An active immunization approach to generate protective catalytic antibodies

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We report that mice immunized with a phosphate immunogen produced polyclonal catalytic antibodies (PCAbs) that catalysed the hydrolysis of carbaryl, a widely used broad-spectrum carbamate insecticide that exerts toxic effects in animals and humans. The reaction catalysed by the PCAbs (IgGs) obeyed Michaelis–Menten kinetics *in vitro* with the following values at pH 8.0 and 25 °C: $K_m \approx 8.0 \mu\text{M}$, $k_{\text{cat}} = 4.8 \times 10^{-3} - 5.8 \times 10^{-1}$, $k_{\text{cat}}/k_{\text{non-cat}} = 5.6 \times 10^1 - 6.8 \times 10^3$ (where $k_{\text{non-cat}}$ is the rate constant of the reaction in the absence of added catalyst). The PCAbs were also active in whole sera under physiological conditions *in vitro*. The PCAbs induced *in vivo* were also active *in vivo*, as

immunization with the phosphate immunogen decreased the mouse blood concentration of carbaryl. To our knowledge, this is the first report demonstrating that active immunization generates antibodies possessing therapeutic catalytic function *in vivo*. We propose that active immunization schemes that induce enzymically active antibodies may provide a highly specific therapeutic approach for degrading toxic substances.

Key words: carbaryl, hydrolysis, immunized mice, polyclonal antibody.

INTRODUCTION

Since the first demonstration of antibody catalysis in 1986, investigators have prepared antibodies that catalyse almost all kinds of known chemical reactions [1–4]. Because catalytic antibodies have high efficiency and specificity, there has been interest in their application for medical treatment. Catalytic antibodies have been prepared that catalyse the hydrolysis of anti-cancer prodrugs *in vitro* and therefore have the potential to diminish the non-specific toxicity associated with many commonly used chemotherapeutic agents [5–7]. A highly active monoclonal catalytic antibody, 15A10, was prepared that hydrolysed cocaine to non-toxic, non-addictive products. Passive immunization with this artificial enzyme blunted the reinforcing effect of cocaine in rats, suggesting that this approach may have therapeutic value [8,9]. Although encouraging, this response was achieved using mouse monoclonal catalytic antibodies, which typically induce deleterious anti-antibody responses in humans, thus limiting their clinical application.

A novel approach for generating therapeutic antibodies is to induce the formation of polyclonal catalytic antibodies (PCAbs) by active immunization *in vivo*. This approach avoids the problematic immune responses associated with monoclonal catalytic antibodies. Several studies have demonstrated that PCAbs active *in vitro* can be generated relatively simply, rapidly and cheaply [10–14]. Here, we provide the first evidence that enzymically active PCAbs can be useful as therapeutic reagents *in vivo*. To achieve this, we immunized mice with an antigen that was designed according to the structural features of carbaryl (Sevin, 1-naphthyl *N*-methylcarbamate) and its hydrolytic transition state. Our rationale was that the elicited antibodies would catalyse carbaryl hydrolysis by stabilizing the hydrolytic transition state of carbaryl via structural and charge complementarity in the antibody-binding pocket.

Carbamate poisoning is a well-known toxicological problem causing morbidity and mortality in both developing and industrialized countries [15]. Carbaryl is a major carbamate insecticide marketed throughout the world; it has been used since 1958 for both outdoor agricultural and indoor house-pet-related insect control [16]. Its acute toxic effects on the central and peripheral nervous systems are due to inhibition of acetylcholinesterase at nerve endings, which causes accumulation of acetylcholine and consequent overstimulation of nicotinic receptors [17]. In addition, recent studies show that chronic exposure to carbaryl may induce neurotoxicity, teratogenesis, tumour initiation, miscarriage and preterm delivery in humans and animals [18–22]. We reasoned that the generation of PCAbs that can catalyse the hydrolysis of carbaryl *in vivo* would degrade carbaryl and reduce its toxic effects.

