

Correlation of Histology and Alpha-1-Fetoprotein Resurgence in Rat Liver Regeneration After Experimental Injury by Galactosamine

W. D. KUHLMANN, K. WURSTER

*Laboratory of Experimental Medicine and Immunocytochemistry, Institut für Nuklearmedizin, DKFZ Heidelberg, Germany, and
Institut für Pathologie, Heidelberg, Germany*

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Summary

Intraperitoneal injections of galactosamine-HCl in rats were followed by transitory elevations of serum alpha-fetoprotein (AFP) concentrations. These were associated with regeneration of the damaged liver; a maximum of serum AFP was reached on day 4 (2.08 ± 0.67 $\mu\text{g/ml}$). In sera of untreated rats, serum AFP levels were <0.1 $\mu\text{g/ml}$ and no cellular AFP was detected in liver sections. Two days after galactosamine injections, AFP was localized for the first time in the cytoplasm of epithelial cells of bile ducts and canaliculi in portal spaces. The intensity of AFP staining reached a maximum between days 3 and 4. In addition, faint but distinct AFP-positive reactions were seen in the cytoplasm of randomly distributed hepatocytes. After day 5, AFP-staining cells rapidly disappeared. A strong correlation was noted between reappearance of AFP in sera, intensity of epithelial bile duct proliferation and cellular AFP staining.

Key words: Alpha-fetoprotein - Galactosamine - Liver regeneration - Immunohistology.

Introduction

Alpha-fetoprotein (AFP) is a normal constituent of fetal serum and amniotic fluid in many species. Sites of AFP synthesis are derived from the endoderm of the embryonic gut: the yolk sac, the gastrointestinal tract and the liver (Gitlin and Boesman, 1967a, b; Gitlin et al., 1967). Under physiological conditions, this protein disappears after birth, and the serum level of AFP in adult individuals decreases to values which can be only detected by methods such as radioimmunoassay (Ruoslahti and Seppälä, 1971; Sell, 1973).

AFP may reappear in substantial quantities in adults during the process of malignant transformation. Measurement of AFP has proved to be of value in the diagnosis of hepatocellular cancer and endodermal sinus tumors (Abelev et al., 1963; Tatarinov, 1964; Abelev, 1968; Laurence and Neville, 1972; Teilum et al., 1975). Elevated serum AFP levels also occur in non-malignant diseases of human liver, i.e., forms of regeneration associated with viral hepatitis, alcoholic cirrhosis and partial hepatectomy (Geffroy et al., 1970; Matray et al., 1972; Delpré and Gilat, 1978). Under experimental conditions in the rat, such as partial hepatectomy, CCl_4 and galactosamine-HCl induced liver injury, temporary elevation of AFP levels have been reported (Sell et al., 1974; Taketa et al., 1975; Sell et al., 1976; Smuckler et

al., 1976). Light microscopic and biochemical investigations have shown similarities between galactosamine-induced liver damage in the rat and acute human viral hepatitis (Keppler et al., 1968; Lesch et al., 1970). Even if morphological changes are different at the ultrastructural level in the two conditions (Medline et al., 1970), this experimental system appears useful for studying relationships between AFP production and liver regeneration. A rationale for the phenomenon of AFP reappearance in such cases has not been given but may be explored by means of immunohistochemistry. From preliminary studies on galactosamine-induced rat liver regeneration we suspected that the bile duct epithelium is involved in temporary AFP production (Kuhlmann, 1978). Thus, our present aim was to investigate in more detail AFP production and cellular fluctuations in galactosamine-induced rat liver regeneration. For this purpose, the amount of AFP in sera was measured. Cellular AFP was localized by immunoperoxidase labelling techniques used in parallel with sections stained by classical histological methods.

Material and Methods

Experimental Models. Eight- (140-180 g = group I) and 14-week-old (250-300 g = group II) male inbred rats (strain BD X) were used. They were fed Altromin standard diet (Altromin, Lage, Germany) and tap water.

