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Co-exposure to pyridostigmine bromide, DEET, and/or permethrin causes sensorimotor deficit and alterations in brain acetylcholinesterase activity

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Abstract

Military personnel deployed in the Persian Gulf War (PGW) were exposed to a combination of chemicals, including pyridostigmine bromide (PB), DEET, and permethrin. We investigated the dose–response effects of these chemicals, alone or in combination, on the sensorimotor performance and cholinergic system of male Sprague–Dawley rats. Animals were treated with a daily dermal dose of DEET and/or permethrin for 60 days and/or PB (gavage) during the last 15 days. Neurobehavioral performance was assessed on day 60 following the beginning of the treatment with DEET and permethrin. The rats were sacrificed 24 h after the last treatment for biochemical evaluations. PB alone, or in combination with DEET, or DEET and permethrin resulted in deficits in beam-walk score and longer beam-walk times compared to controls. PB alone, or in combination with DEET, permethrin, or DEET and permethrin caused impairment in incline plane performance and forepaw grip strength. PB alone at all doses slightly inhibited plasma butyrylcholinesterase activity, whereas combination of PB with DEET or permethrin increased its activity. Brainstem acetylcholinesterase (AChE) activity significantly increased following treatment with combinations of either DEET or permethrin at all doses, whereas the cerebellum showed a significant increase in AChE activity following treatment with a combination of PB/DEET/permethrin. Co-exposure to PB, DEET, and permethrin resulted in significant inhibition in AChE in midbrain. PB alone or in combination with DEET and permethrin at all doses increased ligand binding for m2 muscarinic acetylcholine receptor in the cortex. In addition, PB and DEET together or a combination of PB, DEET, and permethrin significantly increased ligand binding for nicotinic acetylcholine receptor. These results suggest that exposure to various doses of PB, alone and in combination with DEET and permethrin, leads to sensorimotor deficits and differential alterations of the cholinergic system in the CNS.

Keywords: Persian Gulf War; Sensorimotor; Pyridostigmine bromide; DEET; Permethrin; Combined exposure; Acetylcholinesterase; Butyrylcholinesterase; m2 muscarinic acetylcholine receptor; Nicotinic acetylcholine receptor; Brain

1. Introduction

Military personnel deployed in the Persian Gulf War (PGW) were exposed to a unique combination of chemical, biological, and psychological environments. Since their return from the PGW, many of personnel have had symptoms including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability (Institute of Medicine, 1995; Haley et al., 1997a). Combined chemical exposures included a variety of organophosphate compounds, the insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET), and the pyrethroid insecticide, permethrin (Institute of Medicine, 1995). Additionally, these veterans were given a course of twenty-one 30-mg tablets of pyridostigmine bromide (PB) as prophylaxis against organophosphate nerve agents (Persian Gulf Veterans Coordinating Board, 1995).

PB is considered to be relatively safe to humans at the doses given. It is a quaternary dimethyl carbamate that has been used primarily as a treatment for myasthenia gravis at

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higher doses (Breyer-Pfaff et al., 1985, 1990) than that given to PGW veterans (Keeler et al., 1991; Golomb, 1999; Kant et al., 2001). It reversibly inhibits acetylcholinesterase (AChE) in the peripheral nervous system (PNS), thus limiting irreversible inhibition of the enzyme by nerve agents (Blick et al., 1991). AChE activity is restored following spontaneous decarbamylation resulting in near-normal neuromuscular and autonomic functions (Blick et al., 1991). Toxic symptoms associated with PB overdose result from overstimulation of nicotinic and muscarinic receptors in the PNS, resulting in exaggerated cholinergic effects, such as muscle fasciculations, cramps, weakness, muscle twitching, tremor, respiratory difficulty, gastrointestinal tract disturbances, and paralysis (Abou-Donia et al., 1996). Central nervous system (CNS) effects of PB are not expected unless blood–brain barrier (BBB) permeability is compromised Birtley et al., 1966. CNS effects of PB are variable depending on the species and experimental model (mice, Friedman et al., 1996; Grauer et al., 2000; rat, Sinton et al., 2000; Li et al., 2000; Kant et al., 2001; guinea pigs, Lallement et al., 1998, 2001).

The insect repellent *N*,*N*-diethyl-*m*-toluamide (DEET) has been extensively used by humans since its introduction. DEET is commonly used as a repellant against mosquitoes, flies, ticks, and other insects (Robbins and Cherniack, 1986; McConnell et al., 1986; Pollack et al., 2002; Fradin and Day, 2002). Extensive and repeated topical DEET applications can be toxic in humans (Gryboski et al., 1961; Roland et al., 1985; Edwards and Johnson, 1987). The symptoms associated with DEET poisoning include tremors, restlessness, difficulty with speech, seizures, impairment of cognitive function, coma, and death (McConnell et al., 1986). Although the exact mechanisms of DEET toxicity are not known, exposure to extremely high levels of DEET produces demyelination and spongiform myelinopathy in the rat (Verschoyle et al., 1992). DEET efficiently crosses the dermal barrier (Windheuser et al., 1982; Hussain and Ritschel, 1988) and may concentrate in fat (Blomquist and Thorsell, 1977; Snodgrass et al., 1982).

