Chapter 4

# Clinical Relevance of Immunoresistance to Botulinum Therapy

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Additional information is available at the end of the chapter

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**Abstract**

Because botulinum toxin is a bacterial antigen, the therapy with this biological bears the risk that the formation of antibodies is elicited which can neutralize the neurotoxin. Several factors have an impact on this immune response. There are (unknown) patient-related factors but also the dose, the injection interval and the purity of the product play a role in the formation of antibodies. Several assays to detect antibodies are available; immunological assays such as FIA (fluorescence immunoassay) and ELISA, function assays such as MPA (mouse protection assay) and HDA (hemidiaphragm assay), clinical assays such as the EDB assay (extensor digitorum brevis assay), and SCM assay (sternocleidomastoid) which have different sensitivities. Clinical studies with different BoNT/A products demonstrate that the rate of antibody formation is low. Important for the physician is whether the antibody formation has an impact on the responsiveness of the patient. Not all patients with positive sera are nonresponders. The antibody titer is certainly important which might not be high enough to neutralize the injected dose completely but the titer might increase during further treatment with the neurotoxin leading to complete nonresponse. To avoid the formation of antibodies, the lowest dose necessary for the patient should be injected keeping the longest acceptable injection interval.

**Keywords:** botulinum neurotoxin, neutralizing antibodies, secondary nonresponse, immunogenicity, treatment failure

## 1. Introduction

Cervical dystonia (CD) is a chronic disease lasting for the rest of a patient's life and requires a lifelong treatment. Injection of botulinum neurotoxin type A (BoNT/A) is recommended by the

European Federation of Neurological Societies (EFNS) as the first line treatment for primary crandial and cervical dystonia [1]. Currently, three products are approved for medical use in Western markets, and all are approved by the FDA: onabotulinumtoxin A (ONA; Botox/Vistabel®, Allergan Inc., Irvine, CA), abobotulinumtoxin A (ABO; Dysport®Ipsen, Paris, France), and incobotulinumtoxin A (INCO; Xeomin®, Merz Pharmaceuticals GmbH, Frankfurt Germany). The efficacy and safety of these three commercially available BoNT/A preparations have been well established with level A recommendations. Botulinum toxin products are also successfully applied in a broad range of medical indications in the field of other movement disorders (e.g., spasticity, blepharospasm); urological disorders (e.g., overactive bladder) as well as aesthetic indications. This review will focus on clinical studies in CD and spasticity.

There are also several botulinum toxin products originating and approved in Asian countries: in Korea Neuronox (Mecytox Inc), Nabota (Daewong Inc.), Botulax (Hugel Inc.), and from China BTXA or Lantox (Lanzou Institute) with a drug substance similar to ONA but with a different manufacturing process and formulation. Studies conducted with these products will not be further discussed in this chapter. The only approved botulinum toxin type B product (BoNT/B) is Myobloc/Neurobloc (rimabotulinumtoxinB, RIMA, US World Meds, Lousiville, KY). BoNT/B can be used when there is resistance to BoNT/A.

