Original Research

Identification of Carboxylesterase, Butyrylcholinesterase, Acetylcholinesterase, Paraoxonase, and Albumin Pseudoesterase in Guinea Pig Plasma through Nondenaturing Gel Electrophoresis

Geoffroy Napon,^{1,2} Alicia J Dafferner,¹ Ashima Saxena,^{3,†} and Oksana Lockridge^{1,*}

Drugs to protect against nerve agent toxicity are tested in animals. The current preferred small animal model is guinea pigs because their plasma bioscavenging capacity resembles that of NHP. We stained nondenaturing polyacrylamide slab gels with a variety of substrates, inhibitors, and antibodies to identify the esterases in heparinized guinea pig plasma. An intense band of carboxylesterase activity migrated behind albumin. Minor carboxylesterase bands were revealed after background activity from paraoxonase was inhibited by using EDTA. The major butyrylcholinesterase band was a disulfide-linked dimer. Incubation with the antihuman butyrylcholinesterase antibody B2 18-5 shifted the butyrylcholinesterase dimer band to slower migrating complexes. Carboxylesterases were distinguished from butyrylcholinesterase by their sensitivity to inhibition by bis-*p*-nitrophenyl phosphate. Acetylcholinesterase tetramers formed a complex with the antihuman acetylcholinesterase antibody HR2. Organophosphorus toxicants including cresyl saligenin phosphate, dichlorvos, and chlorpyrifos oxon irreversibly inhibited the serine esterases but not paraoxonase. Albumin pseudoesterase activity was seen in gels stained with α - or β -naphthyl acetate and fast blue RR. We conclude that guinea pig plasma has 2 types of carboxylesterase, butyrylcholinesterase dimers and 5 minor butyrylcholinesterase forms, a small amount of acetylcholinesterase tetramers, paraoxonase, and albumin pseudoesterase activity. A knockout mouse with no carboxylesterase activity in plasma is available and may prove to be a better model for studies of nerve agent toxicology than guinea pigs.

Abbreviations: BNPP, bis-nitrophenyl phosphate; CBDP, cresyl saligenin phosphate; HuBChE, human butyrylcholinesterase; isoOMPA, tetraisopropyl pyrophosphoramidate

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Guinea pigs (*Cavia porcellus*) are the currently accepted small animal model for studying the toxicology of organophosphorus nerve agents.^{24,27,33,38,40} The LD₅₀ of soman is 4-fold higher in rats than in guinea pigs, a difference attributed to a higher level of carboxylesterase activity in rat plasma.³⁴ Carboxylesterase in rat plasma protects the animal from nerve agent toxicity by binding and inactivating soman, thus limiting the amount of soman that inhibits acetylcholinesterase in the cholinergic nervous system. Humans and NHP lack carboxylesterase in plasma and serum.²⁹

Guinea pig plasma contains at least 2 classes of carboxylesterase.^{4,7,17} In an earlier study,⁴ plasma proteins separated through preparative column electrophoresis were assayed for hydrolase activity by 8 different esters and for inhibition by physostigmine and tetraisopropyl pyrophosphoramidate (isoOMPA). The

author thus classified the esterases in heparin-treated guinea pig plasma into 3 groups: esterase A (paraoxonase in current terminology), 2 types of B esterases (carboxylesterase), and one C esterase (butyrylcholinesterase). The carboxylesterases in guinea pig and rat plasma are inhibited by isoOMPA,^{4,18} a compound generally regarded as a specific inhibitor of butyrylcholinesterase. Separation of guinea pig plasma esterases through chromatofocusing or polyacrylamide gel electrophoresis followed by assays with a variety of ester substrates and a panel of inhibitors identified carboxylesterases with 3 different isoelectric points, molecular weights of 80 and 58 kDa, and distinct reactivities to monoclonal antibodies against carboxylesterase.^{7,16,17}

No single study has identified all the esterases in guinea pig plasma. One group used polyacrylamide tube gels and identified 4 carboxylesterases and one arylesterase (that is, paraoxonase) but found no cholinesterase in guinea pig serum.²¹ Separation by starch gel electrophoresis identified pseudocholinesterase (that is, butyrylcholinesterase) and carboxylesterase but no arylesterase (that is, paraoxonase) in heparin-treated guinea pig plasma.^{9,14} Aldridge tested sera from 9 species for the

