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α-Fetoprotein in Toxic Liver Injury[1]

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SUMMARY

The temporal sequence of α-fetoprotein appearance in serum was determined in both necrogenic and nonnecrogenic liver injury. Ethionine, thioacetamide, and CCl_4 were used to intoxicate male and female rats for evaluating serum enzyme levels, mitotic indices, and morphological reflections of impairment. Thioacetamide- and CCl_4-induced cell death preceded the mitotic wave in residual hepatocytes, and, in the case of both agents, this intoxicant-mediated necrosis preceded the emergence of α-fetoprotein. Yet, although there was no evidence of either cell destruction or significant mitotic activity in ethionine-poisoned animals, serum α-fetoprotein levels progressively increased. Thus the temporal sequence of α-fetoprotein synthesis and/or release and cellular reorganization for regeneration suggests that reappearance of the protein macromolecule is an expression of the altered phenotype observed during the "step-down" phase of liver regeneration.

INTRODUCTION

α-Fetoprotein is 1 of many hepatocyte-synthesized macromolecules secreted into the serum. Since its emergence is contemporaneous with liver cell proliferation, it may have potential use as a marker for determining growth and, possibly, identifying liver neoplasms (1, 12, 23). While compelling evidence derived from tissue culture experiments has pointed to the association of a specific phase of mitosis with the appearance of this protein (11), the situation in the intact animal, especially during maturity, is less clear (10, 14, 16, 17, 18, 21, 22). After hepatectomy and chemical injury, the polypeptide reappears at the time of liver regeneration, then disappears with maturation and healing, suggesting that α-fetoprotein production coincides with a "step-down" state distinguished by the sacrifice of "luxury" functions.

The mechanisms that regulate α-fetoprotein synthesis are unknown (23). In an attempt to define the prevailing conditions during synthetic induction, we undertook this study involving liver injury in rats, both in organs with loss of hepatocytes and in those without detectable cell loss.

Interestingly, not only those agents associated with the necrogenic effects, *e.g.*, TIAA[3] and CCl_4, but also toxins such as ethionine, which do not produce significant percentages of cell death, raised the levels of circulating α-fetoprotein. Moreover, we discovered a distinct asynchrony between cell death, enzyme release, mitosis, and serum α-fetoprotein levels. These findings intimate that the formation of α-fetoprotein is concomitant with modifications in phenotypic expression during reorganization for regeneration of cells.

MATERIALS AND METHODS

Male and female Sprague-Dawley rats, weighing 225 to 250 g and about 6 to 8 weeks of age, were obtained from Charles River Breeding Laboratory, Wilmington, Mass., or from Tyler Corp., Redmond, Wash. The animals were maintained on Purina laboratory chow and water *ad libitum* for 1 week before experimentation. They were given either CCl_4 (0.25 ml dissolved in an equivalent volume of mineral oil/100 g body weight) or TIAA (20 mg dissolved in water at 2 g/100 ml/100 g body weight) by stomach intubation without anesthesia. A divided dose (0 time and 1 hr) of ethionine (dissolved in 0.9% NaCl solution at 2.5 g/100 ml) was administered i.p. at 0.64 mmole/100 g body weight. The vehicle used for the toxins was delivered to control rats by the same routes as the toxic mixtures. In the case of CCl_4, 0.5 ml of mineral oil was administered per 100 g body weight, while TIAA in water was given in a dosage of 1 ml/100 g body weight. A volume of 0.9% NaCl solution equivalent to the dose of ethionine in 0.9% NaCl solution was used.

We sacrificed animals at intervals ranging to a maximum of 240 hr after intoxication. The rats were killed by exsanguination under light ether anesthesia, and blood was obtained by cardiac puncture for α-fetoprotein analysis and enzyme studies. Sections of the livers were fixed in neutral buffered formalin for histological examination.

For each point in time, a minimum of 3 rats were used, and for each time point or series of them, at least 3 control animals were also sacrificed. α-Fetoprotein was measured by radioimmunoassay, while levels of serum bilirubin, SGOT, SGPT, and alkaline phosphatase were all determined by standard methods (9, 19, 20). Our results are presented as mean ± S.D.

