High-Resolution Epitope Positioning of a Large Collection of Neutralizing and Nonneutralizing Single-Domain Antibodies on the Enzymatic and Binding Subunits of Ricin Toxin

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ABSTRACT

We previously produced a heavy-chain-only antibody (Ab) VH domain-(V_{H})-displayed phage library from two alpacas that had been immunized with ricin toxoid and nontoxic mixtures of the enzymatic ricin toxin A subunit (RTA) and binding ricin toxin B subunit (RTB) (D. J. Vance, J. M. Tremblay, N. J. Mantis, and C. B. Shoemaker, J Biol Chem 288:36538–36547, 2013, https://doi.org/10.1074/jbc.M113.519207). Initial and subsequent screens of that library by direct enzyme-linked immunosorbent assay (ELISA) yielded more than two dozen unique RTA- and RTB-specific V_{H}Hs, including 10 whose structures were subsequently solved in complex with RTA. To generate a more complete antigenic map of ricin toxin and to define the epitopes associated with toxin-neutralizing activity, we subjected the V_{H}H-displayed phage library to additional “pannings” on both receptor-bound ricin and antibody-captured ricin. We now report the full-length DNA sequences, binding affinities, and neutralizing activities of 68 unique V_{H}Hs: 31 against RTA, 33 against RTB, and 4 against ricin holotoxin. Epitope positioning was achieved through cross-competition ELISAs performed with a panel of monoclonal antibodies (MAbs) and verified, in some instances, with hydrogen-deuterium exchange mass spectrometry. The 68 V_{H}Hs grouped into more than 20 different competition bins. The RTA-specific V_{H}Hs with strong toxin-neutralizing activities were confined to bins that overlapped two previously identified neutralizing hot spots, termed clusters I and II. The four RTB-specific V_{H}Hs with potent toxin-neutralizing activity were confined to bins that overlapped the RTA-RTB interface near cluster II. These results provide important insights into epitope interrelationships on the surface of ricin and delineate regions of vulnerability that can be exploited for the purpose of vaccine and therapeutic development.

KEYWORDS

antibody, biodefense, epitope, neutralizing, toxins, vaccines

Ricin is the prototype of the type II ribosome-inactivating protein (RIP) family of toxins (1). Ricin toxin A subunit (RTA) is an RNA N-glycosidase that catalyzes the hydrolysis of a conserved adenine residue within the sarcin/ricin loop (SRL) of 28S rRNA, resulting in ribosome arrest and apoptosis (2–4). Ricin toxin B subunit (RTB) is a galactose- and N-acetylgalactosamine (Gal/GalNAc)-specific lectin that promotes toxin entry into mammalian cells, including the epithelial cells that line the respiratory tract.
Following endocytosis, RTB mediates the retrograde transport of ricin to the trans-Golgi network (TGN) and endoplasmic reticulum (ER). Once within the ER, the disulfide bond that links RTA to RTB is reduced by protein disulfide isomerase and RTA is retrotranslocated (dislocated) into the cell cytoplasm, where it triggers programmed cell death (7, 8). Ricin is classified as a category B biothreat agent by the Centers for Disease Control and Prevention (CDC) because of its potential, when delivered via aerosol, to induce severe lung inflammation and tissue damage (9, 10). Concerns over ricin’s possible use as a biothreat agent stem from the fact that the toxin is relatively easy to procure from its natural source, castor beans (Ricinus communis), as well as from its extreme toxicity following inhalation or injection (11).

As part of a longstanding effort to engineer ricin antitoxins and immunotherapies, we previously produced a library of phage-displayed, heavy-chain-only antibodies (Abs; V_{H}Hs) from two alpacas that had been hyperimmunized with nontoxic mixtures of recombinant RTA, RTB, and ricin toxoid; the library is referred to here as “HobJo” in reference to the names of the two immunized alpacas, Hobbs and Johnnes (12). In our initial study, the phage-displayed V_{H}H library was subjected to high- and low-stringency “panning” on purified native RTA or RTB that had been immobilized on plastic substrates. This initial screening strategy yielded 11 unique RTA-specific V_{H}Hs and 9 unique RTB-specific V_{H}Hs. Five (4 RTA-specific and 1 RTB-specific) V_{H}Hs among the original 20 had strong or moderate in vitro toxin-neutralizing activity (TNA), while the remaining 15 had weak TNA or no detectable TNA. A collection of homo- and heterodimeric (i.e., bispecific) V_{H}Hs were engineered with these original 20 V_{H}Hs and shown to have enhanced avidities for ricin and, in some cases, the capacity to passively protect mice from lethal-dose ricin challenge (12–15). As discussed below, the X-ray crystal structures of 10 different V_{H}-RTA complexes, including 7 V_{H}Hs from the original screen (16–18), have been solved to date.

The original strategy of panning the V_{H}H library onto RTA- and RTB-coated immuno-tubes presented several drawbacks. First, there was a bias for V_{H}Hs directed against a single immunodominant region on RTA that we refer to as cluster I. Cluster I is defined by the murine monoclonal antibody (MAb) PB10, which recognizes a linear epitope encompassing α-helix B (residues 94 to 107) of RTA (see Fig. S1 in the supplemental material) (19–21). Approximately half of the original RTA-specific V_{H}Hs recognized epitopes in cluster I, based on competition enzyme-linked immunosorbent assays (ELISAs) performed with PB10. The second shortcoming associated with the original screen was that it gave rise to V_{H}Hs that recognized cryptic epitopes that are exposed when RTA and RTB are adsorbed to polystyrene but that are not accessible to antibodies when ricin is in its native conformation. Finally, by performing screening with individual subunits, rather than with the holotoxin, we were likely missing antibodies directed against quaternary epitopes. With the long-term goal of generating a saturating B cell epitope map of ricin toxin using the HobJo library, which is estimated to contain ~10^6 independent clones, we reasoned that it would be necessary to derive novel panning strategies.

To generate a more complete antigenic map of ricin toxin and to better define the epitopes associated with neutralizing activity, we have now subjected the HobJo V_{H}H-displayed phage library to pannings on both receptor-bound and antibody-captured ricin holotoxins or subunits. We identified a total of 68 unique ricin-specific V_{H}Hs (31 against RTA, 33 against RTB, and 4 against ricin holotoxin) and present their full-length DNA sequences, binding affinities, and neutralizing activities. We used cross-competition ELISAs with a panel of well-characterized murine monoclonal antibodies (MAbs) as a strategy to localize the epitopes recognized by the new collection of V_{H}Hs. Epitope positioning was validated with available X-ray crystal structures and, in some instances, was followed up with hydrogen-deuterium mass spectrometry (HX-MS). We identified more than 20 different competition bins (a bin was comprised of all V_{H}Hs which shared the same competition profile) that cover ~50% of RTA’s surface area and an indefinite amount of RTB. Having this comprehensive collection of well-characterized, ricin-specific V_{H}Hs provides important insights into epitope interre-
relationships on the surface of ricin and delineates two or possibly three regions of vulnerability that can be exploited for the purpose of vaccine and therapeutic development.

