A Lack of Antinociceptive or Antiinflammatory Effect of Botulinum Toxin A in an Inflammatory Human Pain Model

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Several in vitro and in vivo investigations have shown that botulinum toxin A (BoNT/A) can inhibit the release of substance P and excitatory amino acids. Recently, a marked antinociceptive effect of BoNT/A and inhibition of glutamate release was observed in an animal pain model with inflammatory sensitization. In the present study, we tested the antiinflammatory and antihyperalgetic effect of BoNT/A in a well-characterized human inflammatory pain model. Using a randomized, double-blind, paired study design, we compared the effects of 100 mouse units of BoNT/A versus pure saline. Thermal and mechanical pain testings and superficial skin blood flow measurements were performed at baseline, at 48 h (in normal skin), and at 72 h (in inflamed skin) thereafter. Ultraviolet B irradiation resulted in a local inflammation with significant primary and secondary hyperalgesia. However, despite the evidence of efficacy on sudomotor function, BoNT/A had no effect on pain measures in either normal or inflamed skin. Signs of inflammation and primary and secondary hyperalgesia were found to be unaffected by BoNT. We have confirmed that BoNT/A has no direct effect on acute, noninflammatory pain. Furthermore, despite highly promising data from animal research, we have not observed antiinflammatory or antinociceptive effects of BoNT/A in human inflammatory pain.

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Botulinum toxin (BoNT) is a clostridial neurotoxin that blocks acetylcholine release from peripheral cholinergic terminals. Local BoNT applications have been used to treat neurological diseases with pathologically increased muscle tone such as focal dystonia and spasticity and, more recently, some dermatological conditions such as focal hyperhidrosis and facial rhytides.

BoNT has also been found to alleviate pain associated with pathologically increased muscle tone (1,2). Although this may be considered a secondary effect, dissociation between the pain relief and muscle relaxation have been observed (3). It has been hypothesized that BoNT, in addition to its effect on acetylcholine release, may affect neurotransmitter systems involved in nociceptive transmission or sensitization, and thus, it may have a direct analgesic or antihyperalgesic effect. However, the evidence of BoNT efficacy in pain syndromes without an apparent muscular component is less compelling (4–7). Studies of BoNT in human experimental models of acute nociceptive pain and central sensitization have also failed to detect analgesic or antihyperalgesic efficacy (8–10).

In stark contrast to these human data, a recent study of BoNT in the formalin pain model in rats has demonstrated a significant, dose-dependent reduction of nocifensive behavior and local edema (11). The effect, which was attributed to inflammatory sensitization, was observed in the second phase of the test. As BoNT also inhibited formalin-evoked glutamate release in the periphery, and this mechanism was suggested to be responsible for the effect on inflammatory sensitization (11).

Based on these observations and on the reported ability of BoNT to inhibit release of proinflammatory neuropeptides in vitro (12,13), one can speculate that BoNT may reduce sensitization in human pain states associated with peripheral inflammation. In humans, localized skin inflammation and hyperalgesia can be evoked by controlled irradiation by ultraviolet (UV)-B.
This pain model can reliably detect antiinflammatory effects and antihyperalgetic actions of a range of analgesic mechanisms (14–17). Furthermore, peripheral and central sensitization can be analyzed separately in this model, because they are thought to be represented by primary and secondary hyperalgesia, respectively (18).

Therefore, in the present study, we tested if clinical doses of BoNT can alleviate inflammatory hyperalgesia in human skin. Furthermore, we sought to differentiate if this effect is caused by an antiinflammatory, peripheral, or central action of the toxin.

Methods
The study was approved by the local ethics committee. Written informed consent was obtained according to the Declaration of Helsinki from six healthy, pain-free volunteers aged between 30 and 40 yr old (five men and one woman). Before entering the study, a full medical history was taken; neurologic or psychiatric diseases, hypersensitivity to BoNT/A, intake of aminoglycoside antibiotics or antiinflammatory drugs within the last 4 wk, and (in women) pregnancy were among the exclusion criteria. No clinically significant abnormalities were found in these examinations, and no subjects were excluded on this basis. All subjects had a body mass index in the normal range (15th–85th percentile). The subjects agreed to abstain from alcohol, nicotine, and caffeinated drinks during the study period.

