LOCALIZATION OF ALPHA$_1$-FETOPROTEIN AND DNA-SYNTHESIS IN LIVER CELL POPULATIONS DURING EXPERIMENTAL HEPATOCARCINOGENESIS IN RATS

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Summary

Six-, 12- and 20-week-old rats were fed N-nitrosomorpholine (NNM) at low concentrations (6 mg/kg/day) or high concentrations (20 mg/kg/day) for 6 or 12 weeks. Irrespective of the age of the rats, both NNM schedules resulted in development of hepatomas and during the early stages of hepatoma induction, liver histotoxic patterns depended only on the dose of carcinogen employed. Necrosis of hepatocytes and proliferation of small, oval-shaped cells occurred when high doses of NNM were applied. Parallel to the proliferation of oval-shaped cells, resurgence of alpha$_1$-fetoprotein (AFP) in rat sera was observed and production of this protein was confined to the oval-shaped cells as shown by immunoperoxidase staining. During proliferation of bile duct epithelium, induced by galactosamine injections, those cells could also stain for AFP, and proliferation of oval-shaped cells concomitant with intracellular AFP staining resulted from restitution of heavily damaged liver. In pulse-chase labelling experiments with [^{3}H]thymidine, oval-shaped cells were seen in continuous development towards hepatocytes, and distinct areas of hyperplastic appearance occurred. Normal hepatocytes and hyperplastic areas did not stain for AFP. Upon low-dose feeding of NNM, no cellular AFP and no serum AFP were detected unless hepatoma cells had developed. At the stage of malignant conversion, distinct AFP-staining nodules were localized which consisted of neoplastic hepatocytes. AFP-staining and non-AFP-staining nodules were seen concomitantly in the same animal. AFP was localized only in neoplastic hepatocytes. Pulse-labelling experiments with [^{3}H]thymidine showed the proliferative character of hepatoma cells from which the AFP-staining population in particular was involved. No correlation was found between the presence of AFP and histological grading of hepatomas. The wide range of both serum AFP levels and their rising rates in individual rats indicated the highly heterogenous character of the induced hepatomas with respect to AFP production.

INTRODUCTION

Alpha$_1$-fetoprotein (AFP) is a normal constituent of fetal serum and amniotic fluid in many species. Under physiological conditions its synthesis occurs in the yolk sac, the gastrointestinal tract and the liver of embryos. After birth this protein disappears (Bergstrand and Czar, 1956; Gitlin and Boesman, 1967*a*, *b*; Gitlin *et al*., 1967). The resurgence of antigens characteristic of embryos in human or animal tumors is a general oncologic phenomenon, and oncofetal antigens have been reported for a number of tissues and tumor types (Abelev, 1968; Gold, 1971; Alexander, 1972). AFP is a typical protein of antigenic reversion. It appears

during the process of malignant transformation and proved to be of value in the diagnosis of hepatocellular cancer (Abelev *et al*., 1963; Abelev, 1971; Laurence and Neville, 1972).

Cytological detection of AFP allows the cells responsible for its production to be labelled, and may help to elucidate the importance of its resurgence. In spite of published work on immunofluorescence (Engelhardt *et al*., 1971; Goussev *et al*., 1971; Nishioka *et al*., 1972; Okita *et al*., 1974; Dempo *et al*., 1975) and cell affinity labelling with [^{3}H]estrogens (Uriel *et al*., 1973), a satisfying histological description of cells producing AFP in experimental hepatocarcinogenesis is still lacking. Hence, little is known of the cellular basis of AFP synthesis during malignant growth.

The intention was to employ immunoperoxidase labelling for the localization of cellular AFP. This cytochemical technique reveals histological details with greater ease and with more certainty than corresponding immunofluorescence techniques that we evaluated in preliminary experiments. Furthermore, immunoperoxidase staining had already proven valuable for the detection of various tissue components (for review see Kuhlmann, 1977*a*) including AFP in fetal liver (Kuhlmann, 1975). In the present study, hepatocellular carcinomas were induced in rats by the hepatotropic carcinogen N-nitrosomorpholine (NNM) (Druckrey *et al*., 1967). During the induction phase and at the stage of hepatoma development, tissues were processed for subsequent cellular detection of AFP. Also, double tracer techniques combining immunocytology and autoradiography were used to follow synthesis of AFP and DNA replication during growth of liver cells.

MATERIAL AND METHODS

Animals

NNM was chosen as the carcinogen because it is known to be hepatotropic and to induce hepatomas in rats with a mean induction time of 165 days when continuously given at daily doses of 8 mg/kg (= 1/40 of LD_{50}) (Druckrey *et al*., 1967).

Male inbred rats (strain BD X) were used throughout. Six- and 12-week-old rats were divided into 4 groups, 80-100 animals in each, and given NNM in drinking water: (1) 6 mg/kg/day for 12 weeks or (2) 20 mg/kg/day for 6 weeks. Likewise, 20-week-old rats were given 20 mg/kg/day for 6 weeks. Total carcinogen intake was calculated by daily measurements of water drunk. After ingestion of NNM, rats were kept on a standard diet (Altromin, Lage, Germany) and tap water. From the beginning of NNM treatment, rats were bled weekly for detection of AFP in sera; also, rats were killed at 1-week intervals for histological analysis.

Control animals were kept on a standard diet and tap water. Six-week-old male rats were injected intraperitoneally with D-galactosamine-HCl (Merck, Darmstadt, Germany) (Sell *et al*., 1974) which produced liver cell injury similar to that seen in human viral hepatitis (Reutter *et al*., 1968); these rats served as controls for histological changes in the liver after acute toxic damage of hepatocytes.

Isotope injection

DNA replication was estimated by IP injeetion of [^{3}H]thymidine (spec. act. 6.7 Ci/mmole, NEN, Boston, USA) according to one of these schedules: (1) a 60-min pulse by injecting 500 μCi/rat; (2) a pulse over 24 h by three successive injections of 250 μCi each; (3) a pulse for

60 min by injection of 500 μCi/rat followed by daily injections of unlabelled thymidine for 7 days.

