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HUMAN BUTYRYLCHOLINESTERASE AS A GENERAL PROPHYLACTIC ANTIDOTE FOR NERVE AGENT TOXICITY

IN VITRO AND *IN VIVO* QUANTITATIVE CHARACTERIZATION

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Abstract—Butyrylcholinesterase purified from human plasma (HuBChE) was evaluated both *in vitro* and *in vivo* in mice and rats as a single prophylactic antidote against the lethal effects of highly toxic organophosphates (OP). The variation among the bimolecular rate constants for the inhibition of HuBChE by tabun, VX, sarin, and soman was 10-fold (0.47 to 5.12 × 10^7 M^{-1} min^{-1}; pH 8.0, 26°). The half-life of HuBChE in blood after its i.v. administration in mice and rats was 21 and 46 hr, respectively. The peak blood-enzyme level was obtained in both species approximately 9–13 hr following i.m. injection of HuBChE, and the fraction of the enzyme activity absorbed into the blood was 0.9 and 0.54 for rats and mice, respectively. The stoichiometry of the *in vivo* sequestration of the anti-cholinesterase toxicants was consistent with the HuBChE/OP ratio of the molar concentration required to inhibit 100% enzyme activity *in vitro*. Linear correlation was demonstrated between the blood level of HuBChE and the extent of protection conferred against the toxicity of nerve agents. Pretreatment with HuBChE alone was sufficient not only to increase survivability following exposure to multiple median lethal doses of a wide range of potent OPs, but also to alleviate manifestation of toxic symptoms in mice and rats without the need for additional post-exposure therapy. It appeared that in order to confer protection against lethality nerve agents had to be scavenged to a level below their median lethal dose LD_{50} within less than one blood circulation time. Since the high rate of sequestration of nerve agents by HuBChE is expected to underlie the activity of the scavenger in other species as well, a reliable extrapolation of its efficacy from experimental animals to humans can be made.

Antidotal regimens for anti-cholinesterase (ChE‡) organophosphate (OP) toxicity [1] consist of pretreatment with pyridostigmine followed by post-exposure therapy [1–5] that includes atropine, oxime reactivator and anticonvulsant drugs. The currently approved medical countermeasures for treatment of toxic OPs are near their practical limits [5]. Furthermore, the traditional approach suffers from several disadvantages. The treatment could not prevent the occurrence of severe post-exposure incapacitation such as convulsions [2–4] or behavioral impairments [4, 6, 7]. Proper timing and sequencing of the antidotal treatment were found to be extremely important [5, 8]; however, conditions in emergency cases may not permit such precise timing and immediate post-exposure drug treatment. A possible strategy to prevent toxic manifestations of anti-ChE poisoning is to enhance the sequestration of OPs in the circulation by exogenously administered ChEs [9], thereby detoxifying them before they can inhibit acetylcholinesterase (AChE; EC 3.1.1.7) at physiologically important target sites. Recent advances in the purification of both AChE and butyrylcholinesterase (BChE; EC 3.1.1.8) have allowed the use of relatively large quantities of AChE from fetal bovine serum (FBS) [9–12] and equine serum BChE [13] to demonstrate the feasibility of the enzyme-scavenger approach in protecting experimental animals against nerve agents.

Human serum BChE (HuBChE), like other B-esterases, reacts rapidly and irreversibly with a wide range of anti-ChE compounds [14]. BChE may be considered as an endogenous OP scavenger in a manner analogous to that of carboxylesterases (CaE; EC 3.1.1.1) [15]. However, since only a low level of endogenous BChE is available and since the enzyme/OP ratio of the detoxification of OPs by B-esterases is 1:1 [14], it has a limited effect on preventing the toxicity of nerve agents. Despite its distribution in several organ tissues, the physiological importance of BChE and especially that of plasma BChE is not clear. In fact, 50% inhibition of circulating BChE in monkeys [16] or in humans [17] by anti-ChE drugs did not appear to induce any adverse effects. No untoward symptoms have been reported or linked

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‡ Abbreviations: AChE, acetylcholinesterase; ATC, *S*-acetylthiocholine; AUC, area under curve; BChE, butyrylcholinesterase; BTC, *S*-butyrylthiocholine iodide; CaE, carboxylesterase; ChE, cholinesterase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FBS, fetal bovine serum; HuAChE, human acetylcholinesterase; HuBChE, human serum butyrylcholinesterase; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; and OP, organophosphate.

to BChE following single or multiple transfusions of HuBChE-containing blood to human subjects, despite the fact that this enzyme is known to occur in various natural mutant forms [18, 19].

Preliminary study showed that exogenous administration of BChE-containing lyophilized human serum to mice may be a suitable scavenger when used as a prophylactic antidote against *O*-pinacolyl methylphosphonofluoridate (soman) [20]. Based on immunological and physiological compatibility, human plasma BChE appears to have an obvious advantage over non-human enzyme scavengers for enhancing the level of circulating ChEs in human blood. Thus, this study was undertaken to explore the ability of BChE purified from human plasma to confer prophylaxis against nerve agent toxicity. We report here on the *in vitro* and *in vivo* quantitative characterization of the sequestration of soman, *O*-isopropyl methylphosphonofluoridate (sarin), ethyl - *S* - (2 - diisopropylaminoethyl)methylphosphonothiolate (VX), and ethyl (*N*,*N*-dimethylamido)phosphorocyanidate (tabun) by the purified enzyme. Results showed that HuBChE alone was capable of increasing survival and alleviating post-exposure symptoms in both mice and rats challenged with various OPs, without the need for any post-exposure treatment.

