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DETECTION AND ISOLATION OF ANTIBODY-FORMING CLONES BY LOCAL CYTOLYSIS IN GEL

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To select clones of hybridomas producing monoclonal antibodies (MCA) of required specificity, many different methods have been suggested. However, all, as a rule, are based on testing culture fluid taken from wells in plastic plates, in which the hybridomas are grown. Cultures from positive wells are cloned in semisolid medium [4] or by the finite dilutions method [5], after which screening was repeated. Combining two stages – screening and cloning – into one would evidently result in considerable economies of time, medium, and plastic ware. Köhler and Milstein [6] suggested a method of local hemolysis in gel for detecting hybridoma clones forming antibodies against sheep's red blood cells. We have extended the scope of this method by conducting the reaction in a monolayer by the method suggested previously [2].

A variant of the method whereby nonerythrocytic targets can be used was described previously.

Hybridoma of strain NATF9.9 (F9), obtained by the writers previously and synthesizing IgM MCA against differential antigen Lyt-3.2 of mouse T lymphocytes [1] was used. This allele of the antigen (2nd) is represented on 80% of thymocytes and on some peripheral T cells of CBA and other lines of mice, but is absent in C58 and AKR mice. Line F9 was cultured in vitro for 2 months before the beginning of the experiment.

Before testing, the hybridoma cells and thymocytes were washed 3 times in medium 199 to remove protein, including MCA contained in the culture medium, by centrifugation at 100g for 5 min, then resuspended in medium without serum and kept until required in the experiment in an ice bath. The viability of the cells was estimated by determining incorporation of trypan blue in a Goryaev chamber. It was not less than 95%. Testing and subsequent culture were carried out in plastic Petri dishes 30 mm in diameter (Flow Laboratories, England). The dishes were washed to remove free PLL, and seeded with a suspension of hybridoma cells (10^3 per dish). After about 10 min, the suspension of thymocytes was added to the dishes. After adhesion of the cells the supernatant was drawn off and the mixed monolayer of hybridoma cells and thymocytes was covered with 0.5 ml of 0.3% agarose (from Calbiochem, USA) on medium RPMI-1640 (Flow Laboratories) with 20% fetal calf serum (N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR). After the agarose had solidified, a layer about 5 mm thick was formed. In the other variant, the target cells were added together with agarose. The dishes were incubated in a CO_2 incubator for 1, 2, or 3 h. Next, rabbit complement (from Cedarlane, Canada), diluted in Hanks' solution 1:15, was layered above the agarose and the dishes were incubated for a further 1 h. Zones of cell lysis around hybridoma cells producing MCA were detected by adding a 0.01% solution of the fluorescent dye ethidium bromide (from Serva, West Germany) in a dose of 10 μl to the dish 15 min berore the end of incubation [3]. The liquid above the agarose was drawn off and the dishes ex-

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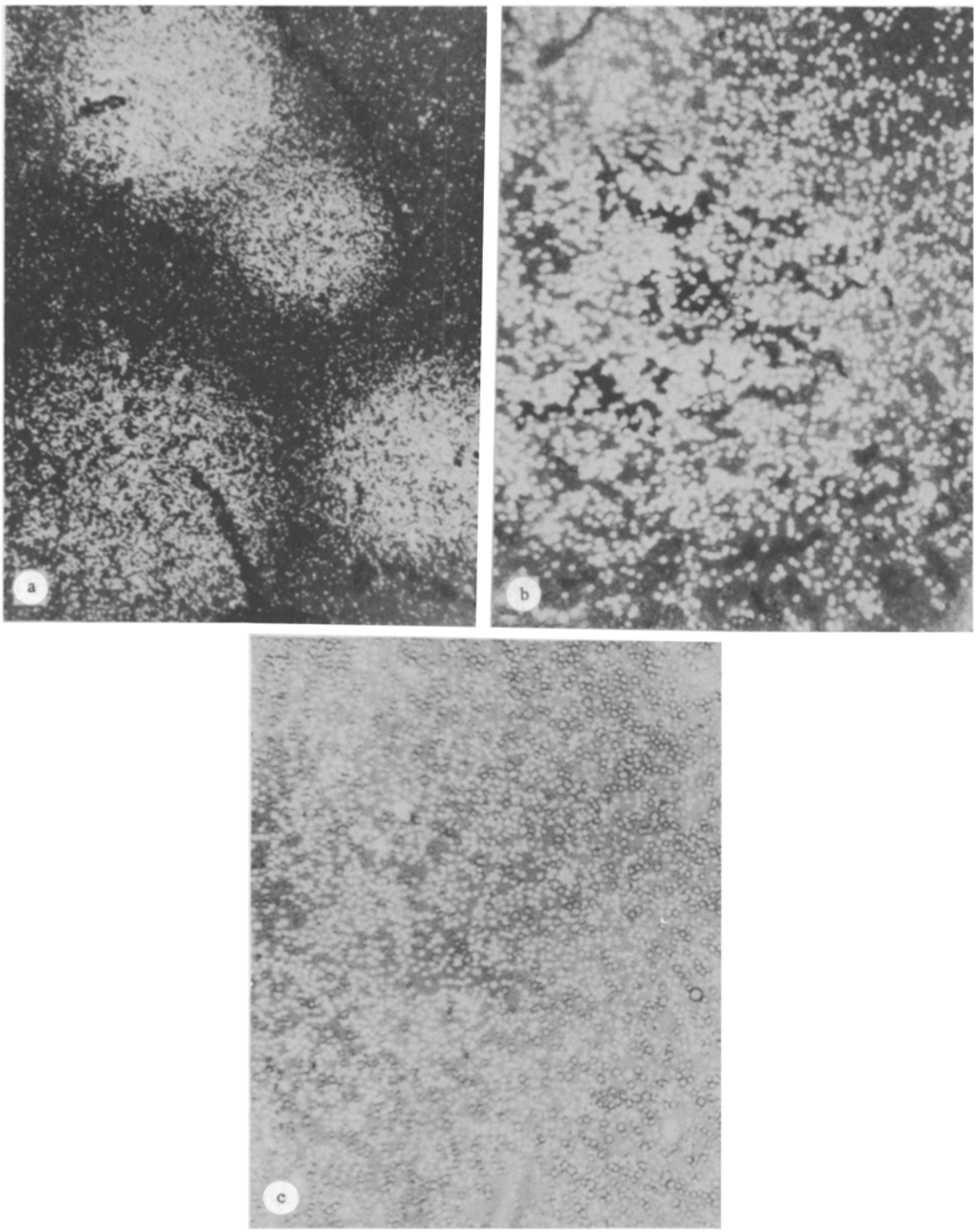