Liver injury was provoked by two intraperitoneal injections of D-galactosamine-HCl (Merck, Darmstadt, Germany) dissolved in 0.9% NaCl, 375 mg/kg body weight per injection: the first dose was given at 8:30 a.m. and the second dose between 8:00 and 9:00 p.m. of the same day (Sell et al., 1976). From each group, three to five rats per day were bled and the livers dissected out: 1, 2, 3, 4, 5, 6, and 10 days after the first galactosamine injection. Control rats were kept on the standard diet and tap water.

Immune Sera and Immunological Procedures. Rat AFP was isolated from amniotic fluid by procedures analogous to those described for the isolation of mouse AFP (Kuhlmann, 1975; Kuhlmann, 1976). Anti-rat AFP immune sera were obtained by hyperimmunization of rabbits with the isolated AFP. A rabbit anti-rat IgG immune serum was prepared by hyperimmunization with rat IgG (Miles, Frankfurt, Germany) in the same manner. Data on the preparation of monospecific sheep anti-rabbit IgG immune sera have also been described (Kuhlmann, 1975; Kuhlmann, 1977).

AFP content in individual rat sera was quantified by the procedure of enzyme-electroimmunodiffusion (Kuhlmann, 1979).

Cellular AFP was detected by an indirect (Sandwich) staining method. For this purpose, pure rabbit anti-AFP and pure sheep anti-rabbit IgG antibodies were isolated from whole immune sera by means of immunoadsorbents. IgG molecules of the "Sandwich" antibodies (sheep anti-rabbit IgG) were conjugated with horseradish peroxidase (HRP; Boehringer-Mannheim, Germany) and purified on a Sephadex G 200 column (Kuhlmann, 1975; Kuhlmann, 1977).

Tissue Processing and Immunocytochemistry. Liver slices about 0.5 cm in thickness were fixed in 96% ethanol - 1% acetic acid for 12-15 h at 0°-4° C, dehydrated in absolute ethanol, cleared in benzene and embedded in paraffin (Kuhlmann, 1975).

Sections 5 to 7 μm thick were mounted on acetone-cleaned slides, deparaffinated 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 (Kuhlmann, 1975). Slides were then washed in PBS and treated by an indirect staining method: incubation was first carried out with unlabelled anti-AFP antibodies (0.05-0.1 mg/ml PBS) for 20 min and followed by HRP-labelled anti-rabbit IgG antibodies (0.1 mg/ml PBS) 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 detected by incubation of samples in 3,3'-diaminobenzidine (Graham and Karnovsky, 1966), 0.5 mg per 1 ml Tris-HCl buffer (0.2 mol Tris/liter buffer) containing 0.01% H_2O_2 . After washing in PBS, sections were treated for 1 min with 0.1% OsO_4 in PBS, then dehydrated and mounted under a coverglass. AFP-labelled sections were also counterstained with haematoxylin.

Controls. Immunocytochemical specificity was checked in parallel sections by incubation in (1) normal rabbit IgG globulins (0.1 mg/ml PBS); (2) rabbit anti-AFP 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 HRP conjugates of sheep anti-rabbit IgG antibodies and enzyme substrate. Details of the methodology of immunoperoxidase cytochemistry with special reference to AFP have been described previously (Kuhlmann, 1975; Kuhlmann, 1978). For routine histology, sections were stained by haematoxylin-eosin. Gomori's silver impregnation was also performed.

Results

Liver Injury and Regeneration

Livers from control rats did not show histopathological changes. In both experimental groups, the extent of liver damage was similar, and the results are summarized in Tables 1 and 2.

