

Anti-peptide antibodies to the P4502D subfamily in rat, dog and man

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1. In order to obtain specific antibodies of the P4502D subfamily, we generated two anti-peptide antibodies against synthetic peptides, DPAQPPRD (peptide A) and DPTQPPRH (peptide B). The sequence of peptide A occurs in rat P4502D2, P4502D4 and human P4502D6, whereas the sequence of peptide B occurs in the dog P4502D subfamily. These sequences are closely related to an epitope of liver/kidney microsomal autoimmune hepatitis.

2. In immunoblotting studies, the anti-peptide antibody against peptide A recognized a 49-KDa protein in microsomes derived from human lymphoblasts expressing P4502D6 and rat liver. It showed no crossreactivity with microsomes from dog liver. In contrast, the anti-peptide antibody against peptide B recognized a 49-KDa protein only in microsomes of dog liver. These indicate that each anti-peptide antibody has the specificity for the respective sequences of the members of P4502D subfamily, with the species investigated herein.

3. In immunoinhibition studies, the anti-peptide antibodies against peptide B inhibited bunitrolol 4-hydroxylation and propranolol 4, 5-hydroxylation, which are mediated by the dog P4502D subfamily. These data suggest that the anti-peptide antibodies against peptide B bind to the native and denatured forms of the P4502D subfamily.

4. The present study has demonstrated that the anti-peptide antibodies against this region are useful for studying the members of the P4502D subfamily.

Introduction

P450 plays an important role in the metabolism of endogenous and exogenous compounds (Gonzalez 1989) and human P4502D6 has received much attention because of its polymorphic expression under genetic control (Gonzalez *et al.* 1988). Immunoblotting and immunoinhibition studies using the antibodies against members of the P4502D subfamily have been performed to determine the expression and the contribution of P4502D6 to drug metabolism (Shimada *et al.* 1994). The purification of the P4502D subfamily is difficult because of the low hepatic contents and non-inducible features (Funae *et al.* 1985). Therefore, the application of immunological methods to the study of the P4502D subfamily has been limited.

Zanger *et al.* (1988) demonstrated that liver/kidney microsomal autoantibodies type 1 (LKM-1 autoantibodies), found in patients with autoimmune hepatitis, exclusively recognized P4502D6. Although the relationship between the polymorphic P4502D6 and the appearance of LKM-1 autoantibodies in patients with autoimmune hepatitis is unknown, they have been proven to be highly valuable

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immunological tools because of their high specificity and potency (Zanger *et al.* 1988). Recently, Manns *et al.* (1991) reported that the common epitope of LKM-1 autoantibodies was a short peptide (DPAQPPRD), corresponding to the residues 263–270 of P4502D6.

Edwards *et al.* (1990) succeeded in generating anti-peptide antibody against the short peptide which can bind P450/A2. This paper describes the production and the use of anti-peptide antibodies against short sequences of the members of 2D subfamily.

Materials and methods

Chemicals

Bunitrolol (BTL) and 4-hydroxy-BTL were gifts from Nippon Boehringer Ingelheim Co., Ltd (Osaka, Japan). Propranolol (PL) was purchased from Sigma Chemical Co. (St Louis, MO, USA). *N*-desisopropyl, 4-, 5- and 7-hydroxy-PL were gifts from ICI Pharma. Co. (Macclesfield, UK). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADPH were purchased from Oriental Yeast Co. (Tokyo, Japan). Freund's complete adjuvant and incomplete adjuvant were purchased from Wako (Tokyo, Japan). *N*- α -florethylmethoxycarbonyl amino acids for peptide synthesis were purchased from Millipore (London, UK). The microsomes derived from human lymphoblasts expressing 2D6 were purchased from Daiichi Pure Chemical Co., Ltd (Tokyo, Japan). The other reagents were of analytical grade.

