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INCREASE IN SERUM ALPHA FOETOPROTEIN LEVEL IN HEPATIC REGENERATION OF THE RAT. EFFECTS OF AGE AND OF MAGNITUDE OF REGENERATIVE ACTIVITY

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Summary.—The effects of variable degrees of liver regeneration induced by two-thirds partial hepatectomy or intoxication with different doses of carbon tetrachloride (CCl_4) on increased production of alpha foetoprotein (AFP) have been studied in very young (5–6 weeks) and older (15 weeks and more) rats by counter-immunoelectrophoresis (sensitivity down to 250 ng/ml). In the young animal adequate regeneration following two-thirds hepatectomy as well as 100 µl CCl_4 successfully induced large increases in serum AFP levels. Smaller doses as well as a large fatal dose of the toxin, all of which resulted in inadequate regenerative activity, failed to excite AFP synthesis and secretion up to detectable levels. The adult animal did not show detectable AFP with any of these procedures. However, necrosis induced in the regenerating adult liver 24 h following partial hepatectomy did result in the detection of small amounts of protein in the serum. It is concluded that in hepatic regeneration of this species, the age of the animal as well as the magnitude of regenerating activity in the liver are critical in the occurrence of high levels of serum AFP.

THE α_1 globulin commonly referred to as alpha foetoprotein (AFP) represents one of the most major proteins in the embryonic and foetal life of a large variety of vertebrates tested so far (Gitlin and Boesman, 1966, 1967; Abelev, 1971; Gitlin, 1974; Zizkovsky and Masopust, 1974). It disappears from the blood at a variable period after birth, depending on the species (Gitlin and Boesman, 1966, 1967; Abelev, 1971; Gitlin, 1974). While the function of this apparently important foetal protein is completely unknown, what is biologically of great interest is the reappearance in the adult of significant amounts of this protein on development of certain tumours, particularly hepatocellular carcinoma (Abelev, 1971). In the foetal and postnatal periods of normal animals (Gitlin and Boesman, 1966; Abelev, 1971; Gitlin, 1974; Gitlin, Periecelli and Gitlin, 1972) liver is the single most important source of this protein and its localization in hepatocytes has been shown by both immunofluorescence and immunohistochemical techniques (Engelhardt *et al.*, 1971; Nayak *et al.*, 1974*a*, *b*; Purtilo and Yunis, 1971).

Though high levels of serum AFP are frequently observed in liver cell carcinoma, smaller elevations also occur in several non-neoplastic hepatic disorders accompanied with regeneration (Abelev, 1971). In the mouse, hepatocytic regeneration following surgical ablation or toxic injury is always associated with elevated levels of serum AFP (Abelev, 1971; Rouslahti *et al.*, 1973; Bakirov, 1968). Attempts to induce the reappearance of an elevation of AFP in the rat by such procedures, however, have led to variable and unpredictable results (Abelev, 1971; Stanislawski-Birencwajg, Uriel and Grabar, 1967; Perova, Elgort and Abelev, 1967, 1971;

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Gilli and Masseyeff, 1974; Nechaud and Uriel, 1971). It has been suggested recently that in the rat AFP reappearance is to a great extent a function of age (Abelev, 1971; Gilli and Masseyeff, 1974; Nechaud and Uriel, 1971). Also, the relatively small concentration of this protein, even in the young animal, needs sensitive techniques for its detection (Gilli and Masseyeff, 1974; Nechaud and Uriel, 1971; Sell *et al.*, 1974).

In our earlier studies on experimental toxic liver injury (Das *et al.*, 1974; Nayak *et al.*, 1975) it was possible to vary the extent and severity of hepatocellular necrosis by administering different doses of toxins. By suitably manipulating the magnitude of hepatocytic regeneration following necrosis in these animals, we observed that even using techniques less sensitive than radioimmunoassay reappearance of AFP in the young rat can be directly related to the degree of regenerative activity and that smaller quantities of this protein are indeed synthesized by regenerating liver in the older animal but cannot be detected by such techniques, except when amplified by other procedures.

MATERIAL AND METHODS

Male Sprague–Dawley strain rats aged 5–6 weeks and 15 weeks and above were used. They were given standard rat cube diet (Lever Brothers, Bombay) and allowed free access to water. Each study group consisted of one or more batches of 4–5 animals. Hepatic regeneration was induced by intoxication with carbon tetrachloride (CCl_4) or by two-thirds partial hepatectomy. Control groups of animals were given injections of equivalent volumes of liquid paraffin or were subject to sham operation respectively.

