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Brief report

Combination of two candidate subunit vaccine antigens elicits protective immunity to ricin and anthrax toxin in mice

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ABSTRACT

In an effort to develop combination vaccines for biodefense, we evaluated a ricin subunit antigen, RiVax, given in conjunction with an anthrax protective antigen, DNI. The combination led to high endpoint titer antibody response, neutralizing antibodies, and protective immunity against ricin and anthrax lethal toxin. This is a natural combination vaccine, since both antigens are recombinant subunit proteins that would be given to the same target population.

1. Introduction

The Department of Health and Human Services and the Centers for Disease Control and Prevention (CDC) have designated more than 30 biological agents and toxins as having the potential to pose a severe threat to human health should they be deliberately released into the public sphere (http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html). In many instances, especially in the case of toxins like ricin, the onset of morbidity can occur within a matter of hours and there may be little if any opportunity to intervene therapeutically [1]. In other instances, like the dissemination of anthrax spores through the US Postal Service, the scope of an event could overwhelm public health response capabilities. For this reason, there is a concerted effort by Public Health and Department of Defense officials to develop prophylactic vaccines that could be administered to at-risk populations, including emergency first responders, medical care staff, and laboratory personnel. With a number of candidate biodefense vaccines now in Phase I and II clinical trials [2], it is an opportune moment to consider the possibility of combination vaccines as a means to ultimately minimize the total number of injections and office visits necessary to achieve protective immunity against more than one pathogen or toxin.

Ricin is a ribosome-inactivating protein (RIP) and a member of the AB family of protein toxins [3]. Ricin's enzymatic subunit (RTA) is an RNA N-glycosidase that cleaves ribosomal RNA, leading to protein synthesis arrest and cell death. Ricin's binding subunit (RTB) mediates binding to cell surfaces *via* terminal galactose and N-acetyl galactosamine moieties on glycoproteins and glycolipids. Because of ricin's recent history as a biothreat agent, the development of countermeasures, including a vaccine, are of significant public health importance [2,4]. One of the leading vaccine antigen candidates is RiVax™, a recombinant derivative of RTA whose enzymatic activity has been largely eliminated through a point mutation in a key active site residue and also contains a mutation in the site attributed to the induction of vascular leak syndrome (VLS) [5]. Parenteral vaccination of RiVax, either as an adjuvant-free formulation or adsorbed to aluminum salts, elicits RTA-specific serum IgG antibodies that are associated with both systemic and mucosal immunity to ricin in mice and non-human primates (C. Roy and R. Brey, unpublished results) [6–8]. Furthermore, the results of two Phase I clinical trials have indicated that RiVax is safe and immunogenic in humans [9,10]. A second RTA-based antigen known as RV*Ec* is also in Phase I clinical trials and has been shown to be as effective as RiVax in eliciting immunity to ricin in a mouse model [11]. However, because of the ready availability of GMP-grade RiVax, we chose RiVax over RV*Ec* for this specific study.

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When considering a putative combination vaccine for biodefense, we postulated that RiVax could be combined with a recombinant protective antigen (PA) vaccine antigen aimed at eliciting immunity to *Bacillus anthracis* infection. PA is an 83 kDa protein secreted by *B. anthracis* that forms hepatmers on host cell surfaces and then non-covalently assembles with two other secreted bacterial proteins, edema factor (EF) and lethal factor (LF), to form edema toxin (ET) and lethal toxin (LT), respectively. ET and LT are the major virulence determinants of *B. anthracis* and blocking their action is essential in counteracting the effects of inhalational anthrax [12]. Indeed, PA is one of the primary antigenic components of the currently licensed anthrax vaccine known as Biothrax™, which consists of formalin-fixed culture filtrates of a nonencapsulated strain of *B. anthracis* that have been adsorbed to aluminum salts adjuvant. With the impending phase out of Biothrax™ in favor of more defined vaccine formulations, there are ongoing efforts to identify recombinant derivatives of PA that are safe (*i.e.*, minimally reactogenic), immunogenic and stable [13]. One possible candidate is dominant negative inhibitor (DNI) or VeloThrax™, which is a derivative of PA with two point mutations (K397D, D425K) that impede the capacity of the protein to undergo conformational changes necessary for translocation of EF and LF into host cells [14]. DNI has been shown in mice to be effective at inducing the onset of PA-specific ET and LT neutralizing antibodies [14]. Moreover, clinical grade lots of DNI are remarkably stable in lyophilized form at high temperatures [15]. Thus, DNI constitutes a promising candidate antigen for a second-generation anthrax vaccine.

