

93. ANTAGONISM OF ORGANOPHOSPHOROUS LETHALITY STERICALLY STABILIZED LIPOSOMES (SL) CONTAINING RECOMBINANT ORGANOPHOSPHOROUS ACID HYDROLASE (OPH)

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ABSTRACT

Sterically stabilized liposomes were employed as a carrier model to antagonize the lethal effects of the anticholinesterase organophosphorous agent, paraoxon. OPH rapidly catalyzes the hydrolysis of paraoxon to the less toxic 4-nitrophenol and diethylphosphate. This recombinant enzyme was encapsulated within SL and administered to mice either alone or in combination with pralidoxime (2-PAM) and/or atropine. These results indicate that this carrier model system provides a dramatic protective effects against the lethal effects of paraoxon. Moreover, when this carrier liposomes were administered with 2-PAM and/or atropine either alone or in various combinations, a striking synergistic protection against paraoxon was observed. The advantage of this new SL system is the high encapsulation efficiency and it can be stored at 2°C. SL containing recombinant OPH alone can protect better than 2-PAM and/or atropine combination against the lethal effect of paraoxon. Application of 2-PAM, atropine and SL containing OPH can protect over 1000 LD50 doses of paraoxon. Phosphotriesterase is being used to increase organophosphate (OP) degradation to phosphotriesterase to antagonize (OP) intoxication. For these studies, sterically stabilized liposomes encapsulating recombinant phosphotriesterase were employed. This enzyme was obtained from *Flavobacterium sp.*, and was expressed in *Escherichia coli*. It has a broad substrate specificity which includes parathion, paraoxon, and other organophosphorous compounds. Paraoxon is rapidly hydrolyzed by phosphotriesterase to the less toxic 4-nitrophenol and diethylphosphate. This enzyme was isolated and purified over 1600 fold and subsequently encapsulated within sterically stabilized liposomes (SL). The properties of this encapsulated phosphotriesterase were investigated. When these liposomes containing phosphotriesterase were incubated with paraoxon, it readily degraded the paraoxon. Hydrolysis of paraoxon did not occur when these sterically stabilized liposomes contained no phosphotriesterase. These sterically stabilized liposomes (SL) containing phosphotriesterases (SL)* were employed as a carrier model to antagonize the toxic effects of paraoxon by hydrolyzing it to the less toxic 4-nitrophenol and diethylphosphate. This enzyme-(SL) complex (SL)* was administered intravenously to mice either alone or in combination with pralidoxime (2-PAM) and/or atropine intraperitoneally. These results indicate that this carrier model system provides a striking enhanced prophylactic and therapeutic protection against the lethal effects of paraoxon. Moreover when these carrier liposomes were administered with 2-PAM and/or atropine, an enhanced protection occurred. (Supported by grants from NIH, NSF, USAMRDC, NOAA, and NATO)

INTRODUCTION

Parathion, an organophosphorous (OP) pesticide, is still as one of the most widely used insecticide worldwide and has caused toxicological problems. It is metabolized to its active metabolite, paraoxon, which is responsible for most of its activity and toxicity. Paraoxon exerts its toxic effect by inhibiting acetylcholinesterase (AChE). This would prevent the hydrolysis of the neurotransmitter, acetylcholine, and result in high accumulation of acetylcholine. These acetylcholine molecules bind to receptors causing overstimulation of the effector cells, resulting in cholinergic intoxication. Organophosphorous compounds are believed to exert their effect by phosphorylating the esteratic site of acetylcholinesterase, forming an almost irreversible complex. There are cholinesterase reactivators, i.e., pralidoxime (2-PAM) which can reactivate the OP-inhibited cholinesterase. This would increase the rate of dephosphorylation of the OP-inhibited enzymes, restoring the hydrolysis of acetylcholine. Atropine, a reversible OP pharmacologic antagonist, acts at the acetylcholine receptor, primarily at the muscarinic sites. One of the most common antidotal combinations now used to treat OP poisoning is atropine and pralidoxime. Atropine itself has severe autonomic side effects which can affect stamina, vision, and body temperature control, etc. Pralidoxime (2-PAM), the biochemical antagonist, has limited absorption and poor physiological disposition factors. It would be desirable to have an organophosphorous antagonist which exerts its effect by actually destroying the toxicant. If there were a mechanism to degrade paraoxon rapidly as it

