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Effect of Thyroid Hormone on the Ploidy of Rat Liver Nuclei as Determined by Flow-Cytometry (40140)

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In mammalian liver, the proportion of polyploid nuclei increases with age until full growth has been reached. Liver cells in newborn animals are primarily diploid; later, tetraploid and octaploid cells predominate (1). The rate at which polyploidy develops can be accelerated by hepatic regeneration in response to liver damage caused by disease, toxins, or by partial hepatectomy (2). Deficiency in growth hormone or thyroid hormone has been shown to prevent the accumulation of polyploid nuclei (3), whereas the administration of thyroid hormone to hypothyroid rats stimulates the formation of tetraploid hepatic nuclei (3, 4).

In earlier studies, ploidy was evaluated by histological methods which relied on measurements of nuclear diameter, or by microspectrometry using the Feulgen staining procedure (3, 4). Only relatively small numbers of nuclei could be evaluated by these two methods. In this report, we describe the use of flow cytometry (5) to determine the influence of thyroid status on the ploidy of rat liver nuclei. This method employs the fluorochromes acridine orange (AO) or propidium iodide (PI), which intercalate between the base pairs of DNA to produce fluorescence of a distinct wavelength. Under the experimental conditions, the intensity of the fluorescence is in proportion to the amount of DNA present (5–7). Simultaneous measurements of light scatter are used to determine the relative nuclear size. With this approach, large numbers of nuclei can be rapidly processed and their ploidy classes distinguished on the basis of their DNA contents.

Materials and methods. Male Sprague Dawley rats (6–12 weeks old) were obtained from the Carworth Division of Becton-Dickinson Laboratories, New York, and maintained on a Wayne Lab Bloc diet (1 μg iodine/g) and tap water *ad libitum.* Animals were rendered hypothyroid by surgical thyroidectomy at 6 weeks of age, followed by the administration of 100 μCi ^{131}I after 1 week of low iodine diet. Hypothyroidism became manifest by cessation of weight gain 3 to 4 weeks after radioactive iodine treatment. In these animals, thyroid hormone levels were undetectable, and TSH was elevated. At 12 weeks, the thyroidectomized animals were killed, and the liver nuclei were isolated according to the method of Blobel and Potter (6). Nuclear ploidy was determined as outlined below, and compared with that of nuclei obtained in the same manner from euthyroid animals 6 and 12 weeks of age.

To determine the effect of the thyroid hormone, the hypothyroid animals were injected at 12 weeks of age with 1 mg/100 g BW of T_3 (triiodothyronine, Sigma Chemicals, Inc., St. Louis, MO) dissolved in 3% serum albumin. Two, three, four, or six days later, the animals were killed, and the ploidy of isolated liver nuclei was analyzed as described below.

The quality of the nuclear preparation was checked by phase microscopy. The purified nuclei suspended in 0.88 *M* sucrose were immediately frozen and stored at −80°. Prior to the analysis, aliquots of frozen nuclei were allowed to thaw on ice. Such frozen/thawed nuclei were found to have the same morphological and staining characteristics as fresh nuclei when examined with light and fluorescence microscopy, and had identical patterns of acridine orange (AO) fluorescence. The fluorochrome AO (Sigma Chemical Corp.) was complexed with isolated nuclei by mixing equal volumes of a suspension of nuclei (2×10^5 nuclei/ml) and a freshly prepared solution of AO (9.5×10^{-6} g/ml) both in TKM

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(0.05 *M* Tris, pH 7.4; 0.025 *M* KCl; 0.005 *M* $MgCl_2$), and allowing them to interact for 5 min at 25°. This procedure was found to result in optimal fluorescence, as determined by concentration and time curves.

