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Maturation of Rat Megakaryocytes Studied by Microspectrophotometric Measurement of DNA.* (30412)

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The maturation process of the megakaryocyte series of cells is not well understood. It is generally accepted that nuclear division occurs without cytoplasmic division during megakaryocyte maturation, with the result that nuclei of mature megakaryocytes contain several times the normal diploid chromosome complement of the species. There has been no completely objective method of grouping megakaryocytes into maturation stages based on their appearance under the light microscope. It seemed, however, that measurement of the quantity of deoxyribonucleic acid (DNA) in individual megakaryocytes might provide a systematic means of classification. With such a method additional insight into the maturation process of megakaryocytes may be gained, and a clearer assessment of effects of various experimental or disease-related perturbations of the megakaryocyte-platelet system may be possible. We have, therefore, measured microspectrophotometrically the DNA content of megakaryocytes in Feulgen-stained preparations of smears of rat bone marrow. Garcia(1) has recently described the DNA content of megakaryocytes of rabbits measured by essentially the same methods.

Methods. Smears of bone marrow of tibias of 8 male Sprague-Dawley rats, 23 to 107 days old, were made. The smears were air-dried for at least 1 hour, fixed in Carnoy's solution (6 parts absolute alcohol: 3 parts chloroform: 1 part acetic acid) for 5 minutes or 1 hour, and subjected to the Feulgen-staining procedure(2,3). In most cases, smears were hydrolyzed for 6 minutes at 60°C in 1 N HCl, stained in decolorized basic fuchsin for 30 minutes, and rinsed twice with SO_2 for 2-3 minutes, and once with tap water for 5 minutes.

The DNA of individual cells was measured microspectrophotometrically. The 2-wavelength method(4,5) was used since it tends to reduce distributional error introduced by the uneven distribution of chromatin material of megakaryocyte nuclei. A Canalco Ultra-Microspectrophotometer with a digital ratio reporter and a Bausch and Lomb grating monochromator as light source were employed. The image of a nucleus was centered on a mirror (the phototube head mirror) seen by a phototube, and the clear area around the nucleus was minimized by using an iris diaphragm and 2 leaf diaphragms. The light intensities passing through the specimen were measured at 2 wavelengths: (A) near the absorption peak of the Feulgen-stained DNA (565 mμ in our work), and (B) at a wavelength (506 mμ) where the absorption coefficient was half of that at the first wavelength. Background was determined by similar measurements in a clear area of the slide. The instrument automatically presents the results as percent transmission at the 2 wavelengths. The iris and leaf diaphragms were then moved out of the field and a sliding plate having a hole of fixed diameter was moved into the groove formerly occupied by the leaf diaphragms. The ratio between the amount of light transmitted through the fixed diaphragm and the diaphragm employed for the nucleus was used as a measure of the

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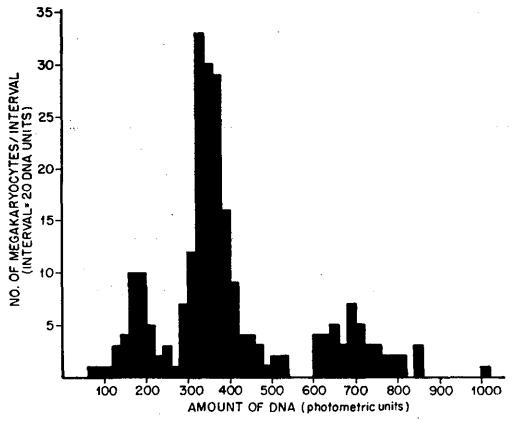

FIG. 1. Distribution of DNA values of megakaryocytes of rat 7 (Table II) determined by microspectrophotometry. To make the histogram, the observed DNA values were grouped into 20-unit intervals. Total number of megakaryocytes measured was 238, all in one marrow smear. There was also one DNA value of 1556, not shown in Figure.

area of the latter. Patau's formula(4) was then used to convert the transmission and area measurements into arbitrary units of DNA.

