Chapter 7

NERVE AGENT BIOSCAVENGER: DEVELOPMENT OF A NEW APPROACH TO PROTECT AGAINST ORGANO-PHOSPHORUS EXPOSURE

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INTRODUCTION

Nerve agents are highly lethal chemical agent threats to the US population. Chemically, they belong to the organophosphorus (OP) compound group and are among the most toxic substances identified. OP compounds were originally developed for use as insecticides, but their extreme toxicity and rapid effects on higher vertebrates have led to their adoption as weapons of warfare. The OP compounds most commonly used as chemical weapons (referred to as "nerve agents") are O-ethyl N,N-dimethyl phosphoramidocyanidate (tabun; North Atlantic Treaty Organization [NATO] designation: GA), diisopropyl phosphonofluoridate (sarin; NATO designation: GB), pinacoloxymethyl-fluorophosphonate (soman; NATO designation: GD), cyclohexylmethyl phosphonofluoridate (cyclosarin; NATO designation: GF), and ethyl-S-diisopropylaminoethyl methylphosphonothiolate (VX). Newer, nontraditional nerve agents pose even greater dangers than these traditional ones.

Nerve agents in aerosol or liquid form can enter the body by inhalation or by absorption through the skin. Poisoning may also occur through the consumption of liquids or foods contaminated with nerve agents. Nerve agents are lethal at extremely low levels; exposure to a high concentration of nerve agent can result in death within minutes. Poisoning takes longer when the nerve agent is absorbed through the skin. The values for the median lethal dose (LD_{50}) in mammals, including estimates for humans, are in the μg/kg dose range for all routes of exposure except skin, in which LD_{50} values are in the mg/kg range.[1] Personnel may also be effected through secondary contact with contaminated victims. Survivors may have long-term central nervous system dysfunction following intoxication.[2]

The acute toxicity of OPs is attributed to their binding to and irreversible inhibition of acetylcholinesterase (AChE). The resulting increase in acetylcholine concentration manifests at the cholinergic synapses of both the peripheral and central nervous systems by over-stimulation at the neuromuscular junctions as well as alteration in the function of the respiratory center.[3–5] This precipitates a cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation, behavioral incapacitation, muscular weakness, and convulsions, culminating in death by respiratory failure.[3]

Nerve agents are stable, easily dispersed, and can be manufactured by readily available industrial chemical processes, including OP pesticide production facilities, which can easily be converted to produce nerve agents. Even the most dangerous forms of nerve agents are within the technical capability of sophisticated terrorist networks. Nerve agents possessed by rogue states and other potential US adversaries have long been known to pose a serious threat to US forces. Aum Shinrikyo's 1995 sarin attack in the Tokyo subway system demonstrated that nerve agents are also a real and potent terrorist threat to civilian populations. Nerve agents are attractive chemical weapons for terrorist use because small quantities are fast-acting and can cause death or harm by multiple routes. Some types of nerve agents are highly persistent, enabling terrorists to construct long-lasting hazards to target populations. For example, the administration of highly persistent nerve agents to frequently used public facilities, like subway trains, can effect mass disruption by causing citizens to fear using those facilities important to everyday life. The use of nerve agents in combination with other weapons may also make differentiating causalities challenging and place first responders and law enforcement personnel at risk when entering a contaminated area.

Current antidotal regimens for OP poisoning consist of a combination of pretreatment with a spontaneously reactivating AChE inhibitor, such as pyridostigmine bromide, and postexposure therapy with an anticholinergic drug, such as atropine sulfate, and an oxime, such as 2-pralidoxime chloride[6] and an anticonvulsant such as diazepam,[7] if needed. Although these antidotal regimens effectively prevent lethality and, in best cases, reverse toxicity following exposure, they do not prevent the exposed individual from becoming a casualty. Moreover, no current therapies for nerve agent exposure can provide sustained protection to an individual; they have to be readministered within minutes or hours and are therefore limited by practical and logistical issues. Treated patients often show signs of postexposure incapacitation, convulsions, and performance deficits or, in the case of recurring seizures, permanent brain damage.[8–10] Some nerve agents, such as soman, present an additional challenge because of the rapid dealkylation of soman-inhibited AChE that is resistant to therapeutic reversal by an oxime.

An urgent need exists for new medical countermeasures to nerve agent exposure that provide higher survival rates, eliminate or reduce enduring adverse effects to survivors, and significantly reduce or eliminate the need for repeated administration of therapeutic drugs. Ideally, medical treatment should be administered within approximately 1 minute after exposure and should be effective for all OP compounds. These challenges stimulated the development of enzyme bioscavengers as a pretreatment therapy to sequester

highly toxic OPs in circulation before they reach their physiological targets.[11]

The use of enzymes as therapeutic agents is not unique; enzymes are used in wound healing, proteolysis, fibrinolysis, and depletion of metabolites in cancer. Enzymes have many advantages; they are specific, highly efficient, operate under physiological conditions, and cause essentially no deleterious side effects. However, there are certain requirements for an enzyme to be an effective therapy for OP toxicity in vivo: (*a*) it should react rapidly, specifically, and irreversibly with all OP nerve agents; (*b*) it should have a sustained half-life in circulation for it to be effective as a scavenger for long periods; (*c*) it should be readily available in sufficient quantities; and (*d*) it should not be immunogenic. The bioscavengers that have been explored to date for the detoxification of OPs fall into three categories: (1) those that stoichiometrically bind to OPs (ie, 1 mole of enzyme neutralizes 1 mole of OP, inactivating both), such as cholinesterase (ChE), carboxylesterase (CaE), and other related enzymes; (2) a group generally termed "pseudo catalytic," such as those combining AChE and an oxime so the catalytic activity of OP-inhibited AChE can rapidly and continuously be restored in the presence of oxime; and (3) those that can naturally catalytically hydrolyze OPs and thus render them nontoxic, such as OP hydrolase, OP anhydrase, and paraoxonase.