Carbon tetrachloride (British Drug House, analytical reagent) was administered by intragastric intubation in doses of 10, 25, 100 or 500 μl/100 g body weight, in a solution in liquid paraffin (Das *et al.*, 1974; Nayak *et al.*, 1975). Partial hepatectomy was performed according to the method of Higgins and Anderson (1931) and a two-third ablation was verified by weighing the liver tissues. Some groups of older animals were given 500 μl of CCl_4/100 g or 5 mg of dimethylnitrosamine/kg 24–48 h after partial hepatectomy. Dimethylnitrosamine (DMN) was prepared synthetically and its purity tested (courtesy of the Department of Biochemistry, V.P. Chest Institute, Delhi University). The 5 mg dose injected intraperitoneally as an aqueous solution, which is equivalent to 15 mg/kg in the normal rat with intact liver, has been shown by us to induce liver necrosis only in the partially hepatectomized animal (Nayak *et al.*, 1975).

A cumulative mitosis count was also carried out in groups of older rats given 10, 25 and 100 μl doses of CCl_4. An aqueous solution of colchicine (1 mg/kg) was injected intraperitoneally 37 h after dosing with CCl_4 and animals were sacrificed 3 h later. Mitosis was counted in paraffin sections of liver stained with haematoxylin and eosin and expressed as number/1000 liver cell nuclei.

Blood was collected during the experimental period from the retrobulbar plexus by capillary tubes (0·5–1·0 ml) and at sacrifice by cardiac puncture (3–4 ml). In most animals a sample before the start of experiment and one or more at 24 h periods afterwards were tested. Animals subjected to single procedures of CCl_4 intoxication or partial hepatectomy were sacrificed 24–48 h later while those to which CCl_4 or DMN was given following partial hepatectomy, were sacrificed 24–48 h after administration of the toxin.

Qualitative detection of AFP in the serum was carried out by the counter immunoelectrophoresis (CIEP) technique of Alpert and his colleagues (Alpert *et al.*, 1971) as well as by radial agar gel diffusion. In our hands the CIEP test detected AFP down to a level of 250 ng/ml. In each test, positive and negative controls were used. Monospecific antiserum against rat AFP was raised in rabbits and prepared according to procedures described earlier (Nayak *et al.*, 1974).

AFP containing cells were looked for in liver tissues obtained at sacrifice by the fluorescence and peroxidase labelled immunochemical localization techniques reported from our laboratory (Nayak *et al.*, 1974, 1975).

RESULTS

Partial hepatectomy

Adult animals, aged 15 weeks and over, never showed detectable AFP in the serum before or after surgery either by the diffusion or the CIEP technique. In young animals of 5–6 weeks age, on the other hand, while AFP was not detected in the intact animal, it appeared 24 h after surgery. The precipitation lines seemed more prominent in the 48 h sample. Tests were positive not

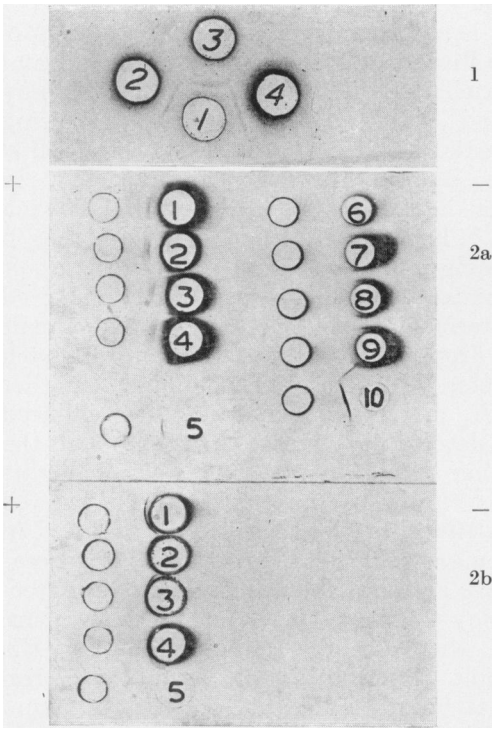

FIG. 1.—AFP in agar gel diffusion. Anti-rat AFP serum (1) has reacted with sera of 5–6 week old rat 48 h after partial hepatectomy (2), 5–6 week old rat 48 h after 100 μl CCl_4 (4) and newborn rat (3).

FIG. 2.—Counter-immunoelectrophoresis for AFP in hepatic regeneration of rats. Left rows of paired wells were filled with anti-rat AFP serum and right wells filled with serum of experimental rats.