Oil of minimum refractive index for bone marrow smears (1.540) was used in mounting the coverslips. Megakaryocytes were measured with a 20-power objective and other cells with either a 20- or 43-power objective. Seventy-three to 346 megakaryocytes from each of 8 rats were measured, totaling 1693. All megakaryocytes on the margins of marrow smears were measured except those that were crowded so close to other cells that very little free area was left around the nucleus. Megakaryocytes were identified by the size and character of their Feulgen-stained nuclei. In some marrow preparations nuclei of cells other than megakaryocytes were also measured. Blood smears of some of the rats were similarly stained with the Feulgen reaction at the same time as the bone marrow smears of those rats, and the DNA of lymphocytes or monocytes was measured.

Results. When the DNA values of megakaryocytes were arranged in a frequency distribution, the megakaryocytes fell into groups with the modal DNA content of successive groups increasing approximately by a factor of two. A histogram depicting DNA content of megakaryocytes of one rat, arranged in 20-unit intervals as shown in Fig. 1, illustrates the modal interval, the number of cells, and the variation about the modal interval for each group. In this specimen there was one value of 1556 DNA units which is not shown on the Figure. The shapes of the histograms of DNA values of megakaryocytes of the 7 other rats were similar.

In some cases the DNA content of marrow cells other than megakaryocytes or of peripheral blood cells was also determined. There appeared to be unimodal distribution with a fairly large variation (Fig. 2). The modal value was assumed to represent the 2N or diploid amount of DNA (Table I). The differences in diploid values between rats seen in Table I are due largely to changes in the Feulgen-staining procedure, mainly in the length of hydrolysis time, which was modified somewhat from rat to rat. The divergences of diploid values of different kinds of cells from the same animal, all stained at one time, are, however, probably due to errors inherent in the 2-wavelength method, which may not correct completely for distributional errors, and may tend to give relatively higher esti-

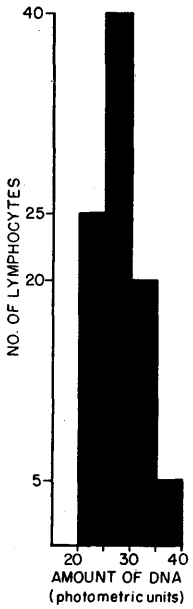

FIG. 2. Distribution of DNA values of lymphocytes determined by microspectrophotometry. To make the histogram, DNA values were grouped into 5-unit intervals. Total number of lymphocytes measured was 90, all in one blood smear (rat 1 in Table I).

TABLE I. DNA Values* (Photometric Units) of Different Kinds of Cells.

Rat	Megakaryocytes		Lymphocytes		Monocytes		Marrow cells	
	Mode of 16N class	Estimate of 2N(16N/8)	Mode	No.	Mode	No.	Mode	No.
1	320	40	27†	90				
5	720	90					78	68
6	470	59	44	21				
7	340	42	32	21	47	26	46	38

* Values can be compared only within each row.

† Lymphocyte values of rat 1 were measured with 43× magnification and multiplied by $20^2/43^2$ to make them comparable with megakaryocyte values that were measured with 20× magnification. Nuclei of all other diploid cells and of megakaryocytes were measured with 20× magnification.

mates of DNA values for larger cells than for smaller cells. In addition, the variation in diploid values within an animal may also reflect a real biological difference, since it has not yet been ascertained conclusively whether the diploid value is quite constant for all cells of an individual or varies around a mean value with somewhat different submeans for different kinds of cells or tissues (6). The ploidy of the various megakaryocyte classes was determined by comparison with the observed 2N values. In Table I are shown the modal DNA values of lymphocytes, monocytes, or marrow cells of 4 of the 8 rats whose megakaryocytes were measured, along with the modal value of what was interpreted thereby to be the 16N megakaryocyte class, and the 2N DNA value that was estimated by dividing the presumed 16N value by 8. Comparison of the 2N values of diploid cells with the 2N values calculated from megakaryocyte measurements indicates that the estimate of 2N that we chose for megakaryocytes agrees overall more closely with the observed 2N values of diploid cells than would one-half (or twice) that number.

