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DNA SYNTHESIS IN POLYPLOID AND BINUCLEATE HEPATIC CELLS IN THE REGENERATING RAT LIVER OF DIFFERENT AGES

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ABSTRACT

In many vertebrate species, polyploid and binucleate cells are found in the adult hepatic parenchyma. The number of polyploid and binucleate hepatic cells increases with age. In this paper, the proliferative capacity of polyploid and binucleate cells were studied, in order to analyse a progressive reduction with age in the regenerative capacity of tissues. Three groups of rats, 4-week, 6-week and 15-week-old, were used in this experiment. The animals were partially hepatectomized, received an injection of tritiated-thymidine at 18 hours after the operation, and were sacrificed 30 minutes later. The livers were then subjected to the combined autoradiographic and microspectrophotometric studies. The labeling index was calculated for each hepatic cell type, which was classified according to nuclear polyploidy and binuclearity. Spatial distribution of polyploid cells in the liver lobule and that of hepatic cells labeled with tritiated-thymidine were also studied, and were taken into consideration to interpret values of the labeling index. The results of these studies indicate that value of the labeling index of polyploid hepatic cells is generally smaller than that of diploid cells, and that value of the labeling index of binucleate cells is also smaller than that of mononucleate cells. These results strongly suggest that the proliferative capacity of hepatic cells is reduced with polyploidization.

INTRODUCTION

Ageing is associated with a progressive reduction in the regenerative capacity of tissues. Young rats are better at restoring their liver mass and forming new cells after partial hepatectomy than adults[2)3)16)]. In many mammalian species, including rat, ageing is also associated with a progressive development of hepatic polysomaty[1)12)13)14)19)]. Diploid nuclei predominate in the livers of newborn rats. As the animals age, tetraploid, octoploid and even hexadecoploid nuclei appear in sequence and rise in frequency. It is also known that such polysomatic tissues contain many binucleate cells with two diploid, two tetraploid or two octoploid nuclei, and it is now considered that, in all probability, the binuclearity is an intermediate stage between a

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given state of polyploidy and the immediately superior one[1)13)]. Post *et al.*[16)] found that successive injuries to the rat liver, separated by intervals of healing, were also associated with an increasing deficit in its restorative capacity and an accelerated shift towards higher levels of ploidy. These findings suggest that polyploidization of hepatic cells may be associated with reduction in the capacity to respond to the growth stimulus, and the present work is an attempt to test the hypothesis.

Bucher *et al.*[4)5)] showed that the capacity of rat liver to initiate DNA synthesis following partial hepatectomy decreases with increasing age. In the present experiment, tritiated-thymidine incorporation into DNA in the regenerating livers was studied autoradiographically shortly after the onset of DNA synthesis following partial hepatectomy in different aged rats. In order to identify ploidy levels of hepatic cell nuclei, the technique of micro-spectrophotometry was used.

MATERIALS AND METHODS

Three groups of male Wistar strain rats of accurately known ages were used: (a) three 4-week-old rats, weighing 50 to 54 g, (b) four 6-week-old rats, 102 to 112 g, and (c) four 15-week-old rats, 305 to 340 g. They were subjected to two-thirds partial hepatectomy by the method of Higgins and Anderson[9)] without anesthesia. They received an intraperitoneal injection of tritiated-thymidine (spec. act. 5.0 c/mmol, Radiochemical Centre, Amersham) in sterile saline solution at 30 min. before sacrifice. The doses of tritiated-thymidine were 2 μc/g body weight for 4-week and 6-week-old rats, and 500 μc/rat for 15-week-old rats. They were sacrificed by decapitation at 18.5 h after partial hepatectomy except two 15-week-old rats which were sacrificed at 20 h after operation. The sacrifices were made between 10.30 to 11.30 a.m. to eliminate any possibility of complication by diurnal variations in mitotic activity.

