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This investigation examined the utility of three recombinant protein-expression systems (COS cells, insect cells and insect larvae) to cost-effectively produce biologically active human butyrylcholinesterase (BuChE). It was determined that baculovirus-infected insect cells (Sf9 and High 5) expressed 3.5- and 8.2-fold, respectively, more active enzyme than COS-7 cells. Baculovirus-infected cabbage looper (*Trichoplusia ni*) insect larvae produced over 26 times more than High 5 cells; in fact, one baculovirus-infected insect larva provided more active protein than 100 ml of insect cell culture. Analysis of the larvally expressed proteins revealed that the vast majority of BuChE expressed was inactive due to extensive degradation that occurred *in vivo*. However, the active form of BuChE does have enzyme kinetics similar to those of its human serum counterpart. Cabbage looper larvae were also examined for their ability to serve as an *in vivo* animal model to study protection against anti-cholinesterase toxicity. This was unsuccessful due to their high tolerance to the very toxic organophosphorus compounds tested. This tolerance may be attributed at least in part to a novel endogenous organophosphorus acid anhydride hydrolase activity that is capable of hydrolysing the chemical-warfare nerve agents sarin (isopropyl methylphosphonofluoridate) and soman (pinacolyl methylphosphonofluoridate). These results show that cabbage looper larvae can serve as an inexpensive recombinant protein-expression system for human BuChE.

Introduction

The cholinesterases and carboxylesterases are a multi-gene family of enzymes that hydrolyse a diverse group of carboxylesters. Enzymes of this family are inhibited irreversibly by many organophosphorus (OP) nerve agents and insecticides [1,2]. The most studied and critical of these events is the inhibition of enzyme acetylcholinesterase (AChE, EC 3.1.1.7) by chemical-warfare nerve agents, such as sarin (isopropyl methylphosphonofluoridate, or GB), soman (pinacolyl methylphosphonofluoridate, or GD) and VX {*O*-ethyl *S*-[2-[bis(1-methylethyl)amino]ethyl]methyl phosphonothiolate}. Inhibition of AChE, which resets the cholinergic system by hydrolysing the neurotransmitter acetylcholine [3], results in loss of neuronal function, paralysis and, soon thereafter, death. It has been shown that the serum enzyme butyrylcholinesterase (BuChE, EC 3.1.1.8) can serve as a scavenger *in vivo* to stoichiometrically bind the OPs prior to the inhibition of synaptic AChE [4]. This stoichiometric scavenger (one molecule of scavenger reacts with one molecule of OP) approach has proven effective but requires a large amount of material to destroy a small amount of OP. Catalytic scavengers (one molecule of scavenger reacts with many molecules of OP) have also been investigated, but the enzymes found thus far in Nature are generally inefficient; they have high K_m values and low turnover numbers [5]. Through site-directed mutagenesis of the human BuChE cDNA we have created novel organophosphorus acid anhydride hydrolases (OPAHs) that are capable of performing as catalytic scavengers *in vitro* [6]; however, there have been insufficient quantities of these enzymes to perform studies *in vivo*. All of these OP-scavenging studies have been limited because they require significant amounts of active recombinant protein, which are currently unobtainable.

To find more efficient and productive recombinant human (rh)BuChE expression systems three eukaryotic systems were examined. Considerations included ease of

Key words: baculovirus, carboxylesterase, nerve agents, protein expression, Sf9 cells.

Abbreviations used: AChE, acetylcholinesterase; AcNPV, *Autographa californica* nuclear polyhedrosis virus; BuSCh, butyrylthiocholine; echothiophate, 2-[(diethoxyphosphinyl)thio]-*N*,*N*,*N*-trimethyl ethanaminium; tabun, ethyl dimethylaminophosphoramidocyanidate; sarin, isopropyl methylphosphonofluoridate; soman, pinacolyl methylphosphonofluoridate; OP, organophosphorus; OPAH, organophosphorus acid anhydride hydrolase; paraoxon, *O*,*O*-diethyl *p*-nitrophenyl phosphate; BuChE, butyrylcholinesterase; (rh)BuChE, recombinant human BUChE; Sf, *Spodoptera frugiperda*; VX, *O*-ethyl *S*-[2-[bis(1-methylethyl)amino]ethyl]methyl phosphonothiolate; wt, wild-type.

