

# Botulinum A Toxin Therapy: Neutralizing and Nonneutralizing Antibodies—Therapeutic Consequences

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**Although muscle-relaxant doses of botulinum A toxin (BoNT/A) are generally lower than doses stimulating the immune system, specific antibodies are raised in a substantial number of patients. As a rule, this necessitates the termination of treatment. Therefore, a reliable determination of specific anti-BoNT/A antibodies is helpful and we introduced, for this purpose, a novel *in vitro* toxin-neutralizing assay based on a nerve-muscle preparation. We measured the antibody titers in four groups of subjects: Group 1 comprised 75 randomly selected patients of a total of 295 who responded to treatment with Dysport in our local clinic. Five patients, in group 2, were nonresponders. Group 3 consisted of 32 untreated volunteers and group 4 of 8 subjects immunized with a toxoid more than 10 years ago. Two of the responders had marginal titers of neutralizing antibodies, while they were present in all nonresponders. The sera of all responders were also tested for nonneutralizing antibodies by ELISA. Their occurrence, however, was of no consequence to the therapeutic success. The blood samples of volunteers were free from specific antibodies, whereas antibodies persisted in the immunized subjects for longer than a decade. Patients from various clinics who had been treated unsuccessfully with the toxin—14 patients had received BOTOX, 7 had been treated with Dysport, and 7 with both products—all had neutralizing antibodies. Whether there was an antibody response depended on the amount of toxin administered. We believe, however, the effective toxin dose can be reduced by so much as to make antibody production highly improbable.** © 1997 Academic Press

## INTRODUCTION

Botulinum A toxin is the drug of choice to improve several forms of focal dystonia (13, 26). Its scope of therapy, however, has widened immensely during the recent years. To date, not only disorders characterized by abnormal contractions of striated muscles, like tremor (14), tics (21), facial wrinkles (9, 20), and spasticity (5, 27), are treated with it, but also vegetative disturbances that manifest themselves in certain gas-

trointestinal ailments (18, 19, 23) and gustatory sweating (25). As a consequence the number of reports of acquired failure of therapy is growing (2, 3, 10). The main reason why nerve endings lost their sensitivity to the toxin was attributed to the production of neutralizing anti-botulinum toxin antibodies (8, 15, 17, 29). But since the toxin in use is not a purified protein, there may also be antibodies directed against the accompanying proteins (e.g., hemagglutinins) which account for at least 70% of the total mass of the complex formed by the toxin and concomitant proteins. Antibodies appeared particularly in patients who had received higher doses than those necessary for the treatment of blepharospasm and facial spasm (6, 16, 29) and the existence of a dose relationship seems probable. Moreover, if antibodies are formed, it is unclear how long their production will be sustained in patients who fail to respond to treatment. Also, botulinum toxins share amino acid sequences with tetanus toxin (22), and polyclonal antibodies have been described that neutralize both toxins by recognizing a common epitope (1). Thus, despite the differing immunogenicities of clostridial neurotoxins, cross-reactivity may exist and patients immunized against tetanus toxoid might also be immune to botulinum A neurotoxin. In the present work we have characterized various types of antibodies (neutralizing and nonneutralizing). We have determined dose-immune response relationships and, judging from the persistence of antibodies in immunized subjects, have estimated how much time may have to elapse until a secondary nonresponder can reacquire his sensitivity to the toxin.

## MATERIALS AND METHODS

### Materials

Botulinum A toxin (BoNT/A) complex (LD<sub>50</sub> 35pg mouse) was from Dr. E. Schantz, Madison, Wisconsin. This toxin was used for the determination of the calibration curve and the antibody titers of sera. The active constituent consists of two molecules of neurotoxin linked to hemagglutinin and a nonagglutinating protein. The complex, also termed the 19 S complex,

has a molecular weight of 900 kDa. The neurotoxin accounts for 30% of the total mass (12). Our own hemagglutinin-free neurotoxin was purified according to DasGupta and Sathyamorthy (4). Its LD<sub>50</sub> was 10 pg/mouse.

Tetanus toxin (TeNT) was from U. Weller, Mainz, Germany. Bovine serum albumin (protease-free) was from SERVA (Heidelberg, Germany). All buffers were prepared with analytical-grade chemicals from Merck (Darmstadt, Germany). Specific anti-botulinum A toxin F(ab')<sub>2</sub> (equine Fermo serum, 750 U/ml) was from Behringwerke (Marburg, Germany). It served as a standard for the construction of the calibration curve. Anti-human IgG from goat and anti-horse IgG from rabbit, both conjugated with peroxidase, were from Dako (Denmark). Krebs–Ringer solution was composed of 118 mM NaCl, 4.75 mM KCl, 2.54 mM CaCl<sub>2</sub>, 1.19 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM NaHCO<sub>3</sub>, 11 mM glucose, and 0.1% bovine serum albumin. The toxins were diluted in Krebs–Ringer solution to a final concentration of 1 ng/ml for the purified neurotoxin and 2 ng/ml for the complex. For ELISA, wells were coated with toxin solutions free of protective albumin.

