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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. Pascual‡*

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 (Hc/βtre) 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) 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 a 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/A555–661 and BoNT/A1150–1289, which conferred ~75% protection, and one minor protective epitope, BoNT/A730–939, 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.
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 R664 to L1295 (GenBank accession no. X52066) was designed for optimized expression in the yeast Pichia pastoris. Factors taken into account for the synthetic genes were yeast codon bias, the redundancy of C. botulinum A/F content, and the necessity for not depleting any particular rRNA 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 pCR1 TOPO cloning vector (Invitrogen Life Technologies), excised with EcoRI and SalI, and cloned into the P. pastoris expression vector pPICZ B cut with EcoRI and XhoI. Such a vector is designed to provide a C-terminal histidine tag for subsequent protein purification. Adenovirus 2 fiber was amplified from genomic adenovirus 2 DNA (a gift from Dr. J. Chroboczek, European Molecular Biology Laboratory, Grenoble, France). Attempts to express the entire Ad2F were unsuccessful; thus, only the C-terminal region, starting from G378 to E582, was cloned. This region encompasses 1) the two amino acids rich in Gly (4 of 15), 2) the region containing the trimerizing domain, and 3) the region containing the globular domain, commonly referred to as the “knob,” which is important for interacting with the coxsackievirus/adenovirus receptor on the cell surface (15). This region proved to be easily expressed in P. pastoris, even when fused to a variety of Ags or fluorescent proteins.

To make Hcβtre-Ad2F, Hcβtre was amplified with primers generating SalI and KpnI ends, cloned, and excised with the corresponding enzymes. The vector pPICZ B was cut with EcoRI and KpnI. Finally, Hcβtre-Ad2F was assembled via a tripartite ligation. Primers were designed to allow in-frame junction between the different components and the formation of a flexible joint between Hcβtre and the Ad2F. To make Red2-Ad2F, a similar strategy was adopted. Red2 gene was amplified with primers generating EcoRI and SalI ends using pDsRed2 (BD Biosciences) as template, and fused to Ad2F as described. These constructs were transformed into P. pastoris by electroporation.

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 with a 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 Tolon column (BD Biosciences), as per the manufacturer’s instruction. Purified proteins were eluted, titrated, 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).

Recombinant Ad2F-biding assay

The mouse fibroblast L929 cells, human HeLa cells, and human 293 cells (American Type Culture Collection) were grown according to the complete medium (CM): RPMI 1640 (Invitrogen Life Technologies), 10% FBS (Atlanta Biologicals), 10 mM HEPES buffer, 10 mM nonessential amino acids, 10 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin. Confluent cells were detached by trypsinization, washed, and resuspended in FACS buffer (10 g/L of Dulbecco’s PBS (Sigma-Aldrich) plus 2% FBS). Cells were incubated with 25 µg of Red2-Ad2F or Red2 (BD Clontech), and incubated at room temperature for 45 min. Cells were washed once with 3 ml of FACS buffer and resuspended in 200 µl of FACS buffer. Bound fluorescence was analyzed with a FACSCalibur (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 µm in a Leica Cryocut 1850 (Leica Microsystems) using Instrumedics Cryotlane Tape 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 Hcβtre. Fecal samples were extracted from the supernatants of 10% (w/v) suspension of sample and PBS with 50 μg/ml soybean 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 centrifugation for 10 min at 10,000 × g. HRP-goat anti-mouse IgA, IgG, IgG1, 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 incubation at 37°C and a washing step, the specific reactivity was determined by addition of an enzyme substrate, ABTS (Moss) at 100 μl/well. Absorbance was measured at 415 nm on a kinetics reader model EL312 (Bio-Tek Instruments). Endpoint titers were defined as the highest reciprocal dilution of sample giving an absorbance at OD<sub>415</sub> 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 Biosciences, San Diego, CA). Wells were coated overnight at 4°C. After washing, wells, 20 μg/ml biotinylated rat anti-mouse IgE was incubated for 2 h at 37°C, washed, incubated with 1/500 dilution of HRP-goat anti-biotin for 1 h at 37°C. Wells were developed, as described, and a specific amount of IgE was extrapolated from a standard curve derived from wells incubated with an IgE anti-trinitrophenyl mAb (BD Pharmingen).