Permethrin [3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester], a type I synthetic pyrethroid insecticide, exists in four different stereoisomers (Casida et al.,1983). It provides insecticidal activity for several weeks following a single application and is used to control fleas, flies, mites, and cockroaches. Permethrin causes modification of sodium channels leading to prolonged depolarization and repetitive discharges in presynaptic nerve fibers after a single stimulus (Narahashi, 1985). This repetitive nerve action is associated with tremor, hyperactivity, ataxia, convulsions, and in some cases paralysis.

We previously reported that subchronic exposure to 0.1 ×, 1 ×, and 10 × doses of DEET (4, 40, and 400 mg/kg, respectively, daily dermal) and permethrin (0.013, 0.13, and 1.3 mg/kg, respectively, daily dermal), either alone or in combination, caused sensorimotor deficits (Abou-Donia et al., 2001a). The 1 × dose of each chemical was based on the estimation by the Department of Defense for the exposure to these chemicals to the deployed military personnel in the PGW in 1991. The present study was carried out to evaluate the interactive effects on sensorimotor performance and the cholinergic system following exposure to various doses of PB, alone and in combination of DEET and permethrin.

2. Materials and methods

2.1. Chemicals

Technical-grade (>93.6%) permethrin 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane carboxylic acid (3-phenoxyphenyl) methyl ester was obtained from Roussel Uelaf (Pasadena, TX). DEET (99.7% *N,N*-diethyl-*m*-toluamide), PB (≥ 99%, 3-dimethylamino carbonyl oxy-*N*-methyl pyridinium bromide), acetylthiocholine iodide, butyrylthiocholine iodide, atropine, and nicotine were purchased from Sigma (St. Louis, MO). 5,5'-Dithio-bis-2-nitrobenzoic acid (DTNB) was purchased from Aldrich. The inhibitor, 1,5-bis-(*N*-allyl-*N,N*-dimethyl-4-ammonium phenyl) pentan-3-one dibromide (BW284C51) was obtained from Sigma. [^{3}H]AF-DX 384, specific activity 106 μCi/mmol and [^{3}H]cytisine, specific activity 32 nCi/pmol were purchased from New England Nuclear (Boston, MA). All other chemicals and reagents were of highest purity available from commercial sources.

2.2. Animals

Male Sprague–Dawley rats weighing 200–250 g were obtained from Zivic-Miller Laboratories (Allison Park, PA) and housed at Duke University Medical Center vivarium. The animals were randomly assigned to control and treatment groups and housed at 21–23 °C with a 12-h light/dark cycle. They were supplied with food and water ad libitum. The rats were allowed to adjust to their environment before starting the treatment. All the experimental studies on the rats were carried out strictly according to the U.S. Army and Duke University Institutional Animal Care and Use Committee.

2.3. Treatment

The rats were randomly divided into following groups ($n = 5$ per group):

1. Controls: received 70% ethanol dermally and water by gavage orally each day.
2. PB alone: PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively) was given daily by gavage for the last 15 days of the experiment, from days 46 to 60.
3. PB + DEET: DEET (0.1 ×, 1 ×, or 10 ×; 4, 40, or 400 mg/kg, respectively, dermal) was given daily for 60 days.

In addition, during the last 15 days, the rats were treated orally with PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.

4. PB + permethrin: Permethrin (0.1 ×, 1 ×, or 10 ×; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal) was given daily for 60 days. In addition, during the last 15 days, the rats were treated orally with PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.
5. PB + DEET + permethrin: DEET (0.1 ×, 1 ×, or 10 ×; 4, 40, or 400 mg/kg, respectively, dermal) and permethrin (0.1 ×, 1 ×, or 10 ×; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal) were given daily for 60 days. In addition, during the last 15 days, the rats were treated orally with PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively) daily by gavage.

DEET and permethrin were applied directly to the preclipped skin on 1 in.^{2} of the back of the neck. The chemicals were applied to give the desired concentration of test compounds in 0.1 ml of vehicle. DEET was given in doses of 4, 40, or 400 mg/kg (0.1 ×, 1 ×, and 10 × dose, respectively) in ethanol with or without permethrin (0.013, 0.13, or 1.3 mg/kg; 0.1 ×, 1 × x, and 10 × dose, respectively). Controls received an equal volume of the vehicle. The treatment with DEET and permethrin was carried out for 60 days, whereas the treatment with PB was during the last 15 days of the experiment.

The 1 × dose of PB, DEET, and permethrin corresponds to an estimate of the military personnel's exposure to these agents during the PGW as provided by the Department of Defense. Animals were sacrificed 24 h after the treatment with the last dose.

2.4. Behavioral studies

A comprehensive battery of standardized tests was employed on day 60 following the beginning of the treatment with DEET or permethrin. These behavioral tests were designed to measure sensorimotor reflexes, motor strength, and coordinated gait (Bederson et al., 1986; Markgraf et al., 1992; Goldstein, 1993, 1995). All behavioral testing was performed by a trained observer blind to the animal's treatment status, and was carried out in a soundproof room with subdued lighting (less than 10.76 lumens/m^2, ambient light). Rats were handled for 2 min daily for 5 days during the week prior to behavioral testing. The behavioral evaluations commenced 2 h following the last treatment with the last dose. All behavioral tests were carried out in each group in the same order to maintain the time elapsed after the last treatment.

2.4.1. Beam walking and beam score

2.4.1.1. Description. The testing apparatus is a 2.5 × 122 cm wooden beam elevated 75.5 cm above the floor with wooden supports. A 20 × 25 × 24 cm goal box with a 9.5 cm opening is located at one end of the beam. A switch-activated source of bright light (75 W Tungsten bulb) and white noise are located at the start–end of the beam and serve as avoidance stimuli. The rats were first trained with a series of three approximate trials (Goldstein, 1993, 1995).