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use, cost, and quantity and quality of the BuChE produced. The systems analysed included two conventional cell-culture systems (COS and insect cells) and a third, unconventional, approach utilizing baculovirus-infected cabbage looper (*Trichoplusia ni*) insect larvae. The COS cell system was initially selected due to its ease of use, reasonable cost and speed in expressing mutant forms of recombinant proteins. The baculovirus system [*Autographa californica* nuclear polyhedrosis virus (AcNPV)] has been shown to be useful for the expression *in vitro* of many eukaryotic proteins and is generally regarded as highly efficient. In addition, most of the recombinant proteins expressed in this system have been found to be similar to their native counterparts in bioactivity, immunoactivity and conformation [7]. Nevertheless, some problems remain with baculovirus-expressed proteins, notably glycosylation patterns, which could impact the expression of the BuChE glycoprotein. Based on reports that other human enzymes have been produced at high levels in *T. ni* [8,9], we postulated that the intact animal would be more likely to produce a fully glycosylated product, though not necessarily with a mammalian pattern, and at higher concentrations than that expressed by insect cell culture. Further, a high concentration of BuChE in an intact animal might efficiently scavenge OP cholinesterase inhibitors, when challenged, and thereby confer protection *in vivo* from insecticides and nerve agents. Hence, the recombinant BuChE–AcNPV-infected larvae might also adequately serve as a useful model to test the efficacy of bioscavengers to protect against OP toxicity. This study has led to the discovery of a novel cabbage looper OPAH.

Materials and methods

Cells, larvae and virus

COS-7 cells were purchased from ATCC (Rockville, MD, U.S.A.). Insect cells (*Spodoptera frugiperda* Sf9 cells and *T. ni* High 5 cells) and wild-type (wt)-AcNPV were purchased from Invitrogen (San Diego, CA, U.S.A.). Fourth- or fifth-instar cabbage looper larvae and all entomological supplies were purchased from EntoPath (Easton, PA, U.S.A.). The BuChE–AcNPV baculovirus was the generous gift of Dr Oksana Lockridge (University of Nebraska Medical Center, Omaha, NE, U.S.A.).

Production of (rh)BuChE in COS-7 cells, insect cells and cabbage looper larvae

COS-7 cell expression Qiagen-purified plasmid DNA containing the human wt-BuChE cDNA cloned into the vector pRcCMV (Invitrogen) was used to transfect COS-7 cells by the DEAE-dextran method [10]. Following transfection, cells were grown in 5% CO_2 at 37 °C in Dulbecco's modified Eagles's medium (Life Technologies, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum. After 24 h, medium was removed, and cells were washed with Hank's balanced salt solution and incubated for an additional 60 h in Opti-MEM I reduced-serum medium (Life Technologies). Control medium was prepared by culturing non-transfected cells under identical conditions. Harvested medium was centrifuged at 2300 ***g*** and concentrated by ultrafiltration.

High 5 and Sf9 expression The BuChE–AcNPV consisted of the human wt-BuChE cDNA placed downstream of the polyhedrin promoter (O. Lockridge, personal communication) and was amplified to high titres using conventional procedures [7]. High-titre BuChE–AcNPV (multiplicity of infection, 10) was used to infect 3.0×10^6 Sf9 or High 5 cells. Monolayer cultures were grown in Sf-900 II serum-free medium (Life Technologies) at 27 °C for 84 h. Control medium was prepared by culturing wt-AcNPV-infected (multiplicity of infection, 10) and -uninfected insect cells under identical culture conditions. After harvesting, media were centrifuged at 2300 ***g***, concentrated by ultrafiltration and assayed for BuChE activity.

