Authors: Sun, Tsieh
Title: *Flow Cytometry and Immunohistochemistry for Hematologic Neoplasms, 1st Edition*

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Dedication

This book is dedicated to my wife, Sue, for her constant support, patience, and understanding.

PREFACE

In recent years, hematopathology has become increasingly complicated. The complexity of hematopathology is due mainly to two reasons. First, the new classifications of lymphomas and leukemias (namely, the Revised European-American Classification of Lymphoid Neoplasms and the World Health Organization Classification) furnish a great variety of disease-specific entities not only on the basis of morphology, on which the older classifications were based, but also in consideration of the immunophenotype, molecular genetic findings, and clinical manifestations. Second, there is a plethora of new techniques emerging in recent years, which not only helps in the accuracy of diagnosis but also provides guidelines for the treatment and indicators for prognosis. In the cytogenetic area, fluorescence in situ hybridization has greatly expanded the versatility of traditional karyotyping. In the field of molecular biology, polymerase chain reaction (PCR) has gradually replaced the time-consuming Southern blot technique. The branching of PCR into quantitative PCR and reverse transcriptase PCR (RT-PCR) brings molecular biology to a new horizon. In situ hybridization and microarrays have gradually entered into the immunohistochemical laboratories of large medical centers. Finally, the recent development of gene expression profiling provides a power tool for stratification of lymphomas and leukemias, and is highly promising for the diagnosis, prediction of prognosis, and guidance of treatment for hematologic neoplasms.

Despite of all the exciting technological advances, immunophenotyping remains the mainstay in the routine diagnosis of hematologic neoplasms. Most laboratories use immunohistochemistry for immunophenotyping, because it is more convenient for morphologic correlation and it can be easily handled by a histology laboratory. However, flow cytometry provides timely, quantitative information and frequently a definitive distinction between malignant and benign lesions (e.g., information of light-chain restriction). Whereas flow cytometry is more helpful in diagnosing leukemias and generates prognostic predictors in some entities (e.g., CD38 and ZAP 70 for chronic lymphocytic leukemia), immunohistochemistry is more useful in diagnosing lymphomas with small numbers of tumor cells (e.g., Hodgkin lymphoma, T-cell-rich B-cell lymphoma) and provides some specific markers that are not available for flow cytometry (e.g., cyclin D1 for mantle cell lymphoma and bcl-6 for lymphomas of follicular center cell origin). Although the utilization of both techniques will undoubtedly yield a complete picture and more definitive diagnoses of hematologic tumors, concerns of cost restraint and specimen size frequently limit the luxury of choice. The major purpose of this book is to discuss the pros and cons of these two techniques in individual diseases so that the reader can make an intelligent choice to achieve an accurate diagnosis.

Whereas immunophenotyping may help diagnosing most cases of hematologic neoplasms, molecular cytogenetic techniques are mandatory for the diagnosis of a few tumors, such as Burkitt lymphoma and chronic myelogenous leukemia. In some tumors, the genotype is decisive in the choice of therapy. For instance, if the karyotype in a case of acute promyelocytic leukemia is not t(15;17)

but its variant, the patient may not respond to the therapy of *alltrans*-retinoic acid, and arsenic trioxide may have to be used. Cytogenetic studies are also important in cases with acute myeloid leukemia, myelodysplastic syndromes, and chronic myeloproliferative disorders, because the karyotypes in many cases dictate the clinical manifestations and the prognosis. The prognostic prediction, in turn, affects the therapeutic decision. In addition, for the detection of minimal residual disease after therapy, molecular genetic techniques are frequently more sensitive than immunophenotyping. Unfortunately, those sophisticated molecular genetic techniques are only available in limited numbers of reference laboratories, and they are frequently costly. Therefore, guiding the reader to select these new techniques in supplementing immunophenotyping is the second important task of this book.

Most hematopathology textbooks emphasize the application of immunohistochemistry, whereas flow cytometry textbooks seldom mention this technique. This book will provide a balanced view on both areas and a convenient way for the reader to study both subjects side by side. As case review is the most efficient way of learning, this book includes a large number of cases, covering all important clinical entities, with detailed clinical history and flow cytometric and immunohistochemical findings preceding the discussion of the individual disease. Abundant color illustrations of immunohistochemical stains, flow cytometric histograms, and tables summarizing diagnostic features and comparing similar diseases are furnished to facilitate the reader's understanding. It is the wish of the author that this book will become a handy guidebook for the hematopathologists and clinical hematologists/oncologists in their busy daily practice.

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Chapter 1
General Introduction

With the progress in subclassification of hematologic neoplasms and the refinement in treatment of these tumors, diagnosis of lymphoma and leukemia has become increasingly complicated. The World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues relies not only on morphology of the tumors but also requires immunophenotyping and genotyping for an accurate diagnosis. Although genotyping with molecular genetic techniques are frequently the tools to make a definitive diagnosis, immunophenotyping is the most important means to provide a prompt diagnosis. Immunophenotyping can be achieved either by flow cytometry (FC) or immunohistochemistry (IH) techniques. Although each of these two techniques has its advantages and disadvantages, each is complementary to the other, and they should be used together. This book will discuss the pros and cons in the application of these two techniques as well as their respective roles in diagnosing various hematologic neoplasms.

The flow cytometer has been hailed as a new product of technical revolution, but the concept of FC has existed for >50 years, and cell counters have been used extensively for >20 years. Nevertheless, the emergence of the fluorescence detector in the new generation of cytometers greatly enhanced their versatility. The availability of a great variety of monoclonal antibodies finally pushed FC to the forefront. The outbreak of acquired immunodeficiency syndrome (AIDS) incidentally accelerated the acceptance of flow cytometers as routine laboratory instruments because of the tremendous demands for testing the helper: suppressor T-cell ratio as a screening technique in the early epidemic of AIDS. However, the flow cytometer has been promptly adopted by hematology and oncology laboratories as a routine tool because most monoclonal antibodies are cell lineage-or developmental stage-specific for blood cells.

FC has the advantage of being more efficient, sensitive, accurate, and reproducible than manual techniques. With FC, multiple specimens can be simultaneously processed with a panel of 10 or more monoclonal antibodies, and tests can be completed within several hours. When there is a sufficient specimen, FC counts 3,000 to 5,000 cells for the study of each antigen, compared with 100 to 200 cells counted in manual techniques. The examination of large numbers of cells enhances the sensitivity and accuracy of FC and makes it possible to detect small numbers of neoplastic cells. The percentage of various cell groups obtained by FC is highly reproducible and is thus comparable between different laboratories. However, the major merit of FC is its capability of simultaneously measuring multiple parameters (forward light scatter, side scatter, and fluorescence), and the data thus obtained can be stored for further analysis. In addition, the electronic gating gadget enables the study of separate cell groups without requiring tedious isolation techniques.

The major limitations of FC, at this stage, are the high cost of the instrument, the special skill required to operate it, and the lack of a morphologic correlation with the markers. The failure of the machine to distinguish a normal cell from a tumor cell leads to the indiscriminate counting of both populations; thus a wrong conclusion may be drawn if not enough tumor cells or parameters are analyzed. However, with the availability of increasing numbers of specific monoclonal antibodies and fluorochromes, and the emergence of four- to five-color analyzers, the function of FC in terms of diagnosis and therapeutic monitoring has been markedly improved in recent years.

The current simplification of this automated instrument makes it feasible to use as a routine laboratory procedure, not only in large medical centers but also in medium-sized hospitals. Although it is now technically possible for clinical laboratories to use flow cytometers, the lack of expertise in interpreting the results still hampers the broad use of this instrument. It is apparent that a wellillustrated guidebook with detailed clinical cases is needed to help flow cytometer workers to understand how to apply FC to identify malignant cells and to diagnose hematologic neoplasms.

However, the more popular technique for immunophenotyping is IH for obvious reasons. IH can be conveniently performed in a histology laboratory with a minimal investment in equipment and reagents. It can be done on frozen as well as permanent sections. The feasibility of performing IH on archived material retrospectively is a great advantage of

IH. Nevertheless, the major merit of IH is the direct correlation of morphology with markers, which makes surgical pathologists more confident in interpreting the results leading to a diagnosis.

At this stage, the application of IH is limited by the availability of monoclonal antibodies that can be used for histologic staining. After fixation and embedding, many antigenic epitopes are altered so that they can no longer react to most antibodies that are used for FC. The major drawback of IH is its inability to demonstrate surface immunoglobulins and thus the inability to demonstrate a clonal B-cell population in most cases of lymphomas. The antigen-retrieving technique using a microwave oven may help to detect immunoglobulins in some tumors, especially in those with abundant cytoplasm, such as immunoblastic lymphoma and plasma cell neoplasms. The inability to distinguish surface from cytoplasmic antigens is another drawback. For instance, cytoplasmic CD3 is present in early T-cell stage (thymocytes) and surface CD3 is detected in mature T-cell stage (peripheral T cells). When a tumor is stained positive for CD3 by IH, the developmental stage of the tumor cells cannot be pinpointed. The same is true for the distinction between natural killer (NK) cells and NK-like T cells.

There are other limitations of IH. Multiple staining cannot be performed on the same cells. Therefore, the characterization of tumor cells by IH is not as comprehensive as by FC, which can detect 2 to 4 antigens on the same cells. IH using sequential horseradish peroxidase and alkaline phosphatase can label two different antibodies, but it is difficult to demonstrate two antigens on the same cell.

Another limitation of IH is the inability to quantify the percentage of tumor cells for therapeutic monitoring. The imaging technique may achieve the effect of semiquantitation, but it is tedious and the information is not as accurate as that obtained from FC. Finally, IH is usually ordered after examination of the hematoxylin and eosin-stained sections, so that a conclusion cannot be made until the third day after the receipt of the specimen. The turnaround time of FC is approximately 3 hours, and the results can be obtained on the day the specimen is received. The advantages and disadvantages of IH and FC are listed in Table 1.1.

Of course, the diagnosis of hematologic neoplasms is so complicated that it cannot depend on immunophenotyping alone to do the job. The importance of correlating immunophenotyping with clinical features, morphologic findings, and other supplementary tests, such as cytochemistry, molecular biology, and cytogenetics, can never be overemphasized. Therefore, the core of this book is the presentation of 39 clinical cases with pertinent information to provide the reader with a comprehensive concept of hematologic neoplasms and the role of FC and IH in diagnosis, classification, and treatment of these disorders. Case review is a most efficient way of learning and is probably a modern trend in learning methods. In each section, a case history and immunophenotypic findings are presented, followed by a concise discussion in morphology, immunophenotyping, molecular genetics, and clinical presentation.

To make this book understandable by people from different disciplines (clinicians, anatomic pathologists, clinical pathologists, and other clinical laboratory scientists) who share an interest in immunophenotyping, three introductory chapters are included to familiarize the reader with the basic principles of FC, IH, and molecular genetics. Preceding the case presentations is a chapter on classification of hematologic neoplasms. There have been many

classifications of this group of tumors, but the World Health Organization (WHO) classification is now universally accepted. The principle of classification again is based on clinical presentation, morphology, immunophenotype, and molecular genetics. For instance, on the basis of clinical presentation, tumors can be divided into predominantly disseminated lymphoma and/or leukemia, primary extranodal lymphomas, and predominantly nodal lymphoma. Immunophenotyping can divide hematologic neoplasms into different maturation stages, such as progenitor B cell, pre-pre-B cell, pre-B cell, immature B cell, mature B cell, activated B cell, and plasma cell. Based on the origin of intranodal B-cell differentiation, tumors can be assigned to germinal center, mantle zone, marginal zone, and parafollicular perisinusoidal areas. Finally, according to the presence or absence of mutation of the variable region of immunoglobulin heavy-chain gene, lymphomas can be divided into pregerminal center, germinal center, and postgerminal center groups.

TABLE 1.1 Comparison of Immunohistochemistry and Flow Cytometry

	Immunohistochemistry	*Flow Cytometry*

Morphologic correlation	Excellent	No visual correlation
Cost	Less expensive equipment needed	Expensive equipment and additional technicians
Specimen required	Paraffin and frozen sections	Blood, marrow, body fluid, fine needle aspirates, and fresh solid tissues
Available antibodies	Limited but gradually expanded	Abundant
Dual stain on same cells	Limited	5- to 6-color staining
Quantitation	Estimation with imaging techniques	Accurate and reproducible
Distinction between surface and cytoplasmic staining	Difficult	Easy
Turnaround time	4 hours after hematoxylin and eosin-stained section is examined	3 hours

Currently, there are many monoclonal antibodies available for identification of different cell lineages or developmental stages. The selection of appropriate antibodies is the important first step for immunophenotyping, not only because of cost containment but also because of the limited size of specimens. As a general panel to cover multiple cell lineage or all clinical situations is impossible to set up, one may select to use a two-tiered approach or targeted approach for monoclonal antibody selection. A two-tiered approach uses a screening panel to determine the final panel of monoclonal antibodies. The targeted approach is based on either the clinical diagnosis or morphology (by examining the tissue imprints, blood or bone marrow aspirate smears) to select the monoclonal antibody panel. To guide the reader in a targeted approach, abundant morphologic illustrations from specimens of blood, bone marrow, lymph node, spleen, and other soft tissue are provided in this book.

The goal of this book is to serve as a handy laboratory guide for immunophenotyping of hematologic neoplasms. The users may want to compare our cases with their own to find out what they need to study and to avoid omission of some important tests. For this purpose, the summary table at the end of each case study should be used as a quick reference. For those who are not familiar with certain theories or techniques, the introductory chapters may serve as short refresher courses. For many special aspects that are not covered by this book, the reader is referred to Suggested Readings.

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Chapter 2

Principles of Flow Cytometry

INSTRUMENTATION

A flow cytometer is versatile in its capability of measuring multiple parameters simultaneously. These parameters include the physical properties of cells (e.g., cell size and cytoplasmic granularity), surface membrane, cytoplasmic and nuclear antigens, and DNA-RNA contents of individual cells in a cell suspension. Surface, cytoplasmic, and nuclear antigens are detected by means of fluorochrome-conjugated antibodies. These antigens are thus the *extrinsic properties* of the cell. The physical properties of the cell are the *intrinsic properties*, because no exogenous reagents are added for their detection. These parameters are measured through an optical system, and the light signal thus generated is registered in an electronic system. The computer system is responsible for data storage, gating, and graphic display on a screen.

Although the Coulter counter can be considered a flow cytometer, the term *flow cytometer* is usually reserved for cell counters with fluorescence detectors, such as the FACSCanto II (Becton Dickinson) (Fig. 2.1) and the Cytomics FC500 (Beckman Coulter, Inc.) (Fig. 2.2). The design of the optical, electronic and computer systems may be somewhat different in various flow cytometers, but they are constructed with essentially the same principles.

With the change of light source from mercury arc to laser, the cooling system from water cooled to air cooled, and the computer system from simple to sophisticated, the size of the flow cytometer has become smaller (table-top) and its function expanded in terms of data storage, number of fluorochromes used, and graphic and data display. However, the basic components of a flow cytometer remain the same: the fluid transport system, the optical system, the electronic system, and the computer system (1, 2, 3, 4, 5, 6, 7 and 8).

FLUID TRANSPORT SYSTEM

The fluid transport system starts with a sample receiving area where test tubes containing patients' specimens and controls are placed in a carousel. The cell suspension is aspirated by means of differential air pressure or vacuum into a tubing system leading to the flow chamber (Fig. 2.3). When in the flow chamber, which is a conical nozzle, the specimen is surrounded by a cell-free stream of sheath fluid, producing a laminar flow configuration. The outer sheath fluid forces the cells in the sample to line up single file. When the sample exits from the flow chamber through a narrow orifice, the flow velocity becomes markedly increased (about 1 to 10 m/s). The cell stream then meets the light source (mercury arc lamp or laser) at the light interception point in the sensing area, and electric or optical signals are generated.

OPTICAL SYSTEM

Electric signals are generated due to changes in electric resistance of the fluid when cells suspended in an electrically conducting medium pass through a light beam in the sensing area. Based on the fact that blood cells have lower electric conductivity than saline solution has, Wallace Coulter designed a cell counter that has dominated the American market for >30 years. The principle he used to measure cell volume is now called the Coulter Principle. This principle was also used in some older models of flow cytometers, such as the fluorescence-activated cell-sorting (FACS) analyzer (Becton Dickinson), which contains a mercury-cadmium arc lamp. The advantages of using mercury and xenon arc lamps are their low cost, ease of operation, and broad range of spectral output. The disadvantages are nonuniform radiance, low brightness, and instability of the arc. Because the disadvantages outweigh the advantages, mercury arc lamps are no longer used in the new models of flow cytometers.

Currently, all flow cytometers use lasers as the light source, and optical instead of electric signals are generated. Lasers can deliver intense, coherent (waves of light that are parallel and unidirectional), monochromatic light (single color or wavelength), thus resulting in low divergence and high brightness of the signals generated. The use of small air-cooled ion lasers has facilitated the development of smaller, less expensive, easier-to-install flow cytometers, such as the FACSCanto II and Cytomics FC500.

FIGURE 2.1 Photograph of FACSCanto II flow cytometer manufactured by Becton Dickinson. (From Becton Dickinson with permission.)

FIGURE 2.2 Photograph of Cytomics FC500 flow cytometer manufactured by Beckman Coulter. (From Beckman Coulter, Inc., with permission.)

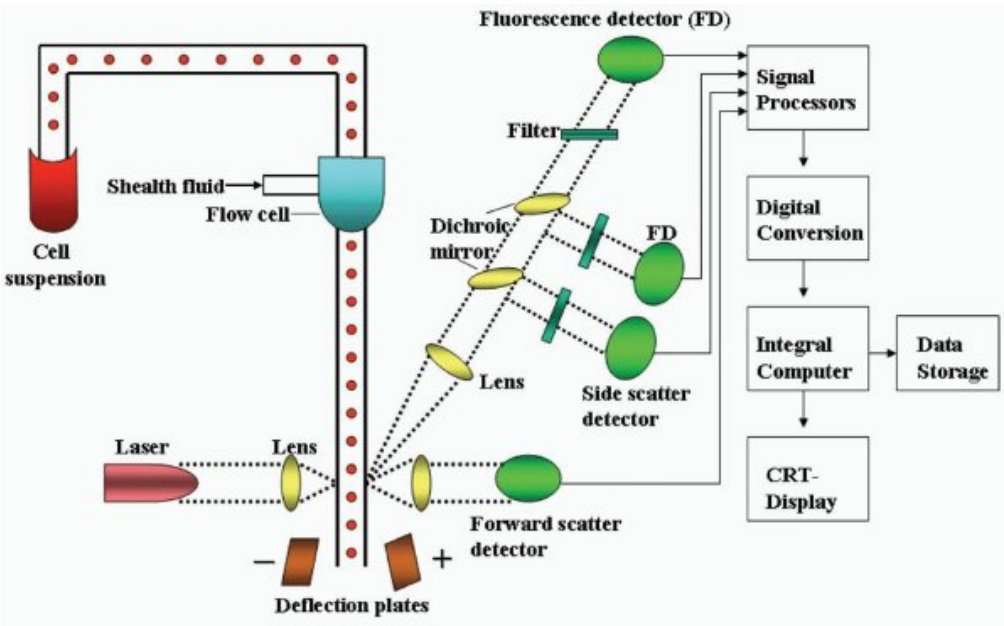

FIGURE 2.3 Basic structure of a flow cytometer showing the fluid transportation system, the optical system, the electronic system, and the cell sorter.

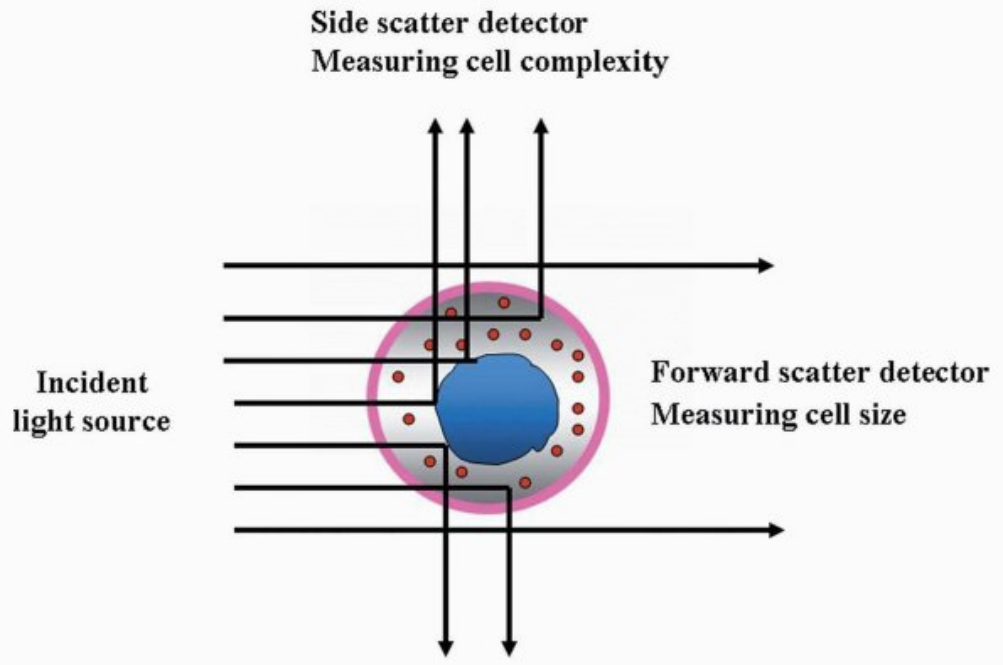

FIGURE 2.4 Schematic illustration showing the relationship of light scatter and cell size/structure.

The most frequently used optical signals are forward-angle (2 to 10 degrees) and right-angle (90 degrees) light scatter (Fig. 2.4). The former is proportional to cell size, and the latter is related to cell characteristics, such as cytoplasmic granularity and nuclear configuration.

Fluorescent Signals

The light scatter signals are usually used for screening. However, the characterization of cells is mainly based on their surface, cytoplasmic, and nuclear antigens, which are detected through a special optical signal: the fluorescent signal. Fluorescent signals are generated through fluorochrome-labeled antibodies that react specifically with various cell antigens, thus facilitating the identification of cell lineage, developmental stage, and special groups of tumor cells. Cellular DNA and RNA can be directly stained by intercalating agents, such as propidium iodide, which can bind DNA and RNA with the resultant generation of fluorescent signals.

Fluorochrome-labeled cells absorb incident light from the laser, resulting in emission of a longer wavelength, which is called fluorescence. The color of light emitted is a function of its wavelength (Table 2.1). As long as the wave-lengths emitted from the various fluorochromes do not overlap, the same specimen can be treated with 2 to as many as 10 fluorochrome-conjugated antibodies. The selection of fluorochrome is determined by the compatibility between the wavelength spectrum of the light source and the excitation ranges of the fluorochromes (Table 2.2). The most frequently used fluorochrome pair is fluorescein isothiocyanate (FITC) and phycoerythrin (PE).

Lens, Filter, and Mirror

A series of optical devices is used to direct the incident light, reflected light, and fluorescence to detectors. A beam-shaping lens is used to focus the laser down to a narrow *beam waist* of 20 to 100 μm in the sensing area. In multicolor analysis, separation of multicolored light is accomplished by the combined use of filters and dichroic mirrors. A short-pass filter allows only light of short wavelengths to pass and blocks that of the longer wavelengths. A long-pass filter acts the opposite way. Dichroic mirrors, in contrast, allow light of certain wavelengths to pass and reflect the light that is not allowed to pass.

ELECTRONIC SYSTEM

After traveling through all the filters and lenses, the photons of light impinge on the detectors and are converted into electrons (photoelectric effect). The detectors include photomultiplier tubes, which are commonly used for side-scatter and fluorescence signals, and photodiodes, which are used for absorption, extinction, and forward-scatter signals. The electronic signals or voltage pulses are analog signals in various magnitudes from 1 to 10 V. Analog signals may be processed as peak amplitude (height), integral (area), width (duration), or shape of the pulse and are expressed in either logarithmic or linear scales.

TABLE 2.1 Wavelength and Color of Fluorochromes

Fluorochrome	*Excitation Max. (nm)*	*Emission Max. (nm)*	*Color*
Propidium iodide	488	620	Red
Peridinin chlorophyll	488	670	Red
Fluorescein isothiocyanate	494	517	Green
Phycoerythrin	495	576	Orange
Rhodamine	545	575	Orange
Texas Red	596	615	Red
Phycocyanin	620	655	Red
Allophycocyanin	620	660	Red
Cyanin 5	633	670	Red

TABLE 2.2 Wavelength of the Light Source and Choice of Fluorochrome Combination

Light Source	*Excitation Wavelength (nm)*	*Fluorochrome Combination*
Mercury arc lamp	485, 546	FITC, PE, RD1
Argon ion laser	488	FITC, PE, RD1
Dye laser	600	Texas Red, APC
Krypton-ion laser	568, 647	APC
Helium-neon laser	633	APC

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; RD1, rhodamine.

The analog signals are digitized in the analog-to-digital converters. The digital signal is a function of instrument resolution. A low-resolution instrument may have 256-channel resolution, whereas a high-resolution instrument may range over 1,024 channels. The

last component of the electronic system is the pulse height analyzer, which analyzes the digital signals and quantifies them for computer display on an oscilloscope screen.

COMPUTER SYSTEM

The flow cytometer can be interfaced with an external computer system that performs three important functions.

1. *List-mode storage*: All parameters measured can be stored permanently on a floppy disk or temporarily on a hard disk. All data can be combined and analyzed later and can be printed in graphic form as a permanent record.
2. *Gating*: A gate is an electronic window that can be set with a cursor on the screen in a rectilinear or amorphous form to circumscribe a group of cells with similar characteristics (e.g., size and cytoplasmic granularity). Gating is performed to isolate electronically this special group of cells for analysis, avoiding the difficult task of purifying the cell population by biologic means. Depending on the software capability, several gates can be set so that information can be gathered on several populations of cells.
3. *Graphic display*: The computer can provide graphic displays in several forms, as described below.

Scattergram (Dot Plot, Cytogram)

This is a graphic display of dots as determined by two related parameters along the *x* and *y* axes. For instance, one can plot forward-angle light scatter against right-angle light scatter; each dot represents a single cell or event. On the basis of cell size (forward angle) and cytoplasmic granularity (right angle), the scattergram usually shows three distinct groups of cells in peripheral blood samples. The lymphocytes are the smallest (with no cytoplasmic granules); the monocytes are the largest; the granulocytes have the most cytoplasmic granules (Fig. 2.5). In lymph node specimens, the distinction between a group of large cells and a group of small cells frequently helps to separate lymphoma cells from reactive lymphocytes or a small cell lymphoma from a mixed small and large cell lymphoma.

Single Histogram

This graph is usually used to display the number of cells (*y* axis) versus fluorescence intensity (*x* axis). When cells are stained with a fluorochrome-labeled antibody, a single histogram provides the percentages of positive and negative populations (Fig. 2.6). An isotopic-negative control should be used to determine the cutoff point between these two populations. For instance, if the monoclonal antibody is mouse immunoglobulin G (IgG), the mouse IgG with no specific antibody function should be used. When DNA stain is used, the single histogram can demonstrate the percentage of cells in different stages of the cell cycle and determine the ploidy of chromosomes with a normal control.

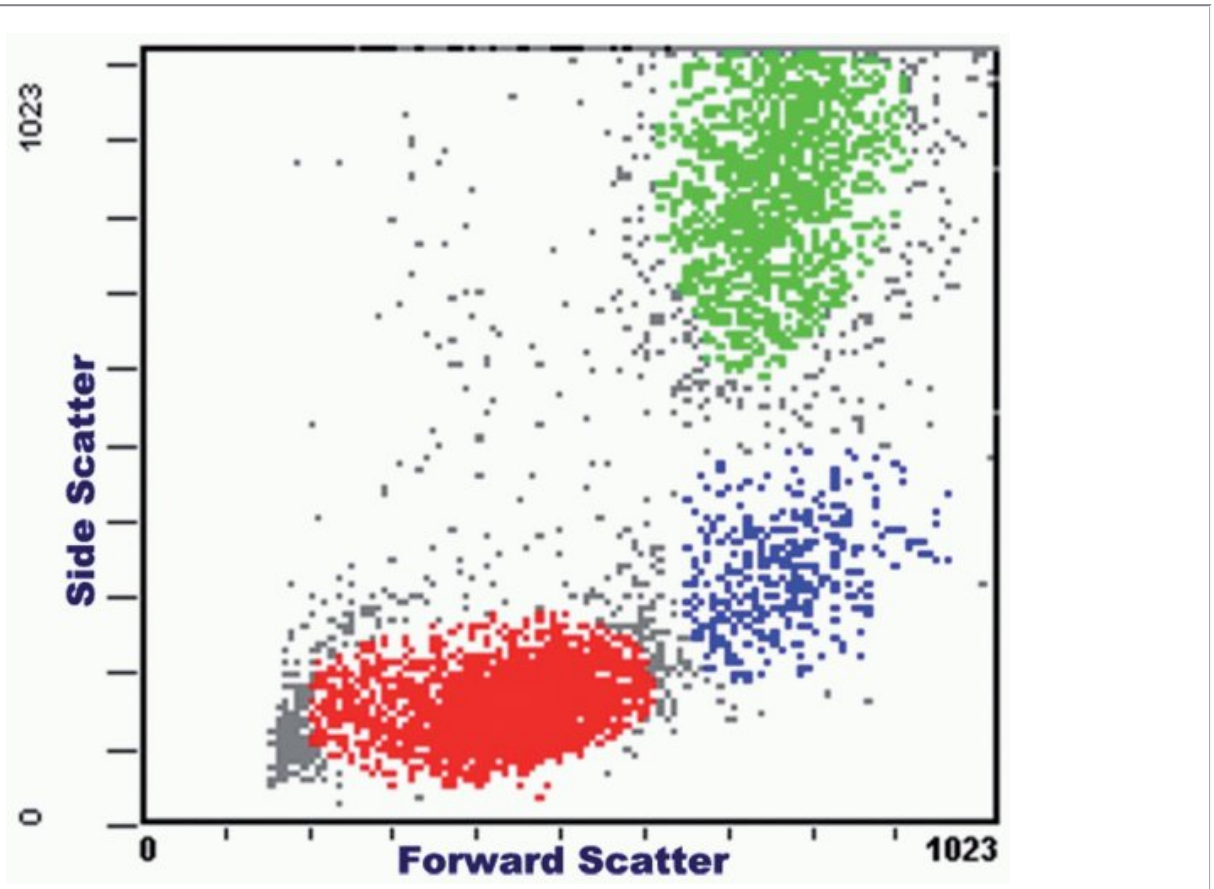

FIGURE 2.5 Scattergram with side scatter plotted against forward scatter, showing clusters of

lymphocytes (red), monocytes (blue), and granulocytes (green) in a peripheral blood specimen.

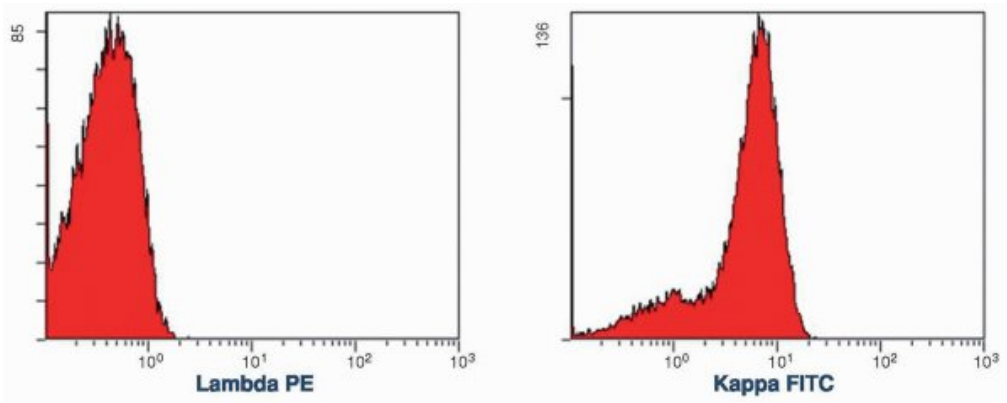

FIGURE 2.6 Histogram of a specimen stained with kappa and lambda antibodies. The lambda population is negative, whereas the kappa population is positive. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

Contourgram (Contour Plot, Contour Map, Contour Histogram, or Two-Parameter Histogram)

The contourgram computes the percentage of cell groups as determined by two parameters (e.g., two monoclonal antibodies or DNA and RNA contents) (Fig. 2.7). In contrast to the dot plot, the contour plot is composed of isocontour lines representing the cross-sections of the peaks and valleys of the data. An isocontour line connects all elements with a similar frequency of events. When the cutoff points of these two parameters are determined, the contourgram can be divided into four quadrants, and the percentages of subpopulations are readily computed. This is a very useful means for further characterization of subpopulations. Dot plots can also be used for this two-dimensional analysis.

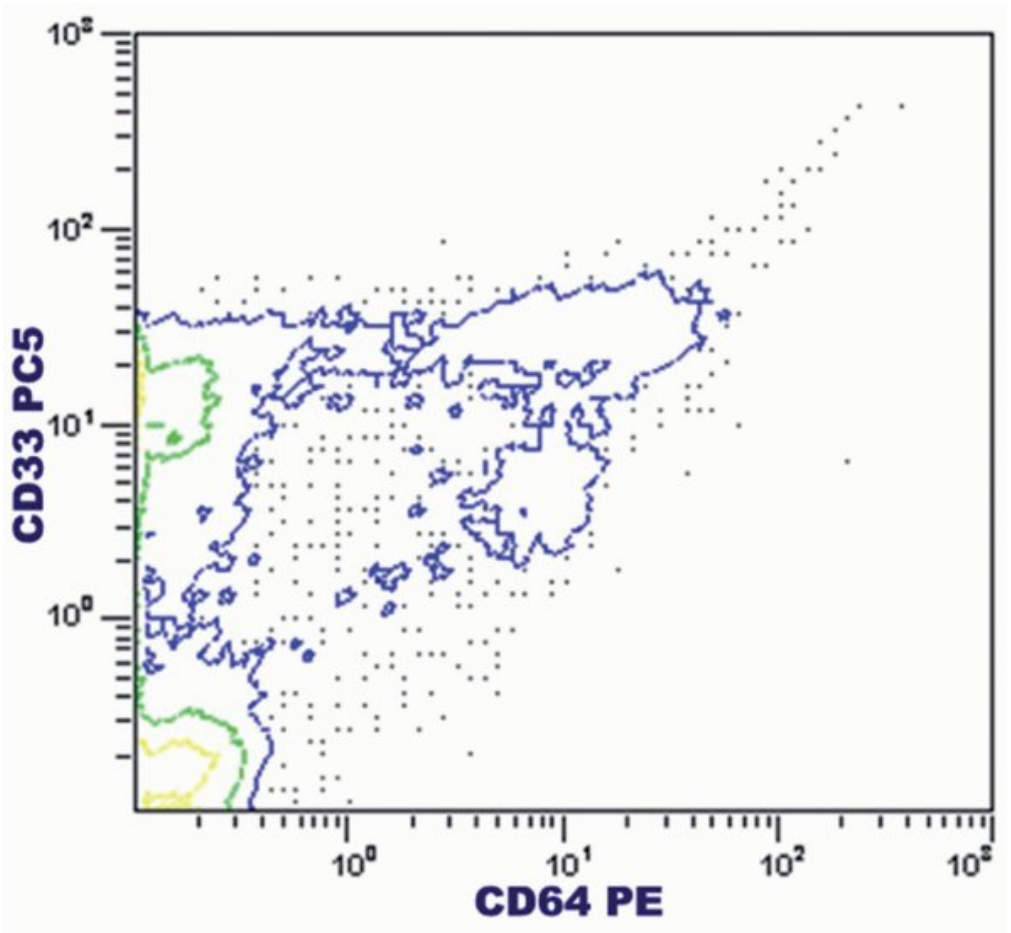

FIGURE 2.7 Contourgram of a case of acute myelomonocytic leukemia showing various populations as defined by CD33 and CD64. PC5, phycoerythrin-cyanin 5; PE, phycoerythrin.

Isometric Plot (Three-Dimensional Isometric Curve)

The isometric plot is most frequently used for simultaneous DNA and/or RNA analysis, but it can be used to display the correlation of any three parameters (Fig. 2.8). An isometric plot is often used to present multifactor analysis in research projects and is seldom used in clinical laboratories for routine phenotypic analysis.

Cell Sorter

Although cell sorting can be based on the principle of electroacoustic or electromechanical fluid switching, most instruments use droplet sorting. For droplet sorting, the stream of cell suspension first passes through an ultrasonically vibrating nozzle so that the stream can be broken up into evenly spaced droplets (e.g., 30,000 to 40,000 droplets per second). At the discretion of the operator, the droplets containing the cells of interest, which have been identified by a monoclonal antibody, are selectively charged electronically, either positive or negative, and then deflected as

they fall through an electromagnetic field formed by two deflection plates. The uncharged droplets fall vertically into a waste container. The cells separated in this way are usually viable and 95% pure. The cell sorter is undoubtedly a useful tool in research laboratories; however, it is not an absolute necessity for a clinical laboratory, because different groups of cells can be separated by electronic gating, and the cell group of interest can thus be selected.

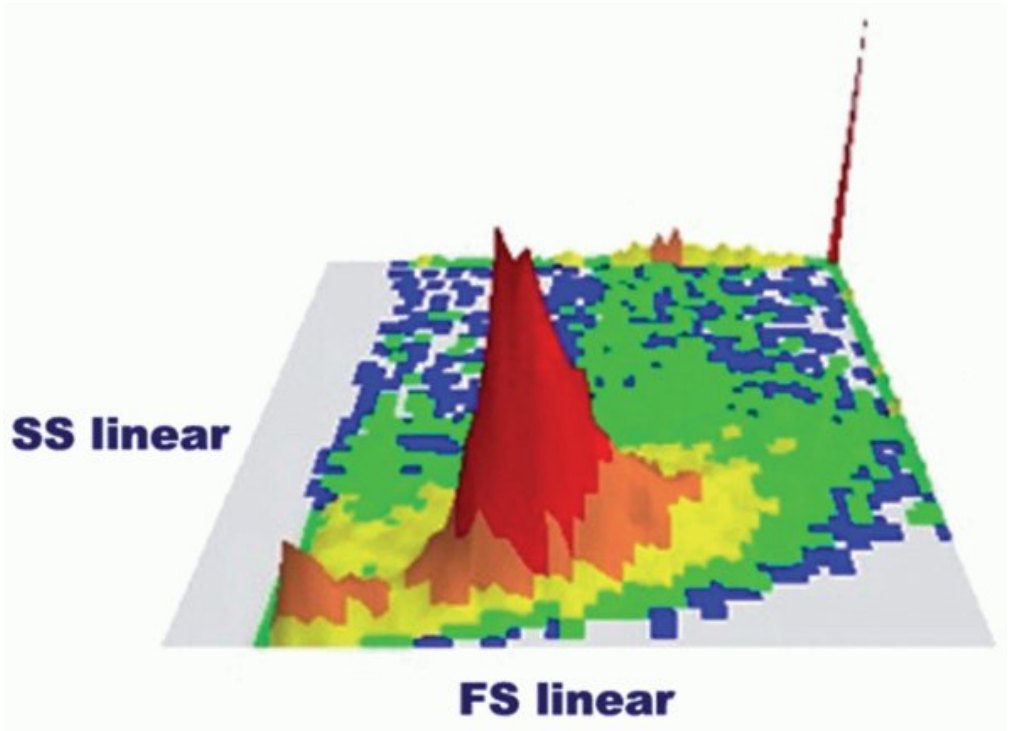

FIGURE 2.8 Isometric plot defined by side scatter (SS) and forward scatter (FS) showing a prominent peak of a leukemic population.

FACTORS AFFECTING FLOW CYTOMETRY RESULTS

A flow cytometer is a highly accurate instrument, provided that adequate quality control is maintained. For the standards and quality assurance concerning specimen collection and instrumentation in flow cytometry (FC), the reader is referred to the guidelines proposed by the National Committee for Clinical Laboratory Standards (9,10). Similar guidelines are also published by the National Institutes of Health, entitled *Guidelines for Flow Cytometric Immunophenotyping* (January 1993). Another excellent source of information concerning standardization and validation of laboratory procedures is the U.S.-Canadian consensus recommendations on the immunophenotypic analysis of hematologic neoplasia by FC (11).

Initial instrument setup and daily performance monitoring are the major quality assurances for the instrument (4,9,10). However, for the latest model of flow cytometers, alignment is optimized by service engineers, and daily adjustment is not required (4).

The problems affecting FC results are discussed as follows:

Specimen Problems

Besides instrumentation, the test results are also affected by inappropriate specimen collection and technical problems (Table 2.3). Recently, flow cytometrists have become increasingly aware that the patient's condition, medication, and biological factors also may cause some artifacts in immunophenotyping by FC (Table 2.4) (9).

From a pathologist's point of view, the most important problem is sampling error. Samples taken from a normal area, from areas containing a small proportion of tumor cells or large numbers of degenerated or dead cells, or taken from specimens with necrosis or fibrosis also may give erroneous results. When a metastatic carcinoma or extensive necrosis is present in the lymph node, the scattergram may show a disarrayed dot distribution (Fig. 2.9). A splenectomy specimen should be handled promptly, because splenic tissue autolyzes rapidly due to high levels of proteolytic enzymes in the blood components.

Peripheral blood or bone marrow specimens are prepared for staining either by the lysis of erythrocytes with lysing agent, such as ammonium chloride, or by density gradient separation with Ficoll-Hypaque. These two methods for immunophenotyping are generally comparable. However, some studies found that certain populations of cells may be lost with the density gradient technique, and the consensus conference on the immunophenotyping of leukemias and lymphomas recommended this method not be used (11). In contrast, the question has been raised as to whether the lysing agent used in the whole-blood lysis may alter certain antigens (4).

TABLE 2.3 Factors Affecting Flow Cytometry Results

Sampling Problems
Sampling error
Low tumor cell/normal cell ratio
Large numbers of degenerated or dead cells
Specimens with necrosis and fibrosis
Delayed processing of splenectomy specimens
Instrument Problems
Improper photomultiplier adjustment for isotypic control
Improper color compensation for double labeling
Improper gating
Reagent Problems
Improper antibody dilutions
Monoclonal vs. polyclonal antibodies (surface immunoglobulins)
Inappropriate fluorochrome conjugate
Miscellaneous
Cytophilic immunoglobulins
Solution: Incubation in RPMI at 37°C for 1 h
Antibody reaction to Fc receptors of tumor cells
Solution: Use Fab fragment antibody
Capping phenomenon
Solution: Use sodium azide as preservative
Phagocytosis of immunoglobulins

Solution: Eliminate monocytes

One should also realize that staining intensities of tumor cells may be much more heterogeneous than staining of cells from healthy individuals because of variability in the antigens per cell (9). Even the fluorescence intensities of normal cells in abnormal specimens may show increased heterogeneity due to enhanced nonspecific binding of some (or all) antibody reagents. Therefore, there may be more overlap between positive and negative populations and more difficulty in selecting an appropriate analytic cutoff point.

Instrument Problems

To guarantee that the results obtained are meaningful, the operator of a flow cytometer should follow a step-by-step procedure. First, evaluate the *viability* of the cells examined with 3% Trypan blue stain; only the dead cells are stained. Viability below 80% of the cells examined may provide unreliable results. Currently, a viability dye, 7-amino-actinomycin

D (7AAD), is mixed with the monoclonal antibodies to demonstrate the nonviable cells in the histogram, and the viability of the cell population is automatically computed.

TABLE 2.4 Potential Sources of Artifacts in Immunophenotyping by Flow Cytometry (9)

Cause	*Effect*	*Resulting Artifact*
Medications of drugs		
Zidovudine (AZT)	Increased granulocyte fragility	Decreased light on scatter resolution, increased granulocyte contamination of mononuclear preparation
Some antibiotics (e.g., cephalosporins)	Increased cellular autofluorescence	False positive if appropriate negative control is not used
Some chemotherapeutic agents (e.g., daunorubicin)	Increased cellular autofluorescence	False positive if appropriate negative control is not used
Nicotine	Increased lymphocyte margination, decreased lymphocyte count	Lower absolute values of lymphocyte subsets
Corticosteroids	Decreased CD4 levels	Overestimation of disease-related alterations
Anti-human lymphocyte antibodies (e.g., CD3 or soluble CD4)	Lymphocytopenia, modulation, or blocking of cell surface receptors	Decreased labeling with antibody reagent
Biological factors		

Reticulocytosis	Incomplete red cell lysis, increased contamination of mononuclear preparations	Decreased light scatter resolution, red blood cell contamination of lymphocyte gates
Strenuous exercise	Increased lymphocyte margination, decreased lymphocyte counts	Lowered absolute values for lymphocyte subsets
Diurnal variation	Variable absolute lymphocyte count	Variable absolute subset values
Specimen age and holding condition	Variable granulocyte preservation and/or leukocyte viability	Increased granulocyte contamination of lymphocyte gates, false-positive nonspecific staining of dead cells

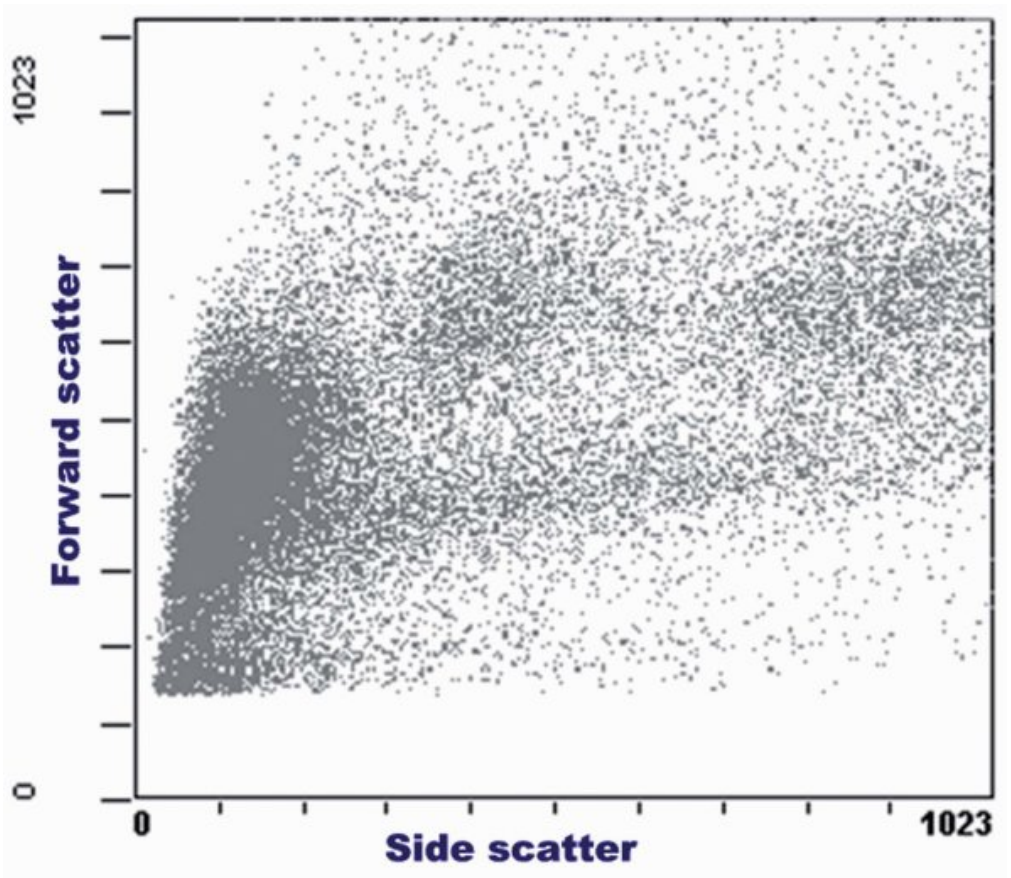

FIGURE 2.9 Scattergram of a lymph node biopsy with extensive necrosis showing a disarrayed dot distribution.

The second step is "*gating*" to select the cell cluster for analysis. For peripheral blood specimens, forward scatter versus side scatter is

still the routine strategy. This plot provides the best visual differentiation between lymphocytes, monocytes, and granulocytes (12). For lymphoma or lymphoid leukemias, the cluster of lymphocytes should be selected electronically for analysis. As will be discussed later, other gating strategies may be considered for specimens other than blood. The third step is “back-gating” to check the correctness of the gated population. The combination of this plot is CD45, the panleukocyte marker also known as leukocyte common antigen (LCA), versus CD14, a monocyte marker (13). As a result, the monocytes and more differentiated hematopoietic precursor cells are represented in quadrant 2 of a two-parameter histogram, as they react to both CD14 and CD45 (14). In quadrant 4, two groups of cells can be identified because they react only to CD45. The CD45-bright group represents mainly lymphocytes and

the CD45-dim group, neutrophils (14). Lymphoblasts and myeloblasts are CD45 dim or CD45 negative. When the lymphocyte cluster is further separated into large and small lymphocyte groups, this can be checked by a single histogram of forward scatter to see if two peaks are clearly separated (Fig. 2.10).

Gating

Gating is the most important step in making a correct diagnosis. Many problematic cases are either the result of “loose” gating (e.g., a big lymphocyte gate including other cell populations) or “tight” gating (only the central part of the cell cluster is gated). A loose gating will result in a lower percentage of the target population. In contrast, tight gating may inadvertently eliminate some B cells and natural killer cells, which have lower and higher forward scatter, respectively, than do T cells.

In addition to this homogeneous gating based on physical properties of cells (light scatter), there is also heterogeneous gating by using fluorescence antibody versus side scatter. The T-gating method used CD3 as a T-cell marker (15), B gating uses CD19 (16), and leukocyte gating uses CD45 (17). The T-gating method should be more specific than the routine homogeneous gating for T-cell subset analysis.

In specimens other than peripheral blood, there is an excess of nonleukocytes exhibiting leukocyte-like scatter characteristics leading to a low fraction of recovered leukocytes (18). Furthermore, even in the leukocyte populations, the cell size and cytoplasmic complexity may overlap, causing some difficulty in their separation (19). Substantial inaccuracy may occur when this gated population is analyzed after specific monoclonal antibody staining (14). This is especially true when the light scatter gate is used to identify a leukemic population (20).

A new gating strategy was first advocated by Stelzer et al. (19), who used a combination of right-angle light scatter and CD45 (panleukocyte antibody) for flow cytometric analysis of normal bone marrow specimens. Subsequently Festin et al. (18) applied the same combination to enumerate lymphoid cells in posttransplant bone marrow and liver biopsy specimens. Borowitz et al. (20) and Rainer et al. (21) used this gating strategy for immunophenotyping of acute leukemia in bone marrow. Nicholson et al. (22) applied the same principle to T-cell subset studies in whole-blood specimens. In our study, we found that this gating strategy is not only suitable for acute leukemia specimens in the bone marrow, but can also be applied to the study of chronic leukemias and lymphomas in the peripheral blood, bone marrow, lymph node, or other soft tissues (23). When comparing this new gating technique with conventional gating, we found that the percentage of tumor cells isolated by the new gating is consistently higher than that isolated by the conventional one, no matter whether it is lymphoma or leukemia or what kind of specimen is involved.

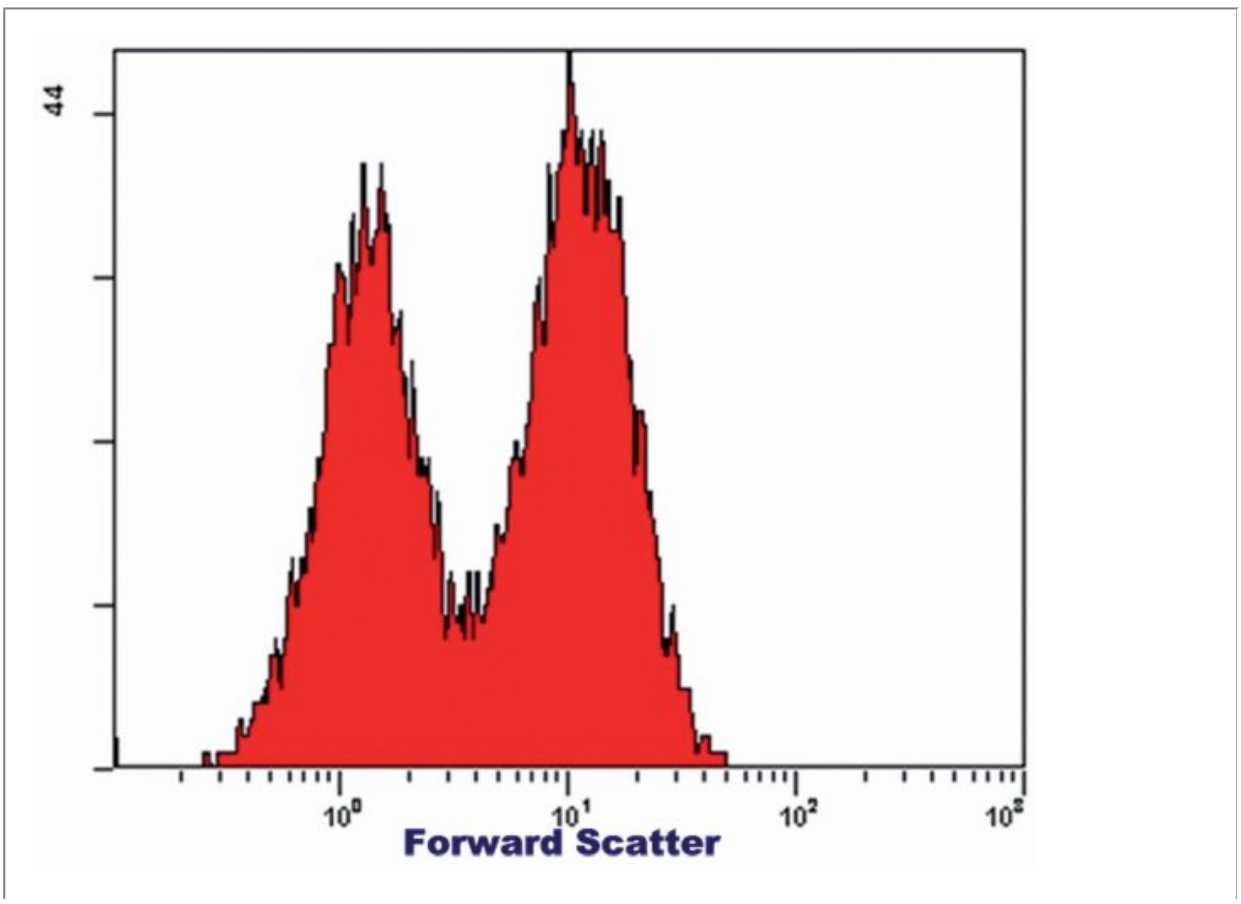

FIGURE 2.10 Histogram of forward light scatter showing small- and large-cell populations from a lymphocyte gate.

Cursor Setting

The second important step is setting the cutoff point (cursor setting) between positive cells and negative cells. The negative cells are the cells from the same patient incubated with mouse immunoglobulin of the same isotype as the monoclonal antibody used for the study (isotypic control). However, cursor setting is sometimes complicated, and certain adjustment with the photomultiplier tube is needed. If the photomultiplier tube is underadjusted, the weakly positive peak may become false-negative. In contrast, overadjustment may put the strongly positive peak off the screen.

As the isotype control is sometimes superfluous and potentially misleading, many authors advocate pattern reading rather than presentation of percentages of various populations (4,11). It is certainly not appropriate to depend blindly on isotype controls to distinguish positive and negative populations, but quantitative report is helpful for therapeutic monitoring and should be retained. In case of doubt, a contourgram is sometimes useful to identify the dim population, which can be mistaken as partial-negative (Fig. 2.11).

Electronic Compensation

In double labeling, two fluorochromes, such as fluorescein and PE, are used. Although both fluorochromes are excited at 488 nm, they emit at different wavelengths. However, the spectra of these emissions overlap. Therefore, electronic adjustment (a procedure known as *electronic compensation*) must be made to correct spectral overlap. Undercompensation may sometimes cause false-positive results showing a double-labeled population. Overcompensation, in contrast, causes false-negative results as the positive populations may be off the screen.

Reagent Problems

The major reagent problems include improper antibody dilution, selection of antibody, and conjugation of fluorochrome. Monoclonal antibodies are generally used for FC except for the study of surface immunoglobulins. Immunoglobulins have many epitopes; therefore, polyclonal

antibodies are more likely to react positively than are monoclonal antibodies. A neoplasm may not show a certain epitope of the immunoglobulin that is specific for a monoclonal antibody. For instance, the antibody may be specific for IgG-1 subclass, whereas the tumor cells may express IgG-4 subclass.

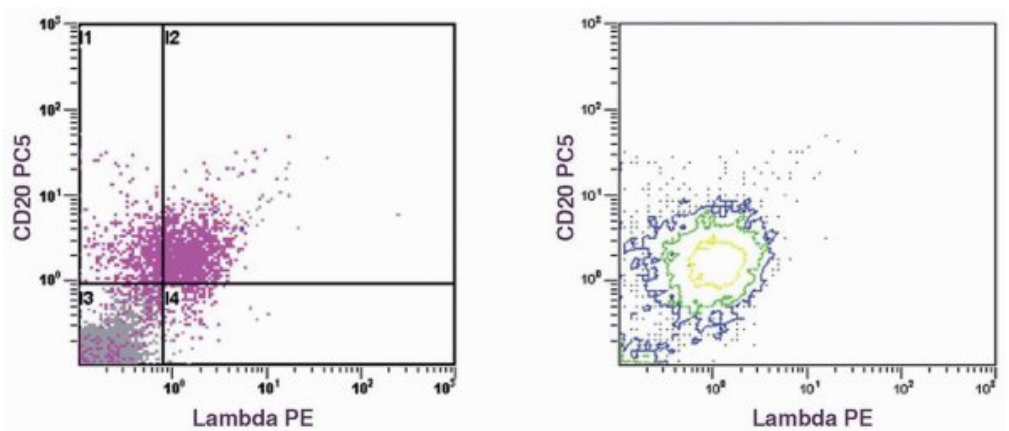

FIGURE 2.11 Scattergram plotted by CD20 versus lambda (**left**). The dividers as determined by isotopic controls incorrectly define the positive population. Contourgram (**right**) correctly identifies an intact cluster of chronic lymphocytic leukemia cells that have dim staining in both CD20 and lambda. PC5, phycoerythrin-cyanin 5; PE, phycoerythrin.

FITC has a low quantum yield, and its spectrum overlaps with autofluorescence of lymphocytes (15,16). Therefore, FITC should be conjugated with an antibody that reacts to a high-density antigen on the cell surface, such as CD45 or human leukocyte antigen (HLA-DR). In contrast, weak antigens, such as CD13, CD10, and CD33, should be conjugated with bright fluorochromes (5). The fluorochrome intensity is brightest among PE and its tandem fluorochromes including PE-Texas Red, PE-cyanine 5 (PE-Cy5), PE-Cy5.5, and PE-Cy7 (24). This is followed by the allophycocyanin (APC) group including APC, APC-Alexa 700, and APC-CY7. The small molecule

fluorochromes, including FITC, peridinin chlorophyll protein (perCP), Pacific Blue, and the Alexa series of dyes, exhibit fluorescence approximately 1 log dimmer than the above fluorochromes (24).

Miscellaneous

Other potential problems can be avoided by using standard procedures (9). For instance, *cytophilic immunoglobulin* (patient's own serum immunoglobulin that adheres to lymphocyte surface) may cause aberrant results by reacting with anti-immunoglobulin antibodies. The cytophilic immunoglobulin can be eliminated by washing after the lymphocytes are incubated in phosphate-buffered saline or RPMI medium at 37°C for 1 hour.

The *Fc receptor* is present on the surface of lymphocytes and monocytes. If the antibody used contains the Fc fragment, false-positive results may be obtained. Because it is now common practice to use antibodies with an Fab fragment, this nonspecific reaction is no longer a problem.

A *capping phenomenon* is frequently seen in surface immunoglobulin reaction, because the anti-immunoglobulinsurface immunoglobulin immune complex can be internalized, leaving only a small portion of the complex on the cell surface (capping). This may lead to a false-negative result. The routine use of sodium azide as a preservative for antibody reagents and as an additive to phosphate-buffered saline diluent may prevent this phenomenon.

Finally, *phagocytosis of immunoglobulin* by monocytes or macrophages may give rise to false-positive results for surface immunoglobulin. Pretreatment to separate the phagocytic cells from lymphocytes and careful gating to eliminate monocyte contamination of the lymphocyte gate are measures to prevent this phenomenon.

In summary, many factors can affect the flow cytometric results. Therefore, strict adherence to standard procedure and to quality assurance is mandatory for obtaining clinically meaningful results.

PARAMETERS FOR CELL DISTINCTION

After ensuring that the instrument, sampling, and reagent problems are under control, one can start to analyze patients' specimens. The major drawback for FC is lack of correlation between morphology and cell markers. Therefore, many parameters are being used to characterize different cell types. The parameters that help to distinguish different cell populations are listed in Table 2.5.

Cell Size

As mentioned before, cell size can be determined by forward-angle light scatter. Cell size is not only important in distinguishing normal leukocytes (lymphocyte versus monocyte versus granulocyte); it can also differentiate large tumor cells from small reactive lymphocytes and a mixed large and small lymphoma cell population.

Cytoplasmic Granularity

Cytoplasmic granularity can be determined by right-angle light (side) scatter. This is the second important parameter.

Cell size combined with cytoplasmic granularity can distinguish the three major types of leukocytes in the blood.

TABLE 2.5 Parameters for Cell Distinction by Flow Cytometry

1.	Cell size
2.	Cytoplasmic granularity
3.	Immunophenotyping: Identify cell lineage, developmental stages, and clonality
4.	Relative percentage of reactive cells: kappa/lambda ratio, selective loss of pan-T-cell markers
5.	Intensity of surface immunoglobulin: Dim immunofluorescence on CLL cells
6.	Double labeling: CD19/CD5 for CLL/mantle cell lymphoma, CD22/CD11c for hairy cell leukemia

7. DNA/RNA contents: Diploidy versus aneuploidy, increased RNA in proliferating cells

CLL, chronic lymphocytic leukemia.

Immunophenotyping

Immunophenotyping is the use of monoclonal antibodies to identify the surface and cytoplasmic antigens in individual cells, thus constructing a phenotype of these cells based on positive and negative reactions to a group of antibodies. For instance, detection of both a monoclonal B-cell population and a polyclonal T-cell population in a lymph node is consistent with a B-cell lymphoma with reactive T lymphocytes. With 5- to 6-color FC, a very accurate immunophenotyping can be achieved (Fig. 2.12).

Comparison of Percentages of Reactive Cells

This is an important parameter used to draw a preliminary conclusion. For instance, either kappa light chain or lambda light chain antibodies can be used to identify B cells, but when the kappa/lambda ratio is calculated, it can determine the clonality of a B-cell population. By the same token, a prominent discrepancy between the percentage of different pan-T-cell markers (CD3, CD5, and CD7) is suggestive of T-cell neoplasms (25).

Intensity of Immunofluorescence of Surface Immunoglobulin

This correlates with the amount of immunoglobulin present on the surface of B cells. As a rule, the follicular center cells stain more brightly than do medullary cord cells (26). Thus cells from follicular lymphoma stain brightly, and those from chronic lymphocytic leukemia stain dimly. Chronic lymphocytic leukemia transforming into a large cell lymphoma (Richter syndrome) may be detected by FC

on the basis of the presence of two patterns of immunofluorescence intensity and immunophenotypes (27).

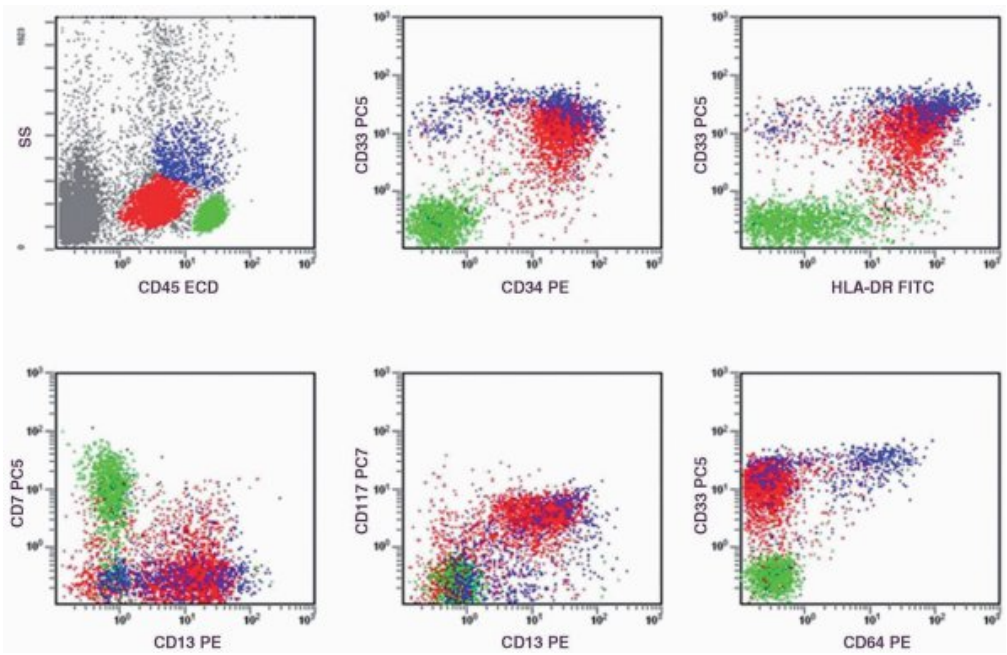

FIGURE 2.12 Five-color flow cytometry demonstrates three cell populations in a case of acute myelomonocytic leukemia (myelocytes, red; monocytes, blue; lymphocytes, green).

Double Labeling

This is accomplished by the two-color analysis setting in flow cytometers. Whereas single-marker labeling may identify the lineage of certain cells, double labeling may define a neoplastic population. The most common example is the coexistence of CD19 and/or CD20 (B-cell markers) with CD5 (T-cell marker) in chronic lymphocytic leukemia and/or small lymphocytic lymphoma and mantle cell lymphoma (28). Another example is hairy cell leukemia, which expresses both a B cell (CD22) and a monocyte marker (CD11c) (29). With the availability of six-color analysis, more accurate and more defined tumor population detection will be achieved.

DNA and RNA Contents

The DNA/RNA ratio may differ between a tumor cell and a normal cell population. For instance, tumor cells may show an aneuploid peak, whereas normal cells express a diploid peak. The increase in RNA content in the cell cycle is a good indicator of cell growth.

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Chapter 3

Principles of Immunohistochemistry

For many years, pathologists had depended on morphology alone to make a histologic diagnosis until the availability of “special stains.” Special stains were developed because pathologists always realized the fallacy of the morphologic approach and felt the need to have some accessory tests to substantiate the diagnosis. The first kind of special stains are cytochemical stains, which mainly identify cell lineage and cellular chemistry. When cytochemical techniques are applied to histologic sections, it is called histochemistry, which further improves the accuracy of a morphologic diagnosis. However, it is the development of immunohistochemistry that finally makes histologic diagnosis highly objective. Immunohistochemistry is particularly indispensable for the practice of hematopathology, but the molecular cytogenetic techniques have also played an increasingly important role in the diagnosis of hematologic neoplasms. The advent of nonradioactive in situ hybridization (NISH) techniques represents the current effort to combine histologic staining with the studies of nucleic acids (DNA and RNA). These various entities will be discussed briefly in this chapter.

CYTOCHEMISTRY

Cytochemistry is an integral part in the diagnosis of acute myeloid leukemia (AML) required by the French-American-British Cooperative

Group (1,2). Although its role has been gradually replaced by immunophenotyping with flow cytometry, cytochemistry is still useful in identifying cell lineages, especially the immature monocytes in the bone marrow, which are frequently difficult to recognize morphologically.

Myeloperoxidase (MPO) is the first screening test to distinguish AML from acute lymphoblastic leukemia (ALL). This enzyme is present in neutrophilic, eosinophilic, and monocytic lineage but not in lymphocytes (3). However, the minimally differentiated myeloblasts may not stain for MPO as seen in the AML-M0 cases, which can be distinguished from ALL only by immunophenotyping. The MPO in eosinophils is resistant to cyanide, so that eosinophil and its immature forms can be identified by this specific reaction. The peroxidase of megakaryocytes and platelets cannot be visualized by light microscopy, but it can be demonstrated by electron microscopy.

Sudan black B reaction is slightly more sensitive than, but similar to, MPO reactions. Sudan black B is a fat-soluble substance, but it probably stains for substances related to MPO, because the Sudan black B reaction becomes negative in patients with MPO deficiency (4). In addition to the MPO-positive cells, Sudan black B also stains fat cells, macrophages, and cytoplasmic vacuoles in Burkitt lymphoma cells.

Specific esterases are a group of enzymes capable of hydrolyzing halogenated naphthol esters (3). The most commonly used substrate is naphthol AS-D chloroacetate. *Chloroacetate esterase* is most frequently used for the identification of neutrophilic series and occasionally used for mast cells. Because chloroacetate esterase is stable even in paraffin-embedded tissue, it can be used for the diagnosis of myeloid sarcoma and extramedullary hematopoiesis. Chloroacetate esterase is negative for monocytes, megakaryocytes, erythroblasts, and lymphocytes. Only abnormal eosinophils, such as those seen in AML with bone marrow eosinophilia, are positive for chloroacetate esterase.

Nonspecific esterases are a group of enzymes capable of hydrolyzing various aliphatic and aromatic short-chain esters (3). They are called nonspecific esterases because these enzymes exhibit a wide range of substrate specificity. The substrates used to detect nonspecific esterase activity include α-naphthyl butyrate, α-naphthyl acetate, naphthol AS-D acetate, and naphthol AS acetate. The first two substrates are most frequently used because they do not stain for granulocytes. Thus sodium fluoride inhibition is not needed to distinguish monocytes from granulocytes. Besides monocytes and histiocytes, α-naphthyl acetate esterase is also positive in megakaryocytes and platelets, and the reaction is sodium fluoride sensitive (4). T lymphocytes usually show a focal paranuclear staining for nonspecific esterases. Because nonspecific esterases are sensitive to heat, storage, and fixative, they cannot be demonstrated in paraffin-embedded tissue sections.

Acid phosphatases (APs) are a group of enzymes capable of hydrolyzing monophosphate esters in an acid environment (3). By electrophoresis, APs can be separated into seven nonerythrocytic isoenzymes. Isoenzymes 2 and 4 are present in neutrophils and monocytes; 3, in lymphocytes and platelets; 3b, in primitive blood cells and blasts; and 5, in hairy cells of hairy cell leukemia. The most important function of AP is to identify hairy cells, which show a strong, diffuse, tartrate-resistant AP. This reaction may be demonstrated occasionally in other lymphomas or leukemias, but the reaction is seldom as intense and diffuse as in hairy cell leukemia. The focal paranuclear staining pattern of AP is helpful in identifying T lymphocytes.

Periodic acid-Schiff (PAS) is capable of reacting with R-CHO groups in tissues to form an insoluble bright-red complex (aldehyde-fuchsin-sulfurous acid compound) (5). Therefore, tissue and cells containing glycoproteins, mucoproteins, and high-molecular-weight carbohydrates are positive for PAS. The PAS reaction is positive in most blood cells; it is detected in 80% to 90% of cases of ALL and in 10% to 15% of cases of AML (3). It is particularly useful when a block pattern is demonstrated in pronormoblasts and lymphoblasts. A positive PAS stain in erythroblasts is a common finding in erythroleukemia; it is a coarsely granular pattern in cells of early stage and a finely granular pattern in cells of later stage. Normoblasts in healthy persons are PAS negative, but they can be PAS positive in erythrodysplasia. PAS staining of the periphery of cytoplasm, especially in cytoplasmic protrusions, is characteristic for megakaryocytes and megakaryoblasts. This pattern, if present, is helpful for the diagnosis of acute megakaryoblastic leukemia.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is the application of a labeled or enzyme-bound antibody to identify a specific antigen, which is visualized under light microscopy by means of a color signal. There are several important milestones in the history of immunohistochemistry development (5). In 1940, Coon first used immunofluorescence techniques to detect antigens in frozen sections (6). Avrameas et al. (7) developed enzymatic labeling to demonstrate the antigenantibody immune complex in tissue sections. Taylor and Burns (8) first applied immunohistochemical techniques to formalin-fixed, paraffin-embedded tissue sections. The subsequent progression from the one-step direct conjugate method to the multiple-step indirect method as well as to the discovery of the hybridoma technique by Kohler and Milstein (9) greatly facilitated the versatility of immunohistochemistry. The staining technique is further enhanced by enzyme digestion (10) and finally by antigen retrieval (AR) techniques (11,12) that make immunohistochemistry the indispensable tool in the practice of surgical pathologists. The current goal is to make immunohistochemistry quantitative so that it is not only a tool for diagnosis, but one for therapeutic monitoring and prediction of prognosis in various tumors (13,14).

Immunohistochemical staining involves multiple and somewhat complicated steps; therefore, there are many technical problems to watch for, and there are many technical decisions to make depending on the target antigens. Due to space limitation, only a few important technical matters are discussed in this chapter. For a comprehensive review, the reader is referred to the textbooks by Dabbs (15) and Elias (16).

Fixatives

There are two groups of fixative: The cross-linking fixatives (e.g., formaldehyde) and coagulant or precipitation fixatives. The latter group includes acid fixatives (e.g., Bouin's solution) and heavy metal fixatives (B5 and Zenker's fluid). Williams et al. (17) compared the

effects of various fixatives in immunohistochemical stain results on tonsil tissue and found that 10% neutral buffered formalin (NBF), 10% zinc formalin, and 10% formal saline gave the most consistent results overall and showed excellent antigen preservation. In contrast, 10% formal acetic acid, B5, and Bouin's fixative showed poor antigen preservation.

Others feel that there is no particular optimal fixative because the staining results depend on a complex interaction among the fixative, pH, osmolarity, temperature, length of treatment, and tissue types (16). For instance, there is no difference between formalin and Bouin's fixative for the staining of insulin, pancreatic polypeptide, and gastrin, but better results are obtained by Bouin's fixative than by formalin for the staining of glucagons and somatostatin (18). Elias (16) suggested dividing specimens into multiple fixatives, including NBF, 10% formal saline, 95% ethanol, Omni, modified methacarn, and B5 for subsequent processing. B5 is considered most suitable for fixation of lymphoid tissues but, because it contains mercury, many laboratories avoid using it.

Immunohistochemical Staining Procedures

Direct Conjugate-Labeled Antibody Method

The direct method is the application of labeled monospecific antibody directly to the tissue section (Fig. 3.1). The label can be an enzyme, biotin, fluorochrome, or colloidal gold. There are several procedures that are used to enhance the direct method. The most common one is the use of biotin to conjugate the antibody, and then the biotin will bind to the receptor of either the labeled avidin or streptavidin. With the second layer of labeling, the staining becomes amplified.

Indirect or Sandwich Method

The indirect method includes two major procedures: The indirect labeled procedure and the indirect unlabeled antibody procedure. The first procedure uses two layers of antibodies; the unlabeled primary antibody directly reacts to the tissue antigen, and the labeled secondary antibody reacts to the primary antibody (Fig. 3.2). The unlabeled antibody procedure uses three layers of antibodies (Fig. 3.3). The tertiary antibody is an anti-enzyme antibody that will conjugate with a specific enzyme, such as peroxidase, alkaline phosphatase, or glucose oxidase. When the enzyme reacts to its specific substrate in the system, a color product

will be demonstrated. The indirect method is generally more sensitive than the direct method, mainly because the polyvalent secondary antibody is able to detect multiple sites on the Fc and Fab portions of the primary antibody, and the primary antibody is more accessible to the secondary antibody than to the tissue antigen, which is being modified by fixation and embedding (16).

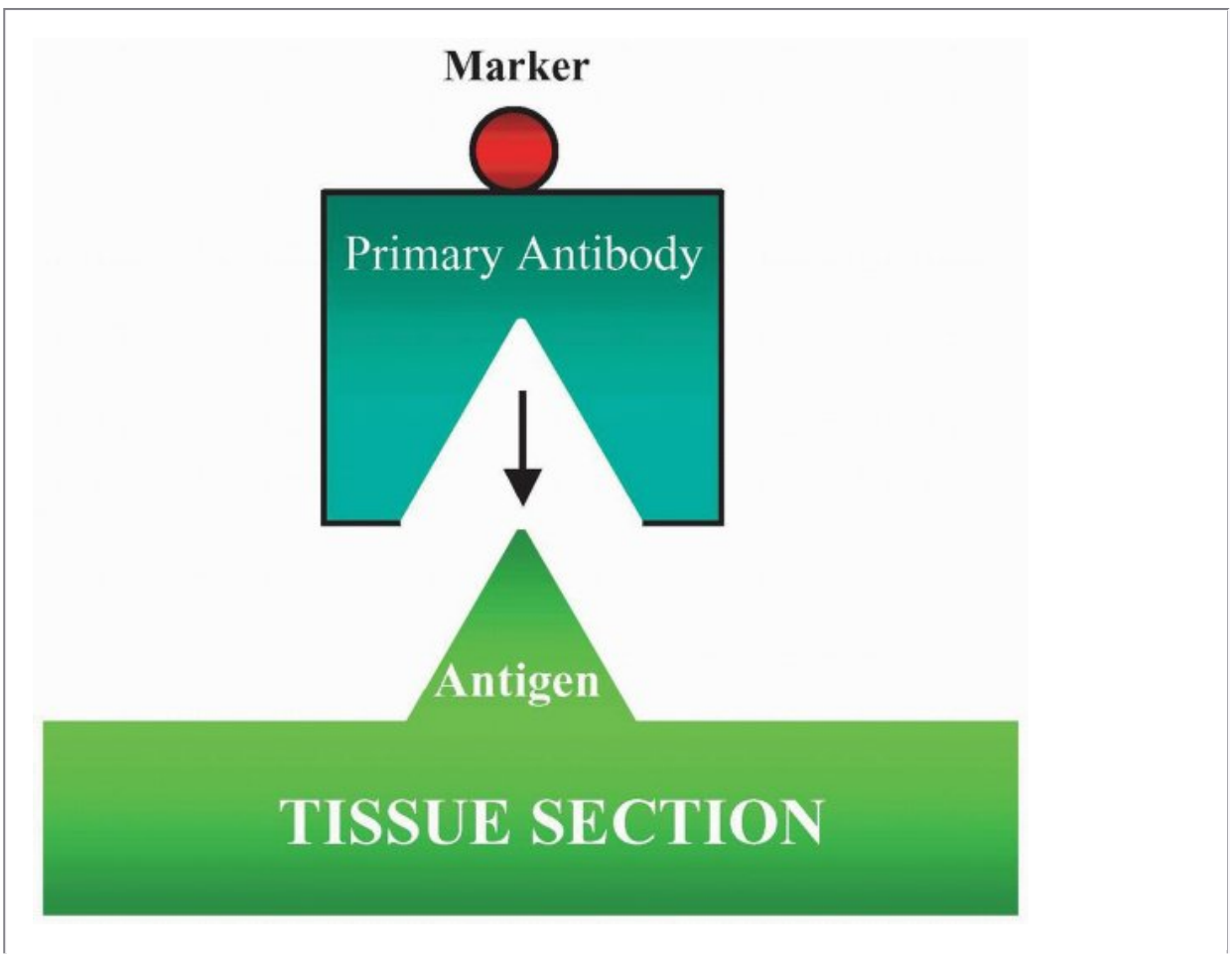


FIGURE 3.1 Direct method is to apply a labeled antibody directly to tissue sections. The marker then demonstrates the cellular location of the antigen in the section.

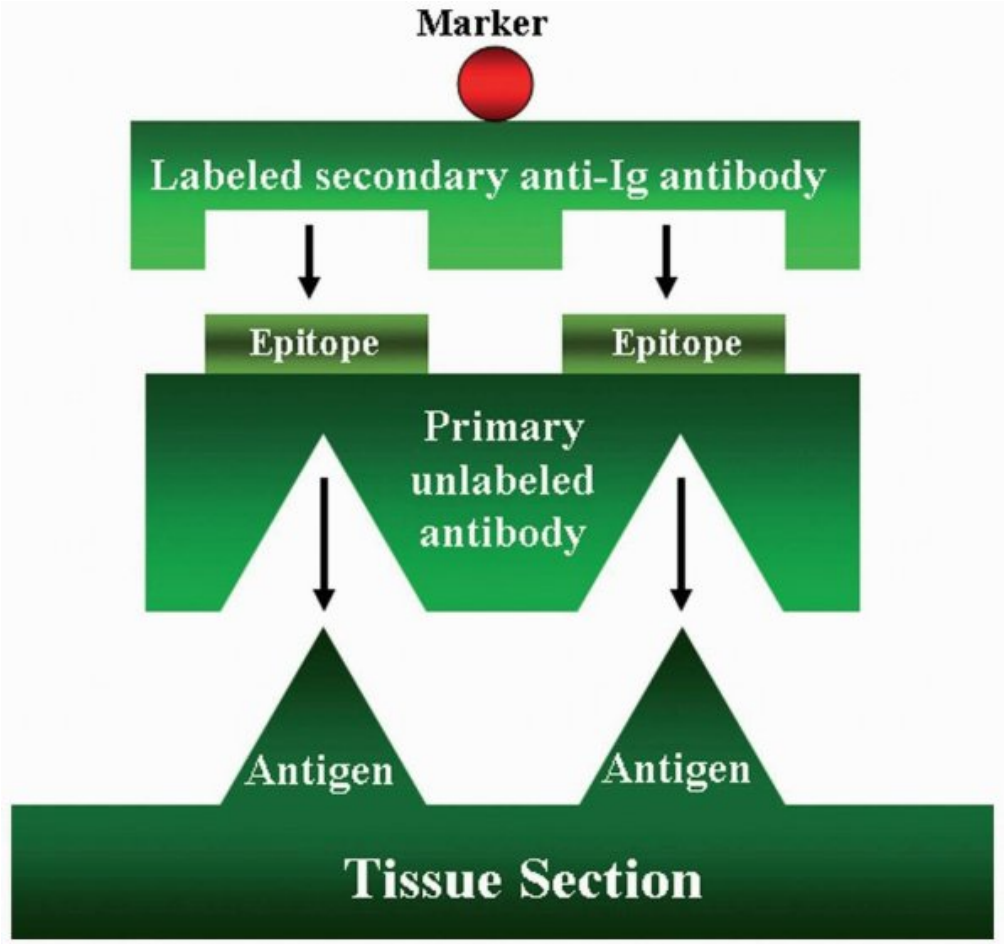


FIGURE 3.2 Indirect labeled method has two layers of antibodies. The primary unlabeled antibody is specific for a particular tissue antigen. The secondary labeled antibody is an anti-immunoglobulin antibody. The marker then identifies the sites of the tissue antigen.

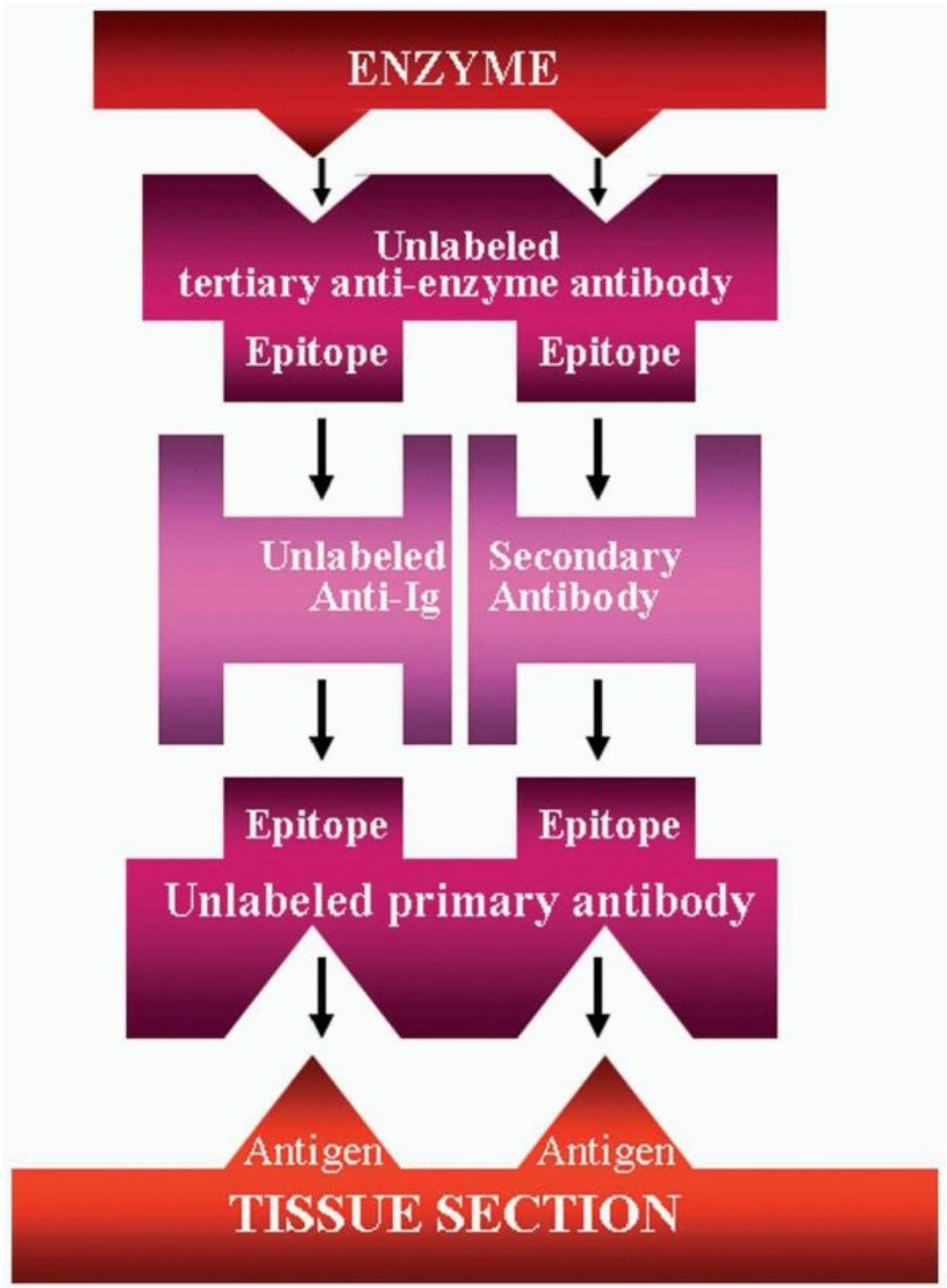


FIGURE 3.3 Unlabeled antibody method has three layers of antibodies. The primary unlabeled antibody is specific for a particular tissue antigen. The secondary unlabeled antibody is an anti-immunoglobulin that reacts to both the primary antibody and to the tertiary antibody. The tertiary antibody is an anti-enzyme antibody, which reacts to a particular enzyme. The enzyme is demonstrated by its reaction with the substrate.

Antigen Retrieval and Amplification Techniques

The antigen of interest in tissue sections can be altered after formalin fixation so that it may not react adequately to immunohistochemical staining. The original thinking was that formaldehyde fixation causes an irreversible reduction or even total loss of some antigenic determinants in paraffin sections (19). It is now known that formaldehyde induces alterations in only the tertiary and quaternary structures of protein, but not the primary and secondary structures, so that the epitopes of interest remain intact after formalin fixation (20). The function of the AR technique is, therefore, to restore the tertiary structure of the epitope, making it more accessible to specific antibodies.

The most common AR technique is heating tissue sections in water. The most popular procedure is the microwave heating method (12).

Briefly, deparaffinized slides are placed in plastic Coplin jars containing AR solution and are heated in the microwave for 10 minutes. After heating, the slides are washed in phosphate-buffered saline for 5 minutes and are ready for immunohistochemical staining. The temperature may vary from 90°C to 120°C.

The AR solution can be distilled water or various buffered solutions. Besides the microwave, the Coplin jars can be placed in a water bath, pressure cooker, steamer, or autoclave and achieve similar results. The major influencing factors in this technique are the heating temperature, heating time, and pH value of the AR solution.

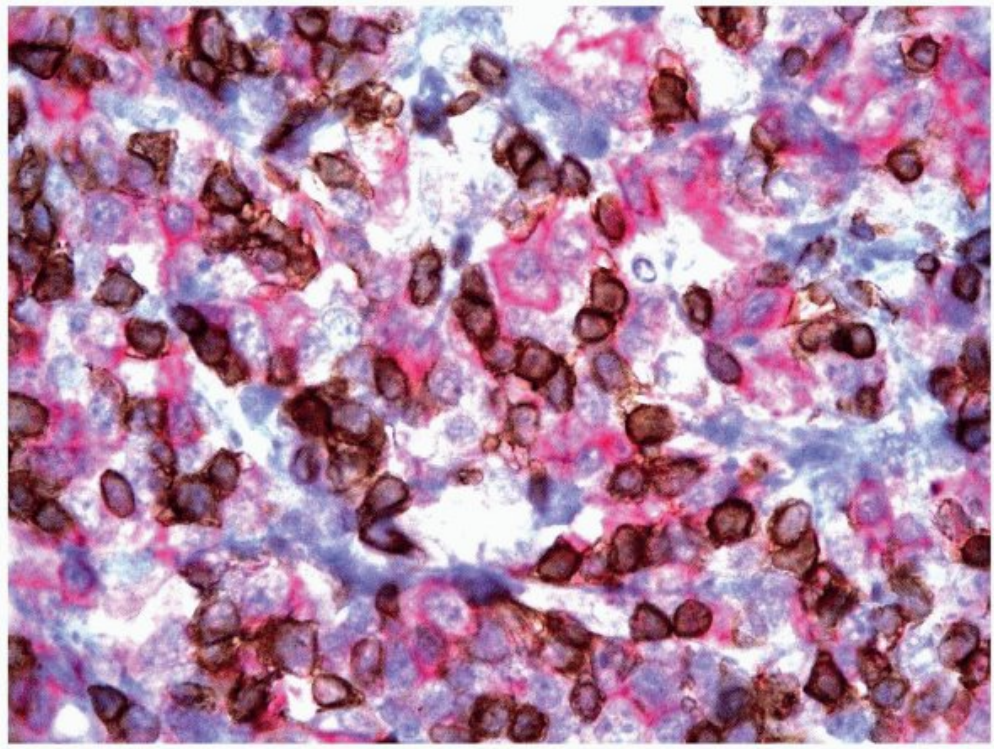


FIGURE 3.4 Lymph node biopsy from a case of T-cell-rich B-cell lymphoma dual-stained for CD3 (*brown*) and CD20 (*red*). The large tumor B cells are positive for CD20, whereas the reactive T cells are positive for CD3. Immunoperoxidase-immunoalkaline phosphatase stain, 60× magnification.

Proteolytic enzyme digestion also facilitates the increase of immunohistochemical staining sensitivity. For instance, digestion with 0.06% trypsin may restore the glucagon immunoreactivity of formalin-fixed rectal tumors (21), and pretreatment with neuraminidase enhances staining of myelin-associated glycoprotein (22).

The catalyzed reporter deposition (CARD) technique can also be used for signal amplification in immunohistochemistry. The deposition of the biotinylated tyramine (reporter) is through free radical formation, which is catalyzed by oxidizing horseradish peroxidase in the staining system (23). The radicalized biotinylated tyramine will be covalently bound to electron-rich protein residues (tryptophan, histidine, phenylalanine) near the antibody binding site, so that more biotinylated molecules are deposited and the signal is amplified.

Double Immunoenzymatic Techniques

Most of the immunohistochemical stains use only one staining system (e.g., immunoperoxidase). However, in some instances, double staining is desirable. For instance, concurrent demonstration of κ- and λ-positive cells and estimation of the κ/λ ratio is useful to determine the clonality of plasma cells. In this circumstance, two immunoenzymatic systems (e.g., immunoperoxidase and immunoalkaline phosphatase) (Fig. 3.4) or one immunoenzymatic system with two different substrates (e.g., immunoperoxidase with diaminobenzidine [DAB] and 4-chloro-1-naphthol [4-CN]) can be used. In fact, in the three immunoenzymatic systems, several different substrates are available to produce various chromogens. Therefore, multicolor staining can be potentially achieved. If necessary, immunogold stain can also be added to identify additional antigen in the same sections. The immunoenzymatic systems and immunogold staining and their color products are listed in Table 3.1 (5).

TABLE 3.1 The Immunoenzymatic Systems/Immunogold and Their Color Products (5)

Procedure	*Color*
Peroxidase	
Diaminobenzidine (DAB)	Brown
DAB with enhancement	Black
3-Amino-9-ethyl carbazole (AEC)	Red
4-Chloro-1-naphthol (4-CN)	Blue-black
Hanker-Yates reagent	Blue
α-naphthol pyronin	Red
3,3′,5,5′-tetramethylbenzidine (TMB)	Blue
Alkaline phosphatase	
Fast blue BB	Blue
Fast red TR	Red
New Fuchsin	Red
BCIP-nitroblue tetrazolium (NBT)	Blue
Glucose oxidase	
Tetrazolium	Blue
Tetranitroblue tetrazolium (TNBT)	Black
Immunogold	
Colloidal gold	Red
With silver enhancement	Black

Quality Control

Immunohistochemical staining is a multistep procedure involving tissue procurement, fixation, processing, sectioning, staining,

interpreting, and reporting. There are many pitfalls in diagnostic immunohistochemistry as summarized by several review articles (5,24,25). For each step, strict quality control should be observed (5). However, the major responsibilities for the pathologist remain to be correct interpretation and reporting. In this aspect, the positive and negative controls are essential. A positive control is a section that contains the antigen of interest and is stained the same way as the patient's specimen. A negative control is the same specimen stained

with the same procedure as the positive control without including the primary antibody. The primary antibody is usually replaced by the antibody diluent (buffer plus bovine serum albumin carrier protein) or by nonimmune immunoglobulin from the same species of the primary antibody.

False-negative results are obtained when the test tissue is negative but the positive control shows appropriate staining. Under these circumstances, the problem is usually prestaining-related, such as improper tissue fixation, processing, pretreatment, or a combination of several factors. When a manual staining procedure is used, the omission of one of the components in the staining may also occur.

False-positive results are obtained when the test tissue and the negative control show positive staining. This is called nonspecific background staining. There are many causes of nonspecific staining, but the most common one is nonspecific ionic binding of antibodies to charged connective tissue elements (5). Sometimes, undissolved precipitates of chromogen or counterstain may also be mistaken as a positive reaction (5).

Selective Use of Monoclonal Antibodies for Staining

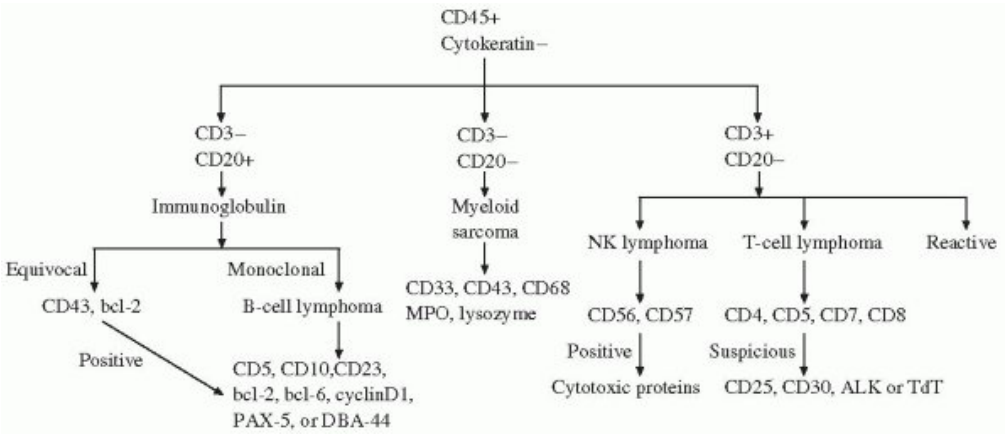
As mentioned before, many antigenic epitopes may become inaccessible after fixation and embedding. Therefore, many of the monoclonal antibodies used for fresh tissue are not applicable for routinely processed tissue sections. Besides using the AR techniques to restore the immunologic reaction, many new antibodies that react to the hidden epitopes are being produced. The most exciting additions are many gene products discovered by recent gene expression studies (26). The increasing numbers of new antibodies for tissue sections greatly enlarge the scope of immunohistochemistry, but some of these antibodies are not yet available for flow cytometry. The currently available monoclonal antibodies are listed in Table 3.2.

Depending on the experience of the pathologist and the difficulty of the diagnosis, variable numbers of antibodies can be selected in individual cases (25, 26 and 27), thus large screening or standard panels for all cell lineages are not necessary and wasteful. There are basically two approaches for antibody selection (24). The first approach is the algorithmic approach by multistep screening. The decision in every step depends on the results of the antibody reactions in the preceding step. The general practice is to work on several steps at a time and to interpret the results according to the algorithmic sequence. The second approach is a panel approach on the basis of a preliminary diagnosis either clinically or morphologically. The antibody panel is construed not only for the targeted diagnosis but also for the possible differential diagnoses. The second approach is more suitable for experienced pathologists.

The following are some suggestions for the algorithmic approach (Algorithm 3.1). In a poorly differentiated tumor, CD45 (LCA) and pancytokeratin are the proper antibodies for screening. Occasionally, S100 should be included to rule out nonmelanotic melanoma. If pancytokeratin is positive, the pathologist will select antibodies to distinguish various types of epithelial tumors. When CD45 is positive, the tumor is considered to be possibly a hematologic neoplasm, and CD3 (Fig. 3.5) and CD20 (Fig. 3.6) should be ordered to identify the T-cell and B-cell lineage. The lymphoid cells being predominantly positive for CD3 usually represents reactive T lymphocytes, unless those positive cells are morphologically neoplastic.

However, most of the lymphomas are of B-cell origin; therefore, if CD20 staining is not confined to the follicular area of the lymph node or the mucosa-associated lymphoid tissue, lymphoma should be considered. Immunoglobulin staining will identify the clonality of the B cells, and the

presence of a monoclonal B-cell population in most occasions represents a B-cell lymphoma. Unfortunately, monoclonal surface immunoglobulin pattern is mainly demonstrated in plasma cell tumors or B cells with abundant cytoplasm, such as immunoblasts. Many lymphomas do not show a definitive immunoglobulin staining. In those cases, bcl-2 and CD43 staining can be helpful. CD43 is a T-cell marker, but its coexpression with CD20 is highly suggestive of a B-cell lymphoma (except in the Payer patch) (Fig. 3.7) (26,28). Bcl-2 is specific for follicular lymphoma, but its expression in a B-cell population is also seen in other B-cell tumors. When a B-cell lymphoma is suspected, additional specific markers such as CD10, CD5, CD23, bcl-6, cyclin D1, PAX5, and DBA-44 can be used for subclassification.



ALGORITHM 3.1 Algorithmic approach for Immunodiagnosis. MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase.

TABLE 3.2 Monoclonal Antibodies Used in Immunohistochemistry

CD/Antigen	*Cell Specificity*	*Clinical Application*
ALK	ALCL cell	ALCL
Bcl-2	B cell	B lymphoma
Bcl-6	B cell	B lymphoma
CD1a	Thymocyte, Langerhans cell	Precursor T-cell lymphoma/leukemia
CD3	T cell	T lymphoma/leukemia
CD4	T-helper cell	T lymphoma/leukemia
CD5	T cell	T lymphoma/leukemia
CD8	T-suppressor cell	T lymphoma/leukemia
CD10	Immature B cell	ALL, lymphoma of follicular center cell origin
CD15	Reed-Sternberg and myeloid cells	Hodgkin lymphoma
CD20	B cell	B lymphoma

CD21	FDC	FDC tumor and follicle identification
CD23	B cell, FDC	B lymphoma
CD30	Reed-Sternberg and activated T/B cells	Hodgkin lymphoma
CD34	Hematopoietic stem cell	Acute lymphoid/myeloid leukemia
CD42b	Platelet/megakaryocyte	Acute megakaryoblastic leukemia
CD43	T cell, B cell subset	T/B-cell lymphoma, myeloid sarcoma
CD45	All leukocytes	Lymphomas, leukemias
CD45RA	T cell, B cell subset	T/B-cell lymphoma
CD45RO	T cell, B cell subset	T/B-cell lymphoma
CD56	NK cell	NK/T-cell lymphoma/leukemia
CD57	NK cell	NK/T-cell lymphoma/leukemia
CD61	Platelet/megakaryocyte	Acute megakaryoblastic leukemia
CD68	Monocyte/histiocyte	Monocyte/histiocyte tumors
CD79a	B cell	B lymphoma
CD79b	B cell	B lymphoma
CD117	Hematopoietic stem cell	Acute myeloid leukemia
Cyclin D1	B cell	Mantle cell lymphoma
DBA-44	B cell	Hairy cell leukemia
Ki-67	Proliferation fraction	High-grade lymphoma
TdT	Precursor T/B cells	Precursor T/B-cell

		lymphoma/leukemia
TRAcp	Lymphoid cells	Hairy cell leukemia

ALK, anaplastic lymphoma kinase; ALCL, anaplastic large cell lymphoma; FDC, follicular dendritic cell; NK, natural killer; CD, cluster designation; TdT, terminal deoxynucleotidyl transferase; TRAcp, tartrate-resistant acid phosphatase.

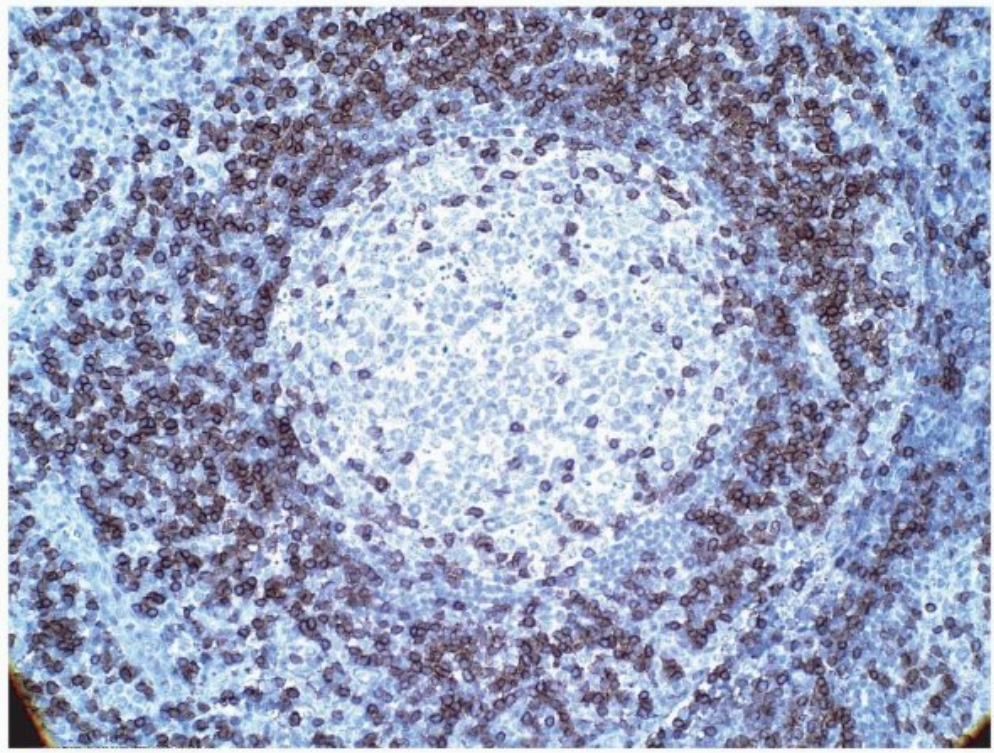


FIGURE 3.5 Normal lymph node biopsy stained for CD3, showing interfollicular staining with partial involvement of the mantle zone. The germinal center is spared. Immunoperoxidase, 20× magnification.

For T-cell tumors, CD4, CD5, CD7, CD8, CD25, CD30, terminal deoxynucleotidyl transferase (TdT), and anaplastic lymphoma kinase (ALK) can be used selectively, depending on the morphology. If natural killer (NK)/T-cell lymphoma is in the differential diagnosis, CD56 and CD57 should be included in the immunohistochemical panel.

An immunophenotype of CD45+, CD3-, CD20- is highly suggestive of myeloid sarcoma, and CD33, MPO, lysozyme, CD68, and CD43 should be ordered. Occasionally, this immunophenotype can be demonstrated in plasmacytoma, but this tumor usually shows weak or negative CD45 staining. For suspected AML in the bone marrow, CD34 and CD117 may help to identify the blasts, but these markers usually are not sensitive enough for a definitive diagnosis of leukemia.

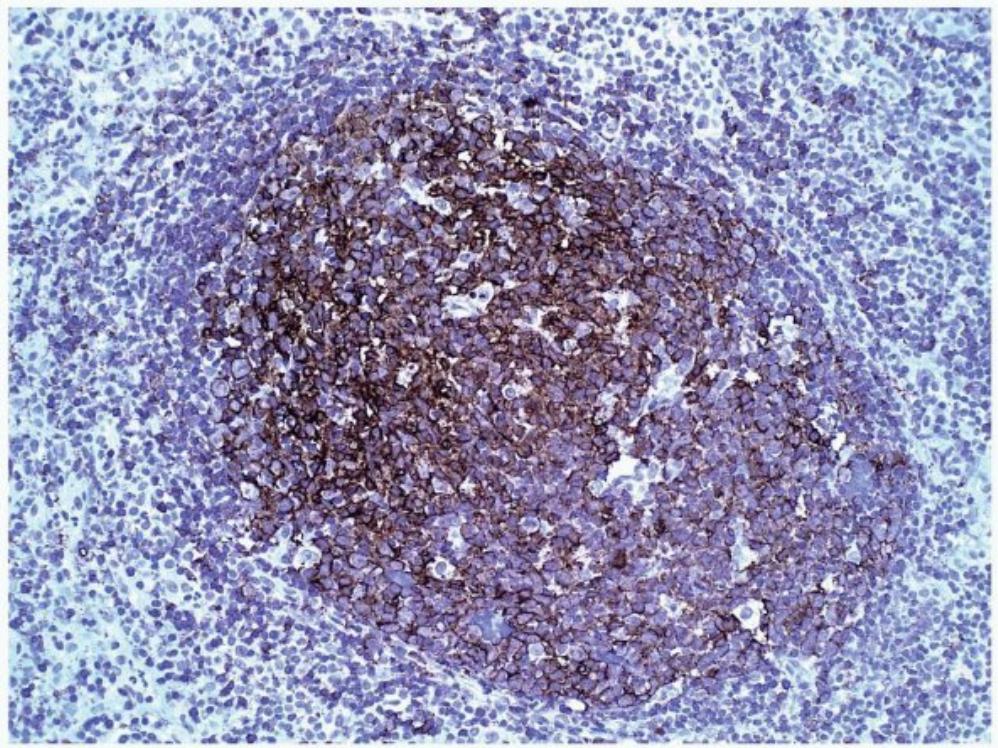


FIGURE 3.6 Normal lymph node biopsy stained for CD20, showing strong staining of the germinal center cells and partial involvement of the mantle zone. Immunoperoxidase, 20× magnification.

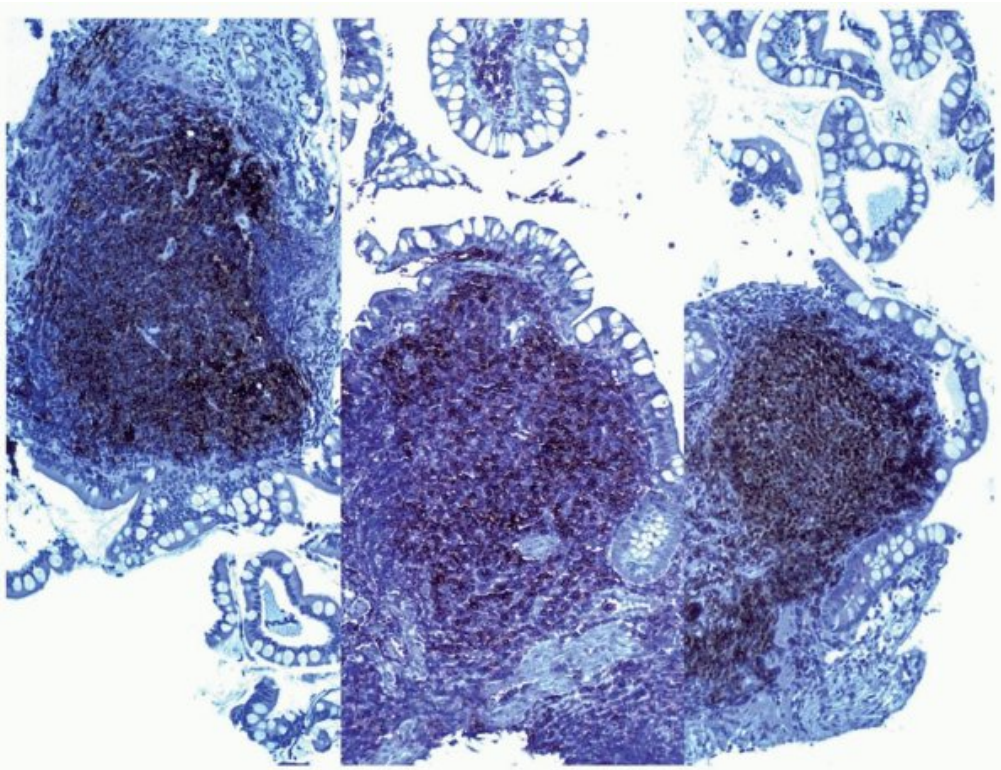


FIGURE 3.7 Biopsy of terminal ileum showing a Payer patch, which reacts to CD20 (**left**), bcl-2 (**center**), and CD43 (**right**). Immunoperoxidase, 10× magnification.

IN SITU HYBRIDIZATION

In situ hybridization is frequently incorporated with immunofluorescence techniques for the demonstration of chromosomal abnormalities, which is called fluorescence in situ hybridization (FISH) and is the most popular technique for cytogenetic studies. Isotopes can also be used for probe labeling to demonstrate different kinds of nucleic acids. In histologic examination, the most useful technique is NISH (16,29). This technique is mainly used to identify DNA and/or RNA of microorganisms, especially viruses. In hematopathology, it is helpful to demonstrate Epstein-Barr virus in various types of lymphomas (Fig. 3.8). The recent application of NISH to identify

immunoglobulin light-chain messenger RNA is particularly useful in cases where regular immunoglobulin staining is weak or equivocal (Fig. 3.9).

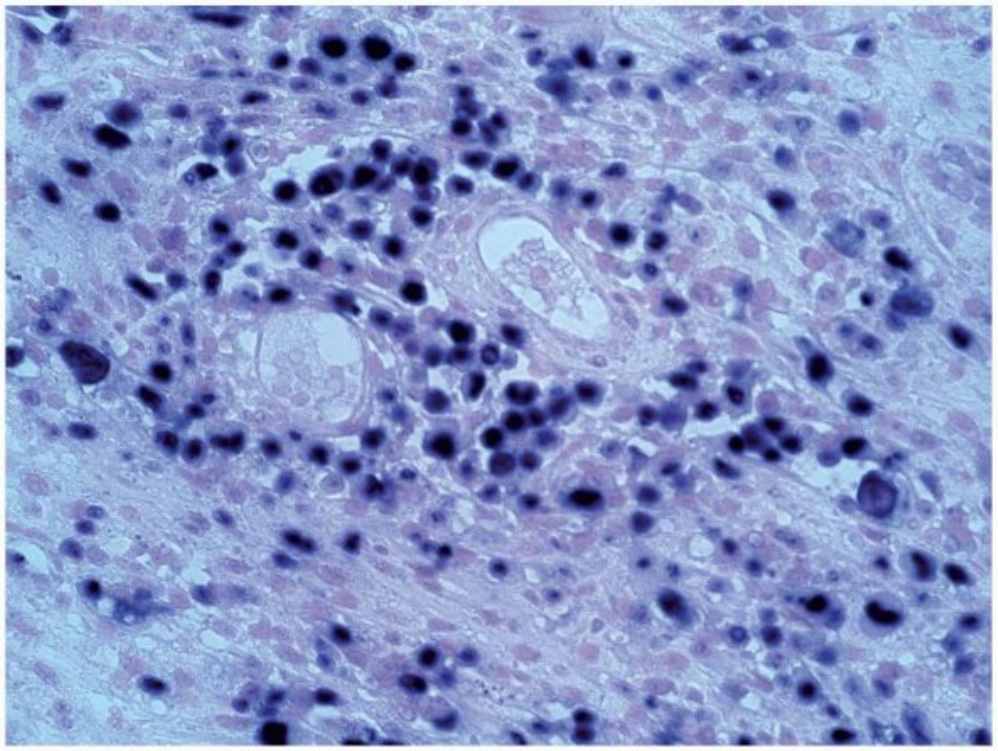


FIGURE 3.8 Brain biopsy of a case of posttransplant lymphoproliferative disorder showing Epstein-Barr virus staining. RNA in situ hybridization, 40× magnification.

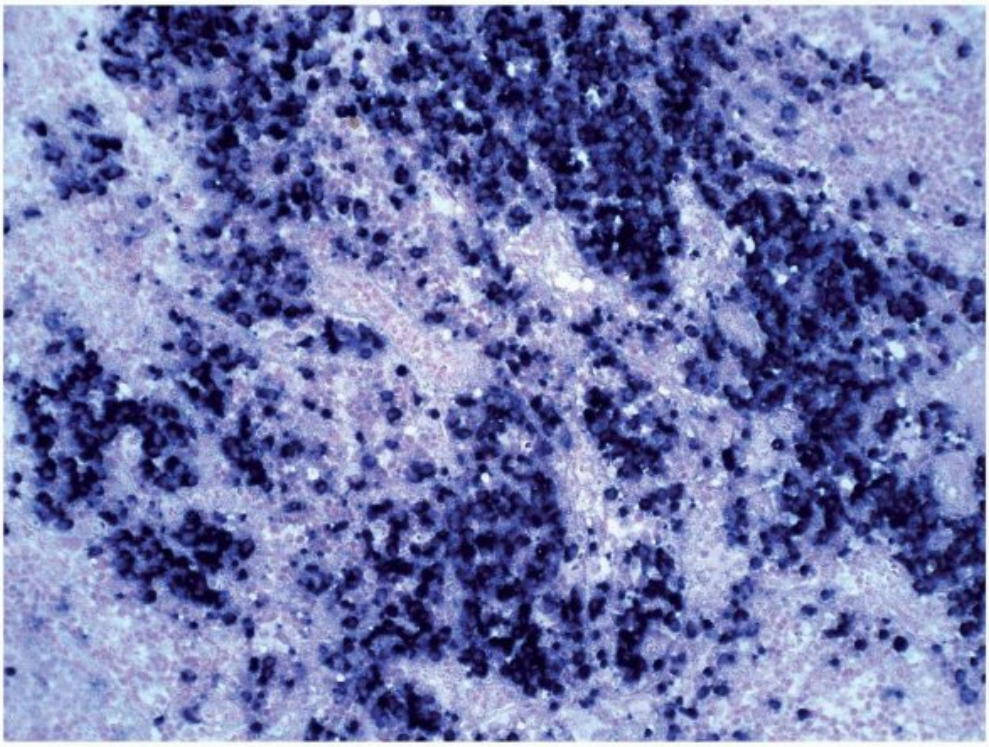


FIGURE 3.9 Lymph node biopsy of a case of lymphoplasmacytic lymphoma, showing diffuse strong κ light-chain staining. RNA in situ hybridization, 20× magnification.

The first step in nucleic acid hybridization is to break the double-stranded DNA into two single strands with high temperature, which is called denaturation. A DNA probe is then added to the preparation, and if it is complementary to the targeted DNA, the probe will hybridize with single-stranded DNA from the patient specimen to reform a double-stranded DNA. This hybridized product is then demonstrated by the probe label, such as biotin or enzymes. This procedure is applicable for both DNA and RNA. Because RNA cannot be cloned, the enzyme reverse transcriptase has to be used to convert RNA to a complementary copy of DNA (cDNA) to make probes for the detection of RNA viruses or immunoglobulin RNA.

There are several strategies for the improvement of in situ hybridization techniques. These strategies include target amplification (in situ polymerase chain reaction, primed labeling, self-sustained sequence replication), signal amplification (tyramide signal amplification, branched DNA amplification), and probe amplification (padlock probes and rolling circle amplification). The potential of this technology is highly promising.

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Chapter 4
Molecular Genetics

Although this book is devoted mainly to immunophenotyping of hematologic neoplasms by flow cytometry and immunohistochemistry, molecular genetics is incorporated in the discussion of all our cases, as it has played an increasingly important role in the diagnosis and prognostication of lymphomas and leukemias (1, 2, 3, 4 and 5). The role of molecular genetics, however, may differ from one entity to another and can be divided into three categories:

1. Mandatory for diagnosis: For the diagnosis of Burkitt lymphoma and chronic myelogenous leukemia (CML), C-MYC oncogene and breakpoint cluster region-Ableson (BCR-ABL), respectively, are the absolute diagnostic markers. There are no substitutes for these markers that are acceptable for a definitive diagnosis. Two subtypes of acute myeloid leukemia (AML) are also defined by cytogenetic karyotypes (6). The first one is t(8;21) (q22;q22) or AML1/eight-twenty-one (ETO), and the second one is inv (16) (p13q22) or t(16;16) (p13;q22) or core binding factor/smooth muscle myosin heavy chain (CBFB/MYH11). These genetic abnormalities are so characteristic that they supersede the blast count for the diagnosis of AML. Both karyotypes also confer a good prognosis.
2. Markers for treatment or prognosis: For the diagnosis of acute promyelocytic leukemia (AML-M3) a morphologic diagnosis is possible, but the identification of t(15;17)(q22;q12) or promyelocytic leukemia/retinoic acid receptor alpha (PML/RARα) is not only important for the confirmation of the diagnosis but also for the treatment. If the leukemic cells carry other variants, such as t(11;17), patients will not respond to all-*trans*retinoic acid treatment (6). For anaplastic large cell lymphoma, the anaplastic lymphoma kinase (ALK) gene is not necessarily present in each case, but the presence or absence of this gene will greatly influence the clinical course, the therapeutic strategy, and the prognosis (7).
3. Confirmation of diagnosis: For mantle cell lymphoma, there are many morphologic and immunologic criteria for the diagnosis, but the results of these tests are frequently equivocal, and the demonstration of t(11;14) (q13;q32) or B-cell lymphoma/leukemia /immunoglobulin H (BCL-1/IgH) is essential for a definitive diagnosis (8). Lymphoplasmacytic lymphoma is usually diagnosed by exclusion of many other lymphomas, but the detection of t(9;14) (p13;q32) or /immunoglobulin H (PAX5/IgH) will confirm the diagnosis in cases of nonsecretory lymphoplasmacytic lymphoma (cases without Waldenström macroglobulinemia) (9).

CYTOGENETICS

Cytogenetics plays multiple roles in relation to hematologic neoplasms (4,10,11). In clinical practice, the most useful occasion for cytogenetics is in determining the nature of a tumor in equivocal cases when immunophenotyping or immunogenotyping demonstrated monoclonality of the tumor and yet morphologic evidence of malignancy is lacking. In these cases, an abnormal karyotype casts a decisive vote for malignancy (10,12). Even when monoclonality is not identified, chromosomal aberration is a strong indication of malignancy or premalignancy (10,11).

Although most lymphomas and leukemias still have no diagnostic chromosomal pattern, karyotyping is the most reliable tool for a definitive diagnosis of many hematologic neoplasms. As mentioned before, it is particularly useful for the diagnosis of CML, Burkitt lymphoma, acute promyelocytic leukemia, mantle cell lymphoma, and anaplastic large cell lymphoma, just to name a few (Fig. 4.1).

Chromosomal aberrations are classified into numeric and structural abnormalities. Structural abnormalities include translocations, deletions, inversions, duplications, and isochromosomes. Among these, reciprocal translocation is most commonly found in hematologic neoplasms. This translocation involves the exchange of segments between two chromosomes; the total number of chromosomes is unchanged.

The numeric abnormalities are subdivided into polyploid and aneuploid. The term polyploid refers to multiplication of the normal haploid number of 23. Thus diploidy refers to 46 chromosomes, triploidy 69, and tetraploidy 92. Aneuploid, in contrast, refers to multiplication of chromosomes by irregular numbers, for instance, monosomy and trisomy. As the result of monosomy in a single chromosome, the total number of chromosome becomes 45. When

trisomy is present in a single chromosome, the number of chromosome becomes 47.

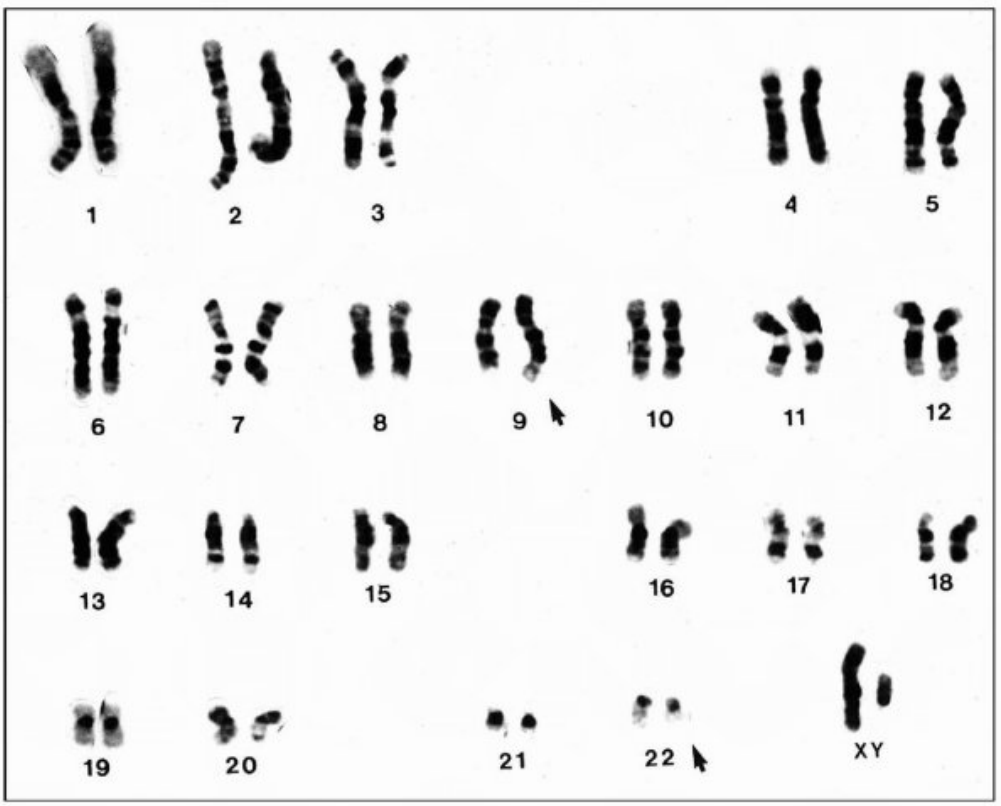

FIGURE 4.1 Karyotype of a case of chronic myelogenous leukemia, showing t(9;22) translocation (arrow), (Philadelphia chromosome). (Courtesy of Dr. P. Koduru, North Shore University Hospital, New York).

In addition, cytogenetics is a reliable predictor for prognosis. Generally speaking, neoplasms carrying a normal karyotype have a better prognosis than those with an abnormal one. There are, however, a few exceptions. For instance, cases of anaplastic large cell lymphoma that express t(2:5) have a more favorable prognosis than those without this karyotype (7). In childhood acute lymphoblastic leukemia (ALL), cases with hyperdiploidy carry a better prognosis than do diploidy cases (13). The prediction of prognosis by karyotyping is sometimes associated with the histologic pattern. For instance, a favorable prognosis with t(14;18) is due to its association with follicular lymphoma, whereas the poor prognosis predicted by t(8;14) is due to its association with Burkitt lymphoma (14). Some numeric chromosomal abnormalities, such as +5, +6, or +8, are related to shorter survival in patients with non-Hodgkin lymphoma (15). In AMLs and myelodysplastic syndromes, karyotyping is routinely performed even in entities without a specific karyotype, because the presence or absence of chromosomal aberration and the presence of a particular karyotype are important to predict the prognosis.

Cytogenetic studies are also useful in monitoring clinical course. When the patient has a relapse, karyotyping is able to determine whether the tumor is recurrent or secondary (16). Structural chromosomal abnormalities usually initiate malignant transformation, whereas numeric chromosomal abnormalities represent chromosomal evolution leading to the progression of disease (17). The appearance of new abnormalities in the karyotype, no matter whether the abnormality is structural or numerical, signals transformation of the tumor to a higher grade malignancy (10).

Finally, cytogenetic analysis is also able to detect residual minimal disease. The detection of residual tumor cells is frequently based on the use of molecular genetic techniques, such as polymerase chain reaction (PCR) or the fluorescence in situ hybridization (FISH) technique.

Fluorescence In Situ Hybridization

Conventional karyotyping requires fresh specimens, which are cultured to harvest cells in the metaphase for chromosome analysis. Therefore, it is time consuming and costly, and it frequently obtains unsatisfactory results because of the failure in cell growth or because the normal population overgrows the tumor cells. FISH overcomes all of these deficiencies of karyotyping (11,18), and can identify the cytogenetic abnormality in cells at the interphase. Therefore, it can be applied to dried smears of bone marrow, peripheral blood, body fluids, and paraffin sections. Because cell culture is not required, the failure rate is very low. However, FISH can also be applied to cultured cells in metaphase under special conditions. A large number of cells can be analyzed by FISH so that a percentage of positive cells can be calculated. Therefore, this analysis is particularly useful in therapeutic monitoring. FISH is more sensitive than

karyotyping as it can detect cryptic or masked translocations. For instance, 5% of patients with CML have a Philadelphia chromosome from a submicroscopic insertion translocation. Therefore, the aberration is not detectable by conventional karyotyping, but is readily detected by FISH (11).

FISH involves the binding of fluorochrome-labeled DNA probes to target DNA or RNA sequences inside fixed

cells. The basic principle is that a single-stranded DNA can bind to a complementary single-stranded DNA, and this hybridization process can be demonstrated with fluorochrome labels. Normal cells contain two alleles of each chromosome; therefore, two signals for a certain chromosome can be demonstrated by a specific DNA probe in normal cells. When one signal or three signals representing the same chromosome are demonstrated in individual cells, this represents numeric abnormalities (monosomy or trisomy) (Figs. 4.2 and 4.3). Chromosomal translocation can be demonstrated by showing two overlapped signals from two different genes (Fig. 4.4). Chromosomal inversion can be detected in the culture cells at the metaphase.

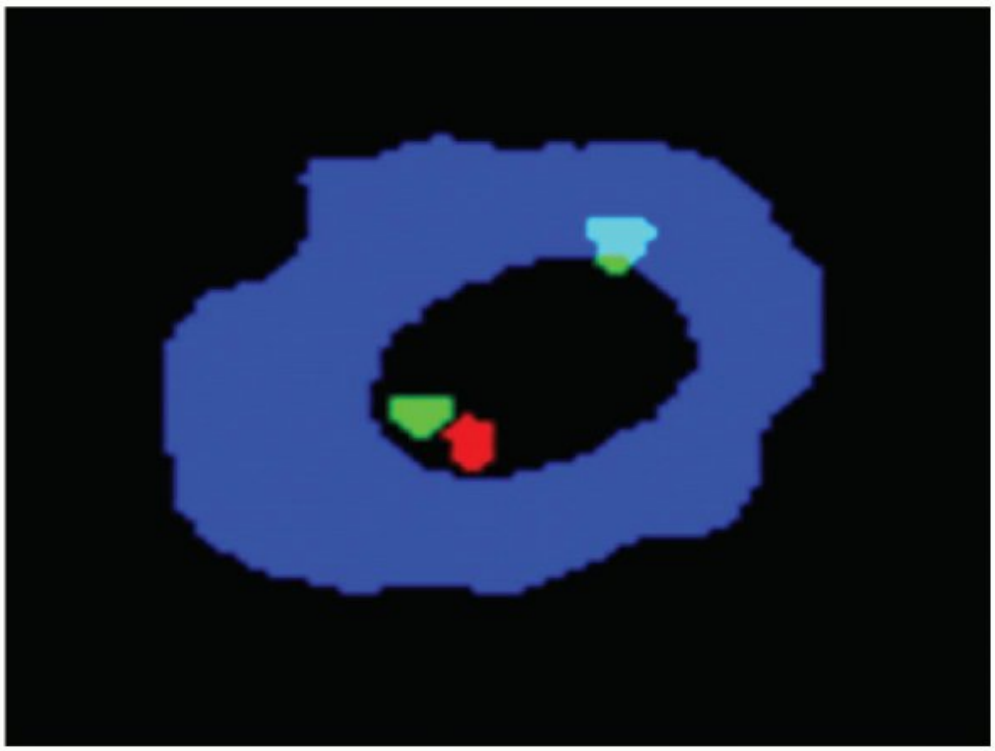

FIGURE 4.2 Fluorescence in situ hybridization technique demonstrates the deletion of one retinoblastoma (Rb) gene (*single red signal*).

Depending on the targeted abnormalities, a certain probe should be selected (11). For the detection of quantitative abnormalities, the centromere-specific probes should be used. For the detection of structural abnormalities, such as reciprocal translocations and inversions, locus-specific probes are most frequently used. Telomere-specific probes are used to detect subtle chromosome abnormalities that involve the ends of chromosomes. Whole chromosome-specific paints with sequence DNA probes are used to identify marker chromosomes and to detect cryptic translocations.

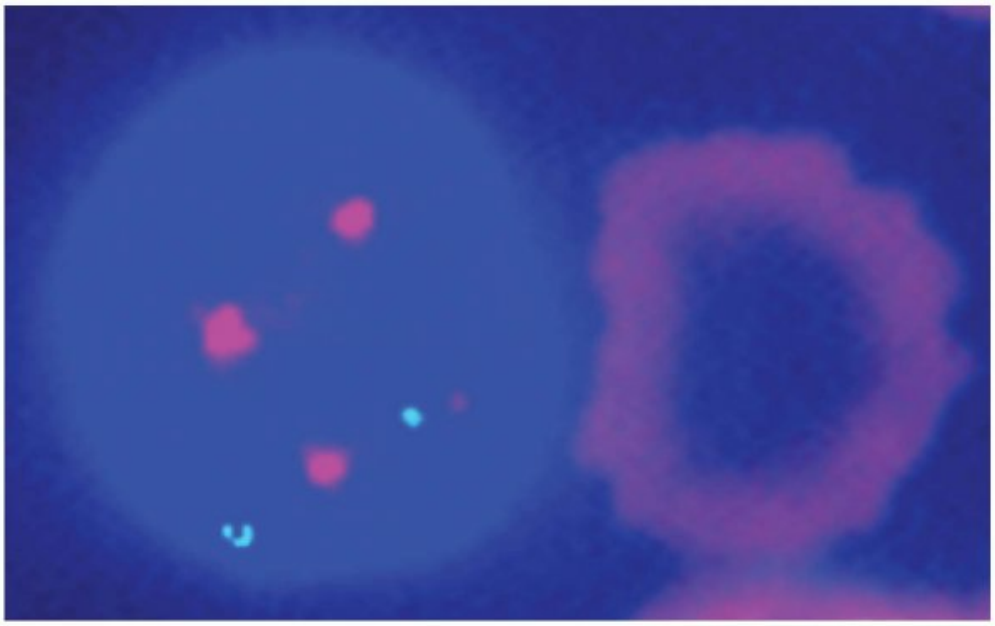

FIGURE 4.3 Fluorescence in situ hybridization technique demonstrates trisomy 12 in a case of chronic lymphocytic leukemia with prolymphocytoid transformation.

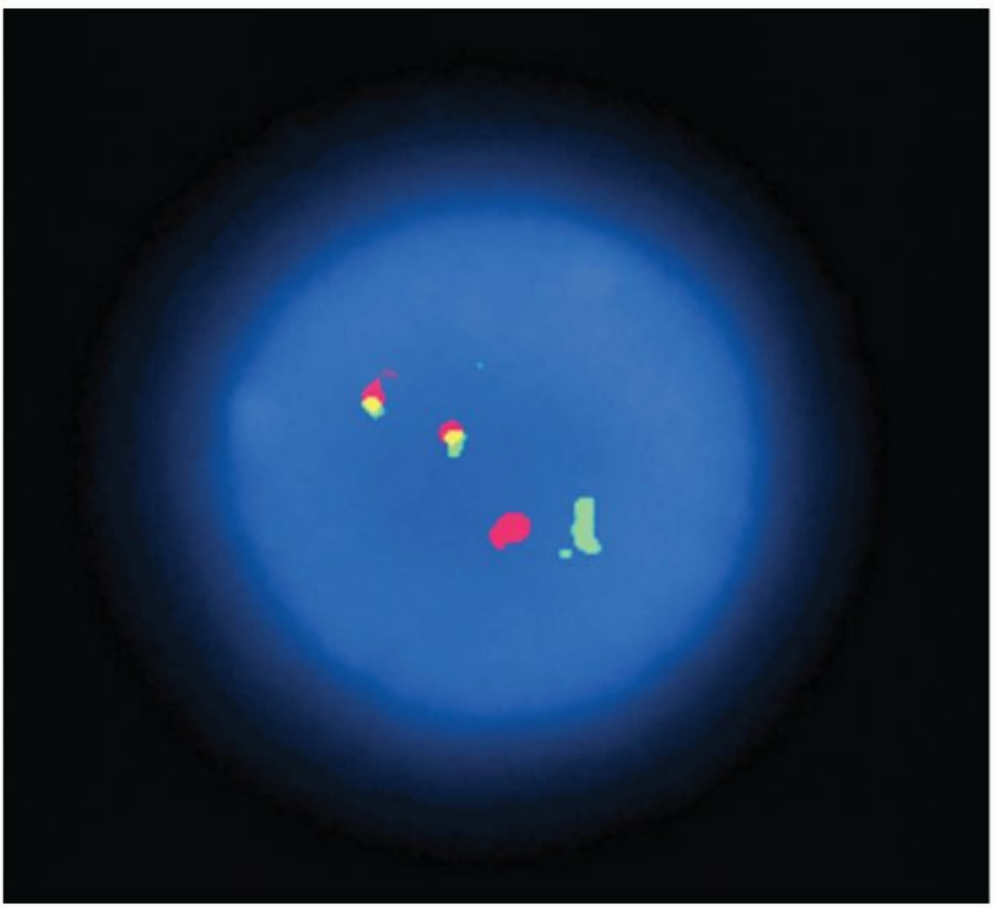

FIGURE 4.4 Fluorescence in situ hybridization technique demonstrates double fusion signals (*yellow*), with one *green* and one *red* signal, representing bcl-1/immunoglobulin H (IgH)

translocation. (From Mary Lowery Nordberg, Ph.D., LSU Health Science Center, Shreveport, LA, with permission.)

FISH probes can be used with different strategies, including single fusion, double fusion, break-apart, and extra-signal strategy. For technical details, the reader is referred to reference 11.

MOLECULAR BIOLOGY

FISH can be considered a hybrid of molecular biology and cytogenetics. In addition to FISH, the most commonly used molecular biology techniques in clinical laboratories are Southern blotting and PCR. The variants of PCR include reverse transcriptase PCR (RT-PCR) and quantitative PCR (real-time PCR). In hematology, molecular biology techniques are used to detect rearrangements of the antigen receptor genes (immunoglobulin and T-cell receptor [TCR]) and oncogenes, to identify clonalities of the suspected tumor cells and oncogene translocations. The most current techniques are the analysis of the variable region of immunoglobulin heavy-chain gene (V_H gene) to determine the developmental stage of a particular B-cell tumor, and gene expression profiling (GEP), which can stratify lymphomas and leukemias by using a large number of gene probes for the purpose of diagnosis and prediction of therapeutic response as well as prognosis.

Immunoglobulin and T-Cell Receptor Gene Rearrangement

Surface immunoglobulin and TCR are the definitive markers of B cells and T cells, respectively. Their production is

controlled by a series of genes (19, 20 and 21). The variable region of the heavy chain of an immunoglobulin molecule is encoded for by three genes, V (variable), D (diversity), and J (joining). The constant region of either heavy chains or light chains is encoded by one gene, C (constant). The heavy-chain (H) gene has about 50 to 100 V_H segments, 25 to 30 D_H segments, and 6 J_H segments (Table 4.1). The κ light-chain gene has about 40 to 80 V_k segments and 1 J_k segment. The λ light chain has about 40 V_L segments and 6 J_L segments. The light-chain genes have no D segments. All of these segments are arranged in an orderly array on various chromosomes: The heavy-chain gene locus is on 14q32, the κ light-chain locus is on 2p12, and the λ light-chain locus is on 22q11. During the differentiation of the B cell, a series of gene rearrangements takes place (Fig. 4.5).

TABLE 4.1 Characteristics of Receptor Genes

	Heavy Chain	*κ Chain*	*λ Chain*	*TCRa*	*TCRß*	*TCRγ*	*TCRδ*
Locus	14q32	2p12	22q11	14q11	7q34	7p15	14q11
Variable segment	50-100	40-80	40	50-100	75-100	8	4
Diversity segment	25-30	0	0	0	2	0	2
Joining segment	6	5	6	50-100	13	2	3
Constant segment	9	1	6	1	2	2	1

TCR, T-cell receptor.

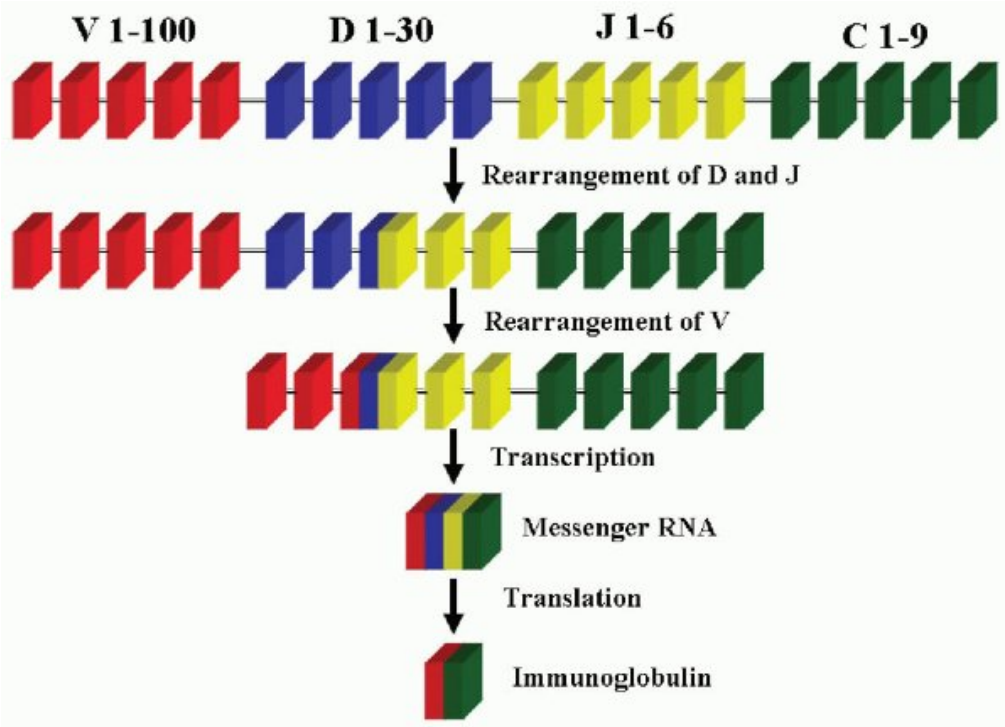

FIGURE 4.5 Immunoglobulin heavy-chain gene rearrangement. The germline heavy-chain gene is composed of four regions: V (variable), D (diversity), J (joining), and C (constant). Each region contains a certain number of segments. DNA rearrangement is the random selection of one segment from each gene from the germline to recombine into a new gene. Selected gene segments are transcribed into messenger RNAs, which are, in turn, translated into proteins (immunoglobulins).

The heavy-chain gene rearrangement takes place in the pre-B cell stage when the recombinases encoded by recombination activating gene (RAG1) and RAG2 genes mediate the random rearrangement of one each of the D and J gene segments. This is followed by the rearrangement of one of the V gene segments. After the completion of the VD-J rearrangement, the DNA is transcribed into messenger RNA (mRNA) with splicing of the rearranged segments next to the constant (C) region gene. This RNA transcript is then translated into heavy-chain immunoglobulin. The light-chain genes go through the same process and translate into light-chain immunoglobulins in a later stage. The light-chain and heavy-chain immunoglobulins will then combine and form an intact immunoglobulin molecule. The κ light-chain gene is usually rearranged first. If the κ gene rearrangement fails to produce a κ light-chain protein, the λ chain will then be rearranged (22,23).

The TCR is a heterodimer, consisting of two polypeptide chains. Most T cells carry the α-ß heterodimer on their cell membrane, whereas some bear the γ-δ receptor (12,24). The α- and δ-chain genes are located on the same locus of chromosome 14. The ß- and γ-chain genes are located on the long arm and short arm of chromosome 7, respectively. During normal T-cell development, the δ and γ chains are probably rearranged at the same time, followed by the ß-chain gene. The α chain gene is the last one to be rearranged.

Somatic Mutation of Immunoglobulin Heavy-Chain Gene

The B lymphocytes that have undergone immunoglobulin gene rearrangement differentiate into mature surface immunoglobulin positive naïve B cells. These naïve B cells circulate in the blood and go to the lymph node, entering the mantle zone and the primary lymphoid follicles (6). After exposure to the antigen, naïve B cells transform into centroblasts and proliferate, forming the germinal center. In the germinal center, somatic mutations occur in the immunoglobulin variable region (V_H) gene. Centroblasts then mature into centrocytes. Centrocytes that have high affinity to the antigen trapped on the processes of follicular dendritic cells survive, but those with low affinity die by

apoptosis. B cells derived from the germinal centers continue to undergo mutation or hypermutation. Somatic mutation of the V_H gene is the hallmark of B cells or tumor cells derived from the germinal center. B cells that do not undergo V_H mutation are called pregerminal center B cells. B cells that undergo V_H mutation but do not have continued mutation show no intraclonal diversity. These cells are called postgerminal center B cells. V_H gene mutation status is determined by DNA sequencing; mutation is defined by <97% homology to germline.

Southern Blotting

Southern blotting is one of the first nucleic acid probe assays used in clinical molecular laboratories. It is so named because Dr. Southern was the first to use this technique to transfer DNA from the gel onto a filter (25). Subsequently, the same procedure was used to transfer RNA and protein from the gel onto a filter; the new procedures were designated Northern blotting and Western blotting, respectively.

DNA is first isolated from a clinical specimen by phenol-chloroform extraction and ethanol precipitation (25). The DNA extract is then digested by restriction endonucleases, such as *Bam*HI, *Eco*RI, and *Hind*III. The resultant restriction fragments are size fractionated by electrophoresis in 0.8% agarose gels. The DNA in the gels is treated with alkali to become single stranded, transferred to a nitrocellulose or nylon membrane, and immobilized by heating. Bands containing DNA of the gene fragment of interest are detected by a DNA probe, usually labeled with a radioisotope, and visualized by autoradiography. Normal lymphocytes show only germline bands, but clonal lymphocytes reveal one or more bands not identical to the germlines, which are called rearranged bands or restriction fragment length polymorphism (RFLP) (Fig. 4.6).

It is important to select the appropriate probes for this procedure (25). The C (constant region) probes were first cloned and used as the probes for heavy-chain gene, light-chain gene, and TCR genes. However, the J segment is involved in most rearrangement and is limited in number; therefore, the use of J probes may have a higher positive yield, and these probes have gradually replaced the C probes for all gene rearrangement studies. The higher detection rate of a J probe than a C probe is partly due to the fact that C_μ is deleted after heavy-chain switch, but J_H remains (26).

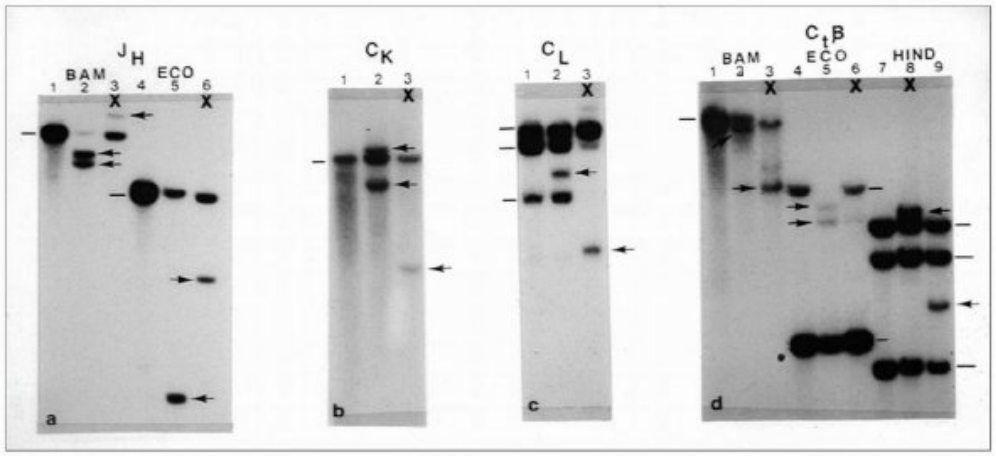

FIGURE 4.6 Southern blotting hybridization analysis of the immunoglobulin heavy-chain gene (J_H), light-chain genes (C_K and C_L), and T-cell receptor ß-chain gene (C_tß) in a case of B-cell acute lymphoblastic leukemia. (From Sun T, et al. Comparison of phenotyping and genotyping of lymphoid neoplasms. *J Clin Lab Anal*. 1989;3:156-162.)

For TCR gene analysis, the TCRα-chain gene is composed of an enormous J region; therefore, it is impractical to test the TCRα-chain gene routinely by using either a J probe or C probe. In contrast, the TCRγ-chain gene is difficult to analyze because of its limited number of V segments. As a result, <10 different restriction fragments are generated after the digestion by restriction endonucleases. Therefore, polyclonal T-cell population may produce a visibly rearranged band and will be misinterpreted as a monoclonal band. In addition, this band may sometimes obscure the neoplastic clone.

Polymerase Chain Reaction

The Southern blotting technique is time consuming, and sometimes the size of clinical specimens is so minute that it is not sufficient for this sensitive technique. The PCR is an in vitro technique for enzymatic amplification of a DNA segment of interest that can provide sufficient material for Southern blotting. Most frequently, the amplified product is simply subjected to electrophoresis, and the electrophoretogram is stained with a DNA dye, propidium iodide, to demonstrate the rearrangement or germline bands. Currently, capillary electrophoresis is used. In an automatic instrument, the amplified product is transferred to the capillary loop for electrophoresis. After this procedure, the electrophoretic pattern is scanned by the detector, and a electrophoretogram is generated for interpretation (Fig. 4.7). This technique is superior to gel electrophoresis in identifying the rearrangement bands.

The PCR procedure is composed of repetitive cycling of three simple reactions: DNA denaturation, primer annealing, and primer extension (Fig. 4.8). All reactions take place in the same test tube (well) at various temperatures. DNA denaturation is accomplished by a high temperature (90°C to 95°C), which breaks the hydrogen bonds of the double-stranded DNA and produces single-stranded DNA. Two single-stranded oligonucleotides (primers), synthesized to be complementary to known sequences of the target DNA, are added with polymerases and excess deoxyribonucleoside triphosphates (nucleotides), as the building block for new

DNA synthesis. At a lower temperature (45°C to 55°C), the two primers anneal to opposite ends of the two single-stranded DNA molecules derived from the first step. The polymerase then catalyzes the synthesis of a complementary second strand of the new DNA at 72°C, leading to the extension of each annealed primer. Two single-stranded DNA copies are produced in the first cycle, but DNA increases geometrically in subsequent cycles. After repeating the cycle 30 times, about 1 million copies of the target DNA segments are generated in 4 hours.

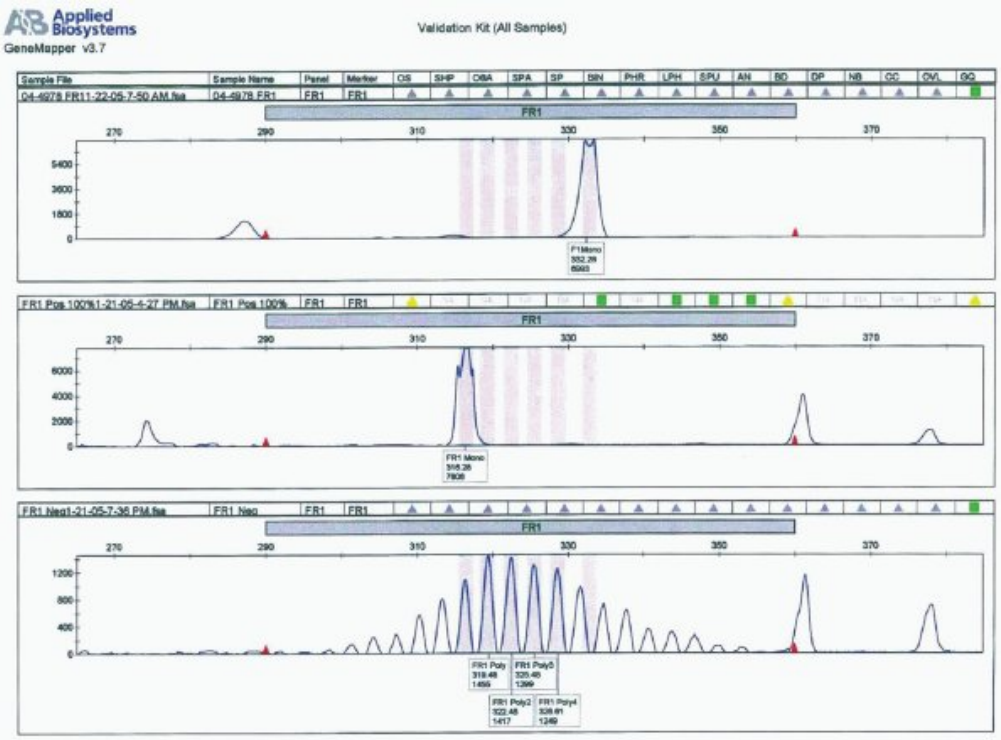

FIGURE 4.7 Polymerase chain reaction with capillary electrophoresis technique using three pairs of primers for the detection of immunoglobulin heavy-chain gene rearrangements. Frameworks I and II show a monoclonal peak, representing gene rearrangement. Framework III shows a polyclonal pattern.

PCR is most desirable for the diagnosis of lymphomas and leukemias with chromosomal translocation, such as CML (27) and follicular lymphoma (28). Because of its sensitivity, PCR is increasingly frequently used for detection of residual leukemia and lymphoma (29). RT-PCR is used to analyze RNA from tumor cells (26). The first step of this procedure is to copy the mRNA into complementary DNA (cDNA). This procedure is particularly useful for the study of gene expression (30).

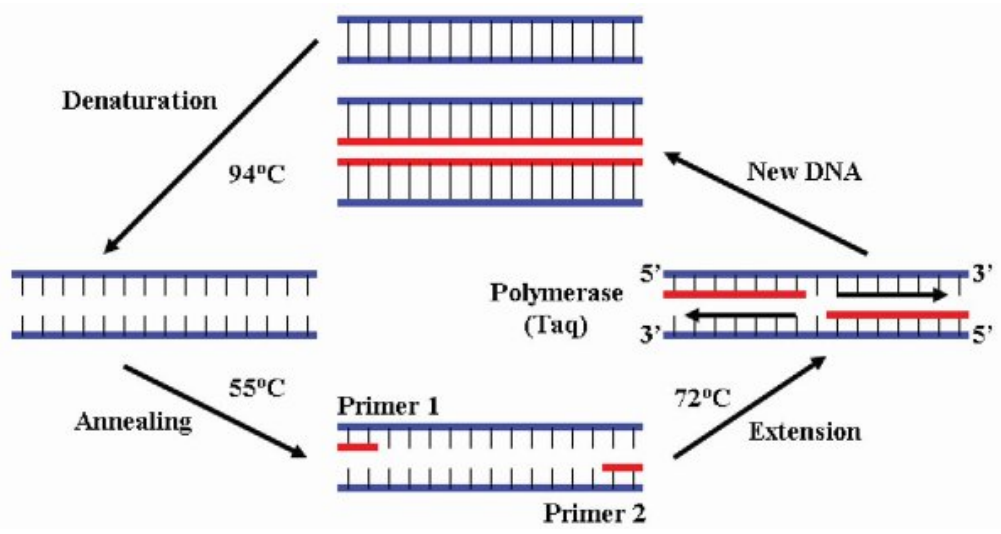

FIGURE 4.8 Scheme of polymerase chain reaction showing the stages of denaturation, annealing, and extension with the reaction of primers and polymerase under different temperatures.

Quantitative Polymerase Chain Reaction

Quantitative PCR is mainly used in the field of infectious diseases, but it is also helpful in the detection of minimal residual disease in different kinds of lymphomas and leukemias (31). The older methodology is competitive PCR, and the current method is real-time quantitative PCR based on the use of fluorogenic probes (32). The fluorogenic probe, also called TaqMan probe, consists of an oligonucleotide to which reporter dye and a quencher dye are attached (Fig. 4.9). When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, forward and reverse primers anneal to specific sequences of the target DNA at the 5′ and 3′ ends. The TaqMan probe anneals to specific sequences in between the forward and reverse primer sites. If hybridization occurs, the probe is cleaved by the 5′ nuclease activity of the DNA polymerase. As a result, the quencher dye and reporter dye are separated; the reporter dye then emits its characteristic fluorescence. The ABI Prism Sequence Detection System measures the increase in the reporter dye's fluorescence during the thermal cycling of the PCR so that a quantitative result is obtained.

Gene Rearrangement in Lymphoproliferative Disorders

Detection of one or more rearranged bands by Southern blotting or PCR techniques indicates a clonal proliferation

of lymphocytes so that each of them has the same sized fragments after digestion and these fragments migrate the same distance. The accumulation of DNA from many cells at the same site makes the rearranged band visible with the isotope label. A monoclonal lymphoid population is usually a neoplastic population.

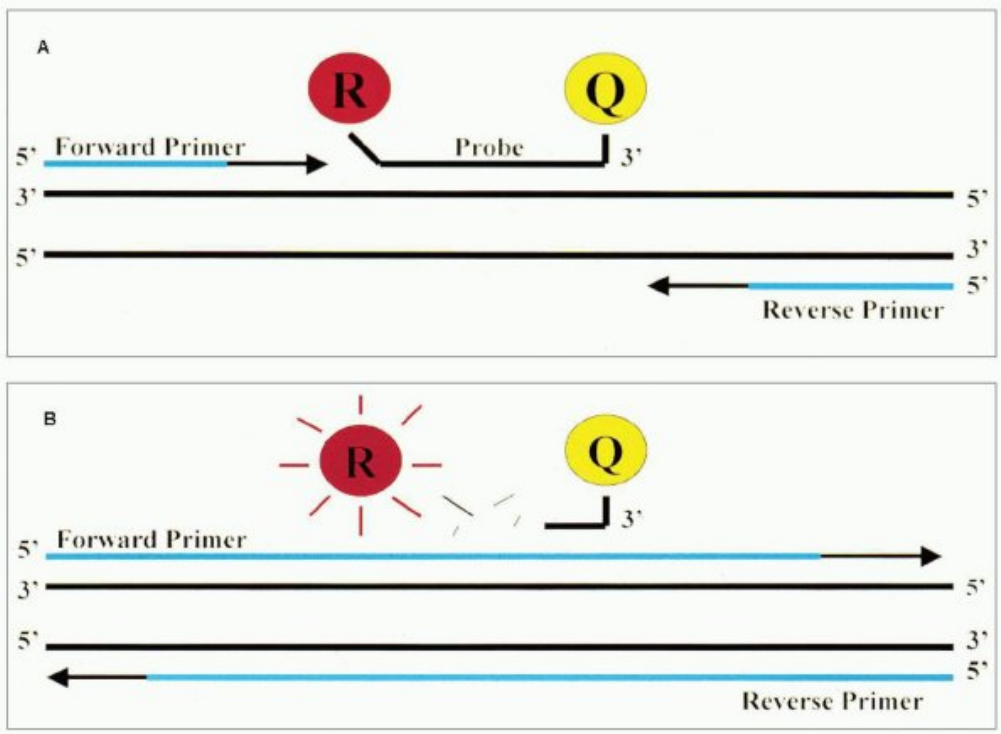

FIGURE 4.9 A: The reporter (R) and the quencher (Q) dyes are attached to the probe. When both dyes are attached to the probe, reporter dye emission is quenched. **B:** During the extension, DNA polymerase cleaves the reporter dye from the probe. When separated from the quencher, the reporter dye emits its characteristic fluorescence. The ABI Prism Sequence Detection System measures the increase in the reporter dye's fluorescence during the thermal cycling of the polymerase chain reaction.

However, there are certain “benign” conditions that show gene rearrangement consistently. Immunoglobulin gene rearrangement has

been found in benign lymphoepithelial lesions in Sjögren disease and TCR gene rearrangement in lymphomatoid papulosis (33,34). Gene rearrangement has also been demonstrated in acquired immunodeficiency syndrome (AIDS)-related lymphadenopathy, lymphadenopathyassociated rheumatoid arthritis, posttransplant lymphoproliferative disorders, pityriasis lichenoides et varioliformis acuta, and systemic Castleman disease (12,35). Nevertheless, most of these benign disorders may be premalignant conditions that are susceptible to transformation of neoplasms (22). Malignant transformation of Sjögren disease (33) and AIDS-associated lymphadenopathy (36) are two better known examples.

Another problem in DNA analysis is cross-lineage rearrangements. Rearrangement of TCR was reported in 25% of patients with non-T-cell ALL (37), whereas rearrangement of heavy-chain gene was seen in 10% of patients with T-cell leukemia (38). TCR and immunoglobulin gene rearrangement may also be demonstrated in about 50% of cases of myelogenous leukemia with terminal deoxynucleotidyl transferase (TdT) expression and in 10% without TdT expression (39). However, the rearranged bands in cross-lineage conditions are usually faint and represent partial (DJ segment) rearrangement.

The immunoglobulin light chain is lineage specific; therefore, the demonstration of light-chain rearrangement, regardless of the existence of TCR rearrangement, should be considered to be a B-cell clonal proliferation unless proven otherwise. Immunogenotyping is needed much more for the diagnosis of T-cell neoplasms than for that of B-cell tumors because there are no reliable immunophenotypic markers for the diagnosis of T-cell neoplasms. Unfortunately, the success rate of demonstrating TCR gene rearrangement in T-cell tumors is much lower than that of immunoglobulin gene rearrangement in B-cell malignancies (40,41).

In addition to the diagnosis of lymphoid tumors, gene rearrangement analysis can also identify the clonal origins of two or more coexistent tumors (e.g., composite lymphoma) (42,43) or the relationship of two consecutively appearing tumors (e.g., Richter syndrome) (44,45). Lymphoma cells can also be detected in the peripheral blood by genotyping despite the absence of morphologic evidence of hematogenous spread (46). Furthermore, the DNA hybridization technique can also detect chromosomal translocation by using a probe aiming at the breakpoint region such as t(9;22) in CML, t(8;14) in Burkitt lymphoma, and t(14;18) in follicular lymphoma.

Oncogenes

Among all the genomic alterations, chromosomal translocation draws most attention. Translocation is frequently a nonrandom change and thus is diagnostic. It also frequently involves oncogenes and antigen receptors in translocation, thus helping to elucidate the mechanism of tumorigenesis, which varies in different tumors (Table 4.2) (47). The mechanism of oncogene activation can be divided as follows:

1. *Fusion transcript*: The classic example is CML, but the same pattern is encountered in cases of ALL. In these cases, the cytogenetic abnormality is t(9;22)(q34;q11), or the so-called Philadelphia chromosome. The translocation results in the fusion of c-ABL, a proto-oncogene,

on chromosome 9q34 and a restriction region on chromosome 22q11 called the BCR, leading to transcription to an aberrant hybrid c-abl-bcr RNA. The bcr domain activates the tyrosine kinase activity of the c-abl protein (47). The abnormal activity of the tyrosine kinase may disturb the normal process of transduction in the cell and cause malignant transformation.

TABLE 4.2 Chromosomal Translocations in Lymphomas

Neoplasm	*Translocation*	*Genes Involved*
Anaplastic large cell	t(2;5)(p23;q35)	ALK;NPM
Burkitt	t(8;14)(q24:q32)	c-MYC;IgH
	t(2;8)(p12;q24)	Igκ;c-MYC
	t(8;22)(q24;q11)	c-MYC;Igλ
Burkitt-like	t(14;18)(q32;q21)	IgH;BCL-2
Cutaneous T cell	t(10;14)(q24;q32)	NFKδ2(LYT-10);IgH
Diffuse large B cell	t(3;14)(q27;q32)	BCL-6;IgH

	t(14;15)(q32;q11-13)	IgH;BCL-8
Follicular	t(14;18)(q32;q21)	IgH;BCL-2
Lymphoplasmacytic	t(9;14)(p13;q32)	PAX5 (BSAP);IgH
Mantle cell	t(11;14)(q13;q32)	BCL-1 (CCND1);IgH
Marginal zone/MALT	t(11;18)(q21;q21)	API2;MLT
	t(1;14)(p22;q32)	BCL-10;IgH
Plasma cell myeloma	t(4;14)(p16;q32)	FGFR3;IgH
	t(14;16)(q32;q23)	IgH;c-MAF
	t(16;22)(q23;q11)	c-MAF;Igλ
Small lymphocytic/CLL	t(14;19)(q32;q13)	IgH;BCL-3

CLL, chronic lymphocytic leukemia; MALT, mucosa-associated lymphoid tissue.

2. *Transcriptional deregulation*: The well-known example is Burkitt lymphoma, in which the c-MYC proto-oncogene is translocated from chromosome 8 to chromosome 14 and juxtaposed with the heavy-chain gene. As a result of the translocation, c-MYC submits to the control of the transcriptional enhancer of the immunoglobulin gene and is thus activated or deregulated. Constitutive MYC expression may prevent cells from entering the resting state (G_0 phase) and differentiating, leading to continuing proliferation of undifferentiated cells (48).
3. *BCL-2 overexpression*: Follicular lymphoma is characterized by the genomic alteration of t(14;18), in which the proto-oncogene BCL-2 (18q12) moves into the proximity of the immunoglobulin heavy-chain enhancer region (14q32). As a result, the proto-oncogene is activated (deregulated) and the functional bcl-2-Ig fusion protein is overexpressed. The BCL-2 gene encodes for an inner mitochondrial membrane protein that plays a role in blocking programmed cell death (apoptosis) (49). Therefore, cells with abnormal expression of this protein remain in the G_0 phase and become immortalized.
4. *PRAD1 overexpression*: In mantle cell lymphoma, the proto-oncogene BCL-1 (11q13) is juxtaposed to an immunoglobulin enhancer sequence located on chromosome 14. This translocation results in deregulation of the PRAD1 gene linked to the BCL-1 locus (50). PRAD1 encodes for cyclin D1, a cell-cycle protein. As a result of PRAD1 activation, the G1-S transition of the cell cycle is disturbed and the t(11;14)-carrying cells cannot exit from the cell cycle, leading to an expanded B-cell department (51).
5. *Activation by point mutation or gene amplification*: Mutation of the RAS genes has been found in some hematologic neoplasms, including AML, CML, ALL, and plasma cell myeloma (17). Amplification of the n-MYC gene has been demonstrated in neuroblastoma and the NEU gene in breast and ovarian carcinoma (47), but this mechanism of activation has not been detected in lymphoid neoplasms.
6. *Deletion or mutation of tumor suppressor genes*: Tumor suppressor genes, such as p53, have been reported to be involved in the tumorigenesis of Burkitt lymphoma and Richter transformation (52).

Gene Expression Profiling

Gene expression profiling (GEP) using DNA microarrays has great potential in the fields of diagnosis, prediction of prognosis, and guidance of treatment for hematologic neoplasms (53, 54 and 55). This technique is used to tether hundreds or thousands of gene-specific probes in arrays on a solid phase, such as glass. RNA is extracted from tissues of interest, labeled with a detectable marker (usually fluorochromes). The samples containing this mRNA are then hybridized with the gene-specific probes on the array. Images are generated by the use of confocal laser scanning, and the relative

fluorescence intensity of each gene-specific probe represents the level of expression of the particular gene.

After the expression data are collected, they are presented in a matrix in which each row represents a particular gene and each column represents a specific tissue sample. To facilitate data interpretation, elements of the data in a matrix are often rendered in color to indicate the level of expression of each gene in each sample. The colors used are based on the log ratio for each sample measured as compared with a control sample. For instance, log ratio values close to zero are represented by black, those with values >0 are represented by red (indicating upregulated genes), and those with negative values are represented by green (indicating downregulated genes). The original expression matrix generally shows no apparent pattern or order. Programs that perform clustering are required to arrange the rows and columns in order.

There are two methods for analysis of data. The supervised method depends on prior knowledge about the sample to search for genes that correlate with a disease state. The unsupervised method disregards prior knowledge of the nature of the specimens, is often used for screening, and divides the data into clusters with either the hierarchical clustering or k-mean cluster analysis.

After data analysis, gene expression signatures can be recognized. A gene expression signature is defined as a group of genes that is characteristically expressed in a particular group of cells belonging to a certain cell lineage, disease entity, or subtype of leukemia and/or lymphoma.

The usefulness of GEP is exemplified by the studies of diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), and Burkitt lymphoma. Approximately 40% of DLBCL patients can be cured, whereas the remaining patients die within a few years despite treatment. These two groups of patients can now be stratified with GEP. The former group shows the germinal center B cell-like (GCB) signature, whereas the latter group, the activated B cell-like (ABC) signature (56). Furthermore, the ABC group (but not the GCB group) reveals a high expression profile of nuclear factor-kappa B (NF-κB) target genes. A new compound, PS-341, may inhibit NF-κB, so that it is potentially a powerful therapeutic agent in combination with other chemotherapeutic regimens (57).

CLL patients can also be divided into two prognostic groups by GEP. The major difference between the two groups is the presence or absence of V_H gene somatic mutation. Cases with unmutated CLL cells represent a naïve B-cell origin, demonstrating a worse prognosis than those with mutations (58). For CLL patients with the V_H mutation subtype, a treatment with cytotoxic drugs should be postponed until the appearance of clinical progression (54).

The distinction between Burkitt lymphoma and DLBCL is sometimes difficult to determine by using morphologic and immunologic criteria, yet it is important to distinguish these two entities because their treatment and prognosis are quite different. Cytogenetic studies are helpful because Burkitt lymphoma has c-myc rearrangement, but DLBCL may have bcl-2 rearrangement. However, two recent studies with GEP showed that even cytogenetics is not entirely reliable: Burkitt lymphoma may not have c-myc or may have bcl-2 rearrangement, and DLBCL may have c-myc gene rearrangement (59,60). Therefore, GEP is the only reliable means to make such an important distinction.

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Chapter 5

Classification of Hematologic Neoplasms

There are two major groups of hematologic neoplasms: Lymphoma and leukemia. Lymphoma is lymphoid tumors initially confined to lymphoid organs or extranodal tissue, whereas leukemia includes lymphoid and myeloid neoplasms originating from the bone marrow and circulating in the peripheral blood. However, with the advent of new technology, especially immunophenotyping and molecular biology, lymphoma cells can be detected in blood and bone marrow even in the relatively early stage, and the demarcation between lymphoma and leukemia is sometimes blurred. Lymphoma and leukemia have their counterparts, such as lymphoblastic lymphoma versus acute lymphoblastic leukemia (ALL) and small lymphocytic lymphoma (SLL) versus chronic lymphocytic leukemia (CLL). The tumor cells of the counterparts may have the same morphology, immunophenotype, immunogenotype, karyotype, and clinical characteristics, such as the presence of a mediastinal mass in both lymphoblastic lymphoma and ALL of T-cell origin. It has recently been found that the difference

in location (tissue vs. blood) between lymphoma and leukemia may be associated with the presence or absence of adhesion molecules or lymphokine receptors on the tumor cells. For instance, the difference in phenotype between SLL and CLL is the presence of the adhesion molecule, CD11a/CD18 (LFA-1), on SLL cells (1). In contrast, CLL cells express the lymphokine receptors, CXCR4 and CCR7, which are not present on the SLL cells (2). The high frequency of the leukemic phase in Burkitt lymphoma is due to its lack of LFA-1 (3).

Leukemia can be further divided into acute and chronic groups. In acute leukemias, the clinical course is rapidly progressive and the leukemic cells are immature blasts. Chronic leukemias are just the opposite: The clinical course is slow and indolent, and the leukemic cells are mature appearing in lymphoid leukemia or intermediate forms (promyelocytes, myelocytes, and metamyelocytes) in myeloid leukemia. Although lymphoma is not divided into acute and chronic forms, lymphoma cell types can also be differentiated on the basis of maturation. For instance, T-cell lymphoma can be divided into thymic and postthymic (peripheral T cell) subtypes. The homogeneity of leukemia and lymphoma cells in terms of their maturation stage prompted the theory of maturation arrest as the mechanism of tumorigenesis (4). The proliferation of new stage-specific monoclonal antibodies will certainly help to pinpoint the hematologic neoplasms in highly defined stages.

The classification of hematologic neoplasms, therefore, depends on many parameters, including cell morphology, size, stage of maturation, and clinical course. With the recent advances in new technologies, the classification is also based on immunophenotypes, immunogenotypes, and karyotypes. However, the basic requirement for diagnosis is still cytologic recognition of the neoplastic cells. Therefore, it should be useful to be acquainted with the ontogeny of blood cells. This is particularly relevant to biphenotypic lymphoma and/or leukemia and to those tumors derived from a pluripotent stem cell or progenitor cells. Having this knowledge can also help us to understand why, for instance, chronic myelogenous leukemia may have a blast crisis of myeloid, lymphoid, erythroid, or megakaryocytoid origin and why erythroid leukemia has coexistent myeloblasts and trilineage dysplasia.

DEVELOPMENTAL STAGES OF HEMATOPOIETIC CELLS

During fetal life, hematopoiesis initially takes place in the yolk sac and occurs later in the liver and spleen. After birth, the hematopoietic function is taken over by the bone marrow. All the blood cells are derived from a pluripotent stem cell that can differentiate into various lineages, including erythrocytes, megakaryocytes, basophils, eosinophils, neutrophils, monocytes, and lymphocytes (Fig. 5.1). The precursors of these lineages are called colony-forming units. Precursors of T lymphocytes must go to the thymus, where they develop through several stages of thymocytes and finally become mature T lymphocytes. The mature T lymphocytes exit the thymus, enter into the blood and lymph, and reach the peripheral lymphoid tissues. B lymphocytes do not go through the thymus but mature in the bone marrow and enter the lymphoid tissue via blood and lymph. The third lineage of lymphoid cells was initially called null

cell, because it lacks surface immunoglobulin and does not form sheep erythrocyte rosettes. It is now recognized that most null cells are large granular lymphocytes that are able to lyse a variety of tumor cells and virus-infected cells, and are thus called natural killer (NK) cells (5). The ontogeny of NK cells is still not clear, but NK cells probably share the same stem cells with T lymphocytes.

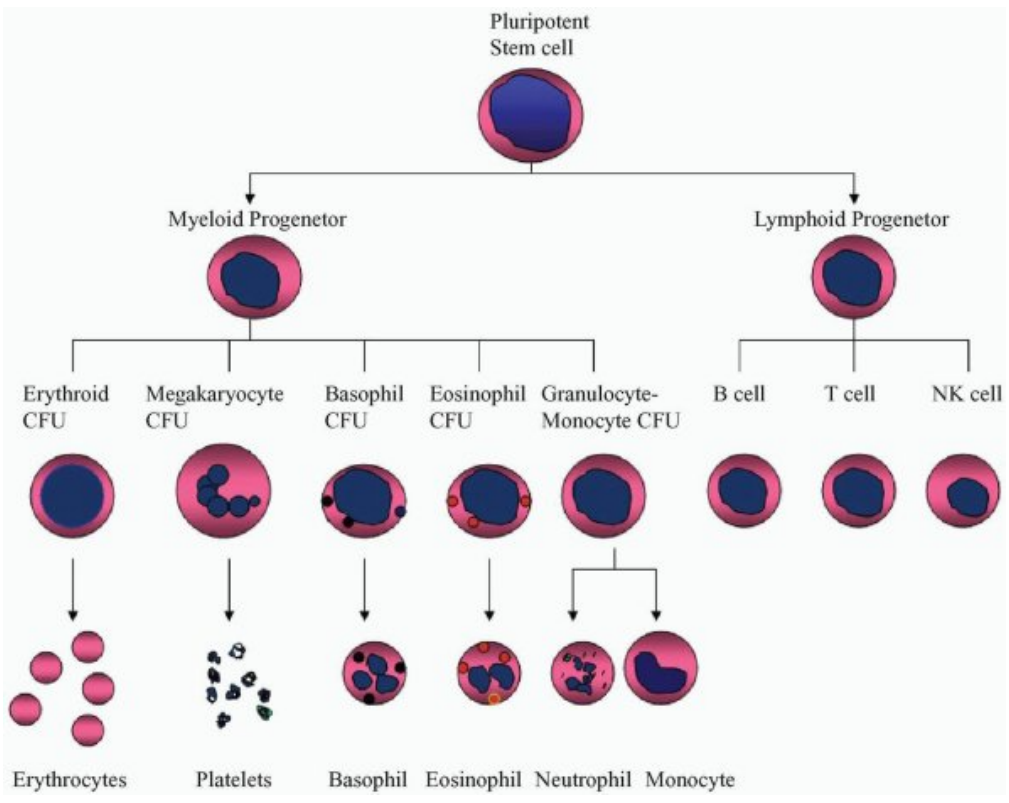

FIGURE 5.1 Development of hematopoietic cells (hematopoietic tree). CFU, colony-forming

unit.

The proliferation and maturation of various precursors are stimulated by cytokines called colony-stimulating factors (CSFs) (5). T lymphocytes produce interleukin-3 (IL-3), which simulates all precursors, and granulocyte-monocyte CSF (GM-CSF). Macrophages and marrow stromal cells also produce granulocyte-monocyte CSF and additional CSFs specific for granulocytes (G-CSF) or monocytes (M-CSF), as well as IL-1 and IL-6. The interleukins can enhance colony formation by hematopoietic precursors in the presence of CSFs. The bone marrow stromal cells can also produce IL-7, which preferentially stimulates the maturation of B lymphocytes.

The development of T lymphocytes starts in the thymus. The earliest stage of thymocyte is called *stage I thymocyte* or *prothymocyte,* which represents 13% of the entire thymocyte population (Table 5.1) (6,7). It expresses terminal deoxynucleotidyl transferase (TdT) in the nucleus; CD3 in the cytoplasm; and CD38, CD71, and the earliest T-cell surface marker, CD7, on the surface. Some studies also indicated the presence of surface HLA-DR, CD34, and possibly CD2 at this stage. *Stage II thymocytes,* also called *common thymocytes*, include subcapsular and cortical cells and represent approximately 75% of the total thymocyte population. In this stage, CD1, CD2, CD5, and CD7 are expressed. CD4 and CD8 are usually coexpressed, or only one of these two is expressed. *Stage III thymocytes,* also called *medullary* or *mature thymocytes,* consist of about 15% of the thymocyte population. The mature thymocytes express surface CD3 and T-cell receptor protein, and start to divide into CD4+, CD8-, and CD4-, CD8+ are two subgroups. When the mature thymocytes enter into the peripheral circulation, they become *postthymic* or *peripheral T cells*, which lose the CD38 and TdT markers.

The development of B cells is confined to the bone marrow. In the *B-cell progenitor stage,* only TdT, HLA-DR, CD34, and cytoplasmic CD79 are expressed (Table 5.2) (6,7). Additional antigens, CD10, CD19, and cytoplasmic

CD22, appear in the next stage, the *pre-pre-B-cell stage*. The *pre-B-cell stage* is characterized by the presence of cytoplasmic μ chain without accompanying light chain and the loss of CD34. CD20 first appears at this stage. In the *immature B-cell stage*, surface immunoglobulin light chains and CD21 start to appear. CD10 usually disappears at this stage, but it is frequently present on Burkitt lymphoma cells, which are considered immature B cells (8). When B cells become mature, immunoglobulin D (IgD) appears on the surface side-by-side with IgM. The *mature B cells* are considered resting or "virgin" until becoming activated by their contact with antigens. As heavy-chain switching occurs on B-cell activation, the activated B cells express surface IgM, IgA, or IgG instead of IgM/IgD. CD21 disappears at this stage, but activation antigens, such as CD38 and CD71, and proliferation-associated antigen, Ki-67, are frequently expressed. Activated B cells finally develop into the terminal stage, *plasma cell*. Plasma cells synthesize cytoplasmic immunoglobulin and express surface CD38, CD138, PCA-1, and PC-1. Some activated B cells may become *memory B cells*, which have an immunophenotype similar to that of either a resting B cell (7) or an activated B cell (9).

TABLE 5.1 Immunophenotype of Different Developmental Stages of T Lymphocytes

Stage I Prothymocyte	*Stage II Common Thymocyte*	*Stage III Mature Thymocyte*	*Postthymic T Lymphocyte*
TdT	TdT	TdT	TCR
cCD3	cCD3	cCD3	CD3
CD7	CD7	CD7	CD7
CD2 (±)	CD2	CD2	CD2
CD34	CD5	CD5	CD5
HLA-DR	CD1	CD4 or CD8	CD4 or CD8
CD38	CD4	CD3	
CD71	CD8	TCR	

	CD38	CD38

cCD3, cytoplasmic CD3; TCR, T-cell receptor (TCRα ß 95%, TCRγδ 5%); TdT, terminal deoxynucleotidyl transferase.

TABLE 5.2 Immunophenotypes of Different Developmental Stages of B Lymphocytes

Progenitor B cell: TdT, HLA-DR, CD34, cCD79

Pre-pre-B cell: TdT, HLA-DR, CD34, CD19, CD10, cCD22, cCD79

Pre-B cell: TdT, HLA-DR, CD19, CD10, CD20 CD79, cCD22, Cμ

Immature B cell: HLA-DR, CD19, CD20, CD79, CD21, cCD22, sIgM

Mature B cell: HLA-DR, CD19, CD20, CD21, CD22, CD79, sIgM/IgD

Activated B cell: HLA-DR, CD19, CD20, CD22, CD79, sIgM or IgG

Plasma cell: cytoplasmic immunoglobulins, CD38, CD138, PCA-1, PC-1

c, cytoplasmic; s, surface; TdT, terminal deoxynucleotidyl transferase; Ig, immunoglobulin.

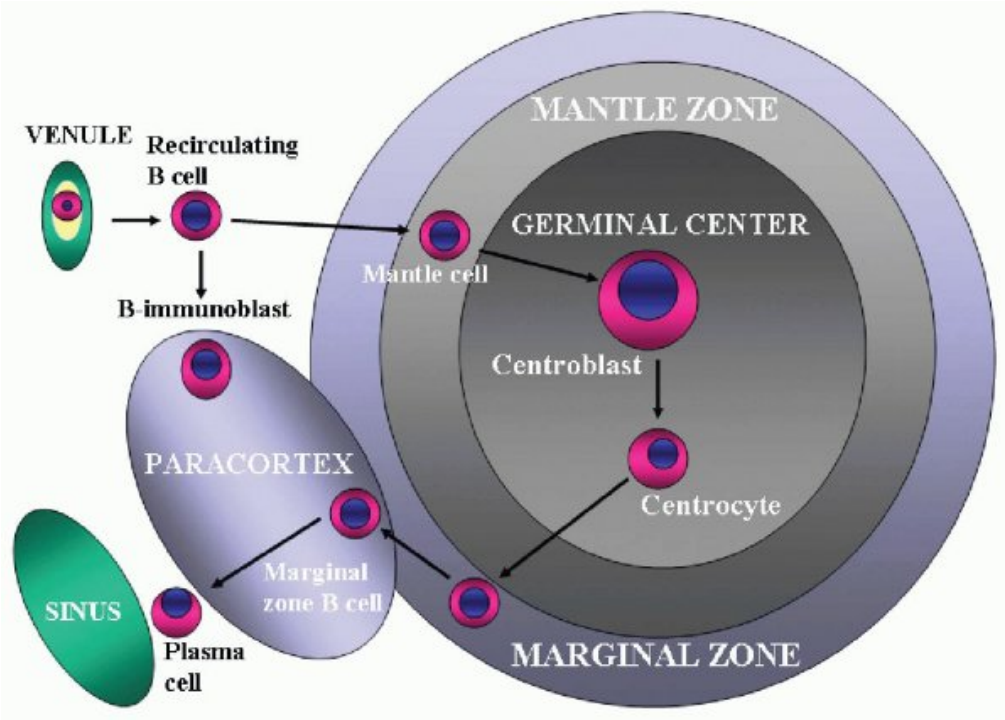

FIGURE 5.2 Intranodal B-cell differentiation (maturation). Recirculating B cells migrate through the highendothelial venule in the hilum of lymph node to mantle zone, germinal center, marginal zone, and finally, the sinus.

The circulating lymphocytes may migrate to the lymph nodes, the mucosal follicles, and other extranodal sites depending on their surface-homing receptors for highendothelial venules in various tissues (10). In the lymph node, lymphocytes travel from one compartment to another, undergoing further morphologic changes.

Intranodal B-Cell Differentiation

The lymph node contains four compartments: (i) the cortex, (ii) paracortex, (iii) medullary cords, and (iv) sinuses. The cortex is composed of lymphoid follicles. A *primary follicle* contains aggregates of resting or virgin B cells (9,11). After antigenic stimulation, resting B lymphocytes become activated, leading to proliferation and blastic transformation. A *germinal center* is formed and is surrounded by a *mantle zone*, which is made up of the same resting B cells as those in the primary follicles. The follicle with a germinal center is called a *secondary follicle*. In the germinal center, B cells are activated by antigens and start to proliferate and undergo somatic mutation. Generation of memory cells and plasma cells and heavy-chain class switch also take place in the germinal center (12).

On the basis of current studies, the sequence of normal B-cell differentiation within the lymph node is suggested as follows (Fig. 5.2) (9,13). In the mantle zone, small lymphocytes develop into intermediate lymphocytes (*mantle cells*) and finally into blastoid lymphoid cells. The blasts then move into the germinal center and evolve through the stages of small noncleaved cells, large noncleaved cells, and small cleaved cells (*follicular center cells*).

Some activated B cells transform into memory B cells and migrate to the marginal zone to become *marginal zone cells* (14). Under certain conditions, the marginal zone cells move to the parafollicular perisinusoidal area and become parafollicular B cells. These cells have ovoid nuclei and relatively abundant clear cytoplasm resembling monocytes and are thus called *monocytoid B cells*. Some B cells transform into effector cells, which are plasma cells. The plasma cell is the terminal stage of the B cell, which moves to the medullary cord and finally migrates back to the bone marrow.

Pregerminal Center, Germinal Center, and Postgerminal Center Lymphomas

Lymphoma can develop at each stage of intranodal differentiation. Accordingly, there are mantle cell lymphoma, follicular lymphoma, nodal marginal zone lymphoma, lymphoplasmacytic lymphoma, and plasma cell myeloma. The origin of a lymphoma can be determined by the status of V_H gene mutation. Lymphomas that show no V_H gene mutation represent a tumor from the pregerminal center. Lymphomas that express V_H gene mutation and intraclonal diversity are derived from the germinal center; whereas those that have V gene mutation but not intraclonal diversity are originated from postgerminal center B cells.

Pregerminal center lymphoma is represented by BCLL/SLL and mantle cell lymphoma. Germinal center lymphoma includes follicular lymphoma, Burkitt lymphoma, and a subset of diffuse large B-cell lymphoma. Postgerminal center lymphoma includes nodal marginal zone B-cell lymphoma, extranodal marginal zone B-cell lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, and

lymphoplasmacytic lymphoma.

CLASSIFICATION BASED ON CLINICAL PRESENTATION

Lymphomas of different origins also may show different clinical presentations. In the World Health Organization (WHO) classification, the mature B-cell neoplasms are divided into three groups, according to their major clinical presentations (13).

