Delayed Neurologic and Behavioral Effects of Subtoxic Doses of Cholinesterase Inhibitors

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ABSTRACT

We tested the hypothesis that pyridostigmine bromide (PB) intake and/or low-level sarin exposure, suggested by some as causes of the symptoms experienced by Persian Gulf War veterans, induces neurobehavioral dysfunction that outlasts their effects on cholinesterase. Adult male Sprague-Dawley rats were treated during 3 weeks with s.c. saline, PB in drinking water (80 mg/l), sarin (62.5 μg/kg; 0.5 × LD50, three times/week s.c.), or PB in drinking water + sarin. Animals were tested for passive avoidance, nociceptive threshold, acoustic startle, and open field activity 2, 4, or 16 weeks after treatment. Two weeks after sarin, acoustic startle was enhanced, whereas distance explored in the open field decreased. These effects were absent with PB + sarin or PB by itself. No effect on any variable was found at 4 weeks, whereas at 16 weeks sarin induced a decrease and PB + sarin induced an increase in habituation in the open field test. Nociceptive threshold was elevated in the PB + sarin group at 16 weeks. No effect of treatment on passive avoidance was noted in any group. Brain regional acetylcholinesterase and cholineacetyltransferase activities were not affected at any time after treatment, but muscarinic receptors were down-regulated in hippocampus, caudate putamen, and mesencephalon in the sarin group at 2 weeks. In conclusion, this study gives further support to the use of PB against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association with low-level sarin exposure.

Many veterans of the Persian Gulf War complain from clusters of symptoms, including cognitive alterations, balance disturbances, and vertigo, and muscle aches and weaknesses (Haley, 2001), which have been ascribed by some authors, among other possible factors, to exposure to the ChE inhibitors pyridostigmine bromide (PB), a carbamate, and/or sarin, a highly toxic organophosphorus (OP) chemical warfare nerve agent.

PB, like other carbamate ChE inhibitors, protects animals from the lethal effect of OP ChE inhibitors when given in anticipation of exposure to these OP agents. The mechanism of this protection seems to be the preoccupation by the carbamate of ChE reactive sites, which become unavailable to the OP ChE inhibitor, with subsequent restoration of enzymatic activity due to the reversible decarbamylation of ChE. This phenomenon is the basis for the use of PB as a prophylactic of nerve agent intoxication (Dirnhuber et al., 1979; Leadbeater et al., 1985; Kluwe et al., 1987; Keeler et al., 1991; Koplowitz et al., 1992). The therapeutic target for this application of PB has been to maintain inhibition of plasma butyrylcholinesterase (BuChE) between 20 and 40%. Large-scale use of this premedication occurred during the Persian Gulf War with relatively few side effects related to cholinergic hyperactivity in some subjects (Keeler et al., 1991). Possible exposure to sarin may have occurred after explosions of ammunition dumps with consequent air contamination at Khamisiyah, Iraq (McCauley et al., 2001). The effects of low-level repeated exposure to OP nerve agents, not associated with acute clinical signs or symptoms, have attracted less attention than the well known effects of acute intoxication with these agents (Sidell, 1974; Ecobichon and Joy, 1982; Chambers, 1992). Behavioral and electroencephalographic alterations in workers exposed to low levels

ABBREVIATIONS. ChE, cholinesterase; PB, pyridostigmine bromide; OP, organophosphorus; BuChE, butyrylcholinesterase; ChAT, cholineacetyltransferase; AChE, acetylcholinesterase; RBC, red blood cell; QNB, quinuclidinyl benzilate; ANOVA, analysis of variance; LSD, least significant difference; F.U., force units.
(not associated with acute intoxication) of nerve agents have been reported (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982). However, a study of human volunteers exposed to low-to-moderate levels of nerve agents has indicated no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer (Panel on Anticholinesterase Chemicals, 1982; Coordinating Subcommittee, 1985). The same conclusion seems to hold for low-level accidental exposures to OP nerve agents (Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit cognitive or neurobehavioral abnormalities in experimental animals. Our initial experiments were aimed at establishing the optimal doses of sarin and PB. For sarin, the optimal dose was defined as the highest dose not associated with toxic signs after single or multiple doses within the 3-week period of treatment. This criterion was adopted because no episodes compatible with symptoms of acute intoxication with ChE inhibitors have been described in soldiers during the Persian Gulf War, although it is possible that low-level exposure to sarin may have occurred. In the case of PB, the optimal dose was defined as one producing 20 to 30% inhibition of plasma BuChE. This is the degree of BuChE inhibition reported for human subjects receiving the same PB dosage as soldiers during the Persian Gulf War (Keeler et al., 1991) (90 mg of PB over 24 h, divided in three oral doses).

