Shimane J. Med. Sci. Vol. 2, pp. 25—31, 1978

Immunohistochemical Localization of Lactate Dehydrogenase Subunits in Porcine Liver ; Effect of Fixatives on Detection of Specific Fluorescence

(LDH subunits/fluorescent antibody/liver)

MOTOHIKO ITO[a], HARUO FUKUDA[a], YUKIO SUZUKI[b], TAKAYUKI HARADA[b], and SHIGERU MORIKAWA[b]

[a]*Department of Thoracic Surgery, Chest Disease Research Institute, Kyoto University, Kyoto 606, and* [b]*Department of Pathology, Shimane Medical University, Izumo 693, Japan*

(Received July 27, 1978)

Immunohistochemical localization of lactate dehydrogenase subunits was studied using a fluorescent antibody technique in porcine liver. Because of the difficulty in demonstrating specific fluorescence of LDH-M in the liver tissue, many fixatives were tested both in frozen and paraffin sections to obtain consistent staining results. Among the fixatives examined, ether-ethanol-acetone mixture in frozen sections gave the best result in demonstrating both subunits in the liver tissue by immunofluorescence. Paraffin sections failed to preserve LDH-M specific fluorescence in this study. Specific fluorescence for LDH-H was observed in the hepatic cell membrane facing the space of Disse, in the lining cells of sinusoids and in the cyto plasm of Kupffer cells. LDH-M specific fluorescence was observed as fine granules in the cytoplasm of hepatic cells.

Lactate dehydrogenase (LDH) is a tetramer composed of two types of subunits, H and M, each of which has a quite different antigenicity (1—3). H- and M- subunits give five possible isozymes by random combination (4). It is well known that the distribution of LDH is variable in tissues or organs. Since most of the tissues consist of different cell types, immunohistochemical analysis of H- and M- subunits in tissues is expected to provide pertinent information concerning function of LDH in tissues.

In a previous paper (5), immunohistochemical localization of LDH subunits was studied using fluorescent antibody technique in acetone fixed bovine tissues. Specific fluorescence for each subunit was observed in heart muscle, renal tissue, and skeletal muscles (5). However, some difficulties were encountered in demonstrating specific fluorescence of LDH-M in the liver tissues under the conditions employed. The importance of the choice of fixatives and/or pretreatment for specific fluorescence in the tissues was emphasized by us (6, 7) and others (8). In the present paper different fixatives were examined as to their ability to demonstrate LDH-H and -M subunits in porcine liver by immunofluorescence, both in frozen and paraffin sections.

MATERIALS AND METHODS

Preparation of Antisera and Conjugates : LDH from porcine heart (LDH-4H) and from porcine skeletal muscle (LDH-4M) were obtained from Boehringer and Soehne GmbH (Mainheim, Germany) and were used as antigens without further purification. Antisera against H- and M- subunit were obtained from male albino rabbits by the same procedure described in our previous paper (5). Globulin fractions of the antisera were prepared by precipitating three times with one-third saturated ammonium sulfate. The globulins were then conjugated with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory, Inc. U. S. A.) by the method of Marshall *et al.*(9). The conjugates were then purified by fractionation on DEAE-cellulose column (0.94 mEq/g, Brown Company, Berlin, U. S. A.)

Assay for Specificity of Antisera : Specificity of antibody was examined by double gel diffusion method of Ouchterlony and by immunoelectrophoresis. Procedures were as described previously (6).

Preparation of Sections : Part of fresh porcine liver was put in a test tube and frozen at −70°C by immersing the tube into an acetone-dry ice mixture. Frozen sections (4−5 μ) were cut in a cryostat at −20°C, placed on clean non-fluorescent slides, allowed to dry for 15−30 min at room temperature, and then fixed in one of the fixatives listed in Table I.

