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ALPHA-FETOPROTEIN AND HEPATOMA

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Tomizo Yoshida, M.D.

PREFACE

Of the methods developed for the diagnosis of cancer using body fluids, the diagnosis of primary liver cancer based on the detection of α-fetoprotein (AFP) by precipitin reaction in gel has been considered the most reliable one. When this protein is detected in the blood, diagnosis of primary liver cancer can, with rare exceptions, be made with reasonable certainty.

In Japan it has been only three years since the study of this protein began, nevertheless the procedure mentioned has been widely employed, and more than several hundred cases of primary liver cancer have already been examined.

The fact that liver cells undergoing cancerous change can resynthesize protein of the embroyonic stage provides an important lead in understanding the true nature of cancer. Considerable advances have been made in experimental studies conducted along this line.

In light of this background it was opportune that the Japanese Cancer Association held a symposium on "Alpha-Fetoprotein and Hepatoma" focussing on the following points:

1. Evaluation of the procedure using AFP for the diagnosis of primary liver cancer;
2. Pathohistological differences between AFP-producing hepatoma and AFP-non-producing hepatoma;
3. Appearance of AFP in diseases other than primary liver cancer;
4. Determination and significance of low levels of AFP under limit of sensitivity of precipitin reaction in gel;
5. Analyses of the mechanism of AFP appearance in animal experiments.

The results of this symposium are given in this monograph. We look forward to a further delineation of the relation of hepatitis to liver cancer through microdetermination of AFP by radio-immunoassay and more sensitive detection of Australia antigen together with the support of pathological findings. Determination of the association among hepatitis, liver cirrhosis, and liver cancer must wait for future studies; in Japan, with a high incidence of these three diseases, it is a matter requiring early resolution.

At this symposium the diagnostic value of AFP based on the studies made to date was established and the direction in which future studies will be pursued was determined.

This volume is a compilation of the presentations made at the 11th Japanese Cancer Association Symposium on "Alpha-Fetoprotein and Hepatoma" held in Tokyo in December 1971 and arranged by us. We wish to express our appreciation to Drs. L. R. Purves, R. F. Masseyeff, J. Uriel, and D. Buffe for contributing, at our invitation, papers on their valuable studies.

June 1972

Hidematsu Hirai
Toru Miyaji

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FACTORS INFLUENCING α-FETOPROTEIN BIOSYNTHESIS IN PATIENTS WITH PRIMARY LIVER CANCER AND OTHER DISEASES

René F. MASSEYEFF

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Factors influencing α-fetoprotein (AFP) production in primary liver cancer patients are reviewed. Considering the frequency of positive blood samples amongst patients and the level of this protein whenever present, age, sex, and size of the tumor definitely influence positivity rates, while geographic or ethnic influences remain to be proven in the author's view. Investigation by fluorescent antibody technique has shown that only a small proportion of cancer cells do produce AFP. α-Fetoprotein levels tend to increase with time in those patients, sometimes with temporary peaks.

Preliminary findings on low rate production of AFP in normal and pathological people as estimated by radioimmunoassay are indicated. Finally hypotheses on the mechanisms of AFP production by cancerous liver cells are reviewed.

Since the demonstration by Abelev (*1*) that experimental liver tumors of mice produce an α-globulin identical with a protein present in fetal blood, and the demonstration by Tatarinov (*62*) that a similar situation occurs in human primary liver tumors, the detection of α-fetoprotein (AFP) has become a valuable aid in the diagnosis of human primary liver cancer (PLC).

A number of studies (*2*, *12*, *40*, *45*, *65*), have shown that a positive test with the conventional Ouchterlony method for the demonstration of AFP is found only in PLC and in a rarer cancer, teratoblastoma of the gonads (TRB) (*5*). Our personal experience in Africa coincides completely with this common conclusion. Only 3 false positive cases were found, out of more than 70,000 tests covering a wide range of normal and pathological individuals. Indeed, these 3 false positives were not documented sufficiently to completely rule out the occurrence of a small, hidden primary liver tumor.

