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COMMENTARY

Protein Structure Facilitates High-Resolution Immunological Mapping

Madison Zuverink, Joseph T. Barbieri

Microbiology and Immunology, Medical College of Wisconsin, Milwaukee, Wisconsin, USA

ABSTRACT Select agents (SA) pose unique challenges for licensing vaccines and therapies. In the case of toxin-mediated diseases, HHS assigns guidelines for SA use, oversees vaccine and therapy development, and approves animal models and approaches to identify mechanisms for toxin neutralization. In this commentary, we discuss next-generation vaccines and therapies against ricin toxin and botulinum toxin, which are regulated SA toxins that utilize structure-based approaches for countermeasures to guide rapid response to future biothreats.

KEYWORDS botulinum toxin, RTA, RV*Ec*, RiVax, antibodies, mass spectrometry, ricin

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Address correspondence to Joseph T. Barbieri, jtb01@mcw.edu.

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This commentary addresses studies by Mantis and coworkers, who in this issue of *Clinical and Vaccine Immunology* describe the continued mapping of the neutralizing epitopes within ricin toxin (RTX) and relate these studies to current research on the botulinum neurotoxins (BoNTs) (1, 2). Our goal is to provide a perspective on how protein structure has facilitated development of vaccines and therapies against regulated select agent (SA) toxins. An overview of the properties of ricin toxin and botulinum neurotoxin is presented in Fig. 1. Ricin toxin and the botulinum neurotoxins are HHS and USDA Select Agents and Toxins (7 CFR part 331, 9 CFR part 121, and 42 CFR part 73).

AB toxin structure/function. AB toxins contain modular domains with distinct functional activities (3). The A domain encodes an enzymatic activity which targets a host protein to modulate host cell physiology, often inactivating a biological process within cells, leading to cell death or occasionally enhancing the action of a cellular process. The B domain often binds a host receptor, such as a protein or glycolipid, via interactions that bind directly to a specific host receptor or that bind a carbohydrate present on a glycosylated host receptor. AB toxins are often synthesized as single-chain proteins in an inactive form and are converted to an active di-chain by proteolysis between the A and B domains, which remain connected by an interchain disulfide (4, 5).

RTX is a category B bioterrorism toxigenic lectin enriched in seeds of the castor bean plant *Ricinus communis*. RTX is posttranslationally processed by host glycosylation and activated by cleavage between A and B domains (6, 7). The ricin toxin A domain (RTA) is a type II ribosome-inactivating protein with N-glycosidase activity, which depurinates 28S rRNA within the 60S host ribosome (8). Depurination stalls protein synthesis and induces a ribotoxic stress response (9). Ricin toxin B domain (RTB) contains at least two homologous lectin binding sites specific for galactose and binds galactose-containing lipids and glycoproteins (10). While binding of galactose results in nonproductive uptake of RTX, binding to mannose moieties on host mannose-containing receptors results in RTX retrograde trafficking to the endoplasmic reticulum, where RTA is delivered into the cytosol to target the ribosome (11). RTX exploits receptors on multiple cell types; therefore, the symptoms vary based on the route of intoxication and include pulmonary edema, respiratory distress, low blood pressure, seizures, and vomiting (12).

