Published in final edited form as:

Expert Rev Vaccines. 2008 February ; 7(1): 55–74. doi:10.1586/14760584.7.1.55.

Analysis of epitope information related to *Bacillus anthracis* and *Clostridium botulinum*

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Financial & competing interests disclosure

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Abstract

We have reviewed the information about epitopes of immunological interest from *Clostridium botulinum* and *Bacillus anthracis*, by mining the Immune Epitope Database and Analysis Resource. For both pathogens, the vast majority of epitopes reported to date are derived from a single protein: the protective antigen of *B. anthracis* and the neurotoxin type A of *C. botulinum*. A detailed analysis of the data was performed to characterize the function, localization and conservancy of epitopes identified as neutralizing and/or protective. In order to broaden the scope of this analysis, we have also included data describing immune responses against defined fragments (over 50 amino acids long) of the relevant antigens. The scarce information on T-cell determinants and on epitopes from other antigens besides the toxins, highlights a gap in our knowledge and identifies areas for future research. Despite this, several distinct structures at the epitope and fragment level are described herein, which could be potential additions to future vaccines or targets of novel immunotherapeutics and diagnostic reagents.

Keywords

anthrax toxin; *Bacillus anthracis*; botulinum toxin; *Clostridium botulinum*; epitope; therapeutic antibodies; vaccine

Bacillus anthracis and *Clostridium botulinum* are Gram-positive soil-dwelling bacteria. *B. anthracis* is a relatively common veterinary pathogen that has emerged recently as a major biological weapon. By contrast, *C. botulinum* does not cause invasive disease but produces a potent neurotoxin that has both therapeutic and weapon potential [1,2]. Given their potential importance as biological weapons, there is intense interest in the generation of vaccines, passive antibody therapies and immune-based diagnostic strategies against both *B. anthracis* and the *C. botulinum* toxin [1-14]. Information related to immune epitopes can be used to advance our understanding of disease pathogenesis and host immunity, as well as applied to the development and characterization of therapeutics and diagnostics, including the establishment of reliable correlates of protection to monitor their performance. This review aims to provide a comprehensive panorama on the current status of knowledge specific to epitopes from these pathogens and to identify areas where additional study is needed.

Bioinformatic tools have greatly enhanced our ability to both catalog and analyze vast amounts of scientific data. The performance of meta-analyses from the data can facilitate in-depth evaluation of existing knowledge in a particular area of research [15]. The Immune Epitope Database and Analysis Resource (IEDB) [101] is a project that is hosted by scientists at the La Jolla Institute for Allergy and Immunology with support from the National Institute of Allergy and Infectious Diseases, a part of the US NIH. The goal of the IEDB is to compile and present immunological data and analysis tools through a single interface [16]. The scope of the IEDB encompasses structures that are targets of adaptive immunoreceptors of humans and other vertebrates (i.e., of epitopes that are reasonably defined in structural terms). More specifically, the IEDB focuses on the inclusion of peptidic B- and T-cell epitopes, either linear or conformational, of less than 50 residues in length and of nonpeptidic epitopes that are less than 5000 Da [17].

The database is populated with reports from the published literature, as well as by direct submission from epitope discovery groups, and contains an extensive collection of B- and T cell epitopes, as well as MHC binding structures, and a detailed annotation of the immunological context in which each epitope was defined (immunized/infected species, source organism and antigen of the epitope, and experimental techniques). Moreover, the compiled and standardized data can then be further evaluated using several analysis tools available at the IEDB website, as part of the Analysis Resource.

Based on the contextual information provided, the epitopes can be classified for their biological function as antigenic, immunogenic, neutralizing and/or protective (Box 1). Epitopes were excluded from this analysis if they only had MHC binding data, negative data, or data from assays where the same structure is used for immunization and detection, for these epitopes cannot be classified in the aforementioned categories.

This study includes all *B. anthracis* and *C. botulinum* epitopes identified as of 30 June 2007. The current epitope knowledge for these pathogens is highly focused on a small subset of antigens and is significantly biased towards antibody epitopes. However, an overview of the epitope information is provided for both B- and T-cell epitopes. In addition, a detailed analysis of the functionality and location of neutralizing and protective immune epitopes is provided.

Basic features of *C. botulinum* & *B. anthracis* pathogenicity & toxicity

B. anthracis and *C. botulinum* are facultative and strictly anaerobic bacteria, respectively and are related. They can each form highly resistant spores that, upon germination, produce toxins. As in other bacterial toxins, these toxins conform to the A/B toxin model, where the cell internalization functions ('B component') are on separate subunit(s) from the toxic enzymatic activity ('A component') [18].

Inhalation of *B. anthracis* spores can result in fulminant pulmonary syndrome that is associated with high mortality, as was shown during the bioterrorism attacks of 2001. *B. anthracis* has two major virulence factors, the anthrax toxin(s) and the poly-D-glutamic-acid capsule. These virulence factors are encoded by two plasmids, pX01 and pX02, and bacterial strains lacking either plasmid are significantly attenuated in virulence. Anthrax toxins are produced upon internalization and germination of spores in macrophages, from where the germinative form of the bacilli disseminates [12]. Although the exact relationship between toxin production and the fulminant sepsis that follows the inhalation of anthrax is unknown, there is general consensus that toxin production is critical for *B. anthracis* pathogenesis, which is supported by the observation that toxin-defective mutants are hypovirulent and that toxin-neutralizing antibodies correlate with protection against disease. The toxin B-component is named protective antigen (PA) because antibodies to this protein mediate protection in animal models. The complete PA molecule, known as PA83, has to undergo cleavage of its N-terminal domain, PA20, by host proteases to become the active, pore-forming heptamer of PA63 molecules [19]. The toxic A-components lethal factor (LF) and edema factor (EF) can then associate with the PA prepore, which subsequently inserts in the target cell membranes producing the pore through which EF and LF gain access to the cytosol and effect their toxicity. LF is a metalloprotease that targets the MAPK family of proteins. EF has Ca²⁺- and calmodulin-dependent adenylyl cyclase activity that affects the endogenous cellular cAMP levels. Hence, the term 'anthrax toxin' involves a cellular binding/transporter molecule (PA) and cytotoxic components (LF and EF). Both EF and LF can associate simultaneously with a PA heptamer to affect the target cell at both proximal and distal parts of the signal transduction pathways. In the case of homogenous LF-PA and EF-PA associations, the toxins are called lethal toxin (LeTx), and edema toxin, respectively [18, 19]. In addition, *B. anthracis* also produces anthrolysin, a cholesterol-dependent cytolysin that has potent activity *in vitro* [20], although its contribution to the pathogenesis of anthrax is unclear.

Two main types of toxins are found in *C. botulinum* and these are responsible for the clinical syndrome of botulism. The most deadly and common form of botulism syndrome follows ingestion of *C. botulinum*-contaminated food where the spores have germinated and produced botulinum neurotoxin (BoNT). Alternatively, the spores can germinate in the

intestinal tract of infants and in wounds. From a biological weapon point of view, there is enhanced concern that this toxin can be aerosolized to deliver paralytic doses [18,21]. On a molar basis, BoNT is the most toxic agent known to exist, and targets motor neurons leading to flaccid paralysis that impair respiration leading to death through asphyxiation. The toxin is produced as a single-chain protein that is then cleaved into two disulfide-linked polypeptide chains: an A component, the light chain, where the proteolytic activity resides, and a B component, the heavy chain, involved in cell binding and internalization [18]. Seven different serotypes (A–G) exist, which differ in their specificity for cellular receptors and in the intracellular targets of the toxin protease activity. Recently, the long-proposed hypothesis of dual receptors for this toxin was validated by demonstrating that BoNT/B needs to bind to both cell-surface gangliosides and a protein receptor to mediate toxin internalization [22]. Once inside the motor neuron, the proteolytic activity of the light chain differs among some serotypes but, in all cases, the targets are proteins involved in exocytosis of presynaptic vesicles, therefore inhibiting neurotransmitter release and causing paralysis.

