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Stepwise Engineering of Heterodimeric Single Domain Camelid V_HH Antibodies That Passively Protect Mice from Ricin Toxin*

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Background: We sought to engineer highly efficacious agents that neutralize ricin toxin.
Results: We identified monomeric single-chain camelid V_H domains (V_HHs) capable of neutralizing ricin *in vitro* and engineered heterodimeric V_HHs that neutralized ricin *in vivo*.
Conclusion: Stepwise engineering of V_HHs resulted in highly potent ricin toxin-neutralizing antibodies.
Significance: This study highlights the potential use of a V_HH platform as a strategy for therapeutics against diverse biological toxins.

In an effort to engineer countermeasures for the category B toxin ricin, we produced and characterized a collection of epitopic tagged, heavy chain-only antibody V_H domains (V_HHs) specific for the ricin enzymatic (RTA) and binding (RTB) subunits. Among the 20 unique ricin-specific V_HHs we identified, six had toxin-neutralizing activity: five specific for RTA and one specific for RTB. Three neutralizing RTA-specific V_HHs were each linked via a short peptide spacer to the sole neutralizing anti-RTB V_HH to create V_HH "heterodimers." As compared with equimolar concentrations of their respective monovalent monomers, all three V_HH heterodimers had higher affinities for ricin and, in the case of heterodimer D10/B7, a 6-fold increase in *in vitro* toxin-neutralizing activity. When passively administered to mice at a 4:1 heterodimer:toxin ratio, D10/B7 conferred 100% survival in response to a 10 × LD_{50} ricin challenge, whereas a 2:1 heterodimer:toxin ratio conferred 20% survival. However, complete survival was achievable when the low dose of D10/B7 was combined with an IgG1 anti-epitopic tag monoclonal antibody, possibly because decorating the toxin with up to four IgGs promoted serum clearance. The two additional ricin-specific heterodimers, when tested *in vivo*, provided equal or greater passive protection than D10/B7, thereby warranting further investigation of all three heterodimers as possible therapeutics.

Ricin, a 65-kDa glycoprotein found in the seeds of the castor bean plant, is a member of the A-B family of protein toxins, which includes cholera toxin, Shiga toxins 1 (Stx1)[3] and 2 (Stx2), botulinum neurotoxins (BoNTs), and anthrax toxin (1, 2). The ricin B subunit (RTB) is a galactose and *N*-acetylgalactosamine (Gal/GalNAc) lectin that promotes toxin attachment and entry into all mammalian cell types (3, 4). Following endocytosis, RTB mediates the retrograde trafficking of ricin from the plasma membrane to the trans-Golgi network and the endoplasmic reticulum. Once in the endoplasmic reticulum, the ricin A subunit (RTA) is liberated from RTB and is dislocated across the endoplasmic reticulum membrane into the cytoplasm, where it functions as an RNA *N*-glycosidase whose sole substrate is a universally conserved adenosine residue within the so-called sarcin/ricin loop of mammalian rRNA (5). Hydrolysis of the sarcin/ricin loop by RTA results in the cessation of cellular protein synthesis, activation of the ribotoxic stress response, and cell death via apoptosis (6).

Ricin, a category B toxin, as defined by the Centers for Disease Control and Prevention, is extremely toxic in purified or semipurified forms by injection, inhalation, or ingestion (7–9). Recent high profile incidents involving ricin-laden envelopes addressed to members of the United States Congress and the President have accelerated efforts by the Department of Defense and the National Institutes of Health to develop countermeasures against the toxin (10, 11). We and others have produced a large collection of RTA- and RTB-specific murine and chimeric mouse-human mAbs with toxin-neutralizing activity *in vitro* and *in vivo* (1, 12–16). Although many of these mAbs have therapeutic potential, funding agencies are increasing moving away from the "one bug, one drug" model of biodefense therapeutics to more broad-based platform technologies that can provide rapid onset against similarly acting biothreat agents.

Camelids produce a class of heavy chain-only antibodies which bind antigen strictly through their V_H domain. Recombinant heavy chain-only V_H domains (V_HHs) are conforma-

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[3] The abbreviations used are: Stx, Shiga toxin; BoNT, botulinum neurotoxins; RTA, ricin A subunit; RTB, ricin B subunit; V_HH, heavy chain-only antibody V_H domain; efAb, effector antibody.

tionally stable, frequently bind to active site pockets, and have excellent commercial properties (17–20). Additionally, monomeric V_HHs can be genetically linked to express heteromultimeric binding agents with improved properties (21, 22). We previously reported a novel antitoxin strategy that promotes both toxin neutralization and serum clearance with two simple protein components (21). One component is a V_HH heterodimer consisting of two toxin-neutralizing V_HHs recognizing nonoverlapping epitopes. The linked V_HHs lead to enhanced neutralization properties compared with the V_HH monomers (22). In addition to toxin neutralization, the V_HH heterodimers can promote toxin clearance from serum by co-administration of an effector antibody (efAb), which is an anti-tag mAb that recognizes two peptide tags separately engineered into sites flanking the V_HH heterodimer. The efAb can bind at the two sites on each V_HH heterodimer, which itself binds the toxin at two sites, thus resulting in toxin decoration with up to four Abs to promote serum clearance (21, 23), presumably by Fc receptor-mediated processes.

In this study, we produced and characterized a collection toxin-neutralizing and non-neutralizing V_HHs specific for the enzymatic and receptor binding subunits of ricin. We next engineered V_HH heterodimers consisting of pairs of V_HH monomers and demonstrate their potential, in the absence and presence of efAb, to confer immunity to ricin in a mouse model. We demonstrate the capacity to stepwise engineer heterodimers with increased affinity and toxin-neutralizing activity and the significant boost in potency that efAb confers on passive protection *in vivo*. In light of our recent success in developing V_HH antibodies against BoNT and Stx, we propose that this antitoxin technology platform may have important applications for biodefense.

MATERIALS AND METHODS

Toxins, Chemicals, and Reagents—Ricin toxin (RCA-II), RTA, and RTB were obtained from Vector Laboratories (Burlingame, CA). A recombinant, attenuated form of the ricin toxin A subunit, known as RiVax™, was kindly provided by Dr. Robert Brey (Soligenix, Inc., Princeton, NJ) (24). Anti-E tag mAb was obtained from Phadia (Uppsala, Sweden), whereas HRP anti-E-tag mAb and HRP anti-M13 antibody were purchased from GE Healthcare. All other chemicals and reagents were purchased from Sigma-Aldrich unless noted otherwise.

