Anticonvulsant-Resistant Seizures following Pyridostigmine Bromide (PB) and N,N-Diethyl-\textit{m}-toluamide (DEET)

Leslie A. Chaney, Robin W. Rockhold, Robert W. Wineman, and Arthur S. Hume

Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi

Received April 3, 1998; accepted January 24, 1999

An acute toxic interaction has been described, in which sublethal doses of pyridostigmine bromide (PB) and the insect repellent N,N-diethyl-\textit{m}-toluamide (DEET), when administered concomitantly, resulted in seizures and lethality. To investigate the possible relationships between seizures and lethality and the role of the cholinergic system in this interaction, PB (5 mg/kg), DEET (200 mg/kg) or PB (3 mg/kg) + DEET (200 mg/kg) were administered ip to male ICR mice, alone or following ip pretreatment, with one of several anticonvulsant agents: diazepam, 10 mg/kg; fosphenytoin, 40 mg/kg; phenobarbital, 45 mg/kg; or dextrorphan, 25 mg/kg, or the anticholinergic agents, atropine (5 mg/kg), atropine methyl nitrate (2.7 mg/kg), or mecamylamine (2.5 mg/kg). The anticonvulsants selected for this study act through different mechanisms to reduce seizures. None of the anticonvulsants was able to reduce the incidence of seizures following treatment with PB, DEET or PB + DEET. Only diazepam delayed the onset of seizures. Fosphenytoin or diazepam significantly prolonged the time to lethality following PB, but only fosphenytoin reduced the incidence of PB-induced lethality. Diazepam or phenobarbital significantly prolonged the time to lethality following PB + DEET. Both atropine and atropine methyl nitrate protected against PB and PB + DEET-induced lethality and PB-induced seizures. Neither agent blocked seizures resulting from DEET or PB + DEET. Mecamylamine reduced seizures and lethality in PB-treated mice, but not in mice treated with DEET or PB + DEET. The results indicate that seizure activity is not a causative factor in the toxic interaction between PB and DEET. Furthermore, PB, DEET and PB + DEET induce seizures that are resistant to standard anticonvulsants, and each appears to operate through different mechanisms to produce seizures. Peripheral muscarinic receptors may play a specific role in lethality caused by PB + DEET.

Key Words: Pyridostigmine Bromide (PB), N,N-diethyl-\textit{m}-toluamide (DEET); cholinesterase inhibitors; seizures; anticonvulsant agents; mice.

An acute toxic interaction has been demonstrated recently in which synergism occurs when a reversible cholinesterase inhibitor, pyridostigmine bromide (PB), is combined with a personal insect repellent, N,N-diethyl-m-toluamide (DEET), in mice (Chaney et al., 1997). This interaction is of interest, because recent reports suggest that these agents may have been involved in the unexplained illnesses that appeared in many veterans of the Persian Gulf War (Haley and Kurt, 1997; NIH, TAWP, 1994). In addition, cholinesterase inhibitors are widely used in agriculture for pest control and, to a lesser extent, in medicine, for treating atony of the intestinal tract and bladder, glaucoma, and myasthenia gravis. They are also used to terminate the effects of competitive neuromuscular blocking agents (Taylor, 1996). DEET is used extensively, worldwide, and may become even more prevalent in the future as a critical public health tool for protecting against insect-borne disease (Osimitz and Grothaus, 1995).