Within 24 h, galactosamine produced foci of hepatocellular necrosis. These were more extensive near the portal tracts than in the central vein area. Numerous acidophilic degenerating cells and Councilman bodies were present. In a few cases, necroses were confluent, giving rise to the picture of liver atrophy. From day 2, areas of necrosis decreased. On day 5, they were only sporadically noticeable and had disappeared by day 10. Beginning on day 2, mitotic activity started in parenchymal epithelial cells, reaching a peak on day 4 and afterwards decreasing rapidly. On day 1 the canaliculi and bile ducts showed signs of epithelial degeneration and cell necrosis. On day 2 a rapidly increasing mitotic activity appeared in the canalicular epithelial cells and the epithelial cells of bile ducts. This reached a maximum on day 3, then decreased rapidly. Also on day 2, a striking increase of canalicular cross-sections was seen along the borders of portal tracts, reaching a maximum on days 3 and 4 (Fig. 1 a), subsequent decrease was slow; canaliculi were always increased over controls on day 10 (Fig. 1 b). Inflammatory infiltrates were present after 24 h, predominantly in the areas of parenchymal necrosis. They were not seen until day 2 in the portal tracts, which showed some oedema. The infiltrates consisted of histiocytes and neutrophilic and eosinophilic granulocytes; lymphocytes were present only in small numbers. Histiocytes and neutrophilic granulocytes predominated after 24

h, the number of neutrophilic granulocytes decreased from day 2 onward. An interesting phenomenon then became apparent: there were increasing numbers of eosinophilic granulocytes for a short time. By day 3 the inflammatory infiltrates were mainly composed of histiocytes whereas neutrophilic and eosinophilic granulocytes were present only in small numbers. By day 5 there were only a few histiocytes still present. Neutrophilic and eosinophilic granulocytes had disappeared by this time. By day 10 all signs of inflammation had disappeared. The marked stimulation of Kupffer cells also began to decrease on day 5 but remained demonstrable on day 10. Oedema of the portal tracts started disappearing on day 3 and was no longer present by day 10. At this time portal tracts showed only some fibrosis and a moderate increase in the number of canaliculi in the sections. In the hepatic parenchyma itself, there was still a slight stimulation of Kupffer cells and in the areas of healed necroses, a minimal increase in argyrophilic fibers was visible.

Table 1. Histological changes of the liver after galactosamine injury

	1. Day	2. Day	3. Day	4. Day	5. Day	10. Day
Necroses	+++	++	++	+	(+)	-
Councilman bodies	+++	++	++	+	(+)	-
Inflammation	+++	++	++	++	+-(+)	-
Canalicules	-	++	+++	+++	++	++
Mitosis in canalicular epithelial cells	-	++	+++	+	-	-
Mitosis in liver cells	-	+	++	+++	+	-
Kupffer cells	++	+++	+++	+++	++	+
Oedema	++	++	+	+	(+)	-
Fibrosis	-	-	-	+	+	+

Table 2. Time-dependent composition of inflammatory infiltrates in the liver after galactosamine injury

	1. Day	2. Day	3. Day	4. Day	5. Day	10. Day
Inflammation						
portal	+	++	++	++	+	-
parenchymal	+++	++	++	++	(+)	-
Histiocytes						
portal	++	++	+++	++	+	-
parenchymal	++	++	+++	++	(+)	-
Granulocytes neutrophilic						
portal	++	+	+	-	-	-
parenchymal	++	+	+	(+)	-	-
Granulocytes Eosinophilic						
portal	(+)	++	+	+	-	-
parenchymal	(+)	++	+	(+)	-	-
Lymphocytes						
portal	-	(+)	+	+	(+)	-
parenchymal	+	+	+	+	(+)	-

Table 3. Serological and cellular detection of AFP after galactosamine injury

Period of study	Serum AFP ($\mu\text{g/ml} \pm \text{SD}$)	Hepatocytes	Canalicular epithelial cells
Day 1	<0.1	– ^a	–
2	0.19 ± 0.04	–	(+)
3	0.54 ± 0.22	(+)	+
4	2.08 ± 0.67	(+)	+
5	1.53 ± 0.39	(+)	(+)
6	1.24 ± 0.48	–	(+)
10	0.16 ± 0.03	–	–
Normal control	<0.1	–	–

