GUEST EDITORIAL

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Banding in Leukemia: Techniques and Implications

The establishment of the normal human karyotype and the universal acceptance of the Denver Nomenclature in 1960 (1) marked the beginning of modern human cytogenetics. Since that time, cytogenetic studies as an aid to medical diagnosis have been utilized with great success in the detection of genetic defects. Recently, such studies have been applied to the investigation of presumably acquired diseases, especially neoplastic diseases such as the leukemias. Since increasing evidence exists that at least some malignant cells possess chromosomes with characteristic structural and/or numerical abnormalities, the precise identification of individual chromosomes has become particularly important for the study of human neoplasia.

As of 1960, the 46 human chromosomes could be grouped into eight broad categories (groups A to G and the sex chromosomes) on the basis of their morphology (e.g., size and position of the centromere), which enabled the cytogeneticist to identify with certainty chromosomes #1, 2, 3, and 16. With the introduction of autoradiographic analysis of human chromosomes in the mid-1960's (2), it became possible to identify chromosomes #4, 5, 13, 14, 15, and X's in excess of one. This method is based on the labeling of newly replicated DNA with [³H]thymidine. DNA replication does not take place simultaneously along an entire chromosome; instead, different segments of an individual chromosome undergo DNA synthesis at different times during the S-phase of the cell cycle. Therefore, when dividing cells are incubated with [³H]thymidine and then harvested and their chromosomes are examined by autoradiography, each chromosome exhibits a characteristic labeling pattern. This technique makes it possible to distinguish between early- and late-replicating chromosomes and has been used to distinguish one late-labeled (hot) X from other chromosomes of the C group in cells with two or more X chromosomes. Although this technique could also be used to distinguish between pairs of B-, D-, and E-group chromosomes, identification of all the chromosomes in the human karyotype was still not possible.

In 1968, Caspersson et al. (3) introduced quinacrine fluorescence staining for the chemical differentiation of metaphase chromosomes. Using this method, one could identify for the first time each pair of chromosomes precisely and characterize details of their structure. This method had the disadvantage, however, of requiring a fluorescent light attachment for the laboratory microscope, which was too expensive for many laboratories. Fortunately, the Giemsa staining technique (4) was soon developed and found to be applicable for most laboratory purposes.

MOST FREQUENTLY USED TECHNIQUES FOR IDENTIFICATION OF CHROMOSOMES

The banding method can be applied to morphologic studies not only of fixed chromosomes but also of those in living cells. The two most widely employed techniques for the study of fixed chromosomes are fluorescence and Giemsa staining.

Fluorescence Staining

This technique exploits the differential affinity of various segments along the longitudinal axis of a chromosome for a fluorescent stain; when exposed to UV light, each chromosome shows a characteristic pattern of fluorescent (Q-) bands. The most frequently used stains are quinacrine dihydrochloride (Atebrine), Arancil (Acranil) (5), quinacrine mustard (6), benzimidazole derivatives (33258 Hoechst) (6), and acridine orange (7). These stains bind by intercalation between the bases in DNA, and those regions of DNA particularly rich in dA-dT residues are enhanced, thus exhibiting greater fluorescence upon exposure to UV light (8).

Giemsa Staining

Chromosomes are stained with Giemsa during or after various chemical and/or physical treatments designed to disrupt nucleoprotein associations. The type of treatment to which the chromosomes are subjected determines the banding pattern produced by Giemsa staining. Presently, four different procedures are in use; they produce characteristic patterns referred to as G-, R-, C-, and T-bands.

ABBREVIATIONS USED: [³H]dATP = deoxyadenylate-deoxycytidylylate; ASC = acetate-saline-Giemsa; CML = chronic myelogenous leukemia; AMML = acute myelomonocytic leukemia; AML = acute myeloblastic leukemia.

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Editor's note: Periodically, the Journal publishes solicited guest editorials as a means of transmitting to investigators in cancer research the essence of current work in a special field of study. The Board of Editors welcomes suggestions for future editorials that succinctly summarize current work toward a clearly defined hypothesis regarding the causes or cure of cancer.
G-banding.—This procedure is based on the preferential binding of stain to regions of the chromosome containing DNA rich in dA–dT residues. In the most commonly used methods of producing G-bands, ASG (9) or the proteolytic enzymes trypsin, pronase, and L-chymotrypsin (10) are used, although denaturation with urea (11), detergents (12, 13), and organic and inorganic salt solutions (14–16) also gives satisfactory results. The ASG method, first described by Sumner et al. (9), involves fixation of cells in methanol, denaturation of the DNA with acetic acid, and preparation of chromosome spreads by air-drying. The mechanism of this method may be as follows: Nonrepetitive DNA is readily denatured by the acid treatment and has little affinity for Giemsa. A 1-hour incubation in 2×SSC (0.3 M sodium chloride and 0.03 M trisodium citrate) at 60°C allows the annealing of repetitive DNA, which then binds the Giemsa stain to produce characteristic dark bands. In contrast, nonrepetitive DNA anneals imperfectly, if at all, during the incubation, and its location on chromosomes is indicated by the light-staining regions. The technique of proteolytic enzyme (e.g., trypsin) digestion was successfully used by Seabright (17) and Wang and Fedoroff (18) in 1971. Wang and Fedoroff suggested that the trypsin hydrolyzed the protein component of nucleoproteins after the DNA was denatured by the fixation procedures used. The exposed DNA was then free to react with the Giemsa stain, thereby producing the banding pattern observed.