MATERIALS AND METHODS

Materials

Organophosphate anti-ChE inhibitors were prepared according to synthetic principles outlined previously: VX [21], soman and sarin [22], tabun [23], and 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) [24]. MEPQ was used as a standard OP to determine the active site concentration of both HuBChE and fetal bovine serum (FBS)-AChE. Purity of neat OPs was evaluated by using (a) ^{1}H- and ^{31}P-NMR spectroscopy, (b) titration of a known amount of FBS-AChE [11], and (c) LD_{50} determinations in mice. The purity of OPs was >95%.

HuBChE and human acetylcholinesterase (HuAChE) were purified in our laboratory from outdated human blood by affinity chromatography procedures (details to be published elsewhere). One milligram of pure HuBChE contained 11 nmol of active site HuBChE. HuAChE was 50% pure and 1 mg contained 8 nmol of active site. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), *S*-butyrylthiocholine iodide (BTC) and *S*-acetylthiocholine (ATC) were obtained from the Aldrich Chemical Co. (Milwaukee, WI).

HuBChE assay

BChE and AChE activities were determined by the method of Ellman *et al.* [25], using BTC and ATC (0.5 mM each) as substrate, respectively. Activity measurements were carried out in a 50-mM phosphate buffer, pH 8.0, at 25–26°. One unit of enzyme activity was defined as the amount required to hydrolyze 1 μmol substrate/min.

Kinetics of inhibition of HuBChE and HuAChE by OPs

Freshly prepared stock solutions of OP inhibitors in water (kept in an ice-water bath) were added to the appropriate enzyme solution (0.3 to 1.0 nM) to a final concentration of 3–200 nM. The inhibition rate was followed by diluting the incubated solution 50–100 times in the assay cuvette and monitoring the residual activity of the enzymes for periods of 1–2 min. Periodically, the concentration of the stock solution of the inhibitor was verified by titrating with FBS-AChE as described elsewhere [11].

In vitro *titration of HuBChE*

To a 0.015 to 0.15 μM active site concentration of HuBChE in 5 mM phosphate buffer, pH 8.0, various amounts of OP inhibitor solution (0.1 to 2 times enzyme equivalents) were added and incubated at 25°. Residual enzyme activity was assayed by the method of Ellman *et al.* as described above. Inhibition was allowed to proceed to completion. The percentage of residual enzyme activity was plotted against the number of equivalents of OPs.

Animals

Male albino ICR mice (20–30 g) and male Sprague–Dawley rats [Charles River (UK), 200–300 g] were housed in a controlled environment with free access to food and water. Their care and maintenance were in accordance with the principles outlined in the "Guide for Care and Use of Laboratory Animals" (NIH publication 85-23, 1985 revision).

Time course of HuBChE in circulation

At various time intervals following the administration of an i.v. (tail vein) or i.m. (thigh muscle) bolus injection of enzyme solution in saline, heparinized blood samples (5–10 μL) were drawn from either the retro-orbital sinus of mice or the tail vein of rats, and diluted 20- to 40-fold in distilled water. HuBChE activity was determined as described above.

In vivo *titration of HuBChE*

Mice and rats were administered intravenously (tail vein) 0.1 to 0.3 mL of a solution of HuBChE in saline containing 750–2700 units of HuBChE (12–45 nmol equivalents of active sites). Approximately 1 to 1.5 hr following the administration of HuBChE, animals were injected with an i.v. bolus solution containing 3–15 nmol OP in 0.1 to 0.3 mL saline. Four to eight successive injections of OPs were administered to each animal at 15-min intervals. Heparinized blood samples (5–10 μL) were drawn and diluted as described above. Blood samples were drawn 2–3 min prior to each injection. Residual activity of HuBChE was plotted against the cumulative dosage of the OP.

Determination of protective ratio in mice

Groups of mice (N = 16/group) were administered HuBChE intravenously. Ten to fifteen minutes later, blood samples were drawn for the determination of circulating HuBChE levels, followed by a single i.v. bolus injection of various doses of OPs in 0.1 to 0.2 mL saline. The 24-hr mortality was recorded and the median lethal dose (LD_{50}) was calculated by the method of Spearman–Karber [see Ref. 26]. No additional therapy was administered to the mice.

Table 1. Second-order rate constants for the inhibition of HuBChE and HuAChE by racemic OPs*

	k_i ($\times 10^7$ M^{-1} min^{-1})			
	HuBChE (serum)		HuAChE (RBC)†	
OP	5 mM	50 mM	5 mM	50 mM
Tabun	0.25	0.47	0.35	0.40
Sarin	0.54	1.23	1.38	1.84
Soman	2.21	5.12	8.75	9.10
VX	1.48	0.82	12.0	3.98
MEPQ	76.0	63.0	142.0	52.0

* Measurements were carried out in 5 and 50 mM phosphate buffer, pH 8.0, at 26°. Results are averages from 5 to 13 determinations. Standard errors from the mean (not shown) were less than 10%.

† Human AChE from red blood cells (3000 U/mg).