2.4.1.2. Scoring. For the testing trials, rats were placed at the start–end of the beam, near the sources of light and noise. Both the latency until the animal's nose entered the goal box (up to 90 s) and the use of the hind paw to aid locomotion was recorded on a seven-point scale: 7 = *normal performance*, 1 = *unable to place hind paw onto the horizontal surface of the beam*. Rats that fell off the beam were assigned latencies of 90 s.

2.4.2. Incline plane

The rats were placed on a platform that was elevated at increasing angles. The angle at which the animal fell off the platform was recorded. The results of two trials were averaged at each time point.

2.4.3. Grip time

Rats forepaw grip strength was assessed by having the animal's forepaw grip a 5-mm diameter wood dowel. Time to release their grip was recorded in seconds.

2.4.4. Statistical analysis

Data for beam-walk time and grip time were analyzed with two-way (drug group and dose) ANOVA with post hoc planned contrasts between each group and controls. Because the data were not normally distributed, data for beam-walk score and incline plane performance were first analyzed with the Kruskal–Wallis test. The Mann–Whitney U test (corrected for multiple comparisons) was then used to compare each group with controls. Two-tailed $P < .05$ was considered significant.

2.5. Tissue retrieval for biochemical evaluations

Twenty-four hours after the treatment with the last dose, the rats were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/kg ketamine and 15 mg/kg xylazine). Blood was drawn by cardiac puncture and brains were dissected out. The brains were washed thoroughly in ice-cold normal saline and brain regions were dissected on ice and frozen immediately in liquid nitrogen. Plasma was separated and frozen at − 80 °C until enzyme studies were done.

2.5.1. Acetylcholinesterase and butyrylcholinesterase

Brain AChE and plasma cholinesterase (BChE) activities were measured by the Ellman assay (Ellman et al., 1961). For AChE assays, dissected brain regions were homogenized in Ellman buffer, centrifuged for 5 min at 5000 × g, and the resulting supernatant used for AChE analysis. AChE activity

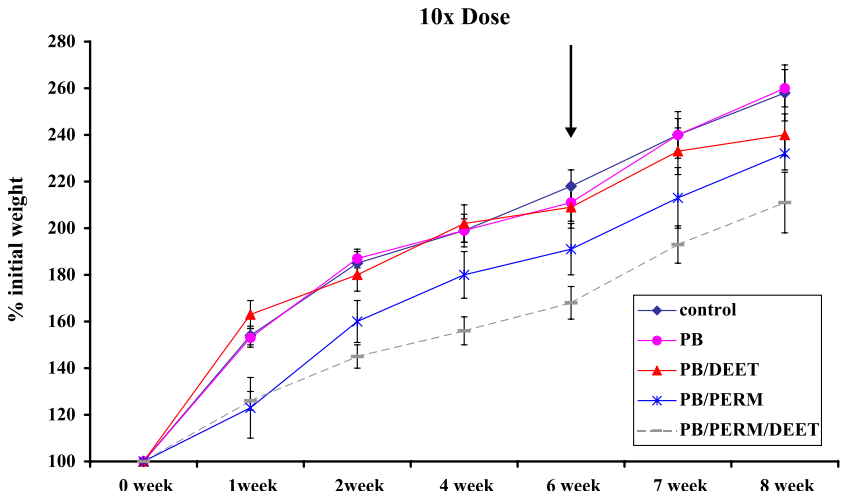


Fig. 1. Body weight gain following treatment with 10 × dose PB, alone or in combination with 10 × dose of DEET and permethrin. The rats were treated with PB (0.1 × , 1 × , or 10 × ; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB + DEET (0.1 × , 1 × , or 10 × ; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB + permethrin (0.1 × , 1 × , or 10 × ; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB + DEET + permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (0.1 × , 1 × , or 10 × ; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. Weight of the rats was recorded and the data are plotted as mean ± S.E. of weight gain as percent initial weight. Arrow represents the beginning of the oral treatment with PB by gavage.

was measured using acetylthiocholine as substrate in a Molecular Devices UV Max Kinetic Microplate Reader at 412 nm as described by Abou-Donia et al. (2001b). The enzyme activity is expressed as nanomoles of substrate hydrolyzed per minute per milligram of protein. Protein concentrations in tissue samples and plasma were determined by the method of Smith et al. (1985).

2.5.2. Muscarinic acetylcholine receptor binding

For the assay of the ligand binding for m2 muscarinic acetylcholine, the tissue was homogenized in 10 mM phosphate buffer, pH 7.4, and centrifuged at 40,000 × *g* for 10 min, and the membranes were suspended in the same buffer at a protein concentration of 1.25–2.5 mg/ml as described by Huff et al. (1994), and the ligand binding was carried out according to Slotkin et al. (1999). The m2 muscarinic acetylcholine binding was carried out by using m2 muscarinic acetylcholine-specific ligand, [^{3}H]AF-DX 384 at room temperature for 60 min. Nonspecific binding was carried out in the presence of 2.2 μM atropine sulfate. Ligand-bound membranes were trapped on glass filters presoaked with 0.1% polyethylenimine using a rapid vacuum filtration system.

