DNA Cytophotometry of Chromosomes in a Case of Chronic Myelogenous Leukemia

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The DNA-stain content of human metaphase chromosomes is measured by use of quantitative cytochemistry, scanning cytophotometry, and image-processing techniques. This approach is applied to chromosomes from a female with chronic myelogenous leukemia. Measurements are normalized for each cell and expressed on a scale by which the sum of the measurements for the 44 autosomal chromosomes is 100 units; the normalized measurements are compared with standard values from eight normal persons. Banding analysis of the leukemic cells shows a 9q+/22q− translocation. This is confirmed by the DNA measurements; 0.42 units are involved. In addition, measurements of both leukemic and nonleukemic cells reveal a 9+/20− translocation that cannot be detected visually; 0.12 units are involved. Neither translocation shows a net loss of DNA; any loss exceeding 0.05 units (6 × 10^6 base pairs of DNA) would be significant (P < 0.05). These results illustrate the power of this approach and bear on the etiology of chronic myelogenous leukemia.

Additional Keyphrases: chromosomal DNA content, quantitative cytochemistry, scanning cytophotometry, chromosomal translocation, Philadelphia chromosome

This presentation is concerned with measuring the relative amount of DNA in the individual chromosomes of human cells in metaphase. Such measurements reveal how the total cellular DNA (12 pg) is partitioned among the 46 chromosomes; because the measurements quantitate DNA, the fundamental component of chromosomes, they constitute a new and powerful tool that complements conventional cytogenetic analysis. We are using this approach to study variations within and among phenotypically normal individuals.

The power of this approach is illustrated by its first application to a clinical condition—a case of chronic myelogenous leukemia (CML)—in which measurements both support the findings of morphological analysis and reveal a new and previously unsuspected effect.

The methodology is complex—invoking quantitative cytochemistry, scanning cytophotometry, computer analysis of digital images, and statistical evaluation of results—but the underlying principle is the straightforward use of optical absorption to quantitate stained chromosomes on a microscope slide (1).

Quantitative cytochemistry involves staining chromosomes with a dye that absorbs in the visible part of the spectrum. Staining is done under conditions that are specific and stoichiometric for DNA, so that the amount of dye bound to the chromosome is directly proportional to the amount of DNA present. Scanning cytophotometry records the distribution of absorbing material within the microscope field. A spot, matching in size the diffraction-limited resolution of the microscope optics, scans the field and systematically divides it into a regularly spaced array of picture elements. Absorbance (optical density) is measured for each element of the field, the measurements are digitized, and the resulting digital image is processed by a general-purpose digital computer to extract the absorbance measurements associated with each chromosome. The sum of such measure-
ments is directly proportional to amount of absorbing material and hence to the amount of DNA present in the chromosome. The staining efficiency varies somewhat from cell to cell and from experiment to experiment; this effect is minimized by statistical techniques that normalize all the measurements on a cell-by-cell basis. The normalized measurements then are compared among individuals and among the cells of each individual.

The rationale and technical aspects of our approach already have been described in detail (2). The methodology is presented briefly in the following section.

Materials and Methods

Preparation Processing

Conventional cytogenetic preparations are processed as follows:

1. The preparations are stained with a fluorescent drug, quinacrine dihydrochloride. Suitable metaphase cells are selected and photographed. In each selected cell, all the chromosomes are mapped on the photographs and are identified by their fluorescent banding patterns (3).

2. The preparations are re-stained for DNA by first treating with neutral formalin and rinsing with distilled water to remove the quinacrine dihydrochloride, then digesting with ribonuclease to remove the RNA, and finally staining with gallicyanin–chrome alum at pH = 1.64. The preparations are mounted to match the refractive index of cells, which is 1.556.

