CHAPTER I

INTRODUCTION

DESCRIPTION

Alpha-fetoprotein (AFP), one of the embryo-specific proteins, is useful in cancer as a model for diagnosis, chemotherapy and research, and in pregnancy as a mean of monitoring fetal development and fetal pathophysiology.

AFP is an albumin-like plasma protein with the molecular weight of approximately 70,000 for the single polypeptide chain (1). It is a major serum protein of the early fetus, and could be identified to its characteristic position on paper chromatography between the zones of albumin and α_1-globulin, and present in low concentrations less than 20 ng per milliliter in the sera of normal adults. AFP is frequently found in abnormal amounts in the sera of patients with hepatoma, teratocarcinoma and occasionally in other varied malignancies (2,3). In fetus, very active AFP synthesis occurs during the 14th week of gestation, serum AFP reaches its maximum concentration of about 3,000 µg per milliliter, and then its level declines slowly until birth, AFP synthesis drops markedly shortly after birth, and reappears in adults only under unusual circumstances. This protein has been detected in the sera of fetal mouse and calf.

The evidence available at present indicates that, during

pregnancy, fetal AFP is transmitted into the maternal circulation system and amniotic fluid (4,5).

The measurement of amniotic fluid, and pregnant sera concentrations of AFP has been used as a potentially important screening test of fetal well-being (6).

Discovery of alpha-fetoprotein

In 1944, Pedersen described a calf embryonic serum globulin which he termed "fetuin". In 1956, Bergstrand and Czar reported a new fraction in the electrophoresis of plasma proteins migrating between the albumin and alpha-1-globulin fraction in a fetus a few weeks old. They called it a "new protein fraction" or "X component". Its proximity to the band of albumin is probably the reason why the existence of this substance was hidden for so long. In 1963, Abelev first reported the transplantable mouse hepatomas synthesized and secreted into the blood an embryo-specific alpha-fetoprotein. Similar observations were made on rats and monkeys (7), dogs and rabbits (8), with chemically induced primary hepatomas.

In 1964, Tatarinov demonstrated the presence of AFP in the sera from patients with primary liver cancer (PLC), and suggested that this protein would be useful in diagnostic purposes for the early detection of PLC. There after, many other workers confirmed the observations of Tatarinov on the diagnosis of PLC (9-14).

In 1965, Masopust and Kotal called it "fetoprotein", Gitlin labeled it "alpha-fetoprotein", which was adopted as the official name of this substance by the International Agency for Research on Cancer (1).

Synonyms which have been applied include:-

alpha-1-embryo-specific protein

protein F

alpha-1-fetoprotein

ESA globulin

alpha-1-globulin

post albumin

This protein had been shown in vitro culture to be produced by fetal liver cells in 1967 (15), since 1969, it had been abbreviated as "AFP" (16).

In 1970, Nishi studied the physicochemical and chemical properties of the pooled fetal and the hepatoma patients sera by immunochemical method, and essentially identical were obtained (17).

In 1973, Hirai et al purified AFP from various sources, such as sera of human and rat fetuses, and sera of hepatoma patients and hepatoma-bearing rats. Some physicochemical, chemical and immunochemical analyses of the purified materials were made. No significant differences were observed, and it was found that during hepatocarcinogenesis in rats fed 4-dimethylaminoazobenzene (DMB), AFP appeared transiently in the blood in an early stage (18).

Chemistry of alpha-fetoprotein

Chemical and physicochemical properties

The physicochemical properties of AFP had been investigated by Gitlin and Boesman (1), Nishi (17), Hirai et al (18), and Alpert et al (19).

In Table 1, page 4, indicates some physicochemical properties of human and rat AFP. The data were very similar to those of human albumin (20), the AFP from hepatoma, and from normal fetal liver, appeared to have identical physicochemical properties.

Hirai et al (18) reported the similarity of chemical compositions of AFP from human and rat, as shown in Table 2, page 5. No marked difference was observed between AFP from sera of human fetuses and hepatoma patients.

Table 3, page 6, shows the amino acid composition from the experiment of Hirai et al (18), and Nishi (17). No difference was observed between human fetal and hepatoma AFP. Though in general the data for rat AFP were also similar in those for human AFP.