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¹Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska;

²University of Nebraska–Omaha, Omaha, Nebraska; and ³Division of Biochemistry, Walter Reed Army Institute of Research, Silver Spring, Maryland

*Corresponding author. Email: olockrid@unmc.edu

[†]Present address. US Military HIV Research Program, Walter Reed Army Institute of Research, Silver Spring, Maryland

ability to hydrolyze paraoxon and reported that guinea pig serum contains arylesterase (that is, paraoxonase) at levels similar to those in rat.¹

We identified the esterases in guinea pig plasma by using nondenaturing gradient polyacrylamide slab gels. Gel-shift assays with monoclonal antibodies revealed bands of acetylcholinesterase and butyrylcholinesterase activity. Paraoxonase was identified through its resistance to inhibition by cresyl saligenin phosphate (CBDP), dichlorvos, and chlorpyrifos oxon and sensitivity to inhibition by EDTA. Carboxylesterase activity was inhibited by CBDP, dichlorvos, chlorpyrifos oxon, and bis-*p*-nitrophenyl phosphate (BNPP) but not EDTA. Albumin pseudoesterase activity was visualized on gels stained with β - or α -naphthyl acetate and fast blue RR dye.

Materials and Methods

CBDP (CAS 1222-87-3) was synthesized by Dr John Mikler (Medical Countermeasures Section, Defense Research Establishment Suffield, Medicine Hat, Alberta, Canada). Dichlorvos Chem Service PS-89. Chlorpyrifos oxon (catalog no. MET-674B) was obtained from Chem Service (West Chester, PA); α -naphthylacetate (N8505), β -naphthylacetate (N6875), fast blue RR dye (F0500), isoOMPA (CAS 513-00-8; catalog no., T1505) and BNPP (N3002) were purchased from Sigma (St Louis, MO). BNPP was prepared as a 20-mM solution in dimethyl sulfoxide. Heparin-treated guinea pig plasma was purchased from Innovative Research (catalog no., 16094; 1.0 U/mL with butyrylthiocholine; Novi, MI). Heparin-treated human plasma (2.9 U/mL with butyrylthiocholine) was obtained from the University of Nebraska Medical Center blood bank (Omaha, NE). Heparin-treated plasma from Sprague-Dawley rats (IRTSD-NaHeparin, catalog no. 16666) was purchased from Innovative Research. Guinea pig acetylcholinesterase was purified from erythrocytes (Dr Ashima Saxena, Walter Reed Institute of Research, Silver Spring, MD). Human butyrylcholinesterase (HuBChE; UniProt accession no. P06276) was purified from plasma by Drs Lawrence Schopfer and Oksana Lockridge (University of Nebraska Medical Center, Omaha, NE). Fetal bovine serum was purchased from Gibco (Gaithersburg, MD). Antihuman acetylcholinesterase monoclonal antibody HR2 (1 mg/mL) was obtained from Thermo Fisher Scientific (catalog no. MA3-042, Waltham, MA). Antihuman acetylcholinesterase antibodies AE1, AE2, 1G, 6A, and 10D have been described previously.¹¹ AntiHuBChE monoclonal antibody B2 18-5 (GenBank accession nos. KT189143 and KT189144; 0.91 mg/mL) has been described previously.³⁶

Nondenaturing 4% to 30% polyacrylamide gels. Polyacrylamide 4% to 30% gradient slab gels (thickness, 0.75 mm), each with a 4% stacking gel were poured in a vertical slab gel apparatus (model SE600, Hoefer, Thermo Fisher Scientific). Electrophoresis was performed at 4 °C and a constant voltage of 320 V for 18 h. Before being loaded on a gel, each plasma sample (volume, 5 μ L) was diluted with 5 μ L of 0.1 M TrisCl pH 7.5 and 10 μ L of 60% glycerol containing 0.1% bromophenol blue.