The profile of toxic symptoms which precede acute lethality following ip administration of PB and/or DEET in mice includes the development of seizure activity within min of drug administration (Chaney et al., 1998a). Inhibition of cholinesterase by organophosphate (OP) cholinesterase inhibitors results in sustained activation of nicotinic and muscarinic cholinergic receptors by acetylcholine, which can lead to hyperexcitability of the central (CNS) and peripheral (PNS) nervous systems and can induce seizures in a dose dependent manner (Maynert et al., 1975; Turski et al., 1989). Since PB is a quaternary ammonium compound, it would not be expected to readily penetrate the blood-brain barrier and thus, would not produce the CNS symptoms common to non-quaternary OP cholinesterase inhibitors (Taylor, 1996). However, Domino (1987) documented the occurrence of gross motor seizures and EEG seizures following sc administration of high doses of either PB (1 mg/kg) or another quaternary cholinesterase inhibitor, neostigmine (0.32–1 mg/kg), to rats. McCain et al. (1997) also reported the development of convulsions in rats following oral administration of PB. Such findings suggest some component of interaction of PB with the CNS. The insect repellent, DEET, on the other hand, readily crosses lipid membranes and gains entry into the CNS (Robbins and Cherniack, 1986). Very little is known about the mechanism of toxicity of DEET, but reports of symptoms related to DEET exposure at high doses in humans and other mammals reflect an apparent action on the CNS causing generalized seizures (Edwards and
The use of anticonvulsants to reduce seizure activity and prevent lethality following exposure to OP cholinesterase inhibitors has been investigated with mixed results. Some success has been documented in preventing OP-induced seizures with benzodiazipines or N-methyl-D-aspartate antagonists (Braitman and Sparenborg, 1989; Lipp, 1972; Rump et al., 1973; Shih, 1991). However, little protection against lethality occurred following benzodiazipines unless anticholinergic agents were co-administered (Bokonjic and Rosic, 1991; Braitman and Sparenborg, 1989; Domino, 1987; Shih et al., 1991). Very few studies have been conducted on the occurrence and prevention of seizures resulting from overexposure to reversible quaternary ammonium cholinesterase inhibitors, and even less information has been reported on prevention of seizures induced by DEET (Domino, 1987; Lipscomb et al., 1992; Longo, 1962; Tenebein, 1987).

The purpose of this study was to investigate the outcome following the administration of anticonvulsants to mice prior to the induction of seizures and lethality by PB and/or DEET. The anticonvulsants that were selected for these experiments act through different mechanisms to reduce seizures. The role of the cholinergic system in the toxic interaction between PB and DEET was investigated further by examining the effects of a systemic muscarinic blocking agent (atropine sulfate), a peripherally-selective cholinergic blocking agent (atropine methyl nitrate), and a nicotinic ganglionic blocking agent (mecamylamine), on the occurrence of seizures and lethality following administration of PB and/or DEET.

MATERIALS AND METHODS

Materials. Pyridostigmine bromide, atropine methyl nitrate, and mecamylamine hydrochloride were purchased from Sigma Chemicals (St. Louis, MO) and dissolved in distilled water immediately prior to dosing, in each experiment. Dextrophan D-tartrate, purchased from Research Biochemicals, Int’l. (Natick, MA), was dissolved in phosphate-buffered saline. N,N-Diethyl-m-toluamide (Sigma Chemicals, St. Louis, MO) was administered undiluted. Diazepam and phenobarbital, purchased from Elkins-Sinn, Inc. (Cherry Hill, NJ), atropine sulfate (Fujisawa USA, Inc., Deerfield, IL), and fosphenytoin (Cerebyx\textsuperscript{R}, Parke-Davis, Morris Plains, NJ) were each administered in the commercially available, injectable-dosage form.

Animals. Male ICR mice (20–24 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in the central animal facility at 22 ± 1°C under a 12-h light-dark cycle. Mice were separated into plastic cages in groups of 10–12, and allowed to acclimate to the animal facility environment for 48 h before beginning experimental protocols. Tap water and laboratory chow were provided ad libitum.