Fig. 1. Lysis of thymocytes from CBA mice by antibodies secreted by single cells of hybridoma F9. a) Fluorescent plaques under low power (Opton microscope, objective 4, ocular 7); b) fluorescence of plaque under magnification of 70; c) the same field of vision as in b, but with combination of fluorescence and phase contrast; single producing cell located in center of plaque, nonproducing cell at periphery (objective 10, ocular 7).

amined under the "Opton" luminescence microscope (West Germany) in blue-violet light. Single cells giving rise to plaques (zones of lysed thymocytes taking up the dye) were outlined with a sharp pointed needle on the outer side of the bottom of the dish. For further culture, 1.5 ml of RPMI-1640 culture medium with 20% fetal calf serum was added to the dishes, which were incubated for 10-13 days. In that time grew clones which could be isolated with a thin capillary tube, using an inverted microscope (Diavert, Leitz, West Germany). The isolated clones were propagated in wells of, initially, 96-well, and later, 24-well plates (from Linbro, England).

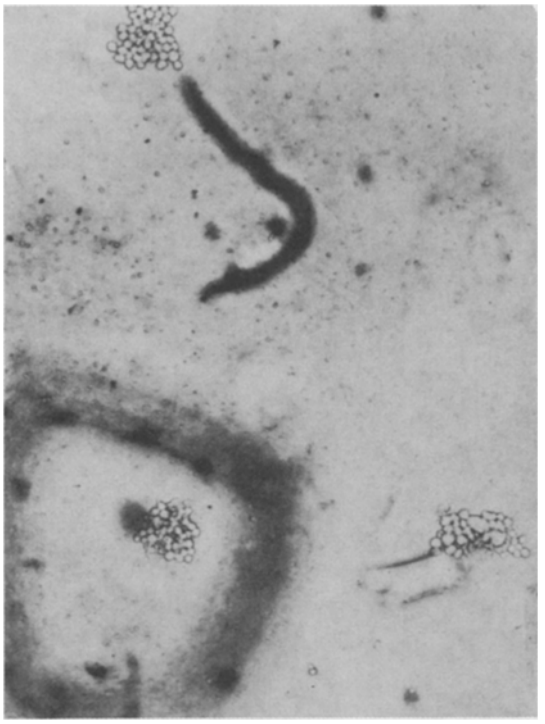

Fig. 2. Clones grown on positive (labeled) cell and from negative cells (7th day of culture). Diavert microscope, objective 4, ocular 7.

The results of testing single hybridoma cells against thymocytes of CBA mice with optimal parameters of the suggested technique are given in Fig. 1. Of the two variants of addition of target cells (to a monolayer with hybrid cells and into the body of the agarose above the cells) the first turned out to be best. Distinctness of outlines of the plaque, on which the sensitivity of the method depends, was much greater in this case and the background of dead cells was significantly lower, not more than 10%. The background is clearly visible in the photograph, in which fluorescence and phase contrast are combind (Fig. 1c).

