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A sensitive blotting system for detection of α -fetoprotein variants with monoclonal and polyclonal antibodies

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Human α -fetoprotein (AFP) variants from cord sera were separated by isoelectric focusing in agarose gels under native conditions, transferred to nitrocellulose paper and detected with polyclonal and monoclonal antibodies (Moabs). Rabbit anti-AFP recognized up to 9 individual electrophoretic variants in the range of pH 4.5 to pH 5.2. The reactivity of 8 Moabs ranged from weak to strong and showed variability in the pattern of AFP bands recognized. Moabs were separated into 3 groups according to the number of bands detected: group 1 detected 6 to 7 bands; group 2 recognized only one band; and group 3 recognized 4 bands. The sensitivity of the system with polyclonal antibodies was 0.15 ng of AFP in complete cord serum and varied between 300 and 0.2 ng with Moabs.

Key words: α -Fetoprotein; Electrophoretic variant; Isoelectric focusing; Immunoblotting; Monoclonal antibody

Introduction

Human α -fetoprotein (AFP) is a major fetal serum glycoprotein of M_r 70 000, synthesized mainly by the yolk sac and fetal liver (Glitin, 1975). After birth the quantity of AFP decreases until it reaches from 0 to 15 ng/ml in normal adult serum (MacDonald and Kelly, 1978). In a number of neoplastic processes of the liver and germinal cells, the synthesis and secretion of AFP may increase up to several milligrams per milliliter of serum, therefore, quantification of this protein

in biological fluids has been useful in detecting and monitoring primary liver cancer and teratocarcinomas (Ruoslahti and Seppala, 1979). However, the significant rise of AFP levels in some patients with benign liver diseases (Chen and Sung, 1979) has limited the value of AFP quantification as an independent specific test to establish the diagnosis of cancer (Taketa et al., 1983).

Like other glycoproteins, AFP has been shown to be heterogeneous by a variety of techniques (Alpert et al., 1972; Lester et al., 1976; Kerckaert et al., 1979). Recent investigations have demonstrated that lectin binding to molecular variants of AFP might be associated with different pathological processes (Aoyagi et al., 1984; Buamah et al., 1984; Ishiguro et al., 1985; Taketa et al., 1985a, b). In this respect, detection of AFP variants might be valuable, since it could be used to dissociate malignant from non-malignant disorders of the liver (Aoyagi et al., 1984).

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Abbreviations: AFP, human α -fetoprotein; Moabs, monoclonal antibodies; IEF, isoelectric focusing; NP, nitrocellulose paper; NBT, *p*-nitro blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; PBS, phosphate-buffered saline.

In the present study, we describe a specific and sensitive system for the detection of AFP electrophoretic variants in their native form, by a combination of isoelectric focusing (IEF) in agarose gels, blotting and immunological detection with polyclonal and monoclonal antibodies.

Materials and methods

Chemical and biological reagents

Agarose (IEF), ampholytes (Pharmalyte) and CNBr-activated Sepharose-4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden); ampholytes (Bio-Lyte), nitrocellulose paper, *p*-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Bio-Rad (Richmond, CA); diethanolamine, Tween 20, sorbitol and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO).

Human cord sera were obtained from children born at normal term pregnancies at a local hospital and stored at -20°C . AFP International Reference Preparation (AFP-IRP) was obtained from the National Institute for Biological Standards and Control (lot 72-227, Holly Hill, London, U.K.), rabbit IgG anti-human AFP (lot 104) was purchased from Dako (Copenhagen, Denmark), goat anti-rabbit IgG (H + L)-alkaline phosphatase conjugate (lot 26655) was obtained from Bio-Rad, goat anti-mouse IgG-alkaline phosphatase conjugate (lot 84F-8832) and goat anti-mouse IgA-alkaline phosphatase conjugate (lot 113F-8855) were purchased from Sigma. Mouse monoclonals anti-AFP (Moabs), codes: 21.1,IgA; 490,IgG1; 21.2,IgG1; 384,IgG1 and 144,IgG2, were obtained through V. Van Heyningen, from the Western General Hospital, Edinburgh, U.K.; Moabs codes 2051,IgG and 2062,IgG were obtained through M. Mayumi, from the Jichi Medical School, and from the Institute of Immunology, Tokyo, Japan; Moab HB134,IgG1 was obtained from supernatant culture fluids of hybridoma cell line HB134 (American Type Culture Collection, Rockville, MD).