1. Predominantly disseminated lymphoma and/or leukemia: This group of tumors usually involves the bone marrow with or without peripheral blood and solid tissues, such as lymph nodes and spleen. It includes CLL, lymphoplasmacytic lymphoma, hairy cell leukemia, splenic marginal zone lymphoma, and plasma cell myeloma.
2. Primary extranodal lymphomas: This group of tumors virtually always presents in extranodal sites. It is represented by extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) lymphoma.
3. Predominantly nodal lymphomas: This group of tumors involves the lymph nodes initially but frequently spreads to the bone marrow, liver, spleen, and peripheral blood. It includes follicular lymphoma, mantle cell lymphoma, and nodal marginal zone B-cell lymphoma.
4. Two aggressive B-cell lymphomas may present with either nodal or extranodal disease. These are diffuse large B-cell lymphoma and its variants and Burkitt lymphoma.

CLASSIFICATION OF ACUTE LEUKEMIAS

The French-American-British (FAB) classification has been used as the basis for the classification of acute leukemia for many years (15,16). However, the WHO classification has

made many changes to the FAB classification. The FAB classification divides ALL into L1, L2, and L3, but the WHO classification considers that the division of L1 and L2 does not serve any clinical purpose and merges them into precursor B-cell and precursor T-cell ALLs (17). L3 is retained as Burkitt cell leukemia. In acute myeloid leukemia (AML), the original FAB categories, M0, M1, M2, M4, M5, M6, M7, are now classified in the category of AML not otherwise categorized (Table 5.3). This category also includes acute basophilic leukemia, acute panmyelosis with myelofibrosis, and myeloid sarcoma. Besides this category, the major addition is the acute myeloid leukemia with recurrent cytogenetic abnormalities, which includes six well-defined clinical entities.

TABLE 5.3 WHO Classification of Acute Myeloid Leukemia

Acute myeloid leukemia with recurrent cytogenetic abnormalities
AML with t(8;21) (q22;q22), (AML1/ETO)
AML with inv(16)(p13q22) or t(16;16) (p13;q22), (CBFβ/MYH11)
Acute promyelocytic leukemia [AML with t(15;17) (q22;q12), (PML/RARα) and variants] (AML-M3)
AML with 11q23 (MLL) abnormalities
Acute myeloid leukemia with multilineage dysplasia
Acute myeloid leukemia and myelodysplastic syndrome, therapy related
Alkylating agent related
Topoisomerase II inhibitor-related

Acute myeloid leukemia not otherwise categorized

AML, minimally differentiated (AML-M0)

AML without maturation (AML-M1)

AML with maturation (AML-M2)

Acute myelomonocytic leukemia (AML-M4)

Acute monoblastic and monocytic leukemia (AML-M5)

Acute erythroid leukemia (AML-M6)

Acute megakaryoblastic leukemia (AML-M7)

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Acute leukemia of ambiguous lineage

French-American-British (FAB) classification in parentheses. AML, acute myeloid leukemia; MLL, multi-lineage leukemia; ETO, eight-twenty-one; CBF, core binding factor; MYH, smooth muscle myosin heavy chain; PML, promelocytic leukemia; RAR, retinoic acid receptor alpha.

CLASSIFICATION OF CHRONIC LEUKEMIA

In the WHO classification, several chronic myeloid leukemias are in the category of myeloproliferative diseases: Chronic myelogenous leukemia, chronic neutrophilic leukemia, and chronic eosinophilic leukemia or hypereosinophilic syndrome (14). The chronic lymphoid leukemias, including CLL, Sézary syndrome, large granular lymphocytic leukemia, prolymphocytic leukemia, and adult T-cell leukemia, are in the category of lymphoid neoplasms in the WHO classification (17, 18 and 19).

CLASSIFICATION OF LYMPHOMA

Modern classification of non-Hodgkin lymphoma started with Rappaport (20), whose classification was based on the histologic pattern (nodular or diffuse), cytology (lymphocyte or histiocyte), and cell differentiation (well differentiated or poorly differentiated). The classification of Lukes and Collins (21) combined immunologic subtypes with morphology and proposed that immunologic phenotypes could be determined by morphologic features. The Kiel (22) classification proposed by Lennert and associates is mainly morphologic with more cell types included. The updated Kiel

classification further divides lymphoma into T-cell and B-cell categories as well as low-grade and high-grade groups (Table 5.4) (22). The lesser known schemes include Dorfman, the British National Lymphoma Investigation, and the “old” WHO (U.N. World Health Organization) classifications. These six different schemes unavoidably caused some confusion among pathologists; thus the National Cancer Institute in the United States organized a team of experts to evaluate the available classifications and establish a “compromised” new scheme. As a result, a working formulation of non-Hodgkin lymphomas for clinical use was proposed (23). The Working Formulation

is relatively simple and yet incorporates all the major components from other schemes (Table 5.5). Its major advantage is dividing the lymphomas into three prognostic groups that make the Working Formulation clinically relevant. It was promptly accepted and has been widely used, especially in North America.

TABLE 5.4 Updated Kiel Classification of Non-Hodgkin Lymphoma

B Cell	*T Cell*
Low-grade malignant lymphoma	**Low-grade malignant lymphoma**
Lymphocytic	Lymphocytic
Chronic lymphocytic leukemia	Chronic lymphocytic leukemia
Prolymphocytic leukemia	Prolymphocytic leukemia
Hairy cell leukemia	Small cell, cerebriform
Lymphoplasmacytic/cytoid (immunocytoma)	Mycosis fungoides/Sézary syndrome
Plasmacytic	Lymphoepithelioid (Lennert lymphoma)
Centroblastic-centrocytic (follicular ± diffuse; diffuse)	Angioimmunoblastic (AILD)
Centrocytic (mantle cell)	T-zone lymphoma
Monocytoid, including marginal-zone cell	Pleomorphic, small cell (HTLV-1 ±)
High-grade malignant lymphoma	**High-grade malignant lymphoma**
Centroblastic	Pleomorphic, medium sized and large cell
Immunoblastic	(HTLV-1 ±)
Burkitt lymphoma	Immunoblastic (HTLV-1 ±)
Large-cell anaplastic (Ki-1 ±)	Large-cell anaplastic (Ki-1 ±)

Lymphoblastic	Lymphoblastic

AILD, angioimmunoblastic lymphoproliferative disorder; HTLV-1, human T-cell lymphotrophic virus type 1.

The Working Formulation, however, does not identify individual disease entities and does not include many new entities, especially in the T-cell lymphoma category, that have appeared in recent years. In addition, the new treatments used currently have changed the outlook of many diseases; thus the prognostic grouping may no longer be valid for some of the lymphomas. Therefore, some American hematologists and oncologists believe that the Working Formulation has outlived its usefulness. Because of this situation, a revised European-American Classification of Lymphoid Neoplasms (REAL classification) was proposed (Table 5.6) (24). This new scheme encompasses many new entities, covers both Hodgkin lymphoma and non-Hodgkin lymphoma, and incorporates immunophenotypes and cytogenetics as integral parts of the diagnosis. Therefore, this new classification has become very popular in large medical centers around the world, where immunophenotyping and molecular genetic analysis can be performed.

The REAL classification, however, contains a number of provisional entities that required additional studies for confirmation or elimination in future schemes. The WHO classification fulfills this function by verifying these provisional entities (Table 5.7) (17, 18 and 19). For instance, the hepatosplenic γδ T-cell lymphoma, nodal marginal zone B-cell lymphoma, and subcutaneous panniculitis-like T-cell lymphoma are retained, whereas Hodgkin-like anaplastic large cell lymphoma, Burkitt-like lymphoma, and T-cell CLL are eliminated because they are resolvable into other definitive diagnoses. Primary effusion lymphoma and intravascular large B-cell lymphoma are added as subtype of diffuse large B-cell lymphoma. In addition, follicular center cell lymphoma has been changed into follicular lymphoma, angiocentric lymphoma becomes nasal T/NK-cell lymphoma, and lymphoplasmacytoid lymphoma reverts to lymphoplasmacytic lymphoma. However, the basic principle of the REAL classification still remains in the WHO scheme, and this new classification is sometimes referred to as the updated REAL classification (15).

TABLE 5.5 Working Formulation of Non-Hodgkin Lymphoma for Clinical Usage

	Low Grade
A.	Malignant lymphoma, small lymphocytic
	Consistent with CLL
	Plasmacytoid
B.	Malignant lymphoma, follicular, predominantly small cleaved cell
	Diffuse area
	Sclerosis
C.	Malignant lymphoma, follicular, mixed
	Small cleaved and large cell
	Diffuse areas

	Sclerosis
	Intermediate Grade
D.	Malignant lymphoma, follicular, predominantly large cell
	Diffuse areas
	Sclerosis
E.	Malignant lymphoma, diffuse, small cleaved cell
	Sclerosis
F.	Malignant lymphoma, diffuse, mixed
	Small/large cell
	Sclerosis
G.	Malignant lymphoma, diffuse, large cell
	Cleaved cell
	Noncleaved cell
	Sclerosis
	High Grade
H.	Malignant lymphoma, large cell, immunoblastic
	Plasmacytoid
	Cleaved cell
	Polymorphous
	Epithelial cell component
I.	Malignant lymphoma, lymphoblastic

Convoluted cell

Nonconvoluted cell

J. Malignant lymphoma, small noncleaved cell

Burkitt

Follicular areas

Miscellaneous

Composite

Mycosis fungoides

Histiocytic

Extramedullary plasmacytoma

Unclassifiable

Other

CLL, chronic lymphocytic leukemia.

MULTILINEAGE PHENOTYPE

A complicated problem accompanying multimarker analysis is the discovery of multilineage phenotypes, which are sometimes referred to as lineage promiscuity (if it is due to the differentiation of a progenitor cell) or lineage infidelity (if due to aberrant gene regulation) (25). Many terms in this area, such as bilineal, biclonal, biphenotypic, and hybrid form, are poorly defined, and their distinction from each other has not been delineated. Therefore, some cases may not be classifiable, and there is an obvious need to standardize these nomenclatures.

In the WHO scheme, this group of leukemias is classified under the heading of acute leukemias of ambiguous lineage. Bilineal acute leukemia is defined as leukemia with a dual population of blasts with each population expressing markers of distinct lineage, i.e. myeloid and lymphoid. Biphenotypic acute leukemia is defined as leukemia with blasts coexpressing myeloid and lymphoid specific antigens (26). However, the coexpression of only one or two cross-lineage antigens is not a sufficient criterion to diagnose biphenotypic leukemia. For instance, the expression of CD13 and CD33 in a case of ALL should be called myeloid-antigen-positive ALL. The European Group for the Immunologic Classification of Leukemia (EGIL) has proposed a scoring system for the relative specificity of various lineage-associated markers (Table 5.8) (27).

TABLE 5.6 Revised European-American Classification of Lymphoid Neoplasms

B-Cell Neoplasms

I. Precursor B-cell neoplasm: Precursor B-lymphoblastic leukemia/lymphoma

II. Peripheral B-cell neoplasms

1. B-cell chronic lymphocytic leukemia/prolymphocytic leukemia/small lymphocytic lymphoma

2. Lymphoplasmacytoid lymphoma/immunocytoma

3. Mantle cell lymphoma

4. Follicle center lymphoma, follicular

Provisional cytologic grades: I (small cell), II (mixed small/large cell), III (large cell)

Provisional subtype: Diffuse, predominantly small-cell type

5. Marginal-zone B-cell lymphoma Extranodal (MALT type ± monocytoid B cells)

6. Provisional subentity: Splenic marginal zone lymphoma (± monocytoid B cells)

7. Hairy cell leukemia

8. Plasmacytoma/plasma-cell myeloma

9. Diffuse large B-cell lymphoma*

10. Burkitt lymphoma

11. Provisional entity: High-grade B-cell lymphoma, Burkitt-like*

T-Cell and Putative NK-Cell Neoplasms

I. Precursor T-cell neoplasm: Precursor T-lymphoblastic lymphoma/leukemia

II. Peripheral T-cell and NK-cell neoplasms

1. T-cell chronic lymphocytic leukemia/prolymphocytic leukemia

2. Large granular lymphocyte leukemia (LGL)

T-cell type

NK-cell type

3. Mycosis fungoides/Sézary syndrome

4. Peripheral T-cell lymphoma, unspecified*

Provisional cytologic categories, medium-size cell, mixed medium/large cell, large cell, lymphoepithelioid cell

Provisional subtype: Hepatosplenic γδ T-cell lymphoma

Provisional subtype: Subcutaneous panniculitic T-cell lymphoma

5. Angioimmunoblastic T-cell lymphoma (AILD)

6. Angiocentric lymphoma

7. Intestinal T-cell lymphoma (± enteropathy associated)

8. Adult T-cell lymphoma/leukemia (ATL/L)

9. Anaplastic large cell lymphoma (ALCL), CD30+, T- and null-cell types

10. Provisional entity: Anaplastic large cell lymphoma, Hodgkin-like

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Hodgkin Lymphoma

I. Lymphocyte predominance

II. Nodular sclerosis

III. Mixed cellularity

IV. Lymphocyte depletion

V. Provisional entity: Lymphocyte-rich classic Hodgkin lymphoma

MALT, mucosa-associated lymphoid tissue; NK, natural killer.

* These categories are likely to include more than one disease entity.

TABLE 5.7 World Health Organization Classification of Lymphoid Neoplasms

B-Cell Neoplasms
Precursor B-cell neoplasms
Precursor B-lymphoblastic leukemia/lymphoma (precursor B-cell acute lymphoblastic leukemia)
Mature (peripheral) B-cell neoplasms
B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Lymphoplasmacytic lymphoma
Splenic marginal zone lymphoma
Hairy cell leukemia
Plasma cell myeloma/plasmacytoma
Extranodal marginal zone B-cell lymphoma or MALT type
Nodal marginal zone B-cell lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma
Mediastinal large B-cell lymphoma
Primary effusion lymphoma

Intravascular large B-cell lymphoma

Burkitt lymphoma/Burkitt cell leukemia

T- and NK-Cell Neoplasms

Precursor T-cell neoplasm

Precursor T-lymphoblastic lymphoma/leukemia (precursor T-cell acute lymphoblastic leukemia)

Mature (peripheral) T-cell neoplasms

T-cell prolymphocytic leukemia

T-cell large granular lymphocytic leukemia

Aggressive NK-cell leukemia

Adult T-cell lymphoma/leukemia (HTLV-1+)

Extranodal NK/T-cell lymphoma, nasal type

Enteropathy-type T-cell lymphoma

Hepatosplenic T-cell lymphoma

Subcutaneous panniculitis-like T-cell lymphoma

Mycosis fungoides/Sézary syndrome

Anaplastic large cell lymphoma, T/null cell, primary systemic type

Anaplastic large cell lymphoma, T/null cell, primary cutaneous type

Peripheral T-cell lymphoma, unspecified

Angioimmunoblastic T-cell lymphoma

Hodgkin Lymphoma (Hodgkin Disease)

Nodular lymphocyte predominance Hodgkin lymphoma

Classic Hodgkin lymphoma

Nodular sclerosis Hodgkin lymphoma

Lymphocyte-rich classical Hodgkin lymphoma

Mixed-cellularity Hodgkin lymphoma

Lymphocyte depletion Hodgkin lymphoma

HTLV-1, human T-cell lymphotropic virus type 1; MALT, mucosa-associated lymphoid tissue.

TABLE 5.8 Scoring System for Markers Proposed by the European Group for the Immunologic Classification of Leukemia (EGIL)

Score	*B-Lymphoid*	*T-Lymphoid*	*Myeloid*
2	cCD79a*	CD3/cCD3	Myeloperoxidase
	cIgM	anti-TCR	
	cCD22		
1	CD19	CD2	CD117
	CD20	CD5	CD13
	CD10	CD8	CD33
		CD10	CD65
0.5	TdT	TdT	CD14
	CD24	CD7	CD15
		CD1a	CD64

c, cytoplasmic; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase; Ig, immunoglobulin.

* CD79a may also be expressed in some cases of precursor T lymphoblastic leukemia/lymphoma.

In a study of 163 cases of acute leukemia, Del Vecchio et al. (28) found that 121 cases (74%) expressed pure phenotype, 29 (18%) showed single ectopic surface antigen (low-grade deviation group), 8 (5%) revealed coexpression of several markers related to different phenotypes (biphenotypic leukemia group), and the remaining 5 cases (3%) expressed no antigen or only HLA-DR (nondiagnostic group). The identification of biphenotypic or multilineage leukemia is not purely an academic curiosity; it is frequently associated with prognosis and sometimes with therapeutic regimen. For instance, cases of CD2-positive AML usually had poor response to chemotherapy for myeloid leukemia but striking responses to drug combinations usually reserved for lymphoid leukemia (25). The association of immunophenotype and prognosis in various hematologic neoplasms is discussed under separate disease entities.

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Chapter 6

Clinical Application

The clinical application of immunohistochemistry is discussed in Chapter 3; this chapter is devoted mainly to flow cytometric applications. By using a panel of appropriately selected monoclonal antibodies, flow cytometric analysis may determine the cell lineage (T cell, B cell, natural killer [NK] cell, or myelomonocytic cell), developmental stage (mature vs. immature, thymic vs. postthymic) and

clonality (monoclonal vs. polyclonal) of a given cell population. It may also determine the heterogeneous and aberrant features and the percentage of the tumor cells (1). The analysis of these parameters by immunologic means is called immunophenotyping.

Immunophenotyping serves many different functions. When the features of cytology and histopathology are not diagnostic, immunophenotyping helps distinguish a benign lesion from a malignant one, thus achieving a definitive diagnosis. Even when diagnosis is not a major problem, immunophenotyping is still needed and plays an essential role in differential diagnosis, subclassification, and prediction of prognosis. These functions are well exemplified in the area of low-grade B-cell lymphomas, such as small lymphocytic lymphoma, chronic lymphocytic leukemia, mantle cell lymphoma, follicular lymphoma, hairy cell leukemia, and various types of marginal zone B-cell lymphoma, to name just a few. The continuing discovery of new monoclonal antibodies enables better refinement for the diagnosis and classification of these diseases. For instance, the availability of CD23 antibody facilitates the distinction between small lymphocytic lymphoma and mantle cell lymphoma. The prognosis for a certain lymphoma can be evaluated by quantifying the proliferation-associated antigen, such as Ki-67 and PCNA (proliferating cell nuclear antigen). A poor prognosis may also be expected when high percentages of activation antigens (e.g., CD25, CD38, CD71, and human leukocyte antigen [HLA]-DR) are present.

CRITERIA FOR FLOW CYTOMETRIC DIAGNOSIS OF HEMATOLOGIC NEOPLASMS

The major drawback of flow cytometry (FC) for the diagnosis of hematologic neoplasms is its inability to correlate the cell morphology with the surface, cytoplasmic, or nuclear markers, because FC does not allow pathologists or other scientists to have a direct view of the cells examined. Therefore, a set of criteria is established to distinguish hematologic neoplasms from normal leukocytes (Table 6.1).

TABLE 6.1

Criteria for Diagnosis of Hematologic Neoplasms by Flow Cytometry

1. Immunoglobulin light-chain restriction.
2. Loss of surface immunoglobulin in a B-cell population.
3. Coexistence of two different cell-lineage markers on the same cell population.
4. Expression of immature cell markers in a large number of cells.
5. Selective loss of one or more cell lineage antigens.

Immunoglobulin Light-Chain Restriction

The surface immunoglobulin (Ig) light-chain ratio is the most commonly used diagnostic criterion, because it defines the B-cell lineage and clonality at the same time. Its importance is based on the fact that 80% to 90% of lymphomas are of B-cell origin. When one light chain is dominant over the other, it is referred to as light-chain restriction and is indicative of monoclonality. The normal κ/γ ratio is about 2:1. The definition of monoclonality on the basis of this ratio varies from different studies. Taylor (2) defined a monoclonal pattern as a κ/λ ratio ≥3:1 or a λ/κ ratio ≥2:1. Samoszuk et al. (3) defined monoclonality as a κ/λ ratio of 5.5:1 and a λ/κ ratio of 1.7:1. By using a higher cutoff point in the λ/κ ratio the specificity is increased, whereas the sensitivity is decreased (false-negative rate was 27%). This false-negative rate is probably too high to be acceptable by clinical laboratories. Our experience is that if the B-cell population is <20% of the total population or if the minor light-chain component (either κ or λ) is >10% (e.g., κ 45% and λ 15%), the value of the κ/λ ratio is not reliable.

Taylor (2) also defined monoclonality as the ratio of the predominant heavy chain to the sum of other heavy chains ≥3:1. The argument against the use of heavy chains as markers for clonality is that heavy-chain switch may take place after gene rearrangement, and thus no predominant heavy chain will be detected in that situation. In addition, for cost effectiveness, most laboratories have discontinued the use of three or five heavy-chain antibodies. However, the demonstration of heavy-chain switch is sometimes associated with lymphoma transformation, such as in Richter syndrome (4). Furthermore, the data obtained from heavy-chain analysis can be used to double check the light-chain results and is useful in borderline cases (5). Nevertheless, for cost containment, most clinical laboratories have discontinued the use of heavy-chain antibodies.

For surface Ig studies, polyclonal antibodies should be used because monoclonal antibodies react only to a single antigenic epitope of a particular Ig and do not react to some subclasses of Ig (such as IgG3 or IgG4) that may be present exclusively on some tumor cells.

Loss of Surface Immunoglobulin in a B-Cell Population

In about 10% to 20% of lymphomas, B-cell antigens are demonstrated on tumor cells that show no surface Ig by immunohistochemical study or a low percentage of Ig-positive cells by flow cytometric analysis (5,6). Because surface Ig is the antigen receptor on normal B cells, the lack of it is found only on neoplastic cells. The common examples that express the surface Ig-negative, B-cell antigen-positive immunophenotype is the primary mediastinal B-cell lymphoma and acute lymphoblastic leukemia of B-cell origin (L1 and L2).

Coexistence of Two Different Cell Lineage Markers on the Same Cell Population

Dual-cell lineage markers have become the hallmark of several lymphoid tumors. For instance, chronic lymphocytic leukemia, small lymphocytic lymphoma, and mantle cell lymphoma carry a B-cell marker (either CD19 or CD20) and a T-cell marker (CD5), whereas hairy cell leukemia bears a B-cell marker (CD22) and a monocyte marker (CD11c). In acute myeloid leukemia, more and more lymphoid markers are being found on the leukemic cells. These doublelabeled leukemic cells previously were considered to be bilineal. However, when a single lymphoid marker (e.g., CD7) is coexistent with myeloid markers, it is now considered to be an aberrant phenotype, which is consistent with leukemia rather than a normal myeloid population.

Expression of Immature Cell Markers in a Large Number of Cells

The demonstration of terminal deoxynucleotidyl transferase (TdT) and CD10 (CALL) on tumor cells of lymphoblastic lymphoma and/or acute lymphoblastic leukemia and CD34 (hematopoietic progenitor cell antigen) and CD117 (c-kit or stem cell factor receptor) on cells of acute myeloid leukemia are good examples. The only exception is the presence of hematogones in pediatric bone marrow or in patients after bone marrow transplantation or chemotherapy (Fig. 6.1). Hematogones are precursors of lymphocytes, which may carry the immature markers, such as CD10, TdT, or CD34, but morphologically they appear like mature lymphocytes and not leukemic blasts (7, 8 and 9). However, immature-looking hematogones can also be present in occasional cases. Therefore, morphologic verification of an immature cell phenotype is always necessary, particularly in pediatric patients or those after chemotherapy or bone marrow transplantation.

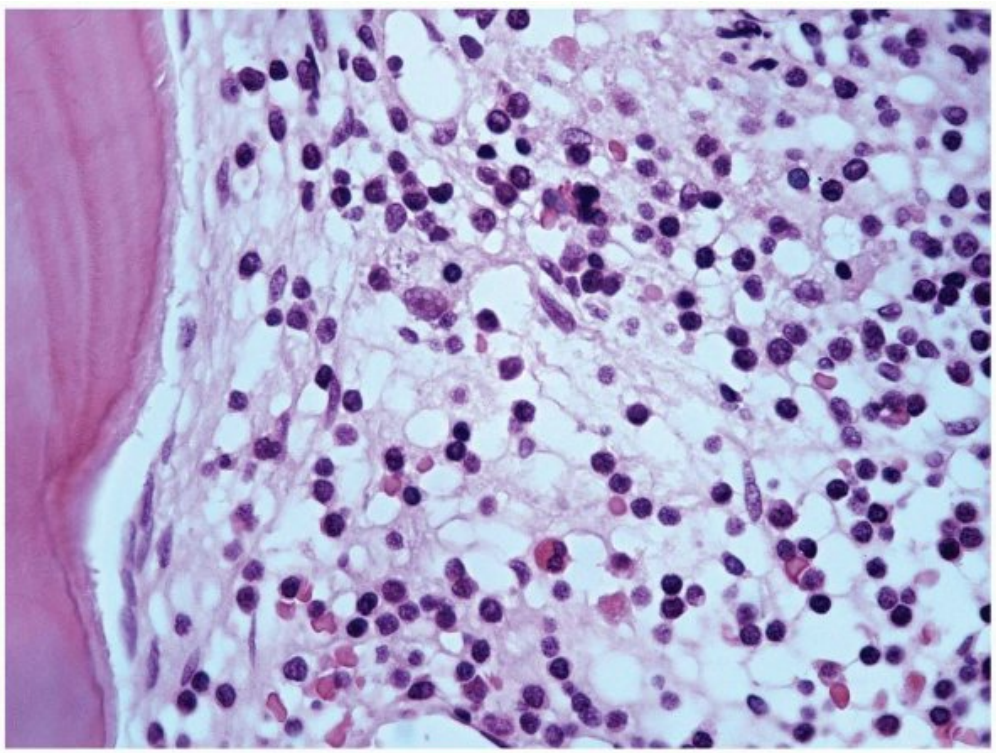

FIGURE 6.1 Bone marrow biopsy from a patient with acute myeloid leukemia after chemotherapy. Note a homogeneous population of small lymphocytes representing hematogones, as proved by flow cytometry. Hematoxylin and eosin, 60× magnification.

There is a tendency for commercial laboratories to offer a blast count based on the percentages of immature cell markers without the knowledge of clinical diagnosis and morphologic correlation. The general practice is to multiply the percentage of the gated population by the percentage of immature cell markers in this particular population. For instance, if the myeloid gate accounts for 50% of the total events registered in the flow cytometric analysis and 30% of the gated population expresses CD34, the blast count is then reported as 15%. This result can be very misleading, because the immature markers, as mentioned above, may be expressed by different kinds of cells and each dot (or event) in the dot plot does not represent an intact cell. These dots may include cell debris, noncell particles,

platelets, and other contaminants.

Selective Loss of One or More Cell Lineage Antigens

Selective loss of one or more cell lineage antigens on a group of lymphoid cells is also an indication of malignancy (6,10). This criterion is particularly useful for diagnosis of T-cell lymphomas because there are no clonal markers for T cells analogous to light-chain restriction for B cells. When three pan-T-cell surface markers (CD3, CD5, and CD7) are included in a study panel, the early-appearing marker (CD7) is more frequently demonstrated in the tumor derived from an early T-cell developmental stage, whereas

the late-appearing T-cell markers (CD3 and CD5) may be decreased. In contrast, the late-appearing marker (CD3) is more frequently seen in peripheral T-cell lymphomas, and the early-appearing marker (CD7) may be absent. The gradual decrease in CD7+ cells in contrast to the persistence of CD3+ cells in cases of mycosis fungoides is one of the most dramatic examples. However, one must distinguish cytoplasmic from surface CD3, because the former is a marker that appears in immature T cells.

TABLE 6.2

Categories of Surface and Nuclear Antigens

Lineage-associated antigens
B cell: CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79, CD138, PCA-1, immunoglobulins (IgA, IgG, IgM, IgD, κ, λ)
T cell: CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD45RA, CD45RO, TCRαß, TCRγδ
Nature killer cell: CD16, CD56, CD57
Myelocyte/monocyte: CD11b, CD11c, CD13, CD14, CD15, CD33, CD64, CD68, CD117
Immature cell antigens: CD10, CD34, CD117, TdT
Activation antigens: CD25, CD26, CD30, CD38, CD54, CD71, HLA-DR
Histocompatibility antigens: HLA-I, HLA-II (HLA-DP, HLA-DR, HLA-DQ)
Adhesion molecules: CD11a/CD18 (LFA-1), CD44, CD56 (NCAM), CD54 (ICAM-1), CD102 (ICAM-2), CD106 (VCAM-1), CD31 (PECAM-1)
Proliferation-associated antigens: Ki-67, PCNA

TdT, terminal deoxynucleotidyl transferase; CD, cluster designation; TCR, T-cell receptor; HLA, human leukocyte antigen; LFA, lymphocyte function-associated antigen; NCAM, neural cell adhesion molecule; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PECAM, platelet-endotheial-cell adhesion molecule.

Another indicator for the existence of a monoclonal T-cell population is the predominance of a T-cell subset, mostly CD4 and occasionally CD8. However, the minor component (CD4 or CD8) usually is not entirely absent. In addition, in viral infections, especially human immunodeficiency virus (HIV) infection, CD8 will be markedly increased and CD4 will drop to a very low percentage. In Hodgkin lymphoma, in contrast, flow cytometric analysis of a lymph node may show predominantly CD4+ cells. Therefore, the selective loss of a

T-cell subset alone is not diagnostic for non-Hodgkin lymphoma unless proven morphologically.

SELECTION OF MONOCLONAL ANTIBODY PANELS

A lymphoma or leukemia is diagnosed not by a single specific marker but by a panel of monoclonal antibodies. Therefore, the selection of a suitable monoclonal antibody panel is one of the most important steps for an accurate diagnosis of hematologic neoplasms. There have been many review articles summarizing the characteristic panels for each hematologic neoplasm (11, 12, 13 and 14). However, the state of the art is to balance between the inclusion of enough monoclonal antibodies to cover the differential diagnoses and the exclusion of excessive monoclonal antibodies to maintain cost effectiveness. There are many monoclonal antibodies available. They can be used to detect surface, cytoplasmic, and nuclear antigens. According to their function, these antigens can be further divided into six categories (Table 6.2).

Lineage-Associated Antigens

This category of antigens is most frequently used in a clinical setting for identifying the lineage of the tumor cells and further narrowing down the differential diagnosis. This category is further divided into B cell, T cell, NK cell, and myelomonocytic cell antigens. The list of B-cell-associated antigens is rapidly expanding. The most common ones include CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79, CD138, and PCA-1. The T-cell-associated antigens include CD1, CD2, CD3, CD4, CD5, CD7, CD8, T-cell receptor αß and T-cell receptor γδ. The NK cell-associated antigens consist of only three antigens—CD16, CD56, and CD57—although other antigens, such as CD11c, are frequently expressed by NK cells. The myelomonocyte-associated antigens include CD11b, CD11c, CD13, CD14, CD15, CD33, CD64, CD68, and CD117.

Immature Cell Antigens

This category includes CD10, CD34, CD117, and TdT. The presence of these markers in a large number of cells usually indicates a hematologic neoplasm, except for the regenerating lymphocytes and/or myelomonocytic cells, hematogones, and dysplastic myeloid cells, which may express one or more immature cell markers.

Activation Antigens

This category is composed of CD25, CD26, CD30, CD38, CD54, CD71, and HLA-DR. These activation antigens may serve as growth factor receptors, may be involved in cellto-cell interaction, and bind to a microenvironment (15).

The activation antigens are usually present on actively proliferating tumor cells, thus conferring a poor prognosis. Some activation antigens may be associated with a particular tumor, such as CD25 in hairy cell leukemia and CD30 in anaplastic large cell lymphoma.

Histocompatibility Antigens

Histocompatibility antigens are important in directing cell-to-cell interaction. For instance, the CD4 cells react with cells carrying HLA-II antigen, whereas the CD8 cells react with those bearing HLA-I antigen. The HLA-II antigen includes HLA-DP, HLA-DR, and HLA-DQ. Among them, HLA-DR is the most frequently used antigen for immunophenotyping. HLA-DR is an early-appearing B-cell antigen, but its presence persists into the mature stage. Therefore, if HLA-DR is demonstrated alone without other cell lineage—associated antigens, it is consistent with a stem cell leukemia or lymphoma. HLA-DR is also present on activated T cells, myeloblasts, and monoblasts, but not on promyelocytes. Its absence on promyelocytes helps to identify acute promyelocytic leukemia.

Adhesion Molecules

Many adhesion molecules have been discovered in recent years, so that the inclusion of all antigens in this category is impossible. Adhesion molecules on lymphocytes play an important role in interactions with vascular endothelium and the extracellular matrix, thus controlling lymphocyte homing and migration (16, 17 and 18). In large cell lymphomas, the expression of the lymphocyte homing receptor, CD44, is frequently demonstrated in neoplasms of stages III and IV, but infrequently among cases of stages I and II (16). It appears that CD44 expression may influence lymphoma dissemination. Small lymphocytic lymphoma and chronic lymphocytic leukemia share the same immunophenotype except for the adhesion molecules CD11a/CD18, which are present in the former but absent in the latter (15). The presence or absence of adhesion molecules may explain why cells of small lymphocytic lymphoma stay in tissue, whereas those of chronic lymphocytic leukemia spread to the bloodstream. In addition, there are neural cell adhesion molecules, intercellular adhesion molecules (types 1 and 2), vascular-cell adhesion molecule type 1, and platelet-endothelial-cell adhesion molecule type 1.

Proliferation-Associated Antigens

The commonly known proliferation-associated antigens include Ki-67 and PCNA. Ki-67 is a nuclear antigen associated with proliferation and is expressed in all phases of the cell cycle except for the G0 phase (19). PCNA, in contrast, is present in low concentrations in G1 and G2-M phases, but in high concentration in S phase (20). Because these antigens are expressed only on the nuclei of proliferative cells, calculation of the percentage of Ki-67- or PCNA-positive cells may give a clue to the aggressiveness of the tumor. Detection of a high percentage of Ki-67-positive cells in a low-grade lymphoma is suggestive of transformation to a high-grade lymphoma.

The approach of monoclonal antibody selection differs in different laboratories under various situations (21). There are essentially three ways to select the panels.

Standard Panel

This approach was most common in the early era of FC when the number of monoclonal antibodies was limited. The standard panel usually included representative antibodies from different cell lineage, including B cell, T cell, monocyte, and HLA-DR. B-cell antibodies include CD19, CD20, and immunoglobulins. T-cell antibodies consist of three pan-T-cell antibodies, CD3, CD5, and CD7. The monocyte marker, CD14, is frequently combined with the panleukocyte antibody, CD45, for a gate check. However, because lymphoma and leukemia are further subclassified, as illustrated by the revised European-American Classification of Lymphoid Neoplasms (REAL) and the World Health Organization (WHO) classification, immunophenotyping has become increasingly complicated. A standard panel of 16 or 19 monoclonal antibodies can no longer meet the demand of the modern trend. Further expansion of the standard panel is not only unpopular under the current climate of cost containment, but also unfeasible because it needs a large sample.

A current trend of creating a computer database to facilitate interpretation of immunophenotyping for hematopoietic neoplasms by FC also falls into this category (22, 23, 24 and 25). On the basis of the computer score, a list of differential diagnoses is generated, and a correct diagnosis usually falls into one of the top four or five choices. This approach requires the use of a large panel of antibodies, yet it only narrows the range of differential diagnosis.

Two-Tiered Approach

This approach includes the use of a simple screening panel to obtain some preliminary information and, on the basis of this information, determines a specific panel. This approach is particularly suitable for acute leukemia. For instance, a few immature markers (e.g., TdT, CD10, CD34, and CD117) are analyzed; if one or more of them are positive, cell lineage markers are added for subclassification. Positive reactions to TdT and CD10 will direct the use of lymphoid markers for the diagnosis of acute lymphoblastic leukemia. In contrast, positive CD117 and CD34 will point to the direction of acute myeloid leukemia. Currently, a few core panels are suggested for the differential diagnosis of small B-cell lymphomas. Examples are panels consisting of CD20, CD10, CD23, and κ and λ light chains (26) or CD5, CD10, and CD23 (27) or double staining for CD23 and FMC-7 (28). In this approach, additional antibodies can be added later for a final diagnosis. Although this approach is economical in the optimal use of monoclonal antibodies, it is time consuming. Unless the screening and corroborative tests are done on the same day, this approach is generally not acceptable clinically.

Targeted Approach

This is the most efficient way to select a panel of monoclonal antibodies. It requires a morphologist to review the blood smear, bone marrow aspirate, or frozen section to make a preliminary diagnosis, and a monoclonal antibody panel will then be set up accordingly. If no specimen is available for examination, a preliminary clinical diagnosis should be obtained to determine the panel. With this approach, usually only six to eight monoclonal antibodies are needed. The remaining part of the specimen should be

saved in the refrigerator for additional monoclonal antibody testing, in the event that the preliminary diagnosis is incorrect or further subclassification is desired. However, Nguyen et al. (29) found that morphologic misinterpretation often occurred, leading to incorrect panel selection. These authors suggested setting up two large standard panels, designated blood/bone marrow/spleen panel and tissue/fluid panel, respectively.

TABLE 6.3

Cell Specificity and Clinical Application of Common Monoclonal Antibodies

Cluster Designation	*Monoclonal Antibodies*	*Cell Specificity*	*Clinical Application*
CD1a	Leu6, OKT6, T6	Thymocyte, Langerhans cells	T-ALL, T-lymphoma, histiocytosis
CD2	Leu5, OKT11, T11	E-rossette receptor	T-ALL, T- lymphoma
CD3	Leu4, OKT3, T3	T-cell receptor complex	T-ALL, T-lymphoma
CD4	Leu3, OKT4, T4	Helper/inducer T cell	Identification of T subset

CD5	Leu1, OKT1, T1	T-cell, B-cell subset	T-ALL, T/B lymphoma, CLL
CD7	Leu9, OKT16, 3A1	T-cell receptor for IgM-Fc	T-ALL, T-lymphoma
CD8	OKT8, T8	Cytotoxic/suppressor T cell	Identification of T subset
CD10	CALLA, OKBcALLa, J5	Immature B cell and T cell	ALL, B-lymphoma
CD11b	Leu15, OKM1, Mo1	Monocyte, granulocyte, NK cell, T-suppressor cell	AML
CD11c	LeuM5, αS-HCL3	Monocyte, B cell from HCL	AML, HCL
CD13	LeuM7, OKM13, My7	Monocyte, granulocyte	AML
CD14	LeuM3, OKM14, MY4, Mo2	Monocyte, granulocyte	AML
CD15	LeuM1, My1	Monocyte, granulocyte, Reed-Sternberg cell	Hodgkin lymphoma
CD16	Leu11	NK cell, granulocyte, macrophage	NK-cell disorder
CD19	Leu12, OKpanB, B4	B cell	B-ALL, B-lymphoma, CLL
CD20	Leu16, B1	B cell	B-ALL, B-lymphoma, CLL
CD21	CR2, OKB7, B2	Follicular dendritic cell, B cell, C3d	B-lymphoma
CD22	Leu14, OKB22, B3,αS-HCL1	B cell	B-lymphoma, HCL
CD23	B6, Leu20	B cell	B-lymphoma, CLL

CD25	IL-2, OKT26a, Tac	IL-2 receptor on T cell (Tac antigen)	HCL, adult T-cell leukemia
CD30	Ki-1, BerH2	Reed-Sternberg cell, activated T or B cell	Hodgkin lymphoma, anaplastic large cell lymphoma
CD33	LeuM9, My9	Monocyte, granulocyte	AML
CD34	HPCA-1, My10	Hematopoietic progenitor cell	Acute leukemia
CD38	Leu17, OKT10, T10	Plasma cell, activated T or B cell	Myeloma
CD41	J15	Platelet GPIIb/IIIa	Megakaryoblastic leukemia
CD42a,b	HPL14, AN51, 10P42	Platelet GPIX and GPIb	Megakaryoblastic leukemia
CD43	MT-1, Leu22, L60	T-cell, B-cell subset	T- or B-cell lymphomas
CD45	HLE-a, LCA	All leukocytes	Lymphomas, leukemias
CD45RA	MT-2	T-cell, B-cell subset	Follicular lymphoma
CD45RO	UCHL1	T cell, B cell, monocyte, granulocyte	T-lymphoma
CD56	Leu19, NKH-1	NK cell	NK-cell disorder
CD57	Leu7, HNK-1	NK cell, T-cell subset	NK-cell disorder
CD61	10P61, VI-PL2	Platelet GPIIIa	Megakaryoblastic leukemia
CD64	FcrP1, gp75	Monocyte	Monocytic disorder
CD68	KP1, PG-M1	Monocyte, histiocyte	Monocytic/histiocytic tumors
CD71	Tr receptor, OKT9, T9	Activated T/B cell, macrophage	Acute leukemias, lymphomas

CD74	LN2	B cell, monocyte	B-lymphoma
CDw75	LN1	B cell, T-cell subset	B-lymphoma
CD79a	HM47, HM56, JAB117	B cell	B-lymphoma
CD79b	SN8, B29/123, CH3-1	B cell	B-lymphoma
CD103	HML-1, B-ly7	B cell	HCL
CD117	C-kit, stem cell factor receptor	Hematopoietic stem cell	AML
CD138	B-B4, 1D4, F59-2E9, M115	B cell, plasma cell	B-lymphoma, myeloma
	FMC-7	B cell	PLL, HCL, B-lymphoma
	HLA-DR	B cell, activated T cell, myeloblast, monoblast	B-cell neoplasms
	PCA-1	Plasma cell, monocyte, granulocyte	Myeloma
	Glycophorin A	Erythroid series	Erythroleukemia
	TCR-1, βF-1, WT31	T cell	T-lymphoma/leukemia
	TCR-δ1, TCS1, antiδ	T cell	T-lymphoma/leukemia

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; IL-2, interleukin 2; NK, natural killer; PLL, prolymphocytic leukemia; Tr, transferrin; Ig, immunoglobulin.

TABLE 6.4

Minimal Monoclonal Antibody Panels for Diagnosis of Hematologic Neoplasms

Panel	*Antibodies*
B-cell lymphoma/chronic lymphocytic leukemia	κ/λ, CD5/CD19, CD23, CD10, FMC-7
T-cell lymphoma/leukemia	CD3/CD4, CD3/CD8, CD5, CD7, CD25, CD30
Hairy cell leukemia	κ/λ, CD11c/CD22, CD25, CD103, FMC-7
NK lymphoma/leukemia	CD2, CD3/CD4, CD3/CD8, CD16, CD56, CD57
ALL	TdT, CD7, CD10/CD19, CD34, Cμ, κ/λ
AML	MPO, CD7/CD13-CD33, CD14, CD34, CD117, HLA-DR
AML-M6 (erythroleukemia)	AML panel plus glycophorin A
AML-M7 (megakaryoblastic leukemia)	AML panel plus CD41, CD42b, CD61
Myeloma/macroglobulinemia	Surface and cytoplasmic κ/λ, CD5/CD19, CD38/CD138, CD56

MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; NK, natural killer; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia.

Since the early 1980s, thousands of monoclonal antibodies specific for leukocyte-differentiation antigens have been developed. These antibodies are categorized into different functional groups, and those that are reacting to the same epitope are assigned the same cluster designation or differentiator (CD). At the 8th International Workshop on Leukocyte Differentiation Antigens held in Adelaide, Australia, in December 2004, the last CD was CD339 (30). These antibodies have been used mainly on fresh and appropriately frozen cells. However, there has been an increasing number of newly developed antibodies that are reactive with antigens in fixed paraffin-embedded sections (31,32) (see Table 3.2). These antibodies are most helpful in morphologic correlation with immunophenotypes and in retrospective studies. The monoclonal antibodies that are commonly used for immunophenotyping of lymphomas and leukemias are summarized in Table 6.3. The minimal monoclonal antibody panels used in different types of lymphoma and leukemia are listed in Table 6.4.

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CASE 1 Chronic Myelogenous Leukemia

CASE HISTORY

A 59-year-old man was admitted to the hospital because of marked leukocytosis found incidentally on routine work-related physical examination. He was otherwise asymptomatic. He denied having fevers, chills, night sweats, weight loss, and other constitutional symptoms. Physical examination on admission showed no hepatosplenomegaly and no lymphadenopathy. He is a radiation health safety officer and was estimated to have 25 rads lifetime exposure. His half-sister died of leukemia at young age.

Peripheral blood examination revealed a total leukocyte count of 42,300/μL with 56% segmented neutrophils, 9% bands, 2% metamyelocytes, 5% myelocytes, 1% blasts, 19% lymphocytes, 3% monocytes, 2% eosinophils, and 3% basophils. His hematocrit was 50.6%, hemoglobin 16.4 g/dL, and platelets 463,000/μL.

A bone marrow biopsy showed 0.5% myeloblasts, 2.3% promyelocytes, 21.8% myelocytes, 8% metamyelocytes, 15% bands, 27% segmented neutrophils, 1.5% monocytes, 4.8% eosinophils, 4.8% basophils, 1.8% pronormoblasts, 1.3% basophilic normoblasts, 2.8% polychromatophilic normoblasts, and 8.3% orthochromatophilic normoblasts. The M/E ratio was 6:1. No myelodysplastic changes were demonstrated.

A core biopsy showed 90% cellularity with a widened cuff of immature myeloid cells along the bony trabeculae. The cellular component is predominantly myeloid cells. However, trilineage hematopoiesis was still present. A diagnosis of chronic myelogenous leukemia (CML) was established by flow cytometry (FC) and fluorescence in situ hybridization (FISH).

The patient was treated promptly with Gleevac. In a 3-month follow-up, the total leukocyte count returned to 4,850/μL with normal hematocrit, hemoglobin, and platelets. FISH analysis revealed no fusion signals. A bone marrow transplant is planned for the patient.

FLOW CYTOMETRY FINDINGS

Flow cytometric analysis of the bone marrow showed myeloperoxidase 78%, CD13-CD33 79%, human leukocyte antigen (HLA)-DR 20%, CD34 13%, CD117 43%, CD14 0%, and CD7 0% (Fig. 6.1.1).

MOLECULAR GENETICS

FISH for breakpoint cluster region/Ableson (BCR/ABL) was performed, which showed fusion signals in 79% of cells.

DISCUSSION

CML was first reported in two patients in 1845, but it was not until 1960 that the association of CML with the Philadelphia chromosome (Ph') was identified (1). The karyotype of t(9;22)(q34;;q11) was reported in 1973, and the molecular characterization of the BCR/ABL fusion gene was established in the 1980s.

CML is now recognized as a clonal myeloproliferative disorder that originates in a hematopoietic stem cell. Therefore, it involves not only the myeloid cells but also monocytes, erythrocytes, megakaryocytes, and lymphocytes. Its stem cell origin is evidenced by the occurrence of blasts of various cell lineages during the blast crisis and by the demonstration of BCR/ABL fusion products in different kinds of cells (2, 3, 4 and 5). This is the first hematologic neoplasm in which the association between cytogenetic aberration and leukemogenesis is established. It is one of the most common leukemias, accounting for 15% of leukemias in adults (2).

Morphology

Clinically, CML is divided into three phases: chronic, accelerated, and blast, which can be identified through morphology. In the chronic phase, the peripheral blood shows leukocytosis, usually over 50,000/µL and most cases exceed 100,000/µL (6,7). The leukocytes are mainly composed of granulocytes of various stages, from myeloblasts to segmented neutrophils, but the major population is composed of myelocytes and segmented neutrophils (Fig. 6.1.2). This phenomenon is sometimes referred to as myelocyte bulge and is characteristic of CML. Peripheral basophilia, in addition to granulocytosis, is probably the most important finding for a morphologic diagnosis of CML because it helps to distinguish reactive granulocytosis. However, the absence of basophilia does not exclude CML. Eosinophilia is also a common feature in CML, but it can also be seen in allergy and many other reactive conditions, so its presence is not specific.

The blast count in the chronic phase is <3%, and the percentage of blasts and promyelocytes combined is <10% (7). The percentage of monocytes is usually below 3%, but, due to the high leukocyte count, an absolute monocytosis may be present (5). The platelet count is usually elevated, and its morphology is often normal. In the minority of cases, giant platelets or platelets with decreased or absent granules may be present. Most patients have mild anemia, but the red cell morphology is essentially normal. Nucleated red blood cells may be detected in the peripheral blood in a small number of cases.

The bone marrow features are similar to those of the peripheral blood with a wide spectrum of myeloid cells

P.53

(Fig. 6.1.3) (5, 6 and 7). However, these cells are usually more immature than those in the blood, showing more promyelocytes and myelocytes. Blasts are usually <5% in the bone marrow. Myelodysplastic changes are not seen in the chronic phase. Megakaryocytes are increased and are characteristically microcytic and hypolobated (Figs. 6.1.4 and 6.1.5). In some cases, megakaryocytic hyperplasia is so prominent that some authors designated it Ph'-positive essential thrombocythemia (7). The degree of myelofibrosis is usually proportional to megakaryocytosis, and reticulin fiber

P.54

is increased in up to 40% of CML patients (5). Erythrocyte precursors are generally decreased, resulting in an M/E ratio as high as 10:1. Sea-blue histiocytes and pseudo-Gaucher cells (Fig. 6.1.6) are frequently present because of an increase of cell turnover. The bone marrow biopsy shows marked hypercellularity with granulocytosis and megakaryocytosis (Fig. 6.1.7). The immature myelocytes normally reside along the bony trabeculae and form a 2- to 3-cell layer. In CML cases, the paratrabecular cuff of immature myelocytes may become widened to up to a 5- to 10-cell layer (5).

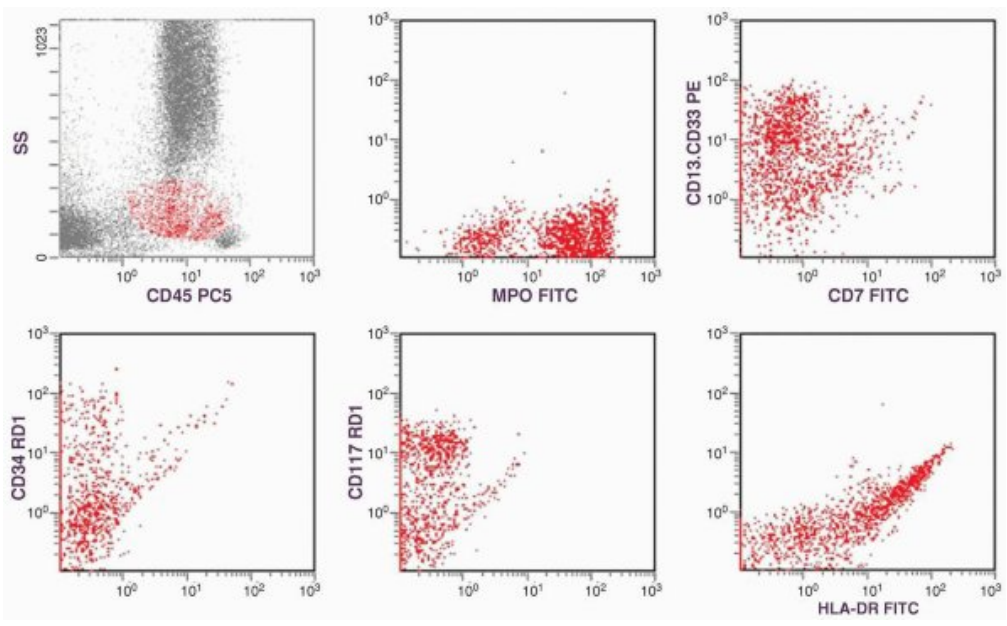

FIGURE 6.1.1 Flow cytometric analysis shows positive myeloperoxidase and CD13-CD33; partially positive CD34, CD117, and human leukocyte antigen (HLA)-DR reactions; but negative CD7 reaction. FITC, fluorescence in situ hybridization; PE, phycoerythrin; MPO, myeloperoxidose.

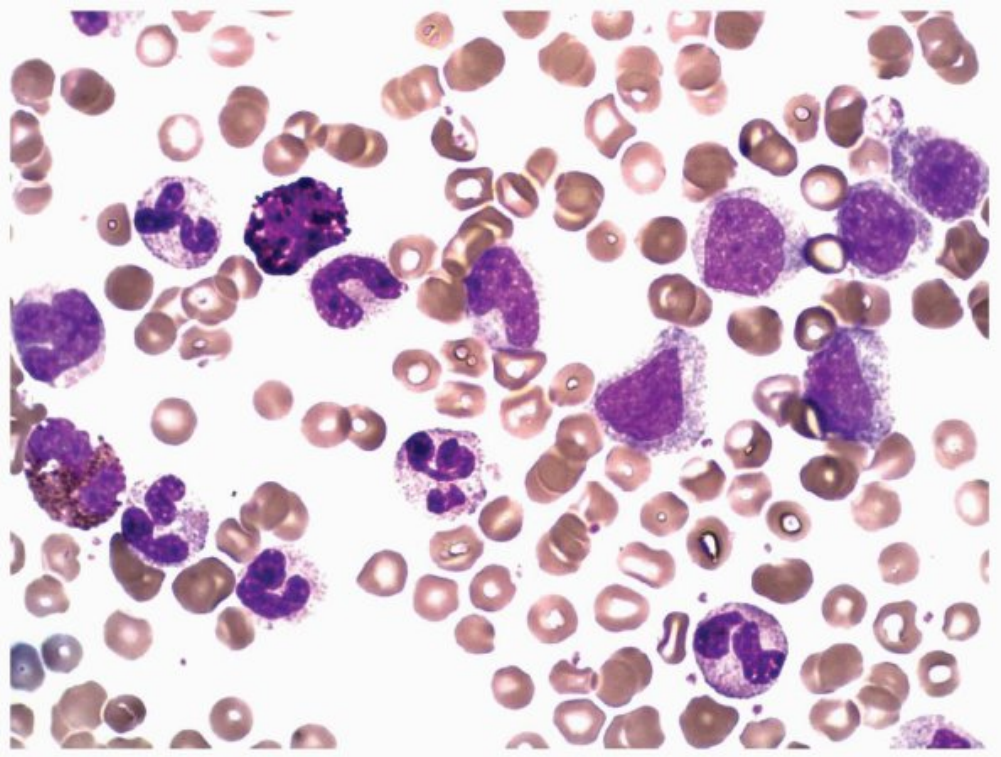

FIGURE 6.1.2 Peripheral blood smear from a patient with chronic phase of chronic myelogenous leukemia (CML) shows predominantly myelocytes and segmented neutrophils. A basophile and an eosinophil are also seen. Wright-Giemsa, 100× magnification.

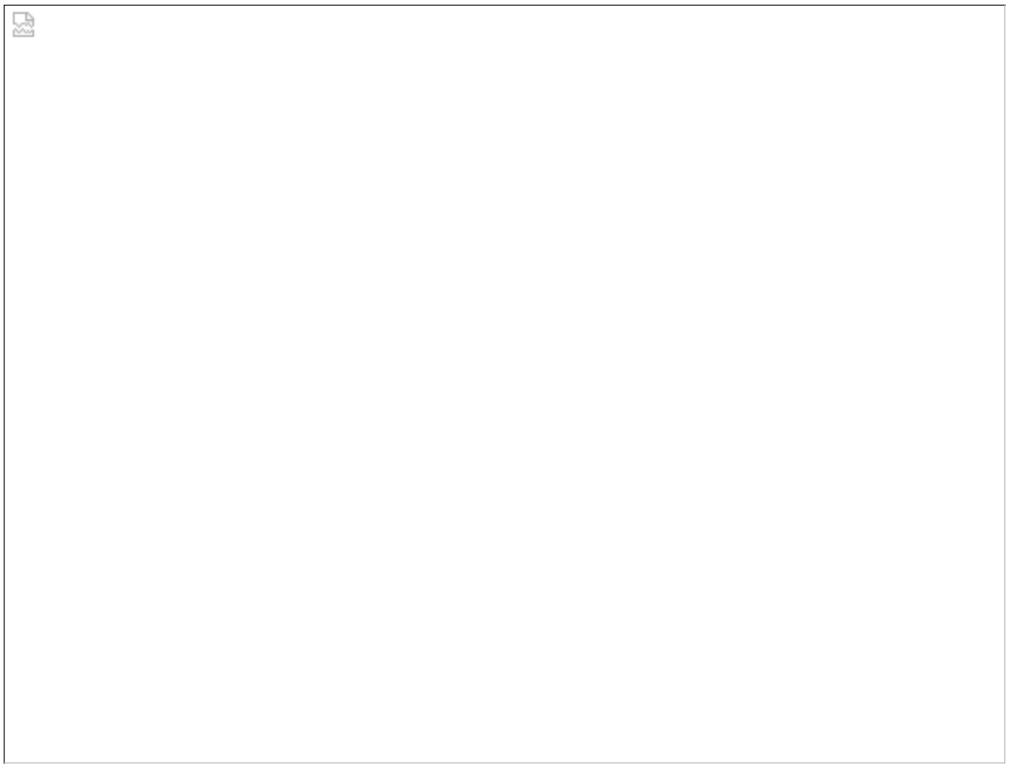

FIGURE 6.1.3 Bone marrow aspirate from a patient with chronic phase of chronic myelogenous leukemia (CML) shows predominantly promyelocytes and myelocytes with a few eosinophils and basophils. Wright-Giemsa, 100× magnification.

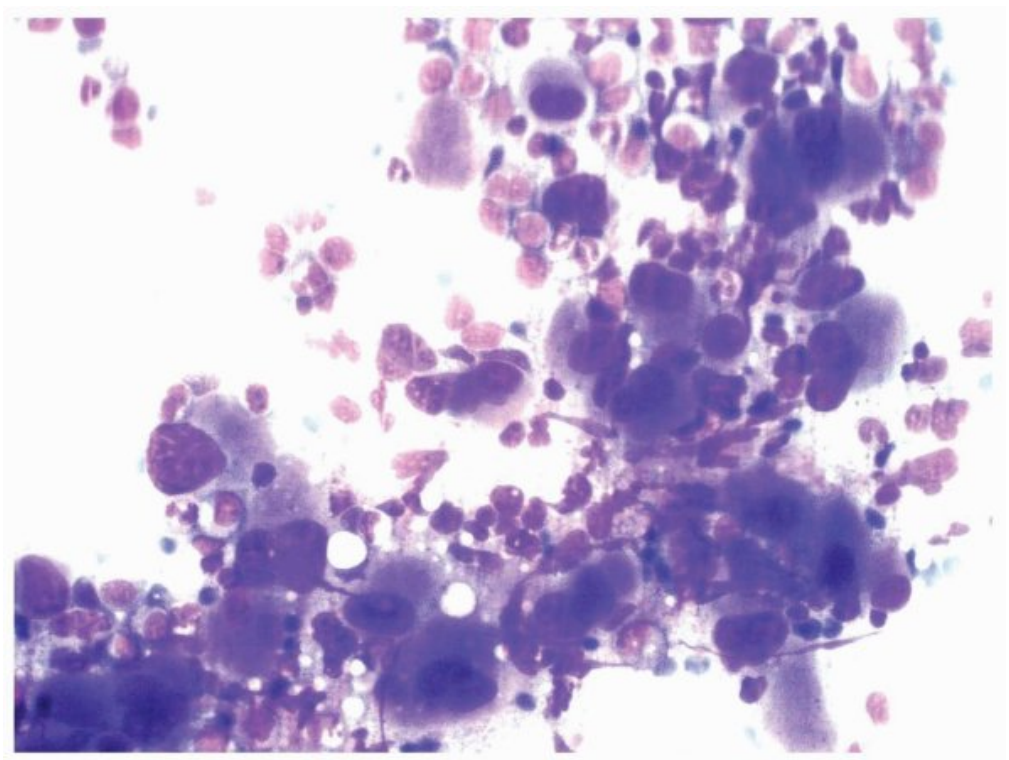

FIGURE 6.1.4 Bone marrow aspirate from a patient with chronic myelogenous leukemia (CML) shows a large cluster of megakaryocytes. Most megakaryocytes are mononucleated or

hypolobated microcytic forms. Wright-Giemsa, 100× magnification.

The progress from chronic phase to accelerated phase is indicated by one or more of the following criteria as defined by the World Health Organization (WHO) (5): (i) a blast count in the peripheral blood and/or bone marrow between 10% and 19% (Fig. 6.1.8); (ii) peripheral blood basophils 20% or above; (iii) persistent thrombocytopenia (<100,000/μL) unrelated to therapy, or persistent thrombocytosis (>1,000,000/μL) unresponsive to therapy; (iv) increasing spleen size and increasing leukocyte count unresponsive to therapy; and (v) cytogenetic evidence of clonal evolution. Marked myelodysplasia and prominent megakaryocytic proliferation with microcytic and hypolobated forms are also suggestive of accelerated phase. However, they have not yet been established as independent indicators of accelerated phase. In addition, nucleated erythrocytes are more frequently seen in the peripheral blood, and reticulin or collagen fibrosis is more prominent in the bone marrow than in the chronic phase.

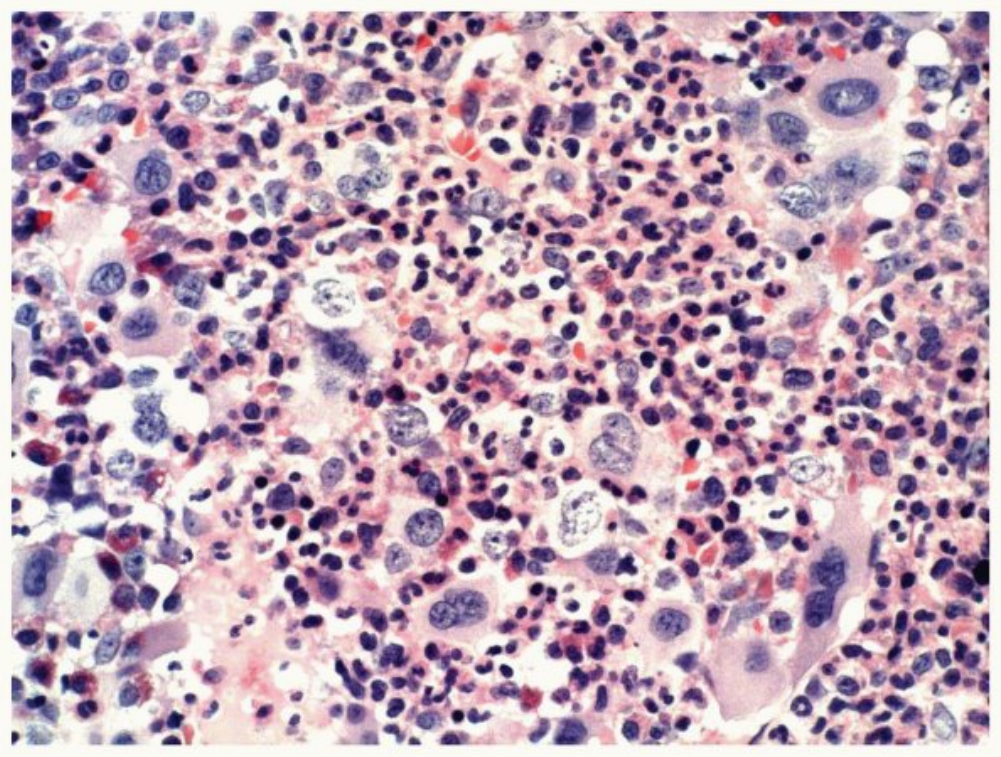

FIGURE 6.1.5 Bone marrow biopsy from a patient with chronic myelogenous leukemia (CML) reveals megakaryocytic proliferation. Hematoxylin and eosin, 40× magnification.

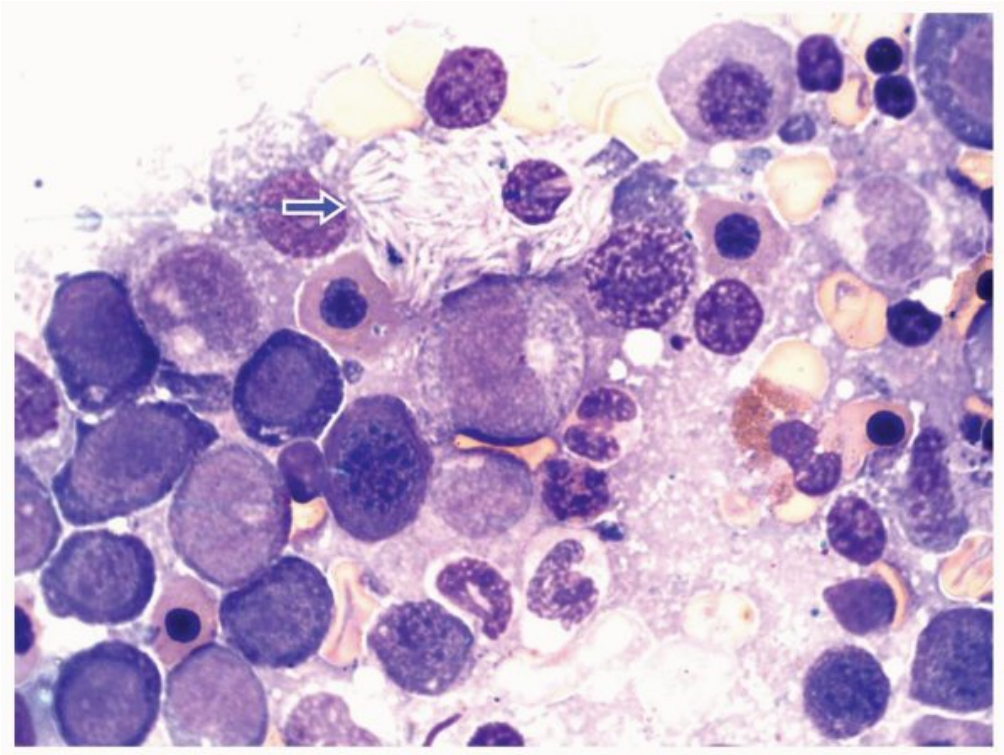

FIGURE 6.1.6 Bone marrow aspirate from a patient with chronic myelogenous leukemia (CML) reveals a peudo-Gaucher cell (*arrow*). 100× magnification.

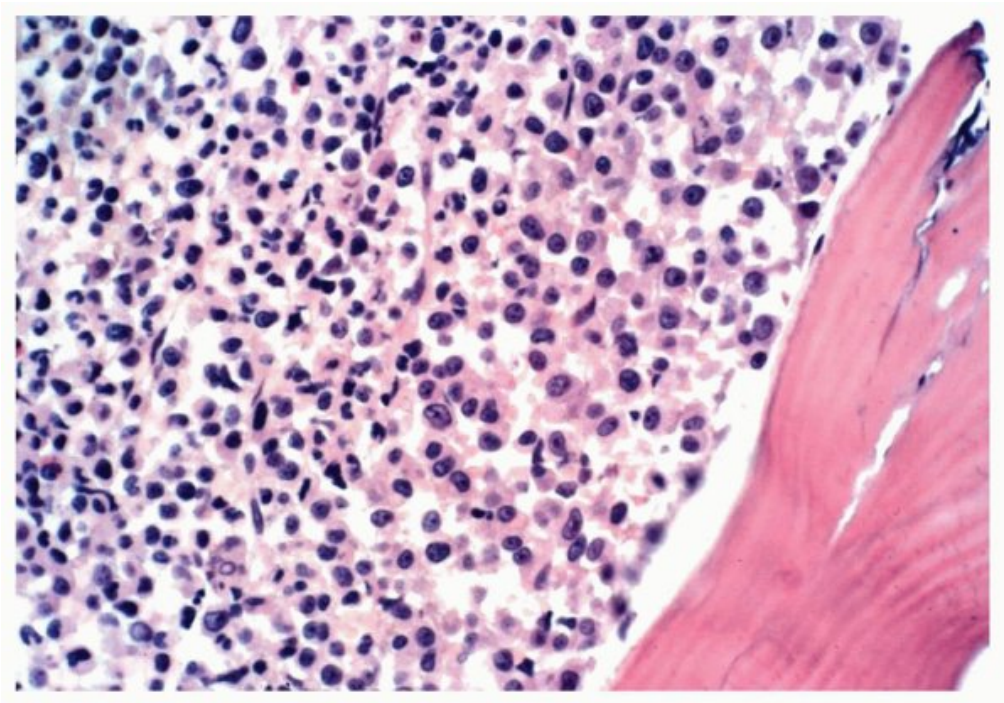

FIGURE 6.1.7 Bone marrow biopsy from a patient with chronic phase of chronic myelogenous leukemia (CML) shows widening of the paratrabecular cuff of immature myeloid cells. Hematoxylin and eosin, 40× magnification.

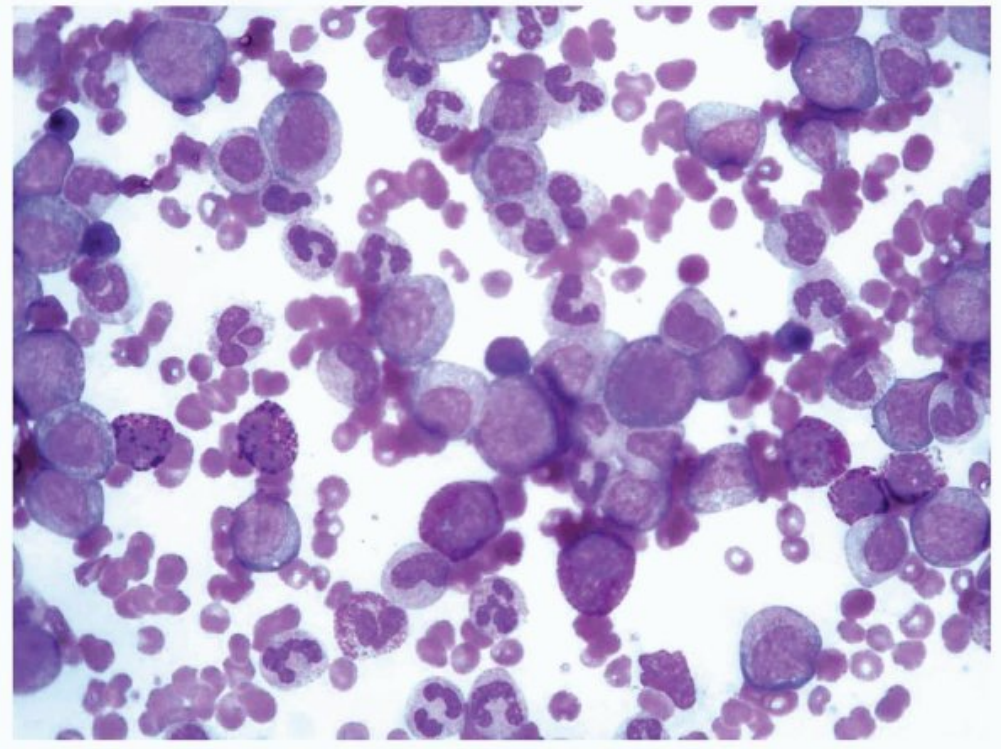

FIGURE 6.1.8 Peripheral blood from a patient with accelerated phase chronic myelogenous leukemia (CML) reveals an increased number of blasts with the presence of eosinophils and basophils. Wright-Giemsa, 100× magnification.

The blast phase is defined as the presence of 20% or more blasts in the peripheral blood and/or bone marrow (Fig. 6.1.9) (4,5). However, blast phase is also indicated when large clusters of blasts are demonstrated in the bone marrow biopsy (Fig. 6.1.10) (5). Under unusual conditions, high blast count is not demonstrated in the peripheral blood or bone marrow, but extramedullary blast proliferation is present (8). This phenomenon was considered to be a predisposing condition of blast crisis, but it is now defined as one of the manifestations of blast phase (5).

In the blast phase, basophilia is still present, but cytopenia occurs in cell lines other than myeloid. Myelodysplastic changes become more prominent in blast crisis than in accelerated phase. About two thirds of the cases are of myeloblastic crisis and one third lymphoblastic. However, monoblastic, megakaryoblastic, promyelocytic, erythroblastic, and multilineage blastic crises have been reported. The identification of the blasts frequently requires immunophenotyping.

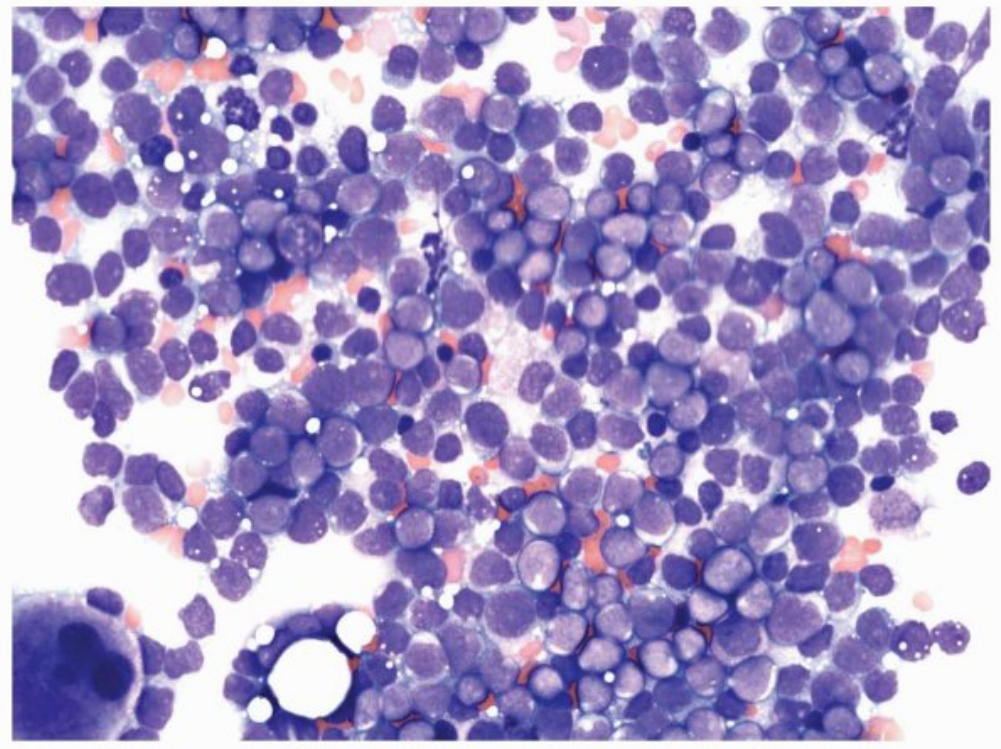

FIGURE 6.1.9 Bone marrow aspirate from a patient with blast phase chronic myelogenous leukemia (CML) shows 90% of blasts. Wright-Giemsa, 40× magnification.

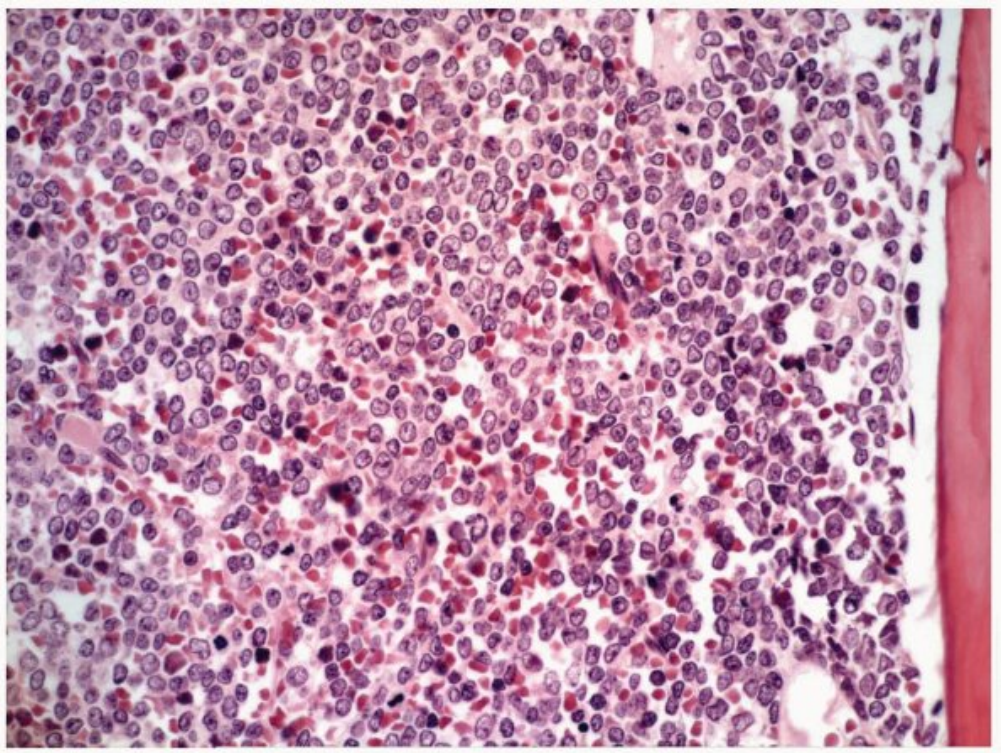

FIGURE 6.1.10 Bone marrow biopsy from a patient with blast phase chronic myelogenous leukemia (CML) shows total replacement of normal hematopoietic cells by the blasts. Hematoxylin and eosin, 40× magnification.

Immunophenotyping

Immunophenotyping does not play an important role in the initial diagnosis or in therapeutic monitoring. A definitive diagnosis and follow-up of the patients depend on the demonstration of t(9;22) or the bcr-abl fusion product.

FC may demonstrate a myeloid population with positive CD13, CD15, CD33, and myeloperoxidase (5). Except in the blast phase, lymphoid and monocyte antigens are generally negative. This immunophenotype is not specific because it cannot distinguish CML from leukemoid reaction. However, immunophenotype can help to distinguish various phases of CML. A flow cytometric study showed that the range of CD34-positive cells is 0% to 26% in the chronic phase, 6% to 64% in the accelerated phase, and 27% to 97% in blast phase (9). An immunohistochemical study revealed the ranges of CD34-positive cells to be 0.1% to 1.1% in the chronic phase, 2.8% to 10.0% in the accelerated phase, and 0.6% to 98% in the blast phase (10). However, due to the small sample size, the difference between the accelerated phase group and the blast phase group was not statistically significant. CD117 is also positive in CML cases, proportional to the blast count. However, no systematic study of CD117 in CML has been reported.

Immunophenotyping is most useful in the distinction between different blasts. CD34 may identify both myeloblasts and lymphoblasts, but CD117 is positive only in myeloblasts. Lymphoblasts are positive for terminal deoxynucleotidyl transferase and CD10; the latter is mainly seen in B lymphoblasts. This distinction is very important because the treatment and prognosis of patients with myeloblast crisis and lymphoblast crisis are markedly

different. The megakaryoblasts can be identified by CD41, CD42, and CD61, and the erythroblasts can be identified by glycophorin A and hemoglobin A staining.

Comparison of Flow Cytometry and Immunohistochemistry

Both FC and immunohistochemistry are not very useful in the initial diagnosis of CML because the immunophenotypes between CML and leukemoid reaction are very similar if the blast count is not high in the CML case. The timehonored test of leukocyte alkaline phosphatase (LAP) score, in contrast, is very useful as a screening technique in distinguishing these two entities. The cytoplasmic granules in the granulocytes of CML cases have low LAP activities, thus the score is lower than the normal granulocytes in leukemoid reaction. However, the LAP activity is inhibited by anticoagulants; therefore, the blood for the test has to be obtained by finger stick and a smear is to be made immediately. This is one of the reasons that the LAP score is not frequently performed. Naturally, it is more convenient for the clinician to order the sophisticated molecular genetic tests (e.g., FISH) for a prompt diagnosis, and the screening test is usually skipped.

Molecular Genetics

CML is characterized by the presence of t(9;22)(q34;q11) as detected by conventional karyotyping in 95% of patients. The shortened chromosome 22 was originally called the Ph' chromosome, a term that is still used today. This genotype has now been verified by molecular biology as the translocation of a proto-oncogene, ABL, on chromosome 9 to juxtapose the BCR gene on chromosome 21. As a result, a BCR/ABL fusion transcript is formed, and its product (a fusion bcr/abl protein) acts as a constitutively active cytoplasmic tyrosine kinase. Because the breakpoint in the BCR gene can be at the minor BCR (m-bcr), major BCR (M-bcr), or micro-bcr, the fusion proteins are sized at 190, 210, and 230 kd, respectively.

All typical CML cases express a 210-kd bcr/abl. A subgroup of CML expressed a larger 230-kd bcr/abl fusion protein and showed clinically a lower white cell count and slower progression than the typical CML (11). This subgroup has been reclassified recently as chronic neutrophilic leukemia (12,13). Ph'-positive acute lymphoblastic leukemia (ALL) cases express either a 210-kd or a 190-kd bcr/abl protein. In childhood ALL, 80% of patients carry the 190-kd bcr/abl protein (13).

The role that the bcr/abl protein plays in CML leukemogenesis is complicated. For this aspect, the reader is referred to several excellent review articles (1, 2 and 3,14, 15, 16 and 17). In this section, only several well-established theories are briefly summarized. First, bcr/abl protein can transform hematopoietic cells in vitro so that their growth and survival become independent of cytokines. The mechanism is through its tyrosine kinase activity to phosphorylate the tyrosine residues of several substrates (1,3,18). As a result, multiple signal transduction cascades affecting cell growth, differentiation, adhesion, and death are activated. These cells then escape normal constraints on growth and become leukemic. Second, bcr/abl protein can protect hematopoietic cells from programmed cell death (apoptosis) in response to cytokine withdrawal and DNA damage (1, 2, 3 and 4,14, 15, 16 and 17,19). This effect is dependent on tyrosine kinase activity of the bcr/abl protein and is reported to be associated with the activation of the Ras gene (14). As a result, these cells become immortal. In contrast, because apoptosis does not occur after DNA damage in CML cells, the accumulated mutations in CML cells may finally lead to blast crisis (14,19). Third, defective adherence of immature hematopoietic CML cells to marrow stroma cells and extracellular matrix may facilitate their release into the blood and home to extramedullary locations or trap inside the blood compartment (1,14,15).

At the time of transformation to the accelerated and blast phases, cytogenetic evolution occurs in 50% to 80% of patients (2,5). The most common change is trisomy 8. In myeloblast crisis, double Ph' chromosome, trisomy 8, trisomy 19, or isochromosome i(17q) may

occur (7). The association between trisomy 8 and c-Myc overexpression and between isochromosome i(17q) and p53 mutation were suspected but have not been established (19). In contrast, the pathologic effects are more clear in t(3;21)(q26;q22) associated with expression of the AML-1/EVI-1 fusion protein and t(7;11)(9p15;p15) associated with expression of the NUP98/HOXA9 fusion protein (19). In addition, deletion or inactivation of tumor suppression genes, such as p53, RB1, and p16, have also been associated with blast crisis in CML (2,15). As p53 is genetically or functionally inactivated in a large fraction of CML cases in blast phase, it obviously plays an important role in CML blast crisis (2,19).

One of the most obvious differences between the normal abl protein and the bcr/abl fusion protein is their subcellular locations (1). The abl protein is located in both the nucleus and cytoplasm, but the bcr/abl fusion protein is located exclusively in the cytoplasm. In contrast, the vast majority of secondary changes involve genes encoding nucleuslocalized proteins that regulate gene transcription (19). It is interesting that when imatinib inhibits the bcr/abl tyrosine kinase in vitro, the bcr/abl protein may enter into the nucleus of the culture cells (1). Therefore, it appears that the aberration of the genes that encode the nucleus-localized proteins, the subcellular location of bcr/abl fusion protein, and the progression of the disease are related sequences.

The current case showed leukocytosis with immature myeloid cells and basophilia in the peripheral blood that led to the suspicion of CML. The high M/E ratio and disproportional high percentage of myelocytes in the peripheral blood and bone marrow also supported the diagnosis. Finally, it was the identification of bcr-abl fusion product by FISH that established a diagnosis of CML. Although the total leukocyte count below 50,000/μL and the absence of splenomegaly are in favor of leukemoid reaction, the presence of many immature myeloid cells, particularly myeloblasts, in the peripheral blood makes leukemoid reaction the unlikely diagnosis. The FISH result is decisive in excluding this entity. If bcr/abl fusion product is not detected, myelodysplastic/myeloproliferative diseases should be considered. The atypical chronic myeloid leukemia may have similar morphology in the peripheral

blood and bone marrow, but the bone marrow should reveal prominent myeloid dysplasia. Chronic myelomonocytic leukemia may also show similar features, but there should be high monocyte counts in both peripheral blood and bone marrow with marked myelodysplastic changes.

TABLE 6.1.1

Salient Features for Laboratory Diagnosis of Chronic Myelogenous Leukemia

1. Leukocytosis: 50,000 to 100,000/μL
2. Wide spectrum of myeloid cells with myelocyte bulge, basophilia, and eosinophilia in the peripheral blood
3. Hypercellular bone marrow with particular increase in myelocytes or promyelocytes, basophilia, and eosinophilia
4. Blast count: chronic phase <5%; accelerated phase 10% to 19%; blast phase >20%
5. Immunophenotype: Positive for CD13, CD15, and CD33, and increased CD34 and CD117 proportional to blast counts
6. Cell lineage of blasts should be determined by flow cytometry or immunohistochemistry.
7. Low leukocyte alkaline phosphatase (LAP) score
8. Philadelphia chromosome, t(9;22) demonstrated by karyotyping
9. Breakpoint cluster region/Ableson (BCR/ABL) gene/messenger RNA/protein detected

by molecular biology techniques

The salient features for laboratory diagnosis of CML are summarized in Table 6.1.1.

Clinical Manifestations

CML is usually seen in patients between 40 and 60 years old, with a median age of 53 years (6,14). The male/female ratio is about 1.4:1. About 40% of patients are asymptomatic, and 50% of patients are diagnosed by routine testing (3). Therefore, most (85%) cases are diagnosed in the chronic phase. Some patients may have a history of radiation exposure or previous chemotherapy, but the cause of CML in most patients is unknown.

The chronic phase is manifested by an indolent clinical course. The symptoms are usually nonspecific, including fatigue, malaise, headache, weight loss, and anorexia. About 50% of patients have splenomegaly caused by extramedullary hematopoiesis (3,13). The high leukocyte number may cause leukostasis. If the patient has a high basophil count, he or she may have flushing secondary to hyperhistaminemia (14). However, the initial diagnosis is usually not based on clinical symptoms, but is due to a high leukocyte count with a wide spectrum of immature myeloid cells and increased numbers of basophils and eosinophils.

The chronic phase may persist for 3 to 5 years and progress to accelerated and blastic phases (2). The accelerated phase may last for 1 year (18). When the patient reaches the blast phase, the median survival is about 18 weeks. The life expectancy of CML patients is about 4 years. However, about 10% of patients may survive for more than 8 years.

A form of atypical CML has been identified (6,7,20,21). Patients with this form have granulocytosis, sometimes monocytosis, but no Ph' and BCR/ABL rearrangement. However, nonrecurrent cytogenetic abnormalities are present in most cases. Its characteristic laboratory findings include marked myeloid dysplasia and erythroid hypoplasia in the bone marrow. Its prognosis is significantly worse than CML. Therefore, atypical CML is considered a separate entity and is classified under myelodysplastic/myeloproliferative disease in the WHO classification (5).

Pediatric patients may have the adult form of CML or juvenile myelomonocytic leukemia (JMML). JMML is Ph' negative, but some cases may show other nonspecific cytogenetic abnormalities (6,22,23). These patients have leukocytosis, monocytosis, thrombocytopenia, and hepatosplenomegaly. The distinguishing features of JMML include frequent skin infiltration and elevation of fetal hemoglobin. The in vitro colony-forming assays, showing abundant spontaneous colony growth, 95% inhibition of colony by antibodies to granulocyte macrophage colony-stimulating factor, and hypersensitivity to granulocyte macrophage colony-stimulating factor, are relatively specific for the diagnosis of JMML. JMML is classified under myelodysplastic/myeloproliferative disease in the WHO classification (5).

The adult form of chronic myelomonocytic leukemia is similar to JMML in clinical and laboratory aspects (4,24). The reader is referred to Case 3 for a detailed discussion.

As mentioned before, the initial diagnosis may be based on the examination of the peripheral blood. The LAP score may help to distinguish the chronic phase of CML from a leukemoid reaction. However, in the accelerated and blast phase, the LAP score may become gradually increased. Thus these two entities should be distinguished on multiple morphologic parameters (Table 6.1.2) (6,7,25). A definitive diagnosis, however, depends on the identification of karyotype by conventional cytogenetics or bcr/abl fusion product by molecular cytogenetic techniques (14,15,26).

The Southern blot analysis is able to identify the rearranged BCR gene (Fig. 6.1.11). The Western blot analysis can detect the bcr/abl protein. The FISH technique can detect the fusion gene in interphase and/or metaphase nuclei (Fig. 6.1.12) (27). This technique can be applied to dried smears of peripheral blood and bone marrow, and it is the method of choice for initial diagnosis as it can identify the fusion product in 100% of CML cases (5). The conventional karyotyping can only identify 95% of CML cases because of the existence of cryptic translocation (1,5,16). However, it is able to demonstrate additional cytogenetic aberrations and is thus particularly useful in identifying patients transforming from chronic phase to accelerated or blast phase (5).

The most sensitive technique is the reverse transcriptase-polymerase chain reaction (RT-PCR), which is used to detect BCR/ABL messenger RNA transcript. The nested RT-PCR is even more sensitive than RT-PCR (21). However, the qualitative RT-PCR techniques are

labor-intensive and difficult to standardize. Therefore, they have been gradually replaced by the quantitative real-time PCR techniques, using either the TaqMan or LightCycle system (28,29).

TABLE 6.1.2

Comparison between Chronic Myelogenous Leukemia (CML) and Leukemoid Reaction

	CML	*Leukemoid Reaction*

Leukocyte count	Near 100,000/μL	Below 50,000/μL
% Promyelocytes/myelocytes	Higher	Lower
Blasts	Present	Absent
Basophilia	Present	Absent
Cytotoxic granules	Absent	Present
Myeloid/erythroid ratio	Near 10:1	Below 10:1
Leukocyte alkaline phosphatase (LAP) score	Low	High
Splenomegaly	Frequently present	Usually absent
Philadelphia chromosome	Present	Absent
Breakpoint cluster region-Ableson (BCR-ABL) fusion product	Present	Absent

For therapeutic monitoring, it is recommended that karyotyping or FISH is used in the early phase of treatment. When Ph' is no longer detectable, serial quantitative PCR studies should be performed at approximately 3-month intervals (29).

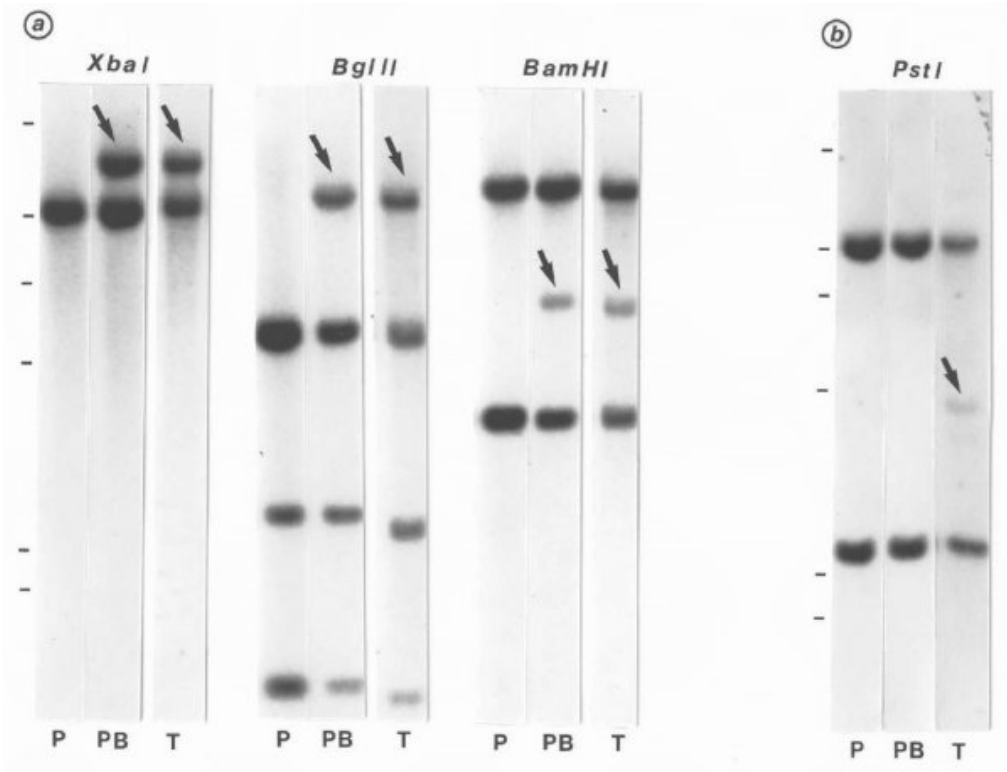

FIGURE 6.1.11 **A:** Southern blot hybridization analysis of DNA from peripheral blood (PB) and tumor tissue (T) with transprobe-1 (for breakpoint cluster region [BCR] or Philadelphia chromosome) showing identical rearrangement bands (*arrows*) after digestion with *Xba*I, *Bgl*II, and *Bam*HI. P, placental control. **B:** Same tissues reacted with T-cell receptor ß chain probe showing a faint rearranged band (*arrow*) in tumor tissue (T) after *Pst*I digestion. (From Sun T, Susin M, Koduru P, et al. Extramedullary blast crisis in chronic myelogenous leukemia. *Cancer*. 1991;68:605-610, with permission.)

During the chronic phase, cytoreductive therapy is needed to prevent leukostasis. Hydrourea and busulfan may induce hematologic but not cytogenetic remission (2,3,14). High-dose chemotherapy followed by allogeneic bone marrow transplantation used to be the only means to achieve cytogenetic cure. In patients who are not suitable candidates for bone marrow transplant, the alternative therapy was the use of interferon alfa, which can induce both hematologic and cytogenetic remission in chronic

phase. However, the recent use of BCR/ABL tyrosine kinase inhibitors has revolutionized the treatment of CML (15,30). The most commonly used drug is imatinib mesylate with the trade names of Gleevec in the United States and Glivec in Europe. It competitively binds to the adenosine triphosphate (ATP)-binding site of the BCR-ABL, and inhibits protein tyrosine phosphorylation (31). Imatinib mesylate induces complete cytogenetic responses in up to 90% of patients and major molecular responses in most of them (30).

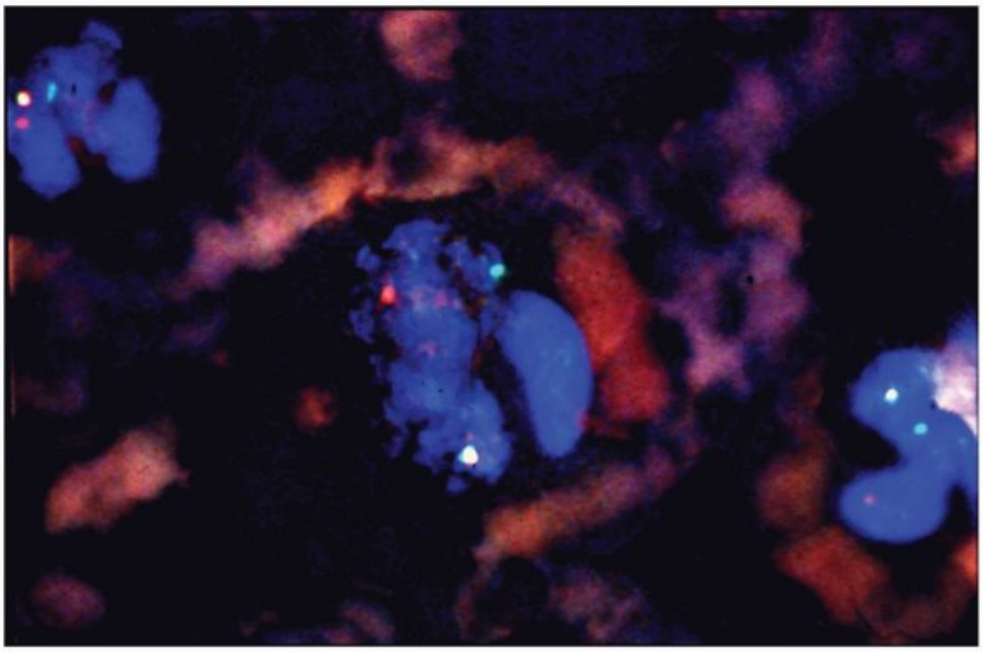

FIGURE 6.1.12 Fluorescence in situ hybridization of peripheral blood from a chronic myelogenous leukemia (CML) patient reacting with breakpoint cluster region (BCR) and Ableson (ABL) probes, showing a green signal (BCR), a red signal (ABL), and a yellow signal, which represents the overlapping of a green and red signal, in all three segmented neutrophils, indicative of BCR-ABL gene fusion product.

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CASE 2 Myelodysplastic Syndromes

CASE HISTORY

An 81-year-old man was admitted because of abdominal pain and diarrhea for 4 days. The patient claimed that he had had anemia for about 20 years, but he had been asymptomatic until several months prior to admission when he developed shortness of breath, fatigue, and palpitations. He was then found to have pancytopenia, and a bone marrow examination revealed hypocellular bone marrow that was consistent with aplastic anemia. Initial workups, including vitamin B12, folate, and antinuclear antibody screening, were all within normal limits. The patient was a sheet-metal worker with frequent exposure to paint sprays.

Physical examination showed lower abdominal pain localized in the suprapubic area with hyperactive bowel sounds. There was no hepatosplenomegaly, and no peripheral lymph node was palpable. The skin demonstrated no petechiae, ecchymoses, or purpura.

Hematologic workup showed a total leukocyte count of 1,200/μL with 29.9% neutrophils, 60.4% lymphocytes, 5.7% monocytes, 3.2% eosinophils, and 0.8% basophils. The hematocrit was 28%, hemoglobin 9.8 g/dL, mean cell volume (MCV) 103.1 fL, and platelets 40,000/μL. The bone marrow aspirate revealed erythroid and megakaryocytic dysplasia with the presence of 12% myeloblasts.

The patient was treated with intravenous fluids with prompt improvement of abdominal symptoms. He responded well with subsequent administration of erythropoietin and granulocyte-colony-stimulating factor (G-CSF; Neupogen). His peripheral blood cell count before discharge showed a total leukocyte count of 7,600/μL with an absolute neutrophil count of 6,400/μL. His hematocrit was 34.2% and platelets 65,000/μL.

FLOW CYTOMETRY FINDINGS

In the bone marrow aspirate, the following results were obtained: T-cell marker: CD7, 0%. B-cell marker: CD19, 0%. Myeloid markers: CD13-CD33, 84%; CD14, 4%; myeloperoxidase, 30%. Major histocompatibility complex (MHC)-II antigen: human leukocyte antigen-(HLA-DR), 76%. Stem cell markers: CD34, 50%; CD117, 55% (Fig. 6.2.1).

CYTOGENIC FINDING

Cytogenetic analysis of unstimulated cultures revealed an apparently normal GTG banding pattern: 46, XY.

DISCUSSION

Myelodysplastic syndromes (MDS) are a heterogenous group of disorders with ineffective hematopoiesis and myeloid dysplastic changes in the bone marrow and peripheral blood. As a result, the bone marrow is usually hypercellular and the peripheral blood is cytopenic in one or more cell lineages.

MDS are usually seen in elderly persons, who may have normal hematopoiesis under normal condition but may have latent age-associated defects that may lead to the development of MDS when under stress (1). The pathogenesis of MDS may be multifactorial, but apoptosis of myeloid cells before their maturation and release from the bone marrow may play an important role (1,2). Other factors include deficiency in humoral promoters, damage of the microenvironment, loss of the ability of progenitors to respond to stimuli, and replacement of the normal marrow with abnormal clones of hematopoietic cells (1).

The French-American-British (FAB) classification of MDS was first established in 1982 and divided MDS into five categories: (i) refractory anemia (RA), (ii) RA with ringed sideroblasts (RARS), (iii) RA with excess blasts (RAEB), (iv) RAEB in transformation (RAEB-T), and (v) chronic myelomonocytic leukemia (CMML) (3).

The World Health Organization (WHO) classification modified the old classification into six categories (4, 5, 6 and 7). First, the blast count for acute myelogenous leukemia (AML) in the FAB classification was 30%, but recent studies have found that patients with 20% to 30% blasts have the same prognosis as those patients with >30% blasts. In addition, 50% to 60% of patients with RAEB-T evolve to AML within 6 months after initial diagnosis (7). Therefore, the subtype of RAEB-T is now classified as AML. Second, CMML has features of both myelodysplastic syndromes and myeloproliferative disorders, so that it is now classified under myelodysplastic/myeloproliferative diseases. Third, on the basis of the blast count, RAEB is further divided into RAEB-1 and RAEB-2 categories. Finally, three new categories were added: Refractory cytopenia with multilineage dysplasia (RCMD), 5q- syndrome, and MDS, unclassifiable (MDS-U).

Morphology

The criteria for classification are based on both quantitative and qualitative changes (Table 6.2.1) (4, 5, 6, 7, 8, 9, 10, 11 and 12). Quantitatively, the major parameters are the percentages of blasts and monocytes in the bone marrow and the peripheral blood and the percentage of ringed sideroblasts among the erythrocyte precursors. Qualitatively, it is the dysplastic changes seen in different cell lineages. Dysplasia is mainly

manifested as the changes of the configuration and lobulation of the nuclei, the size of the nuclei and of the entire cell, and cytoplasmic granularity.

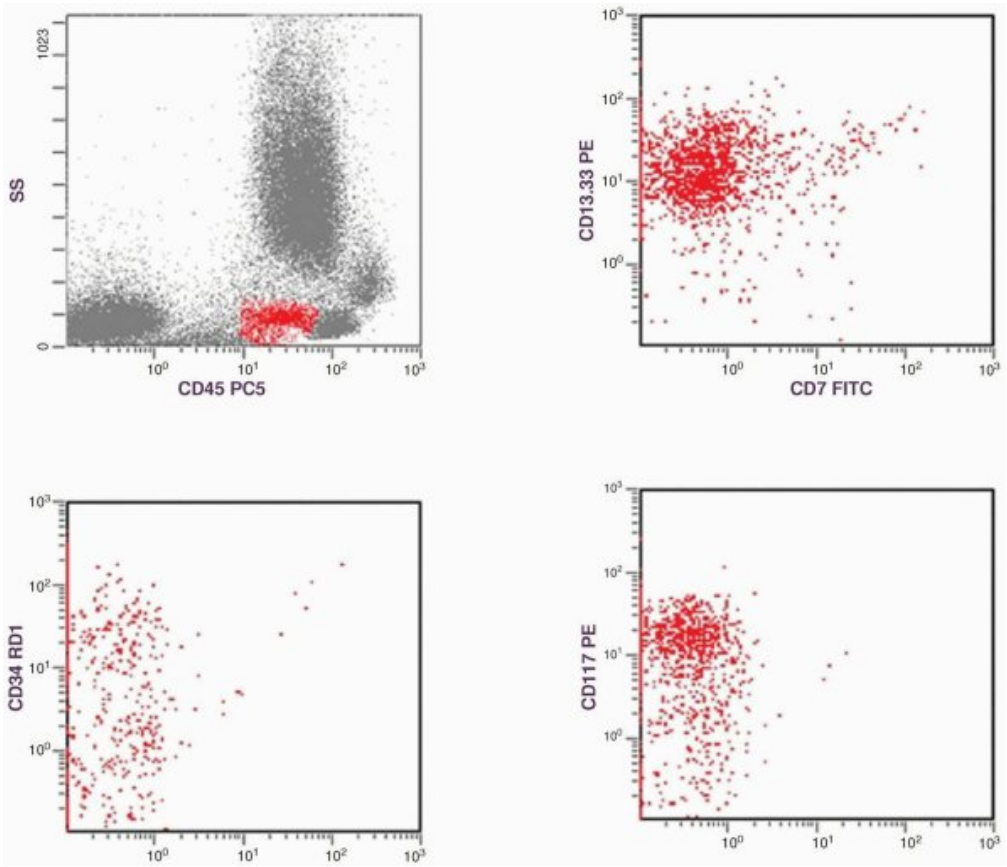

FIGURE 6.2.1 Flow cytometric histograms show the gating of an immature myeloid population with positive reactions to CD13.CD33, CD34, and CD117. This cluster represents the myeloblasts in a case of refractory anemia with excess blasts.

TABLE 6.2.1

Differences in Various Subtypes of MDS

Type	*Blasts in Marrow (%)*	*Blasts in Blood (%)*	*Ringed Sideroblasts (%)*	*Dysmyelopoiesis*
RA	<5	<1	<15	Erythroid
RARS	<5	<1	>15	Erythroid
RAEB1	5-9	<5	Variable	2-3 lineages
RAEB2	10-19	5-19	Variable	2-3 lineages
RCMD	<5	<1	Variable	Multilineages

MDS, myelodysplastic syndrome; RA, refractory anemia; RARS, RA with ringed sideroblasts; RAEB, RA with excess blasts; RCMD, refractory cytopenia with multilineage dysplasia.

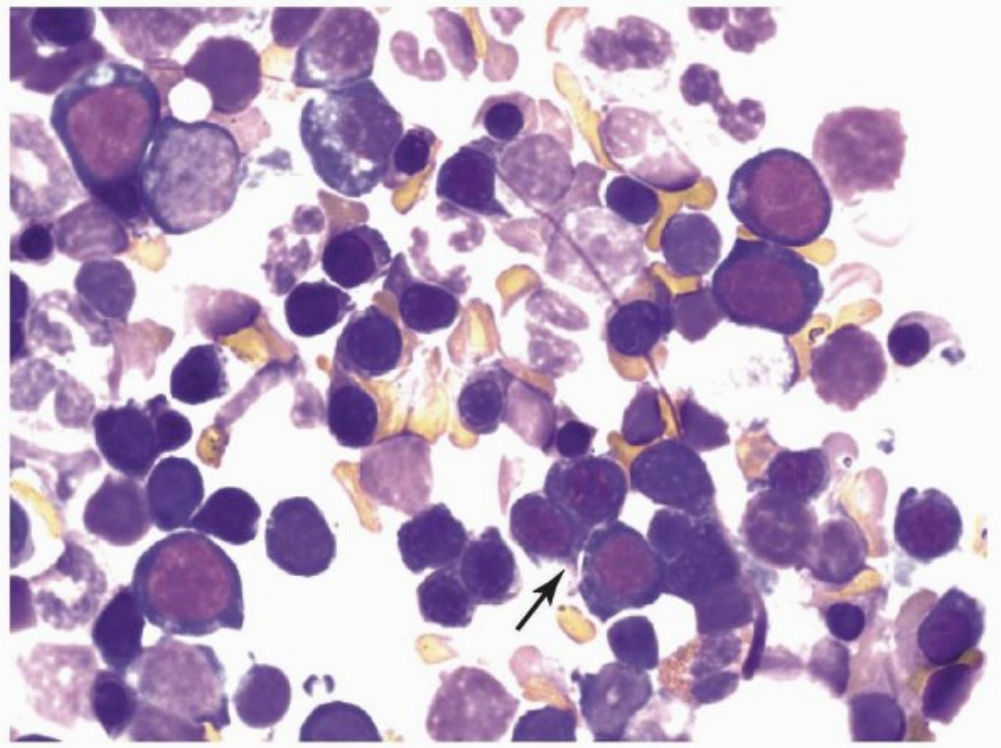

FIGURE 6.2.2 Bone marrow aspirate from a case of refractory anemia shows erythroid hyperplasia with the presence of a cluster of megaloblastoid normoblasts (*arrow*). Wright-Giemsa stain, 100× magnification.

In the erythroid series, the most common findings are megaloblastoid changes (Fig. 6.2.2) and the presence of ringed sideroblasts (Fig. 6.2.3), which is due to the deposition of iron in the mitochondria of normoblasts. A ringed sideroblast is defined by ≥10 iron granules encircling one third or more of the nuclear circumference in an iron-stained smear. The nuclear configuration can be in a bizarre shape (e.g., budding, internuclear bridging), multilobated, fragmented, or karyorrhetic (Fig. 6.2.4). The cytoplasm may contain inclusions, such as Howell-Jolly bodies and Pappenheimer bodies or vacuoles. The normoblasts may become Periodic acid-Schiff (PAS) positive as contrast to the negative staining in normal nucleated red blood cells. Anisocytosis, poikilocytosis, and nucleated red blood cells may be seen on the peripheral blood smears.

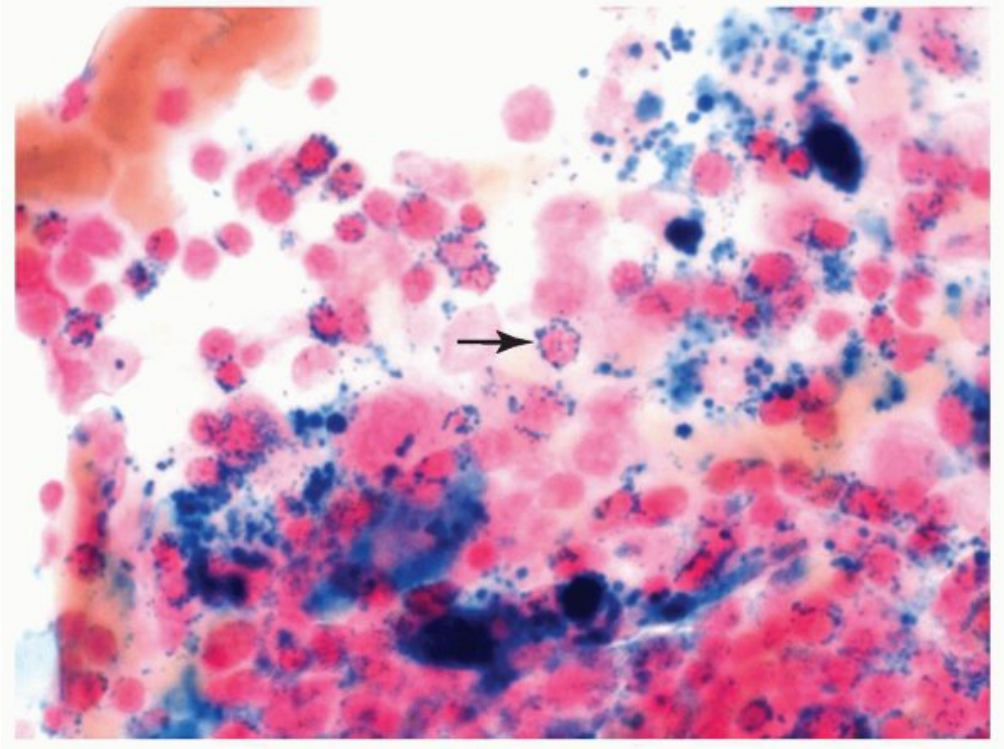

FIGURE 6.2.3 Bone marrow aspirate from a case of refractory anemia with ringed sideroblasts shows many ringed sideroblasts (*arrow*) in the Prussian blue-stained smear. 100× magnification.

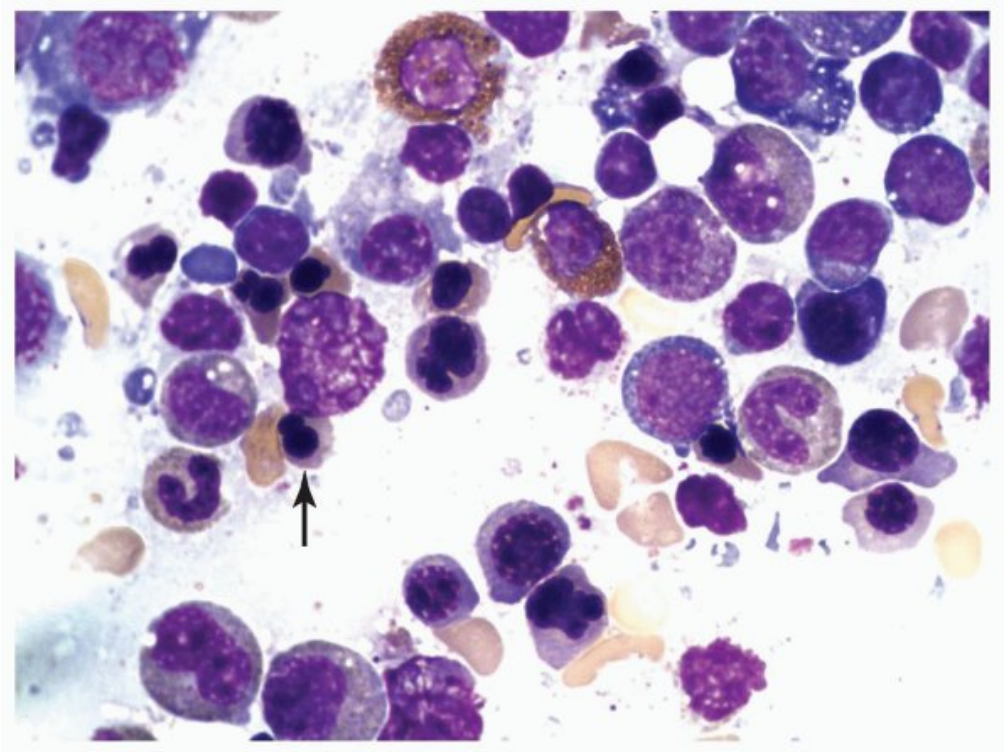

FIGURE 6.2.4 Bone marrow aspirate from a case of refractory anemia with excess blasts shows many dysplastic normoblasts with nuclear budding or bizarre nuclear shapes (*arrow*). Wright-Giemsa stain, 100× magnification.

In the granulocytic series, the most common findings are hypolobulation and hypogranularity. When a bilobed nucleus is present, those cells are referred to as pseudo-Pelger-Huet cells (Fig. 6.2.5). Hypersegmentation (Fig. 6.2.6), hypergranularity, giant nuclei, or huge cell size are also features of myeloid dysplasia, if vitamin B12 and folate deficiency are excluded. Bizarre nuclear configuration, ringed granulocytic nucleus (Fig. 6.2.7), nuclear fragmentation, and separated nuclear lobes may also occur in some

cases. The presence of pseudo-Chediak-Higashi granules has been reported, but this finding is extremely rare.

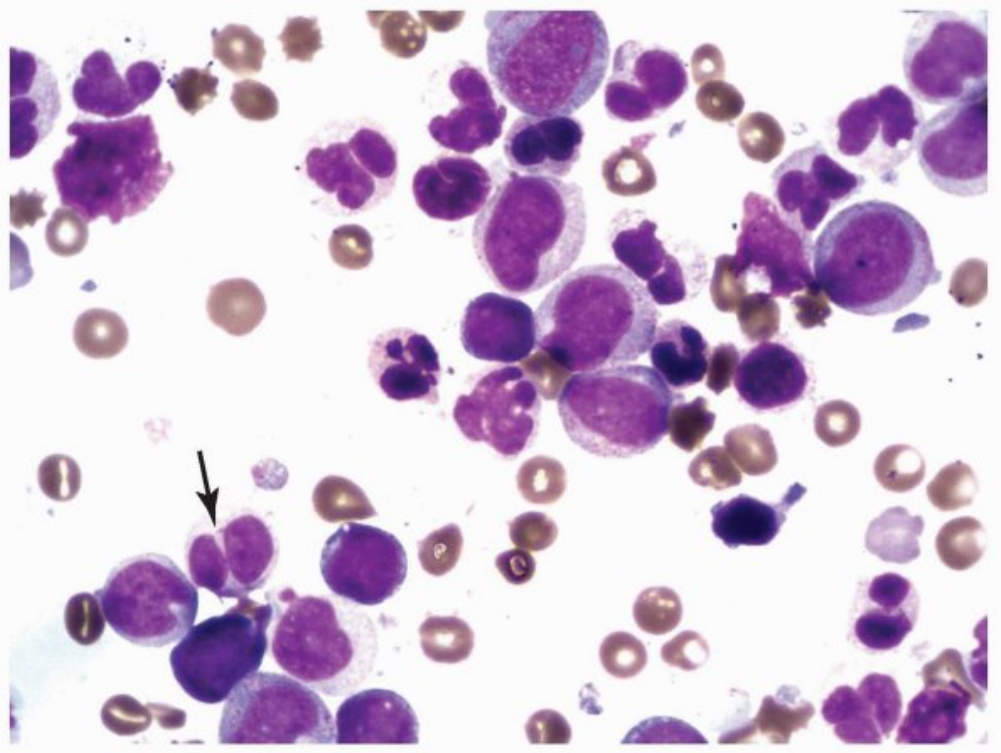

FIGURE 6.2.5 Bone marrow aspirate from a case of refractory cytopenia with multilineage dysplasia shows several hypolobated pseudo-Pelger-Huet cells (*arrow*). Wright-Giemsa stain, 100× magnification.

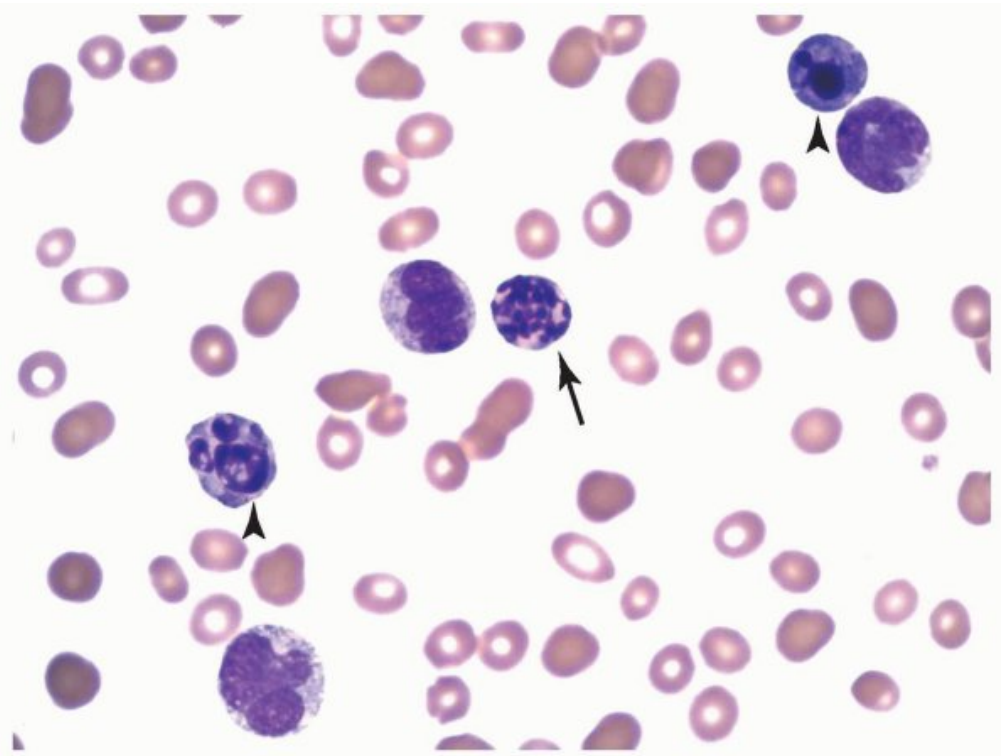

FIGURE 6.2.6 Bone marrow biopsy from a case of myelodysplastic syndrome, unclassifiable, shows a hypersegmented neutrophil (*arrow*) and two apoptotic cells (*arrow heads*). Wright-

Giemsa stain, 100× magnification.

In the megakaryocytic series, the most common findings are micromegakaryocytes, hypolobulation, mononucleation, and the presence of naked nuclei (Fig. 6.2.8). The nuclei may be arranged in a bizarre pattern or in widely separated lobes. Hypogranular megakaryocytes can also be demonstrated in some cases.

All of these dysplastic features should be demonstrated in a high-quality and freshly prepared blood or bone marrow smear. If a smear is made >2 hours after specimen collection, the cell morphology can be distorted and it is invalid for estimation of myelodysplasia. Myelodysplastic changes can be seen in many different conditions, such as vitamin B12 or folate deficiency, heavy metal exposure, paroxysmal nocturnal hemoglobinuria, treatment with G-CSF, and congenital hematologic disorders (4). Therefore, a diagnosis of MDS should not be made until other possible causes are excluded. A few dysplastic cells can sometimes be seen in normal persons, thus at least 10% dysplastic cells should be identified in a particular cell lineage before it is called MDS. In some cases of unilineage cytopenia, a diagnosis is difficult to make; those cases should be observed for 6 months before calling it MDS (5).

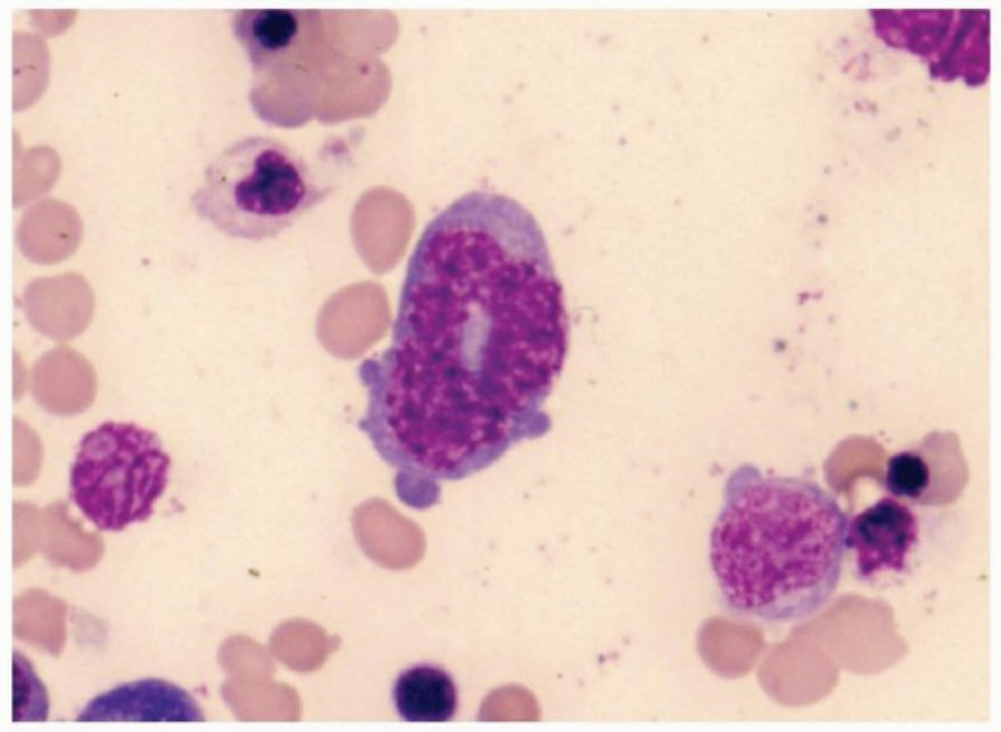

FIGURE 6.2.7 Bone marrow aspirate from a case of myelodysplastic syndrome, unclassifiable, shows a ringed nucleus in a granulocyte. Wright-Giemsa stain, 100× magnification.

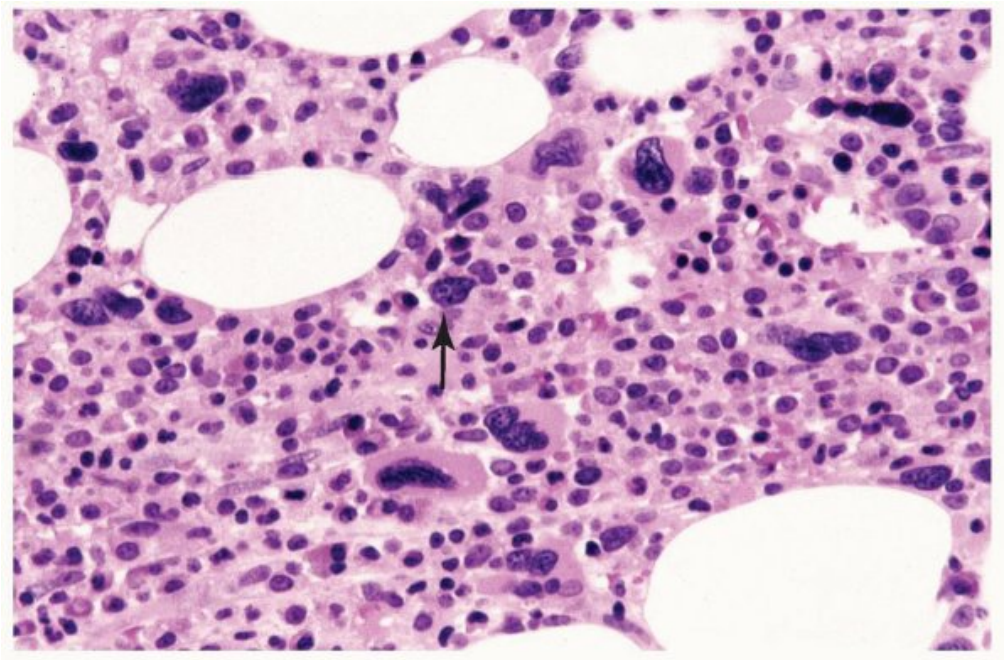

FIGURE 6.2.8 Bone marrow core biopsy from a case of refractory cytopenia with multilineage dysplasia shows many dysplastic megakaryocytes with microcytic and hypolobated morphology. Naked nuclei (*arrow*) are also present. Hematoxylin and eosin stain, 100× magnification.

Histologic examination of core biopsy is not as helpful as aspirate in providing positive identification of MDS. The most distinguishing feature of MDS in tissue sections is the so-called abnormal localization of immature precursors (ALIP), which is usually presented in high-grade MDS and is associated with a more rapid evolution to acute myeloid leukemia (4,13,14). In normal hematopoiesis, the immature myeloid cells first appear along the paratrabecular zone and gradually move to the intertrabecular area as they become mature. The definition of ALIP is the presence of at least three aggregates of three to more than five myeloblasts and promyelocytes in the intertrabecular area (Fig. 6.2.9). On the contrary, erythroid or megakaryocytic precursors are normally present centrally in the bone marrow. Therefore, the detection of clusters of pronormoblasts and immature megakaryocytes in the intertrabecular areas is called pseudo-ALIP (Fig. 6.2.10). In contrast, it is abnormal to find erythroid precursors and megakaryocytes concentrate in the paratrabecular region, which can be seen in MDS. Although ALIP is characteristic of MDS, it can also be seen in chronic myeloproliferative disorders, posttransplantation bone marrow, or in patients receiving granulocyte growth factors (11).

Cellularity is best evaluated by bone marrow biopsy. Most MDS cases have hypercellularity, and the minority has normocellularity. When the cellularity is <30% in patients <60 years or <20% in patients >60 years, it is classified as

hypocellular MDS, which is highly responsive to immunosuppressive therapy (6).

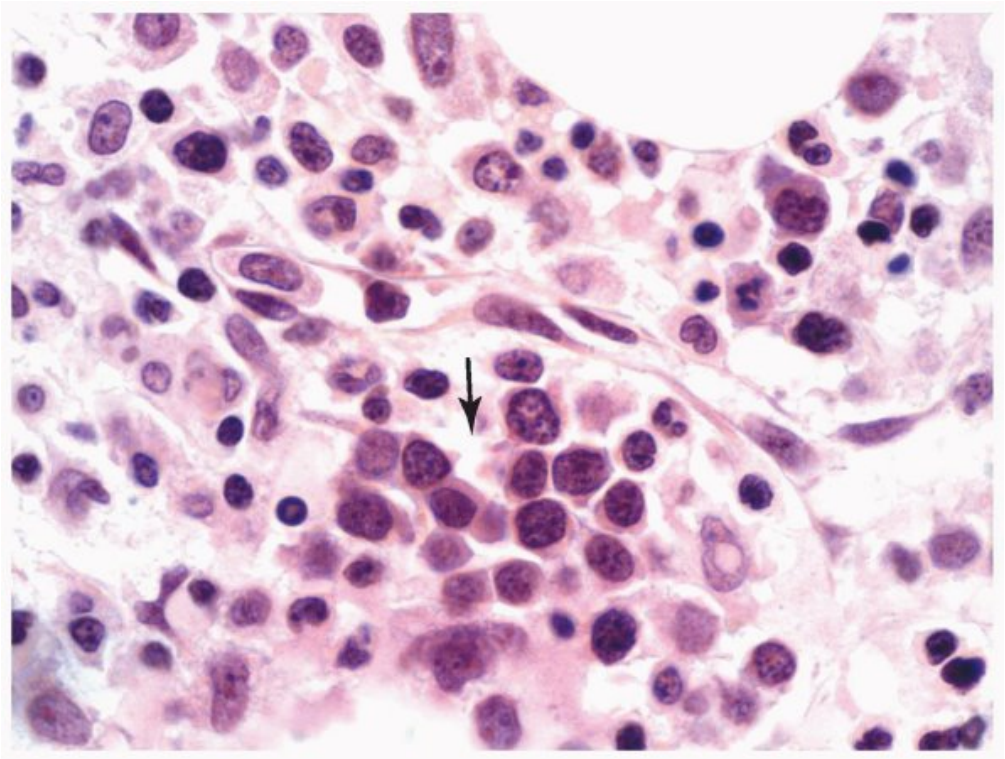

FIGURE 6.2.9 Bone marrow core biopsy from a case of refractory anemia with excess blasts shows a cluster of immature myeloid cells (*arrow*) in between the bony trabeculae representing abnormal localization of immature precursors (ALIP). Hematoxylin and eosin stain, 100× magnification.

Microscopic examination of the spleen in 13 MDS cases showed four histologic patterns, erythrophagocytosis, extramedullary hematopoiesis, red pulp plasmacytosis, and red pulp monocytosis (15).

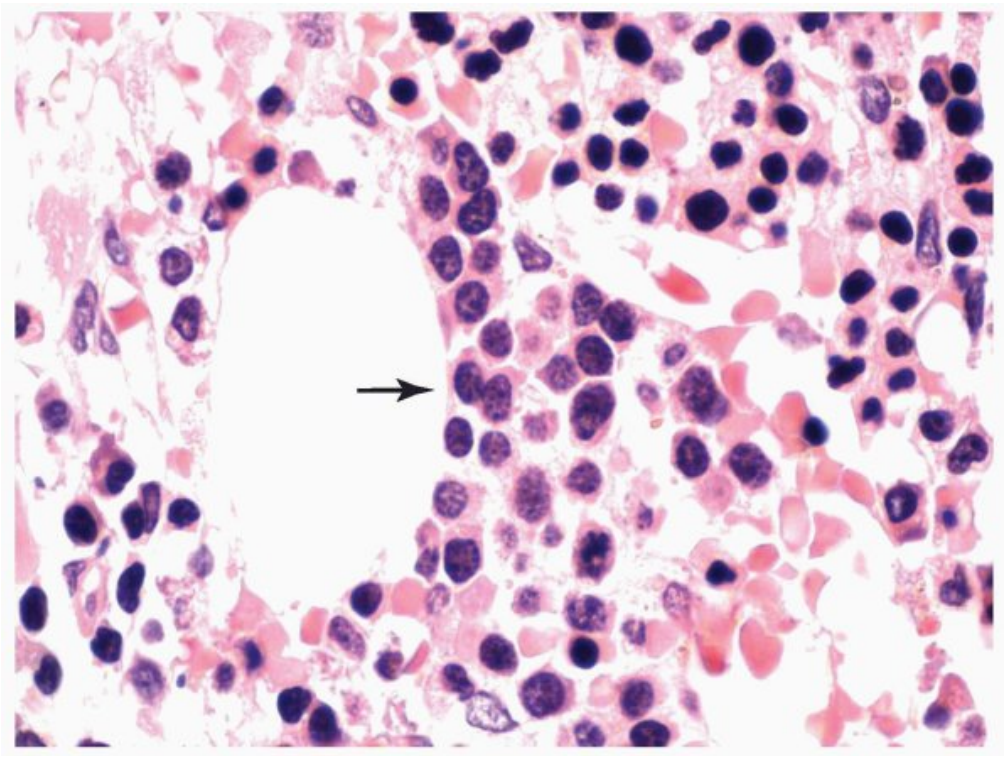

FIGURE 6.2.10 Bone marrow core biopsy from a case of refractory anemia with ringed sideroblasts shows a cluster of pronormoblasts (*arrow*) representing pseudo-abnormal localization of immature precursors (ALIP). The pronormoblasts have regular nuclear contour and erythroid chromatin pattern. The surrounding mature normoblasts also help to identify the erythroid origin of the immature cells. Hematoxylin and eosin stain, 100× magnification.

Refractory Anemia

RA mainly affects the erythroid series. The anemia is usually normochromic and macrocytic but may be normocytic. The granulocytes and platelets are generally normal, but neutropenia and thrombocytopenia may occur in some patients. Blasts are seen in <1% in the peripheral blood and <5% in bone marrow. The bone marrow is usually hypercellular with predominant erythroid precursors. Dyserythropoiesis is inevitably present, but the degree of dysplasia is variable in individual cases. Megaloblastoid changes are frequently seen. The cytoplasm of nucleated erythroid cells is usually PAS positive. Ringed sideroblasts may also be encountered, but they are <15%. Dysplastic changes in granulocytes and megakaryocytes are seldom demonstrated. If the changes are marked, the case should be classified under other categories.

In some cases, the bone marrow may be hypocellular with erythroid hypoplasia resembling aplastic anemia. This condition is more commonly seen in elderly patients. However, the absence of cytopenia in other cell lineage and the increase of immature cells may distinguish hypocellular MDS from aplastic anemia.

Refractory Anemia with Ringed Sideroblasts

RARS is associated with anemia with dimorphic features; hypochromic and normochromic populations are present in the peripheral blood. The red blood cells can be normocytic or macrocytic. Basophilic stippling, Pappenheimer bodies, and nucleated erythrocytes are more frequently demonstrated in the peripheral blood in RARS than in other forms of MDS. The numbers of granulocytes and platelets are normal in most cases, but they may be decreased in some cases. Blasts, if present in peripheral blood, are <1%. The bone marrow is usually hypercellular with predominance of erythroid series. Dyserythropoiesis with megaloblastoid change is present in variable degrees. The major distinction between RARS and RA is the presence of >15% ringed sideroblasts in the bone marrow. Hemosiderin-laden macrophages may be abundant in some cases. Dysplastic changes are absent or mild in myeloid and megakaryocytic series. The number of blasts in the bone marrow is <5%. As in RA, normocellular or hypocellular bone marrow can be demonstrated in rare cases of RARS.

Refractory Anemia with Excess Blasts

RAEB is defined by the presence of 5% to 19% of blasts in the bone marrow and <20% of blasts in the peripheral blood. Because the percentages of blasts in the bone marrow and peripheral blood affects the prognosis of patients (16), RAEB is further classified as RAEB-1 when there are 5% to 10% blasts in the bone marrow and <10% in the blood (4,12). When the bone marrow and blood show 11% to 19% of blasts or Auer rods are seen in the blasts even when the percentage is <11%, it is classified as RAEB-2. Patients with 5% to 19% blasts in the blood and <10% blasts in the bone marrow are also classified as RAEB-2 (4).

RAEB patients are inevitably anemic. The anemia is normochromic and normocytic or macrocytic. Anisopoikilocytosis

is frequently present together with nucleated red blood cells in the peripheral blood. Most patients are pancytopenic with neutropenia and thrombocytopenia. Dysplastic granulocytes and atypical platelets are also present in the peripheral blood.

The bone marrow is hypercellular with panmyeloid hyperplasia. The increase in blasts is usually accompanied by an increase in promyelocytes. Dysplasia can be demonstrated in all cell lineages: Myeloid, erythroid, and megakaryocytes. ALIP is frequently present in this category of MDS. Ringed sideroblasts may be demonstrated in bone marrow, and in some cases may exceed 15% of the nucleated erythrocytes. Cases that show hypocellular bone marrow with erythroid hypoplasia should be distinguished from aplastic anemia. The presence of dysplastic changes in granulocytes and megakaryocytes and the increase in immature cells as demonstrated by CD34 and CD117 should help to distinguish RAEB from aplastic anemia.

Refractory Cytopenia with Multilineage Dysplasia

RCMD shows bicytopenia or pancytopenia and evidence of multilineage dysplasia, but no increases in blasts or monocytes, and no Auer rods are demonstrated. This type of MDS does not fit into any categories of the FAB classification and is designated by the WHO classification as a new entity. The type and degree of dysplastic changes may vary from patient to patient, and no unifying morphologic feature has been established for this category. The ringed sideroblasts are usually <15% of nucleated erythrocytes. If ringed sideroblasts are ≥15%, the case should be classified as RCMD and ringed sideroblasts (RCMD-RS). The bone marrow is hypercellular with bilineage or trilineage hyperplasia, resembling RAEB without an increase in blasts. If blasts are present, they are <5%.

Myelodysplastic Syndrome, Unclassifiable

MDS-U is used for cases that do not satisfy the definition of the above categories. Some patients may have cytopenia but no morphologic evidence of dysplasia. Other patients may have dysplasia in either granulocytic or megakaryocytic cell lines. In those cases,

clonal cytogenetic abnormalities associated with MDS may link them to MDS-U. When there is marked erythroid hyperplasia with marked dyserythropoiesis but no abnormalities are demonstrated in the myeloid and megakaryocytic lineage, those cases may be difficult to distinguish from M6b.

MDS Associated with 5q- Syndrome

When cases of MDS are associated with an isolated del(5q) chromosome abnormality, it is classified as a distinct entity by WHO (4). The clinical significance of identifying this particular karyotype is its association with long survival. Cases in which additional karyotypic abnormalities are found should not be included in this entity. The peripheral blood findings include macrocytic anemia, normal to high platelet count, and normal to slightly elevated leukocyte count. The most characteristic finding is dysmegakaryopoiesis in the bone marrow (17). Megakaryocytes are increased with the presence of micromegakaryocytes or hypolobulated or mononucleated megakaryocytes. Dysplastic granulocytes and increased myeloblasts may be encountered. Erythroid series, in contrast, are hypoplastic. Patients with 5q- syndrome have low frequency of leukemic transformation and relatively long survival.

Immunophenotype

Many flow cytometric studies on MDS have been reported, and most studies claimed that a high percentage of MDS cases showed immunophenotypic abnormalities (18, 19, 20, 21 and 22). Other studies claimed that immunophenotype is a good prognosticator correlating well with the International Prognostic Scoring System (IPSS) (23,24). However, these abnormalities are variable from case to case, and characteristic immunophenotypes have not yet been established. Furthermore, a large panel of antibodies is usually required, and many of those antibodies are not commonly used. Many abnormalities are based on the intensity of certain markers or on the abnormal location or distribution of a cell cluster as determined by a pair of antibodies. Therefore, further standardization is necessary before reliable immunophenotypes are established. So far, there are no reliable markers to evaluate erythroid and megakaryocytic series. Thus only selected myelomonocytic markers are discussed in this case.

The immunophenotypes of MDS are usually based on quantitative changes of surface antigens by comparing MDS cases with normal controls. In general, the antigens expressed on normal myeloid precursors (such as CD34, CD117, and HLA-DR) and those on immature granulocytes (such as CD13 and CD33) are increased in MDS (18, 19, 20 and 21). In contrast, antigens that are expressed on mature granulocytes (such as CD10, CD11b, CD11c, CD16, and CD64) are decreased in MDS (18, 19, 20 and 21, 25, 26).

Among all the quantitative changes, CD34 abnormality is most thoroughly studied (19,21,24,27). The percentage of CD34 cells is usually proportional to the number of blasts. Therefore, its percentage increases progressively from RA and RARS to RAEB and leukemic transformation (19). However, not all blasts express CD34, yet immature myeloid cells may show CD34. One study also found that CD34 expression in nonblast myeloid cells was significantly higher in therapy-related MDS than de novo MDS (21). In the study by Xu et al. (21), the percentage of CD34-positive nonblast myeloid cells was 12±15% in MDS cases with normal karyotype, but it was 23±17% in those with cytogenetic abnormalities (21). Qualitative changes are mainly manifested as aberrant expression of nonmyeloid markers, namely, T-cell, B-cell, and natural killer cell markers. CD7 and CD19 can be detected on maturing myeloid cells or monocytes (23). CD56 may be found on myeloblasts (22) and maturing myeloid cells and monocytes (23). Abnormal patterns of CD11b versus CD16 expression or CD13 versus CD16 expression (20,23,28) and abnormal clustering of various cell markers (24) have also been identified in MDS cases. Finally, the low side-scatter property in the nonblastic myeloid cells in MDS cases represents the presence of hypogranular granulocytes (21).

Immunohistochemical staining may help to determine the cell lineage in core biopsy not only in terms of quantity but also in distribution of different cell types. For instance, it may help to distinguish ALIP from pseudo-ALIP.

Myeloid makers that can be used for immunohistochemical stains include myeloperoxidase, lysozyme, elastase, CD15, and CD68 (11,29). Erythrocytes can be identified by hemoglobin A or glycophorins A and C. Megakaryocytes are positive for CD41, CD42b, CD61, and Factor VIII. CD34 and proliferating cell nuclear antigen can be used to identify hematopoietic precursors with a strong myeloid commitment, which is particularly useful to identify true ALIP (29). The demonstration of CD34-positive aggregates, particularly large aggregates, is a predictor for poor prognosis regarding both leukemic transformation and survival. CD117 staining may serve the same function as CD34.

In the current case, the patient had pancytopenia with macrocytic anemia. The bone marrow was hypercellular showing dysplastic changes in erythroid and megakaryocytic cell lines and 12% myeloblasts. The flow cytometry revealed high percentages of CD13, CD33, CD34, and CD117. The constellation of the clinicopathology findings is characteristic of RA with excess blasts. The patient responded well with erythropoietin and G-CSF and was discharged from the hospital promptly. However, the presence of high percentages of CD34 and CD117 in this patient is associated with an unfavorable prognosis.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry may identify quantitative and qualitative abnormalities in MDS cases, even though they are nonspecific and cannot be depended upon for a definitive diagnosis. Immunohistochemistry, in contrast, is used to demonstrate the distribution of various cell types and is helpful to identify ALIP.

Molecular Genetics

The basic phenomenon in MDS is ineffective hematopoiesis. Therefore, it is important to find out the mechanisms that induce such a phenomenon. The most popular hypothesis is an increase of apoptosis in the early phase of MDS, as supported by histochemical, flow cytometric, and biochemical studies (2,30, 31, 32 and 33). A flow cytometric study, using Annexin V to enumerate apoptotic CD34 cells and Ki-67 to enumerate proliferative cells, found that apoptosis was significantly increased in RA, RARS, and RAEB cases (34). However,

in RA and RARS, apoptosis always exceeded proliferation, whereas in RAEB, apoptosis was equalized with proliferation. The same study also found that the pro-apoptotic (Bax/Bad) versus antiapoptotic (Bcl-2/Bcl-x) protein ratio was increased in RA/RARS, whereas disease progression was associated with significantly reduced ratio.

Apoptosis may be induced by cytokines such as tumor necrosis factor-α and interferon-γ (30,31). Cell culture studies showed that these cytokines can suppress the growth of hematopoietic progenitors and induce Fas expression on CD34 cells (31). The increase of these cytokines and Fas expression has been demonstrated in some MDS patients. These studies implicate the potential role of the Fas/Fas ligand system in the induction of apoptosis. In clinical practice, the inhibition of apoptosis with hematopoietic growth factors and erythropoietin may benefit in improving the blood counts.

MDS has proved to be a clonal disease by molecular genetic techniques. Cytogenetic abnormalities are frequently demonstrated in MDS patients, approximately 30% to 50% in primary MDS cases and in >80% of therapy-related MDS cases (35). Most of the abnormalities are numerical, such as chromosome 5 and 7 monosomy and deletion of the long arm of chromosomes 5 and 7 (32,35,36). Structural aberrations, such as inversion of chromosome 3 and translocations also occur. Translocations include translocation-Ets-leukemia (TEL) fusion, mixed-lineage leukemia (MLL) fusion, nucleoporin abnormality, ecotropic viral integration/ site (EVI-1) family expression, and others (32).

The International MDS Risk Analysis Workshop divided MDS cases into three prognostic cytogenetic groups (16). Good outcomes were those with normal chromosomes, -Y alone, del(5q) alone, and del(20q) alone. Poor outcomes were associated with complex abnormalities (more than three abnormalities) or chromosome 7 anomalies. Intermediate outcomes were associated with cytogenetic aberrations besides those mentioned above. There are a few chromosome abnormalities that are associated with relatively well-defined morphologic clinical syndromes (12,22).

5q- Syndrome

As mentioned above, 5q- syndrome is designated as a separate entity in the WHO classification of MDS. However, 5q-, either isolated or associated with other cytogenetic abnormalities, is seen in a large variety of hematologic disorders, especially myeloid diseases (17). As a sole abnormality, 5q-syndrome is most frequently seen in MDS and AML, particularly in therapy-induced cases. It is predominantly (68% to 74%) seen in female patients. In MDS cases, 5q- syndrome may be seen in RA, RAEB, and RCMD.

Monosomy 7 Syndrome of Childhood

This syndrome is seen in children ages 6 months to 8 years. It is male predominant. The peripheral blood may show anemia, thrombocytopenia, monocytosis, and leukoerythroblastosis. The bone marrow may present with dysplastic changes in erythroid, granulocytic, and monocytic series. Megakaryocytes are usually normal morphologically but may be decreased in >50% of patients. Myeloblasts may be present in the peripheral blood, but they are usually <2%. In the bone marrow, the range of blasts is between 3% and 11%. Because neutrophils have defective chemotaxis, patients may have recurrent infections. The syndrome shares many clinical and hematologic features with juvenile myelomonocytic leukemia, and their distinction may not be possible in some cases. This syndrome is usually associated with a poor prognosis.

TABLE 6.2.2

Salient Features for Laboratory Diagnosis of MDS

1. Peripheral cytopenia in 1-3 cell lineages
2. Dysplasia in 1-3 cell lineages demonstrated in peripheral blood and bone marrow
3. Usually hypercellular bone marrow
4. Increased ringed sideroblasts
5. Increases in myeloid precursor (CD34, CD117, HLA-DR) and immature granulocytic (CD13, CD33) antigens
6. Decreases in mature granulocytic antigens (CD10, CD11b, CD11c, CD16, CD64)

7. Aberrant expression of nonmyeloid markers (CD7, CD19, CD56)

8. Identification of cell lineage and distribution by immunohistochemistry

9. Cytogenetic abnormalities: numerical changes in chromosomes 3, 5, and 7 most frequent

MDS, myelodysplastic syndrome; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

del(17p)

This anomaly is associated with a particular type of dysgranulopoiesis characterized by small neutrophils with pseudo-Pelger-Huet nuclei and cytoplasmic vacuoles. Some mature neutrophils show completely nonlobulated nuclei. These patients usually have a high incidence of p53 mutation and an unfavorable prognosis.

inv(3)(q21-26)

Patients with this abnormality usually have normal or increased platelet counts in the peripheral blood and dysmegakaryocytosis in the bone marrow. The megakaryocytes are increased, showing micromegakaryocytes with hypolobated nuclei. This anomaly may be seen in RARS or RAEB, and is generally associated with an unfavorable outcome.

TABLE 6.2.3

International Prognostic Scoring System for MDS

Score	*0*	*0.5*	*1.0*	*1.5*	*2.0*
% Blasts	<5	5-10	—	11-20	20-30*
Karyotype†	Good	Intermediate	Poor	—	—
Cytopenias‡	0-1	2-3	—	—	—

MDS, myelodysplastic syndrome.

* Current World Health Organization (WHO) range of acute myeloid leukemia.

† See text.

‡ Cytopenias are defined as Hb <10 g/dL, neutrophils <1,500/µL, and platelets <100,000/µL.

Dysregulated Genes in MDS

The pathogenesis of MDS is highly complicated as it involves many dysregulated genes, which include many oncogenes, cell cycle regulatory genes, apoptotic genes, angiogenic genes, genes regulating DNA methylation, genes regulating histone acetylation, receptor

tyrosine kinase genes, and immunomodulatory genes. A detailed description of the involvement of these genes in MDS can be found in a review article by Nishino and Chang (33). The salient features for laboratory diagnosis of MDS are summarized in Table 6.2.2.

Clinical Manifestations

Primary MDS is usually seen in patients >50 years (12). The incidence of MDS increases dramatically after 40 years of age and rises to >20 cases per 100,000 people aged 70 years and older (1). However, it may also be seen in children, particularly those with specific cytogenetic abnormalities, such as monosomy 7 syndrome of childhood. Secondary MDS is usually seen in elderly persons with exposure to radiation or chemotherapy. Patients with secondary MDS fare poorer with more rapidly progressive marrow failure than do those with primary MDS.

Clinical symptoms are related to cytopenia. For instance, dyspnea and pallor are due to anemia; fever, oral pain (agranulocytic angina), and recurrent bacterial or fungal infections are associated with neutropenia; and ecchymoses, petechiae, and mucocutaneous bleeding are related to thrombocytopenia (1). At the end stage, the patient may progress to complete bone marrow failure or leukemic transformation. RA and RARS have a low incidence of leukemic transformation, whereas RAEB has a higher incidence of transformation and rapidly progressive marrow failure. The clinical condition of RCMD is intermediate between these two groups (12).

According to the International MDS Risk Analysis Workshop, the prognosis of MDS depends on cytogenetic abnormalities, percentage of myeloblasts in the bone marrow, and the number of cytopenia (Table 6.2.3) (16). Age and gender also affect survival. Patients >60 years and men have shorter survival.

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CASE 3 Myelodysplastic/Myeloproliferative Diseases

CASE HISTORY

A 63-year-old man presented with a 2-year history of leukocytosis and a 9-kg weight loss during a period of 4 months prior to admission. He saw a hematologist 2 years previously because of elevated leukocyte count, but no conclusive diagnosis was made. The patient did not have a history of exposure to ionizing irradiation or cytotoxic agents. He also denied any fevers, chills, or night sweats. There were no localized symptoms or somatic complaints. Physical examination revealed no splenomegaly or lymphadenopathy.

Hematologic workup revealed a hematocrit of 44%, as compared with 48% recorded 4 months previously. His total leukocyte count was 15,400/μL with 64% neutrophils, 21.9% lymphocytes, 8.1% monocytes, 6.0% eosinophils, and 0.4% basophils. The platelet count was 338,000/μL.

Examination of bone marrow aspirate showed a marked increase in monocytic series (10.25%), which included monocytes, promonocytes, and monoblasts. The total blast count including myeloblasts and monoblasts was 3.8%. There was bone marrow eosinophilia (7.2%) with various developmental stages, up to myelocytes. Dysplastic changes were present within the granulocytic population, such as hypolobation, pseudo-Pulger-Huet cells, and hypogranularity. Megaloblastoid changes were seen frequently in the normoblast populations. The core biopsy revealed a myeloid-erythroid (M/E) ratio of 6:1 with predominance of myelomonocytic cells. A few clusters of immature myelomonocytic precursors were present in the intertrabecular areas. Eosinophilia was also demonstrated.

FLOW CYTOMETRIC FINDINGS

Results in the bone marrow were as follows: CD13-CD33, 82%; CD14, 34%; CD34, 12%; human leukocyte antigen-DR (HLA-DR), 31%; CD45, 100% (Fig. 6.3.1).

IMMUNOHISTOCHEMISTRY AND CYTOCHEMISTRY

Immunohistochemical stain for CD68 (PG-M1) and α-naphthyl butyrate esterase stain highlighted the large population of monocytic series in bone marrow biopsy and bone marrow aspirate, respectively.

MOLECULAR GENETIC FINDINGS

Cytogenetic study demonstrated a 46,XY karyotype, and fluorescence in situ hybridization study showed no rearrangement of the translocation-Ets-leukemia (TEL) ETS variant gene 6 (or ETV6) locus associated with a translocation t(5;12).

DISCUSSION

As discussed in Cases 1 and 2, chronic myeloproliferative disorder (CMPD) and myelodysplastic syndrome (MDS) can be demonstrated separately in the myeloid series. However, there are cases that present with features of both CMPD and MDS. For instance, a study of >500 MDS patients according to the French-American-British (FAB) scheme revealed that 4.4% of cases had features of both MDS and CMPD (1). This is a dilemma for hematologists in terms of management of these patients. Recently, the World Health Organization (WHO) classification created a new entity: Myelodysplastic/myeloproliferative diseases (MDS/MPD) that include diseases presenting with both MDS and CMD features (2). This new entity allows clinicians to have more flexibility in treating patients according to the predominant feature. For instance, chronic myelomonocytic leukemia was previously classified as MDS in the Revised European-American Classification of Lymphoid Neoplasms (REAL classification) (3), but is now included in this new entity and can be treated as MDS or CMPD according to its major clinical features.

Historically, the FAB was the first group to recognize the existence of such diseases, and in 1994 classified them under the name of chronic myeloid leukemias, including chronic granulocytic leukemia, atypical chronic myeloid leukemia (aCML), and chronic myelomonocytic leukemia (CMML) (4). The WHO scheme separates the Philadelphia chromosome-positive chronic myeloid leukemia

(CML) from aCML and CMML. The latter two entities are consistently Philadelphia chromosome-negative, and are designated as MDS/MPD. In addition, the WHO classification includes juvenile myelomonocytic leukemia (JMML) in the MDS/MPD category.

Morphology

The general feature of MDS/MPD is proliferation of one or more of the myeloid lineages in the bone marrow (2,5). This proliferation may produce increased numbers of circulating cells in one or more cell lineages. On the other hand, one or more of the other lineages may be dysplastic, leading to ineffective hematopoiesis in the peripheral blood, such as anemia, leukopenia, or thrombocytopenia. The blast count in the peripheral blood and bone marrow should be <20%. Splenomegaly and hepatomegaly are common.

In this category, CMML is the most common disorder, accounting for 3/100,000 persons annually (2,5,6). It is characterized by a substantial monocytosis in both the

peripheral blood and the bone marrow (Table 6.3.1). The peripheral blood monocyte count should be >1000/μL (Fig. 6.3.2), and the monocyte count in bone marrow should be >10% (Fig. 6.3.3). Immature myeloid cells (myelocytes and metamyelocytes) can be present in the peripheral blood, but they are usually <10%. Unlike CML, CMML shows myelodysplastic changes in one or more myeloid lineages, but the Philadelphia chromosome or breakpoint cluster region/Ableson (BCR/ABL) fusion is not present. If myelodysplasia is absent or minimal, the diagnosis of CMML can still be made when peripheral monocytosis has lasted for >3 months, other causes of monocytosis have been excluded, and clonal cytogenetic abnormality is present in bone marrow cells (2). Unlike acute myeloid leukemia, CMML has <20% blasts in the blood or bone marrow.

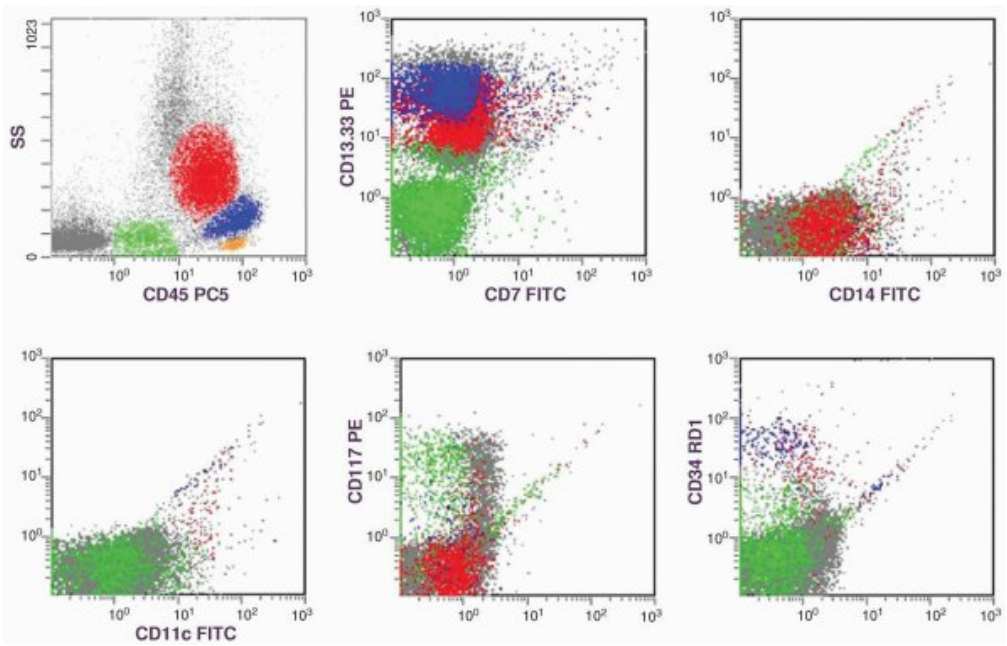

FIGURE 6.3.1 Flow cytometric histograms show three populations: the *blue* and *red* populations are mature and immature monocytes, and the *green* population represents immature myelocytes. The monocyte populations are positive for CD13.33, CD14, CD117, and CD34. The myelocytes are positive for CD13.33, CD117, and CD34. Both populations are negative for CD11c. SS, side scatter; PC5, phycoerythrin-cymin 5; FITC, fluorescein isothiocyanate; RD1, rhodamine.

If the blasts in the blood are 5% to 19% or 10% to 19% in the bone marrow, it is classified as CMML-2, whereas cases with <5% blasts in the blood and <10% in the bone marrow

are classified as CMML-1. The M/E ratio in CMML is lower than that in CML and aCML, as there are >15% of erythroid components in the bone marrow (6). Mild basophilia and mild eosinophilia can be seen in some cases. If eosinophilia is striking (>1,500/μL), it becomes the variant of CMML with eosinophilia, which is associated with specific symptoms due to eosinophilic degranulation and may be associated with a specific karyotype, t(5:12) (7,8). The spleen and lymph node can be infiltrated by the myelomonocytic cells. Some patients may have generalized lymphadenopathy. These lymph nodes are characterized by extensive infiltration by plasmacytoid monocytes (9, 10, 11 and 12). The plasmacytoid monocytes have round nuclei, finely dispersed chromatin, inconspicuous nucleoli, and eosinophilic cytoplasm, but their clonal relationship with the neoplastic cells has not been proven.

TABLE 6.3.1

Salient Features for Laboratory Diagnosis of Chronic Myelomonocytic Leukemia

1. Persistent peripheral monocytosis (>1,000/μL) for >3 mo
2. Presence of immature myeloid cells (<10%) in peripheral blood
3. Bone marrow monocytosis (>10%)
4. Myelodysplasia ≥1 myeloid lineages
5. No Philadelphia chromosome or breakpoint cluster region/Ableson (BCR/ABL) fusion gene, but cytogenetic abnormality is present in 20% to 30% of cases
6. Fewer than 20% blasts (myeloblasts, monoblasts, and promonocytes included) in blood and bone marrow

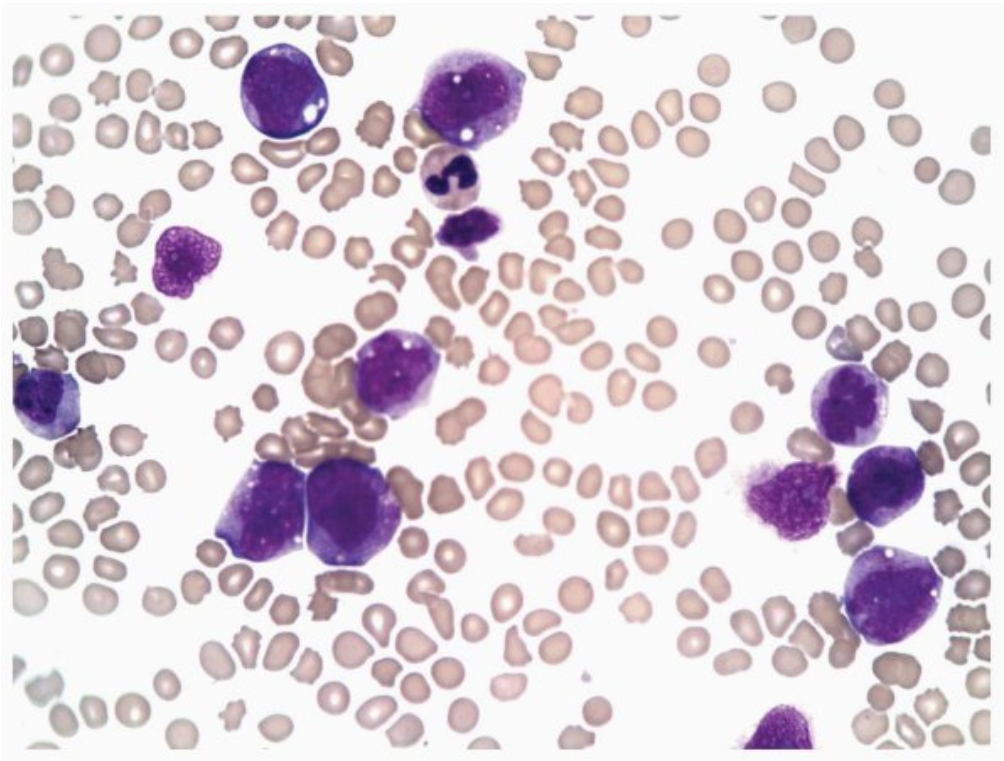

FIGURE 6.3.2 Peripheral blood smear from a case of chronic myelomonocytic leukemia (CMML)-2 shows monocytes of different developmental stages. Wright-Giemsa, 60× magnification.

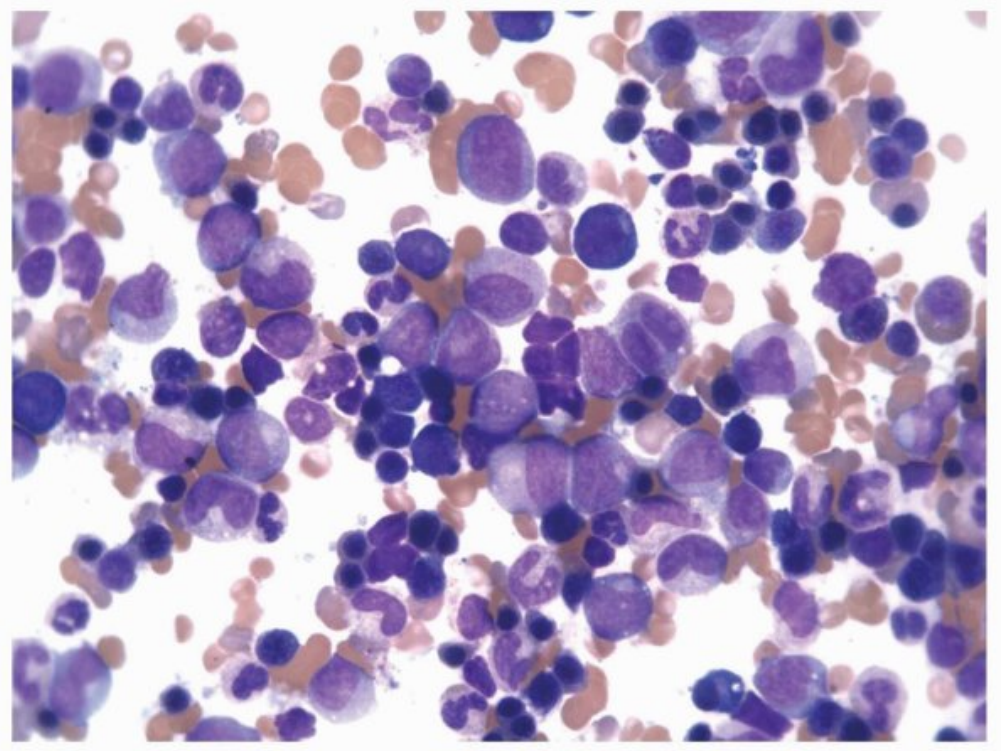

FIGURE 6.3.3 Bone marrow aspirate from a case of chronic myelomonocytic leukemia (CMML)-2 shows many mature and immature monocytes. Wright-Giemsa, 60× magnification.

TABLE 6.3.2

Salient Features of Laboratory Diagnosis of Atypical Chronic Myeloid Leukemia

1. Peripheral leukocytosis with >10% immature myeloid cells
2. Prominent dysplasia in ≥1 myeloid lineages
3. No or minimal absolute monocytosis (<10% in blood)
4. <20% blasts in blood and bone marrow
5. No Philadelphia chromosome or breakpoint cluster region/Ableson leukemia virus (BCR-ABL) fusion gene

aCML has clinical, laboratory, and morphologic features similar to those of CML, but it differs from CML in the absence of the Philadelphia chromosome, BCR/ABL fusion, and the presence of dysplastic changes in one or more myeloid cell lines (Table 6.3.2) (2,5,6,13). aCML can be distinguished from CMML by lower percentage of monocyte count (<10%) and higher percentage of immature myeloid cells (>10%) in the peripheral blood (Fig. 6.3.4). The blast count is usually <5% in the peripheral blood and always <20% in the bone marrow. Because of marked myelopoiesis, the M/E ratio in the bone marrow is frequently >10:1 (Fig. 6.3.5).

As the name indicates, JMML is seen in children <14 years, and 75% of patients are <3 years (2,5,6,14, 15, 16 and 17). It has the lowest incidence in the MDS/MPD group (1.3 per million children per year) (2), but it is most common in pediatric patients with

myeloproliferative syndrome (18). Despite the age difference, JMML is similar to CMML, and some authors consider these two diseases to be synonymous.

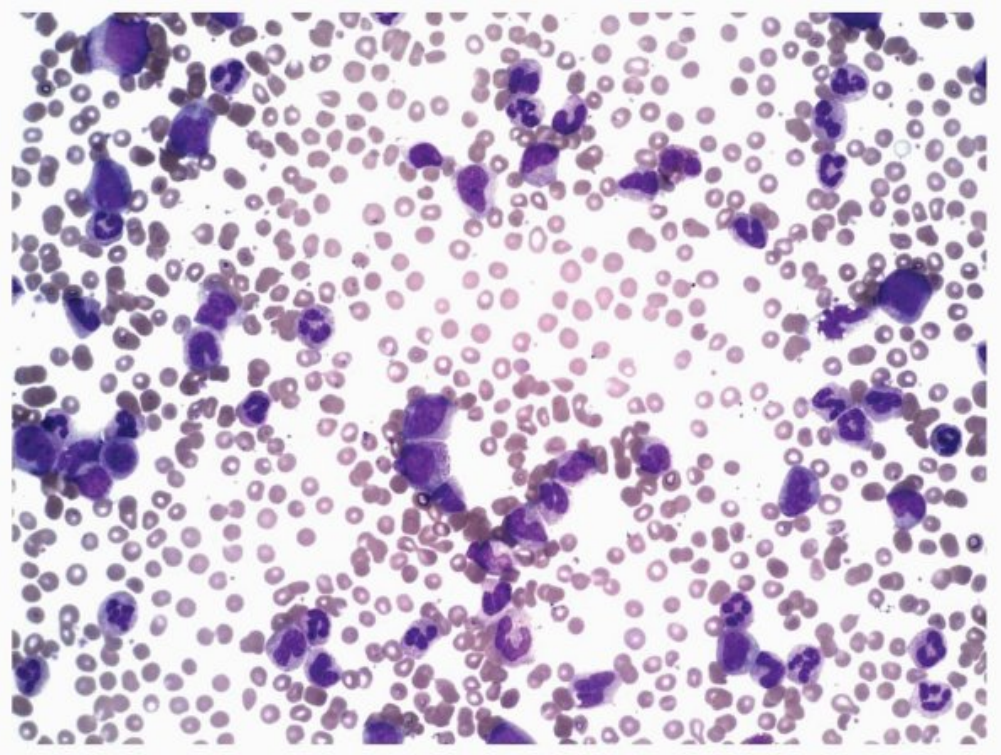

FIGURE 6.3.4 Peripheral blood smear from a case of atypical chronic myeloid leukemia (aCML) shows marked leukocytosis with several immature forms. Wright-Giemsa, 40× magnification.

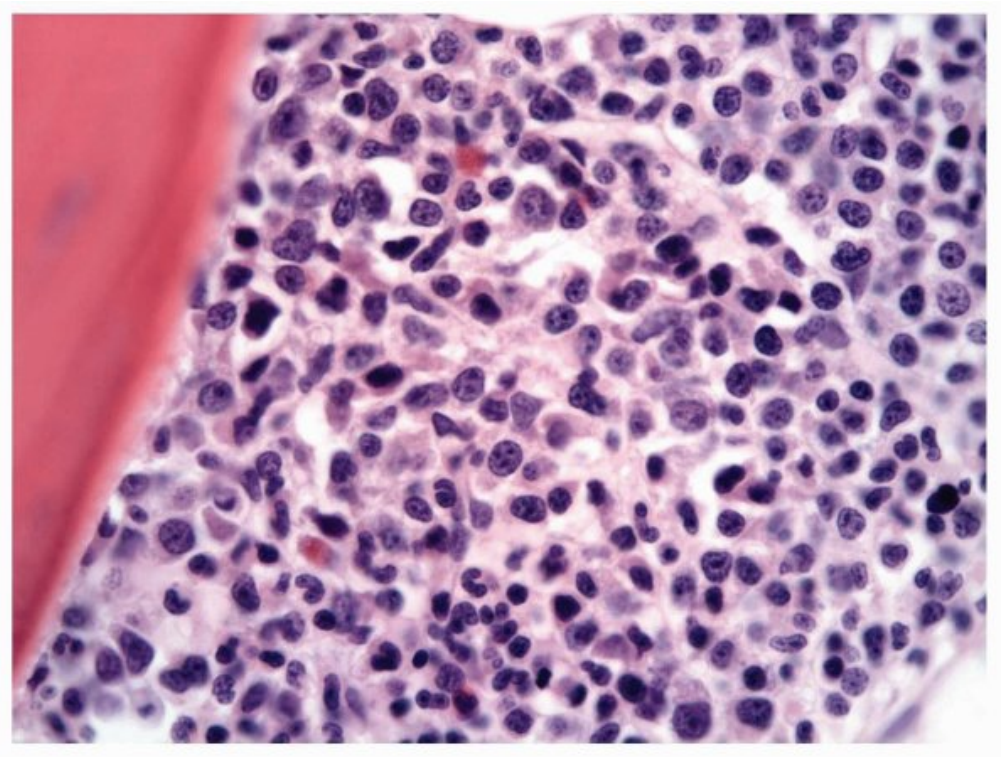

FIGURE 6.3.5 Bone marrow core biopsy shows hypercellularity with a high myeloid-erythroid (M/E) ratio. Hematoxylin and eosin, 60× magnification.

The major laboratory features include peripheral leukocytosis (>10,000/μL), and monocytosis is present in the peripheral blood (>1,000/μL) and bone marrow (5% to 10%) (Table 6.3.3). Immature myeloid cells are also present in the peripheral blood. As in other MDS/MPD entities, the Philadelphia chromosome and BCR-ABL fusion are absent. Unlike CMML, myelodysplastic changes usually are not prominent, and the total leukocyte count is usually higher (2,6,18). Blasts, including promonocytes, are <20% in the peripheral blood and bone marrow, distinguishing JMML from acute myelomonocytic leukemia. However, myelomonocytic cells may infiltrate the skin, lung, liver, and spleen, mimicking acute leukemia. In nearly 70% of patients with JMML, the hemoglobin F level is >10% and the hemoglobin A2 level is low. Experimentally, the JMML cells are able to form spontaneous granulocyte-macrophage colonies in vitro and they have marked hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulation in vitro (2,14). The differences between CMML, aCML, and JMML are summarized in Table 6.3.4.

TABLE 6.3.3

Salient Features for Laboratory Diagnosis of Juvenile Myelomonocytic Leukemia

1. Peripheral monocytosis (>1,000/μL)
2. Bone marrow monocytosis (10% to 20%)
3. <20% blasts (monoblasts and promonocytes) in blood and bone marrow
4. Peripheral leukocytosis of >10,000/μL with <10% immature myeloid cells
5. No Philadelphia chromosome or breakpoint cluster region/Ableson (BCR/ABL) fusion gene but abnormal karyotype frequently present
6. Hemoglobin F level >10% and low hemoglobin A_2 level

TABLE 6.3.4

Comparison of CMML, aCML, and JMML

Feature	*CMML*	*aCML*	*JMML*
Philadelphia chromosome	Negative	Negative	Negative
BCR-ABL	Negative	Negative	Negative
Peripheral monocytes	>1,000/μL	≥3% to <10%	>1,000/μL
Marrow monocytes	≥10%	≤3%	5% to 10%

Peripheral immature myeloid cells	≤10%	10% to 20%	≤10%
Peripheral basophils	<2%	<2%	<2%
Granulocytic dysplasia	Marked	Very marked	Minimal
Marrow erythroid precursors	>15%	Low percentage	Low percentage

CMML, chronic myelomonocytic leukemia; aCML, atypical chronic myeloid leukemia; JMML, juvenile myelomonocytic leukemia; BCR/ABL, breakpoint cluster region/Ableson.

Immunophenotype and Cytochemistry

In one flow cytometric study of CMML, it was found that the monocytes showed decreased expression of monocyteassociated antigens CD13, CD15, CD36, and HLA-DR and aberrant expression of nonmyelomonocytic antigens CD2 and CD56 (19). Aberrant expression of two or more antigens may help distinguish CMML from reactive monocytosis. CMML cases also showed a significantly higher percentage (>20%) of CD14 (moderate) bone marrow monocytes as compared with cases of reactive monocytosis (19). CD14 (moderate) monocytes are CD45 dim, so they represent immature monocytes, whereas mature monocytes are CD14 (strong). The combination of two or more immunophenotypic aberrations and ≥20% CD14 (moderate) bone marrow monocytes was 67% sensitive and 100% specific for the diagnosis of CMML (19).

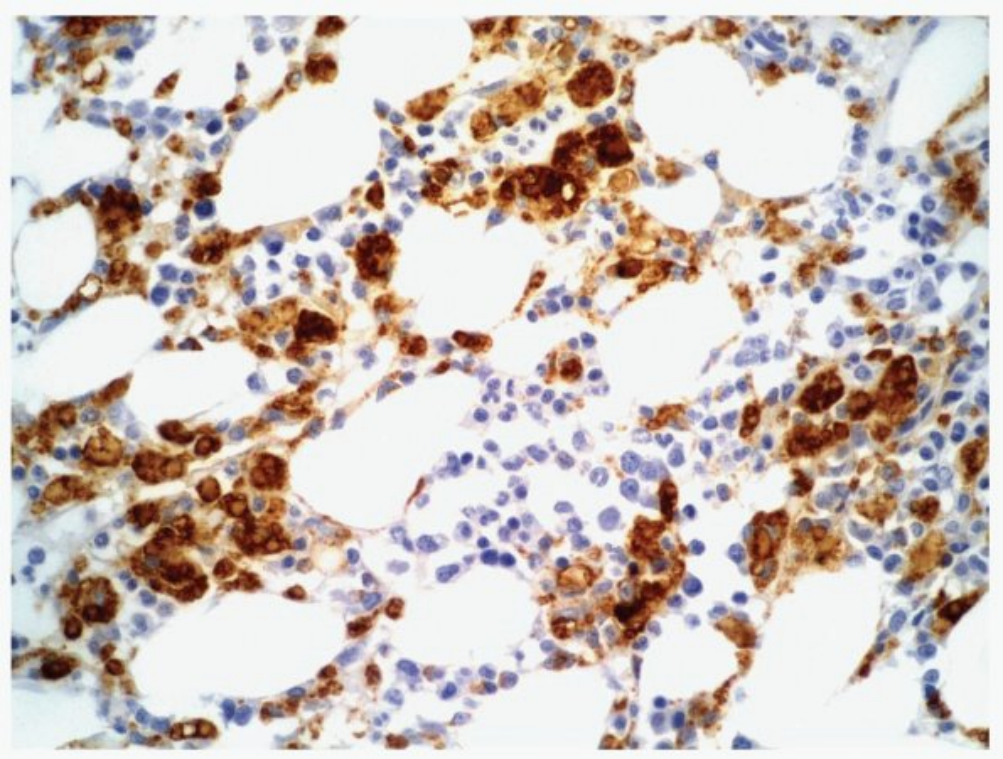

FIGURE 6.3.6 Bone marrow core biopsy from a case of chronic myelomonocytic leukemia (CMML) shows positive CD68 PG-M1 staining in many monocytes. Immunoperoxidase, 40× magnification.

To distinguish CMML from acute myelomonocytic leukemia, the blasts count is the major criteria and 20% of blasts is the cut-off point.

Yang et al. (20) suggested using a panel of markers to separate different stages of monocytes. The entire monocyte population can be isolated by dual bright CD33 and CD64 staining. CD64 is positive for all mature and immature monocytes. CD14 has two epitopes: My4 and Mo2. My4 is present in mature monocytes and promonocytes. Mo2 is only expressed by mature monocytes. Therefore, dual staining with these markers can separate mature monocytes from promonocytes and monoblasts. The plasmacytoid monocytes in the lymph nodes of CMML cases also show a specific immunophenotype. These cells are positive for CD4, CD14, CD43, CD56, and CD68 (9, 10, 11 and 12).

There have been no reports of specific immunophenotypes for aCML and JMML. However, the immunophenotype as described in CMML is also applicable to the monocytes in JMML. In aCML the myeloid cells can be identified by CD13 and CD33, and myeloperoxidase and myeloblasts can be estimated by CD34 and CD117. Monoblasts, however, are negative for CD34 and CD117 in many cases.

Immunohistochemical stains, such as CD33, CD68 (Fig. 6.3.6), and myeloperoxidase, are helpful in demonstrating the myelomonocytic components. However, cytochemical stains with myeloperoxidase, lysozyme, α-naphthyl acetate esterase and α-naphthyl butyrate esterase (Fig. 6.3.7) on peripheral blood or bone marrow smears are most useful in distinguishing myelocytes from monocytes. Leukocyte alkaline phosphatase scores are decreased in 50% of JMML cases, but are variable in aCML cases (2).

Comparison between Flow Cytometry and Immunohistochemistry

Morphologic identification of various abnormal monocytes is difficult and subject to significant variations between observers. Therefore, immunophenotype and cytochemical stains are of utmost importance to facilitate an accurate count of monocytes. With a large panel of myelomonocytic markers, flow cytometry can isolate monocytes from myelocytes and identify different developmental stages, so that it is most helpful in substantiating the diagnosis of CMML and JMML. Cytochemical staining with esterases has the advantage of correlating the markers with morphology, but some immature monocytic cells may not show nonspecific esterase staining thus underestimating the monocyte count.

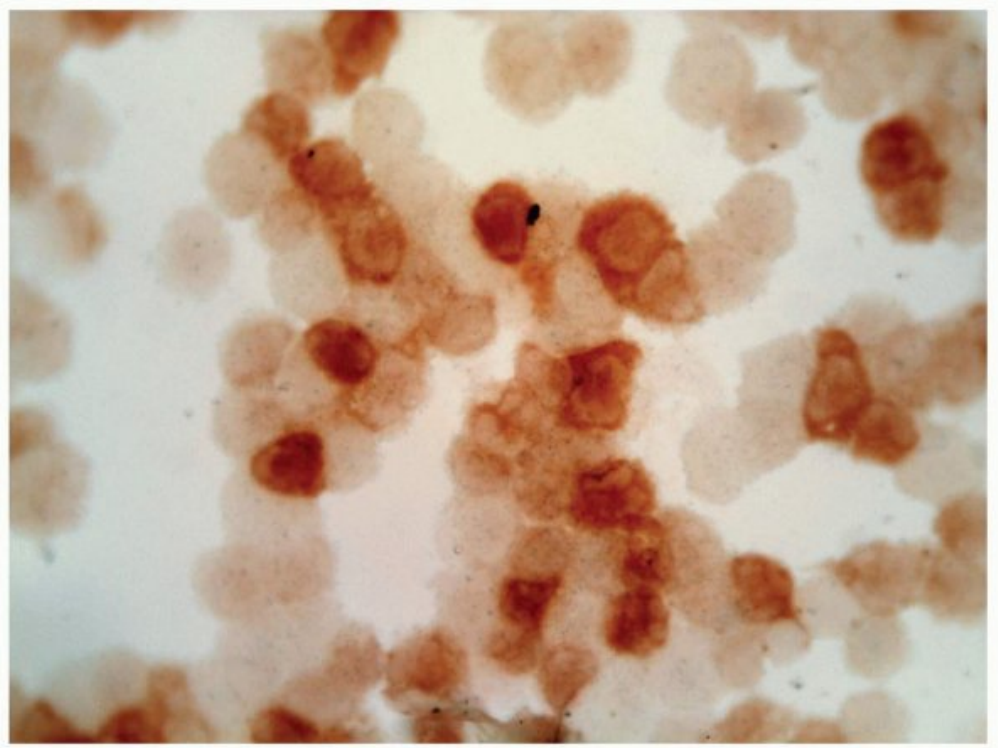

FIGURE 6.3.7 Cytospin smear of bone marrow aspirate from a patient of juvenile myelomonocytic leukemia (JMML) shows positive staining of α-naphthyl butyrate esterase in many monocytes. Cytochemical stain, 60× magnification.

Immunohistochemical staining is not helpful in distinguishing myelocytes and monocytes, because most markers stain both populations. CD68 (KP1) stains both myelocytes and monocytes, but CD68 (PG-M1) stains only monocytes, so the latter marker is more helpful in the differential diagnosis.

Molecular Genetics

Approximately 20% to 30% of CMML cases have aberrant karyotypes, including trisomy 8, del(20q), monosomy 7, and del(11q), but these abnormalities can also be seen in other myeloproliferative or myelodysplastic disorders (2,6). However, a subset of CMML, CMML with blood and marrow eosinophilia, is associated with t(5:12)(q33;p13), which is specific (7,8). Molecular characterization has revealed that this translocation involves the TEL gene on chromosome 12 and the platelet-derived growth factor receptor (PDGFR) gene on

chromosome 5 (21). The resultant TEL-PDGFR fusion transcript may play an important role in the proliferative process, probably through the deregulation of an oncogene derived from rat sarcoma virus (RAS) (6). Point mutation of RAS genes has been found in as many as 40% of CMML patients (2). Translocations of the PDFGR gene to other partner genes have also been reported (22). A recent report demonstrated recurrent somatic activating

mutation in the Janus kinase 2 (JAK2) tyrosine kinase in 9 of 116 CMML/aCML cases (23). CCAAT/enhancer binding protein α (CEBPα) gene mutation is involved in CMML cases transforming into acute myeloid leukemia (24).

As many as 80% of patients with aCML have cytogenetic abnormalities, including +8, +13, +14, del(20q), i(17q), and del(12q), but none of them are specific (2,6). As mentioned above, JAK2 mutation has also been found in aCML patients (23). t(9;15;12) translocation involving the ETV6 gene at 12q13 and the JAK2 gene at 9q24 has been reported in a case of aCML in transformation (25).

Cytogenetic abnormalities occur in 30% to 40% of JMML patients, but none of them are specific (2). Monosomy 7 is frequently associated with JMML, but the relationship of JMML and childhood monosomy 7 syndrome is uncertain (14). The most important genetic finding in JMML is point mutation of the RAS gene, which is present in 20% to 30% of patients (2,6). These point mutations may induce the increase of intracellular levels of RAS-guanosine 5″-triphosphate (GTP), which may alter the RAS signaling pathway. In JMML patients with neurofibromatosis type 1 (NF1), loss of the normal NF1 allele is a common finding in the leukemic cells. The normal NF1 protein, nerofibromin, down-regulates RAS-GTP. Inactivation of NF1 may deregulate the RAS pathway. In addition, somatic mutations of PTRN11 have been reported in 35% of JMML patients (26).

The current case showed both myeloproliferative and myelodysplastic features with peripheral and bone marrow monocytosis, so it was consistent with CMML morphologically. The positive α-naphthyl butyrate esterase staining in the bone marrow aspirate smear and positive CD68 (PGM1) staining in the core biopsy further confirmed this diagnosis. In addition, the patient also had eosinophilia in the peripheral blood and bone marrow. CMML with eosinophilia is associated with t(5:12), but it occurs in only 1% to 2% of such cases (2).

Clinical Manifestations

Patients with CMML may have fatigue, weight loss, fever, and night sweats (2). In addition, they may have recurrent infections due to dysfunctional leukocytes and hemorrhages due to thrombocytopenia. Many patients have splenomegaly and/or hepatomegaly (2,6). The survival time of these patients may vary from 1 month to >100 months with a median survival of 20 to 40 months in most studies (2). The most important prognostic predictor is the number of blasts.

