Ultrastructural and Biochemical Changes Associated with Pyrrolizidine-induced Hepatic Megalocytosis


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SUMMARY

Rats were fed a diet containing from 0.02 to 0.08% ground Crotalaria spectabilis seed for 8 months to evaluate the biochemical and ultrastructural changes that occurred in the livers of chronically intoxicated rats that developed megalohepatocytosis. These rats had small irregularly shaped livers with disrupted architectural patterns. Microscopically, the livers were composed primarily of megalohapatocytes 2.5 times the size of the normal hepatocytes, hyperplastic bile ducts, and regenerative nodules. The ultrastructural features and biochemical changes of these affected livers were similar to those reported in naturally occurring and experimentally induced hepatomas. The megalohapatocytes contained enlarged, irregularly shaped nuclei. Numerous organelle-filled invaginations of the nuclear membrane protruded into the nucleoplasm. Some invaginations separated from the nuclear membrane and formed membrane-enclosed nuclear inclusions. Other partially enclosed invaginations resulted in a mixture of nuclear and cytoplasmic constituents. The nucleolar components were abundant and were dispersed throughout the nucleoplasm. Alterations in the cytoplasmic organelles of the megalohapatocytes included a decrease and a morphological modification of the rough endoplasmic reticulum. Lamellar arrays of smooth endoplasmic reticulum filled large portions of the cytoplasm. An increase in the size and distribution of the Golgi complex and a reduction in attached ribosomes and glycogen granules were apparent. The RNA and nitrogen levels of the affected livers were of the same magnitude as the controls, while the DNA was increased to 200%.

INTRODUCTION

Megalohapatocytosis has been produced in rats by the administration of pyrrolizidine alkaloids (7, 8, 27, 31), either in a single dose or numerous smaller doses over an extended period (8, 33). Following a single injection of the alkaloid, weanling rats developed megalohapatocytosis within 3 to 4 weeks (31, 32), while older rats required larger doses and repeated exposure to the alkaloid (8, 25). The enlarged hepatocytes were capable of synthesizing DNA (8, 29), establishing higher levels of certain enzymes (28), and maintaining their ability to increase in size (27). In this report, the ultrastructural features and biochemical changes that occurred in the livers of monocrotaline-intoxicated rats are presented. Particular attention is given to the similarities of the livers with monocrotaline-induced megalohapatocytosis and those containing hepatomas.

MATERIALS AND METHODS

Fifty 100-g male Sprague-Dawley rats were fed Rockland Rat Diet containing Crotalaria spectabilis seed. These seeds contained the pyrrolizidine alkaloid monocrotaline at levels of approximately 3.5%. Ten additional rats of similar size, breed, and sex were given a control diet. The concentration of C. spectabilis seed in the experimental diet was increased from 0.02 to 0.08% by an 0.02 increment every 45 days during the initial 6 months, with the level maintained at 0.08% for the last 2 months of the experiment. The animals were killed either immediately prior to their anticipated demise or at the termination of the experiment by ether anesthesia and exsanguination by decapitation. After the livers were excised and weighed, portions were taken for light and electron microscopy and biochemical evaluations.

For light microscopy, small sections of the liver were placed in 10% neutral buffered formalin for 24 hr. They were subsequently dehydrated, embedded in paraffin, and sectioned at 5 μ. Sections were stained with hematoxylin and eosin, Masson's trichrome and periodic acid-Schiff stain. Tissues processed for electron microscopy were cut into small cubes and fixed for 1.5 hr in osmium tetroxide buffered with Veronal acetate (9) or sodium phosphate (21). They were subsequently dehydrated through a graded series of ethanol and embedded in an epoxy resin mixture (22). Sections of the tissues were cut on an ultramicrotome, placed on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with an electron microscope. Homogenates of liver tissue and Tris-HCl buffer. pH 7.5, in a ratio of 1 g:2 ml were prepared on a Potter-Elvehjem homogenizer at 0°. Concentrations of nitrogen were determined according to the micro-Kjeldahl procedure (15). Nucleic acids were extracted according to a modification of the Schmidt-Thannhauser procedure as reported by Munro.