The remainder of the liver tissue was cut into smaller pieces and each was place in one of the fixatives listed in Table II in the cold. Then the blocks were dehydrated, cleared and embedded in paraffin according to the procedure of Sainte-Marie (10). Sections (3−4 μ) were cut, deparaffinized with xylene and hydrated through successive ethanol baths.

Staining with FITC-Labeled Conjugates and Microscopy : After washing with PBS, sections were stained with FITC-labeled conjugates in a moist chamber at room temperature for 16 hr. These slides were then washed with PBS, mounted in glycerol containing 10% PBS and examined under a Carl Zeiss fluorescence microscope.

RESULTS

1. *Specificity of Antisera*

Each of the antisera was tested for specificity and the presence of contaminating immune system by both immunodiffusion and immunoelectrophoresis. When each of the antigens used in this study was electrophoresed on agarose plates, and anti-LDH-H antibody was placed in the upper trench and anti-LDH-M antibody was placed in the lower, only a single precipitin arc was observed between corresponding antigen and antibody (Fig. 1-A, B). When the mixture of antigens was electrophoresed in a similar agarose plate, anti-LDH-H antibody formed a single precipitin arc towards the anode, and anti-LDH-M antibody made an arc towards the cathode (Fig. 1-C). These results

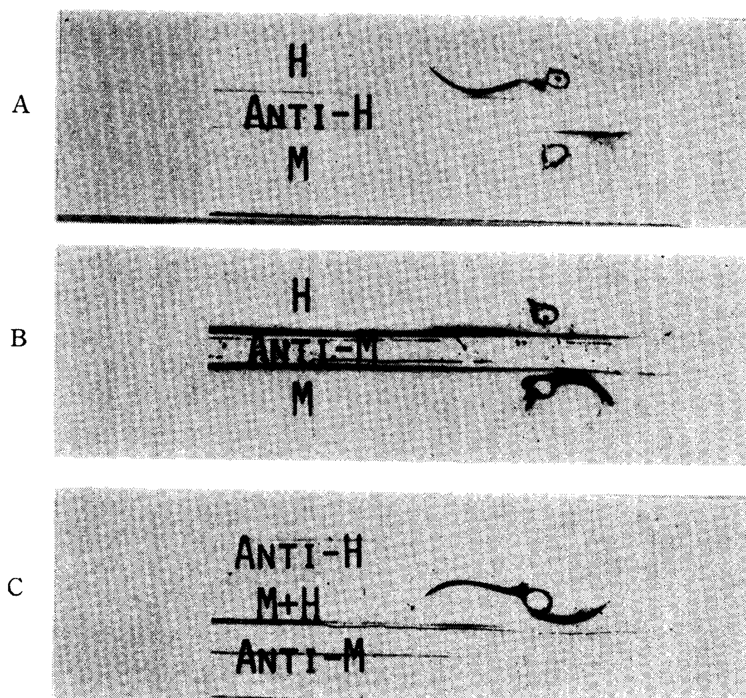

Fig. 1. Immunoelectrophoresis of LDH-H and -M subunits. The upper well was filled with LDH H and the lower was filled with LDH-M. After electrophoresis, anti-LDH-H antiserum was put into the upper trench. Anti LDH-M antiserum was put into the lower (A, B). A mixture of the subunits was put into the center well. Upper trench was filled with anti-LDH-H and the lower was filled with anti-LDH-M. A precipitin arc was observed between the corresponding immune systems (C).

Fig. 2. Double gel diffusion study of the LDH subunits and their antisera. No cross-reactivity was revealed between LDH-H and -M.

suggested that anti-LDH-H antibody and the antigen system are quite different immune systems from anti-LDH-M antibody and antigen. Double gel diffusion studies confirmed this result as shown in Fig. 2. No precipitin band was seen between the different antigen and antibody systems. None of the antisera formed a precipitin arc between normal porcine serum as determined by immunoelectrophoresis.

