LEUKAEMIA CYTOGENETICS

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PREFACE

THE finding in 1958 of a chromosomal abnormality in cells from a patient with acute leukaemia stimulated further cytogenetic studies of the leukaemias and allied disorders. During the 1960s many research groups were active in this field and now after a decade of study the value of chromosome analyses in diagnosis and management, and their contribution to the pathogenesis of the disorders, can be reviewed. This monograph is based on eight years' experience in the cytogenetics laboratory of my department where such studies commenced late in 1962 and on reports and other literature to the end of 1969; I have summarised the present position and considered what further approaches might be profitable.

The abbreviations, symbols and conventions used in the text are those of the *British Journal of Haematology* (1970, **18**, 3-12) and the Chicago Conference (1966).

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H. J. WOODLIFF

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Chapter I

INTRODUCTION

THE term "cytogenetics" is derived from the two parent sciences of cytology, the study of cells, and genetics, the study of transmission of biological information from one generation of organisms or cells to the next. Cytogenetics is largely concerned with the appearance and behaviour of chromosomes, as they are the constituents of the cell nuclei which carry the genes or units of inheritance. Both plant and animal chromosomes have been studied for many years but technical difficulties have prevented extensive studies of human material until comparatively recently. The number of human chromosomes had generally been thought to be forty-eight until 1956 when Tjio and Levan (1956) reported the true number to be forty-six (Chu, 1960). This was rapidly confirmed (Ford and Hamerton, 1956*a* and *b*; Bender, 1957; Hsu *et al.*, 1957) and the modern era of human cytogenetics began. Considerable advances have since been made and there is now a large literature dealing with chromosome abnormalities in congenital and neoplastic disorders.

The diseases considered here are the myeloproliferative and lymphoproliferative disorders, neoplastic proliferations of the haemopoietic and lymphoid tissues which include the leukaemias, lymphomas and their allied disorders; the names and possible inter-relationships are given in Fig. 1.

The first findings of a chromosomal abnormality in human leukaemia by Ford *et al.* in 1958, stimulated many others to undertake research into the subject. Since then a vast amount of information has been published and will be reviewed here. First, however, a brief history of the techniques which have been employed will be given; the methods will then be discussed in more detail, the normal human chromosome complement described and the types of abnormality which might be found considered. Subsequent chapters will deal with the different disorders.

Chromosomes are only apparent in cells whose nuclei are dividing by mitosis or meiosis. Mitosis is the nuclear division stage in the life cycle of individual proliferating somatic cells. Between

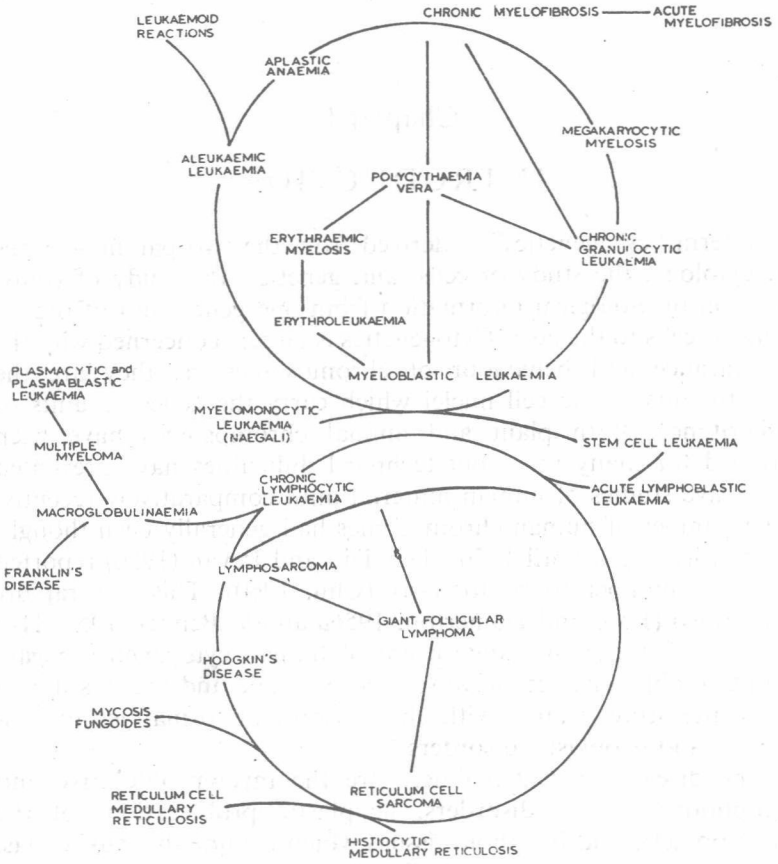

FIG. 1.—The myeloproliferative and lymphoproliferative disorders.

mitoses there are three stages of interphase, a pre-DNA synthesis (G 1) phase, a DNA synthesis (S) phase, and a post-DNA synthesis (G 2) phase. During the S phase, the DNA and nuclear protein of the chromosomes are duplicated.

