Vaccination of Rabbits with an Alkylated Toxoid Rapidly Elicits Potent Neutralizing Antibodies against Botulinum Neurotoxin Serotype B

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New Zealand White (NZW) rabbits were immunized with several different nontoxic botulinum neurotoxin serotype B (BoNT/B) preparations in an effort to optimize the production of a rapid and highly potent, effective neutralizing antibody response. The immunogens included a recombinant heavy chain (rHc) protein produced in Escherichia coli, a commercially available formaldehyde-inactivated toxoid, and an alkylated toxoid produced by urea-iodoacetamide inactivation of the purified active toxin. All three immunogens elicited an antibody response to BoNT/B, detected by enzyme-linked immunosorbent assay (ELISA) and by toxin neutralization assay, by the use of two distinct mouse toxin challenge models. The induction period and the ultimate potency of the observed immune response varied for each immunogen, and the ELISA titer was not reliably predictive of the potency of toxin neutralization. The kinetics of the BoNT/B-specific binding immune response were nearly identical for the formaldehyde toxoid and alkylated toxoid immunogens, but immunization with the alkylated toxoid generated an approximately 10-fold higher neutralization potency that endured throughout the study, and after just 49 days, each milliliter of serum was capable of neutralizing $10^7$ 50% lethal doses of the toxin. Overall, the immunization of rabbits with alkylated BoNT/B toxoid appears to have induced a neutralizing immune response more rapid and more potent than the responses generated by vaccination with formaldehyde toxoid or rHc preparations.

Botulinum neurotoxin (BoNT), the causative agent of botulism, is the most potent of all the known toxins (7). BoNT is a secreted protein produced by the anaerobic soil organisms Clostridium botulinum, Clostridium baratii, and Clostridium butyricum in seven distinct serotypes (serotypes A to G) (9, 23, 28). The BoNT serotypes are all synthesized as single-chain polypeptides with molecular masses of approximately 150 kDa. Posttranslational cleavage of the original polypeptide monomer results in the formation of a disulfide-linked dichain product composed of light chain (LC) and heavy chain (HC) domains. The HC is divided into two distinct functional domains; the first mediates toxin binding and uptake by peripheral neuronal cells, and the second mediates translocation of the LC subunit into the target cell cytosol. Once it is in the cytosol, the zinc metalloprotease of the LC specifically cleaves the soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) responsible for synaptic vesicle docking and neurotransmitter release at the neuromuscular synapse.

Human botulism typically results from the ingestion of contaminated foods (often improperly prepared canned goods), although BoNT intoxication can also result from wound colonization by one or more species of Clostridium. Similarly, infant botulism results from exposure to actively secreted toxin following the germination of ingested Clostridium spores, which proliferate in the immature gastrointestinal tract. Regardless of the route of exposure, BoNT intoxication occurs by the same mechanism, once the toxin enters the circulation. Although there is no cure for botulism after the onset of symptoms, an effective circulating antibody response can completely neutralize an otherwise intoxicating dose of BoNT. Widespread immunization against the toxin is precluded by the growing number of clinical applications of BoNT for the treatment of various neuromuscular spasticity disorders, yet BoNT vaccine development continues for the purposes of immunizing at-risk populations, such as laboratory workers, first responders, and military personnel (26).

A number of BoNT immunogens and a variety of vaccination strategies have successfully been used to elicit neutralizing antibody responses against individual BoNT serotypes (3, 19, 20, 29, 32). The immune responses to BoNT vary according to the animal species, the toxin serotype, and the antigen preparation. Additionally, the development of a potent neutralizing antibody response to BoNT serotype B (BoNT/B) has proven problematic, prompting a demand for alternative toxin-derived immunogens (25, 27).

In the present study, we tested three BoNT/B immunogens in New Zealand White (NZW) rabbits using a rapid vaccination scheme to develop a potent toxin-neutralizing immune response in a short time period (12). Rabbits were immunized with BoNT/B recombinant heavy chain (rHc) or toxoid preparations derived from formaldehyde inactivation or urea-iodoacetamide alkylation of active toxin (15). All three immu-
nogens elicited toxin-neutralizing antibody responses by the end of the study; however, vaccination with the alkylated toxoid preparation induced a more rapid and more potent BoNT/B-neutralizing response than the other immunogens.