2. Results and discussion

To assess the feasibility of a RiVax-DNI combination vaccine, groups of female BALB/c mice, 6–8 weeks of ages ($n = 10$/group; Jackson Laboratories, Bar Harbor, ME) were primed on day 0 and then boosted on day 14 with RiVax (10 μg), DNI (10 μg), or a combination of RiVax and DNI (10 μg + 10 μg), administered by intraperitoneal injection. Lyophilized DNI (batch 803918A; Baxter Pharmaceutical Solutions LLC, Bloomington, IN) was reconstituted in sterile water [15]. RiVax (batch 190-100L-GLP-FF-090105) was stored at −20 °C in 50% glycerol and 50% histidine buffer (10 mM histidine, 144 mM NaCl, pH 6.0) [16]. Each antigen was adsorbed to Alhydrogel® (0.85 mg/mL; InvivoGen, San Diego, CA) in histidine buffer for 3 h at 4 °C prior to immunization. For the combination vaccine, DNI and RiVax were each adsorbed to Alhydrogel independently and then mixed 1-to-1 (v/v) just prior to injection. Following the second immunization, each group of animals (RiVax, DNI, and dual) was arbitrarily divided into two groups, A and B. The A groups were ultimately challenged with LT, while the B groups were challenged with ricin. Sera were collected from all immunized mice *via* the lateral tail veins on days 20 and 200, representing snap shots of the early and late ("memory") responses to the vaccines. All animal experiments were done in strict compliance with protocols approved by the Wadsworth Center's Institutional Animal Care and Use Committee (IACUC). Log transformed paired and unpaired *t* tests and the Mantel–Cox test were conducted with Graphpad Prism version 5.0 (San Diego, CA).

Analysis of sera collected on day 20 indicated that the RiVax-vaccinated mice had anti-ricin holotoxin IgG geometric mean titers (GMT) of $>2.0 \times 10^5$ and, as expected, no detectable serum antibodies against PA (Fig. 1A; Table 1; Table S1). On day 200, the ricin-specific IgG GMT had declined by roughly half, as compared to day 20. On day 20, DNI-vaccinated mice had anti-PA GMT of 1.8×10^5, and no detectable serum antibodies against ricin. Analysis of day 200 sera indicated that PA-specific antibody titers in DNI-vaccinated were unchanged, and in some mice slightly increased, as compared to day 20. Finally, the dual RiVax-DNI vaccinated mice had both ricin- and PA-specific serum antibodies on days 20 and 200, indicating that the subunit antigens were not incompatible with each other (Fig. 1A; Table 1; Table S1). However, on day 20, the ricin- and PA-specific serum IgG GMTs in the group of dual vaccinated mice were roughly half of that observed in mice immunized with the individual antigens, demonstrating that the magnitudes of the antibody responses were negatively affected ($p < 0.01$) by the combination of the two antigens. By day 200, anti-ricin antibody titers in the dual immunized mice were still lower than in the mice that received RiVax only, but these differences were not statistically significant ($p = 0.55$). In contrast, anti-PA titers remained lower in the dual immunized mice, as compared to DNI immunized animals ($p < 0.0001$), indicating that the immunogenicity of DNI is adversely affected when combined with RiVax.

Table 1
Ricin- and PA-specific serum antibody geometric mean titers following immunization.

Immunization	Ricin[a]		PA[a]	
	Day 20	Day 200	Day 20	Day 200
RiVax	204,800	109,750	1[b]	1
DNI	1	1	189,619	175,564
Dual	126,069	95,543	44,572	54,875

[a] Coating antigen used in ELISAs, as described in the text.
[b] When no antigen-specific serum IgG titers were detectable, an arbitrary value of 1 was assigned for the purpose calculating the geometric mean titers.