TABLE 1

Some physicochemical properties of AFP

	Human AFP		Rat AFP	Human
	Fetal liver	Hepatoma	Hepatoma	Albumin
Sedimentation constant	4.50	4.50	4.76	4.6
Diffusion constant	6.18	-	5.68	6.1
Molecular weight	64,600	70,000	75,100	69,000
Isoelectric point	4.75	4.75	4.75-5.0	4.9

TABLE 2

Chemical composition of AFP (%)

	Human		Rat	Human
	Hepatoma	Fetus	Hepatoma	Albumin
Nitrogen	14.9	14.7	13.1	16.0
Sulfur	1.8	1.7	2.0	1.5
Carbohydrate	3.4		5.3	0.08
Mannose	0.68			
Galactose	0.49			
Glucosamine	0.89			
Fucose	±			
Sialic acid	1.3			

TABLE 3

Amino acid composition of AFP

(moles/mole protein)

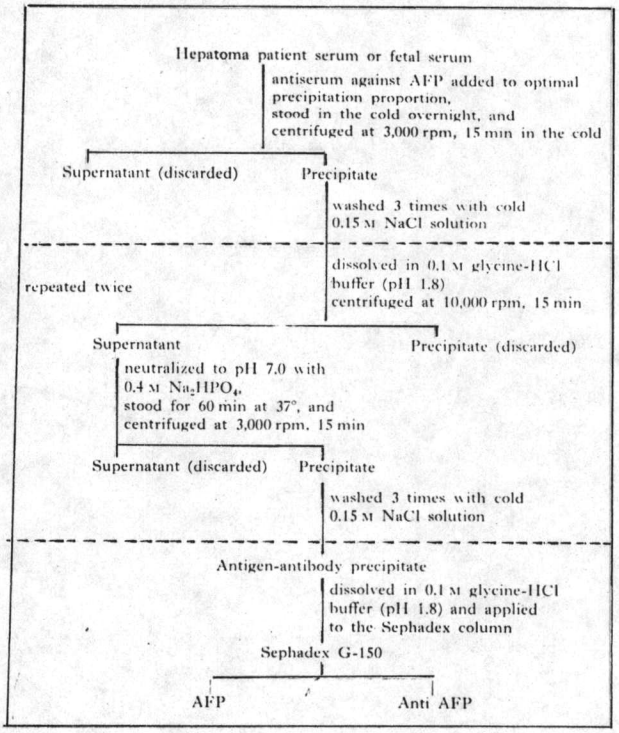
	Human AFP		Rat AFP	Human
	Fetus	Hepatoma	Hepatoma	Albumin
Aspartic acid + Asparagine	49	49	60	52
Threonine	36	35	33	28
Serine	37	36	38	24
Glutamic acid+Glutamine	110	104	103	81
Proline	21	22	32	25
Glycine	26	27	31	12
Alanine	50	49	58	62
Half-cystine	22	26	34	17
Valine	27	29	27	41
Methionine	4	6	14	5
Isoleucine	25	26	31	8
Leucine	53	54	66	60
Tyrosine	16	16	16	16
Phenylalanine	27	29	25	30
Lysine	36	35	54	56
Histidine	12	12	21	15
Arginine	17	17	23	23
Tryptophan	2	2	1	1

Some values, e.g. methionine, histidine, lysine, of human albumin appeared to be different from those of human AFP. Anyhow this data showed a good agreement with Rouslahti (21).

Purification of AFP

AFP had been detected in fetal and maternal sera (22), fetal urine (1), amniotic and cerebrospinal fluid (23). Of these, human fetal blood was considered to be the best source of AFP for purification, cord blood and ascitic fluid were regarded as secondary sources.

The flow chart for the purification procedure as described by Hirai et al (18) was as follow.



Evolutionary trend In the evolutionary scheme, Gitlin (24) calculated that AFP might have been presented in primitive forms like the shark over 400 million years ago. Synthesized from the entoderm of the embryonic foregut, AFP "early in evolution" arose mainly from the gastrointestinal tract, with time, synthesis shifted away from the stomach to outgrowths of the entoderm, liver, and yolk sac.

In the shark, AFP was produced mainly in the stomach, with lesser amounts in the intestinal mucosa and liver and with little production in the yolk sac.