Seizure studies. Mice were randomly divided into groups of 6–8 animals and weighed. Doses of PB and/or DEET were selected to provide a range of seizures and lethality. PB (2–5 mg/kg), DEET (100–700 mg/kg), or combinations of both agents, were administered by contralateral bolus ip injections. In animals receiving pretreatment with an anticonvulsant, an initial injection of the anticonvulsant was given, followed 15 min later by simultaneous contralateral injections of either distilled water and PB (5 mg/kg) or DEET (200 mg/kg), or PB (3 mg/kg) and DEET (200 mg/kg). These particular doses of PB and/or DEET were previously determined to induce seizures in approximately 80% of treated animals. Injection volume varied depending on the desired dose. The maximum injection volume given for any individual drug was 10 ml/kg bw. Doses of anticonvulsants were initially selected to be consistent with established doses for mice (Borchard et al., 1990; Rockhold et al., 1991), and then increased up to 2-fold in an effort to achieve the desired anti-seizure effect: dextrophan, 25 mg/kg; diazepam, 5–10 mg/kg; phenobarbital, 23–45 mg/kg; fosphenytoin, 20–40 mg/kg.

Immediately following injections, mice were placed in an opaque cage and observed continuously for signs of seizure activity for 1–2 h. Onset of seizures was defined as the appearance of frequent clonic limb movements. The time between PB and/or DEET injections and onset of seizure activity was recorded. Time to death was also recorded for mice who died during the observation period. Mortality was assessed 24 h after drug injection.

Separate studies were conducted to investigate peripheral and central cholinergic system involvement in seizure activity and acute mortality resulting from exposure to PB and/or DEET. Mice were given a pretreatment injection of atropine sulfate (5 mg/kg), atropine methyl nitrate (2.7 mg/kg), or a nicotinic ganglionic antagonist, mecamylamine (2.5 mg/kg), 15 min prior to administering PB and/or DEET. Seizure activity and mortality were evaluated as previously described.

Data analysis. On each day of anticonvulsant testing, individual groups of mice were administered PB and/or DEET individually to control for day-to-day variation in response to these agents. Since the incidence of PB and/or DEET-induced seizures or lethality did not differ significantly between these groups, data from multiple studies were pooled. Statistical comparisons between treatment groups for incidence of seizures and mortality were made using Fisher’s Exact test. Latency to onset of either seizures or death was compared between treatment groups using analysis of variance (ANOVA) with Student-Newman-Keuls post hoc tests. A $p < 0.05$ was considered statistically significant.

RESULTS

Seizure behavior. The incidence and time to onset of seizures and lethality following PB and/or DEET administration are presented in Table 1. Severe tonic-clonic seizures began abruptly within 10 min of PB administration, and were preceded by mild to moderate signs of cholinergic activation, including tremors, muscle fasciculations, increased salivation, lacrimation, and diarrhea. Neither muscle paralysis nor obvious signs of respiratory distress were observed prior to the induction of seizures. In most cases, death occurred within minutes of seizure onset. Seizures associated with the administration of DEET were preceded by uncoordinated movement and were less violent in nature, but more prolonged. DEET-induced seizures began with clonic motions in the forelimbs and progressed to intermittent tonic-clonic convulsions with “barrel rolling,” prostration, and loss of righting reflex. After the initial appearance of seizures at sub-lethal doses of DEET, seizure activity occurred intermittently for 30 min to 3 h, depending on the administered dose, while the animal remained prostrate in an unresponsive state, followed by complete recovery. Combinations of PB and DEET at doses that potentiate lethality induced seizure activity that initially resembled those caused by PB, described above, with abrupt onset of violent convulsions quickly followed by death of the animal.

