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Mucosal Vaccine Targeting Improves Onset of Mucosal and Systemic Immunity to Botulinum Neurotoxin A

Massimo Maddaloni,* Herman F. Staats,† Dagmara Mierzejewska,‡ Teri Hoyt,* Amy Robinson,* Gayle Callis,* Shunji Kozaki,§ Hiroshi Kiyono,‖ Jerry R. McGhee,¶ Kohtaro Fujihashi,¶ and David W. Pascual2*

Absence of suitable mucosal adjuvants for humans prompted us to consider alternative vaccine designs for mucosal immunization. Because adenovirus is adept in binding to the respiratory epithelium, we tested the adenovirus 2 fiber protein (Ad2F) as a potential vaccine-targeting molecule to mediate vaccine uptake. The vaccine component (the host cell-binding domain to botulinum toxin (BoNT) serotype A) was genetically fused to Ad2F to enable epithelial binding. The binding domain for BoNT was selected because it lies within the immunodominant H chain as a β-trefoil (Hcβtre) structure; we hypothesize that induced neutralizing Abs should be protective. Mice were nasally immunized with the Hcβtre or Hcβtre-Ad2F, with or without cholera toxin (CT). Without CT, mice immunized with Hcβtre produced weak secretory IgA (sIgA) and plasma IgG Ab response. Hcβtre-Ad2F-immunized mice produced a sIgA response equivalent to mice coimmunized with CT. With CT, Hcβtre-Ad2F-immunized mice showed a more rapid onset of sIgA and plasma IgG Ab responses that were supported by a mixed Th1/Th2 cells, as opposed to mostly Th2 cells by Hcβtre-dosed mice. Mice immunized with adjuvanted Hcβtre-Ad2F or Hcβtre were protected against lethal BoNT serotype A challenge. Using a mouse neutralization assay, fecal Abs from Hcβtre-Ad2F or Hcβtre plus CT-dosed mice could confer protection. Parenteral immunization showed that the inclusion of Ad2F enhances anti-Hcβtre Ab titers even in the absence of adjuvant. This study shows that the Hcβtre structure can confer protective immunity and that use of Hcβtre-Ad2F gives more rapid and sustained mucosal and plasma Ab responses. The Journal of Immunology, 2006, 177: 5524–5532.

Botulism occurs from the infection by Clostridium botulinum or by ingestion of its neurotoxin complex, resulting in flaccid paralysis (1, 2). Due to its potency, the illicit dissemination of botulinum neurotoxins (BoNT)3 poses a major threat to the unprotected populace. Of the seven serotypes, serotypes A, B, and, to a minor extent, E are typically associated with botulism in humans, whereas serotype C mostly affects domestic animals (1, 2). The toxins are each naturally synthesized as a single 150-kDa polypeptide, requiring posttranslational proteolysis to yield a ∼50-kDa fragment or L chain, containing the catalytic activity, and a 100-kDa component or H chain (∼100 kDa), containing the translocation domain in the N terminus and the cell-binding domain in the C terminus (Hc). Upon binding to the target cell, the translocation domain in the N terminus translocates the L chain to the cytosol, and the L chain endopeptidase activity activates a group of proteins (termed SNARE proteins) required for release of acetylcholine at the neuromuscular junction resulting in flaccid paralysis (3–6).

The current vaccine is a pentavalent one with as little at 10% of the toxoid preparation representing neurotoxin (7), suggesting that much of the immune response induced by the vaccine is directed against vaccine components that will not contribute to protection against BoNT lethal challenge. Because formalin is used to inactivate BoNT to produce the toxoids for vaccine formulation, such chemical modification of the toxins results in a considerably altered tertiary structure (8) needed to elicit neutralizing Abs (9–11). Thus, better formulations are needed. To this end, structural analysis of the catalytic and binding sites of C. botulinum BoNT serotype B reveals that the Hc is structurally subdivided into a N-terminal subdomain, forming a jelly-roll motif, and a C-terminal subdomain, forming β-trefoil structure (5). We hypothesize that this β-trefoil structure contains the important neutralizing epitopes. In fact, in a previous study, neutralizing Abs against Hc BoNT serotype A (BoNT/A) exhibited two major protective epitopes, BoNT/A155–661 and BoNT/A1150–1289, which conferred ∼75% protection, and one minor protective epitope, BoNT/A790–939, which conferred 28% protection (9). The neutralizing epitope BoNT/A1150–1289 conferred 75% protection and spanned about two-thirds of the β-trefoil region. Taken together, these results hint that the intact β-trefoil structure would be a potential candidate for an optimized vaccine.

Replication-deficient adenovirus vectors (12) and other viruses (13) have been experimentally used to correct genetic deficiencies, but these viral vectors have remained, in large part, immunogenic.

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3 Abbreviations used in this paper: BoNT, botulinum neurotoxin; Hcβtre, β-trefoil structure of Hc for BoNT; Ad2F, adenovirus 2 fiber protein; AFC, Ab-forming cell; CFC, cytotoxicity-forming cell; CM, complete medium; DAPI, 4′,6-diamidino-2-phe-nylindole; CT, cholera toxin; LN, lymph node; slgA, secretory IgA.
Despite this condition, adenovirus vectors retain the advantage of transfecting the airway epithelium (14, 15). However, examination of the adenovirus components shows that adenovirus adhesion is mediated by the interaction between its attachment or fiber protein and receptors on the cell surface (15). Study of adenovirus (16) and reovirus (17) attachment proteins reveals that they share a common feature in that they are homotrimeric to form a high-energy structure that springs open upon binding its cell receptor, thus triggering a series of events ultimately leading to the entry of the virus into the cell. Therefore, it may be possible that adenovirus 2 fiber protein (Ad2F) could potentially be used in lieu of the intact virus for mucosal targeting.

