Egg Yolk Antibodies for Detection and Neutralization of Clostridium botulinum Type A Neurotoxin

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ABSTRACT

The objective of this research project was to determine the usefulness of an egg antibody platform for producing materials for the detection and neutralization of botulinum type A neurotoxin. Yield estimates for detection and neutralizing antibodies produced using methods described were calculated. Antibody specific to botulinum toxoid A (aToxoid) and toxin A (aBoNT/A) was produced by immunizing hens with botulinum toxoid A (toxoid) followed by increasing amounts of botulinum neurotoxin A (BoNT/A) in Freund incomplete adjuvant. Egg yolks were extracted with polyethylene glycol (PEG) for antibody detection and neutralization experiments. A model aToxoid/toxoid immunoassay using only egg yolk antibody was developed and had a detection limit of 1 pg/ml of toxoid. In an indirect enzyme-linked immunosorbent assay of BoNT/A-specific antibody, the aBoNT/A contained more BoNT/A-specific antibody than did the aToxoid, and aBoNT/A was as effective as commercial rabbit antibody. The aToxoid provided no protection against BoNT/A in a standard mouse neutralization assay; however, 1 mg of PEG-extracted aBoNT/A neutralized 4,000 lethal doses of BoNT/A injected intraperitoneally. Based on these results, we calculated that in 1 month one hen could produce more than 100 liters of antibody detection reagents or enough antibody to neutralize approximately 11.6 million mouse lethal doses of botulinum toxin. Utilization of an egg antibody platform is potentially rapid (28 to 70 days) and scalable to kilogram quantities using current egg production facilities with as few as 1,000 hens.

The laying hen passively transfers 150 mg of immunoglobulin (IgY) to each egg yolk produced (9, 37). Because the typical laying hen produces 300 eggs per year, the total amount of IgY deposited in egg yolks per hen per year is 45 g. As much as 10% of this IgY can be rendered antigen specific (e.g., antibody); hence, use of the hen as an antibody producer has increasingly received commercial interest (37). Several research groups have reviewed commercial applications of egg yolk IgY technology (3, 9, 19, 21, 30, 31); however, the potential use of antibody for biodefense is a more recent idea (5).

Botulinum neurotoxin (BoNT) produced by gram-positive spore-forming Clostridium spp. is widely acknowledged as the most toxic substance known. In addition to its lethality, BoNT is easily produced and transported; therefore, the Centers for Disease Control and Prevention has classified BoNT as a high-risk threat agent for bioterrorism (2). Although BoNT has not been used successfully in bioterrorism, classic foodborne botulism is caused by ingestion of BoNT from improperly preserved food, and the standard validated method for detection of BoNT in food is the mouse bioassay (15). The mouse bioassay has several well-recognized drawbacks, including time requirements, non-specific deaths due to food or environmental materials present, and the need for animals. Therefore, sensitive immunoassays utilizing antitoxin antibodies are frequently used to detect BoNT, e.g., the enzyme-linked immunosorbent assay (ELISA) (29, 34). Currently, the only successful antidote for BoNT exposure in humans is early administration of serum-derived equine botulinum antitoxin. However, the high dose (10 ml) of diluted antitoxin serum required for neutralization can result in serious complications, including complement induction, serum sickness, and occasionally anaphylactic shock (15).

The objective of this research project was to determine the usefulness of an egg antibody platform for producing materials for the detection and neutralization of BoNT type A (BoNT/A). Although production of egg yolk IgY specific to any high-risk threat agent can be accomplished, BoNT/A or denatured BoNT/A (toxoid) was used as the model agent. Others have successfully made chicken antibotulinum toxoid A IgY with BoNT/A binding capabilities (13, 26, 36), but no one has immunized hens with highly toxic BoNT/A. Hence, the first specific objective of this study was to develop an improved protocol for producing egg yolk IgY against BoNT/A (aBoNT/A). The second specific objective was to demonstrate the utility of avian IgY as a detection reagent by developing a sensitive immunoassay for detection of BoNT/A toxoid using only egg IgY reagents (aToxoid). The relative binding of aBoNT/A and aToxoid to BoNT/A was also examined in an immunoblot assay. The third specific objective was to test the ability of aBoNT/A and aToxoid to neutralize BoNT/A in a mouse bioassay. The antibody reagent production capability of one laying hen in 1 month was calculated to provide capacity estimates for biodefense.

