IMMUNOHISTOCHEMICAL STUDIES ON LACTATE DEHYDROGENASE SUBUNITS IN NORMAL BOVINE TISSUES

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The distribution of lactate dehydrogenase (LDH) subunits was investigated in normal bovine tissues such as heart, kidney, liver, and skeletal muscle using fluorescent antibody technique. Fluorescence specific for LDH-H was located in the heart muscle fibers, in the cytoplasm and membrane of hepatic cells, and in the cytoplasm of epithelia of distal tubuli. Specific fluorescence of LDH-M was observed in the skeletal muscle fibers and in the cytoplasm of hepatic cells. Immunofluorescence was in accord with the results of histochemical localization by nitro blue-tetrazolium stain. Cold acetone was the best fixative among those examined. But it failed to locate LDH-M in renal tissue, and this observation was discussed.

Lactate dehydrogenase (LDH) is a tetramer composed of two types of subunits, H-subunit (LDH-H) and M-subunit (LDH-M). The subunits combine randomly to make five isozymes; 4H, 3H1M, 2H2M, 1H3M and 4M. These are numbered LD-1 to LD-5 respectively. It is well known that each subunit is quite different in their immunochemical property (7, 9, 16), electrophoretic mobility, chromatographic property, substrate specificity, heat stability and reaction to inhibitors (for review see Kaplan, reference 8). It is also revealed that the distribution of LDH isozymes is much variable in tissues or organs. For example, heart and kidney are rich in LD-1 and LD-2, muscle and liver in LD-4 and LD-5, and spleen, pancreas and adrenal gland in LD-3. The isozyme pattern of LDH in neoplastic tissue (5) or foetal tissue (4) is reported to be quite different from that of normal adult tissue. Attempt to localize the LDH-H and LDH-M subunit activities histochemically was began by Brody (2) and Brody and Engell (3) using the inhibitors such as urea, excess of lactate *etc.* In spite of the immunochemical difference between LDH-H and LDH-M, however, the immunohistochemical localization of each subunit has not been studied at all. Immunohistochemical method is expected to demonstrate the localization of each subunit separately in a same section. In the present work, the immunohistochemical localizations of LDH subunits have been studied by fluorescent antibody technique and were compared with the enzyme-histochemical localization of LDH.

MATERIALS AND METHODS

Preparation of antisera: Lactate dehydrogenase from bovine heart (LDH-4H) and lactate dehydrogenase from bovine muscle (LDH-4M) obtained from C. F. Boehringer and Soehne GmbH (Mannheim, Germany) were used as antigens without further purification.

Complete Freund's adjuvant (Difco Laboratories, Detroit) plus 2.5 mg of LDH-4H or 10 mg of LDH-4M was injected into the foot pads of male albino rabbits. One month later, following an intravenous injection of 2-(benzhydryl-oxy)-N,N-dimethylethylamine hydrochloride (Benadryl), a booster injection of antigen was given intravenously. The rabbit injected with LDH-4H was bled 7 days after the booster injection and the antiserum was collected. Another booster injection was given into the rabbit injected with LDH-4M, 2 weeks later. The animal was bled 7 days after the last injection and the antiserum was collected.

Preparation of fluorescein conjugates: Globulin fractions of the antisera were prepared by precipitating three times with one third saturated ammonium sulfate. The globulins were then conjugated by the method of Marshall, Evelund and Smith (10) with fluorescent isothiocyanate (FITC) (Baltimore Biological Laboratory, Inc. Baltimore). Following separation from free fluorescent dyes by gel filtration on Sephadex G-50 (Pharmacia, Uppsala) the conjugates were purified by fractionation on DEAE-cellulose (0.93 mEq/g, Brown Company, Berlin) columns. Fractions eluted at pH 7.0 with 0.03 and 0.1 M phosphate buffer was used for staining. After concentration with ethylene glycol (m. w. 6000) the conjugates were dialyzed against several changes of phosphate buffered saline (pH 7.1–7.2, PBS). If necessary, the conjugates were absorbed twice with mouse spleen acetone powder, so as to eliminate non-specific staining completely.

