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Characterization of human serum butyrylcholinesterase in rhesus monkeys: Behavioral and physiological effects

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ABSTRACT

The effects of a large dose of human serum butyrylcholinesterase (HuBChE) were evaluated in rhesus monkeys using a serial-probe recognition (SPR) task designed to assess attention and short-term memory. Each monkey received an intravenous injection of 150 mg (105,000 U or 30 mg/kg) of HuBChE 60 min prior to testing on the SPR task. Concurrent with the cognitive–behavioral assessment, blood was collected at various time points throughout the study and was analyzed for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities, anti-BChE antibody production and gross clinical pathology (i.e., complete blood count and clinical chemistry panel). HuBChE revealed a peak blood activity of 227 U/ml at 5 min after intravenous injection and a mean residence time of approximately 72 h. No cognitive–behavioral decrements of any kind in SPR performance and no toxic signs in clinical pathology were detected in any of the blood assays during the 5 weeks of observation. Anti-HuBChE antibodies peaked at about 14 days after injection, with no concomitant behavioral changes. These results demonstrate the behavioral and physiological safety of HuBChE in rhesus monkeys and support its development as a bioscavenger for the prophylaxis of chemical warfare agent toxicity in humans.

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1. Introduction

Organophosphorus (OP) chemical warfare nerve agents, such as soman, sarin, tabun, and VX, are extremely toxic compounds that pose a serious threat as a possible weapon of mass destruction. These agents produce their toxic effects by irreversibly inhibiting acetylcholinesterase (AChE), leading to acetylcholine (ACh) accumulation at synaptic sites and hyperactivity of the cholinergic system (Fonnum and Guttormsen, 1969; Shih, 1982; Taylor, 1990). Left untreated, nerve agent poisoning results in a cholinergic crisis characterized by miosis, bradycardia, fasciculations, convulsions, hypersecretion, and possibly death due to respiratory failure (Dunn and Sidell, 1989; Shih, 1982). The current regimen for OP intoxication consists of a combination of compounds including pretreatment with pyridostigmine bromide, followed by post-exposure treatment with atropine and 2-PAM chloride (Doctor et al., 1991; Lenz et al., 2001; Wilson and Ginsburg, 1955). This therapy effectively prevents lethality, however, it fails to provide complete protection and these drugs alone produce untoward side effects (Castro et al., 1992; Leadbeater et al., 1985; McDonough et al., 1989; McDonough and Shih, 1997). The goal of preventing OP injury and lethality while eliminating adverse behavioral side effects stimulated the search for new pretreatment drugs.

Enzymes like cholinesterases (ChEs) were identified as potential prophylactic bioscavengers that could sequester highly toxic OPs before they reach physiological targets thus protecting against in vivo toxicity and post-exposure incapacitation (Allon et al., 1998; Ashani et al., 1991; Broomfield et al., 1991; Doctor et al., 2001; Doctor et al., 1991; Maxwell et al., 1992; Raveh et al., 1989; Raveh et al., 1997; Wolfe et al., 1992; Wolfe et al., 1987). Several of these studies in rodents and non-human primates demonstrated that a clear advantage of using this strategy over the current multiple-drug regimen is that a single pretreatment may provide more than adequate protection against OP intoxication. Because bioscavengers have to be used prophylactically, it was critical to demonstrate that these enzymes did not display undesirable side effects. Thus, several studies assessed behavioral and physiological effects of equine serum (Eq) BChE alone in the absence of nerve agent exposure (for review see Doctor et al., 2001; Lenz et al., 2001). Genovese and Doctor (1995) administered EqBChE to rats that were subsequently tested in a battery of behavioral tasks including passive avoidance,

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locomotor activity, and a variable-interval schedule of reinforcement. No significant alterations in behavior were observed in these tasks following exposure to EqBChE. Using rhesus monkeys (*Macaca mulatta*), Matzke et al. (1999) and Broomfield et al. (1991) failed to observe any decrements in cognitive–behavioral performance on a six-item serial probe recognition (SPR) task, a test of attention and short-term memory, following exposure to EqBChE. Performance on a primate equilibrium platform was similarly unimpaired in rhesus monkeys that were administered EqBChE and challenged with soman, despite cumulative soman challenges equivalent to three times the LD_{50} (Wolfe et al., 1992).

Similar studies were conducted to address the safety and efficacy of HuBChE in mice (Ashani et al., 1991), rats (Brandeis et al., 1993), and rhesus monkeys (Raveh et al., 1997). Brandeis et al. (1993) reported that HuBChE protected rats against soman exposure and demonstrated that spatial memory, as assessed by a Morris water maze at 1 week post-treatment, was unaffected by exogenous HuBChE pretreatment. Raveh et al. (1997) examined spatial discrimination in rhesus macaques that were exposed to both HuBChE and VX or soman. Subjects that showed an enzyme to OP ratio near or over 1:1 displayed no/mild signs of toxicity, which largely recovered by the next day. For HuBChE safety specifically, four rhesus macaques that were exposed to either 13 (10,400 U) or 34 mg (27,200 U) of HuBChE did not display any observable deficits. More recently, the behavioral safety of a large dose of HuBChE alone, physostigmine alone, and HuBChE pretreatment followed by physostigmine challenge, were evaluated in mice by Clark et al. (2005). They exposed mice to 2000 U of HuBChE by intraperitoneal injection followed by an assessment of acoustic startle reflex (ASR) and prepulse inhibition (PPI) and demonstrated that a 600-fold increase in circulating blood BChE activity did not alter ASR or PPI behavior. As expected, mice that received physostigmine (0.4 mg/kg) by subcutaneous injection, showed significant decreases in the amplitude of ASR to 50 ms, 120 dB noise pulses. In addition, the time to peak and the amount of PPI of the ASR were significantly increased. This effect was partially mitigated in mice that were pretreated with HuBChE.