PLASMA-DERIVED STOICHIOMETRIC BIOSCAVENGERS

Candidate stoichiometric bioscavengers are naturally occurring human proteins that bind and react with nerve agents, including enzymes such as ChEs and CaEs. Each of these stoichiometric scavengers has the capacity to bind one molecule of nerve agent per molecule of protein scavenger.

Cholinesterases

Wolfe et al were the first to report the use of exogenously administered AChE as a bioscavenger.[12] They demonstrated that pretreatment of mice with fetal bovine serum (FBS) AChE afforded complete protection against VX, while providing a much lower level of protection against soman. However, FBS AChE pretreatment in conjunction with postexposure administration of atropine and 2-pralidoxime protected mice from both VX and soman. The authors also reported that animals displayed no detectable side effects in response to the administration of FBS AChE alone.

Maxwell et al conducted a similar set of experiments with rhesus monkeys.[13] Monkeys pretreated with FBS AChE that were challenged with either 1.5 or 2.5 times the LD_{50} of soman received total protection without decreased performance when assessed by a serial probe recognition task. Subsequently, Maxwell et al compared the relative protection afforded to mice against soman by three different treatment regimens: (1) pyridostigmine pretreatment with postexposure atropine therapy, (2) postexposure asoxime chloride with atropine therapy, and (3) FBS AChE pretreatment alone.[14] The researchers concluded that the FBS AChE pretreatment alone not only prevented the lethality of animals exposed to 8 to 10 times the LD_{50} of soman, but also protected against behavioral incapacitation.

Boomfield et al were the first to study the protection afforded by butyrylcholinesterase (BChE). They reported that a commercial preparation of equine serum (Eq) BChE afforded complete protection to rhesus monkeys against 2 times the LD_{50} challenge of soman, with no supporting therapy, and against 3 to 4 times the LD_{50} challenge of soman when combined with postexposure therapy with atropine.[15] Protection against a single LD_{50} of sarin without supporting therapy was also demonstrated. Furthermore, when animals were assessed for behavioral deficits using a serial probe recognition task, they all returned to baseline performance levels following soman exposure.

Raveh et al conducted the first study demonstrating the in vivo stoichiometry of OP neutralization by the bioscavenger.[16] They demonstrated that approximately 90% to 95% of FBS AChE that was administered by intravenous (IV) injection was found in the circulation of mice. Circulating enzyme concentrations rose to peak levels in 30 minutes to 1 hour and were maintained for up to 6 hours. This provided a window in which OP challenge of animals yielded a linear correlation between the moles of OP administered and the moles of enzyme neutralized. Ashani et al compared the OP scavenging properties of plasma-derived human (pHu) BChE with those of FBS AChE in mice, rats, and rhesus monkeys against several different nerve agents as well as other OPs.[17] They observed that in mice and rats, the same linear correlation existed between the concentration of pHu BChE in blood and the level of protection afforded against soman, sarin, or VX. They further noted that to be effective, a scavenger had to be present in circulation before OP exposure because the nerve agent had to be scavenged within one blood circulation time period. The window to determine stoichiometry of enzyme and OP became useful even when the enzyme was administered by intramuscular (IM) injection and the OP by subcutaneous injection.[18,19] Raveh et al reported that the same

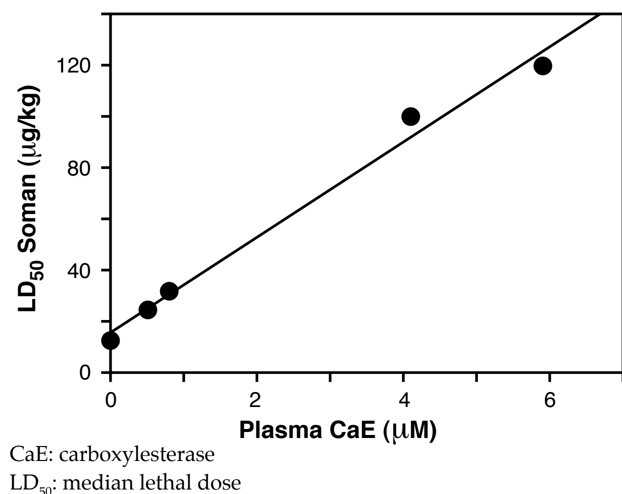
dose of enzyme could protect against 3.3 times the LD_{50} of soman or 2.1 times the LD_{50} of VX in rhesus monkeys.[19] They also reported substantial protection against soman-induced behavioral deficits using a spatial discrimination task.

Wolfe et al assessed the ability of FBS AChE or Eq BChE pretreatment to protect rhesus monkeys against multiple LD_{50} of soman.[20] Survival and the ability to perform the primate equilibrium platform behavioral task were concurrently assessed. Animals pretreated with FBS AChE were protected against a cumulative exposure of 5 times the LD_{50} of soman and showed no decrement in the primate equilibrium platform task. Two of the four monkeys that received purified Eq BChE showed a transient decrement in the primate equilibrium platform task performance when the cumulative dose of soman exceeded 4 times the LD_{50}. All experimental animals were observed for an additional 6 weeks and none displayed residual or delayed performance decrements, suggesting no residual adverse effects.

