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Development of a Guinea Pig Model for Low-
dose Chronic Exposure to Organophosphorus
Nerve Agents

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14. ABSTRACT An animal dosing model and related maximum tolerated dose (MTD) were developed for repeated exposures in guinea pigs to three organophosphorus chemical warfare nerve agents (CWNA). Male animals were injected subcutaneously with sarin (GB), soman (GD) or VX once a day (Monday through Friday) for 2-, 4-, or 13-weeks. An initial 13-week study for each CWNA employed doses of vehicle (normal saline), 0.2, 0.4, 0.6, and 0.8 x the previously established acute LD50. A 2-week and 4-week exposure were also performed for each agent at doses less than the 13-week MTD to verify lack of toxicity. Animals dosed daily for 13 weeks with 0.4 x LD50 of GB or GD or with 0.2 x LD50 of VX did not display signs of acute cholinergic toxicity. In animals dosed daily for either 2- or 4-weeks, the MTDs were 0.4 x the acute LD50 for all 3 CWNA. There were no differences among these groups and their respective vehicle controls for weight gains, body temperature, complete blood cell counts, blood chemistries, nor by histopathology. At the MTD in all groups, red blood cell cholinesterase activity one hour after the last exposure was inhibited up to 90% compared with controls.					
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ABSTRACT

An animal dosing model was developed in guinea pigs for low-dose repeated exposure to three organophosphorus chemical warfare nerve agents. The goal was to establish a maximum tolerated dose (MTD) without exhibiting clinical signs of cholinergic toxicity for subacute, subchronic and chronic durations. Male animals were injected subcutaneously with sarin (GB), soman (GD) or VX once a day (Monday through Friday) for up to 13 weeks. Dose groups were vehicle (normal saline), 0.2, 0.4, 0.6, and 0.8 x the previously established acute LD₅₀ of each agent respectively. One 2-week and one 4-week exposure duration were also performed for each individual agent using MTDs determined from the initial 13-week study to verify lack of clinical signs of toxicity. Animals dosed daily for 13 weeks with 0.4 x LD₅₀ of GB or GD (accumulative up to 26 LD₅₀'s) or with 0.2 x LD₅₀ of VX (accumulative to 13 LD₅₀'s) did not display signs of acute cholinergic toxicity. Thus, these daily doses were the 13-week exposure MTDs for each respective nerve agent. There were no differences among these groups and their respective vehicle controls for weight gains, body temperature changes, complete blood cell counts, blood chemistries, nor by histopathology. In animals dosed daily for either 2 or 4 weeks, the MTDs were 0.4 x the acute LD₅₀ for all 3 nerve agents. Thus, the MTDs were established for GB, GD and VX in our guinea pig low-dose chronic exposure model for three exposure durations. In the 0.2 or 0.4 x LD₅₀ groups red blood cell cholinesterase activity one hour after the last exposure was inhibited significantly, up to 90% compared with controls, for any of the 3 nerve agents at any of the three exposure durations.

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INTRODUCTION

Organophosphorus (OP) chemical warfare nerve agents (CWNA), such as sarin (GB), soman (GD) and VX, produce their clinical effects by inhibiting the enzyme acetylcholinesterase (AChE) (Taylor, 1996). The normal function of this enzyme is to degrade the cholinergic neurotransmitter acetylcholine (ACh). Thus, OP nerve agents exert their toxic effects by causing the accumulation of ACh at the synaptic junction (Shih, 1982), which, in turn, results in overstimulation of the central and peripheral nervous system. The standard U.S. military doctrine has been to preserve the fighting force in the event of chemical warfare attack and to return the war fighters to duty/battle as soon as possible. Therefore, much of the research in support of this doctrine has been on studies of the acute high dose exposure and medical countermeasures for the clinical effects of cholinergic overstimulation. Comparatively little research has been performed on possible health effects of low-dose chronic exposure to CWNA.

The use of GB and possibly other CWNA in the Middle East, the exposure of civilians to GB by terrorists (e.g., Tokyo subway incidence), the worldwide CWNA demilitarization efforts, the disposal and handling of agent wastes, and the increasing awareness of environmental toxicology have led to concerns that exposure of military personnel and civilians to nerve agents for any extended duration may cause long-term health problems distinct from acute toxicity (Moore, 1998; Romano et al., 2001). In light of these concerns it is appropriate that we study the long-term consequences of any possible exposures to these agents.

Ongoing medical research studying the acute toxic effects of CWNA in our laboratory is performed using the guinea pig as the model (Shih and McDonough, 2000). The guinea pig is considered to be a more valid rodent model for the toxicological effects of OP nerve agents than are rats or mice (Inns and Leadbeater, 1983). Rats and mice possess large amounts of plasma carboxylesterase (CaE) enzyme that covalently binds nerve agents such as GB and GD. As a result, they require substantially higher doses of these agents than guinea pigs or nonhuman primates to produce equivalent lethal effects (Sterri et al., 1980; Sterri et al., 1981; Maxwell et al., 1987). The rate of elimination of GD from the rat also appears to be slower than in guinea pigs or non-human primates, which suggests that the guinea pig is a more appropriate rodent model for human exposures (Due et al., 1993)

The purpose of this study was to establish the highest daily exposure dose that does not induce signs of cholinergic intoxication in animals during 2, 4 or 13 weeks of exposure to GB, GD and VX. The dosing model would then be used in the future to study subtle changes in physiological and behavioral parameters of animals subject to similar exposures. More specifically, the objectives of this study were to (1) establish an appropriate dosing regimen (i.e. the maximum tolerated dose, MTD) to enable studies of low-dose chronic exposure to CWNA in male guinea pigs, (2) determine whether there are pathologic effects of low-dose chronic exposure to CWNA at or below the MTD, and (3) determine whether red blood cell (RBC) AChE inhibition is an appropriate indicator of low-dose chronic exposure to CWNA.

MATERIALS AND METHODS

Animals

A total of 296 male Hartley guinea pigs (Crl:(HA)BR), weighing 250 ± 20 g, obtained from Charles River Labs (Kingston, NY) were used for these studies. Upon arrival, the animals were quarantined and tested for evidence of disease. They were housed individually in polycarbonate cages at controlled temperature ($21^\circ \pm 2^\circ\text{C}$) and humidity ($50\% \pm 10\%$). The room was maintained on a 12-h light dark cycle with lights on at 0600 h. Laboratory chow (Harlan Teklad, Indianapolis, IN) and water were available freely when the animals were in home cages. Animals were implanted subcutaneously (sc) on the right side with sterile Implantable Programmable Temperature Transponders (IPTT-200) (BioMedic Data Systems Inc., Seaford, Delaware) for animal identification and body temperature monitoring. Animals were allowed to acclimate in the animal quarters for approximately one week prior to the start of the experiment. Animals weighed approximately 300 g at the beginning of these studies and weights were normalized between groups before the start of exposures.

Nerve Agents

Sarin (GB; isopropyl methylphosphonofluoridate), soman (GD; pinacolyl methylphosphonofluoridate), and VX (O-ethyl S-(2-(diisopropylamino)ethyl)methylphosphonothioate) were obtained from the US Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). The nerve agent was diluted in sterile normal saline (i.e., the vehicle) to varied concentrations to allow for constant injection volume of 1.0 ml/kg body weight. Dilute OP nerve agents were aliquoted into serum vials (one each for daily injection), sealed with Teflon septa and stored at -80°C . Enough OP nerve agent was diluted prior to the start of experiment for the completion of an entire nerve agent study. This insured that the animals were exposed to the same batch and dilution of OP nerve agent among the 2-, 4-, and 13-week groups. Nerve agent was administered subcutaneously between the shoulder blades. The fractions of the LD_{50} doses of GB, GD and VX used in this study were based on the institute database, which indicated that the 24-hour acute LD_{50} doses for GB, GD and VX were 42, 28, and $9 \mu\text{g}/\text{kg}$, sc, respectively (personal communication, Dr. Irwin Koplovitz).

Experimental Protocol

Duration of Exposure

Duration of exposure was based on that described by Eaton and Klaassen (1996) in Casarett & Doull's *Toxicology*, which states, "Subacute exposure refers to repeated exposure to a chemical for 1 month or less, subchronic for 1 to 3 months, and chronic for more than 3 months." Thus, 2-, 4- and 13-week exposure durations, representing subacute, subchronic and chronic exposures, respectively, were chosen for the present study.

Body weight and temperature

On each exposure day, body weight of each animal was recorded just prior to CWNA injection to assess the health condition of the animal and for calculation of injection volume (1.0

ml/kg). Body temperature of each animal was monitored both before and one hour after each CWNA injection using the IPTT-200 transponders.

Thirteen-week Exposure

An initial 13-week (chronic) exposure was performed using a Monday through Friday (M-F) injection regimen. Exposure started on a Monday and ended on the 13th Friday. This study allowed determination of the maximum tolerated doses (MTDs) for the subsequent 2-week (subacute) and 4-week (subchronic) exposure experiments and, thus, decreased the number of animals needed for these later experiments. If a dose was found in the 13-week study to cause animals to show severe signs of intoxication or to die during the first 2 or 4 weeks of exposure, that dose was excluded from subsequent experiments for 2- or 4-week duration. Five dose groups with 4 (for GB) to 6 (for VX and GD) animals per group were used: a vehicle control, 0.2, 0.4, 0.6, and 0.8 x LD₅₀. Guinea pigs were injected with GB, VX, or GD once daily Monday through Friday for 13 weeks. Each animal received the same dose of a nerve agent daily throughout the 13 weeks of exposure. Injections were made each day at 0930 ± 1 hour. Acute signs of cholinergic toxicity (from mild to severe: tremors, hyperactivity, excitability, salivation, lacrimation, prostration, recumbency, loss of righting reflex, dyspnea, convulsions, apnea) were monitored in their home cages for at least 2 hours post-injection. Blood was taken by toe-nail clip (Vallejo-Freire, 1951) at 1 hour after the very last injection to measure RBC AChE activity.