**MATERIALS AND METHODS**

**Animals.** Female CD-1 mice (weight, 20 to 25 g), purchased from Charles River Laboratories (Wilmington, MA), and female NZW rabbits (weight, 3 to 4 kg), purchased from Covance (Princeton, NJ), were housed in animal facilities accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee at SRI International.

**BoNT/B hrc expression and purification.** The BoNT/B hrc expression construct (encoding the C-terminal 448 residues of the Okra strain toxin appended with an N-terminal hexahistidine tag) was cloned into pQE30 (Qiagen, German town, MD) and transformed into *Escherichia coli* M15<pREP4> (Qiagen) or BL21 (CodonPlus, Stratagene, La Jolla, CA) for isopropyl β-D-thiogalactopyranoside (IPTG)-induced overexpression. A 250-ml culture was grown at 37°C in 2× YT medium (tryptone, yeast extract, NaCl, 25 µg/ml k-anamycin, 100 µg/ml ampicillin). When the optical density at 600 nm (OD<sub>600</sub>) of the culture reached approximately 0.7, IPTG was added to a final concentration of 1 mM, and the culture was allowed to grow for an additional 4 h at 25°C. The cultures were then centrifuged, and the cell pellets were stored overnight at −80°C. The cells were lysed by incubation for 10 min at room temperature in bacterial protein extraction reagent (Pierce, Rockford, IL). The cell lysate was centrifuged for 10 min at 9,000 × <i>g</i> (Heraeus 3040 rotor), and the supernatant was discarded. The cell debris pellet was resuspended in 10 ml inclusion body resolubilization buffer (100 mM sodium phosphate, pH 8.0, 400 mM NaCl, 6 M guanidine-HCl, 1% Tween 20) and drawn through an 18-gauge needle and then through a 27-gauge needle to disrupt the clumped debris. Another 15 ml of inclusion body resolubilization buffer was added, and the solution was incubated at room temperature for 60 min with gentle rocking. The solution was centrifuged at 9,500 × <i>g</i> (Heraeus 3046 rotor) for 10 min, and the supernatant was removed and applied to a nickel-agarose column (His GraviTrap; GE Healthcare, Pittsburgh, PA) that was equilibrated with inclusion body solubilization buffer. The column was washed with 15 ml urea wash buffer A (100 mM sodium phosphate, pH 8.0, 400 mM NaCl, 8 M urea, 1% Tween 20, 5 mM imidazole) and then with 30 ml urea wash buffer B (100 mM sodium phosphate, pH 8.0, 400 mM NaCl, 8 M urea, 1% Tween 20, 20 mM imidazole). Purified Hc was eluted with six 1-ml volumes of 100 mM sodium phosphate, pH 8.0–400 mM NaCl–6 M guanidine–HCl, 1% Tween 20 (possibly due to the use of a precipitated slurry), we did not give the animals the day 70 booster dose.

**Active toxin boosting.** Purified active BoNT/B (Okra strain, 1.4 × 10<sup>5</sup> 50% lethal doses [LD<sub>50</sub>sg/ml; Metabiologics, Inc.) was diluted to the appropriate dose in phosphate-buffered saline (PBS) and combined with an equal volume of Freund's incomplete adjuvant. For the formylated and alkylated BoNT/B antigen groups were administered escalating booster doses of 1, 5, 10, 100, and 1,000 ng active toxin. The two animals given a booster vaccination on day 80 were combined with two additional animals for boosting on day 84. The four animals boosted on day 84 were combined with two additional animals for boosting on day 87. All eight animals were boosted on days 90 and 94. Therefore, for all of the eight rabbits used in each group of the study (those immunized with formylated and alkylated antigens), rabbits A and B received 1, 5, 10, 100, and 1,000 ng of the active toxin; rabbits C and D received 5, 10, 100, and 1,000 ng of the active toxin; rabbits E and F received 10, 100, and 1,000 ng of the active toxin; and rabbits G and H received 100 and 1,000 ng of the active toxin. To determine if active toxin boosters were able to inactivate antiserum in the formylated toxin-vaccinated rabbits, a group of rabbits was vaccinated as described above (the final booster vaccination was on day 70) without boosting with active toxin. The two animals from the Hc group dosed with 1 ng of toxin on day 80 showed symptoms of BoNT intoxication on day 84, and they were not dosed a second time. Two additional animals in the rHc group