**RESULTS**

In an effort to obtain a comprehensive map of toxin-neutralizing and nonneutralizing B cell epitopes on the surface of ricin, the HobJo VHH phage display library was subjected to nine different screens (Table 1). Screens 1 to 4 involved panning on immunotubes coated with RTA or RTB and yielded a total of 36 ricin-specific VHHs (Tables 1 and 2). The 20 VHHs resulting from screens 1 and 2 were described previously and included 11 against RTA (6 with TNA) and 9 against RTB (1 with TNA) (12). We successfully solved the X-ray crystal structures of seven of those VHHs in complex with RTA (16–18). Screens 3 and 4 yielded an additional 16 ricin-specific VHHs, including 7 against RTA, 1 of which was successfully crystallized (17), and 9 against RTB (Table 1).

Screens 5 and 6 were designed to circumvent two issues that we had encountered in screens 1 to 4. First, roughly half of the 36 VHHs from screens 1 to 4 failed to bind soluble ricin (R), even though they recognized plate-adsorbed ricin ($R = 100 \text{nM}$), RTA ($A = 10 \text{nM}$), or RTB ($B = 500 \text{nM}$ or undetectable). Second, the majority of the RTA-specific VHHs were directed against cluster I, which is defined functionally by competition with MAb PB10 and is known to be immunodominant (12). To overcome these issues, the HobJo library was panned, in screen 5, on ricin captured with asialofetuin (R-ASF) to approximate the conformation of soluble ricin and, in screen 6, on ricin that was captured with PB10 (R-PB10) to mask immunodominant epitopes within cluster I. VHHs identified in screen 5 were cross-checked for binding to R-PB10, while VHHs identified in screen 6 were cross-checked for reactivity with R-ASF. Only VHHs that were positive in both assays were characterized further (Tables 1 and 2). This strategy yielded a total of 56 VHHs that were reactive with both R-ASF and R-PB10. After we eliminated siblings from the pool, a total of 13 VHHs remained: 10 against RTA, 2 against RTB, and 1 specific for ricin holotoxin.

Screen 7 was designed to identify VHHs that recognize two different linear epitopes on RTA defined by the murine toxin-neutralizing MAbs PB10 and SyH7 (19). Two biotin-tagged 18-mer peptides, E12 and G3, corresponding to the core epitopes of PB10 and SyH7, respectively, were captured on immunotubes via the use of avidin and then subjected to panning with the HobJo library. The screen proved unsuccessful,

### Table 1: Summary of HobJo screens

| Screen | Target(s) | A | B | R | Total | TNA^b |
|--------|-----------|---|---|---|-------|-------|
| 1      | RTA       | 11| 0 | 0 | 11    | 2     |
| 2      | RTB       | 0 | 9 | 0 | 9     | 1     |
| 3      | RTA       | 7 | 0 | 0 | 7     | 0     |
| 4      | RTB       | 0 | 9 | 0 | 9     | 0     |
| 5      | R-ASF     | 5 | 0 | 1 | 6     | 0     |
| 6      | R-PB10    | 5 | 2 | 0 | 7     | 1     |
| 7      | Peptides  | 0 | 0 | 0 | 0     | 0     |
| 8      | RTB-ASF   | 0 | 5 | 0 | 5     | 0     |
| 9      | R-F5 + F6 | 4 | 8 | 3 | 15    | 3     |

^a A, RTA; B, RTB; R, ricin holotoxin.
^b Toxin neutralizing activity, based on IC_{50}, indicated as follows: +++, IC_{50} < 10 nM; ++, IC_{50} < 100 nM; +, IC_{50} < 500 nM; I, IC_{50} > 500 nM or undetectable.
^c VHHs in screens 1 and 2 were reported previously (12).
^d R-ASF, ricin captured by ASF; R-PB10, ricin captured by PB10; RTB-ASF, RTB captured by ASF; R-F5 + F6, ricin captured with VHH JIV-F5 and then blocked with excess JIV-F6.
^e One anti-RTA VHH from screen 9 was not successfully expressed and thus was not characterized for TNA and also was not included in the final VHH total of 68.
### TABLE 2 Properties of ricin-specific V_{H} Hs described in this study

| V_{H} | Screen | GenBank accession no. | PDB ID | TNA | K_{D} (nM) | Specificity | Cluster(s) | Family |
|-------|--------|-----------------------|--------|-----|------------|-------------|------------|---------|
| JIV-F5 | 1 | KF746018 | 4Z9K | +++ | 0.019 | A | I |
| JIV-F6 | 1 | KF746019 |  | 1.86 | A | III | JIV-F6 |
| JIV-G12 | 1 | KF746020 | 4LGS | + | 0.129 | A | I |
| JY-A7 | 1 | KF746021 | 4LHQ |  | 4.3 | A | I | JY-A7 |
| JY-D9 | 1 | KF746022 |  |  | 0.174 | A | I + II | JY-D9 |
| JY-D10 | 1 | KF746023 | 4LGR | ++ | 0.114 | A | I + II |
| JY-E1 | 1 | KF746024 | 3BOZ | + | 0.686 | A | II |
| JY-E3 | 1 | KF746025 |  |  | 0.296 | A | I |
| JY-E5 | 1 | KF746026 | 4LG | +++ | 0.191 | A | I + IV |
| JY-F10 | 1 | KF746027 |  |  |  | A' |
| JY-G11 | 1 | KF746028 | 4LHJ | ++ | 0.553 | A | I |
| JIY-A7 | 1 | KF746021 | 4LHQ |  | 4.3 | A | I | JY-A7 |
| JIY-D9 | 1 | KF746022 |  |  | 0.174 | A | I + II | JY-D9 |
| JIY-D10 | 1 | KF746023 | 4LGR | ++ | 0.114 | A | I + II |
| JIY-E1 | 1 | KF746024 | 3BOZ | + | 0.686 | A | II |
| JIY-E3 | 1 | KF746025 |  |  | 0.296 | A | I |
| JIY-E5 | 1 | KF746026 | 4LG | +++ | 0.191 | A | I + IV |
| JIY-F10 | 1 | KF746027 |  |  |  | A' |
| JIY-G11 | 1 | KF746028 | 4LHJ | ++ | 0.553 | A | I |
| JIY-A7 | 1 | KF746021 | 4LHQ |  | 4.3 | A | I | JY-A7 |
| JIY-D9 | 1 | KF746022 |  |  | 0.174 | A | I + II | JY-D9 |
| JIY-D10 | 1 | KF746023 | 4LGR | ++ | 0.114 | A | I + II |
| JIY-E1 | 1 | KF746024 | 3BOZ | + | 0.686 | A | II |
| JIY-E3 | 1 | KF746025 |  |  | 0.296 | A | I |
| JIY-E5 | 1 | KF746026 | 4LG | +++ | 0.191 | A | I + IV |
| JIY-F10 | 1 | KF746027 |  |  |  | A' |
| JIY-G11 | 1 | KF746028 | 4LHJ | ++ | 0.553 | A | I |