2a. 5–6 week old rats: 1 and 2 = 24 h after partial hepatectomy, 3 and 4 = 24 h after 100 μl CCl_4, 5 = 48 h after partial hepatectomy, 6–9 = before regeneration inducing procedure. 10 = positive control (newborn rat).

2b. 15-week and older rats: 1–3 = DMN (5 mg/kg) following partial hepatectomy. Precipitation lines are close to the right wells. 4 and 5 = 48 h after partial hepatectomy.

only by CIEP but also by agar diffusion when relatively more serum was used in larger wells (Fig. 1, 2).

CCl_4 intoxication

As in the case of partial hepatectomy, animals 15 weeks or older did not show AFP within 48 h of inotixcation at any dose level. In the young animal only the 100 μl dose consistently induced the reappearance of AFP, particularly at the 48 h period. As with partial hepatectomy, detection was possible by both CIEP and agar diffusion using larger volumes of serum (Fig. 1, 2). Following the 25 μl dose, a trace of the protein seemed to reappear in an occasional animal and even then was detectable only by the CIEP technique. The 10 μl and 500 μl doses failed to induce any reappearance. All rats administered 500 μl died within 24–36 h.

Intoxication following partial hepatectomy

This was carried out only in adult animals and was aimed at inducing necrosis in a regenerating liver. With both CCl_4 and DMN, AFP reappearance occurred in three-quarters of the animals within 24–48 h after intoxication, detectable only by the CIEP technique. The large 500 μl dose of CCl_4 is not as lethal to partially hepatectomized animals as to normal rats with intact liver (Nayak *et al.*, 1975), making it possible for the former to survive longer than 48 h.

Mitotic counts

Mean mitotic counts in control rats and in rats administered 10, 25 and 100 μl of CCl_4 40 h before sacrifice were 1, 47, 87 and 127/1000 non-mitotic liver cell nuclei respectively.

AFP in liver cell

In none of the animals studied, irrespective of whether or not they showed AFP in the serum, could the protein be convincingly localized in the hepatocytic cytoplasm or elsewhere.

DISCUSSION

Intoxication with carbon tetrachloride and partial removal of liver tissue are both well known procedures for exciting one or more waves of parenchymal cell regeneration in the liver (Morrison *et*

al., 1965; Carter, Holmes and Mee, 1956; Bucher, 1963; Weinbren, 1959). The magnitude of this regenerative activity seems to depend directly on the quantum of hepatocellular loss, which in turn is related to the dose of toxin administered (Das *et al.*, 1974; Nayak *et al.*, 1975) or the extent of surgical ablation carried out (Bucher, 1963). Increased DNA synthesis in a regenerating liver following partial hepatectomy has been shown to be proportional to the amount of tissue removed (Bucher, 1963), a two-third removal being ideal both for inducing brisk and adequate regeneration and allowing survival. In our present study a 100 μl dose of CCl_4 induced 3 and 1·5 times more regenerative activity, in terms of mitosis, than the 10 and 25 μl dose respectively. A similar correlation between dose level of CCl_4 and hepatic regeneration has also been observed recently in mice (Pihko and Ruoslahti, 1974).

The association of hepatic regeneration with increased levels of AFP seems to be well established now (Abelev, 1971; Ruoslahti *et al.*, 1973; Bakirov, 1968; Perova *et al.*, 1967, 1971; Gilli and Masseyeff, 1974; Nechaud and Uriel, 1971, 1972; Pihko and Ruoslahti, 1974; Sell *et al.*, 1974). Earlier studies had shown that this phenomenon can be easily observed in mice (Abelev, 1971) but not so in rats (Stanislawski-Birencwajg *et al.*, 1967). This is almost certainly because of the relative concentrations of the protein in various species of animals, both during different phases of development as well as following liver regeneration. The AFP level of adult mouse (Pihko and Ruoslahti, 1973) is about 10 times higher (Abelev, 1971; Pihko and Ruoslahti, 1974) than what is seen in rats and humans of corresponding age (Sell and Gord, 1973; Ruoslahti and Seppala, 1971). Using sensitive techniques which achieve detection of very small amounts of the protein, reappearance or variable augmentation of normally low levels of AFP has now been reported not only in very young rats subjected to hepatotoxic agents like CCl_4 and DMN (Gilli and Masseyeff, 1974; Nechaud and Uriel, 1971, 1972) or partial hepatectomy (Sell *et al.*, 1974) but also following partial hepatectomy in adult rats (Sell *et al.*, 1974). However, as expected, in the latter study AFP levels following 70% hepatectomy were 100-fold higher in young rats (5-week old) than in the adult animal, while the 7-week old rats showed intermediate levels. Also in such animals a drop to normal levels occurred much faster. In our older animals neither CCl_4 nor partial hepatectomy induced a detectable increase in AFP. On the other hand, in the 5–6 week old rats two-thirds partial hepatectomy as well as 100 μl of CCl_4 consistently resulted in demonstrable AFP in serum. Regenerative activity following partial hepatectomy is known to be significantly more in the weanling animal than in the adult (Bucher, 1963). It is clear then that failure to detect AFP in the serum of the earlier studies (Stanislawski-Birencwajg *et al.*, 1967) was chiefly because relatively insensitive techniques of detection were employed and adult animals with limited capacities for physiological as well as stimulated production of AFP were used.

