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Mechanisms of enhanced neutralization of botulinum neurotoxin by monoclonal antibodies conjugated to antibodies specific for the erythrocyte complement receptor☆

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ABSTRACT

Immune complexes formed between monoclonal antibodies (mAbs) and toxins can neutralize toxicity *in vivo* by multiple mechanisms. Toxin sequestration and clearance by mAbs may be improved by enhancing their ability to bind to red blood cells (RBCs) through immune adherence. This can be achieved by converting the mAbs to heteropolymers (HPs), which are antigen-specific mAbs cross-linked to mAbs targeting the complement receptor (CR1), a protein that is expressed on the surface of RBCs in primates and mediates delivery of complement C3b-containing immune complexes to tissue macrophages. Conversion of mAbs to HPs has been shown to enhance clearance of multivalent antigens from the blood circulation, but the interaction of HPs with monovalent toxins has not been examined. Using botulinum neurotoxin (BoNT) as a model system, we studied the effect of conversion of a pair of BoNT-specific mAbs into HPs on toxin neutralization and handling *in vivo*. Two HPs given in combination had 166-fold greater potency than un-modified mAbs, neutralizing 5000 LD50 BoNT, when tested in transgenic mice expressing human CR1 on RBC membranes. Improvement required adherence of BoNT to the RBC *in vivo* and 2 HPs, rather than an HP + mAb pair. The HP pair bound BoNT to RBCs in the circulation for 2 h, in comparison to BoNT-neutralizing anti-serum, which induced no detectable RBC binding. HP pairs exhibited enhanced uptake by peritoneal macrophages *in vitro*, compared to pairs of mAbs or mAb + HP pairs. In a post-exposure therapeutic model, HPs gave complete protection from a lethal BoNT dose up to 3 h after toxin exposure. In a pre-exposure prophylaxis model, mice given HP up to 5 days prior to BoNT administration were fully protected from a lethal BoNT dose. These studies elucidate general mechanisms for the neutralization of toxins by HP pairs and demonstrate the potential utility of HPs as BoNT therapeutics.

1. Introduction

Botulinum neurotoxins (BoNT) are a serologically diverse family of molecules produced by organisms of the genus *Clostridium*. BoNTs are the most potent biological toxins known and have been designated as category A select bioterror agents (Arnon et al., 2001). BoNTs induce peripheral neuromuscular and autonomic paralysis by inhibiting cholinergic function. The process of intoxication proceeds by a number of steps, generally beginning with either oral or inhalational exposure. BoNT crosses the intestinal or respiratory epithelium and then transits through the blood circulation to reach its target sites, cholinergic nerve endings at neuromuscular junctions (NMJ) (Simpson, 2013). At the NMJ, BoNT is internalized by the presynaptic neuron through endocytosis. Within the neuron,

Abbreviations: BoNT, botulinum neurotoxin; BoNT/A, serotype A botulinum neurotoxin; CR1, complement receptor; Fab′, mAb antigen binding domain; HC50A, BoNT/A recombinant 50 kD C-terminal domain; FP, a fusion protein consisting of a streptavidin molecule and an scFv specific for glycophorin; hCR1, human complement receptor; HP, heteropolymer; HRP, horseradish peroxidase; i.p., intra-peritoneal; i.v., intravenous; mAb, monoclonal antibody; mAb, monoclonal antibody; NMJ, neuromuscular junction; OPD, o-phenylenediamine dihydrochloride; PBS, phosphate buffered saline; RBCs, red blood cells; RI-BoNT, recombinant inactive BoNT; scFv, single-chain variable fragment.

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the BoNT catalytic light chain domain exits the endocytic vesicle and enters the cytoplasm, where it cleaves proteins that are required for the release of acetylcholine in response to neuronal stimulation. Once BoNT has been internalized by a nerve ending and has cleaved its substrate, the nerve ending is no longer functional. Therefore, BoNT countermeasures need to prevent interaction of the toxin with cholinergic nerve endings. Methods that use monoclonal antibodies (mAbs) to sequester BoNT in the blood circulation and enhance clearance can contribute to BoNT neutralization by interfering with a key step in BoNT intoxication.

Because BoNT exists in 7 known serotypes and multiple sub-serotypes that can differ dramatically in mAb binding and sensitivity, a comprehensive biodefense preparedness strategy for BoNT exposure may require dozens of different mAbs (Hill et al., 2007; Smith et al., 2005). The primary motivation for the present study is that mAbs capable of binding to multiple BoNT serotypes appear to be less potent at neutralization than single serotype-specific mAbs, so optimizing BoNT sequestration and clearance may be important for creating a definitive, poly-specific BoNT therapeutic (Garcia-Rodriguez et al., 2011).

Antibody binding induces rapid clearance of BoNT from the bloodstream through sequestration of BoNT in the liver and spleen (Ravichandran et al., 2006). Clearance requires binding of polyclonal antiserum or at least three distinct antibodies (L. Simpson and F. Al-Saleem, unpublished observations) (Nowakowski et al., 2002; Ravichandran et al., 2006). The mechanism is extremely potent, with a capacity of neutralizing >10,000 LD50 BoNT, and occurs within minutes of intravenous injection (Nowakowski et al., 2002; Ravichandran et al., 2006). This clearance can also be induced with polypeptide-tagged single-chain variable fragments (scFv) that form immune complexes when mixed with a mAb specific for the polypeptide tag (Sepulveda et al., 2010). The mechanism for clearance of BoNT in an immune complex likely involves capture by Fcγ receptor-bearing fixed tissue macrophages (Takai, 2005). Complement-mediated mechanisms may contribute to this process, as a study in humans showed that a proportion of antibody-containing immune complexes can incorporate complement C3b and adhere to red blood cells (RBCs) through complement receptor type 1 (CR1) (Davies et al., 1990).

