Successful development of recombinant human diphtheria antitoxin: a project update

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Introduction

Diphtheria is a disease caused by toxigenic strains of diphtheria toxin-producing *Corynebacterium spp.* Vaccination prevents clinical development of the disease which is consequently rare in regions where immunization campaigns are in place. Regions with limited vaccine coverage nevertheless experience outbreaks that must be treated with diphtheria antitoxin (DAT), an equine serum product that carries the risk of causing adverse reactions including serum sickness or transmission of undetected infectious agents between species. Equine DAT is difficult to stockpile with frequent supply chain and distribution problems in addition to short shelf life.

At the 10th World Congress, we announced our project to develop human monoclonal antibodies that neutralize DT while avoiding the cross-species and shelf life problems associated with equine blood products. Here we report the successful development of those antibodies and our next steps toward the long term goal of replacing equine DAT with a recombinant antibody product of consistent identity that can be produced in cell culture.[1]

Methods and results

Antibody phage display is a validated approach that facilitates selection of sequence-defined DT-binding antibodies that can be manufactured under reproducible conditions in cell culture directly from human antibody gene libraries *in vitro.* Using two naïve human antibody libraries (HAL9 and HAL10) and two immune libraries created for this project (VJN and CD138+), 400 DT-binding antibodies were selected. Of this pool, Vero cell toxin neutralization tests (TNT) yielded 34 DT-neutralizers in IgG format.

Three of these antibodies were selected for additional Vero cell TNT testing, each targeting one of the three DT structural domains (catalytic (C), transmembrane (T), and receptor binding (R) domains), against increasing toxin concentrations. Individual antibodies lost neutralization capacity at higher DT doses, yet showed strong neutralizing activity when the antibodies were used in combinations of two or three.

Subsequently, the neutralization efficacy of these antibodies was assessed using a non-lethal guinea pig intradermal challenge assay based on the method described in the European Pharmacopoeia, confirming that these three antibodies in combination, both in pairs and triples, resulted in a clinically relevant neutralization potency (79 IU/mg, Table 1).

Table 1: Non-lethal *in vivo* neutralization at Lr/100 toxin dose level* for antibodies and antibody combinations

Antibody clone(s)	[IU/mg]
ewe375-D4 (anti R-domain)	<0.72
ewe375-H4 (anti C-domain)	<6.4
ewe372-F6 (anti T-domain)	<1.23
ewe375-D4 + ewe375-H4	79.4
ewe375-D4 + ewe372-F6	79.4
ewe375-H4 + ewe372-F6	79.4
ewe375-D4 + ewe375-H4 + ewe372-F6	79.4

*Lr/100 is the smallest amount of DT that causes a weak but detectable erythema in the presence of 0.01 IU of reference DAT antitoxin.

Discussion

Our results suggest that the neutralizing potency of these three antibodies in combination is comparable and likely superior to the results obtained for single monoclonal antibodies developed elsewhere. The use of antibody combinations that bind more than one domain on the target toxin may represent a robust therapeutic approach and are being explored as candidates for further regulatory and clinical development as a replacement for equine DAT.

Antibody phage display is an efficient and affordable technology that can be applied to the development of antibodies for use in therapeutic and diagnostic contexts. Based on the success of this project, the PETA International Science Consortium is funding similar research to develop recombinant human antibodies that neutralize black widow spider venom as a replacement for equine antivenom.

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[1]Wenzel EV, Bosnak M, Tierney R, Schubert M, Brown J, Dübel S, Efstratiou A, Sesardic D, Stickings P, Hust M. Human antibodies neutralizing diphtheria toxin in vitro and in vivo. Sci Rep. 2020; 10(571): doi:10.1038/s41598-019-57103-5

Introduction

- Eye irritation testing is conducted as part of the overall safety assessment of chemicals.
- While several in vitro and ex vivo methods can identify severe eye irritant and corrosive chemicals and chemicals that do not require hazard classification (i.e., “nonirritants”), no methods are available that can identify all eye irritation hazard categories.
- Results from prospective testing of agrochemicals using in vitro methods have reported discordant results relative to in vivo tests.
- Establishing confidence in new methods requires public-private partnerships that allow cross-sector communication and cooperation. PETA Science Consortium International, CropLife America companies, and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are collaborating to:
  - Assess the applicability of in vitro eye irritation/corrosion methods to agrochemical formulations.
  - Develop a defined testing approach for prediction of U.S. and international irritancy classifications.

