

A novel immunoassay with direct relevance to protection against organophosphate poisoning

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Antiparaoxon immune sera were employed in a new immunoassay based on competition between acetylcholinesterase and antibodies for the binding of paraoxon. Unlike radioimmunoassay, the new assay described herein can be extended to predict the feasibility of antibodies to confer *in vivo* protection of acetylcholinesterase against organophosphate poisoning. The toxicity of paraoxon was reduced in mice which were pre-injected with the immune sera.

<i>Organophosphate</i>	<i>Immunization</i>	<i>Acetylcholinesterase</i>	<i>Paraoxon</i>
	<i>Antiparaoxon antibody</i>	<i>Immunoassay</i>	

1. INTRODUCTION

Organophosphorus compounds are widely used as insecticides. Accidental exposure of humans and domestic animals to such insecticides results in a potentially lethal cholinergic poisoning [1]. Antidotal treatment with atropin and cholinesterase reactivators is not always effective in cases of acute poisoning and rarely, if at all, in some forms of delayed effects [2]. The use of prophylaxis might therefore be advisable in some vulnerable populations.

Organophosphorus insecticides inhibit acetylcholinesterase (AChE). It is this inhibition that is thought to produce their highly toxic effects. Since the affinity of organophosphates towards AChE is relatively high [3], it may be questioned whether antibodies could efficiently compete with the enzyme for the binding of organophosphorus compound. Successful competition may possibly con-

fer protection against poisoning and make immunization a potential prophylactic tool. The production of antibodies against organophosphorus compounds has been described [4–6]. However, one cannot predict as yet how efficient these antibodies would be in reducing the toxicity of organophosphates. We thought that since AChE inhibition can be measured *in vitro*, a concurrent *in vitro* measurement of the enzyme protection by antibodies might serve as a meaningful indicator for *in vivo* protection. To test this hypothesis we prepared antibodies against paraoxon (the oxidized form of the insecticide parathion), which is a potent inhibitor of AChE [7] and examined their ability to protect the enzyme (AChE) from inhibition by this organophosphate. We then tested whether *in vivo* results would be consistent with our *in vitro* data.

2. MATERIALS AND METHODS

2.1. Animals

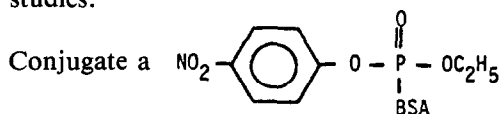
New Zealand white rabbits were used for preparation of antibodies. ICR mice weighing 20–24 g were used to test *in vivo* toxicity and protection by antibodies.

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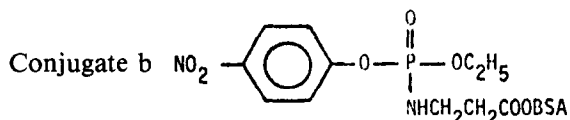
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2.2. Preparation of immunogens

Two different paraoxon derivatives conjugated with a protein carrier were employed in the present studies:



Conjugate a was prepared from *O*-(4-nitrophenyl)-*O*-ethyl phosphorochloridate which was added to a solution of bovine serum albumin (BSA) in 0.1 N Tris buffer (pH 8.8). The *O*-(4-nitrophenyl)-*O*-ethyl phosphorochloridate was prepared by reacting 4-nitrophenyl phosphorochloridate with an equimolar amount of ethanol in the presence of pyridine. This compound was identified by ^{31}P - and ^1H -NMR.



Conjugate b was prepared from the corresponding active ester which was added to BSA in 0.1 N NaHCO_3 (pH 6.7). The corresponding active ester was prepared by reacting equimolar amounts of the corresponding acid with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. The product was purified on a silica gel column eluted with chloroform:hexane (1:1) and identified by ^{31}P - and ^1H -NMR and mass spectrometry.

The corresponding acid was prepared by coupling *O*-(4-nitrophenyl)-*O*-ethyl phosphorochloridate and the trimethylsilyl ester of *N*-trimethyl- β -alanine in the presence of triethylamine. The product was recovered from the reaction mixture and analyzed by ^{31}P - and ^1H -NMR.