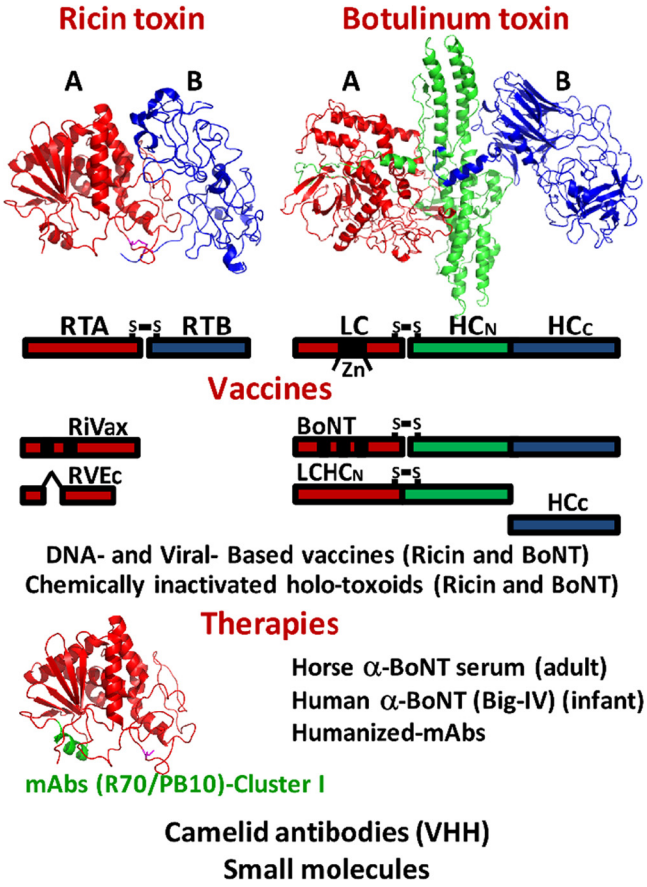

FIG 1 Ricin toxin and botulinum toxin: structure-function organization and vaccines and therapies under investigation. (Upper panel) Crystal structures of ricin toxin (PDB: 2AAI) and botulinum toxin serotype A (PDB: 3BTA) are shown. The A domains (catalytic domains; red) and B domains (binding domains; green and blue) are indicated. The schematics represent the following: ricin toxin (RTA, 267 amino acids [aa]; disulfide linked to RTB, 262 aa); botulinum toxin (LC, 443 aa; disulfide linked to HC [HC_N], 448 to 872 aa; HC_C, 877 to 1,295 aa). (Middle panel) Vaccines. Ricin protein-based vaccines, including RiVax and RV*Ec*, are RTA derivatives modified for optimized production and attenuation and appear to have similar potencies in rodents and nonhuman primates, while viral and chemically inactivated holotoxoid vaccines are less extensively developed. BoNT protein-based vaccines include catalytically inactive BoNT and $LCHC_N$ (due to potency) and HC_C (due to ease of production), along with chemically inactivated holotoxoid vaccines. A bivalent (A/B) HC_C derivative is currently in clinical trials; numerous DNA and viral vectors express HC_C. (Lower panel) Therapies. Several ricin antibody-based MAbs and, subsequently, camelid (V_HH) antibodies have been identified that neutralize ricin within RTA in four distinct clusters. Cluster I comprises 97 to 105 aa, where two neutralizing MAbs (R70/PB10) bind. Camelid therapies and small-molecule inhibitors have been developed for ricin and botulism therapies. Current approved botulism antibody therapies include treatment of adult botulism with pooled horse α-BoNT(ABCDE) serum, while infant botulism is treated with human botulism immune globulin intravenous (Big-IV).

BoNTs are category A bioterrorism agents produced by *Clostridium botulinum*, or by related clostridia, which comprise seven serotypes (namely, serotypes A to G) defined by serotype-specific neutralizing antibodies (Abs) (13). BoNTs are activated by proteolysis. The B domain (HC) contains a receptor binding domain (HC_C) and a translocation domain (HC_N). BoNTs bind dual neuronal receptors: a sialic acid-decorated glycolipid known as a ganglioside and a synaptic vesicle protein or a second ganglioside (14–20). BoNTs enter neurons via synaptic vesicles where HC_N facilitates pH-dependent delivery of the catalytic A domain (light chain [LC]) into the cytosol (21). LCs are Zn^{2+} metalloproteases which target SNARE-family vesicle fusion complex proteins involved in neurotransmitter release. During translocation, the intact interchain disulfide is reduced to facilitate LC delivery into the host cell cytosol to target the SNARE substrates within peripheral motor neurons (5, 21, 22). The proteolysis of SNARE substrates inhibits

acetylcholine release at the neuromuscular junction, a requirement for muscle contraction. This results in the flaccid paralysis of botulism (23).

Structure-based analysis and complementary methods. Design of effective vaccines requires mounting a protective immune response to epitopes within the toxin which stimulates the production of neutralizing antibodies. Serum-derived antibodies are characterized by antigen specificity and affinity and by neutralization of the protein toxin. Biochemical assays, namely, the enzyme-linked immunosorbent assay (ELISA), immunoblotting, and surface plasmon resonance (SPR) analysis, have been used to demonstrate immune response, antigen reactivity, and binding affinity. More recently, structural techniques such as X-ray crystallography and, as described in the studies reported in this issue of *Clinical and Vaccine Immunology* by Mantis and coworkers, hydrogen-deuterium exchange mass spectrometry (HD-X MS) (1, 2) have been used to resolve antibody and antigen complexes (24). The shift from methods that predominantly identify linear epitopes via denatured antigens or small peptides to native, conformational epitopes limits artifacts that arise in solid-phase binding of antigen, as noted by Mantis and coworkers. Additionally, competition ELISA is complicated by steric hindrance and exposure or masking of epitopes in the solid-phase assays. The use of antigenic peptides as screens employing phage or solid-phase assays can also be problematic, since the peptide conformation may not resemble the epitope (25, 26).

