



HHS Public Access

Author manuscript

Expert Rev Vaccines. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

Expert Rev Vaccines. 2016 September ; 15(9): 1213–1222. doi:10.1586/14760584.2016.1168701.

Progress and Challenges Associated with the Development of Ricin Toxin Subunit Vaccines

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Summary

The past several years have seen major advances in the development of a safe and efficacious ricin toxin vaccine, including the completion of two Phase I clinical trials with two different recombinant A subunit (RTA)-based vaccines: RiVaxTM and RVEcTM adsorbed to aluminum salt adjuvant, as well as a non-human primate study demonstrating that parenteral immunization with RiVax elicits a serum antibody response that was sufficient to protect against a lethal dose aerosolized ricin exposure. One of the major obstacles moving forward is assessing vaccine efficacy in humans, when neither ricin-specific serum IgG endpoint titers nor toxin-neutralizing antibody levels are accepted as definitive predictors of protective immunity. In this review we summarize ongoing efforts to leverage recent advances in our understanding of RTA-antibody interactions at the structural level to develop novel assays to predict vaccine efficacy in humans.

Keywords

toxin; biodefense; vaccine; protection; antibody; neutralizing; epitope

A. Perspective

The development of vaccines against lethal biological threat agents (biothreats) like ricin toxin poses unique challenges within the world of vaccinology [1,2]. Most notable among these is the need to assess vaccine worthiness when human efficacy trials are not feasible because of ethical and safety concerns. The Food and Drug Administration (FDA), which oversees vaccine regulatory concerns in the United States recognizes the inherent limitations associated with the development of medical countermeasures (MCM) for biodefense and has put forth guidelines for vaccines that rely on careful documentation of immune correlates in relevant animal models, preferably supported by descriptions of the mechanisms that

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Declaration of Interests

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

underlie protection [3]. A persuasive case for vaccine licensure further necessitates validating specific responses observed in animal models to humans.

In this review we showcase recent advances in the development of ricin toxin subunit vaccines and highlight efforts to validate candidate vaccines using a combination of mouse and human immunology, structural biology and serology-based techniques. We argue that integrated approaches being used to develop a ricin toxin vaccine may serve as a prototype for other biothreat agents for which human efficacy trials are not feasible. This review will specifically focus on recent milestones in ricin vaccine development, notably Phase I clinical trials for two different subunit vaccines and immunity to aerosol challenge in a non-human primate model. The reader is referred to excellent recent reviews on other aspects of ricin, including incidence of human and animal exposures [4,5], biology of the toxin at the cellular and sub-cellular levels [6–8], animal models of ricin intoxication [9], immunity to ricin and preclinical vaccine development [10,11], and the design and development of post-exposure therapeutics [12].

B. Ricin toxin: Structure and function

Ricin is a member of the ribosome-inactivating protein (RIP) family of toxins found throughout the plant and microbial worlds [13,14]. Ricin toxin is found in the beans of the castor oil plant, *Ricinus communis*, which is ubiquitous in tropical and subtropical environments. While castor bean plants are often propagated as ornamentals in private and public garden spaces, they are most commonly grown for industrial applications. It is estimated that more than a million tons of castor beans are produced annually, with cultivation occurring mostly in India and Brazil. The oils extracted from the castor beans, notably ricinoleic acid, an unsaturated omega-9 fatty acid, are used in the production of industrial lubricants, cosmetics and even biofuels [15,16].

In its mature form, ricin is a 65 kDa glycoprotein consisting of two subunits, RTA and RTB, joined by a single disulfide bond [17–20]. RTA (267 amino acids) is an RNA N-glycosidase (EC 3.2.2.22) that selectively inactivates eukaryotic ribosomes through cleavage of a universally conserved ribosomal RNA element known as the sarcin-ricin loop (SRL) [21–23]. RTA assumes three distinct folding domains (residues 1–117, 118–210, and 211–267) [18,20] (Figure 1). The active site consists of a large, polar and solvent exposed cleft situated on one face of the molecule. Five residues, Tyr80, Tyr123, Arg180, Glu177, and Trp211 are critical for RTA's RNA N-glycosidase activity [20,24–27]. One of the candidate ricin toxin subunit vaccines, RiVax, carries a point mutation in Tyr80 that attenuates RTA's RNA N-glycosidase activity more than 1000 fold. The other candidate subunit vaccine, RVEc, is equally attenuated because of a deletion of folding domain 3 (residues 199–267).