#### Experimental Procedures

The left phrenic nerve–hemidiaphragm was excised from male or female NMRI mice (18–22 g), placed in an

organ bath containing 3.5 ml of Krebs–Ringer solution, and stimulated via the N. phrenicus (28). Indirectly stimulated muscles (1 Hz, 1 ms, 1 V) maintained an undiminished contractile response (twitch) over 4 h. In each experiment, the preparation was first allowed to equilibrate for 15 min under control conditions. Then, the incubation medium was exchanged for the toxin-containing serum. Before mixing the toxin with the undiluted sera, sera were dialyzed against the Krebs–Ringer solution. The mixtures were then incubated for 60 min at 37°C. Appropriate toxin concentrations were used to allow the contraction amplitude, in the absence of antibodies, to be reduced by half in approximately 60 min. After application of the mixture, the amplitude remained unchanged for some time (Fig. 1a), then decreased more or less slowly, depending on the antibody titer in the serum. The time required to decrease the amplitude by 50% (paralysis time, always less than 3 h) served to construct the calibration curve using the anti-botulism serum from Behring (750 U/ml) as a standard. When paralysis time exceeded 180 min in the presence of a patient's serum, the serum was diluted to allow paralysis to develop within that time. Taking the dilution factor into account, the antibody titer was determined with the help of the calibration curve.

ELISA tests were performed using 96-well plates

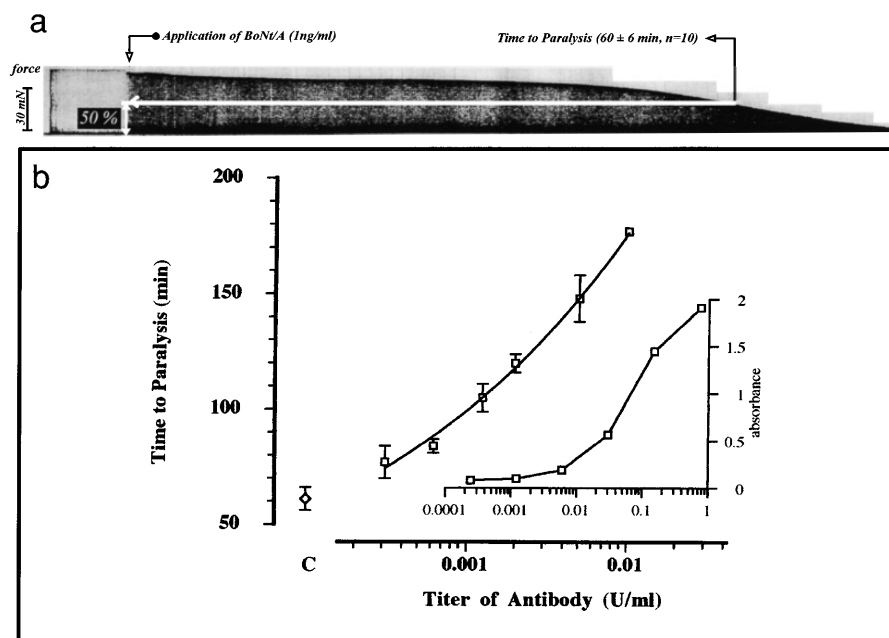


**FIG. 1.** Development of paralysis and antibody titer. (a) A mouse diaphragm was continuously stimulated via the phrenic nerve at a frequency of 1 Hz. After equilibration the muscle was exposed to 1 ng/ml of botulinum toxin. The arrows indicate when the toxin was applied and when the twitch was reduced by 50% of its initial value, respectively. Paralysis time is defined as the time elapsed till the contraction amplitude has been halved. (b) Calibration curves of a standard anti-botulinum A toxin serum: Antibody titer plotted against time to paralysis in an *in vitro* experiment using the mouse hemidiaphragm ( $n = 3 \pm \text{SD}$ ). The diamond symbol C (for control) represents the paralysis time of diaphragms exposed to a mixture of antibody-free human sera and 2 ng/ml of toxin complex ( $n = 35 \pm \text{SD}$ ). Inset, Antibody titer plotted against absorbance in an ELISA test ( $n = 2$ ).

coated overnight with 100 ng/ml of the antigen (TeNT, BoNT/A, and BoNT/A–hemagglutinin complex, respectively) at 4°C in sodium phosphate buffer (20 mM) containing NaCl (150 mM) (PBS). After being washed with the buffer, the plates were incubated with bovine serum albumin (10 mg/ml) in PBS to block nonspecific binding to the plates. The anti-botulism serum from Behring (horse, 750 U/ml) was diluted in PBS and used for the determination of the titer/response curve (Fig. 1b, inset). These dilutions and the human sera, diluted 1:3 and 1:10 with the same buffer, respectively, were applied to the plates and incubated for 8 h. The wells were washed again and incubated with the anti-horse and anti-human IgG peroxidase conjugates, respectively. Upon washing, the plates were developed with benzidine, and the absorbance was measured in an EIA reader at 450 nm.

