Virchows Arch [Pathol Anat] (1981) 393:9–26

Virchows Archiv A

Alpha-Fetoprotein: Cellular Origin of a Biological Marker in Rat Liver Under Various Experimental Conditions

W.D. Kuhlmann

Laboratory of Experimental Medicine and Immunocytochemistry,
Institut für Nuklearmedizin, D.K.F.Z., D-6900 Heidelberg, Federal Republic of Germany

Summary. Alpha$_1$-fetoprotein (AFP) was detected by serological, light and electron microscopic methods in various experimental models. These included (a) liver regeneration after partial hepatectomy or CCl_4 intoxication (mouse and rat); (b) liver intoxication by high doses of N-nitrosomorpholine (NNM) and chemical induction of hepatomas (rat). AFP levels varied greatly according to the animal species and strains used. Low and high AFP-producing species and strains were distinguished. In liver regeneration after hepatectomy or CCl_4 intoxication, cellular AFP was found in the cytoplasm of hepatocytes. In NNM-intoxicated livers, elevated AFP levels were associated with proliferation of canalicular epithelial cells in which AFP was localized. In early stages of hepatocarcinogenesis, significant AFP increase occurred after high-dose carcinogen feeding and AFP was also localized in proliferating canalicular epithelial cells. On low-dose NNM feeding, no cellular AFP was detected unless hepatomas had developed. At the stage of malignant conversion, distinct AFP staining and non-AFP staining hepatocellular carcinomas appeared in livers.

Key words: Alpha-fetoprotein – Liver regeneration – Hepatocarcinogenesis – Immunohistology

Introduction

Proteins characteristic for the fetus emerge in the course of phylogenetic and ontogenetic development and appear to be associated with histogenesis and cytodifferentiation of organs. Among the fetus-associated biomolecules, alpha$_1$-fetoprotein (AFP) has been one of the most intensively studied proteins since its first observation by Bergstrand and Czar (1956) in the human fetus. Under physiological conditions, AFP is synthesized in the yolk sac, the gastrointestinal tract and the liver of the fetus of many species (Gitlin and Boesman 1967a, 1967b; Gitlin et al. 1967). After birth, the protein disappears from body fluids following almost complete suppression of the genes responsible (cf. Ruoslahti and Seppälä 1979).

0340-1227/81/0393/0009/$03.60

The reappearance of AFP in sera of hepatoma bearing adult individuals was first described in mice (Abelev et al. 1963), Subsequent studies substantiated the association of AFP synthesis with malignant growth of organs which have derived from embryonic gut (Abelev 1971; Laurence and Neville 1972; Teilum et al. 1975). Furthermore, numerous studies have also shown the close relationship of this protein with non-malignant liver diseases (Perova et al. 1971; Sell et al. 1974; Taketa et al. 1975; Smuckler et al. 1976; Delpré and Gilat 1978).

Significant production of AFP in liver diseases make this protein a useful biological marker for studies on liver histo- and cytodifferentiation. However, comprehensive data on its functional histology and immunopathology are still incomplete. In previous communications we described procedures for the localization of AFP with horseradish peroxidase as label in immunohistological ligand assays (Kuhlmann 1975; Kuhlmann 1978a, 1978b). Here, we present our studies on AFP production in cell populations during liver regeneration and in the course of hepatoma development.

Material and Methods

Experimental Models. Rats of the inbred strain BD X, C3H/He mice (Zentral-Institut für Versuchstiere, Hannover, Germany) and BALB/c/J mice (The Jackson Laboratory, Bar Harbor, U.S.A.) were used. Studies on liver regeneration and hepatocarcinogenesis were performed on males. The experimental models are summarized in Table 1. Control animals received no treatment, sham operation or exposure to liquid paraffin or tap water. All animals were kept on standard diet (Altromin no. 1324; Altromin GmbH, Lage, Germany) and tap water ad libitum.

Immunological Reagents and Procedures. Isolation of mouse and rat AFP and preparation of rabbit anti-mouse and anti-rat AFP immune sera have been described (Kuhlmann 1975; Kuhlmann 1976). Rabbit anti-mouse IgG and anti-rat IgG immune sera were prepared in the same manner; an anti-rabbit IgG immune serum was produced in sheep. The AFP content of sera was quantified by an enzymo-electroimmunodiffusion method (Kuhlmann 1978a; Kuhlmann 1979b).

Cellular AFP was stained by a direct or by an indirect peroxidase labelled antibody method (Kuhlmann 1975; Kuhlmann 1978b). Antibodies were isolated from crude immune sera by specific immunoadsorbents. For enzyme conjugation, antibodies were coupled with horseradish peroxidase (HRP, RZ 3) (Kuhlmann et al. 1974). The antibody-HRP conjugates were purified in two steps by use of Sephacryl S-200 and Sepharose 4B – Concanavalin A columns (Lannér et al., 1978). Immunocytochemical control sera included unlabelled and HRP labelled normal rabbit IgG globulins, rabbit anti-glucose oxidase, anti-mouse IgG and anti-rat IgG antibodies; HRP labelled anti-AFP antibodies were also absorbed with AFP (Kuhlmann 1978a). For light microscopic studies, the PAP technique (peroxidase – antiperoxidase antibody complexes, Sternberger 1974) was also employed. PAP was purchased from DAKO – Immunoglobulins (Copenhagen, Denmark).

Light Microscopy and Immunohistology. Liver slices were fixed in 99% ethanol – 1% acetic acid for 12–15 h at 0–4° C and embedded in paraffin (Kuhlmann 1975). 5–7 µm thick sections were mounted on acetone cleaned slides, deparaffinated in xylene and passed into phosphate buffered saline (PBS).

Endogenous peroxidases were inhibited by treatment with 1% hydrogen peroxide in PBS for 1 h. Sections were first incubated with unlabelled rabbit anti-AFP antibodies (0.005–0.01 mg/ml) for 24 h at 4° C, followed by HRP labelled sheep anti-rabbit IgG antibodies (0.1 mg/ml) for 20 min. In the PAP technique, sections were first reacted with rabbit anti-AFP antibodies (0.005–0.01 mg/ml), then with sheep anti-rabbit IgG (0.1 mg/ml) followed by PAP (diluted 1:50). Unreacted antibodies were washed off by three successive washings for 5 min each in PBS supplemented with 1% bovine serum albumin and 0.5 M NaCl. Peroxidase activity was revealed by incubation in 3,3′-diaminobenzidine and H_2O_2 (Graham and Karnovsky 1966). After washing in PBS, sections were treated with

Table 1. Experimental models

Experiments	Treatment	Period of study
Partial hepatectomy[a] 20 week-old mice 8 week-old rats	70% resection	1–7 days after operation
CCl_4 poisoning[b] 20 week-old mice 8 week-old rats	100 µl CCl_4/100 g body weight	1–7 days after oral ingestion
NNM poisoning[c] 8, 12 week-old rats	20 mg NNM/kg/day for 4 weeks	7–60 days from NNM start
Hepatocarcinogenesis 8, 12 week-old rats	6 mg NNM/kg/day for 12 weeks 20 mg/kg/day for 6 weeks	induction phase, hepatoma stage

[a] See Brues et al. (1936) for mice, Higgins and Anderson (1931) for rats
[b] Mode of CCl_4 application see Kuhlmann (1979b)
[c] Description of N-nitrosomorpholine (NNM) see Druckrey et al. (1967)

0.1% OsO_4/PBS for 1 min, dehydrated and mounted under coverglass. AFP reacted sections were post-stained with haematoxylin or with Gomori's silver impregnation. In the latter case, sections were first photographed because the AFP specific stain was lost during the impregnation.

