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Adenovirus transduced human butyrylcholinesterase in mouse blood functions as a bioscavenger of chemical warfare nerve agents[&]

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Running Title*:* Characterization of Ad-expressed human BChE in mouse blood

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Abbreviations used: Hu BChE, human butyrylcholinesterase; Ad, adenovirus; AChE, acetylcholinesterase; ACh, acetylcholine; OP, organophosphorus, PRAD, proline-rich attachment domain.

Abstract

Human serum butyrylcholinesterase (Hu BChE) is a promising therapeutic against the toxicity of chemical warfare nerve agents. Recently, we showed that recombinant (r) Hu BChE can be expressed at very high-levels, 400 to 600 U/mL in mouse blood by delivering the Hu BChE gene using adenovirus (Ad). Here, we report the biochemical properties of the Ad-expressed full-length and truncated rHu BChE in mouse blood. The molecular sizes of the full-length rHu BChE subunit and its oligomers were similar to those of native Hu BChE, though only a small portion of the full-length rHu BChE subunit underwent assembly into dimers and tetramers. As expected, Ad-containing the truncated Hu BChE gene transduced the expression of monomeric rHu BChE only. Compared to 415U of rHu BChE per mL in blood, tissues including liver, lung, heart, brain, kidney, muscle, intestine, diaphragm, salivary gland and fat expressed <10 U/g of rHu BChE activity. Ad-expressed rHu BChE in mouse blood neutralized soman and VX at rates similar to those of native Hu BChE and rHu BChE expressed *in vitro*. Since the expression of rHu BChE rapidly declined 6 days post-virus administration, sera were assayed for the presence of anti-Hu BChE antibodies. Anti-Hu BChE antibodies were detected on day 7 and in increased amounts thereafter which coincided with the loss of Hu BChE expression in sera. In conclusion, the delivery of Hu BChE gene using Ad can be a promising strategy that can provide protection against multiple lethal doses of chemical warfare nerve agents *in vivo*.

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Chemical warfare nerve agents are organophosphorus (OP) compounds that are among the most toxic substances known (Dacre, 1984). OPs produce their toxic effects by irreversibly inhibiting acetylcholinesterase (AChE), the enzyme that breaks down the neurotransmitter acetylcholine (ACh) (Taylor, 1990). The accumulation of ACh in response to OP exposure causes an over-stimulation of cholinergic receptors at the neuromuscular junctions and nerve synapses (Marrs et al, 1996), which can lead to muscle weakness, increased secretions, respiratory depression, seizures, coma, and ultimately death resulting from respiratory and/or cardiovascular failure or convulsions. Partial protection against OP toxicity can be achieved by pretreatment with a spontaneously reactivating inhibitor such as pyridostigmine bromide and postexposure therapy with anticholinergic drugs such as atropine sulfate and oximes such as 2-PAM chloride or bispyridinium oximes such as TMB4 and obidoxime (Heath and Meredith, 1992; Sidell, 1997; Marrs et al., 2006).

A novel approach to treating OP poisoning is the use of enzymes to sequester these compounds in the circulation before they reach their physiological target, AChE in the nervous system. Among these, plasma-derived human butyrylcholinesterase (Hu BChE; EC 3.8.1.1; accession # M16541) is the most viable candidate for human use (Ashani et al., 1991; Raveh et al., 1993; Raveh et al., 1997; Allon et al 1998; Lenz et al., 2005; Doctor and Saxena, 2005). Hu BChE is a stoichiometric scavenger in that one mole of the enzyme binds and inactivates one mole of OP nerve agent (Lenz et al., 2005; Doctor et al., 2005). Native Hu BChE is mostly a tetrameric glycoprotein consisting of four identical subunits with a combined molecular weight of 340 kDa (Lockridge et al.,

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1979; Lockridge et al., 1987). The molecular weight of each subunit is 85 kDa, of which 65 kDa is protein and 20 kDa (24-26%) is carbohydrate (Haupt et al., 1966; Saxena et al., 1998). A dose of 200 mg of Hu BChE is envisioned as a prophylactic treatment in humans to protect from 2 x LD_{50} of soman (Ashani and Pistinner, 2004). We recently showed that gram quantities of Hu BChE suitable for use as a bioscavenger can be obtained from Cohn Fraction IV-4 paste, a by-product of the human plasma generated during the production of human blood proteins such as g-globulin, clotting factors and others (Saxena et al., 2008). Sufficient amounts of Cohn Fraction IV-4 paste are generated in the United States by blood processing establishments to produce at least 100,000 doses of the bioscavenger product per year. Although this amount of material may be adequate for use by first responders in case of civilian exposure or, in deliberate or accidental limited combat engagements, it is not sufficient to protect the entire population or the entire military. Therefore, alternate approaches are being sought to identify a more reliable source of Hu BChE.

Recently, we reported that a single i.v. of adenovirus (Ad) containing the genes for truncated and full-length Hu BChE transduced high-levels (400-600 U/mL, 200- to 300- fold higher than the basal activity) of recombinant human butyrylcholinesterase (rHu BChE) in the circulation of BChE knockout mice (Chilukuri et al., 2008a). Gao et al (2005) also reported expression of very high levels of two mutant Hu BChEs with cocaine hydrolase activity in rats following a single injection of Ad containing the genes for these enzymes. In this study, we report on the biochemical properties of rHu BChE expressed by Ad-containing the full-length and truncated gene. We found that the molecular sizes and OP binding properties of full-length and truncated rHu BChE were

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indistinguishable from those of native Hu BChE. We also found that most of the virally expressed rHu BChE entered the circulation suggesting its potential to counteract the toxicity of multiple LD_{50}'s of OPs *in vivo*.

