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Comparison of Polyethylene Glycol-Conjugated Recombinant Human Acetylcholinesterase and Serum Human Butyrylcholinesterase as Bioscavengers of Organophosphate Compounds

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ABSTRACT

Comparative protection studies in mice demonstrate that on a molar basis, recombinant human acetylcholinesterase (rHuAChE) confers higher levels of protection than native human butyrylcholinesterase (HuBChE) against organophosphate (OP) compound intoxication. For example, mice challenged with 2.5 LD_{50} of *O*-isopropyl methylphosphonofluoridate (sarin), pinacolylmethyl phosphonofluoridate (soman), and *O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate (VX) after treatment with equimolar amounts of the two cholinesterases displayed 80, 100, and 100% survival, respectively, when pretreatment was carried out with rHuAChE and 0, 20, and 60% survival, respectively, when pretreatment was carried out with HuBChE. Kinetic studies and active site titration analyses of the tested OP compounds with acetylcholinesterases (AChEs) and butyrylcholinesterases (BChEs) from different mammalian species demonstrate that the superior in vivo efficacy of acetylcholinesterases is in accordance with the higher stereoselectivity of AChE versus BChE toward the toxic enantiomers comprising the racemic mixtures of the various OP agents. In addition, we show that polyethylene glycol-conjugated (PEGylated) rHuAChE, which is characterized by a significantly extended circulatory residence both in mice and monkeys (*Biochem J* **357:**795–802, 2001; *Biochem J* **378:**117–128, 2004), retains full reactivity toward OP compounds both in vitro and in vivo and provides a higher level of protection to mice against OP poisoning, compared with native serum-derived HuBChE. Indeed, PEGylated rHuAChE also confers superior prophylactic protection when administered intravenously or intramuscularly over 20 h before exposure of mice to a lethal dose of VX (1.3–1.5 LD_{50}). These findings together with the observations that the PEGylated rHuAChE exhibits unaltered biodistribution and high bioavailability present a case for using PEGylated rHuAChE as a very efficacious bioscavenger of OP agents.

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The primary role of acetylcholinesterase (acetylcholine acetylhydrolase, 3.1.1.7; AChE) is the termination of impulse transmission in cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. A second acetylcholine-hydrolyzing enzyme, butyrylcholinesterase (alcholine acyl hydrolase 3.1.1.8; BChE), often occurs together with AChE in various tissues. BChE differs from AChE in its substrate specificity and in susceptibility to inhibitors. Organophosphate (OP) compounds, such as the nerve agents *O*-isopropyl methylphosphonofluoridate (sarin) and pinacolylmethyl phosphonofluoridate (soman), inhibit AChE and BChE irreversibly by rapid phosphonylation of the serine residue in the enzyme active site. The acute toxicity of these nerve agents is elicited in motor and respiratory failure after inhibition of AChE in the peripheral and central nervous system. Although the current drug treatment regimens against nerve

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ABBREVIATIONS: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; OP, organophosphate; CW, chemical warfare; VX, *O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate; FBS, fetal bovine serum; EqBChE, equine serum butyrylcholinesterase; HuAChE, human acetylcholinesterase; HuBChE, human butyrylcholinesterase; ChE, cholinesterase; PEG, polyethylene glycol; PEGylated, polyethylene glycol-conjugated; rHuAChE, recombinant human acetylcholinesterase; HEK, human embryonic kidney; PBS, phosphate-buffered saline; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; CBDP, cresylbenzodioxaphosphorin oxide; CaE, carboxylesterase; MRT, mean residence time.

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agent exposure increased survival in intoxicated animals, they failed to prevent severe incapacitation, unless administered immediately after exposure.

The high reactivity of both acetylcholinesterase and butyrylcholinesterase toward CW nerve agents [e.g., *O*-isopropyl methylphosphonofluoridate (sarin), pinacolylmethyl phosphonofluoridate (soman), ethyl *N*,*N*-dimethylphosphoramidocyanidate (tabun), and *O*-ethyl-*S*-(2-isopropylaminoethyl) methylphosphonothiolate (VX)] or toward organophosphate compounds, led to the proposal that these enzymes could be potential bioscavengers for prophylactic treatment of OP poisoning. Previous studies suggest that exogenous cholinesterase can serve as an effective therapeutic agent for sequestration of highly toxic OPs before they reach their physiological target (Raveh et al., 1993, 1997; Lenz et al., 2005, and references therein). Administration of native AChE [e.g., fetal bovine serum (FBS)-AChE] or BChE [e.g., equine serum (Eq)BChE and human serum (Hu)BChE] protected the animals from a variety of highly toxic OPs without any toxic effects or performance decrements in both rodent and nonhuman primate models (Raveh et al., 1993, 1997; Lenz et al., 2005). Based on data from animal experiments, it was estimated that for human BChE, 200 mg would be required to provide protection for an adult human against 2 LD_{50} of soman (Ashani et al., 1998).

The requirement for ChEs in large quantities encouraged the development of large-scale production systems for the generation of recombinant ChEs (Kronman et al., 1992; Fischer et al., 1993; Saxena et al., 1998); however, pharmacokinetic studies have shown that recombinant enzymes generated in either bacterial or mammalian cells are retained in the circulation of experimental animals for much shorter periods than native FBS-AChE or BChE (Kronman et al., 1995; Saxena et al., 1998). Extensive studies have shown that this problem can be effectively resolved by conversion of recombinant ChE into a circulatory long-lived enzyme by either optimization of post-translation modification (e.g., enzyme glycosylation, sialylation, and subunit oligomerization) (Kronman et al., 1995, 2000; Chitlaru et al., 1998, 2001, 2002; Duysen et al., 2002; Cohen et al., 2004) or by chemical modification of the enzyme through controlled appendage of polyethylene glycol (PEG) side chains (Cohen et al., 2001, 2004). Optimally PEGylated rHuAChE displayed very long circulatory residence in both mice and rhesus macaques, exceeding that of native serum-derived cholinesterases (Cohen et al., 2004). However, the use of such a circulatory long-lived PEGylated version of recombinant human AChE as an in vivo bioscavenger of OP compounds requires that additional conditions be met. First, the chemical modification of the enzyme should not alter its reactivity toward various OP compounds. And second, the PEGylated enzyme should display high bioavailability but should not accumulate in vital organs where it may exert some negative biological effects.

In the present study, we examined the ability of PEGylated rHuAChE to protect mice against OP compound intoxication and monitored both circulatory buildup and subsequent biodistribution of the chemically modified recombinant enzyme. These experiments allowed us to conclude that PEGylated recombinant human AChE is highly qualified for serving as a bioscavenger of OP agents in vivo. Moreover, by comparing the ability of serum BChE, rHuAChE, and PEGylated rHuAChE to protect mice against OP compound exposure, we could establish that on a molar basis, rHuAChE as well as PEGylated rHuAChE are superior to native BChE in their ability to protect mice, because of their higher stereoselectivity toward various OP agents. The high stereoselectivity of PEGylated rHuAChE together with its extended circulatory stability suggests that this enzyme form can serve as a superior prophylactic agent against OP intoxication.

Materials and Methods

Enzymes. Procedures of transfection of the human embryonic kidney (HEK)-derived cell line HEK-293 with the expression vector of the C-terminal truncated HuAChE enzymes (Cohen et al., 2001) and the generation of stable cell clones expressing high levels of recombinant product were described previously (Kronman et al., 1992). The method for purification of the secreted rHuAChE was described previously (Kronman et al., 1995). Human serum BChE was purified from outdated screened human plasma, obtained from local blood banks in Israel, as described previously (Kronman et al., 1995). FBS-AChE was purified similarly from fetal calf serum (Biological Industries, Bet-Haemek, Israel).

Attachment of PEG chains to primary amines in rHuAChE was performed using succinimidyl propionate-activated methoxy-PEG (Nektar Inc., San Carlos, CA) as described previously (Cohen et al., 2001). In brief, purified rHuAChE (5 μM) was incubated with PEG-20,000 at a ratio of 30:1 (mol/mol) $[PEG]_0/[AChE\ primary\ amines]_0$ in 50 mM borate buffer, pH 8.5, for 2 h at room temperature. The chemically modified products were dialyzed extensively against PBS and analyzed on 6% SDS-polyacrylamide gel electrophoresis gels. The estimated molecular mass of PEGylated AChE is approximately 145 kDa (average of four PEG chains per AChE molecule).

