METHODS IN HEMATOLOGY

THE LEUKEMIC CELL

Edited by

D. CATOVSKY

CHURCHILL LIVINGSTONE

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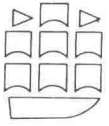

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The Leukemic Cell

METHODS IN HEMATOLOGY

Volume 2

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Foreword

Hematology, par excellence, is the discipline where laboratory and clinical medicine coalesce. Perhaps it is the accessibility of the blood as an organ that makes this possible. The 'Methods in Hematology' series is intended to bring together technical methods related to a particular blood disorder or group of disorders. The description of an investigation will include not only a full account of how it should be done, but also an account of its clinical and scientific basis, of the principles on which the method is based, the problems that may be encountered in its performance and their resolution, and a discussion of the significance of the result.

The publishers, Churchill Livingstone, are advised on the choice of topics and of editors, by a small board from both Europe and the USA. The editor is invariably a leading scientist in his discipline and the contributors each experts in their respective fields. The intention is generally to omit those methods dealt with adequately in standard accounts of methodology and to include those which may pose special problems, newer but potentially valuable technics and technics that may be useful at a research level while, at the time of writing, not yet used in day-to-day practice.

This second volume in the series is concerned with the characterization of the leukemic cell. Leukemia is a field in which new developments are rapidly translated into day-to-day practice. Even where the tests are not available, a frequent practice is to send blood or marrow to a laboratory where they can be performed. Dr Daniel Catovsky, as a member of the Medical Research Council Leukaemia Unit of the Royal Postgraduate Medical School, London, has been at the sharp end of many of these developments and he has brought together a group of eminent workers from the United States of America, United Kingdom, France, Germany and Australia to contribute to this volume. It is hoped that this book will encourage more hematologists to use these technics and that this in turn will lead to a better understanding of these diseases and provide a sounder basis for their clinical management.

London, 1981

I. Chanarin

Preface

The development of new objective criteria for the diagnosis and classification of leukemia has led to a better characterization of the various forms of the disease, and has facilitated the recognition of new disease entities. It has led, too, to a greater insight into the natural history of leukemia and to improvements in patient management. Indeed, therapy and research in leukemia has served as a model for understanding and treating other neoplastic processes.

Much of this progress has resulted from technological advances which, in the past few years, have permitted a more accurate description of the cellular events in hemopoietic malignancies. It has often been difficult for hematologists to keep abreast with the rapid methodological developments. The aim of this book is to make this task easier by setting out the technics currently available for the study of leukemic cells in sufficient detail to make them of practical value. With few exceptions, methods regarded as useful for diagnosis and cell characterization, and which are essential for further research in this field, have been included.

Hematologists, pathologists, clinicians and other scientists actively involved in leukemia research and practice will find an account of the methods used in leading laboratories in Europe and the USA, not easily found in this form elsewhere. This has been achieved by the high standard of work of all the contributors who have combined simplicity of presentation with depth of knowledge of the frequently complex modern technics.

I wish to acknowledge the invaluable secretarial assistance of Mrs Day Haysome and Miss Sue Masterson, and the cooperation of the staff of Churchill Livingstone. The printing of the illustrations was supported by donations from the Société Chimique Pointet Girard, Upjohn Ltd, Raymond A. Lamb and Mercia Brocades Ltd.

London, 1981 D.C.

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Overview

D. Catovsky

INTRODUCTION

For many years cell morphology on Romanowsky stained films was the mainstay in the diagnosis of the acute and chronic leukemias in their myeloid (AML, CML) and lymphoid (ALL, CLL) forms. We are now aware that, within these broad groups, there are numerous entities which differ in their response to therapy and prognosis. The combination of morphology, chromosome analysis and membrane and enzyme markers, for example, has helped the recognition of blast transformation of CML with typical features of AML or ALL[1–4] (Ch. 5 and 7). Another windfall from a multiparameter analysis of leukemic cells has been the recognition of a good correlation between specific chromosome abnormalities and some forms of leukemia (see below).

