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BUTYRYLCHOLINESTERASE AND ACETYLCHOLINESTERASE PROPHYLAXIS AGAINST SOMAN POISONING IN MICE

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Abstract—Human butyrylcholinesterase (BChE, EC 3.1.1.8) or acetylcholinesterase (AChE, EC 3.1.1.7) from fetal bovine serum (FBS), administered i.v. in mice, sequestered at approximately 1:1 stoichiometry the highly toxic anti-ChE organophosphate, 1,2,2-trimethylpropyl methylfluorophosphonate (soman). A quantitative linear correlation was demonstrated between blood-ChE levels and the protection conferred by exogeneously administered ChE. Results presented here demonstrate that either human BChE or FBS-AChE is an effective prophylactic measure sufficient to protect mice from multiple LD_{50}s of soman without the administration of post-treatment supportive drugs.

Treatment of acute intoxication by organophosphorus (OP‖) poisons, especially soman, presents a most difficult medical problem, both in terms of mortality, delayed morbidity and the low index for quality of life among survivors [1, 2]. High mortality rate and late toxicity signs persist despite various pre- and post-exposure treatment modalities (e.g. carbamates, anti-muscarinics, anti-convulsants and oxime reactivators). Thus, rapid sequestration of OPs in the circulation by pretreatment with an exogenously administered scavenger, such as acetylcholinesterase (AChE), has the potential advantage of being a single drug completely adequate to confer protection that will be free of performance-related effects induced by all OPs. Indeed, recent studies have substantiated the potential efficacy of AChE as a single drug pretreatment which protects mice against organophosphorus poisoning. Wolfe *et al.* [3] and Raveh *et al.* [4] reported that AChE from fetal bovine serum (FBS) could protect mice from lethal doses ($3–8 \times LD_{50}$) of the highly toxic OPs, ethyl - *S* - 2 - (*N,N* - diisopropylaminoethyl)-methylphosphonotiolate (VX) and 7-(methyl-ethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ) respectively. However, when soman was used as an OP, only limited protection against an even lower dose ($2 \times LD_{50}$) was obtained in mice; these results may partially be attributed to the experimental protocol in which mice were challenged with soman 22 hr following i.p. pretreatment with FBS-AChE [3].

To further evaluate the concept of cholinesterase prophylaxis against OPs relative to currently used multiple drug therapy, it will be useful to obtain basic data with regard to the ability of the preadministered scavenger to display universal antidotal efficacy towards all types of hazardous OPs. Since the lipophilicity and pharmacokinetic properties of soman are likely to differ from VX and MEPQ, it was important to determine the extent to which pretreatment with a scavenging esterase could confer protection against soman poisoning.

In this report we present the results in mice of *in vivo* titration of both human BChE and FBS-AChE with soman. It is further demonstrated that both esterases could protect mice from multiple LD_{50}s of soman without the administration of any supportive drug, and that such protection could be correlated with the blood-level of exogenously administered enzyme. Since this study provides the first report on protection against OPs by pretreatment with human BChE, study of this enzyme was extended to include protection experiments against the quaternary nitrogen-containing OP, MEPQ [4].

MATERIALS AND METHODS

Materials. Soman was prepared according to the procedure of Monard and Quinchon for the synthesis of isopropyl methylfluorophosphonate [5]. MEPQ was prepared as described before [6]. Stock solutions of soman (20 mg/mL in propylene glycol) and MEPQ (10 mg/mL in acetone) were kept at −20°, and diluted in 0.9% saline to the desired concentration. The toxicity of the diluted solutions was determined prior to each experiment by performing a dose–response study in mice, and calculating the i.v. LD_{50} of OPs.

Lyophilized human serum BChE [*ca.* 7% (w/w) BChE] was purchased from Behring Institute (FRG) and was used as such. Stock solutions (600–

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‡ Posthumous.

