Sensorimotor Deficit and Cholinergic Changes following Coexposure with Pyridostigmine Bromide and Sarin in Rats

Mohamed B. Abou-Donia,*† Anjelika M. Dechkovskaia,* Larry B. Goldstein,‡§ Sarah L. Bullman,‡ and Wasiuddin A. Khan*

*Department of Pharmacology and Cancer Biology and †Department of Medicine (Neurology), Duke University Medical Center, Durham, North Carolina 27710; and ‡Veterans Administration Medical Center, Durham, North Carolina

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A myriad of neurological symptoms including muscle and joint pain, ataxia, chronic fatigue, headache, and difficulty in concentration have been reported by Persian Gulf War (PGW) veterans. A large number of these veterans were prophylactically treated with pyridostigmine bromide (PB) and possibly exposed to sarin. In the present study we investigated the effects of PB and sarin, alone and in combination, on sensorimotor performance and the central cholinergic system of rats. Male Sprague-Dawley rats were treated with PB (1.3 mg/kg, 15 daily doses, oral) and sarin (50, 75, 90, and 100 μg/kg, single im dose on day 15), alone and in combination. The animals were evaluated for postural reflexes, limb placing, orienting to vibrissae touch, incline plane performance, beam-walk time, and forepaw grip time 7 and 15 days following treatment with sarin. Treatment with either PB or sarin alone resulted in significant sensorimotor impairments. Coexposure to sarin and PB resulted in significant sensorimotor deficits that worsened over time. By 15 days following sarin treatment, plasma butyrylcholinesterase (BChE) activity returned to normal levels in the animals treated with sarin alone, whereas in the animals exposed to PB or PB plus sarin, there was an increase in the enzyme activity. Cortical acetylcholinesterase (AChE) activity remained inhibited in the animals treated with sarin alone and in combination with PB. Muscarinic acetylcholine receptor (m2 mAChR) ligand binding with [3H]AFDX-384 in cortex and brain stem showed significant increases (~120–130% of control) following coexposure to PB and sarin at higher doses. To evaluate the potential of PB for augmentation or inhibition of the toxicity induced by acute sarin exposure, the animals were exposed to either 10 or 100 μg/kg sarin (single im injection) with or without pretreatment with PB, and sacrificed 3 h after treatment with sarin. Pretreatment with PB offered slight protection in the plasma as well as brain regional enzyme activities. Pretreatment with PB did not have any effect on sarin-inhibited brain regional AChE activity following treatment with 100 μg/kg sarin. These results show that prophylactic treatment with PB offers some degree of protection in peripheral cholinesterase. Furthermore, these results show that treatment with either sarin or PB alone resulted in sensorimotor impairments, while coexposure to high doses of sarin with PB caused an exacerbated deficit.

Key Words: sarin; pyridostigmine bromide; PB; acetylcholinesterase; muscarinic acetylcholine receptor; neurotoxicity; combined exposure; sensorimotor; Gulf War.

Since their return from the war, many Persian Gulf War (PGW) veterans have complained of symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability (Institute of Medicine, 1995, 2000), as well as sensorimotor complaints including numbness or tingling, weakness, and heaviness of the arms and legs (Knoke et al., 2000). These veterans were exposed to a unique combination of biological, chemical, and psychological environments (Caldwell, 1992; Institute of Medicine, 1995, 2000). Combinations of chemical exposures included a variety of pesticides and nerve agent sarin (Institute of Medicine, 1995, 2000; McCauley et al., 2001). In addition, a majority of U.S. service personnel were given pyridostigmine bromide (PB) as a prophylactic against possible nerve gas attack (Cook et al., 1992; Golomb, 1999). Sarin, an organophosphate agent, and PB, a quaternary dimethyl carbamate, primarily affect the cholinergic system. PB binds to peripheral cholinesterase and thus shields the enzyme from sarin-induced inhibition.