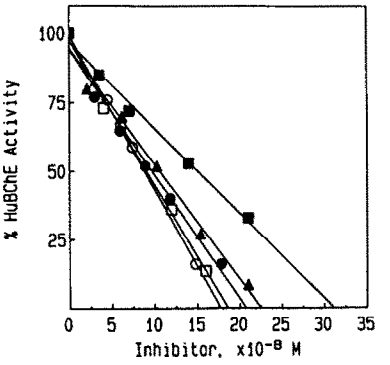

Fig. 1. *In vitro* titration curves of equal concentrations of purified HuBChE (0.175 μM, based on protein assay) by various OPs in 5 mM phosphate buffer, pH 8.0. Inhibition was completed within 30–60 min of incubation at 25°. Data points are average from duplicates (±5%). Key: MEPQ (□); VX (○); sarin (●); soman (▲); tabun (■).

Survivability test and toxic symptoms in rats

Rats (N = 11–12/group) were pretreated i.v. with 0.25 mL of HuBChE in saline, and the blood-enzyme level was determined prior to challenge with OP, as described above for mice. No additional therapy was administered either pre- or post-challenge. Rats were observed continuously for 14 days post exposure for the development of toxic signs and behavioral alterations.

RESULTS

Bimolecular rate constants of the sequestration of OPs by HuBChE

To estimate the ability of HuBChE to sequester OPs *in vivo*, the bimolecular rate constants for the inhibition of HuBChE by sarin, soman, VX, tabun, and MEPQ were determined and compared to data obtained for HuAChE purified from red blood cells. The bimolecular rate constants were calculated as described previously for MEPQ [24] and are summarized in Table 1. The rates of inhibition for sarin and soman suggest that under the same molar concentration HuBChE reacts approximately 1.5-fold slower than HuAChE in 50 mM phosphate buffer. VX, as expected [27], inhibited HuAChE under the same experimental conditions 4- to 5-fold faster than HuBChE. Similar differences in the reactivity of AChE versus BChE from sources other than human have been reported [14, 27, 28]. Values of k_i for tabun varied between 0.25 and 0.47×10^7 M^{-1} min^{-1} for low and high ionic strength, respectively, and thus HuBChE and HuAChE are likely to sequester this OP at similar rates.

Assuming a temperature coefficient $Q_{10} = 2$ for the inhibition of HuBChE by OPs, it is hypothesized that at 37° the corresponding k_i values will vary between 1 and 12×10^7 M^{-1} min^{-1}. It is of interest to compare the kinetic properties of the endogenous scavenger CaE with those of the exogenous HuBChE. The bimolecular rate constants for the inhibition of HuBChE and CaE [15] suggest that an equimolar concentration of the former will sequester tabun, sarin, and soman 5- to 20-fold faster than CaE. VX is a 10^4-fold more potent inhibitor of HuBChE than of CaE. Thus, enhancement of BChE blood level should provide effective protection *in vivo* against toxic OP compounds in a manner analogous to that of a high level of endogenous CaEs in rodents [15]. In view of possible variations in local physiological concentrations of electrolytes at various target sites where HuBChE may act as a scavenger, it is noteworthy that the inhibition of HuBChE by a charged (MEPQ) or a partially charged (VX) OP was slowed at high ionic strength, whereas uncharged OPs (sarin, soman, and tabun) displayed reversed dependence on buffer strength. These observations indicate that electrostatic interactions on the surface of HuBChE may control enzyme activity in a manner analogous to that of AChE [29].

In vitro *titration of HuBChE with OPs*

Results from *in vitro* titration of purified HuBChE with sarin, soman, VX, tabun, and MEPQ are shown in Fig. 1. When the same concentration of enzyme solution was treated with increasing concentrations of either the standard titrant MEPQ [11, 24] or VX, similar titration curves were obtained. The amount of VX and MEPQ required to inhibit 100% of enzyme activity was in good agreement with the active-site concentration of HuBChE estimated from protein assay at 280 nm (0.175 μM). Thus, the two enantiomers of both MEPQ and VX displayed similar rates of inhibition towards HuBChE. Sarin and soman required an excess of 15–25% over the stoichiometric amount needed for MEPQ and VX to inhibit enzymic activity 100%. This may be attributed to different anti-HuBChE activities of the stereoisomers that constitute the racemic mixture of P-F type nerve agents (i.e. sarin and soman) [30, 31]. When the chiral inhibitor tabun was used as a titrant, the molar concentration required to inhibit enzyme activity 100% was approximately 2-fold the active site concentration of HuBChE, suggesting that the

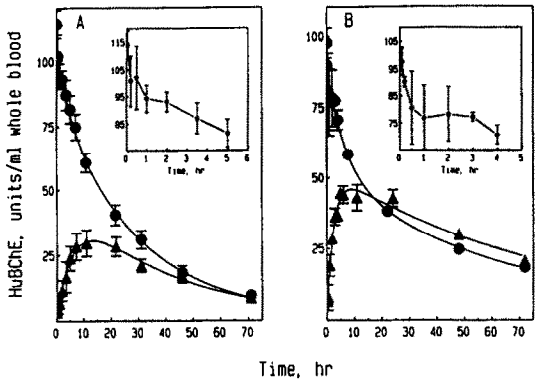

Fig. 2. Average enzyme levels in blood of mice (panel A; N = 4) and rats (panel B; N = 3) following i.v. (●) and i.m. (▲) injections of 13 mg/kg HuBChE. The average endogenous activities of blood BChE (E) of mice and rats were 1.3 and 1.9 U/mL, respectively. Values are means ± SD. Curves were generated by using a bi-exponential decay equation for the i.v. route, U/mL blood = $A \cdot \exp(-k_1 t) + B \cdot \exp(-k_2 t) + E$, and first-order opposing reactions for absorption (ab) and elimination (el) following i.m. injection, U/mL blood = $A \cdot \exp(-k_{el} t) - B \cdot (-k_{ab} t) + E$. Inset: Expansion of the time course between 0 and 5 hr after i.v. injection of HuBChE.

two optical isomers of tabun may inhibit HuBChE at different rates.

