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ORIGINAL ARTICLE

A COMPARISON OF THE SENSITIVITYOF DIFFERENT STRAINS OF MICE TO SARIN

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Summary

Poisoning from chemical warfare agents (CWAs) such as sarin is associated with neuronal degeneration. This damage is thought to result from glutamatergic excitotoxicity such as seen following kainic acid induced seizures. In order to search for novel neuroprotectants it is necessary to select good mouse models for susceptibility to nerve agent-induced seizures and the resulting neurodegeneration. The mouse strains tested (C57BL/6, ICR, DBA/2, SW, and FVB/N, Harlan Laboratory) had widely different sensitivity to sarin as shown by differences in the dose required resulting in 50% mortality, LD_{50}. Differences also were observed among the strains in Fluoro-Jade C staining with the C57BL/6 and DBA having little to no staining when euthanized at 7 days whereas the other strains did. Differences in acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activity were found among the strains as well. The ICR strain was excluded from the FOB and weight data due to difficulty getting a consistent LD_{50}. Weight loss and FOB scores were similar for all strains. All strains had inhibited AChE activity after sarin exposure and exhibited inhibition of CNS BuChE after sarin exposure but only ICR and SW reached significance.

Key words: Mouse models; sarin; acetylcholinesterase inhibitors; strain differences; neuropathology; organophosphate

INTRODUCTION

Poisoning from chemical warfare agents such as sarin causes prolonged seizure activity and is associated with neuronal degeneration within the central nervous system [1]. Sarin inhibits acetylcholinesterase (AChE) to increase synaptic levels of acetylcholine. Seizure activity is believed to begin due to the excessive muscarinic acetylcholine receptor activation. The seizures then transition to a mixture of cholingeric and noncholinergic components before becoming noncholinergic in the final stage. After 40 minutes, the status epilepticus seizure activity is assumed to result from the excessive amounts of glutamate released by the affected neurons [2].

Due to the role of glutamate in the development of status epilepticus, it was assumed that the postsynaptic

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sensitivity to glutamatergic agonists would provide guidance to the relative sensitivity of the mice to sarin-induced neurodegeneration. Studies with the glutamate agonist kainic acid (KA) revealed strain differences in seizure morphology and vulnerability to excitotoxins [3;4]. The strains tested by McLin et al, [4] demonstrated differences in hippocampal degeneration after KA administration as measured by Fluoro-Jade B staining. The FVB/N, DBA, and ICR strains were susceptible while the C57BL/6 (C57) was resistant. Swiss Webster (SW) mice were excluded due to the inability to find a dose that produced tonic-clonic seizures without causing death [5].

In order to search for novel neuroprotectants for treatment of chemical nerve agent poisoning, it is necessary to determine which mouse strain exhibits neuronal degeneration at relatively low dose of sarin. We determined the LD_{50} for sarin in five strains of mice. Initial doses of sarin were intended to be based on their relative susceptibility to KA-induced seizures across strains [5]. Using C57 mice as a baseline, a strain rated as moderately sensitive to glutamate by McLin and Steward [5], doses were calculated up or down to determine the LD_{50} of sarin as 200 µg/kg. Initial estimates of LD_{50} doses in other strains based on their sensitivity to KA and extrapolated to sarin proved inaccurate. Therefore, dose-response curves for sarin were determined to establish the approximate LD_{50} for each strain. Fluoro-Jade C staining of each strain was used as an indicator of neuronal injury.

EXPERIMENTAL PROCEDURE

Animals

Male C57BL/6 (C57), ICR CD-1 (ICR), DBA/2 (DBA), ND4 Swiss Webster (SW), and FVB/N mice were obtained from Harlan Laboratories (Indianapolis, IN) and were received for use at weights of 20-25 g, aged 2-3 months. Mice were maintained under a 12h light:12h dark cycle and housed in individual cages. A standard rodent pellet diet and water were available *ad libitum*. All mice were allowed 7 days of acclimation to facilities and subjected to at least three days of handling before dosing. All procedures were approved by the Laboratory Animal Care and Use Committee of Wright State University, Dayton, OH and reviewed by a DoD veterinarian.

