Microspectrophotometric Estimation of the Desoxyribonucleic Acid (DNA) Content of Individual Normal and Leukemic Human Lymphocytes

By Nicholas L. Petrikis, M.D.

The development of microspectrophotometric methods for the study of constituents of individual cells provides an important tool for cytologic research and for the re-evaluation of many concepts based on purely morphologic hematology. Through the application of ultraviolet absorption microspectrophotometry, a relationship between nucleic acids and protein synthesis has been demonstrated, particularly with respect to ribonucleic acid and the myeloma plasma cell.1-2 Recently an important correlation between the chromosome number and the DNA content of the cell nucleus has been established.3-8 The cellular DNA appears to be confined to the chromosones, and this quantity of DNA is believed to be a constant value in the interphase diploid somatic nuclei of all tissues within a species and is twice the amount of DNA present in the haploid germ cell nuclei. Since the DNA content of the nucleus in nonmitotic cells parallels the chromosome number, it gives in practice a physicochemical measure of the chromosome number in nonmitotic, nonpolytene nuclei, especially when compared with the DNA content of spermatids of the same species.

While chemical determinations of the average DNA content per cell might be adequate in tissues with a very low rate of mitosis and in the absence of nuclear polyploidy, the true distribution of the DNA in individual cells cannot be thus determined in mixed populations of cells characterized by probable varying rates of mitosis and aberrations of mitosis as have been reported in the leukemias.9

In the present study, microspectrophotometric determinations of Feulgen dye were employed to estimate the relative DNA content of individual lymphocytes in smears from the blood, lymph nodes, and bone marrow of normal subjects and patients with acute and chronic forms of lymphocytic leukemia. In addition, imprints from testes were made in order to compare the DNA content of lymphocyte nuclei with that of haploid spermatid nuclei. An evaluation was made of the significance of the findings in terms of recent advances in cytogentices.

Materials and Methods

Lymphocytes were obtained from the blood of nine normal subjects and from fifteen patients with acute and chronic lymphocytic leukemia. Heparinized venous blood was centrifuged at 3000 r.p.m. for 3 minutes and the buffy coats were obtained for smears. The...
### Table 1.—Average DNA Content (Arbitrary Units) of Lymphocytes from Normal and Leukemic Subjects, with Pertinent Clinical Data

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<th>Subject</th>
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<th>Age</th>
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<th>Standard error</th>
<th>No. cells measured</th>
<th>Total leukocyte count/cu. mm.</th>
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**MICROSPECTROPHOTOMETRIC ESTIMATION OF DNA IN LYMPHOCYTES**

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Fig. 1.—Upper graph: Distribution curve of DNA content of normal human blood lymphocytes and spermatids. Lower graph: DNA content of lymphocytes from a normal human axillary node. Ordinate: number of cells measured. Abscissa: DNA in arbitrary units.

Fig. 2.—The DNA content as plotted against nuclear diameter for circulating blood lymphocytes (left) and lymph node lymphocytes (right).
snears, while still moist, were fixed in 100 per cent methyl alcohol for 30 seconds and then were air dried. In addition, bone marrow smears and lymph node imprints from aspiration and biopsy material were obtained from three patients with leukemia and were prepared similarly. Imprints from the testis were obtained from one patient shortly after death and were fixed in the same manner in order to compare lymphocytes with spermatids with respect to their DNA content.

The cells were stained with the Feulgen reagent following hydrolysis with 1N HCl for 12 minutes in a water bath set at 56°C. Staining was carried out at room temperature for 1 hour. Fixation with methyl alcohol rendered the chromatin diffusely homogeneous. The validity of using smears rather than sectioned material was shown by Korson. The microspectrophotometric apparatus and technic employed in this study were similar to those described by Pollister. This apparatus consisted of a 931A photomultiplier tube mounted above a microscope with an interposed iris diaphragm. This arrangement permitted a single nucleus or portion thereof to be projected onto the photomultiplier tube, and the intensity of light transmitted through the nucleus to be recorded on a microammeter. The light transmitted through an empty area on the slide was used for the blank reading. The light source consisted of a tungsten ribbon filament lamp in conjunction with a Farrand interference filter with wave length of 550 mÅ, which is near the maximum absorption of the Feulgen stain.

