An Evaluation of Neutralizing Antibody Induction During Treatment of Glabellar Lines with a New US Formulation of Botulinum Neurotoxin Type A

Ira Lawrence, MD; and Ronald Moy, MD

BACKGROUND: The induction of neutralizing antibodies during the aesthetic application of botulinum neurotoxin type A is rare, but of potential clinical concern. Phase III studies of a new US formulation of botulinum neurotoxin type A, Dysport (BoNTA-ABO [abobotulinumtoxinA]; Medicis Aesthetics, Scottsdale, AZ), have not identified any cases of neutralizing antibody formation during the treatment of glabellar lines in patients who received up to nine treatments.

OBJECTIVE: To provide an in-depth analysis of the potential for induction of neutralizing antibodies in the study population enrolled in phase III trials of BoNTA-ABO in the treatment of glabellar lines.

METHODS: First and last available serum samples from patients in the BoNTA-ABO Glabellar Lines Development Program were screened for BoNTA-ABO antibodies with a radioimmunoprecipitation assay (RIPA), followed by a confirmatory competitive assay (RIPA-C). Confirmed RIPA-C–positive samples were further evaluated for the presence of neutralizing antibodies using a mouse protection assay (MPA), a highly specific bioassay for neutralizing antibodies. We conducted safety and efficacy evaluations, including day 30 responder rate (a rating of no or mild glabellar lines) and duration of response in the last treatment cycle.

RESULTS: Of 1554 patients who received at least one BoNTA-ABO treatment (10 units at five injection points, for a total dose of 50 units/treatment; range one to nine treatments), five (0.32%) were antibody positive on the RIPA-C assay—two at baseline and three at the last treatment cycle. None of the RIPA-C–positive samples tested positive for neutralizing antibodies upon further evaluation using the highly specific MPA. Of note, the RIPA-C–positive group had a responder rate of 100% and a mean response of 103.3 days, while the RIPA-C–negative group had a responder rate of 90% and a mean response of 89.4 days. The safety of BoNTA-ABO did not appear to be altered in the RIPA-C–positive group.

CONCLUSIONS: At the dose and treatment interval used in the correction of glabellar lines, induction of neutralizing antibodies to BoNTA-ABO was not observed. None of the five samples that initially gave positive results in a RIPA-C assay were positive when further evaluated using the MPA. Clinically, RIPA-C–positive status did not correlate with any reduction in efficacy or an altered safety profile, although the small numbers prevent definitive conclusions. These data suggest that the five RIPA-C–positive samples represented false positives. (Aesthetic Surg J 2009;29:S66–S71.)

Botulinum neurotoxin type A (BoNT-A) injections are the most frequently sought nonsurgical aesthetic procedure, accounting for almost 2.5 million procedures in 2008 alone. BoNT-A also has important therapeutic uses, including the treatment of movement disorders such as cervical dystonia and tremors. Both aesthetic and therapeutic uses of BoNT-A require repeated injections to maintain the desired effect(s). Accordingly, the potential of BoNT-A formulations for inducing neutralizing antibodies is of critical importance.

As with other antigens, BoNT-A immunogenicity is influenced by the specific formulation and by the extent of antigenic exposure, including specific activity, frequency of treatment, and dose. The purity and
Neutralizing Antibody Induction During Treatment With BoNTA-ABO

The gold standard assay is the mouse protection assay (MPA), which tests for neutralizing BoNT-A antibodies by determining the ability of sera to prevent the death of mice given a lethal dose of botulinum toxin. Although this assay is highly specific (100%), it has low sensitivity (range 30%-50%). Furthermore, because it relies on the use of live animals, both ethical and cost issues dictate against its use in large-scale antibody screening studies. Enzyme-linked immunosorbent assays, Western blots, and other rapid tests for antibody identification are less cumbersome than the MPA, but they detect both neutralizing and nonneutralizing antibodies and are therefore not as sensitive or specific.

As a key component of the BoNTA-ABO US Glabellar Lines Development Program, the immunogenicity of BoNTA-ABO during aesthetic applications has been closely evaluated. Individual phase III studies of BoNTA-ABO failed to identify any cases of neutralizing antibody formation during treatment of glabellar lines, but the methods used in these analyses have not been fully described. Here we provide a comprehensive, in-depth evaluation of antibody formation in the study population enrolled in phase III trials of BoNTA-ABO for the treatment of glabellar lines. The data reported here indicate that patients treated with BoNTA-ABO did not develop neutralizing antibodies during the course of these studies at the prescribed dose and treatment schedules.

METHODS

The serum samples for antibody testing and the efficacy and safety data analyzed as part of this study were obtained from studies approved by the Institutional Review Boards of centers participating in a Glabellar Lines Development Program. These studies were conducted in accordance with ethical standards for biomedical research, as established by the 18th World Medical Assembly, Helsinki, Finland, 1946 and later revisions, and with US federal regulations and guidelines.

