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ABSTRACT

Percutaneous exposure to the chemical warfare nerve agent VX was evaluated in African green monkeys ($n = 9$). Doses of VX (7.5–100 μg/kg) were applied to the skin for 60 min and residual agent was quantified (before decontamination) to estimate the absorbed dose. Monkeys were evaluated for the presence or absence of clinical signs of toxicity and blood was sampled periodically (30 min–12 weeks) following exposure to measure the degree of circulating acetylcholinesterase (AChE) inhibition. Monkeys were also evaluated for behavioral changes from VX exposure using a serial probe recognition (SPR) task. The lowest observable adverse effect level (LOAEL) for the production of major clinical signs was determined to be 42.22 μg/kg (absorbed dose estimate = 17.36 μg/kg) and the LOAEL for AChE inhibition was 13.33 μg/kg (absorbed dose estimate = 6.53 μg/kg). Behavioral performance was unaffected at doses that, while producing substantial AChE inhibition, did not produce clinical signs. VX represents a substantial threat as a contact hazard and these results complement previous studies using the percutaneous route of exposure with VX and extend the findings to a non-human primate species.

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1. Introduction

VX (*O*-ethyl-*S*-[2-(diisopropylamino)ethyl]methylphosphonothiolate) is a fast-acting, highly potent organophosphorus chemical warfare nerve agent. VX, like the "G" agents such as soman (GD) or sarin (GB) can affect exposure through the inhalation route. VX can be distinguished from the "G" agents, however, in that it is more potent and less volatile at moderate temperatures and generally much more persistent in the environment (Sidell, 1997). Because of these characteristics, percutaneous exposure to VX is a more substantial threat (i.e., as a contact hazard) than with the "G" agents and is probably the route of exposure with the greatest risk of occurrence.

VX inhibits acetylcholinesterase (AChE) resulting in an increase in cholinergic activity that, when exposure is sufficient, produces a cascade of events that include muscle fasciculation, hypersecretion, tremor, convulsions, respiratory arrest and death (Holstege et al., 1997). While the effects of VX have been studied in many laboratory species including rats (Gupta et al., 1991), guinea pigs (Shih et al., 2005), chickens (Wilson et al., 1988), and rhesus monkeys (Raveh et al., 1997), typically, these studies have looked at an injection route of exposure, and sometimes, an inhalation route (Genovese et al., 2007a). Several studies, however, have investigated the effects of VX after percutaneous exposure. For example, van der Schans et al. (2003) evaluated the toxicokinetics of high doses of VX administered via intravenous or percutaneous routes in guinea pigs. Additional studies have looked at the electrophysiological and/or clinical manifestations of percutaneous administrations in guinea pig (Joosen et al., 2008; Mumford et al., 2008) and pig

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(Chilcott et al., 2003). We are unaware of any previously published studies in the literature evaluating a percutaneous exposure to VX in non-human primates. There has been at least one case of suspected VX exposure via a percutaneous route in humans (Nozaki et al., 1995).

The efficiency of the percutaneous route of exposure is susceptible to variability from a large number of factors influencing penetration rates such as the duration of the exposure and the general permeability of the skin, which can vary across species (see Hudson et al., 1979; Noakes and Sanderson, 1969) and can vary within species as a function of different exposure sites (Duncan et al., 2002; Lundy et al., 2004). Environmental temperature has also been shown to greatly influence the absorption of VX from the percutaneous route (Craig et al., 1977). An additional factor is whether the exposure site is occluded or whether the site is exposed to air (e.g., McElligott, 1972).

In the present study, we investigated the effects of percutaneous exposure to VX in African green monkeys. Rather than determining a lethal dose, our major objective was to characterize the threshold doses producing adverse effects defined in several ways. Specifically, we wanted to determine the threshold amounts of VX absorbed from the percutaneous exposures that would produce: (a) inhibition of circulating AChE, (b) mild or just noticeable clinical signs, and (c) major clinical signs and/or major behavioral performance deficits. In order to estimate the amount of VX absorbed, we applied measured amounts of VX to an exposure medium and then quantified the amount of unabsorbed VX remaining on the exposure medium and that wiped from the surface of the skin around the exposure site following a fixed duration (60 min) exposure. While not necessarily comprehensive, we implemented a number of procedures to hold other variables constant, such as using the same exposure area in all subjects. Additionally, to minimize the effects of vaporization of VX, we used an occluded exposure. Finally, we decontaminated the site after exposure using reactive skin decontamination lotion (RSDL) to ensure sufficient safety of the exposures that is necessary to conduct moderately long-term evaluations with the subjects. RSDL is approved by the US Food and Drug Administration for chemical decontamination and its use has been shown to be an effective decontamination procedure for VX (Bjarnason et al., 2008; Lundy et al., 2004).

2. Methods

2.1. Subjects

Nine female African green monkeys [*Chlorocebus sabeus* (formerly *Chlorocebus aethiops sabeus*)] weighing 3.0–4.3 kg were used. Monkeys were individually housed and maintained in a temperature (20–22° C)- and humidity (50 ± 10%)-controlled vivarium under a 12-h light–dark cycle (lights on at 0600). Seven subjects were ~10 years old and had previous pharmacological experience including organophosphorus compounds. The other two subjects were ~4 years old and were experimentally naïve. None of the subjects received any pharmacological agents other than that used for anesthesia during routine physicals for at least 6 months prior to participation in the study.

Water was available ad libitum and all subjects were fed commercial primate rations supplemented with fresh fruit, nuts, vegetables and vitamins. The diet also included banana-flavored nutritional pellets (190 mg, Bio-Serv, Frenchtown, NJ) earned during behavioral sessions. Animals were mildly food restricted to maintain performance motivation by regulating food intake outside of that earned during behavioral sessions.