A stock solution of propidium iodide (PI) was prepared by dissolving 250 mg of PI in 100 ml of distilled H_2O. This solution was stored in a dark brown bottle at 4°. A working solution was prepared by diluting the stock solution 25 times with TKM. The nuclei were stained by mixing 2×10^5 nuclei in 1 ml of TKM with 1 ml of PI working solution. This mixture was kept at 4° for 5 min before fluorescence was measured. This procedure is similar to that used by others (7), except that we did not use sodium citrate as a vehicle to make the cells permeable to PI. We have found that isolated nuclei are equally permeable to PI dissolved in sodium citrate as in TKM. PI is less sensitive to environmental conditions than AO, and is considered a reliable indicator of DNA content (8). Immediately after staining, AO green fluorescence and PI red fluorescence were measured in 10^4 nuclei per aliquot by flow cytometry. A Cytofluorograf, Model 4802 A, connected to a multichannel distribution analyzer, Model 2100 (Bio-Physics, Inc., Mahopac, NY) was used for simultaneous nuclear counts and for the measurement of light scatter and intensity of fluorescence. Results were recorded as histograms, the *y*-axis indicating the numbers of nuclei per channel, and the *x*-axis representing the intensity of fluorescence or the degree of light scatter expressed in channel numbers. Histograms of lymphocytes obtained from normal rat spleen were used as a fluorescence standard of diploid nuclei (9).

Results. Rat liver nuclei invariably showed two distinct peaks of fluorescence intensity. The first peak (Fig. 1, a, b, c) of lower intensity corresponded to the fluorescence of rat lymphocytes (Fig. 1d), and, therefore, was interpreted as representing diploid nuclei (9). The second peak indicated the population of tetraploid nuclei. The few nuclei with fluorescence intensity intermediate between the diploid and tetraplid mode were interpreted as S-phase nuclei. A small number of octaploid nuclei located to the right of the tetraploid peak contributed only insignificantly to the total intensity of fluorescence, and were not included in the analysis. Shown in Fig. 1 are representative histograms of fluorescence and light scatter of liver nuclei isolated from

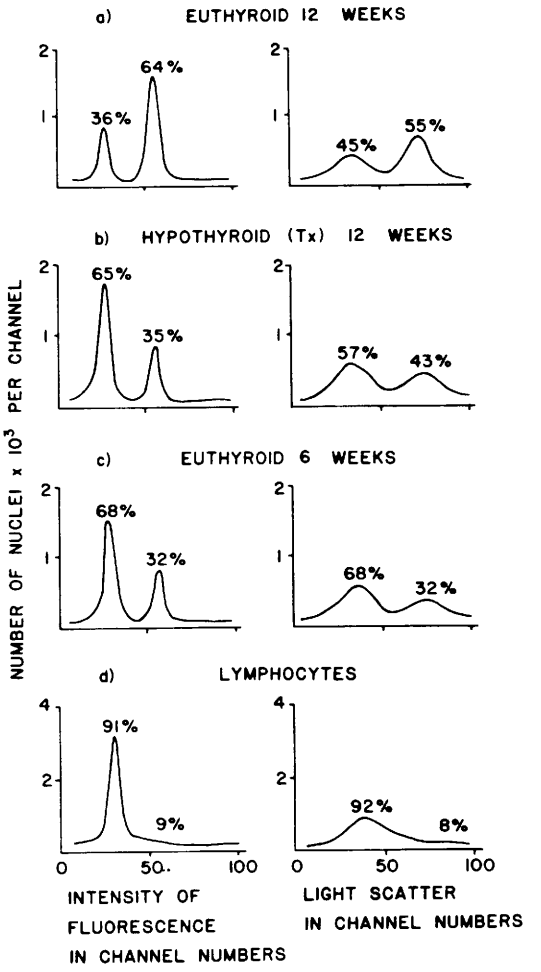

FIG. 1. Histograms of euthyroid and hypothyroid rat liver nuclei. The *x*-axis represents either the intensity of green AO fluorescence, which is proportional to the DNA content of the nuclei (left column), or the amount of light scatter, which is proportional to the size of the nuclei (right column). In both columns, the *y*-axis represents the number of nuclei per channel. Rat liver nuclei show a bimodal distribution. The first peak (lower fluorescence intensity, small nuclei) corresponds to the fluorescence and size of rat lymphocytes (d), a cell type having diploid nuclei only. The second peak of fluorescence and of light scatter corresponds to the tetraploid nuclei. The nuclear population of normal 12-week old rats (a) contains twice the number of tetraploid nuclei as compared to hypothyroid animals of the same age (b), which remains similar to that observed at the time of thyroidectomy (6 weeks of age) (c).