In preliminary experiments the doses of hybridoma cells and of target cells were varied. The number of plaques in the field of vision and, subsequently, the possibility of isolating single cells depended on the number of hybridomas. Target cells had to be added in sufficient numbers to form a continuous monolayer or a dense suspension in the layer of gel. In our case doses of 10^3 hybridoma cells and $2 \cdot 10^7$ thymocytes per dish 30 mm in diameter were optimal.

A sufficient incubation time before addition of complement for our model is 1 h. After longer incubation (2 or 3 h) the plaques increased in size, and with the chosen concentration of hybridoma cells, they often merged.

Specificity of local cytolysis was demonstrated by substituting as the target cells thymocytes from C58 and AKR mice, which do not carry Lyt-3.2. Cells not giving plaques served as the internal control in the experimental dishes (Fig. 1c).

After a few days in culture clones of hybridoma cells grew (Fig. 2). Positive clones were transferred for further culture into 96-well, and later into 24-well plates.

The level of sensitivity of the method was determined by diluting target cells carrying Lyt-3.2 with cells not carrying this antigen. By mixing CBA thymocytes with C58 thymocytes in ratios of 1:1, 1:2, and 1:10 (Fig. 3), it was found that reducing the number of positive target cells led to a decrease in the degree of saturation of the plaque with lysed cells and reduced clarity of its boundaries. Even if 10% of all cells carried the antigen sought, a patch of lysis which differed from the background appeared (Fig. 3c).

The method described enables the operations of selections and cloning of hybridoma cells producing MCA against antigens of the cell membrane to be combined. As a model we chose the differential antigen Lyt-3.2 of T lymphocytes, but it is evident that practically any cells, normal or neoplastic, can be used as target cells. With this modification it is possible to detect hybridomas producing cytotoxic antibodies. Cytolysis, moreover,