Assay for specificity of antisera: Specificity of antibody was examined by double gel diffusion method of Ouchterlony and by immunoelectrophoresis. Procedures were fully described in our previous paper (13).

Preparation of sections: Bovine heart muscle, kidney, liver and skeletal muscle were examined. Small piece of each fresh organ was put in a test tube and frozen at -70°C by immersing the test tube in an acetone-dryice mixture. Frozen sections ($4-5\ \mu$) were cut in a cryostat (International Equipment Company) at -20°C , placed on clean glass slides, allowed to dry for 15–30 min at room temperature and then fixed in one of the following fixatives: (1) Cold 95% ethanol for 30 min, (2) 95% ethanol containing 5% glacial acetic acid (acidified ethanol) for 30 min, (3) 10% formalin in PBS (phosphate-buffered formalin) for 30 min, (4) Formaldehyde gas for 30 min, and (5) Cold acetone for 10 min. The remainders of each sections were stained without fixation. Among them cold acetone gave the best results for localizing the LDH subunits. Acetone-fixed sections were used mainly for staining in this study.

Staining with FITC-labelled conjugates and microscopy: After washing with PBS, sections were stained with FITC-labelled conjugates in a moist chamber at room temperature overnight. These slides were then washed with PBS mounted in glycerol containing 10% PBS and examined under a Carl-Zeiss fluorescence microscope.

Enzyme-histochemical staining for LDH: The histochemical distribution of LDH was investigated by Mori's modified method using nitro blue-tetrazolium (Sigma Chemical Company, St. Louis) as reagent (12). Unfixed frozen sections were stained for 10–30 min in the incubating mixture composed of 3 ml of nitro blue-tetrazolium (5 mg), 4 ml of 1 M DL-sodium lactate solution, 2.5 mg of nicotinamide adenine dinucleotide, 2 ml of 0.1 N KCN and 11 ml of 0.1 M phosphate buffer (pH 7.0). The final pH of the incubation mixture was adjusted to 7.4.

RESULTS

Specificity of antisera

The antisera were tested for the presence of contaminating immune system and the cross-reactivities between the isozyme LDH-4H and LDH-4M by immunodiffusion and immunoelectrophoresis. As shown in Figure 1, anti LDH-H made only one wide precipitin band against LDH-4H and no precipitin band was observed between anti LDH-H and LDH-4M. Same result was obtained in the case of anti-LDH-M and the antigens, respectively (Fig. 2). Cross-reactivity between two subunits of LDH (LDH-H and LDH-M) was not observed in our immune system.

Immunohistochemical and enzyme-histochemical studies

Heart: LDH-H specific fluorescence was observed in the cytoplasm of heart muscle as fine fibrils distributed along the muscle fiber (Fig. 3). Almost all muscle fibers showed specific fluorescence for LDH-H. No specific fluorescence was observed in the nuclei of muscle cells and in the connective tissues. Anti-LDH-M conjugate could not stain any of the heart muscle sections. LDH activity located almost same pattern as the immunohistochemical localization of LDH-H in heart muscle as shown in Fig. 4.

Kidney: The cytoplasm of the cells of the distal tubules was stained diffusely with both anti-LDH-H conjugate and nitro blue-tetrazolium (Figs. 5 and 6). Epithelial cells of the proximal tubules and the cells of glomeruli remained unstained. Anti-LDH-M conjugate could not stain any tissues in the kidney.

Liver: Very feeble fluorescence specific for LDH-M was observed diffusely in the cytoplasm of hepatic cells in the frozen sections fixed with acetone. It was observed as a fine-meshed structure in cytoplasm. However, the fluorescence was too feeble to be demonstrated in a fluorescence microphotograph. The fluorescence specific for LDH-H was observed rather brightly along the cell membrane which faced bile canaliculi (Fig. 7). Some of the hepatic cells showed faint specific fluorescence of LDH-H in their cytoplasm, too. Almost all of the hepatic cells were stained diffusely in their cytoplasm with nitro blue-tetrazolium reagent histochemically (Fig. 8). However, no specific structure was observed in the histochemically stained sections. The epithelia of the bile ducts and Kupffer's cells were not stained either immunohistochemically or enzyme-histochemically.

