Stepwise Engineering of Heterodimeric Single Domain Camelid V_{H}H 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 (VHHs) capable of neutralizing ricin in vitro and engineered heterodimeric V_{H}Hs that neutralized ricin in vivo.

**Conclusion**: Stepwise engineering of V_{H}Hs resulted in highly potent ricin toxin-neutralizing antibodies.

**Significance**: This study highlights the potential use of a V_{H}H 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 VH domains (VHHs) specific for the ricin enzymatic (RTA) and binding (RTB) subunits. Among the 20 unique ricin-specific VHHs we identified, six had toxin-neutralizing activity: five specific for RTA and one specific for RTB. Three neutralizing RTA-specific VHHs were each linked via a short peptide spacer to the sole neutralizing anti-RTB V_{H}H to create V_{H}H “heterodimers.” As compared with equimolar concentrations of their respective monovalent monomers, all three V_{H}H heterodimers had higher affinities for 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 choler toxin, Shiga toxins 1 (Stx1) 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 bioterror 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_{H}Hs) 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_{H}H, 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_{HH}s 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_{HH}s recognizing nonoverlapping epitopes. The linked V_{HH}s 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_{HH}s 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 (Sologenix, 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 the Wadsworth Center and approved by the Wadsworth Center’s 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 \times 10^4 and 5 \times 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 \times 10^9 independent clones (>95% with V_{HH} inserts) were prepared from 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_{HH}s selected as strong positives for RTA binding, and nine unique clones for V_{HH}s 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_{HH}s likely to have common B cell clonal origins. Based on this analysis, eleven RTA-binding V_{HH}s and nine RTB-binding V_{HH}s were selected for protein expression.

We have previously described the protocols used for purification of V_{HH}s 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_{HH}s were engineered to contain a flexible spacer (GGGS \times 3) between the two V_{HH} monomers and two copies of E-tag flanking the V_{HH} heterodimer (21).

ELISA—Nunc-Immuno plates (ThermoScientific, Swedenboro, 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_{HH}s. For competition assays, murine IgGs (10 μg/ml) were added to the ELISA plate wells 1 h prior to the addition of the V_{HH}s (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).

**VₜH Affinity Determinations**—Affinity of VₜHs 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 (Promega, Madison, WI).

Cell viability was assessed 45–48 h later using CellTiter-Glo (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 VₜHs with Ricin Toxic-in-neutralizing Activity—We prepared a VₜH-displayed phage library representing the repertoire of two alpacas that were repeatedly immunized with ricin toxin subunit antigens and then boosted with ricin toxoid (see “Materials and Methods”). The VₜH-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 VₜHs with RTA or RTB binding activity that were then subjected to DNA sequencing as a means to determine their relatedness. Of the 25 VₜHs, there were 11 apparently unrelated RTA-specific VₜHs and 9 unrelated RTB-specific VₜHs (Table 1 and Fig. 1). All 20 unique VₜHs were expressed and purified from *E. coli* as E-tagged thioredoxin fusion proteins.

The 20 unique VₜHs were tested in a Vero cell cytotoxicity assay for the ability to neutralize ricin. Five RTA-specific VₜHs (RTA-F5, RTA-G12, RTA-D10, RTA-E5, and RTA-G11) and one RTB-specific VₜH (RTB-B7) demonstrated a dose-dependent capacity to protect Vero cells from ricin-induced cytotoxicity (Table 2 and Fig. 2A). Three VₜHs (RTA-F5, RTA-E5, and RTB-B7) had estimated IC₅₀ values of 25 nM (≈30:1 molar ratio VₜH:ricin), one (RTA-D10) had an IC₅₀ of 80 nM and two (RTA-G12 and RTA-G11) had IC₅₀ values of >90 nM. The remaining 14 VₜHs had no detectable neutralizing activity, even at 330 nM (≈2,200:1 VₜH:ricin).

To determine the relationship between toxin-neutralizing activity and dissociation constants (Kₕ), the six neutralizing VₜHs were subjected to SPR analysis. All six VₜHs 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 VₜHs to those of the 14 non-neutralizing VₜHs (Table 2 and Fig. 2B). This comparison revealed EC₅₀ 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 VₜH with the highest relative affinity for ricin toxin was RTB-G5 (200 pm), a VₜH 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 VₜHs bind epitopes that overlap those recognized by pre-
Stepwise Engineering of Toxin-neutralizing Antibodies

TABLE 2
Characteristics of RTA- and RTB-specific VHHs

| VHH | EC \(_{50}\), nM | K, nM | IC \(_{50}\), nM | Competitive inhibition assays* |
|------|--------------|------|--------------|------------------------------|
|      | h1 | h2 | h3 | PB10 | SyH7 | IB2 | GD12 |
| RTA-F5 | 1.50 | 2.24 | 5 | ++++ | − | − | − |
| RTA-F6 | 1.00 | | | | | | |
| RTA-G12 | 0.50 | 0.62 | >300 | ++++ | | | |
| 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 | 0.85 | 1.94 | 5 | ++++ | | | |
| RTA-F10 | 13.20 | | | | | | |
| RTA-G11 | 0.83 | 0.35 | 90 | ++++ | | | |

| VHH | EC \(_{50}\), nM | K, nM | IC \(_{50}\), nM | Competitive inhibition assays* |
|------|--------------|------|--------------|------------------------------|
|      | h1 | h2 | h3 | PB10 | SyH7 | IB2 | GD12 |
| 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 | | | | | | |

* VHH or RTB-specific murine mAbs were tested for capacity to prevent indicated VHH 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; ++++, >60% reduction).
* Underlining indicates neutralizing VHHs. Asterisks indicate VHHs used in heterodimer formation.