Synthesis of peptides and coupling to a carrier protein

The peptides (DPAQPPRD and DPTQPPRH) were synthesized automatically with a Shimadzu PSSM-8 Peptide Synthesizer. They were confirmed to have the correct sequences by amino acid sequence analysis using protein sequencer model 476A. To conjugate the peptides with a carrier protein, a cysteine was introduced to the C-terminals of the peptides. The peptides were coupled to keyhole limpet hemocyanine through the C-terminal cysteine of the peptides using the Inject Activated Immunogen Conjugation Kits (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

Preparation of an anti-peptide antibody

Anti-peptide antibodies were raised in female Japanese White rabbits obtained from Kitayama Labes (Kyoto, Japan). The rabbits were subcutaneously injected with 500 μ g conjugated peptides in Freund's complete adjuvant in a total volume of 1 ml at various sites on the back. The rabbits were given boosters subcutaneously with 500 μ g conjugated peptides in Freund's incomplete adjuvant in a total volume of 1 ml at 1, 2, 3, 5 and 8 weeks. Antiserum was collected at 3 days after the last boost. The blood was centrifuged and the resultant serum was applied to an Affi-Gel-Protein A Agarose column (Bio Rad, Richmond, CA, USA) to obtain an immunoglobulin (IgG) fraction.

Enzyme-linked immunosorbent assay (ELISA)

Peptides used as antigens were diluted to a final concentration of 10 μ g/ml and incubated in ELISA microplate wells at 4°C overnight. Plates were washed three times with phosphate-buffered saline plus 0.5% Tween 20, blocked with 0.5% skim milk at 4°C overnight. After washing, the plates were incubated with anti-peptide antibodies diluted to different fold dilutions in phosphate-buffered saline containing 0.5% skim milk at room temperature for 1 h. The plates were washed three times, and peroxidase-conjugated goat anti-rabbit IgG (Serving Science and Medicine, Israel) diluted 1:1000 was added to the plate and incubated at room temperature of 1 h. The plates were washed and developed for 30 min at room temperature with 0.1 ml, 0.2 mg/ml 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphate) in 0.05 M sodium citrate buffer (pH 5.3) containing 0.015% hydrogen peroxide. The reaction was stopped by the addition of 0.1 ml 10% sodium dodecyl sulphate (SDS). The absorbance at 405 nm was read in a plate reader.

Measurement of enzyme activities

In immunoinhibition studies on microsomal BTL and PL oxidation, microsomes (100 μ g protein) from rat liver, dog liver and human lymphoblasts expressing P4502D6 were preincubated with various amounts of the IgG at room temperature for 30 min prior to addition of the substrate (final concentration; 10 μ M) and the NADPH-regenerating system. Metabolites formed were determined by the hplc methods as previously described (Suzuki *et al.* 1992, Fujita *et al.* 1993).

Other methods

The preparation of the microsomal fraction was described previously (Omura *et al.* 1964). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the methods of

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Results

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Laemmli (1970) using 10% polyacrylamide. Proteins separated by SDS-PAGE were blotted on to a nitrocellulose membrane. After incubation with anti-peptide antibodies, the membrane was incubated with peroxidase conjugated goat anti-rabbit IgG. P4502D on the membrane was detected by staining the peroxidase with H_2O_2 and diaminobenzidine. The content of protein was determined by the method of Lowry *et al.* (1951).

Results

Table 1 shows the amino acid sequences of the two synthetic peptides and the members of P4502D subfamily. The sequence of peptide A is identical with that of the epitope of LKM 1 autoantibodies and occurs in both rat P4502D2, P4502D4 and human P4502D6. The sequence of peptide B differs by two residues from that of the epitope of LKM-1 autoantibodies and occurs in the dog P4502D subfamily. These sequences are confirmed to be unique to the respective members of P4502D subfamily among all the protein in the SWISS-PROT, PIR, PRF and Genpept data bases as of March 1994.