The availability of several antibody-antigen structures revealed by X-ray crystallography and provided by Mantis and coworkers permitted structure-based analysis of antibody-antigen complexes, including analysis of the molecular interactions within individual epitopes. Additionally, classification of indirect, structural residues and those involved in direct binding has illuminated interactions involved in neutralization (24). Understanding antibody-antigen contacts at the molecular level will ultimately aid in the engineering of antibodies of higher affinity to their antigens, which has been correlated with neutralization (27, 28) On the other hand, crystal structures of antibody-antigen complexes are not always available and can limit the solid-phase binding analysis to a static snapshot of a complex. Furthermore, molecular contacts do not resolve affinity or predict neutralization. When available, antigen structures can be used effectively to model and interpret both affinity and neutralization data.

Previously, peptide arrays, competitive ELISA, and phage display have been utilized to map B cell epitopes on ricin (29, 30). These methods focused on primary sequences or linear epitopes, while other mapping strategies such as alanine-scanning mutagenesis required cautious interpretation due to potential secondary structure disruption (24). Mantis and coworkers utilized HD-X MS to identify dominant epitopes within ricin for serum analysis of antibody response using competitive ELISA. The four epitope clusters identified by murine monoclonal antibodies (MAb) were further assigned as neutralizing epitopes by employing a library of camelid V_HH antibodies, again using the structure of a subset of these V_HH antibodies in complex with RTA solved by X-ray crystallography as a reference (31, 32). HD-X MS defines epitope regions via detection of a decreased rate of exchange of deuterium for amide hydrogens in the presence of antibody. Though the rate of exchange depends on many factors, identification of tryptic peptides with reduced exchange can lead to identification of broad epitopes. HD-X MS validated X-ray crystallographic data in the camelid study; however, the authors acknowledge that interpretation of intermediate protection was complicated, as one MAb, PB10, affected deuterium exchange on areas distal to the epitope when mapped to the crystal structure. This underscores the importance of utilizing multiple methods to assess protection and to map antigenic regions.

For the botulinum toxins, epitope mapping studies have used single-chain variable-fragment phage libraries which recognize native structure, MAbs, and solid-phase ELISA to confirm reactivity while using competition with short peptides to confirm domain specificity (33–35). One study compared BoNT/A, which contained such a structure, alone or in complex with nontoxic nonhemagglutinins, which are accessory proteins that lacked a crystal structure. The epitopes predominantly mapped to LC and HC_N

when BoNT/A was in complex, which is in agreement with current models of the BoNT complex (36, 37), which have shown the HC_C to be shielded. The isolated translocation domain of the botulinum neurotoxin subunit also contains multiple epitopes, demonstrating the potential for multiple mechanisms of neutralization (35).

Subunit vaccines versus whole-protein vaccines. Factors considered in vaccine design include ease of production, protein stability, potency of the protective immune response to toxin challenge, and potential off-target toxicity. As separation of AB toxins into either the A domain or the B domain results in atoxic fragments, the idea of the use of subunit vaccines is attractive to minimize risks to hosts, and minimal immunogenic domains may facilitate protein production. Mantis and coworkers used the RTA-derived vaccine RiVax to map ricin-neutralizing MAbs. RiVax elicits an antibody response in humans, mice, and macaques and protects macaques from challenge with a lethal dose of aerosolized ricin (38). Note that RTA was demonstrated to be more protective than RTB in active immunization and passive protection in mice and in cellular assays (39). The failure of the RTB vaccine to neutralize RTX is surprising; however, blocking toxin binding and entry is only one of several mechanisms that can neutralize toxin action (40, 41).

Previous studies identified neutralizing epitopes against BoNTs that were present in the receptor binding domain. Additionally, vaccination with HC_C protects against challenge with holotoxin in one-dose vaccination (42). On this principle, an HC_C subunit vaccine directed toward seven serologically unique botulinum toxins, toxins A to G, protected immunized mice from challenge with homologous botulinum holotoxins (43). On the other hand, $LCHC_N$ has also proved to be a potent neutralizing vaccine (44). The use of subunit vaccines has shown mixed effectiveness against heterologous BoNT subtypes. For example, high-dose vaccination was required to protect against challenge with heterologous BoNT/A subtypes (45). Vaccination with a holotoxin may require fewer vaccinations if neutralizing epitopes are present in multiple domains. A recent study demonstrated that BoNT holotoxins are more potent vaccines than their subunit vaccine equivalents (46).

