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Gene-Delivered Butyrylcholinesterase is Prophylactic against the Toxicity of Chemical Warfare Nerve Agents and Organophosphorus Compounds

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ABSTRACT

Gene delivery using an adenoviral system has been effective in introducing therapeutic proteins *in vitro and in vivo*. This study tested the feasibility of using adenovirus to deliver clinically relevant amounts of butyrylcholinesterase (BChE), a proven bioscavenger of nerve agents. The adenovirus construct expressed full-length mouse BChE. Mice were injected with a single dose of adenovirus ($1.5x10^{10}$ infectious units) in the tail vein; plasma was collected through day 11 and assayed for BChE activity. Maximum activity, representing a 300-3400-fold increase over baseline, was found on day 4. Expression levels returned to baseline by day 10. Nondenaturing gel electrophoresis showed the recombinant BChE was a dimer that could be converted to tetramers by addition of polyproline. The toxic compounds chosen for protection studies were positively charged organophosphorus agents, echothiophate and VX. Mice containing elevated blood levels of BChE (300-3000 folds over the control mice) were challenged with incremental doses of echothiophate or VX. Mice showed no signs of toxicity and were protected from up to 30 x LD_{50} dose of echothiophate and from 5 x LD_{50} dose of VX. A good correlation was observed between tolerated echothiophate dose and plasma BChE levels at time of challenge. The absolute increases in levels of circulating BChE and the sustained nature of the response resulted in a very high enzyme concentration, deemed critical in acute toxicity (5 x LD_{50} or more) scenarios. These results suggest that gene-delivered BChE is a prophylactic and affords protection equivalent to that of a multi-milligram injection of the same.

Introduction

Even though its natural substrate(s) and physiological function are enigmatic, human butyrylcholinesterase (HuBChE, EC 3.1.1.8) is universal in tissue distribution (Masson and Lockridge, 2010) and is a major constituent of plasma (Ryhanen, 1983). BChE inactivates man-made and natural organophosphate (OP) chemical toxins. The irreversible binding and inactivating function of BChE against a broad spectrum of OP poisons is an attractive feature for its exploitation as a prophylactic agent to prevent incapacitation and death by OP chemical warfare nerve agents. Hence intense research efforts were devoted in recent years to producing large quantities of HuBChE from a variety of sources and characterizing their OP bioscavenging activity in experimental models *in vitro* and *in vivo* (Saxena et al., 2006; Lenz et al., 2007b; Bird et al., 2010; Masson and Lockridge, 2010).

It has been shown that one molecule of BChE irreversibly binds and inactivates one molecule of the OP agent before it escapes from the circulation and reaches physiological targets (Raveh et al., 1997). Also, it has been estimated that a HuBChE dose of 200 mg/70 kg body weight would be required to provide protection to humans against up to 2 x LD_{50} doses of soman without the need for immediate postexposure therapy (Ashani and Pistinner, 2004). Large amounts of native BChE have been purified from outdated human plasma by a commercial firm for conducting Phase I clinical studies (Lenz et al., 2007a). Functional recombinant (r) HuBChE has been produced using mammalian cell cultures (Duysen et al., 2002; Chilukuri et al., 2005), transgenic goats (Huang et al., 2007), plants (Geyer et al., 2010) and silk worm larvae (Wei et al., 2000; Li et al., 2010). The rHuBChE produced in the milk of transgenic goats (Protexia^{TM}) is under advanced development as a pretreatment drug against OP toxicity (Huang et al., 2007).

Nonetheless, alternate avenues to raise blood levels of BChE must be considered given the cost associated with producing large quantities of GMP quality material and the potential safety/stability issues with glycans in HuBChE made in heterologous systems (Chilukuri et al., 2005; Chilukuri et al., 2008b).

We have been exploring experimental gene therapy approaches for elevating the blood levels of functional BChE using the mouse as a model system. In this approach, the gene carried by adenovirus enters the body's cells, primarily liver cells, and turns them into small factories for producing the therapeutic protein at a high-level and for a prolonged period of time. Using adenovirus numerous proteins have been introduced *in vitro* and *in vivo* and evaluated for their beneficial effects against diseases such as haemophelia, cystic fibrosis, and cancer (Imperiale and Kochanek, 2004). In previous studies using adenovirus type V as the vehicle of gene delivery, we have shown that full-length and truncated HuBChE are expressed at high levels in mice (Chilukuri et al., 2008a; Chilukuri et al., 2009). The adenovirus-expressed rHuBChE in mouse blood neutralizes the chemical warfare nerve agents VX and soman *in vitro* with efficiencies similar to that of native HuBChE, suggesting that gene therapy could mitigate OP toxicity (Chilukuri et al., 2008a; Chilukuri et al., 2009). In the present study, we have utilized a recombinant adenovirus expressing mouse (Mo) BChE to show that the enzyme protects mice from 5 x LD_{50} doses of the nerve agent VX. In addition, we show that mice expressing high levels of MoBChE tolerate increasing LD_{50} doses of the organophosphorus compound, echothiophate.

Materials and Methods

Reagents and cells

Material sources were: AdenoVator vector cloning kit and human embryonic kidney epithelial cells (HEK-293A cells) (QBiogene/MP Biomedicals, Irvine, CA); Pfu DNA polymerase, calf intestinal alkaline phosphatase, and DNA ligase (Promega Corp. Madison, WI); Restriction endonucleases, BamHI, EcoRI, Pme I, and Pac I (New England Biolabs, Beverly, MA); DNA purification kits (Qiagen Inc.,Valencia, CA); Lipofectamine 2000, cell culture media, medium supplements, and trypsin-EDTA (Life Technologies Inc., Gaithersburg, MD); Monoclonal antibody to 6 x histidine tag (AbCam, Cambridge, MA); Nickel affinity resin, PVDF membrane and ECL plus Western blotting detection kit (GE Healthcare Sciences, Piscataway, NJ); Beta-naphthylacetate, Fast blue RR, butyrylthiocholine iodide (BTC), 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB), procainamide-Sepharose 4B gel and all other chemicals (Sigma Chemical Co. St. Louis, MO; Quality Biological Inc., Gaithersburg, MD); Echothiophate iodide (Wyeth-Ayerst, Rouses Point, NY); Nerve agent O-ethyl S-2-N,N-diisopropylaminoethyl methylphosphonothiolate (VX) (Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD). The purity of VX was >98.5% as determined by ^{31}P NMR.

Production of recombinant adenovirus expressing MoBChE

Recombinant adenovirus expressing MoBChE was generated as described in the applications manual (MP Biomedicals/QBiogene Inc., Irvine, CA). Full-length MoBChE cDNA containing a 6 x histidine tag at its carboxyl terminus (Gift from Dr. Palmer Taylor, University of California at San Diego, San Diego, CA; Accession # M99492) was used in a polymerase chain reaction (PCR) to introduce BamHI restriction endonuclease sites at 5' and 3' ends of the gene. The

primers used in the PCR reaction were 5'ATAGGATCCATGCAGACTCAGCATACCAAG'3 (forward) and 5'ATGGATCCTTAGTGATGGTGATGGTGATGGAGAGCTGTAC'3 (reverse). The PCR product was first cloned into ZERO BLUNT PCR cloning vector and later transferred into adenoviral transfer vector pAdenoVator-CMV5-IRES-GFP linearized previously with restriction endonuclease BamHI. The orientation and the complete nucleotide sequence of the insert were identified by nucleotide sequencing using Big-Dye termination kit version 3.0 (Applied Biosystems, Carlsbad, CA). For generation of recombinant adenovirus plasmids, pAdenoVator-CMV5-IRES-GFP-MoBChE plasmid was linearized with PmeI and co-transformed with Ad5dE1/dE3 backbone plasmid into recombination proficient BJ5183 cells. Recombination was confirmed by PacI digestion and one of the recombined clones (pAd-MoBChE) was retransformed into DH5α cells for large-scale plasmid DNA preparation.