Both conjugates were separated from the reactants on Sephadex G-25. Protein [8] and phosphate determinations [9] showed that conjugate a contains 29 hapten residues per BSA molecule, while conjugate b contains 40 hapten molecules per BSA molecule. Rabbit serum albumin (RSA)-paraoxon conjugates were prepared in the same manner as the BSA-paraoxon conjugates described above.

2.3. Preparation of antibodies

The antibodies were produced by injection of the antigens described above into 10 intradermal sites (2 mg protein emulsified in a complete Freund

adjuvant, 1:1); 4 booster injections were given at 2–4 weeks intervals. Immune sera were collected 10 days following the last injection.

For this study, we used two antisera, each of which was collected from different rabbits. No.1 was injected with conjugate a, and no.2 with conjugate b. Determination of the amount of the specific antibodies in these antisera by a quantitative precipitation [10], using RSA-paraoxon conjugate, showed no preference for either of the two antigens described above. In preliminary experiments, we found that paraoxon-inactivating enzymes which are present in rabbit sera [11] could be destroyed by heating the sera to 64°C for 1 h. This treatment, which prevented the destruction of paraoxon, did not affect the binding ability of IgG [12] and thus increased the observed binding of [^3H]paraoxon from 27% with unheated immune sera to 60% with the heated immune sera. Heated sera were therefore used in our studies.

2.4. Radioimmunoassay (RIA)

RIA was performed by determining the amount of [^3H]paraoxon ([*phenyl*-3- ^3H]-, 1.3 Ci/mmol) bound to the antisera in the presence of various concentrations of unlabelled hapten. The reaction mixture for the binding assay contained 10 μl of the tested serum, 90 μl of 3% BGG, 100 μl [^3H]paraoxon (prediluted 1:2000) and 300 μl saline. After 30 min incubation at 37°C and consequent ammonium sulphate precipitation, the bound counts were determined in the pellet.

2.5. Enzyme-antibody competition assay (EACA)

The reaction mixture (100 μl) contained the following: 100 mM Tris buffer, pH 7.4, containing 2 mM NaCl, 0.2 units AChE (purified from electric eel with a specific activity of 1641 units/mg protein), 10 μl immune or control serum (their cholinesterase activity was completely inactivated prior to the assay) and various concentrations of paraoxon as described for each particular experiment.

Incubation of the reaction mixture was at 25°C for 10 min. In some cases the serum was added 5 min prior to the addition of AChE, while in others the serum and AChE were added simultaneously. A second incubation for an additional 10 min was then carried out following the addition of [^3H]acetylcholine chloride ([^3H]ACh, 500 $\mu\text{Ci}/\text{mmol}$).

The reaction was terminated by the addition of 100 μ l chloroacetic acid (1 M) containing 0.5 M NaOH and 2 M NaCl. The [3 H]acetate, which was hydrolyzed during the incubation, was then extracted into a scintillation liquid [13] and its radioactivity determined. The differences between the amounts of [3 H]acetate liberated in the presence of control serum and those liberated in presence of immune serum (at the same concentration of paraoxon) were used to calculate the amount of the paraoxon which was inactivated by the immune serum.

2.6. Toxicity determinations

Four groups of mice (16 animals in each) were used for toxicity and protection tests. The first group did not receive any treatment except for poisoning with paraoxon. The other 3 groups were

injected intravenously (i.v.) with either non-immune or one of the immune sera (0.5 ml). Paraoxon was administered subcutaneously (s.c.), 15 min following the injection of the sera. Before challenging, each of the groups were divided into 4 subgroups ($n = 4$), each of which received a different dose of paraoxon. The LD_{50} values were calculated according to Weil [14].

