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METHOD OF DETECTING ANTIBODIES PRODUCED BY HYBRIDOMAS TO CELL NUCLEAR ENDONUCLEASES

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The study of endonucleases of cell nuclei has aroused increased interest in recent years. The use of various immunochemical and immunologic methods, and in particular, those based on hybridoma technology, may be an effective tool for such investigations.

However, considering the high heterogeneity of the nonhistone proteins of chromatin and the relatively low endonuclease content among them, the starting point for these developments must be the creation of a relatively simple but sensitive method of detection of antibodies produced against the corresponding enzymes.

In this paper one such method is described, whereby antibodies to cell nuclear endonucleases can be detected with high sensitivity and reproducibility in hybridoma culture medium.

EXPERIMENTAL METHOD

Extracts of cell nuclei containing solubilized endonucleases were obtained as follows. Nuclear residues were treated with 10 mM Tris-HCl, pH 7.4, in a volume giving a concentration of nuclear DNA of 2-4 mg/ml, and the samples were carefully suspended. Aliquots of the samples were treated with 3 volumes of 0.4 M KCl and 0.01% Triton X-100 and homogenized for 30 min in the cold. The samples were centrifuged at 12,000-15,000g for 10 min and the supernatants were used as the source of endonucleases.

Supercoiled DNA of plasmid pBR 322 was obtained by the method in [2] with minor modifications. Electrophoresis of the plasmid DNA was carried out in horizontal 1.2% agarose gels with a voltage of 1 V/cm. The conditions of photography of the gels and scanning of the negatives were described previously [1].

EXPERIMENTAL RESULTS

The method is based on the ability of endonucleases to convert closed circular DNA molecules into the open circle form and linear DNA. Since a single- or double-stranded cut is

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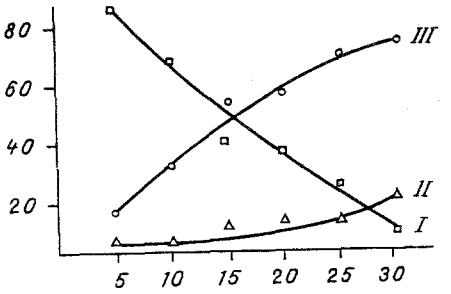

Fig. 1. Time course of degradation of supercoiled DNA of plasmid pBR 322 under the influence of cell nuclear extracts from splenic lymphocytes. Abscissa, incubation time (in min); ordinate, content of different forms of plasmid DNA (in %). I) Supercoiled DNA; II) linear DNA; III) open circle DNA.

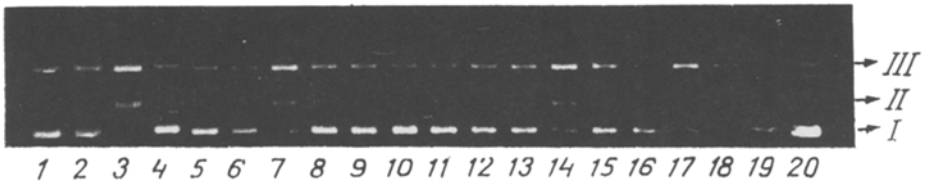

Fig. 2. Detection of antibodies to cell nuclear endo-DNases. Each channel from No. 1 to No. 19 corresponds to a sample with culture medium of a particular hybridoma. Channel No. 20 corresponds to the control preparation of the plasmid. The presence of antibodies to endo-DNases was revealed in channels Nos. 3, 7, 14, 17, and 18. Remainder of legend as to Fig. 1.

sufficient for the corresponding transition, and since the three above-mentioned forms of DNA can be readily separated in agarose gels in the form of three fixed bands, this approach, biochemically speaking, is the best for detecting endonuclease activity. The time course of accumulation of the open-circle and linear DNA of plasmid pBR 322 under the influence of extracts of cell nuclei of mouse splenic lymphocytes is illustrated in Fig. 1. A similar kinetics also is observed for extracts of cell nuclei of mouse thymocytes and partially purified Ca/Mg-dependent endonuclease of human splenic lymphocyte cell nuclei.

Under the influence of endonucleases contained in the extract, rapid degradation of the supercoiled form of pBR 322 DNA and its conversion into the open circle and linear forms, readily detectable by electrophoresis, thus takes place.

The method of detection of antibodies to these enzymes is based on fixation of the antibodies to plastic covered with protein A (Pharmacia, Sweden), subsequent binding of the endonucleases with the fixed antibodies, their incubation with plasmid DNA, and degradation products by electrophoresis. The method has been tested during preparation of hybridomas producing antibodies to Ca/Mg-dependent endonuclease from human splenic lymphocyte nuclei, and as a whole it consists of the following stages.

We used commercial 96-well plates for microtitration. Into each well 50 µl of a solution of protein A in a concentration of 5 µg/ml was introduced. The plates were incubated for 20 h at 0-4°C. At the end of incubation the plates were washed 3 times with distilled water. Plates with immobilized protein A were kept at 0-4°C for up to 3 months. Before use the plates were washed with 0.02 M K,Na-phosphate buffer, 0.15 M NaCl (PBS), pH 7.0. To each well was added 50 µl of a 2% solution of bovine serum albumin (fraction V, from Sigma, USA), and the samples were incubated at 4°C overnight. The plates were washed twice with distilled water and twice with PBS, each time for 4 min. Into each well 50 µl of PBS and 5-10 µl of culture media of antibody-producing hybridomas were introduced. The plates were incubated overnight at 4°C. They were then washed twice with PBS and twice with 10 mM Tris-HCl, pH 7.4. The duration of each washing was 4 min. Into each well 50 µl of extract containing endo-DNase (15-20 g protein) or from 2 to 5 µl of the purified enzyme (about 0.1 ng protein) was introduced and the plates were incubated for 7-10 h at 4°C. The wells were washed for 4 min each time: twice with 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM mercaptoethanol (IB solution). Next, 0.3 µg of supercoiled pBR 322 DNA in 50 µl of IB solution was added to the

wells and the plates were incubated for 2 h at 37°C. At the end of incubation 5 µl of 50% glycerol with bromphenol blue was added to the samples. The samples were applied to a horizontal block of 1.2% agarose measuring 17 × 0.4 × 20 cm. To increase the quantity of the samples to be analyzed at the same time, two or three parallel rows of nests were formed in the block.

The results of testing a series of primary clones of hybridomas obtained after immunization of mice with human splenocyte nuclear extracts containing endo-DNase activity, are given in Fig. 2.

Antibodies fixing endo-DNases and thus, when incubated, converting supercoiled pBR 322 DNA into open circle and linear DNA, were present in the culture media of some clones. The latter is particularly demonstrative, for it shows that, under the incubation conditions used, endo-DNases are sufficiently active to form not only nicks, but also double-stranded cuts.

Further culture of clones giving a positive response (according to the data in Fig. 2) also confirms that they do in fact produce antibodies to endo-DNases.

The method devised by the writers can thus be used for screening hybridomas producing antibodies to various endo-DNases, including to endo-DNases of human lymphocyte nuclei.

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