In addition to BoNT, *C. botulinum* produces C2 toxin, where the A and B components are two separately encoded polypeptides, analogous to other clostridial toxins, such as those found in *C. perfringens* and *C. spiroforme* [18,23]. The A component (component I) has ADP-ribosylating activity, with specificity for actin, leading to cytoskeleton disruption and cytotoxicity [23,24]. The B component (component II) has some degree of homology to the *B. anthracis* PA protein and also forms oligomers that bind to cell receptors. This toxin is produced by some strains of *C. botulinum* types C and D, and induces hypotension, increased intestinal secretion, vascular permeability and lung hemorrhage [24].

Inventory of *B. anthracis*- & *C. botulinum*-derived epitopes

Currently, the IEDB contains 15 references [25-39] dealing with B-cell epitopes and four references [40-43] with data on T-cell epitopes derived from *B. anthracis*. The IEDB contains ten references for *C. botulinum* [23,44-52] dealing solely with B-cell epitopes and three references [53-55] containing both T- and B-cell determinants. These numbers are reflective of all the manuscripts containing epitope-related information that could be identified in PubMed up to 30 June 2007.

These references describe a total of 11 *B. anthracis* and 73 *C. botulinum* unique B-cell epitopes. As for T-cell epitopes, seven structures from *B. anthracis* and 42 from *C. botulinum* have been reported (Table 1, Appendix). It is notable that in a few instances the same structure could be assigned to both T- and B-cell epitope categories. These numbers are small compared with other pathogens from the NIAID A, B and C Priority Pathogens list [102]. For example, 176 B-cell epitopes and 692 T-cell epitopes derived from *Mycobacterium tuberculosis*, and 219 B-cell epitopes and 509 T-cell epitopes derived from Influenza A virus are found within the IEDB. This disparity probably reflects the only recently enhanced interest in *B. anthracis* and *C. botulinum*. Indeed, only two of the 18 epitopes from *B. anthracis* were identified prior to the anthrax bioterrorist attacks of 2001. A systematic analysis of the epitopes contained within both these organisms has not yet been reported in the scientific literature.

The ratio of B- to T-cell epitopes is approximately 1.5:1 for both pathogens, consistent with the notion that antibody responses are the main correlate of protection from toxic syndromes [56]. The known T-cell epitopes from both organisms are exclusively of the CD4 type, and no information is currently available about CDS T-cell epitopes.

In terms of the chemical nature of the epitopes, all the *C. botulinum* epitopes, and all but one of the *B. anthracis* epitopes, are peptidic in nature. The single carbohydrate epitope

described for *B. anthracis* is derived from BclA, the immunodominant antigen of the spore outer coat (exosporium) [10,33,57]. This oligosaccharide contains anthrose, a sugar found, so far, only in *B. anthracis*. To our knowledge, no other nonpeptidic targets for immune recognition have been described in these organisms.

For the peptidic antibody epitopes described, the ratio of linear versus conformational epitopes is clearly in favor of linear epitopes (9:1 for *B. anthracis* and 23:1 for *C. botulinum*). This result probably reflects the relative experimental ease in defining linear B-cell epitopes compared with the identification of conformational epitopes.

Protein antigen sources of the epitopes

B. anthracis is a complex microorganism, whose complete genome has been mapped [58], and encodes approximately 5600 different proteins. However, immune epitopes from a mere five different protein antigens have been described to date (Table 2).

Given the fulminant course of *B. anthracis* pulmonary infection and the mode of action of the toxin, the presence of antibodies to PA, the major active component of the currently licensed vaccine [12,57], is a sought-after correlate of protection. Furthermore, there is considerable interest in the development of anti-PA passive antibody therapy as an adjunct to antimicrobial therapy and for the prevention of disease in individuals at risk for anthrax – at least one therapeutic monoclonal antibody (mAb) to PA is in clinical development [59]. Accordingly, the majority of the peptidic B-cell epitopes reported in the literature are derived from PA. A single epitopic structure has been described for each of the toxin enzymatic components (LF and EF). Besides anthrax toxin components, oligopeptides from the bacillus poly- γ -D-glutamic acid (P- γ -DGA) capsule are targets for antibody recognition. In the case of the T-cell epitopes, seven peptides derived solely from PA have been described.

Future work is required to define epitopes from other candidate antigens that induce protective B- and T-cell responses. For instance, peptidic structures from the BclA glycoprotein and other exosporium proteins are targets of the immune response in vaccinated animals and humans [12,57,60]. Identification of the corresponding epitopes could facilitate studies on the immune response to *B. anthracis* spores and its relationship with the pathogenesis and disease outcome. In this line, it is significant that no information is available on epitopes on anthrolysin.

Similarly, in the case of *C. botulinum*, almost all of the defined immune epitopes are derived from BoNT, with just two epitopes derived from the component II of the C2 toxin. This preponderance in BoNT epitope knowledge is in keeping with the neurotoxin being responsible for botulism syndrome. However, given that over 3400 proteins are encoded by this organism and that *in vivo* spore germination occurs in infant and wound botulism, it seems that additional valuable information could be obtained by studying immune responses associated with those syndromes. Furthermore, BoNT epitopes are described only for *C. botulinum* types A and E and the vast majority of the epitopes are from BoNT/A. No epitopes are described from the other five serotypes of BoNT. This gap in knowledge is of particular concern in the degree of preparation against biological attacks, since each of these toxins has considerable biological weapon potential.

Host distribution of epitope-specific responses

The distribution of epitopes by the organisms in which the specific immune response was detected is shown in Table 3. In this case, since certain epitopes are targeted by more than host, the numbers should not be considered additive.

Most of the epitope-specific immune responses to both *B. anthracis* and *C. botulinum* have been defined in mice, although other species have been studied. In the case of *B. anthracis*, some epitopes have been defined in rabbits and macaques, as well as in vaccinated humans. Clearly, epitopes could be studied in additional species, since veterinary vaccination with the avirulent Sterne strain 34F2, or other Sterne-like strains, is widespread for cattle, and the efficacy of these vaccines is routinely tested in guinea pigs.

Similarly, *C. botulinum* epitope-specific responses have been detected in humans vaccinated with pentavalent toxoid and in individuals inoculated with commercial preparations of BoNT/A for therapeutic purposes; some data also exist from experimental animals, such as chickens, horses and rabbits. A number of epitopes have been described as being recognized across species in mice, humans, chickens and horses (see later). Further definition of epitopes recognized in a diverse set of hosts would be of significant benefit, allowing rigorous comparison of the immune responses and identification of correlates of protection after vaccination of different species, in order to streamline the extrapolation of findings in experimental animal models to human-intended diagnostics and therapeutics.