## RESULTS

### *Determination of Anti-botulinum Toxin Antibodies Using the Phrenic Nerve–Hemidiaphragm Preparation and ELISA*

Botulinum toxin type A complex (2 ng/ml) and hemagglutinin-free neurotoxin (1 ng/ml), added to dialyzed sera of antibody-free controls, both paralyzed the hemidiaphragm and reduced its contraction amplitude by 50% within  $61 \pm 5$  ( $n = 32$ ) and  $60 \pm 6$  min ( $n = 10$ ), respectively (Fig. 1a). The time to paralysis was prolonged depending on the titer of specific antibodies which was adjusted with a standard botulism antitoxin from horse (Fig. 1b). The detection limit of toxin-neutralizing antibodies was approximately 0.0003 U/ml. Using the ELISA technique, the detection limit of neutralizing and nonneutralizing anti-botulinum toxin antibodies was approximately 0.01 U/ml. This means that the ELISA is approximately 300 times less sensitive than the muscle preparation and, moreover, cannot discriminate between toxin-neutralizing and -nonneutralizing antibodies.

### *Quantification of Botulinum Toxins of Various Origins*

One hundred units of Dysport equals 2.5 ng botulinum toxin complex (11), while 100 U of BOTOX resides in 25 ng of the protein complex (24). One molecule of the complex ( $M_r$  of 900,000) contains two molecules of neurotoxin ( $M_r$  of 150,000) (12). On this basis concentration–response curves were calculated (Fig. 2). Dysport was as potent as our own purified neurotoxin. The concentration–response curve of BOTOX was shifted to the right by a factor of 10; i.e., taking account of molarity, BOTOX had to be applied in a 10-fold higher concentration than Dysport to attain an equipotent response.

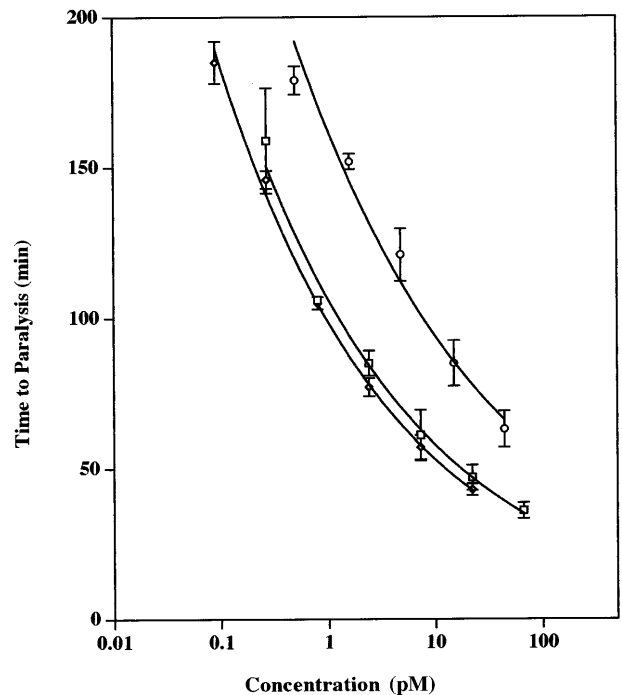


**FIG. 2.** Concentration–response curves for purified botulinum A neurotoxin, BOTOX, and Dysport. Mouse hemidiaphragms were exposed to varying amounts of three different botulinum toxin preparations: purified neurotoxin type A ( $\square$ ), Dysport ( $\diamond$ ), and BOTOX ( $\circ$ ). Concentrations in pmol/liter (abscissa) were plotted against time up to the point when the contraction amplitude had decreased to 50% of its initial value (ordinate). Each point is the mean of three experiments. Variance is expressed as  $\pm$ SD. Where no deviation bar is shown, it is concealed by the symbol.

### *Antibody Titers in Sera of Dysport-Treated Patients from Hannover, Volunteer Blood Donors, and BoNT-Immunized Subjects*

Within a period of 15 months, 300 patients suffering from various forms of dystonia consulted the Neurological Clinic of Hannover Medical School. Females accounted for 58% and males for 42%. The average age was 50.8 years, the mean duration of treatment was 5.5 years, and the mean interval between injections was 3.2 months. Fifty percent of these patients suffered from torticollis spasmodicus, 35% from facial dystonias, 10% from generalized torsion dystonia, and 5% from spasticity. All were treated with Dysport, the doses varying according to their requirements (Table 1). Among these patients were 4 secondary nonresponders and 1 primary nonresponder. Patients were considered nonresponders when they showed neither improvement nor muscle weakness or atrophy after at least two successive treatments. The primary nonresponder, a 42-year-old man, had been suffering from torticollis spasmodicus for 11 years. Then in 1994 he had four unsuccessful treatments with Dysport and two with BOTOX. The intervals between the injections were always 2 months. The sera of 75 responders and all

nonresponders were tested for botulinum toxin complex-neutralizing antibodies, using the nerve-muscle preparation. The sera of those patients, in particular, who received high-dose treatment ( $>600$  U) were selected for the test. In contrast to the sera of nonresponders, all of which contained antibodies of various titers, detectable titers of specific antibodies were found in the sera of only two responders (Fig. 3; Table 1). The mean dose of Dysport administered to secondary nonresponders was  $20 \pm 5$  ng ( $823 \pm 194$  U) (Table 2). The lowest dose that induced antibodies was 15.5 ng (620 U). Thus, of the group of patients receiving doses higher than 15 ng of Dysport ( $n = 40$ ), approximately 10% produced neutralizing antibodies. Serum samples from volunteer blood donors ( $n = 32$ ) were free of botulinum toxin complex-neutralizing antibodies (Fig. 3). Each serum, however, contained anti-tetanus toxin antibodies ( $>0.1$  U/ml), indicating a successful immunization with tetanus toxoid (see Discussion). Subjects immunized with botulinum toxoid ( $n = 8$ ) had neutralizing titers of anti-botulinum toxin A antibodies although they had not received booster injections for 10 years (Fig. 3).