Passive avoidance and open field activity tests were used to assess cognitive function and motor activity, respectively. Auditory startle and nociceptive threshold were assessed to determine the existence of possible neurological dysfunction. In addition, we analyzed, in key brain regions, the activity of ChAT and AChE, the enzymes responsible for acetylcholine synthesis and degradation, respectively, as well as the expression of muscarinic cholinergic receptors in the same animals that were subjected to the neurobehavioral tests mentioned above. Separate groups of animals were studied at 2, 4, or 16 weeks after 3 weeks of exposure.

Materials and Methods

Male Crl:CD(SD)IGSBR Sprague-Dawley rats, weighing 250 to 300 g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Laboratories (Kingston, NY) and housed individually in temperature- (21 ± 2°C) and humidity- (50 ± 10%)-controlled animal quarters maintained on a 12-h light/dark full-spectrum lighting cycle with lights on at 6:00 AM. Laboratory chow and water were freely available. Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense or the Laboratory of Neurophysiology (VA Greater Los Angeles Healthcare System). The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by Association for Assessment and Accreditation of Laboratory Animal Care.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Laboratories, Inc. (Berkeley, CA). Sarin, obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline before injection. Saline or sarin injection volume was 0.5 ml/kg s.c. PB was purchased from Sigma-Aldrich (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3-week period.

Determination of Optimal Doses of Sarin, PB, and Their Combination. A preliminary verification of the LD50 of sarin in rats was conducted by the “up and down” method (Dixon, 1965) using five doses (three animals per dose level) with 120 mg/kg as the initial dose at intervals of 0.05 log10 unit. To find the optimal dose for sarin, animals were administered LD50 doses of this agent in 0.1-unit increments starting from 0.2 and up to 0.7× LD50, three times (Mondays, Wednesdays, and Fridays) per week for 3 weeks in groups of six animals per dose. The highest dose not associated with toxic signs (described in detail below) during this 3-week period was adopted for the main study.

After correction for surface area equivalence between rats and human subjects (Freireich et al., 1966), the PB rat dose equivalent to that used in humans during the Persian Gulf War was calculated as 9 mg/kg/day. Experiments were set up to measure the plasma BuChE activity as well as the possible existence of signs of cholinergic toxicity in animals receiving 2.5, 5, 10, or 20 mg/kg/day PB in the drinking water during 3 weeks. Before this, the average daily drinking volume for the set of rats to be used (as milliliters of water intake per kilogram of body mass per day) was determined by measuring volume of water consumption over a 3-week period. This pilot study indicated that to achieve the desired daily doses described above, animals should be given PB in the drinking water at concentrations of 20, 40, 80, and 160 mg/l, respectively. The effects of PB treatment on plasma BuChE were monitored.

The optimal repeated dose of sarin to be used in combination with PB in drinking water at a concentration determined by the previous study was established as follows. While taking PB in drinking water, animals were administered doses of 0.3, 0.4, 0.5, or 0.6 LD50 sarin s.c., three times (Mondays, Wednesdays, and Fridays) a week for 3 weeks in groups of six animals per dose.