^a Intensity of immunocytochemical AFP reactions: – no; (+) faint; + strong

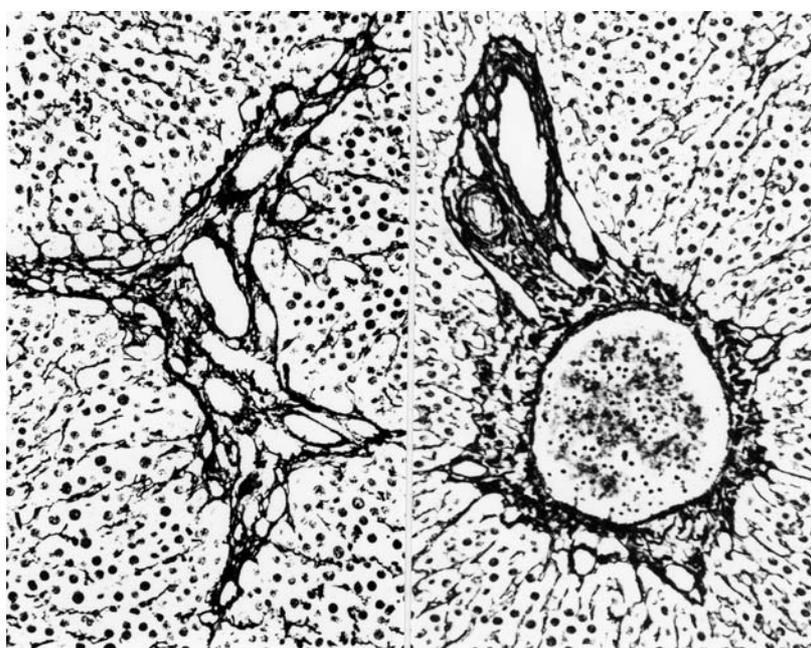


Fig. 1a and b. Canalicular cross-sections reach a maximum on days 3 and 4 after galactosamine injury. Afterwards they decrease, but remain still on a higher level than normal on day 10. Original x 160. **a** Portal tract with increased cross-sections of canaliculi on day 3. They are of various size and are best visible after silver impregnation; Gomori. **b** Portal tract on day 10 showing moderately increased numbers of canalicular cross-sections which are mostly small; Gomori

Resurgence of AFP in Sera

Serum AFP concentrations in untreated rats were $<0.1 \mu\text{g/ml}$. Galactosamine injury was regularly accompanied by an increase of AFP in sera. No differences in AFP elevation were noted between animals of group I and group II, and variations in serum AFP levels followed the same time scale. Data for rats of group I are shown in Table 3 and are representative for both groups of rats (mean values \pm SD). Serum AFP was slightly increased 2 days after poisoning, then it rose and reached a maximum on day 4. Afterwards, concentrations declined steadily.

Cellular Detection of AFP

No AFP could be localized in normal livers of group I and group II rats. Two days after galactosamine injections, AFP was detected for the first time: faint but distinct AFP positivity was seen in the apical cytoplasm of epithelial cells of some bile ducts and canaliculi in portal spaces. On day 3, immunocytochemical AFP staining became prominent in canalicular epithelial cells within portal and periportal areas and also in bile ducts (Figs. 2 and 3 and Table 3). In addition, faint AFP-positive reactions were observed on day 3 and day 4 within parts of the cytoplasm of some randomly distributed typical hepatocytes which did not show signs of degeneration (Fig. 4). On day 5, smaller numbers of AFP-positive canaliculi and bile ducts were found. Between day 6 and day 10, liver structure returned to normal and proliferation of canalicular epithelial cells had come to a standstill; during this time, AFP positivity disappeared rapidly. Finally, canalicular and bile duct epithelial cells and hepatocytes no longer stained for AFP.

In both experimental groups, intracellular AFP and its histotopological distribution were the same. Mesenchymal cells did not stain for AFP. Occasional AFP-stained cells of small size were seen distributed randomly in the section, apparently histiocytes which have phagocytized AFP-containing debris. AFP-positive reactions were also observed in parenchymal cells which showed signs of degeneration and which either formed groups of several cells or tubular-like structures. However, in control sections it was seen that such cells also stained for IgG globulins. The latter proteins are not synthesized by the liver cells and must have been taken from extracellular spaces. Since these cells might have taken AFP also from extracellular spaces, this type of staining was regarded as non-specific and was not taken into account.

