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Monoclonal Antibodies to Different Epitopes of Human Alpha-fetoprotein (AFP)

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Abstract—*Monoclonal antibodies specific for human alpha-fetoprotein (AFP) were produced by the hybridoma technique. By using solid-phase immunofluorescence and radioimmunoassays, the antibodies were proven to distinguish 4 different epitopes on the AFP molecule.*

INTRODUCTION

ALPHA-FETOPROTEIN (AFP) is a glycoprotein with a molecular weight of about 70,000 daltons, which is normally expressed only in the fetal liver and yolk sac [1, 2]. Elevated serum AFP concentrations in adults have been shown to be useful in prenatal diagnosis of several fetal malformations and the diagnosis and follow-up of liver cancer and teratocarcinoma. The characterization of the biological role of human AFP is therefore of great practical interest.

Antibodies have been found to be valuable tools for demonstrating the concentration of different substances in biological fluids and separating them by affinity chromatography. They are also of importance in characterizing the antigenic structure of protein molecules and can therefore allow conclusions on the molecular composition and on the evolution of proteins.

The significance of antibodies increased considerably since the initiation of the hybridoma technique by Köhler and Milstein [3]. This technique allows the production of large quantities of homogenous monoclonal antibodies from malignant cell hybrids against single epitopes of an antigen molecule.

The present paper describes the production of monoclonal antibodies to human AFP which detect 4 different epitopes on the AFP molecule.

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MATERIALS AND METHODS

Antigen and immunization

Human alpha fetoprotein was purified from cord blood serum by preparative polyacrylamide gel electrophoresis and affinity chromatography using conventional rabbit anti-AFP antibodies [4, 5].

The antigenicity of the preparation was checked by immunodiffusion test using rabbit antisera against whole human serum (from the Institute of Hematology and Blood Transfusion, Moscow, U.S.S.R.) and monospecific antiserum against human AFP [6]. No impurities could be detected by immunodiffusion test and by analytical polyacrylamide gel electrophoresis.

For production of conventional mouse anti-AFP antiserum BALB/c female mice were immunized by intraperitoneal injection of 40 μg AFP in complete Freund's adjuvant and boosting 4 weeks later with the same quantity of AFP without adjuvant. For the production of monoclonal antibodies the boost injection was administered intravenously.

Hybridoma technique

The BALB/c myeloma line X 63-Ag 8.653 developed by Kearny *et al.* [7] was used for the experiments. Fusion of myeloma cells with spleen cells was performed 4 days after boost immunization by means of polyethylene glycol. Anti-AFP antibody-producing hybridomas were selected by antigen-labeled solid-phase radioimmunoassay (SRIA) and immunofluorescence assay (SIFA) [8].

Cloning of hybridomas was carried out by a limiting dilution technique.

Solid-phase immunofluorescence assay (SIFA)

The solid-phase immunofluorescence assay (SIFA) was performed according to previous publications [8–10]. Disks 0.7 mm in diameter were cut from SM 11307 cellulose nitrate membrane filters (Sartorius GmbH, Göttingen, F.R.G.) by a punch made from syringe needles. Incubations were performed in microtitration plates, washing was done with tap water.

After finishing the assay and transferring the cellulose nitrate disks to a measuring plate the fluorescence intensity of a defined area of the disks was measured with a microfluorometer. This consisted of a Fluoval 2 fluorescence microscope (VEB Carl Zeiss Jena, Jena, G.D.R.) combined with an MPV photometry device (Leitz GmbH, Wetzlar, F.R.G.).

Two combinations of SIFA were used in the experiments described here: an antigen-labeled SIFA and a sandwich SIFA. For an antigen-labeled SIFA 12 disks were incubated in the first step with 10 μl of a goat anti-mouse Ig (1 mg Ig fraction or purified antibodies per ml phosphate-buffered saline, PBS) and in the second step, after the 12 disks had been distributed to 12 single wells of the microtitration plate, with 10 μl of the hybridoma culture fluid or ascites fluid dilution. All dilutions were performed in PBS containing 10% fetal calf serum. In the third step the disks were incubated with 10 μl containing 50 ng FITC-labeled AFP (5 μg/ml). In a few experiments FITC-labeled human serum albumin (HSA) was used for comparison [8].