The study was performed in a randomized, double-blind, paired design. The overall subject disposition is depicted in Figure 1. For each subject, 2 1-mL syringes were prepared. One syringe contained 100 mouse units of BoNT serotype A (BoNT/A) (Dysport®; Ipsen, Wrexham, United Kingdom) diluted in 0.5 mL of saline. The second syringe was filled with 0.5 mL of saline only. Both syringes were labeled with either “right side” or “left side” according to a computer-generated randomization list (Randlist Version 1, Dat-Inf GbR, Göttingen). Drug preparation was performed by staff members who did not participate in subjects’ observation or data acquisition to maintain double-blind conditions.

BoNT/A was intracutaneously injected in the middle of the upper ventral aspect of one leg at five injection sites (midpoint and vertices of a square with side 3 cm). On the contralateral side, saline was injected in the same way. UVB sunburn spots were induced at both injection sides 48 h after the injection (see below).

Thermal and mechanical pain testings and superficial skin blood flow (sBF) measurements were performed at baseline (before the injection procedure), 48 h thereafter (in normal skin), and 24 h after the UVB irradiation (72 h after the injection procedure) when UVB-induced sensitization is stable (18). Two weeks after the injection procedure, the area of anhidrosis after intradermal BoNT/A application and UVB irradiation was determined by means of the jodine starch reaction. All assessments and analyses were performed by one investigator.

The sunburn was induced according to a standardized procedure, as recently described (18). A calibrated UVB source (Sellasol; Sellas Medizinische Geräte GMBH, Gevelsberg-Vogelsang, Germany; wavelength, 290–320 nm) was used. The individual minimal erythema dose was determined at screening, and circular spots of 5 cm in diameter were irradiated with 3 times minimal erythema dose of UVB light on the previously injected areas on the upper ventral aspect of both legs. The side of first irradiation was randomized. All participants were instructed to avoid additional UV exposure.

The sequence of measurements was standardized. First, superficial skin blood flow was measured, followed by assessment of the area of secondary hyperalgesia, mechanical pain threshold testing, including a stimulus/response (SR) function for mechanical sensitivity, and, finally, thermal pain threshold assessments.

We measured the superficial sBF at the sites of interest using laser Doppler imaging (moorLDI; Moor Instruments Ltd., Axminster, United Kingdom).
device scans with a 2-mW helium laser across the skin surface and registers the shifted frequency from the backscattered light. Thereby, the velocity of moving erythrocytes is calculated and presented as a color-coded picture representing a relative measure of skin perfusion (laser Doppler flux = velocity × concentration of moving erythrocytes) in 2 dimensions. Laser Doppler imaging is well established technique for noninvasively assessing microvascular BF. The laser head was positioned 35 cm above the measurement site. The scan region was 8 × 8 cm. The images were analyzed using a dedicated image-processing software (Moor Instruments Ltd.). A measure of sBF was obtained by calculating the median laser Doppler flux within a square of 2 × 2 cm that was centered in the respective sites. This variable (expressed in arbitrary units [aU]) served as surrogate for inflammation intensity.

Cold pain perception threshold (CPT) and heat pain perception threshold (HPT) were assessed by the method of limits (19) using a commercially available thermal sensory testing device (TSA-2001; Medoc, Ramat Yishai, Israel). The Peltier thermode, sized 18 × 18 mm, was attached to the skin at the measurement sites (within the sites of irradiation) using an elastic band. To minimize variation of probe application pressure, the band was wrapped tightly around the upper leg and then stretched by ~2 cm, and the ends were adhered. Care was taken to consider upper leg curvatures when placing the probe to achieve optimal contact between the probe and the leg surface. Skin adaptation temperature was 32°C, and rate of temperature change was 0.8°C/s with a return rate of 4°C/s. Stimulator temperature range was 0°C–54°C. Subjects were initially trained in a standardized manner to report the thresholds. They were instructed to stop the increase of temperature at the first perception of unpleasant cold (CPT) and heat (HPT). Each test was repeated three times and averaged. There was a 15-s interstimulus rest period between each determination.

