THE BIOCHEMICAL AND IMMUNOREGULATORY PROPERTIES OF A DISTINCT MURINE ALPHA-FETOPROTEIN ISOFORM

BY

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ABSTRACT

Ph D. Nicolai S.C. van Oers Microbiology and Immunology

Alpha-fetoprotein (AFP) is a tumor-associated embryonic serum glycoprotein, existing in the circulation as a heterogeneous population of closely related molecular variants. The biological function(s) of AFP is not known, but the precisely regulated expression of AFP molecules during ontogenetic development and in certain diseases is consistent with an immunoregulatory function.

The present thesis examines the functional significance of murine AFP microheterogeneity. In the initial phase of this study, seven individual AFP isoforms were purified with a novel separation protocol developed on Mono Q anion-exchange columns linked to an FPLC system All seven subspecies were further characterized by isoelectric focusing gels, immunoblot analysis, molecular weight determination, and sialic acid composition studies When all seven variants were tested in several AFP sensitive immune assays, we determined that all the immunosuppressive activity of native AFP was localized to a single distinct molecular variant. This isoform, AFP-1, exhibited an isoelectric point of pH= 5 1, contained 1 mol of sialic acid/mol of protein, and represented approximately 6 % of the total population of naturally occurring AFP isoforms isolated from the amniotic fluid at days 15-19 of murine gestation Further studies indicated that sialic acid expression on the carbohydrate portion of the AFP molecules was unlikely to be involved in the suppressor function.

Since it has been reported that the polyunsaturated fatty acids arachidonic acid and docosahexaenoic acid complexed to AFP molecules may be necessary for the expression of AFP-mediated immunoregulatory activity, we also examined the potential contribution of these polyunsaturated fatty acids to the immunoregulative function of the active isoform. Gas liquid chromatographic analyses, delipidation procedures and fatty acid reassociation experiments indicated that these fatty acids are unlikely to contribute to AFP-mediated immunosuppressive activity. We also determined that MAF-derived AFP from different gestational time points including

days 10.5, 12.5, 14.5, 16.5, and 18.5 exhibits immunosuppressive activity in vitro All the above results are the first direct demonstration that individual molecular variants of murine AFP have distinct immunoregulatory properties. This should facilitate a better comprehension of the relationship of molecular structure to biological function of AFP molecules during fetal development.

RÉSUMÉ

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LES CARACTÉRISTIQUES BIOCHIMIQUES ET LES FONCTIONS IMMUNORÉGULATRICES D'UNE VARIANTE DISTINCTE DE L'ALPHA-FOETOPROTÉINE CHEZ LA SOURIS

L'alpha-foetoprotéine (AFP) est un antigène carcino-embryonnaire sécrété dans le sérum sous forme d'une population de variantes moléculaires qui sont structuralement très voisines. La fonction biologique de l'AFP est inconnue, mais la régulation précise de microhétérogénéité de l'AFP au cours du développement foetal et pendant certaines maladies suggère une fonction immunorégulatrice. La relation exacte entre la microhétérogénéité de l'AFP chez la souris et l'effet immunomodulateur de cette protéine fut étudiée. Nous avons mis au point une technique de purification de sept variantes moléculaires distinctes de l'AFP par fractionnement par chromatographie-anion (Mono Q) avec un système de chromatographie rapide (FPLC). La pureté des sept variantes fut caractérisée par focalisation isoélectrique, électrophorèse en gel d'acrylamide, composition en acide sialique et par des téchniques de buvardage (immuno-blotting)

Des études in vitro sur plusieurs réponses lymphocytaires révélèrent que l'activité immunosuppressive de l'AFP se trouvait exclusivement dans une des sept variantes distinctes. Cette variante, AFP-1, constitue 6% de la microhétérogénéité de l'AFP isolée du liquide amniotique. AFP-1 possèdait une pointe de focalisation de pH 5.1 et contenait une mol d'acide sialique. D'autres part, nos résultats suggèrent que la présence des acides sialiques n'était pas nécessaire aux fonctions immunosuppressives de la protéine.

L'alpha-foetoprotéine possède la capacité de lier les acides gras comme les acides arachidonique et docosahexaenoique. Par conséquent, cette interaction peut être responsable des effets immunomodulateurs de l'AFP Cependant, les extractions d'acides gras, ainsi que la comparaison des propriétés liantes des variantes distinctes de l'AFP par chromatographie de gaz fûrent employées pour examiner cette possibilité Il apparaît que la fonction immunosuppressive de l'AFP-1 ne peut pas être attribuée à la liaison des acides arachidonique et docosahexaenoique à l'AFP.

Il a également été déterminé que l'AFP isolée du liquide amniotique à 10 5, 12.5, 14.5, 16.5, et 18 5 jours de gestation a la capacité de modifier la réponse lymphocytaire. Les résultats obtenus auront servi à mieux comprendre le rapport structure-fonction des variantes distinctes de l'AFP durant l'ontogénèse

ACKNOWLEDGEMENTS

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Finally, I wish to thank Marina Pascali, my sister and my parents for their unending support and encouragement

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. Development of a rapid analytical and preparative scale separation protocol for isolating molecular variants of murine AFP with a Mono Q™ anion-exchange column linked to a fast protein liquid chromatography system (FPLC).
2. Seven distinct isoforms of mouse AFP were identified and purified in quantities sufficient for in-depth functional assays
3. A single molecular variant of mouse AFP, termed AFP-1 was found to be responsible for the immunoregulatory properties normally ascribed to the native population of murine AFP molecules, as determined in T-dependent antibody synthesis, mitogen stimulated CD4+CD8- thymocyte responses, and lymphokine boosted NK cytolytic activity This same molecular variant was capable of exerting the greatest growth promoting activity of murine bone marrow cells.
4. The immunoregulatory isoform, AFP-1, exhibited a molecular weight of 70 kd, an isoelectric point of pI= 5 1, contained 1 mol of sialic acid per mol of protein, and represented approximately 6% of the total population of AFP molecules isolated from the mouse amniotic fluid of days 15-19 fetuses
5. Sialic acid composition studies with a modified thiobarbituric acid assay and neuraminidase digestions of native AFP suggested that the immunoregulatory activity of AFP molecules was not linked to sialic acid expression
6. Mouse AFP was shown to complex 6 distinct fatty acids including palmitic, stearic, oleic, linoleic, arachidonic and docosahexaneoic acid comprising a total composition of 1 0 mol of fatty acid per mol of protein as determined by gas chromatography (GC) All seven AFP subspecies separated by the Mono Q column exhibited an equivalent fatty acid binding composition of 1 mol, but had quantities of arachidonic and docosahexaneoic acid below the GC detection limits
7. Preparations of native AFP delipidated with octadecylsilyl silica cartridges and chloroform/methanol extractions exhibited suppressive activities comparable to control AFP

8 The specific re-association of 2 to 3 moles of arachidonic acid and docosahexaenoic acid was insufficient to convert several non-suppressive isoforms into active forms.

9. MAF-derived AFP exhibits a progressive increase in concentrations with time of development up to a maximum of 2.2 mg/ml at day 18.5 of gestation, as measured by rocket immunoelectrophoresis

10 AFP isolated from MAF at different gestational time points will suppress Con A stimulated $CD4^{+}CD8^{-}$ thymocytes.

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Manuscripts and Authorship

The experimental results in this thesis comprise 2 published manuscripts (van Oers, N.S.C., Boismenu, R., Cohen, B.L., and Murgita, R.A. 1990. Analytical and preparative scale separation of molecular variants of alpha-fetoprotein by anion-exchange chromatography on Monobead resins. J. Chromatogr. 525, 59-69, [Chapter II]; and van Oers, N.S.C., Cohen, B L., and Murgita, R.A. 1989. Isolation and characterization of a distinct immunoregulatory isoform of alpha-fetoprotein produced by the normal fetus J. Exp. Med. 170, 811-825, [Chapter III]).

Chapter IV consists of 1 manuscript submitted for publication (van Oers, N.S C , Wang, T., Powell, W.S , and Murgita, R.A 1990. The fatty acid binding properties of distinct isoforms of mouse alpha fetoprotein Evidence that arachidonic acid and docosahexaenoic acid are not involved in AFP-mediated immunosuppression J Biol. Chem) Chapter V consists of a manuscript in preparation Richard Boismenu performed the immunoblot analysis in Chapter II (Figure 4B). Brenda Cohen performed several functional assays in Chapter III (Figure 9). The fatty acid analyses undertaken in Chapter IV were performed in collaboration with Tao Wang and Dr. W.S. Powell. All of the research was conducted under the supervision of Dr. R.A. Murgita.

LIST OF ABBREVIATIONS

aa:	Amino acid
AA:	Arachidonic acid (20:4)
ACHR:	Acetylcholine receptor
AFP:	Alpha-fetoprotein
ALB:	Albumin
AMLR:	Autologous mixed lymphocyte reaction
APAGE:	Alkaline polyacrylamide gel electrophoresis
APC:	Antigen presenting cell
bp:	base pairs
CAT:	Chloramphenical acetyltransferase
CI:	MHC class I
CII:	MHC class II
Con A:	Concanvalin A
CTL:	Cytotoxic T lymphocyte
DHA:	4,7,10,13,16,19-Docosahexaenoic acid
E2:	Estradiol, estrogen
FA:	Fatty acids
FBS:	Fetal bovine serum
FFA:	Free fatty acids
PG:	Prostaglandin
FPLC:	Fast Protein Liquid Chromatography
Gal:	Galactose
GC:	Gas chromatography
Gc:	Group C component (Vitamin D binding protein)
GlcNac:	N-acetylglucosamine
HPLC:	High pressure liquid chromatography
IEF:	Isoelectric focusing
IMAC:	Immobilized Metal Affinity Chromatography
kb:	kilobase
LCA:	Lens Culinaris agglutinin (lentil)
LN:	lymph node
LPS:	Lipopolysaccharide

MAF:	Mouse amniotic fluid
Man:	Mannose
MG:	Myasthenia gravis
MHC:	Major histocompatibility complex
MLR:	Mixed lymphocyte reaction
mol:	mole
MW:	Molecular weight
NANA:	N-acetylneuraminic acid, sialic acid
NK:	Natural killer cell
NMS:	Normal mouse sera
NS:	Natural suppressor cell
ODS:	Octadecylsilyl
PBS:	Phosphate buffered saline
PEG:	Polyethylene glycol
PFC:	Plaque-forming cell response
PGs:	Prostaglandins
PHA-E:	Phytohemagglutinin E
pI:	Isoelectric point
PUFA:	Polyunsaturated fatty acids
RBP:	Retinol binding protein
RCA:	Ricinus communis agglutinin
SBA:	Soy bean agglutinin
SDS:	Sodium dodecyl sulfate
SDS-PAGE:	SDS-Polyacrylamide gel electrophoresis
SRBC:	Sheep red blood cell
TBA:	Thiobarbituric acid
TD:	T-dependent
TI:	T-independent
WGA:	Wheat germ agglutinin
16:0:	Palmitic acid
18:0:	Stearic acid
18:1:	Oleic acid
18:2:	Linoleic acid

19:2:	10,13-nonadecadienoic acid
20:4:	Arachidonic acid
22:6:	4,7,10,13,16,19-Docosahexaenoic acid

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I. LITERATURE REVIEW

A. ALPHA-FETOPROTEIN

1 Historical Development and Overview

Alpha-fetoprotein (AFP) was first discovered in 1956 following a routine analysis of human serum samples separated by paper electrophoresis (Bergstrand and Czar, 1956). Comparing the separation patterns of adult and fetal samples, Bergstrand and Czar identified a previously undetected fetal protein, migrating between albumin and alpha-1-globulin This novel protein was subsequently named alpha-fetoprotein at a World Health Organization meeting in 1969. A major breakthrough in AFP research occurred in the early 1960's following the attempts of Abelev and co-workers to purify and characterize tumor-specific antigens from hepatic cancers Using a combination of ammonium sulfate precipitation, preparative agar gel electrophoresis and immunofiltration procedures, these researchers became the first group to successfully purify a hepatoma-specific tumor antigen (reviewed in Abelev, 1983) Then, in a remarkable discovery, this same tumor protein was identified in the liver of normal fetuses Biochemical and immunochemical results indicated that the hepatoma antigen isolated was AFP. These results led to the definition of AFP as an onco-fetal molecule (Alexander, 1972), and prompted an enormous research effort to define the chemical and biological properties of AFP (for reviews see Ruoslahti and Seppala, 1979, Abelev, 1971, Smith and Kelleher, 1980, Adinolfi et al., 1975, and Murgita and Wigzell, 1981) In 1973, Parmely and Caldwell published abstracts that led investigators to examine the immunoregulatory properties of AFP (Parmely et al., 1973, Caldwell and Hsu, 1973). They reported that serum fractions rich in AFP were immunosuppressive Two years later, Murgita and Tomasi showed that purified mouse AFP had strong immunosuppressive activity in vitro (Murgita and Tomasi, 1975a) and this finding was subsequently confirmed by several other research groups (Zimmerman et al., 1977, Yachnin, 1983, reviewed in Murgita and Wigzell, 1979, 1981). Of particular relevance to these reports were the subsequent studies of Lester et al correlating the immunosuppressive potency of human AFP samples with the relative amounts of an electronegative subspecies of AFP in these preparations (Lester et al., 1976, 1978b). The above investigations

have raised many important questions about the biological properties of AFP, and in particular, the functional significance of the molecular microheterogeneity of AFP

2. Biochemical Properties

a) Genetic Organization

Mouse AFP is encoded by a single copy gene on chromosome 5, 14 kb downstream of the albumin gene (reviewed in Camper et al., 1989). The nucleotide coding sequence for mouse AFP was derived from yolk sac mRNAs that were reverse transcribed into cDNA clones (Ingram et al., 1981). The complete 584 amino acid (aa) sequence of the mature murine AFP was deduced from the nucleotide sequence of several overlapping cDNA clones (Gorin et al , 1980, 1981) Using the cloned DNA sequences as hybridization probes, Kioussis et al determined that the mouse AFP gene was organized into 15 coding regions interrupted by 14 intervening sequences (Kioussis et al , 1981) Morinaga and co-workers used a similar cloning strategy to characterize the nucleotide and primary aa sequence for human AFP (Morinaga et al., 1983). These authors identified a nucleotide sequence of 1770 bases that encoded the full-length human AFP molecule No allelic polymorphisms of either mouse or human AFP have been identified, but genetic variants have been described in different inbred strains of rat (Gal et al., 1984)

b) Physiological Concentrations of AFP During Development

AFP is synthesized by the liver, yolk sac, and to a lesser extent, the gastrointestinal tract of all mammalian fetuses (reviewed in Ruoslahti and Seppala 1979, Adinolfi et al , 1975). The onset of AFP expression occurs at day 10 of gestation in the mouse and is detected by the fourth week of gestation in the human During the course of fetal development, AFP is secreted into several fluid compartments including the fetal and maternal serum, and amniotic fluid As shown in Table I, the highest concentration of human AFP is present in the fetal serum, with peak levels reaching 2-4 mg/ml at 16 weeks of gestation The concentration then gradually decreases over a 34 week period, reaching levels of 60 ug/ml at birth. The concentration of human AFP in amniotic fluid is 25-250 times lower than that detected in the fetal serum and fluctuates much less over the course of fetal

TABLE I

PHYSIOLOGICAL CONCENTRATIONS OF HUMAN AND MOUSE AFP AT DIFFERENT STAGES OF GESTATION

HUMAN AFP (μg/ml)[a]

BODY FLUID	Gestational Age (Weeks)					Newborn
	8	12	16	20	30	
Amniotic Fluid	20	25	26	14	0.8	n/a
Fetal Serum	500	1200	3400	2600	100	60
Maternal Serum	0.04	0.04	0.05	0.1	0.2	n/a

MOUSE AFP

	Gestational Age (Days)							Newborn (Weeks)		
	10	12	14	16	18	20		1	2	3.5
Fetal Tissue Extract[b] (Units/animal)	0.02	10	250	400	700	800	Serum[c] (μg/ml)	2000	2500	0.001
Fetal Serum[d] (AU/ml)	0	5	20	30	70	100				

[a]Results originally published by Adinolfi et al., 1975, and Ruoslahti and Seppala, 1979

[b]Data compiled from Kahan and Levine, 1971 with the Units/animal derived from a complement fixation assay

[c]Values originally published by Olsson et al., 1977

[d]The quantities of murine AFP are expressed as arbitrary units/ml using a rocket immunoelectrophoresis procedure (Hau et al., 1981)

development. There is an elevated concentration of AFP in the maternal serum during pregnancy, but at values 500-1000 fold less than that detected in the fetal serum. The presence of AFP in the maternal circulation is thought to arise from the fetus by transamniotic and transplacental diffusion.

The physiological concentration of murine AFP at different stages of ontogeny was initially characterized in fetal tissue extracts with a complement fixation assay (Kahan and Levine, 1971). As shown in Table I, Kahan and Levine observed a linear increase in AFP levels from days 10-20 of gestation Hau et al utilized a rocket immunoelectrophoresis procedure to demonstrate a similar increase in fetal serum AFP levels (Hau et al., 1981). Employing the same procedure, Mizejewski and Vonnegut have reported that the AFP concentrations in days 16-18 mouse amniotic fluid are about 1 mg/ml (Mizejewski and Vonnegut, 1984) The most detailed studies on the post-natal concentrations of murine AFP have been conducted by Olsson et al. (Olsson et al., 1977) (Table I) These authors found that the newborn rodent continues to express AFP for 2-3 weeks after birth In the third week, there is a dramatic decrease in the expression of AFP resulting in a concentration drop from 2 0 mg/ml to almost undetectable levels

What becomes obvious from these studies is the marked drop in the concentration of AFP after birth, reaching low ng/ml values which persist throughout adult life However, the re-initiation of AFP expression in adults can occur in association with a selective number of pathological disorders including liver injury, hepatocellular carcinomas, and germ cell tumors (Abelev, 1971, Olsson et al , 1977, Lindahl et al., 1978, reviewed in Ruoslahti and Seppala, 1979) The highly specific association of AFP with developmental and malignant events has attracted much interest on the regulation and tissue-specific expression of this protein Studies have revealed several regulatory elements necessary for the activation and high level expression of AFP (reviewed in Nahon, 1988).

i. Cis- and trans-acting regulatory elements

Mouse AFP is actively transcribed in the fetal liver and yolk sac, constituting approximately 15% and 10% of the total mRNA, respectively. Experiments with cell culture systems have demonstrated that several cis-acting regulatory elements are required for this activation and tissue-specific expression

(reviewed in Camper et al., 1989). To identify the sequences involved, Godbout et al. generated a series of clones containing a systematic set of deletions in the 5' flanking region of the gene and transfected the clones into hepatoma cells, Hela cells and other cell types (Godbout et al. 1986). By comparing the transient transcriptional activity of the clones in different cell types, the authors identified several regulatory regions, including a tissue-specific promoter region containing a TATA box within 200 bp of the transcriptional start site. The region further upstream of the AFP gene was also found to contain 3 enhancer elements at -2.5 kb, -5.0 kb and -6.5 kb, each of which displayed position and orientation independent activity and activated the transcription of heterologous promoters. In further studies, a single enhancer or a combination of all 3 enhancers was found to display equivalent activities in the yolk sac. However, these enhancers had an additive effect on transcriptional activities in the liver since all 3 were necessary to provide maximal activation (Godbout et al, 1988) By constructing chimeras containing the 5' flanking region of murine AFP placed upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene and transfecting these constructs into different cell types, Molne et al recently discovered 2 new regulatory elements (Molne et al, 1989) The first was an enhancer element located in the 0 8 kb region upstream of the AFP gene When a portion of the AFP 5' coding region was inserted into the constructs containing the 0 8 kb enhancer element, CAT expression was reduced to background levels On the basis of this result the authors concluded that a negative regulatory element was present in the 5' intragenic region of AFP that was capable of modulating the 0.8 kb enhancer element The significance of the proximal enhancer element and intragenic negative regulatory element remains unclear at present However, some enhancer sequences are proposed to be potential binding sites of a liver-specific trans-acting factor (reviewed in Camper et al, 1989), and Wang et al have recently identified a nuclear AFP DNA binding protein in a rat hepatoma cell line (Wang et al., 1987) Investigations are currently in progress to characterize the potential trans-acting factors involved in the regulation of AFP expression.

ii. Abnormal levels of AFP synthesis

As previously mentioned, AFP transcription declines sharply in newborns,

resulting in an approximately 10,000-fold reduction in AFP mRNA levels. Yet Olsson et al. have identified one mouse strain (BALB/c/J) which has an elevated level of AFP expression in the adult, with an average serum AFP concentration of 994 ng/ml (Olsson et al., 1977) To determine whether this aberrant expression was due to a genetic factor, Olsson et al crossed the BALB/c/J mice with strains of mice expressing normal, low ng/ml levels of AFP Their results showed that all the F1 mice had low serum AFP levels, indicating that the abnormal expression in BALB/c/J mice was controlled by a single recessive Mendelian gene, termed raf (regulation for AFP synthesis). In a subsequent series of investigations to examine the molecular mechanism of raf regulation, chimeric mice were generated by fusing 8-cell stage embryos of BALB/c/J (raf^b/raf^b) with C57BL/6J embryos containing the raf^a/raf^a allele (Vogt et al , 1987). In these studies, the increase in mRNA levels in the chimeric mice was found to correlate with the relative contribution of the raf^b/raf^b genotype. Based on these results and the previous findings, the raf locus was proposed to regulate the basal level of AFP mRNA in the adult liver The BALB/c/J mice contained a rare raf^b allele which was recessive to raf^a.

iii. AFP re-expression during pathological disorders

The re-expression of AFP during liver regeneration and in certain tumors is of great clinical interest and may provide further insights into the process of malignant transformations (Lindahl et al., 1978, Olsson et al., 1977, reviewed in Abelev 1971). To examine the regulation of AFP re-expression in adult mice following liver damage, Abelev injected different strains of mice with carbon tetrachloride (CCl_4). The CCl_4 injection initially results in extensive liver necrosis followed by a period of liver regeneration during which there are transient increases in serum AFP concentrations ranging from 10-100 ug/ml He found that the level of AFP re-expression was highly strain dependent, and C57BL/6 mice produced 10-fold less AFP after CCl_4 treatment than did C3H mice. To examine the molecular basis of AFP over-expression in the BALB/c/J mice and its underproduction in C57BL/6 mice, Belayew and Tilghman prepared a series of genetic crosses between these 2 strains of mice and C3H mice, and compared the level of AFP mRNA induction in the offspring (Belayew and Tilghman, 1982). Upon CCl_4 treatment, the C3H x C57BL/6 offspring generated AFP mRNA levels equivalent to the C57BL/6

underproducing parent, suggestive of a dominant Mendelian gene. The albumin (ALB) mRNA levels were constant in all the mice, indicating that the difference in AFP mRNA induction was not caused by fluctuations in the degree of liver damage in individual mice. To identify whether the effects were due to the previously characterized raf gene or a separate genetic locus, C57BL/6 (raf^a) were crossed with BALB/c/J (raf^b) and the F1 offspring were injected with CCl_4. The F1 phenotype was identical to the C57BL/6 parent, indicating that low inducibility was dominant. However, the degree of inducibility in F1 x BALB/c/J backcrossed mice did not partition in a 1:1 distribution that would be expected with a raf allele. The results instead demonstrated the presence of two unlinked loci, raf and rif (regulation of AFP inducibility). The rif locus affected the inducibility of AFP mRNA expression during liver regeneration. C57BL/6 mice contained a rare rif^b/rif^b genotype which was dominant over rif^a

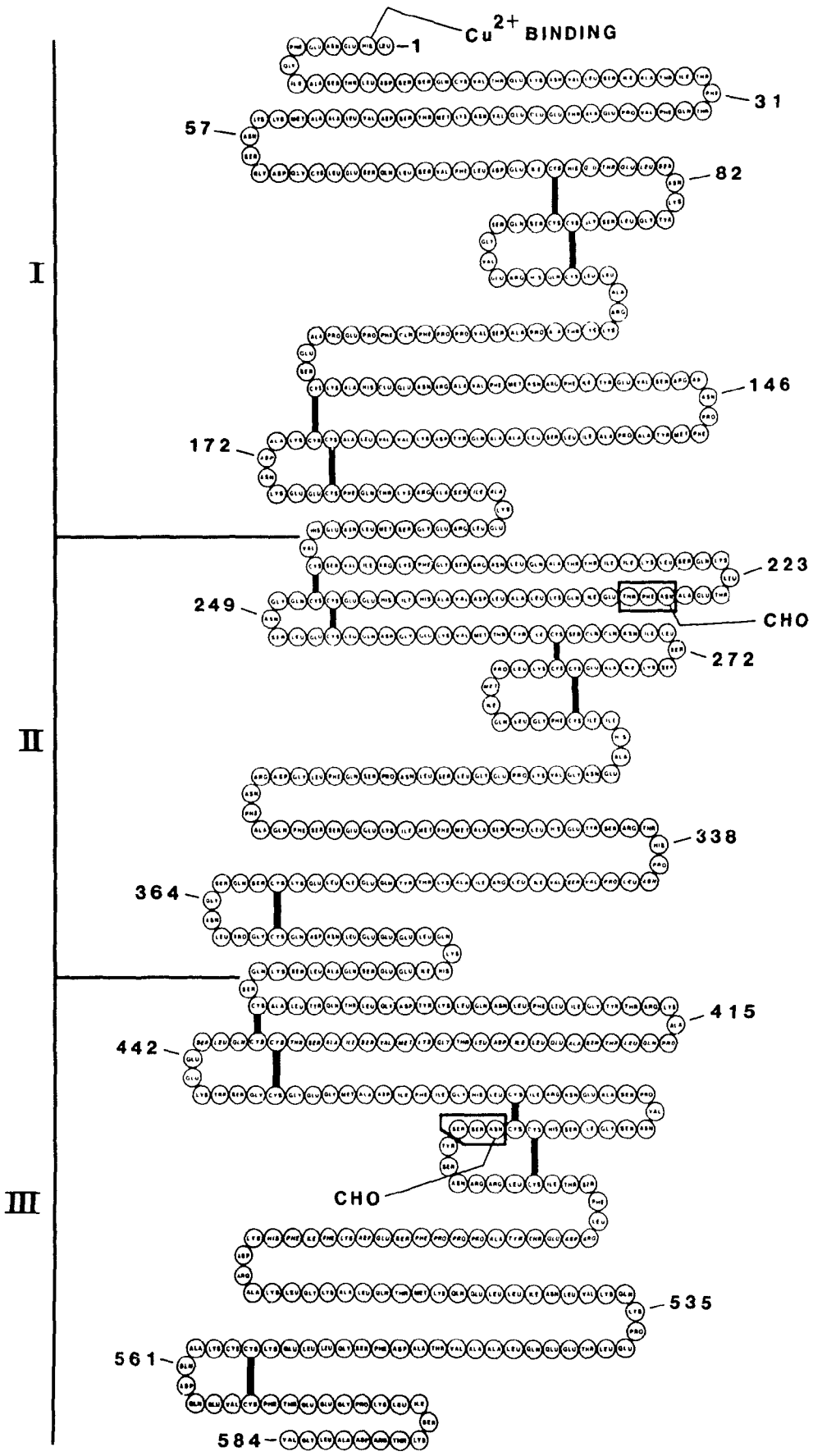
c) Protein Structure

Mammalian AFP is composed of a single polypeptide chain of 584-590 amino acids with a molecular weight ranging from 68,000-74,000 daltons as determined by SDS-PAGE and gel filtration (reviewed in Ruoslahti and Hirai, 1978, Ruoslahti and Seppala, 1979). Based on nucleotide sequence data, the deduced primary aa sequence of murine AFP would suggest a signal sequence of 5 aa followed by 584 aa comprising the mature protein (Gorin et al , 1980, 1981). In contrast, human AFP has a longer signal sequence of 19 aa and a secreted polypeptide chain of 590 aa (Morinaga et al., 1983). N-terminal sequence analysis has revealed some sequence variability in the N-terminal start position for mouse, rat and human AFP These differences have been reported and attributed to limited proteolysis of the AFP N-terminus (Peters et al , 1978b, Ruoslahti and Seppala, 1979). In the mature mouse protein, there are 32 sulfhydryl groups which form 14 disulfide bridges that span the protein (Fig. 1) On the basis of adjacent cys-cys residues and the positions of the disulfide bridges, the entire molecule can be viewed as being composed of three repeating domains which are shown in Figure 1 (Gorin et al , 1981). Each domain can be further divided into sub-domains, consisting of 3 anti-parallel helices held together by the disulfide bridges.

Figure 1.

Disulfide bonding pattern and secondary structure of mouse alpha-fetoprotein

The structure of murine AFP was based on the model originally proposed by Brown for albumin (Brown, 1976). Boxed-in regions indicate the potential glycosylation sites. The histidine involved in metal binding was defined for human AFP by Aoyagi et al., 1978, and Lau et al., 1989.


Cu2+ BINDING
1
31
57
82
146
172
223
249
CHO
272
338
364
415
442
CHO
535
561
584
I
II
III

i. Carbohydrate Structure

Approximately 4 % of the molecular weights of mouse and human AFP are reported to be due to the presence of N-linked oligosaccharides attached to asparagine residues in the polypeptide chain (Gorin et al., 1981, Morinaga et al, 1983). Based on the glycosylation sequence motif [Asn-X-Ser(Thr)], 2 potential glycosylation sites have been identified in the mouse (Fig.1) and 1 in the human To date only the carbohydrate structures for human AFPs from tumor isolates have been elucidated by techniques including lectin and paper chromatography, and sequential exoglycosidase digestions. Human AFP purified from a yolk sac tumor contains a single N-linked bi-antennary oligosaccharide which has an additional N-acetylglucosamine (GlcNac) residue attached to the beta-linked mannose (Fig. 2A) (Yamashita et al., 1983). In contrast, human AFPs isolated from patients with hepatocellular carcinomas lack the bisecting GlcNac (Yoshima et al., 1980) Both types of carbohydrate chains exhibit variability in the degree of sialylation and are occasionally fucosylated (Tsuchida et al., 1989). Although the structure for mouse AFP is not yet elucidated, sugar composition studies would suggest the presence of 2 sugar chains with a similar carbohydrate structure as that for human AFP, without the fucose or bi-secting GlcNac (Zimmerman et al, 1976) Like human AFP, the mouse protein exhibits a high degree of sialic acid (NANA) variability (Zimmerman et al., 1973, 1976).

d) Protein Homologies

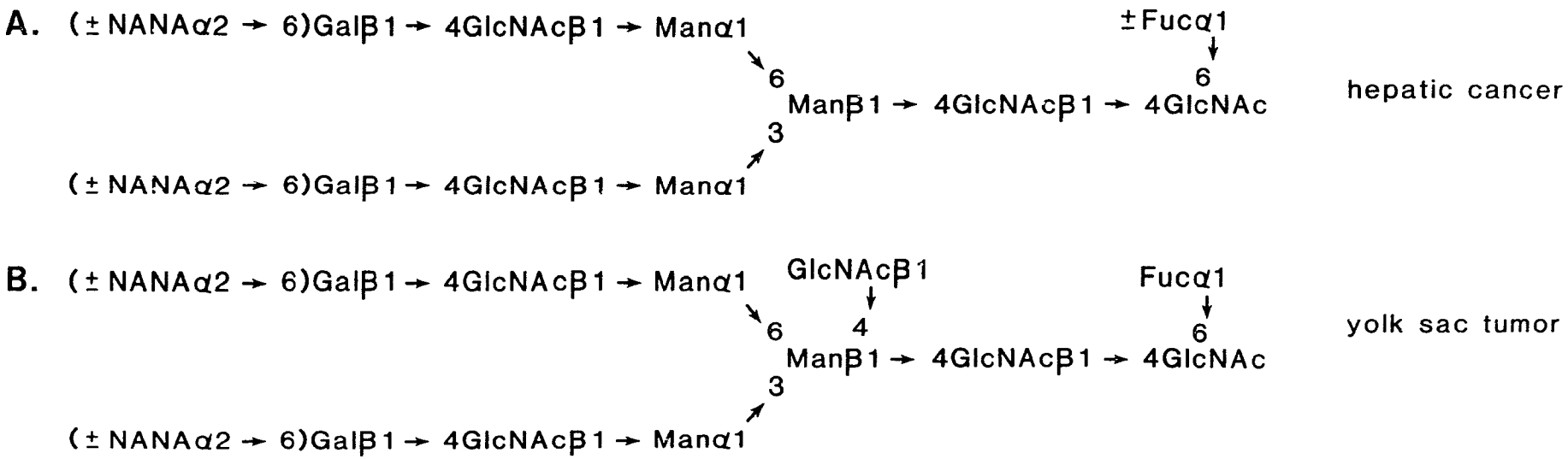
Immunodifffusion analysis has demonstrated that human AFP will cross-react with AFPs from at least 11 other mammalian species (reviewed in Nishi et al., 1975). This is not surprising since amino acid composition studies have shown a very high degree of similarity in aa content of human (Nishi, 1970, Ruoslahti and Seppala, 1979), mouse (Zimmerman et al., 1976), rat (Watabe et al, 1974), and other mammalian AFPs including rabbit, dog, and porcine (reviewed in Ruoslahti and Seppala 1979) Remarkably, fetal specific proteins, potential homologues of mammalian AFP, have been identifed in sharks and birds (Gitlin et al, 1967, 1973), suggesting a strong evolutionary conservation dating some 300-400 million years ago (Gitlin, 1974, Haeflinger et al., 1989).