PB has been used as a treatment for myasthenia gravis at a higher dose range than what was given to Persian Gulf War Veterans (Breyer-Pfaff et al., 1985, 1990). The veterans were given a course of twenty-one 30-mg tablets of PB as prophylaxis against organophosphate (OP) nerve agents (Institute of Medicine, 1995, 2000; Persian Gulf Veterans Coordinating Board, 1995). At this dose, PB reversibly inhibits 30–40% of the AChE in the peripheral nervous system, thus limiting irreversible inhibition of the enzyme by nerve agents (Blick et al., 1991). AChE activity is restored following spontaneous decarboxylation resulting in normal neuromuscular and autonomic functions (Blick et al., 1991; Watts and Wilkinson, 1977). Toxic symptoms associated with PB overdose result from overstimulation of nicotinic and muscarinic receptors in the peripheral nervous system, causing exaggerated cholinergic
effects such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Dinia et al., 1996; Cook et al., 1992). With severe intoxication, death may occur because of asphyxia. The positive charge on the quaternary pyridinyl nitrogen prevents PB from crossing the intact blood-brain barrier (BBB) (Birtle et al., 1966). Therefore, central nervous system (CNS) effects of PB are not expected unless BBB permeability is compromised. Approximately half of U.S. personnel seen at the health care clinics during the PGW complained of muscarinic symptoms that may have been related to chemical exposure, including PB (Cook et al., 1992; Golomb, 1999). The role of PB in the development of signs and symptoms associated with PGW deployment remains poorly understood.

Sarin, O-isopropylmethylphosphonofluoridate, is an organophosphate that has been studied primarily as a potent warfare nerve agent (Taylor, 1985). The main clinical features associated with acute sarin intoxication are seizures, fasciculations, tremors, and hypothermia (Taylor, 1985). The appearance of these symptoms correlates with the inhibition of AChE, both in the CNS and peripheral nervous system (PNS) (Gupta et al., 1991). This is followed by excessive accumulation of acetylcholine, leading to hyperactivation of nicotinic and muscarinic acetylcholine receptors. Excessive accumulation of acetylcholine leads to activation of ligand-gated ion channel, nicotinic acetylcholine receptor (nAChR), and muscarinic acetylcholine receptor (mAChR). These receptors mediate diverse cellular responses by distinct signaling mechanisms (Wess, 1996). Indeed, previous studies from our laboratory and others have shown that organophosphate compounds, including sarin, cause differential regulation of nAChR and mAChR (Huff et al., 1994; Jett et al., 1991; Katz et al., 1997; Khan et al., 2000). In vitro studies by Bakry et al. (1988) suggest that sarin binds to nAChR and modulates its ligand-binding characteristics. Therefore, it is likely that changes in cholinergic pathways play a key role in the toxicity induced by sarin.

In addition to acute cholinergic effects, healthy individuals exposed to low-dose sarin have been reported to exhibit neurological signs and symptoms up to 10 years following initial exposure (Duffy and Burchfiel, 1980; Sidell, 1974). The neurological effects included increased beta activity and increased amount of rapid eye movement sleep (Duffy and Burchfiel, 1980). Abnormal electrophysiological recordings following a single large dose or repeated subclinical doses of sarin in rhesus monkeys have been observed (Burchfiel et al., 1976; Burchfiel and Duffy, 1982). Distal sensory axonopathy has been observed 3 years after sarin intoxication in Tokyo (Himuro et al., 1998). Repeated inhalation exposure to sarin has been shown to cause muscular weakness of the limbs in mice (Husain et al., 1993). Long-term behavioral changes characterized by a decrease in activity, increased morbidity, and changes in gait have been observed in rats following low-level sarin exposure (Kassa et al., 2001).

In the present study, we evaluated the sensorimotor performance and brain regional acetylcholinesterase activities and m2 mAChR ligand binding following treatment with sarin and PB, alone and in combination. These results suggest that treatment with sarin and PB alone resulted in sensorimotor impairment and cholinergic changes and that coexposure of high doses of sarin with PB resulted in exacerbated deficits.

**MATERIALS AND METHODS**

Sarin stock (1.90 mg/ml in saline) was obtained from the U.S. Army Medical Research and Materiel Command, Fort Detrick, Maryland. Pyridostigmine bromide, butyrylthiocholine iodide, and acetilthiocholine iodide were obtained from Sigma Chemical Co. (St. Louis, MO). [3H]AF-DX384(2,3 Dipropylamino) (sp. activity, 100 Ci/m mol) was obtained from NEN (Boston, MA). All other reagents were of highest purity available commercially.

