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Pulmonary delivery of an aerosolized recombinant human butyrylcholinesterase pretreatment protects against aerosolized paraoxon in macaques

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ABSTRACT

Butyrylcholinesterase (BChE) is the leading pretreatment candidate against exposure to organophosphates (OPs), which pose an ever increasing public and military health. Since respiratory failure is the primary cause of death following acute OP poisoning, an inhaled BChE therapeutic could prove highly efficacious in preventing acute toxicity as well as the associated delayed neuropathy. To address this, studies have been performed in mice and macaques using Chinese Hamster Ovary cells (CHO)-derived recombinant (r) BChE delivered by the pulmonary route, to examine whether the deposition of both macaque (Ma) and human (Hu) rBChE administered as aerosols (aer) favored the creation and retention of an efficient protective "pulmonary bioshield" that could scavenge incoming (inhaled) OPs in situ thereby preventing entry into the circulation and inhibition of plasma BChE and AChE on red blood cells (RBC-AChE) and in cholinergic synapses. In contrast to parenteral delivery of rBChE, which currently requires posttranslational modification for good plasma stability, an unmodified aer-rBChE pretreatment given 1–40 h prior to >1 LD50 of aer-paraoxon (Px) was able to prevent inhibition of circulating cholinesterase in a dose-dependent manner. These studies are the first to show protection by rBChE against a pesticide such as paraoxon when delivered directly into the lung and bode well for the use of a non-invasive and consumer friendly method of rHuBChE delivery as a human treatment to counteract OP toxicity.

1. Introduction

Organophosphates (OPs) such as nerve agents and pesticides cause toxicity by inhibiting the activity of acetylcholinesterase (AChE) in neuromuscular junctions [1,2]. Because of this critical targeting of cholinesterases, any efficacious therapeutic candidate for preventing/treating OP poisoning will be a molecule that can bind and competitively scavenge OPs before they reach their physiological target. Plasma-derived BChE currently represents the most advanced pretreatment for protection against OP exposure [3–5] which could potentially take the form of threats to military personnel and first responders, agricultural workers and potentially civilians in the case of deliberate contamination of the environment and critical water supplies. The efficacy of BChE prophylaxis, in terms of survivability and prevention of cognitive impairment, has been clearly demonstrated in rodents and macaques against multiple LD50s of nerve agents [3,5,6]. However because of limited availability and the cost of plasma-derived HuBChE, focus has switched to the development of a recombinant (r) BChE counter-measure. To date, rBChE has been successfully produced in goat milk, mammalian cells and plants [7–9]. However, all forms exhibit poor plasma stability presumably due to host cell-specific glycosylation [10] and must undergo post-translational modification to increase their circulatory retention times following parenteral i.m. (intramuscular), s.c. (subcutaneous) and i.v. (intravenous) delivery [7–9]. In this context, while PEG-ylation of rBChE molecules improves their pharmacokinetic (PK) parameters e.g. AUC, C_{max} and MRT similar to that of the plasma-derived form [10], it also increases the cost and may limit delivery options.

Another challenge to optimal delivery results from the 1:1 stoichiometry between enzyme and OP which necessitates a large rBChE pretreatment dose that must be efficiently transported into the blood and circulate at high concentrations sufficient to scavenge OPs to a level below their median lethal dose within one blood-circulation-time to prevent toxicity [11]. In previous animal PK and protection studies in non-human primates, minipigs and guinea pigs (GPs) [5,6,12], plasma-derived BChE pretreatments of 8.5–30 mg/kg have usually been administered i.m. with reproducible results. However, i.m. and s.c. injections into macaques of large

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tetrameric PEG-rBChE molecules, resulted in variable T_{max} following i.m. injections while C_{max} poorly correlated with dose following s.c. injections [13] due to delayed transport from the injection site.

To optimize the scavenging efficacy of large rBChE molecules, an alternate delivery approach has been developed using an aerosolized form of rBChE (aer-rBChE). This takes advantage of the fact that (i) inhalation of vapors and particles is the predominant form of exposure to insecticides and G-type nerve agents and serves as a major means of intoxication because of rapid accesses of the OP to the blood; (ii) inhaled rBChE molecules will be retained in the lung; being too large to transit the lung endothelium; and (iii) levels of rBChE can be easily maintained in the lungs in a user-friendly way by maintenance "puffs". Thus, in this scenario, BChE, delivered as an aerosol could coat the airways of the lungs forming a "pulmomary bioshield" that can scavenge incoming (inhaled) OPs in situ thereby preventing both their entry into the systemic circulation and their inhibition of RBC-AChE and plasma BChE.

The efficacy of antidotes against OP poisoning cannot be investigated in humans for ethical reasons and will most likely be approved using the Animal rule. To examine the efficacy of pulmonary delivery, mice and macaques were administered different doses of aerosolized PEG-rMaBChE and rHuBChE at 1–40 h prior to 1–2 LD50 aer-paraoxon (Px) and blood cholinersterase activity was monitored at various times thereafter as a measure of protection. The results demonstrate good dose-dependent protection against aer-Px toxicity by both PEG-ylated and unmodified forms of rHuBChE and rMaBChE without immunogenicity.

