Methods in Human Cytogenetics

Edited by
H. G. Schwarzacher and U. Wolf

Coeditor of the English Version
E. Passarge

With Contributions by
W. Gey, W. Krone, S. Ohno, E. Passarge,
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Preface

This volume was originally intended to be an English translation of the book *Methoden in der medizinischen Cytogenetik*, published in 1970. Just about then, however, a number of new techniques were introduced in human cytogenetics and soon acquired the utmost importance, particularly in clinical diagnosis, so that the English edition had to be considerably enlarged. As a result, there are now twelve chapters instead of eight, and two additional authors have been called upon, Dr. KRONE and Dr. SCHNEDL. In addition to the up-to-date presentation of conventional methods of cell culture and techniques for the preparation and identification of human chromosomes, this text covers the various techniques of producing banding patterns and applying them in chromosome identification. Further, it deals with the culture of amniotic fluid cells and gives instructions for handling tissue-culture cells for biochemical analysis; it thus meets the ever-increasing requirements of a modern cell-culture laboratory.

To paraphrase the aims of this book, we quote part of the preface to the German edition: "It was intended to collect the various methods so as to make them accessible for laboratory use. Furthermore, it is hoped that the reader faced with current research problems will be stimulated to modify and supplement the techniques described, instead of merely applying them automatically. In a rapidly developing field, some methods are still preliminary, and no final presentation seems possible."

In preparing the English edition, the German edition was first translated by Mr. YAIER COHEN, whose work is gratefully acknowledged. However, it would have been difficult to edit the book without the help of EBERHARD PASSARGE, who revised all chapters except the last two (XI and XII) and offered many valuable comments. We are much indebted to him.

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H. G. SCHWARZACHER
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CHAPTER I

Cell Cultures from Blood and Bone Marrow

RUDOLF ARTHUR PFEIFFER

1. Introduction

The analysis of chromosomes from nucleated cells of the hemopoietic system, particularly lymphocytes, has become the main method in clinical cytogenetics, because of the ease with which material can be obtained by the physician.

The culture of lymphocytes from peripheral blood is inexpensive, simple to perform, reliable, and therefore suitable for routine work. Stimulated lymphocytes may enter metaphase within 48 hours in vitro thus permitting analysis of their first division.

The immune response of lymphocytes can be tested with unspecific mitogens. Mixed cultures of lymphocytes from different donors may be used as *in vitro* models of histocompatibility.

The direct effects of various agents (viruses, ionizing radiation, poisons, drugs) on chromosomes can thus be very easily assessed in vitro by using lymphocyte cultures. Since the mitotic rate in bone marrow is high, the cells in the metaphase can be prepared without culturing.

The effect of toxic substances on cells and chromosomes in vivo can relatively simply be tested on the bone marrow cell population (Schmid *et al.*, 1970; Arakaki *et al.*, 1970).

Leukemias result from neoplastic transformation of a particular cell type, and the karyotype can be determined during any stage of the disease.

2. Bone Marrow

No tissue or organ in the healthy adult organism contains as many dividing cells directly available for chromosome analysis as does bone

marrow. The majority of metaphases observed in smears of bone marrow from healthy donors is presumably derived from the erythropoietic series: proerythroblasts, erythroblasts, and normoblasts. Less frequently encountered are dividing myeloblasts, promyelocytes, or the various forms of myelocytes. Reticulocyte and plasmacyte, and occasional monocyte and lymphocyte divisions are seen. Polyploid metaphases with 4n, 8 n or even 16 n mainly represent megakaryocytes.

According to Rohr (1960), 10–42% of erythroblasts and 6–11% of myeloblasts are in metaphase at any one time, but these figures vary with different diseases, the highest values being found for erythroblasts in hemolytic anemias. Killmann *et al.* (1962) calculated an average mitotic frequency of 8.86 per 1000 nucleated cells. Of these, 6.15 (69.4%) belonged to the erythrocyte, and 2.5 (28.2%) to the leukocyte series. Rhythmic diurnal fluctuations in mitotic frequency were not observed.

Chromosome preparation destroys the characteristic cytoplasmic structures and it is impossible to identify the dividing cells. Hence, the preparation of at least one marrow smear stained in the usual fashion (e.g. according to Pappenheim) of the preincubated or the incubated bone marrow, or both, is recommended for comparison. The cytogeneticist should be familiar with the morphology of the components of bone marrow.

2.1. Collection of Material

Aspiration of bone marrow is done from the tibia, the iliac crest (in children), the sternum, or from a vertebral process, using the standard methods reviewed by Berman (1953) or Rohr (1960). The presence of fat cells and marrow particles will indicate that bone marrow and not peripheral blood has been obtained.

2.2. Direct Preparation of Chromosomes from Cells in Metaphase or Prometaphase

The bone marrow is suspended in an isotonic salt solution (NaCl-glucose, normal saline, or balanced salt solutions prepared according to Tyrode, Hanks, Gey, Earle, etc.) containing heparin in order to prevent hemagglutination and to break up the marrow fragments and separate the fat cells from the bone marrow. The preparation may then be incubated, usually in the presence of colchicine. Separation of the different types of cells is not necessary.