Experimental Groups. Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into four treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (control group). Group 2 animals received PB in drinking water (80 mg/l) and were injected with saline (PB group). Group 3 animals received tap water and were injected with sarin (62.5 μg/kg s.c., equivalent to 0.5× LD50) (sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB + sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 8:00 AM. At 9:00 AM that Monday morning, injection of either saline (0.5 ml/kg s.c.) or sarin (62.5 μg/kg s.c.) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 5:00 PM on Friday of the 3rd week. Animal dosing procedures were performed at the U.S. Army Medical Research Institute of Chemical Defense Laboratory. After a period of 1, 3, or 15 weeks after treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology (VA Greater Los Angeles Healthcare System) where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB + sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown) have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls) were transported in the same way to cancel out any potential differences due to transportation stress.

Number of animals was 12 per group, and the total number of groups (treatments × times after treatments) was also 12 with a grand total of 144 rats.

Observation of Signs of Intoxication. Animals were observed for signs of cholinergic intoxication for at least 1 h after sarin injec-
tion. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to a rating schedule described previously (Shih and Romano, 1988).

**Blood ChE Measurements.** When animals were received at the U.S. Army Medical Research Institute of Chemical Defense Laboratory, they were allowed to acclimate for a week. During this period, blood was collected from the tail vein (Liu et al., 1999) on two separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period.

Blood was collected into an Eppendorf 1.5-ml microtube containing 50 μl (1000 USP units) of heparin sodium and mixed. Forty microliters of whole blood was transferred to another microtube containing 160 μl of 1% Triton X-100 (in saline) solution, mixed well, and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 rpm (20,000 g relative centrifugal force). Plasma was carefully aspirated off, and 20 μl of RBCs was transferred into a microtube containing 180 μl of 1% Triton X-100 solution. The tube was tapped firmly until RBCs were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at −78°C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous reactivation or additional inhibition of ChE activity. Whole blood and RBC AChE activity was determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Hitachi, Diagnostics, Nutley, NJ). The analytical procedure was based on the manual method of Ellman et al. (1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate. Plasma BuChE activity was measured with the same method, but by using butyrylthiocholine as substrate, and manual readings of kinetic data on a Beckman scanning spectrophotometer.

**Regional Brain Activity of ChAT and AChE, and Quinclidinyl Benzilate (QNB) Binding.** Animals were euthanized by decapitation while under deep halothane anesthesia (2.5% in 30% O₂ balanced with N₂O). The brain was rapidly removed and flash frozen in methylbutane cooled to −70°C. The brain was microdissected in the posterior lobes, cerebellum, and medulla. These tissue samples were homogenized, and aliquots of these homogenates were used to determine tissue AChE activity with the kinetic method of Ellman et al. (1961), ChAT activity with the method of Fonnum (1975), and QNB binding with saturation assays (Yamamura and Snyder, 1974).

**Inhibited (Passive) Avoidance Response.** This was measured in a “step through” apparatus (McGaugh, 1972), consisting of 1) a small compartment made of white plastic; 2) a larger, dark compartment of stainless steel; and 3) a shock delivery unit adjustable for the intensity and duration (1 mA, 0.5 s) of the mild electric shock used as an aversive stimulus. The procedure involved two trials separated by a retention time of 48 h. On trial 1, the animal was placed in the white compartment. Entry into the dark compartment lead immediately to the closing of a door and administration of foot shock. Retention was tested after a 48-h delay, the measure being time taken to enter the dark compartment after release from the white compartment. The time to enter was defined as “retention,” a measure of memory of the single training session. The retention trials were set at a limit of 10 min.

**Open Field Locomotor Activity.** This was measured during a 20-min session in circular open field chambers of 60 cm in diameter, with walls 45 cm in height, under low-level red light illumination. This is done to maximize exploratory activity, which is normally inhibited in rats by daylight or bright illumination, and to eliminate unwanted visual clues from the surrounding environment. The animal movements were recorded with a video tracking and motion analysis system. This consists of a Sony CCD video camera (sensitive to the wavelength of light used), Targa M16 Plus video digitizing board on a microcomputer, and Ethovision software (Noldus, Inc., Costerweg, The Netherlands). Tracking was performed at a rate of 1 Hz during the entire 20-min session and stored in memory. Distance traveled was summed at 1-min intervals, and these values were fitted by nonlinear regression, using the Marquardt algorithm, to the following model: $Y = A \cdot e^{−Bt}$, where $Y$ is distance moved (in centimeters) and $t$ is time after initiation of test (in minutes). The values of parameters $A$ (initial velocity, in centimeters per minute) and $B$ (habituation, per minute) were obtained as described above for every animal. Analysis of variance (ANOVA) was then performed for the two parameters using the factor treatment (control, PB, sarin, and PB + sarin) at every time after treatment (3, 4, or 16 weeks).