In this study, we examined the neurobehavioral effects of a large (150 mg; 105,000 U) dose of HuBChE on trial completion, choice accuracy, and choice latency in a six-item SPR task in rhesus macaques. This procedure has been demonstrated to be a sensitive and reliable assessment of cognitive–behavioral functioning (e.g., attention and memory) following challenges by drugs distressing the cholinergic system (Castro, 1995; Castro, 1997; Myers et al., 2002). To more fully and comprehensively characterize the safety of HuBChE in rhesus monkeys, we analyzed blood samples throughout the study to determine the pharmacokinetics of BChE in blood, the production of anti-BChE antibody, and the manifestation of any clinical blood pathology resulting from HuBChE administration. The absence of cognitive–behavioral decrements of any kind in SPR performance and any obvious signs of clinical pathology in any of the blood assays provide strong support for the behavioral and physiological safety of HuBChE in rhesus monkeys.

2. Materials and methods

This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the WRAIR/NMRC Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

2.1. Materials

All reagent grade chemicals including acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), sodium phosphate, ethopropazine hydrochloride, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). HuBChE was isolated from Cohn fraction IV-4 paste (Saxena et al., 2010) and stored in lyophilized form at −20 °C.

2.2. Subjects

Four adult female rhesus monkeys (*M. mulatta*) weighing between 4.3 and 4.9 kg served as subjects. All monkeys were experimentally naïve with respect to the behavioral procedures and apparatus prior to the start of the study. The monkeys were housed individually in stainless steel squeeze-back cages (61 cm W × 71 cm D × 86 cm H) with free access to tap water. Certified primate rations (Purina Mills, Inc., St. Louis, MO) supplemented with daily fresh fruits and vegetables were provided no sooner than 30 min after the daily session to maintain desired body weights at approximately 90% of free-feeding weight. The colony was maintained on a 12-h light/dark cycle with no twilight (lights on at 0600 h) and at 20–22 °C with a relative humidity of 50% (±15%), using at least 10 complete air changes per hour of 100% conditioned fresh air. The sessions began at approximately 1100 h and were conducted during the light part of the cycle, 5 days per week (Monday–Friday).

2.3. Cognitive–behavioral apparatus

The subjects were tested unrestrained in their home cages. A 35.6 cm (14 in.) capacitive touch screen monitor (GoldStar StudioWorks, model GLD 45I, Microtouch Systems, Inc., Methuen, MA) was attached to the front wall of each cage, with the center of the screen located 38.9 cm above the chamber floor. Because screen touches are difficult to execute around the screen's perimeter, the effective area of the screen was reduced by 1.5 cm on all four sides. Grain-based banana-flavored food pellets (750 mg, Bio-Serv Inc., Frenchtown, NJ) were delivered by a pellet dispenser (BRS/LVE Model QNB-400 1) into a food cup (7.9 × 10.8 × 7.6 cm) positioned in the front of the test chamber, accessible through an aperture (7.6 cm wide × 5.4 cm high) centered 15.1 cm below the lower edge of the touch screen and 11.6 cm above the chamber floor. A computer, running a custom-written Visual Basic® 6.0 routine, was used to control experimental events and collect all data.

2.4. Serial-probe recognition (SPR) procedure

Each daily session consisted of 240 trials. On each trial, 6 unique sample stimuli (list items) were presented sequentially, separated by a 0.5-s interstimulus interval (ISI) during which the screen was blank. Each list item was a compound stimulus comprised of two superimposed, randomly selected ASCII characters of different size and color. The individual characters ranged from about 0.3 to 2.7 cm in length and 0.3 to 2.7 cm in width. Because the same ASCII character could be selected for a particular sample stimulus, one character was 15% smaller than the other and was offset slightly above and to the left of the other to avoid perfect overlap and to achieve a greater diversity of compound sample stimuli. The RGB color saturation of each ASCII character ranged from 0 to 255. To exclude extremely dark characters but not true colors, at least one of the three saturation levels had to exceed 79. Each list stimulus was displayed in the top-center portion of the screen, about 13.5 cm from the left edge of the screen and about 4 cm from the top of the screen to the center of the stimulus. Each list item was presented for 4 s or until it was touched (with the restriction that a minimum of 0.5 s had elapsed), at which point it was terminated and the ISI was initiated. After presentation of the sixth sample stimulus, the screen was blank throughout the 1-s probe delay (retention interval) that preceded the choice period. During the 15-s choice period a probe stimulus was displayed in the lower-left or lower-right portion of the screen, and a standard or default stimulus (a 6.6-cm white square) was presented

in the other portion of the screen, with equal frequencies of presentation on both sides. The probe item was a compound stimulus that matched a list item on half of all trials (120/240). Across these "matching" trials, probe items matched list items at each of the six serial positions with equal frequency (20 at each serial position). On matching trials, touching the probe stimulus was considered correct. In contrast, on "non-matching" trials the probe stimulus was not among those listed (novel) and touching the default stimulus was considered correct. A correct choice response immediately produced the conditioned reinforcer (a white screen for 0.25 s) each time and a food pellet 50% of the time, determined randomly by the computer. Touching the opposite stimulus was considered incorrect. Choice periods that elapsed without a response ended after 15 s and were considered incorrect. A 5-s intertrial interval (or ITI, during which the screen was blank) separated each trial, regardless of whether a choice was correct or incorrect. A response during the ITI reset the interval, although few such responses occurred. Prior to initiating assessment of HuBChE, all animals achieved a criterion of stability on accuracy and choice reaction time. The overall accuracy and median choice reaction time for each of 10 consecutive sessions did not deviate more than 15% from the grand mean of all 10 sessions.

