

86. DIISOPROPYLFLUOROPHOSPHATE (DFP) ANTAGONISM BY RECOMBINANT ORGANOPHOSPHORUS ACID ANHYDROLASE (OPAA) ENCAPSULATED WITHIN STERICALLY STABILIZED LIPOSOMES (SL)

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INTRODUCTION

The investigation was focused on antagonizing organophosphorus (OP) intoxication by employing a new conceptual approach, which is based on encapsulation of recombinant enzymes within biodegradable enzyme carrier systems. OP compounds exert their toxic effects by inhibiting acetylcholinesterase (AChE) causing an excessive accumulation of the neurotransmitter, acetylcholine, and subsequent disruption of cholinergic nervous transmission. Clinical therapy presently uses the OP antidotes atropine and 2-PAM. Atropine antagonizes acetylcholine at the muscarinic sites, and pralidoxime (2-PAM) reactivates the OP inhibited acetylcholinesterase (1). But neither 2-PAM nor atropine degrades the OP agent. One of the fundamental mechanisms for the detoxification of OP compounds involves the degradation with OP-hydrolyzing enzymes so that the OPs are unable to phosphorylate the AChE. Using exogenous enzymes in OP antagonism previously has been reported (2), (3) and (4). However, the injection of purified free enzyme preparations into the blood stream has serious limitations because of immunologic reactions and various adverse physiological disposition factors. Biodegradable liposome carriers, which are permeable to the toxin molecules, can efficiently provide large amounts of the highly purified metabolizing enzymes to remain in the circulation for long periods of time. Carrier resealed annealed erythrocytes (CRBC) were first used as drug/enzyme carriers 2-3 decades ago (5) and (6). Drugs and enzymes were encapsulated into resealed annealed red blood cells by hypotonic dialysis. CRBCs were successively employed as carriers with rhodanese in cyanide antagonism (7), (8), (9), (10), (11) and (12) and in OP antagonism (13) and (14). Encapsulation of squid-type diisopropylphosphorofluoridate-hydrolyzing enzyme (DFPase) into carrier red blood cells was first reported in 1993 (15). Sterically stabilized liposomes have been widely used as targeted drug delivery systems in the clinical therapy (16). In the stealth liposome system, a drug is encapsulated in a biocompatible carrier vesicle that can circumvent the body's immune defenses, thereby circumventing rapid uptake by the macrophage cells of the reticuloendothelial system (16), (17), (18) and (19). Previous studies reported the antidotal effects of OP hydrolase (OPH) when it was encapsulated within sterically stabilized liposomes (20). OP hydrolase (OPH) and OPA anhydrolase (OPAA) have different substrate specificity. OPH hydrolyzes paraoxon and other agricultural insecticides and OPAA degrades DFP, sarin and soman (21). OPAA is a prolidase enzyme, which can hydrolyze dipeptides with a prolyl residue at the carboxyl terminus (E.C. 3.4.13.9). Nucleotide sequence of a gene encoding an OP nerve agent degrading enzyme from *Alteromonas haloplanktis* was reported in 1997 (22). Biodegradable liposome carriers act as protective environments for metabolizing enzymes that antagonize the toxic effects of various OP agents. This study is focused on the application of a recombinant OP hydrolyzing enzyme, OPAA, whose substrate specificity is amenable to hydrolyzing DFP, sarin and soman.

MATERIALS AND METHODS

Chemicals: DFP was purchased from Sigma (St Louis, MO). DFP solution was prepared by diluting in cold (0-2°C) solution of 0.9 % sodium chloride and was chilled on ice not longer than 2 hours before administration. Atropine sulfate and 2-PAM solutions were prepared daily. All other chemicals used were of the highest purity commercially available.

Enzyme: Purification of OPAA (EC 3.1.8.2) from *Alteromonas* strain JD6 was reported in 1993 (21) and in 1991 (22).

Encapsulation: Sterically stabilized liposomes (SL) were prepared as previously described (19). Percentage of encapsulation efficiency was calculated from the amount of encapsulated OPAA divided by the amount of OPAA added multiplied by 100.