In an effort to improve the current BoNT vaccine, we adopted a two-prong approach to create a mucosal vaccine against BoNT/A. First, we questioned whether the β-trefoil structure, which is responsible for the cell-binding activity, would be sufficient to elicit neutralizing Abs because this structure is maintained in all serotypes, as well as in tetanus toxoid (18). Accordingly, our vaccine includes this β-trefoil structure. In addition, we hypothesize that a fusion protein between this β-trefoil structure from BoNT/A genetically fused to adhesin Ad2F attachment protein may enhance its immunogenicity and uptake by the common mucosal immune system.

In this study, we showed that the addition of the mucosal-targeting molecule, Ad2F, enhanced onset of plasma and mucosal Ab responses. Both vaccines (with and without Ad2F) conferred complete protection from systemic challenge with as much as 20,000 LD50 of the native BoNT/A. Upon evaluation of mucosal Abs to the immunodominant Hc as a β-trefoil (Hcβtre) structure, mice dosed with Hcβtre-Ad2F showed the best protection against the BoNT/A complex.

Materials and Methods
DNA manipulations
As shown by others (19), initial attempts to express the C. botulinum gene encoding the Hc BoNT/A gene in yeast failed to yield any protein. Consequently, a synthetic gene encoding for Hc BoNT/A amino acids R864 to L1295 (GenBank accession no. X52066) was designed for optimized expression in the yeast Pichia pastoris. Factors taken into account for the synthetic gene were yeast codon bias, the reduction of C. botulinum ATC content, and the necessity for not depleting any particular tRNA pools. The Hcβtre spans from position S1096 to L1295, as predicted by others (18). To clone the Hcβtre domain, we included 15 more amino acids upstream from the predicted Hcβtre beginning with K1076 to L1295 to facilitate proper folding of the relevant domain.

To clone the Hc BoNT/A gene into the expression vector, the synthetic gene encoding for Hc BoNT/A was amplified with primers containing EcoRI and SalI restriction sites. Likewise, to clone the Hcβtre, this gene was amplified from the synthetic Hc BoNT/A using primers containing EcoRI and SalI restriction sites. In both cases, the 5' primers containing the EcoRI site also provided an ATG initiation codon embedded into an optimal Kozak's sequence. The PCR products were cloned into a pCRII site also provided an ATG initiation codon embedded into an op-

Protein expression, purification, and FACS analysis
Recombinant proteins were produced by shifting the carbon source from glycerol to methanol, as required with P. pastoris. Briefly, yeast cells were cultured in yeast nitrogen base-glycerol until OD 0.5–1.0. Cells were then harvested by centrifugation and resuspended in yeast nitrogen base-methanol for 36–48 h. Pelleted biomass was either used right away or stored at −80°C until needed. Cells were disrupted by bead-beater in ice, and debris were cleared by centrifugation, followed by filtration through a 1.2-μm filter, then by filtration through a 0.45-μm filter under vacuum. The cleared supernatant was then applied to a Talon column (BD Biosciences), as per the manufacturer's instructions. Purified proteins were eluted, tritiated, and loaded onto a 12% polyacrylamide gel. Recombinant Hcβtre proteins were analyzed by Coomassie-stained SDS-PAGE to assess the quality of the protein. The Red2-Ad2F was blotted and probed with a commercial rabbit anti-Red2 Abs (BD Biosciences).

Histological preparation
Mice were nasally dosed with 50 μg Red2-Ad2F, and after 90 min, mice were euthanized to harvest heads. The skin, tongue, and lower jaw were removed, and the remaining portion was embedded into OCT (Sakura Finetek) with the top of turbinates oriented in the bottom of plastic cryomold to avoid cutting teeth. Turbinates were then snap frozen at −80°C. Turbine frozen sections were cut at −30°C in a Leica Cryocut 1850 (Leica Microsystems) using Instrumedics Cryocane Tube Transfer System and a D profile tungsten carbide cryostat knife (Delaware Diamond Knives) to retain the undecalcified bone with epithelial lining on a slide. Frozen sections were air dried 30 min, then cover-slipped unfixed using Prolong Gold ready-to-use Anti-Fade Reagent with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes).

Immunizations
Specific pathogen-free BALB/c mice were obtained from the National Cancer Institute and maintained in the Animal Resources Center at Montana State University (Bozeman, MT). All mice were kept under pathogen-free conditions in individually ventilated cages under HEPA-filtered, barrier conditions and fed sterile food and water ad libitum. The mice were free of bacterial and viral pathogens, as determined by Ab screening and by histopathologic analysis of major organs and tissues. Mice between age 5 and 8 wk were immunized nasally with 50 μg of Hcβtre or Hcβtre-Ad2F plus 5 μg of cholera toxin (CT; List Biological Laboratories), and boosted with their respective vaccines on days 7 and 14 postimmunization with 2 μg of CT. For i.m. immunizations, BALB/c mice were immunized with equimolar amounts of Hcβtre Ag, which represented 50 μg of Hcβtre-Ad2F and 25 μg of Hcβtre on days 0, 7, and 14 with or without 1.0 μg of CT given at the anterior tibial tuberosity to ensure delivery into the muscle (20).