CaE is another enzyme with potential as a good anti-OP scavenger molecule. CaE can be distinguished from ChEs because while ChEs react with positively charged carboxylesters, such as acetylcholine and butyrylcholine, and are readily inhibited by carbamates, CaE does not react with positively charged substrates and is inhibited by carbamates only at high concentrations.[21] These differences in substrate specificity also extend to the reaction of CaE with OP compounds. Positively charged OP compounds, such as VX, react poorly with CaE, while neutral OP compounds such as soman, sarin, and paraoxon, react rapidly. CaE is synthesized in the liver and secreted into circulation.[22] The levels of circulating CaE vary between mammalian species, and animals that have high levels of plasma CaE require much larger doses of OP compounds to produce toxicity than do species with low levels of plasma CaE.[23] For example, the LD_{50} for soman in rats is 10-fold higher than the LD_{50} in nonhuman primates, which correlates with the differences in the plasma concentrations of CaE found in these species. Like nonhuman primates, humans do not express CaE in plasma.[24]

The primary evidence supporting the hypothesis that CaE can function as a stoichiometric scavenger against OPs (especially sarin and soman) but not for V agents was obtained by comparing LD_{50}s of OPs in animals possessing high endogenous plasma levels of CaE to LD_{50} in the same animal species following inhibition of plasma CaE with chemicals (Figure 7-1).[25] For example, inhibition of plasma CaE reduced the LD_{50} of soman in rats approximately 8-fold, suggesting that circulating CaE can be an effective bioscavenger against OPs. Furthermore, investigations of the reactivation of OP-inhibited CaE have suggested that it may be possible to increase its potential as an OP scavenger by exploiting its turnover of OPs. Maxwell et al observed that OP-inhibited CaE did not undergo aging that prevented oxime reactivation of OP-inhibited ChEs,[26] while Jokanovic et al found that OP-inhibited plasma CaE in rats underwent spontaneous reactivation with a half time of 1 to 2 hours.[27] Extensive investigations need to be carried out before considering using CaE as a bioscavenger for humans. Although human CaE has been cloned and expressed,[22] there is no commercial source of highly purified CaE for use in in-vivo testing of protective efficacy.

The absence of immunological or physiological side effects following transfusions of plasma in humans and the lack of adverse reaction to the administration of partially purified pHu BChE suggest that this enzyme would be the most promising prophylactic antidote for human use.[28,29] As an exogenously administered prophylactic, pHu BChE has several advantages for human use.[30] First, it reacts rapidly with all highly toxic OPs, offering a broad range of protection for nerve agents, including soman, sarin, tabun, and VX. Second, its retention time in human circulation is long



CaE: carboxylesterase
LD_{50}: median lethal dose

Fig. 7-1. Effect of plasma carboxylesterase concentration on soman median lethal dose (administered subcutaneously) in different species. Data points (from lower left to upper right of graph) for species were monkey, rabbit, guinea pig, rat, and mouse.
Reproduced with permission from: Maxwell DM, Wolfe AD, Ashani Y, Doctor BP. Cholinesterase and carboxylesterase as scavengers for organophosphorus agents, In: Massoulie J, Bacou F, Bernard E, Chatonnet A, Doctor BP, Quinn DM, eds. *Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology*. Washington, DC: American Chemical Society; 1991: 206.

and it is readily absorbed from injection sites. Third, because the enzyme is from a human source, it should not produce adverse immunological responses upon repeated administration to humans. Fourth, because the enzyme has no known physiological function in the body, it is unlikely to produce any physiological side effects. Because the biochemical mechanism underlying prophylaxis by exogenous pHu BChE was established and tested in several species, including nonhuman primates, results can be reliably extrapolated from animal experiments and applied to humans. A dose of 200 mg of pHu BChE is envisioned as prophylaxis for humans exposed to 2 to 5 times the LD_{50} of soman.

The foremost requirement to advance pHu BChE as a bioscavenger for human use was to obtain sufficient amounts of purified enzyme with which to conduct animal and clinical studies. Although a procedure for the purification of pHu BChE from human plasma, which contains ~ 2 mg of enzyme per liter of plasma, was described, this source is not suitable for producing gram quantities of pHu BChE.[31] Cohn Fraction IV-4 paste (a by-product of human plasma generated during the production of human blood proteins, such as γ-globulin and clotting factors), was identified as a rich source of pHu BChE. Cohn Fraction IV-4 paste contains ~ 150 mg of enzyme per kg, which is much higher than human plasma, and contains much lower quantities of other plasma proteins because of the fractionation steps employed in the production process. A procedure for the large-scale purification of pHu BChE from Cohn Fraction IV-4 paste was developed using batch procainamide affinity chromatography followed by anion exchange chromatography. Approximately 6 g of purified enzyme was obtained from 120 kg of Cohn fraction IV-4.[32]

Pharmacokinetics and the Safety of Plasma-Derived Human Butyrylcholinesterase

Purified pHu BChE displayed high bioavailability in the circulation of mice, guinea pigs, and nonhuman primates when administered by IV, IM, or intraperitoneal (IP) injections. The enzyme displayed a mean residence time (MRT) of about 48 hours in mice (IM, IP), 109 to 110 hours in guinea pigs (IM, IP) and 72 to 74 hours in rhesus (IV) or cynomolgus monkeys (IM).[32–34] The circulatory stability profiles were similar to those previously observed for enzyme purified from human plasma in rats and mice,[17–19] guinea pigs,[35] and rhesus monkeys.[19]