Patients and Treatment

The study population consisted of individuals enrolled in the BoNTA-ABO Glabellar Lines Development Program who had serum antibodies available for testing. Subjects provided written informed consent before enrolling in the original study. All patients in this study received at least one treatment consisting of 50 units of BoNTA-ABO (10 units/0.05 mL at each of five separate injection points in the glabellar region). Subsequent treatments (if any) were given approximately every 12 to 16 weeks, for up to nine retreatments.

Antibody Assessments

For each subject, the first and last available serum samples were analyzed for the presence of BoNTA-ABO antibodies as described below. All samples were analyzed at Ipsen Pharma AA, a subsidiary of Ipsen Biopharm, in Barcelona, Spain.
Samples were initially screened with a radioimmuno-precipitation assay (RIPA) that used an $^{125}$I-labeled recombinant C-terminal fragment of the botulinum toxin heavy chain (Hc) as the ligand, because this is the portion of the molecule that is usually recognized by neutralizing antibodies. Following incubation with serum samples, protein G-coated beads were used to precipitate antibody-ligand complexes and radioactivity was measured in a gamma counter. Positive samples were tested in a confirmatory competitive assay (RIPA-C). Each sample was analyzed twice, with competitor cold unlabeled Hc added to the second sample at 100 times the amount of $^{125}$I-labeled Hc. Samples with greater than 20% inhibition in the presence of competitor were considered to be RIPA-C-positive. All samples that were positive in the RIPA-C assay were further evaluated in the MPA.

To characterize the RIPA used in this study, samples from 197 BoNTA-ABO–treated subjects were analyzed by RIPA and the results were compared with MPA data. The RIPA was found to be highly sensitive, but of low specificity (100% sensitivity compared with the MPA; 66.5% specificity). Its negative predictive value was 100%, whereas its positive predictive value was 24.0%. Addition of the RIPA-C assay improved the specificity and predictive value of the assay. Compared with the MPA, the RIPA-C was 100% sensitive and 94.7% specific. Its negative predictive value was 100% and its positive predictive value was 66.7%.

Efficacy and Safety Evaluations

Efficacy was assessed using a validated four-point photographic scale, the glabellar line severity scale (GLSS; where 0 = no visible glabellar lines, 1 = mild glabellar lines, 2 = moderate glabellar lines and 3 = severe glabellar lines). Responders were defined as subjects with a baseline GLSS score of 2 or 3 and a GLSS score of 0 or 1 at maximum frown at day 30 following BoNTA-ABO treatment. The efficacy endpoints evaluated in this study were the day 30 responder rate in RIPA-C–positive subjects compared with RIPA-C–negative subjects and the duration of response (time to return to GLSS of 2 or 3) in the last treatment cycle in RIPA-C–positive subjects compared with RIPA-C–negative subjects.

All adverse events were recorded. To determine whether the appearance of antibodies correlated with an altered safety profile, the incidences and types of treatment-emergent adverse events (TEAE) in RIPA-C–positive subjects were compared with those in RIPA-C–negative subjects. Particular attention was paid to inflammatory injection site reactions, reactions consistent with a systemic immunologic response, and to ocular events related to muscle paralysis.

Statistical Analyses

Two-sided statistical tests were used to compare RIPA-C–positive and RIPA-C–negative subjects at the 0.050 significance level. Two-sided 95% confidence intervals (CI) were calculated using the normal approximation to the binomial distribution. Where relevant, two-sided 95% CI for the estimated differences between seroconversion groups were calculated with the method described by Agresti and Caffo. The primary $P$ value for differences between RIPA-C–positive and RIPA-C–negative groups was determined by using the Fisher exact test with the exception of duration of response, which was evaluated by the Wilcoxon test for treatment difference.

RESULTS

Antibody Status

Antibody screening was conducted on the 1554 patients who had received at least one treatment with BoNTA-ABO, had baseline and follow-up blood samples that could be evaluated for antibody status, and had received BoNTA-ABO in their final treatment cycle. A total of 847 (54.5%) patients were BoNTA naïve at the time of entry into the original study. Five of the 1554 subjects (0.32%) were antibody-positive as assessed by the RIPA-C assay (Figure). Samples from the remaining 1549 subjects tested negative on the RIPA-C assay.