During mitosis (Figs. 2 and 3), the chromosomes divide and usually each set forms a daughter nucleus; this process, called karyokinesis, is generally accompanied or followed by cytokinesis in which the cytoplasm divides, giving rise to two daughter cells. Mitosis is divided into several phases; during prophase, the chromosomes appear as separate identities, each consisting of two long threads (the chromatids) joined at a centromere. As

prophase progresses, the chromosomes become shorter and more compact and finally the nuclear membrane disrupts. A spindle then forms to which each centromere becomes attached and during metaphase the chromosomes line up on the equatorial plane of the cell. At anaphase the two sister chromatids of each chromosome separate completely to form daughter chromosomes which move

FIG. 2.—Mitotic figure and blastoid cells in a leucocyte culture.

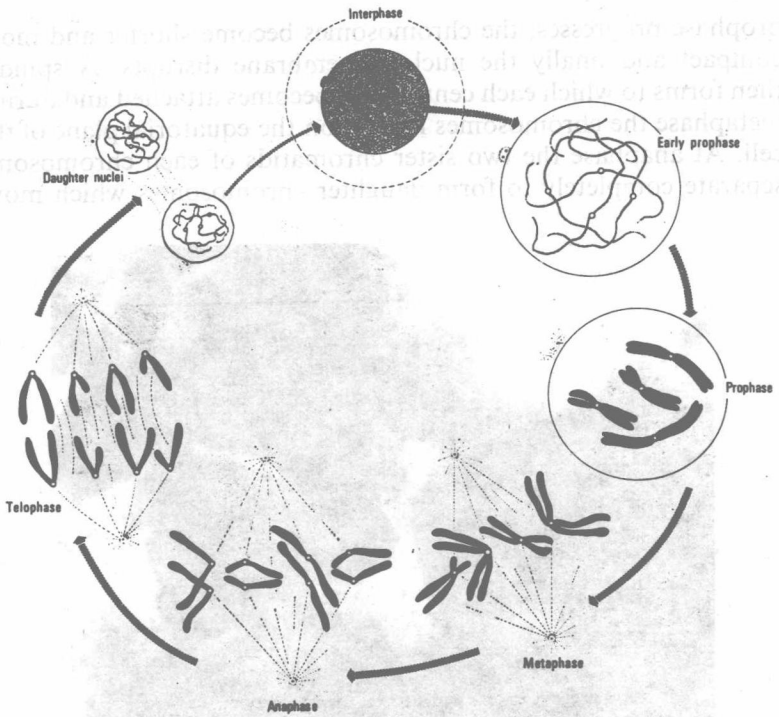

FIG. 3.—Mitotic cycle.

to opposite poles of the spindle. During telophase, each daughter set of chromosomes becomes reconstituted into a nucleus with a membrane.

Meiosis is a special form of nuclear division in which, in two successive phases, four gametes are formed from one somatic cell. A reduction in chromosome numbers occurs so that each gamete contains half the number (haploid) of chromosomes of the somatic cells (diploid). At fertilisation, the fusion of male and female gametes restores the chromosome numbers to diploid. Since this process is not relevant to studies of leukaemic cells, it is not considered further here; those interested will find a description of the process in a textbook of genetics (for example, Berry, 1965; Thompson and Thompson, 1966; Rieger *et al.*, 1968).

Chromosome studies are carried out on material containing cells in mitosis, because it is in metaphase that morphology is

most distinct; these may be obtained either directly from the body or indirectly by culturing cells *in vitro*. The study of direct material is preferable although it has the disadvantage that relatively few dividing cells may be obtained and the recent improvements in techniques were first obtained by culturing cells *in vitro*. Chromosome studies of cultured cells began in 1928 (Kemp) but significant advances did not take place until modern techniques were developed. These consisted of adding colchicine to "stop" mitosis in metaphase, the use of hypotonic solutions to cause the cells to spread and the squashing or air-drying of the metaphases on glass slides to cause spreading of the separated chromosomes (Hughes, 1952; Makino and Nishimura, 1952; Hsu and Pomerat, 1953; Ford and Hamerton, 1956*c*; Tjio and Levan, 1956). Tjio and Levan's important finding on chromosome numbers was made on cells from foetal tissue cultures and in 1958 bone marrow cultures were first used for such studies by Ford and his co-workers. Another commonly used tissue is skin (Harnden, 1960; Hirschhorn and Cooper, 1961), but this is of value in studies in haemopoietic cells only in the demonstration of differences between cells from the two different tissues.