OPAA Activity Determination in (SL): OPAA activity in liposomes was measured by monitoring the production of fluoride from DFP with a fluoride ion sensitive electrode (Orion Research Inc., Boston, MA) (24). The assay solution for the enzyme fractions contained NaCl (70 mM), KCl (280 mM), Tris (70 mM, pH 7.2), and DFP (3.44 mM). The solution for determining enzyme activity of (SL)* was isotonic: It contained phosphate buffer (10 mM), NaCl (0.144M), MgCl₂*6H₂O (2.0 mM) dextrose (5mM), and DFP (3.44mM), and had the osmolality of 290 mosM. The DFP solution was kept on ice before using, and the enzyme activity was determined at 25 ± 1°C in a thermostated titration flask

(Brinkman Instrument, Inc.). The total volume of the solution was 5.00 ml. Electrode potential was recorded as a function of time on a strip chart recorder (Orion Research Inc, Boston, MA), and the potential values were converted to concentration using the Nernst equation. Results are expressed as the mean \pm 1 standard deviation unless otherwise indicated. One unit of OPAA is defined as that amount of enzyme which hydrolyzed 1 μ mol of DFP to fluoride and isopropylphosphate per minute.

Animals

Male Balb/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighting between 18-20 g were housed in a temperature and light controlled rooms (21 \pm 2 °C, 12-h light/dark, full spectrum lighting cycle) and were furnished with water and 4% fat Rodent Chow (Teklad HSD, Inc., WI) ad libitum. All animal procedures were conducted in accordance with the guidelines by The Guide for the Care and Use of Laboratory Animals (National Academic Press, 1996), credited by AAALAC (American Association for the Assessment and Accreditation of Laboratory Animal Care, International). At the termination of the experiments, surviving animals were euthanized in accordance with the 1986 report of the AVMA Panel of Euthanasia.

In vivo experiments: Male mice received 10-20 units of OPAA intravenously (as the free enzyme or encapsulated within SL*) one hour prior to receiving DFP subcutaneously. No gross toxic effects were apparent in mice receiving encapsulated OPAA, atropine and 2-PAM, either alone or in various combinations. Atropine (10 mg/kg) and 2-PAM (90 mg/kg) were administered intraperitoneally to mice 45 and 15 minutes, respectively, prior to receiving OPAA. Each LD₅₀ value was obtained from five or more graded doses of DFP administered to five or more groups of six to eight mice based on 24 h mortality. The LD₅₀ values were determined by the method of Litchfield and Wilcoxon (25) as determined by the computer program PHARM/PC Version 4.1 (26). All statistical procedures were performed at the 95% confidence level.

RESULTS AND DISCUSSIONS

Recombinant OPAA was encapsulated into SL by mechanical dispersion method (19) and (17) with minor modifications with an 80 to 90% encapsulation efficiency. Purification of OPAA (EC 3.1.8.2) from *Alteromonas* strain JD6 has been reported by Cheng, et al. (1993). Sterically stabilized liposome encapsulating OPAA rapidly hydrolyzes DFP to fluoride and diisopropyl phosphate (Figure 1). Enzymatic hydrolysis of DFP with varying amount of (SL)* is shown in Figure 2. Concentration of the (SL)* within each added aliquot was constant and DFP hydrolysis was linear. Formation of fluoride ions was directly proportional to the amount of (SL)* containing OPAA. There was no OPAA activity noted with liposomes containing no enzyme. Increases in amount of (SL)* are equivalent to increases in amount of OPAA in the reaction. DFP hydrolysis catalyzed by the OPAA containing (SL)* was linear over a period of 1 min depending on the concentration of DFP

These sterically stabilized liposome system encapsulated with recombinant OPAA was used in these OP antagonism studies. Prophylactic antidotal protective effects of OPAA (encapsulated and free) with the combination of 2-PAM and/or atropine are summarized in Table 1. (Potency ratio = LD₅₀ antagonized / LD₅₀ unantagonized). The LD₅₀ for the unantagonized DFP is 4.2 mg/kg (control, expt. 1). Atropine alone gave an increase in LD₅₀ to 5.7 mg/kg (expt. 2), and 2-PAM alone increase the protection to 7.7 mg/kg (expt. 3). When 2-PAM and atropine were combined, the LD₅₀ was increased to 29.3 mg/kg (expt. 4). When free OPAA enzyme without liposomes was administered in a combination with 2-PAM + atropine (expt. 5), the protection of 2-PAM and atropine combination was only slightly enhanced to 33.2 mg/kg. When sterically stabilized liposomes encapsulating OPAA was used in combination with 2-PAM and atropine (expt. 6), a dramatic enhancement was detected: The LD₅₀ value was increased to 98.6 mg/kg. The overall protection against DFP intoxication was enhanced from 4.2 mg/kg to 98.6 mg/kg with the 2-PAM, atropine, and (SL)* combination.