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The chicken egg yolk IgY platform provides a rapid method for commercial-scale antitoxin production; therefore, egg yolk IgY may serve as an inexpensive alternative source of antitoxin for detection and neutralization purposes. Due in part to the unique physicochemical characteristics of IgY (14, 35), several researchers have demonstrated the efficacy of IgY as an oral treatment or prophylactic for gastrointestinal diseases (31). Hence, IgY antitoxin may be a useful oral therapy in incidences of known consumption of toxin-contaminated food. Although the safe systemic administration of IgY has not been sufficiently demonstrated, IgY antitoxin should be considered as an alternative to horse antitoxins.

MATERIALS AND METHODS

Birds and housing. Single-comb White Leghorn laying hens 22 weeks of age were the primary egg IgY producers. Hens were individually housed in cages with raised floors and were maintained under a light regimen with a 16-h light:8-h dark cycle. Hens were fed a standard corn and soybean meal–based laying hen diet ad libitum. Quail also were used as egg IgY producers and were housed in groups of three birds per cage in cages with raised floors. The light regimen was a 14-h light:10-h dark cycle, and quail were fed a corn and soybean meal–based quail breeder diet ad libitum. All procedures involving animals (chickens, quail, and mice) were approved by the Animal Care Committee at the University of Wisconsin, Madison.

Egg IgY production. Egg yolk IgY preparations containing aToxoid were produced by immunizing laying hens against botulinum toxoid A, and those containing aBoNT/A were produced by immunizing hens against BoNT/A with several booster immunizations consisting of increasing amounts of BoNT/A. For production of aToxoid, hens received an initial immunization consisting of 100 μg botulinum toxoid A (Metabiologics, Madison, WI) in 500 μl of phosphate-buffered saline (PBS) emulsified with 500 μl of Freund complete adjuvant (Becton Dickinson, Sparks, MD). The emulsion was injected intramuscularly into each breast and thigh (0.25 ml at four injection sites). Seven days after initial immunization, the hens received a booster immunization administered in the same manner as the initial immunization (1 ml per hen with 0.25 ml per site). The booster immunization consisted of 100 μg of botulinum toxoid A in PBS emulsified with an equal volume of Freund incomplete adjuvant (Becton Dickinson). IgY extracted from eggs laid beginning 20 days after the initial toxoid A immunization was considered chicken aToxoid.

Quail aToxoid IgY was generated using the same basic protocol as that for laying hens except emulsions consisted of 100 μg of botulinum toxoid A in 50 μl of PBS emulsified with 50 μl of Freund complete or incomplete adjuvant for the initial and booster injections, respectively. The emulsions were administered intramuscularly into each breast and thigh (25 μl at four injection sites). IgY extracted from eggs laid beginning 20 days after the initial immunization with toxoid A was considered quail aToxoid.

To generate sufficient IgY levels against toxin, laying hens were first immunized with toxoid A as described above and then administered weekly boosters of increasing levels of BoNT/A (Metabiologics) in Freund complete adjuvant. Immunization preparation and administration were as described for toxoid A, except BoNT/A replaced toxoid A at the dose indicated below. The immunization schedule consisted of BoNT/A booster immunizations on days 21, 28, 35, 42, 49, 56, and 63 after initial toxoid A immunization, and the booster contained 0.75, 1.5, 3, 6, 12, 29, and 50 μg of botulinum toxin A on each of these respective immunization days. The use of 130 μg of BoNT/A for hen immunizations was approved by the University of Wisconsin Biosafety Committee and met the Biological Weapons Convention Compliance requirements.

Egg collection. For aToxoid purification, hen and quail eggs were collected 20 to 30 days after initial immunization because these eggs represent peak IgY production (32). Hen eggs were collected from 70 to 100 days after initial toxoid immunization for isolation of aBoNT/A.

