CELL-POPULATION ANALYSIS OF THE INITIAL PERIOD OF HEPATOCYTE PROLIFERATION IN THE REGENERATING MOUSE LIVER

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During the investigation of mammalian mitotic cell cycles cases of deformation of the Quastler–Sherman curve of labeled mitoses [13] frequently arise, when the theoretically demanded "plateau" of this curve is appreciably depressed or reduced to a "peak" [3, 8]. On purely logical grounds such deformation may be the result of lengthening of G_2 (G_2 + M) or shortening the duration of the S phase in a certain proportion of the cells studied. At the present time only the first of these possibilities has been taken into account [7]. The duration of DNA synthesis in the cell is claimed to be constant, namely 8 h [2].

The opinion that heterogeneity of cell populations is single in nature is supported by many experimental facts. However, the facts themselves are sufficiently heterogeneous, and that makes their interpretation difficult. There is an urgent need for a more detailed analysis than has hitherto been undertaken, i.e., many more parameters of the system chosen for study must be taken into account. This is the condition for a stricter interpretation of the experimental results: it would enable the correctness of conclusions formulated to be tested against their consequences, i.e., it would be possible to use true data without resorting to "analogous" situations. One such attempt was made in the investigation described below, devoted to the initial stage of proliferation of mouse hepatocytes, stimulated by partial hepatectomy.

EXPERIMENTAL METHOD

Experiments were carried out on male (CBA × C57BL/6)F_1 mice weighing 18-20 and 24-27 g, two-thirds of whose liver was removed under pentobarbital anesthesia; the operation was performed between 10 a.m. and noon, and the duration of each operation was 4 min. The group of young mice was killed 24, 30, 36, and 42 h after partial hepatectomy, 10-12 mice at each time. Of the 18 adult mice 10 were killed 36 h and eight were killed 42 h after the operation. The schedule of administration of the radioactive DNA marker was as follows: young mice killed 24, 30, and 36 h after the operation – one injection of ^{3}H-thymidine (specific radioactivity 14 Ci/mmole) in a dose of 0.5 μCi/g 0.5 h before sacrifice; young mice killed 42 h after the operation – two injections, the first, of ^{3}H-thymidine (0.5 μCi/g) 6 h before sacrifice, the second of ^{14}C-thymidine (specific radioactivity 60 mCi/mole) in a dose of 5 μCi per mouse 0.5 h later; adult mice received four injections of ^{3}H-thymidine (sessional dose 0.5 μCi/g), 6, 4, 2, and 0.5 h before sacrifice. The mice were killed by decapitation. Pieces of liver were fixed in 10% formalin solution and preparations of isolated cells were obtained by alkaline dissociation of the tissue [1]. The preparations were then covered with type M radiosensitive emulsion and exposed in darkness at 4°C for 18-21 days (in individual cases parallel preparations were exposed for 3-6 months in order to determine the fraction of weakly labeled hepatocyte nuclei). After development and fixation of the autoradiographs the preparations were stained with Mayer's acid hemalum. In mice receiving a single injection of labeled thymidine, the index of labeled hepatocyte nuclei (I_S), the index of unlabeled interphase nuclei of a well-marked premitotic structure (I_{G2} [6]), the mitotic index (MI), and the index of nuclei in the postmitotic reconstruction phase (postMI, [6]) were determined. In mice receiving two different labels, the index of ^{14}C-labeled nuclei (IS), the index of ^{3}H-labeled nuclei and, finally, the index of unlabeled nuclei of three morphological categories, namely premitoses, mitoses, and postmitoses, were determined. In the group of adult mice, the overall ^{3}H-index, the

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TABLE 1. Time Course of Proliferative Process in Parenchyma of Regenerating Mouse Liver (initial period)

Time of regeneration, h	Index of hepatocyte proliferation, promille,				Sum of indices, promille
	I_S	I_{G2}	MI	postMI	
24	8,7±3,8	0,9±0,4	0,0	0,0	9,6±3,9
30	9,8±4,5	2,9±0,7	0,0	0,4±0,2	12,7±2,9
36	325,2±36,3	108,0±29,7	2,6±1,2	10,8±3,7	446,6±55,4
42	234,3±43,7	55,1±10,9	41,1±10,4	290,8±48,4	621,7±31,0

Legend. PostMI — phase of postmitotic reconstruction.