The physico-chemical similarities between AFP and albumin (ALB), and the

Figure 2.

Proposed structures of the N-linked oligosaccharide chains of human AFP.

A) The carbohydrate structure of human AFP isolated from patients with a hepatocellular carcinoma and **B)** the sugar chain of human AFP purified from yolk sac tumor (Tsuchida et al., 1989). The structures were deduced by a combination of lectin reactivity and exoglycosidase digestions. NANA= sialic acid; Gal= galactose; GlcNac= N-acetylglucosamine; Man= mannose; Fuc= fucose.


A.
(±NANAα2 → 6)Galβ1 → 4GlcNAcβ1 → Manα1
6
3
(±NANAα2 → 6)Galβ1 → 4GlcNAcβ1 → Manα1
Manβ1 → 4GlcNAcβ1 → 4GlcNAc
±Fucα1
6
hepatic cancer
B.
(±NANAα2 → 6)Galβ1 → 4GlcNAcβ1 → Manα1
GlcNAcβ1
6
4
3
(±NANAα2 → 6)Galβ1 → 4GlcNAcβ1 → Manα1
Manβ1 → 4GlcNAcβ1 → 4GlcNAc
Fucα1
6
yolk sac tumor

observation that AFP and albumin concentrations in fetal and adult serum are extremely high and inversely related has led to the suggestion that AFP may be the fetal counterpart of albumin (Abelev, 1971, Kekomaki et al., 1971). Both proteins are immunologically cross-reactive when reduced and alkylated (Ruoslahti and Engvall, 1976), exhibit a similar MW and share about 30% amino acid sequence homology (190 out of 584 aa) (Ruoslahti and Terry, 1976, Vander Jagt et al., 1987). The proposed secondary tripartite domain structure of AFP is in fact based on the model originally proposed for ALB (Brown, 1976, Gorin et al., 1980, 1981, Morinaga et al., 1983)

AFP and ALB also exhibit nucleotide sequence conservation with Group-specific component (Gc), the major vitamin-D binding protein in the plasma (Daiger et al., 1975, Cooke and Haddad, 1989). These 3 plasma proteins are proposed to be members of the same multigene family, which is hypothesized to have arisen from a triplication of a primordial gene (Yang et al., 1985). The association of the 3 genes on the same chromosome, their aa sequence homologies and nucleotide sequence relatedness support this common origin (Schoentgen et al, 1986, McLeod and Cooke 1989, Cooke, 1989) The 3-dimensional structure of human serum albumin, using low resolution x-ray crystallography, indicates a predominantly alpha-helical globin protein (Carter et al, 1989). Based on the extensive sequence homologies with ALB, AFP would likely have a similar structure.

3. Characterization of AFP Microheterogeneity

The precisely regulated transcriptional activation of AFP during ontogenesis, its re-expression in association with certain diseases, and its binding similarities and sequence homologies with ALB generated considerable interest in defining the biological properties of AFP. However, many of the serum or tissue enriched sources of AFP contained a large number of other maternal and fetal proteins including albumin which, because of its structural similarities to AFP, is particularly difficult to separate (Pihko et al., 1973, Ruoslahti and Terry, 1976, Johnson et al., 1974, and Pantelouris and Arnason, 1967). An in-depth structure-function analysis of AFP necessitated the development of suitable separation techniques for purifying

AFP, including affinity chromatography, electrophoresis, isoelectric focusing and ion-exchange procedures (reviewed in Smith and Kelleher, 1980).

a) Conventional Separation Techniques

i. Immunoprecipitation

Human AFP was first successfully purified by mixing serum samples from a hepatoma patient with rabbit antisera generated against fetal sera and pre-absorbed with normal human sera. The resulting immunoprecipitates were re-solubilized at low pH and run on gel filtration columns to separate AFP from immunoglobulins (Nishi, 1970). These early precipitation techniques enjoyed widespread use, but are now largely superceded by a variety of affinity chromatography methods.

ii. Affinity Chromatography

Immunoabsorbent affinity chromatography employing anti-AFP Ig coupled to beads is commonly used to purify AFP in large quantities (Wu et al , 1980, Watanabe et al., 1982) One major concern with this procedure has been the use of harsh chaotropic agents such as guanidine or urea in the elution buffers (Ruoslahti and Seppala, 1979). Recent improvements have included the development of a non-linear pH gradient during the elution step, thereby avoiding potential protein denaturation problems (Ferrua and Masseyeff, 1985). Another means of AFP purification is the use of gel-entrapped Ab filtration (Mizejewski et al , 1979) With this method, the Ab is incorporated into a polyacrylamide gel during the polymerization reaction. The gel-Ab is homogenized to form a gel slurry and subsequently employed as an affinity column. The recovery of AFP directly from mouse amniotic fluid (MAF) using this procedure was 30% AFP and ALB also exhibit high affinity binding for a number of heavy metals such as zinc, copper, lead, and nickel (Wu et al , 1987, Freiden 1986). Yet, there are sufficient differences in the metal binding properties between AFP and ALB to resolve these proteins by immobilized metal-affinity chromatography (IMAC) (Sulkowski 1985, Andersson et al., 1987) Andersson et al. have shown that AFP and ALB can be completely separated on immobilized Ni^{2+} -Sepharose 6B columns using mild elution conditions consisting of a falling pH gradient in which AFP is eluted at pH 7.0 followed by ALB at pH 6.0

Since rodent AFPs posses a high affinity binding site for estrogen (Uriel et al., 1975, Hassoux et al., 1977), estradiol affinity columns have been used to purify AFP. Modifications to this procedure have included altering the nature of the ligand by altering the ratios of carbodiimide, diaminononane, and estradiol hemisuccinate coupled to the column (Mizejewski et al., 1980). Mouse AFP can be purified from MAF in a one step batch procedure with recoveries of 29% using defined ligand ratios. Another ligand found to selectively interact with mammalian AFP is the triazine dye Cibacron Blue F3G-A. The F3G-A covalently linked to Sephadex G-100 is deployed as a ligand affinity column However, several passages of AFP are needed to remove contaminating proteins and a second purification step such as DEAE-cellulose is often necessary (Huse et al., 1983). A derivation of this procedure, termed affinity partitioning, has also proven satisfactory in AFP separations. F3G-A is ligated to poly-ethylene glycol (PEG), and this liquid forms one phase of a two phase non-miscible aqueous solution of dextran, PEG, and water. The addition of AFP and ALB results in the partitioning of the proteins between the two phases, with AFP exhibiting a greater partition coefficient for the lower dextran phase than ALB (Birkenmeier et al., 1984).

In the past 2 decades, some of the purification techniques originally intended for separating AFP from other proteins, revealed that AFP from several mammalian species exists as a group of closely related molecular variants (Alpert et al., 1972, Gustine and Zimmerman, 1972a, Bayard and Kerckaert, 1977, Higgins, 1979, Clarke, 1980). Defined by their charge, size and lectin binding differences, the identification and isolation of these molecular variants is currently of some value for clinical diagnostics (Smith and Kelleher 1989, Tsuchida et al., 1989, Kinoshita et al., 1989), and may be important for delineating the biological properties of AFP.

b) Charge Variants

1. Mouse AFP

The serendipitous discovery of charge variants of mouse AFP occurred during a series of investigations examining protein variations which appear during

palate shelf development in fetal mice (Gustine and Zimmerman, 1972a) To better understand normal palate formation and the process of cleft palate, Gustine and Zimmerman isolated 5 closely related proteins considered to be palate specific (Gustine and Zimmerman, 1972b). However, the subsequent analysis of the 5 electrophoretically defined proteins revealed that all were in fact AFP variants which had originated from the fetal plasma and amniotic fluid (Gustine and Zimmerman, 1973). The focus of their research then shifted to the characterization of these 5 isomers, termed Fp1-Fp5. The results published by these authors are summarized in Table II. They noted that early in ontogeny, amniotic fluid consisted mainly of two AFP variants which contained low quantities of sialic acid. At day 14 5, all 5 subspecies were detected. By day 18.5 the proportions of isomers had progressively shifted to the more sialylated variants, particularly Fp5 A similar pattern was noted for the variants in the fetal serum with one major difference (data not shown) By day 16.5, the variant Fp5 already represented 70% of the subspecies, and predominated by day 18.5. Thus, there was a clear distinction in the type of heterogeneity present in MAF versus fetal serum at different gestational stages. These 5 isomers were further defined by their distinct pI values (Table II) which ranged from pH 4.80- 5.2. One variant, Fp5 exhibited 2 distinct isoelectric points

To identify the major tissue group(s) responsible for generating this microheterogeneity, a variety of fetal tissues were extracted and grown in culture in the presence of radioactive leucine and N-acetylglucosamine (Wilson et al , 1976) Five variants corresponding to those normally found in the MAF and fetal serum were identified in the liver and yolk sac. Interestingly, these organs secreted different proportions of AFP subspecies depending on the day they were isolated. Yolk sacs isolated at day 13 synthesized all 5 AFP variants while the fetal liver primarily produced Fp5. If extracted on day 16 5, both organs predominantly secreted the maximally sialylated variant, Fp5. Since day 13 yolk sac cultures produced 7 times as much AFP as the liver, Wilson and Zimmerman suggested that during early ontogeny, the yolk sac is the primary tissue group responsible for the secretion of undersialylated forms into the amniotic fluid and serum This pattern of expression was termed developmental microheterogeneity and was attributed to the levels and specificity of sialyltransferase enzymes in both the liver and yolk sac (Zimmerman and Madappally, 1973, Madappally et al , 1976). More recent

TABLE II

DEVELOPMENTAL HETEROGENEITY OF MOUSE AMNIOTIC FLUID-DERIVED AFP

PERCENTAGE OF INDIVIDUAL ISOFORMS

AFP VARIANT	DAYS OF GESTATION 12.5	14.5	16.5	18.5	Moles NANA/ Moles Protein	pI	
Fp1[a]	52	20	15	5	0.32	5.21	
Fp2	40	28	20	5	1.02	5.13	
Fp3	3	22	15	15	1.37	5.05	
Fp4	3	22	22	15	1.72	4.96	
Fp5	2	4	28	60	2.72	4.86	4.80

	10.5	12.5	14.5	16	17.5
ISO-1[b]	17	8	5	4	4
ISO-2	24	25	12	7	12
ISO-3	28	32	15	13	10
ISO-4	31	26	24	15	0
ISO-5	0	5	28	36	42
ISO-6	0	4	16	24	32

[a]Mouse AFP data obtained from Zimmerman et al., 1973, 1976.

[b]Description of mouse AFP isoforms published by Wong et al., 1988

investigations have supported these findings since glycosylation enzymes have been shown to be expressed in a cell-type specific and developmentally regulated manner (Rademaker et al., 1989, Paulson et al., 1989). For example, Lee et al. have identified 3 distinct sialyltransferases that are differentially expressed in various rat tissues (Lee et al., 1989).

It is also interesting to note that human serum transferrin exists as 5 isoforms differing only in the amount of NANA substituted onto the carbohydrate (Petren et al., 1989) The glycosylation patterns of transferrins are altered in patients with hepatocellular carcinomas due to an increase in highly branched sugars (Yashita et al., 1989).

Charge variants of mouse AFP have also been identified by anion-exchange chromatography. Higgins characterized 4 charge variants of AFP from day 16-19 fetal tissue extracts analyzed on DEAE-ion exchange columns (Higgins, 1979) However, 2 of these charge subspecies co-eluted with ALB, and none of the variants were biochemically characterized. More recently, Wong et al examined the developmental heterogeneity of AFP in samples of MAF run on Mono Q columns linked to an FPLC system (Wong et al., 1988) Six isoforms, termed ISO-1- to ISO-6 were identified and the proportions of each were shown to change through the course of fetal development For comparative purposes, these results are summarized in Table II along with the original results of Zimmerman et al. What becomes strikingly obvious are the gradual changes in the relative concentrations of individual subspecies with time of gestation, with ISO-5 and ISO-6 predominating by day 16. These authors suggested that ISO-1 - ISO-4 corresponded to Fp1 to Fp4 and ISO-5 and ISO-6 conformed with Fp5 as originally identified by Zimmerman

ii. Human AFP

Charge variants of human AFP isolated from the serum of fetuses or hepatoma patients were originally identified by techniques such as isoelectric focusing, ion-exchange chromatography, and agarose gel electrophoresis (Alpert et al., 1972, 1973). AFP purified from these sources exhibited 2 distinct charge variants with unique isoelectric points of 4.85 and 5.20. However, the relative amount of each charge isomer was variable with different tissue isolates and this was partly attributed to the extent of sialylation of the N-linked oligosaccharide.

Digestion with neuraminidase converted the 2 variants to a third form exhibiting a slower electrophoretic mobility. In 1976 Lester et al. defined 3 human AFP electrophoretic variants in fetal liver extracts and hepatoma samples (HAFP1, HAFP2, HAFP3), as revealed by extended agarose gel electrophoresis and crossed immuno-electrophoresis (Lester et al., 1976). Neuraminidase digestion of these variants resulted in the conversion of HAFP3 and HAFP2 to HAFP1 and the generation of a new cathodal migrating band, HAFP0, indicating that sialic acids were only partly responsible for human AFP microheterogeneity (Lester et al., 1977b, 1978a, 1978b, reviewed in Yachnin 1983). Six isoelectric variants of human AFP have been detected on IEF gels containing 8 M urea (Lester et al , 1978a). However, these authors published a subsequent paper suggesting that the increased IEF heterogeneity from 3 to 6 variants was due to HAFP-ampholyte complexes in the urea IEF gels, which resulted in artifactual alterations in the apparent isoelectric points of certain HAFP variants (Lester et al , 1979). Reports have also been published indicating that certain fatty acids bound by human AFP may also contribute to the charge heterogeneity defined by IEF gels. For example, Parmelee et al. showed that the binding of fatty acids resulted in a shift of the pI of human AFP from pH 5 3 to 4 7, and concluded that the pH 5.3 subspecies is fatty acid free (Parmelee et al., 1978). Wu et al have used chromatofocusing procedures on FPLC systems and identified 3 human AFP subspecies that eluted at pH 5.2, 4.5, and pH 4.0 (Wu et al , 1989). The nature of these charge differences was not defined.

c) Lectins

The presence of structural variations in the oligosaccharide portion of AFP molecules has been extensively analyzed by lectins. Lectins are sugar specific, cell-agglutinating or carbohydrate binding proteins that are classified into a number of specificity groups according to the monosaccharide that is the most effective inhibitor of the agglutination reaction (Table III) (Lis and Sharon 1986, Osawa and Tsuji, 1987). A variety of lectins have been used in the analysis of AFP including Concanavalin A, Ricinus communis, Lens culinaris and Wheat germ agglutinin. A combination of lectin affinity immunoelectrophoresis and chromatography procedures is commonly used for the detection and fractionation of distinct glycoforms of AFP from tumor and fetal samples (reviewed in Smith and Kelleher 1980, Krusius and

TABLE III

LECTIN BINDING MOLECULAR HETEROGENEITY

LECTIN[a]	MONOSACCHARIDE	NUMBER OF VARIANTS	
		MOUSE AFP[b]	HUMAN AFP[b]
Concanvalin A	Man	2 (Ruoslahti et al., 1978) 3 (Kerckaert et al., 1979) 4 (Hau et al., 1981)	2 (Mackiewiez et al., 1984) 3 (Wu et al., 1980)
Lens culinaris (LCA)	Man	3 (Kerckaert et al., 1979) 6 (Aussel et al., 1976)	2 (Kerckaert et al., 1980) 3 (Wu et al., 1980)
Ricinus Communis (RCA)	Gal	Multiple (Kerkaert et al., 1979) (Smith et al., 1980)	1 (Smith et al., 1980)
Wheat Germ (WGA)	Glc NAc	1 (Smith et al., 1980)	1 (Smith et al., 1980)
Phytohemagglutin (PHA-E)	-	-	4 (Ishiguro et al., 1985)

[a] Reviewed in Osawa and Tsuji, 1987; and Lis and Sharon, 1986

[b] Lectin binding properties of AFP from different mammalian species was reviewed in Smith and Kelleher, 1980

Additional reviews in Taketa et al., 1983, Kerckaert et al , 1979

Ruoslahti, 1982, Taketa et al., 1983). For example, differences in microheterogeneity patterns detected by Con A and lentil agglutinin have been used to distinguish patients with yolk sac malignancies, benign liver disorders, and hepatomas (Buamah et al., 1981, 1984a, 1984b, 1987, Toftager-Larsen and Norgaard-Pedersen, 1988, Tsuchida et al., 1989, Kinoshita et al., 1989).

Mouse AFP has a larger proportion of lectin reactive isomers than human AFP because of the presence of 2 N-linked sugar chains. Several of the lectin binding variants identified for mouse and human AFP are summarized in Table III and these have been more thoroughly reviewed elsewhere (Smith and Kelleher, 1980, Mackiewicz et al., 1984). There are many mouse AFP variants that appear to react with Ricinis communis agglutinin (RCA) and this probably reflects the extensive NANA substitution on AFP, and the avidity of the RCA lectin for terminal galactose residues. Up to 3 Con A variants of human AFP have recently been identified, but the nature of the carbohydrate differences of each was not determined. The interaction of both human and mouse AFP with these lectins varies considerably if the sugar chains are fucosylated or otherwise modified. For example, Tsuchida et al. showed that Con A could not react with the sugar chain shown in Fig. 2b due to the bi-secting GlcNac Lentil agglutinin was found to react strongly with the fucosylated bi-antennary chain shown in Fig 2a, but this avidity is weakened by the loss of fucose or the addition of the bi-secting GlcNac. Recently, a relatively novel technique termed lectin affinity high performance liquid chromatography has been developed (Green and Baenziger, 1989). By comparing the retention times of glycoconjugates passed over several distinct lectin columns, a fingerprint of that particular oligosaccharide can be obtained and compared to those of known sugar structures Another recent communication has shown that lectin N-linked oligosaccharide cross-linking interactions can form highly ordered lattices which are distinct for different lectins and carbohydrate structures (Bhattacharyya et al., 1989) It will be interesting to see whether these two techniques have future applications in AFP studies.

d) Size variants

Molecular size heterogeneity in several mammalian AFPs hase been detected by both gel chromatography and SDS-PAGE (reviewed in Ruoslahti and Seppala,

1979). A large number of the size differences reported are a result of AFP preparations containing dimers and oligomers (Ruoslahti et al., 1979). Monomeric human AFP exhibiting a MW of 68 kd, can form intermolecular disulfide bonds leading to the appearance of both dimers and trimers (Lester et al, 1978b) The polymers of human AFP could readily dissociate to the monomeric form only upon exposure to disulfide reducing agents. Mouse AFP preparations have also been reported to form large MW aggregates as detected on Sephadex G-200 gel filtration (Hassoux et al., 1977). Wu and Waterhouse proposed that these aggregates are in fact artifacts of the isolation procedure (Wu and Waterhouse, 1982). A second form of size heterogeneity in AFP has been demonstrated by SDS-PAGE, where apparent MWs can vary from 500-2000 daltons (reviewed in Smith and Kelleher, 1980) These differences have been attributed to variable glycosylation patterns or N-terminal polypeptide modifications. For example, two rat AFP variants were identified by SDS-PAGE exhibiting MW of 72 kd and 74 kd, respectively (Peters et al., 1978a). The larger molecule was found to contain an additional 20 aa attached near the N-terminus.

B. LIGAND BINDING PROPERTIES OF AFP

The structural similarities between AFP and ALB prompted many authors to look for similarities in the functional properties of AFP and ALB. The known functions of ALB included the maintenance of intravascular osmotic pressure and the binding and transport of numerous low MW substances in the plasma (reviewed in Kragh-Hansen, 1981). Many comparative binding studies revealed a considerable overlap in the types of ligands complexed by AFP and ALB. Based on its predominant expression during development, AFP is hypothesized to serve as the physiological binding and transport protein in the fetal serum, interacting with numerous endogenous and exogenous substances including free fatty acids (FFA) (Uriel et al., 1987, Torres et al., 1989, Savu et al, 1981, Parmelee et al, 1978), bilirubin (Hsia et al., 1980), trypotophan (Ingvarsson and Carlsson, 1978), metals (Wu et al., 1987), estradiols in the case of rodent AFPs (Savu et al , 1981, Nunez et al., 1987), and retinoic acid compounds (Ruoslahti et al , 1978b)

1 Fatty acids

The knowledge that ALB binds FAs prompted Parmelee et al. to examine the fatty acid binding properties of human AFP isolated from fetal tissue extracts. These authors purified 2 isoelectric variants and analyzed each for the presence of fatty acids. The variant with a pI of 4.7 was found to contain 2.4 moles of FA/mol protein while the pI 5.3 subspecies was fatty acid free (Alpert et al., 1972, Parmelee et al , 1978). The molar ratios of the fatty acids identified and quantitated by gas chromatography are shown in Table IV and include 0.21 for palmitic [16:0], 0.05 for stearic [18:0], 0.66 for oleic [18:1], 0.17 for linoleic [18:2], 0.29 for arachidonic [20.4] and 1.01 for docosahexaenoic acid [22:6]. Fetal ALB purified from the same extract bound only 0.7 moles of FA/mol of protein including 0.05 moles of 20:4 and 0.03 moles of 22:6. Subsequent studies by many investigators have shown that all mammalian AFPs studied to date are able to complex free fatty acids (Parmelee et al , 1978, Ingvarsson and Carlsson, 1978, Berde et al., 1979, Nunez et al., 1987) This ligand binding property is extremely important since free fatty acids are very hydrophobic in nature and are relatively insoluble in the serum. The FA-AFP complex permits their storage, transport, and cell-delivery (Ruoslahti et al., 1979, Kragh-Hansen 1981, Berde et al., 1979). The quantities of FA bound by AFP have ranged from 0.8 - 3.0 mol/mol protein, depending on the species and source of AFP. As illustrated in Table IV, AFP purified from rat amniotic fluid contains about 0.8 mol FA/mol protein, while fetal serum derived rat AFP complexes 1.4 mol of FA/mol protein and rat AFP isolated from fetal tissue extracts has 1.8 mol FA/mol protein (not shown). These variabilities are proposed to be related to the FFA composition of the tissue or fluid from which AFP is purified.

To further define the FFA binding properties of human AFP, Berde and co-workers used a fluorescence enhancement technique (Berde et al., 1979). Cis-parinaric acid, a non-fluorescent polyene FA will fluoresce only upon binding to AFP. By measuring the relative fluorescence intensities of cis-parinaric acid binding at varying protein concentrations and FA/AFP molar ratios, these authors identified 3 FA binding sites on AFP. Scatchard analysis of the data indicated that these 3 sites had decreasing association constants of $2.1 \times 10^7\ M^{-1}$, $9.1 \times 10^5\ M^{-1}$, and 1.4 x

TABLE IV
FATTY ACID COMPOSITION OF HUMAN AND RAT AFP

MOLES FA/MOLE PROTEIN

FATTY ACID	HUMAN AFP Fetal Tissue	HUMAN ALBUMIN Fetal Tissue	RAT AFP Amniotic Fluid	RAT AFP Fetal Serum	RAT ALBUMIN Amniotic Fluid
16:0	0.21	0.1	0.23	0.28	0.81
16:1	-	-	0.09	0.08	0.25
18:0	0.05	0.02	0.04	0.08	0.09
18:1	0.66	0.37	0.17	0.27	0.69
18.2	0.17	0.10	0.06	0.10	0.29
20·4	0.29	0.05	0.06	0.07	-
22.4	-	-	0.03	0.07	-
22 5	-	-	0.02	0.07	-
22 6	1 01	0 03	0.08	0.32	-
TOTAL	**2.39**	**0.70**	**0.8**	**1.4**	**2.3**

*Results summarized from publications of Parmelee et al., 1978 and Calvo et al., 1988

10^5 M^{-1}. In competition experiments, no difference was found in the binding affinities between cis-parinaric acid and 16:0, 18:1 and 22:6, but this has been disputed by several research groups (see later).

The ligand binding properties of mouse AFP were initially characterized by Savu and co-workers (Savu et al., 1981). Employing equilibrium dialysis techniques to measure FA-AFP interactions, these researchers identified 4-5 arachidonic acid binding sites/mol of AFP with an association constant of 0.3 x 10^7 M^{-1}. Mouse ALB was found to bind 20:4 with a 10-fold higher Ka, but had only 1 binding site/mol of protein.

Comparative studies between AFP and ALB have revealed one important distinction in their FA binding properties. AFP predominantly complexes the polyunsaturated fatty acids (PUFAs) such as 20:4 and 22:6 (Aussel et al., 1983, Hsia et al., 1986, Nunez et al., 1987, Savu et al., 1981, Deutsch, 1983). This property is proposed to be of physiological relevance during embryogenesis since the ability of the fetus to synthesize 20:4 and 22:6 from their metabolic precursors is quite limited (Hsia et al., 1987). AFP is hypothesized to bind these FA at the maternal-fetal placental interface and transport them to developing tissues. This is in agreement with recent reports showing that most developing fetal tissues will take up AFP and ALB from the fetal circulation (Laborda et al., 1989). In newborn rodents, AFP is still actively synthesized at several weeks of age and presumably continues to fulfill these transport requirements by binding the essential fatty acids from the mother's milk during the lactation period.

Recently, Uriel et al. have shown that AFP can regulate the transfer of complexed 20:4 into the phospholipid and triglyceride fractions of a rat rhabdosarcoma cell line (Uriel et al., 1987). The FFAs bound by AFP were proposed to enter the cells in a two-component process. The first was a non-saturatable process where the net uptake of the FA was linearly related to the 20:4 concentration This non-saturatable uptake occurred when the AFP concentration was fixed and the concentrations of 20:4 added was greater than 2 mol/mol of protein. If the AFP/FA molar ratio was kept fixed at more physiological values of 0.5, a saturatable binding effect was seen. These results prompted the authors to suggest the existence of AFP specific cell-surface receptors. Upon binding there is an accelerated dissociation of the FA from AFP and the FAs are hypothesized to be

extracted from AFP by plasma membrane FA binding proteins (PM-FABP) (Clarke and Armstrong, 1989).

It is interesting to note that the role of an albumin receptor in the delivery of albumin transported ligands (including FAs) has been the subject of considerable controversy since attempts to isolate and characterize an albumin receptor have been unsuccessful. Weisiger et al. originally reported the presence of an ALB receptor on liver cells which was required for the uptake of fatty acids (Weisiger et al, 1981). However, Reed and Burrington (Reed et al., 1989) have concluded that the albumin receptor effect may in fact be due to a surface-induced conformational change in albumin. Such a surface interaction generates a sub-population of albumin molecules with a higher affinity for the hepatocyte surface. Yet, Schnitzer et al recently identified membrane associated endothelial cell albumin binding proteins (Schnitzer et al., 1988). More research is required to confirm the existence of both ALB and AFP receptors on specific cells, and no membrane receptor proteins for AFP have yet been isolated.

2. Bilirubin

The similarity in the FA binding properties between AFP and ALB prompted several research groups to examine the binding properties for other potential ligands common to the two proteins One such ligand is bilirubin, a catabolic product of hemoglobin and heme proteins. The species of bilirubin generated in the reticuloendothelial system is very hydrophobic in nature and must be transported from the blood to the liver by serum albumin (Brodersen, 1979) Once taken up by the hepatocytes, the bilirubin is enzymatically conjugated with glucuronic acid (Singh and Bowers, 1986) and eventually excreted in the bile The bilirubin binding properties of AFP were initially documented for human and bovine AFP (Ruoslahti et al., 1979, Aoyagi et al., 1979, Hsia et al., 1980) The AFP-bilirubin complexes formed in the fetus may serve an analogous role as that performed by serum albumin in the adult. Since bilirubin is neurotoxic and can cause brain damage in the fetus, Ruoslahti et al. have suggested a protective role for AFP by sequestering bilirubin from developing tissues (Ruoslahti et al, 1978c, 1979). By measuring changes in the bilirubin absorption spectrum in the presence of bovine AFP,

approximately 1.3 sites were identified with an association constant of 1.1 x 10^6 M^{-1}. Fluorescence quenching techniques, which measure the loss of AFP fluorescence in the presence of bilirubin were used to identify one strong and one weak bilirubin binding site on human AFP (association constants 1.1 x 10^7 M^{-1} and 1.8 x 10^5 M^{-1}, respectively). These sites do not compete with FA binding sites and are thought to complex in a region between the first and second domains of AFP (Berde et al., 1979, Aussel et al., 1984).

3. Metals

Metals are another group of natural ligands for ALB and a comparative study with AFP revealed a similar binding activity for zinc, copper, and nickel. Zinc and copper are considered essential trace elements, especially important for the nutritional and metabolic events in neonates and infants (Walravens, 1980). In infants and adults, copper is absorbed by the intestinal mucosa, bound by either albumin or transcuprein, and the Cu-protein complex is transported to the liver. There, the Cu is taken up by the hepatocytes and incorporated into ceruloplasmin or complexed by metallothionein (Freiden 1986). During embryogenesis, AFP is proposed to carry out the short term transport to the liver normally performed by albumin Studies with human AFP have revealed 4-5 high affinity copper binding sites with a Kd= 6-8 x 10^6 M, in contrast to human albumin which has only 2-3 sites and a lower Kd= 1-3 x 10^5 M (Wu et al., 1987). The chelation of copper by AFP is thought to involve the imidazole group of an l-histidine residue located near the N-terminus of the protein. The identification of histidine in the chelation reaction was confirmed by the pH sensitivity of the histidine residue, the loss of copper binding following histidine photooxidation, and the synthesis of an N-terminal peptide containing histidine which was able to bind copper (Aoyagi et al., 1978, Lau et al , 1989). Zinc is another essential metal absorbed in the intestine by the duodenum and then distributed to the various organs. In plasma, 50% of the zinc is loosely bound to albumin while 40% is tightly bound to alpha-2-macroglobulin Wu and co-workers examined the zinc binding properties of both AFP and ALB by gel filtration chromatography (Wu et al., 1987) The affinity of AFP for zinc (Kd= 6-8 x 10^6 M) was found to be 20 fold higher than that for ALB

(Kd= 1-3 x 10^{-5} M). Furthermore, 4-5 high affinity binding sites were identified in AFP in comparison to the 2-3 sites present in ALB. The inhibition studies conducted by these authors suggested that calcium, copper, and lead might be able to bind AFP at the zinc binding sites. Immobilized metal binding studies have shown that both AFP and ALB are able to chelate 0.62 moles of nickel per mol of protein (Andersson et al , 1987).

4. Estradiol

The wide range of distinct ligand binding properties exhibited by AFP were suggestive of an important role in modulating the biological effects of drugs and metabolites, properties which have been well-defined for ALB (Kragh-Hansen, 1981). This hypothesis prompted both Aussel and Savu to examine the potential association of AFP with estrogens since these sterol compounds were particularly prevalent during pregnancy (Aussel et al., 1973, Savu et al., 1981) Studies with mouse AFP have revealed a high affinity binding interaction with estrogens, exhibiting a Ka=8 x 10^{7} M^{-1} with 0 3 binding sites per mol of protein. However these binding properties have only been described for rodent AFP since human, rabbit, and hamster AFP do not display any capacity to bind estrogens (Nunez et al., 1977). In the case of rodent AFPs, both FAs and diethylstilbesterol (DES) can compete for the estradiol binding site and high molar ratios of bound PUFAs can decrease the apparent Ka for estradiol (Vallette et al , 1989) The biological significance of these binding properties has not been fully elucidated In general, it has been observed that organs from immature rodents are insensitive to estrogens while the same adult organs are extremely sensitive to the hormonal effects induced by estradiol. These differences are proposed to be due to the formation of an estradiol/AFP complex which delivers a signal to cells, rendering them refractile to the growth effects of estrogen (Mizejewski et al , 1983, 1989) This can occur in spite of adding a considerable excess of estradiol, much greater than the binding capacity of AFP In order to analyze the biological properties of AFP/estradiol complexes, Mizejewski et al developed a bioassay with adult ovariectomized mice The uterine wet weight of these mice increases 4-fold if they are injected with estradiol, comparable to the normal uterine growth in female mice during their

estrous cycles. If the ovariectomized mice were injected with estradiol in combination with AFP, their uterine growth was suppressed by 82%. During fetal ontogeny, the regulation of uterine proliferation is necessary to permit the successful development of the fetus within the maternal decidual. Based on these results, Mizejewski et al. suggested that AFP binds estradiol, and this complex delivers a signal to cells which prevents their subsequent growth in the presence of estradiol. These authors also noted that aged AFP (several weeks at - 20°C, or repeated freeze thaw) would result in a growth potentiating activity in these mice. In an unrelated study, Aussel and Masseyeff have shown that exogenously introduced estradiol will accumulate in the fetal brain if injected into the maternal circulation (Aussel and Masseyeff, 1983, Aussel et al., 1985). This process is thought to occur via AFP binding and transport, but the physiological importance of this finding is not known.