Egg yolk IgY purification. A simple water extraction method was used to purify IgY to confirm toxoid binding of aToxoid by an indirect ELISA. IgY was water extracted from 200 μl of liquid egg yolk by the addition of 1.8 ml of acidified PBS (pH 5) for 12 h at 4°C. For all other experiments, IgY from hen and quail egg yolks was further purified according to a procedure modified from that of Polson et al. (24). One part egg yolk was gently mixed for 3 min with two parts PBS and 3.5% (wt/vol) polyethylene glycol (PEG) 8000 powder (Fisher Chemicals, Fairlawn, NJ) until the PEG was fully dissolved. The mixture was then centrifuged at 4,000 × g for 20 min. The supernatant was filtered through glass wool to remove the top lipid layer, mixed with 8.5% (wt/vol) PEG, and centrifuged at 2,300 × g for 20 min. The pellet was resuspended in PBS and then precipitated again in 12% PEG and centrifuged as before. The remaining pellet was lyophilized in an AdVantage freeze dryer (VirTis, Gardiner, NY). For experimental use, 50 mg of IgY was dissolved in distilled water. Excess PEG was removed by dialysis of the IgY solution in PBS, and aliquots containing 1 or 5 mg/ml IgY were obtained.

aToxoid indirect ELISA. All ELISAs described in this article have been modified from those of Trott et al. (38). A 96-well Nunc-Immuno Plate with MaxiSorp surface (Thermo Fisher Scientific, Waltham, MA) was coated overnight at 4°C with 100 μl of 50 mM sodium bicarbonate (pH 8.5) containing 0 or 10 μg/ml botulinum toxoid A (Metabiologics). The plate was washed twice and then blocked for 2 h at 22°C with 175 μl per well 1% bovine serum albumin (BSA); fraction 5 with ≥98% purity; Sigma Aldrich, St. Louis, MO) in PBS. Egg yolk IgY containing aToxoid was water extracted with acidified PBS (pH 5) for 12 h at 4°C as described above. The extraction mixture was centrifuged at 1,500 × g for 10 min, and the supernatant was further diluted in 1% BSA, pH ~ 7. Duplicate samples (100 μl per well) of each dilution (1:2 × 104, 1:2 × 105, and 1:2 × 106) were added to both toxoid-coated and noncoated wells for 1 h at 22°C and then washed six times. Goat anti-chicken IgG-Fc conjugated with hors eradish peroxidase (HRP; Bethyl Laboratories, Montgomery, TX) was added to the wells (100 μl per well) for 30 min at a dilution of 1:20,000 in 1% BSA. After washing eight times, the plate was developed for 20 min at 22°C by adding 100 μl per well 50 mM sodium acetate with 0.1 mg/ml tetramethyl benzidine and 3 mM H2O2. The enzymatic reaction was stopped by addition of 50 μl per well 0.5 M H2SO4. The absorbance at 450 nm was measured with an EL800 plate reader (BioTek, Winooski, VT), and data were expressed as optical density (OD).

Development of botulinum toxoid A sandwich ELISA. Three ELISAs were developed as described above except for the use of different sources of aToxoid. The first and second sandwich ELISAs developed used rabbit aBoNT/A (10 μg/ml; Metabiologics) or PEG-extracted quail aToxoid (5 μg/ml), respectively, as coating antibodies. After blocking with 1% BSA, triplicate botulinum toxoid A (Metabiologics) samples serially diluted (1:10) from 100 ng/ml to 0.1 pg/ml were incubated (100 μl per well).
for 1 h and then washed six times. PEG-extracted chicken αToxoid IgY diluted in blocking solution (2.5 μg/ml) was used as a detection antibody. The secondary detection antibody, goat anti-chicken IgG-Fc conjugated with HRP, and substrate reactions were carried out in the same manner as described above. The toxoid detection limits of each ELISA were determined by plotting the OD results from a range of toxoid concentrations (OD = log2[toxoid concentration]) and determining the lowest concentration of toxoid with an OD value within the linear range of the curve.