‖ Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; FBS, fetal bovine serum; MEPQ, 7-(methylethoxyphosphinyloxy) - 1 - methylquinolinium iodide; OP, organophosphate(s); and soman, 1,2,2-trimethylpropyl methylfluorophosphonate.

3000 units/mL) were prepared in 0.9% saline, and enzyme concentration was determined by a titration with MEPQ [6], in accordance with the residual activity method described before [4]. The specific activity of human BChE was approximately 60 units/nmol of active site. FBS-AChE was purified as previously described [7]. The specific activity of FBS-AChE was 400 units/nmol active site.

Animals. Adult male ICR mice weighing 28–30 g were used throughout this study. They were housed, ten in a cage, in a temperature-controlled (20 ± 1°) environment with 12-hr light/dark cycles and free access to food and water. Their care and maintenance were in accordance with the principles enunciated in the "Guide for Care and Use of Laboratory Animals" (NIH Publication No. 85-23, 1985 revision).

AChE and BChE assays. Heparinized blood samples (5–10 μL) were drawn from the retro-orbital sinus and diluted 10- to 20-fold into distilled water. AChE and BChE activities were determined by the method of Ellman *et al.* [8] using acetylthiocholine and butyrylthiocholine as substrates respectively.

Kinetics of inhibition. Bimolecular rate constants for the inhibition of AChE and BChE were determined according to a previously described method [6].

In vivo *titration.* The titration of human BChE and FBS-AChE with soman was carried out according to Raveh *et al.* [4]. Mice were administered (i.v.) either human BChE or FBS-AChE (100–1200 units in 0.15–0.20 mL saline). This was followed 2–4 hr later by repeated 1–2 nmol bolus injections (i.v.) of either soman or MEPQ. Three to six consecutive injections were administered to each mouse at 10- to 15-min intervals. Residual blood ChE activity was determined as described above, 2 min prior to each challenge. The amount of enzyme per mouse was calculated assuming an average blood volume of 7.5% of body weight [9].

Determination of protective ratio. Groups of mice (twelve mice each) were administered (i.v.) various doses of either human BChE or FBS-AChE. Ten to fifteen minutes later, blood samples were drawn for determination of enzyme activity followed by a single i.v. bolus injection of 0.10–0.20 mL of either soman or MEPQ. The 24-hr mortality was determined, and the median lethal dose (LD_{50}) was calculated by the method of Weil [10]. No additional supporting therapy was given to the mice throughout this study.

RESULTS

Bimolecular rate constants and toxicity of soman and MEPQ. The bimolecular rate constants for the inhibition of FBS-AChE and human BChE by either MEPQ or soman are summarized in Table 1. The high rates of inhibition of both esterases by soman and MEPQ enabled rapid completion of the sequestration of these OPs under the experimental conditions employed. Thus, the high toxicity of both MEPQ and soman (Table 1) along with the high bimolecular rate constants permitted the use of relatively small quantities of BChE and AChE per mouse.

In vitro *titrations of human BChE and FBS-AChE.* When the same concentration of BChE solution was incubated with an increasing amount of either soman or MEPQ, different titration curves were observed (Fig. 1A). The molar concentration required for 100% inhibition of BChE or FBS-AChE by soman was approximately 1.5- and 2.0-fold higher, respectively, than the amount required for total inhibition of the same enzyme solution by MEPQ. This observation is consistent with a previous report [11] demonstrating that the ratio of the rate of inhibition of horse serum BChE by the four enantiomers of soman is 1:0.075:0.075:0.003 whereas this ratio for inhibition of bovine erythrocyte AChE is 1:0.23:<0.0001:0.0001. Both enantiomers of MEPQ appear to display a similar rate of inhibition towards human BChE, namely a stoichiometric amount of racemic MEPQ reacts with BChE. A similar observation was reported for the inhibition of eel [6] and FBS-AChE [4] by MEPQ.