2.5. Pharmacological procedures

Monkeys were trained to sit passively in a collar-restraint chair (Primate Products, Inc., Woodside, CA) and present either leg for blood collection/injection procedures. HuBChE (150 mg, ~105,000 U) was dissolved in 5.5 ml of phosphate buffered saline (PBS). SPR testing began exactly 60 min after enzyme administration. The enzyme injection was delivered into the saphenous vein of one leg and all blood samples were collected from the saphenous vein of the other leg to avoid any possible overestimation of circulating HuBChE. The pre-injection (0-time) baseline blood sample was collected 1 week prior to enzyme administration. Post-injection blood samples were taken at 5 and 45 min, at 3.5, 7, 24, 48, and 72 h, and at 1, 2, 3, and 4 weeks following enzyme injection. For all monkeys, a vehicle injection (5.5 ml PBS) was given 5 days prior to enzyme administration and on day 29 (696 h) post enzyme injection. A few days after the second vehicle control, physostigmine hemisulfate (i.e., eserine hemisulfate, Sigma Chemical Co., St. Louis, MO, USA), a pharmacological control to demonstrate sensitivity of the behavioral test to disruption, was injected intramuscularly at a dose of 0.07 mg/kg, 15 min before SPR testing.

2.6. Assay for AChE and BChE activities

BChE activity in purified samples was measured spectrophotometrically at 22 °C by monitoring the change in absorbance at 412 nm, as described (Ellman et al., 1961). All blood samples were diluted 20-fold with water and analyzed for AChE and BChE activities using 1 mM ATC and 1 mM BTC as substrates, respectively. As whole blood samples contain both AChE and BChE activities, samples were incubated with 20 μM ethopropazine, a selective BChE inhibitor, for 20 min, for the determination of AChE activity (Naik et al., 2012; Worek et al., 1999). No AChE inhibitor was applied for the measurement of BChE activity. The formation of product was followed by monitoring an increase in absorbance at 412 nm at 22 °C in the presence of 1 mM DTNB. Activity was reported as U/ml, where 1 U represents 1 μmole of ATC or BTC hydrolyzed per min.

2.7. Analysis of pharmacokinetic parameters

Pharmacokinetic parameters were calculated using a Windows-based computer software for noncompartmental analysis of pharmacokinetic data (Laub and Gallo, 1996). The area under the blood BChE concentration vs time curve (AUC) was calculated by the trapezoidal method. Terminal half-life ($T_{1/2}$) was calculated as $T_{1/2} = \ln(2)/\text{terminal slope}$. C_{max} was defined as the maximum concentration of BChE activity in blood. Mean residence time (MRT) = AUMC/AUC, where AUMC is area under the first-moment curve.

2.8. Assay for anti-BChE antibody

The presence of anti-HuBChE antibodies was examined by ELISA, using 0.2 U of HuBChE per well as the plate-coating antigen and a 1:200 dilution of blood (Maxwell et al., 1992; Rosenberg et al., 2002). Briefly, wells of a 96-well plate were coated with 100 μl HuBChE solution in PBS by an overnight incubation at 22 °C. The plates were washed with PBS containing 0.05% Tween-20 (washing buffer), and the wells were blocked with 250 μl of 1% BSA in PBS (blocking buffer) for 2 h at 22 °C. After washing the plates three times with washing buffer, 100 μl each of 5-fold serial dilutions (ranging from 1:200 to 1:125,000) of monkey blood in PBS containing 0.1% BSA and 0.05% Tween-20 (dilution buffer) were added and incubated overnight at 22 °C. The plates were washed three times with washing buffer. One hundred microliters of horseradish peroxidase (HRP)-conjugated goat anti-monkey IgG (H + L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD), diluted 1:1000 in dilution buffer, was added to each well and incubated for 1 h at 22 °C. After three washes with washing buffer, 100 μl of 2,2′-azino-bis-(3-benzthiazoline-6-sulfonic acid) (ABTS) substrate solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and incubated for 1 h in the dark at 22 °C. The absorbance was measured at 405 nm, and antibody concentrations were calculated from the standard curves. Standard curves were generated from an ELISA run in parallel by coating the plate with various amounts of purified monkey IgG and detecting the bound IgG by HRP-conjugated goat anti-monkey IgG.

2.9. Analysis of hematology and serum chemistry parameters

Hematology parameters (complete blood count, CBC) were determined using an ABX Pentra 60 (ABX Diagnostics, Irving CA). All specimens were tested unaltered using flow cytometry technology. Serum chemistry profiles (CHEM20) were determined using a Johnson & Johnson Vitros 250 Chemistry System (Ortho Clinical Diagnostics, Raritan, NJ). Serum collected from each centrifuged specimen was analyzed using colorimetric and point-rate technology. Blood samples subjected to the CHEM 20 and CBC studies were analyzed by three experts (an experienced clinical pathologist, a medical doctor, and a primate veterinarian) who searched for any signs of possible pathology.

2.10. Subjective evaluation procedures

Throughout the study, all monkeys were observed daily before and after each behavioral session for any clinical signs of toxicity such as lethargy, vomiting, abnormal breathing, hyperactivity, etc. No such signs were observed at any time throughout the study.

2.11. Data analysis

A repeated-measures analysis of variance (ANOVA) was used to evaluate each dependent variable, and when a significant overall effect was observed, Tukey's HSD test was utilized to make post-hoc comparisons. A significance level of $p < .05$ was used for all tests.