#### *Antibody Titers in Sera of Patients Treated Outside of Hannover*

We received the sera of 44 suspected nonresponders from various clinics all over Germany. Detectable titers of botulinum toxin complex-neutralizing antibodies were found in the sera of 29 patients. All of the remaining, antibody-negative, patients ( $n = 15$ ) responded to subsequent injections of BoNT/A, as confirmed by the attending physicians. Of the antibody-positive pa-

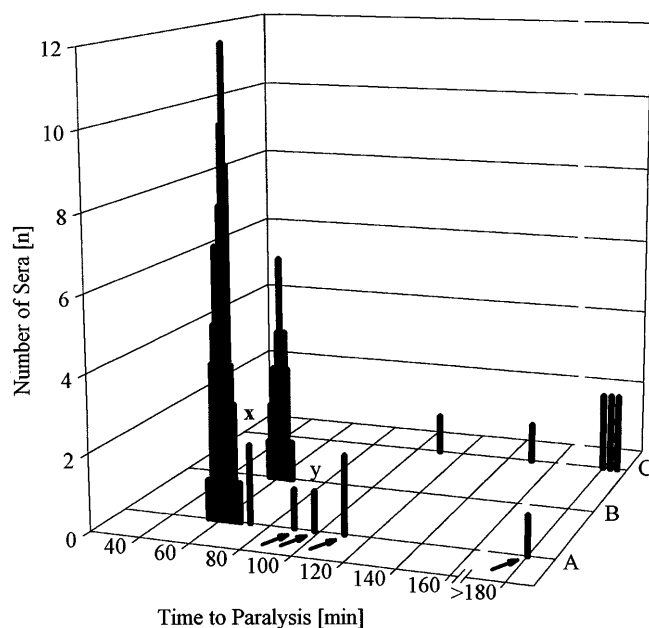


**FIG. 3.** Paralysis times in three groups of subjects. Mouse hemidiaphragms were exposed to 2 ng/ml of botulinum A toxin complex mixed (A) with the dialyzed sera of patients treated with Dysport, (B) with dialyzed sera of volunteer blood donors, and (C) with dialyzed sera of botulinum toxoid-immunized subjects. Columns show numbers of sera (ordinate) that paralyze the diaphragm after the times indicated on the abscissa. The column marked "x" represents the sera of two patients which contained traces of antibodies, the column marked "y" represents the serum of a patient who did not respond to the toxin in the first place, and the arrows mark the columns that represent the sera of nonresponders.

**TABLE 1**

Data for Dysport-Treated Patients from Hannover

|                                                                | Low dose   | Medium dose | High dose            |
|----------------------------------------------------------------|------------|-------------|----------------------|
| Range (ng)                                                     | 1.0–7.4    | 7.5–15.0    | $>15.0$              |
| Range (U)                                                      | 40–299     | 300–600     | $>600$               |
| Patients treated (n)                                           | 140        | 120         | 40                   |
| Sera tested (n)                                                | 20         | 26          | 30                   |
| Nonresponders (n)                                              | 0          | 1           | 4                    |
| Time to paralysis (min)                                        |            |             |                      |
| Responders (means $\pm$ SD)                                    | $61 \pm 3$ | $62 \pm 3$  | $61 \pm 3$           |
| One "primary" nonresponder                                     |            | 98          |                      |
| Secondary nonresponders                                        |            |             | 90, 110, 112, $>180$ |
| Positive ELISA with toxin complex (nonneutralizing antibodies) |            |             |                      |
| n                                                              | 8          | 11          | 14                   |
| in percentage of sera tested                                   | 40         | 42          | 47                   |

*Note.* Patients treated with Dysport were divided into three groups according to the doses applied. Secondary nonresponders received doses of more than 600 U, i.e., 15 ng. Nonneutralizing antibodies (positive ELISA with toxin complex) were found more frequently in sera of patients treated with Dysport in the upper dose range.

tients, 14 had been treated with BOTOX. Twelve of them had received mean doses of  $48 \pm 14$  ng ( $192 \pm 56$  U) each, while we had no information about the remaining 2 patients. Seven patients had received mean doses of  $20 \pm 2$  ng ( $800 \pm 92$  U) of Dysport (Table 2). The latter dose comes close to the mean dose administered to antibody-positive patients from Hannover. Seven patients had received injections of both products, and no information at all was available on the treatment of 1 patient (Table 2). Sex and age of patients, diagnosis, and treatment are shown in Table 3.