For the production of recombinant adenovirus expressing MoBChE (Ad-MoBChE) HEK-293A cells which produce the deleted E1 genes in trans were transfected with 4 μg of Pac-1 digested pAd-MoBChE plasmid using Lipofectamine 2000. Cells were harvested 15 days post-transfection and re-suspended in Hanks Balanced Salt Solution and crude recombinant virus was collected after four freeze-thaw cycles. The crude virus was sent to QBiogene/MP Biomedicals, Irvine, CA for single clone selection, amplification, purification, viral titer determination, and quality testing. The titer of the purified virus was 4.76×10^{12} viral particles per milliliter or 3.16×10^{11} infectious units per milliliter. The control adenovirus lacked the MoBChE gene, but was otherwise identical to the MoBChE expressing virus.

Cell culture and infection with Ad-MoBChE and purification of rMoBChE

HEK-293A cells (1 x 10^6) were seeded in 6-well plates. After 24 h, the cells were infected with 0-2.5 infectious units (IU)/cell of Ad-MoBChE for 1-2 h using 500 µl of infection medium (DMEM containing 2% FBS, antibiotics penicillin and streptomycin, and sodium pyruvate) at 37°C. Following infection, 2.5 ml of growth medium (DMEM containing 10% FBS, 50 µg/ml penicillin and streptomycin, L-glutamine and sodium pyruvate) was added to the cells and incubated at 37°C. The BChE activity in the culture medium was quantified using butyrylthiocholine as the substrate (Ellman et al., 1961).

For large-scale expression and purification of rMoBChE, HEK-293A cells (10 x 10^6) were seeded in 150 mm^2 tissue culture dishes. After 24 h, the cells were infected with Ad-MoBChE (1 IU/cell) for 1-2 h using 10 ml of infection medium at 37^{o}C. To the infected cells, 15 ml of growth medium was added and the cell cultures were incubated for 5 to 6 days at which time BChE activity levels peaked. The spent medium was collected and cleared of cell debris by centrifugation at 2500 rpm for 10 min at 4^{o}C. Purification of rMoBChE was accomplished by subjecting the medium to ammonium sulfate fractionation followed by affinity chromatography using procainamide Sepharose 4B and nickel affinity resin (Chilukuri et al., 2008b).

Animal experiments

BChE null mice (n=7) were used for the time course of expression of rMoBChE following a single injection of the virus. Challenge experiments with echothiophate and VX were performed with wild-type mice. BChE null mice (strain 129sv) lack endogenous BChE activity (Li et al., 2006) and, therefore, allow accurate assessment of the induced BChE expression level. For these studies, female BChE null mice (224±5 days of age, 20-27 g body weight) were housed at 20^{o}C

and fed food and water ad libitum. On day 0, blood was collected via the saphenous vein prior to injection of 50 µL containing 1.5 x 10^{10} IU of recombinant or control virus via the tail vein. Following i.v. injection of the virus, blood was drawn (10 µl) daily for 11 days 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). Plasma samples were analyzed for the presence of BChE tetramers, dimers, and monomers by non-denaturing polyacrylamide gel electrophoresis. Two animals treated with Ad-MoBChE or control virus were euthanized on day 5, 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. Supernatants were transferred to clean tubes and assayed for BChE activity.

Challenge experiments with echothiophate were performed using wild-type strain 129Sv mice. Ad-MoBChE (n=6) and Ad-control (n=4) were injected in the tail vein as described above. Plasma was collected from all mice prior to virus injection and at the indicated intervals through day 11 post virus injection for assay of BChE activity. On day 4 post virus injection baseline temperature, body weights and observations were recorded. Mice were challenged subcutaneously with 2 x LD_{50} of echothiophate (200 µg/kg). Mice were observed continuously through one hour post echothiophate challenge. Axial body temperatures and signs of cholinergic toxicity (straub tail, hunched posture and tremors) were observed periodically through toxicant post challenge. Moribund mice were euthanized immediately. Animals that survived the first 2x LD_{50} challenge were challenged with another 2 or 3 x LD_{50} 3 hours after the first challenge. Animals that survived the combined 4 or 5 x LD_{50} doses of echothiophate were injected several

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hours later with additional LD_{50} doses following the same procedures. Two mice were exposed to 30 x LD_{50} combined doses of echothiophate during a 72 hour period. These mice were observed for cholinergic symptoms for 8 days after the last dose of echothiophate.

Challenge experiments with VX were performed at the Collaborative Research Facility, USAMRICD, Aberdeen Proving Ground, MD, 21010. Ad-MoBChE (n=15) and Ad-control (n=10) viruses were injected into the tail vein of mice as described above. Plasma was collected from all the mice prior to virus injection and on day 4 post viral injection. On day 4 mice were challenged subcutaneously with 2.5 x LD_{50} of VX (28 µg/kg). Mice were observed for signs of cholinergic symptoms through one hour post challenge. Animals that survived the first 2.5 x LD_{50} VX challenge were challenged with another 2.5 x LD_{50} at 1 hour after the first challenge. Surviving animals were observed for 24 h for cholinergic symptoms and subsequently euthanized as mandated by the protocol.

Non-denaturing polyacrylamide gel electrophoresis

The relative amount of BChE tetramers, dimers, and monomers in plasma samples from Ad-MoBChE-treated animals was estimated on 4% to 30% polyacrylamide gradient gels. The gels were subjected to electrophoresis at 120 V for 16 h at 4^0C in a Hoeffer SE600 gel apparatus. Gels were stained for BChE activity in the presence of 2 mM butyrylthiocholine by the method of Karnovsky and Roots (Karnovsky and Roots, 1964).

Sucrose gradient centrifugation

Aliquots of purified rMoBChE or native human BChE (1 to 2 U/ml) were mixed with catalase (11.3S used as a sedimentation marker) and applied to linear 5-20% sucrose gradients prepared in 50 mM sodium phosphate, pH 8.0. Catalase serves as positive control to check for the

integrity of the sucrose gradients. The gradients were centrifuged at 75,000 X g for 18h at 4^{o}C in a SW41Ti rotor (Beckman Instruments, Fullerton, CA) Gradients were fractionated from the top using an AutoDensiflow IIC (Buchler Instruments, Lenexa, KS), and fractions were assayed for BChE activity using the micro-Ellman assay (Doctor et al., 1987).

Assay for BChE activity

Mouse plasma or tissue samples were tested for BChE activity with 1 mM butyrylthiocholine and 0.5mM DTNB in 100 mM potassium phosphate buffer pH 7.0, at 25˚C. Formation of the 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 butyrylthiocholine hydrolyzed per min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

SDS-PAGE was carried out with precast 10% gels. After electrophoresis, the proteins were transferred to PVDF membrane. The membrane was blocked in 4% powdered milk for 1 h, washed once with Tris buffered saline containing Triton X-100 (TTBS) and incubated overnight in primary antibody (rabbit polyclonal antibody to rMoBChE, 1:2000 dilution) in 0.5% milk powder containing 0.01% sodium azide. The membrane was washed with TTBS five times with intermittent shaking for 8 min and incubated with a secondary antibody conjugated with horse radish peroxidase, (Cat # Ab6721, Abcam, Cambridge, MA; 100 ng/ml) in 0.5% milk powder for 1 h. The membrane was washed again as above and the protein bands detected using ECL-Plus reagent. Chemiluminescence was measured in a Bio-Rad image reader.

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Additional Methods

Protein concentration was determined using the BCA protein assay kit (Pierce Co, Rockford, IL) using the enhanced method according to the manufacturer's instructions. Rabbit polyclonal antibody to rMoBChE was produced according to established procedures (Washington Biotechnology Inc., Simpsonville, MD). Immunoglobulin G-type was purified from rabbit serum by affinity chromatography using protein A/G Sepharose following the manufacturer's protocols. The effect of poly L-proline on the oligomerization of rMoBChE in mouse plasma was performed as follows: 1 µl of mouse plasma was incubated with 5 µl of 120 µM polyproline (Sigma, MW 1,000-10,000) in PBS for 1.5 h at room temperature and diluted with 994 µl of 0.05 M Tris-HCl pH 7.5, 25% glycerol, 0.05% bromphenol blue. Ten µl of this mixture (equivalent to 0.01 µl mouse plasma) was used for analysis by non-denaturing gel electrophoresis.

