IMMUNOHISTOCHEMICAL STUDY OF CARDIOLIPIN AND PHOSPHATIDYLINOSITOL IN MOUSE LIVER

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UDC 612.351.1.017.1

The localization of phospholipid haptens (cardiolipin and phosphatidylinositol) in frozen and paraffin sections of mouse liver fixed in acetone and in an acetone-buffer-formalin mixture was studied by the indirect fluorescent antibodies method. Antiphospholipid sera specifically stained the plasma membranes of the hepatocytes, especially the region of the membrane facing the blood sinus. Detection of phospholipid haptens in liver sections with the aid of antiphospholipid sera depends on the method of obtaining and fixing the sections. Depending on the method of immunization, two types of antiphospholipid sera are obtained; they differ in their stability, in the possibility of isolating antibodies on lipid immunosorbents from them, and in their ability to stain liver sections.
KEY WORDS: immunofluorescence; cardiolipin; phosphatidylinositol; liver.

By immunohistochemical investigation of lipid haptens with the aid of monospecific antisera it is possible to determine the cellular and subcellular localization of individual lipid haptens in sections of organs and tissues, so that lipid haptens can be used as specific markers of morphological and functional cell differentiation.

Few immunohistochemical studies of lipid haptens have been published. The distribution of glycolipid haptens (globoside and ceramide lactose in human tissues and globoside in rat tissues [10], Gm_1-ganglioside in human tissues [5], and the phospholipid hapten cardiolipin in rat organs [9]) has been studied.

The aim of the present investigation was to obtain antiserum against two phospholipid haptens and to study the specific localization of these haptens on the hepatocyte membrane of the mouse liver. The work forms part of an investigation of the antigenic structure of the plasma membranes of mouse liver cells.

EXPERIMENTAL METHOD

Mouse liver cardiolipin (MLCL) was isolated by the method of Faure and Coulon–Morelec [6]. Commercial preparations of bovine heart cardiolipin (BHCL), bovine brain phosphatidylinositol (PI), and cholesterol and lecithin from egg yolk (from Sigma) also were used.

Antisera against phospholipid haptens were obtained by two methods. In the first method (type 1 antisera) rabbits were immunized with MLCL and BHCL intravenously on alternate days (10 injections) by the method of Inoue and Nojima [8]. A mixture of lipid haptens with accessory lipids (cholesterol and lecithin) was used for immunization. Methylated bovine serum albumin (MBSA) served as the carrier protein. The total load of hapten per rabbit was 3-5 mg. In the second method (type 2 antisera) the rabbits were immunized repeatedly with MLCL. The first immunization cycle was carried out by the method described above. Two months after the last injection the rabbits were reimmunized by injection of antigen mixed with Freund's complete adjuvant into the popliteal lymph nodes [7]. The immunization mixture was the same as that described in the first method. The total load of hapten per rabbit was 6-10 mg. Blood was taken from the immunized rabbits on the 7th-9th day after the last injection of antigen. The antisera were kept at −20°C. Rabbit antisera against bovine brain PI, obtained by the first method of immunization, were generously provided by M. V. Sitkovskii and I. K. Vardanyan. Antisera against mouse liver cell "ghosts" [1] also were used. Antibodies were isolated from the antisera against MLCL and the "anti-ghost" sera on an immunosorbent prepared from MLCL and BHCL by the method of Coulon–Morelec [4].

Laboratory of Immunochemistry and Diagnosis of Tumors, Oncologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Blokhin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 86, No. 9, pp. 332-335, September, 1978. Original article submitted January 10, 1978.

 0007-4888/78/8609-1206$07.50

Fig. 1. Paraffin sections of mouse liver fixed in ABF: a) treatment with type 1 anti-PI-serum; b) treatment with type 2 anti-CL-serum. Here and in Figs. 2 and 3, objective 40×, ocular, homal 3.

The cellular localization of the lipid haptens in mouse liver sections was studied with the aid of immune sera against lipid haptens by the indirect fluorescent antibodies method [3]. Two methods of obtaining the liver sections were used: a) from frozen tissue fragments in a cryostat [3], b) from fragments of liver embedded in paraffin wax [2]. Paraffin sections were fixed: a) in anhydrous acetone (Ac) and b) in a mixture of acetone with 0.03 M phosphate buffer, pH 6.1, and formalin (ABF) in the ratio of 45 : 25 : 30. The cryostat sections were fixed in acetone. The liver sections were incubated with antiphospholipid sera and with donkey serum against rabbit γ-globulin labeled with fluorescein isothiocyanate. As a control of the specificity of fluorescence, sera taken from rabbits before immunization, and also anti-CL and anti-PI-sera neutralized with homologous haptens and with heterologous lipids, were used.

EXPERIMENTAL RESULTS

All immune sera, both anti-CL and anti-PI, irrespective of the method of immunization, stained paraffin sections fixed in Ac and ABF. The pattern of fluorescence observed in the sections was the same for all immune sera (Fig. 1). Areas of plasma membranes of the hepatocytes facing the blood sinuses were brightly fluorescent. Areas of the membranes between two adjacent hepatocytes gave much weaker fluorescence. The cytoplasm of the cell stained faintly. Individual antisera stained the nuclei more brightly. Fluorescence of the Kuppfer cells and endothelium of the blood vessels was observed. Nonimmune sera did not stain paraffin sections of the liver after the same fixation.

On incubation of cryostat sections of mouse liver fixed in Ac with antisera against CL and PI a difference was found between the antisera obtained by different methods of immunization. The type 2 anti-CL-sera stained mainly the regions of the hepatocyte membranes opposite the blood sinuses in cryostat liver sections just as in paraffin sections (Fig. 2a). On incubation of cryostat sections with type 1 anti-CL and anti-PI-sera, fine and uniform fluorescence of the whole hepatocyte membrane was observed (Fig. 2b). This same type of fluorescence was observed if the cryostat sections were treated with sera of immunized animals.