The new technics have been particularly helpful in the analysis of morphologically 'undifferentiated' blast cells, which includes a high proportion of cases

Table 1.1 Methods for the study of leukemic cells

Morphology	Light microscopy Ultrastructure (TEM, SEM)[a] Cell volume estimations
Cytochemistry	Light microscopy Ultrastructure (TEM)
Histology	Plastic embedding Immunoperoxidase
Membrane markers	RBC rosetting tests Immunofluorescence Antibodies to antigens and Ig[b]
Enzyme assays	Terminal transferase Adenosine deaminase Hexosaminidase; other enzymes
Culture systems	Clonal growth in agar Permanent cell lines
Chromosomes	Banding technics
Hormone receptors	Glucocorticoids
Cell kinetics	Autoradiography Flow microfluorimetry

[a] TEM: transmission electron microscopy; SEM: scanning electron microscopy.
[b] Ig: immunoglobulins (heavy and light chains).

of ALL, particularly the L2 type of the FAB classification.[5] The most important methods for this purpose, at the present time, are listed in Table 1.1.

What follows is a personal account as to how these research tools have facilitated the analysis of patients in the context of clinical hematology.

MORPHOLOGY

The basis of diagnosis is examination of blood and marrow films; most leukemias are diagnosed with this material. In a proportion of cases the need for extra information derives from the knowledge that some prognostic features may be disclosed by other technics. For example, in ALL, surface marker studies may reveal T-cell features which carry a worse prognosis and perhaps indicate a more aggressive therapeutic approach. In most cases of AML, by using the criteria of the FAB group,[5] the patterns of maturation into granulocytic (M2), monocytic (M4, M5), or erythropoietic (M6) cells can be determined on light microscopy only. In the less mature forms (M1 and M5), cytochemical technics (Ch. 2) are important for correct classification.

One of the forms of AML more readily recognized by morphology is hypergranular promyelocytic leukemia (M3), as described in the original FAB paper.[5] But a variant form of M3, characterized by a paucity of granulation also exists.[6] Typical M3 cells are seen, but in very small numbers, and the main distinguishing feature is the bilobed or reniform nucleus. These patients have the hemorrhagic diathesis regularly present in M3, but, in contrast to the typical M3 cases, the variant form has an extremely high WBC count.

The important contribution of ultrastructural analysis by means of transmission electron microscopy (TEM) is detailed in Chapter 4. In my experience TEM is extremely useful in the recognition of Sézary cells, small and large cleaved cells in follicular lymphoma presenting in leukemic phase, poorly differentiated plasmablasts, and, in some instances, in distinguishing prolymphocytic leukemia (PLL) from CLL.[7]

TEM is of particular value in the diagnosis of double leukemias. We have recently been able to study four patients in whom a lymphoproliferative disorder, CLL, follicular lymphoma or hairy-cell leukemia, was associated with AML in three and a chronic myelomonocytic leukemia in another.

Morphological studies on leukemic cells could be taken a step further by the analysis of cell volume histograms obtained by a Coulter model ZBI, linked to a Channelyzer. This system permits the definition of the modal volume of the leukemic population, the presence of heterogeneity and an objective comparison between cell sizes in different leukemias.[8]

CYTOCHEMISTRY

Cytochemical technics are of value in distinguishing between AML and ALL and the various AML subtypes[9,10] (Ch. 2) and hence in their classification,[5] especially when the maturation features are not obvious in Romanowsky stained films. The peroxidase reaction visualized by TEM (as described in Ch. 4) adds important information in cases of CML in blast crisis by demonstrating enzyme

activity in megakaryoblasts, and demonstrates myeloid blasts with small granules not visible by light microscopy as well as basophil promyelocytes.[11]

ACID PHOSPHATASE

By applying the acid phosphatase reaction at ultrastructural level we have been able to demonstrate a small lysosomal granule characteristic of monoblasts. These granules, of 0.05 to 0.2 μm in size, contain acid phosphatase (Fig. 1.1), but lack peroxidase and appear to be one of the earliest features of monocytic differentiation.[12] They are not observed in myeloblasts, where the earliest sign of differentiation is the presence of myeloperoxidase activity in the membranes and primary granules.[12]

Cytochemical reactions for acid phosphatase and alpha-naphthyl acetate esterase have also been helpful in the study of normal and leukemic lymphoid cells.[13] A localized strong paranuclear acid phosphatase reaction is characteristic of human fetal thymocytes[14] and in blast cells in the majority of ALL cases with positive T-lymphocyte markers[15] (Ch. 2). Two recent studies have shown the value of this reaction in diagnosing T-ALL with incomplete or immature

