On the enhancement of anti-neurotoxin antibody production by subcomponents HA1 and HA3b of *Clostridium botulinum* type B 16S toxin-haemagglutinin

In a recent article in this journal, Lee *et al.* (2005) reported that immunization of mice with formaldehyde-treated botulinum neurotoxin B (BoNT/B) in the presence of haemagglutinin subcomponents HA1 and HA3b, normally present in the 16S toxin, produced higher levels of anti-BoNT/B antibodies (Abs) than immunization with BoNT/B alone. Because of the paper's potential clinical therapeutic implications, this comment discusses three major issues in the paper: quality of the antigen, dose and frequency of immunization. I show that these were quite different from those used in therapeutic applications of active BoNT/B (or any BoNT) complex.

BoNTs (mostly types A and B) are used to treat a variety of clinical conditions associated with involuntary muscle spasm and contractions as well as in cosmetic and other therapeutic applications (Jankovic, 2002, 2004; Atassi & Oshima, 1999; Brashear *et al.*, 2004; Dressler & Bigalke, 2005). The therapeutic benefits are of limited duration and toxin injections need to be repeated every 3–6 months. In a very small percentage of patients (less with toxin A than with toxin B) the treatment elicits blocking Ab responses against the correlate toxin, which reduce or completely terminate the patient's responsiveness to further treatment (Göschel *et al.*, 1997; Atassi & Oshima, 1999; Atassi, 2002, 2004;

Jankovic, 2002, 2004; Jankovic *et al.*, 2005; Dressler & Bigalke, 2005). It would be highly desirable to eliminate or substantially reduce the appearance of blocking antitoxin Abs in therapy of dystonia and other patients who become unresponsive to treatment.

In a recent article in this journal, Lee *et al.* (2005), following-up their earlier work on a neurotoxin product free of accessory proteins (Arimitsu *et al.*, 2003), set out to determine if the haemagglutinins (HAs) in the products contributed to enhancement of the immune response. They noted that 'type A and B PTXs (progenitor toxins) have been used for treating patients with many forms of dystonia. In both toxin types, PTXs are used because they are more stable than NTXs (neurotoxins). The treatment is very effective, but has serious side effects for some patients in whom anti-PTX, including anti-NTX Abs, is produced after several injections.' They also reported 'Although we have no quantitative data, there is some evidence that high levels of anti-NTX Ab are produced by immunization of rabbits with PTX alone compared with NTX alone.' Thus, the purpose of the work was to determine if immunization of mice with BoNT/B in the presence of two haemagglutinin subcomponents, HA1 and HA3b, normally present in the 16S toxin, produced higher levels of anti-BoNT/B Abs than immunization with BoNT/B alone. In the Methods section the authors inform the reader that the 'immunization schedule was based on the assumed treatment of patients with dystonia, i.e. recurrent injection of a low dose of antigen (toxin)'.

The reader could, therefore, reasonably conclude that the research was designed to understand the immunological factors responsible for neutralizing Ab formation in patients and to investigate the possibility of decreasing the antigenicity of BoNT. In view of the value of BoNT/A and BoNT/B in various therapies, including dystonias, an effective strategy that could extend their usefulness to immunoresistant patients would be most desirable.

Several factors can also influence the immune response to the toxin. These include the quality of the antigen and its dose, duration of treatment and frequency of immunization (Atassi, 2002, 2004). It has been well established that the immune responses to a protein are determined to a great extent by its form and by the presence of other proteins in the immunogen (Atassi, 2002, 2004). Furthermore, the immune response to the whole toxin is under genetic control, and the response to each epitope is under separate genetic control. The appearance of blocking Abs in treatment might be controlled by the major histocompatibility complex of the host (Atassi & Oshima, 1999; Atassi, 2002, 2004).

I wish to address here the main issues relevant to the paper of Lee *et al.* (2005): antigen quality, dose and frequency of immunization.

Antigen

Lee *et al.*, (2005) treated the three forms of toxins, BoNT/B, and the 12S and 16S progenitor toxins (associated with non-toxic components) with formaldehyde to render them non-toxic. They were reportedly dialysed for 7 days at 37 °C against 0·10 M sodium phosphate buffer at pH 8·0, containing 0·6 % formaldehyde. Lee and colleagues inactivated the toxin in their work to be able to administer the relatively massive toxin doses that would have otherwise been lethal had the toxin not been inactivated. However, the consequences of formaldehyde treatment on these protein preparations need to be considered.