Time course of HuBChE in blood of mice and rats

Similar time courses of HuBChE were observed in the blood of mice and rats following injections of the same dose of HuBChE (13 mg/kg) (Fig. 2). The activity of the enzyme injected i.v. could be accounted for in the blood of both species, assuming a blood volume of 7.5% of body weight. A rapid distribution of 10–20% was seen immediately following i.v. injection of HuBChE. Then a relatively constant level was maintained for 1–2 hr in mice and 1–3 hr in rats, followed by a slow reduction of enzyme activity (Fig. 2, insets). Blood-HuBChE concentration remained significantly higher than the endogenous level of BChE even 72 hr after i.v. injection of HuBChE. The time course of blood-HuBChE levels following an i.m. injection of HuBChE suggests that a peak blood-enzyme level was obtained between 9 and 13 hr after the administration, and was 30–40% of the maximum concentration obtained following the i.v. injection of the same dose of HuBChE. This level was maintained constant for another several hours and declined thereafter at a rate similar to that observed after an i.v. injection. Significant blood-HuBChE levels were observed 72 hr after an i.m. injection.

Results in Table 2 show that the fractions of HuBChE dose that were absorbed following an i.m. injection in mice and rats were 0.54 and 0.9, respectively, of the administered dose [AUC(i.m.)/AUC(i.v.)]. Thus, despite its high molecular weight (360,000) HuBChE could be absorbed effectively via the i.m. route.

Throughout this study, no untoward side effects were observed in mice or in rats following either i.m. or the i.v. injections of as high as 15 mg/kg of purified HuBChE.

In vivo *titration of HuBChE*

Mice. Figure 3 depicts the *in vivo* titration curves of exogenously administered HuBChE (initial blood level, 10.3 to 12.4 nmol HuBChE/animal) by various OPs in mice. All OPs were administered in four to six sequential i.v. injections within 1–2 hr post loading of HuBChE, thus ensuring minimal loss of enzyme activity due to clearance from the circulation. Results for soman, sarin, VX, and tabun showed a linear relationship between HuBChE residual activity in blood and the cumulative dose of the nerve agent. In a few cases after the first 75% of the inhibition of blood-BChE activity, the titration curve tended to set a curvature. This may be attributed to the relatively low molar concentration of HuBChE that could not compete with the rate of either the escape of the OP from the blood or its detoxification by endogenous scavengers. For all cases studied, complete inhibition of circulating HuBChE required the administration of an excess of the nerve agent over the predicted values. The prediction was based on the blood concentration of the exogenously administered HuBChE taken together with the endogenous ability of naive mice to detoxify OPs. The latter is a quantity that can be approximated by the following i.v. LD_{50} values (nmol/mouse): sarin, 18; soman, 9.5 VX, 1.5; and tabun, 42. However, since the anti-ChE titrant was not administered by bolus but rather by sequential injections, the corrected LD_{50} was determined by injection of a near median lethal dose divided into four injections and administered in 10- to 15-min intervals. The amount of OP required to produce 50% mortality was found to increase by 30–50% compared with the LD_{50} of a single i.v. bolus injection. Thus, the *in vivo* stoichiometry was predicted by adding the modified LD_{50} (nmol/animal) in naive animals to the HuBChE blood-level (nmol/animal) prior to exposure. The latter value was normalized in accordance with the stoichiometry of the inhibition obtained by *in vitro* titrations.

The following calculated stoichiometry values for the *in vivo* titrations were compared with the data extrapolated from Fig. 3. (nmol OP/mouse needed to inhibit 100% circulating HuBChE): *tabun*, found: 90, calcd: 83; *sarin*, found: 42, calcd: 40; *soman*, found: 35, calcd: 28; *VX*, found: 15, calcd: 13. Thus, the amount of OP needed to inhibit circulating HuBChE was found to be in reasonable agreement with the predicted values, thereby substantiating the suggested biochemical mechanisms underlying the *in vivo* sequestration of nerve agents by HuBChE.

In all cases studied, the manifestation of toxic symptoms (tremors, fasciculations) occurred after the fourth or fifth injection. At this point the residual activity of circulating HuBChE was 2–3 nmol/mouse (i.e. <25% of its initial activity). Apparently, this enzyme level could not detoxify efficiently the next administered dose which was 2.25, 8.6, 6.7 and 12.4 nmol/mouse for VX, sarin, soman and tabun, respectively.