Hepatic cell suspensions were prepared by the method of James *et al.*[10)] with minor modifications. After decapitation of the rat, the liver was perfused via the aorta with a solution of 0.04 M sodium citrate and 0.15 M sucrose. Small liver tissue was incubated for 45 min. at 37°C, minced with scissors and suspended in a homogenized tube by several strokes with a hand-driven and loose-fitting pestle in 0.25 M sucrose at 4°C. After filtration through 4 sheets of gauze, the suspension was centrifuged for 10 min. at 120 g. Smears were prepared from the sediment and fixed in 100 parts 95% ethylalcohol, 40 parts formalin and 7 parts glacial acetic acid. Stained by the Feulgen method, the slides were coated with liquid emulsion (Sakura NR-M 2). After 2 weeks exposure, the slides were developed.

Consecutive fields of the smears, chosen at random, were mapped and all labeled cells in the fields were marked. Grains were then removed with

potassium ferricyanide, and all hepatic cells in the fields, either labeled or unlabeled, were classified into the several cell types according to polyploidy and binuclearity. Identification of the ploidy classes of nuclei was made by means of the following two methods. One is visual classification according to the combined criteria of nuclear size and staining intensity of Feulgen, which has been described by Alfert and Geschwind[1] and James, Schopman and Delfgaauw[10]. Unlabeled nuclei could be easily classified by means of this method. Labeled diploid nuclei, of which DNA content was much smaller than the amount of DNA of unlabeled tetraploid nuclei, and labeled tetraploid nuclei, of which DNA content was about the middle between the amount of DNA of unlabeled tetraploid nuclei and that of unlabeled octoploid nuclei, could be also identified visually. The other method is microspectrophotometric measurements of Feulgen-DNA contents of nuclei. The method was applied upon the labeled nuclei, which could not be classified visually. In a few experimental animals, DNA contents of all labeled nuclei in the fields were measured microphotometrically. Microphotometric measurements were made by the method described by Ris and Mirsky[18] at wavelength of 570 mμ. The validity of application of the method to the smeared hepatic cells was tested by comparing the DNA values of several nuclei, which were measured by this method, with those of the same nuclei, which were measured by the method of scanning measurements of total nuclear extinction[6][20]. Binucleate cells were easily distinguished from mononucleate cells in the smears[10]. A total of more than 2000 hepatic cells per rat were scored and the labeling index was calculated for each cell type of individual rats.

Tissue blocks of the resting liver excised at operation and those of the regenerating liver taken at sacrifice were fixed in neutral 10% formalin, and embedded in paraffin. From the blocks of the resting liver, 15 to 20 μ thick sections were prepared and stained by the Feulgen method. The spatial distributions of diploid and polyploid parenchymal cell nuclei in the liver lobule were then examined. Discrimination between diploid and polyploid nuclei was made according to the combined criteria of size and staining intensity[1][7][17]. From the blocks of the regeneraing liver, 5 μ thick sections were prepared, coated by emulsion and developed after 1 month exposure. After stained with hematoxylin-eosin, the distribution of labeled nuclei in the lobule were examined.

RESULTS

Fig. 1 shows the frequency distributions of Feulgen-DNA values of labeled mononucleate cells of a 4-week-old rat (Fig. 1-a) and a 15-week-old rat (Fig. 1-b). Unlabeled nuclei fall into three classes, the DNA contents of which are in the ratio of 1:2:4, corresponding diploid, tetraploid and octoploid nuclei

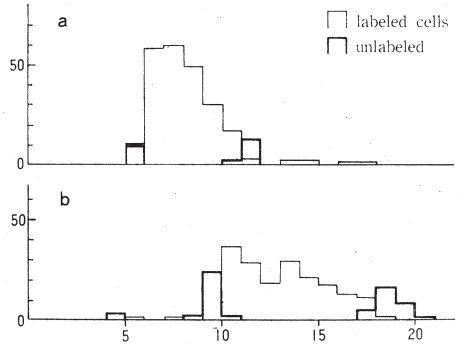

FIG. 1

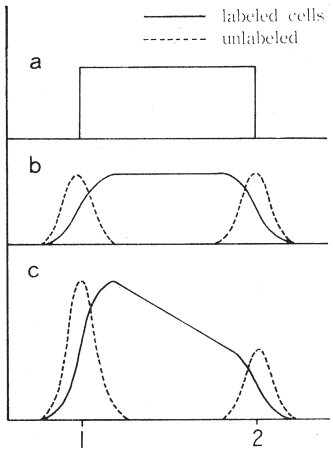

FIG. 2

FIG. 1. Frequency distribution of Feulgen-DNA values of labeled mononucleate cells at 18.5 h after partial hepatectomy. (a) a 4-week; (b) a 15-week-old-rat. *Abscissa*: total nuclear DNA content in arbitrary units; *ordinate*: cell number.

