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Pharmacokinetics and immunogenicity of a recombinant human butyrylcholinesterase bioscavenger in macaques following intravenous and pulmonary delivery

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ABSTRACT

Recombinant (r) and native butyrylcholinesterse (BChE) are potent bioscavengers of organophosphates (OPs) such as nerve agents and pesticides and are undergoing development as antidotal treatments for OP-induced toxicity. Because of the lethal properties of such agents, regulatory approval will require extensive testing under the Animal Rule. However, human (Hu) glycoprotein biologicals, such as BChE, present a challenge for assessing immunogenicity and efficacy in heterologous animal models since any immune responses to the small species differences in amino acids or glycans between the host and biologic may alter pharmacodynamics and preclude accurate efficacy testing; possibly underestimating their potential protective value in humans. To establish accurate pharmacokinetic and efficacy data, an homologous animal model has been developed in which native and PEGylated forms of CHO-derived rMaBChE were multiply injected into homologous macaques with no induction of antibody. These now serve as controls for assessing the pharmacokinetics and immunogenicity in macaques of multiple administrations of PEGylated and unmodified human rBChE (rHuBChE) by both intravenous (IV) and pulmonary routes. The results indicate that, except for maximal concentration (Cmax), the pharmacokinetic parameters following IV injection with heterologous PEG-rHuBChE were greatly reduced even after the first injection compared with homologous PEG-rMaBChE. Anti-HuBChE antibody responses were induced in all monkeys after the second and third administrations regardless of the route of delivery; impacting rates of clearance and usually resulting in reduced endogenous MaBChE activity. These data highlight the difficulties inherent in assessing pharmacokinetics and immunogenicity in animal models, but bode well for the efficacy and safety of rHuBChE pretreatments in homologous humans.

1. Introduction

Exposure to organophosphate (OP) neurotoxins in the form of nerve agents or insecticides has become an ever increasing threat to both military and civilian personnel. In addition to the occupational use of pesticides to control agricultural, household and structural pests, intentional release may result in contamination of the environment and critical water supplies. In this context, Gulf War epidemiologic studies have consistently linked pesticide use with the neurocognitive deficits and neuroendocrine alterations described as Gulf War illness that has afflicted many veterans since the war's end [1].

All OP chemical agents exhibit a common mechanism of toxicity, as a result of AChE inhibition and accumulation of acetylcholine, overstimulation of cholinergic receptors, and consequent clinical signs of cholinergic toxicity such as seizures, brain damage as well as cognitive and behavioral defects [2–4]. In animal studies, these symptoms may be prevented by prior treatment with OP-antidotal bioscavenger molecules [5–7]. Of the many catalytic and stoichiometric enzymes, identified as potent bioscavengers of OP neurotoxins, native and recombinant butyrylcholinesterase (rBChE) are the most advanced candidates in terms of developing a human

Abbreviations: BChE, butyrylcholinesterase; Ma, macaque; aer, aerosol.

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treatment. However, due to the serious or life threatening nature of exposure to OP, potential treatments against OP toxicity cannot be tested in humans and will require extensive efficacy testing in animal models and the "Animal Rule" (21 CFR 601.90 for biological products) for regulatory approval. This is important because the reliance on animal studies to determine the efficacy of biological countermeasures under the "Animal Rule", means that native and recombinant human BChE (rHuBChE) molecules need to be tested in heterologous animal models e.g. rodents, or non-human primates (NHP), which usually result in the induction of a humoral immune response and rapid antibody-dependent elimination of the enzyme following a second injection; potentially making efficacy testing problematic.

To address this issue, a homologous macaque (Ma) model in which native and CHO-derived recombinant MaBChE were administered to macaques, was initially developed in order to obtain accurate pharmacokinetics, safety and efficacy testing without induction of anti-BChE antibody following multiple injections [8,9]. Thus, although the unmodified form was rapidly cleared, three injections of PEG-rMaBChE two months apart resulted in good *in vivo* stability, with a mean retention time (MRT) of ~175 h similar to the highly stable native MaBChE (240 h) in the absence of any immune response. Similarly mouse BChE injected IP into mice exhibited good PK parameters and with no induction of antibody [10]. These homologous animal studies are considered to be predictive of the outcome expected to occur in humans receiving rHuBChE and bode well for development of a rHuBChE countermeasure for human use.

To date, functional studies using native or rHuBChE pretreatments have been shown to protect against nerve agent in rodents, NHP and mini-pigs [7,9,11,12]. In addition, prophylaxis using PEG-rMaBChE, rMaBChE and rHuBChE (5–10 mg/kg) delivered either as an aerosol (aer) [13] or by intravascular (IV) injection [14] has prevented toxicity following exposure with paraoxon (Px) delivered subcutaneously (SC) or by aerosol respectively.

