acid and methyl retinoate. Administration of the compound was accompanied by widening of the paracortical zone of the mesenteric lymph node and by an increase in the number of lymphoid follicles in the spleen.

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CHANGES IN THE CHARACTER OF HEPATOCYTE PROLIFERATION IN THE LIVER AND ADENOMATOUS NODES IN MICE DURING CCl_4-INDUCED CARCINOGENESIS

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Ontogenetic and reparative growth of the rat and mouse liver is characterized by the progressive development of cell polyploidy, as a result of a process of mitotic polyploidization that is typical of hepatocytes [1]. Meanwhile reports of the accumulation of cells with a low level of ploidy in the early stages of neoplastic growth [10] and of the diploid composition of hepatomas [6, 7, 9, 11, 12] have been published.

Nodular proliferation of hepatocytes is a relatively early reaction of the liver to carcinogens. The study of the cell composition of the nodules can provide information on the sources of abnormal growth and on the characteristics of cell division. In the present investigation the DNA content was studied in hepatocytes during the development of nodular proliferation and also separately in cells isolated from large adenomatous nodes and the surrounding liver. The character of the cell transformations in the course of division was judged by measuring the DNA content in undividing cells, cells synthesizing DNA, and postmitotic cells by a combination of cytospectrophotometric and autoradiographic methods with double isotope labeling.

EXPERIMENTAL METHOD

An oily solution of CCl_4 was given perorally to SWR mice in a dose of 0.1 ml twice a week [8]. The animals' liver was studied after 1, 7, 41, and 52 doses of CCl_4, in the last case 4 months after the end of its administration. Two days after the single or last dose of CCl_4 (except in the case of 52 doses), [^{14}C]thimidine was injected intraperitoneally into

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TABLE 1. Changes in Parameters of Cell Proliferation in Liver and Large Nodes in Mice during CCl_4-Induced Carcinogenesis

Number of doses of CCl_4	Mouse	Tissue	Index of thymidine-labeled cells, %		Unlabeled cell population: mean ploidy, ploidy units		Unlabeled cell population: mean ploidy, ploidy units		p_n^*/p_n	p_c^*/p_c
			[^{3}H] thymidine	[^{14}C] thymidine	of nucleus (p_n)	of cell (p_c)	of nucleus (p_n^*)	of cell (p_c^*)		
1	1	Liver	2,7	40,3	5,4	9,1	7,4	12,2	1,4	1,34
	2	"	0,8	37,0	6,2	9,8	8,4	12,0	1,4	1,22
7	3	"	<0,1	10,2	10,3	12,0	8,5	10,0	0,83	0,84
	4	"	0,3	10,5	7,1	8,7	7,2	8,4	1,01	0,97
41	5	"	1,7	24,0	8,5	9,8	10,9	11,6	1,28	1,18
	6	"	0,3	0,2	8,3	10,1	9,8	12,8	1,19	1,26
	6	Node	1,0	0,2	5,6	6,6	8,1	9,2	1,45	1,39
	7	Liver	1,3	14,2	9,8	12,0	10,3	12,9	1,05	1,07
	7	Node	0,3	2,3	4,2	5,4	6,4	7,6	1,5	1,41
52	8	Liver	0,2	0,8	6,9	8,9	6,1	8,7	0,88	0,97
	8	Node	0,2	1,4	6,4	8,3	5,4	6,9	0,84	0,83

the animals in a dose of 1 μCi/g (specific activity 310 mCi/mmole; Czechoslovakia). The mice were decapitated 18 h after injection of the isotope, i.e., after a time interval long enough for passage through the phases of DNA synthesis, the premitotic phase, and mitosis [3]. The ploidy of the newly formed cells was judged from the DNA content in the nuclei labeled with [^{14}C]thymidine. The second label, tagging cells in the phase of DNA synthesis, was injected into the animals 1 h before sacrifice: [^{3}H]thymidine in a dose of 1 μCi/g (specific activity 21.4 Ci/mmole; USSR). Pieces of liver with small nodules under 0.5 mm in diameter and with large nodes 6-8 mm in diameter were fixed separately in 10% neutral formalin. After fixation the pieces were cut into two parts. One part of each piece was embedded in paraffin wax for histological investigation. The other parts were used to make preparations of isolated cells by alkaline dissociation [2]. These cells were stained by the Feulgen method — hydrolysis in 5 N HCl for 10 min at 37°C, stained in Schiff's reagent for 1 h at room temperature, and covered with type M photographic emulsion. After exposure for 2 months the autoradiographs were developed, labeled, and the position of the labeled and unlabeled, mononuclear and binuclear cells was noted. The content of DNA-fuchsin was measured after removal of the label on a Vickers M-86 scanning integrating microdensitometer. The index of labeled cells was determined on films by examination of 500-1000 cells. The total number of animals in the experiment was 24; for detailed analysis eight mice with highest labeling index were selected.