ELISA to detect BoNT/A β-trefoil Abs
Standard ELISA protocols were followed to quantitate the Abs induced to BoNT/A Hcβtre. Recombinant BoNT/A Hcβtre (5 μg/ml) was used to coat Maxisorp microtiter plates (Nunc) overnight at 4°C. Immune plasma, fecal extracts, or nasal washes from individual mice were evaluated for their
activity against the HcBoNT. Fecal samples were extracted from the super-

natants of 10% (v/v) suspension of sample and PBS with 50 μg/ml soy-

bean trypsin inhibitor (Sigma-Aldrich). Nasal washes were collected by

intubation of the trachea to access the nasopharyngeal cavity. An ~1-inch

long tygon tubing (internal diameter 0.010 inches, outside diameter 0.030

inches; Cole-Parmer) was attached to a 1.0-ml syringe, an approach used

to avoid any blood contamination of the nasal washes. A total of 200 μl

of PBS was inserted via the trachea, and the exudate from the nares was
collected into microfuge tubes. Cells and debris were removed by centrifu-
gation for 10 min at 10,000 × g. HRP-goat anti-mouse IgA, IgG, IgG1,
IgG2a, and IgG2b (Southern Biotechnology Associates) or biotinylated rat
anti-mouse IgE mAb (BD Pharmingen) plus HRP-goat anti-biotin Abs (Vector
Laboratories) were used for detection. Following 90 min of incuba-
tion at 37°C and a washing step, the specific reactivity was determined
by the addition of an enzyme substrate, ABTS (Moss) at 100 μl/well.
Absorbance was measured at 415 nm on a Kinetics Reader model EL312
(Multiscan). Endpoint titers were defined as the highest reciprocal
dilution of sample giving an absorbance at OD415 above 0.100 OD

units above negative controls after 1 h of incubation at 25°C. For total IgE
ELISA, a similar protocol was followed as for Ag-specific ELISA already
described, but wells were coated with rat anti-mouse IgE mAb (2.0 μg/ml;
BD Pharmingen). Serum, serum diluted, or IgE anti-trinitrophenyl mAb
(Vector Laboratories) were used for detection. Following incubation for 2 h
at 37°C, washing, serial dilutions of serum or IgE anti-trinitrophenyl were
at 4°C overnight. After washing, the wells were developed with
antimouse IgG antihuman IgG (H+L) (Jackson ImmunoResearch) and
400 μl of AEC (Moss), and the reaction was allowed to continue until spots
were detected. To assess the protective value of the HcBoNT vaccine, BALB/c mice were
immunized nasally with equimolar doses of HcBoNT or HcBoNT-Ad2F with
or without CT as adjuvant, as previously described. For the first
experiment, one group of mice was immunized nasally with 2 μg of BoNTox-
oid/A (BD Biologics), BoNToxoid/A (0.2 μg of CT combined with 1/50
HcBoNT) and BoNT/A. On day 63 or day 96, mice were challenged i.p. with ~20,000
and 2000 LD50 BoNT/A (2 × 106 mouse LD50/mg, lot no. A121004-01; Meta-
biologics), respectively, in 200 μl of PBS containing 0.2% gelatin, as pre-
viously described (23). Mice were monitored hourly for the first 6 h
and then daily for signs of morbidity, including difficulty breathing and lack of
activity. Mice were euthanized when signs of morbidity were observed.
To determine whether nasal immunization with HcBoNT immunogens
was able to induce protective Abs in mucosal secretions against BoNT/A,
we picked a mouse neutralization assay using BoNT/A complex (3.8 × 107
mouse LD50/mg, batch no. MA0305, lot no. A121005-01; Metabiologics).
In this assay, filter-sterile fecal extracts from naive or day 21 samples were
combined with BoNT/A complex (1/2 final dilution of fecal extract) and
incubated at room temperature for 30 min before i.p. injection into naive
BALB/c mice. As a positive control, some of the naive fecal extracts
were spiked with immune sera from mice immunized nasally with Hc BoNT/A
plus CT at a final dilution of 1/110. If the anti-HcBoNT Abs in the fecal
extracts were able to neutralize BoNT/A in the complex, the mice would
not exhibit BoNT/A intoxication. If the fecal extracts did not contain
BoNT/A-neutralizing Abs, the mice would exhibit signs of BoNT/A tox-
icity, including shortness of breath and immobility. These were monitored
twice daily for up to 5 days. Morbid mice were euthanized in accordance
with the Institutional Animal Care and Use Committee of both institutions.

