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Detection of Minor Immunological Differences Among Human "Universal-Type" Alkaline Phosphatases

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Two clones of monoclonal antibodies against swine alkaline phosphatase (ALPase; orthophosphoric monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1), which were useful in distinguishing human kidney and bone ALPases from liver ALPase, were successfully raised in mice. On the other hand, polyclonal antibody cross-reacted not only with human kidney ALPase but also with all other human universal type ALPases. The difference in cross-reactivitiy of monoclonal and polyclonal antibodies may be caused by the specific antigenicity of human enzymes. The monoclonal antibodies were able to recognize minor heterogeneity that could not be distinguished by their enzymatic properties. The present monoclonal antibody preparations will be utilized for clinical as well as basic investigations to detect minor heterogeneity among universal-type ALPases.

Key words: monoclonal antibody, polyclonal antibody, immunoblotting, minor heterogeneities

In our previous paper, we discussed our successful purification of swine kidney ALPase by utilizing polyclonal antibody against the enzyme for immunoaffinity chromatography [1]. The polyclonal antibody we have raised in rabbits cross-reacted with human kidney-type or so-called universal-type ALPases but not with intestinal or placental ALPase. Therefore, the antibody could be used to distinguish the universal-type ALPase clearly from other enzyme types. However, no difference in immunological reactivity was observed among universal-type ALPases, even though there may be minor molecular heterogeneities among them.

Human universal-type ALPases have almost identical enzymatic properties, such as susceptibility to various inhibitors and thermostability. Electrophoretic mobilities have been routinely used to distinguish their difference in clinical laboratories. Recently, growing attention has been paid to the use of monoclonal antibodies against ALPases [2–8]. A monoclonal antibody is specific for a single antigenic determinant of the enzyme; therefore enzyme polymorphism among placental ALPases has been

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revealed by the antibody, as reported by Slaughter et al. [2]. In this study, we raised monoclonal antibodies against swine kidney ALPase that were useful in detecting minor heterogeneity among human universal-type ALPases by their cross-reactivities.

MATERIALS AND METHODS

Enzyme Preparation

Swine and bovine tissues were obtained from a local slaughter house. White rabbits (3–4 kg, male) and Wistar strain rats (250–350 g, male) were used. Human placenta was from normal labor, and bone was from surgical operation of fibrous dysplasia. The other human samples were autopsy materials.

The tissues were homogenized with distilled water (2 l/kg of the tissue) and mixed with 1-butanol (1 l). The aqueous phase was separated, and the precipitates at pH 4.9 with acetic acid were removed. The supernatant was adjusted quickly to pH 6.5, and the precipitates that appeared by acetone addition (50% in final concentration) were collected by centrifugation and dried to acetone powder.

Enzyme Assay

ALPase activity was determined with 10 mM p-nitrophenyl-phosphate as a substrate in 100 mM 2-amino-2-methyl-1,3-propanediol-HCl buffer containing 5 mM $MgCl_2$, pH 10.0, at 37°C [1]. The enzyme activity was also assayed in the presence of various concentrations of inhibitors, such as levamisole, L-homoarginine, and L-phenylalanine. The reaction was performed in 3 ml substrate solution at 37°C and stopped with 1 ml 2N NaOH. The concentration required to reduce ALPase activity at 50% of original activity was calculated. In order to assay thermostability, the tissue extract was treated at 56°C or 60°C for various intervals and then reacted with the substrate and stopped with 2N NaOH. The time required to reduce ALPase activity to 50% of original activity was calculated.

Purification of ALPase as an Antigen

Swine kidney ALPase was purified from acetone powder by a series of chromatographies with DEAE-cellulose, concanavalin A-Sepharose, and gel filtration, as reported previously [1]. Homogeneity of the enzyme was confirmed by polyacrylamide gel and immunoelectrophoresis. Its specific activity was 1,150 units/mg protein [1,9]. Polyclonal antibody against purified swine kidney ALPase was raised in rabbits as described previously [1].