Figure 1 shows the binding of the anti-peptide antibodies to their respective synthetic peptides, which were adsorbed directly to the ELISA plates. Each antibody could bind to their respective peptides. Western blot analysis was performed to determine the reactivities of the anti-peptide antibodies with microsomes of rat liver, dog liver and human lymphoblasts expressing P4502D6 (figure 2). The anti-peptide antibody against peptide A recognized a 49-KDa protein with microsomes from rat liver and human lymphoblast expressing P4502D6. It showed no crossreactivity in microsomes of dog liver. In contrast, the anti-peptide antibodies against peptide B recognized a 49-KDa protein only in microsomes from dog livers.

The effects of the anti-peptide antibody against peptide A and peptide B on the activity of the P4502D subfamily were investigated. PL and BTL are metabolized by the P4502D subfamily at low substrate concentrations in rat (Suzuki *et al.* 1992, Fujita *et al.* 1993) and dog (Nakamura *et al.* 1995). Therefore, the abilities to metabolize PL and BTL were used as an index of the P4502D subfamily. The anti-peptide antibody against peptide B inhibited bunitrolol 4-hydroxylation in dog liver microsomes, whereas it did not inhibit bunitrolol 4-hydroxylation in microsomes of rat liver and human lymphoblast expressing P4502D6 (figure 3). The anti-peptide antibody against peptide A did not have inhibitory actions (figure 3). Furthermore, the anti-peptide antibody against peptide B inhibited propranolol 4, 5-hydroxylation in dog liver microsomes (figure 4).

Table 1. Comparison of aligned sequences of two synthetic peptides with those of the members of P4502D subfamily.

Peptide	P450	Sequence	Reference
A		DPAQPPRD	
Rat	2D1	DPAQPPRN	Matsunaga <i>et al.</i> (1989)
	2D2	DPAQPPRD	Matsunaga <i>et al.</i> (1989)
	2D3	DPDQPPRD	Matsunaga <i>et al.</i> (1989)
	2D4	DPAQPPRD	Matsunaga <i>et al.</i> (1989)
	2D5	DPAQPPRM	Matsunaga <i>et al.</i> (1990)
Man	2D6	DPAQPPRD	Gonzalez <i>et al.</i> (1988)
B		DPTQPPRH	
Dog	2D	DPTQPPRH	unpublished ¹

¹Kirita *et al.* (The Gene Bank, Accession No. D17397).

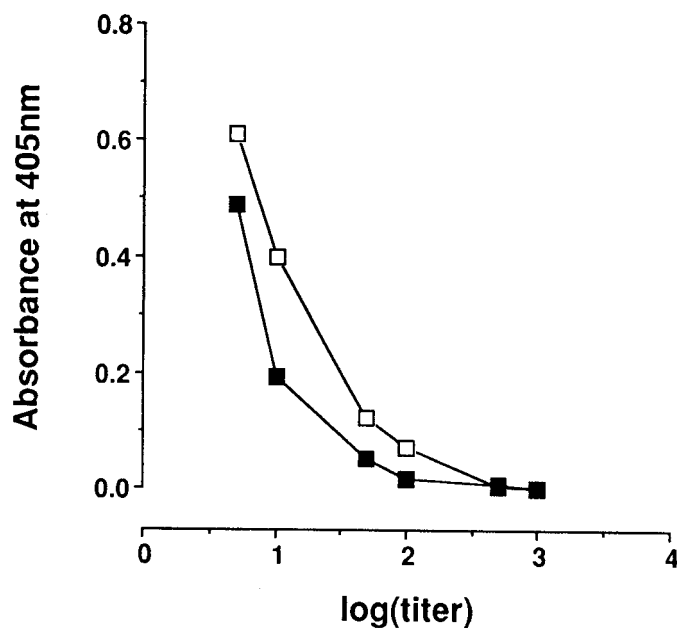


Figure 1. Binding of anti-peptide antibodies to synthetic peptides. Microtitre plates were coated with peptides A (DPAQPPRD, ■) and B (DPTQPPRH, □). A series of dilutions of antisera raised against the peptide was added to the plates and the bindings of antibodies were compared by the methods described in the Materials and methods. Each point is the mean for two determinations.