3. Images of the selected metaphase cells are scanned and digitized by use of the CYDAC (Cytophotometric Data Converter) flying spot microscope system (4). A spot on the face of a cathode ray tube is moved in a continuous rectilinear raster. Light from the spot passes in the reverse sense through a conventional microscope. An 8× ocular and 100× planapochromatic objective (numerical aperture = 1.30) demagnify the spot 800-fold so that it approximates the size of the Airy diffraction-limited spot. A focus-assist device assures optimum focus of the spot in the specimen plane (5, 6). As the demagnified spot sweeps across the specimen, it is modulated by any absorbing material in its path. The modulated light in this data channel is collected by the microscope substage condenser, detected by a photomultiplier tube, and converted into an electronic signal. An optically and electronically equivalent reference channel continuously monitors the intensity of the spot on the face of the cathode ray tube. Analog circuits convert the data and reference signals to their logarithms, and determine the difference between the two logarithmic signals. The difference signal is sampled at 6 kHz and digitized in an eight-bit (255 levels) analog-to-digital converter. The digital values are passed to a small computer and stored on a magnetic tape for subsequent processing. For each scan, 38 400 values are recorded, representing a digital image of 192 scan lines, with 200 picture elements in each line.

The spacing of lines and of elements within lines is 0.25 µm in the specimen plane. Measurements are made with the full spectral bandwidth of the instrument (500 ± 50 nm), the spatial resolution is 1150 lines/mm at 50% modulation transfer, and the signal-to-noise ratio per picture element is about 100. Each digital value is proportional to absorbance and so relates directly to the mass of absorbing material under the measuring spot at the corresponding location in the specimen.

4. The digital images are processed to isolate each metaphase chromosome. Computer processing involves first the generation of a binary image; all picture elements above a threshold value are considered to be potential chromosomal "cores." Hand editing allows touching cores to be separated and fragmented ones to be joined. Each core is named according to the designation used to identify chromosomes in photographs of the quinacrine-fluorescent preparations.

5. The individual chromosomes then are processed to extract total absorbance and other variables. Total absorbance is the sum of the absorbance values of all the picture elements associated with a chromosome, and is directly related to the amount of absorbing material in the chromosome.

Processing is complicated because the chromosomes inherently have ill-defined borders. An iterative procedure is used to ensure that all absorbance values associated with a chromosome are included in the sum and that values are partitioned appropriately between adjacent chromosomes (7). The measurements for each chromosome are corrected for the local background around it (2).

6. Statistical analysis involves assembling the chromosomal measurements and comparing them within cells, within individuals, and across populations. The first stage involves normalizing the measurements to minimize effects of cell-to-cell variations in staining efficiency. This is done by scaling the measurements for each cell so that the sum of the measurements for autosomal chromosomes is 100 relative units. An iterative procedure allows scaling of incomplete as well as complete cells (8). For each individual, the normalized measurements first are tested for detectable differences between paternal and maternal homologs for each of the 22 pairs of autosomal chromosomes and for the pair of sex chromosomes in females (obviously, the X and Y sex chromosomes are different in males). There are serious difficulties associated with the analysis of paired data when there is no way of knowing a priori which member of the pair is being measured (9). This problem has been solved by a test that compares differences between paired measurements to the variance of their mean (10). Inevitably, this test on paired data has little power; differences between homologous chromosomes can be detected only when they are large or when many pairs are examined. When differences are detected, the affected pairs are analyzed separately; otherwise, all the measurements for each chromosome pair are pooled for
comparison with data from other individuals.

At present, an analysis takes several weeks because all computation, including editing of images and monitoring of results, must be done off-line, with use of a large-scale computer. However, we are in the final stages of implementing a real-time image-processing system (11) based on a 64,000-word, 16-bit computer with an 800 ns cycle time. The system will include an interactive display that will allow the operator to edit and to name chromosomes and to monitor the process in real time. When operational, this system should allow completion of an analysis in a matter of days.

Standard Values for the Human Karyotype

Our approach has been used to establish standard values for the distribution of DNA among the 24 chromosomes of the human karyotype. The standard values represent cumulative experience and now include pooled measurements from eight phenotypically normal white adults, four of each sex. Details of these standard measurements are being prepared for publication, but they differ little from preliminary results obtained for two of these eight persons (2, 12).