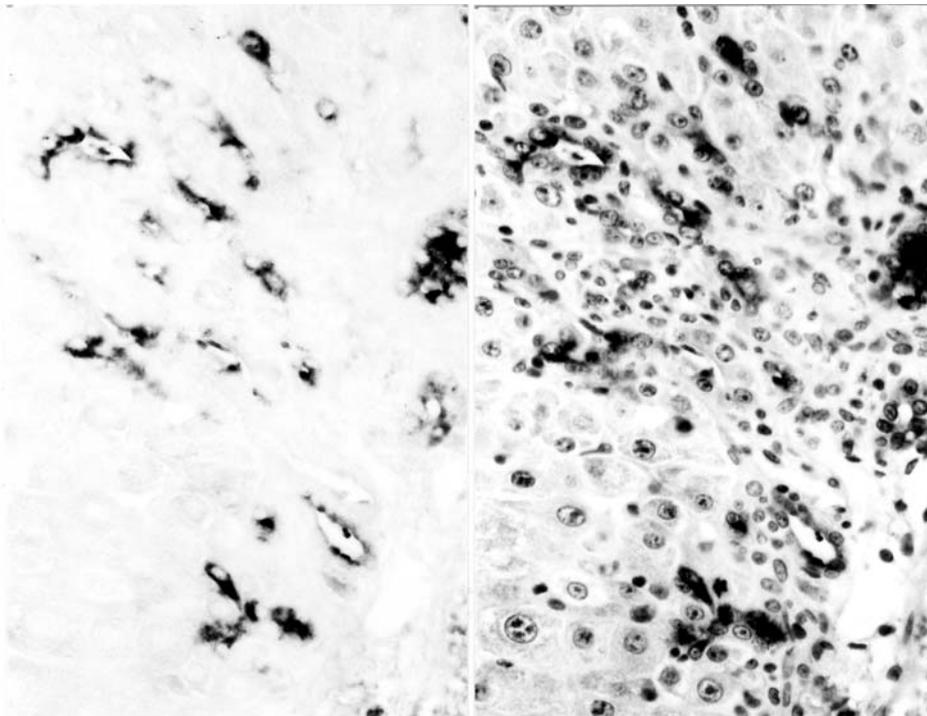


Fig. 2a and b. AFP-positive canalicular epithelial cells within portal tract and periportal area on day 3. Epithelial cells of bile ducts stain also for AFP. Original x 250. **a** Immunoperoxidase. **b** Immunoperoxidase, counterstained with haematoxylin