For the sandwich SIFA 12 disks were first incubated with 10 μl of the globulin fraction from anti-AFP hybridoma ascites fluid (10 mg protein per ml PBS) and then with 10 μl PBS containing 10% calf serum. After the 12 disks had been distributed to single microtitration plate wells they were incubated with 10 μl of either 1:20 diluted AFP-containing human serum (AFP standard serum, about 300 μg AFP/ml; Behringwerke AG, Marburg, F.R.G.) or 1:20 diluted normal human serum. In the last step the disks were incubated with 10 μl containing 1–2 μg (according to the non-specific staining) of FITC-labeled globulin fraction from anti-AFP hybridoma ascites fluid (100–200 μg/ml).

Labeling of antigens and antibodies with FITC was performed according to Gani *et al.* [11] using Na_3PO_4 to stabilize the alkaline pH.

Solid-phase radioimmunoassay (SRIA)

The solid-phase radioimmunoassay (SRIA) was performed by using polyvinyl chloride plates (pill blisters) as solid phase [8].

Three different types of SRIA were used in these experiments: an antigen-labeled SRIA, a competition SRIA and an antibody inhibition SRIA. For the antigen-labeled SRIA 1 μg of isolated goat anti-mouse Ig antibodies in 50 μg PBS were air-dried onto each well. The wells were then incubated with 50 μl hybridoma culture fluid or ascites fluid dilution and in the last step with ^{125}I-labeled AFP (1–2 ng AFP, corresponding to 40,000 counts/min).

To obtain information on the affinity of the monoclonal anti-AFP antibodies a competition radioimmunoassay was performed using the incubation sequence: solid-phase-attached anti-mouse Ig, anti-AFP antibody-containing culture fluid and in the last step a mixture of 25 μl ^{125}I-labeled AFP (0.5–1 ng; 20,000 counts/min) and 25 μl of different dilutions of an AFP-containing human serum (AFP standard serum from Behringwerke AG, Marburg, F.R.G.). The data of the dilutions showing competition were used to prepare a Scatchard plot. The amount of antibody-bound labeled antigen (in counts/min) divided by the amount of unbound free labeled antigen (in counts/min) was plotted vs the concentration of bound (labeled plus unlabeled) antigen. These data were used to calculate the binding constants.

For the antibody inhibition SRIA 1 μg of the Ig fraction from anti-AFP hybridoma ascites fluid (prepared by $(NH_4)_2SO_4$ precipitation) in 50 μl PBS or diluted unfractionated hybridoma ascites fluid were air-dried onto each well of the polyvinyl chloride solid phase. To saturate remaining free sites the wells were then incubated with PBS containing 10% calf serum. In the next step the wells were incubated with a mixture of 0.5–1 ng ^{125}I-labeled AFP (20,000 counts/min) in 25 μl PBS containing 10% calf serum and 25 μl diluted anti-AFP hybridoma ascites fluid or culture fluid. After washing, the single wells were cut out and measured in an NZ-310/A Gamma Counter (Gamma, Budapest, Hungary).

Iodination of AFP had been performed by the chloramine-T method according to Greenwood *et al.* [12].

Determination of the classes and subclasses of monoclonal anti-AFP antibodies

The classes and subclasses of monoclonal antibodies were determined by a solid-phase radioimmunoassay using the following incubation sequence: solid-phase-attached mouse Ig, class- or subclass-specific rabbit anti-mouse Ig (Miles Laboratories, Inc., Elkhard, MD, U.S.A.), monoclonal anti-AFP antibodies and ^{125}I-labeled AFP [13].

RESULTS

Reactivity of monoclonal anti-AFP antibodies in antigen-labeled SRIA and SIFA

After fusion of spleen cells from an AFP-immunized mouse with myeloma cells and distributing them to microtitration plate wells, the supernatants were screened for anti-AFP antibodies by antigen-labeled SRIA. Details of the cultivation and selection procedures will be described in another paper [14].