2.5.3. Nicotinic acetylcholine receptor binding

[^{3}H]Cytisine was used as specific ligand for nicotinic acetylcholine according to the method described by Slotkin et al. (1999). Tissues were homogenized by Polytron in 50 mM Tris–HCl pH 7.4 containing 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 2.5 mM $MgCl_2$. The membranes were sedimented by centrifuging at 40,000 × *g* for 10 min at 4 °C. The resulting pellet was resuspended in the same buffer using Teflon pestle glass homogenizer in a volume to give 1.5–2 mg/ml protein. An aliquot of membrane preparation containing ~ 200 μg protein was used to carry out the incubation with 1 nM [^{3}H]cytisine at 4 °C for 75 min. Nonspecific binding was carried out in the presence of 2 μM nicotine bitartrate. Ligand-bound membranes were trapped on membrane filters using rapid vacuum filtration system.

2.5.4. Statistics

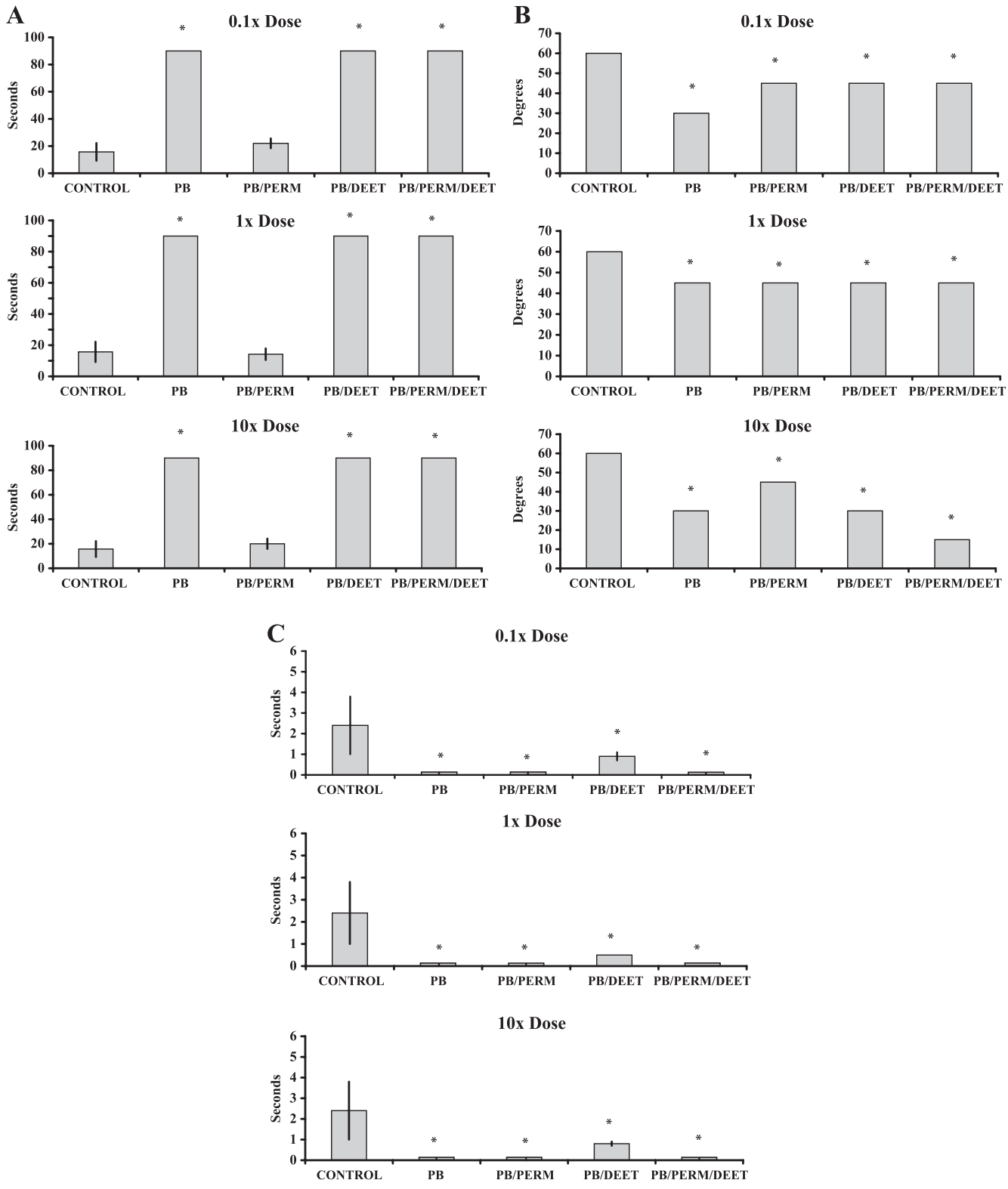
The results were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test. Data for body weight gain were analyzed by two-way ANOVA for the treatment effects and time interaction. $P < .05$ was considered significant.

Fig. 2. Effect of treatment with 0.1 × , 1 × , or 10 × dose of PB alone or in combination with DEET and permethrin on sensorimotor performance: The rats were treated with PB (0.1 × , 1 × , or 10 × ; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB + DEET (0.1 × , 1 × , or 10 × ; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB + permethrin (0.1 × , 1 × , or 10 × ; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB + DEET + permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (0.1 × , 1 × , or 10 × ; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. The rats were tested 2 h after the last treatment. Beam-walk time (A), incline plane (B), and forepaw grip time (C). Top panel: Data were obtained following exposure with 0.1 × dose of PB alone or in combination with DEET and permethrin. Middle panel: Data were obtained following exposure with 1 × dose of PB alone or in combination with DEET and permethrin. Bottom panel: Data were obtained following exposure with 10 × dose of PB alone or in combination with DEET and permethrin. Data in (A) and (C) are presented as mean ± S.E. * Statistically significant ($P < .05$) as compared with controls, corrected for multiple comparisons. Data in (B) are medians, interquartile range = 0 for each condition. For incline plane, all treatment groups were significant when compared with Mann–Whitney *U*, corrected for multiple comparisons.

