# MONOCLONIAL ANTIBODIES AGAINST MOUSE α-FETOPROTEIN

A. K. Yazova, A. I. Gusev, and O. M. Lezhneva

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α-fetoprotein (AFP) is a mammalian embryonic serum protein produced during normal ontogeny by embryonic hepatocytes and the entoderm of the yolk sac. Synthesis of AFP is resumed in hepatocellular carcinomas and germinal teratocarcinomas [1]. AFP is of great interest for research in developmental biology, in carcinogenesis as an estrogen-binding protein, as an immunodepressant, and in the diagnosis of cancer [2]. Determination of AFP in solutions and on sections, as well as its isolation, are carried out by immunologic methods, i. e., with the aid of monospecific antibodies against AFP. Preparation of monoclonal antibodies [8] against AFP, as an absolutely specific and standard reagent, is of great interest in connection with various immunomorphologic and immunochemical investigations, in the preparation of an immunosorbent for AFP isolation, and also for analysis of the antigenic structure of AFP at the submolecular level. Preparation of monoclonal antibodies against human AFP has recently been described [10, 12].

The object of this investigation was to obtain mouse–rat hybridomas producing antibodies against mouse AFP (MAFP).

## EXPERIMENTAL METHOD

A culture of mouse myeloma cells of line P3-NS1Ag4-1 (NS-1), synthesizing but not secretingmouse immunoglobulin (Ig) light chains κ, was generously provided by Dr. Milstein (England). The cells were cultured in DMEM medium (Flow Laboratories, England) with 10% bovine serum in the presence of 8-azaguanine.

Male August rats were immunized twice with a highly purified preparation of MAFP [4]. At the first injection each animal received 30 μg MAFP mixed with Freund's complete adjuvant into the hind footpads. Thirty days later, the rat with the most active primary immune response was chosen for the second injection (the sera were tested by double diffusion in agar with test systems for MAFP and rat AFP (RAFP) [5] and 60 μg MAFP without adjuvant was injected intravenously. The animals were killed after 3 days and blood and the spleens were removed.

Hybridization of spleen and myeloma cells was carried out by the method of Davidson and Gerald [7]. A residue of a mixture of spleen ($1 \cdot 10^8$) and myeloma ($1 \cdot 10^7$) cells, washed to remove serum proteins was obtained in a 100-$cm^3$ flat-bottomed flask, and incubated for 2 min with 3 ml 50% polyethylene-glycol (PEG, from "Schuchardt," mol. wt. 2000), after which the residue was thoroughly washed to remove PEG; the procedures of lowering the PEG and washing to remove it were carried out without agitation of the residue. After incubation of 1 h under a layer of 20 ml DMEM with 20% horse serum (HS, from Gibco, USA) the cell suspension was poured, 0.1 ml at a time, into wells in two 96-well Microplates (Falcon 3040 USA). The hybrid cells were cultured at 37°C in an atmosphere of 5% $CO_2$ during the first two weeks in selective medium (DMEM with 15% HS and with the addition of hypoxanthine, aminopterin, and thymidine), during the following week in the same medium but without aminopterin, and thereafter in DMEM with 15% HS. Colonies of hybrid cells, active as regards producing antibodies against MAFP (anti-MAFP) were cloned and then recloned with limiting dilution. In the first

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TABLE 1. Analysis of Determinant Specificity of Hybridoma Antibodies against MAFP by Solid-Phase IEM

| Hybridomas after recloning | Number of wells with clones | Reaction with AFP | | |
|---|---|---|---|---|
| | | MAFP | RAFP* | HAFP* |
| $C_{10}$ from $F_4$-10 | 27 | 27 | 27 | 1 |
| $E_2$ from $F_4$-10 | 3 | 3 | 3 | 0 |
| $A_{10}$ from $F_4$-1 | 13 | 13 | 11 | 1/11 |
| $F_9$ from $F_4$-1 | 4 | 4 | 4 | 0 |

*Purified preparations of RAFP and HAFP were isolated by the method of sorption-elution on CNBr-Sepharose 4B (from Pharmacia, Sweden), conjugated with purified rabbit antibodies against RAFP and HAFP respectively. For sorption on the wells solutions of AFP in a concentration of $\mu g/ml$ in physiological saline, buffered with phosphate buffer, pH 7.2, were used.