In birds, most AFP synthesis occurred in the yolk sac, with no synthesis in the liver.

In mammals, AFP synthesis was greatest in the liver, followed by the yolk sac, with the least amount produced in the gastrointestinal tract.

In human beings, at 4 to 8 weeks of gestation, the yolk sac rivaled the fetal liver in AFP production. As the yolk sac degenerated at 11.5 weeks, the liver overtook the yolk sac in the production of AFP. The gastrointestinal tract also produced AFP, but in small amounts.

Synthesis by the liver was responsible for the rise in the fetal serum concentration from 70 μg per milliliter at 6.5 weeks to 2,000 μg per milliliter at 9.5 weeks. The fetal serum concentration reached a peak at 14 weeks with 2,000-3,000 μg per milliliter of AFP.

After the fourteen weeks of gestation, the fetal serum concentration falled to levels of 13 to 86 μg per milliliter at birth.

Detection methods

1. Immunodiffusion: Many investigators reported its sensitivity for the detection of AFP of 3-5 μg per milliliter (12,22,25,26,27). By this method it was failed to pick up lower levels of AFP in patients and then led to the erroneous conclusion that AFP did not appear in the serum of normal or pregnant adults.

2. Counterimmunoelectrophoresis (CIEP): This method had also been known as electroimmunodiffusion and immunoosmophoresis. It was approximately 40 times more sensitive than immunodiffusion, and had been described by Alpert et al (28). No false positive were found by the use of this method. It is currently being used as a screening test in a prospective study on every patient in the prenatal clinic in some hospitals, and had been reported its sensitivity to detecting 100 ng per milliliter AFP level.

3. Radioimmunoassay (RIA): The RIA for AFP had the advantage, being more sensitive and more quantitative method for AFP determination. RIA had been reported by several investigators (29,30,31,32,33). The lower limit of sensitivity of the assay was 20 ng per milliliter. It was 20,000 times more sensitive than immunodiffusion.

4. Immunoelectronmicroscopy: AFP was thought to be synthesized in the hepatocytes of the embryo and the fetus. Localization of AFP in the liver carcinoma was investigated by the peroxidase antibody technique

in the rat induced by 3-methyl-4-dimethylaminoazobenzene (3' Me-DAB). Seen through an electron microscope, a peroxidase positive reaction, which was considered as specific for AFP, was found mainly on the rough endoplasmic reticulum (RER). It was especially marked on the one encompassing the mitochondria. On the RER, the reaction was observed as an uneven thick line along the membrane.

A weak positive reaction was also observed in some parts of the membrane of the smooth endoplasmic reticulum (SER) and cell membrane. No positive reaction was detected in the golgi complex (34).

5. Immunofluorescent antibody technique: By means of the immunofluorescent technique with anti-alpha-fetoprotein antiserum, AFP was detected in the cytoplasm of the hepatocytes, even the patient with a slight amount or undetectable of serum AFP by assay method. But only a small proportion of cancer cells do produced AFP. In the same patient some cells produced AFP and some others did not (35,36).

Factors influencing AFP production

AFP was not found in all liver tumours or liver diseases. The rate of positive of AFP varied according to age, sex, and geographical location. By using a sensitive method, abnormal AFP levels were found in only 78 % of histological confirmed cases of hepatocellular carcinoma, 13 % of acute viral hepatitis cases, 44 % of massive necrosis cases, and 23 % of chronic active hepatitis cases. Negative or low AFP results were obtained for other gastrointestinal tumours, acute or chronic non viral diseases. It was suggested that in non-malignant diseases the

AFP elevation might be due not to hepatic regeneration but to viral injury (16).

The sensitivity of assays employed may influence the yield of case with positive AFP results as shown by O'Connor et al (37), that in the same series of cases, 63 % were positive for AFP by immunodiffusion but 85 % were positive by radioimmunoassay. The range of AFP concentrations was from 0.02 to 1000 mg per 100 milliliter (28).

1. Age: The influence of age is clearly shown in Table 4, page 13. Correlation of the presence of AFP with the age of the patients indicated that positive tests occurred mainly in the younger patients, AFP prevalence was slightly less in adults (26, 38, 39).