#### *Botulinum Toxin-Neutralizing vs -Nonneutralizing Antibodies*

The muscle preparation was protected from the action of the toxin complex in the presence of sera containing neutralizing antibodies which, however, may have been directed against any of the various constituents of the complex. We therefore tested the same sera (from 32 patients) on muscle preparations exposed to purified neurotoxin. This way it should be possible to distinguish neurotoxin-specific antibodies from those directed against some other part of the complex. The latter would be unable to prolong time to paralysis in this experiment. The results showed that neutralizing

**TABLE 2**  
Antibody Titers in Sera of Dysport and BOTOX-Treated Nonresponders

| Dysport            |                 |                       |              |                  |               | BOTOX               |                 |                       |              |                 |                |
|--------------------|-----------------|-----------------------|--------------|------------------|---------------|---------------------|-----------------|-----------------------|--------------|-----------------|----------------|
| Patient            | Titer<br>(U/ml) | ELISA<br>(neurotoxin) | Range<br>(U) | Mean dose        |               | Patient             | Titer<br>(U/ml) | ELISA<br>(neurotoxin) | Range<br>(U) | Mean dose       |                |
|                    |                 |                       |              | (U)              | (ng)          |                     |                 |                       |              | (U)             | (ng)           |
| 1                  | 0.09            | +                     | 970–1360     | 1130             | 28.0          | 12                  | >0.05           | +                     | 100–300      | 170             | 42.5           |
| 2                  | 0.002           | –                     | 200–1040     | 700              | 17.5          | 13                  | 0.09            | +                     | 50–240       | 150             | 37.5           |
| 3                  | 0.002           | –                     | 400–760      | 620              | 15.5          | 14                  | 0.5             | +                     | 75–240       | 155             | 39.0           |
| 4                  | 0.005           | –                     | 800–900      | 840              | 21            | 15                  | 0.03            | +                     | 120–350      | 200             | 50.0           |
| 1–4 mean<br>(±SD)  |                 |                       |              | 822.5<br>(194.2) | 20.5<br>(4.7) | 16                  | 0.01            | –                     | 250–300      | 280             | 70.0           |
|                    |                 |                       |              |                  |               | 17                  | 0.01            | +                     | 120–350      | 250             | 62.5           |
| 5                  | 0.04            | –                     | 500–980      | 700              | 17.5          | 18                  | 0.003           | –                     | 100–350      | 250             | 62.5           |
| 6                  | 0.6             | +                     | 500–1500     | 1000             | 25.0          | 19                  | 0.01            | –                     | 80–120       | 100             | 25.0           |
| 7                  | 0.01            | +                     | 480–1040     | 720              | 18.0          | 20                  | 0.004           | –                     | 85–150       | 115             | 29.0           |
| 8                  | 0.02            | –                     | 600–1000     | 825              | 21.0          | 21                  | 0.018           | –                     | 200–360      | 240             | 60.0           |
| 9                  | 0.07            | –                     | 400–1000     | 810              | 20.0          | 22                  | 0.014           | –                     | 100–300      | 200             | 50.0           |
| 10                 | 0.32            | +                     | 700–900      | 800              | 20.0          | 23                  | 0.16            | +                     | 100–250      | 200             | 50.0           |
| 11                 | 0.6             | +                     | 500–1000     | 750              | 18.7          | 24                  | 0.001           | –                     | ?            | ?               | ?              |
|                    |                 |                       |              |                  |               | 25                  | 0.014           | +                     | ?            | ?               | ?              |
| 5–11 mean<br>(±SD) |                 |                       |              | 800.7<br>(92.3)  | 20.0<br>(2.3) | 12–25 mean<br>(±SD) |                 |                       |              | 192.5<br>(56.3) | 48.2<br>(14.0) |
| 1–11 mean<br>(±SD) |                 |                       |              | 808.6<br>(138.7) | 20.2<br>(3.4) |                     |                 |                       |              |                 |                |
| 26                 | 0.004           | –                     | 760–1200     | 1000             | 25            |                     |                 |                       | 10–100       | 60              | 15             |
| 27                 | 0.04            | –                     | 450–960      | 700              | 17.5          |                     |                 |                       | 100          | 100             | 25             |
| 28                 | 0.01            | +                     | 800–960      | 880              | 22.0          |                     |                 |                       | 30–120       | 60              | 15             |
| 29                 | 0.014           | +                     | 680–760      | 720              | 18.0          |                     |                 |                       | 170–230      | 200             | 50.0           |
| 30                 | 0.4             | +                     | 450–1000     | 760              | 19.0          |                     |                 |                       | 50–260       | 215             | 53.8           |
| 31                 | 0.002           | –                     | 540–1000     | 750              | 18.7          |                     |                 |                       | 160–260      | 200             | 50.0           |
| 32                 | 0.12            | +                     | 320–900      | 400              | 10.0          |                     |                 |                       | 60–300       | 100             | 25.0           |
| 33                 | 0.02            | +                     | ?            | ?                | ?             |                     |                 |                       | ?            | ?               | ?              |

*Note.* The titers of nonresponders are juxtaposed with the mean doses administered. Values 1 to 4 are from patients treated with Dysport in our local clinic. Mean values were calculated separately for the patients from Hannover (1–4), from outside Hannover (5–11 and 12–25 for Dysport and BOTOX, respectively), and for both (1–11, Dysport-treated). The values at the bottom (26–32) represent patients treated with both products while no information at all was available for one patient (33). Question marks indicate a lack of information about the doses of BOTOX and Dysport. The ELISA was performed with hemagglutinin-free botulinum A neurotoxin (see Materials and Methods).