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and Flack (23). RNA concentrations were determined by measuring the ultraviolet absorption at 260 \( \text{nm} \) with the extinction coefficient, \( E_{1%} \), equal to 312 (1.00 absorbance unit = 32 \( \mu \text{g/ml} \)). Concentrations of DNA were colorimetrically determined by measuring the absorption at 488 \( \text{nm} \) of the extraction following its reaction with indole as described in a modification (5) of the procedure of Ceriotti (10).

**RESULTS**

The addition of *C. spectabilis* to the diet of growing rats at the concentrations used in this experiment caused a 5 to 10% reduction in growth rate. This difference was most obvious when the animals were on higher concentrations of the seed. The food consumption of the monocrotaline-fed rats was only slightly less than that of the control rats. However, in the rats that died, an appreciable decrease in food intake occurred 1 to 2 weeks prior to death. The average survival time of the 23 rats that died or were killed during the experiment was 169 days (range, 121 to 228 days). The remaining 27 rats were killed at the end of the experiment.

A detailed report on the clinical, gross, and microscopic changes that occurred in these rats was presented in a separate report (3). It will suffice to say that terminally the rats had a decrease in total serum protein, a shift in the albumin/globulin ratio of the serum, and an increase in the blood urea nitrogen. They developed hydrothorax, pulmonary and subcutaneous edema, and ascites. Small, nodular, discolored livers, enlarged hearts, and green, irregularly surfaced kidneys were salient gross observations. Microscopically, megalohepatocytosis and nodular regeneration of the liver, glomerulosclerosis of the kidney and extensive vascular alterations in the lungs, heart, kidneys, and pancreas were observed.

Considerable variation existed in the morphological features of the livers of *Crotalaria*-fed rats. In 17 of the 50 livers, the general architectural pattern was maintained; however, individual lobules varied in size. Differences in the size and staining affinity of the hepatocytes were also observed (Fig. 1). Approximately 10% of the cells had dark eosinophilic cytoplasm and large hyperchromic nuclei. Randomly distributed throughout the lobules were some moderately enlarged hepatocytes with lightly staining cytoplasm and large nuclei. The remaining hepatocytes of this group of moderately affected livers appeared normal.

Thirty-three rats had severely affected livers. As a result of the complete disruption of the microscopic architectural pattern, the central veins, radially arranged hepatic rows, distinct sinusoids, and periportal triads were inapparent. Fifteen of the livers contained well-circumscribed nodules distributed among megalohepatocytes and bile ducts (Fig. 2). These nodules were composed of closely associated, normal appearing, average-sized (mean cell diameter 25 \( \mu \text{m} \); nuclear diameter, 8 \( \mu \text{m} \)) hepatocytes. The nodules were devoid of central veins and were surrounded by a narrow band of connective tissue. Mitotic figures were present in approximately 1.4% of the cells within the nodules.

The remaining severely affected livers were devoid of regenerating nodules. Large hepatocytes, averaging 65 \( \mu \text{m} \) in diameter and containing nuclei with a mean diameter of 30 \( \mu \text{m} \), and proliferating bile duct cells comprised the major parenchymal constituents of these livers (Fig. 3). The megalohepatocytes contained abundant homogeneous cytoplasm and large, centrally located nuclei. The latter had multiple nucleoli and numerous nuclear inclusions. Normal-appearing and bizarre mitotic figures were occasionally observed in these large hepatocytes (Fig. 4). Many of the bile duct cells enclosed well-developed lumens while others assumed a sheet-like array. Thin bands of connective tissue formed an interdigitating network between the bile ducts and hepatocytes. Interspersed within the connective tissue were fibroblasts, macrophages, mast cells, plasma cells, and leukocytes.

Isolated focal areas of necrosis affecting a variable number of cells were present in the severely involved livers. Eosinophilic cellular debris was devoid of cytoplasmic and nuclear organization. Polymorphonuclear leukocytes prevailed in the spaces between the degenerative hepatocytes and stromal tissue.