We next assessed ricin- and LT toxin-neutralizing activities (TNA) elicited by RiVax, DNI, or the combination of RiVax and DNI vaccination regimens. Ricin TNA was determined using a Vero cell cytotoxicity assay [17]. LT-neutralizing activity was determined using a J774 murine macrophage cell-based assay [15,18]. Neutralization activity was defined as the highest dilution of serum that protected ≥50% of the cells from toxin-induced death, a value commonly referred to as the $TCIC_{50}$. In day 20 serum samples, the RiVax-vaccinated mice had no detectable TNA, with the exception of one animal that had a neutralizing titer of 100 (Fig. 1B; Table 2; Table S1). On day 200, however, the neutralizing GMT in this group of animals was >450, with individual titers ranging from 100 to 1600. As expected, RiVax-immunized mice had no detectable serum LT-neutralizing activity at either day 20 or 200. DNI vaccination, on the other hand, was highly effective at eliciting the early onset of LT-neutralizing titers, as evidenced by neutralizing GMT of 1087 and 1728 on days 20 and 200, respectively (Fig. 1B; Table 2; Table S1). No detectable ricin-neutralizing activity was evident in the sera of DNI-vaccinated mice at either time point, confirming the absence of cross-reactivity between RiVax and DNI.

The combination of RiVax and DNI was successful at eliciting TNA against both ricin and LT, although the magnitude of the LT response was significantly dampened as compared to mice that received DNI alone ($p < 0.05$; Table 2; Fig. 1B; Fig. S1). Specifically, the LT-neutralizing GMT in the sera of dual immunized mice was 205 and 800 on days 20 and 200, respectively, as compared to 1087

Table 2
Toxin-neutralizing titers in RiVax-, DNI- and Dual-immunized groups of mice.

Group	Day 20–LT TNA			Day 200–LT TNA			Day 200–Ricin TNA		
	Total	A[a]	B	Total	A	B	Total	A	B
RiVax	1[b]	1	1	1	1	1	459	528	400
DNI	1087	1213	951	1728	1600	1903	1	1	1
Dual	205	264	159	800	606	1056	429	696	264

[a] Each group of mice (left column) was arbitrarily divided into two subsets, A and B. Shown are the neutralizing titers from sera collected on day 20 or 200 from the all mice in a group (as "Total") or as the specific A and B subsets.
[b] Mice lacking detectable toxin-neutralizing serum antibody titers were arbitrarily assigned a value of 1 for the purpose of statistical analysis.