(Continued on next page)
however, as none of the ~100 clones that we examined bound to ricin or either of the E12 or G3 peptides (data not shown). The failure to obtain E12- or G3-specific V_{HH}s was not completely surprising, considering that there are very few examples of single-domain antibodies that recognize linear epitopes, possibly because of the absence of a light chain to provide a cleft for peptide binding (25, 26).

Two final screens were conducted using the HobJo library. Screen 8, which was designed to enrich for V_{HH}s that bind native (not cryptic) epitopes on RTB, involved panning the HobJo library on RTB captured via ASF (RTB-ASF). The screen gave rise to five unique RTB-specific V_{HH}s. In screen 9, the V_{HH} library was panned using two V_{HH}s identified in screen 1; ricin holotoxin was captured with JIV-F5 to obstruct immuno-dominant epitopes in cluster I and then saturated with JIV-F6 to mask a subdominant epitope in cluster III (R-F5 / H11011 R-F6). The screen yielded a total of 15 unique V_{HH}s, including 4 targeting RTA (3 of which were successfully expressed), 8 targeting RTB, and 3 targeting ricin holotoxin.

In total, the nine screens resulted in 68 unique anti-ricin V_{HH}s: 31 specific for RTA, 33 specific for RTB, and 4 specific for ricin holotoxin (Tables 1 and 2). Fourteen additional V_{HH}s (7 anti-RTA and 7 anti-RTB) found in various screens were dropped from further study because of their similarity to other V_{HH}s (i.e., with very few, generally conservative mutations, usually within framework regions) or, in one case, because the V_{HH} failed to be expressed at sufficiently high levels in Escherichia coli to be amenable to analysis (see Table S1 in the supplemental material). The complementarity determining region 3 (CDR3) nucleotide sequences of the entire collection of V_{HH}s were grouped into families based on length and nucleotide identity. There were nine different clonal families encompassing a total of 24 V_{HH}s (Tables 2 and 3); the remaining 44 V_{HH}s were unrelated to each other. The V_{HH} sequences have been deposited in GenBank (Table 2). Within the group of 68 V_{HH}s, CDR3 length ranged from 7 to 22 residues, with an average of 14.8 residues.

### Table 2 (Continued)

| V_{HH} | Screen | GenBank accession no. | PDB ID | TNA | $K_d$ (nM) | Specificity | Cluster(s) | Family |
|-------|--------|-----------------------|--------|-----|-----------|------------|-----------|--------|
| V5G6  | 9      | KY824620              |        |     | 1.24      | R          | SII       |        |
| V5G12 | 9      | KY824621              |        |     | 0.994     | R          | SII       |        |
| V5H2  | 9      | KY824622              |        |     | 2.18      | B          | SII       | V2C11  |
| V5H6  | 9      | KY824623              |        |     | 23.2      | B          |           |        |

\(V_{HH}\)s in screens 1 and 2 were reported previously (12).

Protein Data Bank IDs.

Toxin-neutralizing activity (TNA), as described in the legend of Table 1.

NB, no binding detected in SPR.

\(A, \text{RTA}; A', \text{RTA}'; B, \text{RTB}; B', \text{RTB}'; R, \text{ricin holotoxin}.

Data represent RTA epitope cluster(s) (I to IV) bound by a particular V_{HH}. SII refers to “supercluster II” and is the designation given to RTB- or holotoxin-specific V_{HH}s that are subject to competition from RTA binding cluster II MAb SyH7.

See Table 3 for descriptions of families.

### Table 3 V_{HH} families based on CDR3 similarity

| Family | Member(s) | Specificity |
|--------|-----------|-------------|
| JIV-F6 | V1B10, V1D3 | A           |
| JIY-A7 | JNM-G4    | A           |
| JIY-D9 | V2BB      | A           |
| V1C7   | V289, V2E8, V5C1 | A     |
| V2G10  | V1G6      | A           |
| JIZ-B7 | JIW-D12, JNN-B8, JNN-D1 | B   |
| JIW-G10| JNN-A3    | B’          |
| V2C11  | V2D4, V5H2 | B           |

\(A\) family was defined as composed of two or more V_{HH}s having CDR3 sequences of equal lengths and >90% identity at the nucleotide level.

\(A, \text{RTA}; B, \text{RTB}; B', \text{plate-bound RTB}.

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**Contiguous B Cell Epitope Map of Ricin Toxin**

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Classification and characterization of ricin-specific V_{HH}s. All 68 V_{HH}s in this study (Table 2) were expressed in E. coli as 33- to 35-kDa E-tagged thioredoxin fusion proteins, as described in the supplemental methods and in previous publications (12). The relative purity and integrity of the V_{HH} preparations were assessed by SDS-PAGE, and the resulting gel was stained with Coomassie blue. Molecular mass standards (with kilodalton notations) are shown in the far left lane. The major band (~33 to ~35 kDa) in each lane corresponds to the expected molecular mass of the recombinant V_{HH}s, with an N-terminal thioredoxin fusion partner, a hexa-His tag, and a C-terminal epitope E-tag, as described in the supplemental methods. (B) Toxin-neutralizing activity, as determined in a Vero cell-based microtiter plate assay. The representative set of V_{HH}s consists of two V_{HH}s with strong TNA (IC_{50} = <10 nM) and three with no TNA. (C) Competition ELISA, as described in Materials and Methods. Microtiter plates were coated with the indicated MAbs or ASF (legend on right) and then overlaid with ricin. The plates were probed with V_{HH}s, as indicated along the x axis, and were developed with anti-E-tag secondary antibodies. As such, the sandwich ELISA data provided relative competition values against a panel of RTA- and RTB-specific MAbs. The failure of the V_{HH}s to recognize ricin toxin when bound to ASF is indicative of the antibodies recognizing epitopes near one of the RTB Gal/GalNAc binding sites. The full array of competition ELISAs is presented in Data Set S1.
TNA, while the remainder (~20%) were evenly divided between strong (n = 7) and moderate (n = 7) toxin-neutralizing activities (Tables 1 and 2). The results of the toxin-neutralizing assays performed for the 14 V_{rh}Hs with strong or moderate TNA are shown in Fig. S2 in the supplemental material. Among the seven V_{rh}Hs with the strongest TNA, three were specific for RTA and four were specific for RTB.

