

## Characterization of serum anti-diphtheria antibody activity following administration of equine anti-toxin for suspected diphtheria

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### ABSTRACT

There is a global shortage of equine-derived diphtheria anti-toxin (DAT) for diphtheria treatment. There are few existing data on serum antibody concentrations and neutralizing activity post-treatment to support development of new therapeutics. Antibody concentrations were quantified by ELISA and anti-toxin neutralizing activity by cytotoxicity assay in serum from 4 patients receiving DAT for suspected diphtheria. Using linear mixed effects modeling, estimated mean (SE) half-life was 78.2 (20.0) hours. Maximum serum neutralizing activity ranged from 28.42–38.64 AU/mL with an estimated mean AUC<sub>1–72</sub> of 1396.7 (399.3) AU/mL\*hr. These data provide a standard of comparison for development of novel anti-toxins to replace DAT.

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Diphtheria is a potentially fatal illness caused by infection with *Corynebacterium diphtheriae* and subsequent elaboration of potent exotoxin. Diphtheria toxin causes tissue death at the site of production, while hematogenous toxin dissemination can cause neuropathy and cardiotoxicity.<sup>1</sup> Despite successful implementation of immunization, diphtheria remains a global threat due to relocation of susceptible persons, travel exposures and disruptions in health care infrastructures.<sup>2,3</sup> Recent epidemics have been reported in Haiti, Nigeria, and Laos and are often associated with high case fatality rates (> 10%) in resource-limited countries.<sup>3–5</sup> Fatal cases also occur in developed countries among under-vaccinated populations.<sup>6</sup>

Morbidity and mortality due to diphtheria are greatly reduced by prompt administration of antibodies to neutralize toxin and prevent further tissue damage, in conjunction with antibiotics to eliminate *C. diphtheriae* and stop toxin production.<sup>1</sup> Treatment relies upon equine-derived diphtheria anti-toxin (DAT) that carries the risk of severe allergic reactions; there are currently no alternatives to this equine serum product. The global supply of DAT is extremely limited as most manufacturers have ceased production.<sup>3,7</sup> In the United States, the standard diphtheria treatment is receipt of an unlicensed DAT under an investigational new drug (IND) treatment protocol sponsored by the Centers for Disease Control and Prevention (CDC).<sup>8</sup>

New anti-toxins are urgently needed and human monoclonal antibodies to diphtheria toxin are under development.<sup>9–11</sup> A novel biologic must demonstrate comparable toxin neutralizing

activity to DAT standard of care, yet there are few existing data on the serum neutralizing activity achieved following DAT treatment doses. Current dosing recommendations are based on clinical presentation and duration of disease; there is no established minimum effective serum concentration.

Given that the supply of DAT is precarious to treat patients with suspected disease, a first-in-human trial with healthy volunteers to evaluate a novel diphtheria anti-toxin with a DAT comparator has feasibility and ethical constraints. To address the lack of clinical information regarding anti-toxin neutralizing activity following DAT treatment, we undertook a study to measure serum anti-toxin antibodies in patients receiving DAT for suspected diphtheria.

All study subjects were patients with suspected diphtheria receiving an intravenous infusion of DAT under the CDC-sponsored IND protocol “Use of Diphtheria Antitoxin (DAT) for Suspected Diphtheria Cases.” The CDC diphtheria duty officer provided the treating physician with the dose of DAT recommended in the treatment protocol based on disease location, severity, and duration.<sup>8,12</sup> At the time the informed consent was obtained for DAT treatment, information on an optional study for additional blood draws was provided. Separate, written informed consent for additional blood collection was obtained from each subject. The protocol and informed consents were approved by the CDC’s Investigational Review Board.

Serum samples were requested at 6 timepoints: pre-infusion, 1 hour post-infusion, 1 day post-infusion, 3 d post-infusion,

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7 d post-infusion, and 28 d post-infusion or at the time of hospital discharge (whichever was earlier). Blood was collected into serum separator tubes and sera stored frozen at  $\leq -20^{\circ}\text{C}$ . Additional information submitted with de-identified samples for analysis included subjects' sex, age, date and time of DAT administration and dose of DAT administered.

