INFLUENCE OF AERODYNAMIC PARTICLE SIZE ON BOTULINUM NEUROTOXIN POTENCY IN MICE

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ABSTRACT

For many agents the aerodynamic particle size can affect both the virulence and disease course in animal models. Botulinum neurotoxins (BoNTs), which are widely known as potential bioterrorism agents, are toxic via multiple routes of exposure, including small particle inhalation (1-3 μm MMAD). However, the impact of larger particle sizes on the potency of BoNT has not been previously reported. Outbred mice were exposed to BoNT-containing aerosols with differing mass median aerodynamic diameters (MMADs) of 1.1, 4.9, and 7.6 microns. Collecting aerosolized BoNT onto gelatin filters or into liquid impingers resulted in equivalent estimates of aerosol concentration. Nose-only and whole-body inhalation exposure resulted in nearly identical estimates of the median lethal dose (LD_{50}). The LD_{50} for inhaled BoNT increased approximately 50-fold when the median aerodynamic particle size was increased from 1.1 to 4.9 μm, from 139 (95% CI: 111-185) to 7,324 (95% CI: 4,287-10,891) mouse intraperitoneal median lethal doses ($MIPLD_{50}$).

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LIST OF ABBREVIATIONS

AGI	all glass impinger
APS	aerodynamic particle sizer
BoNT	botulinum neurotoxin
CI	confidence interval
GPB	gelatin phosphate buffer
GSD	geometric standard deviation
HEPA	high efficiency particle arresting
IACUC	institutional animal care and use committee
kHz	kilohertz
LD_{50}	median lethal dose
mg/m^3	milligrams per cubic meter
$MIPLD_{50}$	mouse intraperitoneal median lethal dose
MMAD	mass median aerodynamic diameter
MPA	mouse potency assay
µm	micron(s)

INTRODUCTION

Botulinum neurotoxins (BoNTs) are produced by the Gram-positive spore-forming bacterium *Clostridium botulinum* and related species. These proteinaceous toxins are the most potent toxins known and can cause fatal botulism via ingestion, inoculation into wounds, intestinal colonization, or inhalation of aerosols [Arnon 2001]. The Centers for Disease Control and Prevention (CDC) lists the BoNTs as Tier 1 Select Agents as they are recognized for their potential to pose a severe threat to public health and safety.

The majority of BoNTs are expressed by *Clostridium spp*. as a single inactive polypeptide of 150 kDa, which contains a di-sulfide linkage and is proteolytically cleaved into an active di-chain molecule. The two subunits, a 100 kDa heavy chain and a 50 kDa light chain, possess membrane binding/translocation and proteolytic activities, respectively. The heavy chain encodes specificity for receptor-mediated transcytosis that facilitates mobility through epithelial cells and into cholinergic nerve terminals, thereby delivering the light chain to the axon terminal. The light chain encodes strain-specific proteolytic activity for soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins, as all of the BoNTs exhibit their toxic effects by cleaving SNARE proteins from acetylcholine vesicles and neuronal membranes. This cleavage inhibits the release of acetylcholine at the neuromuscular junction, resulting in a potentially fatal flaccid paralysis [Rusnak 2009, Couesnon 2008].

There are no Food and Drug Administration (FDA) approved vaccines for botulism [Rusnak 2009]. While antitoxins have been developed to treat botulism, the currently available heptavalent botulinum antitoxin must be administered early for

clinical efficacy. Furthermore, the antitoxin does not reverse flaccid paralysis, and therefore many cases require intensive care including hospitalization and mechanical ventilation [O'horo 2017].