2.2. Behavioral testing procedure

All subjects were tested behaviorally using a serial-probe recognition (SPR) task. The SPR is a list memory task wherein sequences of stimuli (sample stimuli) are presented. Subsequently, the subject is presented with a choice stimulus (probe) and, based upon substantial training, responds differentially depending upon whether or not the probe stimulus appeared in the previous sequence. Behavioral sessions were conducted with subjects unrestrained in their home cages using a custom manufactured, computer-controlled, behavioral test panel that contains an interactive touch screen monitor for the presentation of visual stimuli and detection of subject responses. Details of the apparatus and our implementation of the SPR have been published previously (e.g., Genovese et al., 2007b). Performance on the SPR is characterized by indices of accuracy, completed trials, and trial response time and thus, serves as a measure of cognitive and general performance. Sessions were normally conducted Monday–Friday at approximately the same time of day and consisted of 240 trials or until 120 min elapsed. On exposure days, the session began approximately 120 min following the end of the exposure. All subjects had previous experience with the SPR procedure before the start of the study.

2.3. VX exposure procedure

Chemical agent standard analytical reagent material *O*-ethyl-*S*-[2-(diisopropylamino)ethyl]methylphosphonothiolate (VX or EA 1701) (Lot #VX-U-7330-CTF-N) was stabilized by the addition of N,N-diisopropylcarbodiimide (DICDI) (3.34% by weight). Aliquots of the agent were placed into separate vials and refrigerated. Because undiluted VX is known to undergo autocatalytic hydrolysis (Yang et al., 1996), the purity of the agent was determined at the time of every exposure. That is, separate aliquots were opened for each exposure and a sample of the material, at the time of the exposure, was also analyzed by NMR.

For NMR purity analyses, a sample (0.1 ml) was placed into a vial and weighed to an accuracy of 0.1 mg. Internal standard triethyl phosphate (0.1 ml, >99.8% purity, Sigma–Aldrich, part no. 538728, CAS No. 78-40-0) was added and weighed. The mixture was then flame sealed in an insert and placed in a 5 mm tube with D_2O as a lock solvent, and the outer tube was flame sealed. The tube was analyzed by ^{31}P NMR for quantification of phosphorus compounds on a Bruker Avance 300 NMR.

Percutaneous VX exposures were conducted using either one or two sites on the skin. On the day before exposure, an area on the subject mid level on the shoulder blade and lateral to the spine was shaved using an electric veterinary clipper and wiped with isopropyl alcohol (IPA). Care was taken not to abrade the skin and the site was visually inspected to insure that abrasions had not occurred. For one-site exposures, the left side of the subject was used and for two-site exposures the left and right sides of the subject were used. Exposures were conducted using a round stainless steel disc (16 mm diameter and 1.5 mm thick). One side of the disc was first affixed to an Activ-Flex Band Aid (Johnson and Johnson, Inc., Skillman, NJ). A measured amount of VX was then applied to the disc using a calibrated digital syringe (Hamilton Company, Reno, NV) capable of accurately delivering nanoliter (nL) volumes (or μg quantities).

Subjects were taken from the home cage using a pole and collar technique (Anderson and Houghton, 1983), placed in a primate restraining chair, and then into a 2000 L Hazleton exposure chamber with an ambient temperature of 21 °C (±3). The bandage containing the exposure disc was then affixed to the area on the subject previously prepared. Sufficient pressure was applied during the application of the exposure disc to ensure full contact of the disc with the skin surface. In this regard, the exposure disc appeared to have retained good contact with the skin upon its removal at the end of the exposure in each case. The duration of the exposure was 60 min. The entire exposure session was observed, via a video camera and monitor, from a room adjacent to the exposure chamber.

Following the 60 min exposure, the disc was removed from the subject and placed into a scintillation vial for quantification of residual VX. After removing the exposure disc, five IPA-soaked cotton applicators were used sequentially to swab the exposure area. Each swab was placed in a test tube containing IPA for quantification of residual VX. Subsequently, reactive skin decontamination lotion (RSDL) (E-Z-EM Inc., Lake Success, NY) was applied to the exposure site and surrounding area to decontaminate any remaining VX. The subject was then transported back to a holding room for a post-exposure blood draw and then returned to the home cage. Identical procedures were followed for two-site exposures with residual analyses conducted for each disc and swabs at each site.

Residual VX (that retained on the exposure disc and the swabs following exposure) was quantified utilizing isotope dilution and LC/MS/MS analysis conducted on an Agilent Technologies 1100 series Liquid Chromatograph/6410 Triple Quadrupole mass spectrometer (Agilent Technologies, Wilmington, DE). A ten point linear VX calibration curve was developed from analysis of standards ranging from 5.0 to 1000 ng/ml, with 250 ng/ml of the internal standard (IS) $^2H_5$-*O*-ethyl-*S*-[2-(diisopropylamino)ethyl]methylphosphonothioate ($^2H_5$-VX) in each standard. Injections (1 μl) were made with a constant flow of 1 ml/min through a ZORBAX Eclipse XDB-C18, 4.6 mm × 150 mm (5 μm) analytical HPLC column with a C18 precolumn filter. The solvent gradient program was initially composed of 50% organic phase (0.1% formic acid in methanol) and 50% aqueous phase (0.1% formic acid in deionized water). This was then changed with a linear ramp to a composition of 99% organic/1% aqueous at 3 min and held for 1 min, then reversed back to the initial composition from 4 to 5 min. The target compound and internal standard peak eluted at 1.95 min. The MS/MS was equipped with an electrospray ionization source under positive polarity. The nitrogen drying gas temperature was 350 °C at a flow of 10 L/min, a nebulizer pressure of 35 psi, and a capillary voltage of 4000 V, and was operated in multiple reaction monitoring mode (MRM). For the target analyte VX, the MRM program monitored one transition for quantitation (m/z 268 > 128) and one for confirmation (m/z 268 > 86), and for the internal standard $^2H_5$-VX (synthesized in-house), one transition was monitored for quantitation (m/z 273 > 128) (see McGuire et al., 2008). Following analysis of the standards, a weighted regression line ($1/x$) was generated from the response factor (area ana-

lyte × (concentration IS/area IS)) as a function of analyte concentration, and was used to quantify VX. Calibration standards were analyzed weekly and stored frozen where they remained stable for up to 6 months. Quality control samples were analyzed each day with a high, medium, and low standard analyzed along with a matrix spike. These QC samples were required to have <15% error from the target value. An IPA blank and a matrix blank were also run to ensure no interfering peaks were evident.