Of particular interest is the finding that individual

Table 2. Pharmacokinetic constants (hr^{-1}) calculated from the time course obtained following i.v. and i.m. injections of HuBChE*

Animal	Injec. route	k_1	k_2	k_{ab}	k_{el}	t_{max} (hr)	AUC†
Mouse	i.v.	0.18 (0.16)	0.033 (0.007)				1.00
	i.m.			0.17 (0.02)	0.027 (0.003)	12.8	0.54
Rat	i.v.	0.14 (0.09)	0.015 (0.008)				1.00
	i.m.			0.39 (0.05)	0.013 (0.002)	9.0	0.90

* Calculations of k_1 and k_2, and of k_{ab} and k_{el} were based on the equations used to construct best-fitted curves (Fig. 2); t_{max} was calculated according to Gibaldi and Perrier [32]. Figures in parentheses are SEM. HuBChE 13 mg/kg/animal (N = 3–4, each route).

† Ratio between area under the i.m. concentration–time curve (AUC) and AUC under the i.v. curve obtained for same dose of HuBChE.

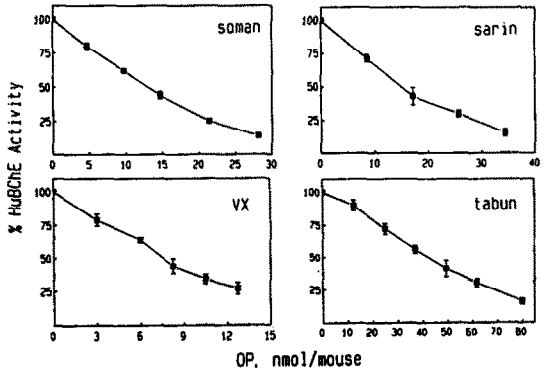

Fig. 3. *In vivo* titration of exogenously administered HuBChE in blood of mice by sequential injections of nerve agents. Results are averages from four mice. Error bars (±SD) are shown only when SD > 3. Blood levels of HuBChE prior to initiation of the titration were (nmol/mouse ± SD): soman, 11.1 ± 0.24; sarin, 11.6 ± 0.28; VX, 10.3 ± 0.48; and tabun, 12.4 ± 0.32.

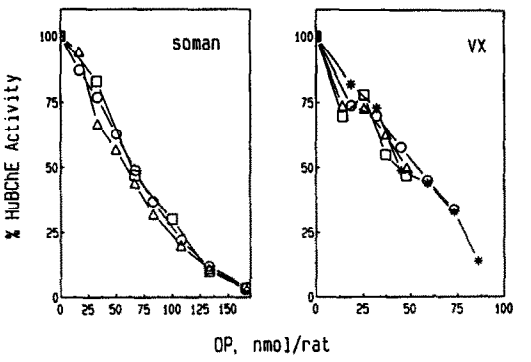

Fig. 4. Individual titration curves of HuBChE in blood of rats. Initial enzyme levels prior to injection of sequential doses of nerve agents ranged between 35 and 41 nmol HuBChE/rat for soman and 70 and 75 nmol HuBChE for VX.

variations in the residual levels of HuBChE in the blood of mice after each administration of the titrant were very small (SD varied less than 6.5% of the mean, N = 4).

Rats. Rats pretreated i.v. with HuBChE were administered with a total dose of approximately 160 nmol soman in eight consecutive i.v. injections. Titration began when enzyme-blood level was 35–41 nmol/animal. Results are depicted in Fig. 4 (left panel). As shown for mice, a linear relationship was obtained between the HuBChE residual activity in the blood of rats and the cumulative dose of soman. Toxic signs of OP intoxication were manifested in HuBChE-treated rats only after the administration of a cumulative dose of 133 nmol soman/animal (seventh injection). None of the animals died after the administration of the eighth injection (total of approximately 2 × LD_{50}). This dose inhibited more than 95% of the activity of the exogenous enzyme.

The i.v. LD_{50} of a bolus injection of soman in non-protected rats used for the *in vivo* titrations was 80 nmol/rat. As described above for mice, it was assumed that the LD_{50} of sequential administration of soman is approximately 100 nmol/rat. This figure taken together with the blood-level of HuBChE normalized to the stoichiometry of soman sequestration by HuBChE predicted a total protection against approximately 150 nmol/rat. The latter value is in good agreement with the total dose of soman actually needed to inhibit circulating HuBChE in rats (160 nmol/rat).

The right panel of Fig. 4 depicts the titration curves obtained for rats containing 70–75 nmol HuBChE/animal prior to injection of sequential doses of VX. Approximately 100 nmol of VX appeared to be required to inhibit more than 95% of HuBChE activity. Since the i.v. LD_{50} of VX administered in sequential doses is estimated to be 18 nmol/rat (1.2-fold the LD_{50} obtained by an i.v. bolus injection of VX), the total dosage of VX

Table 3. Protective ratio conferred by HuBChE against sarin, soman VX and tabun in mice

OP	LD_{50} (i.v.)* (μg/kg)	Protective ratio†		Time§ (sec)
		Observed	Calculated‡	
Sarin	137 (124–152)‖	1.6	1.4	2.2
Soman	124 (112–137)	2.1	1.8	0.6
	133¶ (117–150)	2.4	2.0	0.5
VX	76 (68–84)	4.9	4.8	12.0
Tabun	365 (329–405)	1.8	1.4	1.4

* Determined in animals (N = 16 each group) containing an average of 400 ± 20 U/mouse (0.6 mg or approximately 6.6 nmol/mouse).

† LD_{50} in HuBChE-treated animals divided by LD_{50} in unprotected mice.

‡ For the method of calculation see Results.