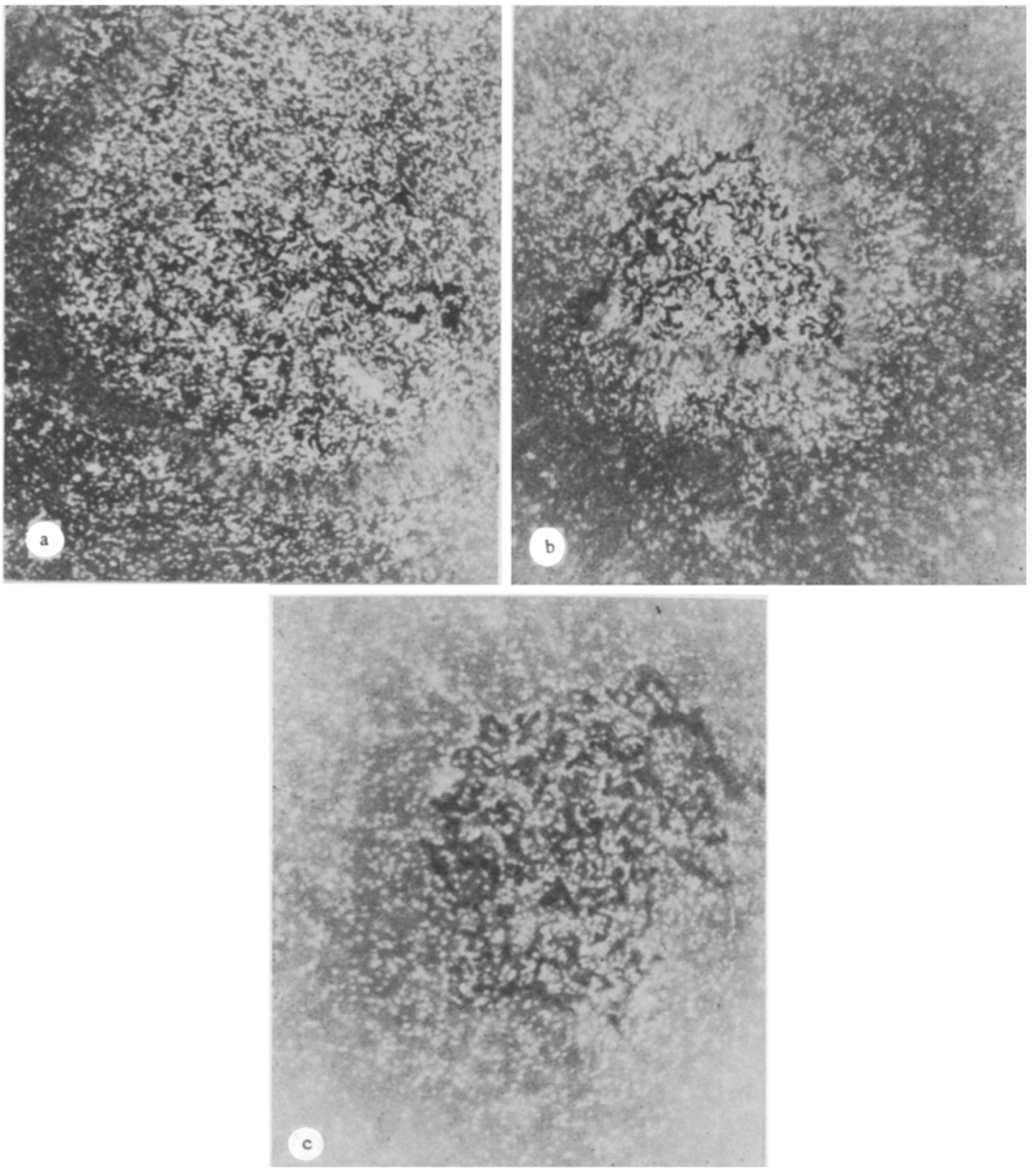

Fig. 3. Change in clarity of boundaries of plaque on dilution of thymocytes from CBA mice, expressing antigen Lyt-3.2 with thymocytes from C58 mice, not expressing that antigen. a) 100%, b) 50%, c) 10% of CBA thymocytes (Opton microscope, objective 4, ocular 7).

is highly reproducible and highly sensitive, so that secretion of MCA by a single hybridoma cell can be determined, and it is also specific (targets not containing Lyt-3.2 do not undergo lysis). In a study of differential antigens, those expressed by part of the cell population are particularly interesting. With our method it is also possible to discover subpopulations even if they do not account for more than 10% of all cells.

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PURIFICATION OF SPECIFIC RAT LIVER ESTROGEN-BINDING PROTEIN BY AFFINITY CHROMATOGRAPHY ON ESTRADIOL-SEPHAROSE

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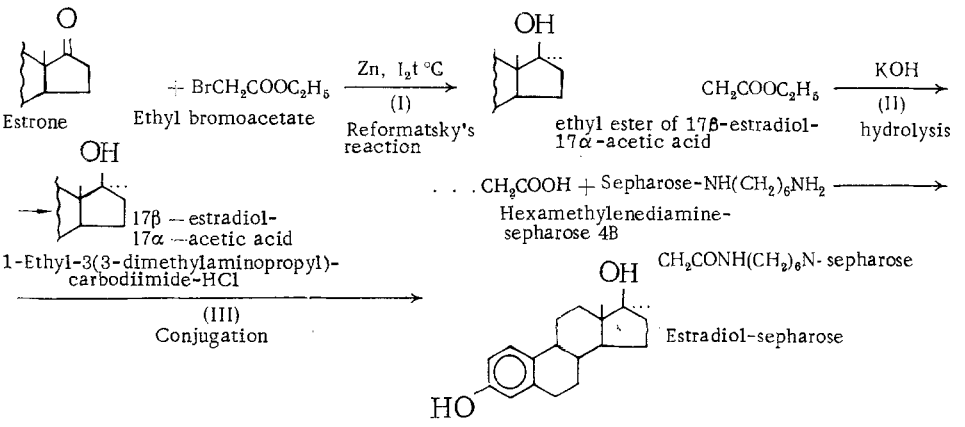
Specific estrogen-binding protein (SEBP) of rat liver differs essentially in certain properties from known receptors and transport proteins [1, 5, 7]. Besides aspects of regulation of the level of this sex-dependent protein in rats already known, new aspects of multifactorial control of determination and regulation of gonad-dependent processes have been discovered [2, 3, 8].

Purification of the SEBP, described in this paper, can be regarded, in particular, as an essential stage in the study of the as yet unknown physiological function of this protein.

EXPERIMENTAL METHOD

Experiments were carried out on sexually mature Wistar or noninbred rats weighing 150-300 g. The animals were decapitated. After perfusion with cold 0.9% NaCl solution the liver tissue from 15 to 20 animals was homogenized in 2 volumes of 10 mM Tris-HCl (pH 7.5) with 10 mM KCl and 1 mM EDTA buffer (TPE buffer), containing 6 mM dithiothreitol. The standard preparation of partially purified SEBP was obtained by precipitation of proteins with ammonium sulfate from the cytosol, gel-filtration on AcA 44 Ultrogel, and ion-exchange chromatography on DEAE-Sephadex A-50 [1]. Before further purification of the SEBP by affinity chromatography, two samples of it were pooled, one of which had previously been dialyzed against TEP-buffer and concentrated 20-30 times on a small (1 ml) column of DEAE-Sephadex A-60.

The stages of synthesis of the affinity sorbent estradiol-sepharose are illustrated in the scheme:



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