Exposure disc samples were prepared for analysis by placing the items into separate 20 ml glass scintillation vials with 5 ml of IPA and sonicated for 20 min. The swab samples were placed into test tubes containing 1 ml of IPA and sonicated for 20 min. An aliquot was removed from each vial/tube and internal standard was added to achieve 250 ng/ml of $^2H_5$-VX before analysis by LC/MS/MS.

Control exposure sessions were conducted at least seven days prior to VX exposure using distilled water and the same general exposure methods. Control sessions, however, did not take place in a chamber used for VX exposures and IPA and RSDL swabbing was not conducted after a control session. While the use of RSDL as part of a control session was considered, we wanted to avoid the possibility that any residual RSDL (either on or under the skin) might be present during the subsequent exposure session.

All subjects received a single control exposure and a single VX exposure. VX doses were administered using a modified up-and-down regimen (Bruce, 1985). Decision criteria of the presence of one or more major signs of toxicity, or a substantial decrease in SPR performance, were considered when determining an increase or decrease in subsequent exposure dose. With respect to the latter criterion, a substantial decrease in performance on the SPR was considered to occur if a 35% or greater change in the number of completed trials or average response time, or a 20% or greater change in accuracy, was observed. These values were chosen since they were clearly outside the normal range of baseline performance observed during the two weeks prior to exposure. Thus, the decision to increase or decrease the dose for a subject, with regard to the dose administered to the previous subject, was based, in large part, on whether the effects of the concentration used with the previous subject had reached the aforementioned criteria. Six subjects received VX and control using one exposure site (i.e., one disc) and three subjects received VX and control using two exposure sites (i.e., two discs). The following applied doses [i.e., the total amount placed on the exposure disc(s)] of VX in μg/kg body weight were administered: single-site, 7.50, 13.33, 23.71, 31.62, 42.22, 100.00 and two-site, 13.33, 23.71, 42.22.

2.4. Subject observation procedure

All subjects were specifically monitored for clinical signs of organophosphorus toxicity for at least 4 h following the beginning of the VX exposure. Subjects were specifically evaluated for the presence of the following signs: ataxia, convulsions, fasciculation, hyperactivity, rhinorhea, salivation, tremors, lacrimation and prostration. Subjects were also evaluated for any other signs that appeared to be unusual. Additionally, subjects were observed daily, throughout the experiment, for any general signs of ill health.

2.5. Blood sampling and cholinesterase assay procedure

Subjects were placed in a primate restraining chair for blood sampling. Approximately 1.25 ml of blood was collected from the lateral saphenous vein into sample tubes containing EDTA (7.5%), at the following time points relative to the end of the 60-min VX exposure: 30 min, 4 h, 25 h, 72 h, 8 days, 4 weeks, 8 weeks, and 12 weeks. Additionally, blood was collected 30 min after control exposures that occurred at least one week before the VX exposure for each subject. Assays for AChE and butyrylcholinesterase (BChE) activity were conducted using whole blood. Details of the procedure which is a modification of the Ellman method (Ellman et al., 1961) have been presented previously (Dabisch et al., 2005). Circulating AChE and BChE activity was measured as units of activity per ml of whole blood (U/ml).

3. Results

VX purity remained stable throughout the study and NMR analyses of samples of the agent at the time of each exposure revealed the average purity to be 92.16% by weight (range = 88.21–94.80). Fig. 1 shows the relationship between the amounts of VX placed on the exposure disc(s) and the estimated amount absorbed. In this regard, the estimated amount absorbed is calculated by subtracting the amount of VX quantified on the exposure discs and the amount of VX quantified from the post-exposure swabs of the skin around the exposure site, from the amount of VX applied to the exposure disc(s). A strong positive correlation between these measures was observed for both one-site and two-site exposures ($r = .99$ and $r = .79$, respectively). The mean proportion of the applied amount that was estimated to have been absorbed was

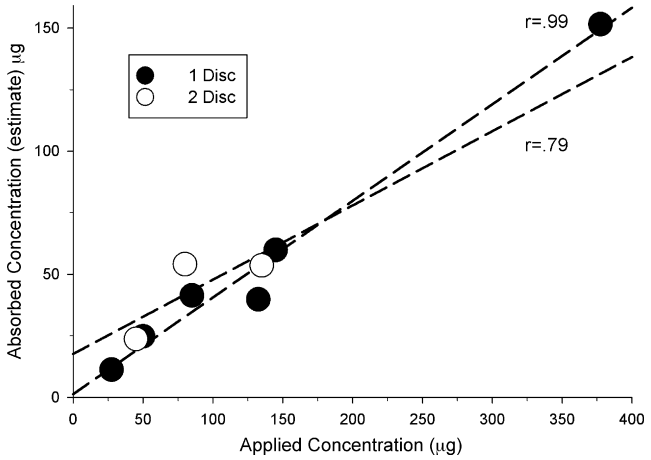

Fig. 1. Relationship of applied dose of VX to absorbed dose (estimated). Each point represents data from a single exposure in a single subject. Absorbed dose was estimated by subtracting the residual amount of VX quantified from the swabs at the exposure site and that remaining on the exposure discs, from the amount of VX applied to the discs. Solid symbols represent exposures using one site and open symbols represent exposures using two sites. Dashed lines represent a linear fit with corresponding Pearson's correlation coefficient (top value is for one-site exposures and bottom value is for two-site exposures).

41.8% (range = 30.0–49.6) for the one-site exposures and 53.3% (range = 39.7–67.6) for the two-site exposures.