Ethics Statement—Studies involving the use of animals were carried out in strict accordance with recommendations from the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. Studies involving alpacas were conducted at Tufts University and were approved by the Tufts University Institutional Animal Care and Use Committee. All procedures involving mice were conducted at the Wadsworth Center and approved by the Wadsworth Center's Institutional Animal Care and Use Committee.

Alpaca Immunizations and V_HH Display Library Preparation—A total of two alpacas (*Vicugna pacos*) were used in this study. One alpaca received five successive multisite subcutaneous injections at 3-week intervals using an immunogen consisting of RiVax (100 μg) and RTB (100 μg), followed by three additional immunizations with RiVax (200 μg) and ricin toxoid (200 μg) and then two immunizations with RiVax (200 μg), RTB (100 μg), and ricin toxoid (200 μg). RiVax was preadsorbed to aluminum salts adjuvant, whereas RTB was combined with alum/CpG adjuvant immediately prior to injection. The second alpaca received three immunizations of ricin toxoid (200 μg) and then two immunizations with RiVax (200 μg), RTB (100 μg), and ricin toxoid (200 μg). Following the final immunizations, animals had end point RTA- and RTB-specific serum IgG titers between 5×10^4 and 5×10^5 and ricin neutralization titers between 1,600 and 3,200. Three days following the final boost, blood was obtained for lymphocyte preparation, and a V_HH display phage library was prepared from the immunized alpaca as previously described (21, 25, 26). 4×10^6 independent clones (>95% with V_HH inserts) were prepared from B cells of the two alpacas and pooled to create the library.

Anti-RTA and anti-RTB V_HH Identification, Expression, and Purification—Panning, phage recovery, and clone fingerprinting were performed essentially as described (21). Two rounds of panning were performed on purified RTA or RTB targets coated onto Nunc Immunotubes. A single low stringency panning using 10 μg/ml target antigen was performed on each subunit target. After phages were eluted, they were amplified and subjected to a second round of panning at high stringency with 1 μg/ml target antigen. Following the second round of panning, ~150 individual *Escherichia coli* colonies were picked and grown overnight at 37 °C in 96-well plates. A replica plate was then prepared, cultured, and induced with IPTG, and the supernatant was assayed for RTA or RTB binding by ELISA.

For each two-cycle panning regimen, >50% of V_HH clones bound to RTA or RTB, as evidenced by ELISA reactivity values that were >2-fold over negative controls. Approximately 60 of the strongest positive binding phage for RTA and RTB were selected for DNA sequence analysis ("fingerprinting"). Sixteen clones with unique DNA fingerprints were identified among the V_HHs selected as strong positives for RTA binding, and nine unique clones for V_HHs were selected as positives for RTB binding. The V_HH coding DNAs from these clones were sequenced and analyzed by phylogenetic tree analysis to identify closely related V_HHs likely to have common B cell clonal origins. Based on this analysis, eleven RTA-binding V_HHs and nine RTB-binding V_HHs were selected for protein expression.

We have previously described the protocols used for purification of V_HHs from *E. coli* as recombinant thioredoxin fusion proteins containing N-terminal hexahistidine and C-terminal E epitope tag (GAPVPYPDPLEPR) (26) and for competition analysis to identify V_HH binding to common or overlapping epitopes (21). Heterodimeric V_HHs were engineered to contain a flexible spacer (GGGGS × 3) between the two V_HH monomers and two copies of E-tag flanking the V_HH heterodimer (21).

ELISA—Nunc-Immuno plates (ThermoScientific, Swedesboro, NJ) were coated overnight at 4 °C with 1 μg/ml target antigen (*e.g.*, ricin), blocked for 2 h with 2% BSA in PBS, and then incubated for 1 h with 2-fold serial dilutions of V_HHs. For competition assays, murine IgGs (10 μg/ml) were added to the ELISA plate wells 1 h prior to the addition of the V_HHs (1 μg/ml). The plates were then washed with 0.1% PBS-T and incubated with HRP-conjugated anti-E tag secondary antibody (1:10,000) for 1 h. The plates were developed with SureBlue

Peroxidase Substrate (KPL, Gaithersburg, MD). The reaction was quenched with 1 M phosphoric acid, and absorbance was read at 450 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

V_HH Affinity Determinations—Affinity of V_HHs for ricin toxin was determined by surface plasmon resonance SPR using a Biacore 3000 (GE Healthcare) instrument, as described previously (16). Ricin was attached to a CM5 chip at a density of 550–650 resonance units. HEPES-buffered saline with EDTA and surfactant P20 (HBS-EP; 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% of the surfactant P20 from GE Healthcare) was employed as the running buffer at a flow rate of 30 μl/min. Serial dilutions of each antibody were made in HBS-EP, pH 7.4, from 600 to 18.75 nM, with each concentration series having at least one cycle of a buffer-alone injection. Injection times were 3–4 min with dissociation times of 10 min. Regeneration of the chip surface was performed at a flow rate of 50 μl/min by two 30-s pulses of 10 mM glycine, pH 1.5. The regeneration was followed by a 2-min stabilization period. All kinetic experiments were run a 25 °C. Kinetic constants were obtained by analysis using the BIA evaluation software.

Vero Cell Cytotoxicity Assays—The Vero cell cytotoxicity assay has been described in detail elsewhere (27). Vero cells grown in DMEM containing 10% FBS were seeded (1×10^4 cells/well) in 96-well cell culture plates and incubated at 37 °C overnight. The cells were then overlaid with ricin (10 ng/ml, 150 pM) in the absence or presence of 5-fold serial dilutions of monomeric or heterodimeric V_HHs and incubated at 37 °C for 2 h. The cells were then washed, and fresh medium was applied. Cell viability was assessed 45–48 h later using CellTiter-Glo (Promega, Madison, WI).

Passive Protection Studies in Mice—Female BALB/c mice aged 8–10 weeks (Taconic Laboratories, Hudson, NY) were separated into groups of five and allowed to acclimate to their surroundings for 1 week prior to initiation of an experiment. Mixtures of ricin (2 μg; 30 pmol; $10 \times LD_{50}$) and V_HH monomers or heterodimers, with or without the anti-E efAb (2:1 efAb:heterodimer ratio), were mixed at defined ratios in sterile PBS to a final volume of 400 ml. The mixtures were incubated at room temperature for 1 h and then administered to mice by intraperitoneal injection. Hypoglycemia was used as a surrogate marker of ricin intoxication (28, 29). Blood (~5 μl) was collected from the tail veins of mice just prior to ricin challenge (time 0) and at 24-h intervals thereafter. Blood glucose levels were measured with an Aviva ACCU-CHEK handheld blood glucose meter (Roche Applied Science). Survival following toxin challenge was monitored for up to 14 days, and animals surviving beyond day 14 were considered fully protected. The mice were euthanized when they became overtly moribund and/or blood glucose levels fell below 20 mg/dl. Statistical differences in survival were tested by the Mantel-Cox test, computed using GraphPad Prism (version 5.0).