Immunoabsorption

To ensure monospecificity, the rabbit IgG anti-human AFP was absorbed with immobilized

normal human serum in a CNBr-activated Sepharose-4B column (Ruoslahti, 1978). The absorbed rabbit IgG anti-human AFP (rabbit anti-AFP) presented one anodic precipitin band in immunoelectrophoresis (Arquembourg, 1975) and a single AFP band immunodetected by SDS-PAGE nitrocellulose transfer (Ramirez et al., 1983).

Hybridoma tissue culture conditions

Hybridoma HB134 cells were grown in RPMI 1640 with 15% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 25 mM Hepes, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5×10^{-5} M 2-mercaptoethanol, all from Gibco (Grand Island, NY). Cell cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air; 150 ml of supernatant fluids were collected at a cell density of $2.6 \times 10^6/\text{ml}$, clarified at $10\,000 \times g$ for 15 min and precipitated with an equal volume of saturated ammonium sulphate. The precipitate was resuspended in 3 ml of phosphate-buffered saline 0.15 M pH 7.4 (PBS) and dialyzed against PBS.

AFP quantification

Rabbit anti-AFP was labeled with ^{125}I (New England Nuclear, Boston, MA) using chloramine-T (Sigma) as oxidant (Hunter, 1978). The quantification of AFP was carried out by a modification of the solid-phase immunoradiometric assay (Hunter et al., 1982). Briefly, plastic beads (Abbott Laboratories, Diagnostics Division, North Chicago, IL) coated with rabbit anti-AFP were incubated for 2 h at 45°C with cord serum or AFP-IRP diluted in PBS with 0.5% Tween 20 and 2% BSA, washed with double distilled water (ddH_2O) and incubated for a further 2 h at 45°C with ^{125}I -rabbit anti-AFP (specific activity 10–12 $\mu\text{Ci}/\mu\text{g}$). The beads were washed and counted for absorbed radioactivity. Dose-response curves were constructed using serial dilutions of AFP-IRP at the range between 1 and 60 ng/ml of AFP, knowing that 1 IU contains 1.21 ng (Sizaret, 1979).

Isoelectric focusing

For IEF, 20 ml of 1% agarose IEF (w/v) in ddH_2O containing 12% sorbitol and 4% Bio-Lyte range pH 3 to pH 10 or a mixture of 10% Pharmalyte range pH 3 to pH 10 and 5% Pharmalyte

range pH 4 to pH 6.5 was poured onto a glass plate (11.5 cm × 7 cm) previously coated with a dry thin layer of 15 ml 1% agarose IEF. A continuous slot in the gel was made by placing a teflon sheet (1 mm × 6 cm) in the middle of the glass plate before the gelification of agarose. Before use, the gel was kept in a moist chamber for 1 h at 4°C. 150 µl of human cord serum containing 95 µg/ml of AFP and diluted 1:2 or more in ddH₂O with 5% ampholytes, was delivered in the continuous well. Alternatively, 10 µl of the diluted cord serum or normal human serum with an AFP concentration of 5 ng/ml were loaded in a 5 mm wide, 1 mm thick and 2.5 mm deep small well. The IEF was carried out according to the manufacturer's instructions (Pharmacia), using a flat-bed IEF apparatus from Pharmacia (FBE 3000) with a cooling system (Coolnics CTR-220, Komatsu-Yamato, Japan) at 4°C in combination with an electrophoretic power supply ECPC 3000/150 (Pharmacia) and a volthour integrator (Pharmacia), for 1500 Vh. After IEF, a strip of the agarose gel across the electrodes was cut out and the pH gradient determined in 0.5 cm gel sections soaked in 1 ml of ddH₂O for 4 h at 22°C.