Materials and Methods

Chemicals: All reagent grade chemicals including butyrylthiocholine iodide (BTC), 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB), potassium phosphate, procainamide-Sepharose 4B gel and bovine serum albumin (BSA) were from Sigma Chemical Co. (St.Louis, MO). Nerve agents O-pinacolyl methyphosphonofluoridate (soman) and O-ethyl S-2-N,N-diisopropylaminoethyl methylphosphonothiolate (VX) were obtained from the U.S.Army Edgewood Chemical and Biological Center, Aberdeen Proving Ground, MD. The purity of soman and VX was >98.5% as determined by ^{31}PNMR. *In vitro* titration of rHu BChE with soman and VX was conducted at the US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland.

Recombinant adenoviruses: Production of recombinant adenoviruses (rAds) expressing full-length Hu BChE (Ad-Hu BChE) and truncated Hu BChE (Ad-tHu BChE) was described previously (Chilukuri et al., 2005; Chilukuri et al., 2008b).

Animal experiments: 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 principle stated in the guide for the Care and Use of

Laboratory Animals, NRC Publication, 1996 edition. BChE knockout mice were made by gene targeting. They tested negative for endogenous BChE activity (Li et al., 2006). The advantage of using BChE knockout mice for this experiment is the absence of background BChE activity. The BChE knockout colony was maintained at the University of Nebraska Medical Center. For these studies, female BChE knockout mice (224±5 days of age, 20-27 g body weight) were housed at 20 ^{o}C and fed food and water *ad libitum.* The viruses were diluted in sterile saline to produce an inoculum of 9 X 10^{9} infectious U in 150 µL of saline. On day 0, blood was collected from the saphenous vein prior to injection of the recombinant or control virus. Following intravenous injection of virus, blood was drawn daily from the animals into heparinized hematocrit tubes and centrifuged at 14,000 rpm for 10 min at 4 ^{o}C. The plasma was removed and immediately assayed for BChE activity (Ellman et al., 1961) or stored at -80 ^{o}C for further studies.

In one experiment, 3 animals treated with Ad-Hu BChE were euthanized on day 6 after the plasma BChE levels had peaked on day 5. The animals were perfused with 50 mL of 0.1 M phosphate buffered saline (PBS) before their liver, lung, heart, brain, kidney, muscle, intestine, diaphragm, salivary glands, and fat were removed and flash frozen. Tissues were homogenized in ice cold buffer containing 50 mM potassium phosphate pH 7.4, 0.5% Tween 20 and centrifuged at 12,000 rpm for 10 min at 4 ^{o}C. The supernatants were removed into clean tubes and assayed for BChE activity (Ellman et al., 1961) and molecular composition by non-denaturing polyacrylamide gel electrophoresis.

Non-denaturing polyacrylamide gel electrophoresis: The relative amount of BChE tetramers, dimers, and monomers, in plasma and tissue samples from recombinant virus-

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treated animals was estimated on 4% to 30% polyacrylamide gradient gels. In case of plasma, 1 µL of sample containing 6 to 1253 U/mL of full-length or truncated rHu BChE was used for non-denaturing polyacrylamide gel electrophoresis. In case of tissues, 30 µL of the tissue extract containing 0.1 to 1.7 U/g of rHu BChE was used for non-denaturing polyacrylamide gel electrophoresis. The gels were subjected to electrophoresis at 120 V for 16 h at 4 ^{o}C in a Hoeffer SE600 gel apparatus. Gels were stained for BChE activity in the presence of 2 mM BTC by the method of Karnovsky and Roots (Karnovsky and Roots, 1964).

Assay for BChE activity: Mouse plasma or tissue samples were tested for BChE activity with 1 mM BTC and 0.5mM DTNB in 100 mM potassium phosphate buffer pH 7.0, at 25 $^{\circ}$C. This assay buffer was used to maintain consistency with the previously published data (Chilukuri et al., 2008a; Li et al., 2006). The formation of product was followed by monitoring the increase in absorbance of 5-thio-2-nitrobenzoic acid at 412 nm using a molar extinction coefficient of 13,600 M^{-1} (Ellman et al., 1961). Activity was reported as U/mL, where 1 U represents 1 µmole of BTC hydrolyzed per min.

