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Evidence for Nonacetylcholinesterase Targets of Organophosphorus Nerve Agent: Supersensitivity of Acetylcholinesterase Knockout Mouse to VX Lethality

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ABSTRACT

The possibility that organophosphate toxicity is due to inhibition of targets other than acetylcholinesterase (AChE, EC 3.1.1.7) was examined in AChE knockout mice. Mice (34–55 days old) were grouped for this study, after it was determined that AChE, butyrylcholinesterase (BChE), and carboxylesterase activities had reached stable values by this age. Mice with 0, 50, or 100% AChE activity were treated subcutaneously with the nerve agent VX. The LD_{50} for VX was 10 to 12 μg/kg in AChE−/−, 17 μg/kg in AChE+/−, and 24 μg/kg in AChE+/+ mice. The same cholinergic signs of toxicity were present in AChE−/− mice as in wild-type mice, even though AChE−/− mice have no AChE whose inhibition could lead to cholinergic signs. Wild-type mice, but not AChE−/− mice, were protected by pretreatment with atropine. Tissues were extracted from VX-treated and untreated animals and tested for AChE, BChE, and acylpeptide hydrolase activity. VX treatment inhibited 50% of the AChE activity in brain and muscle of AChE+/+ and +/− mice, 50% of the BChE activity in all three AChE genotypes, but did not significantly inhibit acylpeptide hydrolase activity. It was concluded that the toxicity of VX must be attributed to inhibition of nonacetylcholinesterase targets in the AChE−/− mouse. Organophosphorus ester toxicity in wild-type mice is probably due to inhibition or binding to several proteins, only one of which is AChE.

The function of acetylcholinesterase is to terminate nerve impulse transmission by hydrolyzing the neurotransmitter acetylcholine. There is overwhelming consensus that acute exposure to organophosphorus (OP) agents inhibits AChE and that toxicity and lethality are due to inhibition of AChE. AChE has such an important role that life without AChE was predicted to be impossible. It was a surprise, therefore, to find that AChE knockout mice live, move, and breathe (Xie et al., 2000).

The 1996 Food Quality Protection Act requires the U.S. Environmental Protection Agency to assess the potential risk of cumulative exposure to related chemicals that share a common mechanism of toxicity (http://www.epa.gov). OP pesticides are considered to have a common mechanism of toxicity, because the initial step in a cascade of reactions is inhibition of AChE (McDonough and Shih, 1997; Mileson et al., 1998; Pope, 1999). However, evidence against a common mechanism of toxicity is mounting. Lush et al. (1998) cloned neuropathy target esterase, a protein that covalently binds mipafox and diisopropylfluorophosphate (DFP). Mipafox and DFP also inhibit AChE, but the degeneration of long axons and paralysis are the consequence of binding to neuropathy target esterase and not to AChE. Richards et al. (2000) found that acylpeptide hydrolase in rat brain is inhibited by 6- to 10-fold lower doses of dichlorvos, chlorpyrifos methyl oxon, and DFP than are required to inhibit AChE. Bomser and Casida (2001) found that chlorpyrifos oxon covalently binds to M2 muscarinic receptors at doses lower than required to inhibit AChE. Carboxylesterase and butyrylcholinesterase covalently bind OP at low doses, but inhibition is thought to have no physiological consequences. The 38 currently approved pesticides vary 1000-fold in the dose that is acutely toxic to rats (Pope, 1999). However, some of this variation can be explained by the need to bioactivate the phosphorothioates to oxons, the scavenging effect of carboxylesterase for some but not all OP, and the variable rates of hydrolysis by

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ABBREVIATIONS: OP, organophosphorus pesticides and nerve agents; AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate; VX, (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate); BChE, butyrylcholinesterase.

paraoxonase (Sweeney and Maxwell, 1999). Toxicologists have noted that each organophosphorus pesticide is associated with a unique set of neurotoxic symptoms (Moser, 1995; Pope, 1999). The picture that is emerging is that a particular OP may be binding to a set of proteins, and the set of proteins differs for each OP.

The AChE knockout mouse provides a new tool for testing the involvement of AChE in OP toxicity. Xie et al. (2000) found that 12-day-old AChE−/− mice were more sensitive to DFP than AChE+/+ and AChE+/− mice, thus demonstrating that AChE was not the only physiologically important target for DFP. In this report we were able to test older animals, because we succeeded in extending the lifetime of AChE−/− mice to young adulthood. The AChE−/− mice were tested with the most potent organophosphorus ester known, the nerve agent VX. The results support the conclusion that OP toxicity is initiated not only by inhibition of AChE but also by interaction with non-AChE targets.

Materials and Methods

Mice. Animal studies have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. The AChE knockout colony is maintained at the University of Nebraska Medical Center (Omaha, NE) by breeding heterozygotes (Xie et al., 2000). The genetic background of the animals is strain 129Sv. Mice of both sexes were treated with VX. The VX-treated mice included 21 wild-type mice ranging in age from 35 to 55 days (average age 48 ± 10.5 days), 18 heterozygous mice ranging in age from 35 to 52 days (average 44.6 ± 7.8 days), and 16 nullizygous mice ranging in age from 34 to 53 days (average 44.4 ± 7.9 days). Weights ranged from 17 to 22 g for AChE+/+ mice, 14 to 24 g for AChE+/− mice, and 11 to 16 g for AChE−/− mice. The untreated control group had eight AChE+/+, six AChE+/−, and six AChE−/− mice in the same age and weight range.