All RIPA-C–positive samples were tested in the MPA for the presence of BoNTA–neutralizing antibodies. None of the RIPA-C–positive samples tested positive in the MPA (Figure).
Neutralizing Antibody Induction During Treatment With BoNTA-ABO

Demographic and Baseline Characteristics by RIPA-C Status

Demographic and baseline characteristics of RIPA-C-positive subjects were compared with those of RIPA-C-negative subjects (Table 1). Although some differences were noted between RIPA-C-positive and RIPA-C-negative subjects, no definitive conclusions can be drawn because of the small number of subjects in the RIPA-C-positive group.

Characterization of RIPA-C–Positive Samples

Characteristics of the RIPA-C–positive samples are summarized in Table 2. Of the five RIPA-C–positive subjects, two had positive samples at baseline and three had positive samples at the last treatment cycle. None of the patients had positive samples at both baseline and last cycle. Three of the RIPA-C–positive subjects were BoNT-A–naive and two had been previously exposed to BoNT-A. Of the two individuals with previous exposure to BoNT-A, one had a positive baseline sample but a negative sample at the last treatment cycle, and one had a positive sample at the last treatment cycle.

All five of the RIPA-C–positive subjects had positive samples within the first three treatment cycles. Two RIPA-C–positive subjects had positive samples at baseline, and one each had a positive sample after one, two, and three cycles of treatment (median of two treatments; Table 2). In comparison, the median number of treatments in the RIPA-C–negative group was three (range one to nine).

Efficacy and Safety

Efficacy evaluations were used to assess the impact of RIPA-C–positive antibodies on treatment outcomes. The number of evaluable patients with a day 30 response (GLSS grade of 0 or 1 at maximum frown) was five of five (100%) for RIPA-C–positive subjects and 1277 of 1412 (90%) for RIPA-C–negative subjects ($P < .999$ using the Fisher exact test). Day 30 responder data were not available for 137 of 1549 patients in the RIPA-C–negative group. The duration of response (time to return to GLSS grade of 2 or 3 at maximum frown) at the last treatment cycle was a mean (standard deviation) of 103.3 (54.0) days for the four evaluable patients in the RIPA-C–positive group and 89.4 days (54.85) for the 1448 evaluable subjects in the RIPA-C–negative group. The difference in response duration between the groups was not statistically significant ($P = .271$ using the Wilcoxon test for treatment difference).

Reports on the short- and long-term safety profiles of BoNTA-ABO have been previously published [12-14,20,21] and summaries are presented elsewhere in this supplement. To assess the impact of RIPA-C–positive status on safety, TEAE occurring in the last cycle of treatment in RIPA-C–positive subjects were compared with those observed in RIPA-C–negative subjects. There did not appear to be any clustering of TEAE in RIPA-C–positive subjects. One individual in the RIPA-C–positive group developed eyelid edema. One subject became pregnant after the initiation of treatment and had a spontaneous

---

**Table 1.** Demographic and baseline characteristics by sera conversion group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>RIPAC–positive (N = 5)</th>
<th>RIPAC–negative (N = 1549)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.8 (1.8)</td>
<td>48.3 (9.9)</td>
</tr>
<tr>
<td>Median</td>
<td>42.0</td>
<td>48.0</td>
</tr>
<tr>
<td>Age category (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 yrs</td>
<td>5 (100%)</td>
<td>932 (60.2%)</td>
</tr>
<tr>
<td>&gt;50 to &lt;65 yrs</td>
<td>0</td>
<td>523 (33.8%)</td>
</tr>
<tr>
<td>≥65 yrs</td>
<td>0</td>
<td>94 (6.1%)</td>
</tr>
<tr>
<td>Gender (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>5 (100%)</td>
<td>1377 (88.9%)</td>
</tr>
<tr>
<td>Male</td>
<td>0</td>
<td>172 (11.1%)</td>
</tr>
<tr>
<td>Race/ethnicity (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>5 (100%)</td>
<td>1367 (88.3%)</td>
</tr>
<tr>
<td>Black or African</td>
<td>0</td>
<td>15 (1.0%)</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>129 (8.3%)</td>
</tr>
<tr>
<td>Hispanic or Latino</td>
<td>0</td>
<td>20 (1.3%)</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>16 (1.0%)</td>
</tr>
<tr>
<td>BoNT-A naïve (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3 (60%)</td>
<td>844 (54.5%)</td>
</tr>
<tr>
<td>No</td>
<td>2 (40%)</td>
<td>705 (45.5%)</td>
</tr>
<tr>
<td>Baseline assessment of glabellar line severity (n [%])</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>3 (60%)</td>
<td>1124 (72.6%)</td>
</tr>
<tr>
<td>Severe</td>
<td>2 (40%)</td>
<td>416 (26.9%)</td>
</tr>
</tbody>
</table>

BoNT-A, botulinum neurotoxin type A; RIPA, radioimmunoprecipitation assay; RIPA-C, RIPA followed by a confirmatory competitive assay; SD, standard deviation.

