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Accumulation of Abnormally High Ploid Nuclei in the Liver of LEC Rats Developing Spontaneous Hepatitis

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Enlarged hepatocytes with huge nuclei were found in LEC rats with hereditary hepatitis. Flow cytometric analysis of the DNA content of nuclei from jaundiced LEC rats revealed the presence of very high polyploids, such as 32n and 64n. At the age of 12 weeks, before the onset of hepatitis, 8n polyploid nuclei were more frequent in LEC rats than in LEA rats, a sibling line of LEC rats. Binucleated hepatocytes were also more frequent in LEC rats than in LEA rats at week 4. Bi-, tri- and tetra-nucleated cells whose nuclei were sometimes different in size were observed when jaundice became manifest. The number of proliferating liver cells, determined by pulse labeling with 5-bromo-2′-deoxyuridine (BrdU), was higher in LEC rats than in LEA rats at 2, 4, 8, 12 and 14 weeks, with a maximum at week 4. A remarkable increase of BrdU uptake was observed at week 16, when jaundice developed. The possible involvement of abnormal cytokinesis and kariokinesis in the manifestation of hepatitis was suggested.

Key words: LEC rat — Polyploid — Polynucleated cell

The LEC (Long-Evans with a cinnamon-like coat color) rat is a new mutant strain with hereditary hepatitis associated with severe jaundice, as reported by Sasaki *et al.*[1] and Yoshida *et al.*[2] Spontaneous hepatitis manifests itself suddenly in adult rats three to four months after birth and about half the rats die of hepatic failure. The clinical symptoms of hepatitis include severe jaundice, a bleeding tendency, oliguria, loss of body weight and elevation of the serum levels of bilirubin and hepatic enzymes, such as GOT and GPT. These findings are similar to those in human fulminant hepatitis. Liver cancers appeared in rats that survive longer.[3] This hepatitis is unlikely to be caused by viruses because no viral particles could be detected by electron microscopic examination of the affected liver, and intraperitoneal injections of cell-free liver homogenates from severely jaundiced LEC rats did not induce hepatitis in neonatal LEA (Long-Evans with an agouti coat color) rats, a sibling line of LEC rats. Genetic analysis showed that a single autosomal recessive gene is responsible for the hepatitis.[4]

The histopathological characteristics of the liver in LEC rats suffering from hepatitis are enlarged hepatocytes with huge nuclei and spotty necrosis with slight infiltration of inflammatory cells.[2] The nuclei of the enlarged hepatocytes are so huge that their DNA content appears to be more than octaploid. From this finding, we supposed that some abnormalities in mitosis and/or cell proliferation occur in the liver of the LEC rats.

In the present study we measured the DNA content of liver cells of the LEC rats at various ages to see whether the DNA content is really increased in the huge nuclei of enlarged hepatocytes. We also examined DNA synthesis and the appearance of binucleated cells to see whether cell proliferation and/or mitosis are disordered in LEC rats.

MATERIALS AND METHODS

Animals Two inbred strains, LEC and LEA, were established from non-inbred Long-Evans rats at the Center for Experimental Plants and Animals of Hokkaido University.[1,2]

Histology The left lateral lobe, sectioned before hepatocyte isolation, was cut into slices 2–3 mm thick with a razor blade. Slices were fixed in Carnoy's solution for staining with hematoxylin and eosin (HE).

Isolation of hepatocytes Cells were isolated from the liver by the collagenase digestion method.[5] During perfusion, the left lateral lobe was sectioned for light microscopic study. After collagenase digestion the liver was removed, minced in a beaker and filtered through layers of 250 μm and 65 μm nylon mesh. The cells were washed twice with Hanks' balanced salt solution by centrifugation for 1 min at 50g, suspended in 40 mM citrate buffer (pH 7.6) and stored in a freezer at −80°C.