BChE blood time–course profile. To determine the optimal conditions for *in vivo* titration of human BChE by either soman or MEPQ, approximately 0.2 mL of enzyme solution containing 3–12 nmol BChE was administered to mice (i.v. or i.p.) and blood-enzyme levels were monitored over a period of 90 hr (data not shown). When human BChE was injected (i.p.), the peak blood-enzyme level was observed after 2 hr. The clearance of human BChE after an i.v. administration was similar to the clearance observed following an i.p. injection. The time–course profiles for BChE are similar to the ones reported for FBS-AChE [4]. The biological half-life of both esterases in mouse circulation is approximately 24 hr. Also, blood levels of both enzymes remained constant between 2 and 4 hr after either i.p. or i.v. injections. Thus, the titration of either human BChE or FBS-AChE with soman or MEPQ was performed between 2 and 4 hr following enzyme administration.

In vivo *titration of BChE and AChE by soman and MEPQ.* Results for *in vivo* titration of BChE with MEPQ (Fig. 2) were essentially similar to the titration curves reported for *in vivo* sequestration of MEPQ by FBS-AChE [4]. An almost linear relationship between BChE residual activity and MEPQ dose was observed. Furthermore, it appeared that in order to completely inhibit the exogenously administered BChE an addition of approximately 2 nmol MEPQ/mouse in excess of the stoichiometric amount of circulating BChE was required. This observation is consistent with the i.v. LD_{50} of MEPQ in untreated mice (2.2 nmol/mouse, Table 1).

The *in vivo* titration of either human BChE or FBS-AChE with soman (Fig. 2) closely resembles the titration of these enzymes with MEPQ. For example, cumulative doses of 20 nmol of soman were sequestered by approximately 8 nmol of human BChE (see Fig. 2, 75% inhibition of 10.2 nmol BChE). The stoichiometry predicted from the *in vitro* titration of BChE by soman (0.65:1), together with the toxicity of soman in untreated mice (9.1 nmol/mouse), suggests that 8 nmol of BChE should sequester *ca.* 22 nmol soman/mouse (8:0.65 + 9.1), a value which is in close agreement with the observed amount. However, complete inhibition of exogenous BChE by soman (Fig. 2)

Table 1. Bimolecular rate constants and toxicity of soman and MEPQ in mice

OP	k_i^* ($M^{-1}\,min^{-1}$)		LD_{50} (i.v.)	
	FBS-AChE	Human BChE	μg/kg	nmol/mouse
Soman	9.1×10^7	7.2×10^7	54 (45–60)‡	9.1
MEPQ	2.0×10^8	3.4×10^8	30 (28–32)	2.2

* 0.05 M phosphate buffer, pH 8.0, 25°.
† Average from three measurements. Estimated error: <15%.
‡ 95% Confidence limits.

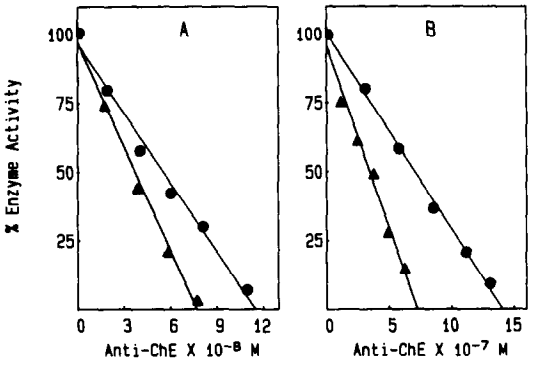

Fig. 1. *In vitro* titration of human BChE (A, 4.5 units/mL) and FBS-AChE (B, 280 units/mL) with anti-ChE OP. To the same amount of enzyme solution in 2 mM phosphate buffer (pH 8.0) increasing amounts of MEPQ (▲) or soman (●) were added and incubated until no changes in enzyme residual activity were observed. Completion of the inhibition reaction occurred between 5 and 30 min of incubation.