The ability of mAbs to sequester antigens in the blood circulation and deliver them to fixed tissue macrophages can be enhanced by directly binding them to RBCs through CR1 binding. "Heteropolymers" (HPs) are cross-linked mAb complexes in which one of the mAbs is specific for CR1 and the other mAb binds to a specific antigen (Lindorfer et al., 2001a). HPs are superior to un-modified mAbs in promoting antigen clearance. HP + antigen complexes bound to RBCs are taken up and processed by macrophages using essentially the same mechanism by which C3b-opsonized antigens bound to RBCs are cleared (Mohamed et al., 2005). This increases the efficiency of clearance of antigen from the circulation. This process of immune adherence may contribute to the defense against bacteria and viral pathogens *via* sequestration, preventing interaction with susceptible tissues.

In a previous study, we induced RBC immune adherence of BoNT + mAb complexes using a fusion protein (FP) that comprised a streptavidin molecule fused to an scFv specific for the RBC membrane protein glycophorin (Adekar et al., 2011). The FP enhanced BoNT neutralization of a pair of mAbs 166-fold by molar ratio. Compared to targeting glycophorin, which primarily plays a structural role on the RBC surface, targeting of CR1 may differ in its mechanism of neutralization because it may replicate aspects of complement-mediated immune complex clearance. HPs may also improve clearance through better interaction with Fcγ receptor-bearing fixed tissue macrophages, because they each contain two Fcγ domains, double that of IgG + FP complexes. We were also interested in studying the interaction of HPs with heterodimeric toxins, such as BoNT, which may behave differently from previously studied HPs that target multivalent antigens, such as phage, bacteria, and IgM (Lindorfer et al., 2001a,b; Mohamed et al., 2005).

2. Materials and methods

2.1. Monoclonal antibodies and conversion into heteropolymers

We used human mAbs specific for either the BoNT serotype A (BoNT/A) heavy chain or light chain A, referred to as 6A and 4LCA, respectively; the anti-CR1 mouse IgGs mAbs 7G9 and HB8592, and the isotype control 7B7 (anti-ΦX174), which have all been described previously (Adekar et al., 2008a,b; Lindorfer et al., 2001a). The HPs were constructed by chemical cross-linking as previously described (Lindorfer et al., 2001b). The final products were subjected to gel filtration in borate saline buffer on Superose 6 (GE Healthcare Life Sciences, Piscataway, NJ), which was calibrated with monomeric IgG, in order to separate cross-linked from monomeric IgG. Cross-linked HP products were pooled and stored at 4 °C. The specific HPs are noted by the conventions we have previously described (Lindorfer et al., 2001a). For example, the anti-botulinum neurotoxin heavy chain A mAb (6A), cross-linked with anti-CR1 mAb (7G9), is 6A X 7G9. Here, these names have been abbreviated, with the suffixes HP, HP-HB, and HP-CTRL denoting HPs containing the 7G9, HB8592, or 7B7 mAbs, respectively (*e.g.*, 6A-HP, 6A-HP-HB, 6A-HP-CTRL, 4LCA-HP, 4LCA-HP-HB, and 4LCA-HP-CTRL).

2.2. Tg-hCR1 transgenic mouse colony breeding and genotyping

Tg-hCR1 transgenic mice (courtesy of Dr. Robert W. Finberg) express the human complement receptor (hCR1) gene under the control of the RBC-specific GATA1 promoter (Repik et al., 2005). Tg-hCR1 heterozygous breeders were mated with C57BL/6 mice (Taconic, Hudson, NY). hCR1 positive animals were detected using PCR of tail DNA extracted using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Amplification of DNA was performed using GoTaq Flexi DNA polymerase (Promega, Madison, WI). CR1 forward and reverse primers reported previously, were (5′-ACCCTTTCTGTCC-TCACA-3′) and (5′-TTTCTCCCTCCGCTTCCAGTTG-3′) (Repik et al., 2005). Thermal cycling consisted of 35 cycles of 94 °C, 30 s, 60 °C 30 s, 72 °C 60 s. RBC hCR1 expression was verified by flow cytometry with the anti-mouse TER-119 FITC (eBiosciences, San Diego, CA) and anti-CR1-PE (Southern Biotechnology, Birmingham, AL) on a BD FACSCantoII (Becton Dickson, Franklin Lakes, NJ), using FlowJo 8.8.6. software (Tree Star, Ashland, OR).

2.3. Analysis in vitro of HP and HP complexes binding to RBCs

Blood from Tg-hCR1 mice was collected in heparinized tubes and RBCs were isolated. The RBCs were washed with 200 μl PBS/1% BSA (PBSA) and centrifuged at 326 × *g* in a microfuge. HC50A, the 50 kD C-terminal domain of BoNT serotype A (13), was biotinylated using a FluoReporter Mini-biotin-XX protein labeling kit (Invitrogen, Carlsbad, CA). Biotinylated HC50A (BIOT-A) was incubated with 1:100 diluted PE-Streptavidin (PE-SA; Jackson ImmunoResearch, West Grove, PA), rotating for 30 min at 4 °C. BIOT-A with PE-SA was then added to RBCs with 20 ng HP and anti-human IgG APC (Jackson Immunoresearch), incubated at RT for 30 min, washed twice in PBSA, resuspended in a final volume of 1 ml PBSA, and analyzed by flow cytometry for RBCs that were "double positive", thus indicating that both HP and biotinylated HC50A were bound to the RBCs.