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Preparation of nuclei for flow cytometry The detergent-trypsin method of Vindelφv *et al.*[6] was used for the preparation of nuclei for flow cytometric DNA analysis. Trypsin (Sigma Chemical Co., St. Louis, Mo.) solution was added to the cell suspension in citrate buffer at room temperature, and 10 min later, trypsin inhibitor (Sigma) and ribonuclease A (Sigma) solution were added. After incubation of the mixture for 10 min at room temperature, ice-cold propidium iodide (PI) (Sigma) solution was added. The sample was wrapped in tin foil to protect PI from light and kept in ice. Samples were examined in a flow cytometer between 15 min and 3 h after the addition of PI solution.

Flow cytometric analysis All studies were performed in an EPICS C flow cytometer (Coulter Electronics, Inc., Hialeah, Fla.). Forward angle scatter and PI-stained nuclei were read at a 450 nm laser line and at the wavelength of 610 nm, respectively. Analysis was carried out on 10^4 nuclei in each sample.

Preparation for the counting binucleated cells Smear preparations of liver cells in citrate buffer were used for visual analysis of bi- and multi-nucleated cells, and at least 500 cells per sample were examined.

Measurement of labeling index using 5-bromo-2′-deoxyuridine (BrdU) BrdU (200 mg/kg body weight) (Sigma) in 1 ml of phosphate-buffered saline (PBS) solution was injected intraperitoneally at 2 P.M. The rats were killed 1 h later and the livers were cut into sections 2–3 mm thick and fixed in Carnoy's solution. Tissue sections were deparaffinized in benzene, passed through a graded alcohol series, and then treated with methanol containing 0.6% H_2O_2 to inhibit endogenous peroxidase. The sections were then treated with 4 *M* HCl for 20 min and incubated with mouse anti-BrdU monoclonal antibody (1:40, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.) overnight. Then, after incubation with biotinylated anti-mouse IgG (1:100, Vector Laboratories, Inc., Burlingame, Calif.) for 30 min, the sections were incubated with an avidin-biotin-peroxidase complex (Vector Laboratory, Inc.) for 1 h. The BrdU incorporation was localized by a final incubation for 2 min with 3,3′-diaminobenzidine tetrahydrochloride. The sections were then counterstained with hematoxylin. Cells that had incorporated BrdU were identified by the presence of amber pigment over their nuclei. The labeling index was counted in three randomly distributed fields including portal and central areas, in each slide. All labeled nuclei of hepatocytes and "oval cells" in the fields and unlabeled nuclei were calculated per area by counts in the middle zone. At least 5000 nuclei per rat were counted.

RESULTS

Histology of LEC rat livers The clinical and histological changes of the LEC rats were essentially as reported,[1, 2, 7] although the appearance of clinical symptoms differed slightly in detail as described briefly below.

No clinical abnormalities were noticed in 14-week-old LEC rats. However, we observed slight alterations in the histology of the liver at 14 weeks. The nuclei of