Results

Characterization of recombinant adenovirus expressing MoBChE

An adenoviral construct incorporating the complete coding sequence for MoBChE was prepared as described in Materials and Methods using standard molecular biology techniques. The MoBChE gene insert was fully sequenced and was observed to have minor changes (D110N, P282S and P410S) which do not involve the active site (S198, H438, and E325) of the enzyme. It appears that these modifications do not significantly influence the enzymatic activity of the recombinant protein towards BTC. The recombinant adenovirus expressing MoBChE was initially characterized using transfection into 293A cells. The expression of MoBChE was both viral dose- and time-dependent (Figure 1). Highest levels of the enzyme (~ 4 U/ml) were found in the medium on day 6 following infection of the cells. The recombinant enzyme was purified using ammonium sulfate fractionation, procainamide and nickel-affinity chromatography (Chilukuri et al., 2005; Chilukuri et al., 2008b) and subjected to SDS-PAGE and sucrose density gradient analysis. The inset in Figure 2 shows that the subunit size of MoBChE produced using adenovirus in 293A cells is similar in size (~85 kDa) to native or rHuBChE. Analysis of the native subunit composition by sucrose gradient centrifugation indicated that rMoBChE generated by adenovirus in 293A cells is a heterogeneous mixture of monomers, dimers and tetramers, with the former two predominating (Figure 2). These results suggest that the recombinant adenovirus is biologically active *in vitro* and that the adenovirus expresses predominantly monomeric and dimeric MoBChE in 293A cells.

Expression of recombinant mouse BChE in mice

We introduced the recombinant adenovirus containing the MoBChE gene into BChE null mice to characterize MoBChE expression *in vivo* over several days. We chose the BChE null mice because they lack endogenous BChE activity (Li et al., 2006) and, therefore, allow accurate assessment of the induced BChE expression level. Following introduction into mice by a single injection through the tail vein, expression of enzymatically active BChE was followed over a period of 11 days. The activity profile of BChE is depicted in Figure 3. Each time point in the figure represents mean +/- SE of values from 5-7 mice. The enzyme activities rose rapidly by day 1, reached peak levels on day 4 and then declined gradually thereafter (Figure 3). Being null for BChE, these mice show no plasma BChE activity prior to the virus injection and hence the fold increase in BChE activity is compared to the typical BChE activity in wild-type mice (~1.5-2 U/ml plasma). The activity in one individual mouse increased 4800-fold on day 4. Overall, 300 to 3000-fold increases in BChE activity levels were achieved over the baseline BChE activity of wild-type mice on day 4 following adenovirus injection. More importantly, mice tolerated extremely high levels of recombinant MoBChE over prolonged periods with no overt signs of toxicity.

We also evaluated the levels of MoBChE activities in various organs of mice when plasma BChE levels were at peak on day 4 and when plasma levels returned to base levels on day 11. Table 1 shows these levels in comparison to day 0 virus preinjection levels in wild-type and BChE null mice. Liver is the only organ that has as much as 10% of the levels found in plasma of BChE expressing mice. All other organs had negligible to ~5% of the plasma levels. The results also show that by day 11, no appreciable BChE activity was present in any of the tissues examined.

Virally expressed recombinant MoBChE is full-length and intact

To determine if the virus-expressed recombinant MoBChE is full-length and intact, plasma from a mouse expressing high levels (2342 units/ml on day 5) was analyzed by Western blotting with rabbit polyclonal anti-rMoBChE antibodies. As shown in Figure 4A, the antibody recognized a doublet of ~80-85 kDa protein in samples from days 1 through 9. The lower migrating form likely represents partially-glycosylated MoBChE since such a phenomenon is well known at very high expression levels of glycoproteins (Clery-Barraud et al., 2009). Plasma from pre-injection bleed (day 0) did not contain readily detectable amount of BChE protein. Also notable is that despite the very high levels introduced into circulation, partial proteolysis was absent or minimal. Figure 4B shows non-denaturing PAGE analysis to identify the subunit assembly of BChE in the same samples depicted in Figure 4A. The results show that rMoBChE expressed in mice is primarily a dimer with <15% detected as tetramers. Monomers are undetectable. This contrasts with the results in 293A cells where monomers are the most abundant BChE form (Figure 2). The BChE activity levels shown in figure 4B appear to be in excellent agreement with the BChE protein levels detected in Figure 4A. These results suggest that virally expressed in MoBChE mice is full-length and intact; the MoBChE remains intact as long as 9 days in the circulation.

It is known that human and horse BChE tetramers contain polyproline peptides in their tetramerization domain (Li et al., 2008b). We tested the hypothesis that the Ad-MoBChE-induced MoBChE in mouse plasma consists primarily of BChE dimers as a consequence of inadequate supplies of polyproline. Three mouse plasma samples containing adenovirus-expressed BChE were preincubated with exogenous polyproline, analyzed on a nondenaturing gel and stained for BChE activity. The results presented in Figure 5 show that the adenovirus-

expressed BChE (lanes 1, 2, and 3) is predominantly dimeric. The BChE dimers were converted to BChE tetramers by preincubating mouse plasma with polyproline (lanes 4, 5, and 6). This demonstrates that BChE dimers can assemble into tetramers in the presence of polyproline. It also explains why the major form of adenovirus-expressed BChE is a dimer; the mouse does not make enough polyproline to allow assembly into tetramers. A polyproline rich peptide is required for assembly into tetramers. The tetrameric adenovirus-produced BChE migrates to the same position as native plasma MoBChE or HuBChE (Figure 5, lanes marked Mo plasma and Hu plasma). This suggests that the glycosylation system of the mouse can add about 24% of the weight of the BChE protein as sugars. The glycosylation system appears to keep up with the demands of the overexpression system, but the polyproline-like factor production does not.

Recombinant mouse BChE protects mouse from echothiophate toxicity: the level of protection is proportional to blood BChE level

To determine the extent and duration of protection offered by adenovirally introduced rMoBChE, a set of 6 mice injected with Ad-MoBChE and a set of 4 mice injected with Ad-control were challenged with escalating doses of echothiophate, which is a chemical warfare nerve agent simulant compound. All control mice died within a few minutes after the first 2 x LD_{50} dose of echothiophate, whereas Ad-MoBChE injected animals survived a cumulative 4 to 30 x LD_{50} doses of echothiophate (Table 2). Remarkably, two animals survived a cumulative dose of 30 x LD_{50} doses administered over a period of two days without any signs of toxicity and remained alive for another 8 days, when they were euthanized. These two animals expressed BChE at ~ 5800 U/ml in plasma at the time of the first 2 x LD_{50} echothiophate challenge. The protection levels offered to the mice are illustrated in Figure 6, showing plasma levels of BChE activity in all 6 mice throughout the experiment. Following each challenge dose, the levels of

BChE dipped in all mice and recovered to varying degrees before the next dose. Lethality was notable in all animals at an echothiophate dose causing a precipitous drop in BChE levels to or below the background level. The two animals that survived 30 x LD_{50} over 3 days and survived for another 8 days maintained blood BChE levels above 100 U/ml. There appears to be a good correlation between tolerated LD_{50} dose and plasma BChE levels at challenge (inset, Figure 6).

Recombinant MoBChE protects mice from VX toxicity

We also carried out experiments to determine the ability of adenovirally produced MoBChE to protect mice from VX toxicity. A set of 15 mice expressing an average of 3522 ± 1155 U/ml plasma BChE were challenged with two consecutive 2.5 x LD_{50} VX doses administered 1 h apart. No lethality or signs of VX toxicity were noted in any of the animals (Table 3). Larger VX doses were not tested. All the animals survived the 24 h monitoring period subsequent to the second VX dose and were euthanized as per the protocol. All animals treated with Ad-control virus died within 3 minutes of the first 2.5 x LD_{50} VX dose.

Echothiophate and VX were selected for these studies because positively charged organophosphorus agents have minimal reactivity with carboxylesterase (Maxwell and Brecht, 2001). The contribution of BChE to detoxify organophosphorus agents is most clearly revealed when carboxylesterase does not compete. A mouse model of detoxication that bypasses plasma carboxylesterase is appropriate as a model for humans because humans have no carboxylesterase in plasma (Li et al., 2005).

Discussion

The results presented here in mice demonstrate the technical feasibility of using adenovirus to introduce functional and meaningful prophylactic levels of MoBChE into the systemic circulation; this strategy protects these mice against a cumulative dose of 4 to 30 x LD_{50} of echothiophate or 5 x LD_{50} of VX.