Thus, to a first approximation, this Ouchterlony test for AFP appears to be highly satisfactory from a diagnostic point of view. Few biological tests, indeed, show such a high specificity. However, this specificity is not absolute and a number of facts show that the process of AFP biosynthesis is quite complex.

Some specific points deserve special comments and studies.

1) A percentage of patients with PLC (ranging from 20 to 60%) do not have

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a detectable level of AFP. Moreover, in the same patient some cells produce AFP and some others do not.
2) The level of AFP is not constant during the evolution of the disease. The dynamics of AFP production raise some interesting questions.
3) An increasing number of "false positive cases" have occurred, *i.e.*, cases where neither PLC nor TRB can be demonstrated in patients with a positive serum AFP test.

α-Fetoprotein Levels in Patients with PLC

We have investigated 304 patients suffering from PLC in Senegal, Africa (*37*, *40*). The diagnosis was made in 177 cases only by clinical examination.

TABLE I. Frequency of Occurrence of Serum AFP in Patients with PLC

	First author	No. of cases	% Pos.	Ref.
Africa				
Congo Kinshasa	O'Conor[a]	22	77	(*45*)
Ghana	Foli[a]	35	72	(*20*)
Kenya	O'Conor[a]	14	71	(*45*)
Mozambique	Portugal	37	68	(*48*)
Nigeria	O'Conor[a]	14	71	(*45*)
Senegal	Masseyeff	304	72	(*40*)
South Africa	Purves[a]	130	78	(*50*)
Uganda	Alpert	40	50	(*10*)
	Alpert[b]	28	86	(*8*)
America				
U.S.A.	Hull Caucasian	32	31	(*27–29*)
	Negro	7	71	(*27–29*)
	Alpert[b] Caucasian	41	51	(*8*)
	Non-Caucasian	21	76	(*8*)
Asia				
Hong Kong	Smith	60	65	(*57–60*)
Indonesia	Kresno[a]	100	87	(*31*, *32*)
Japan	Endo[a]	24	67	(*18*)
Malaya	O'Conor[a]	29	72	(*45*)
Taiwan	Alpert[b]	15	93	(*8*)
	Lin	26	65	(*34*)
Europe				
France	Economopoulos	30	60	(*17*)
	Hirsch-marie	84	59	(*26*)
	Sauger	27	59	(*55*, *56*)
	Uriel	22	55	(*65*)
Great Britain	Foli[a]	35	40	(*20*)
Greece	Economopoulos	35	51	(*17*)
Spain	Teres[a]	17	42	(*63*)
U.S.S.R.	Abelev[a]	28	60	(*4*)

[a] All cases confirmed histologically.
[b] Results obtained by immunoelectrodiffusion.

In 127 cases it was confirmed by microscopic examination of the liver. Using a conventional micro-Ouchterlony method, AFP was present in 72% of these patients, the percentage being nearly identical in both groups. Table I compares this figure with some data collected from the literature. The homogeneity of the results obtained in Africa and Asia is striking.

The prevalence rates calculated from the results obtained in Europe or America are smaller: This point will be discussed later. It was found that 28% of the sera remained negative in our series. Does this mean that they contain AFP, but at a concentration beyond the sensitivity threshold of our method, or are they actually devoid of AFP? The answer to this question can be obtained by using more sensitive methods. Abelev *et al.* (*3*, *6*) have tested 48 negative sera from our collection using a radioimmunodiffusion method which is said to be 16 to 32 times more sensitive than the conventional Ouchterlony method. The presence of AFP was demonstrated in about half of these sera. Thus, with this extremely sensitive method, about 15% of the cases remained negative. From more recent studies using radioimmunoassay methods (*38*) it may be concluded that even in these negative cases AFP can be found in the serum, but at trace levels not differing significantly from those found in other hepatic diseases. Thus it appears to be established that some PLC's are not secreting AFP.