The area of secondary hyperalgesia (sAREA) was determined on the skin surrounding the erythema with a custom-made weighted pinprick probe (force, 256 mN). Volunteers were asked to keep their eyes closed. Stimulation started approximately 8 cm away from the erythema and was repeated along a pattern of eight radial spokes. With movement along each spoke at steps of 5 mm, the subject was asked to report when the sensation of the pricking changed definitely (different, burning, or unpleasant sensation). This spot was marked with a pen, and after the measurement of the distance, the mark was erased to avoid bias during the measurement on the contralateral side. The area of pinprick hyperalgesia was determined from these eight distances by calculating the area of an octagon (in millimeters squared).

This test was performed using a custom-made set of 7 punctate mechanical stimulators (flat contact area of 0.2 mm in diameter) that exert forces between 8 and 512 mN (20,21). Using a “method of limits,” 5 threshold determinations were performed, each with a series of ascending and descending pinprick stimulus intensities. The final threshold was the geometric mean of 5 series of ascending and descending stimuli. Data are presented in millinamometers.

Mechanical pain sensitivity was tested using the custom-made weighted pinprick stimuli described above. Forces from 8 to 512 mN were applied so that a SR function was obtained for pinprick-evoked pain. These seven pinprick stimuli were applied in a balanced order, five times each at each of the irradiation sites, and the subject was asked to give a pain rating for each stimulus on a 0–100 numerical rating scale (0 = no pain and 100 = most intense pain imaginable). To additionally obtain a SR function for dynamic mechanical allodynia, a set of 3 light tactile stimulators (cotton wisp exerting a force of ~3 mN, a cotton wool tip fixed to an elastic strip exerting a force of ~100 mN, and a standardized brush [Somedic, Horby, Sweden] exerting a force of ~200–400 mN) was used (20). The tactile stimuli were applied with a single stroke of approximately 1–2 cm in length over the irradiated skin. Subjects were asked to give a rating on the same scale as for pinprick stimuli. A total of 50 stimuli, tactile and pinprick, were delivered in a balanced order at each site, and the mean pain rating was calculated.

To assess the biological activity of BoNT/A, the focal suppression of sweating was measured 14 days after the injection for each side separately. The subjects were acclimatized in a temperature and humidity-controlled room. To identify the area of anhidrotic skin, we used the iodine starch reaction (22). Sweating was induced by drinking 500 mL of hot tea and bicycle exercise between 100 and 150 W for 10 min. The skin with intact sudomotor activity became dark blue, whereas the anhidrotic skin area remained whitish yellow. To measure the area of anhidrosis, the border was drawn manually on acetate sheets and digitized for further processing. These areas were automatically quantified using home-written software in IDL (RSI, Boulder, Colorado), based on a two-dimensional region-growing algorithm and were normalized to yield millimeters squared values using a 10 × 10 mm reference region. After completion of the study the drug, assignments were unblinded.