BChE activity levels achieved by adenovirus compared to injection of pure BChE

The enzyme levels introduced into systemic circulation by means of adenovirus reached an average of 2163 ± 2043 U/ml in BChE null mice and an average of 3500 ± 1143 U/ml in wild-type mice at peak on day 4 and more importantly, the levels were above 500 U/ml from day 1 to7 post-virus injection. The larger variation in BChE null mice arose due to two mice; one mouse expressing BChE levels of 584 U/ml and the other 6800 U/ml on day 4. This large variation in the expression levels of MoBChE with the same batch and the same amount of Ad-MoBChE is most likely due to interanimal variation and inconsistency in the amount of virus entering into circulation from tail vein injections, which are technically difficult to reproduce. In earlier studies, levels of 374 ±162 U/ml and 574 ±143 U/ml were achieved with full-length and truncated human BChEs, respectively using the same adenovirus system and approach in mice on days 4/5 (Chilukuri et al., 2008). In the earlier studies, a virus dose of 0.9 $x10^{10}$ (IU) for either full-length or truncated HuBChE was injected; this constitutes 60% of the amount of virus injected in the current study (1.5$X10^{10}$ IU/mouse). It is tempting to speculate that homologous expression (i. e. mouse BChE in mouse) is responsible for these higher levels. It has been reported that the mean residence time of BChE is 7 times higher (MRT 225h vs 34h) in a homologous system when compared to a heterologous system (Raveh et al., 1997; Rosenberg et

al., 2002). The levels achieved in this report were 8 to 10-fold higher than those reported using direct injection of purified BChE proteins. For example, injection of 3 mg purified native HuBChE into mice (a human equivalent of ~700 mg/ 70 kg person) by i.m. and i.p. routes produced peak levels of 225 and 364 U/ml, respectively (Saxena et al., 2005).

While the time course of MoBChE gene expression reported here is very much similar to those reported in literature on adenoviral vectors in general, the absolute levels and fold increase in BChE enzyme activity are extraordinarily high. An engineered form of BChE, cocaine hydrolase (CocE), has been studied for expression in rats using adenovirus system (Gao et al., 2005). A direct comparison between our study and the CocE study is not possible because the enzyme assays used are distinctly different. Nonetheless, the CocE study states that the mean highest activity in their system achieved on day 5 with 2.2×10^{10} PFU is equivalent to 27 μg/ml of purified protein. The mean expression level of 3522 U/ml in our VX study achieved on day 4 with 1.5×10^{10} IU translates to ~5 mg/ml of purified BChE protein. Whether the different animal systems used (mouse vs rat), the precise elements present in viral constructs made or homologous vs heterologous gene expression (see also later) contributed to ~185-fold difference between these studies is not clear at present.

Tissue distribution

Tissue distribution analyses when plasma levels were at peak suggest that recombinant MoBChE exist in all tissues analyzed, liver being the organ with the highest amounts. Irrespective of its site of synthesis, it appears that most of the gene-delivered MoBChE is secreted into the circulation. The tissue and plasma levels return to nearly baseline levels by day 11, a key safety consideration for a prophylactic agent intended to be introduced at very high levels for a short-term need.

Duration of expression

The standard adenoviral vector used in the present work expressed MoBChE at high-levels for 9 days. On day 10 the level of recombinant BChE dropped by >99%, but the activity level was still ~5-fold above the pretreatment level of wild type mice. While the peak expression levels were phenomenally high on day 4, the drop soon after peak was equally phenomenal. A similar pattern of expression, with peak on day 4-7 and >90% drop by day 15, was reported for CocE expression mediated by adenoviral vectors (Gao et al., 2005). This pattern of expression is apparently common with adenoviral vectors and is a reflection of immunological reactions, be it humoral or T-cell mediated (Dai et al., 1995; Yang et al., 1995). Both mechanisms are likely to be operative in mice and potential explanations for sharp drop in levels observed. Another potential explanation is that the BChE null mice being more efficient in generating antibodies to exogenously introduced BChE (Hrabovska et al., 2010), the rapid decline is an aberration in null mice. However, this possibility is unlikely to be a major reason for the rapid decline since wild-type mice also showed similar declines and anti-BChE antibodies levels are minimal at best and did not correlate with plasma HuBChE levels (Chilukuri et al., 2009). While we did not explicitly examine the time course in wild type mice, we did follow the time course (day 0 to day 14) of BChE in wild type mice challenged with ecothiophate. The time course did appear to be equally rapid with declines from peak by 92% on day 10 and 98% on day 14. In contrast, prolonged periods of expression up to a year have been achieved in rats by using a new adenoviral vector (Gao and Brimijoin, 2009). The new adenoviral vector expressed a human BChE mutant enzyme that hydrolyzed cocaine and protected from cocaine toxicity.

Subunit assembly

Recombinant MoBChE expressed in mice is a full-length glycoprotein with a subunit molecular weight of 85,000 daltons. Subunits assembled primarily into dimers (~85%) and tetramers (~15%); monomers were present at barely detectable levels in a mouse with expression level of 2342 U/ml. In contrast, full-length human BChE expressed by adenovirus in mice yielded dimers, tetramers and monomers in equal proportion at an expression level of 177 U/ml (Chilukuri et al., 2009). In addition, CocE, a doubly mutated (A328W/Y332A) version of human BChE (Sun et al., 2002), when expressed by adenoviral means in rats, was predominantly (96%) tetrameric in circulation(Gao et al., 2005).

BChE in human and wild-type mouse serum is predominantly tetrameric. The oligomeric forms of rMoBChE reported here from mice are similar to those reported for rHuBChE expressed in the milk of transgenic mice and goats (Huang et al., 2007). It has been suggested (Huang et al., 2007) that the high level of expression of rHuBChE by transgenic mice and goats far exceeded the capacity of the ColQ PRAD peptide protein assembly machinery to form tetramers. Similarly, the very high level of BChE expression driven by the adenovirus system appears to exceed the capacity of the mouse to produce the polyproline peptides required for assembly into tetramers (Li et al., 2008b). However, as discussed earlier, the CocE expression driven by the adenovirus system in rats produced mostly the tetrameric form, perhaps because the absolute levels (27 μg/ml) were rather low, thus having enough polyproline peptides required for assembly. It is also conceivable, albeit unlikely given the observations in both mice and goats, that rats have much higher levels of polyproline levels. Our *in vitro* results indicating nearly complete conversion of plasma BChE dimers into tetramers when incubated with polyproline are consistent with this suggestion. Also consistent with this interpretation is our observation that

the levels of tetramers vary inversely with the levels of rHuBChE expression with adenovirus system (Chilukuri et al., 2009). Co-expression of a polyproline peptide 17-27 residues in length is expected to yield a majority of BChE tetramers (Li et al., 2008b). Irrespective of whether rBChE exists as monomers, dimers or tetramers, their activity to neutralize OP compounds *in vitro* is similar based on a stoichiometric binding (Chilukuri et al., 2009).

Protection from echothiophate toxicity

The adenovirally expressed MoBChE dimer protected mice from up to 30 x LD_{50} doses of echothiophate. The echothiophate challenge experiments illustrate the degree and durability of protection offered to mice when MoBChE was transduced through the adenovirus system. It is remarkable that two animals with pre-challenge BChE levels of >5700 U/ml absorbed 30 x LD_{50} doses of echothiophate administered over a period of two days with no toxic signs There is a good correlation between tolerated LD_{50} echothiophate dose and the plasma MoBChE levels at pre-challenge in much the same manner reported before for increased blood levels due to exogenously administered BChE and levels of protection against OP poisoning (Raveh et al., 1993; Raveh et al., 1997). Our results on continuous monitoring of echothiophate challenged mice for plasma BChE, pre and post-challenge, illustrate the periodic drop in BChE immediately following the echothiophate administration, the sharp drop in BChE to <1 U/ml of mice succumbing to an echothiophate dose, and the protection offered to mice when BChE levels remained above 100 U/ml.