Enzyme-linked immunosorbent assay (ELISA) was performed to measure the concentration of equine antibody to diphtheria toxoid. Briefly, 96-well plates were coated with 100  $\mu\text{l}$  of diphtheria toxoid (MassBiologics) diluted to 1.0 limit of flocculation (Lf)/mL and incubated at  $2-8^{\circ}\text{C}$  for  $\geq 16$  hours. After washing to remove unbound antigen, 100  $\mu\text{L}$  of diluted serum sample was incubated for  $90 \pm 10$  minutes at  $20-25^{\circ}\text{C}$ . After washing, 100  $\mu\text{l}$  of alkaline phosphatase-conjugated goat anti-equine IgG (Jackson, Cat# 109-056-003) at a 1:2000 dilution was incubated for  $90 \pm 10$  minutes at  $20-25^{\circ}\text{C}$  then washed. Substrate solution (100  $\mu\text{l}$  para-Nitrophenylphosphate) was added for  $30 \pm 5$  minutes at ambient temperature. Following addition of stop solution, absorbance at 405 nm was determined using a Molecular Devices Versamax plate reader and SoftMax Pro software.

Serial dilutions of DAT standard obtained from the US Food and Drug Administration [FDA, CBER lot # F4509, 6 antitoxin units (AU)/mL] were used to generate a reference standard curve fit to a 4-parameter logistic regression. The readable range of the standard curve was defined as the points on the curve whose back-calculated values demonstrate precision across multiple independent assays. The concentration of equine anti-diphtheria toxoid antibodies (U/mL) was determined by interpolation from the standard curve. Three-fold serial dilutions of each sample were measured, starting with a 1:50 dilution; dilutions at which the concentration fell within the readable range of the assay were corrected for the dilution factor and averaged to obtain a reportable result. The lower limit of quantification (LLOQ) was 0.10 U/mL; samples with values below the LLOQ were reported as 0.05 U/mL. Samples with initial values above the readable range of the assay were diluted further to allow assessment within the assay's range.

Vero cell cytotoxicity assays were performed to measure anti-diphtheria toxin neutralizing activity. In a 96-well plate, 2-fold serial dilutions of each sample, starting with a 1:100 or 1:1000 dilution were mixed with  $5 \times 10^{-5}$  Lf/mL diphtheria toxin (Lot # C-K561-2010, MassBiologics) and incubated for 1 hour at  $20-25^{\circ}\text{C}$ . Vero cells (ATCC) were added as a  $4.0 \times 10^5$  cells/ml suspension and incubated at  $37^{\circ}\text{C}$  for  $72 \pm 1$  hours. Alamar Blue was added to assess cell viability and fluorescence detected at 590 nm using a Molecular Devices Spectramax plate reader and SoftMax Pro software.

The FDA DAT standard (lot# F4509) was used to generate a reference standard curve fit to a 4-parameter logistic regression and the readable range of the standard curve was defined as the points on the curve whose back-calculated values demonstrate precision across multiple independent assays. The diphtheria toxin neutralizing activity (AU/mL) was determined by interpolation from the standard curve; dilutions at which the concentration fell within the readable range of the assay were corrected for the dilution factor and averaged to obtain a reportable result. For each sample, 2-4 assays were performed with the mean value reported. The lower limit of quantification

was 0.01 AU/mL; samples with neutralizing activity below the LLOQ were reported as 0.005 AU/mL. Samples with initial values above the readable range of the assay were diluted further to allow assessment within the assay's range.

Pharmacokinetic analyses assumed first-order elimination with a logarithmic process ( $\log C_t = \log C_1 - k^*t$ ), where the  $C_t$  (concentration after time  $t$ ) and  $k$  (elimination rate constant) were estimated by the fitted intercepts and slopes in a linear mixed-effects model with restricted maximum likelihood using Vero cell cytotoxicity and ELISA data from all subjects. The half-life was calculated as half-life =  $\log_2/k$  and the partial area under the curve (pAUC) between 1 and 72 hours post-infusion was calculated as  $\text{AUC}_{1-72} = C_1 [\exp(-72k) - \exp(-k)] / k$ . The half-life and pAUC were calculated as the mean of the predicted values for each subject based on the fitted model, with the estimated standard errors accounting for the uncertainty associated with the small number of subjects. Actual hours of sample collection relative to start of DAT infusion were used in the calculations.

Four patients received DAT for suspected diphtheria between May 2014 and December 2015 and consented to have blood drawn before and after DAT administration. None of the patients had diphtheria infection confirmed by culture or PCR. The 2 male and 2 female subjects ranged in age from 35 to 74 y (Table 1). DAT is administered as a fixed dose; 3 subjects received a single 80,000 IU intravenous dose as recommended by the CDC diphtheria duty officer. A 100,000 IU dose was recommended for one subject (#124) who inadvertently received an initial dose of only 10,000 IU. An additional dose of 80,000 IU was administered 3 d later. For this subject, the serum samples obtained just before and after the 80,000 IU dose were used in the analyses. All subjects had a sample obtained pre-infusion and 3 of the 4 subjects had samples collected at the nominal 1 hour post-, 1 day post- and 3 d post-infusion. One subject had a sample collected on nominal day 7 post-infusion and 2 other subjects had samples collected on days 23 and 46, respectively post-infusion (Table 1).