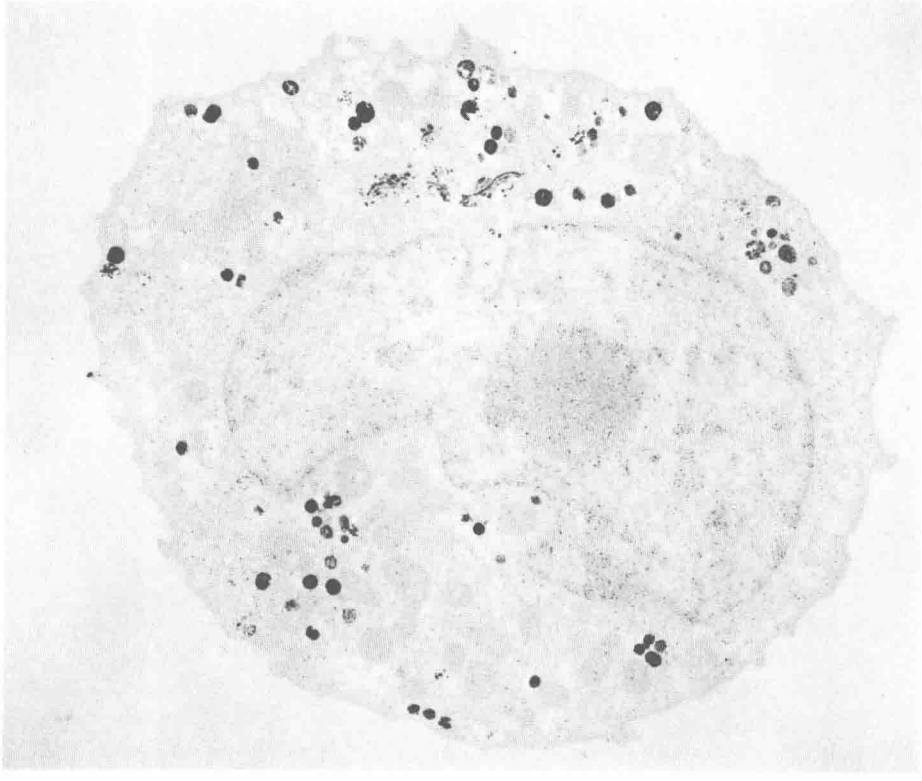

Fig. 1.1 Monoblast from a patient with acute monocytic (M5) leukemia showing acid phosphatase reaction in small cytoplasmic granules and the Golgi membranes (Gomori method,[12] unstained section, × 12 600). The myeloperoxidase reaction was negative in the same case.

membrane phenotype (pre-T) as shown by negative sheep RBC(E)-rosette formation.[16,17] Similar findings were reported in T-lymphoblastic lymphomas of childhood.[18] The acid phosphatase reaction in T-blasts in TEM is seen mainly in the membranes of the Golgi apparatus[13] and differs from that seen in monoblastic leukemia which is in very small granules in the cytoplasm.[12]

Although it is not clear whether there are significant differences in acid phosphatase content between mature B and T lymphocytes, this reaction is useful in characterizing some chronic lymphoid leukemias. CLL of B-cell type is usually negative, whilst most cases of T-CLL and Sézary syndrome show a strong reactivity, which in TEM is confined to lysosomal granules of varying size.[13]

ALPHA-NAPHTHYL ACETATE ESTERASE (ANAE)

There is good correlation between the proportion of peripheral blood lymphocytes with a localized (dot-like) ANAE reaction and the percentage of E-rosettes.[13] Unlike the reaction observed in monocytes, the reaction in lymphocytes is resistant to the inhibition by sodium fluoride. Studies by Grossi et al[19] have suggested that ANAE is positive in the subset of T-lymphocytes with Fc receptors for IgM ($T\mu$), whilst T-lymphocytes with Fc receptors for IgG ($T\gamma$) are negative. In several of our cases with T-CLL in which $T\gamma$ lymphocytes were the predominant population, the ANAE reaction was negative.[13] In contrast, the reaction was consistently positive in the cells of T-prolymphocytic leukemia.

HISTOLOGY

The role of histology in the diagnosis and management of leukemia has lagged behind other technics. A tissue diagnosis is essential in most other tumors including the malignant lymphomas. Not enough attention has been given in the past to the bone marrow structure considering that it is the hemopoietic organ primarily affected in leukemia. Fortunately, considerable advances are taking place at the present time which will hopefully change this situation. New technology such as plastic embedding and semi-thin sections of undecalcified bone marrow, as detailed in Chapter 3, have brought about improved morphological analysis with preservation of the tissue structure.