Formaldehyde adds to the amino groups instantaneously, even at neutral pH and low temperature, to form highly electrophilic immonium cations ($\text{-}\overset{\oplus}{\text{N}}\text{H}=\text{CH}_2 \Leftrightarrow \text{NH-}\overset{\oplus}{\text{C}}\text{H}_2$), which could be reversible under mild conditions. However, in a prolonged reaction period (7 days) at a relatively high temperature and pH (37 °C and pH 8·0) the immonium intermediate would predominantly react with amino acid side chains within favourable distance to form stable methylene bridges (Means & Feeney, 1971). Aldehyde treatment under such conditions irreversibly cross-links amino groups to phenol (i.e. tyrosine), imidazole (i.e. histidine) and indole (i.e. tryptophan) side chains (Atassi, 1977). Formaldehyde would also react with thiol groups and even with the guanidinium side chain of arginine (Means & Feeney, 1971). The high non-selective reactivity of formaldehyde makes it totally unsuitable for employment in immunochemical studies of proteins (Atassi, 1977).

It has been well documented that treatment of proteins with formaldehyde gives products with enhanced antigenicity. For example, cytochrome *c* is a low-molecular-mass protein that is a very weak immunogen. The action of aldehyde causes the formation of intra- and intermolecular cross-links and produces oligomeric species of high molecular mass that exhibit strong immunogenic properties (Reichlin *et al.*, 1970; Jemmerson & Margoliash, 1978, 1979). In the case of the study by Lee *et al.* (2005), treatment of the neurotoxin with formaldehyde at a relatively high temperature and pH (37 °C and pH 8·0) for a prolonged period of time (7 days) is bound to cause the formation of a heterogeneous mixture of oligomers of varying sizes due to intermolecular cross-links. Then to make matters even worse, the authors mixed these products with HA1, HA2 or HA3b without any attempt to remove the excess formaldehyde from the solution of the toxin preparation. Treatment of the 12S and the 16S progenitor toxin preparations with formaldehyde will be expected to generate complex homo- and hetero-oligomers and polymers in which the neurotoxin is covalently cross-linked to itself and/or to the non-toxic components. This means that the neurotoxin-HA complex is no longer formed by protein–protein association, but rather by covalent cross-links within and among the correlate proteins. This would lead to the obliteration of some epitopes and the creation of new epitopes that are not present on the corresponding native proteins. The authors made no effort to isolate and identify any products of this crude treatment.

It is important to point out that patients are not treated with formaldehyde-treated toxin preparations, but with an active toxin B (or toxin A) complex preparation that is not detoxified with any chemical agent and is as close as is possible to a native preparation.

For a control, Lee *et al.* (2005) injected human serum albumin (HSA) by itself or as a mixture with HA1, HA2 or HA3b

and found that each of these mixtures gave higher anti-albumin Abs than immunization with HSA alone (Lee *et al.*, 2005). This might be an acceptable control were it not for the fact that serum albumin is well known to be an insatiable binder of a plethora of proteins and small molecules (for examples of the vast literature, see: Lin *et al.*, 2004; Coyle *et al.*, 2006; Komatsu *et al.*, 2005; Rozak *et al.*, 2005; Chen *et al.*, 2006) and readily undergoes aggregation under a variety of conditions and interactions (again, for examples of the vast literature, see: Vaiana *et al.*, 2004; Militello *et al.*, 2004; Oliva *et al.*, 2003). Serum albumin also acts as a molecular chaperone and can be included in the extracellular chaperone family (Marini *et al.*, 2005). The interactions of HSA with HA subcomponents of *C. botulinum* have not been studied, but with the known properties of HSA, it is not unreasonable to expect that it would associate with HA and/or form aggregates in the presence of these subcomponents. In either case, aggregates of HSA or HA-HSA species would be expected to evoke higher levels of anti-HSA Abs than monomeric HSA. For these reasons, HSA is not an appropriate control for these studies.

It is necessary to point out here other major advantages for the therapeutic application of the BoNT complex over pure neurotoxin. The non-toxin components present in the complex confer on the toxin protection against degradation and rapid clearance *in vivo* during therapeutic application, and its larger size helps in better targeting and keeping more of its activity in the afflicted, injected muscle by reducing its rate of diffusion and hence its spread to distant unaffected muscles (Borodic *et al.*, 2001; Callaway *et al.*, 2002).

Dose

Lee *et al.* (2005) injected detoxified BoNT/B at a concentration of 2 μg in 0·25 ml, mixed with NTNH-HA or different HA subcomponents (total 2 μg in 0·25 ml). The mean weight of 6- to 8-week-old mice is about 23 g. So, on the basis of body weight, the amount injected would be equivalent, in a 70 kg person, to a dose of 6·087 mg per injection.

In treatment of dystonia patients with Myobloc (a BoNT/B complex; Solstice Neurosciences Inc.), the recommended dose range is 2500–10 000 U, which corresponds to 25–100 ng per treatment session (Brashear *et al.*, 1999; Myobloc package insert rev. 11/04). This means that the 2 μg per mouse injected by Lee *et al.* (2005) is about 10^5 times higher than the dose range given to the patient.

