Ultrastructural Changes in Rat Livers Induced by Repeated Injections of Trypan Blue

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SUMMARY

Repeated injections of trypan blue into the s.c. tissue of inbred Wistar rats induced tumors in the liver. A sequence of morphological changes has been observed during induction of the tumors, and these were studied ultrastructurally. The earliest recognized change was the appearance in the portal tracts of focal collections of monocytic cells that resembled Kupffer cells. They appeared either to collect in the periporal connective tissue or to migrate through the endothelial wall and collect within the portal venules or lymphatics. This caused periporal cyst formation and pressure atrophy of the surrounding hepatocytes. No mitotic activity was seen in the monocytic cells, but their numbers increased and occluded lymph flow. The more numerous these cells became the less did they resemble Kupffer cells and the more they came to resemble cells of the established tumors. Collagen fibrils and bundles as well as fibrin appeared among the cells. Subsequently, nonperiodic fibrillar material was also seen around the cells. The cellular areas were then indistinguishable from established primary tumors. The morphological changes seen in the liver resemble those reported in histopathological descriptions of Kupffer cell sarcoma in humans.

INTRODUCTION

In 1949 Gillman et al. (4) described the induction of tumors in the livers of rats by repeated injections of trypan blue. The tumors were classified as reticulum cell tumors. They were transplantable (11), and the cells of transplanted s.c. tumors and metastatic renal tumors were similar morphologically and in enzyme content and were associated with extracellular material similar to those of the primary tumors (7).

A sequence of morphological changes has been shown to occur in the liver during tumor induction. The early changes were present after a total of 50 mg of trypan blue had been administered by s.c. injection and late changes after 150 mg of trypan blue had been administered (4, 5). This paper describes the ultrastructure of these changes and discusses their relevance to other reticuloendothelial tumors.

MATERIALS AND METHODS

Twenty-five male Wistar rats were given repeated injections of trypan blue, and 12 of these rats were used in this study. The rats were from an inbred strain that had been maintained at the Brabraham Laboratories of the Agricultural Research Council for 6 years and had originally been purchased from the Medical Research Council Animal Laboratories, Carshalton, Surrey, England. The trypan blue was from the same batch of Grubler’s dye as used in previous studies (4–6, 8). Each rat received s.c. injections of 10 mg of dye freshly dissolved in 1 ml of glass-distilled water. The injections were given once every other week and commenced when the rats were 9 to 10 weeks old and weighed approximately 220 g. Eight rats, weighing approximately 330 g each at the time of biopsy, were subjected to liver biopsies when they had received a total of 50 mg of trypan blue over 10 weeks. Four rats, weighing approximately 360 g each at the time of biopsy, were subjected to liver biopsies when they had received a total of 150 mg of trypan blue over 30 weeks. At biopsy, pieces of liver tissue were removed from each of these rats under halothane anesthesia. Liver tissue was also removed from two 19-week-old and 40-week-old untreated rats of the same strain. One-half of the tissue was placed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 5 min and reduced in thickness to a maximum of 1.5 mm; the fixation continued at 4° for a total of 3 hr. The tissues were transferred to the phosphate buffer and washed with continuous agitation overnight. They then were embedded in agar, chopped into 50-μm slices, and postfixed in 1% buffered osmic acid for 1 hr. The slices were dehydrated through graded alcohols, infiltrated with Araldite, and embedded (9). After polymerization was complete, the slices were examined by phase-contrast microscopy and portal tracts of pathologically altered liver tissue were marked for examination. Sections 0.5 to 1 μm were cut from these areas with an LKB Ultratome and stained with 0.1% toluidine blue in 0.1% sodium tetraborate at 60° for 5 min; suitable areas then were chosen for ultrathin sectioning. These sections were stained with a solution of 20% uranyl acetate (analytical grade) in absolute methanol for 10 min, washed in deionized water, restained in Reynolds lead citrate (19) for 5 min, and examined with an Hitachi 7S electron microscope. The other one-half of the tissue was fixed in 4% buffered formaldehyde, dehydrated, and embedded in paraffin wax. Sections from these blocks were stained with hematoxylin and eosin to ensure that representative lesions were being studied.

RESULTS

The macroscopic and microscopic changes that occur during the induction period have been described (4, 5). These changes
were not uniformly distributed throughout the liver. The earliest changes could be seen in one area of a liver that showed more advanced changes in adjacent areas. This suggested that a progressive lesion had been induced by trypan blue, and the following morphological descriptions are based on this conclusion.

