ISOLATION AND CHARACTERIZATION OF A DISTINCT IMMUNOREGULATORY ISOFORM OF α-FETOPROTEIN PRODUCED BY THE NORMAL FETUS

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α-fetoprotein (AFP)[1] is a well-characterized onco-fetal protein that is normally expressed during embryogenesis while its reappearance in later life, other than in normal pregnacy, usually signifies disease (1). There are many physico-chemical similarities between AFP and albumin and they may have comparable absorption and transport roles for endogenous and exogenous molecules (2). The two proteins have a significant degree of amino acid sequence homology (3), and they are immunologically crossreactive in the denatured state (4). They are members of the same multigene family that includes human group specific component (Gc), also known as vitamin D-binding protein (5). In contrast to albumin, AFP is a glycosylated protein containing 4% carbohydrate (6). Extensive microheterogeneity of AFP has been demonstrated in many species, including the human, mouse, rat, and rabbit (reviewed in reference 7). The exact biochemical basis of the molecular variation that exists within the native population of AFP molecules has not been resolved, but disparity in carbohydrate units and variable usage of glycosylation sites of AFP are likely to be at least partially responsible (6). Differences in the degree of sialylation among AFP molecules also contribute to the overall pattern of microheterogeneity (8, 9). Molecular variants of AFP have been qualitatively identified on the basis of charge differences using PAGE (8), extended agarose gel electrophoresis (10), immunoaffinoelectrophoresis (11), IEF (12), and anion-exchange chromatography (13). Variants have also been defined by differential lectin-binding properties (7). Examination of AFP profiles during fetal ontogeny and in regenerative and carcinogenic events has indicated that predictable changes in the ratios of isomers can occur in normal development and in certain disease states. This raises the intriguing possibility that quantitative and/or qualitative changes in circulating levels of AFP subspecies may be of clinical diagnostic value (14), and as well, may have functional significance (10, 15). In pursuit of this latter prospect, Yachnin and coworkers (16) were able to

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[1] *Abbreviations used in this paper:* AFP, α-fetoprotein; APAGE, alkaline-PAGE; FPLC, fast protein liquid chromotography; MAF, mouse amniotic fluid; NMS, normal mouse sera; TBA, thiobarbituric acid; TBS, tris-buffered saline, TTBS, TBS containing 0.05% (vol/vol) Tween 20.

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gather convincing evidence that a positive correlation does indeed seem to exist between the relative concentration of certain molecular variants in a given fetal or tumor-derived AFP isolate and its immunosuppressive strength. Findings by Zimmerman et al. (15) suggested that the presence of sialic acid residues on certain AFP molecules was essential for immunosuppression, while Lester et al. (16) reported otherwise, leaving open the important question of the functional relevance of these secondary structures.

In the present investigation we have used an efficient anion exchange column linked to an automated FPLC system, the details of which are to be published elsewhere (van Oers, N. S. C., R. Boismenu, B. L. Cohen, and R. A. Murgita, submitted for publication) to isolate seven molecular variants of murine fetal AFP in quantities suitable for detailed functional studies. We show here that all the immunoregulative activity associated with AFP is localized to a single distinct isomeric form and that this function is not linked to sialic acid expression.

Materials and Methods

Animals. Male and female CBA/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Swiss Webster mice were obtained from Charles River Breeding Laboratories (Montreal, Quebec). All the mice were bred and maintained in our own facilities.

Purification of AFP. AFP was isolated from mouse amniotic fluid (MAF) of pregnant Swiss mice in late-stage gestation (days 16–19). Purification of MAF-derived AFP was accomplished by antibody-agarose affinity chromatography and, when necessary, was followed by a second step using preparative PAGE as described in detail elsewhere (17). Isolated AFP preparations fulfilled strict criteria for purity, including a single Coomassie blue– or silver nitrate–stained protein band on analytical alkaline PAGE (APAGE) and SDS-PAGE, and no detectable contamination with other serum or MAF proteins according to conventional Ouchterlony gel diffusion and immunoelectrophoresis analysis when tested at 2 mg/ml AFP.

Anion Exchange Chromatography. Chromatographic separations were performed on a preparative (Mono Q HR16/10) anion-exchange column coupled to a fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Dorval, Canada). The column was equilibrated with 20 mM L-histidine, pH 5.60 (buffer A), (Sigma Chemical Co., St. Louis, MO), and fractions were eluted with a uniform linear salt gradient of 100% buffer A to 30% buffer B (20 mM L-histidine, 0.5 M NaCl, pH 5.60). 20-mg samples of AFP were applied to the Mono Q HR16/10 column and bound proteins were eluted with the salt gradient at a flow rate of 8 ml/min in a total volume of 325.0 ml. 10-ml fractions were collected, and individual peaks, as monitored by OD_{280} profiles, were pooled and concentrated to 2 mg/ml either on the basis of dry weight of lyophilized samples or by measuring the UV absorbance at 280 nm and determining protein concentration from the OD_{280} reading using a predetermined extinction coefficient of 0.443 for AFP.