Immune sera and immunological procedures

Data on the preparation of monospecific rabbit anti-AFP and monospecific sheep anti-rabbit IgG immune sera were described earlier (Kuhlmann, 1975). For immunocytochemical labelling, pure rabbit anti-AFP and pure sheep anti-rabbit IgG antibodies were isolated from whole immune sera by means of immunoadsorbents (Kuhlmann, 1975). IgG molecules from sheep anti-rabbit IgG antibodies were conjugated with horseradish peroxidase (HRP, RZ 3; Boehringer, Germany) by use of glutaraldehyde and purified on a Sephadex G 200 column (Kuhlmann, 1975; Kuhlmann, 1977*a*).

AFP content in rat sera was quantitated by electro-immunodiffusion (Laurell, 1966) after which immune precipitates were visualized by sheep anti-rabbit IgG being conjugated with glucose oxidase. The threshold for detection of AFP was of the order of 10^{-7} g/ml. When AFP was not measurable, sera were lyophilized, reconstituted in 1/5 of the original volume and tested again.

Immunocytochemistry

Liver blocks of about 1 x 0.5 x 0.5 cm were fixed in 96% ethanol-1% acetic acid for 12-15 h at 4° C, dehydrated in absolute ethanol, cleared in benzene and embedded in paraffin.

Sections, 5 to 7 μm thick, were mounted on acetone-cleaned slides, deparaffinized in xylene and passed from absolute ethanol into 0.1 M phosphate-buffered saline pH 7.2 (PBS). Prior to incubation with antibodies, endogenous peroxidases were irreversibly inhibited by treatment with 1 % hydrogen peroxide in PBS for 1 h at room temperature (Kuhlmann, 1975). Then, slides were washed in PBS and treated by an indirect staining method: incubation was first carried out with unlabelled anti-AFP antibodies (0.01, 0.05, 0.1, 0.5 and 1 mg/ml in preliminary experiments; finally, 0.05 to 0.1 mg antibodies per ml were used in routine incubations) for 20 min and followed by HRP conjugated anti-rabbit IgG antibodies (0.1 mg/ml) for 20 min. Antibodies that did not react were removed by three successive washings of 5 min each, in PBS supplemented with 1 % bovine serum albumin and 0.5 M NaCl.

Peroxidase activity was assayed by incubating samples in 3,3'-diaminobenzidine (Merck, Germany), 0.5 mg/ml 0.2 M Tris-HCl buffer pH 7.2 containing 0.01% H_2O_2 (Graham and Karnovsky, 1966). After washing in PBS, sections were treated for 2-3 min with 0.1 % OsO_4 in PBS, then dehydrated and mounted under cover-glass.

Autoradiographs

Sections were covered with Kodak AR 10 stripping film (Pelc, 1947), exposed for 21 days at 4° C and developed with Kodak D 19 for 5 min. Fixed and dried preparations were mounted without further staining or after hematoxylin staining in glycerol-gelatine. Labelled cells were taken to be those which had more than five developed silver grains above the nucleus.

Histological controls

For routine histology, sections were stained using hematoxylin/eosin. Glycogen accumulation in liver cells was verified using the periodic acid-Schiff (PAS) reaction (modified from McManus, 1946).

Immunocytochemical specificity was controlled on serial tissue sections by incubation in (1) normal rabbit IgG globulins; (2) rabbit anti-AFP antibodies absorbed with an immunoadsorbent which was prepared by coupling AFP to cyanogen-bromide-activated Sepharose 4B (Cuatrecasas, 1970); (3) rabbit anti-rat IgG antibodies. Either procedure was followed by peroxidase conjugates of sheep anti-rabbit IgG antibodies and enzyme substrate.

RESULTS

Induction and development of hepatomas

Important changes in rat liver histology and resurgence of AFP during both the induction phase and the hepatoma stage are summarized in Table I. The induction times indicate the first appearance of hepatomas. Calculations of the mean induction time for the different experimental groups are not included in this study.

TABLE I

EXPERIMENTAL HEPATOCARCINOGENESIS

BD X Rats	Group I 6 weeks	Group II 6 weeks	Group III 12 weeks	Group IV 12 weeks, Group V 20 weeks
Induction phase				
NNM treatment (per kg per day)	6 mg/12 weeks	20 mg/6 weeks	6 mg/12 weeks	20 mg/6 weeks
DNA replication	—*	Oval-shaped cells Hyperplastic areas	—	Oval-shaped cells Hyperplastic areas
Proliferative period (oval-shaped cells)	—	Days 21-56	—	Days 21-56
Intracellular AFP	0	Oval-shaped cells	0	Oval-shaped cells
μg AFP/ml serum	0	1.62±0.47	0	1.41±0.55
Hepatoma stage				
Induction time	200 days	90 days	200 days	90 days
DNA replication	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes
Intracellular AFP	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes	Neoplastic hepatocytes
μg AFP/ml serum	<0.1 to >4,000	<0.1 to >4,000	<0.1 to >4,000	<0.1 to >4,000

* Not significant.

Development of hepatomas did not depend upon the age of the rats at the beginning of NNM feeding. Furthermore, no age-related differences were observed in histotoxic patterns of 6-, 12- or 20-week-old rats. During the induction phase, histotoxic patterns depended largely on the daily dose of NNM. For example, animals of groups II and III ingested approximately the same total amount of NNM (100-120 mg NNM/rat) but at different daily doses and the liver pathology developed quite differently.

In animals which were fed low concentrations of carcinogen (groups I and III), necrosis of original hepatocytes was minimal and significant proliferative activities in the livers were not observed. Areas of pronounced glycogen storage (extensive PAS reaction) without signs of proliferation could be found from day 100.

At high-dose NNM feeding, i.e. in rats of groups II, IV and V, a remarkable shift in histotoxic patterns was observed: extensive necrosis of hepatocytes with loss of glycogen developed within 14 days and reached periportal areas. From day 21, proliferation of small, oval-shaped cells was observed around portal areas with a tendency to form plates and pseudotubular structures (Fig. 1-3). When NNM was removed, cellular proliferations came to a standstill and distortion of the original lobular architecture, concomitant with cirrhosis, occurred. From day 35 foci of proliferated cells (see "Pulse-chase experiments") reconstructed livers which appeared as hyperplastic areas and usually became PAS-positive.

At the final stage, PAS-negative nodules of liver-cell carcinoma with mature or immature cell-types developed (Fig. 4, 5). Irrespective of total NNM ingestion, hepatomas developed in each experimental group. However, in high-dose animals AFP-producing hepatomas were

observed as early as 90 days after initiation of NNM feeding. With low doses of NNM, AFP-producing hepatomas were not observed before day 200.