There are no specific clinical symptoms reported in aCML cases. Those presenting symptoms may be related to anemia, thrombocytopenia, or splenomegaly. The median survival times reported are <20 months (2). About 25% to 40% of aCML cases evolve into acute leukemia.

Clinical symptoms in JMML patients include malaise, pallor, fever, or other evidence of infection (2,6). Other symptoms may be related to bleeding or pulmonary involvement. A maculopapular skin rash may be present in ≤50% of patients. Splenomegaly is virtually always present. Hepatomegaly and lymphadenopathy are found in more than one half of patients (6). Patients with JMML and those with childhood monosomy 7 syndrome have similar clinical symptoms. JMML without monosomy 7 syndrome tend to have higher hemoglobin F levels, more prominent lymphadenopathy, and more severe skin rashes than do patients with monosomy 7 syndrome, whereas the latter have a tendency to develop leukopenia and bacterial infections (14). The median survival times vary from 5 months to >4 years, depending on the treatment the patients received (2). If untreated, 30% of patients die within 1 year of diagnosis.

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CASE 4 Acute Myeloid Leukemia with t(8;21)(q22;q22)

CASE HISTORY

A 19-year-old man presented with a 4-week history of sore throat, fever to 103°F, lethargy, chest congestion, and flu-like symptoms. The day before admission, he noticed the onset of widespread petechiae. He had been seen in the clinic 1 week before, and was given erythromycin for a presumed upper respiratory infection. Physical examination showed pallor, petechiae, diffuse lymphadenopathy with 4+ tonsillar hypertrophy, and mild hepatosplenomegaly. Hematology workup revealed a total leukocyte count of 128,000/μL with 97% blasts, 2% segmented neutrophils, and 1% lymphocytes. The hematocrit was 36% and platelet count, 23,000/μL. Lactate dehydrogenase was 555 U/L. A bone marrow biopsy and aspirate were performed.

FLOW CYTOMETRY FINDINGS

Blood: Myeloid markers: myeloperoxidase 87%, CD13-CD33 97%. Monocyte marker: CD14 0%. Activation antigen: HLA-DR 0%. T-cell marker: CD7 0%. Stem cell marker: CD34 20%.

Bone marrow: Myeloid markers: myeloperoxidase 100%, CD13-CD33 93%. Monocyte marker: CD14 59%. Activation antigen: HLA-DR 86%. T-cell marker: CD7 0%. B-cell marker: CD19 0%. Stem cell marker: CD34 60% (Fig. 6.4.1).

CYTOCHEMICAL FINDINGS

The blasts were positive for myeloperoxidase and chloroacetate esterase, but negative for α-naphthyl butyrate esterase.

MOLECULAR GENETIC STUDIES

Fluorescence in situ hybridization (FISH) for t(15;17) was negative. Karyotyping showed t(8;21)(q22;q22) (Fig. 6.4.2).

DISCUSSION

In the current case, the peripheral blood showed many blasts containing multiple Auer rods, and the bone marrow revealed 92% blasts with >10% of type 3 blasts (blasts that contain >20 cytoplasmic granules) (Figs. 6.4.3 and 6.4.4). The presence of hypergranular myeloid cells with multiple Auer rods and an immunophenotype of negative HLA-DR misled us to consider acute promyelocytic leukemia and triggered the order of FISH for t(15;17), the results of which were negative. The karyotype of t(8;21) together with the above described morphology finally provided a definitive diagnosis of acute myeloid leukemia with maturation (AML-M2). The immunophenotyping of the bone marrow showed a normal percentage of HLA-DR,

indicating that the absence of HLA-DR in the immunophenotype of the peripheral blood specimen was probably a technical error. The identification of this particular karyotype is clinically important because it confirms the diagnosis of AML even when the blast count in the bone marrow is <20% (1). It also confers a favorable prognosis. The presence of type 3 blasts defines the leukemia as AML-M2 irrespective of whether the mature myeloid cell count is below or above 10% (2).

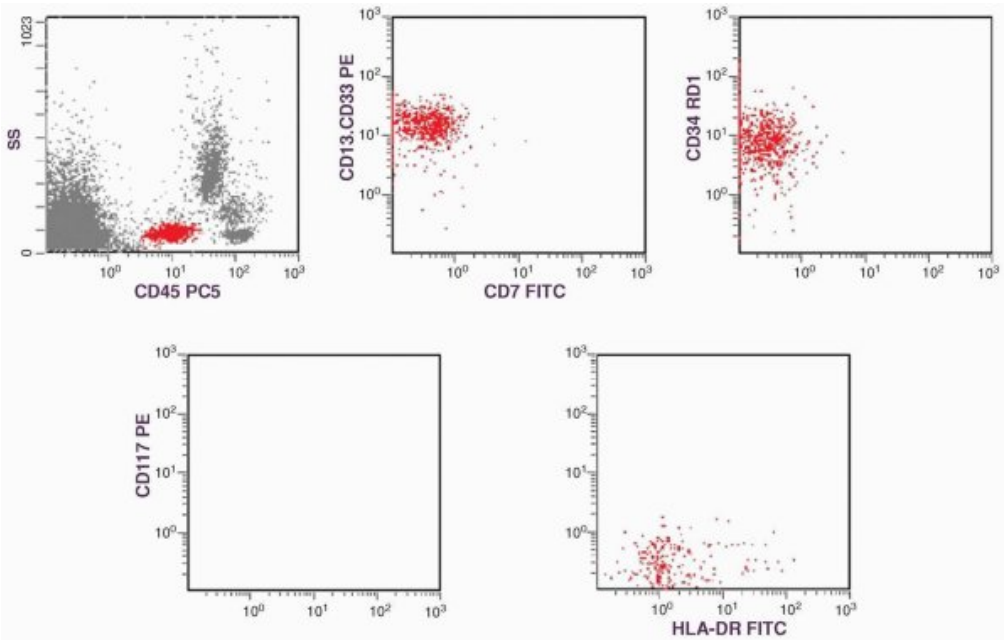

FIGURE 6.4.1 Flow cytometric analysis of bone marrow shows positive CD13.CD33, CD34, and HLA-DR, but negative CD7 and CD117. Note that a large population of mature myeloid cells is present above the gated acute myeloid leukemia (AML) population. ss, side scatter; pc, phycoerythrin-cyanin 5; PE, phycoerythrin; RD1, rhodamine; FITC, fluorescein isothiocyanate.

Morphology

The hematologic features in this particular genotype are characterized by abundant Auer rods (Fig. 6.4.5) or Auer rods with a single long and sharp rod with tapered ends, strong myeloperoxidase activity, salmon-colored cytoplasmic granules, and a rim of basophilic cytoplasm in maturing leukemic cells, large cytoplasmic vacuoles, and bone marrow eosinophilia (3). The eosinophils may show periodic acid-Schiff (PAS)-positive granules. Nucifora et al. (4) added two more parameters: the French-American-British (FAB) M2 subtype and cells containing pink, waxy inclusions approximately 2 to 3 μm in diameter, as the seven predictive criteria for a t(8;21) or AML/ETO (eight twentyone) translocation.

Andrieu et al. (5) developed a weighted score system including FAB-M2 subtype, Auer rods, pseudo-Chediak-Higashi anomaly (Fig. 6.4.6), marrow eosinophilia, large blasts with prominent Golgi (Fig. 6.4.7), and abnormal cytoplasmic granules. The sensitivity of this system is claimed to be 100%, but the false-positive rate is 7%.

In addition, >10% type 3 blasts may be present in AML-M2 including this special subtype (2). Auer rods can be demonstrated in mature granulocytes as well as eosinophils (1). Myelodysplastic changes, such as pseudo-Pelger-Huet cells and hypogranular neutrophils, may be demonstrated in the myeloid series. The special morphologic features are summarized in Table 6.4.1.

The cytochemical characteristics are similar to other myeloid leukemia showing the presence of positive reaction to myeloperoxidase and chloroacetate esterase, but absence of α-naphthyl butyrate esterase.

AML cases with t(8;21) have a high frequency of developing into myeloid sarcoma (6). In a study of 84 patients with t(8;21), 8 had extramedullary myeloid leukemia, mainly involving the spinal cord (7).

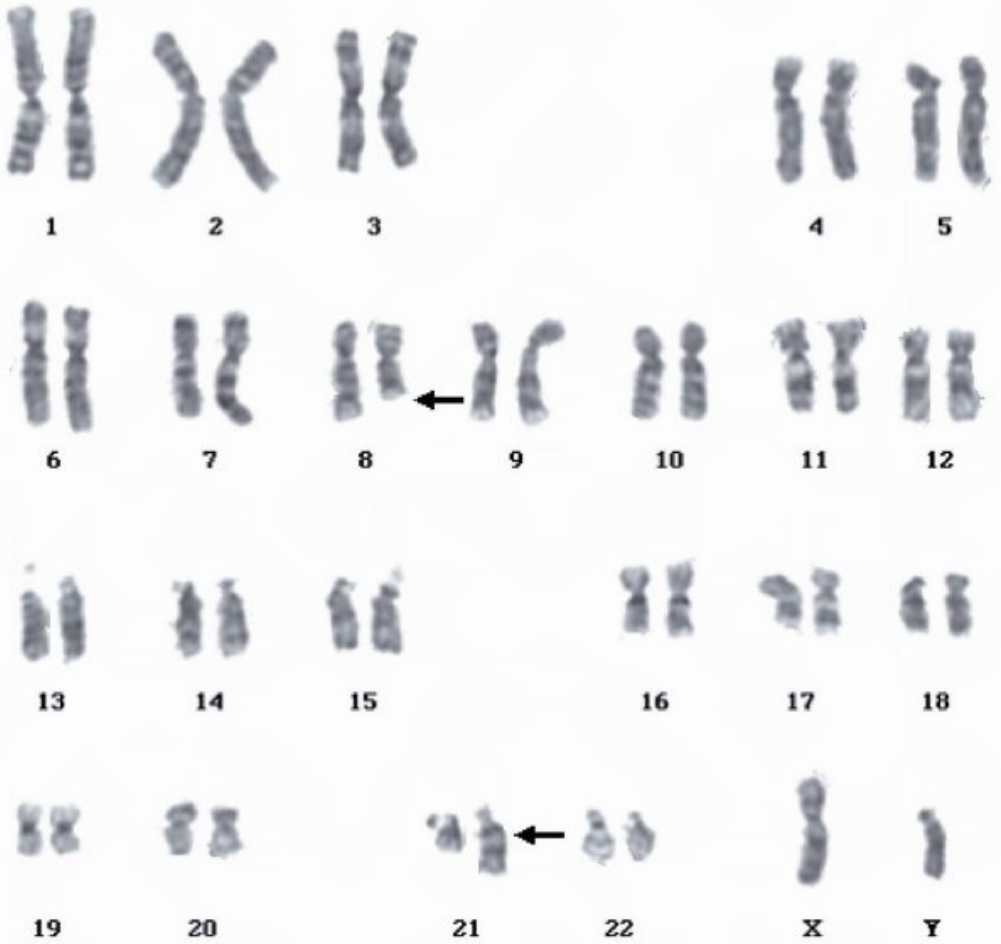

FIGURE 6.4.2 Karyotype of bone marrow reveals t(8;21)(q22;q22) (*arrows*). (Courtesy of Peter Papenhausen, Ph.D., LabCorp of America Cytogenetic Department, North Carolina.)

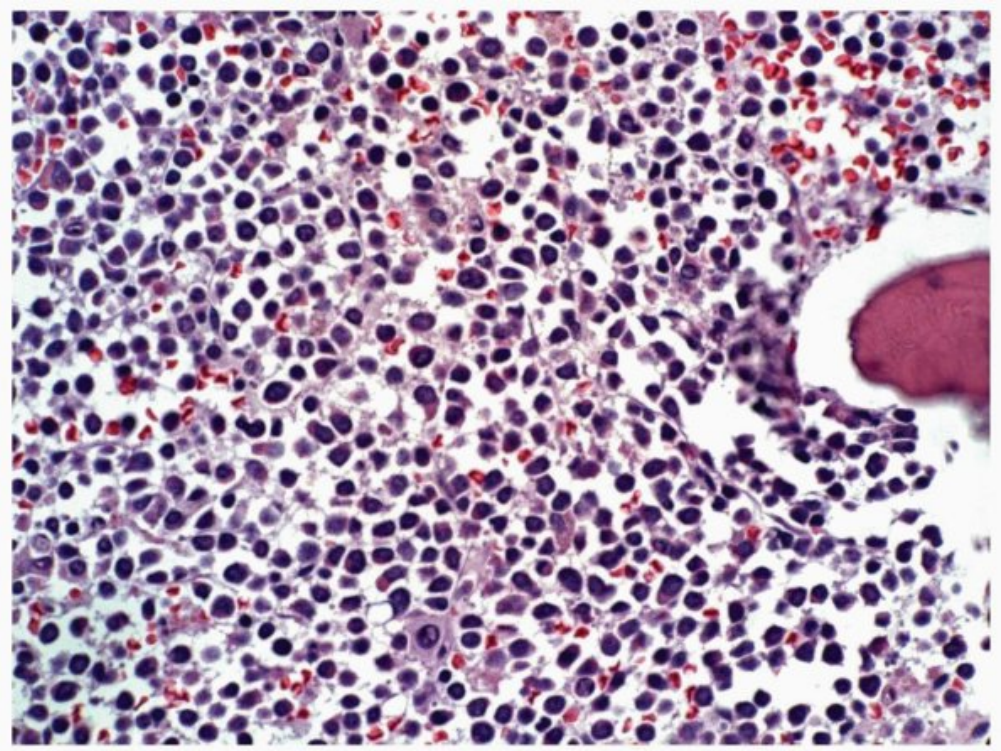

FIGURE 6.4.3 Bone marrow core biopsy shows extensive immature myeloid cell infiltration replacing normal hematopoietic cells. No normoblasts and megakaryocytes are present. Hematoxylin and eosin, 40× magnification.

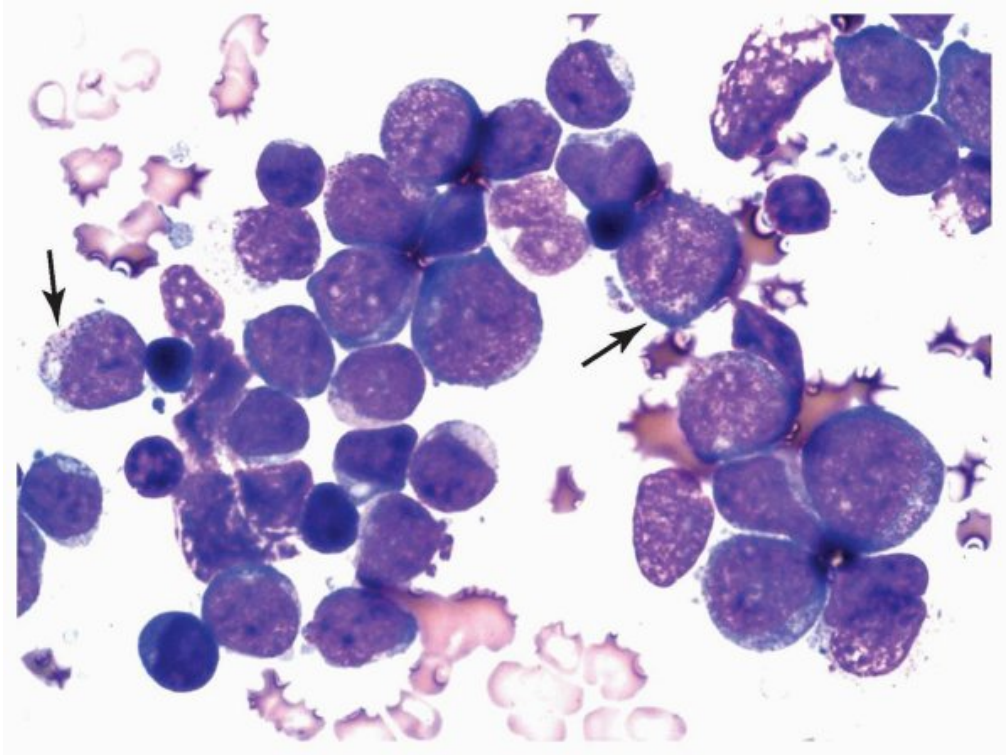

FIGURE 6.4.4 Bone marrow aspirate reveals a cluster of myeloblasts with several showing >20 cytoplasmic granules (type 3 blasts) (*arrows*). Wright-Giemsa, 100× magnification.

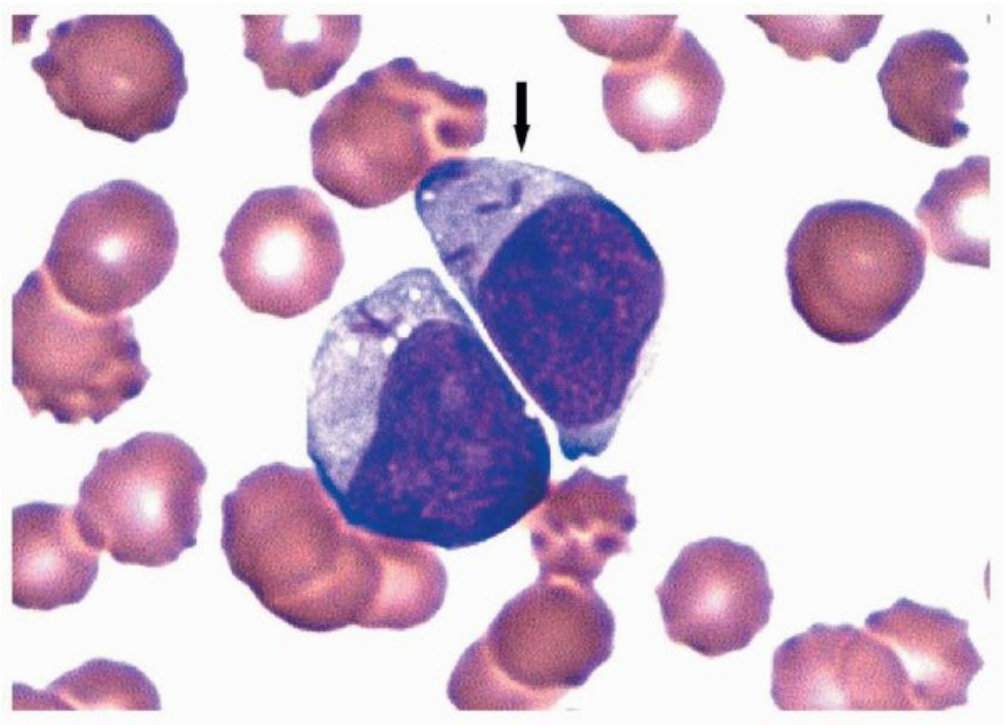

FIGURE 6.4.5 Peripheral blood smear shows two immature myeloid cells with multiple Auer rods (*arrow*). Wright-Giemsa, 100× magnification.

Immunophenotype

An AML case with t(8;21) expresses the same myeloid antigens (such as CD13, CD15, CD33, and myeloperoxidase) as other AML subtypes (1). However, there are also some specific markers for this special subtype. The most important one is the B-cell antigen, CD19, which is present in a subset of blasts (1,8). Another unusual marker is a natural killer cell marker, CD56, which is not as frequently demonstrated as CD19, but its presence confers an adverse prognosis (9). The stem cell marker CD34 is characteristically present and may help to identify its malignant nature. C-KIT gene mutation and overexpression are found in this special subtype of AML (10), but the expression of its protein, CD117, has not been documented in the literature.

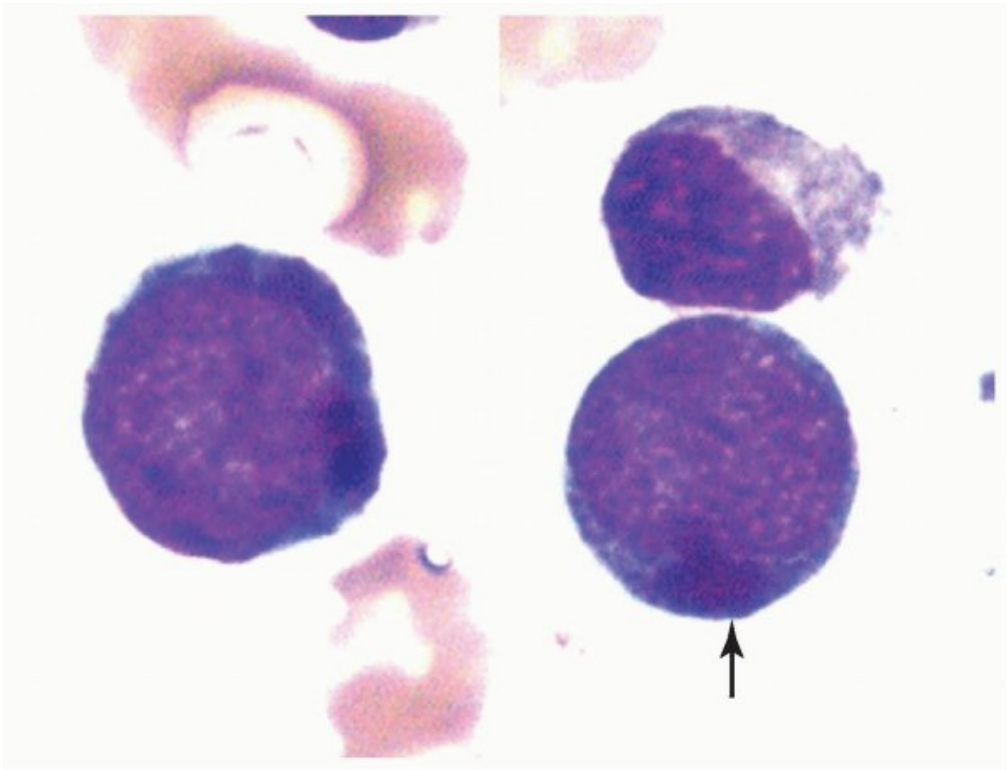

FIGURE 6.4.6 Bone marrow aspirate shows two myeloblasts containing large cytoplasmic lysosomes (pseudo-Chediak-Higashi anomaly) (*arrow*). Wright-Giemsa, 100× magnification.

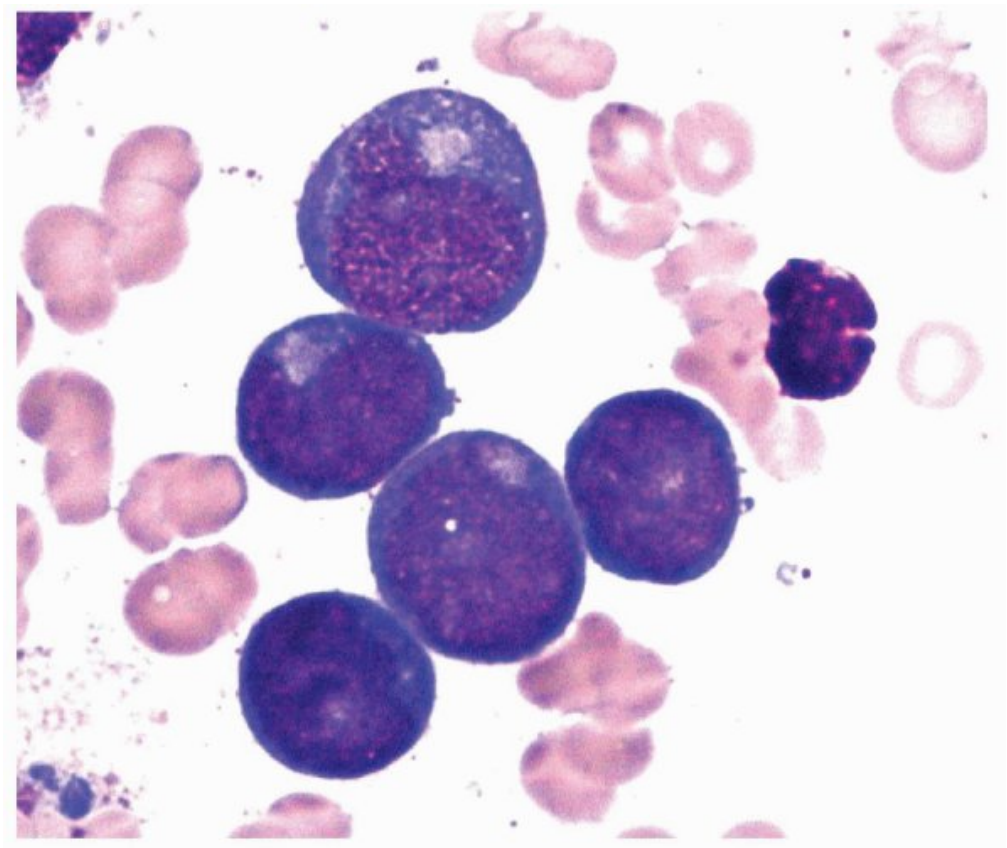

FIGURE 6.4.7 Bone marrow aspirate shows five myeloblasts with prominent Golgi. Wright-

Giemsa, 200× magnification.

In a study of 93 cases of AML with t(8;21), it was shown that the cases are characterized by a significantly higher expression of CD19, CD34, CD56, and CD54 than are other AML subtypes with normal or other abnormal karyotypes (11). Conversely, CD45 RO, CD33, CD36, CD11b, and CD14 were significantly lower in t(8;21) cases than in controls. In other studies, however, CD33 was often expressed in low intensity (5,12,13). T-cell markers, such as CD2 and CD7, are rarely expressed.

One study found that the combination of CD19 and CD34 is most reliable in predicting t(8;21) (8). Using the cutoff of 10% for CD19 and 35% for CD34, this combination correctly classified 92 of 93 AML with t(8;21).

By using immunohistochemistry, the most exciting recent finding is the presence of the PAX5 protein (B-cell-specific activator protein [BSAP]) only in AML cases with

t(8;21), but it was positive in only one third of the cases studied (14). In some t(8;21) cases without a positive immunohistochemical staining for PAX5, up-regulation of PAX5 transcript was identified by real-time reverse transcription-polymerase chain reaction (RT-PCR) studies (14). PAX5 is the master regulator of B-lymphopoiesis through activation of B-cell-specific genes, including CD19 and CD79a (15,16). Therefore, it is not unexpected that CD19 and CD79a are also expressed, though in lower frequency, in PAX5-positive cases. As there is no CD19 monoclonal antibody for immunohistochemistry, the identification of these two B-cell markers, PAX5 and CD79a, is most useful in surgical pathology.

TABLE 6.4.1

Special Morphologic Features in Acute Myeloid Leukemia (AML) with t(8;21)(q22;q22)

1. French-American-British (FAB) AML with maturation (AML-M2) morphology, including type 3 blasts in some cases
2. Abundant Auer rods in mature and immature myeloid cells
3. Salmon-colored granules and a rim of basophilic cytoplasm in myeloid cells
4. Pseudo-Chediak-Higashi anomaly
5. Cells with pink, waxy cytoplasmic globules
6. Large blasts with prominent Golgi area
7. Cytoplasmic vacuoles
8. Bone marrow eosinophilia (>5%)

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry is usually more practical than immunohistochemistry in the study of blood and bone marrow specimens and is capable in identifying this special subtype of AML by showing CD19 and CD34 in addition to myeloid markers. However, immunohistochemistry is most helpful in diagnosing myeloid sarcoma derived from this subtype of AML by using myeloid markers together with PAX5 and CD79a.

Molecular Genetics

Molecular characterization has demonstrated that t(8;21) represents the fusion of the AML1 gene on chromosome 21q22 with the ETO gene on chromosome 8q22. The AML1 gene is also called core binding factor protein α (CBFα), RUNX1, and FEBP2. ETO is also called

MTG8.

The AML1 gene encodes the CBFα protein, which forms a heterodimer with CBFβ that plays an important role in normal hematopoietic differentiation (8,17,18). CBF also cooperates with other basic transcription factors in activating a set of hematopoietic specific genes. The ETO gene is the mammalian homolog of the Drosophila gene nervy, a transcriptional regulator with yet unknown biological function (8).

AML1/ETO encodes a fusion transcript with a primary inhibitory role in normal hematopoietic differentiation. It regulates the expression of both AML1 target and non-AML1 target genes via its interaction with various transcription regulators (17). However, t(8;21) alone cannot induce leukemia. Additional mutations are necessary for the development of AML (17).

Translocation (8;21) is one of the most common AML cytogenetic abnormalities, occurring in 7% to 8% of adult cases and 11.7% of pediatric cases (8). As mentioned before, most cases present with M2 morphology. The incidence ranges from one third to 46% for M2 cases with an abnormal karyotype (19,20). A study of childhood leukemia in a single institute showed that 82% of AML cases with t(8;21) were M2 cases and that 23% had granulocytic sarcoma (21). A German study revealed that, among AML cases with t(8;21), 12.5% were M2, 1.7% M1, 0.09% M3 to M7, and 0% M0 (22). Other studies reported t(8;21) in cases of M1, M4, M4Eo, chronic myeloid leukemia, and myelodysplastic syndrome (4,5,23, 24, 25, 26 and 27). However, in the light of the World Health Organization (WHO) definition, those cases of myelodysplastic syndrome should probably be classified as AML cases (1).

TABLE 6.4.2

Immunophenotypic and Molecular Genetic Features in Acute Myeloid Leukemia (AML) with t(8;21)(q22;q22)

1. Cluster of differentiation (CD)19 (>10%) and CD34 (>35%) in a myeloid population
2. Presence of CD56 predicting unfavorable prognosis with potential development of granulocytic sarcoma
3. Demonstration of PAX5 and CD79a by immunohistochemistry
4. Karyotype: t(8;21)(q22;q22)
5. Molecular biology: AML1/ETO (eight twenty-one)

The positive rate for this abnormality is higher when studied with molecular biology techniques (4,5,28). In a study of 64 patients, Andrieu et al. (5) detected 8% cases with t(8;21) by karyotyping, but 16% of cases showed AML1/ ETO by an RT-PCR assay. In a survey from the Cancer and Leukemia Group B (CALGB), AML1/ETO was detected in other abnormal karyotypes, such as t(8;10)(q22;q26) and t(1;10;8)(p22;p13;q22) (28). Other complex translocations such as t(8;12;21) and t(8;17;21) have been reported (29,30). By the FISH method, the presence of AML1/ETO in other karyotypes was found to be the result of cryptic insertion (28,31). The abnormality can be the AML1 gene inserts into 8q22 or ETO into 21q22. In a large number of patients, additional chromosome abnormalities or complex translocation are identified (1,8). The AML1/ETO fusion product can be detected by RT-PCR even when the patient is in remission for as long as 8 years (19). The immunophenotypic and molecular genetic features of AML with t(8;21) are summarized in Table 6.4.2.

Clinical Manifestation

AML-M2 with t(8;21) is frequently seen in patients younger than 60 years, particularly children. The special chromosomal abnormality is not usually seen in elderly AML patients, with a frequency <2% (8). The majority of cases with t(8;21) occur in primary de novo AML cases. However, it is also present occasionally in secondary AML patients.

The clinical symptoms of AML with t(8;21) are similar to those seen in other acute leukemias, namely bone marrow failure. A particular frequent presentation is granulocytic (myeloid) sarcoma, which involves solid organs, and the bone marrow may show <20% of myeloblasts (1). In one study, the complete remission rate in patients with myeloid sarcoma was 50% as compared to 94% in those without myeloid sarcoma (7).These patients also had a significantly shorter survival.

In general, t(8:21) confers a favorable prognosis; adult patients usually have a good response to chemotherapy, with high remission rates and long-term disease-free survival when treated with high dose cytarabine (1). Pediatric patients, however, have a much less favorable response than adult patients do (24).

Besides age and the presence of myeloid sarcoma, immunophenotype also affects the prognosis. The presence of CD56 usually relates to inferior disease-free survival (9). This study also found that myeloid sarcoma was present exclusively in cases with CD56 expression. The association of leukocyte count and prognosis in this entity is inconclusive. However, the so-called white blood cell (WBC) index, calculated as the product of WBC count by the percentage of blasts in the bone marrow, was a more reliable and independent predictor for relapse-free survival (32).

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CASE 5 Acute Myeloid Leukemia with inv(16)(p13q22) or t(16;16)(p13;q22)

CASE HISTORY

A 52-year-old man presented with shortness of breath, fatigue, and hypersomnolence for 5 months. He was transferred from another

hospital for evaluation of likely leukemia. Physical examination on admission was unremarkable except for pale conjunctivae, ecchymosis on the right hand, and a palpable cervical lymph node. There was no hepatosplenomegaly. Hematology workup showed a total leukocyte count of 38,400/µL with 57% blasts, 3% neutrophils, 32% monocytes, and 8% lymphocytes. A bone marrow biopsy revealed 51% myeloblasts, 24% monoblasts, 13% myeloid cells of various stages, 7% eosinophils, 8% monocytes, and 1.5% erythroid elements. He was then treated with cytarabine and daunorubicin. The clinical course was complicated with neutropenic fever, *Clostridium difficile* colitis, and possible candidiasis in the liver and spleen, as demonstrated by computed tomography (CT) imaging. All the complications were gradually gotten under control by antibiotic therapy and transfusion. The second bone marrow biopsy demonstrated no leukemic cells. The patient was discharged 1 month after admission.

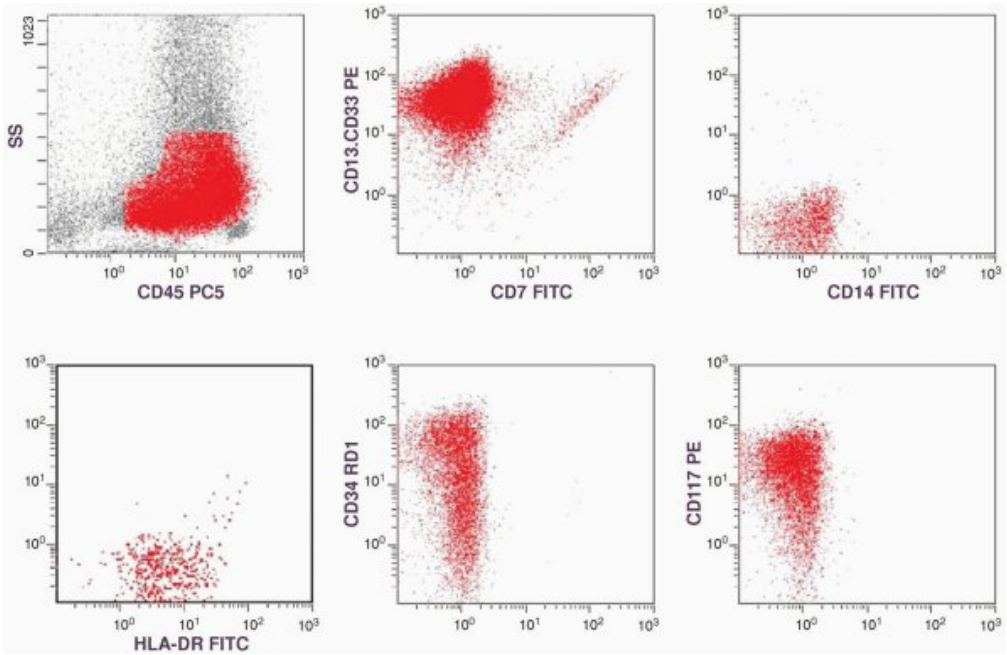

FIGURE 6.5.1 Flow cytometric analysis of bone marrow shows positive reactions with cluster of differentiation (CD)13.CD33, CD7, CD14, human leukocyte antigen-DR (HLA-DR), CD34, and CD117. ss, side scatter; PE, phycoerythrin, RD1, rhodamine; FITC, fluorescence in situ hybridization.

FLOW CYTOMETRIC FINDINGS

Bone marrow: Myeloid cells: Myeloperoxidase 97%, CD13-CD33 96%, CD14 34%, CD13-CD33/CD7 0%, and human leukocyte antigen-DR (HLA-DR) 88%. Stem cell markers: CD34 85%, CD117 96% (Fig. 6.5.1).

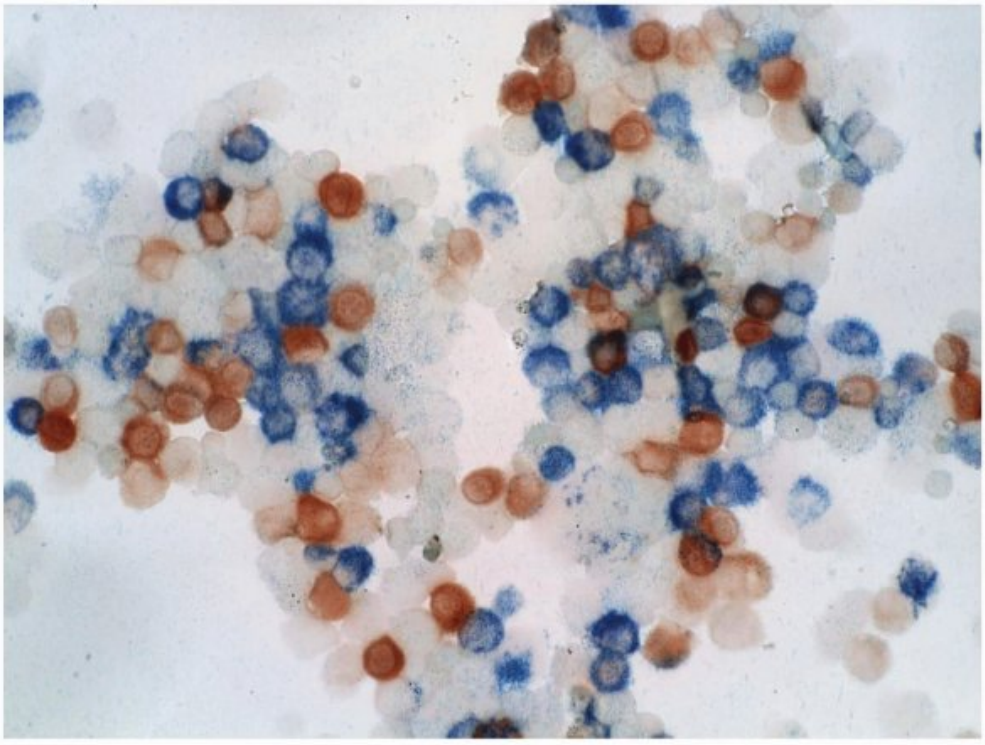

FIGURE 6.5.2 Combined esterase stain of the bone marrow cytospin shows chloroacetate esterase stain (*blue*) of the myeloid cells and α-naphthyl butyrate esterase stain (*brown*) of the monocytoid cells. 40x magnification.

CYTOCHEMICAL FINDINGS

In the bone marrow, the myeloperoxidase stain was positive in both myeloblasts and monoblasts as well as the maturing myelomonocytic cells. The chloroacetate esterase stain identified about 70% myeloid cells, and the α-naphthyl butyrate esterase stain identified 30% monocytic cells (Fig. 6.5.2).

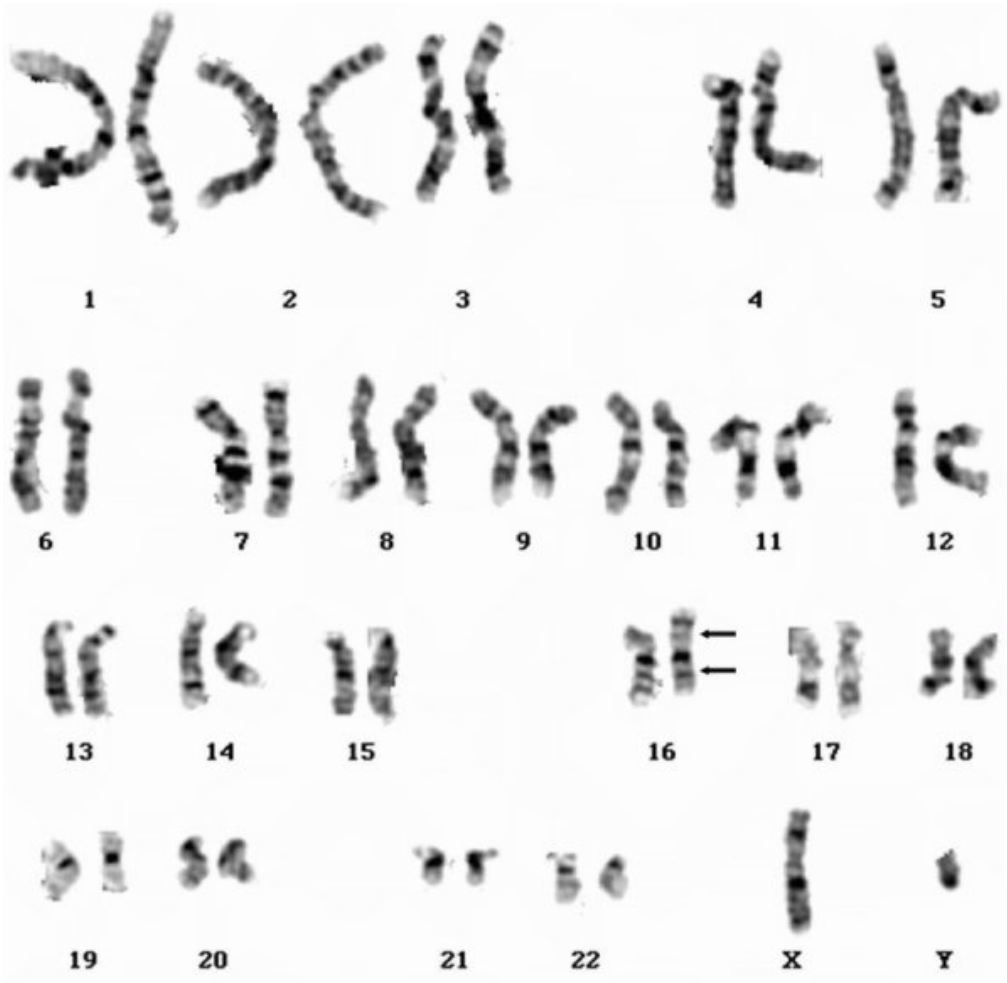

FIGURE 6.5.3 Karyotype of the bone marrow reveals inversion of chromosome 16 (*arrows*). (Courtesy of Peter Papenhausen, Ph.D., LabCorp of America Cytogenetics Department.)

CYTOGENETIC FINDING

The bone marrow showed a karyotype as following: 46,XY, del(7)(q22q34), inv(16)(p13q22) [8]/48,idem, +9, +22 [3]/46, XY [9] (Fig. 6.5.3 shows only inv(16)(p13q22)).

DISCUSSION

Acute myeloid leukemia (AML) with inv(16)(p13q22) or t(16;16)(p13;q22) is seen predominantly in cases of acute myelomonocytic leukemia with eosinophilia (AML M4Eo). It accounts for approximately 5% of all patients with AML and 20% of AML-M4 cases (1). However, this karyotype has also been encountered in other subtypes of AML, chronic myeloid leukemia and myelodysplastic syndromes (2,3). In the study by Mitelman and Heim (4), 206 of the total 241 inv(16) cases were diagnosed as M4 subtype. However, this aberration was also demonstrated in 17 M2 cases, 10 M5 cases, and 1 to 3 cases each of M1, M6, and M7. M0, M1, M2, M4, and M5 have been reported in other studies (2,5,6). In one study, approximately 10% of M4 cases without

eosinophilia showed this karyotype (7). Inv(16) has also been reported in several cases of chronic myeloid leukemia with blast crisis, in which bone marrow eosinophilia was also observed (2).

In the current case, the bone marrow showed approximately 70% myeloid cells and 30% monocytic cells, as identified by cytochemical stain, a ratio that is roughly equivalent to the morphologic differential count. Flow cytometric analysis also showed positive myelomonocytic markers with high percentages of CD34 and CD117, which is consistent with acute myelomonocytic leukemia. Bone marrow eosinophil count is >5%; therefore, it fulfills the definition of M4Eo. Cytogenetic study of the bone marrow shows a complex karyotype including inv(16)(p13q22); thus, it is considered to be AML with inv(16).

Morphology and Cytochemistry

The French-American-British (FAB) definition of M4 is that the myeloid or monocytic component, whichever is the majority, should not be >80% of the nonerythroid population in the bone marrow (8) (Figs. 6.5.4 and 6.5.5). In addition, the percentage of the blast, which is

composed of myeloblasts and monoblasts, should be >30%. The cell lineage identification is based on cytochemical stains: myeloperoxidase, specific esterases, and nonspecific esterases (see Case 7). The World Health Organization (WHO) system lowers the cutoff of the blast count to 20%. However, in AML with inv(16) or t(16;16), the blast count can be <20% and it is still acceptable for AML (9). The peripheral blood may show monocytosis but usually no eosinophilia (Fig. 6.5.6).

The eosinophils in these cases usually show all stages of maturation (9). Abnormal eosinophilic granules are often seen in the myelocyte and promyelocyte stages. In those eosinophils, there are mixed eosinophilic and basophilic, or purple-violet granules (Fig. 6.5.7). These granules are larger than those in normal eosinophils of the same stage, and sometimes the granules are so numerous that they obscure the nucleus.

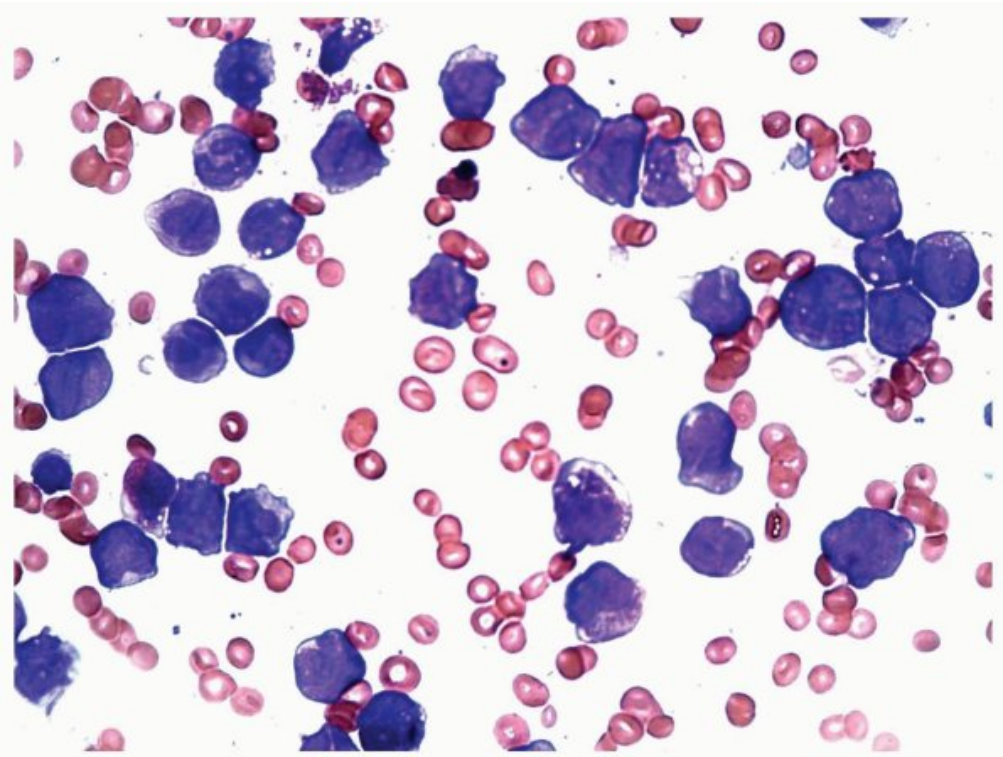

FIGURE 6.5.4 Bone marrow aspirate shows various developmental stages of myelomonocytic cells. Wright-Giemsa, 60x magnification.

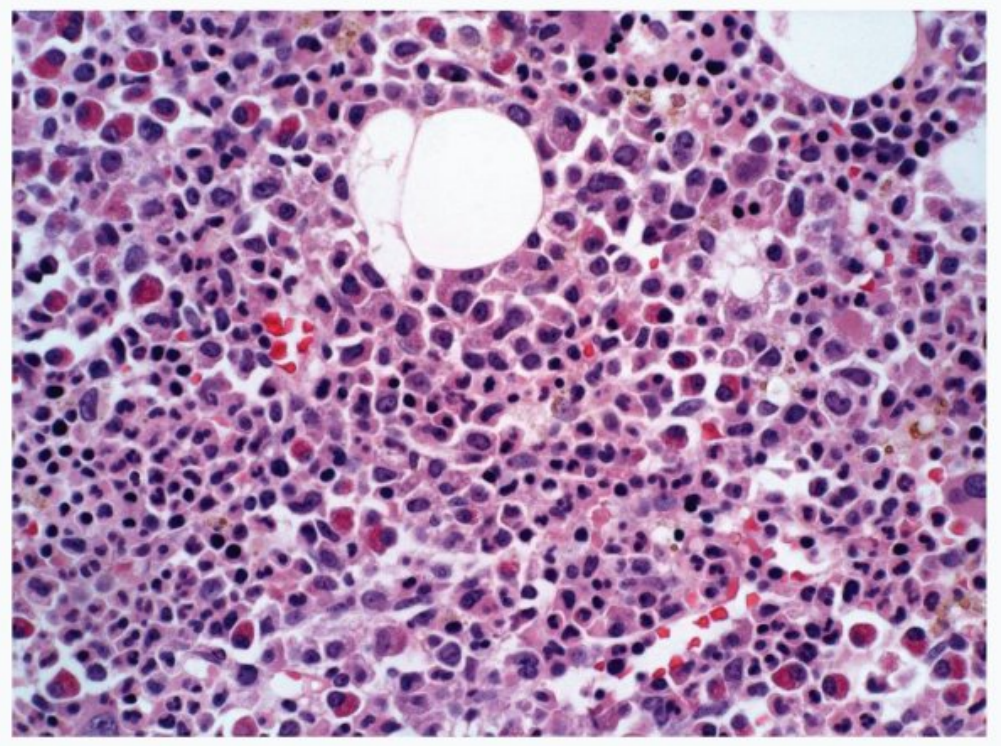

FIGURE 6.5.5 Bone marrow core biopsy reveals hypercellular marrow composed of immature myelomonocytic cells. Eosinophilia is evident. Hematoxylin and eosin, 40x magnification.

Cytochemical stains of these abnormal eosinophils also differ from those of normal eosinophils. Unlike their normal counterparts, these eosinophils react with chloroacetate esterase and periodic acid-Schiff (10). The basophilic granules are positive for myeloperoxidase and negative for toluidine blue; these reactions are opposite of the reactions seen in normal basophils (10). Ultrastructurally, the abnormal eosinophils are characterized by the absence of well-formed central crystalloids in the cytoplasmic granules (10). Eosinophils contain high levels of lysozyme, so that an elevated serum lysozyme value cannot be used as a criterion for monocytic differentiation when eosinophilia is present in an AML case (11). For instance, M2 with eosinophilia may have a high lysozyme concentration in the blood.

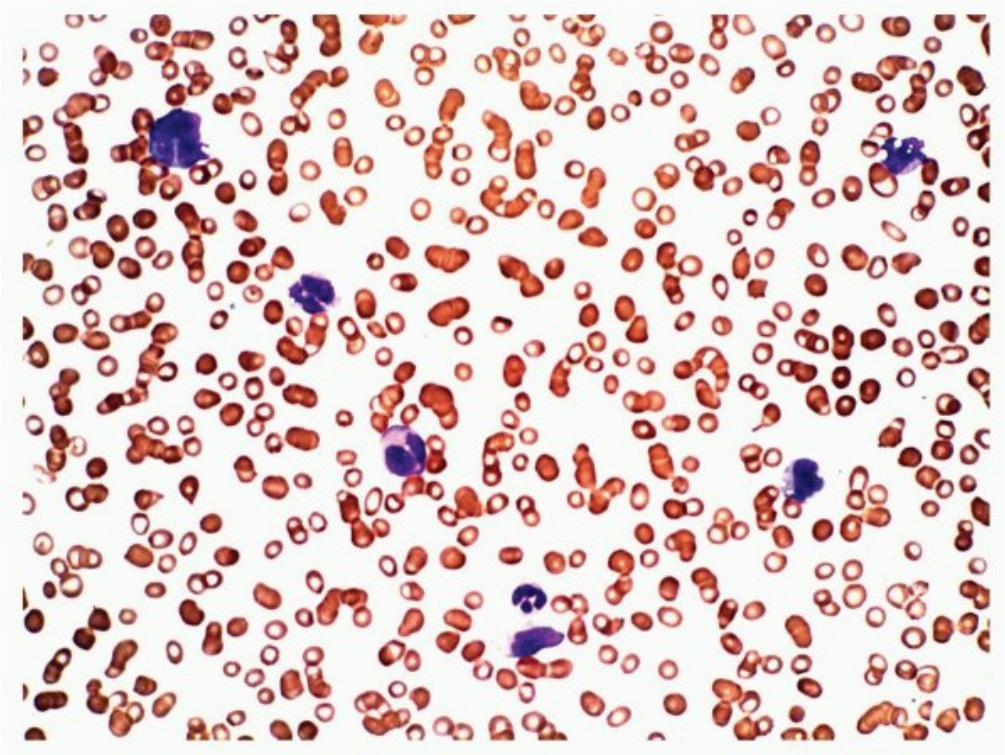

FIGURE 6.5.6 Peripheral blood smear shows monocytosis. Wright-Giemsa, 60x magnification.

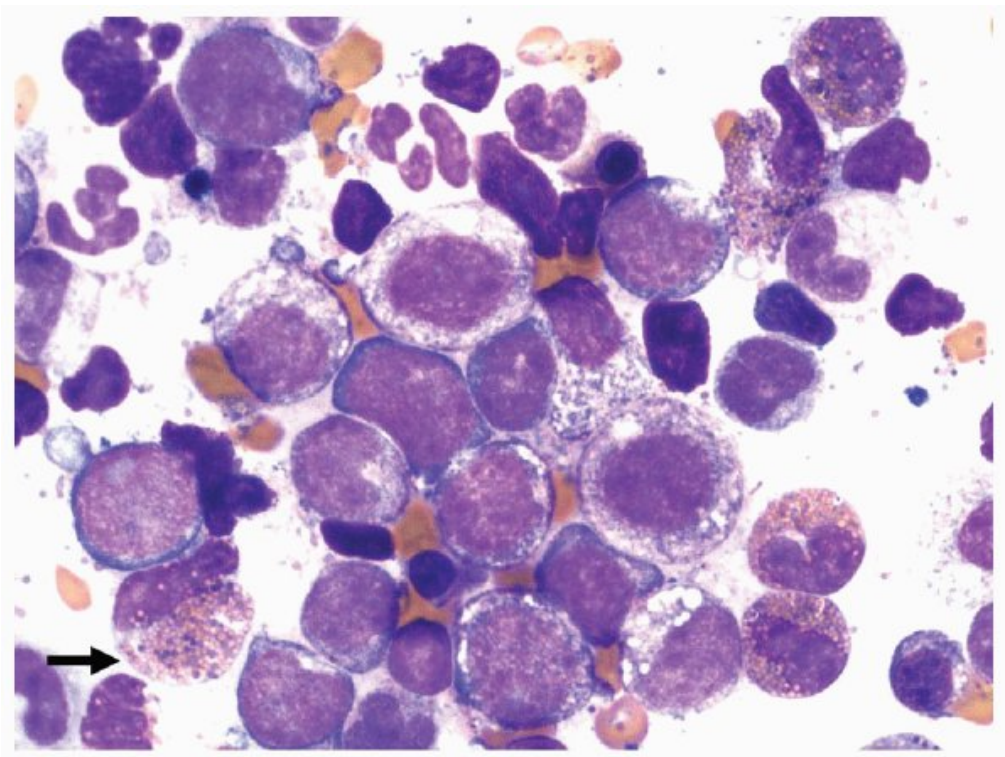

FIGURE 6.5.7 Bone marrow aspirate reveals immature myelomonocytic cells with the presence of basophilic granules in a few immature eosinophils (*arrow*). 100x magnification.

Whether the eosinophils are leukemic cells in M4Eo cases is controversial. There was one report showing inv(16) in the eosinophils of an

M4Eo case (12), but this result has not been confirmed by other studies. Eosinophilia can sometimes behave as a preleukemic syndrome leading to M4 (13,14).

A study of 21 cases showed that dysplasia is a prominent feature in AML with inv(16), which is associated with a significantly higher proliferation rate, as demonstrated by immunohistochemical staining with Mib-1 (Ki-67) (15). However, apoptotic rate in M4Eo is similar to that in other AML subtypes.

Immunophenotype

As in other types of AML, the blasts of M4Eo express CD13, CD33, myeloperoxidase, and HLA-DR. In addition, monocytic component is represented by one or more of the monocytic markers, such as CD4, CD11b, CD11c, CD14, CD36, CD64, and lysozyme (9,16). The malignant nature of the leukemic cells is identified by the presence of CD34 and CD117 (17). These markers, however, can be demonstrated in acute myelomonocytic leukemia with or without eosinophils. The only specific marker for this type of AML is CD2, which is coexpressed with myeloid markers (16). In cell culture of two M4Eo cases, the addition of CD2 antibodies caused reduced cell proliferation. It is therefore assumed that the CD2 molecule may stimulate the proliferation of the leukemic cells and cause a high leukocyte count in M4Eo (16).

Terminal deoxynucleotidyl transferase (TdT) expression has been reported in two studies (16,18). The TdT-positive cells were seen exclusively in the CD34+ CD14-subpopulation (16).

Comparison of Flow Cytometry and Immunohistochemistry

Immunohistochemical staining may demonstrate myeloperoxidase, lysozyme, CD68, CD34, and CD117. In comparison, flow cytometry is preferred to immunohistochemistry, because the histogram of flow cytometry may demonstrate the heterogeneous cell populations with more markers.

Molecular Genetics

In M4Eo cases, three abnormal karyotypes involving chromosome 16 can be demonstrated: inv(16)(p13q22), t(16;16)(p13;q22), and del(16)(q22). Most cases carry the inv(16) karyotype, and del(16q) is least frequently seen. One study showed marked differences in survival and remission duration between the inv(16) or t(16;16) patients and those with del(16q) (3).

Cloning of the 16p and 16q breakpoints identified the core binding factor (CBF)ß and smooth muscle myosin heavy chain (MYH)11 genes located at 16q22 and 16p13, respectively (2). The MYH11 gene codes for a smooth muscle myosin heavy-chain gene. The CBFß gene, also known as polyoma enhancer binding protein (PEBP)2ß, codes for the ß subunit of CBF, a heterodimeric transcription factor. The α subunit of CBF is identical to the AML1 gene, which is involved in t(8;21) translocation (see Case 4). In vitro analysis showed that the murine CBFß gene formed a heterodimeric complex with CBFα thus stabilizing its interaction with DNA (7). As both t(8;21) and inv(16) or t(16;16) are characterized by the disruption and transcriptional deregulation of genes encoding subunits of the CBF, which is involved in the regulation of normal hematopoiesis, these two types of AML are called CBF AML (19). However, the fusion gene CBFß-MYH11 may not be sufficient for leukemogenesis; additional genes may be involved in the pathogenesis (20).

The CBFß-MYH11 fusion gene can be detected by reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR studies demonstrated the existence of marked molecular heterogeneity in terms of breakpoint location, and eight types of fusion transcripts have been reported, with the A type being most common (88%) (2). A J type has been reported recently (21).

Cytogenetic detection of inversions and small deletions of chromosome 16 by standard karyotyping can be difficult (7). The detection of such cytogenetic abnormalities is affected by the ability to obtain adequate metaphases and the coexistence of normal metaphases (7). Several studies have demonstrated the higher sensitivities with fluorescence in situ hybridization (22, 23 and 24) and RT-PCR (2,7) techniques than with the conventional karyotyping. One report showed no cytogenetic abnormality by conventional karyotyping and FISH in a case of M4Eo, but CBFß-MYH11 was identified by RT-PCR (25).

Cases with inv(16) are often accompanied by additional abnormalities with a frequency as high as 50% in one study (3). However, the presence of additional changes does not affect the response to therapy or the survival (26,27). Only one report suggested that coexistence of inv(16) and partial deletion of the CBFß gene could be associated with unfavorable prognosis (24). The salient features for laboratory diagnosis of AML with inv(16) or t(16;16) are summarized in Table 6.5.1.

TABLE 6.5.1

Salient Features for Laboratory Diagnosis of AML with inv(16)(p13q22) or t(16;16)(p13:q22)

1. Karyotype: inv(16)(p13q22), t(16;16)(p13;q22), or del(16)(q22)
2. Molecular characterization by FISH or RT-PCR: CBFß-MYH11 fusion gene

3. Presence of >20% myeloblasts and monoblasts in the bone marrow; <20% blasts is acceptable when typical karyotype or molecular pattern is identified

4. Bone marrow contains myeloid and monocytoid cells with the minor cell component >20%

5. Bone marrow contains >5% eosinophils. Absence of eosinophilia is acceptable. Eosinophils are abnormal in cytoplasmic granules and cytochemical stains.

6. Immunophenotype: Flow cytometry may demonstrate myelomonocytic markers (CD13, CD33, myeloperoxidase, CD14, CD11b, CD11c) and immature cell markers (CD34 and CD117). One special marker is CD2, a T-cell marker that is coexpressed with myeloid markers.

FISH, fluorescence in situ hybridization; RT-PCR, reverse transcription-polymerase chain reaction; CBF, core binding factor; MYH, smooth muscle myosin heavy chain gene; CD, cluster of differentiation.

Clinical Manifestations

AML with inv(16) or t(16;16) has been reported in all age groups, but it is predominantly seen in young patients. CBF AML accounts for up to 20% of young adult cases of de novo AML (25). In a study of 43 pediatric cases of AML in Hong Kong, 5 patients were found to have this abnormality (28).

AML with inv(16) or t(16;16) is usually associated with a favorable prognosis in terms of complete remission and the duration of remission and survival when compared with other AML M4 cases with similar treatment (7). There is no clinical difference between patients with inv(16) and those with t(16;16) (2,3,29). However, patients with del(16q) are different; the outcome of those patients was not better than that of other AML M4 patients in one study (3). In addition, del(16q) cases lack relapse in the central nervous system (CNS) and have lower incidence of eosinophilia and M4 subtype (3).

Clinical symptoms are similar to those seen in other M4 cases. Specific features of AML with inv(16) include a high leukocyte count, hepatosplenomegaly, and high incidence of CNS leukemia, manifested as leptomeningeal disease and intracerebral myeloblastomas (16). Myeloid sarcoma may be present at initial diagnosis or at relapse (9).

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CASE 6 Acute Promyelocytic Leukemia with t(15;17)

CASE HISTORY

A 53-year-old man was admitted to the hospital because of continuous nosebleeds and gum bleeding after tooth brushing over 5 days prior to admission. The patient was in his usual state of health until 2 weeks ago when he developed fatigue, night sweats, fevers, and chills after visiting his dying mother in another state. He also claimed to have lost 5 pounds since then. However, it was his nosebleeds that brought him to the hospital.

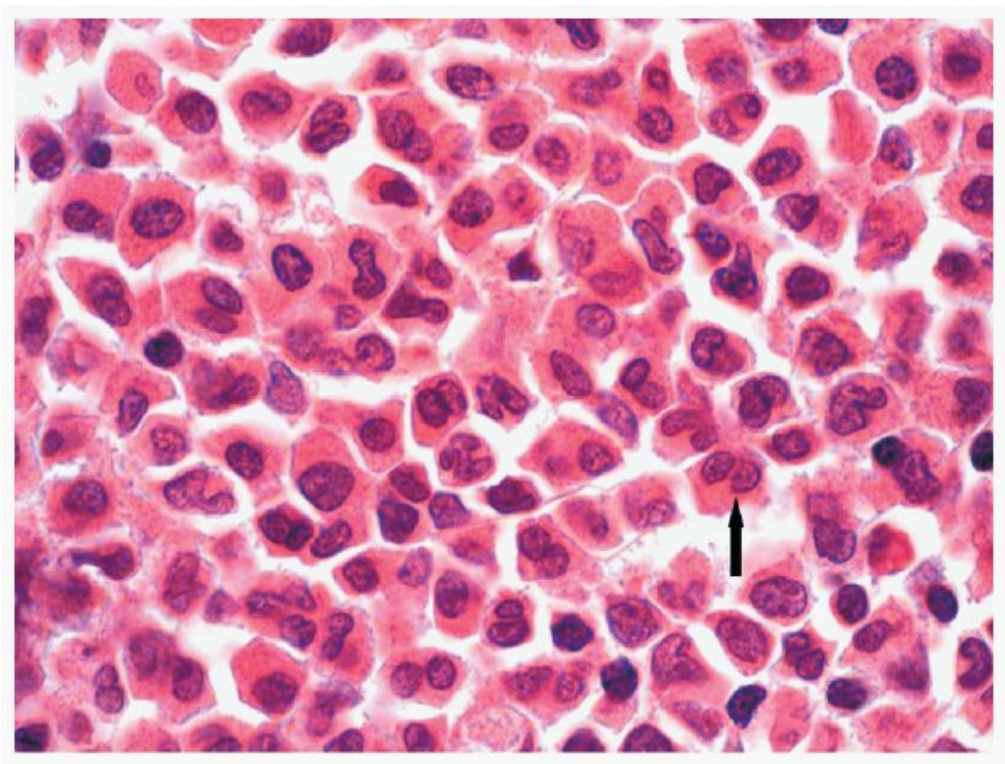

FIGURE 6.6.1 Bone marrow core biopsy shows total replacement of normal hematopoietic cells by the leukemic promyelocytes. Note many bilobed cells (*arrow*) are present. The abundant eosinophilic cytoplasm represents hypergranularity. Hematoxylin and eosin, 100x magnification.

His physical examination on admission showed no bruises or petechiae on the skin and no hepatosplenomegaly. His total leukocyte count was 1,900/μL, hematocrit 24%, hemoglobin 8.5 g/dL, and platelets 19,000/μL. His absolute neutrophil count was 100/μL, lymphocytes 1,600/μL, and monocytes 100/μL. Coagulation studies revealed fibrinogen 257 mg/dL, prothrombin time 15 seconds, and absence of D-dimer. Because of the presence of atypical bilobed cells in the peripheral blood, a bone marrow biopsy was performed (Fig. 6.6.1), which demonstrated features of acute promyelocytic leukemia (APL). Cytogenetic study of the bone marrow revealed t(15;17).

The patient was treated with *all-trans*-retinoic acid (ATRA) leading to complete remission. However, despite consolidation therapy, the patient had a relapse of leukemia 18 months after initial treatment. The patient has since switched to arsenic trioxide (ATO) treatment and is now in complete remission.

FLOW CYTOMETRY FINDINGS

Bone marrow: B-cell markers: CD19 0%, κ 0%, λ 0%; T-cell markers: CD3 0%, CD7 0%; myeloid markers: CD13-CD33 99%, myeloperoxidase (MPO) 75%, CD14 1%, human leukocyte antigen-DR (HLA-DR) 2%. Stem cell markers: CD34 52% (Fig. 6.6.2).

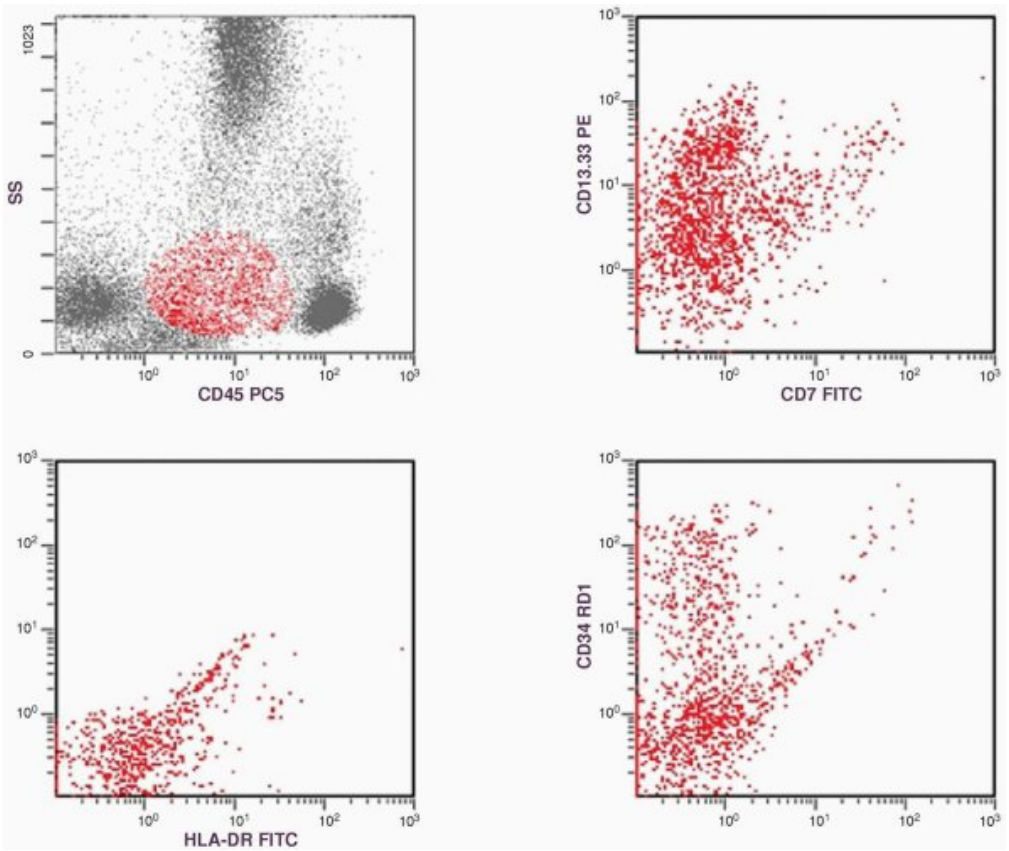

FIGURE 6.6.2 Flow cytometric histograms show strongly positive CD33 and weakly positive CD34 but negative human leukocyte antigen-DR (HLA-DR). ss, side scatter; PE, phycoerythrin; FITC, fluorescence in situ hybridization; RD1, rhodamine; PC5, phycoerythrin cyanin 5.

CYTOCHEMICAL FINDINGS

Leukemic cells from both the peripheral blood and bone marrow were positive for MPO and chloroacetate esterase (CAE) stains, but were negative for α-naphthyl butyrate esterase (NBE) stain. CAE stain also demonstrated single and multiple Auer rods in leukemic cells (Fig. 6.6.3).

DISCUSSION

APL accounts for only 5% to 13% of all cases of acute myeloid leukemia (AML), but it is the most well-defined subtype of AML (1). Cytologically, it differs from other AML in that the leukemic cells are not at the blastic stage but are atypical promyelocytes. Clinically, it is characterized by the presence of leukopenia in most cases rather than leukocytosis as seen in other leukemia and by the frequent existence of a hemorrhagic syndrome at the acute stage. Molecular genetically, it shows a nonrandom karyotype of t(15;17)(q22;q12) with the molecular characteristic of promyelocytic leukemia retinoic acid receptor α (PML/RARα) fusion transcript in the majority of cases. It is one of the rare examples of leukemia for which an effective treatment has been established by understanding its molecular

genetic abnormality. The therapeutic mechanism of ATRA is to induce differentiation of the leukemic cells by reversing the transcriptional repression of PML/RARα.

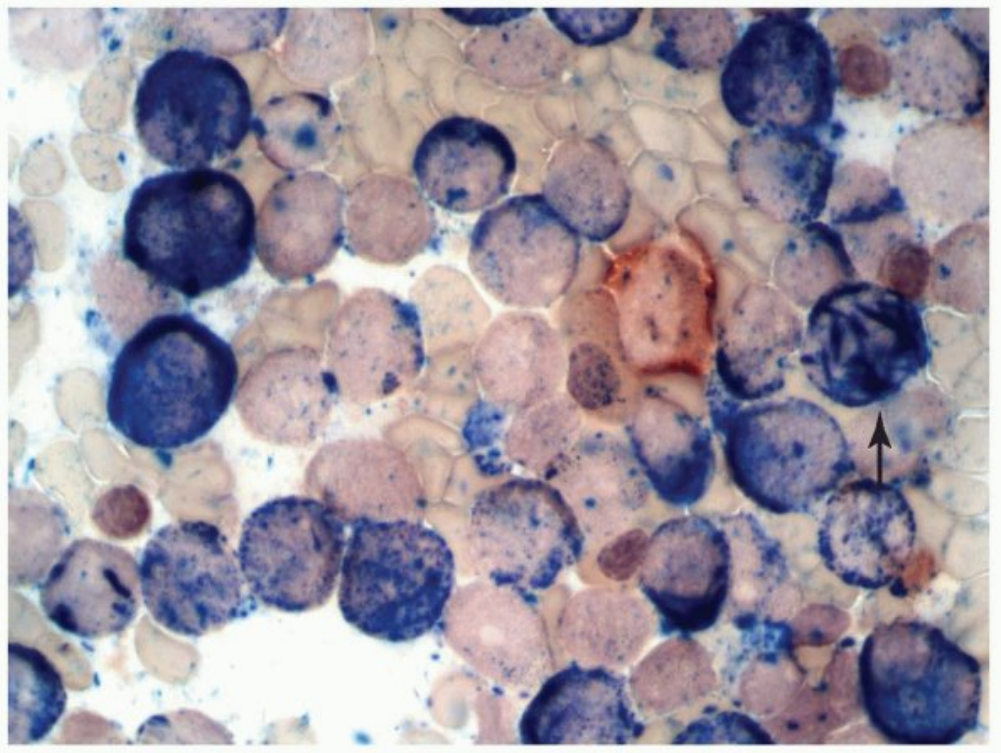

FIGURE 6.6.3 Combined esterase stain of the bone marrow aspirate shows multiple Auer rods demonstrated by chloroacetate esterase (*blue*) stain in a few leukemic cells (*arrow*). 100x magnification.

Morphology and Cytochemistry

The leukemic cells in most cases of APL assume the morphology of hypergranular promyelocytes and are considered the typical cells in AML-M3 (French-American-British [FAB] classification). These leukemic cells are generally larger (14 to 25 μm) than normal promyelocytes and are devoid of a prominent paranuclear clear Golgi region, as is frequently seen in normal promyelocytes (2). The most characteristic feature is the abundance of cytoplasmic granules that cover the entire cytoplasm and mask the nucleus of the leukemic cells (Figs. 6.6.4 and 6.6.5). The nuclei of the APL cells show a great variation both in size and in shape, but many of them are kidney-shaped or bilobed. The cytoplasmic granules are believed to contain MPO, procoagulant substances, and bactericidal enzymes (3).

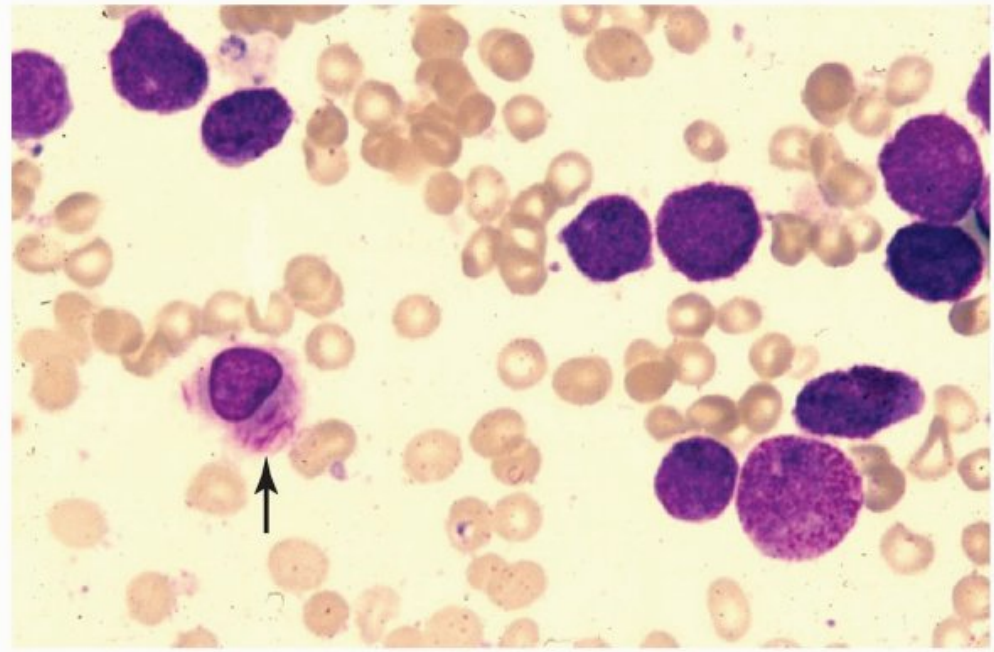

FIGURE 6.6.4 Peripheral blood smear shows several hypergranular promyelocytes with the nuclei being masked by the cytoplasmic granules. Note one promyelocyte contains multiple Auer rods (*arrow*). Wright-Giemsa, 100x magnification.

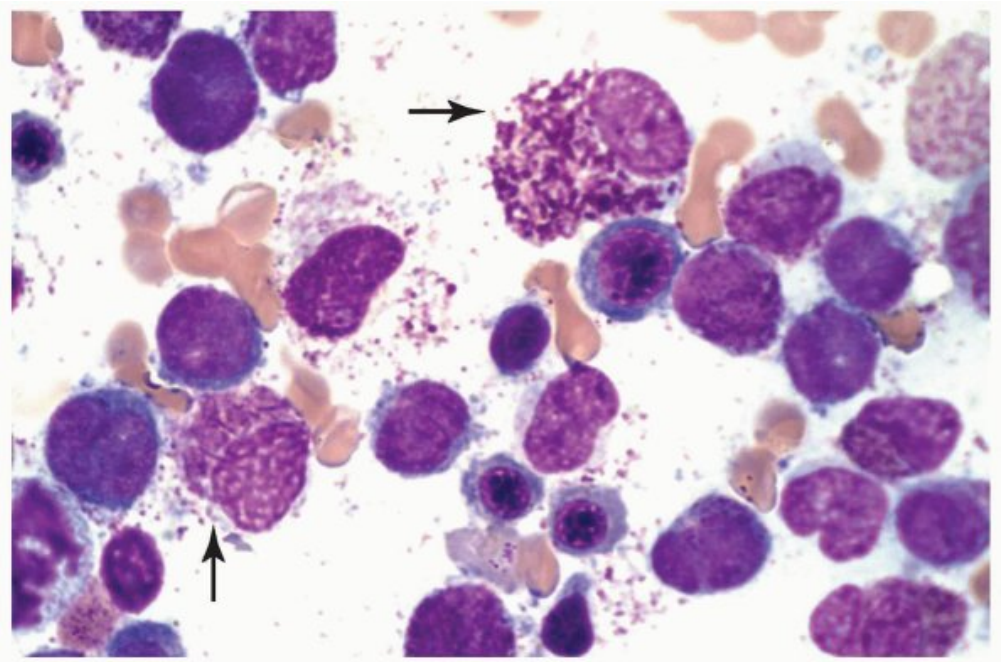

FIGURE 6.6.5 Bone marrow aspirate shows many leukemic promyelocytes, and two reveal multiple Auer rods (*arrows*). Wright-Giemsa, 100x magnification.

However, the above features are not diagnostic for APL unless multiple Auer rods in bundles are demonstrated in the cytoplasm of the leukemic cells. These cells are commonly referred to as faggot cells. The Auer rods in APL cells show an internal, hexagonal tubular structure with a periodicity of 22 to 25 nm in contrast to the 8 to 12 nm periodicity of the Auer rods observed in other types of AML (4).

In approximately 15% to 20% of APL cases, the leukemic cell contains only a few cytoplasmic granules or the granules are so small (<250

μm resolution of light microscopy) that they can only be demonstrated by electron microscopy (5, 6 and 7). These cases are coined hypogranular or microgranular APL, respectively, and are designated AML-M3v in the FAB classification. In M3v cases, the nuclei of the leukemic cells are usually folded or bilobed, mimicking those of monocytes (Fig. 6.6.6).

A rare type of hyperbasophilic microgranular variant is characterized by cells with a high nuclear cytoplasmic ratio, strongly basophilic cytoplasm with sparse or no

granules, and conspicuous cytoplasmic budding mimicking micromegakaryocytes (8,9). A hand-mirror variant of M3v has also been described (10).

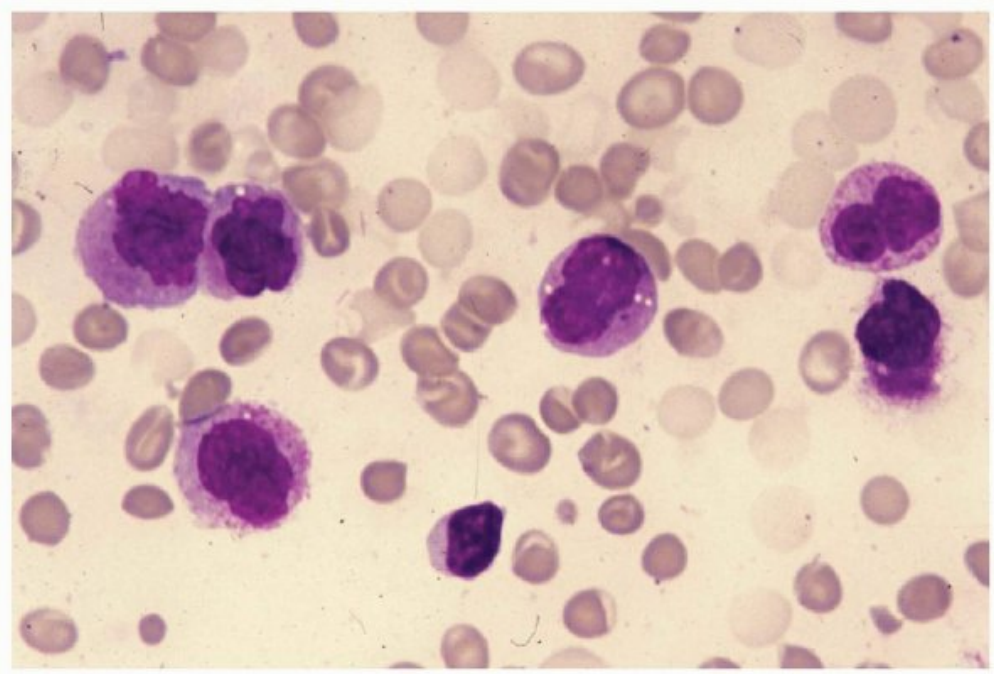

FIGURE 6.6.6 Peripheral blood smear shows several hypogranular promyelocytes with bilobed or folded nuclei, mimicking monocytes. Wright-Giemsa, 100x magnification.

APL cases usually are positive for both MPO and CAE stains. MPO and Sudan black B stains are usually strongly positive (4,9). CAE is particularly helpful in demonstrating the multiple Auer rods. MPO may also serve the same function, but false-positive results may occur due to precipitation of reagents. Among nonspecific esterase, NBE is generally negative in APL cases, but NBE reaction can be seen in certain subgroups of APL cases (11).

Immunophenotype

Because APL may mimic acute monocytic leukemia, flow cytometry is particularly helpful in demonstrating positive myelomonocytic antigens (CD13, CD15, and CD33) but negative monocytic antigens (CD14 including My4, Leu M3, and Mo2) (3,12, 13, 14, 15, 16 and 17). However, the most important diagnostic feature is the absence or low percentage of HLA-DR. HLA-DR is present in myeloblasts and monoblasts but not promyelocytes; therefore, the absence of HLA-DR distinguishes APL from other subtypes of AML. Nevertheless, HLA-DR is also absent in normal promyelocytes, thus APL has to be distinguished from reactive promyelocytosis. Postchemotherapy specimens from other AML subtypes sometimes show synchronous regeneration of promyelocytes that may also mimic APL (Fig. 6.6.7).

The stem cell marker, CD34, is frequently absent in APL cases and is also used to separate APL from other AML cases (13,16, 17 and 18). However, CD34 positivity was demonstrated in 41% of APL cases in one study (19). Those CD34-positive cells harbored the t(15;17) translocation as identified by the fluorescence in situ hybridization (FISH) technique. Another study claimed that the presence of a heterogenous expression of CD13 and dual staining of CD34 and CD15 were highly characteristic of APL with a sensitivity of 100% and specificity of 99% for predicting PML/RARα gene rearrangement (20).

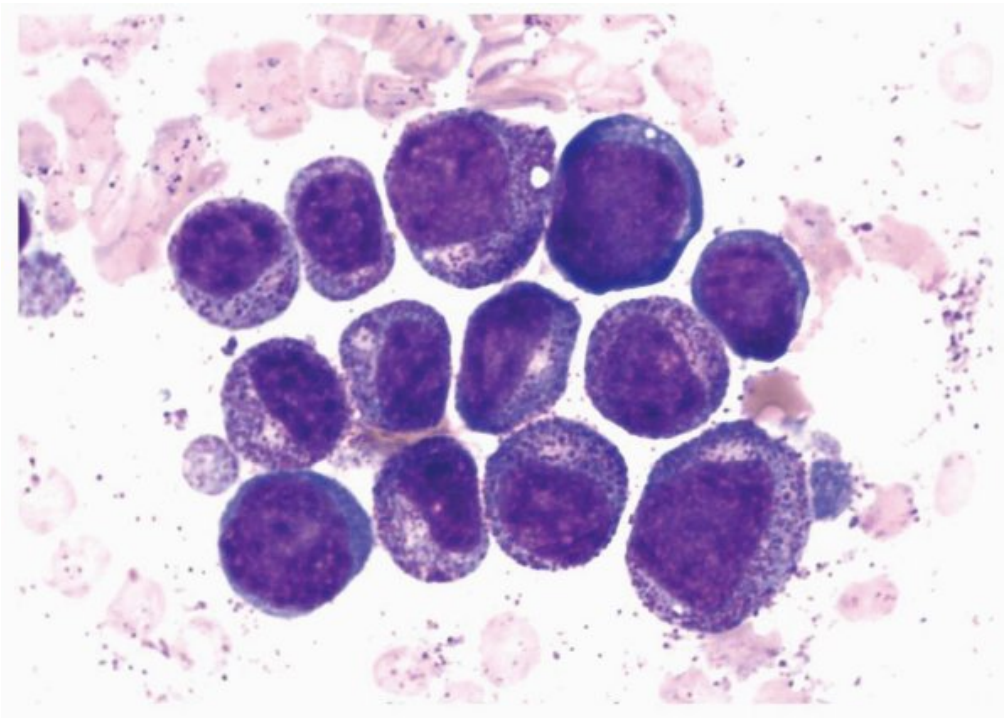

FIGURE 6.6.7 Bone marrow aspirate shows a cluster of hypergranular promyelocytes due to synchronous proliferation of promyelocytes after chemotherapy of M2 leukemia. Wright-Giemsa, 100x magnification.

The recent addition of CD117 (c-kit) to the immunophenotypic panel is very helpful in distinguishing APL from reactive promyelocytosis (21). CD11b will further separate these two entities (21). In a recent study, 77% of APL cases were CD117+ CD11b-, whereas all cases recovered from agranulocytosis were CD117- CD11b+ (21). A panel composed of leukocyte integrin-associated antibodies, including CD11a, CD11b, and CD18 is useful to distinguish between APL and other AML subtypes (22). PML-RARα-positive APL cells typically lack leukocyte integrins and show low percentages of the above markers.

Another useful marker is CD2 (a T-cell marker), which is frequently demonstrated in M3v but rarely in hypergranular M3 (12,13). A recent study showed that expression of CD2 in M3 correlates with the short type of PML-RARα transcript and with poor prognosis (23). CD7, another T-cell marker, may be demonstrated in other subtypes of AML, but is consistently absent in APL (24,25).

Antibodies to the PML gene product are now available for immunohistochemical and immunofluorescent stains (26, 27 and 28). PML protein is present in the nucleus of normal cells and is characterized by a speckled pattern, which is the presence of 5 to 20 nuclear particles (nuclear bodies) per nucleus. The APL cells, in contrast, show a microspeckled or microgranular pattern, which is composed of >50 granules. This phenomenon is the result of disruption of the nuclear bodies and redistribution of the protein in the APL cells. It is a reliable and simple technique and can be used for therapeutic monitoring. After treatment, the PML nuclear pattern may return to being speckled.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry can be used to make the initial diagnosis of APL and for therapeutic monitoring. CD2 may help to predict the prognosis. Immunohistochemical staining for PML protein is considered a reliable technique, but it is not yet commonly used in histology laboratories.

Molecular Genetics

Approximately 99% of APL cases including M3 and M3v show t(15;17)(q22;q21) translocation, which produces a PML RARα or, to a lesser extent, RARα/PML fusion transcript (3,18,29, 30 and 31). The remaining APL cases involve three partner genes translocating with RARα: promyelocytic leukemia zinc finger (PLZF) in t(11;17)(q23;q21), nucleophosmin (NPM) gene in t(5;17)(q23;q12), and nuclear matrix-associated gene (NuMA) in t(11;17)(q13;q21) (1,16,26,31). Leukemic cells with the PLZF/RARα fusion product are resistant to ATRA therapy. In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) demonstrates three isoforms in PML/RARα transcripts: L (long) type, V (variable) type, and S (short) type containing *bcr1, bcr-2,* and *bcr-3,* respectively (26). The *bcr-3* transcript is associated with higher white blood cell counts, M3v morphology, additional karyotypic abnormalities, and the expression of CD34 and CD2 (26). Interestingly, Latin American patients have a high frequency of *bcr-1* subtype, which was also demonstrated in a small cohort of Chinese APL patients and in a Japanese group (32). It was proposed

that this phenomenon might be related to a non-European genetic factor.

RARα regulates transcription of ATRA target genes and recruits the nuclear corepressor (N-CoR)/histone-deacetylase (HD) complex, which lead to a repressive chromatin conformation (26). As a result there is a developmental arrest at the promyelocytic stage. High doses of ATRA release HD activity from PML-RARα but not from PLZF-RARα, because the latter contains a second N-Cor/HD binding site in the PLZF moiety (26). This explains why ATRA is not effective in treating cases with PLZF-RARα. The action of ATRA is to induce the leukemic promyelocyte to differentiate terminally. However, ATRA alone may not be sufficient; therefore, the current protocol is the combination of ATRA and anthracycline-containing chemotherapy (16,26). After treatment, the bone marrow is replenished with terminally differentiated granulocytes, in contrast to the hypocellular bone marrow seen in other leukemia immediately after chemotherapy (29).

Recently, ATO has been used to treat APL patients resistant to ATRA. ATO may be similar to ATRA in inducing APL cell differentiation through disruption of the PML-RARα function (33).

A study of gene expression profiling identified two major clusters in APL cases, corresponding to the two morphologic subtypes (7). The first cluster was represented by cases with M3v morphology, high leukocyte count, *bcr3* PML-RARα isoform, and Flt3-ITDs (Fms-like tyrosine kinase 3-internal tandem duplications). The second cluster was composed of cases with typical M3 morphology, *bcr1* PML-RARα isoform, leucopenia, and Flt3-WT (Flt3-wild-type).

As PML-RARα transgenic mice only develop a nonfatal myeloproliferative disorder, additional mutations are probably required to produce overt leukemia. A candidate gene to play such a role is Flt3 (7). For instance, Flt3-ITDs upregulate the hyperleukocytosis gene and blood coagulation gene clusters. It also down-regulates genes that encode for proteins present in granulocytic granules leading to the hypogranular variant form (7).

Whereas molecular genetic confirmation is mandatory in the diagnosis of APL, treatment should be started as soon as possible even before genetic evidence is available, because the patient may die in the early stage of the disease due to acute hemorrhage. Molecular genetic techniques are useful not only for the diagnosis but also for follow-up of the patients for minimal residual disease (MRD). However, molecular evaluation is reliable only at the end of consolidation and not immediately after induction therapy (28).

Conventional karyotyping may help to diagnose 80% to 90% of APL cases. Its major advantage is its capability to detect additional chromosomal abnormalities besides t(15;17) and to identify other APL variants (26). However, it is a time-consuming procedure, a good quality bone marrow metaphase is not always obtainable for karyotyping, and it is an insensitive technique for the detection of MRD (18,28).

FISH is a rapid and sensitive technique (26,28). It does not require dividing cells, so fresh specimens and culture technique are not needed. FISH is practically applicable to all kinds of specimens: Blood smears, bone marrow, fresh tissues, and paraffin sections (Fig. 6.6.8). However, it cannot detect additional cytogenetic aberrations besides the targeted abnormality [i.e., t(15;17)].

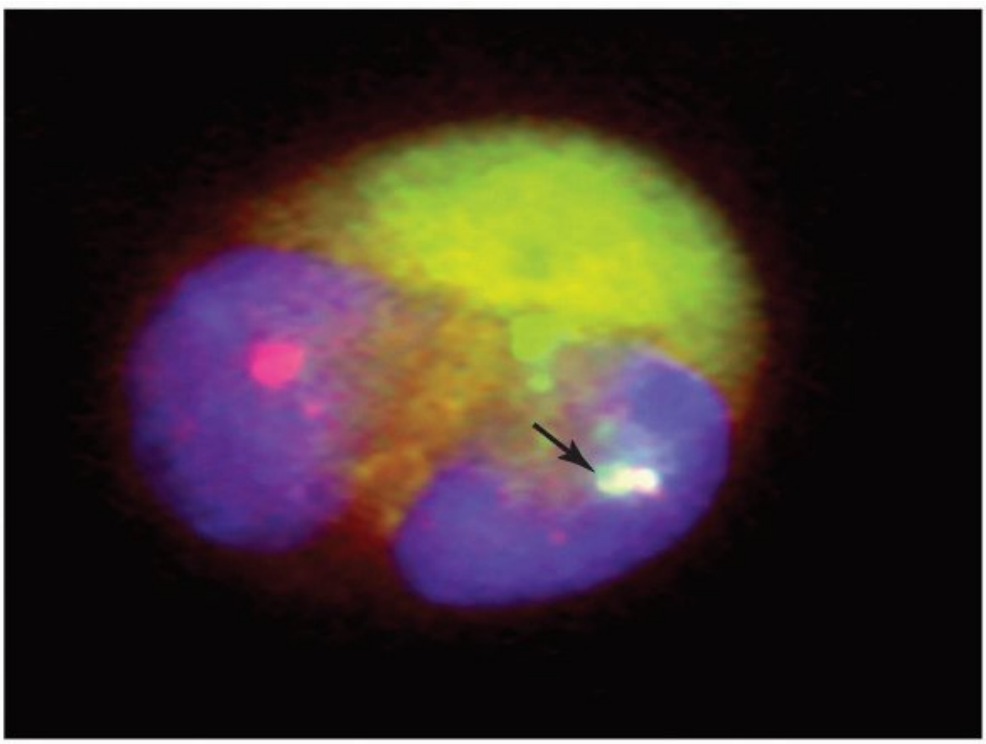

FIGURE 6.6.8 Fluorescence in situ hybridization of bone marrow aspirate with promyelocytic leukemia-retinoic acid receptor α (PML-RARα) probes demonstrated one orange signal, one

green signal, and the PML-RARα fusion product (*arrow*) in a leukemic promyelocyte with a bilobed nucleus. 100x original magnification.

Southern blot is highly specific, but it is time-consuming and laborious (28,30). Additional probes are needed to detect different breakpoints or to rule out a variant translocation.

RT-PCR is the only technique that defines the PML breakpoint type and is suitable for monitoring MRD (16,26,28). After successful treatment, PML-RARα disappears from the leukocytes, and the reappearance of this fusion transcript predicts a relapse. This phenomenon is in marked contrast to t(9;22) in chronic myeloid leukemia and to t(8;21) in AML. In those cases, the transcript may persist for a long time and no relapse occurs (18). However, RT-PCR is prone to contamination and artifacts, and interlaboratory discordance has been reported (26,28). Therefore, real-time PCR is advocated to provide standardization (26).

In the current case, the diagnosis of APL is confirmed by karyotyping. Although the patient had symptoms of hemorrhages, his fibrinogen was normal and D-dimer was negative, so disseminated intravascular coagulation (DIC) was probably not present. The morphology of the leukemic cells is consistent with M3v. The cytochemical staining is most helpful in demonstrating the presence of multiple Auer rods, because treatment can be started with this finding and multiple Auer rods are not easily detected without special staining. The only atypical clinical feature in this case is leukopenia instead of leukocytosis that is commonly seen in M3v cases.

The salient features for laboratory diagnosis of APL are summarized in Table 6.6.1.

TABLE 6.6.1

Salient Features for Laboratory Diagnosis of AML-M4

1. Presence of >20% hypergranular (or microgranular) promyelocytes in the bone marrow
2. Presence of multiple Auer rods in the cytoplasm of leukemic cells
3. Cytochemical staining: strongly positive for myeloperoxidase and chloroacetate esterase, but negative for α-naphthyl butyrate esterase
4. General immunophenotype: positive for myelomonocytic antigens (CD13, CD15, CD33) but negative for monocytic antigens (CD14)
5. Specific immunophenotype: low level or absence of HLA-DR, low level or absence of integrin-associated antibodies (CD11a/CD11b/CD11c/CD18), negative CD34 but positive CD117
6. Abnormal karyotype: t(15;17) detected by cytogenetic technique [rarely t(5;17) or t(11;17)]
7. Identification of PML-RARα by RT-PCR or FISH (rarely PLZF-RARα, NPM-RARa, or NuMA-RARα)
8. PML antibody staining for abnormal PML protein pattern

AML, acute myeloid leukemia; CD, cluster of differentiation; HLA-DR, human leukocyte

antigen-DR; FISH, fluorescence in situ hybridization; NuMA, nuclear matrix-associated gene; NPM, nucleophosmin; PLZF, promyelocytic leukemia zinc finger; PML, promyelocytic leukemia; RARα, retinoic acid receptor α; RT-PCR, reverse transcriptase-polymerase chain reaction.

Clinical Manifestations

The early clinical presentation is leukopenia in typical M3 but marked leukocytosis in M3v, which may reach 200,000/μL. The leukocyte count is an important predictor for the prognosis (7,34). In a study of 239 cases, patients with a leukocyte count <10,000/μL had a higher complete remission rate (85% vs. 62%), reduced relapse risk (13% vs. 35%), and superior survival (80% vs. 57%) than those with a leukocyte count >10,000/μL.

Morbidity and mortality are mainly related to coagulopathy. Many patients die of early fatal hemorrhage, especially intracranial or intrapulmonary hemorrhage. The incidence of early hemorrhage varies from 8% to 47% (29), and the mortality rate due to hemorrhages is still as high as 10% even in patients receiving modern treatment (26). Malignant promyelocytes release procoagulant substances that activate the coagulation cascade and generate thrombin, and deplete fibrinogen, clotting factors, and platelets, so that patients with APL may have DIC, fibrinolysis, and proteolysis (29,35). Clinically, the resolution of coagulopathy is the first sign of response to ATRA (29). Because M3v has higher leukocyte counts and more severe coagulopathy than the typical APL, its prognosis is generally worse than that of the latter (14).

After ATRA treatment, approximately 50% of patients may develop the retinoic acid syndrome, which includes fluid retention, hectic fever, pulmonary infiltrates, and pleural effusions (36). This potentially fatal syndrome should be promptly treated with high-dose corticosteroids. ATO can also induce the same syndrome in about one third of patients (36).

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CASE 7 Acute Myeloblastic Leukemia without Maturation (General Introduction of Acute Myeloid Leukemia)

CASE HISTORY

A 62-year-old man presented with symptoms of unstable angina. He was scheduled to have cardiac catheterization, but the procedure was postponed due to the development of fever of unknown origin for 2 weeks. The fever workup included blood cultures, urine cultures, and computed tomography (CT) scan of the chest and maxilla; all examinations were negative and failed to show any evidence of infection.

Physical examination revealed no hepatosplenomegaly and no lymphadenopathy. The initial peripheral blood examination demonstrated pancytopenia with blasts and several nucleated red blood cells. Further examination showed a total leukocyte count of 24,100/μL with 61% blasts, 11% neutrophils, 24% lymphocytes, and 1% monocytes. The hematocrit was 27.3%, hemoglobin 9.5 g/dL, and platelets 33,500/μL.

A bone marrow biopsy was performed. A 500-cell count showed 85% myeloblasts, 6.4% monoblasts, 1.2% promyelocytes, 0.4% myelocytes, 0.8% metamyelocytes, 1.2% bands, and 1.4% segmented neutrophils. Megakaryocytes were decreased. The core biopsy revealed 80% cellularity with the presence of large sheets of immature myeloid cells. However, small clusters of erythroid cells and mature granulocytes were still visible.

After admission, the patient continued to have cyclical fevers and was started with cefepime. The patient was

informed of the diagnosis of acute myeloid leukemia (AML), the prognosis, and treatment of the disease. He decided to forego chemotherapy and seek possible palliative care at home. The patient was discharged with the instruction to follow up by visiting hematology/oncology, and cardiology clinics.

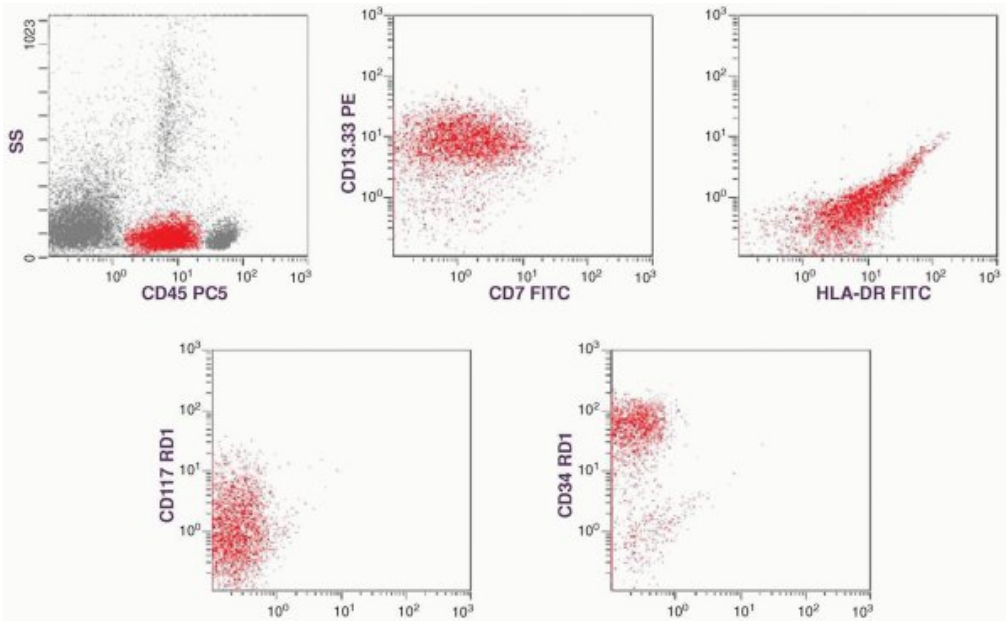

FIGURE 6.7.1 Flow cytometric histograms of the bone marrow show dual CD13-CD33/CD7 staining, with positive HLA-DR, CD117, and CD34. ss, side scatter; PC5, phycoerythrin cyanin 5; FITC, fluorescein isothiocyanate; RD1, rhodamine; PE, phycoerythrin.

FLOW CYTOMETRY FINDINGS

Bone marrow: Myeloperoxidase (MPO) 4%, CD13-CD33 86%, CD13-CD33/CD7 60%, HLA-DR 86%, CD14 0%, CD34 91%, CD117 47% (Fig. 6.7.1).

CYTOCHEMICAL FINDINGS

MPO and chloroacetate esterase (CAE) stains were positive, whereas α-naphthyl butyrate esterase (NBE) was negative in the bone marrow specimen.

DISCUSSION

The first comprehensive classification scheme for AML was proposed by the French-American-British (FAB) group, which was based on the combination of morphology and cytochemistry (1,2). Subsequently, immunophenotypic and cytogenetic criteria were included for substantiation of the diagnosis (3).

The basic requirement for the diagnosis of AML in the FAB system is that >30% of all nucleated marrow cells are blasts and <50% are erythroid precursors, except for erythroleukemia (3). On rare occasions, bone marrow may show <30% blasts, but >30% blasts are present in the peripheral blood. This condition has been accepted as AML by a National Cancer Institute-sponsored workshop (4). The FAB classification includes several subtypes of AML: acute myeloblastic leukemia without maturation (M1), acute myeloblastic leukemia with maturation (M2), acute promyelocytic leukemia (M3), acute myelomonocytic leukemia (M4), acute monoblastic leukemia (M5a), acute monocytic leukemia (M5b), acute erythroleukemia (M6), and acute megakaryoblastic leukemia (M7).

The distinction between M1 and M2 is based on the percentage of blasts in the bone marrow. M1 is diagnosed when >90% of nonerythroid marrow cells are myeloblasts, whereas M2 shows <90% myeloblasts in the bone marrow. The diagnosis of AML was required to have >3% of blasts positive for MPO. However, in those MPO-negative (or <3%) myeloid leukemia cases, the myeloid lineage can be identified by immunophenotyping or electron microscopic detection of MPO. These cases are now called AML with minimal differentiation (M0) (5,6). The incidence of M0

varies from 2% to 22% in different series (5, 6, 7, 8 and 9). M0 is frequently associated with the presence of terminal deoxynucleotidyl transferase (TdT) (9).

TABLE 6.7.1

AML with recurrent genetic abnormalities

AML with t(8:21)(q22;q22); (CBFa/ETO)

AML with abnormal bone marrow eosinophils inv(16)(p13q22) or t(16;16)(p12;q32); (CBFß.MYH11)

Acute promyelocytic leukemia t(15;17)(q22;p22); (PML/RARa) and variants

AML with 11q23 (MLL) abnormalities

AML with multilineage dysplasia

AML and myelodysplastic syndromes, therapy related

AML not otherwise categorized

AML minimally differentiated

AML without maturation

AML with maturation

Acute myelomonocytic leukemia

Acute monoblastic and monocytic leukemia

Acute erythroid leukemias

Acute megakaryoblastic leukemia

Acute basophilic leukemia

Acute panmyelosis with myelofibrosis

Myeloid sarcoma

Acute leukemia of ambiguous lineage

Undifferentiated acute leukemia

Bilineal acute leukemia

Biphenotypic acute leukemia

AML, acute myeloid leukemia; CBF, core binding factor; ETO, eight-twenty-one; MYH, smooth muscle myosin heavy chain; PML, promyelocytic leukemia; RAR, retinoic acid receptor; MLL, mixed lineage leukemia.

The recently proposed World Health Organization (WHO) classification divides AML into four large categories: AML with recurrent cytogenetic translocations; AML with multilineage dysplasia; AML, therapy related; and AML, not otherwise categorized (Table 6.7.1) (10, 11 and 12). The FAB classification is now included in the category of AML, not otherwise categorized. One of the major basic changes in this classification is lowering the diagnostic threshold of blast count from 30% to 20%, because recent studies have indicated that patients with 20% to 30% blasts have a prognosis similar to that of patients with >30% blasts.

The new classification emphasizes the importance of clinical correlation of the AML entities, particularly the correlation of prognosis. Because cytogenetic abnormalities, multilineage dysplasia, and chemotherapy and/or radiation therapy have proved to be intimately related to prognosis in AML patients, they are established as the new categories in the WHO classification.

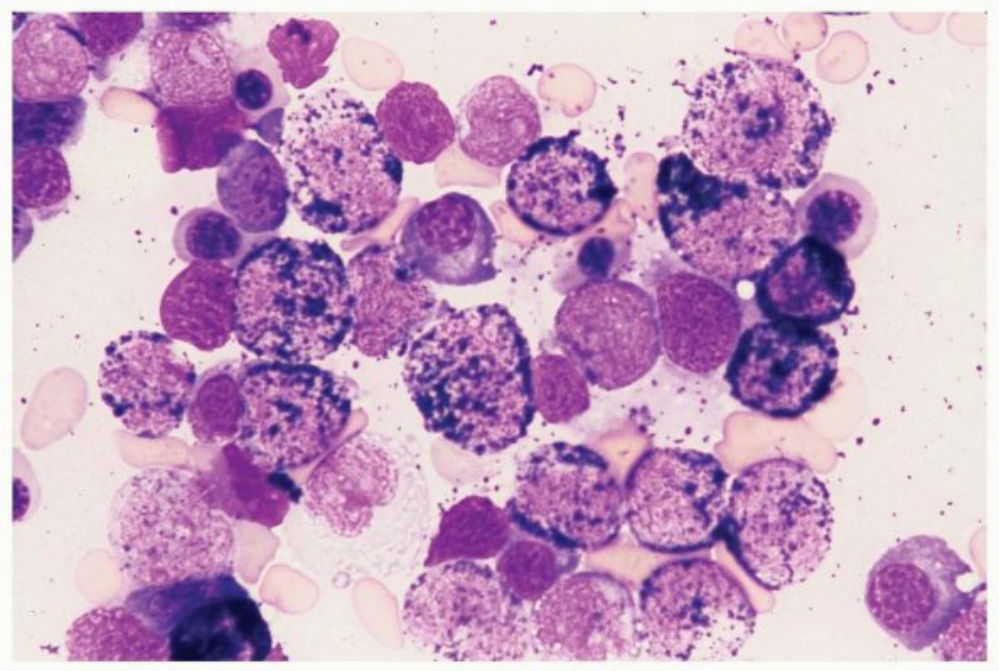

FIGURE 6.7.2 Myeloperoxidase stain of the bone marrow shows positive staining in several myeloblasts as well as maturing myeloid cells. 100x magnification.

Cytochemistry

Routine cytochemical stains for the study of AML cases include MPO (Fig. 6.7.2), specific esterase (e.g., CAE), and nonspecific esterase (e.g., NBE) (10,13,14). The two esterases can be stained simultaneously (combined esterase stain) (Fig. 6.7.3), so that two blood or bone marrow smears are usually sufficient for a routine cytochemical study.

MPO is usually strongly positive in M2, M3, M4, and M6; weakly positive in M1; and weakly positive or negative in M5, but negative in M7 (Table 6.7.2). The peroxidase in megakaryocytes can be demonstrated only by electron microscopy, and is called platelet peroxidase. The eosinophilic peroxidase is characterized by its resistance to cyanide. The basophils are negative for peroxidase.

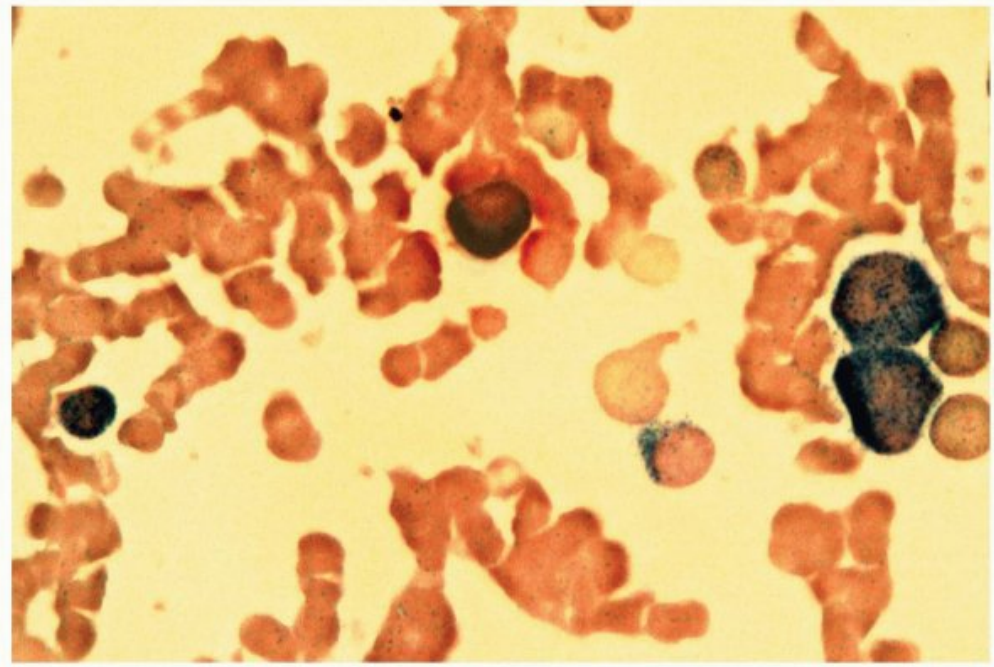

FIGURE 6.7.3 Combined esterase stain reveals chloroacetate esterase staining (*blue*) in two myeloblasts and one granulocyte, and α-naphthyl butyrate staining (*brown*) in a monocyte. 100x magnification.

TABLE 6.7.2

Cytochemical Reactions in FAB Subtypes of AML*

FAB Subtype	*MPO*	*CAE*	*NBE*
M0	-	-	-
M1	+ (>3%)	+	-
M2	+	+ (>80%)	+ (<20%)
M3	+	+	-
M4	+	+ (>20%)	+ (>20%)
M5	+/-	+ (>20%)	+ (>80%)
M6	+	+	-

M7	-	-	-

* Result is based on the reaction of myeloblasts and monoblasts except for M3, which is based on the reaction of promyelocytes. AML, acute myeloid leukemia; CAE, chloroacetate esterase; FAB, French-American-British; MPO, myeloperoxidase; NBE, α-naphthyl butyrate esterase.

MPO-deficient neutrophils are found in about 40% of AML cases. These MPO-deficient neutrophils frequently disappear during complete remission and reappear during relapse (15). MPO deficiency and a low level of MPO activity in AML usually mean a poor prognosis (16).

Sudan black B stain has the same reaction as MPO to various leukocytes. In a study of 1,386 cases of AML, the Medical Research Council of England found that increased Sudan black B positivity predicted a high remission rate and long survival and suggested that >50% of blast with Sudan black B positivity should be used to distinguish M2 from M1 (17).

NBE or other nonspecific esterase (e.g., α-naphthyl acetate esterase) is positive for the monocytic series, and CAE or other specific esterase is positive for the myelocytic series (13). However, about 13% to 37% of promyelocytic leukemia cases may show strongly positive nonspecific esterase (18). A subset of myelomonocytic leukemia displays double staining of specific and nonspecific esterases in all blasts. Another study shows that all types of AML may show double staining in some cases (19).

The periodic acid-Schiff (PAS) stain showing a block pattern is seen in most cases of acute lymphoblastic leukemia (ALL). However, negative PAS staining does not rule out ALL, and positive PAS staining can be seen in occasional cases of AML (13). PAS is probably more useful in distinguishing normal erythroblasts from leukemic erythroblasts. In erythroleukemia, the pronormoblasts and other stages of normoblasts can be positive, but normal nucleated erythrocytes are PAS negative. It should be cautioned, however, that nucleated erythrocytes can be PAS positive in myelodysplastic syndrome (MDS). In megakaryoblastic leukemia, a peripheral PAS staining pattern of megakaryoblasts is characteristic.

Acid phosphatase (AP) staining can be seen in all leukocytes, but tartrate-resistant AP positivity is relatively specific for hairy cell leukemia (13). A focal paranuclear AP stain is characteristic of T lymphocytes and T lymphoblasts. However, in myeloid series, the AP staining is stronger and diffuse. Therefore, AP can be used, but is not particularly helpful in differential diagnosis between AML and ALL.

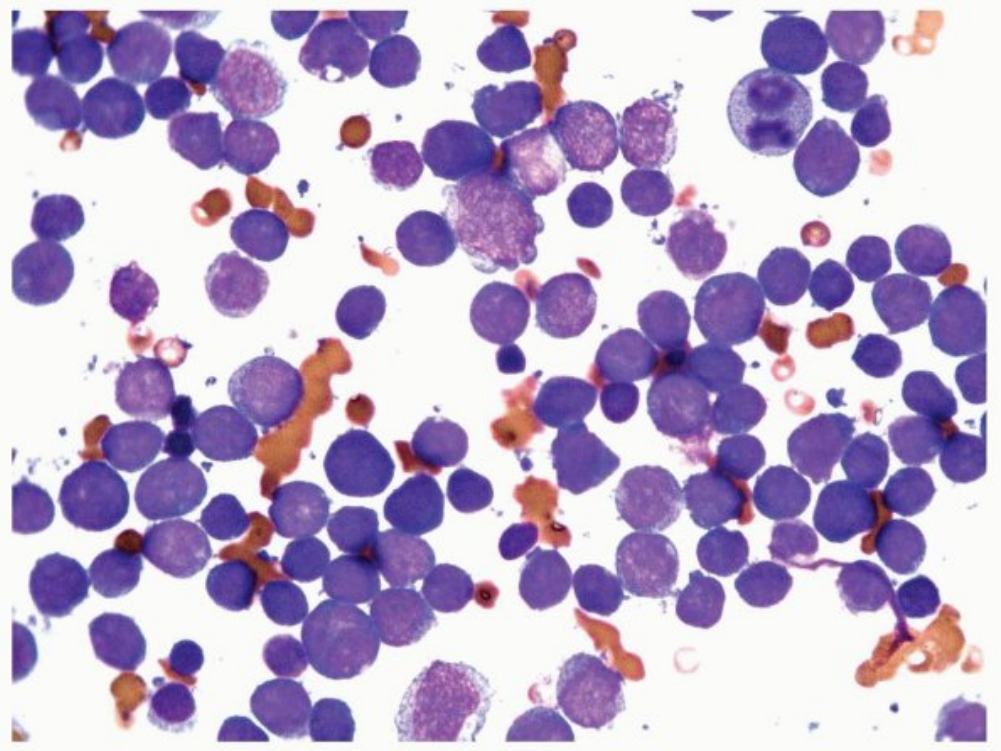

FIGURE 6.7.4 Bone marrow aspirate reveals almost exclusively blasts in the marrow with a high nuclear/cytoplasmic ratio, immature chromatin pattern, and nucleoli. 60x magnification.

With the development of flow cytometry and immunohistochemistry, cytochemical staining is no longer essential in the diagnosis of AML. However, in difficult cases of AML-M4 and AML-M5, the esterase stains are still superior to other techniques in defining the cell lineage with direct morphologic correlation. Unfortunately, cytochemical staining is technically difficult and cannot be done with automated instruments. Furthermore, because of the lack of demand, most commercial or reference laboratories do not offer cytochemical services.

Morphology

In the current case, the bone marrow aspirate showed that >90% of nonerythroid cells were blasts with scanty cytoplasm, immature chromatin pattern, and prominent nucleoli (Fig. 6.7.4). A few of them contained Auer rods in the cytoplasm, and a few cytoplasmic granules were occasionally seen. These features are consistent with myeloblasts. The core biopsy revealed diffuse infiltration of immature myeloid cells replacing the normal hematopoietic cells (Fig. 6.7.5). The same blasts were also found in the peripheral blood smears (Fig. 6.7.6).

The leukemic nature in this case is determined on the basis of extensive infiltration of the bone marrow in the core biopsy and the presence of >20% blasts and <10% nonblastic myeloid cells in the marrow aspirate. Cytochemistry demonstrated positive staining for MPO in about 3% of myeloblasts and CAE in 30% of blasts. The NBE stain was negative for the immature cells. These results confirmed the morphologic impression of myeloblastic leukemia. The flow cytometric result also confirmed the myelocytic lineage by the demonstration of 86% CD13-CD33-positive cells. The percentage of MPO was low and can be considered partial deficiency. In some myeloid leukemia cases, the percentage

of CD13-CD33 is low but that of MPO is high. That is why both of these myeloid markers should be included in flow cytometric studies of AML. The presence of high percentages of CD34- and CD117-positive cells and the dual CD13-CD33/CD7 staining are supportive of a malignant cell population, which will be discussed in the immunophenotype section. With all this information, a diagnosis of AML without maturation (M1) was established.

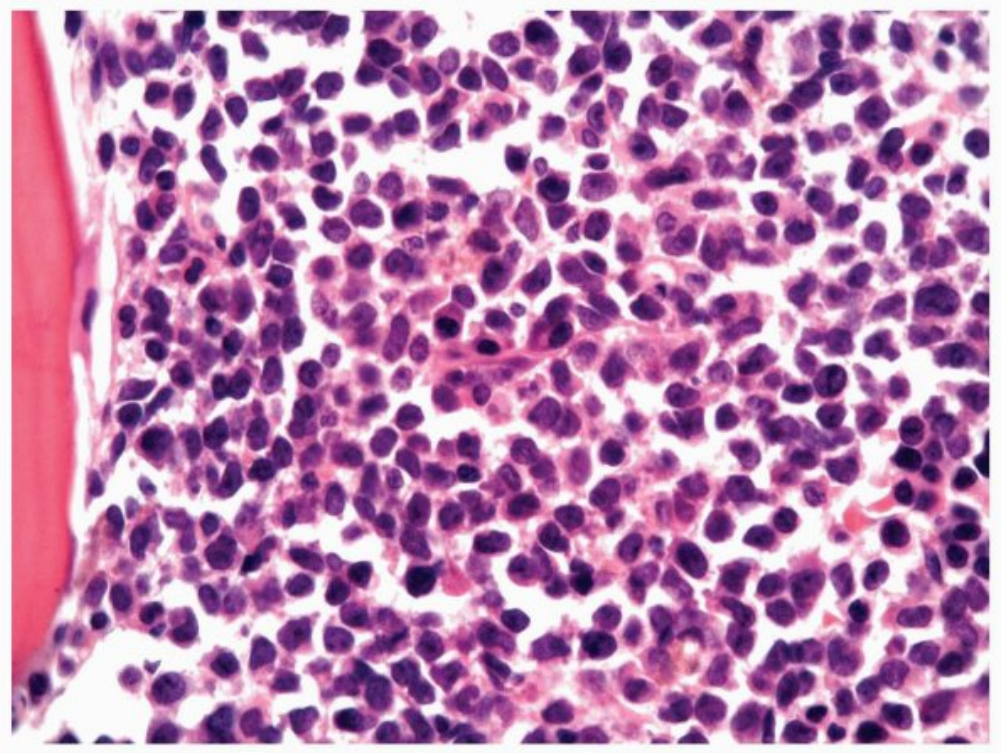

FIGURE 6.7.5 Bone marrow aspirate shows 100% cellularity, and normal hematopoietic cells are almost totally replaced by blastic cells. 60x magnification.

The major differential diagnosis for AML is ALL. Myeloblasts and lymphoblasts can be distinguished by their chromatin pattern, number and prominence of the nucleoli, amount of cytoplasm, and presence or absence of cytoplasmic granules (see Table 6.14.2). Nevertheless, all these morphologic criteria are not absolute: The only reliable morphologic marker is the Auer rod, which, however, is present in the myeloblasts in only 21% of AML cases (20). Therefore, flow cytometry and cytochemistry are needed to help with the diagnosis.

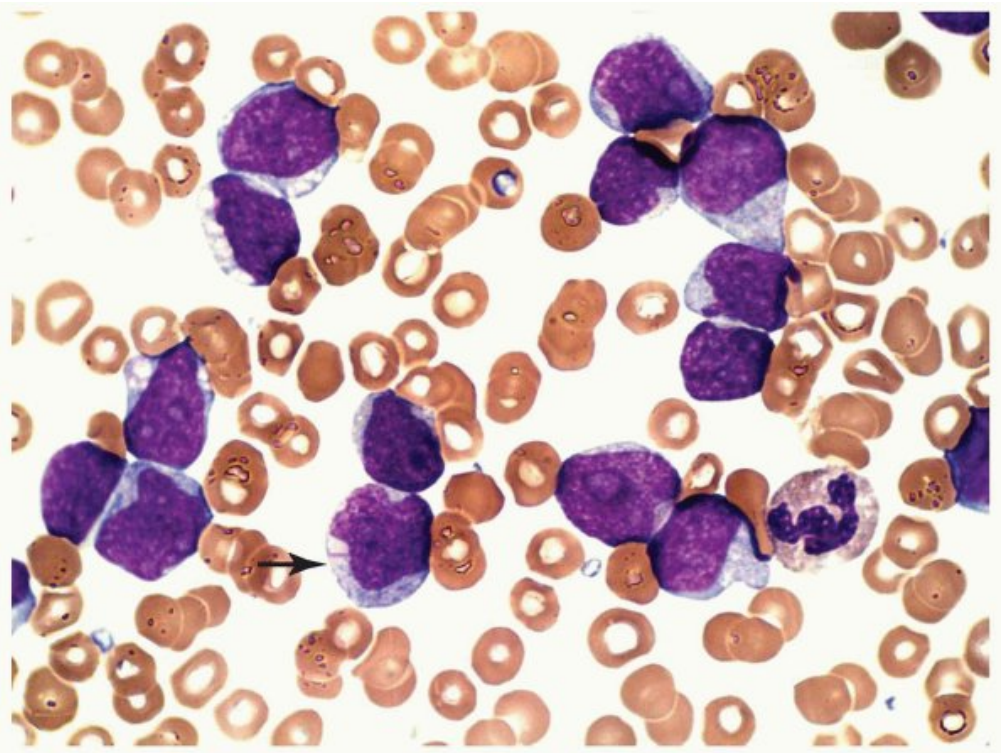

FIGURE 6.7.6 Peripheral blood smear shows multiple myeloblasts with similar features as those seen in the bone marrow. One Auer rod in the cytoplasm of a blast is indicated (*arrow*). 100x magnification.

Immunophenotype

As mentioned before, cytochemical staining can be totally negative in AML, and sometimes the staining is difficult to interpret or inconclusive. Therefore, immunophenotyping is most useful in substantiating the diagnosis (10,14,21,22). There is also evidence that immunophenotypes are frequently reliable predictors for prognosis and sensitive markers for detecting minimal residual disease (23). In addition, immunophenotyping may identify mixed lineage phenotypes, but the clinical significance of these phenotypes is controversial.

Terstappen et al. (24) found that AML cells may differ from normal cells in several aspects: expression of nonmyeloid antigens (e.g., CD2, CD5, and CD7), asynchronous expression of myeloid-associated antigens (e.g., coexpression of CD34 and CD15), overexpression of myeloid-associated antigens (e.g., CD14 and CD34), and absence of expression of myeloid-associated antigens (e.g., CD33, CD11b, CD15). Because selective loss of certain myeloid antigens is helpful in distinguishing leukemia from benign myelocytosis, the use of mixed antibodies (e.g., CD13-CD33) for screening purposes may mask this phenomenon.

Neame et al. (7) recommended the use of seven monoclonal antibodies (CD33, CD13, CD14, CD15, HLA-DR, AML2.23, and polymorphonuclear neutrophil [PMN]6/29) for immunophenotyping, which can help to distinguish the first five types of AML. In our experience, the first five antibodies should be sufficient for differential diagnosis (Table 6.7.3). Essentially, M1 and M5 are all positive for CD33 and CD13, whereas M4 and M5 are also positive for CD14 (Mo2 or My4), a monocyte marker. While the CD14-positive population is >55% in M5, it is <45% in M4. M3 is characterized by the low percentage or complete absence of HLA-DR, which is positive for the blasts but negative for the promyelocytes. The distinction between M1 and M2 is the fact that there is a negative reaction to CD15 in M1, but a positive reaction to CD15 in M2 through M5. The above-mentioned are typical immunophenotypes present in most cases, but exceptions are seen from time to time. For instance, CD14 can be negative for M4 or M5. In that case, CD64, CD11b and CD11c should be used to supplement the phenotyping panel. CD15 can also be positive in M1 and is now seldom used for differential diagnosis.

Immunophenotyping of M6 depends on a positive reaction to glycophorin A or hemoglobin A. The latter can be demonstrated by immunohistochemistry only. M7 can be identified by positive reactions to CD41 and CD61 but negative reactions to CD42 (see Case 12). The reaction in M6 and M7 cases to other myelomonocytic markers is variable and is not dependable for their identification.

The Morphologic, Immunologic and Cytogenetic (MIC) Cooperative Study Group includes CD34 and CD11 in the phenotyping panel to distinguish M1 from other subtypes of AML (3). As mentioned before, CD11b and CD11c are helpful in identifying monocytes. CD34 is now used as an integral component of the AML panel.

Normally, CD34 is present only on stem and/or progenitor cells, but it is expressed in 40% of AML cases (25). Therefore, CD34 helps to distinguish AML from benign myeloproliferative

disorders. Although CD34 may also be detected in cases of MDS, its percentage is usually lower than that in AML cases. When a high percentage is present in a case of MDS, it predicts leukemic transformation (26). CD34 is found more frequently in M0, M1, and M5a (27,28) but is often absent in M3 (29). It is associated with either good or poor prognosis, depending on the cytogenetic alteration in a particular case and the cell lineage (25). Generally, it predicts poor prognosis in AML but good prognosis in ALL (28). When CD34 is related to poor prognosis, it is usually due to the correlation between CD34 and the multiple drug resistance (MDR) protein (25). CD34 may appear at relapse of CD34-negative AML, supporting its being an unfavorable marker (28).

TABLE 6.7.3

Correlation of Immunophenotyping and FAB Classification

Antigen	*M1*	*M2*	*M3*	*M4*	*M5*	*M6*	*M7*
CD33	+	+	+	+	+	+/-	+
CD13	+	+	+	+	+	-	-
CD14	-	-	-	<45%	>55%	-	-

CD15	-	+	+	+	+	+/-	-
HLA-DR	+	+	-	+	+	+/-	+/-
CD41/CD61	-	-	-	-	-	-	+
Glycophorin	-	-	-	-	-	+	-

FAB, French-American-British; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

A relatively new marker for myeloid lineage is CD117 (c-kit or stem cell factor receptor) (30, 31 and 32). This antigen also marks the immature cells so that it can help to distinguish benign myeloid proliferation and myeloid leukemia. CD117 is better than CD34 as an immature cell marker in two aspects. First, CD117 is highly lineage specific; it has been found in lymphoid leukemia or lymphoma only in occasional cases (30, 31 and 32). Second, it can be demonstrated in M3 cases, which usually show negative CD34. CD117-positive AML cases generally carry a favorable prognosis (14).

Lymphoid markers are not infrequently identified in AML cases. As will be mentioned later, its presence may denote specific subtypes of AML. The important lymphoid marker for AML is CD7, which is included in our routine AML panel. CD7 is not present on normal myelomonocytic cells, but is detected on leukemic cells in 9.4% to 37.5% of 5 AML subtypes (M0, M1, M2, M4, and M5) (33,34). Therefore, the demonstration of dual CD7 and CD13-CD33 staining is consistent with AML. CD7-positive AML cases more frequently express the myeloid progenitor-associated antigens, such as CD34, HLA-DR, and TdT than do CD7-negative AML cases (33). This finding may suggest phenotypic immaturity of CD7+ AML and probably explains why none of the promyelocytic leukemia (M3) cases studied showed positive CD7 (28,33,34). A few studies of CD7+ AML showed that patients with this phenotype were younger, predominantly male, had more frequent involvement with the liver and central nervous system, and responded poorly to standard chemotherapy (33).

Two other special lymphoid markers that are present in AML cases are CD2 and CD19. CD2 is frequently associated with M3, and CD19 with M2 subtype (28). In one study of 170 cases of AML, CD2 and/or CD19 were detected in 33% of cases and were associated with good prognosis (35). In another study, CD19 expression was associated with poor prognosis (36). In a third study, CD20 was found to be the most commonly found lymphoid marker in AML cases, but it often was expressed in only a subpopulation of leukemic cells (37).

Previously, the presence of a lymphoid marker on AML cells was considered biphenotypic or mixed lineage leukemia. However, because lymphoid markers are so frequently encountered on AML cells, the presence of a single lymphoid marker no longer constitutes a diagnosis of mixed lineage or biphenotypic leukemia. There is still not a universal criterion to denote a biphenotypic or bilineage leukemia. Some authors consider two or more markers of another lineage as the criterion (38); others use a scoring system based on different combinations of B lineage, T lineage, and myeloid antigens (39). Antigens can also be generated after in vitro culture, leading to a bilineage phenotype (38). Therefore, the significance of identifying a biphenotypic population is still not conclusive. Some studies suggested, however, that the AML with lymphoid markers responded well to ALL therapy and thus convey a better prognosis (35). In addition, when lymphoid markers are present, immunoglobulin genes or T-cell receptor genes may be rearranged (35,40,41).

TdT is demonstrated in about 18% of AML cases (children 19%, adults 21%) (42,43). The percentage of positive cells is usually lower in AML than in ALL. TdT positivity is more common in the immature subtypes of AML (M0 and M1) and is frequently associated with CD34, another immature marker. Although some early reports considered a direct correlation between TdT positivity and immunoglobulin or T-cell receptor gene rearrangement, this finding is not supported by subsequent studies. Therefore, TdT should be viewed as an immature cell marker, but it is not lineage specific. The prognostic significance of the presence of TdT marker is controversial.

CD45, a panleukocyte antigen, is present in normal and leukemic myeloid cells. However, its low molecular isoform, CD45RO, is expressed in normal cells, whereas its high molecular isoform, CD45RA, is expressed almost exclusively in AML cases with or without coexpression of CD45RO (44).

TABLE 6.7.4

Correlation of Cytogenetic and Molecular Abnormalitites with FAB Classification

Cytogenetic Abnormalities	*Genes Involved*	*FAB Type*

	Gene activation	
inv(3)(q21q26)	Ribophorin 1/EVI1	M0, M1, M2, M4, M5, M6, M7
t(3;3)(q21;q26)	Ronphorin 1/EVI1	M1, M2, M4, M6
	Gene fusion	
t(1;22)(p13;q13)	N-RAS/C-SIS	M7 (infantile)
t(6;9)(p23;q13)	DEK/CAN	M1, M2, M4
t(7;11)(p15;p15)	HOXA9/NUP98	M2, M4
t(8;16)(p11;q13)	MOZ/CBP	M5b/M4
t(8;21)(q22;q22)	ETO/AML1	M2
t(9;11)(p22;q23)	AF9/MLL	M4, M5
t(10;11)(p12;q23)	AF10/MLL	M4, M5
+11	ALL1/MLL	M1, M2
t(11;17)(q23;q21)	MLL1/AF17	M5
t(11;19)(q23;p13.1)	MLL1/ELL	M4, M5
t(11;19)(q23;p13.3)	MLL1/ENL	M4, M5
t(15;17)(q22;q11-12)	PML/RARα	M3
inv(16)(p13q22)	MYH11/CBFß	M4Eo
t(16;16)(p13;q22)	MYH11/CBFß	M4Eo
t(16;21)(p11;q22)	FUS/ERG	M1, M2, M4, M5

FAB, French-American-British; EVI 1, ecotropic viral integration site 1; N-RAS, an oncogene derived from rat sarcoma virus; C-SIS, simian sarcoma oncogene; HOXA, hemeobox A; MOZ, monocytic leukemia zinc finger; CBP, CREB-binding protein; ETO, eight-twenty-one; AML, acute myeloid leukemia; MLL, mixed lineage leukemia; ALL, acute lymphoblastic leukemia;

PML, promyelocytic leukemia; RAR, retinoic acid receptor; MYH, smooth muscle myosin heavy chain; CBF, core binding factor.

CD56, a natural killer cell marker, is present in various subtypes of AML. In a study of 80 bone marrow specimens, CD56 was found in 15% M0, 22% M2, 17% M3, 67% M4, and 100% M5 cases (29). There is a unique subtype of CD56+ AML with a phenotype of CD56+, CD33+, CD13+/-, CD34-, HLA-DR-, CD16-. This subtype is characterized by a high white blood cell count and marked nuclear folding with variable cytoplasmic granularity resembling microgranular M3 (M3v) (45). The authors designated these cases as myeloid/natural killer cell acute leukemia.

Comparison of Flow Cytometry and Immunohistochemistry

In general, flow cytometry is preferred to immunohistochemistry mainly because more monoclonal antibodies are available for the former technique. It is difficult to subclassify AML, particularly for M4 and M5, with the latter technique (46). In addition, the sensitivity of detecting CD34 in leukemic cells in AML cases is lower by immunohistochemistry than by flow cytometry, so that it is hard to distinguish leukemic cells from normal hematopoietic cells in tissue sections (46). CD117 can be detected by both techniques, but flow cytometry is quantitative and is more useful in patient following-up.

Molecular Genetics

In recent years, most progress in AML has been made on the molecular genetic front. With the improvement of cytogenetic techniques, clonal chromosome abnormalities can be detected in 55% to 78% of cases of adult AML and in 79% to 85% of childhood AML (47). In these cases, >30 different structural abnormalities, including translocations, deletions, and inversions, have been repeatedly implicated as primary nonrandom chromosome rearrangements (Table 6.7.4) (47, 48 and 49). In contrast to lymphomas, which frequently show complex karyotypes with multiple aberrations, AML often reveals only one chromosome abnormality. Most of these aberrations are leukemia specific because they are not found in nonhematologic neoplasms. Therefore, these abnormalities are considered to represent primary chromosome changes. Additional cytogenetic abnormalities may appear during the clinical course, frequently at the time of relapse. These additional aberrations are called secondary chromosome abnormalities, which are believed

to contribute to disease progression. Primary aberrations often involve structural changes (e.g., reciprocal translocations and inversions), whereas the secondary aberrations usually involve genomic imbalances (trisomies, monosomies, deletions, and unbalanced translocations).

Some of these cytogenetic abnormalities are so specific that they define distinct subtypes of AML, regardless of the blast count (10). These abnormal karyotypes correlate well with the FAB classification. The most striking examples are the association of t(15;17) with M3, inv(16) or t(16;16) with M4Eo, t(8;21) with M2, t(1;22) with infantile M7, and t(8;16) with M4 or M5b with erythrophagocytosis by leukemic cells.

Cytogenetic abnormalities also are accurate predictors for prognosis in AML. For instance, t(15;17), t(8;21), and inv(16) are associated with favorable prognosis. In contrast, t(6:9), 5q-, and 7q- predict poor prognosis (50).

Some karyotypes are associated with certain lymphoid or other surface markers (27,29). The expression of CD2 is associated with M4Eo/inv(16), CD19 is associated with M2/t(8;21), and absence of CD34 and HLA-DR is associated with M3/t(15;17).

Many genes are now known to be converted into leukemia genes by the mechanism of either gene activation or gene fusion (48). Gene activation occurs when a translocated gene is under the control of a new promoter and/or enhancer. This activated or deregulated gene then becomes an oncogene, leading to leukemogenesis. Gene fusion occurs when segments from two different genes are fused together to give rise to a chimeric transcript. The transcript is then translated into chimeric proteins that lead to leukemogenesis through the transduction system. In AML, most karyotypic changes result in gene fusion with only a few abnormalities resulting in gene activation.

Despite the advances in cytogenetic techniques, a proportion of submicroscopic alterations of genetic material can only be detected by molecular techniques. These conditions are called cryptic abnormalities (51). The major examples are fms-like tyrosine kinase 3 (FLT3) mutations, mixed lineage leukemia (MLL) partial tandem duplications, and (WT1) overexpression. The FLT3 mutation is the most commonly mutated gene in AML, accounting for 30% of AML patients (52).

There are also conditions that the cytogenetic results are false-negative due to technical problems. In two large study series, reverse transcription-polymerase chain reaction (RT-PCR) detected chimeric CBFα2/ETO (core-binding factor/eight twenty one) and CBFβ/MYH11 (core-binding factor/smooth muscle myosin heavy chain) transcripts in one third of patients with no detectable cytogenetic abnormalities (53,54). Even in acute promyelocytic leukemia, 15% of cases were found to be cytogenetically negative and molecularly positive in one study (55).

In about 40% of AML cases, no cytogenetic or molecular abnormalities are detected (50). However, the newly used gene expressing profiling (GEP) technique may gradually fill this gap. GEP may help distinguish AML from ALL (56,57). It may also demonstrate specific profiles in various cytogenetic subtypes of AML, such as t(8;21), inv(16), t(15;17), and MLL chimeric fusion genes (58, 59 and 60). In limited studies, GEP has been shown to be able to stratify AML cases with or without cytogenetic abnormalities and to predict the prognosis in these subsets; these are examples of some very attractive applications of this new technology (50).

TABLE 6.7.5

Salient Features for Laboratory Diagnosis of AML-M1

1. >90% myeloblasts in the bone marrow or peripheral blood
2. >3% MPO-positive blasts in the bone marrow
3. Blasts positive for CAE but negative for NBE
4. Blasts positive for CD33/CD13, HLA-DR
5. Blasts in most cases positive for CD117 or CD34
6. Blasts negative for CD14, CD15, CD41/CD61, glycophorin A
7. Immunoglobulin or TCR gene rearrangement in mixed lineage leukemia

Possible cytogenetic aberrations: inv(3)(q21q26), t(3;3)(q21;q26), t(6;9)(p23;q34), +11, t(16;21)(p11;q22)

AML, acute myeloid leukemia; CAE, chloroacetate esterase; MPO, myeloperoxidase; NBE, α-naphthyl butyrate esterase; TCR, T-cell receptor; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

The salient features for laboratory diagnosis of AML-M1 are summarized in Table 6.7.5.

Clinical Manifestations

Clinical symptoms are mainly due to the failure of the leukemic cells to mature and to the inhibition of normal hematopoiesis. Most patients may have anemia and/or thrombocytopenia. As a result, these patients have symptoms of fatigue, malaise, weakness, or hemorrhages. When the mature granulocytes are markedly decreased, superimposed infections are a common phenomenon. A fungal infection can be fatal to the patient.

AML has a bimodal age distribution. The first group, de novo AML, is usually seen in children and young adults with chromosomal abnormalities (mostly translocation) (49). The second group (secondary AML) is seen in elderly persons, associated with MDS, alkylating agent chemotherapy, or Fanconi anemia. It can also be seen in a subset of young patients.

The true de novo AML is characterized by a younger age group, absence of multistep progression, similar cytogenetic abnormalities, and, frequently, good response to chemotherapy. The second group, also called MDS-related AML, is characterized by resistant leukemia, poor marrow reserve with prolonged cytopenia after chemotherapy, early relapse, common cytogenetic abnormalities shared with MDS, and frequent multilineage dysplastic morphology in residual hematopoietic cells. Therefore, these two groups are different not only in age, but also in cytogenetic make-up, therapeutic response, and prognosis.

Adverse prognostic factors in patients with AML include unfavorable karyotype, age >60 years, secondary AML, poor performance score, features of multidrug resistance, leukocyte count >20,000/μL, unfavorable immunophenotype,

CD34 positivity, and elevated lactate dehydrogenase levels (3,61). The identification of FLT3 mutations and overexpression of ecotropic viral integration site 1 (EVI 1) have been included recently as independent indicators for an unfavorable prognosis (14). In general, AML carries a worse prognosis than ALL. In adult AML cases, the cure rates are approximately 40% to 50%, as compared to the 75% to 80% cure rates in pediatric ALL cases (62).

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CASE 8 Acute Myeloblastic Leukemia with Maturation

CASE HISTORY

A 52-year-old man had the chief complaint of shortness of breath and fatigue. He was admitted to another hospital and was found to have anemia. A gastrointestinal workup showed nothing remarkable. A bone marrow biopsy was performed; the diagnosis was myelodysplastic syndrome: Refractory anemia with excess blasts. He was treated with Gleevac and prednisone to no avail. The patient had become transfusion-dependent.

When the patient was transferred to our hospital 1 year later, physical examination showed no hepatosplenomegaly or lymphadenopathy. His total leukocyte count was 56,000/μL with 44% blasts and 38% myeloid cells of various developmental stages, but only 1% of monocytes were demonstrated. Bone marrow examination revealed 68% myeloblasts and 23% other myeloid cells. The monocytes were <1%. No basophils were identified.

FLOW CYTOMETRIC FINDINGS

Bone marrow: Myeloid markers: myeloperoxidase (MPO) 20%, CD13-CD33 97%, CD14 0%, HLA-DR 85%. T-cell

marker: CD7 0%. Immature cell markers: CD34 51%, CD117 74% (Fig. 6.8.1).

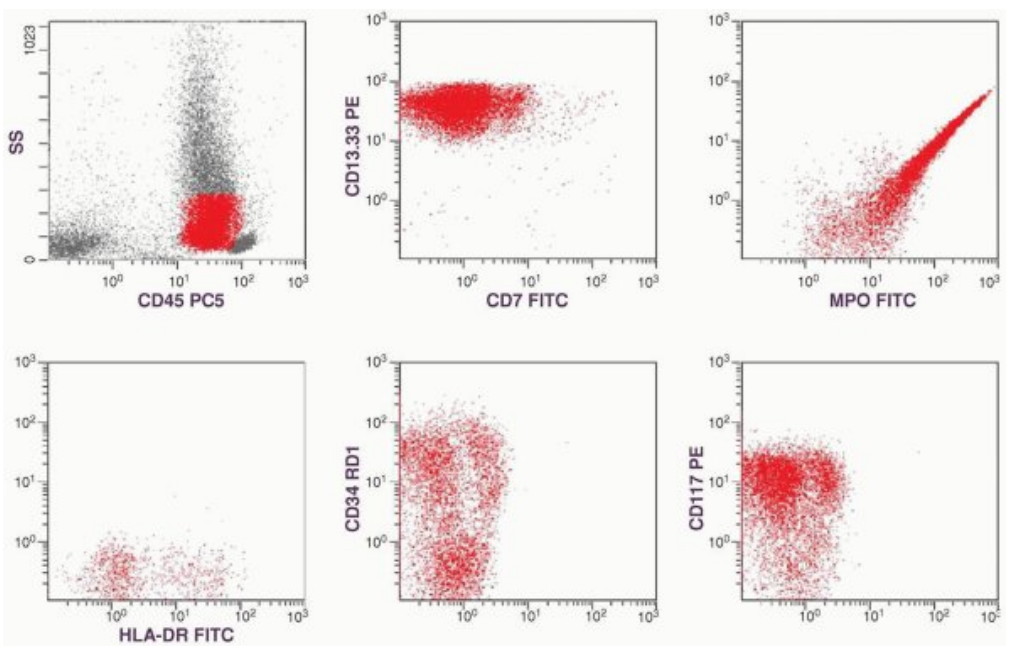

FIGURE 6.8.1 Flow cytometric analysis of bone marrow shows a tight cluster of leukemic cells

in the cluster of differentiation (CD)45/side-scatter gating and a large population of maturing granulocytes with high side scatter. The population of leukemic cells is characterized by myeloid markers CD13-CD33, myeloperoxidase (MPO), and by stem cell markers CD34 and CD117. Human leukocyte antigen-DR (HLA-DR) is weekly positive. ss, side scatter; PC5, phycoerythrin cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; RD1, rhodamine.

MOLECULAR GENETICS

The karyotype was 46, XY, t(6;9)(p23;q34). A (CAN/DEK) fusion gene was detected by reverse transcriptase-polymerase chain reaction.

DISCUSSION

The definition of acute myeloblastic leukemia (AML) with maturation or AML M2 by French-American-British (FAB) classification includes the following criteria (1, 2, 3, 4, 5 and 6): (a) The myeloblast count should be ≥30% in the bone marrow or peripheral blood. The World Health Organization (WHO) system changes the threshold to 20% based on clinical trials. (b) The mature myeloid population from the segmented neutrophil to promyelocyte should be >10% in the bone marrow to distinguish from AML-M2 AML without maturation (AML-M1). (c) Monocytic components should be <20% in the bone marrow and $<5 \times 10^9$/L in the peripheral blood to exclude acute myelomonocytic leukemia.

In addition, the blast count should include blast type 1 (no cytoplasmic granules), blast type 2 (<20 cytoplasmic granules), and blast type 3 (>20 cytoplasmic granules). However, when blast type 3 is >10%, the leukemic case should be classified as AML with maturation, even though the mature granulocytes are <10% (1). Blast types 2 and 3 can be distinguished from promyelocytes by the centrally located nucleus, absence of a prominent Golgi zone, and presence of a fine chromatin pattern. AML with maturation is the most common subtype of AML, accounting for 25% to 45% of AML cases (4,7).

Morphology

In addition to what was described in the definition, this subtype frequently shows dysplastic changes in myeloid cells, including abnormal nuclear segmentation and hypogranulation (4). Eosinophilia may also be present in the bone marrow, but it does not show the cytological or cytochemical abnormalities characteristic of acute myelomonocytic leukemia with eosinophilia (4).

In the current case, the myeloblast count was 44% in the peripheral blood (Fig. 6.8.2) and 68.7% in the bone

marrow (Figs. 6.8.3 and 6.8.4). No monoblasts were demonstrated in the bone marrow, and <1% of monocytes were present. Therefore, morphologically, it fulfils the definition of AML with maturation. However, if monoblasts are present and the distinction between the myeloblast and monoblast populations is not certain, cytochemical staining is required to distinguish M2 from M4.

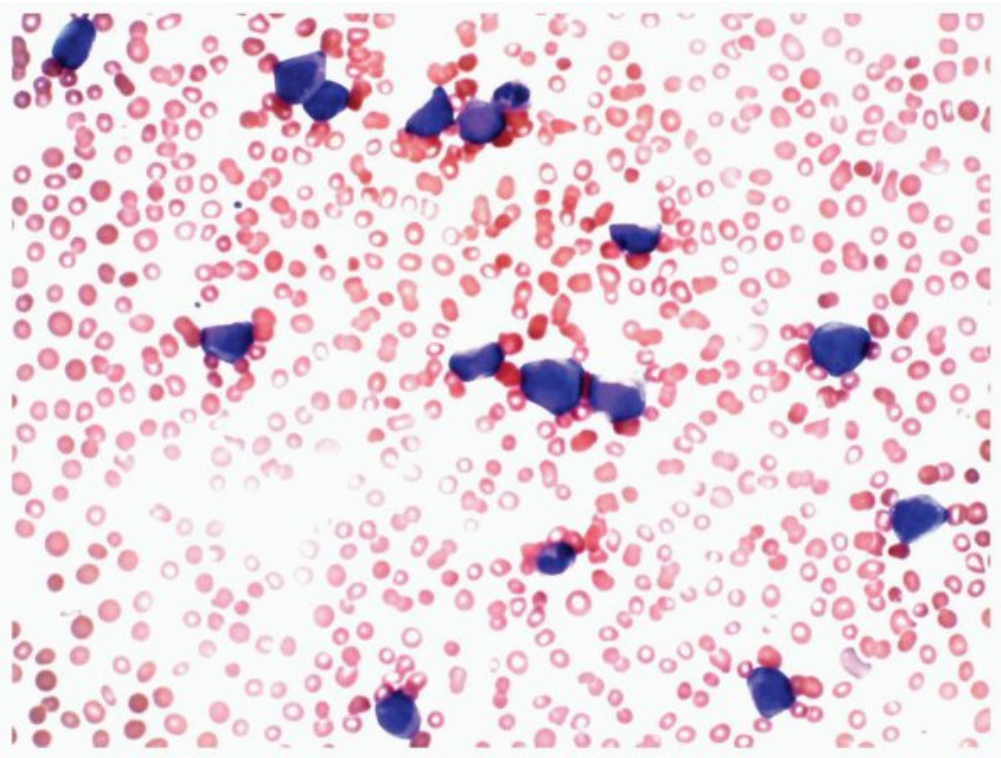

FIGURE 6.8.2 Peripheral blood smear shows myeloblasts and a few mature granulocytes. Wright-Giemsa, 40x magnification.

AML M2 cases with t(6;9)(p23;q34), such as our case, are frequently associated with myelodysplastic syndrome and basophilia in the bone marrow (2). The current case had a history of myelodysplastic syndrome, but basophilia was not demonstrated in the bone marrow.

Cytochemistry

The FAB system requires three cytochemical stains as the basis for AML classification. In M2, the MPO should be positive for >3% of blasts. The specific esterase or chloroacetate esterase (CAE) should be positive, and the nonspecific esterase or α-naphthyl butyrate esterase (NBE) should be negative for the blasts (Fig. 6.8.5). However, in some M2 cases, NBE was negative and yet focal staining of α-naphthyl acetate esterase (another monocyte marker) was demonstrated in most myeloblasts (8).

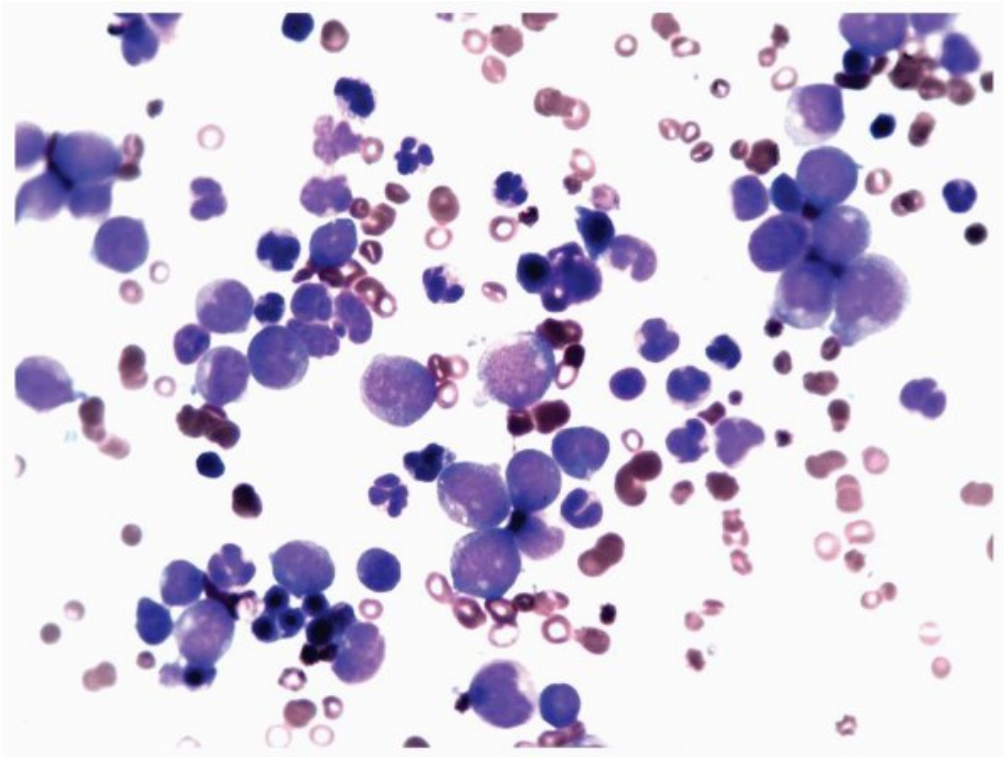

FIGURE 6.8.3 Bone marrow aspirate reveals a high percentage of myeloblasts intermingled with mature granulocytes. Wright-Giemsa, 60x magnification.

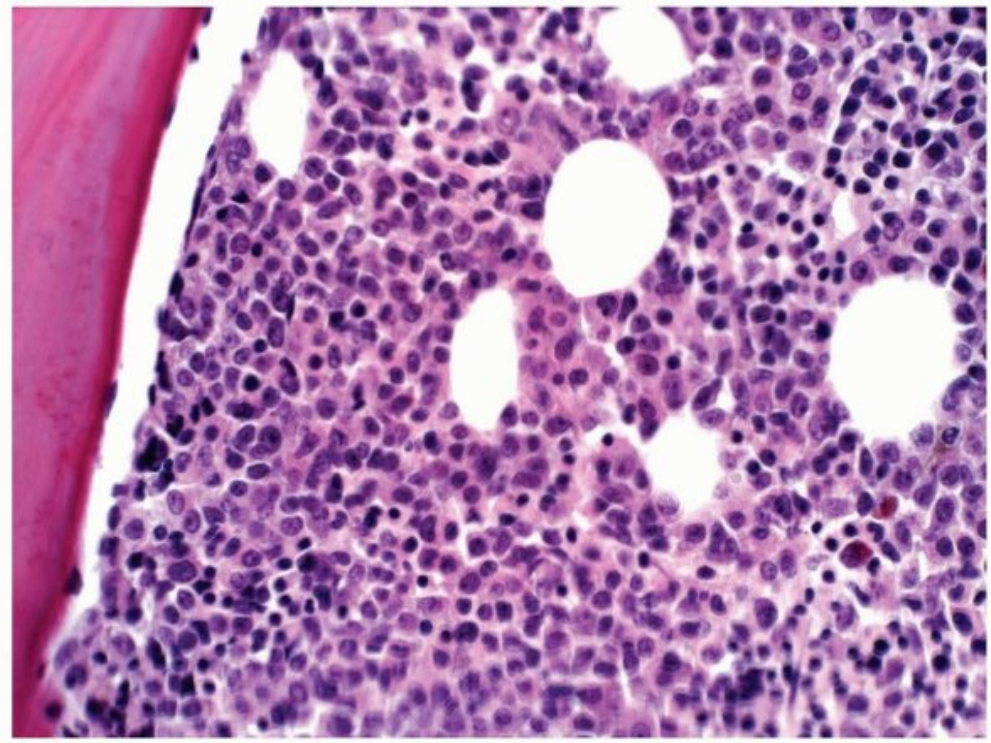

FIGURE 6.8.4 Bone marrow core biopsy demonstrates hypercellularity with mature and immature myeloid cells. No erythroid elements are demonstrated. Hematoxylin and eosin, 40x magnification.

A British group proposed using >50% of blasts with Sudan black B positivity as a cutoff to distinguish M2 from M1; the latter usually had <50% Sudan black B-positive blasts in the bone marrow (9). MPO is frequently stronger in M2 than in M1. When very strong MPO activity and abundant Auer rods are present in neutrophils and eosinophils in an M2 case, t(8;21) should be suspected (10,11) (see Case 4).

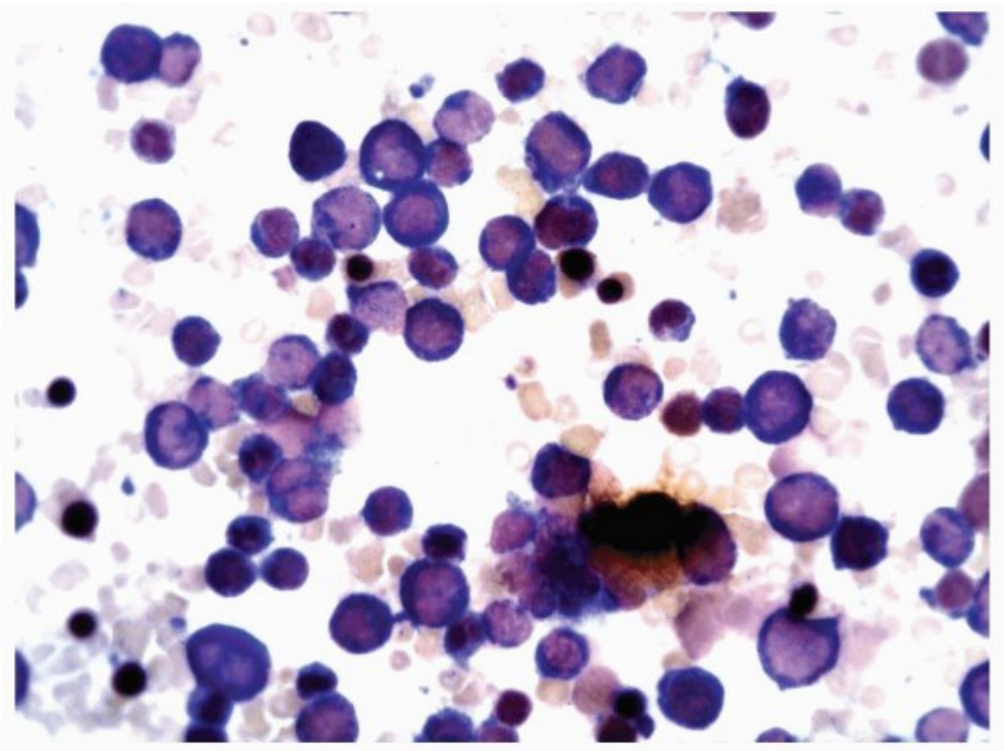

FIGURE 6.8.5 Bone marrow aspirate stained with combined esterases. All myeloblasts and different developmental stages of myeloid cells stained with chloroacetate esterase (*blue*). Only one monocyte is stained by α-naphthyl butyrate esterase (*brown*). Combined esterase stain, 60x magnification.

Immunophenotype

The FAB system uses morphology and cytochemical staining to classify AML. However, because immunophenotyping is now commonly applied to AML classification, cytochemical staining is no longer essential. For instance, in cases of minimally differentiated AML in which myeloperoxidase stain is negative, immunophenotyping by flow cytometry is critical to distinguish it from acute lymphoblastic leukemia.

A monoclonal antibody panel including CD13, CD14, CD15, CD33, CD64 and HLA-DR is sufficient to make a preliminary classification of AML subtypes (12). Whereas CD13 and CD33 are screening markers for AML, others help to differentiate the subtypes. CD14 and CD64 are present in monocytic subtypes (M4 and M5). HLA-DR is low or absent in acute promyelocytic leukemia. CD15 is supposed to be negative in M1 and can help to distinguish other subtypes, but it may be present in some M1 cases and is thus not very specific.

The malignant nature of the myeloid population is identified by two markers, CD34 and CD117. CD34 is a hematopoietic progenitor antigen; therefore, a high percentage of CD34-positive cells is suggestive of leukemia or myelodysplasia (13,14). CD34 is preferentially present in the most immature phenotypes (M1, M2, and M5a) (15). Recently, CD117, a stem cell factor receptor also known as c-kit, is used to supplement CD34 in identifying myeloblasts. CD117 is negative in cases of acute lymphoblastic leukemia, but it is positive in cases of AML including acute promyelocytic leukemia (M3) (16).

AML may also express lymphoid markers, such as CD2, CD7, CD10, CD19, and CD20 (6). The demonstration of a single lymphoid marker in a myeloid population does not indicate a biphenotypic leukemia; rather this aberrant immunophenotype supports the diagnosis of malignancy. CD7 is the most commonly expressed marker, found in 19% to 32% of AML cases depending on the subtypes (6,17). Therefore, CD7 has been included in the diagnostic panel for AML in many laboratories.

Immunohistochemistry may demonstrate myeloid markers, such as myeloperoxidase, lysozyme, and CAE (Leder stain). CD34 and CD117 may also be demonstrated by immunohistochemical stains, which, however, are usually less sensitive than flow cytometry; only partial or negative staining is present in most cases (Fig. 6.8.6). The salient features for laboratory diagnosis of acute myeloblastic leukemia with maturation are summarized in Table 6.8.1.

Molecular Genetics

The most frequent cytogenetic abnormality seen in about 46% of M2 cases is t(8;21)(q22;q22) (18). This is classified as a separate subtype in the WHO system and is described in Case 4.

Another group of M2 is associated with basophilia in the bone marrow. At least two abnormal karyotypes have been found in this group of M2: t/del(12)(p11-13) and t(6;9)(p23;q34) (2). In these cases, the blasts are agranular, but other cells show evidence of maturation toward basophils (19). Basophilic granules can also be detected in a few blasts by electron microscopy. The t(6;9)(p23;q34) results in the formation of a chimeric fusion gene: DEK/CAN (4).

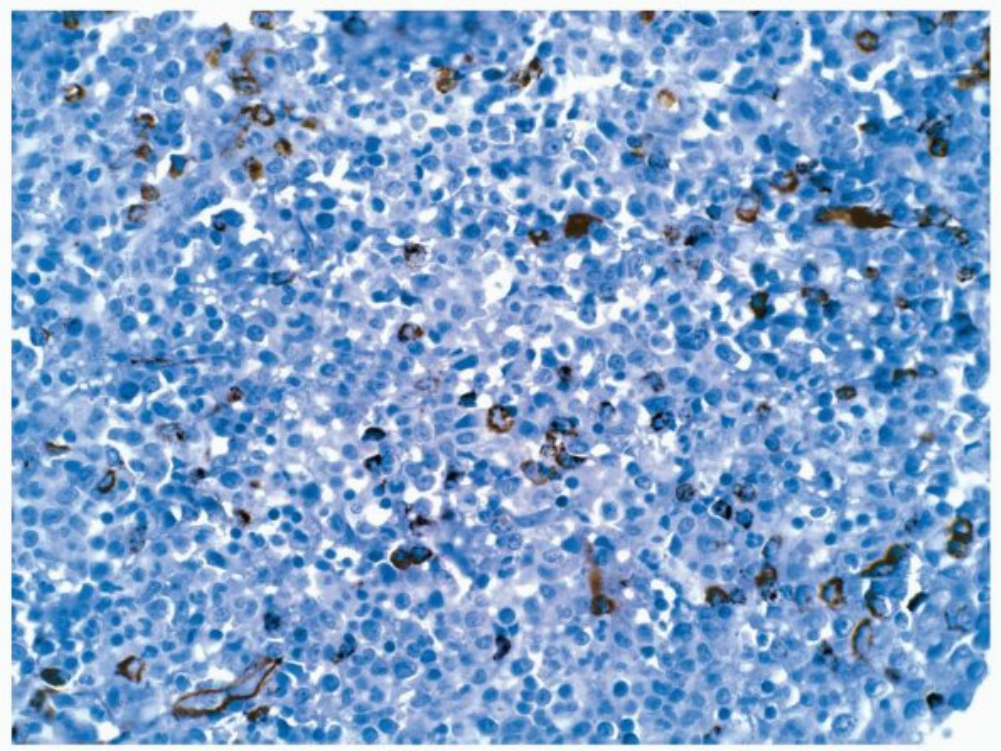

FIGURE 6.8.6 Bone marrow biopsy shows scattered CD34-positive myeloblasts. Immunoperoxidase, 40x magnification.

There are several rare abnormal karyotypes reported in M2 cases. These include inv(3)(q21;q26), t(3;3)(q21;q26), t(7;11)(p15;p15), t(6;21)(p11;q22), +11(2.23), and double minute chromosomes (2,20,21). A rare karyotype of t(8;16)(p21;p13) is associated with hemophagocytosis, particularly erythrophagocytosis (22).

Clinical Manifestations

AML with maturation occurs in all age groups with 40% seen in patients older than 60 years and 25% in patients younger than 25 years (4). The clinical symptoms are not

different from other subtypes of AML. The major mechanisms are failure of the leukemic cells to mature and inhibition of normal hematopoiesis. As a result, the patients may have anemia, neutropenia, and/or thrombocytopenia. This subtype of AML usually responds well to chemotherapy, but the prognosis is frequently associated with the abnormal karyotype of the leukemic cells.

TABLE 6.8.1

Salient Features for Laboratory Diagnosis of Acute Myeloblastic Leukemia with Maturation

1. 20% to 90% of myeloblasts present in bone marrow or blood
2. <20% monocytic components in the bone marrow
3. $<5 \times 10^9$/L monocytic components in the peripheral blood
4. Cytochemical stain for blasts: Positive for myeloperoxidase and chloroacetate esterase but negative for α-naphthyl butyrate esterase

5. Monoclonal antibody panel: Positive for CD13, CD15, CD33, HLA-DR; negative for CD14 and CD64

6. CD34, CD117, and CD7 can be positive to support the malignant nature.

7. Immunohistochemistry: Positive for lysozyme, myeloperoxidase, chloroacetate esterase, CD34, or CD117

CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

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CASE 9 Acute Myelomonocytic Leukemia

CASE HISTORY

A 60-year-old man presented to the emergency room because of a 1-day history of severe epistaxis. He first noticed small amount of bleeding from his nose a few days before, but it stopped spontaneously. The patient also complained of a 40-pound weight loss in the past several months. On admission, he was found to have a hematocrit of 18% as compared with his previous record of 38% a few months ago. His platelet count was 120,000/μL. His leukocyte count was 6,400/μL with 66% neutrophils, 18% lymphocytes, and 18% monocytes. After blood transfusion and intravenous fluids, the patient's condition was stabilized and his bleeding stopped. An ear, nose, and throat (ENT) physician was consulted, but he did not find any visible nasal masses. Because of positive occult blood in the stool, a computed tomography (CT) scan of the abdomen, a colonoscopy, and an upper endoscopy were performed. A sigmoid colon polyp was found, but biopsy showed no malignancy. The patient was discharged 1 week after admission.

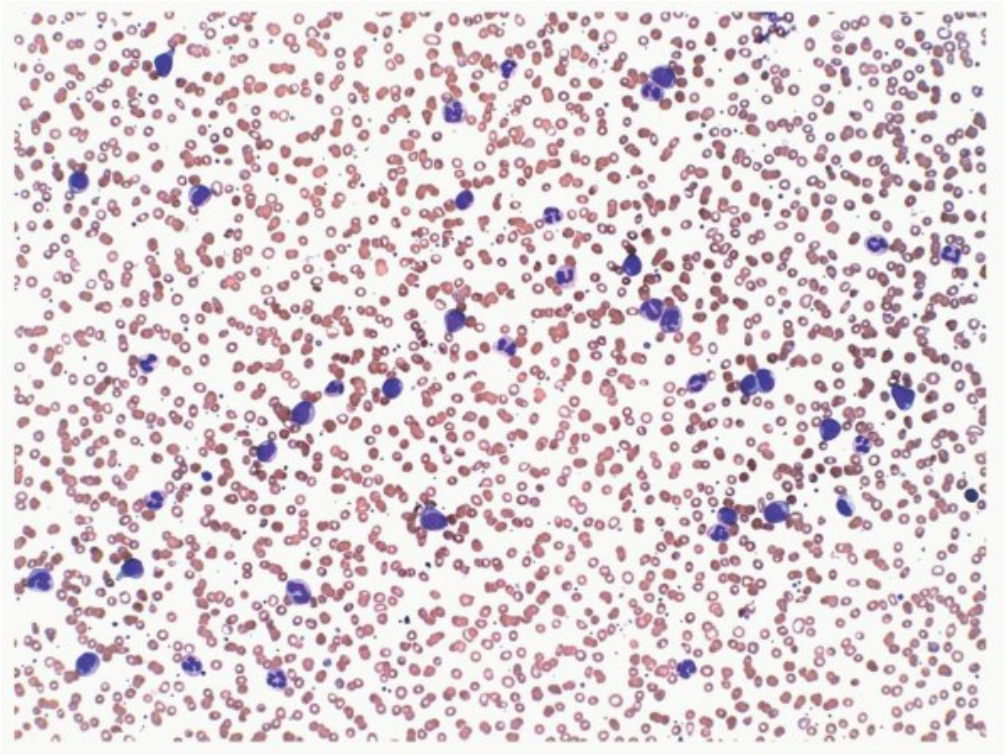

FIGURE 6.9.1 Peripheral blood smear shows leukocytosis consisting of mature and immature myelomonocytic cells. Wright-Giemsa, 20x magnification.

During the subsequent follow-up period, the patient's platelet count gradually returned to normal, but his hematocrit remained at low level (30%) and hemoglobin, 10 g/dL. Hypersegmented and hypogranular granulocytes, giant platelets, spherocytosis, and polychromasia were seen in the peripheral blood. The patient was considered to have myelodysplastic syndrome. However, his leukocyte count rapidly rose to 30,000/μL and the blasts gradually went up to 21% (Fig. 6.9.1). Monocytosis was also present. Bone marrow examination showed 16% myeloblasts, 15% monoblasts and/or promonocytes, 26% monocytes, 2.5% promyelocytes, 6.7% myelocytes, 7.3% metamyelocytes, 12.3% bands, and 5.25% segmented granulocytes. Only 6% of erythroid series was demonstrated (Figs. 6.9.2 and 6.9.3). A diagnosis of acute myeloid leukemia (AML) was established approximately 2 months after first admission.

FLOW CYTOMETRIC FINDINGS

Bone marrow: Myelomonocytic markers: CD13-CD33 98%, CD13-CD33/CD7 40%, CD14 HLA-DR 94%, CD34 89%, CD117 93% (Fig. 6.9.4).

CYTOCHEMICAL FINDINGS

In the bone marrow aspirate smear, myeloperoxidase (MPO) stain was positive in >5% of blasts. The chloroacetate esterase (CAE) and α-naphthyl butyrate esterase (NBE) stains demonstrated approximately the same percentages of myeloid and monocytoid cells.

DISCUSSION

Acute myelomonocytic leukemia (M4) accounts for 20% to 30% of AML cases (1, 2 and 3). Therefore, it is as common as AML M2 in frequency. The diagnostic criterion as defined by the French-American-British (FAB) group is the presence of 30% of blasts in the bone marrow, including type I and type II myeloblasts, monoblasts, and promonocytes (4). The World Health Organization (WHO) classification, however, lowers the cutoff point of blasts in the bone marrow to 20%. In the differential count, both the granulocytic and monocytic components should exceed 20%; below this threshold, the leukemia is classified as M5 or M2, respectively.

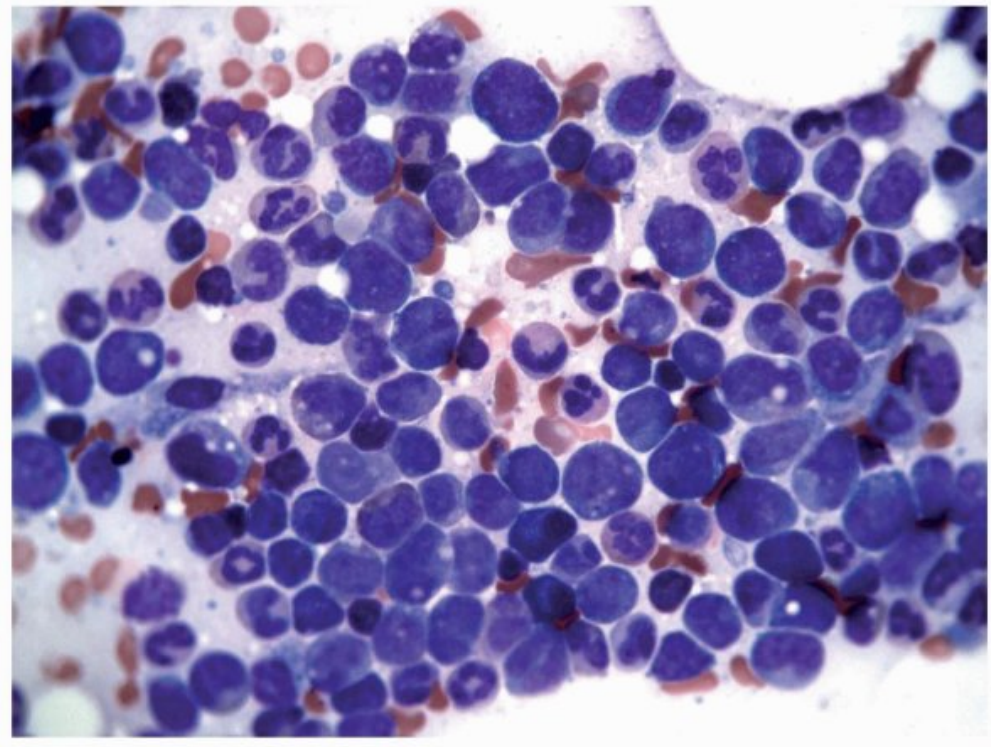

FIGURE 6.9.2 Bone marrow aspirate shows a packed marrow with mostly immature myeloid and monocytoid cells. Wright-Giemsa, 60x magnification.

Cytochemical stains should be used to determine the percentages of myeloid and monocytoid cells. The monocytoid cells can be identified by nonspecific esterase, and the myeloid cells by specific esterase (see Case 7). The identification of monocytoid cells in the bone marrow is

particularly difficult by morphology. However, nonspecific esterase can be weak or absent in monocytoid cells in some cases. If morphologic identification of monocytes is certain, absence of nonspecific esterase does not exclude the diagnosis of M4 (2).

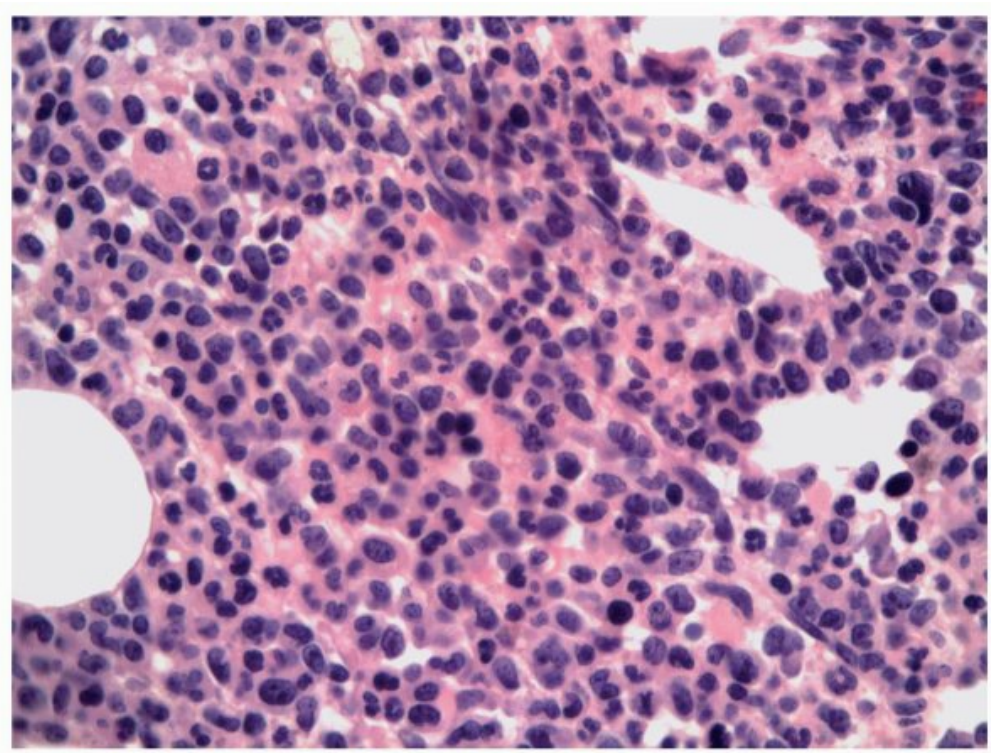

FIGURE 6.9.3 Bone marrow core biopsy reveals hypercellular bone marrow with myelomonocytic leukemic cells replacing the normal hematopoietic components. Hematoxylin and eosin, 20x magnification.

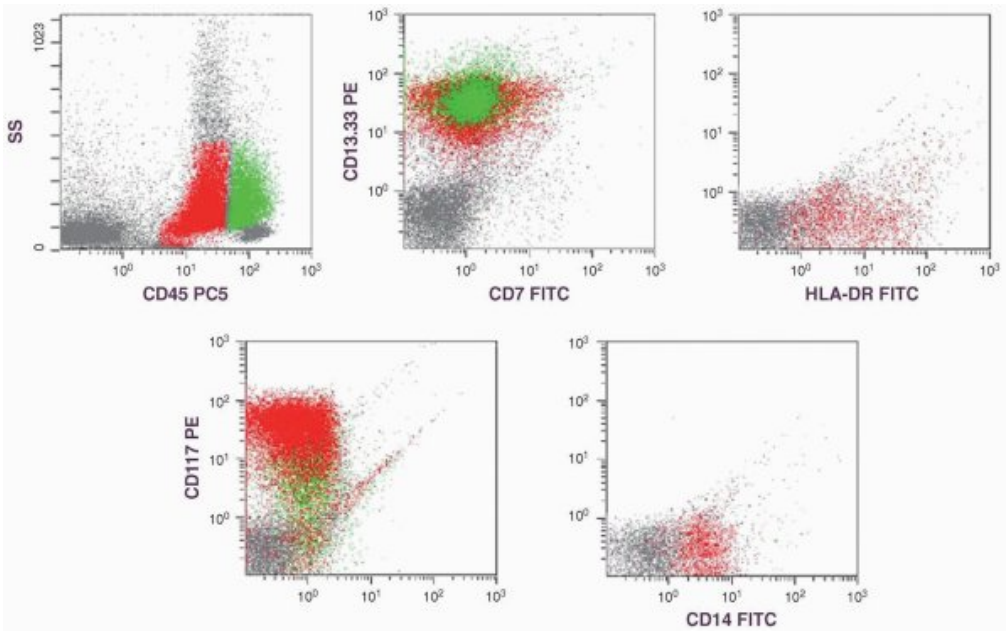

FIGURE 6.9.4 Flow cytometric histograms from a case of acute myelomonocytic leukemia (not the current case) demonstrated two immature cell clusters in the dot plot. Both populations express CD117, CD13-CD33, and (partially) CD7. The red cluster also shows CD14 and human leukocyte antigen-DR (HLA-DR). PC5, phycoerythrin cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; SS, side scatter.

When the percentage of monocytoid cells in bone marrow is <20%, the peripheral blood should have >5 × 10^9/L monocytes to meet the diagnostic criteria (4). When the monocyte count is below that level, a high lysozyme concentration can be used as evidence for a significant monocytosis, thus substantiating the diagnosis of M4 (4). The lysozyme concentrations should exceed three times the normal values in serum or urine to be considered significant. The only exception is the subtype of M2 with eosinophilia that may show a high lysozyme level because eosinophils also contain lysozyme (5).

In the current case, the first presentation in the preleukemic phase was epistaxis, which led to the discovery of anemia and thrombocytopenia in this patient. In the follow-up period, features of myelodysplastic syndrome became apparent, which rapidly evolved into AML that was composed of both myeloid and monocytoid elements. Based on the bone marrow differential count, the cytochemical findings, and flow cytometric results, this case fulfills the diagnostic criteria of M4.

Morphology and Cytochemistry

The leukemic component in M4 includes type I and type II myeloblasts, monoblasts, and promonocytes (4). Type I myeloblasts have no cytoplasmic granules, and type II myeloblasts have <20 azurophilic granules. Monoblast and monocytes may or may not have cytoplasmic granules, but their nuclei differ from those of myeloblasts in a folded or lobulated configuration. However, the very immature monoblast may show round or oval nuclei that are similar to those of myeloblasts, but monoblasts are usually larger than myeloblasts and have abundant cytoplasm with irregular border.

Cytochemical stains are originally required by the FAB system for estimation of the percentages of these two populations, because the distinction between myeloblasts and monoblasts is sometimes difficult. However, with the recent development of flow cytometry and immunohistochemistry, cytochemistry is gradually being replaced.

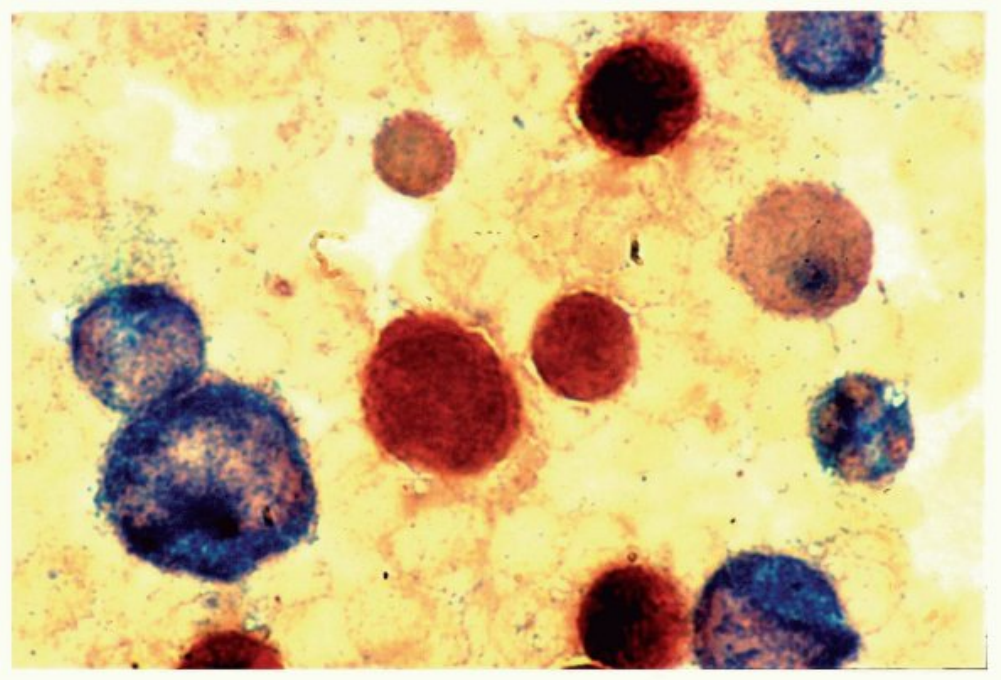

FIGURE 6.9.5 A bone marrow aspirate reveals chloroacetate esterase-positive (*blue*) and α-naphthyl butyrate esterase-positive (*brown*) populations. Note that some cells are positive for both esterases. Combined esterase stain, 100x magnification. (From Sun T. Flow cytometric analysis of hematologic neoplasms, 2002.)

The distinction between monoblasts and promonocytes depends on the nuclear configuration, the chromatin pattern, and the prominence of nucleoli. Promonocytes usually have more obvious lobulation of the nuclei, more mature chromatin pattern, and less conspicuous nucleoli than the monoblasts have.

In the leukemic population, the myeloblasts are positive for MPO, Sudan black B, and CAE. The monoblasts and promonocytes are positive for nonspecific esterases, which include NBE and α-naphthyl acetate esterase. Monocytic series react either weakly positive or negative to MPO and Sudan black B stains.

