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ACTIONS OF PYRIDOSTIGMINE AND ORGANOPHOSPHATE AGENTS ON CHICK CELLS, MICE, AND CHICKENS*

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ABSTRACT

Gulf War veterans were given pyridostigmine bromide (PB) tablets to enhance the therapeutic effect of antidotes to nerve agents in the event of exposure. The goal of this research is to examine whether combined exposure to PB and sarin (agent GB) is more neurotoxic to sensitive surrogate animals, mice and chickens, than if given separately. Scoping trials were performed to establish appropriate dose-response ranges for sarin and control chemicals. IC_{50} values were determined in chickens and mice for in vitro inhibition of acetylcholinesterase (AChE) and neuropathy target esterase (NTE). The results indicated PB neither inhibits NTE nor does it spare sarin's inhibition of AChE. Chick embryo nerve cells in vitro showed more inhibition of AChE activity and no faster recovery when PB treatment was followed by DFP treatment than the other way around. Experiments on chickens also indicated that PB treatment did not inhibit NTE and that it crossed the blood brain barrier inhibiting brain AChE although to a lesser extent than it inhibited blood cholinesterases. Other experiments determined multiple dose levels in chickens for sarin and DFP that inhibited >80% of NTE,

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considered a threshold for triggering organophosphate-induced delayed neuropathy.

INTRODUCTION

Gulf War scenarios often concern the consequences of combined exposures to an organophosphate (OP), e.g., a pesticide and/or chemical agent, and a carbamate, e.g., pyridostigmine bromide.[1–4] This project studies the impact of sarin (*O*-isopropyl, methylphosphono-fluoridate, GB) and pyridostigmine bromide (PB) on the biochemistry and morphology of nerve/muscle systems of mice and chickens.[5] Here we examine inhibitions of brain acetylcholinesterase (AChE) and neuropathy target esterase (NTE) by sarin, diisopropyl phosphofluoridate (DFP) and PB, singly and in combination, using homogenates, cell cultures, mice and chickens. Objectives were to generate baseline information on enzyme and dose levels of the agents and to find out whether the inhibitory effects of an OP and PB are additive, synergistic or antagonistic.

Brain AChE activity is a major target of OP chemical warfare agents. Its inhibition either directly causes or is an indirect indicator of acute CNS and PNS symptoms.[6] Inhibition of brain neuropathy target esterase (NTE) is associated with organophosphate-induced delayed neuropathy (OPIDN).[7,8]

METHODS

Enzyme activities were determined in mouse and chicken brain homogenates and cultures. Freshly dissected or -70°C frozen tissues were weighed and homogenized in 50 mM *Tris*, 0.2 mM EDTA, pH 8.0 buffer (1 : 7.5 w/v). Homogenates were further diluted 1 : 10 in the same buffer for NTE assays and 1 : 10 in a 0.1 M sodium phosphate buffer, pH 8.0 for AChE assays. NTE activity was assayed colorimetrically using phenylvalerate as substrate[9] by a version of the assay of Johnson[10] as modified by Correll and Ehrich.[11] AChE activity was assayed using acetylthiocholine iodide as substrate and the colorimetric method of Ellman et al.[12] with a 96-well microplate reader.[13] Ten day chick embryo cells from mechanically dissociated brains were cultured in a 10% horse serum, 2% embryo extract, MEM medium[14] for 10 to 14 days in a 5% CO_2–air, 37°C incubator. Cells were washed with saline, extracted in a pH 7.3 buffer containing 1 M NaCl, 50 mM *Tris*, 0.2 mM EDTA and 0.5% Triton X-100 and assayed for AChE radiometrically with tritiated acetylcholine iodide according to the method of Johnson and Russell.[15] Protein content was determined by the method of Lowry et al.[16]

All procedures performed on animals and animal care were approved by the UC Davis Animal Care Committee.

RESULTS

Enzyme Homogenates

Both sarin and PB inhibited AChE (Figures 1–3), but only sarin inhibited NTE in the presence or absence of PB (Figures 4 and 5). Inhibitions were similar regardless of whether sarin or PB were added first. The presence of PB did not reduce the extent of the inhibitions.