§ Time required to decrease 95% of OP levels to a concentration produced by 1 × LD_{50} dose *in vivo*. Estimated for 37° on the basis of k_i at 26° (Table 1), and assuming a $Q_{10} = 2$ for the inhibition of HuBChE by OPs. Time was calculated according to Gordon *et al.* [33]. For details, see Discussion and Table 5.

‖ 95% confidence limits.

¶ Determined in mice 16 hr after i.m. injection of 2200 U HuBChE/mouse. Average blood-HuBChE prior to injection of soman was 475 ± 13 U or 7.9 nmol/mouse.

needed to inhibit 70–75 nmol HuBChE was calculated to be 88–93 nmol. This value is reasonably close to the observed ones (i.e. 100 nmol). Manifestation of toxic symptoms occurred after the third or fourth injection of VX; however, animals returned to normal within several hours. As noted in mice, individual variations in the residual levels of HuBChE throughout the titration curve were very small.

Determination of protective ratio in mice

Table 3 summarizes the results for i.v. LD_{50} of sarin, soman, VX, and tabun in HuBChE-pretreated mice. In all cases, the mice had similar levels of HuBChE in their blood (0.6 mg or 6.6 nmol/mouse) prior to the exposure to the challenge. To estimate the time of OP sequestration *in vivo*, the level of HuBChE was monitored between 0.5 and 10 min post-injection of either soman or VX (not shown). The inhibition of enzyme activity was nearly complete within the first 60 sec after administration of the OPs. Calculation of the protective ratio was based on blood levels of HuBChE, on the stoichiometry established from the *in vitro* titrations and on the bolus i.v. LD_{50} in naive mice. The observed protective ratios were found to be in reasonable agreement with the calculated values. Similar results were obtained in mice pretreated i.m. with HuBChE and challenged with soman 16 hr after loading with the enzyme.

Results shown in Table 3 suggest that despite differences in both the initial molar concentration of the OPs and in the bimolecular rate constants of inhibition of the enzyme, HuBChE was able to rapidly sequester all nerve agents. The short time required for the completion of 95% of the reaction between circulating HuBChE and the OPs (Table 3) is consistent with the observed rate of loss of enzyme activity after injection of the challenge.

Survivability and toxic symptoms in HuBChE-treated rats exposed to soman and VX

Soman. Table 4 summarizes the results from two experiments in which 10–15 min after the administration of approximately 4.5 mg (50 nmol HuBChE/rat), animals were challenged with an i.v. bolus injection of a 1.5 × LD_{50} dose of soman (95 nmol/rat). The average blood level of HuBChE prior to injection of soman was 43 and 47 nmol/animal.

Results from experiments A and B show that nearly full protection was conferred to HuBChE-treated rats against soman toxicity. Eight rats (out of nineteen animals) displayed no toxic symptoms. Nine rats showed mild tremors and transient salivation for up to 2 hr. One rat had convulsions that diminished after 3 hr, and one rat died. The level of sequestration of soman in HuBChE-containing blood was 22–30 nmol/rat. This value was calculated from the decrease in blood level of HuBChE 10 min after the exposure to the challenge.

Despite the adequate protection conferred by pretreatment with 43 nmol HuBChE alone (Table 4, Expt. A), it appears that the dosage of HuBChE (or HuBChE to soman ratio) required to completely prevent post-challenge symptoms should be slightly higher. Indeed, an increase of the ratio of HuBChE/soman from 0.44 (Expt. A) to 0.50 (Expt. B) was accompanied by a marked increase in the relative number of sign-free rats after soman exposure.

A third group of rats (N = 11) was injected i.v. with an increasing amount of HuBChE (16–52) nmol/rat) and challenged i.v. with a fixed dose of soman (97 nmol/rat). The amount of soman sequestered by the enzyme (calculated from the observed loss in blood-HuBChE) was plotted against the level of circulating HuBChE prior to the injection of the challenge (Fig. 5). One active site HuBChE detoxified 0.5 mol soman *in vivo*, whereas 1.2 mol soman was required to inhibit 100% enzyme activity *in vitro*. This difference can be attributed to either the rapid transfer of soman from the blood or its detoxification by endogenous scavengers or to both. The linear correlation obtained throughout the entire range of HuBChE concentration supports the hypothesis that the extent of the *in vivo* sequestration of nerve agents can be reasonably approximated on the basis of the HuBChE activity in the blood before administration of the challenge.

It should be pointed out that even though the calculated residual amount of soman (66–72 nmol/rat) exceeded its LD_{50} value (65 nmol/rat), only one out of nineteen rats died (Table 4). This finding suggests that the protection observed cannot be explained solely by the direct sequestration of soman by HuBChE in the blood. A similar indication was obtained from protection experiments in mice that demonstrated consistently higher protective ratios

Table 4. Survivability and toxic symptoms in rats pretreated with 50 nmol HuBChE prior to i.v. challenge with soman*

Expt.	N	HuBChE (nmol/rat)	Soman (nmol/rat)		Symptoms‡			
			Injected	Residual†	None	+	++	Died
A	13	43.1 ± 0.6	96.5 ± 0.5	66.6 ± 0.8	3	8	1	1
B	6	47.1 ± 3.2	94.5 ± 0.4	72.0 ± 2.5	5	1		

* LD_{50} (i.v.) = 46.5 (43.1 to 50.1 at 95% confidence limits) μg/kg or 65 nmol/rat. HuBChE and soman values are expressed as means ± SEM.