2. Sex: Sex was a factor in that more males were positive for AFP than females (28,37). At birth and during the first week of life, serum AFP levels were some 2.0 times higher in boys than in girls (40).

From Table 5, page 13, showing that the positive rate was about 20 % higher in males than in females. The reason for this sex disparity was unknown. There was no evidence of a direct role of male hormone. The recent discovery, that AFP possessed strong binding activity for oestrone and oestradiol, but not for other steroid hormone, since it was possibly related to this fact (39).

3. Pathological features: Patients with large tumours were more often positive than those with smaller ones. In Table 6, page 14, all tumours heavier than 5 kg induced serum positivity. There was no correlation between tumour weight and AFP level (39). Only a small proportion of tumour cells made AFP, less than 5 to 20 % of cells had been found by immunofluorescence to be positive for AFP (22,35).

An assesment of the degree of tumour differentiation was summarized in Table 7, page 14. There was a tendency for the presence of AFP to correlate with degree of undifferentiation. AFP production was lowest in the well differentiated (16 mg per 100 millliliter), highest in the poorly differentiated (32 mg per 100 millliliter) and intermediate (25 mg per 100 millliliter) in the moderately differentiated tumours (29).

4. Geographic location: AFP prevalence in PLC varied significantly in different countries. PLC was relatively infrequent in the United States of America, England, France, Greece and the U.S.S.R. It occurred in high frequency in the Asian countries and Senegal, the Union of South Africa, Mozambigue, Kenya, Uganda and the Congo.

As indicate in Table 8, page 15,the incidence of AFP positivity rate in hepatoma bore out almost the same figure both in Asia and in Africa despite the racial and geographical differences (41).

The differences in AFP test results could be related to differences in aetiology of hepatoma in endemic and non-endemic areas. Since the incidence of hepatoma was similar in American Caucasians and Negroes, these suggested that genetic factors might also play a role in AFP prevalence (42,43).

TABLE 4

AFP tests in different age-group in patients with primary liver cancer (PLC)

Age (year)	No. of patient	No. of AFP positive	% AFP positive
20	5	5	100
21 - 29	29	25	86
30 - 39	56	42	75
40 - 49	40	23	58
50 - 59	28	18	64
60 and older	26	8	31
Total	184	121	65

TABLE 5

Incidence of AFP in the sera of patients with PLC in relation to sex

	Male	Female	Total
AFP - positive	182 (75.9 %)	38 (59.4 %)	220 (72.4 %)
AFP - negative	58 (24.1 %)	26 (40.6 %)	84 (27.6 %)
Total	240	64	304

TABLE 6

Incidence of AFP in the sera of patients with PLC in relation to the weight of the tumoral liver

	Tumoral liver weight					
	Total	Less than 2 (kg)	2-3 (kg)	3-4 (kg)	4-5 (kg)	More than 5 (kg)
AFP - positive	14	3	12	11	8	8
AFP - negative	13	3	6	3	1	0
Percent positive	76	50	67	79	89	100

TABLE 7

AFP frequency as a function of tumour differentiation

Tumour differentiation	Total	AFP positive	
		No.	%
Well	16	9	56
Moderate	51	23	65
Poor	29	21	72

TABLE 8

Reports on serum AFP in patients with hepatoma from different geographic areas

	With histologic confirmation			
	Total	Sero negative	Sero positive	
			No.	%
Africa				
Nairobi	14	4	10	71.4
Kampala	14	5	9	64.3
Kinshasa	22	5	17	77.3
Ibadan	14	4	10	71.4
Dakar	44	9	35	79.5
Bantu	103	1	102	99.0
Singapore	29	8	21	72.4
Hong Kong	60	21	39	65.0
Japan	15	4	11	73.3
Philippines	21	5	16	76.1
Taiwan	48	13	35	72.5
U.S.A.	16	8	8	50.0
Thailand	26	7	19	73.1

AFP and liver diseases

Primary liver cancer (PLC) is found in all parts of the world, but their incidence varies from one region to another, and the variation is marked. It occurs with high frequencies in some parts of Africa and in most countries in East and South-East Asia, and perhaps the highest documental incidence in the world is in Mozambigue (44).

Primary carcinoma of the liver may develop either into a hepatocellular or cholangiocellular type irrespective of the element from which its primary growth originated. This can be explained by the genetic and biological proximity of the cells of liver parenchyma and bile duct epithelium (45).