**TABLE 1. Immunization schedule**

<table>
<thead>
<tr>
<th>BoNT/B antigen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antigen dose (µg)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Immunization schedule&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Active toxin booster dose (ng)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 28</td>
</tr>
<tr>
<td>rHc</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Formalin toxoid</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Alkylated toxoid</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each immunization group consisted of eight female rabbits. Formalin toxoid consisted of formaldehyde-inactivated toxin, and alkylated toxoid consisted of urea-iodoacetamide-inactivated purified active toxin.

<sup>b</sup> The vaccine adjuvants were Freund's complete adjuvant on day 0, and Freund's incomplete adjuvant for all other doses, including boosts with active toxin.

<sup>c</sup> Antigens were administered by the subcutaneous injection of 0.5 ml (as four 0.125-ml injections per animal).

<sup>d</sup> Boosts with active toxin were performed as described in the Materials and Methods. The two animals from the rHc group dosed on day 80 showed symptoms of BoNT intoxication on day 84 and were not dosed a second time. Both animals were found dead on the afternoon of day 84. Two additional animals in the rHc group were dosed on day 84. Both of these animals were found dead on day 87. No further active toxin boosting was performed with the four remaining animals in the rHc group.

<sup>e</sup> Blood samples were collected on day −1 for baseline titer determinations and again on study days 7, 21, 35, 49, 77, and 105.
Enzyme-linked immunosorbent assay (ELISA). Purified active BoNT/B (Metabiologics, Inc.) was diluted to 2 μg/ml in coating buffer (PBS) and added to Immulon 4HBX 96-well plates (ThermoElectron, Rockford, IL) at 100 μl/well. The plates were covered and stored overnight at 4°C. Unbound protein was removed by washing the plates four times with 200 μl/well PBS plus 0.05% Tween 20 (PBST). The wells were then blocked with 100 μl blocking buffer (PBST plus 1% bovine serum albumin) for 1 h. The blocking buffer was removed, and the plates were again washed four times with PBST at 200 μl/well. Rabbit serum was serially diluted (2-fold dilutions) in blocking buffer, added at 100 μl/well, and incubated for 1 h. Unbound antibody was removed by four washes with 200 μl/well PBST, and anti-rabbit IgG-horseradish peroxidase-mouse monoclonal antibody (Sigma) diluted 1:10,000 in blocking buffer was added at 100 μl/well. After 1 h, the plates were again washed four times with 200 μl/well PBST, and 100 μl 3.3',5,5'-tetramethylbenzidine (TMB) was added to each well. HRP activity was quenched after 10 min by adding 50 μl 1% sulfuric acid to each well. The plates were read by measurement of the OD450 with a Spectramax M2 plate reader (Molecular Devices, Sunnyvale, CA). The cutoff titer was defined as the lowest antibody concentration at which the optical density was equal to or greater than 0.4 absorbance units (or a 10% maximum signal) after subtraction of the background reading.