Functional characteristics of *B. anthracis* epitopes

Of the ten peptidic B-cell epitopes described for *B. anthracis* (Table 1, seven are either targets of neutralizing mAbs, which have demonstrated activity in classic *in vitro* and *in vivo* neutralization assays, or are able to induce a protective response upon immunization. The protective capacity of the remaining three epitopes has not been assessed. As expected, five of the seven neutralizing epitopes described are from PA [27,29,34,35,39], consistent with the fact that this protein is the source from which, overall, the most epitopes have been described to date. Besides these PA epitopes, an LF epitope corresponding to the repeat 4 of domain III is recognized by mAbs that can reduce LeTx-induced cytotoxicity in macrophages *in vitro*. One of these mAbs provides protection from mortality following *in vivo* challenge with LeTx in rats [30]. In addition, γ -DGA oligopeptides from the bacilli-capsule antigen have also been used to generate antibodies with proven opsonophagocytic activity [36,37]. Immune responses directed at bacilli-specific antigens, such as the P- γ -DGA capsule, could be clinically useful by hampering the rapid dissemination of the pathogen in the infected host. However, the immunogenicity of γ -DGA is low, requiring carrier-protein conjugates for an effective induction of this protective response.

T-cell epitopes deriving from *B. anthracis* were defined in a PA-immune mouse and vaccinated humans by analyzing the proliferation and cytokine production of lymphocytes to overlapping peptides. Indeed, antibody responses to PA after vaccination are T-helper (Th) dependent [40] and PA peptides are presented by infected macrophages [43], suggesting that the epitope-specific T cells may be required for mounting a protective immune response.

Localization of *B. anthracis* PA epitopes

Since there have been several different PA epitopes described in different experimental contexts and animal hosts, it was of considerable interest to examine the relative localization of these epitopes, and to analyze whether common features could be discerned. A schematic representation of the PA protein and the epitopes recognized is shown in Figure 1. Figure 1A depicts the location of the B-cell epitopes, using different colors for the epitopes recognized in each host organism. Overall, no B-cell epitopes that conform to our 50-amino acid limit have been described in domain 1. The described epitopes tend to cluster in three discrete regions within the remainder of the protein. Each of these regions is recognized in multiple hosts and by at least one neutralizing antibody responses. These three regions correspond to the 2 β 2–2 β 3 loop of domain 2, the domain 3–domain 4 boundary and the

small loop of domain 4. These regions are indicated in Figure 1D, on the 3D model obtained by x-ray crystallography (pdb 1TZN).

The domain 2 2 β 2–2 β 3 loop region, which mediates membrane insertion, is the target of several neutralizing murine mAbs [27,39]. This region is probably also a target for polyclonal rabbit sera after vaccination, as the antisera can compete with an epitope-specific mAb for binding to PA.

A stretch of 20 residues at the boundary of domains 3 and 4, a region that currently lacks defined functional activity, has been broadly assigned as the epitope for an *in vitro* and *in vivo* neutralizing murine mAb, 2D3 [34]. Interestingly, polyclonal antibodies from vaccinated humans and rabbits were shown to compete with mAb 2D3 for binding to PA, suggesting that this epitopic region is also a target of immune recognition after vaccination [34,61].

Two B-cell epitopes for neutralizing mAbs have been described that localize to the small loop of domain 4, the main region involved in cellular receptor binding: one discontinuous epitope defined in mouse by alanine scanning [35] and a linear epitope defined in the crab-eating macaque *M. fascicularis* [29]. The neutralizing activity of the macaque antibody was confirmed to occur via inhibition of the PA–cell receptor interaction. These results indicate that neutralizing antibodies directed to the receptor binding site may be able to inhibit the first step of toxic activity, thereby reducing the risk of residual toxicity inherent to targeting other toxin activities.

A completely different pattern is apparent in the case of T-cell epitopes (Figure 1B), which are found fairly evenly distributed along the whole PA molecule. Domains 3 and 4 contain murine T-cell epitopes [41–43], while single human T-cell epitopes have been described in domains 1 and 2 [40]. Interestingly, the domain-1 epitopes described for PA-immunized mice and a vaccinated human are located within the PA20 region that is cleaved before heptamerization [19], suggesting that this region is still processed for presentation on MHC class II molecules after vaccination, allowing the immune system to respond to the entire PA83 molecule and not just the active PA63.

Analysis of immune responses to anthrax toxin domains & regions

Within the IEDB, an epitope is defined as a molecular structure recognized by immune responses, and narrowed down to a molecular size of 50-amino acid residues (or 5000 Da) or less. Given the small number of defined epitopes described in the literature for *B. anthracis* that fulfil these criteria, we examined the effect of expanding the criteria to include larger fragments or domains that had specific relevance to this analysis, as they represent coarser-grain mappings of the immunologically reactive regions [31,34,42,61–67].

Expanding the criteria for data inclusion provided additional insights into the nature of anthrax-specific immune responses (Figure 1C). First, we found that the entire *B. anthracis* PA83 protein molecule has been studied, and that all the individual domains, including domain 1, could confer protection to toxic challenge upon the immunization of mice. Monoclonal- and polyclonal-neutralizing murine antibodies have been shown to be directed to domains 1, 2 and 4. The anti-domain-1 mAbs activity was demonstrated to occur via the inhibition of LF–PA interaction and slowing the proteolytic digestion of PA by furin, in agreement with the function of this PA region. Domain 1 also exhibits antigenicity in macaques. These findings highlight the surprising lack of fine definition of the epitopes within the highly immunogenic, protective domain 1.

Second, immune recognition of domains 3 and 4 in additional host species (humans, chimpanzees and rabbits) was reported in the literature. Knowledge of the neutralizing ability of antibodies from humans and chimpanzees directed to these domains could not have been derived from the existing data on epitopes of 50 amino acids or less. Therefore, further definition of the individual epitopes that mediate this activity is required in order to establish the degree of conservation among the determinants of neutralizing antibodies elicited in different hosts.

The immunogenicity of the N-terminal region of the enzymatic toxin components, LF and EF, was also studied but, in this case, they were not tested for the neutralizing or protective capacity [1,68-70]. However, even after expanding the molecular size requirement for data inclusion, studies that define additional antigenic or immunogenic regions in other *B. anthracis* antigens besides the toxins were not identified.

Localization of epitopes from botulinum toxin type A

The distribution of the antibody epitopes on the BoNT/A protein is shown in Figure 2A, where a color code defines the peptides reactive in each species. The top panel depicts the domain organization of the BoNT proteins, where the N-terminal light chain is linked through disulfide bonds to the heavy chain. The most N-terminal region of the heavy chain (the Belt) partially covers the light chain in the free state, native conformation. The H_N domain of BoNT is involved in the translocation of the light chain to the cytosol. The H_C domain most C-terminal region (H_{CC}) is involved in the receptor binding, while no clear function has been attributed to the H_{CN} region located at the N-terminal part of the H_C domain. To maximize the biological relevance of the analysis, only peptides recognized strongly (i.e., immunoprevalent epitopes, Box 1) were considered here. The cutoff values for inclusion are listed in Figure 2. Several B-cell epitopes from BoNT/A have been reported in a series of studies performed by the Atassi group [45-47,50,53-55] using overlapping peptides from the heavy chain of BoNT/A1. Focusing the analysis on the more highly antigenic peptides, seven peptides were identified in humans, defining six immunoprevalent sites, two of them located in the H_N domain and four in the H_C domain. In mice, 20 peptides were immunoprevalent and define a total of 13 recognition sites, which were fairly evenly distributed along the heavy chain. Some of these peptides were also shown to induce a BoNT/A1-specific response upon peptide immunization of mice. As many as 18 peptides are recognized in the chicken, defining 11 immunoprevalent sites, again, fairly well-distributed along the heavy chain. By contrast, peptides found to be antigenic in horses are mainly located at the belt and H_N domain (eight peptides defining four antigenic sites), while only one peptide from the H_C domain can be considered highly antigenic.