**Electron Microscopy**

The megalohepatocytes assumed a round, oval, or polygonal conformation which was largely dependent on the surrounding structures. The plasmalemmas of these cells were quite smooth with the exception of the microvillous surfaces that abutted on bile canaliculi. These microvilli were frequently dilated and formed cytoplasmic blebs (Fig. 5). No distinct microvillous border was discernible along the space of Disse. In many instances, the latter space was inapparent and the discontinuous basal lamina was not observed.

The large nuclei of the megalohepatocytes were enclosed by nuclear membranes that contained numerous invaginations. In the less severely affected cells, the nuclei were slightly enlarged and the invaginations were shallow (Fig. 5). In the more severely affected cells, the abundant and voluminous nuclear invaginations included numerous cytoplasmic organelles (Fig. 6).

Evaluations of serial sections prepared from hepatocytes at several degrees of alteration showed that the invaginations likely developed in the schematic manner depicted in Chart 1. Initially, a slight indentation of the nuclear envelope (Figs. 5, 7) deepened to form an invagination. Bleb-like dilations developed at their apex (Fig. 8) and gradually enlarged until the entire invagination assumed the shape of a flask with a long, narrow neck and large base (Fig. 9). Lumens of these "flasks" contained abundant ribosomes and short segments of endoplasmic reticulum. Occasional lysosomes, mitochondria, myelin figures, and lipid droplets were also present. The preponderance of smaller organelles was likely due to the filtering process of the long neck of the "flask."

Some of the invaginations became separated from the nuclear membrane and formed sequestra within the nucleus.

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Chart 1. Proposed sequential changes in the nuclei of megalohepatocytes. The first indication of impending nuclear invaginations is the development of small indentations in the nuclear envelope (A). These indentations gradually deepen, forming finger-like projections into the nucleus (B). During the early stages of development, cytoplasmic organelles are included in the lumen of these invaginations. The finger-like projections balloon at their apices and assume the appearance of a flask with a long, slender neck and large base (C). The lower part of the neck gradually constricts (D and E). These constricted portions fracture and the slender neck separates from the base. The latter becomes a membrane-enclosed sequestrum in the nucleus (arrow). Other invaginations separate from their nuclear membrane attachment, but they are only incompletely enclosed by membranes (double arrow), resulting in a mixture of nuclear and cytoplasmic constituents (F).

(Fig. 10). Fusion of the free ends of the fractured nuclear envelope usually followed. When the free ends failed to fuse, a subsequent mixture of nuclear and cytoplasmic constituents occurred (Figs. 10 and 11). However, in most instances the invaginations did not form sequestra in the nucleus and a direct communication with the cytoplasm was apparent.

The appearance of the nucleoli varied considerably. In the less severely affected nuclei, the oval, compact nucleoli contained irregularly shaped, amorphous, granular, and fibrillar components. The enlarged, irregularly shaped nuclei of the megalohepatocytes contained nucleoli composed of numerous segments of variable size, shape, and content. The fibrillar and granular components were often separated into small packets (Fig. 12) with fibrillar components forming distinct caps and halos around many of the granular aggregates (Fig. 13). In many instances, the fibrillar components were closely associated with the inner surface of the nuclear invaginations (Fig. 12).

The chromatin material consisted of numerous small granules evenly distributed within a fine fibrillar network and embedded in a slightly electron-dense nucleoplasmin. Clumping of the chromatin was apparent only along the membranous portion of the invagination.

The enlarged cytoplasm of these cells contained a proportionate increase in organelles and only isolated areas of organelle-free matrix. The majority of the abundant mitochondria were small, irregular in outline, and contained short cristae with moderately electron-dense matrices. In many instances within the same cell, other mitochondria were 2 to 3 times larger (Figs. 11 and 14). Small electron-dense granules were dispersed in the matrices of most mitochondria. The large mitochondria frequently contained electron-opaque granular aggregates (Fig. 14).