Ricin's binding subunit, RTB is 262 amino acids in length and consists of two globular domains with identical folding topologies [18,28]. Domains 1 and 2 are themselves comprised of three homologous sub-domains (α , β , γ) that probably arose by gene duplication from a primordial carbohydrate recognition domain (CRD) [29]. Only sub-domains 1 α and 2 γ retain functional carbohydrate recognition activity [29,30]. Sub-domain 1 α binds Gal and is considered a "low affinity" CRD, while sub-domain 2 γ binds both Gal

and GalNAc and is considered a “high affinity” CRD [20,31–33]. Both domains are involved in RTB’s ability to attach to cell surfaces [30,34].

The cytotoxic effects of ricin are well documented and have been recently reviewed [35,36]. Ricin is capable of attaching to and entering all mammalian cell types, including epithelial cells that line the respiratory and gastrointestinal tracts. Following attachment, the toxin is internalized into endosomes by clatherin-dependent and -independent mechanisms and then traffics, by a process known as retrograde transport, to the trans-Golgi network (TGN) and eventually the endoplasmic reticulum (ER). In the ER, RTA and RTB separate via a process involving protein disulfide isomerase (PDI) and ER degradation-enhancing α -mannosidase I-like protein 1 (EDEMI) [37–39]. RTA is then retrotranslocated across the ER membrane into the cytoplasm where refolding is facilitated by cytoplasmic chaperons (e.g., Hsc70) and possibly ribosomes themselves [6,40–42].

Once in the cytoplasm, RTA cleaves the *N*-glycosidic bond of a conserved adenosine residue within the SRL of 28S rRNA [23,43]. Hydrolysis of the SRL results in an immediate arrest in ribosome progression and a cessation in translation [22]. Damage to ribosomal RNA also activates the so-called ribotoxic stress response (RSR) involving stress-activated protein kinases (SAPK), leading to the production of pro-inflammatory cytokines and apoptosis-mediated cell death [44,45].

At the whole animal level, ricin is most toxic following injection or inhalation, with the latter route of exposure most relevant to the biodefense community [46,47]. The LD₅₀ of ricin by aerosol exposure is estimated to be 4 μ g/kg in mice [48] and 5.8 μ g/kg in Rhesus macaques [46,49]. Mice and non-human primates succumb to 5–10 LD₅₀ ricin exposure within 1–3 days; the ultimate cause of death has been attributed to respiratory failure. The recent Rhesus macaque studies conducted by Roy and colleagues underscore previous reports documenting the profound effects that ricin has on lung physiology, including massive fluid accumulation, inflammatory exudate, tissue edema, and hemorrhage [50,51]. For these reasons, eliciting a protective mucosal immune response against aerosolized ricin is considered one of the most significant challenges in ricin toxin vaccine development.

C. Preclinical studies of ricin toxin subunit vaccines, RiVax and RVEc

There are two candidate ricin toxin vaccines under development, RiVaxTM and RVEcTM. Both are recombinant, non-toxic derivatives of ricin’s enzymatic subunit, RTA (Table 1; Figure 1). RiVax, developed by Dr. Ellen Vitetta and colleagues at the University of Texas Southwestern (UTSW), is a full-length variant (267 amino acids) of RTA whose enzymatic activity has been eliminated (>1000 fold) through a single point mutation in a key active site residue (Y80). RiVax contains a second mutation at position 76 (V76M) as a means to eliminate RTA’s predilection to induce vascular leak syndrome (VLS) [26,52]. Structurally, RiVax is virtually identical to RTA (Table 1; Figure 1)[53].

RVEc, developed by investigators at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), is a truncated version of RTA that lacks residues 199–267, as well as a small hydrophobic loop in the N-terminus (residues 34–43) (Table 1;

Figure 1) [54–56]. RVEc was engineered to reduce RTA’s intrinsic propensity to aggregate and precipitate from solution. RVEc (also referred to as RTA1-33/44-198) lacks folding domain 3 and is only 188 residues in length. Despite the deletion/truncation, RVEc’s atomic structure is essentially superimposable onto RTA (Table 1: Figure 1)[57]. The fact that RVEc assumes RTA’s normal tertiary structure across folding domains 1 and 2 (despite the absence of folding domain 3) is certainly integral to the success of the vaccine, as a number of potent toxin-neutralizing antibodies are known to target conformation-dependent epitopes within folding domains 1 and 2 [58].

Over the past 10 years, both RiVax and RVEc have been subject to extensive preclinical testing, including efficacy and toxicity studies in mice and rabbits. Dr. Ellen Vitetta and colleagues demonstrated that RiVax elicits protective immunity against systemic and mucosal (aerosol and intragastric) challenges when administered to mice by the subcutaneous, intramuscular, intradermal or intraperitoneal routes [11,26,48,52,59,60]. RVEc is also highly efficacious in the mouse and rabbit models at stimulating systemic and mucosal immunity to ricin [55,56,61,62].