Immunological methods can be applied to paraffin-embedded or frozen bone marrow sections. By means of the immunoperoxidase reaction, particularly the 'unlabeled antibody peroxidase-antiperoxidase (PAP)' method,[20] antigens, immunoglobulin molecules or enzymes can be localized at tissue level with great sensitivity. One example of this is the demonstration of the ALL antigen[2] in neoplastic cell infiltrates in the testis and the cerebellum.[21] The use of the immunoperoxidase technics at ultrastructural level is described in Chapter 4. Other technological achievements such as the 'labeled antigen' procedure which gives a low background on paraffin-embedded tissue sections, and the immunoenzymatic labeling with alkaline phosphatase which allows the detection of two antigens in the same section by combining with immunoperoxidase, were the subject of a workshop report.[22]

Although less essential for routine diagnosis, the study of the bone marrow structure is critical when aspirates are unsatisfactory due to poor or excessive cellularity or a degree of myelofibrosis. The mode in which the bone marrow is infiltrated is often important in the diagnosis of non-Hodgkin lymphomas.[23] The presence of discrete foci of blast cells could be of value in the study of preleukemic states and may facilitate the diagnosis of blast transformation in some patients with CML.[24] A detailed description of the value of bone marrow histology in the diagnosis and classification of leukemia and related disorders is given in Chapter 3.

One of the most important uses of bone marrow biopsy is in the analysis of specimens taken after intensive cytotoxic therapy in AML, particularly during the early stages. It is often a question of deciding whether treatment should continue (if residual blasts are still prominent) or a momentary halt is indicated (if the picture is that of hypoplasia). In our experience these decisions are difficult with bone marrow aspirates only because in half the instances the material obtained is small in quantity and of low reliability.

The process of bone marrow regeneration following induction therapy in AML is preceded by a phase of 'structured fat' which appears to be required to sustain normal hemopoiesis.[25] In contrast, hemopoietic regeneration after ablative therapy of CML in blast crisis followed by autologous reconstitution with buffy-coat cells[26] takes place earlier than in AML and independently of the presence of fat cells.[25]

MEMBRANE MARKERS

Lymphoid cells may differ substantially in the type and quantity of specific binding sites and antigens present on their membrane (Ch. 5). Lymphocyte populations are extremely heterogeneous and numerous stages in the development of T (thymic) and B (bursal) cells are now recognized.[27,28] They can be defined by the presence or absence of certain markers and the chances of determining the surface phenotype of a given cell increases with the number and reliability of the technics employed.

This information is important for the characterization of leukemic cells derived from B and T lines. The concept that phenotypically immature cells correlate with blast cell morphology is roughly correct for the T-cell lineage.[29] It is also applicable to B-cells although less strictly so because the processes of lymphocyte transformation and modulation which take place in lymph nodes (e.g. in the germinal centers[30]) often result in blast-looking cells which may, however, be immunologically mature.[29] These differentiated B-cells are the targets for neoplastic change in most non-Hodgkin's lymphomas.[23,30]

Because the distinction between acute (blastic) and chronic (mature-looking) lymphoid leukemias is still clinically useful, any classification should take into account the morphology and the surface markers of the cells involved. The wider terms of 'lymphoproliferative' or 'immunoproliferative' are still often used, as they embrace related disorders, bridging the gap between leukemia (blood and bone marrow in the strict sense) and lymphoma (lymph nodes or other tissues primarily involved).

CHRONIC LYMPHOID LEUKEMIAS

These are proliferations of differentiated B- or T-lymphocytes, often reflecting normal counterparts present in the peripheral blood. There is a suggestion that, with more sensitive technics for the detection of membrane immunoglobulins (SmIg), such as the mixed antiglobulin and the direct antiglobulin rosetting tests, most 'null' (non-B, non-T) or third population cells in the peripheral blood could be shown to belong to the B-cell lineage.[28]

There are several subsets within the major B and T populations which are represented by very small percentages in the peripheral blood. Cell typing of the chronic leukemias (see below) has been useful in identifying these relatively rare lymphocyte types; this, in turn, may be of value for the purpose of raising monoclonal antibodies which will facilitate studies of their normal function and distribution (Ch. 5).

Chronic T-cell leukemias

These are characterized by lymphocytes which form E-rosettes, are negative for the enzyme terminal transferase and are always SmIg negative. The existence of rare normal T-cell subsets bearing Ia-like antigenic determinants or complement (C3) receptors[31] can be supported by the demonstration of C3[32,33] or Ia[34,35] in E+ leukemic cells.