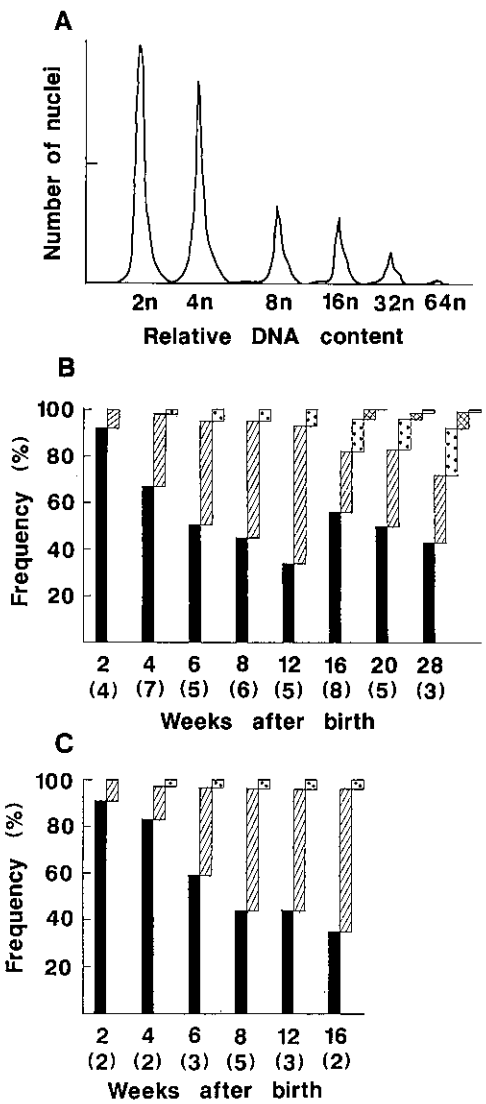

Fig. 1. Ploidy analysis of hepatocyte including "oval cell" nuclei in a flow cytometer. Histogram of DNA of hepatocyte and oval cell nuclei of a 20-week-old LEC rat (A). Ploid distribution in hepatocyte and oval cell nuclei of LEC rats (B) and LEA rats (C). ■ 2n, ▨ 4n, ⊡ 8n, ▩ 16n and □ >16n. Values are means for the numbers of rats shown in parenthesis.

hepatocytes varied in size, and large nuclei possessed several irregular nucleoli and aggregations of chromatin. Binucleate hepatocytes were also observed. Mitosis was rarely detected. Kupffer cells increased in the sinusoid and microgranulomas with Councilman bodies were sometimes detected.

A week 16, loss of weight and anemia were noticed, and jaundice appeared as reported previously.[1-3, 7]

DNA content in hepatocyte nuclei Figure 1A shows a histogram of DNA of hepatocyte nuclei of a 20-week-old LEC rat with jaundice. The nuclei consist of several different classes, and all the classes were found to be euploid. The dominant peak is that of diploid nuclei (2n) and the other peaks are those of 4n, 8n, 16n, 32n and 64n nuclei, respectively.

Figures 1B and C show the average distributions of ploidy in hepatocytes of LEC and LEA rats of various ages. The ploidy pattern of LEA hepatocytes is similar to that of another strain of normal rats.[8-16] However, the ploidy pattern of LEC hepatocytes is significantly different from that of LEA hepatocytes. The percentages of 4n ($P<0.01$) at 4 weeks, 4n ($P<0.01$) and 8n ($P<0.001$) at 12 weeks, and 4n ($P<0.001$) and 8n ($P<0.001$) and 16n ($P<0.001$) at 16 weeks old in LEC hepatocytes are significantly higher than those in LEA hepatocytes. In LEC rats of 16 weeks old, the diploid class became dominant, while the percentage of 16n was 4% and that of highly polyploid nuclei of 32n or 64n was about 0.2%. At 20 and 28 weeks, the percentage of highly polyploid nuclei such as 32n and 64n had increased to 1%. Polyploid nuclei of more than 8n were not detected in hepatocytes of normal LEA rats.

Binucleated cells Figure 2A demonstrates the frequency of binucleated hepatocytes in LEC and LEA rats of various ages. The pattern of LEA rats is similar to that reported for other strains of normal rats.[9-12, 17, 18] At 4 weeks, the frequency of binucleated hepatocytes in LEC rats was significantly ($P<0.001$) higher than that in LEA rats. The proportion of binucleated cells reached a maximum in week 4 in LEC rats, in contrast to week 6 in LEA rats. The maximum proportions of binucleated cells in LEC and LEA rats were not significantly different. Trinucleated and tetranucleated hepatocytes, which have never been observed in normal rats, but sometimes in human cases of postneonatal hepatitis or chronic hepatitis, were observed in jaundiced LEC rats, in addition to mono- and bi-nucleated hepatocytes. In some cases, the sizes of the nuclei in bi- or tri-nucleated LEC hepatocyte differed (Fig. 2B). The percentages of trinucleated hepatocytes in weeks 16 and 28 were 0.53% and 1.07%, respectively.