RESULTS

Identification of RTA- and RTB-specific V_HHs with Ricin Toxin-neutralizing Activity—We prepared a V_HH-displayed phage library representing the repertoires of two alpacas that were repeatedly immunized with ricin toxin subunit antigens and then boosted with ricin toxoid (see "Materials and Methods"). The V_HH-displayed phage library was subjected to rounds of high and low stringency panning on purified RTA or RTB subunits. Ultimately, we identified 25 different phagemids encoding V_HHs with RTA or RTB binding activity that were then subjected to DNA sequencing as a means to determine their relatedness. Of the 25 V_HHs, there were 11 apparently unrelated RTA-specific V_HHs and 9 unrelated RTB-specific V_HHs (Table 1 and Fig. 1). All 20 unique V_HHs were expressed and purified from *E. coli* as E-tagged thioredoxin fusion proteins.

TABLE 1
Nomenclature of V_HHs

V_HH	Protein	Clone	GenBank™ accession number
RTA-F5	JIV-F5	JJS-17	KF746018
RTA-F6	JIV-F6	JJS-20	KF746019
RTA-G12	JIV-G12	JJS-21	KF746020
RTA-A7	JIY-A7	JJS-24	KF746021
RTA-D9	JIY-D9	JJS-25	KF746022
RTA-D10	JIY-D10	JJS-27	KF746023
RTA-E1	JIY-E1	JJS-29	KF746024
RTA-E3	JIY-E3	JJS-31	KF746025
RTA-E5	JIY-E5	JJS-33	KF746026
RTA-F10	JIY-F10	JJS-35	KF746027
RTA-G11	JIY-G11	JJS-37	KF746028
RTB-B1	JIW-B1	JJS-40	KF746029
RTB-C12	JIW-C12	JJS-41	KF746030
RTB-D12	JIW-D12	JJS-43	KF746031
RTB-G5	JIW-G5	JJS-45	KF746032
RTB-G10	JIW-G10	JJS-48	KF746033
RTB-B7	JIZ-B7	JJS-50	KF746034
RTB-B9	JIZ-B9	JJS-51	KF746035
RTB-D8	JIZ-D8	JJS-54	KF746036
RTB-G4	JIZ-G4	JJS-56	KF746037

The 20 unique V_HHs were tested in a Vero cell cytotoxicity assay for the ability to neutralize ricin. Five RTA-specific V_HHs (RTA-F5, RTA-G12, RTA-D10, RTA-E5, and RTA-G11) and one RTB-specific V_HH (RTB-B7) demonstrated a dose-dependent capacity to protect Vero cells from ricin-induced cytotoxicity (Table 2 and Fig. 2*A*). Three V_HHs (RTA-F5, RTA-E5, and RTB-B7) had estimated IC_{50} values of ~5 nM, (~30:1 molar ratio V_HH:ricin), one (RTA-D10) had an IC_{50} of ~25 nM (~150:1 V_HH:ricin), and two (RTA-G12 and RTA-G11) had IC_{50} values of >90 nM. The remaining 14 V_HHs had no detectable neutralizing activity, even at 330 nM (~2,200:1 V_HH:ricin).

To determine the relationship between toxin-neutralizing activity and dissociation constants (K_D), the six neutralizing V_HHs were subjected to SPR analysis. All six V_HHs had similar affinities for ricin, despite having varying degrees of toxin-neutralizing activity (Table 2). Furthermore, using dilution ELISA analysis, we compared the relative affinities of the neutralizing V_HHs to those of the 14 non-neutralizing V_HHs (Table 2 and Fig. 2*B*). This comparison revealed EC_{50} values ranging from 200 pM to 33 nM. Although the four most potent neutralizers (RTA-F5, RTA-D10, RTA-E5, and RTB-B7) were among the best ricin binders, the V_HH with the highest relative affinity for ricin toxin was RTB-G5 (200 pM), a V_HH with no detectable toxin-neutralizing activity. These data suggest that there is a certain threshold affinity required for toxin-neutralizing activity but that other factors like epitope specificity ultimately determine overall potency.

To determine whether any of the five RTA-specific neutralizing V_HHs bind epitopes that overlap those recognized by pre-

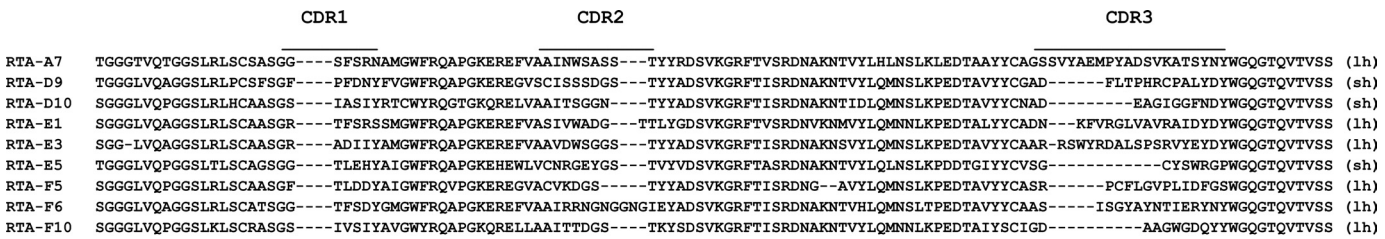

FIGURE 1. **Amino acid sequences of the V_HH variable regions.** Amino acid sequences of RTA-specific (*top*) and RTB-specific (*bottom*) V_HHs are shown. Complementary determining regions (*CDR*) 1, 2, and 3 are indicated by the *horizontal lines*, and the presence of a short (*sh*) or long (*lh*) hinge is indicated in the *right margin*. GenBank™ accession numbers for the V_HH sequences are indicated in Table 1.