Gel Electrophoresis. Analytical IEF-PAGE was performed in 8.5% polyacrylamide gels using a protean vertical slab gel electrophoresis unit (Bio-Rad Laboratories, Mississauga, Canada). Briefly, 25.5 ml of acrylamide stock (30% T: 2.67% C) (Bio-Rad Laboratories) was combined with 45.0 ml of 25% sucrose and 13.5 ml of distilled water. A 5% ampholine solution extending over a pH range of 4.2–4.9 (Pharmalyte; Pharmacia Fine Chemicals) was added to this mixture. Polymerization was initiated with 0.6 ml of ammonium persulfate (20 mg/ml) and crosslinking was achieved with 60 μl tetramethylethylenediamine. 2 μg of protein in a 10-μl volume was applied to the gel, and samples were subjected to electrophoresis for 5 h at 500 V. Densitometric scans of the isoelectric variants were performed with a laser densitometer (2202 Ultroscan; Pharmacia Fine Chemicals). SDS-PAGE was carried out on 12.5% polyacrylamide gels with the Mini-Protean II apparatus (Bio Rad Laboratories). 10-μl samples (2.0 μg of protein) were mixed in a 1:1 ratio with SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2 M dithiothreitol, and 0.1% bromophenol blue) and heated at 100°C for 5 min. Electrophoresis through the resolving gel proceeded for 45–60 min at

250 V. For Western blots, 20-ng samples were prepared in SDS sample buffer and run on 12.5% polyacrylamide gels. APAGE was performed using conditions described previously (17).

Western Blot Analysis. Electroblotting of antigen from SDS-PAGE gels onto Immobilon polyvinylidene difluoride transfer membranes (Millipore Continental Water Systems, Bedford, MA) was performed at 200-mA constant current in a transfer unit (TE-52; Hoefer Scientific Instruments, San Francisco, CA) at 10°C overnight. After electroblotting the transfer membrane was gently agitated for 1 h at room temperature in blocking solution (3% [wt/vol] gelatin in 20 mM Tris, 500 mM NaCl, Tris-buffered saline [TBS] pH 7.5) and subsequently washed twice in TBS containing 0.05% (vol/vol) Tween 20 (TTBS). Membranes were then incubated in a 1:500 dilution of monospecific rabbit anti-mouse AFP antisera in antibody solution (1% [wt/vol] gelatin in TTBS). The membranes were again rinsed twice for 5 min in TTBS and then incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG (1:3,000 dilution in antibody solution) for 1 h. After three successive wash steps (twice in TTBS and once in TBS) the immunoreactive bands were detected with the BCIP/NBT color development solution according to the manufacturer's instructions (Bio-Rad Laboratories).

Neuraminidase Digestion. 10 mg of purified AFP was dialyzed overnight against 0.1 M sodium acetate, pH 5.0, at 4°C. The AFP was then digested with 0.2 U of neuraminidase (*Clostridium perfringens*; 2 U/ml) (no. 101F-8057; Sigma Chemical Co.) at 37°C for 4 h. The amount of sialic acid remaining on AFP preparations after neuraminidase treatment was determined by the thiobarbituric acid (TBA) assay as described in detail below.

Sialic Acid Assay. Sialic acids were liberated from AFP by mild acid hydrolysis using 0.05 N H_2SO_4 and measured by the TBA assay (18). 200 μg of AFP or an equivalent amount of albumin serving as a negative control in a total volume of 100 μl was mixed with 100 μl of 0.05 N H_2SO_4 and incubated at 80°C for 1 h in eppendorf tubes. Aliquots of 75 μl were removed from these digests and oxidized with 37.5 μl of 25 mM periodic acid (in 0.125 N H_2SO_4) at 37°C for 30 min. After this incubation, 30 μl of 2% sodium arsenite ([wt/vol] in 0.5 N HCl) was added to the solution to neutralize excess periodate. After 2 min, the yellow color of liberated iodine disappeared. Next, 200 μl of 0.1 M 2-TBA, pH 9.0, was added to the reaction vessel, the mixture heated in a boiling water bath for 7.5 min, and subsequently cooled in an ice bath. The chromophor was extracted by vigourous shaking with 750 μl of a 5% (vol/vol) 12 N HCl/*n*-butanol solution. The absorbance value of the organic phase was measured at 549 nm with the *n*-butanol/acid solution serving as a blank. *N*-acetyl neuraminic acid (sialic acid; Sigma Chemical Co.) was used to establish a standard curve over the concentration range of 4–48 μg/ml.

In Vitro Anti-SRBC Antibody Response. Primary in vitro antibody responses were generated by culturing 10^7 CBA/J spleen cells with 3×10^6 SRBC in 0.8-ml volumes in 24-well microplates (no. 3524; Costar, Cambridge, MA) according to a modification of the original Mishell-Dutton method as described in detail elsewhere (19).

Mitogen Transformations. Adult CBA/J Lyt-1^+23^- thymocytes obtained by a conventional two-step negative selection protocol with anti-Lyt-2.1 (New England Nuclear, Boston, MA) and rabbit complement (Low Tox; Cedarlane, Hornby, Ontario) were cultured to assay for reactivity to Con A using a previously described microculture system (20). Lyt-1^+23^- thymocytes (2.5×10^5) were cultured in 96-well round-bottomed microculture plates for 48 h with Con A (1 μg/ml, Pharmacia Fine Chemicals). Total volume of cell cultures was 200 μl. Cells were maintained at 37°C in 95% humidified air and 5% CO_2. 6 h before harvesting, cultures were pulsed with 1 μCi [^{3}H]thymidine (66 Ci/mmole sp act) (ICN Biochemicals Canada, St. Laurent, Quebec). Cells were harvested onto glass fiber mats with a multiple sample harvestor (Skatron; Flow Laboratories, Inc., McLean, VA) and water-insoluble [^{3}H]thymidine incorporation was measured with a liquid scintillation counter (1216 Rack Beta; LKB Instruments, Inc.). Results are expressed as mean cpm ± SEM of triplicate cultures.