AFP considered to be specific for PLC. The immunological method, using AFP is now considered a highly specific test for the diagnosis of this disease. Although AFP was also positive in the sera of patients with liver cirrhosis and hepatitis, usually not exceeding 200 ng per milliliter (46).

Hepatocellular carcinoma especially is commonly associated with cirrhosis in about 80 % where as the complication rate with cholangiocarcinoma is as low as 10 to 20 %. The association of hepatocellular carcinoma and cirrhosis seems to vary in different geographical areas. For instance 40 or 50 % of liver cirrhosis cases give rise to hepatocellular carcinoma in Africa and South East Asia, in sharp contrast with the situation in Europe and the United States, the percentage rate is about 5 to 10 %. Furthermore, hepatitis B surface antigen (HBs Ag) was detected in a high percentage of cases as much as 40 %

among patients with hepatocellular carcinoma (47).

AFP appeared to be greater than 10 ng per milliliter in patients with disease of the liver, as shown in Table 9, page 17 (48).

The AFP level in each case is shown in Figure 1, page 18. For hepatocellular carcinoma, 98 out of 125 cases, most of positive cases had a level of AFP over 10,000 ng per milliliter. AFP apparently not only was present in hepatocellular carcinoma, but also in other diseases, however, the higher levels were found most frequently in hepatocellular carcinoma, and when the level was over 400 ng per milliliter, the diagnosis was almost a certainty.

TABLE 9

AFP in liver diseases

Diagnosis	No of cases	10ng/ml		320 ng/ml		400 ng/ml	
		No.	%	No.	%	No.	%
Hepatoma	125	113	90	93	74	86	69
Other malignancies	66	13	20	1	1.5	0	0
Cirrhosis	74	25	34	1	1.4	0	0
Chronic aggressive hepatitis	60	35	58	4	6.7	1	1.7
Chronic persistent hepatitis	12	5	42	1	8.3	0	0
Subacute hepatitis	16	11	69	0	0	0	0
Acute viral hepatitis	36	17	47	2	5.6	0	0
HBs Ag carrier	13	3	23	0	0	0	0

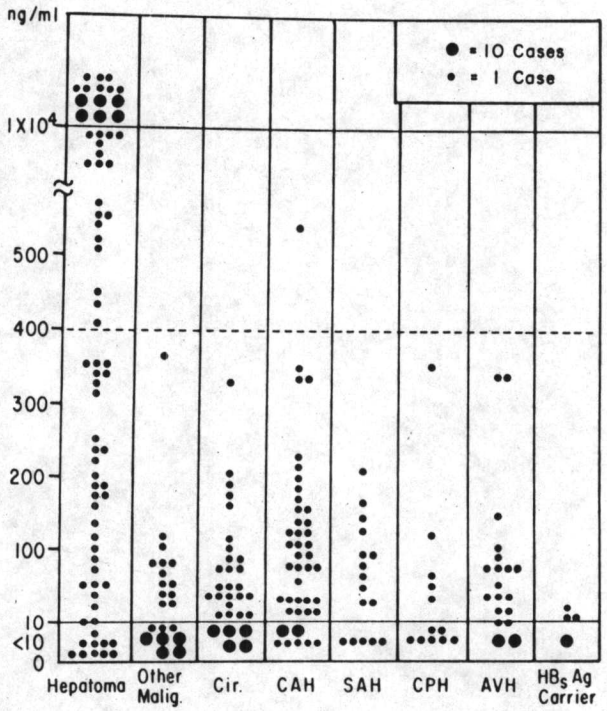


Fig. 1 Serum AFP level in diseases.

AFP in other diseases

AFP had been found in the sera of patients with diseases other than liver, such as embryonal carcinoma of the ovary and testis, gastric and lung cancer with liver metastatis (49).

Masopust et al (12), first reported finding this protein in the sera of some patients with malignant undifferentiated teratoma. Since then other studies have confirmed their findings (9,44), but AFP might not be detectable in the sera of the patients until metastasis was widespread (50).

AFP in pregnancy

AFP passed from the fetus to the mother either transplacentally or via the amniotic fluid, no difference was found between mothers of girls and those of boys both during pregnancy and at the time of delivery (40).