In some cases of M4, the leukemic cells may show both CAE and NBE in the same cells (6,7) (Fig. 6.9.5). This population is considered to be a group of hybrid monocyte-granulocyte. Monocytic components in M4 also show double staining of lactoferrin and lysozyme, characteristic of granulocytes (8). Therefore, the myelocytic and monocytic components in M4 are probably derived from the same precursor cells (8).

A subtype of M4 shows bone marrow eosinophilia. This subtype accounts for 15% to 30% of M4 cases (9) and is designated M4 with eosinophilia (M4Eo). M4Eo is associated with a special cytogenetic karyotype: inv(16) or, less frequently, t(16;16). This entity is described in Case 5.

Immunophenotype

The immunophenotypes of various subtypes of AML were delineated by several groups in the late 1980s (10, 11 and 12). Since then, flow cytometry has become the mainstay for the subclassification. The major myelomonocytic markers are CD13 and CD33, which have been routinely used for screening of myelomonocytic cells. Recently, cytoplasmic staining of MPO also has been included in the panel for AML. The monocytic component, as seen in M4 and M5, is routinely scanned by CD14, which includes several monoclonal antibodies from different manufacturers, such as M02, MY4, and LeuM3. The combined use of MO2, MY4, and CD64 is able to distinguish various maturation stages (13). CD64 is positive for the entire spectrum of monocytes, MY4 for promonocytes and mature monocytes, and MO2 for mature monocytes only.

Other markers, such as CD11b and CD11c, are not as specific for monocytes as CD14, but they are sometimes more sensitive than the latter. CD11b and CD11c are negative for myeloblasts, but can be demonstrated in the more mature forms of myeloid cells. These monocytic markers, however, cannot be relied upon for quantitation; for instance, to distinguish M4 from M5 (11). Other monocyte markers, such as CD32 and CD36, are seldom used for the diagnosis of AML.

In addition to the identification of myeloid and monocytoid lineage, the immunophenotype should include the immature cell markers, such as CD34 and CD117, to identify the malignant nature of the myelomonocytic population (2,14).

HLA-DR is routinely included in the AML panel, because its absence or decrease in percentage is characteristic of acute promyelocytic

leukemia (M3). In the microcytic form of M3, the leukemic cells may show monocytoid nuclei; in those cases, M4 and M5 should be included in the differential diagnosis.

A few studies emphasized the prognostic value of some myeloid markers. For instance, My7 (CD13) and My4 (CD14) are predictors for a low rate of complete remission (15,16). A high CD33/CD13 ratio, in contrast, is a favorable prognostic factor (16). A CD17 antigen, which is seldom included in an AML panel, is a predictor for a shorter survival (12).

T-cell antigens have been detected in 44% of M4-M5 cases (CD2 14%, CD4 12%, CD7 36%) in one study (11). In general, CD7 is the most commonly coexpressed lymphoid antigen in AML cases (3); therefore, it becomes a routine component in the AML panel.

Immunohistochemical staining may demonstrate MPO, lysozyme, CAE (Leder stain), CD15, and CD68. There are two clones of CD68: KP-1 is present in both myeloid and monocytoid cells, whereas PG-M1 is specific for monocytes and/or histiocytes (17). Therefore, the use of PG-M1 is most helpful in identifying the monocytic component in M4 (Fig. 6.9.6). The immature cell markers, CD34 and CD117, can be used to identify the malignant nature of the myeloid cells, if it is not morphologically apparent. In general, flow cytometry is the preferred technique to immunohistochemistry in diagnosing this subtype of AML.

Molecular Genetics

In typical M4 cases, the most common cytogenetic abnormality is the translocation of 11q23 with other partner chromosomes, which is seen in about 20% of M4 and M5 cases (1). In a recent study of 1897 AML cases, the incidence of 11q23 abnormality in M4, M5a, and M5b is 4.7%, 33.3%, and 15.9%, respectively (18). Molecular studies have identified a human homolog of the Drosophila trithorax gene designated mixed-lineage leukemia (MLL) gene, as it can be demonstrated in both acute myeloid and lymphoid leukemias (9).

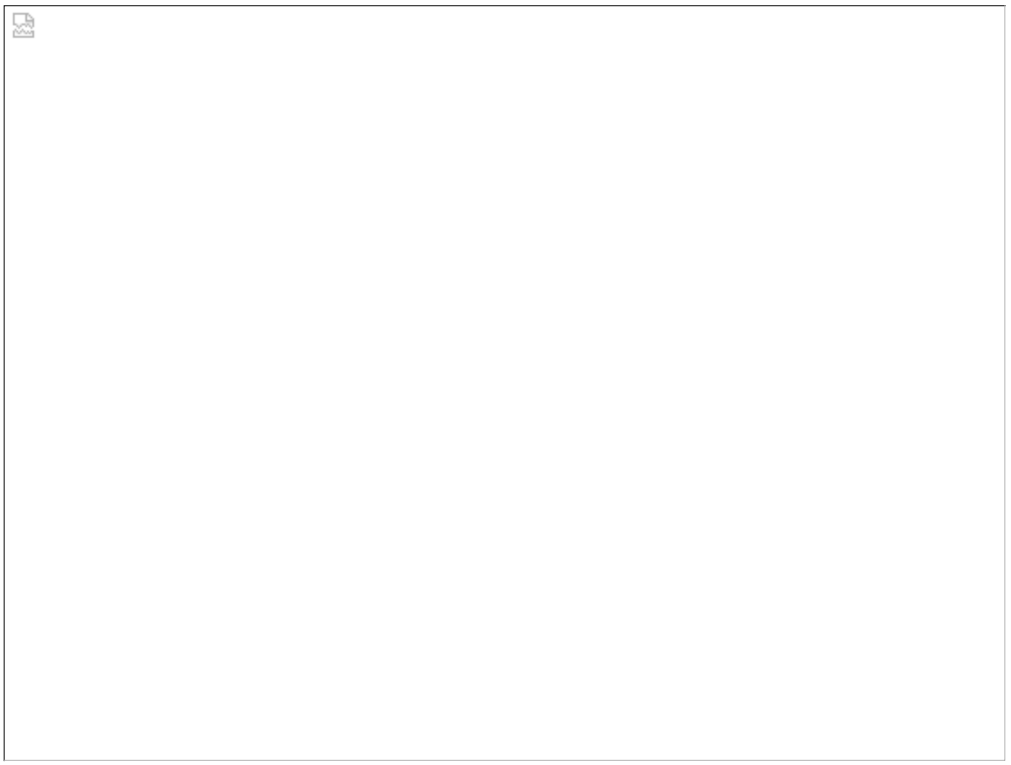

FIGURE 6.9.6 Bone marrow core biopsy shows many cells with CD68 PG-M1 staining. Myeloperoxidase stain, 20x magnification.

More than 30 partners of the MLL gene have been described (19). Among these translocations, t(9;11) is most common. In the Cancer and Leukemia Group B (CALGB) study, AML cases with t(9;11) have longer overall survival than that of other 11q23 rearrangement (20). However, another study found no difference in prognosis between t(9:11) and other forms of 11q23 translocation (18). The incidence of AML with MLL rearrangement is significantly higher in therapy-related AML than in de novo AML (18). In general, AML cases with 11q23 abnormality carry an unfavorable prognosis. The most specific genetic aberration, however, is found in the cases of M4Eo, which show inv(16)(p12q22), t(16;16)(p12;q22), or del(16)(q22). This entity is described in Case 5.

An abnormal karyotype, t(6;9)(p21-22;q34) is seen in M4 with basophilia (21). Recently, t(8;16)(p11;p13) has been found in an increasing number of M4 and M5 cases, which are characterized by the presence of erythrophagocytosis in the leukemic blasts (22, 23 and 24). Other low frequency aberrations include inv(3q26), t(3:3), and +4 (1,21). One study has found high expression of the *c-fos* proto-oncogene in M4 and M5 cases (25). N-*ras* mutation has been reported in a single case of M4 (26).

The salient features for laboratory diagnosis of M4 are summarized in Table 6.9.1.

Clinical Manifestations

M4 occurs in all age groups but is more common in older individuals with the median age of 50 years (2,27). The male/female ratio was 1.4:1 in one study (27).

The clinical presentation is leukocytosis in 85% of patients, and 10% are leukopenic (27). As in the current case, anemia and thrombocytopenia are characteristic features (2). Consequently, patients may have fatigue, fever, bleeding disorders, and gingival hyperplasia. Lymphadenopathy is present in about half the patients and hepatosplenomegaly in 30% to 35% (27). Some patients are preceded with chronic myelomonocytic leukemia (2).

TABLE 6.9.1

Salient Features for Laboratory Diagnosis of AML-M4

1. Presence of at least 20% myeloblasts-monoblasts-promonocytes in bone marrow
2. Both myeloid and monocytoid series should be >20%.
3. If monocytic component is <20% in bone marrow:
   a. Monocyte count in peripheral blood should be $>5 \times 10^9$/L.
   b. Serum lysozyme level should exceed three times the normal value.
4. Myeloperoxidase (Sudan black B) positive cells: >3%
5. Specific and nonspecific esterase-positive cells should be >20%.
6. Flow cytometry: Positive for CD13, CD14, CD33, HLA-DR, myeloperoxidase, and one of the stem cell markers (CD34 or CD117)
7. Immunohistochemistry: Positive for myeloperoxidase, lysozyme, chloroacetate esterase, CD15, CD68, CD34, and CD117
8. Common karyotypes: 11q23 translocation with a partner gene; chromosome 16 abnormalities in M4 with eosinophilia; t(6:9) in M4 with basophilia; t(8;16) in M4/M5 with erythrophagocytosis

AML, acute myeloid leukemia; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

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CASE 10 Acute Monoblastic and Monocytic Leukemia (M5) and 11q23 (Mixed Lineage Leukemia) Abnormalities

CASE HISTORY

A 71-year-old man presented with a history of abdominal pain radiating to his chest for 4 days. The patient had lost 18 pounds in the past 6 months. He was diagnosed with adenocarcinoma of the lung by fine-needle aspiration 2 years ago. Because of his seizure activity, the patient had a biopsy of the right parietal lobe, which proved to be metastatic carcinoma of the lung. The patient received local radiation therapy to his thorax and brain at that time. During a visit to the outpatient clinic, the patient was found to have a leukocyte count of 20,500/µL, with 43% segmented neutrophils, 12% bands, 28% lymphocytes, and 15% blasts. He was then scheduled for admission in 2 weeks.

On the day of admission, the leukocyte count rose to 62,700/µL with a differential of 7% segmented neutrophils, 1% bands, 9% lymphocytes, and 81% blasts. Bone marrow examination revealed 95% cellularity, and the aspirate showed 85% monoblasts, 7% promonocytes, and 3% monocytes. Only 5% of normal hematopoietic cells in the myeloid and erythroid cell lines were present.

Because the patient had stage IV lung cancer and type II Mobitz II heart block at the same time, no specific antileukemic treatment was given. He was treated with hydroxyurea to relieve leukostasis and allopurinol to prevent

tumor lysis. The patient was discharged and died at home on the day of discharge.

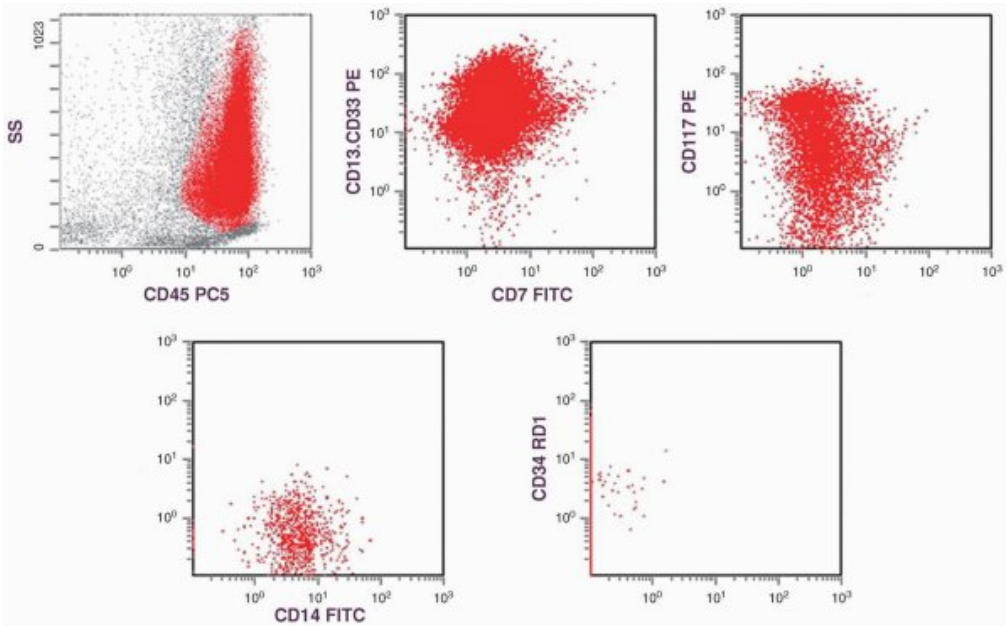

FIGURE 6.10.1 Flow cytometric analysis of bone marrow in an M5 case shows positive reactions to cluster of differentiation (CD)7, CD13.CD33, CD14, and CD117, but negative reactions to CD34. These histograms are not from the current case. SS, side scatter; PE, phycoerythrin; PC5, phycoerythrin cyanin 5; FITC, fluorescein isothiocyanate; RD1, rodamine.

FLOW CYTOMETRY FINDINGS

Bone marrow: Myeloperoxidase (MPO) 30%, CD13-CD33 92%, CD14 48%, CD11c 29%, HLA-DR 87%, CD34 8%, CD7 0% (Fig. 6.10.1).

CYTOCHEMICAL FINDINGS

MPO stain was positive in >10% of blasts. The combined esterase stain showed that >80% blasts in the bone marrow were positive for α-naphthyl butyrate esterase (Fig. 6.10.2).

CYTOGENETIC FINDINGS

Cytogenetic analysis showed two abnormal clones. The first clone of 7 cells revealed t(8;16)(p11.2;p13.3), and the second clone of 4 cells had additional material of unknown

origin on the short arm of the other chromosome 16 in addition to the t(8;16).

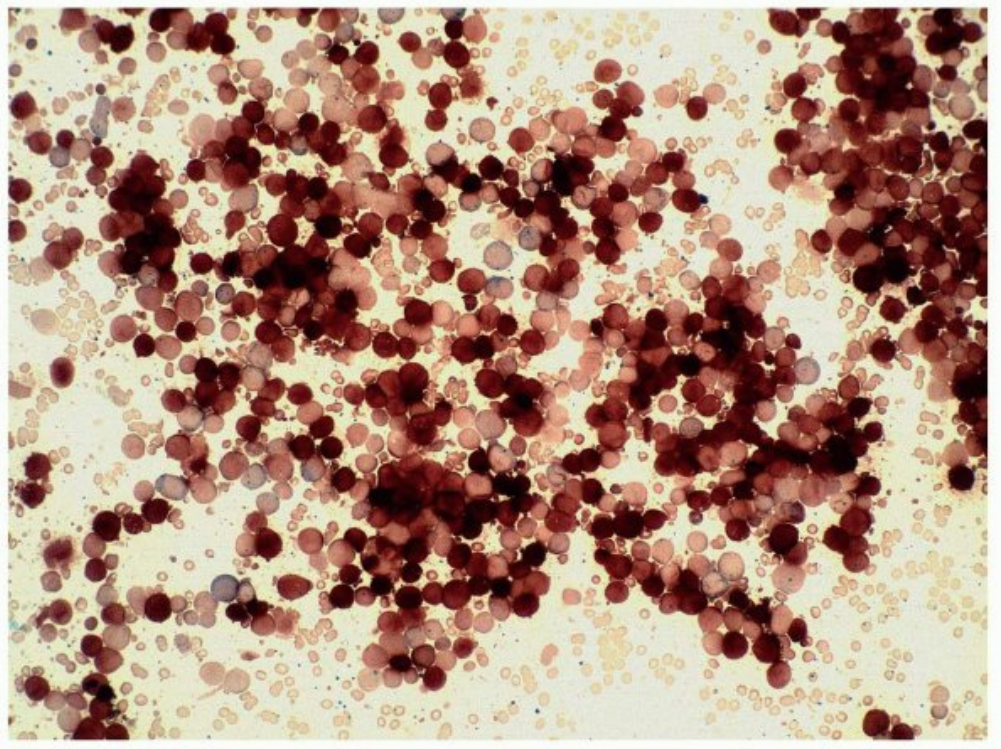

FIGURE 6.10.2 Combined esterase stain of a bone marrow aspirate shows predominantly nonspecific esterase staining of the blasts. Specific esterase stains only a few mature myeloid cells. 40x magnification.

DISCUSSION

The French-American-British criteria for the diagnosis of acute monoblastic leukemia (M5) require 80% or more of the nonerythroid cells in the bone marrow to be monoblasts, promonocytes, or monocytes (1). If the predominant component (>80%) is monoblasts, the condition is designated M5a, whereas the predominant components should be promonocytes in M5b. There are no consistent differences in clinical presentation between these two subtypes (2, 3 and 4). The incidence of these two subtypes combined is approximately 2% to 9% of all cases of acute myeloid leukemia (AML) (5,6).

Morphology

The monoblasts are usually larger than the myeloblasts, measuring about 40 to 50 µm in diameter (Figs. 6.10.3 and 6.10.4). The cytoplasm in most monoblasts is abundant with a grayish-blue tinge. It contains fine or inconspicuous granules, and the cell border is irregular. The nuclei of the very immature monoblasts are round or oval, but folding or creasing is frequently visible in most monoblasts. The promonocyte differs from the monoblast in its smaller size (up to 35 µm) and more prominent folding or creasing or lobulation of the nuclei. The presence of nucleoli in most promonocytes helps distinguish them from mature monocytes. Cytoplasmic vacuolation is frequently seen in monocytic elements. In general, if leukemic bone marrow shows a great variation in the nuclear configuration from cell to cell, the possible diagnosis of M5 should be considered (Figs. 6.10.4 and 6.10.5). Despite all these characteristics, cytochemical stain should be done routinely to definitively identify monocytic elements. For instance, neuroblastoma cells may occasionally be mistaken as monoblasts (7).

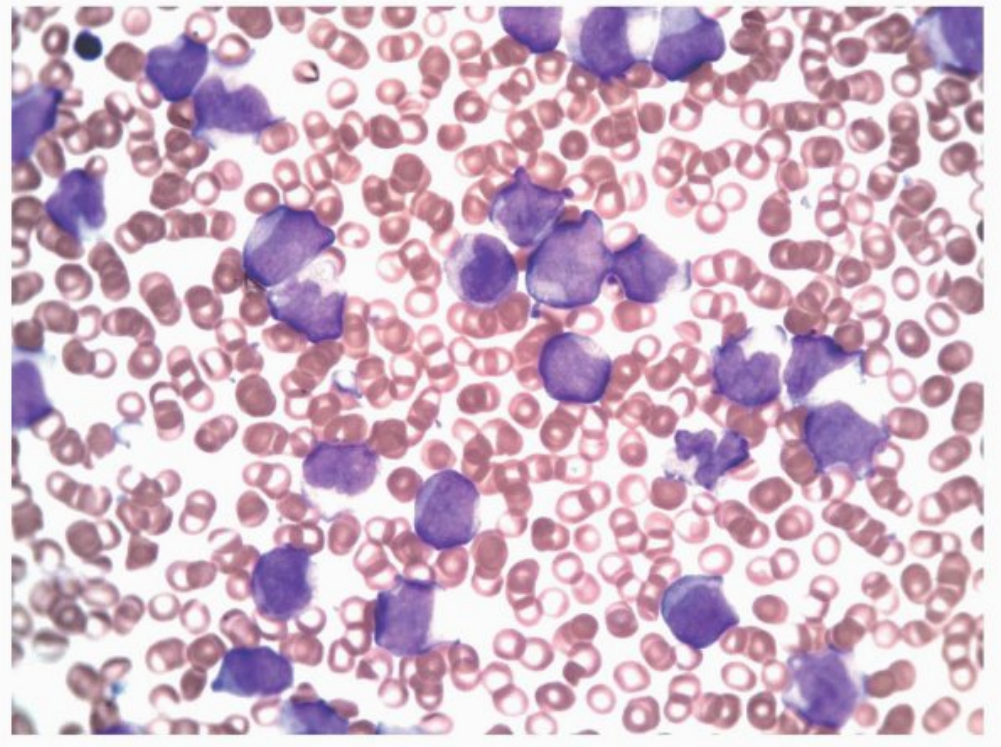

FIGURE 6.10.3 Peripheral blood smear of an M5 case shows various monocytic stages. Wright-Giemsa, 60x magnification.

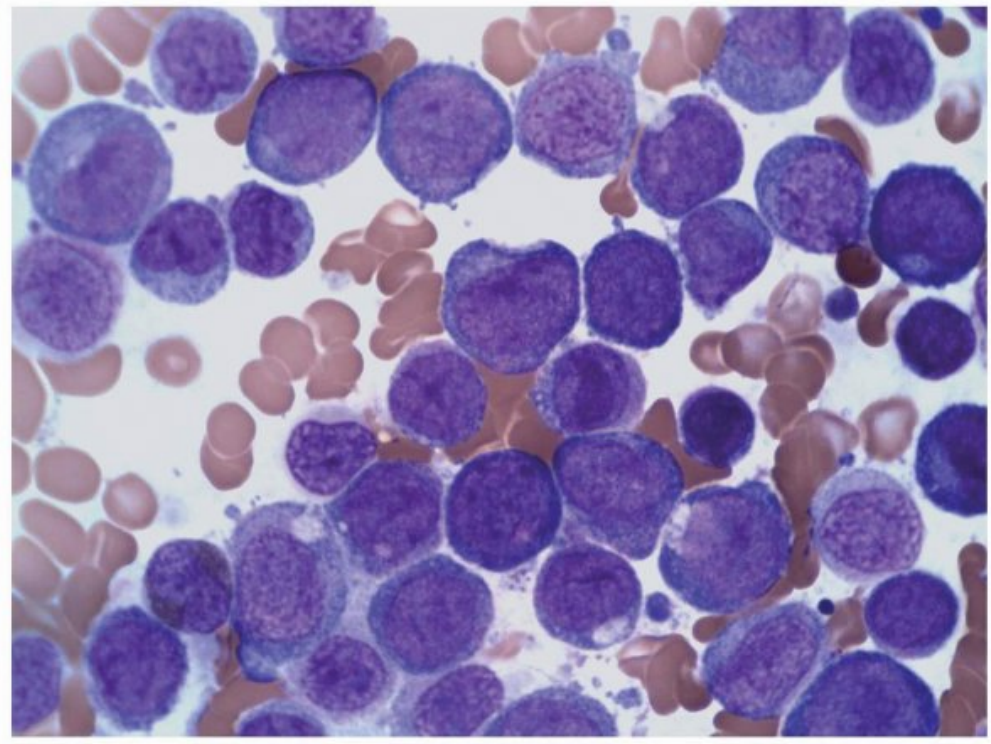

FIGURE 6.10.4 Bone marrow aspirate of an M5 case shows many monoblasts and promonocytes with a few monocytes. Wright-Giemsa, 100x magnification.

For cytochemical staining, M5 is an exceptional subtype of AML that is not required to have more than 3% MPO-positive blasts, because MPO is frequently negative in M5 (8,9). However, the MPO-negative cases should have a strong nonspecific esterase staining to back up the diagnosis. Chloroacetate esterase and periodic acid-Schiff are usually negative in M5, but they can be weakly positive in some cases (8,9). One study found that only one half of the M5 cases were positive for both α-naphthyl acetate esterase and CD14, whereas 25% were positive for α-naphthyl acetate esterase only and another 25% were positive for CD14 only (10). Therefore, a combination of cytochemistry

and immunophenotyping is necessary for an accurate diagnosis of M5.

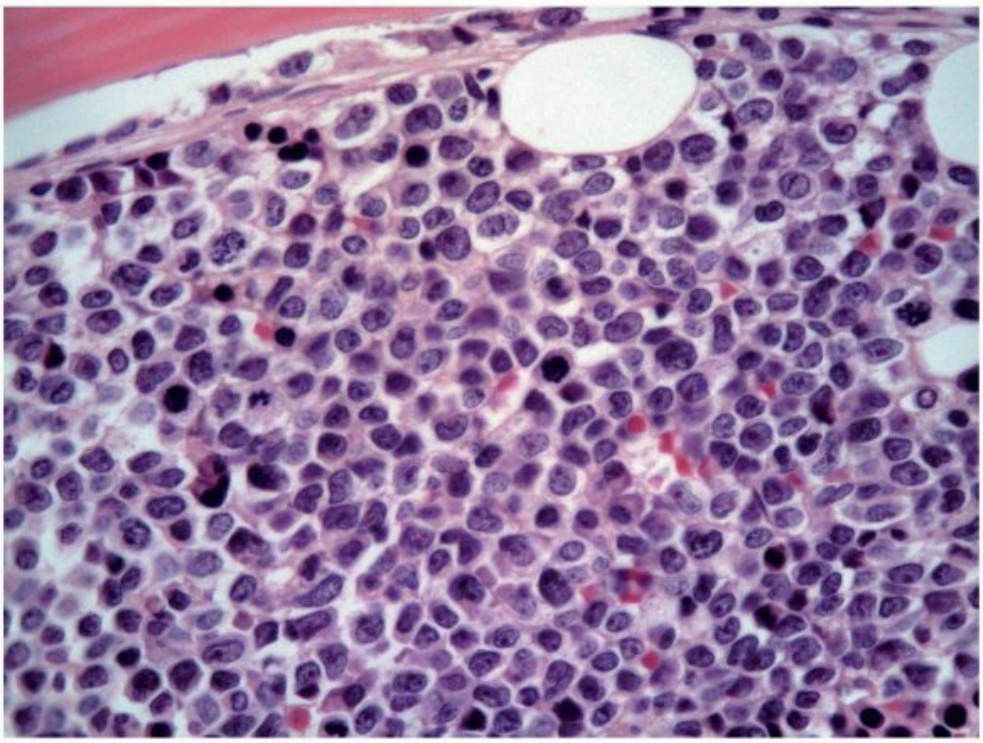

FIGURE 6.10.5 Bone marrow biopsy of an M5 case shows various stages of monocytes, replacing the normal hematopoietic cells. Hematoxylin and eosin, 60x magnification.

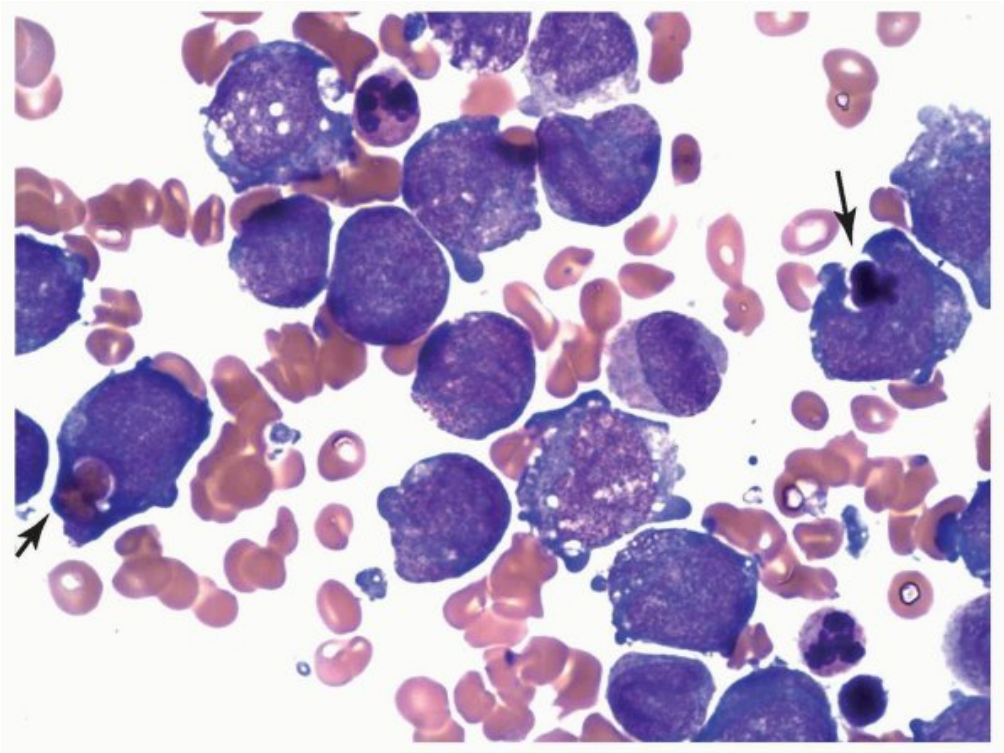

FIGURE 6.10.6 Bone marrow aspirate of an M5 case with t(8;16) shows phagocytosis of erythrocytes and normoblasts by monoblasts (*arrows*). Wright-Giemsa, 100x magnification.

In the current case, the patient's bone marrow contained 95% monocytic cells with 85% monoblasts, which were verified by the nonspecific esterase stain. Therefore, a diagnosis of acute monoblastic leukemia was established. In addition, erythrophagocytosis was demonstrated in the monoblasts (Fig. 6.10.6), and the blasts in the peripheral blood showed prominent cytoplasmic granules (Fig. 6.10.7). These cytologic features are characteristic of a subtype of AML (usually M4 or M5) with the t(8;16) translocation, as documented in our case. As will be discussed later, this karyotype is not very common, but it is worth identifying because it carries a particularly poor prognosis, especially in treatment-related or secondary leukemia (11,12).

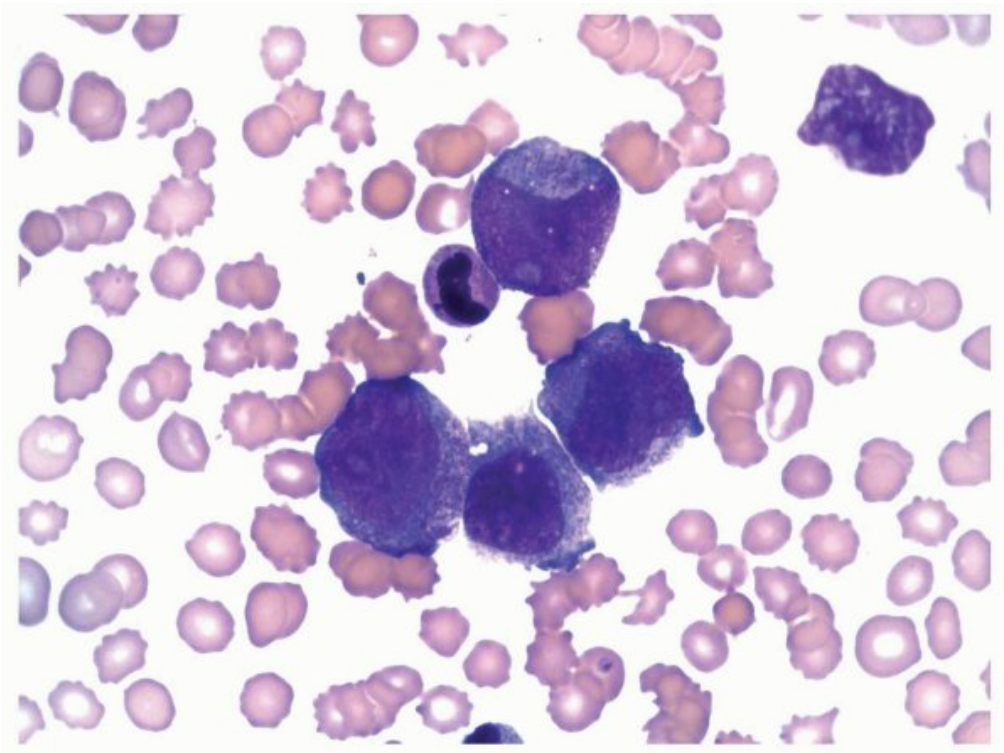

FIGURE 6.10.7 Peripheral blood smear of an M5 case with t(8;16) shows hypergranular monoblasts. Wright-Giemsa, 100x magnification.

Immunophenotype

The immunophenotype of M5 is composed of two sets of antigens. The first set is myeloid markers, which include CD13, CD15, CD33, and CD117 (13). However, myeloid markers, such as CD13 and CD15, can be selectively lost in M5 cases (14). The second set is monocyte markers that include CD4, CD11b, CD11c, CD14 (My4, LeuM3, and Mo2), CD32, CD36, CD64, CD68, and lysozyme. However, CD32 and CD36 are seldom used in routine testing (15,16).

CD34, the stem cell marker, is frequently negative in M5 (13,17), so CD117 is very important to identify the immature cells and to establish the diagnosis of leukemia. Previous studies have emphasized the percentage of CD14 expression as the major tool for distinction between M4 and M5 (18), but this assertion was subsequently challenged by others (19,20).

Although M5a and M5b share all antigens, some antigens are preferentially expressed in mature cells whereas others are demonstrated in immature cells. For instance, CD4 and CD14 are mainly demonstrated in mature monocytes, so they are often present in M5b cases (3,21). In contrast, CD117 is shown mainly in immature monocytes (21). CD11b and CD11c are present in both mature and immature monocytes; therefore, an immunophenotype of CD14- CD11b+ CD117+ is often seen in M5a cases (4). A recent study demonstrates that the combined use of different CD14 epitopes (Mo2 and My4) and CD64 can stratify different maturation stages of monocytes; this strategy appears to be useful in separating M5a and M5b (22). CD64 is positive for all mature and immature monocytes. My4 is present in mature monocytes and promonocytes, whereas Mo2 is only expressed by mature monocytes (22).

Immunoperoxidase antigen can be demonstrated by flow cytometry in M5b cases, but less often in M5a cases (13). However, immunoperoxidase activity is seldom demonstrated by cytochemistry in M5 cases.

CD56, a natural killer (NK) cell marker, is frequently positive in M5 cases, even though it is not specific (14,23). In one study, CD56 was demonstrated in 86% of M5 cases, just second to blastic NK-cell lymphoma and/or leukemia in frequency and far higher than any other AMLs (17).

Lymphoid markers can be detected in certain percentages of M5 cases. T-cell markers (CD2 and CD7) have been demonstrated in M5 cases (17,19). CD20 and CD23 have also been detected in a subpopulation of blast cells in M5 (17,21). Cases with a CD14-negative and T-cell antigen-positive phenotype are associated with leukemic skin infiltration (19). Platelet-megakaryocyte antibodies (CD41, CD42, and CD61) and antierythroid antibody (antiglycophorin A) do not cross-react with M5 cells. However, CD36, another platelet antigen, has been consistently demonstrated in both M5a and M5b cases (21).

A relatively new marker for monocytic lineage is CD163, which is the hemoglobin scavenger receptor. It can be demonstrated in M4 and M5 cases, but is seldom present in other AMLs (24).

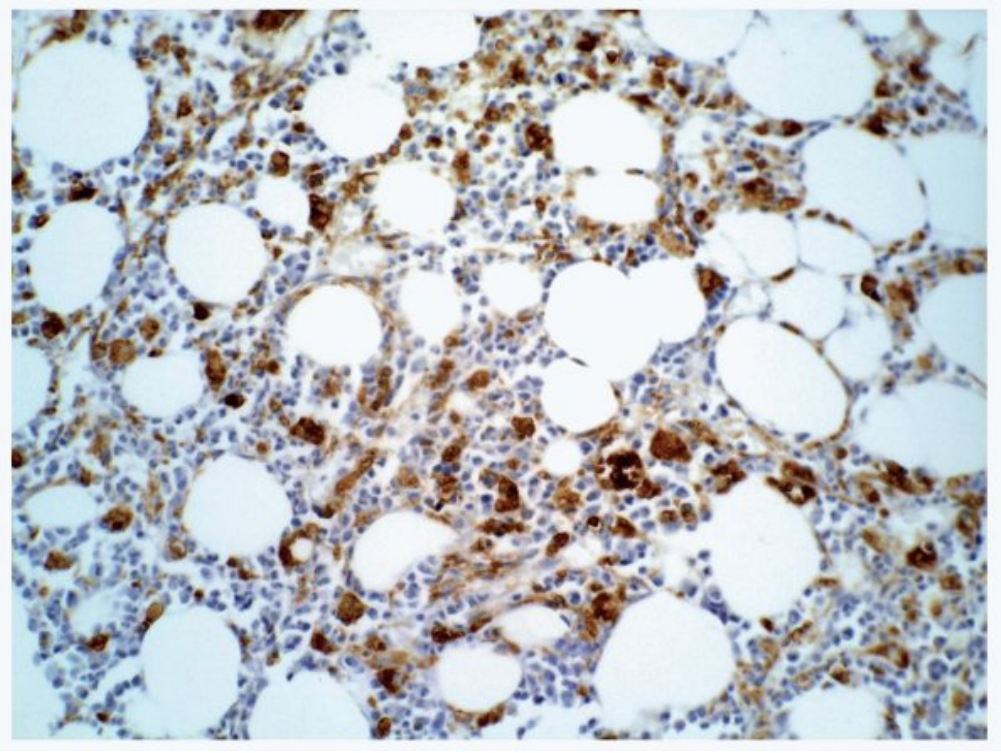

FIGURE 6.10.8 Bone marrow biopsy of an M5 case shows extensive staining of CD68 PG-M1. Immunoperoxidase, 20x magnification.

Comparison of Flow Cytometry and Immunohistochemistry

For the diagnosis of M5, the most difficult part is the identification of the monocytic series. Flow cytometry is able to demonstrate several markers, including CD4, CD11b, CD11c, CD14, and CD64. CD68 is most frequently used in immunohistochemistry, but two clones of CD68 are available. The KP-1 antibody is positive for both myeloid and monocytic cells, whereas PG-M1 is specific for monocytes (Fig. 6.10.8). Lysozyme stain can also be used in tissue stain, but it can be present in both monocytes and myelocytes. If the cell lineage cannot be clearly identified by immunophenotyping, cytochemistry is the final resort. Both α-naphthyl butyrate esterase and α-naphthyl acetate esterase are specific for monocytic series, if the stains are inhibited by sodium fluoride.

Molecular Genetics

The most common cytogenetic aberration in M5 involves 11q23; it accounts for 20% of abnormalities in M5 (25). In the World Health Organization (WHO) classification, 11q23 abnormalities are grouped together as a separate entity, but these abnormalities are mainly present in M5 cases.

Although >30 partner genes have been found participating in translocation of 11q23, only several chromosomal loci, including 6q27, 9q22, 10p12, 27q21, and 19p13.1, are frequently involved (4,25, 26, 27 and 28). The t(6;11) is usually seen in young men who present clinically with localized infection and a moderate leukocytosis (27).

The gene located at 11q23 was originally called *ALL1,* but it was later found that *ALL1* is the same as *MLL* (mixed lineage leukemia) gene (28). Because 11q23-involved abnormalities have been found in AML, acute lymphoblastic leukemia, and lymphomas, MLL is the preferred term to use.

In a study of 58 M5a and 66 M5b patients, 11q23/MLL aberrations were detected in 31% of M5a cases and 12.1% of M5b cases (29). The second most common cytogenetic abnormality in this study was sole trisomy 8, which was found in 22.4% of M5a and 3% of M5b cases (29).

Another cytogenetic abnormality, t(8;16)(p11;p13), is only seen in 2% of M5 cases (25), but it is associated with highly specific pathologic findings, namely erythrophagocytosis by leukemic blasts and hypergranulation in monocytic components (11,30, 31 and 32). The gene located at 8p11 is *MOZ* (monocytic leukemia zinc finger), and that at 16p13 is *CBP* (cAMP response element-binding [CREB]-binding protein). Normally, *MOZ* protein interacts with its cognate complex and joins the general transcription apparatus complex to direct transcription of a certain gene (33). CBP protein, in contrast, interacts with an appropriate DNA binding factor and the transcriptional apparatus complex to direct transcription of another gene (33). As a result of the translocation, the fusion product of these two genes (*MOZ*/*CBP*) may lead to leukemogenesis through three possible mechanisms: the fusion product mis-targets the wrong gene instead of the genes these two individual proteins (*MOZ* and *CBP*) are supposed to target, the fusion product misregulates the targeted gene(s), and the fusion product loses normal function in directing DNA transcription (33).

In patients with a normal karyotype, partial tandem duplication of the MLL gene (*MLL-PTD*) was identified in 1.7% of M5a cases and 4.5% of M5b cases by reverse transcriptase-polymerase chain reaction (RT-PCR) and was confirmed by Southern blot (29). The frequency of Fms-like tyrosine kinase 3 length mutations (*FLT3-LM*) was detected by the same techniques in 6.9% of M5a and 28.8% of M5b cases (29). The *FLT3* gene mutations in other studies were reported to be as high as 40% (4).

The salient features for laboratory diagnosis of M5 are summarized in Table 6.10.1.

Clinical Manifestations

M5 is predominantly seen in adults older than 40 years and in children younger than 10 years (50% of M5 patients are younger than 2 years) (34). Congenital M5 cases have been reported from time to time (34, 35, 36 and 37). The occurrence of M5 in young children appears to be associated with in utero exposure to pesticides and solvents (34). Pediatric patients usually have a worse prognosis than adult patients have.

M5 cases generally have higher leukocyte and platelet counts and more frequent lymphadenopathy than do other subtypes of AML (2,34). Disseminated intravascular coagulation is frequently seen in M5, second only to M3 (2,34). The lysozyme level is elevated in 67% to 100% of M5 cases. As lysozyme is nephrotoxic, 40% of M5 patients in one study had renal insufficiency, which was proportional to the lysozyme levels (38). About one fourth of patients with M5 have leukemic infiltration of skin (Fig. 6.10.9) and/or gum, one half have hepatosplenomegaly with or without lymphadenopathy, 3% to 22% have central nervous system involvement, and 28% have renal failure (2,3,39).

As a result of all these complications, M5 patients have a shorter survival time than patients with other subtypes of AML, although the complete remission rate may be comparable (2,40). The three major factors affecting the survival time in M5 cases are age, renal failure, and serum β2 microglobulin (B_2M) levels, as demonstrated in one study (3). Patients with renal failure or high B_2M levels have a

median survival of 1 and 3 weeks, respectively, whereas patients with no renal failure or low B_2M levels have a median survival of 26 and 29 weeks, respectively. In the same study, lysozyme, lactate dehydrogenase, and B_2M levels were elevated in 88%, 68%, and 81% of M5

cases, respectively, but the first two components did not show statistically significant association with patient prognosis (3).

TABLE 6.10.1

Salient Features for Laboratory Diagnosis of M5

1. Presence of >80% monocytic components among the nonerythroid cells in the bone marrow

   a. M5a: ≥80% of monocytic components are monoblasts.

   b. M5b: Predominantly monocytes and promonocytes

2. Elevation of serum and urine lysozyme levels

3. Cytochemistry

   Myeloperoxidase: May or may not be positive

   Nonspecific esterase: Strongly positive

   Specific esterase and periodic acid-Schiff: Usually negative

4. Immunophenotype

   Myeloid markers: CD33, CD13, CD15 may be positive, but one or more markers may be selectively lost.

   Monocytic markers: CD4, CD11b, CD11c, CD14, CD64, CD68, and lysozyme may be positive, but one or more markers may be selectively lost.

   Immature cell markers: CD34 is usually negative, and CD117 is frequently positive.

   Frequently positive nonspecific marker: CD56

   Consistently negative markers: CD41, CD42, CD61, glycophorin A

5. Cytogenetics: Associated with t/del(11)(q23), t(8;16) (p11;p13), or others

6. Molecular biology: MLL gene on 11q23 and *MOZ*/*CBP* on 8p11/16p13

   FLT3 gene mutation is a frequent finding in patients with a normal karyotype.

CD, cluster of differentiation; MLL, mixed lineage leukemia; MOZ/CBP, monocytic leukemia zinc finger/CREB-binding protein; FLT3, Fms-like tyrosine kinase 3.

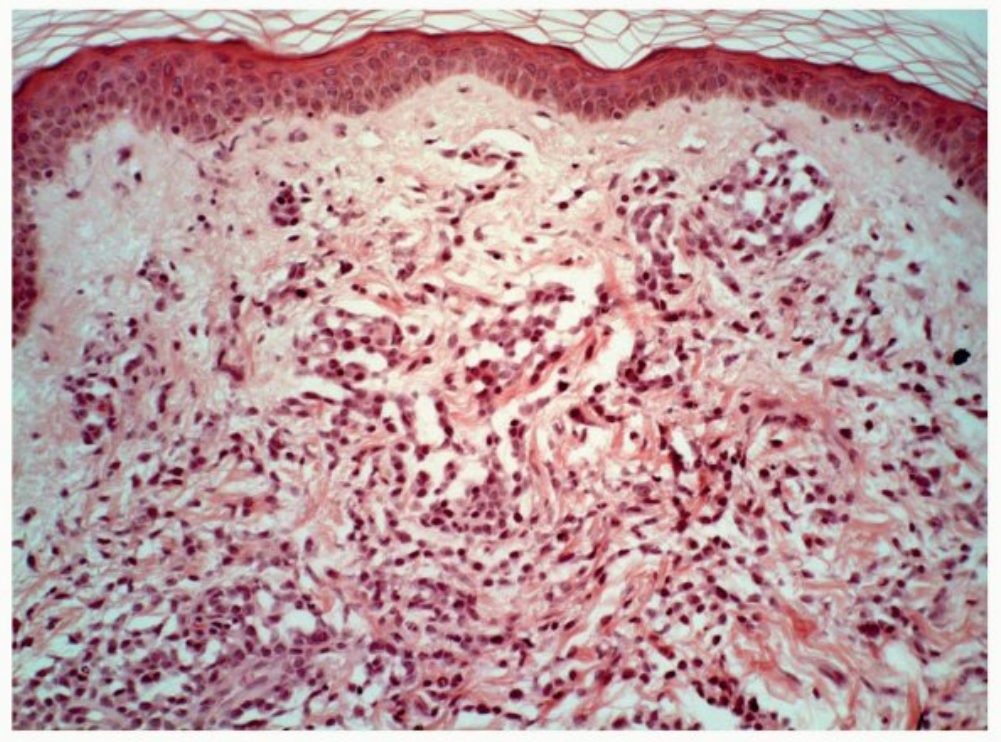

FIGURE 6.10.9 Skin biopsy of an M5 case shows extensive leukemic cell infiltration in the dermis. Hematoxylin and eosin, 20x magnification.

A recent study considered the poor prognosis of M5 associated with cytogenetic abnormalities, such as mutations in the *FLT3* genes (4). This study also found that the disease-free survival and overall survival of patients with M5 did not appear to differ from non-M5 AML cases with currently available therapy.

The clinical features in patients with t(8;16) are similar to those without this aberration. However, patients with t(8;16) have a higher frequency of coagulopathy and extramedullary dissemination (11,30, 31 and 32).

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CASE 11 Acute Erythroid Leukemia

CASE HISTORY

A 69-year-old man was admitted to the hospital for evaluation of anemia and thrombocytopenia. One week prior to admission, the patient started to have low-grade fever, nausea, malaise, and nonbloody diarrhea. He also had several episodes of epistaxis during the past week. The blood work done in the physician's laboratory revealed a hematocrit of 20% and platelets of 30,000/μL and was referred to our hospital for further evaluation. His routine hematology workup 3 months before admission was essentially normal except for a

hematocrit of 34%.

On admission, the patient looked pale, but no petechiae or ecchymosis was found on the skin. Physical examination revealed no hepatosplenomegaly or lymphadenopathy. Laboratory data showed normal serum iron, lactate dehydrogenase, and fibrinogen. D-dimer, fibrinogen split products, and direct Coomb test were negative.

A bone marrow aspirate showed 87.7% normoblasts including 55.7% pronormoblasts. There were 10.3% myeloid

cells, which included no myeloblasts. The pronormoblasts were pleomorphic with multiple intraplasmic vacuoles that were positive for periodic acid-Schiff (PAS) stain. The maturing normoblasts revealed megaloblastoid changes and nuclear dysplasia.

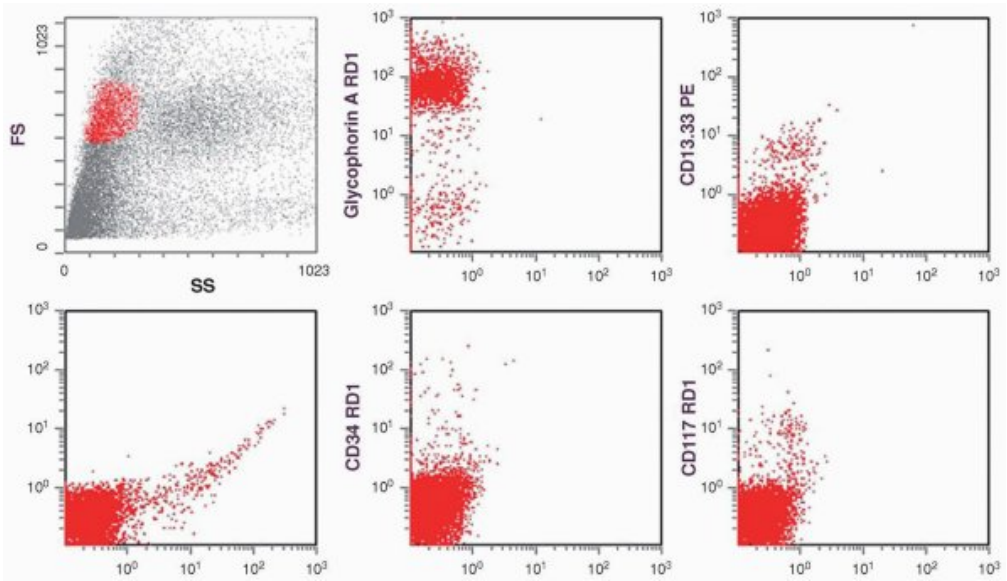

FIGURE 6.11.1 Flow cytometric analysis using the forward scatter (FS) versus side scatter (SS) plot, as the normoblasts are CD45 dim or negative. The histograms show positive glycophorin A reaction, but negative reactions for human leukocyte antigen-DR (HLA-DR), CD34, and CD117. A low percentage of CD13-CD33 is detected. FITC, fluorescein isothiocyanate; RD1, rhodamine; PE, phycoerythrin.

A diagnosis of acute erythroid leukemia was made. The patient's family was informed of the poor prognosis in association with this disease. The patient refused to receive blood products, and the family opted to go home for home hospice care.

FLOW CYTOMETRY FINDINGS

The bone marrow aspirate revealed 3% myeloperoxidase (MPO), 65% CD13-CD33, 0% CD13-CD33/CD7, 2% CD14, 0% human leukocyte antigen-DR (HLA-DR), 44% glycophorin A, and 3% CD34 (Fig. 6.11.1).

CYTOGENETIC FINDINGS

Cytogenetic analysis of the bone marrow detected a complex abnormal karyotype. There was additional material on the short arm of chromosomes 7 and 21 and on the long arm of chromosome 11. A deletion of 5q and 7q and loss of chromosome 16 were also present.

DISCUSSION

Acute erythroid leukemia (AML-M6) is a rare disease, accounting for 4% to 5% of all acute myeloid leukemia (AML). Giovanni di Guglielmo was the first one to recognize this entity (1). In 1917, he described a syndrome with a mixed population of immature erythroid and myeloid cells (later referred to as di Guglielmo syndrome), and in 1926 he reported a disease with pure immature erythroid proliferation (later referred to as di Guglielmo disease) (2). However, this concept of subdivision of erthroleukemia did not draw attention in the field of hematology until recently. The French-American-British (FAB) criteria for M6 is the presence of at least 50% normoblasts (erythroblasts) among the total number of nucleated cells and 30% type I and type II blasts among the nonerythroid population in the bone marrow (Fig. 6.11.2) (3). According to this classification, any case with <30% myeloblasts should be included in the myelodysplastic syndrome. However, Kowal-Vern et al. (4) recognized the prognostic significance of the immature erythroid components; cases with an increased pronormoblast to myeloblast ratio have worse prognosis than those with a higher myeloblast count (4). They suggested the subdivision of M6 into M6a (the original M6) and M6b (pure erythroid leukemia). The same concept was presented by Garand et al. (5) and Hasserjian et al. (6); these groups designated the pure

erythroid leukemia as AML-M6 variant. Mazzella et al. (7) further subdivided AMA-M6 into M6a, M6b, and M6c (7). This group defined M6c cases as those with both myeloblasts and pronormoblasts >30% of the nucleated cells.

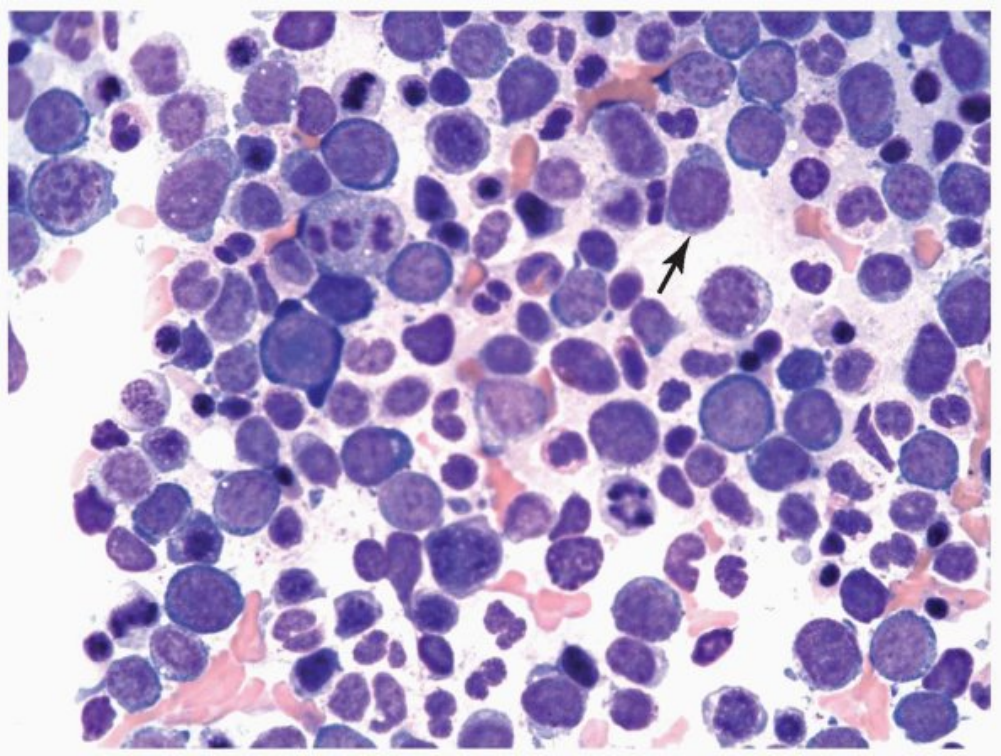

FIGURE 6.11.2 Bone marrow aspirate from a case of erythroleukemia (erythroid/myeloid) shows mainly pronormoblasts with a few myeloblasts (*arrow*). Wright-Giemsa, 60× magnification.

In the World Health Organization (WHO) classification, the original erythroleukemia is now designated erythroleukemia (erythroid/myeloid), and the requirement for the myeloblast count in the nonerythroid population has been reduced to ≥20% (8, 9 and 10). AML-M6b is designated as pure erythroid leukemia, which requires >80% of immature erythroid cells without a significant myeloblastic component in the bone marrow (Fig. 6.11.3).

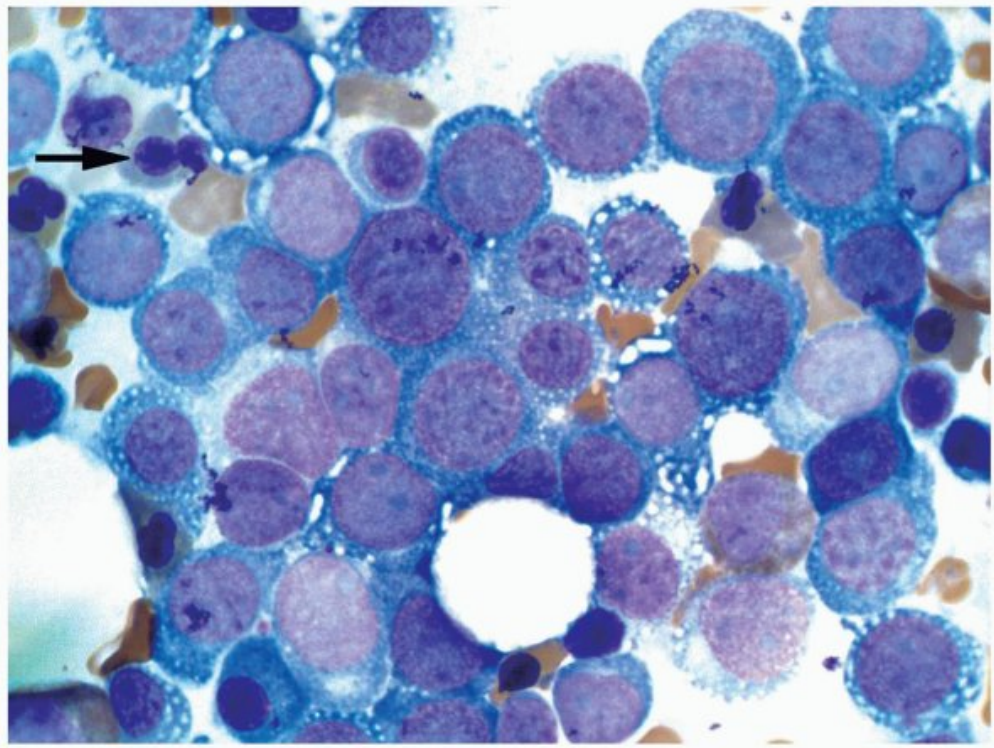

FIGURE 6.11.3 Bone marrow aspirate from a case of pure erythroid leukemia reveals predominantly pronormoblasts with other stages of normoblasts. Myeloblasts are absent. Note cytoplasmic vacuolation in pronormoblasts and a few dysplastic nucleated erythrocytes (*arrow*). Wright-Giemsa, 100× magnification.

Morphology and Cytochemistry

The characteristic morphologic features of acute erythroid leukemia are the predominance of atypical erythroid precursors of all maturation stages and the presence of erythrodysplasia in the bone marrow. As mentioned before, the percentages of the erythroid precursors and myeloblasts distinguish erythroleukemia (erythroid/myeloid) (M6a) from pure erythroid leukemia (M6b). In the peripheral blood, anisopoikilocytosis, macrocytosis, schistocytes, and nucleated erythrocytes may be present, but blasts are seldom encountered.

The erythroid precursors are mainly composed of pronormoblasts and basophilic normoblasts in M6a, but they are usually undifferentiated in M6b; cytochemistry and immunochemistry are often required for identification. The pronormoblasts are of large size with regular cell borders. The cytoplasm is deeply basophilic and devoid of granules. The nucleus is perfectly round with a delicate chromatin pattern, sometimes referred to as a sievelike pattern. The leukemic pronormoblasts and basophilic normoblasts are highly pleomorphic, varying in size and shape. The nuclei can be polylobulated, multiple, fragmented, or extraordinarily large. One to a few prominent nucleoli are usually present. The cytoplasm is characterized by multiple vacuolation and lack of hemoglobinization.

The erythrodysplastic features include megaloblastoid/megaloblastic changes, nuclear budding, nuclear bridging, and other irregular configurations of the nucleus. The frequent presence of erythrodysplasia in M6 may be because most cases of M6 evolve through a myelodysplastic phase (11). M6 is frequently associated with a history of myelodysplastic syndrome, chemotherapy, or exposure to toxin or alcohol (7,12).

The myeloblasts in M6a are not different from those seen in M1 and M2. Auer rods can be found in occasional cases. Dysplastic features are seldom seen in myeloid and megakaryocytic lineages. If a suspicious M6a case shows ≥50% dysplastic myeloid or megakaryocytic cells, it should be classified as AML with multilineage dysplasia (9,10). In cases suspicious for M6b, vitamin B12 and folate deficiency and erythropoietin therapy should always be excluded before a diagnosis is made (2,9). The distinction between M6a and refractory anemia with excess blasts is sometimes difficult, because when there is marked erythroid hyperplasia in myelodysplastic syndrome, the myeloblast count in the small population of nonerythroid nucleated cells may easily reach the 20% cutoff required for the diagnosis of M6a (13).

Cytochemical stain is helpful in substantiating the diagnosis of M6. PAS is normally negative in nucleated red blood cells. However, it is often positive in M6 cases showing coarse granules (block pattern) in pronormoblasts and basophilic normoblasts, and diffuse cytoplasmic staining in polychromatophilic and orthochromic normoblasts (Fig. 6.11.4). A negative PAS stain, however, does not exclude the diagnosis of M6. The MPO, Sudan black B, and chloroacetate esterase stains are negative for normoblasts; if they are positive in M6

stains are myeloblasts (Fig. 6.11.5) (14). Some early normoblasts may show weak focal α-naphthyl butyrate esterase stain, which is, however, not helpful in the diagnosis. The Prussian blue stain for iron is helpful to detect ringed sideroblasts, which are seen more frequently in M6b than in M6a cases (2).

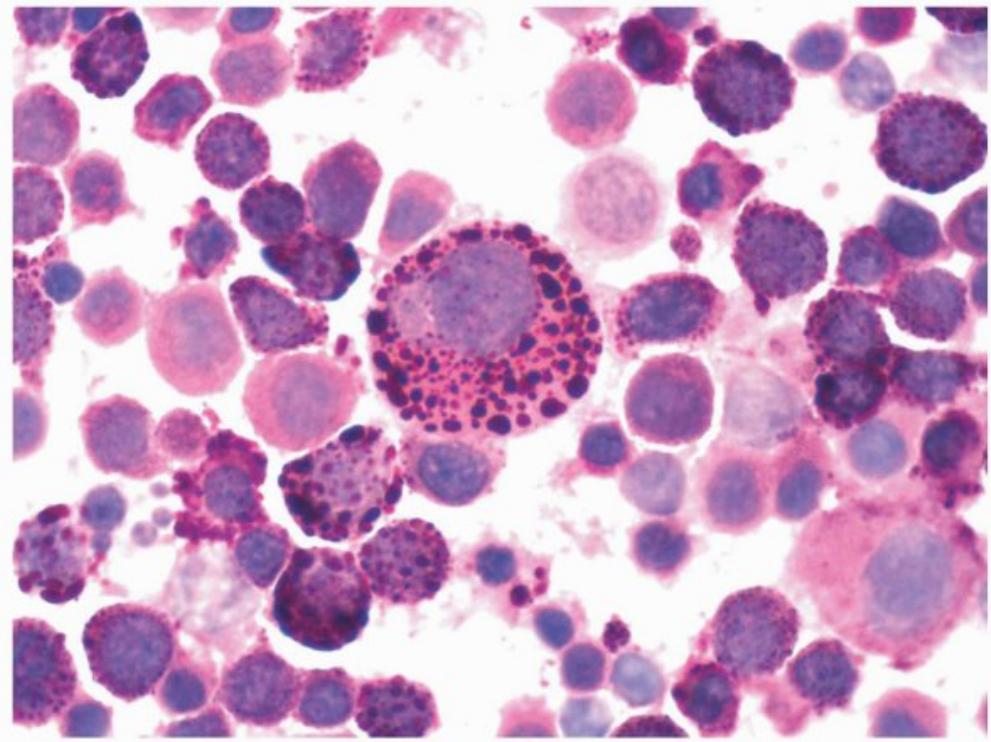

FIGURE 6.11.4 Bone marrow aspirate from a case of pure erythroid leukemia shows coarse periodic acid-Schiff (PAS)-positive granules in the cytoplasm of pronormoblasts and basophilic normoblasts, but diffuse staining in polychromatophilic and orthochromatic normoblasts. PAS, 100× magnification.

In the current case, the bone marrow contained 87% erythroid precursors including 55% pronormoblasts without the presence of myeloblasts. The pronormoblasts were pleomorphic, and the cytoplasm was vacuolated. The normoblasts other than the pronormoblasts showed marked nuclear dysplasia. The bone marrow biopsy was hypercellular with extensive erythroblastic infiltration (Figs. 6.11.6 and 6.11.7). The cytochemical stain demonstrated strong cytoplasmic PAS staining with a block pattern. The flow cytometry revealed 44% glycophorin A. With all this laboratory information, a definitive diagnosis of pure erythroid leukemia (M6b) was established.

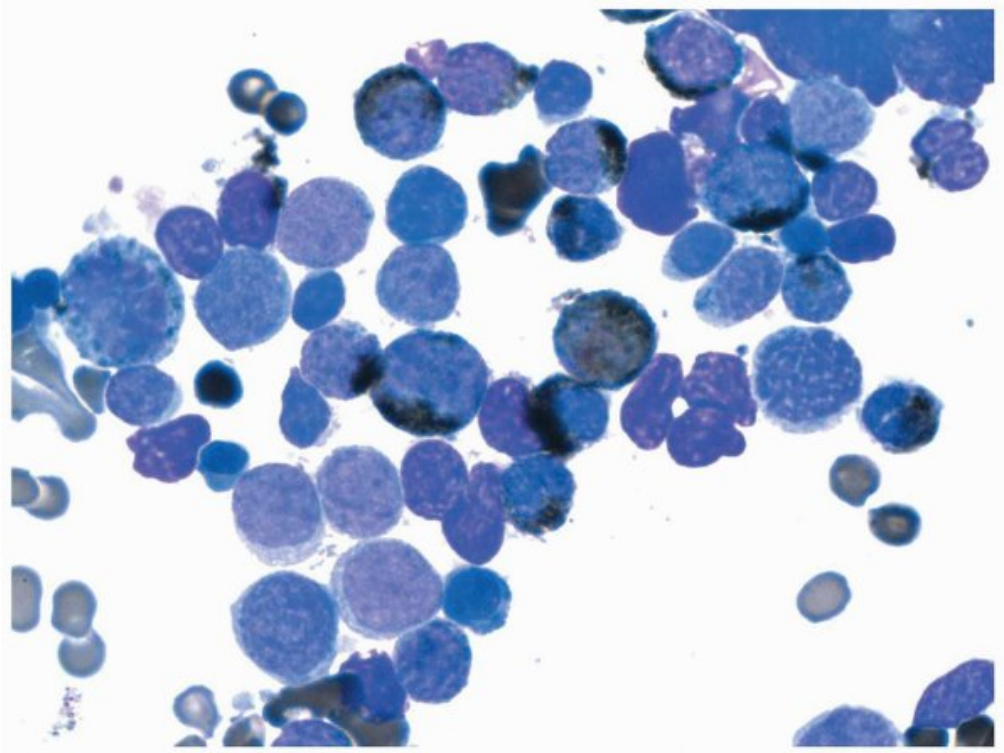

FIGURE 6.11.5 Bone marrow aspirate from a case of M6a reveals myeloperoxidase staining in the myelocytic series including myeloblasts, but the normoblasts are negative. Myeloperoxidase, 100× magnification.

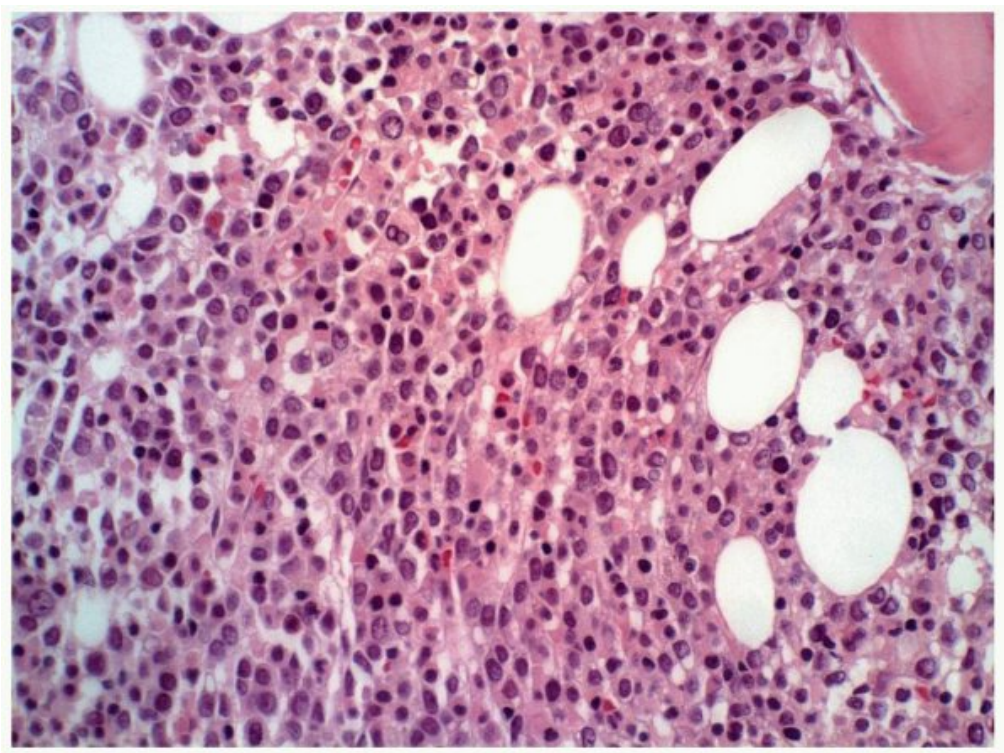

FIGURE 6.11.6 Bone marrow core biopsy from a case of erythroid leukemia shows normal hematopoietic cells are replaced by erythrocytic series. Hematoxylin and eosin, 40×

magnification.

Immunophenotype

In M6a cases, the erythroid precursors are usually recognizable. However, M6b cases often show primitive blasts, and the PAS stain can be negative or equivocal; therefore, immunophenotyping is frequently required for a final diagnosis.

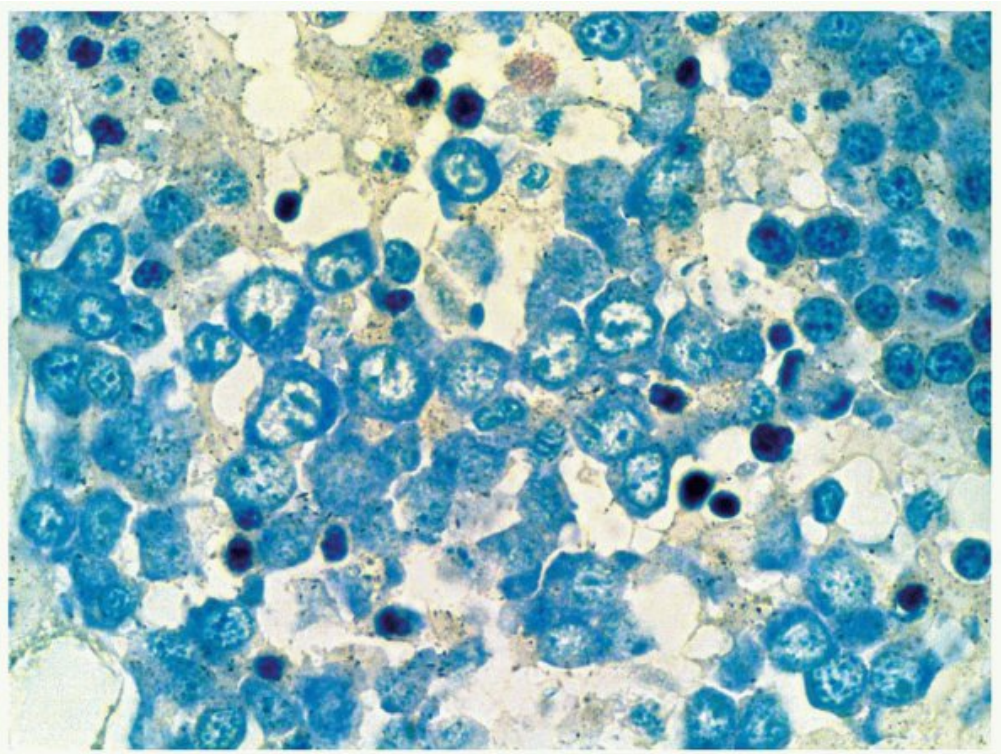

FIGURE 6.11.7 Pronormoblasts in a bone marrow biopsy readily recognizable in a Giemsa-stained preparation. Giemsa, 100× magnification.

Several antibodies can help to identify the erythroid series. Early antibodies include anti-hemoglobin and anticarbonic anhydrase I antisera, which identify the normal components of erythrocytes, but these components may not be present in very immature cells (15,16). The mouse monoclonal antibody, FA6-152, is positive for erythrocyte burst-forming units, erythrocyte colony-forming units, pronormoblasts, and normoblasts, but it is also present in normal monocytes and megakaryocytes (17). A murine monoclonal antibody developed at the Mayo Clinic, RC82.4, is reported to be specific and sensitive in detecting normal and leukemic erythroid cells without cross-reactivity to other cell lineages (16). However, the nature of the antigen with which RC-82.4 reacts is still not clear, and the antibody is not commercially available.

Gupta and Dhond (18) used a panel of monoclonal antibodies specific for different developmental stages (erythrocyte burst-forming units, erythrocyte colony-forming units, normoblasts, erythrocytes) and components (glycophorin A and H antigens) of erythroid cells and found that, in most cases of M6, the phenotype of the pronormoblasts was that of the intermediate stage of maturation (19). Transferrin receptor antibody (CD71) has also been used to detect mature and immature nucleated erythrocytes (18). Although CD71 has been used in the monoclonal antibody panel for the diagnosis of M6 (20), it is an activation antigen, presenting in various conditions, and thus not specific for M6. The erythroid precursors may express CD36, but this antigen is also present on megakaryocytes and monocytes (8).

Greaves et al. (21) found that 78% of 27 cases of M6 reacted with glycophorin-A antibody, but only 3% of 724 cases of nonerythroid leukemias had a positive reaction to that antibody. Because of its specificity and availability, glycophorin-A antibody has become the most widely used for the identification of erythroid cells and diagnosis of M6 (22). Glycophorin-A antibody has been used in flow cytometry and, more recently, in immunohistochemistry.

Using glucose-6-phosphate dehydrogenase isoenzyme analysis, it has been found that M6 is a clonal disorder arising from a multipotent stem cell (23). The human erythroleukemia cell line expresses surface antigens of the erythroid, macrophage, and megakaryocyte lineages (24). This cell line can be induced by different agents into full expression of erythroid, macrophage, or megakaryocyte

phenotypes (24). Therefore, it is not surprising to see that leukemic cells in M6 cases may react to myeloid antigens (CD11b, CD13, CD15, and CD33) and platelet antigen (CD41) (2,11,18). However, the reactions to these antigens are not consistent, and the results are sometimes due to the coexistence of myeloblasts and increased megakaryocytes in the bone marrow; thus, myelomonocytic or megakaryocytic antigens should not be depended on for the diagnosis of M6.

CD34 was present in 26% of M6a and 26.7% of M6b cases in one study (7). However, the percentage of CD34 was proportional to that of myeloblasts rather than pronormoblasts. The expression of CD117 (c-kit) is also associated with myeloblasts (9).

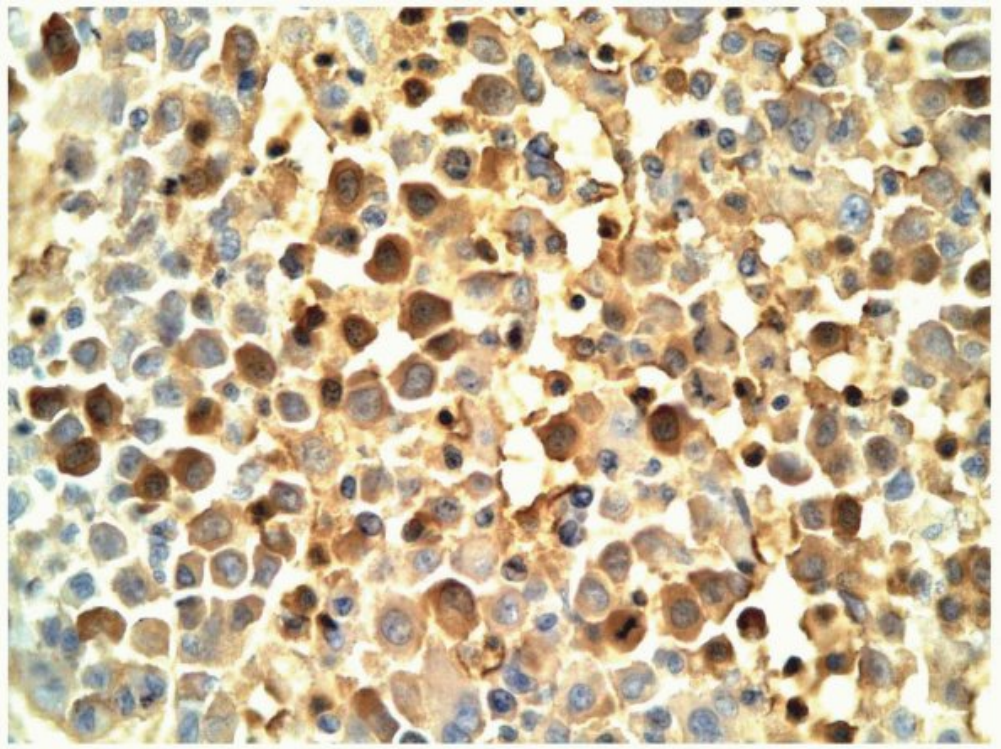

FIGURE 6.11.8 Erythroid cells in an M6 case are highlighted by hemoglobin A staining. 60× magnification.

Comparison of Flow Cytometry and Immunohistochemistry

Immunohistochemical stains can demonstrate erythroid precursors with glycophorin-A and hemoglobin A antibodies (Fig. 6.11.8) (9). Myeloblasts can be detected with CD34 and CD117 together with other myeloid antibodies (e.g., MPO, CD33). In contrast, glycophorin A is the only antibody useful for the diagnosis of M6 by flow cytometry.

Molecular Genetics

The percentage of aneuploidy is particularly high (63%) in M6 (25). Most cases show complex karyotypes with multiple structural abnormalities (9). Monosomy or longarm deletions of chromosome 5 and/or 7 occur most frequently in various studies (7,11,25). The karyotype demonstrated in the current case is characteristic. These two chromosomes are most frequently associated with myelodysplastic syndromes, which may precede the development of M6.

Cuneo et al. (11) divide M6 cases into two groups: Those with three or more cytogenetic abnormalities are designated major karyotype aberrations (MAKA), and those with a single abnormality are designated minor karyotype aberrations (MIKA). The MAKA group is always associated with increased immature erythroid precursors, and the MIKA group with preserved maturation of erythroid cells. The MAKA group has lower hemoglobin levels, lower complete remission rates, and shorter survival than the MIKA group (11). Apparently, this cytogenetic-cytopathologic classification is of prognostic significance. Mazzella et al. (7) found that 8 of 16 M6a cases had a normal karyotype, 4 had MIKA, and 4 had MAKA, whereas 10 of 11 M6b cases had MAKA.

In a case of M6 with monosomy 7, fluorescence in situ hybridization identified the same clonal abnormality in both erythroid and myeloid lineage, and the normal erythroid population coexisted with the leukemic erythroid population (26). These findings substantiate the theory that M6 is a clonal disorder arising from a multipotent

stem cell. Multidrug resistance gene expression and p53 gene mutation were demonstrated in all subtypes of M6, but more frequently in M6b and M6c than in M6a (2).

TABLE 6.11.1

Salient Features for Laboratory Diagnosis of M6

1. M6a: Presence of ≥50% of normoblasts among all nucleated cells and ≥20% of myeloblasts among nonerythroid cells in the bone marrow
2. M6b: Presence of ≥80% immature erythroid precursors with no significant myeloblast component
3. Positive periodic acid-Schiff staining in mature and immature nucleated erythroid cells
4. Glycophorin-A and hemoglobin-A staining by immunohistochemistry and glycophorin-A positivity by flow cytometry
5. Myeloblasts in M6a cases show myeloid markers (e.g., CD13, CD33) and immature cell markers (CD34 and CD117).
6. Complex abnormal karyotypes in most cases, with frequent -5/5q- and -7/7q-

The salient features for laboratory diagnosis of M6 are summarized in Table 6.11.1.

Clinical Manifestations

Clinically, the presenting symptoms are usually associated with severe anemia. M6 cases have the lowest hemoglobin level and leukocyte and platelet counts, compared with other subtypes of AML, partly because M6 is often preceded by a myelodysplastic syndrome (27). Although nucleated red blood cells are frequently present, pronormoblasts are seldom detected in the peripheral blood (12). Hepatosplenomegaly and lymphadenopathy occur in <25% of patients (27).

M6 is often seen in elderly persons, but a subset of younger patients with better clinical outcome has been found in one study (12). Congenital, familial erythroleukemia or erythroleukemia of infancy and childhood have been occasionally reported (28, 29, 30 and 31). M6 may also be presented as a blastic crisis of a myeloproliferative disorder, such as chronic myelogenous leukemia or polycythemia vera (2,5,32).

In a study by Mazzella et al. (7), the increase of proliferation markers (proliferating cell nuclear antigen [PCNA] and Ki-67) correlated positively with pronormoblast count and multiple cytogenetic abnormalities and inversely with survival and erythroid lineage maturation. They also found that patients with increased numbers of myeloblasts and few pronormoblasts had the best prognosis, whereas survival rapidly declined with decreasing myeloblast counts. Accordingly, the authors suggested that chemotherapy for M6 should be directed toward both myeloblasts and pronormoblasts to replace the current regimens that do not affect pronormoblasts.

Therapeutic effects also depend on disease status at presentation. In 19 de novo cases of M6, the remission rate after induction chemotherapy was 95% and the relapse rate was 35%, whereas 8 cases with secondary M6 had a remission rate of 57% and a relapse rate of 75% (33). The reported median survival for M6 varies from 4 to 14 months (34). With autologous hematopoietic stem cell transplantation, the leukemia-free survival (LFS) was 26% ± 5% at 5 years, whereas the LFS was 57% ± 5% for allogeneic hematopoietic stem cell transplantation (34).

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CASE 12 Acute Megakaryoblastic Leukemia

CASE HISTORY

A 48-year-old man presented with fatigue and night sweats for 2 weeks. The patient was previously in good health and went to donate blood in the hospital, just to find out that he had a low leukocyte count. Since then he noticed low-grade fever and night sweats. Upon consulting his primary care physician, he was found to have pancytopenia. He further developed symptoms of dyspnea on exertion while walking up stairs or playing with his children. The patient was referred to a hematologist who admitted him to the hospital for further evaluation.

Physical examination on admission was unremarkable except for the presence of petechiae on his left and right anterior shins. He had no lymphadenopathy or hepatosplenomegaly. Laboratory examination of the peripheral blood showed a total leukocyte count of 1,900/μL, hematocrit of 14.6%, hemoglobin of 4.9 g/dL, and platelet count of 23,000/μL. The differential count revealed 71.1% lymphocytes, 24.3% neutrophils, and 2.3% monocytes, but no immature leukocytes were found.

After admission, a bone marrow biopsy was performed. It revealed 90% cellularity with many megakaryocytes of varying size and shape, which stained positive for CD42b. The bone marrow aspirate showed 52% blasts; many of them had cytoplasmic blebs.

A diagnosis of acute megakaryoblastic leukemia (AMKL; French-American-British [FAB] classification acute myeloid leukemia [AML]-M7)

was established, and induction chemotherapy was started with ARA-C and daunomycin. During the course of treatment, the patient developed neutropenic fever and diarrhea associated with chemotherapy. However, his condition was under control after antibiotic treatment. A repeat bone marrow biopsy showed complete remission, and he was discharged 1 month after admission.

Subsequently, the patient went through multiple cycles of consolidation and salvage therapy, but leukemia relapsed 2 months after the first admission. During this course, he had multiple episodes of neutropenic fever, pulmonary and hepatic aspergillosis, Staphylococcus B bacteremia, and *Clostridium difficile* colonization. Although the infections were treated successfully with various regimens of antibiotics, his leukemia became refractory to chemotherapy. Many blasts finally appeared in the peripheral blood, and the patient died 1 year after the initial diagnosis of M7.

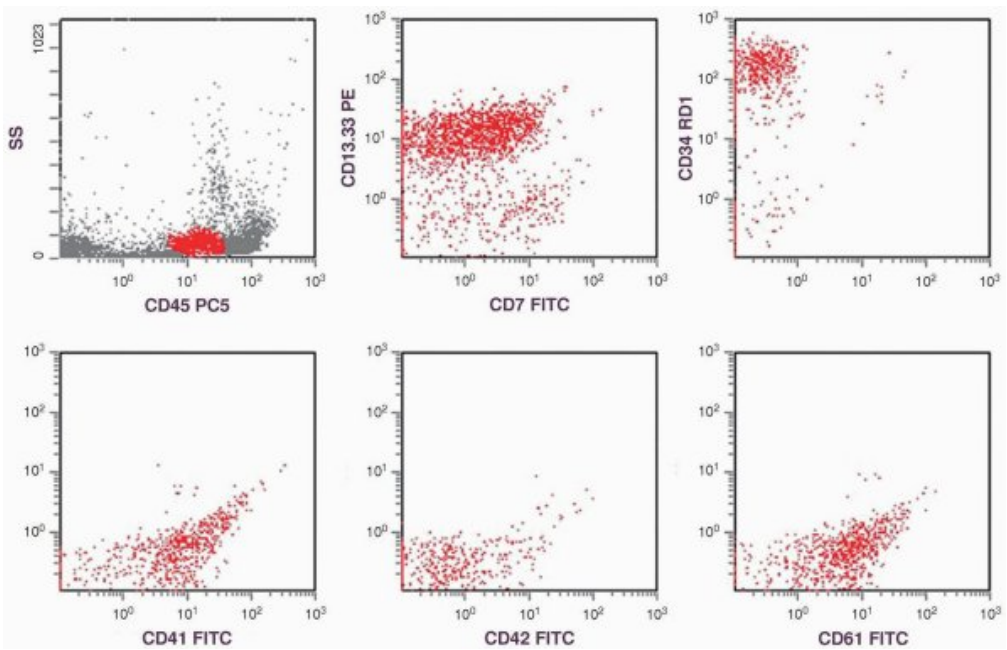

FIGURE 6.12.1 Flow cytometric analysis shows positive CD13.CD33, partially positive CD7, and positive CD34 reactions. The diagnostic feature is the expression of the immature megakaryocyte markers (CD41 and CD61) with low percentage or negative reaction of the mature megakaryocytic marker (CD42). SS, side scatter; PC5, phycoerythrin cyanin 5; PE, phycoerythrin; RD1, rhodamine; FITC, fluorescein isothiocyanate.

FLOW CYTOMETRY FINDINGS

The bone marrow showed 0% myeloperoxidase (MPO), 91% CD13-CD33, 42% CD13-CD33/CD7, 82% CD41, 0% CD42, 75% CD61, 76% CD34, 0% terminal deoxynucleotidyl transferase (TdT), 5% glycophorin A, and 0% CD14 (Fig. 6.12.1).

CYTOCHEMICAL FINDINGS

The leukemic cells in the bone marrow were negative for MPO and α-naphthyl butyrate esterase but positive for periodic acid-Schiff (PAS) stains. The PAS stain showed a typical peripheral pattern with strong staining in the cytoplasmic blebs.

DISCUSSION

AMKL is a rare disease with a bimodal age distribution. The incidence of AMKL differs in various age groups. It accounts for 1% to 10% of AML in adults, 3.1% to 10% in childhood AML, and about 20% in AML of infants (1, 2, 3 and 4). The incidence of AMKL in children with Down syndrome (DS) is estimated to be approximately 500 times greater than that in children without this syndrome (5). There is evidence to indicate that AMKL in these various groups of patients may be biologically different, as they differ in cytogenetic profile and prognosis (6).

AMKL is classified as AML-M7 in the FAB system (7). Its diagnostic criterion is the presence of ≥30% megakaryoblasts in the bone marrow. In the World Health Organization (WHO) classification, the requirement for the blast count is reduced to 20%, but more than one half of the blasts should be identified as of megakaryocytic lineage (8,9). This system also includes the transient myeloproliferative disorder in DS as a variant of AMKL (9).

In contrast to criteria for other subtypes of AML, the FAB scheme requires the identification of megakaryocytic cells not only by

morphology but also by either the platelet peroxidase reaction on electron microscopy or staining with monoclonal or polyclonal platelet specific antibodies. Because myelofibrosis or increased bone marrow reticulin is a common finding in patients with AMKL, satisfactory bone marrow aspirate may be difficult to obtain and characteristic megakaryoblasts are difficult to find. In those cases, a diagnosis of AMKL is allowed on the estimation of the number of blasts in the bone marrow biopsy (7,9).

Under this condition, unequivocal megakaryoblasts should be identified in the peripheral blood and/or bone marrow by immunologic techniques (7).

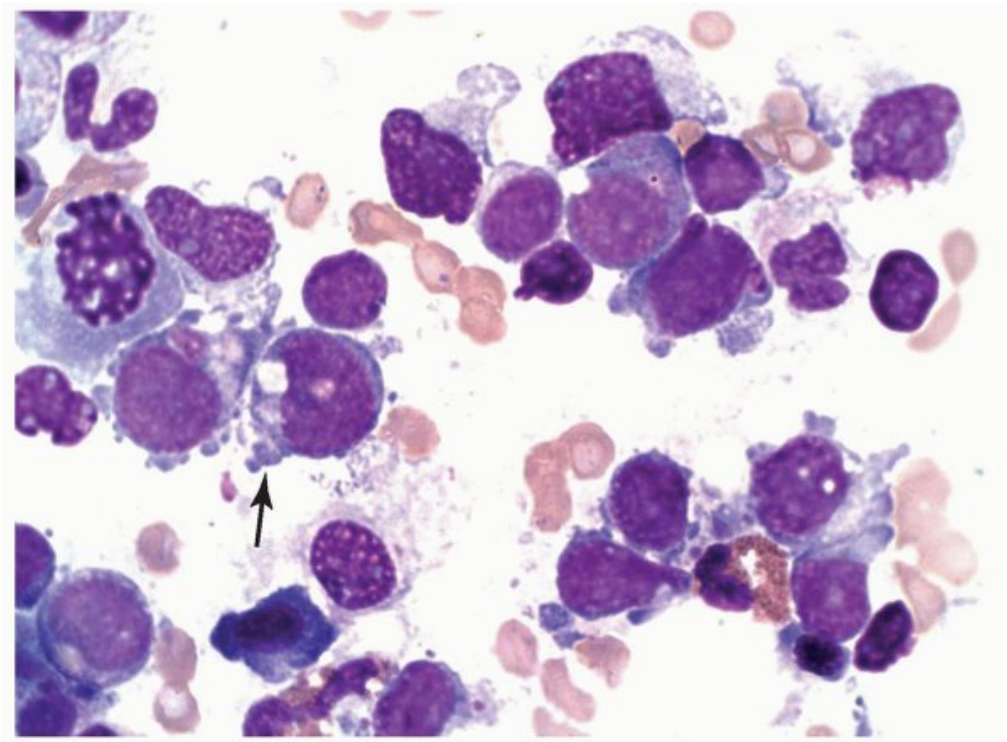

FIGURE 6.12.2 Bone marrow aspirate shows a cluster of megakaryoblasts of varying sizes. Large cells show moderate amount of basophilic cytoplasm with irregular cell border or budding (*arrow*). Chromatin is dispersed and nucleoli are present. Small cells, in contrast, reveal scanty cytoplasm and dense chromatin, resembling lymphoblasts. Wright-Giemsa, 100× magnification.

Morphology and Cytochemistry

The morphology of megakaryoblasts is highly polymorphic. They may appear as small round cells with scanty cytoplasm and dense chromatin, resembling lymphoblasts, or as larger cells with a fine chromatin pattern and prominent nucleoli (Figs. 6.12.2 and 6.12.3) (1,7,10). The large-cell type usually has a moderate amount of basophilic cytoplasm with or without azurophilic granules. The most specific morphologic feature is the presence of cytoplasmic blebs (budding), which represents the process of platelet shedding from the cell surface. However, the real process is seen only in mature megakaryocytes. Cytoplasmic blebs, nevertheless, can be an artifact seen in other subtypes of AML and is thus not pathognomonic for AMKL.

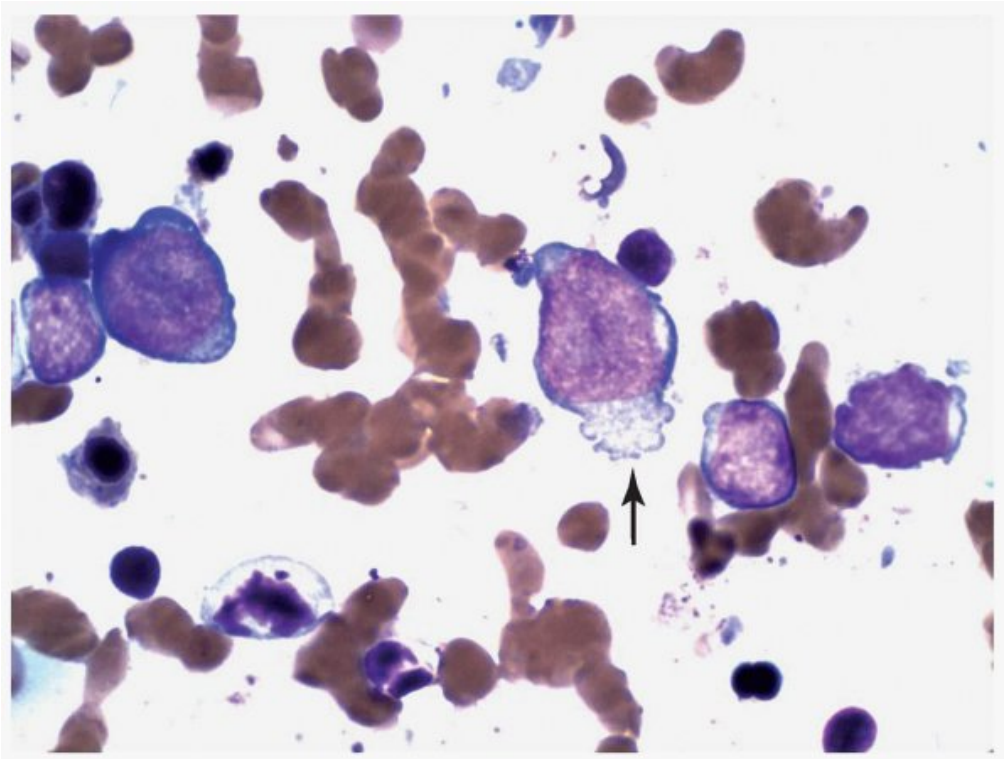

FIGURE 6.12.3 Bone marrow touch imprint shows two large and two small megakaryoblasts. Cytoplasmic projection is clearly visible in the large blasts (*arrow*). Wright-Giemsa, 100× magnification.

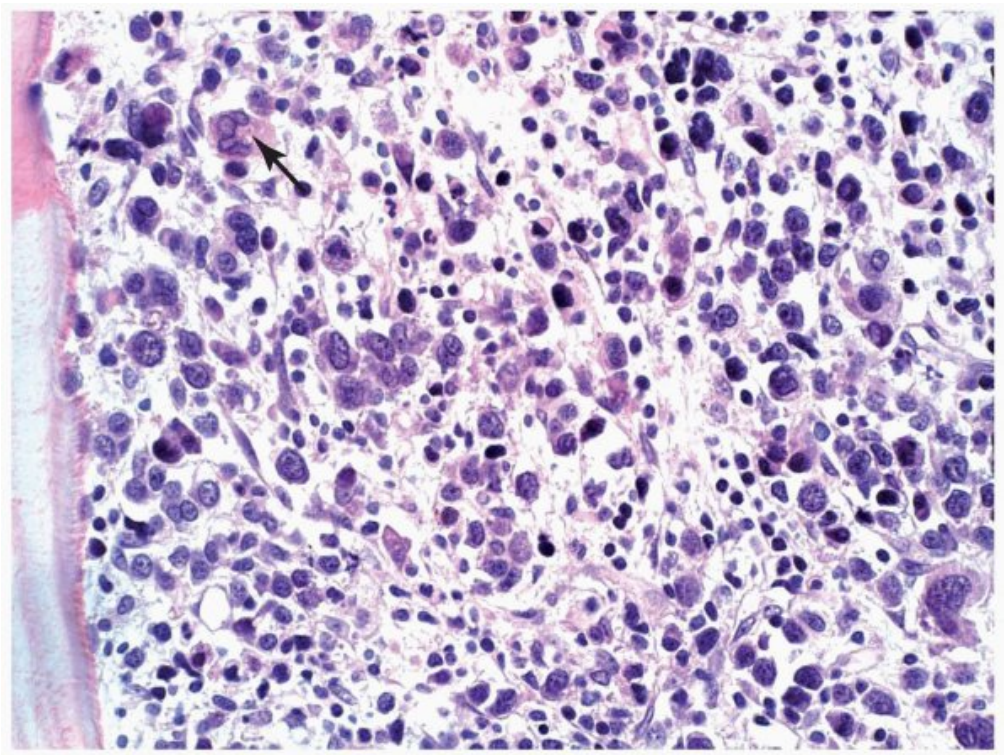

FIGURE 6.12.4 Bone marrow biopsy shows total replacement of the normal hematopoietic cells by megakaryoblasts and a few large megakaryocytes (*arrow*). Marked variation in size of the

blasts distinguishes megakaryoblasts from other blasts. Hematoxylin and eosin, 40× magnification.

Megakaryoblasts may be difficult to identify in bone marrow biopsy, but their presence is usually suggested by the accompanying megakaryocytes and reticular fibrosis (Figs. 6.12.4 and 6.12.5). The megakaryocytes are frequently smaller than normal megakaryocytes, about

7 to 10 μm in diameter, hypolobated, or mononucleated (11). These megakaryocytes are often referred to as micromegakaryocytes or dwarf megakaryocytes. They may be present singly or forming small or large clusters. A reticulin fiber network usually surrounds the mature megakaryocytes, but prominent fibrosis may not be present, especially in cases where immature megakaryoblasts are predominant.

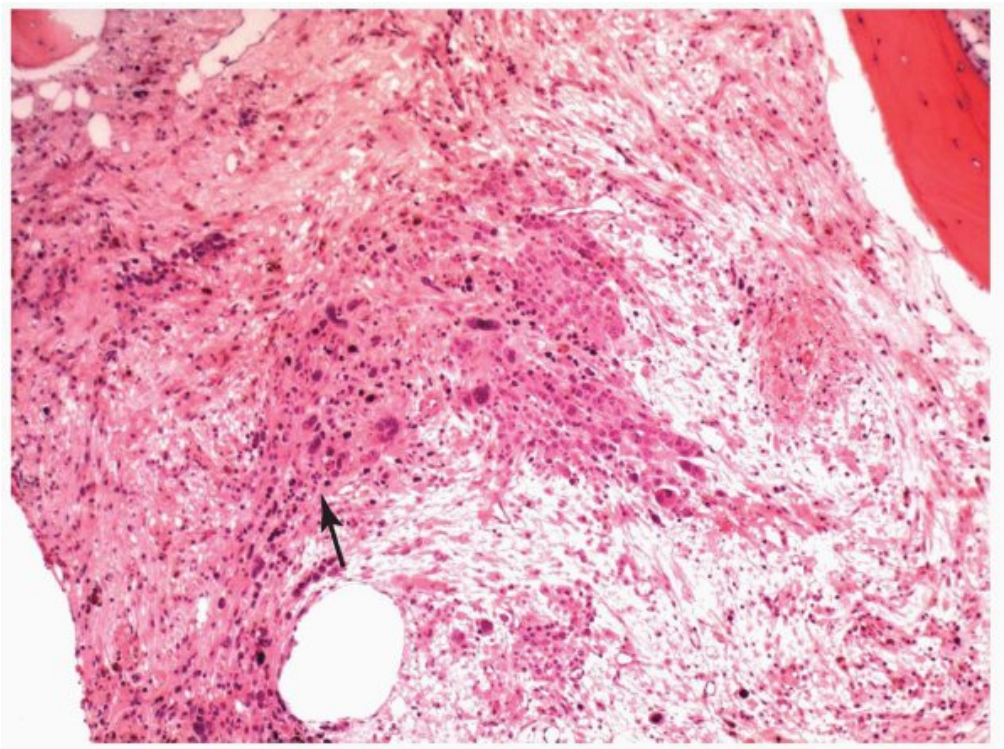

FIGURE 6.12.5 Bone marrow biopsy in a case of M7 with relapse shows a cluster of leukemic cells (*arrow*) on a fibrotic background. Hematoxylin and eosin, 10× magnification.

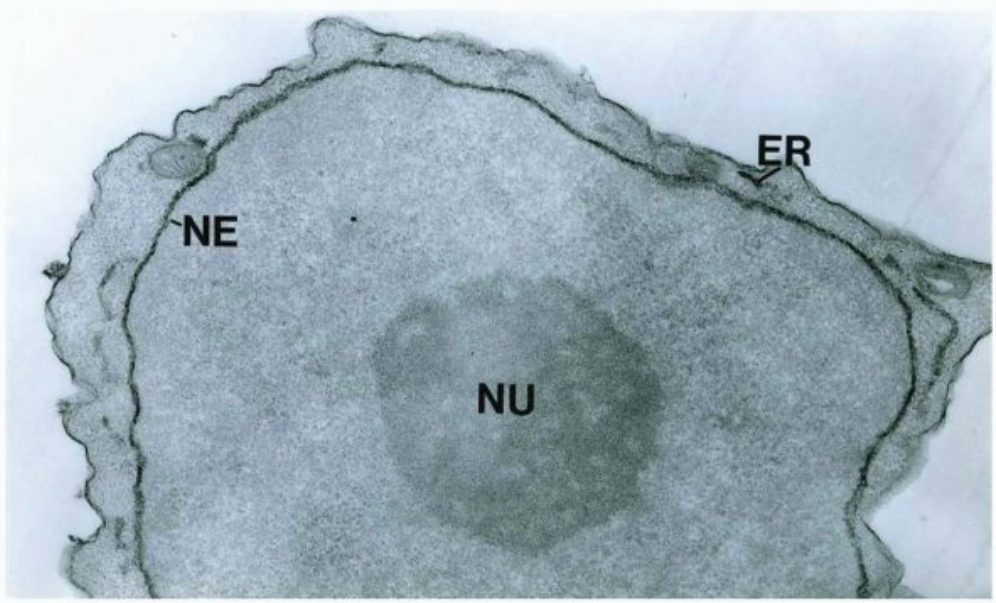

FIGURE 6.12.6 Electron micrograph of a megakaryoblast in a case of M7 leukemia, showing platelet peroxidase activity in the endoplasmic reticulum (ER) including the nuclear envelope (NE). Note the prominent nucleolus (NU) in the nucleus. 30,000× magnification. (Courtesy of Dr. Saul Teichberg, North Shore University Hospital, New York.)

In equivocal cases, electron microscopic identification of platelet peroxidase can be helpful. The platelet peroxidase appears earlier than other platelet antigens as identified by other monoclonal or polyclonal antibodies, discussed later (12). In megakaryocytes and megakaryoblasts, the peroxidase reaction is localized on the nuclear membrane and the endoplasmic reticulum, whereas the reaction in myeloblasts mainly occurs in the Golgi area and cytoplasmic granules (Figs. 6.12.6 and 6.12.7) (7).

In cases with a dry tap, the enumeration of the percentage of megakaryoblasts is impossible and the blast count depends on a rough estimation in the core biopsy. Under this condition, many similar diseases should be carefully excluded, such as myelodysplastic syndromes, acute panmyelosis with fibrosis, idiopathic myelofibrosis, or other subtypes of AML before or after treatment (13,14). In these diseases, megakaryocytes are a reactive component that may become dysplastic, mimicking malignant cells. The distinction of these entities with AMKL requires multiparameter studies. For instance, a recent comparative study of acute panmyelosis with myelofibrosis and AMKL found that the former is characterized by a multilineage myeloid proliferation, smaller blast population, and infrequent expression of megakaryocytic antigen (15). Making the matter more complicated is the fact that these diseases can transform into each other. A case report of chronic idiopathic myelofibrosis transformed into AMKL is a good example (16). In contrast, AMKL with t(1;22) in an infant may be mistaken as metastatic neuroblastoma (9). Cuneo et al. (17) used 20% platelet antigen-positive cells as a cutoff point to distinguish AMKL from other disorders containing megakaryocytic elements.

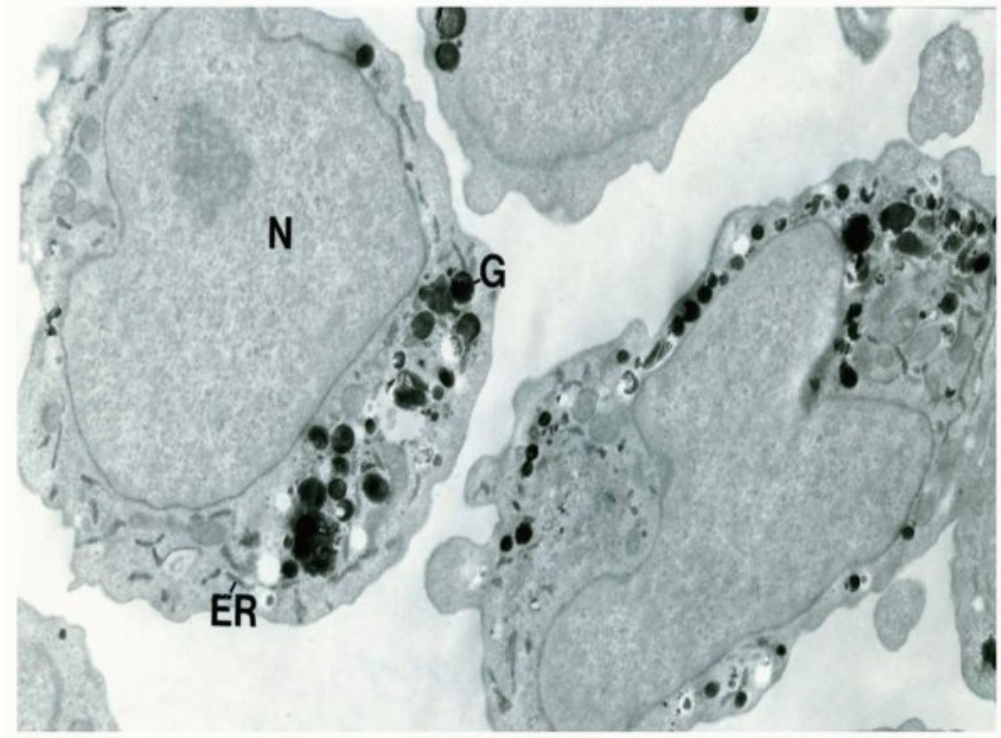

FIGURE 6.12.7 Electron micrograph of a myeloblast showing myeloperoxidase activity in many secretory granules (G) but also in the endoplasmic reticulum (ER) including the nuclear envelope. Note the immature nucleus (N) with a prominent nucleolus in one cell. 11,500× magnification. (Courtesy of Dr. Saul Teichberg, North Shore University Hospital, New York.)

Cytochemical stains are characteristic in the constant absence of MPO and Sudan black B stains but in the presence of PAS (Fig. 6.12.8) and acid phosphatase (18,19). The PAS stain is typical in a peripheral staining pattern with prominent staining of the cytoplasmic blebs (19). The reactions of various nonspecific esterases differ: α-naphthyl butyrate esterase is negative but α-naphthyl acetate esterase and naphthol AS-D acetate esterase are positive, with focal staining in megakaryoblasts.

Immunophenotype

The monoclonal antibodies most commonly used for the identification of megakaryocytes are CD41, CD42, and CD61

(1, 2 and 3,8,13,20). Recently, CD36 has been included in the megakaryocytic profile (4,9). In paraffin sections, factor VIII antibody was frequently used to identify megakaryocytes, but factor VIII may not be present on the leukemic cells in all patients with AMKL (12). Currently, CD42b and CD61 antibodies are available for immunohistochemical staining and provide a more specific identification (9).

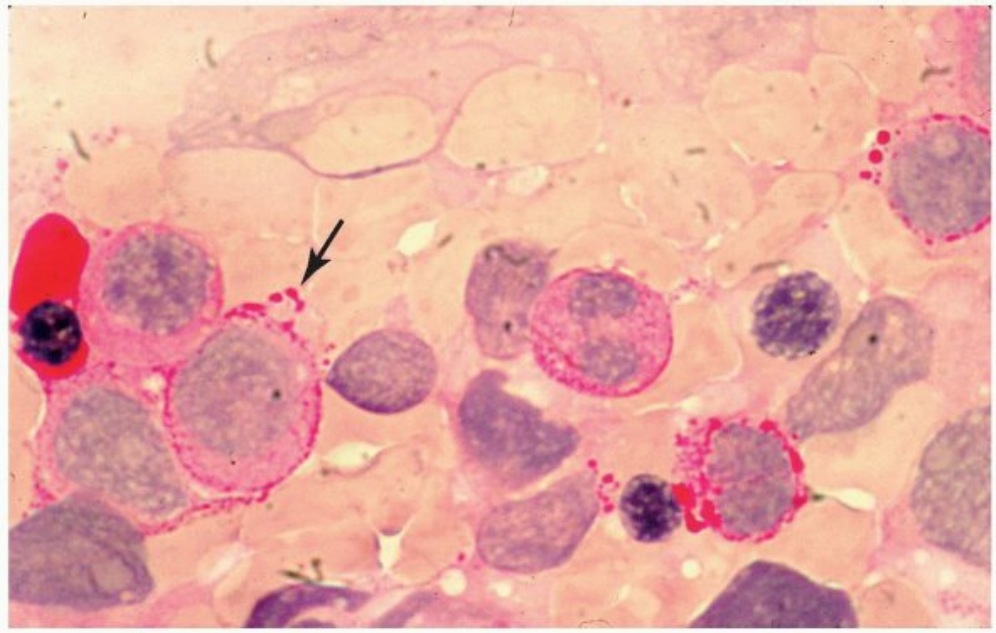

FIGURE 6.12.8 Bone marrow aspirate with periodic acid-Schiff stain shows a peripheral staining pattern with accentuation in cytoplasmic blebs (*arrow*). 100× magnification.

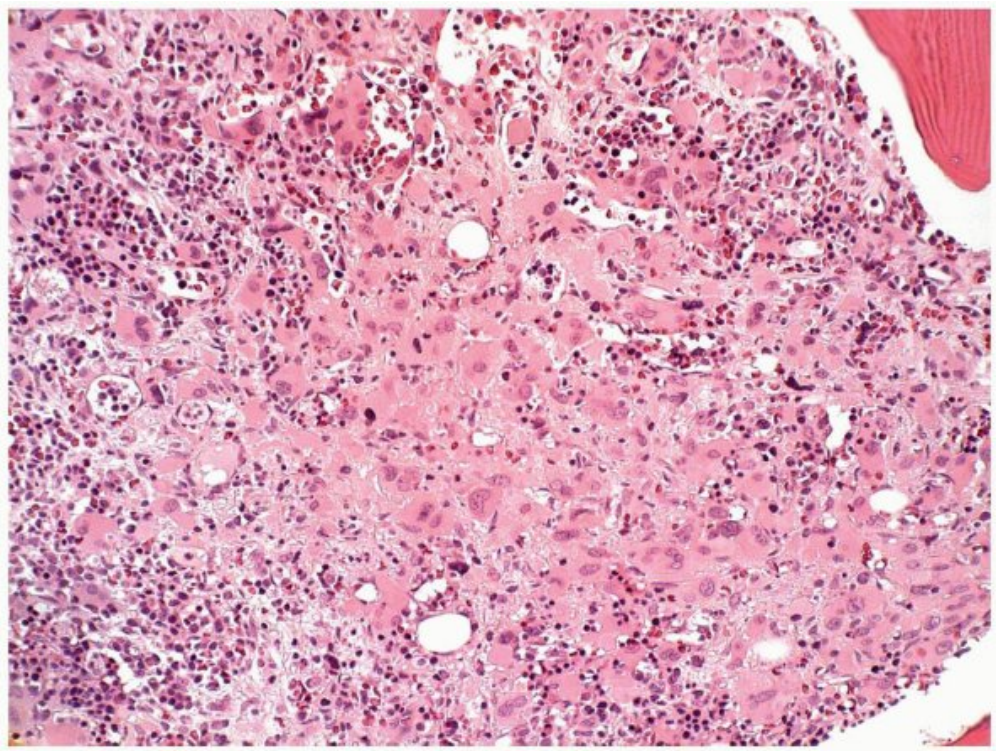

FIGURE 6.12.9 Bone marrow biopsy from a patient with acute myeloblastic leukemia shows extensive megakaryocytic reaction. Hematoxylin and eosin, 20× magnification.

A note of caution: Platelets frequently adhere to myeloblasts and cause falsely high percentages of CD41, CD42, and CD61 (21). Therefore, the study of cytoplasmic platelet antigens may avoid this problem (9). Some studies mentioned that CD42 is positive in mature platelets but negative in some cases of immature megakaryoblasts (13,22,23); thus, the presence of a low percentage of CD42 as compared to CD41 and CD61 may be a clue to the diagnosis of AMKL. In addition, megakaryocytic reaction is occasionally seen in other

subtypes of acute leukemias (Fig. 6.12.9), leading to the demonstration of high percentages of CD41/CD42/CD61-positive populations by flow cytometry.

For myelomonocytic antigens, CD33 is frequently positive, but MPO, CD13, CD14, and CD15 are usually negative (2,19,24, 25 and 26). CD34, a hematopoietic progenitor cell antigen, and CD56, a neural cell adhesion molecule and natural killer cell marker, are frequently expressed on megakaryoblasts (12,19,25, 26, 27 and 28). The expression of HLA-DR is variable (2,18,19). Glycophorin A has been detected in 18% of 41 cases of pediatric AMKL at St. Jude Children's Research Hospital (4).

For lymphoid markers, the B-cell markers (CD19, CD79a, CD10, and CD20) are generally negative (2,19,24,25). The expression of T-cell markers is selective: CD2 and CD7 are often positive, but CD3 and CD5 are usually negative (4,9,18).

In the current case, the diagnosis was based on the demonstration of typical morphology in some of the megakaryoblasts in the bone marrow aspirate. This diagnosis was further confirmed by the flow cytometric study that demonstrated positive reactions in all three megakaryocytic antigens with a much lower percentage of CD42 than of CD41 and CD61. The bone marrow biopsy showed total replacement of normal hematopoietic cells by the small mononucleated megakaryoblasts with scattered megakaryocytes in various degrees of maturation. Myelofibrosis appeared only after chemotherapy. Immunohistochemical stains in bone marrow core biopsy demonstrated CD42b on the relatively mature megakaryocytes, but not on megakaryoblasts. However, when the patient was in remission, CD42b was not demonstrated in the fibrotic bone marrow. The PAS stain also played a role in the early diagnosis of this case by demonstrating the surface staining pattern on the megakaryoblasts.

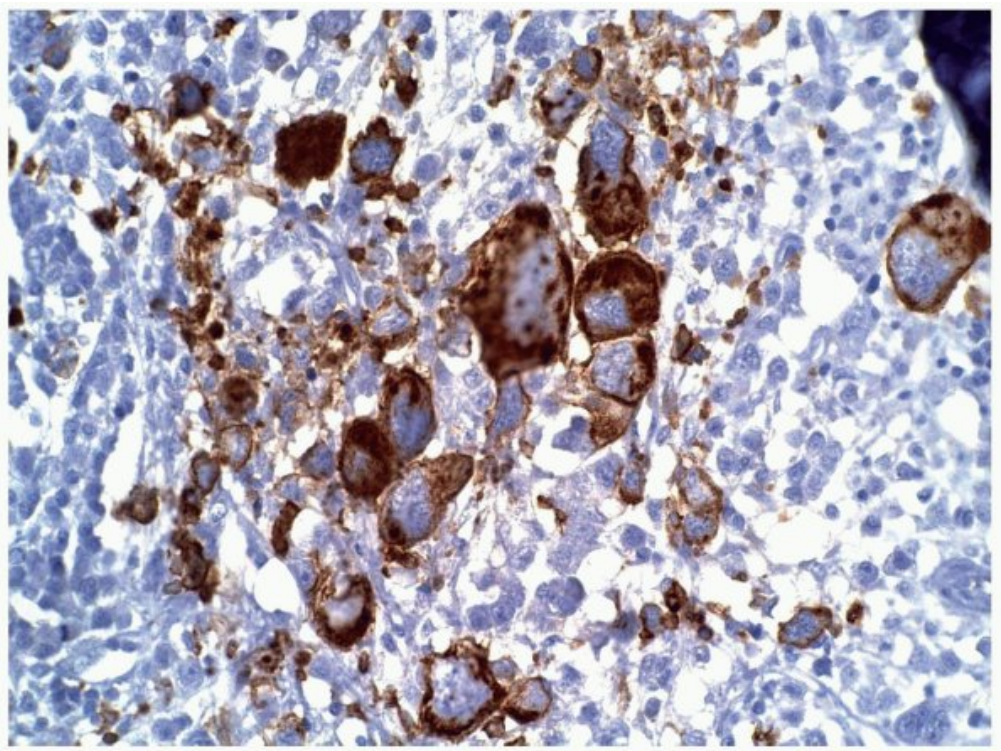

FIGURE 6.12.10 Bone marrow biopsy with CD42b staining shows positive stain in the relatively mature megakaryocytes/megakaryoblasts. Myeloperoxidase, 40× magnification.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry is able to identify three megakaryocytic antigens, CD41, CD42, and CD61. The discrepancy between CD42 and the other two markers is helpful in establishing the diagnosis. In immunohistochemistry, the use of CD42b and CD61 may directly identify the megakaryocytes, but the former only identifies the mature cells and not the blastic cells (Fig. 6.12.10). CD61 staining, in contrast, is affected by the fixation procedure and decalcification (9). Factor VIII staining may be used to supplement CD42b and CD61 in achieving the diagnosis.

Molecular Genetics

As trilineage myelodysplasia is frequently coexistent with de novo AMKL and many of the chromosome aberrations in AMKL are shared by other myeloid neoplasms, this leukemia appears to be derived from a multipotent stem cell (25). The cytogenetic abnormalities are also shared with myelodysplastic syndrome, and dysgranulopoiesis is seen in one third of the pediatric AMKL cases in one study (6). These findings suggest that AMKL may often be a secondary leukemia. Some studies also suggested that megakaryocytes may share a common progenitor cell with erythrocytes (5,26,29).

Cytogenetic analyses have been conducted in many studies of AMKL (3,19,24,25,30,31), but no cytogenetic profile has emerged as a specific marker. By summarizing 31 cases in the literature in addition to their own 15 cases, Cuneo et al. (25) found that -7/7q- and -5/5q- are the most common abnormalities. A more recent French study found AMKL to be characterized by a higher incidence of abnormalities, a higher complexity of karyotypes, and a different distribution of abnormalities among children and adults (6). This study divided AMKL cases into nine cytogenetic groups: (i) normal karyotypes, (ii) patients with DS, (iii) numeric abnormalities only, (iv) t(1;22)(p13;q13), (v) t(9;22)(q34;q11), (vi) 3q21q26, (vii) -5/del(5q) or -7/del(7q) or both, (viii) i(12)(p10), and (ix) other structural changes. Whereas groups 1, 2, 3, and 4 were exclusively seen in children, groups 5, 6, 7, and 8 were mainly seen in adults (6).

Lu et al. (32) found that pediatric patients with DS and those without differ in the karyotypes. In patients with DS, 10 of 43 cases showed no additional cytogenetic abnormalities besides trisomy 21. Most of the patients without DS showed aberrations of chromosome 22, including 16 cases with t(1;22) (q13;q13) and 6 cases with 22q13 translocation variants. The remaining non-DS patients showed frequent cytogenetic changes, including rearrangement of 3q21, 3q26-27, trisomy 21, and other specific changes.

The karyotype aberration t(1;22) has been exclusively reported in pediatric cases; most patients were younger than 12 months (3,19,31,33). This translocation is estimated to be present in 30% of pediatric patients and in >65% of infants with AMKL (34). This translocation is of special interest because it involves two oncogenes and because of its association with myelofibrosis. The N-ras oncogene is located in the breakpoint 1p13, and it may be activated as a consequence of translocation leading to malignant transformation of megakaryocytes (19). The c-sis oncogene is located at chromosome 22q13. A marked increase in c-sis messenger RNA in leukemic cells has been reported in two AMKL cases (35,36). The c-sis gene encodes platelet-derived growth factor B, which plays an important role in the occurrence of myelofibrosis. Recently, however, OTT (RBM15) and MAL (MLK1) genes have been found in 1p13 and 22q13, respectively (37,38). The OTT-MAL fusion transcript can be identified with molecular biology techniques in karyotypically cryptic cases (6). Patients with t(1;22) have a worse prognosis than do those AMKL cases without this translocation because of their poor response to chemotherapy. Therefore, autologous bone marrow transplantation is the therapy of choice, particularly for this group of patients (3).

In AMKL cases with DS, mutation in the gene for globin transcription factor 1 (GATA-1) has been implicated as a major mechanism for leukemogenesis (39). GATA-1 is a transcription factor that is essential for normal megakaryocytopoiesis. Absence of GATA-1 promotes accumulation of immature megakaryocytes. Acquired mutations in GATA-1 have been detected in the vast majority of AMKL patients with DS and in almost all cases of transient myeloproliferative disorder (39).

TABLE 6.1 2.1

Salient Features for Laboratory Diagnosis of Acute Megakaryoblastic Leukemia

1. Presence of ≥20% megakaryoblasts in bone marrow aspirate
2. If bone marrow aspirate is not successful, bone marrow biopsy identification of immature leukemic cell infiltration and immunologic identification of megakaryocytes in blood or bone marrow are required.
3. Flow cytometry shows high percentages of CD41 and CD61 but low percentage of CD42b.
4. Immunohistochemical stains including CD42b, CD61, and factor VIII are available.
5. Electron microscopic identification of platelet peroxidase in leukemic cells
6. A peripheral periodic acid-Schiff staining pattern or staining of the blebs on the blasts
7. t(1;22)(p13;q13) is specific for infantile megakaryoblastic leukemia.

The salient features for laboratory diagnosis of AMKL are summarized in Table 6.12.1.

Clinical Manifestations

As mentioned before, AMKL can be seen in different age groups that may have biological, cytogenetic, and prognostic differences. In adults, AMKL is frequently secondary to chemotherapy, to leukemic transformation of either myelofibrosis or myelodysplastic syndrome, or as megakaryoblastic crisis of chronic myelogenous leukemia (3,7,27). In contrast, AMKL in children generally appears de novo (2).

Children with DS have a 10- to 20-fold increased risk for the development of acute leukemia (23), especially AMKL (25,40,41). In this group of patients, AMKL accounts for approximately 50% of acute leukemia and is frequently preceded by transient leukemia 1 to 4 years earlier (5). In a study of 20 children with DS in Japan, 14 had AMKL and all were younger than 3 years (20). Therefore, the possibility of an association between trisomy 21 and AMKL has been raised. However, trisomy 21 does not frequently appear in AMKL leukemic cells when the patient has a normal constitutional karyotype (20). In contrast, patients with transient leukemia and those with subsequent development to AMKL all have trisomy 21 in their leukemic cells (28). In those patients, if they do not have DS, they may have trisomy 21 mosaicism with a normal karyotype. Because the same cytogenetic abnormalities in addition to trisomy 21 in the leukemic cells of transient leukemia are also found in the leukemic cells of subsequent AMKL, it is suggested that AMKL in these patients arises from transient leukemic cells (28).

Clinically, the patient may have fatigue, weakness, fever, bleeding, ecchymosis, and petechiae, but lymphadenopathy and hepatosplenomegaly are seldom seen

(1,42). However, pediatric patients with t(1;22) may often have organomegaly (34). Patients with AMKL are usually anemic and thrombocytopenic. The leukocyte count may be low at the beginning of the disease, but an abrupt and rapid increase in the number of peripheral blasts is frequently seen in the terminal stage. The platelet aggregation responses may be impaired, and serum lactate dehydrogenase levels are frequently elevated. Bilateral symmetrical periostitis and osteolytic lesions have been observed in children (43).

Some clinical symptoms are defined by cytogenetic aberrations. For instance, patients with t(1;22) translocation have very early onset of AMKL with organomegaly but have no history of transient leukemia and myelodysplastic syndrome (33). Patients with the 3q21 aberration often have a secondary leukemia with weakness, anemia, CD34+ blasts, marked dysmorphic megakaryoblasts, normal or increased platelet counts, and very poor response to chemotherapy (44,45).

AMKL may be coexistent with other leukemias. A spontaneous and simultaneous occurrence of multiple myeloma and AMKL in a case of polycythemia vera has been reported (46). Another case report described coexistence of acute megakaryoblastic and B-lymphoblastic mixed blast crisis of chronic myeloid leukemia with chronic lymphocytic leukemia (47).

AMKL cases usually have a rapidly progressive course. All patients without treatment die within 1 year. At St. Jude Children's Research Hospital, the 2-year event-free survival in patients with DS is 83%, whereas it is 14% for pediatric cases with de novo AMKL and 20% for cases with secondary AMKL (4). Allogeneic transplantation during remission offers the best chance of cure in this hospital.

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CASE 13 Myeloid Sarcoma

CASE HISTORY

A 60-year-old man presented with a 3-week history of increasing fatigue and right neck swelling. Examination of peripheral blood revealed pancytopenia. The patient was treated with antibiotics for neutropenic fever without response. He was admitted to the hospital for further evaluation.

On admission, his total leukocyte count was 9200/μL with 94% myeloblasts and 6% lymphocytes. Granulocytes were not demonstrated in the peripheral blood. The hemoglobin level was 13.9 g/dL, hematocrit 40.3%, and platelet count 57,000/μL. Physical examination showed no hepatosplenomegaly, but right cervical lymphadenopathy was noted. A lymph node biopsy and a bone marrow biopsy were performed after admission.

The patient was started on imatinib (Gleevec), which was followed by a precipitous drop in the leukocyte count from 1,500/μL to 600/μL. Within the ensuing days, the patient developed a disseminated fungal infection, renal insufficiency, and altered mental status. He subsequently died.

FLOW CYTOMETRY FINDINGS

Peripheral blood: Myeloid markers: CD13-CD33 86%, CD13-CD33/CD7 80%, CD14 0%, myeloperoxidase (MPO) 0%. Activation antigen: HLA-DR 91%. Immature cell markers: CD34 85%, CD117 66%.

Bone marrow: Myeloid markers: CD13-CD33 86%, CD13-CD33/CD7 82%, CD14 0%, MPO 0%. Activation antigen: HLA-DR 91%. Immature cell markers: CD34 85%, CD117 66%.

Lymph node biopsy: Myeloid markers: CD13-CD33: 96%, CD13-CD33/CD7 95%, CD34 80%, CD117 62% (Fig. 6.13.1).

IMMUNOHISTOCHEMICAL STAINS

The tumor cells showed a strongly positive staining for CD45 and CD43, but were negative for CD3, CD20, CD34, CD45RO, MPO chloroacetate esterase, and lysozyme.

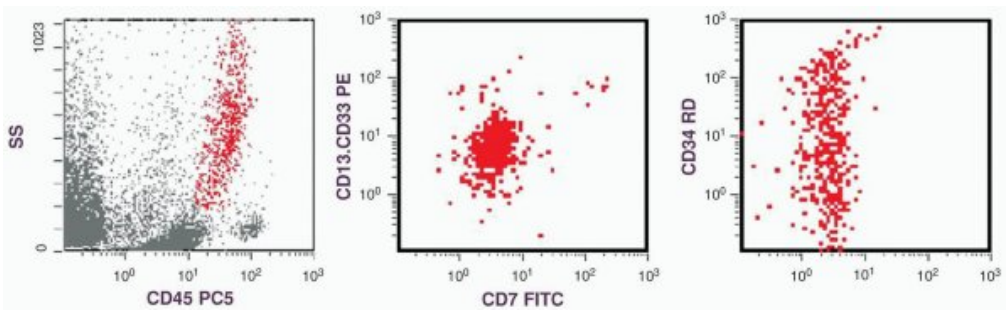

FIGURE 6.13.1 Flow cytometric analysis of a lymph node shows a large cluster of granulocytic cells (*red*) in the dot plot. Gated cell cluster shows a dual CD7 and CD13.CD33 staining as well as a spectrum of CD34 staining, representing mature and immature granulocytes. SS, side scatter; PC5, phycoerythrin cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

CYTOGENETIC FINDING

Cytogenetic study showed a normal karyotype of 46,XY in the bone marrow.

DISCUSSION

Myeloid sarcoma (MS) is a solid tumor of extramedullary myeloid cells localized in soft tissues and in bones (Fig. 6.13.2). Extramedullary myeloid leukemic infiltration can be seen in acute myeloid leukemia (AML) and chronic myeloid leukemia (CML), usually found at autopsy, but as far as a tumor mass is not formed, it should not be considered as MS (1,2). This entity was first described by Burn in 1811 (3). The name of chloroma was designated by King (3) because this tumor sometimes shows a green color that fades when exposed to air. The presence or absence of the green color depends on the concentration of MPO in the tumor. By using the peroxidase stain, Brugess proved the myelogenous origin of chloroma. The term granulocytic sarcoma was introduced by Rappaport (3) and had been generally used for many years until recently when the World Health Organization (WHO) scheme designated this tumor as MS (1). In the literature many other synonyms have been used, including extramedullary myeloid cell tumor, myeloblastoma, myelosarcoma, and monocytic sarcoma.

FIGURE 6.13.2 Splenectomy specimen shows a large solid tumor mass in the center, representing a granulocytic sarcoma.

Morphology

MS is morphologically similar to lymphoma, particularly large cell lymphoma. In three study series, 66%, 75%, and 100% of MS cases, respectively, were initially misdiagnosed as lymphoma (4, 5 and 6). As will be discussed later, a correct diagnosis requires a high index of suspicion and immunophenotyping of the tumor.

The major clue that may lead to the diagnosis is the demonstration of eosinophilic myelocytes (Fig. 6.13.3) in the hematoxylin and eosin-stained histologic sections (4,5). The presence of the mature granulocytes does not count, because this presence may simply represent a leukocytic reaction in a lymphoma, frequently due to necrosis of the tumor cells. Unfortunately, about 50% of MS contains no myelocytes. In those cases, a tissue imprint is more helpful for the distinction between lymphoma cells and immature myelomonocytic cells, including myeloblasts, monoblasts, and promyelocytes. Pure monocytic sarcoma is rare, but most MS contains a certain percentage of monoblasts (5). The presence of cytoplasmic granules and/or Auer rods is particularly helpful in identifying myeloblasts.

On the basis of tumor-cell differentiation, MS can be classified into three groups: well differentiated, immature (poorly differentiated), and blastic (1,4,7). The well differentiated group is composed primarily of promyelocytes, but nearly all stages of granulocyte may be present. The immature group consists of myeloblasts and promyelocytes. In tissue sections, the tumor cells have vesicular nuclei with conspicuous nucleoli (Fig. 6.13.4). The nuclei may be variable

in size and show nuclear grooves, creases, or convolutions (7). The cytoplasm is moderate to abundant, and a small number of tumor cells may show cytoplasmic granules consistent with myeloid differentiation. The blastic group is formed predominantly of myeloblasts. In tissue sections, the nuclei of the tumor cells are uniform and relatively round. The nuclear chromatin is dispersed, and small inconspicuous nucleoli are seen only occasionally. The cytoplasm varies in amount and contains no granules.

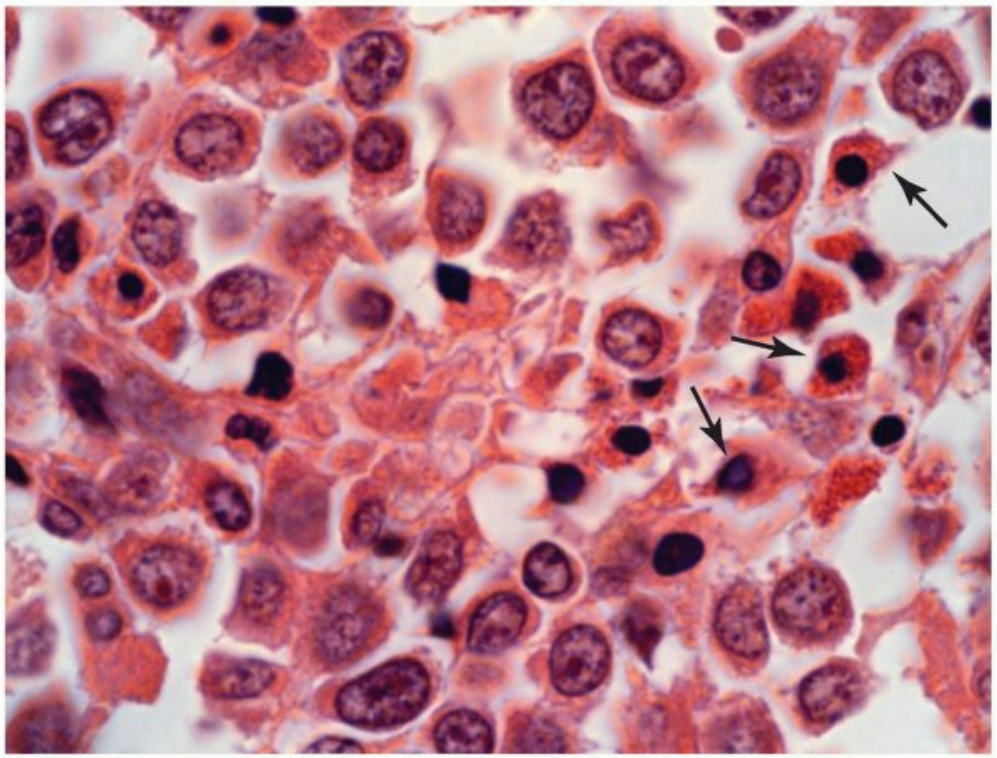

FIGURE 6.13.3 Myeloid sarcoma of bone shows a few eosinophilic myelocytes (*arrows*) scattered among the large tumor cells. Hematoxylin and eosin, 100× magnification.

By immunohistochemical stains, MS can be further divided into different cell lineages, which are designated as granulocytic variant, monoblastic variant, myelomonoblastic variant, megakaryoblastic variant, and erythroblastic variant (8).

MS is usually presented as sheets of leukemic infiltrate, frequently involving adjacent tissues. In the periphery of the tumor mass, tumor cells may form strands and cords (Figs. 6.13.5 and 6.13.6), and sometimes a targetoid pattern, vaguely reminiscent of invasive lobular breast carcinoma (5). The tumor infiltrates by expansion, so that normal tissues, such as the glandular and tubular structures, may be separated but the overall architecture is preserved.

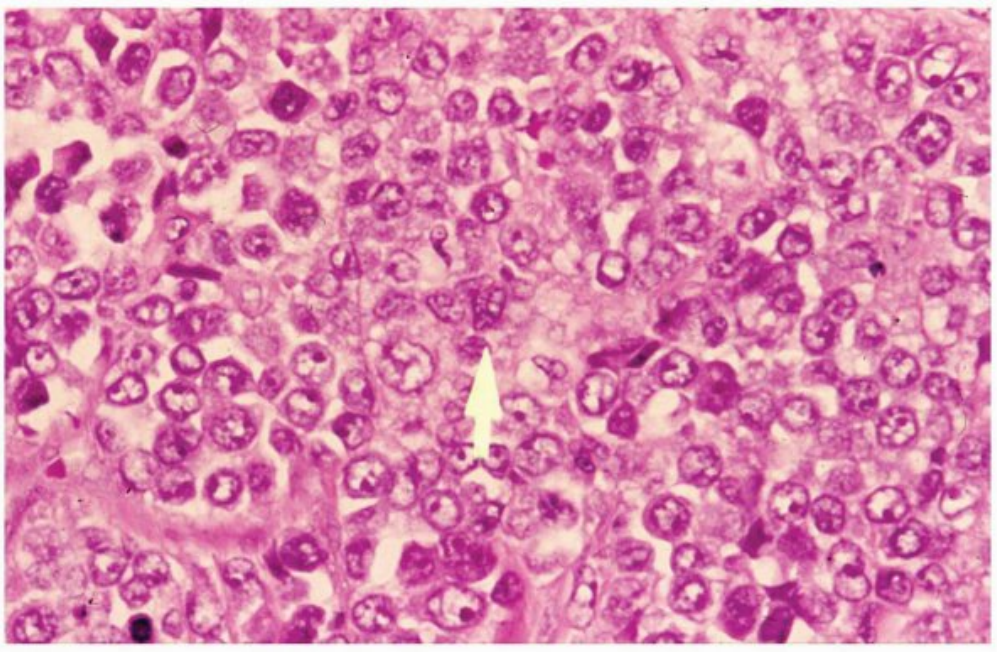

FIGURE 6.13.4 Myeloid sarcoma of the hip shows large tumor cells with vesicular nuclei and conspicuous nucleoli. One cell shows a nuclear groove (*arrow*). Hematoxylin and eosin, 60× magnification.

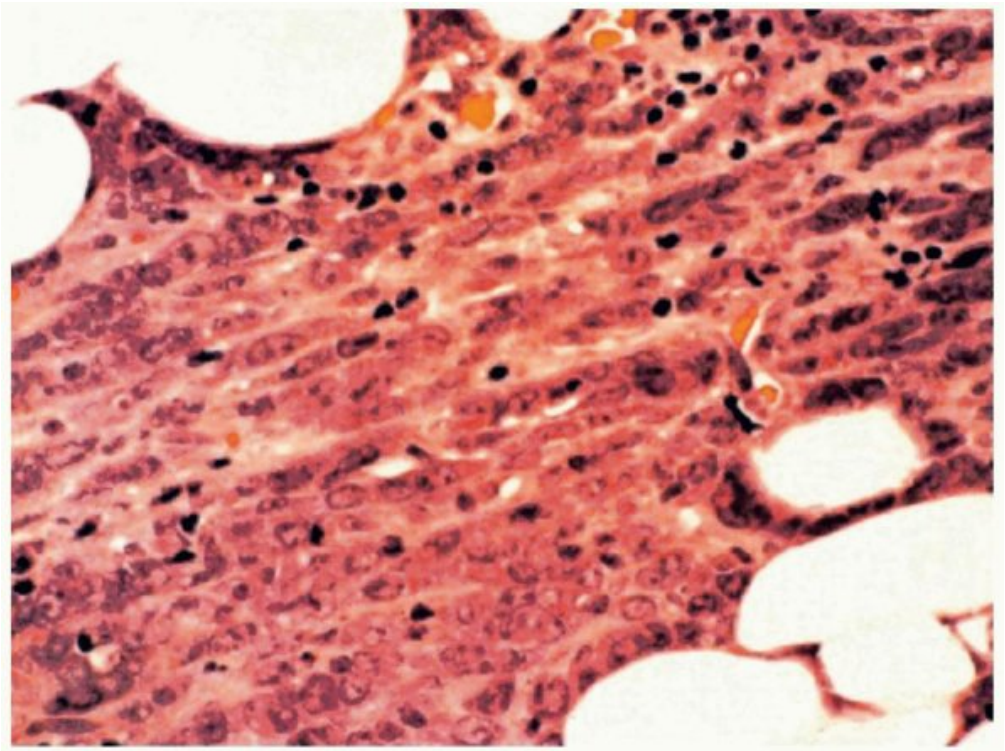

FIGURE 6.13.5 Myeloid sarcoma of a lymph node shows a cording pattern at the periphery of the tumor. Hematoxylin and eosin, 40× magnification.

In lymph nodes, the sinuses and occasionally the paracortex and medulla are infiltrated by leukemic cells, but the germinal centers are preserved. A reported case of monocytic sarcoma showed a myxoid stroma with cording of tumor cells in the lymph node (9). In another case of MS, the tumor cells assumed plasmacytoid features, simulating nonsecretory multiple myeloma (10). The morphologic characteristics of MS are summarized in Table 6.13.1.

MS may show a starry-sky pattern with a high mitotic rate, mimicking lymphoblastic and Burkitt lymphomas (4,5). Occasionally, MS may also simulate embryonal rhabdomyosarcoma, amelanotic melanoma, or undifferentiated

carcinomas (11). Electron microscopy may help in the differential diagnosis by demonstrating specific cytoplasmic granules and/or Auer rods, but the most useful technique for a definitive diagnosis is immunophenotyping by immunohistochemistry or flow cytometry.

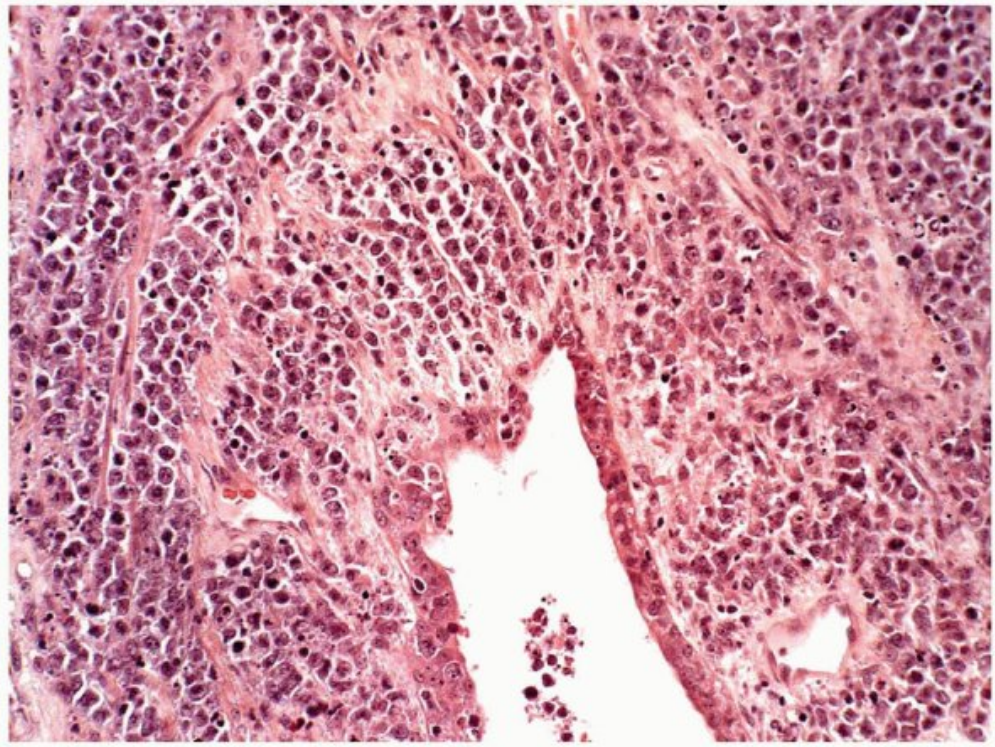

FIGURE 6.13.6 Myeloid sarcoma of the lung shows cords of tumor cells around the bronchus. Hematoxylin and eosin, 20× magnification.

TABLE 6.13.1

Characteristic Morphologic Features of Granulocytic Sarcoma

Histologic pattern	Sheets of tumor cells separate but do not destroy normal tissue. Tumor cells may form strands and cords at the periphery.
Cytology	Tumor cells usually mimic large lymphoma cells, but the scattered eosinophilic myelocytes may give a clue to the diagnosis.
Specific features	Cording arrangement and presence of eosinophilic myelocytes

Immunophenotype

Most immunophenotypic studies of MS were performed with immunohistochemistry. One of the reasons for this is because MS is usually an unexpected diagnosis, the specimen is often fixed, and by the time MS is suspected, flow cytometry cannot be done. Another reason is that there are many immunohistochemical markers that can be used for the identification of myelomonocytic cells, so that it is more convenient to perform immunohistochemical staining alone.

The time-honored Leder stain for chloroacetate esterase is most frequently used. However, this stain is relatively insensitive and is frequently negative in tumors composed predominantly of blasts (12). Other common immunohistochemical markers include CD14, CD15,

CD43 (Fig. 6.13.7), CD68, MPO, and lysozyme. Recently, CD99 and CD117 have been added to the list (13,14). Additionally, elastase, α_1-antichymotrypsin, lactoferrin, and cathepsin have been used in rare reports with various sensitivities, but are not accepted as routine histochemical stains (4,7,11,15, 16 and 17). Audouin et al. (8) have included factor VIII, CD31, and CD61 for the identification of the megakaryoblastic variant of MS and glycophorin C and blood group antigens for the erythroblastic variant.

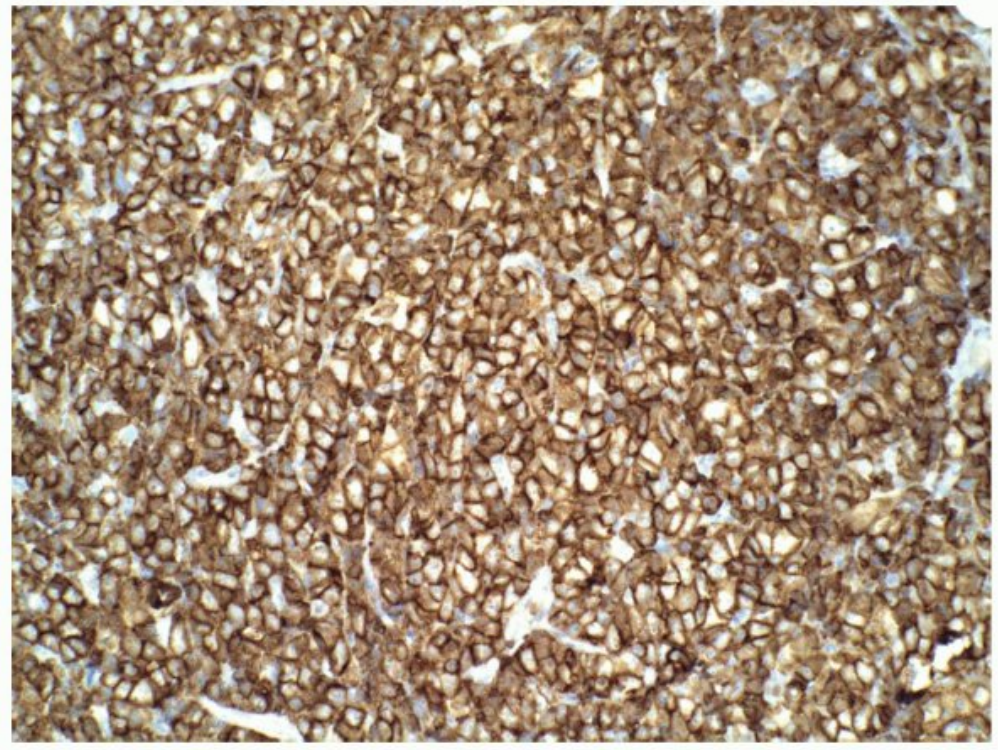

FIGURE 6.13.7 Myeloid sarcoma cells stain strongly with CD43. Immunoperoxidase, 20× magnification.

The sensitivity of some markers depends on the cell type of the tumor. In the study by Traweek et al. (7), CD15 and CD68 (KP-1) were positive for all well differentiated MS and 76% of poorly differentiated MS. However, for the blastic groups, CD68 was positive in only three of five cases, and CD15 was negative in all of five cases. The insensitivity of these two markers in least-differentiated MS was confirmed by Hudock et al. (18).

According to the literature, the most sensitive markers appear to be lysozyme and CD43 (19, 20 and 21). The presence of CD43 in MS has been further confirmed in several other studies (2,8,16,22,23). Although CD43 is a T-cell marker, the diagnosis of MS is valid when the so called “CD43 only” pattern is present, in which other T-cell and B-cell markers are negative (20,24).

A speedy diagnosis can be made if tissue imprints are available. Cytochemical stains for MPO, chloroacetate esterase (for granulocytes), and α-naphthyl butyrate esterase (for monocytes) can be performed on such preparations.

Most of the comparative studies considered flow cytometry superior to immunohistochemistry for the diagnosis of MS. One of the reasons is that some markers (such as CD45) can be negative or weakly positive by immunohistochemical stains but strongly positive by flow cytometry in the same specimen (19,25). This is particularly true for cytospins, smears, and cell blocks, in which immunocytochemical or cytochemical stains are often difficult to interpret (26).

There are several markers which are not myelomonocytic markers but are useful for differential diagnosis. HLA-DR is helpful in identifying MS that is associated with acute promyelocytic leukemia, in which case HLA-DR should be very low or entirely absent. CD34, the stem cell marker, is present in MS cases (particularly the blastic type), but is negative for lymphomas (27). CD34, however, can be negative in well and poorly differentiated types of MS (7). CD56, a neural cell adhesion molecule, can also be found in MS, and its expression usually predicts a poor prognosis (3). In fact, CD56 is probably one of the predisposing factors for the development of MS in patients with AMLs (28). The blasts that express CD56 may bind to tissue expressing the same adhesion molecule, thus forming a solid tumor mass (29).

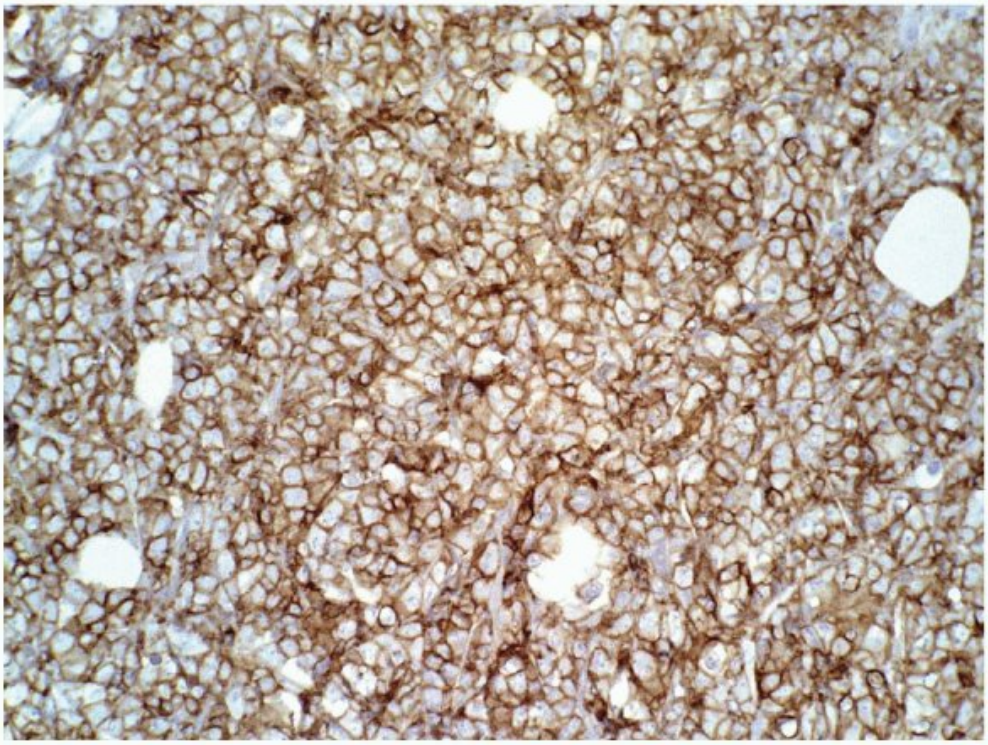

FIGURE 6.13.8 Myeloid sarcoma cells stains strongly with CD45, but negative with CD20 and CD3 (not shown). Immunoperoxidase, 20× magnification.

A panel of CD45 together with T-cell and B-cell markers is a powerful screening tool to distinguish MS and lymphoma, as MS usually only expresses CD45 (but not T-cell and B-cell markers) (Fig. 6.13.8). However, rare cases of MS may demonstrate T-cell markers, such as CD45RO, (UCHL1), CD3, and CD7 (11,15, 16 and 17). Less frequently, B-cell markers, such as CD20, Ki-B3, 4kB5, MB1, and LN2, have been reported in MS cases (11,16). These findings may represent nonspecific cross-reactivity, or, in some cases, they may represent a mixed lymphoid-myeloid phenotype (15,16,30).

Comparison of Flow Cytometry and Immunohistochemistry

Although most cases of MS are diagnosed by immunohistochemistry, flow cytometry is more helpful in some conditions. There are some antibodies that are available only for flow cytometry, such as CD13, CD11b, CD11c, CD14, and CD33. In MS of monocytic lineage, multiple monocytic antibodies (CD11b, CD11c, CD14, and CD64) should be used, because one or two of these markers can be negative in individual cases (19,31).

It is particularly critical to use flow cytometry in minimally differentiated AML (AML-M0) cases, because immunohistochemistry frequently fails to demonstrate diagnostic markers in those cases. The M0 case reported by Amin et al. (32) showed the absence of MPO, lysozyme, Sudan black B, specific and nonspecific esterase, and terminal deoxynucleotidyl transferase (TdT). CD34 was demonstrated in rare cells. However, flow cytometry revealed HLA-DR, CD11c, CD13, CD15, CD34, and TdT. The report from Astall et al. (27) detected no CD15 chloroacetate esterase and lysozyme by immunohistochemistry. The only diagnostic markers were CD45 and CD43. Flow cytometry in the same case, however, demonstrated CD7, CD13, CD33, CD34, and CD43. Finally, the report from Miyata et al. (33) revealed no immunohistochemical reactions to MPO, chloroacetate esterase, and lysozyme, but flow cytometry demonstrated CD7, CD13, CD33, CD41, and CD56.

The current case is also an M0 case, which showed the absence of CD34, MPO, chloroacetate esterase, and lysozyme by immunohistochemical stains, but flow cytometry detected high percentages of CD34 and CD117, as well as dual staining of CD33-CD13/CD7 in the peripheral blood, bone marrow, and lymph node biopsy, thus a diagnosis of MS was established.

Molecular Genetics

The cytogenetic abnormalities most frequently associated with MS include t(8;21)(q22;q22), characteristic of M2; inv(16)(p13;q22) or t(16;16)(p13;q22), characteristic of M4 with eosinophilia; and t(9;11)(p21;q23), characteristic of M5 (3,34,35). In contrast, AML with t(8;21) has a higher incidence of MS, particularly orbital MS (36). In fact, t(8;21) and the expression of CD56 may play a synergistic role in the development of MS (28,37). Translocation between chromosomes 8 and 21 results in the fusion of the *AML1* gene on chromosome 21 to the eleven twenty-one (*ETO*) gene on chromosome 8. The novel chimeric gene (*AML1/ETO*) produces a transcript that may play a role in leukemic transformation (38). A case of coexistence of t(8;21)(*AML1/ETO*) and t(9:22)(*BCR/ABL*) was recently reported in an MS case (39). Yin et al. (40) suggested that the synergistic effect of *BCR/ABL* and *AML/ETO* might provide an additional growth advantage

necessary for neoplastic transformation.

In contrast, pediatric patients with t(8;21) AML and MS usually have a good prognosis (35,41). In general, t(8;21) AML is associated with a younger age of onset, frequent splenomegaly, a high complete remission rate, and long relapse-free survival (29). No cytogenetic abnormalities have been identified in MS associated with myelodysplastic syndrome (MDS) (42).

The salient features for laboratory diagnosis of MS are summarized in Table 6.13.2.

Clinical Manifestations

Most MS cases are associated with AML, CML, other types of myeloproliferative disorders, or MDS. These associated conditions can be present before, during, or after the occurrence of MS. In a few cases, the patient may never show features of leukemia or myelodysplasia. Those patients probably die of MS before leukemia or myelodysplasia starts to surface.

Up to 2004, approximately 800 cases of MS have been reported. According to a study series of 478 patients with myelogenous leukemias, more MS cases were associated with CML (4.5%) than AML (2.5%) (43). Less than 20 MS cases have been reported with MDS.

Among AML cases, the most commonly reported subtypes appear to be AML with maturation (AML-M2) and acute myelomonocytic leukemia with eosinophilia (AML-M4eo). Another source claimed that MS has a significantly increased incidence in M4 and M5 (3), whereas another report mentioned M1 and M2 as the most common leukemias developed after MS (27). However, as most case reports of MS did not subclassify the leukemia, the incidence of the leukemic subtypes cannot be accurately estimated.

TABLE 6.1 3.2

Salient Features for Laboratory Diagnosis of Granulocytic Sarcoma

1. Screening panel is composed of CD45, CD19 or CD20, and CD3. MS cases should be CD45+, CD19/CD20-, CD3-. Lymphoma cases should be CD45+ and either CD19/CD20+ or CD3+.
2. The standard flow cytometry panel for MS may include CD13, CD14, CD15, CD33, and myeloperoxidase.
3. If monocytic sarcoma is suspected, CD11b, CD11c, CD4, and CD64 should be added.
4. Immunohistochemistry panel may include chloroacetate esterase (Leder stain), myeloperoxidase, lysozyme, CD15, CD43, and CD68.
5. Lysozyme and CD43 are considered the most sensitive markers.
6. If monocytic sarcoma is suspected, α-naphthyl butyrate esterase should be added.
7. Two new markers, CD99 and CD117, can be added in equivocal cases.
8. Common cytogenetic abnormalities include t(8;21)(q22;q22), inv(16), and t(9;11)(p21;q23).

CD, cluster of differentiation; MS, myeloid sarcoma.

Among MDS cases, chronic myelomonocytic leukemia and refractory anemia with excess blasts in transformation have a more frequent association with MS and with subsequent development of AML. Nevertheless, MDS-associated MS is not always a forerunner of AML (44). In general, MS is a predictor of poor prognosis in CML and MDS: Patients usually die in a few weeks after the discovery of MS. In CML,

the occurrence of MS is frequently followed by blast crisis.

As mentioned before, MS is frequently misdiagnosed initially. In some MS cases, a correct diagnosis was not made even in subsequent recurrences of the tumor. One patient had a series of 11 episodes of MS in the subcutaneous tissue, lymph nodes, liver, and lumbosacral epidural space over 29 months. However, the recurrences in several episodes were still misdiagnosed for diseases other than MS (28).

The most frequently involved organs and/or tissues are soft tissue, periosteum and bone, lymph nodes, and skin. In female patients, ovaries and the breast are frequently involved (45). One patient had MS in the vagina, both breasts, and ovaries 6 months after the development of AML (46). In pediatric patients, orbital MS is the most frequent finding (35,47). However, many organ involvements were only discovered at autopsy. In autopsied cases, practically all major organs, except for the spleen, have been affected (3).

The age of MS has a wide range, varying from 1 week to 75 years. It has been seen mostly in the middle-aged male population, and less frequently in patients younger than 15 years. The mean age in several reported series is very close: 43 years reported by Eshghabadi et al. (48), 48 years by Neiman et al. (4), and 44 years by Friedman et al. (49).

The importance of making an accurate diagnosis of MS is due to its therapeutic implication. Patients who receive antileukemic therapy with or without local radiation therapy usually have a long remission, whereas other treatments, such as surgical, radiation, and antilymphoma therapy, usually show no effects. Patients who were treated with antileukemic therapy within 4 months from the initial diagnosis of MS achieved complete remission of both MS and leukemia (6). In contrast, patients initially treated for lymphoma usually failed to respond to the subsequent correct treatment and had a dismal prognosis (17). Therefore, the current opinion advocates treating MS as AML, even in the absence of leukemic manifestations (6,17,34,48,49).

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CASE 14 Precursor B-Lymphoblastic Leukemia/Lymphoma

CASE HISTORY

A 20-year-old man was found to have leukocytosis during a preoperative evaluation before a knee operation. Further hematologic workup showed that his total leukocyte count was 55,000/μL with 54% lymphocytes, 33% blasts, 11% neutrophils, and 2% monocytes. The hematocrit was 45% and platelet count 331,000/μL. The only clinical symptom that the patient had at that time was a persistent sore throat.

The patient was initially refractory to chemotherapy, but he was finally in remission after high dose chemotherapy. However, his leukocyte count gradually dropped to 100/μL. His hematocrit dropped to 23.6% and platelets to 30,000/μL. He developed a neutropenic fever with a temperature of 101.2°F. His infection was finally under control, and he received a bone marrow transplant from a matched, unrelated donor.

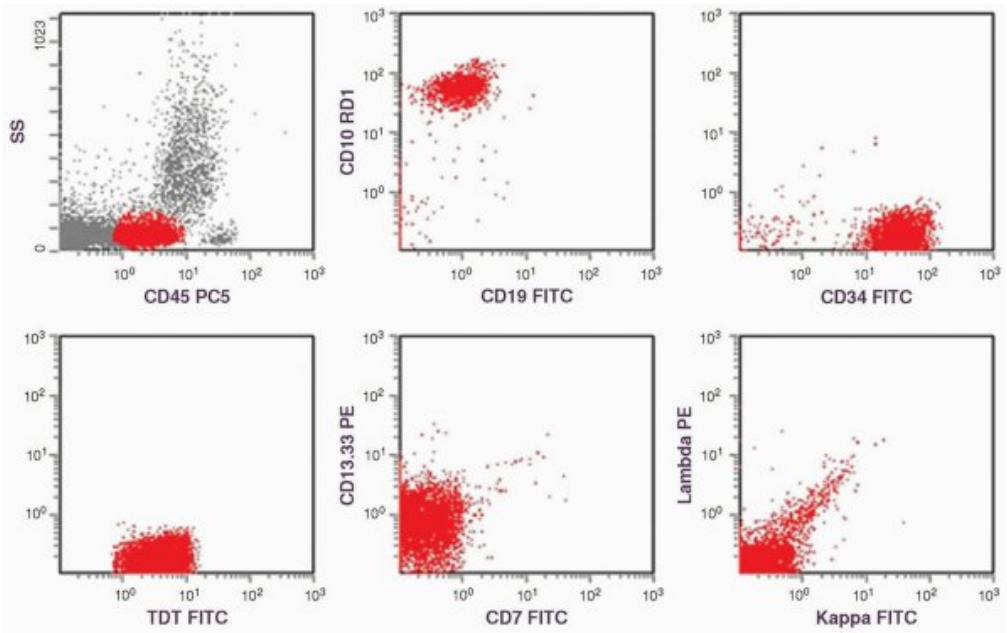

FIGURE 6.14.1 Flow cytometric histograms show dual staining of CD10 and CD19, positive CD34 and terminal deoxynucleotidyl transferase (TdT), partial positive CD13.33, but negative CD7, kappa, and lambda. SS, side scatter; PC5, phycoerythrin-cyanin 5; RD1, rhodamine; FITC, fluorescein isothiocyanate; TDT, terminal deoxynucleotidyl transferase; PE, phycoerythrin.

The patient was doing well for 10 months without any clinical symptoms, but his routine check-up showed multiple blasts at the end of the 10th month. He was again treated with chemotherapy, which achieved a complete remission. However, he developed pulmonary aspergillosis with subsequent spreading to the brain. He had several episodes of left-sided seizures with residual left lower extremity weakness. Despite multiple antibiotic treatments, the patient continued to have spiking fever, and became lethargic and confused. The patient finally died 17 months after the initial diagnosis of acute lymphoblastic leukemia (ALL).

FLOW CYTOMETRIC FINDINGS

Bone marrow aspiration: CD7 0%, CD10 94%, CD10/CD19 84%, CD14 1%, CD13-CD33 53%, CD34 97%, κ 0%, λ 0%, 0%, terminal deoxynucleotidyl transferase (TdT) 85% (Fig. 6.14.1).

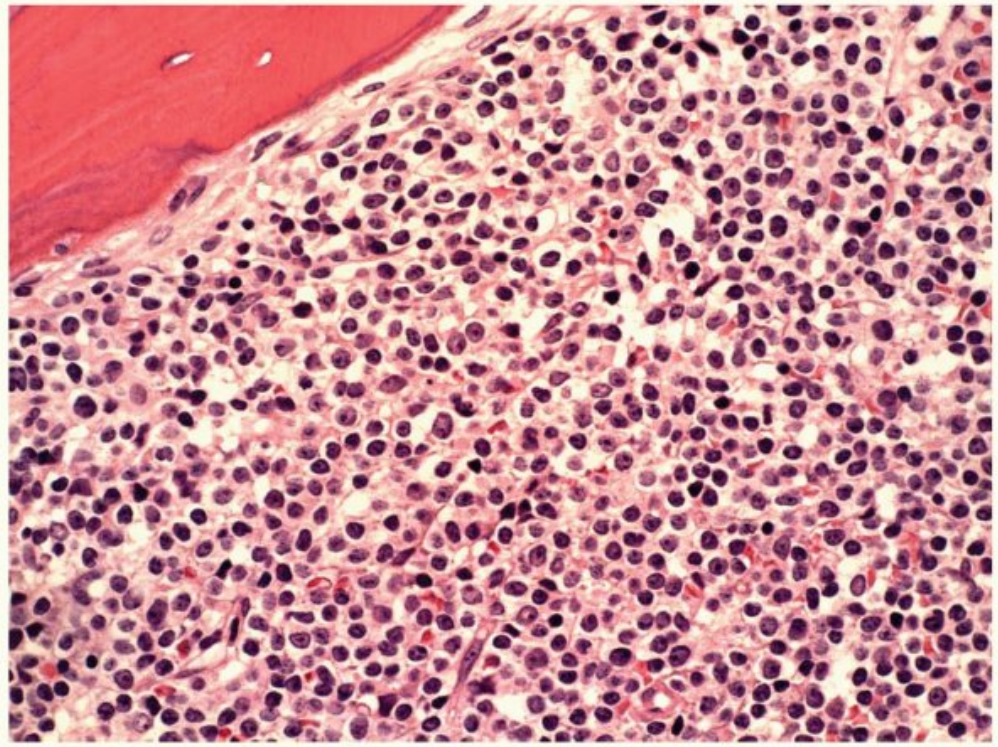

FIGURE 6.14.2 Bone marrow biopsy of a case of B-cell acute lymphoblastic leukemia (ALL) shows diffuse blast infiltration. Hematoxylin and eosin, 40× magnification.

CYTOCHEMICAL STAINS

The blasts were negative for myeloperoxidase (MPO), α-naphthyl butyrate esterase, and chloroacetate esterase but were positive for periodic acid-Schiff (PAS).

DISCUSSION

Precursor B-lymphoblastic leukemia/lymphoma includes ALL and lymphoblastic lymphoma (LBL) of B-cell origin. ALL is a leukemia with proliferation of lymphoblasts involving both the bone marrow (Fig. 6.14.2) and the peripheral blood. The cutoff point for clinical diagnosis of ALL is 25% blasts in the bone marrow (1). A case is diagnosed as ALL when ≥25% of lymphoblasts are present in the marrow. If bone marrow shows <25% of lymphoblasts in a case with lymph node or other soft tissue involvement, it is designated LBL with bone marrow involvement. There is a need to establish an arbitrary threshold to separate ALL from LBL because a leukemic phase may be present in LBL. In contrast, lymphoblasts may be absent in the peripheral blood in occasional ALL cases (aleukemic leukemia). In fact, about one third of ALL patients have a total white cell count of <5,000/μL.

TABLE 6.14.1

FAB Classification for Acute Lymphoblastic Leukemia

	L1	*L2*	*L3*
Size of blasts	Small, uniform	Large, variable	Medium to large, uniform
Amount of cytoplasm	Scanty	Variable	Moderate

Cytoplasmic basophilia	Moderate	Variable	Intense
Cytoplasmic vacuoles	Variable	Variable	Prominent
Nucleus	Regular, occasional clefting, homogenous chromatin	Irregular, clefting common, heterogeneous chromatin	Regular, nonclefted, homogeneous, finely stippled chromatin
Nucleolus	0-1, inconspicuous	≥1, prominent	2-5, prominent
N/C ratio	High	Low	Low

FAB, French-American-British; N/C ratio, nuclear/cytoplasmic ratio.

Morphology and Cytochemistry

The morphology of leukemic lymphoblasts varies between adults and children. The most popular morphologic classification is the French-American-British (FAB) system, which divides ALL into L1, L2, and L3 (Table 6.14.1) (2,3). The leukemic cells in L1 are uniformly small with scanty cytoplasm (Fig. 6.14.3). Their nuclei are regular in shape, with inconspicuous nucleoli. This form is usually seen in pediatric cases. The neoplastic cells in L2 are generally large, but their size is variable, as is the cytoplasm (Fig. 6.14.4). Their nuclei also vary in shape, with prominent nucleoli. This form is more frequently seen in adults than in children. The cells in L3 are uniformly large, with moderate amounts of deep basophilic cytoplasm, which contains many vacuoles. The nuclei are round and regular with prominent nucleoli. This form is rare in comparison with L1 and L2 and is more frequently seen in adults. It can also be the leukemic form of Burkitt lymphoma.

However, not all cases of ALL are easily assigned to the L1 and L2 subgroups, so reproducibility among observers is not high (4). In addition, immunophenotypes and cytogenetic abnormalities play an important role in predicting the prognosis, but these parameters do not correlate well with the L1 and L2 classification. Therefore, L1 and L2 have been combined by the World Health Organization (WHO) into one group: ALL L1/L2 (5). ALL must be distinguished from acute myeloid leukemia (AML). Their distinction is based on morphology, cytochemistry, immunophenotyping, and genotyping (Table 6.14.2).

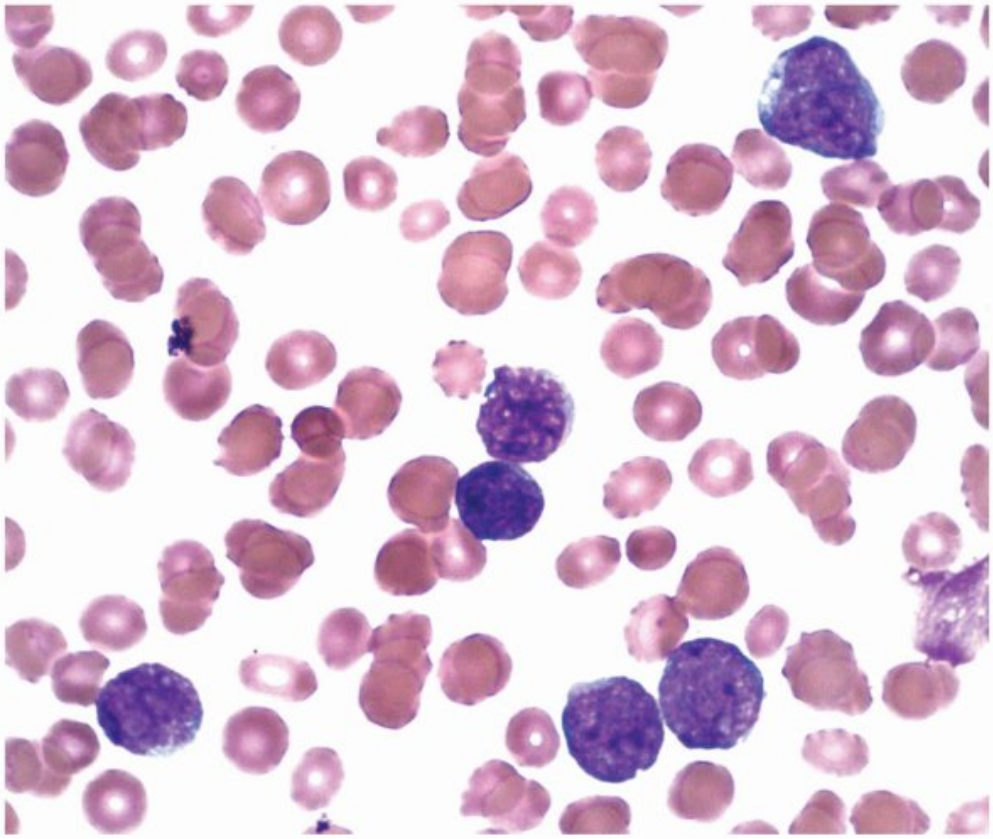

FIGURE 6.14.3 Peripheral blood smear of acute lymphoblastic leukemia (ALL) case shows small, uniform blasts with scant cytoplasm and inconspicuous nucleoli (L1 morphology). Wright-Giemsa, 100× magnification.

Cytochemically, lymphoblasts are only positive for PAS (6). The typical staining pattern for lymphoblasts is called the block pattern, but this pattern is not always present. In fact, some ALL cases can be PAS negative. In contrast, cases of AML may occasionally show a positive reaction to PAS. Therefore, the PAS reaction is not specific. In contrast, MPO, chloroacetate esterase, and α-naphthyl butyrate esterase are relatively specific for AML, so negative reactions to these cytochemical stains are helpful in excluding AML and are thus useful in establishing the diagnosis of ALL.

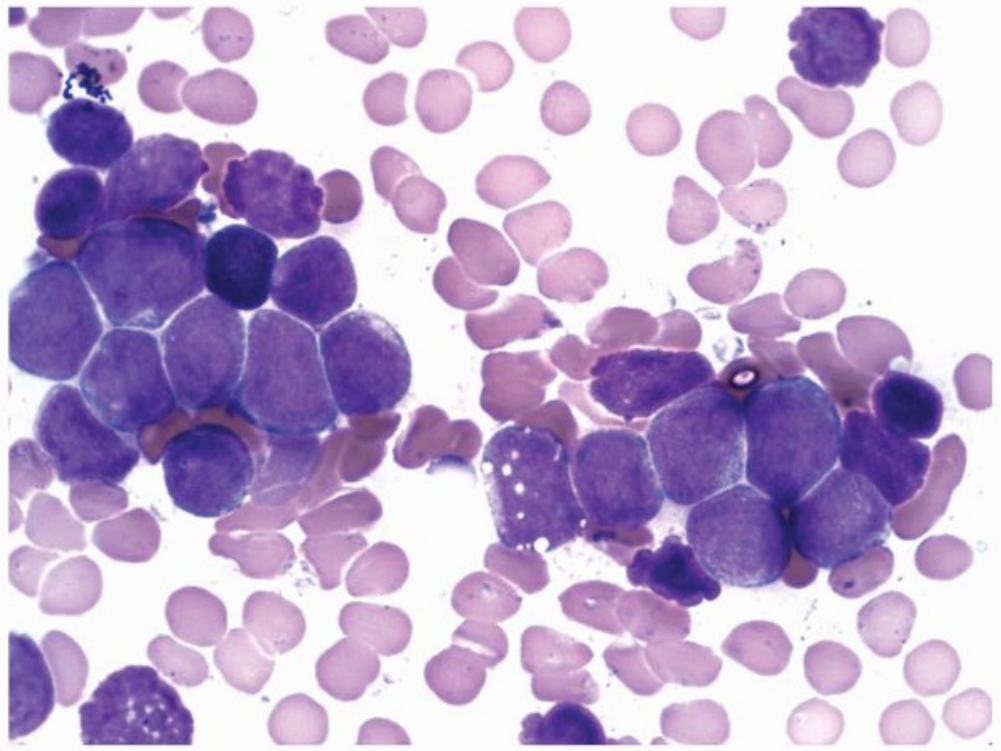

FIGURE 6.14.4 Bone marrow aspirate of acute lymphoblastic leukemia (ALL) case shows large blasts with variable sizes, scant cytoplasm, and inconspicuous nuclei (L2 morphology). Wright-Giemsa, 100× magnification.

Another entity that should be distinguished is the hematogone. Hematogones are normal B-cell precursors that can be demonstrated in pediatric bone marrow or in bone

marrow regenerating after chemotherapy or transplantation. It is, therefore, most important to differentiate hematogones from lymphoblasts, particularly in pediatric ALL cases after chemotherapy. Hematogones are small to medium-sized with a high nuclear cytoplasmic ratio, and can mimic small mature lymphocytes or L1 lymphoblasts. Their major morphologic differences from lymphoblasts are the homogeneous nuclear chromatin pattern and the absence of nucleoli (5). In some cases, however, indistinct nucleoli can be present. A low percentage (0.01% to 1.3%) of hematogones has been detected in the peripheral blood of patients without ALL (7).

TABLE 6.14.2

Differentiating Features between Acute Lymphoblastic and Acute Myeloblastic Leukemias

	Lymphoblastic	*Myeloblastic*
Size of blasts	Variable, depending on subtype	Usually large and uniform
Cytoplasm	Scant	Moderate amount
Cytoplasmic granules	Absent	Frequently present
Auer rods	Absent	Seen in about 1/5 of cases

Nuclear chromatin	Coarse to fine	Delicate and dispersed
Nucleoli	0-2, less prominent	1-4, often prominent
Myelodysplastic changes	Absent	May be present
Myeloperoxidase/Sudan black	Negative	Often positive
Chloroacetate esterase	Negative	Positive in myeloid leukemia
Nonspecific esterase	Negative	Positive in monocytoid leukemia
Periodic acid-Schiff	Often positive	Positive in about 10%-15% of cases
TdT	Frequently positive	Positive in occasional cases
Common ALL antigen (CD10)	Frequently positive	Negative
Myeloid antigens	Negative	Positive
Gene rearrangement	Frequently positive	Occasionally positive

TdT, terminal deoxynucleotidyl transferase; CD, cluster of differentiation.

Immunophenotype

In the Foon and Todd (8) immunologic classification, antibodies against human leukocyte antigen-DR (HLA-DR), CD19, CD10, CD20, Cμ, and surface immunoglobulin M (IgM) are used to subdivide B-cell ALL into six subgroups. However, some of the subgroups may not be relevant in terms of prognosis and treatment. Therefore, the new immunologic classification includes only three subgroups: B-precursor ALL, pre-B ALL, and B-ALL (9). Some authors use the terms of early pre-B, pre-B, and B-ALL to define the same classification (10). Others omit the pre-B stage (1) or add a transitional pre-B subgroup in between pre-B and B-cell ALL (11).

These stages can be distinguished simply by using CD19, Cμ, and surface Ig. B-precursor ALL shows only CD19, pre-B ALL expresses CD19 and Cμ, whereas B-ALL bears CD19 and surface Ig. However, a recent study shows that surface Ig can be occasionally demonstrated in B-precursor and pre-B ALL (12). The malignant nature of the ALL cells is determined by TdT, CD10 (common ALL antigen), and CD34 (hematopoietic progenitor antigen). TdT is present in most cases of ALL except for B-ALL. CD10 is seen in most cases of B-cell ALL. CD34 is present in B-precursor ALL (but not in pre-B ALL) and in some cases of B-ALL.

Additional antibodies that can be helpful in classifying ALL include HLA-DR, CD20, CD22, and CD24 (Table 6.14.3). Cytoplasmic CD22 appears earlier than surface CD22 in the B-cell developmental stage and is consistently positive in B-ALL (13).

A relatively new marker, CD79a, has been routinely used to identify B cells in ALL cases at St. Jude Children's Research Hospital (14). In another study, CD79a was found in the cytoplasm of B cells in most cases of ALL of different categories, including early B cell, pre-B cell, and mature B cell, as well as in common ALL. CD79b is also present in the cytoplasm of B cells in different kinds of ALL, but it is a less sensitive marker than CD79a (15).

TABLE 6.14.3

Immunophenotypic Classification of B-Lineage Acute Lymphoblastic Leukemia

Antigens	*B-Precursor ALL*	*Pre-B ALL*	*B-ALL*
CD10	+	+	±
CD19	+	+	+
CD20	±	±	+
Cyto-CD22	+	+	+
CD22	-	-	+
CD24	+	+	+
CD34	+	-	-
Cyto-CD79a	+	+	+
HLA-DR	+	+	+
Cyto-μ	-	+	-
Surface Ig	-	-	+
TdT	+	±	-

ALL, acute lymphoblastic leukemia; Cyto-, cytoplasmic; Ig, immunoglobulin; TdT, terminal deoxynucleotidyl transferase; HLA-DR, human leukocyte antigen-DR; CD, cluster of differentiation.

TABLE 6.14.4

Correlation between Immunologic Classification, FAB Subgroups, and Cytogenetic Abnormalities

Immunologic Subgroup	*FAB Subgroup*	*Cytogenetic Abnormalities*	*Approximate Frequency*
B-precursor ALL	L1, L2	t(9;22), 11q23 rearr., t(12;21)	50%

Pre-B ALL	L1, L2	t(9;22), 11q23 rearr., t(1;19)	20%
B-ALL	L3	t(8;14), t(2:8), t(8;22)	4%

ALL, acute lymphoblastic leukemia; FAB, French-American-British; rearr., rearrangement.

Mixed lineage ALL cases are encountered occasionally. When CD2 and CD19 are demonstrated, those cases usually represent precursor-B ALL rather than T-cell ALL (T-ALL) because Ig rearrangements are demonstrated in most of these cases, whereas T-cell receptor (TCR) rearrangement is rarely observed (16). One or more myeloid markers can be demonstrated in as many as one fourth of children and one third of adults with ALL (10). However, a diagnosis of acute mixed lineage leukemia should be reserved for cases with definitive evidence of both myeloid and lymphoid characteristics by immunophenotyping and genotyping (15). This mixed lineage feature usually has no prognostic or therapeutic implications, but a few cases may require treatment directed toward both lineages (14).

The immunologic classification also correlates with the FAB subgroups and associates with certain cytogenetic abnormalities (Table 6.14.4) (9,10). B-precursor ALL

constitutes 50% of adult ALL and shows L1 or L2 morphology. It is associated with t(9;22), 11q23 rearrangement, and t(1;19). B-ALL is present in 4% of adult ALL and is the leukemic counterpart of Burkitt lymphoma. Therefore, it shows L3 morphology and is associated with t(8;14), t(2;8), or t(8;22). In B-ALL patients showing no L3 morphology, the cytogenetic changes may also be different, and the prognosis is worse than it is in patients with L3 morphology (9). Because many of these patients carry t(14;18), some authors have suggested that these may represent cases of follicular lymphoma progressing to a leukemic phase with blast transformation (17). However, recent studies show that many of these cases have t(4;11) translocation involving the AF4 and mixed lineage leukemia (MLL) genes (18). The immunophenotype of these cases is characterized by the absence of CD10 and coexpression of myeloid-associated markers, particularly CD15 (19).

One distinct function of immunophenotyping by flow cytometry is to distinguish regenerated lymphoblasts after chemotherapy of ALL versus leukemic lymphoblasts. Wells et al. (20) emphasized the assessment of the dot-plot projections (patterns) using pairs of monoclonal antibodies (CD2/CD19, CD20/CD10, CD22/CD34, HLA-DR/CD11b, CD33/CD13, and cytoplasmic TdT) combined with CD45 peridinin-chlorophyll-protein complex (perCP). In comparison with the pattern of normal lymphoblasts, they found the following aberrations in leukemic lymphoblasts: increased side scatter, increased forward scatter, decreased CD45 expression, overexpression of CD10, underexpression of CD10, absence of CD10, desynchronous CD22/CD34, decreased CD19 expression, myeloid antigen expression, and absence of CD34. Because the aberration differs in each case, such changes can be used to identify tumor cells in the bone marrow of a particular patient and is thus helpful in detecting minimal residual disease (MRD).

As mentioned before, hematogones should be distinguished from lymphoblasts, especially after chemotherapy. Hematogones can be divided into three maturation stages (21). Stage 1 hematogones express TdT, CD34, CD10, CD19, CD22, and CD38. In stage 2, TdT and CD34 are down-regulated and CD10 is partially down-regulated, but CD20 and surface Ig start to appear. Stage 3 hematogones show the same markers as in stage with strong expression of CD20 and surface Igs. The major distinction between hematogones and lymphoblasts is that the former always express a continuous and complete maturation spectrum and lack asynchronous or aberrant antigen expression, whereas neoplastic lymphoblasts often show aberrant immunophenotype (21).

Comparison between Flow Cytometry and Immunohistochemistry

A large panel of antibodies can be used in flow cytometric analysis. Flow cytometry is also able to distinguish cytoplasmic from surface staining (e.g., cytoplasmic CD22 and Cμ). Therefore, this technique is superior to immunohistochemistry for the diagnosis of ALL. However, hematogones can be easily recognized with immunohistochemical stain due to direct morphologic correlation.

In the current case, the negative cytochemical staining in MPO, α-naphthyl butyrate esterase, and chloroacetate esterase but positive PAS staining is not supportive of acute myelogenous leukemia. The positive reactions to TdT, CD10, CD34, and CD19 are consistent with ALL of B-cell lineage. The presence of CD13/CD33 markers can be seen in ALL cases and it does not mean biphenotypic leukemia (5). In terms of stage, the absence of Cμ excludes pre-B ALL, and the absence of surface Igs rules out B-ALL. Therefore, this case should be diagnosed as B-precursor ALL.

