IMMUNOHISTOCHEMICAL CHARACTERISTICS OF INSOLUBLE ANTIGENS OF MOUSE LIVER CELLS

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In most investigations of the antigenic structure of normal and tumor cells, soluble cell antigens have been studied. As a rule, the insoluble antigens have not been investigated. However, they are of great interest, especially because they include components of the cell and intracellular membranes. Haughton and co-workers [2], for instance, who studied the antigenic composition of the insoluble cell fraction, found seven antigens associated with the shadows of cells of ascites tumors. One of these antigens (antigen 4) was an agglutinogen, i.e., it belonged to the cell membrane.

In the present investigation, the antigenic properties of the insoluble fraction of mouse liver cells (cell shadows) were studied by an immunohistochemical method.

EXPERIMENTAL METHOD

Cell shadows were obtained from the liver of noninbred mice by a method based on the successive extraction of the suspension of liver cells by solutions of increasing concentration [3]. After the whole cycle of extractions, 0.00001-0.00002 μg protein was determined in each cell shadow. Tests for soluble nucleic acids were negative. DNA was determined by Dische's methods [4] and RNA by Meibaum's method [7].

The preparations of the cell shadows thus obtained were used for immunizing rabbits. Initially, the antigen was injected with Freund's complete adjuvant subcutaneously (dose 200 million cell shadows), and the same dose was then injected twice without the adjuvant subcutaneously at intervals of 2 and 1 weeks. Blood was taken 1 week after the last injection, 3 times at weekly intervals. For revaccination, the antigen was injected intramuscularly and blood was taken on the 7th and 9th days.

By means of Coons' indirect method of fluorescent antibodies [6], the localization of the immune sera and the γ-globulin fractions obtained from the immune sera exhausted by soluble antigens was studied in liver sections and films of a suspension of the cells.

Usually the immune sera gave hardly any precipitation lines with soluble liver antigens. These sera were exhausted with a liver homogenate prepared in a mixture of Sorensen's buffer (pH 6.8) and physiological saline (1:1) or in distilled water (pH of homogenate 8 according to phenolphthalein). The homogenate was centrifuged for 1 h at 50,000 rpm, and the transparent centrifugate was used for exhaustion. The γ-globulin was precipitated from the exhausted sera with alcohol by Cohen's method [1] and purified by preparative electrophoresis in agar [1]. The γ-globulin, isolated from the exhausted sera, did not react in the precipitation reaction in agar with mouse serum and extracts of organs (liver, kidney, spleen, and lungs).

Nonimmune rabbit sera taken before immunization and the γ-globulin fractions obtained from them, made equal in concentration to the experimental γ-globulins, were used as controls.

The labeled serum consisted of antibodies against rabbit's γ-globulin, the serum of a donkey against rabbit γ-globulin, and was labeled with fluorescein isothiocyanate.

Before the sections themselves were stained, all the sera and γ-globulin fractions were adsorbed with powdered rabbit and mouse liver. Sections of the liver, 3 μ in thickness, were cut from blocks of tissue frozen to –70° in a cryostat (–16°). Before staining, the sections and cells were fixed in 96° ethyl alcohol (15 min) and in acetone (10 min) at 37°. Unfixed sections and cells were also used in the experiment. The liver sections were first treated with immune rabbit sera, and then with labeled donkey antibodies against rabbit γ-globulins.

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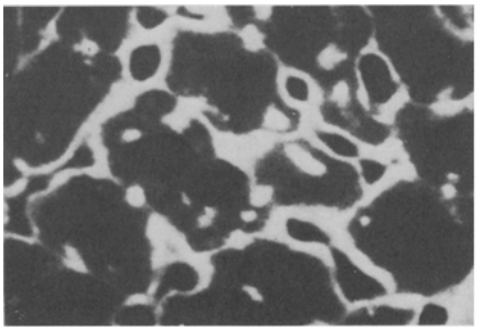

Fig. 1. Section of the liver fixed in acetone and treated with γ-globulins from an immune serum. Magnification 90 × 8.

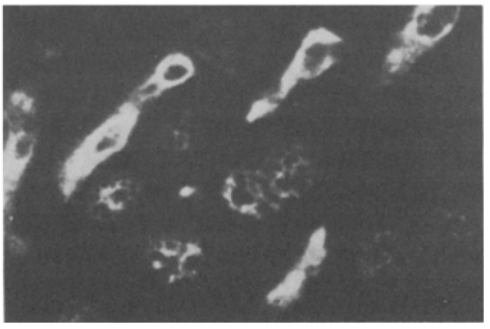

Fig. 2. Section of the liver fixed in alcohol and treated with γ-globulins from an immune serum. Magnification 90 × 8.

EXPERIMENTAL RESULTS

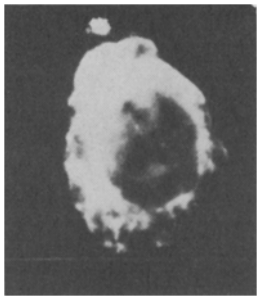

Fig. 3. Liver cell in an unfixed smear treated with γ-globulins from an immune serum. Magnification 90 × 8.