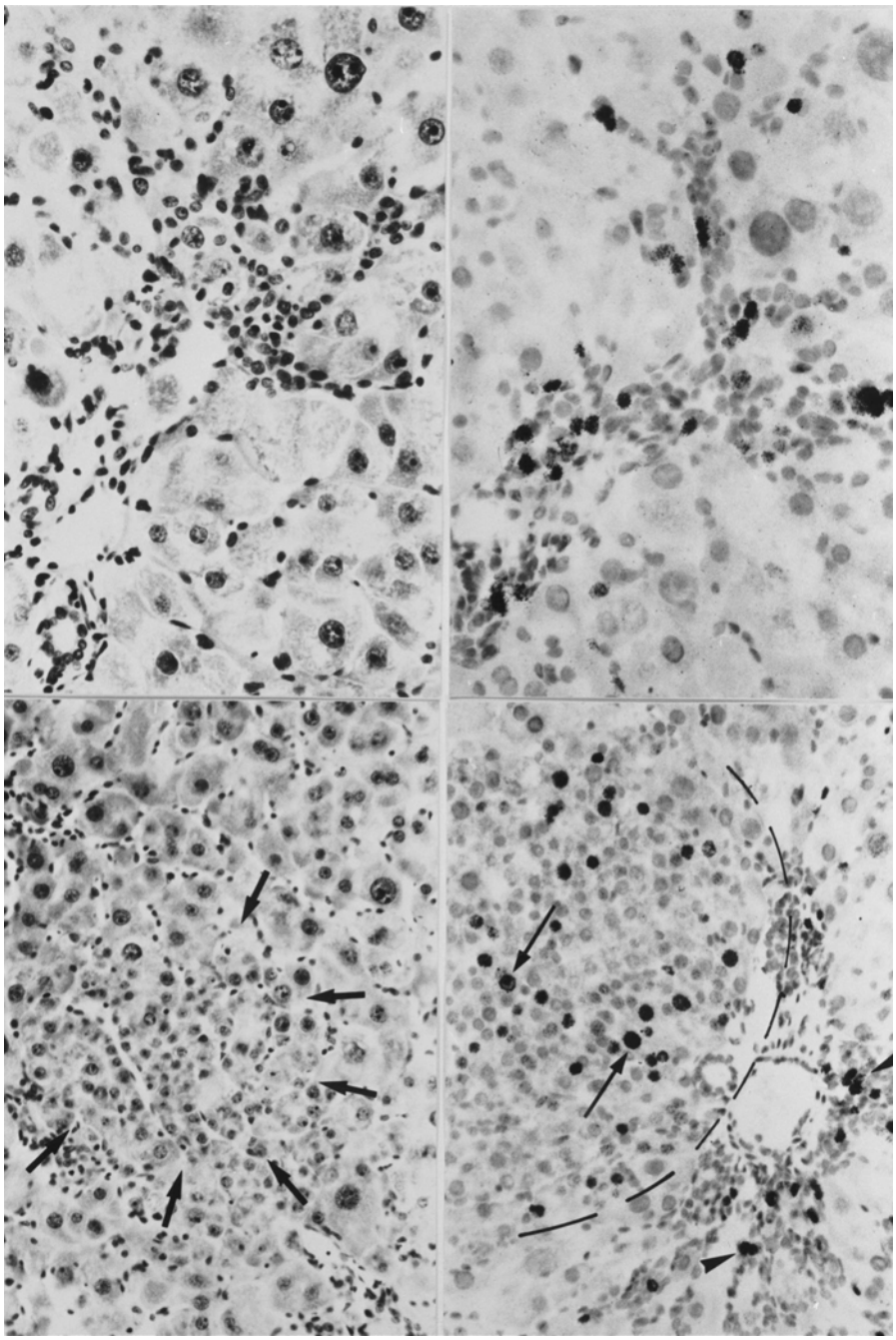

FIGURE 1 – [^{3}H]thymidine incorporation in pulse-labelled rat liver. (*a*) Liver from day 28, HE stained preparation; note numerous small, oval-shaped cells (←). (*b*) Same liver, note pulse-label in proliferating oval-shaped cells (←). (*c*) Liver from day 35, a distinct area of oval cell maturation is indicated (←), hematoxylin/eosin. (*d*) Autoradiograph from same liver after a [^{3}H]thymidine pulse of 1 h; note DNA labelling (←) in an area of hyperplastic appearance (----) and in oval-shaped cells (◄).

[^{3}H]thymidine incorporation in pulse-chase experiments

In rats fed low doses of NNM, significant cell proliferations and DNA replications were not found during early stages of hepatoma induction. In contrast, proliferation of oval-shaped cells was a typical response to high doses of NNM. These cells exhibited high [^{3}H]thymidine

incorporation. When proliferative activity in the liver reached its maximum, i.e. between day 28 and day 35, oval-shaped cells were seen in continuous development towards mature hepatocytes and distinct areas of hyperplastic appearance occurred. Upon pulse-labelling with [^{3}H]thymidine, numerous oval-shaped cells as well as cells from hyperplastic areas were actively engaged in DNA synthesis, and their nuclei were heavily labelled (Fig. 1).

In pulse-chase experiments, about 90% of oval-shaped cells and all those from hyperplastic areas became labelled. In these cases the number of silver grains per nucleus was reduced because of a gradual reduction of initially incorporated [^{3}H]-thymidine by subsequent cell divisions (Fig. 2). Finally, in restituted livers with a cirrhotic pattern significant thymidine incorporation was no longer observed.