An ELISA that utilized reagents derived solely from laying hen egg yolk αToxoid was developed using similar reagents and incubation times as described above except for different coating antibody, blocking solution, and detection antibody. Chicken αToxoid IgY was used as the coating antibody at a protein concentration of 3.33 μg/ml. After washing, the plate was blocked (200 μl per well) for 2 h with 3% nonfat dry milk powder (Bio-Rad Laboratories, Hercules, CA). The detection antibody consisted of chicken αToxoid IgY conjugated to HRP and was diluted to a concentration of 1 μg/ml in blocking solution. The detection antibody was produced by conjugation of HRP to PEG-extracted chicken αToxoid IgY using EZ-Link Maleimide Activated Horseradish Peroxidase Kit (Pierce, Rockford, IL). Substrate development was the same as described above, and absorbance at 450 nm was measured with the BioTek EL800 plate reader.

### aBoNT/A indirect ELISA

A 96-well BoNT/A-coated plate (Metabiologics) was blocked (200 μl per well) for 1 h with PBS containing 1% BSA. PEG-purified IgY solutions containing chicken αToxoid or aBoNT/A were diluted in BSA to 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, and 2.0 μg/ml. Duplicate samples (100 μl per well) of each dilution of αToxoid IgY and aBoNT/A IgY were added to the plate for 1 h and then washed six times. The secondary detection antibody, goat anti-chicken IgG-Fc conjugated with HRP, and substrate reactions were carried out in the same manner as described above.

### Mouse toxin neutralization assay

PEG-purified aBoNT/A IgY (1 or 10 mg/ml) was added to 10 to 1,000 mouse lethal (LD50) doses (0.10 to 10 ng) of BoNT/A1 neurotoxin complex (Hall strain) in a total volume of 0.5 ml of 30 mM sodium phosphate–0.2% gelatin (pH 6.2) and incubated at room temperature for 30 min. The mixture was then injected intraperitoneally into female CD-1 mice (16 to 22 g). Mice were studied in groups of 10 and were observed at least daily. The final death tally was determined 5 days after injection. The neutralization capacity of PEG-extracted IgY was expressed as international units of antitoxin per milliliter of yolk; 1 IU of aBoNT/A neutralizes approximately 10,000 mouse intraperitoneal LD50 doses (17). The number of eggs containing 7,500 IU or the equivalent neutralization capacity of a standard 10-ml vial of horse botulinum antitoxin currently used for treatment of botulism was calculated (12).

### RESULTS

The results of the indirect ELISA revealed that the PBS extract of egg yolks from hens immunized against botulinum toxoid A contained antibody specific to toxoid (Fig. 1). The highest egg yolk dilution required to obtain an OD reading above background was higher than 2 × 10^5 and lower than 2 × 10^6. When added to buffer-coated wells, the lowest egg yolk dilution tested (2 × 10^4) resulted in an OD reading above background (nonspecific binding of IgY to BSA used for blocking the plate). As a result, a solution of 3% nonfat dry milk powder was used for blocking in the final ELISA.

A highly sensitive sandwich ELISA for the detection of botulinum toxoid using only chicken egg yolk reagents was developed after a series of experiments designed to demonstrate the utility of avian antibody as a detection reagent (Table 1). ELISAs using rabbit aBoNT/A or PEG-extracted quail αToxoid (10 or 5 μg/ml, respectively) for coating antibodies with chicken αToxoid for a detection antibody (2.5 μg/ml) resulted in detection limits of 1 μg/ml. In addition to low sensitivity, the ELISAs required a non-avian (goat) secondary detection antibody. An ELISA using

### Table 1. Relative sensitivity of sandwich ELISAs used to detect botulinum toxoid A

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Coating antibody</th>
<th>Detection antibody</th>
<th>Secondary detection antibody</th>
<th>Detection limit</th>
<th>Nonavian reagent</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rabbit toxin A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chicken αToxoid</td>
<td>HRP–goat α-chicken IgY-Fc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 μg/ml</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>2</td>
<td>Quail αToxoid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Chicken αToxoid</td>
<td>HRP–goat α-chicken IgY-Fc&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 μg/ml</td>
<td>Yes</td>
<td>Low</td>
</tr>
<tr>
<td>3</td>
<td>Chicken αToxoid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HRP–chicken αToxoid&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>1 pg/ml</td>
<td>No</td>
<td>High</td>
</tr>
</tbody>
</table>

<sup>a</sup> Metabiologics, Madison, WI.

