day the weight of the PDLN decreased and by the 30th day it was equal to the control value. Doses of 5×10^6-10×10^6 living SC of the parental genotype were thus optimal for induction of the GVHR in the liver, but on the 5th-7th days they were optimal for reading the reaction.

The results show that intrahepatic injection of SC of the parental genotype into F_1 hybrids is accompanied by specific hypertrophy of PDLN caused by the development of a GVHR in the liver. On the 5th-7th days of the reaction the degree of hypertrophy of PDLN depends on the dose of cells injected. Doses of 5×10^6-10×10^6 living SC of the parental genotype are optimal for induction of the GVHR in the liver and development of hypertrophy of the PDLN. On this basis, a new method of quantitative analysis of the strength of GVHR in the liver, allowing the role of the liver to be studied in transplantation immunity, can be suggested.

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ULTRASTRUCTURAL LOCALIZATION OF α-FETOPROTEIN SYNTHESIS IN THE REGENERATING MOUSE LIVER

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KEY WORDS: Liver; regeneration; α-fetoprotein; immunocytochemistry; ultrastructure.

After partial hepatectomy or administration of various hepatotoxins there is a sharp but transient rise in the α-fetoprotein (AFP) level in the blood, and cells containing AFP can be found at these times in liver sections. By means of ordinary immunomorphological methods the AFP-containing cells have been characterized as typical differentiated hepatocytes. In adult mice after treatment with hepatotoxins as a rule such cells are few in number and they are located mainly at the boundary with the injured tissue [3, 5, 6]. These observations suggest that during regeneration of the mouse liver temporary derepression of AFP synthesis takes place in mature differentiated hepatocytes. However, it is only through the use of an immunoenzyme technique for localizing antigens at the electron-microscopic level that cells synthesizing AFP can be reliably differentiated from cells passively accumulating AFP as a result of toxic injury. This technique was used previously to reveal AFP-synthesizing cells in human and murine hepatomas, in the human fetal liver, and in the liver of animals during chemical carcinogenesis [8, 9, 13, 14].

This paper describes the use of an electron-microscopic immunoperoxidase method [10] to identify and characterize AFP-synthesizing cells during regeneration of the mouse liver.

EXPERIMENTAL METHOD

Regeneration of the liver was induced in SWR mice aged 2-3 months by poisoning with CCl_4 vapor [2]. The animals were killed 72 h after poisoning. Rabbit antisera (AS) against a purified preparation of mouse AFP were exhausted with a sorbent of normal mouse serum on a basis of sepharose 4B-CNBr, after which monospecific antibodies (AB) against AFP were iso-