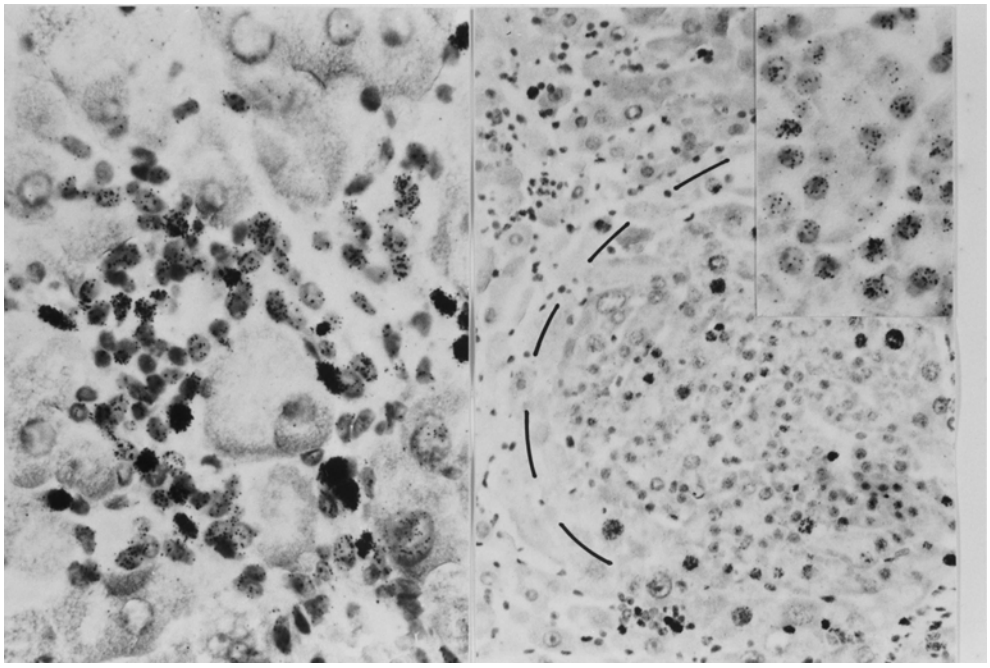

FIGURE 2 – Pulse-chase labelling with [^{3}H]thymidine. (*a*) Liver from day 35; thymidine incorporation in most of the oval-shaped cells. (*b*) Same liver, hyperplastic area (----); inset: higher magnification view of labelled cells.

When the hepatoma stage was reached, 10-20% of the hepatoma cells incorporated thymidine after a 1-h pulse. Following a pulse of 24 h, up to 50% of the neoplastic hepatocytes became labelled. In normal hepatocytes and areas of glycogen accumulation, the percentage of labelled nuclei stayed below 1 % (see "Cellular detection of AFP in hepatomas").

Localization of AFP during the induction phase

Irrespective of their age, rats which were fed high concentrations of NNM produced AFP which was detected in the proliferating, small, oval-shaped cells. AFP was always found in the cytoplasm. Between day 21 and day 28 increasing numbers of AFP-positive cells were observed and a maximum was attained towards day 35. AFP-stained cells either formed groups and tubular structures or were distributed randomly and intermingled with non-stained cells of the same phenotype (Fig. 3).

When proliferation of oval-shaped cells decreased, AFP-positive cells disappeared rapidly. In rats which were given low doses of NNM, no cellular staining of AFP was observed

during the induction phase. Under neither of our experimental conditions could AFP be detected in the "hyperplastic nodules" or areas of hyperplasia.

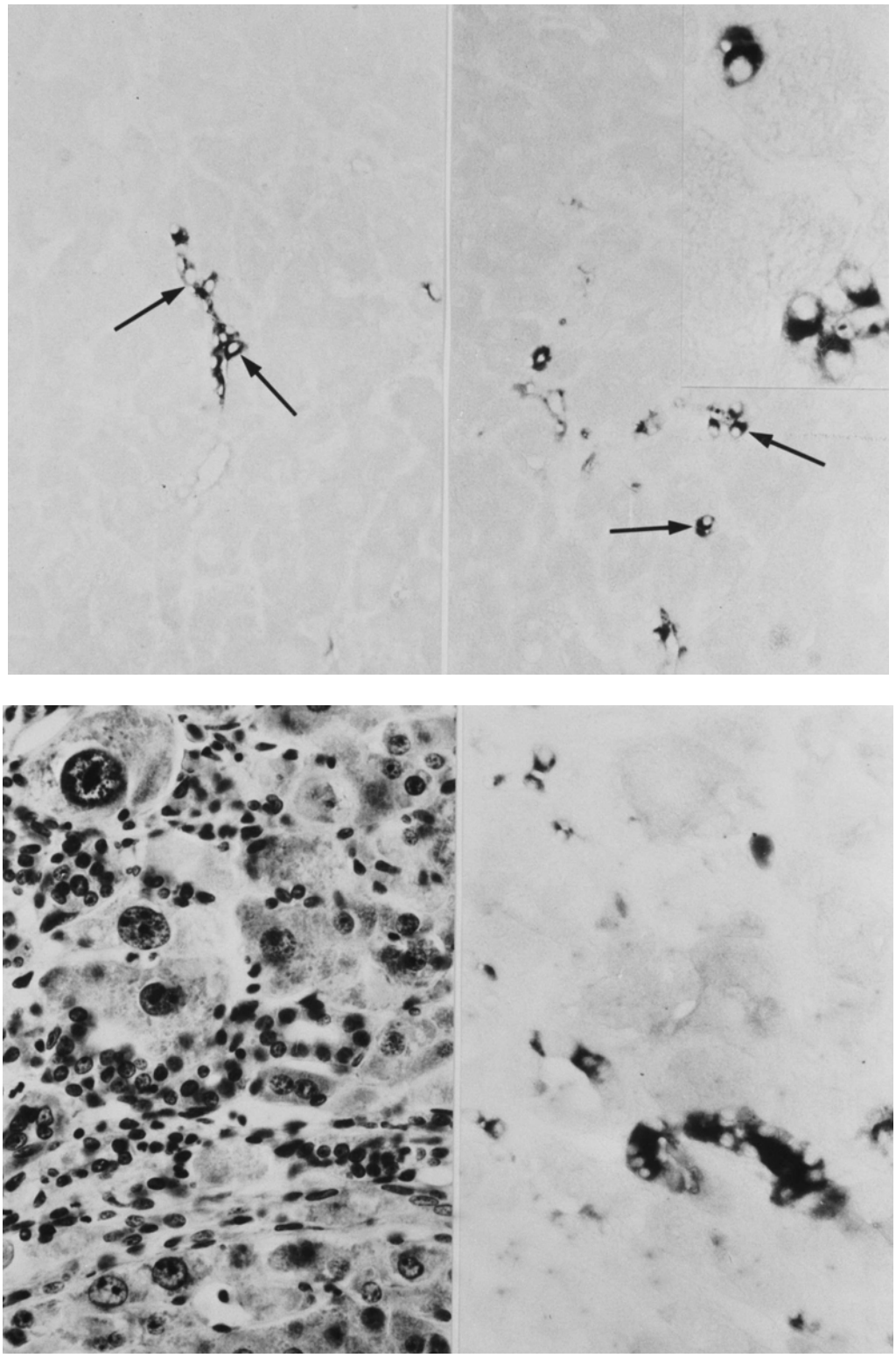

FIGURE 3 – Detection of AFP in liver during early stages of hepatocarcinogenesis. (*a*) Liver from day 28; note intracellular AFP in grouped oval-shaped cells (←). (*b*) Liver from day 35; distinct AFP staining in small cells (←); inset: higher magnification view of AFP-positive oval-shaped cells. (c) Liver from day 42; note numerous oval-shaped cells, hematoxylin/eosin stained preparation. (d) Same liver, serial section stained for AFP; note localization of AFP in grouped cells which form tubular structure.