enters the blood and before it reaches the target sites, paraoxon would possibly be much less toxic. Recombinant enzymes that rapidly hydrolyze OP compounds have been reported by (1) and (2), and have been described to protect animals against OP intoxications (3). However, the potential uses of such free enzyme preparations as antidotes are limited due to their unfavorable physiological dispositions and potential immunological reactions. These disadvantages can be partly overcome by encapsulating the enzyme within a bioprotective environment, i.e., sterically stabilized liposomes. The ability of the CRBC containing recombinant phosphotriesterase to antagonize the toxic and lethal effect of paraoxon was reported by (4). The mechanism of action of this carrier CRBC as an antagonist may be partly attributed to the rapid degradation of paraoxon by this enzyme. The phosphotriesterase is encapsulated within the stealth liposome and exerts no pharmacologic activity until the animal is exposed to the OP agent. Under those conditions, the OP agent is rapidly hydrolyzed before it distributes to the target site.

Sterically stabilized, long circulating, stealth liposomes have found widespread use as model membrane systems, and have been extensively investigated for their potential use as drug carriers (5) and (6). First formulations of long circulating liposomes resulted from attempts to mimic some of the properties of the outer surface of red blood cells. The present research describes the use of sterically stabilized liposomes as a carrier system for recombinant phosphotriesterase as a new approach to developing specific antidotes. The antidotal combination of 2-PAM and atropine are effective in antagonizing OP intoxication; however, the OP agent still remains in the body as neither of these antagonists actually destroys the organophosphates. Because of the potentially rapid action of OP compounds, it seemed appropriate that a major investigative effort be directed towards developing an agent to destroy the OP agent, rather than merely making the animal more resistant to the OP agent. This is being accomplished by encapsulating a phosphotriesterase enzyme within long circulating liposomes.

MATERIALS AND METHODS

Enzyme. A recombinant phosphotriesterase (EC 3.1.8) was purified from an *Escherichia coli* clone containing the plasmid expression vector pjK33 which was isolated from *Flavobacterium* sp (1) and (2). The enzyme was obtained in preparative amounts and purified over 1600 fold.

Encapsulation of Phosphotriesterase within Sterically Stabilized Liposome (SL).* Palmitoylcholine (POPC) and dipalmitoyl phosphatidylethanolamine-N-[poly(ethylene glycol)²⁰⁰⁰] (PEG-PE) were purchased from Avanti Polar Lipid (Alabaster, AL). Purified cholesterol was obtained from Calbiochem (San Diego, CA). Lipids were stored in chloroform under argon at -70°C. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Chloroform solutions of POPC (60 µmol), cholesterol (40 µmol) and PEG-PE (5.4 µmol) were mixed in a round-bottomed flask, and the solvent was removed slowly on a rotary evaporator at 37°C to obtain dry thin lipid film on the flask. Purified phosphotriesterase in HEPES [N-[2 - Hydroxyethyl] piperazine-N¹-[2-ethanesulfonic acid] sodium salt] buffer (concentrated on Amicon Concentrator and clarified by centrifugation if necessary) was added to the dry lipids. The lipid film was hydrated slowly under argon by continuously rotating the flask on a rotary evaporator at 37°C for one hour. The milky liposome suspension was extruded sequentially through 0.2 µ and 0.1 µ polycarbonate membrane filter. Extrusion was repeated five times for each membrane to obtain a homogeneous size distribution of liposomes. Unencapsulated phosphotriesterase was separated from liposomes by gel filtration on sepharose 4B column. The encapsulation efficiency was calculated from the amount of encapsulated phosphotriesterase divided by the amount added times 100.