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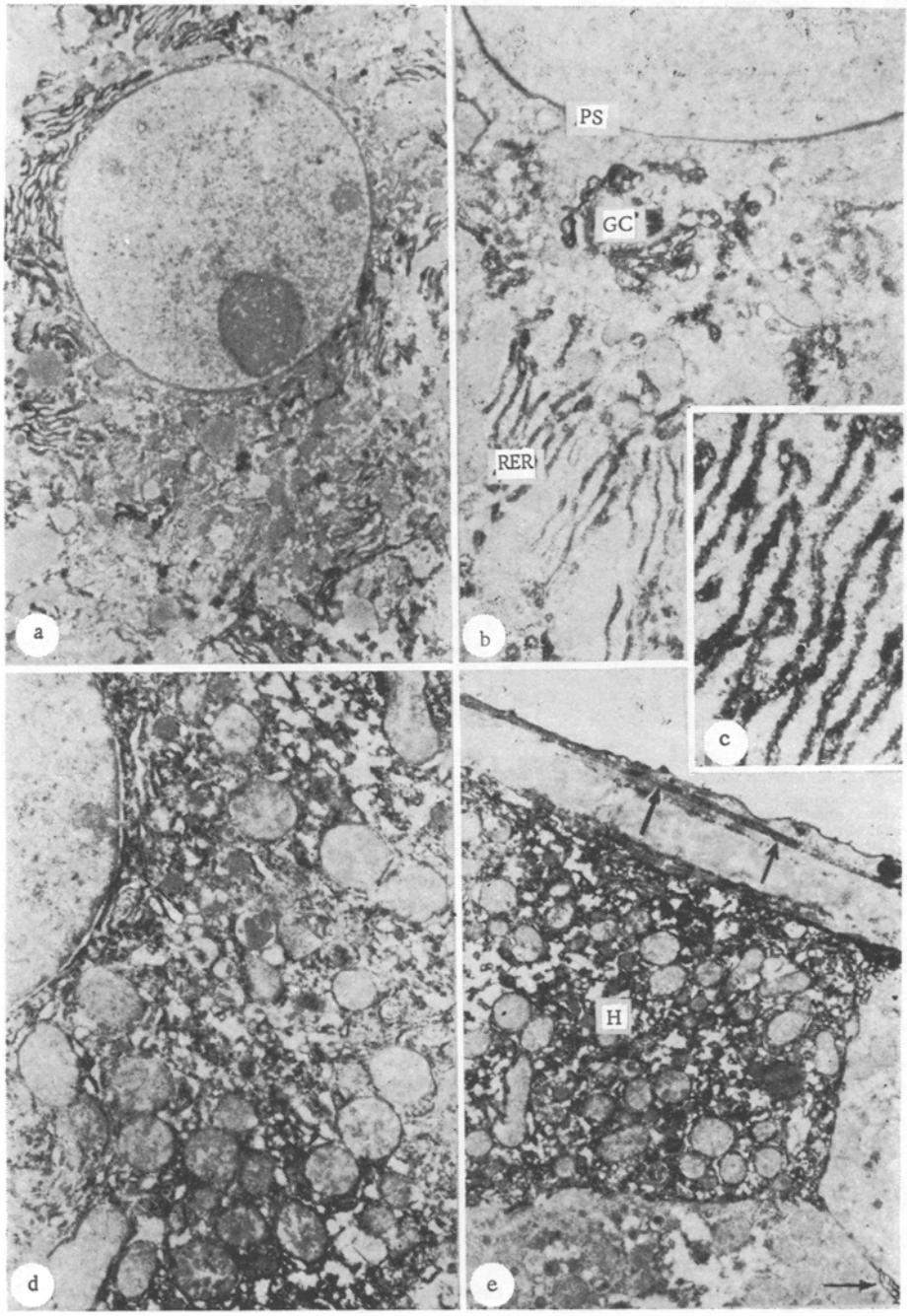

Fig. 1. Immune electron microscopy of mouse liver 72 h after administration of CCl_4. a) Fragment of mature AFP-containing hepatocyte in perinecrotic zone, 5000 ×; b) localization of AFP in perinuclear space (PS), on ribosomes and membranes of rough endoplasmic reticulum (RER), in lumen of cisterns of RER and elements of Golgi complex (GC). 15,000 ×; c) localization of AFP on membranes and ribosomes and in lumen of RER. 30,000 ×; d) fragment of hepatocyte in perinecrotic zone with diffuse deposition of reaction product in cytosol. 12,500 ×; e) Localization of mouse IgG in Disse's space, intercellular spaces (arrows), and cytosol of hepatocyte (H). 5000 ×.

lated on a sepharose sorbent of neonatal mouse serum. Some of the AB against AFP were neutralized with a small excess of pure AFP [1]. Rabbit AS against mouse IgG was diluted in the ratio of 1:100 with buffered physiological saline (BPS). Goat AB against rabbit IgG