In addition, total distance traveled and mean distance to the arena’s border (the inner surface of the chamber’s wall) during the entire test were also calculated for every animal.

**Reactivity (Startle Response).** Reactivity is defined as a response to a sudden, brief, and intense change in the stimulus environment. An acoustic signal served as a stimulus. The apparatus and procedure used to deliver the stimulus and to record the motor reaction of the animals to it has been described previously (Russell and Macri, 1979; Silverman et al., 1988). In this procedure, the animals stand unrestrained on a platform provided with a force sensor that transduces the motor reaction of the animal to the auditory stimulus into electrical pulses detected by an amplifier. A custom-designed computer program delivers a controlled sound and integrates and digitizes the motor-related electrical signal. Quantification of the response is provided in arbitrary force units (F.U.). In the currently reported experiments, 20 trials were performed at fixed intervals of 10 s.

**Nociceptive Threshold.** The procedure to measure nociceptive threshold used in these experiments has been described previously (Crocker and Russell, 1984) and uses reaction to a mild electric foot shock as its measure. It involves the up and down method (Dixon, 1965) for determination of median effective dose from sequential responses to shocks of logarithmically spaced intensity. Animals were placed into a test chamber, the floor consisting of stainless steel rods through which electric shock pulses (60 Hz) of varying intensities could be delivered with a duration of 0.5 s at 10-s intervals. The shock intensities were available in the range from 0.05 to 0.4 mA and were adjusted to a log, scaled 0.1 log units below set at midpoints of the ranges determined by preliminary experiments. The experimenter then adjusted the intensity according to the animal’s response on each trial. A “finch” was defined as an elevation of one or two paws from the grid floor and “jump” as rapid withdrawal of three or more paws from the grid.

**Data Analysis.** Group means and standard deviations of all study variables were obtained for every treatment and time after treatment. Data are presented in graphs as means with S.E. values except when the latter compromised clarity of the graphical display. Differences between group means were tested by ANOVA (general linear model) at each time after exposure to drugs or saline with one factor (treatment) at four levels (control, PB, sarin, PB + sarin). This was followed, if significant (probability for F ratio < 0.05), by multiple contrasts using Fisher’s least significant difference method.

**Results**

**Dose-Finding Studies**

The LD₅₀ of sarin was determined to be 125 μg/kg s.c. An initial evaluation indicated that animals whose drinking water contained PB at a concentration of 80 mg/l had inhibition of plasma BuChE slightly greater than 20% on average. This was within the target effect set for these experiments. (20–
30% inhibition). The next higher PB concentration in drinking water (160 mg/l) induced a larger plasma BuChE inhibition (between 27 and 40%). Thus, the concentration of 80 mg/l PB in drinking water was adopted for the rest of the study. No sign of toxicity, as defined under Materials and Methods, was found in animals drinking water containing PB during 3 weeks.

The dose finding for sarin and the combination of sarin and PB indicated that 0.5 LD50 sarin was the highest dose that did not cause observed acute toxic effects when given alone or in combination with PB (80 mg/l in drinking water) for a period of 3 weeks.

Body Mass

Means of body mass, recorded daily during weekdays, through the 3 weeks of treatment and the subsequent 2 weeks after treatment are shown in Fig. 1. No statistically significant difference was found between treatments. The expected increase in body mass with age was observed at the beginning of the experiments that assessed outcome variables (2, 4, or 16 weeks after treatment), but no difference among treatment groups was found at these time points either.