AFP was determined by radioimmunoassay in sera from women at different stages of pregnancy and after delivery. The AFP concentrations of the pregnancy sera are given in Table 10, page 22. The lowest AFP levels were found during the first trimester, range from 18 to 119 ng per milliliter. Increasing serum AFP concentrations were observed with advancing gestation. During the second trimester, the value ranged from 96 to 302 ng per milliliter, and during the third trimester, from 160 to 550 ng per milliliter were recorded (4).

AFP in the newborn

The newborn sera were taken at birth, and the corresponding maternal sera were obtained during labor and on the first day post partum, as shown in Table 11, page 23.

AFP concentrations of newborn in umbilical cord sera at birth varies from 36 to 175 μg per milliliter. During labor, serum AFP levels were generally lower than during the third trimester. The values during labor varied from 103 to 400 ng per milliliter. AFP values on the first day after delivery were higher than during labor.

As shown in Table 12, page 23, maternal serum AFP concentration fell rapidly during the postpartum period, in the sera of 3 women.

From the level obtained, Seppälä and Rouslahti (4) calculated that the average half-life of maternal AFP during the early postpartum period is about 5 days.

AFP uses in pregnancy

The abnormal levels of serum AFP during pregnancy suggested the presence of various complications such as hydatidiform mole, choriocarcinoma, or twin pregnancy. And that the determination of serum AFP was valuable for prenatal diagnosis, seemed likely a monumental discovery (51).

The use of AFP in pregnancy could be categorized according to indications in early pregnancy and later pregnancy, as shown in Table 13 page 24 (16).

The indications for use of AFP in pregnancy changed throughout all three trimesters.

In the first trimester, AFP values might be used to predict outcome in threatened abortion, after the thirteenth week of gestation, abortion occurred more frequently in women with abnormally elevated AFP results.

In the second trimester, the indications for AFP changed to screening for fetal distress, assessing the severity of diabetes, Rh immunization, toxemia and screening for the development of later birth defects.

In the third trimester, some of the indications for AFP values included the determination of gestational age, the detection of fetal distress, the prediction of fetal death, and the evaluation of innocuous versus serious bleeding.

TABLE 10

AFP concentrations (ng per milliliter) in pregnancy sera

Trimester 1		Trimester 2		Trimester 3	
No.	Serum AFP level	No.	Serum AFP level	No.	Serum AFP level
1	not measurable	23	96	33	160
2	18	24	126	34	163
3	18	25	151	35	196
4	24	26	160	36	222
5	30	27	178	37	270
6	30	28	178	38	275
7	32	29	231	39	275
8	33	30	258	40	285
9	34	31	285	41	285
10	35	32	302	42	298
11	35			43	320
12	39			44	330
13	43			45	340
14	43			46	418
15	44			47	427
16	48			48	490
17	53			49	530
18	54			50	550
19	66				
20	84				
21	116				
22	119				

TABLE 11

Serum AFP concentrations (nanograms per milliliter)

in child-mother pairs

Mother	Newborn AFP	Maternal AFP at labor	First day after delivery
1	36,000	103	118
2	42,000	160	140
3	46,000	153	65
4	55,000	110	160
5	63,000	130	115
6	70,000	155	93
7	130,000	180	140
8	143,000	250	275
9	160,000	400	380
10	175,000	310	305

TABLE 12

Half-life of AFP in maternal serum after delivery

(nanogram per milliliter)

Mother	Post-partum day		
	1	3	5
1	120	108	55
2	145	103	90
3	200	80	75

TABLE 13

Reported indications for AFP assays

Early pregnancy:

- Predict outcome of threatened abortion
- Signal a blighted ovum vs. growing fetus
- Select cases of incompetent cervix for surgery
- Differentiate between mole and normal pregnancy

Later pregnancy:

- Detect fetal distress
- Predict impending fetal death
- Monitor maternal complications, e.g., toxemia, diabetes, Rh immunization
- Screen for twins
- Detect neural tube defects
- Monitor for other birth defects
- Assess uterine bleeding
- Predict (unexpected) stillbirth
- Detect cancer in pregnancy
- Assess gestational age
- Other

Experimental studies on AFP

1. AFP during hepatocarcinogenesis induced in rats by 4-dimethylaminoazobenzene (DAB).

Hirai et al (18) fed rats with a carcinogenic azo dye, DAB, and followed the appearance of AFP, hepatoma usually developed after 4 months of dye ingestion, AFP began to appear in the blood in the 3rd week and increased to a peak at the 6th. The AFP then decreased and disappeared by the 10th to 11th week. After the 12th week AFP reappeared, accompanied by hepatoma development. The phenomenon of the early appearance of AFP designated "primary reaction". In the period of primary reaction no cancer cells could be observed.

