All protein-based products, such as botulinum neurotoxin type A, are potentially immunogenic and can lead to diminished or complete absence of efficacy, especially if administered repeatedly. As such, the product content in botulinum neurotoxin formulations is an important consideration when selecting a product for treatment.

Because formulation data are not always widely accessible, this study analyzed the neurotoxin composition of new toxin formulations that have recently emerged in Asia relative to incobotulinumtoxinA (Xeomin®, Merz Pharmaceuticals GmbH).

Methods

The products analyzed and their properties are described in Table 1. The different protein and neurotoxin contents of the products were measured using a sandwich enzymelinked immunosorbent assay (ELISA) with anti-sera (Figure 1).

Because the manufacturing process for a biological therapeutic should be consistent and every batch representative, the batches were selected arbitrarily.

Care was taken to transport and store the samples at 2°C–8°C, except samples of incobotulinumtoxinA which can be stored at room temperature.

- The composition of the neurotoxin elements of each product compared to those of incobotulinumtoxinA was then analyzed to determine the mean amount of neurotoxin protein.

- All analyses were carried out with an ELISA approved by the FDA (Food and Drug Administration, Silver Spring, MD, USA).

Table 1. Properties of botulinum neurotoxin type A products analyzed in this study

<table>
<thead>
<tr>
<th>Product name</th>
<th>Innotox®</th>
<th>Botulax®</th>
<th>Meditoxin®/Neurotrel®</th>
<th>Nabota®</th>
<th>Relatox®</th>
<th>Xeomin®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>Medytox</td>
<td>Hugel Inc.</td>
<td>Medytox Inc.</td>
<td>Daejong</td>
<td>Microgen</td>
<td>Merz</td>
</tr>
<tr>
<td>Dosage (U)</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Appearance</td>
<td>Liquid</td>
<td>Lyophilizate</td>
<td>Lyophilizate</td>
<td>Lyophilizate</td>
<td>Lyophilizate</td>
<td>Lyophilizate</td>
</tr>
<tr>
<td>Formulation</td>
<td>Polyvalent (no HSA)</td>
<td>0.5 mg HSA</td>
<td>0.9 mg NaCl</td>
<td>0.5 mg HSA</td>
<td>0.9 mg NaCl</td>
<td>6 mg gelatin; 12 mg maltose</td>
</tr>
<tr>
<td>Storage</td>
<td>2°C–8°C</td>
<td>2°C–8°C</td>
<td>2°C–8°C</td>
<td>2°C–8°C</td>
<td>Room temperature</td>
<td>(20°C–25°C)</td>
</tr>
<tr>
<td>Clostridial protein per 100 U (pg)</td>
<td>N/A</td>
<td>5.000²</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>416</td>
</tr>
</tbody>
</table>

Table 2. Determination of content of botulinum neurotoxin type A protein in products by ELISA

<table>
<thead>
<tr>
<th>Product name</th>
<th>Batch name</th>
<th>Doseage (U/vial)</th>
<th>Amount of neurotoxin (pg/vial)</th>
<th>Specific potency (pg/100 U)</th>
<th>Calculated proportion (%) of inactive neurotoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botulax®</td>
<td>HUA 15135</td>
<td>100 U/3</td>
<td>644 ± 43.6</td>
<td>0.115</td>
<td>26.5</td>
</tr>
<tr>
<td>Meditoxin®/Neurotrel®</td>
<td>FAA 1587</td>
<td>100 U/3</td>
<td>575 ± 6</td>
<td>0.114</td>
<td>38.7</td>
</tr>
<tr>
<td>Nabota®</td>
<td>084962</td>
<td>100 U/3</td>
<td>754 ± 11</td>
<td>0.133</td>
<td>81</td>
</tr>
<tr>
<td>Relatox®</td>
<td>0615</td>
<td>100 U/3</td>
<td>578 ± 48</td>
<td>0.173</td>
<td>33</td>
</tr>
<tr>
<td>Xeomin®</td>
<td>31149</td>
<td>416 U/3</td>
<td>0.240</td>
<td>0.002</td>
<td>28</td>
</tr>
</tbody>
</table>

Notes: Additional reagents included O-phenylenediamine dihydrochloride (Sigma-Aldrich Co, St. Louis, MO, USA) and horse antisera reacting with the neurotoxin complex of BoNT/A (UK National Institute for Biological Standards and Control, NIBSC).

For incubation, phosphate buffered saline + 0.1% bovine serum albumin (solution 1) and PBS + 6% gelatuse (Serumwerk Bernburg, Bernburg, Germany; solution 2) (Merck, Darmstadt, Germany, or Reddel-Haen, Seelze, Germany) were used. - Additional reagents included D-phenylalanine dihydrochloride (Sigma-Aldrich Corp, St. Louis, MO, USA) and horse antisera reacting with the neurotoxin complex of BoNT/A (UK National Institute for Biological Standards and Control, NIBSC).

