Increased Neurotoxicity Following Concurrent Exposure to Pyridostigmine Bromide, DEET, and Chlorpyrifos

MOHAMED B. ABOU-DONIA,*1 KENNETH R. WILMARTH,*2 ALI A. ABDEL-RAHMAN,* KARL F. JENSEN,+ FREDERICK W. OEHME,† AND THOMAS L. KURT§

*Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710; †Neurotoxicology Division, U.S. EPA, Research Triangle Park, North Carolina 27711; ‡Comparative Toxicology Laboratories, Department of Clinical Sciences, Kansas State University, Manhattan, Kansas 66506-5605; and §Department of Internal Medicine, University of Texas, Southwestern Medical School, Dallas, Texas 75235

Received January 19, 1996; accepted August 20, 1996

The operating environment of the service personnel during the Persian Gulf War involved psychological, biological, and chemical elements including exposure to pesticides such as the insect repellent DEET (N,N-diethyl-m-toluamide) and the insecticide chlorpyrifos (O,O-diethyl 0-3,5,6-trichloropyridinyl phosphorothioate) and to pyridostigmine bromide (PB, 3-dimethylaminocarbonyloxy-N-methylpyridinium bromide) that was administered as a prophylactic agent against possible nerve gas attack. The present study was designed to determine the toxicity produced by individual or coexposure of hens 5 days/week for 2 months to 5 mg PB/kg/day in water, by gavage; 500 mg DEET/kg/day, neat, sc; and 10 mg chlorpyrifos kg/day in corn oil, sc. Coexposure to various binary treatments produced greater neurotoxicity than that caused by individual exposures and was characterized by severe neurologic deficit and neuropathological alterations. Also, neurotoxicity was further enhanced following concurrent administration of the three chemicals. Severe inhibition of plasma butyrylcholinesterase (BuChE) activity was produced in hens treated with PB (activity 17% of control) compared to those treated with chlorpyrifos (activity 51% of control) or DEET (activity 83% of control). BuChE inhibition was further increased in binary and tertiary treatment groups compared to individual treatment groups. In contrast, a significant inhibition of brain acetylcholinesterase (AChE) was produced in hens administered chlorpyrifos alone (activity 67% of control), while those given chlorpyrifos in combination with other compounds exhibited a significant inhibition of brain AChE activity ranging from 43 to 76%. Brain neurotoxicity target esterase (NTE) was not inhibited in any of the individual treatment groups or PB/DEET, but was significantly inhibited and had activity expressed as a percentage of control in groups administered combined chlorpyrifos with PB of 73% or DEET of 74% and in the tertiary treatment group of 71%. We hypothesize that test compounds may compete for xenobiotic metabolizing enzymes in the liver and blood and may also compromise the integrity of the blood–brain barrier, leading to an increase in their "effective concentrations" in the nervous system to levels equivalent to the toxic doses of individual compounds. This is consistent with the present observation of increases in (1) the inhibition of brain AChE and NTE, (2) the extent of neurologic dysfunction, and (3) the severity and frequency of neuropathologic lesions in the combined treatment groups compared to those administered individual compounds. © 1996 Society of Toxicology.

Between the invasion of Kuwait by Iraq on August 2, 1990, and March 1991, the United States had 697,000 military personnel in the Persian Gulf region (Institute Of Medicine, 1995). Among the troops, women accounted for 7% of the force, while Reserve/National Guard personnel were 17%. Since their return, approximately 30,000 veterans have had unexplained complaints including chronic fatigue, muscle and joint pain, ataxia, rash, headache, difficulty concentrating, forgetfulness, and irritability. Approximately half of the veterans with complaints have been Reservists/National Guard personnel. Among more than 100,000 non-U.S. coalition forces, including Canadian, Saudi, French, Egyptian, Syrian, Moroccan, and British Troops, only several hundred British and Canadian veterans complained of similar illnesses (Persian Gulf Veterans Coordinating Board, 1995).

The Persian Gulf War veterans were exposed in the Gulf area to a unique combination of biological, chemical, and psychological environments. In addition to extreme climatic changes, other exposures may have included sand and dirt, fumes and smoke from military operations, oil well spills and fires, petroleum products, diesel exhaust, depleted uranium, lead, multiple immunizations, and pesticides, e.g., the insect repellent DEET (N,N-diethyl-m-toluamide) and the insecticide chlorpyrifos (O,O-diethyl 0-3,5,6-trichloropyridinyl phosphorothioate) (Fig. 1; Institute Of Medicine, 1995). Furthermore, all U.S., British, and Canadian military personnel were given, at the beginning of the war, 21 30-mg tablets

1 To whom correspondence should be addressed.
2 Present address: ENVIRON Corp., Arlington, VA 22203.
TABLE 1
Summary of the Number of Treated Hens That Died or Were Euthanized during the Study and at Termination and the Number of Animals Used for Neurotoxicity and Histopathological Studies

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals that died before the termination of the study</th>
<th>No. of animals euthanized before the termination of the study</th>
<th>No. of animals euthanized at the termination of the study</th>
<th>No. of animals used for neurochemistry</th>
<th>No. of animals used for histopathological examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Pyridostigmine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromide (PB)</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>DEET</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Chlorpyrifos (CPF)</td>
<td>2 (38, 41)*</td>
<td></td>
<td></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>PB + DEET</td>
<td>3 (15, 17, 20)*</td>
<td>2 (20, 24)*</td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>PB + CPF</td>
<td>1 (17)*</td>
<td>0</td>
<td></td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>PB + DEET + CPF</td>
<td>3 (12, 19, 32)*</td>
<td>2 (34, 41)*</td>
<td></td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

* Hens were given a daily dose, 5 days/week, for 2 months, individual or combinations of 5 mg PB/kg in water by gavage, 500 mg DEET/kg sc, or 10 mg CPF/kg in corn oil sc.