Production and Screening of Monoclonal Antibodies

A female BALB/c mouse was injected intraperitoneally with 100 μg of purified swine kidney ALPase emulsified with Freund's complete adjuvant (Difco), followed by the second injection of the same amount of enzyme 4 weeks later. Cell fusion was performed between the spleen cells of immunized mouse and mouse myeloma cells (line P3-NSl-Ag4-1, Flow Laboratories, Inc.). The culture media of hybridoma cells were screened for anti-ALPase antibody activity according to Rennard et al. [10]. Purified swine ALPase was dissolved to 2 μg/ml in Voller's buffer (50 mM Na_2CO_3 · $NaHCO_3$ buffer, pH 9.2, containing 0.02% NaN_3) [11], and 100 μl was adsorbed onto each well of microtiter plate (Dynatech Laboratories Inc.) for 24 h at 4°C. After washing the wells three times with PBS (10 mM sodium phosphate, pH

7.4, containing 0.15 M NaCl, 0.05% Tween 20), culture medium was added and incubated for 1 h at room temperature. Following another series of washings, a peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories) solution diluted 1,000 times with PBS was added and incubated for 1 h at room temperature. The wells were again washed, and peroxidase activity was developed by adding 175 μl substrate solution (mixture of 10 mg of o-phenylenediamine in 5 ml methanol, 99 ml distilled water, and 10 μl of 30% H_2O_2). After 30 min, the reaction was stopped with 8 M H_2SO_4 (25 μl/well). Absorbance at 492 nm was measured with an automatic multiscanning photometer (Titertek Multiskan MC; Flow Laboratories). The cells in positive wells were passaged and cloned three times. Two stable clones were established and were designated M1 and M2.

The hybridoma cells (1×10^7 per mouse) were inoculated intraperitoneally into BALB/c mice pretreated with 0.5 ml of pristane (2,6,10,14-tetra-methylpentadecane, Aldrich Chemical Co.). When abdominal swelling was detected after 1–3 weeks, the ascitic fluid was collected, fractionated with 50% ammonium sulfate, and stored at −80°C.

The immunoglobulin class and subclass of the monoclonal antibodies were typed by Ouchterlony double diffusion, using commercial antisera specific for the respective classes of murine immunoglobulins (Miles Scientific Laboratories).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Acetone powder of the tissue extract was dissolved in TBS (10 mM Tris-HCl containing 0.9% NaCl, pH 7.4) and electrophoresed with 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate at 2mA/lane for 2 h [12]. Proteins separated in the polyacrylamide slab were transferred onto nitrocellulose membrane (pore size 0.45 μm; BioRad Laboratories) at 5 V/cm for 40 min, according to the method of Towbin et al. [13]. Transfer medium was 25 mM Tris, 192 mM glycine, pH 8.3, containing 20% methanol. Then the nitrocellulose sheet was washed with TBS and blocked with 3% skim milk solution in TBS for 40 min. After incubation with antibody solution diluted 500 times after the buffer for 24 h at 4°C, the membrane was soaked in horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel Laboratories) for 1 h at room temperature. The membrane was washed again with TBS and stained with peroxidase substrate solution (mixture of 30 mg of 4-chloro-1-naphtol dissolved in 10 ml methanol, 50 ml TBS and 100 μl of 30% H_2O_2) [14]. ALPase activity in the gel was stained with β-naphthyl phosphate [15].

Concerning dot-immunobinding assay [14], 50 μl each of the ALPase solution was blotted onto a nitrocellulose membrane with a Minifold (Schleisher & Schuell Inc.). The filter was blocked with 3% skim milk solution in TBS and then treated as described above.

Enzyme-Linked Immunoadsorbent Assay (ELISA)

Immunological examination of monoclonal antibodies was perfomed by the ELISA method according to Harris et al. [5]. A 1:2,000 dilution of rabbit anti-mouse IgG (Cappel Laboratories) in PBS was adsorbed onto each well of microtiter plates, which were next blocked for nonspecific adsorption of protein with 0.5% BSA solution. Diluted antibody solution was then added and incubated overnight. The plates were washed and incubated for 6 h with a 500 mIU/ml solution of each ALPase preparation. The plates were then washed and incubated with the ALPase substrate

solution, and absorbance at 405 nm was measured with an automatic scanning photometer (Titertek Multiscan MC).