Immunohistological specificity was demonstrated on serial sections by incubation in (1) normal rabbit IgG globulin; (2) rabbit anti-AFP absorbed with homologous AFP; (3) rabbit anti-glucose oxidase antibodies and (4) rabbit anti-mouse and anti-rat IgG antibodies. Each procedure was followed by HRP conjugates or by the PAP schedule and the enzyme substrate.

For routine histology, sections were stained with haematoxylin-eosin. Gomori's silver impregnation and toluidine blue staining were also performed. Glycogen accumulation was verified by PAS reaction. Endogenous peroxidase activities were detected by incubation in the medium of Graham and Karnovsky (1966).

Immuno-Electron Microscopy. Livers were sliced into cubes of about 2–3 mm and fixed in cacodylate buffered 6% formaldehyde for 5 h followed by 6% formaldehyde plus 0.25% glutaraldehyde for 60 min at 0° C (Kuhlmann 1978b). Tissue blocks were washed in cacodylate buffer at 0° C for 18 h with several changes of the buffer solution. The buffer was 0.2 molar sodium cacodylate at pH 7.2.

Cellular AFP was stained by pre-embedding immunoelectron microscopy, (cf. Kuhlmann 1977). Briefly, 40 µm thick cryostat sections were incubated at laboratory temperature in the various reagents: (1) HRP conjugates (0.5 mg/ml) for 2 h followed by successive washings; (2) enzyme substrate (Graham and Karnovsky, 1966) for 20 min; (3) postfixation in cacodylate buffered 1% OsO_4 for 30 min after thorough washings.

Specificity was examined as described above. Tissue sections were also treated with 1 or 2% H_2O_2 for 1–2 h prior to incubation in presence of antibodies, in order to abolish endogenous peroxidase activities (Kuhlmann 1975; Kuhlmann 1978b).

Sections were dehydrated in ethanol series and flat embedded in epoxy resin. Ultrathin sections were examined either unstained or after staining with lead citrate (Reynolds 1963) for 30–60 s in an electron microscope.

Results

All observations are summarized in Table 2. A normal adult life span is taken as the age of untreated control animals and corresponds to that of the experimental groups. In C3H/He mice and BD X rats serum AFP concentrations decreased

Table 2. Summary of results

Experiments	AFP in sera[a] (μg/ml)	Cellular localization of AFP	Liver histology (main events)
Normal adult life			
BALB/c/J mice	0.5	–[b]	normal adult liver
C3H/He mice	0.1	–	
BD X rats	0.1	–	
Partial hepatectomy			
BALB/c/J mice	200	hepatocytes	regeneration from residual lobules
C3H/He mice	45		
BD X rats	0.9		
CCl_4 intoxication			
BALB/c/J mice	700	hepatocytes	centrolobular necrosis regeneration from residual hepatocytes
C3H/He mice	80		
BD X rats	5.5		
NNM intoxication			
BD X rats	1.5	canalic. epith. cells	massive liver necrosis prolif. of canalic. epith. cells
Hepatocarcinogenesis BD X rats			
1. induction phase			
6 mg NNM/kg/day	–	–	pre-hepatoma foci[c]
20 mg NNM/kg/day	1.8	canalic. epith. cells	NNM intoxication
2. hepatoma stage	4,000	hepatoma cells	manifestation of hepatocell. carcinoma

[a] Mean from at least 10 samples at days of peak concentrations
[b] not detected
[c] Description by Bannasch (1968) and J. Natl. Cancer Inst. 64, 179–206, 1980

within 4–6 weeks after birth to levels of 0.1 μg/ml and lower. In BALB/c/J mice, AFP decreased much more slowly and reached levels of about 0.5 μg/ml serum within 20 weeks (Table 2). Sham operation and application of liquid paraffin or tap water had no measurable effect on AFP production.

Detection of AFP in Partial Hepatectomy

In mice, AFP increase was slight at 24 h after operation, it then rose steadily and reached a maximum on day 4 (Table 2), after this the concentration declined rapidly. Increase and subsequent decrease of serum AFP was parallel in both mouse strains but concentrations were regularly 5 to 10 times higher in BALB/c/J than in C3H/He mice. In both strains, liver regeneration was similar. Few mitoses were seen 24 h after hepatectomy. A mitotic peak (6%) was seen on day 3, followed by rapid decline.

24 h after partial hepatectomy, faint AFP staining hepatocytes occurred randomly in residual lobules, with preferential localization in centrolobular zones. Through days 2 to 4, a strong AFP reaction was seen in portal and periportal areas (Fig. 1); in centrolobular areas, AFP-positive hepatocytes were

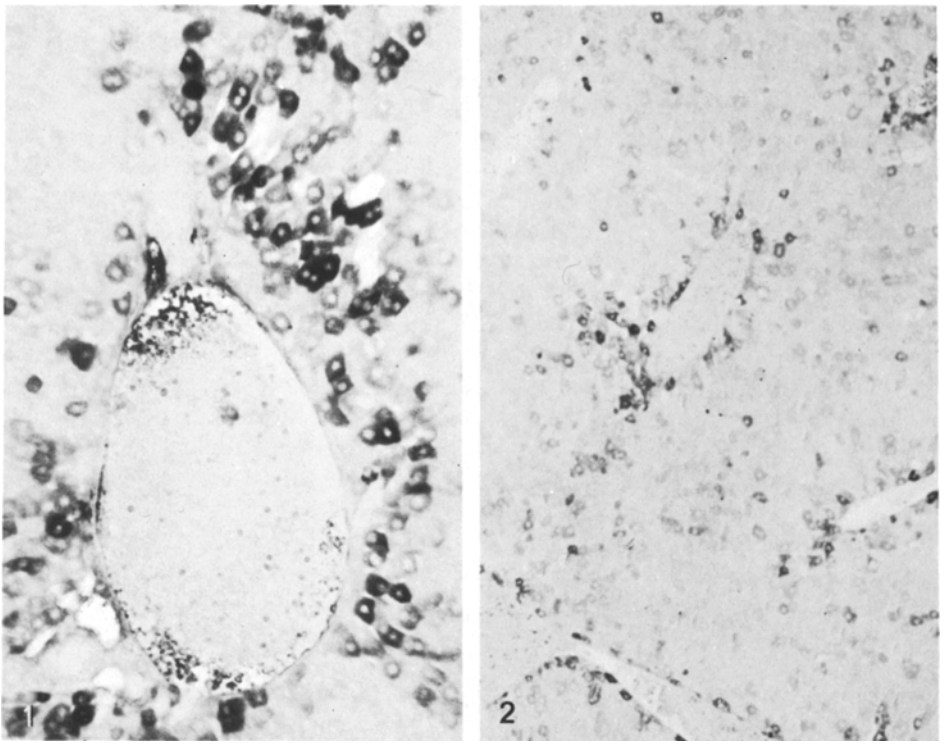


Fig. 1. BALB/c/J liver on day 3 after partial hepatectomy. Note AFP-positive hepatocytes in portal area. Original ×160

Fig. 2. C3H/He liver on day 3 after partial hepatectomy. AFP reactivity is seen in hepatocytes of centrolobular and portal zones; hepatocytes at random distribution stain also. Original ×63

still present. On days 5 and 6, the AFP staining pattern was the same, but the intensity of reactions was significantly reduced.

In C3H/He mice, the histological pattern of AFP-positive hepatocytes was basically the same (Fig. 2). The number of AFP-positive hepatocytes was lower on all days in C3H/He than in BALB/c/J mice. Serum AFP levels and immunohistological AFP corresponded well in all mice of both strains. Generally, few AFP staining hepatocytes were in mitosis.

Partial hepatectomy and subsequent liver regeneration in BD X rats led to a slight increase in serum AFP. The peak concentration was reached on day 4 (Table 2). After this AFP levels declined rapidly to normal values. No cellular AFP was localized at any time.