The extent of AFP production varies in a wide range in PLC patients. AFP concentration was assayed in the sera of 156 patients using a radial immunodiffusion method (*36*). Figure 1 shows the distribution of these levels. Average AFP concentration reaches 0.4 mg/ml (or 0.6 mg/ml if the negative patients are excluded). From the shape of this distribution curve, one may guess that most of the negative cases are, in fact, cases beyond the sensitivity threshold of the

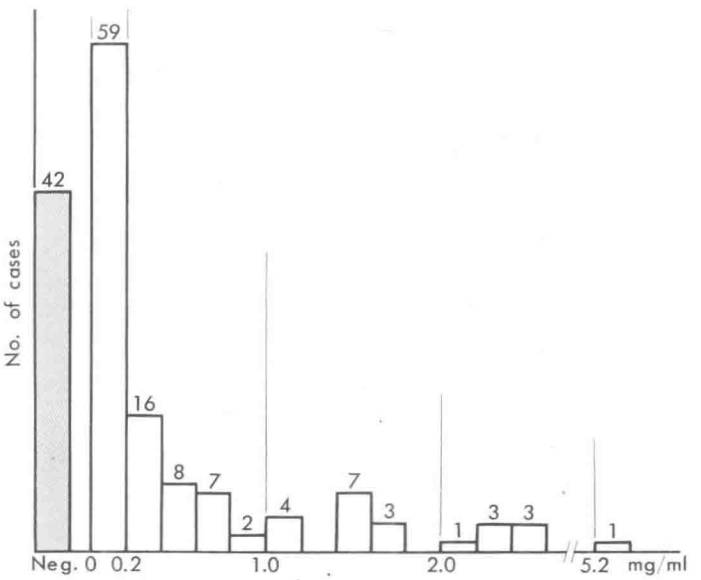

FIG. 1. Distribution of AFP levels in 156 patients with PLC

assay technique. We hoped that analysis of the clinical or biological features of these patients would show some correlation when related to these scattered AFP levels. This analysis was disappointing, since not one characteristic could be related to the AFP level (*40*). However, different conclusions were reached by two other groups. Purves *et al.* (*50*) found that AFP level seems to be related at borderline significance to the levels of cholesterol, hemoglobin, and serum protein, which increase with AFP, while mucoprotein and α_2-globulin decrease. The same group also obtained some evidence that nodular tumors achieved the highest degree of production when compared to massive and diffuse tumors. The microscopic features also seem to influence AFP production, the average serum level being 0.16 mg/ml in the anaplastic forms, 0.32 mg/ml in the poorly differentiated, and 0.25 mg/ml in the well-differentiated tumors. Alpert *et al.* (*8*) also found that the AFP level is influenced by the size of the tumor, being higher in large tumors.

None of these conclusions can be derived from our survey. However, very significant trends can be observed if one considers not the level of AFP but the positivity rate (Ouchterlony method) in different patients.

The influence of age is clearly shown in Fig. 2. Very young patients are most often positive. AFP prevalence is slightly less in adults and decreases distinctly over 40 (*40*). Other groups (*8*, *41*) have confirmed this finding.

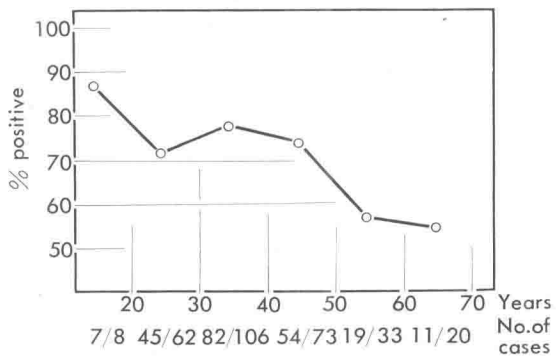

FIG. 2. Frequency of serum AFP-positivity in relation to age in patients suffering from PLC

The influence of sex is clearly apparent from Table II, showing that the positivity rate is 20% higher in males than in females. This fact has been noted by O'Conor *et al.* (*45*) and Alpert *et al.* (*8*). The reason for this sex disparity

TABLE II. Incidence of AFP in the Serum of Patients with PLC in Relation to Sex

	Males	Females	Total
AFP-positive	182 (75.9%)	38 (56.3%)	220 (72.4%)
AFP-negative	58 (24.1%)	26 (43.7%)	84 (27.6%)
Total	240	64	304

From Masseyeff *et al.* (*40*).

is unknown. There is no evidence of a direct role of male hormone. The recent discovery of Nunez *et al.* (*44*) that AFP possesses strong binding activity for oestrone and oestradiol, but not for other steroid hormones, should be mentioned at this point, since it is possibly related to this fact.