When considering BoNTs in the context of an inhalational exposure, it is important to note that the aerodynamic diameter of an infectious or toxic agent-containing aerosol particle is known to affect the quantity of agent required to infect or intoxicate a host [Druett 1953, Day 1972, Roy 2003]. Generally, as the aerodynamic diameter of agent-containing particles increases, the amount of agent required to cause infection or toxicity effects also increases. This observation has been demonstrated with the proteinaceous toxin ricin, where the median lethal dose (LD_{50}) of larger (> 3 μm aerodynamic diameter) aerosol particles containing ricin was determined to be at least four times higher than that of 1 μm particles in a murine model [Roy 2003]. Similarly, Druett et al. demonstrated that the inhalational LD_{50} of *B. anthracis* spores in guinea pigs and nonhuman primates significantly increased when the median aerodynamic diameter of the aerosol was increased from 1 to 12 μm, as did the pathology in the upper respiratory tract [Druett 1953]. The inhalational hazard of BoNT has been characterized in nonhuman primates and mice [Sanford 2010], but all of the prior studies utilized small particle aerosols with aerodynamic diameters < 3 μm. No data exist on the inhalational toxicity of larger particle aerosols containing BoNT. Because aerosol particle size distributions are unknown in biodefense planning scenarios, any medical countermeasures developed against BoNT must protect against inhalation of both small and large particle aerosols containing BoNT. Thus, it is important to understand the toxicity of both small and large particle sizes during vaccine development, and as the

Centers for Disease Control and Prevention ceased offering the investigational pentavalent (ABCDE) botulinum toxoid vaccination for at risk workers, the opportunity to test novel vaccines in appropriate aerosol models has been renewed [CDC *MMWR* 2011]. Therefore, the aim of the present study was to determine the influence of aerosol particle size on the toxicity of BoNT in a murine model of inhalational botulism.

MATERIALS AND METHODS

Animal Use

Female Hsd:ICR (CD-1) mice weighing 18-28 grams were utilized in this study (Envigo, Frederick, MD). All research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* [NRC 2010]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Toxin

Crude BoNT A1 (Hall) was purchased from Metabiologics Inc. (Madison, WI) and received at a concentration of 1.0 mg/mL with a reported activity of 1.5×10^7 $MIPLD_{50}$ per mL. Crude toxin was prepared from *C. botulinum* culture supernatants by acid precipitation and extraction with sodium phosphate. BoNT samples for aerosol generation and mouse potency testing were diluted in gel phosphate buffer (GPB; 33 mM sodium dihydrogen phosphate, 0.2% (w/v) gelatin, pH 6.2 ± 0.1; HiMedia Labs LLC).

Mouse Potency Assay

The quantity of active BoNT utilized for aerosol generation and sampled from the inhalation exposure system was quantified using a mouse potency assay (MPA). Gelatin filters were dissolved in warm GPB and impinger samples collected into GPB were further diluted in the same buffer. Six dilutions of each sample were prepared in GPB and 500 µl per mouse of each dilution was injected intraperitoneally into a group of seven mice. Mice were anesthetized with isoflurane gas and placed on an angled platform prior to injection,

with the head slightly toward the ground so that the head was lower than the hind end to allow the abdominal viscera to shift cranially and minimize the chance of accidental puncture of abdominal organs at site of injection. Following injection, the anesthetized mice were placed back in their cage and observed until they were fully recovered from anesthesia. Mice were subsequently monitored up to three times daily for five days post-injection and humanely euthanized when displaying signs of lethal intoxication that met specific criteria as defined on the IACUC approved clinical scoring sheet.

Inhalation Toxicity Testing

Exposure of mice to BoNT-containing aerosols was performed using a custom whole-body exposure system (Figure 1). A Collison 3-jet nebulizer (CH Technologies, Westwood, NJ) was used for small particle aerosol generation. Large and intermediate particle aerosols were generated with 60 kHz and 120 kHz ultrasonic aerosol generators (Sono-Tek, Milton, NY), respectively. For tests with mice, aerosols were sampled continuously from the breathing zone with 25 mm dissolvable gelatin filters and an Aerodynamic Particle Sizer (APS; Model 3321, TSI Inc.). A TSI model 3302A aerosol diluter configured with a 1:100 dilution nozzle was used in conjunction with the APS for small particle aerosol sizing. For aerosol exposures, mice were placed in individual exposure tubes in which they were able to move freely. Seven mice were exposed at a time in the whole-body exposure chamber. The inhalation exposure period was 10 minutes for all small particle exposures. The inhalation exposure period was increased to 11.5 and 50 minutes for large and intermediate particle sizes, respectively, in order to deliver higher inhaled doses of BoNT.