Protection from VX

The VX challenge data obtained with MoBChE through the adenovirus system compares favorably with those obtained with natural or recombinant purified BChE injected into monkeys, rats, mice, and guinea pigs for protection against sarin, cyclosarin, soman, or VX (Broomfield et

al., 1991; Wolfe et al., 1992; Raveh et al., 1993; Raveh et al., 1997; Cerasoli et al., 2005; Lenz et al., 2005). The protection range, obtained through injection of multiple mg amounts per kg animal body weight, covered 1 x to 5.5 x LD_{50} of the agents. In our experiments, a single injection of MoBChE adenovirus resulted in the production of BChE activity of 3522 ± 1155 U/ml on day 4 post-virus injection and protected 15/15 mice against two successive doses of 2.5 x LD_{50} VX given 1 hour apart. Earlier, we observed that adenovirally expressed HuBChE at levels of ~220 U/ml is sufficient to protect mice against toxicity due to two successive doses of 2.5 x LD_{50} VX toxicity (Chilukuri et al., unpublished data). Thus, BChE activity levels achieved in the current study (3522 ± 1155 U/ml) are expected to be capable of eliminating the acute toxicity of much higher doses (greater than 5 x) of VX. These high-levels of BChE are also expected to protect mice from 5 x LD_{50} or higher doses of soman, sarin and tabun. This expectation was based on 1) BChE levels of ~2300 U/ml protected mice from 19 x LD_{50} of echothiophate (LD_{50} dose, 100 µg/kg sc, Table 2) and 2) LD_{50} values of 124 µg/kg for soman, 137 µg/kg for sarin, and 365 µg/kg for tabun (Raveh et al., 1993) i.v. are comparable to that of the echothiophate LD_{50} dose s.c. Indeed, three mice with elevated blood levels virally expressed Mo BChE (~ 5800 U/ml) were protected from five sequential 1 x LD_{50} doses of soman challenge and showed no toxicity symptoms (Chilukuri N, Unpublished observations). Overall, our results strongly suggest that gene-delivered BChE is as good a prophylactic as multi-milligram amounts of injected enzyme against the toxicity of organophosphorus pesticides and chemical warfare nerve agents. Currently, gene therapy on any delivery platform against OP poisoning, as with any other therapeutic use, is subject to limitations imposed by the host system innate immunity and antigen-specific adaptive immune responses against vector-derived antigens. Vectors

specifically designed to avoid or minimize the host responses would be ideal for high and sustained expression of the transgene product.

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Other: Nageswararao Chilukuri for funding for the work

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FOOTNOTES

K. P and E. G. D have equally contributed to this study. 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. The experimental protocols were approved by the Animal Care and Use Committees (University of Nebraska Medical Center, Omaha, NE and US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD) and all procedures were conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended. This work was presented at the Chemical and Biological Defense Program Enzyme Colloquium Workshop, Falls Church, Virginia, September 21-23, 2010. This work was supported by funds from Defense Threat Reduction Agency, Department of Defense, to Dr. Nageswararao Chilukuri [1.D.0003_09_WR_C].

Figure Legends

Figure 1: Expression of MoBChE in 293A cells as a function of time and adenovirus dose. The assay was done three or more times and similar data was obtained.

Figure 2: Characterization of MoBChE expressed in 293A cells.

Purified MoBChE was analyzed by sucrose density gradient as described in Materials and Methods. Shown are the activity profiles of moBChE (closed circles), natural human BChE (closed triangles) and marker enzyme catalase (open circles) normalized as a percent of the activity detected in peak fractions. Inset shows SDS-PAGE analysis of purified moBChE in comparison to natural and recombinant human BChE

Figure 3: Plasma levels of BChE activity in BChE null mice injected with the mouse BChE gene.

Activities shown are mean +/- SE of 5-7 mice following a single tail vein injection of adenovirus containing the mouse BChE gene.

Figure 4: Polyacrylamide gel analysis of MoBChE expressed in mice.

A, SDS-PAGE and Western blot analysis of day 0-11 plasma samples from a mouse treated with adenovirus expressing the gene for mouse BChE. All Plasma samples were diluted 1:10 in MilliQ water and 1 µl/lane was used. The rMoBChE in the last lane was expressed in 293A cells. Samples were run on 10% SDS-PAGE under reducing and denaturing conditions. The blots were probed with Anti-rMoBChE IgG polyclonal rabbit IgG (200 ng/ml) and Goat Anti-Rabbit IgG HRP Abcam ab6721 (100 ng/ml) followed by detection with ECL. B, Native PAGE and BChE activity staining. The same samples

described for figure 4A were used except that the amounts loaded were 1/10th the amounts (equal to 0.01 µl plasma, day 1-11 samples). The loaded amounts were 5 µl plasma for day 0, wild-type human and untreated wild-type mouse plasma. Processing and staining of the gel for BChE activity is described in materials and methods.

Figure 5: Dimer to tetramer conversion by polyproline.

Three mouse plasma samples containing adenovirus-expressed BChE were incubated with 100 µM polyproline for 1.5h , diluted 1000-fold and analyzed (0.01 µl plasma equivalent per lane) on a nondenaturing gel. The gel was stained for BChE activity with butyrylthiocholine by the method of Karnovsky and Roots (Karnovsky and Roots, 1964). Lanes 1-3, samples without polyproline, Lanes 4-6, samples incubated with polyproline. Human plasma and wild-type mouse plasma samples (5 µl plasma per lane) are shown for comparison.

Figure 6: Plasma levels of BChE activity in mice, pre and post-challenge with echothiophate.

Plasma BChE activities in a set of 6 mice were monitored pre-virus injection, post-virus injection and post-virus/post echothiophate challenge. Arrows indicate the time and the LD50 doses mice received. Inset shows a plot of BChE levels in mice before challenge with echothiophate versus the total LD50 echothiophate doses tolerated.

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Table 1: Tissue levels of BChE activity in mice, pre and post-adenovirus injection (U/ml in plasma; U/mg in other tissues).

	Day 0	Day 0	Day 4	Day 4	Day 11	Day 11
	BChE-/-	BChE+/+	BChE-/-	BChE-/-	BChE-/-	BChE-/-
Plasma	0.0±0	1.46±0.2	996.3	6823.5	0.1	1.8
Liver	0.09±0.02	2.7±0.3	77.1	717.4	0.5	0.5
Brain	0.02±0.01	0.2±0.05	1.5	9.3	0.1	0.2
Salivary Glands	0.02±0.02	0.35±0.03	5.2	182.4	0.1	0.1
Lung	0.01±0.01	0.38±0.1	45.7	330.9	0.1	0.5
Heart	0.02±0.01	0.75±0.1	23.9	156.8	0.1	0.1
Diaphragm	0.03±0.03	0.35±0.1	18.4	147.0	0.2	0.2
Intestine	0.2±0.06	8.5±2.3	9.0	75.1	0.2	0.3
Fat	0.02±0.01	0.40±0.1	19.6	187.6	0.4	0.2
Muscle	0.02±0.02	0.14±0.01	25.8	326.5	0.1	0.2
Ovaries	0.01±0.01	0.12±0.05	ND	267.3	0.1	0.1
Spleen	0.02±0.01	0.69±0.1	23.5	181.5	0.2	0.4
Kidney	0.02±0.03	0.54±0.04	8.4	164.1	0.2	0.2
Skin	0.02±0.02	0.35±0.1	20.3	179.1	ND	0.1
Bladder	0.01±0.02	0.13±0.04	ND	66.6	0.1	0.1

The activity numbers shown are mean of two for day 0 BChE -/- and BChE +/+ mice. The measurements following adenovirus injection were obtained from two different mice on Day 4 and 11.

The residual butyrylthiocholine hydrolase activity in BChE-/- null tissues on day 0 is from carboxylesterase ES-10 (Li et al., 2008a) and is not from BChE. Wild-type strain 129Sv mice are designated BChE+/+. ND, Not determined.

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Table 2: Echothiophate challenge of wild-type mice.