The change of AFP level is shown in Figure 2.

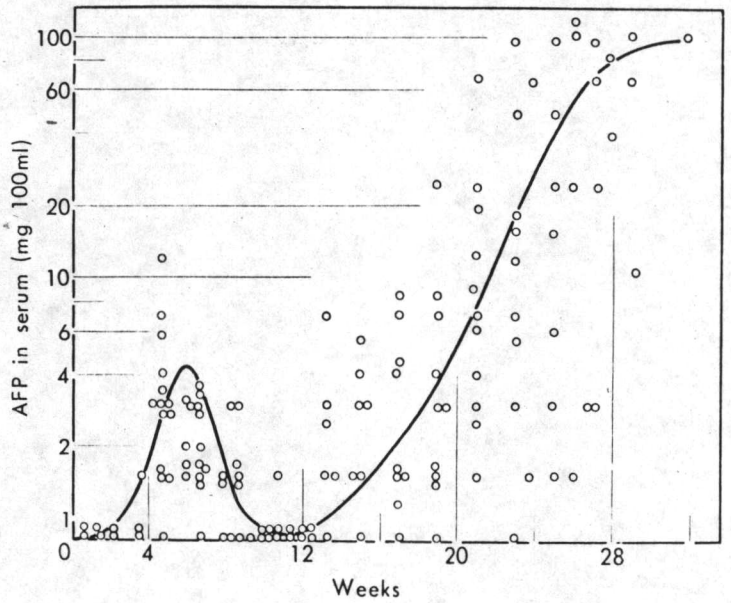


Fig. 2 AFP levels in rat serum during DAB carcinogenesis.

Rats were fed with synthetic standard diet containing 0.06 % DAB for 30 weeks. Blood was collected every week from a tail vein. AFP was determined by immunodiffusion method.

Another significant observation was made by Watanabe. When the DAB containing diet was replaced by a normal diet before 7th week, none of the rat developed hepatoma, but when DAB feeding was stopped at the 11th week, hepatoma developed in all rats. This suggested that DAB feeding must continue throughout the peroid of the primary reaction for the development of hepatoma.

From the experiment of DAB carcinogenesis in rats, administered DAB probably injured the liver cells in the first 3 weeks, and this was followed by regeneration of liver cells. The synthesis of AFP started in the 3rd week, as shown in Figure 2, presumably being associated with the onset of regeneration. Regeneration was most active in the 6th week but the toxicity of DAB continued to exert an effect on the regenerative cells, resulting in the suspension of AFP synthesis. The carcinogenic action of DAB would eventually cause the transformation of regenerative cells. At the 10-11th week, the synthesis of AFP stopped and the transformed cells began to acquire malignancy (18).

2. AFP synthesis in cultured cells

AFP was shown to be produced in tissue culture by human fetal liver and placental tissue (52), and in mice it had also been shown that AFP disappeared from the serum following removal of the primary hepatoma.

Irlin et al (53) observed that during the early stages of cultivation of mouse transplantable ascitic hepatoma, AFP was demonstrated in the tissue culture medium for up to 13 weeks.

Smith et al (36) demonstrated that a few fibroblast like cells in tissue culture of liver biopsies from patients with hepatocellular carcinoma and AFP in their sera also had the protein by indirect immunofluorescent antibody technique.

The present study is an attempt to demonstrate the localization of AFP in the liver biopsy specimens containing cancerous tissue by the direct immunofluorescent antibody technique, and to determine whether AFP is present in all tumour cells or only in certain cells, with particular attention to the forms and the areas of AFP positive localization. The relationship between the localization of AFP in tumour cells, the serum AFP and the histological classification of carcinoma of the liver will be described.