Organophosphate Inhibitors. Soman, sarin, VX, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ), and cresylbenzodioxaphosphorin oxide (CBDP) were prepared according to previously reported procedures (Eto et al., 1962; Levy and Ashani, 1986; Ordentlich et al., 2004). Preparation of VX enantiomers was carried out as reported recently (Ordentlich et al., 2004). The P_S-enantiomer of sarin was obtained in analogy to separation of soman by incubation of the racemic mixture with rabbit plasma, and the corresponding P_R-enantiomer was obtained by titration with HuAChE as suggested in Benschop and de Jong (1988). The purity of OPs (>95%) was determined by ^1H and ^{31}P NMR spectroscopy and by a titration of a known amount of HuAChE. Stock solutions were kept at −20°C and diluted in 0.9% saline to the desired concentration. The toxicity of the diluted solutions was determined before each experiment by performing a dose-response study in mice and calculating the LD_{50} of these OPs, according to the Spearman and Karber method (Finney, 1964). The absolute configurations for the four diastereoisomers of soman, P_SC_S, P_SC_R, P_RC_S, and P_RC_R, were shown previously (Ordentlich et al., 1999) to correspond to the older notations C(+)P(−), C(−)P(−), C(−)P(+), and C(+)P(+), respectively.

Kinetic Studies. HuAChE activity was assayed according to the method of Ellman et al. (1961) in the presence of 0.1 mg/ml bovine serum albumin, 0.3 mM 5,5′-dithio-bis-(2-nitrobenzoic acid), 50 mM sodium phosphate buffer, pH 8.0, and various concentrations of acetylthiocholine or butyrylthiocholine at 27°C and monitored with a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA). Enzyme concentration was determined by enzyme-linked immunosorbent assay (Shafferman et al., 1992) and by active site titration using MEPQ (Shafferman et al., 1996).

Measurements of phosphonylation rates were carried out with at least four different concentrations of OP inhibitor, and enzyme residual activity at various times was monitored. The apparent bimolecular phosphonylation rate constants (k_i) determined under pseudo first-order conditions were computed from the plot of slopes of ln(E)

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versus time at different inhibitor concentrations (Ordentlich et al., 1996, 1999). Rate constants under second-order conditions were determined from plots of $\ln\{E/[OP_0 - (E_0 - E)]\}$ versus time. Interactions of HuAChE or PEG-HuAChE with the organophosphate compounds were analyzed as described previously (Ordentlich et al., 1996, 1999). Stereoselectivity of the enzymes toward various phosphonates was determined by active site titrations, comparing residual activities of enzymes inhibited by the appropriate racemic phosphonate to that of MEPQ (Shafferman et al., 1996).

Animal Procedures. Male outbred ICR mice (Charles River Laboratories, Inc., Wilmington, MA) were maintained at 20–22°C and a relative humidity of 50 ± 10% on a 12-h light/dark cycle, fed with commercial rodent chow (Koffolk Inc., Tel Aviv, Israel), and provided with tap water ad libitum. Treatment of animals was in accordance with regulations outlined in the U.S. Department of Agriculture Animal Welfare Act and the conditions specified in *Guide for Care and Use of Laboratory Animals* (National Institutes of Health, 1996). The local ethical committee on animal experiments approved animal studies.

Challenge Experiments. Molar concentrations of ChEs were determined by active site titration with MEPQ (Levy and Ashani, 1986; Shafferman et al., 1996). ChEs were administered intravenously or intramuscularly to mice (5–10/group) and 1 min or 20–22 h later, the mice were exposed intravenously to OPs. For exposure to soman or sarin, mice were pretreated intramuscularly with CBDP (2 mg/ml from freshly prepared 1 mg/ml solution in dimethyl sulfoxide) 1 h before exposure to inhibit endogenous carboxylesterases (CaEs). The pharmacokinetic profile of HuBChE in mice pretreated with CBDP was virtually identical to that observed in mice that were not pretreated. Toxic signs and mortality were monitored for 24 h after OP administration. In some experiments, body weight was monitored daily for 7 days postexposure.

Pharmacokinetics of HuAChE in the Circulation. Clearance experiments in mice (three to six mice per enzyme sample) were carried out essentially as described previously (Kronman et al., 2000; Chitlaru et al., 2001). Mice were injected either i.v. or i.m. with 200 units/mouse PEGylated rHuAChE, FBS-AChE, or serum HuBChE in 0.2 ml of PBS. Blood samples (5 μl) were drawn from the tail vein, diluted 20-fold in PBS, and centrifuged for 3 min at 3000 rpm for the removal of red blood cells. Residual ChE activity in blood samples was measured at various time intervals, and all values were corrected for background hydrolytic activity in the blood. Pharmacokinetic parameters were calculated using the WinNonlin software (Pharsight, Mountain View, CA).

Tissue Distribution. The C-terminal truncated HuAChE enzyme (ΔC-rHuAChE; Cohen et al., 2001) or PEGylated-rHuAChE (900 U/mouse and 500 U/mouse, respectively) was administered i.v. to mice (15 mice/enzyme sample). At each time point, three mice were sacrificed, and tissues were removed and homogenized in 9 volumes of ice-cold PBS/0.5% Tween 20. AChE activity was measured in the presence of 40 μM iso-tetraisopropylpyrophosphoramide, to inhibit endogenous BChE activity. The weight of the organs removed from mice was liver, 540 ± 70 mg; kidney, 290 ± 30 mg; spleen, 120 ± 20 mg; brain, 430 ± 30 mg; heart, 170 ± 20 mg; and lung, 220 ± 20 mg.

Results

PEGylated rHuAChE May Serve as an Efficient OP Bioscavenger

Reactivity of PEGylated rHuAChE toward Various OP Compounds. The practical use of ChEs as prophylactic agents against OP nerve agents requires that they can be administered hours or days before exposure; therefore, the relatively rapid elimination of recombinant ChEs from the circulation compromises the ability to use these enzyme forms as therapeutic bioscavengers (Kronman et al., 1995, 2000; Chitlaru et al., 1998; Saxena et al., 1998). However, this limitation in circulatory residence can be remedied by various post-translation modifications (Kronman et al., 2000; Chitlaru et al., 2001, 2002; Duysen et al., 2002) or by conjugation of PEG chains to lysine residues of recombinant AChE, resulting in its conversion into a circulatory long-lived molecule (Cohen et al., 2001, 2004). To examine the bioscavenging potential of PEG-modified AChE, recombinant HuAChE devoid of the C-terminal tail was subjected to controlled PEG conjugation under conditions that allow loading an average of four to five PEG moieties per enzyme molecule, without affecting the catalytic activity of the enzyme (Cohen et al., 2001). Comparison of the inhibition constants (k_i, rate constant for phosphonylation; Table 1) of rHuAChE, rHuBChE, and PEGylated rHuAChE toward various OP compounds demonstrated that the reactivity of the PEGylated rHuAChE toward sarin, soman, VX, or tabun is very similar to that of rHuAChE, testifying to the fact that appendage of the PEG chains did not alter the ability of the enzyme to interact in an efficient manner with different OP agents.

Tissue Distribution of Exogenously Administered PEGylated-AChE. In many animal species, BChE serves as the major circulating ChE, whereas AChE is present at considerably lower levels or not at all (Li et al., 2000), raising the question whether administration of large amounts of recombinant AChE or its derivatives may result in altered disposition of the enzyme in various tissues. Furthermore, the presence of polyethylene glycol tails on ChEs may enhance their accumulation in vital organs and thus may exert some negative biological effects. To address these issues, we conducted a comparative biodistribution analysis of the nonmodified and PEGylated forms of C-terminal truncated rHuAChE. Both enzyme forms were administered intravenously to mice, and various organs (brain, liver, kidney, heart, and spleen) as well as serum samples were removed at different times and examined for exogenous AChE enzymatic activity. Taking into account the marked difference in circulatory residence times of the two enzyme forms (Fig. 1A), rHuAChE and PEGylated rHuAChEs were monitored until 90% of the corresponding enzyme forms were removed from circulation (4 and 48 h for rHuAChE and PEGylated rHuAChE, respectively). For rHuAChE (Fig. 1B), exogenous enzyme levels in the liver were approximately 10% of that in the plasma only at 5 and 20 min after enzyme administration, and these values probably reflect rapid hepatic clearance of partially sialylated rHuAChE within minutes of administration by the hepatic asialoglycoprotein receptor (Kronman et al., 1995; Chitlaru et al., 1998). Kidney levels of

TABLE 1
Rate constants of phosphonylation of rHuAChE, PEGylated rHuAChE, and serum HuBChE by various OP agents
Values represent the mean of triplicate determinations with standard deviations.

	k_i		
	Nonmodified HuAChE	PEGylated HuAChE	Serum HuBChE
	$\times 10^{-4}\ M^{-1}\ min^{-1}$		
Sarin	2280 ± 90	3000 ± 120	1200 ± 90
Soman	8600 ± 1900	7900 ± 700	4000 ± 250
VX	11,300 ± 760	8700 ± 580	2300 ± 130
Tabun	1500 ± 60	1280 ± 130	1600 ± 80

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exogenous nonmodified AChE were approximately 2% of plasma levels at all times, most likely representing residual blood in this organ. AChE activity could not be detected at significant levels above endogenous background in the brain, heart, or spleen of the mice at any of the time points examined (always less than 1% of plasma levels). For PEGylated rHuAChE (Fig. 1C), exogenous enzyme levels in all organs were very low at all times and did not exceed 2% of plasma levels. Thus, exogenously administered recombinant human AChE enzyme did not accumulate in any of the organs examined, whether or not the enzyme was chemically modified by PEG conjugation.