Western blotting. Protein samples were mixed with 2× Novex Tris-glycine native sample buffer (Invitrogen, Carlsbad, CA), loaded onto NuPAGE Novex 4 to 12% Bis-Tris gels (Invitrogen), and electrophoresed for 60 min at 200 V. The protein was then transferred to Invitrokon polyvinylidene fluoride (PVDF) membranes (Invitrogen) by electrophoresis in transfer buffer for 60 min at 30 V. The membranes were blocked overnight at room temperature in 5% powdered milk in PBST. On the next day, the membranes were washed four times with 25 ml PBST. Polyclonal rabbit anti-BoNT antibody (Metabiologics, Inc.) was diluted to 0.5 μg/ml in PBST and incubated on the membranes for 60 min (with gentle shaking). The membranes were washed again four times with 25 ml PBST, and detection antibody (anti-rabbit gamma chain, horseradish peroxidase conjugated [Sigma]) was diluted to 1:10,000 in PBST and incubated on the membranes for 60 min (with gentle shaking). The membranes were then washed four times with 25 ml PBST, and SuperSignal West Pico chemiluminescent substrate (Pierce) was used for detection. The membranes were wrapped in plastic wrap and exposed to film for 1 to 5 min.

Mouse lethal toxin challenge assay. The LD₀₅ of the BoNT/B complex was experimentally determined to be 19 pg in CD1 mice (weight, 25 g; Charles River Laboratories). Groups of 10 CD1 female mice were dosed by the intraperitoneal injection of 0.1 ml of undiluted or diluted rabbit serum premixed with 19 ng of BoNT/B complex (Metabiologics, Inc.), which was diluted to 0.1 ml in gelatin phosphate buffer (30 mM Na₂HPO₄, pH 6.3, 0.2% gelatin). The amount of toxin complex used was effectively 1,000 times the experimentally determined LD₀₅. The 0.2-ml serum-toxin mixtures were incubated at room temperature for 30 min prior to injection. The mice were injected with the entire 0.2-ml mixture on day 0 and were monitored for moribundity three times daily for 5 days.

RESULTS

Expression and purification of rHc from E. coli. Expression of the LC and HC domains of recombinant BoNT in E. coli has been reported for serotypes A to D (1, 2, 8, 22, 30, 33). The solubility of the expression products is often poor, and that has posed challenges for the development of vaccines derived from recombinant BoNT subunits. We were able to express BoNT/B rHc in E. coli; however, the protein tended to accumulate in the cells as aggregated inclusion bodies (data not shown). Solubilization of the inclusion bodies in guanidine-HCl enabled nickel column purification of the rHc products (Fig. 1, lane 1). Proper refolding of the protein after purification was difficult to achieve, and the majority of the purified product precipitated during dialysis (data not shown).

Alkylation of active purified BoNT/B. The BoNT toxoids produced by formaldehyde inactivation of toxins from serotypes A to E have successfully been used to produce neutralizing immune responses in humans (24, 25). However, the resulting toxin neutralization capacity of the antisera was less potent for BoNT/B than for BoNT/A (23). Recently, alkylated BoNT/B toxoid (produced by urea-iodoacetamide inactivation of toxin) was used to generate a much higher neutralizing titer of BoNT/B-specific antibodies in mice (15). We sought to test the immunization potency of alkylated BoNT/B toxoid in rabbits. SDS-PAGE and Western blot analyses of the resulting alkylated BoNT/B toxoid confirmed the retention of the size, migration properties, and antibody recognition features of the active purified toxin from which it was derived (Fig. 1, compare lanes 2 and 4). This is in contrast to what was observed for the formulatated toxin (Fig. 1, compare lanes 3 and 4). In fact, treatment with formaldehyde is known to affect the structure of the protein, which causes cross-links between toxin proteins, resulting in aberrant protein migration during electrophoresis, and which can cause the loss of the ability to detect the toxin in Western blots with antibodies specific for the toxin (17).

Antibody response to BoNT/B immunization. (i) ELISA analysis. Serum samples from the individual rabbits in each study group from each bleed day were pooled and analyzed by ELISA for their abilities to recognize BoNT/B. We observed dramatic differences in the immunogenicity of the BoNT/B rHc relative to the immunogenicities of the two BoNT/B toxoid preparations (Fig. 2A). None of the three antigens elicited an appreciable antibody response to the toxin by study day 7. However, by day 21, the ELISA titers...
for the toxoid-immunized groups were 1,000-fold higher than the prebleed levels. The ELISA titers for the sera from these two groups continued to climb severalfold during the course of the study, and the titers leveled off by day 49, despite continued boosting with the active toxin. We obtained nearly identical BoNT/B-specific antibody titers for both the formylated and the alkylated toxoid preparations, indicating that either method of toxin inactivation yields a strong antibody response in rabbits.