EXPERIMENTAL RESULTS

On the 3rd day after a single dose of CCl_4 traces of extensive centrilobular necroses were still present, and usually these were not completely eliminated until after 10-14 days [13]. After frequent doses of CCl_4, scattered areas of micronecroses were found, frequently with signs of cirrhotic changes. In preliminary experiments the first nodules were found macroscopically after 22-24 doses of CCl_4. After 41 and 52 doses the liver had multiple whitish nodules ranging from a fraction of a millimeter to 2-3 mm in diameter, and sometimes there were large nodes up to 6-8 mm in diameter. The index of labeled cells was particularly high after a single dose of CCl_4 and much lower in the case of chronic poisoning, although the picture varied considerably in different animals. The frequency of [^{3}H]thymidine-labeled nuclei as a rule was lower than the frequency of [^{14}C]thymidine-labeled nuclei, possibly on account of decay of the wave of cell divisions on the 3rd day after poisoning (Table 1).

The first dose of CCl_4 caused a burst of proliferation and a high degree of polyploidization of the newly formed hepatocytes, and this was reflected in a shift to the right of the histogram of postmitotic [^{14}C]thymidine-labeled cells with a fall to almost the complete absence of hepatocytes with diploid nuclei, both mononuclear and binuclear (Fig. 1, mouse No. 2). The mean ploidy values of nucleus and cell increased correspondingly (Table 1).

After seven repeated doses of CCl_4, this shift of the histogram of the newly formed cells was not found and the mean ploidy of the postmitotic cells did not increase (Fig. 1, mouse No.

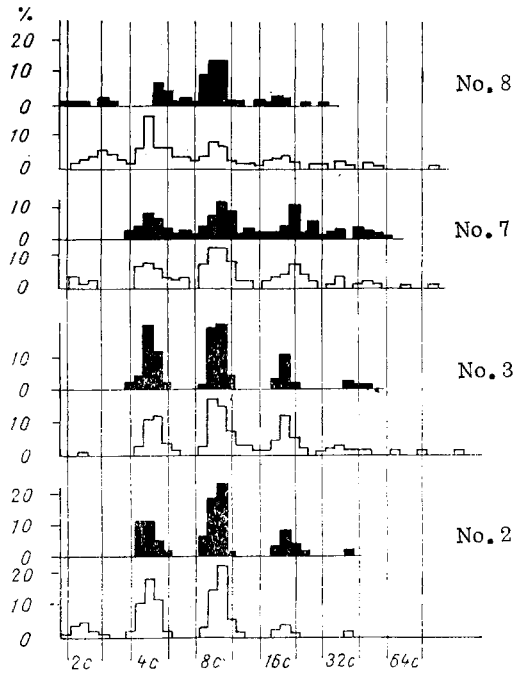

Fig. 1

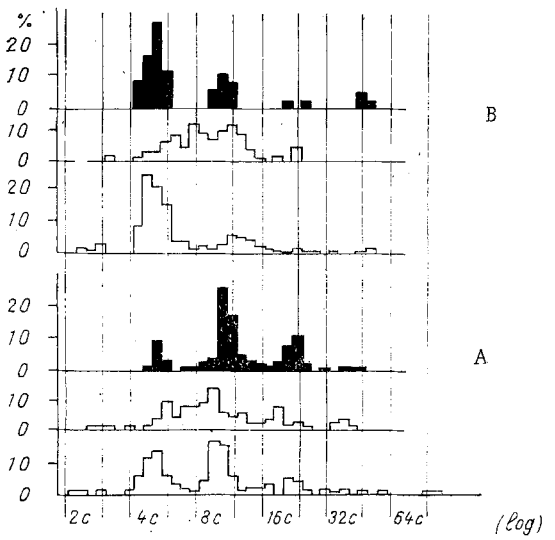

Fig. 2

Fig. 1. Distribution of amounts of DNA in hepatocyte nuclei in liver of mice receiving one dose (mouse No. 2), seven doses (mouse No. 3), 41 doses (mouse No. 7), and 52 doses (mouse No. 8) of CCl_4. Unshaded histograms) unlabeled nuclei, shaded histograms) postmitotic nuclei labeled with [^{14}C]thymidine 18 h before sacrifice. Abscissa, DNA content (in ploidy units); ordinate, number of cells (in %). Vertical lines denote 95% confidence interval calculated from results of measurements on mouse No. 2. Number of measured nuclei for each histogram, from bottom to top: 203, 160, 331, 142, 208, 167, 347, 142.