Statistical analysis
An ANOVA, followed by Tukey’s method, was used to evaluate differ-
ences between variations in Ab titers, Ab AFC, and cytokine-forming cell
(CFC) levels and discerned to the 95% confidence interval. The Kaplan-
Meier method (GraphPad Prism, GraphPad) was applied to obtain the sur-
vival fractions following intoxication with BoNT/A or the BoNT/A com-
plex. Using the Mantel-Haenszel log rank test, the p value for statistical
differences between surviving BoNT/A challenge and HcBoNT plus CT,
HcBoNT-Ad2F plus CT, HcBoNT alone, HcBoNT-Ad2F alone, or BoNToxoid
plus CT vaccinated mice or fecal extracts derived from vaccinated and
naive mice were discerned at the 95% confidence interval. Results
Recombinant Ad2F exhibits cell-binding activity
We hypothesized that the addition of a mucosal-targeting ligand,
i.e., Ad2F, would possibly enhance vaccine uptake. To test for this
response, several recombinant proteins were produced for this study
(Fig. 1A). The Hc fragment of the C. botulinum neurotoxin A (Hc BoNT/A)
is a small molecule subdomain corresponding to the binding domain for
Hc referred to as the β-trefoil (Hcβtre) structure. A fusion protein between the Hcβtre and the Ad2F re-
flected as to the Hcβtre-Ad2F was also assessed. Finally, a fusion
between the Red2 fluorescent protein and Ad2F referred to as Red2-Ad2F was used. The recombinant Hc, Hcβtre, and Hcβtre-
Ad2F migrated with the expected molecular mass of 58, 27.8, and
50 kDa, respectively, as determined by SDS-PAGE (Fig. 1B) or 60
kDa for Red2-Ad2F, as assessed by Western blot (Fig. 1C). To
 assess whether the Ad2F could bind to the mucosal epithelium,
binding studies to human HeLa and 293A cells were performed using the Red2-Ad2F (Fig. 1D). The Red2-Ad2F was able to bind to
both HeLa and 293A cells, but not the recombinant Red2. Nei-
ther Red2-Ad2F nor Red2 were able to bind to mouse L cells,
suggesting that the Ad2F was indeed functional. To test whether this
protein could bind to mouse epithelium, BALB/c mice were
nasally dosed with Red2-Ad2F and after 90 min, nasal passages
were evaluated for protein binding. As depicted in Fig. 1E, the

MUCOSAL BoNT β-TREFOIL VACCINES

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FIGURE 1. Construction of novel BoNT/A vaccines using the β-trefoil (βtre) structure of the BoNT/A Hc adapted with and without the adhesin Ad2F. A, Schematic depictions of BoNT/A Hc, Hcβtre/A (Hcβtre), Hcβtre genetically fused to Ad2F (Hcβtre-Ad2F), and the Red2 fluorescent protein genetically fused to Ad2F (Red2-Ad2F). B, Coomassie blue stained 12% SDS-PAGE of the Hcβtre vaccines. Hc (≈58. kDa), Hcβtre-Ad2F (≈50 kDa), and Hcβtre (≈27.8 kDa). C, The Red2-Ad2F migrates with the expected molecular mass of ≈60 kDa as detected by Western blot analysis using a polyclonal rabbit anti-Red2 Ab. D, FACS analysis of Red2-Ad2F binding to human epithelial HeLa and 293A cells (open histograms), but not to L929 fibroblasts. E, Red2-Ad2F binds to mouse nasal epithelium. BALB/c mice were given 50 μg of Red-Ad2F nasally, and 90 min later, nasal passages were harvested for frozen sections and evaluated for Red2 fluorescence (on apex of turbinate cells) together with DAPI nuclear (blue) stain. F, An adjacent section was cut for H&E staining.

Red2 fluorescence was associated with the nasal epithelium on its luminal surface.

Induction of BoNT-specific mucosal IgA and plasma IgG
To test whether Ad2F could efficiently deliver Hcβtre to the mucosa, mice were immunized nasally on days 0, 7, and 14. A kinetic analysis was performed and showed that the Ad2F could effectively stimulate a rapid secretory IgA (sIgA) Ab response to the Hcβtre, whereas nasal immunization with Hcβtre alone could not (Fig. 2A). Only weak plasma IgG anti-Hcβtre Ab titers were elicited with Hcβtre-Ad2F and none with Hcβtre alone. Thus, in subsequent studies, all immunizations were conducted with CT as mucosal adjuvant. Adjuvanted doses of Hcβtre-Ad2F resulted in a rapid onset of sIgA Ab titers when compared with onset for adjuvanted dosages of Hcβtre, which was delayed by 2 wk (Fig. 2A). In a similar fashion, adjuvanted doses of Hcβtre-Ad2F also resulted in a rapid onset of plasma IgG titers when compared with onset for dosages of Hcβtre plus CT, which was delayed by 1 wk (Fig. 2B). Differences in plasma IgG1 and IgG2a, but not IgG2b responses, were observed between the two immunization groups (Fig. 2B). Hcβtre-specific IgE and total IgE levels were also measured during peak Ab responses on day 28. Mice nasally immunized with Hcβtre, Hcβtre-Ad2F, or Hcβtre-Ad2F plus CT failed to show Hcβtre-specific IgE (Fig. 3C); in the group of mice nasally dosed with Hcβtre plus CT, only one in 10 mice showed an IgE titer of ≥6.

To determine whether immune sIgA anti-Hcβtre Ab titers were present in nasal washes, samples were collected on day 35 postprimary immunization. Mice given nasal Hcβtre-Ad2F plus CT showed significantly higher levels of sIgA in nasal washes when compared with mice given Hcβtre plus CT as adjuvant (Fig. 3A). From these studies, we showed that Hcβtre is immunogenic, and its immunogenicity can be advanced when genetically fused to Ad2F.