In the present investigation, a phosphate analogue of the hydrolytic transition state of carbaryl was conjugated with BSA as the immunogen and injected into mice to produce therapeutic PCAbs in their sera, which in turn catalysed the hydrolysis of carbaryl both *in vitro* and *in vivo*. We propose that active immunization schemes that induce enzymically active antibodies may provide a highly specific therapeutic approach for degrading toxic substances.

EXPERIMENTAL

Materials

1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDAC), *N*-hydroxysuccinimide, sodium oxybate, Tween 20 and chicken egg albumin were obtained from Sigma, St. Louis, MO, U.S.A.; Freund's adjuvant, complete and incomplete, were obtained from Life Technologies, Grand island, NY, U.S.A.; BSA was

Abbreviations used: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide; PCAb, polyclonal catalytic antibody; PNAb, polyclonal non-catalytic antibody; $k_{\text{non-cat}}$, rate constant in the absence of added catalyst.

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Scheme 1 Synthetic routes to generate (a) hapten (product IV), phosphate immunogens (products V and VI) and (b) amide immunogen (product VIII)

CEA, chicken egg albumin; DMAP, 4-dimethylaminopyridine.

provided by Roche Molecular Biochemicals, Mannheim, Germany; Protein A-Sepharose CL 4B was from Amersham Pharmacia Biotech, Uppsala, Sweden. All other chemicals were of analytical grade.

Synthesis of phosphate immunogens V and VI (Scheme 1a)

Preparation of γ -hydroxyethyl butyrate (product I)

Ethyl iodide (16 ml, 0.197 mol) was added to a stirred solution of γ -hydroxy sodium butyrate (16.39 g, 0.13 mol) in *N,N*-dimethylformamide (260 ml), and the mixture was stirred at room temperature for 12 h. The mixture was extracted with ethyl acetate (2 $\times$ 250 ml). The extracts were washed with water three times and then evaporated under reduced pressure (3388 Pa) to remove the solvents. Later, after the temperature rose to 102–106 °C, a fraction was obtained that on redistillation (104–106 °C/3388 Pa) produced a clear colourless liquid (product I). $^1\text{H-NMR}$ (90 MHz; C^2HCl_3): δ 1.60–2.10 (2H, m, CH_2), 2.30–2.60 (2H, t, CH_2CO), 2.95 (1H, s, broad, OH), 3.59–3.75 (2H, t, HOCH_2), 4.05–4.30 (2H, q, OCH_2CH_3).

Preparation of α -naphthyl γ' -(ethyl butyrate) hydrogen phosphate (product III)

Product II was prepared according to the literature [23]. The prepared product was a clear colourless syrup, boiling point 197–201 °C/2711 Pa (reference value [23], 199–201 °C and 2711 Pa). Product II (1340 mg, 5.14 mmol) was added to a stirred solution of product I (220 mg, 1.66 mmol) in a 2:1 (v/v) mixture of pyridine and acetonitrile (4.8 ml) at 0 °C. This was stirred at 0 °C for 1 h, distilled water (4 ml) was added and the reaction mixture was stirred for another 3 h at room temperature. Solvents were removed *in vacuo*, and the residue was dissolved in 0.5 M NaHCO_3 (50 ml) and extracted with diethyl ether (2 $\times$ 50 ml). The aqueous solution was acidified with HCl (pH 1–2) and extracted with ethyl acetate (3 $\times$ 50 ml). The combined ethyl acetate extracts were evaporated *in vacuo* to remove ethyl acetate and give a crude product. The crude product was purified by column chromatography on silica H (gradient elution with chloroform/ethanol, from 10:1 to 3:1, v/v) to give product III as a pale-yellow solid (280 mg, 50%). $^1\text{H-NMR}$ (90 MHz; C^2HCl_3): δ 1.02 (3H, t, OCH_2CH_3), 1.23–3.00 (6H, m, $\text{CH}_2\text{CH}_2\text{CH}_2$), 6.80–8.50 (7H, m, Ar-H). MS (m/z): 399 (M^+ + 1); 237 (M^+ - $\text{CH}_2\text{COOC}_2\text{H}_5$ + 2); 127 [M^+ - $\text{OPO}(\text{OH})\text{O}(\text{CH}_2)_3$]; 115 [M^+ - $\text{C}_{10}\text{H}_7\text{OPO}(\text{OH})\text{O}$].