Intact Animals

Chickens were dosed IM with PB at 1–10 mg/kg in a range-finding experiment and survivors sacrificed after 24 h. Mortality occurred at 2, 5, and 10 mg/kg. There was a clear-cut dose response of brain AChE to PB (Figure 6).

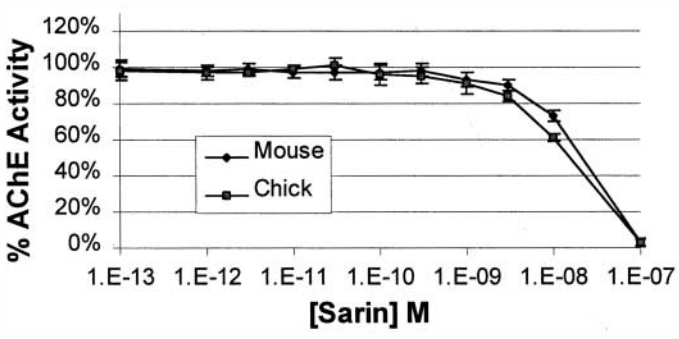

Figure 1. In vitro inhibition of brain AChE by sarin. Means ± S.D. of triplicate assays. Mouse $IC_{50} = 4.0 \times 10^{-8}$ M; Chick $IC_{50} = 2.7 \times 10^{-8}$ M. Incubation time with sarin was 1 h at room temperature.

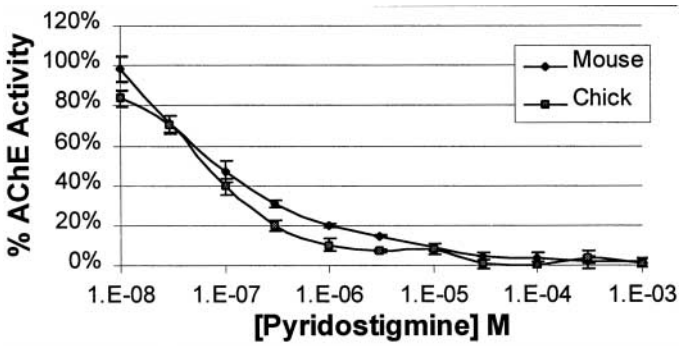

Figure 2. In vitro inhibition of brain AChE by pyridostigmine bromide. Means ± S.D. of triplicate assays. Mouse $IC_{50} = 9.1 \times 10^{-8}$ M; Chick $IC_{50} = 7.6 \times 10^{-8}$ M. Incubation time with PB was 1 h at room temperature.

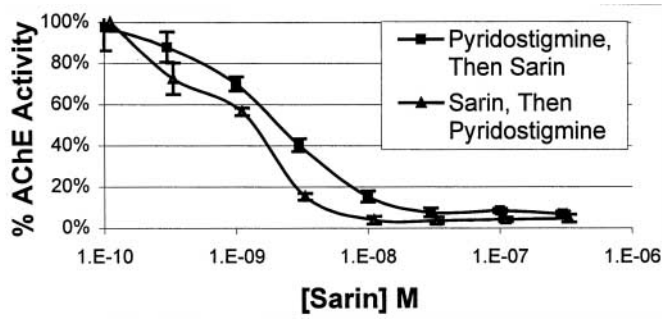

Figure 3. In vitro sarin and pyridostigmine inhibition of mouse brain AChE. Means ± S.D. of triplicate assays. [PB] = 3×10^{-8} M. Incubation times were 1 h at room temperature for each chemical.

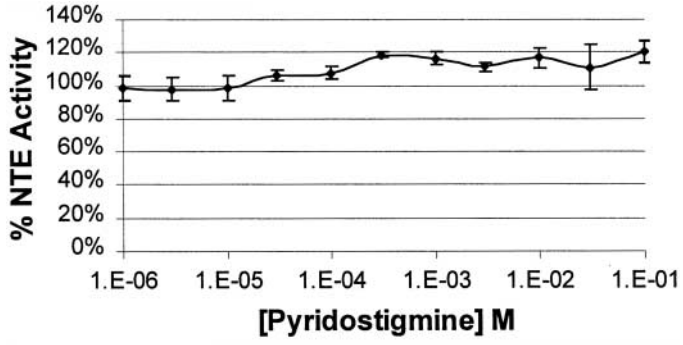

Figure 4. Lack of in vitro pyridostigmine bromide inhibition of chicken brain NTE. Means ± S.D. of triplicate assays. Incubation time for PB was 1 h at room temperature.