Days after beginning of dosing when hens died or were euthanized.

Of pyridostigmine bromide (PB) to protect against organophosphorus nerve gases (Persian Gulf Veterans Coordinating Board, 1995). The troops were to self-administer one PB tablet every 8 hr when the risk of chemical attack was imminent. In the light of the safety record of PB during drug development (Teichman et al., 1987) and of the fact that its oral therapeutic dose for myasthenia gravis is in the range 200–1400 mg a day (Breyer-Pfaff et al., 1985), it was surprising that up to 50% of those seen in health care facilities during the Gulf War complained of PB muscarinic side effects (Institute Of Medicine, 1995; Keeler et al., 1991; Cook and Kolka, 1992).

The prophylactic action of PB against organophosphorus nerve gases is based on its reversible inhibition of 30–40% of acetylcholinesterase (AChE) activity in the peripheral nervous system, thus shielding it from long-lasting inactivation by the nerve gas (Blick et al., 1991). PB, a dimethylcarbamate, has a half-life of elimination of 0.35 hr following a single oral dose of 60 mg in human volunteers (Eiermann et al., 1993).

The aromatic amide DEET is used as a personal insect repellent (Pressley and Longbottom, 1982). Reported human poisonings following dermal application of DEET include two deaths (Gryboski et al., 1961; Zadikoff, 1979; Edwards and Johnson, 1987). Symptoms of DEET toxicity include tremor, restlessness, slurred speech, seizures, impaired cognitive functions, and coma (McConnell et al., 1986). Neuropathologic lesions in rats treated with near-lethal doses of DEET are characterized by spongiform myelinopathy in the brainstem (Verschoyle et al., 1990).

Chlorpyrifos, a phosphorothioate insecticide, undergoes first-pass metabolism to chlorpyrifos oxon (Abdel-Rahman et al., 1993), which inhibits rat brain AChE in vitro with a bimolecular rate constant \( k_{\text{in}} \) of 3.18 \( \times 10^6 \) compared to 3.22 \( \times 10^3 \) for the parent compound (Huff et al., 1994). Chlorpyrifos toxicity results in muscarinic, nicotinic, and central nervous system symptoms (Abou-Donia, 1994). In addition to its acute neurotoxicity, near-lethal doses of chlorpyrifos produce organophosphorus compound-induced delayed neurotoxicity (OPIDN). Recently, Kaplan et al., (1993) have reported sensory neuropathy associated with environmental exposure to chlorpyrifos. Following a suicide attempt with chlorpyrifos, OPIDN was reported in a 42-year-old man (Lotti et al., 1986). Chlorpyrifos at

![Chemical structure of test compounds](https://academic.oup.com/toxsci/article-abstract/34/2/201/1654858/1654858)

**FIG. 1.** Chemical structure of test compounds.
FIG. 2. Effect of daily administration of pyridostigmine bromide (PB), DEET, and chlorpyrifos (CPF) on survival and body weight of treated hens. Hens were dosed daily, 5 days/week, for 2 months with individual compounds or combinations of 5 mg/kg PB in water, po; 500 mg/kg DEET neat, sc; or 10 mg/kg CPF in oil, sc. (A) Number of days hens survived. Solid triangles represent hens euthanized before the termination of study because of severe morbidity. Open circles represent animals euthanized at the termination of the study. The mean and standard deviation of the number of survival days for hens in each treatment group are shown in parentheses. (B) Percentage mortality by treatment group. (C) Percentage of initial body weight at time of euthanization. Bars represent the mean and standard error from each treatment group (n = 5).

doses equivalent to 2–3× LD₅₀ produced OPIDN in the hen (Capodicasa et al., 1991; Abou-Donia and Wilmarth, 1995) and the cat (Fikes et al., 1992). OPIDN is characterized by a delayed and protracted development of ataxia and paralysis accompanied by a Wallerian-type degeneration of the central and peripheral nervous systems (Smith et al., 1930; Abou-Donia, 1981).