Bone Marrow Cell Activation. The mitogenic effect of whole native AFP and its individual isomeric forms on cultured bone marrow cells was measured by methods we have recently described elsewhere (21). In brief, bone marrow cells were collected by flushing the tibias and femurs of CBA/J mice with PBS using a sterile 5-ml syringe and a 25-gauge needle. Cells were cultured in 96-well round-bottomed microtiter plates at a concentration of 2.5×10^5 cells/well in a total volume of 0.2 ml for 48 h, after which cultures were pulsed with [^{3}H]thymidine, harvested, and counted as described above for Con A reactions.

IFN-boosted In Vitro NK Activity. In vitro measurements of spontaneous and lymphokine-boosted anti-YAC NK activity in CBA/J spleen were performed as previously described (22). Cells were cultured in Iscove's modified DME containing 5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin, and supplemented with fresh 0.5% CBA/J normal mouse sera (NMS). Splenic effector cells were stimulated for 24 h with 5,000 U IFN (IFN-α IFN-β from mouse fibroblasts; Enzo Biochem., Inc., New York, NY) in 24-well plates (Costar) at 5×10^6/ml in a total volume of 2 ml. Effector cells at an E/T ratio of 100:1 were then assayed for NK lytic activity against ^{51}Cr-labeled YAC-1 targets.

Results

Definition of AFP Isomeric Forms. An analysis of the protein content of MAF on APAGE revealed as expected the three major protein components (Fig. 1 *A*) previously defined as transferrin, AFP, and albumin (17). The purity of the high-milligram quantities of AFP used throughout these studies was established by demonstrating a single band on analytical APAGE (Fig. 1 *B*), as well as by conventional immunodiffusion tests with a panel of antibodies directed against potential contaminating material present in MAF. In contrast to the single broad band of AFP detected on APAGE, it was routinely possible to visualize six isoelectric variants in IEF gels (Fig 1 *C*), using narrow range ampholines extending over the pH range of 4.2–4.9. The relative amount of each AFP variant present in late gestational-stage MAF was determined by densitometric scans of the IEF gels (Fig. 1 *D*). As shown in Fig. 1 *F*, the proportions of individual AFP subspecies in relation to the total number of native AFP molecules in the test sample was found to range from a minimum of 6% to a maximum of 26%. Fig. 1 *E* denotes the terminology (i.e., AFP-1 through AFP-7) used throughout this study to identify the seven AFP isomers separable on the Mono Q column linked to the FPLC system. It will be noted from data presented

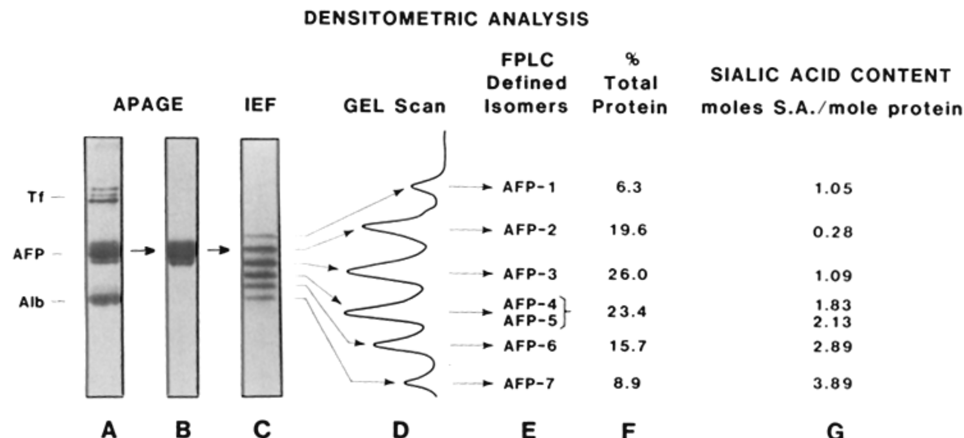

FIGURE 1. Biochemical and electrophoretic analysis of the molecular heterogeneity of AFP. (*A*) Coomassie blue–stained protein pattern of MAF, and MAF-derived purified AFP (*B*) on APAGE gels. The three major protein components of MAF; albumin (Alb), AFP, and transferrin (Tf) are shown. (*C*) Analytical IEF gel analysis of pure AFP showing six isoelectric variants with pIs ranging from 5.1 (*top*) to 4.7 (*bottom*). The densitometer scan of the IEF gel (*D*) was used to calculate the proportion of individual subspecies to the total number of native AFP molecules (*F*). The terminology used throughout this study to define the seven FPLC-generated AFP isomers is shown (*E*). (*G*) The sialic acid content of each isomer as determined by the TBA assay.

in Fig. 2 that the fourth isoelectric isomer defined by its pI of 4.8 on IEF gels splits into two well-defined variants on the Mono Q column. Finally, Fig. 1 *G* shows the sialic acid content of each of the purified FPLC-defined isomers.