A set of exposures with small particle aerosols was conducted using a commercially available nose-only exposure chamber to compare results generated with the custom whole-body system. The nose-only exposure chamber utilized exposure tubes which restrain the mouse. Mice were acclimated to the nose-only exposure tubes on two separate occasions prior to exposure.

Additional tests were conducted to compare the relative sampling efficiency of the all glass impinger (AGI) at 6 liters per minute (lpm) and dissolvable gelatin filters at 1 lpm with BoNT-containing aerosols. In these tests, both devices sampled simultaneously from the whole-body exposure chamber and the aerosol concentration measured by each was determined using a MPA, as described above.

Final mortality data was utilized to estimate the dilution of the liquid from the aerosol sampler that resulted in 50% mortality and the $MIPLD_{50}$ was determined using probit analysis in JMP (v. 11.2.0, SAS Institute Inc., Cary, NC). The total number of $MIPLD_{50}$s present in a sample were utilized to calculate the inhaled dose for each group. The inhaled dose (D_{inh}), in $MIPLD_{50}$ was defined as the amount of BoNT inhaled per mouse and was calculated as the product of the BoNT aerosol concentration in the breathing zone of the subject during the exposure period (C_a) and the respiratory volume (V_r), defined as the volume of air inhaled over the course of the exposure period (Equation 1). The average aerosol concentration in the breathing zone of the subject during the exposure period (C_a) was calculated as the ratio of the product of the BoNT concentration recovered from the gelatin filter utilized during the exposure (C_s; in $MIPLD_{50}$/mL) and the dissolution volume (V_s; in mL) to the product of the sampler flow (Q_s; in mL/min) and the sampling time (t; in min) (Equation 2). Two previously published studies provide significantly different

estimates of the mouse respiratory volume [Guyton 1947, Flandre 2003]. While the study by Flandre et al. (2003) is more recent and utilized modern real-time plethysmography methodologies, the study by Guyton (1947) is still utilized in much of the biodefense community. Therefore, two different estimates of inhaled dose are presented, with each one utilizing a different estimate for the mouse respiratory minute volume (V_r).

$$D_{inh} = C_a \cdot V_r$$

Equation 1. Inhaled dose.

$$C_a = \frac{C_s \cdot V_s}{Q_s \cdot t}$$

Equation 2. Aerosol concentration.

Final dose–mortality data for mice exposed to either large or small particle aerosols containing BoNT were utilized to estimate the inhalational median lethal dose (LD_{50}) and associated 95% confidence intervals using probit analysis in JMP (v. 11.2.0, SAS Institute Inc., Cary, NC) as has been described previously [Druett 1953, Day 1972, Cutler 2011]. Particle size statistics, specifically the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD), were calculated from APS data using Aerosol Instrument Manager (V. 8.1.0.0, TSI, Inc.).