Our hypothesis that some of the Persian Gulf War veterans’ illnesses may have resulted from concurrent exposure to PB and other chemicals such as pesticides was prompted by two observations: (1) failure to identify bacterial, viral, or parasitic diseases as a source of the veterans’ complaints; (2) concurrent exposure to multiple chemicals has been shown to cause increased toxicity of single compounds. For example, the nonneurotoxic chemical iso-buty1 ketone increased the neurotoxicity of the weak neurotoxicant n-hexane (Lapadula et al., 1991) and of EPN (O-ethyl O-4-nitrophenyl phenylphosphonothioate)-induced delayed neuro-
TABLE 2
Clinical Condition and Neuropathological Changes in Hens Following Daily Administration of Individual and Coexposure of Pyridostigmine Bromide (PB), DEET, and Chlorpyrifos (CPF) in Hens*  

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Day of onset (Mean ± SEM)</th>
<th>Severity</th>
<th>Day of onset (Mean ± SEM)</th>
<th>Severity</th>
<th>Spinal cord</th>
<th>Sciatic nerve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>12.8 ± 1.4</td>
<td>1.0 ± 0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEET</td>
<td>9.4 ± 1.4</td>
<td>1.0 ± 0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPF</td>
<td>16.4 ± 3.5</td>
<td>1.0 ± 0.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PB + DEET</td>
<td>5.2 ± 1.4</td>
<td>1.8 ± 0.4</td>
<td>16.0 ± 4.6</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>DEET + CPF</td>
<td>8.2 ± 1.2</td>
<td>1.6 ± 0.2</td>
<td>20.4 ± 3.8</td>
<td>1.4 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>PB + CPF</td>
<td>3.4 ± 0.5</td>
<td>2.4 ± 0.3</td>
<td>20.4 ± 2.1</td>
<td>1.6 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>PB + DEET + CPF</td>
<td>2.8 ± 0.2</td>
<td>2.6 ± 0.2</td>
<td>17.6 ± 2.9</td>
<td>2.6 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

*All values are means ± SEM from each treatment group.

**Hens were given a daily dose, 5 days/week, individual or combinations of 5 mg/kg PB in water po, 500 mg/kg sc DEET, and 10 mg/kg sc CPF.

†Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of euthanization.

‡Severity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues.

§Not different from control.

toxicity (Abou-Donia et al., 1991). Also, propetamphos (1-methylthyl (E)-3-[[ethyaminooxyphosphinooxy]-2-butenoate), an organophosphorus insecticide that does not produce OPIDN, decreased the threshold dose of chlorpyrifos that causes OPIDN (Abou-Donia and Wilmarth, 1995). Consistent with this view is the recommendation of the committee to review the Health Consequences of Service During the Persian Gulf War that “studies are needed to resolve uncertainties about whether PB, DEET, and permethrin have additive effects” (Institute of Medicine, 1995). In fact, we have demonstrated in a previous study that coexposure to these three chemicals resulted in increased neurotoxicity in the adult hen (Abou-Donia et al., 1996).

The present study was carried out concurrently with the above-mentioned companion study to investigate if coexposure to PB, DEET, and chlorpyrifos enhances neurotoxicity compared to exposure to individual compounds.

METHODS

Chemicals

Technical grade (94%) chlorpyrifos (O,O-diethyl O,3,5,6-trichlorpyridinyl phosphorothioate) was obtained from DowElanco Co. (Indianapolis, IN). DEET (≥97%, N,N-diethyl-m-toluamide), pyridostigmine bromide (≥99%, 3-dimethylaminocarbonyloxyl-N-methylpyridinium bromide), ace- tythiocholine iodide, and butyrylthiocholine iodide were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade and obtained from commercially available sources.

Birds

The adult hen was used in this study to allow the investigation of both acute and OPIDN induced by exposure to chlorpyrifos alone and in combination with other chemicals. Although the clinical condition of the Gulf War veterans may not be identical to that of OPIDN, it may have resulted from superimposition of several conditions including OPIDN because of the potential exposure to organophosphorus compounds.

Healthy adult laying hens, 18 months old, weighing approximately 1.5 kg, were obtained from Featherdown Farm (Raleigh, NC). The hens were vaccinated against common chicken diseases and were considered specific pathogen-free, without abnormalities of gait. Control and treated hens were randomly assigned into groups of five hens and housed in single-tier, 3 × 3 × 3-foot stainless-steel cages in a temperature (21–22°C)-controlled room with a 12-hr light, 12-hr dark cycle 1 week before dosing and throughout the study. The hens had a supply of feed (Layena Chicken Feed; Ralston, Purina Co., St. Louis, MO) and water ad libitum.

Dosing

An initial dose–response study was conducted to select the dose used in single and combined treatments. This dose was determined as the maximum dose that produced minimum changes in the clinical parameters following treatment for 5 days/week over a 2-month period that demonstrated the safety of test compounds at relatively large doses. The individual test compound study was carried out using the following dosages for 5 days/week for 2 months: PB, 5 mg/kg/day in water by gavage; DEET, 500 mg/kg/day, neat, sc; chlorpyrifos, 10 mg/kg/day, in corn oil, sc. A group of five hens was given a daily sc injection of the same volume of corn oil on treatment days. Four groups of five hens each were given the following combinations of the test compounds: PB/DEET, PB/chlorpyrifos, DEET/chlorpyrifos, and PB/DEET/chlorpyrifos. Some of the animals died prior to the termination of the study, and their tissues were unsuitable for analysis as the result of autolysis and were replaced. In addition, four animals were euthanized when they became moribund before the end of the study. The number of animals from each treatment group and those that died or were euthanized during the study and at termination are listed in Table 1.