Molecular Genetics

Cytogenetics plays an important role in ALL, because it is the most powerful prognostic predictor that can be used to guide the therapeutic approach. On the basis of cytogenetic findings, childhood precursor-B ALL can be divided into three distinct subgroups (11). The low risk group includes ALL cases with hyperdiploidy (>50 chromosomes), t(12;21), and dic(9:12). The high risk group includes those cases with 11q23 translocations, t(9:22), and hypodiploidy (<46 chromosomes). The remaining cases, including those with t(1;19), are classified in the intermediate risk group. However, another study showed that the poor prognosis associated with pre-B ALL is attributable to its association with the translocation t(1;19) (13). The higher frequency of t(9;22) in adult ALL as compared with childhood ALL partially accounts for the generally poor outcome in adult cases (22). For the high risk group, the patient should be treated aggressively with early bone marrow transplantation. The low risk patients can be treated with less toxic drugs, such as

antimetabolites.

Numerical chromosome aberrations, either alone or in association with structural abnormalities, are present in about half of ALL cases. These changes can be divided into several ploidy groups, namely, low hyperdiploidy (47 to 50 chromosomes), high hyperdiploidy (>50 chromosomes or DNA index >1.15), hypodiploidy, pseudodiploidy (46 chromosomes with structural abnormalities), and gain or loss of a single chromosome (23).

There are more than 30 structural abnormalities, including translocation, deletion, inversion, isochromosome, and dicentric chromosome (dic), known to be present in ALL; the more important ones are listed in Table 6.14.5 (23, 24 and 25). The Groupe Francais de Cytogénétique Hématologique found structural abnormalities in 78% of ALL cases studied (22). In recent years, most genes involved in translocations have been characterized by molecular biology (24,25). Because molecular biology techniques are usually more sensitive than karyotyping, they have become very important tools for diagnosis and prognostic prediction in cases of ALL. The Southern blotting technique has been gradually replaced by polymerase chain reaction (PCR) and reverse transcriptase (RT)-PCR techniques. In addition, the fluorescence in situ hybridization technique is able not only to detect numerical chromosome aberrations but also translocations. Proto-oncogenes are usually involved in chromosomal translocations. As a result, either the proto-oncogene is activated or a fusion transcript/chimeric protein is formed to induce

tumorigenesis (24,25). The loss of tumor suppressor genes is another mechanism in the pathogenesis of ALL.

TABLE 6.14.5

Important Chromosomal Abnormalities and Genes Involved in Acute Lymphoblastic Leukemia

Abnormality	*Genes Involved*	*Approximate Incidence*
t(9;22)(q34;q11)	BCR, ABL	Adults: 30%; children: 3%
t(8;14)(q24;q32)	c-MYC, IgH	1%
t(2:8)(p12;q24)	c-MYC, IgK	<1%
t(8;22)(q24;q11)	c-MYC, IgL	<1%
t(1;19)(q23;p13)	E2A, PBX1	5%
t(17;19)(q22;p13)	E2A, HLF	<1%
t(5;14)(q31;q32)	IL3, IgH	<1%
t(1;11)(p32;q23)	MLL, AF1P	<1%
t(4;11)(q21;q23)	MLL, AF4	Infants: 60%; adults: 5%
t(9;11)(p22;q23)	MLL, AF9	<1%
t(12;21)(p13;q22)	TEL, AML1	Adults: <1%; children: 20%

BCR, breakpoint cluster region; ABL, Ableson; c-MYC, an oncogene derived from avian myelocytomatosis virus; Ig, immunoglobulin; HLF, hepatic leukemia factor; MLL, mixed lineage leukemia; TEL, translocation-Ets-leukemia oncogene; AML1, acute myeloid

leukemia 1.

When an immunophenotype is not conclusive, Ig gene or TCR gene rearrangement should be considered (26). Most B-cell ALL cases show Ig gene rearrangement. However, most B-precursor ALL cases reveal TCR δ chain gene rearrangement (27). Although Ig or TCR gene rearrangements are present in virtually all ALL cases, cross-lineage gene rearrangements occur in >90% of precursor B-ALL and in about 20% of T-ALL, so that a conclusive result may not be obtainable (28). The selection of methods is also important. Because combinatorial diversity is relatively restricted for TCR γ and TCR α rearrangements, Southern blotting is the method of choice for their detection (24). In contrast, IgH, TCR ß, and TCR δ show considerable junctional diversity, and PCR is preferred.

Recent studies show that gene expression profiling has a great potential in stratifying ALL cases, predicting prognosis, and guiding treatment selection (29,30). Gene profiling has also identified unique leukemia-associated markers, which can be monitored by flow cytometry for the detection of MRD (29). For instance, ALL cases with MLL rearrangements are associated with CD10- CD24- CD15+ (30). ALL with t(1;19) is characterized by CD10+ CD34- CD20- Cμ+ (5). B-ALL with t(12;21) shows CD10+ HLA-DR+ CD9- CD20- (5).

The salient features for laboratory diagnosis of B-ALL are summarized in Table 6.14.6.

Clinical Manifestations

ALL is mainly a pediatric neoplasm with an early incidence peak at 2 to 5 years of age that represents about 80% of the childhood leukemia in the United States (23). The incidence in the pediatric group is approximately 30 cases per 1 million children younger than 15 years. However, ALL has a bimodal distribution, with a second peak around age 50 years, and a steady rise in incidence thereafter. The incidence of ALL in adults is about one third that in children. In the United States, ALL is more frequently seen in whites than in blacks (1.8:1) and in boys than in girls (1.2:1) (11).

The clinical symptoms of ALL are due to suppression of hematopoiesis in the bone marrow and, occasionally, extramedullary leukemic infiltration. The most common symptom is anemia, which manifests as pallor, weakness, and excessive tiredness. Hemorrhages, such as petechiae, ecchymoses, and epistaxis, occur in about two thirds of patients. Neutropenia, which may lead to a predisposition to bacterial infections, is less commonly seen. Lymphadenopathy and splenomegaly are seen in three fourths of patients, and hepatomegaly in one half of patients. In ALL cases, central nervous system, testicular, renal, and bone and joint involvement are the most common (3). In B-LBL cases, the skin,

bone, and lymph nodes are most frequently involved (5). However, any organ system can be affected (Fig. 6.14.5).

TABLE 6.14.6

Salient Features for Laboratory Diagnosis of B-Cell ALL

1. TdT positive for precursor-B and pre-B ALL
2. CD10 positive in all subgroups, except for some B-ALL cases
3. HLA-DR positive in all subgroups
4. CD19 frequently present without CD20
5. Cμ positive in pre-B ALL only
6. Monoclonal surface immunoglobulin in B-ALL only
7. Immunoglobulin gene or T-cell receptor gene rearrangements

ALL, acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase; HLA-DR,

human leukocyte antigen-DR; CD, cluster of differentiation.

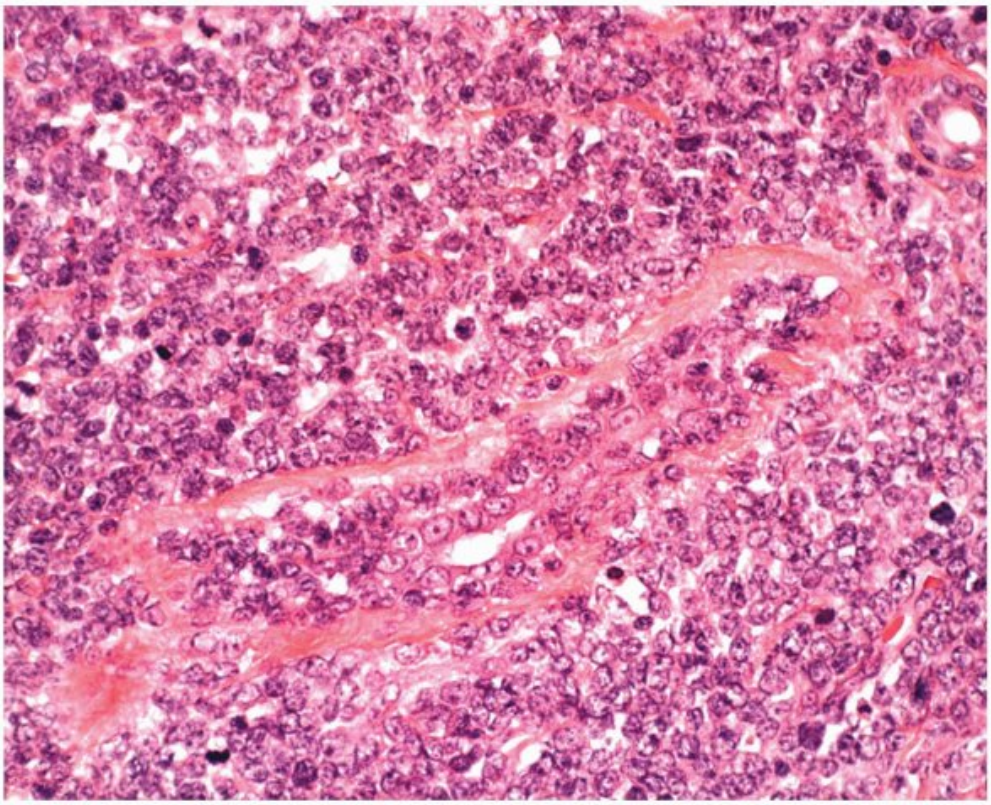

FIGURE 6.14.5 Breast biopsy of acute lymphoblastic leukemia (ALL) case reveals ductal and periductal leukemic infiltration. Wright-Giemsa, 40× magnification.

The current cure rate is about 80% in children but only 30% to 40% in adults (11,31,32). This discrepancy is partly due to the higher frequency of adverse genetic aberrations (e.g., breakpoint cluster region/Ableson[BCR/ABL] fusion gene) and partly due to the usually higher leukocyte count or other factors present in the adult ALL population. In contrast, children aged 1 to 9 years usually have hyperdiploidy and favorable genetic changes (e.g., translocation-Etsleukemia/acute myeloid leukemia [TEL/AML1] fusion gene). The prognosis of infants <12 months old is generally poor. This may be related to both clinical and biological factors, such as high leukocyte counts at diagnosis, irregular or immature phenotypes, and unfavorable molecular and cytogenetic abnormalities (e.g., MLL rearrangement) (33).

The follow-up examination of bone marrow after chemotherapy for the detection of MRD has been advocated in recent years and it has proven to be a powerful tool for the prediction of prognosis (34). There are several sensitive techniques for the detection of MRD, including flow cytometry, PCR, RT-PCR, and fluorescence in situ hybridization (20,25,35). The detection of MRD usually predicts relapse of ALL. However, some recent studies found that PCR analysis may be too sensitive, and long-term remission may be sustained in the presence of MRD detected by PCR (36). Therefore, a threshold of residual disease level should be determined or several techniques should be used to detect MRD at different time points after treatment. One study found that flow cytometric analysis at week 14 postchemotherapy was the most predictive (37).

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CASE 15 Precursor T-Lymphoblastic Leukemia/Lymphoma

CASE HISTORY

A 10-year-old boy was admitted to the hospital because of intermittent cough, dyspnea, progressive wheezing, and orthopnea for approximately 1 month. He was treated for asthma to no avail. Chest x-ray examination revealed a large anterior mediastinal mass with tracheal deviation. Pericardial effusion was also detected. Physical examination found cervical, supraclavicular, and axillary lymphadenopathy. However, the liver and spleen were not palpable. Hematologic workup revealed a total leukocyte count of 512,000/μL with 15% lymphocytes, 12% neutrophils, and 72% blasts. His hematocrit was 35% and platelet count 95,000/μL. The blood chemistry profile was unremarkable except for an extremely high level of lactate dehydrogenase (960 U/L).

After admission, the patient was immediately treated with combined chemotherapy and radiation therapy. However, the size of the mediastinal mass and peripheral lymphadenopathy remained unchanged after treatment. He became increasingly hypoxic and bradycardic and died 5 days after admission.

At autopsy, a large mediastinal mass was found that encased the roots of the aorta, pulmonary artery, and superior vena cava. The tumor also compressed the trachea and invaded the epicardium. The pericardial fluid contained a large number of blasts.

FLOW CYTOMETRIC FINDINGS

The peripheral blood showed 0% CD2, 1% surface CD3, 54% cytoplasmic CD3, 5% CD3/CD4, 10% CD3/CD8, 6% CD5, 98% CD7, 2% CD10, 1% CD19, 3% CD25, 97% CD34, and 85% terminal deoxynucleotidyl transferase (TdT) (Fig. 6.15.1).

DISCUSSION

Precursor T-lymphoblastic leukemia/lymphoma was previously divided into T-lymphoblastic lymphoma (LBL) and Tacute lymphoblastic leukemia (ALL) in the old classifications. However, this new designation has been adopted by both the Revised European-American Lymphoma (REAL) classification (1) and the World Health Organization (WHO) classification (2), because T-LBL and T-ALL are morphologically identical and clinically similar.

LBL was originally called Sternberg sarcoma and was first described by Smith et al. (3) as a T-cell lymphoma derived from thymic lymphocytes. Barcos and Lukes (4) further defined its morphologic and clinical characteristics and considered it a distinct clinical immunopathologic entity. LBL was also called convoluted T-cell lymphoma because its nuclei are convoluted in most cases.

In the Working Formulation of non-Hodgkin Lymphoma, LBL was divided into the convoluted and nonconvoluted subtypes (5).

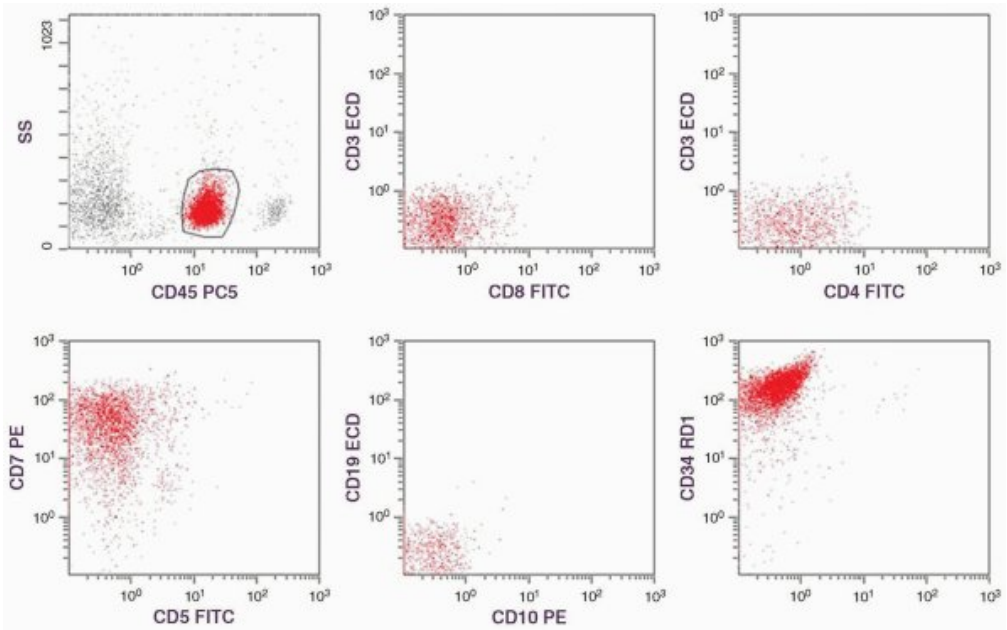

FIGURE 6.15.1 Flow cytometric histograms show that the tumor cells react only to cluster of differentiation (CD)7 and CD34 with low percentages of CD3/CD4 and CD3/CD8. CD5, CD10, and CD19 are negative. SS, side scatter; PC5, phycoerythrin cyanin 5; ECD, phycoerythrin-Texos Red; PE, phycoerythrin; RD1, rhodamine; FITC, fluoresein isothiocyanate.

In children, B-ALL is composed of approximately 85% of ALL cases, whereas T-ALL is identified in about 15% of ALL patients (6,7).

Morphology

The typical morphologic feature of LBL is the presence of a “starry sky” histologic pattern due to the presence of numerous tangible-body macrophages as a result of accelerated apoptosis (Fig. 6.15.2). Mitosis is also prominent (Table 6.15.1). This histologic pattern is indistinguishable from that of Burkitt and Burkitt-like lymphoma. However, these two entities can be differentiated by their cytology (Table 6.15.2). Cells from LBL are intermediate in size with scanty cytoplasm, which shows no vacuolation in imprints. Their nuclei are usually convoluted, containing dusky chromatin and inconspicuous nucleoli. However, LBL may also manifest as a nonconvoluted form or an atypical pleomorphic form (8). In touch preparations, LBL reveals an L1/L2 morphology (9). Cells from Burkitt and Burkitt-like lymphoma

are medium-sized with abundant pyroninophilic cytoplasm, which is deeply basophilic and often vacuolated in imprint preparations. Their nuclei are round or ovoid, containing clumped chromatin and multiple nucleoli in tissue sections (10). Touch preparations of a lymph node may show the L3 morphology with more immature-looking chromatin than in tissue sections. In case of doubt, immunophenotyping is helpful; LBL is predominantly of T-cell origin, but Burkitt and Burkitt-like lymphoma are exclusively of B-cell

type.

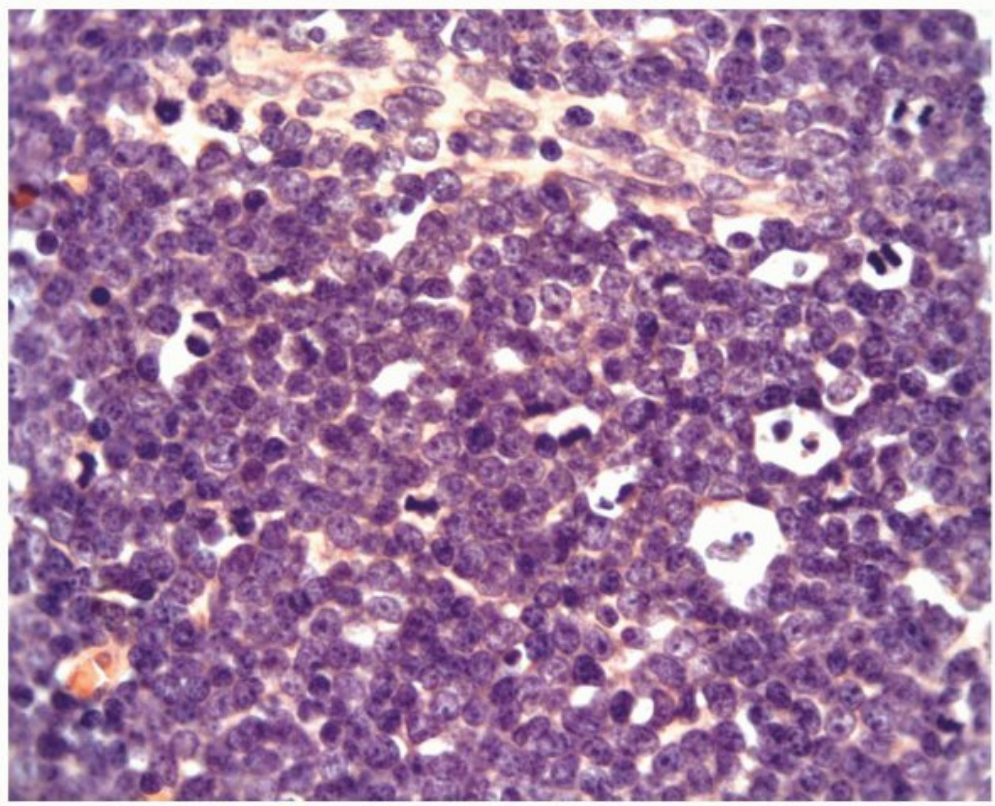

FIGURE 6.15.2 Lymph node biopsy shows a “starry sky” histologic pattern with tangible-body macrophages and mitotic figures in the vacuoles. Hematoxylin and eosin, 60× magnification.

TABLE 6.15.1

Characteristic Morphologic Features of Lymphoblastic Lymphoma

Histologic pattern	Diffuse lymphoid infiltration on a “starry sky” background
Cytology	Small to intermediate size, scanty cytoplasm, convoluted nuclei, dusky chromatin, and inconspicuous nucleoli
Specific features	“Starry sky” pattern and convoluted nuclei with a high mitotic rate

LBL and T-ALL are considered the tissue and leukemic phases of the same disease. Their distinction is rather arbitrary, depending on the distribution of the tumor cells: LBL is mainly in the soft tissue, but ALL is predominantly in the blood and bone marrow (11). However, LBL may have a leukemic phase with bone marrow involvement. On the other hand, ALL may also involve lymph nodes, particularly the mediastinal lymph node. The arbitrary cutoff point for the distinction of these two entities is 25% of lymphoblasts in the bone marrow, above which is designated ALL; otherwise, it is considered LBL with bone marrow involvement (12). Immunophenotyping does not help in their distinction, because their phenotypes are essentially identical. The lymphoblasts in T-ALL show either L1 or L2 morphology (Fig. 6.15.3), whereas L3 is always of B-cell lineage.

TABLE 6.15.2

Comparison of Lymphoblastic Lymphoma and Burkitt Lymphoma

	Lymphoblastic Lymphoma	*Burkitt Lymphoma*
High incidence group	Children	Children
Clinical presentation	Mediastinal mass	Jaw or abdominal lesion
Mitotic rate	High	High
“Starry sky” pattern	Less prominent	More prominent
Cell size	Small to intermediate	Intermediate to large
Cytoplasm	Scanty, pale blue, no vacuoles	Abundant, dark blue, vacuolated
Nuclear shape	Usually convoluted	Round to ovoid
Chromatin pattern	Finely speckled	Clumped
Nucleoli	Inconspicuous	Multiple, distinct
FAB type	L1/L2	L3
Phenotype	Predominantly T cell	Exclusively B cell
Cytogenetic aberration	Not specific	t(8;14), t(8;22), or t(2;8)
EBV related	No	Yes

EBV, Epstein-Barr virus; FAB, French-American-British.

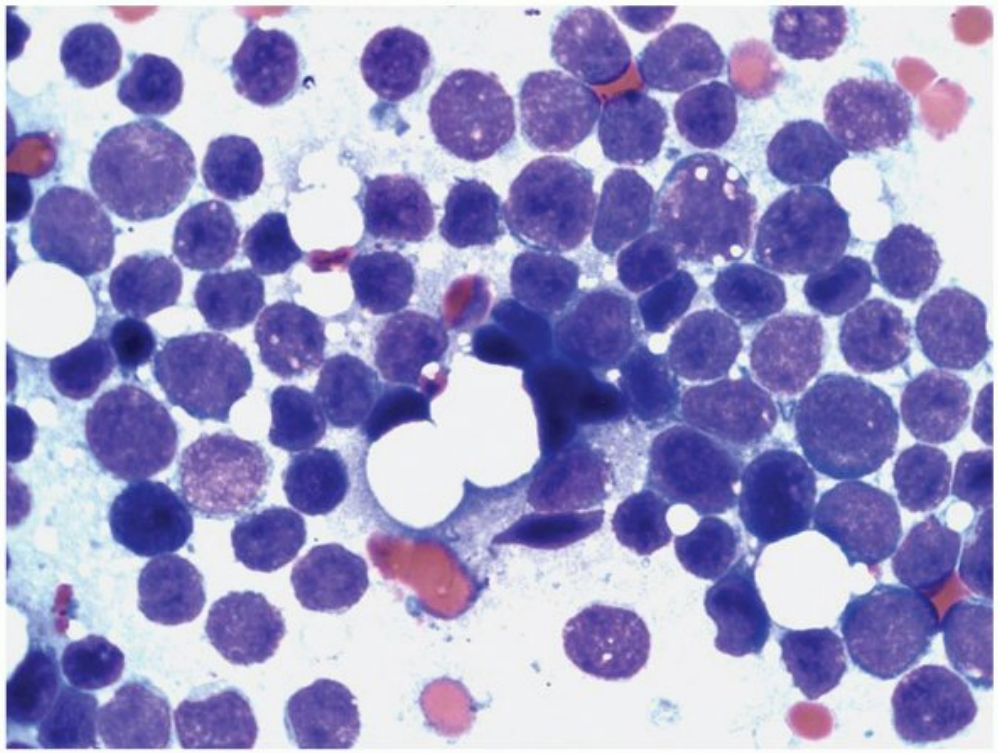

FIGURE 6.15.3 Bone marrow aspirate shows many lymphoblasts with different sizes, immature chromatin pattern, and inconspicuous nucleoli. A few lymphoblasts reveal vacuolated cytoplasm. Wright-Giemsa, 100× magnification.

Cytochemically, the lymphoblasts are periodic acid-Schiff positive, but are negative for myeloperoxidase, as well as for specific and nonspecific esterases. T lymphoblasts may also show focal acid phosphatase staining (2).

TABLE 6.15.3

Immunologic Classification of T-Cell ALL

	CD1	*CD2*	*cCD3*	*sCD3*	*CD4*	*CD5*	*CD7*	*CD8*	*TdT*
Pre-T	-	-	+	-	-	-	+	-	+
Early cortical	-	+ (75%)	+	-	-	+ (90%)	+	-	+
Late cortical	+	+	+	+ (25%)	+ (90%)	+	+	+ (90%)	+
Medullary	-	+	+	+	±	+	+	±	±

CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase; c, cytoplasmic;

s, surface.

Immunophenotype

The traditional immunologic classification divides T-cell ALL into four immunophenotypes (Table 6.15.3) (7). The pre-T-cell phenotype expresses only CD7, cytoplasmic CD3, and TdT without other T-cell antigens. The early cortical phenotype shows CD2, CD5, CD7, and strong TdT. The late cortical phenotype reveals CD1, CD2, CD5, CD7, and dual CD4/CD8 with minimal surface CD3. The medullary phenotype shows CD2, CD3, CD5, CD7, and segregated CD4 or CD8. TdT is not commonly expressed in this phenotype. Cytoplasmic CD3 is expressed in all stages (13). The late cortical phenotype is most commonly encountered, followed by the early cortical phenotype. However, ALL immunophenotypes frequently do not correlate with recognized stages of normal lymphocyte maturation and may not conform to a maturation arrest model. In fact, the common diagnostic feature for T-cell neoplasms is either loss or aberrant expression of T-cell antigens (7,14). The U.S.- Canadian Consensus Recommendation Group indicated that the coexpression of cytoplasmic CD3 and TdT/CD34 alone is diagnostic for T-ALL (15). The phenotypic features of adult T-ALL are similar to those of childhood T-ALL, but human leukocyte antigen-DR (HLA-DR) and CD10 are more frequently positive in adults than in children (13).

On the basis of therapeutic response and prognosis, most authors consider it unnecessary to divide T-ALL into multiple subtypes. The general consensus is either to divide it into pre-T-cell and T-cell ALL or not to divide T-ALL into any subtypes (9,16,17).

However, individual markers may be used to predict the prognosis. Two study groups found that T-ALL cases that expressed CD10 had better prognosis than those without CD10 expression in terms of remission rate and eventfree survival (18,19). CD3 positivity associated with an abnormal karyotype, in contrast, was reported to be a significant adverse risk factor (20). Another report showed statistically significant correlation between the CD2 antigen expression frequency and eventfree survival (6). The comparison of ALL cases with different maturation phenotypes showed no statistical significance in terms of therapeutic response and prognosis (21,22). The coexpression of myeloid markers in T-ALL cases was reported to show a worse prognosis than those without (23,24). This conclusion, however, was not confirmed by other studies (25).

Most cases of LBL are of thymic origin, with approximately one half of the T-cell cases corresponding to common thymocytes and one fourth each to early thymocytes and late thymocytes (12,26). Immature B cell (pre-pre-B cell and pre-B cell), mature B cell, and natural killer (NK) cell types have also been reported (8,11,12,26, 27, 28, 29, 30 and 31). Sheibani et al. (27) divided LBL into five groups: LBL with T-cell phenotype (T-LBL), T-LBL with expression of common ALL antigen (CALLA), T-LBL with expression of NK cell-associated antigens, LBL with pre-B cell phenotype and B-LBL (Table 6.15.4). These immunophenotypes show some clinical correlations, such as the absence of mediastinal mass in the pre-B-cell and B-cell phenotypes and the aggressive clinical course seen in NK-associated antigen phenotypes (27,29,30). However, the NK-associated antigen phenotype is considered to be blastic NK-cell lymphoma/leukemia by other studies (32). Skin involvement is more frequently seen in the CALLA-positive phenotype (28,33), and skin and lytic bone lesions occur more often in the immature B-cell phenotype (8,11,28).

Among all markers, TdT is most useful because it is present in almost all cases of LBL (except for mature B-cell type) and is seldom, if ever, seen in other lymphomas (Fig. 6.15.4) (11,27). In the REAL classification, TdT positivity

is only listed in precursor T- and precursor B-LBL and/or leukemia (1). Therefore, a positive TdT reaction may exclude the diagnosis of Burkitt lymphoma. The reactivities of T-cell monoclonal antibodies depend on the stage of thymocytes to which the tumor cells are related (34). Generally, CD2 is consistently positive in all study series, and CD1 is specific for the common thymocyte stage (11,26,27). The reactivities of CD4 and CD8 are usually used as the criteria for stage identification: early thymocytes are CD4-, CD8-; common thymocytes are CD4+, CD8+; and late thymocytes are CD4+, CD8- or CD4-, CD8+.

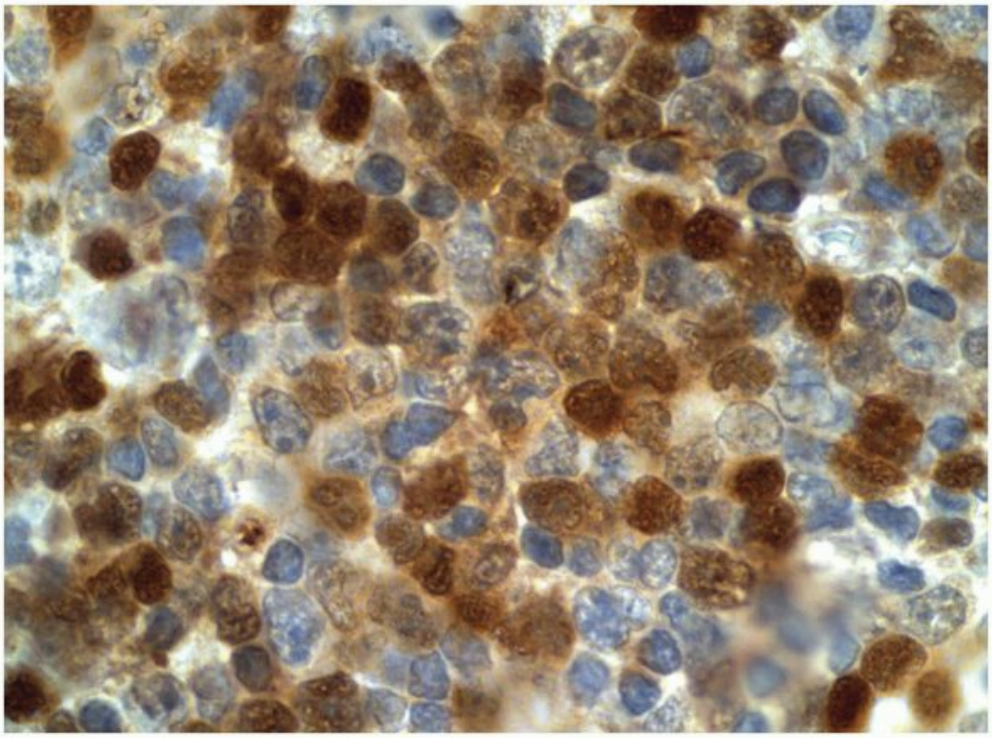

FIGURE 6.15.4 Lymph node biopsy of lymphoblastic lymphoma shows positive staining for terminal deoxynucleotidyl transferase. Immunoperoxidase, 100× magnification.

TABLE 6.15.4

Immunophenotypes of Lymphoblastic Lymphoma

	Immunophenotype	*Special Feature*
I.	T-cell lymphoblastic lymphoma	Mediastinal mass (86%)
1.	Early thymocytes TdT+ CD1- CD2+ CD3-* CD4- CD5+ CD7+ CD8-	
2.	Common thymocytes TdT+ CD1+, CD2+ CD3+ CD4+ CD5+ CD7+ CD8+	
3.	Late thymocytes TdT+ CD1 - CD2+ CD3+ CD4± CD5+ CD7- CD8±	
II.	T-cell lymphoblastic lymphoma with CALLA Thymocyte phenotype with positive CALLA (CD10)	Skin involvement
III.	T-cell lymphoblastic lymphoma with NK antigens**	Nonwhite female

	Thymocyte phenotype with positive CD16 and/or CD57	predominant, aggressive clinical course
IV.	Pre-B-cell lymphoblastic lymphoma TdT- CD10+ HLA-DR+ Cμ+ CD9+ CD24+ T-antigens	Bone or skin lesions No mediastinal mass
V.	B-cell lymphoblastic lymphoma TdT- HLA-DR+ SIg+ T-antigen	No mediastinal mass

CALLA, common acute lymphoblastic leukemia antigen; NK, natural killer; TdT, terminal deoxynucleotidyl transferase; HLA-DR, human leukocyte antigen- DR;.

* Cytoplasmic CD3 is present in all stages.

** Other studies include this entity into NK-cell lymphoma.

CD38, an antigen present in thymocytes and plasma cells, showed a high positive rate in cases of LBL from most studies; thus, it is a useful marker for LBL (11,12,26). CD71, the transferrin receptor antigen, was present in 6 of 15 cases and 3 of 11 cases of LBL, respectively, in two separate series (11,27). In two study series, all cases of LBL were negative for CD25, which represents the α subunit of the interleukin-2 receptor (26,27) but the ß subunit of inter-leukin-2 is widely expressed among T-LBLs (34).

When the thymic element in a thymoma is examined by flow cytometry, the phenotype may mimic LBL. However, in most circumstances, these two entities can be distinguished morphologically. Cytokeratin stain should be positive for thymoma and negative for LBL, although focally positive staining for keratin has been reported in a small group of LBL cases (35).

In rare conditions, pre-B-cell LBL may present as a solitary bone tumor mimicking Ewing sarcoma (35). This group of tumor may express antigens that are positive for Ewing sarcoma, including CD99 and vimentin. To make the matter even worse, B-LBL may be CD45 and CD20 negative, which may mislead to the exclusion of lymphoma. In those cases, however, CD79a and CD19 are positive; thus, these antibodies should be included for differential diagnosis.

For immunohistochemical staining, CD43 (MT-1) is very reliable marker for immature hematopoietic neoplasms; it is 100% positive in T-LBL, 80% in B-LBL, and almost 100% in granulocytic sarcomas (36). The reaction of CD45RO (UCHL-1) in LBL cases is controversial (37,38). CD3 is often present in T lymphoblasts (9), but most cases show localization to the cytoplasm (38). In some cases of LBL, CD3 was coexpressed with CD79a, a B-cell marker (39). However, all these cases showed T-cell receptor (TCR) gene rearrangements, indicating their T-cell lineage (40).

In the current case, the clinical history is typical for the patient's age and the presence of a mediastinal mass. The diagnosis, however, was established by flow cytometric analysis of the peripheral blood, which showed an immunophenotype of precursor-T-lymphoblastic leukemia/ lymphoma. Because of the presence of all the immature cell markers and because both CD4 and CD8 were negative, the tumor was at the stage of early thymocyte. The mediastinal tumor tissue obtained at autopsy showed a typical starry sky pattern with numerous mitotic figures, confirming the diagnosis of LBL. The compression of the large blood vessels and the trachea as well as the invasion of the pericardium finally led to the death of the patient.

Comparison of Flow Cytometry and Immunohistochemistry

For the examination of peripheral blood and bone marrow, flow cytometry is superior to immunohistochemistry because all the antibodies mentioned above are readily available for this technique. Therefore, flow cytometry not only makes the diagnosis but also pinpoints the maturation stage

of the tumor cells. However, for a mediastinal tumor, immunohistochemistry is much more reliable to distinguish between LBL and a thymoma. A TdT staining is most helpful, as LBL is the only lymphoma that expresses TdT. Cytokeratin stain, in contrast, is most helpful for the diagnosis of thymoma.

TABLE 6.15.5

Structural Changes of Chromosomes Associated with T-ALL

Abnormality	*Gene Involved*	*Approximate Frequency*
t(1;14)(p32;q11)	TAL1, TCRα/δ	1% to 3%
t(8;14)(q24;q11)	c-MYC, TCRα/δ	2%
t(10;14)(q24;q11)	HOX11, TCRα/δ	5% to 10%
t(11;14)(p15;q11)	LMO1, TCRα/δ	1%
t(11;14)(p13;q11)	LMO2, TCRα/δ	5% to 10%
inv(14)(q11q32)	TCL1, TCRα/δ	<1%
t(1;7)(p33;q35)	SCL, TCRβ	<1%
t(7;9)(q35;q34)	TAL2, TCRβ	<1%
t(7;19)(q35;p13)	LYL1, TCRβ	<1%
t(7;11)(q35;p13)	TTG2, TCRβ	<1%
t(11;19)(q23;p13)	MLL, ENL	<1%

ALL, acute lymphoblastic leukemia; TAL, tumor-associated lymphocyte gene; TCR, T-cell receptor; cMYC, an oncogene derived from avian myelocytomatosis virus; HOX, hemeobox gene; TCL, T-cell leukemia; LYL, lymphoblastic leukemia gene; MLL, mixed lineage leukemia.

Molecular Genetics

Although T-ALL and B-ALL share a few cytogenetic abnormalities, there are some characteristic changes that are specific to T-ALL. In terms of numerical changes, hyperdiploid karyotype is more frequently demonstrated in B-ALL, whereas pseudodiploid karyotype (normal chromosomal number with structural abnormality) is more often seen in T-ALL. Also, near-tetraploid (chromosomes >65) is more frequently seen in T-ALL than in B-ALL (6). Because hyperdiploidy is usually associated with favorable outcome, whereas pseudodiploidy and near-tetraploidy carry poor outcome, the prognosis of T-ALL is generally worse than that of B-ALL.

The structural changes in T-ALL are characterized by the frequent involvement of the TCRβ locus at 7q32-36 and the TCRαδ locus at 14q11 (6,41,42). The involvement of these two foci comprises approximately 20% of all T-ALL cases (6). The most common structural change is translocation, which leads to a dysregulation of the partner gene under the control of the enhancer of the TCR gene. In rare cases, inv(14)(q11q32) and a tumor-associated lymphocyte gene (TAL1) disruption may be present. This disruption occurs through an interstitial deletion between the SIL (SCL interrupting locus) and the 5′ untranslated region (UTR) of SCL, resulting in a fusion transcript SIL/SCL (6). TAL1 can also be dysregulated by a microscopic deletion of the short arm of chromosome 9, del(9p), resulting in loss of the tumor suppressor gene CDKN2A (2).

The important translocations involving the 14q11 are t(1;14), t(8;14), t(10;14), and t(11;14). Those involving the 7q32-36 include t(1;7), t(7;9), t(7;11), and t(7;19). The structural changes in T-ALL are summarized in Table 6.15.5. The NOTCH1 gene, which is involved in T-ALL with t(7;9) translocation, is of particular importance (43). NOTCH1 point mutations, insertions, and deletions producing aberrant increases in NOTCH1 signaling are frequently present in both childhood and adult T-ALL (43). It is hypothesized that aberrant NOTCH

signaling plays an important role in the pathogenesis of precursor T-lymphoblastic leukemia/lymphoma, and NOTCH inhibitors may prove to be effective for the treatment of this entity (43).

TCR γ-chain or δ-chain gene rearrangement can be detected in most cases of T-cell ALL, and the polymerase chain reaction has been used to monitor minimal residual disease in this leukemia with a certain degree of success (44,45). The salient features for laboratory diagnosis of T-ALL are listed in Table 6.15.6.

TABLE 6.15.6

Salient Features for Laboratory Diagnosis of T-ALL

1. Most cases are positive for TdT.
2. Pediatric cases are frequently negative for CD10 and HLA-DR.
3. CD7 is frequently the only positive T-cell marker, but all T-cell markers can be present.
4. Dual CD4/CD8 positivity is more common than other CD4/CD8 combinations.
5. T-cell receptor gene rearrangement is present in most cases.
6. Translocation frequently involves TCRß at 7q32-36 or TCRαδ at 14q11.

ALL, acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase; CD, cluster of differentiation; HLA-DR, human leukocyte antigen- DR; TCR, T-cell receptor.

TCR gene rearrangement can be demonstrated in most cases of LBL. This analysis is helpful to distinguish LBL from Burkitt lymphoma, which shows immunoglobulin heavy-chain gene rearrangement and thymoma, which reveals germline in genotyping. However, genotyping is not entirely lineage specific, because up to 30% of B-LBLs may show TCRγ gene rearrangements and 10% to 25% of T-LBLs show heavy-chain gene rearrangements (46).

Karyotypic studies in LBL are not as extensive as in T-ALL. In general, these two entities share the same chromosomal aberrations. In a study of 17 cases of T-LBL, 16 had karyotypic abnormalities, most commonly involving 14q11, 7q35, and 7p15, which are similar to those seen in T-ALL (47). Three cases showed a unique t(9;17)(q34;q23) translocation that has not been seen in ALL cases. A specific syndrome showing LBL, eosinophilia, and myeloid hyperplasia and/or malignancy has been reported in fewer than 10 cases, all of them associated with t(8;13)(p11;q11) translocation (48). Recently, a pediatric case with the same syndrome was reported without cytogenetic abnormality detected by conventional karyotyping and by molecular cytogenetic techniques (49). Pre-B LBL is frequently associated with t(1;19) and E2X/PBX (9,50). The t(9;22) translocation is commonly associated with precursor B-cell LBL (50).

The recent investigations of T-ALL/LBL with gene expression profiling (GEP) show a great potential of this technique. GEP can help to stratify patients into different subtypes in relation to their therapeutic responses and prognosis (51, 52 and 53). One study showed that GEP identified prognostically important leukemia subtypes, including T-ALL, E2A-PBX1, BCR-ABL, TEL-AML1, MLL rearrangement, and hyperdiploid >50 chromosomes (51). Another study found that GEP may help to distinguish peripheral and lymphoblastic T-cell lymphomas (52).

The salient features for laboratory diagnosis of LBL are summarized in Table 6.15.7.

Clinical Manifestations

LBL is most frequently seen in male adolescents and accounts for one third of childhood non-Hodgkin lymphomas (9,54). Pediatric patients with a typical T-LBL usually present with a mediastinal mass, which is frequently associated with supradiaphragmatic (cervical, supraclavicular, or axillary) lymphadenopathy. The mediastinal mass may cause airway obstruction, superior vena cava syndrome, pericardial effusions, and pleural effusions (54,55). Constitutional symptoms are seen in about 30% of patients. In adult patients, however, the presentation is frequently extramediastinal, mainly abdominal (56), or subcutaneous lesions (8,27). Other sites of involvement include head and neck, lung, liver, pleura, pericardium, peritoneum, and testes (55). Rare cases involving the uterus and

breasts have recently been reported (57,58). Although bone marrow is frequently involved, pancytopenia or circulating blasts are unusual in LBL cases (55).

LBL is a highly aggressive tumor; patients usually die within 1.5 years (59). The most reliable prognostic factors are serum lactate dehydrogenase (LDH) levels and Ann Arbor disease staging (55). In the low-risk subgroup, characterized by Ann Arbor stage I to III disease or Ann Arbor stage IV disease with serum LDH <1.5 times normal, >90% of patients achieved 5-year disease-free survival (55). When patients are in Ann Arbor stage IV with bone marrow or central nervous system involvement or stage IV with other extranodal site involvement and a high serum LDH level, only 20% of patients achieve a 5-year disease-free survival. A recent evaluation of T-LBL cases showed that the 5-year disease-free survival was 24% (60). Most of the earlier literature suggested that B-LBL involved bone marrow frequently and had a particularly aggressive clinical course (60). However, one study found that bone marrow involvement was less frequently seen in B-LBL than in T-LBL, and the former group was more likely than the latter to achieve a complete remission (61). The unfavorable outcome of B-LBL is frequently associated with certain cytogenetic abnormalities, such as t(1;19) and t(9;22) (50).

TABLE 6.15.7

Salient Features for Laboratory Diagnosis of Lymphoblastic Lymphoma

1. Special marker: lymphoblastic lymphoma is the only lymphoma that expresses TdT.
2. Most T-cell antigens are positive, corresponding to different thymocyte stages.
3. Thymocyte staging depends on the reactions of CD4 and CD8.

   Early thymocyte: CD4- CD8-

   Common thymocyte: CD4+ CD8+

   Late thymocyte: CD4± CD8±
4. TCR antigens are positive.
5. CD38 and CD71 are frequently positive.
6. Immunohistochemistry: CD43 for both T- and B-cell tumors, CD20 and CD79a for B-cell tumors.
7. Small percentages of lymphoblastic lymphoma show B-cell or NK-cell phenotype.
8. TCR gene rearrangement is frequently demonstrated.

NK, natural killer; TdT, terminal deoxynucleotidyl transferase; CD, cluster of differentiation; TCR, T-cell receptor.

In ALL, the influence of the cell lineage is just the opposite of LBL. The T-ALL cases usually have worse prognosis than the B-ALL cases.

In childhood T-ALL, the clinical presentation is characterized by older age, nonwhite race, male gender, higher white cell count (>50,000/μL), mediastinal mass, marked hepatosplenomegaly, lymphadenopathy, and central nervous system involvement (6,7). Older age, high white cell count, and organomegaly are considered high risk factors. Therefore, patients with T-ALL usually have worse prognosis than those with B-ALL. Some reports indicate that pre-T phenotype is worse than other T-cell phenotypes, but other studies showed no differences between these two groups (9). If pre-T-ALL is defined as lack of E-rosette formation but reactive with T-cell

antibodies, then it is not surprising that the absence of CD2 (E-rosette receptor) is associated with adverse clinical outcome (62).

However, the use of highly intensive treatment protocols in recent years has improved the outcome of T-ALL patients. For instance, a comparative study by the Children's Cancer Group showed that the probability of 3-year survival for patients with T-ALL increased from 56% in studies conducted between 1978 and 1983 to 65% in the period between 1983 and 1989 and to 78.8% between 1989 and 1993 (6). The Dana-Farber Cancer Institute study of 125 patients with childhood T-ALL treated between 1981 and 1995 showed the 5-year event-free survival rate to be 75% ± 4% (63).

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CASE 16 Chronic Lymphocytic Leukemia of B-Cell Lineage

CASE HISTORY

A 66-year-old man was found to have an elevated leukocyte count with lymphocytosis on a routine physical examination. The hematologic workup at that time showed a total leukocyte count of 20,200/µL with 41% neutrophils and 53% lymphocytes. The hematocrit was 44% and platelet count 301,000/µL. The patient denied having fever, weight loss, night sweats, and fatigue. He had occasional episodes of upper respiratory infection in the past year. A fine-needle biopsy of the preauricular lymph node 1 year ago showed reactive lymphoid hyperplasia, probably secondary to his ear infection at that time.

Physical examination of the patient revealed no lymphadenopathy and no hepatosplenomegaly. Further laboratory study showed normal levels of lactate dehydrogenase (120 IU/L) and beta-2 microglobulin (1.8 mg/L). Immunoglobulin quantitation demonstrated a low immunoglobulin A (IgA; 32 mg/dL) and IgM (<18 mg/dL).

After flow cytometric analysis of the peripheral blood followed by a bone marrow biopsy, a diagnosis of chronic lymphocytic leukemia (CLL) was established. However, because the patient had no lymphadenopathy, anemia, or thrombocytopenia, the disease was considered to be at Rai stage 0/Binet stage A. The patient was, therefore, not treated and was monitored by the hematology/oncology service with periodic physical and laboratory examination.

In the subsequent 4 years, the patient had several episodes of upper respiratory infection and one episode of rectal bleeding. A colonoscopic examination found no abnormality. The bleeding was considered to be due to his hemorrhoid. A follow-up laboratory examination revealed a total leukocyte count of 28,900/µL with 78% lymphocytes and 17% neutrophils. His hematocrit was 43.2%, hemoglobin 14.7 g/dL, and platelets 269,000/µL. Immunoglobulin quantitation remained at the same levels as that examined 4 years ago.

FLOW CYTOMETRIC FINDINGS

CD5 98%, CD19 85%, CD5/CD19 85%, CD23 87%, CD20 78%, CD20/CD38 10%, CD19/κ 80%, CD19/λ 2%, FMC-7 10%, CD10 2% (Fig. 6.16.1).

DISCUSSION

CLL of B-cell lineage (B-CLL) is the most common leukemia in Western countries, accounting for 40% of all leukemias in adults (1). In the United States, the annual incidence is about 5.17 cases per 100,000 persons (2). On the contrary, the incidence of CLL in Asia is low, especially in India, China, and Japan (1). For instance, the incidence in Japan is 2.5% of all adult leukemias, compared with 38% in Denmark (3). The explanation of this marked geographic difference is unclear, but it is the only leukemia type that has not been associated with occupational exposure to radiation (3).

Morphology

The original requirement for a peripheral lymphocyte count in CLL was 125,000/μL (4). The current criteria are a peripheral lymphocyte count of 5,000 to 10,000/μL and bone marrow lymphocytosis of at least 30% (1). Bone marrow examination is not a mandatory requirement for the diagnosis but it helps to distinguish CLL from peripheral lymphocytosis caused by infections, such as infectious mononucleosis, pertussis and toxoplasmosis, which do not involve bone marrow.

Because there is a specific immunophenotype for CLL, an absolute lymphocyte count is no longer a rigid criterion (1). However, for worldwide clinical applications, particularly in countries where immunophenotyping is not readily available, an absolute lymphocyte count of 10,000/μL is still recommended by the International Workshop on CLL (IWCLL) (5). In addition, because more cases are diagnosed incidentally during routine examination of blood, diagnosis of CLL is more and more often made at an early stage; therefore, clinical manifestation is also not a requirement for the diagnosis.

Most cases of CLL show small mature-appearing lymphocytes in the peripheral blood, bone marrow, and occasionally, internal organs. A small percentage of CLL cases are designated CLL mixed cell type (Fig. 6.16.2) because of the presence of small and large lymphocytes in the peripheral blood (6,7). When the prolymphocytes are composed of 10% to 55% of the cell population, the condition is termed CLL/prolymphocytic leukemia (CLL/PLL). When more than 55% prolymphocytes are present, the diagnosis becomes PLL. The differences between CLL, CLL/PLL, and PLL are summarized in Table 6.16.1 (7). In approximately two thirds of CLL/PLL patients, the disease may evolve and transform into PLL, which is called prolymphocytoid transformation (6,8). In the remaining patients with CLL/PLL, transformation may never occur.

Another better known transformation is Richter syndrome, which is the development of diffuse large cell lymphoma in about 3% to 10% of CLL cases (9). In rare occasions, CLL can also transform into acute lymphoblastic leukemia,

myeloma, paraimmunoblastic variant of B-CLL/small lymphocytic lymphoma, and Hodgkin disease (3,10,11).

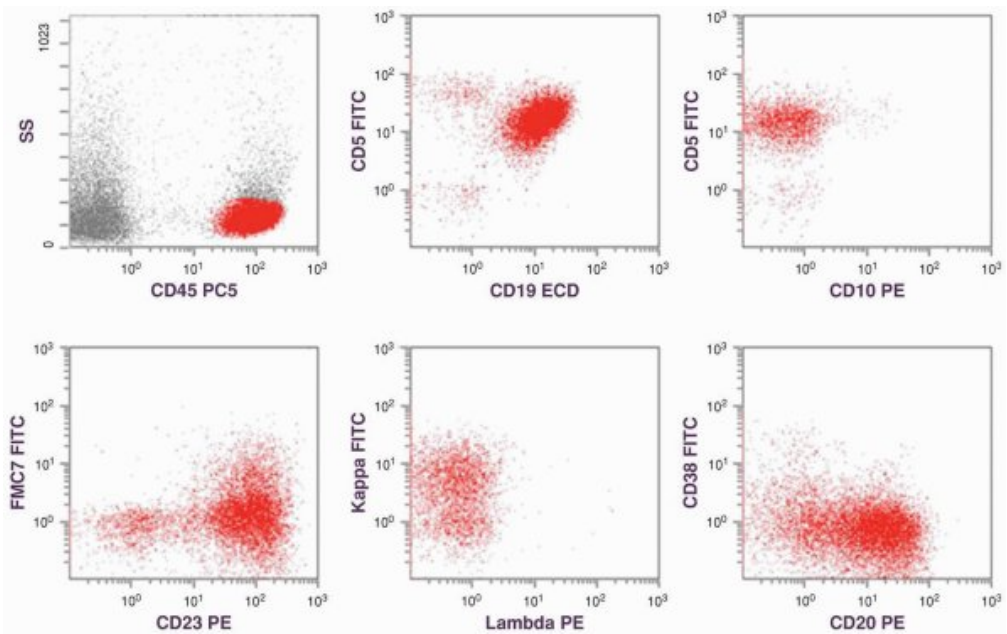

FIGURE 6.16.1 Flow cytometric histograms show dual CD19/CD5 staining and positive reactions to CD20, CD23, and κ. FMC-7 is partially positive, and CD10 and CD38 are negative. The negative CD38 result predicts a favorable prognosis. SS, side scatter; FITC, fluorescein isothiocyanate; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; ECD, phycoerythrin-Texas Red.

In the bone marrow, several infiltration patterns can be seen in CLL (10,11). A nodular pattern is discrete, densely packed collections of

lymphoid cells with infiltrating margins (Fig. 6.16.3); an interstitial pattern shows CLL cells intermingled with normal hematopoietic cells. When the hematopoietic elements are largely replaced by the neoplastic cells, it is called the diffuse pattern (Figs. 6.16.4 and 16.6.5). A diffuse infiltration pattern is usually associated with disease progression and poor prognosis (12). A paratrabecular infiltration is seldom seen in CLL. Bone marrow biopsy is not only helpful in predicting the prognosis, but also useful in evaluating the therapeutic effect and determining the etiology of cytopenia (e.g., leukemic infiltration, autoimmune cytopenia, or aplastic anemia) (13).

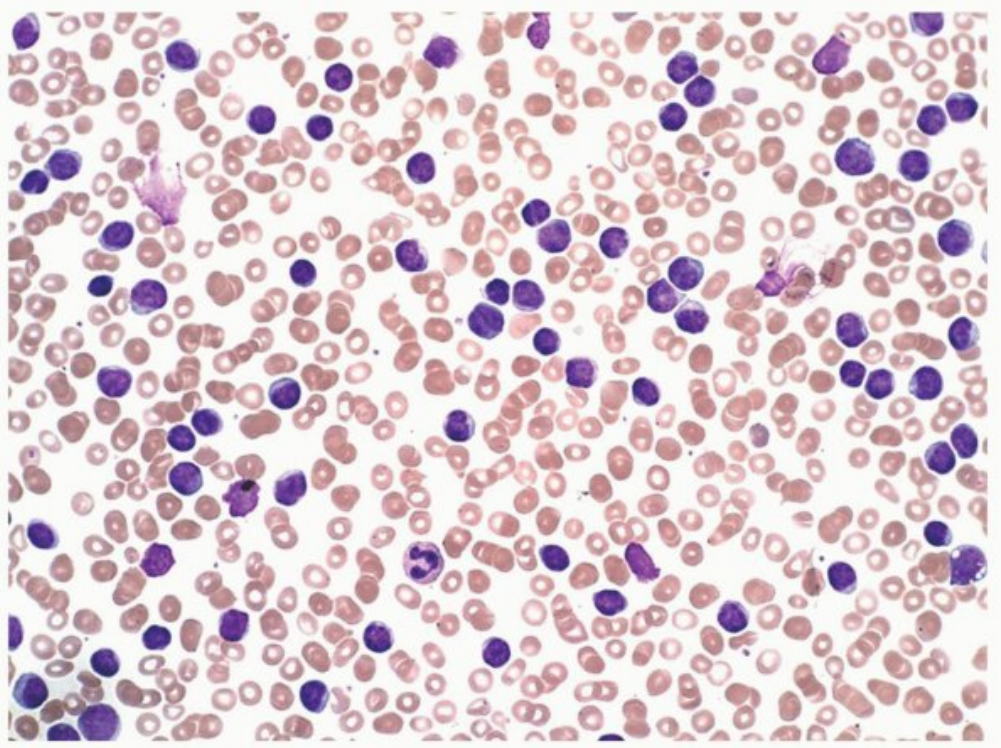

FIGURE 6.16.2 Peripheral blood smear reveals a mixed small and large lymphoid population. Wright-Giemsa, 40× magnification.

In the lymph node, the normal architecture is effaced by diffuse proliferation of uniformly small round cells with variable numbers of plasmacytoid cells. Proliferation centers are present in at least 90% of cases (see Case 18) (11,14).

Immunophenotype

Because most CLL cells are morphologically indistinguishable from normal mature small lymphocytes, marker studies are important for a definitive diagnosis. Although there are many surface markers for CLL, a minimum of four markers is usually sufficient to make the diagnosis.

The first one is a monoclonal surface immunoglobulin pattern, which is almost exclusively composed of IgM-κ or IgM/IgD-κ. The surface immunoglobulin staining is characteristically dim, representing a low density. However, the

total cellular immunoglobulin of a CLL cell is equivalent to that of most normal B cells because they contain increased cytoplasmic immunoglobulin (15).

TABLE 6.16.1

Comparison between CLL, CLL/PLL, and PLL

	CLL	*CLL/PLL*	*PLL*
Leukocyte count ($\times 10^9$)	5-120	Intermediate	>100

% prolymphocytes	<10	10-55	>55
Splenic involvement	Less frequent	Intermediate	Constant
Lymph node involvement	Frequent	Intermediate	Seldom
Surface Ig stain	Dim	Biphasic	Bright
CD5 positivity	Constant	Biphasic	<30%
Mouse RBC rosette formation	Constant	Constant	<30%
FMC-7 positivity	<20%	<20%	Constant
Cytogenetic abnormalities	13q-, 11q-, +12	13q-, 11q-, +12	14q+

CLL, chronic lymphocytic leukemia; RBC, erythrocyte; Ig, immunoglobulin; PLL, prolymphocytic leukemia; CD, cluster of differentiation.

In 10% to 20% of CLL cases, the surface immunoglobulin is too low to be detectable (16); therefore, a B-cell marker (e.g., CD19 or CD20) should be used to identify the B-cell lineage. The intensity of CD20 is also dim in CLL cases, whereas the staining of CD19 is brighter.

The third and most specific marker for CLL is a T-cell marker, CD5, which is present in almost all cases of CLL. A dual staining for CD5 and a B-cell marker (CD19 or CD20) should be performed to ensure that those CD5-positive cells are not the reactive T cells. Because mantle cell lymphoma also expresses a dual CD19 (CD20)/CD5 phenotype, CD23 should be used to distinguish these two entities. CLL is positive but mantle cell lymphoma is negative for this marker (17). Mantle cell lymphoma can also be differentiated from CLL by its brighter fluorescence of the surface immunoglobulin, its larger size than CLL cells, and the presence of cyclin D1 staining. PLL may also show dual CD19 (CD20)/CD5 staining in one third of cases (6).

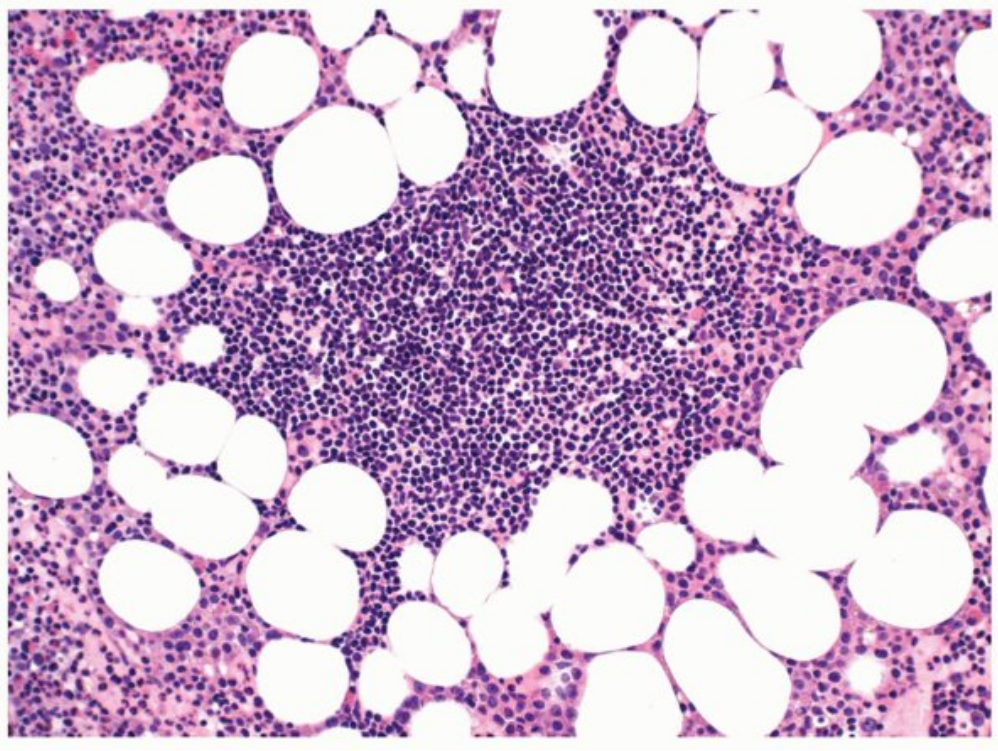

FIGURE 6.16.3 Bone marrow biopsy demonstrates a nodular lymphoid aggregate with infiltrating margin. Hematoxylin and eosin, 20× magnification.

Recently, three more negative markers were added to the CLL panel for differential diagnosis. CD22, CD79b, and FMC-7 are either absent or weakly reactive in CLL cases (1,18). Thus weak surface immunoglobulin, positive CD5 and CD23, negative or weakly positive CD22 or CD79b and FMC-7 are each counted as one point. When the tumor expresses more than three points, it is considered CLL; otherwise, it is likely to be other B-cell neoplasms.

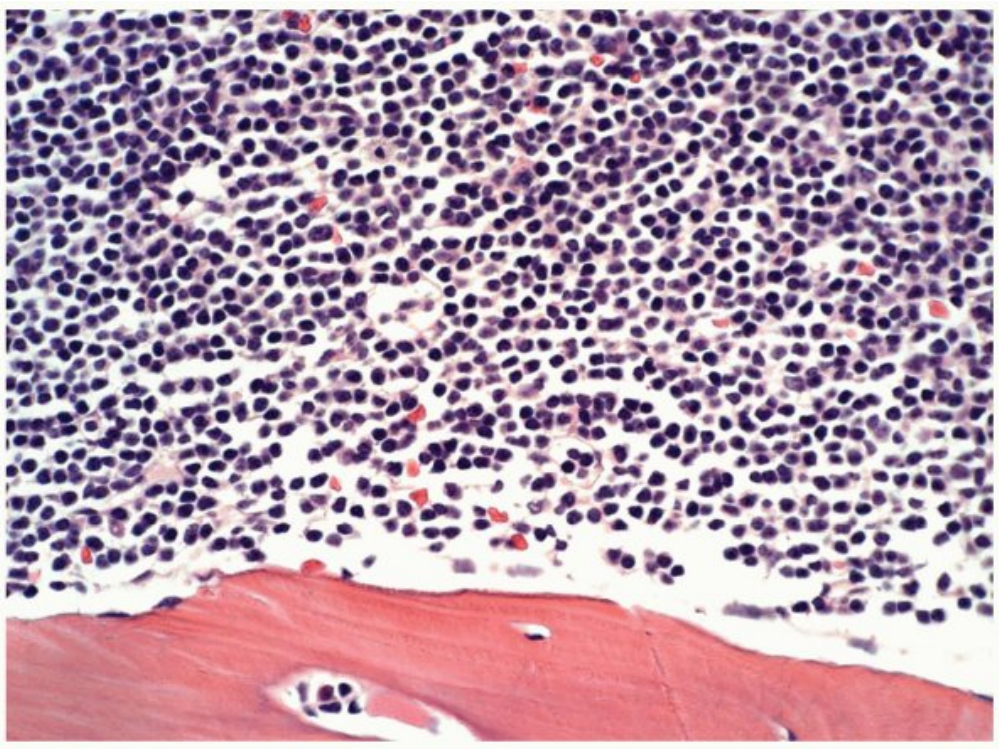

FIGURE 6.16.4 Bone marrow biopsy shows a diffuse infiltration pattern without residual fat vacuoles. Hematoxylin and eosin, 40× magnification.

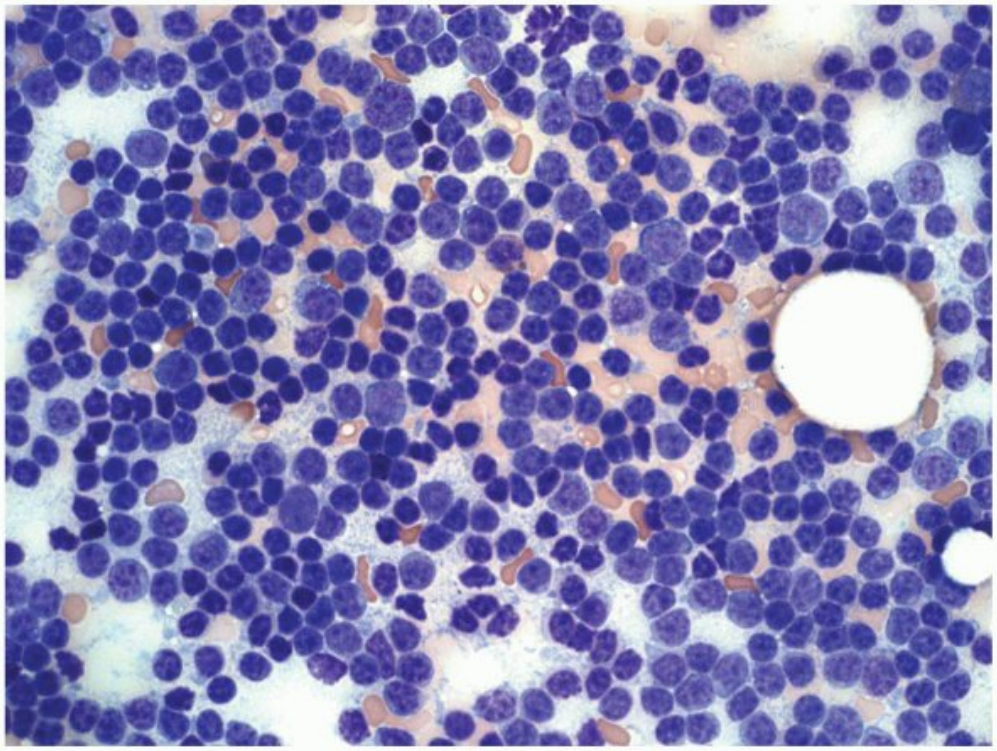

FIGURE 6.16.5 Bone marrow aspirate reveals diffuse lymphocytic infiltration. Wright-Giemsa, 40× magnification.

When FMC-7 becomes positive in >20% of lymphoid cells in the peripheral blood, CLL/PLL should be considered. One study showed that high IgM fluorescence intensity, high FMC-7, and low CD23 expression in CLL cases were associated with a short survival (19). Those cases probably were in the process of prolymphocytoid transformation.

Other B-cell markers, such as CD21, CD24, and CD40, are also positive in CLL cells (3,9,16,20). HLA-DR is consistently demonstrated, but CD10 and CD79b have not been detected on B-CLL cells (11). The terminal B-cell differentiation antigens, plasma cell antigen (PCA)-1 and CD38, are negative in CLL.

CLL cells may also express some myeloid antigens, including CD11b and CD11c, but not T-cell antigens other than CD5 (21). CLL cases expressing CD11c show features of both CLL and hairy cell leukemia (22).

In terms of adhesion molecules, CLL cells usually show low lymphocyte function-associated antigen (LFA)-1 (CD11/CD18) and CD54, intracellular adhesion molecule-1 (ICAM-1), but high Leu-8 (L-selectin) and CD44 (the lymphocyte homing receptor), whereas those of small lymphocytic lymphoma show high LFA-1 (20,23). The difference in the LFA-1 level is considered the mechanism that explains why CLL is disseminated, whereas small lymphocytic lymphoma stays in the lymph node.

Comparison between Flow Cytometry and Immunohistochemistry

As a rule, flow cytometry is most suitable for leukemia without solid organ involvement. The presence of CD5 and CD23 helps to distinguish CLL from many other lymphomas. The absence of CD10 excludes the leukemic phase of follicular lymphoma. However, about 10% to 15% of mantle cell lymphoma may express CD23. In those cases, immunohistochemical stain for cyclin D1 in the bone marrow biopsy is useful to distinguish CLL from mantle cell lymphoma.

The current case is typical for CLL in its natural history, morphology, and immunophenotype. This case, just like many of the CLL cases, was found incidentally during a routine physical examination. The patient was asymptomatic, with no lymphadenopathy, hepatosplenomegaly, anemia, or thrombocytopenia. However, the presence of >10,000/μL of lymphocyte in the peripheral blood and >30% of lymphocytes in the bone marrow met the basic criteria of CLL. Flow cytometric analysis showed an immunophenotype of CD19/CD5+, CD20+, CD23+, dim monoclonal κ pattern, and weak FMC-7. This finding further confirmed the diagnosis of CLL. Because

the patient was asymptomatic, with no lymphadenopathy, hepatosplenomegaly, anemia, or thrombocytopenia, he was not treated despite the fact that the lymphocyte counts increased steadily over a 4-year period. It is expected that this patient may enjoy a normal life span until he has severe superimposed infection or transformation to a high-grade lymphoid neoplasm.

Molecular Genetics

Cytogenetic study is not necessary for the diagnosis of CLL, but an aberrant karyotype may help in differential diagnosis and in prediction of prognosis. With conventional karyotyping, chromosomal abnormalities can be demonstrated in 40% to 50% of CLL cases, but interphase fluorescence in-situ hybridization (FISH) may disclose approximately 80% abnormalities (24). Using karyotyping, trisomy 12 was found to be the most common cytogenetic aberration, but with FISH, 13q- and 11q- are detected more frequently than trisomy 12 in CLL cases (24).

The frequency of trisomy 12 varies from 17% to 57% with an average of 34% in karyotypic studies (25, 26 and 27). When studied with FISH, the frequency ranged from 11% to 55% with an average of 22% (24,25). The prognostic effect of trisomy 12 is controversial (24,28), but it is associated with Richter transformation (29).

The most common structural abnormality is in the long arm of chromosome 13 (7% to 45%), which is often deleted (25,28). A recent study with FISH showed a frequency of 55% (24). The most common locus involved is 13q14. The deletion presumably results in inactivation of a tumor suppressor gene. Although the Rb tumor suppressor gene lies near the 13q14 region, current evidence indicates that it is not the relevant gene (28). More recently, it has been found that the breast cancer susceptibility gene (BRCA2), located at 13q12.3, is also associated with CLL (29).

The second common abnormality occurs in chromosome 11 (14% to 17%) (25). One study found 11q deletion in 20% of CLL cases (30). The frequency of 11q- is 18% by FISH (24). Recent studies showed that the ataxia-telangiectasia mutated (ATM) gene is involved in the deletion at 11q22-23 (24,31). Besides deletion, chromosome 11 may also show translocation with chromosome 14 (32, 33, 34 and 35). One study suggested that CLL cases with t(11;14) translocation may have a higher tendency to transform into high-grade leukemia and/or lymphoma (32). However, this translocation is much more commonly seen in mantle cell lymphoma, which should be excluded with further studies (33).

Deletion of 17q, where p53 (a tumor suppressor gene) is located, has been found in 10% of cases of CLL by

karyotyping (27,30,36,37) and 7% by FISH in one study (24). A higher percentage (42%) of p53 mutation was found in CLL cases with Richter transformation than in those without (15%) (38). p53 abnormality is also more frequently found in CLL cases with >10% prolymphocytes (8 of 15 cases) than those with <10% prolymphocytes (3 of 17 cases) (39). In addition, CLL/PLL cases showed biallelic inaction of p53, whereas typical CLL cases affected only one allele (39). Another study also proposed that both p53 and Rb tumor suppressor genes may play a role in clonal evolution of CLL cases (40).

Chromosome translocations between the bcl-2 oncogene on 18q21 and one of the immunoglobulin genes are found in 1% to 4% of B-CLL cases (28). One of the immunoglobulin genes may involve the heavy-chain gene t(14;18), κ light-chain gene t(2;18), or λ light-chain gene t(18;22). The translocation of t(14;18) is more frequently seen in follicular lymphoma, whereas t(2;18) and t(18;22) are more frequently demonstrated in B-CLL. Despite the rarity of BCL-2 gene rearrangement in B-CLL, BCL-2 messenger RNA (mRNA) and protein expression are quite common. The mechanism of bcl-2 overexpression in CLL is due to BCL-2 promoter region DNA hypomethylation rather than gene rearrangement (41).

Other less common cytogenetic abnormalities include 6q anomaly and t(14;19) (42). In a study of 208 CLL patients, 6q deletion was identified by FISH in 18 patients (9%) (43). The deletion involved band 6q21 in 12 patients and 6q27 in 6 patients. Another FISH study revealed a frequency of 6% for 6q deletion from 325 patients (24).

In terms of prognosis, the most important molecular genetic finding in recent years is the stratification of CLL cases into two groups on the basis of the mutational status of the variable region of the immunoglobulin heavy-chain gene (VH gene). When the VH gene shows >2% difference of nucleotide sequences from germline cells, it is considered mutated. Accordingly, B-cell neoplasms can be assigned into various developmental stages: pregerminal center stage with no somatic mutations, germinal center stage with mutations and ongoing mutational activities, and postgerminal center stage with a stable pattern of mutations (44).

It has been found that CLL cases have either unmated or mutated V_H genes; the former has a poor prognosis, whereas the latter has a good prognosis (45,46). In comparison with normal B-cell differentiation, these two groups are assumed to be derived from naïve B cell and memory B cell, respectively (44). Gene expression profiling studies discovered that both mutated and unmutated groups share common expression levels of many genes, and this profile is distinct from that of normal B cells or other B-cell malignancies (47). However, there are small subsets of genes that are different between mutated and unmutated CLL cases. One of these genes is zeta-associated protein 70 (ZAP-70), which can be identified at RNA and protein levels (44,48).

ZAP-70 directly enhances the B-cell receptor transduction and IgM signaling in CLL cases, leading to increased tyrosine phosphorylation of key signal transduction proteins (49). As a result, the unmutated CLL cells are activated, receiving continued stimulation for division and proliferation (31,44). In contrast, the mutated CLL cells are in the anergic state with downregulated B-cell receptor and reduced signal reception with resultant less aggressive behavior (44).

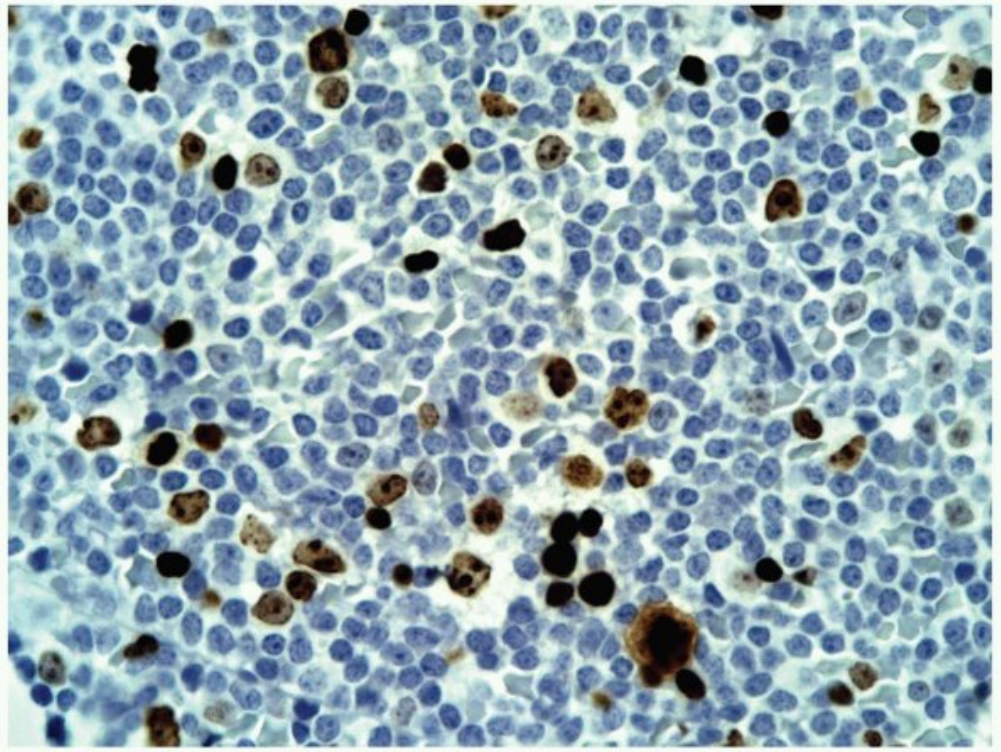

FIGURE 6.16.6 Bone marrow biopsy demonstrates Ki-67-positive large lymphocytes. The Ki-67 stain highlights the nucleoli in some cells. Immunoperoxidase, 60× magnification.

In the microenvironment, the interactions between CLL cells and the stromal cells, or nurselike cells or interactions between CD38 and its natural ligand CD31 may rescue CLL cells from apoptosis (31,50). This phenomenon occurs preferentially in lymph-node pseudofollicles and bone marrow clusters, evidenced by the demonstration of Ki-67 on the leukemic cells in these sites (Fig. 6.16.6) (31).

As mentioned above, the interaction between CD38 and CD31 may inhibit apoptosis. When CD38 is upregulated and reaches its signaling threshold, it can also deliver proliferation signals (50). As a result, the expression of CD38 in CLL cells is usually associated with a poor prognosis. Early studies indicated the close relationship between CD38 and the unmutated status of CLL cells (45). However, subsequent studies found that these two parameters did not always correlate (31). Nevertheless, many studies confirm CD38 as an independent inverse prognosticator when a high percentage (>30%) and an elevated level of antibody-binding capacity (>250) of CD38 are demonstrated (47).

Another finding connecting unmutated V_H gene with poor prognosis is that CLL cells from this group of patients have a shorter telomere length and a higher telomerase activity than those from patients with mutated status (18).

The salient features for laboratory diagnosis of CLL are summarized in Table 6.16.2.

Clinical Manifestations

CLL patients are usually older than 50 years with a male-to-female ratio of 2:1. The organ or tissue involvement depends on the stage of the disease. The Rai staging system is based on the presence or absence of bone marrow involvement,

lymphadenopathy, hepatomegaly, splenomegaly, anemia, and thrombocytopenia to divide CLL into five stages (Table 6.16.3) (3). In terms of prognosis, the five stages of the Rai system can be condensed into three stages: stage 0 predicts good prognosis; stages I and II, intermediate prognosis; and stages II and IV, poor prognosis (51). Binet et al. (52) defined a new staging system, similar to the Rai condensed staging system, with the major differences in counting five involved areas (cervical, axillary, and inguinal lymph nodes; spleen; and liver) and in lowering the cutoff point of the hemoglobin level to 10 g/dL (Table 6.16.4). The IWCLL has recommended the adoption of an integrated Binet-Rai staging system, which they hope will predict the prognosis of CLL more accurately (5).

TABLE 6.16.2

Salient Features for Laboratory Diagnosis of B-CLL

1. Peripheral lymphocytosis with an absolute lymphocyte count of >5,000/μL
2. Bone marrow lymphocytosis of >30% of the total population
3. Diagnostic score points: dim surface immunoglobulin, positive CD5, positive CD23, negative FMC-7, weak CD22 or CD79b (1 point assigned to each marker, >3 points is suggestive of CLL)
4. Positive immunoglobulin gene rearrangement
5. Differentiation markers: CD10 (follicular lymphoma), FMC-7 (prolymphocytic leukemia), CD25-CD103 (hairy cell leukemia), cyclin D1 (mantle cell lymphoma)
6. Cytogenetics: Usually normal; 13q-, 11q-, and +12 are relatively common.

B-CLL, B-chronic lymphocytic leukemia; CD, cluster of differentiation.

TABLE 6.16.3

Rai Staging System for CLL

	Stage 0	*Stage I*	*Stage II*	*Stage III*	*Stage IV*
Peripheral and marrow lymphocytosis	+	+	+	+	+
Lymphadenopathy	-	+	±	±	±
Hepatomegaly or splenomegaly	-	-	+	±	±
Anemia (Hb <11g/dL)	-	-	-	+	±
Thrombocytopenia (platelets <100,000/μL)	-	-	-	-	+

CLL, chronic lymphocytic leukemia.

TABLE 6.16.4

Binet Staging System for CLL

Stage	*Areas Involved*[*]	*Hemoglobin (g/dL)*	*Platelets (× 10^9/L)*
A	<3	≥10	≥100
B	≥3	≥10	≥100
C	Variable	<10	<100

CLL, chronic lymphocytic leukemia.

[*] The areas included cervical, axillary, and inguinal lymph nodes as well as spleen and liver.

With the improvement of diagnostic techniques, many CLL cases are now diagnosed at an early stage. Some patients may survive for many years without therapy, and others may progress rapidly. With the advances in treatments such as purine analogs, monoclonal antibodies, and bone marrow transplant, CLL patients can achieve high response rates, even with molecular remissions (18). Therefore, it has become obvious that patients should be treated before they meet the therapeutic guideline recommended by the National Cancer Institute Working Group (including the development of B symptoms, worsening anemia and/or thrombocytopenia, autoimmune cytopenias, progressive splenomegaly, progressive lymphadenopathy, and lymphocyte doubling time of 6 months) (53).

The recent stratification of CLL patients into two groups based on V_H gene mutational status provides a

reliable indicator for therapeutic targeting: those patients with unmutated V_H gene should be treated, and those with mutated status should be watched and wait (31,45,46). The medium survival was 8 to 9 years for patients with unmutated status but beyond 24 years for those with mutated status (45,46).

TABLE 6.16.5

Prognostic Factors in CLL (1,18,48)

Parameter	*Low Risk*	*High Risk*
Clinical stage	Rai 0, I; Binet A	Rai II, III, IV; Binet B or C
Lymphocyte morphology	Typical	Atypical
Bone marrow pattern	Non-diffuse	Diffuse
Lymphocyte doubling time	>12 mo	<12 mo
Serum markers[*]	Normal	Elevated
CD38 expression	<20%-30%	>20%-30%
ZAP-70 expression	<20%	>20%
Genetic abnormalities	Normal, del 13q	del 11q, del 17p

V_H gene status	Mutated	Unmutated

CD, cluster of differentiation; ZAP-70, zeta-associated protein 70; V_H gene, immunoblobulin variable heavy-chain gene.

* Serum markers include lactate dehydrogenase, beta-2 microglobulin, thymidine kinase, and soluble CD23.

As mentioned before, the unmutated CLL cells usually express ZAP-70 and CD38, which also substantiate the prediction of poor prognosis. Cytogenetic abnormalities are also reliable in defining high and low risk groups (1,18,31,48). For instance, del 13q confers a better survival, whereas del 11q and del 17p predict an ominous outcome. The lymphocyte doubling time of <6 months (53) and a diffuse infiltration pattern in the bone marrow (12) are also bad signs. Serum markers that are useful for monitoring patients include lactate dehydrogenase, beta-2 microglobulin, thymidine kinase, and soluble CD23 levels (1,18,48). The prognostic factors that help stratify the high risk and low risk groups of CLL are summarized in Table 6.16.5.

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CASE 17 Chronic Lymphocytic Leukemia of T-Cell Lineage

CASE HISTORY

A 77-year-old man presented with abdominal pain and was admitted to the hospital on the diagnosis of appendicitis. The patient denied having fever, night sweats, weight loss, or bruising. On admission, the patient had a total leukocyte count of 34,000/μL with absolute lymphocytosis (19,300/μL) and neutrophilia (12,900/μL). After surgery, lymphocytosis persisted with an absolute lymphocyte count of 13,000/μL. Flow cytometric analysis revealed that the lymphocytes were predominantly CD4+ helper T cells, consistent with T-cell leukemia. Physical examination showed no lymphadenopathy but mild splenomegaly. The patient was not treated for leukemia, but was doing well after discharge.

Four months later, the patient presented with red cutaneous papules on the shins, which were considered either leukocytoclastic vasculitis or leukemic involvement of the skin. A skin biopsy demonstrated small mature-looking lymphocyte infiltration of the dermis, consistent with leukemic infiltration (Fig. 6.17.1). The leukocyte count had continued to rise and reached the level of 57,400/μL with an absolute lymphocyte count of 51,000/μL (Fig. 6.17.2). Bone marrow examination showed 43.5% lymphocytes in the aspirate (Fig. 6.17.3) and large lymphoid aggregates in the core biopsy. Flow cytometric analysis of the bone marrow revealed a predominantly CD4-positive population similar to that seen in the peripheral blood analysis. During the second admission, splenomegaly was detected, the clinical course became rapidly progressive, and the patient failed to respond to chemotherapy. He was finally discharged to a hospice.

IMMUNOHISTOCHEMISTRY

Immunohistochemical stains of the bone marrow core biopsy showed that the lymphoid aggregates were positive for T-cell stains (CD3 and CD45RO) (Fig. 6.17.4), but negative for B-cell stain (CD 20). Immunohistochemical stains of the skin biopsy revealed that the leukemic cells were positive for CD3 and CD4 (Fig. 6.17.5) and negative for CD8.

FLOW CYTOMETRY

Flow cytometric analysis of the peripheral blood: T-cell markers: CD2 98%, CD3 97%, CD3/CD4 96%, CD3/CD8 1%, CD5 98%, CD7 99%. B-cell markers: CD19 1%, CD20 1%, CD23 3%, CD19/κ 1%, CD19/λ 0%, CD10 0%. Activation antigen: CD25 6% (Fig. 6.17.6).

Flow cytometric analysis of the bone marrow: T-cell markers: CD2 95%, CD3 91%, CD3/CD4 86%, CD3/CD8 6%, CD5 95%, CD7 98%, TdT 0%. B-cell markers: CD19 3%, CD23 9%. Natural killer (NK) cell markers: CD16 3%, CD56 4%, CD57 2%.

MOLECULAR GENETICS

T-cell receptor γ-chain gene rearrangement analysis of the peripheral blood specimen obtained from the first admission using polymerase chain reaction showed a clonal T-cell population. Cytogenetic study of the bone marrow obtained from the second admission revealed a normal male karyotype of 36XY.

DISCUSSION

Most cases of chronic lymphocytic leukemia (CLL) are of B-cell lineage. The frequency of the T-cell type of CLL (T-CLL) is only about 1% to 2% of the total of CLL cases (1). In the early literature, T-CLL was classified into three types: the knobby type, the azurophilic type, and the adult T-cell leukemia/lymphoma (ATCL) (2,3). The azurophilic type is now included in the large granular lymphoproliferative disorders (LGLDs) (4). The ATCL is also separated from T-CLL (5). The knobby type was so called because of the frequent presence of nuclear protrusions in the tumor cells and still remains in the T-CLL category.

However, because cases of T-CLL usually have a high leukocyte count and an aggressive clinical course and, in some cases, share karyotypes similar to those of T-cell prolymphocytic leukemia (T-PLL), Matutes and Catovsky (6,7) advocated the inclusion of T-CLL into the T-PLL category and called it the small-cell variant of T-PLL. These authors claimed that, although nucleoli are not demonstrated in those T-CLL cells at light microscopic level, they should be visible under electron microscopy. This concept was supported by a few authors (8). The entity of T-CLL is retained in the Revised European-American Lymphoma Classification (9), but it is deleted from the World Health Organization classification (10).

Nevertheless, many authors still consider T-CLL a separate entity because, morphologically, T-CLL cells are indistinguishable from those of B-CLL and the classification should not be based on the results of electron microscopy

and cytogenetics (3,11, 12, 13 and 14). Indeed, many cases of T-CLL do not have cytogenetic abnormalities that are similar to T-PLL, and splenomegaly, if present, is not as prominent as it is in cases of T-PLL.

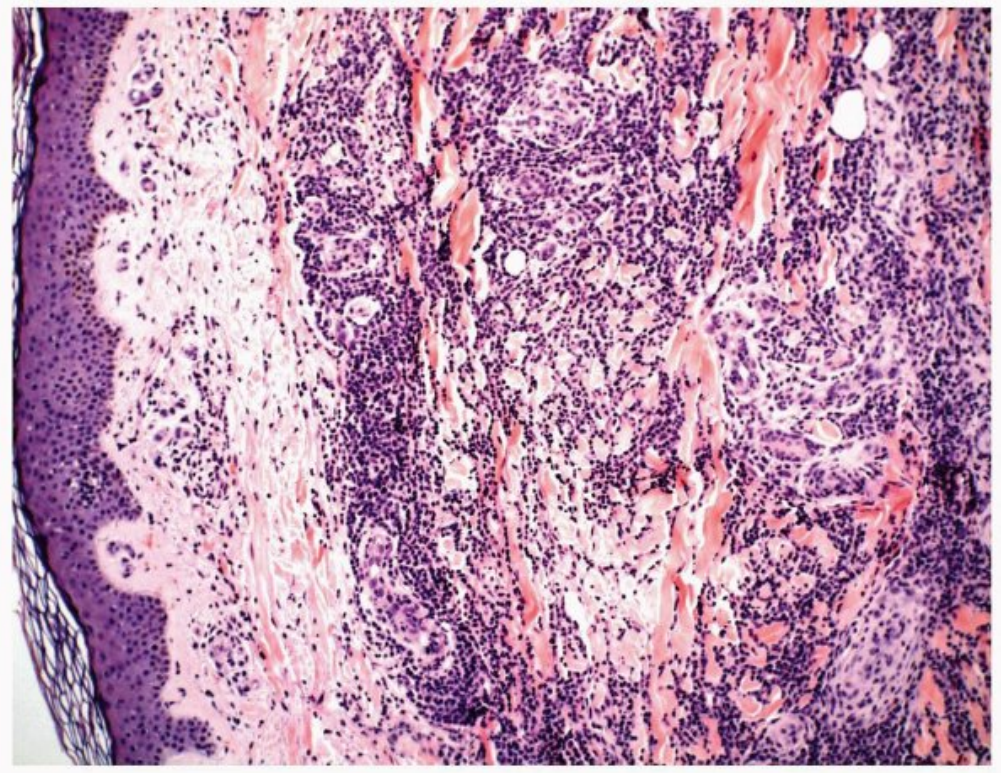

FIGURE 6.17.1 A skin biopsy shows perivascular and periadnexal infiltration by small leukemic cells in the dermis. There is no epidermal involvement. Hematoxylin and eosin, 10× magnification.

In the current case, the tumor cell morphology is indistinguishable from that of B-CLL and the karyotype is not consistent with T-PLL. Although the aggressive clinical course is similar to that seen in T-PLL, it is not justifiable to label it as PLL on the basis of clinical presentation alone. The absence of azurophilic granules in the cytoplasm of the tumor cells and the lack of reactions to CD16, CD56, and CD57 are not consistent with LGLD. The cytology and the histologic pattern in the skin as well as the absence of CD25 reaction are not supportive of the diagnosis of ATCL. Therefore, it is reasonable to consider this case as T-CLL.

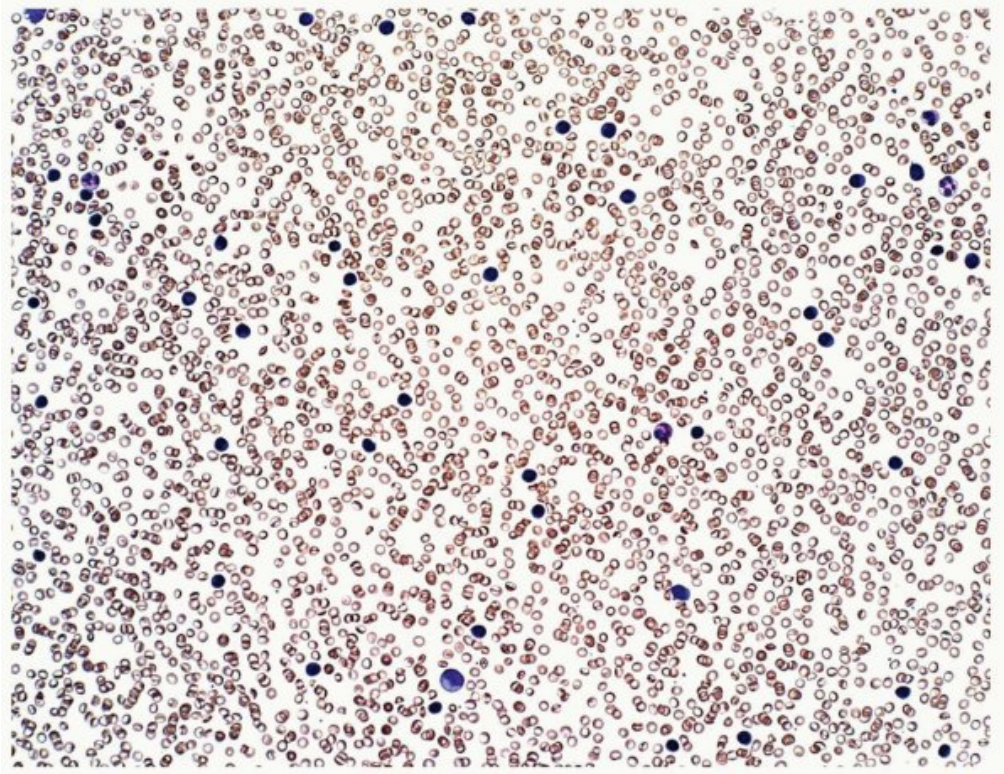

FIGURE 6.17.2 A peripheral blood smear shows numerous small, mature-looking lymphocytes, indistinguishable from those seen in B-cell chronic lymphocytic leukemia. Wright-Giemsa, 20× magnification.

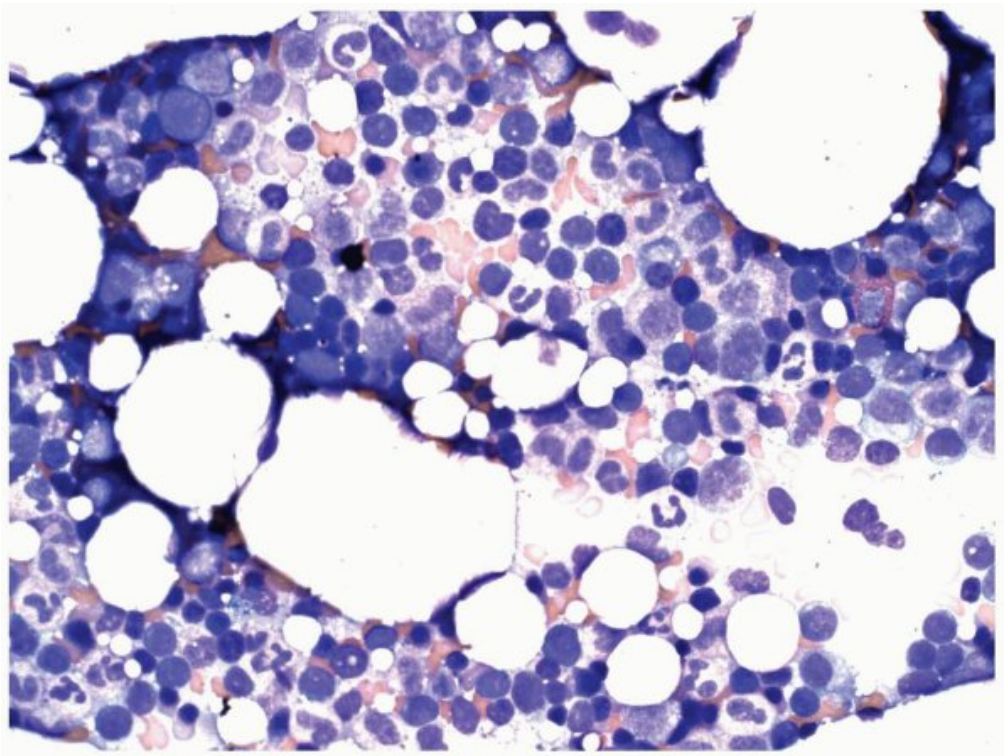

FIGURE 6.17.3 A bone marrow aspirate reveals a high percentage of small lymphocytes. Wright-

Giemsa, 40× magnification.

Morphology

Although early cases of T-CLL were described as knobby type for the presence of nuclear protrusion, most cases of T-CLL assume a morphology indistinguishable from B-CLL (3,11, 12, 13 and 14). In other words, they appear as small, mature-looking lymphocytes. In the study by Hoyer et al. (12), the median presenting lymphocyte count was 36,000/μL. The absence of cytoplasmic granules distinguishes T-CLL cells from large granular lymphocytes. The absence of prominent nucleoli and the high nuclear/cytoplasmic ratio distinguish them from prolymphocytes.

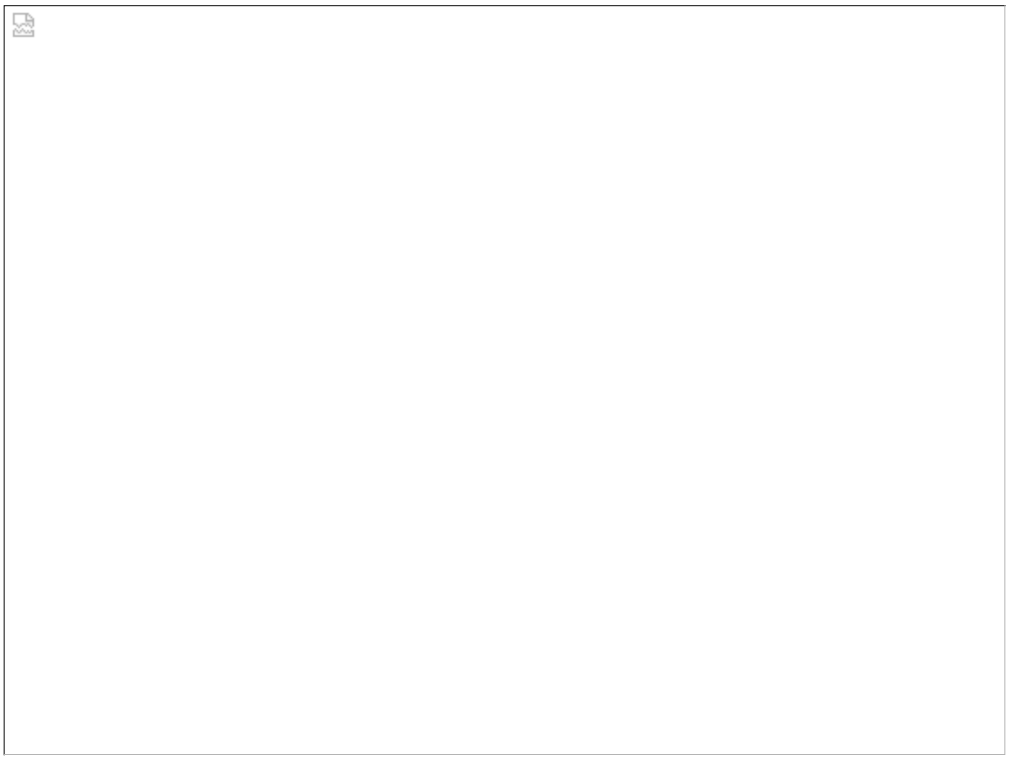

FIGURE 6.17.4 A bone marrow core biopsy shows a large lymphoid aggregate stained positive for CD3. Myeloperoxidase, 10× magnification.

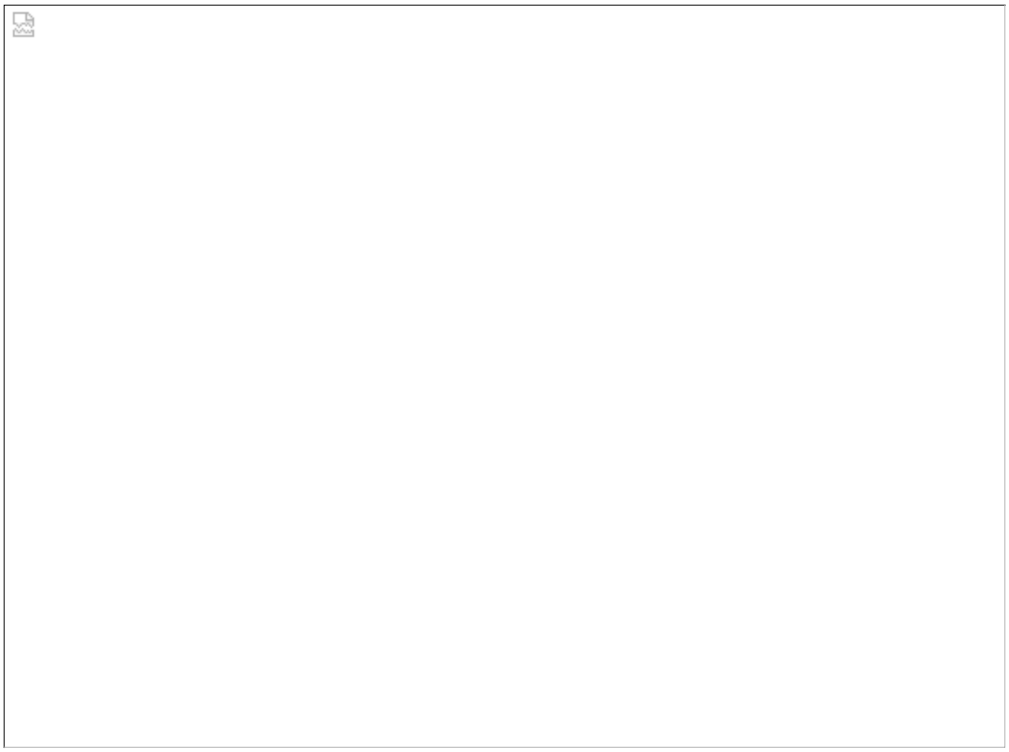

FIGURE 6.17.5 A skin biopsy reveals a leukemic infiltrate that stains for CD4. Immunoperoxidase, 10× magnification.

Sézary syndrome is the most common T-cell leukemia and should be distinguished from T-CLL (15). The typical Sézary cells usually show a cerebriform nucleus and appear in the peripheral blood in the late stage of mycosis fungoides. When Sézary-like cells are present in the peripheral blood without skin involvement, it is called Sézary leukemia (16). This entity is probably a variant of T-PLL.

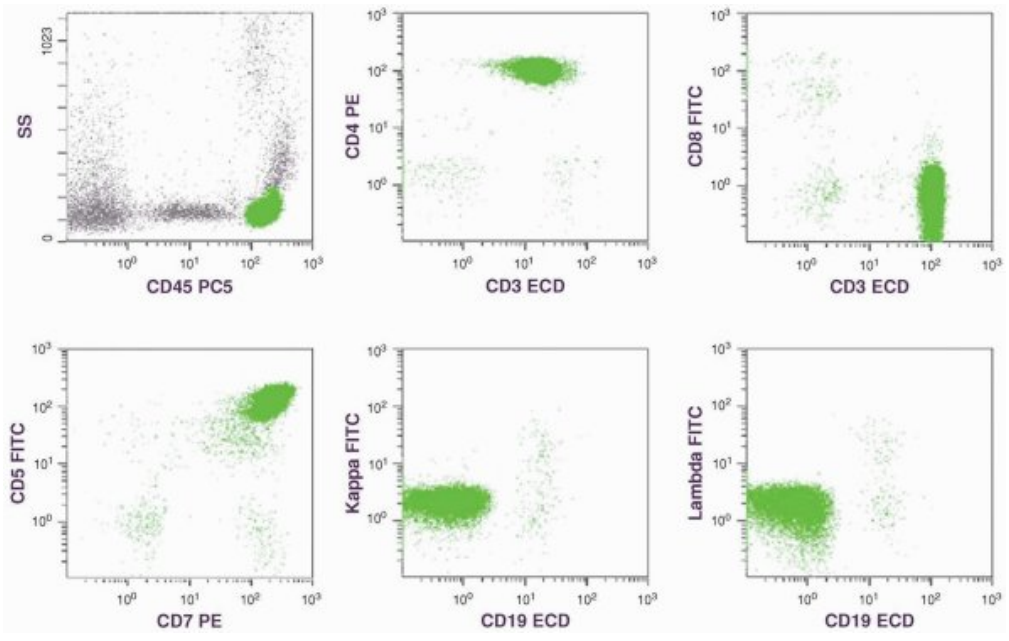

FIGURE 6.17.6 Flow cytometric histograms, with side-scatter (SS) versus CD45 gating, show positive reactions with CD3, CD4, CD5, and CD7 but negative reactions with CD8, CD19, and κ and λ stains. PC5, phycoerythrin-cyamin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; ECD, phycoerythrin-Texas red.

Another differential diagnosis for T-CLL is the ATCL, the tumor cells of which are characterized by the presence of polylobulated nuclei and are generally described as flower cells. These characteristic tumor cells, however, are present only in small numbers, often accounting for only about 5% of peripheral leukocytes (17). Furthermore, the tumor cells may show a cerebriform nucleus, indistinguishable from those of Sézary syndrome.

In the bone marrow, the infiltration pattern of T-CLL is usually interstitial, and the degree of involvement ranges from 15% to 90% (12). This is in contrast to the B-CLL cases, in which bone marrow involvement is usually prominent even at the early stage of the disease (1).

The differences among T-CLL, T-PLL, LGLD, ATCL, and Sézary syndrome are summarized in Table 6.17.1. For further information, the reader is referred to the corresponding Cases 19, 31, 33, and 35, respectively.

Immunophenotype

The immunophenotype of T-CLL is straightforward. It shows all pan-T-cell markers (CD2, CD3, CD5, and CD7), and the majority of cases belong to the helper/inducer subset (CD4+) (3,11, 12, 13 and 14). However, a few cases of the cytotoxic/suppressor

subset have been reported (12,18). When T-cell receptor markers are analyzed, they usually express the T-cell receptor $(TCR)_{\alpha\beta}$ subtype and not the $TCR_{\gamma\delta}$ subtype (14). Thymic T-cell markers, such as terminal deoxynucleotidyl transferase and CD1, are consistently negative in all cases.

TABLE 6.17.1

Differential Diagnosis of Peripheral T-Cell Leukemia

	T-CLL	*ATCL*	*LGLD*	*T-PLL*	*Sézary S*
Cell morphology	**Small mature lymphocytes**	**Lymphocytes with polylobulated nuclei**	**Large granular lymphocytes**	**Prolymphocytes**	**Lymph with cerebr nuclei**
Lymphocytosis	Moderate to marked	Moderate	Relative (neutropenia)	Marked	Modera
Splenomegaly	+	+	+	+	±
Lymphadenopathy	+	+	-	±	+
Skin infiltration	+	+	-	±	+
Pan-T antigens[a]	All positive	All positive except CD7	CD2+, others ±[b]	All positive	All posi except
T-cell subset	CD4+ CD8-	CD4+ CD8-	CD4- CD8+	Variable	CD4+ C

CD25 (IL-2R)	±	+	-	±	±
Activation antigens[c]	±	+	±	±	±
TdT and CD1	-	-	-	-	-
NK-cell antigens[d]	-	-	+	-	-
TCR rearrangement	+	+	±	+	+
HTLV-1 antibody	-	+	-	-	±
Prognosis	Poor	Poor	Generally good	Poor	Poor

[a] Pan-T antigens: Cluster of differentiation (CD)2, CD3, CD5, CD7. Selective loss of one or more antigens occur.

[b] Natural killer (NK)-like T cells (but not true NK cells) express CD3.

[c] Activation-associated antigens: CD38, CD71, human leukocyte antigen-DR (HLA-DR).

[d] NK-cell antigens: CD16, CD56, CD57.

ATCL, adult T-cell leukemia/lymphoma; HTLV-1, human T-cell lymphotropic virus type 1; LGLD, large gra lymphoproliferative disorder; NK, natural killer; T-CLL, T-cell type of chronic lymphocytic leukemia; T-PL T-cell prolymphocytic leukemia; IL-2R, interleukin-2 receptor; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.

The most important negative markers that help to distinguish T-CLL from other lymphoproliferative disorders are the B-cell markers (CD19, CD20, HLA-DR) and NK-cell markers (CD16, CD56, and CD57). In cases where CD8 is expressed, it is particularly important to include the NK-cell markers in the immunophenotypic panel to exclude NK or NK/T-cell lymphoproliferative disorders. True NK-cell leukemia shows absence of surface CD3 but presence of cytoplasmic CD3. A few cases of CD20-positive T-cell lymphoma have been reported (19), but CD20 has never been demonstrated in T-CLL cases.

Another important marker for differential diagnosis is CD25, which is seen in ATCL and occasionally in mycosis fungoides/Sézary syndrome (15,17). In Sézary syndrome and some peripheral T-cell lymphomas, CD7 is usually decreased as compared to other T-cell markers (such as CD3 and CD5).

Other than the above-mentioned markers (CD3, CD7, CD16, CD25, CD56, and CD57), the immunophenotype of T-CLL is similar to other T-cell lymphoproliferative disorders. It is hard to distinguish T-CLL from T-PLL by immunophenotyping, except that T-PLL usually has a variable subset expression (20). In other words, CD8-positive T-PLL is more frequently seen than CD8-positive T-CLL.

A few cases of S100-positive T-cell chronic lymphoproliferative diseases have been reported (21). Those cases have a very aggressive clinical course, with hepatosplenomegaly and central nervous system involvement. However, three of the four cases reported by Hanson et al. (21) expressed CD16, CD56, and CD57 and were probably cases of NK-cell leukemia/lymphoma. Another interesting immunophenotypic finding is that, even in B-CLL, oligoclonal expansion

of the CD4+ CD57+ T-cell subset is consistently demonstrated (22).

Functionally, T-CLL cells frequently show decreased B-cell growth factor and B-cell differentiation factor, which can be the mechanism for hypogammaglobulinemia seen in some patients (23).

Comparison of Flow Cytometry and Immunohistochemistry

Antibodies of CD3, CD4, CD5, CD7, and CD8 are available for immunohistochemical staining and are sufficient for the identification of T-CLL. However, there are more antibodies available for flow cytometry, such as CD2, cytoplasmic CD3, and CD25, that can be incorporated into a large panel for differential diagnoses of various T-cell lymphomas/leukemias.

Molecular Genetics

In the Mayo Clinic study (12), all T-CLL cases that had gene rearrangement analysis showed T-cell receptor ß-chain gene rearrangement, and a few cases showed TCR γ-chain gene rearrangement.

Recurrent cytogenetic abnormalities usually involve 14q11, 14q32, 7p15, and the long arm of chromosome 8 (12,24). The abnormalities are expressed in translocation, conversion, and isochromosome pattern. Because of the high frequency of abnormality in 14q32, the existence of a proto-oncogene at this locus was suspected (25). Finally, a putative oncogene, designated T-cell leukemia 1 (TCL)-1, has been identified at 14q32 (26). 14q11 is the location of the T-cell receptor α/δ locus, which can be fused with TCL-1 by translocation or inversion.

Gene transfer studies of TCL-1 suggest that its overexpression inhibits apoptosis, which is contributory to the neoplastic expansion of T cells (26). TCL-1 is normally expressed in CD4- CD8- immature thymocytes; therefore, its demonstration in T-CLL cells represents an inappropriate gene expression in leukemic cells that have a mature phenotype.

The salient features for laboratory diagnosis of T-CLL are summarized in Table 6.17.2.

Clinical Manifestations

The tumor cells in T-CLL and B-CLL are indistinguishable morphologically. Therefore, it is very important to identify the immunophenotype for their distinction, because these two entities are markedly different in the clinical course. B-CLL usually has an indolent course, whereas T-CLL is often highly aggressive with frequent skin and central nervous system involvement (1,3,12,27). Patients with T-CLL usually are refractory to the therapeutic regimen for B-CLL (12). In T-CLL, mild to moderate splenomegaly is present in about 40% and shotty adenopathy in 50% of cases (12).

In addition, the clinical course of T-CLL is similar to that of T-PLL, and so are their prognoses and cytogenetic abnormalities (20). Splenomegaly is more frequently seen in T-PLL, whereas lymphadenopathy is more common in T-CLL. Cases of T-CLL transforming to T-PLL have been reported; therefore, some cases may represent both entities (1).

TABLE 6.17.2

Salient Features for Laboratory Diagnosis of T-CLL

1. An absolute lymphocyte count in the peripheral blood >5 × 10^9/L
2. Leukemic cells: Predominantly small, mature-looking lymphocytes
3. Lymphocytosis in bone marrow >30%
4. Expression of all pan-T-cell antigens: CD2, CD3, CD5, CD7
5. Mostly helper T-cell phenotype (CD4+ CD8-)
6. TCR gene rearrangement is present.
7. Common cytogenetic abnormalities: t(14;14)(q11;q32), inv(14;14)(q11;q32)
8. TdT and CD1: Negative

T-CLL, T-cell type of chronic lymphocytic leukemia; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.

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CASE 18 Small Lymphocytic Lymphoma

CASE HISTORY

A 75-year-old man presented with shortness of breath and productive cough for several months. The patient had a long history of chronic obstructive pulmonary disease (COPD). Chest x-ray examination revealed right upper lobe and perihilar infiltrate. He was then treated with antibiotics and oxygen. Physical examination showed multiple lymphadenopathy involving occipital, posterior triangle of the neck, anterior triangle, submandibular, submental, supraclavicular, bilateral axillary, and right inguinal regions. Computed axial tomography (CAT) scan also detected large mediastinal masses and complete opacification of the adjacent right lung field. Abdomen revealed possible hydronephrosis of the left kidney. Organomegaly was not found in the patient.

Arterial blood gas analysis showed pH 7.54, pCO_2 29.8, pO_2 57, and oxygen saturation 93%. Blood chemistry was unremarkable except for a high level of lactate dehydrogenase (292 U/dL). A hematology workup revealed a total leukocyte count of 3,000/μL with 27% neutrophils, 71.4% lymphocytes, and 1.1% monocytes. No leukemic cells were detected in the peripheral blood. His hematocrit was 43% and hemoglobin 14 g/dL. A sputum culture grew pseudomonas.

An axillary lymph-node biopsy showed features of small cell lymphoma. The patient continued to receive oxygen and antibiotic treatment. In addition, chemotherapy with rituximab, fludarabine, and cyclophosphamide was started and was complicated with hypotension. This was considered to be the side effect of rituximab, which was then withheld. The patient tolerated one cycle of chemotherapy without further complication. His oxygenation slowly improved, and chest x-ray also showed improvement. The patient was discharged to the nursing home care unit with continued monitoring by the Hematology/Oncology Service.

FLOW CYTOMETRIC FINDINGS

CD5 98%, CD19 85%, CD19/CD5 84%, CD20 77%, CD10 1%, CD23 91%, FMC-7 13%, CD19/κ 88%, CD19/λ 4% (Fig. 6.18.1).

IMMUNOHISTOCHEMICAL FINDINGS

Immunohistochemical stains showed that the tumor cells were positive for CD20, but negative for CD3 and cyclin D1.

DISCUSSION

Small lymphocytic lymphoma (SLL) is a small B-cell lymphoma and the tissue phase of chronic lymphocytic

leukemia (CLL) (1). The term SLL is reserved for those cases with lymph node involvement and without a leukemic phase. However, even in those cases, the bone marrow and peripheral blood may be eventually involved. In patients showing both tissue and leukemic phases, the appropriate term should be CLL/SLL. SLL and CLL have identical immunophenotypes and are considered the same disease in the World Health Organization (WHO) classification (1). This classification is distinguished from the Revised European-American Classification of Lymphoid Neoplasms (REAL classification) (2) by separating prolymphocytic leukemia from CLL/SLL. CLL/SLL accounts for 90% of chronic lymphoid leukemia in the United States and Europe (1). It comprises 6.7% of non-Hodgkin lymphoma (1). The incidence is 5.17 per 100,000 person-years (3).

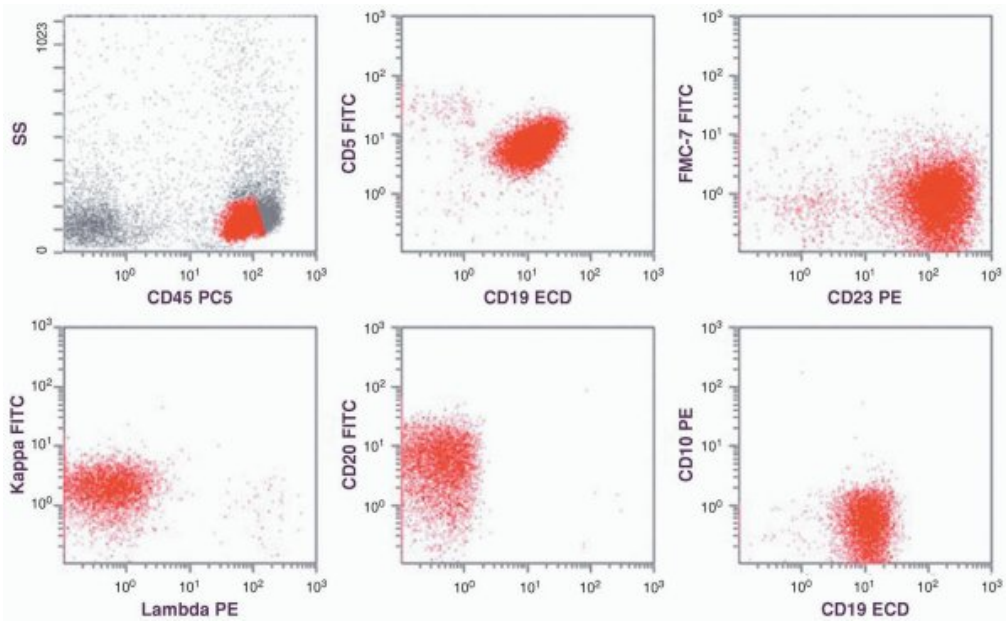

FIGURE 6.18.1 Flow cytometric analysis shows dual cluster of differentiation (CD)19/CD5 staining, strong positive reactions for CD23 and CD20, but dim κ staining. λ, FMC-7 and CD10 are negative. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-Texas Red.

Morphology

In the lymph node, the normal architecture is usually totally effaced by small neoplastic lymphoid cells. In a small percentage of cases, an interfollicular pattern may be present (1). The tumor cells are small lymphoid cells with regular nuclei and a clumped chromatin pattern. Nucleoli are usually not present or inconspicuous. The cytoplasm is scant.

The most characteristic pattern is the presence of proliferation centers or pseudofollicles, which are present in 90% of SLL cases (Fig. 6.18.2) (4). This structure is best detected under low-power magnification as a poorly defined pale stained area. It is composed of prolymphocytes and paraimmunoblasts surrounded by a small

lymphocyte background (Fig. 6.18.3). Prolymphocytes are slightly larger than the small lymphocytes, with a dispersed chromatin pattern and inconspicuous nucleoli. Paraimmunoblasts are larger than prolymphocytes, with dispersed chromatin pattern and one or more conspicuous nucleoli. A pseudofollicle should be distinguished from a residual germinal center, which can be seen in mantle cell lymphoma (MCL) and nodal marginal zone B-cell lymphoma. A germinal center is well-defined and is composed of centrocytes, centroblasts, and macrophages, forming a pattern of polarity. In some instances, a cluster of parafollicular/monocytoid B cells may also mimic the proliferation center (4). The number and size of pseudofollicles differ from case to case, but several studies found that this variation has no correlation with clinical condition and immunophenotype (5,6).

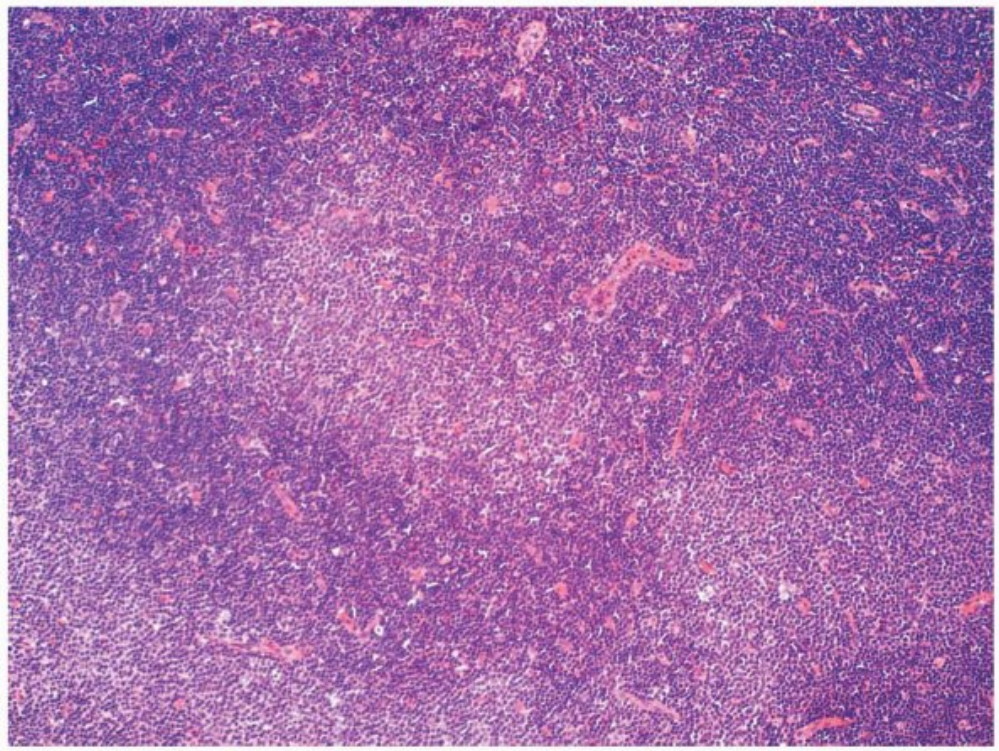

FIGURE 6.18.2 Lymph node biopsy shows total effacement of normal architecture by small lymphocyte infiltration. There are pale-stained areas, representing the proliferation centers. Hematoxylin and eosin, 10× magnification.

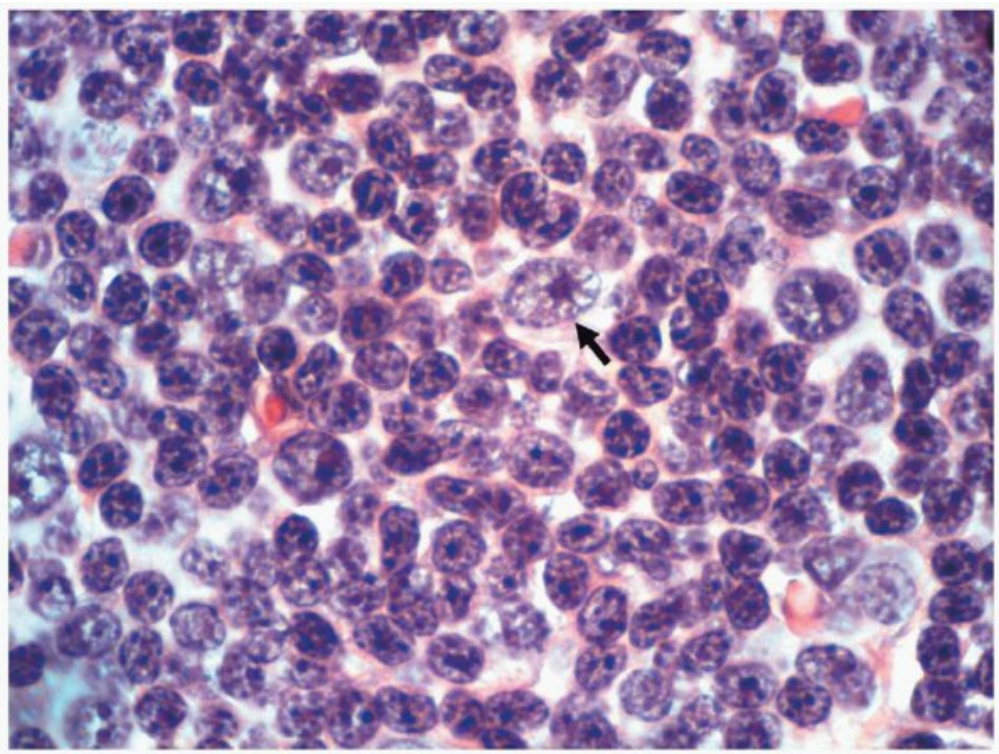

FIGURE 6.18.3 Lymph node biopsy in a case with prolymphocytoid transformation reveals predominance of prolymphocytes with scattered paraimmunoblasts (*arrow*). Hematoxylin and

eosin, 100× magnification.

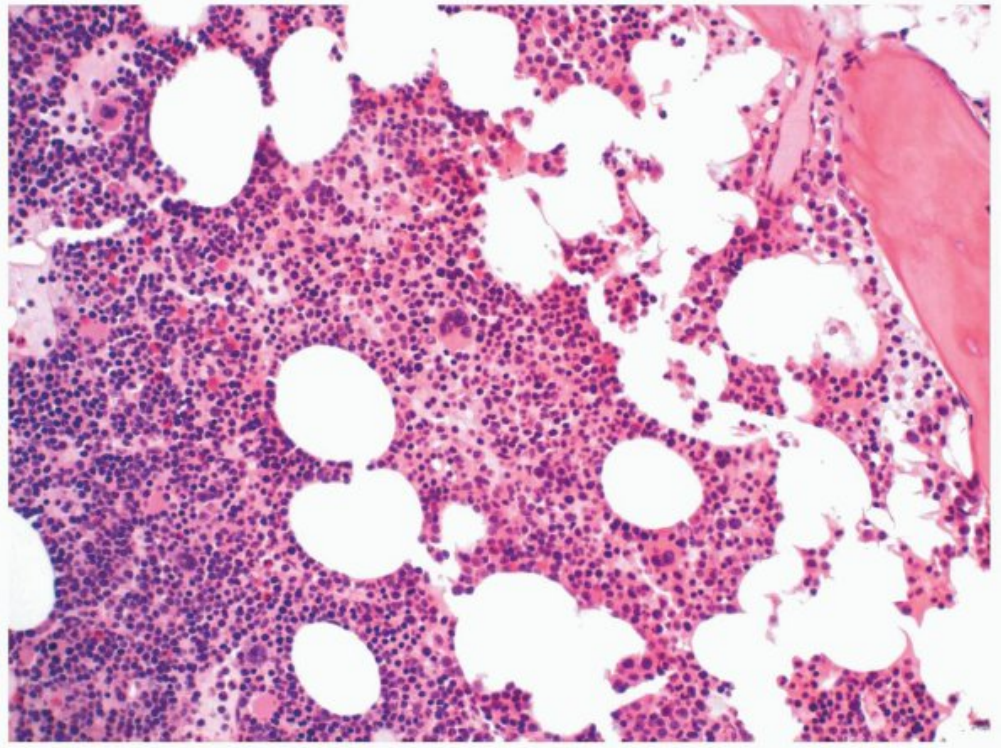

FIGURE 6.18.4 Bone marrow biopsy shows interstitial small lymphocytic infiltration. Hematoxylin and eosin, 20× magnification.

TABLE 6.18.1

Characteristic Morphologic Features in Small Lymphocytic Lymphoma	
Histologic pattern	Diffuse small lymphocytic infiltration replacing normal architecture
Cytology	Small, uniform, regular shaped, mature-looking lymphocytes with dense, clumped chromatin and no conspicuous nucleolus
Specific feature	Presence of proliferation centers

SLL should be distinguished from other small cell lymphomas, particularly the diffuse type of MCL, which has an immunophenotype similar to that of SLL. MCL, however, does not have pseudofollicles, and the tumor cells are usually more irregular than those of SLL (4).

In the spleen, the white pulp is primarily involved, but red pulp infiltration is also present (5). Pseudofollicles may be seen in the spleen, but they are not as conspicuous as they are in the lymph nodes. The bone marrow may show nodular, interstitial (Fig. 6.18.4), or diffuse infiltration pattern (or a combination of the three), but paratrabecular infiltration is seldom encountered (5). A diffuse infiltration pattern is usually associated with a poor prognosis (1). Extranodal involvement is mainly seen in the orbit and lungs. The

diagnostic morphologic features of SLL are listed in Table 6.18.1.

When the cells of the pseudofollicles predominate and proliferate diffusely with resultant nodal replacement, the subtype is designated as a paraimmunoblastic variant of SLL/CLL by Pugh et al. (7) and the tumor-forming subtype of B-CLL in the Kiel classification (8) (Fig. 6.18.5). Paraimmunoblasts should be distinguished from immunoblasts,

because the proliferation of immunoblasts represents large B-cell transformation. The immunoblasts have more vesicular nuclear chromatin, larger and more prominent nucleoli, and darker basophilic (Giemsa), amphophilic (hematoxylin and eosin), or pyronophilic methyl green pyronin ([MGP]) cytoplasm than the paraimmunoblasts have (8).

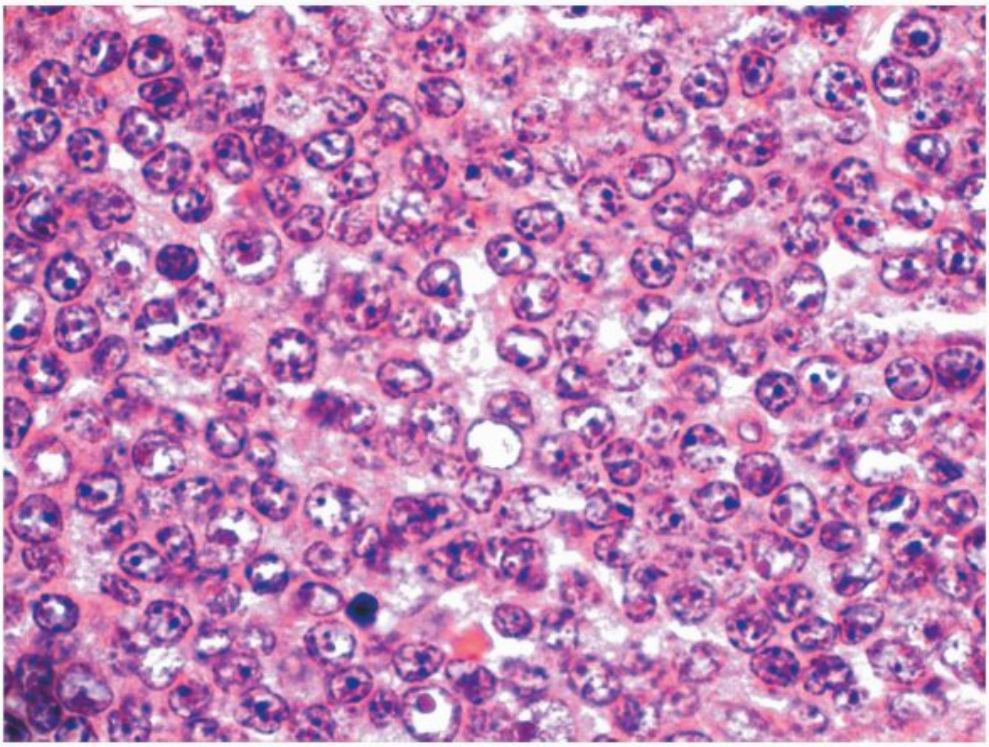

FIGURE 6.18.5 Lymph node biopsy in a case of paraimmunoblastic variant reveals exclusively paraimmunoblastic infiltration. Hematoxylin and eosin, 100× magnification.

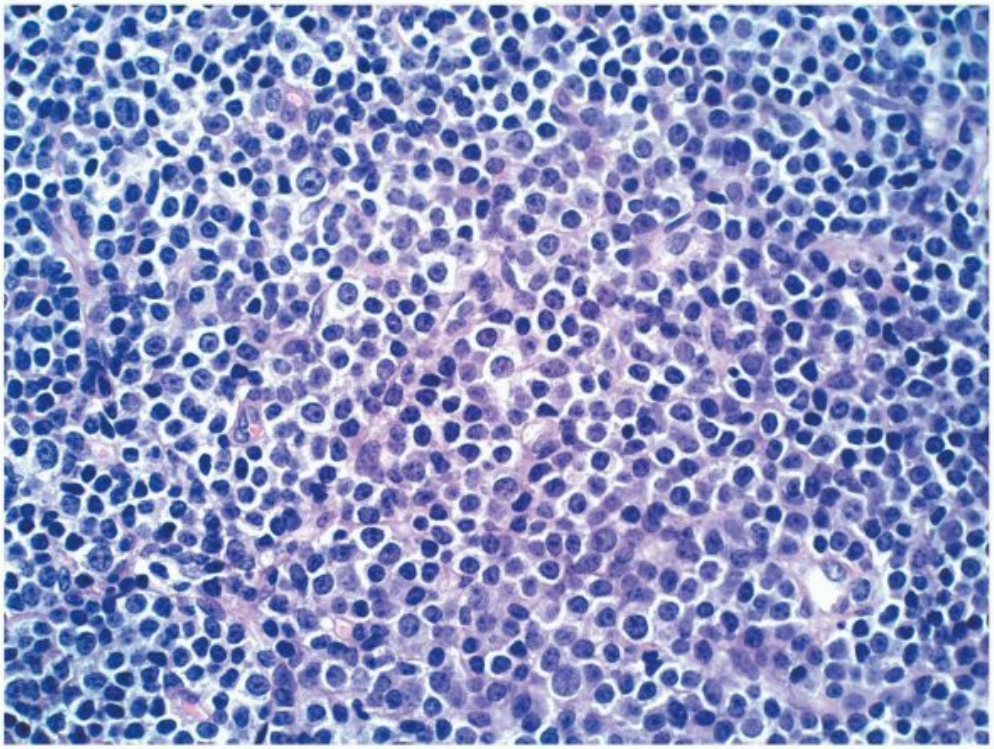

FIGURE 6.18.6 Lymph node biopsy in a case of Richter transformation shows clusters of large lymphoid cells intermixed with small lymphocytic lymphoma cells. Hematoxylin and eosin, 40× magnification.

The recognition of this subtype, although rare, is important because it is frequently mistaken for other types of large cell lymphomas (5), particularly the blastic and large cell variants of MCL (9). This subtype should also be distinguished from prolymphocytic transformation of CLL, which usually shows a large number of prolymphocytes in the peripheral blood and a history of CLL. Histologically, paraimmunoblasts are not a component of prolymphocytic transformation (5). Prolymphocytoid transformation is encountered in approximately 15% of CLL cases (10).

Transformation to large B-cell lymphoma or the so-called Richter syndrome is seen in approximately 3% of patients with CLL (11). These large cell lymphomas are usually diffuse large B-cell lymphoma, including the centroblastic and immunoblastic variants (1,12). Histologically, the transformed large cell lymphoma shows confluent sheets of large tumor cells (Fig. 6.18.6), in contrast to prolymphocytoid transformation, in which the prolymphocytes intermix with the small lymphoid tumor cells. A proliferation fraction exceeding 30%, as demonstrated by Ki-67 staining, is indicative of transformation to large B-cell lymphoma (Fig. 6.18.7) (13).

Immunophenotype

As B-cell neoplasms, SLL cells express most of the B-cell markers (CD19, CD20, CD21, CD23, CD24, CD79a, HLA-DR, and monoclonal surface immunoglobulin [Ig] M and/or IgD), except for CD22, which is usually weak or absent on SLL cells (1,4,14,15). However, the most characteristic feature in SLL is the coexpression of CD19 (CD20)/CD5 (4). Nevertheless, this dual staining is present also in CLL and MCL. CLL is the leukemic counterpart of SLL; thus no routine surface marker can help distinguish them (16). Although the adhesion markers CD11a/CD18 are present on the cells of SLL (but not on those of CLL) (17), adhesion molecules are not routinely tested in clinical laboratories. A recent study showed that the chemokine receptors CXCR4 and CCR7 are expressed at higher levels in peripheral CLL cells than SLL cells in the lymph nodes (18).

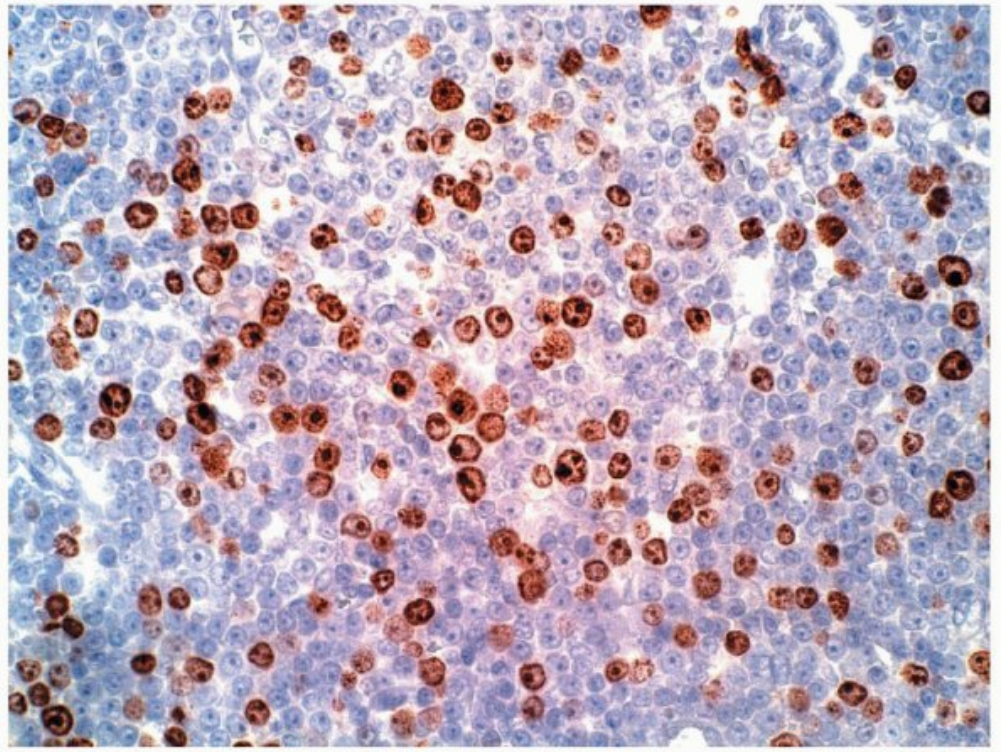

FIGURE 6.18.7 Lymph node biopsy in a case of Richter transformation reveals large numbers of Ki-67 stained cells. Immunoperoxidase, 40× magnification.

SLL/CLL usually shows dim fluorescence intensity of surface immunoglobulin, and the intensity of CD19 is stronger than that of CD20 in SLL/CLL. These features may help distinguish SLL from MCL, which shows moderate intensity of surface immunoglobulin and stronger intensity of CD20 than of CD19 (14). However, because the availability of CD23, the distinction between these two tumors no longer depends on the staining intensity of different antibodies. The SLL/CLL cases are usually positive for CD23, whereas the MCL cases are just the opposite (19, 20, 21 and 22). Nevertheless, about 10% to 15% of MCL is positive for CD23 (5). A higher percentage of CD23-positive MCL cases have been reported in some recent series (23,24). In those cases, a positive cyclin D1 (bcl-1 gene product) staining may help to establish the diagnosis of MCL (5,21,23). SLL generally shows no bcl-1 translocation except for the paraimmunoblastic variant of SLL (9).

CD23 is persistent on large cell lymphoma cells transformed from SLL/CLL, but it remains negative in the blastic variant of MCL; therefore, it helps to distinguish these two entities even when they are transformed (25).

Because CD5 monoclonal antibody is now available for paraffin sections, diagnosis of SLL by immunohistochemistry has become feasible (26). In the plasmacytoid subtype of SLL, which is now designated by WHO as Waldenström macroglobulinemia/lymphoplasmacytic lymphoma, CD5 is consistently negative (8). In a small percentage

of SLL/CLL cases, CD5 is absent (27). Some of those CD5-negative cases could be the result of a negative conversion after chemotherapy (personal observation). In Japan, the incidence of CLL/SLL is low, but CD5-positive B-cell lymphomas/leukemias usually show a poor overall prognosis (28). CD5 has also been demonstrated in a small group of de novo diffuse large B-cell lymphomas and in a few cases of marginal zone B-cell lymphoma and Burkitt lymphoma (29, 30 and 31).

Other markers helpful in differential diagnosis include FMC-7 and CD43. FMC-7, used in flow cytometry, is positive in MCL but negative or weakly positive in SLL (19,22). CD43, used in immunohistochemical staining, is positive in both MCL and SLL, but it can help to distinguish several CD43-negative lymphomas, such as follicular lymphoma, marginal zone lymphoma, and lymphoplasmacytic lymphoma (21,22). CD11c, a monocytic marker that is positive for hairy cell leukemia, was also positive in 83% of SLL cases in one study (22). SLL may also show selective loss of pan-B-cell antigen or negative surface immunoglobulin (32).

A novel combined silver nitrate and immunoperoxidase technique has been reported to be useful in distinguishing SLL/CLL from reactive lymphoid hyperplasia in the spleen (33). This technique helps to demonstrate trabecular infiltration, subendothelial infiltration, and prominent sinus involvement in SLL/CLL but not in lymphoid hyperplasia.

Comparison between Flow Cytometry and Immunohistochemistry

The major immunophenotype of SLL is CD19+/CD20+, CD5+, CD23+, and FMC-7-, which distinguishes SLL from MCL and other non-Hodgkin lymphomas. This immunophenotype can be easily identified by flow cytometry. Nevertheless, CD23 can be present in MCL

cases, and FMC-7 can be positive in SLL cases by flow cytometry (34). Immunohistochemistry can also stain for CD20, CD5, CD23, but not FMC-7. However, bcl-1 can be demonstrated by immunohistochemical stain and is a more powerful tool to distinguish MCL from SLL.

The presenting symptoms in the current case were those of pneumonia, but multiple lymphadenopathy was discovered during physical examination. The diagnosis of SLL was established on the basis of the morphology of the lymph node, showing a small cell lymphoma with multiple pseudofollicles. The flow cytometric analysis and immunohistochemical staining of the lymph node further confirmed this diagnosis. Because the lymphoma involved the mediastinum and spread to the adjacent lung field, lymphoma obstruction of the bronchus was considered the predisposing factor of his pneumonia. The improvement of pulmonary symptoms after chemotherapy supports this assumption. Small cell lymphoma usually has an indolent clinical course; therefore, when the patient becomes symptomatic, the disease is already in the late stage. In this case, the extensive multiple lymph node involvement suggests that the patient might have had the disease for years rather than months. It was the superimposed infection of the lung that drew the attention of the clinicians to make the diagnosis of lymphoma.

TABLE 6.18.2

Salient Features of Flow Cytometric Diagnosis of Small Lymphocytic Lymphoma

1. Monoclonal surface immunoglobulin with dim fluorescence
2. Double staining of CD19 (CD20) and CD5 is characteristic.
3. Positive CD23 distinguishes SLL from mantle cell lymphoma.
4. Positive B-cell antigens: CD19, CD20, CD21, CD24, CD79a, and HLA-DR
5. Important negative antigens: CD22, CD10, FMC-7 (most cases)
6. Immunoglobulin-negative, B-cell antigen-positive pattern may be present.
7. Selective loss of pan-B-cell antigen may be present.

CD, cluster of differentiation; SLL, small lymphocytic lymphoma; HLA-DR, human leukocyte antigen-DR.

The diagnostic features of SLL by flow cytometry are summarized in Table 6.18.2.

Molecular Genetics

In terms of genotyping, immunoglobulin heavy- and light-chain gene rearrangements are usually demonstrated in SLL cases. When SLL cases transform into large cell lymphoma (Richter syndrome), these two tumors often share the same genotype (11,35,36). However, some cases may show nonidentical rearranged bands. This phenomenon may be explained by the existence of two different clones or by the presence of heavy-chain gene switching, postrearrangement gene deletion, and somatic mutation after neoplastic transformation (37, 38 and 39).

Approximately 40% to 50% of SLL cases show no somatic mutation of the immunoglobulin V_H genes, whereas 50% to 60% of cases show somatic mutation (1). Those cases with unmutated VH genes are associated with positive CD38 (>30%) and a poor prognosis (40).

SLL usually shows no cytogenetic abnormalities unless it is evolving into a high-grade lymphoma. The relatively frequent aberrations include deletions at 13q14 (50%), trisomy 12 (20%), deletions at 11q22-23 (20%), deletions at 17p13 (10%), and deletions at 6q21 (5%) (1,15,41). One study of 55 cases of SLL showed that del(6)(q21;q23) was the most common recurring cytogenetic abnormality associated with the presence of large prolymphocytoid cells in the peripheral blood, but the clinical course of this subtype does not differ from the typical cases of SLL (42). Deletion of 17q13 is associated with p53 deletion. Trisomy 12 is associated with unmutated immunoglobulin V_H genes.

Translocation of bcl-2 is not seen in SLL (41), but bcl-2 protein can be demonstrated in the bone marrow from 8 of 10

cases of SLL/CLL (43). Translocation of bcl-1 is also not encountered in SLL except for its paraimmunoblastic variant (9). However, recent studies raised the possibility that those so-called paraimmunoblastic variants may well be the large-cell variants of MCL (31).

Leukemic phase is more commonly seen in SLL than in other lymphomas. Flow cytometry is usually able to detect the lymphoma cells in the blood. When the number of neoplastic cells is small, a data analysis system (Kolmogorov-Smirnov test) was advocated for clonal excess determination (44). However, the availability of the polymerase chain reaction has made the above technique obsolete (45).

Recently gene expression profiling provides a powerful tool for differential diagnosis as well as for stratifying prognostic subgroups of the same lymphoma. Genes associated with cell adhesion, angiogenesis, and inhibition of apoptosis were found to be upgraded in SLL (46). SLL was also found to share with CLL a common signature, including overexpression of L-selectin, P-selectin, titin, interleukin-4 receptor, CCR, adenylate kinase, diacylglycerol kinase, cyclin D2, and bcl-2 (46). However, the transcriptional profile of SLL was clearly distinguished from that of MCL and splenic marginal zone lymphoma (46). Another study of 120 genes showed distinctive gene expression profiling patterns among SLL, reactive lymph nodes, follicular lymphoma, and MCL (47).

Clinical Manifestations

Most SLL patients are >50 years old, and the male to female ratio is about 2:1 (1). Patients are often asymptomatic, and the initial diagnosis is usually due to the presence of painless lymphadenopathy. Some patients may have symptoms of fatigue, autoimmune hemolytic anemia, infections, splenomegaly, hepatomegaly, or extranodal infiltration (1,5). Anemia, weight loss, and night sweats are present in 15% to 43% of SLL cases (5).

The patient may present with only lymphadenopathy at the beginning, but bone marrow and peripheral blood involvement develop eventually. A small percentage of patients may have a low level of monoclonal gammopathy.

When Richter transformation is present, the patient's clinical condition usually changes abruptly, with marked increase of peripheral lymphocyte counts, fever, and the involvement of the central nervous system or other extranodal sites (12). When it occurs, the patient runs a rapidly downhill clinical course and dies within 6 months.

Nodal involvement is seen in 85% of patients at presentation (5). Only 6% of patients are in stage 1 or 2 at the time of diagnosis, whereas 73% of patients have bone marrow involvement (>30% lymphocytes) and 30% have extranodal soft tissue involvement.

The 5-year overall survival rate varies from 51% to 66%, and failure-free survival varies from 23% to 25% in two different series (5,48). The adverse predictors for overall survival include age >60 years, B symptoms, elevated serum lactate dehydrogenase, low hemoglobin (<11 g/dL), and high International Prognostic Index Score (>3) (48).

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CASE 19 Prolymphocytic Leukemia

CASE HISTORY

A 76-year-old man was found to have lymphocytosis during his hospitalization for the treatment of hypertension and congestive heart failure. His total leukocyte count at that time was 51,000/μL with 76% lymphocytes, 18% neutrophils, and 4% monocytes. His hematocrit was 22% and platelet count 420,000/μL. Investigation of his anemia showed that he had both iron and folate deficiencies; he was treated accordingly. Flow cytometric analysis showed that the immunophenotype of the lymphocytes was consistent with chronic lymphocytic leukemia (CLL). The patient had no hepatosplenomegaly or lymphadenopathy. It was decided at his admission that the patient should be followed for his CLL by oncology service but immediate treatment was not necessary.

In the subsequent 5 years, the patient's leukocyte count fluctuated between 40,000 to 60,000/μL, but finally reached 150,000/μL with a hematocrit of 20% and platelet count 110,000/μL. The peripheral blood smear revealed 60% prolymphocytes. Physical examination at that time showed splenomegaly. He was treated with chemotherapy, and his total leukocyte count fell to 23,000/μL. However, his absolute neutrophil count dropped to 300/μL. Subsequently, the patient developed *Pneumocystis carinii* pneumonia and enterococcus bacteremia. Despite multiantibiotic therapy, the patient's condition deteriorated and he died approximately 6 years after the initial diagnosis of CLL.

FLOW CYTOMETRIC FINDINGS

CD5 81%, CD10 2%, CD19 89%, CD19/CD5 80%, CD20 88%, CD23 21%, κ 0%, λ 93%, FMC-7 82% (Fig. 6.19.1).

DISCUSSION

Prolymphocytic leukemia (PLL) can be de novo or secondary to CLL (prolymphocytoid transformation). In the case of transformation from CLL, the peripheral blood may show an increase in the number of both prolymphocytes and small lymphocytes, peripheral lymphadenopathy is frequently present, and the leukocyte counts are not as high as in the de novo cases (1, 2 and 3). Mouse rosette formation with the tumor cells is characteristic for secondary PLL, but this test is no longer a routine test in clinical laboratories. However, in a terminal case when the prolymphocytes become overwhelming, it is difficult to distinguish a primary from a secondary PLL. The definition of PLL is that >55% of the lymphoid cells in the peripheral blood are prolymphocytes (4, 5 and 6). If the prolymphocyte count is between 10% and 55%, this condition is termed CLL/PLL. A prolymphocyte count <10% is not infrequently seen in a typical CLL case, so that does not change the diagnosis. The comparison between CLL, CLL/PLL, and PLL is tabulated in Case 16. In the World Health Organization (WHO) classification, secondary PLL is not included as PLL (5,6).

Morphology

A characteristic prolymphocyte of B-cell lineage is about 10 to 15 μm in diameter with a moderate amount of light basophilic cytoplasm (Fig. 6.19.2). The nucleus has a chromatin density between that of a small lymphocyte and that of a lymphoblast. A single prominent nucleolus is the hallmark of a prolymphocyte (Table 6.19.1). In PLL of T-cell lineage, there are two morphologic variants (6, 7 and 8). The small-cell variant is seen in about 25% of cases. The nucleoli are inconspicuous in those cases that require electron microscopic examination to identify the nucleoli (7). In 5% of T-cell PLL (T-PLL) cases, cerebriform nuclei are demonstrated (Sézary cell-like variant) (6). However, those cases with small lymphoid cells and inconspicuous nucleoli are considered T-cell CLL (T-CLL) by some authors (9,10).

Patients with PLL usually show extremely high leukocyte count in the peripheral blood (>100,000/μL) (5) and extensive leukemic infiltration in virtually every organ (11). In the spleen, both the red and the white pulp are infiltrated. A proliferative nodule with a bizonal appearance (darker at the center and lighter at the periphery) in the white pulp or an inverse pseudofollicular pattern is characteristic of PLL (Figs. 6.19.3 and 6.19.4) (11, 12 and 13). The cells in the center are mature lymphocytes, which are encircled by a rim of prolymphocytes that stain lighter because of their dispersed chromatin. However, in one study of T-PLL cases, the more prominent feature is the expansion of the red pulp with intrasinusoidal lymphoid infiltration (14).

The lymph node, if involved, shows a diffuse infiltration with or without a pseudonodular pattern (11,12). One study of three lymph nodes showed a paracortical distribution of the tumor cells (3). In PLL secondary to CLL, the infiltration may be patchy. When the normal architecture is completely replaced by prolymphocytes and paraimmunoblasts (Figs. 6.19.5 and 6.19.6), it is frequently referred to as a paraimmunoblast variant of small lymphocytic lymphoma/leukemia (12,15). Paraimmunoblasts are cells larger than prolymphocytes with very prominent nucleoli and abundant cytoplasm, and, in some cases, there are only a few of these cells present among the prolymphocytes in the lymph node with this diagnosis (12). Therefore, the term prolymphocytoid paraimmunoblastic transformation

used by Brunning and McKenna (12) is probably more appropriate if prolymphocyte is predominant.

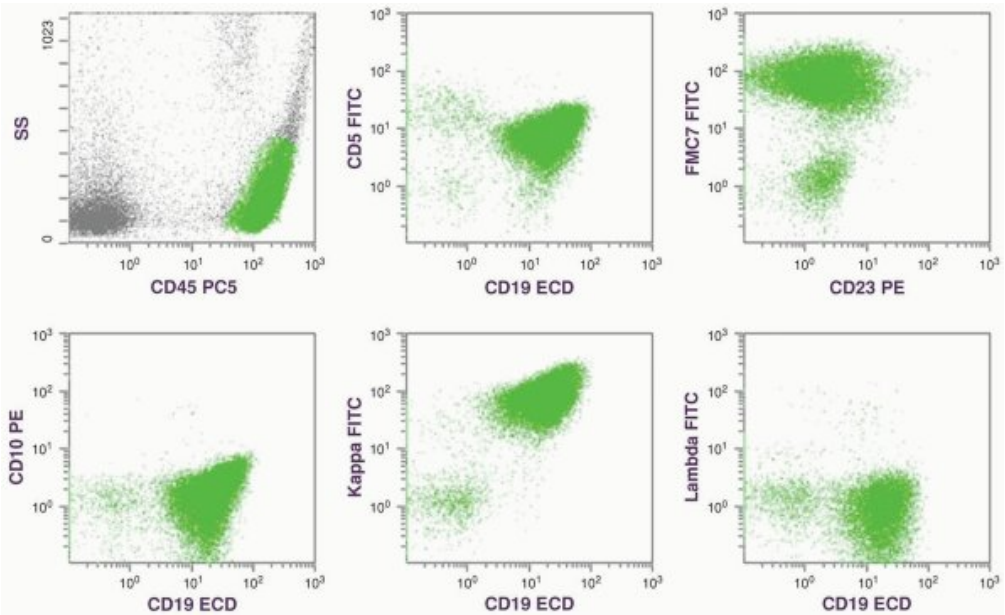

FIGURE 6.19.1 Flow cytometric histograms show dual staining of CD5/CD19, positive FMC-7, partial positive CD23, and monoclonal κ pattern. CD10 and λ are negative. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-Texas Red.

The bone-marrow infiltration is interstitial or diffuse, similar to that seen in CLL, or shows a mixed interstitial-nodular pattern (Figs. 6.19.7 and 6.19.8) (3,11,12). In the skin, the characteristic of T-PLL is perivascular and periappendiceal infiltration by atypical lymphoid cells in the dermis without epidermotropism (16,17).

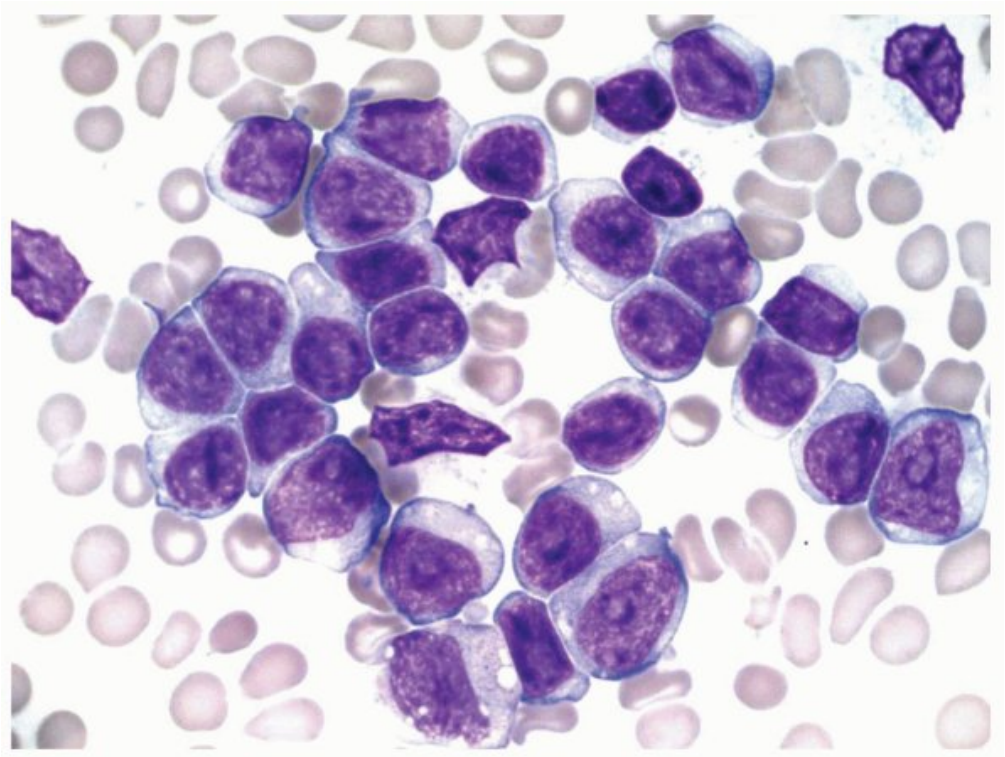

FIGURE 6.19.2 Peripheral blood smear reveals a cluster of prolymphocytes with single

prominent nucleolus and moderate amount of cytoplasm. Wright-Giemsa, 100× magnification.

The prolymphocytes in tissue sections appear medium to large in size with a distinct rim of cytoplasm and a round to oval nucleus. The nuclear chromatin pattern is dispersed, and a single variable prominent nucleolus is usually present (12). Despite the immature appearance of the tumor cells, the mitotic rate is usually low; this combination is considered characteristic of PLL (11).

TABLE 6.19.1

Characteristic Morphologic Features of PLL

Histologic pattern	Diffuse infiltration with or without proliferation centers in lymph nodes; bizonal pattern in white pulp of spleen
Cytology	Chromatin density between lymphoblasts and mature lymphocytes with a single prominent nucleolus and moderate amount of cytoplasm
Specific features	Presence of exclusively immature cells without a high mitotic rate

PLL, prolymphocytic leukemia.

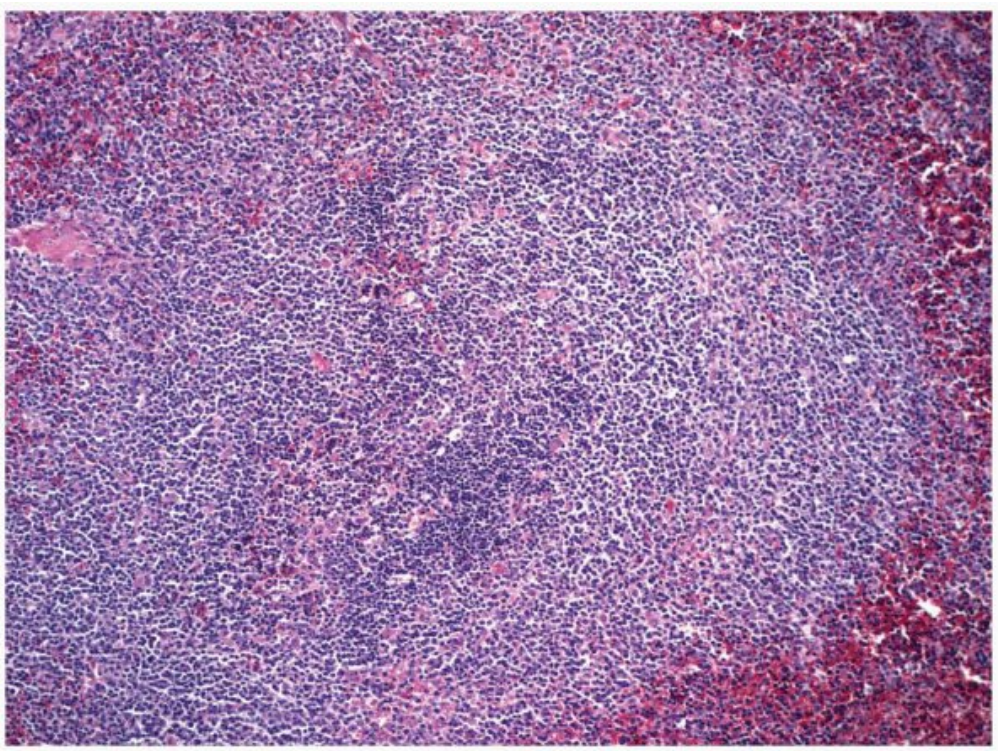

FIGURE 6.19.3 Splenectomy specimen shows a lymphoid follicle with a bizonal pattern (a dense small lymphocyte population in the center and the larger prolymphocytes in the periphery).

Hematoxylin and eosin, 10× magnification.

Immunophenotype

Although typical CLL cells and PLL cells are quite different morphologically, many “transitional” cells with an appearance between those of CLL and PLL, as seen in prolymphocytoid transformation, are not easy to distinguish. Immunophenotyping is helpful in this situation as well as in distinguishing primary and secondary PLL.

Prolymphocytes usually show a bright staining of the surface immunoglobulin, whereas CLL cells typically show dim immunofluorescence. Despite the difference in the density of surface immunoglobulin, both CLL and PLL patients have hypogammaglobulinemia, probably related to imbalance in T-cell subsets (18). In contrast, monoclonal gammopathy is much more frequently seen in PLL than in CLL patients (19).

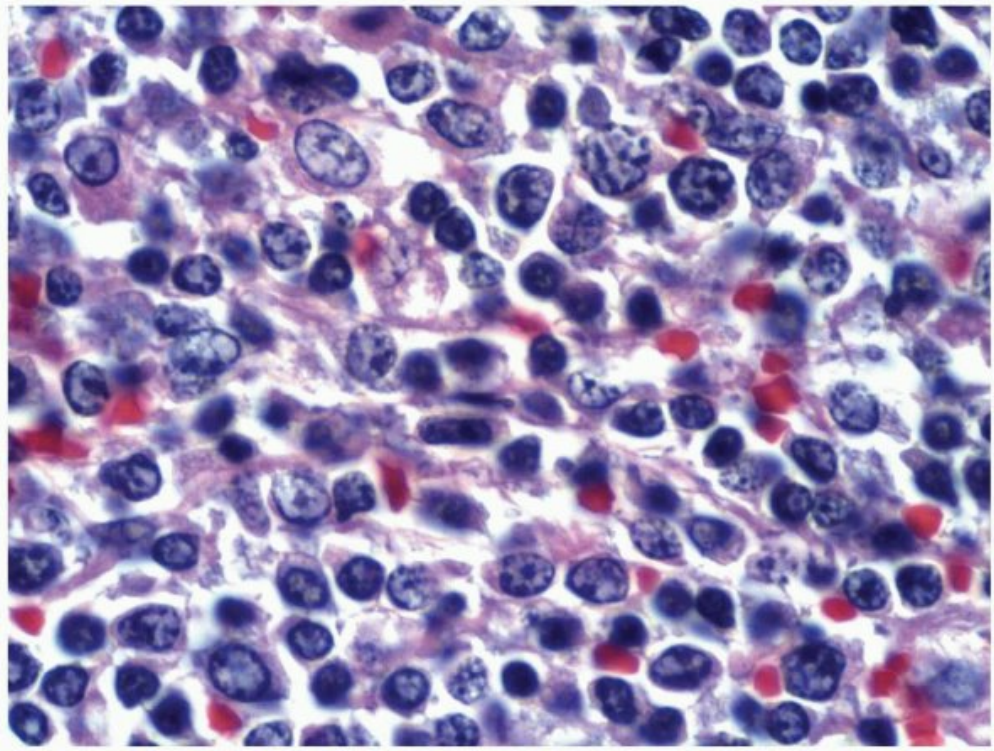

FIGURE 6.19.4 A high-power view of the previous spleen section shows the large prolymphocytes with immature chromatin pattern. Hematoxylin and eosin, 100× magnification.

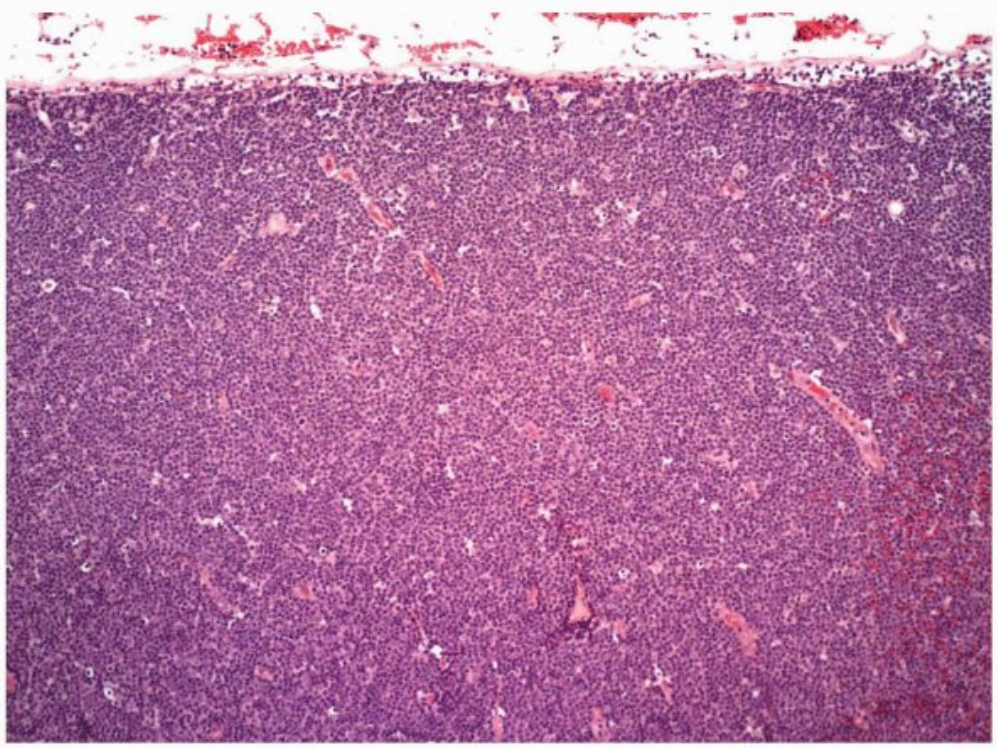

FIGURE 6.19.5 Lymph node biopsy reveals effacement of the normal architecture by a monotonous population of prolymphocytes. Hematoxylin and eosin, 20× magnification.

Another marker that distinguishes between CLL and PLL is the presence of mouse rosetting in CLL cells, which is absent in most PLL cells, except for some cases of secondary PLL (4,12,19). This phenomenon, in addition to the higher density of surface immunoglobulin on PLL cells, indicates that PLL cells are derived from a more mature progenitor than CLL cells are, yet PLL is clinically more aggressive and more refractory to therapy, resulting in a shorter survival for PLL than for CLL patients (20).

Three monoclonal antibodies (CD5, FMC-7, and CD22) are particularly helpful in distinguishing CLL from PLL. CD5

is positive for CLL but is negative for two thirds of PLL cases (21). However, if PLL is transformed from CLL, CD5 may remain positive (12). FMC-7 and CD22 are positive for PLL but negative for most CLL cases. The presence of FMC-7 is particularly important in the diagnosis of PLL (22). CD19, CD20, CD23, and HLA-DR are positive for both (3,20,21).

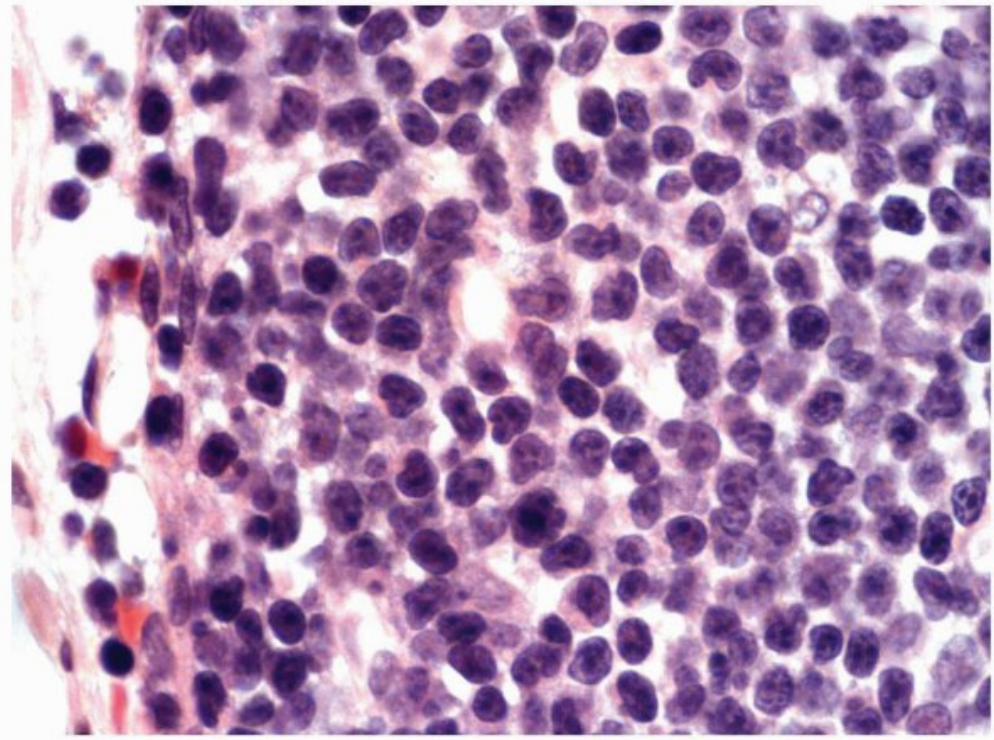

FIGURE 6.19.6 A high-power view of the previous lymph node section shows the immature-looking prolymphocytes. The absence of mitosis and apoptosis among the immature cells is characteristic of prolymphocytoid transformation. Hematoxylin and eosin, 100× magnification.

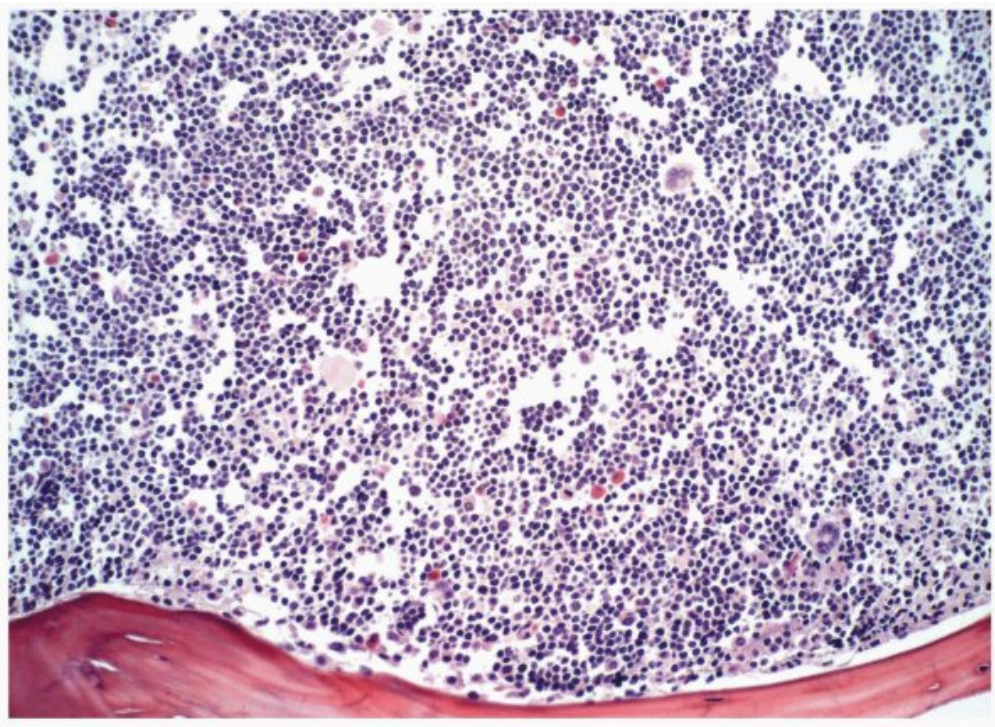

FIGURE 6.19.7 Bone marrow core biopsy shows diffuse prolymphocytic infiltration. Hematoxylin and eosin, 20× magnification.

Moderate expression of CD79b in PLL has been reported (23). The important negative markers for PLL are terminal deoxynucleotidyl transferase (TdT) and CD10, which help to distinguish PLL from lymphoblastic lymphoma/leukemia (4,20). PLL cells are also negative for the terminal B-cell antigens such as plasma cell-associated antigen (PCA-1) and CD38 (4,20,21). Negative CD25 in PLL distinguishes it from hairy cell leukemia, which is positive for CD25 (4,19,20). However, PLL can be induced by phorbol ester into hairy cell leukemia (24), and a hybrid form of hairy cell leukemia and PLL has been reported (25,26).

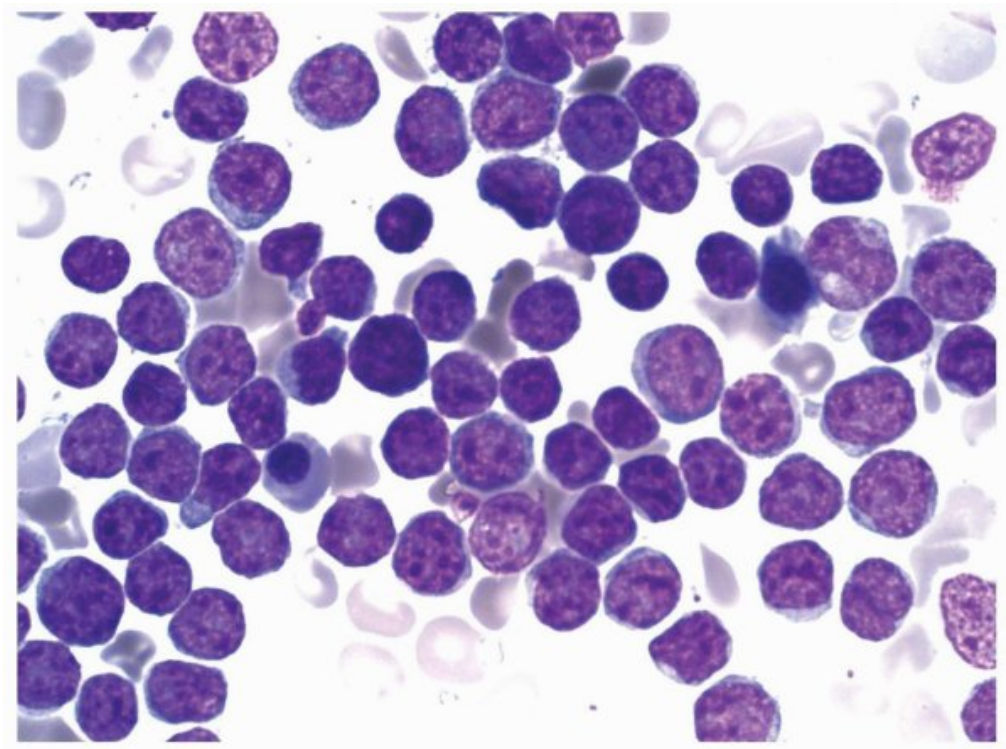

FIGURE 6.19.8 Bone marrow aspirate reveals a pure population of prolymphocytes. The prolymphocytes in the bone marrow usually do not show moderate amounts of cytoplasm. Wright-Giemsa, 100× magnification.

About 20% of PLL cases are of the T-cell type (27). The largest reported series of T-cell PLL included 78 patients studied over 12 years (7). In this series, membrane marker studies defined a post-thymic phenotype (TdT- CD2+ CD3+ CD5+ CD7+) for the tumor cells. Among them, 55% to 65% were CD4+ CD8- 21% to 30% CD4+ CD8+, and 10% to 13% CD4- CD8+ (7,28).

One important antigen that identifies malignant cells of T-PLL is CD52, because patients showing this marker can be treated with alemtuzumab (anti-CD52) with promising results (29). T-cell leukemia-1 protein (tcl-1), the product of TCL-1 oncogene is overexpressed in T-PLL and can be demonstrated by immunohistochemistry (30). It is useful in distinguishing other T-cell neoplasms, but tcl-1 has also been reported in many B-cell lymphomas (17).

Comparison between Flow Cytometry and Immunohistochemistry

Flow cytometry is more helpful than immunohistochemistry in differential diagnosis between various lymphomas that are similar to B-cell PLL (B-PLL). A high percentage of FMC-7 is particularly useful for the diagnosis of PLL, despite the fact that this marker is also present in hairy cell leukemia and various marginal zone lymphomas. However, immunohistochemistry is most helpful in identifying T-PLL by morphologic correlation.

Molecular Genetics

In the Mitleman catalog (31), the most common cytogenetic abnormalities in B-PLL involve chromosomes 1, 3, 6, 8, and 11-14. The most common cytogenetic aberrations include t(11;14)(q13;q32), t(14;17)(q32;q11), t(6;12) (q15;p13), del(3) (p13), and trisomy 12 (31). In a study of nine B-PLL cases, 60% showed 14q+ abnormality (32). Another study of 20 patients revealed monosomy 17 or additions to 17p in 45% of cases (3). Trisomy 12 is more frequently demonstrated in patients with secondary PLL than de novo PLL, as it is the most common cytogenetic abnormality in CLL cases (3). Deletions at 11q23 and 13q14 have been found to be frequent abnormalities in B-PLL by the fluorescence in situ hybridization technique (33).

Recently, several authors have advocated reclassification of PLL with t(11;14) as mantle cell lymphoma, because those cases usually have the immunophenotype of mantle cell lymphoma (CD5+ CD23- cyclin D1+) and are clinically very aggressive (3,34). Because the B-PLL may have cytogenetic abnormalities (e.g., 17q) that are present in other low-grade lymphoproliferative disorders, B-cell leukemia

with prolymphocytic morphology may represent a common end stage of transformation for several B-cell neoplasms (3).

In T-PLL cases, chromosome 14 inversion or translocation with breakpoints at bands q11 and q32 are the most frequent findings (8,28,35). In these abnormalities, the TCL-1 oncogene is juxtaposed with the promoter/enhancer of the T-cell receptor (TCR)αδ locus with resultant tcl-1 expression. Translocation t(X;14)(q28;q11) has also been reported, and the MTCP-1 in Xq28 is juxtaposed with TCRαδ (30).

TABLE 6.19.2

Salient Features for Laboratory Diagnosis of Prolymphocytic Leukemia

1. Total leukocyte count >100,000/μL
2. Presence of >55% of prolymphocytes in peripheral blood lymphoid population
3. FCM shows cells in large lymphocyte gate with bright fluorescence.
4. Monoclonal surface immunoglobulin, usually IgM-κ
5. Immunophenotype for B-cell PLL: Positive for CD19, CD20, CD22, FMC-7, HLA-DR; negative for TdT, CD10, and CD5 (2/3 cases)
6. Immunophenotype for T-cell PLL: TdT- CD2+ CD3+ CD5+ CD7+; 55%-65% CD4+ CD8-, 21%-30% CD4+ CD8+, and 10%-13% CD4- CD8+
7. Immunoglobulin gene rearrangements in B-cell PLL
8. T-cell receptor gene rearrangements in T-cell PLL
9. Cytogenetic abnormalities: 17p abnormalities and 14q+ are frequent for B-cell PLL, inv(14)(q11q32), t(14;14)(q11;q32), and chromosome 8 abnormalities are frequent for T-cell PLL.

FCM, flow cytometry; PLL, prolymphocytic leukemia; TdT, terminal deoxynucleotidyl transferase; IgM, immunoglobulin M; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

The second common cytogenetic abnormality involves chromosome 8, including i(8)(q10), t(8;8)(p21;q11), +8, and 8p+ (8,28,35). Other rare aberrations include del(6q), del(11q), -17, and -22 (28). It is of interest that patients having ataxia telangiectasis with T-cell clones in their blood carrying 14q11 and 14q32 chromosomal rearrangements are at risk of developing T-cell malignancies, including T-PLL (7,36,37). Immunoglobulin gene and TCR gene rearrangements can be detected in B- and T-PLL, respectively (38).

In the current case, the patient had a long history of CLL, but he was not treated until he showed signs of prolymphocytoid transformation—namely, leukocytosis >150,000/μL with 60% prolymphocytes in the peripheral blood. His hematocrit and platelet count also dropped precipitously. Splenomegaly became prominent. His immunophenotype was also characteristic of secondary PLL by showing a high percentage of dual CD19/CD5-positive cells. Although he responded to chemotherapy with decreased leukocytosis, he also became leukopenic with leukopenic fever. The subsequent development of opportunistic infections led to his fatal outcome. He died approximately 1 year after prolymphocytoid transformation.

The salient features for laboratory diagnosis of PLL are summarized in Table 6.19.2.

Clinical Manifestations

PLL is seen about 10% as frequently as CLL, and the median age of PLL patients is 70 years compared with 64 years for those with CLL (19,39). Massive splenomegaly is characteristic of PLL, but hepatomegaly and lymphadenopathy are usually absent or mild in B-PLL (5). In T-PLL, however, lymphadenopathy is present in 26% to 53% of patients, hepatomegaly in 40% to 55%, and skin infiltration in 25% to 27% (7,40). Leukemic meningitis has been reported in a few cases of PLL or CLL/PLL (41). Meningitis may occur in early and advanced stages of the leukemia. Intrathecal chemotherapy alone is effective in clearing up the leukemic cells in the cerebrospinal fluid and eliminating neurologic symptoms caused by leukemic infiltrates (41). At the time of diagnosis, most patients have advanced-stage disease (i.e., Rai Stage IV or Benet Stage C) (42).

A definitive diagnosis is based on the extremely high leukocyte counts, >100,000/μL in about 75% of patients, and the lymphoid cell should be composed of >55% prolymphocytes (4). The median survival in B-PLL is 2 to 3 years compared with 8 years in CLL (43). Patients with PLL of T-cell origin have a median survival of 7 to 7.5 months (7,28) and rarely survive 2 years after diagnosis (44).

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CASE 20 Lymphoplasmacytic Lymphoma

CASE HISTORY

A 65-year-old man was admitted to the hospital for workup of enlarged lymph nodes. Three weeks prior to admission, the patient began to notice multiple enlarged lymph nodes, most predominantly in his right axillary region. One week after, he also noticed the onset of night sweats, fevers, and chills. A lymph node biopsy performed in a local hospital was suspicious of lymphoma. He also reported a 40-pound weight loss in the past year.

Physical examination on admission showed that he had lymphadenopathy in the submandibular, cervical, supraclavicular, axillary, and inguinal regions. These lymph nodes measured from 0.2 to 1.5 cm in diameter, were rubbery and mobile, with mild tenderness but no erythema. No hepatosplenomegaly was present.

Hematologic workup revealed a total leukocyte count of 7,300/μL with a normal differential count. His hematocrit was 36%, hemoglobin 12 g/dL, and platelet count 200,000/μL. Due to the presence of hemagglutination, cold agglutinin was tested, which showed a titer of 3000. Reticulocyte count was 7%, bilirubin 1.4 mg/dL, and lactate dehydrogenase 230 U/L. Immunoglobulin studies revealed immunoglobulin G (IgG) 944 mg/dL, IgM 350 mg/dL, IgA 340 mg/dL, κ 1300 mg/dL, and λ 447 mg/dL. Immunofixation showed a monoclonal IgM-κ pattern (Fig. 6.20.1). A lymph node and a bone marrow biopsy were performed.