TABLE 2
Characteristics of RTA- and RTB-specific V_HHs

				Competitive inhibition assays[a]			
V_HH[b]	EC_{50}[c]	K_d	IC_{50}	PB10	SyH7	IB2	GD12
	nM	*nM*	*nM*				
RTA-F5*	1.50	2.24	5	+++	–	–	–
RTA-F6	1.00			–	–	++	–
RTA-G12	0.50	0.62	>330	+++	–	–	–
RTA-A7	1.20			+++	–	–	–
RTA-D9	3.30			–	–	–	–
RTA-D10*	0.66	0.63	25	–	–	–	–
RTA-E1	3.00			–	+	–	–
RTA-E3	2.00			+++	–	–	–
RTA-E5*	0.83	1.94	5	+++	–	–	–
RTA-F10	13.20			–	–	–	–
RTA-G11	0.83	0.35	90	+++	–	–	–
				SylH3	24B11		
RTB-B1	4.10			–	–		
RTB-C12	1.65			–	–		
RTB-D12	0.83			–	–		
RTB-G5*	0.23			–	–		
RTB-G10	1.65			–	–		
RTB-B7*	0.66	1.33	4	–	–		
RTB-B9*	1.20			–	–		
RTB-D8	3.63			–	–		
RTB-G4	33.00			–	–		

[a] RTA- or RTB-specific murine mAbs were tested for capacity to prevent indicated V_HHs binding to ricin in an ELISA format. The number of plus signs indicates the degree of relative inhibition (−, no reduction; +, 10–30% reduction; ++, 30–60% reduction; and +++, >60% reduction).

[b] Underlining indicates neutralizing V_HHs. Asterisks indicate V_HHs used in heterodimer formation.

[c] The values indicate the effective concentration of V_HH required to achieve 50% maximal binding to ricin by ELISA.

viously identified neutralizing mouse mAbs PB10, SyH7, IB2, or GD12 (12, 30), we performed competitive ricin binding assays by ELISA. We found that V_HHs RTA-F5, RTA-G12, RTA-E5, and RTA-G11 were inhibited by PB10, a mAb known to bind a solvent-exposed, immunodominant α-helix spanning residues 98–106 of RTA (31). RTA-D10, on the other hand, was not inhibited by any of the four murine mAbs tested and may therefore recognize a novel neutralizing epitope on RTA (Table 2).

We next examined whether mAbs 24B11 or SylH3 competitively inhibited RTB-B7 from binding to ricin. 24B11 and SylH3 are two well characterized RTB-specific toxin-neutralizing mAbs that are postulated to recognize epitopes on RTB subdomains 1α and 2γ, respectively (14, 16, 32). RTB-B7 binding to ricin by ELISA was unaffected by preincubation of the toxin with 24B11 or SylH3, indicating that RTB-B7 recognizes a previously unidentified neutralizing epitope on RTB (Table 2).

V_HH Heterodimers Achieve Higher Affinity and Neutralizing Potency in Vitro—In the case of BoNT and Stx1 and Stx2, we have demonstrated that heterodimers created by covalently linking two different toxin-neutralizing V_HH monomers resulted in bi-specific antibodies with increased toxin-specific affinities and improved toxin-neutralizing activities (21, 22). Therefore, we constructed three V_HH heterodimers in which the RTB-specific neutralizing V_HH, RTB-B7, was linked via the flexible peptide spacer $(GGGGS)_3$ to each of the RTA-specific neutralizing V_HHs, RTA-F5, RTA-D10, and RTA-E5; the resulting heterodimers are referred to as F5/B7, D10/B7, and E5/B7 (Table 3). In addition, we created two additional heterodimers. Heterodimer G5/B7 consists of RTB-B7 linked to RTB-G5 which is a high affinity, RTB-specific, non-neutralizing V_HH. The second control heterodimer, G5/B9, consists of two anti-RTB non-neutralizing V_HHs, RTB-G5 and RTB-B9 (Table 3). G5/B7 and G5/B9 are expected to bind to ricin with high affinities but have only moderate or no demonstrable toxin-neutralizing activity.

We first performed dilution ELISAs to determine the apparent affinities of the V_HH heterodimers for ricin, as compared with the individual monomers or a 1:1 mixture of monomers. We found that each heterodimer had a lower EC_{50} than either of the component monomers or corresponding pool of monomers (Fig. 3, *A–E*). In the case of D10/B7, for example, there was a >8-fold decrease in EC_{50} compared with the RTB-B7 monomer alone (Table 3 and Fig. 3*A*). Thus, in this instance, physically linking the monomers resulted in approximately an order of magnitude increase in apparent affinity.

We next sought to determine whether the higher affinity heterodimers would be more potent toxin-neutralizers than their corresponding monomers. Each heterodimer was tested in the Vero cell cytotoxicity assay for ricin-neutralizing activity and compared with its corresponding monomers or a 1:1 pool of the monomers. Linking two non-neutralizing V_HHs (G5/B9) did not result in an agent with any neutralizing activity,

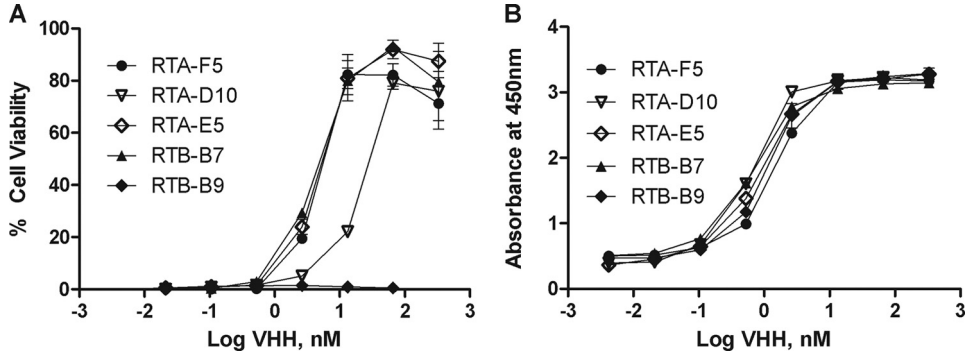

FIGURE 2. **V_HH toxin-neutralizing and binding activities.** *A*, monomeric V_HHs were tested for toxin-neutralizing activity in a Vero cell cytotoxicity assay, as described under "Materials and Methods." V_HHs (at indicated concentrations) were mixed with ricin (10 ng/ml) and applied to Vero cells in triplicate. Cell viability was assessed 48 h later. Shown is a representative experiment with *error bars* indicating S.D. *B*, to assess relative affinity of select V_HHs for ricin, the V_HHs (at indicated concentrations) were applied in duplicate to microtiter plates coated with ricin. The EC_{50} values are defined as the V_HH concentration (nM) that achieved half-maximal binding. Shown is a representative experiment with *error bars* indicating S.D. The experiments described in *A* and *B* were replicated at least three times.