Clinical Evaluation

Control and treated hens were observed daily both inside their cages and when they were allowed to freely move outside the cage. Clinical assessment included walking pattern, leg movement, flying, body tremor, and
entering the home cage. Acute signs of toxicity that developed shortly after
dosing consisted of (1) diarrhea, salivation; (2) reluctance to walk, decreased
activity in cage; (3) shortness of breath; and/or (4) pupil constriction. Persis-
tent signs of neurotoxicity were characterized by locomotor dysfunctions
and were classified into (1) body lowered, mild leg splay while standing
and on landing after flying; (2) leg weakness demonstrated by preference
to fly instead of walking, gait disturbance, e.g., linear or narrow gait, stum-ling while entering the home cage, and moderately impaired flying activity;
(3) leg sprawl out, body unsteadiness, stumbling gait, inability to enter the
home cage, severely impaired flying activity; and (4) partial or complete
paralysis. For quantification, hens with these signs were assigned numerical
values of 1 to 4, corresponding to these conditions and assigned a value of
0 in the absence of any signs.

Histopathological Assessment

At 24 hr following administration of the last dose, treated and control
hens were anesthetized using 0.25% halothane and then euthanized by
decapitation. The spinal cord and sciatic nerve were excised immediately
after euthanization and immersed in 4% phosphate-buffered formalin and
postfixed for at least a week in the same fixative. Cross sections of cervical
and thoracic spinal cord and longitudinal sections of proximal and distal
sciatic nerve were stained for hematoxylin and eosin (H&E) and periodic
acid Schiff’s method. In addition, selected tissues were stained with Mars-
land and Glee’s silver stain. Tissues were assessed with a step-down ap-
proach by first comparing sections from the control and triple treatment
groups and then subsequently examining double and single treatments for
any evidence of alterations observed in the triple treatment groups. Severity
scores for the spinal cord were based on the frequency of enlarged axons
in the ventral, lateral, and columns in cervical and thoracic regions (0, no
detectable differences from control; 1, mild increase in the frequency of
enlarged axons; 2, moderate; 3, severe). Severity scores for the sciatic nerve
also ranged from 0 to 3 and were based on the combined frequency of
prominent enlarged axons in the proximal and distal segments. Photomicro-
graphs of illustrative examples of scores are presented under Results.

Enzymatic Studies

Brains were rapidly removed after euthanization 24 hr after the last dose
and homogenized (10% w/v) in 50 mM Tris–HCl buffer, pH 8.0, with 0.1
mM EDTA. Blood samples were collected into heparinized tubes and spun
in a Beckman Model J2-21 Centrifuge (Beckman Instruments Corporation,Palo Alto, CA) at 5000g for 30 min at 4°C using a JA-20 rotor to separate
plasma and red blood cells. Cholinesterase activities in plasma and brain
homogenates were determined using butyrylthiocholine iodide as substrate
for plasma butrylcholinesterase (BuChE) and acetylthiocholine iodide as
substrate for brain AChE enzyme (Ellman et al., 1961). Brain neurotoxicity
target esterase (NTE) activity was determined immediately after brain ho-
monogentization using phenyl valerate as a substrate, according to the method
of Johnson (1977). Protein concentrations for brain and plasma were deter-
mined using the bicinchoninic acid method (Smith et al., 1985).

Evaluation of Neurotoxicity

Mean rank. Mean rank was calculated for each control and treatment
group to compare the neurotoxicity produced following exposure to individ-
ual or combined treatments. Mean rank was calculated as described pre-
viously (Abou-Donia et al., 1982) based on the method of Jonckheere
(1954), by ranking the hens in the following six categories: (1) time of
onset and (2) severity of acute clinical signs of toxicity, (3) time of onset
and (4) severity of motor dysfunction, and (5) severity of neuropathologic
lesions in the CNS and (6) PNS. Ranks were assigned starting with minimal
categories of the CNS (0, no detectable differences from control; 1, mild
increase in the frequency of lesions; 2, moderate; 3, severe). Severity
scores for the sciatic nerve also ranged from 0 to 3 and were based on the combined frequency of prominent enlarged axons in the proximal and distal segments. Photomicro-
graphs of illustrative examples of scores are presented under Results.

Statistical analysis. For all unpaired data, groups of two or more were
analyzed for overall treatment effect using analysis of variance. If an overall

FIG. 4. Photomicrographs of cross sections of spinal cord from control hens and hens treated with chlorpyrifos (CPF). Ventral (A) and lateral (B) columns from a control animal (severity score, 0). Ventral (C) and lateral (D) columns from a hen treated with CPF exhibiting a mild increase in the frequency of enlarged axons (severity score, 1). Scale bar, 50 µm. H&E.
FIG. 4—Continued
FIG. 5. Photomicrographs of transverse sections through sciatic nerves from (A) a control hen (severity score, 0) and (B) a hen treated with CPF exhibiting a mild increase in the frequency of enlarged axons (arrows) (severity score, 1). Scare bar, 50 μm. H&E.
RESULTS

Treatment with Individual Compounds

Hens treated with PB exhibited transient mild cholinergic signs such as leg weakness and diarrhea with no significant loss of weight (Fig. 2). DEET caused an immediate rapid shallow breathing and inactivity which recovered within 24 hr (Table 2) of dosing daily. Hens treated with chlorpyrifos showed mild acute signs of cholinergic toxicity after 8 to 28 days of treatment such as transient diarrhea and leg weakness. These signs disappeared within 4 weeks despite continued treatment and the hens looked like controls at sacrifice. Hens treated with DEET or chlorpyrifos lost small but significant amounts of weight (Fig. 2). All birds treated with single compounds survived the experimental period (Fig. 2).