Blood Che Activity

Measurements of RBC AChE during the three drug treatment weeks, the pretreatment week (two measurements), and three post-treatment weeks are shown in Fig. 2. PB induced a pronounced decrease in enzymatic activity during the first week, which recovered partially during the following 2 weeks of treatment, with an average AChE activity of 54% of pretreatment levels over the 3 weeks of treatment. Sarin and PB + sarin produced an average decrease in RBC AChE to 35 and 27% of pretreatment, respectively. By the second week after discontinuation of treatment, RBC AChE activity recovered to values not statistically different from the control group.

Nociceptive Threshold

Data are presented in Fig. 3 for both the flinch and jump responses.

Flinch Response. No statistically significant difference among groups was found for the flinch response to the test at 2 or 4 weeks after treatment. In contrast, ANOVA was significant at 16 weeks after treatment and multiple comparisons among groups (Fisher’s LSD test, P < 0.05) indicated that all groups were different from controls (indicated by *) in all those four conditions.

Jump Response. ANOVA showed a significant F ratio at 4 weeks for the jump response, and multiple comparisons showed that nociceptive threshold for this response was significantly lower in the sarin group (0.17 ± 0.017 mA) than in the PB (0.23 ± 0.017 mA) and PB + sarin (0.211 ± 0.016 mA) groups, but not significantly different from controls (0.19 ± 0.016 mA). At 16 weeks after treatment, ANOVA was also significant and multiple comparisons showed that the PB + sarin group had a significantly higher threshold (0.255 ± 0.016 mA) than all other groups (controls = 0.18 ± 0.017 mA, PB = 0.152 ± 0.016 mA, and sarin = 0.17 ± 0.018 mA).

Open Field Locomotor Activity

Parameter A (Initial Velocity). No statistically significant difference among treatments was found at 2 or 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter mean for PB + sarin (360.6 ± 19.9 cm min⁻¹) was significantly higher than for the PB (272.8 ± 19.9 cm min⁻¹) group and sarin (275.3 ± 20.8 cm min⁻¹) group but not different from controls (309.5 ± 20.8 cm min⁻¹) (Fig. 4).

Parameter B (Habituation). No statistically significant difference among treatments was found at 2 and 4 weeks in this parameter. At week 16, ANOVA was significant and multiple contrasts indicated that the parameter means for sarin (0.035 ± 0.0088 min⁻¹) group and PB (0.046 ± 0.0084 min⁻¹) group were lower than for controls (0.072 ± 0.0093 min⁻¹), whereas PB + sarin (0.101 ± 0.0084 min⁻¹) was significantly higher than all other groups (Fig. 4).
Total Distance Moved. ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (3451 ± 207 cm) was significantly lower than controls (4328 ± 338 cm). No difference versus controls was found for the other two treatment groups. No significant difference between group means was found at 4 or 16 weeks after treatment.

Distance to Arena’s Border. ANOVA was significant at 2 weeks after treatment. Multiple contrasts indicated that the sarin group mean (7.78 ± 0.39 cm) was significantly lower than PB (9.58 ± 0.45 cm), and PB + sarin (9.05 ± 0.45 cm), but not different from controls. At 16 weeks after treatment, the sarin + PB mean was significantly higher than controls (indicated by *), whereas sarin + PB was significantly higher than controls and all other groups.

Reactivity (Acoustic Startle)

A significant increase in the average motor response in sarin-treated animals (15.3 ± 1.14 F.U.) against the controls (10.9 ± 1.14 F.U.) over the 20 trials was observed in measurements performed 2 weeks after treatment. This effect of sarin was particularly striking when the maximal response over the 20 trials block was computed (sarin = 62.6 ± 5.49 F.U.; controls 30.0 ± 5.49 F.U.; PB = 37.7 ± 5.02 F.U.; PB + sarin = 31.1 ± 5.01 F.U.). In this case, the mean of the sarin group was significantly higher than all others. No difference among group means was present at 4 or 16 weeks after treatment (Fig. 5).

