Human Bone Marrow Chromosomes in Megaloblastic Anemia

By Edward R. Powsner and Lawrence Berman

With the Technical Assistance of Alma Irene MacLeod

The profound alteration in human erythroblasts in folic acid or vitamin B12 deficiency is manifest to an important degree by the appearance of the interphase nuclear chromatin in the megaloblastic cells. This is reflected in the appearance of the individual chromosomes, and it is generally agreed that the chromosomes of megaloblasts differ in morphologic details from those of normoblasts. Rohr1 refers to the chromosomes in megaloblasts as more delicate and slender than those in normoblasts, and Fieschi2 and Kienle3 have made similar observations. Kiossoglou and Mitus mention "giant" chromosomes in their list of chromosomal abnormalities observed in pernicious anemia.4 Our own experience with Giemsa-, Wright- or Leishman-stained dry films confirms this impression. It seems probable that the alterations in the morphologic appearances of the individual chromosomes are related to structural changes, such as the degree of coiling. These phenomena may in turn be related to the presence of abnormal biochemical end-products or a deficiency of normal substances that are intimately associated with maintaining the physical structure of the chromosomal elements.

If these phenomena are to be studied, it is important that quantitative data based on more reliable evidence than hitherto available be collected on the morphology of individual chromosomes. Although in dry films stained by ordinary methods the chromosomes of megaloblasts appear more delicate and slender than those of normoblasts, it is not possible to use these preparations to measure the difference. This deficiency in stained, dry films has become even more apparent since the application of the hypotonic squash method to human bone marrow. The present report deals with a detailed study of size of the number one chromosome5 in megaloblastic erythropoiesis by methods we have reported previously for direct observation of cells from freshly aspirated human bone marrow.6

Materials and Methods

Bone marrow for these preparations was obtained in the course of diagnostic study of patients referred to the Hematology Services of the Dearborn Veterans Administration Hospital, Dearborn, Michigan.

From the Department of Pathology, Wayne State University, School of Medicine, Detroit, Michigan, and the Radioisotope and Pathology Services, Veterans Administration Hospital, Dearborn, Michigan.

This study has been supported in part by NIH Grant No. AM 09407 (formerly CA 02149) and American Cancer Society Institutional Research Grant No. ICRG 34.

First submitted January 18, 1965; accepted for publication March 13, 1965.

Edward R. Powsner, M.D.: Associate Professor of Pathology, Department of Pathology, Wayne State University, School of Medicine, Detroit, Mich., and Chief, Radioisotope Service, Veterans Administration Hospital, Dearborn, Mich. Lawrence Berman, M.D.: Professor of Hematopathology, Department of Pathology, Wayne State University, School of Medicine, Detroit, Mich.; and Hematopathologist, V. A. Hospital, Dearborn, Mich.
Hospital and the City of Detroit Receiving Hospital. The patients without megaloblastic erythropoiesis were unselected. On the other hand, several of the patients with megaloblastic anemia were initially selected on the basis of peripheral blood or marrow showing megaloblastic erythropoiesis. One patient with normoblastic erythropoiesis had received colchicine for the treatment of gout. In every case, complete blood and marrow study was performed by one or both of us. Diagnosis of megaloblastic erythropoiesis was based on classical appearance of marrow. For the direct squash, about 1 ml. of aspirated marrow was mixed with approximately 1 mg. ethylenediamine tetra-acetic acid, transferred as quickly as possible to a Wintrobe hematocrit tube, and centrifuged at 200 g for 5 minutes. The buffy coat was removed and placed in 1.12 per cent citrate for about 5 minutes at room temperature. No colchicine was used. The mixture was centrifuged at 50 g for 5 minutes, as much as possible of the supernate decanted and a few drops of acetic acid added. The acid concentration was 45 per cent by volume in distilled water. In some preparations the dilute acid contained 1 per cent orcein. During the addition of the acid the cells were gently but thoroughly agitated to prevent excess clumping. The cell suspension was placed on a clean slide, covered with a clean cover slip and squashed by thumb pressure. If necessary, more acetic acid was added to provide adequate dispersion of the cells as judged microscopically. All preparations were examined using phase microscopy and satisfactory ones sealed with wax. Dry films were prepared by Moorhead's method. All acceptable spreads were photographed and printed at a final magnification of 5000×. The criterion for acceptability was simply that the chromosomes of groups A and B (numbers 1 through 5) be separated sufficiently to permit measurement and identification. Chromosomes to be measured were traced and their length measured on the tracings. The average length of the two longest, presumably number one chromosomes, was tabulated and plotted. Mean length and standard deviation of mean were calculated in the usual manner. The reference standard for all measurements was a stage micrometer calibrated by National Physics Laboratory, Teddington, Middlesex.