There was no detectable change in the spinal cords of hens treated with PB or DEET and those of control hens (Fig. 3 and Table 2). In all of the hens treated with chlorpyrifos, there was a small but detectable increase in the frequency of enlarged axons. Figure 4 shows ventral and lateral columns from a hen treated with chlorpyrifos showing a mild increase in the frequency of enlarged axons that had a score of 1. These enlarged axons tended to occur in clusters and were most obvious in the ventral and ventro-lateral tracts.

There were no detectable histopathologic differences in the sciatic nerves of hens treated with PB and those of control hens (Fig. 3 and Table 2). In two of the animals treated with DEET, there was a small but detectable increase in frequency of enlarged axons in the sciatic nerve (Fig. 3 and Table 2). In three of the animals treated with chlorpyrifos, there was a mild increase in frequency of enlarged axons in the sciatic nerves (severity score, 1; Fig. 5).

At the end of the treatment, PB strongly inhibited plasma BuChE activity to 17% of the control value (Fig. 6). DEET produced a slight inhibition of this enzymatic activity which was 83% of the control value. On the other hand, chlorpyrifos inhibited plasma BuChE to 51.3% of control activity. Of the three compounds, only chlorpyrifos resulted in significant inhibition of brain AChE to 67% of control activity. None of the three chemicals had any affect on brain NTE.

Treatment with Two Compounds

Concurrent treatment with both PB and DEET produced intermittent diarrhea, shallow breathing, and diminished leg movement (Fig. 3 and Table 2). Leg splay and altered stance were evident in four hens 9–32 days after the beginning of treatment. The clinical condition of three of these hens progressed to persistent unsteady gait. Chlorpyrifos and DEET produced diarrhea and acute signs similar to those produced by DEET such as body lowered, unsteadiness, reluctance to walk, and rapid shallow breathing. Clinical signs diminished during the course of the study and at euthaniza-
tion three had moderate effects and two exhibited mild signs. Locomotor dysfunction was mild in three hens and moderate in two. Combined treatment with chlorpyrifos and PB resulted in more severe clinical conditions than any other binary treatment. Onset of cholinergic signs was at 2 to 3 days after the beginning of treatment. These signs were severe diarrhea, salivation, reluctance to walk, and falling. With time, hens developed tolerance to the acute effects of the test compounds and by Day 28 had improved to moderate or severe acute toxicity. Locomotor dysfunction, such as change in gait, became evident after 18 to 26 days of dosing and was mild to moderate at euthanization. Hens treated with two compounds exhibited a small but significant loss of weight, despite their ability to reach their feed (Fig. 2). All binary treatments caused death in some of the hens while some were euthanized when they became moribund during the experiment as indicated in Table 1.

In the spinal cord of four of the hens treated with PB and DEET, there was a mild increase in the frequency of enlarged axons (severity score, 1; Figs. 7A and 7B). Three hens treated with PB and chlorpyrifos exhibited a moderate increase in the frequency of axonal swellings in the ventral and ventro-lateral tracts of the spinal cord (severity score, 2; Figs. 7C and 7D). There was also a moderate increase in the frequency of axonal swelling in three hens treated with DEET and chlorpyrifos (severity score, 2). As in other hens treated with chlorpyrifos alone or in combination with other agents, enlarged axons appeared to occur in clusters and were most obvious in the ventral, ventro-lateral, and lateral tracts.

The sciatic nerve of the hens treated with PB and DEET showed a mild increase in the frequency of enlarged axons (severity score, 1; Fig. 8A). In the sciatic nerve of three hens treated with PB and chlorpyrifos there was a moderate increase in the number of enlarged axons (severity score, 2; Fig. 8B) in two animals and in one of the animals, the extent alteration appeared mild (severity score, 1). In the sciatic nerve of the hens treated with chlorpyrifos and DEET, there was a mild but consistent increase in the number of detectable axonal swellings (severity score, 1).

Hens dosed with PB and DEET exhibited great inhibition of plasma BuChE activity to 7.7% of control. Plasma BuChE activity was markedly inhibited in hens dosed with DEET and chlorpyrifos to 50.8% of control while it was reduced to 11.3% of control following treatment with PB and chlorpyrifos (Fig. 6). While PB/DEET treatment did not affect brain AChE activity, the other two combined treatments produced significant inhibition of this enzyme and resulted in activity (percentage of control) of 57.3% for PB/chlorpyrifos and 29.5% for DEET/chlorpyrifos. Brain NTE activity was not significantly affected after coexposure to combined treatment of PB and DEET. Chlorpyrifos in combination with either PB or DEET produced similar inhibition of brain NTE, which had an activity of 73% of control (Fig. 6).