Skeletal muscle: Fluorescence specific for LDH-M was observed in the cytoplasm of only small number of muscle fibers in the cross section of skeletal muscle (Fig. 9). It was not distributed diffusely in the cytoplasm, but bright fine fibril-

FIG. 1. Double gel diffusion study of LDH-5H and antisera. A wide precipitation band was observed between LDH-4H and anti-LDH-H antiserum. No cross-reactivity was seen between LDH-H and anti-LDH-M.

FIG. 2. Double gel diffusion study of LDH-4M and antisera.

FIG. 3. The immunofluorescence of LDH-H in the heart muscle. Specific fluorescence is shown along the muscle fibers.

FIG. 4. LDH activity in the heart muscle.

FIG. 5. The location of LDH-H in the kidney. Bright fluorescence is observed in the cytoplasm of epithelia of renal tubuli.

FIG. 6. LDH activity in the kidney. More deposit of products is observed as compared with the deposit of the fluorescence of LDH-H.

FIG. 7. The immunofluorescence of LDH-H in the liver. Bright fluorescence is observed at the membrane of hepatic cells and feeble fluorescence is observed diffusely in the cytoplasm of hepatic cells. The coarse white granules are nonspecific fluorescence.

FIG. 8. LDH activity in the liver.

FIG. 9. The immunofluorescence of LDH-M in the skeletal muscle. Bright fluorescence is demonstrated in the cytoplasm of muscle fibers.

FIG. 10. LDH activity in the skeletal muscle.

like structure was observed in the fluorescent myoplasm. The brightness of fluorescence was different in each muscle fiber. Skeletal muscle fiber was not stained with anti-LDH-H conjugate in any sections examined. Enzyme-histochemically, LDH-activity was shown in the myoplasm as fine fibers along the myofibrils (Fig. 10). The strength of staining was also different in each muscle fiber. All the muscle fibers were not stained with either anti-LDH-M conjugate or nitro blue-tetrazolium reagent.

DISCUSSION

Nisselbaum and Bodansky (16) reported that anti-human heart LDH rabbit antisera did not react with the LDH from human liver. Markert and Appella (9) and Kaplan and White (7) observed that there were two immunologically different chain components (A, B) in the five isozyme of bovine LDH and that they composed all three chain combinations (AA, AB, BB). It was also confirmed in this study using the purified LDH-4H and LDH-4M as antigens that LDH-H and LDH-M did not crossreact serologically. Therefore, the conjugates used separately show the location of either LDH-H or LDH-M.

Among the fixatives examined, cold acetone gave the most consistent results. However, there were some difficulties in demonstrating LDH-M in the hepatic cells and in the epithelial cells of renal tubuli, even by cold acetone fixation. According to the results of cytochemical analysis of LDH isozymes (1, 15, 17), LD-5 and LD-4, mainly composed of LDH-M, were fractionated in supernatant or soluble fractions of the liver and kidney homogenates. Such difficulty of fixation and different staining pattern due to soluble form of enzymes were observed in the cases of the immunofluorescence studies of catalase (14), lactoperoxidase (6), and xanthine oxidase (unpublished data, Harada and Morikawa). So, the feeble fluorescence of LDH-M in hepatic cells and no fluorescence in renal tissue may be ascribed to difficulty of fixation of frozen sections that contain the isozymes in soluble form.

The immunofluorescence of LDH-H and M was in accord well with the results of enzyme-histochemical staining except with the staining of LDH-M in renal tissue (11). After the resolution of the problem of fixation, the conjugate may be able to locate each subunits more specifically and easily as compared with the histochemical method using inhibitors.

Immunological cross-reactivity between bovine LDH and human LDH was observed in some articles (7, 16), and in our observation. So, the immunofluorescence of each subunit in human normal and neoplastic tissues will be the subjects of further investigation.

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