Prior to DAT treatment, all 4 subjects had detectable diphtheria toxin neutralizing activity, presumably due to endogenous antibody from prior vaccination, whereas no subjects had binding antibody measured in the equine-antibody specific ELISA. Following DAT treatment, the maximum serum concentration ( $C_{\text{max}}$ ) measured one hour post-infusion was available for 3 subjects. The mean  $C_{\text{max}}$  of equine anti-diphtheria binding antibody was 19.48 U/mL (range 16.70-24.15) and the mean  $C_{\text{max}}$  of toxin neutralizing activity was 34.55 AU/mL (range 28.42-38.64). No subject had post-treatment serum values below the level of quantification in either assay (Table 1).

Given the sparse sampling available, we assumed ELISA and Vero cell measurements had different  $C_{\text{max}}$  but a common elimination rate and a linear mixed effects model with subject-specific random intercepts and slopes was used to estimate the pharmacokinetic parameters of equine DAT in the study population.<sup>13</sup> The primary analysis used combined ELISA and Vero cell assay results and their actual time of collection from all 4 subjects 1 hour to nominal day 28 post-infusion, as these were the collection timepoints specified in the protocol. The analysis yielded a mean (SE) elimination rate of  $-0.0114$  (0.0038) with a mean (SE) half-life estimate of 78.2 (20.0) hours (Table 2).

**Table 1.** Serum anti-diphtheria toxoid antibody concentrations and anti-toxin neutralizing activity in DAT-treated subjects with suspected diphtheria.

Subject ID # (Age/Sex)	Nominal Sample Time Point Post-Infusion (hrs)	Actual Sample Time Point Post-Infusion (hrs)	Equine anti-diphtheria toxoid IgG antibody concentrations <sup>a</sup> (U/mL)	Toxin neutralizing activity <sup>a</sup> (AU/mL)
122 (74 yrs/M)	0	-4.50	0.05	1.49
	1	1.00	17.60	28.42
	24	19.50	13.64	19.49
	72	58.58	7.79	17.68
			AUC <sub>1-72</sub> = 790.0 U*hr/mL	AUC <sub>1-72</sub> = 1426.0 AU*hr/mL
123 <sup>b</sup> (35 yrs/M)	0	-0.78	0.05	0.02
	72	77.62	4.57	6.22
	672	572.92	0.21	2.66
124 <sup>c</sup> (47 yrs/F)	0	-35.08	1.00	3.53
	1	1.17	16.70	36.59
	24	28.25	9.20	18.49
	72	97.75	3.60	7.95
	168	192.6	1.10	3.10
			AUC <sub>1-72</sub> = 669.6 U*hr/mL	AUC <sub>1-72</sub> = 1423.3 AU*hr/mL
125 (58 yrs/F)	0	-5.77	0.05	0.58
	1	1.00	24.15	38.64
	24	25.03	13.90	26.94
	672	1117.6	0.12	0.84
			AUC <sub>1-72</sub> = 1176.7 U*hr/mL	AUC <sub>1-72</sub> = 2127.7 AU*hr/mL

<sup>a</sup>Equine anti-diphtheria toxoid binding concentration was measured by ELISA and reported in units (U)/mL and diphtheria toxin neutralizing activity was measured by Vero cell cytotoxicity assay and reported in antitoxin units (AU)/mL based on CBER (FDA) standard diphtheria anti-toxin.

<sup>b</sup>Subject 123 had only 1 sample obtained in the first 72 hours post-infusion therefore AUC<sub>1-72</sub> could not be calculated.

<sup>c</sup>All subjects were treated with 80,000 IU of DAT except subject 124 who received a 10,000 IU dose of DAT, followed 3 d later by an 80,000 IU dose of DAT; the samples obtained following the 80,000 IU dose of DAT (shown in the table) were used in the pharmacokinetic analyses.

The half-life estimated was consistent with the half-life estimates calculated from individual subjects' ELISA and Vero cell assay results, which ranged from 48.9 to 209.6 hours for the 3 subjects with sufficient number of post-infusion samples to permit analysis. The area under the curve for the first 72 hours post-infusion (AUC<sub>1-72</sub>) was also estimated and the mean (SE) equine anti-diphtheria binding antibody AUC<sub>1-72</sub> was 619.3 (177.1) U\*hr/mL and the mean (SE) diphtheria toxin neutralizing activity AUC<sub>1-72</sub> was 1396.7 (399.3) AU/mL\*hr. We also performed a sensitivity analysis by including assay values from the one additional sample collected from one subject at day 46 post-infusion. This analysis increased the mean half-life estimate from 78.2 hours to 117.3 hours with a greater than 2-fold increase in standard error; a more modest increase was observed for the AUC<sub>1-72</sub> values (Table 2).