***In vitro* titration of Ad-expressed full-length and truncated Hu BChE:** Ad-expressed full-length and truncated rHu BChE from mouse plasma were partially purified by affinity chromatography using procainamide-Sepharose 4B gel (Chilukuri et al., 2005; Chilukuri et al, 2008b). This was necessary because mouse plasma contains carboxylesterase which competes with rHu BChE for binding to OPs. The OPs used were soman (0.12 µM stock concentration) and VX (0.15 µM stock concentration). To 1 U/mL

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(100 µL, 0.1 U) of rHu BChE in 50 mM sodium phosphate buffer pH 8.0 containing 0.05% BSA, various amounts of OP nerve agent (2, 4, 6, 8, 10 µL of stock; 0.2 to 1.0 molar equivalents) were added and incubated for 2 h at 25 ^{o}C. Residual enzyme activity was assayed by microEllman assay (Doctor et al., 1987). Briefly, 10 µL of the reaction mixture was mixed with 290 µL of 50 mM sodium phosphate buffer pH 8.0 containing 1 mM BTC and 1 mM DTNB and incubated for 10 min at 25 ^{o}C. Formation of the product was followed by monitoring the increase in absorbance of 5-thio-2-nitrobenzoic acid at 412 nm using a plate reader (SpectraMax Plus, Molecular Devices). The residual enzyme concentration was plotted against the number of equivalents of OP nerve agent in solution.

Enzyme linked immunosorbant assay (ELISA): Anti-Hu BChE antibodies in the sera of mice injected with Ad-tHu BChE were determined by ELISA. Plate wells were first coated with 0.10 mL of 4 U/mL of nativeHu BChE and incubated with mouse sera for 24 h (Chilukuri et al., 2008c) Anti-Hu BChE antibodies were detected with horseradish peroxidase-conjugated goat antibody to mouse IgG using ABTS (2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonicacid]-diammonium salt) substrate. Standard curves were produced using affinity purified mouse monoclonal IgG1 antibody against Hu BChE (Affinity Bioreagents, Golden, CO) for each assay to allow the quantification of antibody response.

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Results

Expression of full-length rHu BChE in mouse tissues

Previously, we reported that rAds containing the full-length and truncated Hu BChE gene transduced very-high levels of rHu BChE activity in the plasma of BChE knock-out mice. Expression of Hu BChE begins to rise on day 2 post-virus administration, reaches peak levels of 400 to-600 U/mL on day 5, and declines thereafter (Chilukuri et al., 2008a). To examine the expression of full-length rHu BChE activity in tissues, three animals were euthanized on day 6 post-virus injection and perfused with 50 mL of PBS. Liver, lung, heart, brain, kidney, muscle, intestine, diaphragm, salivary glands, and fat were processed and assayed for BChE activity. As shown in Table 1, varying levels of rHu BChE activity were found in different tissues. For example, greater than 5 U of rHu BChE per gram of tissue was found in liver, lung, muscle, intestine, kidney, salivary glands, heart, and diaphragm while brain and fat contained lesser amounts of BChE. In comparison, the average plasma BChE level was 411 ± 309 U/mL, a value 40- to 50-fold more than that found in tissues. Thus, > 95% of the virus-expressed BChE was found in the circulation of mice. These high levels of BChE in plasma were found when the adenovirus was injected rapidly (150 µL in 10 seconds) into the tail vein, but not when it was injected intraperitoneally (Chilukuri et al., 2008a).

Molecular sizes and subunit assembly of Ad-expressed rHu BChE in plasma and tissues

Non-denaturing polyacrylamide gel electrophoresis was used to determine the molecular sizes and oligomeric status of the full-length and truncated rHu BChE

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expressed in the plasma and tissues of BChE knock-out mice. Dimeric and tetrameric rHu BChEs were detected in the plasma of those mice injected with Ad-Hu BChE (Figure 1A) but not with Ad-tHu BChE (Figure 1D). Thus, full-length but not truncated rHu BChE underwent subunit assembly. To determine the extent of subunit assembly, plasma from two mice with different levels of BChE activity was analyzed by non-denaturing polyacrylamide gel electrophoresis. The first mouse had a peak plasma BChE level of 177 U/mL on day 5 (Figure 1A) whereas the second mouse had a peak plasma BChE level of 763 U/mL on day 5 (Figure 1B). This large difference observed in the expression levels of truncated or full-length Hu BChE with the same batch and the same amount of recombinant Ad is most likely due to: (1) inter animal variation and (2) inconsistency in the amount of virus entering into circulation from tail vein injections that are technically challenging. We noticed that BChE knock-out mice that received a rapid injection of the virus (< 1 min) expressed higher levels of rHu BChE compared to those that received a slow injection of the virus (> 3 min) (Chilukuri et al 2008a). Nevertheless, this large variation in the expression level of rHu BChE in BChE knock-out mice enabled us to assess the level of sub-unit assembly of rHu BChE in mouse blood. As shown in Figure 1A, the plasma of mouse with 177 U/mL of BChE activity had tetramers, dimers, and monomers with a greater proportion of dimers and tetramers compared to monomers (lanes marked 1-7). Mouse plasma from days 1, 2, and 3 post-Ad-Hu BChE injection contained the highest proportion of dimers and tetramers (Figure 1A, lanes 1-3). In contrast, the plasma from the mouse with 763 U/mL of peak BChE activity contained a greater proportion of monomers as compared to dimers and tetramers (Figure 1B, lanes 1-5). These results suggest that the mouse was capable of supplying the tetramer organizing

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peptide but its supply was limited because only a small percentage of the expressed BChE was assembled into dimers and tetramers. Normal human serum was included in all the gels to locate the positions of Ad-transduced tetrameric, dimeric, and monomeric Hu BChE (lanes marked HS). Plasma from blood drawn prior to virus injection (day 0) did not contain any detectable BChE activity, confirming the loss of BChE expression in BChE knockout mice (lanes marked 0).