2. Methods and materials

Animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations stated in the Guide for Guide for the Care and Use of Laboratory Animals (NRC Publication, 1996). Procedures with animals received prior approval by Institutional Animal Care and Use Committees at Bioqual (mice studies) and the Johns Hopkins University School of Medicine (macaque studies) and were performed at Bioqual, MD and at the JHU Research Animal Resources facilities MD, both of which are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

2.1. Chemicals

Paraoxon (99.1% pure) was obtained from ChemService Inc. (West Chester, PA), diluted in sterile water to obtain a stock solution of 1 mg/ml and maintained at RT protected from light. Inoculation doses were prepared 12–24 h prior to use in 1 ml in PBS pH 6.8. Handling of Px and loading of syringes were performed in a biological safety cabinet.

2.2. Production and purification of rMaBChE and rHuBChE

CHO-derived tetrameric rMaBChE (25 mg/kg) and rHuBChE (>80 mg/kg) were produced in CHO-K1 cells were purified using procainamide Sepharose chromatography as described [9] and stored at 4 °C or in a lyophilized form at −20 °C. The purified enzyme exhibits good stability at RT.

Assays for plasma BChE and RBC-AChE activity were performed as described previously [9] using a modified Ellman assay [14]. Briefly, whole blood samples (20 μl) from mice and macaques were first diluted in 180 μl of water (1/10 diln) and tested for plasma BChE and AChE activity. In the latter assay, 20 μM ethopropazine was used as a BChE-specific inhibitor and 1 mM ATC (SigmaAldrich) as substrate. The specific activity of MaBChE (900 U/mg) is higher than HuBChE (700 U/mg). Background levels of mice ranged from 0.515 to 1.528 U/ml (AChE) and 1.034 to 1.784 (BChE) and in macaques from 2.5 to 6.5 U/ml (AChE) and 3.7 to 8.2 (BChE).

2.3. Efficacy of aerosolized BChE in mice

Mice were anesthetized by i.p. injection of ketamine (100 μg/g) and xylazine (16 μg/g) and suspended from their upper incisors at a 45° incline. Fifty μl aliquots containing known doses of PEG-rMaBChE or Px diluted in PBS were introduced into the distal part of the oropharynx by a pipette [15]. With the nose gently clamped closed with forceps, the mouse aspirated the liquid, which was dispersed as an aerosol and deposited in the lungs. Ten BALB/c mice/group (Jackson Labs, ME) received different doses of aer-Px to determine which caused ~50% inhibition of RBC-AChE. PEG-rMaBChE (100 or 200 U) was administered by aspiration 24 h prior to Px to assess protection. Mice were tail bled before and 0.5, 1, 2, 6, 24, 48 and 72 h after administration of Px.

2.4. Delivery of liquid aerosols into monkeys by microsprayer

The method used was similar to that described [16]. Rhesus macaques were initially given ketamine hydrochloride (10–15 mg/kg i.m.) and moved to the surgery where an endotracheal tube was inserted while the animal was maintained under anesthesia with isoflurane/oxygen. EKG leads and a pulse-oximeter sensor were attached to allow monitoring of heart rate and rhythm; respiratory rate and blood oxygen saturation. Since the animals were too small to permit placement of an endotracheal tube of sufficient size (4 mm ID) for passage of a 3 mm OD pediatric bronchoscope, the procedure was modified to allow use of a rigid microsprayer (Model 1A-1B, Penn Century Inc., PA). The IA-1B MicroSprayer® delivers a plume of aerosol (MMD of 25–30 μm) in a closed system and utilizes a 19-gauge, stainless steel tube and is operated by a specially made 1-ml plastic syringe. In early studies a customized version with a large 30 ml reservoir was used. Prior to endotracheal tube insertion, the rigid microsprayer was with a piece of tape around the proximal shaft of the microsprayer to permit its passage down the inserted ET tube to a point which allow optimal aerosolization of the material. Animals were restrained in dorsal recumbency with the head slightly higher than the tail and hot water heating pads beneath in conjunction with a "Baer" hugger warm air blanket, to maintain normal body temperature. Deposition of the aer-rMaBChE following delivery by microsprayer was examined using a dose of 9 mg/kg rMaBChE admixed with the radioisotope ^{99m}technetium (Tc) and scanned using gamma scintigraphy [16].

2.5. Efficacy of aer-PEG-rBChE and aer-rBChE in monkeys

For the macaque studies, Px was delivered by microsprayer in one ml of PBS at RT. After inoculation, the microsprayer was rinsed with 0.1 N NaOH, followed by 70% alcohol and distilled water. Residues were mixed with 0.1 N NaOH and autoclaved. For protection studies, PEG-rMaBChE and unmodified forms of rMaBChE and rHuBChE at various doses were administered by microsprayer 1–40 h prior to aerPx. For high doses multiple syringes were used. Sixteen monkeys (2/group), ranging in weight from 2.7 to 5.6 kg have been studied. Before enzyme and aer-Px and at 0.5, 1, 2, 4, 8, 24, 48, 72 h after aer-Px administration, 20 μl blood samples from the peripheral vein were diluted in 180 μl water and kept at 4 °C until assayed for AChE and BChE activity.