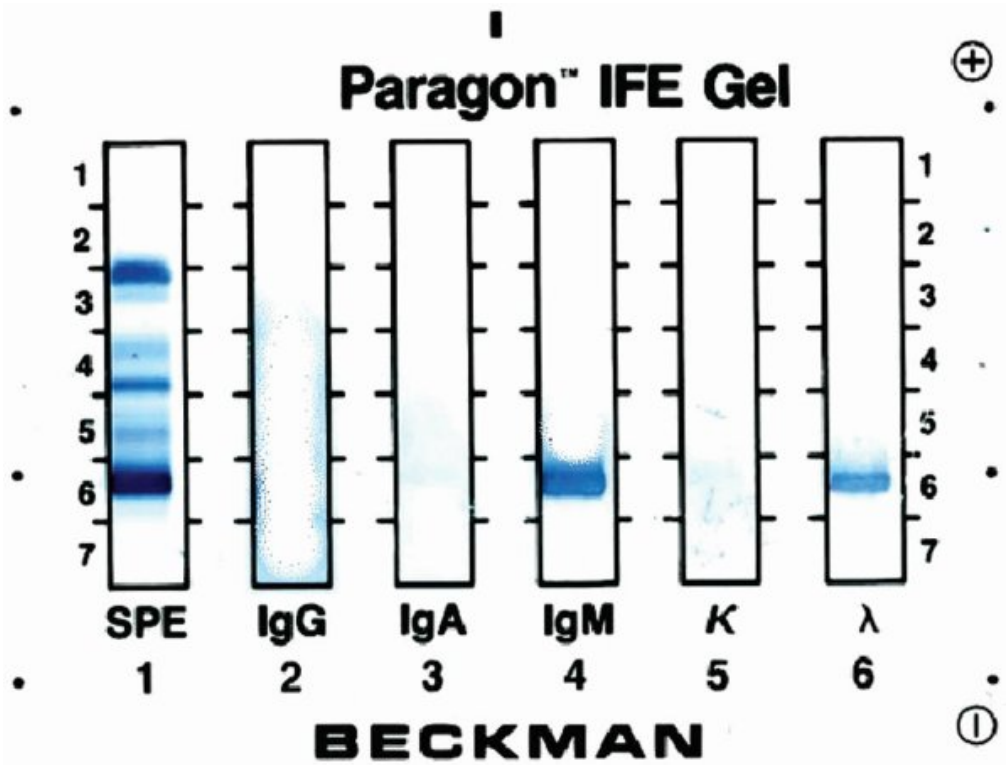

FIGURE 6.20.1 Immunofixation electrophoresis shows a monoclonal immunoglobulin M (IgM)-κ pattern.

FLOW CYTOMETRY FINDINGS

Lymph node biopsy: B-cell markers: CD19 57%, CD20 4%, CD23 8%, surface κ 75%, surface λ 0%, cytoplasmic κ 65%, and cytoplasmic λ 0%. Plasma cell markers: CD38 60%, CD138 10%, CD56 0%. T-cell marker: CD5 0% (Fig. 6.20.2).

IMMUNOHISTOCHEMICAL STAINS

Lymphocytes revealed predominantly CD20 staining with a small percentage of lymphocytes showing CD3 reaction. The plasma cells were positive for κ stain in 90% of cells, and λ in 10%.

CYTOGENETIC STUDY

Karyotyping of the bone marrow specimen revealed a normal male karyotype of 46, XY.

DISCUSSION

In the World Health Organization (WHO) classification, lymphoplasmacytic lymphoma (LPL) and Waldenström macroglobulinemia (WM) are considered synonymous (1). This combined entity is defined as "a neoplasm of small B lymphocytes, plasmacytoid and plasma cells, usually involving bone marrow, lymph nodes and spleen, usually lacking CD5, which has a serum monoclonal protein with hyperviscosity or cryoglobulinemia in most cases" (1). However, the diagnostic criteria are still controversial (2, 3 and 4), and recent cytogenetic evidence appears to indicate that WM is only associated with a subtype of LPL (5,6). LPL cases with t(9;14) are seldom, if ever, associated with WM.

The WHO system also emphasizes that LPL/WM is essentially a diagnosis of exclusion, because many B-cell lymphomas can also mature into plasmacytoid or plasma cells containing immunoglobulin. Making the diagnosis more complicated is the existence of nonsecretory LPL and LPL cases with monoclonal IgG and IgA gammopathy (2,7,8). Therefore, the class of serum monoclonal protein is not specified in the WHO definition.

Another unresolved problem for the diagnosis of LPL/WM is the quantity of the IgM paraprotein used to distinguish WM from other lymphomas and asymptomatic WM or monoclonal gammopathy of unknown significance (MGUS) (4,5). The cutoff suggested for WM ranges from 0.5 g/dL to 3 g/dL (3,4,9,10). However, because patients with a monoclonal spike of <0.5 g/dL may still be symptomatic,

some authors suggested that a cutoff is not necessary as long as a monoclonal IgM protein is present (2,3,5).

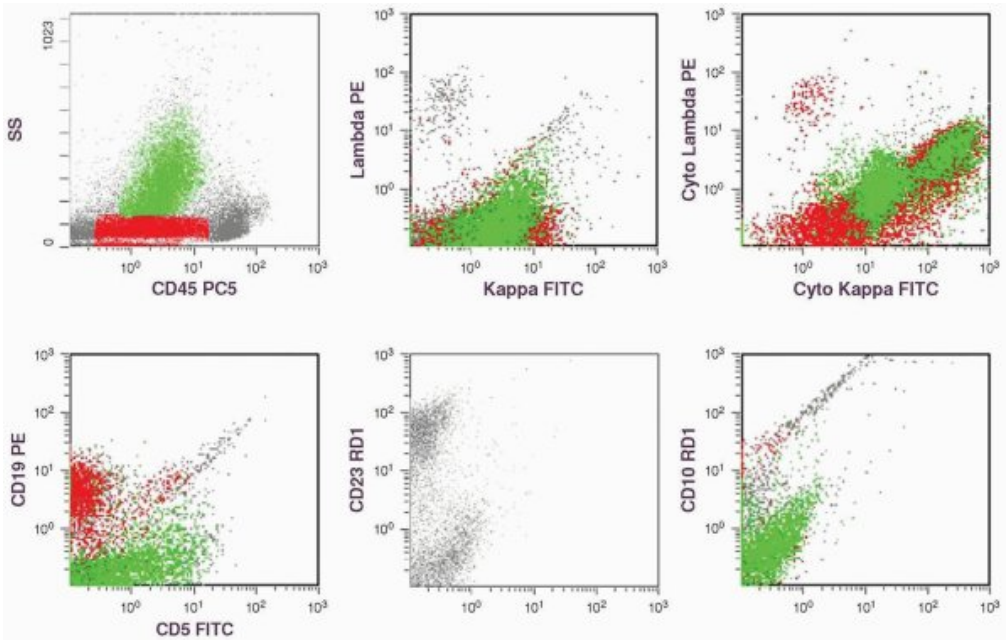

FIGURE 6.20.2 Flow cytometric analysis shows a heterogenous tumor cell population. The *green* cluster represents mainly the cluster of differentiation (CD)19-negative plasma cell population. The *red* cluster represents mainly the CD19-positive B lymphocytes. Both populations show a dual monoclonal surface and cytoplasmic κ pattern and are negative for CD23 and CD10. The *uncolored* cluster represents the normal lymphocyte population. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; RD1, rhodamine.

Although LPL is frequently associated with monoclonal IgM gammopathy, the latter can be seen in many other conditions. In a study of 430 cases, Kyle and Garton (9) found that 56% were MGUS, 17% WM, 7% lymphoma, 5% chronic lymphocytic leukemia, 1% primary amyloidosis, and 14% lymphoproliferative disease. In their classification, the difference between WM and lymphoproliferative disease is based on the quantity of monoclonal IgM protein; the former has >3 g/dL of IgM, whereas the latter has <3 g/dL. In a study of 382 patients, Lin et al. (11) found that 58.9% were LPL/WM, 20.2% were chronic lymphocytic leukemia/small lymphocytic lymphoma, and the remaining were other lymphomas (11).

Morphology

Whereas WM is regarded as a primary bone marrow disorder (Fig. 6.20.3) (2,5,12), LPL frequently presents as a nodebased lymphoma (Fig. 6.20.4). There is no specific histologic pattern to distinguish LPL from other small cell lymphomas, but LPL is characterized by a morphologic continuum of small lymphocytes, plasmacytoid lymphocytes, and plasma cells (4). However, one cell type may be predominant in certain cases, so some authors suggest classifying LPL into lymphoplasmacytoid, lymphoplasmacytic, and polymorphous subtypes (12,13). Owen (5) considers this division as highly subjective and of doubtful prognostic significance.

Because of the lack of pathognomonic features in LPL, the WHO system suggests that LPL should be diagnosed by excluding other lymphomas that may contain considerable numbers of lymphoplasmacytic cells.

Morphologically, small lymphocytic lymphoma/chronic lymphocytic leukemia is most similar to LPL. However, there are subtle differences between these tumors (3,5,14). First, LPL seldom shows proliferation centers. Second, tissue mast cells are usually increased in LPL (Fig. 6.20.5). Third, plasma cells and plasmacytoid cells are more numerous in LPL than in small lymphocytic lymphomas. Fourth, the plasma cells in LPL may contain periodic acid-schiff (PAS)-positive intracytoplasmic and intranuclear inclusions (Russell and Dutcher bodies).

Other small cell lymphomas that show plasma cell or plasmacytoid differentiation include nodal marginal zone B-cell lymphoma, extranodal marginal zone B-cell lymphoma, splenic marginal zone lymphoma, follicular lymphoma, and mantle cell lymphoma (1, 2, 3, 4 and 5,11). The four criteria used to distinguish LPL from small lymphocytic lymphoma

are also applicable in differentiating other lymphomas. In addition, the presence of follicular pattern, the marginal distribution of lymphoma cells, and monocytoid morphology should be looked for to exclude other lymphomas. Plasma cell myeloma should present as exclusively plasma cell infiltration in the bone marrow without lymphadenopathy. When the paraprotein produced is IgM, it should be designated IgM myeloma. In those cases, osteolytic bone lesion is frequently present (15).

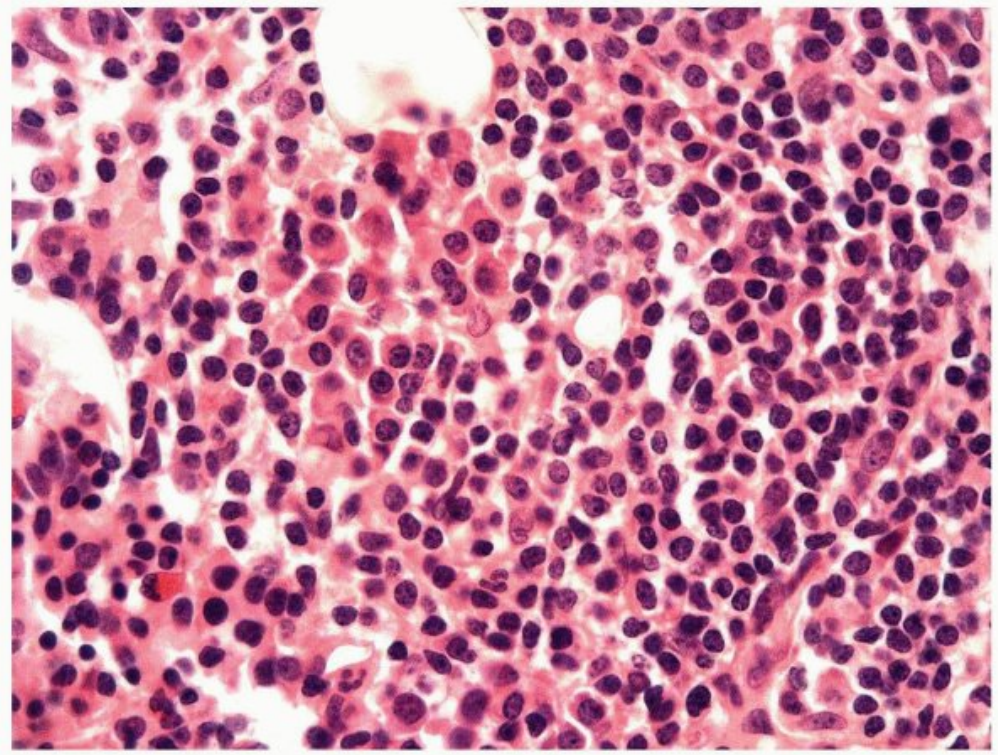

FIGURE 6.20.3 Bone marrow biopsy shows that normal hematopoiesis is replaced by interstitial lymphoplasmacytic infiltration. Hematoxylin and eosin, 60× magnification.

Although small numbers of immunoblasts are frequently present in LPL, the marked increase in immunoblasts usually indicates a transformation to large cell (immunoblastic) lymphoma (Fig. 6.20.6) (1,16, 17, 18 and 19). In rare occasions, LPL may also transform into acute lymphoblastic leukemia (16,20), acute myelogenous leukemia (21, 22 and 23), or chronic myelogenous leukemia (24).

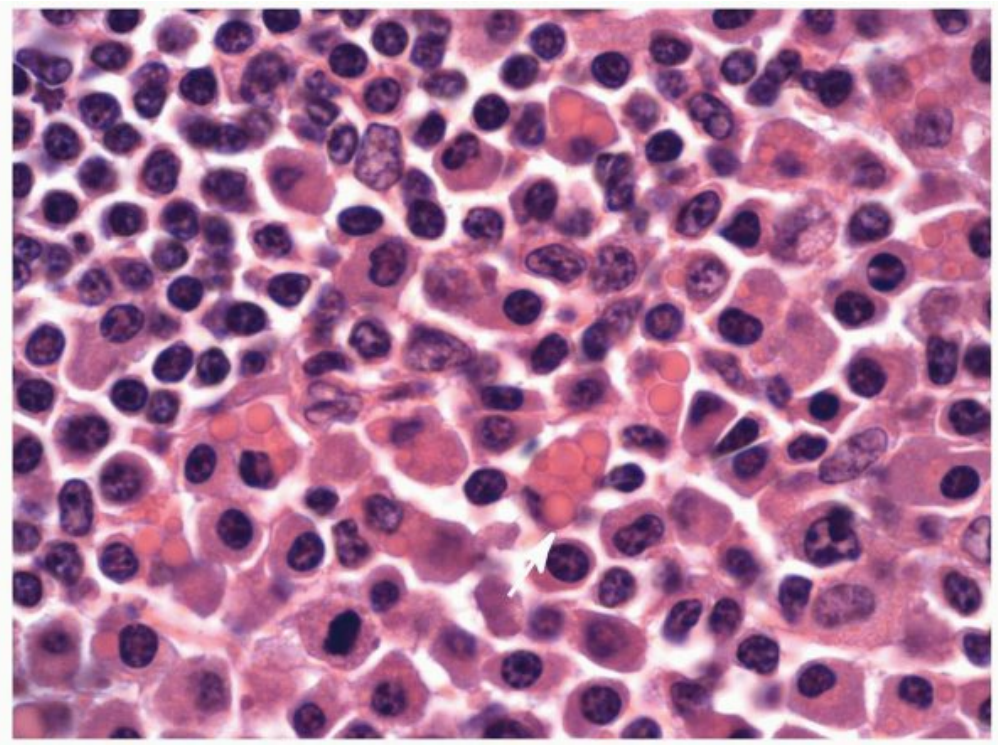

FIGURE 6.20.4 Lymph node biopsy shows a continuum of lymphocytes, plasmacytoid cells, and

plasma cells. Some plasma cells contain numerous Russell bodies (*white arrow*). Hematoxylin and eosin, 100× magnification.

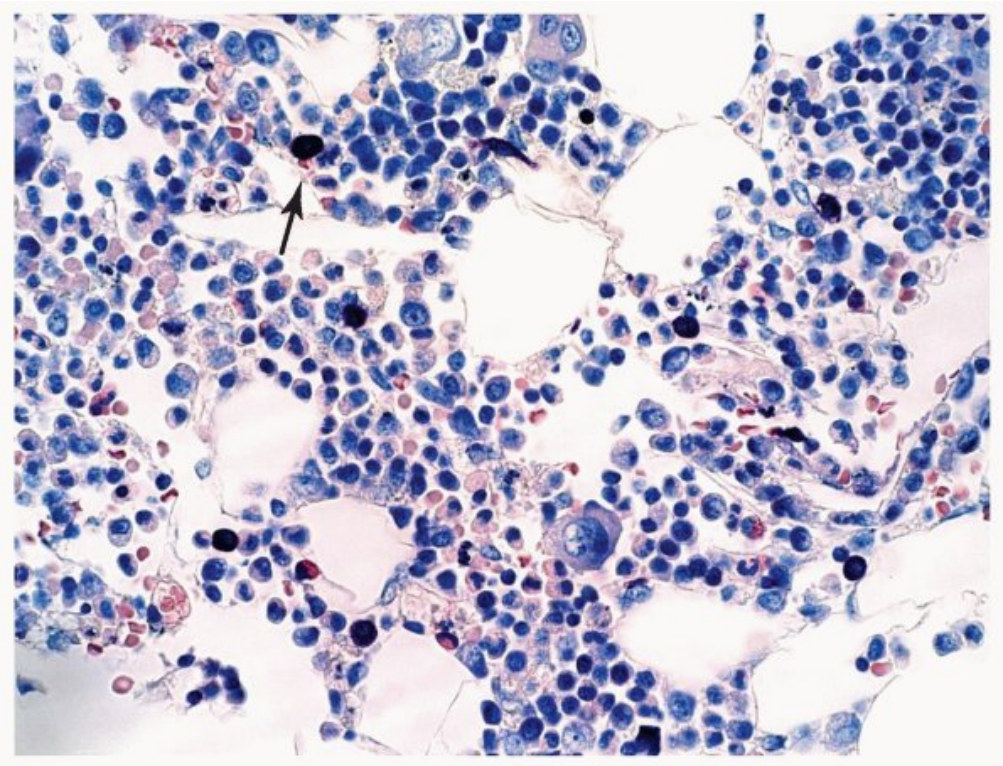

FIGURE 6.20.5 Bone marrow biopsy shows several scattered darkly stained mast cells (*arrow*). Giemsa, 40× magnification.

By definition, WM is a bone marrow disorder, but only 65% of LPL patients have bone marrow involvement in one study (3). However, only 2% of WM patients show osteolytic bone lesions (25). The infiltration pattern in the bone marrow is usually nodular or interstitial, but paratrabecular and diffuse patterns can also be seen in a small number of cases (1,4,5). When a paratrabecular pattern is present in patients with lymphadenopathy, follicular lymphoma should be considered (5).

In the peripheral blood, lymphoplasmacytic cells may be present, particularly in WM and less frequently in LPL. However, more striking features can be demonstrated in the erythrocytes, which may show rouleaux formation, depending on the quantity of the paraprotein. When the monoclonal IgM functions as a cold agglutinin, hemagglutination may occur (26).

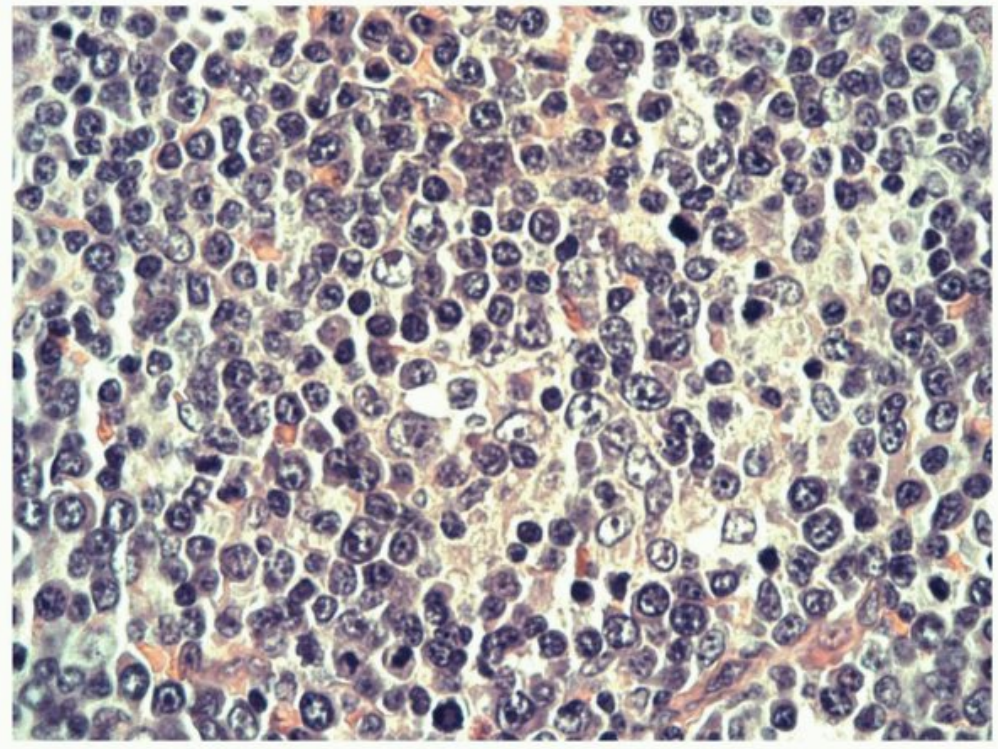

FIGURE 6.20.6 Lymph node biopsy shows immunoblastic transformation of lymphoplasmacytic lymphoma (LPL). Hematoxylin and eosin, 60× magnification.

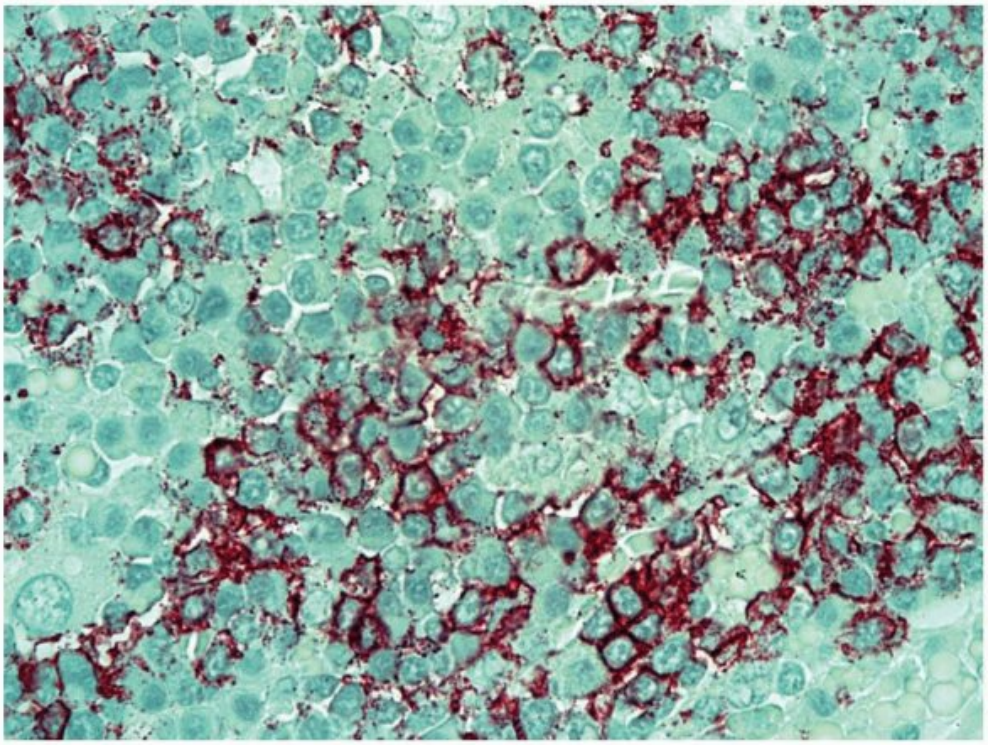

FIGURE 6.20.7 Lymph node biopsy shows CD20 staining on small lymphocytes but not plasma cells. Immunoalkaline phosphatase, 60× magnification.

In the current case, both the lymph node biopsy and the bone marrow biopsy showed a continuum of small lymphocytes, plasmacytoid lymphocytes, and plasma cells. Some of the plasma cells revealed bunches of Russell bodies. Immunohistochemical stains demonstrated a B-cell population with positive CD20 staining and a plasma cell population that presented with a monoclonal κ immunophenotype. However, the dual surface and cytoplasmic monoclonal immunoglobulin pattern, as demonstrated by flow cytometry, is most helpful in diagnosing LPL/WM in this case.

According to the laboratory data, the patient had anemia, elevation of reticulocyte count, serum bilirubin, and lactate dehydrogenase. These findings are consistent with hemolytic anemia. This phenomenon can be explained by the presence of high-titered cold agglutinin, which, in turn, is probably associated with the monoclonal IgM. It is interesting to see that the low quantity of IgM, such as in this case, may cause a disproportionally high titer of cold agglutinin.

Immunophenotype

LPL/WM is characterized by the coexistence of two immunophenotypes: mature lymphocytes and plasma cells (10). In immunohistochemical stains, the lymphocytes show B-cell markers (CD20) (Fig. 6.20.7) and the plasma cells demonstrate a monoclonal cytoplasmic immunoglobulin pattern (Fig. 6.20.8). By flow cytometry, the immunophenotype represents the combination of these two cell types; namely, the coexistence of both B-cell markers and a monoclonal cytoplasmic immunoglobulin pattern. Because of the presence of a spectrum of lymphocytes, plasmacytoid lymphocytes, and plasma cells, both surface and cytoplasmic monoclonal immunoglobulin patterns are present. More specifically, the percentages of cells with monoclonal surface immunoglobulin and monoclonal cytoplasmic immunoglobulin overlap (for instance, 70% of the former and 50% of the latter). This pattern may be explained by the existence of some lymphoplasmacytic cells that express both surface and cytoplasmic immunoglobulin. San Miguel et al. (10) suggest that the coexistence of clonal B lymphocytes and clonal plasma cells is specific for WM and has not been reported in any other lymphoproliferative disorders, including LPL. However, others have found that this phenomenon is also commonly seen in LBL cases (1).

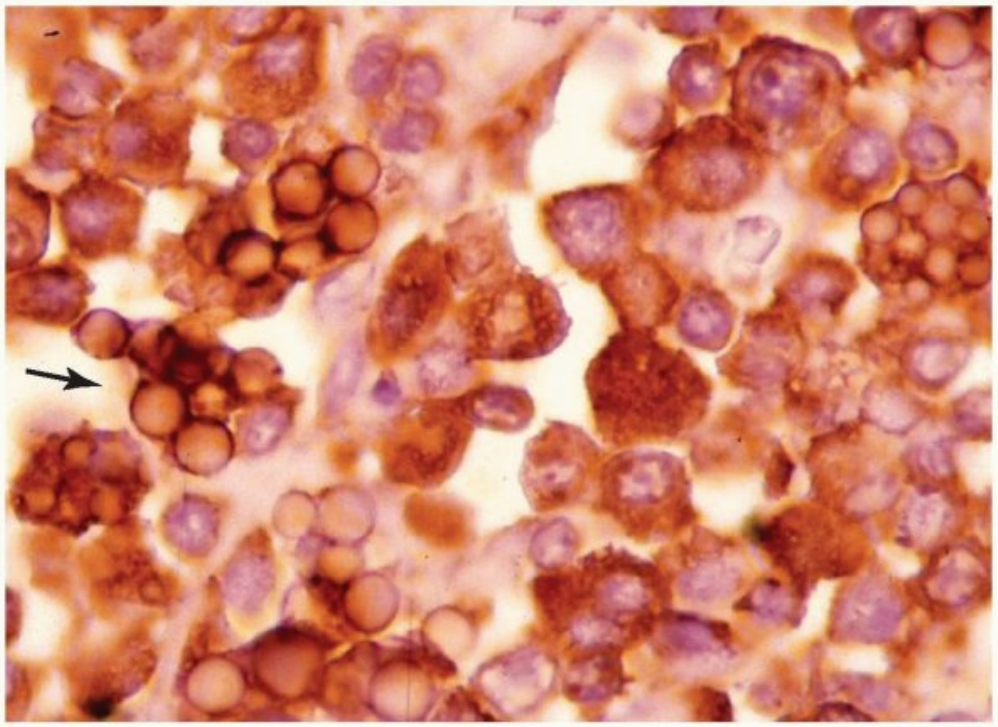

FIGURE 6.20.8 Lymph node biopsy shows κ light-chain staining of the plasma cells, but not the small lymphocytes. Many Russell bodies (*arrow*) are highlighted by the staining. λ light-chain stain is negative (data not shown). Immunoperoxidase, 100× magnification.

The positive B-cell markers demonstrated in the lymphoid cells include CD19, CD20, CD22, CD24, and human leukocyte antigen-DR (HLA-DR) (3,4,9,11,14,16,27). CD27, CD79a, paired Box-containing family of genes (PAX-5), and B-cell lymphoma (BCL)-2 have also been demonstrated by immunohistochemistry (4,5). The presence of a memory B-cell marker (CD27) is consistent with the hypothesis that LPL/WM is derived from postgerminal center B cells (5). A high percentage of cases also express FMC-7 and CD25. The presence of the above markers may raise the possible diagnosis of hairy cell leukemia, but other markers for this disease, such as CD103 and CD11c, are usually negative.

Other negative markers that are useful in the differential diagnosis include CD5, CD10, CD23, and cyclin D1, which may help to exclude small lymphocytic lymphoma/chronic lymphocytic leukemia, mantle cell lymphoma, and follicular lymphoma (4,10). However, San Miguel et al. (10) found that symptomatic WM cases frequently showed upregulation of CD5 and downregulation of both FMC-7 and CD25. Konoplev et al. (28) reported that CD11c and CD23 were present in 81% and 61% of 75 case of LPL/WM, respectively.

Besides the demonstration of a monoclonal cytoplasmic pattern, plasma cells usually show an immunophenotype of CD19+, CD38+, CD45+, CD56- (10). In a subset of plasma cells, CD20 is also positive. This immunophenotype differs from that of the plasma cells in plasma cell myeloma, which usually shows dim or negative CD45, negative CD19, and frequently positive CD56.

TABLE 6.20.1

Salient Features for Laboratory Diagnosis of LPL

1. A small cell lymphoma with significant plasmacytic and/or plasmacytoid cell infiltration

2. No special patterns, such as follicular, marginal zone, and proliferation center, diagnostic for other lymphomas are present.

3. Bone marrow may show interstitial, nodular, diffuse, and occasional paratrabecular infiltration pattern by lymphoplasmacytic cells.

4. Mast cells are frequently increased in bone marrow.

5. The plasma cells may contain prominent Russell bodies or Dutcher bodies.

6. Immunohistochemical stains demonstrate two populations: CD20-positive small lymphocytes and monoclonal κ- or λ-positive plasma cells.

7. Flow cytometry shows a dual monoclonal surface and cytoplasmic immunoglobulin pattern.

8. Characteristic immunophenotype includes positive reactions to CD19, CD20, CD22, CD24, CD38, and HLA-DR and negative reactions to CD5, CD10, CD23, and cyclin D1.

9. A monoclonal IgM gammopathy is supportive of the diagnosis of LPL, but many cases of LPL are nonsecretory for monoclonal immunoglobulin.

10. Fifty percent of the nonsecretory LPL show a karyotype of t(9;14)(p13;q32).

11. The only recurrent genetic aberration in WM cases is deletion of the long arm of chromosome 6 (-6q21).

LPL, lymphoplasmacytic lymphoma; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; IgM, immunoglobulin M; WM, Waldenström macroglobulinemia.

Mast cells are frequently increased in cases of LPL/WM, but they usually reveal a normal immunophenotype (CD2-, CD25-, CD35-, CD63+dim, and CD65+ dim); thus mast cells are not part of the malignant clone (10). However, other studies found that mast cells may induce WM cell proliferation and/or tumor colony formation through, in part, constitutive expression of CD40 ligend (12).

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry can demonstrate the double surface and cytoplasmic immunoglobulin pattern and is very helpful in diagnosing LPL/MW. However, immunohistochemistry can demonstrate two cell populations: The lymphoid cells express CD20, while the plasma cells show a monoclonal immunoglobulin pattern. Therefore, both techniques are helpful in achieving a correct diagnosis, but the results obtained from flow cytometry are sometimes more definitive than those from immunohistochemistry.

Molecular Genetics

Whereas LPL is closely related to WM, the nonsecretory LPL is a biologically distinct entity. A translocation t(9;14) (p13;q32) has been reported in up to 50% of those cases (29,30). However, this abnormality can also be detected in other lymphomas with plasmacytoid differentiation (29,31). This translocation is characterized molecularly as the translocation of the PAX-5 gene in chromosome 9 to the IgH gene in chromosome 14. PAX-5 encodes the B-cell-specific activator protein (BSAP) that is important in early B-cell development. The PAX-5 product abrogates the production of the J peptide (31) and downregulates IgH transcription (32,33). Therefore, no monoclonal IgM gammopathy is present in LBL cases with this translocation. In contrast, t(9;14) and IgH translocation have not been found by karyotyping and fluorescence hybridization technique in pure WM cases (3,5,6).

The only recurrent genetic aberration in WM cases is deletion of the long arm of chromosome 6 (6). Up to 50% of WM cases have this deletion, and most cases have deletion at 6q21 (6,34).

LBL/WM have immunoglobulin heavy- and light-chain gene rearrangement with somatic hypermutation in the variable regions (6,8,35), which indicates that the tumor cells are of the postgerminal center origin. However, the switch μ gene is not rearranged. The lack of

physiologic isotype switching explains why only monoclonal IgM protein is produced in WM (6). In DNA ploidy studies, aneuploidy is seldom found in WM cases except for the aggressive variants of WM (6).

Although plasma cell myeloma seems to be close to WM in terms of production of monoclonal gammopathy and of bone marrow involvement by neoplastic plasma cells, they are very different genotypically. For instance, myeloma may show IgH translocation and switch μ chain gene rearrangement, and is frequently aneuploid (5,6).

The salient features for laboratory diagnosis of LPL are summarized in Table 6.20.1.

Clinical Manifestations

LBL/WM is usually seen in elderly patients with a median age of 63 years (range 25 to 92), and there is a slight predominance of male patients (36). The incidence rate is higher among Caucasians, and only 5% of all patients are of African descent (12). Recent evidence suggests that

LBL/WM may be a familial disease showing multigenera-tional clustering of WM and other B-cell-lymphoproliferative diseases. In a study of 257 patients, approximately 18.7% had a first-degree relative with such diseases (37).

WM differs from plasma cell myeloma in its slowly progressive clinical course; the patient may not require any treatment for several years or may require only plasmapheresis to reduce the concentration of macroglobulin. The initial clinical symptoms are usually vague and non-specific, such as weakness, anorexia, and weight loss. However, in the later stage, there are specific clinical manifestations that are related to the paraprotein and subsequent hematologic changes.

The best known symptom in WM is the hyperviscosity syndrome. However, in most patients this syndrome only appears when the serum viscosity reaches 4 units (4 times as viscous as water) or when IgM concentration exceeds 3 g/dL (25,27). In contrast, patients with a high concentration of IgA or IgG (≥5 g/dL) may also have this syndrome. In addition, the molecular configuration also contributes to high viscosity; for instance, hyperviscosity is frequently associated with the polymers of IgA and with molecular asymmetry of IgG (15). The symptoms of this syndrome include bleeding diathesis, retinopathy, hypervolemia, congestive heart failure, and various neurologic symptoms that may progress from headache and confusion to coma. The blurred vision seen in WM patients is thought to reflect rouleaux formation or “sludging” in ocular vessels (38).

The bleeding tendency in WM is multifactorial. Evidence suggests that coating the platelets by monoclonal IgM protein may cause platelet aggregation defect (39), binding of IgM molecules to fibrin monomers may inhibit fibrin polymerization (39), and immunoadsorption of factor VIII by neoplastic lymphocytes also interferes with the normal coagulation process (40).

Some monoclonal macroglobulins may function as antibodies against red cell antigens I and i (cold hemagglutinins), thus causing hemagglutination (Fig. 6.20.9) in the cold and leading to hemolysis (26). In cases showing these autoantibodies without hepatosplenomegaly, lymphadenopathy, and bone marrow infiltration, the condition is designated as the cold agglutinin syndrome. In 15% of patients, the monoclonal macroglobulin protein may precipitate in the cold (Fig. 6.20.10); this type of protein is called cryoglobulin (type 1) (27,41). Patients with cryoglobulinemia may show the Raynaud phenomenon, cold hypersensitivity, cold urticaria, cold purpura, or glomerulonephritis in <5% of patients (25,27).

Renal insufficiency can be seen in patients with WM, but it is less frequent than in patients with plasma cell myeloma, because Bence-Jones proteinuria is usually <2 g/24 hours or 1.0 g/dL, and hypercalcemia is less frequently seen in WM cases (27,42). In myeloma, tubular degeneration and cast formation are the major changes in the kidney, whereas glomerular lesions due to IgM deposition are the more constant finding in WM (42). Other extramedullary sites involved in WM include spleen, skin, lung, tonsil, colon (Fig. 6.20.11), liver, gallbladder, and soft tissues (12).

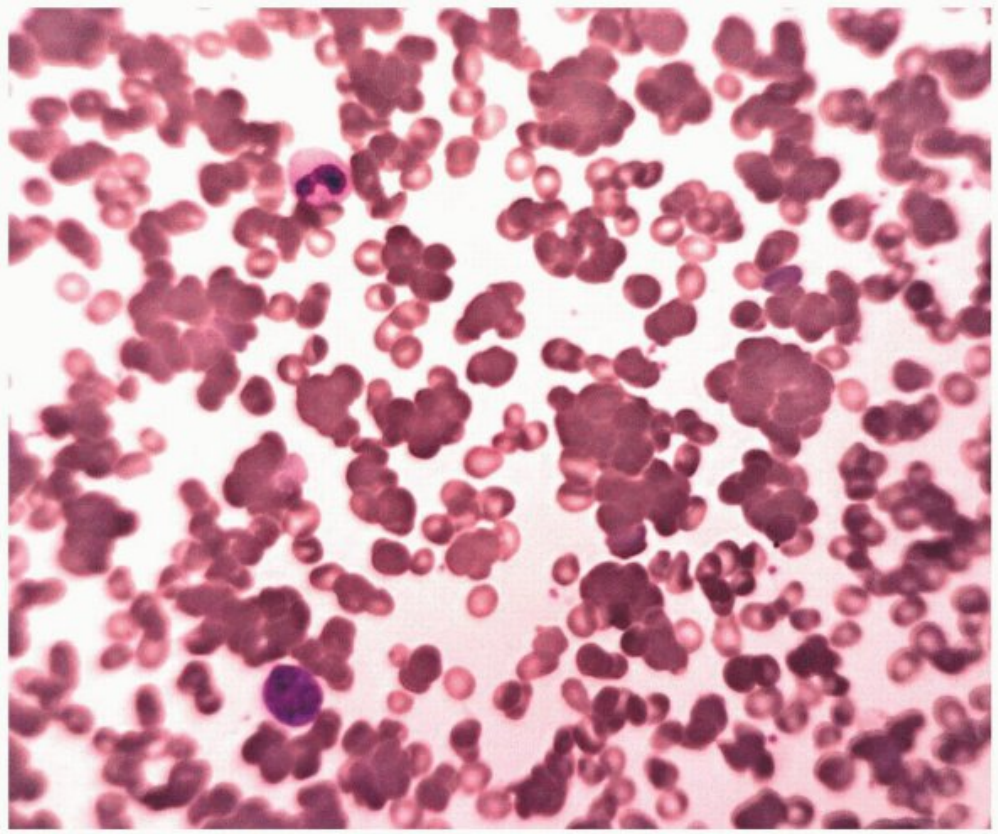

FIGURE 6.20.9 Peripheral blood smear shows hemagglutination. Wright-Giemsa, 40× magnification.

Approximately 10% of patients may develop polyneuro-pathy (27). The pathogenesis of neuropathy may be due to plasma cell infiltration, antibodies against various glycoproteins or glycolipids, or the peripheral nerves and amyloid deposition. Amyloid light chain (AL) amyloidosis should be suspected in all LPL patients with nephrotic syndrome, cardiomyopathy, hepatomegaly, or peripheral neuropathy (12).

In cases of LPL without macroglobulinemia, the above-mentioned clinical symptoms may not be manifested, and the clinical course is similar to other low-grade lymphoproliferative disorders.

As in plasma cell myeloma, Kaposi sarcoma-associated herpesvirus has been identified in bone marrow biopsies from patients with WM, and a viral interleukin-6 produced by Kaposi sarcoma-associated herpesvirus may

participate in the proliferation of malignant cells in WM (16,27). When LPL is associated with type II mixed cryoglobulinemia, it may be associated with hepatitis C virus infection; treatment of patients with interferon to reduce viral load has shown to induce regression of the lymphoma (43).

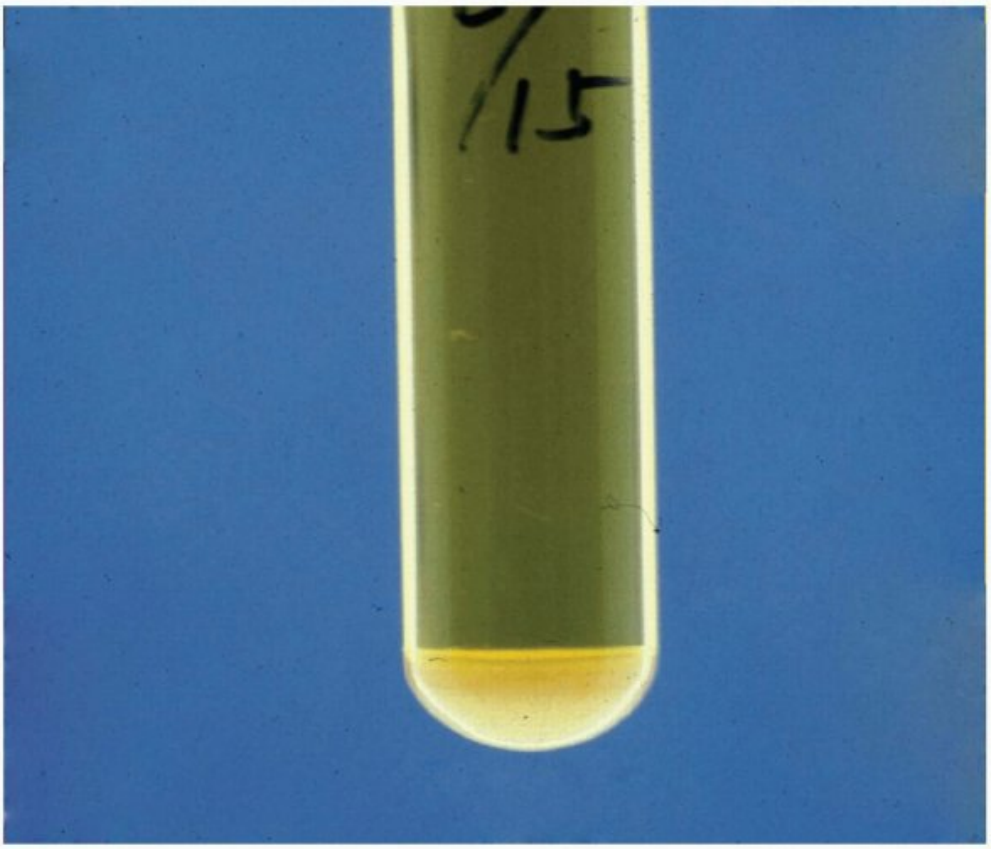

FIGURE 6.20.10 Test tube of serum stored at 4°C overnight showing cryoprecipitate at the bottom.

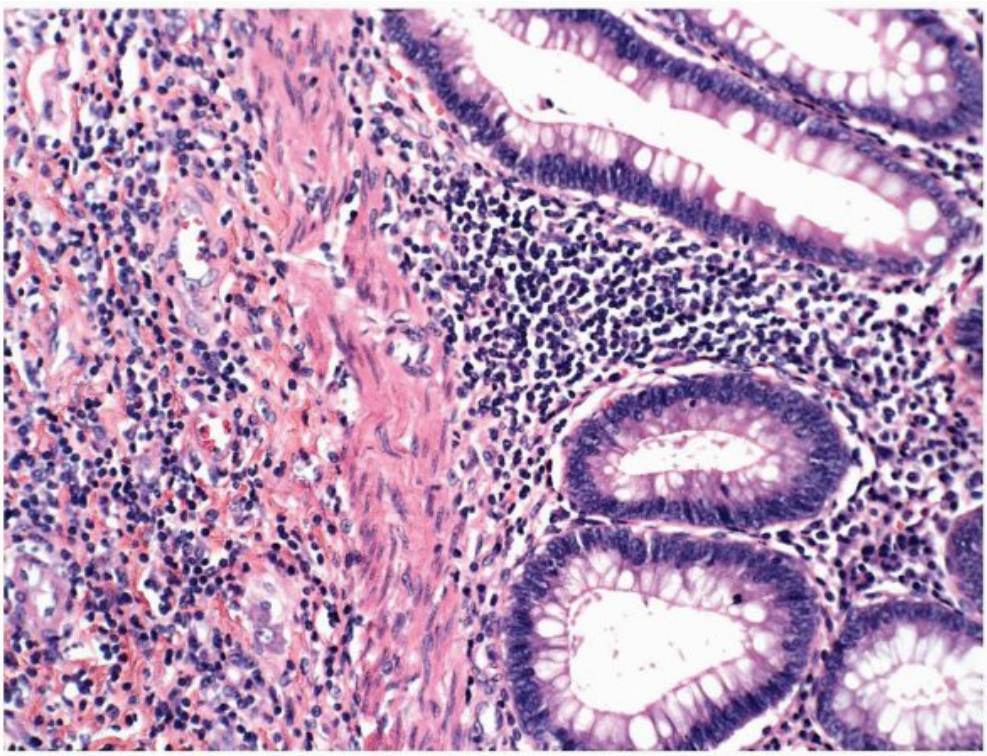

FIGURE 6.20.11 Colon biopsy shows mucosal and submucosal infiltration by lymphoplasmacytic

lymphoma cells. Hematoxylin and eosin, 20× magnification.

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CASE 21 Plasma Cell Myeloma and Plasmacytoma

CASE HISTORY

An 81-year-old man presented with back pain as well as right arm and shoulder pain. He was admitted to the hospital for further examination 3 years ago. During the first admission, a diagnosis of multiple myeloma (MM) was made by bone marrow biopsy. Bone survey showed lytic lesions in the right proximal femur, right humerus, and skull. Degenerative disk disease was found in the cervical spine.

Laboratory finding on admission included decreased immunoglobulin (Ig) levels in IgG (368 mg/dL), IgA (20 mg/dL), and IgM (9 mg/dL). Light-chain Igs were not quantified. However, immunofixation demonstrated monoclonal gammopathy, and urine electrophoresis revealed Bence-Jones protein. Serum calcium at that time was 9.8 mg/dL.

After that, the patient underwent multiple therapeutic regimens, including palliative radiation therapy to the right arm and treatment with prednisone, melphalan, thalidomide, and bortezomib. After extensive treatment, the patient had a complete remission for approximately 1 year with <5% normal-looking plasma cells in the bone marrow on two occasions.

One year later, the patient suffered from compression fracture of the L2 vertebral body, and follow-up study found that he had elevated β_2 microglobulin (B_2M; 10.8 mg/L) and lactate dehydrogenase (LDH; 837 IU/L). Hematologic workup revealed a hematocrit of 27.4%, hemoglobin 9.5 g/dL, and platelets 162,000/μL. The total leukocyte count was 6,900/μL with a normal differential count, but rouleaux formation was present. His serum calcium was 8.8 mg/dL and creatinine 1.2 mg/dL. Serum protein electrophoresis and immunofixation identified a monoclonal λ light-chain gammopathy. A bone marrow biopsy confirmed the relapse of myeloma showing 70% of plasma cells, and the normal hematopoietic cells were largely replaced by sheets of tumor cells. The patient continued to receive chemotherapy for myeloma and awaited vertebroplasty.

FLOW CYTOMETRY FINDINGS

Bone marrow: Cluster of differentiation (CD)38 69%, CD138 62%, CD38/CD138 62%, CD56 62%, CD45 7%, surface κ 2%, surface λ 1%, cytoplasmic κ 2%, cytoplasmic λ 78%.

CYTOGENETIC STUDY

Cytogenetic analysis of unstimulated cultures revealed a male karyotype with an apparently normal GTG banding pattern in all cells analyzed; the karyotype was 46, XY.

TABLE 6.21.1

WHO Criteria for the Diagnosis of Myeloma

Major Criteria
Marrow plasmacytosis (>30%)
Plasmacytoma on biopsy
M component
Serum: IgG >3.5 g/dL, IgA >2 g/dL
Urine: >1 g/24 h of Bence-Jones protein
Minor Criteria
Marrow plasmacytosis (10% to 30%)
M component: present but less than above
Lytic bone lesions
Reduced normal Ig (<50% normal): IgG <600 mg/dL, IgA <100 mg/dL, IgM <50 mg /dL

WHO, World Health Organization; Ig, immunoglobulin.

DISCUSSION

Plasma cell myeloma or multiple myeloma (MM) accounts for 10% of all malignant hematologic neoplasms and about 1% of all cancer-related deaths in Western countries (1,2). In the United States, it is the most common lymphoid malignancy in blacks and the second most common in whites (3). The incidence of MM had been increased 45% during the period from 1940 through 1970, but the incidence appears to have become stable since 1992 (4).

MM is a monoclonal plasma cell neoplasm that produces monoclonal gammopathy in most cases except for the subtype of nonsecretory myeloma and some cases of solitary plasmacytoma. On the basis of clinical symptoms, MM can be divided into symptomatic myeloma and asymptomatic myeloma (5). A premalignant condition of MM is called monoclonal gammopathy of undetermined significance (MGUS) or monoclonal gammopathy, unattributed/unassociated (MG[u]), as recently suggested by the International Myeloma Working Group (5). A more complicated classification is devised by the World Health Organization (WHO) and will be discussed later.

WHO defines the major diagnostic criteria for MM as >30% plasma cells in the bone marrow or plasmacytoma on biopsy with the presence of an M component (paraprotein) in the serum and/or urine (Table 6.21.1) (3). The cutoff level of serum M component for IgG is >3.5 g/dL and for IgA, >2 g/dL. The minor criteria include marrow plasmacytosis in the range of 10% to 30%, the presence of an M component at a lower level than that defined in the major criteria, the presence of lytic bone lesions, and reduction of normal Igs to <50% of normal. Specifically, it is <600 mg/dL for IgG, <100 mg/dL for IgA, and <50 mg/dL for IgM. The diagnosis of MM requires a minimum of one major and one minor criterion or three minor criteria including marrow plasmacytosis and presence of an M component (3).

The International Myeloma Working Group suggests (i) the use of a universal standard of 3 g/dL for all classes of paraprotein and (ii) 10%

clonal plasma cells in the bone marrow as the cutoff for asymptomatic (smoldering) myeloma (5). This classification does not set cutoff points for symptomatic MM, but requires the presence of end organ damage in addition to serum and/or urine paraprotein and bone marrow plasmacytosis as the diagnostic criteria. The rationale behind these criteria is that 40% of patients with symptomatic myeloma may have paraprotein <3 g/dL, and 5% of patients may have <10% plasma cells in the bone marrow (5).

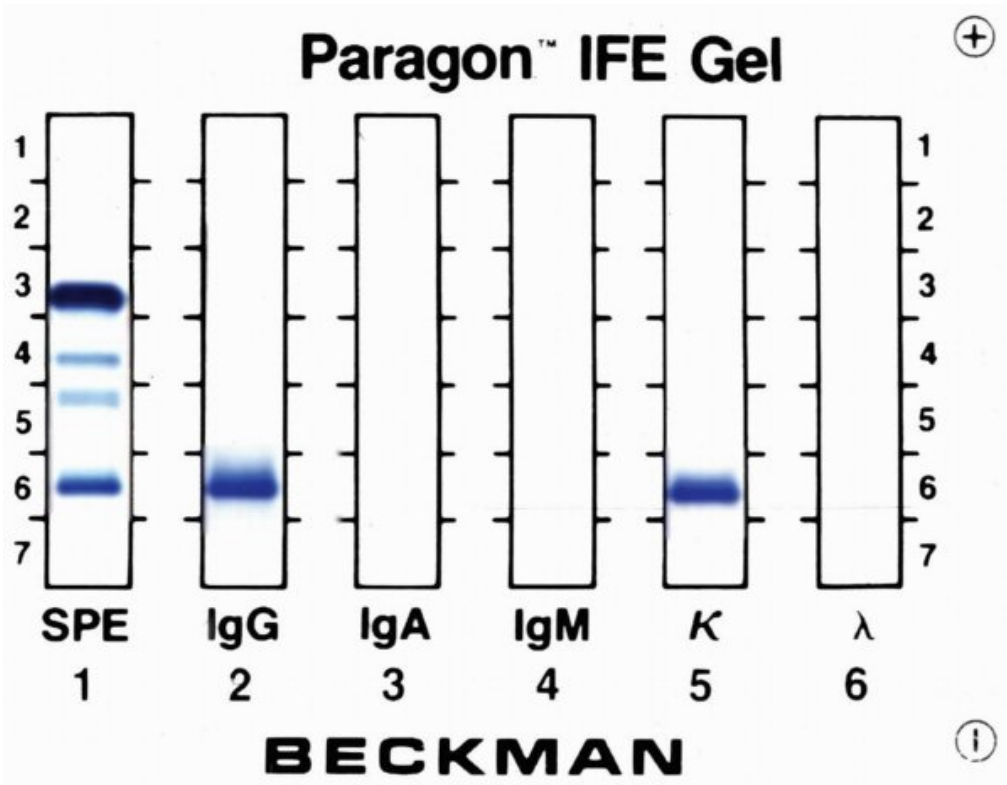

FIGURE 6.21.1 Immunofixation electrophoresis shows that the monoclonal band demonstrated in the first lane by electrophoresis reacts only to immunoglobulin (Ig)G and κ antibodies, indicating monoclonal gammopathy. SPE, serum protein electrophoresis; IFE, immunofixation electrophoresis.

Morphology

The diagnosis of MM is usually suspected when monoclonal gammopathy in the serum (Fig. 6.21.1) and/or Bence-Jones protein in the urine is found (Figs. 6.21.2 and

6.21.3). However, as will be discussed later, several types of plasma cell dyscrasia show no or inconspicuous monoclonal gammopathy. In contrast, cases of amyloidosis may show monoclonal gammopathy, yet it is not necessarily neoplastic. Therefore, the demonstration of myeloma cells is still the most important and definitive criterion for the diagnosis of MM.

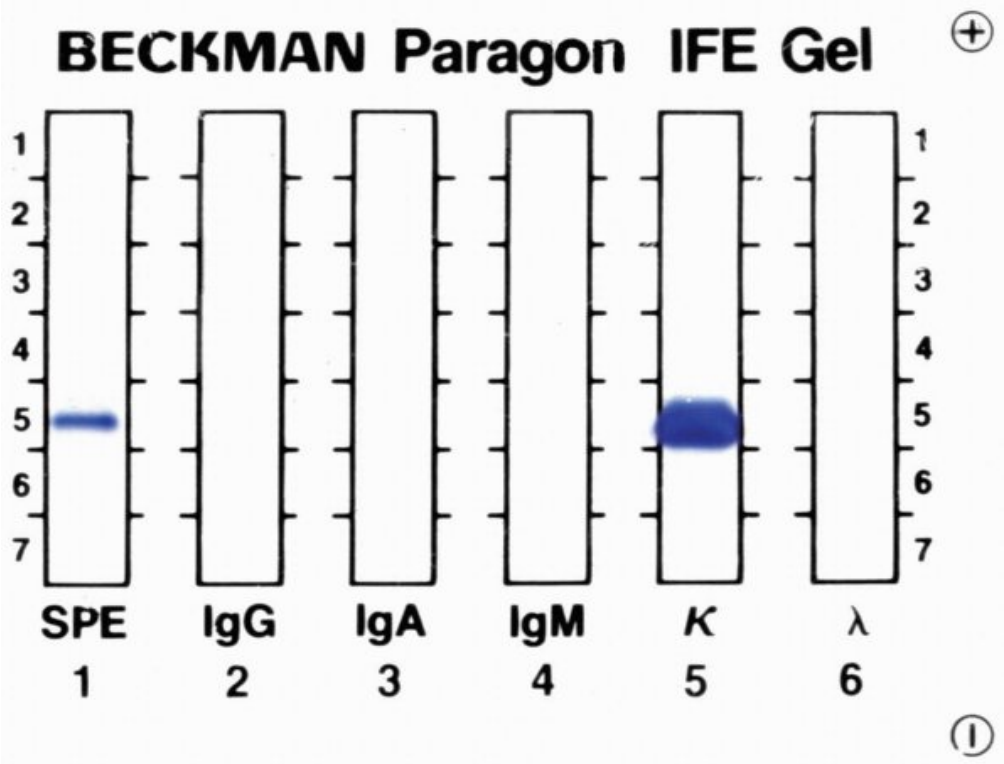

FIGURE 6.21.2 Immunoelectrophoresis shows that the monoclonal band demonstrated in urine electrophoresis (*lane 1*) reacts only to κ light-chain antibody, consistent with Bence-Jones proteinuria. SPE, serum protein electrophoresis; IFE, immunofixation electrophoresis; Ig, immunoglobulin.

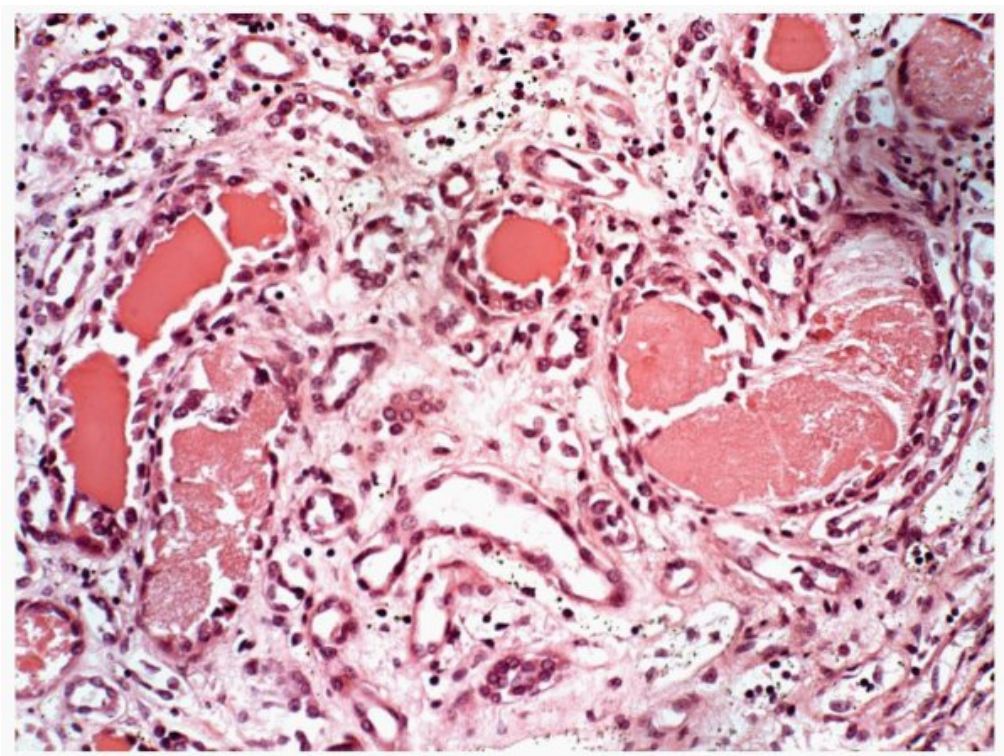

FIGURE 6.21.3 Kidney section from an autopsy case of multiple myeloma shows Bence-Jones

protein casts inside the renal tubules. Hematoxylin and eosin, 20× magnification.

In the peripheral blood, the most striking feature is marked rouleaux formation, which is related to the quantity of paraprotein and the high erythrocyte sedimentation rate that is usually present in MM. Plasma cells are usually not seen in peripheral blood. However, a low percentage of plasma cells can be detected in 15% of MM cases. If >20% or 2,000/μL of plasma cells are present, it is designated plasma cell leukemia (PCL) (Fig. 6.21.4) (5).

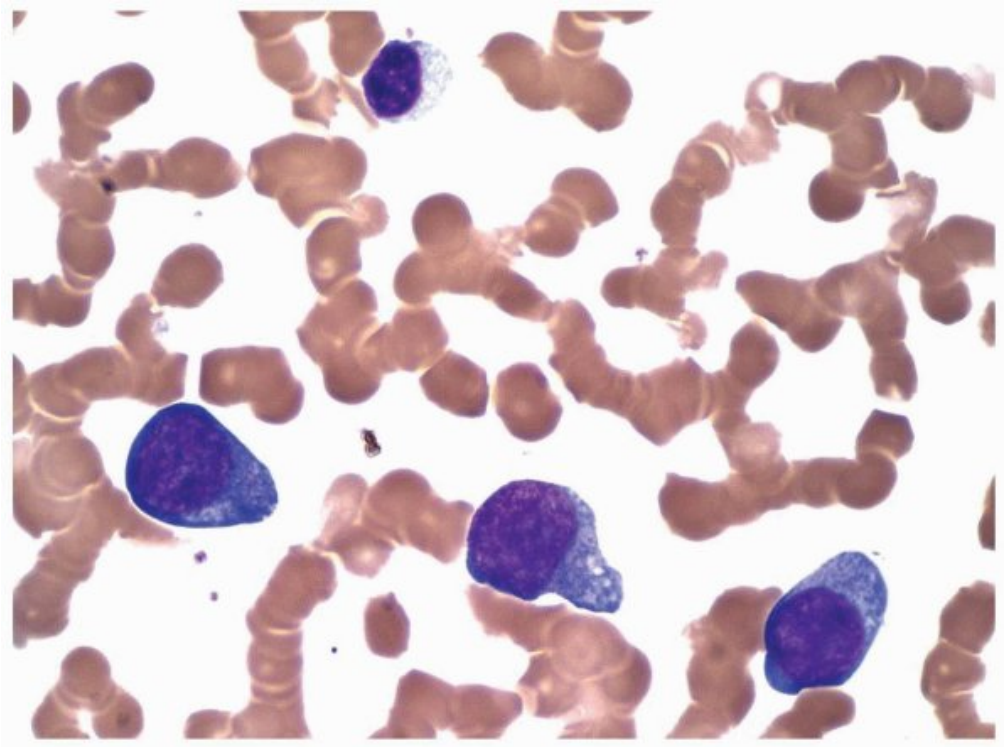

FIGURE 6.21.4 Peripheral blood smear from a case of plasma cell leukemia shows three plasmablasts and one plasma cell with prominent rouleaux formation in the background. Wright-Giemsa, 100× magnification.

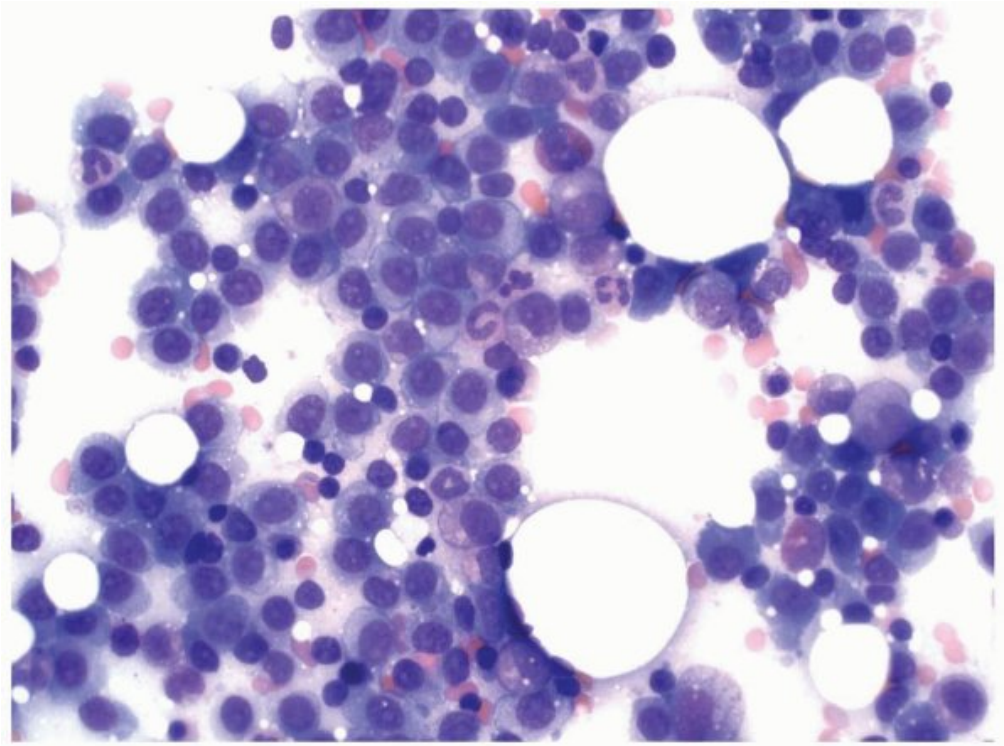

FIGURE 6.21.5 Bone marrow aspirate shows clusters of myeloma cells. Wright-Giemsa, 40× magnification.

The bone marrow usually shows preservation of normal hematopoietic elements, and is only partially replaced by the myeloma cells, with an average of 20% to 36% plasma cells (Fig. 6.21.5) (6). As mentioned before, the percentage of plasma cells in symptomatic myeloma cases may vary (5); therefore, the distribution pattern is a more reliable criterion for the diagnosis. Reactive plasmacytosis usually shows a cluster of 5 to 6 cells around the marrow arterioles or the so-called satellitism. In MM cases, larger clusters, nodules, or sheets of plasma cells are present randomly (mass effect) (Fig. 6.21.6) (3). Normal hematopoietic cells are usually not seen in the "tumor mass." In patients with radiologic evidence of osteolytic bone lesion, osteoclastic activity may be demonstrated histologically (Fig. 6.21.7).

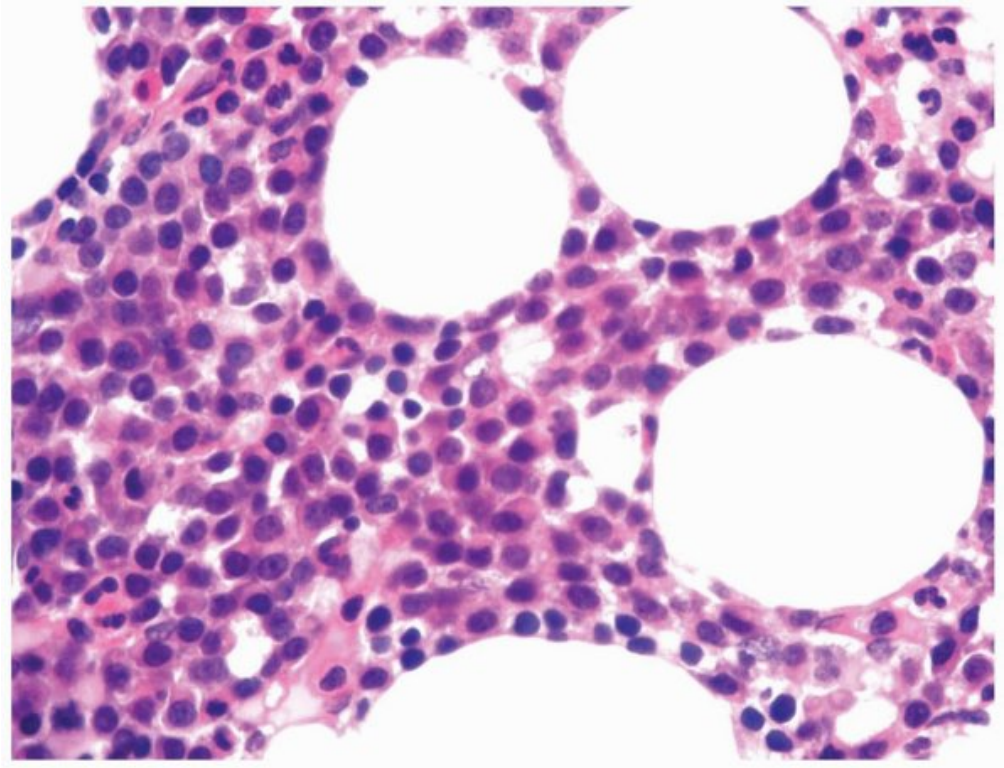

FIGURE 6.21.6 Bone marrow biopsy shows sheets of myeloma cells. Hematoxylin and eosin, 60× magnification.

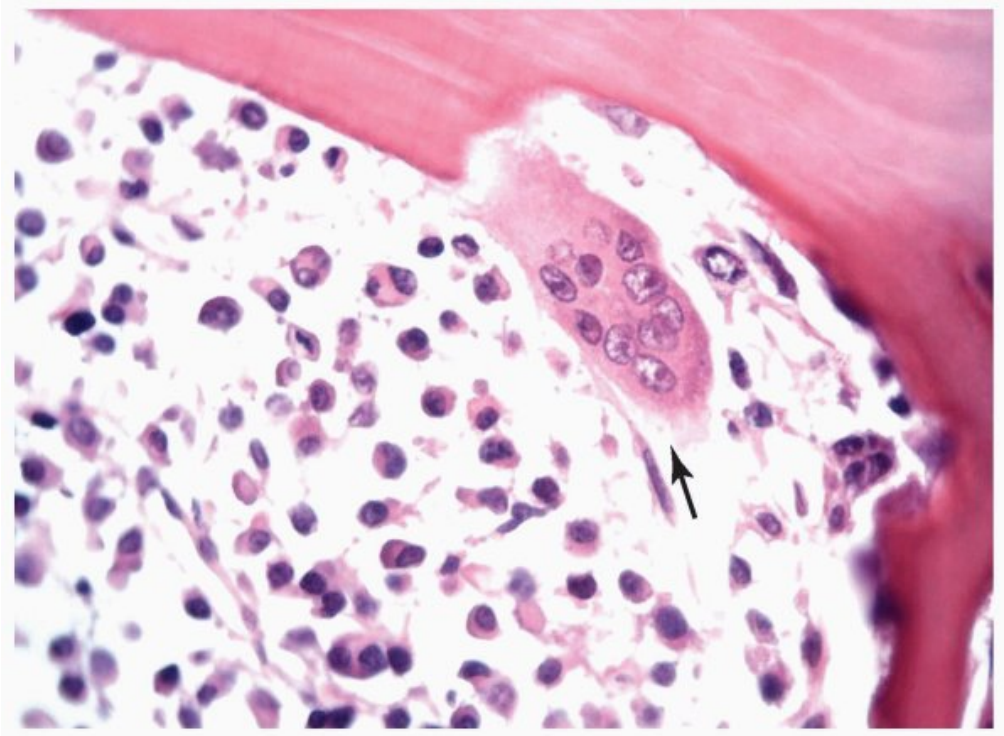

FIGURE 6.21.7 Bone marrow biopsy shows osteoclastic lesion with an osteoclast (*arrow*). Hematoxylin and eosin, 60× magnification.

The morphology of the myeloma cells in most cases is similar to that of normal plasma cells. In other words, the myeloma cells show an eccentric nucleus with a perinuclear hof and deep blue cytoplasm. The nucleus contains a clock face or cartwheel chromatin pattern. However, in some cases of MM, the tumor cells are poorly differentiated or undifferentiated, for which immunologic identification becomes necessary.

On the basis of differentiation, MM can be classified into mature, intermediate, immature, or plasmablastic cytologic type (6). The plasmablastic type shows the worst prognosis, with a medium survival of 10 months compared with 35 months for the other types. However, for the other three types, there appears to be no significant difference in survival (6). MM can also be divided into poorly differentiated, polymorphous, asynchronous, or blastic types (7).

The immaturity of myeloma cells can be manifested as larger nucleus, less condensed chromatin, and presence of nucleoli. The cytoplasm may show fraying borders, cytoplasmic shedding, and the presence of vacuoles, granules, and hyaline and crystalline inclusions (6). The term “Russell bodies” refers to grape-like intracytoplasmic hyaline inclusions that may help recognize the myeloma cells (Figs.6.21.8 and 6.21.9). However, Russell bodies also can be seen in normal, actively secreting plasma cells.

When the inclusion body is present in the nucleus, it is called a Dutcher body (Figs. 6.21.10 and 6.21.11), which is seldom seen in normal plasma cells and is thus helpful for the diagnosis. Both Russell and Dutcher bodies are positive for periodic acid-Schiff stain. Cytoplasmic crystalline inclusions are commonly seen in myeloma patients with the adult Fanconi syndrome (6). Multinucleation and red (flaming) cytoplasm are characteristic of a small number of IgA myeloma. Gaucher-like cells or thesaurocytes that contain fibrillar structures can be seen in a few cases (3).

Because the plasmablastic type of MM carries a particularly poor prognosis, it is important to define the morphology of the plasmablasts (Fig. 6.21.4). According to Rajkumar and Greipp (2), four criteria identify plasmablasts: (i) presence of a fine reticular nuclear chromatin pattern with minimal or no chromatin clumping, (ii) large nuclear size (estimated to be >10 μm), (iii) cytoplasm with little or no perinuclear hof region, and (iv) less abundant cytoplasm comprising less than one half the nuclear area (Fig. 6.21.12).

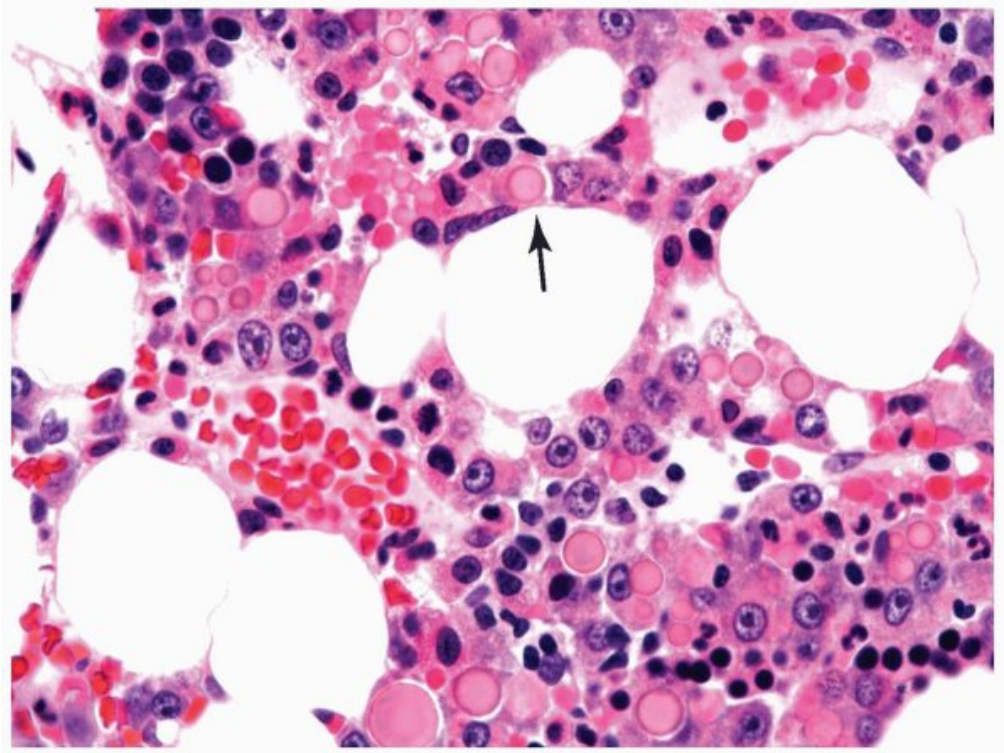

FIGURE 6.21.8 Bone marrow aspirate shows multiple Russell bodies (*arrow*) inside and outside of myeloma cells. Wright-Giemsa, 100× magnification.

The following variants of MM have no or only small amount of paraprotein demonstrated in serum and/or urine:

Nonsecretory myeloma is present in 1% to 5% of all patients with MM. It has all characteristics of MM except that paraprotein is absent in both serum and urine (3,5,8). However, cytoplasmic Ig can be identified with immunocytologic techniques in about 85% of cases. This variant has a lower percentage of plasma cells in the bone marrow and less depressions of normal Ig (3).

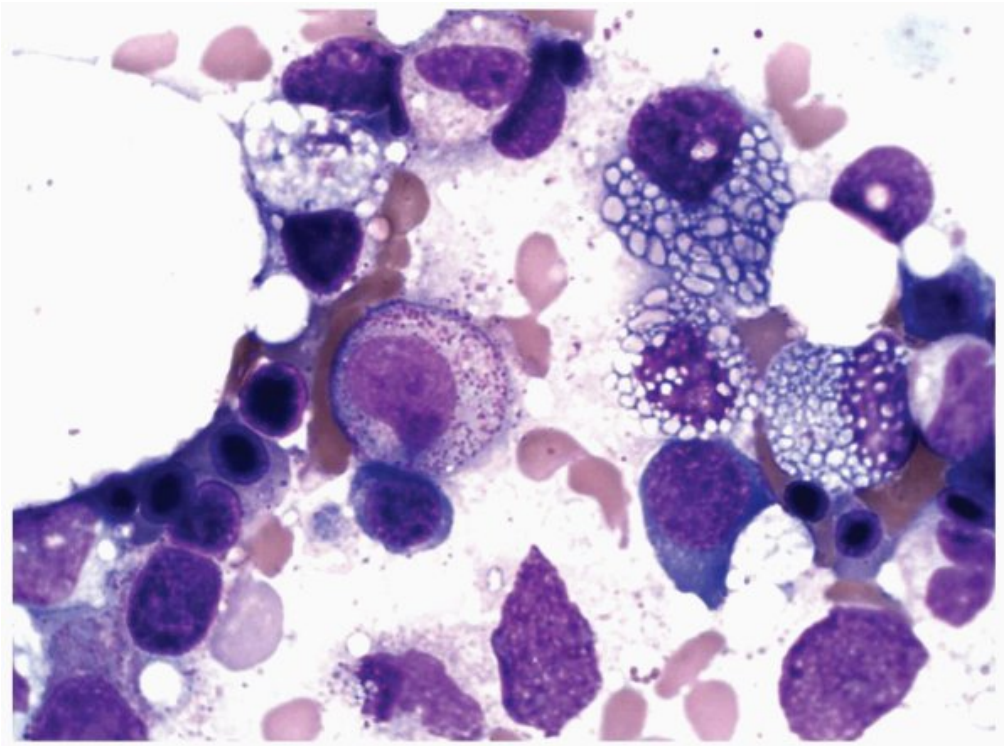

FIGURE 6.21.9 Bone marrow biopsy shows several myeloma cells (Mott cells) containing hyaline cytoplasmic inclusions (Russell bodies). Hematoxylin and eosin, 60× magnification.

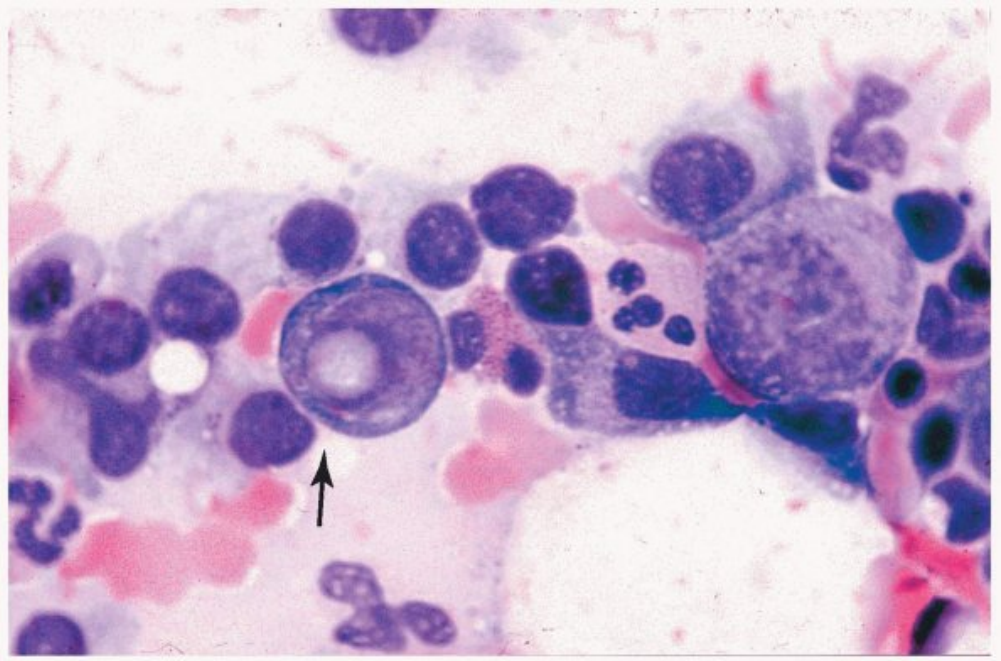

FIGURE 6.21.10 Bone marrow aspirate shows two plasmablasts, one with a huge intranuclear inclusion (Dutcher body) (*arrow*). Wright-Giemsa, 100× magnification.

Solitary plasmacytoma of bone is present in 5% of patients with MM and is characterized by the presence of only one focus of monoclonal plasmacytosis in the bone marrow as detected by radiological examination and confirmed with biopsy (3,5,9). A large proportion of patients with solitary plasmacytoma of bone fail to show a monoclonal paraprotein even on immunofixation studies. In the minority of patients, low level gammopathy can be demonstrated. Local radiation of the solitary lesion usually leads to the disappearance of paraprotein. The persistence of paraprotein after radiation is associated with an increased risk of progression. Almost 50% of patients with this variant progress to overt myeloma (3).

Extramedullary plasmacytoma involves the soft tissue, most frequently the upper respiratory tract, including the nasal cavity and sinuses, nasopharynx, and larynx (Fig. 6.21.13) (3,5,9). Some cases involving the gastrointestinal tract may represent extranodal marginal zone B-cell lymphoma with extreme plasma cell differentiation (3). A monoclonal protein, predominantly IgA, is demonstrated in only 25% of this variant. Extramedullary plasmacytoma may recur in multiple sites including the soft tissue and bone. This condition is called multiple solitary plasmacytoma (5). The conversion rate of extramedullary plasmacytoma to overt MM is approximately 15% (5).

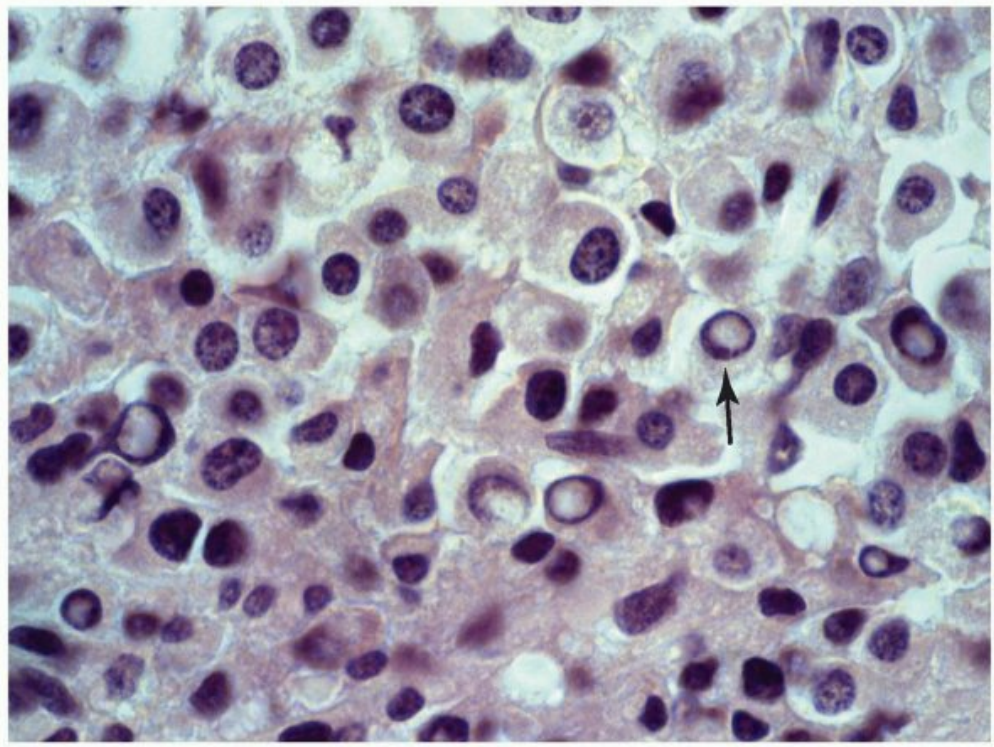

FIGURE 6.21.11 Bone marrow biopsy shows many myeloma cells containing Dutcher bodies (*arrow*). Hematoxylin and eosin, 100× magnification.

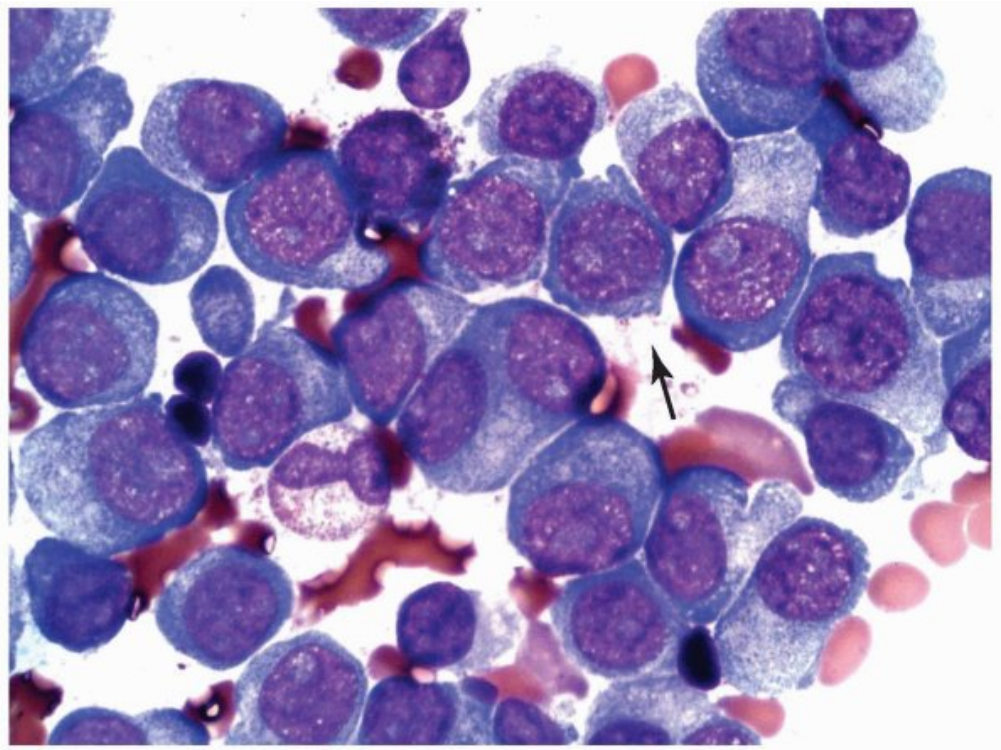

FIGURE 6.21.12 Bone marrow aspirate shows several plasmablasts (*arrow*). Wright-Giemsa, 100× magnification.

In cases of the above three entities, the most reliable diagnostic techniques are immunohistologic, immunocytochemical, and/or immunofluorescent staining to identify a monoclonal κ or λ protein in the tumor cells. Flow cytometry can also help diagnose these entities and is discussed later.

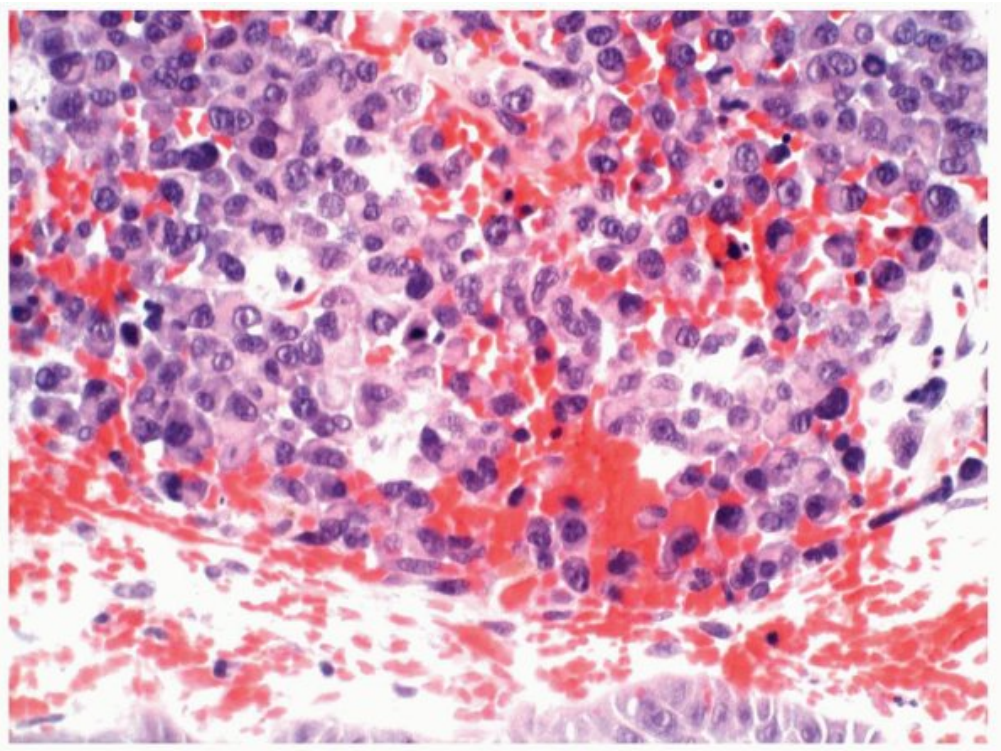

FIGURE 6.21.13 Plasmacytoma from the nasopharynx. Hematoxylin and eosin, 40× magnification.

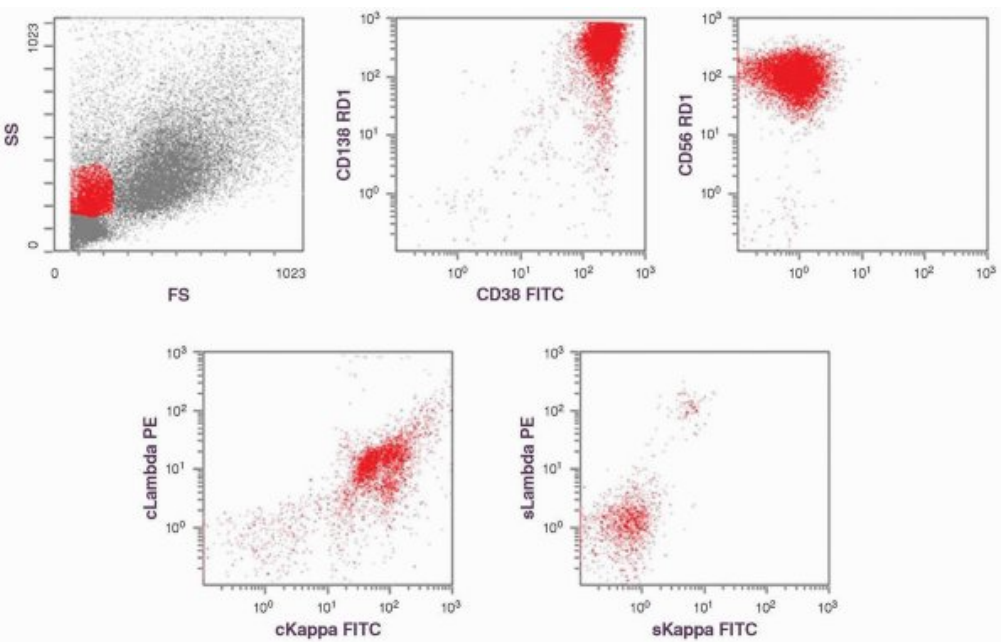

FIGURE 6.21.14 Flow cytometric histograms shows dual staining of CD38/CD138, positive CD56, and monoclonal cytoplasmic κ. No surface immunoglobulins are demonstrated. Side scatter (SS)

versus forward light scatter (FS) plot is used for gating as myeloma cells are usually negative or weakly positive for CD45. RD1, rhodamine; FITC, fluorescein isothiocyanate; PE, phycoerythrin; c, cytoplasmic; s, surface.

Immunophenotype

MM is usually detected by the presence of monoclonal gammopathy in the serum and/or urine or by the detection of osteolytic bone lesions that are further confirmed by bone marrow biopsy. Bone marrow biopsy is performed not only for diagnosis but also for the evaluation of the tumor mass, which is an important predictor for prognosis.

The most important immunophenotype is the presence of monoclonal cytoplasmic Ig and the absence of surface Ig and B-cell antigens (CD19, CD20, and CD22) (Fig. 6.21.14) (10). When surface Ig is present simultaneously with cytoplasmic Ig, it is usually seen in Waldenström macroglobulinemia and/or lymphoplasmacytic lymphoma; otherwise, it can be an artifact due to the presence of cytophilic Ig on the surface of the tumor cells. The only positive surface B-cell marker is CD79a, which is an Ig-associated pan-B-cell antigen (11). Those cases with positive CD20 and surface Ig usually have a poor prognosis (2). CD10 is also considered a poor prognostic predictor by some, but conflicting results have been reported by others (2).

In immunohistochemical staining, the demonstration of the predominance of κ or λ light-chain or a monoclonal κ/λ ratio is most important for the diagnosis. However, when there is coexistence of large numbers of normal plasma cells and macrophages with MM cells, the κ/λ ratio may not be clear cut and other markers may be required to clarify the situation.

Previously, an immunophenotype of positive CD38, negative human leukocyte antigen-DR (HLA-DR), and negative or weakly positive CD45 is considered most specific (12). However, the discovery of two relatively new markers makes the diagnosis much more accurate. The first one is CD56: The presence of this marker distinguishes MM from reactive plasmacytosis, as CD56 is negative in normal plasma cells (13,14). Several studies advocated the use of CD56 and CD19 to distinguish normal from neoplastic plasma cells (14, 15 and 16). Normal plasma cells are CD19+ CD56-, whereas MM cells are CD19- CD56+. In the minority of MM cases, CD56 can be negative, and CD19 can be positive. In cases of MGUS, one report showed no CD56+ cells in 23 patients studied (13), but another study revealed

the coexistence of both CD56+ and CD56- cells in all five patients (15).

The second new marker is CD138, which is a collagen-1-binding proteoglycan, also known as syndecan (16,17). Because of its collagen binding nature, plasma cells may occasionally become negative in fibrotic area as CD138 is shed from the surface membrane into the surrounding fibrotic matrix (18). CD138 can also help to distinguish normal plasma cells (CD19+ CD38+ CD56- CD138+) from Bprogenitor cells (CD19+ CD38+ CD56- CD138-). Therefore, a CD38/CD138 gating provides the best separation of plasma cells from other leukocytes (14).

Other markers that are positive for plasma cells include plasma cell-associated antigen (PCA)-1, PC-1, CD24, CD28, CD30, CD31, CD40, CD44 (homing-associated cell-adhesion molecule), and CD54 (intracellular adhesion molecule 1) (2,10,19). In a study of 49 MM patients, bcl-2 was demonstrated in the tumor cells of 97% cases (20). However, bcl-2 is also present in normal plasma cells. Ki-67 can be expressed in MM cells (particularly those with plasmablastic morphology), but it is not present on normal plasma cells (20). It is associated with higher B_2M and with advanced or relapsed disease.

In addition, myeloma cells may also show T cell, myelomonocytic (CD11b, CD11c, CD13, CD33), megakaryocytic (CD41), and/or erythroid (glycophorin A) antigens. The presence of markers of multiple cell lineages suggests that MM is an early hematopoietic stem-cell disorder manifesting itself at the mature stage of B-cell lineage, analogous to the situation in chronic myeloid leukemia (21, 22 and 23).

Myeloma cell-associated antigen has also been used to identify circulating lymphocytes in MM cases (24). If the circulating cells carry CD38 or PCA-1, the prognosis is poor. These lymphocytes are considered the precursors of the myeloma cells and can also be identified with idiotypic surface Ig, CD10, DNA aneuploidy, or Ig gene rearrangement (21).

Comparison of Flow Cytometry and Immunohistochemistry

In most circumstances, MM can be diagnosed on a morphologic basis. When the bone marrow infiltration is limited or the tumor cells are poorly differentiated, immunohistochemical staining for κ and λ Igs is needed. A predominantly κ or λ light-chain staining should be enough to make the diagnosis unless lymphoma is suspected. When Ig staining is inconclusive, CD38 or CD138 stain may help to highlight the plasma cells. CD56 reaction will confirm the malignant nature of the infiltrate. Ki-67 not only identifies the neoplastic plasma cells; its presence in high percentage also indicates a high-grade tumor, e.g., the plasmablastic form.

Flow cytometry may also demonstrate all of the above markers except Ki-67. The major advantage of flow cytometry is to distinguish cytoplasmic versus surface Ig staining. A monoclonal cytoplasmic Ig pattern can be seen in MM, and a monoclonal surface Ig pattern is seen in various types of non-Hodgkin lymphoma. The coexistence of both monoclonal cytoplasmic and surface Ig is demonstrated in lymphoplasmacytic lymphoma.

The current case had the history of bone pain, and lytic bone lesion was demonstrated by radiology. Flow cytometry revealed a very characteristic immunophenotype with the presence of CD38/CD138, CD56, and a monoclonal cytoplasmic Ig pattern. The negative CD45 and surface Igs were also important findings. However, the patient showed low levels of IgG, IgA, and IgM and no quantitation of Ig light chains. Although immunofixation and flow cytometry demonstrated monoclonal λ light chain in this case, a quantitation of light chain

should have been performed as this would be useful for therapeutic monitoring. In addition, as no monoclonal heavy chain was demonstrated, IgD and IgE quantitation or immunofixation should have been performed to exclude IgD or IgE myeloma. Light-chain disease usually causes severe renal damage; therefore, it is unusual to see that the patient had a normal creatinine level until the late stage of the disease, as evidenced by the presence of 70% plasma cells in the bone marrow.

Molecular Genetics

In early studies, most MM cases were found to be diploid. However, with the advances of modern technology, almost all cases of MM show aneuploidy (1,25). Among the cases with cytogenetic abnormalities, 65% are hyperdiploid, 20% hypodiploid, and 15% pseudodiploid (26).

In MM and MGUS, there appear to be two pathways of pathogenesis. Nonhyperdiploid tumors have a very high incidence of IgH translocations involving five recurrent partners and a relatively high incidence of chromosome 13/13q14 loss (4,25). Hyperdiploid tumors are more frequently associated with multiple trisomies involving chromosomes 3, 5, 7, 11, 15, 19, and 21.

The five recurrent partners in IgH translocation include 11q13 (cyclin D1), 6p21 (cyclin D3), 4p16 (fibroblast growth factor receptor 3 [FGFR3] and multiple myeloma SET domain [MMSET]), 16q23 (c-maf), and 20q11 (mafB) (4). These chromosome translocations may lead directly (11q13-cyclin D1 and 6p21-cyclin D3) or indirectly (4p16, 16q23, cyclin D2) to cyclin D dysregulation. In hyperdiploid tumors, cyclin D1 or, less frequently, cyclin D2 is dysregulated by an undefined mechanism. The dysregulation of cyclin D genes may render the tumor cells more susceptible to proliferative stimuli through interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), or other cytokines produced by bone marrow stromal cells (4).

One of the most common translocations in MM is t(11;14)(q13;q32), which is also found in mantle cell lymphoma. However, the translocation in MM takes place at a γ switch region, and that in mantle cell lymphoma is in or near a JH segment (27). Patients with this translocation usually have a poor prognosis (25).

The presence of deletion or loss of chromosome 13 has been associated with poor prognosis in MM patients. Initially, it was assumed that deletion of the retinoblastoma gene (*Rb*) is responsible for the adverse effect. However, *Rb* protein level is not affected in those patients with 13q; therefore, other genes (i.e., *DBM, BRCA2*) may play a more critical role in the prognosis (26). Nevertheless, disruption of the *Rb* pathway by inactivation of *Rb* or *p18NK4c* can occur in a low frequency in the late stage of the disease (4).

The deletion of the long arm of chromosome 17 (17q-) is also associated with poor prognosis in MM patients. This is directly related to the deletion of a tumor-suppressor gene, *p53* (28). Point mutations of *p53*

have been found more frequently in extramedullary relapse of myeloma (1).

TABLE 6.21.2

Salient Features for Laboratory Diagnosis of Multiple Myeloma

1. Presence of clusters of plasma cells in bone marrow
2. Presence of monoclonal gammopathy
3. Progressive elevation of paraprotein with background immunoglobulin suppression
4. Presence of Bence-Jones proteinuria
5. Monoclonal cytoplasmic immunoglobulin (κ or λ predominant) demonstrated by immunostaining
6. Flow cytometry: Positive markers: CD38, CD138, CD56, monoclonal cytoplasmic immunoglobulin; negative markers: surface immunoglobulins, B-cell markers (CD19, CD20, CD22), CD45 (dim)
7. Immunoglobulin gene rearrangement

8. Common cytogenetic abnormalities: 14q32 translocation with 11q13, 6p21, 4p16, 16q23, or 20q11; 13/13q14 loss; trisomies of chromosome 3, 5, 7, 11, 15, 19, or 21

Several additional oncogenes may be involved in the progress of MM. These include the deregulation of *c-myc,* which is seen in the initial phase of MM, and point mutations of N-*ras* and K-*ras,* which are present mainly in medullary relapse (1). The upregulation of *bcl-2* expression may be due to *ras* activation, loss of *p53* function, or both (26).

Ig gene rearrangement has been found in most cases of MM studied, and its detection rate is proportional to the degree of plasma-cell infiltration in the bone marrow (29). When myeloma cells are <10%, no Ig gene is rearranged. Myeloma cells have a high frequency of Ig VH gene somatic mutation, which is consistent with derivation from a postgerminal center, antigen-driven B cell (3). The salient features for laboratory diagnosis of MM are summarized in Table 6.21.2.

Clinical Manifestations

According to the WHO classification, there are three subtypes of plasma cell dyscrasias that are asymptomatic: MGUS, smoldering myeloma, and indolent myeloma (Table 6.21.3) (3). These three items are distinguished by the percentage of plasma cells in the bone marrow, the quantity of paraprotein, and the presence or absence of lytic bone lesions. MGUS is defined by <10% plasma cells in the bone marrow, with IgG <3.5 g/dL or IgA <2 g/dL, and no lytic bone lesions. Indolent myeloma has >30% of plasma cells in the bone marrow, with IgG in the range of 3.5 to 7 g/dL or IgA in the range of 2 to 5 g/dL, and presence of fewer than three lytic bone lesions. In smoldering myeloma, the percentage of plasma cells in the bone marrow and the paraprotein levels are between these two clinical subtypes, and no lytic bone lesion is present.

TABLE 6.21.3

WHO Classification of Asymptomatic Clinical Subtypes of Myeloma

	MGUS	*SMM*	*IMM*
Plasma cells in BM	<10%	10% to 30%	>30%
M component, g/dL	IgG < 3.5, IgA < 2	IgG > 3.5, IgA > 2	IgG 3.5 to 7, IgA 2 to 5
Lytic bone lesions	None	None	≤3
Symptoms/infection	None	None	None

MGUS, monoclonal gammopathy of undetermined significance; SMM, smoldering myeloma; IMM, indolent myeloma; BM, bone marrow.

The classification of the International Myeloma Working Group differs from that of WHO in the omission of the indolent myeloma subtype (5). Smoldering myeloma is designated asymptomatic myeloma, and the cutoff for both IgG and IgA paraproteins is 3 g/dL.

In the Mayo Clinic studies, approximately one quarter of the MGUS patients developed MM, macroglobulinemia, amyloidosis, or related lymphoproliferative disorders, with an actuarial rate of 16% at 10 years, 33% at 20 years, and 40% at 25 years (5). The risk of MGUS to develop MM or related disorders was about 1% per year. The most important risk factor is high concentration of serum paraprotein. However, patients with Bence-Jones proteinuria, even in a high concentration, may remain in a stable condition for many years (5).

As mentioned before, the International Myeloma Working Group emphasizes end organ damage as the major diagnostic criterion for symptomatic myeloma. The major clinical manifestations in end organ damage are increased serum calcium, renal insufficiency, anemia, and lytic bone lesions, a syndrome referred to as CRAB (5). Recurrent infections are also considered to be an integral part of the clinical symptoms.

There are three major myeloma staging systems—namely, Durie-Salmon, Medical Research Council, and Merlini-Waldenström-Jayakar. The Durie-Salmon system is the most popular and has been adopted by WHO with minor modification. It is based on the hemoglobin

level, serum calcium value, bone x-ray film, and paraprotein quantity to divide myeloma into three stages (Table 6.21.4) (30). The stages are further subdivided into A and B depending on whether the renal function is normal or abnormal. This staging system has a clear relationship to patient survival.

TABLE 6.21.4

Durie-Salmon Myeloma Staging System

	Stage I	*Stage II*	*Stage III*
Hemoglobin (g/dL)	>10	Intermediate	<8.5
Serum calcium (mg/dL)	Normal or <12	Intermediate	>12
Bone x-ray	Normal bone structure or solitary bone plasmacytoma	Intermediate	Lytic bone lesions
Paraprotein			
IgG (g/dL)	<5	Intermediate	>7
IgA (g/dL)	<3	Intermediate	>5
Urine light chain (g/24 h)	<4	Intermediate	>12
Myeloma cell mass ($\times 10^{12}/m^2$)	<0.6	Intermediate	>1.2

As mentioned before, MM patients may develop PCL, which has a frequency between 2% and 4% of all MM cases. PCL may present de novo in patients with no previous evidence of MM (primary form) or as a leukemic phase in the terminal stage of MM (secondary form) (8). About 60% of PCL cases are primary, whereas 40% are secondary. The primary form is seen in younger patients, with a higher incidence of hepatosplenomegaly and lymphadenopathy, a higher platelet count, fewer lytic bone lesions, lower serum Mprotein levels, and longer survival than the secondary form (3,31). The response to chemotherapy in PCL is poor, and the median survival of these patients is about 1 to 2 months. (8).

Most myeloma is of the IgG class (58.6% to 60.3%), whereas the frequencies of plasma cell dyscrasia involving IgA and IgM are similar (about 15%) (32). When IgM is involved, the condition is traditionally called Waldenström macroglobulinemia, because IgM is a macromolecule and, in this entity, osteolytic bone lesion is usually not present. The incidence of light-chain disease ranges from 5% to 15% in different series (32). The incidence of IgD myeloma is about 1%, whereas that of IgE myeloma and heavy-chain diseases is <1% (32).

When two monoclonal components are identified, the condition is called biclonal gammopathy (33). Although its reported frequency is about 2.4% to 4.1%, the real incidence is probably even lower, because the two components in some cases may represent heavy- or, less frequently, light-chain switching, occurring in the same clone of myeloma cells (33,34). An easy way to distinguish biclonal from monoclonal myeloma is to use the double-staining immunofluorescence technique to detect the cytoplasmic Igs with two fluorochromes (Figs. 6.21.15 and 6.21.16) (32). If two monoclonal components are found in the same cells, the conclusion should be monoclonal gammopathy with heavy-chain switching. Among cases of biclonal gammopathy, 65% are MGUS, 19% lymphoproliferative disorders, and 16% MM (35). The clinical features, therapeutic response, and survival of patients with biclonal MM do not differ from those with monoclonal MM (35).

Many parameters can be used to predict the prognosis in MM cases (2). As advocated by the Mayo Clinic, the most sensitive technique is

plasma cell labeling index (PCLI). The mononuclear cells isolated from the patient's bone marrow are incubated with 5-bromo-2-deoxyuridine (BUDURD), and the uptake of BUD-URD is detected by fluorochrome-labeled monoclonal antibody BU-1. BUD-URD is incorporated into the nucleus of cells synthesizing DNA; therefore, cells showing BU-1 antibody are those in the S phase. A PCL1 of ≥1% is classified as high.

The S phase can be directly measured by flow cytometry. Although this technique measures more cells that synthesize DNA than does PCLI, it is time consuming and inaccurate when plasma cells in the patient's bone marrow are <20% (35).

Two helpful tests routinely done in clinical laboratories are serum B_2M and LDH levels. The levels of B_2M reflect both tumor mass and renal function (20). A high level of

B_2M represents a large tumor mass and poor renal function, thus identifying a high-risk group (36). A high level of LDH, in contrast, defines a high-grade myeloma with a rapidly progressive clinical course (20). Therefore, patients with high LDH levels usually have shorter remission and shorter survival.

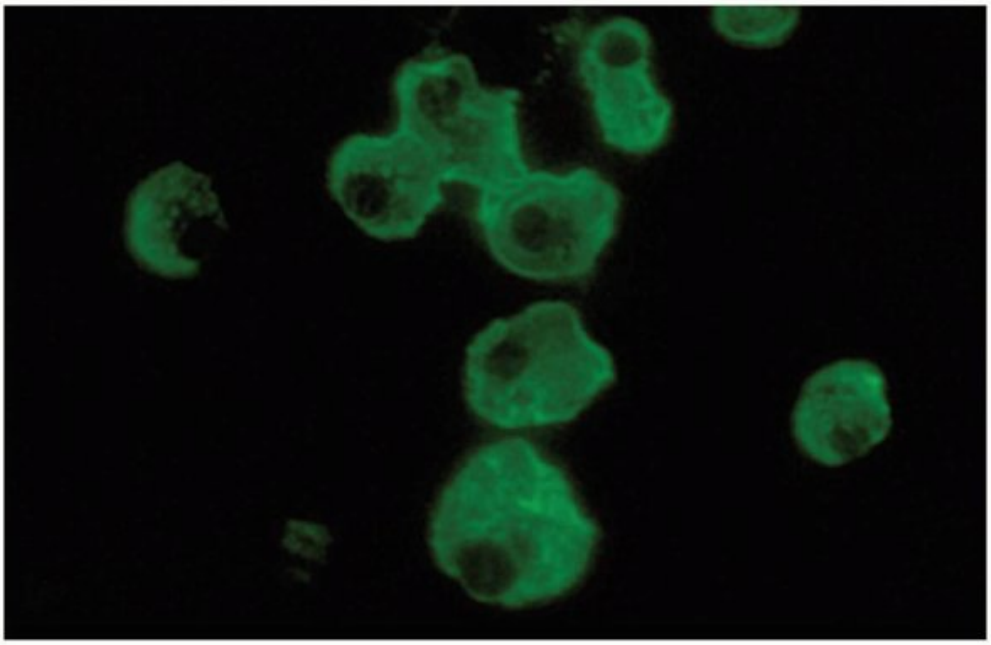

FIGURE 6.21.15 Fluorescein-conjugated antiimmunoglobulin (Ig)G antibody stains positive in the cytoplasm of myeloma cells. Immunofluorescence stain, 100× magnification.

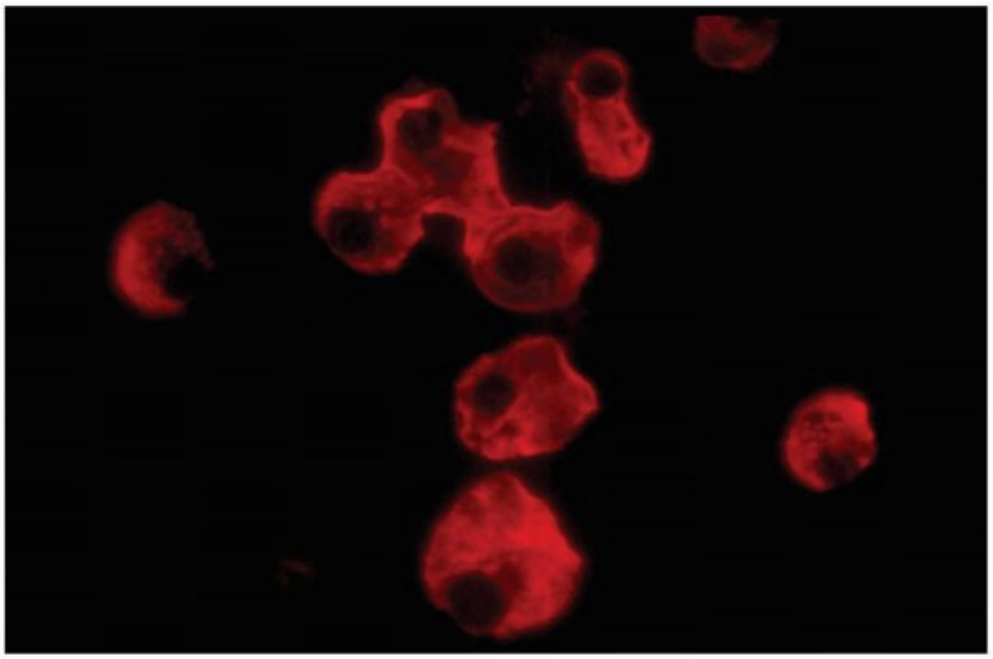

FIGURE 6.21.16 Rhodamine-conjugated antiimmunoglobulin (Ig)A antibody stains positive in the cytoplasm of the same group of myeloma cells as demonstrated in Fig. 6.22.15, indicating that both IgG and IgA are produced by the same clone of myeloma cells. Immunofluorescence stain, 100× magnification.

As mentioned before, cytogenetic abnormalities often carry a poor prognosis, especially those involving chromosome 11 or 13 and translocations. One study found that the median PCLI was 1.5% in patients with cytogenetic aberrations and 0.2% in patients with normal cytogenetics (2). The presence of plasmablastic morphology and circulating plasma cells are also predictors of poor prognosis. Patients with MM show a short survival when cutaneous metastasis occurs (Fig. 6.21.17) (37).

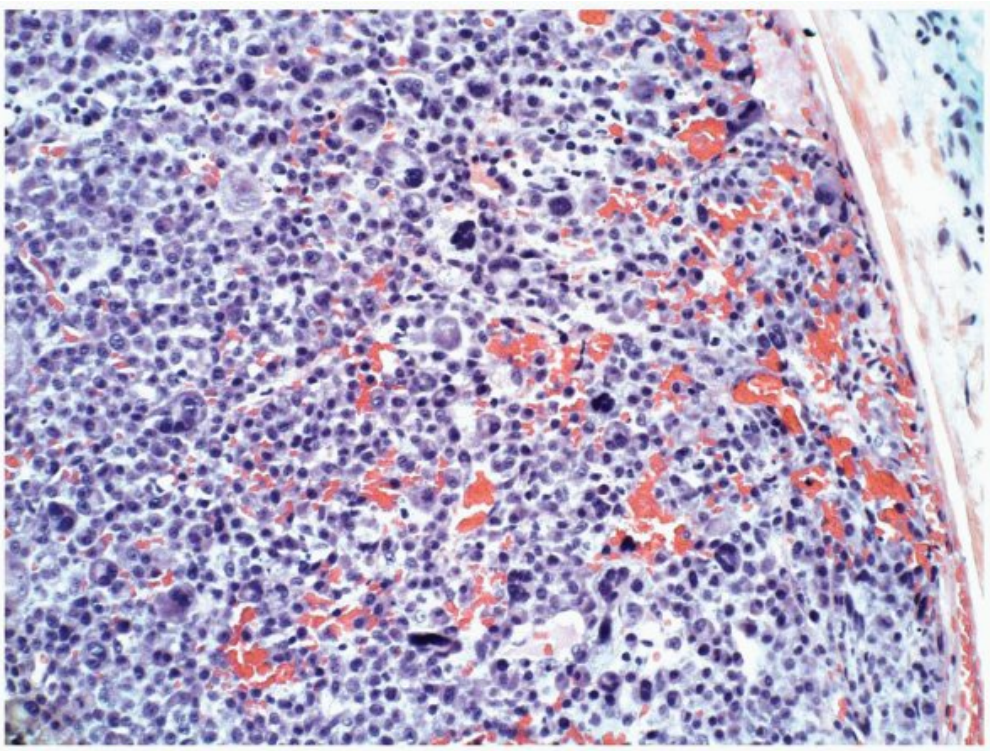

FIGURE 6.21.17 Skin biopsy shows extensive pleomorphic myeloma cell infiltration with many multinucleated giant cells. Hematoxylin and eosin, 20× magnification.

In the terminal stage, MM may transform into lymphoma, acute lymphoblastic leukemia, or acute myelomonocytic leukemia, or it may become drug resistant and actively proliferative. These phenomena are called phenotypic escapes (11).

There are two discoveries that may have potential implications in the pathogenesis of and therapeutic approach to MM (1,38,39). The first one is that IL-6 mediates the autocrine and paracrine growth of MM cells. The bone marrow stromal cells in MM patients secrete large amounts of IL-6; this secretion has a paracrine effect on tumor growth and prevents apoptosis (39). Using murine anti-IL-6 monoclonal antibodies for the treatment of advanced MM has produced some effect but no lasting benefit (40). The second discovery is Kaposi sarcoma herpesvirus or human herpesvirus-8 (HHV-8) in the bone marrow dendritic cells of patients with MM. The bone marrow microenvironment with HHV-8-infected dendritic cells has a growth and antiapoptosis advantage over uninfected stromal cells (39). Thus, HHV-8 may play an important role in the pathogenesis of MM. If this association is confirmed, novel therapeutic strategies targeting this virus would be beneficial to MM patients.

Traditionally, MM is treated with melphalan, vincristine, doxorubicin, and dexamethasone in various combinations. Recently, the use of thalidomide and bortezomib has further improved therapeutic effects (41). Two therapeutic approaches are now being explored simultaneously: The first one is to aim at complete cure, and the second one is to convert MM into a chronic indolent disease.

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CASE 22 Hairy Cell Leukemia

CASE HISTORY

A 52-year-old man presented with fatigue, decreased exercise tolerance, and dyspnea on exertion for 6 months. The patient was in good health 6 months before presenting, and his only medical problem was alcoholism. Upon consultation with his private care physician, he was found to have pancytopenia and was admitted for further evaluation.

Physical examination on admission showed a healthylooking man with no apparent distress. Abdominal examination found a large spleen, but liver was not palpable. There was no peripheral lymphadenopathy.

Hematology workup revealed a total leukocyte count of 1,200/μL with 38.5% lymphocytes, 56.6% neutrophils, 2.6% monocytes, 1.6% eosinophils, and 0.5% basophils. His hematocrit was 33.3%, hemoglobin 11.4 g/dL, and platelets 72,000/μL.

After admission, a bone marrow biopsy was performed and showed 90% cellularity with 70% being “hairy cell.” Myeloid cells were markedly decreased, but megakaryocytes and erythroid series were relatively normal.

He was treated with a cycle of 2-chlorodeoxyadenosine and subsequently developed neutropenic fever. His condition was finally under control with antibiotics and granulocyte colony-stimulating factor (G-CSF). His leukocyte count returned to normal in 2 months. A follow-up examination of peripheral blood and bone marrow showed no leukemic cells 1 year later.

FLOW CYTOMETRIC FINDINGS

Blood: CD5 23%, CD19 77%, CD19/CD5 9%, CD20 80%, CD22 85%, CD11c 95%, CD22/CD11c 85%, CD23 5%, CD25 90%, CD103 97%, FMC-7 92%, CD19/κ 63%, CD19/λ 6%, CD10 77%, CD14 2%, CD45 100% (Fig. 6.22.1).

Bone marrow: CD5 11%, CD19 91%, CD19/CD5 7%, CD22 95%, CD11c 99%, CD22/CD11c 95%, CD23 6%, CD25 91%, CD103 95%, FMC-7 93%, CD19/κ67%, CD19/λ 7%, CD10 90%, CD14 2%, CD45 100%.

IMMUNOCHEMISTRY

Bone marrow biopsy: The tumor cells were positive for CD20, DBA-44, and tartrate-resistant acid phosphatase (TRAP).

DISCUSSION

Hairy cell leukemia (HCL) is a rare disease with an incidence of 2% of lymphoid leukemias (1). However, it is an important entity in the

differential diagnosis of the low-grade lymphoproliferative disorders. Recent advances in the treatment of HCL enable a long-term remission in most patients; thus an accurate diagnosis of this leukemia has become more meaningful than ever before.

Morphology

Typical hairy cells are usually of medium size (10 to 15 μm) with a moderate amount of cytoplasm (Fig. 6.22.2) (2). The cytoplasmic projections are delicate (hairy) and cover the entire cell surface, compared with the villi on the villous lymphocytes of splenic lymphoma, which are polar in distribution (3). The lacelike chromatin pattern in the nucleus of hairy cells is also in contrast to the clumped chromatin patterns in tumor cells from chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), and splenic lymphoma with villous lymphocytes (SLVL or splenic marginal zone lymphoma) (4). Electron microscopy may help to distinguish the villi on hairy cells from those of villous lymphocytes of SLVL and to identify the ribosome-lamella complex in hairy cells (Fig. 6.22.3).

In contrast to other leukemias, the histologic patterns in various organs are very specific for HCL (Table 6.22.1). In the bone marrow, hairy cells are packed “back to back” and show an oval or kidney-shaped nucleus with abundant clear unstained cytoplasm that separates the nuclei widely, and give a pattern variably described as “fried egg,” “sponge,” or “honeycomb” (Fig. 6.22.4) (2,5,6). Because the tumor cells are closely packed, the cell borders appear to be interlocking. A reticulin stain may demonstrate increased reticulin fibers in the area infiltrated by tumor

cells, but collagen fibrosis is typically absent in HCL. An intrasinusoidal infiltration pattern can also be seen in up to 73% of HCL cases, but lymphoid nodules are typically not found in HCL, and this characteristic can be used to distinguish it from SLVL (7).

TABLE 6.22.1

Characteristic Morphologic Features of Hairy Cell Leukemia

Histologic patterns	Bone marrow: Fried egg pattern Spleen: Blood lake or pseudosinus formation Liver: Angiomatous lesion Lymph node: Fried egg pattern surrounding follicles
Cytology	Medium-sized, moderate cytoplasm with cytoplasmic projections, lacelike chromatin pattern of nucleus
Special features	Special cytologic features in blood, and typical histologic patterns in tissues

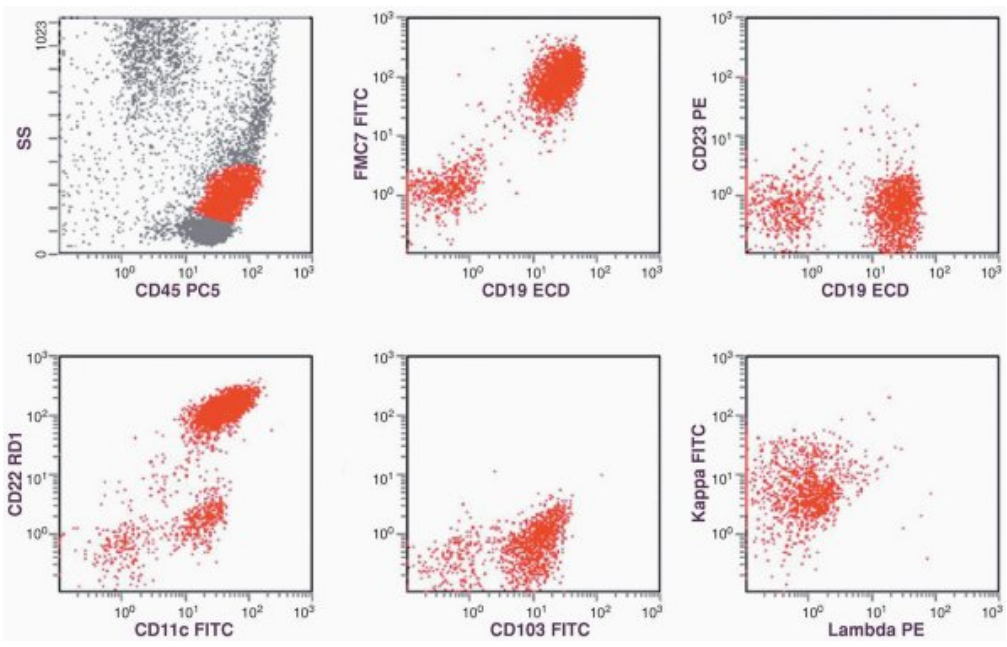

FIGURE 6.22.1 Flow cytometric histograms show CD19, FMC-7, CD11c, CD22, and CD103 reactions in a monoclonal κ B-cell population. CD23 is negative. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-Texas Red; RD1, rhodamine.

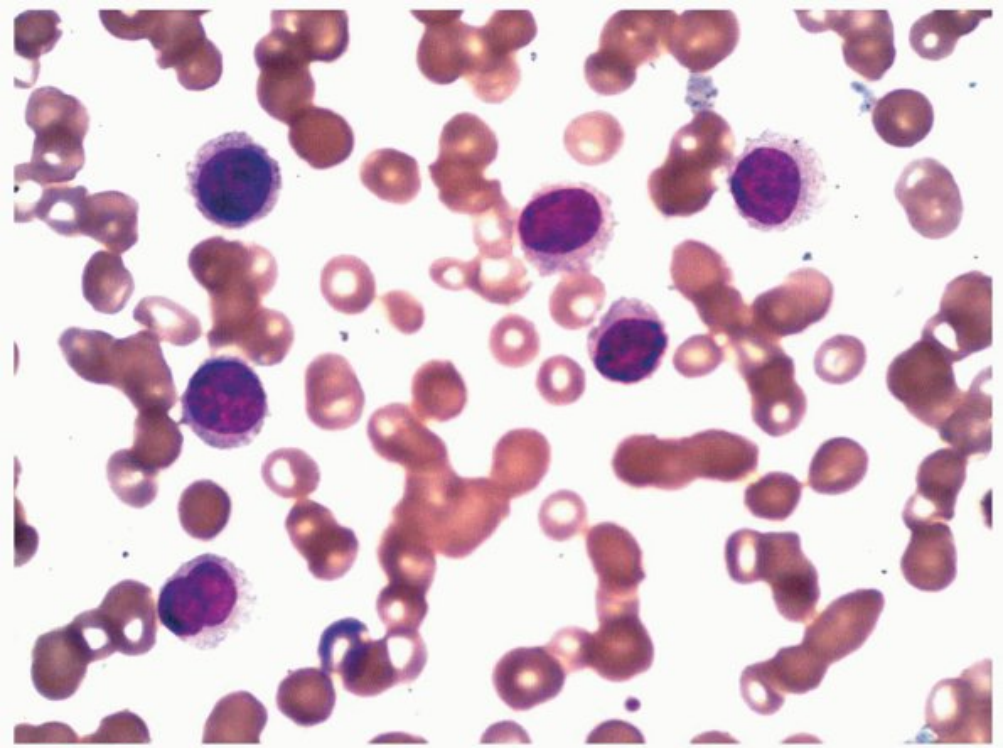

FIGURE 6.22.2 Peripheral blood smear shows six leukemic hairy cells with moderate cytoplasm and multiple cytoplasmic projections. A binucleated cell is present. Wright-Giemsa, 100× magnification.

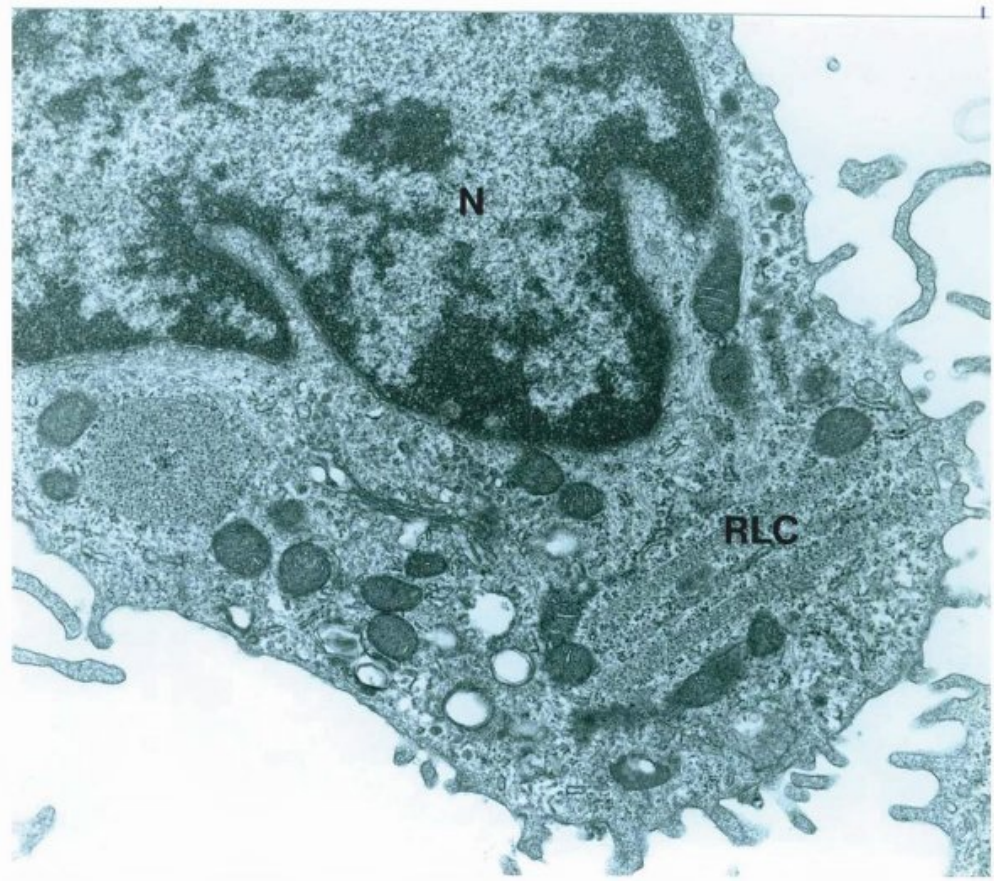

FIGURE 6.22.3 Electron micrograph shows a hairy cell with many cytoplasmic projections and two characteristic ribosome-lamellar complexes (RLC). N, nucleus. 27,500× magnification. (Courtesy of Dr. Saul Teichberg, North Shore University Hospital, New York.)

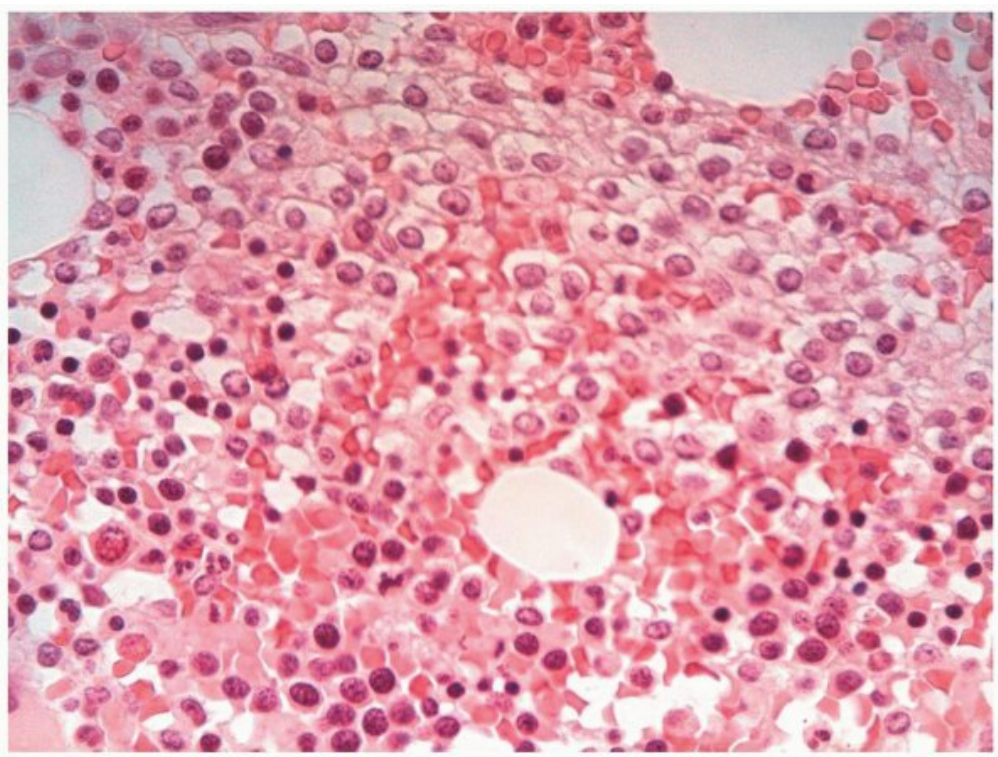

FIGURE 6.22.4 Bone marrow biopsy shows closely packed hairy cells with clear cytoplasm and prominent cell border (honeycomb pattern). Hematoxylin and eosin, 60× magnification.

In the spleen, HCL is characterized by involving solely the red pulp, both the cords and the sinuses. Probably because of the destruction of the splenic cords and atrophy of the white pulp, variably dilated sinuses are filled with erythrocytes and form the so-called blood lakes. When these lakes are lined by hairy cells, they are called pseudosinuses (Fig. 6.22.5) (2,6,8). In full-blown HCL, the red pulp cords are totally destroyed and the entire spleen is composed of the tumor cells intermixing with pools of erythrocytes. Although this pattern has been occasionally encountered in other conditions, such as CLL, chronic myeloid leukemia, and multiple myeloma (9), in an appropriate clinicopathologic setting, this pattern is diagnostic of HCL.

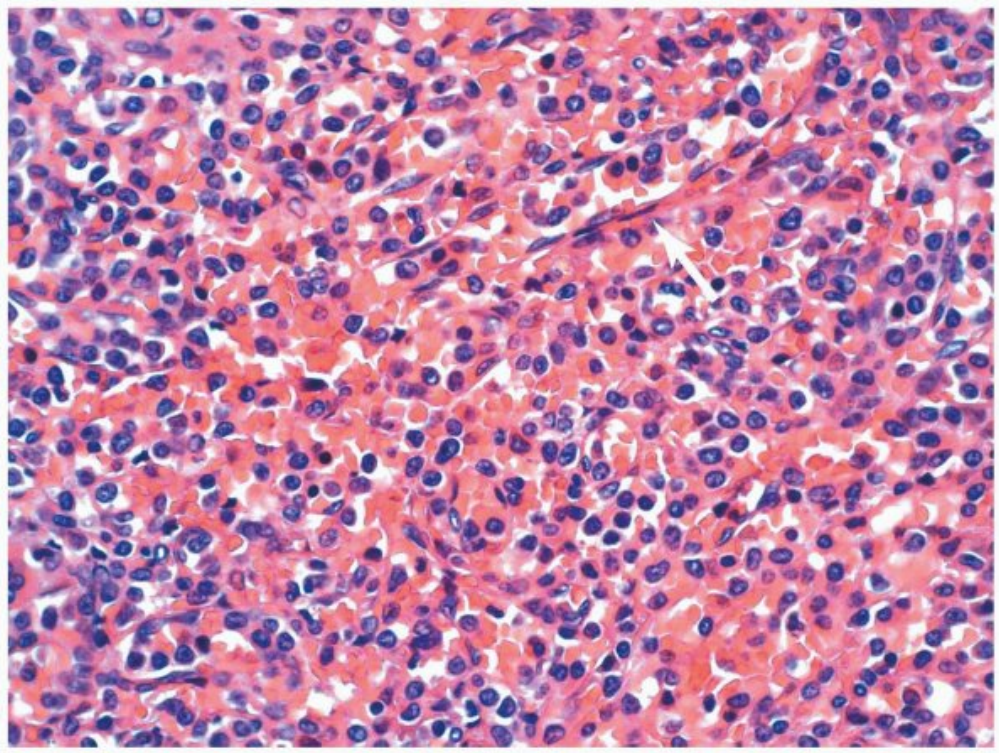

FIGURE 6.22.5 Splenectomy specimen shows pseudosinuses surrounded by leukemic cells, as compared with one residual sinus in this field (*white arrow*). Hematoxylin and eosin, 40× magnification.

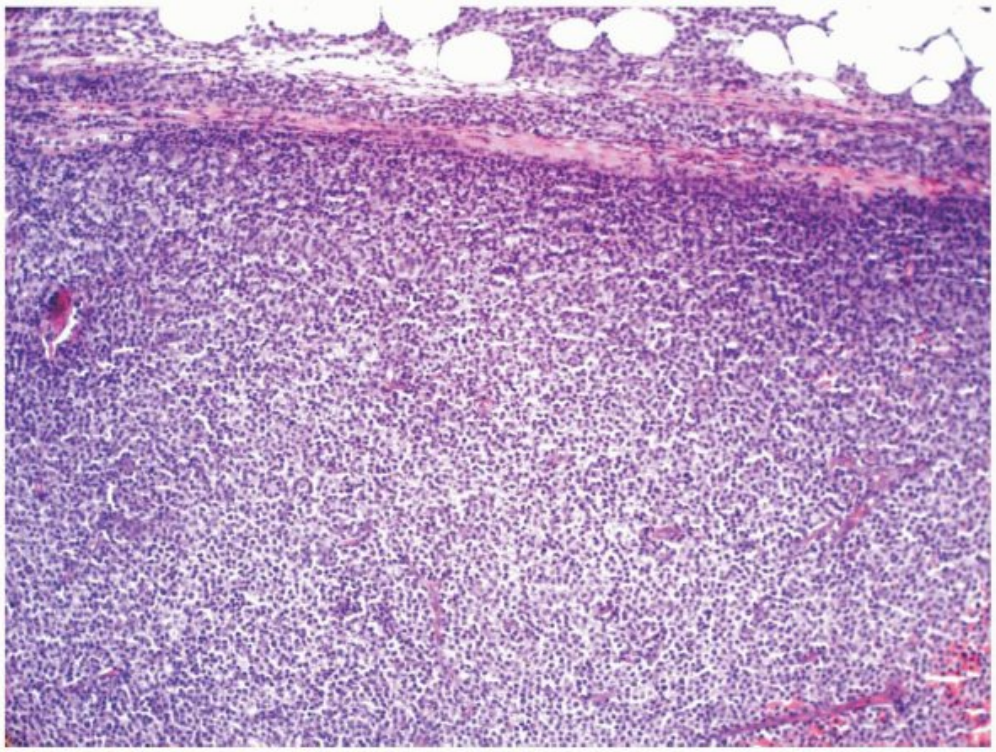

FIGURE 6.22.6 Lymph node biopsy shows total effacement of normal architecture by diffuse leukemic cell infiltrate. Note the wide space between cells representing the clear cytoplasm of leukemic hairy cells. Hematoxylin and eosin, 10× magnification.

In the liver, both portal areas and sinuses are involved by the tumor cells. Leukemic infiltration of the sinusoid wall may produce angiomatous lesions (8,10).

Peripheral lymphadenopathy is observed in only 5% to 10% of patients, but abdominal and mediastinal adenopathies are frequently found at autopsy (6). When peripheral lymphadenopathy is found, high-grade lymphoma transformation should be suspected (11). The normal architecture of lymph nodes is partially or completely replaced by leukemic cells, which often surround the lymph follicles (Fig. 6.22.6.). A fried egg pattern is also frequently seen.

Tumor cells of HCL may also infiltrate skin, lungs, kidneys, stomach, intestine, myocardium, meninges, adrenals, and pancreas, but the histologic pattern is not specific and the infiltrates seldom cause clinical symptoms (12).

The recent improvement of the histologic techniques further facilitates the diagnosis of HCL in tissue sections. Examples include the demonstration of ribosome-lamella complexes in plastic-embedded sections (13) and the visualization of hairy projections on tumor cells in tissue sections stained with monoclonal antibody DBA-44 (Fig. 6.22.7) (14). Immunohistochemical stains for DBA-44 and CD20 are particularly helpful in detecting small numbers of tumor cells in early cases of HCL.

Immunophenotype

Because clinical and laboratory features may overlap among various low-grade B-cell lymphoproliferative disorders, immunophenotyping is particularly useful for differential diagnosis (Table 6.22.2). All B-cell tumors share a

monoclonal surface immunoglobulin (Ig) pattern (IgM in most cases, but IgG in HCL variants). One unique feature of HCL is the expression of preswitched (IgM/IgD) and postswitched (IgG/IgA) Igs by the same cells, as demonstrated by flow cytometry (7) and by RNA transcript analysis of single cells (15,16). In one study, the frequency was as high as 40% (15). Unlike other B-cell tumors, the coexpression of multiple isotypes in HCL is not due to the existence of a heterogenous population, and only a single light-chain type is present.

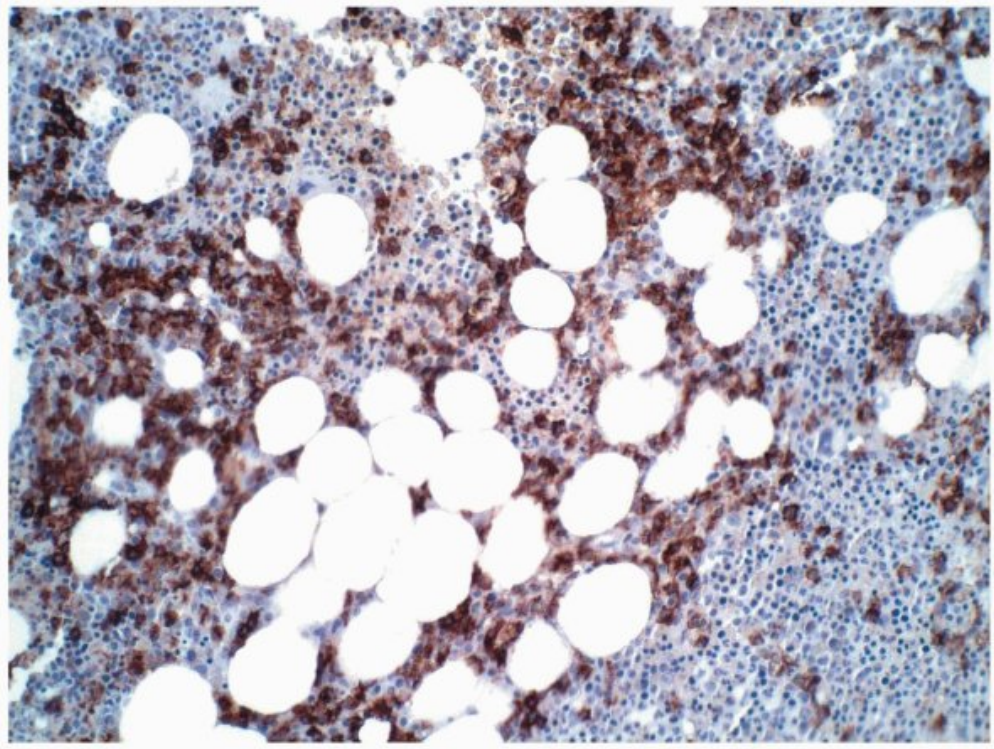

FIGURE 6.22.7 Bone marrow biopsy shows DBA-44 staining of the leukemic cells. Immunoperoxidase, 20× magnification.

TABLE 6.22.2

Comparison of Five Low-Grade B-Cell Neoplasms

	HCL	*CLL*	*PLL*	*SLVL*	*MBL*
Circulating cells	Hairy cells	Samll lymphocytes	Prolymphocytes	Villous lymphocytes	Absent
Leukocytosis	Leukopenia	Moderate	Marked	Moderate	Absent
Splenomegaly	Present	Present	Present	Present	Absent
Major pulp involved	Red	White	White	White	White
Liver involvement	Present	Present	Present	Present	Absent
Lymphadenopathy	Absent	Present	Absent	Absent	Present
Marrow infiltrate	Present	Present	Present	Present	Rare

CD5	-	+	-/+	-	-
CD11c	+	-	-	-/+	-/+
CD19	+	+	+	+	+
CD20	+	+	+	+	+
CD22	+	-	-/+	+	+
CD25	+	-	-	-/+	-
CD103	+	-	-	-	-
TRAP	+	-	-	-/+	-
Monoclonal protein	Rare	RC	RC	Common	RC

CLL, chronic lymphocytic leukemia; HCL, hairy cell leukemia; MBL, nodular marginal zone B-cell lymphoma; PLL, prolymphocytic leukemia; RC, relatively common; SLVL, splenic lymphoma with villous lymphocytes; TRAP, tartrate-resistant acid phosphatase; CD, cluster of differentiation.

B-cell neoplasms also share some common pan-B antigens, such as CD19, CD20, and CD79a, but CD22 was first identified in HCL and is not shared by all B-cell tumors. The Ig-associated antigen, CD79b, in contrast, is characteristically absent in HCL (1,6). The coexpression of a B-cell antigen with CD11c, a monocyte antigen, is another unusual feature of HCL. The finding of dual CD22 and CD11c staining on HCL cells was initially considered highly specific for HCL (17,18). However, this coexpression has been found in >10% of lymphocytes in a variety of B-cell proliferative disorders, including CLL, PLL, and marginal zone B-cell lymphomas (SLVL and monocytoid B-cell lymphoma) (17,19,20). Therefore, one should look for the subtle differences between HCL and other lymphoid neoplasms.

First, HCL usually shows strong fluorescence for both CD22 and CD11c and is well defined in flow cytometric contourgrams, but the staining of CD22 and CD11c in other lymphoid tumors is weaker and less well-defined than HCL (17). Second, other lymphoid tumors show a much higher percentage of CD22+ CD11c- population than HCL cases; most cells are CD11c positive in the latter (17). Another study found that the coexpression of CD20 and CD11c was

even more specific than CD22/CD11C for HCL, because this phenotype was not found in other cases of lymphomas as well as acute and chronic leukemias studied (21).

Another important marker for the identification of HCL is CD25 (Tac antigen or interleukin [IL]-2R) (22). CD25 is present in human T-cell lymphotropic virus type I-associated adult T-cell leukemia/lymphoma (23), and occasionally in Hodgkin lymphoma (24) and diffuse large B-cell lymphoma (25). However, it is very helpful in distinguishing HCL from other low-grade B-cell neoplasms, which are usually negative for CD25. The only exception is SLVL, which showed positive CD25 reaction in 25% of cases in one study (26). The same study revealed that CD103 and hairy cell leukemia-associated antigen (HC)2 were most helpful in distinguishing HCL from SLVL; both markers were generally not positive in SLVL.

In addition, elevated levels of the soluble form of IL-2 receptor (IL-2R; CD25) are present in the sera of HCL patients (27). The IL-2R level correlates well with the tumor cell concentration in the bone marrow and the patient's clinical status.

FMC-7 is usually included in the HCL study panel, but it is not as specific as the above-mentioned markers; it is also positive for PLL and most cases of nodal marginal zone B-cell lymphoma and mantle cell lymphoma (1,28). Bcl-2 antigen is usually positive, but no bcl-2 gene rearrangement is identified in HCL cases (6). Recently, two new antibodies have been used, with promising results, to diagnose HCL. The first one is annexin A1 (ANXA1), which is the product of the ANXA1 gene that is upregulated in HCL cases by gene-profiling analysis (29). Immunohistochemical staining with this antibody was found to be highly specific and sensitive. The second one is CD123, which identifies the α chain of the human IL-3R. Flow cytometry showed that it was positive in 95% of typical HCL cases, but negative in 91%

cases of HCL variant and in 97% cases of SLVL (30).

Other frequently positive but not specific antigens for HCL include human leukocyte antigen-DR (HLA-DR) and plasma cell-associated antigen-1 (PCA-1), which may also help in differential diagnosis (2,21). PC-1 and some early appearing B-cell antigens, such as CD21 and CD24, are usually negative (31,32).

The negative reactions to CD5, CD10, and CD23 are useful in excluding small lymphocytic lymphoma/CLL, mantle cell lymphoma, and follicular lymphoma. However, a recent study showed that CD10 and CD23 can be present in a small percentage of HCL cases (33). In other reports, CD10 has been found in 5% to 26% and CD23 in 20% of HCL cases (33). In contrast, some important markers, such as CD103 and CD25, can be absent in a few cases of HCL (33). Recent studies have indicated that the presence of CD103 and CD11c, absence of CD25, and infrequent presence of TRAP are highly characteristic of HCL variants, which should be distinguished from HCL because of their marked differences in therapeutic response and prognosis (7).

TRAP is a time-honored marker for the diagnosis of HCL. Although many studies claimed to detect acid phosphatase-positive stains in other lymphoid neoplasms, most of these reports did not adhere to the strict definition of real TRAP positivity. In HCL, the acid phosphatase staining is heavy (>40 granules) and diffuse (not focal), and the staining intensity before and after tartrate treatment should be comparable (not markedly different) (Fig. 6.22.8). By summarizing their 15-year experience, Yam et al. (34) found that false-positive or false-negative TRAP results were due to either technical or interpretive errors. Subsensitive techniques and incorrect criteria for result interpretation were the major problems. After eliminating these problems, TRAP is, indeed, highly sensitive and specific. However, in exceptional conditions, positive results can be seen in other lymphoid tumors and negative results in HCL.

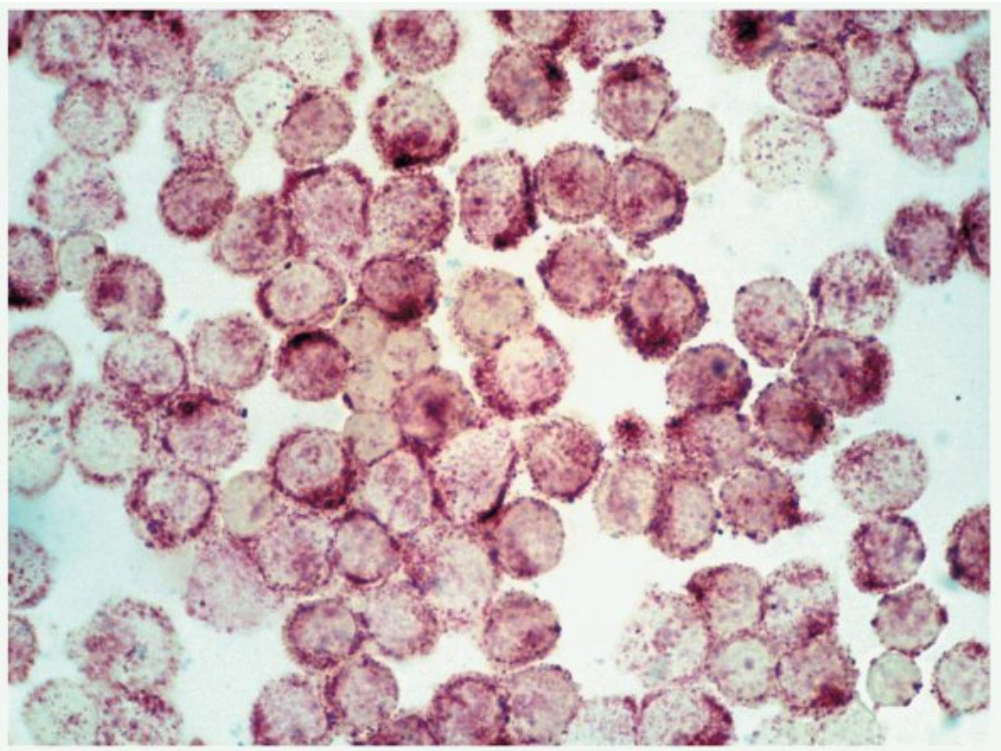

FIGURE 6.22.8 Tartrate-resistant acid phosphatase staining of the leukemic cells on a cytospin of bone marrow aspirate. Note the high percentage and diffuse, heavy staining of the cells. Cytochemical stain, 100× magnification.

Two cases of HCL of T-cell lineage have been reported (35,36). Both cases were believed to be associated with infections by retrovirus human T-cell lymphotropic virus type II (HTLV-II). Re-examination of one of the two cases revealed that the case was actually composed of B-cell HCL and CD8-positive T-cell leukemia (37). The HTLV-II genome was found in the DNA of the leukemic T cells but not in the leukemic hairy cells (38). Severe T-cell dysfunction has been found in HCL cases (39).

Comparison between Flow Cytometry and Immunohistochemistry

The peripheral blood in HCL cases is characteristically pancytopenic, so flow cytometry may not be able to detect the hairy cells. The bone marrow aspirate, in contrast, is difficult to obtain when reticulin fibrosis is present. Therefore, the diagnosis sometimes depends on immunohistochemical staining. DBA-44 and CD20 are frequently used to identify hairy cells in bone marrow; however, CD20 is nonspecific for HCL, and DBA-44 can also be present in other lymphomas, particularly follicular lymphoma (40). The combination of DBA-44 and TRAP stains in tissue sections is considered most sensitive and specific for the diagnosis of HCL. In one study, this combination is positive in all 86 cases of HCL and none of 66 cases of other lymphomas (41). Another study demonstrates this

other non-Hodgkin lymphomas (40). However, if bone marrow aspirate is available or the blood specimen contains many tumor cells, flow cytometry is most reliable for the diagnosis by using a panel of CD11c/CD22, CD25, CD103, and FMC-7.

Molecular Genetics

HCL cases usually show Ig gene but not T-cell receptor gene rearrangements (22). Therefore, despite the frequent demonstration of myelomonocytic markers and of phagocytic activities in hairy cells (21), HCL is still considered a B-cell neoplasm (17). Gene rearrangement does not help in differential diagnosis between HCL and other B-cell neoplasms. However, one study found that HCL may have more than one neoplastic clone; one was sensitive to chemotherapy, but another one was refractory (42). Therefore, genotyping may be occasionally useful for the evaluation of therapeutic problems.

Analysis of the Ig heavy-chain variable (VH) gene in one study showed somatic mutation in most cases of HCL (16). In addition, HCL cases showed multiple isotype expression. Therefore, Forconi et al. (16) hypothesize that HCL cells are arrested at the point where multiple isotypes can be generated but before deletional switch takes place.

A study with comparative expressed sequence hybridization showed a homogeneous gene expression profile in all HCL cases (43). HCL cases also expressed a spleen signature, which was not found in the normal lymph node. As cells that are present in the spleen and not lymph nodes include only sinusoidal lining cells (endothelial cells) and marginal zone B cells, this finding suggested the marginal zone origin of HCL (43).

A study of gene expression profiling by Basso et al. (44) drew a similar conclusion in that HCL cases displayed a homogeneous pattern and were of memory B cell origin. It is known that the marginal zone is composed of a heterogeneous population including memory B cells. This finding explains the possible reason why chromosomal translocation is not found in HCL cases: The mechanism that can generate these aberrations is switched off in memory cells (44).

The study by Basso et al. (44) also identified several genes that could explain the clinical behavior of HCL. For instance, the downregulation of CCR7 gene, a chemokine receptor the deficiency of which hampers the ability of B cells to enter lymph nodes, may explain why HCL cells seldom spread to lymph nodes (44). The upregulation of annexin 1 explains why HCL cells have the capacity of phagocytosis, as annexin 1 is required for phagocytic uptake of *Brucella suis* by human monocytes (44). The overexpression of IL-3Rα and Fms-like tyrosin kinase (FLT)3 may explain bone marrow fibrosis in HCL cases, because the ligands for these two receptors are important for adhesion of B cells to fibronectin (44).

Cytogenetic study may demonstrate various anomalies in HCL, including clonal aberration, numeric changes, and structural alterations in various chromosomes (2,45,46). However, except for one claim of frequent finding of 5q13 (30), no consistent cytogenetic abnormality has been established in HCL (1,2).

TABLE 6.22.3

Salient Features for Laboratory Diagnosis of Hairy Cell Leukemia

1. Hairy projections on leukemic cells
2. Pancytopenia with monocytopenia
3. TRAP positivity
4. Characteristic immunophenotype:
   Positive for CD11c/CD22, CD25, FMC-7, and CD103
   Special immunohistochemical marker: DBA-44
5. Monoclonal surface immunoglobulin pattern
6. Electron microscopy: Cytoplasmic projections and ribosome-lamella complex
7. Immunoglobulin gene rearrangement

TRAP, tartrate-resistant acid phosphatase; CD, cluster of differentiation.

Recently parathyroid adenoma 1 (PRAD1)/CCND1 has been found to be overexpressed in most cases of HCL; in about one third of these cases, its level of expression approaches that seen in mantle cell lymphoma (5). However, this expression is not associated with t(11;14), and the cyclin D1 protein is detected only by Western blotting and not by immunohistochemical staining (6).

The current patient is a typical case of HCL. The clinical presentation is pancytopenia with splenomegaly but not lymphadenopathy. Immunophenotypically, positive CD19, CD20, CD11c/CD22, CD25, CD103, and FMC-7 were demonstrated by flow cytometry; positive CD20, DBA-44, and TRAP were detected by immunohistochemical stains. The only atypical finding is the positive reaction to CD10, which can be seen in some HCL cases as previously reported. A honeycomb histologic pattern was shown in the bone marrow biopsy. The patient promptly responded to 2-chlorodeoxyadenosine therapy with complete remission in subsequent years.

The salient features for laboratory diagnosis of HCL are summarized in Table 6.22.3.

Clinical Manifestations

Clinically, most patients with HCL have splenomegaly; however, 10% to 20% of cases show no palpable spleen (31). About one third of patients have hepatomegaly, but lymphadenopathy is an unusual feature in HCL and occurs mainly in HCL variants (2). Skin, bone, and the central nervous system are seldom involved. The clinical course is usually chronic and indolent: Patients may have a stable condition and do not require treatment until severe pancytopenia occurs. Nevertheless, rare complications may occur, including paravertebral mass, meningitis, mediastinal mass with superior vena cava syndrome, massive abdominal lymph nodes with ascites, gastrointestinal involvement with protein-losing enteropathy, rupture of spleen, and opportunistic infections (31). On rare occasions, HCL may transform into a high-grade lymphoma that is probably associated with p53 mutation (11).

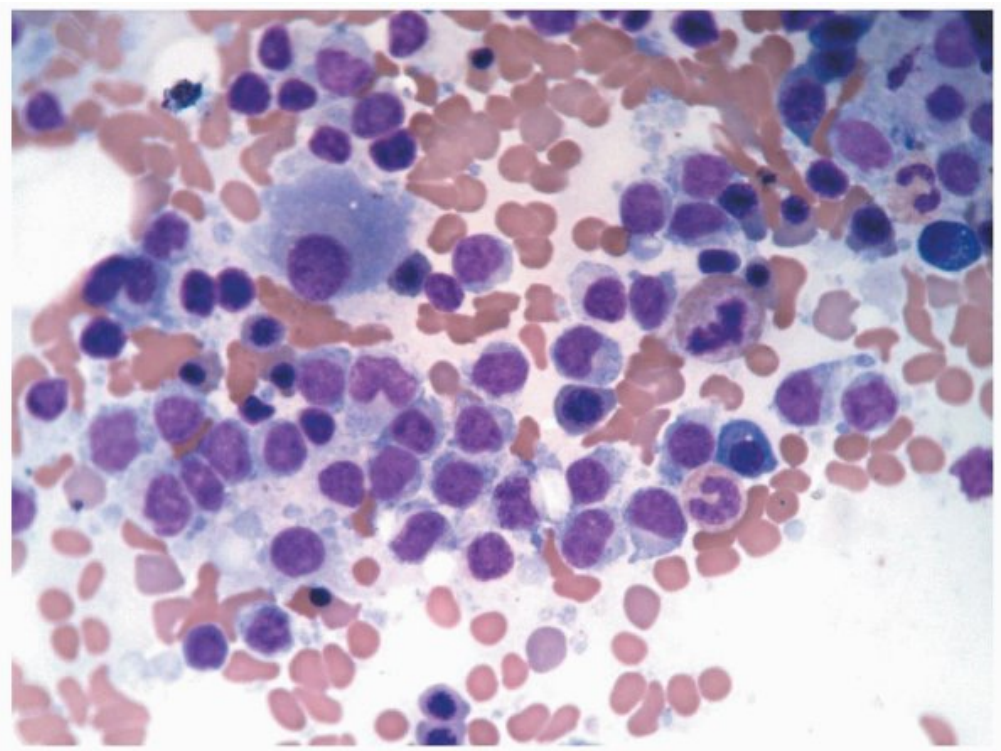

FIGURE 6.22.9 Bone marrow aspirate shows a cluster of leukemic hairy cells intermingled with a few nucleated red cells. Note lymphoid chromatin pattern of the nuclei and the fray cytoplasmic border. Wright-Giemsa, 60× magnification.

The major laboratory feature is pancytopenia with hemoglobin levels between 7 and 10 g/dL and platelet counts <80,000/μL (2). The pancytopenic feature, particularly monocytopenia, is useful to distinguish HCL from other leukemia/lymphoma, such as SLVL. If a monocyte count is normal or increased, a diagnosis of HCL should be questioned, unless it is an HCL variant. Cytopenia is probably the result of splenic sequestration of blood cells as well as inhibition of hematopoiesis in the bone marrow (6).

A dry tap on marrow aspirate is also a characteristic result of reticulin fibrosis in the bone marrow. The morphology of hairy cells in

bone marrow aspirate is frequently not typical (Fig. 6.22.9). Therefore, a bone marrow biopsy should be done routinely, and it is frequently diagnostic.

Cases of HCL variant have been reported with increasing frequency (1,7). In contrast to HCL, the variant usually shows high leukocyte count with no monocytopenia, tumor cells with prominent nucleoli, absence of reticulin fibrosis in the bone marrow, and a slightly different but characteristic immunophenotype, as mentioned before. It is important to recognize the HCL variant because it does not respond to conventional HCL treatments (such as interferon α and 2-chlorodeoxyadenosine) and its prognosis is much worse than that of HCL. HCL variant is also designated as type II HCL, or hybrid form of HCL. Thus far, the hybrid forms are mainly composed of HCL/PLL (47) or HCL/CLL (20,48,49).

Melo et al. (3) suggested that HCL, HCL variant, SLVL, and PLL represent a spectrum of cell types frozen at slightly different stages during late B-cell maturation. This statement has been supported by several in vitro experiments that show induction of CLL and PLL into HCL by tetradecanoyl phorbol acetate (50,51). Therefore, hybrid features are not uncommonly seen in these closely related low-grade B-cell neoplasms, and clear-cut diagnosis may not be always achievable in every case.

The treatment of HCL has progressed rapidly in recent years. In a 1994 study, the 5-year survival rate was 34.4% for untreated patients; 58.8% for patients receiving chemotherapy, steroids, or other drugs; 64.1% for splenectomized patients; and 88.9% for interferon-α-treated patients (52). Recently, the drugs of choice are the purine analogues. Both 2′-deoxycoformycin and 2-chlorodeoxyadenosine induce durable complete remissions in the overwhelming majority of patients (53).

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CASE 23 Splenic Marginal Zone Lymphoma

CASE HISTORY

A 46-year-old woman presented with weakness, fever, and left flank tenderness. She had a long history of Coombspositive hemolytic anemia, requiring frequent transfusions. Physical examination showed marked splenomegaly but no hepatomegaly or lymphadenopathy. Laboratory data revealed a hemoglobin level of 7.1 g/dL, hematocrit 21%, platelets 200,000/µL, and reticulocyte count 19.6%. The leukocyte count was 5,800/µL with 64% lymphocytes. A direct Coombs test was positive. Serum electrophoresis showed no monoclonal gammopathy. The patient was initially treated with steroids and subsequently underwent a splenectomy. Her hemoglobin and hematocrit promptly returned to normal. She became asymptomatic and returned to work full time 1 year after splenectomy.

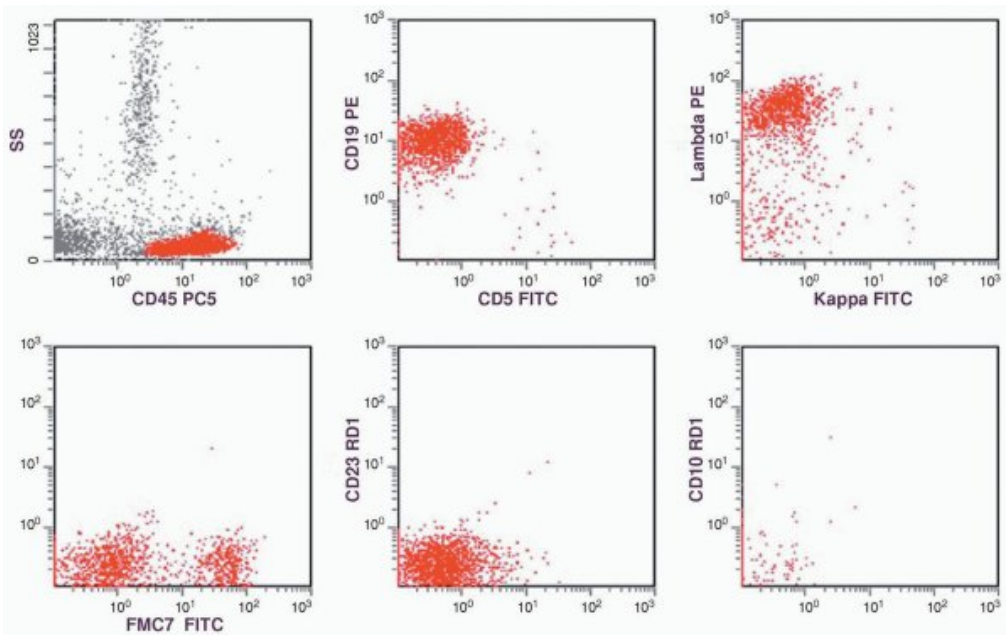

FIGURE 6.23.1 Flow cytometry histograms show positive cluster of differentiation (CD)19 and FMC-7 markers in a monoclonal λ B-cell population. CD5, CD23, and CD10 are negative, excluding mantle cell lymphoma, small lymphocytic lymphoma, and follicular lymphoma. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; FITC, fluorscein isothiocyanate; RD1, rhodamine.

FLOW CYTOMETRIC FINDINGS

Peripheral blood: Polyvalent surface immunoglobulin (Ig) 68%, IgG 3%, IgA 2%, IgM 64%, κ 3%, λ 66%, CD19 65%, CD20 70%, human leukocyte antigen-DR (HLA-DR) 64%, CD3 28%, CD5 29%, CD7 28%, CD11c 32%, CD14 17% (Fig. 6.23.1).

Bone marrow: Polyvalent surface Ig 68%, IgG 2%, IgA 1%, IgM 56%, κ 2%, λ 58%, CD19 67%, CD20 63%, HLA-DR 64%, CD3 32%, CD4 18%, CD5 28%, CD7 30%, CD8 13%, CD11c 25%, CD25 0%.

Spleen: Polyvalent surface Ig 50%, IgG 4%, IgA 2%, IgM 52%, κ 9%, λ 45%, CD3 36%, CD5 34%, CD7 36%, CD11c 20%, CD25 2%.

DISCUSSION

Splenic marginal zone lymphoma (SMZL) has been reported under various names, such as splenomegalic immunocytoma (1,2), malignant lymphoma-simulating leukemic reticuloendotheliosis (3), lymphocytic lymphoma-simulating hairy cell leukemia (HCL) (4), and chronic lymphoproliferative disorder resembling HCL (5). Melo et al. (6,7) first applied the term splenic lymphoma with villous lymphocytes (SLVL) in 1987. The terminology was adopted by the French-American-British (FAB) cooperative group in 1989 (8). Schmid et al. (9) first used the term SMZL, and the same group confirmed that SLVL and SMZL were similar histologically and immunophenotypically (10). Subsequently, SLVL was classified as SMZL as a provisional entity in the revised European-American classification of lymphoid neoplasms (11). This term has been adopted by the World Health Organization (WHO) classification of lymphoid neoplasms (12).

Morphology

SMZL is defined as a small B-cell lymphoma involving the spleen with a characteristic feature in the white pulp, variably described as bizonal or biphasic phenomenon or margination (Fig. 6.23.2) (Table 6.23.1). The germinal center of the white pulp is surrounded and finally replaced by small round lymphocytes (10,12,13). The small lymphocytes are, in turn, surrounded by medium-sized lymphocytes with more dispersed chromatin and abundant pale cytoplasm resembling the marginal zone cells. Scattered transformed large lymphoid cells are also seen in the outer zone. The mantle zone is effaced. Dunn-Walters et al. (14) believed that both small and large cells are of the same neoplastic clone, which is in contrast to the original thinking that the central small cells are the preserved mantle (9). When the germinal center is preserved, follicular colonization, manifested as neoplastic infiltration of the germinal center, can be observed (15).

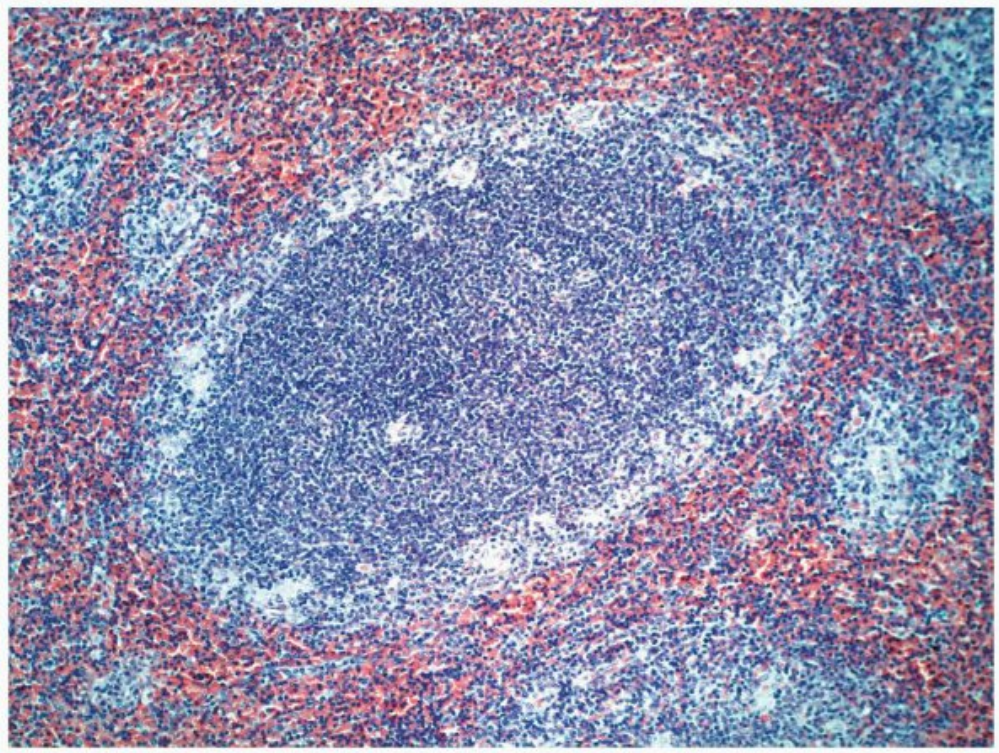

FIGURE 6.23.2 Splenectomy specimen reveals the bizonal phenomenon in the white pulp with the small cells in the center, replacing the germinal center and the marginal zone cells with pale cytoplasm in the periphery. Several cellular nodules composed of both cell types are seen in the red pulp around the follicle. Hematoxylin and eosin, 10× magnification.

TABLE 6.23.1

Characteristic Morphologic Features of Splenic Marginal Zone Lymphoma

Histologic pattern	Mainly splenic white pulp involvement with bizonal phenomenon; tumor nodules present in the red pulp with sinusoidal infiltration; sinusoidal infiltration in the bone marrow; micronodular pattern in the lymph node
Cytology	Small, darkly stained lymphoid cells in the center and pale stained large cells in the periphery of splenic follicles; villous lymphocytes in the peripheral blood
Specific features	Nodular pattern and bizonal phenomenon in the spleen; sinusoidal infiltration in the bone marrow

In the red pulp, besides sinusoidal infiltration, small nodules are sometimes present with the composition of tumor cells similar to that in the periphery of the white pulp follicles (10). Lymphoplasmacytic differentiation may be present, but the frequency is variable in different reports (6,15,16).

In SMZL, peripheral lymph node is seldom involved, but the involvement of the splenic hilar lymph node is more frequent (10,12). In the lymph node, the characteristic feature is a micronodular pattern, without the presence of marginal differentiation (Fig. 6.23.3) (12,17). However, the small lymphoid tumor cells and the marginal zone cells are still present in the lymph node and are intermixed. The sinuses are generally dilated. In the earlier stage, pathology may be confined to the cortical and paracortical areas, but diffuse effacement can be seen in later stages (2,3,16,18,19).

The bone marrow is frequently involved in SMZL. The infiltration can be nodular, interstitial, diffuse, or paratrabecular (Fig. 6.23.4) (2,3,6,16,18,20). However, the most characteristic pattern is intrasinusoidal infiltration, and the demonstration of this pattern may be facilitated by immunohistochemical stains with CD22 or DBA-44 (21). In an appropriate clinical setting, this pattern is considered by some to be diagnostic for SMZL and makes splenectomy unnecessary for diagnostic purposes (22, 23 and 24). Hepatosplenic T-cell

lymphoma may also show sinusoidal infiltration in the bone marrow, but the immunophenotype is entirely different.

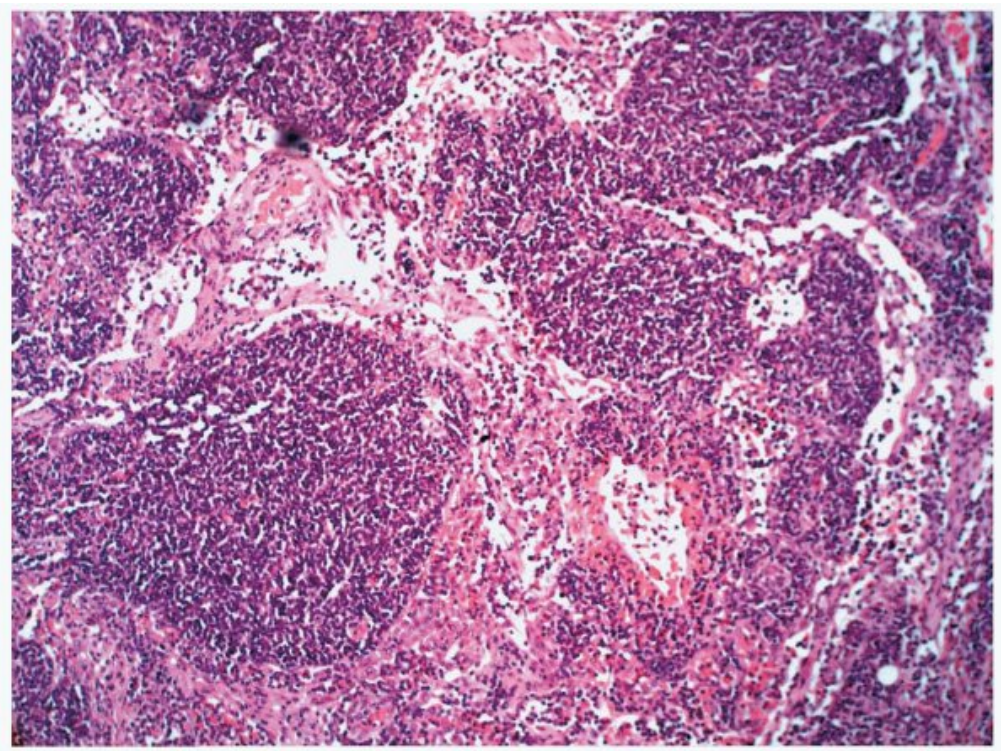

FIGURE 6.23.3 Splenic hilar lymph node biopsy shows a micronodular pattern with dilated sinuses. Hematoxylin and eosin, 10× magnification.

In the peripheral blood, villous lymphocytes may or may not be present in SMZL. However, in SLVL, villous lymphocytes constitute 38% to 99% of all lymphocytes present in the peripheral blood (25). Some authors advocate the use of 20% villous lymphocytes as the cutoff point between SLVL and SMZL (26). Nevertheless, as these two diseases show no significant difference in their clinical features, most studies consider them to be the same entity.

A typical villous lymphocyte is larger than the small lymphocytes found in chronic lymphocytic leukemia (CLL) and generally smaller than prolymphocytes (6,7,25). It has a round or ovoid nucleus with clumped chromatin; in one half of cases, it possesses a small but distinct nucleolus. The cytoplasm is usually moderate and basophilic, but it may be scanty in some cases. However, the striking feature of this cell is the presence of thin and short cytoplasmic villi with uneven distribution (Fig. 6.23.5). In contrast, hairy cells usually have numerous slender cytoplasmic villi (hairy projections) and a uniformly dispersed chromatin pattern. The large size and lower nucleocytoplasmic ratio of hairy cells also help to distinguish them from the villous lymphocytes of SLVL. The small-cell variant of SLVL may show scanty cytoplasm and no nucleoli (25); in those cases, CLL should be excluded by marker studies.

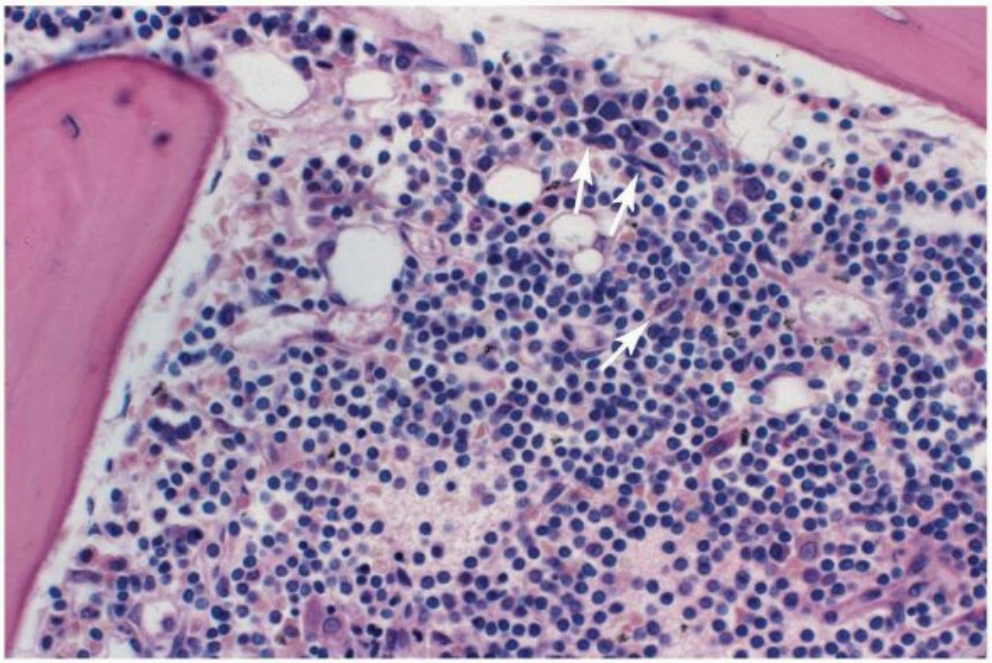

FIGURE 6.23.4 Bone marrow biopsy shows lymphoid infiltration replacing the normal hematopoietic cells. Note the endothelial lining of the marrow sinuses (*white arrows*), representing some intrasinusoidal infiltration. Hematoxylin and eosin, 20× magnification.

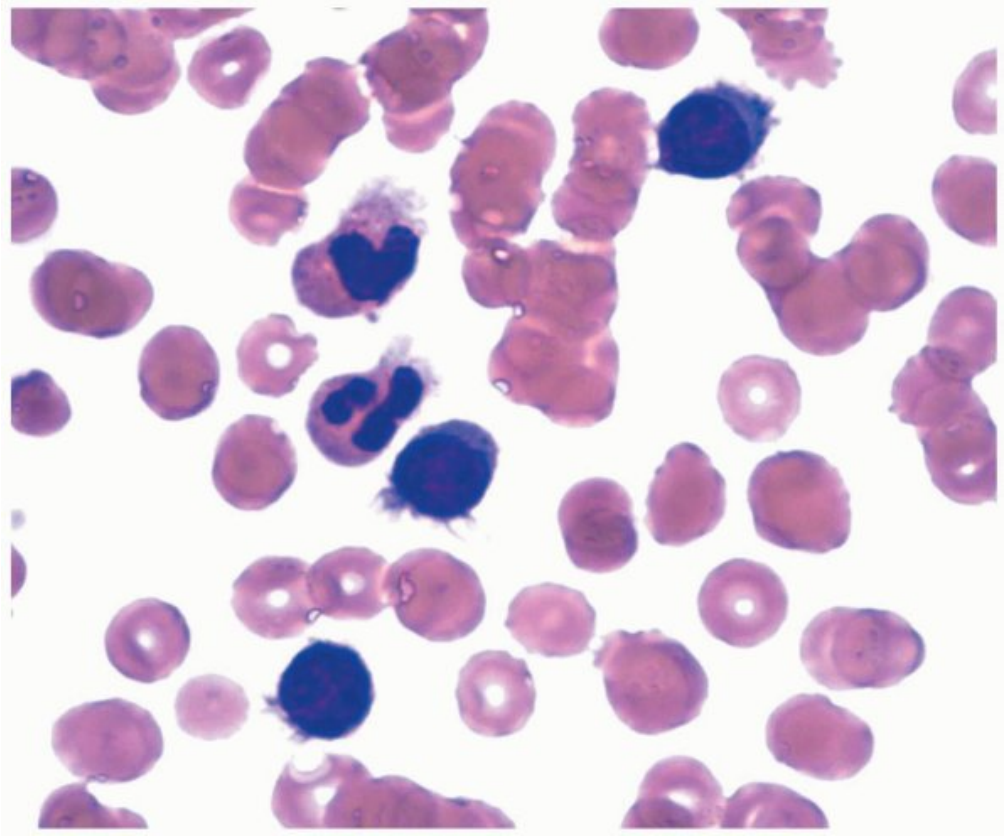

FIGURE 6.23.5 Peripheral blood smear reveals three villous lymphocytes with polar distribution of the villous cytoplasmic projections. Wright-Giemsa, 100× magnification.

Electron microscopy may show a polar distribution of a small number of short and thin cytoplasmic villi in cells of SLVL (Fig. 6.23.6), whereas cells from HCL may show ribosome-lamellar complex and evenly distributed long and slender cytoplasmic projections (Fig. 6.23.7).

In the liver, the portal tracts are mainly involved with nodular infiltration, but sinusoidal infiltration is also present (2,3,16,18,23). Other organs, such as skin, lung, and meninges, have also been reported in a few cases (24). Transformation to large cell lymphoma has been reported in 5% of SLVL cases (13) but in 10% to 13% of SMZL cases (17,26).

The major differential diagnoses of lymphomas involving the spleen include HCL, CLL, prolymphocytic leukemia, and mantle cell lymphoma. HCL involves mainly the red pulp with characteristic pseudosinus formation. Prolymphocytic leukemia shows the bizonal phenomenon similar to SMZL, but the cytology and immunophenotype should help distinguish these two entities. Mantle cell lymphoma is more difficult to distinguish from SMZL, but it involves mainly the white pulp, the tumor cells are monotonous, and plasma cell differentiation is absent (15). CLL involves both the red pulp and white pulp, and the immunophenotype differs from that of SMZL.

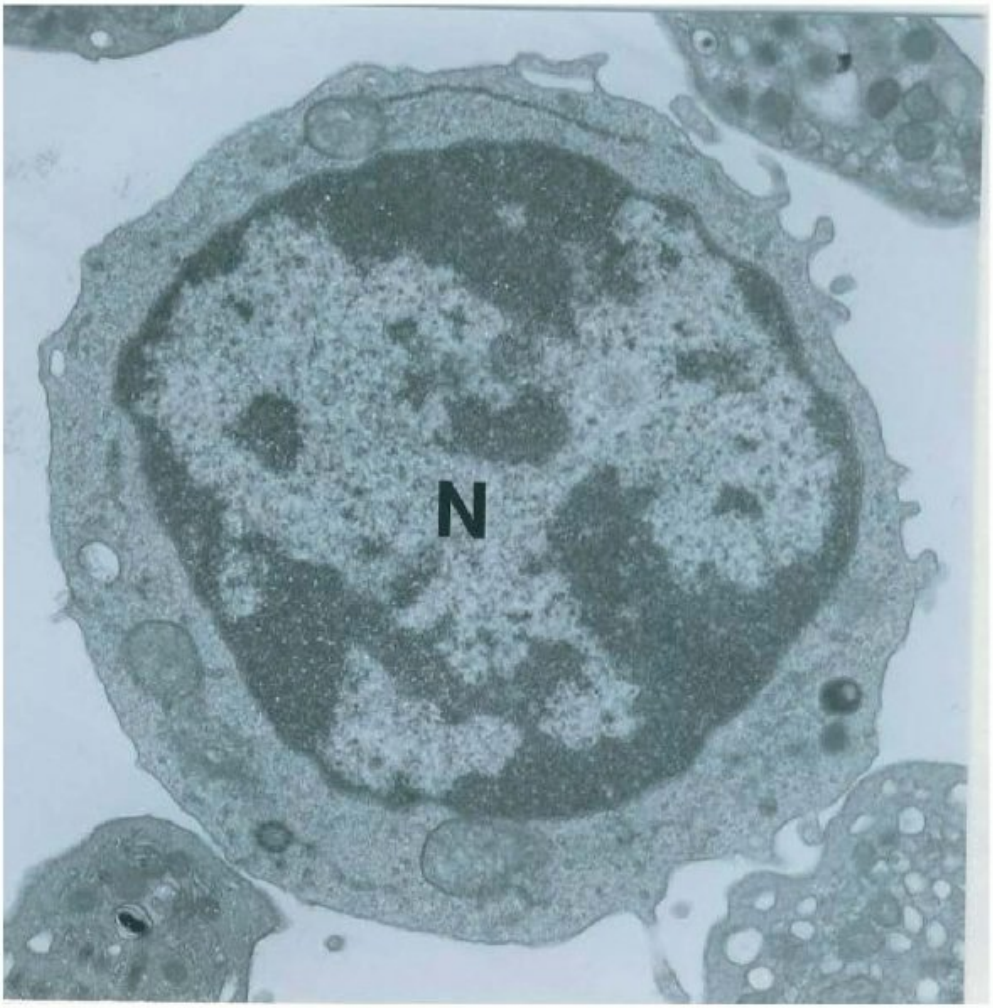

FIGURE 6.23.6 Electron micrograph shows a villous lymphocyte with polar distribution of cytoplasmic projection and no ribosome-lamellar complex. N, nucleus. 13,000× magnification. (Courtesy of Dr. Saul Teichberg, North Shore University Hospital, New York.)