In control rats injected with galactosamine, bile ductular proliferations became prominent between day 2 and day 4, and these formed plates and rosettes. Such cells resembled the oval-shaped cells in NNM-treated animals and cytoplasmic AFP was also found. The intensity of ductular cell regeneration and serum AFP were closely related.

Cellular detection of AFP in hepatomas

When malignant transformation occurs and AFP reappears in rat sera, one or more distinct nodules of hepatoma cells are found by microscopic inspection. Some but not all of these nodules stain for intracellular AFP. No correlation was observed between cellular AFP and the histological grading of the hepatomas. At the border of hepatoma nodules, penetration of neoplastic hepatocytes into normal liver tissue was evident and AFP was found only in the neoplastic hepatocytes. Normal, nearby, adult hepatocytes and bile duct cells did not stain for AFP. Only neoplastic hepatocytes, which did or did not contain AFP, were heavily engaged in DNA synthesis (Fig. 4). In routine histology, AFP-positive cells exhibited a basophilic character and were PAS-negative.

Correlations between serum AFP levels and cellular AFP were difficult to establish. In general, the higher the amount of AFP in sera the higher the number of AFP-staining cells. Nevertheless, few AFP-producing cells were also readily localized in rats with low serum AFP concentrations (e.g. <1 μg/ml) under conditions where the AFP-synthesizing cells were sectioned. In all the cases studied, AFP staining was cytoplasmic and usually without preferential localization in distinct cellular compartments. In some rats with rapidly rising AFP levels, tumor cells showed moderately positive AFP reactions and in these cases a distinct area in the perinuclear region was strongly stained. This localization of AFP corresponded to the area where the Golgi apparatus is known to be located. In hematoxylin/eosin-stained preparations, a large perinuclear halo was readily observed (Fig. 5).

Finally, hepatomas progressed to huge tumor masses containing extensive areas of necrosis. At the periphery of such tumors, groups of AFP-staining as well as non-AFP-staining hepatoma cells were localized. These cells did not stain for rat IgG, whereas cells within and bordering necrotic areas often contained both AFP and IgG. Hence, this type of staining was difficult to judge, but was assumed to be non-specific (Engelhardt *et al*., 1971).

Immunocytochemical controls

Treatment of liver sections with 1 % H_2O_2/PBS for 1 h inhibited irreversibly endogenous peroxidases. Sections did not stain upon incubation in normal rabbit IgG globulins and absorbed rabbit anti-AFP antibodies. Optimal AFP staining was achieved by incubation of sections with 0.05 to 0.1 mg/ml of anti-AFP antibodies. Background reactions due to interaction of immunocytochemical reagents with tissue components were not observed when pure antibodies (instead of γ-globulin fractions or whole immune serum as controlled in preliminary experiments) and purified peroxidase conjugates were employed. Finally, optimal washings of incubated sections were obtained by PBS supplemented with albumin and 0.5 M NaCl.

Passive uptake of plasma proteins by damaged cells could lead to erroneous results but were readily controlled by reaction of serial sections for rat IgG. Non-specific absorption of AFP and IgG from extracellular spaces was found to occur in necrotic areas and their bordering cells when huge tumor masses were reached. Then, adjacent normal hepatocytes could also stain for both proteins. In such cases, AFP localization patterns were not taken into account.