Although RVEc and RiVax have each been under investigation for more than a decade, it wasn’t until just recently that the two vaccines were compared to side-by-side in the same study [63]. Groups of mice were vaccinated by the subcutaneous route across a range of doses (0.3–10 µg) with RVEc and RiVax adsorbed to Alhydrogel. Animals were challenged with 10 LD₅₀ ricin two weeks after the second or third vaccinations. The study revealed that in the mouse model the two vaccines were essentially identical. All vaccinated animals survived challenge, even groups that received only a prime boost regimen with the lowest dose of vaccine. Although RVEc was slightly more effective than RiVax at eliciting toxin-specific serum IgG titers and toxin-neutralizing activity (TNA) after only two immunizations, this trend was not apparent in a more recent study in which RiVax and RVEc were administered to mice by the intradermal routes [64]. While the two vaccines have not been tested head to head in the mouse model using the intramuscular route of vaccination to mimic the route by which the vaccines would be given to humans, there is no reason to expect that they would differ under such circumstances.

D. Outcome of Phase I clinical trials with RiVax and RVEc

The recent completion of two Phase I clinical trials in which RiVax and RVEc, each adsorbed to Alhydrogel, were administered to healthy volunteers suggest that the two vaccines perform similarly to each other in humans [65,66]. The two trials are summarized in Table 2. RiVax was adsorbed to 0.1% Alhydrogel and administered intramuscularly (i.m.) to groups of healthy adults (n=5 per group) at three different dosages (1, 10 or 100 µg) on days 0, 42 and 180. RVEc was adsorbed to 0.2% Alhydrogel and administered i.m. to groups of healthy adults (n=10 per group) at three doses (20, 50 and 100 µg) and on days 0, 28 and 56. In both studies the primary endpoints for assessing safety were occurrences of adverse events (AE). Immunogenicity was based on ricin-specific serum IgG levels determined by ELISA, as well as toxin-neutralizing activity (TNA) measured using mammalian cell-based cytotoxicity assays. Unfortunately, direct comparison of the two studies is slightly complicated because of different methodologies used to assess endpoint titers and TNA.

Vitetta and colleagues report specific IgG ($\mu\text{g/ml}$) based on RiVax-coated ELISA plates, whereas Pittman and colleagues reported geometric mean endpoint titer (GMT) using ricin-coated plates. Vitetta and colleagues assessed TNA using human B cell lymphoma (Daudi) cells [26], while Pittman and colleagues employed mouse T cell lymphoma (EL4) cells [67]. Vitetta reported TNA as the reciprocal endpoint dilution that corresponded to the IC_{50} ; Pittman reported reciprocal endpoint dilution that conferred neutralizing activity above background. Despite these differences in study design and methodologies, there are obvious comparisons that can be made.

Safety

RiVax was similar to RVEc in that the vast majority (80–100%) of study participants reported mild (grade I) systemic AE following vaccination. However, two subjects in the high dose RVEc group (100 μg) did experience grade IV elevation of creatine phosphokinase (CPK) levels after the first vaccination. The reasons for the elevated CPK levels in 2 of 10 subjects is unknown, but Pittman and colleagues make the case that the effects were unlikely to be related to RVEc antigen.

Seroconversion frequencies

Seroconversion frequencies between RiVax and RVEc at comparable dose levels were virtually identical. While the lowest dose of RiVax (1.0 μg) tested did not elicit seroconversion in any of the vaccinees, 4/5 individuals that received 10 μg RiVax and 5/5 that received 100 μg RiVax had seroconverted 2 weeks after the third vaccination. In the RVEc study, 10/10 (100%) and 9/10 (90%) individuals in the 20 and 50 μg groups, respectively, seroconverted after the third vaccination. It is interesting to note that the best seroconversion rates occurred in the middle (not highest) dose ranges. As a case in point, 10 days after the second vaccination, 9/10 individuals in the 20 μg dose RVEc group had seroconverted, whereas only 7/10 (70%) in the 50 μg group had seroconverted.

Peak toxin-specific serum IgG titers

In both the RiVax and RVEc clinical trials, peak ricin-specific serum IgG titers occurred 2–4 weeks after the third vaccination. Because of differences in reporting ricin-specific serum IgG levels between the two studies, a direct comparison of absolute titers is not possible.

TNA

TNA is the indicator most closely associated with immunity to ricin, and therefore a key measure of vaccine efficacy. Unfortunately, neither RiVax nor RVEc were particularly effective at eliciting TNA in human volunteers. In the RVEc study, only 40–50% of the vaccinated individuals in the 20 μg and 50 μg groups had detectable TNA in their sera after the third vaccination [67]. RiVax-vaccinated individuals (10 and 100 μg groups) fared slightly better in that all 8 vaccinated individuals had detectable TNA, although their actual titers were very low (e.g., 20–24) [66].