† Calculated from the amount of HuBChE loss at 10 min following exposure to soman, and the stoichiometry determined from *in vitro* titration.

‡ Observation continued for 14 days: +, light tremors, returned to normal within 3 hr; ++, convulsions, diminished after 3 hr.

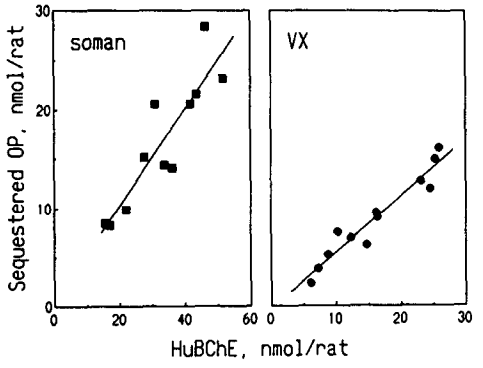

Fig. 5. Correlation between the amount of OP sequestered and the level of HuBChE in rats before an i.v. injection of a fixed dose of the challenge (soman, 97 nmol/rat; VX, 22 nmol/rat). Lines were obtained by least squares linear regression. Soman, slope = 0.49 ± 0.08 (N = 11 r^2 = 0.80). VX, slope = 0.57 ± 0.05 (N = 12 r^2 = 0.93).

than the calculated values (Table 3). It is possible that a non-vascular depot(s) of HuBChE also participates in the detoxification of OPs. More experiments will be required to substantiate such a possibility.

VX. A fixed dose of VX (1.4 × LD_{50}) was injected i.v. into a group of twelve rats pretreated with an increasing amount of HuBChE. As can be seen in Table 5, the ratio of HuBChE/VX calculated in sign-free rats following an i.v. challenge of the OP was approximately 1.1. In comparison, when rats were challenged with 1.5 × LD_{50} of soman, a HuBChE/soman value of 0.5 was found to be sufficient to confer full protection (Table 4), despite the fact that a dose of 1 × LD_{50} soman contains 65 nmol/rat compared with 16 nmol/rat for VX. The early manifestation of OP-poisoning symptoms observed for VX relative to soman during the *in vivo* titrations is consistent with these observations. Thus, differences in the bimolecular rate constants of the sequestration of HuBChE, the pharmacokinetic properties of VX and soman, and/or their detoxification by naturally occurring scavengers could account for the differences observed between HuBChE/VX and HuBChE/soman ratios needed to confer complete protection in rats.

Results shown in Table 5 suggest that in order to completely alleviate post-exposure symptoms in rats challenged i.v. with 1.4 × LD_{50} of VX, HuBChE should be able to reduce 22 nmol VX/rat to a residual level below 10 nmol VX/rat (animals 9–12). This is consistent with the sign-free dose of VX in naive rats (unpublished results). Finally, as demonstrated for soman, a linear correlation was obtained when the amount of VX sequestered in blood (based on loss of HuBChE activity; Table 5) was plotted against the blood concentration of HuBChE prior to exposure to VX (Fig. 5).

DISCUSSION

The time course of the activity of exogenously administered purified HuBChE in mice and rats provided similar concentration–time dependence curves. These were consistent with the time course reported for HuBChE-containing lyophilized human serum [20] and for FBS-AChE following an i.v. administration in mice [11] and monkeys [12]. BChE from equine serum has also been demonstrated to be stable in the blood of rhesus monkeys [13]. The apparent half-life of purified HuBChE in the blood of rodents ranged between 20 and 45 hr. It is of interest to point out that the half-life of HuBChE after blood transfusion in two human subjects, homozygous for the "silent" type of HuBChE, was reported to be 10 days [34] and 3.5 days [35]. Thus, the stability of exogenously administered HuBChE in experimental animals, particularly those with a high metabolic rate (e.g. mice and rats), is considerably lower than might be expected in humans. In contrast, AChE purified from either human erythrocytes (HuBChE) or *Torpedo californica* (both membrane-bound proteins) cleared from the circulation of mice more than 50-fold faster than HuBChE (unpublished data). The relatively high stability of non-membranal soluble forms of either BChE or AChE from plasma sources may be attributed to the number and structure of their sialylated carbohydrate residues [36].

The initial decline in enzyme activity immediately

Table 5. Survivability and toxic symptoms in rats pretreated with increasing amounts of HuBChE prior to i.v. challenge with a fixed dose of VX

Rat No.	VX* (nmol/rat)	HuBChE (nmol/rat) Before exposure	HuBChE (nmol/rat) After exposure	HuBChE/VX	Time† (sec)	Symptoms‡
1	22.0	6.1	3.7	0.28	1.2	+++
2	21.3	7.3	3.4	0.34	2.0	++
3	22.0	8.8	3.5	0.40	2.5	++
4	21.6	10.3	2.7	0.48	3.9	+
5	22.5	12.3	5.3	0.55	2.4	+
6	21.6	14.7	8.4	0.68	1.6	+
7	21.4	16.2	6.6	0.76	2.9	+
8	22.0	16.4	7.3	0.75	2.5	+
9	21.8	23.1	10.3	1.06	3.0	−
10	21.6	24.5	12.5	1.13	2.4	−
11	22.3	25.3	10.3	1.13	3.7	−
12	22.3	25.9	9.8	1.16	4.4	−

* LD_{50} (i.v.) = 16.8 (16.4 to 17.9 at 95% confidence limits) μg/kg or 16 nmol/rat.