Detection of AFP in CCl_4 Poisoning

Liver regeneration after CCl_4 intoxication was accompanied by increase in liver AFP production. On all days and in all animals studied this AFP increase was always higher than after partial hepatectomy (Table 2).

Serum AFP was slightly increased by 24 h after CCl_4 ingestion, then AFP concentrations rose steeply with peak values on day 4 and decreased afterwards. In BALB/c/J and C3H/He mice, the slopes of AFP curves were in parallel but here AFP concentrations in sera of BALB/c/J mice also reached levels 10-fold higher or more than in C3H/He mice. Liver regeneration was similar in both strains. Mitoses were not seen before day 2, then increased mitotic activity was found with a peak on day 3 (8% on average) followed by rapid decline. The marginal zone between viable hepatocytes and central necrotic zone contained most of the dividing hepatocytes.

In BALB/c/J mice, few AFP-positive hepatocytes were seen 24 h after poisoning, in the portal and periportal zones. The number of AFP staining hepatocytes and their staining intensity increased and reached a maximum on days 3 and 4, afterwards AFP positivity decreased. AFP staining was exclusively manifested in hepatocytes of nondamaged liver areas. Throughout the days studied, variably stained hepatocytes were found, either grouped or randomly distributed. On days 3 and 4, hepatocystes adjacent to the necrotic areas often contained the strongest AFP staining (Fig. 3).

In C3H/He mice, cellular AFP was localized from day 2. At all times the histological distribution of positive hepatocytes was the same as seen in BALB/c/J mice, but the number of AFP-positive hepatocytes and its staining intensity were lower than in these mice. Serum AFP levels and the intensity of AFP staining corresponded well in all mice of both strains. Several AFP-positive hepatocytes were in mitosis.

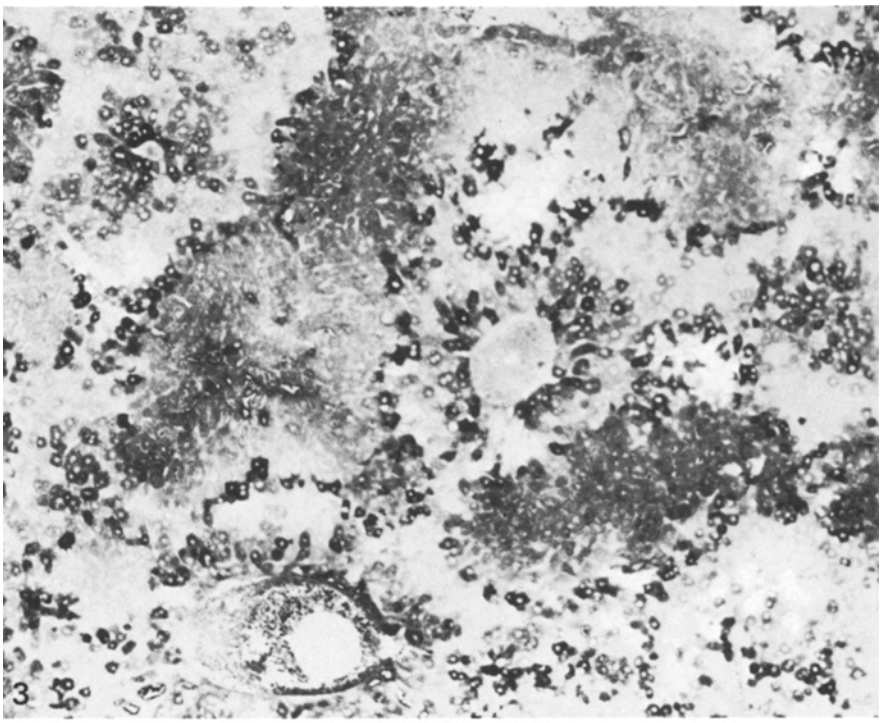
In BD X rats, liver regeneration led to a slight increase in serum AFP with a peak value on day 4. On all days studied, serum AFP levels were higher than those in liver regeneration after partial hepatectomy. AFP concentrations were always much lower in rats than in either mouse strains. Rat liver regeneration was similar to that in mouse liver; the mitotic peak appeared on day 2. Cellular AFP was only detected on days 3 and 4 and positive hepatocytes occurred either as single stained cells or in small groups in midlobular zones near to the necrosis and in portal areas (Fig. 4).

AFP in NNM Poisoning

NNM intoxication led to an increase in serum AFP. AFP rose slightly on day 21 then rose further. Peak values were attained between days 28 and 35 (Table 2), after which they declined.

No age-related differences were found in the histotoxic patterns of 8 or 12 week-old animals. During extensive hepatocyte necrosis, which occasionally reached periportal zones, we observed proliferation of lobular and periportal bile canalicular epithelium (Figs. 5, 6). Such proliferations came to a standstill upon cessation of NNM exposure.

Between day 21 and 35, AFP was detected throughout or in the apical cytoplasm of proliferating canalicular epithelial cells (Figs. 5, 6). AFP stained cells formed groups and tubular structures, or were distributed randomly and intermingled with non-stained cells of the same phenotype. When proliferation of these cells ceased, AFP positivity disappeared rapidly.



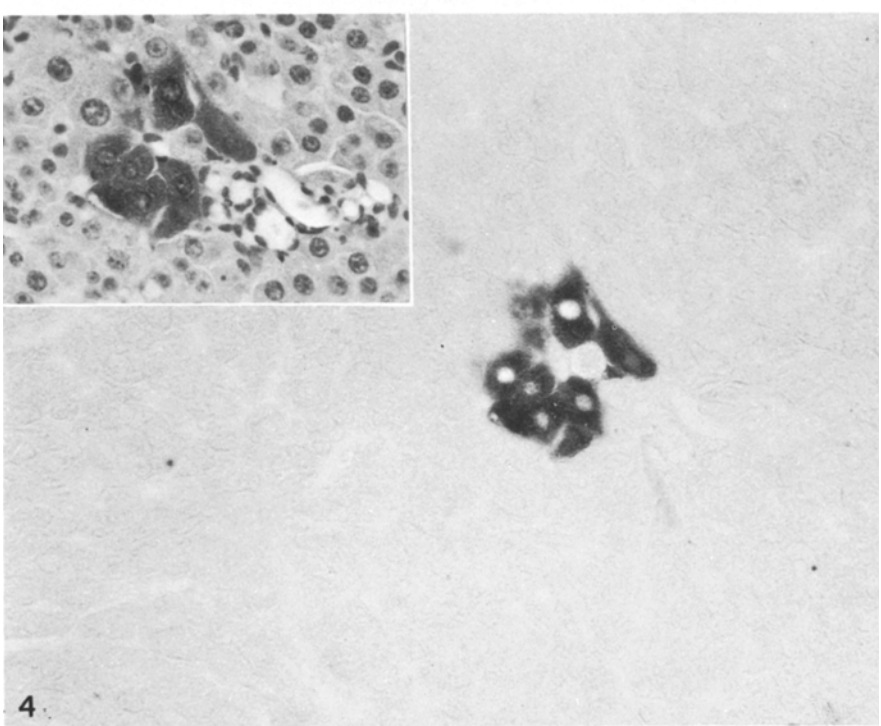


Fig. 3. Localization of AFP in hepatocytes of regenerating BALB/c/J liver; 4 days after CCl_4 treatment. Strong AFP reaction in cells of non-damaged zones and adjacent to the necrosis. Original $\times 63$

Fig. 4. Rat liver on day 3 after CCl_4 intoxication. Detection of AFP in a small group of hepatocytes in a portal zone. Original $\times 250$. *Inset:* Same preparation after counterstaining with haematoxylin. Original $\times 250$