To estimate the proportion of cells in each group a model was constructed assuming the frequency distribution of values of log DNA to be approximated by a normal (Gaussian) distribution for each group and by the sum of normal distributions for the combined groups. It was assumed that the means (modes) of the distribution occurred in the sequence M, 2M, 4M, 8M in the original units and m, m + .3, m + .6, m + .9 in log DNA units, where $m = \log_{10}M$ and $.3 = \log_{10}2$. The conversion to logarithms of the DNA values determined microspectrophotometrically resulted in distributions which were more nearly normal and tended to equalize the variances of the groups. The function used to describe the combination of 3 groups is:

$$F = f_1 e^{\frac{-(x-m)^2}{2s^2}} + f_2 e^{\frac{-(x-m-.3)^2}{2s^2}} + f_3 e^{\frac{-(x-m-.6)^2}{2s^2}},$$

where m, m + .3, m + .6 are the means of the groups, f_1, f_2 and f_3 are proportional to the frequencies in the groups, s^2 is the common variance and x is the $\log_{10}$ DNA value. A fourth term was added for the combination of 4 groups and the parameters m, s^2 and f's and their standard errors were estimated by least squares, utilizing a high speed computer for nonlinear functions. The observed and estimated frequency distributions of the DNA content of the 238 cells of rat 7 are shown in Fig. 3. The estimates of the frequency of cells in each class are summarized in Table II. Very few cells fell into the 4N

TABLE II. Frequency of Megakaryocytes in Ploidy Classes.

Rat	Total No. megs. counted	Relative frequency (%) 4N	8N	16N	32N	Age (days)
1	149	—	12.4	78.1	9.4	23
2	310	0.5	17.4	66.3	15.8	36
3	166	2.2	12.1	72.7	12.9	65
4	73	5.4	9.6	71.0	14.1	65
5	186	0.03	4.9	74.0	21.1	65
6	225	0.9	6.8	67.4	25.0	65
7	238	0.9	12.1	67.7	19.4	107
8	346	—	8.6	72.3	19.1	107
Mean		1.6	10.5	71.2	17.1	
S.E. of mean*		0.80	1.37	1.40	1.77	

* Based on the variation among the estimated frequencies.

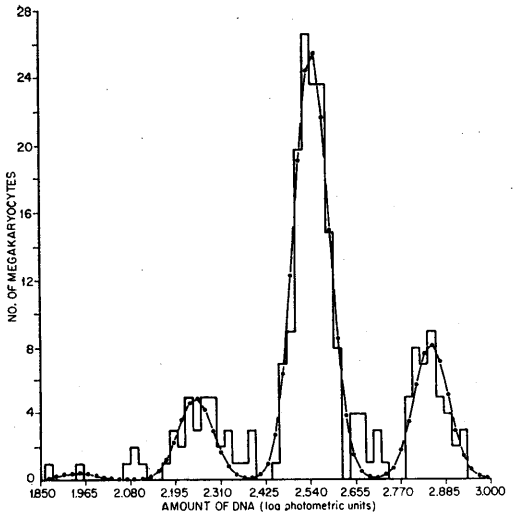

FIG. 3. Observed and estimated distributions of log DNA values of megakaryocytes of rat 7 using the formula given in the text. Histogram indicates observed values, and differs somewhat from Fig. 1 because a different class interval was used.

class. An average of 10% of the cells occurred in the 8N class and 17% in the 32N class. A very large percentage, averaging 71%, was present in the 16N class. Two of the 1693 cells that were measured had DNA values compatible with a 64N class. No differences attributable to age were apparent within the limits of the numbers and ages of the rats studied (Table II).

Discussion. Our results demonstrate that megakaryocytes can be placed in groups according to the amounts of DNA in their nuclei, in agreement with the results of Garcia(1). In addition, the DNA values can be ordered in a 2N, 4N, 8N (2^xN) type sequence, indicating a common DNA synthesis (S) period of the chromosomes in a polyploid megakaryocyte. Upon counting the number of "nuclei" per megakaryocyte in human marrow, using a conventional light microscope, Japa(7) had concluded that "mitosis occurs synchronously in all the nuclei" of a megakaryocyte. Moreover, Kinosita and Ohno(8) observed multipolar mitosis in polyploid megakaryocytes by *in vivo* microscopy through windows in the femurs or tibias of rabbits. It appears therefore that the replicative cycle is to a large extent synchronous among the chromosomes within a polyploid megakaryocyte.