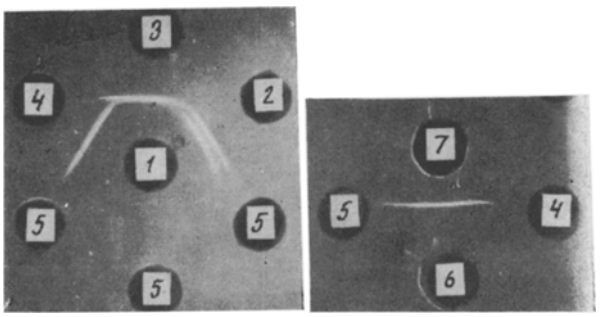


Fig. 1. Identification of nature and activity of hybridoma antibodies against MAFP: 1) rabbit antiserum against rat IgG; 2) adult rat serum; 3) purified preparation of rat IgG; 4) hybridoma anti-MAFP; 5) physiological saline; 6) rabbit monospecific serum against MAFP; 7) newborn mouse serum containing MAFP.

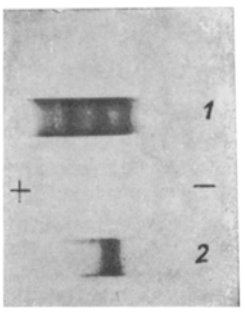


Fig. 2. Immunoisotachophoretic analysis of hybridoma antibodies against MAFP. Immunofixation of membranes after isotachophoresis carried out by method of printing on gel with rabbit antibodies against rat IgG. On application to agar, cathodal end of cellulose acetate membrane located on the right: 1) purified preparation of IgG from normal rat serum; 2) purified preparation of hybridoma antibodies against MAFP.

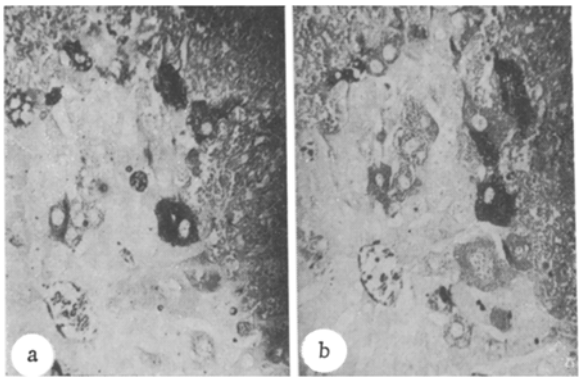


Fig. 3. Detection of MAFP in indirect immunoperoxidase method on mouse liver sections 72 h after poisoning with $CCl_4$. a) Incubation with eluate of hybridoma antibodies against MAFP; b) incubation with rabbit antibodies against MAFP. Magnification 120 ×.

case the seeding density was 10 and 1 cell, in the second case 1 and 0.5 cell per well. Peritoneal exudate cells from BALB/c mice were introduced into 96-well plates $5 \cdot 10^3$ cells into each well, 2-3 days before cloning. The clones were cultured in DMEM with 15% HS.

The culture media of the growing hybridomas were tested for their MAFP content by two methods. The first method was a qualitative version of the solid-phase immunoenzymic method (IEM) [11], in which the working surface of the wells from 96-well microplates (Dynatech microELISA) were incubated with a solution of MAFP in a concentration of 1 μg/ml. After removal of unbound MAFP, the test culture medium was applied. The attached rat IgG (anti-MAFP) were detected by rabbit antibodies against rat IgG, conjugated with peroxidase (C) by the method of Nakane and Kowai [9]. After addition of the substrate (S), namely 5-aminosalicylic acid mixed with hydrogen peroxide, specific coloring of the solution developed. All reagents were applied in a volume of 0.1 ml; the results of the test were read visually 1 h after additon of S. The positive control consisted of rat anti-MAFP-serum. The negative controls were as follows: MAFP + S; MAFP + C + S; MAFP + culture medium for hybridomas + C + S, MAFP + normal rabbit serum + C + S.

The second method was the indirect immunoperoxidase method on liver sections from SWR mice, regenerating after $CCl_4$ poisoning and containing MAFP-synthesizing hepatocytes. Because of the greater throughput of the IEM, all samples were tested in the IEM and some of them tested on sections.