RESULTS

Ethionine intoxication elicited no light microscopic evidence of liver injury, nor was there any chemical evidence of hepatic injury demonstrable by changes in serum transaminase or alkaline phosphatase levels or by increased

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[3] The abbreviations used are: TIAA, thioacetamide; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase.

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serum bilirubin (Charts 1 to 3). Despite the absence of detectable histological injury or serum enzyme alterations in the liver, elevation of α-fetoprotein levels in both male and female animals became apparent 48 hr after the administration of the methionine analog (Chart 3). In both sexes, the polypeptide had reached heights of approximately twice the normal values at 48 hr and 3 times the normal levels at 72 hr. Male animals reacted by releasing more protein than did females.

TIAA, in the strain of rats and the dosage we used, produces a centrolobular necrosis, discernible by light microscopy between 8 and 12 hr after intoxication and more readily identified within 24 hr. Earlier than 8 hr, histological modifications are difficult to detect. Approximately the middle third of the liver lobule represents the site and extent of injury involved.

Changes in serum enzyme values became evident by 24 hr. Whereas SGOT and SGPT levels remained unaltered at 8 hr, by 24 hr they had increased roughly 50-fold (Chart 1, *A* and *B*). By 48 hr, serum enzyme levels had receded toward

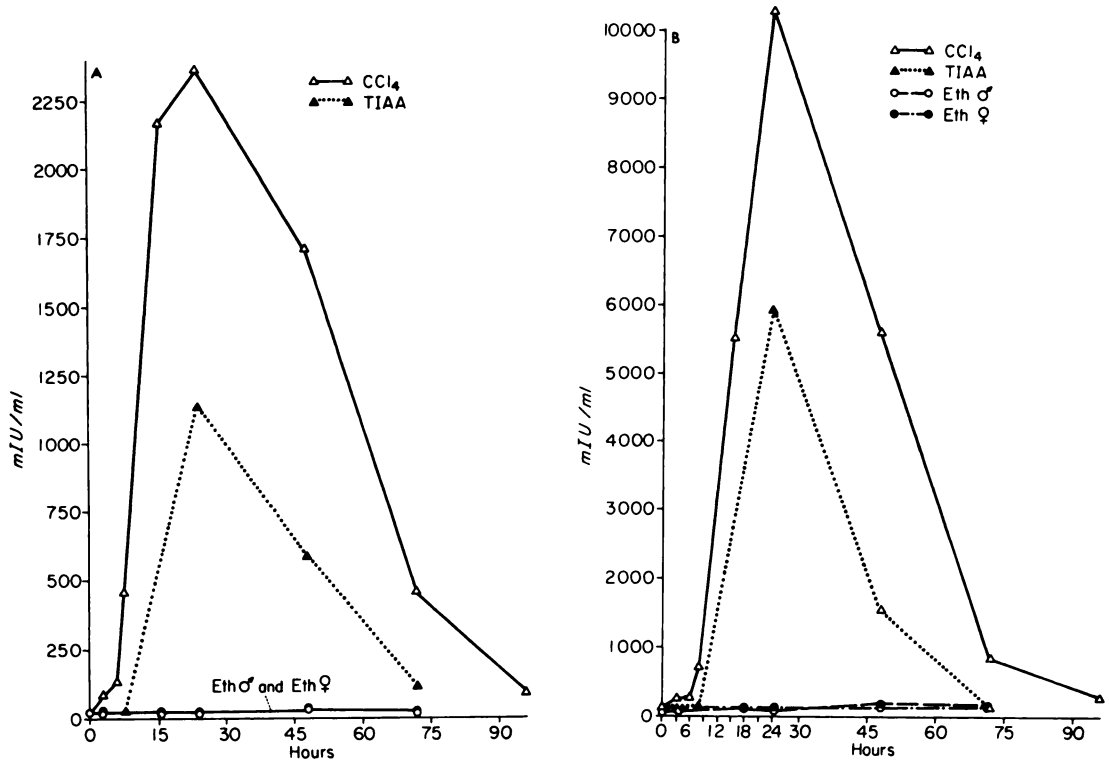

Chart 1. SGOT (*A*) and SGPT (*B*) following CCl_4, TIAA, and ethionine (*Eth*) intoxication. There were no significant differences in ethionine-treated animals and control rats in the levels of either SGOT (*A*) or SGPT (*B*). In contrast, CCl_4 and TIAA treatment resulted in markedly elevated activity in the serum between 15 and 60 hr.