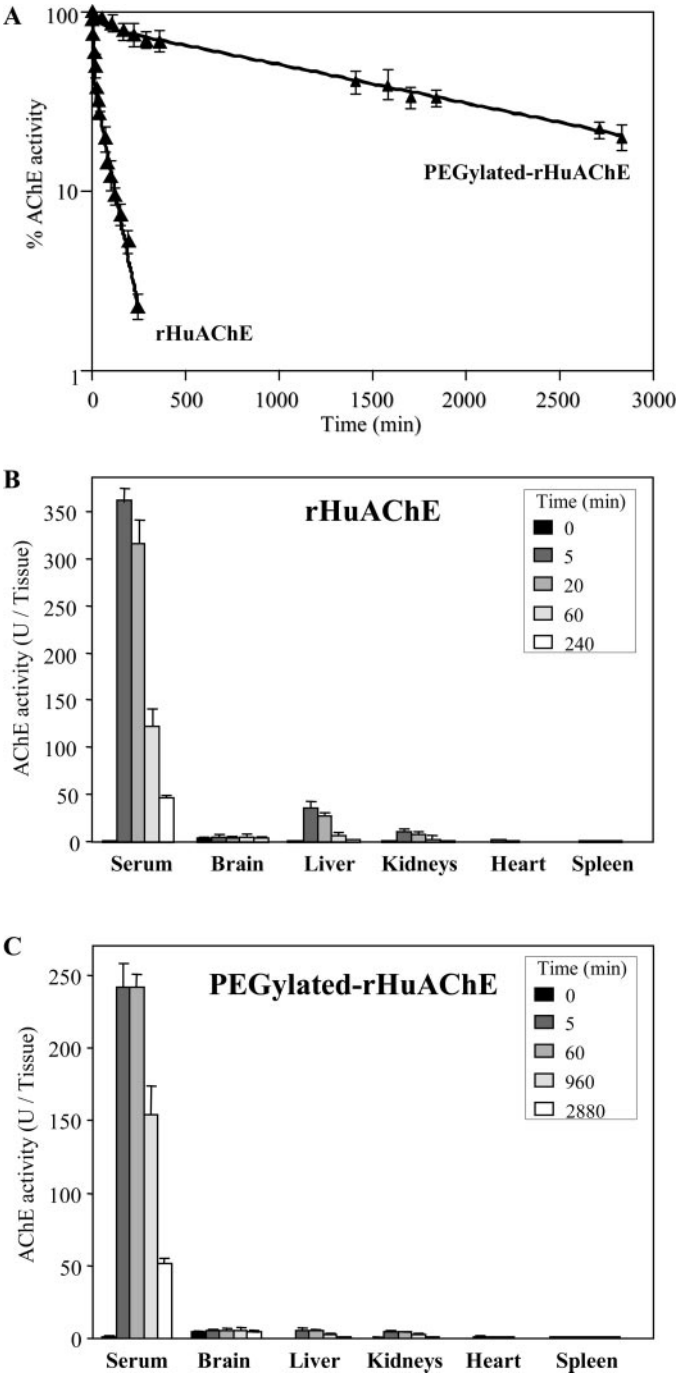

Fig. 1. Circulatory clearance profiles and tissue distribution of rHuAChE and PEGylated-rHuAChE. A, clearance profiles of nonmodified and PEGylated rHuAChE. Exogenous rAChEs (200 U) were administered i.v. to three mice each, at levels that are at least 30-fold higher than background level. Blood samples withdrawn at various time points were assayed for AChE activity, and values were corrected for background hydrolytic activity in the blood. Values are presented as percentage of input AChE determined 1 min after administration. B and C, nonmodified (B) or PEGylated-rHuAChE (C) was administered i.v. to mice and at each time point, tissues from three mice were removed, and AChE activity was measured in tissue homogenates.

Comparison of the Ability of AChE and BChE to Neutralize OP Compounds in Vitro and in Vivo

Enhancement of OP Toxicity in Mice by CBDP. The therapeutic potential of cholinesterases as exogenous scavengers for sequestration of toxic OP agents before they reach their physiological target has been examined in various animal models, including mice, rats, guinea pigs, and monkeys (Raveh et al., 1993, 1997; Lenz et al., 2005, and references therein). Small animals, such as mice, should be ideally suitable for carrying out wide-range comparative studies of the efficacies of different ChEs against a variety of OP agents. However, the susceptibility of mice to certain OP agents (e.g., soman and sarin) is considerably lower than that of primates because of the presence of high levels of endogenous CaEs that bind these OP agents (Maxwell et al., 1987; Li et al., 2005).

Previous studies (Clement, 1984; Maxwell et al., 1987; Shapira et al., 1990) have shown that endogenous CaEs can be effectively inactivated in rats and mice by CBDP and thereby allow for lower quantities of sarin or soman for mice intoxication. However, when administered at high levels, CBDP inhibits cholinesterases as well, and the CBDP dose that would selectively inhibit CaEs in mice without affecting ChE activity has not been determined precisely (Clement, 1984; Maxwell et al., 1987; Jimmerson et al., 1989). To determine exactly the amounts of CBDP that could be administered to mice without affecting exogenously administered cholinesterases, we first measured the bimolecular inhibition constants of CBDP toward AChE (1.4×10^3 M^{-1} min^{-1}) and BChE (2×10^4 M^{-1} min^{-1}; Fig. 2A). Because BChE is at least 20-fold more susceptible to inhibition than AChE, determination of the threshold level of CBDP that could effectively react with endogenous CaEs without affecting cholinesterase activity can be monitored by examining the inhibition of endogenous BChE in mice treated with CBDP at various concentrations (Fig. 2B). At CBDP concentrations of 2 mg/kg or less, endogenous BChE activity was only marginally affected (<2%; Fig. 2B). Furthermore, addition of purified BChE (1 U/ml) to the serum samples removed from mice treated with CBDP within the range of 2 to 10 mg/kg demonstrated that the exogenous BChE was fully active and was not inhibited by the CBDP in a measurable manner (Fig. 2B, inset).

In line with these findings, bioscavenging studies of soman and sarin by AChE or BChE were conducted in the present study after pretreatment of mice with CBDP at a dose of 2 mg/kg 1 h before challenge, because under these conditions the enzymatic activity of both endogenous cholinesterases and exogenously administered cholinesterases was not affected (see above). The LD_{50} values of soman and sarin in mice pretreated with CBDP at 2 mg/kg were, as expected, considerably lower than without CBDP pretreatment (Table 2) and are similar to the corresponding LD_{50} values determined in animal models devoid of serum CaEs, such as

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primates (Raveh et al., 1997). As expected, unlike in the case of soman and sarin, toxicity of VX in mice (LD_{50} of 13.5 μg/kg) was not affected by CBDP treatment, because VX does not effectively bind to carboxylesterases (Maxwell, 1992). Based on these findings, experiments carried out with VX were performed without CBDP pretreatment.