Treatment with Three Chemicals

Hens treated concurrently with PB, DEET, and chlorpyrifos exhibited severe acute cholinergic signs such as diarrhea, salivation, and leg weakness, as well as shallow breathing (Fig. 3 and Table 2). Severity of clinical signs regressed from severe to mild and moderate signs at euthanization. This treatment caused a small but significant loss of weight (Fig. 2). In this treatment group, one hen died and was replaced and two became moribund and were euthanized before the end of the experiment.

There was an increase in the frequency of enlarged axons in the spinal cords of hens treated with PB, DEET, and chlorpyrifos (Figs. 7E and 7F). These enlarged axons appear in clusters and were most obvious in the ventral, ventro-lateral, and lateral tracts. These clusters often appeared in close proximity to small blood vessels. In four of the hens treated with PB, DEET, and chlorpyrifos, the extent of increase in the frequency of these axonal enlargements was severe (severity score, 3; Figs. 7E and 7F) and in one hen the frequency of axonal enlargements was moderate (severity score, 2). The extent to which these alterations are indicative of axonal degeneration was most obvious in silver-stained longitudinal sections of the spinal cord (Figs. 9B and 9C), in which neurofibrillary alterations and axonal fragmentation were apparent.

There was an increase in the frequency of enlarged axons and prominent axonal swellings in the sciatic nerves of hens treated with PB, DEET, and chlorpyrifos (Fig. 3, Table 2). In five of these hens, the extent of this alteration was mild (severity score, 1) and in one it was moderate/severe (severity score, 3). Figure 8C shows a cross section from a hen treated with the three compounds exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). A silver-stained longitudinal section of a sciatic nerve from a hen treated with the three compounds shows many focal enlargements, some of which contain cytoskeletal inclusions (Fig. 10B).

Coexposure to the three compounds PB, chlorpyrifos, and DEET greatly decreased plasma BuChE to 13.2% of control. Brain AChE activity was diminished to 24% while brain NTE was 71% of the control value (Fig. 6).

Relative Neurotoxicity of Individual and Multiple Exposures

The relative neurotoxic effect of individual test compounds and concurrent exposure to two or three chemical combinations were compared by calculating the mean rank. The mean rank encompasses the onset of severity of acute signs of toxicity and locomotor dysfunctions as well as the severity of peripheral nerve and spinal cord damage at the time of euthanization (Fig. 3, Table 3). Control hens were normal and had a mean rank value of 7.0. The mean rank
FIG. 7. Photomicrographs of cross sections of the ventral (A, C, E) and lateral (B, D, F) columns of the spinal cord from a hen treated with PB and DEET. (A, B) Section from a hen treated with PB/DEET exhibiting a mild increase in the frequency of enlarged axons (severity score, 1). (C, D) Section from a hen treated with PB/CPF exhibiting a moderate increase in the frequency of enlarged axons (severity score, 2). (E, F) Section from a hen treated with PB/DEET/CPF exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). Details as in Fig. 4.
FIG. 7—Continued
FIG. 8. Photomicrographs of transverse sections of sciatic nerve. (A) Section from a hen treated with PB and DEET exhibiting a mild increase in the frequency of enlarged axons (severity score, 1) and (B) section from a hen treated with PB/CPF exhibiting a moderate increase in the frequency of enlarged axons (severity score, 2). (C) Section from a hen treated with PB/DEET/CPF exhibiting a severe increase in the frequency of enlarged axons (severity score, 3). H&E. Details as in Fig. 5.
values for individual treatments were significantly higher than the control value. The mean rank values indicated that PB (10.7), DEET (12.9), and chlorpyrifos (14.8) were not significantly different from each other (Table 4). Also, mean rank values indicated that combined exposures were more neurotoxic than individual compounds, with the PB/DEET and DEET/chlorpyrifos combinations having similar values, i.e., 23.8 and 24.3, respectively, and with PB/chlorpyrifos having a greater mean rank value of 30.2. Furthermore, the combined treatment of the three chemicals produced the largest mean rank value of 32.4. Among binary combinations, while the mean rank value for PB/DEET was not significantly different from that for DEET/chlorpyrifos, it was significantly lower than that for PB/chlorpyrifos. The mean rank value of PB/chlorpyrifos was significantly higher than that for DEET/chlorpyrifos (Table 4). On the other hand, the tertiary treatment was significantly higher than all other treatments except for that of PB/chlorpyrifos.