Based on the rationale outlined above, our objective was to test the hypothesis that BoNT treatment can attenuate inflammatory hyperalgesia evoked by UVB. Because preclinical evidence strongly suggested a peripheral mechanism, we decided to power the study on a primary hyperalgesia end-point, namely, HPT. Based on the variability of this end-point from our
previous studies (18), in the paired-design study (assuming a two-sided significance level of 5%), the sample of six volunteers had 80% power to detect a 35% effect of BoNT/A on HPT (the magnitude of BoNT effect in the small-dose group in the formalin pain model in rats (11)). The main outcome variable for secondary hyperalgesia was sAREA. The data were analyzed by means of analysis of variance (ANOVA) with the fixed factors for time (two levels, baseline and 72 h) and group (two levels, BoNT/A-placebo) and the random factor subject. The time-by-group interaction was also included in the model to test whether the changes between the BoNT/A and placebo-pretreated areas after the UVB inflammatory skin reaction were different. The mean group difference of the post–minus premeasurements and the corresponding 95% confidence intervals (CI) were derived from the ANOVA model. This interval is likely to cover the true treatment difference of the underlying population. For the analysis of the variable sAREA, the mean (95% CI) of the BoNT/A-placebo differences within each subject was calculated. Pain ratings to tactile and pinprick stimuli were log-transformed because the distribution of magnitude is not normal. To avoid the loss of zero values because of logarithmic transformation, a constant of 0.1 was added to all raw data (21). The pain ratings of the stimulus response function were compared by ANOVA. Calculations were performed using the SAS software system V9.1 (SAS Institute Inc., 2002–2003, Cary, North Carolina).

Results

All six subjects completed the study. No subject reported pain during the UVB irradiation or spontaneous pain associated with the UVB erythema, which was in all cases in the intended range. No phototoxic blisters were observed. Minor local side effects after the injection procedure such as hematomas, injection pain, and erythema were noted without differences between BoNT/A and placebo. In no case was local weakness reported.

Consistent with previous reports, the UVB-induced inflammation led to a significant decline of HPT compared with baseline (P < 0.001); however, no significant difference between BoNT/A and placebo was observed (P = 0.75; Fig. 2A). The mean difference of HPT change over time between both conditions was −0.34°C (95% CI, −2.53–1.86). The time course of HPT (mean ± sd) for BoNT/A and placebo is depicted in Figure 2A.

UVB irradiation also caused a significant increase in CPT versus baseline (P = 0.002; Fig. 2B). Again, there was no significant difference between BoNT/A and placebo (P = 0.35). The mean difference of change over time was −4.22°C (95% CI, −13.48–5.04). The time course of CPT for both treatment groups is shown in Figure 2B. A significant decrease of mechanical pain threshold (MPT) after UVB sunburn was found (P = 0.009). BoNT/A did not show an effect on MPT compared with placebo (P = 0.8). The mean difference MPT change over time between BoNT/A and placebo was −6.54 mN (95% CI, −61.91–48.84). Mean and sd over time are depicted for both conditions separately in Figure 3A.

After UVB sunburn, a significant increase of SR function values over time was observed (P < 0.001). However, the mean difference SR function levels change over time between BoNT/A and placebo was not significant (−0.12 [95% CI, −0.51–0.27]; P = 0.52; Fig. 3B). Furthermore, the curves of the SR function did not differ between BoNT/A and placebo in inflammatory skin (Fig. 4). Dynamic mechanical allodynia was not observed in both conditions.

Sunburn resulted in large sAREA (mean ± sd; BoNT/A, 13296 mm² ± 9477 mm²); placebo, 13714

![Figure 2. Time courses of (A) heat pain perception threshold (HPT) and (B) cold pain perception threshold (CPT) for the botulinum toxin A (BoNT/A) and placebo pretreated sites. No significant difference between BoNT/A and placebo was observed (P = 0.75 and P = 0.35) for HPT and CPT, respectively (analysis of variance [ANOVA]; n = 6). Data are presented as mean ± SD.](image-url)
However, the difference of $-418$ mm$^2$ (95% CI, $-8207$–$7371$) was not significant.

Twenty-four hours after UVB irradiation, sBF was significantly increased ($P < 0.001$). No antiinflammatory effect of BoNT/A was observed ($P = 0.59$). The mean difference sBF change over time between BoNT/A and placebo was 20.9 aU (95% CI, $-68.5$–$116.2$; Fig. 5).