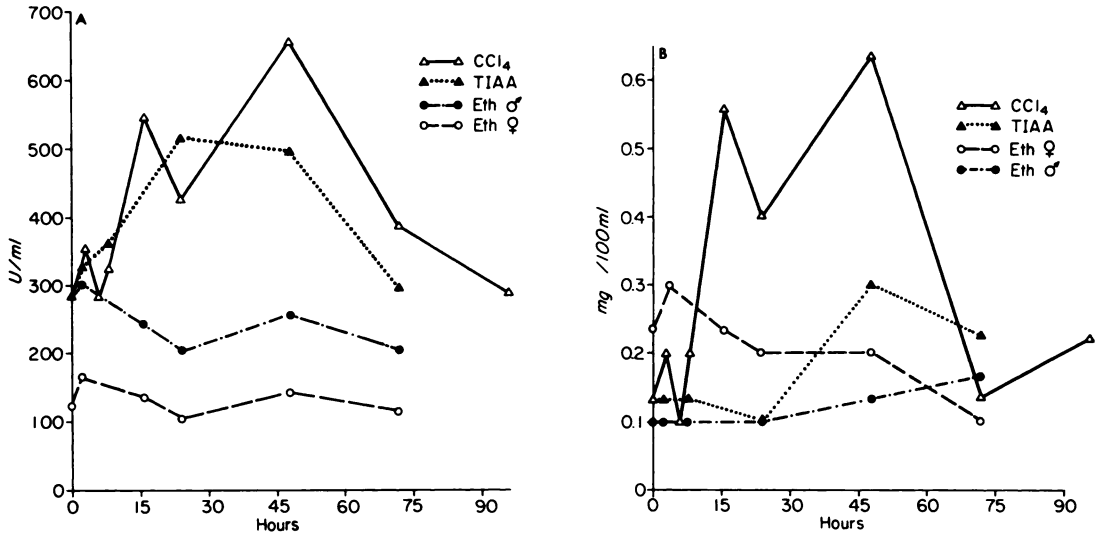

Chart 2. Serum alkaline phosphatase (*A*) and bilirubin (*B*) levels following CCl_4, TIAA, and ethionine (*Eth*) intoxication.

control values (which they attained by 72 hr). We identified a transient hyperbilirubinemia, and an earlier elevation in alkaline phosphatase occurred (Chart 2, *A* and *B*). The rise in α-fetoprotein commenced at 48 hr, when it was observed to be about 3 times the normal value, but by 72 hr, it had become remarkably elevated some 20-fold (Chart 3). This rocketing of the protein did not coincide with the peak of transaminase release or the initial burst of mitosis (which happens at 36 to 48 hr), but instead with the restitution of other chemically defined hepatocellular functions (3, 18).