RESULTS

Enzymatic properties of human ALPases in various tissues, such as kidney, liver, bone, intestine, or placenta, and swine enzymes in kidney, liver, placenta, alveolar bone, enamel organ, dental pulp, and intestine were comparatively investigated by inhibition studies with levamisole, L-homoarginine, and L-phenylalanine and by the thermostability test (Table I).

Universal-type ALPases present in human and swine kidney, liver, bone, and dental tissues were significantly inhibited by levamisole as well as by L-homoarginine. On the other hand, L-phenylalanine inhibited only human and swine intestinal and human placental ALPases. ALPases in other tissues exhibited tolerance to L-phenylalanine. Likewise, in the thermostability test, human and swine intestinal and human placental ALPases showed heat stability, but others lost more than 50% of their activity within 5 min at 56°C or 60°C.

From the result of inhibition experiments, it was concluded that human and swine intestinal and human placenta ALPases differed clearly from other enzymes. However, it was rather difficult to find substantial differences in enzymatic properties among universal-type ALPases. Swine placental ALPase was found to be classified into the universal-type enzyme according to its enzymatic properties.

The immuno-cross-reactivities of ALPases in various swine tissues were investigated by the two monoclonal antibodies. Swine ALPases were applied to SDS-PAGE, and their enzyme activity was stained by the β-naphthyl phosphate method (Fig. 1A). The mobilities of the enzymes were similar except for that of intestine, which exhibited rapid migration (Fig. 1A, lane 7). Figure 1B shows that immunore-

TABLE I. Properties of Alkaline Phosphatases*

		Concentration or time required to reduce ALPase activity to 50% of original activity[a]				
		Lev	L-HA	L-Phe	Heat stability (min)	
Species	Tissue	(mM)	(mM)	(mM)	56°C	60°C
Human	Kidney	0.03	1.18	19	5.49	1.51
	Liver	0.03	1.88	>20	3.56	1.85
	Bone	0.03	1.17	18	2.65	0.72
	Intestine	>1	>10	1.31	>30	>10
	Placenta	0.93	>10	2.26	>30	>30
Swine	Kidney	0.03	1.88	>20	1.08	0.62
	Liver	0.03	2.01	>20	3.17	0.60
	Bone	0.03	2.05	>20	2.49	0.71
	Dental pulp	0.04	1.96	>20	3.14	0.99
	Enamel organ	0.04	2.16	>20	2.37	0.93
	Placenta	0.04	2.38	>20	4.75	1.34
	Intestine	>1	>10	2.10	>30	>10

*Activity was assayed by the rate of hydrolysis of p-nitrophenyl phosphate. The effects of inhibitors were determined in the presence of 5 mM $MgCl_2$ in assay mixture.
[a]Lev, Levamisole; L-HA, L-homoarginine; L-Phe, L-phenylalanine.