Johnson, 1987; Konovalov and Romanov, 1980; Leach et al., 1988; Lipscomb et al., 1992; Poe et al., 1987; de Garbino and Laborde, 1983; Roland et al., 1985; Snyder et al., 1986; Tenebein, 1987; Verschoyle et al., 1992).
PB and DEET. Dose-dependent parallel increases in the incidence of seizures and lethality occurred following PB exposure. A dose-dependent increase in seizure activity was observed in response to DEET, which was not accompanied by an increase in lethality. Combinations of PB (1, 2, or 3 mg/kg) with a subconvulsive, sublethal dose of DEET (100 mg/kg) did not increase lethality, but significantly increased seizure activity at the highest dose of PB (3 mg/kg, data not shown). The seizures produced by this particular dose combination were much less severe than those that occurred when PB was combined with higher doses of DEET. Combinations of PB (2, 3, or 4 mg/kg) with DEET (200 mg/kg) at a sublethal dose, which alone produced seizures in approximately 80% of treated animals, resulted in potentiation of lethality as expected based on previous studies (Chaney et al., 1997). Seizure activity was not increased beyond that expected for DEET alone (80%) at this dose.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>% Seizures</th>
<th>Mean time to onset of seizures*</th>
<th>% Lethality</th>
<th>Mean time to lethality*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB 2 (n, 12)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>PB 3 (n, 12)</td>
<td>8</td>
<td>700</td>
<td>8</td>
<td>780</td>
</tr>
<tr>
<td>PB 4 (n, 12)</td>
<td>42</td>
<td>614 ± 182</td>
<td>42</td>
<td>711 ± 186</td>
</tr>
<tr>
<td>PB 5 (n, 22)</td>
<td>86</td>
<td>362 ± 36</td>
<td>86</td>
<td>460 ± 33</td>
</tr>
<tr>
<td>DEET 100 (n, 20)</td>
<td>0</td>
<td>—</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>DEET 200 (n, 12)</td>
<td>83</td>
<td>241 ± 17</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>DEET 400 (n, 12)</td>
<td>83</td>
<td>181 ± 15</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>DEET 700 (n, 12)</td>
<td>100</td>
<td>146 ± 9</td>
<td>33</td>
<td>617 ± 64</td>
</tr>
<tr>
<td>PB 2 + DEET 200 (n, 11)</td>
<td>100</td>
<td>219 ± 16</td>
<td>18</td>
<td>450 ± 20</td>
</tr>
<tr>
<td>PB 3 + DEET 200 (n, 12)</td>
<td>75</td>
<td>218 ± 16</td>
<td>50</td>
<td>345 ± 28</td>
</tr>
<tr>
<td>PB 4 + DEET 200 (n, 12)</td>
<td>83</td>
<td>216 ± 25</td>
<td>67</td>
<td>347 ± 27</td>
</tr>
</tbody>
</table>

* Time after PB and/or DEET injection (s) ± SE.

**FIG. 1.** Effects of pretreatment with anticonvulsants on the % incidence of seizures and lethality caused by PB and/or DEET administration in mice. Six to 28 animals were tested for each group. Controls received either distilled water and PB (5 mg/kg), DEET (200 mg/kg), or PB (3 mg/kg) and DEET (200 mg/kg). Anticonvulsants were administered at the following doses: diazepam, 10 mg/kg; fosphenytoin, 40 mg/kg; phenobarbital, 45 mg/kg; and dextrorphan, 25 mg/kg. All drugs were administered by ip injection. Statistical comparisons were made on raw data using Fisher’s Exact test. *p < 0.05 vs. control group.

Anticonvulsant pretreatment. Doses of PB were selected for these experiments which either alone (5 mg/kg) or in combination with DEET (3 mg/kg PB + 200 mg/kg DEET) produced seizures and lethality in 50–80% of treated animals. Diazepam (10 mg/kg) produced sedation in control animals, but otherwise no behavioral effects were observed for any anticonvulsant drug at the doses given. Results of anticonvulsant pretreatment on responses to PB and/or DEET are presented in Figures 1 and 2. Pretreatment with either diazepam (10 mg/kg), fosphenytoin (40 mg/kg), phenobarbital (45 mg/kg), or dextrorphan (25 mg/kg) did not reduce the incidence of PB- or DEET-induced seizure activity. Only diazepam significantly delayed the onset of seizures by approximately 80%. Fosphenytoin and diazepam significantly increased the time to lethality by approximately 65 and 95%, respectively, following administration of PB alone, but only fosphenytoin caused a 40% decrease in the incidence of PB-induced lethality. In animals given combinations of PB and DEET, pretreatment with anticonvulsants did not alter the incidence of seizures or lethality. Diazepam significantly prolonged the onset of seizures by approximately 60% in these mice, and both diazepam...
and phenobarbital significantly increased the time to lethality by 170 and 50%, respectively.