DNA synthesis of hepatocytes BrdU uptake was observed almost wholly in hepatocyte nuclei and was randomly distributed in the hepatic lobules of LEC and

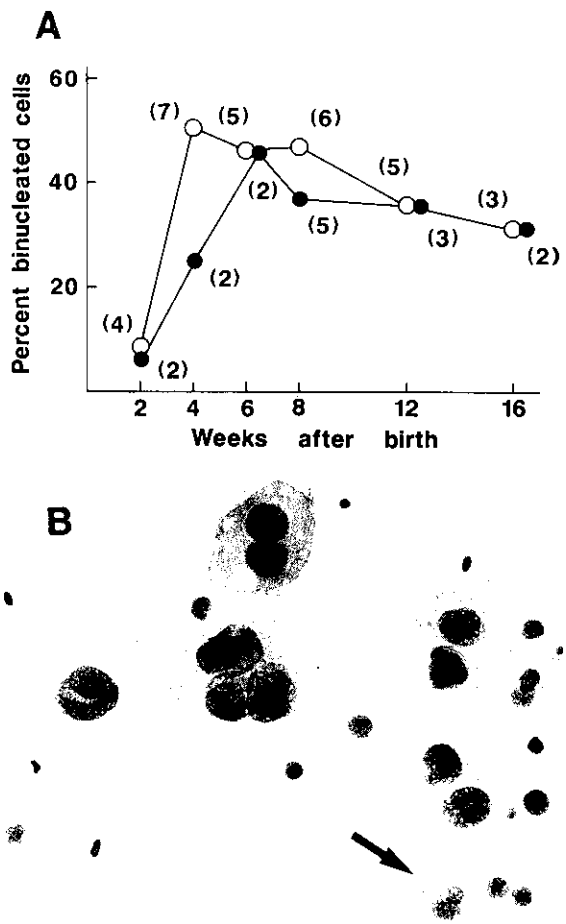

Fig. 2. Frequency of binucleated cells in hepatocytes of LEC rats and LEA rats. Values are means for the numbers of rats shown in parenthesis (A). ○ LEC, ● LEA. Smear preparation of hepatocytes of a 20-week-old LEC rat with jaundice (Giemsa staining, ×340). Large trinucleated hepatocytes and binucleated hepatocytes whose nuclei differ in size (arrow) are observed (B).

LEA rats on day 1 after birth. Figure 3 shows the time courses of change in the labeling indices of LEC and LEA hepatocytes. In both types of rats, they constituted about 10% of the hepatocytes on day 1. The number of labeled nuclei then decreased rapidly within 2 weeks. Subsequently, the labeling indices increased in week 4, decreased again in week 6, and gradually decreased further until week 14 in both types of rats. During this period, labeling indices of LEC rats were significantly higher than those of LEA rats except in week 6. In week 16 when hepatitis became manifested, a marked increase of the labeling index to over 4% was observed in LEC rats, and subsequently a high index persisted until at least

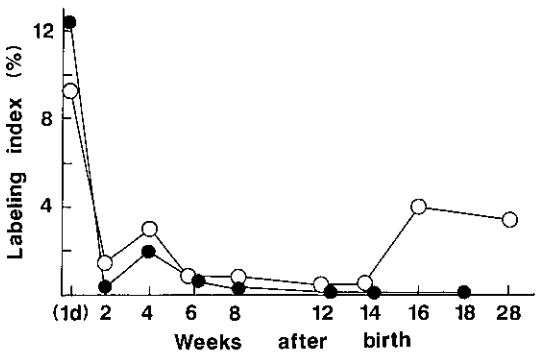

Fig. 3. BrdU labeling index of liver cells of LEC rats and LEA rats. Values are means for 4 or 5 rats. ○ LEC, ● LEA.

week 28. In contrast, in LEA rats, the labeling index remained less than 0.1% after week 14. In livers of LEC rats, not only small nuclei but also fairly large nuclei of hepatocytes were labeled in week 16. In the liver of a 28-week-old LEC rat that had recovered from jaundice, nuclei of "oval cells" were labeled as well as those of small hepatocytes in the periportal area of the hepatic lobule. Only a few huge nuclei of enlarged hepatocytes were labeled.

DISCUSSION

The morphological characteristics of jaundiced LEC rat liver are the appearance of markedly enlarged hepatocytes with huge nuclei and spotty necrosis with only slight inflammatory cell infiltration.[1,2,7] Flow cytometric analysis of the DNA content of hepatocyte nuclei suggested that the enlarged nuclei had high polyploidies, such as 32n and 64n. No aneuploid nuclei were found.

