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Chemical Research in Toxicology

Article

Cite This: Chem. Res. Toxicol. 2017, 30, 1897-1910

pubs.acs.org/crt

Immunopurification of Acetylcholinesterase from Red Blood Cells for Detection of Nerve Agent Exposure

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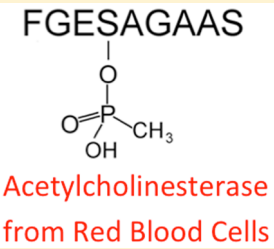
ABSTRACT: Nerve agents and organophosphorus pesticides make a covalent bond with the active site serine of acetylcholinesterase (AChE), resulting in inhibition of AChE activity and toxic symptoms. AChE in red blood cells (RBCs) serves as a surrogate for AChE in the nervous system. Mass spectrometry analysis of adducts on RBC AChE could provide evidence of exposure. Our goal was to develop a method of immunopurifying human RBC AChE in quantities adequate for detecting exposure by mass spectrometry. For this purpose, we immobilized 3 commercially available anti-human acetylcholinesterase monoclonal antibodies (AE-1, AE-2, and HR2) plus 3 new monoclonal antibodies. The monoclonal antibodies were characterized for binding affinity, epitope mapping by pairing analysis, and nucleotide and amino acid sequences. AChE was solubilized from frozen RBCs with 1% (v/v) Triton X-100. A 16 mL sample containing 5.8 μg of RBC AChE was treated with a quantity of soman model compound that inhibited 50% of the AChE activity. Native and soman-inhibited RBC AChE samples were immunopurified on antibody−Sepharose beads. The immunopurified RBC AChE was digested with pepsin and analyzed by liquid chromatography tandem mass spectrometry on a 6600 Triple-TOF mass spectrometer. The aged soman-modified PheGlyGluSerAlaGlyAlaAlaSer (FGESAGAAS) peptide was detected using a targeted analysis method. It was concluded that all 6 monoclonal antibodies could be used to immunopurify RBC AChE and that exposure to nerve agents could be detected as adducts on the active site serine of RBC AChE.



INTRODUCTION

The Centers for Disease Control and Prevention has developed mass spectrometry methods to measure exposure to nerve agents by analyzing adducts on the active site serine of plasma butyrylcholinesterase (BChE) after the BChE has been immunopurified on Dynabeads Protein G.[1−5] An alternative target for measuring exposure is acetylcholinesterase in red blood cells. AChE in RBC would be a useful target because erythrocytes have a longer half-life in the circulation (33 days) than plasma BChE (12 days),[6,7] which means that blood samples drawn weeks after exposure could still have detectable levels of nerve agent adducts on RBC AChE. A second rationale for using AChE as a biomarker of nerve agent exposure is that AChE is more reactive than BChE with the most potent stereoisomers of the nerve agent soman,[8] implying that low level exposure may be more readily detected by measuring adducts on AChE. Evidence for exposure to sarin in 4 victims of the Tokyo subway attack was provided by gas chromatography−mass spectrometry of a sarin hydrolysis product released from RBC AChE by alkaline phosphatase.[9] To date a mass spectrometry method for measuring nerve agent exposure using adducts on RBC AChE has not been developed. In this paper, we present a method for identifying adducts on human AChE based on the current CDC method for identifying adducts on human BChE.

A technical difficulty when working with RBC AChE is that AChE in erythrocytes is membrane bound. AChE is solubilized by treating RBCs with 1% (v/v) Triton X-100. Frozen RBCs after being thawed and treated with Triton X-100 make a viscous solution that traps Sepharose beads and magnetic Dynabeads. This problem was overcome by centrifuging the lysed, Triton X-100 treated cells to remove cell debris. The red supernatant was suitable for immunopurifying AChE. Another drawback is the 10-fold lower concentration of AChE in human RBCs (0.5 mg/L) compared to BChE in human plasma (5 mg/L).[10] The immunopurified soman-treated AChE was digested with pepsin and analyzed by mass spectrometry. A targeted method identified the aged soman adduct on the active site serine of AChE.

Six monoclonal antibodies were characterized: the commercially available AE-1, AE-2, and HR2, and 3 new monoclonal antibodies, 1G, 6A, and 10D, prepared for this project successfully captured human AChE. Binding efficiency was

Received: July 25, 2017
Published: September 11, 2017

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DOI: 10.1021/acs.chemrestox.7b00209
Chem. Res. Toxicol. 2017, 30, 1897−1910

evaluated by Elisa and Bio-Layer Interferometry. A pairing sandwich assay was used to identify overlapping epitopes among the antibodies. The nucleotide and amino acid sequences for five of the antibodies (all except HR2) are reported.

■ MATERIALS AND METHODS

Procainamide Sepharose 4B affinity gel, custom synthesized by Yacov Ashani,[11] had 34 μmol/mL procainamide and was stored in a 20% (v/v) solution of 95% ethanol in water at 4 °C. Ultraculture, serum-free, without L-glutamine was from Lonza (12-725F). Full-length recombinant human AChE (rHuAChE) and truncated rHuAChE were expressed in serum-free Ultraculture by Chinese Hamster Ovary cells and purified on procainamide affinity Sepharose as described below. Dynabeads Protein G were from Life Technologies (no. 10004D). CNBr-activated Sepharose 4 Fast Flow was from Amersham Biosciences (17-0981-01). Protein G Sepharose was from Protein Mods LLC (code PGGH, Madison, WI). The following were from Millipore: Amicon Ultra-15 centrifugal filter, 10 000 MW cutoff (UFC901024); Ultrafree-MC 0.45 μm Durapore PVDF spin filter (UFC30HV00); Protein-Pak Q 8HR (PN35980, SN T50051C03); 0.22 μm GV Durapore centrifugal filter Ultrafree-MC sterile (UFC30GV0S); 0.22 μm Stericup vacuum driven filtration system (SCGPU11RE). Human red blood cells were obtained from the University of Nebraska Hospital blood bank. The whole blood had been collected into citrate phosphate dextrose and adenine-saline. The Sp stereoisomer of the soman model compound, pinacolyl methylphosphono thiomethyl, CBG-1-129, was synthesized by the laboratory of John Cashman.[12,13] It is stored at −80 °C as a neat oil with no additives. The model compound has thiomethyl in place of fluoride but makes the same adduct as soman. All other chemicals and reagents were from Sigma-Aldrich.

Monoclonal Antibodies to Human AChE. Mouse monoclonal HR2 was created by Rakonczay and Brimijoin using human AChE from brain as immunogen.[14] We purchased the HR2 antibody, IgG2b, from GeneTex Inc., Irvine, CA. Monoclonal antibodies AE-1 and AE-2 were created by Fambrough et al. using AChE purified from red blood cells as immunogen.[15] We purchased mouse hybridoma cells that produce AE-1 and AE-2 antibodies from The American Type Culture Collection, Manassas, VA (ATCC HB-72 and ATCC HB-73), and purified the monoclonal antibodies on Protein G agarose (Protein Mods LLC, Madison, WI).

Mouse monoclonal antibodies 1G, 6A, and 10D were produced for this project by Syd Labs, Inc. (Natick, MA) using full-length recombinant human acetylcholinesterase (rHuAChE) as immunogen. Large quantities of monoclonal antibodies 1G, 6A, and 10D were produced in mouse ascites fluid and purified by Syd Labs, Inc.

Purification of rHuAChE. Highly purified full-length recombinant HuAChE (rHuAChE) was used as immunogen. Full-length rHuAChE (accession no. P22303) was cloned into plasmid pGS where the selection marker is glutamine synthetase. HuAChE was expressed in Chinese Hamster Ovary cells (CHO-K1, ATCC CCL-61) in serum-free and glutamine-free Ultraculture containing 25 μM methionine sulfoximine. Culture medium accumulated over a period of 3 months contained 6 mg of full-length rHuAChE (30 000 units) in 764 mL. The culture medium was filtered through Whatman no.1 on a fritted glass funnel to remove particulates, followed by filtration through a 0.22 μm Stericup filter before it was loaded onto a 20 mL column of procainamide Sepharose affinity gel. The procainamide affinity gel was designed for purification of human BChE,[16] but it works even better for purification of rHuAChE. The rHuAChE remains bound in the presence of 1 M NaCl, whereas BChE elutes in 1 M NaCl. Washing with 1 M NaCl elutes contaminating proteins while rHuAChE remains bound, thus yielding a highly purified rHuAChE solution when the rHuAChE is eluted.