Staining gels for acetylcholinesterase and butyrylcholinesterase activity. A previously described histochemical method was adapted for use with polyacrylamide gels.²⁹ The substrate acetylthiocholine iodide revealed brown-red bands for both acetylcholinesterase and butyrylcholinesterase. The band for acetylcholinesterase was identified by gel shift after binding the antiacetylcholinesterase antibody HR2. Samples containing 1.2 μ L guinea pig erythrocyte acetylcholinesterase (0.016 U activity, 0.003 μ g acetylcholinesterase) plus 12.9 μ L 1 mg/mL bovine albumin in PBS and 4 μ L antibody were incubated 2 h at room temperature. The bovine albumin minimized sticking

of acetylcholinesterase to the walls of the microfuge tube. When butyrylthiocholine iodide was used in the staining solution, bands for butyrylcholinesterase but not acetylcholinesterase appeared. Butyrylcholinesterase bands were distinguished from carboxylesterase bands by gel shift due to binding the anti-butyrylcholinesterase monoclonal antibody B2 18-5 and by specific inhibition of carboxylesterase activity by bis-*p*-nitrophenyl phosphate.

Staining gels for paraoxonase activity. The staining solution contained 100 mL of 50 mM TrisCl pH 7.5, 10 mM CaCl₂, 50 mg β -naphthylacetate in 1 mL ethanol, and 50 mg of solid fast blue RR dye.²⁹ The gel was gently shaken at room temperature for 20 min until pink bands due to paraoxonase activity appeared. In addition, a dark band for the major carboxylesterase and pink bands for butyrylcholinesterase and minor carboxylesterase isozymes appeared. Gels were washed with water and photographed on a light table. The stain was specific for paraoxonase when plasma was pretreated with an organophosphorus toxicant (for example, CBDP, chlorpyrifos oxon, or dichlorvos) that inhibited carboxylesterase, butyrylcholinesterase, and acetylcholinesterase.

Staining gels for carboxylesterase activity. Bands for carboxylesterase activity without paraoxonase activity were seen when the gel was stained with α -naphthyl acetate and fast blue RR dye.²⁹ This solution also stained bands for butyrylcholinesterase and albumin but not acetylcholinesterase. The staining solution contained 100 mL of 50 mM TrisCl pH 7.5, 50 mg of α -naphthyl acetate in 1 mL ethanol, and 50 mg of solid Fast blue RR. Bands were dark green.

Counterstaining. Gels previously stained for acetylcholinesterase or butyrylcholinesterase activity were counterstained by using α -naphthyl acetate and fast blue RR dye.

Staining for albumin pseudoesterase activity. Gels stained with α - or β -naphthyl acetate and fast blue RR dye revealed a heavy area of albumin pseudoesterase activity, which migrated faster than other esterases. Counterstaining with Coomassie blue confirmed that this band contained albumin because it was the most abundant protein on the gel and because it migrated to the same position as purified albumin. Albumin pseudoesterase activity was not inhibited by organophosphate esters or EDTA.

Inhibitor treatment to identify esterases. Guinea pig and human plasma samples were incubated with 0.4 mM CBDP, 10 mM dichlorvos, 0.25 mM chlorpyrifos oxon, or 0.4 mM isoOMPA for 2 h at 25 °C and assayed for butyrylcholinesterase activity to assure that butyrylcholinesterase activity was completely inhibited; treated samples then underwent gel electrophoresis. This treatment inhibits serine esterases but has no effect on paraoxonase activity. Plasma treated with 10 mM EDTA has no paraoxonase activity because the EDTA removes the calcium ions that are part of the active site of paraoxonase.^{15,20} Carboxylesterase activity was specifically inhibited by treatment with 1 mM bis-*p*-nitrophenyl phosphate for 1 h.⁷

Results

Antihuman monoclonal antibody HR2 recognized guinea pig acetylcholinesterase dimers and tetramers. Acetylcholinesterase purified from guinea pig erythrocytes (Figure 1) represents disulfide-linked dimers that have been freed of their glycolipid membrane anchor by treatment with phospholipase C. By analogy to human acetylcholinesterase, the C-terminus of guinea pig erythrocyte acetylcholinesterase is alternatively spliced to exon 5, where the addition of a glycopospholipid anchor to Gly587 (GenBank accession no., XP_023420887) deletes 22 amino acids from the C-terminus.^{19,31} We tested a set of 6