Insect larval expression *T. ni* larvae, late in the fourth instar, were infected either by mouth or by direct injection. Infection by mouth involved growing the larvae for $\approx$ 72 h at 30 °C on synthetic medium overlayed with 300 μl of high-titre BuChE–AcNPV or wt-AcNPV. Alternatively, 5 μl of the BuChE–AcNPV or wt-AcNPV was injected with a syringe and the larvae allowed to grow on synthetic medium for 72 h at 30 °C. Harvesting involved homogenizing the larvae in 150 mM NaCl either alone or with a protease-inhibitor cocktail (1 mg/ml EDTA, 0.1 mg/ml (4-amidinophenyl)methanesulphonyl fluoride (APMSF), 0.3 mg/ml E-64, 0.5 μg/ml leupeptin, 0.1 mg/ml soya bean trypsin inhibitor in PBS, pH 7.4). This was followed by centrifugation at 14000 ***g*** for 20 min at 4 °C. The resulting supernatant devoid of haemocytes and cell debris was transferred to a new tube and analysed immediately or stored at 4 °C.

Insect larval animal model Cabbage looper larvae late in the fourth instar were infected by injecting 5 μl of the high-titre BuChE–AcNPV or wt-AcNPV as described above. Larvae were grown on synthetic medium for approximately 72 h at 30 °C and challenged with the chemical-warfare OP nerve agents soman or VX. The challenge was performed either by placing larvae on a 60-mm petri dish saturated with 11 mM soman or 3.5 mM VX or by directly injecting 5 μl of the OP into the larva. The time of death of each larva was measured for BuChE–AcNPV, wt-AcNPV and uninfected controls.

Analysis of expressed proteins

SDS/PAGE was performed in 7.5% polyacrylamide gels using the buffer system of Laemmli [11]. Immunoblotting of nitrocellulose-immobilized proteins was performed following standard procedures, probed with rabbit anti-human BuChE and visualized with goat anti-rabbit IgG horseradish peroxidase. (rh)BuChE enzyme activity was confirmed by staining 7.5% non-denaturing polyacrylamide gels with 1 mM butyrylthiocholine (BuSCh; Sigma, St. Louis, MO, U.S.A.) following the method of Karnovsky and Roots [12]. The molecular mass of the endogenous *T. ni* OPAH activity was determined by applying larval homogenates and known molecular-mass standards (horseradish peroxidase and *Torpedo californica* AChE) to a TSK-GEL 2000 SW gel-filtration column (Supelco, Bellefonte, PA, U.S.A.) and collecting fractions. Proteins were eluted from the column with 150 mM NaCl, pH 7.0, and analysed for activity as described below.

Kinetic assays

Michaelis–Menten constants (K_m) of (rh)BuChE expressed in larvae were determined for the substrates BuSCh and acetylthiocholine (Sigma) using a variation of the Ellman method [13], modified for use in a microtitre plate reader. Briefly, 50 μl of the sample to be tested was placed into each well in a row of wells of a microtitre plate. In another row, the first well was filled with 200 μl of a high concentration (usually 1 mM) of substrate containing 0.22 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M phosphate buffer, pH 8.0, at 25 °C. To each of the remaining wells in the row 100 μl of the phosphate buffer was added, and a series of dilutions was made by pipetting 100 μl of the contents of the first well into the second, mixing, transferring 100 μl of that solution to the next, mixing, transferring 100 μl of that solution to the next and so on to the end of the row. Then, using a multiple pipettor the contents of the entire row were transferred to the row containing the enzyme sample and mixed; the plate was placed in the microtitre plate reader and assayed in the 'kinetics' mode. In this manner reaction rates were obtained for a large range of substrate concentrations simultaneously under the same reaction conditions.