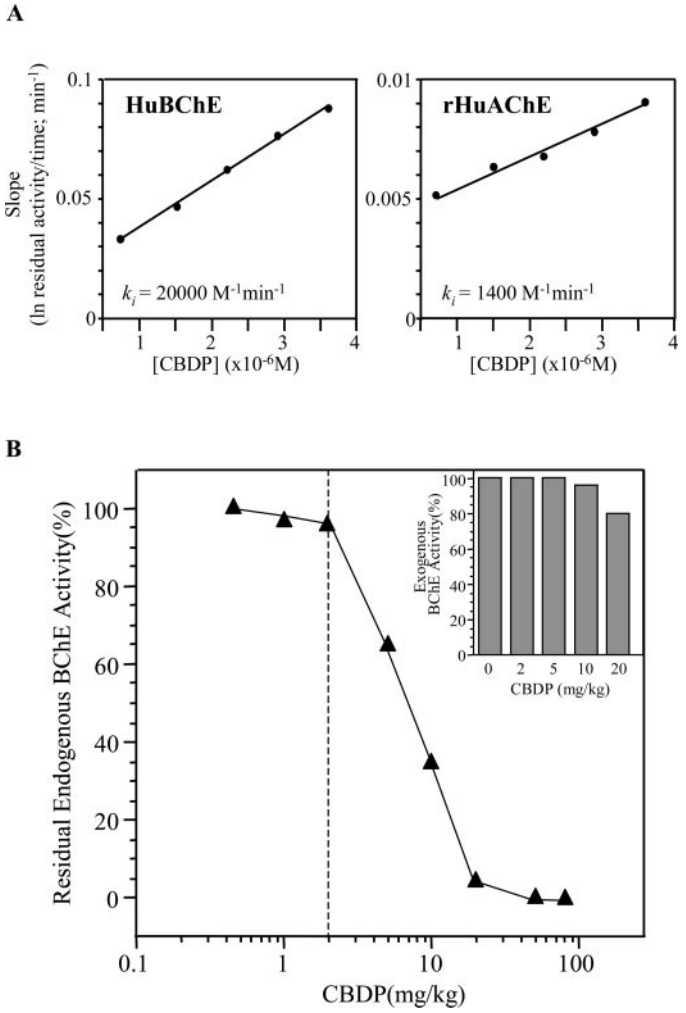

Fig. 2. Reactivity of CBDP toward ChEs, and its effect on ChE activity in mice. A, HuAChE and HuBChE were reacted with CBDP at various concentrations. The apparent bimolecular k_i values determined under pseudo first-order conditions were computed from the plot of slopes of ln(residual enzyme activity) versus time at different inhibitor concentrations. Values represent means of triplicate determination with standard deviations not exceeding 20%. B, residual endogenous BChE was measured in blood samples removed from mice at 1 h after administration of CBDP at various doses (five mice per dose). Values are presented as percentage of activity measured in blood samples of naive animals. Vertical dotted line represents the CBDP dose selected for performing protection experiments against soman and sarin (2 mg/kg). Inset, purified BChE (1 U/ml) was added to serum samples removed from mice pretreated with CBDP within the range of 2 to 10 mg/kg, and BChE activity was determined.

TABLE 2

Determination of LD_{50} values of various OP agents in mice after treatment with CBDP

OP agents were administered at various doses to five or six mice per dose. Figures in parentheses are 95% confidence limits.

OP Agent	LD_{50} i.v.	LD_{50} i.v. after CBDP Treatment
	$\mu g/kg$	
VX	13.6 (12.9–14.3)	13.5 (12.6–14.4)
Soman	54.9 (52.0–57.9)	6.6 (5.9–7.4)
Sarin	82.9 (80.4–85.6)	13.8 (12.6–15.1)

Comparison of the Ability of AChE and BChE to Neutralize Soman in Vivo. To compare the protective efficacy of exogenously administered AChE and BChE, mice were pretreated with equimolar amounts of either ChE and monitored for survival and toxic signs after OP compound challenge. In an initial experiment, recombinant HuAChE and serum HuBChE were examined for their ability to protect mice against an exposure of 1 LD_{50} of soman (1.2 nmol/mouse). Administration of both enzymes 1 min before challenge at a dose of 1.2 nmol/mouse (soman/ChE molar ratio = 1; both OP and ChE administered i.v.) provided protection to all mice from lethality, whereas only 60% of the animals that were not pretreated with ChE survived the 1 LD_{50} soman challenge (Table 3). In contrast, equimolar amounts of recombinant HuAChE and serum HuBChE conferred different levels of protection to mice challenged with soman at a higher dose. Thus, when mice were challenged to 2.5 LD_{50} soman (3 nmol/mouse) after administration of ChEs at a dose of 1.2 nmol ChE/mouse (soman/ChE molar ratio of 2.5), all rHuAChE-pretreated mice survived challenge and exhibited only mild signs of toxicity such as minor tremors and fasciculations (Table 3), whereas four of the five BChE-pretreated mice died within minutes. The single surviving BChE-pretreated mouse exhibited severe signs of toxicity, including tremors, Straub tail, salivation, and respiratory distress. Control animals exposed to the 2.5 LD_{50} soman challenge died within 2 to 3 min. When mice pretreated with rHuAChE or serum HuBChE (1.2 nmol/mouse) were challenged with 2.1 LD_{50} of soman (2.5 nmol/mouse; soman/ChE ratio of 2.1), all 10 mice pretreated with rHuAChE survived, whereas only 5 of 10 mice pretreated with serum HuBChE, survived challenge. rHuAChE-pretreated mice exhibited a reduction in body weight of no more than 5% and regained their initial weight at day 4 postchallenge (Fig. 3). In contrast, serum HuBChE-pretreated mice that survived challenge exhibited a reduction in body weight of 10% and reached their initial weights only on day 7 postchallenge.

The finding that, unlike AChE, BChE confers only partial protection to mice challenged with elevated levels of soman, prompted us to examine the ability of the two ChEs to protect

TABLE 3

Survival of mice after ChE administration and exposure to 1 and 2.5 LD_{50} of soman

Administered Soman	Administered ChE		
	None	rHuAChE	HuBChE
1 LD_{50}			
Administered enzyme (nmol/mouse)		1.2	1.2
Administered soman (nmol/mouse)	1.2	1.2	1.2
Survival (live/total)	60% (3/5)[a]	100% (5/5)	100% (5/5)
2.5 LD_{50}			
Administered enzyme (nmol/mouse)		1.2	1.2
Administered soman (nmol/mouse)	3	3	3
Survival (live/total)	0% (0/5)	100% (5/5)[a]	20% (1/5)[b]

[a] Surviving animals displayed mild symptoms of toxicity: mild tremors and Straub tail.
[b] Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation, and respiratory distress.

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mice from lethality at a wider range of soman/ChE ratios. To this end, mice were exposed to various soman doses 1 min after administration of either rHuAChE or serum HuBChE at ChE doses of 0.9 to 1.3 nmol/mouse (Fig. 4). All BChE-pretreated mice survived challenge when the soman/BChE molar ratios were equal to or lower than 1.6. At higher soman/BChE ratios, the enzyme conferred partial protection only, and survival levels were linearly correlated to the molar ratio of OP compound to enzyme. Thus, only 80, 60, and 20% of the mice survived challenge at soman/BChE ratios increasing from 1.8 to 2.0 and to 2.5, respectively (Fig. 4). In contrast, all AChE-pretreated mice survived challenge even when soman/AChE molar ratios were as high as 2.5. Lethality of AChE-pretreated mice could be observed only at higher soman/AChE ratios, and survival decreased in a linear manner, inversely correlating to the soman/AChE values (Fig. 4). Thus, 20% survival was observed for AChE-pretreated mice, only at the high soman/AChE ratio of 4, equivalent to 4.0 LD_{50}. Taken together, although BChE and AChE equally provide full protection to mice at soman/ChE ratios of 1.6 or less, rHuAChE clearly confers a higher level of protection from lethality at higher soman/ChE ratios.

Comparison of AChE and BChE Reactivities toward Soman, Sarin, and VX. The most probable cause for the lower protective efficacy of BChE against soman intoxication observed in mice (see above) is its lower stereoselectivity toward OP agents, compared with AChE. The limited stereoselectivity of BChE toward the different stereoisomers of soman was manifested for both rHuBChE and equine serum BChE and contrasts the marked stereoselectivity of the human or native bovine AChEs (Table 4) (Benschop et al., 1984; Benschop and de Jong, 1988; De Bisschop et al., 1991; Millard et al., 1998; Ordentlich et al., 1999, 2004). Thus, unlike AChE, which is highly stereoselective toward the toxic P_S-diastereomer, BChE displays considerable reactivity toward both the toxic P_S- and the nontoxic P_R-diastereomers of soman. Judging by the k_i value of either recombinant HuBChE or native serum BChE (Table 4), it seems that as with soman, BChEs have only a limited stereoselectivity (4- to 7-fold) toward the P_S-enantiomers of both sarin and VX. The low stereoselectivity of HuBChE toward sarin is, as for soman, due to its higher reactivity toward the P_R-diastereomer compared with that of HuAChE (>150-fold). Therefore, HuBChE could be expected to react, at least partially, with this less toxic inhibitor.

To examine how the different stereoselectivities of AChE and BChE toward soman may have affected their relative efficacies as in vivo bioscavengers, active site titration analyses with the racemic inhibitor were carried out at concentrations of enzymes and inhibitor simulating the estimated initial concentration ranges in plasma during the in vivo scavenging experiments in mice. Active site concentrations of the two ChEs were determined by titration with MEPQ, a potent OP inhibitor known to react with both AChE and BChE with 1:1 stoichiometry (Levy and Ashani, 1986; Shafferman et al., 1996). The intercepts for zero enzymatic activity, as observed in the actual titration profiles, were normalized to the corresponding intercepts determined in presence of MEPQ (Fig. 5). Under these experimental conditions, 2 mol of soman was neutralized by 1 mol of AChE, whereas only 1 mol of the inhibitor was sequestered by 1 mol of BChE. These results are consistent with the stereoselectivities of the two enzymes toward the P_S-soman diastereomers and seem to be directly correlated with the observed in vivo soman scavenging efficacies of AChE and BChE. As for soman, active site titration analysis demonstrated that 2 mol of sarin was neutralized by 1 mol of AChE, whereas only 1 mol of this inhibitor was sequestered by 1 mol of BChE. In contrast, the differential stereoselectivities of AChE and BChE toward VX were less pronounced. Thus, 1.1 mol of VX was neutralized by 1 mol of AChE, whereas 1.0 mol of VX was neutralized by 1 mol of BChE. Taken together, as for soman, the active site titration profiles of sarin and VX with AChE and BChE are fully consistent with the measured k_i values toward the corresponding enantiomers of these inhibitors. Therefore, we further examined whether the differential stereoselectivities of AChE and BChE toward sarin and VX were also reflected in the ability of the two enzymes to confer in vivo protection against these two OP inhibitors.

