Introduction

The deliberate spread in several American cities in 2001 of viable spores of the anthrax-causing bacterium, *Bacillus anthracis*, has concentrated the attention of the public on the need to counter further possible attacks with biological agents. Defensive strategies include preventative measures such as increased intelligence, law enforcement, public vigilance and education but of equal importance is the participation of the scientific community in developing biosensing technologies, vaccines, drugs and diagnostic tests.

The US-based Centers for Disease Control and Prevention (CDC), an agency of the Department of Health and Human Services, has published a list of disease-causing agents that are considered as plausible infective agents in a terrorist assault. The CDC has classified pathogenic microorganisms and toxins according to perceived ease of dissemination and on their capacities to cause high mortality and social disruption. Research is therefore being concentrated on efforts that will lead to effective protection against the misuse of highest-risk, category A bioagents. In addition to *B. anthracis*, category A-classified agents include botulinum neurotoxins, which are obtained from the common and easily cultivated soil-borne bacterium, *Clostridium botulinum*. As outbreaks of food poisoning have been attributed to *C. botulinum*, there is concern at the possibility of its deliberate introduction into food manufacturing processes. According to a recent bulletin from The National Institute of Allergy and Infectious Diseases in August 2003, the potential misuse of botulinum toxin is an issue of the highest concern.

The symptoms of botulism are induced by a secreted neurotoxin. Once established, therefore, the progress of the disease is unimpeded by antibiotic therapy. Confirmed cases of botulism are currently treated with equine-produced antitoxin but no Food and Drug Administration-approved vaccine has been developed to date. Here, we review work by the group of Sina Bavari at the US Army Medical Research Institute of Infectious Diseases in Frederick, Maryland (Pless *et al.*, 2001) who has developed neutralizing monoclonal antibodies (Mabs) to botulinum toxin A, one of seven serologically defined forms of the toxin known to cause botulism in humans. Biacore’s surface plasmon resonance (SPR) technology was used to measure the kinetics of interactions between neutralizing Mabs and botulinum toxin A (BoNT/A) and to locate functional epitopes.
After inhalation or ingestion, botulinum toxin is rapidly taken up into the bloodstream. It is then carried to peripheral synapses, where it enters the neurons by endocytosis. The toxin is a polypeptide consisting of one 100 kDa heavy chain and one 50 kDa light chain linked by a single disulphide bridge. The toxic light chain is a Zn²⁺-containing endopeptidase that prevents neurotransmitter-containing vesicles from fusing with the neuronal plasma membrane (Figure 1), leading to rapid muscular paralysis. The heavy chain moiety confers the ability of the toxin to irreversibly bind to protein receptors on presynaptic neurons prior to internalization.

**Figure 1.** The mechanism of botulinum toxin. The release of neurotransmitters from neurons to muscle cells is mediated by the fusion of synaptic vesicles in which neurotransmitters are stored, with the neuronal plasma membrane. Membrane fusion is a receptor complex-mediated function involving three proteins that together make up a so-called SNARE complex. One protein of the SNARE complex is located on the outer face of vesicular membranes while the other two are co-expressed in a complex on the internal face of the plasma membrane. In order for fusion between the vesicles and plasma membrane to occur (and hence to effect the transfer of vesicle content to muscle cells) the structural integrity of all three SNARE constituents must be intact. (A) Botulinum toxin, depending on serotype, specifically cleaves one of the SNARE proteins (BoNT/A cleaves SNAP-25, one of the two SNARE proteins expressed on the neuronal plasma membrane). This inhibits formation of the SNARE complex and (B) blocks vesicle interaction with the plasma membrane, (C) preventing membrane fusion and neurotransmitter release. For information on experiments carried out elsewhere in which Biacore was used to probe vesicle:membrane fusion in this context, please refer to the paper by Lévêque et al. published in Biacore Journal and references therein (see citations listed in Further Reading).

Pless et al. raised Mabs to a 50 kDa C-terminal fragment of the heavy chain of botulinum neurotoxin A (BoNT/A H₅). The authors used Biacore to characterize the kinetics of neutralizing antibodies to botulinum toxin and, in an epitope mapping exercise, showed that these antibodies bound two distinct and functionally important sites.
**Materials and methods**

**Immunization strategy**
Mice were vaccinated intraperitoneally over a twenty week period with 0.1 to 2.0 µg of a 50 kDa C-terminal fragment of BoNT/A H_c from *C. botulinum*. The animals were tested for serum antibody titer and splenic cells from high responders were used as fusion partners in generating hybridomas. Hybridoma supernatants were tested by ELISA against whole BoNT/A or BoNT/A H_c, or to the heavy chains of two other human botulism-causing toxins, BoNT/B and BoNT/E. Antibodies were typed for class and subclass and were finally purified using protein G-sepharose.