V_{rh}H association (K_{a}) and dissociation (K_{d}) rates and equilibrium dissociation constants (K_{D}) for ricin were determined by surface plasmon resonance (SPR) using sensor chips coated with native toxin via amine coupling, as described in Materials and Methods. Kinetic constants were fitted using the Langmuir 1:1 binding model (29). We found that K_{D} values ranged from 6 pM to ~32 nM (Table 2; see also Table S2 and Fig. S3 in the supplemental material). In general, the V_{rh}Hs with the highest binding affinity (K_{D}) generally had the strongest TNA (R^2 = 0.6966; Fig. 2A). For example, all 14 V_{rh}Hs with strong and moderate TNA had K_{D} values of <1 nM, while V_{rh}Hs with K_{D} values of ≥1 nM lacked TNA, with one exception. Furthermore, the V_{rh}Hs with the strongest TNA also tended to have the slowest off-rates (K_{d}) (R^2 = 0.6018; Fig. 2B), a relationship that has been noted by other investigators (30). The relationship between binding affinity and TNA was most apparent within V_{rh}H clonal families (Fig. 2C), the members of which would be expected to have epitopes similar to each other (31). The failure of antibodies such as JNM-B5 to neutralize ricin despite its relatively high binding affinity (e.g., 60 pM) demonstrates once again that other factors such as epitope specificity contribute to antibody functionality.

**Localization of V_{rh}H epitopes on RTA.** We next sought to locate the relative binding sites of all V_{rh}Hs that recognized soluble ricin, with the expectation that toxin-neutralizing antibodies would segregate into specific bins or clusters of epitopes on RTA and RTB.

We addressed RTA first. In collaboration with Michael Rudolph (New York Structural Biology Center, New York, NY), all 30 RTA-specific V_{rh}Hs (excepting JIY-F10, which did not recognize soluble ricin) have been subjected to X-ray crystallography trials. To date, we have reported 10 V_{rh}H-RTA cocrystal structures: 6 that involve epitope cluster I, 3 that involve epitope cluster II, and 1 that straddles clusters I and II (Table 2) (16–18). The two cluster I V_{rh}Hs with the strongest TNA, JIY-E5 and JIV-F5, each make considerable contact with RTA’s helix B (i.e., ~472 to ~477 Å^2), an interaction that has been proposed to be critical for toxin inactivation (17–22, 26, 32). The other cluster I V_{rh}Hs, and the cluster I/II V_{rh}H, interacted with β-strand h and α-helix D to various degrees. Within cluster II, the two V_{rh}Hs with weak TNA, V1C7 and JIY-E1, made primary contact with α-helices D and E, while V5E1, which has strong TNA, associated with α-helices A and F and the F-G loop. For perspective, we provide a PyMol surface representation of all 10 solved V_{rh}H structures superimposed onto RTA (see Fig. S4 in the supplemental material).

In the absence of additional X-ray crystal structures, epitope localization on RTA was done by competitive sandwich ELISA with a collection of nine different MAbs against epitopes in clusters I (PB10 and WECB2), I and II (SWB1), I (SyH7, PA1, TB12, and PH12), III (IB2), and IV (GD12) (Fig. 3; see also Table S3 in the supplemental material) (19, 20, 53). As described in Materials and Methods, each of the 30 anti-RTA V_{rh}Hs was tested for the ability to recognize ricin that had been captured by each of the nine cluster I to IV MAbs. The validity of the sandwich ELISA for epitope mapping was evaluated using the following three V_{rh}Hs whose epitopes have been resolved by X-ray crystallography (Fig. 4): JIV-F5 (PDB identifier [ID]: 4Z9K), JIY-D10 (PDB ID: 4LGR), and V1C7 (PDB ID: 5J56) (16–18). In the sandwich ELISA, the binding of JIV-F5 to ricin was affected by PB10, WECB2, and SWB1 but not by SyH7, PA1, PH12, TB12, IB2, or GD12 (Fig. 4A). This competition profile is consistent with the structural epitope of JIV-F5 encompassing α-helix B, β-strand h, and the C-terminal portion of α-helix D (17). By the same token, JIY-D10 was able to bind to ricin that had been captured by GD12, PB10, PA1, SyH7, and IB2 but not WECB2, SWB1, TB12, or PH12, a profile congruent with JIY-D10’s structural epitope being localized to α-helices D and E and β-strand h (Fig. 4B). Finally, V1C7 was
able to bind ricin that had been captured by GD12, PB10, WECB2, and IB2 but not the cluster II-specific MAbs (SyH7, PA1, PH12, and TB12) or the I-II overlap MAb SWB1. This profile is consistent with V1C7 making contact with RTA’s H9251-helix D and H9251-helix E, along with several additional interactions with loops e-d, C-D, and D-E (16) (Fig. 4C). The sandwich ELISA also successfully positioned the epitopes of the seven additional VHHs (JIV-G12, JIY-A7, JIY-E1, JIY-E5, JIY-G11, JNM-F8, and V5E1) whose X-ray crystal structures in complex with RTA have been solved (16–18) (see Data Set S1 in the supplemental material). However, it is important to note that the epitope localization determined by the sandwich ELISA is only approximate, as the assay does not discriminate between direct epitope overlap, steric hindrance, and allostery. Indeed, we noted that the sandwich ELISA overestimated V1C7’s contact on RTA compared to its known structural epitope as defined by X-ray crystallography (see Fig. S5).