Because the major envisaged use of bioscavengers is prophylactic, it was important to demonstrate that pHu BChE was devoid of side effects of its own. Mice and guinea pigs with circulating levels of pHu BChE as high as 300 U/mL did not display any signs of clinical toxicity. Results of necropsy performed on animals, together with the examination of hematology and serum chemistry parameters, did not reveal any clinical signs of pathology following the administration of large doses of pHu BChE.[32,33]

In Vitro and In Vivo Stability of Plasma-Derived Human Butyrylcholinesterase

The thermal stability of purified pHu BChE stored at various temperatures has been evaluated.[32] Enzyme activity was stable when stored in lyophilized form at 4°, 25°, 37°, or 45°C for 2 years. The enzyme was also stable when stored in liquid form at 4° and 25°C for 1 year. The circulatory (in vivo) stability of enzyme stored in lyophilized form at −20°C was evaluated by measuring pharmacokinetic parameters in mice.[32] The pharmacokinetic properties of the enzyme were not affected upon storage at −20°C for 3 years.

Efficacy of Plasma-Derived Human Butyrylcholinesterase

The efficacy of pHu BChE was evaluated in guinea pigs and cynomolgus monkeys against multiple LD_{50} challenges of nerve agents.[34] Guinea pigs were protected against a cumulative dose of 5 times the LD_{50}s of either soman or VX, and there was a decrease in molar concentration of exogenously administered circulating pHu BChE equivalent to the amount of OP administered in a given time period.[36] For example, guinea pigs administered Hu BChE equivalent to 8 times the LD_{50} of soman attained peak blood BChE levels of approximately 300 U/mL. After challenge with 5.5 times the LD_{50} of soman, the enzyme level decreased to approximately 100 U/mL. This approximate 200 U/mL decrease in blood BChE level is equivalent to around 5 to 5.5 times the LD_{50} of soman. With VX challenge, proportionately less enzyme was administered because the LD_{50} of VX is smaller. No signs of poisoning were observed in the experimental animals during the efficacy studies. Animals were subjected to necropsy 7 or 14 days following nerve agent challenge and all tissues were normal upon light microscopic examination. In nonhuman primates, cynomolgus monkeys were protected against a cumulative challenge of 5.5 times the LD_{50} of soman. Of the six animals challenged, one died after the final challenge dose of soman (total 5.5 times the LD_{50} within 4 h) and one was euthanized 48 hours after the final dose of soman. The surviving animals displayed no signs of poisoning. Subsequent examination of these animals did not show any signs of delayed toxicity following examinations of blood chemistry and hematology parameters for less than 20 months.[37]

Most efficacy studies conducted to date have used IV or subcutaneous challenge of OPs. A study in which guinea pigs were administered soman by inhalation challenge following pretreatment with pHu BChE (IV or IM) showed that only 26% to 30% of enzyme was neutralized.[35] Because it is most likely that humans will be exposed to nerve agent through inhalation, more efficacy studies using inhalation are needed before the protective dose of enzyme can be established for humans.

Immunological Safety of Plasma-Derived Butyrylcholinesterase

A critical prerequisite for any potential bioscavenger is a prolonged circulatory residence time and the absence of antienzyme antibodies following repeated injections of the enzyme. Previously, it was demonstrated that multiple injections of Eq BChE into rabbits, rats, or rhesus monkeys resulted in an MRT spanning several days and the induction of antienzyme antibodies.[38–41] In these experiments, blood enzyme activity appeared to correlate negatively with anti-BChE immunogammaglobulin (IgG) levels. On the other hand, administering purified macaque BChE into macaques of the same species resulted in much longer MRT (225 ± 19 h) compared to that reported for heterologous Hu BChE (33.7 ± 2.9 h). A smaller second injection of macaque BChE given 4 weeks later attained predicted peak plasma levels of enzyme activity, although the four macaques showed wide variation in the MRT (54 to 357 h). No antibody response was detected in macaques following either injection of enzyme.[42]

More recently, the consequences of repeated injections of pHu BChE and plasma-derived mouse (pMo) BChE from CD-1 mice were examined in Balb/c[43] and CD-1[36] mice following two IM injections 4 weeks apart. The effects of two heterologous (pHu BChE) and homologous (pMo BChE) injections were monitored by following blood BChE activity and anti-BChE IgG levels. In Balb/c mice, the clearance of pMo BChE activity following the first injection occurred slowly (MRT = 91.8 h), compared to the heterologous pHu BChE injection (MRT = 56.7 h). As expected, the second injection of pHu BChE cleared much faster from the circulation of mice compared to the first injection. Surprisingly, the second injection of pMo BChE did not attain the predicted peak enzyme level, and a shorter MRT (61.6 h) was observed. No circulating anti-pHu BChE IgG was detected following the first pHu BChE injection, and significant levels of antibodies to pHu BChE could be detected 2 days after the second pHu BChE injection. As expected, no circulating anti-pMo BChE IgG was detected following the first pMo BChE injection. However, antibodies to pMo BChE, although 100-fold less than the levels observed with pHu BChE, were detected 5 days after the second Mo BChE injection. This could be due to differences in pBChEs from the two strains of mice and was subsequently resolved by repeating the study in CD-1 mice.