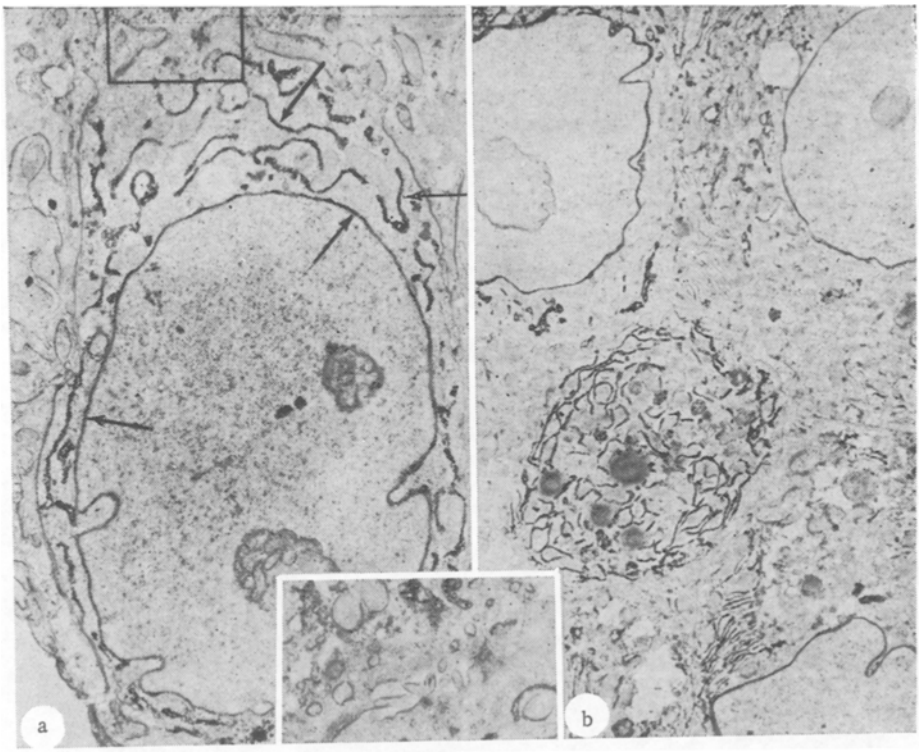

Fig. 2. AFP synthesis in small hepatocytes of perinecrotic zone. a) Localization of reaction product in perinuclear space and cisterns of RER (arrows) of "small hepatocyte." 7500 ×; inset — bile capillary, 15,000 ×; b) group of "small hepatocytes," 4000 ×.

were conjugated with horseradish peroxidase (RZ = 2.75; from Sigma, USA) by a periodate method [11].

Pieces of liver measuring 1 mm^3 were prefixed in a cold mixture of 8% paraformaldehyde and 0.05% glutaraldehyde for 20 min, then fixed with 8% paraformaldehyde for 16 h with constant mixing and at a temperature of 4°C. The fixatives were made up in 0.1 M cacodylate buffer, pH 7.3, with the addition of 7% sucrose. After washing for 24 h with the same buffer, the pieces of liver were frozen in liquid nitrogen and cryostat sections 15 μ thick were cut. From 20 to 25 freely floating sections were incubated in 0.5 ml of a solution of antibodies against mouse AFP for 2 h at room temperature with gentle mixing. As a control of the specificity of the reaction some sections were incubated either with neutralized AB against AFP or with AS against mouse IgG. After incubation the sections were rinsed 3 times with BPS, washed for 1 h in three changes of BPS, and incubated in 0.5 ml of AB against rabbit IgG conjugated with peroxidase. The sections were then washed for 14-16 h in cold BPS, rinsed with deionized water, and stained for peroxidase activity with 3,3-diaminobenzidine [7]. After rinsing 3 times with water, the sections were fixed for 1 h in 1.33% osmium tetroxide solution in 0.1 M phosphate buffer, pH 7.3, rapidly dehydrated in acetone solutions of increasing concentration, and embedded in a mixture of Epon and Araldite. Semithin and ultrathin sections were cut on the LKB III Ultratome. Different areas of the liver were chosen on unstained semithin sections for subsequent electron-microscopic analysis and blocks were trimmed appropriately. Ultrathin sections were examined without additional staining with the JEM-100CX electron microscope.

EXPERIMENTAL RESULTS

On electron-microscopic investigation a positive intracellular reaction for AFP took the form of localized electron-dense precipitates of the reaction product.

Most hepatocytes in the experimental material did not contain AFP. AFP was found only in a small percentage of hepatocytes which, as a rule, were located in a narrow (2-3 layers of cells) zone of parenchyma at the periphery of foci of necrosis in the center of the hepat-

ic lobule. Cells containing AFP were either directly bordering on foci of cell debris or were at a distance of one or, at most, two hepatocytes away from them. Sometimes hepatocytes with AFP were located beneath the endothelium of the venous sinuses, the wall of which was involved in the necrotic focus. Most hepatocytes containing AFP had the ultrastructural features of typical differentiated hepatocytes, with large nuclei, a considerable volume of cytoplasm, and a mean diameter of 20-25 μ (Fig. 1a). AFP in these cells was found in the perinuclear space, on the membranes and ribosomes of the rough endoplasmic reticulum (RER), and in the lumen of its cisterns and of elements of the Golgi complex (Fig. 1b, c). However, the degree and extent of spread of staining of the above-mentioned organelles with reaction product varied from cell to cell. In some hepatocytes AFP was found chiefly in the cisterns of the RER, in others in the Golgi complex, consisting of five or six separate groups of elements. Some hepatocytes were seen in which AFP was present only in the perinuclear space or (in very small quantities) on membranes and ribosomes of single, narrow cisterns of the RER. It is evident that hepatocytes with a trace reaction of this sort could not be detected under the light microscope.

The presence of AFP in the test material was not confined to typical mature hepatocytes. AFP also was found in the perinuclear space and cisterns of the RER of smaller cells, 10-15 μ in diameter, with a large oval nucleus and a small volume of cytoplasm (Fig. 2a). These cells had no definite localization, they were usually single, but sometimes they formed groups of three or four cells (Fig. 2b). These small cells must evidently be regarded as "small hepatocytes," for typical bile capillaries were constantly found both between adjacent mature and "small" hepatocytes (Fig. 2), and in groups of the latter.