The ER was also modified considerably (Fig. 10). The rough ER was composed of short strands which were closely associated with mitochondria and other cytoplasmic organelles. Many cells contained concentrically arranged, laminated, smooth membrane structures which were continuous with segments of the smooth and rough ER. There was also an abundance of polysomes throughout the cytoplasm. Numerous glycogen granules were closely associated with vesicles of smooth ER.

Multiple Golgi complexes were abundant throughout the cytoplasm. They were composed of small vesicles and short, flattened cisternae containing electron-dense material (Figs. 5 and 10). The population of lysosomes and microbodies appeared unaltered in some cells and moderately increased in others. The lysosomes were variable in size and were filled with cell debris, vacuoles, and dense granules. The microbodies were oval and contained irregularly shaped, centrally located, crystallloid structures. The size and number of osmiophilic droplets varied considerably. In some cells, they were the predominant cytoplasmic structure, while in others they were small and distributed throughout the cytoplasm. Multivesicular bodies and cytosegresomes were occasionally present in the enlarged cells.

Only in their irregular distribution did the hepatocytes within the nodules, the bile duct epithelial cells, and the endothelial cells interspersed among the megalohepatocytes differ from those described in the normal rat liver (6). The ultrastructural changes in the necrotic hepatic cells bore no unique features attributable to monocrotaline intoxication.

Biochemistry

The experimental rats were divided into moderately and severely affected groups according to changes observed by light microscopy. The biochemical data from these rats as presented in Table 1 were expressed in relation to the liver weight and the nitrogen content of the liver homogenates. The latter was used in order to eliminate certain dilution factors such as water, glycogen, and lipid content of the tissues. The concentrations of RNA and nitrogen of either group of experimental livers did not differ appreciably from those of the controls. The DNA content of the severely affected livers was increased to 200% that of the control livers. Those experimental livers with less severe morphological changes showed considerable variation in DNA content, with the mean value being slightly greater than that of the controls.

2 The abbreviation used is: ER, endoplasmic reticulum.
hepatomas and 250% in fast-growing hepatomas have been certain nucleic acids (34). Increases in the DNA levels to hepatocytes. appreciably in the livers with hepatomas or megaloid more than one cell population. The continued ability of the content. The nitrogen and RNA levels are not altered reported (35). The livers that contain large numbers of megaloidhepatocytes show an increase to 200% in their DNA livers with megaloidhepatocytosis may reflect alterations in cells (27, 29) would invariably lead to polyploid cells and an increase in DNA. It has also been determined microspectrophotometrically that some highly differentiated hepatomas have DNA values 150 to 200% that of the normal hepatocytes (34). The hyperplastic bile duct epithelium that is commonly present in the livers with megaloidhepatocytosis and in those with hepatomas must also be considered as a source of the increased DNA. A higher level of DNA would result from an increased population of these small ductal cells per unit weight of liver. The exact means by which the pyrrolizidine alkaloids react with constituents of the cell remains to be clarified. It has been proposed that these alkaloids act as alkylating agents and mitotic inhibitors in the rat liver (12, 13), cause mutations in Drosophila (11) and Aspergillus nidulans (2), and produce chromosome breakage in onion roots (4). Esters of dihydropyrrolizine which are formed by rat liver microsomes from pyrrolizidine alkaloids are highly reactive alkylating agents (20). The preponderance of nuclear changes suggests that metabolites of these alkaloids combine with the nucleic acid or protein components to block mitosis without completely inhibiting DNA synthesis. Although quantitative and qualitative ultrastructural and biochemical similarities of the megaloidhepatocytes and neoplastic hepatic cells exist, the potential of the former cells continues to be evasive. The inability of the enlarged hepatocytes to regenerate normally following partial hepatectomy (7), their increased susceptibility to injury (7), their predominantly polyploid status (8), and their ability to synthesize DNA without cell division (1) have been interpreted as being indicative of senility (25). In the presently reported study, the decrease in hepatocyte population without appreciable regeneration and the subsequent hepatic failure is supportive of this view.