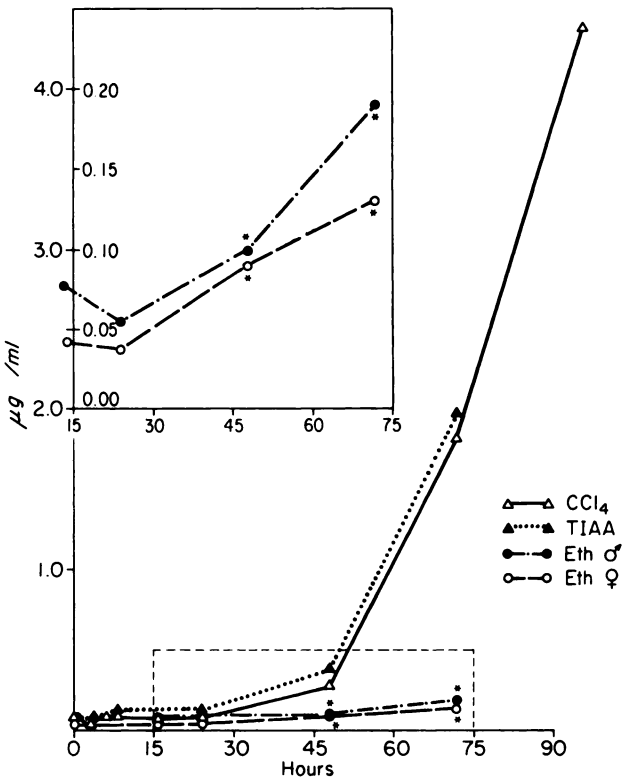

Chart 3. α-Fetoprotein levels following CCl_4, TIAA, and ethionine (*Eth*) intoxication. Ethionine treatment resulted in significant but low-level elevation in α-fetoprotein. *Upper left rectangle*, transformation of scale and expansion of the areas enclosed within the *dotted line*.

The difference in the time sequence between the appearance of α-fetoprotein and mitosis in the case of TIAA injury, as well as the constant increase in levels of the polypeptide during an interval of liver restoration, prompted critical analysis of the injury induced by a 2nd necrogenic agent.

CCl_4, like TIAA, in the dosage and with the rat strain used in our laboratory, produces a centrolobular lesion—with this toxin, notable by 3 hr (19). The 1st group of "dead" cells (that is, their plasma membranes are ruptured and the cytoplasm is severely distorted) is apparent at 6 to 8 hr. Pathological changes continue to develop for the next 12 hr, with further recruitment of dying cells. During the 2nd 24 hr, few, if any, new cells are altered; thereafter, healing occurs, with cellular proliferation. Modified serum SGOT values reflect the morphological changes (Charts 1, 3, and 4), with maximal alterations during the 1st 36 hr. At 24 hr, mitotic figures appear, reaching top frequency at 48 hr, then returning to near-normal levels by 72 hr. In fact, histological evidence of cell death and chemical evidence of enzyme leakage have all but disappeared by 72 hr. α-Fetoprotein, in contrast, does not reach peak activity until 4 days (96 hr) and requires 10 days to drop back to control levels (Chart 4). This is the same period during which specific liver-associated functions are restored (3).

DISCUSSION

All 3 hepatotoxins used in this study produce a liver disease associated with altered synthesis and/or release of α-fetoprotein. The time of its appearance and the quantity of α-fetoprotein in the serum suggest, but do not prove, that this protein is then being synthesized. The 3 agents act via different pathways and evoke different patterns of injury; in fact, ethionine seems to cause little or no cell death. This methionine analog also induces the least elevation of serum α-fetoprotein levels in both male and female rats. CCl_4 and TIAA produce a lesion with a predictable sequence in

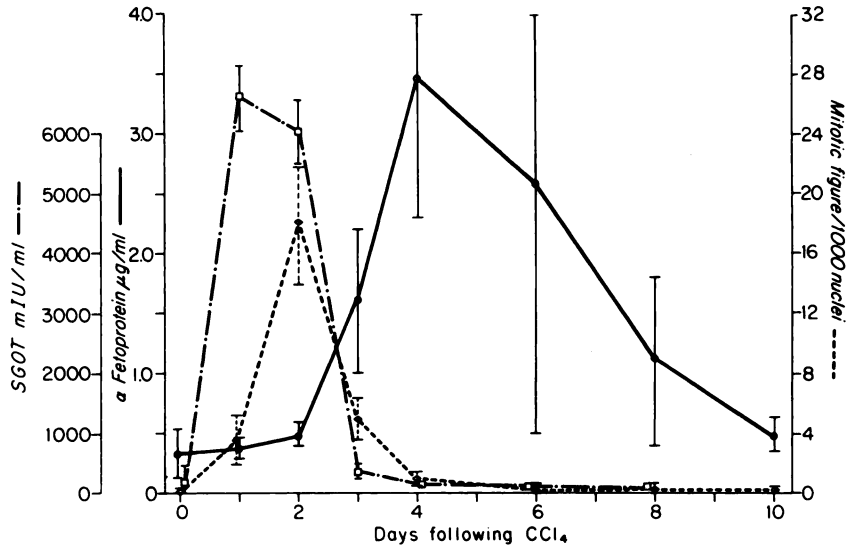

Chart 4. SGOT, mitotic indices, and α-fetoprotein levels in CCl_4 poisoning. Each *point* is the mean of 3 or more animals' values. *Bars*, S.D.