**Table 2.** Characteristics of RIPA-C–positive samples by patient

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>BoNT-A naïve</th>
<th>Timing of RIPAC–positive sample</th>
<th>No. of treatments before positive status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>Baseline</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Yes</td>
<td>Baseline</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Yes</td>
<td>Last treatment</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>Last treatment</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>No</td>
<td>Last treatment</td>
<td>3</td>
</tr>
</tbody>
</table>

BoNT-A, botulinum neurotoxin type A; RIPA, radioimmunoprecipitation assay; RIPA-C, RIPA followed by a confirmatory competitive assay; SD, standard deviation.
abortion. Because of the small number of patients in the RIPA-C positive group, it is not possible to draw any clinically meaningful conclusions from these findings. There were no reports of eyelid ptosis or aesthenopia in the RIPA-C-positive group.

**DISCUSSION**

Because both therapeutic and aesthetic applications of BoNT-A require repeated administration, low antigenicity is an important characteristic of BoNT-A preparations. BoNTA-ABO has high specific activity (115 units/ng) and only very small quantities of protein are required per dose (approximately 0.43 ng per 50 unit dose). It has been proposed that this high specific activity may be associated with low antigenicity. The data presented here support this hypothesis.

In this study, we investigated whether BoNT-A–neutralizing antibodies could be identified in subjects receiving BoNTA-ABO treatment for glabellar lines. Out of 1554 BoNTA-ABO–treated subjects, only five (0.32%) showed evidence of antibodies in the RIPA-C screening assay. Although we cannot definitively rule out the presence of false negatives, in-depth analyses of the RIPA and RIPA-C assays determined a negative predictive value of 100% for both assays.

It is highly likely that all five RIPA-C-positive samples detected in our study represented false positives. None of the five RIPA-C–positive samples tested positive in the MPA (the “gold standard” bioassay for neutralizing antibodies), and no alterations in efficacy or safety were observed in RIPA-C–positive subjects. All five of the subjects recorded a response at day 30. Two of the RIPA-C–positive samples were positive at baseline, even though one of the patients had never been exposed to BoNT-A. Together with the relatively high false positive rate of the RIPA-C assay (33.3%), these observations strongly suggest that the RIPA-C samples did not contain BoNT-A–neutralizing antibodies and were therefore false positives.

BoNTA-ABO treatments for glabellar lines are normally repeated every 12 to 16 weeks. In this study, some patients received up to nine cycles of treatment (median of three). Repeated administration did not increase the number of patients with positive samples. In fact, all RIPA-C–positive samples occurred in patients who had received three or fewer cycles of treatment (median of two), although two patients had previously received BoNT-A treatment.

BoNT-A–neutralizing antibodies are known to be associated with a loss of clinical response. In the case study reported by Borodic, the patient who developed neutralizing antibodies was first identified by a lack of reduction in dynamic facial lines in the glabellar region following BoNTA-ONA injections. In this study, subjects with RIPA-C–positive samples did not show signs of reduced efficacy, as assessed by the proportion of responders at day 30 and the duration of response. Similarly, no obvious alterations were observed in the safety profile, including TEAE likely to be associated with immune reactions. However, firm conclusions concerning the effect of RIPA-C–positive antibodies on efficacy and safety cannot be drawn because of the small number of affected subjects.

On the basis of these data, we conclude that, at the dose and treatment interval used in the correction of glabellar lines, the induction of neutralizing antibodies to BoNTA-ABO was not observed and therefore must be a very rare event. It is likely that none of the RIPA-C–positive samples identified in BoNTA-ABO–treated subjects represented true positives, because all of the samples tested negative in the definitive assay for neutralizing antibodies, the MPA. The low (or absent) incidence of neutralizing antibody induction in subjects treated with BoNTA-ABO may relate to the high specific activity of BoNTA-ABO, which allows minute doses to be used to achieve the reduction of glabellar lines.

**CONCLUSIONS**

Although long-term monitoring is advisable, our data suggest that BoNTA-ABO has very low immunogenicity and is unlikely to induce neutralizing antibodies when used for aesthetic purposes.

**ACKNOWLEDGMENTS**

The authors acknowledge the editorial and writing assistance of Sharon L. Cross, PhD, a writer for Premier Healthcare Resource (Morristown, NJ).

**DISCLOSURES**

The authors were compensated for their contributions in preparing this manuscript and as investigators for this study. The writing of this manuscript was funded by Medicis Aesthetics (Scottsdale, AZ). Dr. Lawrence is employed by Medicis Pharmaceutical, the distributor of Dysport for aesthetic use in the United States. Dr. Moy was a paid investigator for Inamed (Santa Barbara, CA). He has no financial arrangements with Medicis Aesthetics.

**REFERENCES**