The molecular composition and mechanism of action of BoNTs are described in numerous reviews and are only briefly summarized here [2, 3]. The active moiety in all BoNT/A products is the neurotoxin, a 1296 amino acid long protein with a relatively high molecular weight of 150 kD [3, 4]. BoNT/A is synthesized by the anaerobic bacterium *Clostridium botulinum* as a single-chain protein, which is cleaved into two subunits by a clostridial protease resulting in two subunits, a heavy chain and a light chain, linked by a disulphide bridge. The C-terminal domain of the heavy chain binds highly specifically to receptor molecules on the presynaptic membrane of cholinergic neurons. The heavy chain has two binding domains, one for special glycolipids (GT1b) and the other for a protein receptor called SV2 [5]. The receptor-bound BoNT is taken up into the nerve cell by endocytosis. The second domain of the heavy chain called translocation domain then facilitates the release of the light chain out of the endocytotic vesicle into the cytosol of the neuron. The smaller subunit, the light chain, is a highly specific protease which cleaves a neuronal protein, the so-called SNARE protein, SNAP25, required for the secretion of acetylcholine. Cleaved SNAP25 has lost its ability to function in the secretory process. As a result, the acetylcholine-containing secretory vesicle cannot fuse with the presynaptic membrane, acetylcholine is not secreted, and so the muscle cell is no longer activated and becomes paralyzed [6]. By this mechanism, BoNT blocks cholinergic muscular innervation of striated and smooth muscles as well as the cholinergic innervation of exocrine glands (sweat gland, saliva gland). The mode of action is identical for all BoNT/A products. Other BoNT serotypes act in a similar way but their receptor molecules, and substrates are different, for example, BoNT/B, the active substance in RIMA, cleaves a SNARE protein called VAMP [4].

As a bacterial protein, botulinum toxin is a foreign protein to the host immune system, that is, per se an antigen and bears, therefore, the risk to elicit the formation of antibodies particularly because botulinum toxin has to be applied repeatedly after the therapeutic effect has waned

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off. The formation of antibodies against the protein can lead to secondary treatment failure, to nonresponse (secondary nonresponse, SNR), that is, to the termination of the therapy.

This review will discuss the formation of neutralizing antibodies against botulinum toxin particularly in the treatment of CD and spasticity regarding the immunogenicity of this highly efficacious protein and its clinical implications.

## 2. Development of antibodies against botulinum neurotoxin and secondary nonresponse

### 2.1. Immunogenicity of botulinum toxin products

All products contain the 150 kD neurotoxin as the active principle, a foreign protein to the human immune system. This protein is produced by clostridia and is embedded into a complex formed with other bacterial proteins, the so-called complexing proteins or neurotoxin-associated proteins (NAPs) [7]. Some of these proteins are biologically active. They belong to lectins and bind to glycoproteins or glycolipids on cell membranes [8]. They are called hemagglutinins with respect to their capability to agglutinate red blood cells. This specific activity is certainly not required in the treatment of movement disorders. The hemagglutinins are rather necessary in the process of oral intoxication with the toxin. Thus, the complexing proteins have no function in the therapy with botulinum toxin.

Therapeutic proteins which are administered repeatedly can elicit the formation of antibodies, even when these proteins originate from human sources [9]. These antibodies can lead to loss of efficacy [9]. As botulinum toxin products contain bacterial proteins and are administered repeatedly, they have an even higher risk to stimulate the formation of antibodies directed against the neurotoxin, and/or in the case of ONA and ABO against the complexing proteins [10]. In principal antibodies against the active substance will interfere with the therapy. Depending on the antibody titer, it can lead to partial therapy failure or—if the antibody titer is high enough—to a complete treatment failure or secondary nonresponse [11]. In general, partial treatment failure precedes complete treatment failure as demonstrated in a (small) clinical study with 27 patients. Twenty two of these patients showed partial treatment failure. Before comlete nonresponse occurred [12]. It is obvious that antibodies directed against the binding domain of the neurotoxin heavy chain will inhibit the binding of the neurotoxin to the neuron or inhibit the translocation and consequently neutralize the activity [13, 14]. Antibodies directed against the enzymatic domain (light chain) can also inhibit the neurotoxin's activity because of steric hindrance of the translocation process [13].