The results suggest a potentially new conceptual approach for the rational design of antidotal therapy against chemical poisoning. Presently, there are less than six specific antidotes that exceed the LD₅₀ values by five times when protecting against the lethality of chemical poisoning. There is a need for a more successful approach to develop antidotes. Recent developments in molecular biology and biotechnology have made it possible that fast catalytic recombinant enzymes can be rapidly prepared and encapsulated within liposomes with minimal macrophage recognition. By using sterically stabilized long circulating liposomes (SL) the half-life of conventional liposomes of only a few hours can be extended to a half-life of a few days. This permits the development of effective prophylactic and therapeutic agents. These results represent an application of SL as a biodegradable enzyme carrier system for OP hydrolyzing enzyme, OPAA, to degrade OP agents and to antagonize the lethal effects of various OPs including DFP and possibly permit extrapolations can be made from kinetic data with other substrates such as soman and sarin.

Earlier studies (20) indicated that (SL) encapsulating an other OP degrading enzymes (OPH) provide considerable protection either alone or in a combination with 2-PAM and/or atropine to antagonize the lethal effects of paraoxon.

When combined with 2-PAM and atropine, (OPH-SL*) increased the protection over 1000 LD₅₀ doses. Preliminary data with paraoxon indicate that the [2-PAM - atropine - (OPH-SL*)] antidotal system is superior to the [2-PAM-atropine-(OPAA-SL*)] system with DFP. However, of greater importance is that (OPAA-SL*) combined with 2-PAM and atropine greatly potentiated the protection against DFP. If our extrapolation from the enzyme kinetic data is valid, protection with (OPAA-SL*) against the nerve gas, sarin, should be superior to DFP, and the protection against soman should be at least the same as against DFP. The enzyme kinetic data (27) indicates that paraoxon has a high affinity to OP hydrolase (OPH), but this enzyme is less effective for DFP, sarin and soman (Table 2). OPAA has higher affinity to DFP, soman and sarin; however, it does not hydrolyze paraoxon and other agricultural OP insecticides effectively. DFP is chemically and kinetically similar to the chemical warfare agents, soman and sarin, therefore it seems to be an appropriate OP model compound in an attempt to predict the protective effects of (OPAA-SL*) against soman and sarin intoxication.

SUMMARY

Antidotal combination for the antagonism of organophosphorus (OP) poisoning in the USA are 2-PAM and atropine. Atropine prevents muscarinic receptor occupation whereas 2-PAM reactivates the OP inhibited acetylcholinesterase, but they don't degrade the OP molecules. In these studies sterically stabilized liposomes (SL) containing recombinant OP hydrolyzing enzyme (OPAA) were employed as a carrier model to antagonize the lethal effects of DFP. DFP serves as a good substrate for OPAA as well as some chemical warfare agents, e.g. soman and sarin. The in vitro properties of this carrier system and the in vivo antidotal protection was studied when it was used in combination with 2-PAM and/or atropine. The hydrolysis of DFP was determined by measuring the increase of fluoride ion concentration using fluoride sensitive electrode. Recombinant OPAA enzyme originated from *Alteromonas* Strain J6D has a broad substrate specificity for OP compounds, especially for DFP, soman and sarin. The OPAA encapsulated within (SL) could rapidly hydrolyze DFP. The rate of DFP hydrolysis was directly proportional to the amount of (SL)* added to the reaction mixture. (SL) with no enzyme did not hydrolyze DFP. Rate of hydrolysis was first order to fluoride and diisopropylfluorophosphate. The present in vivo studies suggest that the antidotal protection of the classic OP antidotes 2-PAM and/or atropine can be strikingly enhanced when they are used in a combination with the OPAA encapsulated (SL)*. (Supported by grants from NIH, NSF, USAMRDC, NOAA, and NATO)

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KEYWORDS

OPA Anhydrase (OPAA), Sterically Stabilized Liposomes, OP Hydrolase (OPH), Organophorus antagonism, Long Circulating Liposomes

FIGURES AND TABLES

Figure 1. Sterically stabilized liposomes encapsulating OPAA rapidly hydrolyzes DFP.