At baseline, none of the subjects had abnormalities of their sudomotor activity at the relevant skin areas. The saline injections followed by UVB sunburn did not result in a focal anhidrosis as assessed after 14 days. BoNT/A treatment led to substantial anhidrotic areas in all cases ($2008$ mm$^2$ $\pm$ $630$ mm$^2$).

**Discussion**

BoNT inhibits acetylcholine release by interfering with vesicle docking (23). By this mechanism, BoNT exerts its muscle relaxant action, which, in addition to its other uses, may be beneficial in some pain conditions (1,2). BoNT has also been hypothesized to have direct antinociceptive and antihyperalgesic effects by inhibiting the release of pronociceptive neurotransmitters and neuropeptides (9,12,13,24). Recently, a study using the formalin pain model in rats demonstrated a significant, dose-dependent inhibition of nociceptive behavior and local edema after intraplantar BoNT pretreatment in the absence of muscle weakness (11). Because the same doses of BoNT also markedly and dose-dependently inhibited formalin-induced peripheral glutamate release, this mechanism was proposed to be responsible for the observed effects of BoNT on inflammation and nociception. The pattern of efficacy of BoNT in the formalin test appeared fully consistent with the hypothesized mechanism. The antinociceptive action was observed in the second, but not the first, phase; the former, but not the latter, is thought to be dependent on peripheral and central sensitization.
because of release of inflammatory mediators and pronociceptive substances, including glutamate (11). Similarly, peripheral glutamate and glutamate receptors are considered to play a role in inflammatory sensitization but not acute pain (25,26). Furthermore, it can be argued that the antiinflammatory effect may be related to inhibition of release of substance P and calcitonin gene-related peptide. These vasoactive peptides are co-localized with glutamate in primary afferents (27,28) and may be responsible for neurogenic inflammation; BoNT inhibits neuropeptide release in vitro (12,13,24).

In the present study, we have tested the antiinflammatory and antihyperalgetic effect of BoNT/A using a human inflammatory UVB pain model. This pain model provides continuous stimulation of nociceptors by inflammatory mediators and has been shown to provide stable conditions of primary and secondary hyperalgesia, presumed to be markers of peripheral and central sensitization, respectively (18). Various data characterize this pain model as highly sensitive to antiinflammatory drugs (cyclooxygenase inhibitors) and opioids (14–17). Hence, based on the preclinical data mentioned above, we expected that BoNT would inhibit local inflammation and hyperalgesia in this inflammatory pain model in humans. However, despite the evidence of efficacy on sudomotor function, BoNT/A had no effect on pain measures in either normal or inflamed skin. The assessment of primary hyperalgesia by thermal and mechanical pain testings revealed no noticeable effects. Secondary hyperalgesia was also found to be unaffected by BoNT. Finally, inflammation intensity measured with laser Doppler imaging was also unchanged.

The absence of an acute analgesic effect of BoNT/A on normal skin 48 hours after injection is not inconsistent with the hypothesized inhibition of neurotransmitter or neuropeptide release in the periphery because this would not be expected to affect normal afferent function; it also concurs with previous animal and human data. Thus, BoNT did not affect heat-evoked withdrawal responses in rats (11). In humans, under conditions of direct nociceptor stimulation by noxious heat, electrical current, or intradermal capsaicin, no significant difference in any of the outcome measures had been observed between BoNT/A and placebo-pretreated skin (8,10).

The lack of BoNT’s effect on inflammatory sensitization in our study contrasts with the results of the formalin pain model in rats (11) but is consistent with previous human studies under conditions of neurogenic inflammation. Topical capsaicin and high-density electrical stimulation of the skin in humans evoke neurogenic inflammation because of peripheral release of vasoactive neuropeptides such as calcitonin gene-related peptide and substance P (29,30). However, in these human models, BoNT failed to significantly reduce symptoms of sensitization (9,10). Furthermore, there is only limited circumstantial evidence that clinical doses of BoNT/A may reduce neuropeptide release in humans. The area of electrically induced flare reaction as a surrogate of neurogenic inflammation was only marginally reduced (by 13.5%) after BoNT/A treatment compared with placebo (9). Capsaicin-evoked flare was not affected by BoNT (10).