Residues in the belt- H_N boundary and the C-terminal 1275–1296 peptide are recognized consistently in the four species tested, suggesting these regions are broadly antigenic. It would then be of interest to assess the capacity of these regions to induce protective responses, as they may represent common neutralizing antibodies' targets.

The group of Atassi has also studied the antigenicity and immunogenicity of the peptides covering the H_N and H_C domains for murine Th cells (Figure 2B) [53-55]. The overall antigenic potential of these domains appears to be higher in SJL ($H-2^s$) than in BALB/c ($H-2^d$) mice. The receptor-binding region, while a target of antibody responses, was antigenic in SJL mice immunized with BoNT/A toxoid [55], but that result was not seen in mice immunized with a recombinant H_C fragment of BoNT/A [53], while upon direct peptide immunization with determinants from that region, peptide-specific T-cell-proliferative responses could indeed be elicited [55]. The belt and H_{CN} regions, on the other hand, appear to contain a high density of $H-2^s$ determinants. In order to infer which regions

would be of interest to include in vaccines aimed at inducing Th responses, the T-cell epitopes recognized in other species need to be studied as well.

Neutralizing epitopes from BoNT/A

Two linear epitopes and three discontinuous epitopes from BoNT/A have been described as being recognized by neutralizing mAbs. These BoNT/A neutralizing epitopes are represented on the 3D model of BoNT/A obtained by x-ray crystallography (pdb 3BTA) in Figure 2D. The effects of these mAbs on toxin activity correlate with the location of the epitopes on the BoNT/A domains for which discrete functions are known, as described below.

For the heavy-chain epitopes, one structure recognized by five neutralizing murine mAbs raised against the H_C domain [48], co-localizes with the putative ganglioside binding site on the H_{CC} region. Moreover, immunization of mice with this peptide conferred protection in two out of five mice to experimental challenge with ten LD₅₀ doses of BoNT.

The three discontinuous epitopes reported to date are recognized by murine or human mAbs, studied extensively by the group of Marks [52] and found to be capable of synergistically neutralizing the toxin activity in both *in vitro* and *in vivo* mouse models. An analysis of the contribution of individual residues to the total binding energy using a library of BoNT/A1 mutants allowed for the definition of the residues involved in recognition. For the murine mAb S25, three consecutive residues, ¹²⁵⁴QFN¹²⁵⁶, that co-localize with the putative ganglioside binding site were identified. Similarly, for the human mAb 3D12, three consecutive residues, ¹¹²⁹GIR¹¹³¹, on the H_{CC} region were defined that overlap with residues potentially involved in the toxin–protein receptor interaction. The identified residues involved in the recognition by mAb C25 are located on the H_{CN} region, for which there is no defined functionality. Further studies on the mechanism of neutralizing activity of this mAb could help define the role of the H_{CN} region in BoNT toxicity. This set of three mAbs is proposed as an alternative to animal heptavalent antisera for postexposure treatment of toxic syndrome [68,71–73], the only currently available therapy for botulism.

A light-chain peptide is the target of a murine monoclonal IgG_{2b} antibody raised against the isolated light chain [49]. This mAb was not protective *in vivo* when the toxin–mAb complex was formed in the extracellular milieu, but was protective when injected into the presynaptic ganglion neurons 1 h before the addition of BoNT/A. Thus, this response is not of therapeutic interest, but is an example of how studying the basis of the biological activity for inhibitory mAbs allows the confirmation of the functionality of toxin regions deduced from basic toxicological and mutational analysis.

Conservancy & cross-reactive recognition of epitopes among BoNT types & subtypes

Next, we analyzed the conservancy of neutralizing and protective epitopes among the different subtypes and serotypes of botulinum toxin, and reviewed the known cross-reactivity data for these epitopes. When comparing the amino acid sequences derived from the seven different serotypes of BoNT, it is evident that even with the shared general functionality of each domain, a high degree of variability exists that extends throughout the toxin's entire primary structure. Variants are also found within each serotype, which can be defined as strains or subtypes depending on the degree of homology [74].

The amino acid sequences of the known neutralizing epitopes were analyzed for the percentage of identity among subtypes and between serotypes (Table 4) using the Epitope

Conservancy Analysis tool, hosted on the IEDB website [101]. This tool provides all the regions in a target protein that have homology with the query sequence, with an identity threshold level determined by the user.

For the linear BoNT/A epitopes (Table 4), the degree of conservancy of epitopes within the BoNT/A serotype is usually high (88–100%), with a substantial drop in the identity score when the BoNT/A1 epitopes are aligned to other serotypes. For the light-chain epitope, BoNT/A1-28–53 (Table 4), meaningful counterparts can be assigned in the other serotypes because this subunit is more conserved than the heavy chain, of which the linear epitopes cannot always be identified in other serotypes. Still, the mAb specific for this light-chain epitope does not cross-react with BoNT/B – the closest sequence outside the BoNT/A serotype. The heavy-chain linear epitope, BoNT/A1-1230–1254 (Table 4), has similar counterparts within the other BoNT/A subtypes but no significant homologs in other serotypes. Although there is no experimental data on the cross-reactivity of its recognizing mAb, it would then be expected to be BoNT/A specific.

In the case of the BoNT/A epitopes mapped to the individual residues involved in recognition (Table 4), the analysis is restricted to the BoNT/A subtypes since the divergence among serotypes is too profound to allow for a meaningful comparison of discontinuous or short sequences. Epitope conservancy among BoNT/A subtypes is consistent with experimental results relating to cross-reactivity. The residues involved in the recognition by the human mAb 3D12, ¹¹²⁹GIR¹¹³¹, are conserved in all BoNT/A subtypes and for BoNT/A2, a cross-reactive recognition was demonstrated. On the other hand, residues involved energetically in the binding of mAb S25, ¹²⁵⁴QFN¹²⁵⁶, are not conserved in BoNT/A2 and, congruently, this mAb has an affinity three orders of magnitude higher for BoNT/A1 than for BoNT/A2 [74].

The humanized mAb, HuC25, provides an interesting example of how variability can affect cross-reactive recognition. The residues involved in the binding are conserved in BoNT/A1 and BoNT/A2 in seven out of nine instances, but in the presence of T1063P and H1064R substitutions the K_D of HuC25 drops dramatically [52,74]. By artificial molecular evolution of HuC25, mAbs were obtained that have a higher affinity towards BoNT/A1 and are cross-reactive towards BoNT/A2 [75]. This latter activity is mediated by few mutated residues on the mAb, thus, confirming that subtle differences in antigens and antibodies can influence the extent of cross-reactivity.

For BoNT/E (Table 4), the epitopes described in the literature derive from studies of the recognition of the Iwanai strain, the complete sequence of which has not yet been published [51]. An H_N domain epitope, ⁶⁶³VIKAIN⁶⁶⁸, is conserved in the published BoNT/E sequences. The other serotypes containing some homology at this epitope sequence are BoNT/F and BoNTA/4, but in the latter case, the location of the sequence is not conserved. The H_C-domain epitope characterized, ¹²²⁴YLTHMRD¹²³⁰, is not found in the published BoNT/E sequences, which have a tyrosine at position 1225 and are the only sequences with significant homology. Cross-reactive recognition and neutralization by the mAbs directed to these epitopes to other subtypes of BoNT/E and to BoNT/F has not been tested.