**Anticholinergic pretreatment.** Anticholinergic drugs were administered at doses previously shown to counteract intoxication resulting from overexposure to cholinesterase inhibitors (Domino, 1987). Behavioral effects were not observed in control animals at these doses. Results of these studies are presented in Table 2. Both atropine sulfate (5 mg/kg) and peripherally-selective atropine methyl nitrate (2.7 mg/kg) protected against PB and PB + DEET-induced lethality and PB-induced seizures. Neither agent blocked seizures resulting from DEET or the combination of PB and DEET. Mecamylamine, a nicotinic ganglionic antagonist, was able to protect against both seizures and lethality in PB-treated mice, but not in mice treated with DEET or the combination of PB and DEET.

**DISCUSSION**

The mechanism(s) by which PB induces seizure activity by itself, even at high doses, is unclear. Previous research has focused on other cholinesterase inhibitors, mainly on OP chemical warfare agents. These easily penetrate the blood-brain barrier and act within the central nervous system to elicit seizures by increasing the concentration of free acetylcholine in the brain (Maynert et al., 1975; Shih et al., 1991; Turski et al., 1989). Since PB is a positively charged quaternary ammonium compound, it is assumed to act only in the periphery (Taylor, 1996). However, Friedman et al. (1996) recently demonstrated in mice that PB might gain access to the brain when administered peripherally under conditions of stress. Seizures and lethality following PB administration, in the present studies, both appear to be influenced by cholinergic activity, since significant protection is afforded by atropine, atropine methyl nitrate and mecamylamine. Reduction of PB-induced seizures and lethality by atropine methyl nitrate, a peripherally selective muscarinic and nicotinic antagonist, suggests that the PNS has a role in the production of seizures. In fact, rat studies in this laboratory indicate that PB does not enter the CNS, since rat brain acetylcholinesterase activity is not inhibited significantly in either whole brain, or more specifically, in cortex, cerebellum, brainstem, hypothalamus, hippocampus, midbrain, or stri-

| TABLE 2 | Incidence of Seizures and Lethality Following Administration of Pyridostigmine Bromide (PB) and/or N,N-Diethyl-m-Toluamide (Deet) in Mice Pretreated with Atropine (At), Atropine Methyl Nitrate (Amn), or Mecamylamine (Mec) |
|-----------------|-----------------|-----------------|-----------------|
| **Treatment (mg/kg)** | **% Seizures** | **% Lethality** |
| PB 5 (n, 22) | 86 | 86 |
| DEET 200 (n, 12) | 83 | 0 |
| PB 3 + DEET 200 (n, 12) | 75 | 50 |
| AT 5 + PB 5 (n, 9) | 22a | 0a |
| AT 5 + DEET 200 (n, 8) | 63 | 0 |
| AT 5 + PB 3 + DEET 200 (n, 8) | 75 | 0a |
| AMN 2.7 + PB 5 (n, 8) | 25a | 0a |
| AMN 2.7 + DEET 200 (n, 8) | 63 | 0 |
| AMN 2.7 + PB 3 + DEET 200 (n, 17) | 82 | 6a |
| MEC 2.5 + PB 5 (n, 8) | 37.5a | 12.5a |
| MEC 2.5 + DEET 200 (n, 8) | 100 | 0 |
| MEC 2.5 + PB 3 + DEET 200 (n, 8) | 100 | 75 |

