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Botulinum A toxins: units versus units

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Abstract We investigated the efficacies and potencies of two commercial preparations of botulinum neurotoxin type A (BoNt/A) reputed to differ in potency. Tests were conducted in vitro using the mouse phrenic nerve-hemidiaphragm which is an approved tool for measuring clostridial toxicity. In addition, in a double-blind trial on volunteers, varying amounts of one product were injected into the Musculus extensor digitorum brevis of the left foot, while equal amounts, i.e. units, of the other preparation were injected into the same muscle of the right foot. Compound muscle action potentials (CMAPs) were recorded before and at various points in time after the injections. As opposed to wide-spread anecdotal reports, no difference in effectiveness was found. The dose-response curves obtained from the mouse organ preparation with both commercial products equalled one another in potency (number of units) and corresponded to previous toxicity tests in mice conducted elsewhere. Dose-response curves from volunteers were also identical for both commercial preparations. The time course of paralysis and recovery of muscle function did not differ either. At lower concentrations of toxin, however, restoration of muscle function was more rapid than at higher concentrations. Since the results obtained from man and the animal organ preparation are in excellent accord, we conclude that 1 unit of Botox corresponds to 1 unit of Dysport.

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Introduction

Since 1980 when Scott et al. used botulinum neurotoxin type A in the treatment of strabism, its scope in therapy has widened immensely. To date, employment of the toxin includes the treatment of focal dystonias (Jankovic and Brin 1991; Cohen et al. 1989) and certain forms of spasticity (Dengler et al. 1992; Snow et al. 1990) and extends to the treatment of skin to smooth out wrinkles and improve the complexion (Keen et al. 1994; Guyuron and Huddelston 1994), gustatory sweating (Schulze-Bonhage et al. 1996), gastrointestinal disturbances like achalasia, anismus, and anal fissure (Jost 1995; Pasricha et al. 1995; Gui et al. 1994; Jost and Schimrigk 1995). Therefore, it could be foreseen that more than one pharmaceutical company would discover the growing market and offer a toxin preparation for therapy. As with other highly active biological material (insulin, growth factors, antibiotics), dose standardization is a problem (Quinn and Hallett 1989; Schantz and Johnson 1990). The biological activity of the two available commercial products is expressed in units, where one unit is the dose capable of killing 50% of mice of a given weight (Schantz and Johnson 1990; Hambleton and Pickett 1994). On the basis of units, the two preparations should be equal in potency, although they differ in their protein contents (Goodnough and Johnson 1992). Nonetheless, a controversial and heated debate has been conducted in sections of the medical community as to the appropriate dose of each preparation to be used in man. The potency of one unit of one preparation (Botox) is alleged to vary by factors of three (Hambleton and Pickett 1994; Taylor et al. 1991) to approximately seven (Durif 1995) from the potency of one unit of the other preparation (Dysport), with some authors reporting distinct differences down to the first decimal place (Durif 1995). Botulinum neurotoxin,

a protein, is the most powerful biological drug known. Due to resorption from the injection site, systemic side effects caused by the inhibition of acetylcholine release are common (Olney et al. 1988; Lange et al. 1993). The incidence of unintended paralysis of untreated muscles depends largely on the dose injected into the dystonic muscles (Van den Berg et al. 1995). The therapeutic dose is near the threshold level for stimulating the immune system to produce specific antibodies. If this occurs, the toxin will be inactivated without being able to relieve spasm (Borodic et al. 1996; Zuber et al. 1993; Jankovic and Schwartz 1991). Disagreement on the dose equivalence of the two available preparations poses an increased risk for patients through possible side effects and the development of immunity. Therefore we have investigated the efficacies and potencies of the two preparations both in vitro and in vivo . We hope the results may be of assistance to physicians in selecting the minimal effective dose for the benefit of the patient. They may also serve as an incentive to the manufacturers to offer formulations allowing optimum exploitation of biological activity for different therapeutic purposes.