On the Monday following the last CWNA exposure (i.e., 3 days after final injection), animals were deeply anesthetized intraperitoneally with 75 mg/kg pentobarbital sodium. Blood was removed from the caudal vena cava before the animal was euthanized by exsanguination. Blood was examined for complete blood cell counts (CBCs) and blood chemistries. Some tissues (brain, heart, lung, liver, gallbladder, adrenal gland, kidney, sciatic nerve, skeletal muscle, and testes) were harvested and submitted for histopathological examination. Hemi-diaphragms were harvested for determination of AChE activity in homogenate by a colorimetric method (Ellman et al., 1961). Survivability was taken as the number of animals alive 24 hours after CWNA injection.

Two- and Four-week Exposures

Separate groups of animals were studied for 2- or 4-week exposure durations in order to compare blood chemistries, hematology, RBC AChE activity, and tissue histopathological data with those obtained from the 13-week exposure group. Nerve agent dosages (i.e., MTDs) were selected from the responses observed in the initial 13-week studies in which none of the animals displayed signs of intoxication for the desired exposure duration. Vehicle and 0.2 and 0.4 x LD₅₀ of a nerve agent were selected in these experiments with 12 animals per group. These animals were given CWNA once daily with the same dose from Monday through Friday for 2 or 4 weeks, respectively. Home cage observation was made for possible signs of cholinergic toxicity for 2 hours post-injection. Blood was taken at 1 hour after the last injection to measure RBC AChE activity. On the Monday following the last exposure animals were anesthetized and sacrificed and tissues collected in the same manner as that described previously for the 13-week exposed animals.

Blood and Diaphragm AChE Activity

Measurement of RBC AChE activity

RBCs were separated from the plasma by centrifuging 1.0 ml aliquots of whole blood for 5 min at 18,200 x g in a refrigerated centrifuge (4-6° C). Twenty (20) µL RBCs were carefully pipetted and placed into 180 µl 1% Triton X-100 in saline in a microtube and flash frozen. Flash freezing was accomplished by immersing 3/4 of each microtube sample in a slurry of methanol/dry ice. Samples were then stored frozen at -80° C until analysis. RBC AChE activity was determined, using acetylthiocholine iodide as a substrate, by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Inc., Nutley, NJ). The analytical procedure was based on the manual method of Ellman et al. (1961) and modified for the COBAS/FARA by Hobson et al. (1988).

Measurement of diaphragm AChE activity

Diaphragm muscles were dissected free of ribs and tendons and homogenized in ice-cold saline (1:4) containing 1% (v/v) Triton X-100 by three 10 sec pulses of a Polytron homogenizer (Brinkman Instruments, Westbury, NY) at a speed setting of 8. Homogenates were centrifuged at 15,000 x g for 10 min in a refrigerated centrifuge (4-6° C), and the resultant supernatants were stored frozen at -80° C for enzyme and protein analysis. AChE activity was measured, using acetylthiocholine iodide as a substrate, by an automated method using a COBAS/FARA clinical chemistry analyzer (Hobson et al., 1988) based on the manual method of Ellman et al. (1961). Protein was measured by the dye-binding method of Bradford (1976).

Blood Chemistries and Hematology

The blood chemistries (creatinine phosphokinase, lactic acid dehydrogenase, aspartate and alanine aminotransferases, alkaline phosphatase, blood urea nitrogen, total protein, total bilirubin, albumen, creatinine, glucose, sodium, potassium, chloride) were performed on a Hitachi 704 chemistry analyzer using Roche (distributor for Hitachi chemical reagents in USA) reagents. The hematology included differential WBC (neutrophil, lymphocyte, monocyte, eosinophyl and basophyl), RBC (hematocrit and hemoglobin) and platelet measurements performed on a CellDyne 3500 Hematology analyzer.

Histopathological Assessment

Brain, heart, lung, liver, gallbladder, kidney, adrenal gland, skeletal muscle, sciatic nerve, and testes were collected for histopathological evaluation. They were placed into 10% neutral, buffered formalin for fixation. After proper duration of fixation, these tissues were embedded in paraffin blocks and processed routinely for histologic evaluation using a hematoxylin and eosin (H&E) stain. Gross or microscopic examination was performed by a board-certified veterinary pathologist (S.M.D.), who was not aware of the experimental history of a given subject.

Statistical Analysis

Body weights and AChE data are presented as mean \pm SD. Comparisons among different dosing groups within an exposure duration experiment were analyzed for distribution of data; based on that distribution a Dunnett's analysis of variance (ANOVA) was performed. A difference of $p < 0.05$ was considered significant.

RESULTS

Observation of signs of intoxication

The critical element for the successful completion of this study was the ability to identify the clinical signs of cholinergic toxicity. Technical personnel involved in this project were trained extensively to recognize the initial signs induced by OP nerve agents in the guinea pigs. Following daily weighing and injection of saline or CWNA, animals usually exhibited exploratory walking activity, eating and drinking and some had extended grooming activity in the cage. These were not signs of nerve agent toxicity. The first indication of toxic signs of CWNA in guinea pigs was the chewing movements around the lips (excessive chewing activity), which then continue to become fine tremors in the facial area (facial tremors). The animals would then become hyperactive, walking and circling followed by occasional running around the inside of the observation cage. At this stage animals became extremely excitable and any sudden noise (such as a cough) could trigger a jump from the floor. Weakness in head and neck muscles could also be observed at this stage, with the animals having difficulty maintaining an elevated head position. Tremors and excitability are the signs most frequently observed in the animals that were treated with 0.4 and 0.6 x LD₅₀ of VX or 0.6 x LD₅₀ of GD and survived for the 13-week exposure duration. If the exposure dose was high enough (e.g., 0.8 x LD₅₀ for all agents and 0.6 x LD₅₀ for GB and GD), intoxication deepened and signs became severe. Animals became weak in their hind limbs and were unable to maintain an erect, standing posture. This could progress to rigidity in the upper body accompanied by fine eyeball movement (initial nystagmus), both ears twitching with regular rhythm and the head swaying from side to side. These signs indicated convulsions, since these rhythmic movements usually correspond to electrographic seizures (McDonough and Shih, 1997). With severe convulsions the whole body started shaking involuntarily. Forced chewing movement developed as an indication of air-gasping movements. Salivation and lacrimation usually developed after convulsion onset. Rhythmic nystagmus became quite obvious. These convulsive activities lasted for a few hours. Animals soon lost muscle tone and righting reflex. Ears and feet became cyanotic. Respiration became slower and labored and death ensued. Those guinea pigs that received daily 0.6 or 0.8 x LD₅₀ doses of CWNA and died were observed to show the most severe toxic signs.

Sarin Study

Thirteen-week Exposure (N=4 per group)

All 4 guinea pigs exposed to 0.8 x LD₅₀ sarin died after second daily injection (Figure 1). The 0.6 x LD₅₀ animals displayed moderate to severe signs of acute nerve agent toxicity during the first week of exposure (Figure 2). Signs of toxicity occurred between 10 and 20 min after injection (average 14 min) and lasted for several hours. Two of the animals died within the first

two weeks of exposure, and the remaining two died in the third week of exposure. These animals all displayed severe signs of acute nerve agent toxicity prior to their death.

Neither the 0.2 x LD₅₀- nor the 0.4 x LD₅₀-treated groups displayed any outward signs of acute nerve agent toxicity during the 13-week exposure (Figure 2). None of these animals showed any difference in body weight over the 13-week exposure period from that of saline-treated controls (Figure 3). The 0.4 x LD₅₀-treated animals did not appear to gain weight as fast as the 0.2 x LD₅₀ or vehicle-exposed animals, but attained the same weights by the end of the exposure period. Differences in weight between the controls, 0.2 and 0.4 x LD₅₀ groups were not statistically significant at any time interval.

One hour after daily exposure, the body temperature was not different from the baseline value (baseline temperature = 101.5 ± 0.8 °F) in either the 0.2 or 0.4 x LD₅₀-treated groups during the 13-week exposure duration. In those 0.6 or 0.8 x LD₅₀-treated animals that showed signs of intoxication, however, the body temperature occasionally dropped 4-6 °F one hour after exposure.

In the animals receiving 0.2 or 0.4 x LD₅₀ there was no tissue pathology associated with GB exposure when examined 3 days after the last exposure using H&E staining. CBC's and blood chemistries showed no statistically significant differences in any measured parameter between the controls and the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals.

One hour after the last exposure of sarin, RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls. RBC AChE inhibition relative to the age-matched controls was 70% for 0.2 x LD₅₀- and 81% for 0.4 x LD₅₀-treated animals (Figure 4). The level of inhibition between the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals was not statistically significant from each other, although AChE activity was significantly less than controls at both doses.

Two- and Four-week Exposures (N=12 per group)

Due to the signs of toxicity and death with the 0.6 and 0.8 x LD₅₀ doses in the 13-week GB study, these two doses were excluded from subsequent 2- and 4-week studies. Saline vehicle, 0.2 and 0.4 x LD₅₀ doses were evaluated during the 2- and 4-week exposures. Findings observed in these animals were similar to those described for the same dose groups in the 13-week study. There was no GB exposure-induced tissue pathology observed using H&E staining. There was no difference in body weight gains, no body temperature changes, and no changes in CBC's and blood chemistries between the controls and 0.2 or 0.4 x LD₅₀-treated animals.

One hour after the last exposure, RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with age-matched controls. After 2-week exposure RBC AChE inhibition relative to control was 77% for 0.2 x LD₅₀- and 92% for 0.4 x LD₅₀-treated animals. After 4-week exposure RBC AChE inhibition relative to control was 83% for 0.2 x LD₅₀- and 91% for 0.4 x LD₅₀- treated animals. However, the level of inhibition between the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals was not statistically significant in either the 2-week or 4-week exposures (Figure 4). There were also no significant differences in the RBC AChE activities between the 2-week and 4-week exposure durations at any dosage level.

Three days after the last GB dose in the 2-week exposure, the RBC AChE inhibition had partially, but significantly, recovered, with 65% inhibition in the 0.2 x LD₅₀ group and 79% inhibition in the 0.4 x LD₅₀ group. Partial recovery also occurred three days following the 4-week exposure with 68% inhibition in the 0.2 x LD₅₀ group and 79% inhibition remaining in the 0.4 x LD₅₀ group.

Diaphragm AChE measured 3 days after the last exposure was inhibited by 19-26% in 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls for 2- or 4-week exposure durations. However, this inhibition was not statistically significant from controls.