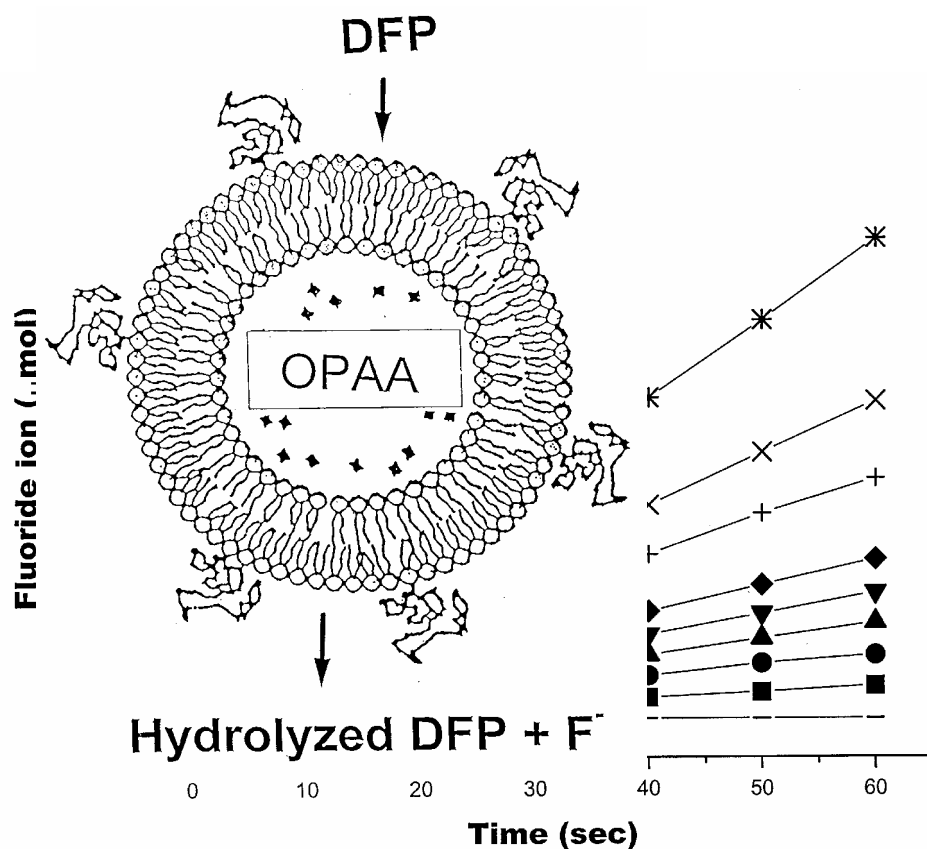


Figure 2. Time course for DFP hydrolysis by OPAA encapsulated within SL.

Table 1. DFP Antagonism on Swiss Webster Mice with OPAA

Exp.	Treatments before DFP	a	b
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	OPAA carriers	OPAA (iv)	2-PAM (ip)	Atropine (ip)		
1	Control (no antagonist)	-	-	-	4.2 (3.9-4.6)	1.0
2	(No Enzyme) (no carrier)	-	-	+	5.7 (5.3-6.3)	1.3
3	(No Enzyme) (no carrier)	-	+	-	7.7(7.4-8.1)	1.8
4	(No Enzyme) (no carrier)	-	+	+	29.3 (28.0-30.6)	6.9
5	Free OPAA (no carrier)	+	-	-	7.3 (6.8-7.5)	1.7
6	Free OPAA (no carrier)	+	-	+	7.4 (6.6-8.2)	1.7
7	Free OPAA (no carrier)	+	+	-	10.3(9.2-11.5)	2.4
8	Free OPAA (no carrier)	+	+	+	33.2(31.1-35.4)	7.8
9	Sterically Stabilized Liposomes	+	-	-	9.6(8.9-10.2)	2.3
10	Sterically Stabilized Liposomes	+	-	+	18.9(16.5-19.5)	4.5
11	Sterically Stabilized Liposomes	+	+	-	21.1 (20.0-23.5)	5.0
12	Sterically Stabilized Liposomes	+	+	+	98.6(86.5-104.5)	23.2

a) DFP (3.0-120 mg/kg) was delivered subcutaneously to mice. Atropine sulfate (10 mg/kg) and 2-PAM-CI (90mg/kg) was given intraperitoneally, 45 and 15 minutes respectively, prior to receiving DFP. (SL)* (20-30 units/mice) was given intravenously through tail vein one hour prior to receiving DFP.

b) Potency Ratio = $\frac{\text{LD50 of DFP with antagonists}}{\text{LD50 of DFP without antagonists}}$

Table 2. Comparison of kinetic behavior of some OP compounds with OPH and OPAA

Substrate	Paraoxon	DFP	Soman	Sarin
Enzyme	kcat			
OPH	41400	410.00	9.6	80
OPAA	0.43	76.8	60.9	281.5