Phosphotriesterase Activity Determination in Sterically Stabilized Carrier Liposomes. Phosphotriesterase activity in liposomes was measured at room temperature, by determining the increase in p-nitrophenol concentration in the presence of excess paraoxon (4). One unit of phosphotriesterase is defined as that amount of enzyme which hydrolysed 1 µmol of paraoxon to p-nitrophenol per min.

Animals. Male Balb/C mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighting between 18-20 g were housed in room temperature and light controlled rooms, and were furnished with water and 4% Rodent Chow (Teklad HSD, Inc., WI) *ad libitum*. All animal procedures were conducted in accordance with the guidelines by the NIH Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services, DHEW Pub No. (NIH) 85-23, 1985).

In vivo experiments. Male mice received 5-10 units of phosphotriesterase intravenously (encapsulated within SL*) one hour prior to receiving paraoxon (in 6% cyclodextrin and propylene glycol solvent system) subcutaneously. The vehicle

employed consists of 40% propylene glycol, 10 % ethanol and 50% water (v/v/v). Animals exposed to paraoxon with antagonists (atropine and/or 2-PAM and/or phosphotriesterase) were determined by 24 hr mortality. Surviving animals were observed for an additional one week for late developing toxicity. No gross toxic effects were apparent in mice receiving encapsulated phosphotriesterase, atropine, and 2-PAM, either alone or in various combinations. Atropine and 2-PAM were administered intraperitoneally to mice 30 and 15 minutes, respectively, prior to receiving paraoxon. The LD₅₀ values with (SL)* were determined by the up-and-down method, and the estimated 95% confidence interval was determined by the method of Bruce, (13). For each experiment, 6-10 mice were used.

RESULTS

This is the first application of Sterically Stabilized Liposomes containing phosphotriesterase (SL)* to antagonize a toxicant. Genes for this phosphotriesterase enzyme had been identified from *Flavobacterium sp.* (12), and had been cloned and expressed in *E. coli*. Use of protamine sulfate treatment, ammonium sulfate fractionations, gel filtration and anion exchange chromatography resulted in the enzyme purification factor of over 1600. This purified phosphotriesterase was encapsulated into sterically stabilized liposomes.

This method for sterically stabilized liposome preparation resulted in approximately 80 % encapsulation efficiency. Phosphotriesterase entrapped in the carrier liposomes was capable of rapidly hydrolyzing paraoxon. Liposomes containing no enzyme, do not hydrolyze paraoxon. Enzymatic hydrolysis of paraoxon with various amount of (SL)* containing phosphotriesterase is shown. The reaction was linear at the time range and at the concentration range in which we conducted these experiments. Increases in p-nitrophenol formation was directly proportional to the amount of (SL)* containing phosphotriesterase. There was a direct linear relationship between the amount of (SL)* and the amount and rate of p-nitrophenol formed in the enzymatic reaction. No increase in p-nitrophenol concentration was detected when liposomes did not contain any enzyme.

Protective effects of phosphotriesterase encapsulated into sterically stabilized carrier liposomes (SL)* either alone, or in a combination of 2-PAM and/or atropine are summarized in Table 1, and are expressed as potency ratios (potency ratio= LD₅₀ antagonized/LD₅₀ unantagonized) to demonstrate the overall efficacy of these antidotal systems to antagonize the lethal effects of paraoxon. The encapsulated phosphotriesterase alone was more effective than the classic antidotal treatment of atropine and 2-PAM. Animals receiving both the encapsulated phosphotriesterase and 2-PAM or atropine showed dramatic increases in the protection, and it was even more highly enhanced when the encapsulated phosphotriesterase was used in combination with atropine and 2-PAM. The antidotal potency ratios, expressed as the protection of the antagonized paraoxon over unantagonized paraoxon, illustrate the dramatic enhanced effects of the (SL)* system in antagonizing paraoxon intoxication not only alone, but particularly in combination with atropine and/or 2-PAM. The LD₅₀ value of paraoxon of the control animal was used as a potency ratio of 1.0. (SL)* alone caused an increase in the potency ratio to 139.0. When 2-PAM (potency ratio=5.0) was used in a combination with (SL)*, the potency ratio was increased to 611.3. Atropine (potency ratio=2.3) used in a combination with (SL)* exhibited an increase in the potency ratio to 600.3. When both atropine and 2-PAM (potency ratio=61.1) were used in a combination with (SL)*, the potency ratio was elevated to 1022.