Turning now to pathological features, one striking fact is apparent from our series: Patients with large tumors are more often positive than those with smaller ones (Table III). All tumors heavier than 5 kg induced serum positivity. However, as already stated, there was no correlation between tumor weight and AFP level.

TABLE III. Incidence of AFP in the Serum of Patients with PLC in Relation to the Weight of the Tumoral Liver

	Tumoral liver weight					
	Less than 2 (kg)	2–3 (kg)	3–4 (kg)	4–5 (kg)	More than 5 (kg)	Total
AFP-positive	3	12	11	8	8	42
AFP-negative	3	6	3	1	0	13
Percent positive	50	67	79	89	100	76

From Masseyeff *et al.* (*40*) (postmortem examination).

Results collected in Table I indicate that AFP prevalence in PLC varies significantly in different countries. While the average frequency is 63%, in Europe it is only 53%, and in the group Africa/Asia, it is 71%. This difference is quite large and suggests an environmental or ethnic influence on the prevalence of AFP. This interpretation has been favoured by Foli *et al.* (*20*). While technical factors may explain some of the observed differences, this is not obviously the case for several studies where samples of different geographic or ethnic origin were examined in the same laboratory (*12*, *27*, *45*, *65*). However, evidence for a geographic or ethnic influence on AFP synthesis by tumoral liver is, in our view, still inconclusive. Some factors, examined above, such as age, sex, or tumor weight, may be responsible for these geographic or ethnic differences. In France, for example, the average age of patients with PLC is 63 (*23*) while in Senegal it is 38 (*46*). In many statistics coming from nontropical areas, sex incidence is quite different from African or Asian series, the predominance of males being reduced (the ratio is between 1/1 to 4/1 in Europe or America while 5/1 to 10/1 is commonly observed in statistics from Africa or Asia). The time of blood examination is also of importance since a late sample is more likely to be positive than an early one: Patients in the tropics very often attend at a very late stage of the disease. It is still possible that genetic factors play only an indirect role in AFP production, *e.g.*, by influencing the evolution of the disease. Tumoral growth seems faster in Africans. Alpert *et al.* (*9*) reported that tumors weighed during postmortem examination were 500 to 1,000 g heavier in Africans than in U.S. patients or Caucasian patients from South Africa. Thus, the influence of genetic or ethnic factors on AFP production is a hypothesis that, in our view, needs more documentation before being accepted.

The experimental results of Stanislawski *et al.* (*61*) suggested that etiological

factors may influence AFP production. This worker showed that, in the rat, hepatomas induced by 3-methyl-4-dimethylazobenzene often produced AFP while those induced by aflatoxin-B_1 did not.

Such experiments are not transposable to human PLC, where the etiological agent is still unknown. However, the recent finding of high prevalence of the hepatitis-associated antigen (HAA) in the sera of patients with PLC has given a new strength to the hypothesis of an oncongenic role for the hepatitis virus. Vogel *et al.* (*66*), in a series of 45 cases, found a definite correlation between the presence of HAA and AFP, while Masseyeff *et al.* (*39*), in a larger series of 209 cases, were unable to confirm this finding.

Factors involved in the biosynthesis of AFP can be investigated at the cellular level. There is a large body of evidence that AFP is produced by the tumor itself. The disappearance of AFP after tumor removal (*11*, *40*), the demonstration by immunofluorescence of AFP in cancerous liver cells (*25*, *49*), and *in vitro* production of AFP by tumor cells in culture (*2*, *53*) are convincing proofs.