In the present studies, the macaque model has been used to assess and compare pharmacokinetic parameters (Cmax, MRT, plasma concentration curve extrapolated to infinity (AUC), elimination half-life (T1/2) and/or immunogenicity following multiple administrations of CHO-derived rHuBChE as well as CHO-derived rMaBChE controls, as a means of predicting the efficacy of protection by rBChE prophylaxis and obtaining animal data required under the Animal Rule. Thus, sera or plasma have been monitored in macaques administered BChE by different routes of delivery. In the first two studies (#1 and #2), macaques received one to three IV injections of either PEG-rHuBChE or PEG-rMaBChE at stoichiometric doses (3 and 5 mg/kg) and in a third study (#3), macaques received one to three administrations of aer-PEG-rMaBChE or unmodified aer-rHuBChE (5–10 mg/kg) via the pulmonary route [13].

Tetrameric forms of MaBChE and HuBChE (MW,345,000) are structurally very similar comprising four identical subunits containing 574 amino acids and 9 carbohydrate chains (23.9% by weight) held together by non-covalent bonds; sharing 96% sequence homology and differing only at 22 amino acids [15]. However, despite the evolutionary closeness of humans and macaques, these results indicate that IV injections of PEG-rHuBChE into macaques results in more rapid clearance than the homologous PEG-rMaBChE and that multiple administrations of PEG-rHuBChE or unmodified rHuBChE induces anti-HuBChE by both IV or pulmonary routes; potentially making an estimation of a human effective dose very challenging.

2. Methods and materials

All Animal studies have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health, and were approved by the Institution's Animal Care and use Committee or local equivalent.

2.1. Expression of rMaBChE and rHuBChE tetrameric proteins

Expression of tetrameric rMaBChE has been described previously [8]. Briefly, MaBChE cDNA (1809 nucleotides) was amplified from total RNA of macaque (*Macaca fascicularis*) liver tissue by RT-PCR. The full length MaBChE cDNA was inserted into the mammalian expression vector pcDNA3.1 carrying a neo (G418-resistance) gene (Invitrogen, CA). For HuBChE expression, the full length HuBChE gene (Genbank ACCESSION NM_000055) was synthesized by GenScript (NJ) and the cDNA cloned into the same pcDNA3.1 vector. For tetramerization, a cDNA fragment of the proline-rich attachment domain (PRAD) of the Colq gene, a 17-residue peptide at the N terminus of the collagen tail [16] was cloned into the pcDNA3.1/Zeo expression vector (Invitrogen CA). CHO-K1 cells, maintained in Ultraculture medium (Lonza, MD), were co-transfected with both MaBChE or HuBChE genes plus the PRAD construct by TransIT-LT1 (Mirus WI) and doubly selected with 400 mg/l G-418 sulfate (Invitrogen, San Diego, CA) and 200 mg/l Zeocin (Invitrogen). Positive colonies were isolated and screened for BChE activity and the 20 highest expressing clones were amplified, verified by RT PCR using MaBChE or HuBChE gene specific primers. Following expansion, expression levels of rHuBChE and rMaBChE were 50 U/ml and 25 U/ml respectively.

2.2. Purification of rMaBChE and rHuBChE

rMaBChE and rHuBChE from transfected CHO-K1 supernatant (SN) were purified using procainamide Sepharose chromatography [17]. Following loading of SN containing the tetrameric proteins, columns were washed with 25 mM sodium phosphate pH 8.0, containing 0.5 mM EDTA and 0.1 M NaCl (rMaBChE) or 0.5 mM EDTA and 0.07 M NaCl (rHuBChE), and the enzyme eluted with a linear gradient of 0.1–1.0 M NaCl in 25 mM sodium phosphate pH 8.0. Fractions were measured for BChE activity at OD280 and those containing BChE were pooled, concentrated and desalted to <0.05 M NaCl in 25 mM sodium phosphate buffer, pH8.0, by ultrafiltration. These steps were then repeated over a second procainamide column.

BChE activity was assayed using 1 mM butyrylthiocholine (BTC) and 0.5 mM5,5-dithiobis 2-nitrobenzoic acid (DTNB) in 50 mM sodium phosphate buffer pH 8.0 at 22 °C. The formation of product was followed by monitoring the increase in absorbance of 5-thio-2-nitrobenzoic acid at 412 nm using a molar extinction coefficient of 13,600 M^{-1}. Activity was reported as U/ml where 1 U represents 1 μmole of BTC hydrolyzed per min.

2.3. PEG-conjugation of tetrameric rMaBChE and rHuBChE

Attachment of the PEG chains to primary amines in rMaBChE was performed using succinimidyl-propionate-activated methoxy-PEG-20 K Sunbright ME-200HS 20 K PEG (NOF, Tokyo, Japan) [18,19]. Purified monomeric or tetrameric rMaBChE (0.5 mg/ml) was incubated by gentle rotation with 25-fold PEG-20 K in 50 mM sodium phosphate, pH 8.0 for 2 h at 23 ± 2 °C. The modified products were dialyzed extensively against 10 mM sodium phosphate, pH 8.0 using a 50 kDa cut off cellulose acetate membrane (Spectrumlabs, Rancho Dominguez, CA). Protein samples were resolved by SDS PAGE on 4–12% SDS PAGE and indicated 4–7 PEG molecules per monomer.