TABLE 2. Kinetics of Population of Unlabeled Nuclei (hepatocytes), Identified at 36 h of Regeneration by Its Well-Marked Premitotic Structure (incorporation of label 35.5-36 h after operation; units of variations of indices shown between parentheses)

Time of regeneration, h	Time after labeling, h	Index of unlabeled nuclei of morphological categories considered, promille			Sum of indices, promille
		premitosis	mitosis	postmitosis	
36	0,5	108,0 (4,1—354,3)	2,6 (0,0—15,4)	10,8 (0,0—45,4)	121,4 (5,7—414,6)
42	6,0	1,4 (0,0—2,1)	1,1 (0,0—6,1)	177,0 (43,0—337,8)	179,5 (43,0—345,2)

index of labeled nuclei (premitoses, mitoses, and postmitoses), and the percentage of labeled mitoses were determined. All parameters were obtained by counting 5000-10,000 hepatocyte nuclei from the mice under magnification of the microscope of 630×. The results were subjected to statistical analysis by the Fisher—Student method.

EXPERIMENTAL RESULTS

It will be clear from Table 1 that the development of hepatocyte proliferation, stimulated by partial hepatectomy, was most clearly defined in its time course by changes in the total of the four parameters studied. This integral parameter is a vector value — it changes from time to time only in the direction of an increase. Under these conditions the character of the change of each of the parameters considered demonstrates the logic of successively linked events during the development of the process, with one exception. The sources of the increase in the fraction of hepatocyte nuclei in the G_2 phase 36 h after the operation is not clear. This increase was not preceded by any corresponding rise in the level of the fraction of DNA-synthesizing nuclei (compare I_S for 24 and 30 h of regeneration). We must briefly examine the question of the validity of using a parameter of proliferation as nontraditional as I_{G2} for the hepatocyte population [4, 6]. In mice, the interphase hepatocyte nucleus becomes premitotic in structure as early as at the beginning of the S phase [10]. This enables the whole mass of premitotically changed nuclei to be divided with the aid of ^{3}H-thymidine labeling into S and G_2 populations, their corresponding indices having been determined. The appropriateness of this method of determining the G_2 index, in the present investigation, receives additional confirmation. It will be clear from Table 2 that 6 h after injection of the ^{3}H-thymidine label into the hepatectomized mice (time of injection 36 h, time of sacrifice 42 h after the operation) the population of unlabeled premitoses disappears almost completely and its place among the interphase nuclei is now occupied by the population of unlabeled postmitotic hepatocyte nuclei. It will be evident that conversion of the unlabeled premitoses into unlabeled postmitoses is the result of progress of the sufficiently synchronous cell population around the mitotic cycle: at 36 h of regeneration, the latter population was in front of mitosis, but in the 6 h immediately following it succeeded in passing through mitosis and changing into the postmitotic reconstruction state. During the same 6-h time interval, the premitotic population was changed and labeled (S): part of it also became postmitotic. It is very important to note that labeled postmitoses recorded after 42 h of regeneration when observed en masse were appreciably brighter, and nearer to mitosis than the unlabeled postmitotic hepatocyte nuclei neighboring them. Hence it follows that the proliferating hepatocyte population, which had not incorporated the label, was formed, premitotic initially and postmitotic later, in the mitotic cycle before the labeled population; at the time of labeling it had therefore left the S phase already, i.e., it was in the G_2 phase.

TABLE 3. Populations of Hepatocyte Nuclei, Unlabeled with ^{3}H-Thymidine, of Three Morphological Categories in Regenerating Liver of Mice Receiving Four Injections of Label — 6, 4, 2, and 0.5 h Before Sacrifice (limits of variations of indices shown between parentheses)

Time of regeneration, h	Index of unlabeled nuclei of morphological categories considered, promille			Sum of indices, promille	% of labeled mitoses
	premitosis	mitosis	postmitosis		
36	25,1 (1,4—88,6)	2,8 (0,0—19,4)	8,4 (0,0—35,8)	36,3 (3,2—135,3)	22,2 (0—93)
42	1,0 (0,0—4,0)	0,6 (0,0—2,2)	32,2 (1,3—139,3)	33,8 (2,7—141,7)	98,5 (95—100)

The increase in the number of G_2 hepatocytes, not after but simultaneously with activation of DNA synthesis in the regenerating mouse liver can be ascribed traditionally to the presence of a so-called "reserve G_2 population" [11] among the hepatocytes of the liver; in that case it must be accepted that the latter, shortly before departure into mitosis, becomes morphologically unidentifiable. However, in that case the basic quality of such a population, namely its departure into mitosis immediately after tissue damage, is not realized. G_2 hepatocytes bound after 36 h depart into mitosis only a little (by not more than 2-3 h) earlier than cells with a G_2 phase of the usual duration. The status of a reserve G_2 population is contradicted also by the extremely high individual variability of the fraction of G_2 nuclei which was found (Table 2).