Study Design

- Agrochemical formulations tested in the study were selected to:
  - Include a range of hazard classifications
  - Focus on common formulation types, including:
    - Suspension concentrates
    - Emulsifiable concentrates
    - Soluble liquid
  - Support comparisons to high-quality in vivo data
- Formulations were categorized using the EPA and GHS classification systems based on historical in vivo animal data.
- **Table 1** lists evaluated in vitro methods, applicable Organisation for Economic Co-operation and Development (OECD) test guidelines (TG), and laboratories that conducted the testing.

Conclusions and Future Directions

- No single test method agreed with the in vivo data classification for all tested formulations (Table 2 and 3).
- Combining multiple tests (e.g., BCOP and NRR, or BCOP and EO) in an integrated testing strategy may be useful in classifying these formulations.
- Additional testing with formulations identified as mild and moderate eye irritants are planned to further identify methods that may be complementary for hazard classification
- Efforts are also underway to:
  - Better understand the human relevance of each of the available alternative test methods
  - Establish how each method aligns with the mechanisms of human eye irritation and where gaps in test method coverage exist

Table 1. Evaluated In Vitro Methods

Test Method	OECD TG	Testing Laboratory
Bovine Corneal Opacity and Permeability (BCOP)	OECD TG 437 (2020)	Institute for In Vitro Sciences
BCOP – Extended Incubation Period*	-	Institute for In Vitro Sciences
Neutral Red Release (NRR)	-	Institute for In Vitro Sciences
Isolated Chicken Eye (ICE)	OECD TG 438 (2018)	Citoxlab
Porcine Cornea Reversibility Assay (PorCORA)	-	MB Research Labs
EpiOcular (EO) (EIT method)	OECD TG 492 (2019)	MatTek
EO (Time-to-toxicity method; ET50-neat protocol)	-	MatTek
EO (Time-to-toxicity method; ET50-dilution protocol)	-	MatTek

Table 2. Phase 1 In Vitro Classification Results Relative to In Vivo Classification Results

	Category IV/Category NC			Category I/Category 1		
	Formulation A	**Formulation B**	**Formulation C**	**Formulation D**	**Formulation E**	**Formulation F**
BCOP-OECD[1]	Concordant	Concordant	Concordant	Concordant	Discordant	Concordant
NRR[2]	Discordant	Concordant	Concordant	Concordant	Concordant	Concordant
ICE-OECD[3]	NPCBM	Concordant	NPCBM	Discordant	Discordant	Concordant
PorCORA[4]	NPCBM	NPCBM	NPCBM	Concordant	Concordant	NPCBM
EO-OECD[2]	Concordant	Concordant	Concordant	NPCBM	NPCBM	NPCBM
EO-neat ET50[5]	Concordant	Concordant	Concordant	Concordant	Discordant	Concordant
EO-dil. ET50[5]	Concordant	Concordant	Concordant	Discordant	Discordant	Concordant
EO-CON4EI[6]	Concordant	Concordant	Concordant	Discordant	Discordant	Concordant

[1]Classification based on most severe response obtained from IVIS or histopathology results.; [2]Classification based on most severe response obtained in two runs.; [3]Classification based on most severe response obtained from ICE score or histopathology results.; [4]Classification based on reversibility.; [5]Classification based on most severe response obtained in 2-3 runs.; [6]Classification presented in Kandarova et al. (2018). Mean of all runs used for decision tree calculations.

Table Abbreviations and Color Key

Abbreviations
CON4EI = Consortium for In Vitro Eye Irritation Testing Strategy Project
dil. = dilution protocol
ET50 = exposure time required to reduce tissue viability to 50%
Form. = formulation
NPCBM = no prediction can be made (see color/term key below).

Color/Term key
Green/Concordant = classification based on in vitro results are concordant with classification based on in vivo data
Red/Discord. = classification based on in vitro results are discordant with classification based on in vivo data
Orange/NPCBM = in vitro classification criteria does not allow for definitive classification of formulation (e.g., EO-OECD classification system indicates no classification prediction can be made when tissue viability ≤60%; therefore, formulations that produce this response cannot be classified).

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A summary of NICEATM and ICCVAM activities at the Eleventh World Congress is available on the National Toxicology Program website athttps://ntp.niehs.nih.gov/go/wc11.