FIG 2 Relationships between VHH binding affinities and toxin-neutralizing activities. (A and B) Using values presented in Table 2, toxin-neutralizing activity (percent viability at 66 nM VHH) of representative VHHs was plotted versus its respective (A) equilibrium dissociation constant (Kd) or (B) off-rate (Kd) value. For simplicity’s sake, individual VHHs are color coded based on their neutralizing activity (i.e., strong, moderate, weak, or no neutralizing [Non] activity). To enable the use of a logarithmic axis, VHHs that lacked TNA were arbitrarily assigned a value of 1 for percent viability (x axis). R² values were calculated using the Power Trendline function in Microsoft Excel. (C) TNA as a function of equilibrium dissociation constant (Kd) for the six VHH clonal families represented in Table 3. Each color represents a family; each dot represents an individual VHH within that family. The downward slope of the lines suggests a relationship between Kd and TNA within a clonal family (i.e., VHHs with the highest affinity for ricin also have the strongest TNA). The percentages of cell viability as plotted are from a single representative experiment done in triplicate.
The sandwich ELISA was next used to approximate the binding sites of the remaining 20 RTA-specific VHHs on the surface of RTA relative to clusters I to IV. All 20 VHHs were inhibited from binding to ricin by at least one MAb, and the majority (16/20) were inhibited by two or more MAbs (Fig. 5; see also Fig. S6 and Data Set S1 in the supplemental material). In total, the 30 anti-RTA VHHs fell into 11 different competition profiles, or “bins” (a bin was comprised of all VHHs which shared the same competition profile). When the bins were aligned vertically and the epitope clusters horizontally, as shown in Fig. 5, it was evident that the putative VHH binding sites were spatially contiguous across the four previously identified epitope clusters. Moreover, each of the 11 competition bins overlapped WECB2 (cluster I; bins 1 to 6), SyH7 (cluster II; bins 7 and 8), or IB2 (cluster III; bins 9 to 11), suggesting that these three MAbs constitute the immunodominant regions on RTA.

Overall, the competition profiles associated with each of the bins enabled us to assign their location on the surface of RTA with confidence, especially if a bin contained one or more VHHs whose X-ray crystal structure in complex with RTA has been solved. Bin 2, for example, is defined by competition with PB10, WECB2, and SWB1. These three MAbs have overlapping epitopes on RTA’s α-helix B, β-strand h, and α-helix D and the D-E loop (53). Moreover, the structural epitopes of five of the nine VHHs in bin 2 have been reported (17, 18). Therefore, we can assume that the remaining four VHHs in bin 2, JIY-E3, JNM-B5, JNM-E4, and JNM-G4, contact α-helix B, β-strand h, and/or α-helix D (Fig. 3; see also Fig. S1).

Similarly, bin 7 is defined by competition with SWB1 and all four cluster II MAbs (PH12, TB12, PA1, and SyH7). PA1 and SyH7 contact the loop before α-helix A and α-helices F and G, while PH12, TB12, and SWB1 contact β-strand d and α-helices D and E. Moreover, as noted above, the structural epitopes of two VHHs in bin 7, JIY-E1 and V1C7,
have recently been reported (16). The remaining three V\textsubscript{H}Hs in bin 7, V2B9, V2E8, and V5C1, are members of the V1C7 family (Table 3). Rosetta-based homology modeling suggests that the V1C7 family members, despite their minor differences in CDR sequences, interact with RTA in similar fashions (31). Thus, the epitopes of all five V\textsubscript{H}Hs in bin 7 can be positioned on RTA with relative accuracy.

In some instances, the results of the sandwich ELISA were more difficult to reconcile. In the case of V1B11 and JNM-D1, representing bins 6 and 10, respectively, considerable cross-cluster competition was involved. V1B11 was competed with by WECB2 (cluster I) and IB2 (cluster III), while JNM-D1 was affected by SyH7 (cluster II) and IB2 (cluster III). When the cross-cluster competition results were taken at face value, the predicted epitopes for V1B11 and JNM-D1 encompassed too large a surface on RTA to localize actual antibody-antigen contact points (Fig. 6). To refine these ambiguous antibody binding sites, we subjected V1B11 and JNM-D1 to epitope mapping by hydrogen-deuterium exchange mass spectrometry (HX-MS), as described recently (31). HX-MS affords peptide-level resolution of antibody binding sites and is therefore an extremely useful methodology for epitope localization (33). As part of this analysis, we also included JNM-C12 (bin 1) since it also demonstrated a degree of cross-cluster competition.

JNM-C12’s binding to ricin was inhibited by cluster I (PB10 and WECB2), cluster I/II (SWB1), and cluster IV (GD12) MAbs (Fig. 6A). This profile differs from that seen with bin 2, for which we have several crystal structures, determined solely by competition with cluster IV (GD12). On the basis of this profile, we predicted that JNM-C12’s core epitope would encompass RTA’s \( \alpha \)-helix B and \( \beta \)-strand h and might also potentially include \( \beta \)-strands b to d. This prediction was partially borne out when JNM-C12 was subjected to epitope mapping by HX-MS. Specifically, JNM-C12 strongly protected RTA peptides corresponding to \( \alpha \)-helix B (residues 92 to 109) and \( \beta \)-strand h (residues 108 to 122) but did not protect \( \beta \)-strands b to d (see Fig. S7A and Table S5 in the supplemental
Thus, competition with GD12 may be steric in nature. In this respect, JNM-C12’s core epitope involves contact with β-helix B and β-strand h and resembles VHHs in bin 2.

The sandwich ELISA predicted that JNM-D1 would recognize β-helices C and G and the F-G loop (Fig. 6B). Again, HX-MS analysis partially confirmed this prediction, as JNM-D1 strongly protected peptides encompassing RTA’s α-helices C (residues 123 to 135) and G (residues 205 to 210) but not the F-G loop (see Fig. S7B and Table S5 in the supplemental material). On the basis of the sandwich ELISA, V1B11 was predicted to recognize an epitope situated between β-strand h and α-helix C (Fig. 6C). HX-MS analysis of V1B11 revealed that it strongly protected RTA’s α-helices C (residues 123 to 135) and G (residues 205 to 210), consistent with the observed IB2 results (see Fig. S7C and Table S5). Thus, HX-MS enabled us to predict with greater confidence the epitopes of V1B11 and JNM-D1 on RTA. Interestingly, despite the differential competition profiles of these two VHHs, their binding epitopes as determined by HX-MS are almost identical. Thus, the sandwich ELISA may provide additional information regarding the orientation of binding of these VHHs to that footprint on RTA, which results in differential steric hindrance with WECB2 and SyH7.

HX-MS also revealed another interesting difference between JNM-D1 and V1B11 in residues 249 to 255, which span a 3/10-helix and a β-bridge in RTA’s C terminus. This region is on the opposite side of RTA from the residues in helices C and G that are...
strongly protected by both VHHs and would not be exposed to solvent in the context of ricin holotoxin. While JNM-D1 induced strong deprotection (i.e., increased flexibility leading to faster hydrogen exchange) in this region, V1B11 induced strong protection (see Fig. S7B and C in the supplemental material). We hesitate to speculate about the significance of the observed deprotection/protection associated with RTA’s C terminus, considering that this region of RTA is prone to unfolding in the absence of RTB. Nonetheless, the observation that two VHHs (JNM-D1 and V1B11) with seemingly identical binding sites (and similar affinities) have opposite effects (protection versus deprotection) on RTA’s C terminus raises interesting questions about potential allosteric changes that can occur within the context of ricin toxin.