5. Retinoic Acid (Retinol)

Retinoic acid is a vitamin A derivative that has been shown to interact with AFP. Usually present in the cell membranes as retinol, this compound is normally transported in the blood as a complex with retinol binding protein (RBP) (Blaner, 1989). Very little data has been published regarding AFP-retinoic acid complexes, but Ruoslahti has performed gel filtration experiments with ^{14}C retinoic acid to show that bovine, mouse, and human AFP all bind retinoic acid (Ruoslahti, 1979). Aussel et al. conducted equil brium dialysis and defined a single binding site on human AFP with a Ka=2.6 x 10^6 M^{-1} on human AFP (Aussel and Masseyeff, 1984). The physiological role for this binding is unclear, and ALB has not been reported to bind retinoic acid.

Recent studies have shown that retinoic acid may be the natural morphogen used to generate digit pattern in vertebrate limb morphogenesis (Maden et al., 1988). The action of cellular retinoic acid binding proteins and nuclear retinoic acid receptors is hypothesized to transduce the retinoic acid signal at the level of gene expression (Dolle et al , 1989) Although RBP is expressed at very high levels in the fetal liver and yolk sac and binds retinol with a much higher Ka than AFP, it will be interesting to see whether AFP has any involvement in the mechanisms of

morphogenesis.

C. IMMUNOREGULATORY PROPERTIES OF ALPHA-FETOPROTEIN

The developing fetal and newborn immune system is relatively immunoincompetent throughout the perinatal period in comparison to the adult, and the acquisition of adult-like immune reactivity occurs late in development (Sterz and Silverstein, 1967). There is a considerable amount of evidence suggesting that the potential for immune responsiveness exists early in ontogeny, but this immune reactivity may be down-regulated by a variety of regulatory mechanisms (reviewed in Murgita and Wigzell 1981). Thus, it is proposed that regulatory factors and strong natural suppressor mechanisms are present to modulate the developing fetal and newborn immune system. At the same time, these regulatory events may also moderate the maternal immmune response to the benefit of the fetus (Purtilo et al., 1972, Thong et al., 1973). One potentially important immunoregulatory factor is proposed to be AFP. The distribution of AFP during ontogenic development and in certain diseases is consistent with a regulatory function. Thus, the physiological decrease in AFP expression in the fetus and newborn rodent is seen to correlate with the onset of adult-like immunological responsiveness (Murgita and Tomasi, 1975a, 1975b, Tomasi et al., 1977, Toder et al., 1979, Murgita and Wigzell, 1981) Furthermore, there are certain pathological conditions where AFP is re-expressed and associated with immunosuppression (Newberry et al., 1973, Hodgson et al., 1978) A brief summary of the immunoregulatory properties of AFP is presented in Table V

1. Regulation of Cell-Mediated Immune Responses

Circumstantial evidence suggestive of an immunoregulatory role for AFP first appeared in 1973, when it was shown that serum fractions rich in AFP could inhibit allogeneic mixed lymphocyte reactions in vitro (Parmely et al., 1973) It has since been shown that AFP-containing samples from fetal, newborn, and pregnancy sera and AFP from patients with gastro-intestinal cancers, hepatitis, and liver failure can inhibit certain immune reactions (reviewed in Murgita and Wigzell, 1981) The in

TABLE V

SUMMARY OF THE IMMUNOREGULATORY ACTIVITIES OF AFP

1. Inhibition of T-Cell mitogen-induced lymphocyte proliferation/transformation
2. Suppression of T-D Ab responses
3. Suppression of autologous MLR
4. Suppression of allogeneic MLR
5. Regulation of macrophage function and accessory cell function and Ia expression
6. Induction of natural T and non-T suppressor cells
7. Inhibition of NK cell activity
8. Stimulatory effects on bone marrow cells
9. Suppression of autoimmune diseases in vivo
10. Inhibition of CTL generation
11. Augmentation of in vitro induced lymphocyte blastogenesis

vivo role of AFP in fetal development was indirectly addressed by injecting sheep Ab against mouse AFP intravenously into pregnant rabbits. If injected on day 21 of gestation, the antibodies would induce fetal resorption in about 65% of the embryonic units while normal sheep sera had no effect (Slade, 1973). These results were confirmed in a subsequent study using pregnant mice injected with rabbit antisera to murine AFP (Mizejewski and Grimley, 1976). In these experiments, the fetuses in pregnant mice receiving either anti-transferrin, anti-ALB, anti-complement, or anti-normal mouse sera (NMS) did not undergo resorption. The authors hypothesized that the neutralization of AFP at the maternal-placental interface may have initiated the fetal rejection through a cell-mediated process. Alternatively, they suggested that the mechanism of pregnancy interruption may have been due to the anaphylactoid contraction of the uterine smooth muscle.

a) T Cell Suppression

The first direct evidence that AFP suppressed immune reactions came from a series of studies of MAF (Murgita and Tomasi, 1975a, 1975b). MAF contains three major protein constituents including transferrin (TF), AFP, and ALB When added at the initiation of primary in vitro T-dependent (TD) antibody responses against sheep red blood cells (SRBC), MAF suppressed the plaque forming cell response (PFC) by 95%. To identify the component(s) responsible for this inhibition, Murgita et al prepared a series of antibody-affinity columns specific for either TF, AFP, or ALB Only AFP containing fractions were suppressive, acting in a non-cytotoxic and dose-dependent manner at concentrations from 200 ug/ml to 1 ug/ml Time course experiments indicated that AFP needed to be incubated with the cells for at least 4 hours and added no later than 48 hours after the initiation of the assay Further experiments revealed that AFP was more effective at inhibiting 2° TD Ab responses since the IgA and IgG PFC responses were inhibited by a 100-fold lower AFP concentration than IgM PFCs In a subsequent paper, Murgita et al showed that purified AFP could also suppress PHA, Con A, and LPS induced lymphocyte transformations and allogeneic mixed lymphocyte reactions (MLR) (Murgita and Tomasi, 1975b). Since all of these experiments suggested that T-cell responses were the target of AFP-mediated suppression, Murgita and Wigzell compared the effects of AFP on TD versus T-independent (TI) Ab PFC. In all cases, in vitro TI

responses to dinitrophenol (DNP)-Ficoll or LPS-induced polyclonal antibody synthesis were unaffected by AFP (Murgita and Wigzell, 1976). Subsequent investigations by Yachnin indicated that human fetal AFP could also inhibit T-cell mitogen responses and MLR but no direct effect was noted on B-cell responses (Yachnin et al., 1976, Murgita et al , 1978a) These in vitro results prompted Murgita et al. to speculate that a potential physiological in vivo role of AFP in the fetus and newborn would be necessary to 1) delay the rejection of the histoincompatible fetus by the maternal immune response, 2) prevent the potential of self-reactivity in the fetus and 3) reduce the capacity of the fetus and newborn to react against potentially beneficial maternal antigens. The subsequent down-regulation of AFP expression would then permit a gradual maturation of Ab responsiveness in the first few weeks of life.

The findings documented by Murgita and Yachnin supported the contention that AFP selectively interferes with T-cell function. Based on the knowledge that the presence of AFP for 8 hours in culture was sufficient to maintain TD Ab suppression in an AFP-free assay for 5 days, Murgita et al. examined the possibility that AFP might indirectly activate regulatory suppressor cells (Murgita et al., 1977). Spleen cells were cultured for 72-96 hours with AFP and then added to a primary TD Ab assay. The addition of the pre-treated spleen cells directly to a primary TD Ab assay resulted in a 60 % suppression of the IgM PFC to SRBC. If Ig-anti-Ig column purified splenic T-cells were used in place of whole spleen cells, the IgM response could be suppressed by 95%. The authors proposed that AFP induces the development of T suppressor cells This hypothesis was supported by the lack of suppressor activity in pre-cultures of spleen cells from nude mice. The suppressor cells were effective in numbers as low as 0.05% of the total cell population.

Since AFP is naturally present in newborn mice, the authors also examined the function of spleen T cells purified from these neonates on TD and TI Ab responses (Murgita et al., 1978a). Newborn spleen cells from 5 day old mice efficiently suppressed primary TD responses by 50%. This activity was age dependent since a gradual diminuition of suppression occurred from 5- 20 days after birth, correlating with the decline in serum AFP levels. Phenotypic studies indicated that these T-cells were of the Lyt1$^+$23 ($CD4^+CD8^-$). Of particular relevance to these reports was the finding that AFP, when incubated in vitro with adult spleen

cells, would induce the generation of inhibitory T cells of the same phenotype as those naturally present in the newborn (Murgita et al , 1981).

All of the evidence described above indicated that the effects of AFP in vitro are restricted to functions requiring T cells. To better define the cellular and genetic restrictions in the immunoregulatory activity of AFP, Peck and co-workers undertook a systematic study of the regulatory influences of AFP on the proliferative and effector phases of T-cell mediated immune reactions, including in vitro MLR and cell-mediated lympholysis (CTL) assays (Peck et al., 1978a) Primary allogeneic MLR were generated between purified splenic T-cells and gamma-irradiated splenic stimulators Proliferative responses between major histocompatibility complex (MHC) disparate strains were suppressed 3-6 fold with AFP concentrations of 150 ug/ml added at the onset of cultures. Allo-antigen reactive T-cell blasts generated in a primary MLR could also be suppressed by AFP if re-stimulated in a secondary MLR. By comparing the inhibitory effects of AFP on other strain combinations, Peck et al. noticed that the degree of suppression was extremely variable and a function of the MHC differences between mouse strains. Using MHC recombinant strains possessing limited genetic differences at either MHC class I, class II, or non-MHC regions, the inhibitory effects of AFP were found to be directed specifically against MHC CII region incompatibilities In certain cases AFP could also suppress MLR involving non MHC antigens including the minor leukocyte stimulatory (MLS) system. Yet, in other mouse strains possessing either MHC, non-MHC, or even MLS incompatibilities, AFP could enhance the proliferative response in the MLR This was particularly evident in strain combinations including B10.D2 anti-DBA/2, CBA/J anti-DBA/2, and B10 BR anti-CBA/J The responding cell susceptible to AFP-mediated regulation was thought to be the Lyt1$^+$23$^-$ ($CD4^+CD8^-$) cell

In this initial phase of investigation, Peck et al. established that MLR generated against MHC CII molecules was actively suppressed by AFP In view of these results, it was predicted that CTL generation would remain unaffected by AFP since these reactions are controlled by MHC CI antigens. To further explore this, Peck et al generated CTLs using the previously outlined strain combinations (Peck et al., 1978b) In most of these experiments, AFP was found to prevent the in vitro activation of CTL irrespective of the H-2 genetic disparity present Kinetic studies indicated that AFP needed to be added within 48 hours and for at least 12 hours of

a 5 day MLR in order to prevent the activation of CTL. The direct addition of AFP to a CTL assay failed to alter the cytotoxic response against any target. As had been observed in the MLR reactions, certain strain combinations were refractive to AFP induced suppression. Interestingly, these authors found that the AFP-induced suppression of CTL development could be circumvented if supernatants derived from normal MLR cultures were added to the AFP suppressed cultures following the removal of AFP at 36 hours of a 5 day culture (Peck et al., 1982). This supernatant activity could be obtained from any H-2 incompatible MLR and was found not to be IL-2. Furthermore, the proliferation of Percoll density gradient purified T cells were refractive to AFP induced suppression in a primary MLR. The authors suggested that AFP was acting on a monocyte/macrophage-like cell.

Although the effects of AFP on allogeneic MLR are variable, Peck et al. clearly established that AFP can selectively interfere with CII associated immune responses. To further delineate the cellular restrictions of AFP-mediated immunoregulation, Hooper and co-workers investigated the effects of AFP on autologous mixed lymphocyte reactions (AMLR) (Hooper and Murgita 1981). While the exact nature of these reactions remains unclear, AMLRs are regarded as <u>in vitro</u> expressions of anti-self reactivity because they occur in the absence of foreign antigen and exhibit memory and specificity. Murine AMLRs mediated by either neonatal or adult T cells are suppressed in a highly efficient manner by AFP at concentrations of 10 ug/ml, well-below that required for allogeneic MLR (Hooper and Murgita, 1981). These authors determined that both newborn and adult murine AMLR involved the recognition of MHC CII gene products by Lyt 1^+ 23^- T cells. Since the ability of AFP to suppress these reactions was well below endogenous serum AFP levels in the mouse, it was hypothesized that an important function of AFP in the fetus and newborn may be to control the expression of self-reactive lymphocytes (Hooper et al, 1982) Subsequent studies with human fetal and hepatoma AFP samples revealed that human AMLRs as opposed to allogeneic MLRs, are highly sensitive to AFP-mediated suppression (O'Neill et al., 1982). Heterologous AFPs from the human and rat were also able to suppress a murine AMLR

b) Role of AFP in the Regulation of Macrophage MHC CII Expression

Macrophages, dendritic cells, and other antigen presenting cells (APC) are essential for the induction and regulation of most types of humoral and cell-mediated immune functions (Weaver and Unanue, 1990). Antigens taken up by these cells either undergo some sort of intracellular processing or are synthesized de novo and re-expressed as small peptides in association with MHC CI or CII molecules (Unanue and Cerottini, 1989). These events coupled with the secretion of lymphokines are necessary for the activation and multiplication of T-cells (Inaba et al., 1988, Houssiau et al., 1989). The central role of these APCs in initiating immune responses and the lack of responsiveness in the fetus, resulted in the initial suggestion that one of the sites of action of MAF may be at the level of the macrophage (Suzuki and Tomasi, 1980). Suzuki and Tomasi utilized a lymph node (LN) T cell proliferation assay to address this possibility. The proliferative response of ovalbumin (OVA) primed LN T cells is marginal in the presence of OVA unless the OVA is added in combination with peritoneal exudate macrophages (PEC) When the PECs were pre-cultured for 24 hours in the presence of MAF followed by a 2 hour OVA pulse, the OVA-pulsed PECs were no longer capable of presenting antigen to the T cells, as measured by a lack of T cell proliferation However, if the LN T cells were initially pre-cultured with MAF, washed and subsequently mixed with OVA-PEC, a normal proliferative response ensued, suggesting that AFP acted directly on the APC. The subsequent experiments of Peck et al. indicated that the effect of AFP on T-cells are the result of AFP acting on a monocyte-enriched cell population (Peck et al., 1982). Based on these reports and the hypothesis that AFP contributes to the immunological hyporesponsiveness of the fetus, Lu et al directly examined the effects of AFP on the expression of MHC CII molecules (Ia) and the antigen presenting capacity of macrophages (Lu et al., 1984, 1989) In these experiments, peptone elicited peritoneal macrophages were harvested from B10 A adult mice and isolated by their adherence to glass cover slips. These cells were incubated for 5 days in the presence of lymphokines, paraformaldehyde fixed, and examined for their cell surface expression of Ia molecules by immunofluorescence AFP, if added at the onset of these cultures at concentrations of 100 ug/ml could inhibit 80% of the surface Ia expression compared to control untreated cells, whereas fetal calf serum (FCS), fetal bovine albumin (FBA), and bovine AFP were without effect. AFP added 2-3 days after the initiation of cultures exerted

progressively less inhibitory activity. These authors noted that AFP did not diminish MHC CI expression, had no effect on cell viability, and failed to impair the binding and opsonization of bacteria. The aforementioned results prompted the authors to speculate that AFP may have significance as an immunoregulatory protein during fetal gestation by delaying the appearance of Ia-bearing cells which would permit the acquisition of tolerance and prevent autoreactive T cell activation. These results would agree with the earlier observation that neonatal macrophages have a defective antigen presenting function (Lu et al., 1979, 1980). This was found to be due to the significantly lower amount of CII expression on the neonatal macrophages versus that shown for comparable numbers of adult macrophages (Lu et al., 1979). Crainie et al. recently reported that AFP could also suppress the constitutive expression of Ia antigens on bone marrow derived macrophages and on a dendritic cell line (Crainie et al., 1989). In further experiments, these authors showed that AFP could inhibit lymphokine-activated Ia expression on macrophages subsequent to their induction.

c) Inhibition of Natural Killer cell Activity

Natural killer (NK) cells are a distinct population of lymphoid cells thought to be involved in tumor cell surveillance, rejection of virally infected cells, and hematopoietic stem cell regulation (Roder et al., 1981). Since cells expressing embryonic antigens such as neonatal thymocytes are also NK sensitive, the activation of NK cells early in ontogeny may be potentially harmful to the fetus (Hansson et al., 1981). In general, developing embryos have a very low level of NK activity until after birth (Roder et al., 1981). Since the increases in NK activity in neonatal mice correlated with the normal physiological decline in AFP expression, Cohen et al examined the potential effects of AFP on NK cells (Cohen et al., 1986). In initial experiments, AFP failed to exhibit any effect on the endogenous or lymphokine activated NK cytolytic mechanism when added directly into the cytotoxicity assay. However, if AFP was added to the cells during the lymphokine induction phase, the responding NK cells exhibited a 2-3 fold reduction in activity, indicating that AFP prevented the induction or activation of NK cells. Although newborn spleen NK cell function is always very low in vitro and in vivo, neonatal NK cytolytic activity can be markedly enhanced in vitro in the presence of

lymphokines. AFP, if added at the onset of these neonatal cultures, suppressed their subsequent induction. The authors speculated that the high endogenous levels of AFP in the fetus and neonate were responsible for the naturally low levels of NK cell activity. Such a regulation would be necessary to prevent the potential destruction of fetal cells and tissues by the NK cells. This hypothesis is strengthened by previous reports showing high NK cell activity in the murine decidua which peaks at day 6.5 and is followed by a rapid decline to negligible levels by day 10 (Croy et al., 1985, Gambel et al., 1985), thus paralleling the onset of AFP expression. Recently, Gendron et al. have shown that infiltrating maternal decidual NK cells are associated with abnormally high spontaneous resorption rate in CBA/J x DBA/J conceptuses (Gendron and Baines, 1988). This is the same strain combination previously shown to be refractory to AFP mediated immunosuppression, as assayed in a MLR (Peck et al., 1982). The high spontaneous abortion rate in these mice was correlated with the deficiency of a lipophilic suppressor factor (Gendron and Baines 1988, 1989, 1990). This would suggest that the regulation of maternal NK cell may involve several biological mediators including prostaglandins, non-T suppressor cells (Clark et al., 1986), and at later stages of gestation, AFP In an unconnected study, chicken AFP has been shown to suppress Japanese quail NK cell activity (Yamada et al., 1983).

d) Stimulatory Effects on Bone Marrow Cells

The knowledge that serum ALB is essential for in vitro growth of activated human lymphocytes (Polet and Spieker-Polet, 1975) and the observation that AFP can induce inhibitory T-cells in vitro (Murgita et al., 1978a), and enhance certain allogenic MLR combinations (Charpentier et al., 1977, Peck et al , 1978) prompted Hoskin et al. to examine the potential growth promoting effects of AFP on different lymphoid cell populations (Hoskin et al., 1985c) A comparative study of various adult lymphoid organs indicated that AFP had a strong stimulatory effect on murine bone marrow cells while spleen, lymph node, thymus and peripheral blood lymphocytes remain unaffected To identify the cell type(s) stimulated by AFP, the bone marrow cells were separated into distinct subsets with the lectin soy bean agglutinin (SBA). AFP was able to exert a strong yet distinct growth promoting effect on both cell populations. The maximal proliferation of the SBA+ subset

occured at 48 hours, while the SBA- cells were maximally stimulated at 96 hours. The differential effects of AFP on these 2 subsets was further distinguished by the observation that the SBA+ fraction was stimulated with as little as 12.5 ug/ml of AFP while the SBA- subset required at least 200 ug/ml. A phenotypic characterization showed that the SBA+ cells were non-T, sIg- bone marrow cells while the SBA- preparation was $Lyt1^{+}23^{-}$ $Thy\text{-}1^{+}$ $MICG^{+}$, very similar to the newborn spleen T suppressor cells and AFP-inducible adult T suppressor cells (Murgita et al., 1977, Hooper et al., 1986).

e) Natural T and non-T Suppressor Cells

The finding that AFP stimulated the growth of distinct bone marrow cell subsets with phenotypic similarities to both T and non-T natural suppressor cells prompted Hooper and associates to propose that AFP may play an important role in inducing these populations during pregnancy (Hooper et al., 1986). The induction of T like suppressor (Ts) cells would be consistent with the original reports of Murgita et al. documenting the presence of AFP-inducible Ts cells in the adult spleen capable of suppressing TD antibody responses (Murgita et al., 1977). The second phenotypic population of cells are the non-T natural suppressor cells (NS), present in the spleens of isopregnant and allopregnant mice These cells are thought to function by inhibiting autoreactive lymphoproliferative responses against fetal and adult tissues (Hoskin et al., 1983, 1985a, 1985b, 1985c, 1989), including tissue specific antigens on the placenta and oncofetal antigens on fetal cells (Hamilton, 1983) These naturally occuring suppressor cells may be recruited by fetally-derived AFP from a pool of pre-existing NS cells present in the bone marrow.

f) Experimental Autoimmune Diseases

AFP has also been shown to prevent and ameliorate experimental autoimmune diseases such as myasthenia gravis (MG), induced in rats by immunization with the acetylcholine receptor (ACHR), and in mice following the passive transfer of human myasthenic immunoglobulin (Brenner et al , 1980, 1984, Bushman et al , 1987). Based on these observations, Brenner et al. suggested that the immunosuppressive effects of AFP may be correlated with the clinical remissions of MG during the second half of pregnancy Since immune responses to

ACHR are dependent on $CD4^+$ T cells and MHC CII expressing APCs, Buschman et al. suggested that the AFP treatment in the mice altered either CII expression on the APC, exerted direct anti-proliferative effect on T-cells, or induced a subset of suppressor cells.

g) Additional Suppressive Properties

In a study unconnected with immunosuppression, Aoyagi et al. examined the effects of AFP in a rabbit reticulocyte lysate cell-free protein synthesis assay (Aoyagi et al., 1982). Normally, a high globulin synthesis, as measured by [^{3}H]-leucine incorporation, is generated in this cell free system. AFP could markedly suppress [^{3}H]-leucine incorporation at levels 50% below control responses, whereas serum albumin had no such effect. A thorough analysis indicated that the suppression was not due to any contaminating ribonuclease activity. Studies with poly U sequences suggested that the inhibition occurred during the initiation of protein translation. Sucrose gradient analysis of ribosome-RNA complexes revealed that this interaction did not occur in the presence of AFP However, the physiological significance of these findings is unknown (Aoyagi et al , 1982)

D. BIOCHEMICAL RELATIONSHIP OF MOLECULAR STRUCTURE TO BIOLOGICAL FUNCTION

During the last 15 decades, a large body of information has been presented supporting the concept that AFP can exert selective immunoregulatory influences on immune responses. However, there are a number of reports which have either failed to demonstrate any immunosuppressive activity by AFP or have documented considerable variabilities in the immunosuppressive potency of different AFP preparations (reviewed in Sell, 1980). These inconsistencies have been variously attributed to a) lack of a role for AFP in immunoregulation, b) differences in the methodology of purification and functional assay, c) loss or absence of biologically active, non-covalently complexed molecules normally bound by AFP, d) source of AFP (tumor versus fetal), and e) post-synthetic modifications contributing to the formation of active (inactive) molecular subspecies of AFP

1. Reconciliation of Putative Failures to Identify Immunosuppressive Activity in AFP Isolates

There are a number of reports documenting an inability of AFP preparations to suppress immune responses, and certain studies have shown that AFP can stimulate particular reactions including blast transformation, MLR, and the generation of cytotoxic T cells (Charpentier et al., 1977, Parmely and Thompson, 1976, Sell et al., 1977) In 1977, Sheppard and Sell first reported in a series of papers that both mouse and rat AFP, isolated from amniotic fluid, fetal serum, or transplantable hepatomas were unable to suppress rat and mouse spleen cell mitogen responses and allogeneic MLRs (Sheppard et al., 1977, Sell et al., 1977) It was also observed that spleen cells, if pre-incubated with AFP would demonstrate a 8-9 fold enhanced cytolytic activity on P815 tumor cell targets. In the same year, Charpentier et al reported that human AFP preparations isolated from a patient with hepatocellular carcinoma augmented human MLR (Charpentier et al., 1977). These groups of reseachers concluded that AFP is not immunosuppressive and that previously published reports describing the inhibitory effects of AFP were a result of impure AFP fractions containing other suppressive materials. However, many of these reports failed to consider the cellular and genetic restrictions in AFP mediated immunoregulation (Peck et al., 1978a, 1978b, 1982, Murgita and Wigzell, 1979).

Many groups examined the suppressive properties of AFP on allogeneic MLRs However, a convincing series of experiments has shown that human and murine AFP are extremely effective at inhibiting MHC CII restricted MLR and in particular autologous MLRs, as opposed to the considerable variability noted for allogeneic MLRs (Peck et al , 1978a, 1978b, Hooper and Murgita, 1981, O'Neil et al , 1982)

Another potential explanation for the aforementioned discrepancies is the mitogen stimulated spleen cell assay employed by several research groups to characterize the suppressive activities of AFP preparations Studies from this lab have shown that mitogen stimulated spleen cells are only marginally suppressed by AFP unless the T cells are initially purified. Furthermore, Hooper et al. have demonstrated that mitogen stimulated CD4$^+$CD8$^-$ thymocytes are the most sensitive population of cells inhibited by AFP while peripheral lymphoid T cells undergo only

a marginal degree of inhibition (Hooper et al., 1987).

The studies of Sheppard did reveal a 2-fold reduction in primary and secondary anti SRBC IgM PFC responses. However, Murgita and Tomasi had previously reported that secondary TD IgA and IgG are markedly suppressed by AFP in contrast to the weak suppression of secondary IgM responses. In many of the studies failing to demonstrate any suppressive effect by AFP, high concentrations of fetal bovine serum (FBS) were used in the assays instead of autologous NMS, and the normal immunosuppressive properties of AFP may have been masked by stimulatory effects present in certain FBS preparations (Murgita et al., 1978a). Finally, the reports that AFP could enhance certain reactions are entirely consistent with the growth promoting properties of AFP on certain lymphoid cell populations (Murgita et al., 1977, Hoskin et al., 1985c, Hoskin et al., 1989b).

a) Effects of the Purification Conditions on AFP

There were also numerous reports suggesting that the conditions of AFP purification could potentially disrupt the functional activity of the protein. In contrast to the conclusions of Sheppard and Sell, Goeken and Thompson reported that AFP isolated from human cord sera could inhibit MLR at concentrations ranging from 50-400 ug/ml (Goeken and Thompson 1977). Albumin purified from the same cord sera preparation exhibited an identical suppressive effect while ALB from other sources showed no effect. These authors also found that the conditions required for eluting AFP or ALB off their respective affinity columns could markedly affect their inhibitory properties. When AFP and ALB were purified under conditions requiring 0.15 M NaCl in the elution buffer, both proteins lost activity. If re-dialyzed against 0.5 M KCl, the suppressive activity of both preparations could be restored. The authors postulated that the 0.5 M KCl might either cleave an inhibitor normally bound by AFP or induce a conformational change in both proteins generating an "active" form. It should be noted that in this report, the purported suppression was a 50-100 fold reduction in the MLR response. Almost all other studies have described reductions of 3-6 fold in an allogeneic MLR. The Goeken and Thompson suppression data should be interpreted with caution.

b) Contributions of Exogenously Bound Molecules

Several groups interpreted the aforementioned reports to be the result of the loss of an exogenously bound moiety during either the isolation or the purification steps in isolating AFP. This argument was further strengthened by the knowledge that AFP is an essential binding protein in the serum during fetal development, complexing a large number of defined and potentially unknown ligands.

i. Estradiol

Keller and co-workers reported that the conditions involved in purifying AFP may be responsible for the immunosuppressive variabilities shown for different AFP preparations (Keller et al., 1977). In contrast to the results of Goeken et al., these authors found that dialyzing mouse AFP against 0.5 M KCl resulted in a loss of activity. These findings, combined with the earlier observations that AFP binds estrogens, prompted Keller et al. to examine the relationship between estrogen binding and immunosuppression. By adding estradiol directly to a non-functional AFP sample (following KCl dialysis), the immunosuppressive activity of the AFP could be restored However, the estradiol-AFP mixture caused a 100-fold decrease in the MLR response which was 10 fold lower than that detected for control untreated AFP. The authors did not comment on these discrepancies and failed to provide any cell viability data which is critical since large amounts of estradiol can be toxic to numerous cell types (Mizejewski et al, 1989). The knowledge that only rodent AFP can complex estradiol, combined with the fact that AFP from many mammalian species is immunosuppressive makes it unlikely that estradiol is an important factor in AFP-mediated immunoregulation. Furthermore, human, rat and mouse AFP can all significantly inhibit a murine AMLR, suggesting a common mechanism of suppression must exist for AFP from all 3 species (Hooper et al., 1982).

ii. Polyunsaturated Fatty Acids

As previously mentioned, mammalian AFPs bind PUFAs with high affinity, and there have been claims that this binding property is required for AFP-mediated immunosuppression (Deutsch et al., 1983, Nunez et al., 1987). Deutsch and co-workers performed mitogen assays on several human AFP preparations including native AFP, AFP defatted by charcoal adsorption, and delipidated AFP reconstituted with arachidonic acid (AA) They found that fatty acid-free AFP could not suppress

the mitogen responses unless reconstituted with AA. Based on these findings, the authors hypothesized that the inhibition of lymphocyte responses by AFP was due to the delivery of the polyene FA 20:4 to the stimulated lymphocytes Unfortunately, Deutsch failed to quantitate the effectiveness of delipidation, and provided no information regarding the amounts of AA that were re-associated with defatted AFP Support for the hypothesis that PUFAs are required for AFP-mediated suppression apppeared in a subsequent series of experiments conducted by Nunez et al (Nunez et al., 1987). These authors found that the inhibitory activity of mouse AFP on PHA-stimulated spleen cells varies from preparation to preparation of AFP. If 1 ug of 22:6 were added to 10 ug of an AFP sample in the mitogen assay, the inhibitory effect of AFP could be increased 1-2 fold. Both Nunez and Deutsch surmised that the fluctuations in the suppressive potency of different AFP preparations was a function of the PUFA to saturated FA ratio complexed to AFP Vallette and co-workers recently described that conformational changes in rodent AFP, as defined by UV-spectral changes, are a function of the PUFA composition of AFP (Vallette et al., 1989). Preparations of AFP that were specifically re-associated with 22:6 exhibited a unique UV-spectral pattern, a lower Ka for estradiol and a loss of immunoreactivity as measured in ELISA and RIA assays. These authors suggested that the tissue sources from which AFP is isolated will vary in PUFA composition, and this would contribute to AFP preparations differing in their PUFA and FFA content. These differences could confer unique conformational states on AFP, resulting in the appearance of AFP "haloforms" The haloforms were proposed to differ in their physiological properties such as immunosuppression

It is now well-documented that the dietary FA composition can modulate the amounts and types of eicosanoids synthesized in tissues Two particularly important PUFAs are AA and docosahexaenoic acid (DHA), of which AA is the direct precursor of the biologically active eicosanoids including prostaglandins (PG), thromboxanes, leukotrienes, and lipoxins, all of which can modulate various immune responses (reviewed in Hwang 1989, Nicosia and Patrono, 1989) Although the main intracellular sources of AA are phospholipids such as phosphatidylcholine and phosphatidylinositol-4,5 bis-phosphate (reviewed in Exton, 1990), the dietary content of AA and DHA can directly influence the eicosanoid pathway utilized (cyclooxygenase versus lipoxygenase) (Hwang, 1989) To address the question as to

whether AA metabolites are involved as secondary messengers of AFP-mediated immunosuppression, Yachnin examined the effects of FAs, PGs and cylcooxygenase inhibitors on mitogen responses (Yachnin et al., 1980b, 1980c). Although FAs had no effect when directly added to these assays, PGE_2 could inhibit the response. However, the kinetics of PGE_2 suppression were distinct from those of AFP, and indomethacin had no ability to abrogate the activity of AFP. Consistent with these results was the finding that AFP-mediated inhibition of class II MHC expression on macrophages was not due to the presence of AA or the production of PGs (Lu et al., 1984). However, these experiments did not rule out the possibilty that the AFP-mediated delivery of PUFAs directly to T-cells caused unresponsiveness. Of relevance to this hypothesis is the finding that AFP can mediate the transfer of AA to cells (Uriel et al., 1987), and blastogenic $CD4^+$ and $CD8^+$ T-cells express receptors for AFP (Laborda et al., 1987, Torres et al., 1989). More recent results have shown that AA can directly affect cellular responses. Examples in the literature have shown that ALB-AA interactions can modulate platelet aggregation (Purdon et al., 1989). Yet the effects of exogenous FAs on T cell responses are unclear. Chow et al. have shown that PUFAs including 18:2, 20:4, and 22:6 can stimulate an increase in intracytosolic Ca^{2+} levels in T cells independent of phophotidylinositide turnover and Ca^{2+} influx (Chow et al., 1990). Contrasting these results, Richieri and co-workers have found that FFA can inhibit both Ca^{2+} release, and degranulation events in a CTL clone stimulated with allogeneic targets (Richiera et al., 1990).

d) Post-Translational Modifications

Several researchers have pursued the possibility that the immunoregulatory activity of AFP may be the result of post-transcriptional and post-translational modifications (Lester et al., 1976, Zimmerman et al., 1977) Many secreted proteins undergo significant co- and post-translational modifications within the lumen of the endoplasmic reticulum, in the Golgi, or during and subsequent to secretion. These modifications may include cleavage of the N-terminal signal sequence, glycosylation, hydroxylation of proline and lysine, gamma-carboxylation of glutamine, fatty acylation, protein phosphorylation, and protease cleavage (Table VI) (reviewed in Wold, 1981). AFP is known to undergo proteolytic processing of its signal peptide, has numerous di-sulfide bonds, and is glycosylated with a variable NANA content, but additional modifications have not been identified

As previously mentioned, AFP exhibits considerable microheterogeneity in many mammalian species. The biochemical properties contributing to this heterogeneity have not been elucidated, but variability in the carbohydrate chains and terminal sialic acid (NANA) residues are partly responsible (Lester et al , 1976, 1977a, 1977b). Furthermore, an analysis of the heterogeneity profiles of AFP variants revealed predictable changes in the ratios of isoforms during fetal development. This raises the intriguing possibility that distinct subspecies of AFP may have functional relevance. These properties prompted Yachnin et al to examine the relationship between human AFP charge heterogeneity and immunosuppressive activity (reviewed in Yachnin, 1983). Their results demonstrated that human AFP samples containing large amounts of a relatively electronegative isomer (HAFP-3, see earlier) had the greatest capacity to suppress mitogen responses (Table VII) Since part of the microheterogeneity of human AFP was already known to be related to the degree of sialylation on the N-linked carbohydrate, Lester et al subsequently examined the role of sialic acid residues in the mechanism of AFP-mediated immunosuppression (Lester et al , 1976) Comparing different AFP preparations, the authors found that no correlation existed between NANA content and the ability of these samples to inhibit mitogen responses. Furthermore, neuraminidase treatment of AFP did not abolish immunosuppressive activity indicating that NANAs were not essential for the bioactivity of AFP A similar investigation with mouse AFP undertaken by Zimmmerman yielded the opposite conclusion (Zimmerman et al., 1977). Having previously identified 5 mouse AFP variants, these authors reported that only the more sialylated variants were active as assayed on TD Ab responses. Neuraminidase digestion of these variants completely abrogated the inhibition, leaving unresolved the role of NANA on AFP.