In contrast to the toxoid preparations, the anti-BoNT/B titers of the sera from the rabbits vaccinated with rHc increased by only approximately 2-fold by day 21. The immune response to this antigen was delayed until day 35, and on day 105, titers that only approximated those of the toxoid preparations were achieved (Fig. 2A). Interestingly, the titers continued to rise in these animals, even though they received the final vaccination on day 42. Unfortunately, boosting of these animals with active toxin was impossible due to the delay in the generation of BoNT/B-specific IgG. In fact, four rabbits that we boosted with a low dose of 1 ng of toxin died 4 days later. This boosting strategy was then discontinued. Given the low yields of soluble protein obtained during purification, it is possible that the rabbits were vaccinated with less than 25 μg of rHc. This could lead to a slower immune response and may have adversely affected the ability of the animals to generate a neutralizing antibody. We are currently optimizing the expression of the protein for future experimentation.

(ii) Toxin neutralization. Pooled serum from each immunization group was tested for BoNT/B neutralization in a series of mouse challenge experiments. A nonlethal local abdominal flaccid paralysis assay (13) was used to assess toxin neutralization, and the results were expressed as IU per milliliter of serum (Fig. 2B). Sera collected throughout the study, on study days 49, 77, and 105, continued to develop improved neutralization potencies, despite an apparent plateau in the anti-BoNT/B ELISA titers for serum from the two rabbit groups immunized with BoNT/B toxoids. Additionally, despite the comparable anti-BoNT/B ELISA titers for the sera from these two groups, throughout the study the neutralization potency was roughly 10-fold higher in sera from animals immunized with the alkylated toxoid antigen, with the levels being as high as 220 IU/ml (95% confidence limit [CL], 184 to 269 IU/ml). Interestingly, the toxin neutralization potencies of sera from vaccination groups 1 (immunized with rHc) and 2 (immunized with formalin toxoid) were very similar at day 105 (25 and 21 IU/ml, respectively [95% CLs, 21 to 30 and 18 to 25 IU/ml, respectively), despite the dramatic lag in the capacity of the rHc-immunized group to generate comparable ELISA titers. We were surprised to discover that boosting with active toxin did not appear to increase the neutralization potential in animals vaccinated with the alkylated toxoid compared with that in animals that did not receive the active toxin booster (Fig. 2B, open triangles with a dashed line). This suggests that the alkylated toxoid alone is an incredibly effective antigen for the generation of antibodies that recognize and neutralize BoNT/B.

In mouse lethality assays, toxin neutralization was consistently most potent in serum from rabbits vaccinated with alkylated toxoid (Table 2). Ten microliters of serum collected on

### TABLE 2. Mouse survival

<table>
<thead>
<tr>
<th>BoNT/B antigen</th>
<th>Day postvaccination of blood draw</th>
<th>Mouse survival after administration of the following amount of serum to each animal: I/100 dilution</th>
<th>1/100 dilution</th>
</tr>
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<tbody>
<tr>
<td>rHc</td>
<td>35</td>
<td>1/10</td>
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<td></td>
<td>49</td>
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<td>105</td>
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<tr>
<td>Formylated toxoid</td>
<td>35</td>
<td>1/10</td>
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<td>49</td>
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<td>Alkylated toxoid</td>
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<tr>
<td></td>
<td>105</td>
<td>1/10</td>
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* The indicated amount of serum was administered to each mouse, and the mice were then challenged with 1,000 times the LD50, which is equivalent to 19 ng of toxin complex per mouse. The data represent the number of mice that survived/total number of mice tested.
study day 35 was protective against BoNT/B at doses up to 1,000 times the LD_{50}, and even a 1:10 dilution (equivalent to 0.1 μl) of the serum from blood drawn on study day 105 neutralized this toxin in 9 of 10 mice that were challenged. The toxin-neutralizing response appeared less rapidly in serum from rabbits vaccinated with formylated toxoid than in rabbits vaccinated with the alkylated toxoid. The anti-formalin toxoid serum from study day 21 failed to effectively protect against challenges with toxin neutralization response appeared less rapidly in serum from rabbits vaccinated with formylated toxoid than in rabbits vaccinated with the alkylated toxoid. The anti-formalin toxoid serum from study day 21 failed to effectively protect against toxin challenges at 5, 10, and 25 times the LD_{50} (data not shown). However, by day 105, 1 μl of this serum was fully protective against challenges with toxin at up to 1,000 times the LD_{50} (19 ng of toxin complex). Serum from rabbits vaccinated with rHc was initially unable to protect against challenges with toxin as little as five times the LD_{50} (data not shown), but by study day 105, 1 μl of the pooled serum from the four surviving rabbits in this group was generating an effective toxin-neutralizing immune response. Unlike the flaccid paralysis assays, we observed little difference in the neutralization potential in antisera collected from animals vaccinated with the alkylated toxoid at days 49, 77, and 105 (compare the results presented in Fig. 2B and Table 2). This is likely the result of the much larger 10-fold dilution range used in the lethality assays compared to that used in the flaccid paralysis assays (a 1.6-fold dilution range).