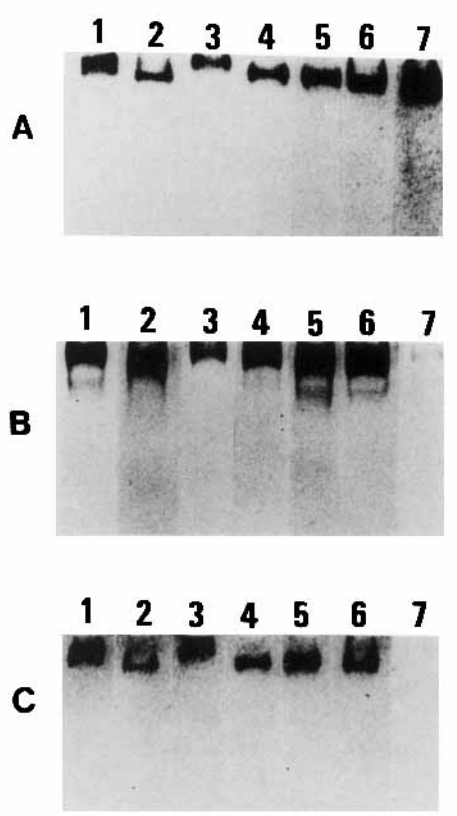

Fig. 1. SDS-polyacrylamide gel electrophoresis **(A)** and transfer immunoblots on nitrocellulose membranes **B** and **C**. Enzymatic activity in the gel was stained by the β-naphthyl phosphate method (A). Nitrocellulose membrane was first incubated with the polyclonal antibody to swine kidney ALPase (B) or with the monoclonal antibody M1 (C). **Lane 1:** Swine kidney. **Lane 2:** Swine liver. **Lane 3:** Swine placenta. **Lane 4:** Swine bone. **Lane 5:** Swine enamel organ. **Lane 6:** Swine dental pulp. **Lane 7:** Swine intestine.

activity with polyclonal antibody after immunoblotting onto a nitrocellulose membrane. ALPases of swine kidney (lane 1), liver (lane 2), placenta (lane 3), bone (lane 4), enamel organ (lane 5), and dental pulp (lane 6) cross-reacted clearly with the polyclonal antibody. Although a band found in intestinal ALPases preparation (lane 7) was stained weakly with the antibody, this may be due to a contaminant in the preparation, because its migration position did not coincide with the band of enzyme activity.

Both M1 and M2 antibodies were identified as belonging to IgM by the immunoglobulin typing assay. Figure 1C shows cross-reactivity of swine ALPases with monoclonal antibodies, and all of swine enzymes except that of intestine cross-reacted with M1 antibody; there were no reactivity differences between polyclonal and monoclonal antibodies.

Cross-reactivity of human ALPases with antibodies against swine kidney enzyme was investigated by the western blot assay after SDS-gel electrophoresis. Human kidney ALPase cross-reacted with both monoclonal and polyclonal antibodies, and the respective immunoreactive bands (Fig. 2D,G) coincided with its enzyme

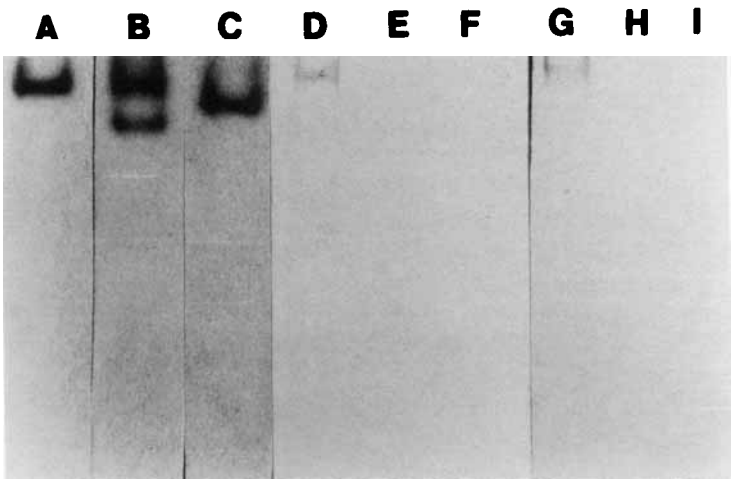

Fig. 2. SDS-polyacrylamide gel electrophoresis **(A–C)** and transfer immunoblots on nitrocellulose membranes **(D–I)**. Enzymatic activity in the gel was stained by the β-naphthyl phosphate method (A–C). Nitrocellulose membrane was first incubated with the polyclonal antibody to swine kidney ALPase (D–F) or with the monoclonal antibody M1 (G–I). **Lanes A, D and G:** Human kidney. **Lanes B, E and H:** Human intestine. **Lanes C, F and I:** Human placenta.