Although the N-linked oligosaccharides are well recognized for their contribution to the stabilization of protein conformation, the regulation of metabolic protein half lives in the circulation, and cell-protein recognition events (Paulson, 1989), the role of these carbohydrates in AFP-mediated immunoregulation remains to be elucidated. Some functional roles for carbohydrates have been reported (Florman et al., 1985). For example, human chorionadotropin (hcG), required for normal development and secretory activity of the gonads, has 4 N-linked oligosaccharides. Deglycosylated hCG will still interact with its receptor, but can no

TABLE VI

POST-TRANSLATIONAL MODIFICATIONS OF PROTEINS

AMINO ACID	TYPE OF MODIFICATION
α-amino terminus	N-acylation
α-carboxyterminus	amidation, ADP-ribosylation
arginine	methylation
asparagine	N-glycosylation
aspartic acid	phosphorylation, methylation
cysteine	intra-, inter-chain di-sulfide bonds
glutamate	carboxylation
glutamine	deamidation (forms glutamate)
histidine	methylation, phosphorylation
lysine	hydroxylation, methylation
proline	hydroxylation
serine	0-glycosylation, phosphorylation
threonine	phosphorylation
tyrosine	halogenation, phosphorylation

References: reviewed in Wold, 1981, and Yan et al, 1989

longer activate intracellular events (Sairam 1989).

Lester et al. concluded that human fetal AFP, initially secreted in an electronegative form (HAFP3), contains all the immunoregulatory activities of the molecule. Upon secretion and release into the extravascular compartments, an undefined post-synthetic modification converts this isoform into electropositive subspecies (Lester et al., 1977a) which lacks the suppressive activity. The authors proposed that the variabilities in inhibitory activities of different AFP preparations was due to the level of HAFP3 in these samples (Table VII).

E. RATIONALE FOR THESIS WORK

In the last two decades, significant advances have been made regarding our understanding of the physical-chemical properties of AFP, its developmental expression and pathological distribution, and its diagnostic potential. Despite these advances, the relationship of molecular structure to biological function(s) has not been fully elucidated. The major objective of this study was to explore the functional significance of the molecular microheterogeneity of AFP In this regard, it became imperative to also examine the potential contribution of non-covalently associated molecules to the immunoregulatory properties of AFP isoforms. The results from this investigation will a) outline the design of an analytical and preparative scale separation protocol for identifying and purifying mouse AFP isoforms (Chapter II); b) define the biochemical characteristics distinguishing individual isoforms (Chapter III), c) assign all of the immunoregulatory activities of AFP to a single unique isomer (Chapter III); d) provide evidence that long chain PUFAs including AA and DHA are not responsible for AFP-mediated immunoregulation (Chapter IV); and e) examine the functional activities of AFP isolated from different gestational time points (Chapter V).

TABLE VII

CORRELATION OF BIOLOGICAL ACTIVITY
WITH HUMAN AFP VARIANTS[a]

HAFP SOURCE	BIOLOGICAL POTENCY (μg/ml)[b]	% OF TOTAL HAFP		
		HAFP1	HAFP2	HAFP3
Fetal Liver	3	-	-	100
Hepatoma 1	20	20	50	30
Hepatoma 2	130	20	51	20
Hepatoma 3	1000	30	41	29
Hepatoma 4	2000	28	53	19

[a]Data obtained from Yachnin et al., 1976a, 1976b

[b]Biological potency is the concentration of AFP required to inhibit a T cell mitogen response by 50%

II. ANALYTICAL AND PREPARATIVE SCALE SEPARATION OF MOLECULAR VARIANTS OF ALPHA-FETOPROTEIN BY ANION-EXCHANGE CHROMATOGRAPHY ON MONOBEAD™ RESINS

A. INTRODUCTION

The classical approaches to AFP detection and purification have included a combination of both chromatographic and electrophoretic techniques (Smith and Kelleher, 1980). Several such separation procedures have indicated that AFP exists as a heterogeneous population of molecules (Alpert et al., 1972, Kerckaert et al., 1979) Thus, molecular variants of AFP have been observed with such procedures as polyacrylamide gel electrophoresis (Zimmerman et al , 1976), isoelectric focusing (Alpert et al , 1972), ion-exchange chromatography (Higgins, 1979), and lectin and hormone affinity chromatography (Bayard et al., 1977, Mizejewski et al , 1980). Furthermore, reproducible changes in the ratios and concentrations of isomers was found to occur during normal development and in certain disease states (Smith and Kelleher, 1980) In addition, investigations by Lester et al. have demonstrated a positive correlation between the presence of certain molecular variants in fetal or tumor derived AFP samples and in vitro immunosuppressive strength (Lester et al , 1976, 1978). A major obstacle to further detailed structure/function studies of AFP molecular variants has been the lack of a suitable procedure for the purification of individual isoforms of AFP In this paper, we describe both an analytical and preparative FPLC™ anion-exchange procedure that is capable of meeting these requirements

The characterization of human AFP microheterogeneity is already of some practical clinical value as changes in the ratios of human AFP isomers have been correlated with several pathological processes (Chan and Miao, 1986, Smith et al., 1979) The separation conditions described herein may therefore have clinical applicability in analytical scale resolution of AFP isomers in biological fluids

B. MATERIALS AND METHODS

Animals

Swiss Webster mice, purchased from Charles River, were bred and maintained in our own animal facilities.

AFP Preparation

Murine alpha-fetoprotein (AFP) was purified from mouse amniotic fluid (MAF) extracted from pregnant Swiss mice in late stage of gestation (days 15-18). The isolation of AFP from MAF was performed by antibody-agarose affinity chromatography as previously described by Murgita and Tomasi (Murgita and Tomasi, 1975a). AFP preparations tested at 2.0 mg/ml failed to show detectable contamination with other serum or amniotic fluid proteins as determined by immunodiffusion and immunoelectrophoresis studies. The purity of AFP was further confirmed by physico-chemical analyses including alkaline and SDS-polyacrylamide gel electrophoresis (APAGE and SDS-PAGE).

Materials

l-histidine, bis-tris, and piperazine were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The equilibration buffer for anion-exchange chromatography consisted of 20 mM *l*-histidine, pH 5.60, while the elution buffer contained 0.5 M NaCl in addition to *l*-histidine. All buffers were prepared with distilled H_2O (Milli Q™ H_2O, Millipore, Canada) and filtered through 0.22 µm filters prior to use. Ampholines required for isoelectric focusing were purchased from Pharmacia (Pharmalyte 4.0-6.5, Pharmacia, Canada). Acrylamide, bis-acrylamide, Tween-20, gelatin, pI markers and the alkaline phosphatase color development reagents were obtained from BioRad (BioRad, Mississauga, Canada).

Anion-Exchange Chromatography

All chromatographic separations were performed on an analytical Mono Q HR5/5 or a preparative Mono Q HR16/10 anion-exchange column linked to a Fast Protein Liquid Chromatography system (FPLC) (Pharmacia Fine Chemicals, Dorval, Canada). For analytical separations, 200 µl of a 2.0 mg/ml AFP preparation in

phosphate buffered saline (PBS) was applied to the Mono Q HR 5/5 column previously equilibrated with *l*-histidine buffer (20 mM, pH 5.60). The pre-determined optimal conditions for eluting individual AFP isomers from the Mono Q column at a constant flow-rate of 1.0 ml/min was initially established with *l*-histidine buffer. After sample application, the column was washed with 0.5 ml of *l*-histidine buffer which allowed for the absorbance (UV-280 nm) to return to the baseline. Bound proteins were eluted with a linear salt gradient extending from 0 to 0.15 M NaCl in 18.0 ml of *l*-histidine buffer. This was followed by a wash step of 0.5 M NaCl for a 2.0 ml volume. The entire procedure was developed with a volume as opposed to time programming base in order to facilitate the scale-up and optimization of runs on the preparative anion-exchange column. Fractions representing individual peaks were collected with a FRAC-100 fraction collector, dialyzed versus PBS overnight, and kept frozen at -20°C until further analysis.

For all preparative scale separations, 10.0 ml aliquots of purified AFP at 2.0 mg/ml in PBS were loaded onto the Mono Q HR 16/10 employing a flow-rate of 8.0 ml/min. Samples were eluted from the Mono Q 16/10 column with a linear salt gradient extending to 0.15 M NaCl over a total *l*-histidine buffer volume of 360 ml. Fractions corresponding to individual peaks were collected using a FRAC-100 fraction collector (Pharmacia). Both the threshold setting and tube advancement control were manually regulated to prevent the potential for cross-contamination between adjacent peaks. Tubes containing UV-absorbing peaks were pooled and concentrated on YM-10 membranes using an Amicon filtration unit (Amicon, Canada). Samples were then dialyzed against PBS and the protein concentration was adjusted to 2 mg/ml by measuring the absorbance at UV-280 nm using an extinction coefficient of 0.443 (Murgita and Tomasi, 1975a). Fractions were analyzed by immunodiffusion, using rabbit antisera against mouse AFP, transferrin, albumin, and NMS.

Isoelectric Focusing

Analytical isoelectric focusing was performed in 5% polyacrylamide gels using a Bio-Rad horizontal mini-IEF cell (Bio-Rad, Canada). Briefly, 2.0 ml of acrylamide monomer concentrate (24.25% (w/v) acrylamide, 0.75% (w/v) bis-acrylamide [25% T, 3% C]) was combined with 5.5 ml Milli-Q H_2O, 2.0 ml of 25% sucrose (w/v)

and 0.5 ml of ampholines (pH 4.0-6.5, Pharmacia). This solution was degassed for five minutes, and polymerization was subsequently initiated with 15 µl of ammonium persulfate (10% w/v), 50 µl of riboflavin-5'-phosphate (0.1% w/v) and 3 µl of TEMED. One microliter samples containing 1 µg of protein were loaded onto the gel and allowed to diffuse into the gel for 10 min. The samples were then electrophoresed at 4°C at a constant voltage of 100 V for 15 min, followed by an additional 15 min at 200 V and finally 450 V for 1 h. The gel was subsequently immersed in a fixative solution (5% (w/v) sulfosalycylic acid, 5% (w/v) trichloroacetic acid in Milli-Q H_2O) for 30 min and stained with Coomassie brilliant blue. The pH gradient was verified by measuring the pH of individual gel slices prior to fixing or by the inclusion of pI markers (Bio Rad).

Western Blotting of IEF Gels

In order to perform immunoblotting of the IEF gel, the gel was poured vertically between 2 glass plates. Following polymerization, one plate was carefully removed, and the gel was run under the identical conditions as outlined for isoelectric focusing gels except that 100 ng of protein was used per sample. After electrophoresis, the IEF gel was removed from the glass plate and equilibrated for 10 min in transfer buffer (12.5 mM Tris, 96 mM glycine, and 20% (v/v) methanol, pH 8.2). The gel was applied to Immobilon (PVDF, Millipore) and proteins were transferred by electrophoresis using a Mini-transblot apparatus (Bio-Rad) in transfer buffer at 40 mA for 18 h. Following the completion of the transfer, the membrane was blocked for 1 hour with gentle agitation in blocking solution (3% (w/v) gelatin in 20 mM Tris, 500 mM NaCl [Tris-buffered saline, TBS], pH 7.5). Prior to incubation with the primary antibody, the membrane was washed twice for 5 min in TBS containing 5% (v/v) Tween-20 (TTBS). The membrane was then incubated for 1 h in a primary antibody solution containing a 1/1000 dilution of a monospecific rabbit anti-mouse AFP antibody preparation in antibody solution (1% (w/v) gelatin in TTBS, pH 7.5). A second wash step of 2 x 5 min in TTBS was followed by a 1 h incubation with 1/3000 dilution of a goat anti-rabbit antibody conjugated to alkaline phosphatase (Bio-Rad). After 3 successive wash steps of 5 min each, twice with TTBS and once with TBS, the membrane was developed with the alkaline phosphatase color development reagents BCIP (bromo-chloro-indolyl phosphate p-toluidine salt) and NBT (p-nitro tetrazolium chloride) (Bio-Rad).

C RESULTS

Qualitative Separations of Mouse AFP Molecular Variants

Native alpha-fetoprotein was purified from mouse amniotic fluid by procedures that have been described in detail elsewhere (Murgita and Tomasi, 1975a). Pure AFP preparations, as verified by conventional APAGE and immunodiffusion techniques, served as the starting material for all subsequent purification procedures Figure 3 illustrates the typical separation obtained for 400 μg of fetal mouse AFP with the 20 mM *l*-histidine buffer system. As shown in Figure 3, AFP is resolved into seven distinct peaks, with one UV-absorbing fraction eluting in the void volume followed by six well-defined peaks that appeared consecutively in the 18 ml linear salt gradient Preliminary experiments indicated that both bis-tris and piperazine buffers could substitute for *l*-histidine (data not shown)

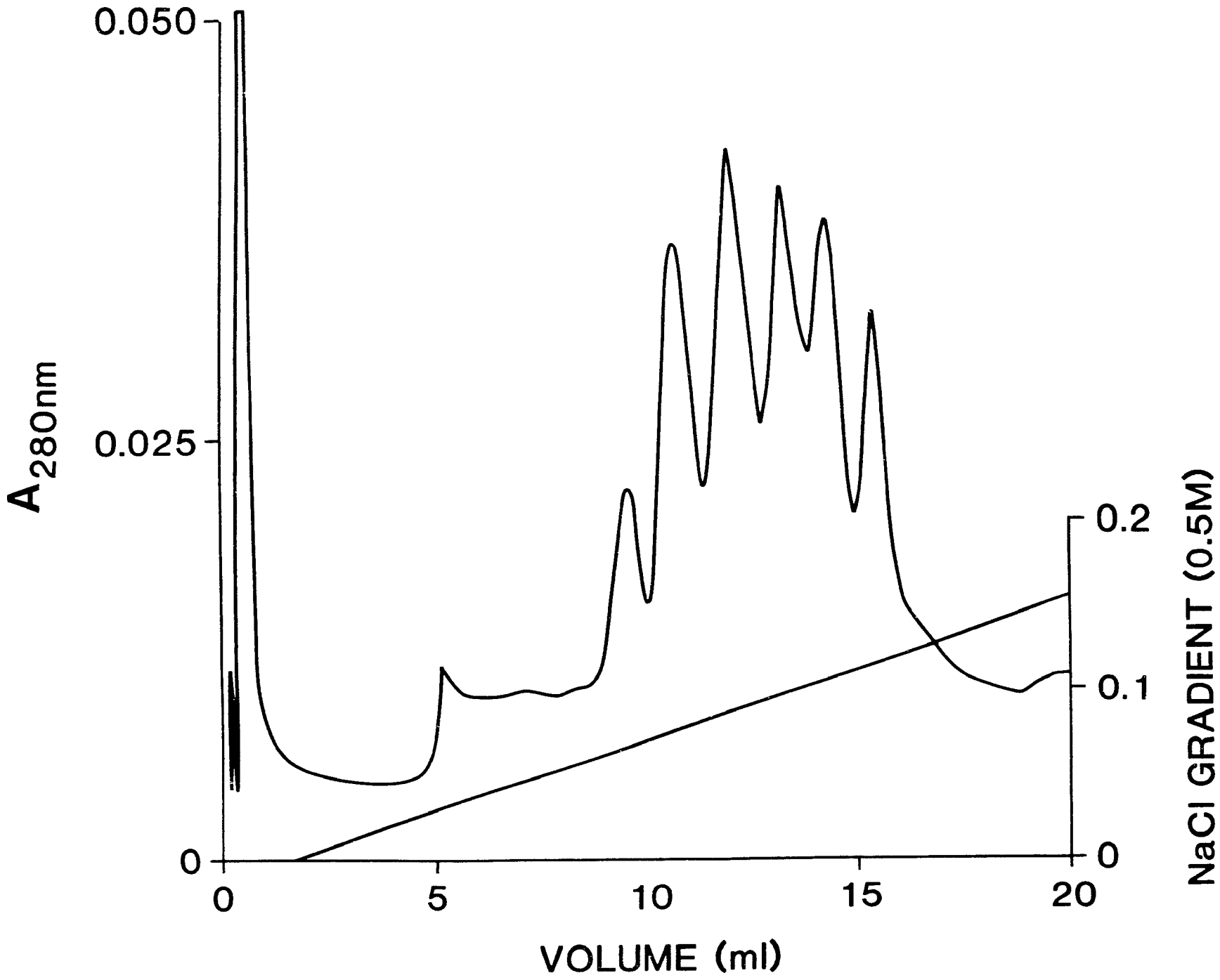
The individual fractions comprising each of the UV-absorbing peaks in the profile shown in Figure 3 were isolated and examined for their AFP content by immunodiffusion studies using monospecific antisera against AFP. Six peaks from the Mono Q column which appeared in succession at retention volumes of 10, 11, 12, 13, 14 and 16 ml developed specific precipitin lines with anti-AFP antisera but not with antisera against albumin, transferrin, or normal mouse sera The six AFP fractions were homogeneous and maintained identical retention volumes when re-chromatographed on the same Mono Q column (data not shown) No amniotic fluid or serum proteins could be detected in the UV-absorbing material present in the void volume or at a retention volume of 5.0 ml A seventh AFP peak was detected in the void volume when individual fractions from several analytical runs were combined. This seventh AFP peak was readily identified when larger quantities of native AFP were applied to preparative anion-exchange columns

Isoelectric Focusing and Immunoblot Analysis of Mono Q Defined AFP Fractions

In order to determine whether the seven distinct peaks separated on the Mono Q column represented unique molecular variants of AFP, the individual fractions were characterized by isoelectric focusing Due to the limited quantities of peaks purified using analytical runs, most isoelectric focusing studies were performed on AFP

Figure 3.

Analytical anion-exchange chromotography of 400 μg of purified mouse AFP with FPLC Mono Q columns. Mouse AFP was applied to the column that had been pre-equilibrated with 20 mM *l*-histidine, pH 5.60 Following sample loading, the column was rinsed and samples were eluted with a linear NaCl gradient extending to 0.15 M NaCl. The elution profile is based on the absorbance at 280 nm.


0.050
0.025
0
A_{280nm}
0
5
10
15
20
VOLUME (ml)
0.2
0.1
0
NaCl GRADIENT (0.5M)

peaks obtained from preparative anion-exchange columns

Unfractionated fetal AFP subjected to narrow range isoelectric focusing (pH 4.0 - 6.5) could be resolved into six distinct variants in a pI range of 4.7 to 5.1 (Figure 4A). The analysis of the seven individual FPLC purified AFP peaks revealed six major isoelectric variants corresponding in mobility to the 6 isoforms of control AFP. The terminology used in this study (ie AFP-1 through AFP-7) defines the seven AFP peaks separable on the anion-exchange column. AFP-1, appearing in the Mono Q fall-through, had the most basic pI of 5.1, whereas AFP-7 was the most acidic (pI 4.7) (Table VIII). It is notable that 2 isomers, AFP-4 and AFP-5, although distinguished by their FPLC retention volumes, exhibited an identical pI of 4.85. Immunoblot analysis of the protein in each of the seven FPLC peaks was performed using a rabbit antisera that recognized the seven AFP isomers. The results showed that the isoelectric microheterogeneity of the seven major immunoreactive bands was identical to control AFP (Fig. 4B) and confirmed that the proteins recovered from each Mono Q peak represented unique isoforms of AFP.

Preparative Scale Separation of Seven Molecular Variants of AFP

Although the protein capacity for the analytical Mono Q column is estimated to be 25.0 mg or 5.0 mg per single peak (Bergstrom et al., 1983), we noted that the fine resolution of individual peaks of AFP began to decrease when quantities of AFP greater than 1-2 mg were loaded onto the column. Therefore a preparative Mono Q HR 16/10 anion-exchange column with a 20-fold greater protein capacity than the analytical column was employed in order to generate larger yields of each isomer for further biochemical studies.

The separation parameters optimized for the analytical column were modified with the FPLC LCC-500 programmable control unit to accommodate the preparative column. In the scale-up mode the gradient volume was expanded 20 fold to 360.0 ml and the flow-rate increased from 1 to 8 ml/min. Figure 5 shows the separation profile of 20.0 mg of AFP on the Mono Q HR 16/10 column equilibrated with 20 mM *l*-histidine, pH 5.60. The seven major UV-absorbing peaks shown in Figure 5 were fractionated and analyzed by double immunodiffusion with a panel of monospecific antisera. One AFP peak was identified in the flow-through on the preparative column. The six major peaks that eluted sequentially in the salt

Figure 4

Isoelectric focusing and Immunoblot analysis of FPLC purified AFP molecular variants. **A)** Native AFP and the FPLC generated AFP isomers designated AFP-1 through AFP-7 were analyzed on narrow ampholine range IEF gels (pI 4.0-6.5) that were Coomassie stained. **B)** For Western blots, 100 ng of control AFP or AFP-1 to AFP-7 was separated on narrow pH range IEF gels (pI 4.0-6.5) and immunoblotted with a monospecific anti-AFP antibody.

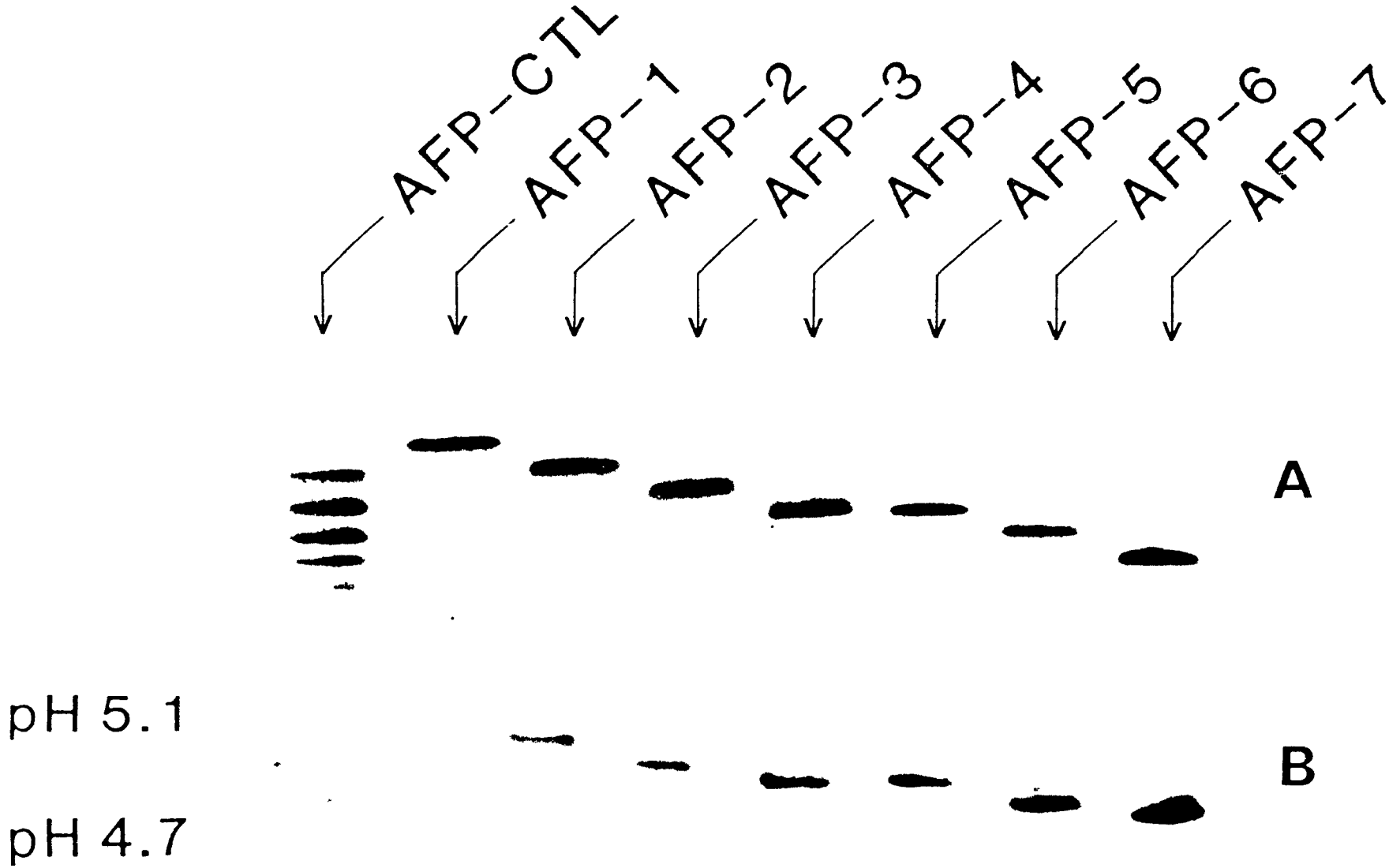
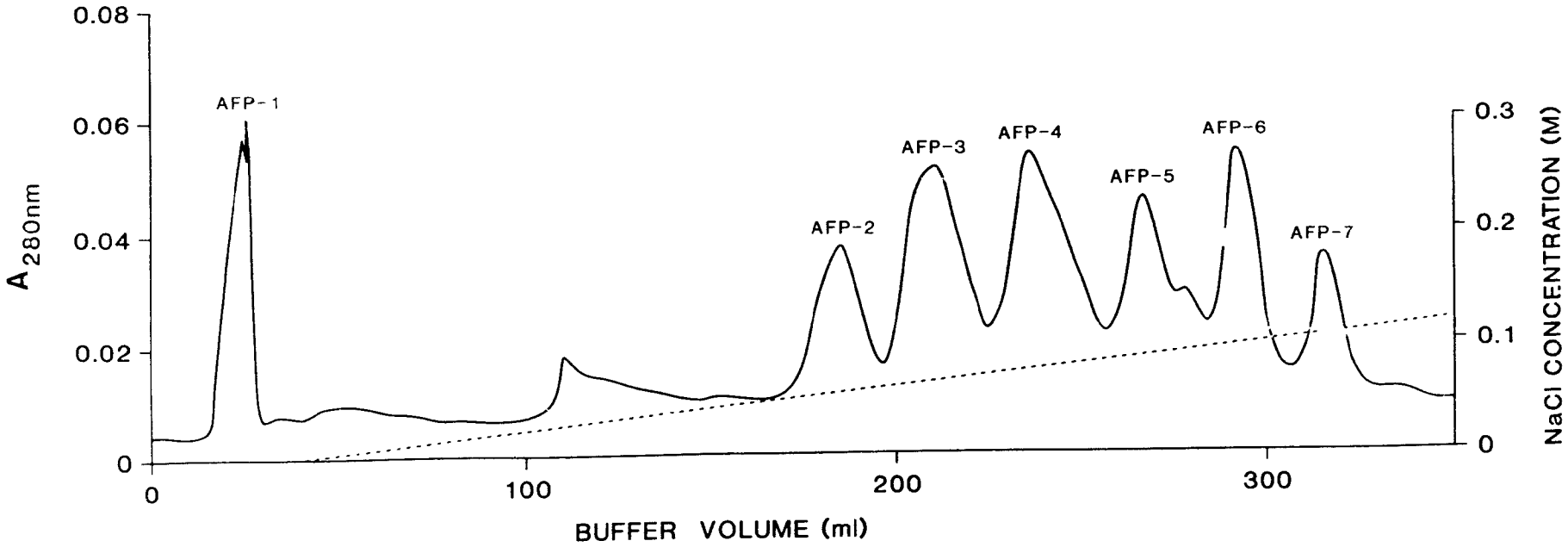

AFP-CTL
AFP-1
AFP-2
AFP-3
AFP-4
AFP-5
AFP-6
AFP-7
A
B
pH 5.1
pH 4.7

Figure 5.

Preparative scale anion-exchange chromotography of 20.0 mg of AFP. Ten ml of mouse AFP at 2.0 mg/ml was loaded onto the Mono Q HR 16/10 column. After protein applications, the column was rinsed with 20 mM *l*-histidine, pH 5.60 and bound proteins were eluted with a linear NaCl gradient over a 360 ml buffer volume. The absorbance was measured at 280 nm and the seven individual AFP fractions are indicated as AFP-1 through AFP-7.


0.08
0.06
0.04
0.02
0
A_{280nm}
AFP-1
AFP-2
AFP-3
AFP-4
AFP-5
AFP-6
AFP-7
0
100
200
300
BUFFER VOLUME (ml)
0.3
0.2
0.1
0
NaCl CONCENTRATION (M)

gradient extending from 0.05 to 0.15 M NaCl were also identified as AFP containing fractions. Therefore, a total of seven AFP containing peaks could be purified in separation times of 50 minutes with the preparative anion-exchangers These seven AFP isoforms, as defined by their specific isoelectric points and FPLC retention volumes (Table VIII), migrated as single 70,000 daltons bands on SDS-PAGE, showing a molecular weight identical to control unfractionated AFP (data not shown)

Quantitative Recoveries of Seven AFP Molecular Variants

In order to calculate the amount of each isoform of AFP purified with the preparative column, individual peaks were fractionated, concentrated, dialyzed against PBS and assayed for their AFP content spectrophotometrically By over-riding the automatic mode on the FRAC-100 fraction collector and manually regulating the fractionation parameters, including the threshold setting and tube switching, it proved feasible to selectively isolate tubes containing homogeneous AFP peaks corresponding to individual isomers while avoiding those areas on the chromatogram containing cross-contaminated isomers Beginning with 20.0 mg of starting material, the amount of each molecular variant purified ranged from a low of 0.2 mg for AFP-1 to a maximum of 1 3 mg for AFP-4 resulting in a total recovery of 25% (Table VIII) Due to the extremely high reproducibility of individual chromatographic separations, larger quantities of each isoform could be obtained by combining corresponding peaks from several 20 0 mg runs of AFP. By manually controlling the fraction collector, many tubes contained cross-contaminated AFP isoforms which had been intentionally excluded on initial chromatographic runs These tubes could be pooled and re-run over the Mono Q column in order to significantly increase the final yield of the seven individual AFP isomers.