Spatial location and secondary elements on RTA associated with neutralizing epitopes. We had originally proposed that toxin-neutralizing antibodies would be confined to a very limited number of sites on RTA, a hypothesis that proved to be somewhat accurate. As shown in Fig. 5, although VHHs with strong and moderate TNA were distributed across six different bins (namely, bins 1, 2, 5, 7, 8, and 9), the six bins likely constitute two hot spots. One hot spot, defined by competition with WECB2 and SWB1 and represented by JNM-C12, JIV-F5, and JIY-D10, among others, involves α-helix B and possibly the C-terminal portion of α-helix D (Fig. 3; see also Fig. S1) (17, 18).
Although \(\beta\)-strand h sits between these structural elements, evidence provided by X-ray crystal structure analysis suggests that the interaction with strand h alone is not sufficient to confer TNA (17, 18). The other hot spot, defined by competition with SyH7 and PA1 and represented by V5E1, involves contact with the loop preceding \(\alpha\)-helix A, \(\alpha\)-helix F itself, and the F-G loop, with the F-G loop likely the critical determinant of strong neutralizing activity (16).

With that in mind, we can account, at the structural level, for strong and moderate TNA associated with other RTA-specific VHHs. VHHs in bins 1 to 6 with detectable TNA (e.g., JNM-E4) would be predicted to engage with \(\alpha\)-helix B and/or \(\alpha\)-helix D, while VHHs in bins 7 and 8 would be expected to contact \(\alpha\)-helix F and the F-G loop. V5C1 (bin 7) sits between these two hot spots, being competed with by all three of SWB1 and SyH7/PA1, and thus may neutralize via contact with either or both hot spots. V1D3 (bin 9) is interesting because it is competitively inhibited by both IB2 and SWB1. On the basis of this profile, V1D3 would be expected to make contact with \(\alpha\)-helices C and G and/or the C-terminal portion of \(\alpha\)-helix D. Its neutralizing activity may therefore be due to contact with \(\alpha\)-helix D and/or proximity to the F-G loop. Alternatively, it may represent a third neutralizing hot spot associated with \(\alpha\)-helix C.

The story is more complicated, however, because even among VHHs with identical competition profiles there were often disparities in TNA that are explained only partially by binding affinities \((K_D)\). In bin 2, for example, JIV-F5, JNM-B5, and JNM-G4 have identical competition profiles and similar binding affinities (0.02 nM, 0.06 nM, and 0.128 nM, respectively) but very different neutralizing activities; JNM-B5 lacks TNA, JNM-G4 has weak TNA, and JIV-F5 has strong TNA. On the basis of the six reported structural epitopes in this area of RTA (17, 18), we hypothesize that JNM-B5 engages solely with \(\beta\)-strand h and not with \(\alpha\)-helix D or \(\alpha\)-helix B. CDR3 of JIV-F5 is relatively short (16 residues) and contains a disulfide bond between Cys50 and Cys97 that renders it spatially constrained; we previously showed that its short length and constrained conformation prevent the formation of an intermolecular \(\beta\)-sheet with \(\beta\)-strand h (17). In contrast, JNM-B5 and JNM-G4 have longer CDR3 sequences (19 and 22 residues, respectively) that are devoid of the cysteine residues necessary for disulfide bond formation. In this respect, JNM-B5 and JNM-G4 resemble JIY-G11 (PDB ID: 4LHJ), JIY-A7 (PDB ID: 4LHQ), and JIV-G12 (PDB ID: 4LGS), whose unconstrained CDR3 elements form strand-strand interactions with \(\beta\)-strand h. However, definitive conclusions about the actual contact points for JNM-B5 and JNM-G4 on RTA await HX-MS and/or X-ray crystal structure analysis.

Epitope localization of toxin-neutralizing and nonneutralizing RTB- and ricin holotoxin-specific VHHs.

We next turned our attention to the 20 VHHs isolated from the HobJo library that were RTB or holotoxin specific and that could bind to soluble ricin. Using a sandwich ELISA with five benchmark anti-RTB MAbs (see Table S4 in the supplemental material), four RTA-specific cluster II MAbs (SyH7, PA12, PH12, and TB12), and the surrogate receptor, ASF, we tentatively localized the epitopes recognized by 16 of the RTB- and holotoxin-specific VHHs (Fig. 1C and 7; see also Fig. S8 and Data Set S1). Four VHHs were unaffected by the panel of MAbs used in the sandwich ELISA, indicating that they recognize epitopes situated outside our existing panel of MAbs.

The 16 RTB- and holotoxin-specific VHHs constituted 12 different bins, based on their competition profiles in the sandwich ELISA. The 11 VHHs in bins 1 to 7 shared the common trait, in the sandwich ELISA, of being inhibited by PA1 and SyH7, a finding that has important implications for epitope localization on RTB. SyH7 and PA1 each make contact with RTA near the interface with RTB’s subdomains 2\(\alpha\) (residues 148 to 183) and 2\(\gamma\) (residues 228 to 262) (Fig. 3) (53). We would predict, therefore, that all 11 VHHs in bins 1 to 7 recognize epitopes within RTB’s subdomains 2\(\alpha\) and/or 2\(\gamma\) and that the observed competition with SyH7 and PA1 in the sandwich ELISA is due to steric hindrance. VHHs in bins 1 to 3 would be predicted to bind epitopes abutting the RTA-RTB interface, based on the observed competition with TB12 and PH12, whose epitopes are considerably “north” of the border. In fact, V5D1, V5G12, and V1B4 (comprising all of bins 1 and 2) bind ricin holotoxin, but not the individual subunits,
indicating that they recognize quaternary epitopes that straddle the RTA-RTB interface. Finally, based on the observed competition with ASF, V11-Hs in bins 5 and 6 are predicted to have epitopes situated in close proximity to (or even overlapping) the Gal/GalNAc recognition element in subdomain 2\(y\) (Fig. 3). Elsewhere, we report results of extensive cross-competition studies performed to refine the epitopes on RTB’s domain 2 recognized by the 11 V11-Hs in bins 1 to 7 (A. Y. Poon, D. Vance, Y. Rong, D. Ehrbar, and N. Mantis, unpublished data).