The DNA values occurring between modal peaks probably belong to cells that had progressed part of the way through the S phase at the time of the sampling. Since the S phase lasts 5 to 10 hours in many mammalian cells, and since the various chromosomes of a single nucleus do not all replicate at exactly the same time(9,10), even though nuclear division may be synchronous, a distribution of DNA values in the intervals between classes would be expected.

To determine the ploidy of the various groups of megakaryocytes, the modal DNA values of these groups were compared with diploid values of lymphocytes and monocytes of blood smears and of cells other than megakaryocytes in the marrow. By our interpretation of the correspondence of peak frequencies with N values, the class with the greatest number of cells is the 16N class, while the 8 and 32N classes include considerably fewer cells, and the 4N class only a very small percentage. The numbers may be low in the 4N class because of failure to recognize some of these cells, since recognition was dependent entirely upon observation of nuclei stained by the Feulgen reaction, without any cytoplasmic counterstaining. If it is assumed that all megakaryocytes follow the same maturation process and pass through all stages, then the relative numbers of cells of each ploidy indicate the relative length of time spent in the various stages. The small number in the 4N class may indicate that this stage turns over very rapidly. Likewise the largest percentage of the maturation time would be spent in the 16N stage and much less time in the 8N and 32N stages. Another possible interpretation, however, is that death of megakaryocytes by platelet production from their cytoplasm can occur in more than one of the polyploid stages. Thus, platelet production by 16N megakaryocytes would reduce the number of cells remaining to continue to the 32N stage. Fewer 32N than 16N cells would, from this viewpoint, indicate that only a part of the megakaryocyte population arrived at the 32N stage. The question whether platelets are produced

at several ploidy stages or only the final stage of megakaryocyte maturation remains to be resolved. Since, however, both 8N and 32N stages have much lower frequencies than the 16N stage, it seems reasonable to hypothesize that megakaryocytes remain in the 16N stage considerably longer than in either the 8 or 32N stages.

The percentage frequencies of the various ploidy classes that we obtained by microspectrophotometric measurement of DNA (4N, 1.6%; 8N, 10.5%; 16N, 71.2%; 32N, 17.1%; 64N, 0.1%) are roughly similar to those reported by Japa(7), who counted the number of nuclei in megakaryocytes in squashes of human marrow (4N, 2.5%; 8N, 25.5%; 16N, 53%; 32N, 18%; and 64N, 1%). The class frequencies seen in Garcia's (1) histograms of DNA values of rabbit megakaryocytes are also similar to ours, especially in the higher ploidy classes, except that the class having the highest frequency in his study was 32N rather than 16N. This may be due to a species difference between rabbits and rats. The maximum chromosome complement of megakaryocytes has been variously reported as 16, 32, or 64N. In our study, only 2 of 1693 cells had 64N values of DNA, making this class very rare.

Classification of marrow megakaryocytes into maturation stages on a morphological basis has varied with investigators, with the method most closely resembling the microspectrophotometric measurement of DNA having been Japa's system of counting number of nuclei per cell(7). Other methods have relied on cytoplasmic as well as nuclear morphology. In studies of megakaryocytes in rats, Feinendegen and associates(11) and Ebbe(12) divided recognizable megakaryocytes into 3 classes or groups. About 18% of the megakaryocytes were placed in Group I, the most immature cells recognized; about 22% in Group II; and about 60% in Group III, the most mature cells. Other studies based on cell morphology(13) have also indicated that about 70% of the recognizable megakaryocyte population is comprised of platelet-producing ("mature") megakaryocytes. It is readily apparent that there is not a simple correspondence between these 3 morphologic groups and either the ploidy groups determined by microspectrophotometry or the groups of Japa determined by counting nuclear lobes. Groupings into 3 morphologic stages may not necessarily separate cells according to ploidy, and such groups may include more than 1 ploidy class.