antibodies were exclusively directed against the neurotoxic constituent of the complex. The titer measured had the same height as the titer against the toxin complex (results not shown). In addition, through the use of the ELISA technique, all sera containing neutralizing antibodies ( $n = 32$ ) were tested for neurotoxin-specific antibodies (Table 2) and, conversely, antibody-negative sera ( $n = 72$ ) for nonneutralizing antibodies (Table 1) aimed at some other part of the complex. Neurotoxin-specific antibodies could be detected in only 43% of the antibody-positive sera which contained titers of 0.01 U/ml or higher (Table 2). In sera of responders antibodies against the complex were present that did not neutralize the neurotoxin (Table 1).

## DISCUSSION

It is common practice to measure by units both the titer of specific antibodies and the activity of botulinum toxin preparations destined for use in therapy. To avoid

confusion, we have calculated the doses of both commercial products, i.e., BOTOX and Dysport, on the basis of protein content. The conversion appears justified because, in contrast to the paralytic action which depends solely on the proteolytic activity of the neurotoxins, the immune response is almost exclusively determined by the protein contents of the preparations. The potency loss of BOTOX which, in relation to protein content, amounted to 90% (Fig. 2), probably resulted from the formation of toxoid. Toxoid formation had previously been attributed to lyophilization of botulinum toxin in a solution containing a high concentration of sodium chloride (7). The 10-fold higher protein concentration per unit of BOTOX may compensate for the loss of potency but, on that order, might adversely affect the immune system.

A very reliable *in vitro* neutralization test has been introduced to detect and quantify antibodies raised in patients during treatment with botulinum A toxin. Toxicity tests in mice are terminated by the paralysis of

**TABLE 3**

Data from Patients Who Did Not Respond to Either Dysport or BOTOX

| Patient | Sex | Age<br>(years) | Diagnosis | Treatment           |                      |
|---------|-----|----------------|-----------|---------------------|----------------------|
|         |     |                |           | Duration<br>(years) | Interval<br>(months) |
| 1       | M   | 37             | GTD       | 4                   | 3.7                  |
| 2       | M   | 33             | GTD       | 3                   | 2.3                  |
| 3       | F   | 80             | SPA       | 2                   | 2.0                  |
| 4       | M   | 61             | TC        | 3                   | 3.0                  |
| 5       | F   | 38             | TC        | 2                   | 2.7                  |
| 6       | M   | 31             | TC        | 5                   | 2.7                  |
| 7       | F   | 55             | GTD       | 2                   | 1.2                  |
| 8       | F   | 56             | TC        | 3                   | 2.8                  |
| 9       | M   | 45             | TC        | 4                   | 2.4                  |
| 10      | M   | 45             | TC        | 3                   | 2.3                  |
| 11      | M   | 50             | TC        | 3                   | 3.3                  |
| 12      | M   | 58             | TC        | 3                   | 4.5                  |
| 13      | F   | 46             | TC        | 2                   | 3.0                  |
| 14      | M   | 44             | TC        | 3                   | 4.5                  |
| 15      | F   | 23             | TC        | 2                   | 4.8                  |
| 16      | F   | 53             | TD        | 2                   | 3.4                  |
| 17      | M   | 23             | GTD       | 5                   | 3.8                  |
| 18      | F   | 69             | TC        | 3                   | 5.1                  |
| 19      | F   | 61             | OMD       | 4                   | 3.7                  |
| 20      | F   | 70             | TC        | 4                   | 4.0                  |
| 21      | M   | 52             | ?         | 2                   | 3.4                  |
| 22      | F   | 59             | TC        | 2                   | 4.8                  |
| 23      | F   | 26             | TC        | 4                   | 5.3                  |
| 24      | F   | ?              | ?         | ?                   | ?                    |
| 25      | F   | 41             | ?         | ?                   | ?                    |
| 26      | M   | 55             | TC        | 7                   | 7.0                  |
| 27      | M   | 68             | TC        | 4                   | 2.8                  |
| 28      | F   | 60             | TC        | 2                   | 2.2                  |
| 29      | M   | 73             | TC        | 3                   | 3.0                  |
| 30      | M   | 35             | TC        | 5                   | 5.0                  |
| 31      | F   | 62             | TC        | 3                   | 3.3                  |
| 32      | F   | 36             | TC        | 4                   | 5.3                  |
| 33      | ?   | ?              | ?         | ?                   | ?                    |

*Note.* The numbers in the first column correspond to the numbers in Table 2. Numbers 1–4 represent the patients from our local ambulance and 5–33 those from outside Hannover. The data refer to 1996. GTD, generalized torsion dystonia; SPA, spasticity; TC, torticollis spasmodicus; OMD, oromandibular dystonia.

the respiratory muscles. Testing in the isolated respiratory muscle is a truncated version of the animal test and has the advantage of avoiding complex pharmacokinetics. This test is approximately 300 times more sensitive than the ELISA technique and can detect amounts of neutralizing antibodies so small as to be of no consequence for therapy. Thus, antibodies near the detection limit (0.0003 U/ml) were found in the sera of two patients who still responded to treatment (see Fig. 3, values marked with "x"). Patients with titers higher than 0.001 U/ml, however, were resistant to therapeutic doses of botulinum A toxin. Neutralizing antibodies, as the cause of therapeutic failure, were also identified with the help of the mouse *in vivo* toxin neutralization

assay (17). Knowledge of the antibody status can be helpful in showing the way to proceed in cases of unsatisfactory responses to therapy. Thus, it was found that neutralizing antibodies were not present in the 15 putative secondary nonresponders. Whatever the causes of initial failure may have been, the resumption of therapy was a success.