FIG. 2. A model for DNA value distribution of labeled cells. (a) A model which is constructed on the assumption that the rate of DNA synthesis is constant: (b) on the assumption that, in addition to (a), DNA values of unlabeled cells of a ploidy class shows a normal distribution; (c) on the assumption that, in addition to (a) and (b), cells beginning DNA synthesis increase in number as time passes. *Abscissa*: DNA in arbitrary units; *ordidate*: cell number.

respectively. In the 4-week-old rat, DNA values of a large proportion of labeled nuclei are noted between DNA values of unlabeled diploid nuclei (2 N values) and those of unlabeled tetraploid nuclei (4 N values), indicating that they are diploid nuclei synthesizing DNA. DNA values of a small proportion are noted between 4 N values and DNA values of unlabeled octoploid nuclei (8 N values), indicating that they are tetraploid. Fig. 1-b shows that, in the 15-week-old rat, a majority of labeled mononucleate cells is tetraploid and a minority is diploid.

DNA values of labeled diploid nuclei which have completed DNA synthesis and DNA values of labeled tetraploid nuclei beginning synthesis overlap in the range of 4 N values. The situation is the same in the range of 8 N values. Therefore the labeled nuclei, of which DNA values are in the range of 4 N or 8 N values, can not be classified into the ploidy classes according to DNA value of each nucleus alone. Estimates were made of the cell number of the two successive ploidy classes in these ranges, using a model for DNA value distribution of labeled cells in the initial stages of regeneration. The model is shown in Fig. 2-c. That was constructed on the assumtion that: (a) the rate of DNA synthesis was constant through DNA doubling, (b) DNA values of unlabeled cells of a ploidy class showed a normal distribution, and

TABLE 1. Number of labeled and unlabeled cells of each cell type in a total more than 2000 liver parenchymal cells per rat and labeling indices calculated therefrom

Age (week)	Body weight (g)	Duration of regeneration (h)	2N			2NBi			4N			4NBi			8N			8NBi			16N			Total LI (%)
			Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	Unlabeled	Labeled	LI (%)	
4	50	18.5	775	170	18.0	995	82	7.6	96	3	3.0	8	0		1	0								12.0
4	54	18.5	638	170	21.0	963	95	9.0	125	5	3.8	15	0											13.4
4	54	18.5	383	447	53.9	918	197	17.4	94	13	13.8	11	0											31.7
6	108	18.5	92	14	13.2	788	69	8.1	1186	111	8.6	71	0	0	2	0		1	0					8.3
6	102	18.5	109	41	27.3	819	96	10.5	1205	158	11.6	90	2	2.2	7	0								11.8
6	112	18.5	75	9	10.7	609	16	2.6	1293	35	2.6	79	1	1.3	7	0								2.9
6	106	18.5	42	13	23.6	491	35	6.7	1378	95	6.4	103	0	0	7	0								6.6
15	305	18.5	51	6	10.5	181	6	3.2	1283	211	14.1	339	9	2.6	160	0	0	20	0		2	0		10.2
15	340	18.5	72	2	2.7	207	1	0.5	1366	44	3.1	305	2	0.7	58	1	1.7	7	0					2.4
15	310	20.0	35	2	5.4	179	3	1.6	1492	81	5.1	364	1	0.3	47	0	0	4	0					3.9
15	330	20.0	60	2	3.2	170	7	4.0	2132	197	8.5	431	8	1.8	176	0	0	8	0		2	0		6.7

2N, 4N, 8N, 16N: mononuclear diploid, tetraploid, octoploid and hexadecoploid cells respectively; 2NBi, 4NBi, 8NBi: binucleate cells with two diploid, tetraploid and octoploid nuclei respectively.