TABLE VIII

QUANTITATIVE RECOVERIES OF AFP ISOMERS

AFP FRACTION[a]	RETENTION VOLUME[b]	ISOELECTRIC POINT[c]	YIELD OF INDIVIDUAL ISOMERS[d]
AFP-1	25 ml	5.1	0.2 mg
AFP-2	189 ml	5.0	0.52 mg
AFP-3	211 ml	4.9	0.79 mg
AFP-4	236 ml	4.85	1.3 mg
AFP-5	268 ml	4.85	0.8 mg
AFP-6	295 ml	4.8	1.3 mg
AFP-7	318 ml	4.7	0.3 mg
		TOTAL	5.21 mg

[a]AFP fractions were defined by the FPLC retention times, immunodiffusion analyses, and isoelectric focusing patterns

[b]The retention volumes are based on the FPLC elution profile of 20.0 mg of AFP similar to that shown in Figure 2.

[c]The isoelectric points of the individual isomers was estimated from isoelective focusing gels which had pI markers alongside native AFP

[d]Fractions representing individual AFP peaks were dialyzed, concentrated, and the amount of protein was calculated as described in Materials and Methods

D. DISCUSSION

The data presented in this paper indicates that an automated FPLC system employing Mono Q anion-exchange columns can be suitably adapted for the detection and recovery of molecular variants of mouse AFP

The optimal chromatographic conditions for separating AFP variants were initially established on an analytical Mono Q column with three aqueous buffers, *l*-histidine, bis-tris, and piperazine, all having pKa values ranging from pH 5.0 - 6.0 At pH values in this range, about 1 pH unit above the range of isoelectric points for the native population of AFP molecules (pH 4.7 - 5.1), the net charge differences between the molecular variants of AFP appeared optimal to effect their resolution on anion-exchange columns Chromatography of mouse AFP in buffers of varying pKa values either more acidic or more basic than the pH 5.0 - 6.0 range confirmed that the pH chosen was essential for effective separations Although similar separation patterns were obtained with all three buffers set at pH 5.6, the *l*-histidine buffer was selected for all chromatographic procedures described herein This is partly because both bis-tris and piperazine are likely to have toxic and/or other adverse effects on lymphocyte tissue culture systems that we are presently employing to study the immunoregulatory properties of AFP isomers

During the course of these investigations, we noted that the resolution of individual peaks began to decrease when protein loads greater than 1.0 mg of AFP were applied to the analytical column This may be partly attributed to the finding that AFP isomers are known to have almost identical amino acid and carbohydrate compositions and thus the distinguishing characteristics of each are likely to be rather subtle (Zimmerman et al., 1976). In addition, the resolution of AFP peaks was severely diminished if separations were performed on mouse amniotic fluid as opposed to purified AFP preparations. To generate larger quantities of individual AFP peaks, the purification conditions developed for the analytical column were adapted for the preparative Mono Q anion-exchange column Deploying the preparative column, mouse AFP was efficiently separated into seven well-resolved isoforms, as defined by the consistent retention volumes, the unique isoelectric points of individual isoforms compared to the typical isoelectric microheterogeneity of AFP, and the immunoblotting studies with anti-AFP antisera That the additional

AFP isomer resolved from the void volume of the HR 16/10 column was not as readily detected in chromatographic runs on the analytical column is probably due to quantitative factors since this isomer represents less than 5% of the total number of native AFP molecules.

The identification of seven distinct mouse AFP isomers described in this study has not previously been reported. Earlier investigations on AFP microheterogeneity revealed the presence of a maximum of six isoelectric variants during normal fetal development which focused over a pI range of 4.8 to 5.2 (Gustine and Zimmerman, 1973). It is notable in this regard that the AFP subspecies defined in this study extended over a range of isoelectric points very similar to that described by Zimmerman. However, two of the FPLC defined isomers identified here, AFP-4, and AFP-5 were found to have identical pI values, and this close isoelectric similarity may have hampered previous efforts to identify all of the native molecular variants of AFP

The variants of mouse AFP have been shown to undergo reproducible changes in their concentrations with ontogeny (Zimmerman et al., 1976, Higgins, 1979, Wong et al., 1988) These precisely regulated developmental shifts in the numbers and ratios of AFP isomers may be consistent with an immunoregulatory function for AFP operative at certain critical stages of gestation Previous investigations have ascribed this potential physiological activity to the broad population of native AFP molecules (Murgita and Wigzell, 1981, Lester et al., 1976) We have recently determined that only one of the seven mouse AFP molecular variants described in this report is able to exert potent immunosuppressive activity in vitro (van Oers et al., 1989) Further studies to characterize this isomer are currently in progress using both biophysical and molecular genetic approaches (Borsmenu et al., 1988)

The detection of human AFP in the adult is a common diagnostic measure for many diseases The measurement of serum AFP in pregnant women is used as a means of risk assessment for certain fetal malformations (Tottager-Larsen and Norgaard-Pederson, 1988). As well, high levels of AFP in adults may indicate acute and chronic liver diseases (Bloomer et al., 1975, Buamah et al., 1984b), and germ cell tumors and gastrointestinal neoplasm (Shuster et al., 1980) It is noteworthy that the detection of differences in the numbers and ratios of individual isomers in relation to certain diseases has been applied for presumptive diagnoses

(Buamah et al., 1987). Based on these findings, it is interesting to speculate that the efficient analytical separation protocol described here of mouse AFP isomers may be extended to human AFP in the classification of malignant versus non-malignant diseases associated with aberrant re-synthesis of AFP.

III. ISOLATION AND CHARACTERIZATION OF A DISTINCT IMMUNOREGULATORY ISOFORM OF ALPHA-FETOPROTEIN PRODUCED BY THE NORMAL FETUS

A. INTRODUCTION

An 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 (Buamah et al., 1987) and as well, may have functional significance (Lester et al., 1976, Zimmerman et al 1977). In pursuit of this latter prospect, Yachnin and his co-workers were able to 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 (reviewed in Lester et al., 1977b). Findings by Zimmerman et al (Zimmerman et al., 1977) suggested that the presence of sialic acid residues on certain AFP molecules was essential for immunosuppression while Lester et al (Lester et al., 1977b) reported otherwise leaving open the important question of the functional relevance of these secondary structures.

In the present investigation we have employed an efficient anion-exchange column linked to an automated FPLC system 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.

B. MATERIALS AND METHODS

Animals

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

Purification of AFP

Alpha-fetoprotein 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 followed by a second step employing preparative polyacrylamide gel electrophoresis as described in detail elsewhere (Murgita and Tomasi, 1975a) Isolated AFP preparations fulfilled strict criteria for purity including a single Coomassie blue or silver nitrate stained protein band on analytical alkaline and SDS-polyacrylamide gel electrophoresis (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™ 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), 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). Twenty 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 Ten millilitre 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 pre-determined extinction coefficient of 0.443 for AFP

Gel Electrophoresis

Analytical isoelectric focusing (IEF-PAGE) was performed in 8.5% polyacrylamide gels using a BioRad Protean vertical slab gel electrophoresis unit Briefly, 25 5 ml of acrylamide stock (30% T· 2 67% C) (BioRad, Mississauga, Canada) was combined with 45 0 ml of 25% sucrose and 13 5 ml of distilled water A five percent ampholine solution extending over a pH range 4 2-4 9 (Pharmalyte, Pharmacia) was added to this mixture. Polymerization was initiated with 0 6 ml of ammonium persulfate (20 mg/ml) and cross-linking was achieved with 60 ul TEMED Two µg of protein in a 10 ul volume was applied to the gel, and samples were electrophoresed for 5 hours at 500 volts Densitometric scans of the isoelectric variants were performed with an LKB laser densitometer (LKB 2202 Ultroscan) Sodium dodecyl sulfate (SDS)-PAGE was carried out on 12 5% polyacrylamide gels with the BioRad Mini-PROTEAN II apparatus (Bio Rad). Ten ul 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 2M dithiothreitol (DTT) and 0 1% bromophenol blue) and heated at 100°C for 5 minutes Electrophoresis through the resolving gel proceeded for 45-60 minutes at 250 V For Western blots 20 ng samples were prepared in SDS sample buffer and run on 12 5% polyacrylamide gels Alkaline PAGE (APAGE) was performed employing conditions described previously (Murgita and Tomasi, 1975a)

Western Blot Analysis

Electroblotting of antigen from SDS-PAGE gels onto Immobilon PVDF Transfer Membranes (Millipore) was performed at 200 mA constant current in a TE-52 Transfer Unit (Hoefer Scientific Instruments) at 10°C overnight After electroblotting the transfer membrane was gently agitated for 1 hour at room temperature in blocking solution (3% [w/v] gelatin in 20 mM Tris, 500 mM NaCl [Tris-buffered saline, TBS] pH 7 5) and subsequently washed twice in TBS containing 0 05% (v/v) Tween 20 (TTBS) Membranes were then incubated in a 1 500 dilution of monospecific rabbit anti-mouse AFP antisera in antibody solution (1% [w/v]) gelatin in TTBS) The nitrocellulose membranes were again rinsed twice for 5 minutes in TTBS and then incubated with alkaline-phosphatase

conjugated goat anti-rabbit IgG (1:3000 dilution in antibody solution) for 1 hour After 3 successive wash steps (twice in TTBS and once in TBS) the immuno-reactive bands were detected with the BCIP/NBT color development solution according to the manufacturer's instructions (BioRad)

Neuraminidase Digestion

Ten milligrams of purified AFP was dialyzed overnight against 0.1M sodium acetate, pH 5.0 at 4°C The AFP was then digested with 0.2U of neuraminidase (Clostridium perfringens, 2 U/ml, Sigma, Lot # 101F-8057) at 37°C for 4 hours The amount of sialic acid remaining on AFP preparations after neuraminidase treatment was determined by the thiobarbituric acid assay (TBA) 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 thiobarbituric acid assay (Aminoff, 1961) Two hundred 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 hour 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 Following this incubation, 30 µl of 2% sodium arsenite [(w/v) 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-thiobarbituric acid, 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% (v/v) 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) was used to establish a standard curve over the concentration range of 4-18 µg/ml.

In Vitro Anti-SRBC Antibody Response

Primary in vitro antibody responses were generated by culturing $10x10^6$ CBA/J spleen cells with $3x10^6$ SRBC in 0.8 ml volumes in 24 well microplates (Costar,

Cambridge, MA, Cat. no. 3524) according to a modification of the original Mishell-Dutton method as described in detail elsewhere (Melancon-Kaplan and Murgita, 1987).

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) and rabbit complement (Low Tox, Cedarlane) were cultured to assay for reactivity to Concanavalin A employing a previously described microculture system (Hooper and Murgita, 1981). Lyt 1+23 thymocytes ($2.5x10^5$) were cultured in 96 well round bottom microtitre plates for 48 hours with Concanavalin A (1 µg/ml, Pharmacia) Total volumes of cell cultures was 200 µl Cells were maintained at 37°C in 95% humidified air and 5% CO_2 Six hours prior to harvesting, cultures were pulsed with 1 uCi tritiated thymidine (ICN, specific activity 66 Ci/mmole) Cells were harvested onto glass fibre mats with a multiple sample harvestor (Skatron, Flow Laboratories) and water insoluble tritiated thymidine incorporation was measured with a liquid scintillation counter (LKB 1216 Rack Beta). Results are expressed as mean counts per minute ± 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 (Hoskin et al , 1985c) In brief, bone marrow cells were collected by flushing the tibias and femurs of CBA/J mice with PBS using a sterile 5 ml syrin and a 25-gauge needle Cells were cultured in 96-well round-bottom microtiter plates at a concentration of $2.5x10^5$ cells per well in a total volume of 0.2 ml for 48 hours after which cultures were pulsed with tritiated thymidine, harvested, and counted as described above for ConA reactions.

Interferon Boosted <u>In Vitro</u> NK Activity

<u>In vitro</u> measurements of spontaneous and lymphokine boosted anti-YAC NK activity in CBA/J spleen were performed as previously described (Cohen et al , 1986). Cells were cultured in Iscove's modified Dulbecco's Eagles medium

containing 5 x 10^{-5}M 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin, and supplemented with fresh 0.5% CBA/J NMS. Splenic effector cells were stimulated for 24 hours with 5000 U IFN (alpha and beta interferon from mouse fibroblasts; Enzo Biochemicals, N.Y.) in 24 well Costar plates 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 labelled YAC-1 targets.

C. 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 6A) previously defined as transferrin, AFP, and albumin (Murgita and Tomasi, 1975a) The purity of the high milligram quantities of AFP used throughout these studies was established by demonstrating a single band on analytical APAGE (Fig 6B) 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 6C), 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 6D). As shown in Fig. 6F, 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 low of 6% to a maximum of 26%. Figure 6E denotes the terminology (ie., 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 in Figure 7 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, Figure 6G shows the sialic acid content of each of the purified FPLC defined isomers.

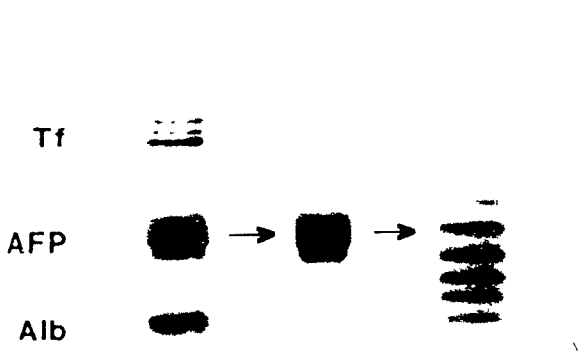
Quantitative Recovery of Individual AFP Isomers

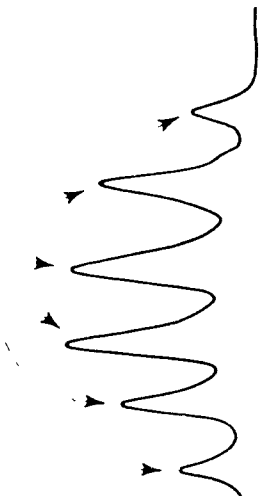
After first establishing optimal separation conditions for individual AFP isomers

Figure 6.

Biochemical and electrophoretic analysis of the molecular heterogeneity of AFP. **(A)** Coomassie blue stained protein pattern of mouse amniotic fluid MAF, and MAF derived purified AFP **(B)** on alkaline PAGE gels. The three major protein components of MAF; albumin (Alb), AFP, and transferrin (Tf) are shown. **(C)** Analytical isoelectric focusing (IEF) gel analysis of pure AFP showing six isoelectric variants with pI's 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 thiobarbituric assay.

DENSITOMETRIC ANALYSIS

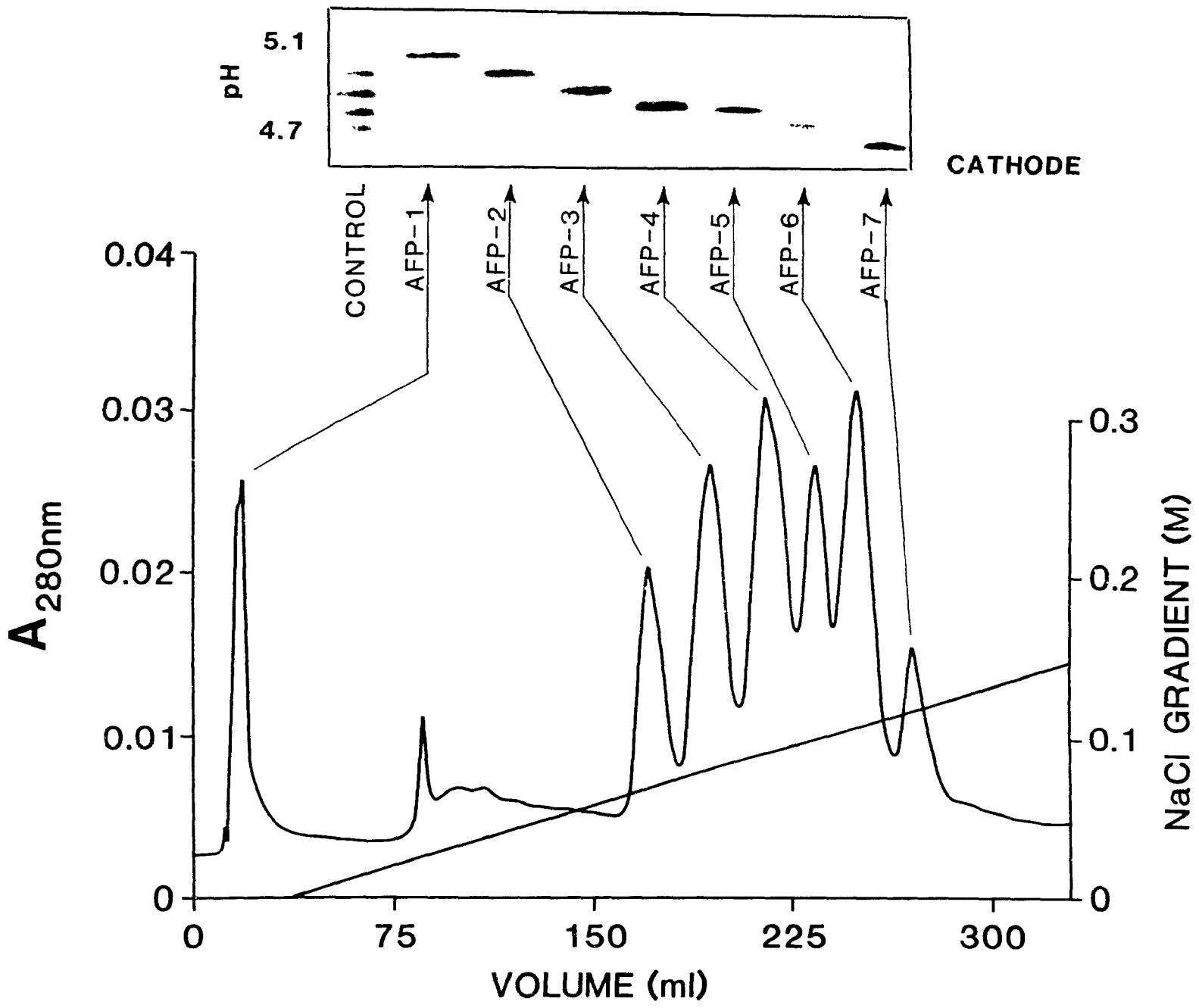




APAGE	IEF	GEL Scan	FPLC Defined Isomers	% Total Protein	SIALIC ACID CONTENT moles S.A./mole protein
			AFP-1	6.3	1.05
			AFP-2	19.6	0.28
			AFP-3	26.0	1.09
			AFP-4	23.4	1.83
			AFP-5		2.13
			AFP-6	15.7	2.89
			AFP-7	8.9	3.89
A	B	C	D	E	F	G

Figure 7.

Resolution of seven isomeric forms of AFP on a preparative anion exchange Mono Q HR16/10 column. Twenty mg of mouse AFP was applied to the Mono Q HR16/10 column equilibrated with 20 mM *l*-histidine, pH5.60. 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 labelled 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.


pH
5.1
4.7
CATHODE
CONTROL
AFP-1
AFP-2
AFP-3
AFP-4
AFP-5
AFP-6
AFP-7
0.04
0.03
0.02
0.01
0
A280nm
0.3
0.2
0.1
0
NaCl GRADIENT (M)
0
75
150
225
300
VOLUME (ml)

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 functional tests. The elution profile shown in Figure 7 for 20 mg of AFP was very similar to that routinely observed on the preliminary analytical scale runs. The minor peak which consistently eluted at approximately 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 in Figure 9 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 Figure 7 (insert), each of the FPLC peaks did indeed represent a unique isomer having a different isoelectric point within the pH range of 4 7 to 5.1, with the exception of AFP-4 and AFP-5 which exhibited identical protein banding at an approximate pI of 4.8 The seven AFP variants, 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. In order 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 M W. on SDS-PAGE and performed immunoblot analysis. As shown in Fig 8A, the proteins recovered from the seven peaks on the chromatogram each had M.W 's of 69 Kd, identical to that for control unfractionated AFP. Western blot analysis using mono-specific anti-mouse AFP antibodies confirmed that the protein recovered from each FPLC generated peak represented purified AFP molecules (Fig 8B). The immunoblots were negative when developed with antisera specific for other known components of MAF (data not shown).

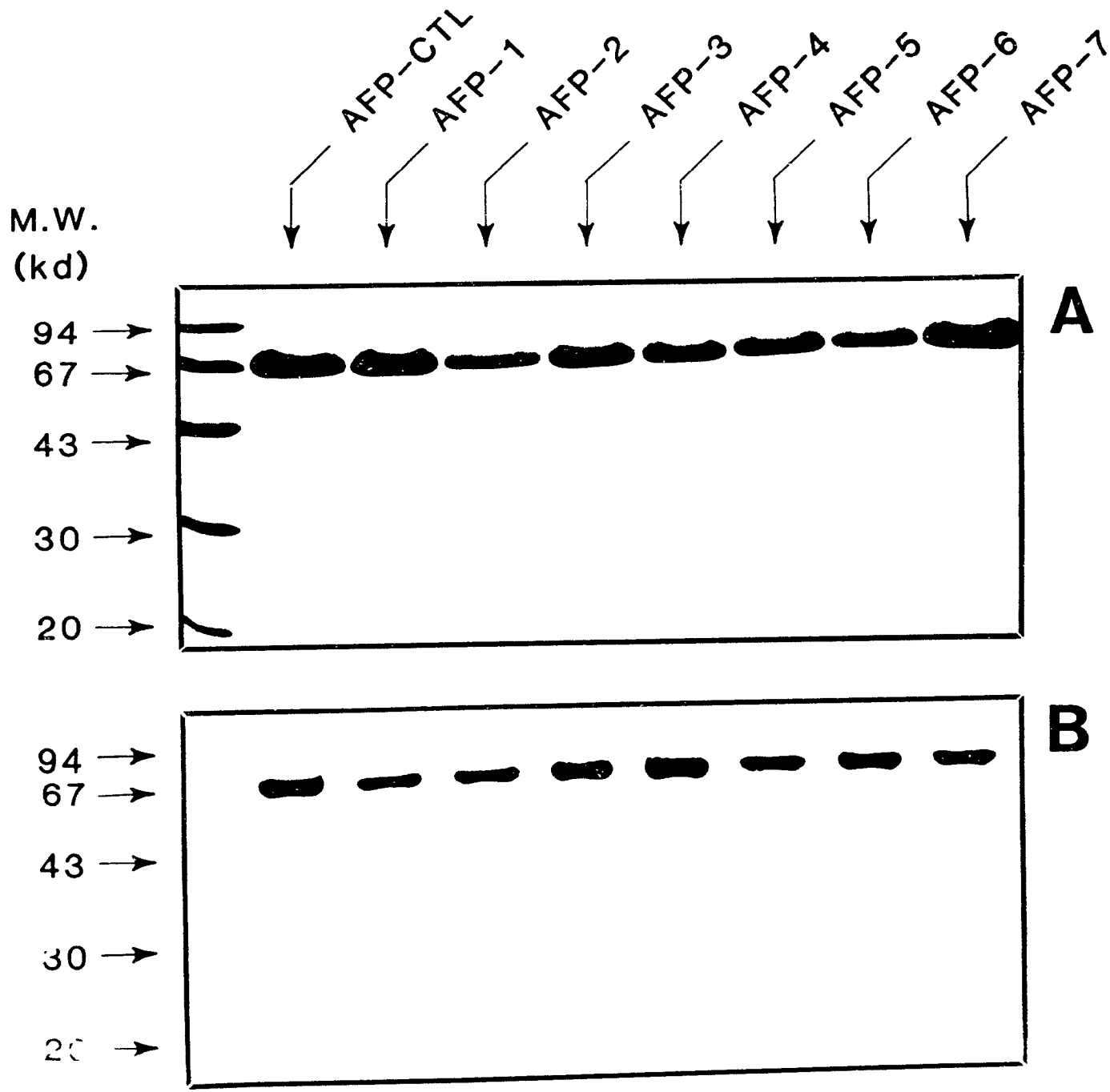
Comparative Immunoregulatory Effects of the Seven Distinct Isomeric Forms of AFP

In earlier studies we had established that AFP is capable of exerting strong immunosuppressive effects on <u>in vitro</u> anti-SRBC antibody responses (Murgita and Tomasi, 1975a), Lyt $1^{+}23^{-}$ Con A reactive thymocyte responses (Hooper et al., 1987)

Figure 8.

Molecular weight characterization and immunoblot analysis of AFP molecular variants purified by FPLC anion-exchange chromatography.

Two 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 (**Figure 8A**). For Western blots (**Figure 8B**), 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 M.W.M. which include phosphorylase b (94 Kd), bovine serum albumin (67 Kd), ovalbumin (43 Kd), carbonic anhydrase (30 Kd), soybean trypsin inhibitor (20.1 Kd).


AFP-CTL
AFP-1
AFP-2
AFP-3
AFP-4
AFP-5
AFP-6
AFP-7
M.W.
(kd)
94
67
43
30
20
A
94
67
43
30
B

and natural killer cell activity augmented in vitro by lymphokines (Cohen et al., 1986).

As shown in Figure 9A, 9B and 9C, 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 to 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 Figure 10. Here a representative dose-response curve extending from 100 µg/ml 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 this reaction. Thus, Con A responses in the presence of various amounts of isomers AFP-2 through AFP-7 always fell within the standard error of the mean for albumin control responses as denoted in Figure 10 by the dotted lines.

Recent studies by Hoskin et al. (Hoskin et al., 1985c) 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 Figure 9D, augmented bone marrow cell proliferation in the presence of AFP-1 is comparable to that mediated by control AFP while the other isomers displayed moderate stimulatory capacity which was still well above control values in the presence of media alone or albumin.

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 (Zimmerman et al., 1976) prompted us to investigate the relative amount of NANA

Figure 9.

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 (**Figure 9A**). CBA/J spleen cells ($10x10^6$) were cultured with $3x10^6$ 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 four days of incubation, anti-SRBC activity was assayed **Figure 9B** shows adult CBA/J Lyt 1^+23 thymocytes ($2.5x10^5$) co-cultured with 1 µg/ml ConA and 200 µg/ml of the indicated proteins. Proliferative responses were measured at 48 hours of culture. In **Figure 9C** mouse albumin, control AFP, and AFP-1 to AFP-7 were tested for their suppressive activity on interferon boosted NK cell activity. Adult CBA/J spleen cells were incubated with interferon and the indicated proteins for 24 hours. After 24 hours, the NK activity of the cells measured against ^{51}Cr-YAC. Bone marrow cell proliferative responses (**Figure 9D**) were measured after culturing normal adult CBA/J cells ($2.5x10^5$) with the indicated proteins for 48 hours, as described in the materials and methods.

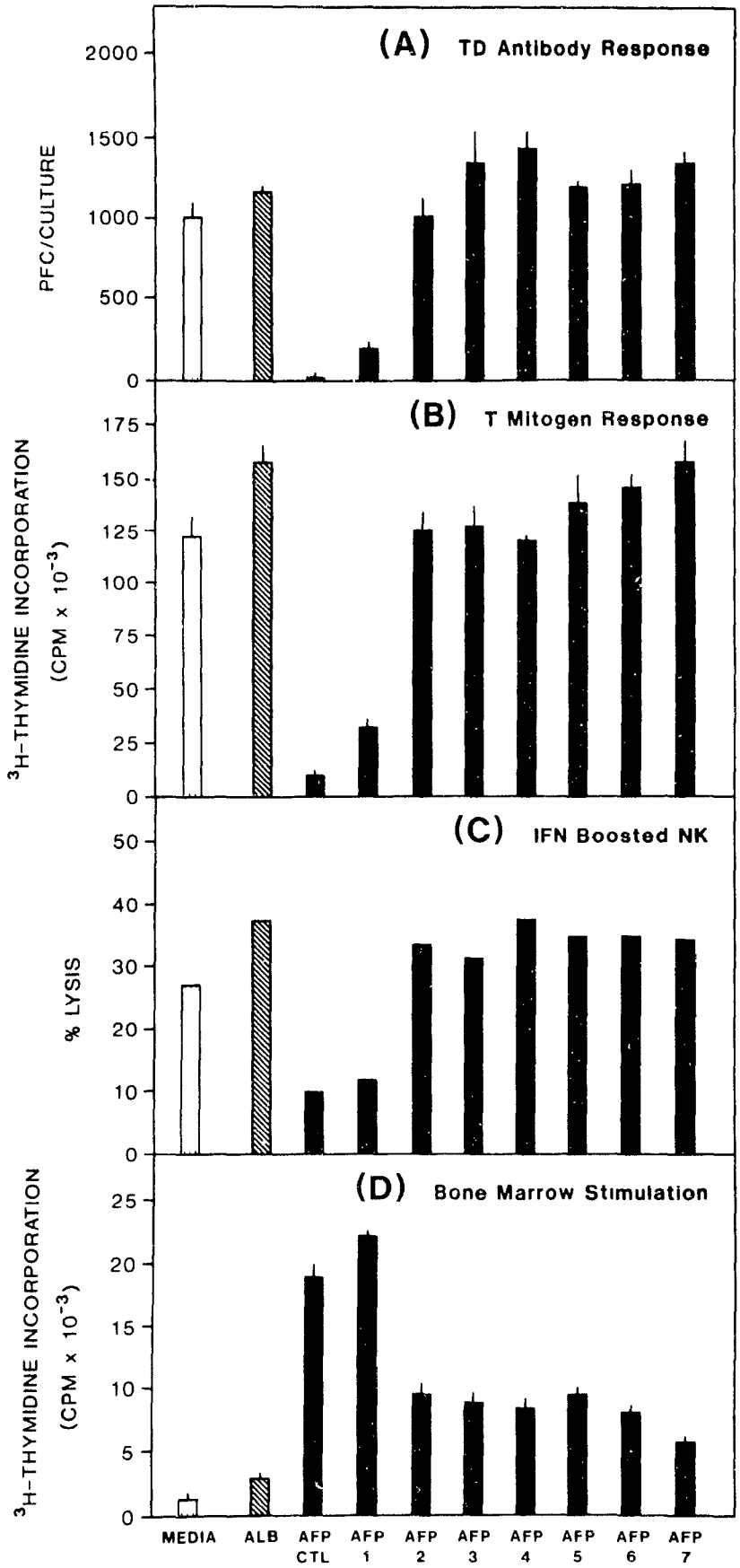
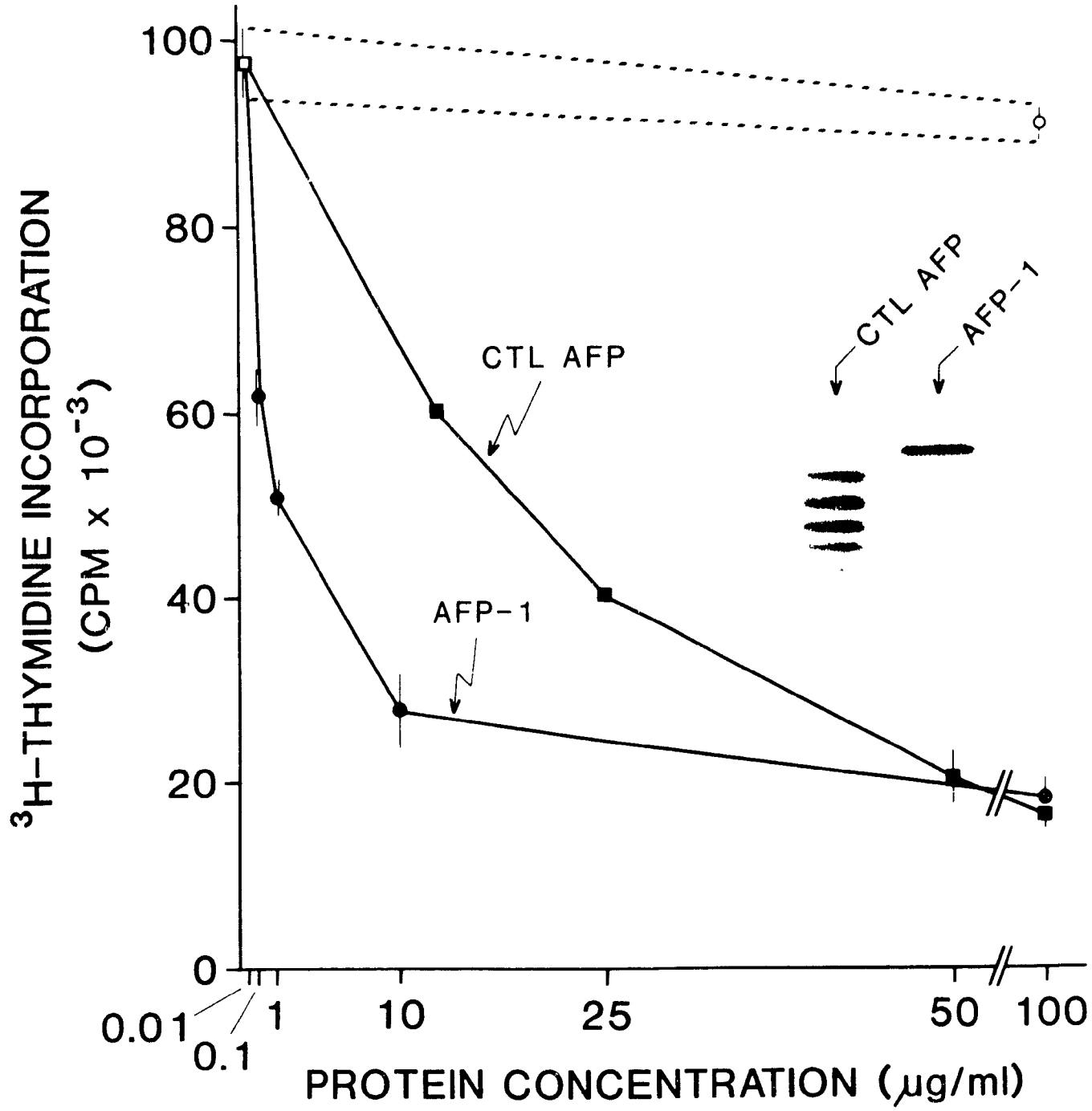

(A) TD Antibody Response
PFC/CULTURE
2000
1500
1000
500
0
(B) T Mitogen Response
^{3}H-THYMIDINE INCORPORATION (CPM x 10^{-3})
175
150
125
100
75
50
25
0
(C) IFN Boosted NK
% LYSIS
50
40
30
20
10
0
(D) Bone Marrow Stimulation
^{3}H-THYMIDINE INCORPORATION (CPM x 10^{-3})
25
20
15
10
5
0
MEDIA
ALB
AFP CTL
AFP 1
AFP 2
AFP 3
AFP 4
AFP 5
AFP 6
AFP 7
ADDITIONS TO CULTURES (200 μg/ml)

Figure 10.