In CD-1 mice, the clearance of homologous pMo BChE activity following the first injection also occurred slowly (MRT = 73 h), compared to the heterologous Hu BChE injection (MRT = 48 h). As expected, the second injection of Hu BChE cleared much faster from the circulation of mice compared to the first injection (MRT = 26 h). The second injection of homologous Mo BchE, on the other hand, attained a peak enzyme level that was similar to that observed following the first injection and a similar MRT of 79 hours. As expected, circulating anti-Hu BChE IgG could be detected 5 days following the first pHu BChE injection, which increased dramatically after the second injection. No significant antibody response was detected following either of the two homologous pMo BChE injections. The absence of antibody responses following either injection in a homologous system are in agreement with the long retention times and the absence of significant adverse effects following administration of homologous macaque BChE into macaques. The observation that the second injection of pMo BChE resulted in a pharmacokinetic profile that was similar to that of the first injection is in agreement with the lack of a humoral response to the injected enzyme. The observed extended stability of exogenously administered pMo BChE into mice and macaque BChE into macaques suggests that even a single injection of homologous BChE is sufficient to maintain the enzyme at a long-lasting therapeutic level. The results of both studies with two injections of BChE have clearly demonstrated the utility of homologous BChE as an effective and safe scavenger, exhibiting high stability and low immunogenicity in recipient animals. With respect to the potential use of pHu BChE in humans, these results are consistent with a reported in-vivo half-life of 8 to 11 days and the absence of reported untoward immunological and physiological side effects following blood transfusions and IV injections of partially purified pHu BChE into humans.[28,29,44,45]

Behavioral Safety of Plasma-Derived Butyrylcholinesterase

Because the major use of bioscavengers is prophylactic, administered days or weeks prior to a potential exposure, it is essential that the enzyme be devoid of undesirable effects. Thus, several studies have evaluated the behavioral and physiological effects of pBChE administered alone as well as prior to nerve agent

exposure.[46] For example, Genovese and Doctor evaluated the effects of highly purified Eq BChE on learned and unlearned behavior in rats.[47] Administration of 500 to 7500 U of Eq BChE (resulting in circulating BChE levels as high as ~ 55 U/mL) did not affect acquisition or retention of a passive avoidance task. Additionally, no disruption of performance of a food-maintained operant behavior task was observed. To evaluate unlearned performance, 24-hour spontaneous motor activity was evaluated before and after administration of Eq BChE. There was no disruption of either the total number of activity counts nor the circadian pattern of activity when monitored for 10 days following administration. Finally, the enzyme was shown to provide significant protection against performance degradation produced by 7-(methylethoxyphosphinyloxy)-1--methylquinolinium iodide (MEPQ), a peripherally active OP compound. The safety and efficacy of FBS AChE and Eq BChE was also evaluated in rhesus monkeys using a memory-intensive serial probe recognition task, in which subjects were required to recall a list of stimuli.[13,15,19] Repeated administration of a commercial preparation of Eq BChE that produced a 7- to 18-fold increase in circulating BChE levels did not systematically affect task performance,[41] and rhesus monkeys pretreated with 460 to 503 nmol of Eq BChE were protected against 2 or 3 times the LD_{50}s of soman or sarin.[15]

Similar studies were conducted to address the safety and efficacy of pHu BChE in mice,[17] rats,[48] guinea pigs,[35] and rhesus monkeys.[19] In all cases, doses of pHu BChE sufficient to protect against OP exposure were devoid of behavioral side effects. Brandeis et al demonstrated that pHu BChE was protective against soman and had no apparent effect on spatial memory as assessed by a Morris water-maze task.[48] Similarly, Raveh et al evaluated the safety of pHu BChE and its therapeutic efficacy against VX and soman toxicity using standardized observations and behavioral performance on a spatial discrimination task in rhesus monkeys.[19] For subjects in which the ratio of enzyme to OP was near or greater than 1, no or mild signs of toxicity were observed, largely with recovery by the next day. Regarding the safety of pHu BChE, three of four monkeys exposed to either 13 mg (10,400 U) or 34 mg (27,200 U) of pHu BChE did not show any observable deficits resulting from pHu BChE administration alone.[19] The transient behavioral effect observed in the fourth monkey was attributed to a nonspecific malaise induced by this enzyme preparation.

More recently, the behavioral safety of large doses of pHu BChE alone were evaluated in mice and rhesus monkeys. Clark et al showed that in mice, 2000 U of pHu BChE (the equivalent of 30 times the dose required for protecting humans from 2 times the LD_{50} of soman) did not significantly alter acoustic startle or prepulse inhibition behavior.[49] Similarly, administration of 30 mg/kg of pHu BChE was devoid of any adverse effects in rhesus monkeys when performance was assessed using a six-item serial probe recognition task.[50] Taken together, these studies demonstrate that pHu BChE pretreatment can provide protection against OP exposure while being devoid of adverse behavioral and physiological effects.