**DISCUSSION**

**BoNT vaccine development.** The development of effective vaccine antigens derived from the botulinum neurotoxins is critical for the rapid and effective immunization of select populations at risk for intoxication (including researchers and medical professionals working with the toxins, as well as first responders and military personnel who might be exposed to a BoNT bioweapon). Among the seven known BoNT serotypes, serotypes A, B, E, and F are the most relevant to traditional human epidemiology, with BoNT/A being the most potent, followed closely by BoNT/B. The Centers for Disease Control and Prevention (CDC) maintains a pentavalent BoNT vaccine (serotypes A to E) and a monovalent vaccine (serotype F), both of which are made available as needed under investigational new drug (IND) protocols. These vaccines consist of formaldehyde-inactivated BoNT toxin preparations (4). Several other bivalent and multivalent BoNT subunit vaccines currently under development offer the promise of being more effective and better-characterized products. Human neutralizing antibody responses to BoNT/B have traditionally been less robust than the responses to BoNT/A (24, 25). This difference may be a function of the quality of the BoNT/B toxoid used in the vaccine, the inherent antigenicity of BoNT/B, or a combination of these and other not yet well understood factors. A recently described methodology for the production of a new, nontoxic toxoid form of BoNT/B with an improved capacity to elicit neutralizing antibodies was employed in this study (15).

Vaccination of animals to generate neutralizing antibodies against BoNT is also useful for the production of immunotherapeutics. A heptavalent non-species-specific equine F(ab’)\_2 antitoxin with IND status is available through the CDC for the treatment of botulism by passive immunotherapy. Unfortunately, adverse side effects, predominantly due to the carryover of impurities, have been observed in up to 20% of patients treated with this antitoxin (10). Human polyclonal antibodies produced in transgenic animals offer an alternative to non-species-specific antibodies, and several groups are developing such platforms (11, 21). Additionally, trivalent mixtures of recombinant human monoclonal antibodies with high neutralization potencies for BoNT/A are also being developed for therapeutic use (6, 18), and researchers plan to adopt the same strategy for the neutralization of the other six BoNT serotypes.

**Rapid vaccination of rabbits.** Knowledge of the efficacy of an accelerated vaccination scheme may be useful for achieving the optimal production of human antibodies in transgenic animal platforms. The antigenic potencies of three nontoxic BoNT/B derivatives (rHc and two toxoid preparations) were examined in a vaccination study with three groups of NZW rabbits. An accelerated vaccination scheme of biweekly dosing was used to rapidly elicit the most potent immune response possible. Rabbits were also boosted with active purified toxin during the later stages of the study to determine whether the toxin neutralization response could be further enhanced. Serum was collected from immunized animals 1 week after each vaccination dose and assessed for toxin recognition by ELISA and for neutralization by mouse challenge assays. By the conclusion of the 15-week study (all animals were exsanguinated on study day 105), rabbits from all three vaccination groups had produced neutralizing antibodies of various potencies to BoNT/B.