VX Study

Thirteen-week Exposure (N=6 per group)

All six 0.8 x LD₅₀-exposed animals had died after the fifth injection (Figure 5). The 0.6 x LD₅₀-exposed animals displayed signs (up to prostration and recumbency) of acute nerve agent toxicity during the first week of exposure (Figure 6). These signs of toxicity occurred between 34 and 50 min after injection (average 43 min) and lasted for 1.5-5.0 hours. Interestingly, these clinical signs became less pronounced during the 6th-8th weeks, but reappeared in the 9th week. These signs became severe following subsequent exposures. Two of the six animals died in the 10th week, 2 more died during the 12th week and 1 more died one hour after the very last (i.e., 65th) exposure (Figure 5). These animals all displayed severe signs of acute nerve agent toxicity prior to their death. One animal of the 0.6 x LD₅₀ group survived all 13 weeks, with an accumulative exposure of 39 LD₅₀'s; this animal did display moderate signs of acute agent poisoning.

The 0.4 x LD₅₀-treated animals did not display signs of acute nerve agent toxicity until the 9th weeks of exposure (Figure 6). The first sign of toxicity occurred around 60 min after injection. These signs were mild, usually of tremors and hyperactivity in nature, and lasted for 1.0-1.5 hours. They displayed these mild signs of nerve agent toxicity on and off (i.e., not every animal had signs) during the remaining 4 weeks of exposure. None had developed into more severe toxic signs. All 6 of the 0.4 x LD₅₀ animals survived the 13 weeks of exposure. The 0.4 x LD₅₀-exposed animals survived a cumulative dose of 26 LD₅₀'s of VX. The 0.2 x LD₅₀-treated animals did not display any obvious signs of acute VX toxicity during the 13-week exposure (Figure 6).

One hour after daily exposure, the body temperature was not different from the baseline value (baseline temperature = 101.9 ± 1.0 °F) in either the 0.2 or 0.4 x LD₅₀-treated groups during the 13-week exposure duration. In those 0.6 or 0.8 x LD₅₀-treated animals that showed signs of intoxication, however, a body temperature drop of 4-6 °F in one hour after exposure was observed in some animals.

The body weights of the 0.2 x LD₅₀-exposed animals were not different from those given vehicle alone during the 13-week exposure period (Figure 7). The 0.4 x LD₅₀ animals weighed less than either the 0.2 x LD₅₀- or vehicle-exposed animals by the end of the 9th week of exposure, corresponding to the time when signs of toxicity were observed (Figure 6). The 0.6 x

LD₅₀-treated animals weighed significantly less than the other groups by the end of the second week of exposure.

Three days after the last exposure, there was no VX exposure-related pathology observed, even in the only remaining 0.6 x LD₅₀-treated animal, using H&E staining. CBC's and blood chemistries showed no differences in parameters among the saline control, 0.2, and 0.4 x LD₅₀ VX-treated animals, except for an elevation of Na⁺ in the 0.4 x LD₅₀-treated animals; however, those values were within the range of normal values (Manning et al., 1984).

One hour after last exposure, RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls (Figure 8). RBC AChE inhibition relative to control was 90% for 0.2 x LD₅₀- and 92% for 0.4 x LD₅₀-treated animals. The difference in inhibition between the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals was not statistically significant.

Diaphragm AChE three days after the last exposure was inhibited by 27-33% in 0.2, 0.4 or 0.6 (N=1) x LD₅₀-treated animals compared with controls. However, these changes were not statistically different from controls.

Two- and Four-week Exposures (N=12 per group)

Due to the signs of toxicity seen with the 0.6 and 0.8 x LD₅₀ doses in the pilot study those doses were excluded from subsequent 2- and 4-week exposures. The findings observed in these animals were very similar to those described earlier in the 13-week experiment. There was no VX exposure-related pathology observed using H&E staining. One animal treated with 0.2 x LD₅₀ for 4 weeks demonstrated mild, multifocal axonal degeneration with myelin dilatation in the sciatic nerve. The significance and nature of this singular lesion is uncertain. A toxic etiology associated with nerve agent exposure is unlikely. Other possible causes include trauma and neuromuscular disorder. There was no difference in body weight, body temperature, CBC's and blood chemistries between the controls and 0.2 or 0.4 x LD₅₀-treated animals.

RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls (Figure 8). After 2-week exposure RBC AChE inhibition relative to controls was 85% for 0.2 x LD₅₀- and 90% for 0.4 x LD₅₀-treated animals. After 4-week exposure RBC AChE inhibition relative to controls was 89% for 0.2 x LD₅₀- and 91% for 0.4 x LD₅₀-treated animals. There was no statistically significant difference in inhibition between the 0.2 and 0.4 x LD₅₀-treated animals at the end of either the 2- or 4-week exposures. There was also no significant difference between the RBC AChE activities between the 2-week and 4-week study durations at any dosage level.

Three days after the last VX exposure in the 2-week study, the RBC AChE inhibition had been reduced to 60% in the 0.2 x LD₅₀ group and 66% inhibition in the 0.4 x LD₅₀ group. Partial recovery also occurred three days following the last VX exposure in the 4-week study with 60% inhibition remaining in the 0.2 x LD₅₀ group and 65% inhibition remaining in the 0.4 x LD₅₀ group. All of these recoveries were statistically significant.

Diaphragm AChE measured three days after last exposure was inhibited by 11-31% in 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls for 2- or 4-week exposure durations. However, these differences were not statistically significant from controls.

Soman Study

Thirteen-week Exposure (N=6 per group)

All six of the 0.8 x LD₅₀-exposed animals died after the 7th injection (Figure 9). The 0.6 x LD₅₀ animals displayed signs of acute nerve agent toxicity during the first week of exposure (Figure 10). Initial signs of toxicity occurred between 15 and 33 min after injection (average 20 min). Of the 0.6 x LD₅₀ exposed group the first animal died in the first week, a second died in the second week, a third animal died in the third week, and the fourth animal died in the ninth week of exposure (Figure 9). These animals all displayed severe signs of acute nerve agent toxicity prior to their death. Two remaining animals survived the 13-week exposure period and developed only mild tremors and hyperactivity occasionally throughout this period.

Neither the 0.2 x LD₅₀ nor the 0.4 x LD₅₀-treated animals displayed any outward signs of acute nerve agent toxicity during the 13-week exposure. None of these animals had a difference in body weight over the exposure period from that of saline-treated controls (Figure 11). It is interesting to note that the 0.6 x LD₅₀ animals did not differ in body weight at any time from the control group over the course of the study. The 0.4 x LD₅₀ animals were exposed to an accumulative dose of 26 LD₅₀'s without displaying any obvious signs of acute soman toxicity.

One hour after daily exposure, the body temperature was not different from the baseline value (baseline temperature = 101.6 ± 0.9 °F) in either the 0.2 or 0.4 x LD₅₀-treated groups during the 13-week exposure duration. In those 0.6 or 0.8 x LD₅₀-treated animals that showed signs of intoxication, however, a body temperature drop of 5-6 °F in one hour after exposure was observed in some animals, usually associated with subsequent death.

There was no agent-related pathology when examined 3 days after the last exposure using H&E staining in those animals that survived the 0.2 or 0.4 x LD₅₀ doses. The two 0.6 x LD₅₀-treated animals that had signs of intoxication and survived in the 13-week pilot studies showed multifocal neuronal degeneration and necrosis accompanied by spongiosis in the cerebral cortex, thalamus, caudate nuclei and hippocampus. CBC's and blood chemistries showed no difference between the controls and the 0.2 x LD₅₀- or 0.4 x LD₅₀-treated animals.

One hour after the last exposures, RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls (Figure 12). RBC AChE inhibition relative to controls was 85% for 0.2 x LD₅₀- and 91% for 0.4 x LD₅₀-treated animals. The difference in inhibition between the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals was not statistically significant.

Diaphragm AChE measured three days after last exposure was inhibited by 18-35% in 0.2, 0.4 or 0.6 (N=2) x LD₅₀-treated animals compared with controls. However, these changes were not statistically significant from controls.

Two- and Four-week Exposures (N=12 per group)

Due to the signs of toxicity and death with the 0.6 and 0.8 LD₅₀ doses, these 2 doses were excluded from subsequent definitive 2- and 4-week exposures. Vehicle, 0.2 and 0.4 x LD₅₀ treatments were evaluated during the 2- and 4-week exposure experiments. Very similar findings were observed in these animals as were described earlier in the 13-week experiment at the same exposure doses. There was no GD exposure-related pathology observed using H&E staining. There was no difference in body weight, body temperature, CBC's and blood chemistries between the controls and 0.2 or 0.4 x LD₅₀-treated animals.

RBC AChE was significantly inhibited in both 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls (Figure 12). After the 2-week exposure, RBC AChE inhibition relative to controls was 89% for 0.2 x LD₅₀- and 91% for 0.4 x LD₅₀-treated animals. After the 4-week exposure, RBC AChE inhibition relative to controls was 86% for 0.2 x LD₅₀- and 88% for 0.4 x LD₅₀-treated animals. However, the level of inhibition between the 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals was not statistically different for either the 2-week or 4-week studies. There was also no significant difference in RBC AChE activities between the 2- and 4-week study durations at any dosage level.

Three days after the last GD exposures in the 2-week study, the RBC AChE activities had recovered to 70% inhibition in the 0.2 x LD₅₀ group and to 72% inhibition in the 0.4 x LD₅₀ group. Partial recovery also occurred three days following the last GD exposures in the 4-week study with 72% inhibition remaining in the 0.2 x LD₅₀ group and 77% inhibition remaining in the 0.4 x LD₅₀ group. All of these recoveries were statistically significant from the inhibition levels at one hour after the last exposure.

Diaphragm AChE measured three days after the last exposure was inhibited by 23-32% in 0.2 x LD₅₀- and 0.4 x LD₅₀-treated animals compared with controls for 2- or 4-week exposure durations. However, these changes were not statistically significant from controls.

