



## Technical note

## Active immunization induces toxicity of diphtheria toxin in diphtheria resistant mice – Implications for neuroinflammatory models

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## ARTICLE INFO

## Article history:

Received 13 August 2009

Received in revised form 11 December 2009

Accepted 26 January 2010

Available online 4 February 2010

## Keywords:

Diphtheria toxin

Experimental autoimmune encephalomyelitis

Active immunization

## ABSTRACT

Cell-type specific expression of the human diphtheria toxin receptor in generally toxin resistant mice represents an innovative approach for the selective depletion of pre-defined cell populations. We demonstrate that in wildtype mice diphtheria toxin – in concentrations otherwise well tolerated – is highly toxic and lethal together with active immunization irrespective of the immunogenic peptide applied. We found increased lung cellularity as only pathological abnormality. Animal models of inflammatory diseases requiring active immunization including experimental autoimmune encephalomyelitis may thus not be applicable in diphtheria receptor transgenic mice pointing to a major limitation of this otherwise technically interesting approach.

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### 1. Introduction

Depletion of specific cell populations *in vivo* can provide essential information on their respective functions and different methods have been developed. One technical approach for experimental cell depletion is based on the fact that rodents – unlike humans – are resistant to the infectious agent causing diphtheria. In humans infections with exotoxin secreting strains of *Corynebacterium diphtheria* lead to necrosis of respiratory epithelial cells. The responsible diphtheria toxin (DTx) binds a toxin receptor on the surface of toxin-sensitive cells and inhibits RNA translation and protein synthesis of infected cells (Kimata and Kohno, 1994). Rodents are insensitive to DTx due to species specific differences of the cell surface DTx receptor (Cha et al., 1998), which has been identified as a membrane-anchored form of the heparin-binding epidermal growth factor-like growth factor (HB-EGF) (Naglich et al., 1992). HB-EGF from mice and rats does not bind DTx (Cha et al., 1998) and the

transgenic expression of human HB-EGF can be used to confer DTx sensitivity to rodent cells and to ubiquitously expressing transgenic mice, which die from cardiac failure within several days after treatment with DTx (Cha et al., 2003).

Additionally, mouse lines have been generated, which express human HB-EGF under the control of cell-type specific promoters making defined cell populations sensitive to exogenously added DTx (Saito et al., 2001; Buch et al., 2005). Such selective depletion of specific cell populations in animal models of immune mediated human diseases promises insights into disease mechanisms. We therefore studied whether the application of DTx in combination with active immunization – as required for the active induction of experimental autoimmune encephalomyelitis (EAE) (Gold et al., 2006) and neuritis (EAN) (Meyer zu Hörste et al., 2007a) – is sustained by the commonly used C57BL/6 mouse strain.

### 2. Materials and methods

#### 2.1. Animal experimentation

All animal experiments were conducted according to local regulations. Inbred female C57BL/6 mice aged 8–12 weeks

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weighing 26–31 g from our local breeding colony received intraperitoneal injections of 500 ng pertussis toxin (PTx) (Sigma-Aldrich, Taufkirchen, Germany) dissolved in 200  $\mu$ l sterile phosphate buffered saline (PBS) on the day of immunization (d0) and on day 2 after immunization (d2). Diphtheria toxin (DTx) from three different suppliers (MerckDTx, Merck, Darmstadt, Germany; ListDTx, List Biological Laboratories, Campbell, CA, USA; SigmaDTx, Sigma-Aldrich, Taufkirchen, Germany) was reconstituted according to the manufacturer's protocol, stored in aliquots at  $-80^{\circ}\text{C}$  and thawed only once before usage. Two different batches of MerckDTx were tested. DTx was dissolved in 100  $\mu$ l sterile

