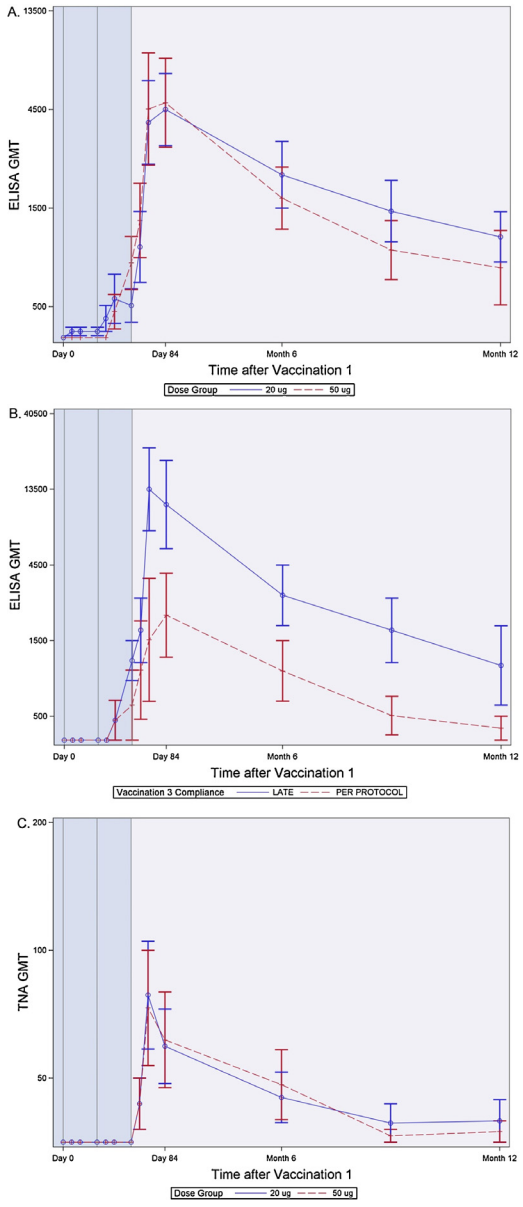

Fig. 1. (A) ELISA geometric mean titer (GMT) of anti-ricin IgG in sera from subjects in Group 1 (20-μg dose) and Group 2 (50-μg dose). Note: error bars represent 1 standard error above and below the geometric mean. For calculation purposes, ELISA titers <500 were set to 353.6 (500/$\sqrt{2}$). $N = 10$ per group for all time points except month 12. $N = 9$ for the 20-μg group (Group 1) at month 12; $N = 6$ for the 50-μg group (Group 2) at month 12. (B) ELISA GMT of anti-ricin IgG in sera from subjects in Group 2 who received RV*Ec*™ dose 3 per protocol or delayed by 27 days. Note: error bars represent 1 standard error above and below the geometric mean. For calculation purposes, ELISA titers <500 were set to 353.6 (500/$\sqrt{2}$). $N = 5$ per group for all time points except month 12. $N = 4$ for the late group at month 12; $N = 2$ for the per-protocol group at month 12. (C) GMT of neutralizing anti-ricin antibodies in sera from subjects in Group 1 (20-μg dose) and Group 2 (50-μg dose). Note: error bars represent 1 standard error above and below the geometric mean. For calculation purposes, TNA titers <50 were set to 35.4 (50/$\sqrt{2}$). $N = 10$ per group for all time points except month 12. $N = 9$ for the 20-μg group (Group 1) at month 12; $N = 6$ for the 50-μg group (Group 2) at month 12.

Table 5 shows the ELISA endpoint titer developed over time after the booster for each of these four subjects. All four booster recipients developed neutralizing anti-ricin antibodies. Three subjects (75%) developed a TNA titer on day 7 (with a GMT of 1:317), and 100% of the subjects developed a TNA titer by day 14 (with a GMT of 1:800). One subject (0002) who did not develop a measurable TNA titer after the primary series achieved a 1:200 TNA titer by day 14 after the single booster. Neutralizing anti-ricin antibody titers for each subject are shown in Table 5.