When exposed to purified botulinum A neurotoxin or botulinum toxin complex, respectively, the nerve-muscle preparation allowed discrimination between the sera that contained antibodies directed against the neurotoxin and those with antibodies against the stabilizing proteins like the hemagglutinins which do not contribute to paralysis. Anti-hemagglutinin antibodies were detected with the ELISA technique and were found in patients who still responded to the toxin. In contrast to patients with neutralizing antibodies, those testing positive for nonneutralizing antibodies will continue to benefit from the toxin.

One patient did not respond to botulinum toxin from the very beginning. In searching for an explanation for this failure, it had to be considered that he might have had cross-reactive anti-tetanus antibodies, because both toxins share a 35% homology in amino acid sequence (22) and, at least under experimental conditions, neutralizing antibodies against a common epitope have been found (1). Therefore, we tested the sera of a group of volunteer blood donors, successfully immunized against tetanus, for their capacity to neutralize botulinum toxin. None of the sera, however, showed anti-botulinum reactivity, so that cross-reactive antibodies are improbable to account for refractoriness. Moreover, it is unlikely that the non-responder may have acquired immunity through an undiagnosed botulism. Possibly, he may have reacted to the small initial doses of toxin with an unusually rapid production of antibodies.

The initiation of the production of neutralizing antibodies is more probable when large amounts of protein (neurotoxin) are applied, as could be shown for patients receiving Dysport. Of all local patients treated with high doses (>15 ng) 10% became resistant to the toxin by raising antibodies. A dose correlation was also seen for antibodies formed against the concomitant proteins in the complex (Table 1). Corresponding data of patients treated with BOTOX are not available yet because we had sera only from putative secondary nonresponders at our disposal. However, it is reasonable to surmise that, also in the case of BOTOX, the production of antibodies is linked to the amount of protein applied.

Since detectable antibodies were raised only when the mean dose (in nanograms) of BOTOX was more than twice as high (48 ng) as the mean dose of Dysport (20.2 ng), it has to be considered that the ratio between the protein contents of the two products may be 1:5 rather than 1:10. In any case, based on the number of units, the mean dose of Dysport (808 U) to give rise to

antibodies is approximately four times as high as the mean dose of BOTOX (192 U) (see also Table 2). This advantage of Dysport over BOTOX is forfeited by the current practice of administering doses three to five times higher than is necessary to achieve the therapeutic goal (28). Needless overdosing can do considerable harm to the patients because they may have to discontinue the treatment if antibodies are formed. These may persist for more than a decade, as tests on immunized subjects have shown. Therefore, a patient who develops immunity would possibly be denied treatment for the rest of his life.

Based on the present experience a second generation of botulinum toxin preparations should answer the following criteria: (i) The preparations should be devoid of toxoid. This will keep the risk of sensibilizing the immune system to a minimum. (ii) The toxin should also be purified from concomitant proteins. This will reduce the load of foreign substances that might lead to untoward reactions. [A case of rash linked to Dysport has recently been communicated to the authors (Christian Figge, unpublished).] Also, the concomitant proteins might serve as adjuvants that stimulate the production of anti-neurotoxin antibodies. (iii) Package sizes should be adequate to allow maximum exploitation of biological activity, as has been discussed recently elsewhere (28).

### ACKNOWLEDGMENTS

We thank Ms. Ulrike Fuhrmann for her excellent assistance. We are grateful to G. Erdmann for reading the manuscript and for his advice and constructive criticism. Our thanks are also due to both distributors, Speywood, UK, and Merz GmbH + Co KG, Germany, for supplying their respective toxin preparation and to E. Schantz for the BoNT/A complex. Last but not least, we acknowledge the effort of those physicians who have spared no pains to provide us with all the information on nonresponders whose sera were tested in our laboratory for antibody titers. This article satisfies part of the M.D. thesis of H. Göschel.