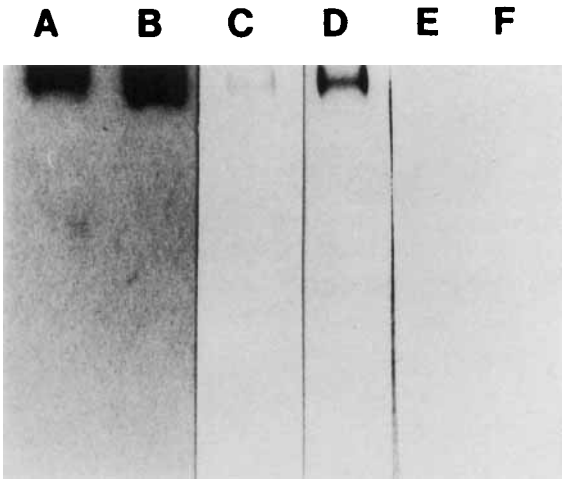

Fig. 3. SDS-polyacrylamide gel electrophoresis **(A, B)** and transfer immunoblots on nitrocellulose membranes **(C–F)**. Enzymatic activity in the gel was stained by the β-naphthyl phosphate method (A, B). Nitrocellulose membrane was first incubated with polyclonal antibody to swine kidney ALPase (C, D) or with monoclonal antibody (E, F). **Lanes A, C and E:** Human bone. **Lanes B, D and F:** Human liver.

activity band (Fig. 2A), but human intestinal and placental ALPases did not react (Fig. 2E,F,H,I). Human bone and liver enzymes (Fig. 3A,B) also cross-reacted with polyclonal antibody (Fig. 3C,D) but not with monoclonal antibody (Fig. 3E,F).

In addition, the enzyme antigenicity was also studied by the ELISA and dot-blot immunobinding assay. The results are shown in Table II and Figure 4. Human kidney

TABLE II. Immuno-Cross-Reactivity of Human Alkaline Phosphatases With Monoclonal Antibodies M1 and M2*

Enzyme source	Dot-blot assay		ELISA	
	M1	M2	M1	M2
Human				
Kidney	1/10,000	1/10,000	1/1,000	1/100
Liver	1/100	1/100	1/10	1/10
Bone	1/10,000	1/10,000	1/1,000	1/1,000
Placenta	—	—	—	—
Intestine	—	—	—	—

*Values are means of maximal dilution factors of the monoclonal antibodies for the immunoreaction. —, not cross-reacted at any concentration.

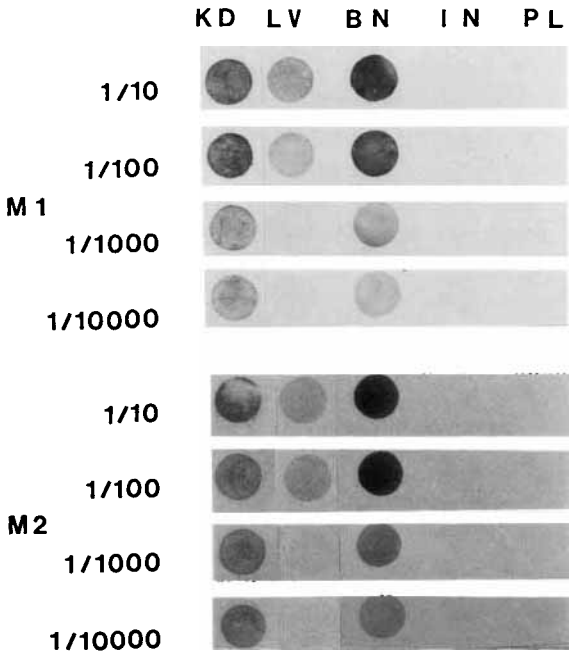

Fig. 4. Dot blot immunoassay of monoclonal antibodies M1 and M2. Human ALPase solutions (50 μl each, 0.2U/ml) were blotted onto the filter, and a serial dilution assay (×10–×10,000) of the antibodies was performed. KD, human kidney; LV, liver; BN, bone; IN, intestine; PL, placenta.

and bone ALPases cross-reacted strongly with both M1 and M2, but human liver enzyme reacted weakly. There was general agreement about the results of the western blotting, dot-blotting, and ELISA, except for human bone. Human bone ALPase cross-reacted clearly with monoclonal antibodies M1 and M2 when examined by the ELISA and dot-blot methods. This disagreement may be due to the diminution of antigenicity of human bone ALPase during SDS-PAGE procedures. The antigenicity of human bone ALPase was found to be especially unstable when compared with those of other ALPases and was easily diminished even by the treatment of butanol extraction. Therefore we used human bone homogenate instead of acetone powder in our immunological experiments.