Lymphocyte isolation

Lymphocytes were isolated from nasal passages, submaxillary glands, small intestinal lamina propria, Peyer’s patches, mesenteric lymph nodes (LN), spleens, naso-associated lymphoepithelial tissue, and head and neck LN. Splenic, Peyer’s patch, mesenteric LN, naso-associated lymphoepithelial tissue, and head and neck LN mononuclear cell suspensions were obtained by conventional methods using dounce homogenization (21, 22) and yielding >95% viability by trypsin blue exclusion. To isolate mononuclear cells from naso passages and submaxillary glands, the tissues were minced and suspended in medium containing 300 U/ml Cladosthrium histolyticum type IV collagenase (Worthington Biochemicals), followed by two 30-min digestions at 37°C or a single digestion for 45 min, respectively, in spinner flasks. The small intestinal lamina propria samples were isolated, as previously described (21, 22). After incubation, the digestion mixtures were passed through Nitex mesh (Fairview Fabrics) to remove undigested tissues. Mononuclear cells were subjected to Percoll (Amersham Biosciences) density gradient centrifugation, and the cells were interfaced between 40 and 75% Percoll (21, 22). Viability of >95% was noted for lymphocytes isolated from each tissue, as determined by trypsin blue exclusion.

B cell Ab ELISPOT

Ab-forming cells (AFC) were enumerated using IgA and IgG Ag-specific ELISPOT assays similar to those previously described (21–23). Mixed cellulosic ester membrane-bottomed microtiter plates (MultiScreen-HA; Millipore) were coated with 5.0 μg/ml recombinant He BoNT/A in sterile PBS. For total IgA or IgG AFC determinations, wells were coated with 5 μg/ml goat anti-mouse IgA or IgG (Hc-specific) Abs (Southern Biotechnology Associates) in sterile PBS overnight at 4°C. The overnight incubation was blocked at 37°C for 2 h in CM. A total of 100 μl of cells from each tissue at varying concentrations was added to the wells, and the plates were incubated at 37°C overnight. Cells were removed, and the plates were washed, as previously described (21–23). For detection of AFC responses, 100 μl of 1.0 μg/ml goat anti-mouse IgA and IgG HRP conjugates (Southern Biotechnology Associates) were added to the wells, and the plates were incubated overnight at 4°C. After washing, the wells were developed with 100 μl of AEC (Moss), and the reaction was allowed to continue until spots developed (~30 min). The reaction was stopped with H<sub>2</sub>O, the plates were allowed to dry overnight, and AFC were enumerated by counting under a low-power dissecting microscope (Leica).

Cytokine ELISPOT

Groups of BALB/c mice were euthanized via CO<sub>2</sub> (5–10/group) 3 wk after the last immunization to collect spleens. Total spleen, head and neck LN, and mesenteric LN mononuclear cells (5 × 10<sup>6</sup> cells) were resuspended in CM, and restimulated with 10 μg/ml recombinant He BoNT/A, OVA (tissue-culture grade; Sigma-Aldrich), or media in the presence of 10 U/ml human IL-2 (PeproTech) for 2 days at 37°C. Cells were washed and resuspended in CM. Stimulated lymphocytes were then evaluated by IFN-γ, IL-4, IL-5, IL-6, and IL-10-specific ELISPOT assays, precisely as previously described (22, 24).

BoNT intoxication challenge and mouse neutralization assay

To assess the protective value of the Hcβtre vaccine, BALB/c mice were immunized nasally with equinimolar doses of Hcβtre or Hcβtre-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 BoNToxoid/A (List Biological Laboratories) plus 2 μg of CT on days 0, 7, 14, and 21. Mice were monitored for Ab titer to Hcβtre, He BoNT/A, and BoNT/A. On day 63 or day 96, mice were challenged i.p. with ~20,000 LD<sub>50</sub> BoNT/A (2 × 10<sup>6</sup> mouse LD<sub>50</sub>/mg, Lot no. A121000-01; Metabiologics), respectively, in 200 μl of PBS containing 0.2% gelatin, as previously described (23). Mice were monitored hourly for the first 6 h and then daily for signs of morbidity, including difficulty breathing and lack of vitality. Mice were euthanized when signs of morbidity were observed.