Mouse	**Type of Virus**	**BChE activity (U/ml)**	**Echothiophate dose**	**Outcome**	**Total LD_{50} survived**
1	Ad-control	1.2	2X LD_{50}	Died 13 min	NA
2	Ad-control	1.0	2X LD_{50}	Died 8 min	NA
3	Ad-control	1.3	2X LD_{50}	Died 3 min	NA
4	Ad-control	1.3	2X LD_{50}	Died 2 min	NA
5	Ad-MoBChE	2395.6	2x LD_{50} 5x LD_{50} 12x LD_{50} 9x LD_{50}	Survived Survived Survived Died 6 min	19
6	Ad-MoBChE	405.3	2x LD_{50} 2x LD_{50} 12x LD_{50}	Survived Survived Died 3 min	4
7	Ad-MoBChE	586.5	2x LD_{50} 2x LD_{50} 12x LD_{50}	Survived Survived Died 3 min	4
8	Ad-MoBChE	5897.1	2x LD_{50} 5x LD_{50} 12x LD_{50} 11x LD_{50}	Survived Survived Survived Survived[1]	30
9	Ad-MoBChE	878.2	2x LD_{50} 3x LD_{50} 12x LD_{50}	Survived Survived Died 4 min	5
10	Ad-MoBChE	5717.6	2x LD_{50} 5x LD_{50} 12x LD_{50} 11x LD_{50}	Survived Survived Survived Survived[1]	30

[1.] These animals were alive and symptom-free 8 days post last dosing.

1 x LD_{50} for echothiophate is 100 µg/kg sc. The time interval between the first and second dose of echothiophate was 3 hours, and between the second and third dose was 4 hours. Animals that survived three doses were treated with a fourth dose on the following day, about 18 hours after the third dose. BChE activity is the activity in plasma before treatment with the first dose of echothiophate.

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Table 3: VX challenge of wild-type mice

Mouse#	Virus	BChE U/ml	VX exposure and signs of toxicity	
			1st 2.5 X	2nd 2.5X
1	Ad-control	1.5	Died in 3min	
2		1.8		
3		2.2		
4		1.2		
5		1.6		
6		2.7		
7		3.3		
8		3.4		
9		3.3		
10		2.9		
1	Ad-MoBChE	4150	No deaths or VX toxicity signs	No deaths or VX toxicity signs
2		2560		
3		4050		
4		2280		
5		2290		
6		2956		
7		2190		
8		3870		
9		4290		
10		2980		
11		5760		
12		4480		
13		5419		
14		2397		
15		3160		

On day 4 post virus injection mice were challenged subcutaneously with 2.5 x LD50 of VX (28 µg/kg). The BChE activities shown are before challenge with VX.

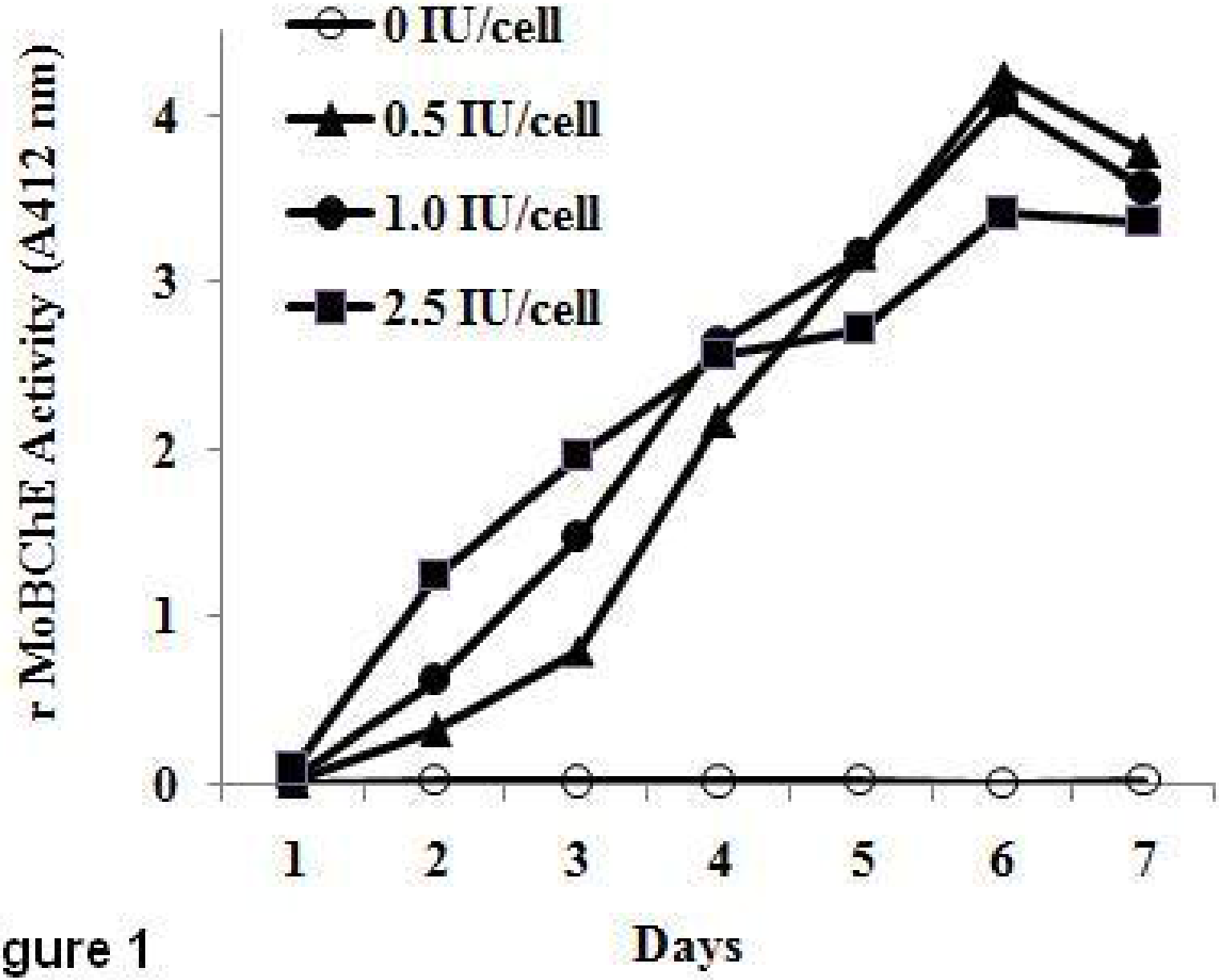
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Figure 1