In general, this analysis confirms the observation that polyclonal antibodies recognize serotype-specific determinants of neutralization [76]. Here, mAbs generated against the entire BoNT/A or BoNT/E toxins were analyzed, and were also found to lack cross-protective capacity between serotypes. These results suggest that vaccine and therapeutic approaches to this toxin may need to be strain specific.

Analysis of immune responses to BoNT/A domains & regions

As in the case of the PA protein of *B. anthracis*, we analyzed the available literature pertaining to the immune response to BoNT/A regions that fall outside our 50-amino acid limit for inclusion in the database as epitopes. The fragments that have been defined as being antigenic or immunogenic in the analyzed references [44,73,74,77-83] are shown in Figure 2C.

In mice, fragments that encompass the entire BoNT/A molecule were shown to have antigenic and immunogenic potential. The isolated heavy chain is the target of neutralizing antibodies and can elicit a protective immune response, while no such activities are associated with the light-chain. This observation is in agreement with what has been described previously for epitope-specific responses, where a light chain epitope is the target of a mAb whose neutralizing capacity is observed only upon intraneuronal injection. In addition, by immunization with overlapping fragments covering the entire BoNT/A1 sequence, it has been shown that protective immunity can be achieved not only by the most C-terminal H_{CC} region where the receptor binding activity resides, but also by the region of the belt-H_N boundary, which, as mentioned previously, is also immunoprevalent in humans, chickens and horses. This finding reinforces the need for further study on the basis of the protection afforded by this region, as it would be a potentially valuable addition to future synthetic vaccines.

In the case of horses, antisera generated against BoNT/A is shown to recognize all the fragments tested, suggesting that upon toxoid/toxin immunization, all the domains induce a specific response. This information is of value since the published epitope-specific responses did not include any data on the antigenicity or immunogenicity of the light chain in this species.

Two regions have been shown to be targets of human antibodies. Specifically, a fragment of the H_{CC} region is recognized by a neutralizing mAb that was later mapped to its defined epitope. Similarly, the H_N domain is confirmed to be a target of antibodies generated by pentavalent toxoid vaccination, as was already shown at the analysis of individual epitopes. In the case of rabbits, the analysis of longer fragments reveals that anti-H_C domain antibodies raised in this species have neutralizing capacity, as is the case in humans and mice.

In general, there is good agreement between the data obtained at the epitope and large-fragment levels of analysis, and the results of the latter point to the need for further definition of the regions involved in protective responses in species other than the mouse.

Analysis of immune responses to domains & regions from other serotypes of BoNT

Data relating to recognition of fragments from other serotypes of BoNT besides BoNT/A is of interest given the almost complete lack of information about the immune response to them that can be obtained by analyzing the epitope-related published data. Regions of more than 50 amino acids from serotypes BoNT/B to BoNT/F were also studied, in five references for BoNT/B [84-88], three for BoNT/C [89-91], two from BoNT/D [89,91], four from BoNT/E [51,78,92,93] and two from BoNT/F [93,94]. No references were identified as containing data regarding immune recognition of BoNT/G fragments. The individual domains 1, 2 and 4 of the component II of C2 toxin have also been tested for both antigenicity and immunogenicity in a single reference [23].

By considering larger fragments, we found, consistent with the data derived from the BoNT/A analysis, that the heavy chains from BoNT/B, C, D and F are targets of neutralizing antibodies. Furthermore, the BoNT/B- and BoNT/E-H_C domain and the BoNT/F H_{CC} region were shown to confer protection from toxic challenge.

Novel neutralization data was also obtained from this analysis, showing that the light chain is a target of neutralizing antibodies in the case of BoNT/B, C and D. This kind of activity had not been demonstrated for BoNT/A, and is also apparently absent in BoNT/E. The basis of this difference in neutralizing epitopes in the different serotypes warrants further studies.

The ability of individual domains of component II of *C. botulinum* toxin C2 to induce neutralizing antibodies has also been studied. The C2 toxin cytotoxicity can be inhibited by anti-domain-4 sera, consistent with this domain's activity, to mediate binding to the cellular receptor.

We also analyzed the information on the cross-reactive recognition of the different BoNT serotypes obtained when expanding our size limit. Sera raised in mice to BoNT/A fragments were in many cases able to cross-recognize other serotypes. In particular, cross-reactivity towards serotypes B, C, D, E and F was observed for antisera raised in CB6F1 mice to recombinant fragments, representing the light chain N-terminal region, the H_N domain, and the N-terminal part of the H_{CC} region, linked to a Th peptide from cholera toxin. In other studies, it was found that cross-reactivity of the BoNT/A fragment-specific sera is not so widespread among serotypes, with titers to heterologous toxins usually lower than to the immunogen, and the results varied depending on the immunization protocol. Furthermore, the protective capacity upon heterologous challenge of the immunized animals was not assessed in these studies. Indeed, the H_C domain of BoNT/A1, which confers protection to homologous challenge, did not protect mice and rabbits from BoNT/B and/or BoNT/E challenge.

A different scenario is seen for BoNT/C and BoNT/D, where anti-heavy-chain cross-neutralizing mAbs were obtained, while their anti-light-chain counterparts failed to exhibit cross neutralization. This result is in accordance with the significant homology in the heavy chain for these two serotypes. For these cross-neutralizing mAbs, it would be of interest to further refine their cognate epitopes so that the basis of their activity could be deduced. At this point, the goal of inducing cross-protective responses among BoNT serotypes has not been met.

Expert commentary

Meta-analysis of epitope-specific immune responses, such as the one performed for this review, can generate a comprehensive picture on the current status of the research regarding specific pathogens of interest. This knowledge can be applied to enhance the definition of correlates of immunity, protection and desirable targets for detection reagents.

The knowledge derived from the immunological research on toxin-producing organisms, such as *B. anthracis* and *C. botulinum*, tends to focus primarily on the immune response to the toxin components, whether at the level of whole proteins, domains or defined epitopes. This focus is expected since the antibody-mediated inhibition of toxicity is the first desirable outcome of a protective vaccination. Anti-toxin antibodies are also useful tools for diagnostic and detection purposes. Congruently, the bulk of the information on epitopes from these two organisms is found at the level of toxin peptides recognized by antibodies. For both anthrax and botulinum toxins, most of the neutralizing and protective antibodies are directed to the region involved in binding to the cellular receptors, which are able to inhibit the first step of toxic activity. Other regions of the 'B component' of these toxins,

such as those important for translocation of the enzymatic components to the cytosol, also have implicit protective capacity, as can be determined by analyzing the reported epitopes and domains that are targeted by neutralizing antibodies.

The usefulness of epitope-derived information for the generation of novel vaccines and detection reagents depends partly on their recognition across species, specifically when correlates of protection detected in experimental animal models are to be applied to humans. In the case of the PA protein from *B. anthracis*, some of the neutralizing epitopes defined in mice are thought to be recognized in rabbits and humans, since the polyclonal sera from vaccinated individuals compete with neutralizing epitope-specific mAbs for binding to PA. However, the epitope specificity and neutralizing capacity of the competing mAbs has not been directly tested and, therefore, it remains to be proven whether these epitopes are actually conserved across species.