Equine-derived polyclonal diphtheria anti-toxin (DAT) is the current standard of care treatment of diphtheria; however there is a worldwide shortage of this product with no defined solution.<sup>3</sup> A logical replacement for DAT would be human monoclonal antibodies produced from DNA recombinant technology given the challenges associated with producing equine anti-toxin in an era where horses are not commonly maintained for production of biologics and the inherent risk of severe allergic reactions associated with equine-derived antibodies. A human monoclonal antibody against the receptor-binding domain of diphtheria toxin is under development and this approach overcomes the difficulties in achieving sufficient neutralizing potency in plasma from human donors without antigen-affinity purification.<sup>14</sup>

Development of a replacement product is hampered by lack of data on the serum neutralizing activity and pharmacokinetics of DAT treatment. While the minimum level of antibody considered protective against diphtheria infection in immunized individuals is generally regarded to be 0.01 IU/mL, much higher concentrations of antibody are required to effectively neutralize the toxin elaborated by *C. diphtheriae* once infection is established. In one of the few historical reports of serum antibody activity after treatment doses of DAT, peak levels in the range of 10–50 AU/mL were observed, varying with the dose of DAT administered and the number of doses.<sup>15</sup> Correlation of serum anti-toxin neutralizing activity units with mitigation of respiratory diphtheria or prevention of systemic complications is not known nor has a minimum effective dose of diphtheria anti-toxin therapy been established.<sup>16,17</sup> Therefore, an antibody therapy to replace equine-derived DAT will likely need to show comparable serological activity. Since it is challenging to assess comparability of epitope binding of polyclonal versus monoclonal antibodies in addition to source species differences by ELISA methodology, functional activity assessed in a cell-based toxin neutralization assay such as the Vero cell assay described in this report will likely be most useful. Equine anti-toxin products are assigned a minimum unit/mL potency relative to a reference standard using either the Vero cell assay (US Pharmacopoeia)<sup>18</sup> or an in vivo erythrodermic assay (European Pharmacopoeia)<sup>19</sup> before release for clinical use and this unit/mL potency is used to dose the product.

**Table 2.** Estimated antibody half-life and partial area under the curve (pAUC) from DAT-treated subjects using linear mixed effects model.

	Elimination rate Mean (Standard Error)	Half-life (hours) Mean (Standard Error)	Vero AUC <sub>1-72</sub> (AU*hr/mL) Mean (Standard Error)	ELISA AUC <sub>1-72</sub> (U*hr/mL) Mean (Standard Error)
Modeled on actual timepoints through day 28	-.0114 (0.0038)	78.2 (20.0)	1396.7 (399.3)	619.3 (177.1)
Modeled on all actual timepoints	-.0072 (0.0033)	117.3 (44.6)	1593.9 (471.5)	643.1 (190.3)

This laboratory study was designed to obtain serological data from patients administered DAT per current recommendations for treatment of suspected diphtheria in the United States. Not unexpectedly, given high rates of vaccine coverage in the United States, only 4 individuals were treated with DAT over an 18 month period of time at a dose (80,000 IU) recommended for treatment of systemic disease. A non-linear mixed effects model was used to accommodate the sparse sampling and estimate the pharmacokinetic parameters of the study population. We used the model to provide an estimate of the half-life of DAT, as well as the peak concentrations achieved and antibody drug exposure for the first 3 d post-infusion ( $AUC_{1-72}$ ), a crucial time for prevention of systemic toxin dissemination. The mean half-life observed (approximately 3 days) is consistent with the half-life reported for other equine polyclonal antibody preparations, such as botulism anti-toxin.<sup>20</sup>

A limitation of the study was that the small number of subjects and sparse sampling affects the precision of the estimated parameters. The estimated half-life result was more variable when the number of timepoints included in the model changed whereas the partial area under the curve parameter was a less sensitive, more robust parameter. Since none of the subjects receiving DAT for suspected diphtheria in this study ultimately had confirmed *C. diphtheriae* infection, these data cannot be used to analyze clinical response to treatment at a given serum concentration.

In summary, this report presents a pharmacokinetic characterization of serum antibody concentrations and toxin neutralizing activity observed with current standard of care treatment, equine-derived diphtheria anti-toxin. If additional data can be acquired from other patients treated for suspected diphtheria, the population pharmacokinetic profile of DAT could be further refined and used as a comparator for evaluations of investigational new therapies.

### Disclosure of potential conflicts of interest

HLS, GS, ML, and DCM are current or past employees of MassBiologics of the University of Massachusetts Medical School which is developing a human monoclonal antibody to diphtheria toxin.

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