Nasal immunization with Hcβtre-Ad2F enhances IgA AFC in nasal passages and submaxillary glands
To determine the distribution of Ag-specific B cells following nasal vaccination, an Ab ELISPOT was performed on lymphoid tissues for Hcβtre-Ad2F plus CT or Hcβtre plus CT-treated mice (Fig. 4). Nasal immunization with Hcβtre-Ad2F plus CT stimulated a pronounced number of Ag-specific IgA AFC in nasal passages and submaxillary glands when compared with mice dosed with the adjuvanted Hcβtre (p < 0.001) (Fig. 4A). Ag-specific IgA...
AFC were also significantly increased in Peyer’s patches (p < 0.05). The remaining Ag-specific IgA AFC response for the small intestinal lamina propria, spleen, and nasal-associated lymphoreticular tissue was the same for both groups. Evaluation of Ag-specific IgA AFC showed only subtle changes in small intestinal lamina propria, Peyer’s patches, spleen, nasal passages, and nasal-associated lymphoreticular tissue (Fig. 4C).

**Nasal vaccination confers protection against systemic BoNT intoxication**

Two challenge studies with BoNT doses that would cause intoxication in humans were conducted using immunized mice. In the first experiment, groups of BALB/c mice were nasally dosed with equimolar amounts of Hcβtre (25 μg) and Hcβtre-Ad2F (50 μg) with CT (2 μg). One control group was given BoNTA toxoid/A (2 μg) with CT, and another control group was left unimmunized. Mice were immunized four times at weekly intervals and assessed for immune titers on day 57 postprimary immunization. ELISA were performed using Hcβtre, Hc BoNT/A, or native BoNT/A as coating Ags (Fig. 6A). Both groups of mice dosed with either Hcβtre or Hcβtre-Ad2F showed elevated Ab endpoint titers against all three Ags; however, the Hcβtre-Ad2F-dosed group showed a slightly reduced titer against Hc BoNT/A. Mice immunized with BoNTA toxoid showed poor Ab reactivity against the immunizing immunogen, as well as to Hcβtre and Hc BoNT/A (Fig. 6A). On day 63, mice were challenged i.p. with 20,000 mouse LD50, which is equivalent to 1.0 human LD100 (3), and both the Hcβtre-dosed or Hcβtre-Ad2F-dosed groups showed complete protection (Fig. 6B). In addition, neither group showed signs of BoNT intoxication. Surprisingly, the mice immunized with the BoNTA toxoid failed to show any protective efficacy and succumbed to intoxication as quickly as naive mice (all within 3 h).

A second challenge study was performed using 10-fold less BoNT/A for the challenge. As described with our immunogenicity studies, mice were given only three doses of vaccine at weekly intervals. BALB/c mice were nasally dosed with 25 μg of Hcβtre or 50 μg of Hcβtre-Ad2F in the absence or presence of 2 μg of CT. The control group was only given three nasal doses of CT. On day 82, sera were collected and evaluated for IgG Abs specific for
Mucosal Abs protect against lethal challenge with the BoNT/A complex

To determine whether mucosal secretions contain neutralizing Abs can passively protect against BoNT/A, an in vivo mouse neutralization assay was used with the BoNT/A complex. The BoNT/A complex was used in these studies to mimic natural exposure to BoNT. In this assay, sterile-filtered fecal extracts were combined with 6.5 LD$_{50}$ of the BoNT/A complex, and incubated at room temperature for 30 min before i.p. injection into naive BALB/c mice. Sterile fecal extracts were prepared from mice immunized with HcBoNT/A or HcBoNTre. Mice nasally immunized with HcBoNTre plus CT and HcBoNTre-Ad2F and CT survived the ~2000 LD$_{50}$ lethal challenge. Thus, these studies showed that immunization with the HcBoNTre, either alone or fused to Ad2F, could elicit neutralizing Abs that protect against systemic lethal challenge.

Intramuscular immunization with HcBoNTre or HcBoNTre-Ad2F elicits elevated Ab titers

To assess how the Ad2F-targeting molecule may enhance the immunogenicity of the HcBoNTre, i.m. immunization studies with Hc BoNT/A and HcBoNTre. Each of the vaccinated groups showed elevated Ab titers to Hc BoNT/A and HcBoNTre, which were significantly greater ($p < 0.001$) than the Ab titers in the CT only immunized group (Fig. 7A). On day 96, mice were challenged i.p. with ~2000 LD$_{50}$. Most of the CT-dosed mice succumbed to the BoNT/A intoxication within 4 h, and none survived (Fig. 7B). Mice dosed with HcBoNTre or HcBoNTre-Ad2F alone succumbed to BoNT/A intoxication within 2 h. Mice nasally immunized with HcBoNTre plus CT and HcBoNTre-Ad2F and CT survived the ~2000 LD$_{50}$ lethal challenge. Thus, these studies showed that immunization with the HcBoNTre, either alone or fused to Ad2F, could elicit neutralizing Abs that protect against systemic lethal challenge.

FIGURE 5. Mucosal targeting enhances head and neck LN IFN-γ and IL-13 production following nasal immunization with HcBoNTre-Ad2F. Three weeks after their final immunization, HcBoNTre-Ad2F plus CT-dosed and HcBoNTre plus CT-dosed mice were evaluated for CFC responses in the spleen (A), head and neck LN (HNLN) (B), and mesenteric LN (MLN) (C) by a cytokine ELISPOT method. Immune lymphocytes were isolated and cultured with Hc BoNT/A (Hc), OVA, or media only for 2 days, and then evaluated for IFN-γ, IL-4, IL-5, IL-10, and IL-13 CFC. Data shown are mean ± SEM of CFC/10^6 lymphocytes from a total of three experiments. *, $p < 0.001$; **, $p ≤ 0.011$; ***, $p < 0.05$.