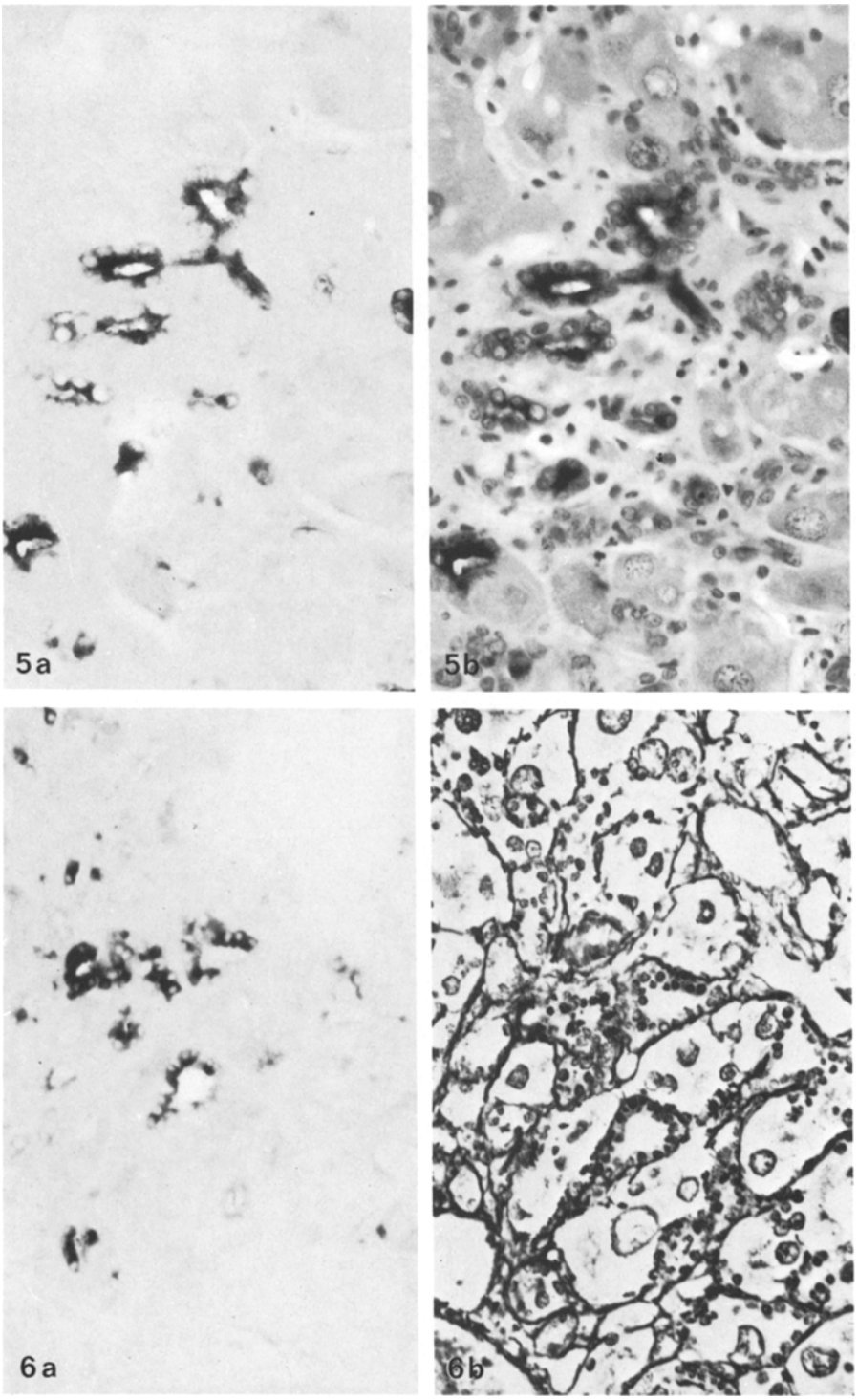


Fig. 5a, b. AFP-positive canalicular epithelial cells on day 28 of NNM intoxication. Original ×250. **a** Immunoperoxidase reaction. **b** Same preparation but counterstained with haematoxylin

Fig. 6a, b. Liver from day 35 of NNM intoxication. Note localization of AFP in grouped cells which form canalicular epithelial structures. Original ×160. **a** Immunoperoxidase reaction. **b** Same preparation after Gomori's silver impregnation

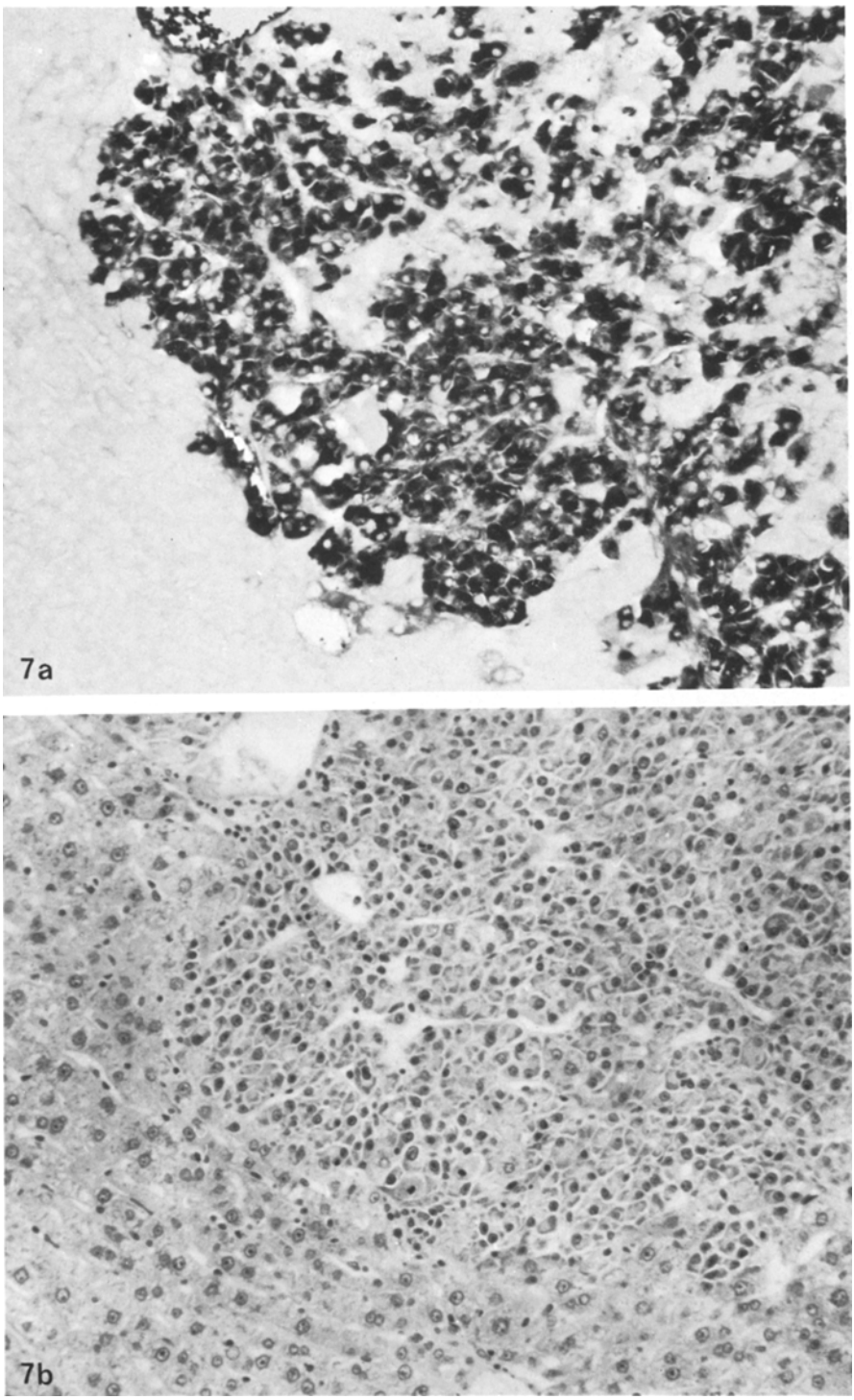


Fig. 7a, b. AFP producing hepatocellular carcinoma. Serial sections, original × 160. **a** Immunoperoxidase reaction for AFP. **b** Haematoxylin-eosin