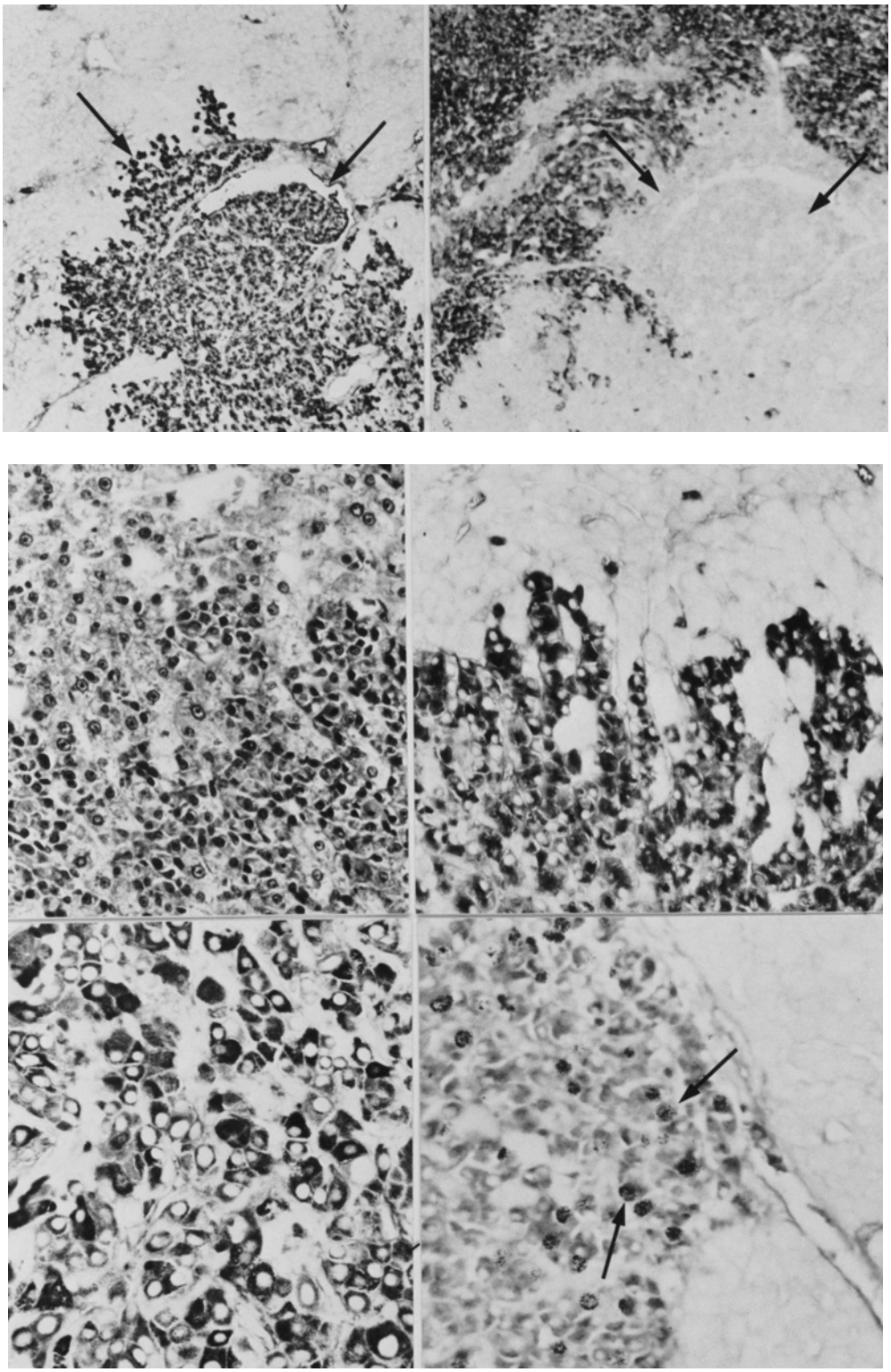

FIGURE 4 – Localization of AFP at the hepatoma stage. (*a*) Low magnification view of a hepatoma nodule after staining for AFP (←). (*b*) Serial section from same liver was reacted by PAS; normal hepatocytes are strongly stained, whereas the AFP-positive hepatoma cells remained PAS-negative (←). (*c*) Higher magnification from the border of a hepatoma nodule and stained by hematoxylin/eosin. (*d*) Serial section from same hepatoma region and reacted for AFP; neoplastic hepatocytes penetrate normal liver, and AFP reaction is only in the hepatoma cells. (*e*) Another view from an AFP-staining hepatoma. (*f*) Thymidine incorporation in AFP-staining hepatoma cells (←); adjacent normal liver is not labelled.

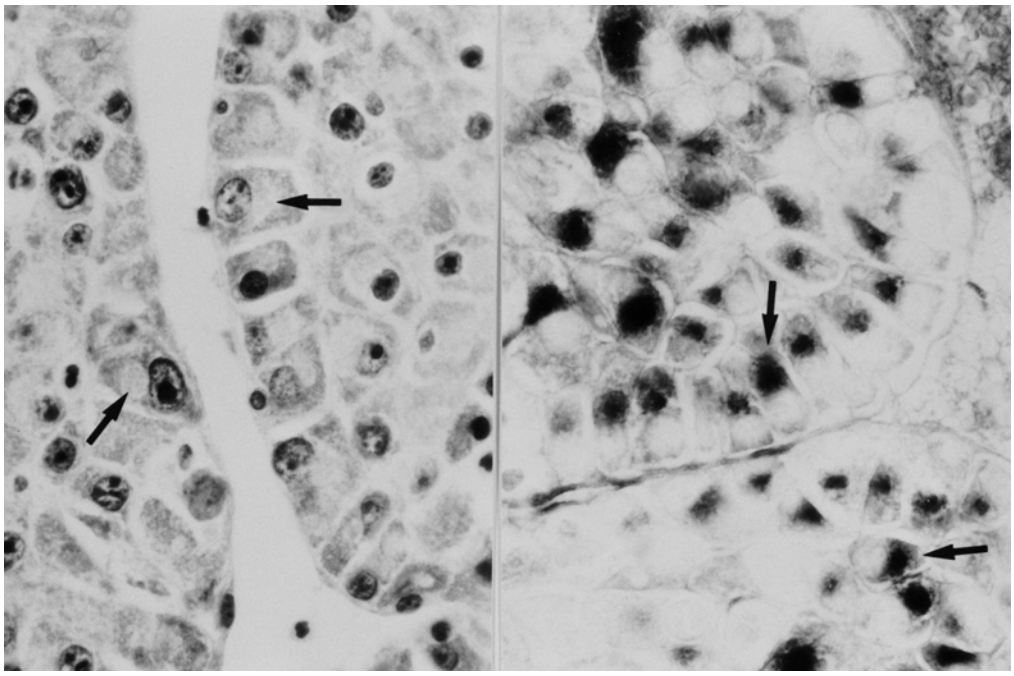

FIGURE 5 – High magnification view of a hepatoma with steep rising rates of serum AFP. (*a*) Hematoxylin/eosin stained preparation; note prominent perinuclear halo (←). (*b*) Serial section from same hepatoma stained for AFP; note strong AFP reaction in perinuclear area (←).

Resurgence of AFP in sera

Reappearance of AFP in sera was coincident with the proliferation of oval-shaped cells during the induction phase and when hepatoma stages were reached (Table I).

Small amounts of AFP were detected for the first time on day 21. Between days 28 and 49, serum AFP levels in individual rats rose and concentrations from 0.7 µg/ml to 2.3 µg/ml could be measured. No age differences were observed in these rats; mean values ±SD are given in Table I. Histological examination showed serum AFP levels to be closely related to the presence of oval-shaped cells. Two weeks after NNM ingestion was stopped, AFP disappeared from the circulation. By the method employed, no AFP could be detected in sera of low-dose NNM-fed rats during early stages of hepatoma induction.

When hepatomas emerged, and irrespective of NNM feeding schedules, a wide range of serum AFP levels was measured, varying from <0.1 µg/ml to concentrations up to and exceeding 4 mg/ml serum. No clear-cut correlations could be made between serum AFP and the histological type of hepatoma or intracellular AFP localization (see "Cellular detection of AFP in hepatomas").

The dynamics of serum AFP levels were also monitored during the course of hepatoma development by collecting serum samples at weekly intervals (Fig. 6). The wide range of both serum AFP levels and rising levels of AFP in individual rats indicated the highly heterogeneous character of the induced hepatomas with respect to AFP production. By comparison of the slope of individual AFP curves, a typical observation in hepatoma-bearing rats was the continuous increase in their serum AFP levels. But rats were also seen in which the following pattern occurred: elevated AFP levels with transient rises, followed by a plateau and further

rise. During hepatoma development, AFP levels never decreased to values which were measured at previous time points.