2. *Effect of Fixatives and Paraffin Embedding on Demonstration of Specific Fluorescence for LDH Subunits in Porcine Liver Tissue*

Differences in the staining results were observed depending upon the staining

TABLE I. *Staining of the Porcine Liver Tissue with Anti-LDH-H and Anti-LDH-M Conjugate in Frozen Sections Using Various Fixatives*

Fixative	Specific Fluorescence*	
	H	M
Acetone	+	±
Acidified acetone (1% acetic acid)	±	±
Ether-acetone (1 : 1)	+	±
Ether-ethanol-acetone (1 : 1 : 1)	++	++
Ether-ethanol (1 : 1)	+	+
95% ethanol	±	±
Acidified ethanols (5, 10, 20% acetic acid)	−	−
10% Phosphate buffered formalin (pH 7.1)	±	±
Formaldehyde gas	±	±
Carnoy's solution	++	±
Carnoy ; modified**	+	±
Carnoy ; modified***	+	±
Glutaraldehyde (2.5%)	+	±
No fixation	−	−

* The symbols used represent brightness and frequency of fluorescence : −, no specific fluorescence observed in any sections. ±, occasional faint fluorescence.
** Acetone 60%.
*** Chloroform 10%, acetic acid 30%.

TABLE II. *Staining of the Porcine Liver Tissue with Anti-LDH-H and Anti-LDH-M Conjugate in Paraffin Sections Using Various Fixatives*

Fixative	Specific Fluorescence*	
	H	M
Ether-ethanol (1 : 1)	++	−
Ether-caetone	+	−
Acetone	+	−
Carnoy's solution	+	−
Carnoy ; modified**	±	−
Carnoy ; modified***	−	±

* The symbols used represent brightness and frequency of fluorescence : −, no specific fluorescence observed in any sections. ±, occasional faint fluorescence.
** Acetone 60%.
*** Chloroform 10%, acetic acid 30%.

procedure and fixatives employed, as illustrated in Tables I and II. Among those examined, cryostat sections fixed with ether-ethanol-acetone mixture gave the best result in demonstrating specific fluorescences for both LDH-H and -M subunits. In general, antigenicity of LDH-M subunit in sections was much more sensitive to fixation and paraffin embedding than that of LDH-H. Paraffin sections failed to preserve LDH-M specific fluorescence except for sections fixed with modified Carnoy's solution in which faint fluorescence was observed only occasionally.

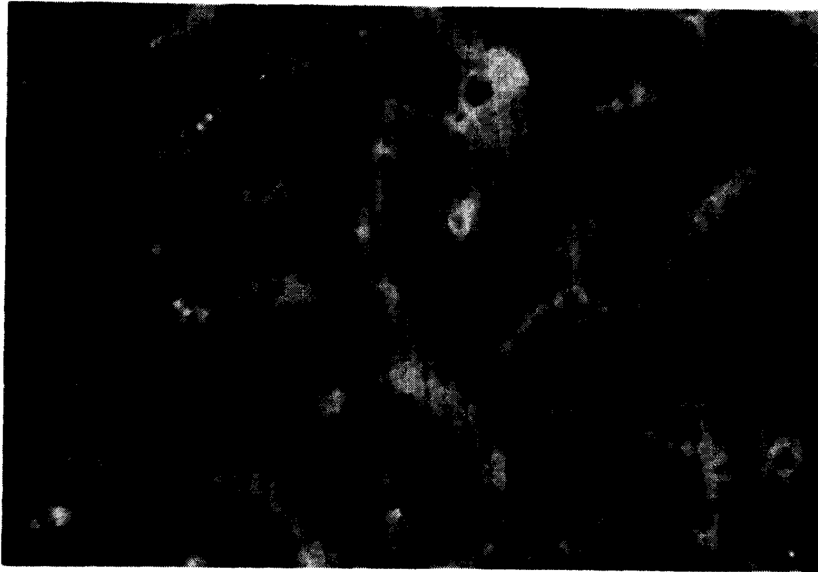

Fig. 3. The location of LDH-H subunit in porcine liver. Bright fluorescence was observed to surround the area of sinusoids including Kupffer cells. Frozen section fixed with ether-ethanol-acetone mixture.