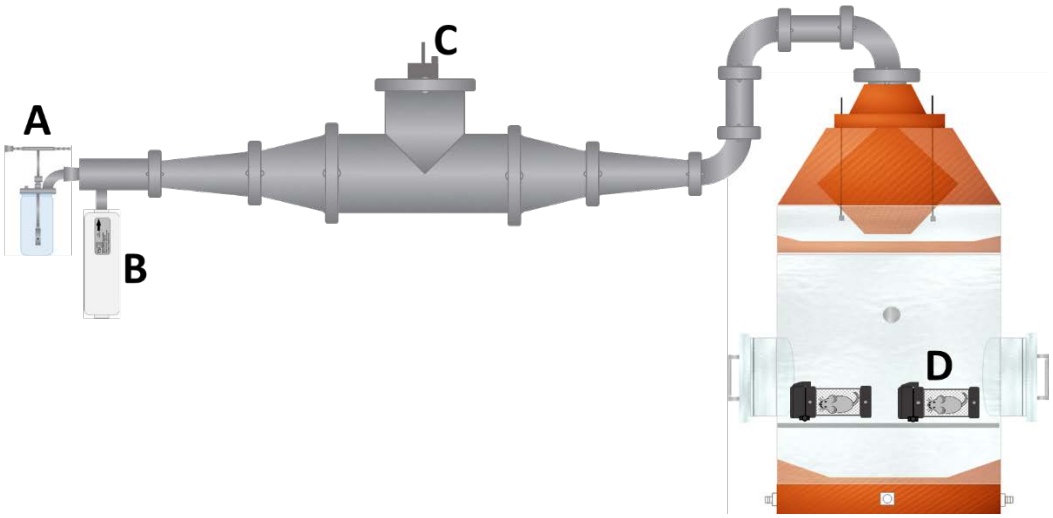

Figure 1: Whole-body mouse exposure system. Aerosols were generated with a Collison 3-jet nebulizer (A) for exposures involving small particle aerosols. A HEPA-filtered passive air inlet (B) provided additional clean airflow into the system. Intermediate and large particle aerosols were generated with Sono-Tek 120 kHz and 60 kHz ultrasonic spray nozzles (C), respectively. Mice were exposed in single mouse tubes located in the exposure chamber (D).

RESULTS

Following inhalation exposure or intraperitoneal injection of BoNT, some mice displayed changes in appearance indicative of intoxication included ruffled fur, hunched posture and piloerection. Changes in natural behavior included isolation, lethargy, increased vocalization, self-mutilation, immobility, and subdued responses when provoked. Lethally intoxicated mice were humanely euthanized once endpoint criteria were met. The aerodynamic particle size was monitored continuously from the breathing zone of the mouse exposure chamber during each exposure. Particle size statistics by nominal size class are presented in Table 1. An overlay of the small and intermediate particle sizes illustrates the discrete particle size distributions in Figure 2.

Table 1. Particle size distributions of BoNT aerosols. Values are arithmetic mean ± standard deviation.

Exposure System	Particle Size Class	MMAD (μm)	GSD
Whole Body	Small	1.1 ± 0.06	1.6 ± 0.08
	Intermediate	4.9 ± 0.11	1.4 ± 0.01
	Large	7.6 ± 0.28	1.4 ± 0.04
Nose Only	Small	1.1 ± 0.04	2.2 ± 0.09

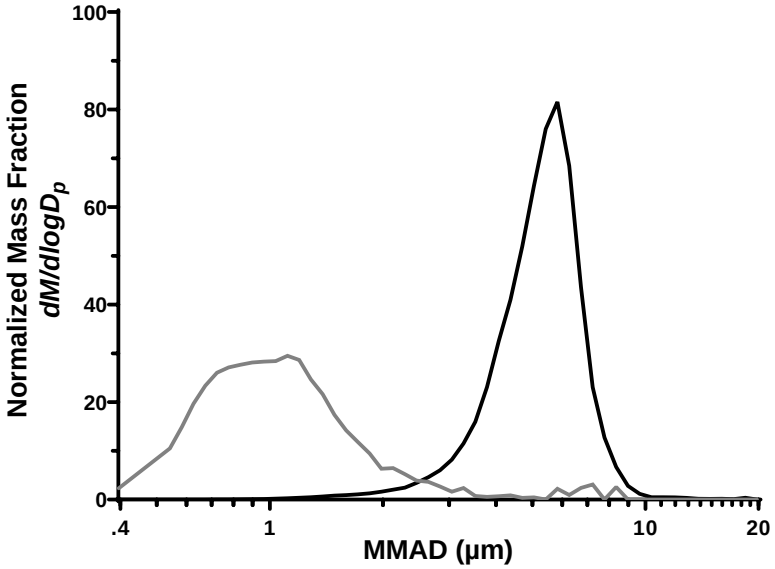

Figure 2: Particle size distributions of small (1.1 µm, grey) and intermediate (4.9 µm, black) BoNT-containing aerosols.