a Significant p < 0.05, Fisher’s Exact test on raw data.
It was demonstrated in the present experiments that PB-induced seizures were resistant to standard anticonvulsant drugs. This included the benzodiazepine, diazepam, which has been reported by others to give significant protection against seizures caused by OP inhibitors of cholinesterase (Bokonjic and Rosic, 1991; Domino, 1987; Lipp, 1972; Rump et al., 1973; Shih, 1991). In contrast, even at doses shown efficacious by others, diazepam administration produced only a prolongation of the time to onset of PB-induced seizures. A second anomaly that deserves mention is that fosphenytoin was able to significantly reduce lethality following PB administration, while the incidence of seizure activity was unaffected. Fosphenytoin is rapidly converted to phenytoin, an anticonvulsant drug which alters numerous physiological functions through actions on several second messenger systems (DeLorenzo, 1995). Phenytoin has been shown to prolong survival in several animal models under conditions of reduced oxygen availability (Cullen et al., 1979; Baldy-Moulinier, 1971; Hoff and Yahm, 1944; Naiman and Williams, 1964). This may be analogous to the present situation in which paralytic effects on respiratory muscles and increased bronchoconstriction following the high individual doses of PB (5 mg/kg) are likely to have induced hypoxia, leading ultimately to death. In support of these ideas, Artru and Michenfelder (1980) have reported that phenytoin prolongs survival time in a hypoxic mouse model without suppressing convulsions.

The mechanism of acute neurotoxicity caused by DEET has not been elucidated. In studies by Verschoyle et al. (1992), a neuropathological examination following acute administration of near-lethal oral doses of DEET (1–3 g/kg) in rats revealed the formation of a spongiform myelinopathy in cerebellar roof nuclei and the formation of neuronal cytoplasmic clefts. Partially controlled motor seizures were elicited by DEET in those animals. EEG recordings also revealed the development of spike discharges that were accompanied by abnormal changes in auditory evoked response, indicating an excitatory component in DEET toxicity (Verschoyle et al., 1992). Schoenig et al. (1993) conducted neurobehavioral studies in rats following either acute or chronic oral administration of DEET at much lower doses (50–500 mg/kg) and found only a slight increase in locomotor activity. They concluded that the nervous system is not a selective target in DEET toxicity since symptoms only appear at doses where other, more systemic toxic effects occur. Generalized seizures, the most common symptom in cases of human overexposure to DEET, have reportedly been managed by emergency intervention with diazepam, phenobarbital, or phenytoin (Osimitz and Grothaus, 1995; Tenebin, 1987). In this study, standard anticonvulsant drugs all failed to protect against DEET-induced seizures in mice. Anticholinergic agents also were not able to reduce seizures caused by DEET. This suggests that seizure activity is mediated through a different (non-cholinergic) pathway than that of PB.

The toxic interaction between PB and DEET has drawn recent attention as a potential causative factor in Gulf War illnesses (Chaney et al., 1997; Haley and Kurt, 1997; McCain et al., 1997; NIH, TAWP, 1994). Abou-Donia et al. (1996a,b) have shown that chronic concurrent administration of PB and DEET in chickens results in greater neurotoxic effects (clinical signs, locomotor dysfunction, tremor) than those caused by administration of individual agents, without inhibiting brain acetylcholinesterase activity. In the present study, the interaction between PB and DEET resulted in seizures that were resistant to both anticonvulsant and anticholinergic treatment. The occurrence of seizures appeared to be dominated by the effects of DEET, since PB-induced seizures respond to anticholinergic pretreatment. On the other hand, PB + DEET-induced lethality may be mediated through cholinergic pathways, particularly by peripheral muscarinic receptors. Both atropine, a systemic anti-muscarinic agent, and its peripherally-selective analog, atropine methyl nitrate, effectively reduced the incidence of lethality of the combination, but a nicotinic ganglionic blocker, mecamylamine, did not.

In summary, these studies demonstrate that seizure activity does not play a primary causative role in the acute toxic interaction between PB and DEET. Seizures that result from administration of these agents are resistant to standard anticonvulsants at the doses tested. PB and DEET appear to operate through different pathways to generate seizures, since anticholinergic drugs were able to protect against seizures induced by PB but not DEET. Peripheral muscarinic receptors may be involved selectively in the toxic interaction between PB and DEET. This was demonstrated by the protective effect of atropine methyl nitrate, which significantly reduced lethality following administration of PB + DEET at doses that potentiate lethality in mice.

REFERENCES