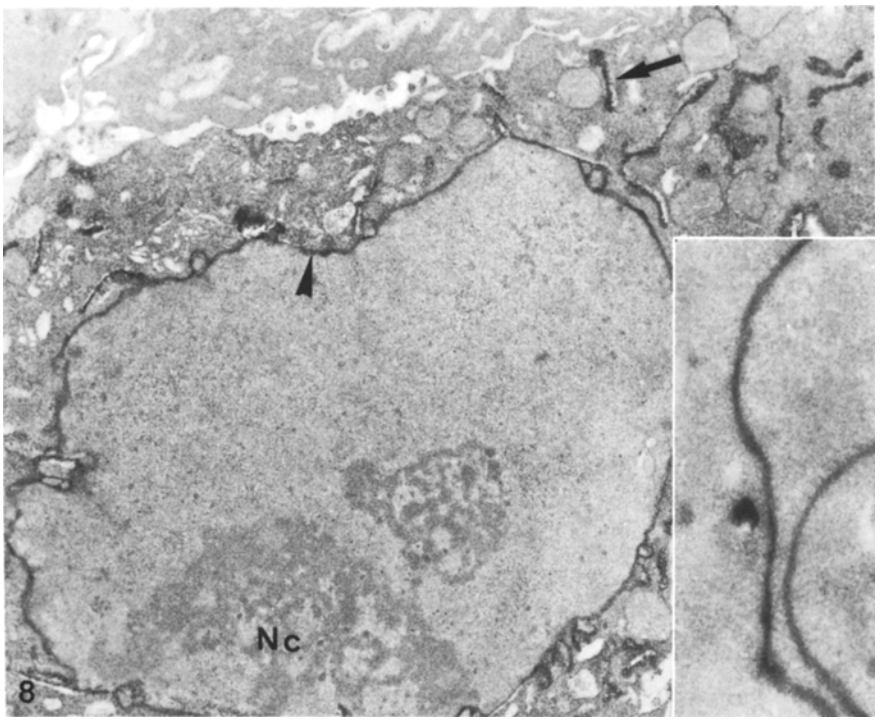


Fig. 8. Hepatoma cell with AFP in its flat RER lamellae (←) and in the PNS (⇐). Nucleolus (*Nc*); lead citrate counterstain. ×7,800. *Inset*: Higher magnification view of AFP containing RER lamellae. ×21,600

Detection of AFP in Hepatocarcinogenesis

No age-related differences were found in histological changes during the induction phase and hepatoma stage. High-dose NNM feeding led to pictures described above ("NNM poisoning") which were not seen upon low-dose NNM feeding. Irrespective of low- or high-dose NNM feeding, hepatocellular carcinomas developed in each experimental group.

In both groups of rats, a wide range of serum AFP levels was measured at the hepatoma stage. The dynamics of AFP concentrations varied greatly in the course of progressive hepatoma development: continuous increase or transient rises followed by plateau and further rise could be seen in individual rats.

At the time of the reappearance of AFP, small hepatomas were found, in which the cytoplasm of malignant hepatocytes stained for AFP (Fig. 7). AFP staining hepatocellular carcinomas were poorly differentiated, showed marked basophilia and no PAS reactivity. Penetration of hepatomas into normal tissue was obvious, very often hepatoma cells adjacent to normal hepatocytes contained strongest AFP reactions. At the border of carcinomas, non basophilic and non AFP staining liver adenomas without signs of malignancy were frequent-

ly observed. The higher the amount of AFP in sera the greater the number of AFP staining cells. Normal adult hepatocytes, bile duct and Kupffer cells did not stain for AFP. At early time intervals, small AFP staining and non AFP staining carcinoma could be distinguished in the same histological preparation. In both AFP staining and non AFP staining hepatocellular carcinomas we observed strong cytoplasmic basophilia, enhanced mitotic and nucleolar activities, decreased endogenous peroxidase activity and loss of PAS reactivity.

In late stages, huge tumors were found, composed of a variety of cell types and showing extensive necrosis. In the adjacent but non malignant parts, liver cells occasionally stained for AFP. These cells, however, also stained for serum IgG (controls in parallel sections) and were suspected to have imbibed proteins non-specifically from extracellular spaces (see "Immunohistological Technique", Discussion).

At the electron microscopic level, AFP staining hepatocellular carcinomas consisted of malignant cells with pleomorphic appearance, often with interdigitating cell borders. The cytoplasm was irregular in shape and possessed abundant free ribosomes in the cytoplasm which did not stain for AFP. The latter was exclusively localized in the rough-surfaced endoplasmic reticulum (RER) which consisted of short flat lamellae (Fig. 8). The perinuclear space (PNS) and the Golgi apparatus also stained for AFP. The nucleus, also irregular in shape, contained one or more well-developed nucleoli.

Discussion

Immunohistological Technique

Quantitative and qualitative data on preparation of immunohistological reagents and tissues have already been described (Kuhlmann 1975; Kuhlmann 1977; Kuhlmann 1978a, 1978b). The optimal amount of antibodies and conjugates in the various incubation steps was determined empirically. In this study we also compared antigen localization by the PAP and by the indirect peroxidase labelled antibody techniques. Immuno-staining by PAP was never superior to that by the HRP labelled antibody procedure. Immunohistological reactions are ambiguous due to the threshold of detection, thus, the non-reactivity of normal adult hepatocytes is understandable: the quantity of AFP produced by a single cell may be too low to be detected by the methods employed. Neither the indirect peroxidase labelled antibody method nor the PAP technique could resolve this problem.

Adequate control incubations were important in order to exclude false positive reactions, e.g. due to passive uptake of excreted AFP from extracellular spaces. In normal and regenerating mouse livers, small areas of focal necrosis can occur (Yokoyama et al. 1953), and artefactual leakage of proteins into hepatocytes can be expected (Le Bouton 1978). Thus, artefactual staining due to passive uptake of serum was checked in this study on serial sections by incubation for IgG globulins (Engelhardt et al. 1971), a globulin which is not synthesized by hepatocytes (Miller and Bale 1954). Tissue sites in which both AFP and IgG positivities occurred were not taken into account. Furthermore,

interaction of tissue with non-antibody globulins, with unrelated antibodies and with HRP conjugates was controlled as described above, but no staining was found under experimental conditions.

AFP in Hepatectomy and CCl_4 Poisoning of Mice

In mice, an AFP regulating principle (*Raf*, regulation of alpha-fetoprotein) was considered to be a single recessive Mendelian gene which modulates AFP levels in adult individuals (Olsson et al. 1977). Because similar regulatory systems can be expected to act in other species, AFP synthesis in response to liver injury and subsequent regeneration was studied in a high AFP producing (BALB/c/J) and a low AFP producing (C3H/He) mouse strain. For histological details see Stowell and Lee (1950), Tsuboi and Stowell (1951), Yokoyama et al. (1953), Heby and Lewan (1971), Schultze et al. (1973), Kuhlmann (1979b) and for previously described serological data on AFP see Bakirov (1968), Pihko and Ruoslahti (1974).