3. RESULTS

As can be seen from fig.1, antibodies prepared from immune rabbits were specific for paraoxon without cross-reactivity with *p*-nitrophenol or diethyl phosphate. Parathion, which is structurally related to paraoxon, was also recognized by these antibodies. The lowest limit of detection with these antibodies was 300 pg paraoxon (~ 1 pmol).

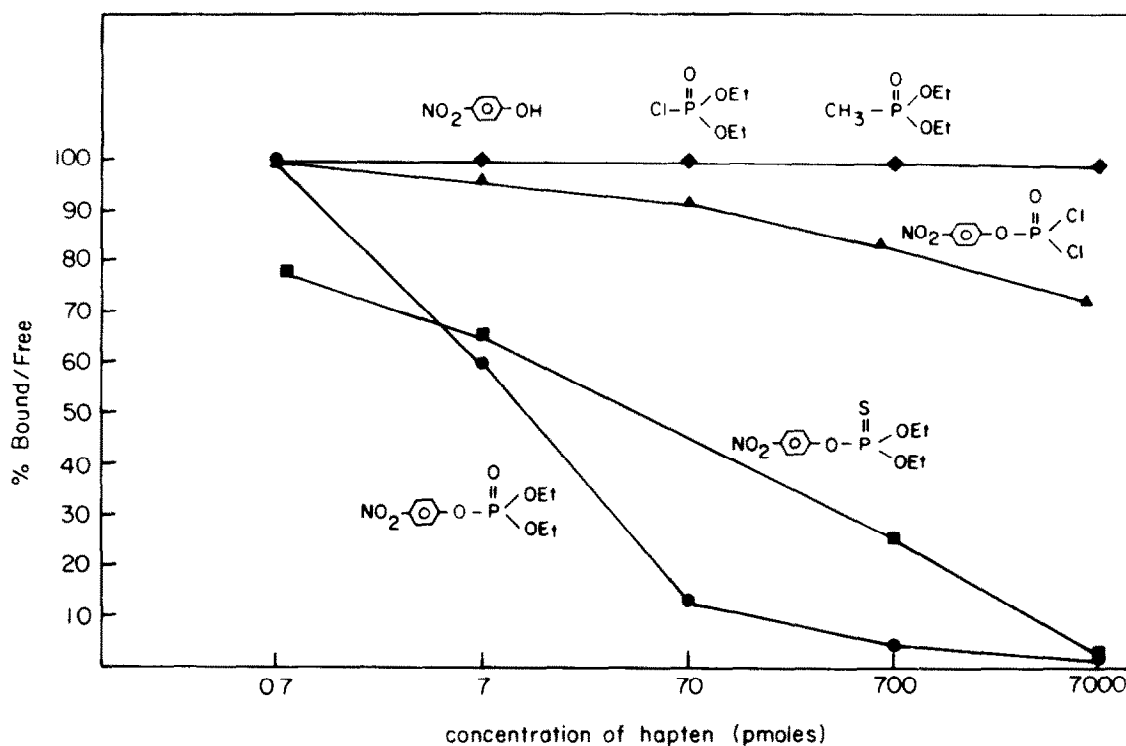


Fig.1. Standard curves for radioimmunoassay of paraoxon and related compounds. 20 μ l of a 1:5 dilution of antiparaoxon sera (from a rabbit which was immunized with antigen a) were preincubated for 30 min at 37°C with 100 μ l [3 H]paraoxon (10000 cpm; 0.8×10^{-7} M). 100 μ l of various concentrations of unlabelled hapten (as indicated by the formula for each curve on the graph) were then added together with 80 μ l of 20% normal sheep serum and 200 μ l of 0.9% NaCl and incubated at 25°C for 1–2 h. Antigen-antibody complex was then precipitated with 50% ammonium sulfate, washed twice and counted.

The ability of the antisera to prevent paraoxon from inhibiting AChE was then tested. It was found that the inhibition of AChE by paraoxon was significantly reduced if the organophosphate had been preincubated with immune sera as compared to normal sera (fig.2A). From the maximal difference in the inhibitions produced by the

various concentrations of paraoxon preincubated with normal vs immune sera (inset of fig.2A), it was possible to calculate both the amount of paraoxon bound and the concentration of the antibodies in the immune serum.