Table 1 summarizes the clinical signs of the one-site VX exposures. The largest exposure (absorbed dose = 39.89 μg/kg) produced profound clinical signs of toxicity that included severe tremor, salivation, fasciculation and respiratory disruption. Rescue therapy was administered to this subject and although the subject survived, recovery was minimal. A detailed report on that subject appears in Appendix A. The next lowest dose examined (absorbed dose = 17.36 μg/kg) also produced substantial clinical signs, including salivation and tremor, and rescue therapy was provided. Additionally, a nearly complete inhibition (~95%) of circulating AChE was observed at 30 min post exposure. At this dose, however, recovery was complete with a normal appearance within 24 h. Lower doses (absorbed doses of 9.48 μg/kg and 11.34 μg/kg) did not produce any clinical signs other than local fasciculation at or around the exposure site. These two doses, however, did produce a substantial decrease in circulating AChE activity. While also producing a substantial decrease in circulating AChE activity, an absorbed dose of 6.53 μg/kg did not produce any clinical signs of toxicity. The lowest dose tested (absorbed dose = 2.92 μg/kg) did not produce any clinical signs and the impact on circulating AChE was negligible.

Three doses of VX tested (42.22 μg/kg, 23.71 μg/kg and 13.33 μg/kg) were also administered using two exposure sites (i.e., by splitting the application between the two exposure discs). These applied doses resulted in absorbed doses of 16.74 μg/kg, 16.39 μg/kg and 7.08 μg/kg, respectively. Thus, the proportion of the applied dose that was absorbed in the two-site exposures was similar to that of the one-site exposures (see Table 1). None of the two-site exposures, however, produced any clinical signs of toxicity, including local fasciculation. Furthermore, while all three of the two-site exposures produced inhibition of circulating AChE, the peak amount of AChE inhibition observed (67.8%, 15.2% and 13.6%, respectively) was less than that observed with the corresponding one-site exposures.

Except for the two highest doses of VX examined, SPR performance was not substantially degraded after all other doses. That is, performance on the SPR on the day of exposure and subsequent days did not meet the criteria for disruption (see Section 2) and was, in general, within the range of baseline values for accuracy,

Table 1
Effects of single percutaneous (one-site) exposures to VX in African green monkeys.

Absorbed dose (μg/kg)	Applied dose (μg/kg)	Effects	Rescue therapy	Threshold
39.89	100.00	Clinical signs within 30 min of exposure. Severe signs by the end of the 60 min exposure including whole body tremors, profuse salivation, fasciculation, prostration and shallow/irregular/labored breathing. Minimal recovery observed.	Atropine, 2-PAM, diazepam administered multiple times	
17.36	42.22	Clinical signs including salivation, tremor and fasciculation. AChE inhibition ~95% at 30 min post exposure. Recovery (including behavioral performance) 24 h post exposure.	Atropine, 2-PAM, diazepam administered at 2 h post-exposure	*LOAEL* – major clinical signs
11.34	23.71	Local fasciculation. No other clinical signs. No SPR deficit. Peak AChE inhibition ~65%, 4 h post-exposure.	None	
9.48	31.62	Local fasciculation. No other clinical signs. No SPR deficit. Peak AChE inhibition ~59%, 4 h post exposure	None	*LOAEL* – minor clinical signs
6.53	13.33	No clinical signs. No SPR deficit. Peak AChE inhibition ~35%, 4 h post exposure.	None	*LOAEL* – AChE inhibition
2.92	7.50	No clinical signs. No SPR deficit. Peak AChE inhibition ~8%, 4 h post exposure.	None	

completed trials, and trial response time. The subject receiving the highest dose had persistent deficits in function (see Appendix A) which precluded further SPR testing after exposure. The subject receiving an absorbed dose of 17.36 μg/kg was symptomatic and received IM injections of atropine sulfate (0.29 mg/kg), 2-PAM HCl (17.4 mg/kg) and diazepam (1.0 mg.kg) after the exposure as an antidote treatment and no SPR session was conducted on the day of exposure. Performance on the following day, and subsequent days, was not degraded. In general, all surviving subjects continued to maintain performance on the SPR during the weeks following exposure and no delayed performance deficits were observed.

Average cholinesterase activity measured from blood sampled after control exposure sessions was 3.90 U/ml (±0.12 SEM) for AChE and 0.47 U/ml (±0.03 SEM) for BChE. Fig. 2 shows AChE activity (as a percentage of control values) from blood samples taken 30 min, 4 h and 25 h following exposure in all subjects except the subject receiving the highest dose (see Appendix A). VX exposures produced dose-dependent decreases in circulating AChE activity with positive correlation coefficients observed between dose and activity at all three time points. AChE inhibition was, in general, greater at 4 h after exposure as compared to either 30 min or 25 h after exposure. One subject receiving a 17.36 μg/kg absorbed dose showed peak inhibition of AChE activity at 30 min post exposure. That subject, however, received rescue therapy that included the oxime 2-PAM HCl (which would be expected to regenerate VX-inhibited AChE) after the 30 min post-exposure blood sample but before the 4 h blood sample. Additionally, the one-site exposures produced more AChE inhibition than the closest counterpart absorbed dose administered as a two-site exposure in every case, except for the aforementioned subject after 2-PAM HCl was administered. Recovery of AChE activity was complete in all surviving subjects by 4 weeks following exposure with the mean activity of 101.78 (±3.58 SEM) percent of control values. Fig. 3 shows the corresponding BChE activity values (as a percentage of control values) from blood samples taken at the same time points as Fig. 2. VX exposures also produced dose-dependent decreases in circulating BChE activity with positive correlation coefficients observed between dose and activity at all three time points. As with AChE, BChE inhibition was, in general, greater at 4 h after exposure as compared to either 30 min or 25 h after exposure. In general, however, BChE inhibition appeared to be less than for AChE. Also similar to what was observed with AChE, the one-site exposures generally produced more BChE inhibition than the closest counterpart absorbed dose administered as a two-site exposure.