In both types of experiments, typical hepatocytes of adult phenotype were involved in AFP production. Because AFP was synthesized in small quantities before mitoses reached their maximum, a direct connection between AFP and DNA synthesis was not evident. Engelhardt et al. (1976) and Mohanty et al. (1978) suggested that initiation of cell division is not a prerequisite for AFP production in hepatocytes, and the latter may already produce AFP before they enter the S phase. Schultze et al. (1973) reported that DNA replication has not started on day 1 after CCl_4 intoxication, thus the rapid increase in serum AFP on day 1 will exclude a direct relationship. On the other hand, the peak of regeneration in CCl_4 and in hepatectomized mice preceded the peak of AFP synthesis by about 24 h. Hence, liver regeneration and enhanced AFP production showed some correlation.

The present immunohistological and serological results favor the view that the great majority of enhanced AFP was correlated with growth and cell multiplication of hepatocytes (Sell et al. 1976). The immunohistological staining patterns could be considered to be the result of gradual differentiation from asynchronous proliferation (Guillouzo et al. 1978). For example, in partial hepatectomy, DNA synthesis followed a gradient from portal to centrolobular zones (Bade et al. 1966). It is known that restoration of mouse liver proceeds at a variable rate in different individuals (even in mice selected as to sex, age and strain) and a wide range of variations must be expected (Yokoyama et al. 1953). This would explain the fluctuations of immunohistological AFP staining pattern which occurred with time in hepatocytes of periportal and centrolobular areas. The observation that the majority of AFP-positive hepatocytes was seen in interphase suggested AFP production before cells enter the S phase or after the S phase, with or without mitosis.

The main difference between the two mouse strains was the consistently higher serum AFP levels and the stronger immuno-staining in the BALB/c/J. In partial hepatectomy, subsequent regeneration occurred to the same extent in both strains. Hence, the much higher AFP levels were not the effect of liver regeneration alone. The *Raf* regulation principle seen in normal liver ob-

viously played an important role in AFP synthesis (Lindahl et al. 1978; Kuhlmann 1979b) and, BALB/c/J mice were a "high AFP-inducible" and C3H/He mice a "low AFP-inducible" mouse strain.

A further interesting observation was that serum AFP levels and immunohistological AFP staining was always higher after CCl_4 intoxication than after partial hepatectomy, here also the highest values were obtained in the BALB/c/J strain when compared with C3H/He mice. Hence, the relationship of AFP synthesis to liver regeneration and *Raf* regulation was submitted to further modulation, and in the CCl_4 model a direct or indirect gearing of gene activation was suspected, in view of the products of the CCl_4 metabolism (see next section). Finally, BALB/c/J mice also proved to be a "high AFP-inducible" and C3H/He mice to be a "low AFP-inducible" mouse strain in the CCl_4 experiment.

AFP in Hepatectomy and CCl_4 Poisoning of Rats

As in mice, rats responded with enhanced AFP production (for histological details and appearance of AFP in sera see Post et al. 1960; Grisham 1962; Rabes and Tuczek 1970; Perova et al. 1971; Sell et al. 1974; Sell et al. 1976; Smuckler et al. 1976; Watanabe et al. 1976).

Immunohistological AFP stainings have shown that in both species and under both experimental conditions increased AFP synthesis occurred in adult-type hepatocytes and was caused by AFP gene derepression. Most probably, the *Raf* principle worked very efficiently in rats under both normal and pathological conditions. This conclusion is also supported by observations in which the induction of AFP is significantly impaired with age in rats (Sell et al. 1974). In comparison with mice, serum AFP levels were higher after CCl_4 intoxication than after partial hepatectomy and the response of AFP synthesis to liver regeneration was also further enhanced: gearing of gene activation by CCl_4 metabolic products at the level of transcription and de novo synthesis of mRNA. Possible mechanisms for this were discussed by Watanabe et al. (1976) from their mitomycin and 8-azaguanine studies on rat after CCl_4 poisoning.

AFP in NNM Poisoning

One of the most prominent changes in liver was megalocytic degeneration of hepatocytes in the centrolobular and intermediate zones of the livers. Concomitantly, proliferation of canalicular epithelial cells was seen, which correlated well with AFP increase in sera. Such proliferations might have corresponded to a true increase in canaliculi or to prolongation of canaliculi as a phenomenon of regeneration, followed by marked bending and twisting for spatial reasons. This would have the effect that more cross-sections of the same canaliculi were seen under the microscope. Histological, immunohistological and serological data permitted the conclusion that proliferating canalicular epithelial cells synthesized AFP. When NNM feeding was stopped, proliferative activity came to a standstill and AFP was no longer detected.

The histogenesis of bile duct proliferation is still unclear. The main reason is most probably toxic necrosis of parenchyma (Popper et al. 1957; Bannasch

1975). In the present model, massive and prolonged hepatocyte necrosis appeared to be the primary event in such proliferations, but it is also thought that high doses of NNM or products of its metabolism may play an important role by action on the bile duct system. In contrast to acute hepatocyte necrosis due to CCl_4 this effect would be superimposed on parenchymal necrosis. Interestingly, similar canalicular epithelial cell proliferations and AFP staining occurred in galactosamine-HCl injured rat liver (Kuhlmann and Wurster, 1980). Following feeding of ethionine in a choline deficient diet on which no significant liver necrosis occurred, proliferation of AFP-positive "oval cells" (immunofluorescent studies) was observed. The latter were considered to have originated from bile duct cells (Shinozuka et al. 1978).

Our immunohistological results have shown that not every newly formed canalicular epithelial cell produced AFP at any one time, apparently a minimal proliferation rate was necessary. It is suggested that a certain, and still undefined, level of cytodifferentiation had to be reached in which fetal genes became activated for subsequent AFP synthesis. The reason for this is not understood but may be related to the origin of hepatocytes and bile duct epithelium from common progenitor cells in liver anlage and embryonic endoderm.

AFP in the Hepatoma Induction Phase

For histological and other considerations see Price et al. (1952), Inaoka (1967), Bannasch (1968), Onoé et al. (1973), Bannasch (1975), Institute of Laboratory Animal Resources (1980). Metabolic data are considered in Kuhlmann (1978a).

When NNM was applied in high doses, the same histological changes were observed as described above ("NNM poisoning"). In our material, a progression of AFP-positive canalicular epithelial cells into normal and AFP staining hepatocytes was not seen. Typical hepatocytes failed to stain for AFP in the course of hepatoma induction. By immunofluorescent studies, so-called "transitional cells" and "small hepatocytes" were responsible for AFP synthesis (Dempo et al. 1975; Tchipysheva et al. 1977). However, the term transitional or small hepatocyte is not comprehensible with regard to our immunohistochemical description of AFP staining hepatocytes in toxic and nontoxic liver regenerations.

When high-dose NNM feeding was stopped, proliferation of canalicular epithelial cells rapidly came to a standstill. AFP levels then decreased in parallel and cellular AFP was no longer localized unless hepatocellular carcinomas emerged. In contrast to other reports (Okita et al. 1974) we did not observe AFP staining in hyperplastic nodules.

AFP at the Hepatoma Stage

There was no causal relationship between hepatoma development and proliferation of AFP staining canalicular epithelial cells in the tumor induction phase. Malignant transformation to hepatocellular carcinomas was preceded by AFP-negative foci of decreased glycogen, enhanced cytoplasmic basophilia and increased mitosis (for details see Bannasch and Müller 1964; Bannasch 1975).

Finally, AFP staining and non AFP staining hepatocellular carcinomas emerged. The importance of the frequently seen liver cell adenomas (benign, AFP-negative, at the border of carcinomas) in the process of hepatoma development could not be clarified.