A significant advance was the use of peripheral blood leucocyte cultures. Although the use of such cultures had been suggested in 1932 (Haldane quoted by Chrustchoff, 1935) and metaphases from such cells published in 1935 (Chrustchoff) lit le progress was made until 1960. This was because blood cultures usually contain few if any cells capable of undergoing mitosis (Chrustchoff, 1935; Bond *et al.*, 1959; Alexander and Spriggs, 1960; Woodliff, 1962). It was then discovered that phytohaemagglutinin induced mitosis in cultures of normal leucocytes. Whilst studying cultures of leukaemic cells, using the gradient technique of Osgood and Krippaehne (1955) in which leucocytes were separated from the blood after precipitation of the erythrocytes by PHA, Nowell noticed that mitoses were present in some of his control cultures of normal leucocytes. He investigated the circumstances favourable to mitosis and found that moderate variations of temperature, pH, oxygen tension, carbon dioxide tension, plasma and cell concentration, as well as the amount of agitation, had little effect. Mitoses were found in all cultures which contained PHA but in none when it was excluded. Nowell concluded that the PHA was an initiator of mitosis in cultures of normal human leucocytes. This finding was soon confirmed and has been used extensively

by cytogeneticists in the study of human chromosomes (Moorhead *et al.*, 1960).

Since artefacts might be produced by *in vitro* culture, direct studies of haemopoietic cells are preferred whenever possible. This is the method of choice for leukaemic bone marrow but cells from blood, lymph nodes and spleen may require to be cultured in order to provide sufficient metaphases for analysis.

Chapter II

METHODS

Introduction

Biological material containing cells which are dividing *in vivo* or which will divide in subsequent *in vitro* culture requires to be specially treated for chromosome studies. The steps may include the following procedures: the use of a spindle poison, such as colchicine, to arrest mitosis at the metaphase stage, hypotonic treatment to swell the cells and cause discrete separation of the chromosomes, fixation, spreading or squashing onto a glass slide, and staining. The chromosomes are then examined microscopically and often photomicrographed. These procedures are considered in this chapter together with a short account of some special methods which are used mainly in research.

Direct Preparations

Introduction

Direct preparations are preferred when there are sufficient dividing cells present in the tissue at the time of collection as this avoids any artefacts due to *in vitro* culture.

Peripheral blood usually contains very few or no dividing cells; however, in some cases of leukaemia or allied disorder, circulating malignant cells may be present and an occasional mitotic figure found; a technique similar to that used for bone marrow (see below) can then be tried.

Lymph nodes may yield a few dividing cells suitable for study but more can usually be found following culture. The nodes are dissected and a cell suspension made which is then treated as for bone marrow. *Splenic tissue* can be dealt with similarly (Yam *et al.*, 1968) but culture is usually required to produce sufficient mitoses for analysis.

Body fluids containing suspended cells are occasionally suitable for processing by a direct method but *skin* and other *solid tissues* are not.

Bone Marrow

Bone marrow may be prepared directly since it usually contains dividing cells. Some workers have pre-treated their patients with a spindle poison prior to aspiration in order to obtain a greater number of metaphases (Bottura and Ferrari, 1960, 1961; Meighan and Stich, 1961*a* and *b*; Traczyk, 1963). Usually, the "colchicine" derivative, desacetylmethyl-colchicine (demecolcine, Colcemid) is injected intravenously in a dose of 0·05 to 0·1 mg per kg of body weight and the bone marrow aspirated 1 to 24 hours later. The ethics of the procedure have been questioned (Stewart, 1960) and such pre-treatment should only be given to patients with established malignant disease. Since chromosomal abnormalities may be produced by such drugs (Hansteen, 1969), their routine use *in vivo* is not recommended. Most workers use a spindle poison *in vitro* but some prefer to omit this stage altogether (Sandberg *et al.*, 1960).

Each laboratory develops its own favoured method of dealing with bone marrow specimens, generally based on the techniques described by Sandberg *et al.* (1961) and by Tjio and Whang (1962, 1965). After hypotonic treatment, fixation and staining, some workers use a squash technique (Makino and Nishimura, 1952); although such preparations can be made permanent this is relatively difficult (Schultz *et al.*, 1949) and air-dried preparations are usually preferred (Rothfels and Siminovitch, 1958; Tjio and Whang, 1965). Spreading may be enhanced by "flaming" (Saksela and Moorhead, 1962; Moorhead, 1964).