In the present study, UVB irradiation resulted in a highly significant inflammation and peripheral nociceptor sensitization, as evidenced by flare and primary hyperalgesia, yet no antiinflammatory or antihyperalgesic effect of BoNT/A was detected. Secondary hyperalgesia, which is believed to result from central sensitization by continuing peripheral nociceptive barrage (31), was also unchanged by BoNT/A, consistent with data from other studies using models of central sensitization (9,10).

The most likely explanation for the observed differences between BoNT’s effects in animal and human models of inflammatory pain is the different role that neuropeptides or excitatory amino acids play in these models. Thus, species differences are likely to be implicated if the attenuation of formalin-evoked nociception by BoNT is mediated via reduction in substance P release, as suggested by in vitro data (12,13). Substance P plays a major role in plasma extravasation, neurogenic inflammation, and inflammatory hyperalgesia in rodents but does not seem important in inflammatory sensitization in humans (29,30,32). Neurokinin receptor antagonists have shown efficacy in a wide range of animal models of inflammatory pain, including the formalin test, but failed to alleviate pain in humans (33). Furthermore, if BoNT antinociception in the formalin test is mediated by reduced glutamate release in the periphery, this mechanism may not play an important role in peripheral sensitization of human skin under conditions of neurogenic inflammation (34).

Another potential explanation is that neurogenic inflammation may be of importance in the formalin model in the rat, but its contribution to inflammatory sensitization after UVB irradiation in humans may be limited. Nonetheless, as discussed above, the effects of BoNT on neurogenic inflammation in humans seems minor (9,10).

Despite the lack of impairment of rotarod performance (11), inhibition of formalin-evoked nociception by BoNT may still be explained by alleviation of a painful local muscle spasm (which may remain undetected by this test); this would not be expected to play any role in the human model of cutaneous inflammation. We intradermally injected BoNT/A at the ventral aspect of the upper leg, whereas rats were injected...
through the surface into the plantar footpad. BoNT/A easily diffuses in the tissue for several centimeters (35); hence, in our study, we chose intradermal injections directly over the large upper leg musculature to exclude any secondary effects on pain and hyperalgesia by reduction of muscular tone. In contrast, because of the large doses applied and the relatively small anatomical structures, this mechanism cannot be excluded in the rat hind paw.

One might also speculate that the UVB irrigation and the ensuing inflammatory reaction might have reduced the tissue concentration of BoNT/A and thus resulted in reduced efficacy. However, BoNT/A was injected 48 hours before the irradiation. Moreover, we assessed the biological activity of BoNT/A 14 days after injection and found relevant areas of focal anhidrosis as indicators of its preserved efficacy.

The timing of testing also seems to be appropriate for detecting the maximum effects of BoNT/A. In the present study, BoNT/A was injected 48 hours before pain testing in normal skin and 72 hours before testing in inflamed skin. These time points were chosen according to the formalin pain model study results (11), where BoNT/A was found to have a maximum antinociceptive action when injected 1–12 days before the injection of formalin. Furthermore, in humans, a marked anhidrotic effect, as a surrogate for BoNT/A’s effects, was already found two and three days after intradermal injection (9).

The present study is limited by the relatively small sample of participants. Nevertheless, because of the small within-subject variability in this model, low subject numbers are required to detect even rather small treatment differences using a crossover design (18). The sample size calculation was based on the large effects found in the formalin pain model (11). Although we cannot exclude that a small antinociceptive and antinflammatory effects of BoNT were not detected in this study because of low statistical power, we did not find any consistent trend in any outcome variable.

In conclusion, our study has confirmed that BoNT/A has no direct effect on acute, noninflammatory nociception in humans. Furthermore, under conditions of inflammatory sensitization in humans, we have not observed the large antihyperalgesic effect of BoNT/A reported in an inflammatory pain model in rats.

References

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