Recombinant Hu BChE activities were found in tissues as well as in plasma. To determine whether the tissue forms of full-length rHu BChE were the same as in plasma, the mouse expressing 763 U/mL of peak plasma BChE activity was euthanized on day 6, and its tissues were analyzed by non-denaturing polyacrylamide gel electrophoresis. As shown in Figure 1C, all tissues contained an intense band corresponding to monomeric rHu BChE and very weak bands corresponding to dimers and tetramers. This was in contrast, to the plasma findings of more intense dimers and tetramers (Figure 1B).

These results suggest that Ad-Hu BChE expressed full-length rHu BChE is competent to form oligomeric forms whose formation is limited by the supply of tetramer organization peptide in the mouse plasma. Ad-tHu BChE expressed truncated rHu BChE on the other hand can only form monomers. In addition, the finding that the expression of both genes results in the expression of products whose molecular sizes were similar to that of native Hu BChE suggests that the structural integrity of Ad-expressed rHu BChE is preserved in mouse plasma.

In vitro neutralization of OPs by Ad-expressed rHu BChEs

To determine if rHu BChE expressed by Ad-Hu BChE and Ad-tHu BChE could bind and neutralize OP nerve agents, the enzymes were partially purified by procainamide affinity chromatography and titrated with soman and VX. Native Hu BChE and full-length and truncated rHu BChE expressed *in vitro* in 293A cells were simultaneously assayed for comparison, and the data are shown in Figure 2. Binding curves for rHu BChE expressed by Ad-Hu BChE and Ad-tHu BChE were very similar to those of rHu BChE expressed in 293A cells and native Hu BChE, suggesting that the OP binding activity of Ad-expressed rHu BChE in mouse plasma was fully preserved.

Presence of antibodies to Ad-transduced rHu BChE

Since the levels of Ad-expressed rHu BChE declined rapidly 6 days post-virus administration, sera from mice injected with Ad-tHu BChE were evaluated for the presence of anti-rHu BChE antibodies by ELISA. As shown in Figure 3, anti-rHu BChE antibodies were detected in the serum of mice 7 days post-virus administration and the antibody concentration gradually increased thereafter. In particular, mouse # 13 had high-levels of circulating anti-Hu BChE antibodies compared to the other four animals. Plasma BChE activity in mouse # 13 was 330 U/mL, which is 4-fold lower than that in mouse # 11 and is somewhat closer with the level observed in other mice (400 to 600 U/mL). Thus, anti-Hu BChE antibody production coincided with the decline of Hu BChE activity but antibody levels did not correlate with Hu BChE levels. The two animals injected with empty vector did not show any anti-BChE antibodies (data not shown).

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Discussion

Hu BChE is a lead candidate as a prophylactic against chemical warfare OP nerve agents. However, large-scale production of the native enzyme has been hampered due to the limited supply of outdated human plasma and the high cost of the purification process. The use of recombinant enzyme is also difficult because the protein lacks *in vivo* stability and methods to improve it for repeated administrations are still under development (Duysen et al., 2002; Huang et al., 2007, Chilukuri et al., 2008a, Chilukuri et al., 2008c,). Recently, delivery of proteins of therapeutic interest via gene transfer using Ad has become an attractive alternative approach (Walther and Stein, 2000). Ad type V has been shown to infect almost all types of mammalian cells (mouse, rat, human, guinea pig, swine, and monkey) *in vitro* and *in vivo*. Ad has been proven to be highly effective in transducing proteins *in vivo* that are structurally and functionally similar to their native counterparts. Host cells infected with Ad, specifically hepatocytes, are converted into small factories such that the recombinant protein will be produced for extended periods and in high amounts in circulation. Using Ad, numerous proteins have been transduced *in vitro* and *in vivo* and evaluated for their beneficial effects against diseases such as atherosclerosis, wound healing, cystic fibrosis, cancer, diabetes, and blood clotting disorders. Earlier, we reported that a single intravenous injection of Ad-Hu BChE or Ad-tHu BChE into BChE knock-out mice resulted in persistent high serum levels of Hu BChE expression which is higher than that reported for any other method reported to date (Chilukuri et al., 2008a). Whereas Hu BChE expression levels of 400-600 U/mL were achieved on day 4/5 of post-virus injection, an i.m. or i.p. injection of three milligrams of native Hu BChE yielded peak activity levels of only 225 U/mL and

363 U/mL, respectively (Saxena et al., 2005). Thus, a single injection of 9 X 10^9 infectious units of virus expressing truncated or full-length Hu BChE is equivalent to a single i.p or i.m injection of 4 to 8 mg of native Hu BChE in mice. Results of this study indicate that almost all of the Ad-expressed full-length and truncated rHu BChE is secreted and is similar to native Hu BChE in molecular size and binding to OPs, soman and VX, suggesting the suitability of this form of the enzyme as a bioscavenger.