Preparation of α -naphthyl γ' -butyrate hydrogen phosphate (product IV)

Distilled water (1.8 ml) and 1 M NaOH (400 μl) were added to a stirred solution of product III in tetrahydrofuran (2.5 ml). After 5 min, a second aliquot of 1 M NaOH (400 μl) was added. The reaction mixture was stirred at room temperature for 1 h, then acidified with HCl to pH 1.0, and finally extracted with ethyl acetate (3 $\times$ 20 ml). The combined ethyl acetate extracts were dried and concentrated *in vacuo* to give the crude product. Recrystallization from ethyl acetate gave pure product IV (40 mg, 65%). $^1\text{H-NMR}$ (90 MHz; C^2HCl_3): δ 1.5–2.5 (6H, m, $\text{OCH}_2\text{CH}_2\text{CH}_2\text{CO}$), 2.0–4.0 [1H, s, $\text{OPO}(\text{OH})$], 7.0–8.0 (7H, m, Ar-H), 8.5–10.5 (1H, broad, $\text{OCO}(\text{OH})$).

Preparation of the immunogens V and VI

EDAC (100 mg, 0.52 mmol) was added dropwise over 5 h to a stirred solution of product IV (40 mg, 0.15 mmol), *N*-hydroxy-succinimide (100 mg, 0.78 mmol) and BSA (60 mg) in redistilled water (4 ml) and pyridine (100 μl). The reaction mixture was further stirred at room temperature for 16 h and then dialysed against running tap water for 3 days. The dialysis residue was freeze-dried to give immunogen V as a pale-yellow powder (56 mg). Immunogen VI (50 mg) was synthesized in the same manner as immunogen V except that chicken egg albumin was used instead of BSA.

Preparation of the amide immunogen VIII (Scheme 1b)

Preparation of *N*-(1-naphthyl) 4'-hydroxycarbonyl butamide (product VII)

1-Aminonaphthalene (31 mg, 0.22 mmol) was coupled with glutaric anhydride (38 mg, 0.33 mmol) in dichloromethane (10 ml) using 4-dimethylaminopyridine (30 mg, 0.24 mmol) as a base. The reaction was monitored by TLC. The crude product was dissolved in 0.05 M NaOH (10 ml), and the suspension was removed by filtration. The filtrate was acidified with HCl to pH 1.0, and the resulting suspension was precipitated by centrifugation to yield a pellet. Recrystallization of the pellet from ethyl acetate gave the pure product VII (30 mg, 52%). $^1\text{H-NMR}$

(90 MHz; C^2HCl_3): δ 1.9–2.3 (6H, m, $\text{OCCH}_2\text{CH}_2\text{CH}_2\text{CO}$), 7.5–8.1 (7H, m, Ar-H), 8.5–10.5 (1H, broad, OCOH).

Preparation of immunogen VIII

Immunogen VIII (40 mg) was synthesized in the same manner as immunogen V except that product VII, a non-toxic analogue of carbaryl, was used instead of product IV.