FIGURE 6. Nasal immunization with adjuvanted HcBoNTre-Ad2F or HcBoNTre, but not with BoNToxoid/A, confers complete protection in BALB/c mice subsequently challenged i.p. with 20,000 LD$_{50}$ BoNT/A on day 63 postprimary immunization. Immunizations were conducted on days 0, 7, 14, and 21, and the groups were subdivided as follows: Group 1 ($n = 3$ mice) given 25 μg of HcBoNTre plus CT (2 μg); Group 2 ($n = 4$ mice) given 50 μg of HcBoNTre-Ad2F (to ensure that equivalent amount of HcBoNTre was given); Group 3 ($n = 3$ mice) given 2 μg of BoNToxoid/A; and Group 4 ($n = 4$ mice) was left as naive. A. On day 57, plasma IgG titers from each group were measured against HcBoNTre, BoNT/A Hc, or native BoNT/A. The mice dosed with adjuvanted HcBoNTre showed a greater endpoint titer against Hc BoNT/A than mice dosed with HcBoNTre-Ad2F; however, both dosing groups showed similar endpoint titers against native BoNT/A. *, $p = 0.002$. B. On day 63, mice were challenged i.p. with 20,000 mouse LD$_{50}$ and monitored for survival for 5 days. Both the adjuvanted HcBoNTre-dosed and HcBoNTre-Ad2F-dosed mice showed a 100% survival rate. **, $p < 0.05$. 

Intramuscular immunization with HcBoNTre or HcBoNTre-Ad2F elicits elevated Ab titers
formed. BALB/c mice were dosed with equimolar amounts of 0.002 and Hcβtre with mice given naive fecal samples. Only. A. On day 84, plasma IgG titers from each group were measured against Hcβtre and BoNT/A He, and equivalent endpoint titers were obtained between groups 2 and 3 and between groups 4 and 5. Plasma IgG titers from each group were significantly greater than titers in the control CT-dosed group. * p < 0.001. B. On day 96, mice were challenged i.p. with 2000 mouse LD50 and monitored for survival for 5 days. Both the adjuvanted Hcβtre-dosed and Hcβtre-Ad2F-dosed mice showed a 100% survival rate, unlike the mice dosed with Hcβtre or Hcβtre-Ad2F alone, which did not. *, p ≤ 0.002 and **, p = 0.015.

Hcβtre or Hcβtre-Ad2F with and without CT adjuvant were performed. BALB/c mice were dosed with equimolar amounts of Hcβtre vaccine given alone or together with 1.0 μg of CT adjuvant on days 0, 7, and 14. Ab titers were assessed weekly and showed that two doses of Hcβtre-Ad2F were sufficient to produce elevated IgG Ab titers when compared with mice similarly dosed with Hcβtre by day 14 (Fig. 9A). In fact, the Ab titers induced by Hcβtre-Ad2F remained significantly greater through day 28. The addition of adjuvant modestly improved the IgG Ab response induced by Hcβtre-Ad2F. In addition, the Hcβtre-Ad2F plus CT group showed enhanced IgG Ab titers when compared with the Hcβtre plus CT group (Fig. 9A). Secretory IgA Ab titers were induced by all groups, but clearly mice dosed with Hcβtre-Ad2F with or without CT showed the greatest sIgA response peaking on day 21 (Fig. 9B), but these Ab responses subsequently deteriorated to sIgA levels obtained in mice immunized with Hcβtre or Hcβtre plus CT. Thus, these data show that the inclusion of the Ad2F molecule improves the immunogenicity of Hcβtre when given parenterally even in the absence of adjuvant.

**FIGURE 7.** Nasal immunization with adjuvanted Hcβtre-Ad2F or Hcβtre confers complete protection in BALB/c mice subsequently challenged i.p. with 2000 LD50 BoNT/A on day 96 post-primary immunization. Immunizations were conducted on days 0, 7, and 14, and the groups were subdivided as follows: Group 1 (n = 5 mice) given 25 μg of Hcβtre plus CT (2 μg); Group 2 (n = 5 mice) given 50 μg of Hcβtre-Ad2F (to ensure equivalent amount of Hcβtre was given) plus CT (2 μg); Group 3 (n = 8 mice) given 2 μg of CT only; Group 4 (n = 5 mice) given 25 μg of Hcβtre; and Group 5 (n = 5 mice) given 50 μg of Hcβtre-Ad2F. A. On day 84, plasma IgG titers from each group were measured against Hcβtre and BoNT/A He, and equivalent endpoint titers were obtained between groups 2 and 3 and between groups 4 and 5. Plasma IgG titers from each group were significantly greater than titers in the control CT-dosed group. * p < 0.001. B. On day 96, mice were challenged i.p. with 2000 mouse LD50 and monitored for survival for 5 days. Both the adjuvanted Hcβtre-dosed and Hcβtre-Ad2F-dosed mice showed a 100% survival rate, unlike the mice dosed with Hcβtre or Hcβtre-Ad2F alone, which did not. *, p ≤ 0.002 and **, p = 0.015.

