# DETERMINATION OF THE SPECIFICITY OF MONOCLONAL ANTIBODIES BY MIXED PRECIPITATION IN GEL

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UDC 616.153.962.4-097-078.733.4

KEY WORDS: precipitation; gel; monoclonal antibodies; antigen.

Detection and determination of the specificity of monoclonal antibodies (MA) are key tasks in the selection of hybridomas of given specificity, and also in the detailed study of MA themselves. To test MA by the solid-phase immunoenzyme method, which is usually used for these purposes, highly purified antigens are needed, and their degree of purity entirely determines the specificity of the test. Immunohistochemical methods of detection of MA in sections or in living cells likewise do not enable the identity of the antigens revealed by different MA to be judged, for different antigens may have a similar histological localization, and individual determinants of the same antigen may differ in the extent of their preservation when different fixing agents are used.

In the present investigation specific precipitates in gel were used as carriers of the antigen to determine the corresponding MA. In this way MA to a given antigen could be determined without the need to have it in the pure form, yet at the same time, with absolute specificity and very high sensitivity. Because of the simplicity of the method it can be used for mass screening of hybridomas. The method was developed on a system to α-fetoprotein (AFP).

## EXPERIMENTAL METHOD

The principle of the method is based on the formation of a mixed precipitate, consisting of antigen, MA, and polyclonal precipitating serum. Polyclonal rabbit antibodies and rat MA were used. Incorporation of MA into the precipitate was revealed with rabbit antibodies against rat IgC, conjugated with peroxidase.

Precipitating test systems for mouse AFP (MAFP), rat AFP (RAFP), and a commercial diagnostic immune serum against human AFP (HAFP), produced by the N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR, were used.

The test system consisted of monospecific rabbit antisera (AS) against AFP [3] and unpurified preparations of MAFP, RAFP, and HAFP (AG), forming a clear precipitation line in the zone of equivalence in agar.

As a control of specificity of incorporation, a test system for mouse serum albumin (MSA), consisting of rabbit monospecific antiserum against it (AS), obtained by the scheme of immunization described above [3], and the equivalent dilution of adult mouse serum (AG), was used.

Antibodies against rat IgG were isolated by means of an immunosorbent from rabbit antiserum against globulin fraction of rat serum (N. F. Gamaleya Institute of Experimental Medicine), previously exhausted by adult mouse serum to remove cross-reacting antibodies against mouse IgG, which excludes the demonstration of endogenous mouse IgG in liver sections. IgG isolated from adult rat serum by reprecipitation with ammonium sulfate and conjugated with CNBr-Sepharose 4B was used as the immunosorbent. Fab'-fragments were obtained from the isolated antibodies [8]. The Fab' preparation was conjugated with peroxidase (Sigma, USA) by the periodate method [10]. Activity of the resulting conjugate was tested for all culture media by the indirect immunoperoxidase method [9] on slices of regenerating mouse liver [5].

Culture media from five subclones of mouse-rat hybridomas, synthesizing rat MA against MAFP [6, 7], and culture media from two different subclones of mouse-rat hybridomas synthe-

Laboratory of Immunochemistry, Research Institute of Carcinogenesis, All-Union 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. Trapeznikov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 4, pp. 492-494, April, 1985. Original article submitted July 29, 1984.

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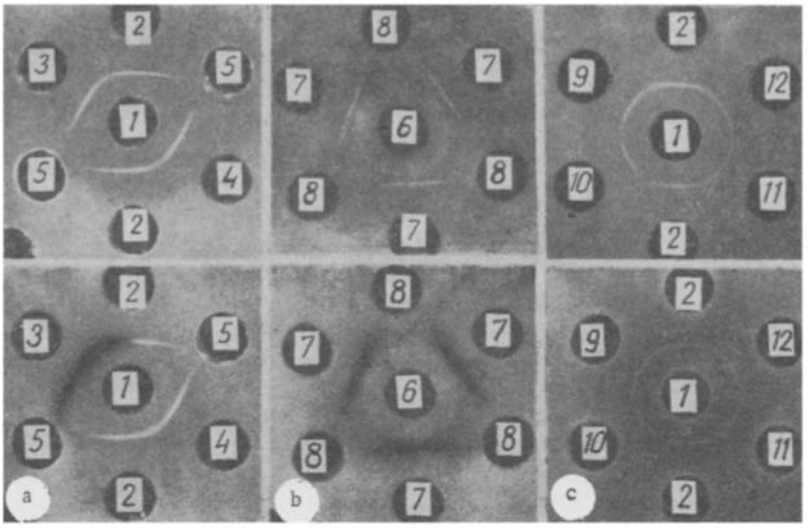