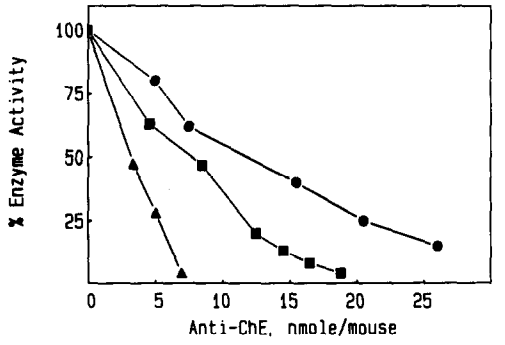

Fig. 2. *In vivo* titration of blood-ChE in mice administered BChE or FBS-AChE intravenously. Results are averages from three mice per titration. Key: (▲) BChE (5.16 nmol/mouse) titrated with MEPQ; (●)BChE (10.2 nmol/mouse) titrated with soman; and (■) FBS-AChE (6.2 nmol/mouse) titrated with soman. Titrations were initiated 2–4 hr after the administration of enzyme.

will require an additional amount of soman over the stoichiometry predicted from the combination of *in vitro* titration and soman detoxification in untreated mice. FBS-AChE also sequestered soman *in vivo* as one would expect from *in vitro* stoichiometry. For

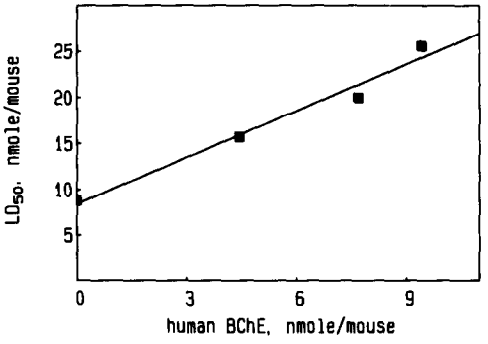

Fig. 3. Correlation between i.v. LD_{50} of soman in mice and blood levels of exogenously administered (i.v.) human BChE. Each dose group contained twelve mice. Line equation ($LD_{50} = 1.68 \times [BChE] + 8.62$) was calculated by the method of linear regression ($r^2 = 0.97$). The LD_{50} of soman in unprotected mice was found to be 9.1 nmol/mouse.

example, 90% of 6.2 nmol FBS-AChE (i.e. *ca.* 5.5 nmol active site) neutralized *in vivo* a cumulative dose of approximately 17 nmol soman (Fig. 2) compared to a calculated value of 20.1 nmol soman ($5.5 \times 2 + 9.1$). The shape of the *in vivo* titration curve in this particular case suggests that a slight excess (over the calculated value) of soman will be required to neutralize completely circulating FBS-AChE.

Protection experiments. The intravenous LD_{50} of soman in unprotected mice (Table 1) was found to be 54 μg/kg (9.1 nmol/mouse weighing 30 g). Pretreatment with 4.2, 7.5 and 9.2 nmol BChE/mouse increased the LD_{50} of soman to 93, 126 and 143 μg/kg respectively. Figure 3 demonstrates the high correlation between i.v. toxicity of soman and blood-BChE levels expressed in nanomoles per mouse. The results in Table 2 show that almost full protection occurred at a BChE/soman ratio of approximately 0.5. After applying a correction for detoxification of soman by naive mice (approximated by the LD_{50} value of soman in unprotected mice, i.e. 9.1 nmol soman/mouse), the stoichiometric ratio of BChE to soman required to confer protection was approximately 1.0.