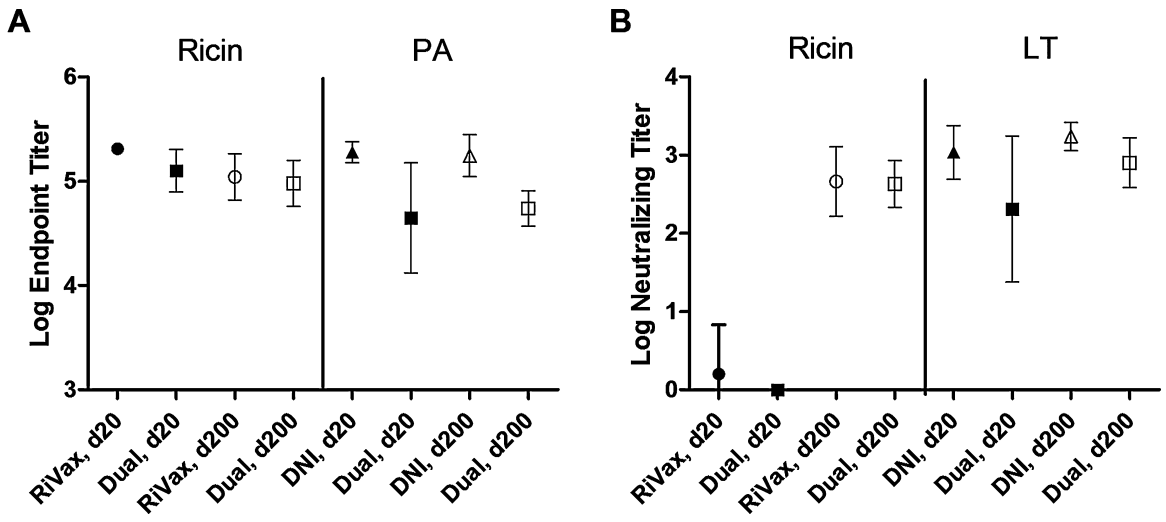

Fig. 1. Endpoint and toxin-neutralizing serum antibody titers elicited in mice following RiVax, DNI, and dual immunizations. Mice were immunized with RiVax, DNI, or the combination of RiVax and DNI adsorbed to aluminum salts on days 0 and 20, as noted in the text. Sera were collected on days 20 and 200 and examined for (A) ricin- and PA-specific IgG antibodies and (B) ricin and LT toxin-neutralizing activities. Symbols and error bars represent means and standard deviations.

and 1728 in the sera of mice that received only DNI. The reduction in TNA in the dual immunized mice was proportional to the total PA-specific antibodies, suggesting that the combination vaccine was simply less potent at eliciting PA-specific antibody titers and not altered in its capacity to elicit TNA *per se*. Interestingly, the combination vaccine did not affect the onset of ricin toxin neutralizing antibodies, as evidenced by the fact that on day 200 the ricin-specific TNA were virtually identical between the RiVax and dual immunized groups of mice (GMT 459 *versus* 429, respectively; $p = 0.86$). It should be underscored that neither the RiVax or dual immunized groups of mice (with one exception) had any detectable ricin-specific TNA on day 20 (Table 2; Fig. 1B).

We next wished to determine whether the dual RiVax and DNI immunization regimen was sufficient to protect mice against lethal dose toxin challenges. Each group of mice that had been immunized with RiVax, DNI, or the combination of RiVax and DNI was randomly divided into two groups ($n = 5$ per group), A or B, and then challenged on day 202 with $10 \times LD_{50}$ ricin (0.1 mg/kg; Group B) or $2.5 \times LD_{100}$ of lethal toxin (1:1 PA:LF by weight; Group A). Challenge doses of ricin ($10 \times LD_{50}$) and LT ($2.5 \times LD_{100}$) were based on previous studies from our laboratory and other laboratories [11,18,19] and validated (whenever possible) with pilot studies (Fig. S1) [11,18]. As shown in Table 3 and Table S1, RiVax-immunized mice survived ricin toxin challenge, but not LT challenge. Conversely, DNI-immunized mice were protected from LT, but succumbed to ricin toxin. Among the mice that received the combination of RiVax and DNI, 5 of 5 survived ricin challenge, whereas 4 out of 5 survived LT challenge (Fig. S2). The single dual-immunized mouse that succumbed to LT challenge had the lowest LT-neutralizing titer (*i.e.*, 200) of all 20 mice that received DNI, suggesting that death was due to the effects of the toxin and not an experimental aberration. As expected, mice that received vehicle only (*i.e.*, aluminum salts) succumbed to ricin and LT challenges (Table S1).

Finally, to further interrogate the efficacy of the combination vaccine, the four mice that had been immunized with the RiVax and DNI combination and that survived LT challenge were then secondarily challenged two weeks later (day 216) with $10 \times LD_{50}$ ricin. As controls, the DNI-immunized mice that survived a primary LT challenge were also secondarily challenged with ricin. All four dual-immunized mice survived ricin challenge, while all DNI-immunized mice succumbed to ricin intoxication, thereby demonstrating that the combination vaccine did in fact confer specific immunity to both LT and ricin toxins. It should be noted that we also attempted the inverse experiment: mice that had been immunized with the dual vaccine and then challenged with ricin were secondarily subject to an LT challenge. Unfortunately, the challenge was unsuccessful due to insufficient potency of a new lot of LT (data not shown).