**Efficacy testing of Mabs**
Antibodies were mixed with increasing doses of BoNT/A and were administered to the mice by intraperitoneal injection. Mabs were incubated for 1 h with 10 x LD_{50} of BoNT/A or with 5 x LD_{50} of BoNT/B and BoNT/E before injection. Mortality was scored after 7 days.

**Biacore-based affinity and kinetic measurements**
Purified anti-BoNT/A H_c Mabs were captured using anti-mouse Fc antibodies immobilized on sensor chips. Kinetic analyses were performed by injecting between 30 nM and 300 nM BoNT/A H_c over the prepared sensor chip surface at a flow rate of 25 µl/minute for 2 minutes. Sensorgrams were subtracted with data from a Mab-free control surface and results were evaluated using BIAevaluation software. The sensor chip was regenerated by injecting 2 x 30 s pulses of 10 mM glycine, pH 1.8.

**Biacore-based epitope mapping**
Anti-BoNT/A H_c Mabs were captured on a sensor chip as described above and unreacted anti-mouse Fc was blocked by injecting a saturating concentration of unrelated antibody. 200 nM BoNT/A H_c in HBS was then injected over the Mab, followed by a second anti-BoNT/A H_c Mab (Figures 2 and 3). All antibody pairings (see Table 1) were tested.

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**Figure 2.** Biacore-based epitope mapping principles. The capture molecule in the study by Pless et al. was one of a pair of neutralizing Mabs raised against BoNT/A H_c. After immobilization of the first Mab, BoNT/A H_c was injected over the prepared surface, followed by a second Mab. A Mab with specificity identical or highly similar to its partner in a dual combinatorial trial will not bind BoNT/A H_c because the epitope is masked by the first Mab (A). In contrast, a Mab that binds a different epitope on the antigen will bind the antigen (B), causing an increase in signal in the corresponding sensorgram. In this way, by using all combinations of the eleven Mabs in the study, the authors were able to define the relative position of at least two functional epitopes on the toxbin. (For more details on epitope mapping, see Biacore Application Note 1: Characterization of monoclonal antibody epitope specificity using Biacore’s SPR technology).
Results

Mab generation and isotype characterization

All hybridoma supernatants were tested using ELISAs in which BoNT/A or BoNT/A HC were used as capture molecules. Of the 660 generated Mabs that were reactive to BoNT/A HC, 488 (74%) also recognized the whole native toxin, BoNT/A. Some of these positive pre-clones were then expanded and the supernatants were tested for their ability to protect mice from the lethal effects of BoNT/A. Hybridomas were further expanded, screened and subcloned and eleven were finally selected for further characterization. Two Mabs of isotype IgG1 and nine of isotype IgG2a were purified on a protein G column. The pattern of Mab specificity was estimated by testing five of these eleven Mabs in ELISAs using alternative BoNT HC isotypes (BoNT/A, BoNT/B and BoNT/E) as capture molecules. All Mabs bound BoNT/A HC and did not cross-react with either BoNT/B HC or BoNT/E HC.

Toxin-neutralizing function of Mabs

Four of the five selected Mabs fully prevented mortality in mice infected with native toxin. They were, however, specific to intoxication with BoNT/A and were completely ineffective against either BoNT/B or BoNT/E.

Interaction of BoNT/A HC with neutralizing Mabs

All neutralizing antibodies tested had similar on-rates (k_a between 3.1 x 10^5 M^-1 s^-1 and 17 x 10^5 M^-1 s^-1) while the off-rates were more varied (k_d between <0.2 x 10^-4 s^-1 and 6.7 x 10^-4 s^-1). All neutralizing antibodies tested interacted with BoNT/A HC with high affinity (K_D < 0.86 nM).

Epitope mapping

All combinations of Mabs were tested in sandwich assays (first Mab : BoNT/A HC : second Mab). An increased sensorgram signal on addition of the second Mab indicated that the Mabs bound distinct epitopes. Absence of a signal increase indicated shared or overlapping epitopes (Figure 3). No increase in signal was seen if the first and second antibodies were identical.