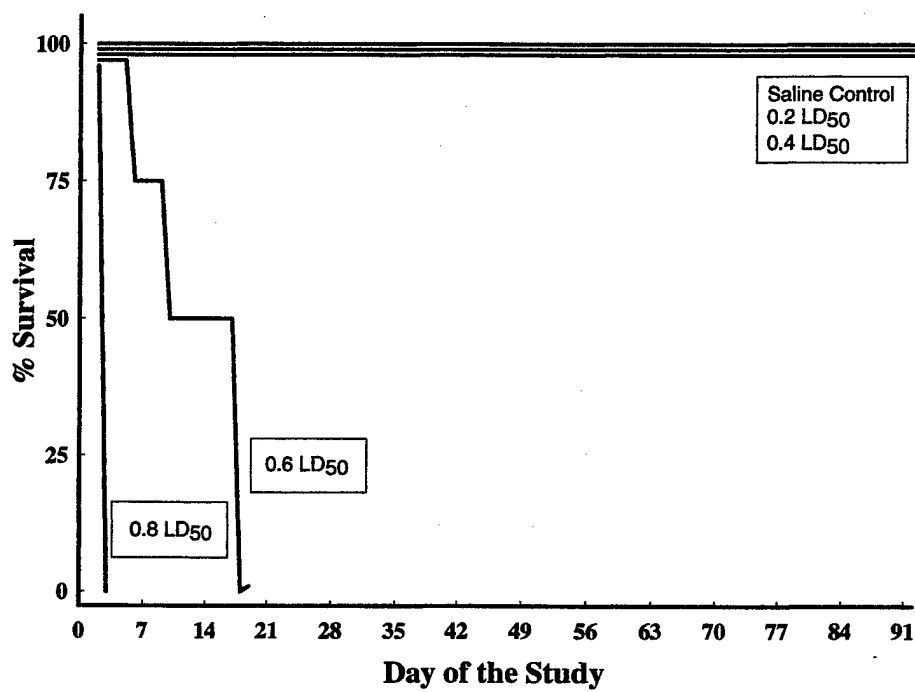


Figure 1. Survival and mortality for a pilot 13-week sarin (GB) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of GB daily from Monday – Friday for 13 weeks. N = 4 per dose group. LD₅₀ for GB is 42 ug/kg, sc.

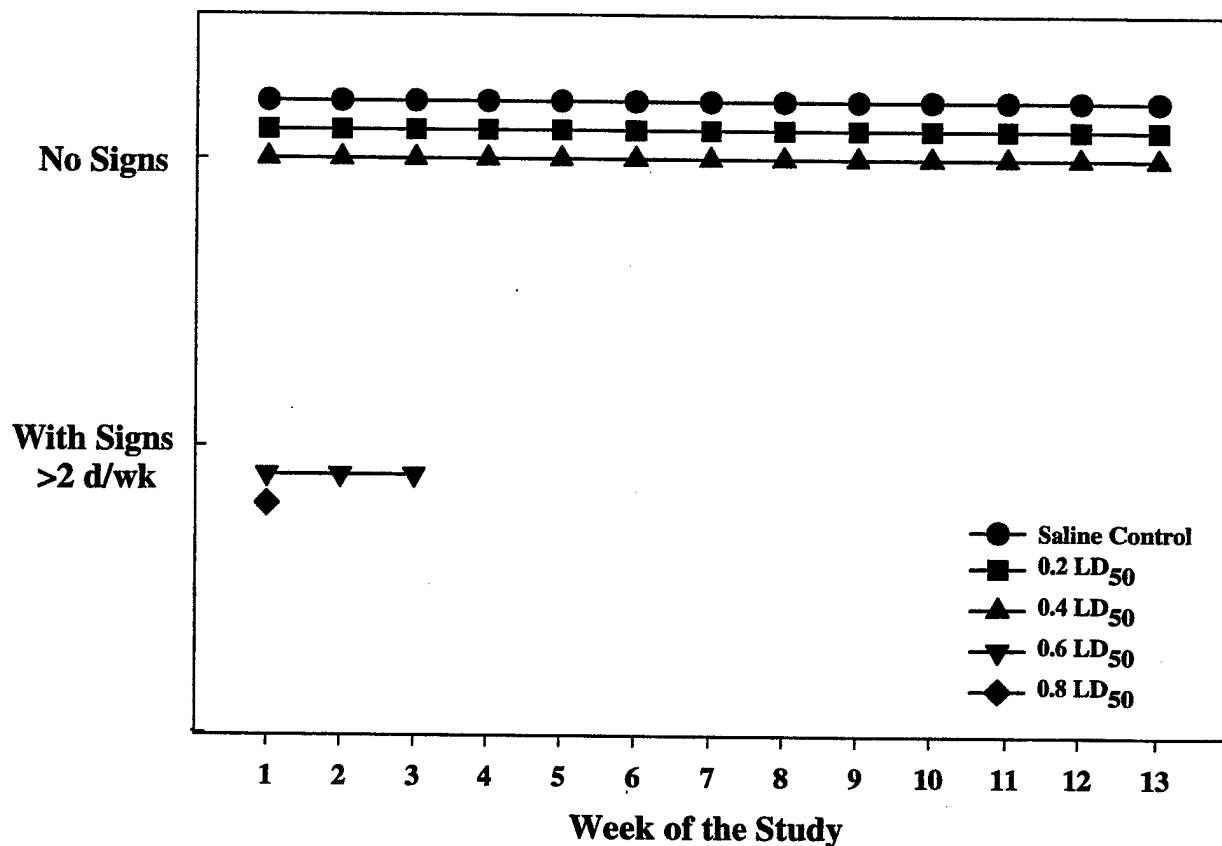


Figure 2. Weekly records of signs of nerve agent toxicity in the 13-week sarin (GB) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6, and 0.8 x LD₅₀ doses of GB daily from Monday – Friday for 13 weeks. N = 4 per dose group. Graph shows weeks of the study when signs of intoxication were displayed by an exposure group for at least 2 days each week. Clinical signs of toxicity are defined as the presence of any or all of the following: tremors, hyperactivity, excitability, convulsions, salivation, lacrimation, recumbency, loss of righting reflex, or dyspnea. No sign was displayed by the saline, 0.2 or 0.4 x LD₅₀ groups over the 13 weeks.

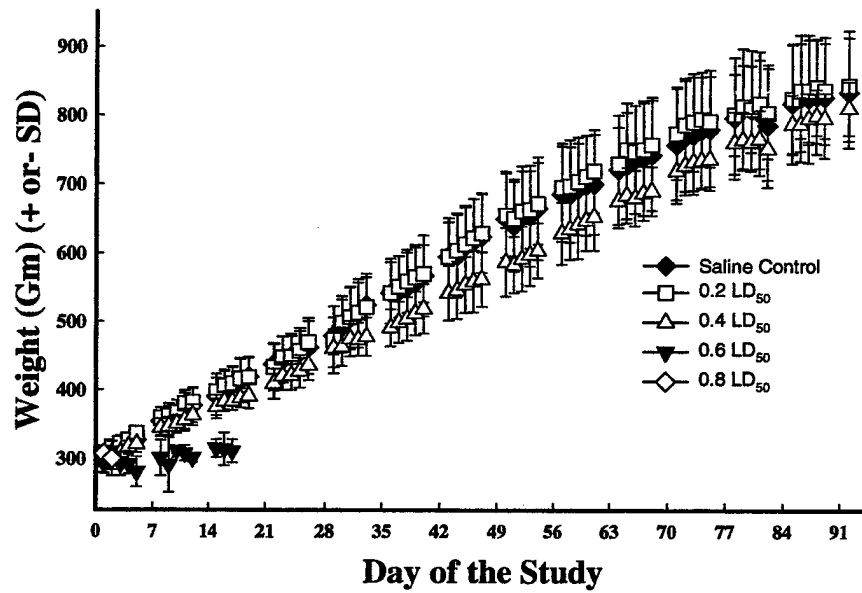


Figure 3. Daily body weight records for a pilot 13-week sarin (GB) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of GB daily from Monday – Friday for 13 weeks. Values represent means \pm SD (N = 4 per dose group).

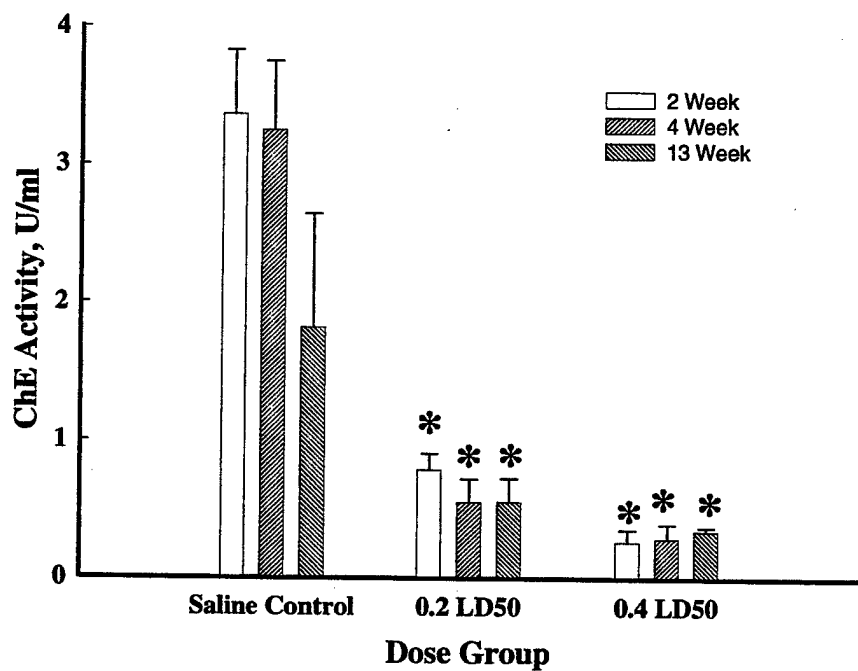


Figure 4. RBC AChE activity for low dose chronic exposure to sarin (GB) in male guinea pigs. Animals were administered subcutaneously with saline or 0.2 and 0.4 x LD₅₀ doses of GB daily from Monday – Friday for 2, 4 or 13 weeks. Blood was taken one hour following the last GB exposure on a Friday. Values represent means ± SD (N = 12 per group for 2 or 4 weeks and 4 per dose group for 13 weeks). * P<0.05 vs. respective saline control group.