In our first article on the AChE knockout mouse (Xie et al., 2000) the average life span of AChE−/− mice was 14 days. Since then, their life span has been extended to an average of 60 to 80 days by feeding the dams a high-fat diet during the nursing period, and by feeding the pups liquid Ensure Fiber with FOS, Vanilla Flavor (Ross Products Division Abbott Laboratories, Columbus, OH) after weaning. Our oldest AChE−/− mouse is 354 days old as of July 6, 2001.

Transportation of Mice. Three of 19 nullizygous mice did not survive the trip from Omaha to Baltimore, an overnight trip by air with the services of Bax Gobal (1-800-CALL-BAX, Irvine, CA). In contrast, all wild-type and heterozygous mice survived. Housing during the trip consisted of a plastic box divided into four sections with plastic dividers. Air intake was filtered through Hepa filters located on the sides and cover. These boxes are rodent shipping boxes sold by Taconic Farms (Germantown, NY). A mouse house, consisting of a plastic pipette box top with a side hole for a door, was fixed in place with duct tape in each of the four sections. The mouse house helped the AChE−/− mice to stay warm during the trip. Another reason for the mouse house was to minimize stress for the AChE−/− mice, by giving them a place to hide. When the box was opened, all AChE−/− mice were in their houses. Food for the trip for nullizygotes consisted of Ensure Fiber with FOS solidified with gelatin, whereas food for AChE+/+ and AChE+/− animals was standard mouse food pellets. Liquid was available as Napa Nectar, a sweet gelatinous commercial preparation.

VX. The nerve agent VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) (mol. wt. = 267.36) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). VX was determined by gas chromatography to be greater than 98% pure. VX was dissolved in saline and injected subcutaneously in the back of the neck in a volume of 20 μl or less. Mice were observed up to 20 h. VX was chosen for this study because VX is known to inhibit AChE and BChE, but to react poorly with carboxylesterase (Maxwell et al., 1994). Rodents have 100 times more carboxylesterase in their body than cholinesterases (Maxwell et al., 1987a). The nerve agents sarin and soman react with carboxylesterase and must therefore be given in high doses to inactivate carboxylesterase in plasma before toxic signs are seen (Boskovic, 1979; Gupta and Dettbarn, 1987; Maxwell et al., 1987a,b; Grubic et al., 1988). VX was expected to discriminate better among the three AChE genotypes than sarin or soman.

Tissue Extraction. Blood, brain, lungs, liver, intestine, heart, and quadriceps muscle were collected at time of death or 3 to 20 h after treatment with VX. Serum was separated from other blood components by centrifugation. Tissues and sera were stored frozen. Tissues were weighed and then homogenized in 10 volumes of 50 mM potassium phosphate, pH 7.4, containing 0.5% Tween 20, in a Tissumizer (Tekmar, Cincinnati, OH) for 10 s. The suspension was centrifuged in a microfuge for 10 min, and the supernatant was saved for enzyme activity assays. The extraction buffer contained Tween 20 rather than Triton X-100 because mouse BChE activity was inhibited up to 95% by 0.5% Triton X-100, but was not inhibited by 0.5% Tween 20 (Li et al., 2000).

Enzyme Activity Assays in Tissue Extracts. AChE and BChE activity was measured by the method of Ellman (1961) at 25°C, in a Gilford spectrophotometer interfaced to MacLab 200 (ADInstruments Pty Ltd., Castle Hill, Australia) and a Macintosh computer. Samples were preincubated with 5,5-dithio-bis (2-nitrobenzoic acid) in 0.1 M potassium phosphate buffer, pH 7.0, to react free sulfhydryl groups before addition of substrate. AChE activity was measured with 1 mM acetylthiocholine after inhibiting BChE activity with 0.1 mM tetraisopropyl pyrophosphoramide (liver required 1 mM for complete BChE inhibition). BChE activity was measured with 1 mM butyrylthiocholine.

Acylpeptide hydrolase activity was measured in a SpectraMax 190 microtiter plate reader (Molecular Devices, Sunnyvale, CA). *N*-Acetyl-L-alanine *p*-nitroanilide (Sigma Chemical, St. Louis, MO) was dissolved in 0.1 M Bis-Tris, pH 7.4, to make a 4 mM solution. The pH dropped to 7.3 and had to be adjusted back up to pH 7.4 to get the compound completely into solution. The rate of hydrolysis of 4 mM *N*-acetyl-L-alanine *p*-nitroanilide was measured at 405 nm (Scaloni et al., 1994) at 25°C. Each 200 μl of assay solution contained 5 μl of tissue extract. Absorbance was read every 5 min up to 40 min. Micromoles of substrate hydrolyzed per minute were calculated from the slope of the line by using the extinction coefficient of 7530 M^{-1} cm^{-1} at 405 nm.

Units of activity for AChE, BChE, and acylpeptide hydrolase are defined as micromoles of substrate hydrolyzed per minute. Units of activity were calculated per gram wet weight of tissue. Tissue extracts from the 55 treated and 20 untreated mice were assayed in duplicate for AChE and BChE activity, and in triplicate for acylpeptide hydrolase activity for a total of 3450 assays.