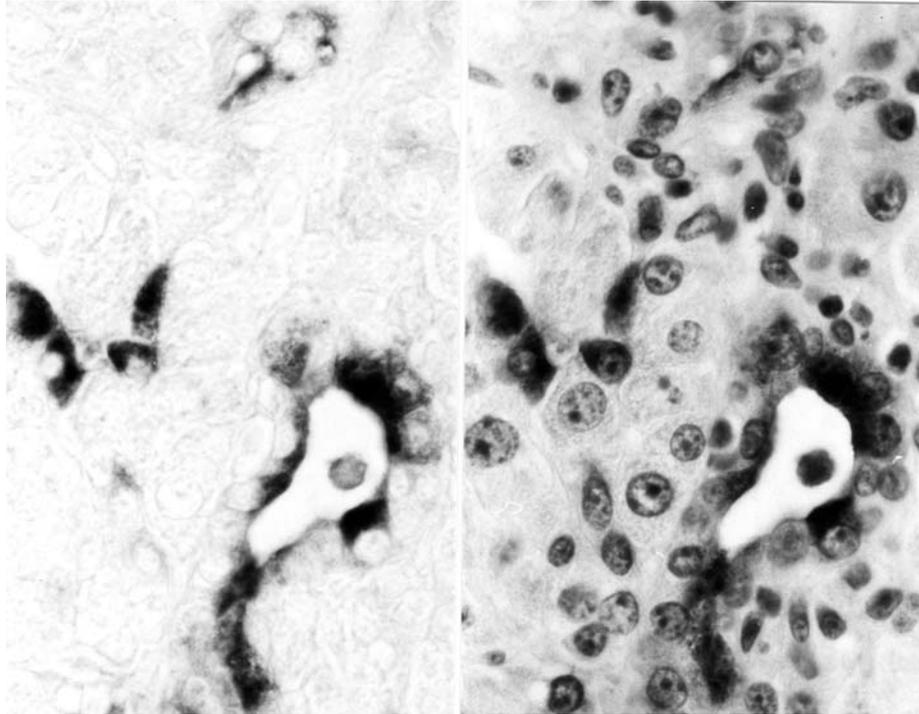


Fig. 3a and b. AFP-positive canalicular epithelial cells on day 4 in a portal tract area. Original x 540. **a** Immunoperoxidase. **b** Immunoperoxidase, counterstained with haematoxylin

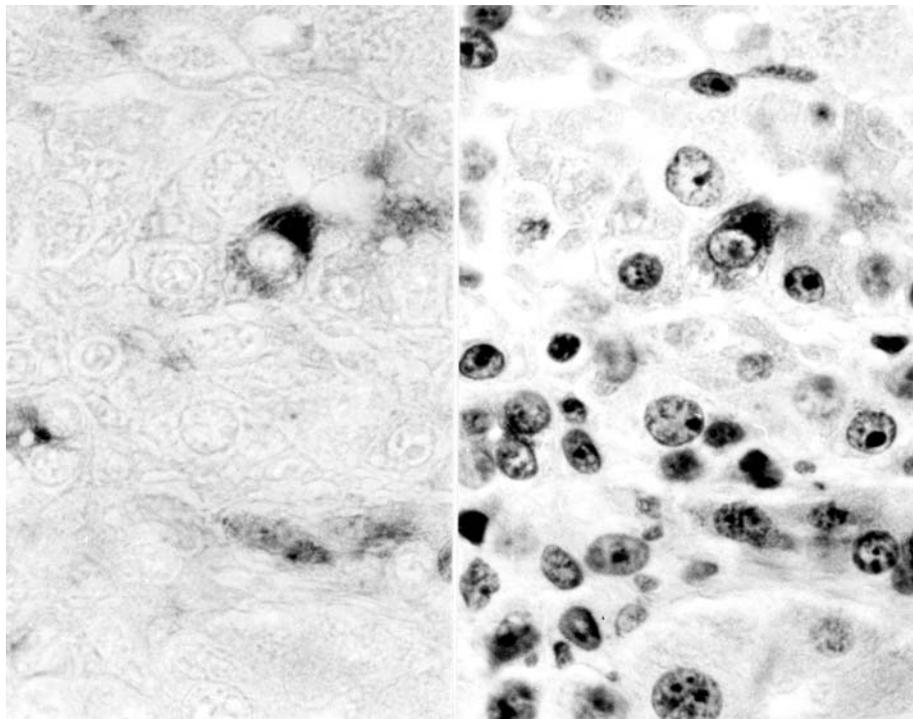


Fig. 4a and b. Randomly distributed hepatocytes show faint AFP positivity on days 3 and 4. Original x 540. **a** Immunoperoxidase. **b** Immunoperoxidase, counterstained with haematoxylin