Figure 1. Monoclonal antibody HR2 binds guinea pig (GP) erythrocyte G2 acetylcholinesterase (AChE) dimers. The nondenaturing gradient polyacrylamide gel was stained for acetylcholinesterase activity by using acetylthiocholine.²³ After the addition of 60% glycerol and 0.1% bromphenol blue, samples were immediately loaded on the gel to minimize photoinactivation of acetylcholinesterase by bromophenol blue.

monoclonal antibodies to human acetylcholinesterase for binding to guinea pig erythrocyte acetylcholinesterase in gel-shift assays. Treatment with the HR2 antibody shifted the position of purified guinea pig erythrocyte acetylcholinesterase dimers, whereas the other 5 antibodies had no effect (Figure 1). In addition, HR2 bound acetylcholinesterase tetramers in guinea pig plasma (Figure 2, lanes 5 and 9). These results confirmed that antibody HR2 binds guinea pig erythrocyte acetylcholinesterase dimers³⁹ and plasma acetylcholinesterase tetramers.

Acetylcholinesterase tetramers in guinea pig plasma. The presence of acetylcholinesterase tetramers in guinea pig plasma was visualized by comparing the migration of guinea pig G2 acetylcholinesterase dimers (Figure 2, lane 4) and fetal bovine acetylcholinesterase tetramers (lane 6) with bands in guinea pig plasma (lane 5). Guinea pig plasma (Figure 2, lane 5) had no band at the position of guinea pig dimer (lane 4) but did have a band at the position of fetal bovine acetylcholinesterase tetramers (lane 6). The acetylcholinesterase bands (Figure 2, lanes 4 through 6) disappeared from their original position after incubation with antibody HR2 (Figure 2, lanes 8 through 10), forming complexes that migrated near the top of the gel.

The presence of the G4 acetylcholinesterase tetramer band in guinea pig plasma treated with 0.4 mM isoOMPA supported the conclusion that guinea pig plasma contains acetylcholinesterase (Figure 2, lane 13). Acetylcholinesterase was resistant to inhibition by isoOMPA, whereas HuBChE was sensitive to inhibition by isoOMPA. Likewise, isoOMPA inhibited guinea pig carboxylesterase (B esterase in previous terminology⁴). IsoOMPA-treated guinea pig plasma lacked all butyrylcholinesterases and

Figure 2. Gel-shift assay identifies acetylcholinesterase tetramers in guinea pig (GP) plasma. Antihuman acetylcholinesterase monoclonal HR2 binds guinea pig acetylcholinesterase (AChE) dimers (lane 8), guinea pig acetylcholinesterase tetramers (lane 9), and bovine acetylcholinesterase tetramers (lane 10). The nondenaturing gel was stained with acetylthiocholine to reveal acetylcholinesterase and butyrylcholinesterase (BChE) activity. Each well contained 10 μ L plasma. FBS, fetal bovine serum; Hu, human.

carboxylesterases (Figure 2, lane 13), leaving only the band corresponding to isoOMPA-resistant G4 acetylcholinesterase tetramer. The band-free lane containing human plasma treated with isoOMPA confirms that 0.4 mM isoOMPA inhibited butyrylcholinesterase. The blue bands in Figure 2 are albumin complexes with bromophenol blue. The G2 and G4 acetylcholinesterase bands (Figure 2, lanes 4 and 5) did not stain with butyrylthiocholine (data not shown), consistent with the specificity of butyrylthiocholine as a substrate for butyrylcholinesterase. We therefore concluded that guinea pig plasma has G4 acetylcholinesterase tetramers and no other acetylcholinesterase isozymes.