Even in the young rats used by us smaller doses of CCl_4 (10 and 25 μl) which excited much less regenerative activity than the 100 μl dose failed to induce detectable increase of AFP. Very mild elevation of AFP in the odd animal receiving the 25 μl dose may well be due to individual variation in susceptibility to this dose of CCl_4 resulting in somewhat excessive regeneration. Following intoxication with varying amounts of CCl_4 in mice Pihko and Ruoslahti (1974) demonstrated a direct correlation between mitotic counts and serum levels of AFP. Administration of the large, fatal 500 μl dose, both in their mice as well as in our rats, on the other hand, failed to result in a significant rise in the serum AFP level. This is possibly

due to the extensive liver cell necrosis induced by this dose (Das *et al.*, 1974) leading to only minimal regeneration and early death. The magnitude of hepatic regeneration following fractional hepatectomy depends directly on the extent of surgical ablation (Bucher, 1963). From the different degrees of hepatocellular necrosis produced by varying doses of CCl_4 we believe that 10, 25 and 100 μl doses of the toxin may be considered equivalent in terms of loss of hepatic parenchyma, to 10, 25 and 70% hepatectomy (Das *et al.*, 1974) respectively. Gilli and Masseyeff (1974) in a recent study using sensitive radioimmunoassay, demonstrated elevation of serum AFP levels in 5-week old rats only when they were subjected to CCl_4 inhalation but not when a 25% hepatectomy was performed. Obviously, this amount of hepatic ablation was insufficient to excite adequate regeneration and can be compared with our model of 5–6 week old rats receiving 25 μl of CCl_4 in which AFP was not generally detected.

An interesting finding in the present study was the presence of AFP in a number of partially hepatectomized adult animals when necrosis was induced in the regenerating liver by either a large dose of CCl_4 or by DMN. As indicated earlier, at this age partial hepatectomy alone fails to induce production of enough AFP detectable by CIEP. Extensive and massive necrosis unaccompanied by regeneration also does not bring about an increase of AFP, even in younger rats. It is possible, then, that adult regenerating livers do synthesize traces of AFP but the amount secreted is too small to be detected by less sensitive techniques until necrosis of such livers helps in releasing larger amounts of the protein to blood from the damaged cells resulting in a significant rise in serum levels and its detection. That a distinct though small (about 3-fold) increase in the output of AFP follows hepatic regeneration in adult rats induced by 70% hepatectomy has been lately demonstrated more directly by the use of sensitive radioimmunoassay (Sell *et al.*, 1974).

Using techniques in which AFP can be localized in hepatocytes of foetal and newborn livers under light microscopy (Nayak *et al.*, 1974, 1975; Purtilo and Yunis, 1971) we could not detect the protein in regenerating liver cells. This may not be so surprising since in such techniques only infinitely small portions of the total hepatocytic mass are examined whereas serum levels represent the output of the entire liver. In any case, serum levels in rats with regenerating livers are generally not as high as those during the 3rd to 4th week of normal postnatal life in this species, when, even though the protein is detectable in serum, its intrahepatic localization is seldom, if at all, apparent in random 3 μm sections of the liver (Nayak *et al.*, 1975). Also, the distribution of AFP synthesizing cells can indeed be very random (Nayak *et al.*, 1975). Immunoelectron microscopy may provide crucial information in this connection. Alternatively, regenerating liver in the mouse, which synthesizes quantitatively far more AFP than that of the rat, may be more ideal for such studies.

Human serum AFP levels in both physiological and pathological states have been shown to be closely comparable with those in the rat (Pihko and Ruoslahti, 1973, 1974). Several studies reported recently have revealed increases in these levels in human patients recovering from diffuse liver diseases, and seem again to suggest a direct correlation between the degree of regeneration and serum AFP values (Pihko and Ruoslahti, 1974; Ruoslahti *et al.*, 1975).

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