4. Discussion

RV*Ec*™ was well tolerated and immunogenic at 20-μg and 50-μg dose levels. A single boost vaccination of RV*Ec*™ appears to greatly enhance immunogenicity of the vaccine in human subjects.

The frequencies and types of AEs experienced after RV*Ec*™ administration were similar to those reported by Vitetta et al. [17,18] after RiVax administration. In our study, AEs did not appear to be dose related and thus are unlikely to be related to antigen; rather, they may be related to the adjuvant or preservative.

Although the elevated CPK levels noted in 2 of 10 subjects who received a single 100-μg dose cannot be attributed with certainty to RV*Ec*™, such attribution cannot be ruled out with certainty. Notably, these two individuals are the only subjects whose CPK levels rose to more than six times the upper limit of normal. However, the study team accepted that the 100-μg dose was above the tolerable dosage for this vaccine and that further evaluation of RV*Ec*™ should include only significantly lower doses. That the two subjects with grade 3 elevated CPK with onset at day 27 and day 1 after dose 1 are maternal first cousins is curious. They reported no family history of muscle or neuromuscular disease. Both work in the same environment, but no common exposure was identified outside of work. Even after thorough medical histories, no individual or common source was identified. Although the elevation occurred on different days after dose 1, these two subjects experienced the elevation within one calendar day of each other. This may suggest a commonality, environmental or genetic, that has not been uncovered [30–33]. Subject 0053 moved heavy furniture the night before the beginning of his dramatic CPK elevation. Some reports suggest that rhabdomyolysis after eccentric exercise may be associated with polymorphisms in genes coding for proteins such as α-actinin 3 and myosin light chain kinase [34]. One could speculate that it is possible that one cousin could be homozygous and the other heterozygous for one of the familial protein genotypes associated with muscle damage. It seems unlikely that there would still be RV*Ec* in the system of the subject whose CPK elevation started 27 days after vaccination.

Vitetta et al. found that seven individuals vaccinated with RiVax had mild to moderate CPK elevations and one individual had a CPK elevation that rose to 30 times the upper limit of normal for the test [17]. The RiVax recipient with the highest CPK elevation was in the group receiving the lowest dose and experienced the elevation on the same day as the third dose and just prior to receiving the third dose. The CPK elevations were ruled unlikely related to RiVax.

SIRVA has been observed with the IM administration of other vaccines, such as vaccines against influenza and human papillomavirus [35,36]. In the case of SIRVA diagnosed in present study, we postulate that the vaccine was inadvertently injected into the deltoid bursa (damaged by repeated injury) and spread to other shoulder bursii, setting off a cascade of reactions that caused inflammation of the bursii and surrounding areas of the shoulder.

Significant ricin-specific ELISA antibody levels were seen in 100% of vaccinees after the third dose of RV*Ec*™. Ricin-specific ELISA titers tended to be higher in the 20-μg group compared to the 50-μg group. In each group, 50% of vaccinees developed detectable neutralizing antibodies by 2 weeks after dose 3 (at day 70). In Vitetta et al.'s study [17], one of five, four of five, and five of five vaccinees receiving three doses of, respectively, 10, 33, or 100 μg of RiVax seroconverted after the third dose; all subjects with detectable total antibody levels also had neutralizing antibodies.

Of interest, five Group 2 vaccinees in our study received dose 3 on time, and five were delayed by 27 days. The mean ricin-specific

Table 3
ELISA anti-ricin antibody endpoint titers in human serum collected at the time points shown.