<sup>b</sup> Produced in the laboratory.

<sup>c</sup> Bethyl Laboratories, Montgomery, TX.
TABLE 2. Calculated yield of toxin reagents produced in one month from one hen immunized against botulinum toxin

<table>
<thead>
<tr>
<th>Product</th>
<th>Possible uses</th>
<th>Effective concn</th>
<th>Reagent yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified IgY&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Indirect ELISA</td>
<td>2–31 μg/ml</td>
<td>94–1,450 liters</td>
</tr>
<tr>
<td></td>
<td>Capture antibody</td>
<td>3.33 μg/ml</td>
<td>870 liters</td>
</tr>
<tr>
<td></td>
<td>Neutralization</td>
<td>2.9 IU/ml</td>
<td>1,162 IU</td>
</tr>
<tr>
<td>IgY-HRP conjugate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Detection antibody</td>
<td>1 μg/ml</td>
<td>2,320 liters</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated based on 85% egg production rate and polyethylene glycol–purified IgY content of 110 mg per yolk.

<sup>b</sup> Calculated based on 80% conjugation efficiency of IgY to HRP.

chicken aToxoid IgY for a coating antibody (3.33 μg/ml) and HRP-conjugated chicken aToxoid IgY for a detection antibody (1 μg/ml) eliminated the need for the goat secondary detection antibody and had a detection limit of 1 pg/ml (Table 1).

Extraction of aBoNT/A IgY from 20 15-ml hen egg yolks yielded 2.2 g of lyophilized IgY or an average of 110 mg per yolk. In proof-of-concept experiments, aToxoid IgY suspended in water, dialyzed with PBS, and diluted to 1 mg/ml was used as a capture antibody or neutralizing antibody. The aToxoid IgY (5 mg) was conjugated to HRP, diluted to 1 mg/ml, and used as a secondary antibody in a detection ELISA for nontoxic botulinum A toxoid. Based on these results, we calculated that one laying hen in 1 month can produce enough aToxoid IgY to make 870 liters of capture antibody reagent or 2,320 liters of detection antibody reagent (Table 2).

A comparison of aBoNT/A IgY and aToxoid IgY binding to BoNT/A in vitro is shown in Figure 2. The aBoNT/A IgY was capable of binding a fixed amount of BoNT/A at a much lower concentration (approximately 10%) of antibody (2 to 31 μg/ml) than aToxoid IgY (16 to 250 μg/ml) to obtain similar OD values (Fig. 2). The aBoNT/A IgY contained in the egg yolks from one hen in 1 month would yield 94 to 1,450 liters of reagent if used at working concentrations of 2 to 31 μg/ml (Table 2).

In a mouse neutralization assay, aToxoid IgY failed to neutralize BoNT/A. The neutralization capacity of PEG-extracted aBoNT/A IgY was 4,000 LD<sub>50</sub> doses per mg IgY, or 2.9 IU/ml yolk, or 44 IU per yolk. Therefore, one hen in 1 month produced antibody with the neutralization capacity of 1,162 IU (Table 2). We calculated that 170 eggs from immunized hens would contain antitoxin with the equivalent neutralization capacity (7,500 IU) of a standard 10-ml vial of horse antitoxin.

**DISCUSSION**

An immunization protocol was successfully developed for the production of significant amounts aBoNT/A IgY beginning 70 days after initial immunization. When antibodies are produced to most immunogens in the laying hen, the peak antibody titer in egg yolk typically occurs 21 to 28 days after initial immunization administered at an optimal dose ranging from 50 μg to 1 mg (32). However, when immunogens highly toxic to the hen (e.g., BoNT/A) are used, multiple immunizations with toxoid followed by low doses of toxin sequentially increased in concentration can ensure both hen survival and adequate antibody response to toxin. The use of toxin is an improvement compared with protocols describing methods for antibody production against toxoid (13, 26, 36). Our results suggest that there were marked differences in the quality of the antibody to toxoid versus toxin as a useful detection or neutralization reagent of BoNT/A. New immunogenic BoNT/A toxoids that more closely resemble BoNT/A may be useful for generating toxin-specific antibody (18, 20). Further research is needed to provide a means for shortening the time lag between initial immunization and peak antibody. Horses are immunized with increasing doses of botulinum toxin for 2 years to produce trivalent (types A, B, and E) botulinum antitoxin (12), and a comparable antitoxin probably can be produced by immunizing laying hens in a similar amount of time.