DISCUSSION

The present results demonstrate that individual chemicals used to protect Gulf War soldiers from nerve-gas poisoning and insect-borne diseases at doses that produced mild toxicity to hens were highly toxic when used in combination. The insect repellent DEET, the insecticide chlorpyrifos, and the anti-nerve gas agent PB at large but mild doses when used alone caused neurologic deficits in the adult hen following concurrent subchronic exposure. Increased neurologic dysfunctions were accompanied by significant inhibition of plasma and brain cholinesterases and brain NTE activity as well as neuropathologic lesions. These findings suggest that an agent such as PB, which normally does not reach the brain, can augment the neurotoxicity produced by chlorpyrifos that does. Similarly, DEET that does not have cholinergic action can enhance those actions of an agent that does, such as chlorpyrifos. These findings have three important implications. First, concurrent exposure to test chemicals may decrease the body's normal mechanisms to remove these chemicals and/or increase their transport to the neurotoxicity target. Second, the use of PB concurrently with exposure to pesticides and other chemicals in the Gulf War may be related to some of the complaints of the service personnel. Third, genetic variations in plasma and liver esterases having reduced activity may be useful in identifying a subpopulation particularly susceptible to neurotoxic chemicals such as chlorpyrifos.

Although the three test compounds adversely affect the nervous system, they all have a distinct mode of action. The acute oral LD₅₀ of PB in male rats is 61.6 mg/kg (McCain, 1995) and that of DEET is 3000 mg/kg (Ambrose et al., 1959). The acute oral LD₅₀ of chlorpyrifos in male rats is
FIG. 9. Photomicrographs of transverse sections of spinal cord through the lateral columns stained with Marsland and Glee's silver stain. (A) Section from control hen. (B, C) Sections from hens treated with PB/DEET/CPF exhibiting fragmentation of axis cylinders (arrows) and focal enlargements. Scale bar, 50 µm.
150 mg/kg and in hens is 50 mg/kg (Lotti et al., 1986; Abou-Donia and Wilmarth, 1995). Individual test compounds, under the present dosing regimen, produced inhibition of plasma BuChE in the following descending order: PB > chlorpyrifos > DEET. The large inhibition resulting from PB and chlorpyrifos is consistent with their anticholinesterase effect. On the other hand, DEET, an aryl amide, caused a slight but significant inhibition of plasma BuChE. Neither PB nor DEET alone or together had any effect on brain AChE, consistent with their properties that PB does not cross the blood–brain barrier (Birtley et al., 1966) and that DEET does not have anticholinesterase activity. On the other hand, as expected, chlorpyrifos administration produced significant inhibition of brain AChE (Pope et al., 1992; Richardson et al., 1993; Huff and Abou-Donia, 1995). Furthermore, combined exposure to chlorpyrifos with PB, DEET, or both greatly increased brain AChE inhibition.

The effect of test compounds, singly and in combination, on NTE is also consistent with their mode of action and clinical manifestation. NTE is the putative target and a biomarker for OPIDN (Johnson, 1969). A single or multiple exposure of an organophosphorus ester that causes at least 70% (Johnson, 1969; Lotti, 1992) or 54% (Sprague et al., 1981) inhibition of NTE, respectively, followed by “aging” results in OPIDN. The result that neither PB nor DEET inhibited hen brain NTE is in agreement with the fact that these compounds do not produce OPIDN. Also, the chlorpyrifos dosage when used alone neither inhibited NTE nor produced OPIDN, indicating that it was below the threshold level to cause OPIDN. On the other hand, chlorpyrifos in combination with PB, DEET, or both resulted in a significant inhibition of NTE activity (27 to 29% inhibition). Since this inhibition was below the threshold level for NTE inhibition, neurologic deficits and neuropathologic lesions cannot be related to OPIDN. Neurologic dysfunctions resulting from coexposure to chlorpyrifos and other chemicals may have resulted from superimposition of neurotoxic effects of administered compounds. Inhibition of AChE in the PNS by the three compounds increases ACh in the synapse, leading to increased skeletal and smooth muscle contraction (Abou-Donia, 1994). This effect may be augmented by neuromuscular toxicity induced by PB (Hudson et al., 1985). Acute and subacute exposure of rats to PB caused morphological alterations in the neuromuscular junction from the diaphragm, soleus, and extensor digitorum longus muscles. Combined treatment with the three chemicals caused less severe alterations in the cervical columns than those seen below the cervical level that included the descending tracts, i.e., corticospinal columns of the lateral and ventral regions.

The increased neurotoxicity of the test chemicals resulting in severe neurologic deficits in the experimental animal model reported in this study may explain some of the unex-
FIG. 10. Photomicrographs of transverse sections of sciatic nerve stained with Marsland and Glee's silver stain. (A) Section from a control hen. (B) Section from a hen treated with PB/DEET/CPF. Note the many focal enlargements, some of which exhibit cytoskeletal inclusions (arrows). Scale bar, 50 μm.
TABLE 3

Ranked Neurotoxicity Scores Following Daily Administration of Individual and Coexposure of Pyridostigmine Bromide (PB), DEET, and Chlorpyrifos (CPF)*

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Clinical signs</th>
<th>Locomotor dysfunction</th>
<th>Histopathological changes</th>
<th>Mean rank* (NTI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day of onset</td>
<td>Severity</td>
<td>Day of onset</td>
<td>Spinal cord</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>PB</td>
<td>13</td>
<td>15</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>DEET</td>
<td>19</td>
<td>15</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>CPF</td>
<td>12</td>
<td>15</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>PB + DEET</td>
<td>28</td>
<td>25</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>DEET + CPF</td>
<td>21</td>
<td>24</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>PB + CPF</td>
<td>33</td>
<td>33</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>PB + DEET + CPF</td>
<td>35</td>
<td>34</td>
<td>36</td>
<td>36</td>
</tr>
</tbody>
</table>