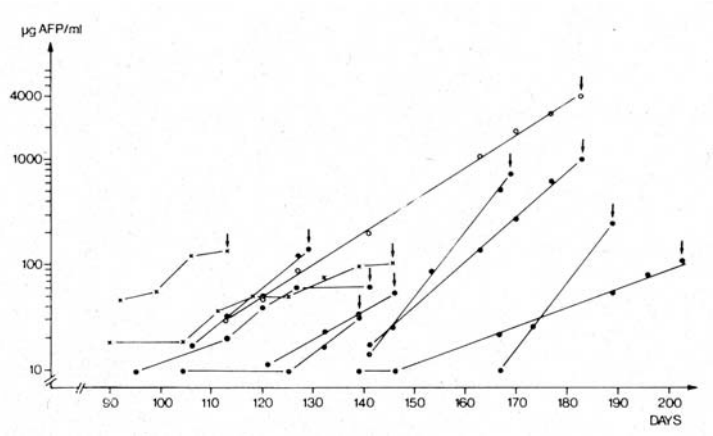

FIGURE 6 – Reappearance of AFP in sera at the time of malignant conversion and examples for dynamics of serum AFP levels during subsequent growth of hepatomas. Finally, animals were killed and submitted to immunocytochemical AFP staining (↓).

DISCUSSION

Numerous descriptions of histogenesis and classification of experimental liver cancer have been published since Yoshida (1932) and will not be referred to here (for references see Bannasch, 1975). Rather, attention will be paid to the major histological changes and the phenomenon of AFP resurgence. In this paper we present our results from immunoperoxidase labelling of intracellular AFP at the various stages of hepatoma development. The advantage of immunoperoxidase techniques over immunofluorescence lay in observing tissue preparations by ordinary light microscopy with the possibility of counterstaining. Routinely, sections were treated with OsO_4, thus enhancing the specific cytochemical reaction and the overall contrast. Implications of immunocytochemistry and the necessary steps for the present study have been described elsewhere (Kuhlmann, 1975; 1976; 1977*a*, *b*).

The following main points arose from our study. (1) Hepatomas were induced in rats by feeding either low or high doses of NNM, but prominent proliferation of oval-shaped cells with subsequent development of hepatocytes occurred only at high doses of NNM. (2) When high doses of NNM were fed, AFP reappeared during the early phases of carcinogenesis, before hepatomas were found, and this protein was detected in sera and in the cytoplasm of proliferating oval-shaped cells. (3) At the final stage of carcinogenesis, neoplastic hepatocytes proliferated and nodules of AFP-staining hepatoma cells were found together with nodules of non-AFP-staining hepatoma cells. (4) The wide range of both serum AFP levels and increasing levels of serum AFP in individual rats indicated the heterogeneous character of induced hepatomas with respect to AFP production.

Cellular fluctuations during hepatocarcinogenesis

At high doses of NNM, prominent changes in livers occurred as megalocytic degeneration of adult hepatocytes and concomitant proliferation of PAS-negative, small, oval-shaped cells within 3 weeks of NNM feeding. By [^{3}H]thymidine labelling in pulse-chase experiments, oval-shaped cells were shown to be actively engaged in DNA synthesis and, furthermore,

their development into hepatocytes was demonstrated. These cells constituted areas with hyperplastic appearance still active in thymidine incorporation, but during further progression DNA synthesis came to a standstill and glycogen accumulation comparable to that seen in normal hepatocytes was observed. Hence, proliferation of oval-shaped cells, of unknown origin, was a significant response to the acute hepatotoxic damage, and restitution of the liver occurred by subsequent maturation of these cells into hepatocytes. Our findings were in accordance with observations made with other carcinogens where development of proliferating "oval cells" into hepatocytes was suggested (Price *et al*., 1952; Farber, 1956; Inaoka, 1967; Iwasaki *et al.*, 1972; Onoe *et al*., 1973). Finally, cirrhotic distortion of the original liver structure was a regular feature. The fact that no age differences were found in the sequential fluctuations of liver cells indicated common pathways of liver regeneration in rats during and after the provoked liver damage.

In contrast, neither proliferation of oval-shaped cells nor extensive necrosis of hepatocytes were detected when low concentrations of NNM were fed. Consequently, areas of hyperplastic appearance as described above were not observed. During later stages of hepatocarcinogenesis, but before hepatomas developed, we were able to distinguish areas of enhanced glycogen storage which may be regarded as irreversible precancerous lesions (Bannasch, 1975). In our material, such cells did not show signs of malignancy (like enhanced proliferation), and their role in later development of hepatomas was not clear.

Localization of AFP during the induction phase

By routine microscopy and immunoperoxidase staining of serial sections it was clearly shown that the emerging oval-shaped cells were responsible for AFP production during early stages of carcinogenesis with high doses of NNM. These results support immunofluorescence work (Dempo *et al*., 1975; Tchipysheva *et al*., 1977) in which transitional cells and "small hepatocytes" were thought to produce AFP in the early stages of azo-dye and 2-acetylaminofluorene carcinogenesis. From the 1-h pulse labelling experiments with [^{3}H]thymidine we found that a small proportion of the AFP-staining oval-cell population was actually in S phase, whereas the majority of AFP-staining cells were not labelled by [^{3}H]thymidine. The importance of this observation is still under study and may reflect AFP synthesis after one or more mitotic cycles of the oval-shaped cells which in turn retained the capacity for DNA replication during stages preceding hepatocyte maturation (see "[^{3}H]thymidine incorporation in pulse-chase experiments"). Finally, a correlation between the reappearance of AFP in sera and intensity of oval-cell proliferation concomitant with AFP-positive staining was noted in individual rats. Similar serological observations were reported with other chemicals (Kitagawa *et al*., 1972).

In contrast to these observations we found that, during carcinogenesis with low doses of NNM, no significant proliferation of oval-shaped cells, no AFP staining of liver cells and no reappearance of AFP in sera (by the method employed) were seen. Thus, serological, histological and immunocytochemical data suggested that oval cells were indeed the cells which produced AFP at this stage of carcinogenesis. Since both NNM schedules (low and high doses) led to hepatomas, oval-cell proliferation with concomitantly transitory AFP synthesis was not regarded as a prerequisite for conversion to cancer. Merely, oval cells resulted from restitution of damaged liver due to acute toxic injury.