Comparison of the Ability of AChE and BChE to Neutralize Sarin and VX in Vivo. In view of the similar profiles of the titration curves of soman and sarin (Fig. 5), the protective potential of AChE and BChE against sarin toxicity was examined in mice at a sarin/ChE molar ratio of 2.5. To

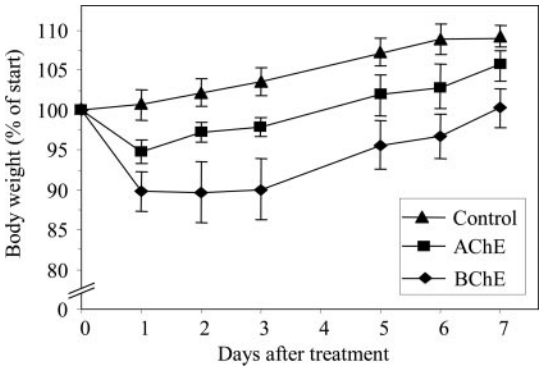

Fig. 3. Toxic effects in ChE-pretreated mice after exposure to soman. rHuAChE or serum HuBChE (1.2 nmol/mouse) was administered to mice, which were then exposed to soman (2.1 LD_{50}). Body weight of survivor mice was monitored for 7 days after challenge. Body weight of control mice treated with PBS without subsequent exposure to soman is shown for comparison.

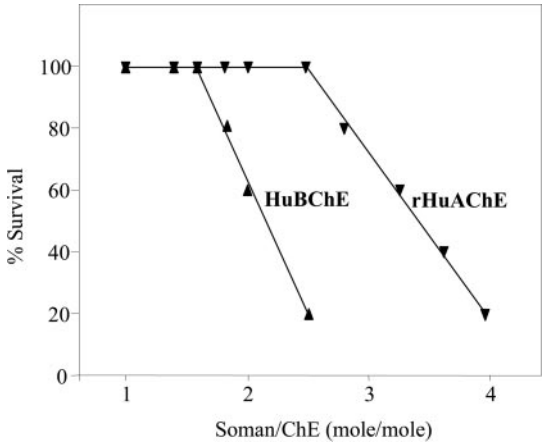

Fig. 4. Survival of AChE- and BChE-pretreated mice after exposure to soman at a wide range of soman to ChE molar ratios. rHuAChE or serum HuBChE (0.9–1.3 nmol/mouse) were administered to CBDP-pretreated mice and mice were then exposed to soman at various doses. Each point is based on 5 to 10 mice per group. Values are presented as percentage of surviving mice for each soman to ChE molar ratio.

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this end, rHuAChE or serum HuBChE (2.6 nmol/mouse) was administered to mice, and after 1 min, the ChE-treated mice were challenged with 2.5 LD_{50} of sarin (6.5 nmol/mouse). When serum HuBChE was administered, all mice died within minutes after exposure to sarin. In contrast, when rHuAChE was administered, 80% of the mice survived challenge, yet they displayed severe toxic symptoms, including severe tremors and respiratory distress (Table 5). In an additional experiment, rHuAChE and serum HuBChE were compared for their ability to protect mice against VX intoxication. In this case, greater molar quantities of the ChEs (3.2 nmol/mouse; VX/ChE molar ratio of 1.25:1) were administered, because as suggested by the active site titrations, rHuAChE exhibited a lower stereoselectivity toward VX than to soman or sarin (Table 4; Fig. 5). Control animals exposed to a 2.5 LD_{50} VX challenge without ChE pretreatment died within 10 to 15 min (Table 5). All mice pretreated with rHuAChE survived and did not exhibit any symptoms of toxicity. In contrast, serum HuBChE provided only partial (60%) protection against this agent, whereas survivor animals displayed severe toxic symptoms. Taken together, the bioscavenging experiments described above clearly demonstrate that rHuAChE is more effective than serum HuBChE, on a molar basis, in its ability to protect mice against soman, sarin, and even to some extent VX poisoning.

The Longevity of PEGylated rHuAChE Makes It an Effective Prophylactic Bioscavenger of OP Compounds. Based on the similar reactivities of rHuAChE and its PEGylated form toward OP agents (Table 1), one could expect that the circulatory stable PEGylated rHuAChE will be as effective as the short-lived nonmodified rHuAChE in its ability to neutralize OPs in vivo. Indeed, when PEGylated rHuAChE was administered to mice at a dose of 1.2 nmol/mouse and mice were exposed after 1 min to 2.5 LD_{50} of soman, the animals were fully protected and displayed no more than minor symptoms of intoxication (Table 6). Thus, the appendage of PEG moieties to rHuAChE did not compromise the ability of the enzyme to effectively protect mice against soman poisoning.

The therapeutic value of an OP bioscavenger as a prophylactic agent in some realistic scenarios depends on its ability to provide protection even when it is administered a long time before the exposure to the CW agent. To examine the prophylactic potential of the PEG-modified AChE, equal amounts (3.2 nmol/mouse) of either rHuAChE, PEGylated rHuAChE or serum HuBChE were administered to mice, and 22 h later the mice were exposed to 1.5 LD_{50} of VX (Table 7). All mice pretreated with the circulatory short-lived rHuA-

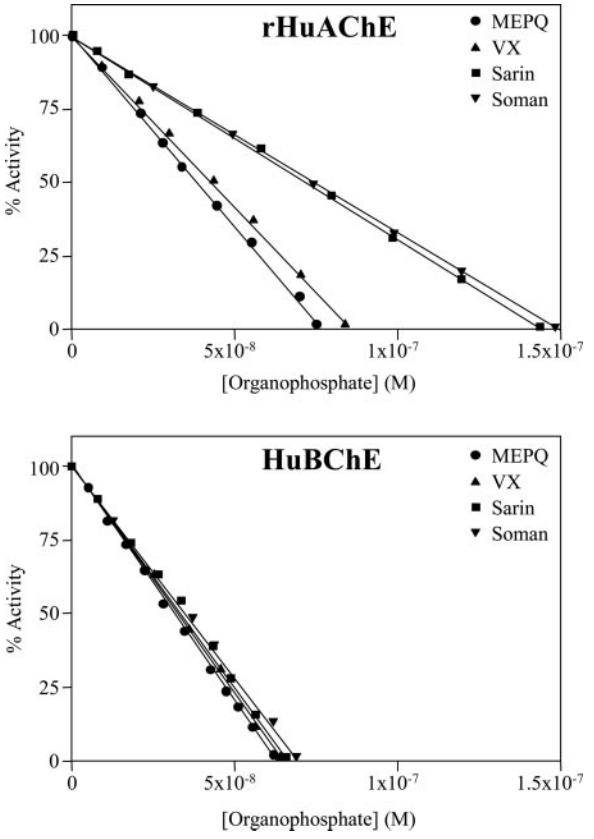

Fig. 5. Active site titration of rHuAChE and serum HuBChE with soman, sarin, VX, and MEPQ. In all titration experiments, the concentration of the catalytic subunit of rHuAChE and HuBChE was 60 to 80 nM. The residual activity was determined after incubation at 27°C in the presence of various concentrations of the different compounds for 30 and 60 min.

TABLE 4

Compilation of k_i of phosphonylation of ChEs by enantiomers of VX, sarin, and soman

All values determined in the present study represent the mean of triplicate determinations with standard deviations not exceeding 20%. P_S/P_R is ratio of bimolecular rate constants of phosphonylation of the stereoisomers. For soman, values were determined for the pairs P_SC_S/P_RC_S and P_SC_R/P_RC_R. Bimolecular rate constants for HuAChE with sarin were determined in the present study. Bimolecular rate constants for HuAChE with VX and soman are based on Ordentlich et al. (1999, 2004). Bimolecular rate constants for BoAChE with VX, sarin, and soman are based on Benschop et al. (1984) and Benschop and de Jong (1988). Bimolecular rate constants for HuBChE with sarin and VX were determined in the present study. Bimolecular rate constants for HuBChE with soman are based on Millard et al. (1998). Bimolecular rate constants for EqBChE with sarin and VX were determined in the present study. Bimolecular rate constants for EqBChE with soman are based on Ordentlich et al. (1999). The bimolecular rate constant for other native serum-derived BChEs (human, canine, and porcine) with stereoisomers of soman are similar (De Bisschop et al., 1991) with the P_S/P_R values in the range of 2.5 to 120.