TABLE 3
Characteristics of V_HH heterodimers

Heterodimer	Constituents	EC_{50}[a]	IC_{50}[b]	Protection[c]
		nM	nM	
D10/B7	RTA-D10/RTB-B7	0.08	0.15	4 of 20
F5/B7	RTA-F5/RTB-B7	0.20	1.00	4 of 5
E5/B7	RTA-E5/RTB-B7	0.30	1.00	5 of 5
G5/B7	RTB-G5/RTB-B7	0.20	0.90	0 of 5
G5/B9	RTB-G5/RTB-B9	0.10	NA[d]	0 of 5

[a] The values indicate the concentration of V_HH required for 50% maximal ricin binding by ELISA.
[b] The values indicate the concentration of V_HH required to neutralize 50% ricin in Vero cell assay.
[c] The values indicate the number of mice passively administered V_HH (3 μg/mouse) that survived a 10 × LD_{50} ricin challenge.
[d] NA, not applicable.

although G5/B7, composed of one neutralizing and one non-neutralizing V_HH, was slightly more potent than its constituent neutralizing monomer (Fig. 4).

Despite their increased apparent affinities for ricin, F5/B7 and E5/B7, each consisting of two neutralizing V_HH monomers, did not demonstrate enhanced toxin-neutralizing activity as compared with the pooled monomers (Fig. 4). On the other hand, D10/B7 did have markedly improved toxin-neutralizing activity, as compared with its respective individual monomers (~30-fold) or with a 1:1 pool of monomers (>5-fold) (Table 3 and Fig. 4). D10/B7 was ~7-fold more effective at neutralizing ricin *in vitro* than either F5/B7 or E5/B7 (Table 3).

In Vivo Passive Protection Afforded by V_HH Heterodimer D10/B7 without and with a Secondary efAb—Because of its high *in vitro* neutralizing potency, we wished to test D10/B7 for its ability to passively protect mice from a 10 × LD_{50} dose of ricin toxin. D10/B7 was mixed with ricin at a heterodimer:toxin molar ratios of 1 (1.5 μg), 2 (3 μg), 4 (6 μg), and 8 (12 μg); incubated *ex vivo* for an hour; and then injected into mice via the intraperitoneal route. We also performed *in vivo* challenge studies in which we added a mouse monoclonal anti-E epitope tag IgG_1 (effector antibody or efAb) to the heterodimer-toxin mixtures prior to injection into mice. We have previously shown that co-injection of the efAb with BoNT-specific VHH heterodimers improved toxin clearance (23) and the protective efficacy of VHH heteromultimers (21, 22). As controls for these studies, groups of mice received 10 μg (10:1 V_HH:ricin ratio) of the individual V_HH monomers (RTA-D10 or RTB-B7) or a mixture of 10 μg of each of RTA-D10 and RTB-B7. A final control group of animals received ricin but no antitoxin agents.

Mice that received monomeric V_HHs alone had only a slightly greater time to death as compared with ricin-only treated animals. Mice that received a 1:1 mixture of monomers had a significantly longer time to death ($p < 0.01$), but eventually all mice in these groups succumbed as well (Fig. 5). However, the heterodimer D10/B7 demonstrated a dose-dependent capacity to protect mice against ricin challenge. Mice that received D10/B7 at 4- (6 μg) or 8-fold (12 μg) molar excess over ricin were completely protected from toxin challenge, whereas only 20% of the mice that received D10/B7 at 2-fold (3 μg) molar excess survived challenge. This dose, however, had a significantly longer time to death than ricin alone ($p < 0.0001$). A 1:1 ratio of D10/B7 to toxin provided no protection, although, again, there was a significant increase in mean time to death over ricin alone ($p < 0.01$). These data reveal that D10/B7 at 4-fold molar excess over ricin is sufficient to fully neutralize ricin *in vivo*. The addition of the efAb to the mixture markedly improved the performance of D10/B7 *in vivo*. In particular, whereas 2-fold (3 μg) molar excess D10/B7:ricin conferred only 20% protection in the challenge model, the same heterodimer: toxin ratio plus the efAb (2:1 efAb:heterodimer) conferred 100% protection ($p = 0.0001$) (Fig. 6). Note that, because the heterodimer binds twice to each toxin, a 2:1 ratio of agent:toxin is needed to saturate ricin binding. The addition of the efAb to a 1:1 D10/B7:ricin molar ratio resulted in 40% (two of five mice) protection, a significant improvement over D10/B7 alone at this dose ($p < 0.05$). This treatment also resulted in a prolonged time to death in the remaining mice (three of five mice), a significant improvement over animals that received ricin alone ($p < 0.001$).

The observed improvement in protection afforded by efAb treatment is presumably the result of enhanced Fc receptor-mediated clearance of ricin-heterodimer complexes (23). However, we wished to investigate whether Fc receptor-mediated clearance is sufficient to promote ricin toxin-neutralization *in vivo*. We reasoned that if Fc receptor-mediated clearance is sufficient, then the addition of efAb to the non-neutralizing heterodimer G5/B9 would be expected to afford protection against ricin challenge, as compared with G5/B9 alone. Mice