To determine whether nasal immunization with Hcβtre immunogen was able to induce protective Abs in mucosal secretions against BoNT/A, we picked a mouse neutralization assay using BoNT/A complex (3.8 × 10<sup>7</sup> mouse LD<sub>50</sub>/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 He BoNT/A plus CT at a final dilution of 1/110. If the anti-Hcβtre Abs in the fecal extracts could not 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 toxicity, including shortness of breath and immobility. Five mice 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 differences between variations in Abs 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 survival fractions following intoxication with BoNT/A or the BoNT/A complex. Using the Mantel-Haenszel log rank test, the p value for statistical differences between surviving BoNT/A challenge and Hcβtre plus CT, Hcβtre-Ad2F plus CT, Hcβtre alone, Hcβtre-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 (He 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 referred to as 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 He, 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. Neither 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
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 (~58.8 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-AdF 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 staining. 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. 3B). 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 26.

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

FIGURE 2. The addition of the mucosal-targeting ligand, Ad2F, enhances time for mucosal Ab production. BALB/c mice nasally immunized on days 0, 7, and 14 with Hcβtre or Hcβtre-Ad2F, either alone or in combination with CT, and fecal IgA (A) and plasma IgG (B) Ab titers against the Hcβtre were determined by standard ELISA methods. In the absence of CT, nasal immunization with Hcβtre-Ad2F elicited a robust and rapid onset of secretory IgA (sIgA) Abs, whereas mice nasally dosed with Hcβtre only produced a weak sIgA Ab response. Both groups produced weak plasma IgG anti-Hcβtre titers. Upon CT coadministration, a rapid onset and sustained sIgA and plasma IgG Ab titers were produced when nasally vaccinated with Hcβtre-Ad2F, but this rapid onset was not evident with the Hcβtre-dosed mice. Thus, for the remaining studies, only responses in adjuvanted mice were evaluated. Data represent the mean ± SEM (n = 10 mice). *, p = 0.001; **, p = 0.015; and ***, p = 0.02 represent the statistical differences in sIgA and IgG anti-Hcβtre levels between mice given the same vaccine with CT as adjuvant compared with mice dosed with their respective vaccine without CT.
Nasal immunization with Hcβtre-Ad2F and CT shows increased secretory IgA (sIgA) (A) and Hcβtre titers with significant differences in plasma IgG1 and IgG2a titers (B) but no changes in total IgE levels (C). Data represent mean ± SEM (n = 10) sampled at day 35 postprimary immunization or sampled at day 28 for total IgE levels. Only one of 10 mice from the Hcβtre plus CT-dosed group showed a plasma IgE anti-Hcβtre titer of 2^+; none of the other groups showed a Hcβtre-specific IgE titer. *, p <= 0.001 and **, p = 0.003 represent the differences in sIgA, IgG1, and IgG2a Ab titers between mice nasally dosed with Hcβtre-Ad2F plus CT and mice dosed with Hcβtre plus CT, respectively.

Nasal immunization with Hcβtre-Ad2F plus CT as adjuvant elicits a mixed Th cell response

To learn what are the supportive Th cells for the observed Ag-specific B cell responses, adjuvanted Hcβtre-Ad2F-dosed or Hcβtre-dosed mice were evaluated for CFC. Lymphocytes from spleen, head and neck LN, and mesenteric LN were cultured and assayed for the production of IFN-γ, IL-4, IL-5, IL-10, and IL-13. Following 48 h of stimulation, lymphocytes were analyzed by cytokine ELISPOT method. Although the responses varied among the tissues examined, for the most part, a mixed Th cell phenotype was observed when triggered with Hc BoNT/A. In the spleen, IFN-γ, IL-4, and IL-5 CFC were induced to equivalent levels for both dosing groups, but increased numbers of IL-10- and IL-13-producing cells were detected in the Hcβtre-Ad2F-dosed mice (Fig. 5A). In the head and neck LN, IL-4, IL-5, and IL-10 were not significantly different between either dosing group; however, IFN-γ (p < 0.001) and IL-13 (p < 0.05) were particularly enhanced in Hcβtre-Ad2F-dosed mice (Fig. 5B). In the mesenteric LN, IL-5 and IL-10 were similar for both groups; however, Hcβtre-Ad2F-dosed mice exhibited significantly less IFN-γ (p < 0.011), IL-4 (p < 0.05), and IL-13 (p < 0.001) CFC (Fig. 5C).

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 BoNToxoid/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 BoNToxoid 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 BoNT/A 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 Hcβtre-Ad2F in the absence or presence of 2 μg of CT. The control group was only given three nasal doses of CT.
FIGURE 5. Mucosal targeting enhances head and neck LN IFN-γ and IL-13 production following nasal immunization with Hcβtre-Ad2F. Three weeks after their final immunization, Hcβtre-Ad2F plus CT-dosed and Hcβtre 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/1 × 10^6 lymphocytes from a total of three experiments. *, p < 0.001; **, p ≤ 0.011; ***, p < 0.05.