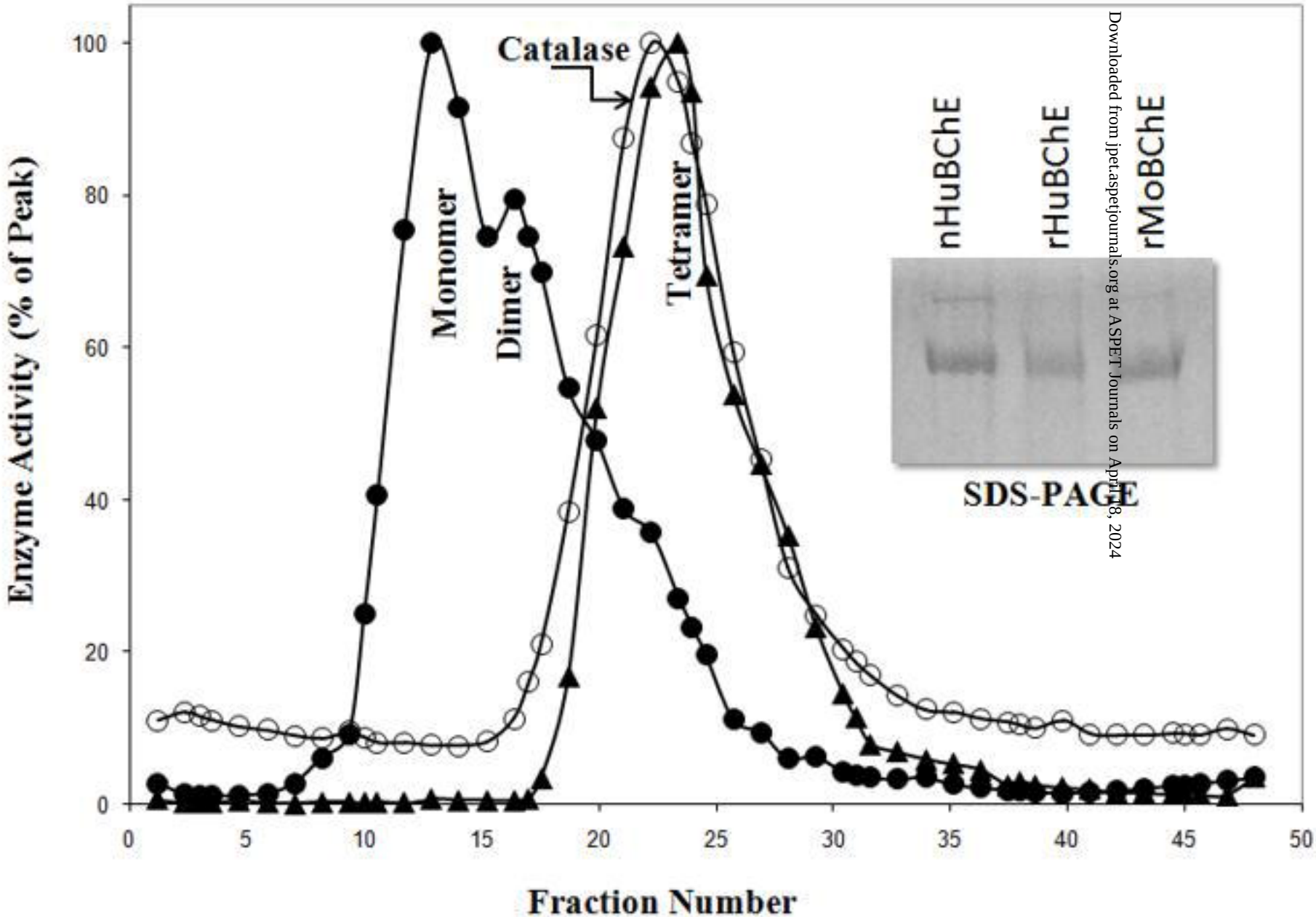


Figure 2

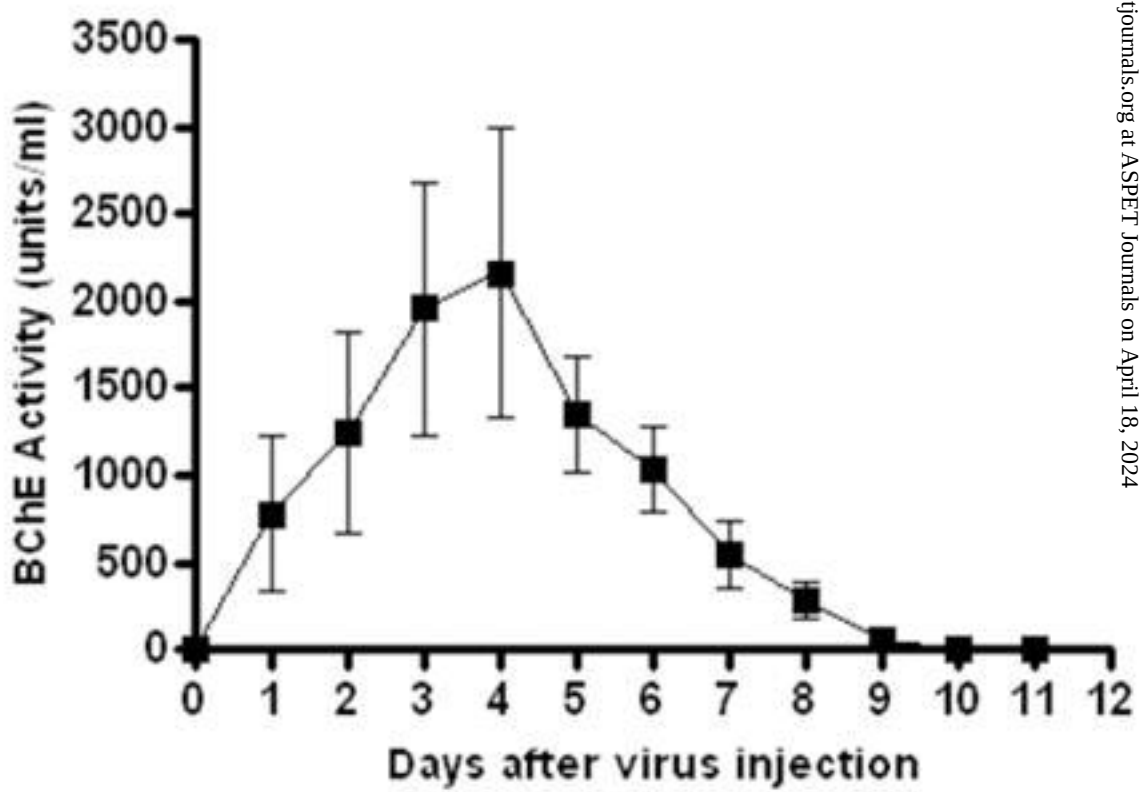


Figure 3

Figure 4