DISCUSSION

This research is an attempt to use fast catalytic enzymes encapsulated within biodegradable carriers to antagonize the lethal effects of toxicants. These results represent the first attempt to use sterically stabilized liposomes containing recombinant phosphotriesterase (SL)* to hydrolyze paraoxon. Hopefully this approach may serve to protect against the toxic effects of other chemicals with other enzymes. This is being accomplished by encapsulating recombinant enzyme with high catalytic activity within a sterically stabilized liposome. This phosphotriesterase has high affinity to paraoxon ($K_m=0.05$ mM); therefore, it is a good substrate to study OP antagonism on this enzyme. There is no significant difference in K_m values between the free and the encapsulated phosphotriesterase; therefore paraoxon readily penetrates through the membrane of the carrier cells, -what is a critical requirement for the application of our approach.

Phosphotriesterase is being encapsulated within (SL)* to accomplish several factors. First, this places the phosphotriesterase in a bioprotective environment so that the enzyme will not be rapidly degraded in the body. Secondly, this prolongs the activity of the enzyme which is important as the OP agent is lipid soluble and can remain in the fatty depot for a long time. In order to remove the OP agent, an antagonist with long duration of action is desirable. Thirdly, the mechanism of action of this antagonist is quite different from the classic antagonist of 2-PAM and atropine. The combination of the latter two antagonists merely prevent the pharmacologic actions of the OP agent by making the

animals more resistant to the OP agent, but the toxic agent still remains in the body. By the use of phosphotriesterase within (SL)*, the OP agent could be degraded in the body, thereby terminating its action. OP hydrolyzing enzymes have been found in various human and animal tissues (9) and (10) and bacteria (11). When phosphotriesterase preparation is administered intravenously, it can protect animals against OP poisoning (12) and (3) but the enzyme is rapidly; therefore, the therapeutic value of the free enzymes alone as an antagonist is very limited.

This new conceptual approach which uses carrier cells containing highly purified recombinant enzymes to rapidly destroy the organophosphorous (OP) compound before it reaches the target site provides striking results. Although the free purified enzyme preparations can protect animals against certain OP intoxications, their clinical uses are very limited in scope due to their rapid degradation and elimination by body defense systems. The half life of free peptide preparations is only about 20 minutes; therefore, it is impractical to use them to prevent or to treat exposures. The encapsulation of the enzyme of interest into carrier cells, enhances the stability of the enzyme and prolongs the life-span of the carrier cells. Once the phosphotriesterase encapsulated into a carrier cell is administered to the body, the enzyme within the carrier cells could continue to hydrolyze toxic OP agents. The cell carrier system may be useful not only in prevention and/or treatment of acute OP toxicity, but also it may prevent the delayed toxicity; in this case, preventing toxicities from chronic exposure to OPs, which would not be achievable by other approaches. This could be particularly important to agricultural workers and pesticide manufacturing personnel. It may also play a significant role for military personnel as the OP nerve gases are one of the most potent chemical weapons. It permits a practical prophylaxis in our combat troops as the protection can last for days to months, depending on the carrier system, without affecting the mobility and physical performance of our troops. Furthermore, this cell carrier system may be useful in combination with the traditional antidotes, atropine and 2-PAM, since the encapsulated phosphotriesterase protects by an entirely different antidotal mechanism.

This approach has developed a prophylactic agent which is superior to the antidotal combination of 2-PAM and atropine. This study indicates that the magnitude and duration of protection could be greatly increased when highly purified phosphotriesterase was entrapped into sterically stabilized liposomes. The (SL)* allows permeation of the paraoxon molecules to freely enter and leave the carrier vehicle cells reaching a rapid equilibration and resulting in a rapid detoxication when the enzyme still retains its activity in the protected intracellular environment.