RECOMBINANT STOICHIOMETRIC BIOSCAVENGERS

Plasma-derived Hu BChE represents a first-generation biological scavenger. This material is obtained from outdated human plasma (Cohn Fraction IV-4 paste), and the overall availability is related to the quantity of fraction of the processed human plasma available at any given time. Sufficient amounts of Cohn Fraction IV-4 paste are generated in the United States by blood processing establishments to produce at least 100,000 doses of the bioscavenger product per year. Although this amount of material may be adequate for use by first responders in case of civilian exposure or deliberate, accidental, or limited combat engagement, it is not sufficient to protect the entire population or even the entire military. To identify a more reliable source of Hu BChE, recent research efforts focused on the development of Hu BChE from recombinant expression systems. If successful, such efforts will allow for a constant supply of material of reproducible purity and activity without dependence on the supply of outdated plasma. There are a variety of potential sources of recombinant Hu BChE (rHu BChE), including transgenic plants,[51] transgenic animals,[52] transfected insect larvae,[53] or algae.[54] In addition, rHu BChE can be expressed in cell lines.[55,56] The cell-derived rHu BChE was shown to be a mixture of monomers, dimers, and tetramers and contained incomplete glycan structures.[57] Similarly, goat-milk–derived rHu BChE is primarily a dimer, with some protein present as monomers and tetramers. In contrast, pHu BChE is predominantly tetrameric and possesses mostly biantennary complex and some high mannose glycan structures. Also, goat-milk–derived rHu BChE has a different glycosylation pattern than that of pHu BChE and contains a carbohydrate moiety that has been demonstrated to be immunogenic in humans.[58] Because of the lack of subunit assembly and complete glycan structures, rHu BChE has a much shorter circulatory half-life than pHu BChE.[57] To enhance its

biological residence time, rHu BChE was modified to include polyethylene glycol adducts. The polyethylene glycolylated material had a pharmacokinetic profile similar to that of the pHu BChE,[55,59] suggesting that differences in pharmacokinetics between plasma-derived and recombinant enzymes can be addressed using in-vitro posttranslational modifications. Efficacy studies using rHu BChE from transgenic goat milk in guinea pigs against soman and VX have yielded results similar to those previously described that used pHu BChE.[59] These results suggest that effective recombinant stoichiometric bioscavengers can be developed, potentially providing a source for sufficient material for military members and civilians (such as first responders, emergency medical personnel, and agricultural workers) that may be occupationally exposed to OP pesticides.

CATALYTIC BIOSCAVENGERS

Although stoichiometric scavengers are able to afford good protection as long as the enzyme level in the body is higher than the amount of OP, they have a relatively high molecular weight; a comparatively large quantity is required to neutralize a small amount of nerve agent. A catalytic scavenger, even having the same high molecular weight, could be administered in smaller quantities and would potentially produce the same or greater extent of protection. It would also be advantageous because it would not be consumed in the process of detoxifying the nerve agent, making it available to protect against multiple OP exposures. Enzymes with intrinsic, catalytic, anti-OP activities come from a variety of sources, such as the OP hydrolase from *Pseudomonas diminuta*,[60] the OP anhydrase from A*lteromonas haloplanktis*,[61] and human paraoxonase 1 (Hu PON1).[62–66] Recombinant OP hydrolase from *Pseudomonas diminuta* was shown to protect mice against behavioral side effects and lethality caused by soman.[67] Similarly, pretreatment with only OP hydrolase purified from *Pseudomonas* species was shown to protect mice from lethality due to paraoxon, diethylfluorophosphate, and tabun.[68,69] Most of these enzymes possess short circulation times in vivo, and none has the ability to hydrolyze all known toxic OPs, nor do any have the high turnover required to dispose of the OPs from blood in one circulation time. In addition, these bacterial enzymes are likely to initiate potent immune responses in humans; therefore, they are not suitable for repeated use. Bacterial enzymes could conceivably be useful for skin protection as active components of topical skin protectants or covalently bound to the cornified layer of epidermis.[70] OPs can also be detoxified through enzymatic oxidation of their alkyl chains. In particular, breakdown of VX by horseradish peroxidase[71] or by *Caldariomyces fumago* chloroperoxidase[72] could be used in a polyfunctional active topical skin protectant and for skin decontamination.

Conversely, Hu PON1 can possibly afford protection without the potential complication of inducing an immune response. However, Hu PON1 does not possess the desired catalytic activity at a rate that is fast enough for use as a nerve agent pretreatment. Because agent must be cleared from the bloodstream within one circulation time (1 to 2 minutes) before it reaches critical targets,[15] a functional catalytic scavenger must have both a lower K_m (a measure of the strength of binding of a substrate to the enzyme) and a high turnover number (k_{cat}). Research efforts were directed toward creating such an enzyme by specific mutation of enzymes such as Hu BChE and Hu PON1. Hu BChE mutation designs were based on the fact that OP inhibitors are hemisubstrates for this enzyme. The acylation reaction is similar to that of normal substrates, but the subsequent reaction, equivalent to deacylation of the active site serine, cannot be affected because the amino acid group responsible for dephosphylation is not in the appropriate position.[73,74]

The perceived solution to this problem was to insert a second catalytic center into the active site specifically to carry out the dephosphylation step of the reaction.[74] Applying this rationale, wild-type Hu BChE was mutated in the oxyanion hole to create a mutated enzyme, G117H, with the ability to catalyze the hydrolysis of sarin, diisopropylfluorophosphate (DFP), paraoxon, VX, and other nonaging nerve agents.[74,75] Aging and reactivation are parallel first-order reactions in phosphylated enzymes. In the reactivation reaction, the phosphoryl group is removed from the active site serine residue (Ser198), restoring activity, whereas in the aging reaction one of the alkyl groups is removed from the phosphoryl group, rendering the inhibited enzyme nonreactivatable. To catalyze the hydrolysis of rapidly aging nerve agents such as soman, it is necessary to slow the rate of the aging reaction so that reactivation is faster. This was accomplished by replacing the carboxyl group Glu197 adjacent to the active site serine with an amide group.[76] Although these efforts were successful, the mutants have catalytic activities that are still too slow for practical use.