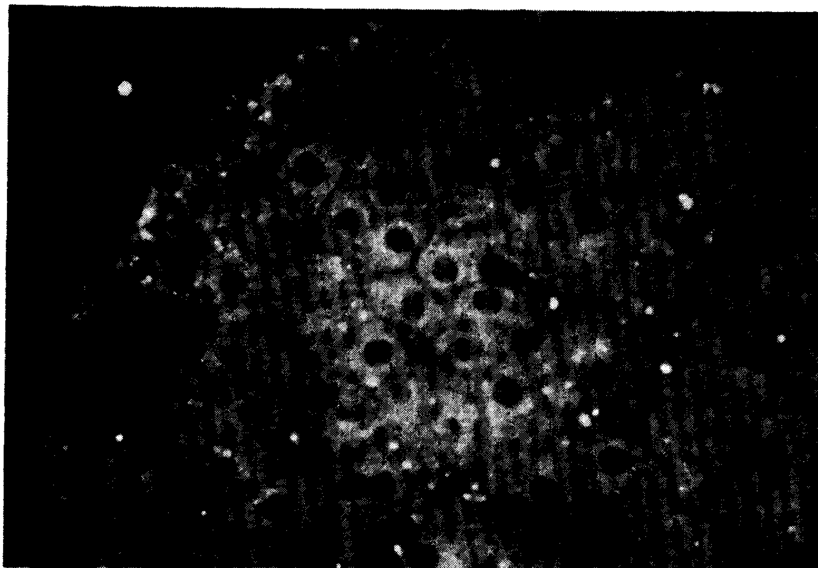

Fig. 4. The location of LDH-M subunit in porcine liver. Specific fluorescence was observed in the cytoplasms of hepatic cells as fine granules. Frozen section fixed with ether-ethanol-acetone mixture.

3. Staining Results of LDH Subunits in Porcine Liver Frozen Sections Fixed with Ether-Ethanol-Acetone

Only the frozen sections fixed with ether-ethanol-acetone mixture could demonstrate the location of both LDH-H and -M subunits in porcine liver, immunohistochemically. Specific fluorescence for LDH-H was observed in the hepatic cell membrane facing the space of Disse, in the lining cells of sinusoids and in the cytoplasm of Kupffer cells (Fig. 3). Most of the fluorescence, specific for LDH-M, was observed diffusely in the cytoplasm of hepatic cells

as fine granules (Fig. 4). It was clearly demonstrated that LDH-H and -M subunits locate quite differently in liver tissue.

DISCUSSION

In a previous paper, we reported the immunohistochemical localization of LDH subunits in bovine tissues by fluorescent antibody technique (5). Here the location of LDH-M subunit could not be demonstrated in acetone-fixed frozen sections from liver tissue, though Plegemann *et al.*(11) and Wróblewski and Gregory (12) reported that liver tissues are rich in LDH-M as determined by the biochemical and electrophoretical analysis. In our previous studies (6, 7), it was suggested that fixatives employed were critical for immunohistochemical demonstration of the enzymes in soluble forms. LDH-M is reported to be distributed in soluble fractions in liver tissue (13, 14). Except for the ether-ethanol-acetone mixture, almost all the fixatives examined failed to reveal the location of LDH-M in hepatic cells. Why the ether-ethanol-acetone fixation gave the best results is uncertain, however, quick insolubilization and removal of fat from the specimen may be one of the factors influencing the conditions of fixation. Contrastingly, mild fixatives, such as buffered formalin, glutaraldehyde, gave poor results in demonstration of LDH-M.