Butyrylcholinesterase in guinea pig plasma. The most intense butyrylthiocholine-hydrolyzing band in guinea pig plasma was the G2 butyrylcholinesterase dimer (Figure 3, lane 1). This band was identified as butyrylcholinesterase by its reaction with the antiHuBChE antibody B2 18-5. After incubation with B2 18-5, the G2 butyrylcholinesterase dimer band shifted to a slower migrating position (Figure 3, lanes 2 through 4). Butyrylcholinesterase was assumed to be a dimer because it migrated at the level of the G2 butyrylcholinesterase dimer in human plasma (Figure 3, lane 5). This band dissociated after treatment of guinea pig plasma with 10 mM dithiothreitol, confirming that it is a disulfide-linked guinea pig G2 butyrylcholinesterase dimer. Human G1 monomers, G2 dimers, and G4 tetramers of butyrylcholinesterase formed complexes with antibody B2 18-5 that shifted to slower migrating positions on the gel (Figure 3, lane 6). The location of the epitope that binds antibody B2 18-5 is

Figure 3. Gel-shift assay identifies G2 butyrylcholinesterase (BChE) dimers in guinea pig (GP) plasma. The gel was stained with butyrylthiocholine for BChE activity. Hu, human.

unknown, but because the antibody bound HuBChE monomers (Figure 3, lane 6), the epitope is independent of subunit contacts.

Five additional bands in Figure 3 (lanes 1 through 4) are isozymes of guinea pig butyrylcholinesterase, given that they hydrolyzed butyrylthiocholine and acetylthiocholine, were inhibited by isoOMPA, and were not inhibited by the specific carboxylesterase inhibitor BNPP (Figure 4). We conclude that guinea pig plasma has G2 butyrylcholinesterase dimers and 5 additional butyrylcholinesterase isozymes.

The B2 18-5 monoclonal antibody was created by using butyrylcholinesterase purified from human plasma as antigen.⁶ AntiHuBChE monoclonal antibodies mAb2 and 11D8³⁵ did not recognize guinea pig plasma butyrylcholinesterase.

Carboxylesterase in guinea pig plasma. The inability of antibody B2 18-5 to shift the minor butyrylcholinesterase bands (Figure 3 lanes 2-4) led us to examine the possibility that these bands are carboxylesterase rather than butyrylcholinesterase. Heparin-treated plasma from guinea pigs, rats, and humans was treated with the specific carboxylesterase inhibitor BNPP. The butyrylcholinesterase isozymes in guinea pig (Figure 4 A, lanes 3 and 4) and human (lane 8) plasma were not inhibited by BNPP. The butyrylcholinesterase activity in rat plasma (Figure 4 A, lanes 5 and 6) was too low to show butyrylcholinesterase bands from 5 µL plasma. We concluded that the BNPP-resistant bands (labeled as 'BChE' in Figure 4 A and B) are butyrylcholinesterase and not carboxylesterase.

The gel was counterstained with α -naphthyl acetate and fast blue RR (Figure 4 B), revealing intense bands for carboxylesterase activity in control samples of guinea pig plasma (labeled

Figure 4. Inhibition of carboxylesterase activity with 1 mM BNPP. (A) Gel stained for butyrylcholinesterase (BChE) activity by using butyrylthiocholine. (B) Same gel counterstained with α -naphthyl acetate and fast blue RR to show carboxylesterase (Ces). Alb, albumin; Hu, human.

'Ces'; lanes 1 and 2) and control rat plasma (lane 5). Incubation with 1 mM BNPP reduced carboxylesterase staining in both guinea pig plasma (Figure 4 B, lanes 3 and 4) and rat plasma (lane 6). Human plasma lacks carboxylesterase²⁹ and showed no change in the pattern of stained bands after treatment with BNPP. The intense band of carboxylesterase activity migrated behind albumin (Figure 4); together, these data confirm that BNPP inhibits carboxylesterase but does not inhibit butyrylcholinesterase activity.

A weak double band of carboxylesterase activity was inhibited by BNPP (labeled 'Ces' in Figures 4 B and 5) and was more intense in the gel stained with β -naphthyl acetate after background staining for paraoxonase was removed (Figure 5).