These conclusions were supported by immunocytochemical AFP staining in rats with galactosamine-induced experimental hepatitis. One of the prominent morphologic features was the intensive proliferation of bile-duct epithelium as already described by Lesch *et al*. (1970) and which was followed by the appearance of AFP in serum (Sell *et al*., 1974). In our

animals, we also observed bile ductular proliferations from day 2 after injections of galactosamine. Such proliferations could be stained for intracellular AFP. Rats in which few regeneration plates were observed contained AFP serum levels that were below the detectable limit of the method employed. It could be that a minimal proliferation rate of oval-shaped cells was necessary for the re-expression of AFP.

When NNM feeding was stopped, oval-cell proliferation came rapidly to a standstill and, furthermore, AFP was no longer detected afterwards until hepatomas developed. In contrast to other reports (Okita *et al*., 1974) we did not observe AFP staining in hyperplastic nodules.

Cells producing AFP at the hepatoma stage

At the hepatoma stage, AFP was localized in the cytoplasm of cells which by routine histology were typical basophilic and PAS-negative neoplastic hepatocytes, and which were clearly distinguished by size and shape from normal adult hepatocytes. These neoplastic hepatocytes did not accumulate glycogen as was usually the case in normal hepatocytes. PAS-positive globules within intracellular spaces might occur in malignant and normal hepatocytes of individual rats, but these findings did not follow a regular pattern. Recently, intracellular PAS-positive globules were found to be related to the deposition of a_1-antitrypsin in human primary liver carcinoma cells (Palmer and Wolfe, 1976). However, in our NNM-induced hepatoma cells, no clear-cut relationship between production or storage of AFP and PAS-positive globules could be established: upon immunocytochemical control incubations, such PAS-positive globules exhibited enhanced contrast, a phenomenon which needs further consideration.

It is important to note that AFP staining never occurred in adjacent normal liver cells. In the usual hematoxylin/eosin preparations, AFP-positive and AFP-negative nodules were seen as circumscribed areas of liver cell carcinoma of grade II/III with trabecular pattern or as grade IV liver cell carcinoma (Edmondson and Steiner, 1954), but no correlation between histology and serum AFP levels was possible.

Our studies support the findings of others (Goussev *et al*., 1971; Nishioka *et al*., 1972) that not every hepatoma cell was an AFP producer cell. Rather, AFP-staining cells represented populations of distinct neoplastic hepatocytes with a certain degree of retrodifferentiation (clones). This suggestion was supported by the fact that AFP-staining and non-AFP-staining nodules could be observed side by side. Following pulse labelling of hepatoma-bearing rats with [^{3}H]thymidine for 1 h, 10-20% of the hepatoma cells, whether AFP-positive or not, were observed to be in S phase. However, after a further chase of 24 h, >50% of the AFP-staining cells exhibited DNA replication, whereas the number of labelled nuclei from non-AFP-staining hepatoma cells in the same animal was much lower. It must be mentioned, however, that in other animals non-AFP-staining nodules could be also heavily engaged in DNA synthesis. The importance of these observations is not yet clear.

Preferential localization of AFP-staining cells at the border of sinusoid capillaries, along bile ducts, blood or lymphatic vessels (Goussev *et al*., 1971; Uriel *et al*., 1973) was not observed in distinct nodules, but occurred when huge tumor masses were reached. In the latter, more or less necrotic areas and damaged cells were also present which stained for both AFP and IgG. This staining pattern was considered to be atypical (Engelhardt *et al*., 1971).

Reappearance of AFP during hepatocarcinogenesis

Early elevated AFP levels were described as being a response to the carcinogenic diet, and this phenomenon was correlated with carcinogenicity of the chemical agent, accumulated

dose, and subsequent liver alteration including oval cell proliferation (Watabe, 1971; Kitagawa *et al*., 1972; Kroes *et al*., 1972, 1973, 1975; de Néchaud and Uriel, 1973). Because elevated serum AFP levels without oval-cell proliferation could be measured during and after feeding of very small quantities of N-2-fluorenyl-acetamide, hepatocarcinogenic agents were also shown to have metabolic effects in common which lead to selective derepression of AFP synthesis (Kroes *et al.*, 1972; Becker and Sell, 1974). Because the method employed for AFP measurements in the sera of our rats did not permit the detection of very low amounts of AFP (<0.1 μg/ml) it was possible that in the present studies low amounts of AFP also resulted from a selective derepression of AFP synthesis. But, more importantly, the elevated serum AFP concentrations described here were only observed in rats given high doses of NNM, which resulted from AFP synthesis by oval-shaped cells.

Reappearance of serum AFP in the later stages of carcinogenesis was unequivocally caused by hepatoma cells as demonstrated in the present work by immunoperoxidase staining. Similar results were reported from immunofluorescence studies (Goussev *et al*., 1971; Nishioka *et al*., 1972). The occurrence of AFP in primary liver cancer was considered to be the direct consequence of retrodifferentiation during neoplastic change in which stages may be reached where synthesis of AFP takes place (Uriel, 1969, 1976). Then, it may be argued that various degrees of retrodifferentiation would generate neoplastic hepatocytes of clonal origin leading to AFP-positive and AFP-negative hepatomas. This suggestion was supported by our immunoperoxidase staining technique which permitted a clear-cut distinction between AFP-staining and non-AFP-staining hepatoma nodules. The phenotype of neoplastic hepatocytes was of ancillary importance and did not reflect the potential of a neoplastic hepatocyte to produce AFP.

Serum AFP levels resulted from synthesis, secretion and turnover of this protein, and the rising rates of serum AFP in the course of hepatoma development were useful indicators for production and especially for secretion of AFP by the responsible cells. In rats with steeply rising AFP levels, signs of pronounced secretion could be observed, and in these cases the Golgi complexes were heavily stained for AFP.

From the foregoing it can be deduced that immunoperoxidase labelling of AFP in combination with autoradiographic studies of proliferating cells enabled us to give a concise description of the cellular basis of AFP synthesis. Furthermore, results from measurements of serum AFP levels and from monitoring their rising rates in the course of hepatoma progression strongly indicated that all these diagnostic procedures together were useful tools to elucidate phenomena of production and secretion of AFP. This may be also true for other tumor-relevant substances in other systems.

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