3. Results

3.1. General health and clinical condition

Animals showed no overt clinical signs of toxicity observed throughout the study. There were no significant effects on the body weight gain in the rats treated with 0.1 × or 1 × dose. The rats treated with 10 × dose of PB and permethrin and that of 10 × dose of PB/DEET/permethrin gained less weight than controls or those treated with PB and DEET (Fig. 1). Two-way ANOVA for the treatment effects and time interaction did not show any significant effects.



3.2. Effect of treatment with 0.1×, 1×, or 10× dose of PB alone or in combination with DEET and permethrin on sensorimotor performance

Fig. 2 shows the results of the behavioral tests after 60 days exposure to 0.1 ×, 1 ×, or 10 × dose of DEET and permethrin in combination with PB. PB treatment was carried out during the last 15 days of the experiment.

3.2.1. Beam-walk score

There was a significant difference among the groups at each dosing level (0.1 × dose, Kruskal–Wallis $H=29$, $P<.0001$; 1 × dose, Kruskal–Wallis $H=28$, $P<.0001$; 10 × dose, Kruskal–Wallis $H=25$, $P<.001$). Except for the combination of PB and permethrin (median score = 7, interquartile range = 0 for each dose), each group (PB, PB + permethrin, PB + permethrin + DEET) significantly differed from controls (median score 7, interquartile range = 0) at each dosing level (median score 1, interquartile range = 0, for each group at each dose level; Mann–Whitney U corrected for multiple comparisons, $P<.002$ for each comparison).

3.2.2. Beam-walk time

Two-way ANOVA showed a significant effect of drug group [$F(3,52)=1572$, $P<.00001$] but no effect of dose [$F(2,52)=2.02$, $P=.14$] and no Group × Dose interaction [$F(6,52)=2.05$, $P=.08$]. Fig. 2A shows the planned com-