The values indicate the effective concentration of VHH required to achieve 50% maximal binding to ricin by ELISA.

![FIGURE 1. Amino acid sequences of the VHH variable regions.](image-url)
Stepwise Engineering of Toxin-neutralizing Antibodies

FIGURE 2. V_{1},H toxin-neutralizing and binding activities. A, monomeric V_{1},Hs were tested for toxin-neutralizing activity in a Vero cell cytotoxicity assay, as described under “Materials and Methods.” V_{1},Hs (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_{1},Hs for ricin, the V_{1},Hs (at indicated concentrations) were applied in duplicate to microtiter plates coated with ricin. The EC_{50} values are defined as the V_{1},H 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_{1},H heterodimers |
|---------|----------------------------------------|
| Heterodimer | Constituents | EC_{50} | IC_{50} | Protection |
| D10/B7 | RTA-D10/RTB-B7 | 0.08 | 0.15 | 4/20 |
| F5/B7 | RTA-F5/RTB-B7 | 0.20 | 1.00 | 4/5 |
| E5/B7 | RTA-E5/RTB-B7 | 0.30 | 1.00 | 5/5 |
| G5/B7 | RTB-G5/RTB-B7 | 0.20 | 0.90 | 0/5 |
| G5/B9 | RTB-G5/RTB-B9 | 0.10 | NA | 0/5 |

The values indicate the concentration of V_{1},H required for 50% maximal ricin binding by ELISA.

The values indicate the concentration of V_{1},H required to neutralize 50% ricin in Vero cell assay.

The values indicate the number of mice passively administered V_{1},H (3 µg/mouse) that survived a 10 × LD_{50} ricin challenge.

NA, not applicable.

Although G5/B7, composed of one neutralizing and one non-neutralizing V_{1},H, 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_{1},H 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_{1},H 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 VH heterodimers improved toxin clearance (23) and the protective efficacy of VH heterotrimers (21, 22). As controls for these studies, groups of mice received 10 µg (10:1 V_{1},H:ricin ratio) of the individual V_{1},H 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_{1},Hs 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 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
were passively administered G5/B9, without or with efAb, and then challenged with a 10 × LD₅₀ 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 V₅H Heterodimers—We tested the remaining V₅H heterodimers, F5/B7, E5/B7 and RTB-G5/RTB-B7, for the ability to passively protect mice from a 10 × LD₅₀ dose of ricin toxin. The heterodimers were mixed at a 2-fold molar excess (3 μg) with 10 × LD₅₀ 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 V₅H heterodimers display enhanced potency to protect animals from ricin exposure compared with V₅H monomers, especially when both V₅H components are toxin-neutralizing.

DISCUSSION

In this study, we engineered, using a unique platform technology, novel antitoxins against the category B toxin ricin. We
In vivo activity of monomeric VHHs upon ricin challenge. RTA-D10 and RTB-B7 VHH monomers (or an equimolar mixture of the monomers) were premixed at a 10:1 molar ratio (10 μg/g) with the equivalent of 10 LD_{50} of ricin toxin (2 μg/g) 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).

In vivo protection conferred by VHH heterodimer D10/B7. D10/B7 (with or without efAb) was premixed at the indicated amounts 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, except the ricin control group (n = 20), the 3-μg dose group (n = 20), and the 3-μg dose + efAb group (n = 10).
Stepwise Engineering of Toxin-neutralizing Antibodies

initially identified 11 RTA-specific V\textsubscript{H}Hs and 9 RTB-specific V\textsubscript{H}Hs from a V\textsubscript{H}H library generated from two immunized alpacas. Among the 20 unique ricin-specific V\textsubscript{H}Hs, we identified six (five RTA-specific and one RTB-specific) that had clear toxin-neutralizing activity \textit{in vitro}. We next engineered a series of V\textsubscript{H}H heterodimers consisting of RTB-B7, the single neutralizing anti-RTB V\textsubscript{H}H, linked to one of three neutralizing anti-RTA V\textsubscript{H}Hs. One heterodimer in particular, D10/B7, had a 6-fold increase in toxin-neutralizing activity \textit{in vitro} as compared with an equimolar mixture of the component monomers. \textit{In vivo} analysis revealed that D10/B7 was able to fully protect mice from a 10 × \text{LD\textsubscript{50}} ricin challenge at a V\textsubscript{H}H: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\textsubscript{H}H 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 for the engineering protective V\textsubscript{H}H heterodimers against BoNT/A and E5/B7, which 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.\textsuperscript{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.\textsuperscript{5} V\textsubscript{H}H monomers that neutralize ricin \textit{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\textsubscript{H}H 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\textsubscript{H}H 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.

\textsuperscript{4} D. J. Vance, J. M. Tremblay, N. J. Mantis, and C. B. Shoemaker, unpublished data.
\textsuperscript{5} C. Herrera, D. J. Vance, and N. J. Mantis, unpublished observations.
\textsuperscript{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 V₄₅Hs, 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 V₄₅H 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. Based on the results presented in this study, we propose that V₄₅H heterodimers and the inclusion of efAbs will extend the therapeutic window beyond these time points. Finally, the V₄₅Hs identified in this study may also prove useful in ricin toxin detection. Indeed, Anderson et al. (36) developed a ricin-specific immunoassay using camelid V₄₅Hs 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 V₄₅H, B4, with an affinity for ricin of 2 nm. In our study, RTB-G5, an anti-RTB V₄₅H, had an EC₅₀ of 231 pm. Therefore, the use of RTB-G5 alone or in combination with other V₄₅Hs may enable the development of a more sensitive detection assay.

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