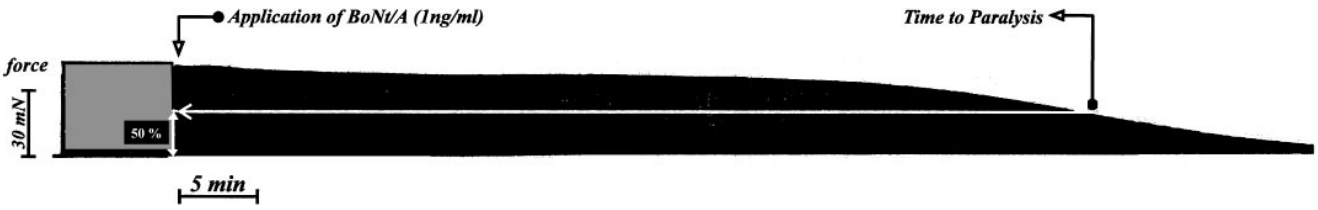
Materials and methods

Materials. Botulinum A neurotoxin (Dysport, batch no. 114/05/97; Botox, batch no. CGB 004/10/97) was kindly supplied by the respective distributors, Speywood, Maidenhead, UK, and Merz GmbH & Co., Frankfurt, Germany. The active component consists of two molecules of neurotoxin linked to hemagglutinin and non-agglutinating protein forming a high-molecular complex of 900 kD, also termed the 19 S complex. The neurotoxin accounts for 30% of the total mass of the complex (Inoue et al. 1995). Both products differ in their specific biological activity. While 100 units reside in 2.5 ng of Dysport, the same amount of Botox contains only 10 units (Schantz and Johnson 1990). Our own neurotoxin type A was purified according to DasGupta and Sathyamorthy (1984). Its LD_{50} was 300pg/kg mouse. Human serum albumin (5% solution) was from the German Red Cross Society, bovine serum albumin (protease-free) from Sigma. Krebs-Ringer solution was composed of 118 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM KH_2P0_4, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 11 mM glucose, and 0.1% bovine serum albumin. For the in vitro experiments the lyophilised commercial toxins were redissolved and stepwise diluted in Krebs-Ringer solution supplemented with bovine serum albumin (0.1%). For the in vivo experiments, Botox and Dysport were redissolved in appropriate volumes of physiological saline containing 0.05% human serum albumin resulting in concentrations of 80 U/ml for Botox and 400 U/ml for Dysport. To meet experimental requirements, both toxins were further diluted in the same solutions.

Experimental procedures. The left phrenic nerve-hemidiaphragm was excised from male or female NMRI mice (18–22 g) and placed in an organ bath (Bülbring 1946; Habermann et al. 1980) containing 3.5 ml of Krebs-Ringer solution. The pH was adjusted to 7.4 by gassing with 95% O2 and 5% CO2. The phrenic nerve was continuously electrostimulated at a frequency of 1 Hz via two ring electrodes. The pulse duration was 0.1 ms, the amplitude supramaximal (3 mV). Isometric contractions were recorded with a force transducer (Scaime, France) and a pen recorder (Hellige, Germany). The resting tension of the diaphragm was approximately 15 mN. Indirectly stimulated control muscles 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 solution. Toxin concentrations were such as to allow reduction of the contraction amplitude by 50% within 3 h. After toxin application, the amplitude remained unchanged for some time (Fig. 1), then decreased more or less slowly depending on the toxin concentration. The time required to decrease the amplitude by 50% (paralysis time, always less than 3 h) was used to construct the dose-response curves.

The study with researchers performing the experiments on themselves fulfill the requirements of the local ethic committee. In these experiments compound muscle action potentials (CMAPs) were recorded from the M. extensor digitorum brevis by conventional surface techniques. The N. peroneus was stimulated at the ankle joint by applying supramaximal electric shocks, at a distance of 8 cm from the recording electrodes. The distance between the stimulating and recording electrodes was kept constant. This was controlled by measuring the latency between stimulation and evoked CMAPs which remains unchanged if the distance between the sites of stimulation and recording is not altered. The recording and the indifferent electrodes were 5 cm apart. Before injecting the toxin, a control CMAP was recorded. Then 1 ml of a toxin solution of Botox was injected into the extensor muscle of a volunteer's left foot, while 1 ml of the other toxin solution (Dysport), containing the same number of units, was injected into the corresponding muscle of the right foot. To ensure the correct injection site, myographic guidance was employed. Syringes were coded and neither the volunteer nor the injecting physician had knowledge of which preparation they contained. Each toxin concentration was tested in three volunteers. Seven days later , the extent of paralysis was ascertained, and control examinations were done every 4 weeks. To quantify the effects, the quotient of amplitude after toxin application/amplitude before toxin application was calculated (If both amplitudes are of the same height, there is no paralysis, and the quotient is the maximum value of 1.). The dose-response curve was constructed for day 7 on the basis of CMAP inhibition calculated as $(100\text{-}S_2/S_1 \times 100)$. In another experiment, 5 U/ml of each toxin preparation were injected into the respective muscles and CMAPs were recorded, first after 6 h and then at intervals over a period of 2 weeks.