The distribution of megakaryocytes through a series of ploidy classes, based on DNA measurements, suggests that replication of DNA may continue throughout the process of megakaryocyte maturation. In contrast, the results of DNA labeling experiments(11, 12), in which only megakaryocytes of the earliest stage recognizable under the light microscope (Group I) were labeled for the first few hours after injection of tritiated thymidine (H^3Tdr), suggested that DNA replication occurred only in these younger megakaryocytes, and concomitantly that Groups II and III have the same ploidy. However, different criteria were used to determine the stages of maturation of the megakaryocytes in the 2 cases: quantity of DNA in the former, and morphological appearance of the cell, especially the cytoplasm, in the latter. It is possible that development of polyploidy by nuclear replication and maturation of the cytoplasm may occur independently of each other with the result that any degree of polyploidy might be found in company with any degree of cytoplasmic differentiation. Thus, nuclei of varying ploidy might appear in megakaryocytes with completely differentiated cytoplasm as suggested by the results of Ebbe(12) who reported that nuclei of Group III were either single, multiple, or segmented. Likewise, nuclear replication might proceed to a highly polyploid state in megakaryocytes having immature cytoplasm. Indeed, Feinendegen *et al,* and Ebbe saw mitosis only in Group I megakaryocytes. Thus, the possibility is raised that a very active period of DNA synthesis leading to nuclear polyploidy takes place prior to cytoplasmic maturation of megakaryocytes. The labeling experiments with tritiated thymidine (11,12) suggest that a major proportion of the cells destined to enter the compartment of morphologically-recognizable megakaryocytes in the 2 days after injection of H^3Tdr,

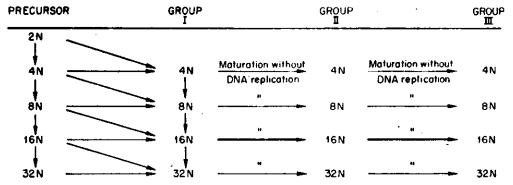

FIG. 4. Hypothesis of megakaryocyte maturation (see text). Groups I, II, and III refer to the system of megakaryocyte classification used by Feinendegen *et al*(11) and Ebbe(12).

or whose progeny would enter that compartment, were synthesizing DNA at the time of isotope injection (the H^3Tdr pool is said to last less than 30 minutes), since the percentage of the megakaryocyte population that was labeled reached nearly 100% within 48 hours, although only 20-30% of the megakaryocytes in Group I and none in Groups II and III were labeled within 30 minutes after injection of H^3Tdr. This interpretation further suggests that the DNA synthesis phase may occupy a large percentage of the generation cycle of these cells.

As a working hypothesis, we propose that nuclei of megakaryocytes become polyploid independent of and prior to cytoplasmic maturation. This also requires that nuclear replication (but not necessarily morphological changes in the nucleus) permanently cease at various ploidy stages whenever the cytoplasm commences to mature (Fig. 4), with the further implication that platelet production and megakaryocyte death may occur at any ploidy stage from 4 to 32N.

In conclusion, measurement of DNA by microspectrophotometry provides a quantitative means of classifying megakaryocytes into groups. Whether the groups of successively higher ploidy parallel stages of cytoplasmic maturation of the megakaryocytic series of cells is still uncertain. This method will be used in further studies of the megakaryocyte life cycle and studies of the mechanisms that regulate megakaryocytes and platelets.

Summary. The amount of DNA in the nuclei of individual megakaryocytes of male Sprague-Dawley rats was measured microspectrophotometrically in marrow smears stained by the Feulgen reaction. When the DNA values were arranged in a frequency distribution, the megakaryocytes fell into groups with the modal DNA content of successive groups increasing approximately by a factor of 2. Ploidy of the megakaryocyte groups was determined by comparison with DNA values of diploid cells. Frequencies of 4N, 8N, 16N, and 32N megakaryocytes were, respectively, 1.6%, 10.5%, 71.2%, and 17.1%. The results indicate synchronous division of the nucleus of polyploid megakaryocytes. The relative numbers of cells in ploidy stages may indicate the relative length of time spent in the stages. An hypothesis of megakaryocyte maturation is proposed. The method may prove useful in studies of megakaryocyte maturation and regulation.

ADDENDUM: In a recent microspectrophotometric study, de Leval observed a frequency distribution pattern of DNA of polyploid megakaryocytes of guinea pigs very similar to that of rats described here (de Leval, M., Compt. Rend. Soc. Biol., 1964, v158, 2198).

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