### REFERENCES

- Bartels, F., H. Bergel, H. Bigalke, J. Frevert, J. Halpern, and J. Middlebrook. 1994. Specific antibodies against the Zn<sup>2+</sup>-binding domain of clostridial neurotoxins restore exocytosis in chromaffin cells treated with tetanus and botulinum A neurotoxin. *J. Biol. Chem.* **269**: 8122–8127.
- Borodic, G., E. Johnson, M. Goodnough, and E. J. Schantz. 1996. Botulinum toxin therapy, immunologic resistance, and problems with available materials. *Neurology* **46**: 26–29.
- Critchley, E. M. R. 1995. Non-responders. *Mov. Disord.* **10**: 392.
- DasGupta, B. R., and V. Sathyamorthy. 1984. Purification and amino acid composition of type A botulinum neurotoxin. *Toxicon* **22**: 415–424.
- Dengler, R., U. Neyer, K. Wohlfarth, U. Bettig, and H. H. Janzik. 1992. Local botulinum toxin in the treatment of spastic drop foot. *J. Neurol.* **239**: 375–378.
- Gonnering, R. S. 1988. Negative antibody response to long-term treatment of facial spasm with botulinum toxin. *Am. J. Ophthalmol.* **105**: 313–315.
- Goodnough, C. M., and E. A. Johnson. 1992. Stabilization of botulinum toxin type A during lyophilization. *Appl. Environ. Microbiol.* **58**: 3426–3428.
- Greene, P., S. Fahn, and B. Diamond. 1994. Development of resistance to botulinum toxin type A in patients with torticollis. *Mov. Disord.* **9**: 213–217.
- Guyuron, B., and S. W. Huddelston. 1994. Aesthetic indications for botulinum toxin injection. *Plast. Reconstr. Surg.* **5**: 913–918.
- Hambleton, P. 1994. Immunogenicity aspects of botulinum toxin. Pages 16–19 in *Botulinum Toxin: Challenges and Developments*. LMB Public Relation Survey.
- Hambleton, P., and A. M. Pickett. 1994. Potency equivalence of botulinum toxin preparations. *J. R. Soc. Med.* **87**: 719.
- Inoue, K., Y. Fujinaga, T. Watanabe, T. Ohyama, K. Moriishi, H. Sunagawa, K. Inoue, and K. Oguma. 1995. Molecular composition of progenitor toxins produced by C. botulinum types A and D. *Jpn. J. Med. Sci. Biol.* **48**: 263–264.
- Jankovic, J., and M. F. Brin. 1991. Therapeutic uses of botulinum toxin. *N. Engl. J. Med.* **324**: 1186–1194.
- Jankovic, J., and K. Schwartz. 1991. Botulinum toxin treatment of tremors. *Neurology* **41**: 1185–1188.
- Jankovic, J., and K. Schwartz. 1991. Clinical correlates of response to botulinum toxin injections. *Arch. Neurol.* **48**: 1253–1256.
- Jankovic, J., and K. Schwartz. 1993. Longitudinal experience with botulinum toxin injections for treatment of blepharospasm and cervical dystonia. *Neurology* **43**: 834–836.
- Jankovic, J., and K. Schwartz. 1995. Response and immunoresistance to botulinum toxin. *Neurology* **45**: 1743–1746.
- Jost, W. H. 1995. Botulinum toxin in the treatment of anismus and spastic pelvic floor syndrome. *Mov. Dis.* **10**: 404.
- Jost, W. H., and K. Schimrigk. 1995. Botulinum toxin in the therapy of anal fissure. *Lancet* **344**: 1127–1128.
- Keen, M., A. Blitzer, J. E. Aviv, W. Binder, J. Prystowsky, H. Smith, and M. Brin. 1994. Botulinum toxin A for hyperkinetic facial lines: Results of a double-blind, placebo-controlled study. *Plast. Reconstr. Surg.* **94**: 94–99.
- Krack, P., U. Teschendorf, and W. Dorndorf. 1995. Modification of a facial tic with botulinum toxin. *Mov. Disord.* **10**: 401.
- Niemann, H. 1991. Molecular biology of Clostridial neurotoxins. Pages 303–348 in J. E. Alouf and J. H. Freer, Eds., *Sourcebook of Bacterial Protein Toxins*. Academic Press, San Diego.
- Pasricha, P. J., W. J. Ravich, T. R. Hendrix, S. Sostre, B. Jones, and A. N. Kalloo. 1995. Intraspincteric botulinum toxin for the treatment of achalasia. *N. Engl. J. Med.* **332**: 774–778.
- Schantz, E. J., and E. A. Johnson. 1990. Dose standardisation of botulinum toxin. *Lancet* **335**: 421.
- Schulze-Bonhage, A., M. Schröder, and A. Ferbert. 1996. Botulinum toxin in the therapy of gustatory sweating. *J. Neurol.* **243**: 143–146.
- Scott, A. B., B. G. Kennedy, and H. A. Stubbs. 1985. Botulinum A toxin injection as a treatment for blepharospasm. *Arch. Ophthalmol.* **103**: 347–350.
- Snow, B. J., J. K. Tsui, M. H. Bhatt, M. Varelas, S. H. Hashimoto, and D. B. Calne. 1990. Treatment of spasticity with botulinum toxin: A double blind study. *Ann. Neurol.* **28**: 512–515.
- Wohlfarth, K., H. Göschel, J. Frevert, R. Dengler, and H. Bigalke. 1997. Botulinum A toxins: Units vs. units. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **355**: 335–340.
- Zuber, M., M. Sebal, N. Bathien, J. de Recondo, and P. Rondot. 1993. Botulinum antibodies in dystonic patients treated with type A botulinum toxin: Frequency and significance. *Neurology* **43**: 1715–1718.