**FIGURE 8.** Abs in mucosal samples are protective against challenge with the BoNT/A complex. Day 21 mucosal samples were evaluated for their BoNT-neutralizing capacity using a mouse neutralization assay. Filter-sterilized soluble fecal extracts (diluted 1:2) from Hcβtre-Ad2F plus CT dosed (n = 4 mice) (O) and Hcβtre plus CT dosed (n = 4 mice) (□), from naive mice (n = 8) (●), or from naive mice, but spiked with immune mouse plasma (n = 7) (□) were reacted with ~6.5 LD50 BoNT/A complex for 30 min, then were injected i.p. into naive BALB/c mice. All mice treated with naive fecal extract and the BoNT/A complex became moribund within 1 day, and all of the mice given the naive fecal extract spiked with immune plasma survived. Complete protection was obtained in all mice treated with the BoNT/A complex plus fecal extracts from mice nasally immunized with Hcβtre-Ad2F plus CT. This survival fraction was not statistically different from mice given fecal extracts from Hcβtre plus CT only. *, p = 0.005 for survival fractions from treatment groups compared with mice given naive fecal samples.

**FIGURE 9.** Parenteral immunization with Hcβtre-Ad2F enhances serum IgG and mucosal IgA Ab responses to Hcβtre. BALB/c mice i.m. immunized on days 0, 7, and 14 with Hcβtre or Hcβtre-Ad2F, either alone or in combination with CT. Plasma IgG (A) and fecal IgA (B) Ab titers against the Hcβtre were determined by standard ELISA methods. In the absence of CT, i.m. immunization with Hcβtre-Ad2F elicited a robust and rapid onset of IgG and sIgA Abs, whereas mice i.m. immunized with Hcβtre produced a delayed IgG and a weak sIgA Ab response. By day 35, both groups had equivalent IgG and sIgA anti-Hcβtre titers. Upon CT coadministration with Hcβtre-Ad2F or Hcβtre, a rapid onset of plasma IgG Ab titers were produced, but mice vaccinated with Hcβtre-Ad2F showed greater Ab responses. sIgA Ab titers were greater in the Hcβtre-Ad2F plus CT-dosed group than titers in mice similarly dosed with Hcβtre. Thus, these studies show that targeting with Ad2F enhances Ab responses to Hcβtre. Data represent mean ± SD (n = 5 mice). *, p ≤ 0.001; ***, p = 0.005; and ****, p = 0.024 represent the statistical differences in sIgA and IgG anti-Hcβtre levels between mice given the same vaccine with or without CT. †, p ≤ 0.001 and ††, p ≤ 0.009 represent differences between mice given Hcβtre-Ad2F or Hcβtre or given Hcβtre-Ad2F plus CT or Hcβtre plus CT.
adsorbed to alum. In addition, there is a second monovalent vaccine for BoNT/F (3). Although the conventional vaccine is efficacious, it bears a number of disadvantages, and an improved vaccine could allay these limitations. First, the BoNTToxoids used for the preparation of the pentavalent vaccine are impure, with as little as 10% toxoid present in the vaccine (7). As a result, the vaccine is exposed to irrelevant Ags unnecessary for eliciting protection. Second, the process of formalin inactivation of BoNT to produce toxoids chemically modifies the toxin resulting in a considerable loss of tertiary structure (7), thus reducing the number of viable antigenic and protective epitopes expressed by native toxins (7, 8). This latter point was evident in our study in which commercially prepared toxoid failed to elicit elevated Ab titers to either Hc BoNT/A, Hcβtre, or the native BoNT/A. Moreover, the residual formalin present in the vaccine is very painful to the recipient (25) and may deter compliance with the regular boosts required. The immunization protocol is rather elaborate, with vaccinations required at 0, 2, and 12 wk, a booster at 12 mo, and additional boosters every 2 years to maintain the desired level of immunity. Consequently, a new vaccine with conformationally preserved epitopes may lessen the number of immunizations and require less frequent boosters to maintain protective immunity. Third, some formalin remains in the vaccine to keep the toxin in the denatured form (19, 26). This result is problematic because formalin is carcinogenic (27-29), thus making its presence in a vaccine less desirable. Fourth, there are safety considerations for producing native toxins for formalin inactivation, resulting in higher costs associated with preparing the toxoid vaccine (25). Fifth, natural botulism, as well as illicit dissemination of BoNT (3), would almost surely occur via mucosal surfaces, and a vaccine developed for mucosal delivery would be advantageous to induce secretory IgA, as well as systemic, IgG Ab responses (23). This additional level of protection is far superior to that of the conventional pentavalent BoNT vaccine not optimized to include mucosal anti-BoNT immunity. A final disadvantage is that, although traditionally BoNTs have been grouped as one of seven serotypes, it has become apparent that each serotype is actually composed of a family of closely related subtypes in which the primary sequence of each BoNT/A subtype can vary by as much as 32% (30). Therefore, it would be advantageous to produce a vaccine that can preserve as many of the neutralizing and cross-neutralizing epitopes among serotypes and serotype variants.

In an effort to develop an alternative BoNT vaccine with improved efficacy, we adopted a two-prong strategy using BoNT/A as a vaccine prototype. We and others have recently identified the Hc containing the cell-binding domain in the C terminus as a potential vaccine candidate (11, 31) that contains a β-trefoil structure (18). The Hcβtre is a structure common to all serotypes (18), and this motif is even repeated in the progenitor toxin complex (32). This Hcβtre domain has been implicated in previous studies using a peptide analysis approach to determine the protective epitopes for Hc BoNT (9, 10). In the first study (9), three Hc regions in BoNT/A were identified for eliciting protection: BoNT/A1205–661, BoNT/A780–939, and BoNT/A1150–1289, conferring 78, 29, and 75% protection, respectively, after challenge with two LD99 dosed. In the second study, the synthetic peptide corresponding to Hc BoNT/A1230–1253 was ≈40% protective in mice against a lethal BoNT/A challenge (10). Thus, it appeared from these studies that, at a minimum, peptides encompassing a portion of the Hcβtre were protective. In addition, mAbs to Hc BoNT/A, some of which contain epitopes within the β-trefoil structure, have also been shown to be protective (10, 30).