Two satisfactory methods are given here:

(1) Marrow is aspirated by a conventional technique and a portion of the specimen (often all that can be spared since some of the aspirate is usually needed for diagnostic purposes) added to 2 ml of a magnesium and calcium free phosphate buffered saline. The specimen is usually processed immediately but if this is impractical it is stored overnight at 4°C. If many large fragments of marrow are present an aliquot is transferred to a further tube of collection fluid and the specimen broken up by pumping with a Pasteur pipette to form a suspension of separated cells. 0·1 ml (10 micrograms) of a demecolcine solution (1 mg in 10 ml of distilled water) is then added to the cells and incubated for one hour at 37°C. In preparations with a high mitotic index this stage may be omitted and still yield suitable metaphases. The specimen is then transferred to a graduated centrifuge tube and spun at low speed (800

rpm) for five minutes when the supernatant fluid is removed and replaced by Hank's solution. After further centrifugation, supernatant is removed and the button of cells re-suspended in 5 ml of 0·075 M potassium chloride solution and incubated at 37°C for five minutes. All but 2 ml of the supernatant is removed, freshly mixed fixative (methanol 3 volumes: glacial acetic acid 1 volume) is layered onto the fluid and the underlying solution gently removed with a Pasteur pipette so that the fixative bathes the button of cells. The deposit is then vigorously pipetted to obtain a uniform cell suspension and after centrifugation and two washings in fresh fixative, they are finally suspended in about 0·5 ml of fixative depending on their concentration. Two or three drops of the suspension are pipetted onto a cleaned wet slide, which is ignited by passing through a flame and, after burning, any excess fluid is wiped or flicked off the slide. The preparation should be checked microscopically and if necessary the cells concentrated by centrifugation and re-suspension in a lesser amount of fixative, or diluted by adding a greater volume. Suitable preparations are stained as described on p. 14.

(2) Marrow aspirate is added directly to 20 ml of cold Earle's solution and rocked to suspend the cells; 7 ml of the suspension is then added to 28 ml of 0·44 per cent sodium citrate solution and mixed by repeated inversion. This hypotonic treatment is allowed to continue at room temperature for 15 minutes, after which the preparation is centrifuged lightly and the supernatant removed; 5 ml of fixative (methanol acetic) is added and mixed with the cells and the suspension is then treated as described for Method 1.

Culture Methods

Peripheral Blood

Mention has already been made of the circumstances leading to the widespread use of peripheral blood cultures for chromosome studies (p. 5) and the many methods in use today are based on that described by Moorhead *et al.* in 1960 (Moorhead, 1964; Mellman, 1965). Mitotic figures are obtained from normal blood cells by culturing them with PHA or some other mitogenic agent which induces some of the lymphocytes to divide (leukaemic cells may mitose without stimulation). Whole blood can be used and micro-techniques based on adding a few drops to a culture medium have been described (Arakaki and Sparkes, 1963; Tips *et al.*,

1963); however, in most methods the leucocytes are first separated from the red cells and although this can be applied to small quantities of blood (Edwards, 1962; Froland, 1962) most techniques use from 2 to 10 ml. The blood is usually collected into heparin but citrate anticoagulant can also be used. Leucocyte-rich plasma can be obtained by allowing the red cells to settle by gravity or centrifugation alone or following the addition of fibrinogen, dextran or PHA (Skoog and Beck, 1956). Some workers like to remove granulocytes by magnetism following their ingestion of iron particles; other methods include filtration, differential absorption and density gradient techniques (Hastings *et al.*, 1961; Carstairs, 1962; Cooper and Hirschhorn, 1962; Speed and Lawler, 1964; Yam *et al.*, 1968; Pentycross, 1968; Boyle and Chow, 1969). Cultures are generally set up as soon as practicable but separated leucocytes in their own plasma may be stored at 5°C for up to four days (Mellman *et al.*, 1962) and mitoses may even be obtained from whole blood stored for up to 22 days at 4°C (Petrakis and Politis, 1962). Preservation of leucocytes in a special medium and storage at −180°C is also possible (Ducos and Colombies, 1968).

A variety of media have been used, most containing about 20 per cent of a protein solution such as autologous plasma, human AB serum or foetal calf serum and 80 per cent of an amino-acid and dextrose containing salt solution, such as Medium 199 or Eagle's medium (Woodliff, 1964).

Small screw-cap containers are usually used as culture vessels and leucocyte suspensions cultured at 37°C. Many media contain a phenol red pH indicator and a carbonate dependent buffer; the pH of these can be adjusted by gassing with 5 per cent CO_2 in air (to make it more acid) or by loosening the cap to allow CO_2 to "blow off" to make it more alkaline. Humidified incubators with an adjustable gas phase are useful especially when culturing cells in open petri dishes but are not necessary for routine work.

PHA is obtained commercially (Difco M) as a sterile powder which is reconstituted with distilled water. Each new preparation should be tested for potency by comparing it with a previous batch known to be mitogenic. Some workers prepare their own material but those commercially available are now so satisfactory that this is hardly worthwhile.

The following method has given consistent and satisfactory results (Woodliff, 1962).

Method.—Blood from patients with a high erythrocyte sedimen-