For Hu BChE to efficiently function as a bioscavenger it must circulate at high concentrations for extended time periods and bind to OPs similar to plasma-derived native Hu BChE. When injected intravenously, Ad primarily enters the liver and infects liver cells to transduce proteins (Shah et al., 2000, Connelly and Mech., 2004). The expression of full-length rHu BChE in the liver as well as in nine other tissues including lung, heart, brain, kidney, muscle, intestine, diaphragm, salivary glands and fat obtained 5 days after administration of the recombinant virus was forty-to-several hundred-fold lower than that present in the circulation, suggesting that almost all of the Ad-transduced rHu BChE entered the circulation. Low levels of rHu BChE in all tissues suggest that circulating BChE may be binding to blood vessels and/or it may have entered the lymphatic system. These observations suggest that Ad-transduced full-length and truncated rHu BChE are efficiently secreted into the circulation.

Native Hu BChE is mostly tetrameric, while the enzyme produced *in vitro* using recombinant DNA technology is a mixture of tetramers, dimers, and monomers (Duysen et al., 2002; Chilukuri et al., 2005). Full-length rHu BChE underwent subunit assembly in mice whereas truncated rHu BChE, as expected, failed to undergo assembly due to absence of the tetramerization domain. The fact that rHu BChE underwent tetramer

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assembly in mouse suggests that the mice contributed a BChE tetramer-organizing peptide similar to the tetramer-organizing peptide present in native Hu BChE tetramers (Li et al., 2008). However, it appears that the mouse was able to supply an inadequate amount of this peptide because only a small percentage of the transduced Hu BChE formed dimers and tetramers.

The OP binding properties of Ad-expressed full-length and truncated rHu BChE and native Hu BChE were compared by titrating equimolar amounts of these enzymes with two nerve agents, soman and VX. The identical binding curves for full-length and truncated rHu BChE expressed in mouse blood when compared to those for truncated and full-length rHu BChE expressed in 293A cells and native Hu BChE suggest that the OP binding activity of rHu BChE is not affected by the expression system or its oligomeric status. Thus, Ad-expressed full-length and truncated rHu BChE are identical to native Hu BChE in their OP binding properties.

Time courses of full-length and truncated rHu BChE in mouse plasma show that their activities began to rise steeply on day 2, reached peak levels at day 4/5 and then declined. By day 10, very little or no Hu BChE activity could be detected in the serum (Chilukuri et al., 2008a). This phenomenon has been attributed to immunological reactions including T cell-mediated attack on vector-containing host cells, humoral inactivation of transduced proteins, and antibody production to transduced human proteins (Dai et al., 1995; Yang et al., 1995). Indeed, anti-rHu BChE antibodies were detected in mouse sera on day 7 following the virus injection, rose thereafter and peaked on day 10 in two animals and remained the same in the remaining three animals. In particular, one animal contained 5 to 10-fold higher levels circulating anti Hu BChE

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antibodies (30µg/ml) compared to all other animals. Reasons for this large variation could be due to inter animal variation with the response of the immune system to foreign antigens. These low anti-Hu BChE antibody levels were similar to those produced following a first injection of purified full-length or truncated rHu BChE (100 U or 150 µg) into mice and were 50- to 200- fold lower than those produced by the second injection of the enzyme (Chilukuri et al., 2008c). A lack of correlation between anti-Hu BChE body levels and plasma rHu BChE levels suggests that a combination of processes including immunological reactivity to virus infected host cells and to viral coat proteins, the induction of anti-Hu BChE antibodies, and humoral inactivation of rHu BChE, all contribute to the rapid decline of Hu BChE in mouse plasma (Dai et al., 1995; Yang et al., 1995). This is supported by the observation that long-term expression of human proteins *in vivo* could be attained in immunocompromised mice (Yang et al., 1994, Okuba et al., 2000),

In summary, we demonstrated that Ad-expressed full-length and truncated rHu BChE in mouse plasma are structurally intact and bind to OP compounds in identical stoichiometry to that of native Hu BChE. Most of the Ad-expressed rHu BChE is found in the circulation suggesting its suitability as a bioscavenger of OP nerve compounds. It is expected that these high levels of rHu BChE in mouse plasma are capable of eliminating the toxicity of multiple LD_{50} doses of chemical warfare nerve agents.

References

Allon N, Raveh L, Giat E, Cohen E, Grunwald J, and Ashani Y (1998) Prophylaxis against soman inhalation toxicity in guinea pigs by pretreatment alone with human serum butyrylcholinesterase. *Toxicol Sci* **43:** 121-128.

Ashani Y, Shapira S, Levy D, Wolfe AD, Doctor BP, and Raveh L (1991) Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. *Biochem Pharmacol* 41: 37-41.

Ashani Y and Pistinner S (2004) Estimation of the upper limit of human butyrylcholinesterase dose required for protection against organophosphate toxicity. *Toxicol Sci* **77:** 358-367.

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

Chilukuri N, Duysen EG, Parikh K, Sun W, Doctor BP, Lockridge O, and Saxena A (2008a) Adenovirus-mediated transfer of human butyrylcholinesterase results in persistent high-level transgene expression in vivo. *Chem Biol Interact* **175:** 327-331.

Chilukuri N, Sun W, Naik RS, Parikh K Tang L, Doctor BP, and Saxena A (2008b) Effect of polyethyleneglycol modification on the circulatory stability and

immunogenicity of recombinant human butyrylcholinesterase. *Chem Biol Interact* **175:** 255-260.

Chilukuri N, Sun W, Parikh K, Naik RS, Tang L, Doctor BP, and Saxena A (2008c) A repeated injection of polyethyleneglycol-conjugated recombinant human butyrylcholinesterase elicits immune response in mice. *Toxicol Appl Pharmacol* **231:** 423-429.