which cell destruction ceases by 24 to 48 hr. Mitotic waves precede the emergence of α-fetoprotein; indeed, cellular proliferation is complete before the highest values of this macromolecule can be measured in the serum. Indications of a similar elevation in α-fetoprotein before easily discernible changes are present have also been reported in studies of carcinogens (4, 5, 15, 25).

Healing of the liver after acute injury or partial hepatectomy involves both cellular replacement and restitution of specific cellular functions (7). The liver appears to be unique in the category of "mitotic" organs in that a clearly defined germinal or replicative pool cannot be histologically identified (8); nonetheless, its restorative potential is astonishing. Cells in the periportal and midzones (and perhaps even in the central area) all retain their capacity for proliferation, an observation emphasized by labeling patterns during regeneration (7). The morphological expression of singularity in these potential replicative or germinal cells may be particularly subtle or nonexistent. It may be that with the right stimulus, all hepatic parenchymal cells can proliferate.

In certain circumstances, liver cells destined for proliferation become altered in functional activity, and although the extent of such changes is considerable, their lobular distribution remains enigmatic (7). The extent of modification can be interpreted as a marked change in only a few cells or as a slighter modulation in many. Since the relative uniformity of cytoplasmic structural alteration and the random nature of mitotic cell recruitment suggest a more congruous change, we would conjecture that multiple rather than few cells are affected. The observable manifestation of this step-down is the sacrifice of the luxury functions acquired later in evolution (6), a pattern consistent with the liver's response in healing chemically induced injuries, in coping with partial hepatectomy, and in drug-mediated hypertrophy. The xenobiotic system's mode of restitution is a case in point. During the initial phase of regeneration, following either surgically or chemically induced loss of hepatocytes, mixed-function oxidase levels are modified (2, 24); and after hepatectomy, such an adjustment must reflect a physiological restructuring of this system, since no chemically induced cell injury is involved. The retrenchment results in structurally and functionally less "differentiated" cells.

The comparable presence of α-fetoprotein under similar circumstances implies that synthesis of the polypeptide also reflects the less differentiated phenotype. In accordance with this notion are the appearance and departure of α-fetoprotein during development, as well as in the newborn (1, 23), within an established time course following hepatectomy and observed in this report. The time curves of serum α-fetoprotein levels and liver mixed-function oxidase activity are chronologically related. While this formulation does not specify the biological significance of α-fetoprotein during healing, it does direct attention to a temporal-functional association. To date, only the role of an immunosuppressant has been proposed (13). Whether the emergence of this macromolecule during the healing of liver lesions is purposeful and allied with this event or whether α-fetoprotein merely reflects an accessory manifestation of the altered phenotypic expression of regeneration remains unresolved.