Treatment

Sarin (USAMRICD, Aberdeen Proving Ground, MD) was diluted with 0.9% saline with an injection volume of 0.5ml/100g. Mice were injected subcutaneously with higher or lower doses as appropriate determine the dose response curve. Dose response curves were constructed based on mortality. Controls received an injection of saline only. Animals were euthanized after 7 days for collection of blood and tissue. Previously published work reported positive Fluoro-Jade B staining up to 30 days post soman exposure [6]. We therefore choose the encompassing 7 day time point for the sarin exposures.

Functional observational battery

A functional observational battery (FOB) was performed using the method of Garrett et al [7] to determine the severity of poisoning, including seizure activity. Animals were observed every 15 minutes for an hour post-injection. The animals were monitored for five different sympathetic and motor measures including posture, motor behavior, gait, eyelid closure, and breathing depth. After the monitoring period the ani-mals were placed back on the cage rack and monitored for morbidity. The scores range from 6 for a normal animal to 21 for severe poisoning symptoms (seizure activity). For each category the animal was given a score from 1 being non-symptomatic to 4 being severe symptoms and a sixth category of sur-vival was scored as alive (1) or dead (2). All categories were then summed. The maximum score of the ani-mal over the hour was divided by 21 (the maximum score achievable) and multiplied by 100 to get the percent score. Higher percentages indicate an increase in signs and toxicity.

Animal weights

Animals were weighed prior to injection, baseline weight (BW), and for three days post injection, post-exposure weight (PW). If an animal lost more than 25% of its baseline weight it was euthanized. Percent change in weight was determined by subtracting BW from PW and dividing by BW and multiplying by 100: (((PW-BW)/BW)*100).

Histology

Mice were sacrificed seven days after exposure. Brains were removed, flash frozen in isopentane and stored at -80°C until sectioned. Frozen 10 µm sections were collected between -1 to -2.92 Bregma

(coordinates from[8]) and stained for Fluoro-Jade C (Millipore, Temecula, CA) using the method of Schmued et al, [9]. Tissue sections were collected on gelatin coated slides. The sections were immersed in a solution consisting of 1% sodium hydroxide and 80% ethanol for 5 minutes, then in 70% ethanol for 2 minutes, and distilled water for 2 minutes. The slides were then incubated in a 0.06% potassium permanganate solution for 10 minutes. A 0.0001% solution of Fluoro-Jade C solution made in 0.1% acetic acid was left on for 10 minutes. The slides were rinsed with distilled water, and placed on the slide warmer at 50°C for 5 minutes. Prior to coverslipping with Permount mounting medium (Fisher Scientific, Pittsburgh, PA), the slides were cleared with xylene for one minute. Slides were examined using a Leica microscope fitted with an Optronics camera. Neuronal degeneration was searched for by examining each brain section.

Cholinesterase activity

Frontal Cortex tissue was assayed for AChE and butyrylcholinesterase (BuChE) using a modified Ellman method [10]. The tissue was stored at -80 ºC until the assay was completed. Prior to the assay, the tissue was homogenized in 0.1M $NaPO_4$ with a pH of 7.4 and 0.5% Tween-20, then centrifuged at 16.2K g at 4 ºC for five minutes. Half of each sample was inhibited with 10mM iso-OMPA (tetraisopropyl-pyrophosphoramide) and incubated for an hour to determine the AChE activity. Acetylthiocholine was used as a substrate and dithionitrobenzoate to achieve a color change. Cholinesterase (ChE) activity was determined using the uninhibited sample in triplicate using a Biotek™ EL808 Microplate Analyzer. A Bradford protein assay was performed to determine protein concentration. BuChE activity was calculated by subtracting AChE activity from ChE activity. Results were reported as nmol/microgram protein/min.