The role of 2-PAM and/or atropine in this marked synergism is still not clear. For example; if the acetylcholinesterase inside the nervous system plays a major role, the 2-PAM has to distribute to the nervous tissues. 2-PAM has an electrical charge of 1, it is unlikely that it can penetrate the blood brain barrier. It should form a neutrally charged structure to do this. One possible explanation for the OP antagonism is, that the peripheral tissues outside the vascular system probably plays the major role of the OP intoxication. It should be noted, that the phosphotriesterase in combination with atropine make up the most striking potentiation. Whereas 2-PAM is enhanced over 100 fold with phosphotriesterase, atropine is enhanced over 200 fold. Since atropine occupies predominantly the muscarinic receptors for acetylcholine, the mechanism involved the reactivation of inhibited cholinesterase must still play a major role. To explain this dramatic protection probably does involve a series of factors. For example, to assume the construction of the OP inhibited acetylcholinesterase is partially reactivated, and the lowering of the acetylcholine concentration will permit the atropine to act at a more enhanced level. Since the atropine and acetylcholine compete for the receptor occupation, with the decrease in acetylcholine, the role of atropine becomes more prominent. An enzyme with a high turnover number may be necessary for these dramatic protections observed. With lower turnover number the dramatic enhancement may not be apparent, as the cascade of events would not be accelerated.

In summary, a new concept has been presented for the antagonism of OP intoxication. This general approach has great conceptual significance, as it suggests the potential for encapsulating other enzymes as a drug delivery system to degrade other chemical toxicants.

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KEYWORDS

Phosphotriesterase, OPA anhydrase, paraoxonase, paraoxon antagonist, paraoxon hydrolysis, sterically stabilized liposomes, stealth liposomes, long circulating liposomes

ACKNOWLEDGMENTS

This study was supported by research funds from Texas A&M University, NATO, USAMRMC, OTKA and NIEHS. We would like to acknowledge Dr Jeffrey S. Karn for supplying us with clone of the parathion hydrolyzing gene.

FIGURES AND TABLES

Table 1. Effects Of 2-Pam, Atropine And Phosphotesterase Encapsulated Within SL On The LD₅₀ Of Paraoxon In Mice

Experiment # Treatment before paraoxon ^a	LD ₅₀ (mg/kg) ^b	Potency Ratio ^c
1. Control	0.9 (0.57-1.23)	1.0
2. Atropine	2.08 (1.51-2.84)	2.31
3. 2-PAM	4.50 (3.28-6.16)	5.0
4. SL	125.2 (91.39-170.5)	.0
5. Atropine + 2-PAM	55.0 (40.15-68.5)	61.1
6. Atropine + SL	540.3 (394.5-740.21)	600.3
7. 2-PAM + SL	550.2 (401.6-753.77)	611.3
8. 2-PAM + Atropine + SL	920.0 (671.6-1260.4)	1022.2

a Paraoxon (0.6 to 1,200 mg/gk) was delivered subcutaneously to mice in 6% cyclodextrin and/or propylene glycol solvent solutions. The Propylene glycol solvent consisted of 40% propylene glycol, 10% ethanol, and 50% water. Atropine sulfate (10 mg/kg) and 2-PAM-Cl (90 mg/kg) was given intraperitoneally 30 and 15 minutes respectively to receiving paraoxon. SL (0.2-0.4 mL/mice) was intravenously through tail vein 1 hr prior to receiving paraoxon.

b LD₅₀ values were determined by the up and down method (simulated up-and-down study, Dixon, 1965), and the estimated 95% confidence interval was determined by the method of Bruce, (1985). For each experiment, 6-10 mice were used. The LD₅₀ values were calculated from the equation: $\log(LD_{50}) = \log(Dose_{final}) + k \log(d)$, where dose final is the final dose administered, k is the tabular value from table (Dixon, 1965), and d is the interval between doses.

c

$$\text{potency ratio} = \frac{LD_{50} \text{ of paraoxon - antagonized}}{LD_{50} \text{ of paraoxon - unantagonized}}$$