Apart from patient-related factors (sensitivity of the patient's immune system), several product-related factors may influence the immunogenicity of biological proteins (see **Table 1**). In case of BoNT products, the manufacturing process, the antigenic protein load, and the presence of complexing proteins contribute to the immune response but treatment-related factors, for example, the interval between injections, booster injections, and prior exposure may also be involved. The role the protein load and treatment intervals play in the process of

antibody formation is convincingly demonstrated by the first generation of ONA. It contained 10 times more antigenic protein (50 ng of clostridial protein) than the current formulation. Consequently, it generated a high rate of antibody formation and secondary nonresponders [15, 16]. Therefore, physicians were advised to keep the intervals between single treatments as long as acceptable for the patient to prevent the formation of antibodies [17]. The amount of neurotoxin protein in ONA has since been markedly reduced to 5 ng clostridial protein, (corresponding to 0.83 ng neurotoxin protein) leading to a marked decrease in antibody formation [18] (see further discussion in Section 2.3.1).

| According to CHMP guideline EMEA/CHMP/BMWP/14327/2006; 2008 |
|---|
| Factors that may influence the development of an immune response against a therapeutic protein |
| • Patient and disease-related factors |
| • Genetic factors modulating the immune response |
| • Genetic factors related to a gene defect |
| • Age |
| • Disease-related factors |
| • Concomitant treatment |
| • Duration, route of administration, treatment modalities |
| • Previous exposure to similar or related proteins |
| Product-related factors of immunogenicity |
| • Protein structure |
| • Formulation |
| • Aggregation and adduct formation |
| • Impurities |

**Table 1.** Factors influencing immunogenic response.

Complexing proteins do not play a role in the mechanism of action of BoNT once it has reached body fluids; thus, antibodies directed against these proteins cannot block the activity of BoNT, they cannot neutralize the neurotoxin. It has been reported that approximately 50% of patients (treated for a therapeutic indication) develop antibodies against the complexing proteins without therapeutic consequences [19]. From this standpoint, complexing proteins are just inert proteins without any effects on BoNT therapy. However, new data suggest that this might not be the case. A growing body of evidence shows that complexing proteins can interact with the host immune system and, therefore, be clinically relevant [20].

To produce antibodies, the immune system needs to be activated. Not only the antigen has to be present, but also an activating signal [21]. The first cells that recognize the antigen (i.e., BoNT) are dendritic cells. These cells present the antigen to T-lymphocytes. These lymphocytes are then activated by cytokines secreted by the dendritic cells. The activated T-lymphocytes subsequently activate B-lymphocytes which produce the antibodies [21]. Dendritic cells have exposed pattern recognition receptors (toll-like receptors), which react with different bacterial products such as bacterial DNA, parts of the bacterial cell wall, and bacterial proteins such as

flagellin and hemagglutinins [21]. The latter are known to act as adjuvants, which bind to and activate dendritic cells [22].

The first step of the binding to immune cells has been demonstrated by analyzing the interaction of the BoNT complex, and the purified BoNT, free from complexing proteins, with lymphoblasts, fibroblasts and a human neuroblastoma cell line (as a control) [20]. It was shown that both the complexing proteins and the BoNT complex reacted with the lymphoblasts, in contrast to the pure BoNT [20]. And moreover, whereas the purified BoNT did not influence the release of inflammatory cytokines, BoNT complex and the complexing proteins lead to a dramatic increase in release [20]. Thus, the complexing proteins can affect the formation of antibodies against BoNT by stimulating dendritic cells.

### 2.2. Assays to detect antibodies against botulinum neurotoxin

The simplest way assays to detect antibodies are immunoassay-based procedures such as enzyme-linked immunosorbent assay (ELISA), the fluorescence immunoassay (FIA), and the immunoprecipitation assay [23]. These assays are often used in clinical studies to screen sera of a large patient population. Positive samples are then further evaluated in functional assays because immunassays cannot discriminate between neutralizing and non neutralizing antibodies; they only detect antibodies that bind to the antigen, botulinum neurotoxin. Whether these antibodies inhibit the activity of the neurotoxin cannot be assessed with these assays, and therefore, the assay results cannot be correlated with therapeutic response.