By contrast, in the case of the anti-BoNT/A antibodies, the recognition of heavy-chain epitopes across different species has been studied extensively, but such information is not available for the particular epitopes targeted by antibodies with proven neutralizing capacity. Nevertheless, the H_{CC}- and the belt-H_N-domain boundary regions have been shown both to be targets of neutralizing antibodies in specific organisms and to be immuno-prevalent sites for antibody recognition in four different species. Therefore, it could be expected that the immune response to these regions may represent good correlates of protection to BoNT/A toxic challenge. Further studies aimed at addressing these issues are still needed. Indeed, basic work is still required, for instance, to define the function and exact location of the belt-H_N-domain neutralizing epitope. In this sense, it is important to note that, either in mAb-mediated neutralization of *in vivo* toxicity or in peptide vaccination experiments, no single epitope has been shown to induce significant protective responses to BoNT challenge. This result points out the need to include several epitopes in the formulation of synthetic vaccines to confer full protection to toxic exposure.

Given that both anthrax and botulinum toxins act intracellularly on their substrates, we wanted to assess the extent and value of an immune response to the enzymatic subunit (A components). In this regard, we found that the enzymatic active subunits are also desirable targets for protective antibodies. In the case of anthrax toxin, a single protective epitope from the enzymatic toxic components has been described, located in the domain III of the LF, which is involved in the interaction of LF with its substrate. Antibodies to this region are able to confer passive immunity to LeTx. Nevertheless, when studying the reported immune response to larger fragments, we found that antibodies to the N-terminal, PA-binding domains of both LF and EF have also been implicated in protection to toxic challenge.

In BoNT, the light chain could only be found to be the target of protective immune responses when we analyzed fragments of over 50 amino acids, and this activity was not found in all the serotypes. This information suggests that there is potential value in inducing anti-light-chain antibodies, but the epitopes that are important in this response are yet to be defined. Given that, in general, the light chain is better conserved among BoNT serotypes than the heavy chain, the finer definition of these epitopes is desirable, as is the further clarification of the apparent difference among serotypes in the protection to toxic challenge conferred by this domain.

There is also an urgent need to explore the possibility of obtaining cross-neutralizing antibodies based on epitope-specific recognition. The analysis of the mere seven neutralizing epitopes described in the published literature so far (five from BoNT/A and two from BoNT/E), has not enabled the definition of potential sites of cross-neutralizing activity.

The required antibodies could be aimed at regions whose immunogenicity is masked in the course of protein vaccinations, in which case, further work at the epitope level of recognition would still be required.

A separate point should be made to the fact that subsets of antitoxin antibodies, as shown for anti-PA mAbs [95] can, in some instances, mediate enhancement of toxicity. An epitope-based approach to the development of vaccines and therapeutic reagents is, therefore, extremely useful in that it would avoid this kind of undesirable reactivity.

In conclusion, the present analysis has provided a review of the status on the immunological research on *C. botulinum* and *B. anthracis* with regards to immune-epitope information to date. Several epitopes are identified that can serve as starting points for producing the next generation of vaccines and detection reagents.

Five-year view

This revision of the published data on the epitopes from *B. anthracis* and *C. botulinum* has allowed us to pinpoint areas where work is needed in order to afford understanding of the pathogenesis of these organisms and the ability of the immune system to effectively restrain their actions.

Two main areas of future work seem evident from this analysis. First, is the study of other targets of protective immunity in addition to those defined for the toxins themselves, as they may serve as additional correlates of protection. In this sense, the spores of anthrax have been shown to be opsonized *in vitro* by anticapsule antibodies, but how effective, or even desirable, such a response would be *in vivo* has yet to be fully determined. In principle, spore and bacillus antigens from *B. anthracis* could be possible additions for obtaining protective and therapeutic antibody preparations for the management of anthrax infection [96-98].

The immune response to *C. botulinum* surface antigens has not been studied. Since the spores germinate *in vivo* in wound and infant botulism, the course of those syndromes could be modified with prophylactic and/or therapeutic reagents aimed at the spores and/or the bacteria themselves. However, these approaches would not be applicable for cases of direct intoxication with the toxin, either in a food-borne or aerosolized form.

Second, further progress needs to be made in the study of T-cell determinants from the toxins and the whole organisms, and how the T-cell reactivity might affect the outcome of infection and toxic shock. The Th-dependent antibody response to the toxins has already prompted some investigations on the CD4⁺ T-cell immunity and its epitope specificity, but the immunoprevalency and immunodominancy of these epitopes still needs to be addressed for this information to be applied to the development of effective vaccines. Although the cytotoxic responses are not the predominant immunological arm for fighting bacterial infections or toxic syndromes, these toxins act intracellularly and, possibly, are presented on MHC-class I molecules to generate CD8⁺ T-cell responses. It is noteworthy that, to the best of our knowledge, no studies have been conducted to assess the existence of class I epitopes on these toxins. We hope that future lines of work will address these issues.

In the case of the BoNTs in particular, another future area of work is research aimed at the development of vaccines and detection/diagnostic reagents that are able to work across serotypes. The lack of cross-neutralizing epitopes among serotypes, except for the known cross-neutralizing determinants on BoNT/D and C, has led to the development of multivalent toxoid preparations as prospective vaccines. The use of epitope-derived

information, on the other hand, could overcome this limitation and prove the existence of cross-neutralizing determinants.

Appendix

Antigen	Epitope	Category	Ref.
<i>Bacillus anthracis</i>			
Edema factor	342 GVATKGLNVHKGSSDWG 358	B cell	[26]
Lethal factor	380 DSLSEEEKELLNRIQVDS 397	B cell	[30]
Protective antigen	340 (A)SFFD 344	B cell	[27,39]
Protective antigen	442 SKNLAPI 448	B cell	[62]
Protective antigen	610 IKLNAKMNIIRDKRFHYDRN 630	B cell	[31,34,61]
Protective antigen	679 EGLKEVINDRYDMLN 693	B cell	[25]
Protective antigen	700 DGKTFIDFKKYNDKPLYISNPYKVNVA 729	B cell	[32]
Protective antigen	715 PLYISNPY 723	B cell	[29]
Protective antigen	N682, K684, L685, P686, L687, Y688	B cell	[35]
Protective antigen	93 IWSGFIKVKKSDEY 106	T cell	[41]
Protective antigen	141 RLYQIKIQYQRENPT 156	T cell	[40]
Protective antigen	183 PELKQKSSNSRKKR 196	T cell	[41]
Protective antigen	411 SLVLGKNQTLAT 422	T cell	[40]
Protective antigen	573 GKDITEFDNFDDQTSQNIK 592	T cell	[43]
Protective antigen	576 ITEFDNFDDQTSQ 589	T cell	[41]
Protective antigen	688 RYDMLNISSLRQDG 701	T cell	[41]
Protective antigen	746 STNGIKKILIFSKK 759	T cell	[41]
<i>Clostridium botulinum</i>			
C2-II	182 ANANRDTDRDGIPDE 196	B Cell	[24]
C2-II	707 RLSGVFLIELDKLII 721	B cell	[24]
BoNT type A	2 PFVKNQFNYKDPVNGV 17	B cell	[44]
BoNT type A	28 GQMGPVKAFKIHNKIWPVPERDFTN 53	B cell	[49]
BoNT type A	218 AVTLAHELIHAGHR 231	B/T cell	[46,47,55]
BoNT type A	449 ALNDLCIKVNNWDLFFSPS 467	B cell	[46,47,50]
BoNT type A	449 ALNDLCIKVNNWDLFFSPSEDNFTN 473	B cell	[49]
BoNT type A	463 FFSPSEDNFTNLDNKGEEI 481	B/T cell	[46,47,50,55]
BoNT type A	477 KGEEITSDTNIEAAEENIS 495	B/T cell	[46,47,50,55]
BoNT type A	491 EENISLDLIQYYLTFNFD 509	B cell	[46,47,50]
BoNT type A	505 TFNFDNEPENISLENLSSD 523	B/T cell	[46,47,50,55]
BoNT type A	519 NLSSDIIGQLELMPNIERF 537	B/T cell	[46,47,50,55]
BoNT type A	533 NIERFPNGKKYELDKYTMF 551	B/T cell	[46,47,50,55]
BoNT type A	547 KYTMFHYLRAQEFEGKSR 565	B/T cell	[46,47,50,55]
BoNT type A	561 HGKSRIALTNSVNEALLNP 579	B/T cell	[46,47,50,55]
BoNT type A	575 ALLNPSPRVYTFSSDYVKK 593	B/T cell	[46,47,50,55]
BoNT type A	589 DYVKKVKNKATEAAMFLGWV 607	B/T cell	[46,47,50,55]
BoNT type A	603 FLGWVEQLVYDFTDETSEV 621	B cell	[46,47,50]