The column was washed with (1) 440 mL of 20 mM TrisCl, pH 7.5, 0.05% (w/v) NaN_3, thus eluting all the pink color, (2) 200 mL of 0.2 M NaCl in buffer, (3) 200 mL of 0.5 M NaCl in buffer, and (4) 100 mL of 1 M NaCl in buffer. The rHuAChE was eluted with 0.5 M tetramethylammonium bromide in buffer, with a yield of 74%. Purification trials with additional samples of culture medium found that rHuAChE could be eluted with 0.5 M choline chloride in buffer or with 0.15 M procainamide in buffer. In preparation for additional purification by anion exchange chromatography, the rHuAChE was desalted and concentrated in an Amicon Ultra-15 centrifugal filter and clarified by filtration through a 0.45 μm Ultrafree centrifugal filter. The sample (1 mL) was loaded onto a Protein-Pak Q 8HR anion exchange column operated at 300 psi in a Waters high performance liquid chromatography (HPLC) system. The Protein Pak column was washed at a flow rate of 1 mL/min for 30 min with 20 mM TrisCl, pH 7.5, followed by 30 min with 0.1 M NaCl in buffer. No rHuAChE was released by these washes. rHuAChE was eluted with 0.19 M NaCl in 20 mM Tris buffer. A small peak of contaminating protein eluted with 0.5 M NaCl in Tris buffer. The yield from 6 mg of full-length rHuAChE was 3.8 mg of highly purified full-length rHuAChE. A larger scale purification yielded 31 mg of rHuAChE from 4.5 L of culture medium.

HuAChE in RBCs is a dimer of identical subunits. RBC AChE has 557 amino acids[17] per subunit and a glycoinositol phospholipid membrane anchor at the C-terminus.[18] In contrast HuAChE in nerve synapses and muscle is a tetramer of 4 identical subunits assembled around proline-rich motifs in PRiMA and ColQ proteins.[19] Each subunit has 583 amino acids (P22303) and no phospholipid anchor. To more closely mimic the AChE in erythrocytes, we used a truncated, monomeric rHuAChE, Q552stop, missing 32 amino acids from the C-terminus. The truncated, monomeric rHuAChE was used in the following assays: to screen culture media from hybridoma cells; to measure AChE binding to immobilized monoclonal antibodies; to determine K_d values (both by ELISA and OctetRED96); for antibody pairing analysis; and in gel-shift assays. Truncated, monomeric rHuAChE was expressed and purified as described for full-length rHuAChE.

Classical Method for Preparing Crude RBC AChE. Red cell ghosts prepared by the method of Dodge[20] were solubilized by addition of 1% Triton X-100.[21,22] The ghosts from 5 mL of frozen packed red blood cells yielded 3.9 μg of AChE. The quantity of AChE protein was calculated from AChE activity assayed at pH 7.0, where 5 units of activity represent 1 μg of AChE protein. The yield from various preparations was 0.4, 0.5, 0.7, 0.8, and 1 μg of AChE per milliliter of packed RBC. It has been estimated that packed human red blood cells have 0.5−1 μg of AChE per milliliter of cells.[10,23] In contrast, human plasma has 4−5 μg of BChE per milliliter of plasma. RBC AChE prepared by the method of Dodge was used for testing AChE binding to monoclonal antibodies immobilized on Dynabeads Protein G and on Sepharose beads.

No-Ghost RBC AChE. The classical method of Dodge[20] for preparation of red cell ghosts requires centrifugation in a floor-mounted high speed refrigerated centrifuge, such as the Sorvall RC5, to remove the hemoglobin. We also prepared RBC AChE using a microfuge. Our simplified method did not involve separating hemoglobin from lysed cells. Since red cell ghosts were not prepared, we named the solubilized AChE "no-ghost RBC AChE". Frozen RBCs (20 mL) were thawed and diluted with 30 mL of 1% Triton X-100 in phosphate buffered saline (PBS), 0.1% azide. This solubilized the membrane-bound AChE but left a small amount of insoluble debris. The debris was removed by centrifugation in microfuge tubes for 30 min at 14 000 rpm (12 000*g*), 4 °C. The solubilized no-ghost RBC AChE solution was red but not viscous. No-ghost RBC AChE had an activity of 1.8 u/mL with 1 mM acetylthiocholine. The AChE activity was stable for months at 4 °C. The yield of no-ghost RBC AChE was 0.9 μg per milliliter of packed RBCs. The no-ghost preparation was used for immunopurification of soman labeled RBC AChE.

Protein Concentration. The total protein concentration of the RBC AChE solution (from the Dodge preparation method) in 1% Triton X-100 was measured with the Pierce BCA protein assay kit (catalog 23225) using bovine albumin as standard. The protein concentration of pure rHuAChE (both full-length and truncated, monomeric forms) was calculated from absorbance at 280 nm where a

DOI: 10.1021/acs.chemrestox.7b00209
Chem. Res. Toxicol. 2017, 30, 1897−1910

1 mg/mL solution had an absorbance of 1.8 or from AChE activity using a specific activity of 5000 units/mg for pure rHuAChE.[21]

AChE Activity. AChE activity was measured in 0.1 M potassium phosphate, pH 7.0, at 25 °C with 1 mM acetylthiocholine in the presence of 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) on a Gilford spectrophotometer interfaced to a MacLab data recorder (ADinstruments, Inc.). The increase in absorbance at 412 nm was converted to micromoles of acetylthiocholine hydrolyzed using the extinction coefficient 13 600 M^{-1} cm^{-1}.[24] Units of activity are expressed as micromoles per minute. AChE units per milliliter (pH 7) were converted to milligrams per milliliter using a specific activity of 5000 units/mg at pH 7. When activity was measured at pH 8, the specific activity was 1.3-fold higher.

Gel Electrophoresis. Polyacrylamide 4−30% (w/v) gradient gels, 0.75 mm thick, were poured and run in an SE600 vertical slab gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). SDS gels were electrophoresed at 150 V constant voltage for 20 h. Nondenaturing gels were electrophoresed at 250 V constant voltage for 20 h at 4 °C. Nondenaturing gels were stained for AChE activity by the method of Karnovsky and Roots.[25] The staining solution contained 180 mL of 0.2 M maleic acid adjusted to pH 6.0 before use, 15 mL of 0.1 M sodium citrate, 30 mL of 0.03 M $CuSO_4$, 30 mL of water, 30 mL of 5 mM potassium ferricyanide, and 0.15 g of acetylthiocholine iodide. Gels were incubated in the staining buffer for 1−2 h with gentle shaking until brown-red bands appeared.

ELISA Screening of Hybridoma Cell Culture Medium. Immulon 2HB 96-well plates (Thermo Fisher Scientific, Waltham, MA) were coated with 1 μg of goat anti-mouse IgG in 200 μL of pH 9.6 sodium carbonate−bicarbonate buffer per well at 4 °C overnight. Wells were washed three times with PBS containing 0.05% (v/v) Tween-20 (PBST). Eight rows were incubated with 100 μL of conditioned culture medium in 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000, and 1:1100 dilutions (w/v) in PBS for 1.5 h at room temperature. Wells were washed 3 times with PBST, followed by incubation for 1 h at 24 °C with 100 μL of truncated, monomeric rHuAChE. The truncated, monomeric rHuAChE concentrations ranged from 2 to 40 ng in 100 μL of 1 mg/mL bovine serum albumin (BSA), 0.01% NaN_3, 25 mM sodium phosphate, pH 8.0. It was essential to dilute the rHuAChE into buffer containing albumin immediately before use, because AChE activity disappeared from dilute AChE solutions prepared a day earlier. Unbound rHuAChE was removed by washing the wells 3 times with PBST. Bound rHuAChE was detected as the yellow color that developed in the presence of 100 μL of Ellman reagent (19 mL of 0.1 M potassium phosphate pH 7.0 + 0.5 mL of 20 mM 5,5′-dithiobis(2-nitrobenzoic acid) + 0.2 mL of 0.1 M acetylthiocholine). Absorbance at 405 nm was recorded on a BioTek 96-well plate reader (Winooski, VT) after allowing the yellow color to develop for 10 min. Binding affinity was estimated from plots of 1/absorbance at 405 nm versus 1/nM AChE. All 57 culture media were positive in the assay to detect binding of rHuAChE to monoclonal antibodies in culture medium. Three hybridoma clones were selected for expansion.

K_d Values Measured by ELISA. K_d values for binding of monoclonal antibodies to truncated, monomeric rHuAChE were measured using the protocol for screening culture media. In place of culture medium, wells were incubated with 100 μL of purified monoclonal antibodies diluted in PBS to 0.0001, 0.0004, 0.001, and 0.01 mg/mL for 1.5 h at room temperature. Truncated, monomeric rHuAChE amounts ranged from 2 to 40 ng in 100 μL of 1 mg/mL bovine serum albumin (BSA), 0.01% NaN_3, 25 mM sodium phosphate, pH 8.0. Bound AChE activity was measured with Ellman reagent as above. Binding affinity was estimated from plots of 1/absorbance at 405 nm versus 1/nM AChE. Figure 2 shows data for 0.1 mL of 0.001 mg/mL AE-1 monoclonal per well. The slope of the line in the double reciprocal plot was divided by the intercept on the y axis to yield a value for the dissociation constant, K_d.[26]

K_d Values Measured by Bio-Layer Interferometry on OctetRED96. The measurements were performed by Dr. Udaya Yerramalla at Precision Antibody, Columbia, MD, on an OctetRED96 instrument. Murine monoclonal antibodies at 20 μg/mL in PBS containing 1 mg/mL bovine serum albumin were captured using anti-mouse IgG Fc Capture dip-and-read biosensors. For measurement of association rate constants, sensors were dipped into 2-fold serial dilutions of truncated, monomeric rHuAChE in PBS containing 1 mg/mL bovine serum albumin. The rHuAChE concentrations ranged from 50 to 1.56 nM. For measurement of dissociation rate constants, the probes with attached rHuAChE were dipped into PBS containing 1 mg/mL bovine albumin. The response (nm shift) from a ligand free sensor dipped in rHuAChE was used as a reference for subtraction. A control sample, pooled purified mouse IgG captured on sensor and dipped into the rHuAChE was used to test for nonspecific binding. A minimal response close to baseline was observed with the control sample, thus confirming the specificity of the test antibodies for the antigen. A total of 7 association curves and 7 dissociation curves were recorded for each monoclonal antibody. Curve fitting analysis was performed to determine k_{on}, k_{off}, and K_d. The truncated rHuAChE, Q552stop, is a monomer with a MW of 65 kDa produced by deletion of the C-terminal tetramerization domain.