The second attribute of our approach is the addition of a mucosal-targeting molecule Ad2F. Replication-deficient adenovirus vectors have widely been used in vaccination regimens (33, 34), and these vectors have been proven efficacious in their ability to stimulate protective immune responses (35, 36). However, as with any vaccine platform, concerns arise as to whether repetitive application of these vectors can limit their use (33, 34). Similar concerns are applicable with our methods, as well; however, again their application may be beneficial in a prime-boost strategy involving vaccination with the Hcβtre, both with or without Ad2F. It was evident from this study that the recombinant fusion protein with Ad2F could bind to the mouse nasal epithelium enabling uptake of the Hcβtre-Ad2F vaccine. Its use rapidly stimulated both mucosal IgA and systemic IgG Abs when Ad2F was present. Because nasal immunization with Hcβtre ultimately developed to similar anti-Hcβtre Ab titers as with Hcβtre-Ad2F, subsequent immunizations may only require Hcβtre. These studies will be pursued. When tested parenterally, our results showed that the inclusion of Ad2F-binding moiety enhanced plasma IgG and fecal IgA responses. This finding is of interest because i.m. immunization in the absence of CT adjuvant showed elevated IgG Ab titers as early as after two doses of Hcβtre-Ad2F and remained elevated at least until day 35 postprimary immunization. Although the coadministration of CT adjuvant clearly enhanced the IgG Ab titer, this increase was, at best, 9.2-fold. Additional studies will be taken to discern whether more vaccine can enhance this Ab response in the absence of adjuvant or whether the amount of adjuvant can be reduced. Such study will require evaluation of more suitable adjuvants approved for human use. How Ad2F is improving the systemic IgG response after i.m. immunization, at this point, can be speculated. Ad2F binds the coxsackie-adenovirus receptor (reviewed in Refs. 37, 38) and is expressed in a variety of tissues (39, 40). Thus, the parenteral delivery of Hcβtre-Ad2F may be taken up more efficiently because of coxsackie-adenovirus receptor expression. Alternatively, the Ad2F may enhance the immunogenicity of Hcβtre. Moreover, the Hcβtre also induced elevated Abs alone, albeit, lesser than Hcβtre-Ad2F. However, mucosal IgA Abs were induced to greater levels when using the Hcβtre-Ad2F than when using Hcβtre, although both showed only low mucosal Ab levels after day 35, suggesting long term mucosal memory was not induced. Nonetheless, these results show the feasibility of using either Hcβtre-Ad2F or Hcβtre in parenteral immunization paradigm.

Our study showed that the Hcβtre is strongly immunogenic when delivered nasally. As was evident, the Hcβtre stimulated 100% protection to mice when challenged i.p. with either 20,000 or 2,000 LD50. This immunity was found to be long lasting as noted by the day 96 challenge. In addition, we found that the Abs within mucosal secretions were protective. Using a mouse neutralization assay, sterile fecal extracts protected against 6.5 LD50 of the BoNT/A complex. Intoxication with the BoNT complex is 100-fold more toxic than purified BoNT (41, 42), when using the gastric route of exposure. This BoNT complex would most likely represent the form of toxin dissemination in bioterrorism. Our results suggest that the protective value of these mucosal Abs, at a minimum, approximates the lower end protection (2,000 LD50) found with systemic Abs. It also shows that our vaccine formulation is better than using BoNTToxoid as a nasal vaccine immunogen for the induction of protective humoral immunity in mucosal secretions. For example, fecal extracts from 75 to 100% of mice nasally immunized with Hcβtre plus CT or Hcβtre-Ad2F plus CT, respectively, protected mice against a lethal BoNT/A complex challenge. However, when a similar assay was performed with fecal extracts from mice nasally immunized with BoNTToxoid, only one-third of the mice were protected against one LD50 of BoNT/A complex (23). Clearly, this evidence showed that immunization
with the Hcβtre is protective and is a better immunogen than the toxoid.

Although no differences in protection were noted between mice dosed with the Hcβtre or the Hcβtre-Ad2F, it is important to note that the addition of the Ad2F acted as a mucosal accelerant in which anti-Hcβtre sIgA, and plasma IgG Ab titers were more quickly induced. Although Hcβtre-Ad2F given without adjuvant was able to induce some Ab titers to the Hcβtre, elevated plasma IgG Ab titers did not become elevated as was in the presence of an adjuvant. It may be more advantageous to give Hcβtre-Ad2F without adjuvant as a booster, rather than to initiate the Ab response, and thus lessen the need for mucosal adjuvants. Studies are currently addressing this possibility. The use of the Hcβtre immunogen may also be beneficial to elicit a polyclonal antisera to neutralize against BoNT intoxication. Because a portion of the neutralizing epitopes are retained in the Hcβtre, it may be more advantageous to give Hcβtre as a recombinant vaccine candidate.

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Disclosures

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