R-banding.—This technique, described by Dutrillaux and Lejeune (4), is based on thermal denaturation of DNA. Air-dried slides are immersed in a phosphate buffer (20 mM, pH 6.5) bath for 10–12 minutes at 87°C and are then stained with diluted Giemsa at room temperature for 10 minutes. The banding pattern of chromosomes is observed by phase contrast microscopy. With the exception of the secondary constriction of chromosome 9, the colored bands correspond exactly to the nonfluorescent regions seen after Q-banding and are opposite in intensity to G-bands.

C (constitutive heterochromatin)-banding.—The procedure involves denaturing of DNA by treatment with HCl, RNase, and NaOH, and then renaturation of DNA in a saline citrate solution at 65°C. The centromeric areas (heterochromatic regions), believed to be composed of the highly repetitive DNA known as constitutive heterochromatin, produce a dark band after Giemsa staining. The facultative heterochromatin regions, such as the repressed X in female cells, do not respond to this stain (19).

T (terminal)-banding.—Dutrillaux (20) described two methods of T-banding, both of which are similar to the R-banding technique. The first method differs from R-banding by requiring a longer incubation time (up to 30 min). In the second method, the pH of the buffer is reduced from 6.5 to 5.1. After either Giemsa or acridine orange staining, terminal bands can be observed on the chromosomes. The origin of these bands and their exact chemical nature is not known, although it is possible that they are the parts of the R-bands most resistant to the denaturation conditions used. T-banding is especially helpful to precisely localize juxta-telomeric break points.

The relationship between patterns of DNA replication, Q-bands, and G-bands has been examined by Gann and Evans (21). In general, patterns of late replications reflected the patterns of fluorescence and G-bands. The only exception was the heavily labeled (hot) X chromosome in cells from females; this newly synthesized X chromosome could best be identified by autoradiography. Moreover, the patterns obtained after G-banding are almost identical to those produced by Q-banding. The major exception is the Y chromosome; Q-staining reveals brilliant fluorescence on the distal part of the long arm, whereas two distinct bands in this region are produced by the ASG method. Other areas, such as near the centromere of chromosomes #1, 16, and 22, show a dull fluorescence, whereas in G-bands all except #22 show a strong Giemsa-positive region.

NEWER TECHNIQUES OF BANDING IN LIVING CELLS

The banding methods described above all involve the staining of chromosomes after fixation. New banding methods have been developed, however, which allow for the treatment of chromosomes in living cells before harvest, fixation, and Giemsa staining. The substances used, which include actinomycin D (22, 23), azure B (24), and tetracycline (23), all band in the G1-phase of the cell cycle. The mechanism by which these compounds produce banding patterns on chromosomes is not entirely clear. Actinomycin D is thought to bind specifically to guanine bases in DNA and to compete for a protein-attachment site. Therefore, the light-staining bands observed in chromosomes from actinomycin D-treated cells may result from the displacement of protein from nucleoprotein complexes. The relative content or distribution of adenine–thymine versus guanine–cytosine base pairs along the chromosome may also play a role in the banding patterns produced by actinomycin D. However, actinomycin D, like azure B and tetracycline, possesses planar molecular structures and is able to intercalate between the base pairs of DNA. Such intercalations could disrupt or prevent associations between DNA and protein, thereby allowing the Giemsa or other stain to interact with DNA. The bands produced on chromosomes treated in living cells by actinomycin D, azure B, or tetracycline are identical to G-bands.

At present, most laboratories favor the G-banding technique because it is easy, reproducible, and does not require special equipment. Many laboratories have made minor modifications of the basic technique. In our laboratory, we favor the Giemsa–trypsin banding method, although we use the fluorescence method for some preparations. The methods for Giemsa–trypsin staining and for fluorescence banding used in our laboratory are described below.