LI: labeling index.

Total LI: labeling index for a total cell population.

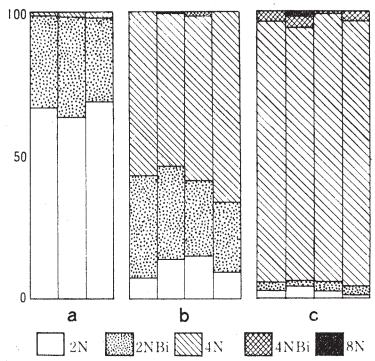

FIG. 3. Distribution of label among cell types in the regenerating rat liver of different ages, shown in histograms of cumulative percentages. (a) 4-week; (b) 6-week; (c) 15-week-old rats. *Ordinate*: %. 2N, 2NBi, 4N, etc., see in Table 1.

(c) cells beginning DNA synthesis increase in number as time passes. The figure of the model has a fairly good agreement with those in Fig. 1. The cell number of each ploidy class was estimated by superimposing the model upon the figure of DNA value distribution of individual rats.

The number of labeled and unlabeled cells of each cell type in a total of more than 2000 hepatic cells per rat are presented in Table 1. During 4th to 15th postnatal week, marked changes take place in the proportion of the cell types in the total population, both labeled and unlabeled. These changes are due to the development of hepatic polysomaty. The proportion of cell types in the population of labeled hepatic cells is shown in histograms of cumulative percentages (Fig. 3). Labeled cells of increasing degrees of ploidy appear in sequence with age, and their percentages increase roughly in proportion to the development of polysomaty. In 4-week-old rats, about two-thirds of labeled cells are mononuclear diploid, and one-third binuclear diploid. After the age of 4 weeks, the percentage of mononuclear tetraploid cells increases. In 15-week-old rats, most of labeled cells are mononuclear tetraploid. Besides these three cell types, DNA synthesis was also found in binuclear tetraploid cells of 6-week and 15-week-old rats and in a mononuclear octoploid cell of a 15-week-old rat.

The labeling index was calculated for each cell type of individual rats and is presented in Table 1. The same values were plotted in Fig. 4 to show the relationship between value of the labeling index and degree of ploidy of cell types. Cell types were arranged according to degree of ploidy in such a sequence as in Fig. 4, since the binuclearity was regarded as an intermediate stage between successive states of polyploidy of mononucleate cells[1)13)]. Fig. 4 indicates that: (a) value of the labeling index tends to decrease as nucleus becomes higher in degree of ploidy with the exception of the values of mononuclear and binuclear diploid cells of 15-week-old rats, (b) values of the labeling index of mononuclear and binuclear diploid cells decrease with age, and (c) value of the labeling index of binucleate cells tends to be smaller

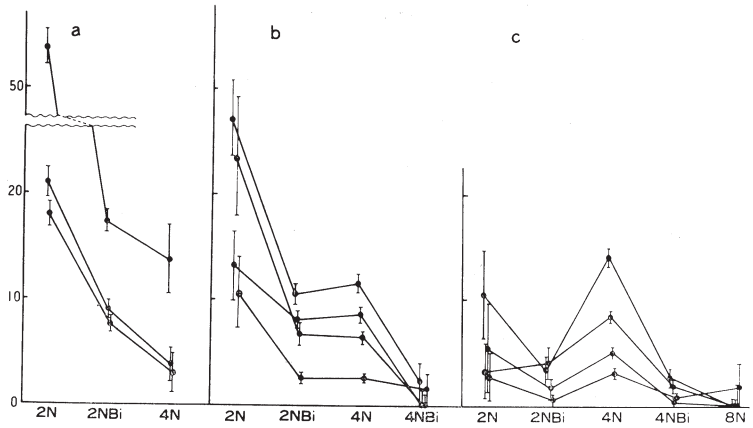

FIG. 4. Relation between value of the labeling index and degree of ploidy of cell types. (a) 4-week; (b) 6-week: (c) 15-week-old rats. The values of same rat are linked by a line. The standard error is represented by vertical bar. *Abscissa*: cell type arranged according to degree of ploidy. 2N, 2NBi, 4N, etc., see in Table 1. *Ordinate*: the labeling index, %.

than that of mononucleate cells with a nucleus of the same degree of ploidy.