It has also been postulated that the megaloidhepatocytes are instrumental in producing hepatomas. Schoental et al. (29–31) have observed hepatomas in rats that survived for an extended period following the administration of pyrrolizidine alkaloids. It was theorized that the megaloidhepatocytes divided abnormally, giving rise to daughter cells capable of producing hepatomas. Scheuer (28) observed an

<table>
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<th></th>
<th>No. of animals</th>
<th>DNA (mg/100 mg liver)</th>
<th>RNA (mg/100 mg liver)</th>
<th>Nitrogen (mg/100 mg liver)</th>
<th>DNA/nitrogen (mg/100 mg N)</th>
<th>DNA/nitrogen (mg/100 mg N)</th>
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<tr>
<td>Control rats</td>
<td>6</td>
<td>0.223 ± 0.018</td>
<td>0.738 ± 0.024</td>
<td>3.39 ± 0.13</td>
<td>6.57 ± 0.54</td>
<td>22.0 ± 1.8</td>
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<tr>
<td>Monocrotaline-intoxicated rats</td>
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<tr>
<td>Severely affected liversa</td>
<td>15</td>
<td>0.375 ± 0.019</td>
<td>0.683 ± 0.024</td>
<td>2.89 ± 0.11</td>
<td>13.1 ± 2.9</td>
<td>23.5 ± 2.1</td>
</tr>
<tr>
<td>Moderately affected liversa</td>
<td>11</td>
<td>0.283 ± 0.081</td>
<td>0.742 ± 0.011</td>
<td>3.10 ± 0.15</td>
<td>9.34 ± 3.09</td>
<td>23.8 ± 1.3</td>
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aLivers composed primarily of megaloidhepatocytes with hyperplastic bile ducts or regenerative nodules.
bLivers containing hepatocytes which vary only in size and staining affinity.

DISCUSSION

Many morphological and biochemical features of livers with hepatomas are similar to those with pyrrolizidine-induced megaloidhepatocytosis. Cells of the fast-growing hepatomas, like megaloidhepatocytes, contain large irregularly shaped nuclei (14, 16–19, 26). Numerous organelle-filled nuclear invaginations and nuclear inclusions are characteristic of both groups. The nucleolar components are abundant, frequently fragmented, and widely distributed in the nucleoplasm. Changes that occur in the cytoplasm of these cells are also similar. There is a decrease in amount and a modification of the arrangement of the rough ER (18, 19). The lamellar arrays of ER disappear and are replaced by small cisternae which are randomly distributed throughout the cytoplasm (17, 19). There is a reduction in attached ribosomes and a concomitant increase in the free variety. Concentric membrane arrays of smooth ER are frequently present in both groups of cells (24). Dense inclusions in irregularly shaped mitochondria of variable size and a decrease in glycogen granules are also observed in the livers with megaloidcytosis and hepatomas (26).

Quantitative biochemical similarities of the livers with megaloidhepatocytosis and hepatomas also exist. The pattern of nucleic acid metabolism in hepatomas indicates a gradual increase in the biosynthesis and decrease in the catabolism of certain nucleic acids (34). Increases in the DNA levels to 140% in slow-growing hepatomas, 177% in medium-growing hepatomas and 250% in fast-growing hepatomas have been reported (35). The livers that contain large numbers of megaloidhepatocytes show an increase to 200% in their DNA content. The nitrogen and RNA levels are not altered appreciably in the livers with hepatomas or megaloidhepatocytes.

The increased level of DNA reported for hepatomas and for livers with megaloidhepatocytosis may reflect alterations in more than one cell population. The continued ability of the megaloidhepatocytes to synthesize DNA (27, 29) would invariably lead to polyploid cells and an increase in DNA. It has
increase in succinic dehydrogenase, \( \beta \)-glucuronidase, and esterases in the megalohepatocytes. He suggested that these cells possessed preneoplastic tendencies rather than degenerative characteristics.

Although this report does not permit extrapolation of the data to determine the potentials of the megalohepatocytes, it does elucidate the morphological features of these cells. It was also determined that the livers with megalohepatocytosis had a sizable increase in DNA which was attributed to the polyploid status of the enlarged hepatocytes and hyperplastic bile ducts. It was of particular interest that many similarities existed between the megalohepatocytes and the experimentally induced and naturally occurring tumors.