Quantitative Recovery of Individual AFP Isomers. After first establishing optimal separation conditions for individual AFP isomers on an analytical size Mono Q HR5/5 column (data not shown), we proceeded to perform separations on a preparative Mono Q HR16/10 column for the purpose of obtaining the milligram quantities of each isomer needed for eventual use in the planned function tests. The elution profile shown in Fig. 2 for 20 mg of AFP was very similar to that routinely observed on the preliminary analytical scale runs. The minor peak that consistently eluted at ~80 ml into the run failed to react with any of our antisera directed against MAF or NMS components and did not show a discernable Coomassie blue- or silver-stained band on APAGE or IEF gels. Moreover, the unidentified material in this peak was inactive in the functional tests described below. To help confirm that the seven major peaks generated on FPLC represented distinct isomeric forms of AFP, each individual peak was examined on narrow range IEF gels. As shown in Fig. 2, *insert*, each of the FPLC peaks did indeed represent a unique isomer having a different pI within the pH range of 4.7–5.1, with the exception of AFP-4 and AFP-5, which exhibited identical protein banding at a pI of ~4.8. The seven AFP variants,

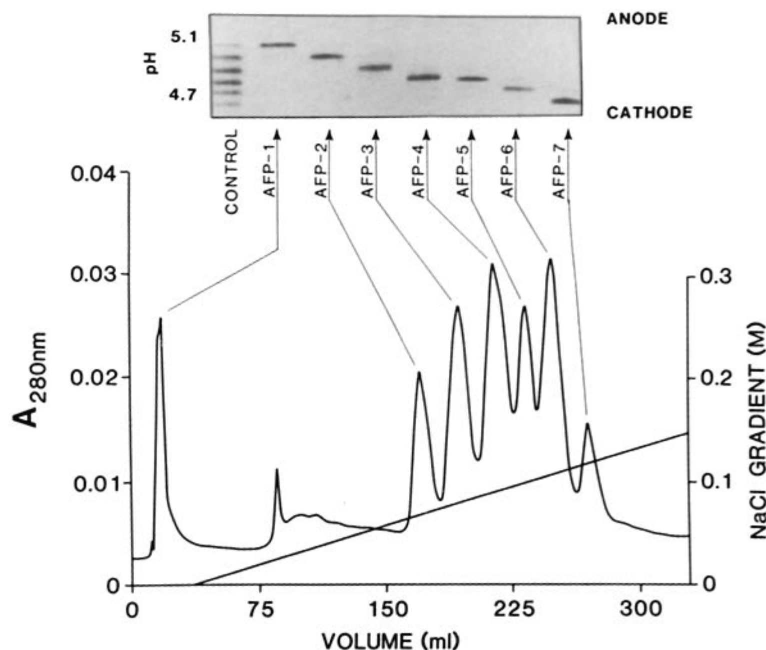

FIGURE 2. Resolution of seven isomeric forms of AFP on a preparative anion exchange Mono Q HR16/10 column. 20 mg of mouse AFP was applied to the Mono Q HR16/10 column equilibrated with 20 mM L-histidine, pH 5.60. AFP isomers retained on the column were eluted sequentially with a linear salt gradient up to 0.15 M NaCl over a total buffer volume of 325.0 ml. Peaks containing AFP are indicated and labeled as isomeric forms AFP-1 to AFP-7. The minor peak at the 80-ml elution volume is not AFP and is unidentified. The insert shows a Coomassie blue-stained IEF gel of control AFP and the AFP isomers identified in the chromatogram.

defined by their highly reproducible retention volumes on Mono Q anion-exchange column chromatography, were therefore termed AFP-1 through AFP-7, with AFP-1 appearing in the void volume. To further verify that each protein band seen in the IEF gel and defined by the FPLC chromatogram was an AFP variant, we compared their molecular masses on SDS-PAGE and performed immunoblot analysis. As shown in Fig. 3 *A*, the proteins recovered from the seven peaks on the chromatogram each had molecular masses of 69 kD, identical to that for control unfractionated AFP. Western blot analysis using monospecific anti-mouse AFP antibodies confirmed that the protein recovered from each FPLC-generated peak represented purified AFP molecules (Fig. 3 *B*). The immunoblots were negative when developed with antisera specific for other known components of MAF (data not shown).

Comparative Immunoregulatory Effects of the Seven Distinct Isomeric Forms of AFP. In earlier studies we had established that AFP is capable of exerting strong immunosuppressive effects on in vitro anti-SRBC antibody responses (17), Lyt-1^+23^- Con A-reactive thymocyte responses (23), and NK cell activity augmented in vitro by lymphokines (22).

As shown in Fig. 4, *A–C*, a purified control AFP sample containing the full spectrum of isomeric forms performed as expected from our previously published studies by efficiently inhibiting each of the in vitro lymphocyte functions tested. However, what is equally clear and particularly striking in this series of experiments is that of the seven AFP isomers tested in parallel, only AFP-1 was able to mediate a suppressive effect comparable with control unfractionated AFP. Isomers AFP-2 through AFP-7 were entirely without effect in these assay cultures, even when tested at concentrations as high as 200 and 400 μg/ml. The distinction in functional activity between AFP-1 and the other isomers is even more apparent from dilution analysis data shown in Fig. 5. Here a representative dose-response curve extending from 100 to 0.01 μg/ml of each purified isomer shows that AFP-1 is a more efficient suppressor of Lyt-1^+23^- Con A thymocyte responses than is unfractionated control AFP, while the remaining six isomers again failed at all concentrations to influence