3. Results

3.1. Physiological safety of HuBChE

The time-course of circulating BChE activity is shown in Fig. 1A. Mean baseline BChE activity at 0-min equaled 4.3 U/ml. At 5-min post injection, the mean level of BChE in blood was elevated to 227 U/ml. After this peak at 5 min, BChE levels started declining and reached baseline levels by 2 weeks post injection. The inset in Fig. 1A shows BChE levels across the first 24 h period. Using a Windows-based program

for non-compartmental analysis of pharmacokinetic data (Laub and Gallo, 1996), the following parameters were calculated: MRT = 72.1 ± 4.1 h, C_{max} = 227 ± 24 U/ml, $T_{1/2}$ = 22.4 ± 4.1 h, and AUC = 16,080 ± 2180 U h/ml. Fig. 1B shows circulating AChE activity following administration of a large dose of HuBChE. Increases in baseline AChE activity (25–50%) were observed in blood samples taken during the first 3.5 h following HuBChE administration in all animals, which returned to baseline levels by 48 h. Fig. 1C shows anti-HuBChE antibody levels in the blood of monkeys following administration of HuBChE. Mean IgG levels approximated 25 ng/ml for up to 72 h after injection, and then rose to about 50 ng/ml by the end of first week. During the second week, levels continued to rise and peaked at 100 ng/ml. These IgG levels were maintained for the next two weeks.

Tables 1 and 2 show results from the serum chemistry and hematology analyses, respectively. For the most part, values were within the expected and normal ranges for adult female rhesus monkeys and did not show any robust or consistent changes following administration of HuBChE. For neutrophils (NEU # and NEU %), mean levels were slightly elevated in the second and third weeks after HuBChE injection and mean lymphocyte (LYM # and LYM %) levels were slightly elevated at 72 h after injection. However, these measures were characterized by considerable variability (high standard deviations

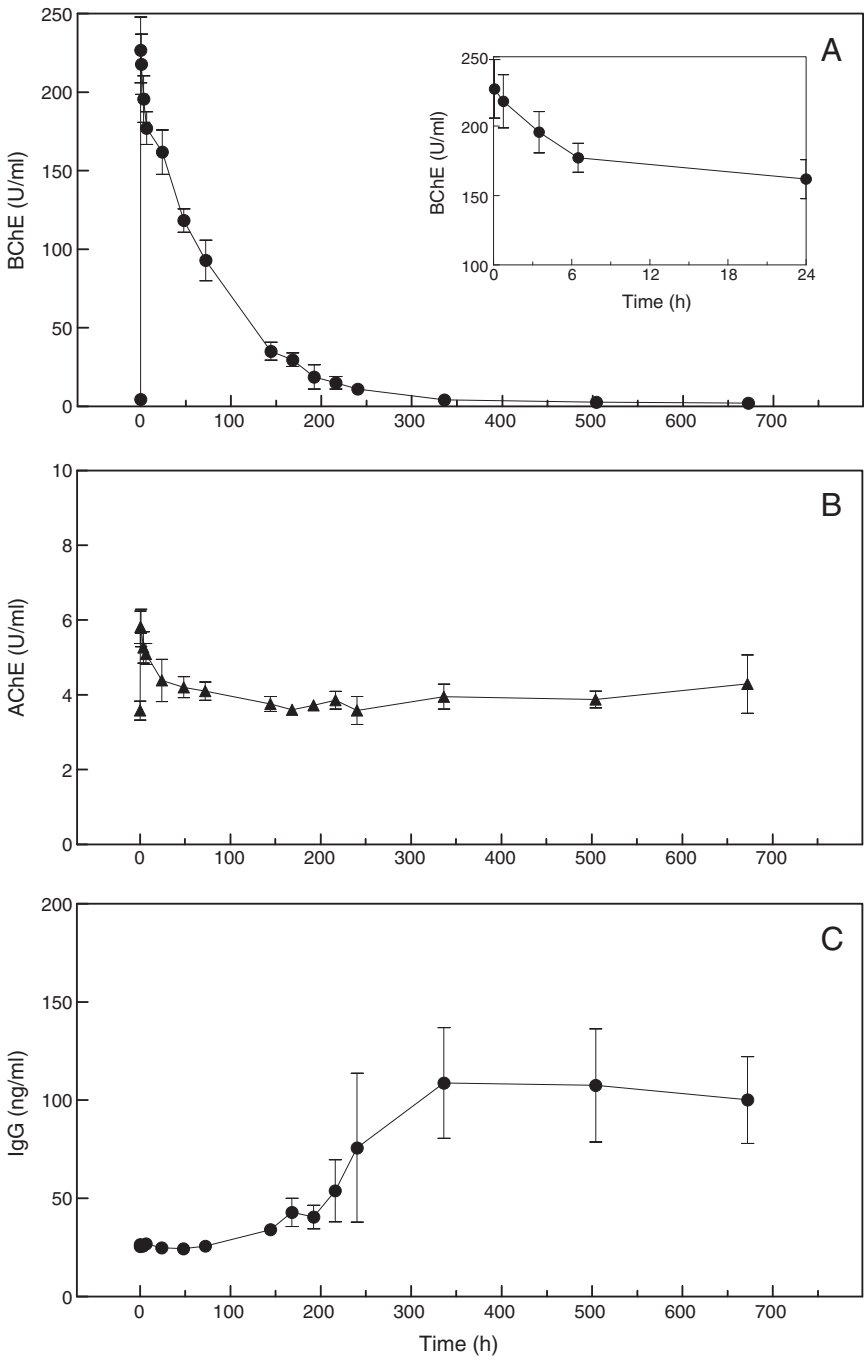

Fig. 1. Time courses of ChE activity and antibody levels in the blood of macaques following administration of HuBChE. Four rhesus macaques were administered HuBChE (30 mg/kg) by i.v. bolus injection. Blood samples were collected at various time intervals and assayed for AChE and BChE activities as described (Naik et al., 2012; Worek et al., 1999). Circulating BChE activity (U/ml) is shown in panel A. The inset shows the same data for the first 24 h. Circulating AChE activity (U/ml) is shown in panel B. The presence of anti-HuBChE antibodies in the blood of macaques was determined by ELISA (panel C). Values in each panel are mean ± SEM.

relative to the mean) and all mean values fell well within the normal physiological ranges, casting doubt on the putative biological relevance of the observed mean changes across time.