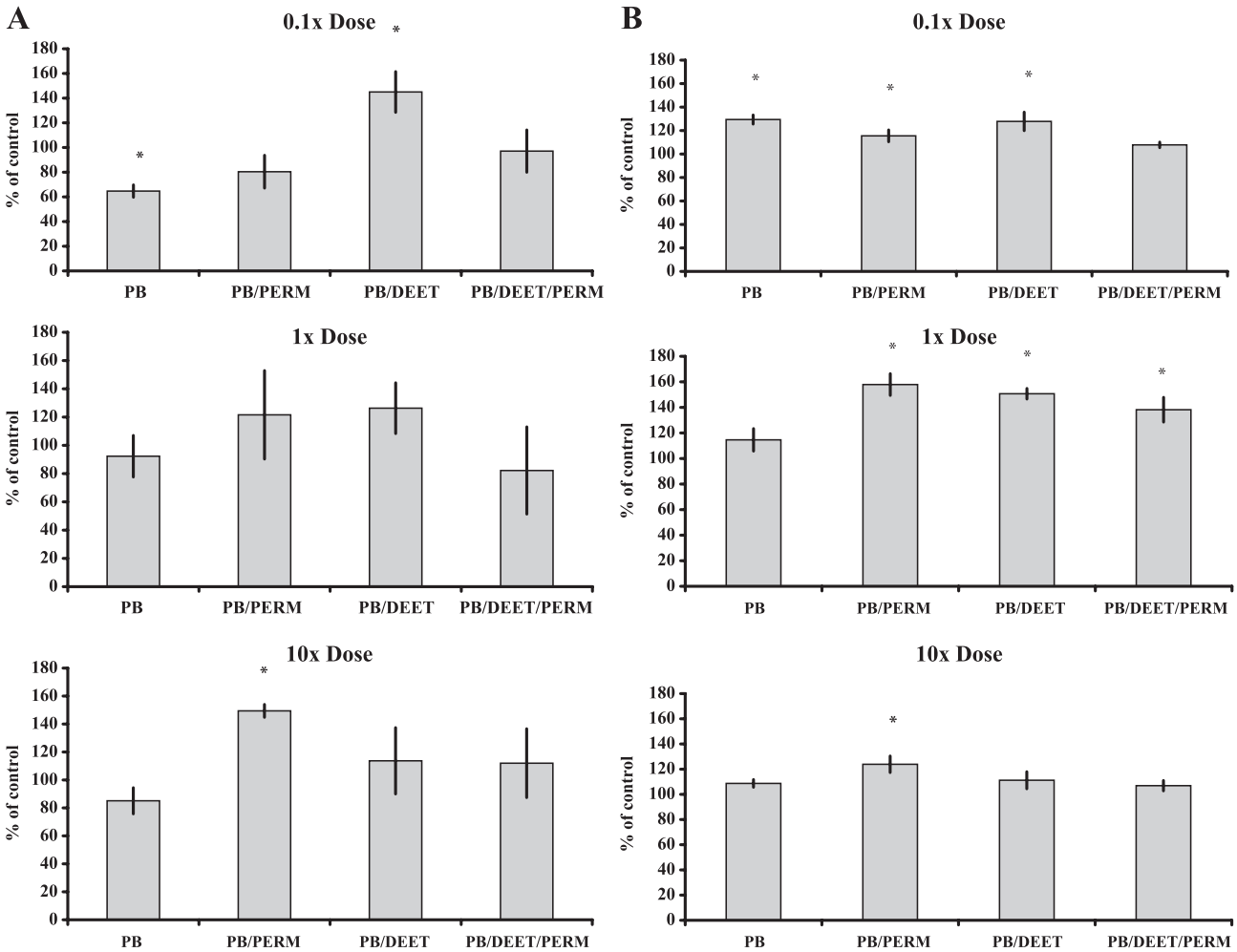


Fig. 3. Effect of treatment with 0.1 ×, 1 ×, or 10 × dose of PB alone or in combination with DEET and permethrin on plasma butyrylcholinesterase and brain regional AChE activity. The rats were treated with PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage), PB + DEET (0.1 ×, 1 ×, or 10 ×; 4, 40, or 400 mg/kg, respectively, dermal, daily for 60 days), PB + permethrin (0.1 ×, 1 ×, or 10 ×; 0.013, 0.13, or 1.3 mg/kg, respectively, dermal, daily for 60 days), or PB + DEET + permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (0.1 ×, 1 ×, or 10 ×; 0.13, 1.3, or 13 mg/kg, respectively, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed, and plasma and brain regions were evaluated for the enzyme activities. (A) Plasma and (B) brainstem. Top panel: Data were obtained following exposure with 0.1 × dose of PB alone or in combination with DEET and permethrin. Middle panel: Data were obtained following exposure with 1 × dose of PB alone or in combination with DEET and permethrin. Bottom panel: Data were obtained following exposure with 10 × dose of PB alone or in combination with DEET and permethrin. Enzyme activity (nmol/min/mg protein) in the control animals: plasma, 0.04+.001; brainstem, 1.29 + 0.09. Data are presented as mean ± S.E. (percent of control). * Statistically significant ($P<.05$).

parison contrasts for each treatment group at each dose level vs. control [$F(1,52)$]. All comparisons were significant except for the combination of PB and permethrin at the 1 × dose.

3.2.3. Incline plane

There was a significant difference among the groups at each dosing level (0.1 × dose, Kruskal–Wallis $H=29$, $P<.0001$; 1 × dose, Kruskal–Wallis $H=28$, $P<.0001$; 10 × dose, Kruskal–Wallis $H=25$, $P=.001$). Fig. 2B shows the median angles for each treatment group at each dose level vs. control. Each group significantly differed from controls at each dosing level (Mann–Whitney U, corrected for multiple comparisons, $P<.002$ for each comparison).

3.2.4. Forepaw grip

Two-way ANOVA showed a significant effect of drug group [$F(3,52)=5.34$, $P<.003$] but no effect of dose [$F(2,52)=0.14$, $P=.87$] and no Group × Dose interaction [$F(6,52)=0.15$, $P=.99$]. Fig. 2C shows the planned comparison contrasts for each treatment group at each dose level vs. control [$F(1,52)$]. Each group significantly differed from controls at each dosing level.

3.3. Effect of treatment with 0.1×, 1×, or 10× dose of PB alone or in combination with DEET and permethrin on plasma BChE and brain regional ache activity

Fig. 3A shows the effects of treatment with 0.1 ×, 1 ×, or 10 × dose of PB alone or in combination with DEET and permethrin on plasma BChE activity at three different doses (top panel, 0.1 × dose; middle panel, 1 × dose; bottom panel, 10 × dose). Data on the effects of single chemical treatment are presented in Fig. 3 (top panel). Treatment with 0.1 × PB alone produced significant inhibition (~ 65% of controls) of plasma BChE activity. Treatment with PB and DEET at 0.1 × dose produced a significant increase (~ 145% of control) whereas no significant changes were observed in combination with PB and permethrin or a combination with PB, DEET, and permethrin. At 10 × dose (bottom panel), a combination of permethrin and PB caused a significant increase (~ 149% of control) in plasma BChE activity.

Fig. 3B shows the effects of treatment with 0.1 ×, 1 ×, or 10 × dose of PB alone or in combination with DEET and permethrin on AChE activity in the brainstem (top panel, 0.1 × dose; middle panel, 1 × dose; bottom panel, 10 × dose). There was a significant increase in the brainstem AChE activity at 0.1 × dose of PB alone or in combination with DEET or permethrin (upper panel). At 1 × dose, a combination of PB/DEET or PB/permethrin or PB/DEET/permethrin resulted in a significant increase (~ 138–150% of control) in AChE activity (middle panel), whereas at 10 × dose only the combination of PB/permethrin caused a significant increase (~ 123% of control) (lower panel). Cerebellum AChE activity showed a significant increase at 1 × dose following treatment with a combination of PB/DEET/permethrin (data not shown) or 10 × dose with a combination of PB/permethrin (data not shown). A combination of PB/DEET/permethrin at 10 × dose resulted in a significant decrease (~ 88% of control) in cerebellum AChE activity (data not shown).