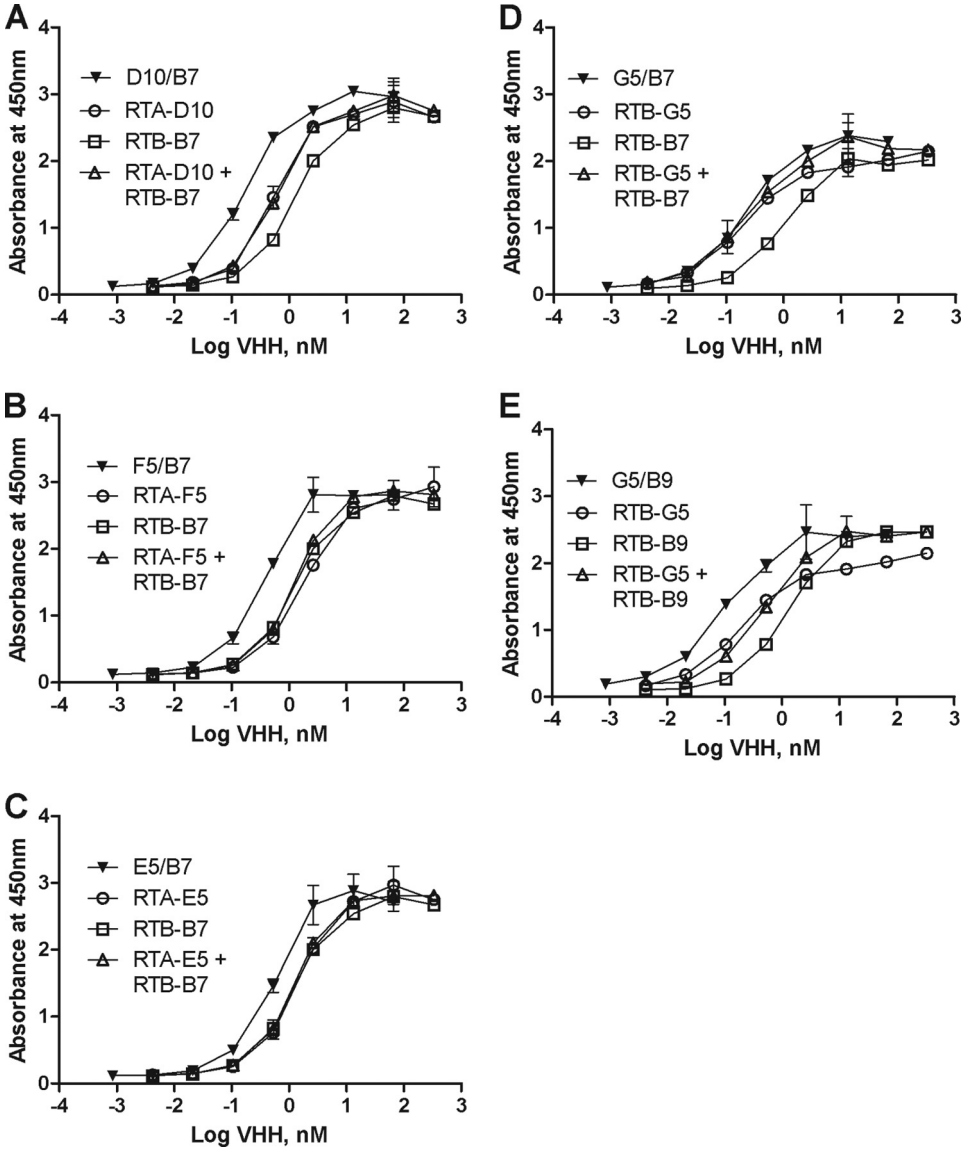

FIGURE 3. **Relative affinities of V_HH heterodimers for ricin.** To assess relative affinity of select V_HH heterodimers (F5/B7, D10/B7, E5/B7, G5/B7, and G5/B9) and compare them to their monomeric constituents and equimolar mixtures of those monomers, the V_HHs were applied at indicated concentrations (nM) in duplicate to microtiter plates coated with ricin, as described in the legend of Fig. 2. *A–E* show a single pair of monomeric V_HHs (*open symbols*) and their corresponding heterodimers (*solid symbols*). Each panel represents one heterodimer and its corresponding monomers, as indicated in the legends embedded within the graphs. Shown are representative experiments with *error bars* indicating S.D. All experiments were replicated at least three times.

were passively administered G5/B9, without or with efAb, and then challenged with a 10 × LD_{50} dose of ricin toxin. We found that the efAb afforded no benefit to G5/B9, because mice treated or not with efAb succumbed to ricin intoxication with similar kinetics (Table 2 and Fig. 7). Therefore, Fc receptor-mediated clearance is itself not sufficient to neutralize ricin *in vivo*.

In Vivo Passive Protection Afforded by Other V_HH Heterodimers—We tested the remaining V_HH heterodimers, F5/B7, E5/B7 and RTB-G5/RTB-B7, for the ability to passively protect mice from a 10 × LD_{50} dose of ricin toxin. The heterodimers were mixed at a 2-fold molar excess (3 μg) with 10 × LD_{50} of ricin, a heterodimer:toxin ratio that in the case of D10/B7 afforded 20% protection, as shown in Fig. 5. Heterodimers F5/B7 and E5/B7 were able to protect 80% ($p < 0.05$) and 100% ($p < 0.01$) of mice, respectively. Heterodimer G5/B7 conferred no protection, although there was a significant increase in time to death over ricin alone ($p < 0.05$) (Table 2 and Fig. 7). Interestingly, even though D10/B7 is a more potent neutralizer *in vitro*, F5/B7 and E5/B7 were better able to protect mice from ricin intoxication at the 2:1 heterodimer:toxin ratio, in which both binding sites on ricin can be bound. Future experiments will be done to determine the most potent heterodimer and the lowest dose required for protection. Combined, these experiments show that V_HH heterodimers display enhanced potency to protect animals from ricin exposure compared with V_HH monomers, especially when both V_HH components are toxin-neutralizing.

DISCUSSION

In this study, we engineered, using a unique platform technology, novel antitoxins against the category B toxin ricin. We