Production of antisera

Female Balb/c mice, weighing 20–25 g, were each immunized with an emulsion of 0.1 mg of immunogen V dissolved in water (0.2 ml) mixed with Freund's complete adjuvant (0.2 ml). The immunization was repeated after 10 days using Freund's incomplete rather than complete adjuvant. Thereafter, the mice were immunized twice more with 0.05 mg of immunogen V in 0.4 ml of water at 10-day intervals. A control group of non-immunized mice were treated in the same way using saline only. The mice were bled on the 35th day of the immunization schedule. The blood was incubated at 37 °C for 2 h, allowed to clot overnight at 4 °C, and then centrifuged. The serum was extracted and stored at –20 °C. The antiserum dilution curves were measured by ELISA, using immunogen VI as the antigen to coat the wells. Immunogen VI has the same hapten as immunogen V but has a different carrier protein to reduce non-specific binding.

Purification of IgG from antisera

The antiserum ($\approx$ 0.6 ml) from four mice (M-61, M-62, M-63 and M-64) was precipitated separately by $(\text{NH}_4)_2\text{SO}_4$ according to the method of Thomason and Hebert [24], dialysed and then concentrated using 60% poly(ethylene glycol). The resulting solution (2 ml) was subjected to chromatography on a Protein A–Sephacrose CL 4B fast-flow column (5 ml), and the eluate was monitored at 280 nm using a spectrophotometer (model ES-1; Bio-Rad). The absorbed IgG was eluted with 0.1 M citrate buffer (pH 3.5), and 0.5 ml fractions of the eluate were collected in tubes containing 1 M Tris/HCl buffer (pH 9.0, 0.05 ml/tube). The combined fractions for each mouse containing IgG (2 μM , $\approx$ 2 ml) were subjected to dialysis with distilled water for 3 days, and the dialysis residue was freeze-dried to produce PCABs (PCAb-61, PCAb-62, PCAb-63 and PCAb-64, respectively) in the form of an off-white powder. Control polyclonal non-catalytic antibody (PNAb) preparations from (PNAb-61, PNAb-62, PNAb-63 and PNAb-64) were prepared from four non-immunized mice using the same method. The concentration of IgG was determined by absorbance at 280 nm assuming a specific absorption coefficient ($A_{1\text{cm}}^{1\%}$) of 14 [11], and the purity was demonstrated by SDS/PAGE. The concentration of IgG in whole blood was determined based on the assumption that the serum represents 50% of the blood volume and that 90% of the IgG was recovered after purification.

Kinetics of carbaryl hydrolysis

The rate of carbaryl hydrolysis was calculated by measuring the increase in the product α -naphthol with time. The concentration of α -naphthol was calculated from the absorbance of carbaryl and α -naphthol in 30 mM Tris/HCl buffer (pH 8.0) containing 0.4% (v/v) ethanol at 25 °C. We chose to measure α -naphthol absorbance at 270 nm, as this wavelength afforded the best distinction between α -naphthol and carbaryl. The concentration of α -naphthol in the reaction mixture was calculated from the equation:

$$C = (A_{270}/L - [S]_0\epsilon_1)/(\epsilon_2 - \epsilon_1)$$

where C is the concentration of α -naphthol in the reaction mixture, L is the distance to the light source (1 cm), $[S]_0$ is the initial concentration of carbaryl, and ϵ_1 ($9.08 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$) and ϵ_2 ($2.66 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$) are the molar absorption coefficients of carbaryl and α -naphthol at 270 nm respectively. The substrate was prepared in ethanol to a volume of 8 μl and was then mixed with 992 μl of water to a final concentration of 0–24 μM . In some cases, the reaction mixtures contained 0.2 μM PCAb or 0.2 μM PNAb. Figure 1 provides the A_{270} value over time for PCABs with and without the substrate carbaryl present. The data show that the low concentrations of PACBs used in all experiments (0.2 μM IgGs) did not appreciably affect the measurement of absorbance changes. The initial rate of α -naphthol release obtained in the presence of PNAb was subtracted from that obtained at the same value of carbaryl concentration ($[S]$) in the presence of PCAb to provide the rate (v) of the PCAb-catalysed reaction. The kinetic parameters V_{max} and K_m and their associated S.E.M. values were obtained by fitting the data to the Michaelis–Menten equation by non-linear regression of v versus $[S]$ [10]. To determine the antibody-catalysed reaction rate of immunized sera (as compared with non-immunized sera) with the background reaction in sera, 64 μl of serum was added to a 1 ml reaction mixture containing 10 μM carbaryl. The reaction was carried out at pH 7.4 and 38 °C, with 7.7 mM K^+ , 152 mM Na^+ , 2.5 mM Ca^{2+} and 4.1 mM Mg^{2+} , which is similar to physiological conditions in the mouse [25]. The *in vitro* inhibition experiments involving hapten IV, a transition-state analogue, were carried out by adding different concentrations of hapten IV to a 1 ml reaction mixture containing 0.2 μM PCAb (IgGs) and 10 μM carbaryl at pH 8.0 and 25 °C.