It may be, however, that the G_2 hepatocytes analyzed, while not a reserve G_2 population, nevertheless have a lengthened G_2 phase. These cells may enter the S phase after the operation sooner than the others, but later, for some unknown reasons, they are delayed before mitosis. This hypothesis does not agree with the presence of individual variants with a high G_2 index in the sample corresponding to 36 h of regeneration (in four of 12 mice the G_2 index was 170-354 promille).

Considering these high values of the G_2 index, it must be assumed that initial activation of DNA synthesis in the parenchyma of the regenerating mouse liver is (and is frequently) very considerable, which in reality is not so [9].

Let us now examine the possibility of shortening the time of the *S* phase of these cells to 3-4 h, i.e., to the time interval between the 30th and 36th hours of regeneration. Such a possibility was not confirmed autoradiographically (Table 3). Injection of the label in fractions between 30 and 36 h evidently did not convert the unlabeled premitotic hepatocyte nuclei into labeled. This is proved by the low percentage of labeled mitoses at 36 h, and also by coincidence of the sums of the three indices of unlabeled nuclei (premitoses, mitoses, and postmitoses) at two times of regeneration — a situation analogous to that recorded for a single injection of the label (Table 2).

We know, however [5], that labeled thymidine is not always incorporated into the *S* nucleus; this depends on the pathway of thymidine triphosphate biosynthesis. The hepatocyte population, extremely heterogeneous morphologically and physiologically, may also be heterogeneous with respect to the chemistry of DNA synthesis. Some cells passing through the *S* phase may not take up the ^{3}H-thymidine label, i.e., may be undetectable autoradiographically. Absence of labeling of the *S* phase and shortening of its duration may be linked events, related to the same cells. The latter do not constitute a population of one particular size among the *S* hepatocytes, for the ratio between the fractions of labeled and unlabeled premitotically changed hepatocyte nuclei varies very considerably among the sample. In that case it is not the size of the population with reduced duration of the *S* phase that is determined, but the formation, by the latter, of the proliferative response of the hepatocytes induced by partial resection of the liver at a particular time point, namely, at its beginning.

LITERATURE CITED

1. L. N. Belov, M. E. Kogan, T. A. Leont'eva, et al., Tsitologiya, **17**, No. 11, 1332 (1975).
2. O. I. Epifanova, Hormones and Cell Multiplication [in Russian], Moscow (1965).
3. A. A. Zavarzin, DNA Synthesis and the Kinetics of Cell Populations in Mammalian Ontogeny [in Russian], Leningrad (1967).
4. P. A. Karmanov, N. A. Milyutina, M. Ya. Neklyudova, et al., Byull. Éksp. Biol. Med., No. 11, 119 (1983).
5. V. M. Kotel'nikov, Éksp. Onkol., **8**, No. 6, 10 (1986).
6. N. A. Milyutina, Ontogenez, **3**, No. 4, 421 (1972).
7. Yu. A. Romanov, Circadian Rhythms of Physiological Processes [in Russian], Moscow (1972), pp. 48-51.
8. L. K. Romanova, Regulation of Repair Processes [in Russian], Moscow (1984).
9. V. F. Sidorova, Z. A. Ryabinina, and E. M. Leikina, Regeneration of the Liver in Mammals [in Russian], Leningrad (1966).

10. H. W. Altmann, Verh. Dtsch. Ges. Path. Abt. C, **50**, 15 (1966).
11. S. Gelfant, Exp. Cell Res., **26**, 395 (1962).
12. N. A. Milyutina (N. A. Miljutina), E. M. Kogan, and M. A. Neklyudova (M. A. Nekhljudova), Virchows Arch. Abt. B, Cell Path., **29**, 179 (1978).
13. H. Quastler and F. G. Sherman, Exp. Cell Res., **17**, 420 (1959).