Although the protective ratio, i.e. complete LD_{50} of MEPQ in BChE-treated mice, was not determined, four mice pretreated with 5.16 nmol BChE/mouse could be protected against three consecutive (30-min intervals) doses of 3.36, 1.68 and 1.92 nmol

Table 2. Correlation between BChE/soman ratio and survival of mice pretreated with human BChE and challenged i.v. with soman*

Mode of calculation†	BChE/soman (nmol ratio)	Survival ratio‡
I	0.19–0.29	4/11
	0.30–0.39	2/9
	0.40–0.44	8/11
	0.45–0.49	8/8
II	0.30–0.49	0/6
	0.50–0.69	7/14
	0.70–0.84	5/8
	0.85–1.10	10/11

* Correlation between BChE/soman ratio and survival of mice was statistically significant at $P < 0.01$.

† (I) Nanomoles BChE per mouse divided by total amount (nanomoles) of i.v. administered soman. (II) Nanomoles BChE per mouse divided by corrected amount of soman dose (total amount of i.v. administered soman less 9.1 nmol which corresponds to 1 LD_{50} of soman in unprotected mice).

‡ Survivors (24-hr observation) over number of animals in each group. Data were collected from protective ratio experiments.

MEPQ/mouse (total of 6.96 nmol MEPQ or $3.2 \times LD_{50}$). However, when a group of similarly pretreated mice (N = 5) were challenged i.v. with 6.8 nmol MEPQ/mouse ($3.1 \times LD_{50}$) in a single bolus injection, only two mice survived.

The LD_{50} (i.v.) of soman in a group of mice (N = 12) increased from 54 (45–60, at 95% confidence limit) μg/kg in naive mice to 130 (116–144) μg/kg in mice pretreated with a single dose of 7.3 nmol FBS-AChE per mouse. Since only half of the racemic mixture of soman is sequestered by FBS-AChE, a dose-level of 7.3 nmol/mouse FBS-AChE is predicted to provide a protective ratio of 2.6 [(7.3 × 2 + 9.1):9.1], which is in close agreement with the observed increase in the LD_{50}.

DISCUSSION

The results obtained in this study show that 1 mole of either human BChE or FBS-AChE was sufficient to neutralize in mice approximately 1 mole of soman and MEPQ, potent anti-ChE OPs. Furthermore, it was demonstrated that exogenously administered BChE or AChE is an effective prophylactic measure sufficient to protect mice from multiple LD_{50}s of the highly toxic soman without the administration of supportive drugs. In view of the immunogenic properties of ChEs [12], we considered the possibility of immune response which could influence the interpretation of the data with respect to the protection conferred by the administered enzymes. A single injection of either esterase did not show any clinical symptoms during an observation period of 48 hr. Furthermore, the availability of the enzyme for reacting with the OP challenge 2–4 hr following the administration of the exogeneous esterases suggests that untoward immunogenic response, if it exists at all, did not affect severely the stoichiometry of the protection afforded by either ChE.

When a high dose of either soman or MEPQ was administered in an i.v. bolus injection to mice pretreated with either human BChE or FBS-AChE, protection afforded by the exogenous esterase was slightly but definitely less effective than the same amount of OPs administered in three to five consecutive doses. This observation suggests that protection of animals against OP poisoning with scavenger prophylaxis depends, among other factors, on the rate of its *in vivo* sequestration by the circulating esterase and also the pharmacokinetic properties of the OP challenge. For example, although both soman [13] and MEPQ [4] hydrolyze very rapidly in mouse plasma, unlike soman the amount of MEPQ neutralized by BChE could be accounted for on the bais of *in vitro* titration profiles and its detoxification by naive mice. Since MEPQ distribution is limited to peripheral tissues [4], it may be argued that it is more available to the circulating esterases, compared to the lipid-soluble soman. It is anticipated that the progressive inhibition of exogenously administered BChE or AChE in mice by repeated administration of OPs will eventually decrease the apparent rate of the scavenging reaction; however, in the case of the lipid-soluble soman such a decrease may allow more OP to escape the scavenger and reach other active target sites which are capable of interacting with soman. Thus, for different OPs a varying enzyme : OP ratio in the circulation may be necessary to confer the same protective ratio in OP-challenged animals.

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