Male Sprague-Dawley rats (200–250 g) were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed in the Duke University Medical Center vivarium on 12-h dark-light cycle. The animals were allowed food and water ad libitum. The animals were treated with PB or water or sarin between 7:30 and 11:00 AM. All animal treatments and procedures were carried out strictly according to the recommended guidelines by the Army and the Duke University Medical Center institutional animal care and use committee.

To evaluate the neurobehavioral and biochemical effects of exposure to sarin and PB, two different sets of treatments were carried out.

**Treatment 1**

Animals in this treatment were subjected to neurobehavioral evaluations on days 7 and 15 following treatment with sarin and finally sacrificed on day 15 for biochemical determinations.

**Control:** Animals (n = 5) received daily water (orally by gavage) for 15 days and then a single im injection of normal saline on day 15. PB alone: animals in this group (n = 5) received PB (1.3 mg/kg orally by gavage) for 15 days and a single im injection of normal saline on day 15.

**Sarin alone:** animals in this group (n = 10) received water (orally by gavage daily for 15 days), and on day 15 they were treated with single im dose of 50, 75, 90, and 100 μg/kg, sarin (0.50, 0.75, 0.90, and 1 × LD50, respectively). There were 15 animals in the group treated with 100 μg/kg sarin.

**PB and sarin:** the animals received pretreatment with PB (1.3 mg/kg, daily orally by gavage) for 15 days and then single im treatment with 50, 75, 90, and 100 μg/kg sarin on day 15. The number of animals treated in each group was the same as for sarin alone.

**Treatment 2**

Animals were sacrificed 3 h following treatment with sarin for biochemical determinations only.

**Control:** the animals (n = 5) received water daily (orally by gavage) for 15 days and then a single im injection of normal saline on day 15.

**PB alone:** animals (n = 5) received PB (1.3 mg/kg, oral) for 15 days and a single im injection of normal saline on day 15.

**Sarin alone:** animals in this group (n = 5) received water (orally by gavage daily for 15 days) and on day 15 they were treated with single im dose of 10 and 100 μg/kg sarin (0.10 and 1 × LD50, respectively). There were 10 animals in the group treated with 100 μg/kg sarin.

**PB and sarin:** the animals received pretreatment with PB (1.3 mg/kg daily orally by gavage) for 15 days and then single im treatment with 10 and 100 μg/kg sarin on day 15. The number of animals treated in each group was the same as for sarin alone.

At the termination of the experiment, the animals in both the treatment schedules were anesthetized with 0.2 ml ketamine/xylazine (100 mg/kg ket-
amine, 15 mg/kg xylazine), and blood was drawn in a heparinized syringe. Brains were removed and washed thoroughly with ice-cold normal saline to remove blood. Brain regions, cortex, midbrain, cerebellum, and brain stem were dissected on ice and snap frozen in liquid nitrogen. Plasma was separated and frozen at −80°C for enzyme studies.

**Behavioral Testing Battery**

The behavioral tests employed in these studies evaluate sensorimotor reflexes, motor strength, and coordination. All behavioral testing was performed by an observer blind to the animal’s treatment status and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m², ambient light) between 7 and 11:30 AM. Behavioral testing was carried out 7 and 15 days after sarin administration.

**Reflexes.** Postural reflexes (Bederson et al., 1986; Markgraf et al., 1992), visual, tactile, and proprioceptive forelimb-placing responses (Markgraf et al., 1992), and orienting to vibrissae touch (Whishaw et al., 1985) were carried out as described by Abou-Donia et al. (2001).

**Inclined plane.** Rats were placed on a flat plane in the horizontal position, with the head facing the side of the board to be raised (Abou-Donia et al., 2001; Yonemori et al., 1998). The angle at which the rat began to slip downward was recorded. The results of the two trials were averaged for each testing session.

**Forepaw grip time.** The rats’ forepaw strength was assessed by having them grip a 5-mm diameter wood dowel that was held horizontally and raised so that the rat supported its body weight, as described by Andersen et al. (1985) and Abou-Donia et al. (2001). Time to release grip was recorded in seconds. The results of the two trials were averaged for each testing session.