Epitope Mapping by Pairing Analysis. The purpose of this analysis was to identify monoclonal antibodies that bind to different epitopes on HuAChE. Measurements were performed by Dr. Udaya Yerramalla at Precision Antibody on a PALL ForteBio OctetRED96 instrument. A biosensor coated with a goat anti-mouse Fc antibody was used to capture the first murine monoclonal. Then the sensors were dipped in wells containing truncated, monomeric rHuAChE at a concentration of 50 nM in PBS containing 1 mg/mL BSA. Binding of the detection monoclonal antibody to rHuAChE was evaluated with Octet sensorgrams. Multiple pairing sets were arranged. The response obtained from the self-pairing analysis was taken as the baseline, and any significant response above the baseline was evidence that both monoclonal antibodies bound to rHuAChE as a pair.

Immunomagnetic Capture of RBC AChE and rHuAChE on Dynabeads Protein G. The protocol developed by Sporty et al. for human BChE[4] was adapted for capture of human AChE. In brief, 300 μL of Dynabeads Protein G suspension was added to 6 microfuge tubes. The Dynabeads were washed three times with 200 μL of PBS, followed by incubation overnight with 100 μg of a monoclonal antibody (AE-1, AE-2, HR2, 1G, 6A, or 10D) in 0.6 mL PBS. The Dynabead−antibody complex was washed twice with 200 μL of triethanolamine buffer (0.2 M triethanolamine, 0.025% NaN_3, pH 7.8), before the antibody was cross-linked to Protein G with 200 μL of dimethyl pimelimidate (5.4 mg/mL) in triethanolamine buffer for 30 min. The cross-linking solution was discarded, and residual reagent was inactivated by incubating the beads for 15 min in 0.1 M TrisCl, pH 7.5. Beads were washed 3 times with 0.5 mL of PBS containing 0.05% Tween-20 (PBST). Each washed Dynabead−antibody complex was suspended in 300 μL of PBS and aliquoted at 50 μL per tube to give 6 samples. A total of 36 tubes were prepared, 6 tubes for each antibody.

Binding of AChE to the immobilized monoclonal antibodies was tested against RBC AChE (Dodge method preparation) and truncated, monomeric rHuAChE. Assays were performed in triplicate in the presence of 1% Triton X-100 in PBS, 0.1% azide. The RBC AChE and the rHuAChE solutions were adjusted so that the AChE activity was identical at 2.9 units/mL. All 36 tubes received 1.8 mL of 1% Triton X-100 in PBS, azide, and either 0.2 mL of RBC AChE in 1% Triton X-100 or 0.2 mL of rHuAChE in 1% Triton X-100 to total 2 mL per tube. Tubes were rotated overnight at room temperature. The quantity of AChE bound was evaluated by measuring AChE activity in the unbound fraction, compared to AChE activity in control samples that had not been exposed to antibody.

Binding of AChE to Monoclonal Antibodies Immobilized on Sepharose Beads. Monoclonal antibodies AE-1, AE-2, 1G, 6A, and 10D were immobilized on Sepharose beads by reacting 5 mg of monoclonal antibody with 3 mL of CNBr-activated Sepharose in pH 8 coupling buffer. The antibody-bound beads were washed with PBS and stored in 15 mL of PBS, 0.1% sodium azide. For most preparations, 0.2 mL of suspension contained 66 μg of monoclonal antibody bound to 40 μL of beads. Monoclonal HR2 from GeneTex was immobilized to

DOI: 10.1021/acs.chemrestox.7b00209
Chem. Res. Toxicol. 2017, 30, 1897−1910

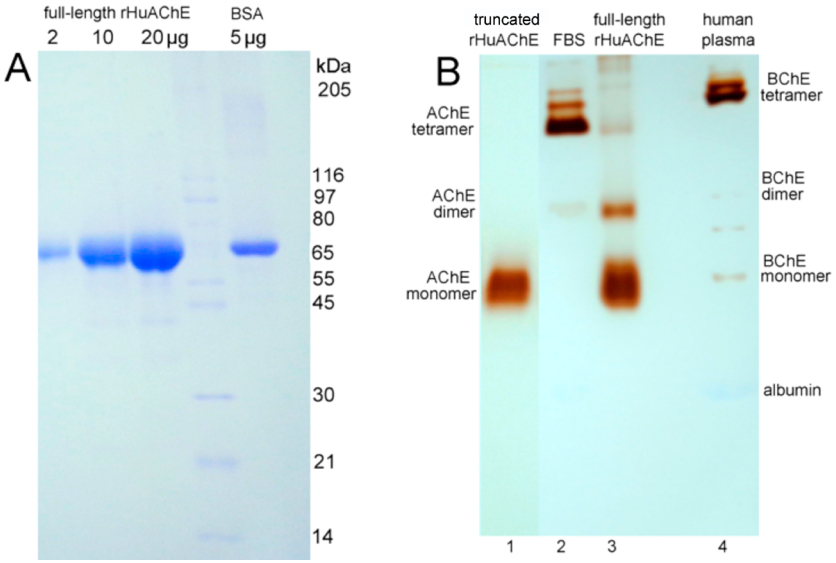

Figure 1. (A) Reducing SDS gradient gel showing purity of the full-length rHuAChE used as immunogen. The molecular weight of rHuAChE is 65 kDa and that of albumin is 67 kDa. (B) Nondenaturing gradient gel stained for AChE and BChE activity with acetylthiocholine. Truncated, monomeric rHuAChE (lane 1) consists entirely of monomers, whereas full-length rHuAChE (lane 3) consists of tetramers, dimers, and monomers. Fetal bovine serum (FBS in lane 2) is a marker for AChE tetramers. Human plasma (lane 4) is a marker for BChE tetramers, dimers, and monomers.

CNBr-activated Sepharose at a ratio of 100 μg of HR2 to 100 μL of swollen beads.

Binding of AChE to monoclonal antibodies immobilized on Sepharose was tested in triplicate in the presence of 1% Triton X-100 using the same protocol described for Dynabeads. The incubation volume of 2 mL contained 1.8 mL of 1% Triton X-100 in PBS, azide, plus 16.5 μg of monoclonal antibody immobilized on Sepharose beads and either 0.12 μg of RBC AChE (0.6 units) in 0.2 mL of 1% Triton X-100 dissolved in PBS, azide, or 0.16 μg of truncated monomeric rHuAChE in 0.2 mL of 1% Triton X-100 dissolved in PBS, azide.

Inhibition of RBC AChE by a Soman Model Compound. Noghost RBC AChE in 0.6% Triton X-100 with an activity of 1.8 u/mL was treated with a soman model compound that had a thiomethyl group in place of the fluoride ion in authentic soman.[13] The soman model compound had been diluted into dimethyl sulfoxide so that a 1 μL aliquot inhibited 40−50% of the AChE activity in 16 mL. The residual AChE activity in the soman-inhibited sample allowed measurement of percent binding to immobilized antibody (1.8 u/mL ÷ 5000 u/mg = 3.6×10^{-4} mg/mL × 16 mL = 5.8×10^{-3} mg total AChE).

Immunopurification of Soman-Inhibited RBC AChE. Monoclonal 10D immobilized on Sepharose beads (330 μg of 10D on 200 μL beads) was rotated overnight with 16 mL of soman-inhibited noghost RBC AChE in 0.6% Triton X-100, PBS azide. Activity assays of the unbound AChE indicated that 90% of the AChE had been captured by the immobilized antibody. Hemoglobin and other proteins were removed from the suspension by washing the beads 6 times with 3 mL of 1% Triton X-100, PBS, while the beads were in a 0.45 μm spin filter in a 50 mL tube (ChemTech Inc.50 mL filter tube CTF-CA045-03). At the end of the washes with 1% Triton X-100, the beads and the flow through were colorless.

Triton X-100 interferes with mass spectrometry analysis of peptides.[27] The detergent was removed by washing the beads with 1 M NaCl in 0.1 M TrisCl, pH 8, until absorbance at 280 nm of the wash was less than 0.02. The beads were checked for the presence of bound AChE by addition of acetylthiocholine in Ellman's reagent; the yellow color that developed immediately indicated that the wash steps had not eluted AChE from the immobilized antibody. The yellow color was washed off the beads with 1 M NaCl in 0.1 M TrisCl, pH 8 before the beads were desalted by washing with water.