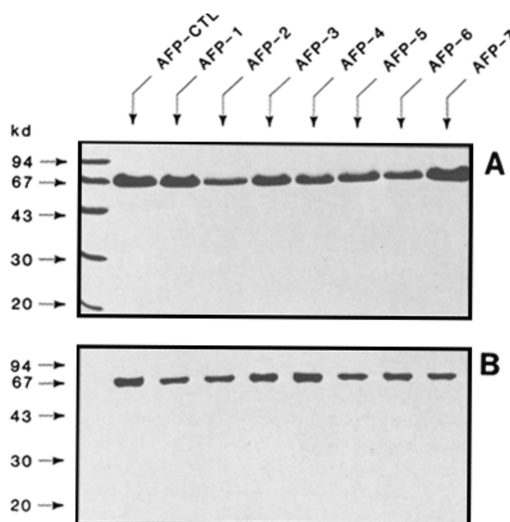

FIGURE 3. Molecular mass characterization and immunoblot analysis of AFP molecular variants purified by FPLC anion-exchange chromatography. 2 μg of control AFP or Mono Q-defined AFP isomers AFP-1 to AFP-7 were separated on 12.5% SDS-PAGE and stained with Coomassie blue (*A*). For Western blots (*B*), 20 ng of protein containing control AFP or AFP-1 to AFP-7 was separated on 12.5% SDS-PAGE and immunoblotted with a monospecific anti-AFP antibody. Arrows indicate location of molecular masses, which include phosphorylase b (94 kD), BSA (67 kD), OVA (43 kD), carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20.1 kD).

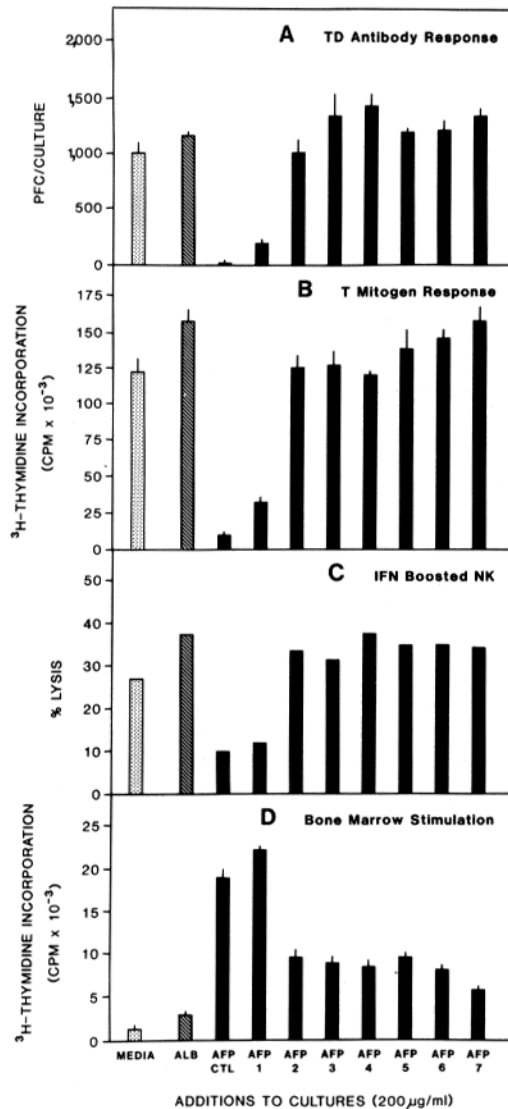

FIGURE 4. Comparative immunoregulatory effects of the seven FPLC-defined AFP isomers on in vitro lymphocyte functional assays. Control AFP and each individual AFP isomer were tested for functional activity on T-dependent antibody responses (*A*). CBA/J spleen cells (10^7) were cultured with 3×10^6 SRBC and 200 μg/ml of NMS, control AFP, or the indicated isomers in a total volume of 0.8 ml RPMI, 10% FCS. After 4 d of incubation, anti-SRBC activity was assayed. *B* shows adult CBA/J Lyt-1^+23^- thymocytes (2.5×10^5) cocultured with 1 μg/ml Con A and 200 μg/ml of the indicated proteins. Proliferative responses were measured at 48 h of culture. In *C*, mouse albumin, control AFP, and AFP-1 to AFP-7 were tested for their suppressive activity on IFN-boosted NK cell activity. Adult CBA/J spleen cells were incubated with IFN and the indicated proteins for 24 h. After 24 h, the NK activity of the cells measured against ^{51}Cr-YAC. Bone marrow cell proliferative responses (*D*) were measured after culturing normal adult CBA/J cells (2.5×10^5) with the indicated proteins for 48 h, as described in Materials and Methods.

this reaction. Thus, Con A responses in the presence of various amounts of isomers AFP-2 through AFP-7 always fell within the SEM for albumin control responses as denoted in Fig. 5 by the dotted lines.

Recent studies by Hoskin et al. (21) have shown that cultured bone marrow cells undergo a strong proliferative response in the presence of AFP. We therefore examined the effect of each of the FPLC-separated isomers on cultured bone marrow cells to determine which of the molecular variants could demonstrate growth-promoting activity. As shown in Fig. 4 *D*, augmented bone marrow cell proliferation in the presence of AFP-1 is comparable with that mediated by control AFP, while the other isomers displayed moderate stimulatory capacity that was still well above control values in the presence of media alone or albumin.