Comparison of these data with those obtained by the RIA showed that the two techniques are

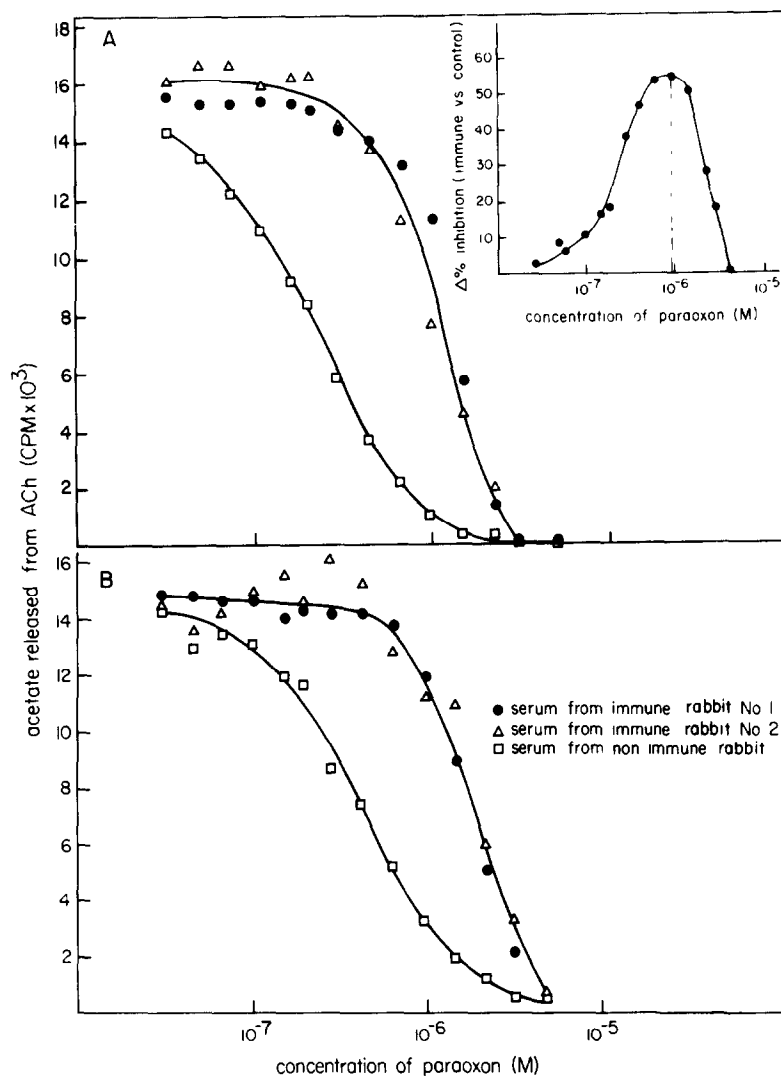


Fig.2. The prevention of paraoxon-elicited enzyme inhibition by antiparaoxon immune sera. (A) Rabbit sera [either normal (\square); or immune for conjugate a (Δ), and conjugate b (\bullet)] were preincubated with various concentrations of paraoxon for 5 min at 25°C. AChE was then added and an additional 10 min incubation was carried out. The differences between the inhibitions of AChE obtained with non-immune vs immune sera are given in the inset. (B) Rabbit sera (same as in A) and AChE were simultaneously incubated with various concentrations of paraoxon for 10 min at 25°C. Activities of AChE in both cases (A,B) were determined after the addition of [3 H]ACh, by measuring the radioactivity within [3 H]acetate which was released during 10 min at 25°C.

compatible. Thus, the concentration of the anti-paraoxon specific antibodies in the immune sera were 78 $\mu\text{g/ml}$ as calculated from the data obtained by RIA and 69 $\mu\text{g/ml}$ as calculated from the EACA.