Carboxylesterase Activity in Mouse Serum. Carboxylesterase activity was assayed in serum from mice of various ages by measuring hydrolysis of α-naphthyl acetate (Yang and Dettbarn, 1998). The α-naphthyl acetate was dissolved in ethanol to make a 0.02 M stock solution, which was stored frozen. Mouse serum contains four esterases that hydrolyze α-naphthyl acetate. To inhibit AChE and BChE, the mouse serum was preincubated with 10 μM eserine. To inhibit paraoxonase, the mouse serum was preincubated with 12.5 mM EDTA. A 2-ml reaction contained 1.83 ml of 0.1 M potassium phosphate, pH 7.0, 0.05 ml of 0.5 M EDTA, 0.02 ml of 1 mM eserine, and 5 μl of mouse serum. After a preincubation period of 20 min to allow complete inhibition, 0.01 ml of 0.02 M α-naphthyl acetate was added. Change in absorbance at 321 nm was recorded on MacLab interfaced to a Gilford spectrophotometer. Units of activity, defined as micromoles of substrate hydrolyzed per minute, were calculated from the extinction coefficient of 2200 M^{-1} cm^{-1} for α-naphthol at pH 7.0. The

spontaneous rate of hydrolysis was subtracted from the observed rates.

Temperature. Surface body temperature was measured with a digital thermometer, Thermalert model TH-5, and a surface Microprobe MT-D, type T thermocouple (Physitemp Instruments, Clifton, NJ).

Grip Strength. The inverted screen test was used to measure grip strength. A mouse was placed on top of the screen. The screen was rotated 180° so the mouse was upside down, and the time until the mouse fell off or climbed to the top was measured.

Statistical Analysis. The up and down method of Bruce (1987) was used for toxicity assays to minimize the number of animals. LD_{50} values in Table 1 were estimated using a probit regression analysis in SPSS (SPSS, Inc., Chicago, IL). An analysis of covariance was also used to compare the estimated probit regression lines from each group to determine equal slopes and intercepts. Tissue enzyme levels in Table 2 were tested for statistical significance by multivariate analysis of variance in the Excel program of Microsoft Office 98. The Bonferroni correction ($p \leq 0.025$) was applied to adjust for multiple comparisons.

Results

Detoxifying Enzyme Levels as a Function of Age

The levels of AChE, BChE, and carboxylesterase activities were measured in mouse sera as a function of age. These enzymes are scavengers of organophosphorus nerve agents. It was important to determine when these enzymes reached stable activity values so that animals could be grouped by age. Figure 1A shows that AChE activity in serum of AChE+/+ and AChE+/− mice was low (0.1 and 0.04 U/ml) 5 days before birth but increased during the postnatal period, reaching a plateau value by postnatal day 8 to 12. The time to reach the plateau value was similar in AChE+/+ and AChE+/− mice. The plateau value for AChE+/+ mice was 0.6 U/ml, and for AChE+/− mice was 0.3 U/ml. Thus, AChE+/− mice have about 50% of the AChE activity of wild-type mice. Nullizygous mice had no AChE activity at any time. There was no sex difference in the levels of AChE activity (Fig. 1B), not even in mature mice at 1 year of age.

Figure 2A shows that BChE activity in serum was 0.2 U/ml 5 days before birth and that BChE activity increased every day during the nursing period, reaching a plateau value of about 1.5 U/ml by postnatal day 21, the day of weaning. The BChE activity levels were similar in mice of all three genotypes and were unaffected by the absence of AChE. There were no sex differences in BChE activity for animals up to 55 days of age. However, female wild-type mice achieved a 2-fold higher BChE activity in serum by 1 year of age (Fig. 2B) compared with male wild-type mice.

Carboxylesterase activity in serum (Fig. 3) followed a pattern similar to that of BChE in Fig. 2A, in that activity was low but detectable 5 days before birth (0.4 U/ml) and increased to a plateau value of 15 to 20 U/ml by postnatal day 30. Neither AChE genotype nor sex influenced carboxylesterase activity.

It was concluded that AChE, BChE, and carboxylesterase enzyme levels had reached stable values by postnatal day 30, justifying the grouping of animals age 34 to 55 days. AChE genotype had no effect on BChE or carboxylesterase activity levels or on pattern of expression during postnatal development. This had been a matter of concern because AChE is thought to have a role in development (Layer and Willbold, 1995; Greenfield, 1998). Paraoxonase, an enzyme that hydrolyzes organophosphorus esters, reaches a plateau value on postnatal day 20 in mice (Li et al., 1997).

TABLE 1

Response of three AChE genotypes to VX injected subcutaneously

LD_{50} values were 24 μg/kg for AChE+/+, 17 μg/kg for AChE+/−, and 10 to 12 μg/kg for AChE−/− mice.

Dose of VX	AChE+/+		AChE+/−		AChE−/−	
	Dead	Live	Dead	Live	Dead	Live
μ/kg						
25	7	1				
24	1	1			1	
22		4	3			
20		2	2			
18		2	2	1		
16		1	1	4		
14		1	1	4	1	2
12		1			3	2
10					2	3
8						2

LD_{50} Values for VX

The nerve agent VX was injected subcutaneously into 55 mice of various AChE genotypes. Significant differences between dose-response curves were observed. The LD_{50} value for AChE+/+ mice was 24 μg/kg (confidence interval 18–25). The LD_{50} for AChE+/− mice was 17 μg/kg (confidence interval 15–20). The LD_{50} for AChE−/− mice was 10 to 12 μg/kg (Table 1). This result shows that mice with 100% of the normal AChE activity (AChE+/+ mice) were better protected from VX than were mice with 50% of the normal AChE activity (AChE+/− mice) and were much better protected than mice with zero AChE activity (AChE−/− mice).

Toxic Signs

Mice were observed for signs of toxicity to see whether the absence of AChE revealed a novel response to VX in AChE−/− mice.