Pepsin Digestion. Immunopurified AChE was released from the beads with 200 μL of 50% acetonitrile, 1% trifluoroacetic acid while the beads were in a 50 mL 0.45 μm spin tube. The flow through was transferred to a microfuge tube and dried. The dry protein was dissolved in 150 μL of 0.6% formic acid and digested for 2 h at 37 °C with 20 μL of a freshly prepared 2 mg/mL pepsin solution in 0.6% formic acid. The digested sample was filtered through a prerinsed 0.45 μm spin filter to remove particulates. The flow through was dried, dissolved in 20 μL of 0.1% formic acid, and centrifuged at 14 000 rpm in a microfuge for 60 min (5.8 μg of starting AChE ÷ 20 μL = 0.29 μg of AChE per microliter, assuming no losses). The top 10 μL was transferred to an autosampler vial for analysis by liquid chromatography tandem mass spectrometry.

Liquid Chromatography Tandem Mass Spectrometry. Data acquisition was performed with a Triple-TOF 6600 mass spectrometer (ABI Sciex, Framingham, MA) fitted with a Nanospray III source (AB SCIEX, Framingham, MA) and a Pico Tip emitter (no. FS360-20-10-N-5-C12, New Objectives, Woburn, MA). The ion spray voltage was 2700 V, declustering potential 60 V, curtain gas 30 psi, nebulizer gas 10 psi, and interface heater temperature 150 °C.

Peptides were introduced into the mass spectrometer using ultrahigh pressure liquid chromatography. A splitless Ultra 1D Plus ultrahigh pressure chromatography system (Eksigent, Dublin, CA) was coupled to the Triple-TOF via a cHiPLC Nanoflex microchip column system (Eksigent, Dublin, CA). The Nanoflex system uses a replaceable microfluidic trap column and a replaceable separation column. Both are packed with ChromXP C_{18} (3 μm, 120 Å particles; Trap = 200 μm × 0.5 mm; Separation = 75 μm × 15 cm). Chromatography solvents were water/acetonitrile/formic acid (A 100/0/0.1%, B 0/100/0.1%). Picomole amounts of sample, in a 5 μL volume, were loaded. Trapping and desalting were carried out at 2 μL/min for 15 min with 100% mobile phase A. Separation was obtained with a linear gradient 5%A/95%B to 70%A/30% B over 60 min at a flow rate of 0.3 μL/min.

Peptides present in the data were identified by matching to the Swiss Prot or National Center for Biotechnology Information (NCBI) nonredundant databases, and corresponding proteins were identified. The Paragon algorithm in Protein Pilot v 5.0 (AB SCIEX) was used to search the databases. Database search parameters specified the protease used for digestion, the state of cysteine alkylation, the species from which the sample was derived, the "ID focus", which was typically "Biological modifications", the database, and the type of "Search effort", which was typically "Thorough". Protein Pilot software was used to inspect the database search results.

A targeted method was used to detect the soman-labeled active site peptide of AChE. Product Ion data acquisition was performed on the

DOI: 10.1021/acs.chemrestox.7b00209
Chem. Res. Toxicol. 2017, 30, 1897−1910

Table 1. Comparison of Sepharose Beads to Dynabeads Protein G as Solid Supports for Anti-HuAChE Monoclonal Antibodies[a]

mAb	isotype	% RBC AChE bound to Dynabeads	% rHuAChE bound to Dynabeads	% RBC AChE bound to Sepharose	% rHuAChE bound to Sepharose
AE-1	IgG1 kappa	84.3 ± 2.5	71.4 ± 5.4	82.4 ± 2.2	80.0 ± 2.9
AE-2	IgG1 kappa	33.7 ± 7.5	14.0 ± 6.1	82.4 ± 2.2	68.3 ± 5.0
HR2	IgG2b kappa	83.3 ± 1.9	93.0 ± 1.3	54.6 ± 1.7	70.7 ± 4.5
1G	IgG1 kappa	37.6 ± 2.6	22.4 ± 4.0	87.0 ± 2.0	84.0 ± 2.9
6A	IgG1 kappa	45.6 ± 1.7	35 ± 2.7	84.5 ± 1.5	84.7 ± 3.1
10D	IgG1 kappa	87.3 ± 1.2	82.3 ± 2.6	82.0 ± 1.0	79.0 ± 3.3

[a]Expressed as % bound + standard deviations (n = 3).

singly charged mass for the active site peptide labeled with aged soman in positive mode (FGESAGAAS + 78 Da + H^+ = 874.35 Da, where 78 Da is the added mass for aged soman). A data acquisition cycle consisted of two steps. An MS survey scan was made over a mass range of 400−900 Da with an accumulation time of 1000 ms. This was followed by a Product Ion MSMS scan whenever the 874.35 Da parent ion appeared in the survey scan. By targeting a single mass, more time could be devoted to data accumulation. Consequently the quality of the MS and MSMS spectra was improved. Data were analyzed with Peak View v 2.1 (AB SCIEX). An Extracted Ion Chromatogram for the 874.35 Da mass was constructed from both the mass spectral and MSMS fragmentation data.

Nucleotide and Amino Acid Sequences of Monoclonal Antibodies. Syd Labs, Inc. (Natick, MA) amplified the variable and constant regions of mouse anti-HuAChE monoclonal antibodies AE-1, AE-2, 1G, 6A, and 10D by polymerase chain reaction of cDNA derived from hybridoma cells. The amplicons were sequenced and cloned into expression plasmids. Recombinant antibodies were expressed by HEK293 cells and purified on Protein A beads. The reliability of the PCR-based sequencing results was confirmed by testing the recombinant antibodies for binding to full-length rHuAChE. The HR2 monoclonal antibody was not sequenced because the hybridoma cell line was not available. Nucleotide and amino acid sequences were deposited in GenBank.

Statistical Analysis. Quantitative results are expressed as means ± SD from at least 3 independent experiments. Data acquired by biolayer interferometry on an OctetRED96 instrument yielded traces for association and dissociation of the ligand. Biosensor response traces were processed using ForteBio Data Analysis Software (version 8.0, Pall ForteBio, CA, USA). The data were fit by nonlinear regression analysis using a simple 1:1 Langmuir interaction model.

RESULTS

Purity of Full-Length rHuAChE Used as Immunogen. The purity of full-length rHuAChE was checked by gel electrophoresis and by mass spectrometry of trypsin-digested protein. The SDS gel in Figure 1A shows a single band for rHuAChE at 65 kDa. Mass spectrometry analysis confirmed that the rHuAChE is highly purified. Protein Pilot software identified 566 amino acids in full length HuAChE for 97% coverage of the mature HuAChE sequence (accession number P22303 minus 31 residues in the signal peptide). The most abundant protein in the digest was HuAChE with a peptide count of 673. The next most abundant protein, albumin, was identified with a peptide count of 13. The 67 kDa molecular weight of albumin is similar to that of rHuAChE. The mass spectrometry result indicated that the preparation contained less than 2% albumin (13/673 × 100 = 1.9%).

Lane 3 in Figure 1B shows that full-length rHuAChE consists predominantly of monomers but includes dimers and tetramers. The paucity of tetramers is explained by the fact that polyproline peptides are required for assembly to tetramers.[28,29]

CHO cells provide an inadequate supply of polyproline peptides. Pure full-length rHuAChE was concentrated to 1 mg/mL and filter sterilized on a 0.22 μm centrifugal filter before 9.8 mg was sent to Syd Labs to use as immunogen.

As expected, the truncated, monomeric rHuAChE used for screening (Lane 1 in Figure 1B) consists exclusively of monomers. Albumin separates from AChE on a nondenaturing gel (Figure 1B), but not on an SDS gel (Figure 1A).

AChE Sticks to Surfaces. When rHuAChE (full-length or truncated, monomeric) was stored in glass or plastic tubes at a concentration of 100 to 500 u/mL (20 to 100 μg/mL) in 0.19 M NaCl, 20 mM TrisCl pH 7.5, the AChE activity was stable for at least 3 years at 4 °C. However, when either type of rHuAChE was diluted to 0.3 u/mL in PBS (0.08 μg/mL), the activity the next day was 0.03 u/mL, a loss of 90%. Diluting rHuAChE to 0.03 u/mL in 10 mg/mL BSA in PBS resulted in no loss of AChE activity in 4 weeks. Truncated, monomeric rHuAChE diluted in 1 mg/mL BSA, 20 mM sodium phosphate, pH 8, to concentrations of 2 to 40 ng per 100 μL for ELISA screening lost all activity by the following day. Therefore, rHuAChE was diluted into 1 mg/mL BSA just before use in ELISA. After the truncated, monomeric rHuAChE was captured by immobilized antibody, the complex retained AChE activity. The plastic and glass surfaces that bound rHuAChE had no AChE activity. A red RBC AChE solution (Dodge preparation) in 1% Triton X-100 in PBS with a protein concentration of 1.5 mg/mL and an activity of 2.9 units/mL, retained that level of activity for at least one month at 4 °C. Loss of AChE activity by AChE binding to surfaces has been recognized for many years. Laboratories that routinely assay AChE activity stabilize their dilute AChE solutions with albumin.[30−32]

Binding Efficiency Is Higher for Monoclonal Antibodies Immobilized on Sepharose than on Dynabeads Protein G. Mouse anti-human AChE monoclonal antibodies were tested for ability to bind RBC AChE (Dodge preparation) and truncated, monomeric rHuAChE in 1% Triton X-100 in PBS. The monoclonal antibodies were immobilized on Sepharose beads or on Dynabeads Protein G. Table 1 shows that more than 80% of RBC AChE was captured by monoclonal antibodies AE-1, AE-2, 1G, 6A, and 10D immobilized on Sepharose beads, but only AE-1, HR2, and 10D captured greater than 80% of RBC AChE immobilized on Dynabeads Protein G. Only monoclonal HR2 captured more AChE when bound to Dynabeads Protein G than when bound to Sepharose. The same pattern of differential binding capacity was shown for truncated, monomeric rHuAChE.