RESULTS

Measurements for all acceptable cells are plotted in figure 1. The mean length in the 62 cells from marrow of patients with megaloblastic erythropoiesis is 8.0μ. The mean for 119 nonmegaloblast marrow cells is 6.2μ. The standard deviations of these means are 0.16 and 0.082, respectively. Note that the observed difference between the means is approximately 6 times the standard deviation of this difference.

In one patient examined twice, at the first examination, while erythropoiesis was megaloblastic, the mean length in 18 cells was 7.2μ. At the second examination, 16 days later when erythropoiesis had become normoblastic, the mean length in 11 cells was 5.8μ. The standard deviations of the means were 0.4 and 0.3, respectively.

No attempt was made to measure the width of the chromatids. The apparent width of a chromatid in this type of preparation is actually the diameter of the morphologic major helix. In most cases the turns of the helix are not resolved by the microscope. Because the apparent width is of the same order of magnitude as the wave length of green light, 550 mμ, it is impossible to provide more than a crude estimate of chromosome width. To the limited extent that this estimate is meaningful, no difference was noted between the two groups.

DISCUSSION

The difference between chromosome lengths in megaloblastic and normal marrow cells had long been observed in stained smears. The squash technic
Fig. 1.—Distribution of lengths of the number one chromosome for cells from normal and megaloblastic marrow. The means and standard deviations of the means are indicated.

provides a preparation in which the length of a single chromosome can be measured with considerable precision. Our inability to identify the cell type in these preparations is a minor disadvantage inasmuch as the metabolic abnormality of megaloblastic anemia is a general one affecting, at least, all the major cell types in the marrow and the mucosa of the digestive tract. Nevertheless, to interpret the significance of the apparent difference between the mean lengths of the two sets of chromosomes we must consider (1) whether
the difference is real and (2) if real, whether the difference represents one of chromosome mass or chromosome form.

The reason for the first question will be obvious to all workers in this field. There is a marked variation of chromosome length between preparations or even between cells in the same preparation. This is also shown here by the range of lengths within the two groups of cells. For nonmegaloblastic cells a similar range was reported by Sasaki. He attributed the variation to contraction induced by colchicine, and his data support the accepted observation that contraction is increased by longer exposure to colchicine. Colchicine is not the only or even the most important factor. Except for marrow obtained from one patient, our material was not exposed to colchicine. Chromosomes from the one patient treated with colchicine were, if anything, longer than those from the other nonmegaloblastic patients. No cells were exposed to colchicine in vitro. We assume that the observed variation, at least in part, results from the continuously changing shape of the chromosomes during mitosis coupled with our inability to identify the moment of maximum contraction. In an earlier report of a portion of this work we attempted to select only those cells in which the chromatids were widely separated and presumably maximally contracted in late metaphase. We abandoned this when we found that the criterion of wide separation was too subjective to be useful and when separate analysis of measurements of (1) chromosomes of this selected group of cells with widely separated chromatids and (2) the chromosomes of all cells in which measurement was possible, led to entirely similar differences between the chromosomes from megaloblastic and normal marrows. Whatever the reason, the analysis of measurements of chromosome length is greatly complicated by this variation.