Temperature and Vasodilation. For AChE+/+ and +/− mice, surface body temperature briefly rose about 1°, at 2 to 3 min after injection of VX (Fig. 4). The increase in body temperature was followed by reddening of the paws, snout, and inner ears, suggesting vasodilation. The reddening of the extremities disappeared after about 2 h. Animals that survived VX were observed to lose body temperature. At 8 min after injection of VX, surface body temperature had started to drop, decreasing to a low of 30°C in survivors 1 to 6 h after VX treatment. After 20 h, body temperature had returned to a normal 36–37°C. A hypothermic state of nearly 24-h duration is a characteristic feature of OP intoxication that has been documented for DFP and chlorpyrifos (Gordon and Grantham, 1999). The temperature response of AChE−/− mice was different. After VX treatment, the temperature dropped to a low of 33°C at about 1 h, and returned to normal within 2 h. The more rapid return of normal body temperature in AChE−/− mice probably reflects a decrease in M2 muscarinic receptor levels. Studies in M2 muscarinic receptor knockout mice have shown that in the absence of M2 receptors the mouse does not respond to drugs expected to decrease body temperature (Gomeza et al., 1999).

Motor Activity and Grip Strength. Another sign of toxicity was immobility. Mice stopped walking and remained fixed in one spot, sitting hunched with eyes open. As time

TABLE 2

AChE, BChE, and acylpeptide hydrolase activity in tissues of VX-treated mice

Units of enzyme activity are micromoles of substrate hydrolyzed per minute per gram wet weight of tissue. The standard deviations were 3 to 30% of the average values shown.

Genotype	Tissue	AChE			BChE			Acylpeptide Hydrolase		
		Control	VX Live	VX Dead	Control	VX Live	VX Dead	Control	VX Live	VX Dead
+/+	Brain	1.71	1.04[a]	0.86[a]	0.13	0.09[a]	0.05[a]	0.0046	0.0047	0.0047
+/−	Brain	0.91	0.50[a]	0.53[a]	0.11	0.09	0.05[a]	0.0052	0.0046	0.0054
−/−	Brain	0	0	0	0.10	0.09	0.05[a]	0.0051	0.0045	0.0045
+/+	Muscle	0.28	0.20	0.15[a]	0.26	0.19	0.14	0.0047	0.0052	0.0047
+/−	Muscle	0.22	0.13[a]	0.11[a]	0.25	0.15[a]	0.11[a]	0.0074	0.0052[a]	0.0054
−/−	Muscle	0	0	0	0.27	0.19	0.11[a]	0.0055	0.0050	0.0053
+/+	Liver	0.06	0.06	0.01[a]	5.36	5.3	3.10[a]	0.0184	0.0172	0.0188
+/−	Liver	0.06	0.06	0.05	3.93	4.25	2.64	0.0224	0.0172[a]	0.0192
−/−	Liver	0	0	0	3.69	4.08	4.15	0.0207	0.0185	0.0203
+/+	Intestine	0.21	0.20	0.20	5.97	3.13	5.08	0.0148	0.0089[a]	0.0106
+/−	Intestine	0.15	0.11	0.14	4.37	3.92	3.77	0.0149	0.0119	0.0122
−/−	Intestine	0	0	0	7.95	7.52	6.28	0.0154	0.0180	0.0132
+/+	Lungs	0.20	0.20	0.13	0.79	0.51[a]	0.41[a]	0.0116	0.0097	0.0112
+/−	Lungs	0.14	0.10[a]	0.10	0.55	0.41	0.38[a]	0.0139	0.0118	0.0113
−/−	Lungs	0	0	0	0.65	0.44[a]	0.44[a]	0.0114	0.0110	0.0117
+/+	Heart	0.14	0.11	0.12	0.81	0.63	0.46[a]	0.0089	0.0073	0.0098
+/−	Heart	0.08	0.09	0.07	0.61	0.50	0.37[a]	0.0104	0.0094	0.0107
−/−	Heart	0	0	0	0.60	0.44[a]	0.41	0.0103	0.0085[a]	0.0087
+/+	Serum	0.54	0.35[a]	0.31[a]	2.79	1.95[a]	1.64	N.D.	N.D.	N.D.
+/−	Serum	0.28	0.17[a]	0.21	1.75	1.61	1.14[a]	N.D.	N.D.	N.D.
−/−	Serum	0	0	0	1.88	1.63	1.48	N.D.	N.D.	N.D.

N.D., not determined.

[a] Significantly different from control $p \leq 0.025$ by analysis of variance with Bonferroni's correction to adjust for multiple comparisons.

progressed mice assumed a flattened posture with head extended and hind legs protruding; this posture reflected weakening of muscles. Most +/+ and +/− mice did not lose the ability to grip a screen, although some did lose the ability to climb to the top of an inverted screen. Twenty hours later, survivors appeared healthy: they were active and eating, posture had returned to normal, and they were able to pass the inverted screen test. Nullizygotes never passed the inverted screen test because they are devoid of grip strength even before VX treatment.

Pulsating Paws. VX treatment caused the paws of +/+ and +/− mice to pulsate in a manner similar to the pulsating seen in the paws of untreated −/− mice. This characteristic motion was seen when mice were held by the scruff of the neck. All four paws moved in unison in a steady pulsating outward and inward motion.