Apart from some congestion, the external surface of the liver appeared macroscopically normal even after 5 injections of 10 mg of dye had been given. On section, however, blue areas 0.5 to 1 mm in diameter were seen on the cut surface. Microscopically, the earliest detectable lesion in the liver sections consisted of collections of a few monocytic cells in 1 corner of several portal tracts (Fig. 1). As the lesions progressed, the monocytic cells became more numerous and lay in cystic spaces round the portal tracts (Fig. 2). The cysts became larger and sometimes more cellular and replaced parts of the surrounding hepatic lobules (Fig. 3). An eosinophilic material appeared within the cysts which then became more cellular. Elongated cells with large, pale nuclei appeared among the monocytic cells, filled up the cysts, and extended into the hepatic lobules. Mitotic activity was not seen in any of these cellular areas until they were densely cellular and several hepatic lobules had been destroyed. Liver biopsies from rats that had received 15 injections of 10 mg of dye showed that the liver was partly replaced by a variable mixture of small and large cysts and tumors. These occurred throughout the liver and most appeared to have originated in a portal tract rather than in a hepatic lobule. The tumors consisted of pleomorphic cells loosely arranged in fibrillar connective tissue (Fig. 4). Numerous small cysts and tumors were present also immediately under the serosal surface.

Electron Microscopy

**Portal Tract of Untreated Rats.** The ultrastructure and arrangement of structures in the portal tracts from the 2 untreated rats were similar to those described by Rouiller and Jezequel (20). In the perportal connective tissue 2 types of cells were recognized. One type (Fig. 5, A) had a densely heterochromatic irregular nucleus and a small amount of cytoplasm that contained a few mitochondria and some dilated endoplasmic reticulum. These were similar to “adventitious connective tissue cells” described by Schnack et al. (23). The other cell type (Fig. 5, R) was larger, with an oval or elongated, less heterochromatic nucleus and with abundant cytoplasm. The perinuclear cytoplasm contained a few mitochondria, a little rough endoplasmic reticulum, and numerous ribosomes. The peripheral cytoplasm contained many filaments but few organelles and extended into short or long pseudopodial processes between collagen bundles and other portal structures. These cells were most numerous round bile ducts. They were similar to the reticuloendothelial cells described by Nicolson and Rouiller (16), and Rouiller et al. (21).

**Portal Tracts of Treated Rats.** The earliest abnormality to be detected was the presence of a few monocytic cells in a space lined by endothelial cells in the portal tracts. The predominant monocytic cell had an irregular nucleus, approximately 5 μm in diameter, with abundant margination of heterochromatin and very rarely a nucleolus. The cytoplasm was extensive and contained numerous vacuoles with electron-dense contents, free ribosomes, rough and smooth endoplasmic reticulum, and a few mitochondria. The cytoplasm that extended into long or short pseudopodial or filamentous processes protruded in many directions from the cell surface and entwined with similar processes of adjoining cells. Occasional lymphocytes were present also; but red cells, polymorphonuclear leukocytes, and platelets were not seen. The spaces around the cells were free of electron-dense material and appeared empty (Fig. 6). The space was limited by flattened cell processes of endothelial cells devoid of basement membrane and by the monocytic cells or their processes which were in direct contact with underlying collagen bundles. Similar monocytic cells were present in the surrounding connective tissue. The other structures in the portal tracts were normal. The hepatocytes and the sinusoidal endothelial cells adjacent to the portal tracts were normal. Kupffer cells formed part of the sinusoid wall. The cells had similar nuclei and their cytoplasm contained similar organelles to those in the monocytic cells. The Kupffer cells, however, did not have the numerous pseudopodial processes of the monocytic cells.

The monocytic cells became more numerous in the perportal connective tissue and migrated into portal venules but not into the spaces of Disse or along the sinusoids. The cells became more pleomorphic; some cells contained numerous phagosomes, whereas others contained none. The spaces surrounding them contained finely granular material and fibrin. Collagen fibers and their bundles appeared in the extracellular spaces (Fig. 7). Hepatocytes adjacent to these areas underwent both atrophic and hypertropic changes but finally became necrotic. The pleomorphic monocytic cells extended along the sinusoids as the hepatocytes disappeared. From this stage onwards one cell type became predominant. The cells had elongated, irregular nuclei with some margination of heterochromatin and nucleoli. The cytoplasm was extensive and contained numerous mitochondria, scattered profiles of smooth and rough endoplasmic reticulum, occasional vacuoles, and few phagosomes. Pseudopodial processes extended from the cell surfaces into the amorphous surrounding ground substance (Fig. 8). The cells, some of which were binucleate, still varied considerably in size and shape. They were either packed closely together or arranged loosely in a granular ground substance in which electron-dense, nonperiodic fibrillary material was seen (Fig. 8, inset). As the tumors enlarged, the tumor cell nuclei became larger and the cytoplasm contained fewer organelles.