Studies by immunofluorescence were undertaken in cooperation with Abelev's group. One aim of these experiments was to verify whether all tumor cells were producing AFP or only some of them. One drawback of this immunofluorescence technique is that it does not indicate whether or not a protein found in a cell has actually been produced by the cell or whether it has passively entered the cell. This is likely to occur if tissue fixation has been delayed, especially when cells have fragile membranes, like hepatocytes. Our studies were made using the controls suggested by Engelhardt *et al.* (*19*). Since hepatocytes do not synthesize IgG, they must not be positive in immunofluorescence using an anti-IgG serum. Staining then means that their membrane has been damaged. A positive staining with anti-AFP serum is thus not significant. This technique has been applied to consecutive cuts, one being stained with anti-IgG, the next with anti-AFP. Thus, the only cells accepted as specifically positive are those which are AFP-positive and IgG-negative.

The results were quite unexpected (*60*). Only a small proportion (5%) of cancer cells were found to synthesize AFP. Of course, the method of analysis tends to underestimate the number of cells actually producing AFP, but even if IgG-positive cells are counted, the proportion of AFP-positive cells does not exceed 10–15%.

The cell arrangement is characteristic: They form sorts of sleeves around capillaries of tumor sinuses. The intensity of fluorescence is very heterogeneous, but usually the brighter cells are located close to the capillary.

A different pattern was obtained in 2 metastases in the lungs. The majority of cells were AFP-positive and the intensity of fluorescence was less heterogeneous. However, the cells located near vessels were still more fluorescent. No morphological difference was observed between AFP-positive and AFP-negative cells.

Thus the main conclusion reached as a result of this work is that only a small proportion of tumor cells produced AFP and that the only difference between positive and negative cells lies in their environmental conditions, the former being located around vessels.

Dynamics of AFP Production

We were interested in the dynamics of AFP production for theoretical and practical reasons. One of the practical questions is, does AFP detection allow an early diagnosis of PLC, early enough to offer a possibility of surgical cure?

The observation of currently attending patients is unlikely to solve this question, since PLC is an insidious disease and tumors are frequently quite large when patients report the first symptoms. Thus, it was decided to follow up a sample of susceptible population with the AFP Ouchterlony test. This was done in Senegal on approximately 9,000 male workers, 3 times a year for 2 years (*64*). Nine cases of PLC were identified during this period, 6 of which were AFP-positive. Of these cases, 3 were found in apparently healthy people: In fact, liver tumors could be found. It was only in 1 case that the tumor was small and limited enough to permit surgical removal. However, 1 month after partial hepatectomy, the patient died from extensive metastases. The rather pessimistic conclusion of these experiments is that the gel immunodiffusion test is usually positive, too late to give a chance for curative treatment. However, a more sensitive test may permit a different conclusion.

Some patients with a clinically demonstrable tumor, AFP-negative on hospital admission, became positive after 1 to 2 months. This observation, made in 3 patients out of 14 in our series, is interesting since it raises the question of the cellular origin of this protein. It may be produced either by the existing tumoral cells becoming less differentiated, or by new cancerous cells, these latter cells being either daughter cells of the existing cancerous cells or new cancerous cells arising from noncancerous surrounding hepatocytes. The last hypothesis is not unlikely, since the observation of liver cancer patients in Senegal often gives the impression of a cancerous disease beginning at nearly the same time in different parts of the liver. Thus, the idea of the emergence of new cancerous clones, starting from less differentiated cells, seems acceptable. However, it is still impossible to prove it.

The wide dispersion of AFP levels observed in different patients is an indication of the absence of a mechanism resulting in regulation of AFP biosynthesis.

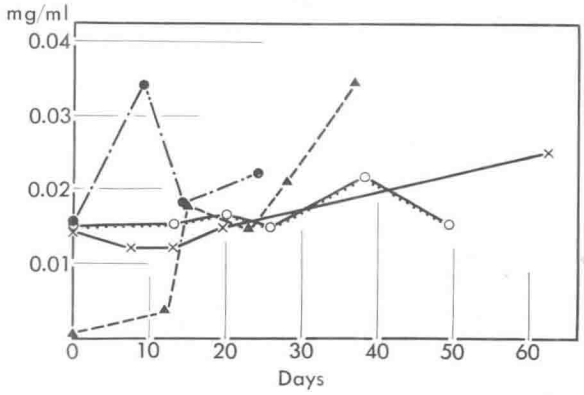

FIG. 3. Evolution of serum AFP levels in 4 patients with PLC