Antigen	Epitope	Category	Ref.
<i>Bacillus anthracis</i>			
BoNT type A	617 ETSEVSTDDKIADITIIP 635	B/T cell	[46,47,50,55]
BoNT type A	631 TIIPYIGPALNIGNMLYK 649	B cell	[46,47,50]
BoNT type A	645 NMLYKDDDFVGALIFSGAVI 663	B cell	[46,47,50]
BoNT type A	659 SGAVILLEFIPEIAIPVLG 677	B/T cell	[46,47,50,55]
BoNT type A	673 IPVLTGFALVSYIANKVLT 691	B cell	[46,47,50]
BoNT type A	687 NKVLTVQTIDNALSKRNEK 705	B cell	[46,47,50]
BoNT type A	701 KRNEKWDEVYKYIVTNWLA 719	B/T cell	[46,47,50,55]
BoNT type A	715 TNWLAKVNTQIDLIRKKMK 733	B/T cell	[46,47,50,55]
BoNT type A	729 RKKMKEALENQAETKAI 747	B cell	[46,47,50]
BoNT type A	743 TKAIINYQYNQYTEEEKNN 761	B/T cell	[46,47,50,55]
BoNT type A	757 EEKNNINFNIDDLSSKLNE 775	B cell	[46,47,50]
BoNT type A	771 SKLNEKINAMININIKFLN 789	B/T cell	[46,47,50,55]
BoNT type A	785 NKFLNQCSVSYLMNSMIPY 803	B cell	[46,47,50]
BoNT type A	799 SMIPYGVKRLDFDASLKD 817	B/T cell	[46,47,50,55]
BoNT type A	813 ASLKDALLKYIDNRGTLI 831	B cell	[46,47,50]
BoNT type A	827 RGTLLIGQVDRLLKDKVNTL 845	B cell	[46,47,50]
BoNT type A	841 VNNTLSTDIPQLSKYVDN 859	B/T cell	[46,47,50,55]
BoNT type A	855 KYVDNQRLSTFTEYIKNI 873	B cell	[45-47,50,53]
BoNT type A	869 YIKNIINTSILNRYESNH 887	B cell	[45-47,50,53,54]
BoNT type A	883 YESNHLIDLSRYASKINIG 901	B cell	[45-47,50,53,54]
BoNT type A	897 KINIGSKVNFDPIDKNQIQ 915	B/T cell	[45-47,50,53-55]
BoNT type A	911 KNQIQLFNLESSKIEVILK 929	B/T cell	[45-47,50,53-55]
BoNT type A	925 EVILKNAIVYNSMYENFST 943	B/T cell	[45-47,50,53-55]
BoNT type A	939 ENFSTFWIRIPKYFNSIS 957	B/T cell	[45-47,50,53-55]
BoNT type A	953 FNSISLNNEYIINC MENN 971	B/T cell	[45-47,50,53-55]
BoNT type A	967 CMENNSGWKVSLNYGEIHW 985	B/T cell	[45-47,50,53-55]
BoNT type A	981 GEIHWTLQDTQEIKQRVVF 999	B cell	[45-47,50,53-55]
BoNT type A	995 QRVVFKYSQMINISDYINR 1013	B/T cell	[45-47,50,53-55]
BoNT type A	1009 DYINRWIFVTITNRLNNS 1027	B/T cell	[45-47,50,53-55]
BoNT type A	1023 RLNNSKIYINGRLIDQKPI 1041	B/T cell	[45,46,47,50,53,55]
BoNT type A	1037 DQKPISNLGNIHASNNIMF 1055	B/T cell	[45,46,47,50,53,55]
BoNT type A	1051 NNIMFKLDGCRDTHRYIWI 1069	B/T cell	[45-47,50,53-55]
BoNT type A	1065 RYIWKYFNLFDKELNEKE 1083	B/T cell	[45-47,50,53,55]
BoNT type A	1079 LNEKEIKDLYDNQNSGIL 1097	B/T cell	[45-47,50-55]
BoNT type A	1093 NSGILKDFWGDYLYQYDKPY 1111	B/T cell	[45-47,50,53-55]
BoNT type A	1107 YDKPYMLNLYDPNKYVDV 1125	B/T cell	[45-47,50,53,55]
BoNT type A	1121 KYVDVNVVGIRGYMYLKG 1139	B/T cell	[45-47,50,53,55]
BoNT type A	1135 YLKGPRGSVMTTNIYLNSS 1153	B/T cell	[45-47,50,53-55]
BoNT type A	1149 YLNSSLYRGTKFIKKYAS 1167	B/T cell	[45-47,50,53,55]
BoNT type A	1157 GTKFIKKYASGNKDNIVRNDRVY 1181	B cell	[48]
BoNT type A	1163 KKYASGNKDNIVRNDRVY 1181	B/T cell	[45-47,50,53,55]

Antigen	Epitope	Category	Ref.
<i>Bacillus anthracis</i>			
BoNT type A	1177 NDRVYINVVVKNKEYRLAT 1195	B/T cell	[45-47,50,53-55]
BoNT type A	1191 YRLATNASQAGVEKILSAL 1209	B/T cell	[45-47,50,53,55]
BoNT type A	1205 ILSALEIPDVGNSQVVVM 1223	B cell	[45-47,50,53,55]
BoNT type A	1219 QVVVMKSKNDQGITNKCKM 1237	B cell	[45-47,50,53]
BoNT type A	1230 GITNKCKMNLQDNNGNDIGFIGFHQ 1254	B cell	[48]
BoNT type A	1233 NKCKMNLQDNNGNDIGFIG 1251	B/T cell	[45-47,50,53,55]
BoNT type A	1247 IGFIFGHQFNNAKLVASN 1265	B/T cell	[45-47,50,53,55]
BoNT type A	1261 LVASNWYNRQIERSSRTL 1279	B cell	[45-47,50,53,55]
BoNT type A	1275 SRTLGCSEWIFVDDGWERPL 1296	B/T cell	[45-47,50,53-55]
BoNT type A	G1129, I1130, R1131	B cell	[52]
BoNT type A	N918, L919, E920, F953, R1061, D1062, T1063, H1064, Y1066	B cell	[52]
BoNT type A	Q1254, F1255, N1256	B cell	[52]
BoNT type E	663 VIKAIN 668	B cell	[51]
BoNT type E	1224 YLTHMRD 1230	B cell	[51]

BoNT: Botulinum neurotoxin.

Acknowledgments

We thank the Immune Epitope Database and Analysis Resource curation team for their excellent work in curating the cited epitope information and Alison Deckhut Augustine for helpful suggestions and a critical review of the manuscript.