For each individual, the number of cells measured ranges from five to 13. The standard values are based on measurements from 70 cells containing 3197 chromosomes, of which 3136 were analyzed. The remaining 1.1% were excluded from the analysis either because they crossed one another or because they were overlaid with debris.

The standard deviation of the normalized measurements for each of the 24 types of chromosomes for any individual estimates the overall variation associated with the measurements and includes any differences between homologs and any cell-to-cell variation, as well as variation associated with the instrument. The standard deviation of the measurements varies with the size of the chromosome, and so a pooled estimate is given by the average of the coefficients of variation for all the types of chromosomes. The mean value of this average coefficient for all the individuals is 3.9%. This corresponds to a standard deviation for the measurements of 0.09 units for a chromosome of average size, and this figure can be used as an estimate of the overall precision of our measurements. Because a typical experiment involves measurements from 10 cells, the sensitivity of our approach is about a third of this, or 0.03 units. It should be noted that a diploid human metaphase cell contains about 12 pg of DNA. Thus one unit on our normalized scale is equivalent to 120 fg of DNA. One femtogram (10⁻¹⁵ g) of DNA corresponds to 6 × 10⁸ daltons, which is about 1 × 10⁶ base pairs of DNA.

We detect effects that involve as little as 0.06 units or 7.2 × 10⁵ base pairs of DNA, and this number should be halved when extrapolated to effects in postmitotic cells.

The means of the measurements for each of the auto-

tosomal pairs and for the sex chromosomes were compared among the eight individuals. Three of these eight persons had one or more chromosomes whose measurements differed significantly from the measurements for the other individuals at the 99% confidence level. At present, no biological significance can be assigned to these chromosomes that contain variant amounts of DNA stain. They can be considered as marker chromosomes, and they can be used for cytogenetic studies in the same way as any other marker chromosome.

Chromosomal Measurements in Chronic Myelogenous Leukemia

Measurements of relative DNA stain content were used to study the chromosomes of a patient with chronic myelogenous leukemia (CML). Mammalian cancer cells are characterized by chromosomal rearrangements and aneuploidy that generally appear to vary from tumor to tumor; CML is the human malignancy that shows the most consistent pattern of chromosomal abnormality. In this disease, the distal part of the long arm of a chromosome No. 22, the larger of the small acrocentric chromosomes, is deleted to form the so-called Philadelphia chromosome (13). Recently, Rowley used fluorescent banding patterns to analyze chromosomes from patients with CML and reported that the material deleted from chromosome No. 22 appeared to have been translocated to the distal part of the long arm of a chromosome No. 9 (14). This observation now has been confirmed in 72 of 73 patients with the Philadelphia chromosome, the exception being a possible translocation to a chromosome No. 2 (15). Measurements of chromosomal DNA for one of Rowley’s cases, a 24-year-old white woman, were used to examine the presumed 9q+/-22q− translocation of CML.

Metaphase spreads of leukemic cells were obtained from a bone-marrow preparation that had been previously analyzed by fluorescent banding and that contained a sufficient number of high-quality spreads for DNA staining and measurement. Leukemic cells were identified by the presence of the small Philadelphia chromosome. Nonleukemic cells were obtained from preparations made from a 72-h culture of peripheral blood from the same patient. Measurements were made of the chromosomes of 11 leukemic cells from the bone-marrow preparation and of 12 nonleukemic cells from the blood preparation. All measurements were compared with the standard measurements and were found to be within normal limits except for chromosomes No. 9, 20, and, in the leukemic cells, 22. Figure 1 illustrates the fluorescent banding patterns for chromosomes No. 9, 20, and 22 from three leukemic and three nonleukemic cells; Table 1 gives the means of the measurements for chromosomes No. 9, 20, and 22 for the 11 leukemic and 12 nonleukemic cells, together with the corresponding standard measurements.