The mean dystonia dose of Botox (a BoNT/A complex; Allergan Inc.) in the USA is approximately 236 U or 11·8 ng BoNT/A complex per session (Aoki, 2002; Botox package insert 71390US12.J). So the dose used by Lee *et al.* (2005) was $5{\cdot}16 \times 10^5$ higher than is used in dystonia therapeutic applications. In a recent study of 929 dystonia and other patients (Yablon *et al.*, 2005) using a current BoNT/A formulation (Botox), dystonia patients ($n=326$) were given 1–15 treatments of BoNT/A (mean per treatment: 187·3 U = 9·365 ng; range per treatment: 20–500 U = 1–25 ng; maximum total per patient: 4210 U = 210·5 ng). Other patients received similar dose ranges. It was reported that neutralizing Ab appeared in 5 (less than 0·6 %) of the 929 patients.

The mean recommended dose of Dysport (a BoNT/A-complex product; Ipsen Ltd) for treatment of dystonia is about 500 units. Currently, the reported BoNT/A complex amount is 12·5 ng per 500-unit vial. Therefore, on normalized weights the dose administered by Lee *et al.* (2005) was $4{\cdot}87 \times 10^5$ higher than that used in dystonia patients.

Both the Ab and T cell immune responses to a protein antigen are dose-dependent (Young & Atassi, 1982; Atassi, 1984, 2004). In the same manner, there is an optimum dose of BoNT for a maximum response by the host (Atassi *et al.*, 1996; Rosenberg *et al.*, 1997; Poewe *et al.*, 1998). In BALB/c, the optimum dose of inactivated toxin has been reported to be 1 μg per mouse (Rosenberg *et al.*, 1997). The dose of 2 μg per mouse used by Lee *et al.* (2005) was close to the optimal range for the Ab response. However, it is evident that the doses used in patient therapy are extremely suboptimal. Thus, the Ab response will increase with an increase in antigen dose and frequency of immunization. Therefore, increasing the BoNT dose by 10^5-fold would exert an enormous boost to the Ab titre. Studies of dystonia patients have shown that the Ab response is dependent on the toxin dose (Göschel *et al.*, 1997).

On another level, the injection of humans with a dose equivalent to 2 μg active neurotoxin per mouse would be perilous. It is equivalent to about 3×10^5-fold LD_{100} values (based on mouse LD_{100} values in our hands of BoNT/B = 6·25 pg per mouse and BoNT/A = 6·5 pg per mouse). Finally, it should be noted that that antigen dose could even influence the genetic control of the immune response (Young & Atassi, 1982).

Frequency of immunization

Lee *et al.* (2005) injected the mice once a week for 6 weeks. Dystonia patients receive a neurotoxin-complex injection once every 3–6 months, with variations being dependent on the patient. It is well established that the immune response is influenced by the frequency of immunization (Atassi, 2004). As with all antigens, the appearance of Ab responses against BoNT is closely linked to duration of treatment and frequency of immunization. The Ab response will increase with the increase in antigen dose and frequency of immunization. In dystonia patients treated with toxin complex, the appearance of blocking Abs seems to correlate with the cumulative toxin dose, the time interval between injections and the total protein load (Göschel *et al.*, 1997; Inagi *et al.*, 1997; Jankovic, 2002). In addition, Ab recognition and epitope recognition have been shown to improve with duration of treatment and frequency of immunization (Rosenberg *et al.*, 1997; Dolimbek *et al.*, 2005).

Type of antibody

It is important to point out that the Ab level, although suggestive, does not necessarily always equate with blocking activity. Blocking activity is only partially determined by Ab level, while Ab affinity, the epitopes recognized by these Abs, immunoglobulin class and isotype play major roles (Atassi, 2004; Atassi *et al.*, 2005). Lee *et al.* (2005) do not consider these other factors. Also, they do not determine whether the differences observed in Ab levels are equivalent to differences in blocking activity.

In conclusion, the work of Lee *et al.* (2005) used a chemically altered antigen not used in therapy, and injected it at a much higher dose and more frequently. Unfortunately, the findings have little or no relevance to the therapeutic applications of active BoNT/B (or any BoNT) complex.

Acknowledgements

The author wishes to thank the Welch Foundation for the award of the Robert A. Welch Chair of Chemistry.

we all make mistakes :)

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DOI 10.1099/mic.0.28862-0

Authors' reply

We would like to express our opinion on the above article by Dr Atassi with regard to our paper (Lee *et al.*, 2005).