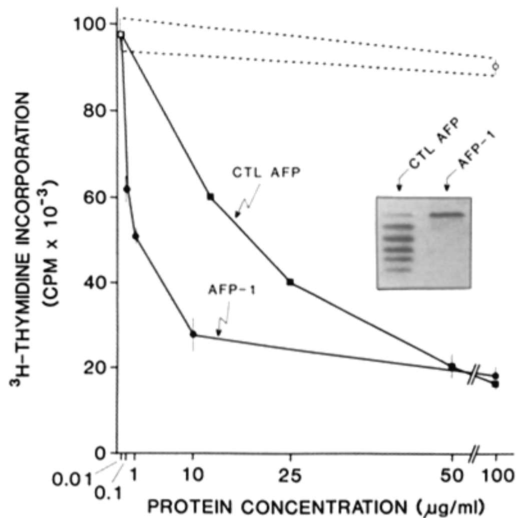

FIGURE 5. Dose-response effect of control AFP vs. AFP-1 and the nonsuppressive isomers on Con A-stimulated Lyt-1$^+$23$^-$ thymocytes. Adult CBA/J Lyt-1$^+$23$^-$ thymocytes were cultured with Con A in the presence of control AFP and purified isomers at the concentrations indicated. The boxed in area shows control Lyt-1$^+$23$^-$ responses in the presence of either mouse albumin of the Mono Q-defined isomers AFP-2 to AFP-7. After 48 h of culture, proliferative responses were measured by [^{3}H]thymidine incorporation as described in Materials and Methods. The insert shows the IEF pattern of control AFP and AFP-1.

The Role of Sialic Acid in AFP-mediated Immunosuppressive Activity. Evidence that some of the microheterogeneity of mouse AFP could be attributable to variability in the sialic acid (NANA) composition of its subspecies (9) prompted us to investigate the relative amount of NANA present in each purified AFP isomer. Using a modification of the TBA assay (18), the mol of NANA per mol of protein were calculated for each FPLC-defined AFP isomer. As noted in Fig. 1 *G*, there was a progressive increase in sialic acid residues on AFP-2 to AFP-7, with AFP-7 having 4 mol of NANA per mol of protein. AFP-2 was assumed to lack NANA entirely because it gave low values similar to that of mouse albumin, a protein known to have no covalently coupled NANA residues. It is notable that AFP-1 has a sialic acid content similar to AFP-3 even though the two isomers exhibit distinct pIs and FPLC retention volumes. This finding indicates that both the anion-exchange column and IEF gels are resolving charge differences in these isomers that are independent of sialic acid content.

Conflicting results have emerged regarding the contribution of covalently linked sialic residues to the suppressive activity of AFP (15, 16). Experiments were therefore performed in which the immunosuppressive activity of AFP was assessed after cleavage of the sialic acids by neuraminidase digestion. The effectiveness of removal of these residues was confirmed by the TBA assay, and the data shown in Table I indicate that there was an 80% reduction in sialic acid residues after enzyme treatment of two separate AFP preparations. Thus, according to our measurements enzymatically treated AFP preparations usually retained between 15 and 20% of their sialic acid content, which might suggest the existence of an enzyme-resistant population. More exhaustive attempts to achieve complete digestion, including increased enzyme to protein ratios, longer incubation times, recycling on Sepharose-bound neuraminidase columns, and the use of neuraminidase derived from *Vibrio cholera*, failed to increase the efficiency of digestion. The functional activity of two different enzyme-treated AFP preparations was determined by measuring their effect at 200 μg/ml on Con A-stimulated Lyt-1$^+$23$^-$ thymocyte responses. As shown in Table I, the desialylated AFP preparations were only slightly less suppressive than the un-

TABLE I
Effects of Neuraminidase Digestion on AFP-mediated Suppressive Activity

Sample	NANA/protein*	Lyt-1+23− Con A thymocyte response‡ Exp. 1	Exp. 2	Exp. 3
	mol		*cpm ± SE*	
Media		77,384 ± 5,148	31,531 ± 3,637	94,962 ± 6,522
Control AFP	3.16 ± 0.50§	2,919 ± 293	1,749 ± 450	7,887 ± 523
AFP desialylated‖	0.69 ± 0.12	5,969 ± 734	4,719 ± 1,253	ND
AFP desialylated¶	0.75 ± 0.14	6,373 ± 783	4,005 ± 479	9,877 ± 526

* Sialic acids were liberated from the protein by mild acid hydrolysis, and the free sialic acid content was measured by a modification of the TBA assay.

‡ Adult CBA/J Lyt-1+23− thymocytes (2.5×10^5) were cocultured with 1 μg/ml of Con A and 200 μg/ml of the indicated proteins. Proliferative responses were measured at 48 h of culture.

§ The moles of NANA per mole of AFP was determined from three different preparations of AFP.

¶ The experiment described above was repeated under the identical conditions.

‖ 10 mg of control AFP was digested with 0.2 U neuraminidase (*C. Perfringens*) for 4 h at 37°C in 0.1 M Na acetate, pH 5.0.

treated control AFP. While a possible contribution to the suppressor mechanism by the small amount of enzyme-resistant sialic acid residues remaining on the desialylated samples cannot be excluded, the most likely conclusion from these experiments is that sialic acids play no central role in mediating the immunosuppressive effect of AFP. This conclusion is supported by our findings that the immunosuppressive AFP-1 isomer expresses essentially the same amount of sialic acid as does the nonsuppressive AFP-3 isomer.