Trunk blood was collected in heparinized tubes at the time of euthanasia, stored on ice and assayed the same day. Fresh whole blood was diluted 1:100 with 0.1M $NaPO_4$ with a pH of 7.4 buffer and stored at 25°C. AChE levels were determined by inhibition of BuChE using iso-OMPA. BuChE activity was calculated by subtracting AChE activity from the total ChE activity in the blood samples. Results were reported as nmol/µl/ min.

Statistical analysis

All statistics were performed using Statistica, version 7.0. FOB scores were analyzed using a Kruskal-Wallis ANOVA. Weight loss data was analyzed using repeated measures ANOVA. AChE and BuChE data were analyzed using a one-way or two-way ANOVA followed by a Fisher LSD post-hoc test. Significance was determined using $p<0.05$. Statistics could not be performed on the dose response curve data to determine the statistical LD_{50} due to the steepness of the dose response curve, which at most was 0.21 log steps between zero and 100 percent mortality. Doses reported are those closest to the LD_{50}. The bars on the figures are mean (M) with error bars representing standard error of the mean (SEM).

RESULTS

Dose response, mortality, and histology

The C57 strain had the lowest LD_{50} (195 µg/kg), followed by DBA (260 µg/kg), SW (320 µg/kg), FVB/N (360 µg/kg) and undetermined ICR (<300 µg/kg, table 1). The SW, FVB/N, and ICR strains were variable in the dose-dependent percent mortality. The FVB/N strain was administered five doses of sarin ranging from 260 to 380 µg/kg. They had 17% mortality at the dose of 280 µg/kg,

Table 1. Mouse strain differences in LD_{50} dose of sarin and Fluoro-Jade C staining compared to sensitivity to KA. *Note*. Sensitivity to KA adapted from McLin et al. (2006).

Mouse Strain	N	LD_{50} Sarin Dose	Sensitivity to KA by Dose	FJ-C Staining	Negative Controls	ChE Data
C57	4	195 µg/kg	moderate	no	9	4
DBA	4	260 µg/kg	low	no	-	4
SW	6	320 µg/kg	N/A	yes	6	6
FVB/N	4	360 µg/kg	high	yes	4	4
ICR	4	<300 µg/kg	high	yes	6	4