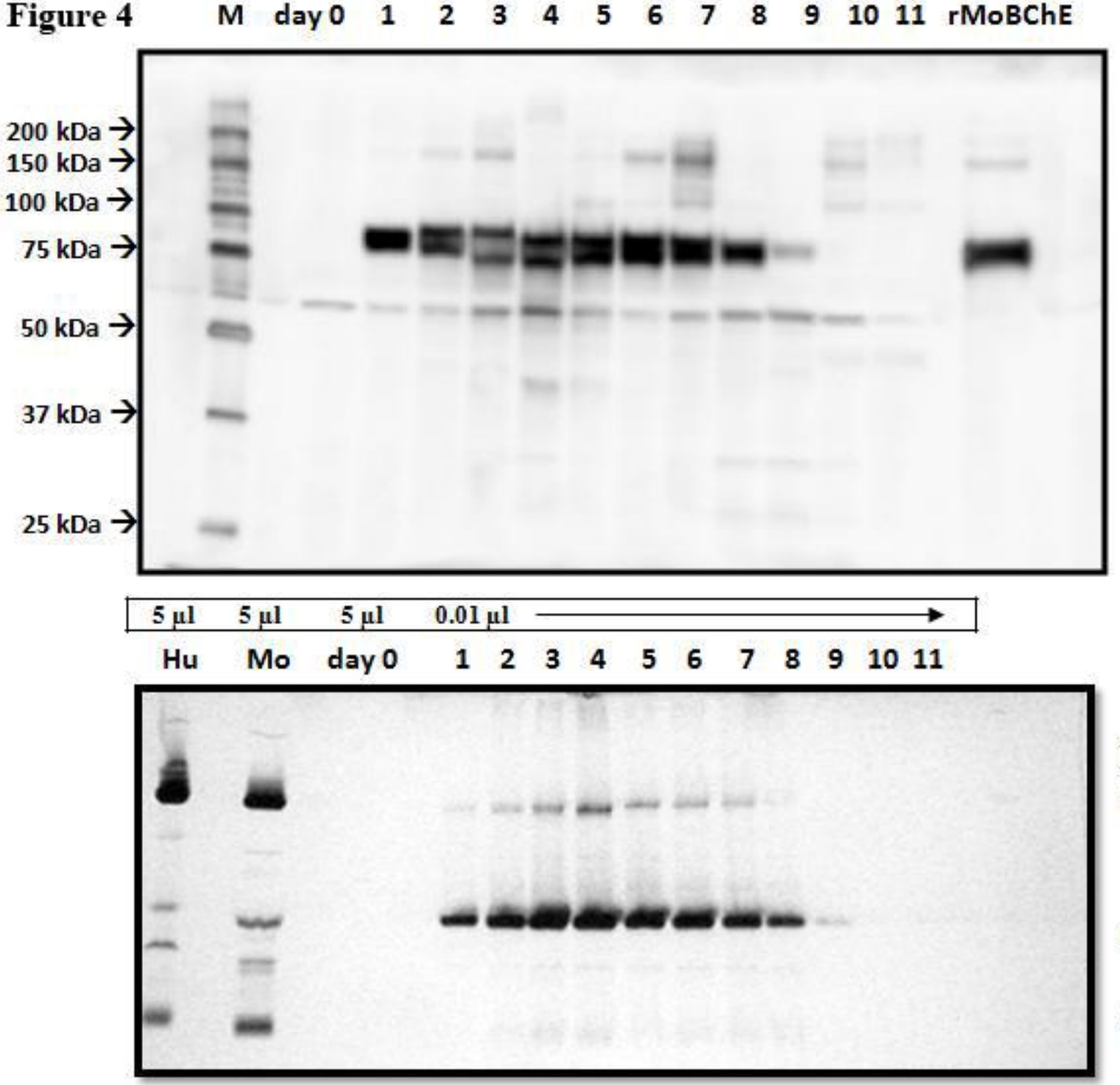
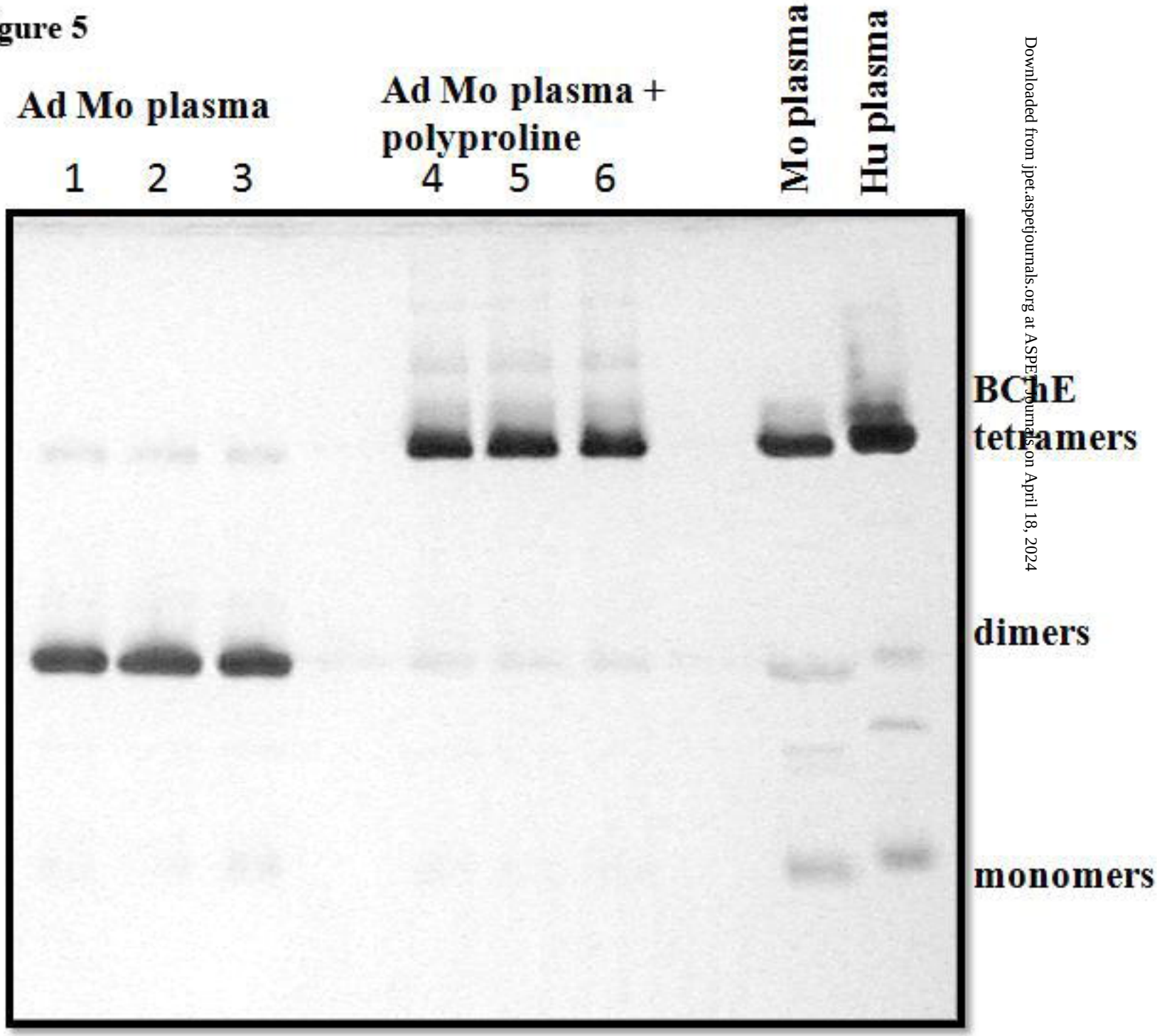


Figure 5



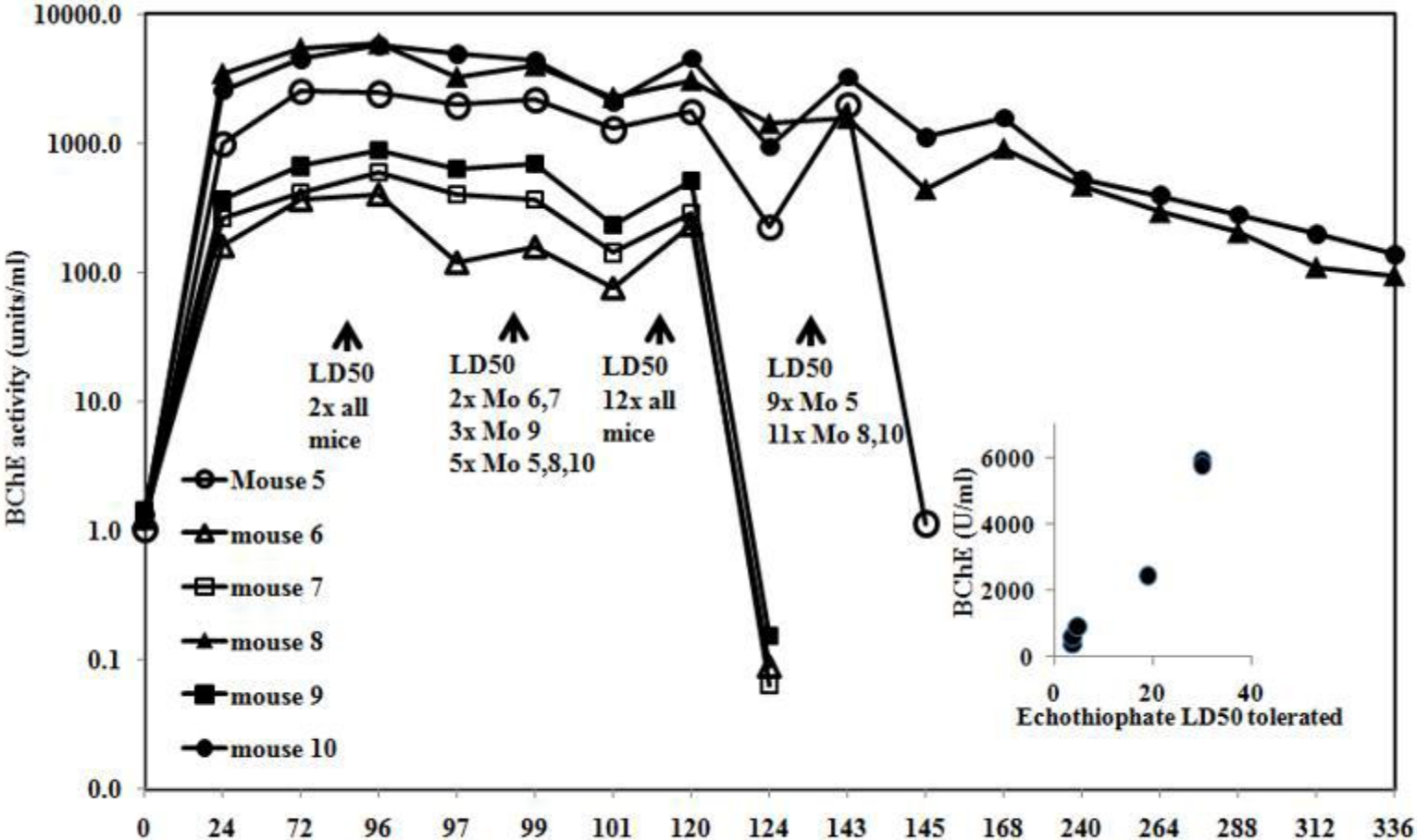


Figure 6