The development of BoNT/B antiserum, as assessed by ELISA, was rapid for animals vaccinated with the two different toxoid preparations. This apparent parallel immune response proved to be quite distinct when toxin neutralization was examined. Compared with formalin toxoid, alkylated toxoid elicited neutralizing titers more rapidly, and the titers were of roughly 10-fold greater potency. These results are consistent with the results of a previous study in which mice vaccinated with alkylated toxoid produced BoNT/B-neutralizing antibodies of 600-fold greater potency than those produced by mice vaccinated with formalin toxoid (15). Boosting of rabbits with up to 1 μg of active toxin was unable to increase the neutralization titers in formalin-toxoid-vaccinated animals to equal those observed in the alkylated-toxoid-vaccinated animals, even though the formalin-toxoid-vaccinated rabbits survived the active toxin boost. Additionally, the neutralizing titers in a fourth group of rabbits vaccinated with alkylated toxoid but not boosted with active toxin essentially paralleled those in the active-toxin-boosted group. These results, together with the comparable toxin-neutralizing capacities of the study day 105 sera from the rHc-vaccinated rabbits (which did not receive active toxin boosting) and formalin-toxoid-vaccinated rabbits (which did receive active toxin boosting), suggest that active toxin boosting does not contribute appreciably to the generation of high toxin-neutralizing titers in rabbits.

In contrast to the anti-BoNT/B responses observed in the rabbits vaccinated with the two toxoid preparations, the response elicited in rabbits vaccinated with the rHc preparation arose much more slowly. The expression of BoNT recombinant heavy chain derivatives in E. coli has traditionally resulted in the production of proteins of poor solubility and, hence, poor purification yields (5, 16, 31). While other
laboratories have had good success in generating soluble BoNT rHc proteins (33), our preparation of BoNT/B rHc precipitated. The precipitated rHc was nevertheless emulsified in Freund’s adjuvant and administered as a vaccine to rabbits. The BoNT/B rHc titers determined by ELISA climbed slowly but steadily in these rabbits and ultimately reached the BoNT/B recognition levels comparable to those observed in rabbits vaccinated with the toxoid preparations, even though the rabbits received only four vaccination doses, the last of which was administered on study day 42. The toxin neutralization titers progressed similarly, with no observable signs of protection against toxin in these rabbits being detected until the final blood draw. The appearance of neutralization potency in those serum samples was a surprise, given that four of the eight rabbits in the vaccination group had succumbed to 1-ng active toxin doses during booster dosing 3 weeks earlier. Although this study was not continued long enough to do so, it may be worth exploring the benefits of the possible long-term toxin immunity afforded by vaccination with insoluble rHc.

We observed that alkylated toxoid proved to be an antigen at least comparable to other BoNT/B antigens, when alkylated toxoid was compared to formylated toxoid and rHc, in both the speed with which the toxin-neutralizing responses were elicited and the potencies of the responses. The alkylated toxoid appears to be superior to either our rHc preparation or the formylated toxoid in neutralizing the toxin in both mouse flaccid paralysis assays and lethality assays. However, given the insolubility of rHc and the possibility of overformylation of the commercially available toxoid, we did not conduct a precise, quantitative comparison of the three antigens used in this study. Despite the problems inherent in our study, these results highlight the importance of the choice of antigen to be used for immunization to obtain neutralizing antibodies to BoNT/B. We are currently examining the long-term efficacy of the alkylated BoNT/B toxoid, and we are testing whether alkylation of the other BoNT serotypes generates neutralizing antisera more effective than formylated toxoid preparations. Knowing the most effective method for toxoid preparation that will give the best neutralizing antibodies will potentially allow a lower dose of antiserum to be administered for the treatment of BoNT intoxication, lowering the risk of adverse reactions in patients. Additionally, different forms of the toxoid may be better immunogens when they are used as vaccines in humans, leading to a faster response to BoNT intoxication for those who are at risk of being exposed, such as military personal and scientists who work with the toxin.

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