3.2. Behavioral safety of HuBChE

Rhesus macaques were trained on a 6-item SPR task prior to administration of HuBChE. Fig. 2 summarizes critical aspects of SPR performance for the entire duration of the experiment. The three measures of performance shown are the number of trials completed (top panel), accuracy (center panel), and choice response latency (bottom panel). Values represent the mean of each daily session for all four monkeys. The first vehicle injection is indicated by V and post HuBChE data are indicated by solid symbols, beginning at 1 h. It should be noted that a second saline vehicle injection was given on day 29 (696 h after enzyme injection). The farthest right data point within each panel represents performance on the day physostigmine was injected at a dose of 0.07 mg/kg (indicated by a triangle). For accuracy and choice latency, only trials in which a choice response was made were included in the analysis. A repeated-measures analysis of variance (ANOVA) was conducted separately on each dependent variable and Tukey's HSD test was utilized to make post-hoc comparisons between time points when a significant overall effect of time was observed.

The mean number of trials completed was consistently high and near the maximum of 240 throughout the experiment, but equaled only 88.5 on the day of physostigmine administration. A significant main effect of time was observed, $F_{(24,72)} = 27.412$, $p < .05$. Post-hoc comparisons revealed that the number of trials completed following physostigmine injection was significantly lower than all other sessions. No other significant differences between sessions were observed. Accuracy was approximately .838 for all sessions except following physostigmine administration, which equaled .666. A significant main effect of time was observed, $F_{(24,72)} = 2.042$, $p < .05$. Post-hoc comparisons revealed that accuracy following physostigmine injection was significantly lower than all other sessions. No other significant differences between sessions were observed. Mean choice response latencies were consistent for each subject, but differed considerably between subjects. Therefore, these latency data were analyzed and plotted as a proportion of the mean latency of each subject on the first vehicle day. A value equivalent to the vehicle performance would equal 1.0. Proportional latencies were near 1.0 for all sessions except on the day physostigmine was injected when it equaled 1.71 (a relative increase of 71% from vehicle). A significant main effect of time was observed, $F_{(23,69)} = 2.9135$, $p < .05$. Post-hoc comparisons revealed that the proportional choice latency following physostigmine injection was significantly higher than all other sessions except those at 384, 552, 648, 672, and 696 h after injection. Although a slight increase in mean proportional latency was observed across the last few sessions of the experiment, this increase was not significant compared to earlier sessions and resulted largely from increased response latencies for one monkey. No other significant differences between sessions were observed. Thus, for all three performance measures, no statistically significant differences were observed between the first and second vehicle injections or between the vehicle and enzyme injections. In contrast, performance following physostigmine administration differed significantly from the first vehicle administration for all three measures and differed significantly from the second vehicle administration for number of trials completed and accuracy, but not proportional choice latency.

4. Discussion

HuBChE is currently the most viable candidate enzyme for use as a bioscavenger in humans because of the extensive research program showing its efficacy and safety in a variety of species. The present experiment is an important part of that program because of the choice of species (rhesus monkey), the large dose of HuBChE administered, and use of a sensitive neurobehavioral test with concomitant measures of pharmacokinetics, clinical chemistry, and antibody production. It should be noted that ongoing research efforts to advance recombinant human AChE as a bioscavenger of poisonous OP compounds are in progress and this approach may also hold considerable promise in the future (Cohen et al., 2006; Evron et al., 2007). Because the major use of a bioscavenger is prophylactic, i.e., the bioscavenger will be administered days or weeks prior to a potential exposure, it is essential that the enzyme be devoid of undesirable effects. The safety of HuBChE was previously assessed in mice (Ashani et al., 1991), rats

Table 1
Hematological profiles of rhesus monkeys following HuBChE administration.