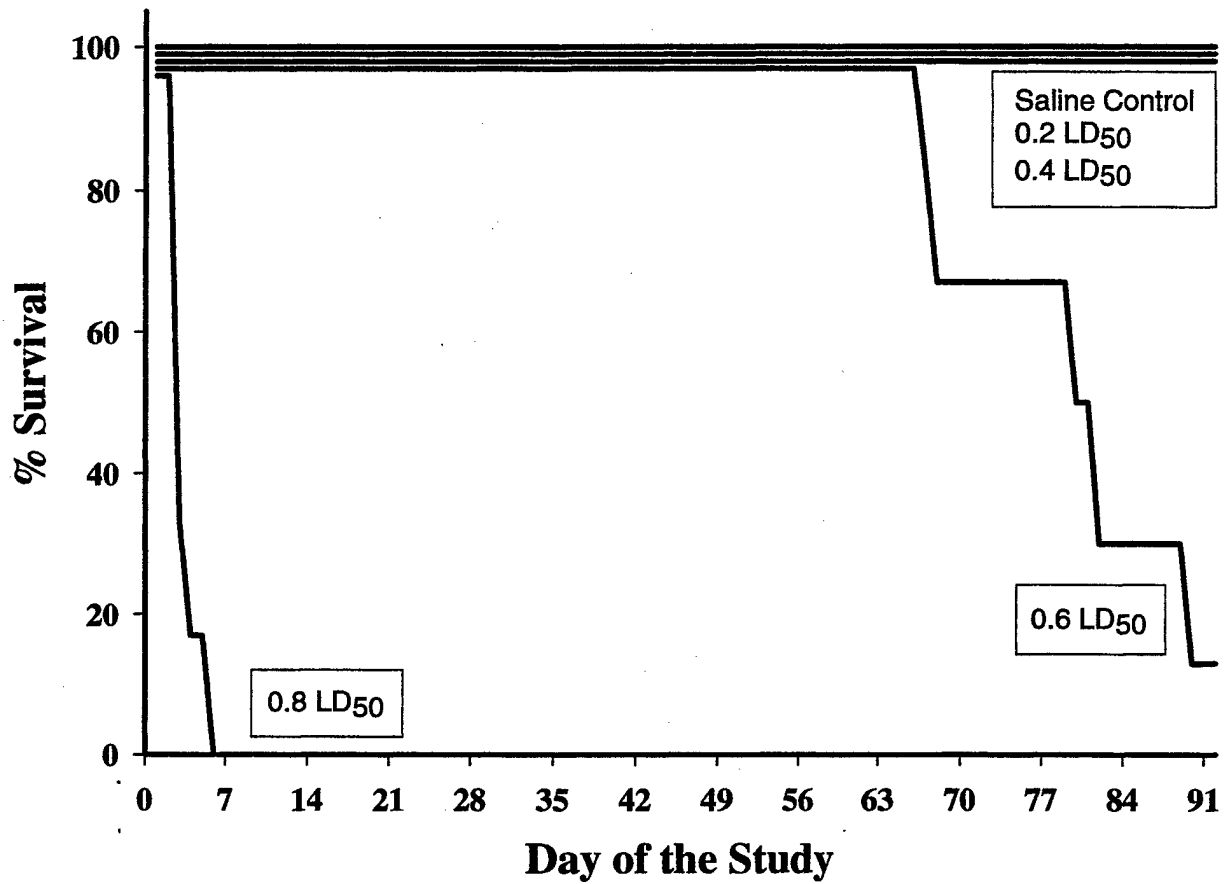


Figure 5. Survival and mortality for a pilot 13-week VX exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of VX daily from Monday – Friday for 13 weeks. N = 6 per dose group. LD₅₀ for VX is 9 ug/kg, sc.

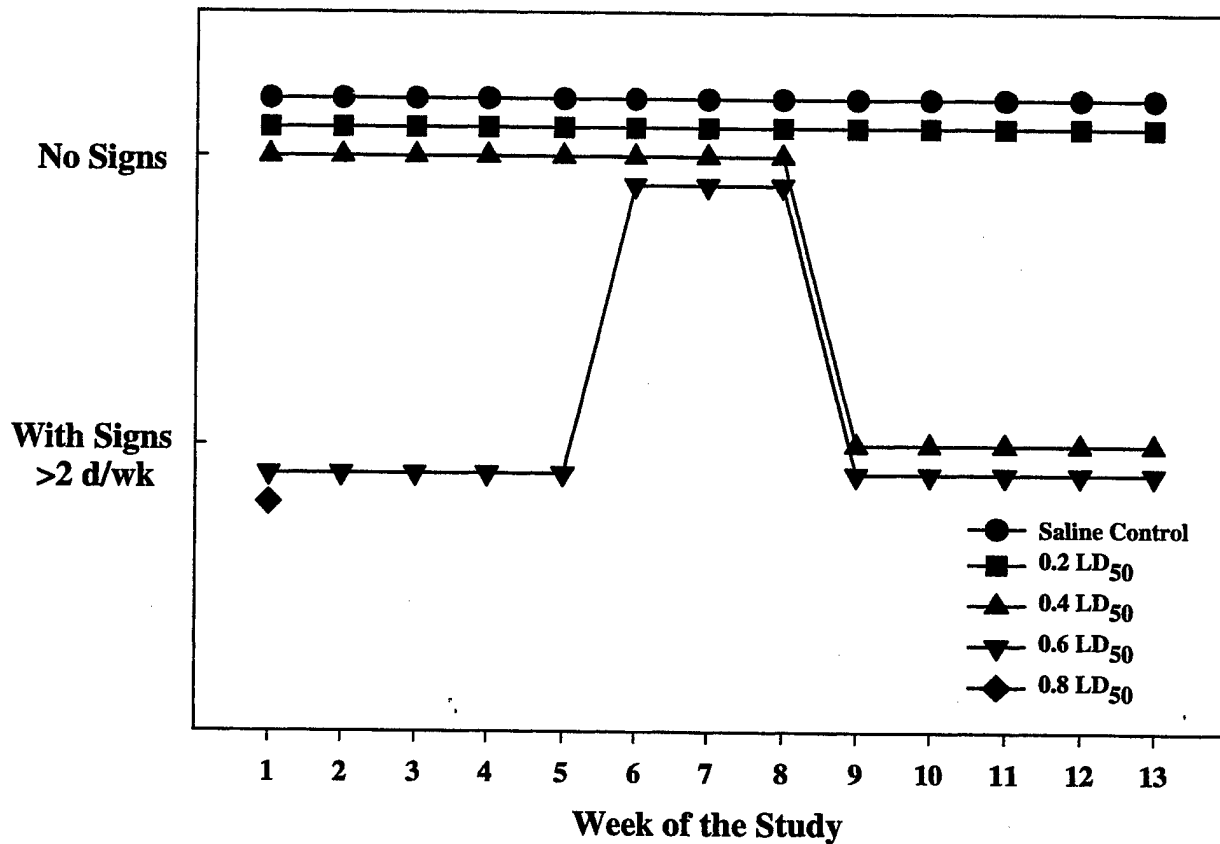


Figure 6. Weekly records of signs of nerve agent toxicity in the 13-week VX exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of VX daily from Monday – Friday for 13 weeks. N = 6 per dose group. Graph shows weeks of the study when signs of intoxication were displayed by an exposure group for at least 2 days each week. Clinical signs of toxicity are defined as the presence of any or all of the following: tremors, hyperactivity, excitability, convulsions, salivation, lacrimation, recumbency, loss of righting reflex, or dyspnea. For details see the Results.

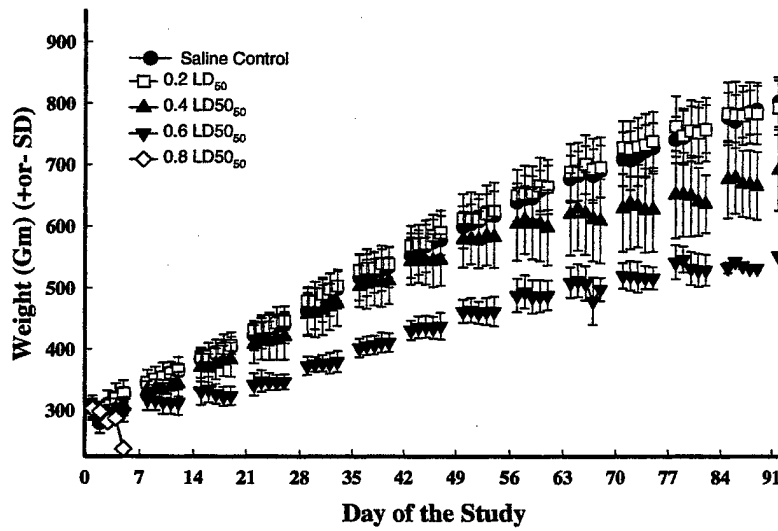


Figure 7. Daily body weight records for a pilot 13-week VX exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of VX daily from Monday – Friday for 13 weeks. Values represent means \pm SD (N = 6 per dose group).

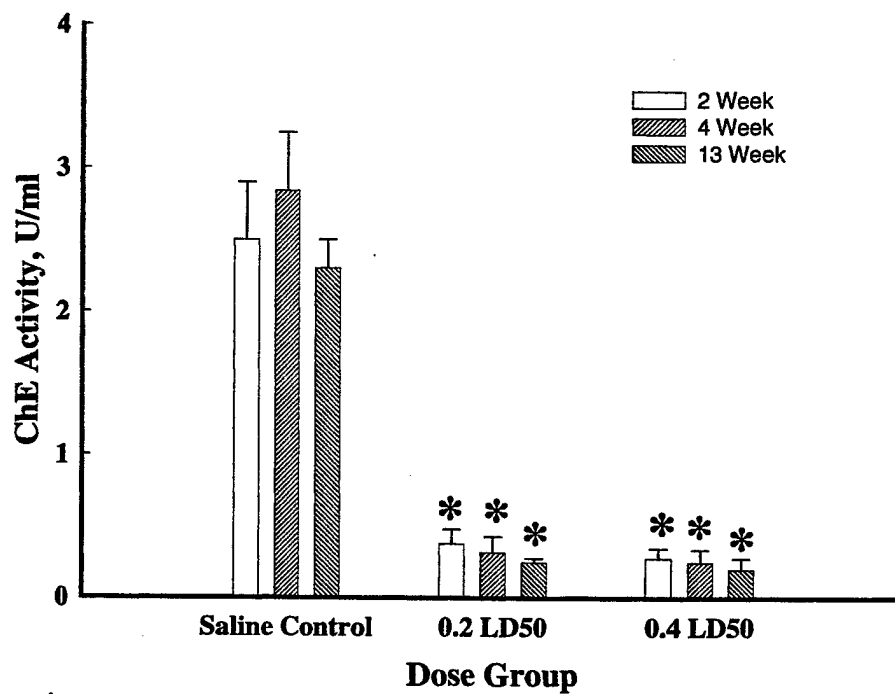


Figure 8. RBC AChE activity for low dose chronic exposure to VX in male guinea pigs. Animals were administered subcutaneously with saline or 0.2 and 0.4 x LD₅₀ doses of VX daily from Monday – Friday for 2, 4 or 13 weeks. Blood was taken one hour following the last VX exposure on a Friday. Values represent means ± SD (N = 12 per group for 2 or 4 weeks and 6 per dose group for 13 weeks). * P<0.05 vs. respective saline control group.