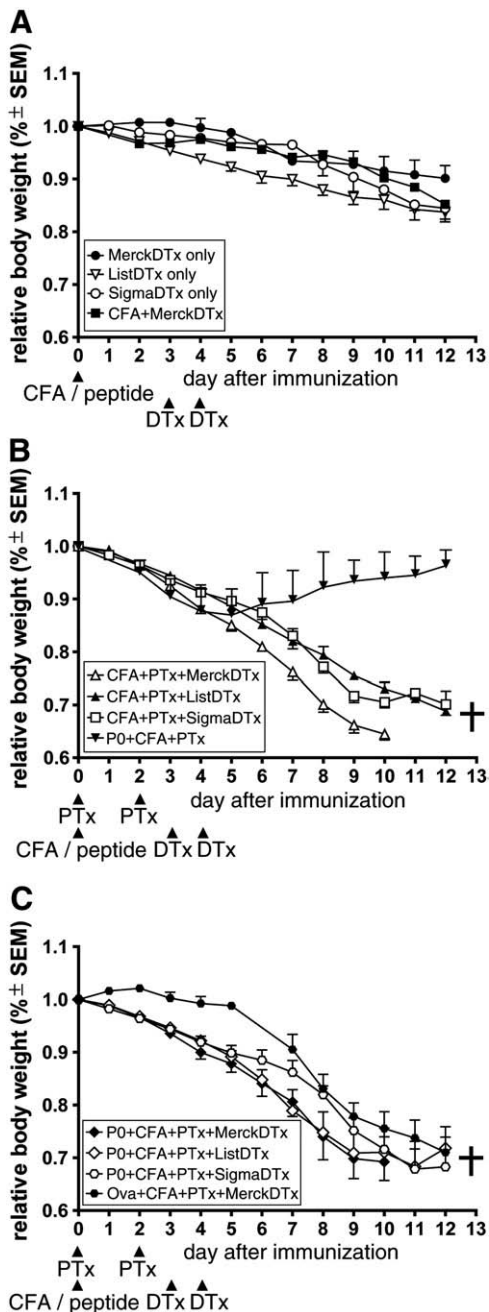
PBS and applied intraperitoneally on d3 and d4 at 50  $\mu\text{g}/\text{kg}$  (1  $\mu\text{g}$  per mouse), at 25  $\mu\text{g}/\text{kg}$  (0.5  $\mu\text{g}$  per mouse) and 5  $\mu\text{g}/\text{kg}$  (0.1  $\mu\text{g}$  per mouse). Animals received subcutaneous injections of 100  $\mu$ l complete Freund's adjuvant (CFA) (Sigma-Aldrich) containing 1 mg/ml heat inactivated *Mycobacterium tuberculosis* strain H37RA mixed with 100  $\mu$ l PBS into the flanks. The CFA/PBS mixture was injected either alone or containing 200  $\mu\text{g}$  Ovalbumin peptide spanning amino acids 323–339 (Ova<sub>323–339</sub>) (Peptides International, Louisville, KY, USA) or 200  $\mu\text{g}$  myelin protein zero peptide spanning amino acids 180–199 (PO<sub>180–199</sub>) (Peptides International). Three independent experiments were performed.

## 2.2. Histology

Animals were fixed in 4% paraformaldehyde solution and the heart, lung, liver, spleen, kidney and sciatic nerve were dissected, paraffin embedded and cut to 10  $\mu\text{m}$  sections. All sections were subsequently stained with haematoxylin–eosin (H&E) and lung sections stained with Elastica–van Gieson and Alcian blue–PAS (all from DAKO, Hamburg, Germany). Lung sections were stained against CD3 (1/1,500, ab5690, Abcam) and CD68 (1/500, MCA1957, AbD Serotec) using DAB based detection. Sciatic nerves were postfixed in glutaraldehyde based fixation medium, epoxy resin embedded and cut to semithin sections as previously described (Meyer zu Hörste et al., 2007b). Sections were evaluated by a specialized veterinary pathological laboratory blinded to the treatment applied (Department of Veterinary Pathology, Ludwig-Maximilians-University, München, Germany).