REFERENCES

1. Abelev, G. I. Alpha-fetoprotein in Ontogenesis and Its Association with Malignant Tumors. Advan. Cancer Res., *14:* 295–358, 1971.
2. Barker, E. A., Arcasoy, M. A., and Smuckler, E. A. A Comparison of the Effects of Partial Surgical and Partial Chemical (CCl_4) Hepatectomy on Microsomal Cytochrome b_5 and P_{450} and Oxidative *N*-Demethylation. Agents and Actions, *1:* 27–34, 1969.
3. Barker, E. A., and Smuckler, E. A. Altered Microsome Function during Acute Thioacetamide Poisoning. Mol. Pharmacol., *8:* 318–326, 1972.
4. Becker, F. F., Horland, A. A., Shurgin, A., and Sell, S. A Study of α_1-Fetoprotein Levels during Exposure to 3′-Methyl-4-dimethylaminoazobenzene and Its Analogs. Cancer Res., *35:* 1510–1513, 1975.
5. Becker, F. F., and Sell, S. Early Elevation of α_1-Fetoprotein in *N*-2-Fluorenylacetamide Hepatocarcinogenesis. Cancer Res., *34:* 248–249, 1974.
6. Bonney, R. J., Hopkins, H. A., Walker, P. R., and Potter, V. R. Glycolytic Isoenzymes and Glycogen Metabolism in Regenerating Liver from Rats on Controlled Feeding Schedules. Biochem. J., *136:* 115–124, 1973.
7. Bucher, N. L. R., and Malt, R. A. Regeneration of Liver and Kidney. New York: Little, Brown & Co., 1973.
8. Goss, R. J. Hypertrophy *vs.* Hyperplasia. Science, *153:* 1615–1620, 1966.
9. Henry, R. J., Cannon, D. C., and Winkelman, J. W. (eds.), Clinical Chemistry: Principles and Techniques, Ed. 2. Hagerstown, Md.: Harper and Row, 1974.
10. Hirai, H., Nishi, S., and Watabe, H. Radioimmunoassay of Alpha-Fetoprotein. Protides Biol. Fluids, *20:* 579–587, 1973.
11. Leffert, H. L., and Sell, S. $Alpha_1$-Fetoprotein Biosynthesis during the Growth Cycle of Differentiated Fetal Rat Hepatocytes in Primary Monolayer Culture. J. Cell Biol., *61:* 823–829, 1974.
12. Masseyeff, R. Human Alpha-Protein. Pathol. Biol., *20:* 703–725, 1972.
13. Murgita, R. A., and Tomasi, R. B., Jr. Suppression of the Immune Response by α-Fetoprotein. J. Exptl. Med., *141:* 269–286, 1975.
14. Nishi, S., and Hirai, H. Radioimmunoassay of α-Fetoprotein Hepatoma, Other Liver Diseases and Pregnancy. Gann, *14:* 184–192, 1973.
15. Onoe, T., Dempo, K., Kaneko, A., and Watabe, H. Significance of α-Fetoprotein Appearance in the Early Stage of Azo-Dye Carcinogenesis. Gann, *14:* 233–248, 1973.
16. Perova, S. P., Elgart, D. A., and Abelev, G. I. Alpha-Fetoprotein in the Sera of Rats after Partial Hepatectomy. Biull. Eskp. Biol. Med., *71:* 45–71, 1971.
17. Reddy, J., Chiga, M., and Svoboda, D. Initiation of the Division Cycle of Rat Hepatocytes following a Single Injection of Thioacetamide. Lab. Invest., *20:* 405–411, 1969.
18. Ruoslathi, E., Salaspuro, M., Pihko, H., Anderson, L., and Seppälä, M. Serum α-Fetoprotein: Diagnostic Significance in Liver Disease. Brit. Med. J., *393:* 527–529, 1974.
19. Sell, S. Radioimmunoassay of Rat α_1-Fetoprotein. Cancer Res., *33:* 1010–1015, 1973.
20. Sell, S., and Gord, D. Rat α-Fetoprotein. III. Refinement of Radioimmunoassay for Detection of 1 ng Rat α_1F. Immunochemistry, *10:* 439–442, 1973.
21. Sell, S., Nichols, M., Becker, F. F., and Leffert, H. L. Hepatocyte Proliferation and α-Fetoprotein in Pregnant, Neonatal, and Partially Hepatectomized Rats. Cancer Res., *34:* 865–871, 1974.
22. Sell, S., Reynolds, R. D., and Reutter, W. Rat $Alpha_1$-Fetoprotein: Appearance after Galactosamine-Induced Liver Injury. J. Natl. Cancer Inst., *53:* 289–291, 1974.
23. Sell, S., and Wepsic, H. T. α-Fetoprotein. *In:* F. F. Becker (ed.), The Liver: Normal and Abnormal Functions, Vol. 5, Part B, pp. 773–820. New York: Marcel Dekker, Inc., 1974.
24. Smuckler, E. A. Alterations Produced in the Endoplasmic Reticulum by Carbon Tetrachloride. Minerva Med., in press.
25. Taketa, K., Watanabe, A., and Kosaka, K. Different Mechanisms of Increased α-Fetoprotein Production. Ann. N. Y. Acad. Sci., *259:* 80–89, 1975.

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