**DISCUSSION**

The lesions produced in the liver of inbred Wistar rats by trypan blue are usually present before histological abnormalities are seen in lymph nodes, bone marrow, or peripheral blood (4–6); but local infiltration by small numbers of mononuclear cells would be easier to detect in portal tracts of the liver, which are almost devoid of such cells, than in lymph nodes or bone marrow, which already contain large numbers of mononuclear cells. The 1st change detected in the...
tumor process was the focal collection of cells that resembled Kupffer cells (15). The subsequent tumor was derived from these cells and so could be classified as a Kupffer-cell sarcoma (3). This tumor has been described in Moselle wine growers (22), patients receiving Fowler's solution (18) (both due to long exposure to arsenic), patients exposed to Thorotrast (13, 17), and patients exposed to urethan (24). In all of these conditions ingestion of foreign material by Kupffer cells could occur. A standard procedure for demonstrating these cells in situ is by their intravital uptake of trypan blue. The histopathology of the liver in Kupffer-cell sarcoma and the involvement of portal lymph node, spleen, and lung has been reviewed recently by Blackwell and Joske (1). There was no evidence of leukemia in any of these cases, but the histological appearance of the liver tumors resembled those seen in the trypan blue-induced tumors in the livers of rats.

The origin of Kupffer cells is not known. Evidence suggests that they arise from either thoracic duct cells (10) or from precursors in the bone marrow (12). If trypan blue initiated neoplastic transformation of these precursors, it would be difficult to detect in situ because of the problem of locating such cells in diffuse cellular tissues. Early in the induction period abnormal Kupffer cells collected in the portal areas, while surrounding adjacent hepatocytes appeared morphologically normal. The abnormal cells were not, therefore, part of the response of the rat to hepatocellular damage, but their presence led to local alteration and possibly blocking of blood flow in terminal portal tracts. This in turn led to hepatocellular damage. Some abnormal cells contained numerous phagosomes in their cytoplasm, others contained a few, and some cells were without phagosomes. The longer the rats were exposed to repeated doses of trypan blue the less well differentiated did the abnormal cells become. No mitotic activity was seen in the abnormal cells in the portal tracts (4). Trypan blue appeared to stimulate Kupffer cell precursors, either directly or indirectly, to produce cell lines with different cytoplasmic capabilities. Cells of one line that had retained a phagocytic capability may have been stimulated to become hypertropic and actively phagocytic when surrounded by necrotic and fragmenting hepatocytes. This proliferative stimulus would be lost once the cellular debris had been removed. Then the proliferation may have been stimulated of another cell line that had not retained a phagocytic capability and that preferred to grow in semisolid rather than fluid conditions that followed the formation of fibrin and collagen in the cysts. The appearance of fibrin and collagen in sinusoids has been reported in toxic conditions (2) and appears to be a feature of hepatocellular disorders that affect the endothelium of the liver sinusoids (23). Reticuloendothelial cells in the portal tract connective tissue the morphology of which was intermediate between that of endothelial cells and Kupffer cells have been reported (21). Such cells become hyperplastic after injections of zymosan, a yeast hydrolysate, and resemble morphologically the abnormal monocytic cells seen in trypan blue-treated rats (4).

In previous papers, Gillman et al. (5, 6) have presented evidence suggesting that the neoplastic cells of tumors induced in rats by trypan blue are abnormal histiocytes that arise from endothelium. The present paper does not demonstrate an endothelial origin for the tumor, but it does show morphological similarities between this tumor and those induced by arsenic or Thorotrast. Trypan blue is not a pure chemical compound (14). The isolation of a carcinogenic fraction or fractions from this dye might simplify further studies on a dye that is both a teratogen and an inducer of proliferative changes in reticuloendothelial tissues of rats.

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REFERENCES


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Fig. 1. Part of a portal tract showing a portal vein (v) surrounded by numbers of monocytic cells (m) situated in periportal connective tissue (50 mg trypan blue). Toluidine blue, × 500.

Fig. 2. Cyst in liver containing large monocytic cells (m). Three hepatocytes (h) in various stages of atrophy are present (50 mg trypan blue). Toluidine blue, × 500.

Fig. 3. Three portal veins (v) and a bile duct (d) completely surrounded by mononuclear cells. The remains of at least 1 hepatocyte (h) is present (50 mg trypan blue). Toluidine blue, × 500.

Fig. 4. Pleomorphic cellular and fibrillar tumor replacing liver (150 mg trypan blue). Toluidine blue, × 500.

Fig. 5. Normal rat hepatic portal tract. In the portal connective tissue between bile ducts (B), portal vein (V), and hepatocytes (H) are 3 reticuloendothelial cells (R) and an adventitial connective tissue cell (A). × 4,000.

Fig. 6. Trypan blue-treated rat (50 mg). The portal connective tissue contains a focal collection of monocytic cells (M) resembling Kupffer cells within an endothelial-lined (E) space adjacent to a bile duct (B). × 4,000.

Fig. 7. Trypan blue-treated rat (50 mg). Several pleomorphic monocytic cells are present among collagen fibers (C) adjacent to a bile duct (B). × 4,000.

Fig. 8. Trypan blue-treated rat (150 mg). The periportal tissues are replaced by pleomorphic cells. Collagen fibers (C) are present in the intercellular spaces as well as a single group of nonperiodic fibrils (arrow) characteristic of trypan blue-induced tumors. × 4,000. Inset, detail of these nonperiodic fibrils. × 20,000.
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