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Alpha-fetoprotein and liver cell proliferation in rats fed choline-deficient diet*)

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With 3 figures

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Synthesis of alpha-fetoprotein (AFP) is enhanced in rats with hepatocellular carcinoma (1, 10, 19) as well as after toxic liver damage or partial hepatectomy (4, 16, 22). It appears that increased production of this so-called oncofetal antigen (2) is closely coupled to proliferation, whether neoplastic or otherwise, of the hepatic cells (22, 23). In the wake of lesser noxious effects, liver involvement may also be associated with elevated serum AFP levels, per example, in rats fed Miller's hypoproteic diet (4) and in pyridoxine-deprived baboons (7). The purpose of the present communication is to report on the occasional reappearance of AFP in the serum of rats fed a lipotrope deficient diet and to offer an explanation, based on the hepatic alterations occurring under these circumstances.

Materials and methods

Animals. Female albino rats of the Hebrew University (Sabra) strain, aged 60 days at the beginning of the experiment, were used. The animals had free access to food and tap water at all times.

Production of anti-AFP serum. Immunization, absorption procedures, testing and evaluation of antisera were described previously (14, 17). Briefly, antisera were raised in rabbits against rat amniotic fluid. The antisera were exhaustively absorbed with adult male rat blood and an organ pool. The absorbed rabbit antisera did not react with normal rat serum or saline extracts of diverse organs. They gave one precipitation line when reacted, by *Ouchterlony*'s double immunodiffusion test, with amniotic fluid, embryonic blood and serum of pregnant or hepatoma-bearing rats (fig. 1).

Experimental design. Two groups of 61 rats each were examined. Group 1. Control rats were fed *Amrod*[1]) standard laboratory diet, comprising 18.6% protein[2]), 4.4% fat and 58.5% carbohydrates[3]). Group 2. Experimental rats were kept on a semisynthetic choline deficient diet. The latter consisted of 73%

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[1]) Ambar-Yissum, Jerusalem.

[2]) In addition to proteins of fish and vegetable derivation, bovine milk powder was supplemented.

[3]) These figures are the averages computed from several analyses carried out during 1974/1975.

starch, 18% casein ("vitamin-free"), 5% vegetable oil and 4% salt mixture (U.S.P. No. II). To each 1000 gm were added 3 mg of thiamine, 2 mg of riboflavin, 7 mg of calcium panthotenate, 2.5 mg of pyridoxine and 30 mg of choline chloride. As of the age of 5 months, 4 to 6 rats were killed at biweekly intervals, the oldest animals being about 11 months of age. Two hours prior to sacrifice, 100 μCi of tritiated thymidine (^{3}HTdR 5Ci/mM, Amersham) in 0.5 ml of saline were injected intraperitoneally. The animals were anesthetized with pentobarbitone, blood was drawn by cardiac puncture and liver specimens were fixed in *Bouin's* solution.

Histological and serological examinations. Liver sections were cut at 6 μ and stained with hematoxylin and eosin. Consecutive sections were coated with Ilford K-5 liquid emulsion, exposed for 14 days, developed in Kodak D-19 and stained with hematoxylin and eosin. The number of labelled nuclei of hepatocytes was counted in 50 successive high-power fields (HPF); labelled inflammatory, mesenchymal or ductal cells were not considered. Rat sera were reacted by immunodiffusion with anti-AFP serum; the plates were observed for 3 days, washed, stained with amido black and rechecked.

Results

The livers of all rats fed the standard laboratory diet were essentially normal. There were some small intralobular aggregates of mononuclear cells in most specimens; these are of frequent occurrence in our control population of rats (3). Similar foci were also found in the animals of Group 2. The livers of rats fed the choline deficient diet revealed mild, diffuse, fatty change characterized by the presence of small vacuoles in many hepatocytes. In about one third of the rats there was, in addition, marked periportal fatty metamorphosis characterized by large solitary vacuoles distending the hepatic cells (fig. 2). No correlation was evident between the degree of fatty change and the duration of the experiment.