Bins 8 to 12 each contained a single V11-H. On the basis of competition with SytH3 and JB4, we would propose that V5H6 (bin 8) and V5D5 (bin 10) also recognize epitopes in RTB domain 2. Moreover, the fact that V5D5 is unable to recognize R-ASF suggests that its epitope is occluded upon RTB-receptor interaction and is therefore in close proximity to the Gal/GalNAc recognition domain but on the opposite side from V11-Hs in bins 5 and 6. In contrast, V5C4 (bin 11) and V5B1 (bin 12) were each competitively inhibited from binding to ricin by 24B11, which is postulated to recognize RTB subdomain 1 (34–36). V5B1 was also unable to recognize R-ASF, presumably because its epitope is near the domain 1 Gal-specific binding element (5). We recently identified three additional MAbs specific for RTB’s domain 1, although they were not included in this study (37).

Among the 20 RTB- and holotoxin-specific V11-Hs isolated from the HobJo library, four were classified as having strong TNA, one moderate TNA, and three weak TNA (Fig. 7 and Table 2). The four V11-Hs with strong TNA had \(K_d\) values of <110 pM and were located in bins 3 (JIZ-B7), 4 (VSE4 and VSG1), and 5 (V2C11). They are also defined by competition with the two anti-RTA MAbs, SytH7 and PA1. On the basis of their competition profiles, we hypothesize that JIZ-B7, VSE4, VSG1, and V2C11 recognize a neutralizing hot spot at the RTA-RTB interface. Two V2C11 family members, V2D4 and V5H2, are also predicted to target this hot spot but have only weak TNA, probably because of their relatively low binding affinities.

Along those same lines, it should be underscored that VSE1 and JIZ-B7 have identical competition profiles, even though VSE1 is classified as an RTA-specific antibody and JIZ-B7 as an RTB-specific antibody (Fig. 5 and 7). Had we conducted the epitope binning studies with ricin holotoxin rather than the individual RTA and RTB
subunits, V5E1 and JIZ-B7 would theoretically have been in the same bin. We speculate that JIZ-B7 recognizes a quaternary epitope that straddles the RTA-RTB border, possibly even in a configuration that mirrors V5E1 (16).

**DISCUSSION**

In this study, we sought to advance our longstanding efforts to generate a comprehensive B cell epitope map of ricin toxin with a focus on localizing the epitopes recognized by toxin-neutralizing antibodies. Ricin’s enzymatic subunit (RTA) is of particular interest to the biodefense research community as two recombinant RTA-based vaccines, RiVax and RVEC, are being evaluated in preclinical and clinical studies (38–40). Predicting the efficacy of these two vaccine candidates in humans is challenging, because neither the anti-ricin serum IgG titer nor the TNA is necessarily a reliable predictor of protective immunity (41). However, there is evidence that epitope-specific antibody responses may be more useful measures in this regard (42). The availability of a high-resolution epitope map of RTA would enable a targeted analysis of specific antibody responses following vaccination, possibly even resulting in the identification of a serum antibody profile that correlates with immunity. Understanding the antibody response to RTA is also relevant to the development of RTA-based immunotoxins, whose efficacy (in some instances) has been limited by the onset of one or more toxin-neutralizing antibodies that render the immunotoxin inactive (43, 44).

We estimate that the current collection of 68 VHHs described in this study covers slightly more than 50% of the surface area of RTA, with a particular bias toward two immunodominant regions of RTA, referred to as epitope clusters I and II (20). Areas not covered in the current VHH repertoire include the entire “backside” of RTA (Fig. 3, right panel; see also Fig. S1 in the supplemental material). In terms of RTB, we identified an aggregate of neutralizing epitopes localized at the RTA-RTB interface positioned in close proximity to the Gal/GalNac recognition element of RTB’s domain 2 γ (5). In a separate report, we refer to this region of RTB as “supercluster II” and demonstrate that toxin-neutralizing activity is associated with antibody binding in the vicinity of the RTA-RTB interface (as manifested through competition ELISA performed with SyH7 and V5E1) rather than with obstruction of the Gal/GalNac recognition element (A. Y. Poon, D. Vance, Y. Rong, D. Ehrbar, and N. Mantis, unpublished data). This interfacial region of ricin toxin is also apparently the target of a number of holotoxin-specific VHHs (e.g., VSD01) whose epitopes likely involve residues on both sides of the border. Unfortunately, our current report does not address the mechanism(s) by which the supercluster II antibodies neutralize ricin. We can only speculate that they interfere with a region of the toxin that is required for retrograde transport from the plasma membrane to the ER and/or retrotranslocation from the ER to the cytoplasm (45–48).

Nonetheless, our results are consistent with there being two putative neutralizing hot spots on RTA, with “hot spot” being defined as a cluster of closely spaced epitopes recognized by antibodies with potent toxin-neutralizing activity. The first hot spot encompasses RTA’s α-helix B and α-helix D. The importance of α-helix B as a target of neutralizing antibodies has been recognized for more than 2 decades (22, 49), although it is only recently that the critical holdfasts have been identified (16, 18, 21, 26, 32). The second hot spot (as noted above) is located at the RTA-RTB interface, defined by RTA’s α-helix F and the F-G loop and (possibly) RTB’s subdomains 2α and/or 2γ (16). At present, it is not clear why these regions render ricin vulnerable to antibody attack, although evidence is mounting that neutralization occurs intracellularly rather than extracellularly (13, 45–47).

The HobJo VHHs described here should be a valuable resource for the ricin toxin research community and may have important applications not only for vaccine development but also for diagnostics and therapeutics. For example, Turner et al. recently showed that pairing a llama-derived anti-ricin VHH (D12fneg) with a modified version of alpaca-derived VHH JIV-F6 (F6m+ +) yielded a highly sensitive assay that could detect less than 1 ng/ml of ricin (12, 50). Here we have described two new JIV-F6 family members, V1B10 and V1D3, each with higher affinities for ricin than JIV-F6. In another
application, Legler et al. used JIV-F6, JIV-D10, and JIV-E1, along with several of their own anti-ricin V_{HH}, to show that the ability of a V_{HH} to increase the melting temperature of RTA correlated with its neutralization ability (26). The availability of dozens of additional V_{HH} coding sequences, affinities, and TNAs (Table 2) enables those types of analyses to be extended. Finally, the V_{HH}s are amenable to recombinant engineering and may provide a platform for therapeutic development. We demonstrated, for example, that select V_{HH} heterodimers effectively aggregate ricin in solution and inactive the toxin in vitro and in vivo (12–14). With the available epitope maps derived from the current study, it is now possible to rationally design different V_{HH} bispecific antibodies with appropriate linkers intended to target notable hot spots on RTA and RTB.