4. Discussion

African green monkeys were administered high purity VX using a fixed duration percutaneous route of exposure that allowed for the estimation of the absorbed dose of VX. The percutaneous route of exposure is particularly susceptible to variability from a large number of factors and any laboratory model of percutaneous exposure is subject to qualifications that must be considered when extrapolating the results outside of the laboratory and for comparison to other laboratory studies. In the present study, we were able to estimate an absorbed dose of VX by quantifying the amount of VX that could be unequivocally determined to not have been absorbed (i.e., the residual) and subtracting that amount from the amount applied to the exposure site. The estimate, however, does not account for the amount of VX that was decontaminated by the RSDL applied after the 60 min exposure. Nevertheless, we believe that this procedure represents a substantial increase in accuracy for determining dose-effect relationships with percutaneous exposures to VX.

Clinical signs, circulating AChE and BChE, and cognitive behavior were used to evaluate adverse effects. From the results, threshold doses can be determined as the lowest observable adverse effect level (LOAEL) for three categories of adverse effects as described in Table 1. First, a 6.53 μg/kg absorbed dose is the LOAEL for the inhibition of circulating AChE. This dose resulted in a peak inhibition of ~35%. The dose below this level produced a small amount of inhibition (~8%) but can generally be considered to be within a range of measurement error and normal day-to-day variability. The 9.48 μg/kg absorbed dose is the LOAEL for minor clinical signs. In this regard, the only minor clinical sign observed was muscular fasciculation at and around the site of exposure. The 17.36 μg/kg absorbed dose is the LOAEL for major clinical signs. The subject receiving this dose showed the major clinical signs of salivation, tremor and fasciculation and 2 h following the end of the exposure the subject was treated with rescue therapy. Recovery, however, was observed on the following day with all clinical signs resolving and behavioral performance on the SPR task not showing a deficit.

The effects of the highest absorbed dose (39.89 μg/kg) evaluated were severe, and although the subject survived, this dose can be considered to be in the lethal range, as extensive rescue therapy was implemented and it is reasonable to infer that such therapy enabled survival. Furthermore, this dose produced persistent effects, including severe muscular dysfunction, and muscular paral-

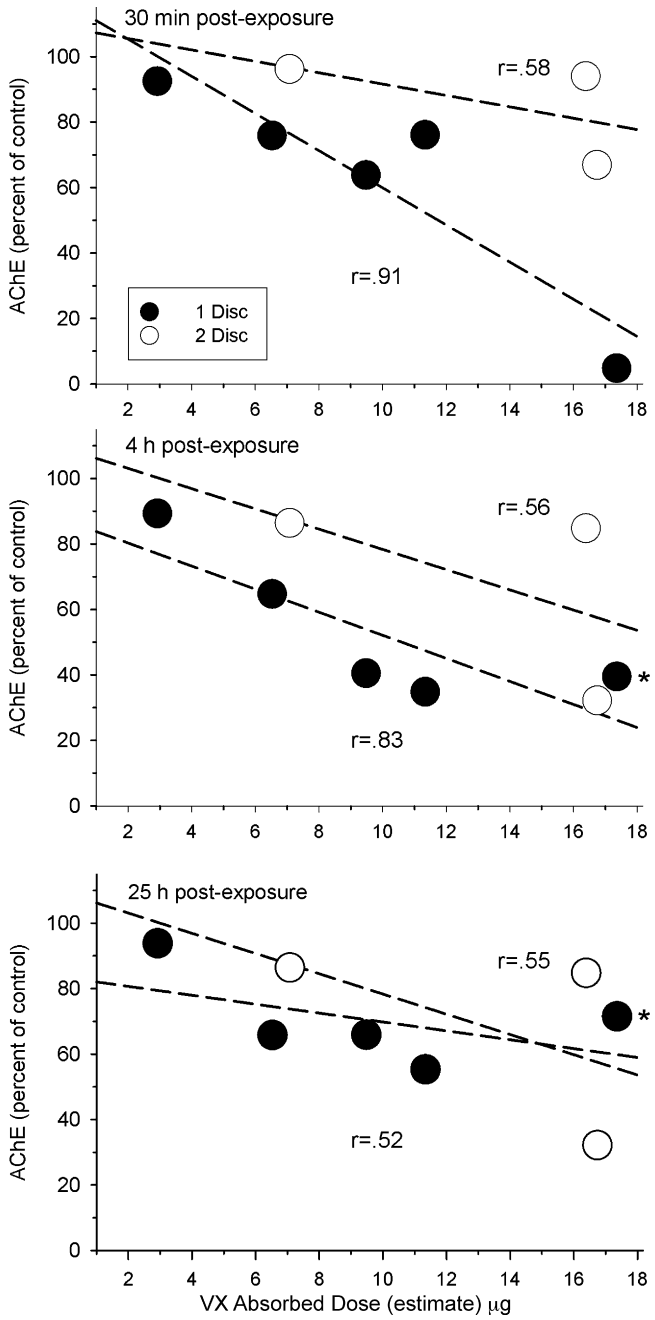

Fig. 2. Circulating AChE activity in blood sampled 30 min (top), 4 h (middle) and 25 h (bottom) following a 60 min percutaneous exposure to VX in African green monkeys. Abscissae: estimated absorbed dose of VX (μg/kg). Activity is expressed as a percentage of each subject's control activity (U/ml) assessed during a control exposure occurring at least seven days prior to VX exposure. Each point represents the value from a single subject. Closed symbols represent exposures with one site and open symbols represent exposures with two sites. Dashed lines represent a linear fit with corresponding Pearson's correlation coefficient (lower value corresponds to closed symbols and upper value corresponds to open symbols). Asterisks denote that the subject had received rescue therapy including the oxime 2-PAM HCl before the sample was drawn.