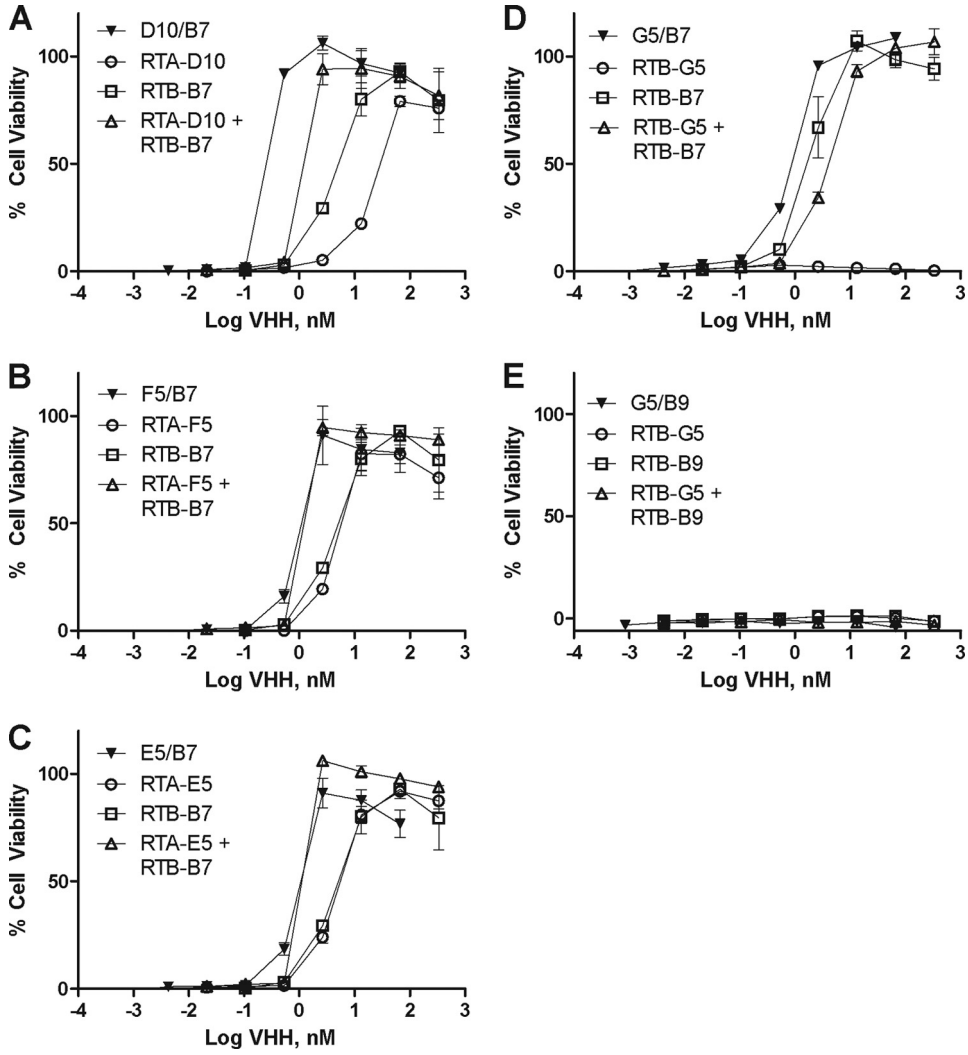

FIGURE 4. **V_HH heterodimers *in vitro* toxin-neutralizing activity.** To assess toxin-neutralizing activities of select V_HH heterodimers (F5/B7, D10/B7, E5/B7, G5/B7, and G5/B9) and compare them with their monomeric constituents and equimolar mixtures of those monomers, the V_HHs were tested in a Vero cell cytotoxicity assay, as described in the legend of Fig. 2. V_HHs (at indicated concentrations, nM) were mixed with ricin (10 ng/ml) and applied to Vero cells in triplicate. *A–E* show a single pair of monomeric V_HHs (*open symbols*) and their corresponding heterodimers (*solid symbols*). Each panel represents one heterodimer and its corresponding monomers, as indicated in the legends embedded within the graphs. Shown are representative experiments with *error bars* indicating S.D. All experiments were replicated at least three times.

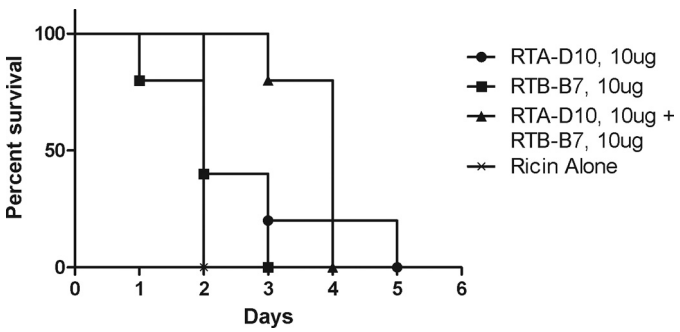

FIGURE 5. ***In vivo* activity of monomeric V_HHs upon ricin challenge.** RTA-D10 and RTB-B7 V_HH monomers (or an equimolar mixture of the monomers) were premixed at a 10:1 molar ratio (10 μg) with the equivalent of 10 $\times$ LD_{50} of ricin toxin (2 μg) and injected intraperitoneally into BALB/c mice. Survival was monitored over a 2-week period. Moribund mice with blood glucose levels less than 20 mg/dl were euthanized, as described under "Materials and Methods." Each experimental group consisted of five mice ($n = 5$).

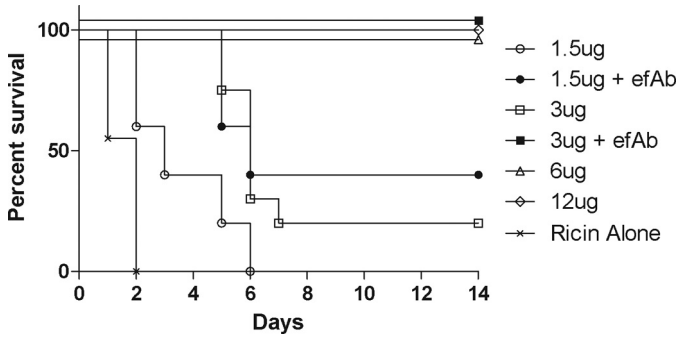

FIGURE 6. ***In vivo* protection conferred by V_HH heterodimer D10/B7.** D10/B7 (with or without efAb) was premixed at the indicated amounts with the equivalent of 10 $\times$ LD_{50} of ricin toxin (2 μg) and injected intraperitoneally into BALB/c mice. Survival was monitored over a 2-week period. Moribund mice with blood glucose levels less than 20 mg/dl were euthanized, as described under "Materials and Methods." Each experimental group consisted of five mice, except the ricin control group ($n = 20$), the 3-μg dose group ($n = 20$), and the 3-μg dose + efAb group ($n = 10$).

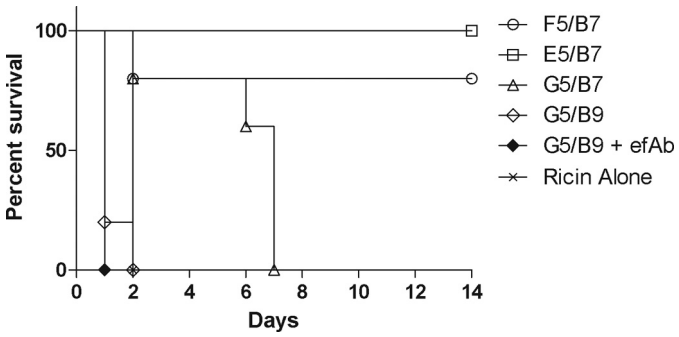

FIGURE 7. ***In vivo* protection by additional V_HH heterodimers.** Indicated heterodimers (with or without efAb) were premixed at a 2:1 molar ratio with the equivalent of 10 LD_{50} of ricin toxin (2 μg) and injected intraperitoneally into BALB/c mice. Survival was monitored over a 2-week period. Moribund mice with blood glucose levels less than 20 mg/dl were euthanized, as described under "Materials and Methods." Each experimental group consisted of five mice ($n = 5$).

initially identified 11 RTA-specific V_HHs and 9 RTB-specific V_HHs from a V_HH library generated from two immunized alpacas. Among the 20 unique ricin-specific V_HHs, we identified, six (five RTA-specific and one RTB-specific) had clear toxin-neutralizing activity *in vitro*. We next engineered a series of V_HH heterodimers consisting of RTB-B7, the single neutralizing anti-RTB V_HH, linked to one of three neutralizing anti-RTA V_HHs. One heterodimer in particular, D10/B7, had a 6-fold increase in toxin-neutralizing activity *in vitro* as compared with an equimolar mixture of the component monomers. *In vivo* analysis revealed that D10/B7 was able to fully protect mice from a 10 $\times$ LD_{50} ricin challenge at a V_HH:ricin ratio as low as 4:1. Co-administration of D10/B7 with an efAb improved the protective potential significantly, thereby demonstrating our capacity to engineer high affinity toxin-neutralizing antitoxins against ricin toxin. Two other neutralizing heterodimers, F5/B7 and E5/B7, appear to be slightly more potent than D10/B7 and will be tested further in the future. In light of our success in engineering protective V_HH heterodimers against BoNT/A (21) and Shiga toxins Stx1 and Stx2 (22), these data reveal the power of this antitoxin technology to apply to a broad range of toxins. Therefore, we conclude that the technology is applicable to ricin and that this may have important implications as a general strategy for therapeutics against category A–C toxins.