A method used for the detection of BoNT neutralizing antibodies must test the function of each domain of the neurotoxin: binding, translocation, as well as the catalytic (proteolytic) activity of the light chain in one assay or in a set of assays, because antibodies can be directed against only one domain and its affinity might not be high enough to inhibit the binding to the neuronal receptor or prevent the uptake of the neurotoxin into the nerve cell. If a single functional assay is applied, it should be based on intact cellular systems. The easiest test is the mouse protection assay (MPA). Mice are treated with increasing doses of BoNT and their survival rate is determined. This assay is presently considered the gold standard because the median lethal dose (MLD) can be determined very accurately [24]. The MLD increases when BoNT antibodies are present. By means of a calibration curve, based upon standard antibody titers, titers in patients' sera can be calculated. However, exact titers determined with the MPA are rarely published. Often the MPA assay is carried out by injecting a mixture of a LD100 dose of the toxin with the serum of a respective patient into five mice. If all mice survive, the serum is antibody-positive [18]. The correlation between the MPA and clinical responsiveness was investigated in an early study with 51 patients (34 nonresponder and 17 responder [25]); the specificity of the assay was high (100%), no patient with a negative titer in the MPA showed a clinical nonresponse. A disadvantage of the MPA is its low sensitivity. Only half of the patients with nonresponse showed a positive MPA result. This result may be explained by the fact that the detection limit of the assay is too high [25] or the nonresponse might be caused by non-immunological factors.

The MPA has many disadvantages. The test is costly, requires several days before it can be evaluated and exposes the test animals to prolonged agony including respiratory failure. Since

the endpoint of the test is the paralysis of the respiratory muscle, a truncated version of the test is represented by an isolated nerve muscle, the phrenic-hemidiaphragm preparation (mouse hemidiaphragm assay; HDA). In this assay, the serum of the patient is incubated with a fixed amount of the neurotoxin followed by the measurement of the effect on the contraction amplitude of an indirectly stimulated mouse hemidiaphragm mounted into an organ bath [26]. This assay is quantitative and highly sensitive, about 25 times more sensitive than the MPA [27].

A disadvantage of the assay is that a high amount of patient's serum is required (4.0 mL) so that in most cases, the serum sample is only determined once.

Because cell-based assays (CBA) are now approved to replace the LD50 assay, the CBA could also be adapted to determine antibodies.

All these functional assays analyze the antibody titer in sera and not in the muscle in which the neurotoxin is applied and potentially neutralized. The antibody titer in sera and in muscles must not necessarily be identical. This fact could explain why patient with an antibody titer still respond to neurotoxin. To circumvent this disadvantage, clinical assays were developed that measure—indirectly—the inhibitory activity of antibodies in muscles. These include the extensor digitorum brevis test (EDB) [28] the unilaterally brow injection test (UBI) [29], the sternocleidomastoid test SCM [30], and the sudomotor sweat test [31]. They are generally not quantitative (results are "positive" or "negative" based on the judgment of the investigator) and do not determine the antibody titer directly but it's clinical effect and principally show a satisfactory correlation with the responsiveness of the patient, which means that they do not provide assay-positive results for patients who are still sensitive to botulinum toxin therapy. Although very valuable for the clinical practice these assays are usually not applied in large clinical studies because patients have to be reinjected when nonresponse occurred and judged in a visit after additional days.

### 2.3. Factors which determine the formation of antibodies

The CHMP guideline on immunogenicity assessment of biotechnology-derived therapeutic proteins lists factors, which determine the formation of antibodies in patients (**Table 1**) [32]; some of them are patient related and some are based on the product and its application. Until today, no investigations are published why patients develop antibodies against botulinum neurotoxins. It is unclear whether for instance the specific HLA repertoire of a patient favors the formation of antibodies or other patient-related (e.g. genetic) factors. There could be a higher incidence of antibody formation in children as found in an early study, in which 35 of 110 treated children developed an antibody titer and became secondary nonresponder [33]. More research, however, is needed to confirm this risk factor. In the following, some factors related to the products and clinical procedures are discussed.