Out of the positive wells 6 hybridomas were cloned, recloned and subjected to multiplication in culture and growth as ascites (after pretreating the mice with pristane). Results of antigen-labeled SIFA showed the specificity of the hybridoma antibodies for AFP (Table 1). Culture fluids of the hybridoma clones designated 2, 9, 23, 32, 38 and 41 reacted with FITC-labeled AFP but not with FITC-labeled HSA. The results of experiments with a 7th hybridoma designated 31, which was selected because of slower growth in later experiments, are not included in the table.

The classes and subclasses of the monoclonal anti-AFP antibodies were determined by a solid-phase radioimmunoassay. All antibodies belonged to the IgG class with the following subclass specificities: IgG 2a for Mo 2, 9, 41 and 31, IgG 1 for Mo 23 and 38 and IgG 2 b for Mo 32 [13].

The relative antibody concentrations of the ascites fluids of the hybridomas were determined by titration in antigen-labeled SRIA and SIFA. The results of these experiments are shown in Fig. 1. The strongest reactivity was observed with ascites antibodies from clone 9. In SRIA positive results could still be obtained when diluted 10^{-9}, and in some cases even 10^{-12}. In general, monoclonal anti-AFP antibodies showed a stronger reactivity in SRIA than in SIFA. But hybridoma antibodies 23 and 41 reacted significantly stronger in SIFA than in SRIA.

The binding constants (in l/mol) obtained by competition solid-phase radiommunoassay were 1.2×10^7 for Mo 2, 3.4×10^8 for Mo 9, 1.6×10^7 for Mo 23, 8×10^7 for Mo 32, 7.3×10^7 for Mo 38, 1.5×10^8 for Mo 41 and 6.2×10^7 for Mo 31.

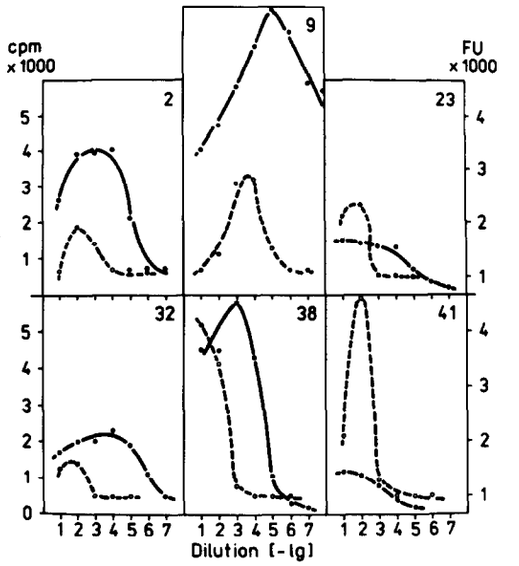

Fig. 1. Reaction of different monoclonal anti-AFP antibodies in antigen-labeled solid-phase radioimmunoassay (●——●) and immunofluorescence assay (O----O). Results are in counts/min and arbitrary fluorescence units (FU). Designation of the corresponding antibody in the right upper corner. Incubation steps: (1) goat anti-mouse Ig (isolated antibodies), (2) diluted monoclonal antibodies, (3) ^{125}I- or FITC-labeled AFP.

Characterization of the specificity of the monoclonal anti-AFP antibodies in two-site sandwich SIFA

To check which of the selected hybridoma anti-AFP antibodies reacted with the same epitope or antigenic determinant sandwich SIFAs were performed using each monoclonal antibody at the solid phase in combination with each FITC-labeled antibody. The difference was ascertained for each pairing between the values for AFP-containing human serum and normal human serum. In all cases no significant difference was obtained if the same antibody was used as solid-phase and labeled antibody. For comparison, this value was taken as 0% and the highest value for each group: solid-phase antibody/all FITC-labeled antibodies, was regarded as 100%. The relative reactivity of each pairing was determined according to these values. The results of the

Table 1. Results of the solid-phase immunofluorescence assays to verify the specificity of the monoclonal anti-AFP antibodies

Labeled antigen	Monoclonal antibodies*						
	2	9	23	32	38	41	Anti-HSA†
FITC-AFP	1900	3700	2900	1300	2600	3600	400
FITC-HSA	300	400	200	300	200	400	1600

Results are in arbitrary fluorescence units (FU; minus values of negative control).

*Culture fluid of the corresponding hybridomas. Incubation steps: goat anti-mouse Ig (Ig fraction), culture fluid, labeled antigen.