Besides the local deposits of reaction product described above a large area of deposits or diffuse precipitation of the product in the cytoplasmic matrix (cytosol) and on membranes of organelles was observed in a few isolated hepatocytes, without any reaction product to be seen in the lumen of the organelles (Fig. 1d). In this same zone a similar diffuse distribution of reaction product was found in some of the hepatocytes after incubation of the sections with AB against IgG (Fig. 1e), evidence in support of the passive penetration of IgG and AFP into these cells from the blood as a result of their toxic injury. Outside the cells IgG was present on the surface of the endothelium of the venous sinuses, in Disse's space, and in the intercellular spaces of the hepatic trabeculae as far as the zone of dense junctions (Fig. 1e).

Sometimes hepatocytes containing AFP could be observed both in the cytosol and in the lumen of the cisterns of the RER. These cells did not have evident ultrastructural signs of injury. This type of localization of AFP could be attributed either to necrobiotic changes in the AFP-synthesizing cells or their inadequate fixation.

The reaction product was found in the control series of sections, treated with AB against AFP neutralized with pure antigen, both in peroxisomes and lysosomes of hepatocytes (endogenous peroxidase) and in erythrocytes (peroxidase-like activity).

The investigation thus confirmed the results of light-optical observations [3, 5, 6] and showed that derepression of AFP synthesis does in fact take place in the regenerating mouse liver. The main site of AFP localization is the cytoplasm of a small number of mature differentiated hepatocytes in the perinecrotic zone. The presence of AFP in the perinuclear space, on membranes and ribosomes of the RER, and in the lumen of cisterns of the RER and elements of the Golgi complex suggests that these cells synthesize AFP. In addition, smaller cells, which the writers have called "small hepatocytes," also take part in AFP synthesis under these conditions. In their ultrastructure these cells resemble the oval cells described in the rat liver during chemical carcinogenesis [14]. The discovery of the role of "small hepatocytes" in the regeneration process in the liver and in hepatocarcinogenesis is of great interest and requires further study.

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ACTION OF FIBRONECTIN ON MORPHOLOGY AND CYTOSKELETON OF HEPATIC EPITHELIAL CELLS IN CULTURE

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Fibronectin is one of the basic surface proteins of different types of cells [1, 3]. There is considerable evidence that it participates directly in the mechanism of adhesion of cells to a substrate. For instance, its addition to certain cultures of both nontumorigenic and tumorigenic fibroblasts, which have weak adhesive properties and do not possess fibronectin on their surface, leads to an increase in their adhesiveness and, evidently as a result of this, to their morphological normalization [5, 7]. Additionally, as a rule in such normalized cells the bundles of microfilaments which are absent in transformed cells are restored [5].

Most investigations into the role of fibronectin in cell biology have been carried out in the past on cultures of mesenchymal origin [7, 11,]3]. The action of fibronectin on epithelial cells has not been investigated. To examine this problem, in the research described below nontumorigenic and tumorigenic lines IAR of hepatic epithelium were used. The morphological features, cytoskeleton, surface proteins, and many other characteristics of these cells have been fully described previously [2, 4, 9].

EXPERIMENTAL METHOD

Hepatic epithelium of line IAR 2 was obtained in culture in 1973 [8] from a primary culture of rat hepatocytes of line BD4. After treatment in culture with N-methyl-N-nitro-N-nitrosoguanidine, tumorigenic line IAR 2-31 was obtained from this line [6, 8]. Line IAR 6-IRT 7A was obtained from a clone grown in methyl cellulose from cells of adenocarcinoma IAR 6-IRT 7. The history of this line has been fully described previously [2]. All cells were grown in Williams E medium (from Flow Laboratories, England), with the addition of 10% embryonic calf serum (from Gibco, England) and 100 units/ml monomycin.

Fibronectin was isolated from the plasma of noninbred albino rats, using two-stage affinity chromatography on gelatin-sepharose and on arginine-sepharose by the method in [12]. The final preparation contained electrophoretically pure fibronectin with a molecular mass of about 250 kilodaltons. It was concentrated to a fibronectin concentration of 1 mg/ml and dialyzed overnight against Hanks' solution.

Fibronectin was added to the cultures in a final concentration of 50 μg/ml, either directly to the medium to the already growing cells, or to the substrate for 30 min before

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