Determination of carbaryl hydrolysis by catalytic antibody *in vivo*

Two groups of Balb/c mice (10 mice for each group), weighing 20–25 g each, were immunized with either immunogen V or VIII for 5 weeks (as described above) before carbaryl administration. A control group of 10 mice that were not immunized were housed in parallel. The mice were given water but no food for 16 h prior to carbaryl administration. Each mouse was given carbaryl intragastrically at a dose of 9.7 mg/kg of body weight. The mice were bled completely 1 h after carbaryl administration. The concentration of carbaryl in the blood was determined by an assay involving the coupling of *p*-nitrobenzenediazonium fluoroborate with α -naphthol, a product of the alkaline hydrolysis of carbaryl [26]. To avoid the contamination of free α -naphthol, 0.1 ml of 3% KMnO_4 was incubated with the blood for 2 min, extracted with chloroform three times and dried *in vacuo*. The concentration of free α -naphthol in the blood was expected to be very low since it is known to be efficiently transformed into α -naphthyl glucoside, α -naphthyl sulphate or another water-soluble substance, all of which are separated from carbaryl by chloroform extraction [27].

RESULTS

Antibody production

A previous study identified a monoclonal antibody capable of hydrolysing the *p*-nitrophenyl *N*-aryl carbamate through the $\text{B}_{\text{Ac}2}$ pathway [28]. This was regarded as a major advance in the catalytic antibody field, as previous studies had indicated that carbamate ester hydrolysis occurs predominantly via an $\text{E}_{1\text{c}}\text{B}$

Figure 1 Hydrolysis rates of carbaryl by purified IgGs

Shown is a comparison of hydrolysis rates of 20 μM carbaryl in the presence of 0.2 μM PCAb-61 (Δ) and 0.2 μM PNAb-61 (○) at pH 8.0 and 25 °C. Also shown is 0.2 μM PCAb-61 in the absence of carbaryl (□) at pH 8.0 and 25 °C. Each data point represents the mean ± S.D. for three independent measurements.

mechanism instead [29]. Here, we investigated whether the related carbamate, carbaryl, could also be hydrolysed through the highly disfavoured B_{Ac}2 pathway by PCABs (Scheme 2). With this goal in mind, we used a stable tetrahedral-phosphate analogue of the hydrolytic transition state of carbaryl generated through the B_{Ac}2 pathway as the hapten determinant to produce PCABs (Scheme 1, product IV). This hapten IV was conjugated to BSA (Scheme 1, immunogen V) and injected multiple times into four mice. The average antiserum titre was 1/36000 5 days after the last immunization, as determined by ELISA. The average concentration of IgG in the whole blood was 3.6 μM. The PCABs (IgG) from the antisera were isolated by (NH₄)₂SO₄ precipitation and chromatography on Protein A-Sepharose. Control PNAB (IgG) preparations from non-immunized mice were prepared using the same procedure.