3.4. Effect of treatment with 0.1×, 1×, or 10× dose of PB alone or in combination with DEET and permethrin on brain m2 muscarinic and nicotinic acetylcholine receptor ligand binding

To evaluate the effects of treatment with 0.1 ×, 1 ×, or 10 × dose of PB, alone and in combination with DEET and permethrin, on muscarinic and nicotinic acetylcholine receptor ligand binding studies were carried out with membrane preparations. For m2 muscarinic acetylcholine receptors, m2-specific ligand, [^{3}H]AFDX was used. The data on 1 × dose are presented in Fig. 4. PB treatment alone or in combination with DEET or a combination of PB/DEET/permethrin produced a significant increase in ligand binding density in the cortex (~ 130–145% of control). Similarly, at 0.1 × and 10 × dose, treatment with PB alone or in combination with DEET or permethrin or DEET and permethrin resulted in significant increase in the ligand binding (data not shown); however, there was no difference among the doses.

Ligand binding for nicotinic acetylcholine receptors using [^{3}H]cytisine was carried out in the cortex membranes

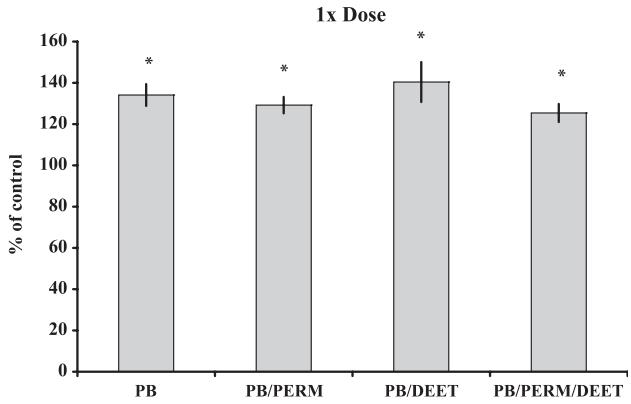


Fig. 4. Effect of treatment with 1 × dose of PB alone or in combination with DEET and permethrin on m2 muscarinic acetylcholine receptor ligand binding in the cortex. The rats were treated with PB (1 × ; 1.3 mg/kg, daily by gavage), PB + DEET (1 × ; 40 mg/kg, dermal, daily for 60 days), PB + permethrin (1 × ; 0.13 mg/kg, respectively, dermal, daily for 60 days), or PB + DEET + permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (1 × ; 1.3 mg/kg, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed. The details of membrane preparation and [^{3}H]AFDX 384 ligand binding assay are elaborated in Materials and Methods. Ligand binding in control membranes: 51 + 4.1 fmol/mg protein. Data are presented as mean ± S.E. (percent of control). * Statistically significant ($P<.05$).

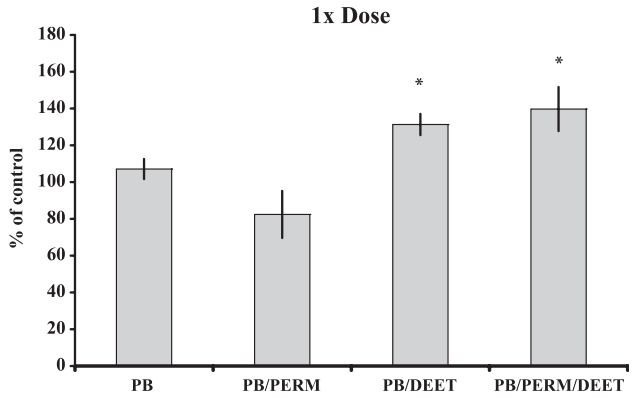


Fig. 5. Effect of treatment with 1 × dose of PB alone or in combination with DEET and permethrin on nicotinic acetylcholine receptor ligand binding in cortex. The rats were treated with PB (1 ×; 1.3 mg/kg, daily by gavage), PB + DEET (1 ×; 40 mg/kg, dermal, daily for 60 days), PB + permethrin (1 ×; 0.13 mg/kg, respectively, dermal, daily for 60 days), or PB + DEET + permethrin as above. The rats in the PB-alone group or in the combination with PB groups were treated with PB during the last 15 days orally with PB (1 ×; 1.3 mg/kg, daily by gavage) as described in Materials and Methods. Twenty-four hours after the last treatment, the animals were sacrificed. The details of membrane preparation and [^{3}H]cytisine ligand binding assay are elaborated in Materials and Methods. Ligand binding in control membranes: 23.9 + 4.1 fmol/mg protein. Data are presented as mean ± S.E. (percent of control). * Statistically significant ($P < .05$).

prepared from the animals treated with 0.1 ×, 1 ×, and 10 × dose of PB, alone and in combination with DEET and permethrin. The data are presented in Fig. 5 for 1 × dose only. Treatment with PB/DEET or PB/DEET/permethrin caused a significant increase in the ligand binding (~ 130% of control). Treatment with PB alone at any dose did not produce any significant change in ligand binding. There was no dose response.

4. Discussion

In the present study, we evaluated the neurotoxic response following exposure with 0.1 ×, 1 ×, and 10 × dose of PB (0.13, 1.3, and 13 mg/kg) alone or in combination with DEET (4, 40, and 400 mg/kg) and permethrin (0.013, 0.13, and 1.3 mg/kg). PB was administered, daily by gavage only on the last 15 days of the experiment. Our results show that treatment with PB alone led to significant impairment in the behavioral performance on all tests as compared to controls. There was no apparent dose effect on behavioral testing and no interaction between treatment group and dose level for the two behavioral tests for which this could be analyzed. The combination of PB and DEET as well as the combination of PB, permethrin, and DEET also led to poorer performance on each test. Combined exposure to PB and permethrin had a lesser impact. Furthermore, these data also suggest that treatment with PB, alone and in combination with DEET and permethrin, caused differential effects on AChE and m2 muscarinic receptors in the CNS.