Ricin-specific IgG and TNA

A historically confounding issue in mouse studies and a pilot Phase I study with RiVax antigen alone (not adjuvanted) has been an apparent disconnect between ricin-specific endpoint titers and TNA [63,68]. For example, in the RiVax pilot Phase I trial, volunteer 13's ricin-specific serum IgG levels were 4.7 µg/ml and toxin-neutralizing titers were 1.4 µg/ml [68]. Volunteer 7, on the other hand, had 22 µg/ml of ricin-specific serum IgG but only 1.7 µg/ml toxin-neutralizing titers. This disconnect was evident in the subsequent Phase I clinical trials. Pittman performed a Pearson correlation analysis examining ELISA units versus TNA from RVEc vaccinated individuals, which yielded an r value of 0.71 [65]. A similar analysis of RiVax-vaccinated individuals revealed an r value of 0.81, although that number was adjusted to eliminate a pair of outliers from the analysis [66]. The exact relationship between ricin-specific endpoint titers and toxin-neutralizing activity is an issue that needs to be resolved in future clinical studies.

Half-life of ricin-specific serum IgG

In both cases, toxin-specific serum IgG titers dropped steadily 2–4 weeks after the final vaccination, with half-lives between 100 and 180 days.

Passive transfer studies

Unfortunately, neither of the recent Phase I clinical trials included passive transfer studies to examine whether sera from humans vaccinated with RiVax or RVEc are sufficient to neutralize ricin in a mouse challenge model. Passive transfer studies were, however, done as part of a former pilot Phase I clinical trial with RiVax (without adjuvant), which demonstrated that high amounts (e.g., 25–60 µg) of human RiVax IgG antibodies were able to neutralize the effects of ricin in a mouse model[68].

Conclusions from RiVax and RVEc Phase I clinical trials

In summary, RiVax and RVEc were deemed comparatively safe and immunogenic in humans. However, toxin-specific antibody levels demonstrated short half-lives and neither vaccine proved particularly effective at eliciting toxin-neutralizing activity. While both these issues could potentially be resolved with more potent adjuvant(s) (see Section F), the ultimate success of the ricin vaccine program also necessitates more established surrogate and/or correlate markers of protection that can be used to assess vaccine efficacy in humans.

E. Vaccine-induced immunity to aerosolized ricin challenge in non-human primates

A recently completed RiVax vaccination/aerosol challenge study conducted in Rhesus macaques constitutes a milestone in the development of ricin subunit vaccine [47]. The study consisted of two cohorts of nine Rhesus macaques (Table 2). In each cohort six animals received 100 µg RiVax adsorbed to Alhydrogel and administered by intramuscular injection on days 0, 30 and 60. Three animals in each cohort served as controls and were not vaccinated. On day 110, the macaques were subject to aerosolized ricin challenge (1.5–6.5

LD₅₀). Serum samples collected over the course of the study were examined for seroconversion, ricin-specific IgG levels and TNA.

Serum analysis studies indicated that all 12 RiVax-vaccinated animals had seroconverted two weeks after the second vaccination (day 45), with peak endpoint titers observed two weeks after the third immunization (1534 µg/ml RiVax-specific IgG; range 1190–1977 µg/ml). Toxin-neutralizing titers achieved a geometric mean TNA endpoint titer of 427 (163–1132 range) two weeks after the third immunization. The TNA values increased an additional 3-fold (1280) in the ensuing month, indicating that toxin-neutralizing antibodies continued to mature for weeks after the third vaccination, a phenomenon that we have observed in mice [69]. Following aerosolized toxin challenge, control animals expired or were euthanized within 3 days, The RiVax-vaccinated animals all survived, except for one which died from complications of a secondary infection and not a direct consequence of ricin intoxication.

The study by Roy and colleagues is the first report in the literature demonstrating that parenteral vaccination of non-human primates with a recombinant, aluminum salts adsorbed RTA-based subunit vaccine is sufficient to confer protective immunity to an aerosolized ricin challenge. All previous vaccination/aerosol challenge studies were conducted in mice, rats or rabbits [48,59,62]. The demonstration that Rhesus macaques can be rendered immune to inhaled ricin justifies the continued development of RiVax (and/or RVEc) for use in humans and downplays the need to identify alternative vaccine antigens. In our opinion, resources can now be allocated to better defining (i) correlates of immunity, (ii) optimizing vaccine formulations, (iii) defining route and optimal timing of vaccinations, and (iv) identifying compatible adjuvants [2].