† Time required to complete the observed loss of HuBChE following i.v. injection of VX was calculated according to Gordon *et al.* [33] using the following equation:

$$t = (k_i VX_0[1 - E_0/VX_0])^{-1} \cdot \ln[(1 - FE_0/VX_0)/(1 - F)].$$

F is the fraction of HuBChE (E) inhibited by VX. Molar concentrations were calculated assuming 17 mL of blood/rat and t was estimated using $k_i = 1.97 \times 10^7\ M^{-1}\ min^{-1}$ at 37°.

‡ Ranking of post-exposure symptoms: (+++) convulsions and severe fasciculations; (++) moderate fasciculations, tremors and ataxia; animals returned to normal 3–24 hr after exposure to challenge; (+) light fasciculations, tremors, and ataxia; returned to normal within 3 hr; and (−) no clinical symptoms.

after its i.v. injection ($t_{0.5}$, 3.8 to 4.9 hr; see k_1 values, Table 2) may be due to an equilibration process between the blood and another compartment(s). Based on the second-order bimolecular rate constants for the inhibition of HuBChE (k_i) and the rapid loss of blood-HuBChE after exposure to the challenge, it appears that all four nerve agents were scavenged extremely rapidly by blood containing HuBChE. Therefore, HuBChE that may have been distributed into another compartment(s) could not be re-distributed into the plasma fast enough to compete effectively with the rate of transfer of the challenge from blood to target organs. However, the possibility that HuBChE in a non-vascular compartment(s) participates in the detoxification of nerve agents cannot be ruled out. More experiments will be required to assess the relative contribution of non-vascular HuBChE to the overall detoxification of OPs. In addition to its relative stability in the blood, HuBChE was shown to be absorbed into the circulation following i.m. injection in two species. This finding, taken together with the results from protection experiments in mice pretreated i.m. with HuBChE, suggests that the i.m. route is likely to produce significant therapeutic levels of HuBChE for a relatively long period of time even after a single administration of the enzyme.

The initial concentrations of HuBChE and the challenge *in vivo* were at near stoichiometry (micromolar range); therefore, the velocity of the detoxification of the OPs by the enzyme can be approximated by the product of $[HuBChE]_0$ $[OP]_0 k_i$. An increase in this expression reduces the time of sequestration. Thus, for some nerve agents the relatively high molar concentration ($[OP]_0$) produced in the blood by a less potent inhibitor of the scavenger could compensate, within certain limits, for the low k_i value. The successful protection against tabun, which is 10 times less potent an inhibitor than soman but has an equitoxic dose in mice that is 3.5-fold higher (Table 3), is consistent with this hypothesis. In contrast to tabun, the $[OP]_0$ for VX was 2.4-fold less than that of soman (Table 3). Despite the relatively low molar concentration of VX and its low bimolecular rate constant [k_i (soman)/k_i(VX) = 6], reasonable protection was conferred in HuBChE-treated animals against multiple toxic doses of VX. Thus, the k_i values of the sequestration of various nerve agents by HuBChE are sufficiently high to minimize the effects of possible pharmacokinetic differences among nerve agents on the antidotal efficacy of the enzyme. This qualifies HuBChE as a universal scavenging antidote against OPs.

To establish further the hypothesis that HuBChE detoxifies OPs *in vivo* extremely rapidly, a modification of the standard bimolecular second-order rate equation [33] was used to estimate the time required to complete the reaction between circulating HuBChE and the concentration of OP produced *in vivo*. Assuming a homogeneous distribution of both reactants in blood at $t = 0$, molar concentrations were normalized to a blood volume of 7.5% of body weight. Indeed, calculations suggest that the sequestration time of all nerve agents in both species should be completed within 5 sec at 37°.

Only in one case (VX in mice, Table 3) was more time (12 sec), required to complete the reaction between the OP and circulating HuBChE. In addition, the residual amount of HuBChE following an i.v. bolus injection of the challenge was relatively high in all cases studied. These observations suggest that nerve agents may be removed rapidly from the blood into other compartments and/or detoxified rapidly by endogenous blood constituents. Dedrick *et al.* [37] estimated the time of blood circulation in mice and rats to range between 8 and 13 sec. Thus, it appeared that in order to confer protection against lethality, nerve agents had to be scavenged to a level below their LD_{50} dose within less than one blood circulation time. These estimates are consistent with previous findings showing that more than 98% of the toxic isomers of soman were "cleared" from the blood within 1 min after administration of $1 \times LD_{50}$ soman in anesthetized Wistar rats [38].

In general, linear correlations were established between the HuBChE residual activity and either the amount of nerve agents used to construct the *in vitro* and *in vivo* titration curves or the dose administered in a single i.v. bolus injection. Although k_i values for inhibition of HuBChE varied 10-fold, the level of protection observed in either i.v. or i.m. HuBChE-treated animals could be predicted from the stoichiometry of the sequestration and the concentration of the circulating exogenous enzyme alone. Since the high rate of sequestration of nerve agents by HuBChE is expected to underlie the activity of the scavenger in other species as well, a reliable extrapolation of its efficacy from experimental animals to humans will be permitted.

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