Subject #	Dose (μg)	Time after vaccination 1									
		Day 0	Day 7	Day 14	Day 28	Day 56	Day 70	Day 84	Month 6	Month 9	Month 12
Group 1											
0002	20	<500	<500	<500	<500	<500	13500	13500	4500	1500	<500
0007	20	<500	500	500	500	500	4500	4500	4500	4500	1500
0008	20	<500	<500	<500	<500	1500	40500	13500	13500	13500	4500
0014	20	<500	500	500	500	500	4500	13500	1500	1500	1500
0017	20	<500	<500	<500	<500	<500	13500	13500	4500	1500	1500
0019	20	<500	<500	<500	<500	<500	4500	4500	4500	1500	1500
0022	20	<500	<500	<500	<500	1500	4500	4500	1500	500	<500
0023	20	<500	< 500	<500	<500	<500	500	500	<500	1500	1500
0024	20	<500	<500	<500	<500	<500	1500	4500	1500	500	500
0026	20	<500	<500	<500	<500	<500	<500	500	<500	<500	ND[a]
GMT[b] (SEM)		353.6 (1.00)	378.9 (1.05)	378.9 (1.05)	378.9 (1.05)	505.9 (1.21)	3894.5 (1.59)	4500.0 (1.49)	2171.9 (1.45)	1448.9 (1.41)	1088.0 (1.32)
Responders		0/10 (0%)	2/10 (20%)	2/10 (20%)	2/10 (20%)	4/10 (40%)	9/10 (90%)	10/10 (100%)	8/10 (80%)	9/10 (90%)	7/9 (78%)
Group 2											
0025	50	<500	<500	<500	<500	4500	13500	13500	4500	1500	500
0029	50	<500	<500	<500	<500	<500	<500	<500	500	<500	NS[c]
0030	50	<500	<500	<500	<500	<500	<500	1500	500	500	<500
0031	50	<500	<500	<500	<500	<500	<500	1500	500	<500	NS[c]
0033	50	<500	<500	<500	<500	1500	13500	13500	4500	4500	1500
0035	50	<500	<500	<500	<500	1500	4500	4500	1500	1500	500
0036	50	<500	<500	<500	<500	1500	121500	121500	13500	4500	4500
0037	50	<500	<500	<500	<500	<500	4500	4500	1500	<500	NS[c]
0041	50	<500	<500	<500	<500	<500	13500	4500	1500	<500	NS[c]
0045	50	<500	<500	<500	<500	1500	13500	4500	1500	1500	<500
GMT[b] (SEM)		353.6 (1.00)	353.6 (1.00)	353.6 (1.00)	353.6 (1.00)	812.8 (1.34)	4526.6 (1.87)	4851.5 (1.64)	1674.2 (1.41)	939.2 (1.39)	771.5 (1.51)
Responders		0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	5/10 (50%)	7/10 (70%)	9/10 (90%)	10/10 (100%)	6/10 (60%)	4/6 (67%)

Note: each subject received three doses of 20 μg (Group 1) or 50 μg (Group 2) RV*Ec* vaccine at days 0, 28, and 56.

[a] Not determined due to subject withdrawal.

[b] Response was defined as an ELISA titer ≥1:500. Samples with titers <1:500 were classified as nonresponders. For statistical calculations, ELISA titers <1:500 were set to 353.6 (500/$\sqrt{2}$).

[c] No sample collected.

Table 4
TNA neutralizing anti-ricin antibody endpoint titers in human serum collected at the time points shown.

Subject #	Dose (μg)	Time after vaccination 1									
		Day 0	Day 7	Day 14	Day 28	Day 56	Day 70	Day 84	Month 6	Month 9	Month 12
Group 1											
0002	20	<50	<50	<50	<50	<50	200	100	<50	<50	<50
0007	20	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0008	20	<50	<50	<50	<50	<50	400	200	100	100	100
0014	20	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0017	20	<50	<50	<50	<50	<50	100	50	<50	<50	<50
0019	20	<50	<50	<50	<50	<50	100	100	100	<50	<50
0022	20	<50	<50	<50	<50	<50	200	100	50	<50	<50
0023	20	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0024	20	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0026	20	<50	<50	<50	<50	<50	<50	<50	<50	<50	ND[a]
GMT[b] (SEM)		35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	78.5 (1.34)	59.5 (1.22)	45.1 (1.15)	39.2 (1.11)	39.7 (1.12)
Responders		0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	5/10 (50%)	5/10 (50%)	3/10 (30%)	1/10 (10%)	1/9 (11%)
Group 2											
0025	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0029	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	NS[c]
0030	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
0031	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	NS[c]
0033	50	<50	<50	<50	<50	<50	100	100	100	<50	<50
0035	50	<50	<50	<50	<50	<50	100	<50	<50	<50	<50
0036	50	<50	<50	<50	<50	<50	800	400	200	50	50
0037	50	<50	<50	<50	<50	<50	100	100	50	<50	NS[c]
0041	50	<50	<50	<50	<50	<50	<50	<50	<50	<50	NS[c]
0045	50	<50	<50	<50	<50	<50	100	100	<50	<50	<50
GMT[b] (SEM)		35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	35.4 (1.00)	73.2 (1.37)	61.6 (1.30)	48.3 (1.21)	36.6 (1.04)	37.5 (1.06)
Responders		0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	5/10 (50%)	4/10 (40%)	3/10 (30%)	1/10 (10%)	1/6 (17%)