* All values are means from each treatment group.
* Hens were given a daily dose, 5 days/week, individual or combinations of 5 mg/kg PB in water po, 500 mg/kg sc DEET, and 10 mg/kg sc CPF.
* Animals were assessed daily for clinical signs, locomotor dysfunctions, and tremor and scored as indicated under Methods. Severity scores indicate the condition of the animal at the time of euthanization.
* Severity of histopathological alterations was determined by examination of the cervical and thoracic spinal cord and sciatic nerve tissues.
* Mean rank or neurotoxicity index (NTI) is the mean score of clinical signs, locomotor dysfunction, tremor, and histopathological alteration shown in Table 2. Every parameter was ranked according to severity scores from least to greatest, and within the severity according to time of onset to obtain ranked scores.

expected neurologic complaints of some of the Persian Gulf War veterans (Keeler et al., 1991). Although this experimental study was not designed to determine the cause of the Persian Gulf War veterans' illnesses, its findings suggest that coexposure to test compounds may have contributed to their complaints. In an attempt to approximate actual exposure conditions, PB which was taken orally by service personnel as a 30-mg tablet three times daily (equivalent to 1.3 mg/kg/day) was administered (5 mg/kg) to hens by gavage (Institute of Medicine, 1995). Exposure to DEET and chlorpyrifos by the service personnel was through the skin and by inhalation. In this study both chemicals were applied subcutaneously for accurate quantification of the dose. DEET is efficiently absorbed through the skin; thus, over 90% of DEET was diffused across the hairless mouse skin in 24 hr (Windheuser et al., 1982) and its dermal absorption was nearly complete in 48 hr in rats (Valdez et al., 1996), while 50% of the applied dose to human skin was absorbed (Spencer et al., 1979). Thus, absorbed DEET following dermal application may approximate that of subcutaneous injection. The levels of exposure to the two pesticides during the Gulf War are not known. A 1986 NIOSH study, however, estimated the upper 1% of the weekly application dose of DEET by the employees in the Everglades National Park to be 1122 mg/kg/day (392.6 g/week).

Although the mechanisms of increased neurotoxicity following concurrent exposure to test chemicals are not known, we hypothesize that they are related to the increased effective

TABLE 4

Differences in the Mean Rank of Clinical Signs, Locomotor Dysfunction, and Neuropathological Alterations of Hens Administered Pyridostigmine Bromide (PB), DEET (D), or Chlorpyrifos (CPF) Either Individually or in Combination

<table>
<thead>
<tr>
<th>Control</th>
<th>Single treatments</th>
<th>Binary treatments</th>
<th>Tertiary treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB</td>
<td>DEET</td>
<td>CPF</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>5.9</td>
<td>7.8</td>
</tr>
<tr>
<td>PB</td>
<td>13.1</td>
<td>13.9</td>
<td>19.5</td>
</tr>
<tr>
<td>DEET</td>
<td>10.9</td>
<td>11.4</td>
<td>17.3</td>
</tr>
<tr>
<td>CPF</td>
<td>9.0</td>
<td>9.5</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PB + DEET</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEET + CPF</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PB + CPF</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
exposure to chemicals. Thus, individuals with diminished chemical alterations, and pathological lesions varied and dependent on both the nature and the dosage of each chemical. These studies also suggest that the idiosyncracy of individuals may play a major role in their reaction to concurrent exposure to chemicals. Thus, individuals with diminished ability to metabolize drugs and pesticides, such as those with the "atypical" genetic variant of plasma BuChE (Viby-Mogensen, 1981), are potentially vulnerable and at high risk to anticholinesterase and related chemicals and this may account for the more severe signs seen in some of the Gulf War veterans. This suggestion is supported by the recent finding that a soldier, homozygous for atypical BuChE, who had exhibited succinylcholine-induced apnea during surgery, also suffered severe cholinergic symptoms following prophylactic treatment with PB during the Persian Gulf War (Loewenstein-Lichtenstein et al., 1995).

This study has expanded and confirmed our previous finding (Abou-Donia et al., 1996) that coexposure to subneurotoxic doses of PB and pesticide chemicals resulted in increased neurotoxicity characterized by neurologic dysfunction, inhibition of plasma BuChE and brain AchE, and NTE and neuropathologic lesions. Further studies are needed to investigate the potential neurologic deficits resulting from coexposure to these chemicals at dosages to which the Gulf War veterans may have been exposed. Currently, we are investigating the specific mechanisms by which concurrent exposure to chemicals results in increased neurotoxicity.

ACKNOWLEDGMENTS

This study was supported in part by a grant from the Perot Foundation. The technical assistance of Jeanene Olin, the statistical expertise of Judy Schmid, and the secretarial assistance of Diane Brooks are appreciated.

REFERENCES


pyrifos and saffron on the development of delayed neurotoxicity in the hen. *Toxicologist* 15, 205.