Fig. 1. Testing MA by the mixed precipitation in gel method. Top row — preparations before treatment with substrate, bottom row — after treatment. a) Reaction of MA with test system to MAFP: 1) monospecific polyvalent AS + Fab' conjugate, 2) AG of test system in dilution 1:2, 3) AG of test system + culture medium containing MA against MAFP, 4) AG of test system + culture medium containing MA against membrane antigen of mouse hepatocytes, 5) physiological saline; b, c) control for specificity of incorporation of MA into precipitate, 6) AS against MAFP + AS against MSA + conjugate, 7) AG of test system against MAFP + culture medium containing MA against MAFP, 8) AG of test system against MFA + culture medium containing MA against MAFP, 9-12) AG of test system against MAFP + adult rat serum in dilution of 1:2 to 1:16 respectively.

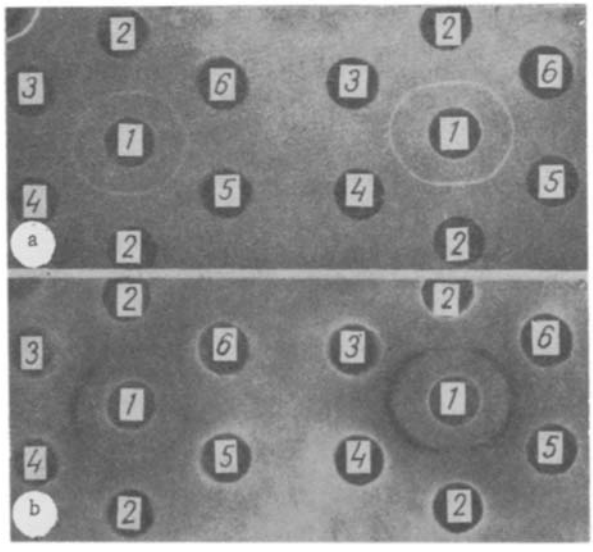

Fig. 2. Semiquantitative determination of MA in culture medium. Top and bottom rows — preparations before and after treatment with substrate respectively. 1 and 2) See Fig. 1a; 3-6) AG of test system to MAFP + culture medium containing MA against MAFP in dilution of 1:2 to 1:16; a, b) two different culture media containing different concentrations of MA against MAFP.

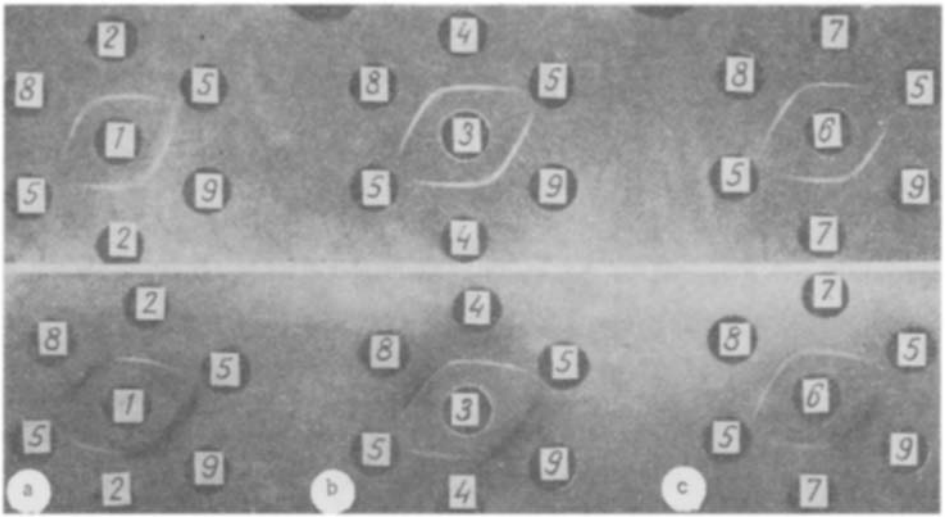


Fig. 3. Analysis of MA against MAFP with cross-reacting antigens (a — MAFP, b — RAFP, c — HAFP). Top and bottom rows show preparations before and after treatment with substrate respectively. 1, 2, 5) See Fig. 1a; 3) AG of test system for RAFP + Fab' conjugate, 4) AG of test system for RAFP in dilution of 1:2, 6) AS of test system for HAFP + Fab' conjugate, 7) AG of test system for HAFP in dilution of 1:2, 8) AG of corresponding test system + culture medium containing MA against MAFP, 9) AG of corresponding test system + culture medium containing MA against MAFP.

sizing rat MA against two membrane antigens of mouse hepatocytes [4] were tested. The precipitation test was carried out in 2% agar in physiological saline by the standard method [2].

