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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.

The conclusion that HA1 and HA3b, but not HA2, have adjuvant activity was also confirmed by control experiments using human Alb. We have no definitive reason for using human Alb as a control, but our reasons for choosing it were (1) human Alb is added as a stabilizer in toxin preparations for treatment, (2) ovalbumin is usually employed as an antigen in experiments demonstrating the adjuvant activity of some substances (Duverger *et al.*, 2006; Sun, 2006; in both papers 100 μg ovalbumin was used as immunogen – since the procedure performed by Duverger and colleagues was similar to ours, it is explained below), and (3) we thought that the immunological behaviour of human Alb might be the same as ovalbumin in mice. The mice were immunized three times (we found three injections were enough in this case) with Alb (2 μg per 0·25 ml) or Alb mixed with HA1, HA2 or HA3b (1 μg Alb and each HA subcomponent per 0·25 ml). The anti-Alb Ab levels in the mice immunized with Alb + HA1 or HA3b were significantly higher than those of mice immunized with Alb alone or Alb + HA2. Dr Atassi misunderstood these data. He mentioned that immunization with each of three mixtures, Alb + HA1, HA2 or HA3b, produced larger amounts of anti-Alb Abs than Alb alone immunization. We think our control experiments work well and lead to the conclusion that HA1 and HA3b, but not HA2, have adjuvant activity.

Immunization schedule, antigen dose and type of the Ab

We immunized the mice six times at 1 week intervals with 1 or 2 μg antigen per 0·25 ml (since similar data were obtained with 1 or 2 μg, the data obtained using 1 μg were eliminated in the manuscript, according to the suggestion of one of the reviewers). The mice were immunized at 1 week intervals because 1 week in mice corresponds to several months in a human. In our previous experiments in mice, high titres of Abs were produced when they were immunized with approximately 100 μg type C or D toxoid preparation without adjuvant (Oguma *et al.*, 1982, 1984), and therefore we employed a 1 or 2 μg dose at this time. Of course, the amounts used, 1 or 2 μg, are very high compared with the dose employed in patients for treatment. However, we thought that this level of dose may be necessary to analyse the adjuvant activity of HA subcomponents. When we checked the serum Ab titre after three injections, little difference was observed among the mice immunized with different antigens, and therefore we continued the injections up to six times. Duverger *et al.* (2006) demonstrated that the oedema toxin of *Bacillus anthracis* has adjuvant activity by immunizing mice nasally three times at weekly intervals with 100 μg ovalbumin alone, ovalbumin plus 5 μg recombinant protective antigen, or ovalbumin plus oedema toxin (5 μg recombinant protective antigen together with 5 μg recombinant oedema factor). They demonstrated that oedema factor also has adjuvant activity: it enhanced immunity to the protective antigen itself and promoted high levels of plasma IgG and IgA responses, as well as neutralizing Ab against the protective antigen. Similarly to these experiments, we can conclude that anti-NTX Ab production is enhanced by co-administration of NTX with HA1 or HA3b from the results of the experiments we have performed so far. However, we will carry out further experiments in which mice are immunized with reduced doses and/or

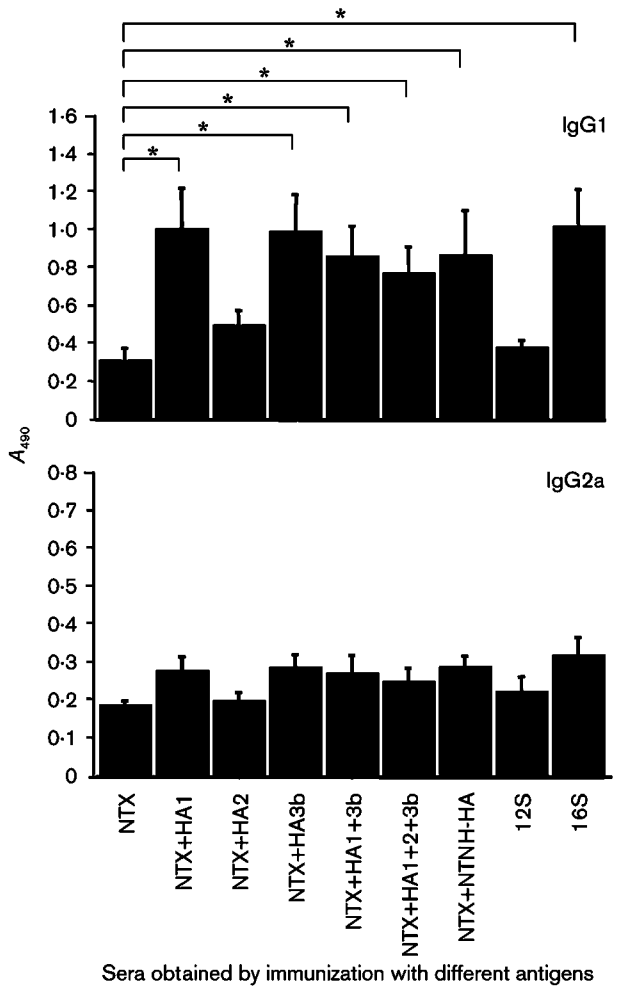

Fig. 1. Subclass of Abs against NTX. Subclass of anti-NTX Abs obtained by immunization with different antigens was determined by ELISA with 96-well plates. The wells were coated with 1 μg NTX, and then successively reacted with 0·1 ml 1 : 100 diluted mouse sera and 1 : 1000 diluted horseradish-peroxidase (HRP)-conjugated rabbit anti-mouse IgG1 (MP Biomedical, LLC) or HRP-conjugated goat anti-mouse IgG2a (Santa Cruz Biotechnology). *$P < 0·05$.

reduced injection times with longer intervals, if necessary.

The toxin-neutralizing titre (reciprocal of the highest dilution of the serum to neutralize 10 MLD toxin) of mice immunized with 16S was 80–90, and that of pooled sera immunized with NTX alone was 20–30. These data were obtained by injecting each reaction mixture (diluted sera and 10 MLD toxin) into two mice intraperitoneally. Since (1) the number of injected mice was insufficient, and (2) these neutralization data were not necessary to lead to the conclusion that HA1 and HA3b have adjuvant activity, we did not describe them in the manuscript. The subclass of the Abs was not determined. Since the publication of our paper we have determined cytokine production and the data are presented in Fig. 1. Production of anti-NTX IgG1 Ab was enhanced by co-administration of NTX with HA1 or HA3b, but not HA2.

The purpose of our manuscript was to demonstrate that HA1 and HA3b have adjuvant activity. We speculate that adjuvant activity of HA1 and HA3b might be one of the reasons why anti-NTX Ab is sometimes produced in patients treated with botulinum PTX several times. We would like to confirm this by doing additional experiments and hope other researchers will also conduct similar experiments, utilizing the most suitable procedures available. Also, we think that many experiments are still necessary to determine which type of toxin is the best for treatment. We are now planning to do experiments to clarify this point by employing highly purified type A and B NTXs and HA-positive PTXs obtained in our laboratory.

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