Connelly S and Mech C (2004) Delivery of adenoviral vector DNA to mouse liver. *Methods Mol Biol* **246:** 37-52.

Dacre JC (1984) Toxicology of some anticholinesterases used as chemical warfare agents -a review, in *Cholinesterases, Fundamental and Applied Aspects,* Brzin, M., Barnard, E.A. and Sket, D., Eds., de Gruyter, Berlin, Germany, pages 415-426.

Dai Y, Schwarz EM, Gu D, Zhang WW, Sarvetnick N, and Verma IM (1995) Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization to factor IX adenovirus vector antigens allows for long-term expression. *Proc Natl Acad Sci* (USA) **92:** 1401-1405.

Doctor BP, Toker L, Roth E, and Silman I (1987) Microtiter assay for acetylcholinesterase. *Anal Biochem* **166:** 399-403.

Doctor BP and Saxena A (2005) Bioscavengers for the protection of humans against organophosphate toxicity. *Chem Biol Interact* **157-158:** 167-171.

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

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

Gao Y, Atanasova E, Sui N, Pancook JD, Watkins JD, and Brimijoin S (2005) Gene transfer of cocaine hydrolase suppresses cardiovascular responses to cocaine in rats. *Mol Pharmacol* **67**:204-211, 2005.

Haupt H, Heide K, Zwigler O and Schwick HG (1966) Isolation and physico-chemical characterization of cholinesterase in human serum. *Blut* **14:** 65-75.

Heath AJW and Meredith T (1992) Atropine in the management of anticholinesterase poisoning, in *Clinical and Experimental Toxicology of Organophosphates and Carbamates*, Eds., Ballantyne B. and Marrs, T.C, Butterworth, Oxford, pages 543-560.

Huang YJ, Huang Y, Baldassarre H, Wang B, Lazaris A, Leduc M, Bilodeau AS, Bellemare A, Cote M, Herskovits P, Touati M, Turocotte C, Valeanu L, Lemee N, Wilgus H, Begin I, Bhatia B, Rao K, Neveu N, Brochu E, Pierson J, Hockley DK, Cerasoli DM, Lenz DE, Karatzas CN and Langerman S (2007) Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. *Proc Natl Acad Sci (USA)* **104:** 13603-13608.

Karnovsky MJ and Roots L (1964) A "direct-coloring" thiocholine method for cholinesterases. *J Histochem Cytochem* 12: 219-221.

Lenz DE, Maxwell DM, Koplowitz I, Clark CR, Capacio BR, Cerasoli DM, Federko JM, Luo C, Saxena A, Doctor BP and Olson C (2005) Protection against soman poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys. *Chem Biol Interact* **157-158:** 205-210.

Li B, Duysen EG, Saunders TL, and Lockridge O (2006) Production of butyrylcholinesterase knock-out mice. *J Mol Neurosci* **30:** 193-195.

Li H, Schopfer LM, Masson P, and Lockridge O (2008) Lamellipodin proline rich peptides associated with native plasma butyrylcholinesterase tetramers. *Biochem J* **411:** 425-432.

Lockridge O, Eckerson HW and La Du BN (1979) Interchain disulfide bonds and subunit organization in human serum cholinesterase. *J Biol Chem* **254:** 8324-8330.

Lockridge O, Bartles CF, Vaughan TA, Wong CK, Norton SE and Johnson LL (1987) Complete amino acid sequence of human serum cholinesterase. *J Biol Chem* **262:** 549-557.

Marrs TC, Maynard RL and Sidell ER (1996) Chemical warfare agents: Toxicology and treatment John Wiley, Chichester, pp 221-230.

Marrs TC, Rice P and Vale JA (2006) The role of oximes in the treatment of nerve agent poisoning in civilian casualities. *Toxicol Rev* **25**: 297-323.

Okuba Y, Bessho K, Fujimura K, Lizuka T and Miyatake S-I (2000) Osteoinduction by bone morphogenetic protein-2 via adenoviral vector under transient immunosuppression. *Bichem Biophys Res Commun* **267**:382-387.

Raveh L, Grunwald J, Marcus D, Papier Y, Cohen E and Ashani Y (1993) Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. *Biochem Pharmacol* **45:** 2465-2474.

Raveh L, Grauer E, Grunwald J, Cohen E and Ashani Y (1997) The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol* **145:** 43-53.

Saxena A, Ashani Y, Raveh L, Stevenson D, Patel T, and Doctor BP (1998) Role of oligosaccharides in the pharmacokinetics of tissue-derived and genetically engineered cholinesterases. *Mol Pharmacol* **53:** 112-122.

Saxena A, Sun W, Luo C, and Doctor BP (2005) Human serum butyrylcholinesterase: In vitro and in vivo stability, pharmacokinetics, and safety in mice. *Chem Biol Interact* **157-158:** 199-203.

Saxena A, Luo C, and Doctor BP (2008) Developing procedures for the large-scale purification of human serum butyrylcholinesterase. *Protein Expr Purif* **61:** 191-196.

Shah V, Chen A, Hendrickson H, Weiler D, Smith Y, Yao J and Katusic Z (2000) Gene transfer of recombinant endothelial nitric oxide synthase to liver in vivo and in vitro. *Am J Physiol* **279:** G1023-G1030.