The second important aspect of the study by Roy and colleagues is the demonstration that parenteral vaccination is sufficient to confer local immunity to a toxic agent like ricin, which is known to affect the respiratory epithelium and elicit massive pulmonary inflammation [46]. The suggestion from the study is that ricin was neutralized in the lung by toxin-specific serum IgG antibodies that had gained access to the respiratory compartment before or immediately following toxin exposure through transudation or possible FcR-mediated transport [70]. While a formal role for ricin-specific IgA in protection cannot be ruled out, parenteral vaccination is known not to stimulate a secretory antibody response. To our knowledge, there is only one other report that demonstrates in a non-human primate model that respiratory immunity to a biological toxin can be achieved through parenteral immunization. The other report entailed three intramuscular vaccinations of Rhesus monkeys with staphylococcus enterotoxin B (SEB) toxoid, which afforded protection against subsequent aerosol challenge [71]. Protective immunity correlated with circulating anti-SEB IgG antibody titers, further substantiating the importance of serum IgG in mucosal immunity to biothreat toxins.

Finally, some comparisons can be made regarding the relative magnitudes of the toxin-specific responses between the Rhesus macaques and human volunteers that received RiVax [47,66]. However, it must be underscored that the human vaccine study was conducted with freshly prepared RiVax antigen adsorbed to Alhydrogel on the day of vaccination (E. Vitetta,

personal communication) [66], whereas the NHP study was conducted using a lyophilized glassy solid formulation of RiVax adsorbed to aluminum salts adjuvant [47,72]. The monkeys had peak toxin-specific serum IgG levels following RiVax vaccination that were on average 10 times greater than what was observed in RiVax-vaccinated humans (Table 2). At the time of challenge, however, toxin-specific serum IgG levels had declined to 267–499 µg/ml and were therefore just 2–3 times over the levels that were achieved in several of the humans that had received three vaccinations with RiVax (256–280 µg/ml). Because TNA in the monkeys were reported as endpoint titers, while the human samples were reported as micrograms per milliliter it is not possible to directly compare the two species in terms of toxin-neutralizing antibodies [47,66]. This fact underscores the need for the field as a whole to establish standardized and validated assays so that critical parameters like toxin neutralization can be compared across species and across different clinical trials.

F. Key challenges moving forward in ricin toxin vaccine development

In addition to the need to establish standardized and universally accepted assays in the ricin research community, there are at least three additional challenges associated with the further development of a ricin toxin subunit vaccine.

The first pertains to subunit stability. RTA is purportedly highly unstable in solution and has a propensity to unfold and aggregate, a characteristic that would severely limit the shelf life of a candidate ricin vaccine [55,56,73]. In the case of RVEc, the issue of protein stability has been addressed at the genetic level through elimination of domains and loops involved in unfolding [56]. RVEc has been further engineered to include stabilizing disulfide bonds [74]. In the case of RiVax, the stabilization issue has been addressed through lyophilization technology [72]. RiVax adsorbed to aluminum hydroxide solutions in histidine or ammonium acetate buffers were lyophilized with trehalose as a stabilizing excipient. The lyophilized vaccine preparations were stable at 40°C for more than 15 weeks without an effect on immunogenicity or potency, as measured in the mouse model. While a rigorous systematic stability/potency study of lyophilized RiVax has not been reported in the literature, it is clearly efficacious because the lyophilized version of RiVax was administered to the Rhesus macaques that survived aerosol challenge [47].

The second major challenge associated with ricin toxin subunit vaccine development relates to adjuvanticity. It is apparent from the Phase I trials that neither RVEc nor RiVax adsorbed to Alhydrogel is sufficiently robust in humans, as evidenced by the fact that serum IgG titers were slow to peak and TNA were either not detectable or just above baseline even after a prime and two boosts [65,66]. Investigations into the effects of different “next generation” adjuvants like AS01 on RiVax and RVEc is clearly warranted [75–77]. Towards this goal we recently demonstrated that LT-IIa and LT-IIb_{T13I}, two related ADP-ribosylating enterotoxins from *E.coli* are potent adjuvants when combined with RiVax and RVEc and administered to mice by the intradermal route [64,78]. It remains to be determined whether either of these adjuvants is active in humans.

The third obstacle associated with ricin toxin subunit vaccine development relates to assessing RiVax and RVEc’s efficacy in humans in the absence of human challenge studies,

especially considering that the exact correlates of immunity to ricin remain poorly understood. On the one hand, the importance of toxin-neutralizing antibodies in protection against systemic and mucosal ricin exposure is indisputable. In just the past ten years, there have been more than a dozen reports demonstrating that passive administration of a toxin-neutralizing monoclonal antibody (mAb), polyclonal antibody (pAb) mixture or Fab fragments is sufficient to render mice impervious to a lethal dose of ricin administered by injection, ingestion or inhalation [79–88]. On the other hand, toxin-specific serum IgG levels, at least in mice, are not reliable indicators of immunity. We have observed that mice with similar or even identical toxin-specific endpoint titers, typically ranging from 1,000–10,000, experience different fates upon ricin challenge: some survive while others succumb at rates only marginally better than unvaccinated control animals, suggesting that the “quality” rather than the “quantity” is the primary factor in determining protection.