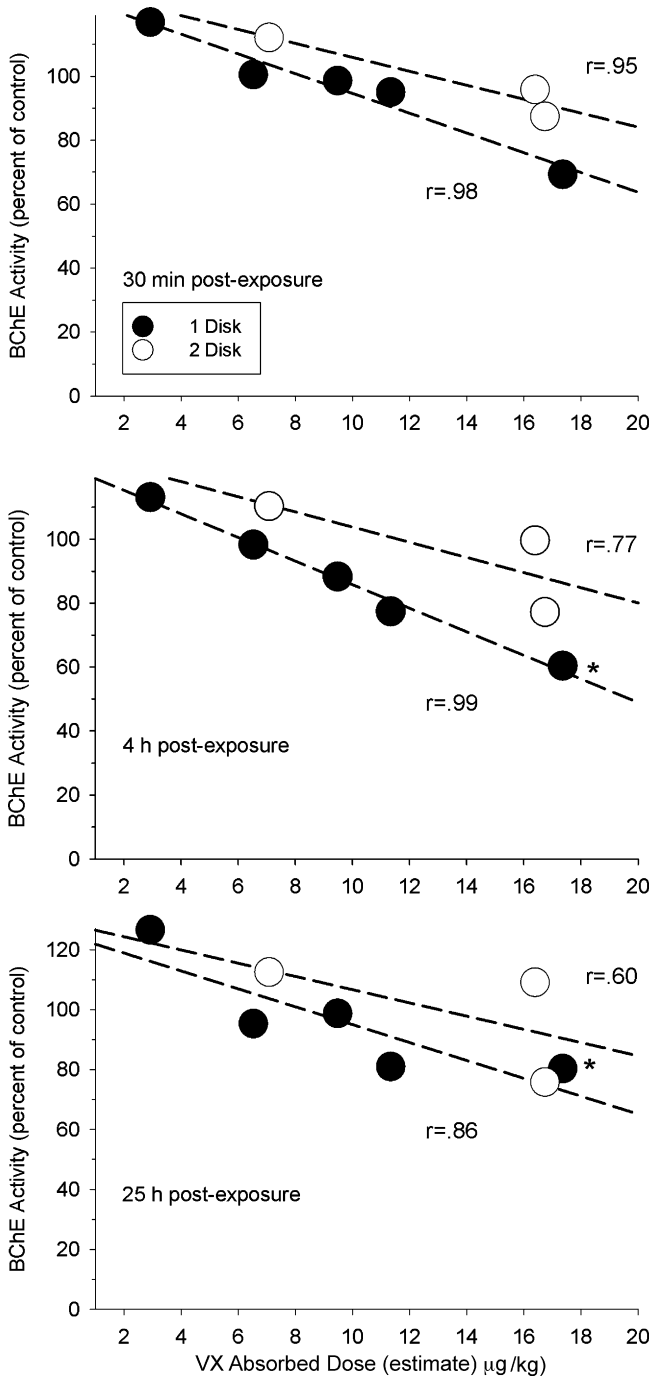

Fig. 3. Circulating BChE activity in blood sampled 30 min (top), 4 h (middle) and 25 h (bottom) following a 60 min percutaneous exposure to VX in African green monkeys. Abscissae: estimated absorbed dose of VX (μg/kg). Activity is expressed as a percentage of each subject's control activity (U/ml) assessed during a control exposure occurring at least seven days prior to VX exposure. Each point represents the value from a single subject. Closed symbols represent exposures with one site and open symbols represent exposures with two sites. Dashed lines represent a linear fit with corresponding Pearson's correlation coefficient (lower value corresponds to closed symbols and upper value corresponds to open symbols). Asterisks denote that the subject had received rescue therapy including the oxime 2-PAM HCl before the sample was drawn.

ysis has been reported previously from VX in guinea pigs (Bide et al., 2005). Subsequent histology revealed CNS pathology not unlike that seen following high dose exposure to the nerve agent soman (see Appendix A). While experimental procedures vary across studies, the absorbed dose administered to this subject is well below the LD50s of 79.6 μg/kg for percutaneous VX in swine (Bjarnason et al., 2008), 60 μg/kg reported for the white pig (from Chilcott et al., 2003) and 125 μg/kg in the guinea pig (van der Schans et al., 2003). In one sense, these results suggest that primates may be more sensitive than the pig, guinea pig or swine. The 39.89 μg/kg absorbed dose in the monkey, however, resulted from a 100 μg/kg applied dose – a value more consistent with the aforementioned studies

where the LD50 values also refer to applied doses and where no absorbed dose estimates were made.

Three of the doses evaluated were administered using one and two exposure sites in separate subjects. The absorbed doses from equivalent applied doses were similar, but not the same in these cases. Nevertheless, in general, all three of the two-site exposures produced less AChE inhibition than the nearest equivalent absorbed dose for a one-site exposure. Furthermore, none of the two-site exposures produced any clinical signs. The contrast was most pronounced at the 42.22 μg/kg applied dose, which resulted in less than a 0.65 μg/kg difference in absorbed dose, but produced clinical signs when administered with a one-site exposure and no clinical signs when administered as a two-site exposure. It is generally thought that two factors relating to the degree of absorption for the percutaneous route are the concentration gradient and the surface area of the exposure (see Hudson et al., 1979). In the present comparison, the two-site exposure could be expected to have a greater surface area but, perhaps, a lower concentration gradient than the analogous one-site exposure, suggesting that concentration gradient is a dominant factor in this instance. It is notable, however, that approximately twice as much RSDL was used for the decontamination with the two-site exposures. Since RSDL is known (see Manufacturer Safety Data Sheet) to be absorbed beneath the skin, it is possible that a greater amount of VX below the skin was decontaminated after the exposure in the two-site instance. In the absence of determining the exact absorbed doses, only limited conclusions can be drawn in the present case.