Following a modified protocol, the 150 kDa neurotoxin purified from the "Hall Strain", C. botulinum type A was confirmed by western blot as complexing protein-free and detoxified by 0.4% formaldehyde treatment to produce the nontoxic antigen for antibody preparation.

- Complexing proteins (excluding the botulinum neurotoxin protein) were prepared as previously published.

- The purified toxin was dialyzed against 50 mM TRIS (tri(hydroxymethyl) aminomethane)/HCl pH 7.9, Q-Sepharose column chromatography-purified (GE Healthcare, Munich, Germany) and column-bound complexing proteins were eluted.

- Antibodies against Botulax were immobilized on a CNBr sepharose matrix (GE Healthcare).

- BoNT/A was removed through affinity chromatography, eluted, and its composition checked for integrity.

Figure 1. Sandwich ELISA Technique

- Monoclonal antibody-coated well
- Antigen binds to antibody
- A monoclonal antibody, linked to enzyme, binds to immobilized antigen
- Substrate is added and color reaction measured. The rate of color formation is proportional to the amount of antigen

Results

- Highly sensitive sandwich ELISA was used to quantify the amount of BoNT/A protein in Botulax, Meditoxin, Nabota, and Relatox. InconbotulinumtoxinA was independently analyzed in parallel as a control and found to have a mean toxin content of 416 pg/vial, comparable to reports from another batch.

- It should be noted that this variation from published values is due to these batches of toxin being no longer approved for the present analysis and the use of a different batch of incobotulinumtoxinA, as well as a 5% inter-vial variability during the manufacturing process (unpublished data, Merz Pharmaceuticals GmbH).

- Although Botulax, Nabota, Meditoxin and Relatox had more neurotoxin than Xeomin in an equivalent volume, they contained greater amounts of inactive neurotoxin.

- This inactive neurotoxin cannot be taken up by neurons and hence has no clinical efficacy but might represent an immunogenic impurity leading to immunostimulation and antibody production.

- In the future, comparative studies on the efficacy, drug endurance, and safety profile of all neurotoxin products, particularly on the incidence of secondary treatment failures due to neutralizing antibody formation in patients undergoing long-term treatment with BoNT/A, will be necessary.

Discussion

- All BoNT/A formulations contain the 150 kDa neurotoxin (active molecule). Xeomin, however, consists solely of the 150 kDa neurotoxin.

- All products are based on the botulinum toxin complex with about sixfold more additional bacterial proteins, assuming a molecular weight of 4900 kDa for the complex.

- Some of the complexing proteins are carbohydrates and glycoprotein binding proteins.

- In contrast to the 150 kDa neurotoxin, these complexing proteins have the potential to bind to dendritic cells, the sentinel cells of the immune system.

- These cells must be activated as the first step of the initiation of an immune response.

- Complexing protein-containing products have a higher potential to cause an immune response.

- The formation of antibodies in patients treated with complexing-protein-containing products in aesthetic medicine has been reported.

- In contrast, antibody formation was not observed in patients treated with Xeomin free of complexing proteins.

- A further important factor determining the potential for immunogenicity of BoNT/A formulations is the amount of neurotoxin protein present. This is associated with increased antigen levels and, consequently, a greater risk of antibody production.

- It was demonstrated in cervical dystonia patients treated with BoNT products that the specific potency (U pg neurotoxin) is correlated with the antibody-induced immune response.

- That is, therefore, helpful for the clinician to receive information about the specific potency of different botulinum toxin products.

- As Xeomin's manufacturing process isolates only the active 150 kDa neurotoxin, Xeomin is entirely free of complexing proteins.

- Xeomin remains the only BoNT product marketed as containing "purified neurotoxin" that has been registered with regulatory authorities in the USA and Europe.

- One can conclude that the lower specific potency of Botulax, Nabota, Meditoxin, and Relatox may actually indicate the presence of significant amounts of inactive, rather than active, neurotoxin.

- High neurotoxin protein levels detected in this study were not due to biologically efficacious neurotoxin, but due to inactive toxin provided that all products were equivalent in containing 100 U per vial.

- This inactive neurotoxin cannot be taken up by neurons but might represent an immunogenic impurity. These inactive components, which have no clinical efficacy, per se, may stimulate antibody production.

Conclusions

- Although Botulax, Nabota, Meditoxin and Relatox had more neurotoxin than Xeomin in an equivalent volume, they contained greater amounts of inactive neurotoxin.

- This inactive neurotoxin cannot be taken up by neurons and hence has no clinical efficacy but might represent an immunogenic impurity leading to immunostimulation and antibody production.

- In the future, comparative studies on the efficacy, drug endurance, and safety profile of all neurotoxin products, particularly on the incidence of secondary treatment failures due to neutralizing antibody formation in patients undergoing long-term treatment with BoNT/A, will be necessary.