In the liver sections, a characteristic pattern of localization of the fluorescent antibodies was obtained. The specific distribution of the antigens was seen particularly clearly in sections fixed with acetone (Fig. 1).

It can be seen in Fig. 1 that every cell was outlined. The boundary between the parenchymatous cells was less bright than the boundary between the cells bordering on the capillary. The cross section of the bile capillaries was clearly fluorescent at the boundary between two parenchymatous cells. The longitudinal and transverse sections of the capillaries were clearly outlined, but the membranes of the Kupffer cells were not fluorescent. The cytoplasm of the cells was fluorescent, but much less intensely than the boundaries. Inclusions were distinctly visible in the cytoplasm, more especially near the nuclei, but sometimes also at the borders of the cells. The nuclei and the nuclear membranes were not fluorescent; on examination in the phase-contrast microscope, the nuclei were optically empty. The epithelium of the vessels was not fluorescent.

In the unfixed section, the pattern of staining was less clear than in those treated with acetone, but the general character of the fluorescence was the same. The cytoplasm was stained more brightly than in the section fixed with acetone, but the same contrast in the fluorescence of the cell boundaries bordering on the capillaries and between the parenchymatous cells themselves was not present. The nuclei and the epithelium of the cells were not fluorescent.

The pattern of fluorescence in the sections fixed with alcohol was of a different character (Fig. 2). The general intensity of fluorescence was much reduced. The cell borders fluoresced irregularly. The capillaries were well outlined, and the fluorescent membranes of the Kupffer cells could be seen in them. Hardly any fluorescence was observed in the cytoplasm. When examined in the phase-contrast microscope, the nuclei were full. The epithelium of the cells was fluorescent.

Sometimes during staining the fluorescence of the connective-tissue element (membranes of the Kupffer cells, endothelium of the blood vessels) was lost, and only the fluorescence of the nuclei and a feeble, chaotic fluorescence of the cytoplasm remained.

In unfixed sections, the control sera and γ-globulin fractions produced a diffuse background staining of the cytoplasm and borders of the cells, which were not stained in sections fixed with alcohol and which gave a feeble and irregular fluorescence in sections fixed with acetone.

To discover whether the fluorescence of the membrane of the parenchymatous cells belonged to the connective tissue, the sections were treated with collagenase and then stained by Coon's method. A glass slide with the sections was treated with 250 μg of the enzyme dissolved in Palitch's buffer (pH 7) [5]. The section was incubated for 4 h at room temperature (20-22°) in a moist chamber. The fluorescence of the membranes remained in the sections fixed in acetone and then treated with collagenase.

As a control of the action of collagenase, sections of the liver before and after incubation with the enzyme were fixed in acetone and impregnated with silver. In the untreated sections, the endothelium of the cells and

a loose reticular network were stained. After the action of collagenase, the connective-tissue elements were not impregnated with silver.

By obtaining a suspension of isolated parenchymatous cells from the liver and staining them with fluorescent antibodies, the localization of labeled antibodies could be studied in preparations freed as far as possible from connective-tissue elements.

The relationship between the intensity and character of fluorescence and the nature of the fixing agent was the same for the cells as for the sections of the liver. The unfixed cells gave a bright superficial fluorescence (Fig. 3), and the intensity of the fluorescence fell considerably after treatment with alcohol. In the acetone-treated preparations, the surface of the cells was fluorescent, and particularly clearly if the cells touched one another. Cells treated with the control sera did not show the specific fluorescence of the structure on the surface whatever the method of fixation.

The results of these experiments show that the membrane staining in the unfixed liver sections treated with acetone, presisting after the action of collagenase, and the surface fluorescence of the cells, both unfixed and fixed in acetone, belonged to the membrane antigens of the parenchymatous cells of the liver. The results of the experiment in which the preparations were fixed in alcohol and acetone showed that these antigens were evidently phospholipoproteins in nature, for they were extracted with alcohol but not with acetone.

Hence, the antibodies developing in rabbits after immunization with the insoluble "skeleton" of the liver cell are mainly directed against the antigens of the cell membranes. This provides a methodological basis for the analysis of the antigenic specificity of the cell surface*.

LITERATURE CITED

1. L. A. Zil'ber and G. I. Abelev, The Virology and Immunology of Cancer [in Russian], Moscow (1962), p. 198.
2. W. Boyle, D. A. Davies, and G. Haughton, Immunology, 6, 499 (1963).
3. N. C. Bruemmer and L. E. Thomas, Exp. Cell Res., 13, 103 (1957).
4. E. Chargaff and J. N. Davidson (ed.), The Nucleic Acids, Vol. 1, New York (1955), p. 301.
5. M. W. Clark, The Determination of Hydrogen Ions, Baltimore (1920).
6. A. H. Coons and M. H. Kaplan, J. Exp. Med., 91, 1 (1950).
7. W. Meibaum, Hoppe-Seylers Z. Physiol. Chem., 258, 117 (1939);

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