#### *2.3.1. Dose of the neurotoxin*

In general, the dose of an antigen determines the formation of antibodies: a higher dose bears a higher risk of antibody formation. But so far, there is no defined threshold below which no antibodies are produced.

The first approved botulinum toxin product (ONA) contained a high amount of the neurotoxin complex: 25 ng (100 U) corresponding to 4.2 ng of the neurotoxin protein because a high amount of neurotoxin (ca 80%) was inactivated during manufacturing. Up to 17% of the patients treated with this product developed antibodies [18, 34, 35]. Based on an improved manufacturing process in 1997, a new lot of ONA became available which contains a substantially lower amount of the botulinum toxin complex leading to an about sixfold decreased rate of antibody formation [18]. Nevertheless, there is still inactive neurotoxin present in ONA which could enhance the risk of antibody formation [36].

The dose of RIMA in the treatment of CD is about 50 times higher than the dose of INCO and ONA, which means that a markedly higher amount of antigen is injected (about 12 times more neurotoxin protein). In addition, about 30% of the neurotoxin is inactive (because it is not processed into heavy and light chain) [37]. Therefore, approximately between 35 and 44% of patients treated with RIMA develop antibodies against BONT/B [38].

**Table 2** provides an overview over the different amounts of bacterial protein and neurotoxin protein injected in comparable doses. The differences between the products are obvious: the high amount of neurotoxin protein in RIMA seems to be responsible for the high percentage of antibody formation. One would expect that the product with the lowest amount of antigen bears lowest risk of antibody formation (see below).

| Product | Dose | Amount of protein (ng) | Amount of neurotoxin protein (ng) |
|---|---|---|---|
| ABO* | 250 U | 2.17 | 1.62 |
| INCO* | 100 U | 0.44 | 0,44 |
| ONA* | 100 U | 5 | 0.73 |
| RIMA** | 5000 U | ≈55 | ≈11 |

* Calculated from Ref. [36].
** Calculated from Ref. [37].

**Table 2.** Amount of neurotoxin protein (antigen) injected into patient.

A higher cumulative dose seems to correlate with a higher risk of antibody formation: in an early study, Jankovic and Schwartz observed that patients with a titer of neutralizing antibody were treated with a higher cumulative dose (1709 U ± 638 U over 2.5 years) than patients with a lower cumulative dose (1066 U ± 938 U over 2.4 years) [16]. Göschel et al. found a correlation between a high dose and a high proportion of antibody formation in patients treated with ONA and/or ABO, but only a small population of 28 patients was analyzed [19]. Some indications require a higher dose than other and, therefore, bear a higher risk of antibody formation. This was confirmed by Lange et al. who showed that in patients treated for CD and spasticity higher doses correlate with a high rate of antibody formation [27]. This is in accordance with another study with patients suffering from CD. The patients with SNR received a higher dose than the responders. Also these authors concluded that a higher dose is a risk factor for the development of antibodies [39].

*2.3.2. Injection interval*

A second injection after a short injection interval might act as a "booster" injection. Greene et al. observed that patients whose sera were positive in the MPA had been treated with markedly shorter injection intervals [17]. An evaluation of sera of secondary nonresponders treated with ABO and ONA demonstrated that patients treated within 1–2 months showed a higher proportion of antibody formation than patients with an injection interval of 4–13 months [27]. Thus, a short treatment interval seems to correlate with a higher risk of antibody formation and secondary nonresponse. Greene et al. suggested to inject as infrequently as possible ideally no more frequently than 3 months [16]. This procedure is still standard clinical practice. In the prescribing information of all products, it is recommended not to reinject earlier than 12 weeks. Unfortunately, this interval is not adequate for all patients, some require an earlier treatment because of termination of the therapeutic effect. In a long-term prospective CD study, it was demonstrated that 22.5% of the patients reinjected with INCO <10 weeks and 24.6% between 10 and 12 weeks did not develop antibodies [40]. Although more long-term studies are required, the strict adherence to an injection interval of 12 weeks seems to be obsolete at least for INCO.