Fig. 1 Development of paralysis by purified BoNt/A. A mouse diaphragm was continuously stimulated via the phrenic nerve at a frequency of 1 Hz. After equilibration the muscle was exposed to 1ng/ml of botulinum neurotoxin. The arrows indicate when the toxin was applied and the twitch was reduced by 50 % of its initial value, respectively



Results

Dose dependency of the action of Botox and Dysport in vitro

The time course of muscle paralysis was identical for both commercial toxin preparations. A latent period was followed by a progressive decline in the contraction amplitude down to zero. The time to paralysis, i.e. the period that passed to attain half maximal twitch (see 'Methods'), depended strictly on the toxin concentration. Figure 2 shows the dose-response curves for Botox and Dysport which coincide completely. Thus, both toxins have the same efficacy and potency, and the in vitro experiments are in complete accord with toxicity tests in mice (definition of unit).

Time course and dose dependency of the action of Botox and Dysport in man

Figure 3a shows representative recordings of CMAPs obtained from a volunteer prior to and 6, 24, and 72 h after the injection of 5 U/ml of either Botox or Dysport into the left and right M. extensor digitorum brevis, respectively. The injection volume was 1 ml each. Paralysis induced by this low dose became significant about 24 h later. It appeared there may have been a minutely lesser effectiveness of Botox. However, this difference disappeared at higher concentrations. A plateau, which was in the range of 0.4 – 0.5, was reached approximately 100 h after the toxin injections (Fig. 3b). Restoration of muscle function began after 8 weeks. It was a slow process, and a restitutio ad integrum did not occur within 16 weeks (Fig. 4). Although muscles injected with the low toxin concentrations (5 U) appeared to recover more rapidly from Botox than from Dysport, the time courses of restitution were identical at higher concentrations. Dose-response curves were calculated from the values obtained on day seven after the injection and from the values obtained just prior to the injection. They coincide completely (Fig. 5). The maximum of paralysis, with the initial muscle function reduced to 15%, was achieved with 15 U/ml; the highest dose injected was 45 U/ml. CMAP decreases were identical for both toxin preparations. So, once aspirated into the syringes, the toxins could not be distinguished with respect to their origin: dose-reponse and development of paralysis were identical for each product.

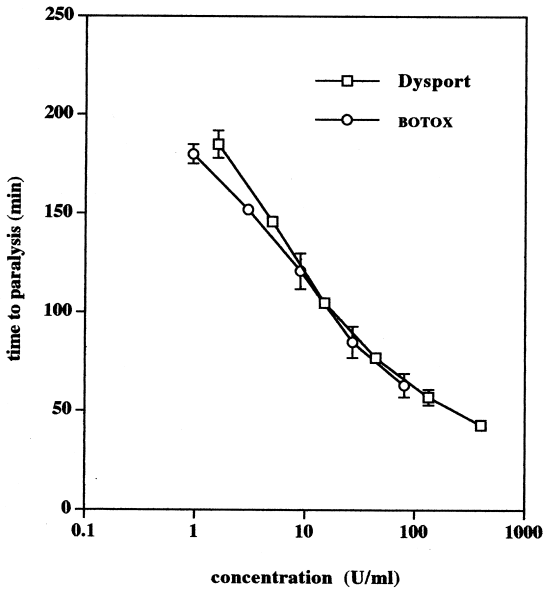


Fig. 2 Concentration-response curves for Botox and Dysport. Mouse diaphragms were exposed to varying amounts of the two commercial toxin preparations. Concentrations in U/ml (*abscissa*) were plotted against time up to the point when the contraction amplitude had decreased to half its initial value (*ordinate*). Each point is the mean of three experiments. Variance is expressed as ±SD. Where no deviation bar is visible, it is concealed by the symbol