Small Particle LD_{50} Estimation

For mice exposed using the whole-body exposure system, the inhaled doses calculated using Guyton's estimate of respiratory minute volume and survival data were used to estimate an inhalational LD_{50} of 139 $MIPLD_{50}$ with 95% confidence intervals of 111 – 185 $MIPLD_{50}$. In contrast, the inhalational LD_{50} calculated using Flandre's estimate of respiratory minute volume and survival data was 405 $MIPLD_{50}$ with 95% confidence intervals of 324 – 538 $MIPLD_{50}$. A summary of inhaled doses and survival results for small particle whole-body inhalation exposures is presented in Table 2.

Table 2. Small particle (1.1 µm MMAD) whole-body inhalation dosimetry and survival summary. Two different estimates of inhaled dose are presented, each based on a different published estimate of murine respiratory minute volume, and labeled as either

Guyton or Flandre in reference to the first author of the relevant publication. The 95% CIs for the Guyton- and Flandre-based estimates of inhaled dose were 111-185 $MIPLD_{50}$ and 324-538 $MIPLD_{50}$, respectively.

Inhaled dose ($MIPLD_{50}$)		Number exposed	Number succumbed	Inhalation LD_{50} ($MIPLD_{50}$)	
Guyton	**Flandre**			**Guyton**	**Flandre**
84.1	245.1	7	0	139	405
97.2	283.0	7	0		
125.9	366.6	7	4		
131.1	382.0	7	6		
236.7	689.4	7	5		
470.3	1,369.9	7	7		

For mice exposed using the nose-only system, the inhaled doses calculated using Guyton's estimate of respiratory minute volume and survival data were used to estimate an inhalation LD_{50} of 133 $MIPLD_{50}$. In contrast, the inhalational LD_{50} calculated using Flandre's estimate of respiratory minute volume and survival data was 385 $MIPLD_{50}$. Confidence intervals for both estimates were incalculable in JMP. A summary of inhaled doses and survival results for small particle nose-only inhalation exposures is presented in Table 3.

Table 3. Small particle (1.1 µm MMAD) nose-only inhalation dosimetry and survival summary. Two different estimates of inhaled dose are presented, each based on a different published estimate of murine respiratory minute volume, and labeled as either Guyton or Flandre in reference to the first author of the relevant publication. Confidence intervals were incalculable in JMP.

Inhaled dose ($MIPLD_{50}$)		Number exposed	Number succumbed	Inhalation LD_{50} ($MIPLD_{50}$)	
Guyton	**Flandre**			**Guyton**	**Flandre**
6.2	18.2	7	0	133	385
17.2	50.2	7	0		
57.9	168.6	7	0		
96.5	281.0	7	0		
135.8	395.6	7	4		
210.6	613.3	7	7		

Intermediate and Large Particle LD_{50} Estimation

Three groups of mice were challenged with aerosols with a mean MMAD of 7.6 µm containing BoNT, and none of the mice were lethally intoxicated. The highest achieved inhaled dose of 884 $MIPLD_{50}$, estimated using Guyton's estimate of respiratory volume, was 4.8 times greater than the upper 95% confidence interval calculated from the small particle LD_{50} estimated in the whole body exposure chamber.

For mice exposed to aerosols with a mean MMAD of 4.9 µm using the whole-body exposure system, the inhaled doses calculated using Guyton's estimate of respiratory minute volume and survival data were used to estimate an inhalational LD_{50} of 7,324 $MIPLD_{50}$ with 95% confidence intervals of 4,287 – 10,891 $MIPLD_{50}$ (Table 4). In contrast, the inhalational LD_{50} calculated using Flandre's estimate of respiratory minute volume and survival data was 21,973 $MIPLD_{50}$ with 95% confidence intervals of 13,399-33,685 $MIPLD_{50}$. A summary of inhaled doses and survival results for intermediate particle whole-body inhalation exposures is presented in Table 4. A comparison of the dose-lethality relationships for aerosols with a mean MMAD of 1.1 µm and 4.9 µm are shown in Figure 3.

Table 4. Intermediate particle (4.9 µm MMAD) whole-body inhalation dosimetry and survival. Two different estimates of inhaled dose are presented, each based on a different published estimate of murine respiratory minute volume, and labeled as either Guyton or Flandre in reference to the first author of the relevant publication. The 95% CIs for the Guyton and Flandre-based estimates of inhaled dose were 4,287 – 10,891 $MIPLD_{50}$ and 13,399-33,685 $MIPLD_{50}$, respectively.