![Figure 3](https://example.com/figure3.png)

Figure 3. Binding profiles of Mabs to overlapping and discrete epitopes on BoNT/A HC using combinations of Mab pairings. HEPES-buffered saline was injected over the surface between each treatment. In the sensorgram shown here, a Mab designated 4A2-2 was used as the capture species. Irrelevant IgG (blocking reagent), BoNT/A HC and a second Mab were then added in sequence. Of the four partners tested, only Mab 6B2-2 bound BoNT/A HC captured by 4A2-2, strongly indicating the existence of a second epitope defined by 6B2-2.
Antibodies fell into three classes in the epitope mapping experiments, identifying two and possibly three epitopes on the antigen. Mab 6B2-2 bound independently of all other antibodies and thus addresses a distinct epitope. All other Mabs except 6C2-4 prevented each other’s binding and thus identify a second epitope. The interaction pattern of Mab 6C2-4 suggests a site partially overlapping these other two epitopes.

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Table 1. Composite epitope mapping of BoNT/A Hc using all combinations of neutralizing Mabs.

| Unimpeded binding of second Mab to BoNT/A Hc after capture by first Mab (recognition of distinct epitopes) |
| Partly impeded binding of second Mab to BoNT/A Hc after capture by first Mab (recognition of overlapping or proximal epitopes) |
| Fully impeded binding of second Mab to BoNT/A Hc after capture by first Mab (recognition of identical epitopes) |

Figure 4. Interpretation of the combinatorial epitope mapping studies.
The binding data in Table 1 indicated a discrete epitope on BoNT/A that was recognized by Mab 6B2-2: all other Mabs in the study were still able to bind BoNT/A Hc after capture with 6B2-2. In addition, a separate epitope was recognized by 6C2-4, the occupancy of which partly impeded binding by Mabs of the 6E9/10 designation but which completely inhibited binding by Mabs 4A2-2 and 4A2-4. In summary, the data suggested the existence of two distinct epitopes, one of which was defined solely by 6B2-2, while the other was defined largely by 6C2-4 (which shared overlapping sites defined by 4A2-2 and 4A2-4, and possibly a proximal epitope defined by Mabs of the 6E9/10 designation).
The work of Pless et al. described herein shows how Biacore can be used to select high affinity antibodies and also to define epitopes on botulinum toxin that may expedite the development of effective peptide-based antagonists. Indeed, the consensus in the scientific literature supports a move to generate high potency engineered Mabs or oligoclonal antibody cocktails, a strategy that may be realised by the application of Biacore-based assays to provide a rapid and reliable means to characterize the binding kinetics and affinities of new reagents for a range of particular toxins.

Studies using mice and primates indicate that botulism can be prevented by intravenous administration of botulinum toxin heavy chain and neutralizing antibodies, respectively. The present antitoxin treatment regime in humans consists of polyclonal antibodies, which although effective if given early, may sensitize the patient over extended periods. In addition, as vaccines require time to induce protective immunity, passive antibody treatment may be the only viable, short-term solution in a critical situation such as the aftermath of a suspected terrorist attack. It is even suggested that in preparation for such a scenario, specific antibodies should be stockpiled.

As the half-life of human IgG in human serum is approximately one month, one attractive strategy is the development of humanized antibodies containing complementarity determining regions from optimal neutralizing Mabs grafted into a human antibody framework. Recently, Nowakowski et al. showed how BoNT/A could be potently neutralized in vivo using an “oligoclonal” cocktail of three recombinant Mabs derived from antibody single chain variable fragments (scFv) (scFv fragments have proven unsuitable for in vivo toxin neutralization due to rapid clearance of these relatively small proteins from serum). Nowakowski et al. used Biacore to compare the binding kinetics and affinities of recombinant Mabs compared with the source scFv fragments. In the three cases tested, the affinities of the constructed Mabs for BoNT/A were up to three orders of magnitude higher than those of the source scFvs, with increased association rate accounting for most of the difference. By administering the Mabs in combinations of two and three together with BoNT/A, the protective potency of the Mabs was increased enormously, reducing mortality to the extent that the full oligoclonal Mab cocktail offered complete protection to mice exposed to as much as 5000 ID$_{50}$ of toxin (and even gave a 50% survival rate at 20000 ID$_{50}$). These data strongly support the authors’ proposition that efforts should be concentrated on the generation of stockpiles of oligoclonal Mabs, and that this strategy should be considered for further category A toxins including other BoNT serotypes and toxins released by B. anthracis and possibly Yersinia pestis.
References

Citations in boldtype contain detailed accounts of Biacore experiments.

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   Bioterrorism: a clear and present danger
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Further reading

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2. Lévêque, C., Ferracci, G., Seagar, M. and Miquelis, R.
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3. Biacore Application Note 1, BR-9000-36
   Characterization of monoclonal antibody epitope specificity using Biacore's SPR technology