As expected, VX produced a dose-dependent decrease in circulating AChE activity. Within the time points sampled, peak inhibition was generally noted 4 h after the exposure, but was still substantial 25 h after the exposure. These results are consistent with the relatively slow rate of AChE inhibition from percutaneous VX exposure observed in previous studies (e.g., van der Schans et al., 2003). It is notable that, as expected, in the cases where the oxime 2-PAM HCl was administered as part of a rescue therapy, the inhibition appeared to lessen. Recovery of AChE activity was a relatively gradual process but was complete with all surviving subjects, showing a return to pre-exposure levels of AChE activity when blood was sampled 4 weeks following the exposure. In general, the effects of VX on BChE were similar, albeit to a lesser extent, to those observed for AChE. That is, VX also produced a dose-dependent decrease in circulating BChE activity and, within the time points sampled, peak inhibition was generally noted 4 h after the exposure.

Cognitive and general performance measures from the SPR behavioral task were unaffected by any doses of VX that did not produce major clinical signs. Even when circulating AChE was substantially inhibited (35–65%) when blood was sampled 1.5 h before the behavioral session and approximately 1 h after the behavioral session, performance on the SPR task was maintained. This result is consistent with earlier studies in which SPR performance was maintained with equivalent and even greater AChE inhibition following exposure (either vapor or IM injection) to the nerve agents soman and sarin in African green and rhesus monkeys (Genovese et al., 2007b, 2008, 2010). Clearly, a substantial decrease in circulating AChE activity alone does not correlate with a performance decrease. In the case of the highest symptomatic dose tested, the general health of the subject precluded further testing on the SPR task. In the other case when the VX dose was symptomatic, treatment precluded testing on the day of exposure, but SPR performance on the next (and subsequent) days did not show a deficit, further evidencing that recovery was substantial in that subject. For the duration of the study we did not observe any case of a delayed onset deficit on the SPR task.

The results of the present study characterize the effects of VX from the percutaneous route of exposure in African green monkeys. As a chemical warfare nerve agent, the characteristics of VX make it a likely risk as a contact hazard, and these results extend results from previous studies using the percutaneous route with rodents and swine to a non-human primate species. Threshold exposure doses for multiple adverse effect criteria, including major clinical signs, were determined and expressed as the applied dose and also as an estimate of the absorbed dose. The latter approach, in particular, can facilitate interpolation of the results to other laboratory studies and extrapolation of the results to humans.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Case report for animal receiving percutaneous VX (39.89 μg/kg estimated absorbed dose)

Subject was a healthy adult female (~10 years of age) weighing 3.8 kg with no previous history of clinical disease and serologically negative for Simian retrovirus (SRV), Simian immunodeficiency viruses (SIV), Simian T-cell leukemia (STLV), and Simian foamy virus (SFV). The subject received a percutaneous exposure to 100 μg/kg (377.5 μg) VX. Following the 60 min exposure, the residual amount of VX (that which was quantified on the application disc and from IPA swabs from the skin around the exposure site) following the exposure was 225.9 μg, yielding an absorbed dose estimate of 39.89 μg/kg.

Approximately 30 min after the start of the exposure, chewing and tremor were observed and at 50 min after the start of the exposure salivation was also observed. Symptom severity increased dramatically during the last minutes of the exposure, with shallow and irregular breathing noted and prostration occurring in the last 10 min of the exposure, followed by subsequent collapse. Convulsions were not observed at any time.

After removal of the applicator and decontamination of the exposure site with RSDL, rescue therapy began and included three successive IM administrations of atropine sulfate (0.1 mg/kg × 3). Additionally, 2-PAM HCl (30 mg/kg) and diazepam (4 mg/kg) were also administered IM. Additional injections of atropine sulfate (0.1 mg/kg) were given approximately 60 min and 4 h following the end of the exposure. Eight hours following the exposure, atropine sulfate (0.05 mg/kg) and 2-PAM HCl (30 mg/kg) were again administered IM. Lactate Ringer's solution was administered (approximately 75 ml total) in several injections 2–8 h post-exposure. At approximately 12 h post-exposure, major symptoms had subsided.

At this time, the subject exhibited clinical signs of generalized weakness, and slight dyspnea with an inability to maintain normal erect posture or to initiate the voluntary intake of food or water, but did retain the ability to ingest food and water if presented orally. The second day following exposure the subject showed slight improvement in condition, being more alert and responsive with the ability to maintain an approximately normal sitting posture but still exhibiting muscular weakness and reluctance to eat or drink without assistance. The next four days the subject showed slight improvement with increased awareness and energy to more actively take food when directly offered, but still did not initiate

feeding or drinking. Although the subject regained some muscular strength and was able to move around the pen, the subject lacked coordination, appeared to have some degree of hind-limb paralysis and fatigued quickly, returning to a recumbent position.

AChE activity from blood sampled approximately 4 h post-exposure (and notably, after 2-PAM HCl administration) was measured at 27.7% of control values. Activity from samples taken 72 h, 6 d and 10 d following exposure were observed to be 89.8%, 73.8% and 90.5% of control values, respectively.

Serum chemistry analysis taken six days after exposure showed an increase of aspartate aminotransferase (AST) (386 U/L), alanine aminotransferase (ALT) (205 U/L), lactate dehydrogenase (LDH) (7146 U/L) and creatinine kinase (CK) (14,352 U/L). Although elevations in AST, ALT and LDH can be associated with hepatocellular injury, the combination with CK deviations suggests degeneration or necrosis with increased membrane permeability from myopathy. Serum chemistry also showed a slight azotemia (44 mg/dl) without corresponding increase of creatinine consistent with the effects of anorexia. On the eighth day after exposure, the clinical presentation reversed, with return to generalized lethargy, recumbent posture and hypothermia (96.4 °F). A second serum chemistry analysis (with hemolysis present) showed further elevation in AST (791 U/L), ALT (235 U/L), an elevation of total bilirubin (1 mg/dl), but a decline in LDH (3118 U/L) and CK (12,000 U/L).