To identify the MA, culture media of their dilutions were mixed in the ratio of 1:1 with AG of the test system, and peroxidase-labeled Fab' fragments were added in the same ratio to rabbit AT (the dilution of the labeled conjugate was chosen in preliminary experiments to give optimal staining of the precipate). In these mixtures AG and AS of the test system continued to give a clear reaction in the zone of equivalence. The mixtures prepared were introduced into appropriate wells (Figs. 1-3).

The agar plates were transferred to Kapron grids 18-20 h after incubation in a humid chamber and rinsed with physiological saline to remove nonreacting substances, the liquid being mixed for 15-20 h. The plates were then transferred to diaminobenzidine tetrachloride substrate (DAB, from Sigma, USA): 5 mg DAB to 10 ml of 0.05 M Tris-HCl buffer, pH 7.4, and 10 µl of 30% $H_2O_2$) for 15-30 min, rinsed with distilled water, photographed, and dried under filter paper. The stain remained stable in the dried preparations.

## EXPERIMENTAL RESULTS

MA against MAFP were specifically incorporated into the precipitate of the test system to MAFP (Fig. 1a) but were not incorporated into the precipitate of the test system for the foreign antigen. It will be clear from Fig. 1b that when a double test system was used (for MAFP and MSA) the stained reaction product was revealed specifically in the composition of the precipitate formed by MAFP and rabbit AS against it, whereas the albumin precipitate remained absolutely unstained (the inner precipitation band). With this arrangement the albumin precipitate was ring-shaped, since AT against albumin gave a reaction of complete immunologic identity both with AG of the test system for albumin and with the albumin contained in the MAFP preparation.

Rat MA were incorporated strictly specifically into the precipitate of the test system. Neither rat IgG (Fig. 1c) nor MA against surface antigens of mouse liver, which also are rat IgG, were incorporated into it.

Specific incorporation of MAFP into the precipitate was discovered in all five specimens of culture medium containing MA against MAFP. The samples of culture medium analyzed contained various concentrations of MA, as was clearly revealed by titrating them (Fig. 2).

All the culture media studied, including those against membrane antigens of mouse hepatocytes, revealed the the specific location of each antigen on the liver slices [4, 7] by the use of the conjugate obtained as described above in the indirect immunoperoxidase method.

The method of mixed precipitation in gel can not only identify MA in the reaction with homologous antigen, but it can be used to analyze particular MA for cross-reacting antigens. Incorporation of MA into precipitates of MAFP, RAFP, and HAFP is illustrated in Fig. 3. Under these circumstances it was shown that all MA against MAFP tested gave a cross-reaction with heterologous antigens (RAFP and HAFP). More intensive staining was observed in this case with the homologous precipitate, slightly weaker with RAFP, and weaker still with HAFP (Fig. 3). These results agree fully with data obtained previously on analysis of these same MA by the solid-phase immunoenzyme method [6].

The main advantages of the method are its high specificity and sensitivity for determination of MA, comparable with the sensitivity of the solid-phase immunoenzyme method.

To detect complexes formed by MA and antigen it is not necessary to obtain Fab'-fragments, for conjugates of whole molecules of antibodies against rat IgG with peroxidase can be used. In that case the reaction is carried out in two stages: The precipitate formed with incorporated MA after careful washing to remove soluble complexes is incubated for 18-20 h with conjugate poured into the central well in the gel, and treated with substrate after washing.

It is evident that IgG incorporated into the conjugate must not contain antibodies cross-reacting with AG of the test system. It is therefore unnecessary first to verify the test system with the conjugate, and should a color appear due to cross-reacting antibodies, the conjugate must be exhausted.

The method of mixed precipitation in gel described above can thus be used for simple and highly specific testing of hybridoma antibodies against any antigen precipitating in agar. In this case purified preparations of antigens are not required and 10-20 µl of culture medium is sufficient for analysis. The time taken to perform the test can evidently be considerably shortened by the use of more rapid methods of washing, for example, those described by Axelsen et al. [1].

The authors are grateful to Senior Scientist A. I. Gusev for constant interest in the work and to Senior Scientist T. D. Rudinskaya for useful discussions.

The work was partly subsidized by the Immunologic Division of the World Health Organization.

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