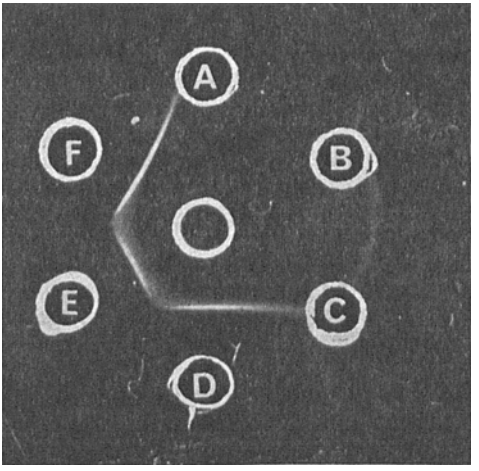

Fig. 1. Immunodiffusion pattern of anti-AFP serum (center well) with sera of rats fed choline deficient diet (A, C and E), serum of control rat (B), serum of pregnant animal (D) and serum of hepatoma bearing rat (F). Note reaction of complete identity corresponding to the presence of AFP in sera D, E and F.

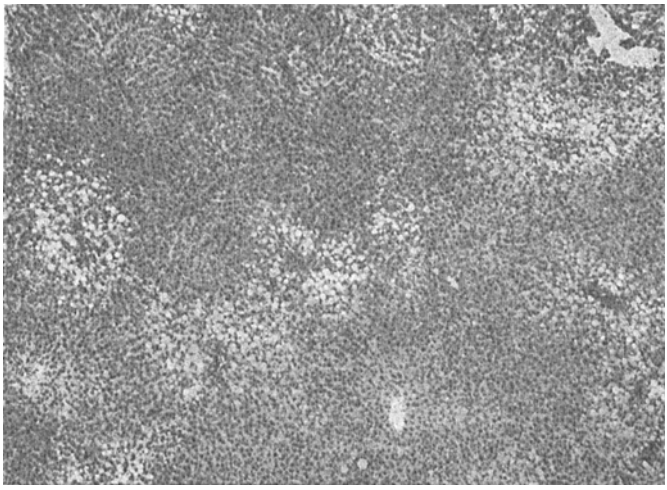

Fig. 2. Histological section of the liver of a rat fed choline deficient diet for 7 months. There is marked peripheral fatty change with distension of the hepatic cells. Hematoxylin and eosin 60.

The frequency of the number of ^{3}HTdR labelled nuclei per 50 HPF in the livers of control and experimental rats is depicted in the accompanying histogram (fig. 3). The number of labelled cells per 50 HPF in the livers of rats fed the standard diet ranged from 0 to 12 and in the livers of animals on the deficient diet from 0 to 31. Moreover, 13 or more labelled nuclei per 50 HPF were found in 16 of the 61 (26%) animals of the latter group. Since thymidine uptake, as determined by the number of labelled nuclei per 50 HPF, was not related to the age of the rats at sacrifice, the results were combined for each of the two groups. The mean number of labelled hepatic cells per 50 HPF was 3.12 (S.E. 0.43) and 7.33 (S.E. 0.99) in the rats fed the standard and choline deficient diet, respec-

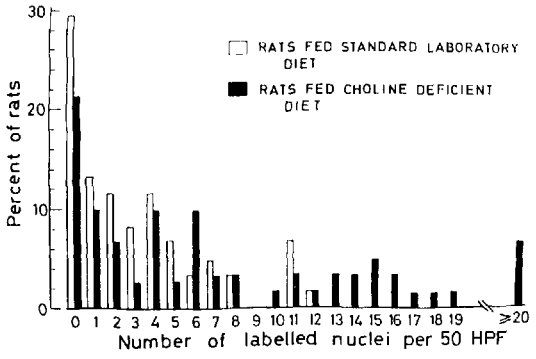

Fig. 3. Frequency histogram of number of ^{3}HTdR labelled cells per 50 HPF in livers of rats fed standard laboratory or choline deficient diet.

tively. The difference between the groups, calculated by Student's t-test, was statistically highly significant ($t = 3.90$, $p < 0.001$).

Alpha-fetoprotein was not detected in the serum of rats of Group 1. Four of the 61 sera of rats of Group 2 were found to contain AFP by the immunodiffusion test (fig. 1). The number of labelled nuclei in the livers of these 4 rats was 6, 6, 7 and 19 per 50 HPF; these four animals were 6 or more months on the deficient diet.