Common characteristics of both AFP-positive and AFP-negative hepatomas were loss of PAS reactivity, decreased endogenous peroxidase activity (impaired haeme metabolism of unknown significance) and increase in cytoplasmic basophilia. AFP staining hepatomas showed strong basophilia in particular. The morphological expression of the degree of cellular differentiation can be seen in the organization of ribosomes and RER (Porter 1961). In this context, the electron microscopic picture of AFP staining hepatocellular carcinomas was interesting, there was an abundance of free ribosomes and few lamellae of RER in the cytoplasm. Thus, AFP staining hepatomas were classified as poorly differentiated. However, studies are still in progress to determine whether there is a correlation between the grade of differentiation and AFP synthesis. The significance of the strong AFP reactions in cancer cells adjacent to normal liver tissue is not yet known.

The occurence of PAS-positive globules in the cytoplasm of hepatoma cells was recently related to the deposition of α_1-antitrypsin in human primary liver carcinoma (Palmer and Wolfe 1976). In rare cases, we also observed PAS-positive globules in the cytoplasm of hepatoma cells. However, for AFP there was no clear-cut relationship between its production/storage and such globules. When PAS-positive globules stained for AFP, they also stained for serum IgG, but became negative in diastase digested sections.

The heterogenous character of AFP producing hepatomas was deduced from measurements of transient rises and plateaus in serum AFP levels and from immunohistological staining. Using the latter technique, more or less stained carcinomas were found in a given animal; apart from the finding of AFP-positive and negative carcinomas side by side in one histological preparation. Thus, AFP might or might not reappear at the hepatoma stage and was not a stable biological marker, its resurgence merely reflected hepatoma-association rather than hepatoma-specificity.

Our studies support the finding that not every hepatoma cell produces AFP (Goussev et al. 1971; Nishioka et al. 1972). From our immunohistological studies we deduced that at the final stage of cell transformation towards malignancy, only some of the neoplastic hepatocytes aquire the capacity for AFP synthesis. De-repression of fetal genes was considered to be the direct consequence of retrodifferentiation during transformation (Uriel 1976; Uriel 1979). Various degrees of retrodifferentiation would therefore generate distinctive malignant hepatocytes and their growth would lead to AFP-positive or AFP-negative hepatomas. Such developments are suggested by the emergence of small and circumscribed AFP-positive and AFP-negative carcinomas in the early stages of hepatoma. Whether and to what extent a *Raf* regulatory principle is involved in all these processes is not known.

Acknowledgement. This study was supported by the Deutsche Forschungsgemeinschaft (Ku 257/3 and SFB 136) Bonn, Germany. I thank Mrs. M. Kuhlmann for excellent assistance.

References

Abelev GI (1971) Alpha-fetoprotein in ontogenesis and its association with malignant tumors. Adv Cancer Res 14:295–358

Abelev GI, Perova SD, Khramkova NI, Postnikova ZA, Irlin IS (1963) Production of embryonal α-globulin by transplantable mouse hepatomas. Transplantation 1:174–180

Bade EG, Sadnik IL, Pilgrim C, Maurer W (1966) Autoradiographic study of DNA-synthesis in the regenerating liver of the mouse. Exp Cell Res 44:676–678

Bakirov RD (1968) Appearance of embryonal serum α-globulin in adult mice after inhalation of carbon tetrachloride. Byull Eksp Biol Med 2:45–47

Bannasch P (1968) The cytoplasm of hepatocytes during carcinogenesis. In: Recent results in cancer research, vol 19. Springer, Berlin Heidelberg New York, pp 1–105

Bannasch P (1975) Die Cytologie der Hepatocarcinogenese. In: Altmann HW, Büchner F, Cottier H, Grundmann E, Holle G, Letterer E, Masshoff W, Meesen H, Roulet F, Seifert G, Siebert G (eds) Handbuch der allgemeinen Pathologie, vol VI/7. Springer, Berlin Heidelberg New York, pp 123–276

Bannasch P, Müller HA (1964) Lichtmikroskopische Untersuchungen über die Wirkung von N-Nitrosomorpholin auf die Leber von Ratte und Maus. Arzneim Forsch (Drug Res) 14:805–814

Bergstrand CG, Czar B (1956) Demonstration of a new protein fraction in serum from the human fetus. Scand J Clin Lab Invest 8:174

Brues AM, Drury DR, Brues MC (1936) A quantitative study of cell growth in regenerating liver. Arch Pathol 22:658–673

Delpré G, Gilat T (1978) Revue générale. L'Alpha-foeto-protéine. Deuxième partie. Gastroenterol Clin Biol 2:193–214

Dempo K, Chisaka N, Yoshida Y, Kaneko A, Onoé T (1975) Immunofluorescent study on α-fetoprotein-producing cells in the early stage of 3′-methyl-4-dimethylaminoazobenzene carcinogenesis. Cancer Res 35:1282–1287

Druckrey H, Preussmann R, Ivankovic S, Schmähl D (1967) Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitroso-Verbindungen an BD-Ratten. Z Krebsforsch 69:103–201

Engelhardt NV, Goussev AI, Shipova LJ, Abelev GI (1971) Immunofluorescent study of alpha-foetoprotein (αfp) in liver and liver tumors. I. Technique of αfp localization in tissue sections. Int J Cancer 7:198–206

Engelhardt NV, Lazareva MN, Abelev GI, Uryvaeva, IV, Factor VM, Brodsky V Ya (1976) Detection of α-foetoprotein in mouse liver differentiated hepatocytes before their progression through S phase. Nature (London New Biol) 263:146–148

Gitlin D, Boesman M (1967a) Fetus-specific serum proteins in several mammals and their relation to human α-fetoprotein. Comp Physiol 21:327–336

Gitlin D, Boesman M (1967b) Sites of serum α-fetoprotein synthesis in the human and in the rat. J Clin Invest 46:1010–1016

Gitlin D, Kitzes J, Boesman M (1967) Cellular distribution of serum α-fetoprotein in organs of the foetal rat. Nature (London) 215:534

Goussev AI, Engelhardt NV, Masseyeff R, Camain R, Basteris B (1971) Immunofluorescent study of alpha-foetoprotein (αfp) in liver and liver tumors. II. Localization of αfp in the tissues of patients with primary liver cancer (PLC). Int J Cancer 7:207–217

Graham RC, Karnovsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302

Grisham JW (1962) A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine-H^3. Cancer Res 22:842–849

Guillouzo A, Belanger L, Beaumont C, Briggs R, Chiu JF, Fleischer B, Fleischer S, Valet JP (1978) Light and electron microscope immunolocalization of alpha-fetoprotein in rat liver cells in vivo and in vitro. Scand J Immunol 8: suppl 8:289–296

Heby O, Lewan L (1971) Putrescine and polyamines in relation to nucleic acids in mouse liver after partial hepatectomy. Virchows Archiv (Cell Pathol) 8:58–66

Higgins GM, Anderson RM (1931) Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. Arch Pathol 12:186–202

Inaoka Y (1967) Significance of the so-called oval cell proliferation during azo-dye hepatocarcinogenesis. Gann 58:355–366

Institute of Laboratory Animal Resources (1980) Histologic typing of liver tumors of the rat. J Natl Cancer Inst 64:179–206

Kuhlmann WD (1975) Purification of mouse alpha$_1$-fetoprotein and preparation of specific peroxidase conjugates for its cellular localization. Histochemistry 44:155–167

Kuhlmann WD (1976) Immunocytological studies on alpha$_1$-fetoprotein producing cells under normal and pathological conditions. In: Peeters H (ed) Protides of the biological fluids, vol 24. Pergamon Press, Oxford, pp 269–276

Kuhlmann WD (1977) Ultrastructural immunoperoxidase cytochemistry. Progr Histochem Cytochem 10:1–57