Improving the safety of vaccines against ricin and botulinum also focused on engineering mutations within the toxin subunits. RiVax and RV*Ec* are composed of atoxic ricin catalytic domains whose sequences extend through residue 267 and are truncated after residue 199, respectively. An additional mutation within a tripeptide (L74-D75-V76) at V76M in RiVax eliminates symptoms associated with vascular leak syndrome without disrupting structural integrity, while RV*Ec* was engineered to remove a hydrophobic loop and was truncated to stabilize the protein (47). For the subunit vaccines utilizing HC_C of BoNT/A, a mutation in the receptor binding pocket (W1266A) retained vaccine potency, showing that receptor binding function is not required to maintain vaccine potency (48). Thus, utilizing protein structure and function allows the use of reverse genetic approaches to optimize vaccine potency.

Chemically inactivated formalin toxoid vaccines continue to be used to neutralize diphtheria toxin and tetanus toxin. This strategy was used for botulinum toxoids, but long-term storage resulted in decreased potency and removal of the vaccine from application. Catalytically inactive holotoxins and subunits produced in *Escherichia coli* and yeast have gained favor due to their ease of purification and engineering (49). Ricin toxoids have demonstrated protection in mice but, given concerns over reversion of cytotoxic effects and delivery, would benefit from recombinant production (50). Analysis of BoNT/A in complex with nontoxic nonhemagglutinin resulted in the mapping of 44 single-chain variable fragments from a phage library of the complex. In this analysis, 2 were identified in the receptor binding domain, 15 in the light chain, and 3 in the translocation domain. Although not focused on identification of neutralizing epitopes, that study supported the idea of the utility of recombinant holotoxin vaccines.

Neutralizing versus nonneutralizing serum. Eradication of many communicable diseases has relied on both an effective vaccine and a campaign to target susceptible populations. The primary approaches have included vaccination and population mon-

itoring; however, diseases resulting from ingestion of seeds of the castor bean plant or toxigenic clostridial species are not considered to represent transmittable infectious diseases. Human exposure to ricin or botulinum toxin is uncommon and is primarily due to ingestion of contaminated foodstuffs. Thus, strategies for effective vaccines and therapies anticipate targeting populations at risk.

Detection of serum antibodies corresponding to purified antigen can be readily performed using ELISA. However, for RTX and BoNT, the ELISA titer does not correspond to neutralization capacity, especially in comparisons between serotypes. For this reason, animal model systems have been utilized to correlate protection data for both toxins. While recombinant ricin vaccines generate an immune response and show promise as vaccine candidates in animal models, screening of B cell hybridoma libraries generated from immunized mice indicated <10% neutralizing antibodies (51). The lack of correlation between detection of an immune response and the presence of neutralizing antibodies necessitates understanding how to shift this balance in humans. As a result, attempting to predict protection in an immunized human group by antibody reactivity alone is not likely to prove helpful. In contrast, the use of tetanus toxoid vaccine has almost completely eradicated tetanus in developed nations (52). Found in soil, *Clostridium tetani* is a prevalent cause of maternal and infant mortality due to spastic paralysis in most of the unvaccinated developing world (53). The toxoid was created before a structure of tetanus toxin was available for use in conducting structure-based analysis; however, tetanus toxoid has proven to be effective because it elicits a protective response. This correlation between protection and the presence of antibody reactivity in immunized populations has allowed accurate monitoring and disease eradication in many developing countries (54).

Overall, access to protein structure provides a reference point for integrating affinity and mapping data to accurately locate binding sites for neutralizing antibodies within a targeted protein toxin, as described by Mantis and coworkers, in comparison to the results previously possible using static approaches such as competitive solid-phase binding assay. We anticipate the utilization of additional high-resolution approaches to continue to enhance dissection of the molecular aspects of host-pathogen interactions beyond that of an antibody-antigen interaction with the continued enhanced efficiency of structural biological approaches, including cryo-electron microscopy (55) and structure-based protein informatics (56), as well as development of novel molecular tools such as camelid-based antibodies (57, 58).

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