Experiments in which antisera and AChE were exposed simultaneously to various concentrations of paraoxon are presented in fig.2B. As can be seen, the two immune sera competed successfully with the enzyme for the binding of paraoxon and reduced its inhibitory effect. These data suggest that the tested antibodies possess a higher affinity toward paraoxon than does the enzyme and that the paraoxon which is bound by the antibodies is no longer available for inhibiting AChE.

To test the ability of the antibodies to protect against paraoxon poisoning in vivo, we injected immune sera into mice and determined the toxicity values of paraoxon in these animals as compared to naive mice or mice which were injected with non-immune sera. The results of these experiments are presented in table 1. It can be seen that the immune sera significantly reduced the toxicity of the paraoxon (row 3, table 1).

However, when the maximal amounts of paraoxon that could have been inactivated by the total amount of the injected immune sera were calculated from our EACA data, we found that

about 40-times more paraoxon was inactivated in vivo than was expected from the in vitro data (rows 4 and 5, table 1). Therefore, we concluded that passive immunization can be achieved even with a relatively small amount of antibodies. Indeed, as can be seen from table 1 (rows 1 and 2), the survival of mice which were injected with the immune sera and consequently poisoned with paraoxon was significantly higher than that of the control mice. We also found that in the surviving mice the signs of poisoning of those injected with immune sera were less severe than those observed in the control group.

4. DISCUSSION

The work described in this article presents the first direct demonstration that antibodies can successfully compete with AChE for binding an organophosphorus compound. Moreover, the direct measurement of enzyme protection in the presence of antibodies constitutes a simple and novel in vitro technique that can evaluate qualitatively the in vivo immune capacity of an immunized individual. This novel immunoassay, the EACA, is fully compatible with the conventional RIA but unlike RIA can also provide information as to the actual potential of antibodies to confer in

Table 1

The ability of antisera injected intravenously to inactivate paraoxon and reduce its toxicity in vivo

Parameter measured	Type of serum injected			
	No serum	Control serum (non-immune)	Antiserum from rabbit no.1	Antiserum from rabbit no.2
Survival ratio with 700 $\mu\text{g/kg}$ paraoxon	2/4	1/4	4/4	—
Survival ratio with 800 $\mu\text{g/kg}$ paraoxon	0/4	0/4	3/4	4/4
LD_{50} values and their 95% confidence limits (in parentheses)	684.3 (625.0–749.2)	632.8 (566.3–707.1)	888.0 714.7–1103.4)	1007.1 875.5–1158.6)
Extra amount of paraoxon (ng) needed to reach LD_{50} in the experimental groups	—	0	4590	6970
Amount of paraoxon (ng) inactivated by 0.5 ml sera as determined in vitro by EACA	—	0	144	180

vivo protection against organophosphorus poisoning. In vivo protection should not be expected in cases where antibodies would only bind organophosphorus compounds (as can be determined by both RIA and EACA), but would not be able to compete successfully with AChE (as can be found only by EACA).

Our study clearly indicates that in vivo protection was achieved when the EACA demonstrated that possibility. However, a significant quantitative difference was found when the ability of the antibodies to bind and inactivate paraoxon in vitro (EACA) was compared to their ability to protect animals against paraoxon poisoning in vivo.

A larger amount of paraoxon was inactivated in vivo than could have been expected from the EACA. This difference can be explained if we take into account that only a small fraction of the injected paraoxon reached the target sites responsible for the cholinergic poisoning. Most of the injected paraoxon is probably destroyed by endogenous hydrolytic activity and by binding to 'non-specific' sites, before reaching those targets. From our data it is possible to estimate that no more than 3–5% of the injected paraoxon reaches the 'target sites', and if so, it would be safe to assume that active immunization against paraoxon would be even more efficient in the protection against poisoning than the passive immunization tested there.

By using the EACA it will be possible to determine whether an immune subject is potentially immune to organophosphate poisoning and to estimate roughly the degree of protection that should be expected in this immune individual.

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