Catalytic specificity

We tested whether the purified PCAB preparations were capable of hydrolysing carbaryl *in vitro*. Figure 1 shows the initial rates of carbaryl hydrolysis by PCAb-61 compared with the negative control PNAb-61 preparation. The data show that PCAb-61 produced a greater rate of carbaryl hydrolysis than PNAb-61. The similar first-order rate constants for PNAb-61 ($k_{\text{PNAb}} = 9.10 \times 10^{-5} \text{ s}^{-1}$) and Tris/HCl buffer alone ($k_{\text{non-cat}} = 8.59 \times 10^{-5} \text{ s}^{-1}$; where $k_{\text{non-cat}}$ is the rate constant in the absence of added catalyst) indicated that the IgG from non-immunized mice had no catalytic activity or contaminating esterases. Because PCABs and PNABs were prepared using the same procedure, we reasoned that PCAB preparations did not have contaminating esterases. Further evidence for specificity was the finding that the catalytic activity of PCABs was inhibited by 80% when co-incubated with the transition-state analogue hapten IV *in vitro* (at a hapten IV/IgG ratio of 0.075; results not shown). We conclude that catalytic antibodies inhibitable by a specific hapten were responsible for specifically catalysing the hydrolysis of carbaryl.

Scheme 2 The proposed mechanism for antibody-catalysed hydrolysis of carbaryl through the B_{Ac}2 pathway

Kinetic characteristics of PCAB

The initial rates of hydrolysis of carbaryl in the presence of 0.2 μM PCAB were determined as a function of substrate concentration, represented by a plot of corrected v versus $[S]$. We found that the IgG fractions from the four immunized mice (PCAb-61–64) catalysed the hydrolysis of carbaryl with typical Michaelis–Menten kinetics (Figure 2 and Table 1). Because there are two active sites in each antibody molecule, the concentration of catalytic sites in the PCAB would be twice the total IgG concentration. Meanwhile, due to the fact that only a small fraction of the total IgG sample was anti-hapten IgG, the concentration of PCAB in the IgG preparation would be much less than the total concentration of the IgG sample. Depending on the estimate of the efficient PCAB concentration, which could vary between 1 and 100% of the total IgG concentration [10], the values for the rate constant k_{cat} and the ratio $k_{\text{cat}}/k_{\text{non-cat}}$ were in the ranges 4.8×10^{-3} – $5.8 \times 10^{-1} \text{ s}^{-1}$ and 5.6×10^1 – 6.8×10^3 (Table 1). This demonstrates that despite the existence of structural heterogeneity in the PCABs, the catalytic properties of the active species of antibodies were similar and that the differences among them were not readily detectable as deviations from the single-site saturation mode.

Catalytic activity of whole sera under physiological conditions

To determine whether the catalytic antibodies were active in whole sera (rather than as purified IgGs) under physiological conditions, the reaction was carried out at pH 7.4 and 38 °C, under ionic conditions similar to those found in mouse sera (see the Experimental section). We found that sera from immunogen V-immunized mice catalysed the reaction twice as quickly as sera from non-immunized mice, indicating that the induced antibodies indeed had catalytic activity under physiological conditions (Figure 3). In contrast, when we used immunogen VIII (a conjugate of a non-toxic analogue of carbaryl and BSA; Scheme 1) to immunize the mice, the sera from these control mice catalysed the reaction at a rate similar to that of the sera from non-immunized mice (results not shown). This indicates that immunogen VIII-immunized mice did not generate antibodies catalysing the hydrolysis of carbaryl.

Figure 2 Demonstration of adherence to the Michaelis–Menten equation of the hydrolysis of carbaryl catalysed by PCAb-61

Each data point represents the mean $\pm$ S.D. for three independent measurements.

Figure 3 The serum from immunogen V-immunized mice catalysed carbaryl hydrolysis under physiological conditions

Shown is a comparison of hydrolysis catalysed by sera from immunogen V-immunized (●) and non-immunized (○) mice. Each data point represents the mean $\pm$ S.D. for three independent measurements.