In thick Feulgen-stained sections, the spatial distributions of diploid and polyploid parenchymal cell nuclei in the liver lobule were examined. Polyploid nuclei appear and increase mostly in the periportal areas within the lobule with increasing age. In 15-week-old rats, most of diploid nuclei are located

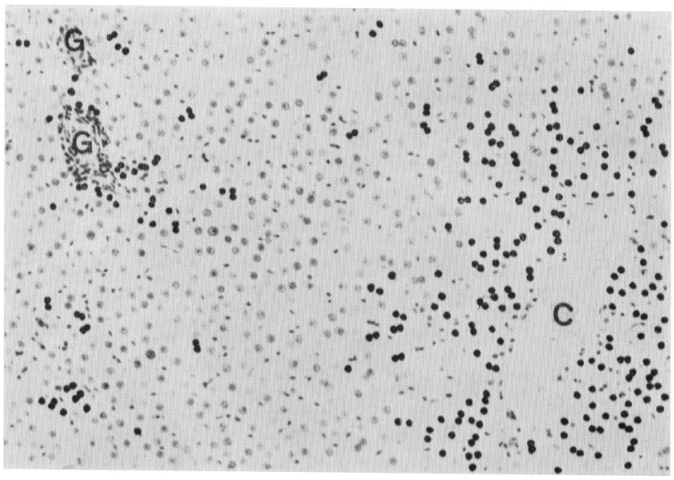

FIG. 5. The spatial distribution of diploid and polyploid hepatic nuclei within the liver lobule of a 15-week-old rat. Discrimination between diploid and polyploid nuclei was made according to the combined criteria of size and staining intensity of Feulgen. Diploid nuclei are represented by black circle. C, central vein; G, Glisson's capsule.

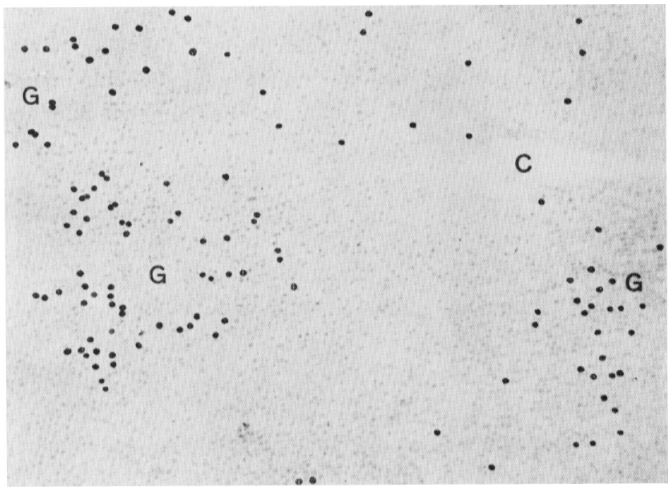

FIG. 6. The spatial distribution of labeled nuclei within the liver lobule of a 15-week-old rat, same case as in Fig. 5. Labeled nuclei are represented by black circle. C, Central vein; G, Glisson's capsule.

in the central parts of the lobule and a few of diploid nuclei are seen adjacent to interlobular connective tissues (Fig. 5).

An autoradiographic study of tissue sections of the regenerating livers showed that labeled parenchymal cells were distributed principally in the periportal areas within the lobule in all age groups (Fig. 6).