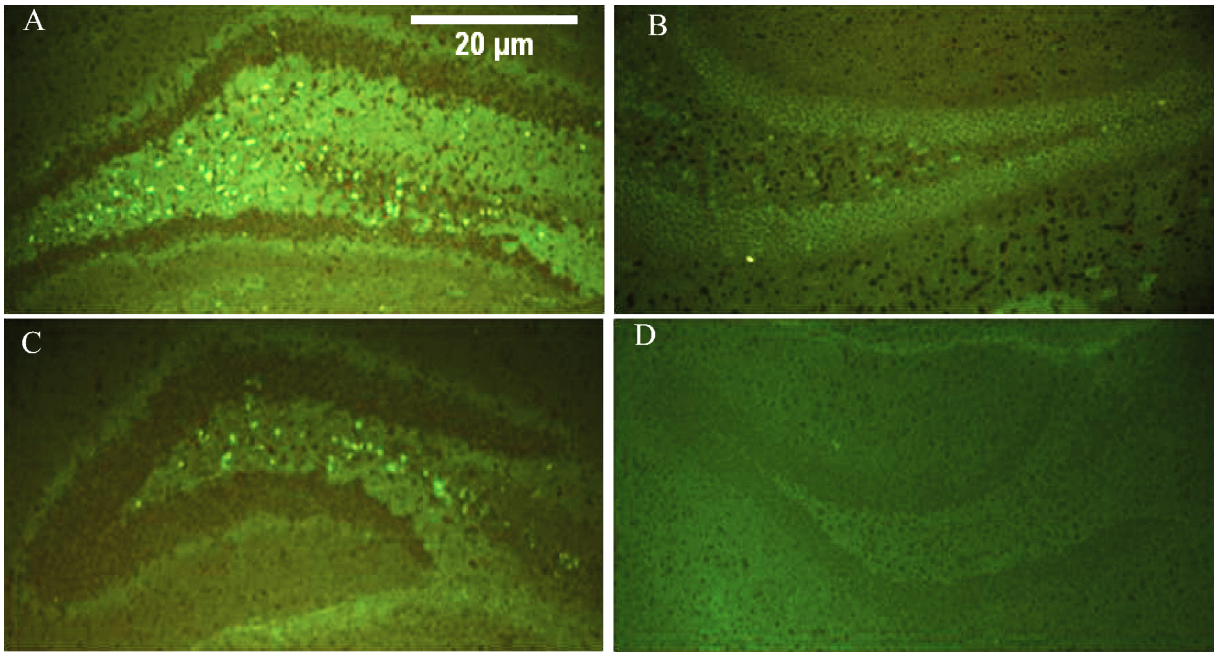

Figure 1. Dentate gyrus of (A.) FVB/N, (B.) C57, (C.) SW, and (D.) DBA mice at the LD_{50} dose of sarin showing neuronal damage with Fluro-Jade C. Bright green fluorescent cells are dead or dying.

no mortality at 320 µg/kg and complete mortality at 380 µg/kg. SW strain was given five different doses of sarin that ranged from 280 µg/kg to 360 µg/kg. Percent mortality for each dose increased dose-dependently from 280 µg/kg to 360 µg/kg except for the 340 µg/kg dose which had slightly lower mortality. The C57 strain was administered two doses of sarin, 190 and 200 µg/kg. There were no deaths at 190 µg/kg and 100% mortality at 200 µg/kg. The DBA strain was given four doses of sarin and suffered no mortality until the LD_{50} dose 260 µg/kg. The ICR strain was given five doses ranging 230 to 300 µg/kg. The ICR strain rapidly gained weight over a short time period and became more sensitive to each dose when weighing above 30g. This made the dose responses very inconsistent where 100% of the animals over 30g would die within 30 min while at the same dose the animals under 30g would all survive. When analyzing the data without those animals over 30g, the dose response curve is incomplete. We did not achieve a 50% mortality rate with this strain of mouse up to 300 µg/kg. The surviving animals closest to the LD50 dose was analyzed for Fluoro-Jade C positive cells. Two strains, C57 and DBA displayed no positive staining using Fluoro-Jade C, while the ICR, SW, and FVB/N exhibited positive staining (examples of staining in Figure 1).

FOB

The FOB scores are a behavioral indication of sarin toxicity including seizure activity. FOB data were analyzed at the dose closest to the LD_{50} from each strain. The ICR strain was eliminated from the FOB analysis due to not achieving the LD_{50} dose. Of the strains, the C57 (M=96.2% ± 0.01 SEM) and SW (M=98.4% ± 0.01 SEM) had higher FOB percent scores, though not significantly different from the other strains (data not shown). The DBA strain had a mean of 92.9% ± 0.04 SEM and the FVB/N had 90.5% ± 0.04 SEM. While not quantitatively different using our scoring methods, the SW and C57 strains had visibly different seizure patterns. The C57 displayed prostration with clonic then tonic seizure activity while SW displayed clonic bouts while moving about the cage periphery.