Discussion

Accumulating evidence continues to support the concept put forth by Murgita and Tomasi in 1975 (17, 24) that immunoregulation is one of the biological functions of AFP. The most well-studied modulating influence of AFP on lymphocyte responses is immunosuppression (reviewed in reference 25). However, AFP may also exert a mitogenic or growth-promoting influence on in vitro cell growth (21), which is to some extent reminiscent of previously observed effects by albumin and transferrin (26), two serum proteins shown to be related to AFP in terms of immunologic cross-reactivity of their unfolded polypeptide chains (4). Recent evidence points to the fact that the immunosuppressive effects of AFP are most pronounced on autoimmune reactions as measured both in vitro (20, 27–29) and in vivo (30, 31). This has led us to propose that in order to ensure normal embryonic development, AFP molecules may have evolved a specialized regulatory function to selectively control potentially harmful expressions of autosensitization (32). According to this reasoning, the developing immune system of the fetus and the newborn, as well as certain adult maternal immunoresponsive elements (33), are subdued by circulating immunoregulatory AFP molecules in such a manner that they function sufficiently well to protect against such external insults as infectious agents and alloaggressive maternal lymphocytes, while at the same time allowing for appropriate checks on unwarranted autoaggressive self-recognition events.

AFP is known to exist as a group of closely related molecular variants, and studies

by Zimmerman et al. (8, 9) have demonstrated patterns of what appear to be precisely regulated developmental shifts in the numbers and ratios of individual isomers throughout the perinatal period. This raises the important question as to whether the immunosuppressive activity that up to now we and many other investigators in this area have generally ascribed to the broad population of AFP molecules produced by the fetus can be attributed instead to what is perhaps a unique molecular variant that is under tight ontogenetic control during normal physiological conditions. It is notable in this regard that there have been previous reports concerned with attempts to correlate immunoregulatory activity with the presence of one or more naturally occurring isomers in a given preparation of AFP (10, 15, 16).

Lester et al. (10, 16) were the first to provide evidence suggesting a connection between the proportions of certain electronegative isomeric forms present in human fetal- and tumor-derived AFP isolates and the ability of those isolates to suppress mitogen- and alloantigen-induced lymphocyte transformation. Thus, fetal liver AFP was noticed to have a relatively high content of electronegative isomers, and was found to be strongly immunosuppressive in contrast to the generally less functionally active tumor-derived product that was shown to contain lower levels of the negatively charged molecular variants of AFP.

Lester et al. (10, 16) also tested the immunosuppressive potency of fetal liver AFP isolates after enzymatic desialylation and showed that functional activity of these isolates was not altered by removal of sialic acid residues. Ontogeny studies in the murine system by Zimmerman et al. (8, 9) showed reproducible changes in the concentrations of individual electrophoretic variants of AFP and this was attributed to increasing levels of sialyltransferase activity in both the yolk sac and fetal liver. Thus, late gestational-stage AFP isolates contained a higher proportion of more sialylated variants than did AFP preparations isolated earlier in ontogeny. When assessing the functional activity of sialylated vs. nonsialylated forms of AFP on primary T cell–dependent antibody responses, these authors provided evidence suggesting that only the more sialylated variants of AFP were immunosuppressive (15). This was in contrast with the findings of Lester et al. (10, 16), and thus, the role of sialic acids in AFP-mediated immunosuppression has remained contentious. Clarification and extension of these earlier findings have been hampered in the past largely due to the lack of a suitable procedure to isolate individual molecular variants of AFP in quantities sufficient for detail functional studies.

While many techniques have been described for analyzing the microheterogeneity of AFP molecules, most have been developed and deployed primarily for analytical purposes and, as such, are either not readily adaptable to upscaling or fail to have the necessary resolving power required to separate on a preparative scale each of the closely related molecular variants. To overcome such limitations we have developed a purification protocol for separating AFP into seven distinct isomeric forms using Mono Q anion exchange columns coupled to an automated FPLC system. The separation profile shown in Fig. 2 demonstrates the efficacy of this method for detecting individual molecular variants of AFP and for recovering each isomer in pure form. The use of L-histidine buffer at pH 5.60 resulted in elution of the AFP-1 isomer in the void volume while the remaining six FPLC-defined isomers were retained on the column and emerged sequentially with a linear increase in salt. The six isomers eluted with the salt gradient show a linear increase in sialic acid content

(Fig. 1 *G*) extending from the AFP-2 isomer, which lacks sialic acid, to a maximum of four residues/mol of protein for AFP-7, raising the obvious possibility that the observed microheterogeneity is largely due to differences in sialic acid content. However, studies by Yachnin and coworkers (16) and by us (unpublished results) clearly show that desialylation reduces but does not abolish AFP microheterogeneity. Moreover, isomers AFP-1 and AFP-3 as well as AFP-4 and AFP-5 express similar contents of sialic acids (Fig. 1 *G*) and yet are easily distinguishable on the Mono Q column separation profiles. We have also determined in preliminary experiments not shown here, as has Yachnin's group previously (34), that delipidation of a native AFP isolate has no significant effect on its isoelectric or FPLC-generated pattern of microheterogeneity. Much of the biochemical bases for the various distinct subpopulations of AFP molecules may therefore involve genetically determined amino acid substitutions, rather than or in addition to ligand binding and post-synthetic changes in chemical compositions.