Discussion

The observations presented herein confirm previous findings inasmuch as AFP could not be demonstrated in the serum of the healthy control rats by Ouchterlony's double immunodiffusion test, a technique by which amounts below 3–10 μg per 1 ml are undetectable (12, 20). That AFP is synthesized by the liver of the adult healthy rat has been convincingly shown by *Sell,* who ascertained that under physiological conditions the concentration of AFP is consistently below 0.06 μg per 1 ml of serum; a manyfold increase in serum concentrations occurs in hepatoma bearing rats and after partial hepatectomy, i.e., in conditions associated with hepatocellular proliferation (19, 22). In the experiments described herein, AFP was demonstrated by immunodiffusion in the serum of 4 of 61 rats fed a choline deficient diet. To the best of our knowledge, only *DeNechaud, Foy* and their associates have heretofore reported on elevated serum AFP levels in animals fed deficient diets (4, 7). Their investigations, however, have not shed light on the underlying hepatic process responsible for the increased synthesis of AFP. In the present study, hepatocellular proliferation, as evinced autoradiographically by the number of ^{3}HTdR labelled cells, was found in rats fed a lipotrope deficient diet: ^{3}H-thymidine uptake was significantly higher in the livers of rats fed the choline deficient diet than in the livers of animals on the standard diet. We would like to suggest that waves of hepatocyte proliferation could account for the elevated serum AFP levels in rats on the deficient diet, since AFP appears to be produced at some time during or after the cell cycle (21). Since increased concentrations of serum AFP follow and accompany peaks of hepatocellular proliferation in rats poisoned with one hepatotoxic agent or another *(Boss,* unpublished observations), it is not surprising that a correlation between thymidine uptake and the serological findings does not exist for each individual animal. It is possible that a higher percentage of positive sera would have been found, had more serum samples been obtained from each rat or a more sensitive serological technique been employed.

It is well known that fatty change and cirrhosis of the liver result from chronic choline deficiency (11). The lobular distribution of fat deposition in rats is variable, being centrilobular, peripheral or diffuse (8). In all our animals, there was diffuse fatty change in the form of small vacuoles, whereas large fat globules, present in only one third of the animals, were restricted to the periphery of the lobules. The diet tendered to our rats contained 30 mg of choline chloride per 1 kg, an amonut which is one tenth that recommended by *Miller* et al. (13). Nevertheless, cirrhosis did not develop, possibly because the amount of casein in the diet was adequate

and fat was not given in excess. Since casein contains 2.8% of methionine (5), the rats were probably not in a state of extreme deficiency in lipotropic factors (9). Be that as it may, the present experiments show that rats maintained on a choline deficient diet for a long period of time develop a liver disorder, which, in addition to fatty change, is characterized by enhanced hepatocyte proliferation and increased synthesis of AFP. These observations are consistent with those on liver injury in pyridoxine-deprived baboons, the serum AFP levels of which are also elevated in a certain proportion of the animals (7). Pyridoxine must be present for the normal functioning of choline as a lipotropic agent (6).

In a previous report from this laboratory, elevated serum AFP levels have been described in glomerulonephritis (15). It is surmised that under these circumstances increased synthesis of AFP is coupled to compensatory hepatocyte hyperplasia (18) secondary to hypoproteinemia due to heavy protein loss in the urine of the nephritic rats. Further, raised serum AFP levels have also been detected in rats fed *Miller*'s hypoproteic diet (4).

In conclusion, it might be inferred that enhanced liver cell proliferation occurring in rats on a choline deficient regimen, even though the content of the diet in protein and fat is adequate, compensates for an increased loss of hepatocytes. The elevated serum AFP levels are compatible with the currently held belief that this so-called onco-fetal antigen (2) is synthesized by regenerating hepatocytes. It seems of interest that a comparatively mild hepatic disorder, such as one resulting from deficiency in a lipotropic factor, may be associated with serum AFP concentrations detectable by immunodiffusion.

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Summary

Liver ^{3}H-thymidine labelling index and serum AFP were determined in rats fed a standard laboratory or choline deficient diet. A significant increase in the rate of liver cell proliferation was evidenced in animals on the deficient diet. Furthermore, elevated serum AFP levels were detected in a proportion of these rats. It is suggested that increased synthesis of AFP corresponds to liver cell regeneration secondary to hepatic damage.

Zusammenfassung

Der ^{3}H-Thymidin-Aufnahmeindex in der Leber und das Serum AFP wurden bei Ratten, welche bei einer standardisierten Laboratoriumsdiät oder mit einer Cholinmangeldiät ernährt wurden, bestimmt. Es wurde festgestellt, daß die Proliferation der Leberzellen bei Tieren, die die Mangeldiät erhalten haben, signifikant erhöht war. Weiterhin wurde bei einigen dieser Ratten eine erhöhte Serumkonzentration des AFP gefunden. Die Befunde deuten darauf hin, daß die vermehrte Synthese des AFP einer Leberzellregeneration, welche sekundär zu einem Leberschaden entsteht, entspricht.

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