**Beam-walking.** The testing apparatus was a 122 cm wooden beam elevated 75.5 cm above the floor, with wooden supports as described by Goldstein (1993) and Abou-Donia et al. (2001). Beam-walking ability was measured with a seven-point scoring system scale as previously described by Goldstein (1993, 1995): 1, the rat is unable to place the affected hindpaw on the horizontal surface of the beam; 2, the rat places the affected hindpaw on the horizontal surface of the beam and maintains balance for at least 5 s; 3, the rat traverses the beam while dragging the affected hindpaw; 4, the rat traverses the beam and at least once places the affected hindpaw on the horizontal surface of the beam; 5, the rat crosses the beam and places the affected hindlimb on the horizontal surface of the beam to aid less than half its steps; 6, the rat uses the affected hindpaw to aid more than half its steps; and 7, the rat traverses the beam with no more than two foot slips. In addition, the latency until the animal’s nose enters the goal box (up to 90 s) is recorded for the final trial. Rats that fell off the beam or did not enter the goal box were assigned latencies of 90 s.

**Statistical analysis.** Comparisons across treatment groups for postural reflexes, limb placing, and vibrissae touch orientation were analyzed with nonparametric analysis of variance (Kruskal-Wallis test). Data for the remaining behavioral tests were compared among groups by one-way or two-way repeated measures ANOVA as appropriate. A three-way repeated measures ANOVA was used to compare the effects of increasing doses of sarin alone or in combination with PB. Treatment with 100 μg/kg sarin alone resulted in 7 deaths out of 15 animals, whereas in the group pretreated with PB, only 5 animals died out of 15. Similarly, 2 animals out of 10 died in 90 μg/kg sarin alone treatment group as compared with only 1 in the group treated with 90 μg/kg sarin and PB.

**Enzyme and Receptor Assay**

**Cholinesterase determination.** AChE in brain regions and BChE in plasma activities were determined according to the method of Ellman et al. (1961) modified for assay in a Molecular Devices UV Max Kinetic Microplate Reader as previously described (Abou-Donia et al., 1996, Khan et al., 2000). Protein concentration was determined by BCA method according to Smith et al. (1985). The enzyme activities are expressed as micromoles substrate hydrolyzed per minute per milligram protein for brain regions and nanomoles substrate hydrolyzed per minute per milligram protein for plasma (percent of control).

**Muscarnic acetylcholine receptor (mAChR) binding assay.** For the assay of mAChR, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × g for 10 min; the membranes were suspended in the same buffer at the protein concentration of 1.5–2.5 mg/ml as described by Huff et al. (1994). The m2 mAChR binding was carried out by using m2-selective ligand [3H]AFDX 384 as described earlier (Slotkin et al., 1999; Khan et al., 2000). The results are expressed as specific binding (dpm/mg protein (percent of control).

**RESULTS**

**Clinical Signs**

The animals were observed for the development of clinical signs of toxicity. Treatment with 100 μg/kg sarin resulted in convulsions and cholinergic toxicity. The onset and magnitude of seizure was greater in the animals treated with a combination of PB and sarin (100 μg/kg). There was no mortality in the group of animals treated with 50 or 75 μg/kg sarin, alone or in combination with PB. Treatment with 100 μg/kg sarin alone resulted in 7 deaths out of 15 animals, whereas in the group pretreated with PB, only 5 animals died out of 15. Similarly, 2 animals out of 10 died in 90 μg/kg sarin alone treatment group as compared with only 1 in the group treated with 90 μg/kg sarin and PB.

**Behavioral Results**

Figures 1 and 2 give the effects of PB and dose-response comparisons for rats treated with increasing doses of sarin and increasing doses of sarin with a fixed dose of PB for each behavioral test on days 7 and 15, respectively, following treatment with sarin. Control rats began to slip off the incline plane when it was raised to 60° from horizontal. Forepaw grip time improved by an average of 3 s between the two testing sessions in controls. Beam-walk scores were perfect for all control rats at both time points. Beam-walk times decreased by an average of 4 s between the two testing days in controls.

Each treatment (PB, sarin, and PB + sarin) resulted in significant sensorimotor impairments compared with controls, as reflected in each behavioral test at each time point. Treatment with PB alone resulted in a greater behavioral impairment than sarin alone only for grip time and only on day 7 (Fig. 1, lower panel). Exposure to the combination of PB and sarin did not result in further deterioration in grip time than that caused by treatment from PB alone. However, the combination of PB and sarin resulted in greater impairment than sarin alone for incline plane performance on both days 7 and 15 and for forepaw grip on day 7 (Figs. 1 and 2).