Giemsa–Trypsin Staining Method

This method is a modification of the techniques of Seabright (17). Store air-dried slides that are 1 week old in a 37°C incubator for 4–5 hours. Immers the slides
While still warm in 0.025% trypsin (Gibco) for a specified time (bone marrow requires from 15 to 30 seconds, peripheral blood from 10 to 20 seconds). Then immerse the slides immediately in cold saline and rinse them well. Stain in 2% Giemsa R66 (G. T. Gurr, #482) in citrate buffer (pH 6.8; G. T. Gurr, #654) for 30-45 minutes. Rinse in tap water and dry.

**Q-Staining**

This method was described by Caspersson et al. (26), and has been modified slightly in our laboratory. To prepare the quinacrine staining solution, dissolve 0.5 mg quinacrine mustard in 10 ml McIlvaine disodium phosphate–citric acid buffer, pH 7.0. Place a few drops of the staining solution on the material to be stained, put a cover slip over it, and stain for 20–30 minutes. Place slides in a Coplin jar, and fill and empty the jar two or three times under running distilled water. Allow the slides to remain in distilled water for 3 minutes, then air-dry. For use, put a few drops of buffer solution or distilled water on the slide, place a clean cover slip over it, and blot the slide with filter paper to remove excess water. Slides are then ready to examine under fluorescent optics.

**CLINICAL IMPLICATIONS OF CHROMOSOME BANDING**

Much has been learned about chromosome structure and configuration with the use of different banding methods. For example, the Ph1 chromosome was first described by Nowell and Hungerford (27) in patients with CML as a G-group chromosome with a partially deleted long arm. Early in 1973, Rowley (28) used fluorescence and Giemsa banding techniques to demonstrate that this marker chromosome actually represents a translocation of the distal portion of the long arm of chromosome #22 to the end of the long arm of chromosome #9. Other workers have described additional translocation sites, including chromosomes #2 (29), #6 (30), #7 (31), #13 (32), #17 (33), #19 (34), and #22 (35, 36). From these reports, one may conclude that the deleted portion of the Ph1 chromosome is usually but not always translocated to chromosome #9.

A new marker chromosome, characterized by a partial deletion of the long arm of chromosome #5 (5q–), was first described by Van den Berghe et al. (37) in the bone marrow cells of 3 patients with a distinct type of refractory anemia; a total of 6 such cases were studied. By using the Giemsa banding method, Verhees et al. (38) were able to determine precisely that the deleted portion was interstitial: del(5)(pter–q12::q31–qter).

We first described the 45 chromosome syndrome in 1964 (39) in three patients with refractory anemia, thrombocytopenia, peripheral leukocytosis, and normal-to-low alkaline phosphatase. All cells in their bone marrow contained 45 chromosomes, the missing chromosome belonging to group C. The bone marrow of the patients showed granulocytic hyperplasia with or without megaloblastoid erythropoiesis, and all of them terminated in AMML. A similar case was described by Macdougall et al. in 1974 (40); using the Giemsa banding technique, they identified the missing C-group chromosome as #7. Cells from a patient with erythroleukemia (41), one progressing from preleukemia to AML (42), and another patient with pancytopenia without leukemia (43) were also reported to be missing chromosome #7.

The presence of an extra C-group chromosome is one of the most frequently occurring aberrations in leukemia cells. With the new banding methods, the precise identification of this extra chromosome has been possible. Thus an extra #8 chromosome has been found in cases of AML, AMML, and erythroleukemia (44), as well as in patients with CML in blast crisis and in those with polycythemia vera (45, 46). In contrast, the extra C-group chromosome has been identified as chromosome #9 in cells from patients with myelosclerosis (47), AMML (48), and thrombocytosis (49); in 2 with polycythemia vera (50); and in 1 with massive splenomegaly and active marrow (disease not diagnosed) (51).

A long acrocentric chromosome marker was first described by Jacobs et al. in 1963 (52) in an African patient with Burkitt's lymphoma, and a similar case was described by Stewart et al. in 1965 (53). With the use of Giemsa banding, Manolov and Manolova (54) were able to identify this acrocentric marker in 10 of 12 African patients with Burkitt's lymphoma as chromosome #14 with an extra terminal band. However, no such marker has been noted in our studies of American patients with Burkitt's lymphoma. Wurster-Hill et al. (55) reported one case each of plasma cell leukemia and multiple myeloma in which two extra terminal bands were present on chromosomes #14. Philip (56) reported another case of multiple myeloma with one additional dark band on chromosome #14.

Another specific structural chromosome rearrangement, noted in a wide spectrum of myeloproliferative and lymphoproliferative disorders, is now known to be the isochromosome of the long arm of chromosome #17, designated as i(17q). In a recent review, Engel et al. (57) described this chromosome in cells from patients with AML, Hodgkin's disease, and CML as a metacentric chromosome of C-group size. Using ASG banding techniques, Lobb et al. (58) conclusively identified this marker as i(17q) in three of their Ph1-positive CML patients undergoing a blast crisis. The i(17q) marker has also been found in a case of eosinophilic leukemia (59).