Parameters (reference range)	0 h	24 h	48 h	72 h	1 wk	2 wk	3 wk	4 wk
RBC (4.63–6.50 $10^6/mm^3$)	5.63 ± 0.37	5.65 ± 0.61	5.47 ± 0.43	5.41 ± 0.51	4.94 ± 0.83	5.12 ± 0.48	5.03 ± 0.60	5.31 ± 0.36
HGB (10.7–15.4 g/dl)	13.1 ± 1.2	13.3 ± 1.7	12.9 ± 1.3	12.8 ± 1.4	11.6 ± 2.0	12.2 ± 1.2	12.0 ± 1.5	12.8 ± 0.8
HCT (33.1–47.3%)	38.2 ± 3.6	39.1 ± 7.0	38.5 ± 5.0	37.7 ± 5.6	33.7 ± 5.5	35.9 ± 3.5	35.2 ± 4.2	37.3 ± 2.2
MCV (66–78 μm^3)	67.8 ± 2.1	69.8 ± 4.3	70.3 ± 4.0	69.5 ± 4.4	68.3 ± 2.5	70.3 ± 1.3	70.0 ± 1.4	70.5 ± 1.0
MCH (21.3–25.8 pg)	23.3 ± 0.6	23.6 ± 0.6	23.6 ± 0.6	23.7 ± 0.7	23.4 ± 0.6	23.8 ± 0.3	23.8 ± 0.9	24.2 ± 0.9
MCHC (31.7–33.4 g/dl)	34.3 ± 0.3	33.8 ± 1.4	33.6 ± 0.9	34.1 ± 1.2	34.3 ± 0.5	33.8 ± 0.7	34.0 ± 0.7	34.4 ± 1
RDW (11.1–14.1%)	13.7 ± 0.7	12.8 ± 1.8	13.0 ± 1.9	12.7 ± 1.7	13.5 ± 0.9	13.1 ± 0.5	13.1 ± 0.4	13.0 ± 0.5
PLT (200–510 $10^3/mm^3$)	272 ± 102	229 ± 75	278 ± 96	171 ± 78	236 ± 74	272 ± 63	208 ± 79	240 ± 111
MPV (7.6–10.9 mm^3)	9.4 ± 0.5	9.8 ± 0.8	9.2 ± 0.8	9.3 ± 2.0	9.1 ± 1.0	9.3 ± 0.9	9.6 ± 0.5	9.6 ± 0.9
PCT (0.19–0.42%)	0.23 ± 0.09	0.22 ± 0.06	0.25 ± 0.08	0.17 ± 0.08	0.21 ± 0.05	0.25 ± 0.04	0.20 ± 0.07	0.23 ± 0.11
PDW (8.9–18.8%)	15.4 ± 2.3	16.9 ± 4.2	14.8 ± 3.5	17.7 ± 3.7	14.5 ± 4.1	14.8 ± 2.4	15.9 ± 1.9	15.8 ± 3.3
NEU # (1.04–10.2 $10^3/mm^3$)	1.61 ± 0.96	1.60 ± 0.51	1.31 ± 0.26	1.15 ± 0.35	1.85 ± 0.68	2.37 ± 1.60	2.42 ± 1.64	2.32 ± 1.94
LYM # (0.87–4.50 $10^3/mm^3$)	1.49 ± 0.76	1.77 ± 0.64	1.88 ± 0.82	1.96 ± 0.78	1.18 ± 0.41	1.53 ± 0.25	1.45 ± 0.35	1.75 ± 0.52
MON # (0.07–0.46 $10^3/mm^3$)	0.12 ± 0.06	0.22 ± 0.10	0.18 ± 0.07	0.12 ± 0.06	0.14 ± 0.03	0.16 ± 0.06	0.17 ± 0.06	0.16 ± 0.12
EOS # (0.03–0.30 $10^3/mm^3$)	0.04 ± 0.010	0.06 ± 0.05	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.05 ± 0.02	0.05 ± 0.03
BAS # (0.00–0.07 $10^3/mm^3$)	0.02 ± 0.00	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.06	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02
ALY # (0.00–0.10 $10^3/mm^3$)	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.02
NEU% (26.0–81.0)	47.8 ± 20.7	44.1 ± 8.2	39.7 ± 9.5	35.6 ± 10.8	56.5 ± 14.9	53.8 ± 13.2	54.7 ± 16.3	48.9 ± 11.5
LYM% (14.0–64.0)	46.3 ± 18.8	47.8 ± 6.0	53.3 ± 8.9	59.1 ± 11.0	36.9 ± 12.9	40.9 ± 11.7	39.2 ± 14.8	45.7 ± 11.4
MON% (1.0–7.5)	4.2 ± 3.0	5.9 ± 1.5	5.1 ± 0.8	3.7 ± 1.5	4.4 ± 1.4	4.2 ± 1.8	4.4 ± 2.3	3.7 ± 1.1
EOS% (0.5–6.0)	1.2 ± 0.3	1.5 ± 0.9	1.2 ± 0.5	1.0 ± 0.5	1.0 ± 0.4	0.6 ± 0.3	1.1 ± 0.3	1.3 ± 0.3
BAS% (0.0–0.8)	0.6 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	1.3 ± 1.8	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.2
ALY% (0.0–1.8)	0.5 ± 0.4	0.6 ± 0.2	0.8 ± 0.3	0.7 ± 0.3	0.5 ± 0.1	0.5 ± 0.2	0.7 ± 0.3	0.6 ± 0.3
LIC% (0.0–2.1)	0.6 ± 0.5	0.9 ± 0.9	0.4 ± 0.3	0.8 ± 0.9	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.2	0.4 ± 0.3

Values are MEAN ± SD. RBC: red blood cell count; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; PLT: platelet count; MPV: mean platelet volume; PCT: plateletcrit; PDW: platelet volume distribution width; NEU: neutrophils; LYM: lymphocytes; MON: monocytes; EOS: eosinophils; BAS: basophils; ALY: atypical lymphocytes; LIC: large immature cells.

Table 2
Serum chemistry profiles of rhesus monkeys following HuBChE administration.