Antibody generated to toxoid and used in an indirect ELISA resulted in a significant amount of egg antibody with a working dilution of PBS-extracted IgY of 1:200,000.
(Fig. 1). At this working dilution, a single hen can generate multiple liters of antibody for ELISA use (Table 2). However, when the αToxoid IgY was compared with aBoNT/A IgY, a clear distinction in the working concentration of the two antibodies for binding BoNT/A was evident (Fig. 2). Hence, to achieve the high yields of quality egg antibody for detecting BoNT/A shown in Table 2, toxin-specific antibody produced using the protocol shown above may be useful.

Using toxoid as a model for BoNT/A, the utility of egg yolk IgY as a reagent for highly sensitive immunoassays was demonstrated by developing a sandwich ELISA using only chicken egg yolk–derived reagents. In the first sandwich ELISA experiments, the use of egg αToxoid IgY with commercial rabbit aBoNT/A was investigated as an initial proof of concept. When plates were coated with rabbit aBoNT/A and the chicken αToxoid IgY was used to complete the sandwich, sensitivity (>1 μg/ml) was insufficient. This lack of sensitivity was likely due to the marked difference in binding affinity between rabbit aBoNT/A and toxoid. There was a marked difference in binding of toxin when egg antibody was directed against toxoid versus toxin (Fig. 2). Because a major goal of this work was to manufacture large quantities of antibody reagents using eggs, an attempt to replace the rabbit aBoNT/A with egg antibody from another species was attempted. Antibody to toxoid was developed in laying quail and was extracted for use in a sandwich ELISA. When plates were coated with the quail αToxoid IgY as the capture antibody and chicken αToxoid IgY was used as the detection antibody, sensitivity was not improved (>1 μg/ml). The poor sensitivity appeared to be the result of cross-reactivity of the goat anti-chicken IgG-Fc HRP conjugate secondary antibody with the quail antitoxoid capture antibody (data not shown). Although the cross-reactive goat secondary antibody could be removed by affinity purification (data not shown), the goat antibody was viewed as the limiting reagent for developing a model toxoid detection system. A chicken detection antibody was developed (αToxoid IgY conjugated to HRP) to replace the goat secondary detection antibody.

Although quail αToxoid IgY could have been used as the coating antibody, a simplified sandwich ELISA with high sensitivity (1 pg/ml) was developed using antibody derived solely from chicken egg yolk. In this ELISA, plates were coated with chicken αToxoid IgY capture antibody. Test samples were added containing the toxoid, and the chicken αToxoid IgY capture antibody conjugated to HRP served as both the primary and secondary detection antibody. Toxoid was used as a model antigen to develop this ELISA, and these results support those of previous work (10, 26), demonstrating that highly sensitive detection assays for BoNT/A or any other toxin can be developed with the appropriate egg yolk antibodies. Additional research should be conducted to determine whether the appropriate toxin-specific antibodies would have improved the sensitivity (0.25 ng/ml) compared with the BoNT detection ELISA utilizing egg yolk toxoid-specific antibody as developed by Rocke et al. (26).

In previous studies, the ability to produce BoNT/A neutralizing antibodies by injecting hens with botulinum toxoid A has been demonstrated (13, 36). The inability to neutralize toxin with the antibody to toxoid in the present study was probably related to the immunization schedule. Antibody to toxoid was extracted from eggs laid 21 days after initial toxoid immunization and after only one booster injection, whereas in previous studies eggs with neutralizing capacity were collected after several weeks of repeated injection with toxoid (13, 36). Gomez et al. (13) obtained eggs 94 to 115 days after initial toxoid immunization, and these eggs had an average neutralizing antitoxin capacity of 0.93 IU/ml of yolk. In comparison, in the present study egg yolks containing 2.9 IU/ml were obtained from eggs laid 70 to 100 days after initial immunization. By neutralizing against toxin rather than toxoid, egg yolks contained three times more neutralization capacity and were obtained 20 days sooner than those reported by Gomez et al. (13).