This work was supported by the US NIH National Institute of Allergy and Infectious Disease Contract HHSN26620040006C (Immune Epitope Database and Analysis Program). Arturo Casadevall is supported, in part, by grant 5U54AI057158-05.

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Websites

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Box 1**Definition of terms used**

- **Epitope:** A structure that is the specific target of a T- or B-cell receptor, with a size limit of 50 amino acids or 5000 Da.
- **Antigenic epitope:** An epitope recognized by the immune system after immunization with the complete antigen or organism that contains it.
- **Immunoprevalent epitope:** An antigenic epitope that is recognized by the immune system more frequently or more strongly than other epitopes from the same antigen.
- **Immunogenic epitope:** An epitope able to elicit antigen- or organism-specific responses when used as immunogen.
- **Neutralizing epitope:** An antigenic epitope that is recognized by antibodies or T cells with proven neutralizing or cytotoxic activity.
- **Protective epitope:** An immunogenic epitope able to confer protection from disease/infection by the pathogenic antigen or organism that contains it.

Key issues

- The current knowledge on *Bacillus anthracis* and *Clostridium botulinum* epitopes, analyzed from the data deposited in the Immune Epitope Database and Analysis Program, is largely limited to antibody epitopes from the toxin components.
- The total number of epitopes described from these organisms is significantly lower than those from other organisms important for human health and disease.
- The knowledge derived from the epitopes described could be valuable in the development of vaccines, immunotherapeutics and detection reagents. Combinatorial approaches based on this knowledge could enhance their potency synergistically.
- Expanding the analysis for larger fragments involved in immune recognition allowed for the identification of additional regions of potential value, but this information is difficult to analyze with regards to structure–function mechanistic perspectives.
- Gaps in knowledge, identified by the analysis, highlight areas where future research is required, such as discovery of B-cell epitopes and their functions, identification of T-cell epitopes and analysis of epitopes from antigens other than the bacterial toxins.

Figure 1. *Bacillus anthracis* protective antigen (PA) structure and its immunological recognition
 Top panel: schematic representation of PA primary structure, its different domains and most relevant features, including the strain-related polymorphism. **(A)** Representation of the location of the antibody (B-cell) epitopes described in the literature. A separate color is used for each host species, and the individual features of each epitope are represented as in the legend. **(B)** Representation of the T-cell epitopes described in the literature, coded similarly as the B-cell epitopes. **(C)** Representation of the fragments described in the literature involved in antibody recognition, coded similarly as the B-cell epitopes. **(D)** Neutralizing epitopes from PA depicted in the 3D structure. A monomer from the heptamer prepore structure (PDB 1TZN) is shown, in complex with its cellular receptor, CMG2, which is shown in gray lines with white background. The PA domains and epitope coloring corresponds to that of the top panels.

Table 1Total number of *Bacillus anthracis* and *Clostridium botulinum* epitopes.

	Bacillus anthracis	Clostridium botulinum
B-cell epitopes	11	73
– Carbohydrate epitopes	1	-
– Linear peptidic epitopes	9	70
– Discontinuous peptidic epitopes	1	3
T-cell epitopes	7	42

Table 2

Epitope distribution by antigen.

Epitopes	<i>Bacillus anthracis</i>				<i>Clostridium botulinum</i>			
	PA	LF	EF	P- γ -DGA*	BclA	BoNT/A	BoNT/E	C2II
B cell	7	1	1	1	1	69	2	2
T cell	7	-	-	-	-	42	-	-

* γ -linked D-glutamic acid oligopeptides of different lengths have been studied, but are counted as a single epitopic structure here.

BclA: *Bacillus anthracis* collagen-like protein; BoNT/A: Botulinum neurotoxin type A; BoNT/E: Botulinum neurotoxin type E; C2II: Component II of *Clostridium botulinum* toxin C2; EF: Edema factor; LF: Lethal factor; P- γ -DGA: Poly- γ -D-glutamic acid; PA: Protective antigen.

Table 3

Distribution of epitopes by host.

	Bacillus anthracis	Clostridium botulinum
<i>Mus musculus (mice)</i>		
B-cell epitopes	8	59
T-cell epitopes	5	42
<i>Homo sapiens (humans)</i>		
B-cell epitopes	1	53
T-cell epitopes	2	–
<i>Oryctolagus cuniculus (rabbits)</i>		
B-cell epitopes	5	3
<i>Macaca fascicularis (macaques)</i>		
B-cell epitopes	1	–
<i>Equus caballus (horses)</i>		
B-cell epitopes	–	42
<i>Gallus gallus (chickens)</i>		
B-cell epitopes	–	38

Table 4

Conservancy of BoNT neutralizing epitopes across serotypes and subtypes.

Serotype/Subtype	Position	Sequence	Identity (%)
BoNT/A1	28–53	G Q M Q P V K A F K I H N K I W V I P E R D T F T N	
BoNT/A2	28–53		100.00
BoNT/A3	28–53	 E G V	88.46
BoNT/A4	28–53	. K V I	88.46
BoNT/B	29–54	. T G R Y Y T D R . . I Y . . G Y	53.85
BoNT/D	29–54	L I T T M . T Q N F S S D T	50.00
BoNT/C	29–54	L A N E . E . . . R . T G N D . F S R N S	42.31
BoNT/E1	25–50	. C Q . F Y . S . N . M K N . . I N V I G T	42.31
BoNT/G	29–54	. P G T Y Y . . . R . I D R . . I V . . . F . Y G F	42.31
BoNT/E2	25–50	. C Q E F Y . S . N . M K N . . I N V I G T	38.46
BoNT/F	29–54	K S K K Y Y . . . E . M R N V . I N . I G T	38.46
BoNT/A1	1230–1254	G I T N K C K M N L Q D N N G N D I G F I G F H Q	
BoNT/A4	1230–1254		100.00
BoNT/A2	1230–1254	. . R L	92.00
BoNT/A3	1226–1250	. . R V . . . L	88.00
BoNT/F	1215–1239	S . G . N . T . . F . N . . . S N . . L L . . S	56.00
BoNT/E1	1191–1215	N S V G . . T . . F K N N . . L L . . K A	44.00
BoNT/E2	1191–1215	N S V G . . T . . F K N N . . L L . . K A	44.00
BoNT/A1	1129–1131	G I R	
BoNT/A2	1129–1131	. . .	100.00
BoNT/A4	1129–1131	. . .	100.00
BoNT/A3	1125–1127	. . .	100.00
BoNT/A1	1254–1256	Q F N	
BoNT/A4	1254–1256	. . .	100.00
BoNT/A2	1254–1256	L Y D	0.0
BoNT/A3	1250–1252	L Y D	0.0
BoNT/A1		N918 L919 E920 F593 R1061 D1062 T1063 H1064 Y1066	
BoNT/A4		 P1063	88.89
BoNT/A2		 P1063 R1064	77.78
BoNT/A3		 P1063 R1060	77.78
BoNT/E Iwanai	663–668	V I K A I N	
BoNT/E1	663–668	V I K A I N	100.00
BoNT/E2	663–668	V I K A I N	100.00
BoNT/A4	590–595	Y I K A I N	83.33
BoNT/F	681–686	A I K A I N	83.33
BoNT/E Iwanai	1224–1230	Y L T H M R D	
BoNT/E1	1224–1230	Y Y T H M R D	85.71
BoNT/E2	1224–1230	Y Y T H M R D	85.71
BoNT/A4	1135–1141	Y L K G P R D	57.14

BoNT: Botulinum neurotoxin.