ChE	k_i										
	VX			Sarin			Soman				
	P_S	P_R	P_S/P_R	P_S	P_R	P_S/P_R	P_SC_S	P_SC_R	P_RC_S	P_RC_R	P_S/P_R
	$\times 10^{-4}\ M^{-1}\ min^{-1}$										
AChE											
rHuAChE	13,700	120	115	2000	<1	>2000	15,000	8000	0.2	0.2	$(4–7.5) \times 10^3$
BoAChE	40,000	200	200	1400	<0.3	>4600	18,000	2700	<1	<1	$(0.3–1.8) \times 10^3$
BchE											
rHuBChE	1600	280	6	1100	150	7	4000	500	~120[a]	600	0.8–33
EqBChE	670	170	4	1030	140	7	6000	1000	120	170	14–50

[a] The bimolecular rate constant for rHuBChE with the P_RC_S stereoisomer of soman was not determined experimentally. However, because the bimolecular rate constants of phosphonylation of the stereoisomers of the two BChEs presented were in most cases very similar, we used for computation, the corresponding k_i value determined for EqBChE as the value of the P_RC_S stereoisomer of soman for rHuBChE.

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ChE died within minutes of the challenge, like control mice, which were not pretreated with enzyme. This was as expected, because the nonmodified version of rHuAChE, which is characterized by a circulatory half-life time of 42 min, would be essentially eliminated from the bloodstream by the time of exposure to VX. In contrast, the PEGylated rHuAChE- and serum HuBChE-pretreated mice displayed 100 and 80% survival rates, respectively (Table 7), demonstrating that recombinant AChE in its PEGylated version confers protection to mice against OP compounds even many hours after administration of the enzyme. To further assess the ability of these two enzyme forms to provide protection against multiple exposures to OPs, these mice were subjected 2 h later to a second challenge of 0.9 LD_{50} VX. After re-exposure, 75% of the mice pretreated with serum BChE died, whereas all the PEGylated rHuAChE-pretreated mice survived (Table 7). Overall, during this experiment a total dose equal to 2.4 LD_{50} of VX was administered to mice, and under these conditions, PEGylated rHuAChE conferred greater protection (100% survival) than HuBChE (25% survival).

Each series of experiments described above was carried out with ChEs administered via the intravenous route. A more realistic approach to prophylactic treatment against OP intoxication, however, would be to administer the therapeutic enzyme of choice i.m. To evaluate the feasibility of intramuscular pretreatment with PEGylated rHuAChE, we first monitored the pharmacokinetic performance of i.m.-administered PEGylated rHuAChE (Fig. 6). After administration of 300 U of PEGylated rHuAChE by intramuscular injection to mice, an initial phase of rapid increase in AChE activity in the serum was observed, followed by a moderate absorption phase, which reached peak levels (T_{max}) at 12 h. The rate of elimination from the serum of the PEGylated rHuAChE ($t_{1/2}$ = 29 ± 3 h) was very similar to that exhibited for this enzyme form after intravenous administration ($t_{1/2}$ = 26 ± 2 h). The bioavailability of the PEG-modified AChE was calculated to be 56%. In comparison, serum HuBChE and FBS-AChE displayed bioavailability values of 54 and 29%, respectively, after intramuscular administration to mice (Fig. 6, inset). Thus, PEG conjugation does not seem to deleteriously affect the ability of the enzyme to be directed to the circulation. This relatively high bioavailability determined for PEGylated rHuAChE suggests that extravascular pretreatment with PEGylated rHuAChE may serve as an effective mode for protection against OP toxicity. To further examine this issue,

TABLE 5

Survival of mice after ChE administration and exposure to sarin and VX

Animals challenged with sarin were pretreated with CBDP 1 h before enzyme administration.

Administered OP Agent	Administered ChE		
	None	rHuAChE	HuBChE
2.5 LD_{50} sarin			
Administered enzyme (nmol/mouse)		2.6	2.6
Administered OP (nmol/mouse)	6.5	6.5	6.5
Survival (live/total)	0% (0/5)	80% (4/5)[a]	0% (0/5)
2.5 LD_{50} VX			
Administered enzyme (nmol/mouse)		3.2	3.2
Administered OP (nmol/mouse)	4	4	4
Survival (live/total)	0% (0/5)	100% (5/5)	60% (3/5)[a]

[a] Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation, and respiratory distress.

TABLE 6

Protection of mice against OP agents by PEGylated rHuAChE: survival of mice after exposure to 2.5 LD_{50} soman

Animals challenged with soman were pretreated with CBDP 1 h before enzyme administration.

	Administered ChE		
	None	rHuAChE	PEG-rHuAChE
Administered enzyme (nmol/mouse)		1.2	1.2
Administered soman (nmol/mouse)	3	3	3
Survival (live/total)	0% (0/5)	100% (5/5)[a]	100% (5/5)[a]

[a] Surviving animals displayed mild symptoms of toxicity: mild tremors and Straub tail.

TABLE 7

Protection of mice against OP agents by PEGylated rHuAChE: survival of mice exposed to VX 20 to 22 h after intravenous administration of ChEs

	Administered ChE		
	None	PEG-rHuAChE	HuBChE
First exposure (1.5 LD_{50}; 20 h)			
Administered enzyme (nmol/mouse)		3.2	3.2
Administered VX (nmol/mouse)	2.4	2.4	2.4
Survival (live/total)	0% (0/5)	100% (5/5)	80% (4/5)
Second exposure (0.9 LD_{50}; 22 h)			
Administered VX (nmol/mouse)		1.4	1.4
Survival (live/total)		100% (5/5)[a]	25% (1/4)[b]

[a] Surviving animals displayed mild symptoms of toxicity: mild tremors and Straub tail.

[b] Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation, and respiratory distress.

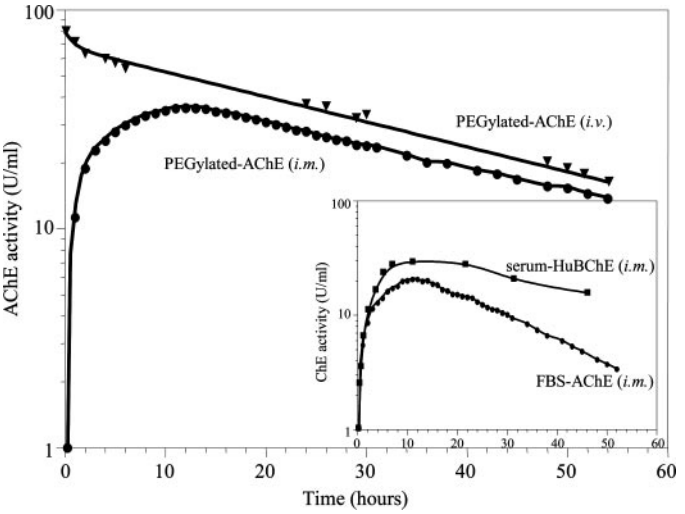

Fig. 6. Circulatory clearance profiles of PEGylated-rHuAChE administered by different routes. PEGylated AChE (200 U) was administered i.v. and i.m. to three mice each. Blood samples withdrawn at various time points were assayed for AChE activity, and values were corrected for background hydrolytic activity in the blood. Values are presented as AChE units per milliliter of blood. Bioavailability of i.m.-administered PEGylated rHuAChE was calculated as area under $curve_{i.m.}$/area under $curve_{i.v.}$ Inset, Clearance profiles of FBS-AChE and serum human BChE administered i.m. Bioavailability values of these two enzymes were calculated by comparing these clearance profiles to those obtained after i.v. administration of the corresponding enzyme forms (Raveh et al., 1993; Kronman et al., 2000).

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mice were intramuscularly administered with 3 nmol of either serum HuBChE or PEGylated rHuAChE, and after 20 h, they were challenged intravenously with 1.3 LD_{50} VX (Table 8). Nearly 90% of mice pretreated with PEGylated rHuAChE survived challenge and exhibited only mild symptoms of toxicity. In contrast, only 60% of the serum HuBChE-pretreated mice survived, while exhibiting severe signs of toxicity, including tremors, Straub tail, salivation, and respiratory distress.

In conclusion, it seems that appendage of PEG chains transforms rHuAChE into a very efficient bioscavenger, displaying a protective potential comparable and maybe even better than that of the native serum-derived HuBChE after i.m. administration.