Discussion

Intraperitoneal administration of galactosamine is known to produce dose-dependent liver cell injury. A low dose, e.g., 200 mg/kg body weight, causes biochemical disturbances in the liver without necrosis, whereas doses in the order of 350-400 mg/kg also provoke liver cell necrosis within 12 h (Farber et al., 1973). Thus, in the present study doses were chosen which have both effects. Histopathological descriptions of rat liver damage due to galactosamine and subsequent regeneration have been presented by Keppler et al. (1968), Lesch et al. (1970) and Medline et al. (1970). In general, their results are comparable with ours. The most striking histological changes were present in the first 4 days (cf., Tables 1 and 2). The cellular composition of the inflammatory infiltrates changed very fast. The predominant cells were histiocytes throughout the inflammation. These appeared very early and were the last cell type to disappear. Neutrophilic granulocytes predominated only after 24 h for a short time. At day 2, when neutrophilic granulocytes had already decreased, eosinophilic granulocytes were preeminent for 1 day, when their numbers decreased. Plasma cells were extremely uncommon in our material, they do not appear to be engaged in inflammation after liver injury by galactosamine.

Another interesting observation was the increasing number of cross-sections of canaliculi along the borders of portal tracts until day 4, when marked epithelial damage had occurred. This phenomenon might correspond to a true increase in canaliculi stimulated by the influence of regeneration, or alternatively correspond to prolongation of canaliculi as a phenomenon of regeneration, followed by marked bending and twisting. This would have the effect that histological slides showed more cross-sections of the same canaliculi. We favor the latter explanation because we did not find solid epithelial strands indicating formation of new ducts. Probably, marked mitotic activity of canalicular epithelial cells during the phases of regeneration between day 3 and day 5 led to hyperplasia of the duct epithelium. This resulted in prolongation, bending and twisting of canaliculi for reasons of space. Popper and Schaffner (1961) have observed that this increase in length may occur as a possibility of regeneration of canaliculi and bile ducts.

Optimal conditions for the immunolocalization of AFP were defined in previous papers and will not be referred to here (Kuhlmann, 1975; Kuhlmann, 1978). However, it must be emphasized that adequate control incubations are important in excluding false positive reactions due to passive uptake of secretory materials from extracellular spaces. Furthermore, immunocytochemical results are (like all cytochemical stainings) to some extent ambiguous due to limitations on the threshold of detection. Bearing this in mind, the non-reactivity of normal hepatocytes in the untreated controls is readily understood and can be reconciled with the fact that small amounts of serum AFP circulate in normal adult rats. These can be measured only by methods such as radioimmunoassay (Sell, 1973).

Enhanced serum AFP levels were found on day 2 after injection of galactosamine. Routine histology and immunoperoxidase staining of serial sections showed that bile canalicular epithelia were responsible for AFP production at this time. Later, on day 3 and day 4, immunohistological AFP reactions were at their most prominent in such epithelia. In both experimental groups, a strong correlation between reappearance of AFP in sera and intense signs of bile canalicular proliferation with concomitant AFP-positive staining was noted in individual rats. Liver sections from rats which were given low doses of galactosamine and which did not develop necrosis or regeneration plates (in experiments not described here) did not stain for AFP.

In addition to AFP staining in bile canalicular epithelia, faint but distinct AFP reactions were observed in randomly distributed hepatocytes between day 3 and day 5, although most of the hepatocytes remained unstained. The reason for the unstainability of the latter could be that the quantity of AFP produced by a single hepatocyte was too low to be detected. Generally, immunocytochemical reactions did not permit conclusions about quantities of AFP produced by either of the epithelia involved.

During the course of liver regeneration, the serum AFP levels observed were attributable to enhanced production of this fetus-characteristic protein in two distinct cell types: cells originating from the bile duct system and the hepatocytes themselves. AFP production is thought to result from (a) cellular repair and (b) alteration of the control of fetal genes. Adult hepatocytes regain the capacity to express a fetal protein (AFP) normally coded by genes in ontogenesis, but the mechanisms ("growth controlling factors") which led to temporary fetal gene expression remain unknown. Furthermore, immunocytochemical results have shown that in this experiment cells of the bile duct system proliferated and differentiated to a level of cytodifferentiation encompassing concomitant reactivation of the fetal genes which led to AFP synthesis. The reason for such bile duct differentiation is not clear. Ultimately, cellular AFP was no longer detectable in restored livers.

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