Polyploidy is generally observed in the livers of old animals,[8–16] and in regenerating liver after partial hepatectomy[10,16,19] or treatment with various chemicals.[20–25] Even in those cases, however, as high polyploidy as the 64n found in LEC rats has never been observed in rats, except after treatment with diethylnitrosamine, which induced 64n nuclei as reported by Wiest.[20] The polyploid pattern observed in the liver of untreated LEC rats is thus quite unusual. It is unknown whether this unusual polyploidy is a cause or a result of hepatitis in LEC rats.

Three mechanisms, mitotic polyploidization,[26] a G2 block and cell fusion,[27] have been proposed for polyploidization. In the first mechanism, the initial stages of mitosis up to chromosome separation proceed normally, but then mitosis stops during cytokinesis (acytokinetic mitosis). Acytokinetic mitosis of a diploid cell leads to the formation of a binucleated cell. In the next mitosis of the binucleated cell, two mononucleated tetraploid cells might be formed, if normal cytokinesis occurred. In this case binuclearity is probably a prerequisite for polyploidy. In the second mechanism, blocking of the cell cycle occurs immediately before mitosis (G2 block). Then endoreduplication occurs and repeated reduplication leads to polyploidy. When G2 block occurs, no mitotic figures can be seen. In the case of LEC rats, mitotic polyploidization seems to play a main role in the formation of high-polyploid hepatocytes, because binucleated hepatocytes with large nuclei and some mitotic figures in enlarged hepatocytes were observed in the present study. Cell fusion was probably not the cause of polyploidization, because it would result in production of aneuploid cells, such as those of 6n, 10n or 12n and so on, which were very rarely seen among LEC rat hepatocytes.

The labeling indices of LEC hepatocytes were significantly higher than those of control LEA rats before the onset of hepatitis. One possible mechanism for this is an elongation of the S phase, and another is more rapid proliferation of hepatocytes in LEC rats than in LEA rats. The second mechanism is supported by the finding that binucleated hepatocytes, which appeared following DNA synthesis in early postnatal life, showed a tendency to increase more repidly in LEC rats than in LEA rats. In LEA rats, the DNA synthesis in early postnatal life is reflected in increase in liver weight. The liver weights of LEC and LEA rats were almost the same at week 4 (1.3 g). However, in LEC rats, subsequent increase in liver weight was much less than that in LEA rats, and in week 6 their liver weights were 2.5 g and 3.7 g, respectively. It is possible that the labeling index was higher in LEC rats than in LEA rats all the time from week 2 because the hepatocytes of LEC rats were dying progressively. Councilman bodies, which resulted from cell death, were detected in week 14. After the onset of hepatitis, DNA synthesis increased markedly and Councilman bodies were frequently observed. The increase of DNA synthesis in LEC rats during this period might be due to regeneration of hepatocytes.

We previously reported[4] that a single autosomal recessive gene is responsible for hepatitis in LEC rats and proposed to name this gene *hts* (hepatitis), although its function and structure have not yet been determined. In this study, we found that polyploid cells increased before onset of hepatitis. As mentioned above, the high labeling index suggested loss of hepatocytes before the onset of hepatitis. But no cell death was detected by histological analysis until week 14. That is, neither activation of Kupffer cells nor Councilman bodies was found until week 14. We suppose that progressive alterations occur insidiously in liver nuclei of LEC rat livers after birth, and when such alterations accumulate and exceed a

certain threshold,[28,29] necrosis of hepatocytes begins. The presence of tri- and tetra-nucleated cells, and unequal-sized nuclei in bi- and tri-nucleated cells in hapatocytes of jaundiced LEC rats also suggest abnormality in cytokinesis or in karyokinesis, though the possibilities that these abnormalities result from cell fusions can not be ruled out.

High polyploidization and the consequent cell death seem to be involved in hereditary hepatitis of LEC rats. LEC rats should be useful in studies on polyploidization, growth, aging and death.

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