Kay et al. (60) first reported finding a correlation between an abnormally small F-group chromosome and polycythemia vera. A small F-group chromosome has also been found in cells of patients with various other erythroid disorders, such as idiopathic sideroblastic anemia (61), Di Guglielmo syndrome (62), AML (63), and subacute myeloid leukemia (61). We have noted a small F-group chromosome in 5 of our patients with various myeloproliferative and lymphoproliferative diseases and some degree of erythroid dyspoiesis. In 1972, Reeves et al. (64) reported that the small F-chromosome in their patients with polycythemia vera was 20q12–-. However, of the 4 patients in our series studied by Giemsa banding, only one had a similar marker. This
patient had hyperplastic marrow and erythroid hyperplasia for a year prior to developing AML.

A missing Y chromosome, easily detected by the Q-band technique, has been found in leukemias and solid tumors (65). To date, at least 28 Ph1-positive CML patients missing the Y chromosome have been described (66), as have fewer cases of Ph1-negative CML (67).

CONCLUSION

The presence of the Ph1 chromosome in the hematopoietic cells of individuals with apparent hematologic disorders is virtually diagnostic of CML. In patients without any hematologic or clinical symptoms, the presence of the Ph1 chromosome can be an indicator of the preleukemic state. For example, a small percentage of Ph1-positive cells was found in the bone marrow of a preleukemic patient 5 years prior to any significant hematologic change and the development of blastic leukemia (68). Baccarani et al. (69) reported a similar case in which all the metaphases in the bone marrow had a Ph1 chromosome. Eventually, these cases of Ph1-positive preleukemia develop a typical leukemic phase.

Published data in cytogenetic studies of acute leukemia (70-77) have shown that approximately half the patients exhibit chromosomal abnormalities in their bone marrow. Rowley and Potter (44) reviewed the available banding data for acute nonlymphocytic leukemia and noted that the incidence of chromosomally abnormal patients was underestimated by at least 10-20%. They found various nonrandom chromosome changes including an additional #8 chromosome, the loss of chromosome #7, a gain or loss of #21, frequent structural rearrangements of #8 and 21, and the loss of a sex chromosome. Less banding data are available for acute lymphocytic leukemia. In a recent paper on this subject (78), I noted that the chromosome group most frequently involved in aneuploidy was the G-group, with the next most common being the B-group. The clinical significance of aneuploid cells in the bone marrow of acute leukemia patients is still unknown. Some investigators feel that the presence or absence of aneuploidy has no apparent bearing on the clinical course or prognosis of disease (63, 70, 73, 77), whereas others believe that aneuploidy indicates a poor prognosis (76).

The presence of aneuploid cells in the bone marrow before and/or after treatment did not necessarily indicate a poor prognosis if adequate treatment was given to eradicate the aneuploid cells (78). In fact, aneuploid cells are useful indicators, the disappearance of which suggests successful treatment leading to long-term survival and possible cure. In contrast, the aggressive persistence of aneuploid cells in the bone marrow is a grave prognostic sign. Nowell (79) proposed that a positive marrow chromosome study in nonirradiated patients with preleukemic symptoms suggests that a leukemic phase is imminent, whereas a negative study may indicate a protracted nonleukemic course.

Although the new banding techniques appear to be highly useful in cytogenetic studies, relatively little banding data has been accumulated for such leukemia patients. This paucity of banding data is due, at least in part, to a number of technical difficulties encountered in cytogenetic studies of leukemia patients. For example, bone marrow samples from such patients frequently have an abnormally small number of cells undergoing mitosis. In other cases, marrow samples have a predominance of hyperdiploid or near tetraploid cells, yielding too many chromosomes for a good spread. Chromosomes from leukemia patients often have a peculiar stickiness, which also makes it difficult to obtain usable spreads. Some of these difficulties can be obviated with 1-day peripheral blood cultures. These provide a greater number of cells in mitosis, since proliferating leukemic cells are usually abundant in the peripheral blood; moreover, the cells are capable of dividing without mitogenic stimulation. Even when a sufficient number of metaphase spreads is obtained, however, the quality of the banding is frequently poor. This problem is usually due to aged slides or to inadequate banding treatment and can be overcome by application of the banding techniques to relatively fresh (1- to 4-week-old) slides. Preparation of a sufficient number of spreads and/or preleukemic bone marrow for a sample is also prudent, so that some steps in the banding procedure can be varied to arrive at an optimal treatment for each individual sample. Finally, improvements in the techniques used to obtain samples will undoubtedly help to resolve many of the problems encountered in cytogenetic studies of leukemia patients.

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