DISCUSSION

In the present studies, significant differences in values of the labeling index were found among the groups of hepatic cells, which were classified according to polyploidy and binuclearity. It seems likely that these differences resulted mainly from differences in the following two matters. One is the capacity of cells to respond to the growth stimulus. The other is uneven distribution of hepatic cells within the liver lobule. Grisham[8] reported that the labeled nuclei were located in the periportal areas at the peak of DNA synthesis (20 to 24 h) after partial hepatectomy. This periportal distribution of labeled nuclei were observed in all age groups of the present studies. The present study on the spatial distribution of diploid and polyploid cells showed that most of diploid nuclei became located in the central parts of the lobule with increasing age. Therefore it is considered that a notable decrease of values of the labeling index of mononuclear and binuclear diploid cells with age is due to changes in the spatial distribution of the diploid cells with age.

The results of the present studies indicate that value of the labeling index tends to decrease as nuclei become higher in degree of ploidy with the exception

of values of mononuclear and binuclear diploid cells of 15-week-old rats. Since it is considered that the exception results from uneven distribution of hepatic cells within the liver lobule, it is reasonable to presume the capacity of cells to respond to the growth stimulus reduces with increasing degrees of ploidy. In addition, the results indicate that value of the labeling index of binucleate cells tends to be smaller than that of mononucleate cells with a nucleus of the same degree of ploidy. It is also presumed that this is due to differences in this capacity.

Bucher, Di Troia and Swaffield[4,5] investigated biochemically DNA synthesis in the regenerating rat livers of various ages, and reported that an initial rise in the rate of DNA synthesis occurred progressively more slowly as age increased, and that peaks of DNA synthesis were correspondingly delayed. Based on the findings of the present studies, it is concluded that these phenomena are, at least to some extent, due to increase of polyploid and binucleate cells with age.

In respect to the position of polyploid cells within the liver lobule, Swartz[19] reported that the larger (polyploid) nuclei were usually concentrated in small areas of the lobule, most often near the portal area and at the periphery, and less frequently near the central vein in the human liver. The results of the present study of the spatial distribution of polyploid cells in the lobule also showed that the majority of the polyploid nuclei were located in the periportal areas of the lobule in the 15-week-old rats. But, in these rat livers, polyploid nuclei were not concentrated in small areas, but distributed fairly diffusely in the periportal areas. This discrepancy may be due to a difference in species of the animals used.

There are a few reports which presented data about the cell composition of hepatic cells synthesizing DNA in the regenerating rat livers after partial hepatectomy. Looney[11] reported that incorporation of tritiated-thymidine was primarily confined to tetraploid nuclei between 18.5 to 26 h after partial hepatectomy in the rats of 200 to 300 g body weight, aged 4 to 5 months. Oehlert, Hämmerling and Büchner[15] presented data of grain number distribution of labeled nuclei at the different times after partial hepatectomy in the rats of 120 to 150 g body weight. Their data seem to indicate that about a half of labeled nuclei are diploid and another half are tetraploid at 18 or 25 h after operation. The differences in the proportion of labeled polyploid cells between two reports may be due to a difference in body weight of the animals used, since the results of Looney agree roughly with the results of the 15-week-old rats of the present studies, and those of Oehlert *et al.* agree with those of the 6-week-old rats of the present studies.

SUMMARY

1. Tritiated-thymidine incorporation by polyploid and binucleate hepatic

cells in the initial stages of regeneration (about 18.5 h) after partial hepatectomy has been studied autoradiographically in 4-week, 6-week, and 15-week-old rats. Smears prepared from hepatic cell suspensions have been used to analyse the population of labeled and unlabeled hepatic cells.

2. Changes in the composition of labeled hepatic cells are as follows. In 4-week-old rats, about two-thirds of labeled cells are mononuclear diploid and about one-third binuclear diploid. Thereafter, the proportion of mononuclear tetraploid cells increases with age. In 15-week-old rats, most of labeled cells are mononuclear tetraploid.

3. Value of the labeling index tends to decrease as nuclei become higher in degree of ploidy with the exception of values of diploid cells of 15-week-old rats.

4. Value of the labeling index of diploid cells decreases with age. It is considered that this decrease is mainly due to decrease of diploid cells in the periportal areas with age, where most of labeled cells are located in the initial stages of regeneration.

5. Value of the labeling index of binucleate cells tends to be smaller than that of mononucleate cells with a nucleus of the same degree of ploidy.

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