Sidell FR (1997) Textbook of military medicine: Medical aspects of chemical and biological warfare (eds. R. Zajtchuk and R.F. Bellamy, Washington D.C. Office of the Surgeon General, Department of the Army, pp129-179.

Taylor P (1990) Anticholinesterase agents, in *The Pharmacological Basis of Therapeutics*, Gilman A.G., Rall T.W., Nies A.S. and Taylor P., Eds., Macmillan, New York, Pages 131-149.

Yang Y, Ertl HC and Wilson JM **(**1994) MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* **1**:433-442.

Yang Y, Li Q, Ertl HCJ and Wilson LM (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* **67:** 2004-2015.

Walther W and Stein U (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs* **60:** 249-271.

Footnotes

[&] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. This work was supported by a grant (1.D0003_05_WR_C) from Defense Threat Reduction Agency (DTRA), Department of Defense.

Figure Legends

Figure 1: Molecular sizes of Ad-Hu BChE and Ad-tHu BChE expressed rHu BChE in mouse plasma and tissues. One µL of mouse plasma containing 6 to 1253 U/ml of full-length or truncated BChE or tissue extract representing 1.7 to 0.1 U of BChE activity was loaded onto 4-30% native polyacrylamide gels and electrophoresed at 100 V and 4 ^{o}C for 36 to 60 h. The gels were stained for BChE activity with butyrylthiocholine iodide. Positions of tetramers (T), dimers (D) and monomers (M) based on migration of tetramers, dimers, and monomers in human plasma (lane marked HS) are shown. **Panels A** and **B** show the molecular sizes of Ad-Hu BChE expressed as full-length rHu BChE in mouse plasma. The peak level of full-length rHu BChE in mouse plasma in panel A was 177 U/mL on day 5 and in the mouse in panel B was 763 U/ml on day 5. **Panel C** shows the expression of full-length rHu BChE in various tissues of mice expressing peak plasma levels of 763 U/mL. The tissues processed in panel C were: 1, diaphragm; 2, salivary glands; 3, lung; 4, liver; 5, heart; 6, intestine; 7, kidney; 8, muscle; 9, fat; and 10, brain. **Panel D** shows the molecular size of Ad-tHu BChE expressing truncated rHu BChE. The peak levels of truncated rHu BChE on day 4 were very high, 1250 U/mL.

Figure 2: *In vitro* binding curves of Ad-expressed Hu BChE and soman (A) and VX (B) in 50 mM phosphate buffer, pH 8.0, 0.05% BSA. Binding was allowed to go to completion by incubating samples for 2 to 3 h at 25 ^{o}C. Data points are average from triplicates (± 4%). Key: native Hu BChE (●); *in vivo* expressed tetrameric (■) and monomeric rHu BChE (▲); and *in vitro* expressed tetrameric (□) and monomeric rHu BChE (▼).

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Figure 3: Anti-Hu BChE antibody levels in the sera of mice following Ad-tHu BChE administration. Mouse serum obtained from animals injected with Ad-tHu BChE was analyzed by indirect ELISA to detect the presence of anti-Hu BChE antibodies. Antibody profiles shown were from five individual mice in the descending order # 13 (▲), 11 (■), 16 (●), 14 (▼), and 15 (♦).

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Table 1

BChE activity in tissues from Ad-Hu BChE treated mice (n=3) on day 6 after i.v. administration of the virus

	BChE activity, U/g Tissue
Liver	11.7 ± 1.93
Lung	10.7 ± 4.2
Muscle	6.7 ±1.2
Fat	3.3 ± 2.0
Diaphragm	10.5± 6.8
Intestine	6.7 ± 3.6
Heart	8.1 ± 4.9
Salivary Glands	9.7 ± 6.5
Kidney	5.7 ± 3.5
Brain	0.9 ± 0.2
Plasma	411 ± 309 (U/mL)

Values are means ± S.D for three independent determinations.