K_d Values Measured by ELISA. The binding affinity of monoclonal antibodies for truncated, monomeric rHuAChE was measured by ELISA, using amounts of rHuAChE between 2 and 20 ng per well. Monoclonal antibody-bound rHuAChE was assayed by a 10 min incubation with acetylthiocholine and Ellman reagent in pH 7 buffer. Absorbance values for wells

DOI: 10.1021/acs.chemrestox.7b00209
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containing more than 8 ng of AChE in 100 μL (1.2 nM) reached a plateau. A representative plot of the primary data is in Figure 2. The K_d value was calculated from the double

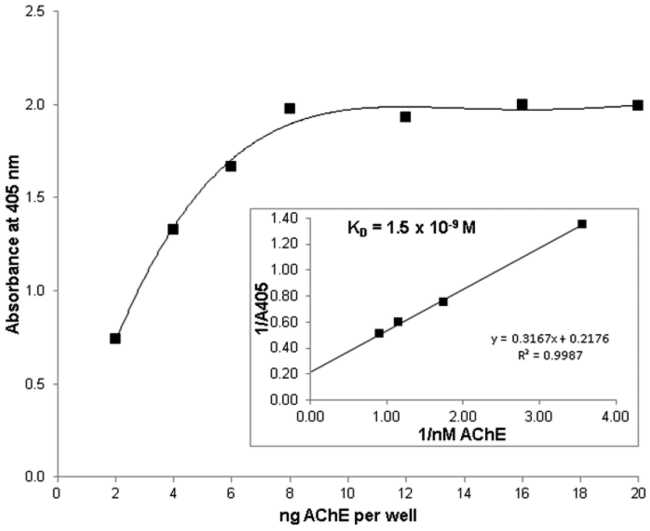

Figure 2. ELISA for determination of the dissociation constant of monoclonal AE-1 and truncated, monomeric rHuAChE. The x-axis in the primary data shows nanograms of AChE in 100 μL. The x-axis in the inset shows 1/nM AChE. The AChE protein concentration in nanograms per 100 μL was converted to nanomolar AChE using a molecular weight of 65 kDa for rHuAChE. The K_d value was calculated by dividing the slope of the line from the inset by the intercept on the y-axis[26] (n = 3).

reciprocal plot shown in the inset by dividing the slope by the intercept on the y axis.[26] A summary of K_d values in Table 2 shows that monoclonal antibodies 1G, 6A, and 10D have nanomolar K_d values that are lower than literature values for monoclonal antibodies AE-1, AE-2, and HR2.

K_d Values Measured by Bio-Layer Interferometry on OctetRED96. Dissociation constants measured for truncated, monomeric rHuAChE by Bio-Layer Interferometry on the OctetRED96 instrument are listed in Table 2. K_d values measured with the OctetRED96 were 2−200-fold smaller than those measured by ELISA. The smaller the K_d, the larger the discrepancy. It should be noted that the Benesi−Hildebrandt method for K_d calculation assumes that the total antigen concentration (rHuAChE) is much higher than the total antibody concentration, such that the contribution of complex to the free rHuAChE concentration can be ignored. If this assumption is not true, then Benesi−Hildebrandt will overestimate the K_d.

The OctetRED96 method shows that monoclonal HR2 binds rHuAChE orders of magnitude more tightly than any of the other monoclonal antibodies. This trend is supported by the ELISA values. Both the ELISA and OctetRED96 values are substantially lower than those reported using the Pansorbin assay. For example, ELISA with monoclonal HR2 yielded a K_d value that was 300-fold lower than the K_d determined for HR2 and human brain AChE (Hu brain AChE) in the Pansorbin assay. These differences in K_d values could originate from differences in the aggregation states of the AChE forms used in the assays. Our ELISA and OctetRED96 assays used truncated, monomeric rHuAChE. The Pansorbin assays used either human brain AChE, which consists mostly of tetramers,[33] or RBC AChE, which consists of dimers.[21] The highest affinity monoclonal according to the OctetRED96 assay is HR2, followed by 10D. Monoclonal antibodies 6A, 1G, AE-1, and AE-2 have similar affinities for truncated, monomeric rHuAChE.

Epitope Mapping. The pairing sandwich assay in Figure 3 identifies monoclonal antibodies that bind to 3 different epitopes on human AChE. Monoclonal antibodies AE-1, 1G, 6A, and 10D share the same epitope, based on the observation that they cannot pair with each other (white boxes). Monoclonal AE-2 can pair with AE-1, HR2, 1G, 6A, and 10D (green boxes). Monoclonal HR2 can pair with AE-1, AE-2, 1G, 6A, and 10D (green boxes). Three antigenic sites on HuAChE are defined by (1) monoclonal antibodies AE-1, 1G, 6A, and 10D, (2) AE-2, and (3) HR2.

Antibody−AChE Complexes Visualized on Nondenaturing Gel. The question in this experiment was whether antibodies that bind to different epitopes or have different binding affinities can be recognized by the behavior of the antibody−AChE complex on a nondenaturing gel stained for AChE activity. Figure 4 shows that all 6 monoclonal antibodies retarded the migration of truncated, monomeric rHuAChE. AE-

Table 2. K_d Values for Monoclonal Antibodies to HuAChE

mAb	K_d, nM	isotype	AChE type	assay	ref
1G	1 ± 1	IgG1 kappa	rHuAChE	ELISA	present report
1G	0.32 ± 0.01	IgG1 kappa	rHuAChE	OctetRED96	present report
6A	1 ± 1	IgG1 kappa	rHuAChE	ELISA	present report
6A	0.12 ± 0.01	IgG1 kappa	rHuAChE	OctetRED96	present report
10D	0.4 ± 0.4	IgG1 kappa	rHuAChE	ELISA	present report
10D	0.07 ± 0.004	IgG1 kappa	rHuAChE	OctetRED96	present report
HR2	0.2 ± 0.2	IgG2b kappa	rHuAChE	ELISA	present report
HR2	0.001 ± 0.0001	IgG2b kappa	rHuAChE	OctetRED96	present report
HR2	58	IgG2b kappa	Hu brain	Pansorbin	14
HR2	140	IgG2b kappa	RBC AChE	Pansorbin	14
AE-1	14	IgG1 kappa	RBC AChE	ELISA	34
AE-1	1.5 ± 0.5	IgG1 kappa	rHuAChE	ELISA	present report
AE-1	0.33 ± 0.01	IgG1 kappa	rHuAChE	OctetRED96	present report
AE-2	50	IgG1 kappa	RBC AChE	ELISA	34
AE-2	1 ± 1	IgG1 kappa	rHuAChE	ELISA	present report
AE-2	0.43 ± 0.01	IgG1 kappa	rHuAChE	OctetRED96	present report

DOI: 10.1021/acs.chemrestox.7b00209
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Capture Ab (1st) in pair	Detection Ab (2nd) in pair						
	AE-1	AE-2	HR2	1G	6A	10D	Mouse IgG
AE-1	0.0502	0.4148	0.419	0.039	0.0187	0.0222	0.1333
AE-2	0.2501	0.1299	0.3373	0.2447	0.2175	0.233	0.1282
HR2	0.3722	0.441	0.0376	0.3585	0.3075	0.3252	0.1451
1G	0.0613	0.3505	0.3867	0.0533	0.0373	0.0366	0.0947
6A	0.0637	0.3609	0.3988	0.0649	0.0533	0.0457	0.1063
10D	0.0686	0.4111	0.4192	0.0575	0.0526	0.0457	0.0996
Mouse IgG	0.1083	0.1418	0.1015	0.0914	0.0623	0.0629	0.1217

Figure 3. Epitope mapping by pairing analysis. Antibodies that pair are highlighted in green. Self-pairing is highlighted in red and is used as the threshold to determine strong pairs. Antibodies that do not pair are in white boxes. Mouse IgG was used as a negative control. Pairing analysis used truncated, monomeric rHuAChE.