At this time, the amount of neutralizing capacity of one egg using the method described is considerably below the BoNT/A neutralizing capacity of a standard 10-ml treatment vial of horse serum containing 7,500 IU and approximately 2 g of protein (12). Based on these results, we calculated that affinity purification of toxin-specific antibody from 170 eggs would contain the same BoNT/A neutralizing capacity (7,500 IU) and protein content (2 g) as the horse serum antitoxin. Antigen-specific antibody is usually 2 to 10% of the total IgY (37); therefore, 170 eggs containing 110 mg of IgY per egg would yield 0.4 to 1.9 g of toxin-specific antibody. In as little as 1 year, a flock of 1 million layers could produce the equivalent of 1.5 million antitoxin doses for treatment of botulism caused by BoNT/A.

The current horse serum treatment is trivalent, with antibodies specific to botulinum type A, B, and E neurotoxins. Further research is needed to develop egg yolk antitoxins specific to type B and E neurotoxins and to increase the neutralizing activity per milligram of protein. Strategies for increasing neutralizing activity per milligram of protein include the use of adjuvants (38) or new immunogenic toxoids (18, 20) during immunization and the use of affinity purification and antibody fragmentation (Fab isolation) (30) to purify neutralizing activity. Additional research is necessary to develop safe and effective treatment of botulism using chicken IgY. Limited research has been conducted on the safety of peripherally administered IgY as a human therapeutic. The safety of IgY has been debated, although IgY-induced hypersensitivity reactions have not been measured directly. Researchers have suggested that IgY is less likely to induce serum sickness than is mammalian antibody because IgY does not activate the mammalian complement system (36). Other researchers have suggested that circulating anti-IgY antibodies increase the probability of hypersensitivity reactions (33). Animal models must be developed to investigate the safety of IgY as a human therapeutic before preclinical human trials can be conducted. Regardless, egg yolk antitoxin IgY has potential as a detection reagent or an oral treatment of toxicosis in cases of known consumption of toxin-contaminated food.

Egg antibodies are a useful alternative to mammalian...
antibodies, and antibodies have been successfully produced against a wide range of antigens, from microbial species (4, 25) to toxins (29, 34). Egg antibodies also have unique binding characteristics that may render them as or more useful than mammalian antibodies in certain systems. Chicken IgY does not bind mammalian complement components, rheumatoid factors, or bacterial and human Fc-receptors (22, 27). As a result, chicken antibodies may have increased sensitivity for detection of toxins in certain food matrices containing animal products.

Another advantage of the laying hen compared with mammals for antibody production is decreased animal handling. Except for colostrum antibodies harvested after birth in milk secretions, mammalian antibody collection requires blood sampling, whereas large quantities of chicken antibody can be obtained easily from egg yolks on a daily basis (Table 2). Consistently high antibody titers are obtained 3 to 4 weeks after primary immunization and can be easily maintained for several months or years by administering booster immunizations (32). Variation of antibody titer between eggs from the same hen is minimal, and titers fluctuate according to a septadian biological rhythm (31).

In this study, each egg yolk contained an average of 110 mg of PEG-purified IgY containing anti-BoNT/A, which is comparable to the reported value of 150 mg of IgY per egg yolk (37). Egg yolk IgY yields are impressive relative to that reported for other species (3, 21). Jensenius et al. (16) reported that IgY corresponding to 0.5 liters of serum could be recovered from one hen in 1 month, a finding in agreement with our calculation that nearly 3 g of IgY could be easily obtained from the eggs of a single hen in 1 month. Lyophilized egg yolk can be stored indefinitely at refrigeration temperature (28, 30). Yields and storage characteristics of IgY may vary depending on the extraction method. In addition to the PEG extraction method developed by Polson et al. (24), other methods of extracting antibody have been published (1, 6–8, 11, 23, 30). Each method has a different purpose and a different intended use for the final product. Dried egg yolk powder or IgY purified from egg yolk may be useful for numerous detection and neutralization applications including biodefense, and antigen-specific IgY can be rapidly mass produced (kilogram quantities) using the existing infrastructure in many regions of the world.

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