**2.4. Antibody formation and secondary nonresponse in clinical studies**

Generally, a two tier approach is used in clinical studies to identify patients with neutralizing antibodies: screening with an immunoassay and confirmation of positive results with a functional assay. Data for antibody formation in clinical studies cannot really be compared because the applied assays have a markedly different sensitivity, the mouse protection assay is at least 5 times (or even 25 times [27]) less sensitive as the mouse hemidiaphragm assay. In addition, there is no published information about the validation of the assays particularly concerning sensitivity and specificity of the assay. In the most cases studies with ONA, ABO, and RIMA apply the MPA whereas in studies with INCO only the HDA is used for the detection of neutralizing antibodies.

In the following, results derived from clinical studies with the different products are summarized. Studies with ONA before 1997 (the "old Botox") will not be discussed because it is no longer on the market. A most recent overview over neutralizing antibody formation in clinical studies is presented by Fabbri et al. [41].

*2.4.1. Cervical dystonia*

*2.4.1.1. OnabotulinumtoxinA*

Brin et al. conducted an open-label long-term observational study with ONA in 326 neurotoxin naïve patients over a mean of 2.5 years [42]. They reported the formation of antibodies during the course of up to 15 treatment cycles in 4 patients (1.2%) resulting from a mouse protection assay. Three of these patients lost responsiveness. One of these patients was also antibody positive in a clinical assay (FTAT), and the other two patients were not tested [42]. In a comparison of the current ONA with the pre-1997 product, it was found that none of the 119

patient treated with the current ONA developed neutralizing antibodies by applying the MPA [16].

#### *2.4.1.2. AbobotulinumtoxinA*

In a randomized, placebo-controlled study with 116 patients Truong et al. found one patient with neutralizing antibodies in the MPA, but this patient still responded to ABO [43]. Coleman et al. reported that 3 patients out of 136 developed neutralizing antibodies as tested with the MPA (2.2%), and two of these patients still responded to the treatment [44]. In a long-term open-label study conducted by Kessler et al., 303 patients with CD were treated with ≥6 injections of ONA. Nine of 17 secondary nonresponders were antibody positive in MPA, HDA, or EDB [45]. The rate of antibody formation was calculated with 2.5% referring to a total of 357 patients (54 patients discontinued the study) [45]. Antibody positive patients were treated with higher doses in shorter intervals and had a higher number of "booster" injections. In a long-term study (10–12 years) conducted by Haussermann et al. Three patients out of 90 patients became nonresponder (3.3%) but were antibody negative in the HDA [46].

#### *2.4.1.3. IncobotulinumotoxinA*

In a placebo-controlled study, four out of 233 patients developed antibodies during the placebo-controlled phase of the trial, four in the open-label phase according to the testing in the HDA, and none of these patients became secondary nonresponder [47]. It has to be mentioned that the antibody-positive patients were treated with other products before the study so the antibody formation might be primed by the other products. No naïve patient developed antibodies. This was also demonstrated in other studies with INCO [28–50]. In an open-label study conducted by Benecke with 100 patients previously treated with ONA, ABO or RIMA, no patient developed antibodies or secondary nonresponse during treatment with INCO for up to 2 years [51]. No patient developed new neutralizing antibodies in a trial with 76 patients with CD based on testing with the HDA. Three patients had neutralizing antibodies at screening (prior to treatment with incobotulinumtoxinA), two of whom experienced no loss of treatment effect 4 weeks after repeated injections of INCO, while a third patient did experience a loss of treatment effect after the second and subsequent INCO injections [52].