Tremor. Whole body tremors were a sign of toxicity, but body tremors did not inevitably lead to death. All animals that died, regardless of genotype, had clonic convulsions; that is, whole body tremors, followed by a tonic convulsion in which all limbs were extended. Tonic convulsions were invariably followed by death. Nullizygotes have a persistent whole body tremor, without ever having been treated with OP, and this tremor is compatible with life.

Eyes. Pinpoint pupils were seen in wild-type mice 10 to 30 min after VX injection. Untreated adult nullizygotes always have pinpoint pupils. VX treatment did not change the pinpoint pupils in −/− mice.

Hair. Piloerection was not seen after VX treatment.

Salivation, Lacrimation, Urination, and Defecation. OP intoxication in humans leads to characteristic signs of toxicity summarized by the mnemonic, sludge: salivation, lacrimation, urination, defecation, gastroenteritis, and emesis. For rodents the mnemonic is salivation, lacrimation, urination, and defecation because rodents do not vomit and their gastric symptoms are difficult to detect. VX treatment of mice caused salivation, lacrimation, and urination but only at lethal doses; salivation, lacrimation, and urination were followed within minutes by tonic convulsions and death. All mice that survived had excessive salivation within 1 h of VX treatment. A white mucus was observed in the eyes of survivors 2 to 18 h after VX treatment. At 20 h eyes were no longer covered with mucus. Excessive urination and defecation were not observed in survivors. Excessive defecation was not observed at any dose of VX.

Toxic Signs in Knockout Mouse

The untreated knockout mouse has several behaviors that make it look like a VX-treated mouse, even when the knockout mouse has not been exposed to VX or to any OP. Pulsating paws, pinpoint pupils, body tremors, a film of white mucus on the eyes when it is handled, and lack of grip strength are characteristic of the untreated nullizygote. The toxic signs in the −/− mice after treatment with VX were similar to those in the +/+ and +/− mice and included loss of motor activity, flattened posture, peripheral vasodilation, and hypothermia. Whole body tremors, although always present in AChE−/− mice, became more pronounced after VX treatment. Lethal doses of VX caused salivation, mucus in the eyes, heaving, agonized breathing, urination, and tonic convulsions in the last minutes of life.

The VX-treated AChE−/− mice showed no novel signs of toxicity that were not also manifest in the AChE+/+ and +/− mice. This is important because the AChE−/− mouse does not have AChE, which means inhibition of an alternative target produced the same symptoms attributed to inhibition of AChE. A notable difference in response to VX was the more rapid return of normal body temperature in the AChE−/− mouse (Fig. 3).

Time to Death

Death occurred within 7 to 22 min after injection of VX. Time to death was independent of AChE genotype and dose of VX (range 10–25 μg/kg) and probably reflects the time it

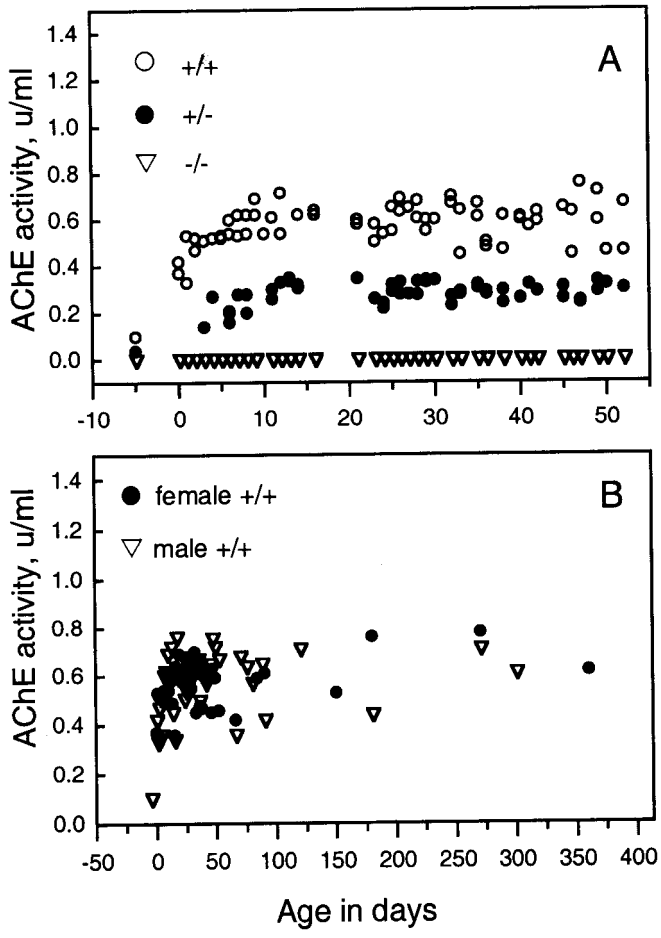

Fig. 1. AChE activity in untreated mice as a function of mouse age and AChE genotype. A, mouse blood was collected from AChE+/+, +/−, and −/− mice starting on embryonic day 16 (−5 on graph). Serum was separated from other blood components by centrifugation and tested for AChE activity. There were 190 animals in the AChE+/+ group, 198 in the AChE+/− group, and 61 in the AChE−/− group. AChE activity was independent of sex; therefore, male and female data were pooled. B, serum from 280 AChE+/+ mice, ranging in age from −5 to postnatal day 370, was assayed for AChE activity. No sex differences in AChE activity were found.