Parameters (reference range)	0 h	24 h	48 h	72 h	1 wk	2 wk	3 wk	4 wk
Metabolism and renal function								
BUN (11–27 mg/dl)	16 ± 0	16 ± 1	14 ± 2	14 ± 1	17 ± 2	16 ± 2	16 ± 2	16 ± 2
Calcium (8.7–10.5 mg/dl)	9.7 ± 1.0	10.3 ± 0.6	9.8 ± 0.8	10.1 ± 0.7	10.4 ± 0.5	10.8 ± 0.1	9.9 ± 0.2	10.7 ± 0.2
Carbon dioxide (19–29 mmol/l)	21 ± 7	20 ± 4	23 ± 5	21 ± 3	20 ± 3	19 ± 3	19 ± 5	19 ± 1
Chloride (110–120 mmol/l)	115 ± 5	115 ± 5	110 ± 4	112 ± 7	112 ± 4	112 ± 2	114 ± 3	113 ± 2
Creatinine (0.7–1.6 mg/dl)	1.0 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
Glucose (39–81 mg/dl)	89 ± 21	77 ± 6	71 ± 12	84 ± 10	78 ± 18	78 ± 5	82 ± 9	70 ± 24
Phosphorus (1.8–6.0 mg/dl)	3.7 ± 1.2	4.0 ± 0.4	4.0 ± 0.5	4.5 ± 0.9	3.5 ± 0.2	4.2 ± 0.4	3.6 ± 0.8	4.3 ± 1.1
Sodium (147–158 mmol/l)	154 ± 4	154 ± 4	153 ± 4	153 ± 5	151 ± 6	155 ± 3	155 ± 0	155 ± 6
Potassium (3.2–4.4 mmol/l)	4.7 ± 0.7	5.0 ± 0.3	4.9 ± 0.2	5.0 ± 0.4	4.7 ± 0.7	5.3 ± 0.6	4.7 ± 0.1	4.6 ± 1.0
Lipid profile								
Triglycerides (35–137 mg/dl)	48 ± 13	67 ± 15	56 ± 9	46 ± 1	54 ± 18	43 ± 12	45 ± 12	41 ± 4
Cholesterol (115–207 mg/dl)	172 ± 36	169 ± 46	169 ± 43	173 ± 48	187 ± 41	184 ± 35	197 ± 49	203 ± 25
Liver function								
Albumin (3.1–4.6 g/dl)	4.5 ± 0.4	4.2 ± 0.1	4.3 ± 0.1	4.3 ± 0.2	4.5 ± 0.3	4.7 ± 0.4	4.6 ± 0.3	4.7 ± 0.4
ALKP (97–688 U/l)	103 ± 24	108 ± 22	99 ± 13	102 ± 20	105 ± 18	103 ± 25	105 ± 15	111 ± 24
ALT (20–91 U/l)	67 ± 6	71 ± 11	66 ± 15	59 ± 18	64 ± 20	63 ± 28	79 ± 26	76 ± 35
AST (29–64 U/l)	49 ± 8	46 ± 11	51 ± 3	41 ± 13	42 ± 5	55 ± 13	42 ± 7	58 ± 24
GGT (51–144 U/l)	97 ± 11	105 ± 24	92 ± 8	92 ± 8	90 ± 7	96 ± 7	101 ± 11	100 ± 10
LDH (638–3012 U/l)	957 ± 407	1086 ± 675	1660 ± 907	1141 ± 531	1113 ± 652	923 ± 238	668 ± 131	849 ± 310
Total bili (0.1–0.4 mg/dl)	0.4 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
Total protein (6.7–8.0 g/dl)	7.7 ± 0.4	7.7 ± 0.6	7.6 ± 0.3	7.5 ± 0.4	7.8 ± 0.4	8.0 ± 0.5	7.8 ± 0.5	8.0 ± 0.6
Other								
CK (69–972 U/L)	58 ± 15	113 ± 58	51 ± 15	80 ± 35	84 ± 63	79 ± 53	58 ± 26	66 ± 33

Values are MEAN ± SD. ALKP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CK: creatine kinase; GGT: gamma-glutamyl transpeptidase; LDH: lactate dehydrogenase.

(Brandeis et al., 1993), and rhesus monkeys (Raveh et al., 1997). These studies were conducted using low doses of HuBChE. For example, Brandeis et al. used a dose of 4 mg or 48 nmole for each rat (250 g), while Ashani et al. used a dose of 4.2–9.2 nmole per mouse. In rhesus monkeys, the dose used was 13 and 34 mg per animal weighing 3.5–10 kg (Raveh et al., 1997). However, HuBChE is a stoichiometric bioscavenger in that 1 mole of enzyme binds to 1 mole of OP. A dose of 200 mg is predicted to protect an individual against $2 \times LD_{50}$ of soman (Ashani and Pistinner, 2004), which means that large quantities of purified enzyme are needed for protection. Therefore, the objective of this investigation was to determine the behavioral and physiological toxicity, if any, of a large dose of purified HuBChE when administered into rhesus monkeys. The dose chosen for these investigations was 30 mg/kg, which is 10 times the clinically relevant dose in humans. This dose should be sufficient to protect rhesus monkeys from an exposure of $10 \times LD_{50}$ of soman ($1 \times LD_{50}$ of soman = 6.5 μg/kg; Raveh et al., 1997).

A large dose of HuBChE administered into rhesus macaques by i.v. injection demonstrated pharmacokinetic profiles similar to those observed previously in mice and rats (Clark et al., 2005; Raveh et al., 1993; Saxena et al., 2005), guinea pigs (Allon et al., 1998; Lenz et al., 2005), and monkeys (Lenz et al., 2005; Raveh et al., 1997). The enzyme circulated with a MRT of 72 h, which is consistent with the observed MRT of 50 and 110 h in mice and guinea pigs, respectively (Doctor and Saxena, 2005). A shorter MRT = 30–37 h in rhesus monkeys was observed by Raveh et al. (1997). Despite circulating levels of BChE as high as 227 U/ml, none of the monkeys displayed any signs of clinical toxicity. Analysis of blood samples collected for a period of four weeks post-HuBChE administration did not reveal any clinical signs of pathology. These results are consistent with those reported in mice and guinea pigs (Saxena et al., 2005; Saxena et al., 2011). A transient increase in AChE activity observed for the first 3.5 h following the administration of HuBChE is an artifact of high concentrations of HuBChE in blood. AChE activity in whole blood was measured in the presence of 20 μM ethopropazine, which is effective in inhibiting >98% BChE activity in blood without inhibiting AChE activity. This method can accurately determine AChE activity in blood that contains up to 60 U/ml of BChE activity (Naik et al., 2012). When BChE activity in monkey blood samples exceeded this concentration, the activity of 2% uninhibited BChE was significantly larger as compared to baseline AChE activity and caused a transient increase in AChE activity because BChE can also hydrolyze ATC. Consistent with this fact, the time course of the observed increase in AChE activity directly mirrored changes in BChE activity, and only when BChE activity was at its highest levels (in the first 3.5 h immediately following injection).

As expected, the administration of HuBChE into monkeys induced the production of anti-HuBChE antibodies on day 6 following enzyme administration. Antibody levels peaked on day 14 and remained constant thereafter, but produced no behavioral changes. The production of anti-ChE antibodies following heterologous administrations of ChEs (i.e., the administration of a ChE isolated from one species into an animal of a different species) has been observed in many studies (Genovese and Doctor, 1995; Gentry et al., 1993; Matzke et al., 1999). Although these antibodies are not toxic to animals, they expedite the clearance of enzyme from circulation. This is why monkey BChE displays an MRT of 227 h in monkeys (Rosenberg et al., 2002), while HuBChE circulates with a shorter MRT of 72 h.