Since (1) it seems that high levels of anti-neurotoxin (NTX) antibody (Ab) are produced by immunization of rabbits with progenitor toxin alone (PTX; a complex of a NTX and non-toxic components), compared with NTX alone, and (2) we found that HA1 and HA3b, but not HA2, bind specifically to the glycoprotein or glycolipid of the cells, we speculated that HA1 and HA3b might have adjuvant (or immuno-enhancing) activity. To clarify this hypothesis, we first observed that when mice were immunized with formalin-detoxified NTX, 12S (HA-negative PTX) or 16S (HA-positive PTX) (type B), a significantly greater amount of anti-NTX Ab was produced in the mice injected with 16S than in NTX- or 12S-injected mice. Next, we demonstrated that immunization with NTX mixed with HA1 and/or HA3b (described here as NTX + HA1 and/or HA3b), but not HA2, also increased anti-NTX Ab production, compared with NTX alone or NTX + HA2. Finally, adjuvant activity of HA1 and HA3b was confirmed by employing human albumin (Alb) instead of NTX. Thereafter, we investigated the mechanism of adjuvant activity of HA1 and HA3b. We found that 16S, HA1 and HA3b significantly increase interleukin 6 (IL6) production from normal mouse spleen cells, compared with NTX alone, and that this IL6 production was mediated by p38 MAPK, PI3-K and PKA, but not by ERK1/2. The percentage of B cells also increased following stimulation with 16S or NTX + HA1 or HA3b, compared to stimulation with NTX alone.

In these experiments, toxins detoxified by treatment with 0·6 % formaldehyde (actually, this was 0·6 % formalin as mentioned below) at 37 °C for 1 week were employed. For immunization, each antigen preparation (2 μg in 0·25 ml) was injected subcutaneously six times at 1 week intervals into the dorsal side of 10 female BALB/c mice (in the control experiment using human Alb, the mice were immunized three times). Six days after the last injection, the mice were sacrificed and blood was collected. This immunization schedule was based on the assumed treatment of patients with dystonia, i.e. recurrent injection of a low dose of antigen (toxin), because it has also been reported that anti-PTX Ab, including anti-NTX, is sometimes produced in patients treated with PTX several times.

Formaldehyde treatment of toxins and control experiment with human Alb

Dr Atassi claims that formaldehyde treatment is not suitable. However, we always use formalin (a solution containing 37 % formaldehyde) for detoxifying the botulinum toxin, and we can obtain high titrations of Abs reacting with the native toxin, including toxin-neutralizing Ab, in animals by immunization with such preparations. Usually, toxins are treated with 0·2–0·7 % formalin at 20 °C (room temperature) up to 37 °C for 4–14 days and at pH 6·0–8·0 (Sugiyama *et al.*, 1974; Hatheway, 1976; Hatheway *et al.*, 1981; Oguma *et al.*, 1980, 1982; Vertiev *et al.*, 2001; Torii *et al.*, 2002). Therefore, in our study we used 0·6 % formalin (containing 0·22 % formaldehyde) at 37 °C for 7 days at pH 8·0 (we apologize for our mistake in describing 0·6 % formalin as 0·6 % formaldehyde in our manuscript). Also, although we did not describe it in the manuscript, the preparations were dialysed against 0·01 M PBS (pH 7·4) after formaldehyde treatment to eliminate the formaldehyde (we thought that it was not necessary to describe this because elimination of formaldehyde is done as a matter of course). After elimination of formaldehyde, the NTX preparation was injected into mice with or without mixing with HA subcomponents. The mice immunized with NTX + HA1 and/or HA3b produced significantly higher anti-NTX Ab than those immunized with NTX alone or NTX + HA2. Furthermore, in *in vitro* tests with mice spleen cells, IL6 mRNA transcription levels and the amounts of IL6, as analysed by RT-PCR and ELISA, respectively, were significantly increased by stimulation with HA1 or HA3b, compared with HA2 or NTX. On the contrary, the stimulation of IFNγ production by these three subcomponents or NTX was not significantly different (IFNγ production was increased only by the 16S toxin). It also became clear by using flow cytometry analysis that the percentage of B cells (CD19-positive cells) was higher following stimulation by 16S or NTX + HA1 or HA3b, compared with stimulation by NTX alone.

Therefore, we can conclude that HA1 and HA3b demonstrate adjuvant activity via increasing IL6 production, even though the NTX preparation may form intra- and intermolecular cross-links due to the formaldehyde treatment. Recently, we demonstrated that native type D NTX is unable to bind any other components except the non-toxic non-HA component (NTNH) (Suzuki *et al.*, 2005). We think that a detoxified NTX preparation may not bind any HA subcomponents under conditions where almost all of the formaldehyde has been removed, although we have no data supporting this at the present time. If there are more suitable agents than formaldehyde that can be used for detoxification, we would like to do experiments using these agents in the future.