A related problem is the use of a single chromosome as the index of overall chromosome length. We chose the number one chromosome primarily for simplicity but also for the technical advantage afforded by its greater length. In using this index it would have been helpful if the number one chromosome were a fixed fraction of total length of all the chromosomes of the cell. The existence of such a fixed relationship is assumed in the Denver classification. In fact, this relationship is only approximate, and the range over which the fraction varies may be considerable. Despite these problems we believe that the difference between chromosomes of cells from megaloblastic and nonmegaloblastic marrow is real. The validity of the comparison is increased by the careful attention which was given to maintenance of uniformity in preparation of chromosomes. The interference caused by variation between chromosomes within groups has been largely overcome by measuring many cells. In confirmation of this, note that our mean length for chromosomes from nonmegaloblastic marrows is very close to that of Sasaki (our calculation), 6.2μ and 5.9μ, respectively. Further note that in the subject studied twice, the mean lengths during megaloblastic erythropoiesis and later during normoblastic erythropoiesis, 7.2μ and 5.8μ, respectively, are similar to the corresponding values for the series as a whole. Finally and most important, these measurements are intended to be confirmatory of the well-established observation that there is morphologic difference between megaloblastic and normal cell
chromatin and chromosomes. The fact that the measurement of chromosome length supports these observations strongly suggests that the measurement is meaningful despite the difficulty imposed by experimental variation.

Granting the reality and reproducibility of the observed difference in length, we must now consider whether this difference is due to a difference in chromosome mass or in chromosome form. The older observation from stained smears that megaloblast chromosomes are thinner suggests that the difference is simply one of form, not mass. Unfortunately, we could not confirm this observation. The appearance of chromosomes in Romanowsky-stained material is quite different from that in squash preparations. The chromosomes of a Romanowsky-stained cell are comparatively thick clubs in which the separate chromatids are often not visible. It is our observation that Giemsa staining of dry chromosome spreads does not greatly alter the appearance of the chromosomes. This suggests that the major factors responsible for the club-like form in ordinary Romanowsky-stained smears is simply the omission of hypotonic treatment and of squashing. It is difficult to assess the reality of the comparative delicacy of megaloblast chromosomes in ordinary smears. No measurements have been reported and we have been unable to perform any, not only because of the poor quality of the chromosomes, but also because of the limitations of the light microscope. One possible explanation for the apparent difference between megaloblastic and normal is that the real increase in length gives the illusion of decrease in width. Independent evidence that alteration of form, not mass, is responsible are the microspectrophotometric measurements which indicated that interphase nuclei of megaloblasts contain the same amount of DNA as normoblasts. It may be presumed that this is also true for the corresponding chromosomes. If the alteration is one of form, an explanation may be that the chromosomes of megaloblastic marrow cells are less tightly coiled than normal. Astaldi and Rondanelli and their respective coworkers have shown that the duration of mitosis in these cells is reduced. A difference in mitotic duration may suggest a difference in chromosome coiling during mitosis, but beyond this suggestion we have no information. Another approach to this problem is to use the electron microscope to measure chromatid width or the diameter of the major helix. A pilot attempt has indicated that this may be possible (work performed in cooperation with Dr. M. Bernstein, Department of Anatomy, Wayne State University).

**Summary**

The long-recognized difference between chromosomes of megaloblastic and normal human marrow cells can be measured in the cells studied by us. The chromosomes from patients with megaloblastic erythropoiesis are approximately one-third longer than the corresponding normal chromosomes.

**Summario in Interlingua**

Le differentia, ab longo recognoscite, inter le chromosomas de megaloblastic e normal cellulas de medulla human pote esser mesurate in le cellulas studiate per nos. Le chromosomas ab patientes con erythropoiese megaloblastic es ap-
proximativamente un tertio plus longe que le correspondente chromosomas normal.

ACKNOWLEDGMENTS

Figure 1 and all photographic prints used for chromosome measurements were made by the Medical Illustration Service, Veterans Administration Hospital, Dearborn, Michigan.

REFERENCES

Human Bone Marrow Chromosomes in Megaloblastic Anemia

EDWARD R. POWSNER, LAWRENCE Berman and Alma Irene MacLeod