Paraoxonase in guinea pig plasma. Whereas paraoxonase activity is maintained in the presence of heparin, paraoxonase is irreversibly inactivated when blood is treated with EDTA, because EDTA chelates the calcium ions that are a critical component of the active site of paraoxonase.²⁶ Heparin-treated human plasma (Figure 5, lane 5) showed a broad band of paraoxonase activity, reflecting the heterogeneity of the HDL complex that includes paraoxonase 1.²³

Paraoxonase is distinguished from the serine esterases acetylcholinesterase, butyrylcholinesterase, and carboxylesterase by

Figure 5. Identification of paraoxonase in a gel stained with β -naphthyl acetate and fast blue RR. Paraoxonase is visible after background activity from butyrylcholinesterase and carboxylesterase is inhibited by using CDBP (lane 3). Weak bands for carboxylesterase (Ces) activity are visible in lane 2 after paraoxonase activity is inactivated by EDTA.

its sensitivity to inactivation by EDTA and its resistance to inhibition by organophosphorus toxicants.^{10,26} We used this information to identify paraoxonase in guinea pig heparin plasma. Guinea pig plasma treated with EDTA (Figure 5, lane 2) retained bands for carboxylesterase and butyrylcholinesterase but lost 2 broad bands corresponding to paraoxonase. After treatment of guinea pig plasma with CDBP, the paraoxonase bands were clearly visible (Figure 5, lane 3), whereas the background activity from carboxylesterase and butyrylcholinesterase was eliminated. Treatment with CDBP and EDTA (Figure 5, lane 4) inactivated all esterases, with the exception of the pseudoeesterase activity of albumin. Similar results were obtained when guinea pig plasma was treated with the organophosphorus toxicants dichlorvos and chlorpyrifos oxon.

The broadband of paraoxonase activity in heparinized human plasma (Figure 5, lane 5) disappeared when plasma was treated with 10 mM EDTA (lane 6) but was retained in the presence of CDBP (lane 7). These results for human plasma confirmed that paraoxonase could be identified by these treatments. We concluded that guinea pig plasma has paraoxonase.

Discussion

The most abundant esterase in guinea pig plasma is the carboxylesterase that migrates behind albumin. This carboxylesterase stains with β -naphthyl acetate and α -naphthyl acetate but not with butyrylthiocholine or acetylthiocholine. In addition, 2 weak bands of carboxylesterase activity are revealed when background activity from paraoxonase is inhibited with EDTA. The carboxylesterases are inhibited by organophosphorus toxicants including CDBP, dichlorvos, and chlorpyrifos oxon. Guinea pig carboxylesterases are inhibited by isoOMPA, a finding also reported previously.⁴ Guinea pig carboxylesterases are inhibited by BNPP,⁷ thus distinguishing them from butyrylcholinesterase, which is not inhibited by BNPP. An isoelectric point of pI 3.9 was reported for the most abundant carboxylesterase

and of pI 6.2 for a minor carboxylesterase.⁷ Guinea pig plasma carboxylesterases previously were segregated into 2 groups according to their recognition by monoclonal antibodies raised against rat lung carboxylesterase.¹⁷

CBDP is considered as a specific inhibitor of carboxylesterase activity when carboxylesterases are defined as esterases that hydrolyze uncharged esters, including *p*-nitrophenyl acetate, *o*-nitrophenyl butyrate, phenyl butyrate, phenyl acetate, α -naphthyl acetate, and β -naphthyl acetate. This definition groups butyrylcholinesterase with carboxylesterases, because butyrylcholinesterase hydrolyzes the same neutral esters. Sequencing of the human genome has clearly established that butyrylcholinesterase is different from the family of carboxylesterases. A single gene on human chromosome 3q26.2 codes for butyrylcholinesterase.² A cluster of genes on human chromosome 16 codes for 5 families of carboxylesterases.²² CDBP inhibits both butyrylcholinesterase and plasma carboxylesterases. The crystal structure of HuBChE covalently modified by CDBP on the active site serine is available in PDB (code 4BBZ).⁸

In agreement with previous findings, we found butyrylcholinesterase in guinea pig plasma.⁹ The dominant guinea pig butyrylcholinesterase appears to be a disulfide-linked dimer with an estimated molecular weight of 170 kDa. This G2 butyrylcholinesterase dimer is recognized by the antibutyrylcholinesterase antibody B2 18-5. Minor butyrylcholinesterase isozymes include a G4 tetramer, a G1 monomer, and 3 other weak bands of unknown structure. Monoclonal antibody B2 18-5 does not form a stable complex with the minor butyrylcholinesterase forms in guinea pig plasma. Bands of butyrylcholinesterase activity stain with butyrylthiocholine and acetylthiocholine, are inhibited by organophosphorus toxicants and isoOMPA, but are unaffected by EDTA (a specific inhibitor of paraoxonase) and BNPP (a specific inhibitor of carboxylesterase).