Figure 1

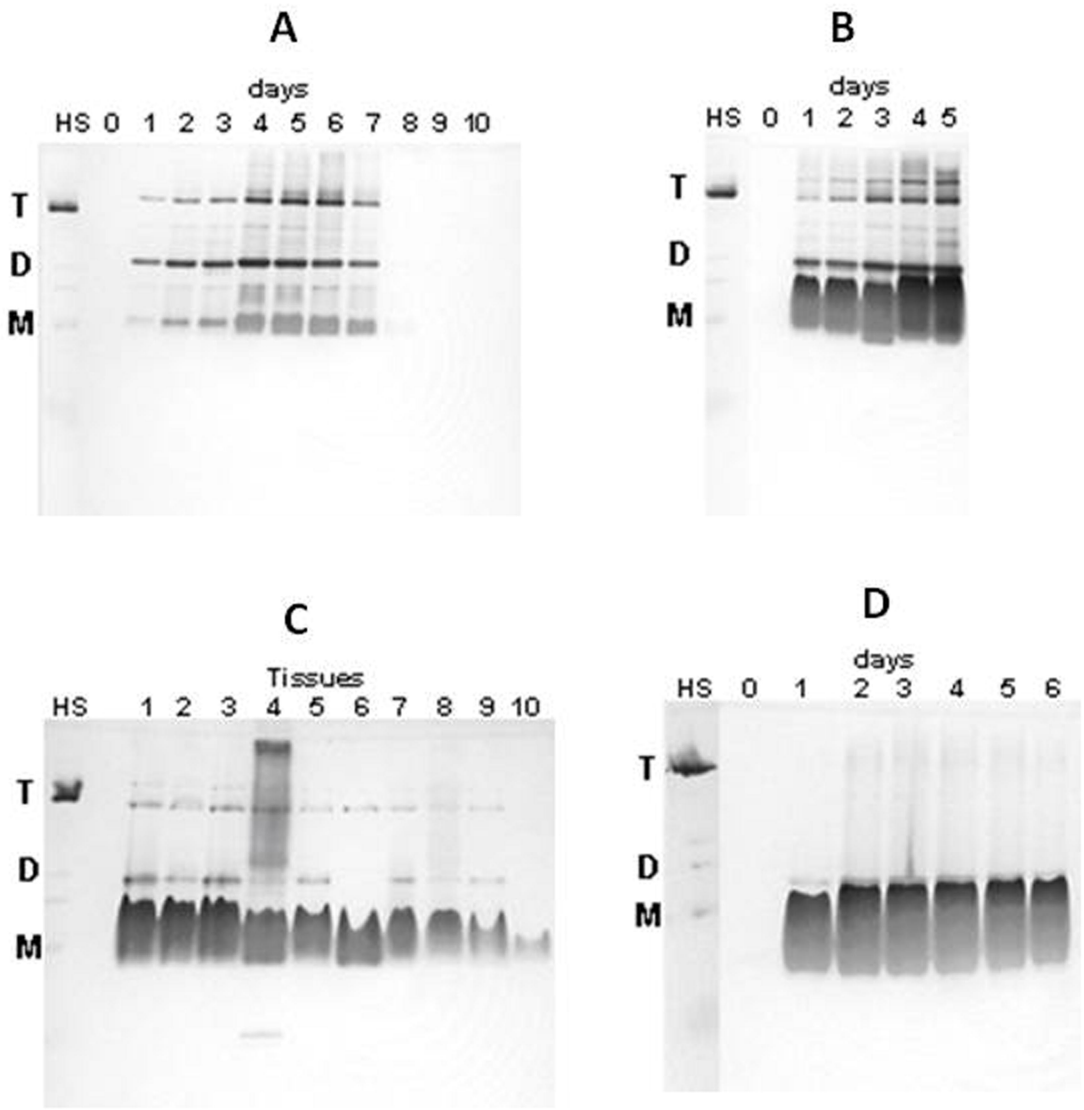
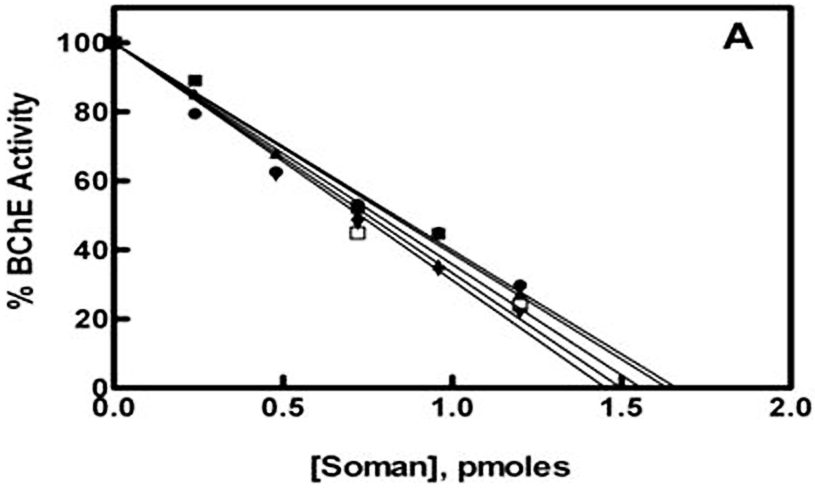


Figure 2



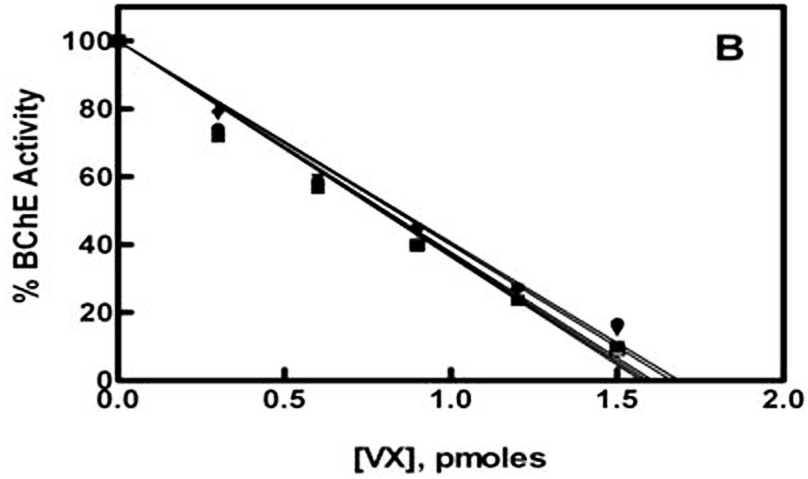


Figure 3