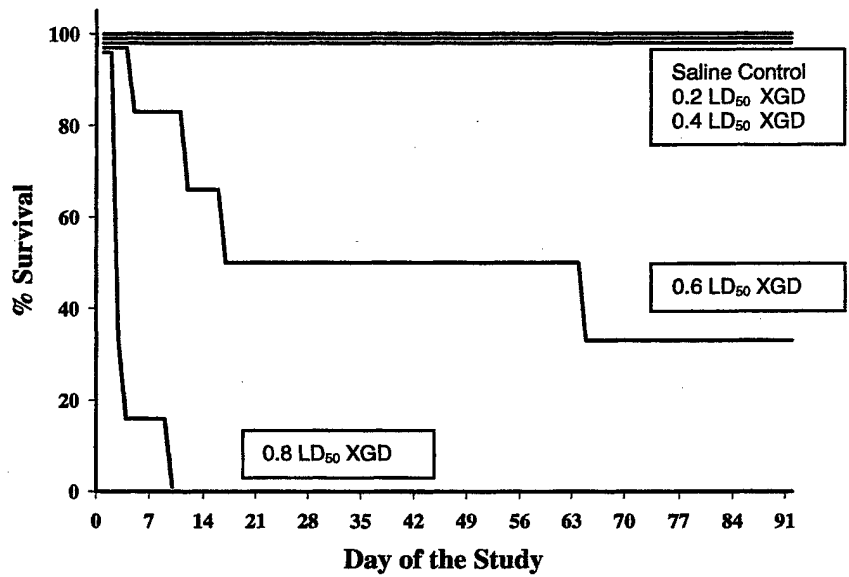


Figure 9. Survival and mortality for a pilot 13-week soman (GD) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of GD daily from Monday – Friday for 13 weeks. N = 6 per dose group. LD₅₀ for GD is 28 ug/kg, sc.

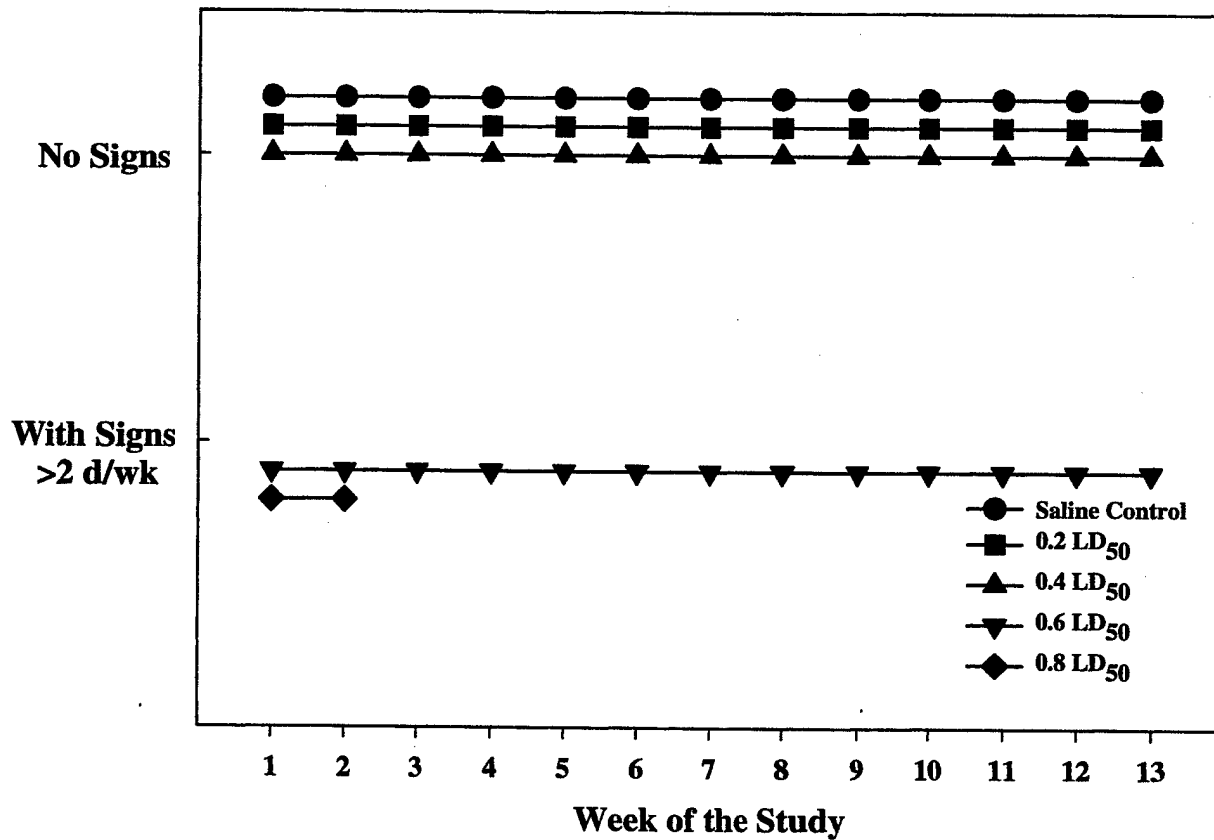


Figure 10. Weekly records of signs of nerve agent toxicity in the 13-week soman (GD) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of GD daily from Monday – Friday for 13 weeks. N = 6 per dose group. Graph shows weeks of the study when signs of intoxication were displayed by an exposure group for at least 2 days each week. Clinical signs of toxicity are defined as the presence of any or all of the following: tremors, hyperactivity, excitability, convulsions, salivation, lacrimation, recumbency, loss of righting reflex, or dyspnea. No sign was displayed by the saline, 0.2 or 0.4 x LD₅₀ groups over the 13 weeks.

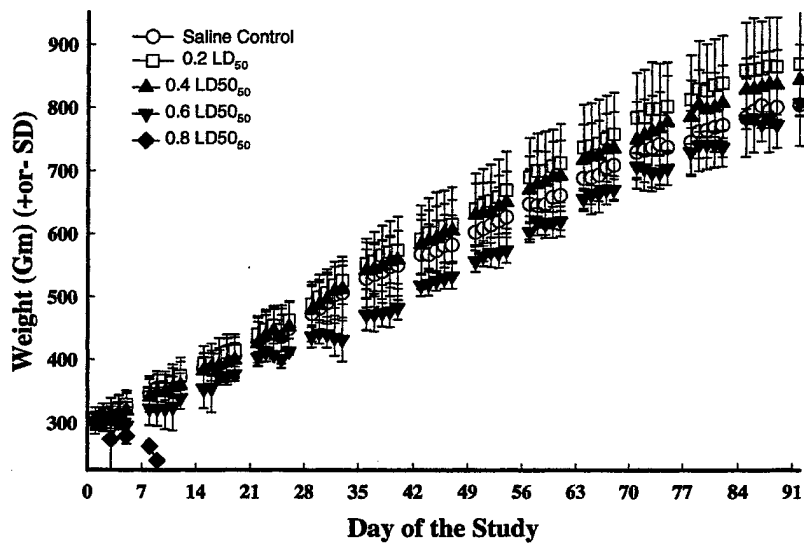


Figure 11. Daily body weight records for a pilot 13-week soman (GD) exposure experiment in male guinea pigs. Animals were administered subcutaneously with saline or 0.2, 0.4, 0.6 and 0.8 x LD₅₀ doses of GD daily from Monday – Friday for 13 weeks. Values represent means \pm SD (N = 6 per dose group).

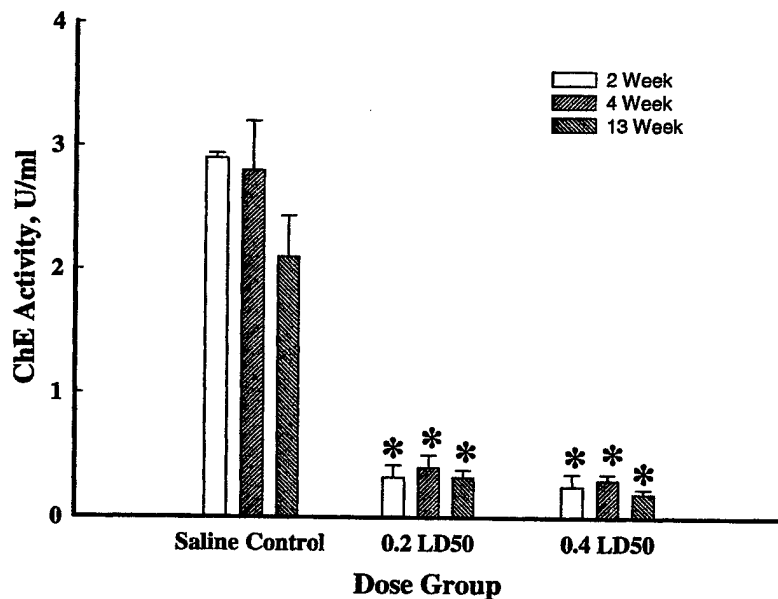


Figure 12. RBC AChE activity for low dose chronic exposure to soman (GD) in male guinea pigs. Animals were administered subcutaneously with saline or 0.2 and 0.4 x LD₅₀ doses of GD daily from Monday – Friday for 2, 4 or 13 weeks. Blood was taken one hour following the last GD exposure on a Friday. Values represent means ± SD (N = 12 per group for 2 or 4 weeks and 6 per dose group for 13 weeks). * P<0.05 vs. respective saline control group.