Dose-response effect of control AFP versus AFP-1 and the non-suppressive isomers on ConA stimulated Lyt 1$^+$23$^-$thymocytes. Adult CBA/J Lyt 1$^+$23$^-$ thymocytes were cultured with ConA in the presence of control AFP and purified isomers at the concentrations indicated. The area within the dotted lines shows control Lyt 1$^+$23$^-$ responses in the presence of either mouse albumin or the Mono Q defined isomers AFP-2 to AFP-7. After 48 hours of culture, proliferative responses were measured by ^{3}H-thymidine incorporation as described in the materials and methods. The insert shows the isoelectric focusing pattern of control AFP and AFP-1.


100
80
60
40
20
0
^{3}H-THYMIDINE INCORPORATION
(CPM x 10^{-3})
CTL AFP
AFP-1
CTL AFP
AFP-1
0.01
0.1
1
10
25
50
100
PROTEIN CONCENTRATION (μg/ml)

present in each purified AFP isomer. Using a modification of the thiobarbituric acid assay (TBA) (Aminoff, 1961), the moles of NANA per mole of protein were calculated for each FPLC defined AFP isomer. As noted in Figure 6G, there was a progressive increase in sialic acid residues on AFP-2 to AFP-7 with AFP-7 having four moles of NANA per mole 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 pI's 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 (Zimmerman et al , 1977, Lester et al , 1977) Experiments were therefore performed in which the immuno-suppressive activity of AFP was assessed following 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 IX indicate that there was an 80% reduction in sialic acid residues following enzyme treatment of two separate AFP preparations Thus, according to our measurements enzymatically treated AFP preparations usually retained between 15 and 20 percent 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 IX the desialylated AFP preparations were only slightly less suppressive than the untreated 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

TABLE IX

EFFECTS OF NEURAMINIDASE DIGESTION ON AFP MEDIATED SUPPRESSIVE ACTIVITY

Sample	Moles of NANA PER mole of protein[a]	Lyt 1+2- ConA Thymocyte Response[b]		
		Exp. 1	Exp. 2	Exp. 3
		(^{3}H cpm ± SE)		
Media		77,384 ± 5,148	31,531 ± 3,637	94,962 ± 6,522
Control AFP	3.16 ± 0.50[c]	2,919 ± 293	1,749 ± 450	7,887 ± 523
AFP-Desialylated[c]	0.69 ± 0.12	5,969 ± 734	4,719 ± 1,253	ND[f]
AFP-Desialylated[d]	0.75 ± 0.14	6,373 ± 783	4,005 ± 479	9,877 ± 526

[a]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.

[b]Adult CBA/J Lyt 1+23- thymocytes ($2.5X10^5$) were co-cultured with 1 µg/ml of Con A and 200 µg/ml of the indicated proteins. Proliferative responses were measured at 48 hours of culture.

[c]Ten mg of control AFP was digested with 0.2 U neuraminidase (*C. Perfringens*) for 4 hours at 37°C in 0.1 M Na acetate, pH 5.0.

[d]The experiment described in (c) was repeated under the identical conditions

[e]The moles of NANA per mole of AFP was determined from three different preparations of AFP

[f]Not done

that the immunosuppressive AFP-1 isomer contains essentially the same amount of sialic acid as does the non-suppressive AFP-3 isomer.

D. DISCUSSION

Accumulating evidence continues to support the concept put forth by Murgita and colleagues (Murgita and Tomasi, 1975a, Murgita and Wigzell, 1981) that immunoregulation is one of the biological functions of AFP. The most well studied modulating influence of AFP on lymphocyte responses is immunosuppression (Murgita and Wigzell, 1981). However, AFP may also exert a mitogenic or growth promoting influence on in vitro cell growth (Hoskin et al., 1985c), which is to some extent reminiscent of previously observed effects by albumin and transferrin (Iscover and Melchers, 1978), two serum proteins shown to be related to AFP in terms of immunologic cross-reactivity of their unfolded polypeptide chains (Pekkala-Flagan and Ruoslahti, 1977) Recent evidence points to the fact that the immunosuppressive effects of AFP are most pronounced on autoimmune reactions as measured both in vitro (Hooper et al., 1981, 1987, O'Neill et al., 1982, Brenner et al., 1980) and in vivo (Brenner et al., 1984, Buschman et al , 1987). This has lead 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 (Hoskin and Murgita, 1985b). According to this reasoning, the developing immune system of the fetus and the newborn as well as certain adult maternal immunoresponsive elements (Murgita, 1976) 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. (Zimmerman et al., 1973, 1976) 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 (Lester et al., 1976, 1977b, Zimmerman et al , 1977)

Lester et al. (Lester et al., 1976, 1977b) 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 which was shown to contain lower levels of the negatively charged molecular variants of AFP.

Lester et al. (Lester et al , 1976, 1977b) 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 (Zimmerman et al., 1973, 1976) 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 versus non-sialylated 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 (Zimmerman et al , 1977). This was in contrast with the findings of Lester et al. (Lester et al , 1976, 1977b), 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 detailed 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. In order to overcome such limitations we have developed a purification protocol for separating AFP into seven distinct isomeric forms employing Mono Q anion exchange columns coupled to an automated FPLC system. The separation profile shown in Figure 7 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 (Figure 6G) extending from the AFP-2 isomer which lacks sialic acid molecules to a maximum of four residues per mole 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 Lester et al. (Lester et al., 1977b) 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 contain similar amounts of sialic acids (Figure 6G) 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 (Yachnin et al., 1980) 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 earlier work of Lester et al. (Lester et al., 1976, 1977b) and Zimmerman et al (Zimmerman et al , 1976, 1977) 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 which 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 (Murgita and Tomasi, 1975a, 1975b, Hoskin et al., 1985c, Cohen et al., 1986, Hooper et al., 1987) to be highly sensitive to AFP-mediated immunoregulatory effects. Thus, we show in Figure 9 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 Figure 10) 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 (Lester et al., 1978, Peck et al., 1978a). 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 more than 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 (Attardi and Ruoslahti, 1976). Experiments are presently underway to examine these possibilities.

Also shown in Figure 9 are data on the comparative in vitro growth promoting effect of control unfractionated AFP versus each of the seven purified isomers on cultured bone marrow cells. We have reported previously on studies showing an immunoenhancing effect of AFP (Peck et al., 1978a) with one important functional manifestation of this activity being the induction of T (Murgita et al., 1977, 1978a, 1981, Hooper et al., 1986) and B cell-like (Hamel et al., 1987, Gronvik et al. 1987, Hoskin et al., 1989a) natural suppressor cells. The growth stimulating properties of AFP, as measured by increased tritiated 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 (Figure 9D). We have previously suggested (Hoskin et al., 1985, Hamel et al., 1987) 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 (Polet and Spieker-Polet, 1976).

The essential biochemical distinction(s) between the immunoregulatory AFP-1 molecules and the other six isomers which lack this functional property remains to be determined We have stated previously (Murgita and Tomasi, 1975a, Murgita and Wigzell, 1981), as has Lester et al. (Lester et al , 1977b), 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 (Parmelee et al., 1978, Deutsch, 1983), to a putative non-covalently bound moiety which 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 (Yachnin et al., 1980) 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 co-factor 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 (Boismenu et al., 1988) are aimed at reaching a definitive answer to this central question.

IV. THE FATTY ACID BINDING PROPERTIES OF DISTINCT ISOFORMS OF MOUSE ALPHA-FETOPROTEIN

EVIDENCE THAT LONG-CHAIN POLYUNSATURATED FATTY ACIDS ARE NOT INVOLVED IN ALPHA-FETOPROTEIN MEDIATED IMMUNOSUPPRESSION

A. INTRODUCTION

Alpha-fetoprotein and albumin have comparable adsorption and transport roles for many endogenous and exogenous molecules including non-esterified fatty acids (Savu et al., 1981, Parmelee et al., 1981), bilirubin (Hsia et al., 1980), metals (Wu et al., 1987), and estradiols in the case of rodent AFPs (Savu et al., 1981, Nunez et al., 1987). The physiological role of the binding properties of AFP are proposed to include the transport and cell delivery of long chain fatty acids to developing tissues (Laborda et al., 1989, Uriel et al., 1987, Torres et al., 1989), maternal-fetal transplacental transfer of polyunsaturated fatty acids (Hsia et al., 1987) (PUFA) and the regulation of estrogen-stimulated uterine growth in immature mice (Mizejewski and Warner, 1989).

One common property shared by all mammalian AFPs studied to date is their high affinity binding of certain polyunsaturated fatty acids (PUFA) including arachidonic acid (20.4, n-6) and docosahexaenoic acid (22:6, n-3) (Savu et al., 1981, Parmelee et al., 1978, Hsia et al., 1987, Calvo et al., 1988). This finding prompted several researchers to propose that the immunosuppressive activity of AFP may be related to the quantities of complexed 20:4 and 22:6 (Parmelee et al., 1978, Nunez et al., 1987, Uriel et al., 1987, Deutsch et al., 1983). Arachidonic acid, at sufficiently high concentrations, has been shown to directly inhibit mitogenic responses of T cells (Kelley and Parker, 1979), regulate Ca^{2+} homeostasis in T cells (Chow et al., 1990), and prevent cytotoxic T lymphocyte degranulation (Richieri et al., 1990). Furthermore, 20:4 is the precursor of the biologically active eicosanoids such as prostaglandins and other enzymatic metabolites which are able to regulate immune responses. The amounts and types of eicosanoids synthesized can in turn be modulated by the uptake of PUFAs such as 22:6 (reviewed in Hwang, 1989). For example, prostaglandin E_2 can inhibit mitogen-induced T cell proliferation (Yachnin et al , 1980c) and major histocompatibility Class II antigen expression on macrophages (Lu et al., 1984). Deutsch and co-workers first reported in 1983 that defatted human AFP could no longer suppress mitogen responses unless reconstituted with 20:4 (Deutsch, 1983) In addition, Nunez et al found that the immunosuppressive potency of mouse AFP preparations could be increased with the addition of exogenous 22:6 directly into the mitogen assay (Nunez et al., 1987).

However, Yachnin et al. reported otherwise, concluding that the suppression of lymphocyte transformations by human AFP could not be attributed to PUFAs or prostaglandins (Yachnin et al., 1980a, 1980b, 1980c), leaving unresolved the role of these PUFAs in AFP-mediated immunoregulation.

Vallette et al. (Vallette et al., 1989) have recently demonstrated that the relative content of unsaturated fatty acids bound by rat AFP contributes to the generation of transient forms of AFP differing in their endocrine and immune functions and Aussel et al. (Aussel et al., 1973) previously showed that distinct rat AFP isoforms differ in their estrogen binding properties. This raises the important question as to whether the immunosuppressive activity of AFP is an intrinsic property of certain AFP molecules rather than being attributable to a putative non-covalently bound ligand such as 20:4 or 22:6. Thus, although PUFAs such as 20:4 and 22:6 can modulate immune responses (Hwang, 1989, Lokesh et al , 1988), their role in AFP-mediated immunosuppression has remained a contentious issue.

In the present investigation, we have analyzed the quantities of fatty acids complexed with native mouse AFP, seven natural isomeric forms of AFP, delipidated AFP preparations, and mouse albumin by gas chromatography, and assayed the preparations for immunosuppressive activity. We show here that the association of the long-chain PUFAs, 20:4 and 22:6, with AFP molecules is not responsible for the immunoregulatory activities of the protein.

B. MATERIALS AND METHODS

Animals

Male and female C3H and Swiss Webster mice were purchased from Charles River Laboratories (Montreal, Canada) and were bred and maintained in our own animal facilities.

AFP Purification

AFP was isolated from mouse amniotic fluid (MAF) extracted from pregnant Swiss mice in late stage gestation (days 15 - 18). The purification of AFP was performed by passaging MAF through anti-normal mouse sera affinity columns as previously described (Murgita and Tomasi, 1975a). Purified AFP was adjusted to a concentration of 2 mg/ml in phosphate buffered saline (PBS); and the purity of AFP preparations was confirmed by alkaline and SDS polyacrylamide gel electrophoresis, and immunodiffusion.

Separation of AFP Isoforms

Seven distinct molecular variants of mouse AFP were separated and purified by preparative anion-exchange chromatography with Mono Q columns linked to a fast protein liquid chromatography system as previously described (van Oers et al., 1990). The seven isoforms were defined by their respective chromatographic retention volumes, isoelectric points, and immunoblotting patterns with anti-AFP antisera.

Extraction and Gas Chromatography of Fatty Acids

Fatty acids in alpha-fetoprotein and albumin preparations were extracted by the method of Folch et al. (Folch et al., 1956) after the addition of 8 µg of 10,13-nonadecadienoic acid (19.2) In some experiments, the protein precipitate which formed at the aqueous interface was retained and dialyzed versus PBS to effect resolubilization. After conversion to their methyl esters by treatment with diazomethane, the fatty acids were quantitated using a model 3300 Varian gas chromatograph with a flame ionization detector. The column employed was a J&W fused silica capillary column (DB23, 30 m x 0.32 mm and 0.25 µM film thickness).

The amounts of fatty acids were calculated by comparison of their peak areas with that of the internal standard, 19:2.

Removal of Free Fatty Acids from AFP

Free fatty acids were extracted from AFP preparations using cartridges of ODS silica (Waters C_{18} Sep-Paks, Millipore Canada Ltd.) as described by Powell (Powell, 1982). AFP (5 mg) in PBS was mixed with absolute ethanol to a final concentration of 30% ethanol, and this solution was acidified to a pH of ca 3.0 with the addition of 1.0 M citric acid. After loading the sample onto the cartridge, unadsorbed protein material was rinsed through the cartridge with 30% ethanol in water (20.0 ml). Fatty acids retained on the column were then eluted with 80% ethanol in water (20.0 ml), and absolute ethanol (10.0 ml). The material in the acidified 30% ethanol fractions was lyophilized, reconstituted in water, and dialyzed versus PBS. In some cases, the acidified 30% ethanol fractions were dialyzed immediately versus PBS followed by concentration on YM-5 membranes with an Amicon filtration unit (Amicon, Canada). Proteins in these fractions were adjusted to a concentration of 1 mg/ml. In certain experiments, fatty acids were extracted from AFP using a charcoal adsorption procedure described by Chen (Chen, 1967) Briefly, 2 mg of AFP or albumin were dialyzed versus distilled water (Milli-Q H_2O, Millipore Corporation), carefully adjusted to pH 3.0 with the addition of 1 M citric acid, and subsequently stirred for 4 hours at 4°C with pre-washed charcoal (Norit A, Fisher Scientific Ltd). The solution was then centrifuged at 20,000g for 15 min The supernatant containing the lipid-free protein was filtered through 0.22 µm filters (Millex-GS filters, Millipore) and the pH was re-adjusted to pH 7.0 by dialysis versus PBS

Preparation of Fatty Acid - Protein Complexes

Arachidonic acid and 4,7,10, 13,16,19-docosahexaenoic acid (Calbiochem Corp.) were purified on an open column of silicic acid (Biorad Laboratories) and adjusted to a concentration of 10^{-1}M in 100% ethanol before use. Each fatty acid was added to native unfractionated AFP, purified AFP isoforms and mouse albumin (2mg/ml) at a final concentration of 2.86 mM, and incubated for 24 hours at 4°C These amounts correspond to a 100-fold molar excess of FFA compared to the 1.0 mole

FFA normally bound by MAF-derived AFP. After the completion of the 24-hour incubation, the protein preparations were dialyzed versus PBS to remove unbound fatty acids Complexed FFAs were subsequently quantitated by GC. In certain experiments, unbound fatty acids were removed by density gradient centrifugation. Briefly, 2.5 ml of protein (2-4 mg of protein) was mixed with 1.42 ml of a solution containing 1 87 M NaCl and 2.06 M KBr (density = 1 239) to generate a final density of 1.15 These preparations were centrifugated at 100,000 X g for 4 hours. Under these conditions, non-protein containing fatty acids micelles floated at the top of the gradient. The lower phase containing the protein was dialyzed against PBS, and concentrated with Centricon-10 ultrafiltration units (Amicon Canada Ltd.) prior to GC analysis

A mixture of (30 nM) arachidonic acid and docosahexaenoic acid (30 nM) were also incubated directly with $CD4^+$ $CD8^-$ T lymphocytes ($2.5x10^6$ cells/ml) for 48 hours at 37°C. These concentrations approximate the quantities of 20.4 and 22:6 that are normally complexed with native AFP preparations present in tissue culture experiments Experiments were also performed with samples of AFP-4 (1.0 mg/ml) that had been dialyzed for 20 hours at 4°C in 10.0 ml of mouse amniotic fluid.

Mitogen Transformations

Adult C3H $CD4^+$ $CD8^-$ thymocytes obtained by a conventional two step negative selection protocol with anti-Lyt 2.1 antisera (New England Nuclear) and rabbit complement (Low Tox, Cedarlane) were cultured to assay for reactivity to Concanavalin A employing a previously described microculture system (Hooper and Murgita, 1981). $CD4^+$ $CD8^-$ thymocytes ($2.5x10^5$) were cultured in 96-well round bottom microtitre plates for 48 hours with Concanavalin A (1 µg/ml, Pharmacia Fine Chemicals). Total volumes of cell cultures were 200 µl. Cells were maintained at 37°C in 95% humidified air and 5% CO_2. Six hours prior to harvesting, cultures were pulsed with 1 µCi [^{3}H] thymidine (66 Ci/mmole, ICN Biomedicals Canada). Cells were harvested onto glass fibre mats with a multiple sample harvestor (Skatron, Flow Laboratories) and water insoluble [^{3}H] thymidine incorporation was measured with a liquid scintillation counter (LKB 1216 Rack Beta, Pharmacia Fine Chemicals). Results are expressed as mean counts per minutes ± SEM of triplicate cultures.

C. RESULTS

Fatty Acid Composition of Mouse AFP

Previous reports have indicated that free fatty acids (FFA) are the only type of lipids associated with mouse and rat AFP (Savu et al., 1981, Calvo et al , 1988). To identify the fatty acids associated with mouse AFP, all the non-esterified FFAs bound by MAF-derived AFP were extracted with organic solvents, converted to methyl ester derivatives and analyzed by gas chromatography (GC). Figure 11A shows a representative profile of the fatty acids separated on a DB-23 fused silica column. Six major components were detected and subsequently identified by comparing their retention times with those from a mixture of methylated fatty acid standards. The major non-esterified fatty acids identified included palmitic acid (16:0), stearic acid (18.0), oleic acid (18:1), linoleic acid (18 2), arachidonic acid (20:4), and 4,7,10,13,16,19-docosahexaenoic acid (22 6) The types of FFA bound to mouse AFP are very similar to that reported for human AFP (Parmelee et al , 1978). The peak with a retention time of 17.0 min in Figure 11 is 19 2, an internal standard added prior to the extraction of the fatty acids from the protein

Quantitative Measurements of Fatty Acids Bound to AFP

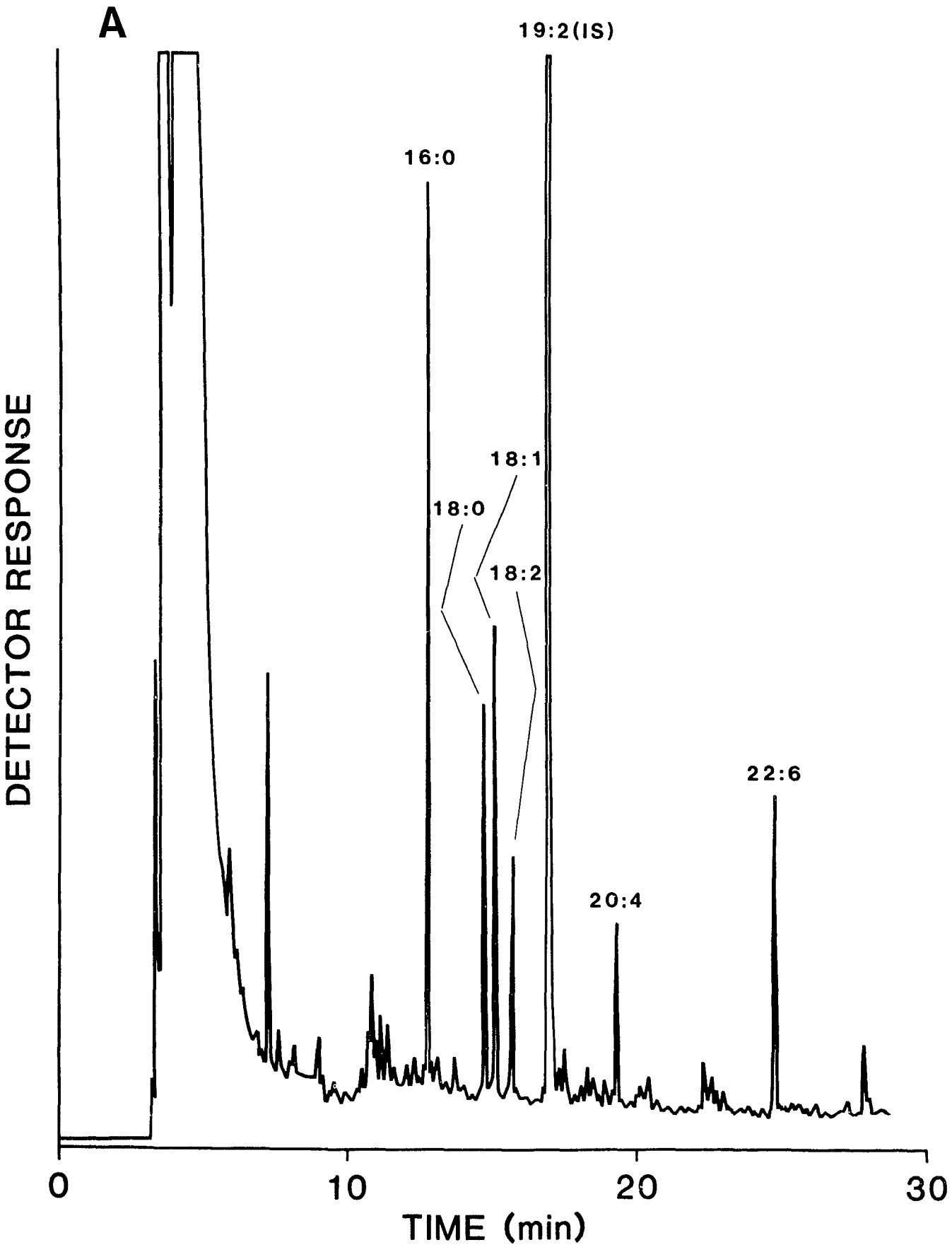
The concentrations of the six fatty acids identified in Figure 11A were calculated by comparing the relative peak area of each to that of the internal standard. The molar ratios of each fatty acid extracted from AFP are shown in Table X The quantities ranged from a low of 0 09 mol arachidonic acid and linoleic acid per mol of AFP to a maximum of 0.34 mol for palmitic acid, comprising a total of 1 0 mol of fatty acid per mol of AFP. Mouse albumin exhibited a total fatty acid content of 0.70 mol, but contained only 0.03 mol of each of 20.4 and 22·6, respectively, levels which were near the GC detection limits of 0 01 mol of FA/mol of protein (Table XI). Human fetal albumin was previously reported to contain 0 05 and 0 03 mol of 20:4 and 22·6, respectively (Parmelee et al , 1978)

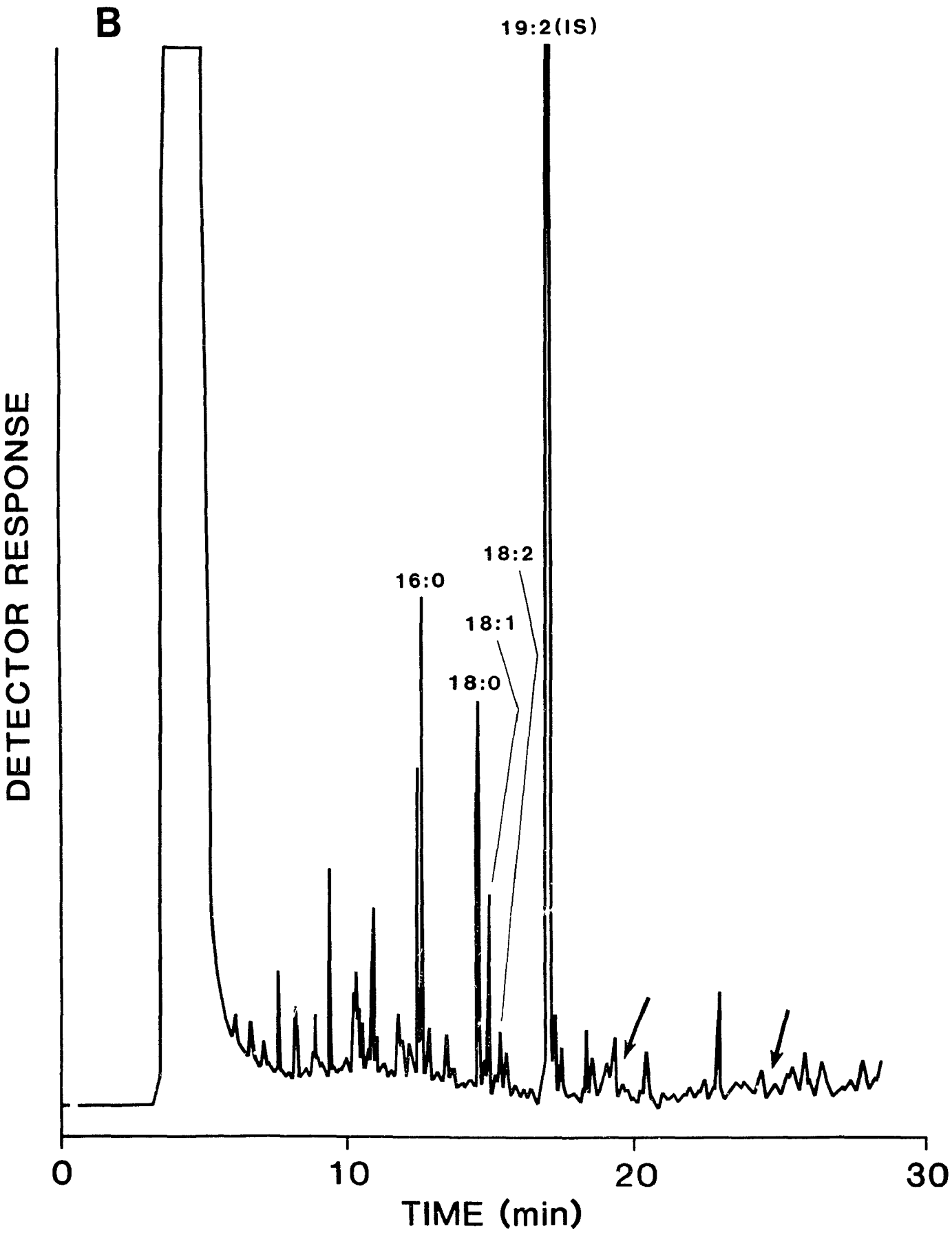
The Role of Arachidonic Acid and Docosahexaenoic Acid in AFP-Mediated Immunosuppressive Activity

It has been previously established that AFP is capable of exerting strong

Figure 11.

Gas chromatograms of the fatty acids from mouse amniotic fluid-derived alpha-fetoprotein. **A**) Alpha-fetoprotein (2.0 mg) was incubated with 8 μg of 19:2 as an internal standard. Fatty acids were extracted from AFP by chloroform:methanol, converted to their methyl esters, and chromatographed on a fused silica capillary column as described in "Material and Methods". The identity of individual fatty acid components was confirmed by the addition of fatty acid standards and are labelled accordingly. **B**) Preparations of AFP were passed over ODS silica cartridges and the residual fatty acids remaining on AFP were extracted, methylated, and run on the GC. **C**) In one experiment, fatty acids were extracted from AFP by 3 successive chloroform/methanol extractions. Following each extraction, the protein was re-solubolized by dialysis against PBS Residual fatty acids remaining complexed to AFP were analyzed by GC during the third extraction. For comparative purposes, the approximate peak areas of the internal standard are 875,000 (A), 1,000,000 (B), and 540,000 (C).