Animal weights

The dose closest to the LD_{50} for each strain was used for the analysis of the weights. The two and three day weights were combined in the groups where there were insufficient survival rates on the third day. There were no differences in weight loss among the strains. All strains lost a significant amount of weight on day one and it continued on day

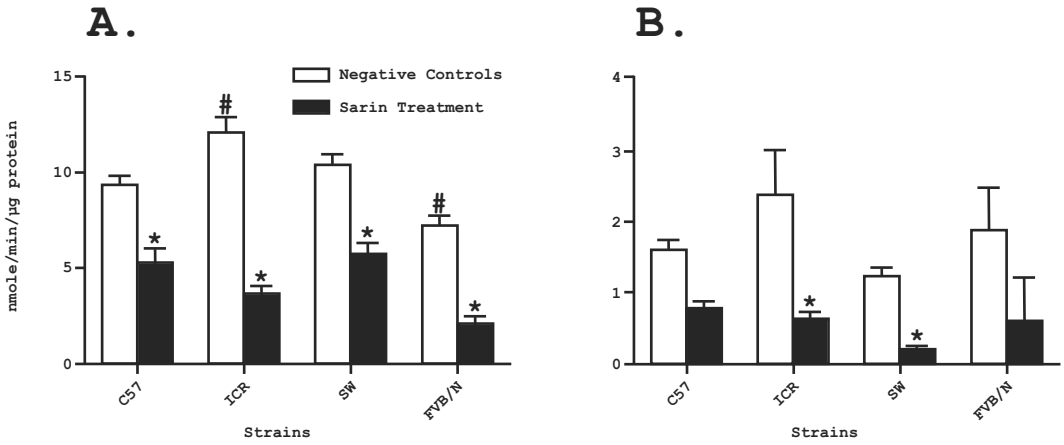

Figure 2. (A.) Frontal cortex (FC) acetylcholinesterase (AChE) and (B.) FC butyrylcholinesterase (BuChE) activity seven days post-exposure for negative controls and the dose closest to the LD_{50} of sarin for each strain.

* Indicates differences from saline treated controls for the same strain
\# indicates differences between ICR and FVB/N negative controls

two or three ($F_{2,32}$=22.04, p=0.002). The percent of weight loss on day one ranged 0.5% to 21.3% with a mean of 7.7% ± 1.52 SEM. The percent of weight loss on day two or three ranged from a 3.2 % to 31.2% with a mean of 9.3% ± 2.74 SEM.

AChE and BuChE

Mice were euthanized seven days post-injection. Trunk blood and frontal cortex (FC) tissue was collected for determination of AChE and BuChE. The dose closest to the LD_{50} for each strain was analyzed for differences (see table 1 for sample sizes). There were no differences in blood AChE activity in any strain (data not shown). Differences were seen in brain AChE activity among groups ($F_{3,35}$=11.15, p<0.001) and BuChE levels among groups ($F_{3,27}$=2.52, p<0.05). AChE activities (see figure 2a) were reduced at the LD_{50} for every strain (p<0.05). BuChE activity (see figure 2b) was reduced in all strains but only significantly in the ICR and SW strains after sarin exposure (p<0.05). Comparing only the negative controls across strains, the ICR strain had a higher AChE level than the FVB/N strain (p<0.05). The DBA strain was not included in the analysis due to a lack of control for comparison.

DISCUSSION

This study revealed mouse strain differences in sensitivity to sarin-induced lethality. Further, there were strain differences in cholinesterase activity and histology. However, there were no differences in acute signs as reflected by similar FOB scores. Weight loss has been correlated with increased neuropathology in B6D2F mice and Sprague Dawley rats [11;12]. This occurred in all the strains in this study at one day and three days after exposure with no difference among the strains. While inhibition of AChE occurred with all strains, there were differences in the baseline measurements among strains. The ICR strain had higher basal AChE compared to the FVB/N strain. All strains exhibited decreased BuChE activity but it was only significantly reduced SW and ICR strains, even though the ICR mice received less than a LD_{50}. Neither mortality rate nor AChE/BuChE activities alone predict weight loss at three days across these strains. Three of the strains did exhibit Fluoro-Jade C staining while C57 and DBA mouse strains did not.