While the earilier work of Lester et al. (10, 16) and Zimmerman et al. (8, 9) cited above certainly points to the conclusion that immunosuppression is a property of some but not all molecular variants of AFP synthesized by the normal fetus, the results of the present investigation are the first to show conclusively and in a direct manner that AFP-mediated immunoregulatory activity is in fact a unique property associated with a single distinct isomeric form that represents only 6% of the fetal population of AFP molecules. This firm conclusion was reached by purifying each of the seven molecular variants of fetal AFP to homogeneity followed by characterization according to their distinct retention volumes on FPLC elution profiles, their sialic acid content, and their molecular weight and display of specific AFP antigenic determinants by Western blotting. Each isomer was then thoroughly tested for functional activity using several different in vitro lymphocyte assay systems known from our previous studies (17, 21–24) to be highly sensitive to AFP-mediated immunoregulatory effects. Thus, we show in Fig. 4 that the AFP-1 isomer suppresses in vitro antibody synthesis, T cell mitogen reactivity, and lymphokine-boosted NK activity with equal or greater efficiency (see Fig. 5) than does a whole unfractionated control AFP preparation. The fact that suppression never reaches 100% in these functional assays agrees with earlier conclusions that a subpopulation of lymphocytes is resistant to the inhibitory effect of AFP (16, 35). The remaining six isomeric forms, AFP-2 through AFP-7, were entirely without effect in these functional assays, leaving open the interesting question as to what other roles these variants may play in normal and disease states. It is possible to surmise that AFP isomers 2 through 7, which represent >90% of the total number of fetal AFP molecules in the circulation, may have some functions in common with albumin, such as maintaining osmotic pressure and, through ligand-binding properties, serving as plasma carrier molecules important for both transport and for detoxification, as in the case of estrogens where the estrophilic properties of AFP is hypothesized to serve a protective role on fetal tissues from the effects of circulating maternal estrogens (36). Experiments are presently underway to examine these possibilities.

Also shown in Fig. 4 are data on the comparative in vitro growth-promoting effect of control unfractionated AFP vs. each of the seven purified isomers on cultured bone marrow cells. We have reported previously on studies showing an immunoenhancing effect of AFP (35) with one important functional manifestation of this ac-

tivity being the induction of T (37–40) and B cell-like (41–43) natural suppressor cells. The growth-stimulating properties of AFP, as measured by increased [^{3}H]thymidine incorporation in cultured bone marrow cells, resides most strongly with the AFP-1 isomer, although the other isomers show some ability to maintain bone marrow cell growth as does albumin (Fig. 4 *D*). We have previously suggested (21, 41) that the growth-promoting effect of AFP, which is particularly evident on the bone marrow, may be an exaggerated form of a shared property with albumin, which is known to be important for mammalian cell growth in vitro (44).

The essential biochemical distinction(s) between the immunoregulatory AFP-1 molecules and the other six isomers that lack this functional property remains to be determined. We have stated previously (17, 45), as has Lester et al. (16), that with available data it seems more likely that immunosuppression will prove to be an intrinsic property of certain AFP molecules rather than being attributable, as some have suggested (46, 47), to a putative noncovalently bound moiety that somehow imparts functional activity to the complex. The rationale for this reasoning is based on our collective experience in the use of stringent purification procedures for AFP, which have included exposure to strong denaturing agents, pH extremes, and high salt followed by extensive dialysis. While all these conditions would tend to favor dissociation and removal of low molecular weight moieties bound to AFP molecules, they fail, in our hands, to diminish functional activity of purified AFP. Moreover, Yachnin et al. (34) have successfully performed further careful studies designed to exclude any ligand binding role for such known factors as hydrocortisone, prostaglandins, fatty acids, or oxygenated sterol compounds as elements contributing to the immunosuppressive action of AFP. Nevertheless, it is still not possible to formally discharge the possibility that AFP may act in conjunction with other, as yet unidentified factors in a complicated manner in order to impart effective lymphocyte-inhibiting activity. However, on the basis of the findings in the present investigation, any alleged cofactor involved in the immunosuppressive mechanism of AFP would presumably have to show specificity for the functionally active AFP-1 molecular variant. Experiments presently underway in our laboratory, including functional analysis of modified synthetic and recombinant AFP peptides (48), are aimed at reaching a definitive answer to this central question.

Summary

In this report, we examine the functional significance of the molecular microheterogeneity of α-fetoprotein (AFP). In doing so, we have taken the direct approach of purifying the naturally occurring isomeric forms of fetal-derived AFP using a preparative anion exchange column linked to an automated fast protein liquid chromatography (FPLC) system followed by parallel testing of each isolated molecular variant for in vitro immunoregulatory activity. The data obtained demonstrate the presence of seven distinct variants of AFP as defined by their retention volumes on FPLC elution profiles, by their pIs on analytical IEF gels, and by Western blot analysis. Molecular mass determination by SDS-PAGE showed each isomer to be equivalent in size to 69,000-dalton native unfractionated AFP molecules. All the immunosuppressive activity of AFP was localized to a single variant representing only 6% of the total composition of native AFP. The immunoregulating isomer termed AFP-1 was the least acidic of the seven isolated variants with a pI of 5.1 and displayed a

sialic acid content of 1 mol/mol of protein. The inhibitory activity of AFP-1 could be readily measured on T cell-dependent antibody synthesis, Con A-induced stimulation of Lyt-$1^{+}23^{-}$ thymocyte DNA synthesis, and lymphokine-activated NK cell activity. All other isomers were without effect in these test systems. The immunosuppressive AFP-1 isomer also displayed the strongest growth-promoting influence on cultured bone marrow lymphocytes. There was no correlation between functional activity and degree of expression of sialic acid residues on the AFP molecules. These findings demonstrate that the immunoregulating function of AFP is confined to a distinct and relatively small subpopulation of native AFP molecules and should therefore contribute to the resolution of outstanding questions regarding the structure/function relationship of this onco-fetal glycoprotein.

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