In summary, we have demonstrated that DNI and RiVax antigens can successfully be combined following adsorption to aluminum salts adjuvant, into a single vaccine that is capable of eliciting protective immunity to ricin and anthrax LT in mice. The combination vaccine generally resulted in high endpoint serum IgG antibody titers to each antigen, as well as toxin-neutralizing antibodies, which constitute the two critical determinants associated with protective immunity to ricin and *B. anthracis*. However, the combination of RiVax and DNI did result in significantly lower antitoxin serum antibody titers as compared to mice that received the vaccines individually. While the differences in anti-ricin titers between RiVax alone and dual-immunized mice were not significant on day 200, the differences in anti-PA titers persisted. Moreover, a single mouse that received the combination vaccine succumbed to LT challenge, indicating that the resultant immunity to LT was compromised to some degree when combined with RiVax. It will be critical to determine whether the combination vaccine is in fact

Table 3
Mouse survival following primary and secondary toxin challenges.

Group[b]	1° CHL[a]		2° CHL
	Ricin	LT	Ricin
RiVax A	–	0/5	–
RiVax B	5/5*	–	–
DNI A	–	5/5*	0/5
DNI B	0/4[c]	–	–
Dual A	–	4/5	4/4*
Dual B	5/5	–	–
Vehicle A	–	0/5	–
Vehicle B	0/5	–	–

[a] CHL, challenge.
[b] Each group of mice (left column) was arbitrarily divided into two subsets, A and B. The A subset received a primary challenge with LT, while the B subset (shaded) received a primary challenge with ricin. The DNI-immunized and the dual-immunized A subsets of animals that survived LT challenge received a secondary challenge with ricin.
[c] A single mouse in this group of animals died of unknown causes prior to the animal's second immunization.
* Indicates statistical significance ($p < 0.05$) based on the Mantel–Cox test.

sufficient to elicit serum antibodies levels capable of providing protection against an actual *B. anthracis* spore challenge.

It is unclear whether the dampened antibody response to DNI when combined with RiVax was a result of immunological interference (*e.g.*, B or T cells competing for similar epitopes on RiVax and DNI) or antigen saturation at the level of processing or presentation [20–23]. Considering DNI and RiVax are not similar at the primary sequence level, it is unlikely that direct interference accounts for the difference in serum antibody titers. To address the issue of antigen overload, it will be critical to perform comprehensive dose-response and time course studies with DNI, RiVax and the combination to determine what actually constitutes antigen saturation in this model and at what time points toxin-neutralizing antibodies reach their maximal titers. Finally, it is imperative to examine what effect (if any) the combination of antigens has on the biophysical properties (*e.g.*, deamidation or unfolding) and/or relative bioavailability of DNI or RiVax, which in turn may influence the onset of antigen-specific antibody responses [16,24–26].

An interesting facet of the data presented in this report is the notable difference in the onset of toxin-neutralizing antibodies following DNI and RiVax immunizations. On day 20, which corresponds to 6 days after the booster immunization, 95% (19/20) of the mice administered DNI had detectable LT-neutralizing antibodies, whereas on the same day only 5% (1/20) of the RiVax-immunized mice had detectable ricin toxin-neutralizing antibodies. By day 200, toxin-neutralizing antibodies were detected in all DNI and RiVax immunized animals. It is interesting to speculate that the threshold for eliciting neutralizing antibodies may be lower for PA than RTA, due to different mechanisms by which antibodies neutralize LT and ricin. In other words, it may be easier to neutralize LT than ricin. For example, anti-PA antibodies have been shown to neutralize LT by at least five different mechanisms, including interference with receptor attachment, inhibition of furin-mediated cleavage of PA, blocking PA heptamerization or EF/LF engagement, and interruption of pore formation in the endosomal membrane [27,28]. In contrast, anti-RTA antibodies do not affect toxin attachment or internalization, but rather interfere with intracellular toxin trafficking [29–31]. Moreover, there is evidence to suggest there are only a limited number of “neutralizing” epitopes on the surface of RTA [32], which is in contrast to PA, where neutralizing epitopes have been identified on each of PA’s four domains. If our model is correct, then efforts to accelerate the onset of ricin toxin-neutralizing antibodies may need to be aimed on “focusing” the antibody response to the most relevant epitopes on the surface of RTA [33,34]. In addition, there may be benefits to complexing RiVax with RTB as a means to elicit toxin-neutralizing antibodies that interfere with ricin-receptor interactions. These studies are ongoing in the laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.11.036.

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