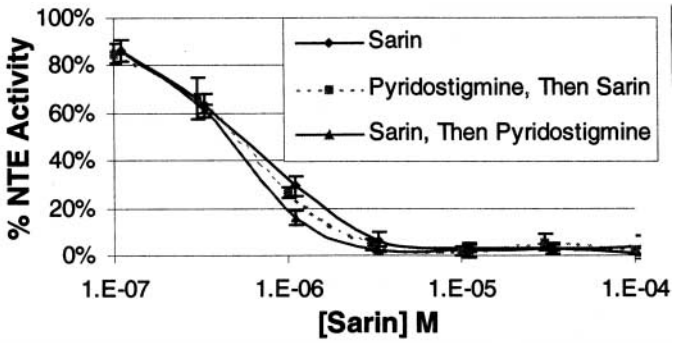

Figure 5. In vitro sarin and pyridostigmine inhibition of chicken brain NTE. Means ± S.D. of triplicate assays. Sarin $IC_{50} = 6.4 \times 10^{-7}$ M; [Pyridostigmine] = 10^{-2} M.

Blood cholinesterase levels were depressed to 34% at 1 mg/kg and to −4.6% at 2 mg/kg after 1 h. Twenty-four hours later the 1 mg/kg birds had recovered to 72% of controls and the surviving 2 mg/kg bird had recovered to 58% of its initial plasma ChE activity (there is no AChE activity in bird red blood cells; the plasma ChE hydrolyzes both acetyl and butyryl esters; Wilson, 1999). PB did not inhibit brain NTE activity regardless of dose level.

Nerve Cell Cultures

PB was added in saline one hour before DFP to chick embryo brain cells. The cells were washed, some sampled and others reincubated in complete media. Both DFP and PB inhibited AChE activity (Table 1). PB may have spared AChE inhibition by the higher concentrations of DFP.

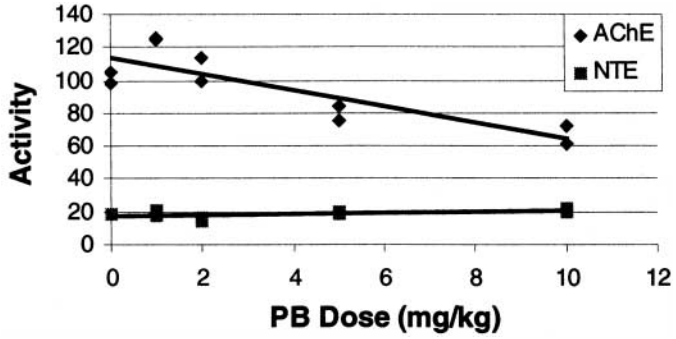

Figure 6. Brain enzyme levels in pyridostigmine-exposed birds. Paired samples. Activity is nmol/min/mg protein.

Table 1. Zero Time AChE Levels of Chick Nerve Cells After PB and DFP Treatments

	0 PB	10^{-8} PB	5×10^{-7} PB	10^{-5} PB
Trial 1				
0 DFP	26.1 ± 0.47	18.5 ± 0.79	4.93 ± 0.42	2.34 ± 0.14
Percent	100	70.9	18.9	9.0
10^{-7} DFP	19.6 ± 0.43	13.0 ± 0.71	5.00 ± 0.26	2.57 ± 0.17
Percent	75.1	49.8	19.2	9.8
Trial 2				
0 DFP	8.34 ± 0.34	4.91 ± 0.40	0.27 ± 0.02	0.46 ± 0.05
Percent	100	58.9	3.3	5.5
10^{-4} DFP	0.074 ± 0.04	0.076 ± 0.11	1.00 ± 0.11	0.29 ± 0.03
Percent	0.9	0.9	11.9	3.5

Activity is nmol/min/ng protein. Mean ± SEM; $n = 3$. Zero time refers to time immediately after treatment with DFP and/or PB.