## EXPERIMENTAL RESULTS

This paper gives the results of one experiment on hybridization of immune rat spleen cells and mouse myeloma cells. The serum of this rat reacted in the precipitation test with MAFP in a titer of 1:16 and with RAFP in a titer of 1:4. On the 10th day of culture colonies of hybrid cells were grown in 152 of 192 wells. On the 21st day, of the colonies which still remained, 22 from 130 wells were positive for anti-MAFP in the IEM (17%). On sections anti-MAFP were found in culture medium of 13 of 22 wells. Five hybrid cultures were selected for cloning. Culture $F_4$ was found to be most effective in cloning: on the 25th day of culture, in a microplate with seeding density of 10 cells per well ($F_4$-10), hybridomas grew in 34 of 96 wells, whereas with a seeding density of 1 cell per well ($F_4$-1), they grew in 14 of 96 wells. All 48 samples contained anti-MAFP according to the results of both methods of testing. It must be pointed out that the mixture of culture media from 48 wells with hybridomas reacted more actively, both in the IEM and on sections, than each sample separately. Microscopic observation on growth of these hybridomas showed that on the 13th day from 1 to 5 clones grew in the different wells. Of 48 hybridomas, five were selected for recloning: $C_{10}$, $E_2$, and $G_1$ from $F_4$-10 and $A_{10}$ and $F_9$ from $F_4$-1. All hybridomas were recloned in one microplate with a seeding density of 1 cell (48 wells) and 0.5 cell (48 wells) per well. One hybridoma died during recloning and no cells grew in any of the 96 wells. The growing clones of four recloned hybridomas continued to produce anti-MAFG, which were found on testing by

both methods. The results of analysis of the specificity of these antibodies with respect to MAFP, RAFP, and human AFP (HAFP), which have common antigenic determinants [6], are given in Table 1.

Of the 47 culture media 45 contained antibodies reacting both with MAFP and with RAFP, in two cases the antibodies reacted only with MAFP, and in two samples a weak reaction with HAFP was found. It can be tentatively suggested that either the initial $F_4$ culture contained several hybrid cells producing antibodies against different cross-reacting determinants of MAFP, or that clones secreting antibodies against one determinant, common for MAFP, RAFP, and HAFP, but characterized by different degrees of avidity relative to these antigens, were selected.

Hybridoma $A_4$ from $F_4$-10, grown from a microscopically distinguishable single clone, was propagated in three 96-well microplates with seeding densities of 10, 10, and 5 cells per well, respectively. After ten days rapid growth of multiple clones was observed in virtually all 288 wells; 93 of the 96 culture media of the microplate with seeding density of 5 cells per well were active in the IEM and 18 of 93 randomly chosen samples were positive also in the reaction on the sections. During culture, 100 ml culture medium was taken from all active wells, concentrated with PEG (from Serva, West Germany, mol. wt. 40,000) to 8 ml, and by sorption-elution on CNBr-Sepharose 4B, conjugated with 5 mg purified MAFP preparation, and the anti-MAFP eluate was obtained. It must be pointed out that the hybridoma culture medium after incubation with the sorbent still preserved its antibody activity. The anti-MAFP eluate was concentrated to 3 ml with the aid of PEG (mol. wt. 40,000). In the agar precipitation test antiserum against rat IgG reacted with the eluate so as to reveal, evidently, one of the IgG subclasses in it: The eluate formed one precipitation line, which merged into the line formed by an IgG preparation heterogeneous for subclasses and antibodies against it (Fig. 1a). In immunoisotachophoresis on cellulose acetate membranes [3] one homogeneous zone was found in the eluate, compared with heterogeneous polyclonal rat IgG (Fig. 2). The anti-MAFP eluate did not precipitate MAFP in agar, but it inhibited appreciably and specifically the precipitation line formed by MAFP and rabbit antibodies against MAFP, in that part of it facing the well containing eluate of hybridoma anti-MAFP (Fig. 1b). These results are evidence of the monoclonal nature of the antibodies isolated. In the IEM, the anti-MAFP eluate reacted in a dilution of 1:128 in the same way as the undiluated eluate: The reaction with MAFP and RAFP was practically equal in strenght, but that with HAFP was much weaker. In sections of regenerating mouse liver, anti-MAFP eluate in a dilution of 1:12 reacted with comparable intensity and with same cells as eluate of rabbit antibodies against MAFP (Fig. 3). An after-reaction was observed to a dilution of 1:256.

Consequently, monoclonal antibodies were obtained against one of the determinants of MAFP common to RAFP and HAFP also, specifically active in three immunologic reactions. Besides the opportunities for application of hybridoma antibodies which have been discovered in different fields of immunology, rat monoclonal IgG is of interest on its own account also as an immunogen for obtaining heterologous antisera against different subclasses of rat IgG.

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