In an ongoing study aimed at defining the cell populations in these disorders (in collaboration with Drs M. F. Greaves, G. Janossy and M. Pepys and Professor A. V. Hoffbrand) we have identified two T-CLL cases with the phenotype: E +, Fcγ +, Ia +, C3 − [34] which support the findings in normal blood of a subset of Fcγ lymphocytes bearing the Ia-antigen.[36,37] A similar phenotype, but negative for Ia, was demonstrated in two other T-CLL's, presumably corresponding to a proliferation of the Ia−, Tγ subset.[37] In two other patients we have observed membrane phenotypes which have not yet been identified in normal blood lymphocytes. One of them, with an unusual T-CLL 'lymphoma', was E +, Fcγ −, Fcμ −, Ia +, C3 + and the other, a T-PLL, was similar except that the Ia-antigen was absent.

Further advances in the characterization of T-cells resulted from the studies by Moretta et al[38] showing that two T-lymphocyte subsets which have either helper or suppressor activity can be identified respectively by the presence of Fcμ and Fcγ receptors (Tμ and Tγ lymphocytes possessing distinct morphological and cytochemical features).[20] Studies with these markers have shown that these immunoregulatory T-lymphocytes not only proliferate in some T-cell leukemias,[34,37] but also that they may be present in abnormal proportions in some B-cell leukemias (see below). In addition to the rosetting technics for the identification of Tγ and Tμ cells (see Ch. 5), these lymphocytes may have a different expression of the TH_2 antigen(s).[40] Thus, leukemias of the TH_2 negative subset (presumably Tμ) have now been reported[41] and one of our T-CLL patients with the Fcγ +, Ia − subset was already found to be TH_2 positive (Dr G. Janossy, personal communication).

Chronic lymphocytic leukemia

B-CLL, the common form of the disease, results from the monoclonal proliferation of B-lymphocytes with weak expression of SmIg, usually IgM ± IgD, binding of mouse RBC,[29,34] Ia antigen and receptor sites for C3.[27] In a proportion of cases the detection of SmIg is below the threshold of routine immunofluorescence testing, although it might be demonstrated by more sensitive technics. This may be important because B-cells without detectable SmIg, but with receptors for C3 (CR_1 only) have been demonstrated in 2.4 per cent of normal peripheral blood cells[31] and in B-CLL the complement detected is CR_2, without CR_1.[27] As CR_2 is not present in the SmIg-negative B-cells of normal blood, the B-lymphocyte of CLL may thus originate from a separate subset.

Studies with mouse RBC rosetting, the test most consistently positive in this disease,[34] suggest that the B-CLL lymphocyte represents a cell which has stopped at an early stage in the process of B-cell maturation.[27] We have shown a marked difference in the expression of this receptor between peripheral blood (high) and other tissues such as lymph nodes and bone marrow (low), suggesting that it might reflect the recirculation property of some lymphocytes.[42]

The various membrane properties of the B-CLL lymphocyte, which include abnormal cap formation when stained for SmIg and incubated at 37°C,[43] help in the differential diagnosis with other B-lymphoproliferative disorders, namely B-PLL and the leukemic phase of non-Hodgkin's lymphomas.[34,43,44] The difference in SmIg density between B-CLL (low) and the other disorders (high) can now be quantitated accurately by various technics, including rapid flow microfluorimetry[45,46] (see Ch. 5 for uses of the fluorescence activated cell sorter).

Although the percentage of 'normal' T-lymphocytes in B-CLL is low as a rule, relating largely to the height of the WBC, when calculated in absolute numbers they often are increased.[47] This finding, and the possibility of assessing the T-cell subsets with relatively simple rosetting technics[30] has stimulated interest in the role played by the T-cell subsets in this disease.

Kay et al[48] and ourselves[49] have observed a significant and persistent increase in the proportion of $T\gamma$ (suppressor) lymphocytes, and, conversely, a decrease in $T\mu$ (helper) cells in B-CLL compared with normal controls. Similarly, a helper defect in purified T-cells from CLL was suggested by functional studies.[50] Our initial observation in 13 patients[49] has now been extended to 40 patients and, in addition, we have evidence suggesting that the higher values of $T\gamma$-lymphocytes are seen in the most advanced stages of the disease, implying that it may be a secondary phenomenon. Nevertheless, the imbalance in the T-cell subsets may be significant in relation to some of the complications commonly seen in B-CLL such as hypogammaglobulinemia and autoimmunity, as it has been documented in other diseases.[51]

Hairy-cell leukemia (HCL)

This condition, despite its relative rarity, has attracted considerable interest, firstly because of the initial conflicting reports on the origin of the hairy cell, and, secondly, because of the lack of information regarding a normal counterpart for the hairy cell within the known leukocyte populations.