DISCUSSION

The incapacitation and lethality after exposure to high doses of CWNA have been well documented. These exposures result in inhibition of AChE activity, increases in synaptic ACh levels, disruption of cholinergic neurotransmission in both the peripheral and central nervous systems (Taylor, 1996). On the other hand, the sub-symptomatic low-dose, repeated or chronic exposures, and the post-exposure long-term effects to CWNA have received relatively little attention. For the past decade since the Persian Gulf War, concern about repeated low-dose exposures to CWNAs has increased and has led to an effort to better identify the health hazards associated with these exposures. Events such as low-dose acute exposure at Khamisiyah, Iraq, in 1991 (Weese, 2001), Aum Shinrikyo's sarin attacks in Japan in Matsumoto City in 1994 (Nakajima et al., 1998) and Tokyo subway sarin release in 1995 (Yokoyama et al., 1998), and residual CWNA after partial decontamination illustrate that low-dose exposure to CWNA could potentially occur to both soldiers and civilians. Furthermore, worldwide CWNA demilitarization efforts, increased environmental concerns, and the possibility of CWNA terrorism have led us to focus our attention on studying the long-term consequences of any possible exposures to CWNA.

A substantial amount of literature has focused on the acute effects of CWNA, and how to treat and protect the soldier from those agents in order to preserve the fighting force (Dunn and Sidell, 1989; Moore et al., 1995; McDonough and Shih, 1997). This research can provide initial guidance in the development of dosing models to study single or repeated exposures to low-doses of CWNA, but completely new criteria, dosing methods, and protocols must be developed to optimize the study of such exposures. The main objectives of this study were to establish daily dosing regimens to be utilized in an animal model for low-dose repeated exposure to CWNA for 2 weeks (subacute), 4 weeks (subchronic) or 13 weeks (chronic) and to establish the maximum tolerated dose that would set the upper bound for such exposures. The intent is to provide the animal model to medical defense research scientists to aid in the investigation of behavioral, physiological, biochemical, cellular and molecular effects. The model will also be useful in intervention studies to identify efficacious countermeasures to minimize such effects of low level CWNA exposure.

In the present study, the MTDs were established for GB, GD and VX in our guinea pig low-dose repeated exposure model for three exposure durations: 2-week (subacute), 4-week (subchronic), and 13-week (chronic). Guinea pigs dosed daily (Monday through Friday) for 13 weeks with 0.4 x LD₅₀ of GB or GD (accumulative up to 26 LD₅₀'s) or with 0.2 x LD₅₀ of VX (accumulative to 13 LD₅₀'s) did not display signs of acute cholinergic toxicity. Thus, these daily doses were the 13-week exposure MTDs for each respective nerve agent. There were no differences among these groups and their respective vehicle controls for weight gains, body temperature changes, CBC and blood chemistries, nor by histopathology. In animals dosed daily for either 2 or 4 weeks, the MTDs were 0.4 x LD₅₀ for all 3 nerve agents. In the 0.2 or 0.4 x LD₅₀ groups RBC AChE activity one-hour after the last exposure was inhibited significantly, up to 90% compared with controls, for any of the 3 nerve agents at any of the three exposure durations.

To begin this study, a decision had to be made on the choice of animal species and of the route of CWNA exposure. Rodents are the preferable order of animals because most of the

earlier CWNA or other OP pesticide studies were done in this species, and a large database is available. Guinea pigs are preferred to rats or mice because interference from high levels of blood carboxylesterases (CaE) in rats and mice makes it difficult to reproduce CWNA LD₅₀ determinations (Maxwell et al., 1987). This problem is least severe in the guinea pig, however. The variation in GD LD₅₀ between rats (118.3 µg/kg: Jimmerson et al., 1989) and guinea pigs (28 µg/kg: Shih and McDonough, 2000) is believed to be associated with the high concentration of CaE in the blood of rats and the significantly lower level of CaE in the blood of guinea pigs (Maxwell et al., 1987). It has been reported that of the three rodent species guinea pig is inherently the most sensitive to OP agents and is a better model for predicting the efficacy of treatments for OP poisoning in primate species (Inns and Leadbeater, 1983). For the purposes of establishing a subacute or subchronic exposure model, the use of a selective CaE inhibitor to mimic non-human primate levels of CaE would seem contraindicated in a sub-lethal study. The most commonly used selective CaE inhibitor, CBDP, is a metabolite of triortho cresyl phosphate (Clement, 1984) and could reasonably be expected to induce neuropathy upon repeated dosing (Crowell et al., 1989), which would invalidate such a model.

As to the route of exposure, since the objective is to develop an animal model to study the systemic effects of low-dose repeated exposure to CWNA in various laboratories, the subcutaneous route of administration is appropriate and convenient. Subcutaneous exposure reflects the percutaneous absorption of CWNA, a likely route of exposure in either the battlefield or a chemical terrorism scenario, and is easier to quantify than non-parenteral dosing such as inhalation or oral routes. Subcutaneous administration can be administered daily without the dangers and unpredictability of long-term inhalation exposures/challenges. The investigators feel confident of the amount of agent administered to each animal daily and thus cumulatively. There is virtually no chance of vapor exposure from animal's fur and off-gassing of nerve agent to laboratory personnel, and, thus, it is safer for scientific investigation. Direct percutaneous exposure of nerve agent to animals poses a potential safety problem to both laboratory personnel and animal caretakers/handlers, and the absorbed dose can vary markedly with ambient temperature, humidity, and the volatility of the CWNA. For parenteral exposures, nerve agent is kept either in a vial or in a syringe prior to administration to the animal with care taken to prevent any agent from contaminating either the personnel or the skin of the animal at injection. CWNA exposed-animals can be removed at any time after injection for behavioral, physiological, biochemical, or toxicological assessments or examinations.

The three nerve agents studied here elicited different toxic responses. The average latency to onset of clinical signs with GB and GD was 14 and 20 min, respectively, whereas the onset of signs observed with VX was later at about 43 minutes following 0.6 or 0.8 x LD₅₀. The longer latency for developing signs of intoxication following VX in comparison with GB or GD has been reported previously in this species (Shih and McDonough, 2000). If animals survived the initial dose, the onset of signs following the subsequent exposures gradually decreased. The animals that received 0.4 x LD₅₀ dose of GB or GD did not display any signs of acute toxicity throughout the 13-week exposure, whereas the animals that received 0.4 x LD₅₀ dose of VX did show signs of acute nerve agent toxicity from the 9th through 13th weeks of the exposure. This would suggest that VX (or its metabolites) may be cleared from the body more slowly and accumulate in tissue, resulting in signs of toxicity after an acquired dose of 16 LD₅₀'s. By contrast, GB, GD, and their metabolites may have been cleared from the body much more

quickly, thus permitting animals to tolerate an accumulated dose of up to 26 LD₅₀s in 13 weeks without obvious signs of intoxication. Such a difference between the clearance of VX and the other CWNA tested could also account for the rate of cumulative lethality with 0.6 x LD₅₀ doses. All GB-treated animals died within three weeks of such treatment (Figure 1), while the cumulative rate of lethality was much lower for VX (Figure 5). The cumulative rate of mortality for GD-treated animals seemed to lie between these extremes and most died during the first 3 weeks after exposure (Figure 9), while in VX-treated animals no mortality was observed until after the 9th week of exposure.

With the additional 2- and 4-week exposure groups and the initial 13-week exposure group, we were able to obtain tissues and blood samples from three exposure durations of each nerve agent for post-exposure toxico-pathological and RBC AChE evaluation and comparison of effects. The results indicated that those animals that survived 2-, 4- or 13-week daily CWNA exposure without showing any signs of intoxication were free of evidence of gross and histopathology. Their body weight gain, body temperature change, CBCs and blood chemistries were as normal as the controls. The two 0.6 x LD₅₀ soman-treated animals that showed signs of intoxication and survived the 13-week daily exposure displayed multi-focal neuronal degeneration and necrosis accompanied by spongiosis in the cerebral cortex, thalamus, caudate nuclei and hippocampus. These lesions are consistent with acute toxicity associated with soman exposure (McLeod et al., 1984; Baze 1993; Britt et al., 2000). It is interesting to note that none of the animals that was treated daily with 0.4 x LD₅₀ (6 animals, signs developed after 9th week) or 0.6 x LD₅₀ (only one survivor) dose of VX, showed signs of intoxication and survived for 13-week exposure displayed any histopathological abnormality.

The cumulative inhibition of RBC AChE activity was similar after the last exposure, despite differences in the duration of the repetitive dosing, either 2, 4, or 13 weeks, and across all agents studied. With daily 0.2 x LD₅₀ exposure, the RBC AChE was inhibited by 70-83% for GB, 85-90% for VX, and 85-89% for GD. There was no statistically significant difference among these three nerve agents. Similarly, with 0.4 x LD₅₀ exposures, RBC AChE was inhibited by 81-93% for all three nerve agents and for all three exposure durations. There was no difference between or among 0.2 and 0.4 x LD₅₀ treatment groups for any of the three nerve agents. Thus, an equilibrium level of inhibition was established within the first 2-weeks of each exposure study, and this equilibrium level of inhibition was consistent across the three CWNA investigated, when these agents are dosed to the same physiological effect. Hulet et al. (2002) reported a graded reduction of RBC AChE reaching approximately 10% of baseline activity at the 10th injection following an identical daily 0.4 x LD₅₀ dose of GB exposure regimen in this species. The establishment of such an equilibrium inhibition is consistent with the concept that "the poison is cumulative and if taken into the body slowly can be accommodated for without the appearance of critical illness." (Sim, 1975). The accommodation that prevents cumulative inhibition of RBC AChE from reaching completion probably involves a combination of both clearance of the CWNA and recovery of AChE activity. The combined rate of these processes then establishes the MTD for a fixed rate of administration, such as the daily dosing used here. Of these processes, it is likely that recovery of AChE activity represents a major factor in establishing the MTD.