12-week old euthyroid animals (Fig. 1a), from 12-week old hypothyroid animals (thyroidectomized at the age of 6 weeks) (Fig. 1b), and from euthyroid 6-week old rats (Fig. 1c). It can be seen that the distribution of diploid and tetraploid populations of nuclei in 12-week old thyroidectomized animals was similar to that of 6-week old euthyroid animals.

The distribution of tetraploid nuclei in euthyroid and hypothyroid animals of the same age was examined in a group of seven animals (Table I). In euthyroid animals, the mean of 61.4% ± (SD) 7.3 of the nuclei were found to be tetraploid, as contrasted with 31.9% ± 4.0 in hypothyroid animals. The differences were statistically significant ($P < 0.001$). Identical differences in ploidy were found when PI instead of AO was used as a fluorescent stain. The examination of nuclear size by light scatter also revealed a bimodal distribution (Fig. 1). In euthyroid animals, with progressing age, the proportion of nuclei of larger size increased, whereas in thyroidectomized animals, this process became arrested. The distribution of nuclei into the different size classes generally followed the ploidy distribution as demonstrated by histograms of fluorescence.

The effect of thyroid hormone administration to hypothyroid animals on nuclear ploidy is shown in Fig. 2. The dose used (1 mg of T_3/100 g of BW) was very large in comparison to a daily replacement dose, and was chosen to saturate the nuclear T_3 receptor sites (10).

Three days after T_3 injection, an increase

TABLE I. VARIATIONS IN PERCENT TETRAPLOID NUCLEI IN LIVER OF EUTHYROID AND HYPOTHYROID ANIMALS.[a]

	% Tetraploid	
Animal number	Euthyroid	Hypothyroid
1	49	33
2	66	36
3	72	33
4	60	27
5	60	26
6	58	36
7	65*	32
Mean (7 animals)	61.4	31.9
SD	7.3	4.0
P	0.001	

[a] The ploidy of liver nuclei (10,000 nuclei per sample) from 12-week old euthyroid and hypothyroid rats was determined by using acridine orange or propidium iodide in flow cytometry. The table shows that euthyroid rats had almost twice as many tetraploid nuclei ($P < 0.001$) as hypothyroid rats. *Propidium iodide stained nuclei. In all other experiments, acridine orange was used.

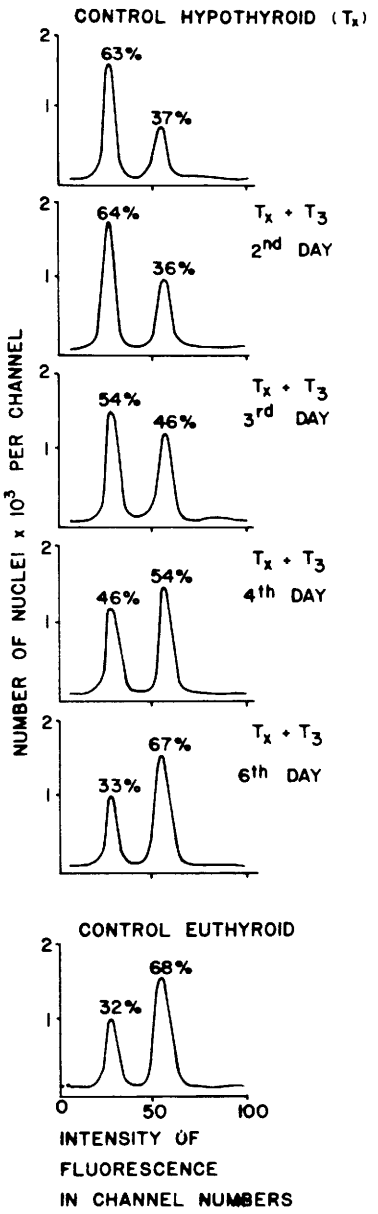

FIG. 2. Effect of T_3 administration on nuclear ploidy. Histograms of green AO fluorescence (coordinates as in Fig. 1, left column). Hypothyroid (Tx) rats (12 weeks old) were injected with 1 mg T_3/100 g BW, and nuclei were isolated 2, 3, 4, and 6 days after T_3 injection. Six days after T_3 injection, the distribution of nuclei between the diploid and tetraploid fraction is similar to that of euthyroid animals of the same age.

in the tetraploid fraction from 37% to 46% was noted. On the fourth day after T_3 injection, the majority of nuclei were tetraploid. After 6 days of hormone administration, the histogram was similar to that found in euthyroid animals of the same age.