For rats treated with sarin alone, there was a dose × time interaction for incline plane performance and grip time, indi-
FIG. 1. Effect of treatment with sarin and PB, alone or in combination, on sensorimotor performance on day 7 following treatment with single im injection of various doses of sarin. The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg). The animals were tested for beam-walk score, beam-walk time, incline plane, and grip response. Each treatment (PB, sarin, and PB + sarin) resulted in significant impairments compared with controls on each behavioral test at each dose of sarin. The data are presented as mean ± SE, n = 5. *Indicates statistically significant.

FIG. 2. Effect of treatment with sarin alone on sensorimotor performance on day 15 following treatment with single im injection of various doses of sarin. The animals were treated with various doses of single im injection of sarin (50, 75, 90, and 100 µg/kg). The animals were examined blindfolded for beam-walk score, beam-walk time, incline plane, and grip response. Each treatment (PB, sarin, and PB + sarin) resulted in significant impairments compared with controls on each behavioral test at each dose of sarin. The data are presented as mean ± SE, n = 5. *Indicates statistically significant.
cating poorer performance on these tasks on day 15 versus day 7 for at least one dose. For incline plane, rats that had received 75 or 100 µg sarin had significantly poorer performance on day 15 compared with day 7. For grip time, the difference between the two time points was significant only for rats that had received 75 µg sarin. For the rats treated with PB + sarin, there were significant dose × time interactions for each behavioral task (Figs. 1 and 2).

Three-way repeated measures ANOVA comparing increasing doses of sarin with increasing doses of PB + sarin showed significant differences for both incline plane performance and beam-walking scores. For incline plane performance, rats given PB + 90 µg sarin had poorer performance than those given 90 µg sarin alone, but only on day 15 (Fig. 2). The combination (PB + 100 µg sarin) resulted in poorer incline plane performance versus the 100-µg dose of sarin given alone on both days 7 and 15 (Figs. 1 and 2). For beam-walking score, the combination of PB + sarin resulted in improved performance at the 50-µg dose of sarin on day 15 only (Fig. 2) and at the 75-µg dose on day 7 only (Fig. 1). In contrast, the 100-µg dose of sarin resulted in poor beam-walk scores at both time points (Figs. 1 and 2).

**Effect of Treatment with Sarin and PB, Alone and in Combination, on Plasma Cholinesterase Activity**

Sarin exposure results in inhibition of plasma cholinesterase at various levels depending upon the dose and duration of exposure. The data presented in Figure 3A are from the animals 15 days after treatment with single im injection of 50, 75, 90, and 100 µg/kg sarin alone and in combination with 15 days of pretreatment with oral PB. Treatment with PB alone resulted in a significant elevation in the enzyme activity (~160% of control). A combination of treatment with a 100-µg dose of sarin and PB also resulted in a significant increase (~126% of control) in the plasma enzyme activity. To study the effect of PB on acute treatment with sarin, in a separate set of experiments we evaluated plasma cholinesterase activity 3 h following treatment with either 10 µg or 100 µg/kg single im injection of sarin alone or in combination with pretreatment with 15 days of daily oral doses of PB (Fig. 3B). The experiments at 3 h after exposure with sarin were carried out to evaluate any protection by PB of sarin toxicity involving the cholinergic system during acute exposure. Animals treated with 100 µg/kg sarin exhibited a significant decrease in plasma cholinesterase activity (~37% of control) that was less following coexposure with PB (~67% of control). There was no significant change in the enzyme activity in the animals treated with 10 µg/kg sarin, either alone or in combination with PB. These results suggest that pretreatment with PB provided some protection in plasma cholinesterase activity in rats treated with higher-dose sarin following acute exposure.