Inhaled dose ($MIPLD_{50}$)		**Number exposed**	**Number succumbed**	**Inhalation LD_{50} ($MIPLD_{50}$)**	
Guyton	**Flandre**			**Guyton**	**Flandre**
470.4	1,370.0	7	0	7,324	21,973
2,660.8	7,749.7	7	0		
6,098.3	17,761.3	7	2		
11,314.7	32,954.3	7	6		

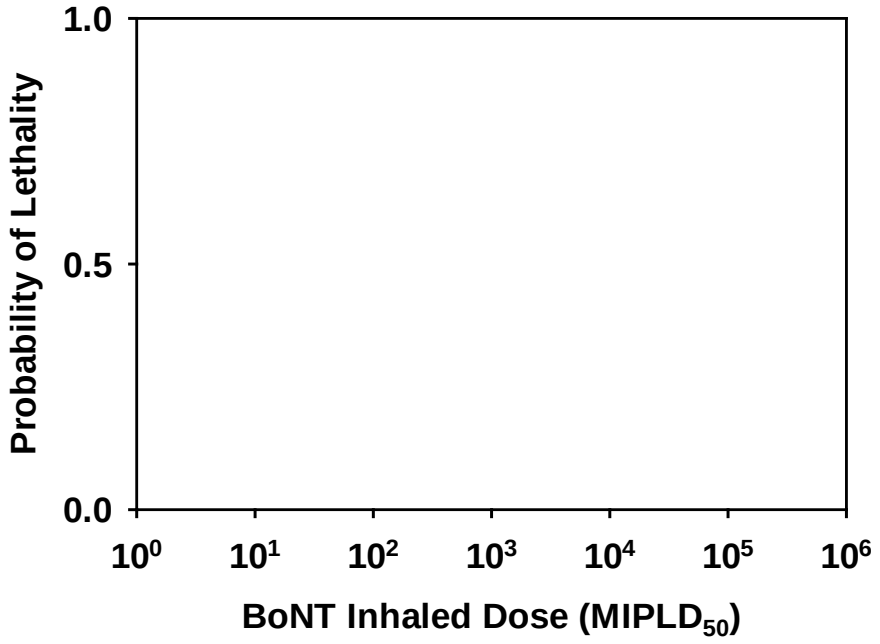

Figure 3. Dose-response for mice exposed to 1.1 µm (left) or 4.9 µm (right) BoNT aerosols in a whole-body exposure system. Guyton's estimate of respiratory minute volume was used to estimate inhaled dose. The inhalation LD_{50} increased 53-fold from 139 to 7,324 $MIPLD_{50}$ as particle size was increased. Dashed lines represent 95% confidence intervals.

Time to Death

The time to death of all aerosol exposed mice that were lethally intoxicated was recorded, and summary statistics are shown in Table 5. It should be noted that animal observations were conducted three times daily, and, therefore, the resolution on the lethality data was 8 hours. No significant differences were observed in the median time to death for the different aerosol particle size exposure groups. Animals exposed to small particle aerosols in the nose-only and whole-body exposure systems had equivalent

median times to death of 46 hours, with a range of 22 – 82 hours. Animals exposed to intermediate particle aerosols also had a median time to death of 46 hours, with a range of 22-70 hours.

Table 5. Median time(s) to death following inhalation exposure to BoNT A1.

Exposure System Type	Particle Size (MMAD)	Median Time to Death (Hours)	Range (Hours)
Nose Only	1.1 µm	46	28-82
Whole Body	1.1 µm	46	22-82
	4.9 µm	46	22-70
	7.6 µm	NA†	NA†

†NA – not applicable as no large particle exposed mice succumbed to intoxication.

Aerosol Sampler Comparison

To compare the relative sampling efficiency of the AGI and the dissolvable gelatin filter, both samplers were connected to the whole-body exposure system to sample aerosol simultaneously without mice present. Both the AGIs and gelatin filters measured similar concentrations of aerosolized BoNT (Figure 4 P=0.0614 when compared using a paired t-test).