OPAH activity of the potential substrates soman, sarin and tabun (ethyl dimethylphosphoramidocyanidate, or GA) was measured in a pH stat (Radiometer, Copenhagen, Denmark) using 2 ml of a 1 mM solution of the test compound in 150 mM NaCl. The activity against VX and echothiophate {2-[(diethoxy phosphinyl)thio]-*N*,*N*,*N*-trimethyl ethanaminium} was measured in a microtitre plate using a variation of the Ellman method described above, with the OP compound replacing the BuSCh as substrate. The hydrolysis of paraoxon (*O*,*O*-diethyl *p*-nitrophenyl phosphate) was followed by measuring the release of *p*-nitrophenol spectrophotometrically at 405 nm, usually in a microtitre plate reader. All chemical-warfare nerve agents (tabun, sarin, soman and VX) were obtained from the Edgewood Chemical and Biological Center (ECBC), Aberdeen Proving Ground, MD, U.S.A., and determined by gas chromatography to be $>$ 98% pure. These agents are extremely toxic and were handled according to established regulations governing chemical-warfare surety materials.

Results and discussion

This project was designed to examine the ability of three expression systems to cost-effectively overexpress biologically active human BuChE. It was determined that baculovirus-infected insect cells (Sf9 and High 5) expressed 3.5- and 8.2-fold, respectively, more active enzyme than COS-7 cells. However, based on the activity per unit volume of the larval homogenates, the production of BuChE activity by baculovirus-infected *T. ni* larvae far surpassed all three of the cell culture lines, expressing more than 26 times that of the High 5 cell media. In fact, one baculovirus-infected insect larva produced more active BuChE than 100 ml of infected insect cell-culture medium. This equates in cost to US$0.05 for the larva versus $\approx$ $5.00 for 100 ml of the medium, a saving of well over 100-times when one includes the additional expenses associated with maintaining the cell line (i.e. sterile plasticware and medium, etc.).

Background levels of endogenous larval cholinesterase were very limited based on Western blots (Figure 1, lane 6), activity gel stains (Figure 2, lane 10) and kinetic assays of controls (results not shown). As in humans, acetylcholine in insects serves as a major excitatory neurotransmitter, so this finding was not expected. This result may be due to the larval cholinesterase being membrane-bound or to a signifi-

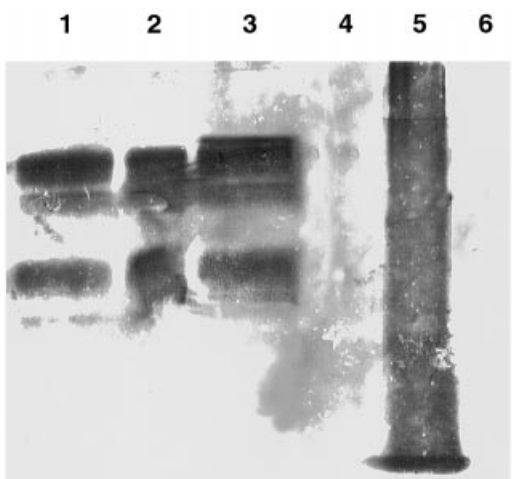

Figure 1 Western-blot analysis of insect larval homogenates

Lanes 1–3, 5, 1 and 15 units of BuChE; lane 4, empty; lane 5, 10 μl of infected *T. ni* homogenate; lane 6, 10 μl of uninfected *T. ni* homogenate.

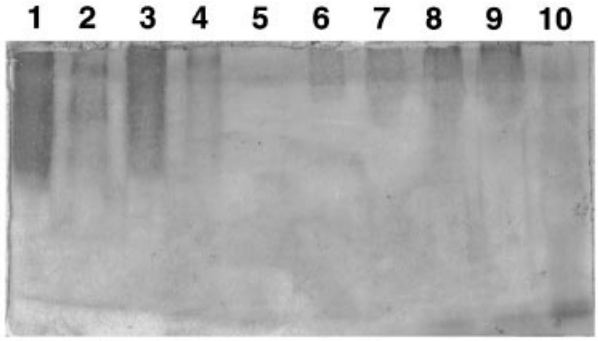

Figure 2 Activity gel stain of insect larval homogenates

Lanes 1–4, 15, 1.5, 9 and 0.15 units of BuChE; lane 5, empty; lanes 6 and 7, 1 μl of infected larvae homogenate; lanes 8 and 9, 10 μl of infected larvae homogenate; lane 10, 10 μl of uninfected *T. ni* homogenate.

cant dissimilarity between human BuChE and the larval cholinesterase in shape and/or function. For example, work on the *Drosophila* cholinesterase has shown that the insect carries one enzyme that has intermediate features between AChE and BuChE in its encoded protein sequence [14] as well as its catalytic specificity [15].