†Monoclonal anti-HSA (F8 B6) described in [8].

sandwich SIFAs are shown in Fig. 2 and Table 2. In addition to the non-reactivity observed if the corresponding antibody was combined with itself, some other combinations either yielded no reaction (2 and 9; 32 and 38) or only a weak reaction (23 and 41).

Characterization of the specificity of the monoclonal anti-AFP antibodies in antibody inhibition SRIA

To confirm the results of the sandwich SIFA, antibody inhibition SRIAs were performed which can also prove the reactivity of antibodies with identical epitopes.

As in the sandwich SIFA, each monoclonal anti-AFP antibody was tested when bound onto the solid phase in combination with each antibody in solution. The strongest inhibition resulting in the lowest binding of ^{125}I-labeled AFP by the solid-phase antibody was generally found in the pairing of the same antibody at the solid phase with itself in solution. The lowest value was taken as 0% and the value of ^{125}I-AFP and binding without antibody as 100%. The relative reactivity of each pairing was determined according to these values, as in the preceding assay. Different combinations were used in several tests with globulin fractions or diluted ascites fluid bound to the solid-phase or diluted ascites or culture fluid used as inhibitory antibody. The trend was the same, as has been found in the experiments shown in Fig. 3 and Table 2. If globulin fractions (1 μg per well) from clones 23 and 41 were used as solid-phase antibodies, no binding of ^{125}I-labeled AFP was observed. By using diluted ascites of clones 23 and 41, significant binding was observed in SRIA which enabled the performance of inhibition assays. As in the sandwich SIFA, the inhibition SRIA showed an identical reactivity of antibodies 2 and 9 and 32 and 38. Some degree of cross-inhibition was observed in the pairing 23 and 41, and also if antibodies 23 and 32 were bound to the solid phase and antibody 9 (in the first case also 2) was used for inhibition.

No inhibition was found by monoclonal anti-HSA antibodies (results not shown).

DISCUSSION

The characterization of the reactivity of different monoclonal antibodies against human AFP was performed by two different immunoassays. Altogether seven independent clones were selected. Experiments for the characterization of six hybridoma antibodies were shown in detail.

Several tests showed the specificity of the antibodies for AFP. Since HSA impurities are often found in different serum protein preparations and AFP and HSA show some degree of sequence homology [15], special attention was given to a possible cross-reaction of the monoclonal antibodies with HSA. Such a possibility could be excluded since no reaction had been found with FITC-labeled HSA, and anti-HSA antibodies had no effect in antibody inhibition tests. Since in the sandwich SIFAs

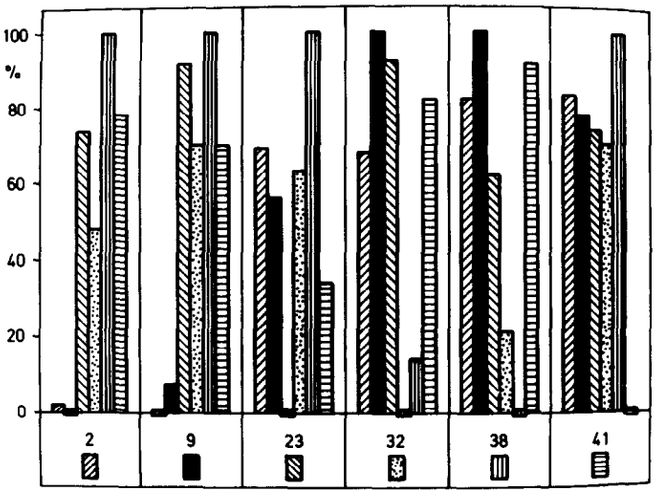

Fig. 2. Results of the two-site sandwich solid-phase immunofluorescence assays for checking the epitope specificity of monoclonal anti-AFP antibodies. Incubation steps: (1) monoclonal anti-AFP antibody (Ig fraction from ascites fluid; plotted on the abscissa); (2) diluted AFP-containing normal human serum; (3) FITC-labeled anti-AFP antibody (represented by the different columns, marked as indicated under the solid phase antibody). The highest FU values of the difference: AFP-containing serum minus normal serum for the corresponding solid-phase antibody combined with each FITC-labeled antibody was taken as 100% (for antibody pairing 2/38: 2700 FU; 9/38: 4400 FU; 23/38: 3200 FU; 32/9: 2200; 38/9: 3400; 41/38: 2300 FU), the lowest value being regarded as 0%. The other values were calculated as a percentage of the maximum value.