Passive Avoidance

No difference between experimental groups was found in the time to enter the dark compartment 48 h after exposure to the aversive stimulus, measured in this test as an indication of acquisition and retention of the avoidance response (data not shown).

Brain Regional AChE Activity

Areas rich in cholinergic nerve cells and terminals were found to have, as expected, the highest AChE activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (Table 1). Central AChE activity was not significantly modified with respect to controls at the time of measurements of tested variables. Sarin-treated animals studied at the end of outcome variables evaluation had evidently recovered from central AChE inhibition. This is in
agreement with the substantial recovery of blood ChE activity recorded for this group at about the same time after treatment (Fig. 2).

Brain Regional ChAT Activity
Areas rich in cholinergic nerve cells and terminals were found to have, as in the case of AChE, the highest ChAT activity levels. No difference between controls and drug treatment groups was found for any of the regions at the three post-treatment time points studied (data not shown).

Brain Regional QNB Binding
Two weeks after treatment, there was a generalized decrease in QNB binding of the sarin group, compared with controls, that was statistically significant in caudate putamen, hippocampus, and mesencephalon (Table 2). This phenomenon reversed at 4 weeks after treatment, when a statistically significant increase in QNB binding was found in somatosensory cortex of sarin-treated animals. No statistically significant changes from control were found at 16 weeks post-treatment in any treatment group.

Discussion
Previous experimentation has shown that some functions can be affected at levels of nerve agents (such as soman and sarin) below the threshold for clinical toxicity (Chippendale et al., 1972; Russell, 1982; Wolthuis and Vanwersch, 1984). Repeated low-level exposures to soman (0.3 LD50) in rats induce initial decreases in body temperature, temporal perception, and locomotor activity. Tolerance was observed to all these effects, except soman-induced hypoalgesia. No effect of soman on memory was found by these authors (Russell et al., 1986). In another study, animals treated with low-level soman (0.4 LD50), and followed up to 6 weeks while in the treatment regime, exhibited a hyper-reactivity condition (Shih et al., 1990). In none of these cases were animals studied beyond the period of drug administration. Effects of low-dose soman on an equilibrium test in rhesus monkeys were reported to wear off 24 h after exposure (Switzer et al., 1990). Exposure to low-dose sarin has been recently reported to induce a decrease in activity and mobility, alteration of gait, and increase in stereotyped behavior and excitability in rats that persisted 3 to 12 months (Kassa et al., 2001a), as well as a deficit in Y-maze performance that subsided 3 weeks after exposure (Kassa et al., 2001b).

In the present series, the initial experiments were successful in finding reproducible effects on plasma BuChE activity of a PB concentration of 80 mg/l in the drinking water, with an estimated dose of about 10 mg/kg body mass/day. This is close to the rat equivalent (9 mg/kg body mass/day) of the dose used in humans for prophylaxis of OP poisoning (1.29 mg/kg body mass/day), based on surface area dosage conversion (Freireich et al., 1966). The degree of plasma BuChE inhibition obtained with this dose was within the range reported for humans taking 90 mg of PB orally per 24 h, divided in three doses (Keeler et al., 1991).

Sarin and PB/H11001
Sarin produced more pronounced and stable inhibition of RBC AChE than did PB. AChE inhibition recovered completely by the end of the 2nd week after discontinuation of treatment for all groups. Animals did not show signs of acute toxicity during or after treatment. The conditions established for this experimental model, i.e., exposure to the highest dose of sarin, alone or in combination with PB devoid of acute toxicity, were thus met.

Sarin-treated animals expressed decreased locomotor activity in the open field and increased reactivity to the acoustic startle test 2 weeks after the discontinuation of treatment. These two phenomena have been observed with central cholinergic hyperactivity caused by ChE inhibition (Overstreet, 1977; Russell et al., 1986). However, in the present experiments both blood and tissue ChE had recovered to normal levels at the time these outcome variables were evaluated. QNB binding, however, showed a generalized decrease, compared with controls, particularly pronounced in caudate putamen, hippocampus, and mesencephalon. Down-regulation of muscarinic receptors may have played a role in the behavioral phenomena described above because this was their only neurochemical correlate.