Figure 2. Western blot analysis of liver microsomes from beagle dog, rat and human lymphoblasts. Proteins separated by SDS-PAGE were blotted on to a nitrocellulose membrane and immunostained with anti-peptide antibodies. (a) Blotting with the anti-peptide antibody against peptide A; and (b) blotting with anti-peptide antibodies against peptide B. Lanes 1 and 4, male SD rat (50 μ g); 2 and 5, male beagle dog (50 μ g); and 3 and 6, microsomes of lymphoblast expressing P4502D6 (50 μ g).

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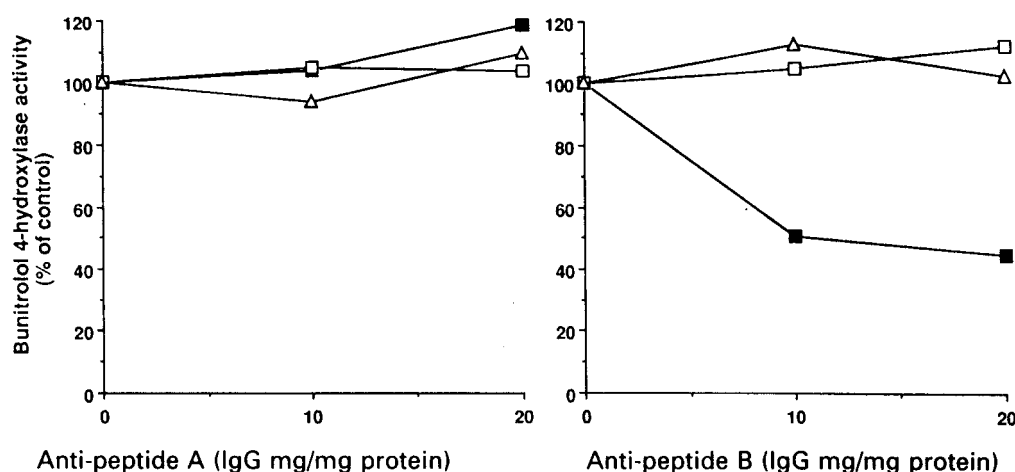


Figure 3. Effects of anti-peptide antibodies against peptides A (rat/human antibody; left) and B (dog antibody; right) on BTL 4-hydroxylation in microsomes of rat liver (□), dog liver (■) and human lymphoblasts expressing P4502D6 (△). The experimental conditions were described in the Materials and methods. Results are expressed as percentage of the activities in control incubations containing an equal amount of pre-immune IgG. The activities of BTL 4-hydroxylase in the control incubations were 1.82 nmol/min/mg for rat liver microsomes, 0.635 nmol/min/mg for dog liver microsomes, and 0.057 nmol/min/mg for microsomes derived from human lymphoblast expressing 2D6. Each point is the mean for two determinations.