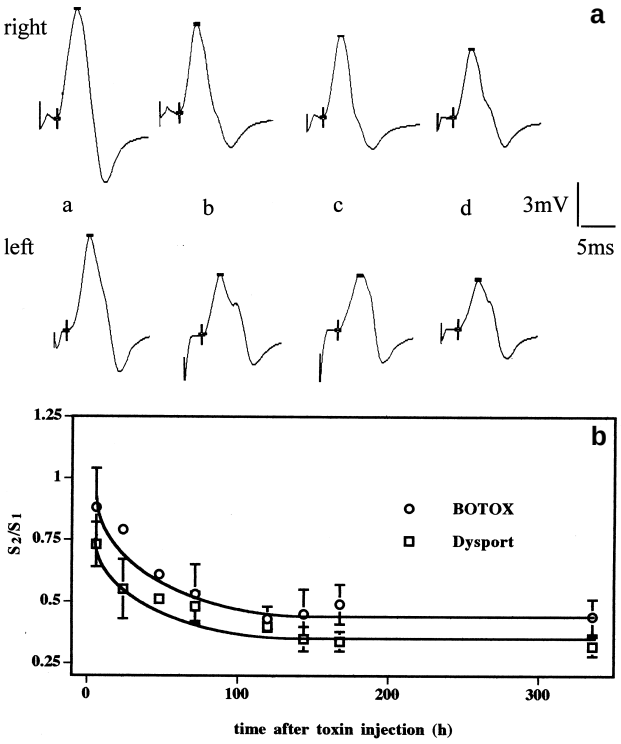


Fig. 3a, b Development of paralysis of the M. digitorum brevis in volunteers. Three volunteers received 5 U/muscle of Dysport and Botox into the right and left toe muscles, respectively. **a** Representative recordings of CMAPs of left and right toe muscles are shown. The first two CMAPs a were recorded before toxin was injected into the muscles. 6, 24, and 72 h after the injections further CMAPs were recorded (*b*, *c*, and *d*, respectively). **b** The decreases in CMAPs caused by the toxins are expressed as quotients (amplitudes at various points of time after the injection divided by amplitude immediately before the injection) (*ordinate*). The *abscissa* indicates the time elapsed after the injection. First point on curves: 6 h after injection. Values are presented as the means of determinations from three volunteers ±SD. Control recordings differ significantly from recordings at 24 h and later points of time, according to the paired Student's *t*-test ($p<0.05$)

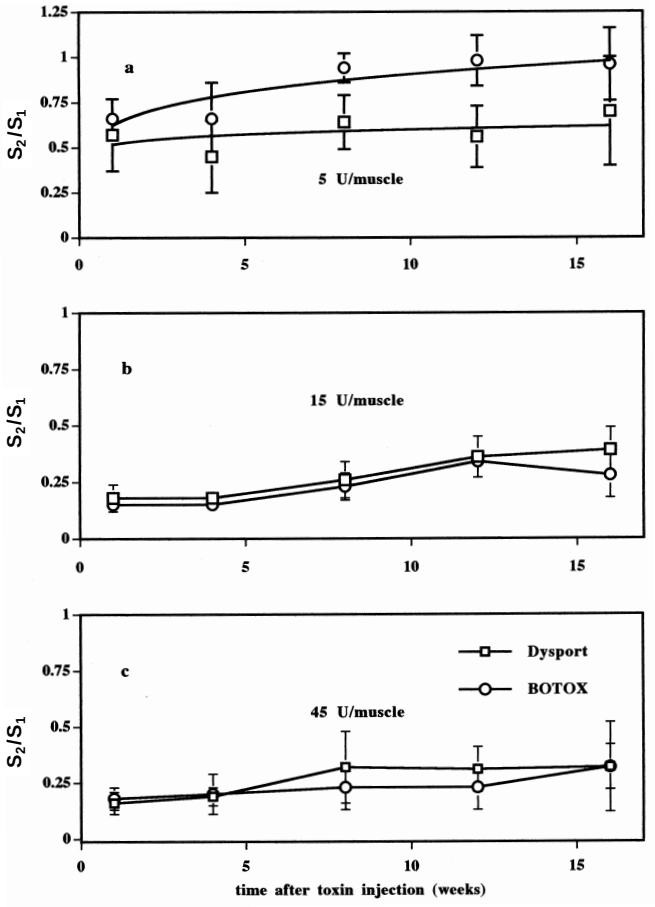


Fig. 4a–c Time course of restoration of muscle function. CMAPs of M. extensor digitorum brevis were recorded on day 7 and then every 4 weeks following application of 5 U (**a**), 15 U (**b**), and 45 U (**c**) of each commercial product to the muscles in volunteers. One volunteer of the 5 U group left the trial after the first week. The difference between the right and the left toe muscle is not statistically significant at this low dose of toxin