Discussion

Various studies have demonstrated that both AChE and BChE can potentially serve as bioscavengers of OP compounds (Lenz et al., 2005, and references therein). In the present study, comparative protection experiments allowed us to determine that on a molar basis, AChE conferred a higher level of protection than BChE against soman and sarin intoxication (Fig. 4; Tables 3, 5, and 6). This marked difference in the ability of AChE and BChE to provide effective protection most likely stems from the higher stereoselectivity of AChE toward the P_S-stereoisomers of the racemic mixtures of soman and sarin (Table 4). Indeed, compilation of the apparent bimolecular rate constants of phosphonylation of different species of AChE (rHuAChE, native serum-derived bovine AChE) by purified stereoisomers demonstrates that this enzyme is characterized by very high P_S/P_R phosphonylation rate constant ratios within the range of 5000 to approximately 60,000. In contrast, the corresponding P_S/P_R phosphonylation rate constant ratios for different BChEs (rHuBChE, native serum-derived equine BChE) are considerably lower: 0.8- to 50-fold. Further support for the assumption that the differential stereoselectivity results in the higher consumption of BChE was obtained from active site titration experiments carried out at enzyme and OP compound concentrations simulating the estimated initial values in the in vivo protection experiments. In these experiments, neutralization of 1 mol of soman was effectively achieved by 0.5 mol of AChE, whereas neutralization of the same amount of soman by BChE required 1 mol of enzyme (Fig. 5), indicating that under these in vitro conditions, AChE reacts effectively with only half of the stereoisomers, whereas BChE reacts at similar efficiencies with both the toxic and nontoxic stereoisomers comprising the racemic mixture of soman. A similar active site titration pattern was observed also when AChE and BChE were reacted with sarin (Fig. 5). We may therefore conclude that at a less than 50-fold difference between the phosphonylation rate constants of the P_S- and P_R-enantiomers (of soman and sarin), as observed for BChE (Table 4), effective discrimination of the enantiomers under the experimental conditions is precluded. In contrast, the greater than 2000-fold difference between the phosphonylation rate constants of the P_S- and P_R-enantiomers of soman and sarin toward AChEs, may be sufficient to restrict in vivo the reactivity of this enzyme to the P_S-enantiomer only. In line with these findings, we expected that the 115- to 200-fold difference between the phosphonylation rate constants of the P_S- and P_R-enantiomers of VX toward AChE (Table 4) may lead to a much less pronounced discrimination, in the reactivity of AChE toward the VX enantiomers in vivo. Indeed, for VX, the amount of AChE required for the in vitro neutralization of the racemic mixture of VX was only slightly lower than that of BChE (Fig. 5). Furthermore, at a VX/ChE molar ratio of 1.25, AChE provided only slightly higher protection than BChE against VX intoxication in vivo; 100 and 60% of AChE-pretreated and BChE-pretreated mice, respectively, survived challenge with 2.5 LD_{50} VX.

Taken together, these experiments suggest that BChE is partially consumed by reaction with the nontoxic P_R-enantiomers of soman and sarin or the less toxic P_R-enantiomers of VX. This seems to be the major cause for the lower amount of AChE, versus BChE required to confer protection against soman and sarin and even against VX. However, one also should consider in scavenging studies the pharmacokinetic rates of the OP diastereoisomers themselves. It has been reported that the nontoxic enantiomers of soman are hydrolyzed by the plasma in vitro at very high rates (de Jong et al., 1988). Yet, it was also shown by the same group that in vivo, approximately 50% of ^{14}C-labeled P_R-diastereomers are eliminated by hydrolysis, whereas the rest is sequestered by covalent binding (Benschop and de Jong, 1991). Therefore, after administration of exogenous BChE, which displays very high affinity toward the P_R-enantiomer (Table 4), P_R hydrolysis is not expected to significantly affect the differential in vivo efficiencies manifested by AChE and BChE.

If indeed 200 mg of BChE is required to protect humans against 2 LD_{50} of soman (Ashani et al., 1998), it seems, based on the present study, that only 78 mg of AChE will be needed to confer the same extent of protection. Taking into account the requirement to minimize quantities of exogenously administered protein in humans, the advantage of AChE over BChE as a therapeutic bioscavenger is evident.

The use of cholinesterases in prophylactic treatments against OP agents requires that these reside in the circulation for sufficiently long periods. Chemical modification of various recombinant proteins by covalent conjugation of PEG chains was shown to increase their circulatory residence (Harris and Chess, 2003); however, in many instances, the conjugation of PEG moieties to various proteins was accompanied by a concomitant loss of biological activity (Harris and Chess, 2003). We have demonstrated in the past that under a certain set of conditions, rHuAChE can be efficiently PEGylated without compromising its catalytic activity and that PEGylation of rHuAChE under these conditions resulted in a 50-fold increase in circulatory retention in mice compared

TABLE 8

Protection of mice against OP agents by PEGylated rHuAChE: survival of mice exposed to 1.3 LD_{50} VX 20 h after intramuscular administration of ChEs

	Administered ChE		
	None	PEG-rHuAChE	HuBChE
Administered enzyme (nmol/mouse)		3	3
Administered VX (nmol/mouse)	2	2	2
Survival (live/total)	0% (0/8)	88% (8/9)[a]	57% (4/7)[b]

[a] Surviving animals displayed mild symptoms of toxicity: mild tremors and Straub tail.

[b] Surviving animals displayed severe symptoms of toxicity: severe tremors, Straub tail, salivation, and respiratory distress.

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with the nonmodified rHuAChE, displaying mean residence time (MRT) values of 2100 min (Cohen et al., 2001). In an additional study, we demonstrated that the PEGylated rHuAChE displayed a 150-fold increase in circulatory retention relative to nonmodified rHuAChE in rhesus macaques, exhibiting MRT values of approximately 10,000 min (Cohen et al., 2004).

In the present study, it is shown that the reactivity of rHuAChE toward various OP agents (soman, sarin, tabun, and VX) was not affected after its PEGylation (Table 1). The reactive PEGylated rHuAChE, by virtue of its improved pharmacokinetic performance, could efficiently protect mice from a VX challenge carried out 20 h after administration of PEGylated rHuAChE. Indeed, the prophylactic performance of PEGylated rHuAChE was even better than that of native serum-derived HuBChE, as manifested by the observation that upon re-exposure to VX, 75% of the BChE-pretreated mice died, whereas all of the PEGylated rHuAChE-pretreated mice survived (Table 7). The superior protection of PEGylated rHuAChE was also manifested when this enzyme was administered intramuscularly to mice 20 h before VX challenge, resulting in nearly 90% survival of the animals. Administration of similar amounts of native serum-derived HuBChE protected 60% of the mice (Table 8). The marked difference in prophylactic efficacy of AChE versus BChE may be explained by the combined effect of the slightly higher stereoselectivity of the AChE toward VX (Table 4) and its better pharmacokinetic performance relative to native human BChE (MRT of PEGylated rHuAChE = 2100 min and of BChE = 1820 min; Fig. 6; Raveh et al., 1993) in this animal model system.

In the present study, we demonstrate for the first time that recombinant AChE can be effectively used as a soluble OP bioscavenger, conferring protection against soman, sarin, and VX. Although soluble AChE is prevalent in the circulation of some animal species, its presence in the circulation of other mammals such as mice and primates is considerably lower than that of BChE (Li et al., 2000), and therefore one may speculate whether the administration of exogenous AChE would result in its altered disposition in various organs. We decided to test this possibility, even though the recombinant HuAChE variant used in the present study is devoid of the C-terminal tail of the enzyme responsible for its assembly into multiunit enzyme forms as well as for its anchoring to biological membranes (Massoulie et al., 2005). Extensive tissue distribution studies were therefore carried out to determine the fate of the exogenously administered rHuAChE and PEGylated rHuAChE. These studies clearly demonstrate that as for nonmodified enzyme, PEGylated rHuAChE did not accumulate in any of the organs examined and did not affect tissue biodistribution (Fig. 1).

The generation of a bioactive PEGylated ChE is dependent on a delicate balance between efficient PEG appendage and enzyme activity preservation and therefore needs to be carried out in a highly controlled manner (Cohen et al., 2001). Recently, it was reported that PEG conjugation of rHuBChE resulted in the generation of a heterogeneous set of products (Chilukuri et al., 2005). Unlike PEGylated rHuAChE, which exhibits a circulatory duration comparable or even exceeding that of native serum-derived BChE (Cohen et al., 2004), the reported PEGylated rHuBChE was eliminated much more rapidly than native serum-derived BChE. This limitation may stem from suboptimal PEGylation, which was shown to result in an insufficient extension of the circulatory lifetime of cholinesterases (Cohen et al., 2001). Furthermore, one should note that PEGylated BChE products may be more prone to heterogeneity than PEGylated AChEs because BChE contains many more lysine residues, which may serve as candidate target sites for PEG conjugation, than AChE.

In conclusion, the observation that the circulatory long-lived PEGylated rHuAChE confers superior prophylactic protection to mice exposed to OP compounds together with the finding that the chemically modified enzyme displays unaltered biodistribution and high bioavailability presents a case for using PEGylated rHuAChE as a highly effective bioscavenger of CW OP agents.

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References

Ashani Y, Grauer E, Grunwald J, Allon N, and Raveh L (1998) Current capabilities in extrapolating from animal to human the capacity of human butyrylcholinesterase to detoxify organophosphates, in *Structure and Function of Cholinesterases and Related Proteins* (Doctor BP, Quinn DM, Rotundo RL, and Taylor P eds) pp 255–260, Plenum Press, New York.