#### *2.4.1.4. RimabotulinumtoxinB*

Jankovic et al. conducted a 42 months lasting observational study: Patients who were previously treated with BoNT/A and partly developed antibodies against BoNT/A were treated with RIMA [38]. According to results of the MPA, 34.4% of the patients developed antibodies against BoNT/B whereas the proportion of patients with antibodies against BoNT/A declined from 13 to 2.5% during the course of the study [38]. Also in this study, the development of immunoresistance correlated with the dose of toxin. The rate of antibody formation after treatment with RIMA was high in three long-term studies, 33.0, 42.0–44.0, and 28% over 2, 4 or 7 years, respectively) [53]. Remarkably, most of the patients who discontinued the study because of poor efficacy did not develop antibody, precise numbers were not given. The authors assume that the development of antibodies did not correlate with the lack of efficacy. In contrast, in a

small study by Dressler and Bigalke, four of the nine patients treated with RIMA became resistant to the therapy and developed antibodies against BoNT/B as shown in the HDA [54].

*2.4.2. Upper limp spasticity*

*2.4.2.1. OnabotulinumtoxinA*

In a pooled analysis of three studies with 191 post-stroke spasticity patients, Yablon et al. reported the development of antibodies in one patient assessed with the MPA (0.5%) [55]. The patient did not respond to ONA at any time of the study. Elovic et al. reported one patient out of 224 developed neutralizing antibodies according to the MPA (0.45%) [56]. At the end of the study, this patient was a nonresponder who was also confirmed in a clinical assay (FTAT).

*2.4.2.2. AbobotulinumtoxinA*

Baktheit reported in the only published study that no patient developed neutralizing antibodies in an open-label trial with 41 patients with post-stroke spasticity [57]. There was no information about secondary nonresponse.

*2.4.2.3. IncobotulinumtoxinA*

Applying the HDA after FIA screening, no neutralizing antibody formation was observed in a study by Kanovsky et al. Seventy three patients were treated in a single cycle with INCO [58]. No patient showed secondary nonresponse. This was also observed in another trial with 47 patients with arm spasticity reported by Dressler et al: no patient of developed antibodies or secondary treatment failure [59].

*2.4.2.4. RimabotulinumtoxinB*

Brashear et al. did not find antibody formation in 10 patient treated with10,000 U of RIMA in a 16 week study followed by a 12 week open-label extension period [60]. Secondary nonresponse was not observed.

In conclusion, the studies demonstrate that the formation of antibodies after treatment with BoNT/A products based on MPA or HDA is low and only very weakly correlated with secondary nonresponse. Patients with neutralizing antibodies were not necessarily nonresponders, and not every nonresponder was antibody positive. But it cannot be excluded that the analyzed titer is too low to neutralize the amount of injected neurotoxin. Because the immune system was already stimulated, it can be speculated that the antibody titer will increase over time with further injections (“booster”) of the antigen (neurotoxin) leading to secondary nonresponse when the titer is sufficiently high. Long-term studies are warranted to observe antibody titer development and therapeutic responsiveness of antibody positive and responsive patients. Complete therapy failure seems to be preceded by partial therapy failure characterized by a low antibody titer which is also characterized by a shorter duration of effect [61].

Secondary nonresponse is not only caused by the formation of neutralizing antibodies. Many patients nonresponsive to BoNT have no detectable antibody titer. Lange et al. analyzed sera of 503 patients treated with ABO or ONA with the HDA and showed that 224 patient's sera (44.5%) contained neutralizing antibodies [27]. Of course, it cannot be excluded that assay results were false negative (the assays were carried only once for each serum, there was not confirmatory assays applied) or the sensitivity of the HDA was too low to detect antibodies which neutralize BoNT after injection in the muscle. More plausible is that lack of response might be caused by other factors like inadequate dosing, failure to inject the appropriate muscle or change in the pattern of muscle hyperactivity and changes in the disease state. Other more subjective factors may also be the cause to determine nonresponsiveness (e.g., too high expectations of patients, the "honeymoon effect" [27]). Further information about secondary nonresponse and antibody development in other indication can be found in recent reviews [62, 63].