2.2.1. Direct Preparation of Cells Immediately Following Biopsy

The aspirate is suspended as described above, and chromosome preparations are obtained directly. Sandberg *et al.* (1960) recommended washing the bone marrow in a cold solution of 0.6% glucose and 0.7% NaCl, leaving the cells in suspension for 10–15 min before preparation of the chromosomes, or suspending the bone marrow in cold isotonic Earle solution.

The mitotic index can be increased by injecting a mitotic inhibitor such as colchicine or its semisynthetic derivative Colcemid (Schär *et al.*, 1954) prior to the biopsy. Several authors (Bottura and Ferrari, 1960; Stewart, 1960; Kinlough *et al.*, 1961; Meighan and Stich, 1961) have applied this procedure to patients. The administration of mitotic inhibitors to patients should be limited to selected cases. However, for the study of animal bone marrow it can be very useful. In mice the mitotic index increased within one and a half hours after injection of colchicine (0.05–0.1 mg/kg body weight), the maximum being around 4 hours (Cardinali *et al.*, 1961). Vinblastine sulphate (Velban; Lilly) has also been shown to be a useful mitotic inhibitor in mice when injected at a single dose of 0.1 mg/kg of body weight before biopsy. The mitotic rate in the bone marrow of the rat is reported to increase after intraperitoneal injection of human blood group 0 plasma 16 hours before aspiration (De Vries and van Went, 1964). The induced hemolysis is followed by a compensatory hyperactivity.

2.2.2. Cell Preparation Following Short Term Incubation with Colchicine

Short-term cultures of suspensions of bone marrow grown in the presence of colchicine were developed by Lajtha (1952) and applied by Ford *et al.* (1958).

Details of the procedure:

Method a (Ford). The bone marrow is suspended in a prewarmed isotonic solution containing heparin (1:20,000) and mixed carefully by drawing it in and out of a syringe with a long needle without forming foam.

Saunders flasks (so-called "Universal Containers"), with a capacity of about 25 ml and screw-top lids with rubber seals, are suitable for all bone marrow and blood cultures. The rubber seals should be coated with collodion: a slightly viscous collodion solution derived from a commercially available stock solution is applied with a fine brush and dried at 100° C (unless the seals are siliconized or made from silicone). The rubber

lid may be sterilised with 70% ethyl alcohol, and then perforated with a sharp needle.

The cells in the closed flask are carefully centrifuged at 400–800 rpm., the supernatant is discarded and the sediment resuspended in Gey's salt solution and mixed well in order to ensure a reliable cell count. The suspension is then incubated in a 4:1 mixture of Gey's salt solution and human serum, devided into several subcultures of 3 ml each; 2000–4000 cells per mm^3 are regarded as optimal. The time of incubation may vary from 5–15 hours, but 7 hours is usually optimal. Two hours before harvesting 0.1 ml of 0.04% Colcemid in NaCl-glucose per ml of culture is added.

The following methods dispense with longer incubation periods:

Method b (Tjio and Whang, 1962). The bone marrow is washed in 0.85% NaCl adjusted to pH 7.0 by 6.6×10^{-3} M phosphate, containing either colchicine or Colcemid at a concentration of 1 µg/ml, and allowed to stand for 1–2 hours at room temperature until processed.

Method c (Kiossoglou *et al.*, 1964; Forteza-Bover *et al.*, 1965). The aspirate is mixed with heparin and centrifuged at 300 rpm. for 5 min in a siliconised tube; the supernatant is discarded. The cells are resuspended in 3 ml of a buffered 0.85% NaCl solution, pH 7.0–7.2, containing colchicine (solution 1:1000), and incubated at 37° C or at room temperature for $1–1^1/_2$ hours.

Method d. This procedure could be especially useful for bone marrow obtained post mortem. The culture medium consists of 40% human serum, 30% medium 199 (Difco), and 30% Hanks' solution (Tolksdorf *et al.*, 1965). 5 ml of fetal calf extract is added to 100 ml of the mixture. Bone marrow cells are suspended in 3 ml of the medium with the addition of 0.5 ml phytohemagglutinin and 200 units of heparin, and kept in the refrigerator for 3 hours. After 24 hours the medium is pipetted off and replaced by fresh medium. The culture is harvested 48 hours after commencement.

The differences between these various procedures are minor and mostly concern the interval between initiation of suspension and preparation. With the exception of method d they all make use of colchicine. Sterile procedures are not necessary in any of them and leukocytes need not to be counted if no long-term culture is intended.

Investigations which compare these modifications systematically have not been published. The following factors appear to be important for obtaining an adequate rate of excellent metaphases: a) the pH and the composition of the medium used for washing, suspension, and incubation, b) the duration of incubation, c) the absolute number of dividing cells present in the specimen.

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