Ten days following the exposure, the decision was made to perform euthanasia. The animal was placed in a deep surgical plane and was euthanized using intravenous injection of sodium pentobarbital. Upon verification of death, a thoracotomy was performed to expose the heart. Exsanguination began by opening the right atrium. The left atrium was cannulated to begin transcardial perfusion. Heparinized saline (500 ml) was infused via the left ventricle as a buffered clearing solution and followed by 2 L of the fixative solution (Bouin's fluid). Following perfusion the brain was removed intact and placed in formalin for 24 h. Using the anterior commissure (ac) as a landmark, the brain was serially sectioned and routinely processed for histological examination using hematoxylin and eosin (HE).

A full necropsy was performed with the following tissues examined grossly: brain, heart, lung, liver, digestive tract, kidneys, and skeletal muscle from both arms and legs. There were no gross lesions in any of the tissues examined. Sections of the left brain at 10, 7, and 3 mm rostral to ac and 2, 7, 9, 11, and 13 mm caudal to ac were examined microscopically. In all sections examined there were multifocal areas of neuronal necrosis. Neurons were shrunken and hypereosinophilic with pyknotic nuclei (see Fig. 4A). Surrounding neuropil was often vacuolated and there was prominent gliosis (Fig. 4B). Occasionally multiple glial cells surrounding degenerate neurons (neurophagocytosis) were observed (see Fig. 4A). Neuronal changes were predominately confined to the deeper layers of the neocortex and constituted less than 10% of neurons examined. Occasionally neuronal necrosis was evident in other areas such as CA1 of the hippocampus. In general, however, neurons outside of the cortex were relatively unaffected. White matter lesions were mild and included axonal degeneration characterized by vacuolation, axonal swelling and digestion chambers (see Fig. 4C). These lesions were most evident in the longitudinal pontine fibers but more rarely were evident in the corpus callosum and other white matter tracks. Subsequent to these findings the corresponding sections of right brain were similarly examined. Lesions were bilateral but not necessarily symmetrical.

The histopathologic brain lesions in this animal are consistent with previous reports of soman-induced neurotoxicity in rhesus macaques. The characteristics of the neuronal necrosis were morphologically consistent with nerve agent-induced hypoxic-ischemic injury as reported by Petras (1994) and Baze (1993). In this case, however, the extent of the lesions was milder and the distribution appears more limited. This result likely reflects, and is consistent with, the clinical signs exhibited by this animal, which did not include convulsions, as well as the extensive rescue therapy administered. In this regard, it is notable that the presence of convulsions following nerve agent exposure has been correlated with a more extensive neuronal necrosis (e.g., Baze, 1993). Furthermore, this case illustrates that a symptomatic, non-convulsive and surviving exposure to nerve agent VX produces detectable and consistent histopathologic lesions observable 10 days following exposure.

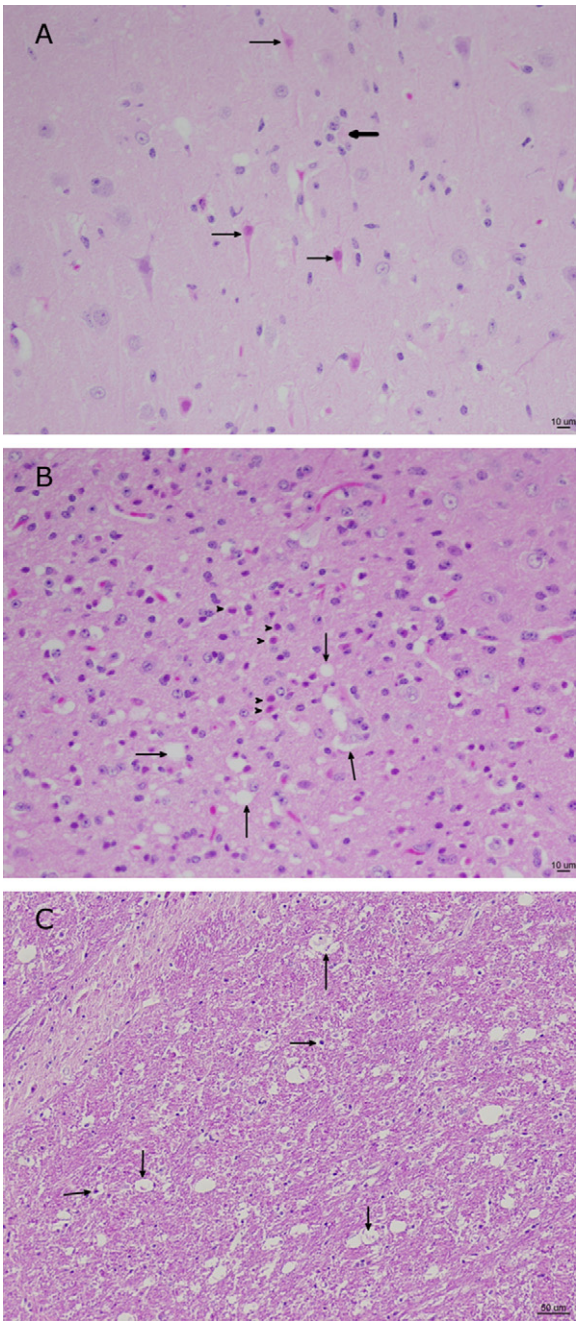

Fig. 4. Photomicrographs depicting various types of neuronal damage in an African green monkey following percutaneous exposure to VX. (A) CA1 hippocampus: multiple neurons are shrunken and hypereosinophilic with pyknotic nuclei (thin arrows). Degenerating neuron is surrounded by phagocytic cells (neuronophagia) (thick arrow). (B) Cerebral cortex, ac +10: vacuolation of the neuropil (arrows) with prominent gliosis and multiple necrotic neurons (arrow heads). (C) Longitudinal pontine fibers: white matter tracks are vacuolated and contain cellular debris (arrows). All sections are HE.

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