The DNA content per cell, in arbitrary units, was calculated following the procedure of

![DNA Content Plot](attachment:image.png)

**Fig. 3.**—The DNA content of individual lymphocytes in four cases of chronic lymphocytic leukemia.
Korson. The extinction coefficient of the nucleus when multiplied by its area gives the DNA content in relative units.

Blank unstained cells were found to have extremely low extinctions and were accordingly neglected from the calculations. Care was taken to keep the field of illumination at approximately 30 μ in diameter in order to reduce the error contributed by light scatter.

The lymphocytes measured had intact nuclei without mitotic figures. In most instances they were round, and in those cells with elongated nuclei, the long and short diameters were averaged. With the exception of the spermatids, the cells were selected at random.

The smears were examined for mitotic figures, but attempts to make chromosome counts were abandoned because of the poor definition of the material. The mean and standard error was calculated for the cells from each patient; curves showing the distribution of the DNA in individual cells were also made.

![DNA Content of Lymphocytes in Acute Leukemia](image)

**Fig. 4.**—The DNA content of lymphocytes in acute lymphocytic leukemia

### Results

**Normal lymphocytes:** The average DNA content per nucleus, expressed in arbitrary units, in circulating lymphocytes from nine normal subjects with corresponding blood lymphocyte count, age, and sex, is shown in Table 1. The circulating lymphocytes from all normal subjects had essentially the same values, averaging 4.33 ± 0.15 units. The DNA content of human spermatids averaged 2.19 ± 0.05 units, which is half the mean value of the lymphocytes. The distribution of DNA in individual normal lymphocytes and spermatids is shown in figure 1. The DNA content of lymphocytes from two normal human lymph nodes was also determined. In conformity with the designation of Swift, the average DNA value for the circulating lymphocytes was designated as class I (diploid). Nuclei
containing twice this value of DNA were designated as class II nuclei (tetraploid). In this tissue, in addition to nuclei with the class I DNA content, many nuclei were found to contain twice the common value present in circulating lymphocytes. Many nuclei were also found with DNA values falling intermediate between the class I and class II levels. Practically no cells measured contained values markedly elevated above the average for class II (fig. 1). In the lymph nodes, the cells of class II were invariably of greater diameter and possessed from one to three nucleoli. These cells resembled those in the germinal centers,

![DNA Content of Blood and Marrow Lymphocytes in Chronic Lymphocytic Leukemia](image)

**FIG. 5.—Comparison of DNA content of blood and marrow lymphocytes from two patients with chronic lymphocytic leukemia.**

while the smaller class II nuclei corresponded to those from the secondary areas of the nodule. A direct relationship between DNA content and cell size was found to occur in the lymph node lymphocytes, but no definitive relationship of cell size and DNA content was present in circulating blood lymphocytes (fig. 2).

Leukemic lymphocytes: The average DNA content per nucleus of leukemic lymphocytes expressed in arbitrary units, with peripheral lymphocyte counts and pertinent clinical data, is shown in table 1. Individual leukemia lymphocytes were found to have a wide variation in DNA content, ranging from values similar to normal lymphocytes, up to 4 times the class I values. The majority of these nuclei with elevated values were in the intermediate range between class I and class II, but many were found with values above the class II level. In six
of the nine patients with chronic lymphocytic leukemia, the circulating lymphocytes were of the class I variety. Clinically these patients were characterized by having long histories of leukemia, and were in remission with their disease (table 1). In the three remaining patients the disease was clinically exacerbated. Patients PAR, VAL and FLU were in terminal stages of chronic lymphocytic leukemia. Patient PAR had a progressive leukemia cutis, associated with marked leukocytosis, anemia and thrombocytopenia. In these patients, the DNA values of individual cells were elevated above the class I level, falling predominantly between class I and II. In patient PAR many cells were found to contain DNA

![Comparison of DNA Content of Lymphocytes in Peripheral Blood, Bone Marrow, and Lymph Node in a Patient with Acute Leukemia](image)

**Fig. 6.**—Comparison of the DNA content of blood, marrow, and lymph node lymphocytes from a patient with acute lymphocytic leukemia.

values above the average class II values. Figure 3 graphically depicts examples of the distribution of DNA in the individual cells from four patients with chronic lymphocytic leukemia. Morphologically these cells were similar to normal lymphocytes and had no, or at most one, nucleolus.