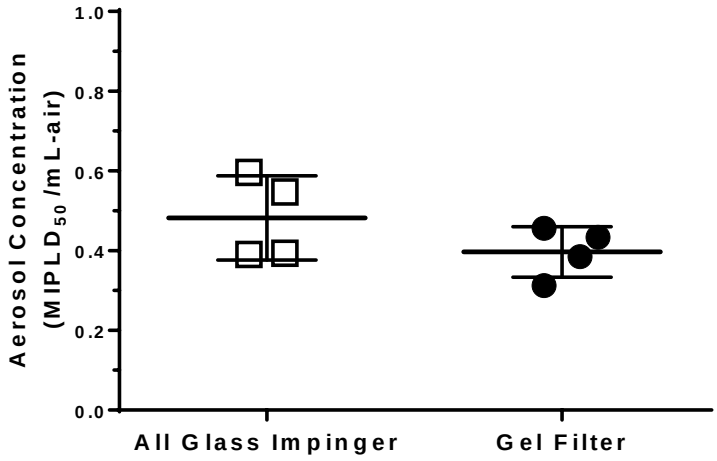

Figure 4. Aerosol sampler comparison. The mean aerosol concentration of small particle BoNT was sampled simultaneously with an AGI and a gelatin filter on four occasions. The efficiency of the samplers for sampling BoNT aerosols were not significantly different via a paired t-test (P=0.0614). Error bars represent one standard deviation from the mean.

DISCUSSION

Previous studies examining the toxicity of inhaled BoNT have utilized small particle aerosols, with MMADs of 1-3 µm. This study assessed the toxicity of inhaled BoNT-containing aerosols with differing MMADs, specifically 1.1 µm, 4.9 µm, and 7.6 µm, in a murine model. The results demonstrate that the lethality of aerosolized BoNT is inversely related to aerodynamic particle size, with larger particle sizes requiring greater amounts of toxin to produce similar proportional lethality in mice. The estimated LD_{50} for mice exposed to an aerosol with an MMAD of 4.9 µm in a whole body exposure system was 7,324 $MIPLD_{50}$, which was 53-fold higher than the inhalation LD_{50} determined for mice exposed to an aerosol with an MMAD of 1.1 µm in the same exposure system. Exposure of mice to an aerosol with an MMAD of 7.6 µm at a dose almost five-fold greater than the upper 95% confidence interval for the small particle LD_{50} did not result in lethal intoxication in any of the exposed animals, again demonstrating that a significantly greater quantity of toxin is required to result in lethal intoxication relative to small particle aerosols. Additional groups utilizing higher doses of toxin were not possible at the 7.6 µm size, as this would have required significantly more toxin to be acquired. The observation that the median lethal doses increases as a function of aerodynamic diameter is in agreement with previous studies with the toxin ricin, as well as various other microorganisms [Druett 1953, Day 1972, Roy 2003]. The large particle exposure model developed in the present study may also be useful for screening the efficacy of potential medical countermeasures against a range of relevant particle sizes. In addition, these data will be useful to inform modeling of the inhalational hazard of aerosolized BoNT in biodefense planning scenarios where large particle aerosols are

potentially relevant. Modeling scenarios with larger aerosol particle sizes would result in lower rates of infection or intoxication and a protracted disease course as has been observed in models of tularemia, plague, anthrax, and melioidosis. Generally, modeling these scenarios would indicate that lesser amounts of medical countermeasures would be required, but caution must be applied as the disease may present greater upper respiratory tract pathogenicity [Thomas 2013]. As the present study utilized a murine model, it is first necessary to determine the most appropriate methodology for extrapolation of these data to humans prior to its utilization in modeling.