In the leukemic cells, measurements of chromo-
Table 1. Chromosomal Measurements for a Case of Chronic Myelogenous Leukemia

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Standard values</th>
<th>Leukemic cells</th>
<th>Nonleukemic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 9</td>
<td>2.37</td>
<td>2.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.42</td>
</tr>
<tr>
<td>No. 9+</td>
<td>2.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.54&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>No. 9q+</td>
<td>1.16</td>
<td>1.19</td>
<td>1.12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>No. 20</td>
<td>0.90</td>
<td>0.87</td>
<td>0.88</td>
</tr>
<tr>
<td>No. 22</td>
<td>0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Comparison of relative DNA-stain content from leukemic and nonleukemic cells with standard values derived from the pooled means of eight healthy persons. Measurements are in relative units of DNA stain content and are normalized so that 100 units is the sum for the 44 autosomal chromosomes. Values are given for each homolog whenever they differ significantly from one another (P < 0.01).

We interpret these results as indicating that this patient has the expected 9q+/22q− translocation only in her leukemic cells. This is a balanced translocation and involves about 0.42 percent of her autosomes No. 22 show a highly significant (P < 0.01) homolog effect. The smaller chromosome always is the one that has been identified as the Philadelphia chromosome. It contains only 0.47 units of DNA, or 0.43 units less than the standard (0.90 units), and the difference is highly significant (P < 0.01). The unaffected homolog contains 0.87 units, which is not significantly different from the standard.

Measurements of chromosome No. 9 in the leukemic cells also show a highly significant (P < 0.01) homolog effect. In every cell, the larger of the pair is the chromosome that has been identified by banding analysis as the one involved in the presumed translocation with the Philadelphia chromosome. It contains 2.78 units of DNA; this is 0.41 units greater than the standard (2.37 units) and the difference is highly significant (P < 0.01). This increase matches the loss of 0.43 units from the Philadelphia chromosome, as differences of less than 0.05 units are not significant (P > 0.05). Thus, these measurements are compatible with a balanced translocation between chromosomes No. 22 and 9 in CML, with no net loss of DNA.

In addition to the 9q+/22q− translocation, the leukemic cells from this patient reveal a further and unexpected abnormality involving the other chromosome No. 9 and one chromosome No. 20. The smaller chromosome No. 9 contains 2.48 units of DNA; this is 0.11 units greater than the standard (2.37) and the difference is highly significant (P < 0.01). The homologs of chromosome No. 20 show a highly significant (P < 0.01) difference from one another. The larger homolog contains 1.19 units of DNA, not significantly different from the standard (1.16 units). However, the smaller homolog contains 1.04 units, 0.12 units less than the standard, and the difference is highly significant (P < 0.01). This loss matches the increase of the smaller chromosome No. 9. It suggests the presence of a second balanced translocation in these cells, again with no net loss of DNA.

Measurements on the nonleukemic blood cells of this patient show, as expected, no difference between the homologs of chromosome No. 22. The mean for these chromosomes is 0.88 units; this is not significantly different from the standard. However, the homologs of chromosome No. 9 show a highly significant (P < 0.01) difference. The smaller of the homologs contains 2.42 units of DNA; this is larger than the standard but is not significantly so. The larger of the homologs contains 2.52 units of DNA; this increase is 0.15 units greater than the standard and the difference is highly significant (P < 0.01). However, this increase is not significantly different from the increase found for the smaller homolog in the leukemic cells, and we feel that the larger homolog in the nonleukemic cells is the same chromosome as the smaller homolog in the leukemic cells. Measurements of chromosome No. 20 show no statistically significant (P > 0.05) homolog effect, but on inspection the measurements appear to segregate into classes of large and small homolog with little overlap. The mean measurement for these chromosomes is 1.12 units of DNA; this is highly significantly (P < 0.01) less than the standard but is the same as the mean of the homologs of chromosome No. 20 in the leukemic cells. This suggests that the 9+/20− translocation found in the leukemic cells also is present in these nonleukemic cells.