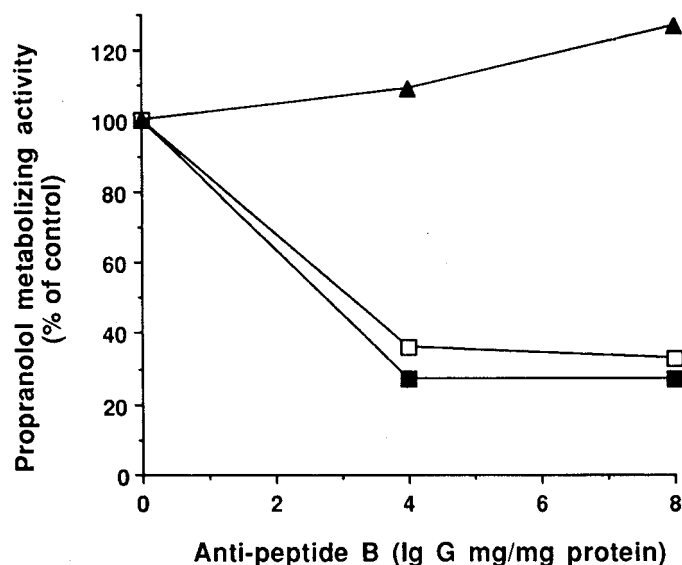


Figure 4. Effects of anti-peptide antibody against peptide B (dog antibody) on PL-metabolizing activities (PL 4-hydroxylation; □, PL 5-hydroxylation; ■, PL N-desisopropylation; ▲) in hepatic microsomes from dogs. The experimental conditions were described in the Materials and methods. Result are expressed as percentage of the activities in control incubations containing an equal amount of pre-immune IgG. The propranolol-metabolizing activities in the control incubations were 0.06 nmol/min/mg for PL 4-hydroxylase, 0.03 nmol/min/mg for PL 5-hydroxylase, and 0.09 nmol/min/mg for PL N-desisopropylation. Each point is the mean for two determinations.

Discussion

Two anti-peptide antibodies against synthetic peptides, DPAQPPRD (peptide A) and DPTQPPRH (peptide B), were generated in the current study. These sequences are closely related to the epitope of LKM-1 autoantibodies, which are found in patients with autoimmune hepatitis (Manns 1989). LKM-1 autoantibodies

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exclusively recognize P4502D6, as judged by their recognition of the protein expressed cDNAs of P4502D6 and by the capacity of the antibodies to inhibit the reactions catalysed by P4502D6 (Zanger *et al.* 1988). Recently, Manns *et al.* (1991) reported that the common epitope of LKM-1 antibodies was a short peptide (DPAQPPRD), corresponding to the residues 263–270 of P4502D6. Therefore, the region corresponding to the residues 263–270 of P4502D6 was considered to be a suitable sequence for generating the anti-peptide antibody. The anti-peptide antibody against peptide A, which is targeted to rat P4502D2, P4502D4 and human P4502D6, bound to a 49-KDa protein in microsomes of human lymphoblast expressing P4502D6 and rat liver. The lack of binding of the anti-peptide antibody against peptide A to dog liver microsomes can be explained by the fact that this region of the dog P450 subfamily differs by two amino acid residues from the sequence of the rat and human P4502D subfamily. In contrast, the anti-peptide antibodies against peptide B, which is targeted to the dog P4502D subfamily, bound to a 49-KDa protein only in microsomes from dog. The binding ability of the anti-peptide antibody against peptide B only to microsomes from dog may be also due to the difference by two residues from the sequences of the rat and human P4502D subfamilies. These indicate that each anti-peptide antibody has the specificities for the respective sequences of the members of the P4502D subfamily.

Interestingly, the anti-peptide antibody against peptide B was found to inhibit BTL 4-hydroxylase and PL 4, 5-hydroxylase activities. We recently purified a new P450 (P450CF1) belonging to the P4502D subfamily from microsomes of dog (Nakamura *et al.* 1995). P450CF1 can mediate BTL 4-hydroxylation and PL-4,5 hydroxylation (Nakamura *et al.* 1995). These data suggest that the anti-peptide antibody against peptide B binds to the native forms of the P4502D subfamily as well as to denaturated forms of the P4502D subfamily. The role of this region in the oxidation reaction has not yet been demonstrated. This region of members of the P4502D subfamily are highly conserved among various animal species. Edwards *et al.* (1989) predicted the location and sequences of α -helices in mammalian P450 from their homology with those of P450cam. Based on this model, the epitope recognized by LKM-1 antibodies would occur between helices corresponding to G and H of P450cam, which is a protruding loop on the surface of the protein and is postulated to play a role in binding to the electron donor protein.

In conclusion, we have succeeded in generating the anti-peptide antibodies that bind specifically to the P4502D subfamily. Studies of the P4502D subfamily are hampered by the difficulty in purification from liver and an antibody against the P4502D subfamily is not commercially available. We have demonstrated an alternative way of obtaining specific antibodies to the P4502D protein herein. In particular, the anti-peptide antibody for DPTQPPRH will be useful for studying the dog P4502D because of its specificity and inhibitory properties.

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