A
19:2(IS)
16:0
18:1
18:0
18:2
22:6
20:4
DETECTOR RESPONSE
0
10
20
30
TIME (min)


B
19:2(IS)
18:2
16:0
18:1
18:0
DETECTOR RESPONSE
0
10
20
30
TIME (min)

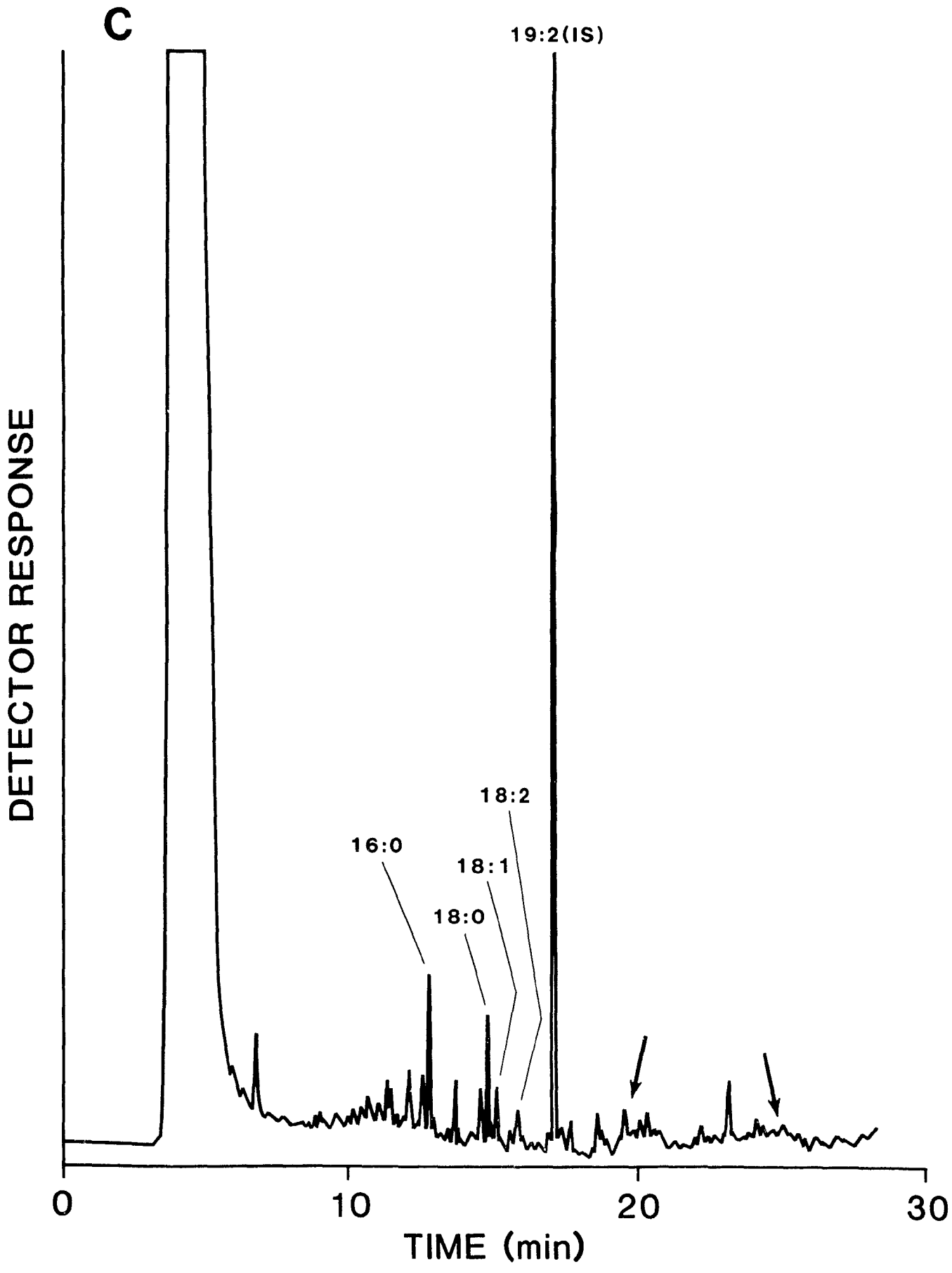

C
19:2(IS)
DETECTOR RESPONSE
16:0
18:0
18:1
18:2
0
10
20
30
TIME (min)

TABLE X

FATTY ACID COMPOSITION OF NATIVE AFP AND ITS SEVEN INDIVIDUAL MOLECULAR VARIANTS

	MOLES FATTY ACID/MOLE PROTEIN*						
PROTEIN SAMPLE	**16:0**	**18:0**	**18:1**	**18:2**	**20:4**	**22:6**	**TOTAL**
Native AFP (6)	0.34 ± 0.18	0.18 ± 0.10	0.17 ± 0.08	0.09 ± 0.02	0.09 ± 0.05	0.14 ± 0.03	1.00 ± 0.46
AFP-1 (2)	0.39 ± 0.18	0.24 ± 0.02	0.18 ± 0.02	0.13 ± 0.03	<0.02	<0.02	1.02 ± 0.25
AFP-2 (3)	0.66 ± 0 34	0 61 ± 0.40	0.14 ± 0.10	0.06 ± 0.03	<0.01	<0.01	1.47 ± 0.87
AFP-3 (3)	0.42 ± 0.07	0.49 ± 0.10	0.12 ± 0.08	0.04 ± 0.03	<0.01	<0.01	1.08 ± 0.28
AFP-4 (3)	0.39 ± 0.08	0.54 ± 0.11	0.12 ± 0.08	0.05 ± 0.01	<0.01	<0.01	1.10 ± 0.28
AFP-5 (3)	0.44 ± 0.20	0 49 ± 0.09	0.08 ± 0.02	0.03 ± 0.02	<0.01	<0.01	1.04 ± 0.33
AFP-6 (3)	0.40 ± 0.14	0.55 ± 0.17	0.08 ± 0 04	0.02 ± 0.01	<0.01	<0.01	1.04 ± 0.36
AFP-7 (3)	0.42 ± 0.02	0.48 ± 0.10	0.08 ± 0 02	0.04 ± 0.03	<0.01	<0.01	1.02 ± 0.17

*Free fatty acids were extracted with chloroform: methanol. The fatty acids in the extracts were methylated and quantitated by gas chromatography as described under "Materials and Methods". The moles of fatty acid per mole of protein for 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 are means ± S.E. of determinations carried out on two to six different preparations of the proteins indicated. The numbers in parentheses are the number of determinations per protein.

immunosuppressive effects on several in vitro functional assays (Murgita and Wigzell, 1981) and more recently we have demonstrated that all this immunoregulatory activity is associated with only one of seven natural isomeric forms of AFP (van Oers et al., 1989). Conflicting results have emerged regarding the contribution of the PUFAs 20:4 and 22:6 complexed to AFP in mediating the immunosuppressive functions of the protein (Nunez et al., 1987, Hsia et al., 1987, Deutsch, 1983, Yachnin et al., 1980c, Vallette et al., 1989). To assess the potential contribution of these fatty acids in AFP-mediated immunosuppression, we compared the fatty acid composition of the biologically active molecular variant of mouse AFP, termed AFP-1, with the six non-suppressive mouse AFP isoforms, AFP-2 through AFP-7 The seven individual isoforms of mouse AFP were separated on a Mono Q anion-exchange column as previously described (van Oers et al , 1990) and the non-esterified fatty acids bound by each were extracted with chloroform:methanol and analyzed by gas chromatography. As shown in Table X, the total amount of FFAs bound by the different isoforms was relatively constant at 1.0 mol FA/mol of protein but the levels of arachidonic acid and docosahexaenoic acid were below the GC detection limits. These results suggest that 20:4 and 22:6 are lost during the purification of the AFP isoforms by anion-exchange chromatography. The low content of 20:4 and 22:6 in purified AFP-1 would suggest that its immunoregulatory activity does not require the presence of these fatty acids. As shown in Table XI, the PBS solution used for dialyzing the AFP isoforms contained residual quantities of 16:0 and 18:0 which may have contributed to some of the increases noted in the isoforms.

Immunosuppressive Activity of AFP Preparations Following Fatty Acid Adsorptions and Re-associations with Arachidonic Acid and Docosahexaenoic Acid

To determine the role of PUFAs such as 20:4 and 22.6 in AFP-mediated immunoregulation, experiments were performed in which the immunosuppressive activity of AFP was assessed following the removal of complexed fatty acids by adsorption to ODS silica cartridges Figure 11B shows the GC fatty acid profile of residual FFAs associated with AFP following ODS silica adsorption. There was a 40% reduction in the combined molar content of 16:0, 18.0, 18 1, and 18.2. More

TABLE XI

QUANTITIES OF FATTY ACIDS BOUND BY AFP, ALBUMIN, DELIPIDATED PROTEIN PREPARATIONS, AND DEFATTED SAMPLES RE-ASSOCIATED WITH 20:4 AND 22:6

	MOLES FATTY ACID/MOLE PROTEIN*						
PROTEIN SAMPLES	16:0	18:0	18:1	18:2	20:4	22:6	TOTAL
Native AFP	0.33 ± 0.18	0.18 ± 0.10	0.17 ± 0.08	0.09 ± 0.02	0.09 ± 0.05	0.14 ± 0.03	1.002 ± 0.46
Mouse Albumin	0.18 ± 0.04	0.06 ± 0.01	0.10 ± 0.02	0.31 ± 0.06	0.03 ± 0.01	0.03 ± 0.01	0.70 ± 0.14
AFP-ODS Silica Adsorbed	0.15	0.15	0.09	0.07	<0.01	<0.01	0.46
AFP-Delipidated by Chloroform/ methanol	0.03	0.01	0.02	0.01	<0.02	<0.01	0.10
AFP-4 to AFP-7 + 20:4, 22:6	0.33	0.16	<0.01	<0.01	0.77	1.82	3.08
Albumin + 20:4, 22:6	0.24	<0.01	<0.01	<0.01	0.79	1.11	2.15
Native AFP + 20:4, 22:6	0.15	0.09	0.03	<0.02	0.28	0.83	1.38
PBS + 20:4, 22:6	0.15	0.07	<0.01	<0.01	<0.01	<0.01	0.22

*Non-esterified fatty acids bound by the indicated proteins were extracted and analyzed as described under "Materials and Methods". Preparations of AFP were delipidated by chloroform methanol extractions or using ODS silica cartridges as indicated in "Materials and Methods" Defatted protein samples and PBS were incubated with a 100-molar excess of 20:4 and 22:6, and the FFA-protein complexes which formed were quantities by GC analysis

importantly, a quantitative analysis of the remaining FFAs indicate that the quantities of 20:4 and 22:6 are below the GC detection limits of 0.01 mol of fatty acid per mol of protein (Table XI). The functional activity of AFP passed over ODS silica cartridges was assayed by measuring its suppressive effects at concentrations ranging from 100 µg/ml to 1 5 µg/ml on Con A-stimulated $CD4^+$ $CD8^-$ thymocyte responses. As shown in Figure 12, AFP depleted of 20.4 and 22:6 shows a titratable suppressive activity comparable to control untreated AFP. Samples of AFP that had been exposed to either 30% ethanol or 30% ethanol at pH 3.0, conditions required for fatty acid adsorption to ODS silica, maintained suppressive activities equivalent to control AFP. The $CD4^+$ CD8 thymocyte responses were also unaffected by the addition of albumin controls adjusted to 30% ethanol, titrated to pH 3.0, and passed over ODS silica cartridges.

In an attempt to increase the efficiency of fatty acid depletions, native AFP preparations were also delipidated by chloroform/methanol extractions. Following 3 successive chloroform/methanol extractions on the same sample of AFP, roughly 90% of the FFAs were removed (Table XI). More importantly, the levels of 20:4 and 22:6 were again below the GC detection units (Fig. 11C). As shown in Table XII, the fatty acid-depleted AFP preparation inhibited the ConA induced $CD4^+$ CD8 thymocyte response by 82%, similar to the 89% suppression seen with native AFP. Due to the large protein loss which occurred with the chloroform/methanol extraction procedures, this method was not suitable for generating larger amounts of delipidated AFP which would be needed for further functional studies.

Experiments were also performed to directly assess the effects of 20:4 and 22:6 on T-cell mitogen responses, and the functional contribution of these PUFAs on several non-suppressive protein preparations. As shown in Table XII, the addition of $3X10^{-8}M$ AA and DHA directly to the mitogen assay had no noticeable effect on $[^3H]$ thymidine incorporation. These concentrations approximate the quantities of 20:4 and 22:6 normally complexed on native AFP preparations added to the tissue culture assay. Control AFP, a mixture of AFP-2 to AFP-7, a single isoform AFP-4, and mouse albumin were also incubated with a 100-molar excess of 20 4 and 22 6 Non-complexed FFAs were removed by dialysis, and the effectiveness of FFA binding was confirmed by GC analysis. As shown in Table XI, cell proteins exhibited a significant increase in the combined molar content of 20:4 and 22:6

Figure 12.

Dose-response effect of control AFP and PUFA depleted AFP preparations on Con A-stimulated $CD4^+$ $CD8^-$ thymocytes. Adult C3H $CD4^+$ $CD8^-$ thymocytes [$2.5x10^5$] were cultured with 1 μg/ml ConA in the presence of varying concentration of control AFP (●), AFP exposed to 30% ethanol (0), AFP titrated to pH 3.0 in 30% ethanol (■), and AFP passed over ODS silica cartridges (▲) as described in "Materials and Methods". Control albumin, albumin exposed to 30% ethanol at pH 7.0 or 3.0, and albumin eluted through ODS silica were all without effect in this assay (△). Proliferation responses were measured at 48 hours of culture by [^{3}H] thymidine incorporation as described in "Materials and Methods".

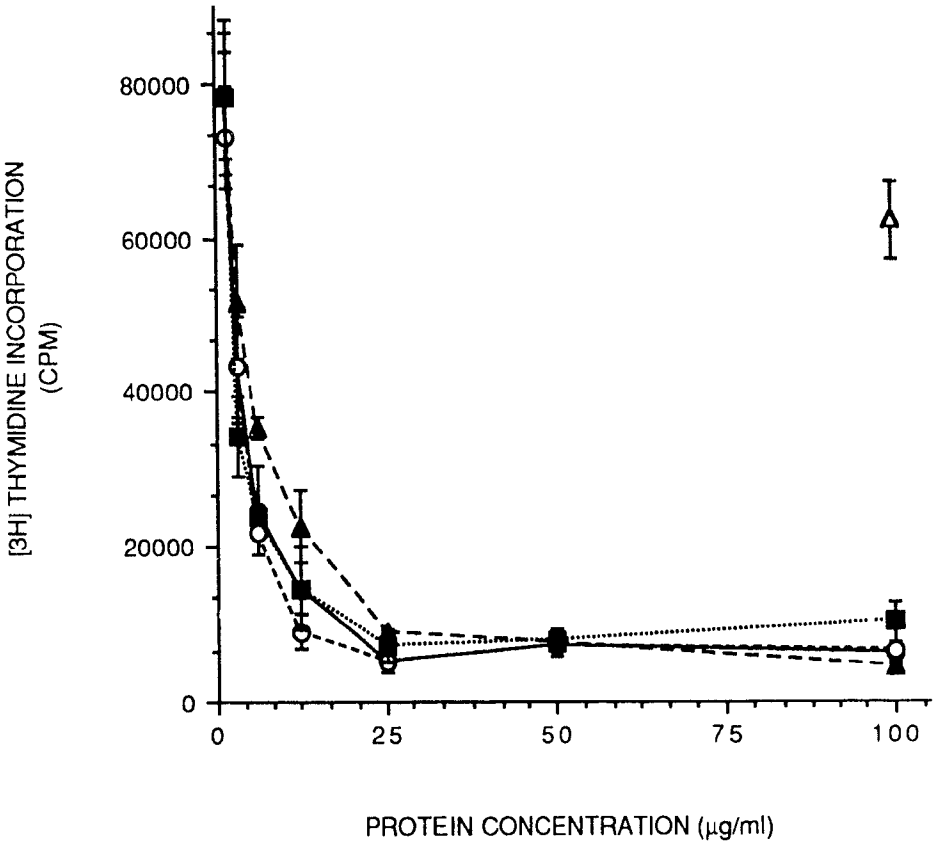

[3H] THYMIDINE INCORPORATION (CPM)
80000
60000
40000
20000
0
0
25
50
75
100
PROTEIN CONCENTRATION (µg/ml)

TABLE XII

REGULATION OF THE CONA STIMULATED CD4+ CD8- THYMOCYTE RESPONSE

			CD4+ CD8- CON A THYMOCYTE RESPONSE[a]		
	SAMPLE	TREATMENT	RESPONSE	CONTROL RESPONSE	% SUPPRESSION
Exp't I	Native AFP	None	4058 ± 1328	35,432 ± 7479	89
	Delipidated AFP[b]	Chloroform/Methanol	6210 ± 1833	35,432 ± 7479	82
	20:4 (3 X 10^{-8}M)	None	37,952 ± 4525	35,432 ± 7479	0
	22:6 (3 X 10^{-8}M)	None	34,516 ± 4162	35,432 ± 7479	0
Exp't II[c]	Native AFP	None	9800 ± 2118	87,062 ± 13,172	89
	Native AFP	20:4; 22:6 Reassociated	7534 ± 967	87,062 ± 13,172	91
	AFP-2 - AFP-7	None	109,130 ± 6793	87,062 ± 13,172	0
	AFP-2 - AFP-7	20:4; 22:6 Reassociated	77,220 ± 4750	87,062 ± 13,172	10
	Albumin	None	148,650 ± 35,221	135,276 ± 8240	0
	Albumin	20:4; 22:6 Reassociated	137,025 ± 1591	135,276 ± 8240	0
	AFP-4	None	123,378 ± 33,788	135,276 ± 8240	9
	AFP-4	20:4; 22:6 Reassociated	177,195 ± 18,172	135,276 ± 8240	0
	PBS	None	135,276 ± 8240	135,276 ± 8240	0
	PBS	20:4; 22:6 Reassociated	152,342 ± 12,142	135,276 ± 8240	0
Exp't III	MAF-CTL	None	4318 ± 619	68,408 ± 16,256	94
	AFP-4 (MAF)	20 hour dialysis against MAF	72,291 ± 1676	68,408 ± 16256	0

[a]Adult C3H CD4+ CD8- thymocytes (2.5 X 10^5) were co-cultured with 1 µg/ml ConA and 100 µg/ml of the indicated proteins or 3 X 10^{-8}M of the indicated fatty acids. Proliferative responses were measured at 48 hours and control response is maximum ^{3}H-thymidine incorporation in the presence of media alone.

[b]Delipidations were performed as described in "Materials and Methods"

Native AFP complexed 1.11 moles, AFP-2 to AFP-6 bound 2.12 moles of these FFAs, AFP-4 had a molar binding content of 2.59 moles whereas mouse albumin re-associated with 1.9 moles of these fatty acids per mole protein. When PBS controls containing 20:4 and 22:6 were prepared in a similar manner, these PUFAs were not detected following dialysis. It is notable that the quantities of 20:4 and 22:6 bound by the AFP isoforms are near the saturable fatty acid binding capacity published for AFP (Savu et al., 1981, Parmelee et al., 1978, Uriel et al., 1987, Torres et al., 1989, Hsia et al., 1987, Calvo et al., 1988). The functional activities of FFA complexes of control AFP, AFP-4, AFP-2 to AFP-7, and albumin (100 µg protein/ml) were determined by measuring their effects on ConA-stimulated $CD4^+$ $CD8^-$ thymocyte responses. As shown in Table XII, only control AFP preparations were immunosuppressive, supporting the conclusion that 20.4 and 22:6 play no central role in AFP-mediated immunosuppressive activity. To help rule out the possibility that some other associated molecule was responsible for AFP-mediated immunosuppression, AFP-4 was directly dialyzed versus mouse amniotic fluid. Following 20 hours of dialysis, the immunosuppressive properties of the AFP in MAF could not be transferred to AFP-4.

D. DISCUSSION

Extensive investigations in both the human and murine systems continue to support the concept that one of the major functions of AFP is immunoregulation (reviewed in Murgita and Wigzell, 1981, Yachnin, 1983). The major purpose of this study was to determine whether the immunosuppressive activity of AFP is an intrinsic property of certain AFP molecules or whether it is due to the association of non-covalently complexed PUFAs.

AFP, like serum albumin, possesses 2 or 3 binding sites for FFAs and is considered the major physiological binding and transport protein of PUFAs in the serum during fetal development (Savu et al., 1981, Parmelee et al , 1978, Laborda et al., 1989, Uriel et al., 1987, Calvo et al., 1988). It is noteworthy that AFP, in contrast to albumin, has a preferential affinity for PUFAs including arachidonic acid and docosahexaenoic acid (Savu et al., 1981, Parmelee et al., 1978, Hsia et al., 1980, Imgvarsson and Carlsson, 1978). These properties, combined with the observation that AFP can mediate the transfer of 20:4 into cells (Uriel et al., 1987, Torres et al., 1989), the identification of AFP receptors on activated T cells (Torres et al., 1989), and the knowledge that PUFAs can modulate immune responses (Hwang, 1989) prompted several researchers to propose that the immunoregulatory activities may be related to the AFP-PUFA complex. Thus, Deutsch suggested that defatted human AFP preparations would not suppress mitogen-induced T-cell responses unless the protein was reconstituted with arachidonic acid (Deutsch, 1983). Nunez et al. reported (Nunez et al., 1987) that the exogenous addition of 1 μg of 22:6 directly into a spleen cell mitogen assay containing AFP could increase the inhibitory properties of the protein 2-3 fold These authors attributed previously reported variabilities in the immunosuppressive activities of different AFP preparations (Lester et al., 1976) to the relative concentration of the saturated and unsaturated fatty acids bound to AFP (Nunez et al., 1987, Deutsch, 1983) Therefore, samples of AFP containing a high molar content of 20:4 and 22:6 were proposed to demonstrate more enhanced immunosuppressive activities (Nunez et al., 1987).

The fact that many of these conclusions were based on indirect evidence with no quantitative analysis of the FA-protein complexes used in the assays, leaves

unresolved the contribution of PUFAs in AFP-mediated immunoregulation In contrast to these reports, Yachnin et al. have found no correlation between the individual or total fatty acid content of fetal or tumor-derived human AFP samples, and the capacity of these preparations to inhibit T-cell mitogen responses (Yachnin et al., 1980c). Furthermore, these authors demonstrated that FFAs at concentrations of 10^{-4} M had no significant suppressive effect on lymphocyte transformations In this investigation, we have demonstrated that the immunosuppressive activity of mouse AFP is not mediated by complexed long-chain PUFAs. GC analysis showed that MAF-derived AFP complexes with 6 distinct fatty acids comprising a total of 1.0 mol of fatty acid per mol of protein (Table X), similar to the quantities associated with amniotic fluid-derived rat AFP (0.8 mol/mol protein) (Calvo et al , 1988). We have recently succeeded in separating mouse AFP into seven distinct isoforms by anion-exchange chromatography (van Oers et al., 1990). Since we determined that all immunoregulative activity of mouse AFP was associated with only one of these seven molecular variants (van Oers et al , 1989), it is conceivable that this isoform (AFP-1) had FFA binding characteristics distinct from the 6 non-suppressive variants which contributed to its activity. Using a combination of anion-exchange chromatography and GC analysis we have found similar quantities of 16:0, 18:0, 18:1 and 18:2 for all seven purified isoforms comprising a total of 1 mole of FFA/mole of protein (Table X). These results are consistent with previous reports showing that distinct rat AFP subspecies have identical fatty acid compositions (Calvo et al., 1988). More importantly, there was no detectable 20:4 and 22:6 bound to any of the seven isoforms in comparison to native AFP. This may be a result of the chromatographic procedure developed for separating the molecular variants of mouse AFP. Consistent with this hypothesis is the report of Calvo et al. documenting a selective loss in the relative proportions of 20 4 and 22:6 bound by rat and pig AFP following affinity chromatography procedures (Calvo et al., 1985). We found that all seven isoforms had an elevated molar content of 16:0 and 18:0. This may be due to contamination with exogeneous saturated fatty acids during the isolation and purification procedures since we detected small amounts of 16:0 and 18:0 in our preparations of PBS (Table XI)

The aforementioned results would suggest that the immunoregulatory activity of AFP does not require the complexed PUFAs 20:4 and 22:6 This interpretation was

substantiated by subsequent experiments showing that AFP batches delipidated by passing through ODS silica cartridges suppressed mitogen responses whereas equivalent amounts of untreated or defatted albumin were without effect. In addition, we were unable to convert non-suppressive molecular variants into an active isoform by re-associating AFP subspecies with approximately 3 moles of 20:4 and 22:6 per mole of protein. It is worth noting that the molar content of FFA in different AFP preparations ranges between 1 and 3 depending on both the tissue source and gestational time point of AFP isolation (Uriel et al., 1987, Calvo et al., 1988). For example, rat amniotic fluid-derived AFP contain less FFA (0.8 mol/mol protein) than that of fetal serum (1 4 mol/mol protein) These differences are hypothesized to be due to the lower levels of 20:4 and 22:6 in the amniotic fluid (Calvo et al , 1988) This observation would be in agreement with our results showing that the addition of exogenous 20:4 and 22 6 to MAF-derived AFP-4 increased its FFA molar content to the maximal binding capacity of 3 moles/mol of protein (Parmelee et al , 1978, Hsia et al., 1981). In spite of the normally low quantities of 20.4, and 22:6 in amniotic fluid, MAF-derived mouse AFP is very effective at suppressing mitogen responses at concentrations as low as 6.25 μg/ml. In fact, the concentrations of 20 4 and 22 6 bound by AFP in these assays are at least 50-fold lower than that required to directly inhibit T cell mitogen responses (Kelley and Parker, 1979) The conclusion that 20:4 and 22:6 are not essential for AFP-mediated immunoregulation is in agreement with results presented by Yachnin et al (Yachnin et al , 1980c) showing no correlation between fatty acid composition and the immunosuppressive potency of different human AFP preparations. It is possible that the previously reported loss of immunoactivity could have been due to the delipidation procedure utilized for defatting AFP Yachnin and co-workers attempted to repeat the charcoal adsorption experiments of Deutsch et al (Deutsch, 1983), but found that the prolonged exposure of AFP at pH 3.0. a condition required for charcoal adsorption of FFAs, resulted in a loss of immunosuppressive activity. Although, we have found that mouse AFP, titrated to pH 3.0 was equally effective as native AFP in suppressing mitogen responses, we were unable to generate consistent results with charcoal adsorption experiments (unpublished observations). There is some evidence to suggest that AFP exposed to charcoal at pH 3 may undergo a profound denaturation in its hydrophobic binding sites

(Parmelee et al., 1978). Fluorescence spectroscopy and circular dichroism studies have revealed dramatic conformational changes in the AFP molecule at acidic and basic pH values (Zizkovsky et al., 1983). These observations suggest that the charcoal treatment itself may in fact denature the protein, resulting in a loss of biological activity. It is also known that the direct addition of FFA to cell cultures may have strong cytotoxic effects on the cells if internalized in excess (Kelley and Parker, 1979, Chow and Jondal, 1990) Since no viability data were reported in the experiments of Deutsch (Deutsch, 1983), and Nunez et al (Nunez et al , 1987), it is possible that the suppressive effects measured in their assays may have been a result of cytotoxic effects of exogenously added FFAs

Although we have shown here that fatty acids are unlikely to contribute to the immunosuppressive activity of AFP, we cannot exclude the possibility that some other non-covalently bound molecule or undetected fatty acid metabolite may be involved. Throughout the course of our investigations, mouse AFP was subjected to temperature and pH extremes, denaturing agents, and high-salt concentrations. All these conditions, which normally favor the dissociation of small molecules, failed to diminish the functional activity of mouse AFP. Moreover, preparations of AFP passed over ODS silica cartridges at ethanol concentrations of 15%, conditions used to absorb prostaglandins, thromboxanes, and monohydroxyeicosatetraenoic acids (Powell, 1982), were as equally suppressive as control AFP (unpublished observations).

Recent experiments by Torres et al. have revealed the presence of AFP receptors on activated T lymphocytes (Toress et al , 1989), and Uriel and co-workers have shown that AFP can mediate the transfer of arachidonic acid into cultured cell lines (Uriel et al., 1987). Since 20.4 is the precursor of the eicosanoids, these authors proposed that the arachidonic acid metabolites such as prostaglandins may regulate lymphocyte functions. However, Yachnin and co-workers have demonstrated that AFP-mediated suppression of T-cell mitogen responses is not affected by indomethacin, a cyclooxygenase inhibitor preventing prostaglandin synthesis (Yachnin et al., 1980c), ruling out the possibility that AFP acts by stimulating prostaglandin production Consistent with these results is the report showing that AFP-mediated inhibition of major histocompatibility complex Class II antigen expression on macrophages is not due to the presence of arachidonic acid or the

synthesis of prostaglandins (Lu et al., 1984). It is also relevant to this line of reasoning that prostaglandin synthesis in the human placenta and fetal membranes is actually inhibited by AFP (Aussel, 1984). The essential biochemical distinction between the immunoregulatory AFP isoform and the remaining six non-suppressive molecular variants remains to be elucidated. In a series of reports correlating biological activity with the presence of specific human AFP electrophoretic variants (Lester et al., 1976), a post-translational modification was postulated to control the immunosuppressive potency of human AFP isomers (Lester et al., 1977a). Experiments are presently underway in our laboratory to determine whether a similar post-transcriptional or post-translational modification may modulate the immunoregulatory activity of mouse AFP isoforms. These studies will include functional analysis of modified synthetic and recombinant AFP peptides (Boismenu et al., 1988).

V. GESTATIONALLY REGULATED EXPRESSION OF A BIOLOGICALLY ACTIVE ALPHA-FETOPROTEIN ISOFORM

A. INTRODUCTION.

Alpha-fetoprotein is a well-characterized onco-fetal molecule, normally expressed by embryonic tissues including the fetal liver, yolk sac, and gastrointestinal tract (reviewed in Ruoslahti and Sepppala, 1979, Abelev, 1971). The transcriptional activation of AFP occurs at about day 10 of fetal development in the mouse, resulting in a linear increase in the quantities of AFP detectable in fetal serum and tissue extracts (Kahan and Levine, 1971, Hau et al. 1981, Nahon, 1987). Several weeks after birth, murine AFP transcription declines sharply, resulting in an approximate 10,000 fold reduction in AFP mRNA levels, so that the concentration of serum AFP decreases from 2 mg/ml to less than 200 ng/ml at 3 weeks of age (Olsson et al., 1977, Camper et al., 1989). Many of the classical approaches for detecting and purifying AFP have indicated that AFP exists as a family of closely-related molecular variants (Kerckaert et al., 1979, Smith and Kelleher, 1980, Lester et al., 1976, 1978b). In several mammalian species, there is a predictable pattern of isoform expression which is gestationally regulated, as process referred to as a developmental microheterogeneity (Gustine and Zimmerman, 1973, Higgins, 1979, Smith and Kelleher, 1980, Wilson and Zimmerman, 1976).

In the present investigation, we have examined the immunosuppressive activites of murine AFP purified from mouse amniotic fluid isolated at specific stages of fetal development, on $CD4^+CD8^-$ thymocyte mitogen responses. We show here that the most effective immunosuppressive AFP preparations were isolated from fetuses at days 10.5, 12.5 and 14.5 of gestation, time points which exhibited the greatest relative amount of an immunoregulatory isoform, AFP-1.

B. MATERIALS AND METHODS

Animals and Amniotic Fluid Collection

CFW Swiss Webster mice, purchased from Charles River (St. Constant, Quebec, Canada) were bred and maintained in our own animal care facility regulated at 25°C with a 12-h light/dark cycle. Pregnancies were obtained by caging 5 CFW females with 1 male overnight. The morning of detection of a vaginal plug was designated as day 0.5 of gestation. Mouse amniotic fluid (MAF) was collected from day 10.5, 12.5, 14.5, 16.5 and day 18.5 embryonic sacs. The fluids from different fetuses were pooled, centrifuged, and the supernatants were stored at -20°C until further use. It should be noted that day 10.5 fetuses contained very little MAF (less than 5 µl per fetus) and the MAF was easily contaminated with fetal serum components. Day 18.5 MAF was extremely viscous, and also proved difficult to extract.

Purification of Alpha-fetoprotein

AFP was purified from MAF by antibody-agarose affinity chromatography as described in detail elsewhere (Murgita and Tomasi, 1975a). Where necessary, AFP was purified by a second chromatographic separation on a Mono S HR5/5 cation-exchange column coupled to a fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals, Baie d'Urfe, Canada). The column was equilibrated with 50 mM formate, pH 4.25. Samples containing between 2-4 mg of AFP, previously dialyzed against the formate buffer, were loaded onto the column, and fractions were eluted with linear salt gradient extending to 1.0 M NaCl in 50 mM formate, pH 4.25. Fractions were collected and individual peaks, as monitored by the OD_{280} absorption profile were pooled and concentrated with Centricon-10 ultrafiltration units (Amicon, Canada). Fractions containing AFP were identified by a combination of sodium dodecyl sulfate and alkaline polyacrylamide gel electrophoresis, and ouchterlony double immunodiffusion analysis outlined in detail elsewhere (van Oers et al., 1990).

Isoelectric Focusing

Analytical isoelectric focusing was performed in 5% polyacrylamide gels

using a Biorad horizontal mini-IEF cell according to the manufacturer's instructions (Biorad Ltd., Mississauga, Canada). One µl samples containing 0.1-1 µg of MAF or purified AFP were loaded onto the gel, and electrophoresed at a constant voltage of 100V for 15 minutes, followed by an additional 15 min. at 200V and finally 450V for 1 hour. The best resolution of distinct AFP isoelectric variants was obtained in pH 4.0-6.5 ampholine gradients (pH 4.0-6.5 Pharmalyte, Pharmacia Fine Chemicals, Baie d'Urfe, Canada). At the completion of electrophoresis, the gel was immersed in a fixative solution [5% (w/v) sulfosalicylic acid, 5% (w/v) trichloroactic acid in distilled water] for 30 min. and stained with Coomassie brilliant blue, or silver stained.

Mitogen Transformations

Adult C3H $CD4^+CD8^-$ thymocytes were obtained by a one-step negative selection protocol with anti-CD8 antisera (kindly provided by Dr. M.H. Julius; McGill University) and rabbit complement (Low-tox, Cederlane Laboratories, London, Canada) Thymocytes ($10x10^7$) were incubated with anti-CD8 (1/50) and rabbit complement (1/10 dilution, 10.0 ml volume) for 60 min. at 37°C. Viable lymphocytes were washed several times in phosphate-buffered saline (PBS), and resuspended in RPMI-1640 (Flow Laboratories, Canada) supplemented with 0.5% autologous normal mouse sera, plus 20 mM Hepes, $5x10^{-5}$ M 2-mercaptoethanol, 4 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. $CD4^+CD8^-$ thymocytes ($2.5x10^5$) were cultured in 96-well round bottom plates for 48 hours with Con A (1 µg/ml, Pharmacia Canada Ltd.) and MAF or AFP added at the initiation of cultures at concentrations ranging from 100 µg/ml to 0.005 µg/ml. Cultures were maintained at 37°C in 95% humidified air and 5% CO_2. Six hours before harvesting, cultures were pulsed with 1 µCi [^{3}H] thymidine (66 Ci/mmole specific activity) (ICN Biomedicals, St. Laurent, Canada). Cells were harvested onto glass fibre filter mats with a multiple sample harvestor (Skatron; McLean, Virginia) 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.