Immunophenotype

Many markers have been studied in cases of SMZL and SLVL. The tumor cells are positive for surface Igs, CD19, CD20, CD22, CD24, FMC-7, HLA-DR, and CD45 (Table 6.23.2) (10,13,15,16,21,27, 28 and 29). Immunohistochemical stains may demonstrate DBA-44, CD79a, CD79b, bcl-2, paired box-containing family of genes/B-cell specific activator protein (PAX/BSAP) and CD45 (CD45RA/MT2) (10,13,21,23,29,30). Several immunohistochemical negative markers (CD10, CD23, CD43, bcl-6, and cyclin D1) are helpful in differential diagnosis (23).

HCL is characterized by its expression of CD11c, CD25, FMC-7, CD103, HC2, and tartrate-resistant acid phosphatase, and these markers used to be considered helpful to distinguish HCL, SMZL, and SLVL. However, current studies have found that one or more of these markers can be positive in some cases of SMZL and/or SLVL, but that only CD103 and HC2 are specific enough to make a definitive diagnosis of HCL (10,28). However, in SMZL, not all HCL markers are present in the same case (24).

TABLE 6.23.2

Comparison of Markers in Five Low-Grade B-Cell Neoplasms

	CD5	*CD10*	*CD11c*	*CD19*	*CD20*	*CD22*	*CD23*	*CD25*	*FMC-7*	*bcl-2*

SMZL	-	-	±	+	+	+	-	±	+	+
HCL	-	-	+	+	+	+	-	+	+	-
SLL	+	-	-	+	+	-	+	-	-	-
PLL	±	-	-	+	+	±	-	-	+	-
MCL	+	-	-	+	+	+	-	-	+	-

HCL, hairy cell leukemia; MCL, mantle cell lymphoma; PLL, prolymphocytic leukemia; SMZL, splenic marginal zone lymphoma; SLL, small lymphocytic lymphoma; CD, cluster of differentiation.

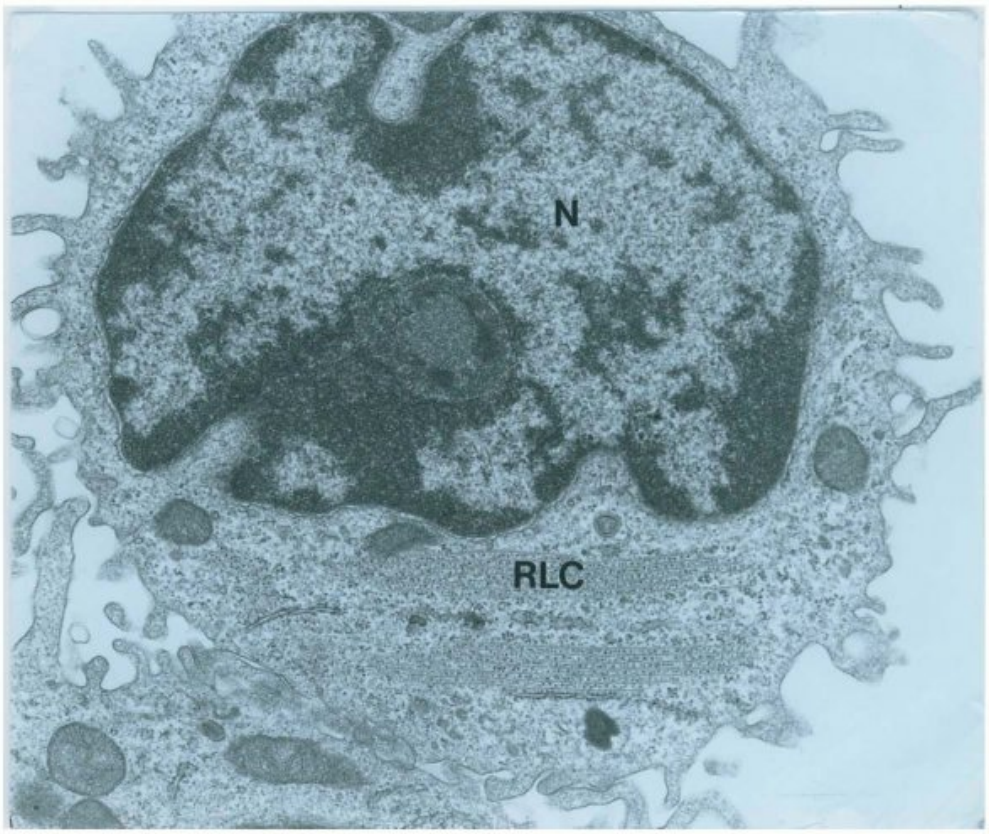

FIGURE 6.23.7 Electron micrograph shows a hairy cell with many fairly uniformly distributed long and slender cytoplasmic projections and two ribosome-lamellar complexes (RLCs). N, nucleus. 25,000× magnification. (Courtesy of Dr. Saul Teichberg, North Shore University Hospital, New York.)

SMZL and SLVL are usually negative for CD5 and CD23, which can help to distinguish small lymphocytic lymphoma and CLL (CD5+ CD23+) and mantle cell lymphoma (CD5+, CD23-) (13,15,21,27,29). Mantle cell lymphoma is also positive for cyclin D1 and CD43, but both markers are negative in SMZL (23). However, CD5 and CD23 can be positive in 20% to 30% of SMZL/SLVL cases (13).

Similar to SMZL, prolymphocytic leukemia is positive for FMC-7 and B-cell markers, but negative for CD23. CD5 is positive in one third of

prolymphocytic leukemia cases (7); these cases are most likely transformed from CLL.

TABLE 6.23.3

Salient Features for Laboratory Diagnosis of Splenic Marginal Zone Lymphoma

1. Mild to moderate peripheral lymphocytosis is present with or without villous lymphocytes.
2. The villi on tumor cells are thin and short with uneven distribution.
3. Tumor cells in both peripheral blood and spleen show monoclonal surface immunoglobulin pattern.
4. Positive B-cell antigens: CD19, CD20, CD22, CD24, FMC-7, HLA-DR
5. Specific markers CD11c, CD25, and tartrate-resistant acid phosphatase are positive in some cases.
6. Negative CD103 and HC2 in SMZL are helpful to distinguish from HCL.
7. Peripheral plasmacytosis and monoclonal gammopathy may be present.

HCL, hairy cell leukemia; SMZL, splenic marginal zone lymphoma; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; HC2, Hairy cell leukemia-associated antigen.

The immunohistochemical stains for CD10 and CDw75 (LN1) are usually negative in SMZL and SLVL cases, which distinguish them from follicular lymphoma (10,15,27,30). Although bcl-2 protein can be present in SMZL/SLVL, and follicular lymphoma, BCL-2 gene rearrangement is not detected in the former (10,27,30,31). The negative reaction to bcl-6 in immunohistochemical stain in SMZL and SLVL cases also helps to distinguish them from follicular lymphoma (23).

The other negative markers in SMZL and SLVL include CD2, CD3, CD14, CD35, CD43, CD45RO, and CD68 (10,27). CD38 can be present in 30% to 38% of SLVL and SMZL cases (10,28). The expression of CD38 usually predicts an unfavorable prognosis (32).

The salient features for laboratory diagnosis of SMZL are summarized in Table 6.23.3.

Comparison between Flow Cytometry and Immunohistochemistry

A diagnostic immunophenotype is not identified by either flow cytometry or immunohistochemistry. However, some cases of SMZL lymphoma may be positive for CD11c and CD25, so that may help distinguish other lymphomas besides HCL. Immunohistochemistry has the advantage of correlating morphology and immunophenotype; therefore, it is usually more useful in differential diagnosis.

Molecular Genetics

As a B-cell lymphoma, Ig gene rearrangement has been demonstrated in SMZL and SLVL cases, but T-cell receptor ß-chain gene and bcl-2 are in germlines (27). Of more interest is the mutation status of the Ig heavy-chain gene. Earlier literature considered that SMZL originated from postgerminal center memory B cells because the tumor cells have mutated Ig heavy-chain variable (V_H) genes (33). However, recent studies have found that SMZL cases may also have unmutated V_H genes and positive surface IgD that are consistent with a naïve B-cell origin (34). These two cell populations are normally present in the splenic marginal zone. Cases with unmutated V_H genes have been reported to have a more aggressive clinical course (35). In addition, several studies have demonstrated the nonrandom use of VH gene segments in SMZL cases (34). This finding suggests that at least some cases of SMZL are antigen driven, such as infections. Indeed, SMZL cases associated with malarial or hepatitis C virus infections have been reported (36,37).

The most common cytogenetic abnormality in SMZL involves allelic loss at 7q21-32, or translocation involving this region and the κ-chain region on chromosome 2, accounting for 40% to 45% of cases (17,24,26). As a result, the CDK6 gene located on 7q22 may be deregulated and might contribute to the pathogenesis of SMZL. For numeric abnormalities, whole or partial trisomy 3 is the most frequent finding in marginal zone B-cell lymphomas, accounting for 36% in SMZL (13,26,24).

There are many cytogenetic abnormalities reported, but the most controversial is t(11;14)(q13;q32), which has been described variably in 15% to 26% of patients with SMZL (17,26,38,39). t(11;14) is mainly seen in mantle cell lymphoma, representing bcl-1/IgH translocation, and bcl-1 encodes cyclin D1 or CCND1 protein. However, in the series of Troussard et al. (38), only 8 of 30 cases expressed CCND1, and 1 of 62 cases had bcl-1/IgH rearrangement. Cyclin D1 was not demonstrated by an immunoperoxidase technique in all 9 and 17 cases of SMZL in two series, respectively (30,40). Therefore, these cases are probably not misdiag-nosed mantle cell lymphoma. As the breakpoints at 11q13 differ between SMZL and mantle cell lymphoma, molecular biological analysis may furnish a correct diagnosis (38).

Gene expression profiling and tissue immunohistochemical microarray provide promising results for stratification of different patient subgroups in terms of prognosis (32). Shorter survival was associated with CD38 expression, naïve V_H genes, and the expression of a set of nuclear factor-κB (NF-κB) genes (32). In addition, gene profiling displays a largely homogenous signature, implying the existence of a single molecular entity (32).

Clinical Manifestations

SMZL is a low-grade non-Hodgkin lymphoma with a stable or slowly progressive clinical course (23,24,26,41). Most patients are older than 50 years. The presenting symptoms are usually mild weakness, fatigue, and abdominal discomfort caused by the enlarged spleen (6). Mild anemia and thrombocytopenia are common at presentation, usually due to hypersplenism (24).

Lymphocytosis, ranging from 10,000 to 40,000/μL, is a consistent feature of SMZL that is helpful to distinguish it from HCL, which often shows cytopenia in the peripheral blood. In addition to the circulating villous lymphocytes, 3% to 12% of plasma cells or plasmacytoid cells may be present in the peripheral blood in SMZL/SLVL (25). Monoclonal gammopathy is also frequently demonstrated, varying from 30% to 60% of cases (6,23,24,26). In some small series, monoclonal gammopathy was reported to be rare (16,27). Plasmacytosis and monoclonal gammopathy, if present, are useful but not specific for differential diagnosis.

In about 10% of patients, autoimmune phenomenon may be the presenting feature. Immune hemolytic anemia, immune thrombocytopenia, rheumatoid arthritis, cold agglutinins, anti-cardiolipin antibodies, lupus anticoagulant, and acquired von Willebrand disease have been reported in SMZL cases (23,24,26).

Asymptomatic patients with mild lymphocytosis but without cytopenia need only to have close follow-up (13,17,23,24,26). This group of patients may survive 10 to 15 years without treatment (13). When splenomegaly causes marked discomfort and/or hypersplenism, splenectomy is the treatment of choice (2,17,23,24,26,41). Most patients may have a long remission period after splenectomy. Only when patients have a progressive clinical course or when the disease has transformed into a large cell lymphoma should chemotherapy be considered. A French study group showed that when patients received chemotherapy as firstline treatment or had a high leukocyte count (>30,000/μL) or lymphocytopenia (<4,000/μL), the prognosis was worse than it was in control groups (29). Other adverse factors include lymphocytosis (>9,000/μL), raised $ß_2$ microglobulin, and the presence of a paraprotein or autoimmune phenomenon (26). In a study of 100 patients with SLVL, the 5-year overall survival was 78% (29).

In the current case, the immunophenotypes of the tumor cells from the peripheral blood, bone marrow, and spleen were comparable, showing a monoclonal IgM-λ population with positive CD19, CD20, and HLA-DR, but no dual staining of CD19/CD5 was demonstrated. This immunophenotype can only help to exclude small lymphocytic lymphoma and mantle cell lymphoma. The diagnosis was made by examining the histologic features in the spleen. The woman showed symptoms of autoimmune hemolytic anemia, but lacked other adverse prognostic factors such as thrombocytopenia, leukocytosis, or lymphocytopenia. As a result, she had a complete remission after splenectomy and went back to work full time 1 year after splenectomy.

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CASE 24 Extranodal Marginal Zone B-Cell Lymphoma

CASE HISTORY

A 60-year-old man was admitted to the hospital because of dyspnea on exertion, shortness of breath, and pleuritic chest pain for 3 months. The patient also had a decreased appetite and an approximate 12-pound weight loss over a 6-month period prior to admission. Upon questioning, the patient admitted to having had fevers, chills, and occasional night sweats. Chest x-ray examination detected bilateral lung mass. The patient had undergone transbronchial biopsies and brushings, which were nondiagnostic. He was then referred to our hospital for a diagnosed wedge biopsy of his lung mass.

Physical examination on admission determined that his lungs were clear to auscultation bilaterally. There was no superficial lymphadenopathy, and his liver and spleen were not palpable. Hematologic workup showed a total leukocyte count of 5,200/μL, hematocrit 40%, and platelets 259,000/μL. His liver function tests, creatinine, and lactate dehydrogenase were within normal ranges. The chest computed tomography (CT) scan revealed a 2 × 6 cm mass in the left lower lobe and a 2 × 3 cm mass in the right lower lobe. A video-assisted lung wedge biopsy was done, and a diagnosis of lymphoma was established.

The patient was treated with nine cycles of chlorambucil, and he continued to improve clinically. Successive CT scans revealed that his left lung lesion disappeared, but the right lung mass gradually increased in size. He also showed increasing fatigue and night sweats. A complete excision of the right lung lesion was performed 1 year after the first admission, and the patient was observed in the oncology clinic on a regular basis.

FLOW CYTOMETRY FINDINGS

Lung biopsy showed 34% CD2, 35% CD5, 1% CD10, 69% CD19, 0% CD19/CD5, 61% CD20, 10% CD23, 4% κ, 56% λ, and 2% CD14 (Fig. 6.24.1).

DISCUSSION

In 1983, Isaacson and Wright (1) developed the concept of lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) for some low-grade lymphomas in the gastrointestinal (GI) tract. This concept was later extended to lymphomas of other extranodal sites (2). Since then it has been

found that these tumors are derived from the marginal zone B cells (3) and are related to monocytoid B-cell lymphoma (4,5). In the revised European-American Lymphoma and World Health Organization (WHO) classifications, monocytoid B-cell lymphoma and MALT lymphoma are designated nodal and extranodal marginal zone lymphoma, respectively (6,7). The relationship of splenic marginal zone lymphoma to these two tumors is still disputable (4).

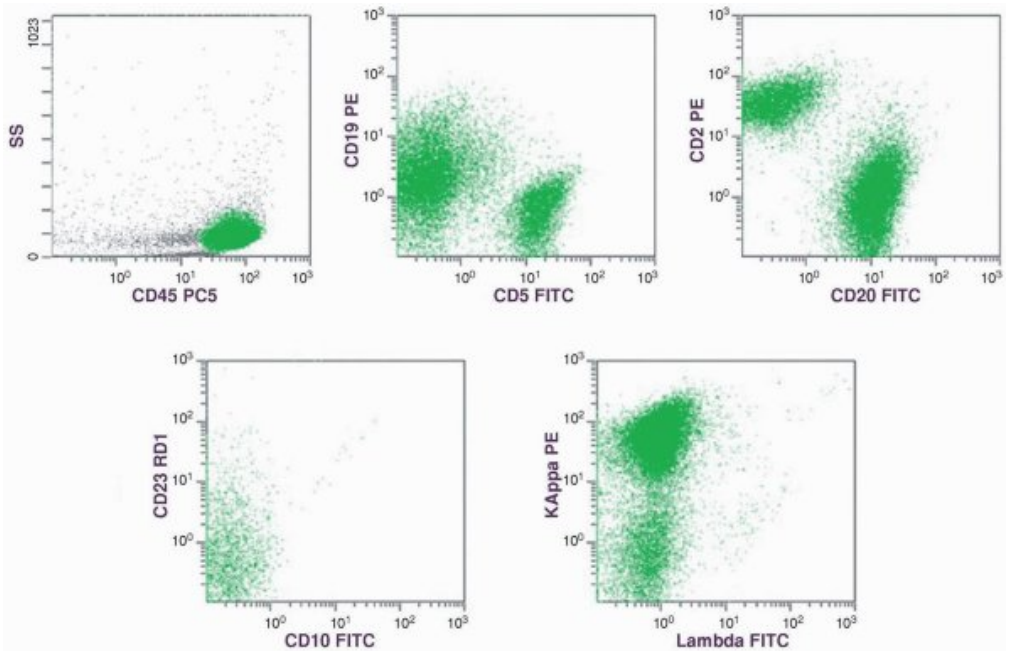

FIGURE 6.24.1 Flow cytometric histograms show positive reactions to CD19, CD20, and monoclonal κ immunoglobulin. A T-cell component, as demonstrated by CD2 and CD5, is also present in the gated population. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; RD1, rhodamine.

In light of the current knowledge, it is apparent that most, if not all, of the MALT lymphomas develop after a specific antigenic stimulation, such as infection by *Helicobacter pylori* in gastric MALT lymphoma, or on an autoimmune background, such as those seen in the salivary glands of patients with Sjögren syndrome (myoepithelial sialadenitis) and in the thyroid gland of patients with Hashimoto thyroiditis (8). There are several other suspicious infectious agents associated with MALT lymphoma, but their etiologic role has not been established. These include *Borrelia burgdorferi* in cutaneous MALT lymphoma, *Campylobacter jejuni* in intestinal MALT lymphoma, *Chlamydia psittaci* in ocular adnexal MALT lymphoma, and hepatitis C virus in nongastric MALT lymphoma (9,10). Furthermore, MALT lymphoma usually occurs in organs where lymphoid tissue is not present normally, such as the stomach, salivary gland, thyroid gland, and the bronchus. In these organs, the lymphoid tissue is called acquired MALT.

Morphology

MALT lymphoma is most commonly seen in the GI tract accounting for 50% of all cases (11). The stomach is the leading location, comprising 85% of the GI cases. Other relatively common sites include lung (14%), head and neck (14%), ocular adnexa (12%), skin (11%), thyroid (4%), and breast (4%) (12). On rare occasions, the central nervous system and the genitourinary tract can also be involved (13, 14 and 15). In fact, MALT lymphoma is considered the most common lymphoma in the urinary bladder (15).

Before the era of immunophenotyping and molecular genetics, MALT lymphoma in the above organs was probably misdiagnosed as pseudolymphoma. Because several criteria for lymphoid hyperplasia, such as polymorphous infiltration, intermixing plasma cells with a lymphoid population, and presence of follicular centers, overlap with the diagnostic features of MALT lymphoma, the confusion is understandable (16).

The major type of tumor cells may appear as centrocytes (small to medium-sized lymphocytes with cleaved nucleus

and moderately abundant cytoplasm), monocytoid B cells (cells with abundant pale cytoplasm and a centrally located bean-shaped or round nucleus), or small lymphocytes. The tumor population is polymorphous with the presence of transformed blasts and plasma cells (8). When plasma cells are present, the existence of an intranuclear inclusion (the Dutcher body) is characteristic of this entity (Fig. 6.24.2). When blasts are >10% of the lymphoid population, it is defined as high-grade MALT lymphoma by some authors (17). However, only the low-grade lymphoma with predominance of small lymphocytes is included in the definition of MALT lymphoma by the WHO group (7). When a large number of blasts present in a low-grade MALT lymphoma, it is considered to be transformation of MALT lymphoma to a diffuse large cell lymphoma rather than a high-grade MALT lymphoma (8).

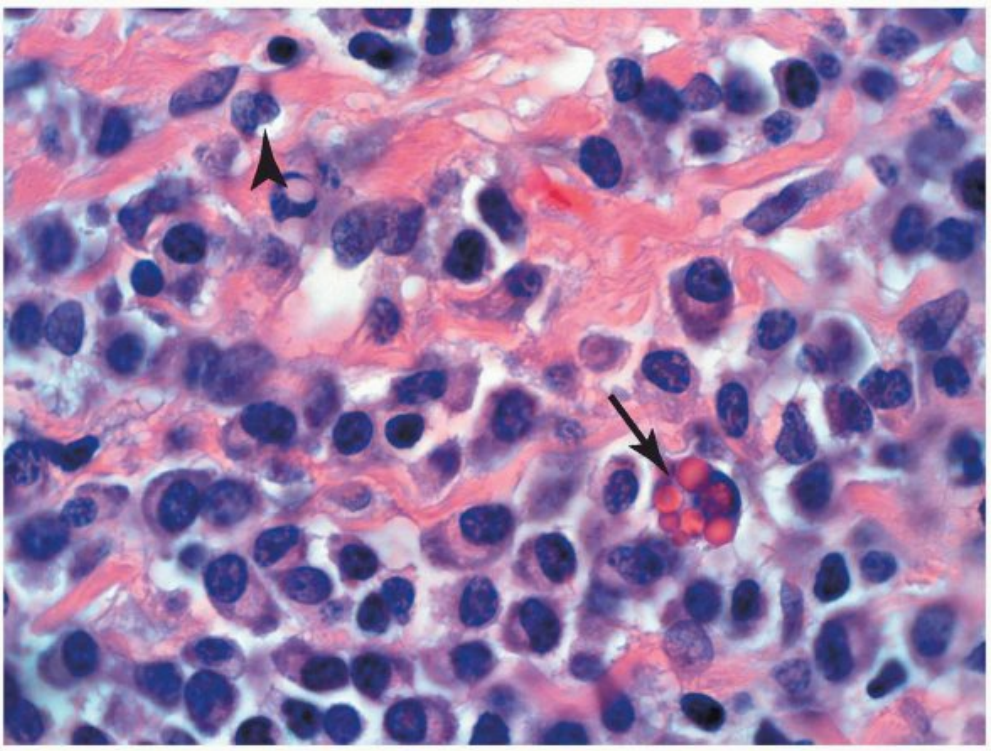

FIGURE 6.24.2 Intranuclear Dutcher body (*arrowhead*) and intracytoplasmic Russell bodies (*arrow*) are demonstrated among a cluster of lymphoplasmacytic cells in a case of lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). Hematoxylin and eosin, 100× magnification.

The cellular component may vary from organ to organ. Centrocyte-like cells are predominant in the stomach, monocytoid B cells are

commonly seen in the salivary glands, small lymphocytes are frequently present in the lung, and plasma and/or plasmacytoid cells are mainly seen in thyroid and skin (18).

These cells infiltrate around the germinal centers at the marginal zone with or without the separation by an attenuated mantle zone (8,16,19,20). The tumor cells expand from the germinal center to form a dense infiltrate, causing effacement of the normal architecture (Fig. 6.24.3). One of the characteristic features of MALT lymphoma is the invasion of epithelial structures to form lymphoepithelial lesions (Figs. 6.24.4 and 6.24.5). The tumor cells may also invade the germinal center, which is referred to as follicular colonization (Fig. 6.24.6). The demonstration of the lymphoepithelial lesion can be facilitated by the use of cytokeratin and B-cell stains, whereas the detection of follicular colonization can be facilitated by the use of CD21 or CD35 to show the follicular dendritic meshwork (11,18).

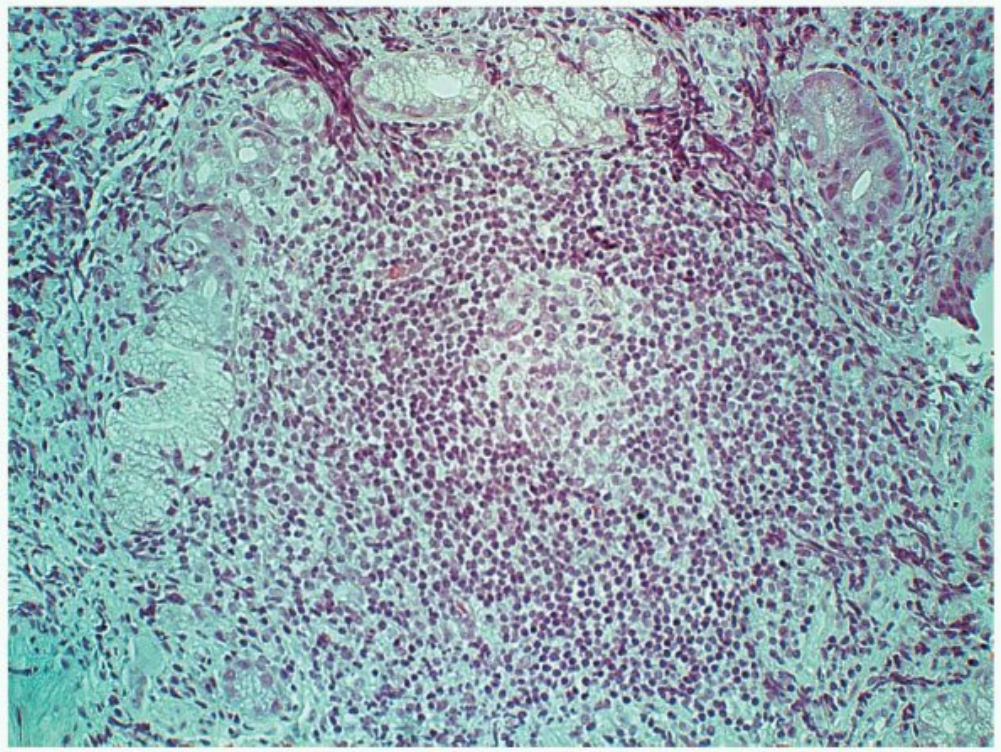

FIGURE 6.24.3 Germinal center is surrounded by a broad mantle zone of small lymphocytes, infiltrating the adjacent gastric mucosa in a case of lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). Hematoxylin and eosin, 20× magnification.

However, the pathologic features may vary from site to site (18). In the stomach, lymphoepithelial lesions are considered essential to the diagnosis of MALT lymphoma. In the lungs, it is characterized by the presence of a nodular pattern with lymphangitic tracking at the periphery (Fig. 6.24.7). Lymphoepithelial lesions can be seen in the bronchus (Fig. 6.24.8). In the salivary gland, the monocytoid B cells form broad interconnecting strands that surround and invade epimyoepithelial islands and displace the germinal center (18,19). The thyroid gland may show extensive lymphoid infiltrates around residual germinal centers, effacing the normal architecture. Follicular colonization and lymphoepithelial lesions may be present but are not essential for the diagnosis.

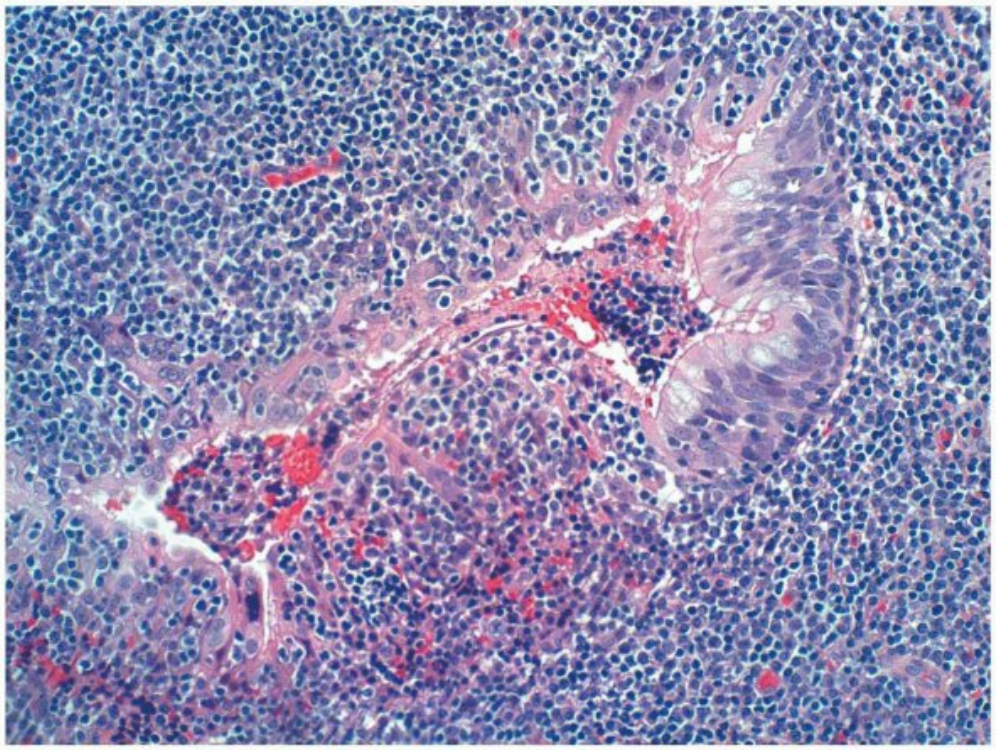

FIGURE 6.24.4 Prominent lymphoepithelial lesion shows a large gland being destroyed by small tumor cells. Hematoxylin and eosin, 20× magnification.

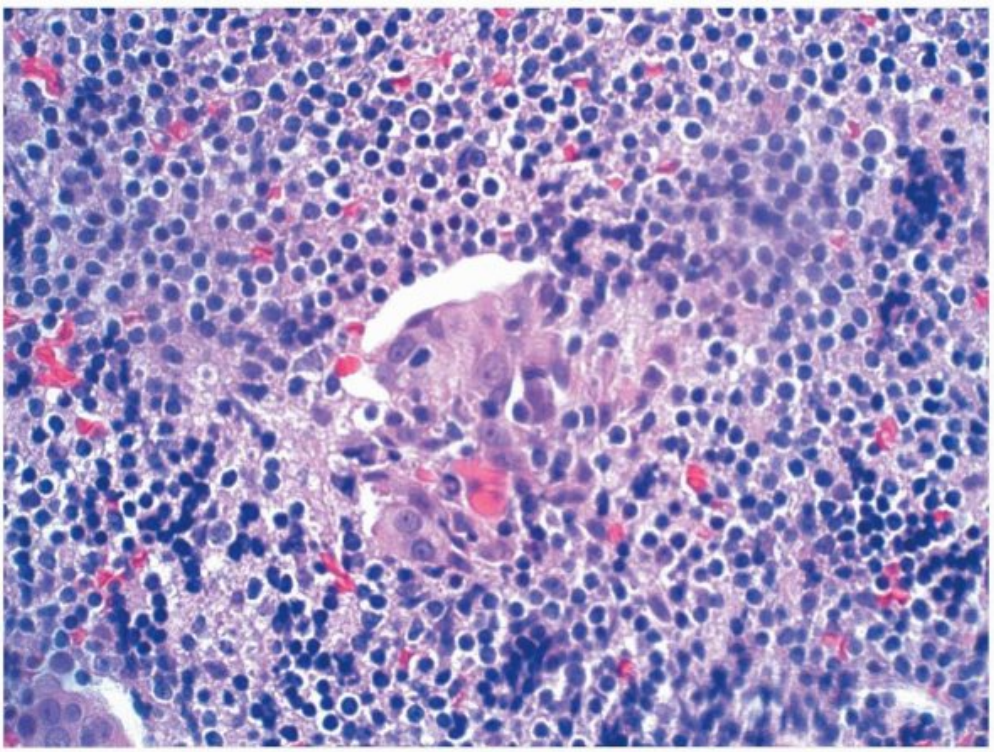

FIGURE 6.24.5 Residual epithelial fragments are surrounded by centrocyte-like cells in a case of lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma). Hematoxylin and eosin,

40× magnification.

There are no specific features of ocular MALT lymphoma except for the paucity of lymphoepithelial lesions. Cutaneous MALT lymphoma is characterized by lymphoplasmacytic infiltration in the upper dermis with lymphoepithelial lesions involving hair follicles, eccrine sweat glands, and sebaceous glands (18,20,21). Mammary MALT lymphoma shows no specific features (18,22). The cellular components are mainly monocytoid B cells with admixed plasma cells surrounding germinal centers. Lymphoepithelial lesions and follicular colonization may be present.

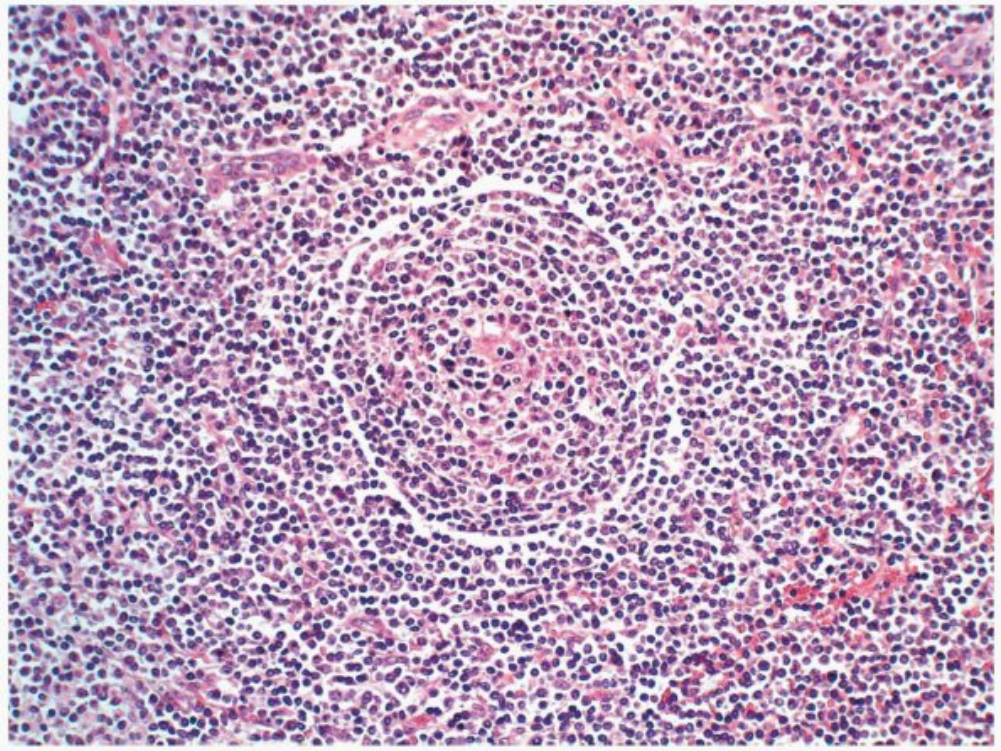

FIGURE 6.24.6 A colonized lymph follicle is surrounded by numerous small tumor cells. Hematoxylin and eosin, 20× magnification.

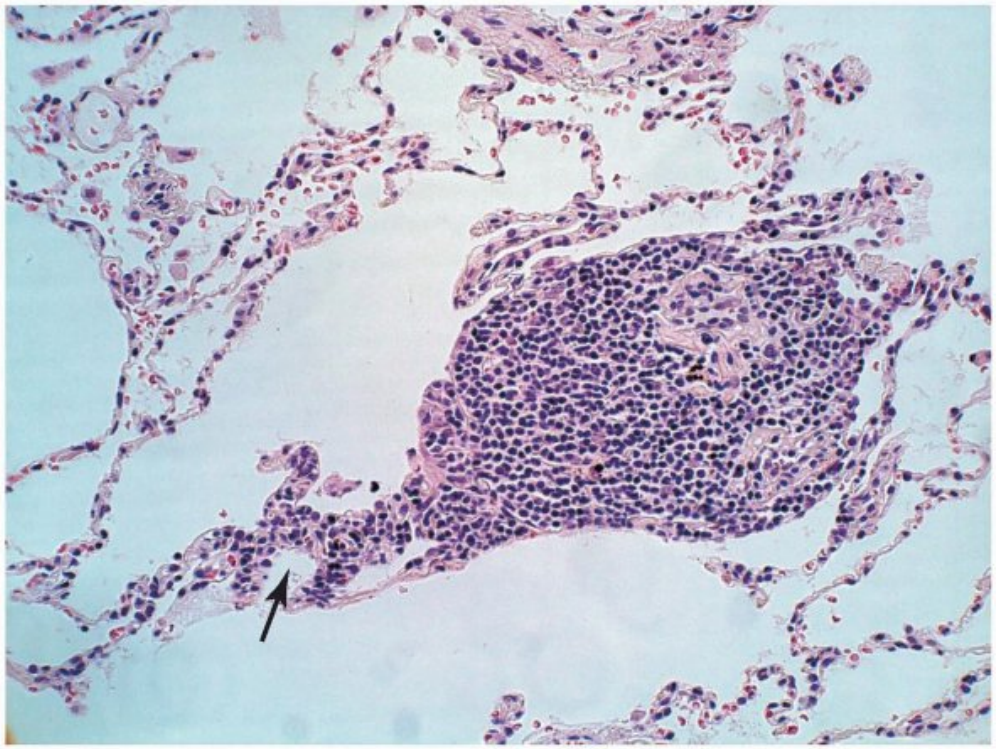

FIGURE 6.24.7 Pulmonary lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) case shows nodular tumor cell infiltration with lymphangitic tracking in the alveolar septum (*arrow*). Hematoxylin and eosin, 20× magnification.

A special form of MALT lymphoma in the small intestine is associated with α-chain disease, which is now designated immunoproliferative small intestinal disease (IPSID) (23,24). This subtype of MALT lymphoma is histologically similar to other subtypes, but presents with striking plasmacytic differentiation.

Several non-Hodgkin lymphomas may mimic MALT lymphoma. When follicular colonization is prominent, the histologic features may be similar to those of follicular lymphoma. However, the residual germinal centers show a polyclonal immunoglobulin (Ig) pattern and are not reactive to bcl-2, whereas follicular lymphoma shows no expansion of marginal zone and is positive for bcl-2 with a monoclonal Ig pattern (25). When lymphoplasmacytic infiltration is prominent, the differential diagnosis is lymphoplasmacytic lymphoma/ immunocytoma. However, if the tumor is located in an extranodal location, a diagnosis of MALT lymphoma is preferred (19). Immunocytoma also shows a monomorphous appearance and absence of germinal centers (18). Mantle cell lymphoma usually shows a monomorphous infiltration without the presence of plasma cells and transformed large cells. Small lymphocytic lymphoma may mimic MALT lymphoma with complete effacement of the normal architecture. The presence of proliferation center and the immunophenotype can help to establish the diagnosis of the former. The characteristic morphologic features in MALT lymphoma are summarized in Table 6.24.1.

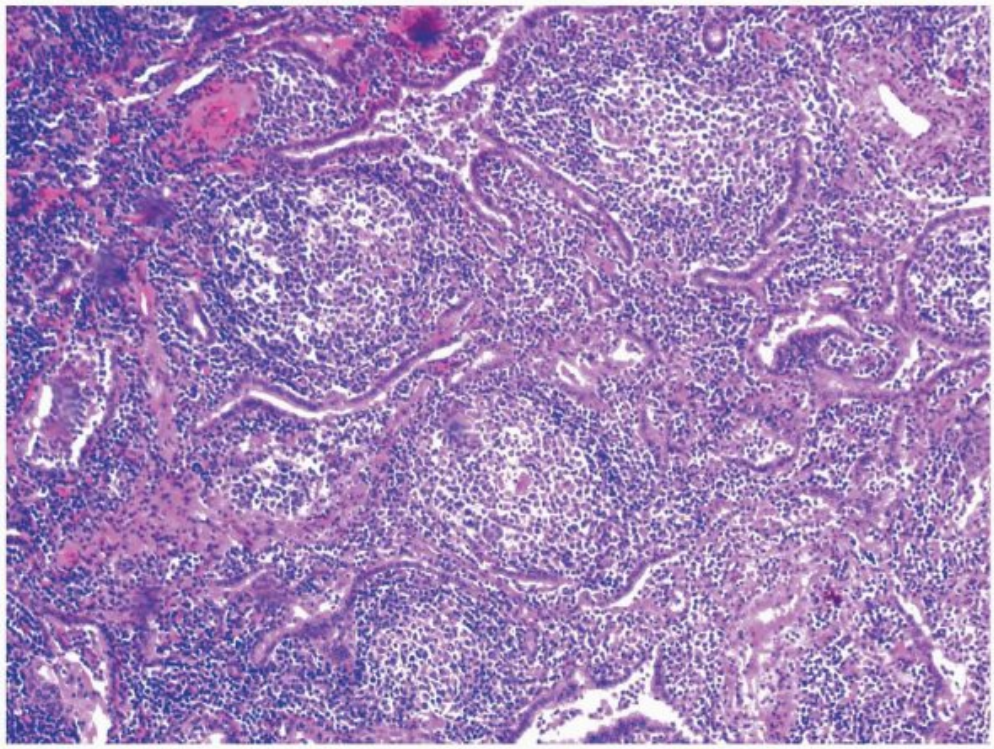

FIGURE 6.24.8 Pulmonary case of lymphoma of the mucosa-associated lymphoid tissue (MALT lymphoma) reveals multiple lymphoid follicles with prominent germinal centers, replacing the normal parenchyma and compressing the bronchioles. Hematoxylin and eosin, 10× magnification.

TABLE 6.24.1

Characteristic Morphologic Features of MALT Lymphoma

Histologic pattern	Lymphoepithelial lesions, marginal zone infiltration, and follicular colonization
Cytology	Polymorphic population with mainly monocytoid B cells, centrocyte-like cells, or small lymphocytes Plasma cells with Dutcher bodies and a few large blasts may be present.
Specific features	Combination of histologic pattern and cytology

MALT, lymphoma of the mucosa-associated lymphoid tissue.

Immunophenotype

There are no specific markers for MALT lymphoma. The role of immunophenotyping is to demonstrate a monoclonal B-cell population

(CD19+, CD20+, CD22+, CD79a+, bcl-2+) with the absence of specific markers for other non-Hodgkin lymphomas (11,19,26). For instance, the absence of CD5 and CD23 excludes the diagnosis of small lymphocytic lymphoma and chronic lymphocytic leukemia. The absence of CD5 and bcl-1 and/or cyclin D1 rules out mantle cell lymphoma. Negative reactions to CD10 and bcl-6 in the germinal centers are against the diagnosis of follicular lymphoma (27). The surface Ig on the tumor cells is most frequently IgM, but in the case of α-chain disease, only α Ig is present without the light-chain expression.

MALT lymphoma is difficult to distinguish from nodal and splenic marginal zone B-cell lymphomas by immunophenotyping, but IgD is positive in splenic marginal zone lymphoma and some cases of nodal marginal zone B-cell lymphoma, whereas it is consistently negative in MALT lymphoma (28). The reaction to CD43 is variable, and CD23 and CD5 can be positive in a few cases of MALT lymphoma (1,29). However, when CD5 is positive, the diagnosis of MALT lymphoma should be made only with definitive support by other parameters. In the few reported cases of CD5-positive MALT lymphoma, a tendency of recurrence, dissemination, and leukemic manifestation was shown (29). Nevertheless, cases of CD5-positive MALT lymphoma with an indolent clinical course have also been reported (27).

As mentioned before, the demonstration of a follicular meshwork by CD21 or CD35 is helpful in recognizing follicular colonization, and the demonstration of the epithelial component by cytokeratin stain is useful in detection of the lymphoepithelial lesions. As a low-grade lymphoma, MALT lymphoma usually shows a low proliferation fraction as expressed by Ki-67 staining, which is in marked contrast to the residual germinal centers that show a high proliferation fraction (25). DNA analysis also displays low S-phase fractions (≤3%) in MALT lymphoma, consistent with the above finding (26).

The current case showed the characteristic pulmonary symptoms with multiple lesions in the lung. The lung biopsy revealed a multinodular pattern and lymphangitic spreading of the tumor cells. The bronchial epithelium was extensively infiltrated by the tumor cells with prominent destruction and replacement of the normal architecture. The flow cytometric findings of negative reactions to CD5/CD19, CD10, and CD23 are helpful in distinguishing it from other low-grade small cell lymphomas. A single lung lesion can be treated effectively with surgical excision, but the patient had bilateral lesions and was initially treated with chemotherapy until the left lung lesion disappeared. The patient had an uneventful recovery after excision of the right lung lesion and was in a long-term remission.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometric analysis may help to exclude other lymphomas by the negative reactions to CD5, CD10, and CD23. Immunohistochemistry, in addition, can also help to detect follicular colonization by the demonstration of the follicular dendritic meshwork and lymphoepithelial lesion by the demonstration of the cytokeratin-positive epithelial cells. Recent findings of the presence of gene products in the tumor cells may provide some positive markers for the diagnosis of MALT lymphoma (9). In cases with t(11;18) and t(1;14), nuclear bcl-10 can be demonstrated in the tumor cells. In contrast, cytoplasmic bcl-10 is shown in cases with t(14;18). In addition, cytoplasmic malt1 protein is present in cases with t(14;18) and nuclear foxp1 protein in cases with t(3;14).

Molecular Genetics

MALT lymphoma shows somatic hypermutation of Ig heavy-chain genes and extensive ongoing somatic mutation that put it into the late memory B-cell stage, presumably due to interaction with the follicular center and the effect of antigenic stimulation (8,30).

Because the distinction in morphology between MALT lymphoma and lymphoid hyperplasia is sometimes blurred, molecular genetic techniques are helpful for the differential diagnosis. The identification of Ig gene rearrangements by polymerase chain reaction in frozen or paraffin sections is particularly useful in establishing the diagnosis of MALT

lymphoma (31). However, Ig gene rearrangements can be demonstrated in uncomplicated gastritis, and false-negative results are sometimes encountered in confirmed lymphoma cases (8,32). Therefore, the interpretation of polymerase chain reaction results should be in conjunction with morphology and immunophenotyping. Nevertheless, a study showed that, even a lesion with a low score according to the Wotherspoon-Isaacson histologic scoring system, if monoclonality is demonstrated, the lesion may relapse in further follow-up (31).

TABLE 6.24.2

Characteristics of Molecular Genetic Findings in MALT Lymphoma

Translocation	*Genes Involved*	*Additional Aberrations*	*Anatomic Sites*
t(11;18) (q21;q21)	API2-MALT1	None	GI tract, lung, head, and neck
t(1;14)(p22;q32)	BCL10-IgH	None	Lung, stomach, skin

t(1:2)(p22;p12)	BCL10-IgL_k	None	Lung, stomach, skin
t(14;18) (q32;q21)	IgH-MALT1	Trisomy 3, 12, or 18	Lung, liver, ocular adnexa, skin
t(3;14)(p14;q32)	FOXP1-IgH	Trisomy 3	Thyroid, ocular adnexa, skin

MALT, lymphoma of the mucosa-associated lymphoid tissue; GI, gastrointestinal; Ig, immunoglobulin.

There have been extensive molecular genetic studies in MALT lymphoma (Table 6.24.2). The accumulated data demonstrate a close correlation between the karyotypes and clinical manifestations, including the site of the lymphoma, therapeutic response, and the potential for high-grade transformation.

t(11;18)(q21;q21)

This translocation is the most common chromosomal aberration demonstrated in MALT lymphoma, with a frequency varying from 13.5% to 35% (9,33). It has been mostly seen in MALT lymphoma of the GI tract and lung, but also has been reported in the head and neck. Molecular characterization has demonstrated the inhibitor of apoptosis 2 (API2) gene on 11q21 and the MALT lymphoma-associated translocation (MALT1) gene on chromosome 18q21. As a result of the translocation, an API2-MALT1 fusion gene is formed, which encodes for the api2-malt1 protein. This fusion gene and protein lead to increased inhibition of apoptosis with a resultant survival advantage of the tumor cells, independent of antigen (33). The api2-malt1 fusion protein may also activate the nuclear factor-κB (NF-κB) pathway, which is considered the unifying mechanism for several cytogenetic aberrations (9). NF-κB appears to drive antigen-independent growth of the lymphoma cells so that eradication of *H. pylori* or other antigens will no longer be effective in the treatment of the patients (34).

Cases with t(11;18) are unresponsive to *H. pylori* eradication therapy, partly because of the autonomous growth of the tumor cells and partly because most such cases are *H. pylori* negative (34). Another characteristic feature of t(11;18) is the association of nuclear bcl-10 protein expression, in contrast to the normal expression of cytoplasmic bcl-10 protein in the germinal center cells (34).

Clinically, cases with t(11;18) usually present with advanced stage disease, but are unlikely to develop secondary chromosomal abnormalities or to transform into diffuse large cell lymphoma (9,30). t(11;14) is highly specific for MALT lymphoma, as it has not been reported in nodal or splenic marginal zone lymphomas or other B-cell neoplasms (27).

t(1;14)(p22;q32) or t(1;2)(p22;p12)

This translocation occurs in 1% to 2% of MALT lymphoma and has been reported in cases involving the lung, stomach, and skin (9). As a result of the translocation, the BCL10 gene on chromosome 1p22 is relocated to chromosome 14, under the control of the IgH enhancer region, or relocated to chromosome 2, under the control of the IgL_k region. As a result, bcl-10 protein is overexpressed in the nuclei of the tumor cells. The bcl-10 protein, in turn, activates the NF-κB pathway.

t(1;14) and t(1;2) are specific for MALT lymphoma and have not been reported in other lymphomas. Because these aberrant karyotypes activate the same pathway as t(11;18), patients also present with advanced stage disease and are unlikely to respond to *H. pylori* eradication (35).

t(14;18)(q32;q21)

This karyotypic abnormality occurs in 15% to 20% of MALT lymphoma, mainly involving the non-GI MALT lymphomas, such as the liver, lung, ocular adnexa, and skin (9,33). As a result of the translocation, the MALT1 gene on chromosome 18q21 is juxtaposed with the IgH enhancer region, resulting in MALT1 overexpression. This overexpression leads to the activation of the NF-κB pathway. Bcl-10 protein is also expressed in the cytoplasm of the tumor cells. Cases with t(14;18) frequently harbor additional karyotypic abnormalities, including trisomies 3 and/or 12 and 18 (9).

t(3;14)(p14;q32)

This translocation involves the forkhead box protein P1 (FOXP1) genes on chromosome 3p14 relocating to chromosome 14 under the control of the IgH enhancer. As a result, foxp1 protein is overexpressed, but it is unclear how this overexpression induces tumorigenesis (33). The overexpression of foxp1 protein has also been found in a subset of diffuse large B-cell lymphoma, and this finding raises the possibility that MALT lymphoma with this translocation may be at risk to transform to diffuse large B-cell lymphoma (9).

This aberration has been encountered in 10% of MALT lymphoma cases, and is present in the thyroid, ocular adnexa, and skin (9,33). Most t(3;14) positive cases also harbor additional genetic abnormalities, such as trisomy 3 (33).

TABLE 6.24.3

Salient Features for Laboratory Diagnosis of MALT Lymphoma

1. Monoclonal B-cell population, positive for the following B-cell markers: CD19, CD20, CD22, CD79a, bcl-2
2. Absence of the following markers: CD5, CD10, CD23, bcl-1/cyclin D1, and IgD
3. Immunohistochemical stain may demonstrate bcl-10 and malt1 protein in cases with special karyotypes.
4. Rearrangement of immunoglobulin genes
5. No rearrangement of BCL-1, BCL-2, and c-MYC, but rearrangement of BCL-6 can be seen in a subset of MALT lymphoma.
6. Demonstration of t(11;18), t(1;14), or t(1;2) is diagnostic for MALT lymphoma.

CD, cluster of differentiation; Ig, immunoglobulin; MALT, lymphoma of the mucosa-associated lymphoid tissue.

The mechanism underlying the transformation from low-grade MALT lymphoma to a high-grade lymphoma is still unclear. However, a number of genetic aberrations have been present in cases with histologic transformation, such as p53 allelic loss and mutation, hypermethylation of p15 and p16, and p16 deletions (9,36).

Although bcl-2 antigen is present in MALT lymphoma, rearrangement of BCL-2 gene has not been demonstrated in this tumor. BCL-1 and c-MYC rearrangement are also not present (36,37), but c-MYC and p53 mutations are encountered in MALT lymphoma (36, 37 and 38). In the earlier literature, BCL-6 rearrangement and mutation were demonstrated only in high-grade MALT lymphoma (38,39). However, a recent study with the fluorescence in situ hybridization technique confirmed BCL-6 translocation in 6 of 306 cases of MALT lymphoma, involving the stomach, salivary gland, lung, skin, and thyroid (40). The salient features for laboratory diagnosis of MALT lymphoma are summarized in Table 6.24.3.

Clinical Manifestations

Low-grade gastric and pulmonary MALT lymphomas are usually seen in individuals older than 50 years, but younger patients have been encountered more frequently in recent years (8). For instance, MALT lymphoma has been reported in pediatric cases with and without human immunodeficiency virus infection (41,42).

As mentioned before, one of the unique features of MALT lymphoma is the demonstration of antigenic stimulation as the mechanism of tumorigenesis. The most striking example is *H. pylori* infection in gastric MALT lymphoma. It is hypothesized that *H. pylori* infection may stimulate both the neoplastic B cells and the *H. pylori*-specific tumor-infiltrating T cells (43). These T cells provide the microenvironment for tumor cell proliferation. This theory is supported by the experiment that the cultured tumor cells maintained in standard conditions die in 5 days without the coexistence of *H. pylori* and T lymphocytes (8). Therefore, even if the tumor cells spread to distant sites, they would fail to grow there in the absence of *H. pylori* and the T cells activated by the organism.

The most important evidence to support the etiologic role of *H. pylori* in MALT lymphoma is that the eradication of this organism results in regression of the tumor in 50% to 100% of cases in various series (8,27,44). However, as mentioned before, cases with t(11;18) are significantly less likely to respond to *H. pylori* eradication therapy (34).

The clinical symptom depends on the involved organ. For instance, patients with gastric MALT lymphoma may have nonspecific dyspepsia and epigastric pain (34), and those with pulmonary MALT lymphoma may have cough, dyspnea, chest pain, and hemoptysis (45). Monoclonal gammopathy is present in about one third of patients with MALT lymphoma in two studies, but its clinical relevance is controversial (46,47).

Most patients have an indolent clinical course with localized symptoms. However, a recent study showed that dissemination of MALT

lymphoma occurred in 54 of 158 patients, but the 5- and 10-year overall survival rates were similar in patients with or without dissemination (48). Therefore, extensive staging to assess dissemination is probably not necessary (49).

As *H. pylori* infection is prevalent in gastric MALT lymphoma cases, it is recommended that all patients should be treated with a course of eradication therapy, except those with t(11;18) or large cell transformation (34). For localized disease, local treatment with either radiation therapy or surgery is effective (20,49). In patients with disseminated disease at presentation, chemotherapy should be used (49). MALT lymphomas have the best 5- and 10-year survival among all non-Hodgkin lymphoma subtypes in the study by the International Lymphoma Study Group (50). The 10-year survival rate of patients with low-grade MALT lymphoma is approximately 80% (17). Non-GI MALT lymphomas appear to progress more rapidly than GI MALT lymphomas (12).

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CASE 25 Nodal Marginal Zone B-Cell Lymphoma

CASE HISTORY

A 74-year-old man had a history of oligodendroma in the left frontal lobe for 14 years. Because of the patient's refusal, no surgical, radiation, or chemotherapy treatment was given. He only received dilantin and phenobarbital to control his petit mal seizures

secondary to the brain tumor. In a routine check-up of dilantin level and complete blood count, he was found to have a total leukocyte count of 20,000/μL with 78% lymphocytes. A subsequent flow cytometric analysis of the peripheral blood identified a monoclonal B-cell population.

Physical examination revealed that the patient had bilateral axillary and left inguinal lymphadenopathy. A computed tomography (CT) scan also documented enlarged lymph nodes in the mediastinum and para-aortic site. However, he did not have hepatosplenomegaly. A biopsy was performed on the right axillary lymph node. Morphologic examination and flow cytometric analysis identified a small cell lymphoma. As dilantin is known to be able to induce lymphoma, this medication was discontinued. Nevertheless, because the patient had no B symptoms (fever, weight loss, and night sweats) and because the lymphoma was low-grade, no treatment of the lymphoma was given. The patient remained asymptomatic for 2 years without any sign of lymphoma progression. He finally died of unrelated illness.

FLOW CYTOMETRIC FINDINGS

Peripheral blood: CD5 36%, CD19 56%, CD19/CD5 0%, CD20 57%, CD23 13%, κ 2%, λ 56%, CD10 0%, CD14 0%.

Lymph node biopsy: CD5 0%, CD19 98%, CD19/CD5 0%, CD20 96%, CD23 4%, FMC-7 23%, κ 0%, λ 94%, CD10 4%, CD14 1% (Fig. 6.25.1).

DISCUSSION

Nodal marginal zone B-cell lymphoma (NMZL) was originally called monocytoid B-cell lymphoma (MBCL) (1), or parafollicular B-cell lymphoma (2). However, it was later found that MBCL was similar to lymphoma of the mucosa-associated lymphoid tissue (MALT) in morphology, immunophenotype, and cytogenetics (3,4). Since then, MBCL and MALT type lymphomas have been designated as nodal and extranodal marginal zone B-cell lymphomas, respectively, in the revised European-American Lymphoma and World Health Organization classifications (5,6). Splenic marginal zone lymphoma is also similar to the above tumors, but their relationship is controversial (4). The frequency of NMZL is low, consisting of 1.8% of lymphoid neoplasms (6).

Morphology

The original description of MBCL includes three histologic patterns: the interfollicular and/or mantle zone-like pattern, the sinusoidal pattern, and the diffuse pattern (7). The last pattern is rare, and a diagnosis should be made with an immunologic confirmation (8). Campo et al. (3) described two histologic patterns in primary NMZL. The first one, designated splenic type, is characterized by a nodular proliferation of large and small lymphoid cells surrounding and infiltrating residual germinal centers (Figs. 6.25.2, 6.25.3, 6.25.4). The mantle corona is absent in most cases. The nodal architecture is totally effaced. The second pattern, designated MALT type, is characterized by a predominantly perivascular, perisinusoidal, and parafollicular infiltration. The mantle zone is preserved between the germinal centers and the tumor cells. Reactive follicles are always present. The MALT type was the predominant pattern in this study. However, both splenic and MALT types can be present in the same patient (9).

Nathwani et al. (8) emphasized that reactive follicles were present in >85% of their cases. However, their report did not elaborate the relationship between the presence of reactive follicles and the different tumor cell infiltration patterns (whether a sinus pattern, interfollicular pattern, or marginal zone pattern). Mollejo et al. (10), in contrast, emphasized the presence of marginal zone pattern in NMZL.

Unlike the description by Campo et al. (3), theirs indicated that the tumor cells were separated from the germinal center by a mantle zone in the “splenic type” (10). Traverse-Glehen et al. (11) emphasized that the splenic type of NMZL is distinguished from splenic marginal zone lymphoma in the absence of a biphasic pattern. In some cases, the germinal centers are invaded or totally replaced by the tumor cells; a process designated follicular colonization (4,8).

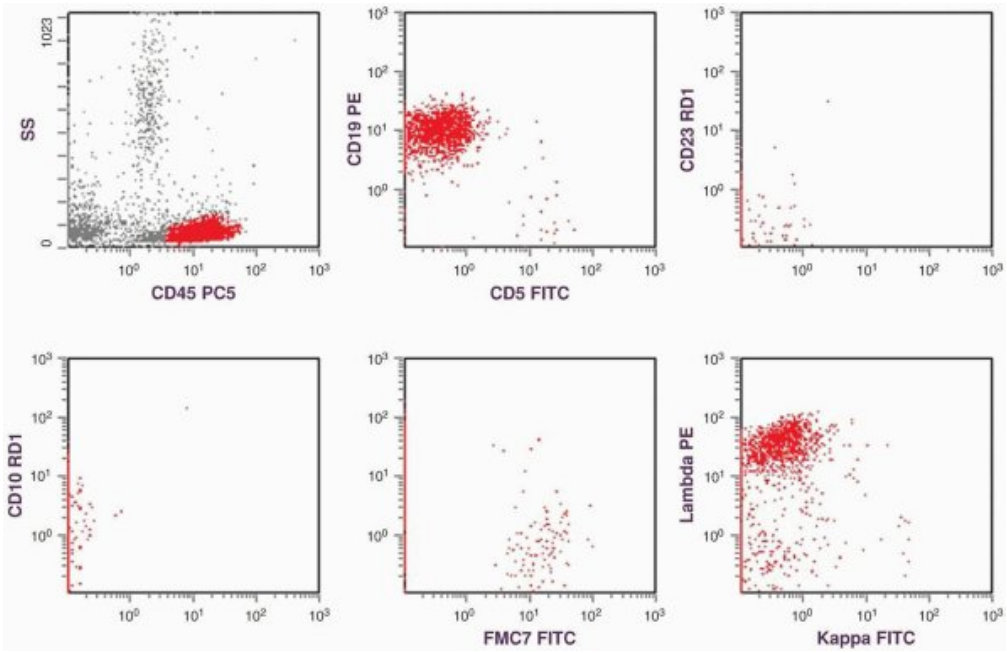

FIGURE 6.25.1 Flow cytometric analysis of the lymph node biopsy shows a CD19-positive monoclonal λ B-cell population. FMC-7 is partially positive, but CD10, CD23, and λ are negative. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; RD1, rhodamine.

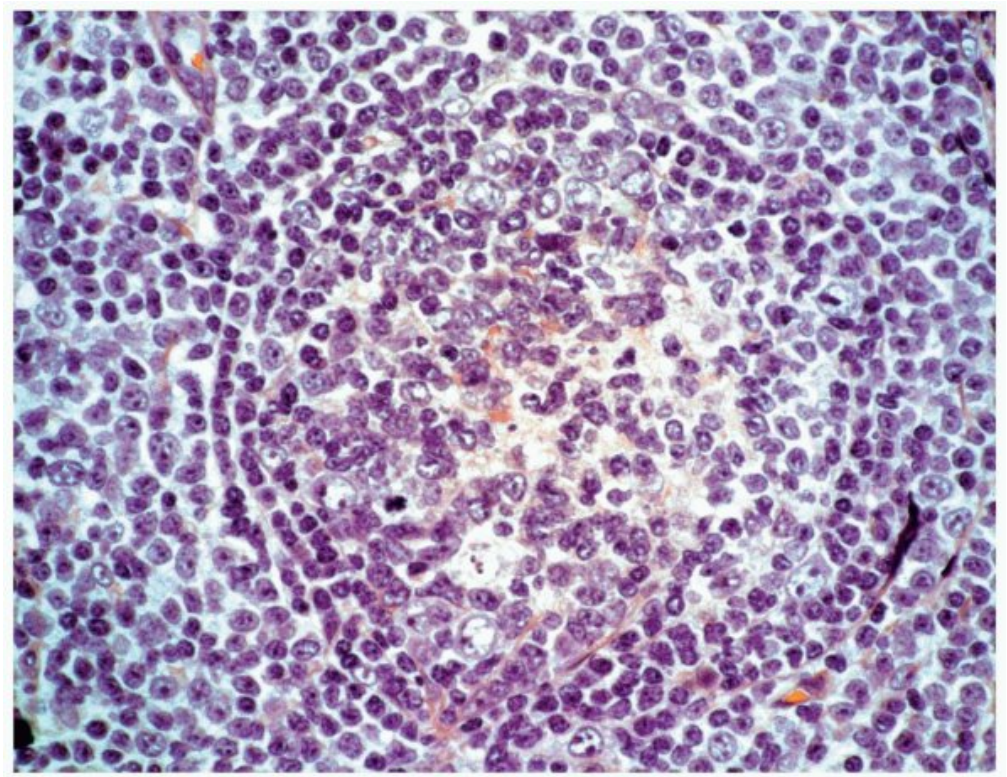

FIGURE 6.25.2 Lymph node biopsy shows monocytoid B-cell infiltration surrounding a germinal center with a wellpreserved mantle zone. The tumor cells have immature chromatin pattern and abundant cytoplasm. There are some large cells intermingled with the small cells.

Hematoxylin and eosin, 40× magnification.

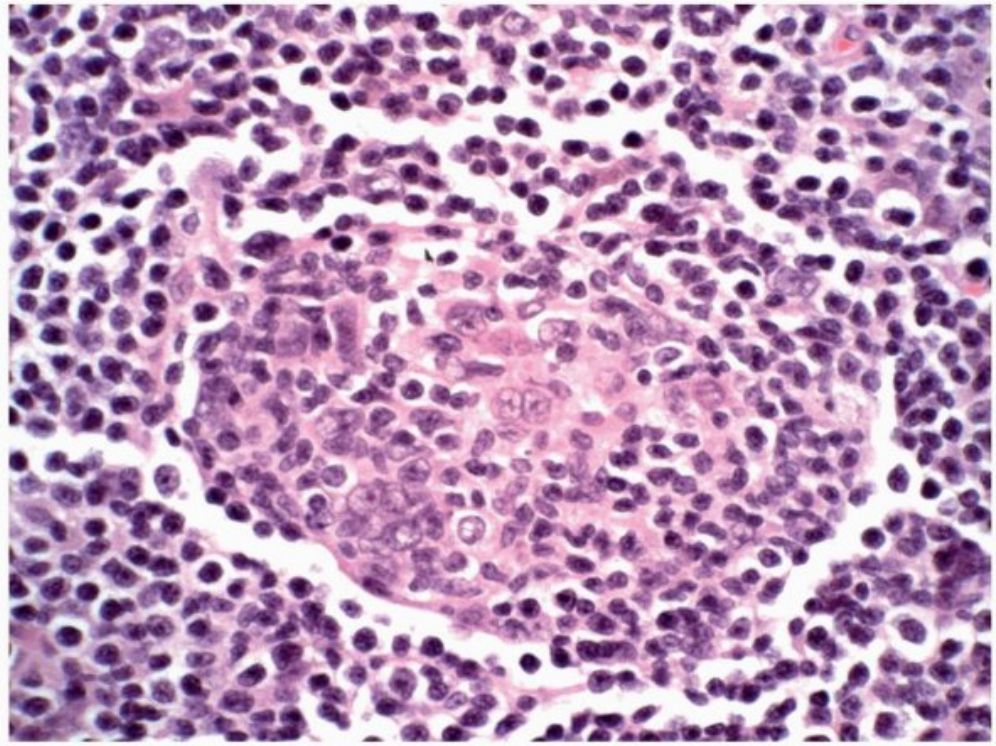

FIGURE 6.25.3 Lymph node biopsy reveals a germinal center surrounded by centrocyte-like tumor cells, which are small with irregular nuclei and dense chromatin pattern. A mantle zone is absent. The residual germinal center is partially colonized. Hematoxylin and eosin, 40× magnification.

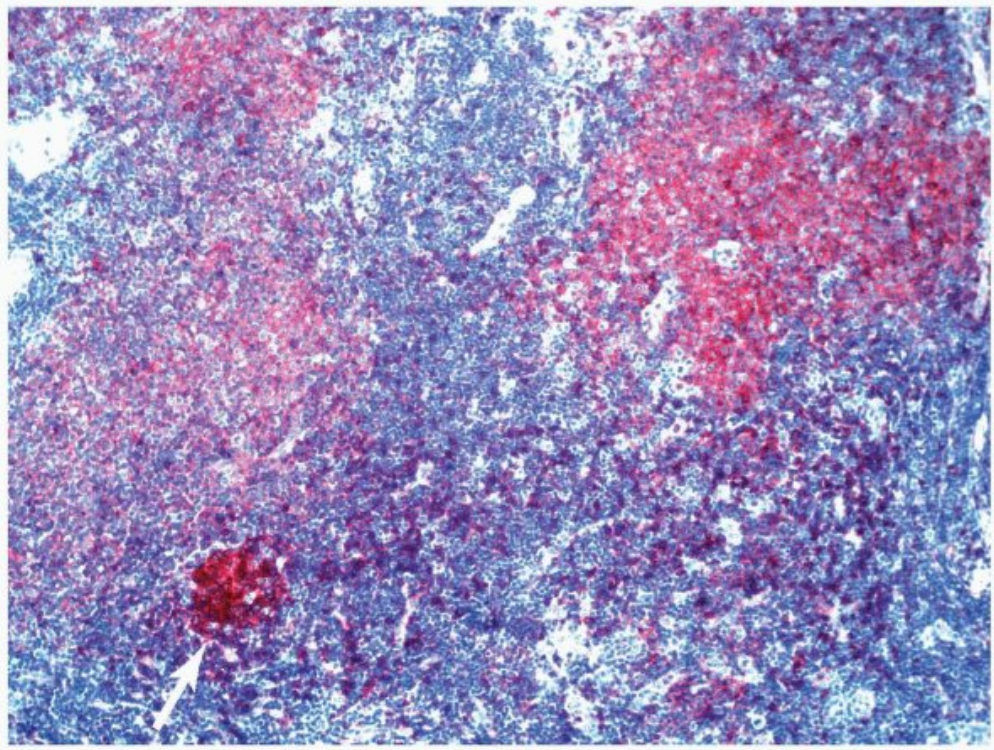

FIGURE 6.25.4 Lymph node biopsy shows that CD20 (B-cell marker) staining highlights the nodular aggregates of tumor cells. A residual germinal center reveals darker staining (*white arrow*). Immunoperoxidase, 10× magnification.

The cytology of this tumor is characterized by a polymorphic infiltration. The size of the tumor cells may be small, medium, or large. If both small cells and large blasts are present, the large cells are often present at the periphery of the nodules (3,12). Neutrophils and plasma cells are frequently present among the tumor cells. However, two major cell types are most frequently described (12,13). The monocytoid B cells are characterized by their abundant cytoplasm, centrally located bean-shaped or round nuclei, and fine chromatin pattern. The centrocyte-like cells have irregular nuclei and scant cytoplasm. When the marginal zone B cells show relatively scant cytoplasm, they can be recognized by the clear space separating each cell (8).

Other characteristic features of NMZL are frequent plasmacytic differentiation and the presence of composite lymphoma. In some cases, clusters and sheets of normal and abnormal plasma cells become the dominant feature that resembles a plasmacytoma or lymphoplasmacytic lymphoma. Monoclonal gammopathy may be present in those cases. However, the presence of monocytoid B-cell features or a nodular pattern in NMZL may help to exclude the diagnosis of lymphoplasmacytic lymphoma (8,11). The composite lymphoma is frequently composed of NMZL and follicular lymphoma. Composite NMZL with Hodgkin lymphoma (14) and with plasma cell myeloma (15) have also been reported recently. Cases with a high percentage of large tumor cells are sometimes considered a composite lymphoma. However, some authors consider that this so-called composite lymphoma may simply represent the heterogeneity of the tumor, and prefer to call it a large cell variant (16).

TABLE 6.25.1

Characteristic Morphologic Features of NMZL

Histologic pattern	Nodular proliferation around germinal center (splenic type) and perivascular, perisinusoidal, and parafollicular infiltration (MALT type)
Cytology	Polymorphic population with major components of monocytoid B cells or centrocyte-like cells

Specific features	Combination of histologic pattern and cytology

NMZL, nodal marginal zone B-cell lymphoma; MALT, lymphoma of the mucosa-associated lymphoid tissue.

A floral variant of NMZL has been reported recently (17). It is similar to the floral variant of follicular lymphoma in the presence of a prominent mantle zone forming a scalloped outline. In some cases, a clear zone is present around the mantle zone. Five of the six cases reported involved cervical lymph nodes. All patients were asymptomatic with a stage I disease and showed no recurrence after surgical excision of the lymph nodes (17). In children and young adults, disruption of residual follicles resembling progressive transformation of germinal centers (PTGC) is a common feature in NMZL and is similar to the floral variant of NMZL (18).

NMZL has a propensity to transform into large cell lymphomas (7,19). High-grade transformation may be seen in 20% of NMZL cases and can be recognized by the presence of one or more well-defined monomorphic clusters or sheets of large B cells or when >20% of large cells are mixed with the smaller tumor cells (8). The transformed large tumor cells also show a high mitotic index and high Ki-67 labeling (12). The characteristic morphologic features of NMZL are summarized in Table 6.25.1.

Differential diagnosis includes mantle cell lymphoma, small lymphocytic lymphoma, and follicular lymphoma; all of which may occasionally show a marginal zone distribution (8,12). Mantle cell lymphoma usually shows monotonous cellular proliferation, absence of large blasts, and absence of plasma cell differentiation (3,10,20). Distinction from follicular lymphoma is mainly based on the benign nature of the reactive follicles and residual germinal centers in NMZL as determined by morphology and immunophenotyping—namely, negative bcl-2 but a high percentage of Ki-67 (10). The lack of clusters of prolymphocytes and paraimmunoblasts and the immunophenotype may help distinguish small lymphocytic lymphoma from NMZL (3).

Monocytoid B-cell hyperplasia also should be distinguished from NMZL (Fig. 6.25.5). The malignant nature of NMZL

can be recognized by the prominent confluent proliferation in the tissue, nuclear irregularity, high mitotic figures, and more transformed large cells (21). The tumor cells are bcl-2 positive, but bcl-2 is negative in benign monocytoid B cells (12).

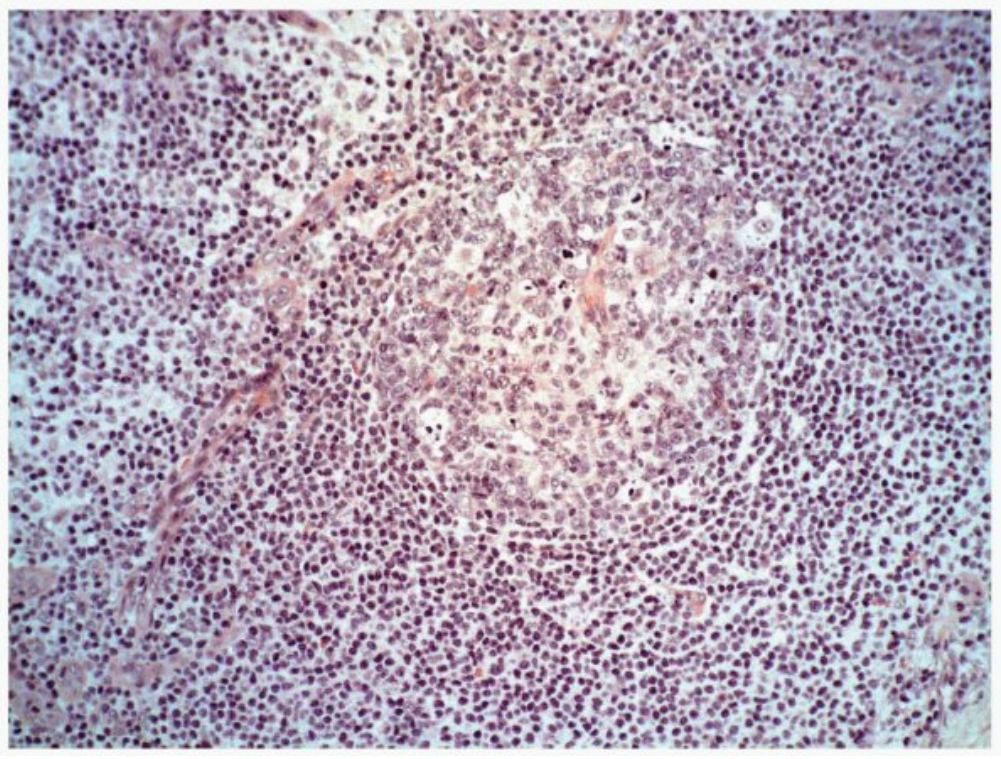

FIGURE 6.25.5 Lymph node biopsy reveals monocytoid B-cell proliferation surrounding a germinal center, mimicking the pattern of nodal marginal zone B-cell lymphoma. These monocytoid B-cells are bland looking, with no mitosis and large cells. Most part of the lymph node shows normal architecture. Hematoxylin and eosin, 20× magnification.

Immunophenotype

The immunophenotype of NMZL is that of a monoclonal B-cell proliferation—namely, the presence of CD19, CD20, CD22, CD79a, and a monoclonal κ or λ pattern (6,9,16,22). CD45RA, CD74 (LN2), and CD75 (LN1) have also been detected in some cases (12). However, the characteristic findings are rather the absence of CD5, CD10, CD23, CD43, and cyclin D1, which may help to distinguish NMZL from other non-Hodgkin lymphomas. For instance, CD5, CD23, and CD43 are positive in small lymphocytic lymphoma and mantle cell lymphoma; cyclin D1 is positive in mantle cell lymphoma; and CD10 is positive in follicular lymphoma (8). Nevertheless, some cases of NMZL may express CD43 (8,12). CD23 may highlight the residual germinal centers in NMZL (9).

A follicular dendritic cell meshwork can be demonstrated by CD21 in NMZL (8,10). Immunoglobulin D (IgD) is positive in some reports (10) but negative in others (22). Campo and coworkers (3) and Campo and Jeffe (12) found that IgD is positive in the splenic subtype but negative in the MALT subtype of NMZL. Because IgD is positive for mantle zone cells but negative for marginal zone cells, the positive reaction in the splenic subtype of NMZL and the splenic marginal zone lymphoma argues against a marginal zone derivative for these tumors (3).

Bcl-2 is positive for tumor cells in most cases, and its absence in the residual germinal center is helpful in excluding follicular lymphoma (10,12). In contrast to follicular lymphoma, bcl-2 gene is not rearranged in NMZL (4,16,22).

In the current case, the lymphoma was suspected to be induced by the long-term dilantin treatment. Although the patient had multiple lymphadenopathy and peripheral blood involvement, he did not show signs of disease progression in 2 years. Unfortunately, he died of unrelated illness at the end of 2 years, so no long-term follow-up was achieved. For some unexplained reason, patients with NMZL frequently have para-aortic lymph node involvement (8), as with this patient. The immunophenotype of the peripheral blood and lymph node was consistent with but not diagnostic of NMZL. Notably, CD10 and CD23 are negative, but CD5 is positive in the peripheral blood specimen. However, the positive CD5 cells may account for the normal T lymphocytes and not part of the tumor cell phenotype, as no CD19/CD5 dual stain was identified. It was the combination of the morphology in the lymph node and the immunophenotype that allowed a diagnosis of NMZL to be established.

Comparison of Flow Cytometry and Immunohistochemistry

The immunophenotypes as demonstrated by both flow cytometry and immunohistochemistry are not specific for the diagnosis of NMZL. However, it may help to establish the diagnosis by excluding other non-Hodgkin lymphomas. In this regard, immunohistochemical staining is more helpful than flow cytometry as it can distinguish tumor cells from residual follicles. For instance, CD21, CD23, and CD35 are positive in colonized follicles (6,9), but the positive staining for these surface antigens may be mistaken as the markers for the tumor cells. The absence of BCL-2 in the follicles distinguishes residual germinal centers from follicular lymphoma. Similar to hairy cell leukemia, DBA-44 has been demonstrated in some cases of NMZL (23).

Molecular Genetics

Ig gene rearrangements are present in NMZL. Most of the common oncogenes, such as BCL-1, BCL-2, BCL-3, and BCL-6 are negative in NMZL (4,13,16,22). C-MYC rearrangement is also not present in NMZL (4).

Analysis of Ig variable heavy chain (V_H) usage and mutation patterns has proved that NMZL is a heterogeneous tumor, arising from different subsets of marginal zone B lymphocytes (23, 24 and 25). In other words, the tumor cells seem to arise from virgin B cells that express unmutated V_H genes, memory B cells that show somatic mutations, and germinal center B cells that undergo somatic hypermutation (25). The pattern of somatic mutation and the V_H gene segment usage were found to be different between NMZL and splenic marginal zone B-cell lymphoma, suggesting that these two diseases are distinct entities (11). Another study showed that the use of V_H gene differed between hepatitis C virus-positive and -negative NMZL cases, and in both groups there was evidence of a clonal antigen-positive selection driven by antigens (26). In other words, the tumor cell proliferation is probably due to hepatitis C virus and, in the other group, an unknown pathogen stimulation.

Trisomy 3 is the most frequent cytogenetic aberration in NMZL, occurring in 56% to 78% of cytogenetically abnormal

cases (13,16). Trisomy 18 was the second most common, reported in 21% of MALT lymphoma cases (27) and in 25% of non-MALT type marginal zone lymphoma cases (16). Structural abnormalities of chromosome 1 is a relatively common finding (4,8). Translocation t(11;18)(q21;q21) has also been reported in some cases of NMZL and is the most common translocation in extranodal low-grade MALT lymphoma (12). This translocation leads to a fusion of apoptosis inhibitor-2 (API2) gene on chromosome 11 and MALT lymphoma-associated translocation (MLT) gene in chromosome 18 (28). However, a study with reverse transcriptionpolymerase chain reaction (RT-PCR) and genomic long and accurate PCR analysis revealed that none of the nine NMZL cases had API2 and/or MLT, whereas 17 of 95 MALT lymphoma cases were positive (29). It appears that NMZL, MALT, and splenic marginal zone lymphoma share the same chromosomal abnormalities, but the frequencies of these anomalies differ in each entity (4,13,30).

TABLE 6.25.2

Salient Features for Laboratory Diagnosis of NMZL

1. Positive for CD19, CD20, CD21, CD22, CD79a, and bcl-2 in a monoclonal population
2. Negative for CD5, CD10, CD23, CD43, and cyclin D1
3. IgD can be positive or negative, depending on the tissue type.
4. Rearrangement of Ig genes
5. No rearrangement for bcl-1, bcl-2, bcl-3, and bcl-6
6. Trisomy 3 and trisomy 18 are the most common cytogenetic abnormalities.

NMZL, nodal marginal zone B-cell lymphoma; CD, cluster of differentiation; Ig, immunoglobulin.

There is a variety of numeric cytogenetic abnormalities identified in marginal zone B-cell lymphoma by karyotyping and fluorescence in situ hybridization, but they are of rare occurrence (11,30). Many of these abnormalities are included in complex karyotypes (30). Some cytogenetic abnormalities, such as trisomy 12 and t(14;18), are probably associated with a composite lymphoma containing small lymphocytic lymphoma or follicular lymphoma, but they are not specific for NMZL (12). The salient features for laboratory diagnosis of NMZL are summarized in Table 6.25.2.

Clinical Manifestations

Patients with NMZL are usually diagnosed at age 60 to 65 years, with a male-to-female ratio of 1:5 (12). The lymph nodes involved are usually in the head and neck, and less frequently in the inguinal and retroperitoneal areas. Patients are usually asymptomatic, but fever, night sweats, and weight loss can be occasionally seen in patients with tumor progression. NMZL is frequently associated with autoimmune diseases, including Sjögren syndrome, systemic lupus erythematosus, and Raynaud phenomenon (19,31).

Bone marrow involvement has been observed in 32% of patients in one study series (32). Primary NMZL, by definition, is not accompanied by splenomegaly. It may spread through lymphatics to other lymph nodes, but peripheral blood is seldom involved (16). However, a leukemic subtype of marginal zone lymphoma with only blood and bone marrow involvement has recently been identified (33). A comparative study of three types of marginal zone B-cell lymphomas showed that MALT lymphoma usually had stage I disease, NMZL showed stage II or III disease at diagnosis, and splenic marginal zone lymphoma was presented with stage IV disease (4). However, NMZL in children and young adults usually presents with stage I disease (18).

Although NMZL is a localized disease, its progression is rapid, with a median progression time of 1 year, as compared with >5 years in the splenic marginal zone lymphoma in one study (16). However, the survival time for both types of lymphoma is comparable (median survival 9 years). Recently, Camacho et al. (23) showed that a shorter, failurefree survival is associated with loss of survivin and active caspase 3 in the tumor cells of NMZL.

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CASE 26 Follicular Lymphoma

CASE HISTORY

A 50-year-old man was admitted for further evaluation of lymphoma after chemotherapy. The patient noticed lumps on both sides of his groin 1 year ago and was considered to have an inguinal hernia. A hernia belt helped relieve the pain, but the lumps continued to grow. His private physician then suggested a biopsy, which showed lymphoma. He underwent four cycles of chemotherapy with minimal response. Instead, he noticed an increase in size of bilateral inguinal lymph nodes.

Physical examination on admission revealed lymphadenopathy in the supraclavicular, axillary, and inguinal regions, but the cervical lymph node was not enlarged. The liver and spleen were not palpable. His blood chemistry was unremarkable except for an elevated lactate dehydrogenase (LDH) (411 U/L). Liver and renal function tests were within normal limits.

Computed tomography (CT) scan revealed multiple enlarged lymph nodes in the mediastinal and retroperitoneal regions. The spleen was also enlarged to 14 × 6 cm.

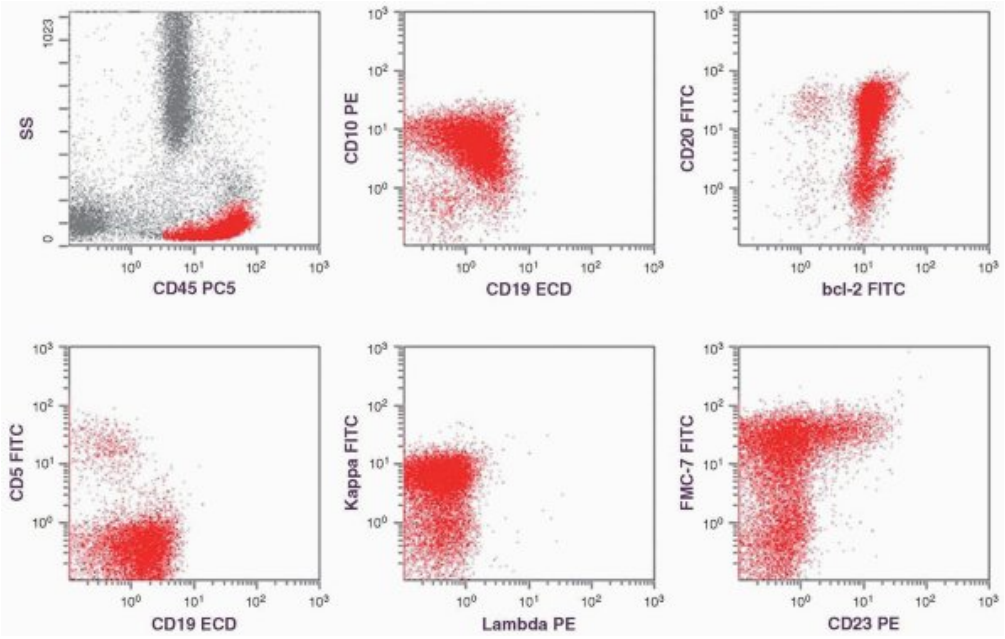

FIGURE 6.26.1 Flow cytometric histograms show dual staining of cluster of differentiation (CD)10 and CD19, as well as CD20 and bcl-2. A monoclonal κ pattern, positive FMC-7, and partial positive CD23 are also demonstrated. A small percentage of T cells are also present in the gated population, as represented by the CD5-positive and bcl-2-positive/CD20-negative clusters. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; ECD, phycoerythrin-Texas Red; FITC, fluorescein isothiocyanate.

The biopsy of the left inguinal lymph node confirmed the previous diagnosis of follicular lymphoma (FL) with large cell infiltration in the interfollicular area. A bone marrow biopsy showed multiple lymphoid aggregates in the paratrabecular areas.

FLOW CYTOMETRY FINDINGS

Lymph node biopsy: CD5 18%, CD19 71%, CD19/CD10 30%, CD20 96%, CD20/bcl-2 69%, CD23 9%, FMC-7 86%, CD19/κ 74%, CD19/λ 2%.

Bone marrow biopsy: CD5 7%, CD19 63%, CD19/CD10 45%, CD20 92%, CD20/bcl-2 76%, CD23 11%, FMC-7 78%, CD19/κ 73%, CD19/λ 0% (Fig. 6.26.1).

IMMUNOHISTOCHEMICAL FINDINGS

The malignant follicles in the lymph node showed positive staining for CD20, bcl-2, and bcl-6 but negative staining for CD3 and CD5.

CYTOGENETIC FINDINGS

Cytogenetic analysis showed a balanced translocation between chromosomes 14 and 18 in all cells analyzed. Trisomy 21 was also noted in the abnormal clone.

DISCUSSION

FL is one of the most common types of non-Hodgkin lymphoma, accounting for 20% to 30% of all non-Hodgkin lymphoma and 40% to 50% of adult non-Hodgkin lymphoma (1). In European countries, the incidence ranges from 13% to 22% (1,2). However, the incidence of FL in Asia, including Japan, is relatively low (3.8% to 12%) (1,3,4).

The term FL was originally used in the Working Formulation (5). In the Kiel classification, this lymphoma is called centroblastic-centrocytic lymphoma (6). The Revised European-American Lymphoma (REAL) classification changed the name to follicle center lymphoma (7), but the World Health Organization (WHO) scheme retains the original term, FL (8,9).

TABLE 6.26.1

Characteristic Morphologic Features of Follicular Lymphoma	
Histologic pattern	Presence of evenly distributed uniform follicles with incomplete or absent mantle zones and back-to-back pattern
Cytology	Predominantly centrocytes, mixed centrocytes and centroblasts, and predominantly centroblasts
Special features	Same as histologic pattern

Morphology

In FL, the normal architecture of the lymph node is usually partially or completely effaced (Table 6.26.1). The neoplastic follicles are often uniform in both size and shape (1,9, 10 and 11). These follicles are evenly distributed, frequently with a back-to-back pattern so that the density of follicles is higher than that in follicular hyperplasia (FH), and there is sparse intervening interfollicular tissue. The margin of the tumor follicles is usually poorly defined. The mantle zone is usually absent or attenuated. In some cases, the follicular pattern is very subtle and requires immunophenotyping to substantiate the diagnosis. The capsule of the lymph node is frequently infiltrated by the lymphoma cells, which may spread into the perinodal soft tissue. The subcapsular and medullary sinuses are often obliterated by the tumor infiltrate.

TABLE 6.26.2

Differentiation between Follicular Hyperplasia and Follicular Lymphoma		
	Follicular Hyperplasia	*Follicular Lymphoma*
Lymph node architecture	Well preserved	Completely or partially effaced
Size and shape of germinal center	Variable	Uniform
Distribution of follicles	Irregular, well separated	Even, back-to-back pattern
Density of follicles	Low	High
Margin of follicles	Sharp	Poorly defined
Mantle zone	Intact	Absent or incomplete
Infiltration of capsule	Absent or minimal	Present
Cells within germinal centers	Polymorphic	Monomorphic

Polarization in germinal centers	Present	Absent
Mitotic rate	High	Low
Tingible-body macrophages	Prominent	Rare
Cells in interfollicular area	Normal lymphocytes	Atypical lymphoid cells
Surface immunoglobulin	Polyclonal	Monoclonal
CD45RA (MT2)	Negative	Positive
CD10	Negative	Positive
bcl-2 protein	Negative	Positive
bcl-6 protein	Negative	Positive
% Ki-67	Low	High
t(14;18)	Absent	Present
Molecular biology	No oncogene	bcl-2
Immunoglobulin gene	Germline	Rearrangement

Modified from Sun T, Susin M. *Differential Diagnosis of Lymphoid Disorders*. New York: Igaku-Shoin; 1996:91, with permission.

Cytologically, FL is composed of small cleaved lymphocytes (centrocytes) and large transformed lymphoid cells (centroblasts) in varying proportions, but it usually shows a monomorphic appearance without a polarization pattern in the follicle. In most cases of FL, the mitotic rate is low and tangible-body macrophages are seldom seen except for the large cell subtype. The presence of atypical lymphoid cells in the interfollicular region is helpful for the diagnosis of FL, although inflammatory cells may also be present.

In FH, the nodal architecture is usually well preserved (Table 6.26.2) (12). The hyperplastic follicles are more

irregular in size, shape, and distribution than are the follicles of FL. In addition, the benign follicles have lower density than the malignant ones. The demarcation between the germinal center and mantle zone is distinct, and the mantle zone is intact. The capsule of the lymph node is usually not involved in FH.

Cytologic features may further distinguish FH from FL. In FH, the germinal centers contain mixed large and small cleaved lymphocytes (centrocytes) and large and small noncleaved lymphocytes (centroblasts) as well as phagocytic macrophages. Polarization of the lymphoid cells is frequently demonstrated in the germinal centers in FH; small and large cleaved lymphoid cells are on one pole (light zone), and small and large noncleaved transformed lymphoid cells are on another pole (dark zone). In the dark zone, the mitotic rate is high and there are many tangible-body macrophages, forming a “starry-sky” pattern.

In the Working Formulation, FL is divided into three subtypes: predominantly small-cleaved cell type (large cell <20%), mixed small-cleaved and large cell type (large cell 20% to 50%), and predominantly large cell type (large cell >50%) (5). The predominantly small-cleaved cell subtype is the most common form, accounting for 40% to 50% of all FL cases. The predominantly large cell subtype is the

least common among the three subtypes.

The International Lymphoma Study Group is of the opinion that these three subtypes are a continuous gradation in the number of large cells and are difficult to reproduce. Therefore, this group proposes these subtypes to be a grading system rather than subclassification (7). However, the grading system is still based on the proportion of cell components: grade I, small cell predominant; grade II, mixed small and large cells; and grade III, large cell predominant.

The WHO scheme adopts the Berard cell-counting method for grading. The calculation is based on the average number of centroblasts in 10 neoplastic follicles examined under a 40× high-power field (hpf) (9). Grade 1 FL is defined by the presence of 0 to 5 centroblasts/hpf (Fig. 6.26.2); grade 2, 6 to 15 centroblasts/hpf; and grade 3, >15 centroblasts/hpf (Fig. 6.26.3). Grade 3 is further divided into grades 3a and 3b. In the former, centrocytes are still present, whereas the latter shows solid sheets of centroblasts. The experts on the WHO classification panel consider that, because there are only minor differences in natural history and response to treatment between grade 1 and grade 2, their division is clinically insignificant. However, the threegrade system is retained in the WHO classification to avoid confusion (8).

The accuracy of this grading system depends on the clear distinction between centrocytes, centroblasts, and follicular dendritic cells. The characteristics of a centrocyte are mainly based on nuclear configuration, which may show prominent clefts, indentation, or linear infoldings. The nuclear chromatin is condensed, and the small nucleoli are inconspicuous. The cytoplasm is scant. The centroblasts are two to three times as large as normal lymphocytes. Their nuclei are usually round but occasionally irregular in configuration, with vesicular chromatin and one to three nucleoli, which are typically opposed to the nuclear membrane. Their cytoplasm is scant and is basophilic on Giemsa stain. The follicular dendritic cells show nuclei with a size similar to those in centroblasts and distinct eosinophilic nucleoli in a vesicular chromatin background. The cytoplasm is indistinct. However, the most characteristic feature is the presence of double nuclei with flattening of the adjacent nuclear membranes (Fig. 6.26.4).

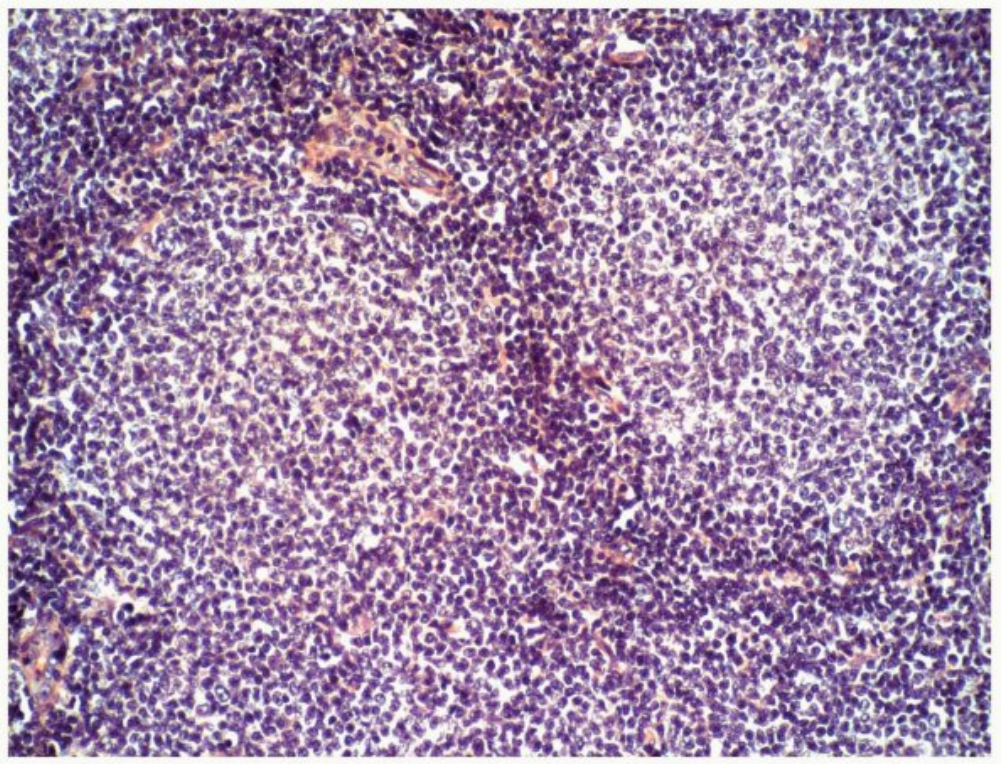

FIGURE 6.26.2 Lymph node biopsy shows a grade 1 follicular lymphoma composed predominantly of centrocytes. The two malignant follicles appear similar in size and shape with a back-to-back pattern. Hematoxylin and eosin, 20× magnification.

Diffuse area is frequently present in various proportions with the follicles. A follicular pattern is defined by the presence of >75% of follicular area. A follicular and diffuse pattern should be reported when the follicular area is between 25% and 75%. When follicular area is <25%, it is designated as minimally follicular. The grade and percentages of follicular and diffuse areas should be included in the diagnosis. When diffuse large cell lymphoma is present, it should be reported as such, instead of calling it diffuse area (Fig. 6.26.5). A worse prognosis can be predicted in cases with large diffuse areas (7). Sclerosis may be present in diffuse areas, particularly in cases after therapy, forming a nodular sclerotic pattern (Fig. 6.26.6). The association of sclerosis with prognosis is controversial (10).

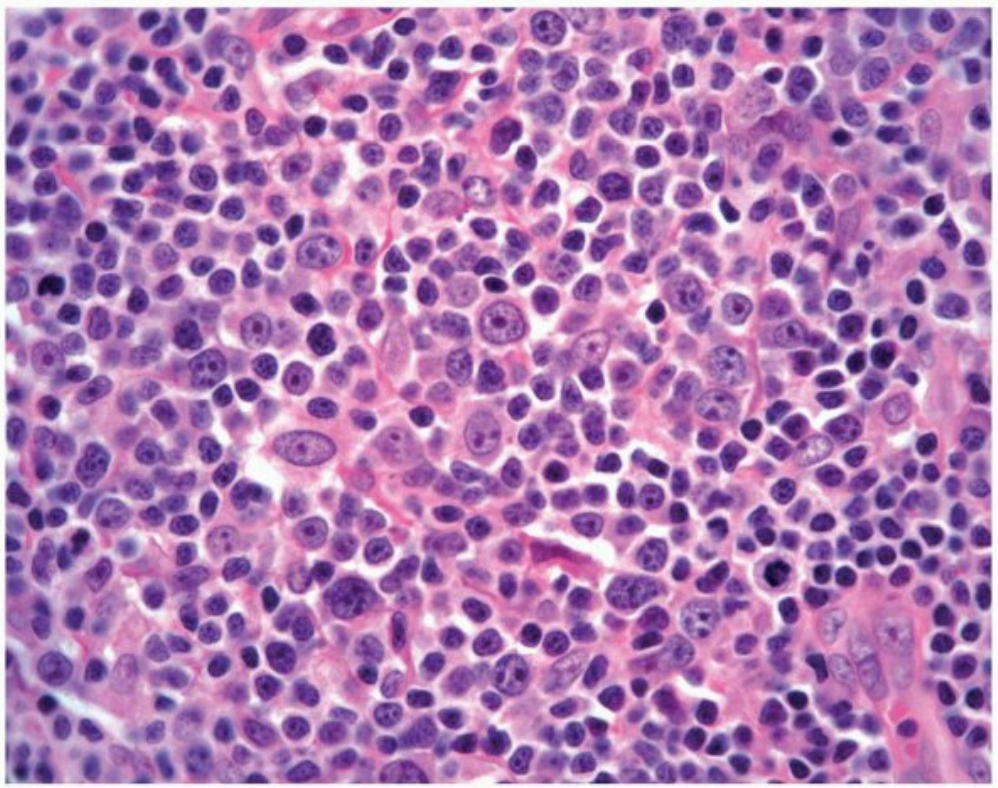

FIGURE 6.26.3 Lymph node biopsy reveals a grade 2 follicular lymphoma with several centroblasts present in the center of the field. Hematoxylin and eosin, 60× magnification.

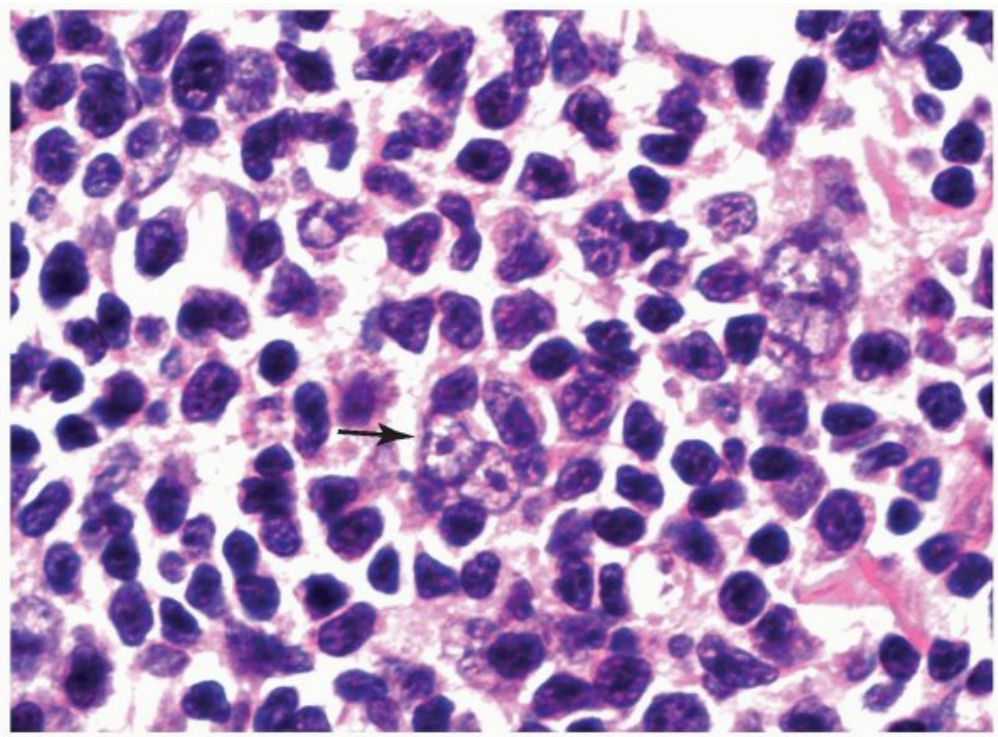

FIGURE 6.26.4 Lymph node biopsy shows a pair of follicular dendritic cells in the center (*arrow*). Note flattening of the adjacent nuclear membranes. There are a few large centrocytes

above the follicular dendritic cells. These cells should be distinguished from the centroblasts. Hematoxylin and eosin, 100× magnification.

There are several variants of FL. The presence of extrafollicular monocytoid B cells is considered a predictor of worse prognosis with shorter failurefree survival and overall survival (13). The marginal zone variant or “reverse” variant is seen in about 10% of FL cases (9,14) (Fig. 6.26.7). Other rare variants include the signet-ring cell variant, the floral variant, and the variant with amorphous extracellular precipitate (9,14).

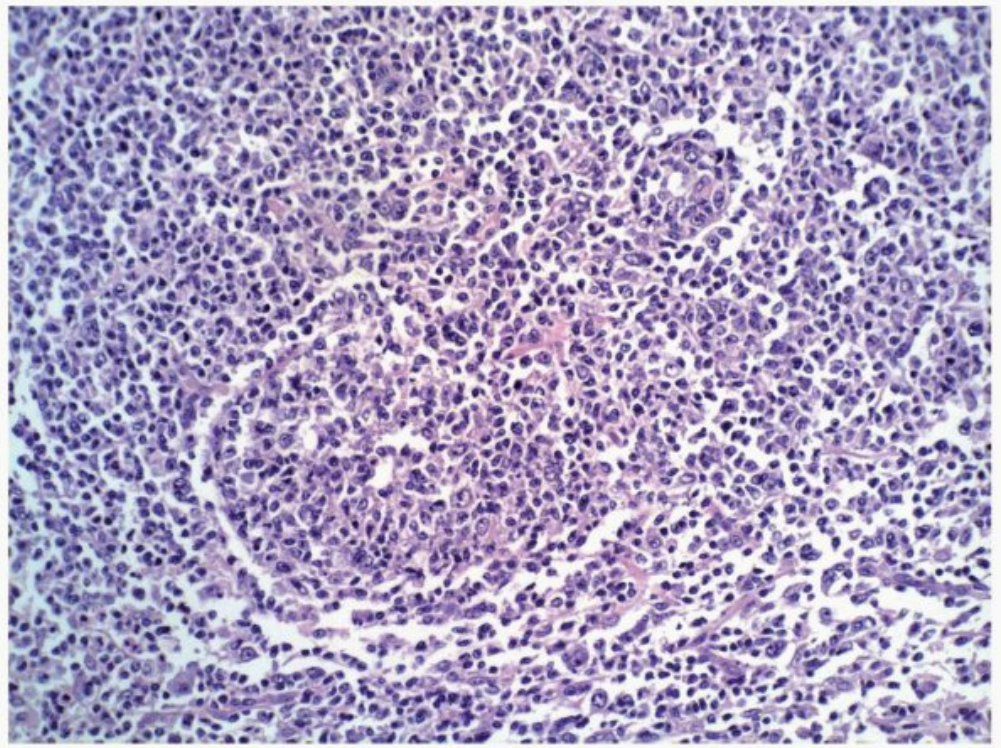

FIGURE 6.26.5 Lymph node biopsy reveals two residual follicles surrounded by diffuse large lymphoma cells, representing transformation to diffuse large B-cell lymphoma. Hematoxylin and eosin, 20× magnification.

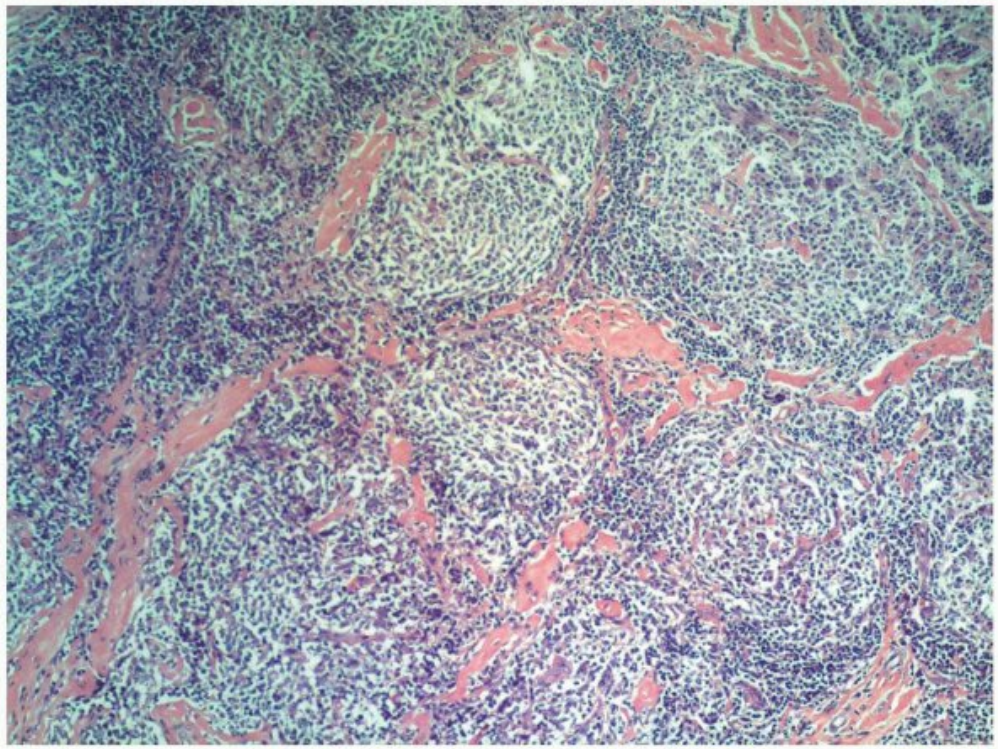

FIGURE 6.26.6 Lymph node biopsy shows broad hyaline fibrous bands surrounding the follicles, forming a nodular sclerosing pattern. Hematoxylin and eosin, 10× magnification.

The initial diagnosis of FL should not be made in the extranodal sites, because a follicular pattern may not be present. In contrast, a nodular lymphoid aggregate in an extranodal site is not necessarily an indication of FL. In the bone marrow, FL is characterized by the presence of a well-defined paratrabecular lymphoid aggregate rather than a nodular aggregate in the intertrabecular area (Fig. 6.26.8) (7,10). The cytologic features may be identical to those of the nodal tumor, but tumor cells in the bone marrow often appear more mature than those in the lymph node. Multiple large paratrabecular lymphoid follicles containing atypical lymphoid cells with infiltration of the surrounding normal bone marrow are features in favor of malignancy.

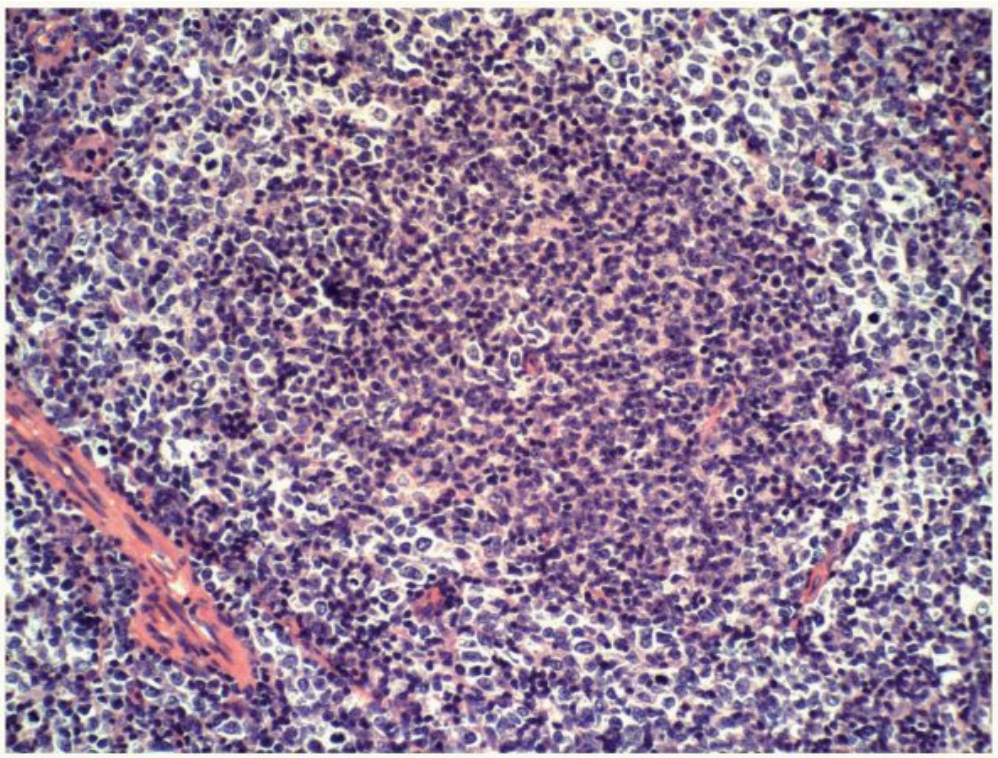

FIGURE 6.26.7 Lymph node biopsy reveals a marginal zone pattern in a follicular lymphoma. Hematoxylin and eosin, 20× magnification.

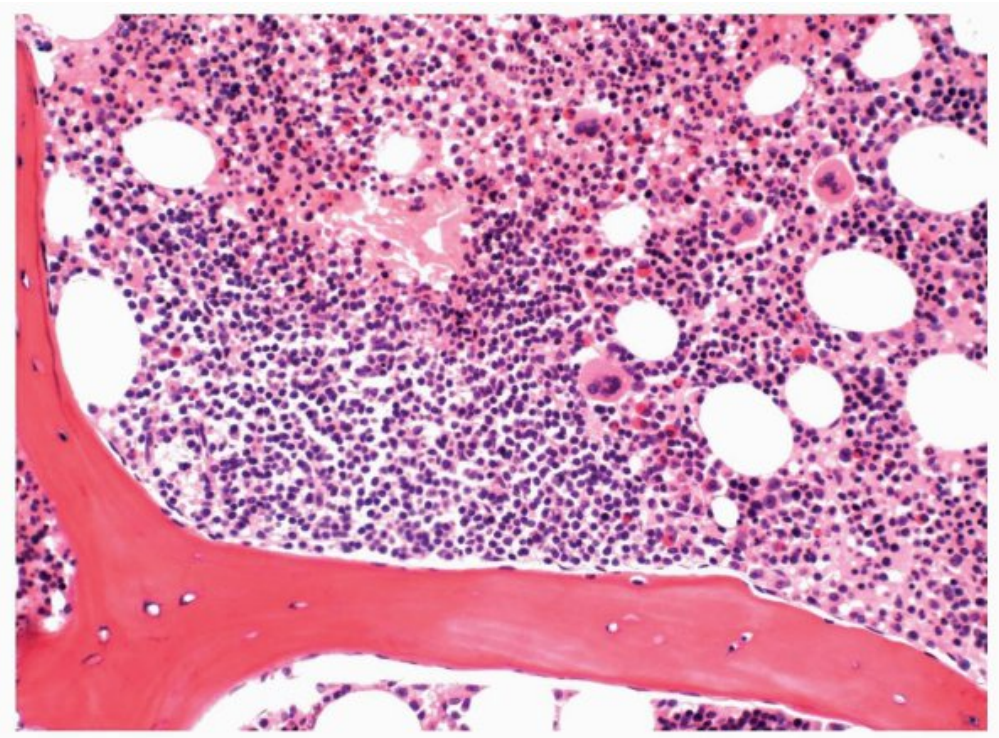

FIGURE 6.26.8 Bone marrow biopsy shows a paratrabecular lymphoid infiltration pattern, characteristic of follicular lymphoma. Hematoxylin and eosin, 20× magnification.

In the spleen, evenly distributed, uniform, white nodules may be present on the cut surface in cases of FL. Microscopically, these nodules are located in the lymphoid follicles of the splenic white pulp. In the large cell subtype, the tumor nodules may have an irregular shape, and their distribution may be uneven (10). The distinction between FL and FH in the spleen can be very difficult if cytologic atypia is not obvious.

In the liver, FL involves primarily the portal tracts of the liver and may spread beyond the limiting plate of the lobules. The infiltration pattern is the same as other types of lymphomas. Its distinction from FH depends on cytologic atypia, hepatic parenchymal involvement, and the absence of plasma cells in the infiltrate (10).

Primary FL of the gastrointestinal tract is rare and accounts for <7% of all non-Hodgkin lymphomas at that location (15). However, in a study of 35 patients with lymphomatous polyposis, 14 cases were found to be FL (16).

FL cells are relatively frequently demonstrated in the blood. The identification of these tumor cells depends on the demonstration of a monoclonal B-cell population with a positive CD10 antigen. The presence of characteristic lymphocytes with cleft nucleus detected by light or electron microscopy may also help to make a specific diagnosis of FL (Fig. 6.26.9) (17).

FL has the tendency to transform into high-grade lymphomas. The transformation rate varies from 10% to 80% in different reports, depending on the follow-up period, frequency of biopsies, and inclusion of autopsy data (18, 19 and 20). Most cases show transformation into diffuse large B-cell lymphoma (18). It may also transform into CD30-positive anaplastic large cell lymphoma (18) or blastic and/or blastoid form (20) on rare occasions.

By using fine-needle aspirates, the diagnosis of FL is based on the presence of lymphoid aggregates, two-nucleilike cleaved cells, irregular nuclei, and nucleoli (21). In a good aspirate, grading can be attempted by estimation of the percentages of centroblasts (22). For instance, centroblasts are <10% in grade 1, averaging 25% in grade 2, and >50% in grade 3. However, morphology alone is usually not sufficient to make a definitive diagnosis; flow cytometry, immunocytochemistry, or fluorescence in situ hybridization is frequently needed to substantiate the diagnosis (21,22).

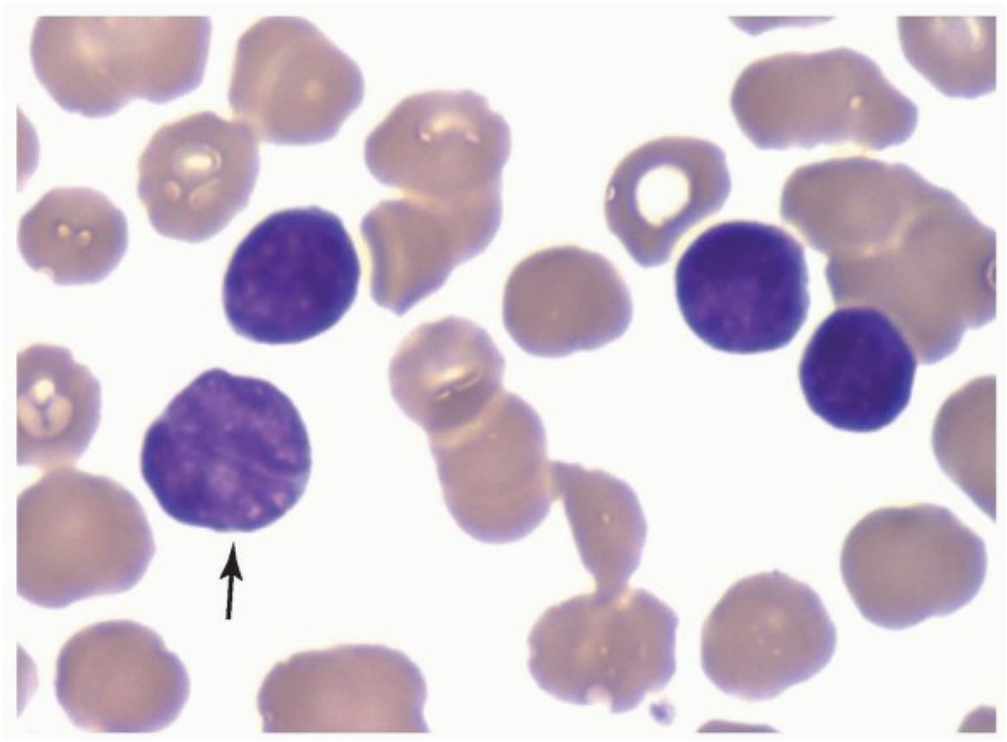

FIGURE 6.26.9 Peripheral blood smear reveals a cleaved small lymphocyte (*arrow*). Wright-Giemsa, 100× magnification.

Immunophenotype

A large monoclonal antibody panel can be used for immunophenotyping of FL. All the common B-cell-associated antigens (CD19, CD20, CD22, CD24, CD79a) are present in FL (1,9,23). The specific antigens for FL are CD10, bcl-2, and bcl-6. In addition, the follicular dendritic cell markers (CD21, CD23, CD35) can demonstrate the dendritic meshwork among the tumor cells and are useful to substantiate the diagnosis of FL (3,9). Follicular dendritic cells may provide a favorable microenvironment for FL in extranodal sites such as the bone marrow (24), and may play an important role in the prognosis of the patients (25).

The immunophenotype of FL reflects its germinal center cell origin, which is shared by Burkitt lymphoma and a subset of diffuse large B-cell lymphoma. Burkitt lymphoma, however, usually shows an absence of bcl-2 (9,26). The diffuse B-cell lymphoma with this immunophenotype can be the result of transformation from FL (27).

Mantle cell lymphoma can be bcl-2 positive, with the presence of a dendritic meshwork, but it is CD5+, CD10-, bcl-6-, CD43+ (1,23). The dendritic meshwork, as demonstrated by CD21 and CD23 staining, in FL is arranged in a nodular spherical pattern, but the meshwork in mantle cell lymphoma shows a loose pattern (28). FL is usually negative for CD43, but a small subset of FL expresses CD43 staining. In those cases, the tumor cells were predominantly large cell type with focally diffuse areas (29).

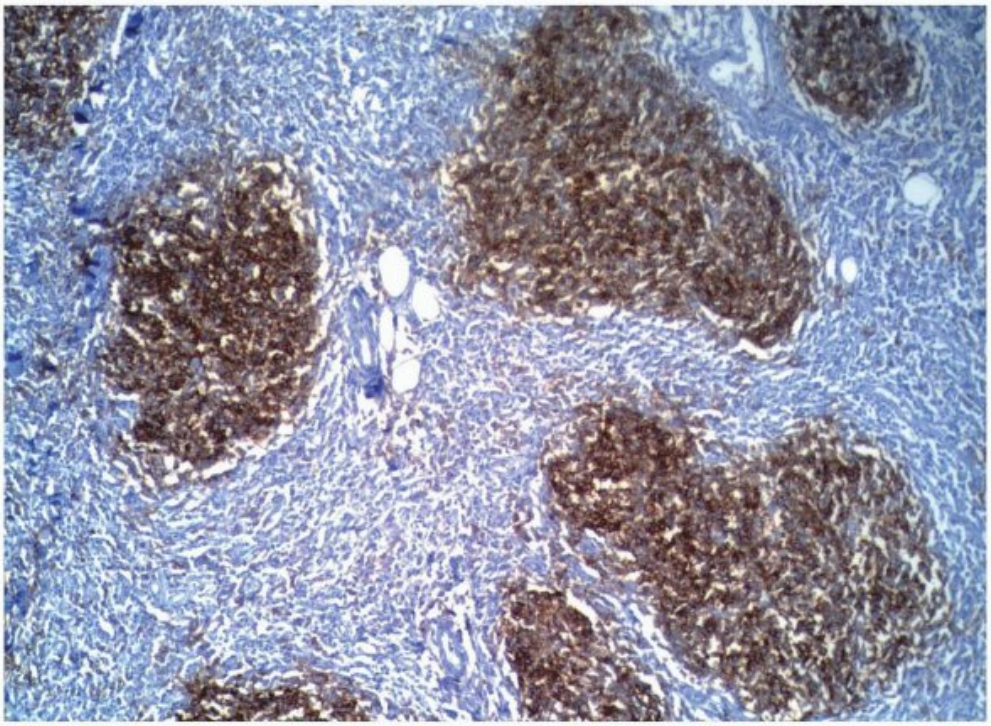

FIGURE 6.26.10 Lymph node biopsy shows a nodular pattern as demonstrated by CD10 staining. Immunoperoxidase, 10× magnification.

FH can be easily distinguished from FL by the lack of monoclonality. It is also negative for bcl-2, bcl-6, CD10, and CD45RA (9,30).

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry can demonstrate a monoclonal B-cell population with a positive reaction to CD10 and bcl-2. However, immunohistochemical staining has the advantage in recognizing the follicular pattern with the special staining of CD10, bcl-2, and bcl-6 identified in the follicular structure (Fig. 6.26.10). It is important that the staining is not outside the malignant follicles unless there are extensive diffuse areas. Bcl-2 can cross-react with interfollicular T cells; cross-reaction should not be counted as positive reaction. The ability of demonstrating the follicular dendritic meshwork by CD21 and CD23 stains is another advantage of immunohistochemistry.

The percentage of Ki-67-positive cells (proliferation index or PI) generally correlates with the FL grades. However, there is a subset of low-grade FL that shows a high PI (31). These low-grade, high-PI cases behave clinically like grade 3 FL. It has been found recently that the S-phase kinase-associated protein 2 (SKP2), an inhibitor of p27, can also help grading and predict transformation of FL (32).

In the current case, the patient had a typical clinical presentation of multiple lymphadenopathies and a waxing and waning clinical course. Histologically, the lymph node showed a typical follicular pattern, and the malignant follicles showed positive CD20, bcl-2, and bcl-6, but were negative for CD3 and CD5. Flow cytometry showed a similar phenotype, i.e., positive CD19, CD10, and bcl-2 but negative CD5. Therefore, the diagnosis of FL was established. The bone marrow in this case showed paratrabecular lymphoid infiltrates with a flow cytometric phenotype similar to that of the lymph node, indicating a stage 4 disease. The second lymph node biopsy revealed large cell infiltration in the interfollicular area representing transformation to a diffuse large B-cell lymphoma.

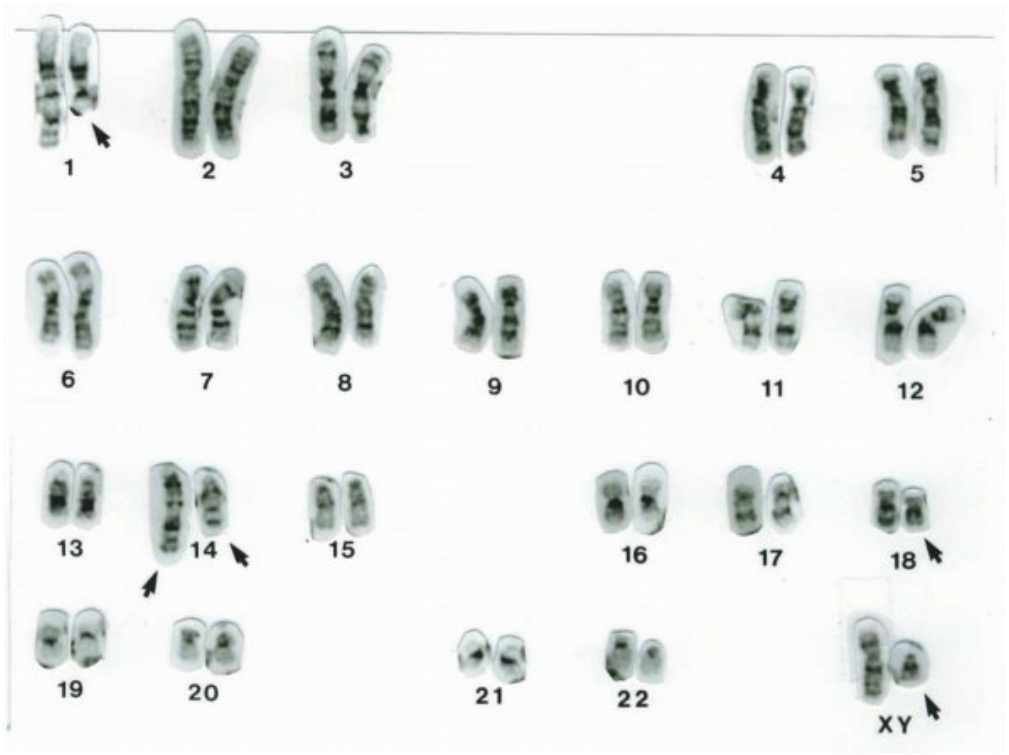

FIGURE 6.26.11 A karyotype of lymph node cells shows chromosomal t(14;18) translocation with additional abnormalities: inv(q21;q32), t(1;14)(q23;q32). (Courtesy of Dr. Prasa Koduru, North Shore University Hospital, New York.)

Molecular Genetics

FL is characterized by t(14;18)(q32;q21) translocation (Fig. 6.26.11). The heavy-chain gene is located at 14q32, whereas the proto-oncogene, bcl-2, is located at 18q21. When bcl-2 moves into the proximity of the immunoglobulin (Ig) heavy-chain gene enhancer region, it becomes deregulated or activated, and the functional bcl-2-Ig fusion protein is overexpressed. The bcl-2 gene encodes for an inner mitochondrial membrane protein that plays the role of blocking programmed cell death (apoptosis) (33). Therefore, cells with abnormal expression of this protein remain in stage G_0 in the cell cycle and become immortalized (1,10). Because the protein does not promote proliferation, it serves to explain why most patients with FL have an indolent clinical course.

Studies with transgenic mice show that t(14;18) translocation alone does not induce tumorigenesis. A second oncogene, such as the c-myc gene, or other chromosomal abnormality is required for neoplastic transformation (23). Furthermore, t(14;18) is present in small clones of B cells in 30% to 50% of healthy individuals (23). Therefore, the results of using molecular techniques to detect t(14;18) for the diagnosis of minimal residual disease should be interpreted with caution. In fact, circulating cells carrying the t(14;18) translocation can be demonstrated in some FL patients even after a continuous complete remission of >10 years (23).

Generally, 80% to 90% of cases of FL in the United States and Europe express t(14;18) (34). However, the frequency of this translocation is lower in Asia. In a study of Japanese patients, only 59.6% of FL cases showed t(14:18) (4). In North America and Europe, the frequency of t(14:18) in grade 3b FL is also low (35).

The variation in reported incidence also depends on the techniques used. Two studies have found cytogenetic analysis to be more sensitive (73% to 89% sensitivity), followed by Southern blotting (68% to 75%), with polymerase

chain reaction being the least sensitive (49% to 65%) (36,37). Recent studies indicate that the fluorescence in situ hybridization technique is the most sensitive and has been used routinely in many laboratories (38,39).

This translocation can also be demonstrated in 30% of diffuse large B-cell lymphomas and occasionally in other lymphoproliferative disorders. The demonstration of bcl-2 protein with dual B-cell antigen staining is reliable for the diagnosis of FL (40). However, bcl-2 protein can be shown in other B-cell lymphomas; in those cases, t(14;18) may not be demonstrated (41).

Sole t(14;18) aberration is only present in 10% of FL cases (9). The remaining cases may have additional breaks or additions. Common abnormalities include +7, +18, 3q27-28, 6q23-26, and 17p (9). In some FL cases with negative bcl-2 gene rearrangement, bcl-6 aberrations can be demonstrated (4). The rearrangement of bcl-6 was found in about 15% of FL, and 5′ mutations of the bcl-6 gene are found in approximately 40% of FL (1). In a recent study, the bcl-6 protein was demonstrated in all 31 grade 1 and 2 FL cases by immunohistochemistry and in all 5 FL cases by Western blotting (42). In the same study, bcl-6 was positive in only 1 of 13 cases with small

lymphocytic lymphomas, 1 of 12 mantle cell lymphomas, but none of 16 marginal zone lymphomas.

Another important question is cytogenetic evolution in the transformation of FL to diffuse large B-cell lymphoma. The involvement of single genes, such as c-myc, p53, CDKN2A/B, and others, has been reported, but none of them can be held accountable for all sets of patients (43).

FL shows immunoglobulin heavy and light chain gene rearrangement with hypermutation in the variable region of the immunoglobulin heavy chain gene (Hv). There is also intraclonal heterogeneity. These findings prove that FL is of follicle center cell origin.

The recent application of gene expression profiling is most promising in stratification of FL patients for predicting the clinical course and prognosis. An unexpected finding has been reported by the National Cancer Institute group, which has identified two discrete gene expression profiles (25). The immune response 1 signature encodes genes expressed by T cells and macrophages and confers a favorable prognosis. The immune response 2 signature includes genes expressed predominantly by monocytes and follicular dendritic cells and confers an unfavorable prognosis. In this study, it appears that the clinical course is mainly influenced by the infiltrating nontumor cells and not the genes of the lymphoma cells.

Another gene expression profiling study found that genes involved in cell-cycle regulation and DNA synthesis, including CXCL12, NEK2, and MAPK1, were differentially expressed in indolent and aggressive cases (44). The salient features for laboratory diagnosis of FL are summarized in Table 6.26.3.

Clinical Features

FL is a lymphoma of older age groups. It is seldom seen in patients younger than 20 years and almost never encountered in young children (1). It occurs with equal frequency in both sexes.

FL has an insidious onset. Therefore, when a diagnosis is made, the disease is already in an advanced stage: About 67% of patients are in stage III or IV at the time of diagnosis (2). Constitutional symptoms, such as fever, weight loss, and night sweats, are present in only 17% of patients. The major clinical presentation is peripheral lymphadenopathy, involving mostly the cervical and inguinal lymph nodes (1,2). Extranodal involvement is rare but can be present in the spleen, Waldeyer ring, or gastrointestinal tract. Skin and soft tissue presentations are very rare: Bone and central nervous system have not been reported as primary sites. In advanced stages, however, bone marrow and liver are most commonly invaded (1). About 33% of patients show a leukemic blood picture (45), but circulating lymphoma cells have been demonstrated with polymerase chain reaction in 75% of patients who are in stages I and II (46).

TABLE 6.26.3

Salient Features for Laboratory Diagnosis of Follicular Lymphoma

1. Monoclonal surface immunoglobulin with bright fluorescence
2. Positive B-cell antigens: CD19, CD20, CD22, CD24, CD79a, HLA-DR
3. Characteristic markers for diagnosis: CD10, bcl-2, bcl-6
4. Additional markers: Ki-67 to demonstrate proliferation index; CD21 and CD23 to demonstrate intrafollicular meshwork
5. Characteristic cytogenetics: t(14;18)(q32;q21)
6. Characteristic molecular biology: IgH/bcl-2
7. Immunoglobulin gene rearrangement with hypermutation in Hv region
8. bcl-6 gene rearrangement or 5′ mutation

CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR; Ig, immunoglobulin;

Hv, variable region of immunoglobin heavy chain gene.

Although patients with FL have an indolent clinical course with a medium survival of 9 to 10 years, FL is generally incurable (34). At the beginning, most patients may respond well to chemotherapy or radiation therapy, but the disease gradually becomes refractory to treatment with repeated relapses and finally leads to the death of the patient (1). The cause of death in most cases is due to transformation to high-grade lymphomas. Some patients may develop acute lymphoid or myeloid leukemia (1).

There have been many studies on the prognostic predictors. The International Prognostic Index (IPI) identified advanced age, elevated serum LDH, poor performance status, advanced stage, and multiple extranodal sites as adverse prognostic features (47). The Follicular Lymphoma International Prognostic Index (FLIPI) proposed five adverse prognostic factors, which include age ≥60 years, hemoglobin <12 g/dL, LDH above normal range, stage III or IV disease, and the presence of >3 nodal sites (48).

The detection of bcl-2 in patients after treatment is a reliable marker to predict prognosis (49). Further study of the breakpoint site in tumor cells shows that cases with a breakpoint at the minor cluster region have a better 3-year failurefree survival than do cases with a breakpoint

at the major breakpoint region. Cases showing no rearrangement of the bcl-2 gene have the worst prognosis (50). As mentioned in the Molecular Genetics section, gene expression profiling is a promising technique for stratification of FL cases to guide the treatment and predict the prognosis.

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CASE 27 Mantle Cell Lymphoma

CASE HISTORY

A 64-year-old man was diagnosed with chronic lymphocytic leukemia and was treated with chemotherapy for 1 year. Three years later, the patient developed splenomegaly and splenectomy with a hilar lymph node biopsy was performed. Two years later, he was referred to another hospital. Upon reviewing the splenectomy specimen and lymph node biopsy, with further flow cytometric analysis of the peripheral blood, the pathologist in the second hospital considered this case to be mantle cell lymphoma (MCL). This new diagnosis was confirmed by a fluorescence in situ hybridization (FISH) study, which demonstrated IgH/BCL-1 translocation.

Subsequently, the patient had a relatively stable clinical course. However, 9 years after the initial diagnosis, his leukocyte count was elevated to 120,000/μL with the presence of blastoid cells. A bone marrow biopsy confirmed blastoid transformation. The patient was started with a course of oral chlorambucil to no avail. A computed tomography (CT) scan revealed abdominal lymphadenopathy. He began to experience diplopia, and examination of cerebrospinal fluid (CSF) demonstrated lymphoma cells. Chest x-ray also revealed pulmonary infiltration with bilateral pleural effusion. Bronchoalveolar lavage showed lymphoma cells with an immunophenotype of MCL by flow cytometry. Despite aggressive chemotherapy, the patient's condition deteriorated rapidly and he died 9 months after the finding of blastoid cells in the peripheral blood. An autopsy was performed and disclosed extensive tumor dissemination, involving multiple abdominal lymph nodes, the lungs, kidneys, liver, small intestine, colon, testes, prostate gland, adrenal glands, and spinal cord.

FLOW CYTOMETRIC FINDINGS

Peripheral blood: CD5 90%, CD19 96%, CD19/CD5 92%, CD20 99%, CD23 4%, CD10 2%, FMC-7 98%, CD19/κ 98%, CD19/λ 2% (Fig. 6.27.1).

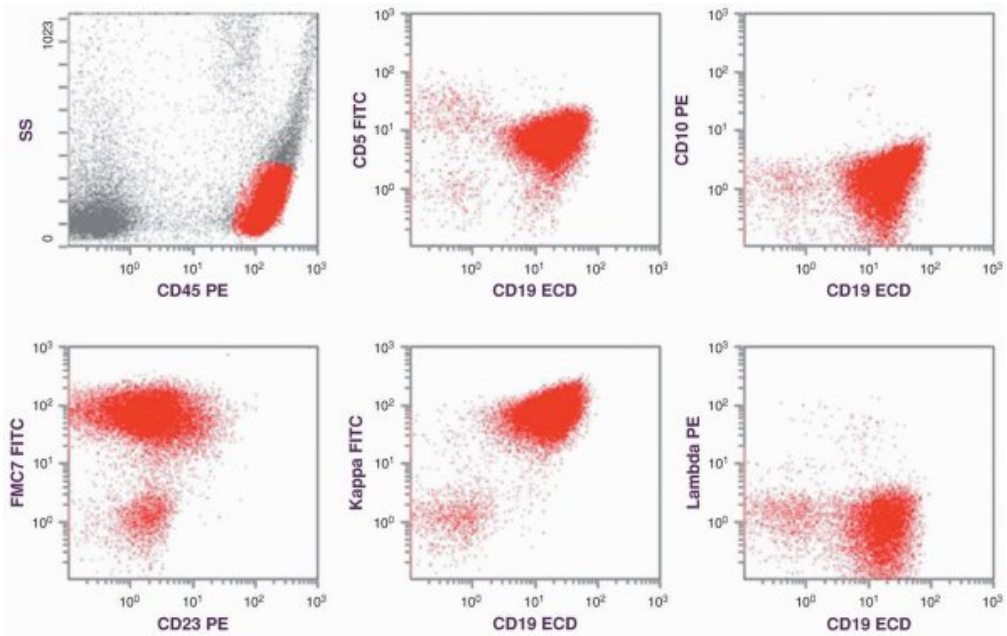

FIGURE 6.27.1 Flow cytometric histograms show dual CD19/CD5 staining and positive FMC-7 in a monoclonal κ population. CD23 is partial positive and CD10 is negative. SS, side scatter; FITC, fluorescein isothiocyanate; ECD, phycoerythrin-Texas Red; PE, phycoerythrin.

Bronchoalveolar lavage: CD5 96%, CD19 91%, CD19/CD5 91%, CD20 94%, CD23 3%, CD10 4%, FMC-7 94%, CD19/κ 88%, CD19/λ 2%.

CYTOGENETIC STUDIES

Karyotype of the bone marrow showed 46,XY,t(11;14) (q13;q32)[2]/46, XY[18]. FISH of the bone marrow revealed 56.1% of cells with a cyclin D1-IgH gene fusion with apparent clonal evolution. It was reported as: nuc ish 11q13 (CCND1×3),14q32(IgH×3) (CNND1 con IgH×2) [29]/11q13 (CCND1×3),14q32(IgH×3) (CCND1 con IgH×1)[17]/11q13 (CCND1×2),14q32(IgH×2)[36].

DISCUSSION

MCL was initially designated by Berard and Dorfman as lymphocytic lymphoma of intermediate differentiation for a group of non-Hodgkin lymphomas that could not be classified into either well-differentiated or poorly differentiated lymphocytic lymphomas according to Rappaport's classification (1). Because about 50% of lymphocytic lymphoma of intermediate differentiation cases showed membrane-associated alkaline phosphatase activity, which is normally found on the membrane of follicular cuff lymphocytes, the alternative term mantle zone lymphoma was proposed for this tumor (1). It was later found that mantle zone lymphoma was identical to centrocytic lymphomas in the Kiel classification (2). In 1982, the International Lymphoma Study Group renamed this tumor MCL lymphoma (3), a term that has since been accepted by the Revised European-American Classification of Lymphoid neoplasms (REAL) (4) and the World Health Organization (WHO) Classification of Hematological Malignancies (5).

Morphology

There are three histologic patterns of MCL: diffuse, mantle zone, and nodular (1, 2 and 3,5). The diffuse type is most frequently encountered and is usually difficult to diagnose without immunophenotyping (Fig. 6.27.2). The mantle zone type shows a residual or naked germinal center surrounded by an expanded mantle zone (Fig. 6.27.3), and the nodular type may represent colonization of the germinal center (6) or arise from the primary lymphoid follicle (Fig. 6.27.4) (1).

According to the WHO classification, there are three morphologic variants: Small cell, monocytoid B-cell, and blastoid cell (5). The small cell variant shows slight or

moderate nuclear irregularity or cleft, with clumped chromatin pattern, no nucleoli, and scant cytoplasm. The monocytoid B-cell has abundant pale cytoplasm resembling the tumor cells of marginal zone B-cell lymphoma. The blastoid variant is divided into classic and pleomorphic subtypes. Tumor cells in the classic subtype resemble lymphoblasts with dispersed chromatin and a high mitotic rate (Fig. 6.27.5). Tumor cells in the pleomorphic subtype are heterogeneous with large cleaved to oval nuclei and pale cytoplasm on Giemsa or methylene green pyronin stain. Nucleoli may be prominent in this subtype (5). The characteristic morphologic features of MCL are summarized in Table 6.27.1.

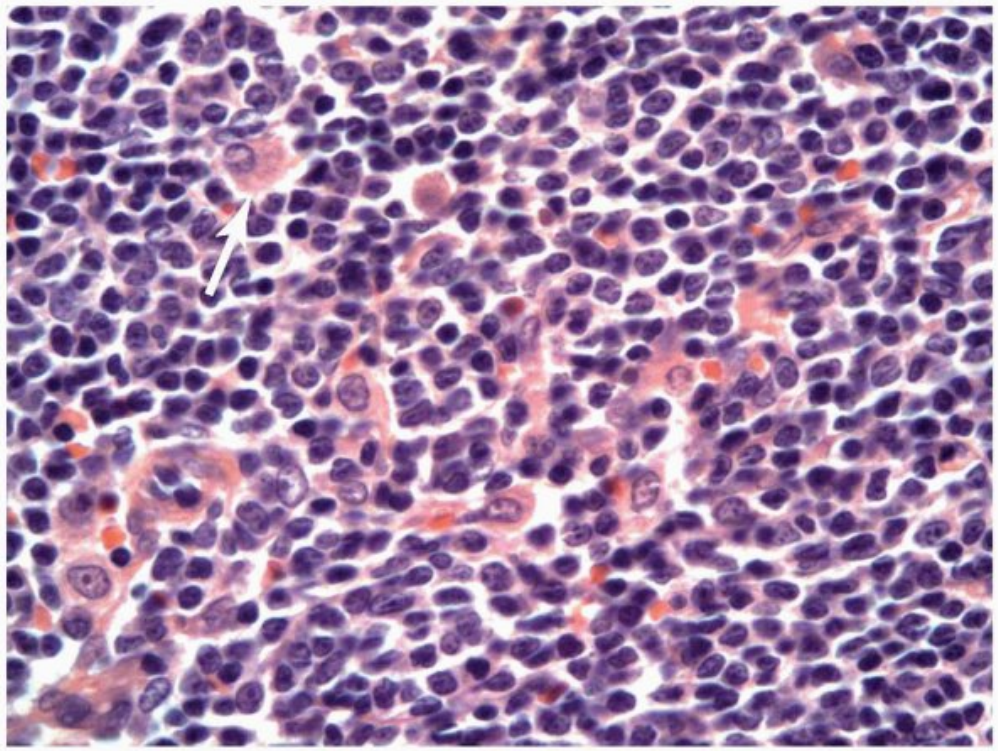

FIGURE 6.27.2 Diffuse type of mantle cell lymphoma shows diffuse infiltration of small lymphoid cells with slightly irregular nuclear configuration. Multiple pink histiocytes (*white arrow*) are present. Hematoxylin and eosin, 60× magnification.

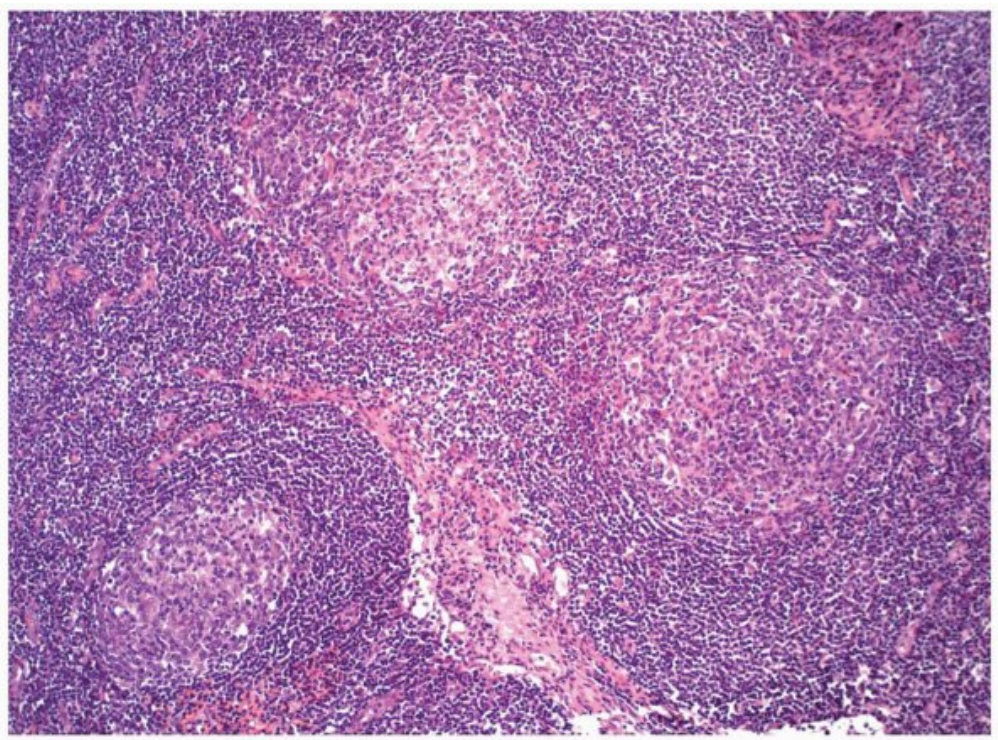

FIGURE 6.27.3 Mantle zone type of mantle cell lymphoma reveals residual germinal centers with greatly expanded mantle zones, which coalesce with each other. Hematoxylin and eosin,

10 × magnification.

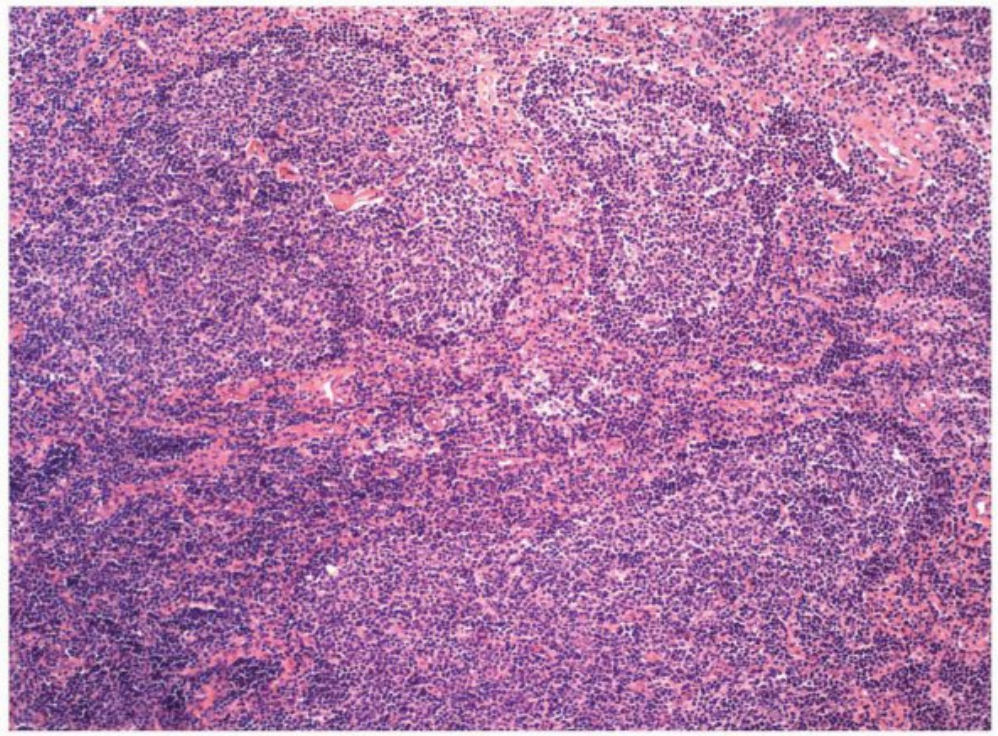

FIGURE 6.27.4 Nodular type of mantle cell lymphoma shows multiple nodules with irregular margins. Hematoxylin and eosin, 10× magnification.

In about two thirds of the cases, histiocytes with granular eosinophilic cytoplasm (pink histiocytes) are intermingled with the tumor cells, imparting a “starry sky” pattern (7). Unlike the tingible-body macrophages seen in Burkitt lymphoma and lymphoblastic lymphoma, these histiocytes do not contain nuclear debris.

One study showed that, in the bone marrow, 82% had a nodular pattern, 50% interstitial, 45% paratrabecular, and 32% diffuse (8). Another study revealed a similar order of frequency, with 68% showing nodular or interstitial pattern, 46% paratrabecular, and 23% diffuse (9). In the spleen, the white pulp is markedly expanded by the tumor cells, but reactive follicles with prominent germinal centers may be also coexistent (10). Liver involvement is mainly confined to the portal areas.

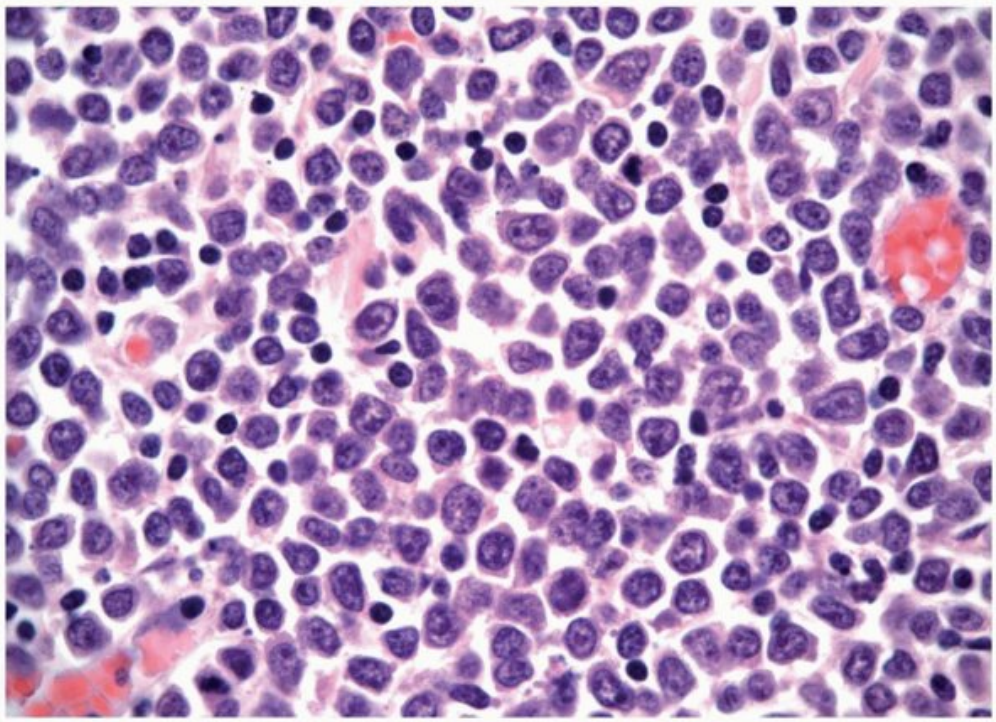

FIGURE 6.27.5 Blastoid variant of mantle cell lymphoma reveals medium-sized lymphoid cells with large nuclei and a dispersed chromatin pattern. Nucleoli are seen in a few tumor cells. Please compare with Fig. 6.27.2. Hematoxylin and eosin, 60× magnification.

TABLE 6.27.1

Characteristic Morphologic Features of Mantle Cell Lymphoma

Histologic pattern	Diffuse, mantle zone, and nodular
Cytology	Small cell, monocytoid B-cell, and blastoid (classic and pleomorphic)
Specific features	Naked germinal center surrounded by an expanded mantle zone in mantle zone subtype; scattered “pink histiocytes” in diffuse subtype

Lymphomatous polyposis of the gastrointestinal tract is most frequently due to MCL (Figs. 6.27.6 and 6.27.7) (11,12). When MCL is present in the gastrointestinal tract, polyposis is the most common presentation.

MCL may mimic several non-Hodgkin lymphomas (10). When the diffuse type of MCL shows minimal nuclear irregularity, it should be distinguished from small lymphocytic lymphoma (SLL). The latter is characterized by the presence of proliferation centers, which should be distinguished from the residual germinal centers in the mantle zone type of MCL (3). The residual germinal centers contain a mixture of small and large centrocytes and centroblasts, but the proliferation centers contain prolymphocytes and paraimmunoblasts. When MCL shows a nodular pattern, it should be distinguished from follicular lymphoma (FL), and the diffuse type of MCL should be distinguished from the diffuse type of FL. Large transformed cells with vesicular nuclei and prominent nucleoli and small cells with markedly cleaved nuclei are usually present in both types of FL, but are not seen in MCL (10,13).

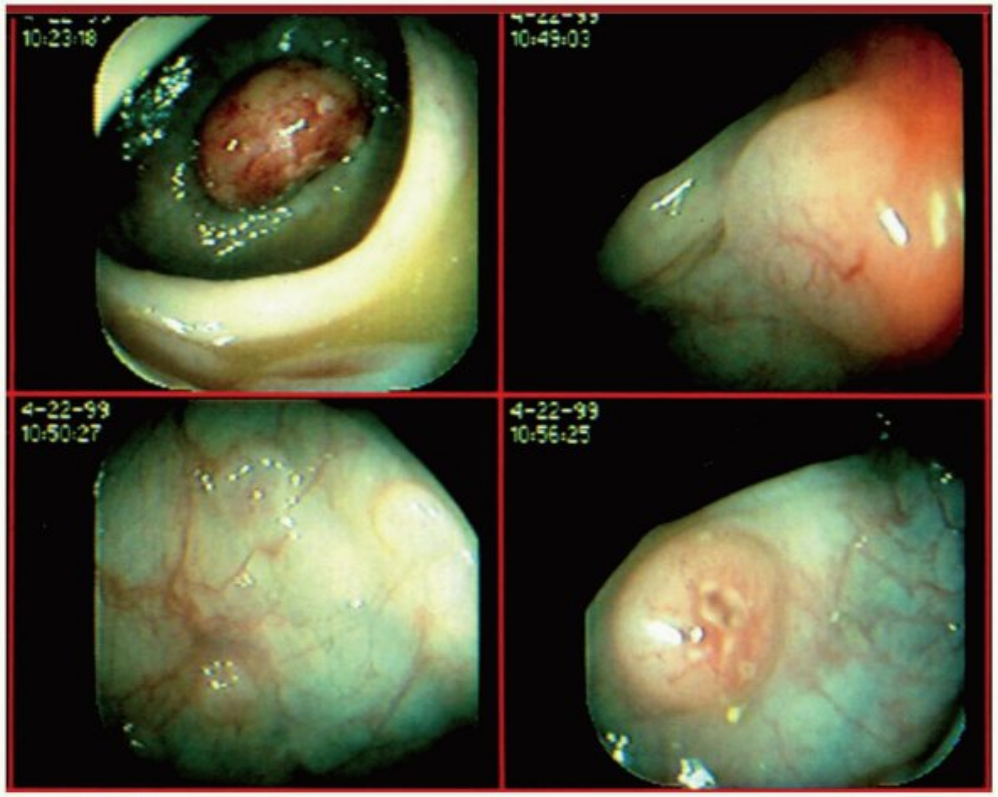

FIGURE 6.27.6 Colonoscopy demonstrates a few polyps with ulcerative surface. (Courtesy of Dr. Stacy Weiland, VA Medical Center, Denver, CO.)

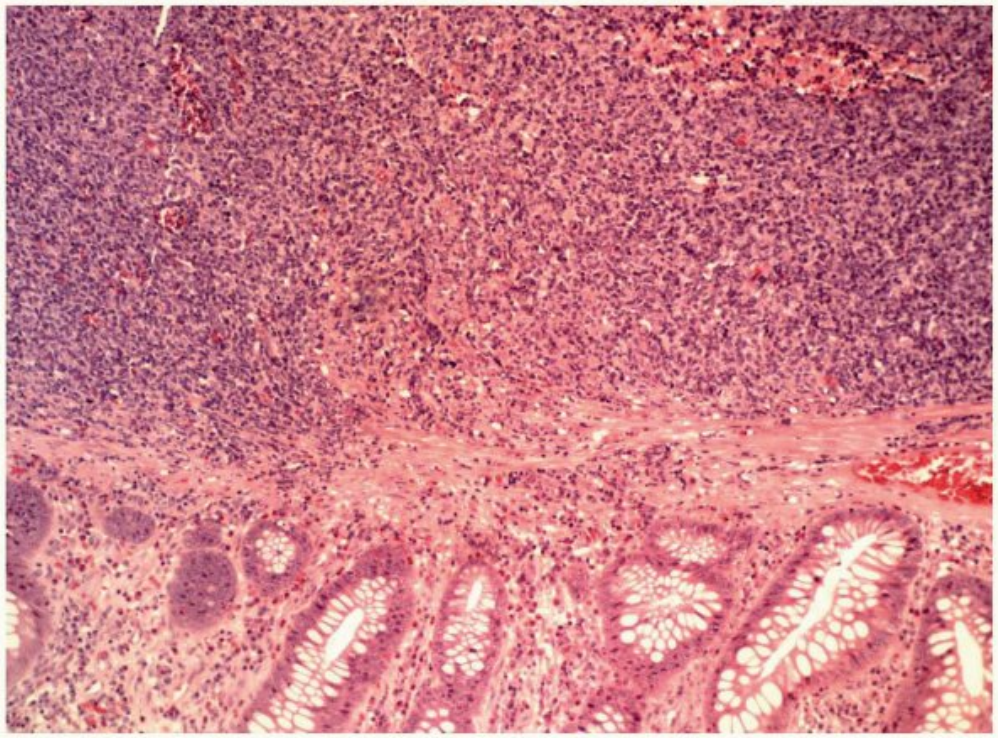

FIGURE 6.27.7 Colonic polyp shows extensive lymphoma cell infiltration in the submucosa. The lamina propria is not involved. Hematoxylin and eosin, 10× magnification.

The blastoid variant of MCL can be divided into the lymphoblastoid and pleomorphic subtypes, which may mimic lymphoblastic lymphoma or large cell lymphoma, respectively (14,15). Their distinction is mainly based on immunophenotyping. Finally, when a leukemic phase of MCL appears (Fig. 6.27.8), the differential diagnoses include chronic lymphocytic leukemia (CLL), prolymphocytic leukemia, acute leukemia, leukemic phase of FL, and leukemic phase of diffuse large B-cell lymphoma (16). The differences between MCL, FL, and SLL/CLL are summarized in Table 6.27.2.

Immunophenotype

Immunophenotyping is most helpful in distinguishing MCL from other lymphomas. However, the immunophenotype of SLL/CLL is similar to that of MCL. Both groups have coexpression of pan-B-cell antigens (CD19 or CD20) with a T-cell antigen, CD5 (Fig. 6.27.9) (7,10). The major difference is the reaction to CD23: It is negative for MCL but positive for SLL/CLL. However, CD23-positive cases (17, 18 and 19) and CD5-negative cases (20) of MCL have been reported. In addition, CD22 is positive in MCL cases but is negative in most cases of SLL/CLL. MCL also has brighter staining than SLL/CLL for surface Ig and CD20. FMC-7 is frequently demonstrated in MCL, but not in SLL/CLL cases unless prolymphocytic transformation occurs. The follicular dendritic cells, as demonstrated by CD21, are present in MCL and not in SLL/CLL (1). In contrast to most lymphomas and leukemias, two thirds of MCL cases express surface λ rather than κ light chain.

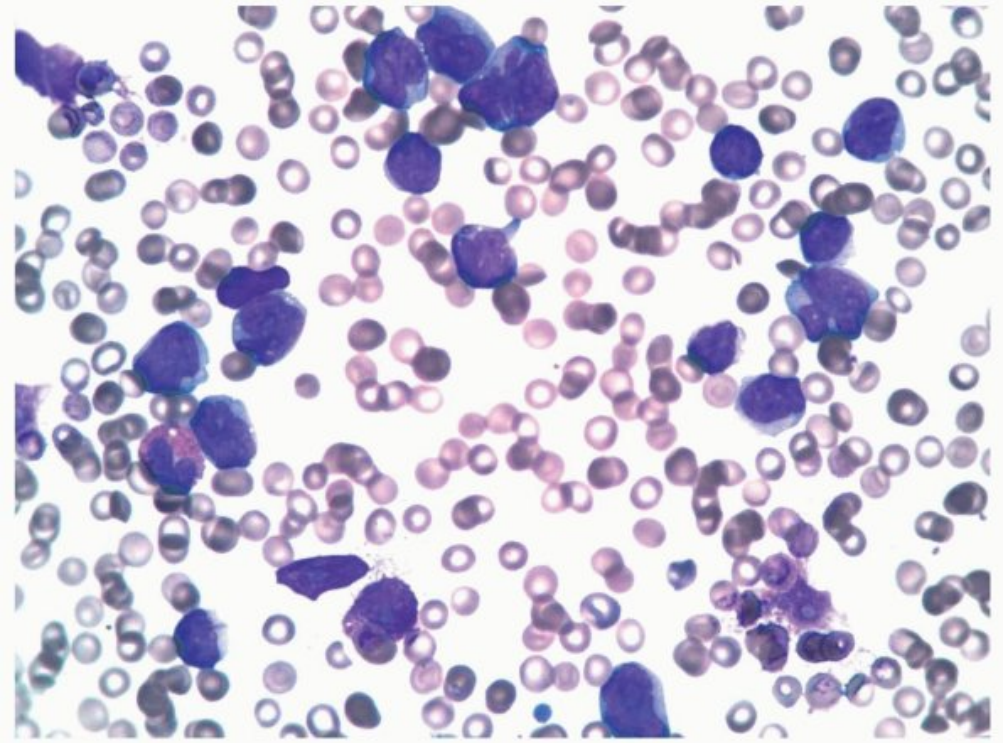

FIGURE 6.27.8 Peripheral blood smear shows many lymphoma cells with variable sizes, irregular configuration, high nuclear-cytoplasmic ratio, and prominent nucleoli. Wright-Giemsa stain, 60× magnification.

TABLE 6.27.2

Differentiation between MCL, FL, and SLL/CLL

	MCL	*FL*	*SLL/CLL*
Cell size	Intermediate	Small	Small to large
Surface immunoglobulin (Ig)	IgM-λ(2/3)	IgM-κ	IgM-κ
Fluorescence intensity	Moderate	Bright	Dim
CD5	+	-	+
CD10	-	+	-
CD19	+	+	+
CD20	+	+	+

CD22	+	+	±
HLA-DR	+	+	+
CD23	-	-	+
CD43	+	-	+
CD45RA	-	+	-
CD74	+	+	+
CDw75	-	+	-
CD79b	+	-	-
CD62L	+	±	+
IgD	+	-	+
Cyclin D1	+	-	-
Alkaline phosphatase	+	-	-
Cytogenetics	t(11;14)	t(14;18)	+12
Proto-oncogene	bcl-1	bcl-2	None

CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MCL, mantle cell lymphoma; SLL, small lymphocytic lymphoma; CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

The distinction of MCL from FL is mainly based on the reactions to CD5 and CD10: MCL is positive for CD5 but negative for CD10; the opposite is true for FL. Although some studies showed that blastic MCL cases were less likely to express CD5 and may express CD10, most investigators found that the blastic subtype had a phenotype similar to that of other subtypes of MCL (14, 15 and 16). In immunohistochemical studies, there are additional markers that may help to distinguish MCL from FL (10,21,22). MCL is positive for IgD, CD43, CD74 (LN2), CD62L (Leu-8), and CD79b but negative for CD45RA and CDw75 (LN1). The phenotype of FL is IgD-, CD43-, CD74+, CD62L+, CD79b-, CD45RA+, CDw75+.

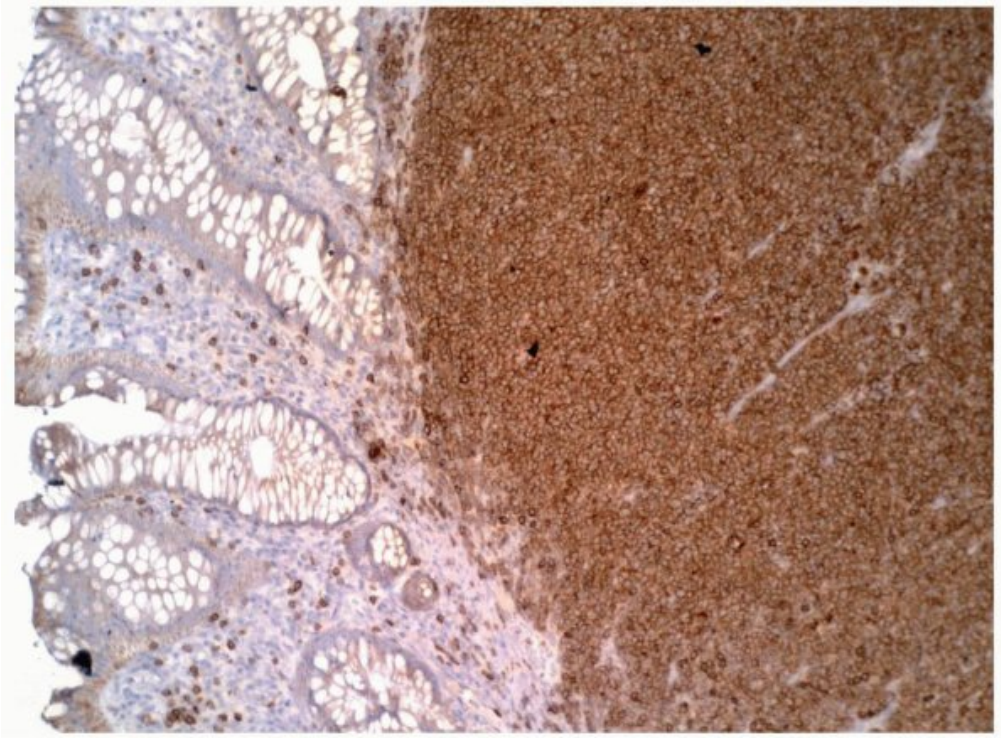

FIGURE 6.27.9 Colonic polyp reveals CD5 staining of 100% tumor cells. CD20 staining (not shown) demonstrates the same pattern. Immunoperoxidase, 10× magnification.

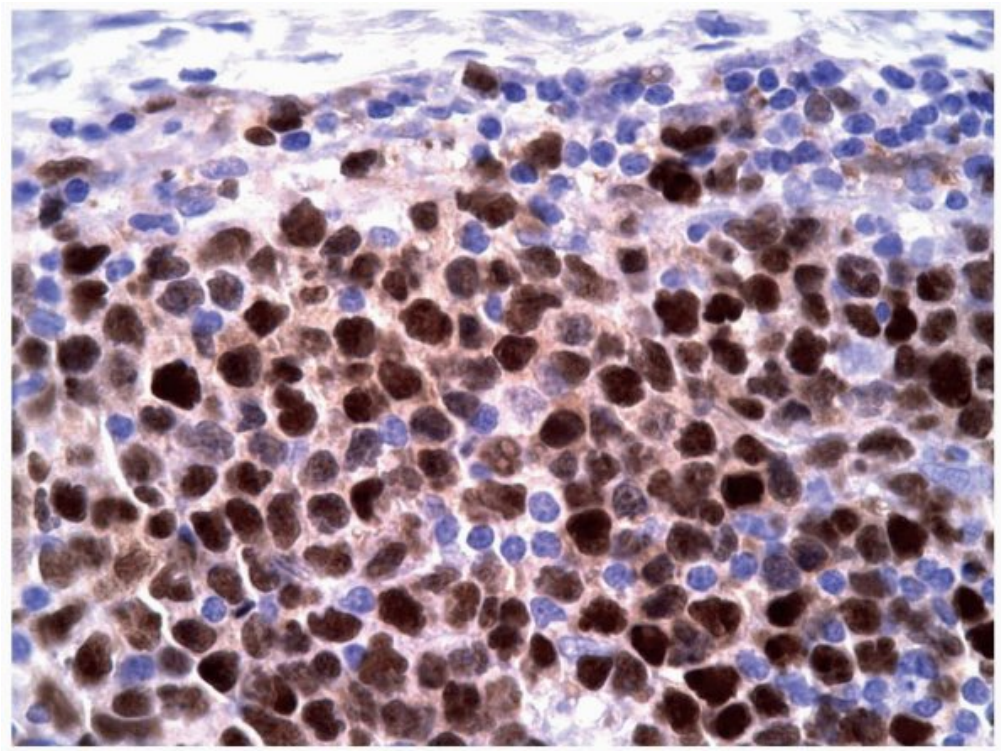

FIGURE 6.27.10 Lymph node biopsy of mantle cell lymphoma case shows positive nuclear staining of cyclin D1 in the tumor cells. Note that a few small lymphocytes are negative for cyclin D1. Immunoperoxidase, 60× magnification.

The presence of surface Ig and absence of terminal deoxynucleofidyl transferase (TdT) and CD10 in blastic MCL are helpful in distinguishing it from lymphoblastic lymphoma. In fact, most cases of lymphoblastic lymphoma are of T-cell lineage, so the presence of several T-cell markers can readily distinguish lymphoblastic lymphoma from MCL, which is of B-cell lineage. Diffuse large B-cell lymphoma may mimic the pleomorphic subtype of MCL but is usually CD5 negative (16), although CD5-positive diffuse large B-cell lymphoma has been reported (23,24).

Cyclin D1 protein as demonstrated by immunohistochemical stains has been considered the most reliable marker for MCL (Fig. 6.27.10). The positive rates varied from 72% to 100% in MCL cases, but it can be also positive in a few cases of prolymphocytic leukemia, splenic lymphoma with villous lymphocytes, and occasional SLL/CLL, hairy cell leukemia, and plasmacytoma/myeloma (23,25). Cyclin D1 has not been found in FL, splenic marginal zone lymphoma, monocytoid B-cell lymphoma, lymphoplasmacytic lymphoma, Sézary syndrome, reactive lymphoid hyperplasia, or high-grade lymphomas (25,26). Cases with positive cyclin D1 staining generally have worse prognosis than those with negative staining (27,28).

Cyclin D1 also can be detected by flow cytometry, but it has been used only on a research basis (29). Similar to immunohistochemical staining, cyclin D1 has been demonstrated in subsets of CLL, prolymphocytic leukemia, hairy cell leukemia, and multiple myeloma by flow cytometry.

Two prognostic predictors, Ki-67 and survivin, can be demonstrated by immunohistochemistry. In patients with Ki-67 expression in >50% of tumor cells, the median survival is 9 months. In contrast, the medium survival is 62 months for patients with expression in <50% (30). Survivin is a member of the inhibitor of apoptosis protein (IAP) family that is expressed in G2/M phase. Cases with nuclear survivin expression in >20% of tumor cells have a survival time of 8 months; it is 60 months for those with expression in <20% (30).

MCL cells may also express surface CD40. The importance of detecting CD40 is that CD40-ligating agents, such as CD40 ligand (CD40L) and CD40 monoclonal antibody, can be used for immunotherapy of MCL, and the increase of soluble CD40 in a patient's blood is associated with a poor prognosis (31).

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry is able to distinguish MCL from other lymphomas in most cases, but immunohistochemistry is more reliable because there are more markers available for this technique. A panel of CD5, CD20, CD23, CD79b/CD79a, and cyclin D1 staining on tissue sections often provides a reliable diagnosis. As mentioned before, a positive cyclin D1 staining is most reliable, but this staining is technically difficult (19,32); special antibodies should be used to demonstrate the nuclear staining (33). In contrast, a few true cyclin D1-negative MCL cases have been identified by gene expression profiling (GEP) and may be the result of replacement by cyclin D2 or D3 (34). A high percentage of cyclin D1 in conjunction with Ki-67 and survivin staining also helps in predicting a poor prognosis. The identification of the blastoid variant of MCL is facilitated by the demonstration of cyclin D1, Ki-67, and cyclin-dependent kinase (CDK)4 on the tumor cells (35).

Molecular Genetics

MCL is characterized by the presence of t(11;14)(q13;q32), which can be detected in about 75% of cases by karyotyping (36). This represents the translocation of the proto-oncogene BCL-1(11q13) juxtaposed to the heavy-chain gene (14q32). A PRAD1 (parathyroid adenoma 1) or CCND1 gene is linked to the BCL-1 gene at its telomeric border. This gene encodes for cyclin D1, a cell-cycle regulatory protein. As a result of the translocation, the CCND1/cyclin D1 genes are deregulated. Therefore, the t(11;14)-carrying cells cannot exit from the cell cycle, resulting in an expanded B-cell department with developmental arrest (10,37). However, the recent finding of the deletion of the ATM (ataxia telangiectasia mutated) gene in MCL suggests that ATM may serve as a tumor suppressor gene for MCL and that its deletion may be the mechanism leading to oncogenic transformation of the expanded B-cell population (38,39).

For the diagnosis of MCL, the detection of t(11;14) either by karyotyping or by molecular technique (Southern blot or polymerase chain reaction [PCR]) is highly specific, but generally the positive rate is not high. The low sensitivity of Southern blot and PCR is due to the large number

of 11q13 breakpoints scattering over a region of >120 kb (36,40). The conventional karyotyping is hindered by the low yield and quality of tumor metaphases (36,40). Therefore, the method of choice for the detection of the translocation is the FISH technique using various probes (IgH/CNND1, CyclinD1/cep11, IgH/bcl-1).

According to the current literature, this technique is highly sensitive and specific for the diagnosis of MCL because the probes used cover a broad area so that t(11;14) can be detected irrespective of the location of the breakpoints within the BCL-1 region (19,36,40, 41, 42 and 43). In one study, only 4 of 32 non-MCL cases showed positive results by FISH (41). These false-positive results were found to be due to the presence of extra copies of chromosome 11 in two cases. Another report showed positive results in 11 of 21 “atypical” CLL cases with an MCL immunophenotype (41). These cases may well represent the leukemic phase of MCL. The additional advantage of FISH is that it can be performed on dried blood and bone marrow smears, fine-needle aspirates, and paraffin-embedded tissues.

The PRAD1/cyclin D1 messenger RNA (mRNA) can be detected by Northern blot or reverse transcriptase-PCR (RT-PCR) (42,43). These

techniques are sensitive for the diagnosis of MCL. RT-PCR, however, also showed cyclin D1 in 65% of non-MCL cases and 43% of atypical lymphoid hyperplasia cases in one study (43).

In addition to t(11;14), there have been many recurrent secondary karyotypic abnormalities reported (44). A few of them are of particular interest because of their possible association with particular genes. For instance, del 17p13 may be associated with p53, del 11q22-23 with ATM, and del 9p21 with p16/p15/p14. In contrast, amplification of 8q22-24 is associated with C-MYC and amplification of 10p12 is associated with the BMI-1 gene. BCL-6 gene is not amplified in MCL cases (44). The gain of BCL-2 gene in MCL is controversial (44,45). The blastoid variant is frequently associated with p53 mutation, p16 deletion, and tetraploidy (14,35,46).

It is hypothesized that the pathogenesis of MCL is essentially the result of dysregulation of the cell cycle through complex cytogenetic events. These include cyclin D1 upregulation, genomic amplification CDK4, deletions of the CDK inhibitor $p16^{INK4a}$, and overexpression of BMI-1 (47).

Gene expression profiling (GEP) is able to stratify MCL cases into various subtypes in terms of IgH gene mutation status, proliferation rate, and blastoid morphology (48). GEP may also help to distinguish MCL from SLL, diffuse large B-cell lymphoma, and splenic marginal zone lymphoma (34,49). One GEP study identified a B-cell-associated tyrosine kinase gene, SYK, which was also demonstrated at RNA and protein levels in MCL cells (45). This gene serves as a possible therapeutic target in MCL- and Syk inhibitor suppresses tumor cell proliferation and induces apoptosis in tumor cell lines.

As mentioned before, most MCL cases express surface λ light chain, which correlates well with the gene rearrangement studies that show the deletion of the κ light-chain gene or rearrangement between the κ deletion element and the recombinant signal sequence (44,50).

The vast majority of MCL cases, with the possible exception of blastoid variant, show unmutated Ig heavy-chain variable (V_H) gene, suggesting that the tumor cells are derived from naïve B cells (44,48). However, approximately 25% of MCL cases have mutated V_H genes. One study found that MCL cases with mutated status were associated with the absence of lymphadenopathy and good prognosis (51). However, most studies revealed no significantly different outcomes between mutated and unmutated cases (44,48). Unlike CLL cases, unmutated cases are not associated with Zeta-chain-associated protein kinase (ZAP)-70 expression. The salient features for laboratory diagnosis of MCL are summarized in Table 6.27.3.

Clinical Manifestations

MCL is seen in elderly patients with a median age of about 60 to 63 years, and the male-to-female ratio ranges from 2.3:1 to 5:1 (6,9,50). The incidence of MCL in the United States is 2.5% to 4.0% of all non-Hodgkin lymphomas, but it is 7% to 9% in Europe.

Most (73%) patients present with generalized lymphadenopathy, and about 50% of patients have systemic symptoms (5.8%). About 50% of patients have splenomegaly at presentation, and 80% of patients with mantle zone subtype of MCL may show prominent splenomegaly. Most patients with MCL are in stage III or IV at diagnosis, and bone marrow and liver involvement are common findings. Hepatomegaly is seen in 20% of patients. Primary extranodal presentation was considered an infrequent finding, but a report showed an incidence of 25% (51). The extranodal presentation is most commonly seen in the gastrointestinal tract and Waldeyer ring (10). Peripheral lymphocytosis of >4,000/μL occurs in 20% to 40% of cases but seldom exceeds 20,000/μL (10). Mild anemia and thrombocytopenia are seen in some cases. Hypogammaglobulinemia, monoclonal gammopathy, and positive Coombs test have been occasionally reported.

MCL has the worst prognosis among all B-cell lymphomas, because it assumes an aggressive clinical course and yet is incurable. In terms of prognosis, most studies emphasize the association with the histologic subtypes, the blastoid morphology, and the cell markers. GEP studies support the stratification based on the above factors. One study found that del 8p21 and 13q14 were associated with inferior survival, whereas del 9p/p15-p16 showed a trend for decreased overall survival (52). Unlike CLL cases, the mutation status of the V_H gene does not predict the prognosis in MCL patients.

Clinically, several factors appear to be adverse prognostic indicators, including leukemic presentation, bone marrow involvement, advanced stage disease, B symptoms, and poor performance status (13,51,53,54).

The relationship between histologic types and prognosis is controversial. Weisenburger and Armitage (10) suggested that nodular and mantle zone types with a median survival of >5 years should be considered a low-grade lymphoma; the diffuse type with a median survival of 3 years, an intermediate grade lymphoma; and the blastoid variant with a median survival of <2 years, a high-grade lymphoma. Majlis et al. (55) found that the 3-year survival

rates were 100%, 50%, and 55% for patients with mantle zone, nodular, and diffuse histologic patterns, respectively. In contrast, Argatoff et al. (51) failed to show any significant differences in the median overall survival among the mantle zone, nodular, and diffuse types. However, the more recent study by the Nebraska group showed that the diffuse type had the shortest median overall survival of 16 months, as compared to 55 months in the nodular type and 50 months in the blastoid variant (56).

TABLE 6.27.3

Salient Features for Laboratory Diagnosis of Mantle Cell Lymphoma

1. Monoclonal surface immunoglobulin (Ig) pattern with intermediate staining intensity
2. Positive B-cell antigens: CD19, CD20, CD22, CD79b, HLA-DR
3. Important negative B-cell antigen: CD23
4. Characteristic marker for diagnosis: CD5
5. Immunoglobulin-negative B-cell antigen-positive pattern may be present.
6. Selective loss of pan-B-cell antigen may be present.
7. Characteristic cytogenetic abnormality: t(11;14)
8. Molecular characterization: bcl-1 (CNND1)/IgH rearrangement
9. Nuclear cyclin D1 identified by immunohistochemistry or fluorescence in situ hybridization

CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR.

The general consensus is that the blastoid variant is unequivocally associated with the worst prognosis (13,15,35,51,57). As mentioned before, the blastoid variant presents with high mitotic rates, and expresses with cyclin D1, Ki-67, CKD4, p53 mutation, and p16 deletion. The blastoid variant is also characterized by the presence of extra copies of CCND1 signals (35). High levels of lactate dehydrogenase (>450 U/L) and/or leukocytosis (>10,000/μL), high percentage of Ki-67, and high mitotic score at presentation are predictors for blastoid transformation (57).

The current case is unusual for a relatively indolent clinical presentation for 9 years. It was until the development of blastoid transformation that an aggressive clinical course followed, leading to a rapidly fatal outcome within 9 months. In the final months, the patient was found to have central nervous system, pulmonary, and abdominal involvement clinically, but the autopsy showed that almost all internal organs were involved, exemplifying a highly aggressive malignancy. Peghini and Fehr (17) reported an MCL patient similar to ours who survived for 19 years.

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CASE 28 Diffuse Large B-Cell Lymphoma

CASE HISTORY

A 65-year-old man was admitted to the hospital for splenectomy. The patient noticed a mass in his left upper quadrant 7 months prior to admission, and the mass appeared to progress in the last 4 months. He denied having any constitutional symptoms such as fever, night sweats, and/or weight loss. A fine-needle aspiration was performed on an axillary lymph node, which showed only reactive cells with no evidence of lymphoma.

Laboratory examination revealed a total leukocyte count of 8,400/μL with 27% neutrophils, 58.1% lymphocytes, 13.2% monocytes, 1.4% eosinophils, and 0.3% basophils. The hematocrit was 48%, hemoglobin 16.3 g/dL, and platelets 141,000/μL. Serum lactate dehydrogenase

was 159 IL/L.

During his hospital stay, a diagnosis of diffuse large B-cell lymphoma (DLBCL) was made on the splenectomy specimen. The patient was started with chemotherapy and continued to receive 8 cycles of CHOP in the subsequent years. Since then, he had been in complete remission for 5 years with one bout of pneumonia and one episode of deep venous thrombosis during this period of time. However, an enlarged left axillary lymph node was discovered on a follow-up examination. A biopsy of the lymph node was taken, and the diagnosis proved to be recurrent DLBCL.

The patient then received fludarabine without effect as the lymphoma spread to the cervical, thoracic, and lumbar spine. He switched to another chemotherapeutic regimen as well as radiation therapy with resultant partial remission. However, the patient developed postradiation esophagitis, and his condition deteriorated rapidly. He died approximately 6 years after the initial diagnosis of lymphoma.

FLOW CYTOMETRY FINDINGS

Lymph node: CD5 3%, CD19 94%, CD20 98%, CD10 4%, CD23 1%, FMC-7 98%, κ 86%, λ 0%, CD19/κ 87%, CD19/λ 5%, CD45 100% (Fig. 6.28.1).