At the present time the sole measure of the “quality” of an anti-ricin antibody response is based on *in vitro* toxin-neutralizing activity (TNA) using mammalian cell-based cytotoxicity assays [89]. In dozens of experiments involving hundreds of mice conducted with RiVax and RVEc over the past three years, we have found that animals with detectable serum TNA are virtually guaranteed to survive a lethal dose ricin challenge [63,64,69,78]. In this respect, TNA can be considered an absolute correlate of protection, which Plotkin defines as “a quantity of a specific immune response to a vaccine that always provides near 100% protection” [90].

The problem is that TNA in and of itself may be too stringent a threshold by which to judge protection, because it is well documented in mice that survival following a lethal dose ricin challenge can occur in the absence of detectable TNA [63,64,69,78]. As a case in point, O’Hara and colleagues vaccinated groups of mice (n=10 per group) two times at monthly intervals with 0.3 microgram of RiVax or RVEc adsorbed to Alhydrogel, resulting in mean reciprocal endpoint titers that were 40–80,000 [63]. As it turns out all vaccinated animals survived 10xLD₅₀ ricin toxin challenge, even though none had detectable TNA in their sera. We speculate that the levels of circulating toxin-neutralizing antibodies are simply below the limit of detection in our current cell-based assays [81]. Alternatively, we cannot formally rule out the possibility that a polyclonal mixture of “non-neutralizing” antibodies (as determined using current *in vitro* assays) may have the potential to inactivate ricin *in vivo* through mechanisms that have not yet been identified. For example, neutralization of anthrax toxin is modulated by FcR-mediated clearance though the action of specific IgG subclasses [91,92]. In the case of ricin toxin, the contribution of Fc-mediated neutralizing activity *in vivo* remains up for debate [84,87,93]. Indeed, very little is known about the exact mechanisms by which ricin is neutralized *in vivo*.

G. Establishing surrogate markers of immunity to ricin

The concept of serum antibody profiling or immune signature analysis is an emerging application in vaccinology and may be ideally suited to the ricin vaccine community [94–96]. Based on work from our laboratory, as well as several other laboratories, evidence suggests that, in the case of ricin, toxin-neutralizing antibodies constitute a small fraction (10%) of the antibody response elicited by RiVax and RVEc, and neutralizing antibodies

target a very limited number of “hotspots” on the surface of RTA, which we refer to as epitope Clusters I-IV (Figure 2) [58,81,97]. So-called Cluster I was first identified by Lemley and colleagues as the target of the potent ricin-neutralizing mouse mAb UNIVAX 70 or simply R70 [98]. Peptide array analysis indicated that R70 recognizes a linear epitope corresponding to RTA’s α -helix B (residues 97–107) [99,100]. We identified a second murine mAb known as PB10 that binds the same peptide, and is as effective as R70 at neutralizing ricin *in vitro* and *in vivo* [81]. High resolution epitope mapping studies using a phage displayed peptide library demonstrated that PB10 and R70 recognize slightly different epitopes, even though they are focused on the same α -helix and bind the same peptide [101]. Recent X-ray crystallographic studies of toxin-antibody complexes have enabled atomic level resolution of epitopes within Cluster I. Zhu et al. solved the structure of RTA in complex with Fab fragments from a PB10-like mAb called 6C2 [102], which revealed antibody contact primarily with α -helix B (residues 97–107).

To interrogate Cluster I in more detail, we screened a library of single domains camelid antibodies (VHH) from RiVax- and ricin toxoid-vaccinated alpacas [103]. Of the eleven anti-RTA VHHs identified in our first screen, seven were directed against Cluster I. Six were originally classified as Cluster I binders based on competitive ELISAs with PB10, while the seventh’s epitope was revealed through X-ray crystallography [97]. A second screen of the same VHH library identified an additional seven novel anti-RTA VHHs, six of which are Cluster I-specific (D. Vance, J. Tremblay, C. Shoemaker, N. Mantis, unpublished results). To date, seven X-ray crystal structures of Cluster I VHHs in complex with RTA have been solved [97] (M. Rudolph, D. Vance, M. Cassidy, Y. Rong, C. Shoemaker, and N. Mantis, manuscript submitted). All seven VHHs differ in the degree to which they make contact with β -strand h (residues 113–117), α -helix D (residues 150–157), and α -helix B (residues 97–107).