**Effect of Treatment with Sarin and PB, Alone and in Combination, on Brain Regional AChE**

The data presented in Figures 4–7 show the inhibition pattern of AChE in cortex, brain stem, midbrain, and cerebellum after single im injection of 50, 75, 90, and 100 µg/kg sarin. Data in panels 4A–7A are from the animals 15 days after treatment with sarin with and without pretreatment with 15 days of daily oral doses of PB. The data in panels 4B–7B are from the animals 3 h after treatment with 10 or 100 µg/kg single-dose sarin, with and without pretreatment with 15 days of daily oral doses with PB. The enzyme activity remained significantly inhibited in the cortex (~43–69% of control) 15 days after treatment with 50, 75, 90, and 100 µg/kg sarin (Fig 4A). Pretreatment with 15 daily oral doses of PB did not have any effect on sarin-inhibited cortical AChE activity, as the enzyme activity in the animals treated with sarin and PB remained significantly inhibited compared with the control. PB treatment alone caused a significant increase in the cortex AChE (~162% of control). These data further underscore the
potential of sarin to inhibit any increase in the enzyme activity in response to treatment with PB. Data in Figure 4B emphasize that acute sarin treatment with 100 \( \mu \text{g/kg} \) sarin caused a significant inhibition in the enzyme activity and that PB pretreatment resulted in significantly less inhibition in the cortical enzyme activity than treatment with sarin alone. However, the enzyme activity in the animals coexposed with sarin and PB still remained significantly inhibited (~63% of control). A similar pattern of significantly increased AChE activity following treatment with 50, 75, 90, and 100 \( \mu \text{g/kg} \) sarin, alone or in combination with 15 days of oral pretreatment with PB, was observed in midbrain (Fig. 6A) and cerebellum (Fig. 7A), suggesting a differential effect of sarin or combination of sarin with PB on brain stem, midbrain, and cerebellum compared with cortex following 15 days after treatment with sarin. Data shown in Figures 6B (midbrain) and 7B (cerebellum) indicate that 3 h following treatment with sarin alone or in combination with PB resulted in significant inhibition in the enzyme activity, and pretreatment with 15 daily oral doses of PB provided some degree of protection in sarin inhibitable enzyme activity. However, the enzyme activity in the animals coexposed with sarin and PB still remained significantly inhibited (~40 and ~58% of control for midbrain and cerebellum, respectively) compared with controls.

FIG. 4. Effect of treatment with sarin and PB, alone or in combination, on cortex AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 \( \mu \text{g/kg} \)) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 \( \mu \text{g/kg} \)) and sacrificed 3 h following treatment with sarin. The control activity was 40.1 ± 9.05 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean ± SE (% control), \( n = 5 \). *Indicates statistically significant.

FIG. 5. Effect of treatment with sarin and PB, alone or in combination, on brain stem AChE activity. Top panel (A): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and various doses of single im injection of sarin (50, 75, 90, and 100 \( \mu \text{g/kg} \)) and sacrificed on day 15 following treatment with sarin. Bottom panel (B): The animals were pretreated with PB (1.3 mg/kg, oral, daily for 15 days) and single im injection of sarin (10 or 100 \( \mu \text{g/kg} \)) and sacrificed at 3 h following treatment with sarin. The control activity was 40.1 ± 8.01 nmoles acetylthiocholine hydrolyzed/min/mg protein. Data are presented as mean ± SE (% control), \( n = 5 \). *Indicates statistically significant.
Effect of Treatment with Sarin and PB, Alone or in Combination, on \( m_2 \) Muscarinic Acetylcholine Receptors in the Cortex and Brain Stem

In the animals coexposed to sarin and PB, there was a significant increase in ligand binding at 90 and 100 \( \mu \)g sarin dose (Fig. 8A). There was a significant increase in cortical ligand binding at 3 h following treatment with 10 \( \mu \)g/kg sarin and a nonsignificant increase at 100 \( \mu \)g/kg sarin (Fig. 8B). A similar significant increase was observed following treatment with PB alone. Brain stem ligand binding from the animals treated with 50, 75, 90, and 100 \( \mu \)g/kg sarin, alone or in combination with 15 days of oral pretreatment with PB, are shown Figure 9A. There was no significant effect of sarin treatment alone, whereas coexposure with sarin PB resulted in a significant increase in ligand binding at 75, 90, and 100 \( \mu \)g/kg sarin doses. There was no change in brain stem ligand binding at 3 h following treatment with 10 or 100 \( \mu \)g/kg sarin with or without treatment with PB (Fig. 9B).