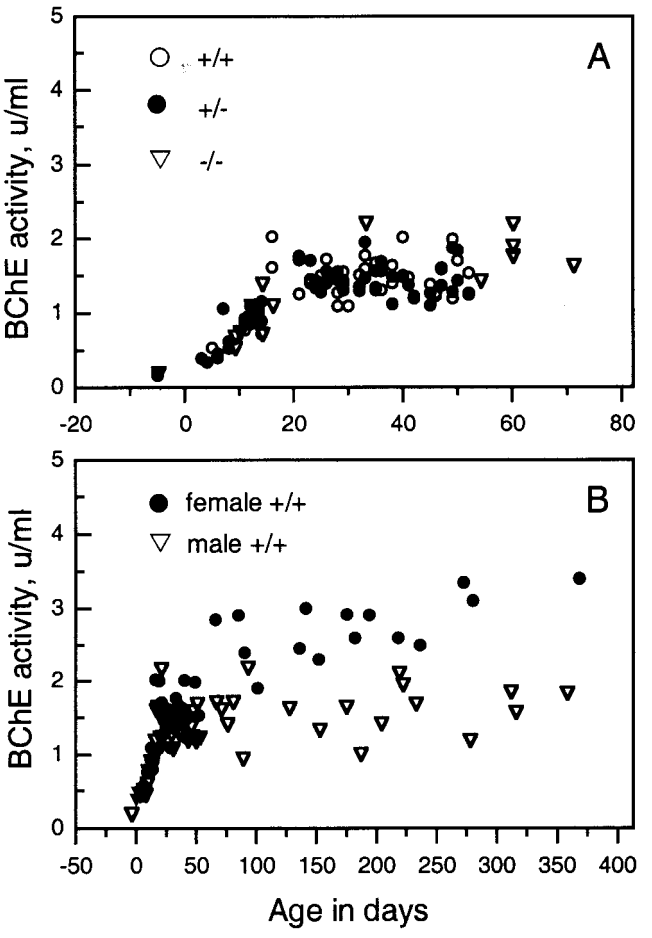

Fig. 2. BChE activity in untreated mice as a function of mouse age and AChE genotype. A, BChE activity in serum of AChE+/+, +/−, and −/− mice, from embryonic day 16 (−5 on graph) to postnatal day 53 was independent of AChE genotype. No sex differences in BChE activity were present in this young age group. B, serum from wild-type mice was assayed for BChE activity. BChE activity remained constant from postnatal day 21 to 370 in male mice, but doubled during this time in female mice.

takes VX to travel from the subcutaneous site of injection on the back of the neck to the brain, muscles, and other organs. Nullizygotes did not die in a shorter time. Mice were considered to have survived VX if they were alive 3 to 20 h after receiving VX.

Enzyme Inhibition

Tissues were extracted from VX-treated and untreated animals and tested for AChE, BChE, and acylpeptide hydrolase activity. Carboxylesterase activity was not tested because VX does not inhibit carboxylesterase (Maxwell et al., 1994). The results are shown in Table 2.

AChE. In untreated mice, levels of AChE activity were highest in brain, followed by serum, muscle, intestine, lungs, heart, and liver. These results confirm the tissue distribution results reported by Li et al. (2000). AChE activity in brain was inhibited about 50% in VX-treated mice in the genotypes AChE+/+ and +/−. Fifty percent inhibition of AChE activity in brain was associated with death or serious signs of toxicity. Untreated AChE+/− brain had only 50% of the AChE activity present in wild-type brain, but this 50% level caused

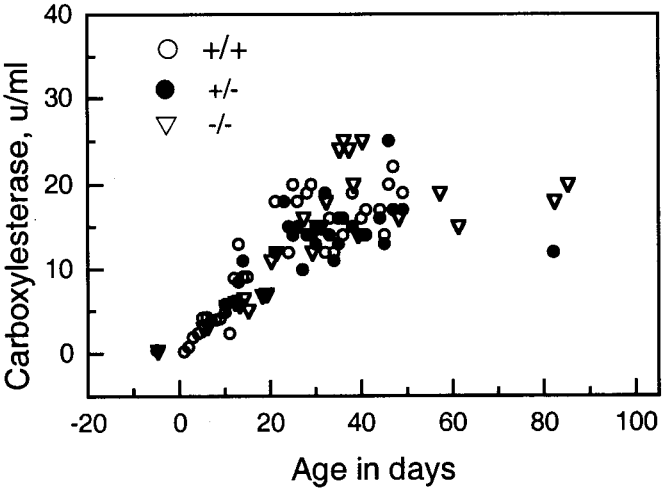

Fig. 3. Carboxylesterase activity in untreated mice as a function of mouse age and AChE genotype.

no signs of toxicity in the absence of VX. The AChE−/− mice have no AChE activity, and therefore no AChE inhibition by VX.

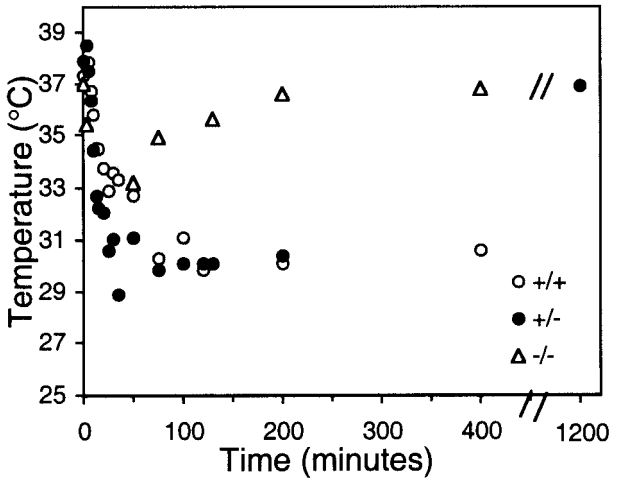

Fig. 4. Surface body temperature after treatment with VX. Body temperature in nullizygous mice (AChE−/−) did not drop as far and recovered faster than temperature in AChE+/+ and +/− mice.