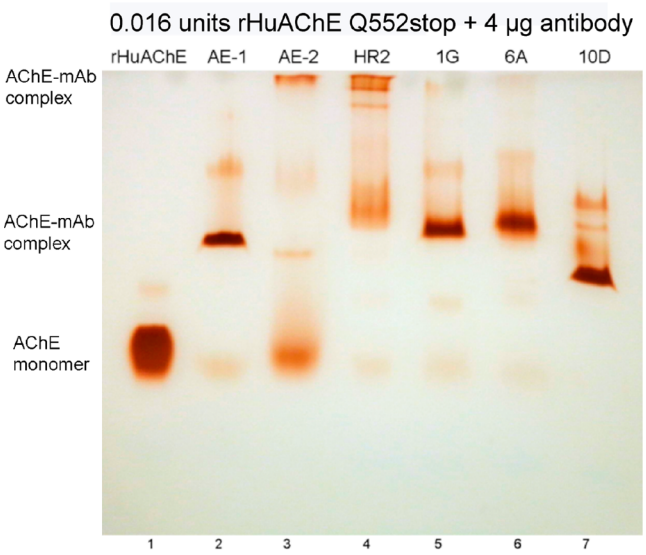

Figure 4. Antibody−AChE complexes visualized on a nondenaturing gradient gel stained for AChE activity. Truncated, monomeric rHuAChE, 0.016 units (0.003 μg) in 20 μL of 1 mg/mL BSA in PBS, was incubated with 4 μg of monoclonal antibody for 2 h at room temperature. Upon addition of glycerol and bromphenol blue samples were immediately loaded on the gel. Prolonged incubation with bromphenol blue was avoided because AChE is readily photooxidized by bromphenol blue in the presence of light, with loss of AChE activity (unpublished observation) similar to the photoinactivation by methylene blue.[35]

2 captured fewer AChE molecules than the other monoclonal antibodies, indicated by significant AChE activity staining (lane 3) at the position of the AChE monomer. This could mean the binding affinity of AE-2 for AChE is poor. However, Table 2 does not support this interpretation because K_d values for AE-2 are similar to those for AE-1. AE-2 shows a band at the top of the gel suggesting that AE-2 formed high molecular weight complexes with AChE. The AE-1, 1G, and 6A complexes with AChE migrated similar distances into the gel, but the 10D complex (lane 7) migrated further. Pairing analysis had shown that AE-1, 1G, 6A, and 10D share the same epitope on AChE. Despite binding to the same epitope, the band pattern for the 10D complex suggests that 10D affects the structure or charge state of AChE differently from AE-1, 1G, and 6A. This might be related to the slightly lower K_d value for 10D compared to AE-1, 1G, and 6A. The HR2 complex stands out as particularly different (lane 4). AChE activity bands for the HR2 complex are diffuse and more slowly migrating than those for AE-1, 1G, 6A, and 10D. In addition, there is a substantial band at the top of the gel, suggesting that HR2 formed complexes too large to enter the gel. This result could reflect the higher binding affinity of HR2 for AChE, which could promote formation of high molecular weight complexes.

Nucleotide and Amino Acid Sequences of Monoclonal Antibodies. NCBI accession numbers for the nucleotide and amino acid sequences of 5 anti-HuAChE monoclonal antibodies are listed in Table 3. The HR2 hybridoma cell line was not available to us and therefore sequence information for HR2 could not be obtained.

Table 3. Accession Numbers for Anti-HuAChE Monoclonal Antibodies in the NCBI Database

mAb	chain	accession no.
1G	heavy	KY684069
1G	light	KY684070
6A	heavy	KY684071
6A	light	KY684072
10D	heavy	KY684067
10D	light	KY684068
AE-1	heavy	KY684073
AE-1	light	KY684074
AE-2	heavy	KY684075
AE-2	light	KY684076

The amino acid sequences of the constant regions of the heavy and light chains in Figures 5 and 6 define all 5 monoclonal antibodies as isotype IgG1 kappa. The variable regions of the heavy chains (residues 1−121) for monoclonal antibodies 1G and 6A are 99% identical, differing at only two positions. However, the variable regions of their light chains (residues 1−113) differ by 47%, thus providing evidence that they are nonidentical monoclonal antibodies. The complementarity-determining regions (CDR) are hypervariable domains that determine antibody binding specificity. The CDR in the heavy chains of 1G and 6A are 100% identical, however the CDR in their light chains are only 25% identical. Since CDR3 of the heavy chain is responsible in large part for binding specificity,[36] we had predicted that monoclonal antibodies 1G and 6A would bind to the same epitope of AChE, a prediction supported by pairing analysis.

The heavy chain CDR3 sequence of AE-2 is unique, consistent with the finding that AE-2 binds to a different epitope of AChE than AE-1, 1G, 6A, and 10D. Previous studies using sucrose density centrifugation or competition binding support the conclusion that AE-2 and AE-1 bind to different antigenic site on AChE.[15,34]

DOI: 10.1021/acs.chemrestox.7b00209
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VH1 region of Fab (1-121)                CDR1                                CDR2
DVQLVESGGGLVQPGGSRKLSCAASGFTFSSFGMHWVRQAPEKGLEWVAYISSGSDTIYY   1G
DVQLVESGGGLVQPGGSRKLSCAASGFTFSSFGMHWVRQAPEKGLEWVAYISSGSDTIYY   6A
EVQLQQSGPELVKTGASVKISCKASYSSFTSYYIHWVKQSHGKSLEWIGYISCYNGATTY   10D
EVQLQQSGPEVVKTGASVKISCKASGYSFTGYYIHWVKQSHGKSLEWIGYISCYNGAASY   AE-1
DVKVVESGGGLVKPGGSLKLSCAASGFTFSRFTMSWVRQTPEKRLEWVATISSGGSYIYY   AE-2
                                                CDR3
ADTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYFCARPLYGSSPGWFAYWGQGTLVTVS   1G
ADTVKGRFTISRDNPKNTLFLQMTSLRSEDTAMYYCARPLYGSSPGWFAYWGQGTLVTVT   6A
NQKFKGKATFTVDTSSNTAYMQFNSLTSEDSAVYYCARWFGS-TGAMD-YWGQGTSVTVS   10D
NQRFKGKATFTVDTSSSTAYMQFNSLTSEDSAVYYCARFESMTTGAMD-YWGQGTSVTVS   AE-1
ADSVKGRFTISRDNAKNTLYLQMNSLKSEDTGMYYCTRDAHF-----D-YWGQGTTLTVS   AE-2
     126          CH1 domain of Fab (126-218)
AAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS   1G
AAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS   6A
SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS   10D
SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS   AE-1
SAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQS   AE-2
  Fab binds Protein G
                                        218     hinge region (219-237)  238
DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFI   1G
DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFI   6A
DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFI   10D
DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFI   AE-1
DLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFI   AE-2
                              Fab binds Protein G
FPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS   1G
FPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS   6A
FPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS   10D
FPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS   AE-1
FPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS   AE-2
          CH2 domain of Fc (238-335)      335             350
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDK   1G
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDK   6A
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDK   10D
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDK   AE-1
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDK   AE-2
          CH3 domain of Fc (350-436)
VSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNT   1G
VSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNT   6A
VSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNT   10D
VSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNT   AE-1
VSLTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNT   AE-2
                436
FTCSVLHEGLHNHHTEKSLSHSPGK*                                     1G
FTCSVLHEGLHNHHTEKSLSHSPGK*                                     6A
FTCSVLHEGLHNHHTEKSLSHSPGK*                                     10D
FTCSVLHEGLHNHHTEKSLSHSPGK*                                     AE-1
FTCSVLHEGLHNHHTEKSLSHSPGK*                                     AE-2
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Figure 5. Heavy chain sequences of 5 mouse monoclonal antibodies to HuAChE. Amino acid sequences are translated from cDNA clones. The complementarity-determining regions (CDR) were defined using VBASE2, http://www.vbase2.org/. The constant region of the mouse IgG1 heavy chain starts at position 122 and ends at position 445. The mouse heavy chain constant sequence of our clones matches accession no. P01868 and classifies the monoclonal antibodies as isotype IgG1. Protein G binds IgG through contact with the two Fab regions indicated in the figure,[37] and through contact with the Fc domain.

Mass Spectrometry Detection of RBC AChE Inhibited by a Soman Model Compound. When the active site peptide FGESAGAAS from human RBC AChE is covalently modified by aged soman, the mass of the peptide is increased by 78 Da. Seventy-eight daltons is the residual mass remaining after the pinacolyl group of soman is enzymatically removed during the aging process.

Preliminary studies demonstrated that the active-site, aged-soman peptide, FGESAGAAS + 78 + H^+ (874.35 Da), eluted at 15.4 min. The most prominent peaks in the MSMS fragmentation spectrum were at 874.36 Da (parent ion), 778.35 Da (minus 96 Da from the parent ion due to loss of methylphosphonate plus a molecule of water from the catalytic serine via β-elimination); 673.31 Da (minus 105 Da from 778.35 due to loss of the C-terminal serine, in a b-ion series); and 602.27 Da (minus 71 Da from 673.31 due to the loss of alanine + serine from the C-terminus). The intensities for all of the remaining fragments were substantially lower than that of these four (data not shown).