An attempt to estimate the recombinant BuChE protein concentration by immunoblotting resulted in a smear indicative of extensive degradation of the majority of protein recognized by anti-BuChE polyclonal antibody (Figure 1, lane 5). Homogenization of larvae in a protease-inhibitor cocktail resulted in a similar smear, suggesting that the degradation events occurred *in vivo* and not during harvesting. This degraded protein is apparently inactive since native gels stained for cholinesterase activity showed that the BuChE activity was limited to a small high-molecular-mass stain at the top of the gel (Figure 2, lane 6–10). However, whereas the majority of the recombinant protein expressed was inactive, the active species does have similar enzyme kinetics to its human counterpart. K_m values for acetylthiocholine and BuSCh were determined to be 0.050 and 0.025 mM, respectively, which are very similar to those of human serum BuChE (0.043 and 0.0177 mM, respectively) [16].

The extensive degradation *in vivo* observed here may largely be due to the process of N-linked glycosylation in insects differing from that in mammals [7]. Human BuChE is heavily N-glycosylated (23.9% by weight [17,18]) with at least nine out of ten potential N-linkage sites carrying an oligosaccharide chain [19]. It seems plausible that altered glycosylation may cause improper folding of the macromolecule, resulting in a general lack of secretion, build-up of high intracellular levels leading to inclusion bodies and heightened protease susceptibility.

The toxicities of the chemical-warfare OP nerve agents soman and VX in *T. ni* were also examined to determine whether the overexpressed (rh)BuChE could enhance survivability. No significant difference was found between the BuChE–AcNPV-infected, wt-AcNPV-infected or the uninfected larva controls (results not shown). However, the cabbage looper was found naturally to possess a high resistance to these compounds. Larvae survived for several hours when exposed to 11 mM soman or 3.5 mM VX by contact, but only for several minutes when injected directly with the OP. When lower concentrations of nerve agent were injected, survival times were extended, to $\approx$ 15 min for 5.5 mM soman and 1.755 mM VX and to several hours for both OPs at 1 mM. The absence of enhanced survivability by the overexpression of BuChE–AcNPV larvae was probably due to the very small amount of OP that can be stoichiometrically scavenged by the active form of the BuChE compared with the high lethal dose of the agents in *T. ni*.

Subsequent experiments focused on why the cabbage looper larvae have such a high tolerance to the highly toxic OPs. Homogenates of uninfected *T. ni* larva were analysed for the ability to hydrolyse soman and VX and several other anti-cholinesterases. It was determined that the larvae contained an enzyme(s) capable of hydrolysing soman and sarin but not VX, tabun, echothiophate or paraoxon. Apparently the novel OPAH possesses the ability to hydrolyse the P–F bond found in the phosphorus centre of soman and sarin but not the P–CN, *p*-nitrophenol or P–S bonds found in tabun, paraoxon or VX, and echothiophate, respectively. Characterization of the *T. ni* OPAH revealed that soman was hydrolysed $\approx$ 1.44 times faster than sarin and that this activity was enhanced by the addition of manganese, but not calcium, in the assay mixture. Further, size fractionation revealed that this OPAH has a molecular mass similar to that of tetrameric *T. californica* AChE and a dissimilar mobility to the endogenous *T. ni* carboxylesterase. The observed larval tolerance to VX cannot be explained based by an endogenous OPAH activity but instead may result from mechanisms similar to those reported to cause heightened insect resistance to OP insecticides [20].

It is concluded that the cabbage looper larvae system may be an economical means of overproducing recombinant BuChE and other proteins in a eukaryotic system.

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