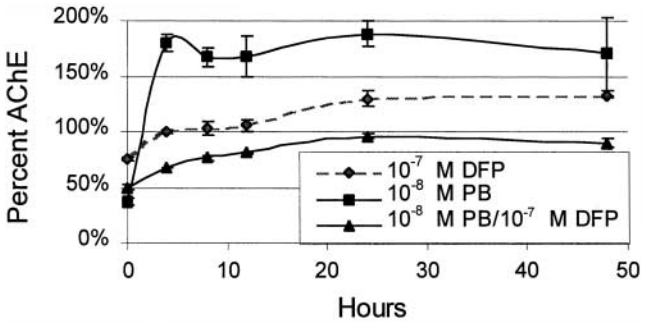

Figure 7. Recovery of AChE activity in chick nerve cell cultures after treatment with DFP and/or pyridostigmine. Mean ± SEM; $n = 3$. "0" hour refers to time of removal of DFP and/or PB.

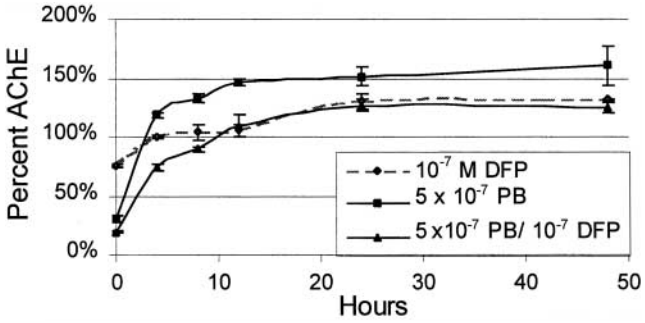

Figure 8. Recovery of AChE activity of chick nerve cell cultures after higher concentrations of PB than shown in Figure 7. Mean ± SEM; $n = 3$.

Inhibition of AChE was additive after treatment with DFP and PB. AChE levels in cells treated with 10^{-7} M DFP and 10^{-8} M PB did not reach the AChE levels of cells treated with DFP or PB alone during recovery (Figure 7). Cells treated with 10^{-7} M DFP and 5×10^{-7} M PB had AChE levels during recovery that were similar to those of DFP alone and approaching those of PB alone (Figure 8).

DISCUSSION

Pyridostigmine tablets was distributed to service personnel during the Gulf conflict at least in part because it had been shown to provide some measure of protection from soman[17,18] and the idea that its quaternary nitrogen structure lessened its ability to cross the blood brain barrier as compared to other carbamates such as physostigmine.[4] PB clearly crossed the blood brain barrier at the levels used here. The large inhibition of cholinesterase activity in the blood of chickens treated with 1 and 2 mg/kg PB suggests that

doses lower than those used in this range-finding study will be required to examine the possible interactions between PB and sarin.

PB did not provide any obvious protective effect on the enzyme, cell or animal levels of organization in so far as AChE activity was concerned. In fact, some cell and animal experiments indicate that the combination of PB and DFP causes greater inhibition of the enzyme than either alone. The notion that a more readily rehydrolyzable anticholinesterase agent could partially protect cholinesterase activity from inhibition by a more firmly bound and rapidly aging OP was not borne out, at least at the concentrations used in this study.

Cell cultures may be useful in studies of PB and OP interactions, especially to sort out toxicities due to direct actions at the receptor level and toxicities due to inhibition of target enzymes.[19,20] Recovery of enzyme activity in nerve and muscle cells in culture after DFP treatment has been shown to be due to new enzyme formation rather than reactivation of existing enzyme.[14,21] Whether recovery shown in cell cultures here is due to reactivation or new synthesis needs study.

Dose-response, combined chemical effects, biochemistry and morphometry experiments on animals dosed with neuropathic OPs, including tri-ortho cresyl phosphate and sarin, are in progress to determine whether PB may serve as a "promoter" of OPIDN[5,7] and exacerbate neuromuscle damage.[5,22]

CONCLUSION

Studies on tissue homogenates, cultured cells and intact animals revealed little direct interaction between PB and OPs on AChE of isolated cells and enzymes. Sarin but not PB inhibited NTE indicating PB is unable to induce OPIDN. Comparison of AChE levels in blood and brain of chickens indicate PB crossed the blood brain barrier at the levels used.

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Care and Use of Laboratory animals'' prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No 86-23, Revised 1985). Parts of the research were conducted at the UC Davis Institute of Toxicology and Environmental Health.

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