Benschop HP and de Jong LPA (1988) Nerve agent stereoisomers: analysis, isolation and toxicology. *Acc Chem Res* **21:**368–374.

Benschop HP and de Jong LPA (1991) Toxicokinetics of soman: species variation and stereospecificity in elimination pathways. *Neurosci Biobehav Rev* **15:**73–77.

Benschop HP, Konings CAG, Van Genderen J, and de Jong LPA (1984) Isolation, anticholinesterase properties, and acute toxicity in mice of the four stereoisomers of the nerve agent soman. *Toxicol Appl Pharmacol* **72:**61–74.

Chitlaru T, Kronman C, Velan B, and Shafferman A (2001) Effect of human acetylcholinesterase subunit assembly on its circulatory residence. *Biochem J* **354:**613–625.

Chitlaru T, Kronman C, Velan B, and Shafferman A (2002) Overloading and removal of N-glycosylation targets on human acetylcholinesterase: effects on glycan composition and circulatory residence time. *Biochem J* **363:**619–631.

Chitlaru T, Kronman C, Zeevi M, Kam M, Harel A, Ordentlich A, Velan B, and Shafferman A (1998) Modulation of circulatory residence of recombinant acetylcholinesterase through biochemical or genetic manipulation of sialylation levels. *Biochem J* **336:**647–658.

Chilukuri N, Parikh K, Sun W, Naik R, Tipparaju P, Doctor BP, and Saxena A (2005) Polyethylene glycosylation prolongs the circulatory stability of recombinant human butyrylcholinesterase. *Chem Biol Interact* **157–158**:115–121.

Clement JG (1984) Importance of aliesterase as a detoxification mechanism for soman (pinacolyl methylphosphonofluoridate) in mice. *Biochem Pharmacol* **33:** 3807–3811.

Cohen O, Kronman C, Chitlaru T, Ordentlich A, Velan B, and Shafferman A (2001) Effect of chemical modification of recombinant human acetylcholinesterase by polyethylene glycol on its circulatory longevity. *Biochem J* **357:**795–802.

Cohen O, Kronman C, Velan B, and Shafferman A (2004) Amino acid domains control the circulatory residence time of primate acetylcholinesterases in rhesus macaques. *Biochem J* **378:**117–128.

De Bisschop HCJV, Michiels KW, Vlaminck LBC, Vansteenkiste SO, and Schacht EH (1991) Phosphonylation of purified human canine and porcine cholinesterase by soman. *Biochem Pharmacol* **41:**955–959.

de Jong LPA, Van Dijk C, and Benschop HP (1988) Hydrolysis of the four stereoisomers of soman catalyzed by liver homogenate and plasma from rat, guinea pig and marmoset and by human plasma. *Biochem Pharmacol* **37:**2939–2948.

Duysen EG, Bartles CF, and Lockridge O (2002) Wild-type and A328W mutant human butyrylcholinesterase tetramers expressed in Chinese hamster ovary cells have a 16-h half-life in the circulation and protect mice from cocaine toxicity. *J Pharmacol Exp Ther* **302:**751–758.

Ellman GL, Courtney KD, Andres V, and Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* **7:**88–95.

Eto M, Casida JE, and Eto T (1962) Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. *Biochem Pharmacol* **11:**337–352.

Finney DJ (1964) The Spearman-Karber method, in *Statistical Methods in Biological Assay*, pp 524–530, Charles Griffin, London.

Fischer M, Ittah A, Liefer I, and Gorecki M (1993) Expression and reconstitution of biologically active human acetylcholinesterase from *E. coli*. *Cell Mol Neurobiol* **13:**25–38.

Harris JM and Chess RB (2003) Effect of PEGylation on pharmaceuticals. *Nat Rev Drug Discov* **2:**214–221.

Kronman C, Velan B, Gozes Y, Leitner M, Flashner Y, Lazar A, Marcus D, Sery T, Grosfeld H, Cohen S, et al. (1992) Production and secretion of high levels of

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recombinant human acetylcholine esterase in cultured cell lines: microheterogeneity of the catalytic subunit. *Gene* **121:**295–304.
Jimmerson VR, Shih TM, and Mailman RB (1989) Variability in soman toxicity in the rat: correlation with biochemical and behavioral measures. *Toxicology* **57:**241–254.
Kronman C, Velan B, Marcus D, Ordentlich A, Reuveny S, and Shafferman A (1995) Involvement of oligomerization, N-glycosylation and sialylation in the clearance of cholinesterases from circulation. *Biochem J* **311:**959–967.
Kronman C, Chitlaru T, Elhanay E, Velan B, and Shafferman A (2000) Hierarchy of post-translational modifications involved in the circulatory longevity of glycoproteins. *J Biol Chem* **275:**29488–29502.
Lenz DE, Maxwell DM, Koplovits I, Clark CR, Capacio BR, Cerasoli DM, Federko JM, Luo C, Saxena A, Doctor BP, et al. (2005) Protection against soman or VX poisoning by human butyrylcholinesterse in guinea pigs and cynomolgus monkeys. *Chem Biol Interact* **157–158:**205–210.
Levy D and Ashani Y (1986) Synthesis and in vitro properties of a powerful quaternary methylphosphonate inhibitor of acetylcholinesterase. A new marker in blood-brain barrier research. *Biochem Pharmacol* **35:**1079–1085.
Li B, Sedlacek M, Manoharan I, Boopathy R, Duysen EG, Masson P, and Lockridge O (2005) Butyrylcholinesterase, paraoxonase and albumin esterase, but not carboxylesterase, are present in human plasma. *Biochem Pharmacol* **70:**1673–1684.
Li B, Stribley JA, Ticu A, Xie W, Schopfer LM, Hammond P, Brimijoin S, Hinrichs SH, and Lockridge O (2000) Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. *J Neurochem* **75:**1320–1331.
Massoulie J, Bon S, Perrier N, and Falasca C (2005) The C-terminal peptides of acetylcholinesterase: cellular trafficking, oligomerization and functional anchoring. *Chem Biol Interact* **157–158:**3–14.
Maxwell DM (1992) The specificity of carboxylesterase protection against the toxicity of organophosphorous compounds. *Toxicol Appl Pharmacol* **114:**306–312.
Maxwell DM, Brecht KM, and O'Neill BL (1987) The effect of carboxylesterase inhibition on interspecies differences in soman toxicity. *Toxicol Lett* **39:**35–42.
Millard CB, Lockridge O, and Broomfield CA (1998) Organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase: synergy results in a somanase. *Biochemistry* **37:**237–247.
Ordentlich A, Barak D, Kronman C, Ariel N, Segall Y, Velan B, and Shafferman A (1996) The architecture of human acetylcholinesterase active center probed by interactions with selected organophosphate inhibitors. *J Biol Chem* **271:**11953–11962.
Ordentlich A, Barak D, Kronman C, Benschop HP, de Jong LPA, Ariel N, Barak R, Segall Y, Velan B, and Shafferman A (1999) Exploring the active center of human acetylcholinesterase with stereomers of an organophosphorus inhibitor with two chiral centers. *Biochemistry* **38:**3055–3066.
Ordentlich A, Barak D, Sod-Moriah G, Kaplan D, Mizrahi D, Segall Y, Kronman C, Karton Y, Lazar A, Marcus D, et al. (2004) Stereoselectivity toward VX is determined by interactions with residues of the acyl pocket as well as of the peripheral anionic site of AChE. *Biochemistry* **43:**11255–11265.
Raveh L, Grauer E, Grunewald J, Cohen E, and Ashani Y (1997) The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol* **145:**43–53.
Raveh L, Grunwald J, Marcus D, Papier Y, Cohen E, and Ashani Y (1993) Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. *Biochem Pharmacol* **45:**37–41.
Saxena A, Ashani Y, Raveh L, Stevenson D, Patel T, and Doctor BP (1998) Role of oligosaccharides in the pharmacokinetics of tissue-derived and genetically engineered cholinesterases. *Mol Pharmacol* **53:**112–122.
Shafferman A, Ordentlich A, Barak D, Stein D, Ariel N, and Velan B (1996) Aging of phosphylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active center. *Biochem J* **318:**833–840.
Shafferman A, Velan B, Ordentlich A, Kronman C, Grosfeld H, Leitner M, Flashner Y, Cohen S, Barak D, and Ariel N (1992) Substrate inhibition of acetylcholinesterase: residues affecting signal transduction from the surface to the catalytic center. *EMBO (Eur Mol Biol Organ) J* **11:**3561–3568.
Shapira S, Kadar T, Cohen G, Chapman S, and Raveh L (1990) Effects of CBDP and MEPQ on the toxicity and distribution of [^{3}H]-soman in mice. *Arch Toxicol* **64:**663–668.

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