Catalytic antibodies decrease the concentration of carbaryl *in vivo*

It was reported previously that the majority of carbaryl administered through the stomach accumulates in the blood and thus is an available target for hydrolysis by serum PCABs [30]. If serum PCABs catalyse the hydrolysis of carbaryl *in vivo*, we reasoned that the concentrations of carbaryl in the blood should be decreased in immunogen V-immunized mice. To test this, 10 immunogen V-immunized mice (which had an average antiserum titre of 1/35 500 and a catalytic activity similar to that shown in Figure 3) and 10 non-immunized mice were each administered 9.7 mg/kg carbaryl. After 1 h, the mice were bled completely, and the concentration of carbaryl in the blood was measured. We found that the average concentration of carbaryl in the immunized mice was 245.4 μ M, which was 121 μ M lower than the average value in non-immunized mice. To test whether the decrease in carbaryl serum concentration in the immunized mice was due simply to antibody binding rather than catalysis, we immunized 10 mice with a non-toxic analogue of carbaryl (immunogen VIII) that does not induce catalytic antibodies, but which does induce antibodies that bind to carbaryl (average antiserum titre, 1/30 000), and administered carbaryl to them by the same method. The resulting average concentration of carbaryl was 359.4 μ M, which was similar to the concentration of carbaryl in non-immunized mice. Collectively these results

suggest that the decrease in carbaryl concentration in mice immunized with immunogen V was due to catalysis and not to antibody binding.

DISCUSSION

Our study is the first to demonstrate that an active immunization procedure can be used to generate catalytic antibodies active *in vivo*. We found that a synthetic immunogen possessing the structural features of a carbaryl hydrolytic transition state induced the generation of PCABs that were catalytically active and protected mice against a challenge with the insecticide carbaryl.

We provided several lines of evidence that suggest that immunization *in vivo* decreased the level of carbaryl mainly as a result of carbaryl hydrolysis catalysed by the induced antibodies. First, we demonstrated that the antibodies induced *in vivo* possessed specific catalytic activity. Purified IgG from the immunized mice catalysed the hydrolysis of carbaryl with typical Michaelis–Menten kinetics (Table 1 and Figure 2). The rate of catalysis was much greater for sera from immunized mice than that for sera from non-immunized mice under physiological conditions (Figure 3). We showed that this catalytic activity was derived from the polyclonal antibodies induced by immunogen V

Table 1 Kinetic parameters for the hydrolysis of carbaryl catalysed by murine PCABs

The parameters K_m and V_{max} were measured as described in the Experimental section. It was assumed that [active centre] = $2 \times 100\%$ [IgG] or $2 \times 1\%$ [IgG]. k_{cat} was calculated using the formula $k_{cat} = V_{max}/[\text{active centre}]$. PCAB concentration was 0.2 μ M. Means $\pm$ S.D. are shown.

PCAb	K_m (μ M)	V_{max} ($\times 10^9$; $M \cdot s^{-1}$)	k_{cat} (s^{-1})		$k_{cat}/k_{non-cat}$	
			100% IgG	1% IgG	100% IgG	1% IgG
PCAb-61	7.3 ± 0.55	2.2 ± 0.16	$(5.5 \pm 0.4) \times 10^{-3}$	$(5.5 \pm 0.4) \times 10^{-1}$	6.4	6.4×10^3
PCAb-62	6.5 ± 0.62	2.1 ± 0.14	$(5.3 \pm 0.3) \times 10^{-3}$	$(5.3 \pm 0.3) \times 10^{-1}$	6.2	6.2×10^3
PCAb-63	7.5 ± 0.68	1.9 ± 0.11	$(4.8 \pm 0.3) \times 10^{-3}$	$(4.8 \pm 0.3) \times 10^{-1}$	5.6	5.6×10^3
PCAb-64	8.2 ± 0.72	2.3 ± 0.19	$(5.8 \pm 0.5) \times 10^{-3}$	$(5.8 \pm 0.5) \times 10^{-1}$	6.8	6.8×10^3

and not from non-specific IgGs or an adventitious enzyme impurity (Figure 1). The specificity of catalytic activity was demonstrated by the observation that specific hapten inhibited the catalytic activity of the *in vivo*-induced antibodies. Secondly, immunogen V-immunized mice had lower serum carbaryl levels than non-immunized mice 1 h after carbaryl administration. In contrast, mice immunized with a non-toxic analogue of carbaryl (immunogen VIII) did not exhibit a decrease in serum carbaryl levels.