The apparent dimer structure of the dominant form of butyrylcholinesterase in guinea pig plasma was a surprise because the dominant form of butyrylcholinesterase in human and equine plasma is the G4 tetramer. The tetramer-organizing domain in human and equine butyrylcholinesterase consists of 40 amino acids at the C-terminus of the 574 amino acid subunit³ (Table 1). Four butyrylcholinesterase subunits assemble into a tetramer by interaction with a polyproline-rich peptide.^{5,30,37} The polyproline-rich peptide is tightly bound to the butyrylcholinesterase tetramer through noncovalent contacts with 12 tryptophans (3 per subunit) in the tetramerization domain. The guinea pig butyrylcholinesterase tetramerization domain (Table 1) contains the conserved 3 tryptophans that stack with polyproline.¹³ This finding suggests that the tetramerization domain of guinea pig butyrylcholinesterase is capable of binding polyproline and that the dearth of butyrylcholinesterase tetramers is explained by an inadequate supply of polyproline peptides.

A weak band of G4 acetylcholinesterase tetramers in guinea pig plasma was identified based on a gel shift assay with the antiacetylcholinesterase antibody HR2. The commercially available HR2 antibody was created by using human brain acetylcholinesterase as antigen.³⁹ Additional evidence for the presence of G4 tetramers in guinea pig plasma includes the following: 1) guinea pig plasma acetylcholinesterase migrated to the same position as acetylcholinesterase tetramers in fetal bovine serum, 2) guinea pig acetylcholinesterase was resistant to inhibition by iso-OMPA, an established characteristic of acetylcholinesterase, 3) guinea pig acetylcholinesterase activity was unaffected by EDTA, and 4) the band of guinea pig acetylcholinesterase stained with acetylthiocholine but not butyrylthiocholine.

Bands for paraoxonase activity are seen by staining gels with β -naphthyl acetate and fast blue RR. Paraoxonase activity in

Table 1. Tryptophan (W) residues in the tetramerization domain important for assembly of 4 subunits into tetramers through interaction with a polyproline-rich peptide

	Tetramerization domain	Accession no.
Guinea pig butyrylcholinesterase	NLDEVEQKWKAGFHLWNNYMTDWKNQFNDYISKKESCVDL	XP_003469406
Human butyrylcholinesterase	NIDEAEWEWKAGFHRWNNYMMDWKNQFNDYTSKKESCVGL	P05276
Guinea pig acetylcholinesterase	DTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQDRCSL	NP_001171362.1
Human acetylcholinesterase	DTLDEAERQWKAEFHRWSSYMVHWKNQFDHYSKQDRCSL	P22303

Table 2. Plasma esterase activity ($\mu\text{L CO}_2/0.10 \text{ mL plasma}/30 \text{ min}$) in human and guinea pig by using various substrates

Substrate	ACholine AChE + BChE	BCholine BChE	BzCholine BChE	PhAcetate PON + Ces	Tributyrin Ces + BChE	PhButyrate Ces + BChE
Human	135	360	50	3300	55	260
Guinea pig	50	170	20	1700	530	1200

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; Ces, carboxylesterase; PON, paraoxonase.

Substrate concentration was 10 mM in bicarbonate- CO_2 buffer pH 7.4. Data are from reference 4, where A esterase is PON1, B esterase is carboxylesterase, and C esterase is butyrylcholinesterase. The esterases in human plasma are paraoxonase (50 mg/L), butyrylcholinesterase (4 mg/L), acetylcholinesterase (0.004 mg/L), and carboxylesterase (0 mg/L).

human plasma is readily detected as a broad band, but paraoxonase is revealed in guinea pig plasma only after background activity from carboxylesterase and butyrylcholinesterase is inhibited with CDBP, dichlorvos, or chlorpyrifos oxon. Confirmation that an esterase band represents paraoxonase is the observation that paraoxonase is irreversibly inactivated by EDTA, whereas the serine esterases are unaffected by EDTA.¹⁵