When RBC ChE activity was measured 72 hours after the last exposure, there was a significant recovery of activity from the previous measurement, taken 1 hour after the last exposure. This recovery averaged $14.0 \pm 2.4\%$ for GB, $15.8 \pm 3.9\%$ for GD, and $25.5 \pm 2.6\%$ for VX (mean \pm SD, $n = 4$ groups). It is commonly accepted that the only way that RBC AChE can be replaced once it is irreversibly inhibited by OP nerve agents is by *de novo* synthesis (Harris et al., 1971) of new red cells, which is thought to occur at the rate of 1-2% per day (Grob and Harvey, 1958; Sim, 1975). Our observed rates of recovery were substantially higher than this and probably represent a combination of *de novo* RBC/AChE synthesis and variable rates of spontaneous reactivation of RBC AChE (Lanks et al., 1977). A strong indication that spontaneous reactivation is involved comes from the observation that although the rates of recovery for GB and GD are similar, at about 5% per day, the rate for VX was significantly higher with a rate of 8.5% per day, and thus this recovery could not all be due to the same rate of RBC replacement. A rough analysis for GB assuming only a 40% inhibition of all unoccupied RBC ChE with each $0.4 \times LD_{50}$ dosage (Hulet et al., 2002) would lead to less than 1% residual activity at the end of two weeks, and essentially no enzyme activity at 4 or 13 weeks, which is substantially below the levels observed. Adding a recovery of 5% of the total AChE activity per day, an equilibrium is reached within two weeks that oscillates between 8% and 13% per day indefinitely. This calculated activity fits well within the range of our experimental observations and suggests that recovery of enzyme activity plays a major role in the MTD. It is interesting to note that the level of inhibition of AChE activity in skeletal muscle, measured at the 72-hour interval after the last dose, was not significantly different from baseline. This suggests that either the muscle was not significantly inhibited at the equilibrium MTD or that the rate of *de novo* AChE synthesis is relatively higher in the targeted tissue.

Previous work suggests that "analysis of blood for ChE is useful for occupational monitoring, but in an exposed patient, one treats the patient, not the ChE activity (Sidell, 1992)." The current study is completely supportive of this clinical observation. Although free of obvious symptoms of cholinergic intoxication and not evidencing demonstrable pathology, the repetitively dosed animals receiving the MTD for all three agents studied had RBC ChE that was profoundly inhibited, certainly well beyond levels that would be cause for clinical concern. The present findings support the notion that the rate of inhibition, not the degree of inhibition of AChE, determines the clinical manifestation of toxic symptoms following OP nerve agents. Thus, RBC AChE inhibition could not be used to differentiate the exposure lengths and dosages of low-dose exposure to CWNA.

In summary, a guinea pig dosing model for low-dose repeated subcutaneous exposure to OP nerve agents was developed. The MTDs for GB, VX and GD were established for subacute, subchronic, and chronic exposure durations. As a guide, the $0.4 \times LD_{50}$ dose was the highest dose that could be given daily with no observable adverse effects with GB, GD, and VX over 2-week (subacute) or 4-week (subchronic) exposure durations. For the 13-week chronic exposure period, the highest daily dose that could be given with no observable adverse effect was the $0.4 \times LD_{50}$ dose for GB and GD, but only $0.2 \times LD_{50}$ dose for VX. Our results indicate that there were no abnormalities in daily body weight gains and body temperature changes, and post-exposure blood chemistries, hematology, and tissue histology in these groups of male guinea pigs. However, AChE activity in RBC was markedly inhibited, one-hour after final exposure, to about 10% of controls regardless of dose, nerve agent and duration of daily subcutaneous exposure. Therefore, RBC AChE activity may not be a good diagnostic indicator for the severity of low-dose chronic exposure to CWNA. Nonetheless, the data may support the idea that AChE is present in great excess and the system exhibits great plasticity.

REFERENCES

- Baze, W.B. 1993. Soman-induced morphological changes: an overview in the non-human primate. *J. Appl. Toxicol.* 13(3):173-177.
- Bradford, M.M. 1976. A rapid and quantitative method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-54.
- Britt, J.O., Martin, J.L., Okerberg, C.V., and Dick, E.J., Jr. 2000. Histopathologic changes in the brain, heart, and skeletal muscle of rhesus macaques, ten days after exposure to soman, an organophosphorus nerve agent. *Comp. Med.* 50(2):133-139.
- Clement, J.G. 1984. Role of aliesterase in organophosphate poisoning. *Fund. Appl. Toxicol.* 4:S96-S105.
- Crowell, J.A., Parker, R.M., Bucci, T.J., and Dacre, J.C. 1989. Neuropathy target esterase in hens after sarin and soman. *J. Biochem. Toxicol.* 4(1):15-20.
- Dunn, M.A., and Sidell, F.R. 1989. Progress in medical defense against nerve agents. *JAMA* 262:649-652.
- Eaton, D.L., and Klaassen, C.D. 1996. Chapter 2: Principles of Toxicology. In *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 5th Ed., ed. C.D. Klaassen, M.O. Amdur, and J. Doull, 13-33. New York: McGraw-Hill.
- Ellman, G.L., Courtney, K.D., Andres, V., and Featherstone, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88-95.
- Grob, D., and Harvey, J.C. 1958. Effects in man of the anticholinesterase compound sarin (isopropyl methyl phosphonofluoridate). *J. Clin. Invest.* 37:350-368.
- Harris, L.W., Yamamura, H.I., and Fleisher, J.H. 1971. De Novo synthesis of acetylcholinesterase in guinea pig retina after inhibition by pinacolyl methylphosphonofluoridate. *Biochem. Pharmacol.* 20:2927-2930.
- Hobson, D.W., Joiner, R.L., and Dill, G.S. 1988. Pre-Task Pilot Study 87-10: Technicon and COBAS/FARA Analytical Method Comparison for the Determination of Erythrocyte Acetylcholinesterase in the Primate, Battelle Laboratories, Columbus, OH.
- Hulet, S.W., McDonough, J.H., and Shih, T.-M. 2002. The dose-response effects of repeated non-acute sarin exposure in guinea pigs. *Pharmacol. Biochem. Behav.* 72:835-845.
- Inns, R.H., and Leadbeater, L. 1983. The efficacy of bispyridium derivatives in the treatment of organophosphonate poisoning in the guinea-pig. *J. Pharm. Pharmacol.* 35(7):427-433.

- Jimmerson, V.R., Shih, T.-M., Maxwell, D.M., and Mailman, R.B. 1989. Cresylbenzodioxaphosphorin oxide pretreatment alters soman-induced toxicity and inhibition of tissue cholinesterase activity of the rat. *Toxicol. Letters* 48:93-103.
- Lanks, K.W., Lieske, C.N., and Papirmeister, B. 1977. Spontaneous reactivation of acetylcholinesterase following organophosphate inhibition. *Biochim. Biophys. Acta* 483:320-330.
- Manning, P.J., Wagner, J.E., and Harkness J.E. 1984. Chapter 6: Biology and Diseases of Guinea Pigs. In *Laboratory Animal Medicine*, ed. J.G. Fox, B.J. Cohen and F.M. Loew, 153. San Diego: Academic Press.
- Maxwell, D.M., Brecht, K.M., and O'Neill, B.L. 1987. The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. *Toxicol. Letters* 39:35-42.
- McDonough, J.H., and Shih, T.-M. 1997. Neuropharmacological mechanisms of nerve agent-induced seizures and neuropathology. *Neurosci. Biobehav. Rev.* 21:559-579.
- McLeod, C.G., Jr., Singer, A.W., and Harrington, D.G. 1984. Acute neuropathology in soman poisoned rats. *Neurotoxicology* 5(2):53-57.
- Moore, D.H. 1998. Health effects of exposure to low doses of nerve agent – A review of present knowledge. *Drug Chem Toxicol.* 21(Suppl 1):123-130.
- Moore, D.H., Clifford, C.B., Crawford, I.T., Cole, G.M., and Baggett, J.M. 1995. Review of nerve agent inhibitors and reactivators of acetylcholinesterase. In *Enzymes of the Cholinesterase Family*, ed. D.M. Quinn, A.S. Balasubramanian, B.P. Doctor and P. Taylor, 297-304. New York: Plenum Press.
- Nakajima, T., Ohta, S., Morita, H., Midorikawa, Y. Mimura, S., and Yanagisawa, N. 1998. Epidemiological study of sarin poisoning in Matsumoto City, Japan. *J. Epidemiol.* 8:33-41.
- Romano, J.A., Jr, McDonough, J.H., Sheridan, R., and Sidell, F.R. 2001. Health effects of low-level exposure to nerve agents. In *Chemical Warfare Agents: Toxicity at Low Levels*, ed. S.M. Somani and J.A. Romano, 1-24. Boca Raton: CRC Press.
- Shih, T.-M. 1982. Time course effects of soman on acetylcholine and choline levels in six discrete areas of the rat brain. *Psychopharmacology* 78:170-175.
- Shih, T.-M., and McDonough, J.H. 2000. Efficacy of biperiden and atropine as anticonvulsant treatment for organophosphorus nerve agent intoxication. *Arch. Toxicol.* 74:165-172.
- Sidell, F.R. 1992. Clinical considerations in nerve agent intoxication. In *Chemical Warfare Agents*, ed. S.M. Somani, 155-194. San Diego: Academic Press.

Sim, V.M. 1975. Anticholinesterase poisoning. In *Cholinergic Mechanisms*, ed. P.G. Waser, 395-398. New York: Raven Press.

Taylor, P. 1996. Anticholinesterase agents. In *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., ed. J.G. Hardman, L.E. Limbird, P.B. Molinoff, R.W. Ruddon and A. G. Gilman, 161-176. New York: McGraw-Hill.

Vallejo-Freire, A. 1951. A simple technique for repeated collection of blood samples from guinea pigs. *Science*. 114:524-525.

Weese, C.B. 2001. Gulf War Syndrome: Questions, some answers, and the future of deployment surveillance. In *Chemical Warfare Agents: Toxicity at Low Levels*, eds. S.M. Somani and J.A. Romano, Jr., 261-299. Boca Raton: CRC Press.

Yokoyama, K., Araki, S., Murata, K., Nishikitani, M., Okumura, T., Ishimatsu, S., and Takasu, N. 1998. Chronic neurobehavioral and central and autonomic nervous system effects of Tokyo subway sarin poisoning. *J. Physiol. (Paris)* 92: 317-323.