Hu PON1 is currently being subjected to mutation in efforts to generate faster catalytic antinerve agent enzymes. Because OPs are "accidental" substrates for paraoxonase,[62,64] it is likely that activity improvement can be realized through protein engineering. Two of the major difficulties in designing appropriate site-

directed mutations in Hu PON1, the lack of knowledge on the residues at the active site and the enzyme's three-dimensional structure, were recently overcome by the work of Josse et al,[65,66] Harel et al,[77] Aharoni et al,[78] and Yeung et al.[79,80] Based on site-directed mutations of amino acids believed to be at or near the active site of Hu PON1 and on limited sequence homology with a DFPase, Josse et al had postulated that the molecule had the shape of a 6-fold beta propeller. Using a mouse-rat-rabbit-human chimera of paraoxonase 1 obtained through gene shuffling experiments and expressed in bacteria, Harel et al[77] and Aharoni et al[78] confirmed the postulated structure through X-ray crystallographic studies. Yeung et al have subsequently carried out site-directed mutation studies to identify and "map" amino acid residues critical for binding and involved in catalytic activity.[79,80] Further studies have revealed a degree of stereospecificity in the hydrolysis of soman by native Hu PON1, with the least toxic soman stereoisomer (C+P+) being hydrolyzed ~ 6 times more efficiently than the most toxic one (C−P−).[81] The observed stereospecificity is primarily due to preferential binding rather than to enhanced turnover of the (C+P+) stereoisomer by Hu PON1. All of these recent findings support the goal of designing a recombinant version of a naturally occurring human enzyme that can be developed as a catalytic biological scavenger to protect against nerve agent poisoning.

INTERAGENCY PARTNERSHIPS: PROJECT BIOSHIELD

Project BioShield was signed into law by President George W Bush on July 21, 2004. It grants the secretaries of the US Department of Health and Human Services and the US Department of Homeland Security authority to present the president and the director of the US Office of Management and Budget with recommendations for developing and procuring countermeasures to chemical, biological, radiological, and nuclear threats. Funding over 10 years was appropriated to the Department of Homeland Security for Project BioShield, establishing a new spending authority to spur development and procurement of "next generation" medical countermeasures (vaccines, therapeutics, and diagnostics) against chemical, biological, radiological, and nuclear agents. It also authorizes the National Institutes of Health to speed research and development in promising areas of medical countermeasures to these agents, grants increased flexibility and authority to award contracts and grants under expedited peer review procedures, and allows more rapid hiring of technical experts deemed necessary for research and development efforts. The Department of Defense is joining in this effort to leverage interagency resources. The objectives are to develop dual-use technologies and products that can be used to expand target populations (military and civilians) for US Food and Drug Administration licensure. Project BioShield legislation requires that products are manufactured under current Good Manufacturing Practices (practices recognized world-wide that ensure the safe manufacturing, management, testing, and control of goods, foods, and pharmaceuticals) and have completed a successful Phase I human clinical safety trial. Plasma-derived Hu BChE is currently being produced from human Cohn Fraction IV-4 and will be used for preclinical safety and toxicology testing with the intention of large-scale production and more extensive testing to be carried out leading to licensure. The bioscavenger countermeasure has been identified as a potential candidate for Project BioShield.

Collaborating in the BioShield process requires the Department of Defense to expand the concept of use to first responders, healthcare workers, and civilians. One way to protect those groups may be to stockpile sufficient amounts of pHu BChE, which could then be used in conjunction with extensive decontamination measures and personal protective equipment when indicated. In some settings, pHu BChE may replace the need for pyridostigmine bromide as a pretreatment medical countermeasure. Most studies tested the enzyme as a preventive countermeasure because once the nerve agent has reached the nerve synapse, pHu BChE becomes ineffective; at that point, intervention would include the traditional countermeasures (atropine, pralidoxime, and anticonvulsant). Although the majority of bioscavenger use will be in the preexposure setting, bioscavenger may also be useful in neutralizing on-going postexposure risks following skin absorption, which could lead to prolonged systemic exposure (ie, the "depot effect").

SUMMARY

OP nerve agents represent a very real threat not only to service members in the field but also to the public at large. Nerve agents have already been used by terrorist groups against civilians and, because of their low cost and relative ease of synthesis, are likely to be used again in the future. In addition, many commonly used pesticides and chemical manufacturing by-products can act as anticholinesterases and may

be a low-dose exposure threat to workers in a variety of professions. Anticholinesterase pesticides may also be used against civilians in a terrorist context. Current therapeutic regimes for acute nerve agent exposure are generally effective in preventing fatalities if administered in an appropriate time period. For acute multi-LD_{50} levels of OP exposure, pyridostigmine pretreatment coupled with postexposure administration of an oxime, atropine, and an anticonvulsant does not prevent substantial behavioral incapacitation or, in some cases, permanent brain damage. It is therefore important from both military and domestic security perspectives to develop novel defenses against nerve agents, including the use of bioscavenger molecules, that avoid many of the difficulties associated with current treatments. While the use of nerve agents on the battlefield may be somewhat predictable, nerve agent use in a terrorist situation will be, in all probability, a surprise event. The potential to afford long-term protection to first-responders exposed to toxic or incapacitating concentrations of OPs is a notable advantage of biological scavengers.