To test the specificity of the fluorescence, antisera against MLCL were exhausted with the homologous hapten (MLCL) and also with cholesterol, lecithin, and MBSA, since lecithin and cholesterol were used as the accessory lipids and MBSA as the carrier protein during immunization of the rabbits with MLCL. Addition of cholesterol and lecithin in doses of 10 mg and of 0.025 ml of 0.5% MBSA to 0.025 ml of antiserum did not exhaust the anti-CL-serum, which continued to stain the liver sections. After addition of 3 mg MLCL to 0.025 ml of antiserum and treatment of the liver sections with adsorbed antiserum, no fluorescence was found.

Fig. 2. Cryostat sections through mouse liver fixed in Ac: a) treatment with type 2 anti-serum; b) treatment with type 1 anti-CL-serum.

Fig. 3. Cryostat sections through mouse liver fixed in Ac, treated with antibodies isolated on CL-immunosorbents from type 2 anti-CL-serum (a) and "antighost" serum (b).

The specificity of fluoresence of the anti-CL-sera was confirmed by treating the liver sections with antibodies eluted from immune sera with CL-immunosorbents. On incubation of antibodies isolated from type 2 anti-CL-sera on immunosorbents prepared from MLCL with paraffin and cryostat liver sections the pattern of fluorescence was the same as that observed in sections treated with native type 2 anti-CL-sera. Of all the structures, the sinus granules of the hepatocytes gave the brightest fluorescence (Fig. 3a). In the same way, paraffin and cryostat liver sections were stained by antibodies eluted from "anti-ghost" sera with CL-immunosorbents (Fig. 3b). These antibodies were active in the complement fixation test with MLCL. No antibodies could be isolated from type 1 anti-CL-sera on immunosorbents prepared from CL. Frac-

tions eluted from the immunosorbents did not stain mouse liver sections. The CL-immunosorbent was incubated with antiserum against mouse γ-globulin and anti-CL-serum incubated with immunosorbent prepared from mouse liver glycolipids. Fractions eluted from the immunosorbents did not stain mouse liver sections. These experiments confirmed the specificity of the CL-immunosorbents and the antibodies obtained from them.

A very important factor in the immunohistochemical study of lipid haptens is the method of obtaining the sections and the choice of reagents for their fixation. Differences in fluorescence between immune and nonimmune sera were found on paraffin sections fixed in Ac and ABF, for all antisera tested, and on cryostat sections fixed in Ac for the type 2 sera only. If a mixture of acetone and buffer (1:1) was used to fix the paraffin and cryostat sections, the fluorescence of all immune sera was indistinguishable from that of nonimmune. This fluorescence was probably due to the presence of normal antibodies in the nonimmune and immune sera. Recent investigations have shown that unimmunized animal and human sera contain antibodies against the glycoproteins of the cell membranes of erythrocytes and certain other tissues [11]. The pattern of fluorescence in liver sections when the same antiserum is used depends not only on the fixative, but also on the method of obtaining the sections. Type 1 antisera against phospholipid haptens in paraffin sections fixed in AC specifically stained liver cell membranes with the strongest fluorescence in the sinus regions of the membranes. The same antisera stained the whole hepatocyte membrane faintly and uniformly in cryostat liver sections fixed in Ac; the pattern of fluorescence, moreover, was the same as that obtained with nonimmune sera. Depending on the method of obtaining the sections and fixing the tissue, the same antiserum probably reveals different antigens in the sections.

Immunization of rabbits with phospholipid haptens by the two different methods yielded two types of antisera which differed from each other in various respects. According to the results of the complement fixation test, all antisera of both type 1 and type 2 tested by immunofluorescence contained antibodies against homologous haptens. Nevertheless, the type 1 antisera revealed lipid haptens in paraffin sections of the liver fixed in both Ac and ABF, but did not reveal them in cryostat liver sections fixed in Ac. Type 2 antisera reacted with lipid haptens in both paraffin and cryostat liver sections fixed in Ac. Antibodies against lipid haptens contained in these types of antisera also differed in their stability: in type 2 antisera the antibody titer was maintained for a long period (3-4 years), whereas the type 1 antisera were quickly inactivated (6 months). Specific antibodies were isolated from the type 2 antisera on lipid immunosorbents, and no antibodies could be obtained from the type 1 antisera. Antibodies were isolated on MLCL immunosorbents from "anti-ghost" antisera obtained by the same method of immunization as the type 2 antisera. "Anti-ghost" sera maintained their activity longer during keeping.

All the antiphospholipid sera, both anti-CL and anti-PI, which were investigated reacted with antigens in mouse liver sections to give the same pattern of fluorescence. In the complement fixation test all anti-CL-sera gave cross reactions with phosphatidylinositol and, conversely, all anti-PI-sera reacted with cardiolipsin; the same antibody titer, moreover, was found in some antisera when tested with both homologous and heterologous haptens. It can tentatively be suggested that the anti-CL- and anti-PI-sera in the immunofluorescence test revealed determinants in the liver sections common to these phospholipid haptens.

The authors are grateful to Professor G. I. Abelev for constant interest in the work and for valuable advice, to M. V. Sitkovskii (Laboratory of Physical Chemistry of Biomembranes, Moscow State University), and to K. V. Vardanyan (Laboratory of Transplantation of Organs and Tissues, Academy of Medical Sciences of the USSR) for generously providing the antisera and to O. A. Gavrilov for technical help with the experiments.

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