Hc BoNT/A and Hcβtre. Each of the vaccinated groups showed elevated Ab titers to Hc BoNT/A and Hcβtre, 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 LD50. Most of the CT-dosed mice succumbed to the BoNT/A intoxication within 4 h, and none survived (Fig. 7B). Mice dosed with Hcβtre or Hcβtre-Ad2F alone succumbed to BoNT/A intoxication within 2 h. Mice nasally immunized with Hcβtre plus CT and Hcβtre-Ad2F and CT survived the ~2000 LD50 lethal challenge. Thus, these studies showed that immunization with the Hcβtre, either alone or fused to Ad2F, could elicit neutralizing Abs that protect against systemic lethal challenge.

Mucosal Abs protect against lethal challenge with the BoNT/A complex

To determine whether mucosal secretions contain neutralizing Abs that 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/A. In this assay, sterile-filtered fecal extracts were combined with 6.5 LD50 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 Hcβtre-Ad2F plus CT or Hcβtre plus CT, from naive mice, or from naive samples spiked with immune anti-Hcβtre plasma. All of the mice given the naive fecal extracts succumbed within 24 h (Fig. 8). Mice given the naive fecal extract containing the added anti-Hcβtre Abs were protected against challenge with the BoNT/A complex (p = 0.005). Likewise, all mice given the fecal extracts from Hcβtre-Ad2F plus CT-dosed mice survived, and 75% of the mice given fecal extracts from mice dosed with Hcβtre and CT survived the challenge with the BoNT/A complex (p = 0.005). These collective results showed the feasibility of nasal immunization with our novel BoNT/A immunogens to induce neutralizing Abs in mucosal secretions that protect against intoxication with BoNT or BoNT complex.

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

To assess how the Ad2F-targeting molecule may enhance the immunogenicity of the Hcβtre, i.m. immunization studies with BoNT/A 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 Hcβtre plus CT (2 μg); Group 2 (n = 4 mice) given 50 μg of Hcβtre-Ad2F (to ensure that equivalent amount of Hcβtre 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 Hcβtre, BoNT/A Hc, or native BoNT/A. The mice dosed with adjuvanted Hcβtre showed a greater endpoint titer against Hc BoNT/A than mice dosed with Hcβtre-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 LD50 and monitored for survival for 5 days. Both the adjuvanted Hcβtre-dosed and Hcβtre-Ad2F-dosed mice showed a 100% survival rate. **, p < 0.05.
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.

**Discussion**

The current vaccine to prevent botulism is a pentavalent BoNT-oxoid comprised of toxoids for five serotypes (A, B, C, D, and E) coadministration with Hcβtre-Ad2F showed equivalent endpoint titers. Thus, data show that targeting with Ad2F enhances Ab responses to Hcβtre. 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. S IgG 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 BoNT toxoids 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 (32). 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/A(255-661), BoNT/A(780-939), and BoNT/A(1150-1289) conferring 78, 29, and 75% protection, respectively, after challenge with two LD_{50}dosed. In the second study, the synthetic peptide corresponding to Hc BoNT/A(1230-1253) 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 LD_{50}. 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 LD_{50} 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 LD_{50}) found with systemic Abs. It also shows that our vaccine formulation is better than using BoNT toxoid 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 BoNT toxoid, only one-third of the mice were protected against one LD_{50} of BoNT/A complex (23). Clearly, this evidence showed that immunization...
with the Hc\breve{b}tre is protective and is a better immunogen than the toxoid.

Although no differences in protection were noted between mice dosed with the Hc\breve{b}tre or the Hc\breve{b}bre-Ad2F, it is important to note that the addition of the Ad2F acted as a mucosal accelerant in which anti-Hc\breve{b}tre sIgA, and plasma IgG Ab titers were more quickly induced. Although Hc\breve{b}bre-Ad2F given without adjuvant was able to induce some Ab titers to the Hc\breve{b}tre, elevated plasma IgG Ab titers did not become elevated as well in the presence of an adjuvant. It may be more advantageous to give Hc\breve{b}bre-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 Hc\breve{b}tre immunogen may also be beneficial to elicit a polyclonal antiserum to neutralize against BoNT intoxication. Because a portion of the neutralizing epitopes are retained in the Hc\breve{b}tre, it is important to note that sequence alignment for the BoNT toxigenic strain reveals evolutionary relationship to adeno-
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