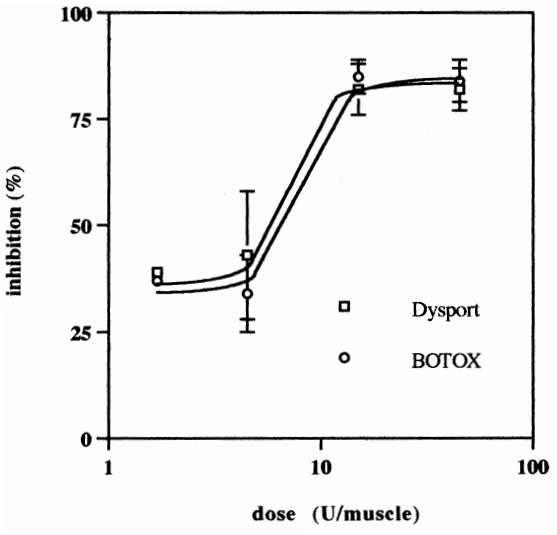


Fig. 5 Dose-response curves of Botox and Dysport in volunteers. The inhibition of CMAPs (*ordinate*) in the M. digitorum brevis was calculated from the data obtained on day 7 after toxin application. A dose of 1.7 U/muscle was administered to one individual who did not participate further in this trial. Since one milliliter was injected into each muscle, the concentration of the toxin equals its dose. Values are presented as the means of three determinations ± SD (except for the lowest dose)

Discussion

The presynaptic side of the extrafusal neuromuscular junction is the site of binding and uptake of the neurotoxic protease, BoNt/A, and it also harbours the toxin's substrate, SNAP 25 (Blasi et al. 1993; Schiavo et al. 1993). When the latter is cleaved by proteolysis, transmitter-containing vesicles can no longer fuse with the plasma membrane, and neuromuscular transmission is blocked (Ahnert-Hilger and Bigalke 1995; Simpson 1981). Muscles are paralysed, and death ensues from respiratory failure. Neuromuscular paralysis has been measured and characterized in vivo (Herrero et al. 1967; Scott and Suzuki 1988) and in vitro (Simpson 1981; Dreyer and Schmitt 1983). Although in vitro paralysis resembles in vivo paralysis in qualitative and quantitative terms, the situation is much more complex in vivo than in vitro, where an isolated organ is exposed directly to defined concentrations of toxin, and pharmacokinetic barriers are largely non-existent. In whole animals, on the other hand, variations in the injection site may occur, and diffusion from different tissues may lead to different concentrations of the toxin within its target organ. Moreover, indirect effects of the toxin can cause death, as for instance from the infection with a pathogenic organism of an insufficiently ventilated lung. Despite these drawbacks of using whole animals, toxicity of clostridial toxins is generally determined in mice and expressed as the median lethal dose (LD_{50}). One LD_{50} equals one unit in the commercial toxin preparations.

Two commercial products are currently used in patients suffering from various forms of dystonia. In relation to units, they were reported to have different potencies (Quinn and Hallett 1989; Hambleton and Pickett 1994). In quest of an explanation for this inconsistency it was maintained that the vials may not contain the number of units declared on the label (Pearce et al. 1995). Another point was that toxicity tests in mice are subject to variation (McLellan et al. 1996). But even if they do vary to some extent, the major problem is that a dose-dependent therapeutic success is more difficult to judge in patients. Whatever the reason, it remains that the therapeutic success in patients appears to correlate relatively poorly with the dose applied. The difficulties lie in the very limited number of patients available that can be matched for bodily constitution, identity and degree of ailment, muscles involved etc. To conduct a controlled, double-blind study of the clinical success of low doses of BoNt, treatment would have to involve also a group of patients receiving doses too low to relieve spasms, and that would raise ethical questions. The issue may be further complicated by development of specific antibodies

to the toxin, necessitating higher doses to relieve a patient's pain. Also, assessment of the therapeutic success depends partly on the patient's subjective report which may be tainted, if the outcome of the treatment is not up to his expectations. Things are more straight forward in animal experiments when a clone of mice of equal weight is used, injections are placed in the same tissue, and the lethal dose is determined.

It is hardly surprising the two commercial preparations were identical when tested for their capacity to cause half-maximal paralysis in a mouse nerve-muscle preparation. Although the test can not ascertain the number of toxin units (a unit being defined as the amount that kills 50% of mice), it is a very reliable and efficient tool to compare the potencies and efficacies of toxins of different origins.