## 3. Results

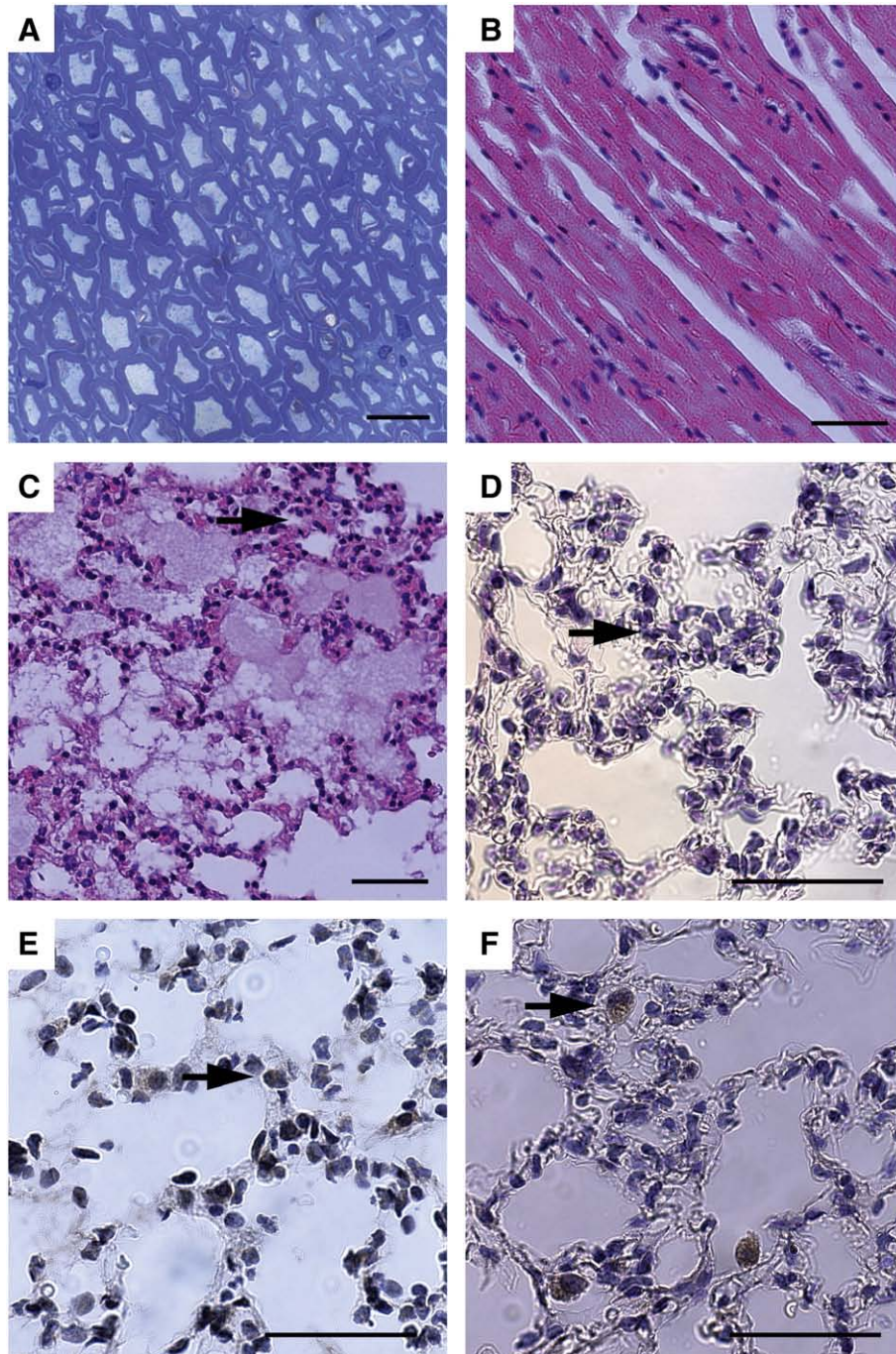
Experimental animals were randomized to receive injections of different combinations of peptides, CFA, PTx and DTx (50  $\mu\text{g}/\text{kg}$ ) from three different sources ( $n = 5$  each). Injection of CFA alone and of PTx alone did not cause any relevant change in body weight, while the combination of CFA and PTx resulted in a minor transient weight decrease of 10% (data not shown). This group quickly began to regain weight after day 7. Injection of DTx from all three providers alone and the injection of CFA together with DTx caused a gradual weight decrease of up to 15% (Fig. 1A) without any further signs of toxicity. A complete EAN immunization protocol against PO<sub>180–199</sub> in CFA together with PTx resulted in a transient 15%