Overall, five RTA-specific and one RTB-specific ricin-neutralizing V_HHs were identified. Among the RTA-specific neutralizing V_HHs, RTA-D10 appears to recognize an epitope that is distinct from the known neutralizing clusters (12), suggesting that it may recognize an as yet uncharacterized neutralizing epitope. However, four of the five were competitively inhibited from binding to ricin by the neutralizing murine mAb PB10, suggesting that these V_HHs recognize epitopes that overlap or are identical with the epitope of PB10. We have previously defined the PB10 epitope at high resolution, showing that it recognizes a linear, solvent-exposed, immunodominant α-helix spanning residues 98–106 of RTA (31). This α-helix (called α-helix B) is a well known target of toxin-neutralizing antibodies (33) and is thought to be conserved among virtually all the ribosome-inactivating proteins (34). Interestingly, PB10 also competitively inhibited two non-neutralizing V_HHs (RTA-A7 and RTA-E3) from binding to ricin. The failure of RTA-A7 and RTA-E3 to neutralize ricin could be because their epitopes, although overlapping the PB10 epitope, do not contact important neutralizing residues within the footprint of the contact region of PB10. Although the inability to neutralize ricin could be related to the V_HH affinity for target, other results make this unlikely. For example, RTA-G12 and RTA-D10, both with affinities for ricin ~600 pM, display vastly different neutralizing activities. RTA-G12 did not achieve 50% neutralization at the highest concentration tested (330 nM), whereas RTA-D10 had an IC_{50} of ~25 nM. These results support the hypothesis that, beyond a certain affinity threshold, toxin-neutralizing activity is dictated by epitope specificity (1, 12).

From the nine RTB-specific V_HHs identified, only one, RTB-B7, was able to neutralize the toxin *in vitro*. This is consistent with previous work from our lab with murine mAbs indicating that the vast majority of antibodies elicited to RTB are non-neutralizing and that neutralizing antibodies to RTB are extremely rare (1, 14). Moreover, as is the case for the RTA-specific V_HHs, we found that affinity is not the sole factor in determining neutralizing activity. This was illustrated by the fact that RTB-B7 was one of the best neutralizers (IC_{50} of 4 nM), despite having a relatively low affinity for ricin (1.3 nM), and the fact that RTB-G5 failed to neutralize the toxin even though it has the highest relative affinity for ricin (EC_{50} of 230 pM) of all 20 V_HHs identified in this study. At present, the epitope recognized by RTB-B7 has not been identified. Based on competitive binding assays, this epitope is clearly distinct from those recognized by the previously characterized RTB-specific neutralizing mAbs 24B11 and SylH3 (14, 16, 32). We speculate that RTB-B7 likely recognizes a conformation-dependent epitope based on its poor binding in Western blot analysis.[4]

We have recently reported that there are two types (I and II) of RTB-specific neutralizing mAbs (1, 15). Type I neutralizers are postulated to prevent RTB from binding to its receptors, thereby inhibiting toxin internalization. Type II neutralizers, however, recognize ricin when it is already bound to cell surfaces and are thought to interfere with toxin uptake and/or intracellular trafficking. Preliminary data suggest that RTB-B7 is a type II neutralizer.[5] In the case of 24B11, we have evidence that the mAb shunts ricin away from the trans-Golgi network and promotes degradation through the lysosomal machinery.[6]

V_HH monomers that neutralize ricin *in vitro* were unable to protect mice from ricin-induced death at the concentrations tested. In contrast, by covalently linking the monomers, the resulting V_HH heterodimers were clearly effective in protecting mice from ricin intoxication. Furthermore, we showed that addition of efAb to the formulation significantly increased the protective efficacy. Because the V_HH heterodimers each contain two copies of E-tag and because heterodimers can bind at two sites on the toxin, up to four efAbs can bind each toxin molecule. We have postulated that decorating the heterodimer-ricin complex with multiple efAbs leads to increased anti-toxin potency through the promotion of toxin clearance

[4] D. J. Vance, J. M. Tremblay, N. J. Mantis, and C. B. Shoemaker, unpublished data.
[5] C. Herrera, D. J. Vance, and N. J. Mantis, unpublished observations.
[6] A. Yermakova, T. I. Klokk, K. Sandvig, and N. Mantis, manuscript submitted.

via low affinity FcγR (23). However, FcγR-mediated clearance is not itself sufficient to confer immunity to ricin, as evidenced by the fact that a high affinity heterodimer, G5/B9, consisting of two non-neutralizing V_HHs, afforded no protection to mice against ricin challenge in the presence or absence of efAbs. This is in contrast to a previous study with BoNT in which co-administering efAb was able to improve the *in vivo* efficacy of non-neutralizing antitoxin V_HH heterodimers (21). The differential effects of efAb could be due to the different cell tropisms exhibited by ricin and BoNT. BoNT toxicity is restricted to neurons, and uptake of non-neutralized toxin-antibody complexes into FcγR-bearing cells like macrophages should not cause pathology. In contrast, ricin intoxicates all cell types and is known to preferentially target macrophages, including Kupffer cells in the liver (35). Therefore, accelerated FcγR-mediated clearance of ricin in the absence of neutralization may not improve the clinical results and could even enhance the toxicity.

Our future studies will be aimed at testing D10/B7 as well as two other neutralizing heterodimers, F5/B7 and E5/B7, as possible therapeutics for ricin intoxication. We have shown using murine and murine-human chimeric mAbs that antibody treatment within 4–6 h of toxin exposure is sufficient to rescue mice from the effects of ricin administered by intraperitoneal injection (13) or aerosol.[7] Based on the results presented in this study, we propose that V_HH heterodimers and the inclusion of efAbs will extend the therapeutic window beyond these time points. Finally, the V_HHs identified in this study may also prove useful in ricin toxin detection. Indeed, Anderson *et al.* (36) developed a ricin-specific immunoassay using camelid V_HHs that can differentiate between ricin and the closely related protein RCA I. With this assay, they were able to achieve sensitive ricin detection (<100 pg/ml) using an anti-RTB V_HH, B4, with an affinity for ricin of 2 nM. In our study, RTB-G5, an anti-RTB V_HH, had an EC_{50} of 231 pM. Therefore, the use of RTB-G5 alone or in combination with other V_HHs may enable the development of a more sensitive detection assay.

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