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mal DNA. In addition, both her leukemic and non-leukemic cells reveal a 9+/20− translocation involving the other chromosome No. 9. This also is a balanced translocation and involves about 0.12% of her autosomal DNA. This second translocation is not detectable morphologically, even when the observer is alerted to its presence. Each translocation is primarily a rearrangement with little or no loss of genetic material. With measurements from 12 cells, the net loss of DNA for either translocation would have to exceed 0.05 units to be significant (P > 0.05). (Note that 0.05 units corresponds to about 6 \times 10^{-15} \text{g} of DNA or 6 \times 10^{6} base pairs in these dividing cells.)

**Discussion**

Our findings in this case suggest more questions than they answer—questions that underscore the futility of drawing general conclusions from a single case, but questions that suggest avenues for further study:

1. How constant is the amount of DNA involved in the 9q+/22q− translocation of CML? Studies on further cases of CML will be necessary to answer this question. To our knowledge, the DNA content of the Philadelphia chromosome has been measured only once before, and it is relevant to compare our present findings with those of Rudkin et al. (16) in which a photographic technique was used to measure the ultraviolet absorption of chromosomes in 11 leukemic cells from a 68-year-old woman. They (16) could identify chromosomes only by groups, as their measurements were reported in 1964 before the advent of the banding methods. They found that the Philadelphia chromosome contained 61% of the mean DNA content of the other three G-group chromosomes. When our present measurements are expressed in the same way, we find that the Philadelphia chromosome contains 55% of the mean DNA content of the other three G-group chromosomes. Thus the two sets of measurements agree closely and suggest that the Philadelphia chromosome may contain the same amount of DNA in the two cases. If the translocation is found to involve a constant amount of DNA it raises important questions about the etiology and mechanism involved in producing such a specific and invariant chromosomal rearrangement.

2. Is the 9+/20− effect really a translocation? Chromosome No. 9 is one of the more variable human chromosomes, particularly with respect to the amount of centromeric heterochromatin as revealed by the C-band technique (H. J. Lubs, personal communication). Unfortunately, we have not had preparations available for C-band studies on this patient. We are studying the C-band patterns for our eight standard persons and have found several polymorphisms. However, as yet we have found no corresponding heterogeneity in DNA stain content. Thus it seems unlikely that the excess DNA for the chromosome No. 9 is due to a variant with a large amount of centromeric heterochromatin.

3. Is the 9+/20− translocation an inherited or an acquired effect? Studies on the patient's blood relatives might reveal the presence of one or both of these variant chromosomes in other members of her family, and so indicate an inherited effect. Alternatively, if cells from other tissues of this patient (e.g., fibroblast cultures) fail to show this effect, it means that this patient is a chromosomal mosaic and indicates that this translocation was acquired at some time after conception. Such a finding would have strong implications for the possible etiology of CML. It is noteworthy that deletion of part of chromosome No. 20 has been reported as occurring sporadically in bone marrow spreads from patients with polycythemia vera (17). This hematopoietic condition may be prodomal to CML, but there is no evidence that it preceded the appearance of CML in this particular patient.

4. Is the 9+/20− translocation a chance finding with no relationship to CML? This would be our conclusion if no other cases of CML show this 9+/20− translocation. However, if other cases are found with this translocation, or if it is found consistently associated with some other clinical conditions, then it would bear importantly on the etiology of CML, and may even lead to the identification and treatment of future victims before they develop overt symptoms of CML. Alternatively, examination of other patients with CML may reveal further subliminal translocations and other chromosomal effects.

Obviously, this one case and her family should be studied further and many other cases must be examined before we will be in a position to answer such questions. Although complex, our approach is the only cytogenetic method presently available for such studies. It quantitates DNA, the most fundamental chromosomal attribute, and it complements banding analysis and the other approaches of conventional cytogenetics. We see it as a powerful new tool of unparalleled precision for basic research into human chromosomes and into chromosome-related disease; and we envision its future use in selected clinical conditions where its unique attributes will make it the investigative procedure of choice.

*Note added in proof:* The patient died before further studies could be done.

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**References**