The other three epitope Clusters (II-IV) were originally defined based on our collection of “legacy” mAbs SyH7, IB2 and GD12 [58,79,81]. Cluster II is defined by SyH7, which recognizes a linear epitope situated within α -helix F (residues 187–198), Cluster III by IB2, which recognizes a discontinuous epitope involving α -helices C and possibly G (R. Toth, D. Weis, D. Volkin and N. Mantis, manuscript in preparation) and, finally, Cluster IV by GD12, which recognizes a linear epitope situated within α -helix E (residues 163–174). The identification of additional Cluster II- and III-specific mAbs, as well as numerous VHHs that target epitopes in or adjacent to those regions, has enabled us to define using X-ray crystallography, hydrogen deuterium exchange (HDX) and competition ELISAs, neutralizing and non-neutralizing epitopes within these two regions of RTA (D. Vance, M. Rudolph, G. Van Slyke, D. Weis, R. Toth, D. Volkin, C. Shoemaker, N. Mantis, manuscripts in preparation). Cluster IV is interesting because it was first identified as a target of toxin-neutralizing antibodies elicited in Hodgkin’s lymphoma patients who had received RTA-immunotoxin therapy [104]. However, GD12 remains the only mAb known that is directed against this region.

To begin to assess the utility of immune profiling analysis in ricin vaccine, sera from RiVax-vaccinated macaques and Phase I clinical trial volunteers were subjected to direct competition ELISAs with toxin-neutralizing mAbs directed against Cluster I (WECB2,

PB10, R70) and Cluster II (PA1, SyH7) [47]. We reasoned that the degree to which an antiserum reduced binding of Cluster I and II-specific mAbs would serve as an indirect indicator of epitope utilization. Indeed, we observed that antisera from RiVax-vaccinated Rhesus macaques and humans reduced binding of Cluster I and II-specific mAbs by 10–80% depending on the specific mAb. Monkeys and humans had similar inhibitory profiles suggesting the antibody response is conserved between the two species. While these data are encouraging and suggestive of the possibility of serum antibody profiling being useful in assessing vaccine efficacy, the connection between mAb competition, toxin-neutralizing activity and protection has yet to be made.

One final point worth mentioning on the topic of B cell epitopes relates to how it is that RVEc is as effective as RiVax at eliciting protective immunity to ricin, considering that RVEc lacks RTA's C-terminus (residues 199–267) and contains a deletion of residues 33–43. We would argue that the apparent immunodominant nature of Cluster I explains why RiVax and RVEc are essentially identical to each other. Specifically, β -strand h (residues 113–117), α -helix D (residues 150–157), and α -helix B (residues 97–107) are completely intact in RVEc. If Cluster I antibodies dominant the response to RiVax and RVEc vaccination, then one would not expect there to be a qualitative difference in the two vaccines. Indeed, that was exactly what we observed experimentally. If anything, we have suggested that the compact nature may actually focus the antibody response against α -helix B, although this has yet to be substantiated [63].

H. Expert Commentary and Five-year view

In summary, the past five years has seen major advances in terms of development of a ricin toxin subunit vaccine. Two clinical trials were completed with two different recombinant RTA-based vaccines, RVEc and RiVax [65,66]. In addition, a non-human primate study with RiVax demonstrated the vaccine's potential to elicit immunity to aerosolized ricin challenge [47]. Finally, the first atomic level insights into the nature of neutralizing B cell epitopes on RTA emerged [97,102].

Acknowledgments

This work was supported in part by Contract No. HHSN272201400021C from the National Institutes of Allergy and Infectious Diseases.

We thank Dr. Michael Rudolph at the New York Structural Biology Center, and Drs. Ronald Toth IV, David Weis, David Volkin at the University of Kansas allowing us to cite unpublished results in this review. We thank Greta Van Slyke, Yinghui Rong, and Jennifer Westfall at the Wadsworth Center for thoughtful discussions.

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Reference annotations

* Of interest

** Of considerable interest

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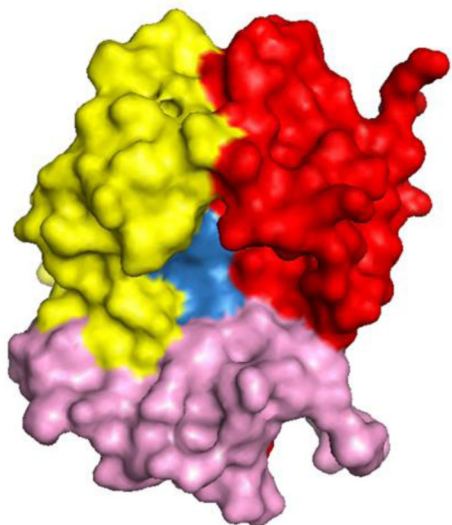
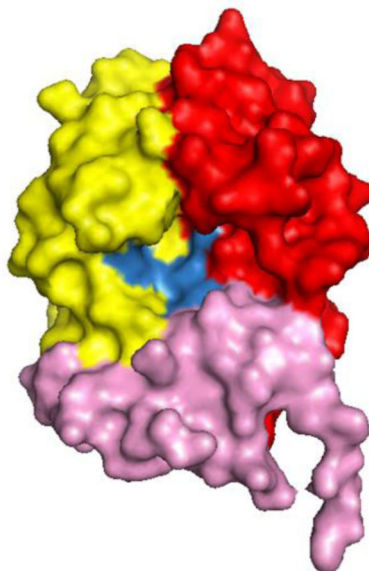
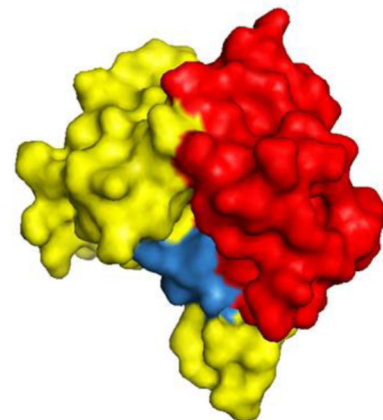
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Key Issues