Table 2. Summary of the results of the sandwich immunofluorescence and inhibition radioimmunoassays

Solid-phase antibody	FITC-labeled or inhibitory antibody*					
	2	9	23	32	38	41
2	3 ± 14† 12‡	0 ± 7 0	74 ± 26 >100	48 ± 30 >100	100 ± 26 >100	78 ± 22 >100
9	0 ± 2 20	7 ± 2 0	91 ± 68 77	70 ± 16 90	100 ± 41 74	70 ± 21 70
23	69 ± 19 38	56 ± 22 24	0 ± 31 0	63 ± 22 91	100 ± 28 >100	34 ± 6 16
32	68 ± 45 >100	100 ± 32 26	91 ± 27 >100	0 ± 14 0	14 ± 5 5	82 ± 23 93
38	82 ± 38 >100	100 ± 32 78	65 ± 12 >100	21 ± 3 0	0 ± 12 19	91 ± 15 >100
41	83 ± 35 91	78 ± 17 55	74 ± 39 34	70 ± 17 84	100 ± 30 68	0 ± 4 0

*Values <40% of the maximum fluorescence or radioactivity set off.

†Upper line: immunofluorescence assay results in percentage of maximum fluorescence value; means and standard deviations of 4 independent experiments.

‡Lower line: radioimmunoassay results in percentage of value of positive control (without inhibitory antibodies); values of 1 representative experiment.

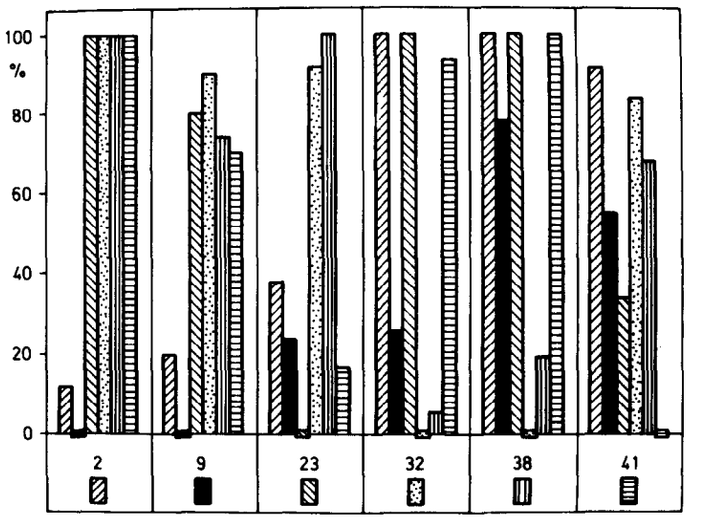

Fig. 3. Results of the antibody-inhibition solid-phase radioimmunoassays for checking the epitope specificity of monoclonal anti-AFP antibodies. Incubation steps: (1) *monoclonal anti-AFP antibody (antibody 2: globulin fraction from ascites fluid; 9: ascites fluid* 10^{-3} *diluted; 23, 32, 38 and 41: ascites fluid* 1:50 *diluted; plotted at the abscissa);* (2) 10% *calf serum;* (3) *a mixture of* ^{125}I*-labeled AFP and monoclonal anti-AFP antibody (hybridoma culture fluid* 1:50 *diluted; represented by the different columns, marked as indicated under the solid phase antibody). The values of the reactivity of solid-phase antibody with* ^{125}I*-labeled AFP without inhibiting antibody were taken as* 100% *(for antibody* 2: 1330 counts/min; 9: 5126 counts/min; 23: 1775 counts/min; 32: 3182 counts/min; 38: 2446 counts/min; 41: 1781 counts/min), *the strongest antibody inhibition being regarded as* 0%. *The values for the other antibodies were calculated as a percentage in relation to the* 100 *and* 0%.

normal human serum was taken as a negative reference antigen and each antibody showed a strong reactivity in at least one combination with AFP-containing serum, the AFP-specificity can be regarded as proven.