Discussion. The influence of thyroid hormone on the formation of polyploid nuclei in rat liver was evaluated by rapid flow cytometry. This method permits the determination of ploidy by direct quantification of nuclear DNA content and estimation of nuclear size by the degree of light scatter. The measurements can be performed rapidly (10,000 nuclei per sample in 10 sec), and with great accuracy.

It should be noted, however, that while the use of isolated nuclei, rather than whole cells, eliminates interferences from satellite DNA and cytoplasmic RNA, it does not provide information on the frequency of binucleated cells.

Ploidy distribution represented by fluorescence histogram measuring the DNA content was found to be similar, though not identical, to those obtained by light scatter reflecting the many factors, other than DNA content, which may influence the size of nuclei (3).

The results of these studies, which use entirely new methods to determine nuclear ploidy, confirm the observation of Carrière (3) and Nadal and Zadjela (4), that the thyroid hormone exerts a marked influence on the formation of polyploid nuclei in rat liver. Thyroidectomy was found to arrest the ploidy pattern at the level observed at the time of surgery, indicating interference with the increase in polyploid fraction normally occurring during the period of growth. These events could be reversed by the administration of thyroid hormone which, within 6 days, led to the distribution of diploid and tetraploid nuclei characteristic of euthyroid animals of the same age. This rapid change in ploidy would require a high mitotic rate because new tetraploid nuclei are formed only during mitosis. Although the mitotic rate was not determined in this study, it has been reported (3) that the administration of thyroid hormone to hypothyroid rats increased the mitotic index from 0.0004 to 0.2%, considerably greater than that of normal rats, which ranged from 0.03% to 0.05%. In this study, we have not examined the question of whether the effects of thyroid hormone were derived from its direct interaction with specific hepatic nuclear receptors (11), or were mediated indirectly by growth hormone. The stimulating effect of T_3 on growth hormone production has been known for many years and has recently been confirmed by radioimmunoassay procedures (12). The influence of growth hormone on hepatic ploidy has been well documented: polyploidy does not develop in dwarf mice nor in congenital pituitary insufficiency in man (13). Hypophysectomy in young animals arrests the development of ploidy (14), a characteristic of normal growth which can be stimulated by partial hepatectomy (4). Furthermore, thyroid hormone administration to hypophysectomized animals increases the number of tetraploid nuclei, demonstrating an effect of thyroid hormone itself (15).

It is of interest that any factor which can cause the slowing of growth of the organism is capable of affecting hepatic ploidy (4). Thus, it appears that a constant equilibrium exists between the total body mass and the amount of liver parenchyma. This equilibrium appears to be regulated by factors capable of stimulating or inhibiting hepatic mitoses necessary for the development of polyploidy. The nature of these factors remains to be clarified. The advantage of the method described in this paper is that it permits a significantly more rapid and accurate determination of hepatic ploidy than do the microscopic procedures. It may, therefore, be applicable to a large-scale study of liver ploidy in various physiologic and pathologic states.

Summary. The ploidy distributions of rat liver nuclei isolated from normal and thyroidectomized rats were determined after conjugation with fluorochromes acridine orange and propidium iodide by rapid flow cytometry on the basis of their DNA content. Using this technique, we have confirmed the marked influence of thyroid status on the ploidy of rat liver nuclei as compared to euthyroid rats of the same age. No further development of tetraploid nuclei occurred from the time of thyroidectomy. However, administration of 1 mg T_3/100 g BW to hypothyroid rats led within 6 days to ploidy pat-

terns identical with those observed in euthyroid animals of the same age.

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