AChE activity in muscle and serum was also inhibited by VX. However, AChE in liver, intestine, lungs, and heart was inconsistently inhibited. AChE activity in animals that died from VX treatment tended to be lower than AChE activity in animals that survived VX treatment, but this was not a consistent pattern.

BChE. Levels of BChE activity were highest in intestine and liver, followed by serum, heart, lung, muscle, and brain. BChE activity was higher than AChE activity in all tissues except brain and muscle. As previously reported by Li et al. (2000), BChE activity was independent of AChE genotype. BChE activity in brain and muscle was inhibited about 50% in animals that died from VX treatment. BChE in serum was inhibited 30 to 40% in mice that died from VX. BChE inhibition was more consistent than AChE inhibition, because all tissues with the exception of liver showed BChE inhibition. Animals that died from VX showed slightly more BChE inhibition than animals that survived VX. Studies in VX-treated humans (Sidell and Groff, 1974) and VX-treated rats (Gupta et al., 1991) have shown preferential inhibition of AChE.

Acylpeptide Hydrolase. Acylpeptide hydrolase was of interest because Richards et al. (2000) found that acylpeptide hydrolase was inhibited by doses of dichlorvos, chlorpyrifos methyl oxon, and diisopropylfluorophosphate that did not inhibit AChE. It was unknown whether acylpeptide hydrolase would be preferentially inhibited by VX. Acylpeptide hydrolase levels were highest in liver, intestine, lungs, and heart, followed by muscle and brain. Acylpeptide hydrolase activity was independent of AChE genotype. VX treatment inhibited acylpeptide hydrolase activity 0 to 40%, but inconsistently. For example, the liver of AChE+/− mice showed 23% inhibition in VX survivors but only 15% inhibition in VX lethalities, whereas liver from AChE+/+ mice was inhibited 7% in survivors and not at all in VX lethalities. Inhibition of acylpeptide hydrolase was less pronounced than inhibition of AChE and BChE.

Could inhibition of acylpeptide hydrolase account for the sensitivity of AChE−/− mice to VX? The results in Table 2 indicate that acylpeptide hydrolase was slightly inhibited in mice of all three AChE genotypes, with no preference for inhibition in the AChE−/− mouse. It is concluded that acylpeptide hydrolase is not a major target of VX and that inhibition of acylpeptide hydrolase does not explain the supersensitivity of AChE−/− mice to VX.

Atropine

Atropine protects from nerve agent toxicity by blocking overstimulation of muscarinic acetylcholine receptors. To determine the mechanism of action of VX in AChE−/− mice, we pretreated mice with atropine. If VX were acting through muscarinic receptors, either by directly binding to the receptor (Silveira et al., 1990; Rocha et al., 1999) or by causing an increase in acetylcholine levels then pretreatment with atropine should protect the AChE−/− mouse from VX. Two wild-type and three AChE−/− mice were pretreated with 12 mg/kg atropine. The wild-type mice challenged with 30 μg/kg VX survived, but the AChE−/− mice challenged with 20, 18, and 16 μg/kg VX died. Thus, atropine did not protect AChE−/− mice from VX lethality.

The time interval between pretreatment with atropine and injection of VX was 40 min. To determine whether death of the AChE−/− mice was the result of atropine or VX intoxication five AChE−/− mice were treated with 12 mg/kg atropine alone. None of the mice died, although they became hyperactive for 3 h. Wild-type mice showed no effects from 12 mg/kg atropine alone.

Discussion

Nonacetylcholinesterase Targets of VX. The present results show that mice with no AChE in any tissue are more sensitive to VX than mice that have AChE (Table 3). Heterozygous mice are intermediate in sensitivity to VX and have about 50% of the normal AChE activity in all tissues. Previous results (Xie et al., 2000) have shown that AChE−/− mice are supersensitive to the organophosphate DFP. Because AChE−/− mice have no AChE, the toxicity of VX and DFP must be attributed to inhibition of nonacetylcholinesterase targets.

AChE−/− mice are not normal. Their body weight is low. They live an average of 60 to 80 days. Their muscles have poor grip strength, and their tremor suggests the presence of excess acetylcholine. It could be argued that their fragile status sensitizes AChE−/− mice to toxicants in general and that their increased sensitivity to VX and DFP is not special.

In this regard, the intermediate sensitivity of AChE+/− mice to VX is important, because AChE+/− mice are phenotypically normal. They have normal body weight, life span, reproductive abilities, and grip strength. They do not appear to have excess acetylcholine, because they have no body tremor. Yet they are more sensitive to VX than are wild-type mice. The finding that healthy AChE+/− mice have intermediate sensitivity to VX indicates that the level of AChE is the important factor in determining the degree to which a mouse

TABLE 3
Supersensitivity of AChE knockout mice to VX and DFP
DFP results are from Xie et al. (2000).