In four of five patients with acute lymphocytic leukemia, the DNA content of individual nuclei was found to be increased above the class I (diploid) value of $4.33 \pm 0.15$. One patient (GIL) had cells whose contents of DNA were entirely of the class I value (table 1 and fig. 4). No blast cells were present in this patient's blood. The predominant cells present in the remaining four patients were of greater diameter than normal lymphocytes. They possessed varying numbers of nucleoli, ranging from 1 to 6, and morphologically were considered to be lymphoblasts. The clinical symptoms in all of these patients were acute, and the duration of life was uniformly short (table 1).
Comparative studies were made on the DNA content of lymphocytes from the bone marrow and peripheral blood of three patients, VAL, PIT and LAB. Lymph node lymphocytes were also studied from LAB. These data are graphically depicted in figures 5 and 6. A number of lymphocytes in the marrow were found to contain more DNA than morphologically similar cells from the circulating blood.

**DISCUSSION**

The data obtained in the present study demonstrate that the DNA content of individual normal human lymphocytes from venous blood averages twice the amount present in the human spermatids. These findings indicate that normal lymphocytes have the diploid chromosome number, and are in agreement with the findings of Kempe in man and Swift in mice. No alteration of DNA content from the diploid value was found in normal circulating lymphocytes suggesting that the morphologic classification of large and small lymphocytes is not due to differences in DNA content (fig. 2). The findings on the normal lymph node reported here are indicative of the presence of two classes of nuclei, one corresponding to the diploid class I DNA content and the other containing twice this amount, or tetraploid. This confirms the finding of Marinone who reported two classes of normal human lymph node lymphocytes as determined by the microspectrophotometric technic. The normal nodes studied in this report contained many cells with a DNA content intermediary between the diploid and tetraploid value with few cells with an increase in DNA above the tetraploid value.

In chronic lymphocytic leukemia, six of the nine patients studied had blood lymphocytes which were uniformly of a diploid nature, as is indicated by their normal DNA distribution and average DNA content. These cells morphologically resembled normal lymphocytes. The findings agree with the chemical determinations of DNA phosphorus in leukemic leukocytes reported by Davidson, Leslie, and White and by Metais and Mandel, in which no increase above normal in average DNA content was found. However, in the remaining three patients with chronic lymphocytic leukemia, and in four of five patients with acute lymphocytic leukemia, many cells were found in the blood with DNA values above the diploid value, as is shown by the alteration of the DNA distribution curve and the increase in average DNA content per cell. The cells found in acute leukemia, in the majority of patients, were morphologically lymphoblasts, but numerous small lymphocytes were also found. The studies made of marrow, blood, and lymph nodes from leukemic patients revealed cells with diploid, tetraploid, and intermediate values. It is likely that the previous reports of lack of significant increase in the DNA content of leukemic leukocytes are based in part on the clinical status of the patients at the time of the study, and in part on the inability of chemical technics to detect varying degrees of mitosis and/or polyteny which might be present in mixed populations of various cell types as occur in blood and bone marrow. The microspectrophotometric technic enables one to select for measurement the specific individual cells under consideration.

From the viewpoint of cytogenetics the demonstration in this study of nuclei containing increased DNA contents may be interpreted in the following ways:

1. **Mitotic activity.** It has been shown by a number of investigators that
a build up of DNA occurs in a cell during the preprophase period of mitosis, until at onset of prophase the DNA content is double that of the intermitotic value. The resulting daughter nuclei possess the diploid chromosome number and the average DNA value for that class of nuclei. The proportion of cells in a sample showing increased DNA is related to the mitotic activity of the tissue, and can be expressed chemically by the average DNA content per nucleus, in the absence of extensive polyploidy.

2. Polyploidy and polyteny. Polyploidy is characterized by an increase of chromosome number in a cell nucleus in a progressive fashion, 2, 4, 8, etc. times the diploid (2N) number of chromosomes. Polyteny can be considered as a condition characterized by reduplication of the chromonemata without reduplication of the chromosomes, resulting in chromosomes of increased size, and without an increase in total chromosome count above the diploid number. In such instances, the DNA content might be markedly increased, depending upon the degree of polyteny, as has been demonstrated photometrically in the Drosophila salivary gland nuclei. The distinction between these two forms of ploidy is not possible in DNA measurements on interphase nuclei where the chromosomes are not visible, but the absence of tetraploid chromosome numbers in the mitotic figures reported in lymphatic leukemia would indicate that the increases in DNA present in class II nuclei are due to the presence of polyteny, rather than polyploidy. The possible physiologic roles of these forms of ploidy is unknown.