Strain sensitivity to sarin-induced lethality was initially assumed to be similar to that found with KA based on the assertion that the neurodegeneration from nerve agent-induced seizures resulted from glutamatergic overstimulation. The rank order for KA dose sensitivity from most sensitive to resistant is as follows: DBA, C57, ICR, and FVB/N [5]. The SW strain was excluded due to a lack of conclusive results on obtaining a dose that caused repetitive seizures without leading to death. In contrast, the rank order for the LD_{50} of sarin was: C57, DBA, SW and FVB/N. The LD_{50} could not be determined for the ICR strain because of their rapid weight gain

but it was over 300 µg/kg. The differences could result from differences in either degradative enzymes or in the ability to release glutamate. The mouse strain sensitivity to sarin is similar to that of diisopropyl fluorophosphates (DFP), an organophosphate insecticide, in which the C57 mouse strain was more sensitive than the DBA strain [13;14].

Though the C57 and DBA strains were sensitive to sarin-induced seizures they did not exhibit Fluoro-Jade C staining. This finding alone does not indicate a lack of neural injury. It may indicate an improper timing of euthanasia, although three strains did exhibit staining and Fluoro-Jade B staining is reported to occur starting at 24hrs and up to a full month after administration of soman in rats and B6D2F1/j@rj mice [6;7;15]. Alternatively, Fluoro-Jade C may not be the best marker to use in these strains. However in C57 mice, Fluoro-Jade B staining was not observed when given KA. This strain does exhibit neural injury following sarin administration as measured by glial fibrillary acidic protein (GFAP), a marker for astrocytic activation evident at seven and fourteen day [7;16] and neuronal nuclei (NeuN) staining of healthy neurons in the hippocampus at fourteen days (manuscript in preparation). An induction of reactive gliosis was evident in the hippocampus of C57 mice following KA-induced seizures as measured by GFAP and lectin staining while no Fluoro-Jade B staining was evident [3]. When C57 and FVB/N mice underwent pilocarpine-induced seizures hippocampal degeneration was observed using Nissl staining [17]. These observations indicate that stains other than Fluoro-Jade are needed in order to measure degeneration in some of these strains of mice.

Weight loss is an indicator of the severity of convulsions and degeneration in the hippocampus with a threshold of 20% weight loss over three days correlating with severe long term damage in B6D2F1/j mice [6]. The strains tested in this study support this observation. The higher baseline levels of AChE and BuChE might explain the difficulty in obtaining an LD_{50} for the ICR strain. However, they still exhibited staining for neuronal injury. All the strains showed inhibition of AChE levels in the frontal cortex after sarin exposure but the C57 and SW strains had a greater percent response. The differences seen with sarin on AChE inhibition are similar to those seen after DFP exposure. After DFP exposure among several mouse strains, DBA/2lbg, C57BL/6lbg, and C3H/2lbtg, no differences in nicotinic and muscarinic acetylcholine receptor densities were found [14]. Due to the similarities with AChE inhibition, it is unlikely that differences among strains after sarin exposure are due to receptor densities alone.

Determining the best animal models for sarin exposure will aid in the development of novel therapeutics for control of seizure activity and neuroprotectants against the excitotoxic degeneration seen after exposure. Discovering the cause of the strain differences in sensitivity could lead to a better understanding of the mechanism by which the damage occurs. More work is needed to evaluate the mechanism underlying the differences in response to histological stain for neuropathology across strains. In previously published work, this laboratory achieved consistent results in seizure activity, weight loss, FOB scores, and AChE inhibition with C57 mice [7;16]. Due to the lack of positive Fluoro-Jade C stained cells in the C57 mouse strain and inconsistent positive Fluoro-Jade staining with the SW strain (unpublished observations), other immunohistochemical stains such as: GFAP and NeuN, have been employed [7;18;19] (manuscript in preparation). The current data emphasizes the need for thorough investigation of the differences between strains and to assess the histological and symptomatic response across the strains.

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