DISCUSSION

These results suggest that both PB treatment alone for 15 days at a physiologically relevant route and dose and coexposure to sarin and PB caused significant sensorimotor deficits. Pretreatment with PB may afford a moderate level of protection in peripheral as well as central nervous system AChE following acute exposure with 100 \( \mu \)g/kg sarin. Interestingly, PB treatment alone caused significant increases in plasma BChE and brain region AChE activities long after the treatment was discontinued. The dose of PB (1.3 mg/kg) and the route of exposure in the current experiments was chosen based on the information provided by the U.S. Department of Defense to approximate the exposure conditions with PB during the PGW. Sarin dose range (50–100 \( \mu \)g/kg) was used to evaluate whether PB treatment would afford protection in neurobehavioral deficits, as organophosphate nerve agents are known to exhibit extended neurological deficits (Baille et al., 2001; Blick et al., 1991; Duffy and Burchfiel, 1980). The changes in neurochem-
ical and behavioral functions observed in our studies may be related to a direct effect of PB and sarin on the cholinergic system. However, the possibility exists that long-term effects associated with PB exposure may be due to an indirect effect on the central nervous system.

Neurobehavioral data show that each treatment (PB, sarin, and PB + sarin) resulted in significant sensorimotor impairments compared with controls, which were reflected in incline plan performance, forepaw grip time, beam-walk scores, and beam-walk times at each dose and at each time point (Figs. 1 and 2). This was true even for the lowest dose of sarin used in the experiments (50 μg). As a result, it was difficult to detect a consistent effect of increasing doses of sarin when given either alone or in combination with PB (Figs. 1 and 2), although dose effects were found for at least some of the tested behavioral parameters at either of the two time points.

The control rats had stable or improving performance over the two testing sessions. Because the sensorimotor impairments at the time of the first assessments at 7 days were severe, it was difficult to detect any further worsening over time. This worsening was significant for only some behavioral parameters.

The effect of combination PB + sarin on sensorimotor performance did not result in change compared with PB alone, suggesting little effect on sensorimotor performance of sarin when given in combination with PB. However, the combination of PB + sarin caused exacerbated effect compared with sarin alone for inclined plane performance on both days 7 and 15 and for forepaw grip on day 7, suggesting that PB added to the impact of sarin on these behavioral parameters. When these treatment groups were compared as a function of sarin dose, poorer behavioral performance was most evident only for the highest dose of sarin. In fact, for beam-walk score, PB in combination with lower doses of sarin resulted in relatively improved performance, suggesting some protective effect of the combination at lower dose. The reasons for this effect are...
unknown. Thus, these data suggest the possibility of sensorimotor deficits in the veterans of PGW, who may have been exposed to PB or sarin. These results are consistent with the epidemiological studies reported by Knöke et al. (2000) and Storzbach et al. (2000).

Pretreatment with PB for 15 days afforded mild protection in sarin-inhibited plasma BChE at 3 h of treatment, whereas 15 days after the cessation of treatment this inhibitory potential of PB diminished (Figs. 4A and 4B). Instead, there was a significant increase in plasma activity, suggesting that PB might be affecting the BChE biosynthesis in liver, the main source of the secreted enzyme in plasma (Chambers and Carr, 1993). Because PB does not readily cross the blood-brain barrier under normal conditions, it is believed that PB could not inhibit CNS AChE activity unless BBB permeability is compromised. Our data suggest that PB treatment for 15 days could cause an increase in AChE activities in cortex, brain stem, midbrain, and cerebellum. Although not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling, as has been shown recently (Bigbee et al., 2000; Guizzetti et al., 1996; Sternfeld et al., 1998). Our data are consistent with a recent finding by Servatius et al. (1998), which reported that Wistar-Kyoto (WKY) rats exhibited persistently exaggerated startle response following treatment with PB, suggesting that PB-associated neurotoxicity may have a central nervous system component. Therefore, it is possible that sarin and PB treatment alone may cause subtle changes that are reflected in increased synaptic modeling and repair.

Our results also suggest that there are regional differences in the brain severity to inhibition of AChE by various doses of sarin, in that the cortical activity (Fig 4A and 4B) remained significantly inhibited for 15 days following treatment with 50, 75, 90, and 100 μg/kg sarin, alone or in combination with PB, whereas the activity recovered in brain stem, midbrain, and cerebellum (Figs. 5A–7A). From the acute exposure data it is clear that sarin inhibition of AChE showed dose response at 10 and 100 μg/kg sarin, whereas there was no apparent dose response at 50, 75, 90, and 100 μg/kg sarin after 15 days. A possible explanation for this differential response may be because of “survival effect,” in that the evaluation was carried out only in the surviving animals and therefore the most severely affected at 90 or 100 μg/kg may not have survived. In the case of brain stem, there was a significant increase in the enzyme activity at all the doses. This implies that long-term consequences of exposure to sarin alone or in combination with PB may be region specific. Our results on the effects of PB on the CNS are at variance from those reported by Friedman et al. (1996) in that we did not detect an inhibition in brain region AChE activity. It is possible that under stress, passage of PB would cause a direct inhibition of AChE, whereas under normal circumstances, such as under our experimental conditions, the effects of PB on the brain region AChE are mediated by indirect mechanisms. However, whether PB could have a direct access to CNS AChE is still debatable (Grauer et al., 2000; Lallement et al., 1998; Sinton et al., 2000).