By comparing the titration curves of the different monoclonal anti-AFP antibodies, striking differences were observed in the reactivity which seems to be inherent for the corresponding hybridoma. These differences are obviously both

of a qualitative and quantitative nature; i.e. the antibodies have different affinities and the hybridomas produce different amounts of antibodies. Although the method used for the determination of the binding constants has several disadvantages (no free movability of the reaction partners; the possibility that anti-AFP antibodies dissociate from the solid-phase-attached anti-mouse Ig; binding of only a small portion of ^{125}I-labeled AFP by the solid phase antibodies), the data obtained allow a comparison of the different antibodies in relation to each other. According to these data, Mo 9 had the highest affinity and Mo 2 the lowest one. Antibodies 23 and 41 showed in general only a weak binding of ^{125}I-labeled AFP but a pronounced binding of FITC-labeled AFP. The same phenomenon was observed with a monoclonal anti-HSA antibody [8, 16]. It was concluded that the antibodies reacted with an epitope which either contained tyrosine or was sterically hindered by the iodine labeling. The latter seemed to be more probable because different batches of ^{125}I-labeled AFP showed different binding intensities to Mo 23 and Mo 41. Relatively strong binding was observed with the batch used for the determination of the binding constants. The high value obtained for Mo 41 seems to be coincidental with the strong binding of FITC-labeled AFP.

The combination of two-site sandwich and antibody-inhibition solid-phase immunoassays showed that the monoclonal antibodies detected 4 different epitopes on the AFP molecule designated a, b, c and d (Table 3). Similar assays have already been used to characterize monoclonal antibodies to AFP and other proteins [17, 18]. The variations observed in our sandwich immunofluorescence assays were very great, so that definite conclusions based only on these tests could hardly be drawn. However, when combined with the antibody-inhibition radioimmunoassays the definition of 4 epitopes seems to be reliable.

According to the data from both tests, epitopes c and d obviously show some degree of overlapping. This result cannot be interpreted as due to the different affinities of antibodies 23 and 41 since in that case the inhibition would work only in one direction.

Table 3. Epitopes on the AFP molecule detected by seven monoclonal antibodies

Epitope	Monoclonal antibodies
a	2, 9 (5)*, 31†
b	32, 38
c	23
d	41

*5 in parentheses is a subclone of 9.
†Experiments with the independent clone 31 were not quoted in the other tables and in the figures.

Some degree of inhibition was also observed by antibodies to epitope a on the binding of labeled AFP to antibodies reactive with epitope b and c. In this case the different affinities seem to play a role at least in the combination Mo 9 as inhibitor and Mo 23 and Mo 32 at the solid phase. A definite answer can, however, be given only in further tests using antibodies standardized for identical concentrations and taking into consideration the probably different affinities of the antibodies when in solution or fixed at the solid phase.

The availability of antibodies to different epitopes of AFP allows the performance of several evolutionary studies. It should be possible to check whether AFPs from different human races and at different ontogenic stages differ in their antigenic structure.

The AFPs of different mammalian species show a high degree of cross-reactivity [19, 20]. Monoclonal antibodies should enable clarification of which epitope(s) is present in most mammalian species and is therefore highly conserved. Experiments in this direction are in progress.

The antibody-inhibition solid-phase radioimmunoassay performed in these experiments can be used for the selection of antibodies to other epitopes of the AFP molecule and of antibodies with higher affinity. The sensitivity is high enough that diluted hybridoma culture fluids give pronounced inhibition values. Since the AFP molecule was described to carry at least seven epitope areas [17], it would also be useful to compare our monoclonal antibodies with the monoclonal anti-AFP antibodies already selected by other groups [16, 17, 21–24]. The detection of monoclonal antibodies to prominent epitopes is especially important for the development of a sensitive immunoassay which should be of practical value for the demonstration of AFP in human sera. First experiments with antigen-competition solid-phase radioimmunoassays using antibody 9 at the solid phase and ^{125}I-labeled AFP showed a sensitivity of up to 10 ng AFP/ml (results not shown). A better standardization is, however, necessary since variations of the results of tests performed on different days were considerably high. The search for an optimum assay variant is therefore still a main task of our present work.

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