**Fig. 1.** Body weight relative to the initial values was assessed in wildtype C57BL/6 mice receiving different combinations of toxins and immunization. A. Intraperitoneal application of DTx (50  $\mu\text{g}/\text{kg}$ ) from Merck (filled circles), List (empty inverted pyramid) and Sigma (empty circle) alone or in combination with subcutaneous CFA (filled squares, A) caused a gradual loss of ~15% body weight. B. Subcutaneous application of myelin protein zero (PO) peptide spanning amino acids 180–199 emulsified in CFA and in combination with PTx caused a transient ~10% weight loss (filled inverted pyramid). Application of DTx obtained from Merck (open pyramid), List (filled pyramid) and Sigma (open square) together with CFA and PTx caused a dramatic ~35% weight loss and was lethal in all animals between day 10 and 12 after immunization. C. Active immunization with PO peptide in CFA together with PTx and DTx from Merck (filled diamond), List (open diamond) and Sigma (open hexagon) resulted in comparable weight loss and mortality. Also ovalbumin peptide spanning amino acids 323–339 (Ova) in combination with PTx and MerckDTx instead of PO resulted in weight loss and mortality (filled hexagon). Five animals per group were used, weight is depicted as percentage of initial weight  $\pm$  SEM and one out of three representative experiments is shown.

weight loss, which quickly regenerated after day 5 (Fig. 1B). The application of CFA together with PTx and DTx resulted in a severe continuous weight loss to a maximum of 35% and finally spontaneous death between days 10 and 12 (Fig. 1B). The lethal DTx effects in combination with CFA and PTx occurred in principle along with DTx from all three providers, although

earliest with MerckDTx followed by ListDTx and SigmaDTx (Fig. 1B). Also, immunization against P0<sub>180–199</sub> together with PTx and DTx from all three suppliers caused rapid and severe weight loss of up to 30% (Fig. 1C) and spontaneous death or required to sacrifice the animals. Control immunization against Ova<sub>323–339</sub> derived from the non mammalian model protein



**Fig. 2.** Animals receiving subcutaneous injections of P0 peptide emulsified in CFA in combination with PTx and DTx intraperitoneally exhibited generalized weakness and reduced spontaneous movement. A. Semithin sections from peripheral nerves did not demonstrate any cellular infiltrates or demyelination. Scale bar represents 10  $\mu$ m. Weakness was thus not caused by peripheral neuropathy. B. Organ histology did not demonstrate any cardiac pathology. C, D. Within lung tissue increased mononuclear cellularity was detectable (arrow), forming infiltrate clusters. Lung sections were stained against CD3 (E) and CD68 (F). E. Cellular infiltrates stained positive for CD3 indicative of T cells. F. Few – mainly intraalveolar cells – stained positive for CD68 indicating macrophages. Scale bars represent 50  $\mu$ m.

Ovalbumin resulted in a comparably severe weight loss, an identical clinical picture and also 100% mortality (Fig. 1C). Significantly reducing the MerckDTx dosage to 25 µg/kg and to 5 µg/kg together with active PO<sub>180–199</sub> peptide immunization including CFA and PTx resulted in an equally rapid and complete mortality (data not shown). Applying MerckDTx two days before immunization was equally lethal between 5 and 6 days after immunization, while MerckDTx injection at day 10 post immunization was lethal between days 16 and 17 post immunization (data not shown). All spontaneously deceasing animals appeared severely ill with generalized weakness and reduced spontaneous movement between days 10 and 12. This severe clinical phenotype was independent from specific immunization and could not be explained by peripheral neuropathy, which can be induced by PO<sub>180–199</sub> immunization (Meyer zu Horste et al., 2007a). In fact histology did not reveal any relevant cellular infiltration or demyelination in the animals' peripheral nerves (Fig. 2A). None of the other groups developed any complications except for the previously described minor weight decrease. We further performed organ histology and found no relevant cardiac pathology (Fig. 2B) and no abnormalities of the liver, spleen or kidney (data not shown) in the animals, who had received both active immunization and DTx. Lung histology demonstrated increased mononuclear cell numbers (Fig. 2C, D). Immunohistochemistry demonstrated these cells to be mainly CD3+ T cells (Fig. 2E) and to a lower extent CD68+ macrophages (Fig. 2F).