Blotting and immunodetection

A sheet of nitrocellulose paper (NP) soaked in transference buffer (0.025 M Tris, 0.193 M glycine, 20% methanol, pH 8.3 (Towbin et al., 1979)) was placed over the gel previously covered with 10 ml of the same buffer. Air bubbles between the gel and the NP were carefully removed. The blotting was performed by covering the NP with four sheets of filter paper, ten sheets of paper towel and a glass plate under 2 kg pressure for 30, 45 or 60 min at room temperature. After transference the NP was incubated in coating buffer (PBS, 0.3% Tween 20 and 4% BSA) for 4 h at room temperature or overnight at 4°C. The coated NP was cut into 0.5 cm strips and treated with 3 ml of rabbit anti-AFP (1 mg/ml) or Moabs (500 µg/ml) diluted 200–300-fold in PBS with 0.3% Tween 20 and 2% BSA. The strips were incubated 2 h at room temperature and then washed three times, 5 min each, with 6 ml of PBS with 0.3% Tween 20. 3 ml of the respective anti-rabbit IgG (15 mg/ml), anti-mouse IgG (0.77 mg/ml) or anti-mouse IgA (1.7 mg/ml)-alkaline phosphatase conjugates di-

luted 350-fold in PBS with 0.3% Tween 20 and 2% BSA were added. After incubation for 2 h at room temperature, the strips were washed four times in 6 ml of PBS with 0.3% Tween and once with 6 ml of 1 M diethanolamine buffer, pH 9.8. The strips treated with the alkaline phosphatase conjugates were developed with 3 ml of 1 M diethanolamine buffer, pH 9.8 containing 0.3 mg of NBT, 0.2 mg of BCIP and 10 µl 1 M MgCl₂ (NBT/BCIP). The reaction was completed within 10 min and the strips rinsed extensively with distilled water to avoid the development of a blue background. The NP strips were dried and stored at room temperature. The photographs of the NP transfers were taken after the strips were soaked in water for 1 h.

Results

Optimal setting for IEF of complete cord serum separated under native conditions was found to be at 15 W, 650 V and 30 mA using 0.05 M H₂SO₄ and 1 M NaOH as anode and cathode electrode solutions respectively. Under the same conditions Bio-Lyte ampholytes gave better separation of AFP electrophoretic variants than Pharmalyte ampholytes, even when the Pharmalyte ampholytes were used up to three times more concentrated than that recommended in the manufacturer's instructions (Pharmacia).

Blotting of IEF-separated cord serum proteins was performed immediately after electrophoresis in order to avoid diffusion of the bands in the agarose gel. Amounts of AFP between 500 ng and 0.15 ng (in diluted cord serum) per small well, were completely transferred from the agarose gel, while saturation of the NP was obtained with higher AFP concentrations. The resolution of the bands improved when the diluted sample contained a concentration of AFP lower than 30 µg/ml. Higher amounts produced spreading and fusion of the AFP bands. In addition, when cord serum was centrifuged and filtered through a Millipore filter (0.45 µm) before dilution, the migration and separation of the bands improved. Better separation of the AFP electrophoretic variants was obtained when the sample was loaded in a continuous well, since the migration front was less wavy than when the sample was applied in the

small well or soaked in a filter strip. Blotting buffer used for Western blot transfers (Towbin et al., 1979), was more efficient than sodium phosphate buffers. The same blotting efficiency was observed when NP of pore sizes 0.2 μm or 0.45 μm were used. No differences in the results were observed with blotting temperatures of 4°C, 10°C or 22°C. Decreasing resolution of the blotting system was obtained when the transference time exceeded 45 min. Blotting times shorter than 30 min did not allow complete transference.

Different blocking agents containing 1% casein hydrolysate (Sigma) and 10% FCS in PBS, 1% casein hydrolysate and 1% BSA in PBS or barbiturate buffer (0.07 M, pH 8.6) were tested. However in our hands the best results were obtained with PBS, 0.3% Tween 20 and 4% BSA. After incubation with coating buffer the NP could be stored dry at 4°C up to 15 days without diminishing the immunoreactivity of AFP bands. Storage of the blots after developing with NBT/BCIP substrate for more than one month decreased the intensity of AFP bands.