Tissue ALPases of other animals, such as bovine, rabbit, and rat, were investigated. As shown in Table III, bovine universal-type ALPases (kidney, liver, bone, and placenta) and rabbit liver ALPase did not cross-react with M1 antibody. All kinds of universal-type ALPases cross-reacted with polyclonal antibody and with M2 except that of rabbit liver.

DISCUSSION

Human ALPases are classified into three main types; kidney-liver-bone, or "universal"-type, intestinal, and placental [16,17]. However, placental enzyme found in mammals lower than hominidae is classified into the universal type [18,19]. Three types of human ALPase are believed to be synthesized by genetically different information, and there are distinct differences between their enzymatic properties. In the inhibition and thermal inactivation studies, swine universal-type ALPases (kidney, liver, bone, dental pulp, enamel organ, and placenta enzymes) showed similar properties, which were compatible with those of human universal-type ALPases in kidney, liver, and bone.

In this report, we have succeeded in establishing two clones (M1 and M2) of monoclonal antibodies by selecting 16 candidates against purified swine kidney ALPase. These two monoclonal antibodies as well as polyclonal antibody showed common immunological properties of cross-reactivity with swine unniversal-type ALPases but not with intestinal and human placental ALPases.

However, there was a slight difference in cross-reactivity when antibodies were examined in detail. Human liver ALPase had very little cross-reactivity with antibod-

TABLE III. Western Blot Assay of Alkaline Phosphatases*

	Enzyme Source of ALPase						
Antibody	Kidney	Liver	Bone	Enamel organ	Dental pulp	Placenta	Intestine
Human							
M1	+	−	−	/	/	−	−
M2	+	−	−	/	/	−	−
PCA	+	+	+	/	/	−	−
Swine							
M1	+	+	+	+	+	+	−
M2	+	+	+	+	+	+	−
PCA	+	+	+	+	+	+	−
Bovine							
M1	−	−	−	/	/	−	−
M2	+	+	+	/	/	+	−
PCA	+	+	+	/	/	+	−
Rabbit							
M1	+	−	+	/	/	+	−
M2	+	−	+	/	/	+	−
PCA	+	+	+	/	/	+	−
Rat							
M1	+	+	+	/	/	+	−
M2	+	+	+	/	/	+	−
PCA	+	+	+	/	/	+	−

*/, not determined; PCA, polyclonal antibody raised in rabbits.

ies M1 and M2. Sussman et al. [20] reported that human bone ALPase did not cross-react with polyclonal antibody against human liver ALPase, and they concluded that human liver ALPase is antigenically a distinct type. The present results lend support to the concept that human bone and kidney ALPases have antigenic specificity different from that of human liver enzyme. In addition, bovine universal-type ALPases did not cross-react with M1 antibody. The slightly different cross-reactivity between M1 and M2 may be caused by their difference in antigenic determinants. Therefore the present monoclonal antibody preparations will be useful in distinguishing human kidney and bone ALPases from liver ALPase. These data suggested that there exist minor heterogeneity among human universal-type ALPases, and it is possible to recognize the difference by cross-reactivity with monoclonal antibodies.

We examined the cross-reaction of human serum ALPases with the monoclonal antibodies as a preliminary step in our investigation. Human serum was treated with protein A (Pansorbin, Calbiochem-Behring Corp.) in order to remove IgG, and then immunoreactivities of ALPases present in the serum (liver, bone, placenta, and intestinal enzymes) were tested. Although those enzymes cross-reacted weakly with monoclonal antibodies, the difference in types was not clearly distinguishable.

Since monoclonal antibody can be raised in substantial quantities and in homogeneous quality, compared with polyclonal antibody, more monoclonal antibody preparation will become available for the purpose of ALPase discrimination in the future.

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