Fig. 2. Distribution of amounts of DNA in hepatocyte nuclei of mouse No. 6 in liver (A) and in a large node (B). Middle histograms denote nuclei in phase of DNA synthesis, labeled with [^{3}H]thymidine 1 h before sacrifice. Remainder of legend as to Fig. 1. Number of nuclei measured, from bottom to top: 305, 98, 314, 94.

3; Table 1). After 41 and 52 doses of CCl_4, no marked degree of polyploidization occurred and most cells as before were hepatocytes with tetraploid and octaploid nuclei, although the frequency of cells with high ploidy was increased (Fig. 1, mice Nos. 7 and 8; Fig. 2). Attention is drawn to the phenomenon of nuclei with peridiploid and aneuploid, intermediate amounts of DNA. This may reflect the development of true chromosomal aneuploidy and fragmentation of chromosomes as a result of mitosis of cells carrying chromosomal aberrations. The frequency of such cells varied from 0 to 3-4% independently of the number of doses of CCl_4 given, and it was higher in the newly formed cells than in the rest of the population. Cells with other abnormalities of shape of the nucleus also were found, often multinuclear cells (up to four nuclei) with nuclei unequal in their DNA content, possibly on account of a disturbance of the late stages of mitosis. Another cause of the occurrence of intermediate amounts of DNA mainly among unlabeled cells could be the admixture of cells injured by hepatotoxin and degenerating cells.

Measurement of the hepatocytes in the large nodes revealed two types of DNA distributions: those starting with the diploid class (Fig. 2, Fig. 3, mouse No. 7) and with the tetraploid class (Fig. 3, mouse No. 7). These results may mean that the rapid growth and formation of nodes were attributable to multiplication and polyploidization of a single stem line of peridiploid cells in the first case and of peritetraploid cells in the second case. As Fig. 2B shows, cells at all levels of ploidy, up to those with a DNA content of 16 c, began to synthesize DNA, as was also the case in the surrounding parenchyma (Fig. 2A).

The results of this investigation thus show that even in the early stages of induced carcinogenesis in the liver the process of mitotic polyploidization characteristic of hepatocytes is disturbed, and this leads to the reproduction and accumulation of cells with low

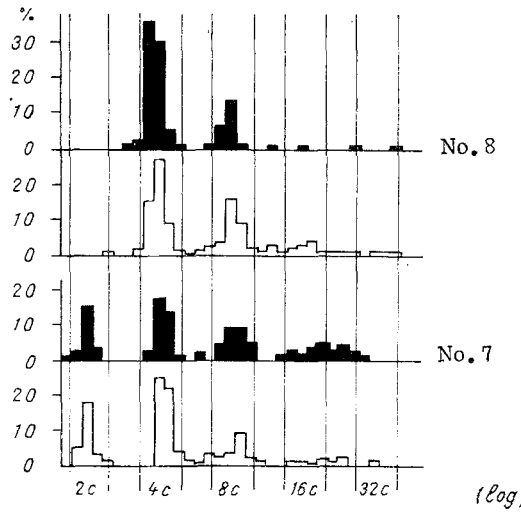

Fig. 3. Distribution of amounts of DNA in hepatocyte nuclei in large nodes in mice Nos. 7 and 8. Legend as to Fig. 1. Numbered of measured nuclei, from bottom to top: 271, 143, 356, 174.

ploidy values. Next, the appearance and active multiplication of cells with a peridiploid DNA content will be noted. Usually during stimulation of reparative growth, which does not subsequently develop into nodular hyperplasia, after a few repeated exposures to the carcinogen polyploidization of practically all cells with diploid nuclei was observed, and the frequency with which they were found fell below 1% [4, 5]. The appearance of many diploid nuclei in some of the present experiments may be due to stimulation of reproduction of single hepatocytes remaining in the tissue or to activation of hypothetical precursor cells. Yet another important circumstance is the presence of chromosomal aberrations and their development on the basis of a defective aneuploid genome. It can be tentatively suggested that active multiplication of cells of relatively low ploidy (with di-, tetra-, and octaploid nuclei) with chromosomal aberrations provides a constantly changing material for the selection of cells with altered properties. A peridiploid stem line is found most frequently in nodular growths and hepatomas [6, 7, 9-12], although other variants may also be found if enough cases are studied.

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