### 2.5. Development of antibody titer after termination of therapy

How the antibody titer developed after cessation of the Botulinum toxin therapy was analyzed in small clinical studies [64]. Thirteen patients treated for various distonic symptoms and with complete secondary treatment failure were analyzed overt time [64]. By applying the HDA to quantitate the titer of neutralizing antibodies in sera Dressler&Bigalke found that in 8/13 patients the titer decreased in the period between 500 and 1750 days. After between 1250 and 2250 days to such a low titer that complete therapy failure is unlikely as the authors assume. Five of the patient did not show decreasing antibody titer. The authors suggest that for some of the patients, botulinum toxin therapy could be reinitiated [64].

Hefter et al. analyzed the development of the antibody titer in a prospective, blinded cohort study including 37 patients with CD who had developed neutralizing antibodies and partial secondary nonresponse to prior therapy with ABO or ONA [65]. The patients received continuous treatment with INCO with 200 U and after 24 months with 300 U for up to 50 months. Ten patients (27%) in this study had a transient increase in titers of such antibodies in the first 24 months of treatment with INCO. However, for the majority of patients (84%), antibody titers declined to levels below the initial. At the end of the study, tests for neutralizing antibodies were either negative or below the lower detection limit in 23 patients (62%). The titer of 24 nonresponders not treated with INCO was also analyzed. Both cohorts, INCO treated and nontreated, showed a similar decline in antibody titer [65]. However, it is unclear whether the patients whose antibody titers decreased regained complete treatment benefit. In any case, the injection of INCO did not increase the antibody titer and one can assume that it was not recognized as an antigen in this long-term study because of the low protein load with this product.

## 3. Conclusions

The development of neutralizing antibodies in patients with CD or spasticity treated with botulinum toxin is rare. However, due to the large number of patients treated worldwide, it

can be assumed that approximately 50,000 individuals become nonresponder. However, because the large difference in the sensitivity of the assays used it is not possible to assess the percentage of patients who will develop antibodies during the course of treatment. With respect of the high antigenic dose in the treatment with the BoNT/B product, neutralizing antibodies are detected much more frequently than with BoNT/A products. Whether the antibody titer is high enough to neutralize the injected dose of the neurotoxin might be different from patient to patient depending on the condition in the injected muscle, for example, how fast the neurotoxin molecules migrate to the motor endplate and escape from the antibody molecules, it might also depend on the distribution of the antibody molecules in the muscle tissue. Therefore, an antibody titer in a respective serum does not necessarily mean that the patient is unresponsive to botulinum toxin, and this is certainly a matter of the concentration of the antibodies in the muscle. The antibody concentration and the binding capacity of the antibodies to BoNT molecules determine whether the injected dose of the neurotoxin is only partly or completely neutralized. But if the immune system is already activated and a low titer is generated it can be assumed that further injections will increase the titer leading eventually to therapy failure. Unfortunately, systematic studies which analyze the development of low titers after further injections are missing.

Whether there are differences in the antigenic potential of botulinum toxin products which is not analyzed in head to head clinical studies and probably never will. All products containing complexing proteins generated antibody induced secondary nonresponse with various rates. There are no reports until now revealing that INCO induced secondary nonresponse in naïve patients. It can be assumed that the absence of other bacterial proteins and substances and a high specific potency of the product favor a low immunogenic potential. As already suggested since the beginning of botulinum toxin therapy, some obvious factors should be taken into account to avoid the formation of neutralizing antibodies: the dose should be as low as possible, just high enough to achieve the therapeutic effect and the injection should be as infrequent as possible. In the event that antibodies associated with nonresponsiveness are already present a different serotype could be applied. It has been shown that the antibody titer decreases over time; therefore, it could be advisable to monitor the antibody titer and reinitiate the therapy after the titer is low. This might take two or more years.

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