The aerodynamic diameter of an inhaled particle is a known determinant of regional deposition in the respiratory tract. As aerodynamic diameter increases, the probability of deposition in the upper respiratory tract increases. Generally, toxin that reaches the peripheral lung would be expected to be absorbed directly into the bloodstream and cause systemic intoxication, while the majority of toxin deposited in the upper respiratory tract would be expected to ultimately be ingested. A previous study in mice quantified the deposition of radiolabeled aerosol particles using single-photon emission computed tomography (SPECT-CT) and demonstrated that 30-fold less mass deposits in the lungs for particles with an MMAD of 5.0 μm relative to those with an MMAD of 1.0 μm [Kuehl 2012]. In the present study, it was found that the LD_{50} for an aerosol with an MMAD of 4.9 μm was 53-fold greater than that of an aerosol with an MMAD of 1.1 μm. This difference in the LD_{50}'s is of the same order of magnitude as that observed for pulmonary deposition of similar particle sizes. Therefore, it is possible that the increased LD_{50} for the 4.9 μm group may be primarily due to decreased deposition in the peripheral lungs. Toxin deposited in the upper respiratory tract, which is subsequently

cleared to the gastrointestinal tract, has less toxicological effects than toxin deposited in the lungs as the oral LD_{50} has been estimated to be 100 times greater than the inhalational LD_{50} [Arnon 2011]. The times to death/euthanasia were similar for mice exposed to particle sizes with an MMAD of either 1.1 or 4.9 µm, which would be expected if the toxicity of inhaled BoNT was primarily due to toxin deposition in the peripheral lungs.

To determine if exposure modality influenced potency of small particle BoNT aerosols, whole-body and nose-only exposure systems were compared. Some researchers have observed mixed results in these two types of exposure systems based upon the aerosolized agent, [Stephenson 1988, Hu 2015] while others have found similar results with both modalities [Yeh 1990]. The current study found the inhalation LD_{50}'s estimated for the nose-only and whole-body exposure systems were similar for aerosols with an MMAD of 1.1 µm – 133 and 139 $MIPLD_{50}$, respectively. These data suggest that differences in the restraint method between the two systems did not significantly affect the toxicity estimates for inhaled toxin. Further, the results suggest that ingestion of toxin deposited on fur during whole-body exposure as a result of grooming post-exposure does not significantly contribute to the observed systemic toxicity. Serotype differences in nerve terminal binding, endocytic internalization, translocation of the light chain across the vesicle membrane, and cleavage of SNARE proteins have been demonstrated [Pirazinni 2017], and these factors are likely to have more significant impact on observed variations in LD_{50} values than exposure modality.

Selection of an appropriate aerosol sampler can influence the estimation of aerosol concentration, and some devices display bias for certain particle size fractions. Researchers have sought to determine the most suitable bioaerosol sampler for their

specific agent and system, such as the work performed by Fabian et al. (2009) wherein the authors demonstrated a several-fold increase in the recovery of infectious virus with the SKC biosampler as compared a compact cascade impactor, Teflon filters, and gelatin filters. Such differences in the efficiency of sampling devices have the potential to significantly affect estimates of inhaled dose in inhalation studies, as well as any estimates of risk derived from such doses. In the present study, the performance of the AGI and gelatin filters were compared for sampling small particles aerosols containing BoNT. The results demonstrate that the two sampler types performed equivalently, and therefore, dose estimates determined using either sampler type can be directly compared for small particle aerosol. However, the present study only evaluated the sampling efficiency of small particle aerosols and differences in efficiencies may be observed with larger particle sizes.

The present study provides novel data demonstrating that aerodynamic particle size is a significant determinant of the lethality of inhaled BoNT. While it is reasonable to assume that this generalized finding applies to other species, including humans, care should be taken when extrapolating the absolute LD_{50} values presented here to human risk assessments, as a number of factors have the potential to influence such an extrapolation, including species differences in respiratory tract physiology, and toxicokinetics post-absorption. A limited assessment of the shift in the LD_{50} as a function of aerosol particle size on toxicity of inhaled BoNT in a large animal model, such as nonhuman primates, would provide additional data to inform selection of appropriate values for use in human risk assessment, and biodefense preparedness planning. The large particle exposure model developed in the present study may also be useful for

screening the efficacy of potential medical countermeasures. Future studies examining the influence of BoNT serotype on the magnitude of the increase in LD_{50} as a function of aerosol particle size are needed to more thoroughly characterize the hazard posed by aerosolized BoNT.

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