REFERENCES

All of the tissues from which the following photographs were made came from the livers of rats that were fed a diet containing finely ground C. spectabilis seed for 8 months.

Fig. 1. Observe the variation in size and staining affinity present in the less severely affected livers. Many of the cells are enlarged and contain hyperchromic nuclei (arrow). Normal appearing hepatocytes are near the large cells. H & E, X 420.

Fig. 2. Many of the affected livers are composed of regenerating nodules (N), hyperplastic bile ducts, and megalohepatocytes (arrow). Note the distorted architectural pattern assumed by the hepatocytes. H & E, X 48.

Fig. 3. Megalohepatocytosis and proliferating bile ducts are the predominant cells in the severely affected livers. Observe the numerous configurations assumed by the bile ducts. Enlarged nuclei, multiple nucleoli, and abundant cytoplasm are common features of the megalohepatocytes. H & E, X 168.

Fig. 4. Randomly distributed chromosomes are present in isolated megalohepatocytes that are in mitosis. H & E, X 1.600

Fig. 5. In the less severely affected megalohepatocytes, the nuclear membrane exhibits only shallow indentations (arrow) and the internal features of the nuclei are unaltered. These cells contain prominent Golgi complexes (G), rough and smooth ER, free ribosomes, and dilated microvilli (mv). X 8,300.

Fig. 6. In the more severely affected megalohepatocytes, the nuclei contain abundant organelle-filled nuclear invaginations. Note the close association of the fibrillar (f) and granular (g) components of the nucleolus with the membranous surface of these invaginations. X 9,360.

Fig. 7. The invaginations of the nuclear envelope begin as shallow projections of the nuclear membrane into the nucleus. Depending on the plane of sectioning, communication of the invagination with the outer nuclear membrane surface is apparent (arrow). However, in a second invagination cut at a different angle, it appears as an oval structure (double arrow). Cross-sectional views are given on invaginations which contain lipid droplets. X 41,090.

Fig. 8. The invaginations of the nuclear envelope increase in size and incorporate cytoplasmic components within their boundaries. Observe the somewhat dilated base and constricted neck of the invagination. Note the electron-dense vesicles of the Golgi complex (G), mitochondria (M), and abundant free ribosomes adjacent to the invagination. X 25,430.

Fig. 9. Many of the invaginations eventually develop into flask-shaped structures with large bases and small slender necks. Note the small opening (arrow) of the invagination to the cytoplasm proper. The cytoplasmic components within these invaginations are primarily ribosomes, ER, and lipid droplets. X 6,760.

Fig. 10. Occasionally one of these nuclear invaginations is fractured from its nuclear membrane attachment. Note the 2 free ends of the fractured invagination (arrow) and a discharge of organelles into the nucleoplasm. Other invaginations appear intact within the nucleus. X 3,970.

Fig. 11. Approximately equal portions of nuclear and cytoplasmic components are present within this intact nucleus (N). These nuclear alterations occur following the fracture of the invaginations. Observe the large mitochondria and abundant short segments of ER and free ribosomes in the cytoplasm. X 11,080.

Fig. 12. Most of the invaginations (I) of the nuclear envelope contain portions of the nucleolus (N) attached to or closely associated with their inner surface (arrow). In most instances, the nucleoli are composed of fibrillar, granular, and amorphous components. Observe the numerous free ribosomes and isolated segments of smooth and rough ER in the invagination. X 25,430.

Fig. 13. Nucleolar caps (arrow) are formed by the fibrillar element of the nucleolus. The depicted cap partially surrounds a segment of the granular component. A circular, granular aggregate is present in the upper right (double arrow). X 57,000.

Fig. 14. The mitochondria vary considerably in size, shape, and content. Frequently these mitochondria contain large electron-dense irregular shaped inclusions (arrow). The inset clarifies the morphological features of these electron-dense collections. X 4,900. Inset X 33,400.
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