Since the two preparations, with respect to units, had the same potency in two mouse test systems, the question arose as to why they should differ in potency in man. With the help of healthy volunteers we tried to provide an answer. Volunteers have some advantages over patients because they can be freely selected for uniformity. The investigator is not left with a heterogenous collective of patients with different muscles involved in their disorders. In volunteers tests can be performed on identical muscles and the effects quantified by recording CMAPs from supramaximally stimulated muscles (Sloop et al. 1996). This is analogous to the recording of muscle twitches in the isolated organ. The results of our investigations show that there is no substantial difference in the efficacies and potencies of the two commercial products in volunteers. Not only do the dose-response curves coincide totally with one another, but the development of paralysis and restoration of muscle function followed also the same time course. A difference in the size of amplitudes of toe muscle contraction is seen only when the low dose of 5 units is applied and may thus be due to statistical deviations rather than different potencies of the two preparations. Since the development of paralysis was more rapid in the isolated organ, and neuromuscular blockage was evident within minutes of the uptake of the toxin by the nerve endings (Dreyer and Schmitt 1983; Schmitt et al. 1981), the delay in the reduction of CMAPs in volunteers indicated that toxin was being taken up over a long time. The incomplete reconstitution of muscle function could result from atrophy due to inactivation of the muscle. Nevertheless, motor nerve endings both in animals and in healthy volunteers did not discriminate between the two commercial products. Therefore, there is no obvious reason to assume they would be more choosy in patients suffering from dystonia. So, how can the difference in the magnitude of the responses observed in patients be accounted for? Can it be explained solely on the basis of inponderabilities which lurk wherever small numbers of patients are involved? This certainly is a point and the reported large differences in equivalent doses stress the problem of dose dependence studies in patients. The high incidence of side effects reported for Dysport (Jankovic and Brin 1991; Anderson et al. 1992; Brans et al. 1995; Brin and Blitzer 1993) may be linked to overdosage of this more highly concentrated preparation. On the other hand, there are clinical trials in which patients with dystonia had received adequate treatment with low doses of Dysport (Van den Berg et al. 1995; Brans et al. 1995). These doses mirror closest our result which, with respect to units, show that Botox and Dysport are equivalent.

Differences in the formulations of the two products may also contribute to the observed discrepancies. The package sizes of Botox and Dysport are different concerning both the number of units they contain and their volumes. Prior to use, the preparations, which are lyophilisates essentially containing sodium chloride, human albumin, and lactose, apart from botulinum neurotoxin, have to be dissolved and diluted with saline in the vials in which they are sold. The vial volumes are such that they do not allow the dilution of the preparations to identical concentrations. Using larger vials of different origin for dilution is not recommended for fear of possible contamination due to the additional handling. Apart from this, there is the well-known problem of protein adhesion to the surface of glassware and plastic material which is to be avoided or kept to a minimum by adding serum albumin in excess. If diluted too much, however, the albumin may lose some of its protective effect and a substantial amount of the therapeutic agent will stick to the surface of the vial. In practise, this will result in undefined concentrations of toxin being applied in the first place. Second, if differing concentrations of toxin are injected into the muscle, diffusion will almost certainly differ, which may result in varying degrees of availability at the site of action. These imponderabilities may finally add up to produce the putative difference in effectiveness of the two preparations. To circumvent these pitfalls the two commercial products were tested under rigorous conditions. We diluted them down to the same number of units per volume and used equal concentrations of protective albumin. We tested the toxin in the highly sensitive and reliable mouse nerve-diaphragm preparation and also in volunteers whose little-toe muscles received equal amounts of toxin contained in equal injection volumes. The results obtained from volunteers corroborate those from the animal organ experiments. They show that a difference in potencies and efficacies of the commercial products does not exist; it is highly unlikely that it does in patients with neuromuscular disorders. Rather, we can be confident that equal numbers of units will produce identical effects, given identical conditions. Thus, dose reduction in Dysport by approximately 80% would not only lower costs substantially but, moreover, would minimize the risk of stimulating the immune system into antibody production.

On the basis of our findings we would finally like to direct the following recommendations to the manufacturers: We suggest the use of larger package vials to allow larger volumes of dilution for Dysport in order to better exploit its biological activity, thereby reducing the danger of raising antibodies, and avoiding the risk of con-

tamination through the use of additional vials for adequate dilutions. The tests necessary to ascertain the appropriate therapeutic dosage should be conducted both in vitro and in vivo.

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