A 5.8 μg of human RBC AChE sample was inhibited 50% by a soman model compound, immunopurified, digested with pepsin, dried, and resuspended in 20 μL of 0.1% formic acid (5.8 μg ÷ 20 μL = 0.29 μg of total AChE per μL or 0.145 μg of soman-labeled AChE per μL, assuming no losses). Five microliters of this preparation was subjected to liquid chromatography tandem mass spectrometry on a Triple TOF 6600 mass spectrometer. Data acquisition employed a Product Ion method. Product Ion acquisition is a high sensitivity method comparable to the more familiar Multiple Reaction Monitoring method. We used Product Ion acquisition because the Triple TOF 6600 is not capable of Multiple Reaction Monitoring.

DOI: 10.1021/acs.chemrestox.7b00209
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                                CDR1                                CDR2
DILLTQSPAILSVSPGERVSFSCRASQSIGTS------IHWYQQRKNGSPRLLIKHASES    1G
DVVMTQTPLTLSVTIGQPASISCKSSQSLLYSNGKTY-LNWLLQRPGQSPKRLIYLVSKL    6A
DIQMTQTTSSLSASLGDRVTISCSASQGISNY------LNWYQQKPDGTVYLLIYYTSSL    10D
DIQMTQTTSSLSASLGDRVTISCRASQDISNY------LNWYQQKPDGTVKLLIYYTSRL    AE-1
DIVISQSPSSLAVSAGEKVTMSCKSSQSLLDSRTRKNYLAWYQQKPGQSPKLLIYWASTR    AE-2
                                CDR3
MSGIPSRFSGSGSGTDFTLSINSVESEDIADYYCQQSNSWPTTFGAGTKLELKRADAAPT    1G
DSGVPDRFSGSGSGTDFTLKISRVEAEDLGVHYCEQGTHFPLTFGAGTKLELKRADAAPT    6A
QSGVPSRFSGSGSGTDYSLTISNLESEDIATYYCQQYSEPPFTFGGGTKLEIKRADAAPT    10D
HSGVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQGKTFPYTFGGGTKLEIKRADAAPT    AE-1
ESGVPDRFTGSGSGTDFTLTISSVQAEDLAVYYCKQSYNHMYTFGGGTKLEIKRADAAPT    AE-2
constant region
VSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYS    1G
VSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYS    6A
VSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYS    10D
VSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYS    AE-1
VSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYS    AE-2

MSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*                       1G
MSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*                       6A
MSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*                       10D
MSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*                       AE-1
MSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC*                       AE-2
```

Figure 6. Light chain sequences of 5 mouse monoclonal antibodies to HuAChE. Amino acid sequences are translated from cDNA clones. The complementarity-determining regions (CDR) were defined using VBASE2, http://www.vbase2.org/. The constant region of the light chain starts at position 114 and ends at position 219. The mouse light chain sequences determined for our clones match accession no. P01837 and classify the light chains as kappa.

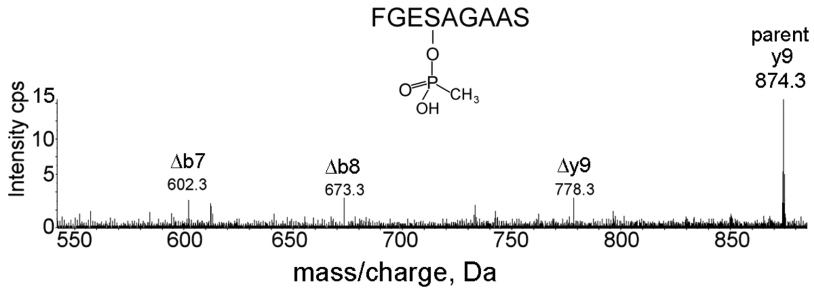

Figure 7. MS/MS spectrum of the active site peptide of human RBC AChE modified on the active site serine with aged soman. The triangle symbol designates fragment ions that have lost the methylphosphonate group and a molecule of water during fragmentation of the parent ion in the mass spectrometer. Immobilized monoclonal 10D was used to immunopurify AChE from no-ghost RBC AChE.

Figure 7 shows the MS/MS spectrum of the active site peptide covalently modified by aged soman. An extracted ion chromatogram was made from the MSMS data between 15.3 and 15.5 min. The three major fragment ions and the parent ion for the active-site, aged-soman peptide of AChE were detected, establishing the presence of this peptide.

■ DISCUSSION

Red Blood Cell Acetylcholinesterase As a Biomarker for Exposure to Soman. We provide a method for using RBC AChE as a biomarker for exposure to organophosphorus nerve agents. The method works with frozen red cells and requires only one centrifugation in a microfuge to prepare samples for immunopurification. Three commercially available and 3 new anti-human AChE monoclonal antibodies immobilized on Sepharose or Dynabeads selectively extract AChE from a crude preparation of solubilized erythrocytes in quantities sufficient for mass spectral analysis. A volume of 6.5 mL of packed red blood cells yielded 5.8 μg of immunopurified AChE, which was sufficient to identify the soman-labeled active-site peptide in a preparation that was 50% inhibited.

Monoclonal Antibodies Immobilized on Sepharose Beads and Dynabeads Protein G. Over a period of 3 years, we tested a variety of RBC AChE preparations in 1% Triton X-100 for binding to monoclonal antibodies immobilized on Dynabeads Protein G. We consistently observed that Dynabeads Protein G bound only 10−50% of the AChE, depending on the immobilized monoclonal antibody. We tested and ruled out the possibility that Triton X-100 interfered with AChE binding to monoclonal antibodies. When we used a different solid support for immobilization, namely, Sepharose beads, we found that monoclonal antibodies bound more than 80% of the AChE. An explanation for the difference in AChE binding efficiency can be offered.

We propose that the difference lies in the manner in which the monoclonal antibodies are attached to the beads. Monoclonal antibodies are immobilized on Sepharose by making a covalent bond between random lysine residues in the monoclonal antibody and hydroxyl groups on CNBr-activated Sepharose. Monoclonal antibodies are immobilized on Dynabeads Protein G via interactions with several constant regions in the heavy chain of IgG. Domain II of Protein G intercalates into a groove between the CH2 and CH3 domains of the Fc region of the heavy chain.[38] In addition Protein G binds to two Fab regions in the CH1 domain of the heavy chain indicated in Figure 5.[37] AChE binds to the variable region in

DOI: 10.1021/acs.chemrestox.7b00209
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the Fab of the heavy and light chains. We hypothesize that access of AChE to the variable Fab region of some antibodies can be partly masked by the interaction of Protein G with Fab. Masking is particularly noteworthy with monoclonal antibodies AE-2, 1G, and 6A (see Table 1). These monoclonal antibodies bind AChE with 33−45% efficiency when they are immobilized on Dynabeads Protein G.

Biomarker Proteins for Detection of OP Exposure. Blood is the most convenient source of proteins for diagnosing exposure to organophosphorus (OP) pesticides and nerve agents. Humans poisoned by OP have been demonstrated to have adducts on plasma BChE and albumin.[39−42] BChE and albumin biomarkers have unique advantages and disadvantages.

BChE reacts readily with low levels of OP, forming characteristic adducts on the active site serine. The CDC has developed a robust screening method, based on BChE, that is capable of testing large numbers of samples for nerve agent adducts.[1−5] However, BChE is not as reactive as AChE with the most toxic isoforms of chemical warfare agents.[8] BChE adducts undergo an aging process that converts unique adducts diagnostic for exposure to a particular nerve agent into a common adduct. Oximes commonly used to treat OP intoxication can remove nonaged adducts from BChE.

OP adducts of serum albumin do not age; therefore they are diagnostic for the original OP that was the source of the exposure.[43,44] Albumin has a longer residence time than BChE in the circulation; therefore albumin adducts can be detected after BChE adducts have disappeared from the circulation.[40,45] Adducts on albumin are not destroyed by oxime drugs commonly used for treatment of OP exposure;[45,46] therefore useful samples can be obtained from patients after they have been treated. However, OP reacts poorly with albumin and the level of adduct formation is typically low. Isolation of the labeled albumin is aided by monoclonal antibodies specific to nerve agent adducts on Tyr411 of albumin.[47]

RBC AChE has its own set of advantages and disadvantages. It is established that RBC AChE is inhibited in humans exposed to nerve agents.[9,48] The active site serine of RBC AChE makes a covalent bond with OP in the same manner as AChE in the nervous system. RBC AChE is considered the most faithful biomarker for nerve agent exposure.[49,50] That is to say that RBC AChE is representative of AChE in the nervous system.[51] This is perhaps the most important reason for developing an RBC AChE method for assessing exposure to OP. Red blood cells have a longer residence time in the circulation than do either BChE or albumin.[6,7] AChE reacts readily with the most toxic stereoisomers of nerve agents soman, sarin, and VX.[8,52] However, OP adducts on AChE, like OP adducts on BChE, age to a common adduct and thereby lose useful information regarding the identity of the OP involved in the exposure. Furthermore, the concentration of RBC AChE in blood is 10-fold lower than the concentration of BChE.[10] Perhaps the most severe disadvantage is that RBC AChE is bound to the membrane of red blood cells. This increases the amount of handling necessary to obtain an analyzable preparation.

Other biomarker proteins in human blood have been proposed as biomarkers but have not yet been developed, namely, acylpeptide hydrolase (P13798) in the cytosol of RBC and neuropathy target esterase (Q81417) in lymphocytes.[53,54]

Anti-Human AChE Monoclonal Antibodies in the Literature. The key to the work in the current report is the use of monoclonal antibodies against RBC AChE. We used three commercially available anti-human AChE monoclonal antibodies from mouse[14,15] and three anti-human AChE monoclonal antibodies from mouse that were prepared in-house. Mouse anti-human AChE monoclonal antibodies have been produced by several laboratories, some of which are commercially available and were used in our study while some are not available.

Human Erythrocyte AChE Immunogen. The first anti-human AChE monoclonal antibodies, AE-1 (IgG1) and AE-2 (IgG1), were produced in mice by Fambrough et al. using pure human erythrocyte AChE as immunogen.[15] These monoclonal antibodies have been used for immunohistochemistry to detect AChE in the neuromuscular junction of human intercostal muscle and guinea pig muscle.[15] An immunocytochemical study identified surface AChE in unfixed rat dorsal root ganglion neurons[55] and showed that antibody binding inhibited neurite outgrowth. AE-2 partially inhibits the activity of human AChE and bovine AChE.[34,56,57] AE-1 and AE-2 do not recognize SDS-denatured AChE and therefore are unsuitable for Western blotting.[57,58] Monoclonal antibodies AE-1 and AE-2 can be purchased from a variety of vendors including EMD Millipore Corp catalog MAB303 and MAB304. The hybridoma cell lines that make AE-1 and AE-2 are available through the American Type Culture Collection catalog HB-72 and HB-73.

Other laboratories have also raised mouse monoclonal antibodies against human erythrocyte AChE. Bjerrum et al. cross-linked monoclonal F18 to Sepharose beads and used the beads to immunopurify AChE from human erythrocytes.[59] Pure AChE with a specific activity of 3800 units/mg and a yield of 88% was eluted with 0.05 M phosphate, pH 11.3, containing 0.5% Triton X-100. Rasmussen et al.[60] made 9 new monoclonal antibodies and in addition tested AE-1 and AE-2 for binding dimeric RBC AChE and tetrameric brain AChE. Three mAbs preferentially bound erythrocyte AChE, while 8 preferentially bound brain AChE in a microplate immunoassay using AChE activity. One antibody was particularly useful for identifying AChE of neuronal origin in amniotic fluid and could therefore be used to diagnose pregnancies with neural tube defects.[60]

Olson et al. made 16 mouse monoclonal antibodies to human RBC AChE.[34] Monoclonal C1B7 (IgG1 kappa) partially inhibits human RBC AChE activity. AE-2 and C1B7 bind to distinct sites on AChE, though both monoclonal antibodies inhibit AChE activity. They decrease catalytic rate but not substrate binding. Dissociation constants for the monoclonal antibodies and RBC AChE were reported to be 20 nM for C1B7, 14 nM for AE-1, and 50 nM for AE-2. C1B7 is available from the Developmental Studies Hybridoma Bank at the University of Iowa.

Johnson and Moore raised monoclonal antibodies to human erythrocyte AChE in mice.[61] These antibodies were shown to inhibit cell adhesion and neurite outgrowth in human neuroblastoma cell line N2α.[61]

Human Brain AChE Immunogen. The laboratory of Stephen Brimijoin prepared mouse monoclonal antibodies to AChE purified from human brain cerebral cortex.[14,62] HR2 (IgG2b kappa) bound AChE from human brain and human RBC. HR2 also bound AChE solubilized from livers of rabbit, guinea pig, sloth, cat, and cow but did not bind AChE from mouse, rat, chicken, frog, or electric eel. Alzheimer brain samples (cortex) were shown to have 41% of the AChE content compared to controls. HR2 does not detect BChE. HR2 has been successfully used in immunohistochemistry, immunoprecipitation, and ELISA procedures[63−65] but cannot be used in Western blot to detect denatured AChE. HR2 is available from

DOI: 10.1021/acs.chemrestox.7b00209
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a variety of commercial sources including GeneTex (GTX22803), Thermo Fisher Scientific (MA3-042), and Abcam (ab2803).

C-Terminal Residues of Tetrameric Human AChE Immunogen. Boschetti et al. designed a monoclonal that could be used to diagnose neural tube defect in amniotic fluid.[66] Neuronal AChE is released into amniotic fluid in neural tube defect but not in normal pregnancies. However, amniotic fluid could be contaminated with RBC AChE. Since tetrameric AChE has a different C-terminal sequence than RBC AChE, Boschetti et al. used the unique 10 C-terminal residues of tetrameric AChE to make monoclonal 190-01. This monoclonal antibody achieved the goal of diagnosing neural tube defect pregnancies. Monoclonal 190-01 also recognized denatured brain AChE on Western blots but, as expected, did not recognize erythrocyte AChE. Monoclonal 190-01 is available from Bio-Porto cat no. HYB 190-01.

A mouse monoclonal antibody against the same 10 C-terminal residues of human AChE was produced by Su et al.[67] Western blot results showed that 2E2 recognized AChE from normal rat brain and muscle, from apoptotic human HeLa cells, and rHuAChE expressed in 293T cells. A rhodamine-conjugated 2E2 stained AChE in rat muscle and rat brain cells.

A mouse monoclonal to the 40 C-terminal residues of human AChE was produced by Cottingham et al.[68] Monoclonal 55C bound native wild-type human AChE but not denatured AChE.

Polyclonals to Human AChE. Polyclonal antibodies produced in goat and rabbit using synthetic peptides as immunogen are available commercially. Silveyra et al. used polyclonal antibodies to immunoprecipitate AChE from Alzheimer brains and to visualize human AChE on Western blots.[69]

Hydrophobic Anchor of Human Brain. Liao et al.[70] raised monoclonal antibodies that specifically recognize the 20 kDa hydrophobic anchor of human brain AChE. The immunogen was heat-denatured amphiphilic detergent soluble G4 AChE from human brain carrying the 20 kDa hydrophobic anchor. Monoclonal antibodies 132-4 (IgG1), 132-5 (IgG1), and 132-6 (IgG3) reacted with native and denatured detergent-soluble AChE from human, bovine, mouse, river trout, and lake trout. No cross-reaction was detected with salt-soluble G4 AChE carrying no anchor, with glycophospholipid-anchored G2 AChE from human and bovine erythrocytes, nor with human BChE. After reduction of brain detergent-soluble AChE with dithiothreitol, the monoclonal antibodies no longer reacted with the antigen because the hydrophobic anchor, which is attached to the catalytic subunit of AChE through a disulfide link, was released when the disulfide bond was reduced. The monoclonal antibodies reacted with tetrameric forms but not with dimeric and monomeric forms. Western blot analysis, after SDS/PAGE under nonreducing conditions, showed reaction with AChE subunits carrying the hydrophobic anchor.[70] The antibodies were at one time available from Statens Seruminstitut, Copenhagen, Denmark, but are not listed on their Web site in the year 2017.

Sequences of Anti-AChE Monoclonal Antibodies. To date the only published sequences of monoclonal antibodies to human AChE are for the 5 monoclonal antibodies in the present report. However, oligonucleotide and amino acid sequences are available for 3 monoclonal antibodies to electric eel AChE.[71,72] The anti-electric eel AChE monoclonal antibodies share less than 50% sequence identity with the variable regions of the anti-human AChE monoclonal antibodies.

■ CONCLUSION

A protocol is presented for using human erythrocyte AChE as a biomarker of nerve agent exposure. Membrane-bound AChE is solubilized from frozen red blood cells and immunopurified by binding to immobilized monoclonal antibody. The immunopurified AChE is digested with pepsin and analyzed by mass spectrometry to identify adducts on the active site serine of AChE, indicative of nerve agent exposure. The same protocol is expected to be suitable for analyzing exposure to organophosphorus pesticides.

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Funding

Supported by DLS/NCEH/CDC Contract 200-2015-87939 (to O.L.), Fred & Pamela Buffett Cancer Center Support Grant P30CA036727, and Centers for Disease Control and Prevention, Office of Public Health Preparedness and Response, and Defense Threat Reduction Agency 11-005-12430 (to T.A.B. and R.C.J.).

Notes

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the U.S. Department of Health and Human Services.

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Mass spectrometry data were obtained with the support of the Mass Spectrometry and Proteomics core facility at the University of Nebraska Medical Center. Journal cover artwork was created with ChemDraw, PyMol, and PowerPoint and is the product of a combined effort by Brooke G. Pantazides, Jonas W. Perez, and Thomas A. Blake, all of the Centers for Disease Control and Prevention.

■ ABBREVIATIONS

AChE, acetylcholinesterase; BChE, butyrylcholinesterase; BSA, bovine serum albumin; CDR, complementarity determining region; FGESAGAAS, PheGlyGluSerAlaGlyAlaAlaSer; HuAChE, human acetylcholinesterase; MSMS, mass spectral fragmentation; NCBI, National Center for Biotechnology Information; OP, organophosphorus toxicant; PBST, phosphate buffered saline with 0.05% Tween-20; PCR, polymerase chain reaction; rHuAChE, recombinant human acetylcholinesterase; RBC, red blood cell; mAb, monoclonal antibody

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DOI: 10.1021/acs.chemrestox.7b00209
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