As for location of LDH-H and -M subunits in liver tissue, each of the fluorescent antibodies stained distinctively the corresponding antigen in frozen sections fixed with ether-ethanol-acetone mixture. Specific fluorescence for LDH-H was observed in the surrounding portion of sinusoids, including the cytoplasms of Kupffer cells. On the other hand, LDH-M specific fluorescence was seen as fine granules in the cytoplasm of the hepatic cells, diffusely. This result corresponds well to the observation by Berg and Blix, who studied the LDH isozyme patterns in highly purified fraction of rat liver parenchymal and Kupffer cells (15). It was observed by disc electrophoresis that parenchymal cells contained only LDH-5 (4M) isozymes and that Kupffer cells contained LDH-2, -3, -4 and LDH-5. To obtain even more precise localization of each subunit, immuno-electron microscopic analyses are now in progress.

The authors are grateful to Mr. T. Matsushita for technical assistance and to members of the Department of Pathology, Chest Disease Research Institute, Kyoto University, for making various facilities available. Thanks are due Miss Kameda for typing the manuscript.

REFERENCES

1) Nisselbaum, J. S. and Bodansky, O. (1961) Reaction of human tissue lactic dehydrogenases with antisera to human heart and liver lactic dehydrogenases. *J. Biol. Chem.* **236**, 401–404
2) Kaplan, N. O. and White, S. (1963) Immunological properties of lactate dehydrogenase isozymes. *Ann. N. Y. Acad. Sci.* **103**, 835–848

3) Markert, C. L. and Appella, E. (1963) Immunochemical properties of lactate dehydrogenase isozymes. *Ann. N. Y. Acad. Sci.* **103**, 915–929
4) Pesche, A., Fondy, T. P., Stoltenbach, F., Castillo, F., and Kaplan, N. O. (1967) The comparative enzymology of lactic dehydrogenases. *J. Biol. Chem.* **242**, 2151–2167
5) Ito, M., Harada, T., Baba, M., and Morikawa, S. (1973) Immunohistochemical studies on lactate dehydrogenase subunits in normal bovine tissues. *Acta Histochem. Cytochem.* **6**, 183–192
6) Morikawa, S. (1967) Studies on alkaline and acid ribonucleases in mammalian tissues: Immunohistochemical localization and immunochemical properties. *J. Histochem. Cytochem.* **15**, 662–673
7) Morikawa, S. and Harada, T. (1969) Immunohistochemical localization of catalase in mammalian tissues. *J. Histochem. Cytochem.* **17**, 30–35
8) Yasuda, K. and Coons, A. H. (1966) Localization by immunofluorescence of amylase, trypsinogen and chymotrypsinogen in the acinar cells of the pig pancreas. *J. Histochem. Cytochem.* **14**, 303–313
9) Marshall, J. D., Evelund, W. C. and Smith, C. W. (1958) Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application. *Proc. Soc. Exp. Biol. Med.* **98**, 898–900
10) Sainte-Marie, G. (1962) A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* **10**, 250–256
11) Plegemann, P. G., Gregory, K. F. and Wróblewski, F. (1960) Distribution of lactic dehydrogenase in rabbit and human tissues. *J. Biol. Chem.* **235**, 2288–2293
12) Wróblewski, F. and Gregory, K. F. (1961) Lactic dehydrogenase isozymes and their distribution in normal tissues and plasma and in disease states. *Ann. N. Y. Acad. Sci.* **94**, 912–932
13) Agostoni, A., Vergani, C. and Villa, L. (1966) Intracellular distribution of the different forms of lactic dehydrogenase. *Nature* **209**, 1024–1025
14) Nemchinskaya, V. L., Ganelian, L. Sh. and Braun, A. D. (1968) Differences in isozyme patterns of nucleic and cytoplasmic lactate dehydrogenase of rat liver cells. *Nature* **217**, 251–252
15) Berg, T. and Blix, A. S. (1973) Distribution of lactate dehydrogenase isozymes in rat liver cells. *Nature: New Biol.* **245**, 239–240