The roles played by various anatomical brain regions and the molecular mechanisms involved in the behavioral effects observed in the present study are not known because these behavioral effects are mediated by a complex array of peripheral and central mechanisms. We have previously shown that exposure to DEET and permethrin at various doses alone and in combination causes neurobehavioral deficits (Abou-Donia et al., 2001a,b). Furthermore, our laboratory has also shown that 1 × dose exposure to DEET and permethrin for 60 days causes neuropathological cell loss in somatosensory cortex and cerebellum (Abdel-Rahman et al., 2001). It is recognized that somatosensory and motor responses are mediated by three adjacent areas in the cortex. These are located in the granular, pyramidal, and posterior agranular layers of the cortex (Barth et al., 1990, Hurwitz et al., 1990, Kolb, 1984). Some of these areas respond to deep changes in tendons and muscles (Donoghue and Wise, 1982). Different lesion studies have shown that severe sensorimotor impairment occurs in the animals with lesions of anteromedial and caudal forelimb cortex (Barth et al., 1990). Similarly, studies with bilateral large lesions in the rat somatic sensorimotor cortex have shown impairment in limb placing response. Additionally, it has also been suggested that limb placing is a function of corticospinal tract (Hicks and D'Amato, 1975). Thus, it is possible that long-term treatment with various doses of PB alone or in combination with DEET or PB/DEET/permethrin could affect these innervations, leading to neurobehavioral deficits.

PB provides protection against organophosphate nerve agents by shielding the peripheral AChE by reversibly binding to it. Thus, the toxic effects of PB are thought to be mediated through peripheral nicotinic and muscarinic acetylcholine receptors (Albuquerque et al., 1997). Indeed, Chaney et al. (1999) found that PB induced seizures in the mouse were mediated via PNS muscarinic and nicotinic receptors. On the other hand, other studies also suggest that PB toxicity is mediated through CNS ACh receptors as well as through the PNS (Servatius et al., 1998). Consistent with the reported effects of PB in the PNS, our results indicate that PB treatment moderately inhibited plasma BChE activity, while the CNS AChE activity did not show any significant effects. Treatment with PB/DEET caused an increase in AChE activity that may be caused by an increase in AChE protein levels. PB, a cholinergic carbamate, may increase AChE expression, similar to that caused by sarin, a cholinergic organophosphate nerve agent (Damodaran et al., 2003). While not universally accepted, an increase in AChE protein may reflect an increased axonal repair and synaptic modeling (Bigbee et al., 2000; Sternfeld et al., 1998; Guizzetti et al., 1996). Therefore, it is possible DEET and permethrin treatment with PB/DEET may cause subtle changes that are reflected in increased synaptic modeling and repair. The behavioral observations following treatment with PB/DEET are consistent with this notion. The up-regulation of m2 muscarinic and nicotinic acetylcholine

receptor ligand binding by treatment with PB at each dose in our study is intriguing because PB does not cross the BBB. An alternate possibility is that there may be certain changes occurring at the cerebrovasculature endothelium leading to the passage of PB into the CNS. Although speculative, this amount of PB may not be sufficiently high to cause AChE inhibition, but it may cause the down-regulation of the muscarinic and nicotinic acetylcholine receptors, and as a compensatory mechanism, an increase in the ligand binding density. It is possible that PB entry into the CNS and the consequent inhibition of CNS AChE may enhance the toxic potential of neurotoxic agents. In the case of the CNS, however, combined exposure of PB with other potentially neurotoxic chemicals may prove additive based on its availability to inhibit AChE activity and a consequent regulation of muscarinic and nicotinic acetylcholine receptors. Furthermore, metabolic competition between these chemicals may also result in differential effects in the CNS, e.g., bioavailability of each of these compounds or their metabolites is affected by liver and plasma esterases that play a major role in metabolic inactivation of these compounds (Abou-Donia et al., 1996). This may explain why permethrin treatment negatively affected the effects of PB in our studies. Other studies also demonstrate that treatment with chemicals that cause inhibition of AChE lead to m2 muscarinic acetylcholine up-regulation (Witt-Enderby et al., 1995; Majocha and Baldessarini, 1984). The CNS effects of PB treatment alone are intriguing in that PB does not cross the BBB. Several recent studies suggested that PB could or could not elicit CNS effects in a variety of animals, such as mice (Friedman et al., 1996; Grauer et al., 2000), rat (Sinton et al., 2000; Li et al., 2000; Kant et al., 2001), and guinea pigs (Lallement et al., 1998; 2001). In our laboratory, we have observed that certain brain areas seem to be affected by PB more than the other areas. Since it is known that cholinergic system is present at the endothelial lining of the BBB vasculature, it is possible that local effects of PB on the BBB endothelial cells cholinergic pathway may enhance the delivery of PB in the CNS. However, this possibility needs further studies.

In summary, our results suggest that exposure to various doses of PB alone or in combination with DEET or a combination of PB, DEET, and permethrin resulted in sensorimotor deficits and alteration in the cholinergic system in rats. These changes may involve a combination of mechanisms related to central and peripheral or neuromuscular system. Such alterations may explain the symptoms and complaints of some of the veterans of the PGW.

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