3. Amitosis. This condition is characterized by an unequal assortment of chromosomes resulting from a direct cell division without the normal formation of the spindle. The extensive occurrence of this process in the present material should be reflected by the presence of cells with DNA values below the diploid average. Lymphocyte nuclei with decreased DNA contents were not observed in the present study, suggesting that amitosis did not play a significant role.

At the present state of our information regarding nuclear DNA, the most plausible explanation of the presence of increased DNA in the nuclei of lymphocytes is that it represents the DNA build-up prior to mitosis, and in those cells where doubling of DNA exists it is strongly indicative of polyteny and superimposed mitotic activity.

The scarcity of mitotic figures in the circulating blood in lymphocytic leukemia has been noted for many years. Dock extensively reviewed the subject in 1902 and was unable to find mitotic lymphocytes in two carefully studied cases of small-cell (lymphocytic) leukemia. In a recent cytologic study, Polli reported that myeloid cells contained the diploid chromosome number, but he did not report on lymphocytes. Bowcock and Dickson and Groat have reported the finding of haploid lymphoblasts in lymphoblastic leukemia. Mitotic figures suitable for chromosome counts were not observed in the present material, but of greater significance was the fact that the photometric determinations of individual lymphocytes and lymphoblasts did not reveal the presence of nuclei with haploid DNA values. Abnormalities of mitosis such as haploidy are of interest when observed, but their presence does not necessarily imply that most of the mitoses are abnormal. Koller found that while the incidence of abnormal mitoses in tumors was occasionally as high as 25 per cent, the preponderance of mitotic figures was morphologically normal. Furthermore, it has been questioned whether such abnormal cells continue to function and multiply.
MICROSPECTROPHOTOMETRIC ESTIMATION OF DNA IN LYMPHOCYTES

The absence of mitotic figures in circulating cells might possibly result from the fact that such cells are filtered from the circulation by the lung capillaries. A greater percentage of lymphoblasts and young lymphocytes has been found in the pulmonary artery than is found in simultaneous blood samples from the aorta.\cite{1}

In most patients with chronic lymphocytic leukemia studied here, the circulating lymphocytes were probably intermitotic cells, as was indicated by their normal DNA content. In four of five patients with acute leukemia, and in three of nine with chronic lymphocytic leukemia, the cells released into the circulating blood were characterized by an increased DNA content of the nucleus, indicating that they were in the process of active mitosis, and in some cases were polytene as well. Analogous increases in the average DNA content per cell of splenic lymphocytes from leukemic C58 black mice were found by Petermann and Schneider\cite{2} in the rapidly growing transplanted leukemia. In the slow-growing spontaneous leukemia in the same strain, the leukemic splenic lymphocytes had an average DNA content per cell similar to the normal nonleukemic spleen lymphocytes. The present study demonstrates an alteration in the state of DNA metabolism of lymphocytes in many of the patients with the clinically more active forms of lymphocytic leukemia. In such patients, the lymphocytes appear to be released into the circulation in an active phase of mitotic activity, as is shown by their increased DNA content. Such increases in DNA content are not found in the circulating lymphocytes from nonleukemic normal subjects and other patients with chronic lymphocytic leukemia. Whether such “mitotically active” leukemic cells represent metastatic foci which potentially might colonize and proliferate in other organs cannot be determined from these studies.

SUMMARY

The DNA content, in arbitrary units, of individual circulating lymphocytes from nine normal subjects, nine patients with chronic lymphocytic leukemia, and five patients with acute lymphocytic leukemia was estimated microspectrophotometrically with the Feulgen dye. Normal circulating lymphocytes were found to contain twice the average DNA content of normal human spermatids, corroborating their diploid chromosome number. Lymphocytes from normal and leukemic lymph node and bone marrow were frequently found possessing four times the average spermatid DNA value. Three out of nine patients with chronic lymphocytic leukemia, and four of five patients with acute lymphocytic leukemia had significantly increased numbers of circulating lymphocytes containing DNA values which were elevated above the normal diploid value. The patients demonstrating cells with elevated DNA values were in a clinically exacerbated phase of their disease. The significance of these findings is discussed.

REFERENCES

N. L. PETRAKIS

30 BIERMAN, H. R.: Personal communication.
Microspectrophotometric Estimation of the Desoxyribonucleic Acid (DNA) Content of Individual Normal and Leukemic Human Lymphocytes

NICHOLAS L. PETRAKIS