Recent efforts have focused on identifying proteins that can act as biological scavengers of OP compounds and can remain stable in circulation for long periods of time. By prophylactically inactivating OPs before they inhibit central nervous system AChE, this approach avoids the side effects associated with current antidotes and the requirement for their rapid administration. Ideally, the scavenger should enjoy a long residence time in the blood stream (11–15 days), should be biologically inert in the absence of nerve agent, and should not present an antigenic challenge to the immune system. Taken together, pharmacological safety, toxicity, stability, and efficacy data strongly support pHu BChE as a safe pretreatment for chemical agent intoxication. Pharmacokinetic parameters of pHu BChE in mice, guinea pigs, and monkeys suggest that a single dose of enzyme can maintain blood BChE at a therapeutic concentration for at least 4 days. Safety and toxicity studies demonstrate that pHu BChE, even at a dose that is 30 times the therapeutic dose, is devoid of tissue toxicity and is safe for human use. Plasma Hu BChE has a long shelf life (2 years) in lyophilized

TABLE 7-1

PROTECTION BY HUMAN BUTYRYLCHOLINESTERASE AGAINST NERVE AGENT POISONING

Treatment	Test Species	Nerve Agent	Protection* (LD_{50})	Impairment	Recovery
pHu BChE	Rat	GD	1.5	None	Immediate
pHu BChE	Guinea pig	GD	5.5	None	Immediate
pHu BChE	Guinea Pig	VX	5.0	None	Immediate
pHu BChE	Rhesus monkey	GD	3.3	4 of 8	15 min to 2 h
pHu BChE	Rhesus monkey	VX	2.1	2 of 4	20 min to 20 h
pHu BChE	Cynomolgus monkey	GD	5.5	1 of 5	4 of 6†
rHu BChE	Guinea pig	GD	5.5	None	Immediate
rHu BChE	Guinea pig	VX	5.5		Immediate
ATR/2-PAM/DZP	Guinea pig	GD	1.5	4 of 4	2 of 4, days‡
ATR/2-PAM/DZP	Guinea pig	VX	1.5	10 of 10	10 of 10, days‡

*Values represent multiples of median lethal doses (LD_{50}s) of nerve agent survived after BChE administration.
†One animal died after the third dose of soman and one was impaired and later euthanized after 48 hours. The remaining four animals were normal, survived, and were held for long-term observations.
‡Two animals died in the first hour, while the other two remained impaired for 2 to 4 days.
2-PAM: 2-pyridine aldoxime methyl chloride
ATR: atropine
DZP: diazepam
Hu BChE: human butyrylcholinesterase
LD_{50}: median lethal dose
pHu BChE: plasma-derived human butyrylcholinesterase
rHu BChE: recombinant human butyrylcholinesterase
Data sources: (1) Genovese RF, Doctor BP. Behavioral and pharmacological assessment of butyrylcholinesterase in rats. *Pharmacol Biochem Behav.* 1995;51:647–654. (2) Lenz DE, Maxwell DM, Koplovitz I, et al. Protection against soman or VX poisoning by human butyrylcholinesterase in guinea pigs and cynomolgus monkeys. *Chem Biol Interact.* 2005;157–158:205–210. (3) Raveh L, Grauer E, Grunwald J, Cohen E, Ashani Y. The stoichiometry of protection against soman and VX toxicity in monkeys pretreated with human butyrylcholinesterase. *Toxicol Appl Pharmacol.* 1997;145:43–53. (4) Garcia GE, Moorad-Doctor D, Doctor BP, et al. Glycan structure comparison of native human plasma butyrylcholinesterase (Hu-BChE) and transgenic goat produced Hu-BChE. *FASEB J.* 2005;19:A867.

form at temperatures 4° to 25°C. Similarly, the pharmacokinetic properties of the enzyme were not affected upon storage at – 20°C for 3 years. Pretreatment with pHu BChE protected guinea pigs against a 5 times the LD_{50} of soman or VX. As expected, pHu BChE injection in mice or monkeys elicited the production of high levels of anti-BChE antibodies. No antibody response was detected following either of the two homologous mouse or monkey BChE injections. The observation that the second injection of homologous BChE resulted in a pharmacokinetic profile that was similar to that of the first injection is in agreement with the lack of a humoral response to the injected enzyme.

By nearly all criteria, the use of biological scavengers to protect against exposure to a lethal dose of a nerve agent offers numerous advantages over conventional treatment therapies (Table 7-1). Developing an effective prophylactic to nerve agent exposure will greatly reduce, if not eliminate, the need to know the precise length of exposure in a crisis situation. Successful prophylaxis will also preclude the need to repeatedly administer a host of pharmacologically active drugs with short durations of action. Also, the need to use personal protective equipment to protect against nerve agent exposure could be greatly reduced, which is particularly significant for first responders handling known casualties of nerve agent exposure. Finally, the appropriate scavenger would protect against all current nerve agent threats, including those that are refractory to treatment by atropine and oxime therapy. In cases of lower doses of nerve agents or in response to agents that potentially exert a time-release depot effect, pHu BChE could be used as a postexposure treatment to combat continued toxicity of the absorbed agent.

Several challenges must be met before bioscavengers can augment or replace the current therapeutic regimes for nerve agent intoxication. The immunogenicity and serum half-life of the scavenger must be determined in humans, and efforts may be required to minimize any immune consequences and maximize the residence time in circulation. Additionally, appropriate dosages of scavenger must be determined that will, based on animal models, protect against concentrations of nerve agents likely to be encountered in a wide range of scenarios. While research efforts to date have resulted in the successful transition to preclinical trials of stoichiometric scavengers, the use of either naturally or genetically engineered enzymes with catalytic activity to hydrolyze OPs holds the greatest theoretical promise for the development of a broad specificity, high efficacy, prophylactic scavenger. Current research efforts are focused on designing and expressing such enzymes and characterizing their in-vivo, antinerve agent efficacy in animal models acceptable to the Food and Drug Administration.

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