MATERIALS AND METHODS

Ricin-specific V_{HH} phage display library. The single-domain-antibody (V_{HH}) library has been described previously (12). The library is referred to as “Hoblo” here in reference to the names of the two immunized alpacas, Hobbs and Johnnes. The V_{HH}-pIII phagemid library was maintained in E. coli TG1 at –80°C. The library was subjected to nine different screens, as described in Table 1 and in Results. Each screen consisted of low-stringency (round 1) and high-stringency (round 2) panning procedures in which target antigen was present at 10 μg/ml and 1 μg/ml, respectively. To begin each screen, an aliquot of the library was used to inoculate Super Broth (SB) medium supplemented with 100 μg/ml ampicillin and 2% glucose. The culture was grown at 37°C to an absorbance at 600 nm (OD_{600}) of ~0.6 to ~0.8 and then infected with VCSM13 helper phage carrying kanamycin resistance (Agilent Technologies, Santa Clara, CA). After a 2-h infection at 37°C, the bacteria were pelleted at 8,000 rpm, reconstituted in SB medium supplemented with ampicillin (100 μg/ml), kanamycin (70 μg/ml), and 0.1% glucose, and incubated overnight at 30°C. The next morning, the bacteria were pelleted by centrifugation and discarded. The resulting supernatants containing V_{HH}-displaying phages were precipitated with 1:5 (vol/vol) 20% polyethylene glycol (PEG 8000)–2.5 M NaCl at 4°C for 2 h. The supernatant was subjected to centrifugation at 8,000 rpm for 20 min to pellet the phages, which were then resuspended in phosphate-buffered saline (PBS) and used for round 1 of panning. Further phage panning protocols are presented in detail in the supplemental material. Chosen V_{HH} were expressed as fusion proteins with an N-terminal thioepitope tag, a linker containing a hexa-His purification tag, and a C-terminal epitope E-tag (GAPVP YPDPLEPR) in E. coli Rosetta-gami 2(DE3)pLacI (EMD Millipore, Billerica, MA) as previously described (14, 15) and presented in detail in the supplemental material.

Peptides. Biotin-tagged peptides E12 (HPDNLQAEAEITHLFTDV) and G3 (IEGEMRTRIRYNRRSAPD) used in screen 7 were synthesized by Genemed Synthesis, Inc. (San Antonio, TX).

ELISA. For cross-competition sandwich ELISAs, Nunc microtiter plates were coated with competitor capture MABs (1 μg/ml in PBS) or ASF (4 μg/ml in PBS) overnight at 4°C and then blocked for 2 h with 2% goat serum–0.1% Tween 20 phosphate-buffered saline (PBST). Ricin (1 μg/ml), RTA (0.5 μg/ml), or RTB (0.5 μg/ml) was then captured by the MABs or ASF and probed with V_{HH} analytes at 330 nM. Bound V_{HH}s were detected with an anti-E tag–horseradish peroxidase (HRP) secondary antibody (Bethyl Labs, Montgomery, TX) and developed with SureBlue TMB (3,3′,5′,5′-tetramethylbenzidine) substrate (KPL). After quenching was performed with 1 M phosphoric acid, absorbance was read at 450 nm on a VersaMax microplate reader ( Molecular Devices, Sunnyvale, CA).

Vero cell cytotoxicity assays. Relative IC_{50}s were determined in Vero cell cytotoxicity assays, as previously described (28). Vero cells were detached from culture dishes with trypsin, adjusted to ~5 × 10^6 cells per ml, seeded (100 μl/well) into white 96-well plates (Corning Life Sciences, Corning, NY), and allowed to adhere overnight. The cells were then treated with ricin (0.01 μg/ml; 154 pM), ricin-V_{HH} mixtures, or medium alone (negative control) for 2 h at 37°C. The cells were washed and then incubated for 48 h at 37°C, after which cell viability was assessed using CellTiter-GLO (Promega, Madison, WI). All treatments were performed in triplicate. Relative IC_{50}s were determined following published guidelines in which 100% viability was derived from cells treated with medium only, while 0% viability was defined as the average value obtained from ricin-treated cells (51). Reported relative IC_{50}s for each V_{HH} were derived from at least three independent toxin-neutralizing assays (each done in triplicate).

SPR and affinity determinations. V_{HH} association and dissociation rates for ricin toxin were determined by surface plasmon resonance (SPR) using a ProteOn XPR36 system (Bio-Rad Inc., Hercules, CA), as described previously (18, 37). For ricin immobilization, general layer compact (GLC) chips were equilibrated in running buffer (PBS–0.005% Tween) at a flow rate of 30 μl/min. Following EDAC [N-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride] (200 mM)–sulfo-NHS (N-hydroxysulfosuccinimide) (50 mM) activation (3 min), ricin was diluted in 10 mM sodium acetate (pH 5.0) at either 4 μg/ml or 2 μg/ml and coupled for 2 min. A third vertical channel received only acetate buffer and served as a reference channel. The surfaces were deactivated using 1 M ethanolamine for 5 min. A ProteOn array system multichannel module (MCM) was rotated to the horizontal orientation for affinity determination experiments. Each V_{HH} was serially diluted in running buffer and then injected at 50 μl/min for 180 s, followed by 1 to 3 h of dissociation. After each experiment, the chip was regenerated with 10 mM glycine (pH 1.5) at 100 μl/min for 18 s until the response unit (RU) values had returned to baseline. All kinetic experiments were performed at 25°C. Kinetic constants for the antibody/ricin interactions were obtained with ProteOn Manager software 3.1.0 (Bio-Rad Inc.) using the Langmuir fit model.
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Epitope mapping by hydrogen deuterium exchange–mass spectrometry (HX–MS). The HX–MS method for epitope mapping V_{H} antibodies is described in more detail elsewhere (R. Toth IV and D. D. Weis, unpublished results) (31). For safety reasons, epitope mapping studies were conducted with a nontoxic variant of RTA known as RiVax (52). Briefly, regions of RiVax where the rate of HX was significantly lower (protection) or higher (deprotection) in the presence of bound V_{H} were identified using k-means clustering and significance testing based on time-averaged HX measurements (ΔHX).

However, unlike that previous work, here the results were not filtered for the solvent accessibility of the RiVax residues. Using k-means clustering, the regions that were assigned as showing strong and intermediate protection were used to define the epitope. The RiVax peptides are numbered sequentially from the N terminus to the C terminus and described in Table S5 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00236-17.

SUPPLEMENTAL FILE 1, PDF file, 3.6 MB.
SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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