C. RESULTS

Concentration of Mouse AFP in Amniotic Fluid At Different Days of Gestation

The concentration of murine AFP in MAF isolated from several gestational time points was measured by rocket immunoelectrophoresis. As shown in Table XIII, the AFP concentration in CFW mice increased progressively from approximately 260 µg/ml at day 10.5 of gestation to a maximum of 2.2 mg/ml by day 18.5. The total volume of extractable MAF, estimated by pooling the contents of 40-80 fetuses, was very low at day 10.5 (5 µl/fetus). By day 16.5, the fetus contained approximately 100 µl of isolatable MAF. However, the volume of MAF decreased markedly by day 18.5 with individual fetuses containing between 10-20 µl/fetus.

Analysis of AFP Isoforms at Different Gestational Time Points

An analysis of the protein content of MAF on APAGE revealed 3 major protein components previously defined as transferrin (TF), AFP, and albumin (ALB) (Figure 13). Densitometric scans of the APAGE gels indicated that the relative percentages of AFP, ALB, and Tf ranged from 43-61%, 30-43%, and 7-13%, respectively, from days 10.5 to 18.5 of ontogeny (Table XIII) The relative proportions of AFP increased from 43% to 61% by day 18.5 of gestation in relation to transferrin (Tf) and albumin (ALB). These values are very similar to the composition of days 16-19 mouse amniotic fluid reported by Murgita and Tomasi (Murgita and Tomasi, 1975a).

Native AFP was purified from MAF that had been isolated from murine fetuses at days 12.5, 14.5, 16.5, and 18.5 of gestation by procedures that have been described in detail elsewhere (Murgita and Tomasi, 1975a). Pure AFP, as verified by conventional APAGE (Figure 13) and immunodiffusion techniques, was subsequently characterized by isoelectric focusing As shown in Figure 14, fetal mouse AFP purified from a pool of MAF (days 15-19 fetuses), and subjected to narrow range isoelectric focusing (pH 4.0-6.5), could be resolved into 6 distinct variants, as previously reported (Zimmerman et al., 1976, Wong et al., 1988, van Oers et al., 1990). A comparative analysis of the isoelectric variants of AFP from day 12.5, 14.5, 16.5, and 18.5 MAF revealed a clear distinction in the pattern of

TABLE XIII

CONCENTRATION OF MOUSE AMNIOTIC FLUID-DERIVED ALPHA-FETOPROTEIN AT DIFFERENT STAGES OF FETAL DEVELOPMENT

MOUSE AMNIOTIC FLUID (DAYS)[a]	AFP CONCENTRATION (µg/ml)[b]	RELATIVE PERCENTAGE OF TOTAL PROTEIN[c]		
		AFP	TRANSFERRIN	ALBUMIN
10.5	259 ± 63	43%	13%	43%
12.5	657 ± 55	51%	7%	43%
14.5	1170 ± 180	56%	10%	34%
16.5	1580 ± 200	58%	12%	30%
18.5	2220 ± 180	61%	12.5%	38%

[a]Gestational time points at which MAF was isolated.

[b]The concentrations of mouse AFP in MAF were calculated with a rocket immunoelectrophoresis procedure.

[c]Samples of MAF were run on 10% alkaline polyacrylamide gels. The gels were scanned with a Bio-rad densitometer to calculate the relative proportions of individual proteins.

Figure 13.

Polyacrylamide gel electrophoresis of mouse amniotic fluid isolated from different gestational time points. **A)** Mouse amniotic fluid and **B)** purified AFP were analyzed on 10% poylacrylamide gels that were Coomassie blue stained.

MAF (days 16-19)

MAF (day 10.5)

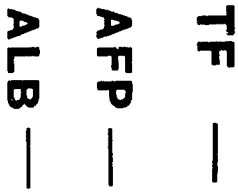
MAF (day 12.5)

MAF (day 14.5)

MAF (day 16.5)

MAF (day 18.5)

Native AFP



MAF (days 16-19)

AFP (day 12.5)

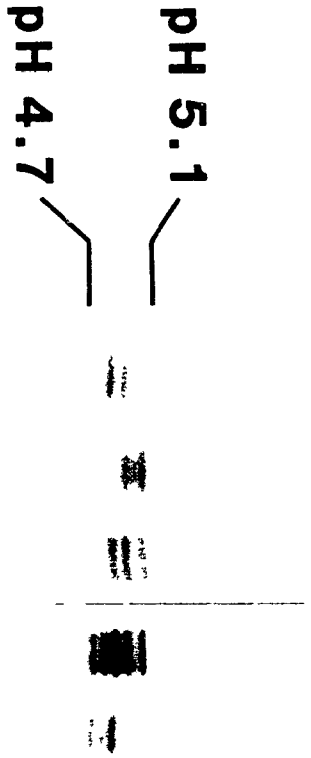
AFP (day 14.5)

AFP (day 16.5)

AFP (day 18.5)

Figure 14.

Comparative isoelectric focusing analysis of AFP preparations purified from several days of murine gestation. AFP, purified from day 12.5, 14.5, 16.5, and 18.5 MAF, was analyzed on narrow ampholine range IEF gels (pH 4.0-6.5) that were silver stained.



CONTROL AFP

AFP (day 12.5)

AFP (day 14.5)

AFP (day 16.5)

AFP (day 18.5)

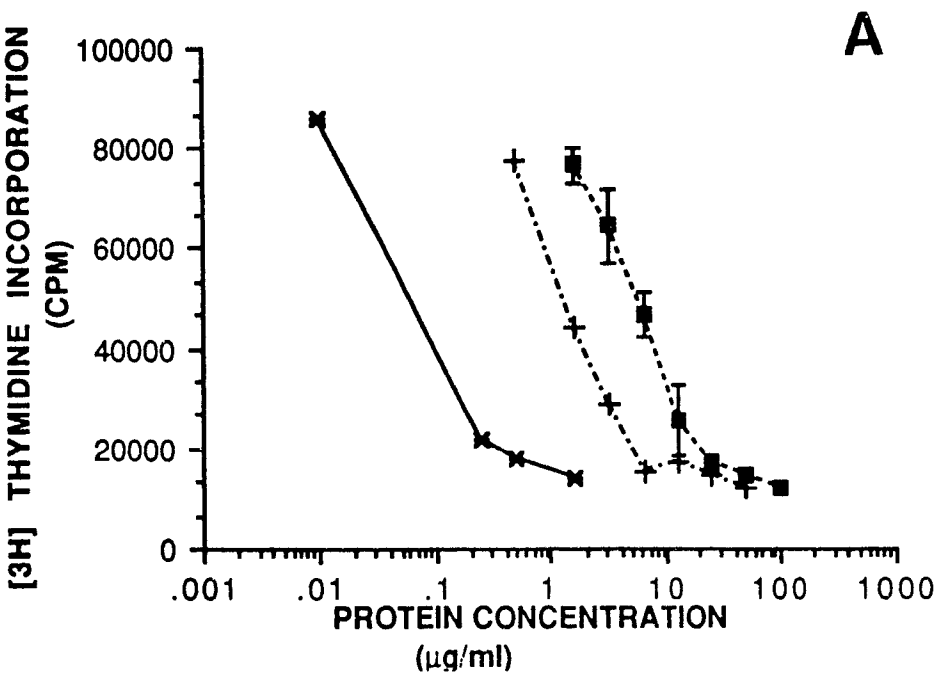
isoelectric heterogeneity during this perinatal period. At day 12.5 of fetal ontogeny, there are only 4 detectable AFP isoelectric variants with pI's ranging from pH 4.85-5.1. By day 14.5, all six isoelectric variants (pH 4.7-5.1) are present, but the relative amounts of the pI 4.9-5.1 subspecies decreased relative to day 12.5. By day 18.5, the AFP isomer exhibiting a pI of 5.1 was barely detectable on silver stained IEF gels. These results indicate that the relative proportions of the more basic isoelectric variants of murine AFP decrease during the course of fetal ontogeny. The fluctuations in isomer distribution at the time points analyzed is similar to that originally noted by Zimmerman et al. (Zimmerman et al., 1976), who defined the changes in AFP isomer concentrations as a developmental microheterogeneity (Zimmerman and Madappally, 1973).

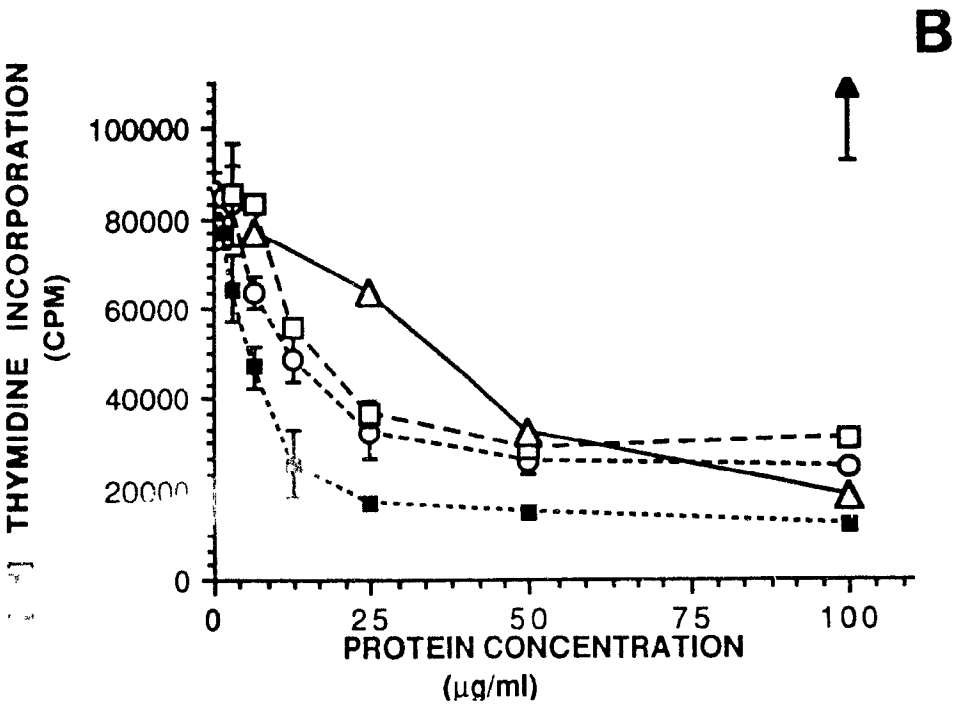
Comparative Immunosuppressive Properties of AFP Isolated From Several Time Points in Fetal Development

Extensive investigations from this lab have shown that AFP is capable of exerting selective immunoregulatory effects in vitro (Murgita and Wigzell, 1981), and we have recently identified a single natural subspecies of mouse AFP which contains all the immunosuppressive activity normally ascribed to the heterogenous population of AFP molecules (van Oers et al., 1989). This isoform, termed AFP-1, exhibited a basic isoelectric point of pH 5.1, and comprised approximately 6% of the native AFP molecules purified from the MAF pooled from day 15-19 fetuses. As shown in Figure 14, the appearance and relative quantities of AFP-1 in the different AFP samples was developmentally regulated. Experiments were therefore performed to compare the immunosuppression properties of MAF-derived AFP isolated from different gestational days. Insufficient quantities of day 10.5 MAF prevented us from purifying the AFP from these samples, and the MAF was added directly to the assays. As shown in Figure 15A, native AFP (days 15-19 MAF) effectively suppresses a $CD4^+CD8^-$ Con A thymocyte assay in a dose-dependent manner at concentrations extending from 100 µg/ml to 6.25 µg/ml. However, the dose-response effects of day 10.5 MAF, and day 12.5, 14.5, 16.5 and 18.5 AFP on mitogen stimulated $CD4^+CD8^-$ thymocytes varied considerably. Day 10.5 MAF exhibited the greatest inhibitory activity, effectively suppressing the mitogen reaction at concentrations below 1.0 µg/ml. Day 12.5 AFP demonstrated suppressive effects

Figure 15.

Dose-response effect of native AFP purified from days 15-19 MAF versus day 10.5 MAF and day 12.5, 14.5, 16.5, and 18.5 AFP on Con A stimulated $CD4^+CD8^-$ thymocytes. Adult C3H $CD4^+CD8^-$ thymocytes were cultured with Con A in the presence of **A)** native AFP (days 15-19) (■) and the gestational stage specific day 10.5 MAF (x) and day 12.5 AFP (+); or **B)** native AFP (■) and day 14.5 AFP (○); day 16.5 AFP (□); and day 18.5 AFP (△) at the concentrations indicated. After 48 hours of culture, proliferative responses were measured by [^{3}H] thymidine incorporation as described in the Materials and Methods.


A
[3H] THYMIDINE INCORPORATION (CPM)
100000
80000
60000
40000
20000
0
.001
.01
.1
1
10
100
1000
PROTEIN CONCENTRATION (µg/ml)


B
THYMIDINE INCORPORATION
(CPM)
100000
80000
60000
40000
20000
0
0
25
50
75
100
PROTEIN CONCENTRATION
(µg/ml)

below 10 ug/ml. For comparative purposes, these results are shown on a logarithmic scale in Figure 15A. In contrast, AFP purified from day 14.5 MAF exhibited suppressive activities comparable to control unfractionated AFP (days 15-19 pool) (Figure 15B). The AFP preparations from day 16.5 were less effective at suppressing the mitogen response in comparison to control AFP while day 18.5 AFP exhibited inhibitory properties only at the higher concentrations of 50 and 100 µg/ml. Therefore, the distinction in functional activity between these preparations appears to be directly related to the normal physiological declines in the relative concentrations of the immunoregulative isoform, AFP-1 in the AFP preparations purified from gestational stage specific MAF.

D. DISCUSSION

The major purpose of this study was to characterize the immunoregulatory properties of murine AFP molecules purified from specific stages of fetal development.

Alpha-fetoprotein is the major fetally-derived protein component in mouse amniotic fluid (MAF), comprising approximately 50% of the total protein content in MAF (Gustine and Zimmerman, 1973, Murgita and Tomasi, 1975a). We determined that the concentration of AFP in MAF increases progressively through the course of gestation, reaching levels of 2.2 mg/ml by day 18.5 of murine ontogeny. Our present findings are in general agreement with earlier studies showing a linear increase in AFP levels in fetal tissue extracts (Kahan and Levine, 1971) and fetal serum samples (Hau et al., 1981). Furthermore, Mizejewski et al. have estimated that the concentration of AFP in MAF isolates pooled from days 16-19 of gestation to be 1 mg/ml.

Numerous investations have revealed that mammalian AFPs from several species are present in the circulation as a naturally occurring heterogeneous population of molecules (reviewed in Smith and Kelleher, 1980). Furthermore, many groups have characterized predictable changes in ratios of AFP subspecies during normal embryonic development (Gustine and Zimmerman, 1973, Higgins, 1979, Kerckaert et al., 1979, Hau et al., 1981, Wong et al., 1988). Using isoelectric focusing techniques, we observed the presence of 4 isoelectric variants (pI 4.9-5.1) at day 12.5 of ontogeny. This was followed by a gradual increase in more acidic AFP isoelectric variants (pI 4.7-4.9) throughout the time course studied. These result are consistent with the original observations of Gustine and Zimmerman describing the developmentally regulated expression of murine AFP subspecies leading to the maximal expression of acidic variants (pI 4.8-4.9) by day 18.5 (Gustine and Zimmerman, 1973)

The results of the present investigation provide strong evidence supporting our previous conclusion that AFP-mediated immunoregulatory activity is a property uniquely associated with a single distinct molecular subspecies of murine AFP, termed AFP-1 (van Oers et al , 1989, 1990) Thus, in Figure 15, we have shown a clear difference in the immunosuppressive properties of AFP, purified from distinct

gestational time points, on mitogen stimulated $CD4^+CD8^-$ thymocytes. The most potent inhibitory preparations of murine AFP appear at days 10.5-14.5 of fetal development. Thereafter, a gradual diminution in immunoregulatory activity occurs, with AFP preparations from day 18.5 fetuses exhibiting marginal suppressive activities. A comparative analysis of these preparations on IEF gels indicated that the highest quantities of AFP-1 relative to the non-suppressive isoforms appear at days 10.5, 12.5, and 14.4 of gestation.

These findings also confirm the reports of Lester et al. who initially suggested that a relationship existed between immunosuppression and the presence of specific molecular subspecies of human AFP (Lester et al., 1978b). These authors found that fetal liver preparations containing high proportions of an electronegative AFP isomer were more functionally active than tumor-derived samples (Lester et al., 1976). It should be noted that the immunoregulatory AFP isoform that we have identified is present in mouse amniotic fluid. The heterogeneity of murine AFP, comprising all 6 isoelectric variants, as detected in MAF, is proposed to originate from the fetal yolk sac as early as day 10 of murine ontogeny (Wilson and Zimmerman, 1976). In contrast, the fetal liver is reported to generate a more restricted pattern of AFP subspecies which are released directly into the fetal serum. Thus, as reported by Zimmerman et al., the liver contributes a very minor quantity of the the more basic isoelectric variants (pH 4.9-5.2), including AFP molecules with a pI= 5.1 (Zimmerman et al., 1976). However, the functional properties of fetal serum AFP isoforms arising from the liver remains to be determined.

The results that we have obtained may account for some of the putative failures to identify immunosuppressive activity in AFP isolates (Sheppard et al., 1977, Sell et al., 1977). It is possible that these investigations may have used AFP preparations lacking the functional isoform. Therefore, isolates of AFP from MAF isolated later than day 16 of gestation would exhibit negligible suppressive effects on immune responses.

The developmentally regulated expression of a unique immunoregulatory AFP isoform in the fetus may comprise one of the several dominant immunosuppressor mechanisms necessary in regulating both fetal and maternal immune responses (van Oers et al., 1989, reviewed in Murgita and Wigzell, 1979, 1981). In addition, the

presence of six non-suppressive isoforms (van Oers et al., 1989) throughout the course of fetal development may reflect the important binding and transport roles for AFP molecules (reviewed in Ruoslahti and Seppala, 1979). Thus, the AFP isoforms may be involved in the uptake and tissue distribution of numerous ligands including fatty acids (Uriel et al., 1987), and metals (Wu et al., 1987).

In conclusion, our results would suggest an essential immunoregulatory role for AFP-1 at critical stages of fetal development. These findings should therefore contribute to the resolution of outstanding questions regarding the structure/function relationships of this onco-fetal molecule. Experiments including biophysical and recombinant DNA techniques are currently in progress to delineate the biochemical characteristics of this unique AFP isoform.

VI. GENERAL CONCLUSIONS

Alpha-fetoprotein is a well-characterized onco-fetal molecule that has attracted considerable attention for its potential as a diagnostic marker in a multiplicity of diseases (Abelev, 1971, Smith and Kelleher, 1989), and because of the accumulating evidence supporting the concept that AFP has an essential immunoregulatory role during normal embryogenesis (reviewed in Murgita and Wigzell, 1979, 1981) The AFP molecules characterized in several mammalian species have been shown to exist in the circulation as a heterogeneous population of isoforms (Alpert et al , 1972, Gustine and Zimmerman, 1972b, Bayard and Kerckaert, 1977, Clarke, 1980) The precisely coordinated appearance of closely related molecular subspecies of AFP during fetal development and in several diseases raises the intriguing possibility that particular patterns of isoform expression may be of functional relevance (Zimmerman et al , 1973, 1977, Lester et al , 1976, Wong et al , 1988) Indeed, Lester et al. reported a positive correlation between the relative content of a specific AFP subspecies in fetal or hepatoma-derived human AFP samples, and the capacity of these preparations to suppress <u>in vitro</u> immune responses (Lester et al , 1976, 1977a, 1977b)

To definitively address the relationship of AFP microheterogeneity to biological function, we devised a rapid protocol for purifying molecular variants of mouse AFP with Mono Q anion-exchange columns linked to an FPLC system (van Oers et al., 1990). We reported the first successful method for identifying and isolating seven AFP subspecies in quantitative amounts sufficient for in-depth functional studies. The seven isoforms described herein, termed AFP-1 to AFP-7, were defined by their reproducible FPLC retention profiles, distinct isoelectric points, immune reactivity with anti-AFP antibodies, and common MWs of 70 Kd corresponding to native AFP. We determined that AFP-2 to AFP-7 had a progressive increase in sialic acid content from 0-4 residues/mol This is consistent with an earlier proposal by Zimmerman et al that the relative extent of sialylation of AFP molecules would contribute to the formation of 5 murine AFP charge variants (Zimmerman et al , 1973, 1976) It is notable in this regard that AFP-1 and AFP-3 exhibited a similar NANA content of 1.0 mol/mol of protein, while isoforms AFP-4 and AFP-5 had a composition of approximately 2 moles/mol of protein Therefore, although a

proportion of murine AFP microheterogeneity may be accounted for by NANA variability, additional undefined biochemical properties are likely to have contributed to the formation of the 7 isomers.

The immunoregulatory activity of the seven AFP isoforms were examined in several in vitro functional assays including T-D antibody responses, $CD4^+CD8^-$ thymocyte mitogen reactivity, lymphokine boosted NK cytolytic activity and bone marrow cell proliferation (Murgita et al., 1981, Hooper et al., 1983, Hoskin et al., 1985c, Cohen et al., 1986). All four assay systems are particularly sensitive to the immunoregulatory effects of the native population of unfractionated AFP molecules. We determined that all the immunosuppressive activity of native AFP was localized in a single isomeric form of the molecule, AFP-1, representing 6% of the total population of native AFP molecules. Furthermore, all seven subspecies stimulated bone marrow cell growth with AFP-1 exhibiting the greatest growth promoting effect (van Oers et al., 1989) These findings are the first direct demonstration that a unique subspecies of mouse AFP is capable of mediating all the immunoregulatory properties normally ascribed to native AFP Since Zimmerman et al. had previously suggested that only the more sialylated forms of murine AFP were suppressive while Lester et al. demonstrated no relationship between sialic acid content and function, we examined the potential contribution of the NANA residues (Lester er al , 1976, Zimmerman et al., 1976). We determined that AFP-1 contains 1 mol NANA/mol AFP, considerably less than the 3-4 moles associated with the suppressive population of AFP molecules described by Zimmerman et al. (Zimmerman et al , 1977) Furthermore, neuraminidase digestion of native AFP did not affect the inhibitory functions, making it unlikely that sialic acid residues are necessary for the in vitro expression of AFP-mediated suppressive function. This is in agreement with the earlier findings of Yachnin et al. who noted no loss in the immunosuppressive potency of desialylated human AFP preparations (Yachnin et al., 1976).

An important physiological role for AFP during gestation is proposed to be the transport and cell delivery of FFAs, in particular AA and DHA, to developing tissues (Laborda et al., 1989). Furthermore, and of particular relevance to the present research, are reports suggesting that the physiological interaction of AFP with AA and DHA is necessary for the expression of AFP-mediated immunoregulation

(Parmelee et al., 1978, Nunez et al., 1987, Deutsch, 1983). It is conceivable that AFP-1 has a higher molar content of AA and DHA in comparison to the other isoforms which would contribute to its immunoregulative function. To address this potentially important association, we initially elucidated the fatty acid composition of native AFP and the seven AFP isoforms by extracting the FFAs and analyzing them by GC. All the AFP subspecies had a similar FFA composition including 16:0, 18:0, 18:1, and 18:2 comprising a total of 1.0 mol FFA/mol protein, indicating that no FFA binding variations existed among the isoforms. However, all seven variants had quantities of AA and DHA below the GC detection limits, at least 8-fold lower than that detected in native AFP. This observation is consistent with the reports of Calvo et al. noting a selective loss of PUFAs on rat and pig AFP following affinity chromatography (Calvo et al., 1985). Despite the absence of detectable quantities of AA and DHA, AFP-1 was extremely effective at inhibiting mitogen stimulated $CD4^+CD8^-$ thymocyte responses. In additional experiments, we determined that native AFP preparations depleted of AA and DHA by octadecylsilyl silica cartridges exhibited comparable functional activities as untreated AFP. Furthermore, preparations of non-suppressive AFP isoforms specifically re-complexed with a 2-3 molar excess of AA and DHA were without effect in the functional assays. Taken together, these results suggest that the complexed PUFAs AA and DHA are not required for AFP-mediated immunoregulation. This is further supported by the observation that mouse albumin, complexing 0.05 moles and 0.03 moles of AA and DHA per mol of protein, respectively, has no known immunosuppressive functions.

The cumulative series of experiments we have conducted (van Oers et al., 1989, 1990), coupled with the findings of several other investigators (Zimmerman et al., 1977, Lester et al., 1976, 1978a, Yachnin, 1983, Lu et al., 1984) would suggest that the immunoregulatory activities of AFP are intrinsic to the protein molecule. We have noted from preliminary ongoing studies that the suppressive properties of AFP are protease sensitive (unpublished observations). In addition, human, rat, and mouse AFP are all able to suppress murine AMLRs (Hooper et al., 1982), while pig AFP has been shown to inhibit PHA-stimulated human, pig, mouse, and frog lymphocytes (Kovaru et al., 1978). These results would suggest that a strong phylogenetic conservation of biological activity exists among the AFP molecules from several species.

The central finding of the present investigation is that all the immunoregulatory activity is localized to a single molecular variant, AFP-1. The biochemical features contributing to the formation of a unique immunosuppressive variant in the fetal circulation remain to be elucidated. We have investigated the possibility that an aa sequence variability and/or protein modification(s) may account for the formation of AFP-1 by comparing N-terminal aa sequences of AFP-1 and several non-suppressive variants. Preliminary results have revealed a variability at position 15 of AFP-1 which does not correspond to the published serine residue also identified in the 2 non-suppressive subspecies. In one preparation, an asparagine was identified while the sequence of a second AFP-1 sample suggested the presence of a lysine. The significance of this finding is currently being assessed, but may indicate the presence of a post-translational modification at the serine residue Comparative 2-d protease mapping and microsequencing may facilitate the identification of this and other amino acid sequence differences.

The heterogeneous population of murine AFP molecules in the amniotic fluid appear to be synthesized by the fetal yolk sac (Wilson and Zimmerman, 1976). In contrast, the fetal liver is thought to primarily secrete maximally sialylated variants into the plasma Since the fetal serum also contains a proportion of undersialylated AFP subspecies, Wilson and Zimmerman suggested that both the yolk sac and the liver contributed to the group of AFP subspecies in the fetal circulation. We would propose that the population of AFP subspecies in the fetal serum would comprise the immunoregulatory isoform that we have identified in the amniotic fluid We speculate that the regulated expression of AFP molecules during development may be important in modulating fetal/maternal immune responses However, the functional properties of AFP isoforms in the fetal and newborn serum warrant further investigation

The emerging pattern of immune responsiveness in the fetus is preceded by quantitative and qualitative maturational changes in different lymphoid cell populations For example, the murine T cell receptor and immunoglobulin gene rearrangements are initiated near days 13-14 of gestation, followed by the subsequent surface expression of their rearranged gene products which are detectable from day 16 onwards (Levitt and Cooper, 1980, von Boehmer, 1988, Strominger, 1989, Ferrick et al , 1989) However, significant manifestations of cell-mediated and

humoral immune responses appear to lag considerably behind the functional maturation of the cellular components (reviewed in Murgita and Wigzell, 1981) The demonstration in this thesis that AFP-1 can suppress several in vitro immune reactions continues to support the original concept of Murgita and Tomasi that immunoregulation is one of the physiological functions of AFP

The appearance of immunoregulatory AFP molecules early in ontogeny may be important for regulating the antigen presenting functions of several cell types As originally proposed by Lu et al., certain AFP molecules may prevent the induction of cell-mediated and humoral immune responses by down-regulating the expression of Ia antigens on APCs (Lu et al., 1989). This would prevent the potential activation of T cells until the acquisition of self-tolerance is complete in the fetus and newborn (von Boehmer et al , 1989, Lo et al., 1986) Lu et al have reported a delayed ontogenesis of Ia-positive macrophages, splenic dendritic cells, and B lymphocytes in the fetus and neonatal rodent until about 3 weeks of age in the newborn (Lu et al , 1979, 1980) The absence of Ia bearing APCs was not found to be the result of any intrinsic functional immaturity since the cells displayed phagocytic activity, secreted IL-1, and expressed surface Ia when cultured in vitro with gamma-interferon (Lu, 1984, Hardy et al., 1973) The regulation of APC function in vivo was thus attributed to several immunoregulatory factors including prostaglandins and AFP (Lu, 1984). This hypothesis was supported by the findings that AFP could down-regulate constitutive and lymphokine-induced MHC CII antigen expression on macrophages and dendritic cells (Crainie et al , 1989, Lu et al., 1984). Furthermore, the onset of APC function in the newborn correlated with the physiological decline in serum AFP concentration (Olsson et al , 1977, Mizejewski et al , 1983)

It is also interesting to note that fetal and newborn mice have a deficiency in NK cell activity In fact, functional NK cells can only be detected 3 weeks after birth in the newborn rodent (Roder et al., 1981) These reports are consistent with the findings of Cohen et al. that AFP can down-regulate lymphokine activated NK cell activity (Cohen et al., 1986), and the observation that NK function rises after birth (Roder et al., 1981) in proportion to the normal decline in AFP expression (Olsson et al., 1977). The regulation of fetal NK cell function by an immunoregulative AFP molecule may be one of several suppressive mechanisms

necessary to prevent autodestruction of fetal tissues which are potential NK cell targets (Hanson et al , 1981) The early appearance of a suppressive AFP molecule may also play a role in controlling the activation of decidual NK-like cells. Croy et al. have noted a transient increase in murine decidual NK cell activity during murine embryogenesis. However, this activity declines to negligible levels after day 10 of gestation (Croy et al., 1985, Gambel et al., 1985). Gendron and Baines have presented preliminary evidence suggesting that the modulation of this activity may be attributable to a combination of uncharacterized high MW compounds and low MW suppressive compounds such as prostaglandins (Gendron and Baines, 1989, 1990). Clark and co-workers have reported that non-T suppressor cells may be involved in the control of these decidual NK cells (Clark et al., 1986). It is conceivable that a combination of suppressor cells, arachidonic acid metabolites, and the immunoregulative AFP isoform expressed as early as day 10 in gestation are required for the control of decidual NK cell activation.

We have determined that all seven AFP isoforms exhibit immunoenhancing activities, with AFP-1 demonstrating the greatest stimulatory effects on murine bone marrow cells. The growth promoting activities of AFP are hypothesized to reflect an initial phase of suppressor cell induction in vivo (Hamel et al., 1987). For example, experiments from this laboratory have shown that native unfractionated murine AFP can stimulate the proliferation of two distinct bone marrow cell populations (Hoskin et al., 1985c). One population consists of non-T natural suppressor (NS) cells which are normally present in newborn spleen, adult bone marrow, and the spleens of isopregnant and allopregnant adult mice (Hoskin et al., 1990). The second cell subset comprises the T-like cells, which are phenotypically identical to the natural newborn T suppressor cells and the inducible population of adult spleen T inhibitory cells (Hoskin et al., 1985c, Hamel et al , 1987, Murgita et al., 1977, 1981) Both populations of AFP-inducible cells are capable of suppressing newborn autologous MLRs in vitro, reactions which are thought to reflect potential autoreactive events in fetal and newborn animals (Hamel et al , 1987, Hooper and Murgita, 1981). These results led to the speculation that the AFP-induced proliferation of bone marrow-derived T- and non-T- cells in vitro may represent the induction of NS cells in vivo, which would be necessary for regulating potential autoreactive responses in the fetus and neonate (Hoskin et al , 1985c).

In addition, Hoskin and Murgita have suggested that the presence of relatively high maternal AFP levels during pregnancy may in fact act as efficient inducers of the NS cells in isopregnant and allopregnant mice (Hoskin et al, 1990). Recent studies have also shown that the maternal immune system is capable of responding to a variety of syngeneic antigens on fetal and placental tissues (Hamilton, 1983). During certain stages of pregnancy, the maternal immune system appears hyper-responsive as demonstrated by the increased mitogen responsiveness of thymocytes and bone marrow cells, and the increased proliferation of T cells recognizing self antigens as measured in AMLRs (Hoskin and Murgita, 1985a, 1985b). These specific maternal anti-fetal responses may be potentially harmful to the development and survival of the fetus unless properly controlled. The naturally occurring NS cells in the spleens of isopregnant and allopregnant mice would function by inhibiting the maternal reactions against autologous antigens (Hoskin et al., 1989, 1983, Gronvik et al., 1987).

In conclusion, we have described the existence of a single murine AFP isoform in the developing fetus which exhibits well-defined immunoregulatory properties previously associated with the population of unfractionated AFP molecules. These findings have addressed some of the central questions concerning the structure/function properties of AFP molecules, and have important implications in our understanding of the immunological basis of the maternal/fetal relationship

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