Kuhlmann WD (1978a) Localization of alpha$_1$-fetoprotein and DNA-synthesis in liver cell populations during experimental hepatocarcinogenesis in rats. Int J Cancer 21:368–380

Kuhlmann WD (1978b) Ultrastructural detection of alpha$_1$-fetoprotein in hepatomas by use of peroxidase-labelled antibodies. Int J Cancer 22:335–343

Kuhlmann WD (1979a) Immuno-electron microscopy of α_1-fetoprotein during normal development of rat hepatocytes. J Ultrastruct Res 68:109–117

Kuhlmann WD (1979b) Immunoperoxidase labelling of alpha$_1$-fetoprotein (AFP) in normal and regenerating livers of a low and a high AFP producing mouse strain. Histochemistry 64:67–75

Kuhlmann WD, Avrameas S, Ternynck T (1974) A comparative study for ultrastructural localization of intracellular immunoglobulins using peroxidase conjugates. J Immunol Methods 5:33–48

Kuhlmann WD, Wurster K (1980) Correlation of histology and alpha$_1$-fetoprotein resurgence in rat liver regeneration after experimental injury by galactosamine. Virchows Arch (Pathol Anat) 387:47–57

Lannér M, Bergquist R, Carlsson J, Huldt G (1978) Purification of enzyme-labelled conjugate by affinity chromatography. In: Hoffmann-Ostenhof O, Breitenbach M, Koller F, Kraft D, Scheiner O (eds) Affinity chromatography. Pergamon Press, Oxford, pp 237–241

Laurence DJR, Neville AM (1972) Foetal antigens and their role in the diagnosis and clinical management of human neoplasms: a review. Brit J Cancer 26:335–355

Le Bouton AV (1978) Extrusion of nascent albumin into the cytosol: an artifact. Anat Rec 190:457

Lindahl G, Olsson M, Ruoslahti E (1978) Mouse alpha-fetoprotein: genetic studies. Scand J Immunol 8: suppl. 8:209–212

Miller LL, Bale WE (1954) Synthesis of all plasma protein fractions except gamma globulins by the liver. The use of zone electrophoresis and lysine-ε-C^{14} to define the plasma proteins synthesized by the isolated perfused liver. J Exp Med 99:125–132

Mohanty M, Das PK, Mittal A, Nayak NC (1978) Cellular basis of induced α-fetoprotein synthesis by hepatocytes of adult mouse after hepatotoxic injury and partial hepatectomy. Int J Cancer 22:181–188

Nishioka M, Ibata T, Okita K, Harada T, Fujita T (1972) Localization of α-fetoprotein in hepatoma tissues by immunofluorescence. Cancer Res 32:162–166

Okita K, Gruenstein M, Klaiber M, Farber E (1974) Localization of α-fetoprotein by immunofluorescence in hyperplastic nodules during hepatocarcinogenesis induced by 2-acetylaminofluorene. Cancer Res 34:2758–2763

Olsson M, Lindahl G, Ruoslahti E (1977) Genetic control of alpha-fetoprotein synthesis in the mouse. J Exp Med 145:819–827

Onoé T, Dempo K, Kaneko A, Watabe H (1973) Significance of α-fetoprotein appearance in the early stage of azo-dye carcinogenesis. Gann Monogr Cancer Res 14:233–247

Palmer PE, Wolfe HJ (1976) α_1-Antitrypsin deposition in primary hepatic carcinomas. Arch Path Lab Med 100:232–236

Perova SD, El'gort DA, Abelev GI (1971) α-Fetoprotein in rat sera after partial hepatectomy. Byull Eksp Biol Med 71:45–47

Pihko H, Ruoslahti E (1974) Alpha fetoprotein production in normal and regenerating mouse liver. In: Masseyeff R (ed) L'alpha-foetoprotéine. INSERM, Paris, pp 333–336

Popper H, Kent G, Stein R (1957) Ductular cell reaction in the liver in hepatic injury. J Mt Sinai Hosp 24:551–556

Porter KR (1961) The ground substance; observations from electron microscopy. In: Brachet J, Mirsky AE (eds) The cell. Academic Press, New York London, pp 621–675

Post J, Klein A, Hoffman J (1960) Responses of the liver to injury. Effects of age upon the healing pattern after acute carbon tetrachloride poisoning. Arch Pathol 70:314–321

Price JM, Harman JW, Miller EC, Miller JA (1952) Progressive microscopic alterations in the livers of rats fed the hepatic carcinogens, 3′-methyl-4-dimethylaminoazobenzene and 4′-fluoro-4-dimethylaminoazobenzene. Cancer Res 12:192–200

Rabes H, Tuczek HV (1970) Quantitative autoradiographische Untersuchung zur Heterogenität der Leberzellproliferation nach partieller Hepatektomie. Virchows Arch (Cell Pathol) 6:302–312

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

Ruoslahti E, Seppälä M (1979) α-Fetoprotein in cancer and fetal development. Adv Cancer Res 29:275–346

Schultze B, Gerhard H, Schump E, Maurer W (1973) Autoradiographische Untersuchung über die Proliferation der Hepatocyten bei der Regeneration der CCl_4-Leber der Maus. Virchows Arch (Cell Pathol) 14:329–343

Sell S, Becker FF, Leffert HL, Watabe H (1976) Expression of an oncodevelopmental gene product (α-fetoprotein) during fetal development and adult oncogenesis. Cancer Res 36:4239–4249

Sell S, Nichols M, Becker FF, Leffert HL (1974) Hepatocyte proliferation and α_1-fetoprotein in pregnant, neonatal, and partially hepatectomized rats. Cancer Res 34:865–871

Shinozuka H, Lombardi B, Sell S, Iammarino RM (1978) Early histological and functional alterations of ethionine liver carcinogenesis in rats fed a choline-deficient diet. Cancer Res 38:1092–1098

Smuckler EA, Koplitz M, Sell S (1976) α-Fetoprotein in toxic liver injury. Cancer Res 36:4558–4561

Sternberger LA (1974) Immunocytochemistry. Prentice-Hall, Englewood Cliffs

Stowell RE, Lee CS (1950) Histochemical studies of mouse liver after single feeding of carbon tetrachloride. Arch Pathol 50:519–537

Taketa K, Watanabe A, Kosaka K (1975) Different mechanisms of increased α-fetoprotein production in rats following CCl_4 intoxication and partially hepatectomy. Ann NY Acad Sci 259:80–84

Tchipysheva TA, Guelstein VI, Bannikov GA (1977) α-Fetoprotein containing cells in the early stages of liver carcinogenesis induced by 3′-methyl-4-dimethylaminoazobenzene and 2-acetylaminofluorene. Int J Cancer 20:388–393

Teilum G, Albrechtsen R, Nørgaard-Pedersen B (1975) The histogenetic-embryologic basis for reappearance of alpha-fetoprotein in endodermal sinus tumors (yolk sac tumors) and teratomas. Acta Pathol Microbiol Scand (A) 83:80–86

Tsuboi KK, Stowell RE (1951) Enzyme alterations associated with mouse liver degeneration and regeneration after single carbon tetrachloride feeding. Cancer Res 11:221–228

Uriel J (1976) Cancer, retrodifferentiation, and the myth of Faust. Cancer Res 36:4269–4275

Uriel J (1979) Retrodifferentiation and the fetal pattern of gene expression in cancer. Adv Cancer Res 29:127–174

Watanabe A, Miyazaki M, Taketa K (1976) Differential mechanisms of increased α_1-fetoprotein production in rats following carbon tetrachloride injury and partial hepatectomy. Cancer Res 36:2171–2175

Yokoyama HO, Wilson ME, Tsuboi KK, Stowell RE (1953) Regeneration of mouse liver after partial hepatectomy. Cancer Res 13:80–85

Accepted May 6, 1981