- We would argue that the success of a ricin vaccine will only occur if the research community continues along this multidisciplinary and highly integrated approach to vaccine development, as bulleted below:
- It is imperative that the ELISA and TNA methodologies are standardized across institutions and that uniform criteria are applied to what constitutes neutralizing activity and protection;
- It is critical that the relationship total toxin-specific antibody levels, TNA, and protective immunity be better defined;
- It is essential that the quantitative thresholds associated with protection be established in mice and non-human primates, and validated in humans.
- Novel approaches like serum antibody profiling may afford an alternative or at least complement cell based toxin-neutralizing assays;
- A head-to-head comparison between RVEc and RiVax in non-human primates and/or humans should be conducted;
- RVEc and/or RiVax should be tested in combination with new adjuvants in an effort to accelerate the onset and magnitude of TNA.

A. RTA (1RTC)**B. RiVax (3SRP)****C. RVEc (3MK9)****Figure 1. Structures of RTA, RiVax and RVEc**

The structures of recombinant (A) RTA, (B) RiVax and (C) RVEc are shown as surface representations generated using PyMOL from the PDB files indicated in parentheses and described in Table 1. RTA's three folding domains (1–3) are colored in red, yellow and pink, respectively. The structures are orientated relative to RTA's active site, which is highlighted in blue. The C-terminal truncation of RVEc relative to RTA and RiVax is evident by the absence of folding domain 3 (pink) and a slightly smaller "active site" region.

Table 1

Crystal structures relevant to ricin toxin vaccine development

PDB^a	Description	Reference
2AAI	ricin	[20]
1RTC	recombinant RTA	[105]
3SRP	RiVax	[53]
3MK9 ^b	RVEc (RTA1–33/44–198)	[57]
Antibody-ricin Interactions		
4LGP	VHH E5-RTA complex	[97]
4KUC	Fab 6C2-RTA complex	[102]

^aIdentification number assigned by the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Database (PDB);

^b, 3MK9 is a disulfide bond-stabilized variant of RVEc, as Compton and colleagues were unable to obtain X-ray crystallographic data of RVEc (1–33/44–198) itself.

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Table 2

Comparison of RiVax and RVEc Phase I and NHP Studies

	Humans		<i>R. macaques</i>
	RVEc ^a	RiVax ^a	RiVax ^a
Trial ID ^b	NCT01317667 NCT01846104	NCT00812071	
Group size (n=)	10	5	12
Groups (µg)	20, 50, 100	1,10,100	100
Vaccination-days	0, 28, 56	0, 42, 180	0, 30, 60
AE ^c	2/30	1/14 (Grade III)	<i>n.a.</i>
Peak endpoint-day	84	210	75
Peak titers	4500–4800 ^d	25–260 µg/ml	1534 µg/ml
Peak TNA-day	70	<i>n.r.</i>	110
Peak TNA-titer	73–78 ^e	20–24 ^f	1280 ^f
Ab half life-day	100	180	<i>n.r.</i>
Reference	[65]	[66]	[47]

^a Adsorbed to Alhydrogel;

^b clinicaltrials.gov identifier number(s);

^c Adverse events (AE). In the RiVax trial, s all volunteers experienced Grade I toxicities, one individual suffered Grade II headache and Grade III nausea. In the RVEc trial, one individual suffered shoulder injury related to vaccine administration, while another suffered rhabdomyolysis;

^d refers to GMT of reciprocal endpoint determined by ELISA using ricin-coated plates;

^e reciprocal toxin-neutralizing endpoint titers (IC₅₀) established in Daudi cells;

^f reciprocal dilution of serum that conferred positive toxin-neutralizing activity in EL4 cells; *n.a.*, not applicable, *n.r.*, not reported.