The minimum amount of AFP detected by the blotting system using rabbit anti-AFP and goat anti-rabbit conjugate as second antibody, was 0.15 ng. This value was obtained by loading 10 μl of serial dilutions of a cord serum sample with a known amount of AFP in small wells. With the use of Moabs the sensitivity varied from 300 ng of

AFP per small well (490,IgG1) to 0.2 ng of AFP per small well (2062,IgG, HB134,IgG1 and 2051,IgG). Adult normal human sera containing AFP concentration of 5 ng/ml (0.05 ng/10 μl of AFP per small well) did not react in the NP transfers with rabbit anti-AFP or Moabs at the dilutions used; goat anti-rabbit IgG, goat anti-mouse IgG, and goat anti-mouse IgA-alkaline phosphatase conjugates did not react with human cord serum proteins on NP transfers. Under conditions of detection, the NBT/BCIP substrate did not react with serum phosphatases on immunoblots.

Fig. 1 presents the immunodetection of AFP electrophoretic variants on nitrocellulose transfers from separated cord serum in a IEF agarose gel, and corresponds to NP strips obtained from the same immunoblot, developed with different antibodies (except lanes 1 and 10) under the same experimental conditions. Rabbit anti-AFP recognized nine individual electrophoretic variants in the range of pH 4.5 to pH 5.2. This range has been previously reported before for AFP (Smith and Kelleher, 1980). In contrast to bands A-H, protein band I, close to pH 5.2, was weakly detected by rabbit anti-AFP (Fig. 1, lanes 1 and 10). The resolution of bands G and H was difficult since they focused at a very close pH range and both reacted strongly with rabbit anti-AFP. These bands were the most abundant AFP variants in IEF.



Fig. 1. Immunodetection of AFP variants on nitrocellulose transfers from cord serum separated by isoelectric focusing in agarose gel. Lanes 1 and 10: rabbit anti-AFP; lane 2: Moab 144,IgG2; lane 3: Moab 21.2,IgG1; lane 4: Moab 384,IgG1; lane 5: Moab 21.1,IgA; lane 6: Moab 490,IgG1; lane 7: Moab 2062,IgG; lane 8: Moab HB134,IgG1; and lane 9: Moab 2051,IgG.

TABLE I
RELATIVE INTENSITY OF THE REACTIVITY OF ANTIBODIES AGAINST AFP ELECTROPHORETIC VARIANTS

AFP band	Anti-AFP								
	Rabbit (1, 10) ^a	144 (2)	21.2 (3)	384 (4)	21.1 (5)	490 (6)	2062 (7)	HB134 (8)	2051 (9)
A	+ ^b	-	-	-	-	-	-	-	-
B	++	+	+	+	-	-	-	-	-
C	+++	-	-	-	-	-	-	-	-
D	++	±	-	-	-	-	-	-	-
E	++	+	±	±	-	-	++	++	++
F	+++	++	±	±	-	-	++++	++++	++++
G	++++	++++	+	±	-	-	++++	++++	++++
H	++++	++++	++++	++++	+++	+++	++++	++++	++++
I	±	±	++	±	-	-	-	-	-

^a The lane numbers from Fig. 1 are indicated in parentheses.

^b The semiquantitative evaluation of the bands is based in scans obtained by densitometry of the strips (Ramirez et al., 1983).

The reactivity of Moabs ranged from weak to strong and showed variability in the pattern of the AFP bands recognized (Fig. 1). Excess of Moab (100 µg in 3 ml of PBS with 0.3% Tween 20 and 2% BSA per strip) did not detect additional AFP protein bands. Table I summarizes the AFP electrophoretic variants recognized by eight different Moabs and rabbit anti-AFP. Moabs could be separated into three groups according to their reactivity in immunoblots: group 1 (lanes 2, 3 and 4) recognized 6-7 bands; group 2 (lanes 5 and 6) recognized only one band, and group 3 (lanes 7, 8 and 9) recognized four bands. None of the Moabs reacted with bands A and C, however, band H was strongly recognized by all of them. Bands G and H could be clearly resolved by Moabs from group 1. No differences in the number and pattern of AFP electrophoretic variants were observed among 25 human cord sera analyzed. However, different numbers of bands and migration profiles were observed in sera from hepatoma patients (manuscript in preparation).