No effect of PB on locomotor activity was found. A previous
report (Hoy et al., 1999) had indicated a decrease in locomotor activity in rats given PB, but this effect was observed immediately after treatment with doses higher than used in the present study.

Both the depressed locomotor activity and enhanced reactivity induced by sarin were prevented by the simultaneous administration of PB. This is in line with the well known protective effect of PB from OP cholinesterase inhibitors lethality (Harris and Stitcher, 1984).

Previous experimentation (Servatius et al., 1998) has reported a delayed enhancement of the acoustic startle response in Wistar-Kyoto, but not Sprague-Dawley rats, with lower doses and shorter exposure times of PB than those reported here. The Wistar-Kyoto rats in those experiments were reported to have a basal plasma BuChE activity 27% lower than the Sprague-Dawley rats. These authors speculated that this fact might have caused a greater penetration of PB into the central nervous system, on account of the diminished scavenging effect of BuChE, and by that mechanism mediated the exaggerated acoustic startle response. In our experiments, we have used a dose almost 10 times higher than the lower dose at which Servatius et al. (1998) reported enhancement of acoustic startle, for a longer period of time (21 days as opposed to 7), but we still did not observe any effects of PB on this response. In fact, as stated above, PB protected sarin-treated animals from the delayed behavioral effects (decreased locomotor activity and hyper-reactivity) of sarin administration.

Nociceptive threshold is a very sensitive indicator of central cholinergic activity. This threshold is reduced (hyperlgesia) in hypocholinergic states (Russell et al., 1990), and the reverse is true of hypercholinergic states (Russell et al., 1986; Shih and Romano, 1988). A delayed elevation of nociceptive threshold for both the flinch and the jump response was found in the animals that had received PB + sarin, a phenomenon most clearly demonstrated 16 weeks after treatment. These results are difficult to interpret in light of the current knowledge of ChE inhibitors effects on pain, because no central ChE inhibition has been detected at this late time. These intriguing findings deserve further exploration with other methodologies for pain threshold evaluation.

The lack of changes in the passive avoidance paradigm indicates that none of the treatments induced alterations in the acquisition or retention of the learned response. Possible cognitive effects of the three treatments will be tested at later stages of this project by two other learning paradigms, conditioned avoidance response, and Morris water maze. Learning impairments have been previously described in rats re-
receiving PB (Shih et al., 1991; Liu, 1992). However, the doses used were considerably higher (6 to 24 mg/kg as a single oral dose) than the one reported in this study (10 mg/kg/day), equivalent, on the basis of body surface area conversion between species, to that taken by soldiers as prophylactic treatment against nerve agent poisoning (1.29 mg/kg/day). Moreover, in the two previous studies referenced above, behavioral tests were performed within minutes of dosing, with no long-term follow-up as in the present experiments. Similarly, behavioral changes have been described after administration of OP ChE inhibitors at doses devoid of acute symptomatology, but assessment was limited to the period immediately after treatment (Wolthuis and Vanwersch, 1984; Russell et al., 1986).

In conclusion, this study was designed to mimic the conditions of soldiers in the battlefield that are taking PB as a prophylactic treatment against nerve agents intoxication, with or without exposure to subsymptomatic levels of these agents. PB was administered in the drinking water so as to achieve a stable dosing regime at levels adjusted to reproduce the doses used in humans. The results have shown that under these conditions, PB did not produce adverse delayed neurobehavioral effects. Moreover, at 2 weeks post-treatment, simultaneous administration of PB and sarin prevented the development of decreased exploratory activity and enhanced response to an acoustic startle test that were associated with sarin exposure without PB protection. Thus, this study gives further support to the use of PB as one of the therapeutic resources against nerve agent poisoning and does not support the hypothesis that delayed symptoms experienced by Persian Gulf War veterans could be due to PB, alone or in association, with low-level nerve agent exposure. Further experimentation is planned to determine the possible effects of this treatment protocol on other physiological and neurobehavioral parameters.

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