Albumin stains with α - and β -naphthyl acetate and fast blue RR. Mass spectrometry has shown that the apparent esterase activity of albumin is explained by stable acetylation of 82 residues including 59 lysines.³² The concentration of albumin in plasma is 40 mg/mL, such that a 5- μL aliquot contains 200 μg protein. Adducts with 200 μg protein release enough naphthol to form visible amounts of insoluble azo dye with fast blue RR. Albumin pseudoesterase activity was not significantly reduced by treatment of plasma with 10 mM EDTA, 0.4 mM CDBP, 10 mM dichlorvos, 0.25 mM chlorpyrifos oxon, or 0.4 mM isoOMPA. The pseudoesterase activity of albumin is too low to contribute significantly to nerve agent detoxication.²⁸

The in vivo toxicity of soman varies markedly between species.³⁴ Pretreatment of animals with 2 mg/kg CDBP—a nontoxic dose that inhibits plasma carboxylesterase and butyrylcholinesterase—abolishes interspecies differences. The bioscavenger capacity of sera from rats, guinea pigs, and NHP was compared by measuring the quantity of radiolabeled soman bound in the absence and presence of 10 μM CDBP; bioscavenger capacity was equated as carboxylesterase concentration.²⁷ From these experiments, the authors concluded that rat serum has a carboxylesterase concentration of 4 μM , but guinea pig and NHP sera have none.

Our current results show that guinea pig plasma does in fact have carboxylesterase. The failure of guinea pig serum to bind radiolabeled soman²⁷ suggests that guinea pig carboxylesterase may have a very poor affinity for soman, such that no soman was covalently bound during the 5-min reaction time.

The concentration of each esterase in guinea pig plasma is unknown. A rough estimate can be deduced by comparing the esterase activity in guinea pig plasma to that in human plasma where the concentrations are known. In a previous study,⁴ esterase activity in human and guinea pig plasma was measured by using a variety of substrates. Acetylcholine (ACholine) is hydrolyzed by both acetylcholinesterase and butyrylcholinesterase. Because human plasma has negligible concentrations of acetylcholinesterase but

considerable butyrylcholinesterase, the 135 value for ACholine hydrolysis (Table 2) is attributed to butyrylcholinesterase activity. In addition, guinea pig plasma has very low acetylcholinesterase activity.⁴ Butyrylcholine (BCholine) and benzoylcholine (BzCholine) are hydrolyzed specifically by butyrylcholinesterase. Human plasma has about 2-fold more butyrylcholinesterase per milliliter than guinea pig plasma. Phenyl acetate is hydrolyzed by PON1, with only a small contribution by carboxylesterase. Paraoxonase activity in human plasma is about 2-fold higher than in guinea pig plasma. Tributyrin and phenyl butyrate are hydrolyzed by carboxylesterase and butyrylcholinesterase, respectively.⁴ Because human plasma has no carboxylesterase, the values in Table 2 (55 and 260) reflect the hydrolytic activity of butyrylcholinesterase. Carboxylesterase activity in guinea pig plasma yields values of 530 for tributyrin and 1200 for phenyl butyrate.

In conclusion, the major esterases in guinea pig plasma are paraoxonase and carboxylesterase, followed by butyrylcholinesterase, albumin pseudoesterase, and acetylcholinesterase. The antihuman acetylcholinesterase monoclonal antibody HR2 recognizes guinea pig acetylcholinesterase. AntiHuBChE monoclonal antibody B2 18-5 recognizes guinea pig butyrylcholinesterase. In contrast, the esterases in human plasma are paraoxonase (50 mg/L), butyrylcholinesterase (4 mg/L), acetylcholinesterase (0.004 mg/L), and albumin (40,000 mg/L). Human plasma contains no carboxylesterase. The presence of carboxylesterase in guinea pig plasma makes this species a sub-optimal model for evaluating human responses to nerve agent toxicity. A knockout mouse devoid of carboxylesterase activity in plasma is being developed as a more suitable model for studying the toxicology of nerve agents.¹²

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