Discussion

This work describes a method which enables the separation and identification of native AFP electrophoretic variants in complete serum samples. The technique is simple in comparison with other methods for the identification of AFP

variants, detects AFP quantities up to 0.15 ng, and it is specific since polyclonal and monoclonal antibodies do not show reactions with normal adult human components other than AFP. Koch et al. (1985) reported a similar method for complement factors C₃ and factor B. The assay is suitable for diagnostic purposes since it can be performed in complete serum without the interference of other components, does not require the use of lectins in addition to specific antibodies, or the use of denaturing agents for the IEF separation, which are known to induce some artifacts (Righetti and Drysdale, 1974) or destruction of epitopes (Koch et al., 1985). In addition to the immunodetection of protein variants, the assay can be adapted for the identification of different isozymes by adding precipitable substrates after blotting or, for the detection of immunoglobulin isotypes, allotypes or idiotypes, by adding a radioactive or enzymatic labeled ligand after transference (manuscript in preparation).

Several techniques have been reported for the analysis of AFP variants in complete serum, in other biological fluids or in tissue extracts. Earlier assays required purification of AFP by chromatographic methods and isoelectric focusing, or electrophoresis with and without crossed immunoelectrophoresis of the purified material (Lester et al., 1976 and 1978). Other methods included preparative isoelectric focusing, ion exchange chromatography, ampholyte displacement chromatog-

raphy, or column lectin affinity followed by immunodetection of the AFP variants by immunoelectrophoresis, counter immunoelectrophoresis, RIA or ELISA (Alpert et al., 1972; Smith and Kelleher, 1973; Young and Webb, 1978; Smith et al., 1983; Buamah et al., 1984). Recently, several authors have used immuno-affino-electrophoresis with lectins (Taketa et al., 1983; Aoyagi et al., 1984; Ishiguro et al., 1985) or antibody-affinity blotting for detection of AFP separated by lectin affinity electrophoresis in agarose gels (Taketa et al., 1985c). These techniques are usually more elaborate and detect from two to seven AFP variants.

As shown in Fig. 1, we have been able to resolve a total of nine different variants. Mono-specific rabbit polyclonal antibody proved to be very useful in the detection of the maximal number of bands. On the other hand, Moabs are valuable for discerning among different AFP bands. The pattern identified by Moabs is partially in agreement with the epitopes recognized by these immunoglobulins: Moabs from group 1 (144,IgG2; 21.2,IgG1; 384,IgG1) are directed to one set of proximal epitopes, and Moabs from group 2 (21.1,IgA and 490,IgG1) recognize a second set of proximal epitopes (Brock et al., 1984). Although Moabs 2051 and 2062 seem to be directed against different epitopes (Nomura et al., 1982), they react on the blots with the same protein bands. The epitope recognition of Moab HB134,IgG1 is not known, nevertheless, it identifies the same AFP variants with which Moabs 2051 and 2062 react. Based on this fact, these three Moabs 2051, 2062 and HB134 were clustered in the same group.

Chan et al. (1986) suggested that some AFP lectin-binding variants may be recognized by polyclonal antibodies, but not by Moabs. With the exception of bands A and C, all Moabs together were able to detect the same number of variants recognized by rabbit anti-AFP. Bands A and C could correspond to cross-reacting molecules absent in adult human serum, but present in cord serum. We believe that this possibility is unlikely since these variants seem to be present in other biological fluids (manuscript in preparation). Moreover, band C is a strong reacting component with an isoelectric point in the range of pH re-

ported for AFP (Yachnin et al., 1977).

It is generally accepted that most, if not all of the human AFP heterogeneity, is due to differences in the carbohydrate moiety of this molecule (Smith and Kelleher, 1980). Some of the Moabs could be directed against these different sugar determinants or protein carbohydrate determinants, as demonstrated by Feizi and Childs (1985) and Watanabe et al. (1985) for Moabs against other tumor-associated antigens. Alternatively, the different electric charges of the AFP variants could influence some regions of the molecule, hiding the epitopes. Finally, the association of AFP with other ligands (Parmelee et al., 1978) could affect the migration and the antibody recognition of some determinants.

Recent investigations with monoclonal antibodies directed against various epitopes of the AFP have suggested that specific recognition of the variants is possible (Bellet et al., 1984). To our knowledge this is the first work describing a collection of Moabs that can discriminate among different AFP electrophoretic variants in the same sample. The combination of isoelectric focusing and immunodetection with polyclonal and monoclonal antibodies opens the possibility of a specific and simple test for the performance of the differential diagnosis of hepatocarcinoma and benign liver diseases.

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