Using a six-item SPR task we did not detect any significant changes in the performance of macaques following the administration of a large dose of HuBChE. This observation is particularly compelling in light of the fact that these animals showed a mean circulating BChE level of 227 U/ml, which is more than 50-fold above the physiological level. Similar observations were previously made by Raveh et al. (1997), who did not observe deficits on a spatial discrimination task resulting from the administration of lower doses of HuBChE alone in rhesus macaques. The current results also support our previous study in which we demonstrated that a 600-fold increase in circulating blood BChE activity did not alter ASR or PPI behavior in mice. The effect of physostigmine on performance was also examined to demonstrate sensitivity of the behavioral test to disruption. As expected, physostigmine, a short-acting and effective carbamate inhibitor of ChEs (Triggle et al., 1998), caused a significant decrease in the number of trials completed as well as the

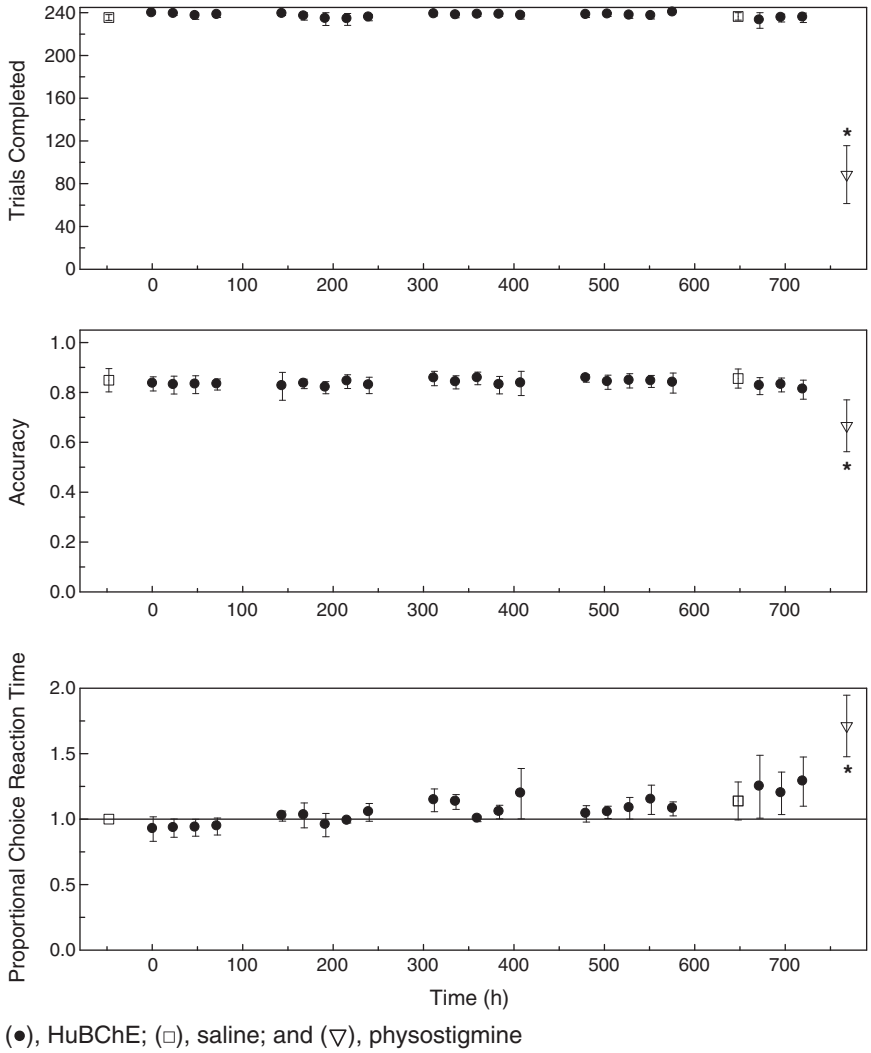

Fig. 2. Effects of HuBChE administration on the performance of rhesus macaques in a six-item serial-probe recognition task. Three measures of performance are shown for each daily serial-probe recognition session: total number of trials completed (top panel; maximum equaled 240), accuracy (center panel), and proportional choice reaction time (bottom panel). The saline vehicle controls are indicated by empty squares. The enzyme was administered only once (at 0 h) and this session and subsequent non-injection sessions are indicated by filled circles. Physostigmine (0.07 mg/kg) was administered at the end of the study and is indicated by an empty triangle. Values are mean ± SEM. Calculations of accuracy and reaction time were based only upon trials in which a choice response was made. Trials completed, accuracy, and proportional choice reaction time differed significantly from the saline vehicle only in the physostigmine session.(●), HuBChE; (□), saline; and (▽), physostigmine.

accuracy with which the trials were completed. Further, a significant increase in choice reaction time was observed following exposure to physostigmine. Physostigmine in rhesus monkeys is fairly well characterized and the decarbamylation rates of physostigmine-inhibited ChEs are believed to be similar to that observed in humans, with a peak physiological effect obtained within 30 min and a typical half-life of ChE inhibition approximating 60 min (Asthana et al., 1995; Wetherell and French, 1991).

With respect to behavioral evaluation in particular, the performance-impairing effects of physostigmine in rhesus monkeys have been shown using a variety of cognitive–behavioral tasks, including attention and short-term memory tasks analogous to the present task (Frederick et al., 1995; Penetar and McDonough, 1983) and behavioral performance generally (Frederick et al., 1995) at doses comparable to the one used here. The performance-impairing effects of physostigmine have also been extended to other nonhuman primates, including squirrel monkeys (Hudzik and Wenger, 1993) and marmosets (Wolthuis et al., 1995) and levels of impairment comparable to those in the present study were observed. Taken together, these results demonstrate the behavioral and physiological safety of HuBChE in mice and rhesus monkeys and support the development of HuBChE as a bioscavenger for use in humans.

Conflict of interest

None.

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