#### 4. Discussion

Mice are generally resistant to DTx and expression of the human DTx receptor under the control of a tissue specific promoter confers toxin sensitivity allowing the depletion of pre-defined cell populations in transgenic mice *in vivo*. This has enabled a successful depletion of various cell populations in experimental animals including hepatocytes (Saito et al., 2001) and neurons (Gropp et al., 2005). This system appeared especially suitable to analyze immunologically mediated disease models as different genetically engineered mice allowing to deplete defined immune cell populations are available including CD11c+ dendritic cells (Jung et al., 2002), CD11b+ monocytes (Duffield et al., 2005), CD4+ T cells and CD19+ B cells (Buch et al., 2005), NKp46+ NK cells (Walzer et al., 2007) and FoxP3+ regulatory T cells (Kim et al., 2007; Lahl et al., 2007). Here, we surprisingly demonstrate that in wildtype mice, which should be fully resistant to DTx, the application of DTx in combination with a commonly used active immunization protocol is 100% lethal – irrespective of an immunogenic peptide being applied. Separate application of DTx or active immunization without DTx did not result in any observable toxicity. Even a 10-fold reduction of the DTx dosage compared to published values did not prevent DTx toxicity. Well established animal models requiring active immunization such as EAE (Gold et al., 2006) and EAN (Meyer zu Horste et al., 2007a) may thus not be applicable in DTx receptor transgenic mice. Our findings may also have relevance for other actively induced animal models of e.g. rheumatoid arthritis (Kannan et al., 2005). Adoptive transfer approaches may be required to circumvent this problem.

Mice receiving immunization in combination with DTx succumbed between days 7 and 9 after the application of DTx

(i.e. days 10 and 12 after immunization). This is comparable to the occurrence of DTx mortality in transgenic mice ubiquitously expressing the human DTx receptor (Cha et al., 2003), but earlier than mortality would be expected in severe experimental autoimmune disorders of the nervous system (Gold et al., 2006; Meyer zu Horste et al., 2007a). We also did not find any evidence of peripheral neuropathy in mice immunized against peripheral myelin protein arguing against DTx amplifying the immune activation during immunization. We rather speculate that active immunization increases DTx toxicity. We tested DTx from three different sources and two different batches of MerckDTx, which argues against a lot or production specific effect. We did not observe cardiac pathology, but mononuclear lung infiltration by mainly T cells, which may reflect septic reactions. We observed minor weight loss in animals receiving DTx only, although previous works did not describe any adverse effects of high DTx dosages in wildtype animals (Cha et al., 2003; Gropp et al., 2005). We thus cannot offer a conclusive mechanism for the rapid and complete lethality of DTx, which only occurred if combined with active immunization. Our observations are still highly relevant for planning future studies in experimental models of autoimmune disorders of the central and peripheral nervous system.

#### Competing interests

The authors declare that they have no competing interests.

#### Acknowledgement

We thank Achim Weber, Institute of Surgical Pathology, University of Zurich for critical discussions. The technical assistance of Tatjana Males and Bianca Wolff is gratefully acknowledged. This study was supported in part by a grant from the Forschungskommission of the Heinrich-Heine-University (to G.M.z.H.), the Fritz-Thyssen-Stiftung (to G.M.z.H.), and by the Deutsche Forschungsgemeinschaft (to H.W.).

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