AChE Genotype	AChE Activity	VX LD_{50}	DFP
		μg/kg	*2.5 mg/kg i.p.*
AChE+/+	100%	24	All lived (n = 8)
AChE+/−	50%	17	7 died, 8 lived
AChE−/−	0%	10–12	All died (n = 3)

TABLE 4
Amount of AChE and BChE in a wild-type mouse
The male, strain 129Sv, 45-day-old mouse weighed 21.75 grams.
Units of activity were converted to nanomoles by using a kcat value for mouse AChE of 195,000 min^{-1} and for mouse BChE of 61,000 min^{-1} (Maxwell et al., 1987a; Vellom et al., 1993). Tissue activities are from Li et al. (2000).

Tissue	Weight	AChE Activity	Total AChE	BChE Activity	Total BChE
	g	*units/g*	*units in tissue*	*units/g*	*units in tissue*
Brain	0.37	4.63	1.71	0.15	0.06
Lung	0.15	0.19	0.03	0.77	0.12
Muscle	8.0	0.40	3.20	0.36	2.88
Intestine	1.15	0.18	0.21	5.3	6.10
Heart	0.19	0.12	0.02	1.33	0.25
Liver	1.05	0.09	0.09	4.3	4.51
Serum	1.74	0.59	1.03	1.9	3.31
Total units			6.29		17.23
Total nmol			0.03		0.28

will be sensitive to VX. The more AChE a mouse carries, the less sensitive it is. Thus, the supersensitivity of the AChE−/− mouse is not simply a consequence of its overall fragile status, but is due to the absence of AChE.

The observation that the more AChE activity a mouse carries the less sensitive it is toward VX suggests that AChE protects other targets of VX against inhibition. In this sense, AChE appears to act as a scavenger of VX.

Is BChE a Physiologically Important Target of VX? BChE has long been considered a nonfunctional, vestigial cousin to AChE. This is largely based on two findings. First, people lacking BChE show no adverse symptoms. Second, measurements of BChE in tissues of normal animals indicated that the BChE level was low relative to AChE level. Recently, improved assay procedures have demonstrated that the BChE level in all tissues of the mouse (except for brain and muscle) is actually higher than the AChE level (Li et al., 2000). A wild-type mouse has 10 times more BChE than AChE in its body (Table 4). This has encouraged us to suggest that BChE may have a physiological role in mice. One possibility is that BChE may function as a backup for AChE in neurotransmission. BChE is located in the synapse of the neuromuscular junction, although at lower levels than AChE (Silver, 1963; Chapron et al., 1997). In the brain, BChE is found in glia cells and axons of white matter, whereas AChE is found in cholinergic synapses (Friede, 1967; Graybiel and Ragsdale, 1982). These locations make it possible for BChE to participate in acetylcholine hydrolysis.

If BChE serves as a backup for AChE in the synapse of the AChE−/− mouse then VX toxicity could arise from inhibition of BChE. This would make BChE the critical alternate target for VX. In wild-type and heterozygous mice, VX was equally effective at inhibiting AChE and BChE. Fifty percent of each enzyme was inhibited under the conditions of our experiments.

A test for a possible role for BChE in neurotransmission was the experiment in which mice were treated with atropine and VX. Atropine binds to the muscarinic receptors and reduces the sensitivity of the postsynaptic membrane to stimulation by excess acetylcholine. It was expected that atropine would protect AChE−/− mice from VX toxicity if the toxicity were initiated by excess acetylcholine. The excess acetylcholine would have come from inhibition of BChE. This is similar to the way in which atropine protects synaptic transmission against AChE inhibition in the wild-type mouse.

We found that atropine did not protect AChE−/− mice against VX toxicity, suggesting that excess acetylcholine might not have accumulated in AChE−/− synapses. This suggests, but does not prove, that BChE does not function as a backup for AChE. We did notice that body tremor in the AChE−/− mice intensified after VX treatment, which is consistent with the accumulation of excess acetylcholine in VX-treated AChE−/− mice. However, the tremor could have been mediated by some other mechanism. An alternative interpretation for the lack of protection by atropine is that muscarinic receptors in the AChE−/− mouse may have become desensitized to excess acetylcholine. Thus, our results do not provide a definitive answer to the question of whether BChE is a physiologically important target of VX. If BChE is not the critical alternate target for VX then the VX toxicity in the AChE−/− mouse is mediated by some as yet unidentified target, a target that is not sensitive to atropine intervention.

Additional Targets. Our findings strengthen the observations of others that OP have sites of action in addition to AChE (Moser, 1995; Pope, 1999). Acetylcholinesterase is definitely a target of VX and other OP, but it is not the only physiologically important target. The list of nonacetylcholinesterase targets for OP includes muscarinic receptors (Silveira et al., 1990; Jett et al., 1991; Ward and Mundy, 1996; Rocha et al., 1999; Bomser and Casida, 2001), adenylyl cyclase (Huff et al., 1994; Song et al., 1997; Auman et al., 2000), acylpeptide hydrolase (Richards et al., 2000), and neuropathy target esterase (Lush et al., 1998).

Another argument in support of noncholinesterase targets is the observation that at low doses, each OP has different behavioral effects. For example, fenthion decreased motor activity but did not alter the tail-pinch response, whereas parathion did not lower activity but did decrease the tail-pinch response (Moser, 1995).

Hypothesis. It is our hypothesis that unknown OP targets exist. Inhibition of these unknown targets allows entry into the multireceptor pathway described by McDonough and Shih (1997), wherein overstimulation of glutamate receptors leads to convulsions, respiratory arrest, and cardiac collapse. Anaphylactic shock caused by massive histamine release may be another mechanism of OP toxicity (Cowan et al., 1996). Identification of these putative unknown targets may lead to new strategies for treating OP toxicity and may explain chronic illnesses attributed to low-dose exposure.

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