Treatment with muscarinic antagonists induces receptor up-regulation (Ben-Barak and Dudai, 1980; Coccini et al., 2000; Majocha and Baldessarini, 1984; Smiley et al., 1998). Wang et al. (1996) reported an increase in muscarinic receptor ligand binding by repeated treatment with nicotine. Increased m2 AChR receptor ligand binding density in the brain stem in response to treatment with PB alone and in combination with 75, 90, and 100 μg/kg sarin could reflect a compensatory mechanism for a reduced ability of these receptors to bind their respective ligands due to desensitization. The increase in ligand binding densities for m2 AChR in the brain stem may be related to the changes in the AChE levels in the cortex and brain stem following treatment with sarin and PB, alone or in combination, because of depletion of acetylcholine pool. Recently we showed that acute exposure with sarin alone at various doses differentially modulates the m2 mAChR ligand binding (Khan et al., 2000). Studies by Ward et al. (1993) and Silveira et al. (1990) also have shown that organophosphate compounds selectively regulate m2 mAChR ligand binding. The data in the current study showing increased m2 mAChR-specific ligand binding in the brain stem following treatment with PB and in combination with various doses of sarin could regulate the ligand binding in vivo. Previously, Chaudhuri et al. (1993) and Liu and Pope (1996) reported an increased m2 mAChR ligand binding in response to chlorpyrifos treatment. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 mAChR upregulation (Majocha and Baldessarini, 1984; Nostrandt et al., 1997; Witt- Enderby et al., 1995). Increased ligand binding for m2 muscarinic receptor results in the inhibition of adenylate cyclase activity through a pertussis toxin–sensitive G-protein resulting in an inhibitory postsynaptic response (Branh et al., 1993; Wess, 1996). The inhibitory nature of the m2 receptor may have regulatory response on GABAergic system in the cortex. It is known that cholinergic input in certain brain regions tonically inhibits the GABAergic system that it is inhibitory to vasomotor glutamatergic neurons. Thus, an increase in m2 AChR in response to treatments with PB alone or in combination may regulate the glutamergic pathway, leading to a impaired motor response.

Prenaptic m2 mAChR has also been shown to cause changes in acetylcholine release via a feedback inhibitory mechanism (Gurantz et al., 1993; Marchi et al., 1990; Margiotta et al., 1987; Raiteri et al., 1984). Thus, our results suggest that the effect of sarin and PB on m2 mAChR may have modulatory effects on other processes, such as acetylcholine release and other second messenger system, that could influence the toxicity of sarin. In this context, it is noteworthy that it has been shown recently that PB induced neuronal apoptosis in rats through the activation of the muscarinic pathway (Li et al., 2000). Thus, it is possible that PB exposure alone may cause CNS dysfunction by activating apoptotic pathway mediated by muscarinic receptors.
PB AND SARIN COEXPOSURE IN RATS

In summary, our results show significant effects of sarin and PB, alone or in combination, on sensorimotor performance as well as changes in cholinergic system in rats. Pretreatment with PB afforded protection in the PNS as well as in the CNS AChE following acute treatment with sarin, while significant neurobehavioral deficits followed 15 days of treatment with PB and sarin, alone or in combination. The anatomic and physiologic mechanisms of the effects of PB and sarin on sensorimotor performance and the role of biochemical changes are uncertain and could be central, peripheral, or nonspecific. Further work is necessary in order to elucidate the mechanism of neurobehavioral deficits following exposure with PB and sarin, alone or in combination.

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REFERENCES


Himuro, K., Murayama, S., Nishiyama, K., Shinoe, T., Iwase, H., Nagoa, M.,