Although difficult to determine directly *in vivo*, the kinetics of catalytic antibodies can be estimated by using *in vitro* kinetic parameters. Our results showed that 1 h after administration of 9.7 mg/kg carbaryl, the carbaryl concentration in the blood of the immunogen V-immunized mice was 245.4 μM , which far exceeds the K_m values (7–8 μM) of the catalytic antibodies (Table 1). Therefore, we estimated that the hydrolytic velocity of the catalytic antibodies was close to their maximum velocity [$V_{\max} = (1.9\text{--}2.3) \times 10^{-9} \text{ M} \cdot \text{s}^{-1}$; Table 1]. Because the total IgG concentration in the blood averaged 3.6 μM , the concentration of the substrate carbaryl in the blood exceeded that of the catalytic antibodies (1–10% of total IgG [14]). Consequently, the hydrolytic velocity should be related directly to the concentration of the catalytic antibodies, and thus the catalytic velocity in the blood was likely to be close to the value of $\approx 3.8 \times 10^{-8} \text{ M} \cdot \text{s}^{-1}$ ($3.6/0.2 \times V_{\max}$; Table 1). According to this estimate, the catalytic antibodies were capable of decreasing the carbaryl concentration by 135 μM in 1 h. This value is in close agreement with the difference between the concentrations of carbaryl in the blood of the immunogen V-immunized and non-immunized mice (121 μM) and thus provides further support for the notion that catalytic antibodies catalysed the degradation of carbaryl *in vivo*. However, it should be noted that endogenous enzymes may also participate in the degradation of carbaryl. The existence of such enzymes would explain why we detected hydrolytic activity in non-immunized mice (Figure 3). The role of exogenous enzymes in carbaryl degradation may be limited, as carbaryl is known to inhibit the activity of most esterases [31]. Regardless of the presence of endogenous catalytic activities, our study clearly shows that immunization *in vivo* increases the catalytic activity in serum as a result of induced antibodies.

It should be emphasized that our estimate for enzyme kinetics *in vivo* was based on reaction conditions *in vitro*, which are different from those *in vivo*. Moreover, we performed our *in vitro* reaction in 30 mM Tris/HCl buffer (pH 8.0) at 25 °C, whereas mouse blood has a pH of ≈ 7.4 and a temperature of 38 °C. There are many metal ions in the blood, including K^+ , Na^+ , Ca^{2+} and Mg^{2+} , which were not present in our *in vitro* reactions [25]. However, we found that $k_{\text{cat}}/k_{\text{non-cat}}$ was not significantly affected by a change of pH from 7.1 to 8.5 (results not shown). Also, we found that $k_{\text{cat}}/k_{\text{non-cat}}$ was only increased 1.4-fold at 40 °C compared with the ratio at 25 °C (results not shown). Thus, for the particular catalytic antibodies studied here, the kinetic values determined *in vitro* were a good predictor of PCAb activity *in vivo*.

We propose that the active immunization approach for generating catalytic antibodies described herein can be used to protect against many target substrates, including pesticides, other toxic agents and narcotic drugs. Furthermore, this strategy can be used for treatment of enzyme deficiencies and cardiovascular diseases. Although the catalytic activity of PCAbs generated in past studies was modest [10,13], improvements in hapten design methods and immunization strategies may lead to an increase in the catalytic activity of antibodies and broaden the application of catalytic antibodies for therapeutic strategies *in vivo*.

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