Note: each subject received three doses of 20 μg (Group 1) or 50 μg (Group 2) RV*Ec* vaccine at days 0, 28, and 56.
[a] Not determined due to subject withdrawal.
[b] Response was defined as a TNA titer ≥1:50. Samples with TNA titers <1:50 were classified as nonresponders. For statistical calculation purposes, TNA titers <1:50 were set to 35.4 ($50/\sqrt{2}$).
[c] No sample collected.

Table 5
ELISA and TNA endpoint titers in human serum collected at specified time points after a single RV*Ec* booster vaccination.

Subject ID[a]	Time after boost									
	Day 0	Day 1	Day 3	Day 7	Day 14	Day 28	Day 42	Day 56	Day 84	Month 6
ELISA										
0031/0002	<500	<500	<500	<500	13500	13500	13500	13500	13500	4500
0033/0001	<500	500	1500	40500	121500	121500	121500	121500	121500	40500
0041/0004	<500	<500	<500	40500	121500	121500	364500	121500	121500	121500
0045/0003	<500	<500	<500	4500	121500	121500	121500	121500	ND[b]	ND[b]
TNA										
0031/0002	<50	<50	<50	<50	200	200	200	200	400	200
0033/0001	<50	<50	<50	400	1600	1600	1600	1600	1600	800
0041/0004	<50	<50	<50	400	1600	1600	1600	1600	3200	3200
0045/0003	<50	<50	<50	200	800	1600	1600	1600	ND[b]	ND[b]

Notes: each of four subjects received three 50-μg doses of RV*Ec* vaccine followed by one 50-μg booster dose 20–21 months after the first dose.
[a] Each volunteer receiving a booster dose was assigned one subject identification number for the initial protocol (FY09-03), shown on the left, and one for the follow-up/boost protocol (FY12-16), shown on the right.
[b] Not determined due to subject withdrawal.

IgG antibody levels in the delayed group were higher than those in the on-time group and remained so for the duration of the study. The one vaccinee in the Vitetta et al. study [17] who was delayed in receiving the third dose of RiVax had the highest titer. Increasing the intervals between doses of other vaccines has similarly resulted in higher antibody levels in the delayed groups [37]. RV*Ec*™ and RiVax behaved similarly in mouse studies [38].

Future studies should optimize dose and schedule and investigate the relationship between the duration of delay and antibody response. As an adjunct to these studies, the immunologic characteristics of antibodies produced against RV*Ec*™ should be investigated. The purpose for the development of a vaccine against ricin toxin is to enable its use as a prophylactic countermeasure. Theoretically, it should be feasible to use ricin-specific antibodies in a postexposure treatment scenario as well. To have some assurance about the usefulness of ricin-specific antibodies in the form of human ricin vaccine immune plasma or human ricin vaccine immune globulin, the efficacy of such antibodies must be demonstrated in animal models. It is imperative to perform passive transfer studies in the near future as proof of concept that human antibodies protect against ricin intoxication.

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. Research on human subjects was conducted in compliance with U.S. Department of Defense, federal, and state statutes

and regulations relating to the protection of human subjects and adheres to principles identified in the Belmont Report (1979). All data and human subjects research were gathered and conducted for this publication under an IRB-approved protocol, number FY09-03.

Acknowledgements

The authors wish to thank Elizabeth S. Brown, Ph.D., for her editorial contributions to this manuscript. This work was supported by the Defense Threat Reduction Agency, Joint Science and Technology Office for Chemical-Biological Defense.

Conflict of interest: None.

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