IMMUNOHISTOCHEMICAL STAINS

Lymph node: Positive reactions are demonstrated for CD20 and CD30 (Fig. 6.28.2), but negative reactions for CD3 and bcl-2.

MOLECULAR GENETICS

Lymph node: 46,XY, del(6)(q15),del(13)(q12q14), add(14) (q32) [9]/46,XY[7]. Fluorescence in situ hybridization for an oncogene derived from avian myelocytomatosis virus (C-MYC): Negative in all 200 interphase cells analyzed.

DISCUSSION

DLBCL is a non-Hodgkin lymphoma of B-cell origin showing diffuse proliferation of large neoplastic lymphoid cells. A large cell is defined by the World Health Organization (WHO) classification and others as having a nucleus equal to or exceeding normal macrophage nuclei or more than twice the size of a normal lymphocyte (1). As will be discussed later, this lymphoma group is morphologically heterogeneous and can be divided into several variants with minor differences in immunophenotypes but having similar clinical outcomes.

In the Working Formulation, DLBCL includes diffuse large B-cell lymphoma and immunoblastic lymphoma (2). The Revised European-American Lymphoma (REAL) classification includes all aggressive large B-cell lymphomas other than lymphoblastic lymphoma and Burkitt lymphoma in the category of DLBCL (3). However, because primary mediastinal B-cell lymphoma differs from other DLBCL variants, it stands out as a special subtype of DLBCL in the REAL classification. The WHO classification adds two more subtypes of DLBCL: Intravascular large B-cell lymphoma and primary effusion lymphoma.

DLBCL is one of the most common lymphomas, accounting for 30% to 40% of adult non-Hodgkin lymphoma in Western countries (1), and 7 per 100,000 person-years in the United States (4). It can be de novo or secondary, being transformed from low-grade lymphomas, such as small lymphocytic lymphoma, follicular lymphoma, lymphoplasmacytic lymphoma, extranodal marginal zone B-cell lymphoma, and splenic marginal zone lymphoma.

Morphology

There are four common variants and two rare variants recognized by the WHO system. Some unusual variants with myxoid stroma, fibrillary matrix, pseudo-rosettes, spindly cells, signet ring cells, cytoplasmic granules, microvillous projections, and intercellular junction are not listed as distinct entities (1). The histologic pattern is characterized by a diffuse large cell infiltration with effacement of the normal architecture, but it can also be interfollicular or intrasinusoidal infiltration. It is recognized that these variants are not easily reproducible between different observers.

Centroblastic Variant

This variant is composed of tumor cells similar to the centroblasts seen in reactive germinal centers. These cells vary from medium to large size and are characterized by large round or oval nuclei with a vesicular chromatin pattern and two to four membrane-bound nucleoli (Fig. 6.28.3) (1,5). The cytoplasm is scanty and amphophilic to basophilic. This variant can be further divided into monomorphic, pleomorphic, and multilobated subvariants. The monomorphic type is composed of predominantly centroblasts. The pleomorphic type comprises a mixed population of centroblasts, immunoblasts, and/or multilobated cells. When pleomorphic type has more than 90% of immunoblasts, it is difficult to distinguish pleomorphic from immunoblastic variant. The multilobated type shows cells with the nuclei composed of more than three lobes and usually inconspicuous nucleoli (5). This variant is the most common among the variants.

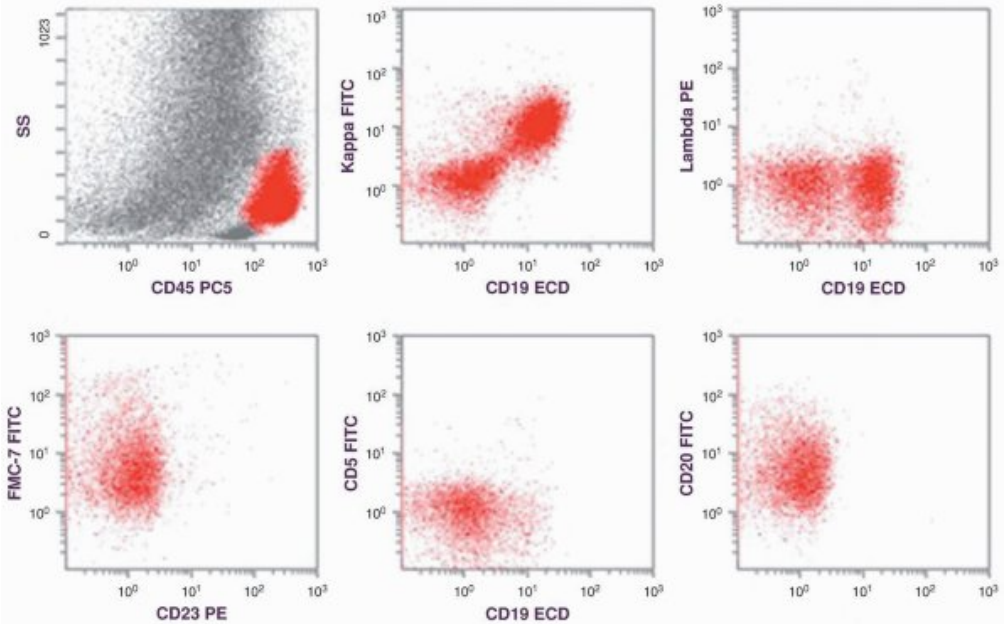

FIGURE 6.28.1 Flow cytometric histograms show positive CD19, CD20, κ, and FMC-7 but negative CD5, CD23, and λ reactions. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; ECD, phycoerythrin-Texas Red; PE, phycoerythrin.

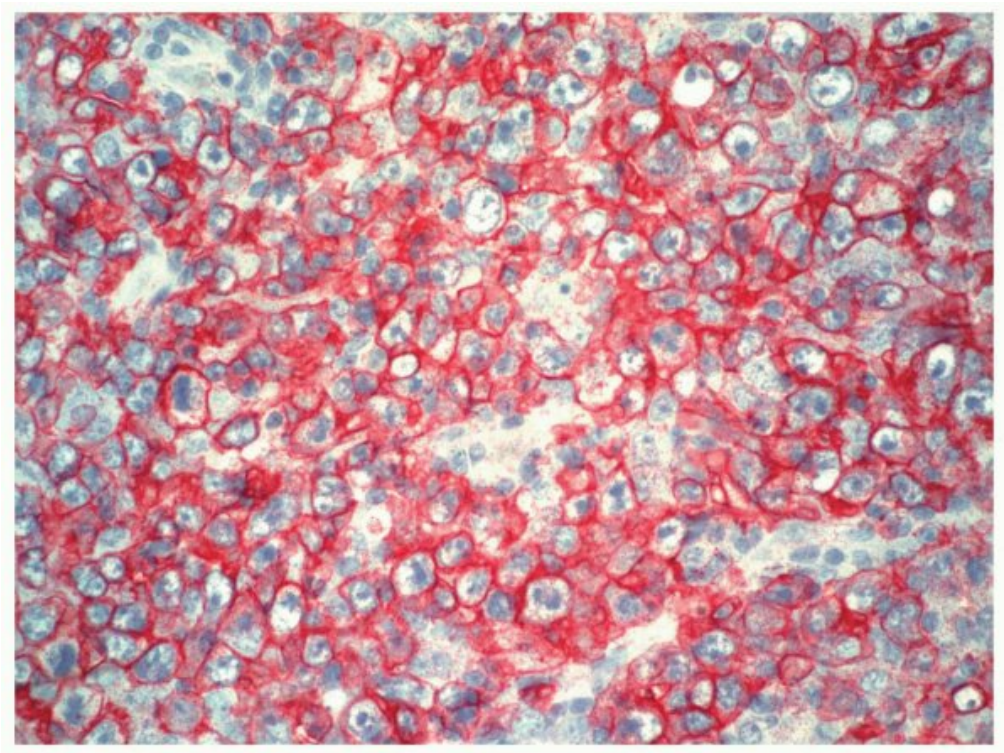

FIGURE 6.28.2 Lymph node biopsy shows positive staining of CD30 on the large tumor cells. Immunoalkaline phosphatase, 40× magnification.

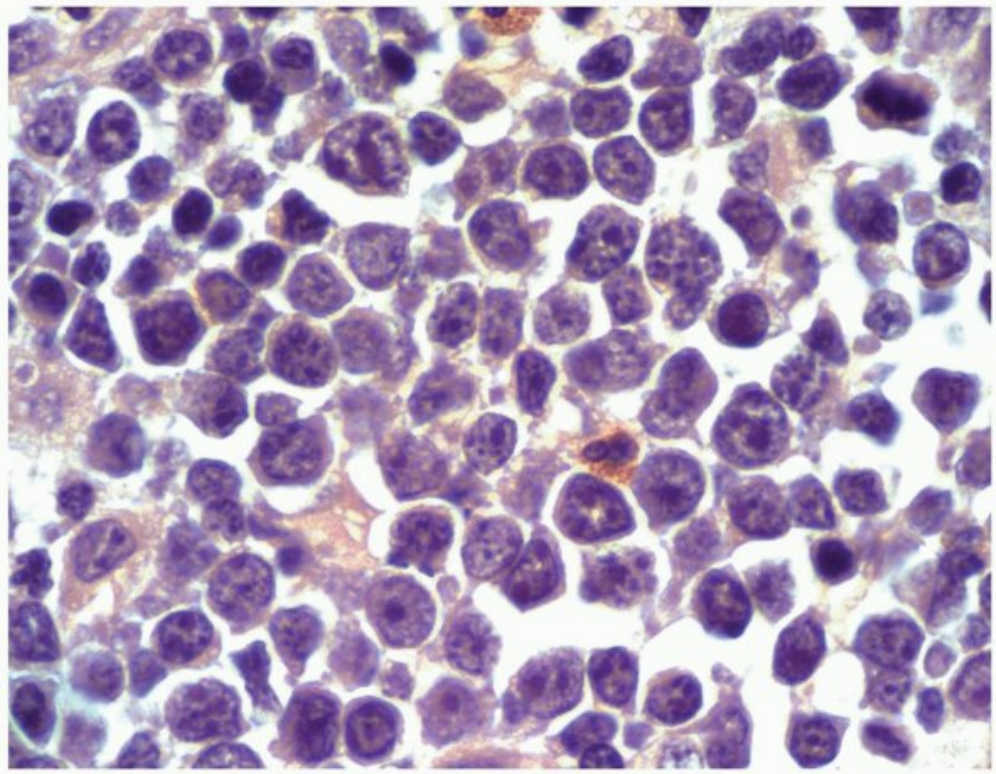

FIGURE 6.28.3 Centroblastic variant of diffuse large B-cell lymphoma in a lymph node biopsy. Tumor cells show large round or oval nuclei with multiple nucleoli and scanty cytoplasm. Hematoxylin and eosin, 100× magnification.

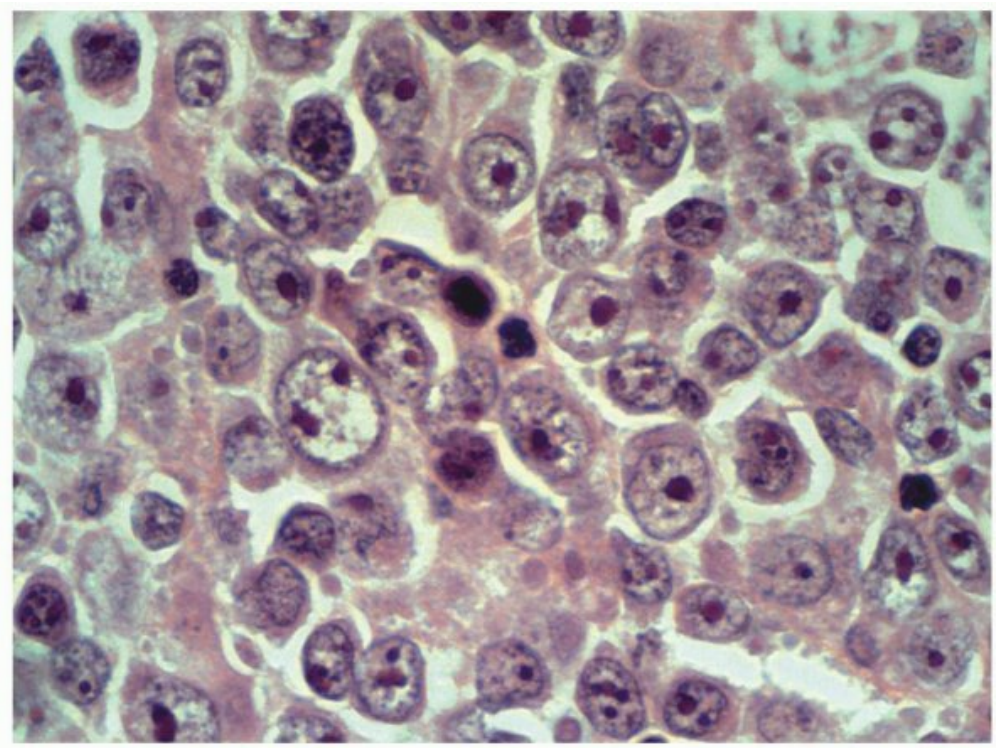

FIGURE 6.28.4 Immunoblastic variant of diffuse large B-cell lymphoma in a lymph node biopsy.

Tumor cells show large vesicular nuclei with single prominent nucleolus and moderate amount of cytoplasm. Hematoxylin and eosin, 100× magnification.

Immunoblastic Variant

This variant encompasses >90% of immunoblasts (1,5). The immunoblasts are large cells with large vesicular nuclei and a single prominent, centrally located nucleolus (Fig. 6.28.4). There is a moderate amount of basophilic cytoplasm. Centroblasts, if present, should be <10% of the population, or it should be classified as a centroblastic variant. Plasma cells and plasmablasts may be seen in this variant. The immunoblastic variant accounts for only 4% of non-Hodgkin lymphomas in the Kiel classification (6).

T-Cell-/Histiocyte-Rich Variant

T-cell-rich B-cell lymphoma was poorly defined in the early literature, but it is now generally accepted as a B-cell tumor accompanied by >90% of T lymphocytes (Fig. 6.28.5) (1,5). The histiocyte-rich variant was later added to this group as representing the same entity (Figs. 6.28.6 and 6.28.7) (7,8). The background T cells or histiocytes are considered by some authors to be the reactive component induced by lymphokines released from the tumor cells. Some studies considered this variant to represent an early immune response that would finally progress into a full-blown DLBCL. However, this variant may have an aggressive clinical course and stage IV disease at the time of diagnosis, and is not necessarily an early stage of this lymphoma (5). The tumor cells may resemble those in the centroblastic variant, immunoblastic variant, lymphocytic and histiocytic (L&H) cells, or Reed-Sternberg cells (7,9). Therefore, this variant has to be distinguished from Hodgkin lymphoma by immunohistochemical staining in some cases.

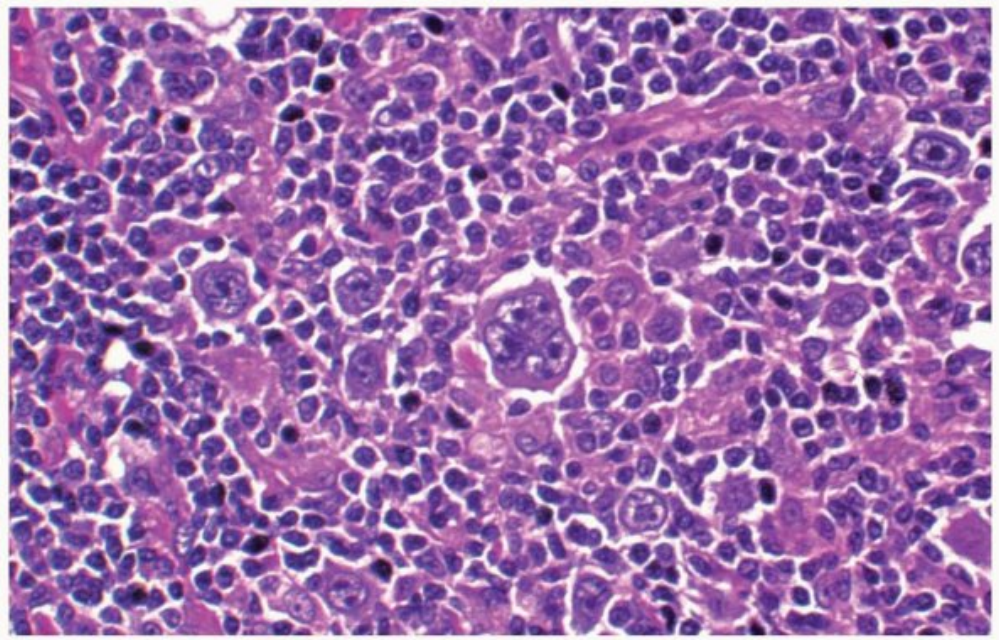

FIGURE 6.28.5 T-cell-rich B-cell lymphoma in a lymph node biopsy shows a few large tumor cells scattered among the small lymphocytes. A multinucleated large tumor cell is present in the center of this field. Hematoxylin and eosin, 60×. (Courtesy of Myron Susin, M.D., of North Shore University Hospital, New York.)

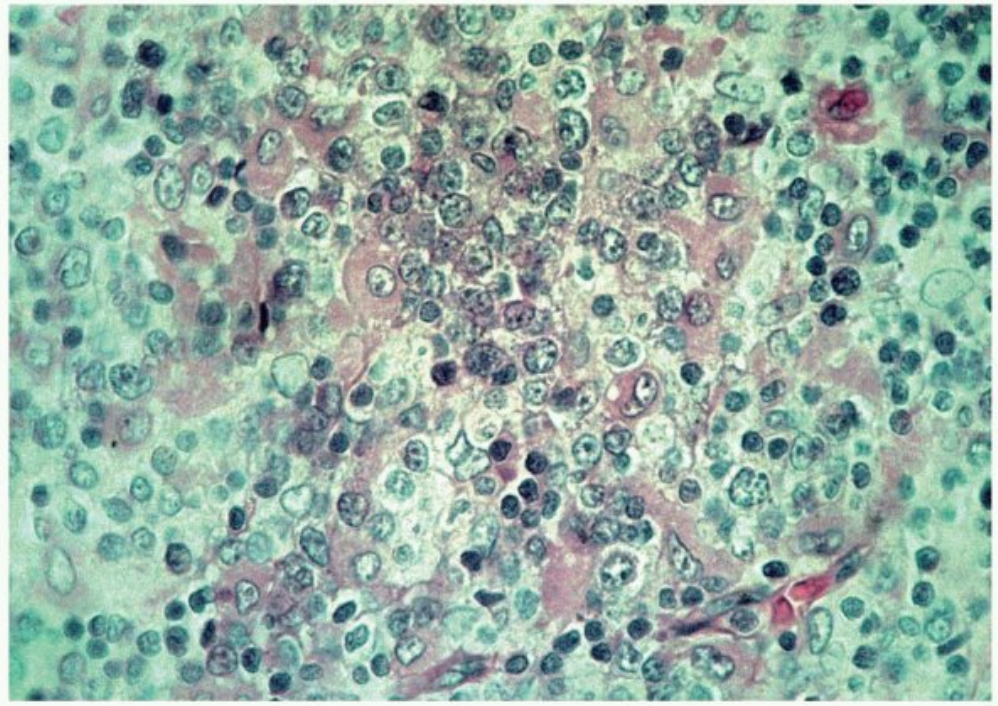

FIGURE 6.28.6 Histiocyte-rich B-cell lymphoma in a lymph node biopsy shows many histiocytes with pink cytoplasm intermingled with the lymphoma cells. Hematoxylin and eosin, 60× magnification.

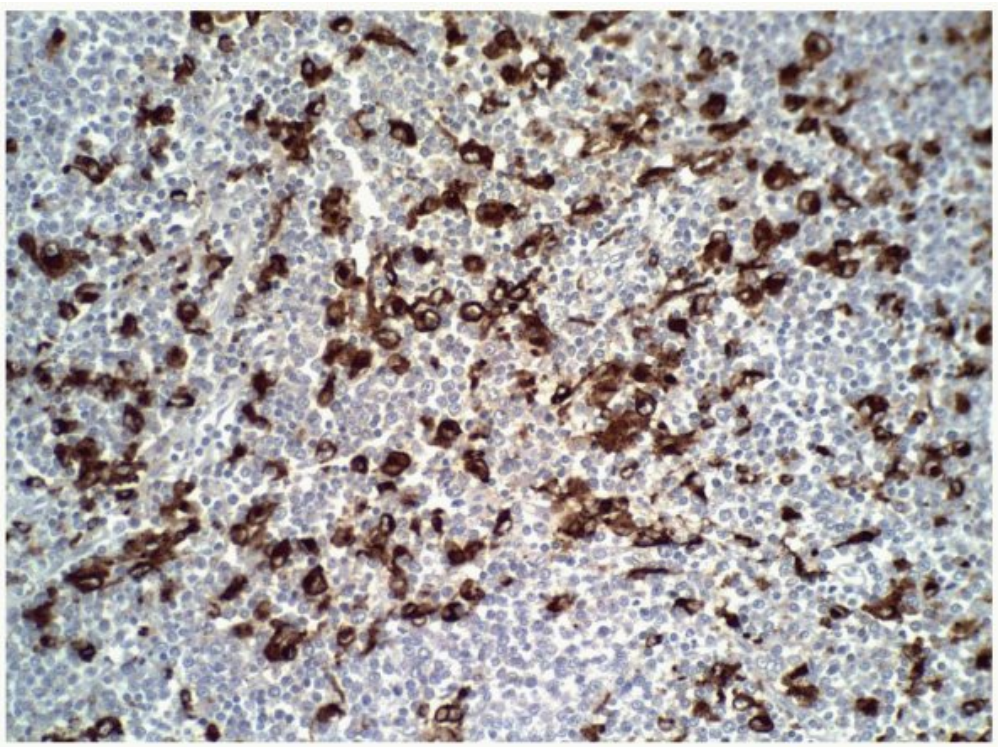

FIGURE 6.28.7 Histiocyte-rich B-cell lymphoma in a lymph node biopsy shows positive CD68 staining in the histiocytes. Immunoperoxidase, 20× magnification.

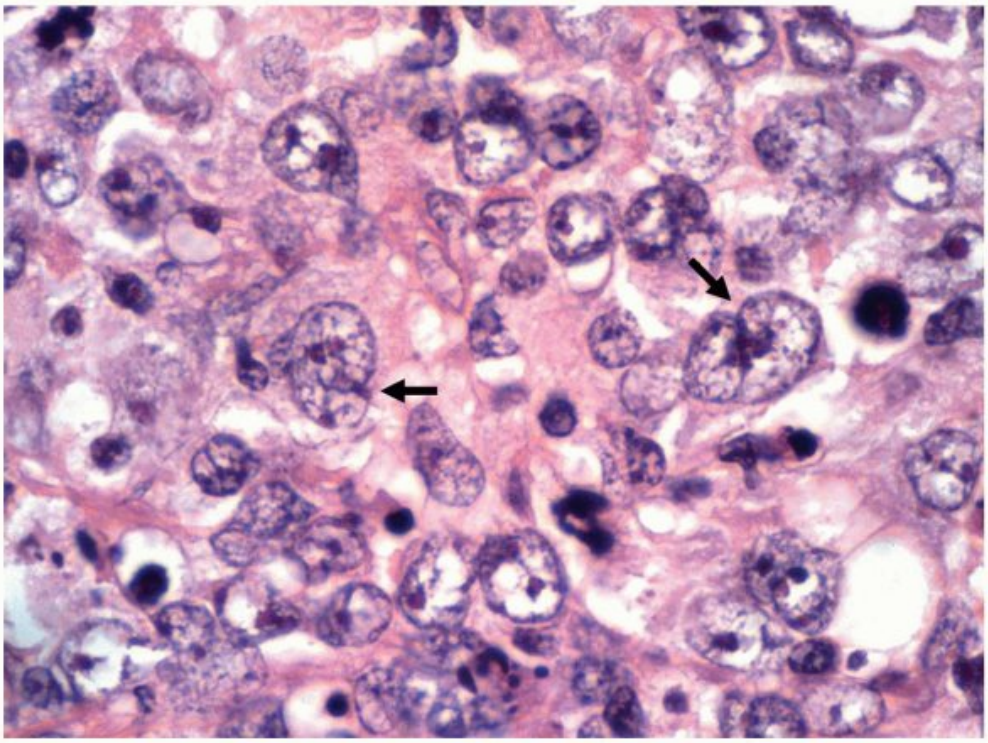

FIGURE 6.28.8 Anaplastic variant of diffuse large B-cell lymphoma in a lymph node biopsy shows large anaplastic tumor cells with vesicular nuclei and prominent nucleoli. There are two characteristic kidney-shaped nuclei in the center of this field (*arrows*). Hematoxylin and eosin, 100× magnification.

Anaplastic Variant

This variant is characterized by a proliferation of large, anaplastic, and bizarre neoplastic cells (Fig. 6.28.8) (1,5). It is similar to anaplastic large lymphoma of T-cell or null cell origin not only morphologically but also in the positive staining with CD30 antigen. However, this variant usually does not have the same molecular cytogenetic characteristics of the T-cell/null cell type of anaplastic large cell lymphoma, so they are considered biologically different. In addition, B-cell anaplastic variant is clinically more similar to other variants of DLBCL, thus it is classified under this entity. The anaplastic variant may show a cohesive growth or a sinusoidal infiltration pattern and should be distinguished from metastatic carcinoma by immunophenotyping.

There are two variants that require immunophenotyping to distinguish them from the above variants:

1. *Plasmablastic variant*: This variant is morphologically indistinguishable from the immunoblastic variant, but its clinical presentation of an oral lesion in human immunodeficiency virus (HIV)-infected patients gives the clue to this diagnosis (10,11). The immunophenotype is closer to that of plasmacytoma than to that of DLBCL (5). The tumor cells are probably in the differentiation stage between a B-immunoblast and a plasma cell. This variant can be considered an extranodal DLBCL.
2. *DLBCL with expression of full-length ALK*: This variant comprises monomorphic immunoblastlike cells but can also encompass plasmablasts in some cases (12). Histologically, a prominent sinusoidal infiltration pattern is frequently demonstrated. Its unique feature is the expression of ALK protein with granular cytoplasm and the Golgi staining pattern. However, the immunophenotype and the molecular genetic characteristics of anaplastic large cell lymphoma are not present in this variant.

TABLE 6.28.1

Characteristic Morphologic Features of DLBCL

Histologic pattern	Diffuse large cell infiltration, can be interfollicular or sinusoidal

Cytology	Centroblastic, immunoblastic, or anaplastic
Specific feature	Combination of histologic pattern and cytology

DLBCL, diffuse large B-cell lymphoma.

The characteristic morphologic features of DLBCL are summarized in Table 6.28.1.

Immunophenotype

DLBCL expresses all B-cell markers, including CD19, CD20, CD22, CD79a, and surface immunoglobulin (Ig). Cytoplasmic Ig is demonstrated in cases when plasmacytic differentiation is present. In a subset of DLBCL, CD10, bcl-2, and bcl-6 are present and make it difficult to distinguish from follicular lymphoma (13, 14 and 15). In fact, this subset of DLBCL may well be transformed from follicular lymphoma (15). In contrast, the CD5-positive cases in 10% of patients represent primary DLBCL rather than transformation from small lymphocytic lymphoma/chronic lymphocytic leukemia (16). The absence of cyclin D1 expression also distinguishes this CD5+ subset from blastoid variants of mantle cell lymphoma (1).

Besides CD5, DLBCL cells do not express T-cell markers, but the infiltrating T cells have great influence on the prognosis. The presence of >20% of infiltrating CD4+ T cells in the pretreatment biopsy is associated with longer relapse-free and overall survival (17), whereas the presence of 15% cytotoxic T cells in the biopsy is associated with a poor progression-free and overall survival time (18).

In the anaplastic variant, CD30 is positive with a membrane or cytoplasmic staining, but CD30 can also be expressed in other variants of DLBCL (19). Other activation antigens, such as CD21, CD23, CD25, CD38, and CD71, can also be shown in this variant (5).

In the T-cell-/histiocyte-rich variant, the number of tumor B cells may be too low to be detected by flow cytometry. However, one study showed that when gated at the monocyte region, small percentages (2.5% to 12%) of monoclonal B-cell populations were detected (20). Immunohistochemical staining is most helpful in detecting the small percentage of B cells that express the CD20 and CD79a (21). This technique can also demonstrate J chain (18.7%), CD45RA, and CD45RB (21). The epithelial membrane antigen (EMA) reactivity varies from 0% to 100% in different series (9,22). CD15, CD30, and vimentin are usually negative, which can help to distinguish Hodgkin lymphoma (21,23,24).

In the plasmablastic variant, the tumor cells are generally negative or weakly positive for CD20 and CD45, but are positive for CD79a and the plasma cell markers, such as VS38c, CD138, and cytoplasmic Ig (10,11). Ig heavy-chain (IgH) gene rearrangement is demonstrated in all case studied (10). The absence of mature plasma cells and of serum monoclonal protein distinguishes it from plasmacytoma.

In DLBCL with expression of full-length ALK, the ALK gene or its product can be demonstrated by immunohistochemistry, Western blotting, and polymerase chain reaction (PCR) (12). The tumor cells are negative for CD30, but positive for CD45 and EMA (12). Despite the fact that the tumor cells lack B-cell antigens (CD19, CD20, CD22, CD79a), their cytoplasm contains IgA with light-chain restriction (1).

Comparison between Flow Cytometry and Immunohistochemistry

Flow cytometric analysis usually can identify a monoclonal B-cell population so that it substantiates the diagnosis of DLBCL in conjunction with the presence of large cells in tissue sections. However, it may not detect the tumor cell population when the tumor cells are <10% in the T-cell-/histiocyte-rich variant. Immunohistochemistry, in contrast, can demonstrate the small number of tumor cells in the T-cell-/histiocyte-rich variant. In addition, immunohistochemistry is instrumental in identifying other variants, such as demonstration of CD30 in the anaplastic variant, detection of VS38c and CD38 in the plasmablastic variant, and identification of ALK in the DLBCL with expression of full-length ALK variant.

Molecular Genetics

All variants of DLBCL show somatic mutations in the variable region of the IgH gene; these mutations suggest that the DLBCL cells are derived from germinal center or postgerminal center B cells (1,5). However, IgH gene rearrangement was not demonstrated with the PCR technique in 30% to 50% of DLBCL cases in one study; the authors considered that the high number of somatic mutations might inhibit primer annealing (5). One study found that patients with IgH gene rearrangement detected in the blood or bone marrow had a lower complete remission rate and a significantly poorer overall survival than the patients with negative results (25).

Using DNA microarrays, two gene expression profiles were demonstrated by Alizadeh et al. (26): the germinal center B-cell-like subgroup and the activated B-cell-like subgroup. The former was associated with a good outcome and the latter with a poor outcome. A subsequent study by Rosenwald et al. (27) identified a third group, which did not express either set of genes from the above two groups. This study suggested that the difference in the prognosis is associated with the activity of the nuclear factor-κB signaling pathway. This pathway interferes with the apoptotic effect of chemotherapy. The germinal center B-cell-like subgroup inhibits and the activated B-cell-like subgroup enhances this pathway; thus the latter subgroup could block the apoptosis induced by chemotherapy,

leading to a poor outcome (27).

Rosenwald et al. (27) also identified t(14;18) translocation and amplification of the c-REL locus exclusively in the germinal center B-cell-like subgroup, indicating that it is a distinct disease entity. By using selective “predictive” genes, these authors further stratified four biologic groups with distinctly different prognoses.

The morphologic variants distribute variably in different gene expression profiling subgroups (27). The centroblastic monomorphic variants are seen mainly in the germinal center B-cell-like subgroup, whereas the centroblastic polymorphic and immunoblastic variants are more common in the activated B-cell-like subgroup.

The gene expression profiling subgroups are associated with different immunophenotypes (28,29). The germinal center B-cell-like subgroup expresses bcl-6+/CD10±/MUM1-/CD138- or bcl-6-/CD10+/MUM1-/CD138-. The nongerminal center B-cell-like subgroup shows bcl-6+/CD10-/MUM1+/CD138-. In another study, the activated B-cell phenotype, as defined by CD20+, cIgM+, MUM-1+, CD138±, bcl-6-, was found to be associated with the plasmablastic variant (30). This group of patients had a high frequency (85%) of tumor protein p53 (TP53) deletions, leading to a poor response to chemotherapy and short survival.

In terms of oncogenes, BCL-6 is the most common cytogenetic defect in DLBCL (5). It was initially considered specific for this lymphoma, but it was later found that this anomaly can also be demonstrated in a significant number of follicular lymphomas. Bcl-6 may be translocated with IgH, with a resultant karyotype of t(3:14)(q27;q32) or with other partner genes, such as t(3;6)(q29;p15) or t(3;22)(q27;q11) (5). Another oncogene, BCL-2, is also commonly present in DLBCL with a frequency of 20% to 30% in the form of t(14;18)(q32;q21) (31). BCL-1 aberration is absent in DLBCL. C-MYC expression is generally considered uncommon, but, with the real-time reverse transcription (RT)-PCR technique, 30% of DLBCL cases showed overexpression of C-MYC (32). C-MYC/IgH or t(8;14)(q32;q21) has been reported in occasional cases of DLBCL. For numerical aberrations, the most frequent changes are gains of X, 3q, 7, 12q, and 18q and loss of 6q and 17p (33).

Epstein-Barr virus (EBV) has been identified in as high as 60% of plasmablast variant cases by in situ hybridization for EBV-encoded RNA (EBER) (10). However, EBV is only found in small percentages of other variants.

The current case is unusual in that the patient initially presented with splenomegaly. The morphologic diagnosis was anaplastic variant of DLBCL. The immunophenotype by flow cytometry was that of a B-cell lymphoma with negative CD5 and CD10, whereas immunohistochemical study revealed the characteristic CD30 marker for the anaplastic variant. The patient was treated successfully for 5 years until he had recurrent lymphoma discovered in the lymph node. The complex cytogenetic karyotype identified in the lymph node was suggestive of a high-grade lymphoma. The patient died rapidly after the recurrence. The salient features for laboratory diagnosis of DLBCL are summarized in Table 6.28.2.

TABLE 6.28.2

Salient Features of Flow Cytometric Diagnosis of DLBCL

1. General expression of B-cell antigens: CD19, CD20, CD22, CD79a, and monoclonal surface immunoglobulin
2. A germinal center subset may express CD10, bcl-2, and bcl-6.
3. Plasmacytoid subsets may express CD138, VS38, and intracytoplasmic immunoglobulin.
4. Anaplastic subset or occasionally other subsets may express CD30.
5. Usually negative for CD5 and cyclin D1
6. Monoclonal immunoglobulin heavy-chain gene rearrangement
7. BCL-2 or BCL-6 gene rearrangement is present in certain subsets.

DLBCL, diffuse large B-cell lymphoma; CD, cluster of differentiation.

Clinical Manifestations

DLBCL is commonly seen in old people with a median age in the seventh decade, but it may be present (with a low frequency) in children (1). The characteristic clinical presentation is a rapidly growing symptomatic mass, mostly in the neck or abdomen due to lymphadenopathy (34). Systemic B symptoms, such as fever, night sweats, and weight loss of >10%, are seen in one quarter of patients, and elevated serum lactate dehydrogenase is present in one third of patients. Approximately 20% of patients have stage I or stage IE disease (35), whereas 40% of patients present with stage IV disease at diagnosis (36,37).

Extranodal dissemination of DLBCL can be seen in 40% of patients (34). The sites involved include gastrointestinal tract, genitourinary system, central nervous system, skin, liver, paranasal sinuses, endocrine glands, and others. Bone marrow involvement occurs in 10% to 20% of cases and is usually associated with later spread to the central nervous system (37,38).

Although there are sporadic reports of poor prognosis for some morphologic subtypes, the prognosis of DLBCL is generally not correlated with morphology. The international prognostic index (IPI) including age, Eastern Cooperative Oncology Group performance status, tumor stage, lactate dehydrogenase level, and the number of extranodal sites involved has proved to be valuable to predict the prognosis of DLBCL patients (27). The expression of CD138 (29) and a high proliferative index demonstrated by Ki-67 staining (1) are also predictors for a poor outcome. However, molecular genetic markers are most reliable. For instance, the BCL-2 expression and p53 overexpression are associated with poor prognosis, whereas BCL-6 translocation is associated with a favorable prognosis (1). The most powerful tool is the gene expression profiling, which stratifies patients into prognostic groups (26,27). A recent study pinpoints six genes in the gene expression profile that correlate well with the prognosis of DLBCL (39). The combination of LMO2, BCL-6, and FN1 is associated with a better prognosis, whereas that of BCL-2, SCYA3, and CCND2 is associated with worse prognosis.

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CASE 29 Mediastinal (Thymic) Large B-Cell Lymphoma

CASE HISTORY

A 16-year-old girl was admitted to the hospital because of low-grade fever, respiratory distress, and orthopnea for several days. After admission, a chest roentgenogram showed a large mediastinal mass accompanied by a left pleural effusion. Echocardiogram revealed significant compromise of venous return and decrease in myocardial contractility. These findings were consistent with the superior vena cava syndrome. An abdominal sonogram and computed tomography demonstrated multiple intrarenal lesions. The bone marrow and the central nervous system were, however, not involved. A biopsy of the mediastinal mass was taken, and a diagnosis of lymphoma was established.

She received several courses of chemotherapy and radiation therapy. After each course, the mediastinal mass shrank for a short period of time but recurred repeatedly. The patient finally underwent high-dose chemotherapy and autologous bone marrow transplant. After such treatment,

chest roentgenogram and computed tomography showed no mediastinal abnormality, and an abdominal sonogram demonstrated disappearance of the kidney lesions. One year after bone marrow transplantation, the patient showed no evidence of tumor recurrence.

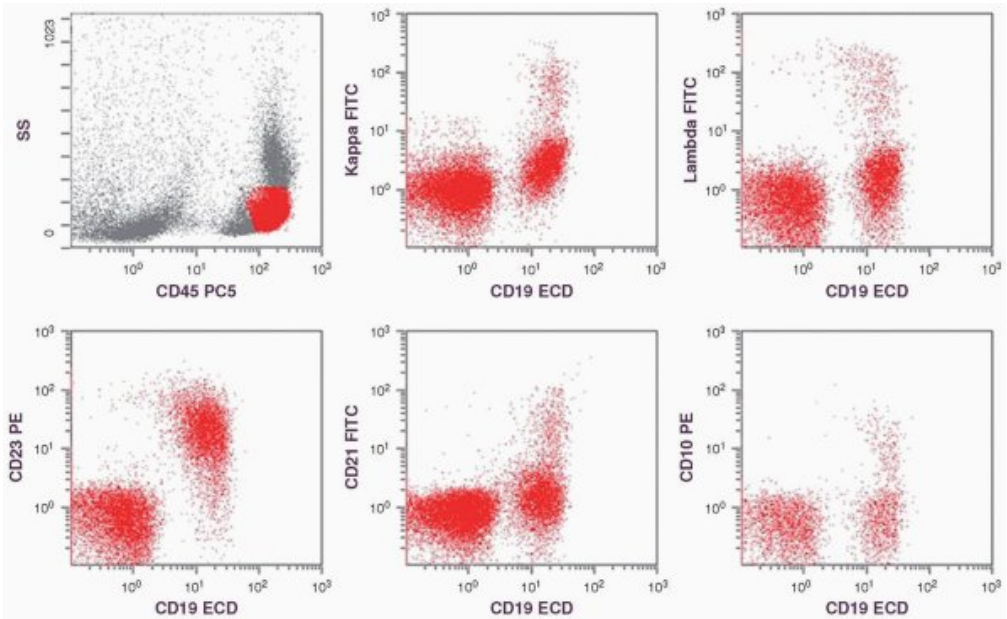

FIGURE 6.29.1 Typical flow cytometric immunophenotype shows positive reactions to CD19 and CD23, but negative reactions to κ, λ, CD21, and CD10. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; ECD, phycoerythrin-Texas Red; PE, phycoerythrin.

FLOW CYTOMETRY FINDINGS

Lymph node biopsy: Large cell gate: B-cell markers: IgG 1%, IgA 0%, IgM 2%, κ 1%, λ 1%, CD19 98%, CD20 99%, HLA-DR 100%. T-cell markers: CD3 3%, CD5 4%, CD7 2%. Monocyte markers: CD11c 13%, CD14 9% (Fig. 6.29.1 not the same case).

Small cell gate: B-cell markers: IgG 0%, IgA 0%, IgM 7%, κ 5%, λ 3%, CD19 23%, CD20 25%, HLA-DR 77%. T-cell markers: CD3 88%, CD5 92%, CD7 84%.

IMMUNOFLUORESCENT STAIN

Terminal deoxynucleotidyl transferase (TdT) was negative.

DISCUSSION

Mediastinal (thymic) large B-cell lymphoma (MLBCL) was first described by Lichtenstein et al. (1) in 1980. It is the most frequently found non-Hodgkin lymphoma in the mediastinum, mainly in the anterior mediastinum. The tumor cells are derived from the medullary B cells in the thymus. Recent evidence suggests that MLBCL is closely related to the nodular sclerosis subtype of classical Hodgkin lymphoma because of the similarity in clinical, morphologic, immunophenotypic, and genetic aspects between these two tumors. In fact, there are cases of mediastinal tumors that are intermediate between these two neoplasms and are, therefore, designated mediastinal gray zone lymphoma (2,3). MLBCL is included in the Revised European-American Lymphoma classification as a separate entity under B-cell neoplasm (4). In the World Health Organization (WHO) classification, this tumor is classified as a subtype of diffuse large B-cell lymphoma (5). Diffuse large B-cell lymphoma is composed of a heterogenous group of B-cell lymphomas with large tumor cells, but MLBCL is a distinct clinicopathological entity clearly distinguished from other large B-cell lymphomas in light of modern technologies (6).

Morphology

The tumor cells of MLBCL may show great variation both in size and in shape among different patients or within the same patient (5). However, a typical case usually shows large tumor cells with clear cytoplasm and extensive

fibrosis that compartmentalizes the tumor cells (Figs. 6.29.2 and 6.29.3) (7). Therefore, in the early literature, this tumor was described as sclerosing, large clear cell lymphoma. However, some reported cases showed medium-sized tumor cells without clear cytoplasm and/or no sclerosis (8,9). The tumor cells can also be cleaved, non-cleaved, centroblastic, or immunoblastic. As mentioned before, some tumors may be difficult to distinguish from Hodgkin lymphoma in morphology (e.g., the presence of Reed-Sternberg-like cells), and others may show an immunophenotype mimicking Hodgkin lymphoma (CD30+ CD15+) (2,3). These variations are probably due to the fact that some tumors in the mediastinum may be secondary/metastatic and that mediastinal B-cell lymphomas may be derived from different tissues, namely, the thymus and the lymph node (10). Evidence supporting the thymic origin of this tumor is based on the fact that epithelium-lined cysts, expanded thymic lobules, Hassall corpuscles, or isolated epithelial cells are found in some cases (7). With the help of the cytokeratin stain, thymic remnants or fibrous tissue reminiscent of thymus is detected in one third to one half of

specimens. The immunophenotype of this tumor is also similar to that of the medullary B cells in the thymus, which will be discussed later.

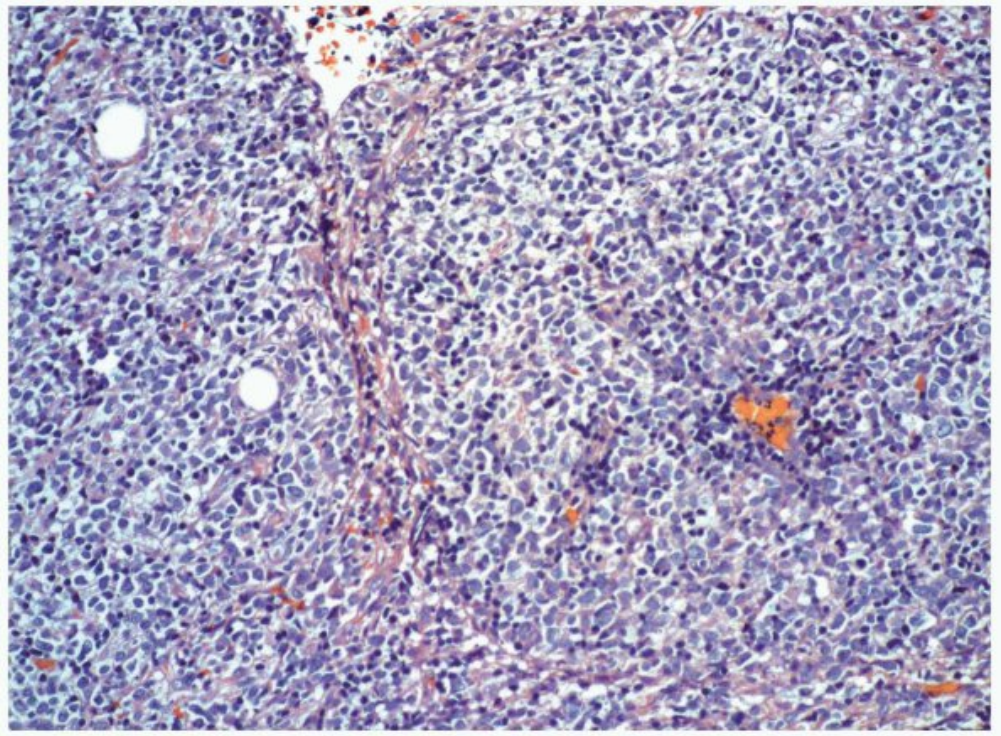

FIGURE 6.29.2 Mediastinal biopsy reveals diffuse, large clear cell infiltration separated by fibrous bands (compartmentalization). Hematoxylin and eosin, 20× magnification.

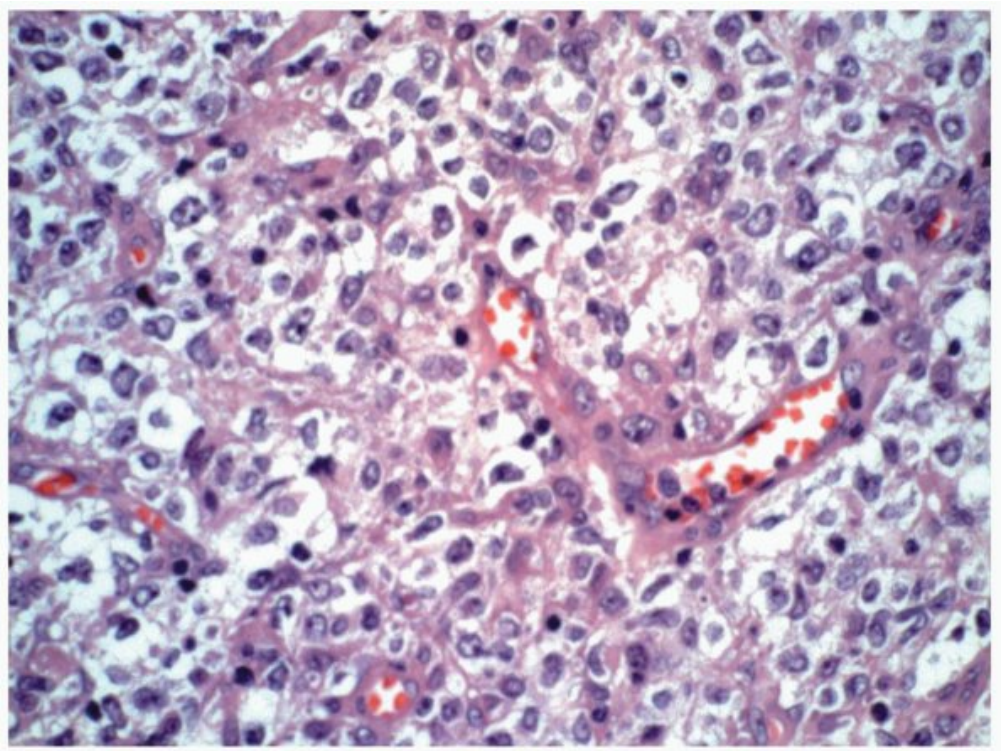

FIGURE 6.29.3 Mediastinal biopsy shows large clear cells arranged in a lobular pattern,

mimicking seminoma. Hematoxylin and eosin, 40× magnification.

TABLE 6.29.1

Characteristic Morphologic Features of Mediastinal Large B-Cell Lymphoma

Histologic pattern	Diffuse large cell infiltration encircled by fibrous bands
Cytology	Large cell with clear cytoplasm
Specific features	Bulky mediastinal tumor with the above histologic and cytologic features

The heterogeneity in morphology is also because the diagnostic criteria used in various series are different. Therefore, the Nebraska Lymphoma Study Group defined a “primary” MLBCL as “a mediastinal mass of at least 5 cm in maximum dimensions, with no extramediastinal mass larger than that in the mediastinum” (8). With this definition, most secondary tumors spread from other locations can be excluded. The diagnostic morphologic features of MLBCL are summarized in Table 6.29.1.

Immunophenotype

The most important markers for the immunophenotype are the B-cell markers. CD20 and CD19 are consistently positive either by immunohistochemistry (Fig. 6.29.4) or by flow cytometric analysis. Among other B-cell markers, CD22 and CD23 are positive in the vast majority of MLBCL, but CD10 and CD21 (C3d receptor) are negative. CD23 is

expressed in a subpopulation of large, dendritic cells (asteroid cells) among thymic B cells (11), and the absence of CD21 is also characteristic for the medullary thymic B cells (12, 13, 14 and 15). However, the hallmark of the immunophenotype in MLBCL is the absence of surface Igs (12, 13, 14 and 15).

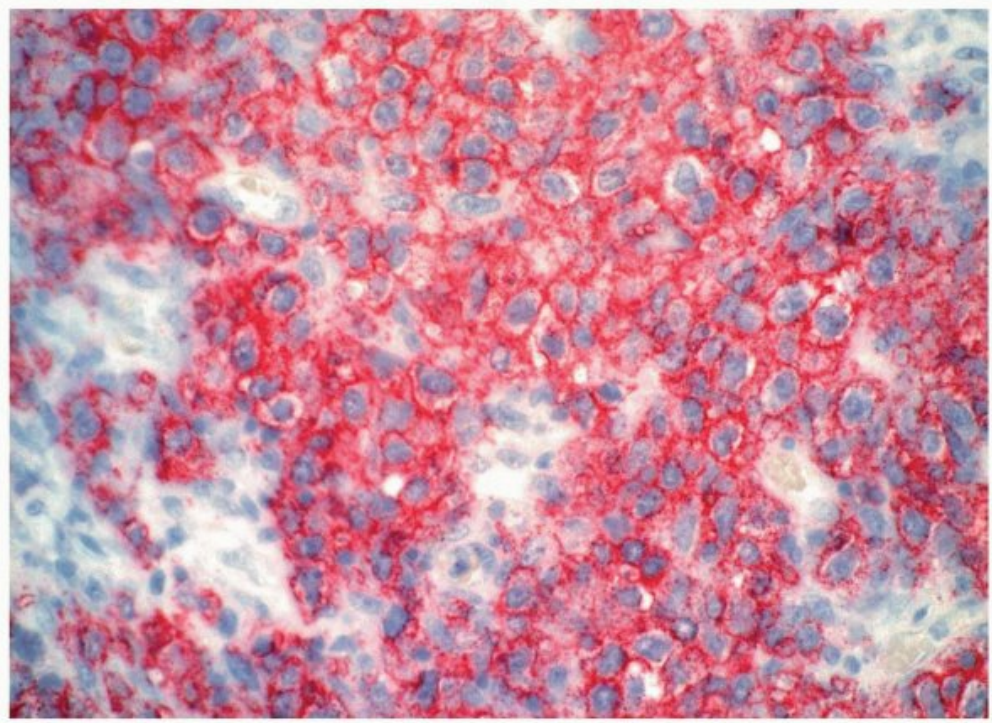

FIGURE 6.29.4 Mediastinal biopsy reveals positive CD20 staining of the tumor cells. Immunoalkaline phosphatase, 40× magnification.

Surface Ig on human B cells is noncovalently associated with a disulfide-linked heterodimer (designated CD79), which is composed of mb-1 (CD79a) and B29 (CD79b). Because the expression of surface Ig requires these polypeptide chains, the surface Ig/CD79 complex is also called the B-cell antigen receptor complex. One of the characteristic features in MLBCL is the discordant expression of Ig and its associated molecule mb-1/CD79a. In other words, CD79a is expressed despite the absence of surface Igs (12). In the same study, this immunophenotype (Ig-/CD79a+) was found in only 5 cases of follicular lymphoma among 110 cases of various types of lymphoma studied. Another discordant phenomenon is the expression of two transcription factors, Oct2 and BOB.1, which are required for germinal center formation and Ig production (2).

Other B-cell-related antigens, including bcl-2, bcl-6, HLA-DR, B-cell specific activator protein Pax5/BSAP, have been reported positive in MLBCL cases (6,16). The plasma cell-associated antigen CD38 is negative in this tumor (6). There is also a defect in the expression of HLA class I and II molecules (10,13).

Among mediastinal tumors, the differential diagnoses include lymphoblastic lymphoma, Hodgkin lymphoma, thymoma, and seminoma. Lymphoblastic lymphoma is usually of T-cell phenotype, but MLBCL is positive for B-cell markers and consistently negative for T-cell markers (CD3, CD5, CD43, CD45RO) (7,17). In addition, CD10, the common acute lymphocytic leukemia (ALL) antigen that is positive in some lymphoblastic lymphomas, is negative in MLBCL. The absence of TdT in the current case also contradicts the diagnosis of lymphoblastic lymphoma. However, TdT has not been included in the study of MLBCL in the literature.

Hodgkin lymphoma also does not express B-cell antigens except for the nodular lymphocyte predominance and the lymphocyte-rich subtypes. Some studies, however, have shown positive CD30 reactions in MLBCL cases (2,3,13,18), which may cause some confusion with Hodgkin lymphoma. However, CD45 is positive and CD15 is generally negative in MLBCL, whereas the Reed-Sternberg cells usually show the opposite reactions. Nevertheless, molecular genetic evidence shows that MLBCL and Hodgkin lymphoma may be transformable to each other, and transitional cases (mediastinal gray zone lymphoma) have been reported (2,3).

Thymoma usually expresses the lymphoblastic lymphoma phenotype, but its reaction to cytokeratin is most helpful in excluding MLBCL. The lobular pattern and large clear cells in seminoma may sometimes be mistaken as MLBCL, and human chorionic gonadotropin ß -(ß-HCG) has been detected in a case of MLBCL (19); therefore, seminoma should always be included in the differential diagnosis. However, MLBCL shows extensive B-cell staining, whereas seminoma is positive for placental alkaline phosphatase.

The current case showed typical demographic findings and characteristic histology of MLBCL. Immunophenotyping revealed the presence of B-cell antigens and absence of surface Ig, T-cell antigens, and TdT. The renal lesion might represent metastatic tumor, and kidney is a common site for MLBCL involvement. Therefore, it is a typical case of MLBCL. The diagnostic features for MLBCL are summarized in Table 6.29.2.

TABLE 6.29.2

Salient Features for Laboratory Diagnosis of Mediastinal Large B-Cell Lymphoma

1. Absence of surface immunoglobulin
2. B-cell antigen phenotype: CD10-, CD19+, CD20+, CD21-, CD22+, CD23+, CD79a+, bcl-2+, bcl-6+, HLA-DR+, Pax5/BSAP+
3. Large cell size as determined by forward light scatter
4. T-cell markers are negative.
5. Immunoglobulin gene rearrangement is present.
6. Expression of MAL gene

CD, cluster of differentiation; HLA-DR, human leukocyte antigen- DR; BSAP, B-Cell specific activator protein.

Comparison of Flow Cytometry and Immunohistochemistry

The basic immunophenotype of MLBCL is that of a B-cell antigen-positive and surface Ig-negative lymphoma. This immunophenotype can be demonstrated by both flow cytometry and immunohistochemistry. However, immunohistochemistry can correlate various markers —B-cell, surface Ig, T-cell, CD30, CD15, and placental alkaline phosphatase—with the tumor cells, so this technique is more helpful in establishing a definitive diagnosis.

Molecular Genetics

Although MLBCL shows no surface Ig, rearrangement of heavy-chain and light-chain genes has been regularly detected. This discrepancy reflects nonproductive Ig gene rearrangement (12). Multiple molecular genetic abnormalities, such as point mutation of the *c-myc* oncogene and *p53* suppressor gene, amplification of the REL proto-oncogene, and gains of 9p, 12q, and Xq have been detected, but these findings are confined to small numbers of tumors (14,16,20).

The molecular characteristics of MLBCL have been gradually established by current studies with gene expression profiling. The two major findings are (i) that MLBCL is distinctly different from other diffuse large B-cell lymphomas and (ii) that it closely resembles Hodgkin lymphomas.

The most specific molecular marker for MLBCL is the expression of the MAL gene, and its messenger RNA can be identified by Northern blot and reverse transcription-polymerase chain reaction (21,22). The MAL protein can also be detected in MLBCL cells but not in other diffuse large B-cell lymphomas. As MAL expression is restricted to a minor

subpopulation of thymic medullary B cells, these findings provide further support for the thymic B-cell origin of MLBCL (22).

In the earlier literature, the absence of BCL-6 rearrangement/mutation in MLBCL was considered a major distinction between MLBCL and other diffuse large B-cell lymphomas (6,15,23,24). However, a recent study claimed that 54% of MLBCL cases showed hypermutation of BCL-6, but the hypermutation sites and mutational spectrum of BCL-6 in MLBCL differed from those found in diffuse large B-cell lymphomas and follicular lymphoma (25). Another recent study also found that more than half of MLBCL cases displayed BCL-6 gene mutation, which usually occurred along with variable region of Ig heavy-chain (IgV_H) gene mutations or MUM1/IRF4 expression (26). Akasaka et al. reported that BCL-6 translocation involving the IgV_H gene was associated with a favorable prognosis, whereas those involving a non-Ig partner were not (27).

The absence of BCL-2 rearrangement/mutation in MLBCL is still considered an important characteristic distinguishing MLBCL from other diffuse large B-cell lymphomas (6,15,24). Paradoxically, bcl-2 protein overexpression has been demonstrated in MLBCL cases in the same frequency as in other diffuse large B-cell lymphomas (23,24).

Gene expression profiling studies have found that the signature gene expression profile of MLBCL is more closely related to classic Hodgkin lymphoma than to other diffuse large B-cell lymphomas (28). In one such study, more than one third of the genes that were more highly expressed in MLBCL cases than in other diffuse large B-cell lymphoma cases were expressed in cases of Hodgkin lymphoma (29). Other negative molecular findings in MLBCL include the absence of BCL-1 rearrangements, the *ras* oncogene, and the Epstein-Barr virus (EBV) genome (15).

Clinical Manifestations

The clinical features of MLBCL are so characteristic that they usually help the pathologists arrive at an accurate diagnosis. In a study conducted by the Non-Hodgkin's Lymphoma Classification Project, 85% of the expert pathologists made the correct diagnosis when clinical history was available, whereas only 58% of them arrived at the same diagnosis without the history (30).

The characteristic clinical history is that of a young woman (age 15 to 45 years) showing a bulky mediastinal mass (>10 cm in diameter in three fourths of patients) and pulmonary symptoms, such as cough, chest pain, and dyspnea (7,31). The tumor usually spreads locally, invading lungs, superior vena cava, pleura, pericardium, and chest wall. About one half of the patients manifest superior vena cava syndrome, and one third have pleural and pericardial effusions (17,32,33). Distant spread seldom occurs. When present, the tumor usually involves internal organs rather than lymph nodes. The most commonly involved organ is the kidney, but invasion of the liver, adrenal glands, pancreas, ovaries, and brain also has been reported (17,34,35).

Nonspecific laboratory findings include moderate to marked increase in the level of serum lactate dehydrogenase and a normal or only slightly elevated serum level of $ß_2$-microglobulin (36). As the tumor is usually bulky, the latter finding is unusual. A possible explanation is that $ß_2$-microglobulin is the light-chain component of HLA-I, which is deficient in MLBCL (7).

Therapeutic failure was frequently encountered in the early reported cases of MLBCL. However, since the current application of aggressive chemotherapy, radiation therapy, and/or bone marrow transplant, the prognosis of MLBCL has improved (7,32,33). According to the Non-Hodgkin's Lymphoma Classification Project, the 5-year overall survival is 50% and the 5-year failurefree survival is 48% (30). A recent study of 141 consecutive patients with a median follow-up of 10.9 years in the Memorial Sloan-Kettering Cancer Center showed an event-free survival and overall survival of 50% and 66%, respectively (37). Other studies revealed that the survival rates of MLBCL and

other diffuse large B-cell lymphomas were very similar, despite their cytogenetic differences (24).

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CASE 30 Burkitt Lymphoma/Leukemia

CASE HISTORY

A 79-year-old man presented to the emergency room with a 7-day history of increasing bowel distention and mild abdominal pain. His past medical history did not reveal relevant symptoms. Physical examination showed distended abdomen with signs of free fluid. Liver and spleen were not palpable. A computed tomography (CT) scan of the abdomen demonstrated free air with a scant amount of free fluid in the abdominal cavity. A paracentesis yielded 50 mL of bloody fluid, which grew *Clostridium difficile*. A Gastrografin enema demonstrated a cecal mass without extravasation of contrast.

The patient was diagnosed with perforated cecum and underwent an exploratory laparotomy 5 days after admission. A cecal mass was resected, and an ileostomy was performed. Biopsy of the cecal mass was diagnosed as a lymphoma. After the operation, the patient had persistent absence of bowel function, but a CT scan of the abdomen did not detect intra-abdominal abscess. However, the patient's clinical condition deteriorated, and he had progressive worsening of oxygenation saturation, hemodynamic instability, and respiratory distress. The patient died 2 weeks after admission.

FLOW CYTOMETRY FINDINGS

B-cell markers: CD19 73%, CD19/CD5 0%, CD20 73%, CD23 9%, CD10 72%, FMC-7 36%, κ 0%, λ 74%, CD19/κ 4%, CD19/κ 54%. T-cell markers: CD3 28%, CD5 25%, CD7 25%. Activation antigen: CD25 58% (Fig. 6.30.1).

IMMUNOHISTOCHEMICAL STAINS

The tumor cells were positive for CD20 but negative for CD3 and bcl-2. Further studies revealed that >99% of tumor cells were positive for Ki-67, and most of them were also positive for c-myc protein. Approximately 60% of tumor cells were CD10 positive and 30% were bcl-6 positive.

MOLECULAR GENETIC FINDINGS

A polymerase chain reaction (PCR) was performed on the paraffin section and showed a c-myc oncogene rearrangement.

DISCUSSION

In 1958, Burkitt first discovered this tumor in Africa (1). He characterized this tumor as a sarcoma involving the jaws of African children. Subsequently, many cases of “African lymphoma” were reported outside the African continent and caused much confusion and controversy. In 1967, the World Health Organization (WHO) formed a committee of experts to investigate this tumor, and the definition of Burkitt lymphoma (BL) was then established (2). In the Working Formulation, BL falls under the category of small noncleaved cell lymphoma, as the tumor cells resemble the small noncleaved cells in the normal germinal centers of lymphoid follicles (3). However, these tumor cells are actually of intermediate size, between the sizes of large cell lymphoma and small lymphocytic lymphoma. Because BL occurs in a certain endemic area in Africa but is sporadic outside Africa, this tumor is also divided into endemic and sporadic type (Table 6.30.1). In the Revised European-American Lymphoma (REAL) classification, this tumor is classified as BL (4). The WHO classification of lymphoid malignancies subdivides BL into endemic, sporadic, and immunodeficiency-associated subtypes (5,6). The originally proposed atypical BL subtype has changed back to Burkitt-like lymphoma (BLL), as that in the REAL classification.

Morphology

The morphologic features in the endemic, sporadic, and immunodeficiency-associated subtypes are indistinguishable (7). An earlier report described the observation of an apparent transition from reactive follicles to follicular and diffuse BL in the sporadic form, but not in the endemic form (8). This difference, however, is not recognized in current studies.

The tumor cells are of medium size, approximately the same as those of the tingible-body macrophages invariably present in BL (Table 6.30.2). In tissue sections, the nuclear chromatin appears coarsely clumped with two to four small nucleoli and a thin rim of cytoplasm. The histologic features show monotonous cohesive sheets of tumor cells with multiple mitotic figures and apoptotic tumor cells. The apoptotic bodies are frequently ingested by phagocytic cells, which are commonly referred to as tingible-body macrophages. The scattered tingible-body macrophages impart the so-called “starry sky” pattern, which is characteristic of but not pathognomonic for BL (Fig. 6.30.2). This feature can also be seen in lymphoblastic lymphoma, immunoblastic lymphoma, and occasionally other non-Hodgkin lymphomas. Therefore, using a touch preparation to identify the cytologic features of the tumor cells is particularly helpful for the diagnosis.

In touch preparations or bone marrow aspirates, the tumor cells are characterized by the moderate amount of deep blue cytoplasm containing multiple cytoplasmic vacuoles (Figs. 6.30.3 and 6.30.4). The cytoplasm stains for

methyl green pyronin (Fig. 6.30.5) due to its high content of polyribosome, whereas the lipid contents of cytoplasmic vacuoles are positive for Oil Red O (Fig. 6.30.6). The vacuoles are negative for periodic acid-Schiff (PAS), indicating the absence of glycoprotein or glycogen. The nuclei are regular, round, or oval with distinct nuclear membrane and more dispersed chromatin pattern than what is perceived in tissue sections (9).

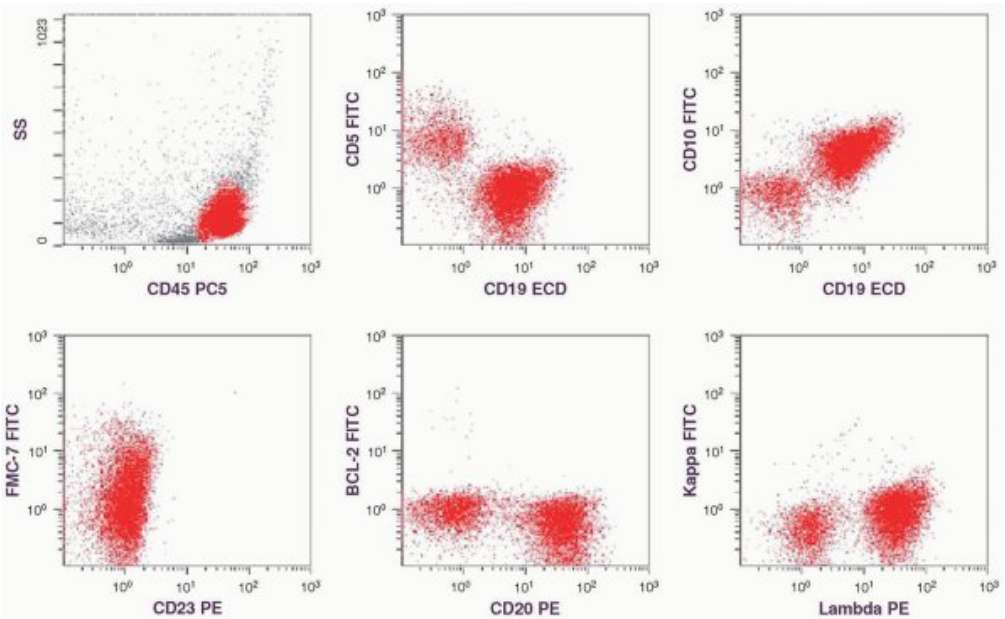

FIGURE 6.30.1 Flow cytometric analysis shows positive CD19, CD20, CD10, and FMC-7 in a monoclonal λ population. The negative bcl-2 reaction helps to distinguish Burkitt lymphoma from follicular lymphoma and diffuse large B-cell lymphoma. SS, side scatter; PC5, phycoerythrin-cyanin 5; ECD, phycoerythrin-Texas Red; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

TABLE 6.30.1

Comparison of Endemic and Sporadic BL

	Endemic Subtype	*Sporadic Subtype*
Annual incidence per 100,000 population	2.3-3.8	0.1-0.3
Age group with high incidence	4-8 years	Bimodal
Male/female ratio	2.1:3.1	2.31:3.71
Initial presentation	Jaw lesion	Abdominal tumor
Bone marrow involvement	About 8%	16%-20%

Central nervous system involvement	About 30%	5%-20%
Leukemic form	Absent	Present
Positive serologic test for EBV	88%-97%	20%
EBV receptor on tumor cells	Common	Rare
EBV genome in tumor cells	100%	11%-20%

BL, Burkitt lymphoma; EBV, Epstein-Barr virus.

TABLE 6.30.2

Characteristic Morphologic Features in BL	
Histologic pattern	Monotonous cohesive sheets of tumor cells with multiple mitotic figures and scattered tingible-body macrophages (starry sky pattern)
Cytology	Medium-sized cells with immature chromatin pattern; blue, vacuolated cytoplasm on Wright-Giemsa-stained preparations
Specific features	Starry sky pattern with characteristic cytology on touch preparations

BL, Burkitt lymphoma.

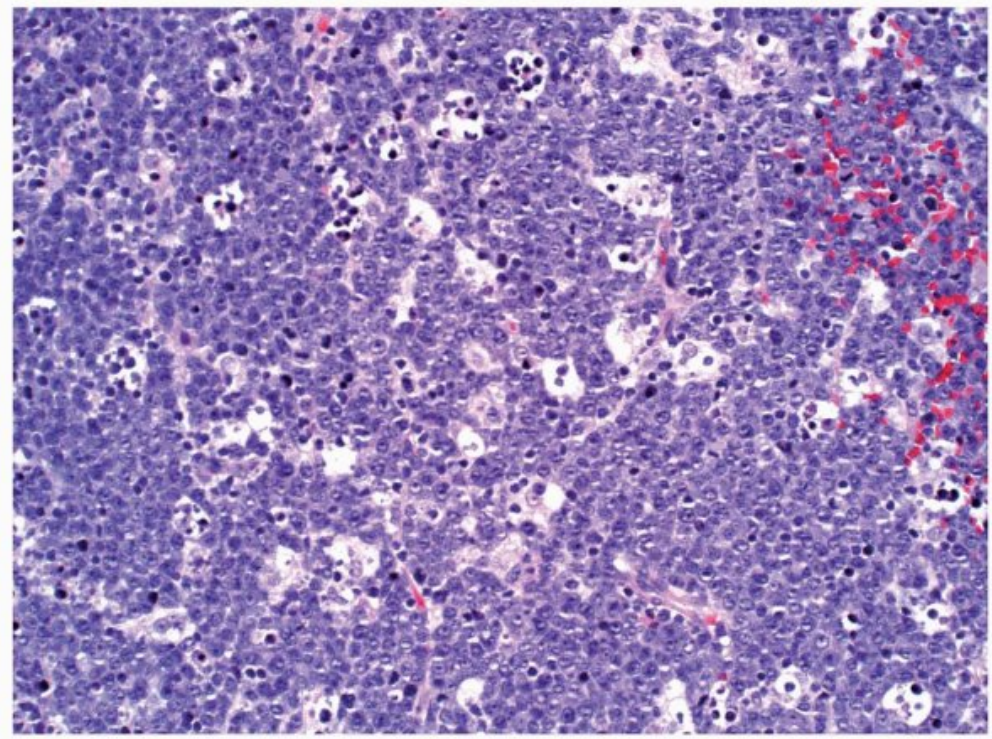

FIGURE 6.30.2 Histology of a lymph node biopsy is characterized by the prominent starry sky pattern due to the presence of multiple tingible-body macrophages. Hematoxylin and eosin, 20× magnification.

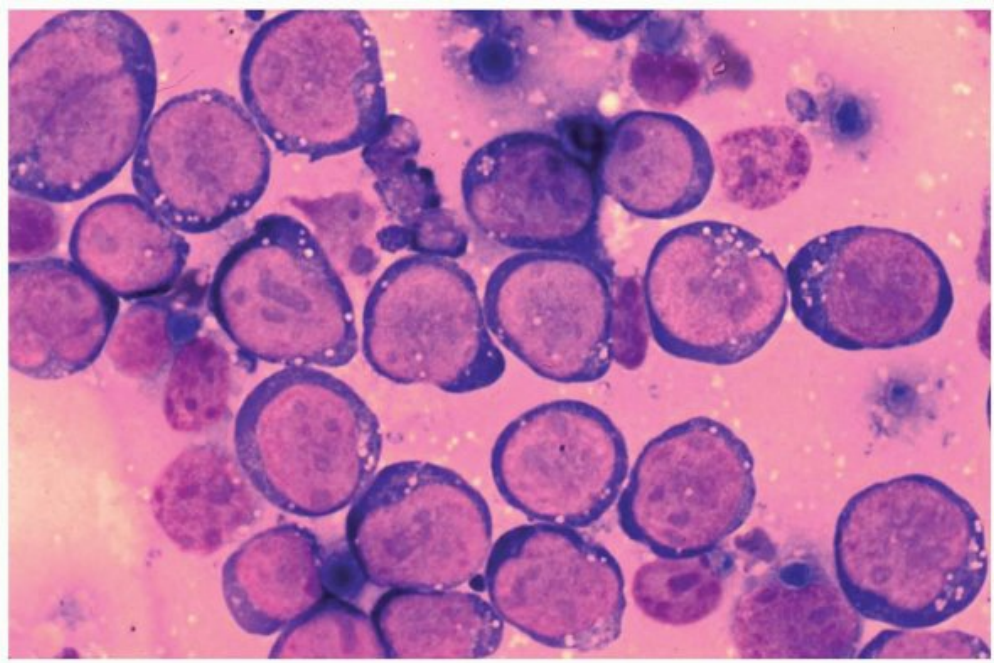

FIGURE 6.30.3 Touch imprint from a lymph node reveals the characteristic cytology of deep basophilic cytoplasm with vacuolization, and immature chromatin pattern with inconspicuous multiple nucleoli. Wright-Giemsa, 150× magnification.

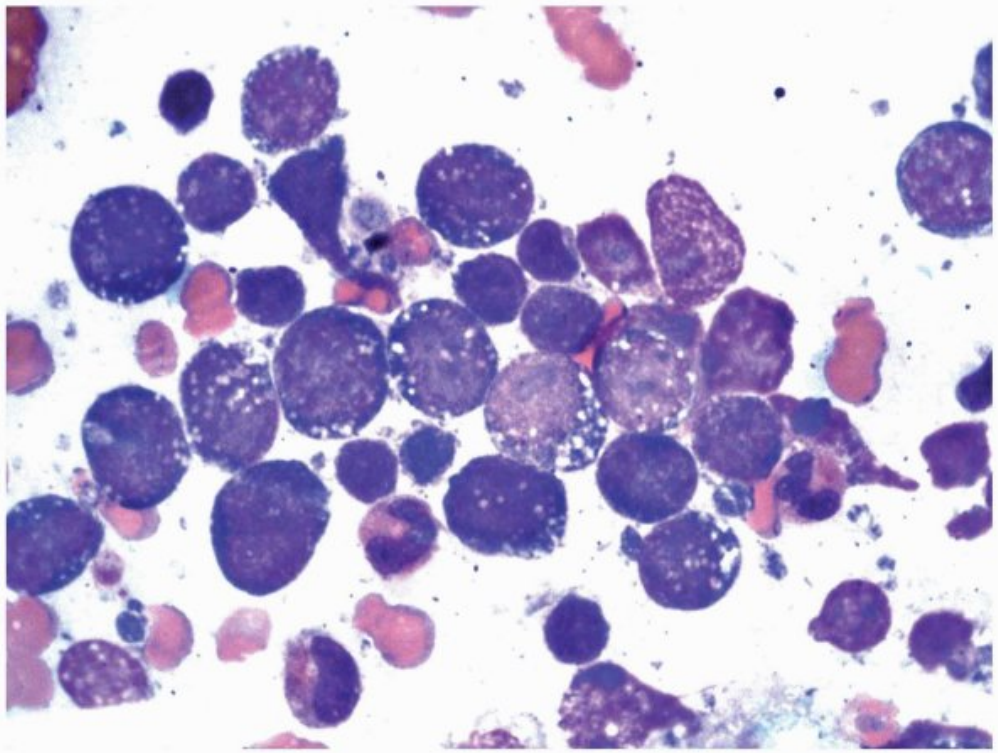

FIGURE 6.30.4 Bone marrow aspirate shows a cluster of Burkitt lymphoma cells with the same characteristics as those seen in the lymph node imprint. Wright-Giemsa, 100× magnification.

Lymphomas with a morphology between that of BL and that of diffuse large B-cell lymphoma are called non-BL in the Working Formulation (3) and BLL in the REAL (4) and WHO (5,6) classifications. To identify BLL and distinguish it from diffuse large B-cell lymphoma is important clinically because the treatments of these two tumors are quite different. The major morphologic differences between BL and BLL are the presence of pleomorphic nuclei in BLL that are irregular in configuration and vary in size, shape, and number of nuclei (varying from one to three) in each cell (Table 6.30.3, Fig. 6.30.7). The

nucleoli in BLL cells are usually more prominent than BL cells, and are frequently eosinophilic (7). One study found that the most distinguished morphologic feature in BLL is the presence of a single prominent nucleolus (10) (Fig. 6.30.8). Another study showed that the pleomorphic features in BLL are also due to the existence of various cell populations (11). In the 19 cases studied, the tumor cells were composed of at least 10% of Burkitt cells and various proportions of centroblasts and immunoblasts with plasmacytic differentiation. BL with plasmacytoid differentiation is classified as a variant of BL and is separated from BLL in the WHO classification (5). BL and BLL also differ in their molecular cytogenetic feature, which will be discussed later.

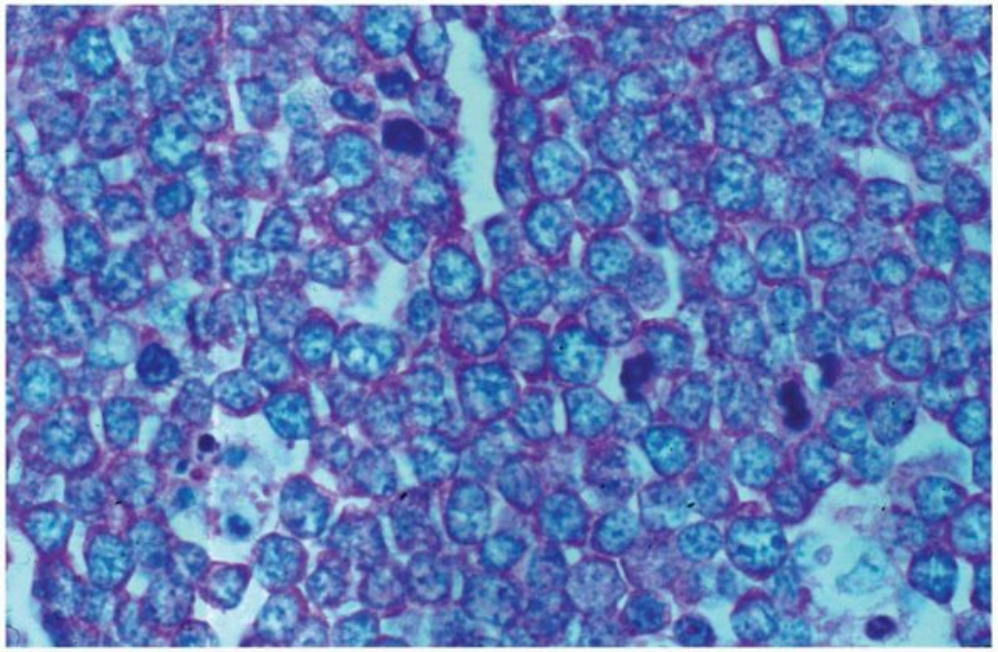

FIGURE 6.30.5 Lymph node biopsy shows positive methyl green pyronin stain in tumor cells. 60× magnification.

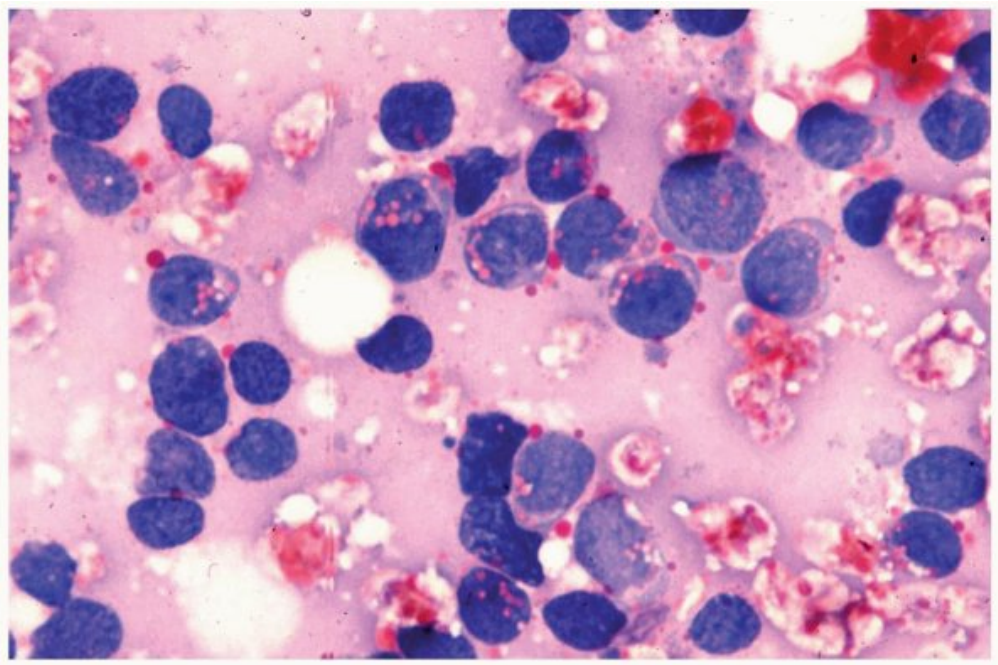

FIGURE 6.30.6 Lymph node imprint reveals positive Oil Red O stain of the cytoplasmic vacuoles of tumor cells. 100× magnification.

TABLE 6.30.3

Comparison of Burkitt Lymphoma (BL) and Burkitt-Like Lymphoma (BLL)

	BL	*BLL*
Nuclei	Uniform, round	Variable size, irregular shape
Multinucleation	Absent	Present
Nucleoli	Small, 2-5, basophilic	Larger, 1-2, eosinophilic
Cytoplasm	Deep basophilic with vacuoles	Light basophilic with vacuoles
Age	Mainly in children	Mainly in adults
Sex ratio	Male predominant	Nearly equal
Extranodal presentation	Frequent	Less frequent
Abdominal involvement	Frequent	Frequent
Bone marrow involvement	Rare	More frequent
Oncogene involved	c-myc	c-myc or bcl-2
Stages III and IV	About 2/3 patients	About 4/5 patients
Median survival	<1 year	<1 year
Five-year survival*	About 42%	About 11%

* The current survival rate is much higher.

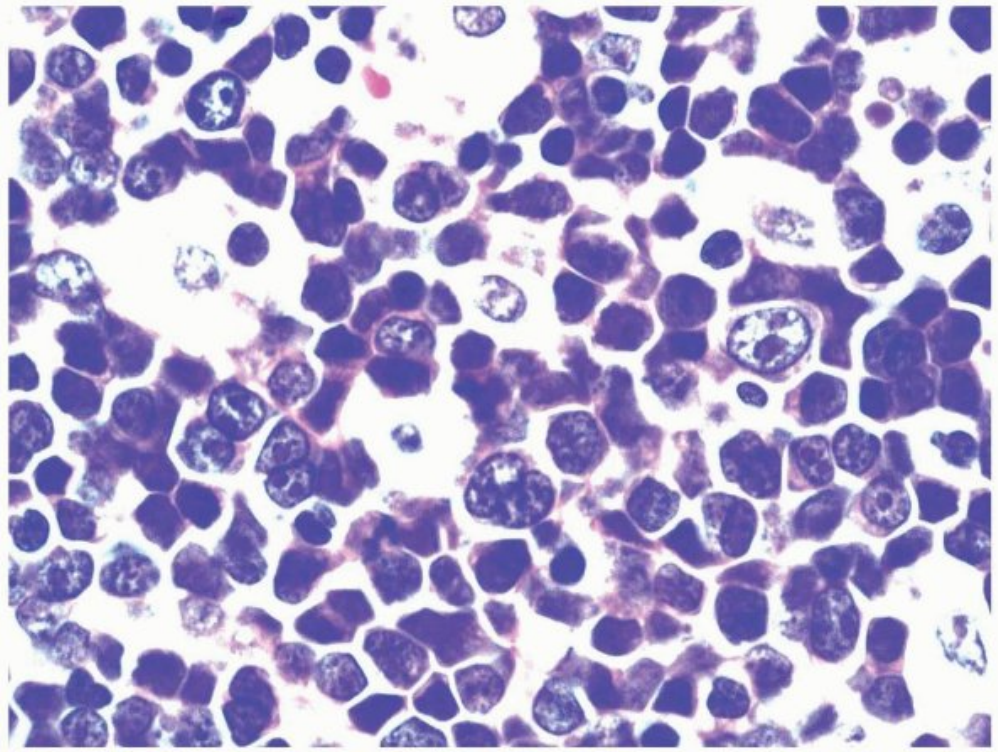

FIGURE 6.30.7 Lymph node biopsy from a case of Burkitt-like lymphoma shows pleomorphic morphology with a few multinucleated cells. Note the tingible-body macrophages. Hematoxylin and eosin, 100× magnification.

Immunophenotype

BL is a tumor of follicular center cell origin. Therefore, the immunophenotype of BL is similar to that of follicular lymphoma. Besides B-cell markers (i.e., CD19, CD20, CD79a, and monoclonal surface immunoglobulin [Ig]), BL also expresses other markers that are specific for follicular lymphoma (i.e., CD10 and bcl-6) (5,12). However, bcl-2 is negative for BL, so this marker may help to distinguish these two tumors (5,12).

Some cases of diffuse large B-cell lymphoma, particularly those transformed from follicular lymphoma, may also share the same immunophenotype with follicular

lymphoma (12,13). These cases are especially difficult to distinguish from BLL. A high proliferation fraction (Ki-67 >99%) is the only acceptable marker for the diagnosis of BL by the WHO without cytogenetic evidence (5) (Fig. 6.30.9). However, some diffuse large B-cell lymphomas may also have a very high percentage of Ki-67. In those cases, the morphology determines the diagnosis. A recent study showed that c-myc protein was demonstrated in 91% of BL cases, but only 25% of diffuse large B-cell lymphoma cases, so this marker can be helpful for differential diagnosis (12).

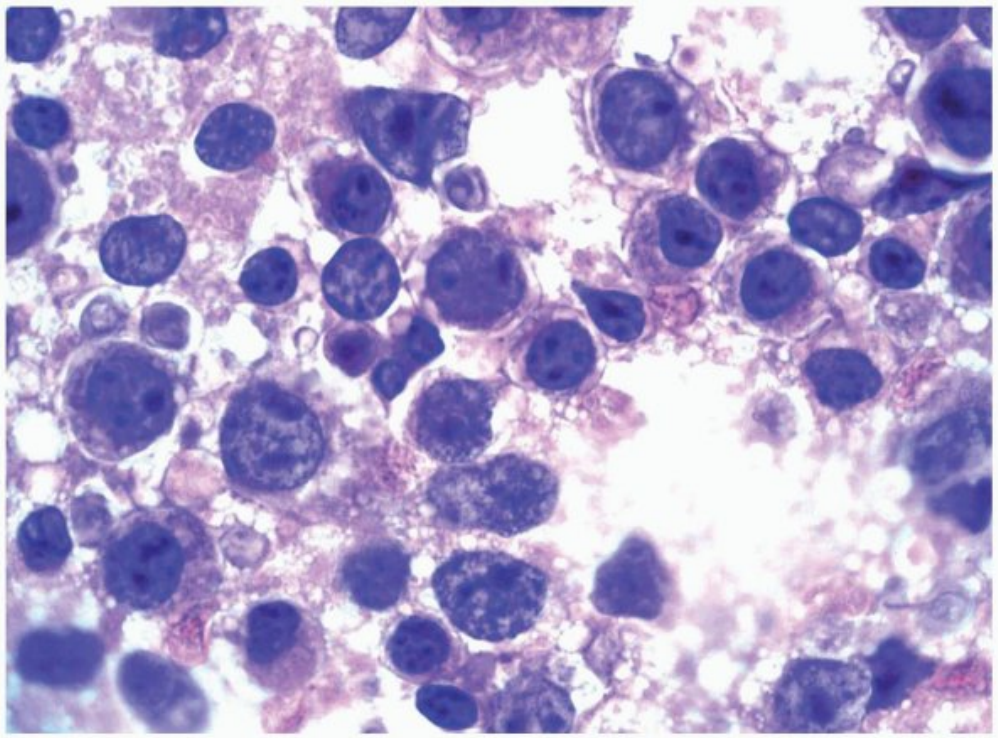

FIGURE 6.30.8 Fine-needle aspirate from a case of Burkitt-like lymphoma reveals prominent single nucleolus in many tumor cells. Hematoxylin and eosin, 100× magnification.

The reactivities of other B-cell markers included 28% for CD21, 66% for CD22, and 75% for FMC-7 in one study (14). CD21 is the Epstein-Barr virus (EBV)/C3d receptor, so its expression depends on the EBV status of the tumor. Therefore, the endemic BL cases express CD21, but the majority of sporadic BL cases are CD21 negative (5).

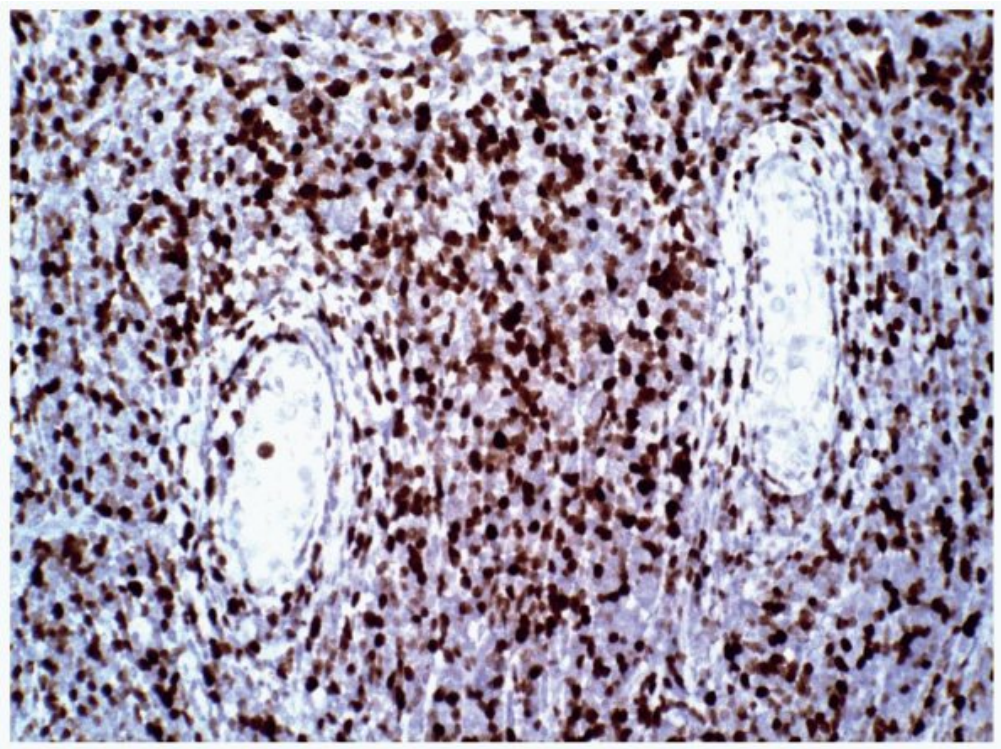

FIGURE 6.30.9 Testicular Burkitt lymphoma shows 100% proliferation fraction as demonstrated

by Ki-67 staining. 20× magnification.

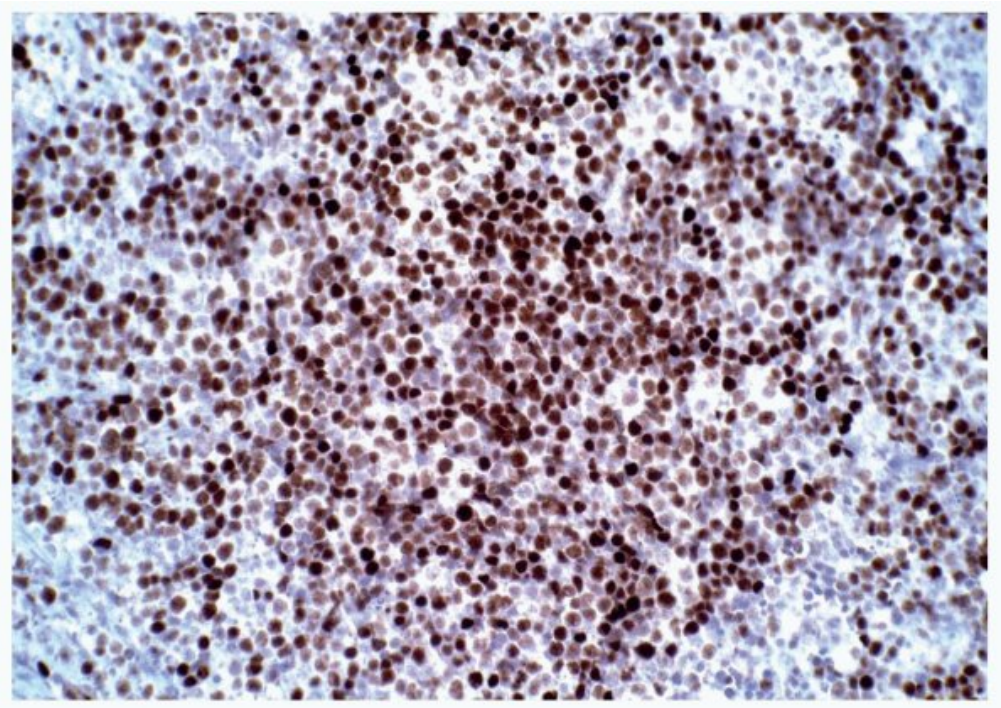

FIGURE 6.30.10 Lymph node biopsy shows bcl-6-positive tumor cells. 20× magnification.

In a comparison of 19 cases of BLL and 25 cases of BL in human immunodeficiency virus (HIV)-infected patients, BLL cases were found to have statistically higher percentages of activation antigens (CD39 and CD70) and of the CD11a/lymphocyte function-associated antigen (LFA)-1 adhesion molecule than the BL cases (11). Another study revealed that BL contains higher percentages of bcl-6 (Fig. 6.30.10) and c-myc (Fig. 6.30.11) but a lower percentage of bcl-2 protein than BLL does (15). The immunodeficiency-associated BL is also specific in the expression of CD45RO, (a T-cell marker) in tumor cells (16).

The sporadic subtype but not the endemic subtype of BL may have a leukemic phase, which is identical to the L3 type of acute lymphoblastic leukemia. L3 can be distinguished from L1 and L2 by the presence of surface Ig and the absence of terminal deoxynucleotidyl transferase (TdT) and CD34 (5).

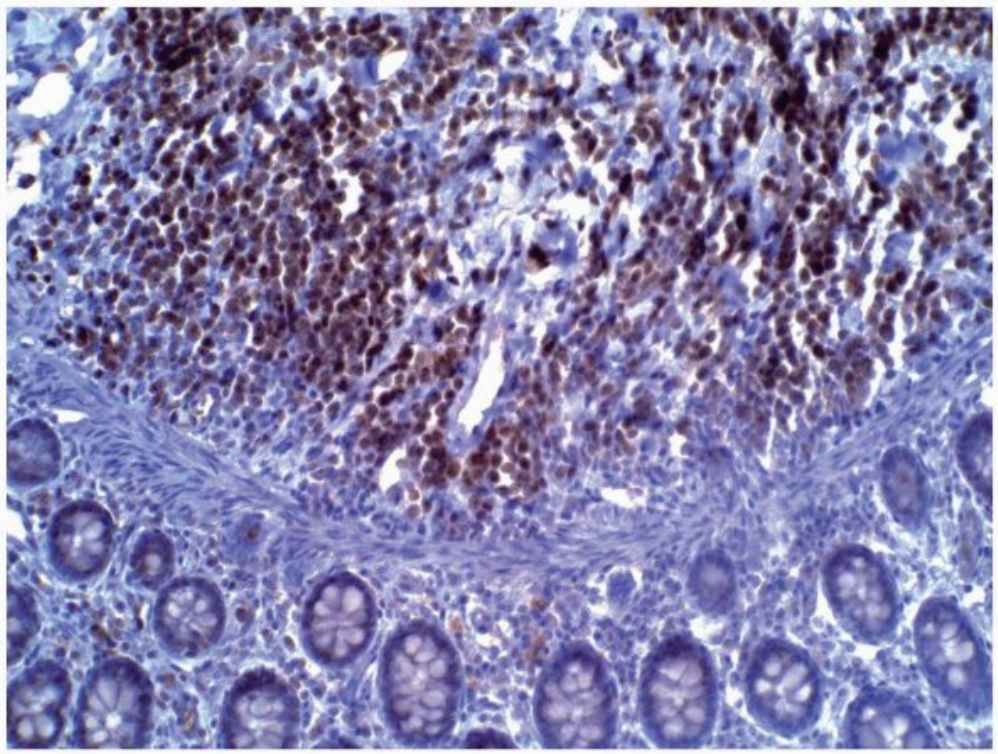

FIGURE 6.30.11 Colon biopsy reveals positive c-myc protein staining in tumor cells in the submucosal region. 20× magnification.

Four cases of de novo CD5-positive BL/leukemia have been reported (17). These cases showed dual CD5/CD19 or CD5/CD20 with positive FMC-7 and negative CD23 and CD10; their immunophenotype mimics that of mantle cell lymphoma. The folded nuclei in the blasts are another feature similar to the blastoid form of mantle cell lymphoma. In these cases, only molecular genetics can accurately identify the tumor as BL and exclude mantle cell lymphoma.

Comparison of Flow Cytometry and Immunohistochemistry

For the diagnosis of BL, immunohistochemistry is superior to flow cytometry because flow cytometry can only demonstrate CD10 and bcl-2 for a specific diagnosis. Additional markers, such as bcl-6, c-myc, and Ki-67, can be demonstrated by immunohistochemistry and make the diagnosis more reliable.

Molecular Genetics

Because of the similarity between BL and other lymphomas in immunophenotype and/or morphology, the WHO classification does not accept immunophenotyping as definitive evidence for diagnosis, and requires molecular cytogenetic documentation to establish the final diagnosis. Rearrangement or translocation of the c-myc oncogene is the hallmark of BL. In 80% of BL cases, the abnormality is t(8;14)(q24;q32), representing the translocation of the c-myc oncogene on chromosome 8 to juxtaposing the heavy-chain gene on chromosome 14 (18). The remaining cases involve the κ (15%) or λ (5%) light-chain gene, showing t(2;8) (p11;q24) or t(8;22)(q24;q11), respectively.

This translocation results in deregulation of the c-myc gene, which leads the tumor cells remaining constantly in the cell cycle (19). However, c-myc gene translocation can also be seen in other lymphomas and/or leukemias, such as diffuse large B-cell lymphoma, transformed follicular lymphoma, blastoid mantle cell lymphoma, plasma cell myeloma, lymphoblastic lymphoma, Richter's transformation, and prolymphocytoid transformation from chronic lymphocytic leukemia (20, 21, 22, 23, 24 and 25).

These cytogenetic abnormalities can now be detected not only by karyotyping but by Southern blotting, PCR, and fluorescence in situ hybridization (26).

Although endemic and sporadic BL and BLL all carry the same cytogenetic translocation (20), the breakpoints are different in various tumors (8). In sporadic BL, the breakpoint in chromosome 8 is often within the c-myc gene, whereas the breakpoint in 75% of endemic BL is upstream of the c-myc gene (27). In contrast, the breakpoint on chromosome 14 involves the heavy-chain joining region in endemic BL, whereas sporadic BL involves the heavy-chain switch region (28,29).

In South America, the predominant breakpoints in BL cells differ from those of sporadic and endemic BL cells (30). Furthermore, the

breakpoints of the BLL cells may lie far from the c-myc gene (31). In some studies, c-myc rearrangement was demonstrated in only a small percentage (32) or in none (33) of the BLL cases studied.

Another striking early discovery is the association of BL with EBV, a relationship that has now been found in many lymphoid tumors (11). EBV has been isolated from virtually all tumor samples from Africa. EBV receptor has also been found in most of the BL cell lines originating from Africa. In contrast, sporadic BLs do not usually carry EBV and EBV receptor, with a frequency of only 11% to 20% (18). EBV is identified in only 25% to 40% of immunodeficiency-associated cases (5). EBV antibodies are detected in 88% to 97% of patients in the endemic area but in only 20% of patients in the nonendemic area (34).

Conflicting results are demonstrated in studies of the association between EBV and BLL. One study showed that EBV DNA was detected by in situ hybridization and Southern blot techniques in 79% of BLL cases but in only 48% of sporadic BL cases (11). Another study failed to demonstrate EBV DNA by PCR in all 12 BLL cases (15). EBV is also more commonly seen in HIV-positive than HIV-negative BL cases (35). Because disrupted and aberrant expressions of the viral genome have recently been found in cases of sporadic BL in the United States that were interpreted as EBV-negative in standard screening, the high negative rate in sporadic BL or BLL may be due to the low sensitivity of the test (36).

Another caution is that the results obtained from BL-derived cell lines may differ from those of primary tumor tissue for molecular analysis of EBV genomes (37). A study of EBV-BL association in Taiwan found that EBV-encoded small RNAs were seen mostly in head and neck BL, but seldom in abdominal BL (38). This finding may partly explain the difference in EBV positivity between the endemic and sporadic BLs.

The etiologic role of EBV in BL, however, is controversial. The demonstration of monoclonality of EBV infection in BL by molecular analysis of EBV terminal repeats supports this assertion (39). However, because BL cells failed to express the EBV-encoded antigens LMP-1 (latent membrane protein-1) and EBNA-2 (EBV nuclear antigen-2) in tumor cells in several studies (40,41), the etiologic role of EBV is challenged. In fact, the predisposing factors of BL may be multiple, including bacterial, viral (EBV, HIV), and parasitic (malaria), leading to defective T-cell regulation of EBV-infected B cells (5).

Another controversial issue concerns bcl-2 rearrangement. Yano et al. (33) found that 3 of 11 BLLs showed bcl-2 rearrangement with comigration of the heavy-chain gene, indicating t(14;18). The rearrangement of c-myc was not detected in any of these cases. In another study, the bcl-2 gene was not demonstrated in 12 cases of acquired immunodeficiency-associated BLL (11). However, a recent study found a high frequency of t(14;18) in Burkitt leukemia (L3) (42).

BL and BLL may also show p53 protein overexpression or p53 mutation (43,44). Inactivation of wild-type p53 function can render the BL cell line significantly more tumorigenic in athymic mice (45). The Rb2/p130 gene is also mutated in most cases of endemic BL but in fewer cases of sporadic BL (46).

In terms of distinction between BL and diffuse large B-cell lymphoma, two recent studies with gene expression profiling (GEP) showed that even cytogenetics was not entirely reliable. The former may or may not have c-myc but may have bcl-2 rearrangement, and the latter may

have c-myc gene rearrangement (47,48). However, they have distinctly different signatures in GEP.

TABLE 6.30.4

Salient Features for Laboratory Diagnosis of Burkitt Lymphoma

- Monoclonal surface immunoglobulin pattern
- Positive B-cell antigens: CD19, CD20, CD22, CD79a
- Specific markers: Ki-67 (>99% cells), CD10, bcl-6, c-myc protein, CD21 (in endemic type)
- Negative markers: CD5, CD23, bcl-2, TdT
- Cytogenetic karyotypes: t(8;14), t(8;22), t(2;8)
- Molecular markers: c-myc/IgH, c-myc/IgK, c-myc/IgL translocations

- EBV genome: Present in 100% endemic subtype, 11%-20% sporadic subtype, 20%-40% immunodeficiency-associated subtype

EBV, Epstein-Barr virus; CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase; Ig, immunoglobulin.

The current patient is a typical case of sporadic BL showing an ileocecal location and a rapidly progressive clinical course. The biopsy of the cecal mass showed characteristic features of "Burkitt cells" and, interestingly, a starry sky pattern, even in the wall of the cecum. All layers of the cecum were involved. Peritonitis was secondary to the perforation of the colon due to tumor cell infiltration; this further accelerated the demise of the patient.

The salient features for laboratory diagnosis of BL are summarized in Table 6.30.4.

Clinical Manifestation

The annual incidence of BL in the endemic area used to be about 20 to 40 times higher than that in nonendemic areas (49). In Africa, BL is seen mostly in boys, and its initial presentation is frequently a jaw lesion (50). The sporadic type has a bimodal age distribution. The first peak is in children and young adults, with a later peak in elderly individuals (51). The initial presentation in this type is frequently an abdominal tumor (70% to 90%) (49). However, the demographic characteristics in Africa are changing, with a decrease in new cases, an increase in patient age, and an increase in the number of abdominal tumors (36). In the Middle East, North Africa, and South America, BL cases exhibit intermediate features of the disease: Some features are similar to African BL, and other features mimic American BL (36). The Far East (Hong Kong and Japan) shows its own forms of BL, distinguishing it from the BL of other areas.

After the outbreak of acquired immunodeficiency syndrome (AIDS), the incidence of BL increased dramatically in the nonendemic area. In HIV-infected patients, its incidence is about 35% to 40% of all cases of malignant lymphoma (52,53), compared with 1% to 2% in the general population (3). Burkitt leukemia (or the L3 type of acute lymphoblastic leukemia) has also been encountered in HIV-infected patients (54). Although BL is seldom seen in other immunodeficiency states (5), BL from HIV-infected patients is designated generally as immunodeficiency-associated BL. This clinical subtype shares clinicopathologic features with those cases without HIV infection (35).

Besides the initial presentation, the endemic and sporadic BLs also differ in the distribution of pathologic lesions. Bone marrow involvement is rarer, but central nervous system involvement is more common in the endemic than in the sporadic subtype (55). A leukemic presentation of Burkitt tumor is specific for the sporadic subtype (56). The sporadic form also frequently involves the testis (Fig. 6.30.9), ovary, pleura, cerebrospinal fluid, peripheral lymph nodes, and pharynx in 10% to 20% of patients (7). Sporadic BL may also be presented in the form of primary effusion lymphoma (57).

BLL is more frequently seen in adults, with no sexual predilection and less frequent extranodal but more frequent bone marrow involvement than BL. In both BLL and BL, the stage at presentation is usually III or IV, and the median survival is within 1 year (58,59). However, the 5-year survival rate of BL is higher than that of BLL from most studies (59). In BL cases, the HIV-infected patient has a significantly shorter overall survival than the patient without HIV infection (35). With the current improvement in the treatment of BL, the prognosis in pediatric cases is excellent. In the early stages of the disease (stages I and II), the event-free survival rate ranges from 85% to 100%, whereas in the advanced stages (III and IV), the survival rate is 75% to 85% (60).

BL is a highly aggressive tumor, probably due to its short doubling time of 24 hours. Therefore, prompt diagnosis and immediate treatment is required. The therapeutic strategy is a very intensive chemotherapy applied within a short duration. With this new approach, even patients with advanced stage BL are curable (61).

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CASE 31 T-Cell Large Granular Lymphocyte Leukemia

CASE HISTORY

A 77-year-old man with a history of cyclic neutropenia was admitted to the hospital because of worsening neutropenia. The patient was found to have pancytopenia 9 years prior to the current admission during an investigation of his cardiac problem. At that time, his total leukocyte count was 3,900/μL with 73% lymphocytes and 11% neutrophils. The hematocrit was 38.7%, hemoglobin 13.3 g/dL, and platelets 93,000/μL. The patient had not been treated despite cyclic drops of all cell counts. He had no major infections except for diverticulitis. His arthritis was considered nonspecific.

On admission, no lymphadenopathy or hepatosplenomegaly was found. An automated complete blood count revealed a total leukocyte count of 1,900/μL with 5.4% neutrophils and 72% lymphocytes. His hematocrit was 38%, hemoglobin 13 g/dL, and platelets 130,000/μL. Moderate numbers of large granular lymphocytes were found on the peripheral blood smear.

A bone marrow biopsy was performed and showed a marked decrease of myeloid cells with a myeloid to erythroid precursor (M/E) ratio of 0.7:1. The differential count revealed marked maturation arrest at the myelocytic stage (14.5% myelocytes, 0.5% metamyelocytes, 1% bands, and 2% segmented neutrophils) and an increase in lymphocytes (36%). A small number of large granular lymphocytes was also identified. Multiple lymphoid aggregates as well as interstitial lymphoid infiltration were found in the core biopsy.

The patient was treated with methotrexate and prednisone. At the time of discharge, the total leukocyte count was 2,400/μL with 43% neutrophils, 30% lymphocytes, and 25% monocytes. The hematocrit was 39.2%, hemoglobin 13.5 g/dL, and platelets 247,000/μL. The patient was treated with the same regimen continuously on an outpatient basis.

FLOW CYTOMETRY FINDINGS

The bone marrow biopsy revealed 98% CD2, 97% CD3, 96% CD5, 23% CD3/CD4, 81% CD3/CD8, 3% CD19, 6% CD20, 5% κ, 4% λ, 35% CD16, 51% CD3/CD57, and 2% CD3/CD56 (Fig. 6.31.1).

MOLECULAR GENETIC FINDINGS

T-cell receptor gene rearrangement analysis of the bone marrow showed T-cell receptor ß-chain gene rearrangement.

Cytogenetic study revealed a normal male karyotype of 46, XY.

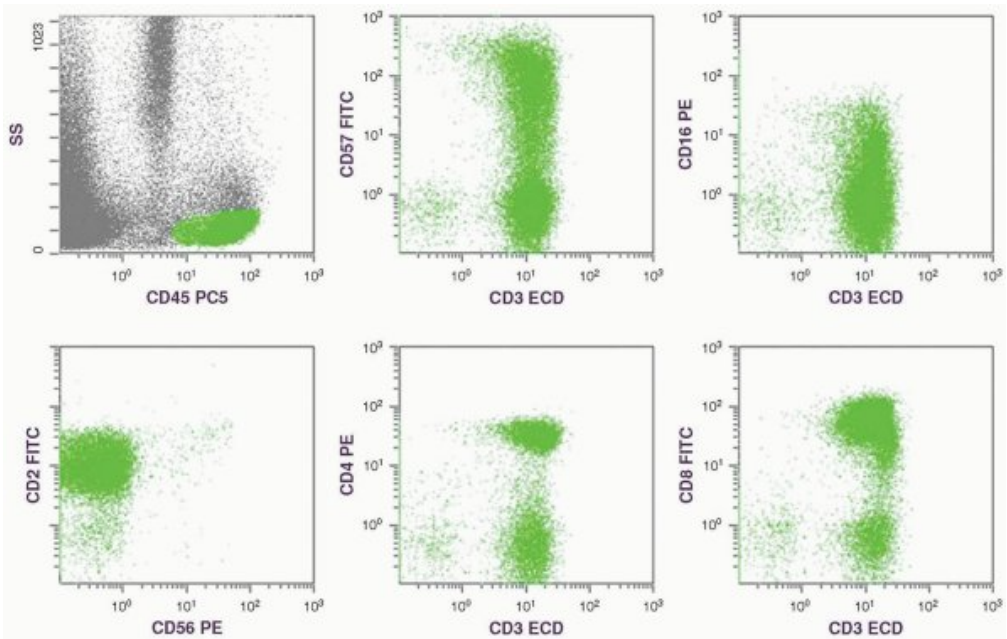

FIGURE 6.31.1 Flow cytometric histograms show positive CD2, CD3, CD57, and CD16 reactions but negative CD56 reaction. There is a reverse CD4/CD8 ratio. The CD4-positive population represents the normal T lymphocytes. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-Texas Red.

DISCUSSION

Large granular lymphoproliferative disorder (LGLD) was first reported by Brouet et al. (1) in 1975 and was defined by McKenna et al. (2)

in 1977 to be a distinct clinicopathologic entity. However, LGLD as defined in the early literature was composed of a highly heterogenous group, which includes chronic leukemia, acute leukemia, lymphoma, and reactive lymphocytosis. In terms of cell lineage, it contains tumor cells of the natural killer (NK) cell lineage or the NK-like T-cell lineage. It is, therefore, not surprising to find the wide clinical spectrum, from indolent to highly aggressive, in the early reported cases. The current definition of LGLD is confined to the leukemic type without lymph node involvement. By this definition, LGLD can be divided into chronic T-large granular lymphocyte (T-LGL) leukemia, chronic NK lymphocytosis (indolent NK-granular lymphoproliferative disorder), and NK LGL leukemia, which includes mainly the aggressive NK-cell leukemia (3, 4 and 5). Chronic LGLD is much more common than the aggressive type. Among the chronic types, 85% of cases are chronic T-LGL leukemia, and 5% of cases are chronic NK lymphocytosis (5). These two types of chronic LGLD are mainly distinguished by immunophenotypes, but are similar in morphology and clinical manifestations. The immunophenotypes of these three groups of LGLD are summarized in Table 6.31.1.

In the World Health Organization (WHO) classification, chronic T-LGL is renamed T-LGL leukemia, and cases with NK-cell phenotypes are classified as NK disorders (6). The WHO classification defines T-LGL leukemia as "a heterogeneous disorder characterized by a persistent (>6 months) increase in the number of peripheral blood large granular lymphocytes, usually between 2 and 20×10^9/L, without a clearly identified cause."

Morphology

The morphology of the tumor cells in T-LGL leukemia is generally similar to that of the normal large granular lymphocytes (LGL), but considerable variation in size and morphology of the tumor cells has been reported (7,8). These cells are larger than the small normal lymphocytes, and their size varies from 15 to 18 μm (Figs. 6.31.2 and 6.31.3). The cytoplasm is usually pale or transparent, containing three or more azurophilic granules, but some LGL may not contain any visible granules. The normal range of large granular lymphocytes is 200 to 400/μL (or 10% to 15%) among the mononuclear cells in the peripheral blood (7,8). When a patient has >2,000/μL of LGL or LGL is >40%

of the lymphocyte fraction for >6 months, T-LGL leukemia should be suspected (9, 10 and 11). However, a few patients may have LGL of <1,000/μL in the peripheral blood (10). There is no agreement on the lymphocyte count in T-LGL leukemia, but 5,000/μL is suggested as the cutoff from reactive lymphocytosis (6).

TABLE 6.31.1

Major Immunophenotypes of LGLD

Marker	*T-LGL Leukemia*	*Chronic NK Lymphocytosis*	*NK-LGL Leukemia*
CD3	Positive	Negative	Negative
CD16	Positive	Positive/negative	Positive/negative
CD56	Negative	Positive	Positive
CD57	Positive	Negative	Positive/negative
CD4	Negative	Negative	Negative
CD8	Positive	Negative	Positive/negative
TCR protein	Positive	Negative	Negative

LGLD, large granular lymphoproliferative disorder; NK, natural killer; TCR, T-cell receptor; CD, cluster of differentiation.

All possible causes of reactive large granular lymphocytosis should be excluded before further studies by phenotyping or genotyping are conducted. The presence of neutropenia is important for the diagnosis of T-LGL leukemia, because reactive large granular

lymphocytosis may have an immunophenotype similar to that of LGL (12). Anemia and thrombocytopenia are also frequently seen. Anemia in some patients is associated with pure red cell aplasia, which is relatively common in Chinese and Japanese patients with T-LGL leukemia (7). The criteria for pure red cell aplasia are anemia, reticulocytopenia (<0.1%), and selective erythroid hypoplasia and/or aplasia with normal myeloid and megakaryocytic cell lines in the bone marrow.

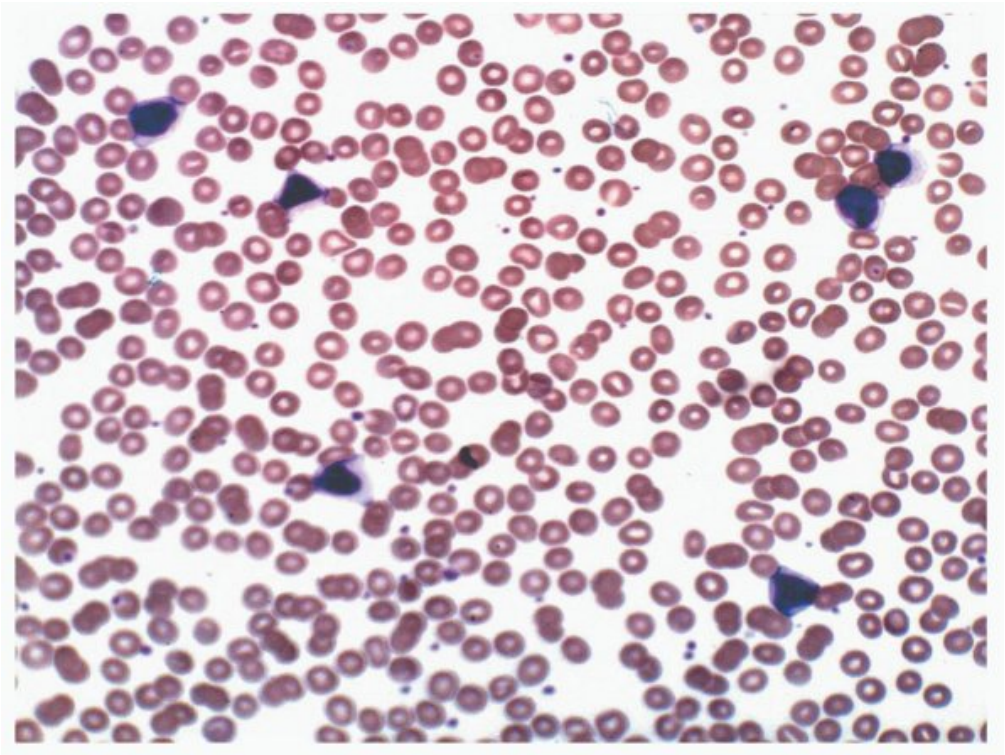

FIGURE 6.31.2 Peripheral blood smear shows several large granular lymphocytes with abundant transparent cytoplasm. Wright-Giemsa, 40× magnification.

The bone marrow is frequently involved showing interstitial or nodular lymphocytic infiltration (Fig. 6.31.4). The lymphoid nodule formation is perceived by some authors as a non-neoplastic reaction to the lymphokines secreted by the tumor cells (13). Morice et al. described a linear accumulation of cytotoxic T cells within marrow microvascular structures, which was considered highly specific for T-LGL leukemia (14). The same study showed that the interstitial infiltrates were composed of T-LGL cells, whereas the large lymphoid aggregates were composed of non-neoplastic lymphocytes, a mixed T- and B-cell population. However, immunohistochemical stains are often needed for the identification of the LGLs, particularly because lymphocytic infiltration may be subtle. Some investigators found maturation arrest of the granulocytes and considered this to be the mechanism of neutropenia secondary to lymphokine secretion by the tumor cells (13,15). Bone marrow aspirates may demonstrate LGLs (Fig. 6.31.5), but LGLs in the marrow are less voluminous

with inconspicuous azurophilic granules (14). It is the immunophenotype that determines the diagnosis (14,16).

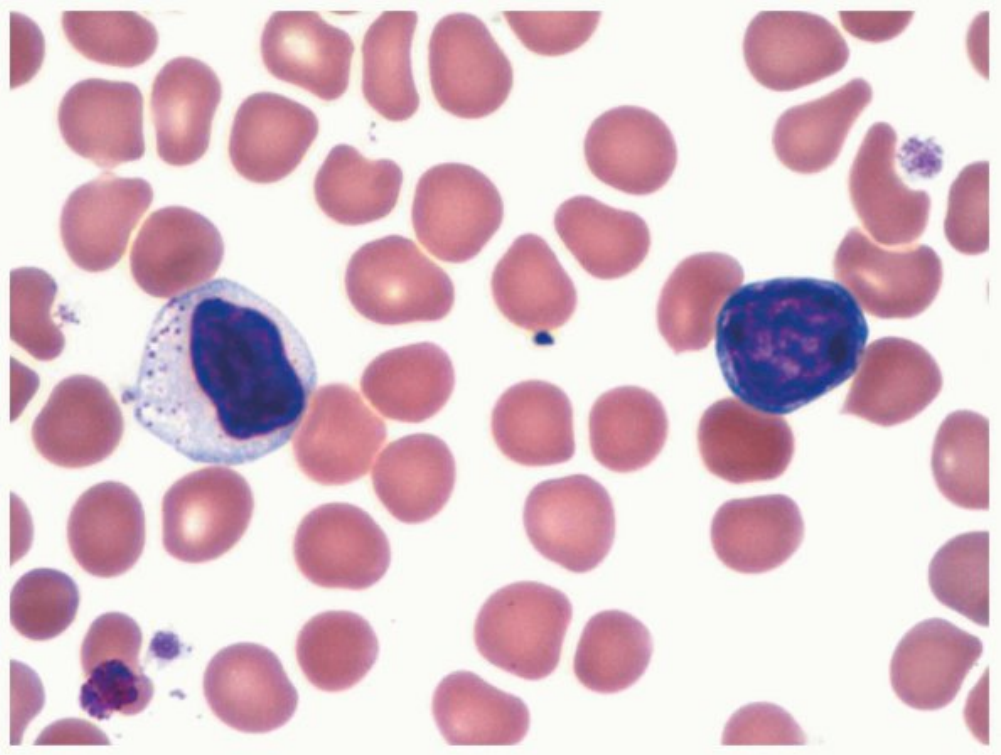

FIGURE 6.31.3 Peripheral blood smear shows one large granular lymphocyte with azurophilic granules in its transparent cytoplasm. A normal lymphocyte is by its side. Wright-Giemsa, 200× magnification.

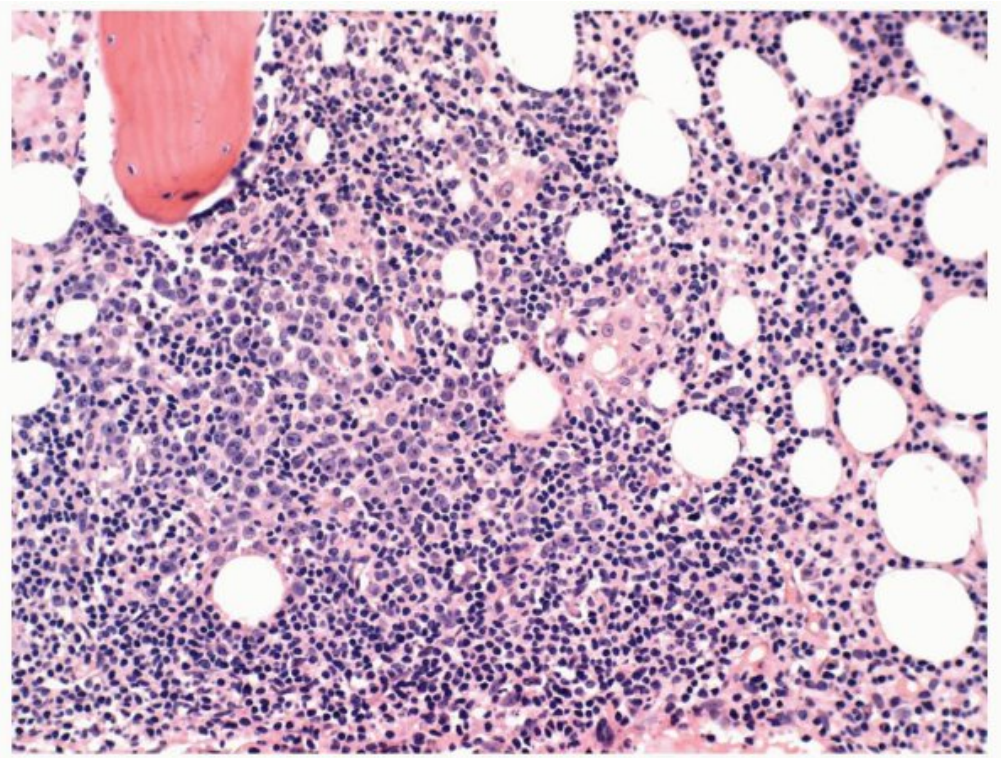

FIGURE 6.31.4 Bone marrow core biopsy reveals a lymphoid aggregate with a pale germinal center and interstitial lymphoid infiltration in the adjacent area. Immunohistochemical stains

identify the lymphoid aggregate as reactive and the interstitial infiltrate as T-large granular lymphocytes (T-LGL). Hematoxylin and eosin, 20× magnification.

The spleen usually shows leukemic infiltration of the red pulp and prominent reactive germinal follicles consisting of polyclonal B cells (4). Unlike other red pulp involving leukemias, such as T-prolymphocytic leukemia and chronic lymphocytic leukemia, cells from T-LGL leukemia do not invade the white pulp, so the follicles may show germinal center hyperplasia and expansion of the mantle zones (13). In the liver, lymphoid infiltration is mainly seen in hepatic sinusoids, but infiltration of the portal areas may also be seen in severe cases (4).

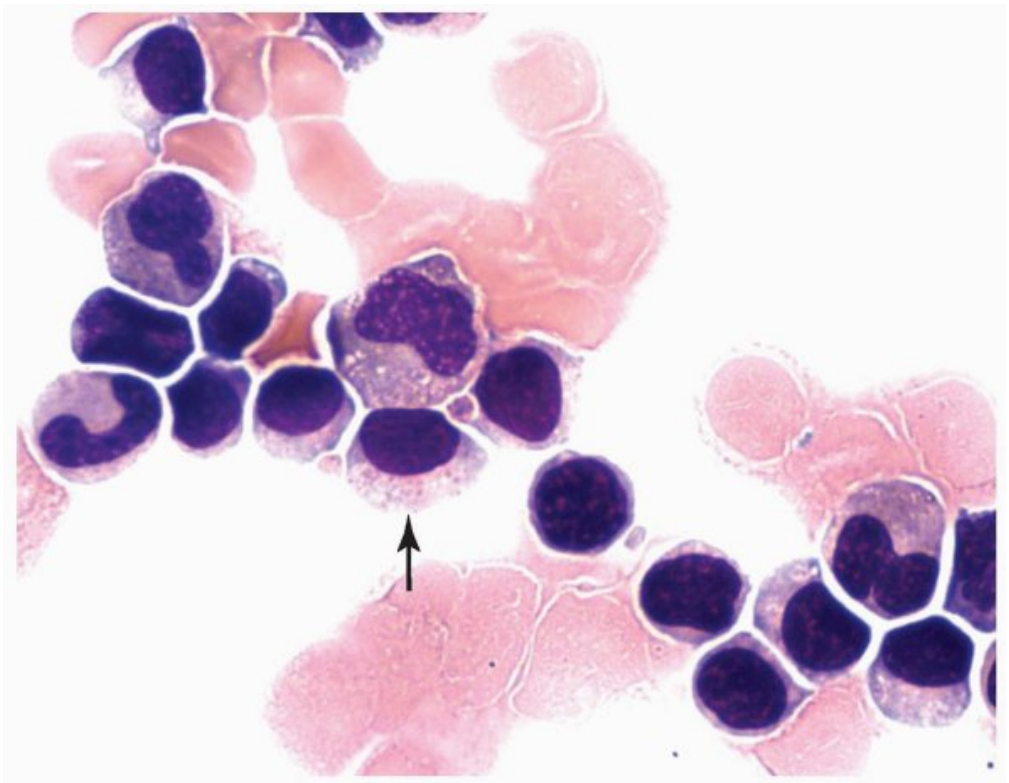

FIGURE 6.31.5 Bone marrow aspirate shows a few large granular lymphocytes (*arrow*) that have less voluminous cytoplasm and inconspicuous cytoplasmic granules. Wright-Giemsa, 100× magnification.

Immunophenotype

The typical immunophenotype is CD3+ CD16+ CD56- CD57+ CD4- CD8+ (3,6,8,12). For T-cell receptor (TCR) proteins, most cases express $TCR_{\alpha\beta}$. However, in cases that have a CD3+ CD4- CD8- phenotype, $TCR_{\gamma\delta}$ is expressed (17,18). Rare cases may have a phenotype of CD3+ CD4+ CD8- or CD3+ CD4+ CD8+ (3).

Among other T-cell markers, CD2 is consistently expressed, whereas the expression of CD5 and CD7 is variable. Recent studies have shown that the CD5 level is consistently lower than those of other T-cell markers, whereas CD7 may also be expressed at a lower level in some cases (12). The myeloid markers, CD11b and CD11c, are also frequently expressed in T-LGL cells (19,20). The differences in the phenotype between NK cells and NK-like T cells (T-LGL) are discussed in Case 32.

The T-LGL cells are cytotoxic T cells as evidenced by the presence of cytotoxic granule proteins T-cell intracellular antigen 1 ([TIA]-1, granzyme B, and perforin) in their cytoplasms (20). Besides the cytotoxic granule protein, the Fas molecule (CD95) and the Fas ligand (CD95L) may also play a role in mediating cell killing in T-LGL leukemia (21). On the other hand, Fas molecule also mediates apoptosis, which is facilitated by interleukin-2 (5). Because there is an interleukin-2 defect in T-LGL leukemia, the tumor cells from most patients are resistant to Fas-induced apoptosis (5). This can be one of the possible mechanisms for tumorigenesis in T-LGL leukemia.

The recent discovery of the NK receptor (NKR) system has great potential in helping to stratify various NK-cell leukemia and T-LGL leukemia. The NKR system includes two groups: killer cell immunoglobulin-like receptors (KIRs) and the C-type-lectin-like receptors that consist of heterodimers of CD94 and NKG2 molecules (12). A recent report by Lundell et al. (12) identified CD94 and at least one of the

KIR antigens in their study of 23 cases of T-LGL leukemia.

Comparison of Flow Cytometry and Immunohistochemistry

The advantage of flow cytometry is its capability in distinguishing NK cell from T-LGL by the presence or absence of surface and cytoplasmic CD3. In tissue sections, a positive CD3 staining can be due to the presence of either surface or cytoplasmic CD3 antigen. However, immunohistochemistry can demonstrate the cytotoxic granule proteins, thus facilitating the identification of the T-LGL cells in tissue (8). In the bone marrow, LGLs are best identified by CD8, TIA-1, and granzyme B staining (14,22). Immunohistochemical stains for CD57 and CD56 were found to be noncontributory in one study of 36 cases of T-LGL leukemia (14), but CD57 was considered helpful in another study of 9 cases (22). In the spleen, the T-LGL cells are negative for CD5 and CD45RO, but positive for cytotoxic granule proteins, whereas the normal T cells in the spleen are positive for CD5 and CD45RO, but negative for cytotoxic granule proteins (13).

Molecular Genetics

TCR gene rearrangement demonstrated by either Southern blotting technique (Fig. 6.31.6) or polymerase chain reaction

(PCR) is one of the most important criteria for the diagnosis of T-LGL leukemia. In most cases, the TCR_B gene is rearranged (6). When both CD4 and CD8 are negative, such as in cases with CD3+ CD4- CD8- CD16+ phenotype, the TCR_γ-chain gene is rearranged (2,18).

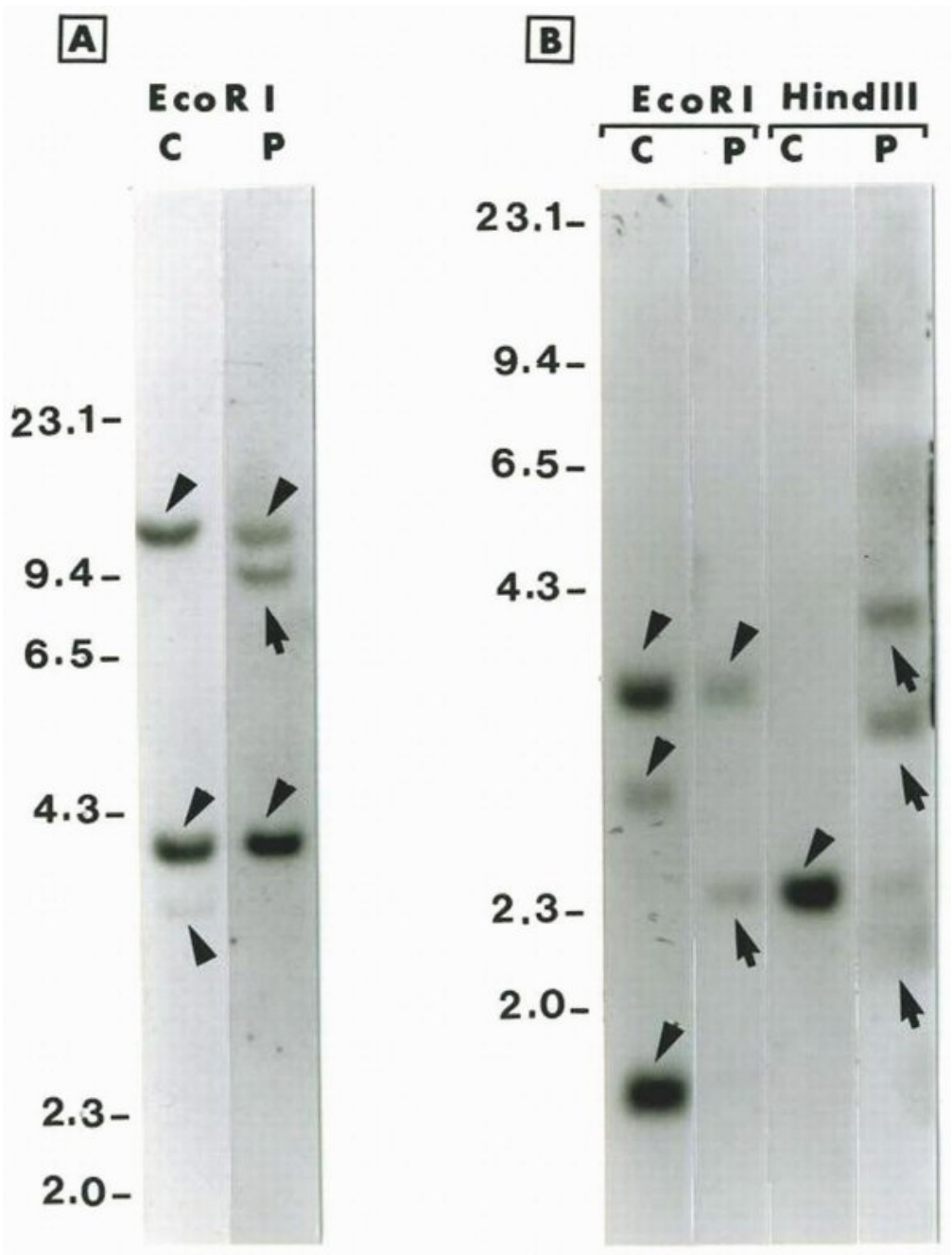

FIGURE 6.31.6 Southern blot hybridization of control (C) and leukemic DNA from the patient (P)

with DNA probe for T-cell receptor ß (**A**) and for T-cell receptor γ (**B**) genes. Rearranged DNA bands in the leukemic DNA are identified by *arrows*. Germline bands are indicated by *arrowheads*. (From Sun T, Brody J, Koduru P, et al. Study of the major phenotype of large granular T-cell lymphoproliferative disorder. *Am J Clin Pathol*. 1992;98:516-521, with permission.)

Alternatively, the clonality of T cells can be demonstrated by flow cytometric analysis of the T-cell antigen receptor ß-chain variable region ($V_ß$) protein expression. One early study of 12 cases of T-LGL leukemia showed a heterogeneous distribution of $V_ß$- and $J_ß$-chains similar to that seen in normal peripheral blood (5). Another study, in contrast, showed a limited usage of TCR $V_ß$-chain in only three families in T-LGL cases (23). A recent study of 23 cases of T-LGL leukemia demonstrated direct or indirect evidence of clonal T-cell populations in 22 cases by analysis of $V_ß$ expression (12).

Aberrant karyotypes have been reported in only a few cases of T-LGL leukemia. A recent report of two cases is particularly interesting (24). One case showed a novel inv(7)(p15q22) as the sole chromosome abnormality, and the other showed an inv(14)(q11;q32) with evidence of clonal evolution. The breakpoints 7p14-p15 and 14q11 in these cases coincide with the $TCR_γ$ and $TCR_α/TCR_δ$ genes, respectively. These were the first cases showing TCR gene involvement in aberrant karyotypes of T-LGL cases, and this involvement may represent the mechanism of pathogenesis of T-LGL.

TABLE 6.31.2

Salient Features for Laboratory Diagnosis of T-LGL Leukemia

1. Presence of 40% large granular lymphocytes or an absolute count of >2,000/µL in the peripheral blood for >6 months without a clearly identified cause
2. Neutropenia or pancytopenia
3. Rheumatoid factor, antinuclear antibodies, neutrophil antibody, and platelet antibody may or may not be present.
4. Typical immunophenotype: CD3+ CD16+ CD56- CD57+ CD4- CD8+ TCR protein +
5. Genotype: TCR gene but not heavy-chain gene is rearranged.

T-LGL, T-large granular lymphocyte; CD, cluster of differentiation; TCR, T-cell receptor.

The current case is typical for T-LGL leukemia. The patient had an indolent clinical course, being asymptomatic without treatment for 9 years. His major clinical finding was cyclic neutropenia, but recurrent infections did not occur. The immunophenotype of the lymphocytes was CD3+ CD8+ CD56- CD57+, but cytotoxic granule proteins were not investigated. $TCR_ß$ gene rearrangement established the T-cell clonality of this disease. The patient also had arthritis, but it was not characterized as rheumatoid arthritis. The absence of splenomegaly was atypical, but it does not exclude the diagnosis of T-LGL leukemia. Finally, the patient responded to methotrexate and prednisone therapy. The salient features of laboratory diagnosis of T-LGL leukemia are summarized in Table 6.31.2.

Clinical Manifestations

Large granular lymphocytosis is frequently a reactive process seen in various diseases. The most common causes are viral infections (human immunodeficiency virus [HIV] and human T-lymphotropic virus type 1 [HTLV1]) and transplantations (bone marrow and solid organs) (8). It has also been reported in patients with chronic bacterial infections and other neoplastic diseases and in patients postsplenectomy (11,25, 26 and 27). Therefore, other underlying diseases should be excluded before a leukemic process is considered. In contrast, patients with T-LGL leukemia may be asymptomatic; a clonal disorder should not be ruled out on this basis.

The major clinical manifestations in T-LGL leukemia are chronic large granular lymphocytosis, neutropenia, splenomegaly, and

rheumatoid arthritis; therefore, it is difficult to distinguish T-LGL leukemia from Felty syndrome.

These two entities are similar in age and sex of the patients and frequency of infections. Furthermore, patients of both diseases have good response to methotrexate. Therefore, some authors consider T-LGL leukemia and Felty syndrome to represent a spectrum of the same disorder (5,8).

Recurrent bacterial infections are secondary to neutropenia. These infections usually involve skin, oropharynx, sinuses, and perirectal areas. Other symptoms may be related to anemia and thrombocytopenia. B symptoms (fever, night sweats, and weight loss) are seen in 20% to 30% of cases (5). Hepatomegaly is seen in 20% of patients. Lymphadenopathy is rare; if present, other subtypes of NK-cell tumors, such as aggressive NK-cell lymphoma/ leukemia, should be considered.

Autoimmune antibodies, such as rheumatoid factor, antinuclear antibody, antineutrophil antibody, and antiplatelet antibody, are frequently found in these patients. Interestingly, T-LGL leukemia is occasionally associated with systemic lupus erythematosus, Sjögren syndrome, and Sicca syndrome, in addition to rheumatoid arthritis (5). As mentioned before, T-LGL leukemia patients may also have pure red cell aplasia, which may be due to inhibition of the red cell precursors burstforming unit and colony forming unit (BFU-E and CFU-E) by LGL clonal expansion (28).

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CASE 32 Natural Killer Cell Lymphoma

CASE HISTORY

A 62-year-old man presented with a history of gunshot wound to his head, which occurred a few years ago, with residual left upper extremity weakness, seizure disorder, and right eye blindness. He was admitted to the hospital because of purulent nasal drainage and increasing periorbital swelling and edema for 2 weeks. Physical examination on admission showed right periorbital swelling with greenish nasal discharge from the left nostril and blood in the right nostril. Computed tomography (CT) scan of the maxillary sinuses revealed extensive swelling and enhancement in the soft tissue of the periorbital region with fluid accumulation in both sides of the maxillary sinuses.

The patient was treated with antibiotics and became afebrile; in addition, his facial cellulitis diminished. However, he developed a necrotic area on his skin overlying the right maxillary sinus, and the area was severely infected. Because of the failure of his infection to become completely clear, a biopsy of the necrotic tissue was taken.

Biopsy of the facial tissue showed extensive necrosis, hemorrhages, prominent blood vessels, and multifocal cellular infiltration (Fig. 6.32.1). Higher magnification revealed thrombosis in some blood vessels, fibrinoid necrosis on the vessel wall (Fig. 6.32.2), and perivascular coagulative necrosis in other vessels. Well-confined perivascular infiltrations, the so-called angiocentric pattern, were prominent. Angioinvasive and angiodestructive lesions were also present. The major cellular components in the infiltrates were atypical lymphocytes with a small number of eosinophils. Neutrophils and plasma cells were not demonstrated. Most of the lymphoid cells had an angulated, convoluted, or cleaved nuclear contour.

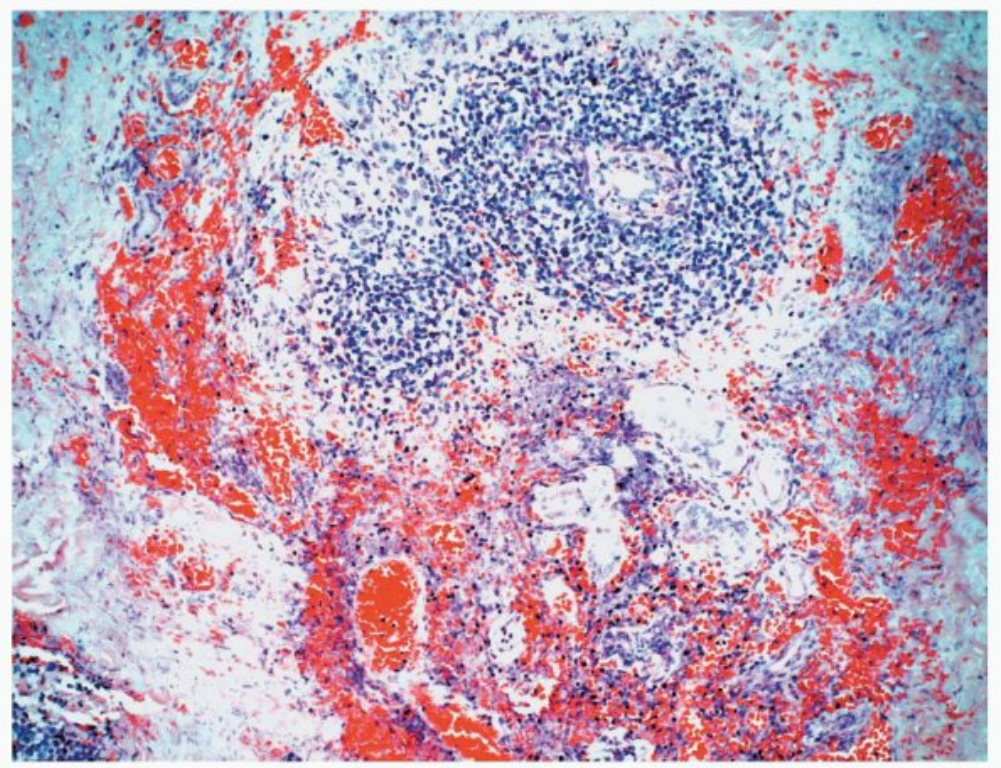

FIGURE 6.32.1 Case of extranodal natural killer (NK)-and/or T-cell lymphoma shows angiocentric lesion with zonal necrosis and hemorrhages. Hematoxylin and eosin, 20× magnification.

FLOW CYTOMETRY

Flow cytometric analysis of the biopsy showed essentially negative B-cell markers: CD19 1%, CD23 0%, FMC-7 0%, κ 3%, λ 2%. T-cell marker analysis revealed CD3 7%, CD3/CD4 4%, CD3/CD8 3%, CD5 6%, CD7 25%, CD8 26 %, CD25 0%. Natural killer (NK)-cell marker study demonstrated CD16-CD56 93%, CD57 11%. Monocyte markers were CD14 1% and CD11c 86% (Fig. 6.32.3).

IMMUNOHISTOCHEMISTRY

Immunohistochemical stains of the biopsy showed negative cytokeratin but positive CD45 leukocyte common antigen ([LCA]) staining for the lymphoid cells. Further studies with lymphoid markers revealed negative CD20 (B-cell

marker) and positive CD3 (T-cell marker) (Fig. 6.32.4). In the T-cell subset study, CD8 was demonstrated exclusively in the lymphoid cells, and CD4 was entirely absent. Based on these findings, NK-cell markers were analyzed; analysis showed a positive staining of CD56 (Fig. 6.32.5) but negative staining of CD57 (Fig. 6.32.6) on the lymphoid cells.

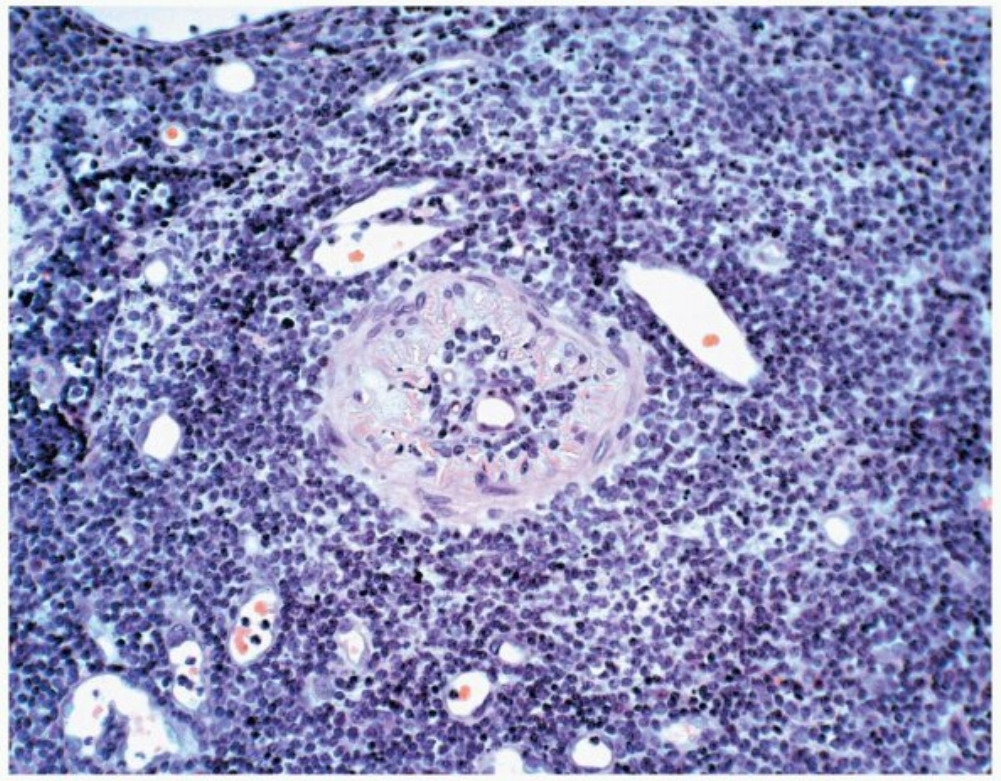

FIGURE 6.32.2 Case of extranodal natural killer (NK)-and/or T-cell lymphoma reveals angiocentric tumor cell infiltration. The artery in the center shows fibrinoid necrosis in the vessel wall. Hematoxylin and eosin, 60× magnification.

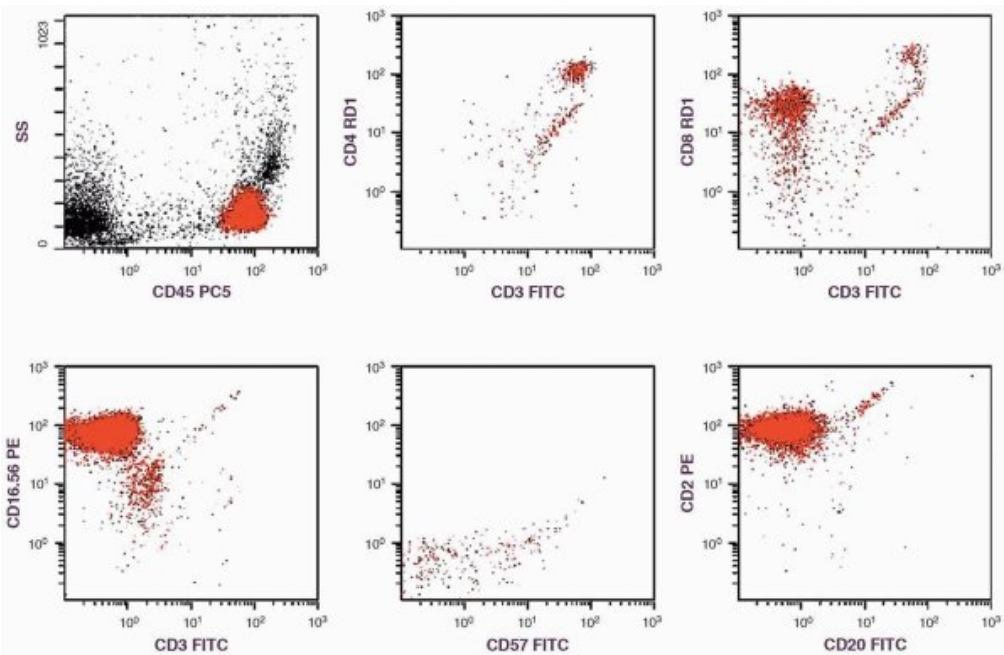

FIGURE 6.32.3 Flow cytometric histograms show positive reactions to CD2, CD8, and CD16-CD56, but negative reactions to CD3, CD4, CD20, and CD57. A side scatter versus CD45 plot is used for gating. SS, side scatter; PC5, phycoerythrin-cyanin 5; RD1, rhodamine; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

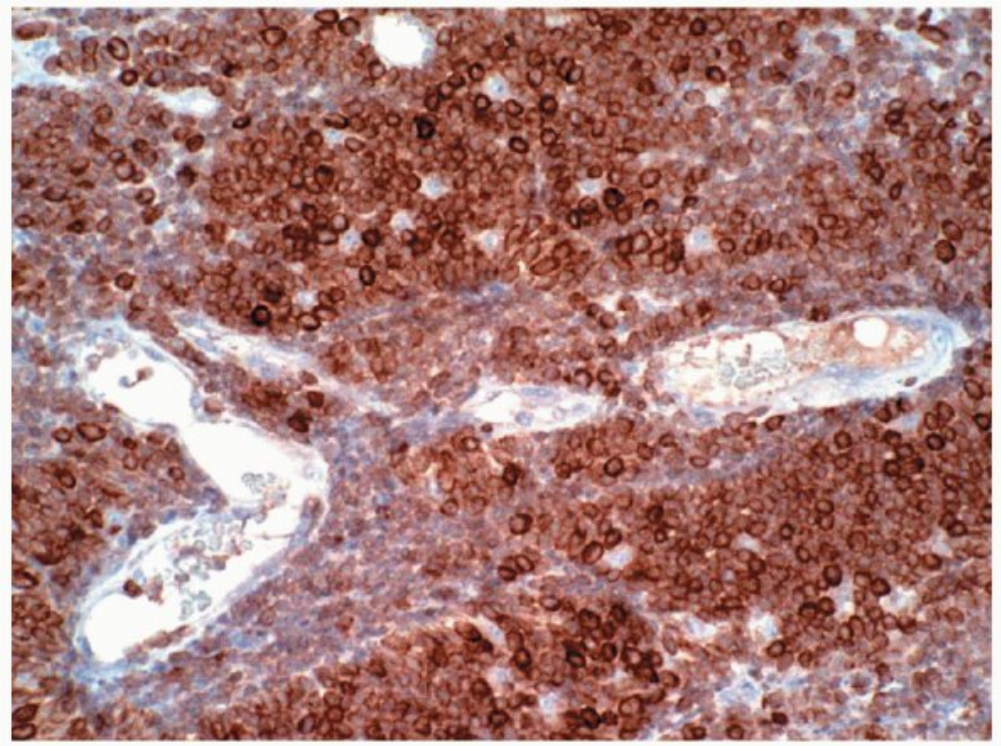

FIGURE 6.32.4 Case of extranodal natural killer (NK)/ T-cell lymphoma reveals angiocentric tumor cell infiltration with positive CD3 staining. Immunoperoxidase, 60× magnification.

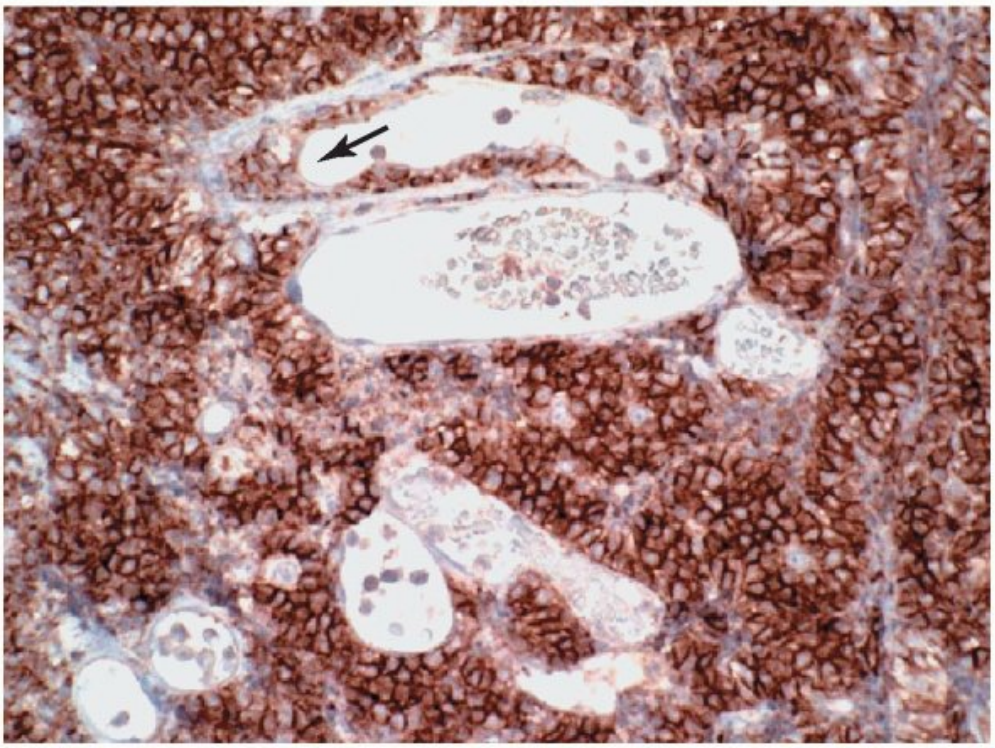

FIGURE 6.32.5 The same case as Figure 6.32.4 shows positive CD56 staining. The positive staining highlights the invasive pattern of the tumor cells in the blood vessel wall (arrow). Immunoperoxidase, 60× magnification.

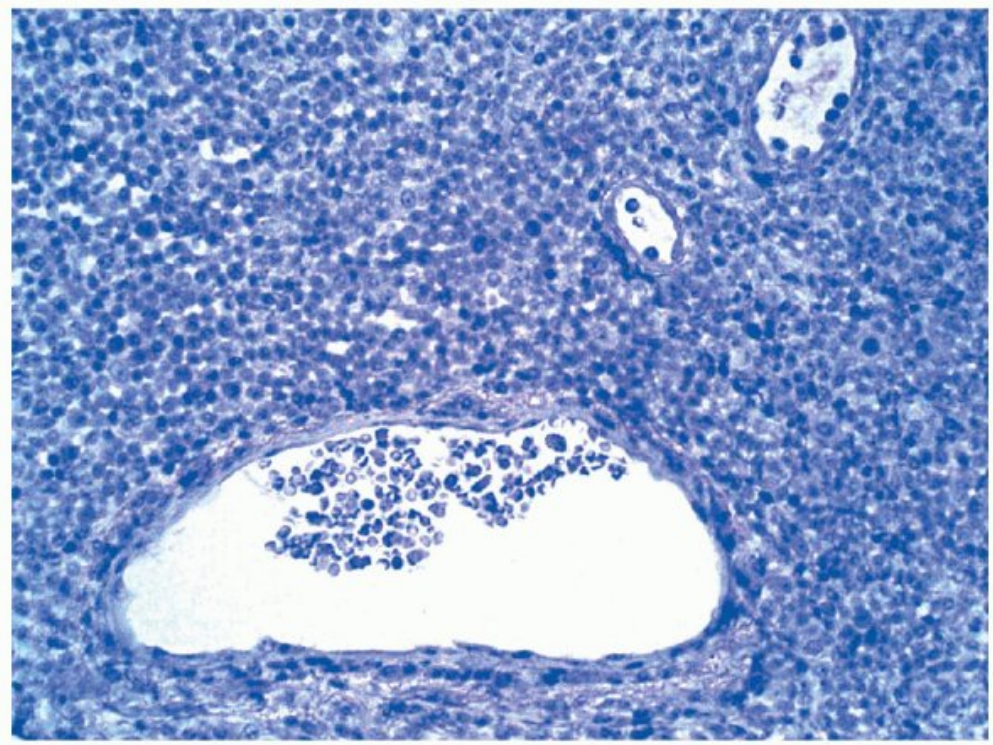

FIGURE 6.32.6 The same case as Figure 6.32.4 shows negative CD57 staining.

Immunoperoxidase, 60× magnification.

MOLECULAR GENETICS

Cytogenetic karyotyping of the tissue biopsy demonstrated a normal male karyotype of 46, XY. Germline configuration was detected with the T-cell receptor (TCR) gene probes and immunoglobulin (Ig) heavy-chain and light-chain gene probes.

DISCUSSION

This case illustrates the requirement of a high index of suspicion in the diagnosis of NK-cell lymphomas. First, the extensive necrosis and hemorrhages, frequently with superimposed infection in the angiocentric lymphoma, are frequently dismissed as an infectious process. Second, the identification of positive T-cell markers in the cellular infiltrate can be conceived as an inflammatory infiltration or a T-cell lymphoma, if T-cell subset study is performed.

It is particularly difficult to make the diagnosis of NK neoplasm in its early stage, because of the presence of a polymorphic cellular infiltration in the early stage of the disease (1, 2, 3, 4 and 5). It is understandable, therefore, that the early literature frequently referred to this disease as indetermine malignancy or polymorphic reticulosis. The location in the midline of the head in the current case provides a clue to the possibility of an NK tumor, particularly nasal NK/T-cell lymphoma. Because of this particular location, early literature also referred to this tumor as malignant midline reticulosis, lethal midline granuloma, or midfacial destructive disease. However, these terms probably encompass a great variety of diseases, including lymphomatoid granulomatosis and Wegener granulomatosis (3,5). The similarity in morphology among these diseases is another cause of difficulty in the diagnosis of NK-cell neoplasms.

The presentation of tissue necrosis is mainly due to the angioinvasive and angiodestructive behavior of the tumor cells; therefore, it is often presented as zonal necrosis. Because of this histologic pattern, this tumor was designated by the Revised European-American classification of lymphoid neoplasms (REAL) as angiocentric T-cell lymphoma. However, angiocentric lesions can also be detected in other lymphomas, so the World Health Organization (WHO) classification changed it to extranodal NK/T-cell lymphoma, nasal type. In contrast, an angiocentric pattern is not invariably present in all NK-cell neoplasms, thus the necrotic lesion is considered to be induced by more than one factor. Obviously, the release of cytotoxic proteins, T-cell intracellular antigen (TIA)-1, granzyme B, and perforin from the NK cells is another contributing factor (6). Finally, the increased expression of the chemokines Ip-10 and Mig in the tumor cells, which is probably induced by Epstein-Barr virus (EBV) infection, is an additional contributing factor (7).

The predisposing factor of NK-cell tumor in this patient is not clear, but there is a strong association of nasal NK/ T-cell lymphoma with EBV, which is present in >95% of patients, irrespective of the ethnic origin (3,5,8,9). On the contrary, nasal lymphomas of T-cell or B-cell origin have only a weak association with EBV; this fact further strengthens the argument for the etiological role of EBV in NK-cell tumors. Monoclonal EBV genome rearrangement is detected in NK tumor cells and is one parameter to verify the neoplasmic nature of this lesion.

Similar to nasopharyngeal carcinoma and Hodgkin lymphoma, nasal T-/NK-cell lymphoma has the type II EBV latency pattern, as manifested by the presence of EBV nuclear antigen (EBNA1) and the latent membrane protein 1 and 2 (3,5). Wegener granulomatosis is EBV negative, whereas lymphomatoid granulomatosis is an EBV-positive B-cell lymphoma (3,5). Therefore, the study of EBV is helpful in the differential diagnosis.

There are many different forms of NK-cell neoplasm reported. The nomenclature is confusing and overlapping. The current WHO classification of NK-cell tumor is probably not comprehensive, but it provides the baseline for a unifying designation. The WHO scheme enlists three NK-cell tumors: blastic NK-cell lymphoma, extranodal NK/T-cell lymphoma, nasal type, and aggressive NK-cell leukemia (10) (Table 6.32.1).

Among the precursor NK-cell neoplasms, there are myeloid/NK-cell precursor acute leukemia and myeloid/ NK-cell acute leukemia reported in the literature (11, 12 and 13). CD4+ CD56+ lymphoma or leukemia is a recently described clinical entity that is now included in the category of blastic NK-cell lymphoma (14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). However, despite the expression of CD56, its cell lineage is still not well established. Current evidence indicates that it may be of the plasmacytoid dendritic cell (pDC) origin (24, 25 and 26). Because of the uncertainty of the cell lineage and the frequent cutaneous involvement, some authors coined this tumor as CD4/CD56 hematodermic neoplasm (26).

TABLE 6.32.1

Classification of NK-Cell Neoplasms

Precursor NK-cell neoplasms

Blastic NK-cell lymphoma/leukemia (CD4+ CD56+ cutaneous neoplasm, agranular CD4/CD56 hematodermic neoplasm)

Myeloid/NK-cell precursor acute leukemia

Myeloid/NK-cell acute leukemia

Mature NK-cell neoplasms

Indolent NK large granular lymphocyte leukemia (chronic NK-LGL lymphocytosis, chronic NK lymphocytosis)

Extranodal NK/T-cell lymphoma, nasal type (nasal and nasal type NK/T-cell lymphomas)

Aggressive NK-cell lymphoma/leukemia (NK-LGL leukemia)

NK, natural killer; CD, cluster of differentiation.

In the mature NK-cell neoplasms, the extranodal NK/T-cell lymphoma, nasal type includes both nasal and extranasal NK/T-cell lymphomas. The term CD3- NK leukemia or NK-LGL leukemia is in the category of aggressive NK-cell leukemia. The indolent NK large granular lymphocyte (NK-LGL) leukemia should also be included as one of the mature NK-cell tumors.

Morphology

NK cells assume the morphology of LGLs, which show abundant pale or transparent cytoplasm with azurophilic granules and a round or kidney-shaped nucleus with a mature chromatin pattern (Fig. 6.32.7). In indolent NK-LGL leukemia, the LGLs appear normal and are indistinguishable from those seen in T-LGL leukemia. In many cases of CD4+ CD56+ lymphoma/leukemia, the tumor cells are agranular. The cytoplasmic granules can be demonstrated in peripheral blood smears and bone marrow aspirates, but not in tissue sections. Therefore, it is important to make touch imprints from lymph node, spleen (Fig. 6.32.8), and other solid tissue masses to look for cytoplasmic granules before the specimen is fixed.

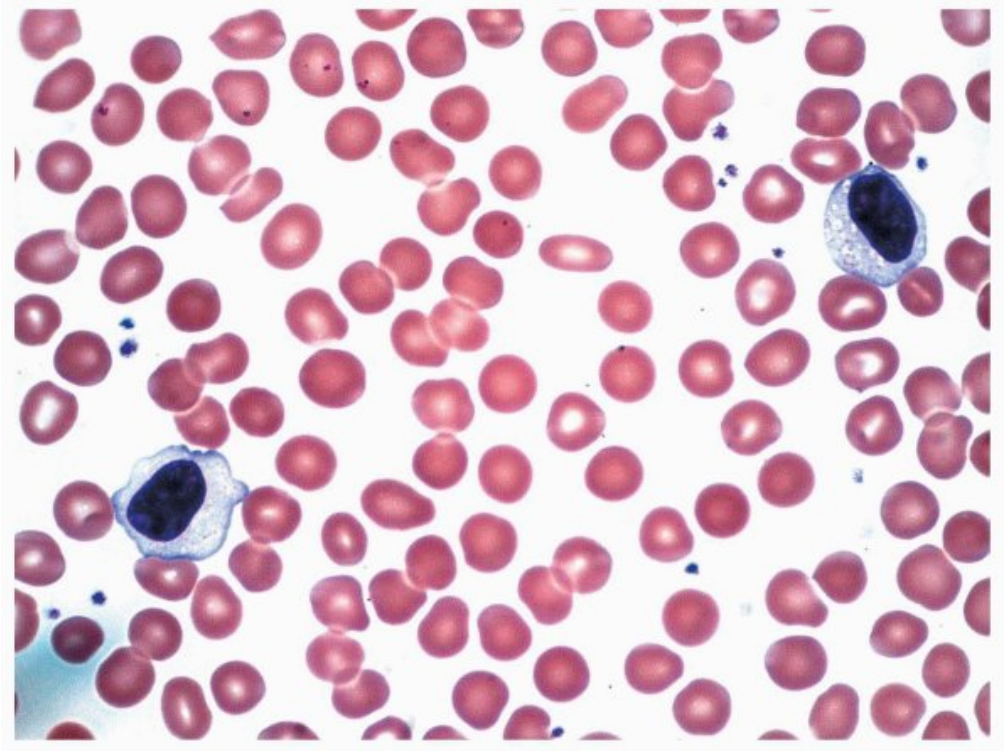

FIGURE 6.32.7 Peripheral blood smear shows two normal large granular lymphocytes with delicate cytoplasmic granules. Wright-Giemsa, 150× magnification.

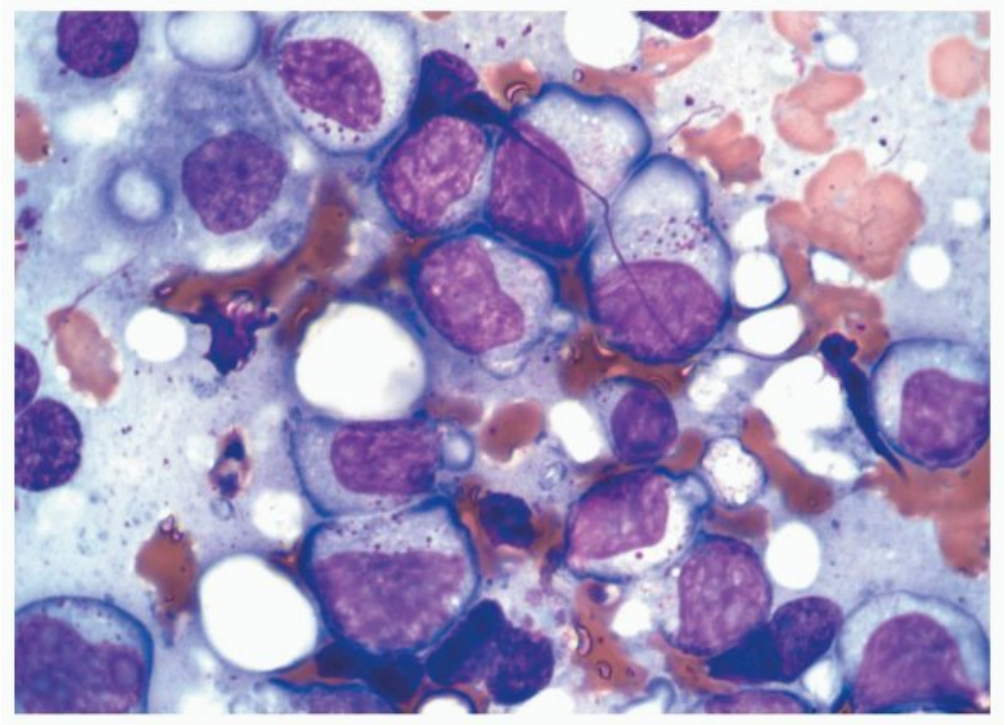

FIGURE 6.32.8 Spleen imprint from a case of aggressive natural killer (NK)-cell lymphoma/leukemia shows a cluster of leukemic cells with cytoplasmic granules. Wright-Giemsa, 100× magnification.

The original criteria for NK/T-LGL leukemia is the persistent presence of 2,000 LGL/μL or >40% of the lymphocyte fraction for at least 6 months, and that all possible causes for reactive lymphocytosis are excluded (27,28). The current cutoff for the diagnosis of T-LGL leukemia is 500/μL or 520/μL of CD3+/CD57+ cells (12,29). The new cutoff for NK-LGL leukemia is 600/μL (13).

Extranodal NK/T-cell lymphomas have a broad cytologic spectrum with variable cell size (1). Therefore, the only diagnostic feature is the presence of cytoplasmic granules, which can be identified only in the touch preparation of the tumor. In the early stage, the lymphoma cells are intermixed with reactive inflammatory cells, including small lymphocytes, histiocytes, polymorphs, eosinophils, and plasma

cells (1, 2, 3, 4 and 5). Therefore, these cases would be easily mistaken as chronic inflammatory diseases. In the late stage, a fullblown feature of pleomorphic tumor cells with irregular nuclei and granular chromatin may finally prevail (30).

The histologic pattern is more helpful than cytology in the diagnosis of this type of NK tumor. Regardless of the location, the pathognomonic features are angioinvasion, angiodestruction, and zonal necrosis in the majority of cases (1,4,30, 31, 32, 33, 34 and 35).

The tumor cells of the aggressive NK-cell lymphoma/ leukemia are usually highly pleomorphic and often contain irregular cytoplasmic azurophilic granules, which are more prominent than those seen in normal LGLs (Fig. 6.32.9) (32,35, 36, 37, 38, 39 and 40). However, the leukemic cells may appear in a later stage (37). Angioinvasion and angiodestruction can be observed in this tumor (4). Hemophagocytosis is also a common feature (1,4,30,35). In the spleen, this leukemia shows predominantly red pulp involvement (41).

In the early literature, blastic NK-cell lymphoma/ leukemia were loosely defined as lymphoblastic morphology with expression of CD56 but not B-cell and myeloid markers (18). With this definition, many cases of acute lymphoblastic leukemia and lymphoblastic lymphoma with CD56 expression might have been included in this category. However, a characteristic pattern has emerged in recent reports, which includes blastic morphology, primary skin involvement, and an immunophenotype of CD4+, CD8-, CD56+, CD57-(14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). The leukemic cells in many cases were agranular (15, 16 and 17). As mentioned before, the cell lineage of this entity may be originated from plasmacytoid dendriotic rather than NK cells (24, 25 and 26).

The skin lesion of this entity usually shows dense lymphoblastoid infiltration in the superficial and deep dermis (Fig. 6.32.10) (14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). Subcutaneous adipose tissue may also be involved. There is usually a Grenz zone of separation from overlying epidermis, and the epidermis is often spared of tumor cell infiltration. The lesion in the lymph node is characterized by interfollicular expansion with sheets of tumor cells. The bone marrow involvement is manifested as clusters of lymphoid-appearing blastoid cells. The pathology of the spleen is usually confined to the red pulp (15, 16 and 17,19, 20, 21, 22 and 23). No angiocentric lesions have ever been reported in this entity.

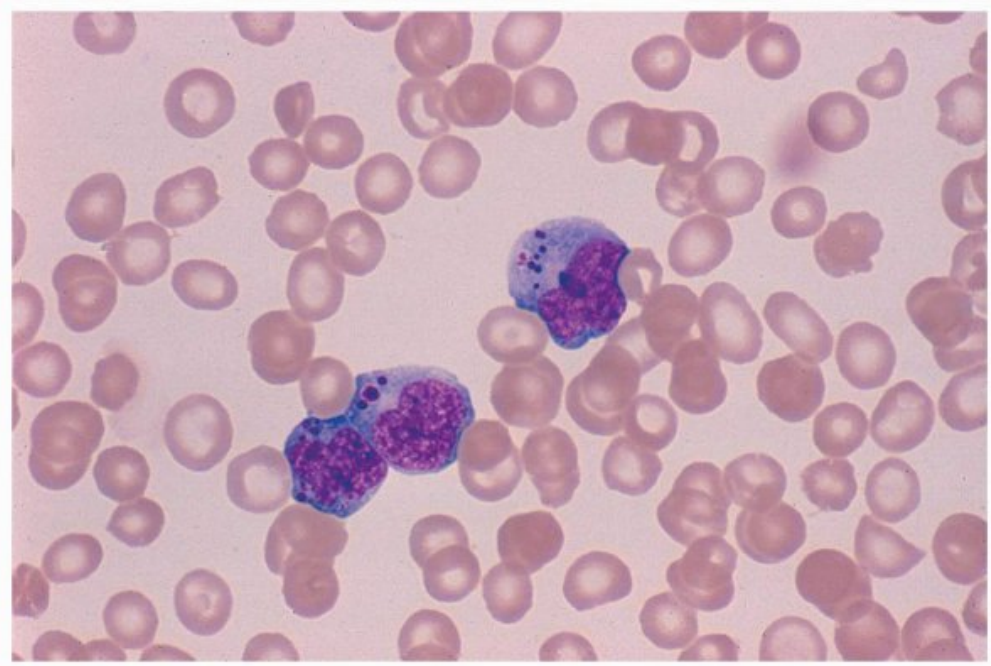

FIGURE 6.32.9 Peripheral blood smear from a case of aggressive natural killer (NK)-cell lymphoma/leukemia shows three tumor cells with prominent cytoplasmic granules. Wright-Giemsa, 150× magnification.

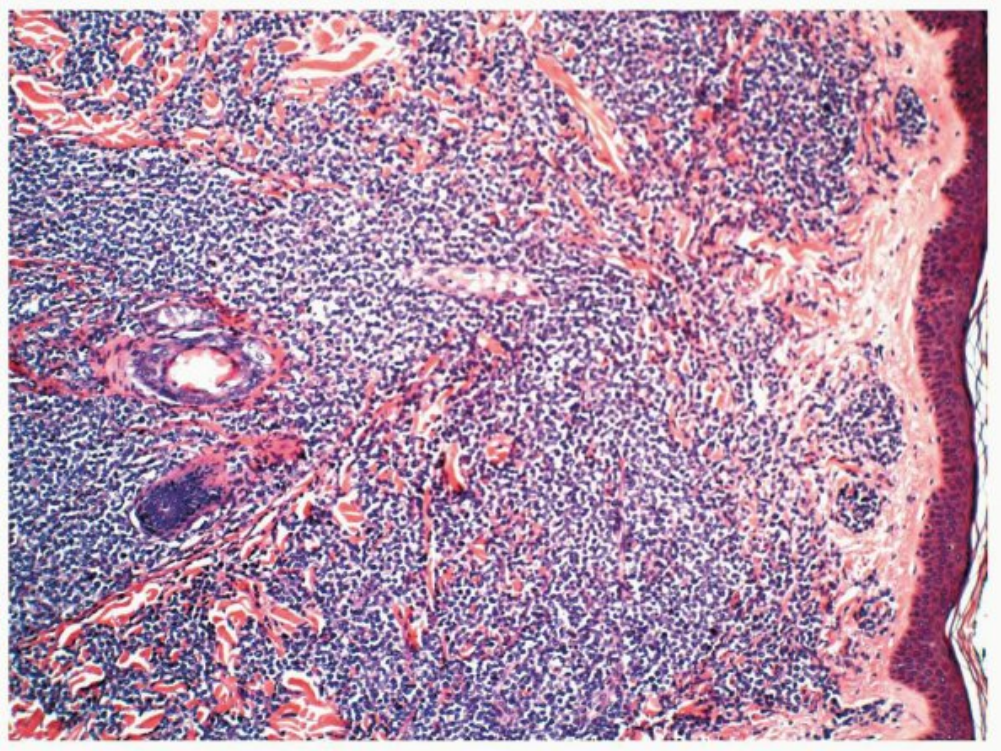

FIGURE 6.32.10 Skin biopsy from a case of CD4+ CD56+ cutaneous lymphoma shows extensive lymphoid infiltration in the dermis. Note a Grenz zone is present separating the epidermis from the leukemic cells. Hematoxylin and eosin, 40× magnification.

Myeloid/NK-cell precursor acute leukemia also assumes a lymphoblastic morphology with the expression of the myeloid and NK markers but without myeloperoxidase reactivity (18,42, 43, 44, 45, 46, 47 and 48). Therefore, it is equivalent to acute myeloblastic leukemia without cytologic maturation (AMLM0) in the French-American-British (FAB) classification. However, the tumor cells show the FAB-L2 morphology, with various sizes, moderately irregular nuclei, prominent nucleoli, and pale cytoplasm. Most cases demonstrate no azurophilic granules in the tumor cells (43). Lymph node biopsy reveals diffuse proliferation of large blastic cells.

The myeloid/NK-cell acute leukemia differs from myeloid/NK-cell precursor acute leukemia by its promyelocytic morphology and the presence of myeloperoxidase reactivity (48, 49, 50, 51, 52, 53, 54 and 55). The tumor cells show deeply invaginated nuclear membranes and scant cytoplasm with fine azurophilic granules, mimicking those seen in the microgranular variant of acute promyelocytic leukemia (Fig. 6.32.11). However, morphology similar to other types of acute myelogenous leukemia has also been observed.

Nevertheless, these three types of immature NK-cell tumors are not necessarily clearly distinguishable from each other. A recently reported case of cutaneous lymphoma expresses a CD56+ CD4+ immunophenotype that is consistent with the blastic NK-cell lymphoma/leukemia (48). However, the morphology of the tumor cells is similar to those of leukemic promyelocytes as seen in M3v, a feature characteristic of myeloid/NK-cell acute leukemia. Furthermore, the presence of myeloid and NK markers but absence of myeloperoxidase is consistent with the myeloid/ NK-cell precursor acute leukemia. Tumors such as this can probably be considered a hybrid form or a stem cell tumor with various differentiations.

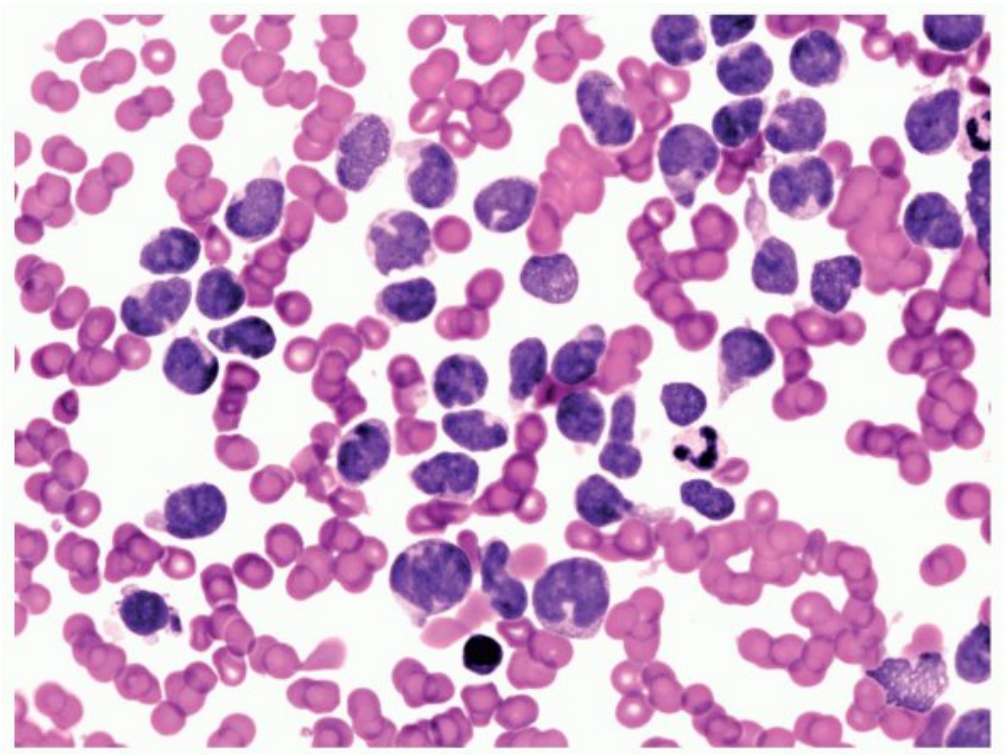

FIGURE 6.32.11 Bone marrow aspirate from a case of myeloid/natural killer (NK)-cell acute leukemia reveals a cluster of leukemic cells with deeply invaginated nuclei and scant cytoplasm. Wright-Giemsa, 60× magnification.

Immunophenotype

Indolent NK-LGL leukemia and T-LGL leukemia can be distinguished only by immunophenotyping (56). The usual phenotype of the former is CD3-, CD4-, CD8-, CD16+, CD56+, and CD57- and that of the latter is CD3+, CD4-, CD8+, CD16+, CD56-, and CD57+ (3,29,51). However, NK cells can show CD57 and CD8, whereas T-LGL (NK-like T cells) may express CD56. The major difference between these two entities is the presence or absence of surface CD3; NK cell is surface CD3 negative but cytoplasmic CD3 (CD3ε) positive. Therefore, in immunochemical stains, NK-cell tumors always express CD3 and cause some confusion for the classification.

TABLE 6.32.2

Differences between NK Cells and NK-Like T Cells

	NK Cell	*NK-Like T Cell*
$_S$CD3	-ve	+ve
$_C$CD3	+ve	+ve
CD4	-ve	-ve
CD8	-/+ve	+ve

CD16	+/-ve	+/-ve
CD56	+ve	-ve
CD57	-ve	+ve
Cytotoxic proteins	+ve	+ve
NK-cell receptors	+ve	-/+ve
$TCR_{\alpha\beta}/TCR_{\gamma\delta}$ proteins	-ve	+ve
TCR gene rearrangement	-ve	+ve

NK, natural killer; CD, cluster of differentiation; TCR, T-cell receptor.

CD3 and TCR form a complex on all T lymphocytes. Accordingly, T cells and NK-like T cells express TCR proteins ($TCR_{\alpha\beta}$ or $TCR_{\gamma\delta}$) that are associated with TCR gene rearrangements. NK cells, in contrast, show neither TCR protein nor TCR gene rearrangements. In addition, CD2, CD11b, and CD11c are consistently present on NK cells, but the expression of CD5 and CD7 is variable. However, one report emphasized the absence of CD5 in NK-cell lymphomas (57). Another important characteristic of NK and NK-like cells is that both kinds of cells contain cytotoxic proteins (TIA-1, granzyme B, and perforin) (3,58). The differences between NK and NK-like T cells are summarized in Table 6.32.2.

Most cases of extranodal NK/T-cell lymphoma, particularly those found in Asian countries, have an NK-cell phenotype and are CD56 positive (3,5,9,30,32). Virtually all of these cases are EBV positive. In immunochemical staining, the routine T-cell markers, such as CD43 and CD45RO, in addition to cytoplasmic CD3 (CD3ε), are all positive. These results frequently cause confusion as to whether these tumors represent NK-like T-cell lymphoma or NK lymphoma. Cutaneous CD56+ NK/T-cell lymphomas frequently express cell adhesion molecules (CD2, CD11a, and CD49d) and their ligands (CD58, CD54, and CD106), which are more frequently associated with an angiodestructive histopathologic pattern than those without the expression of these molecules (59).

The immunophenotype of aggressive NK-cell lymphoma/leukemia is similar to that of extranodal NK/T-cell lymphoma, and EBV is almost always present in the tumor cells (3,13,32,35). The distinction between these two types of tumors is their clinical manifestations.

As in other NK-cell tumors, myeloid/NK-cell precursor acute leukemia shows a CD3-, CD56+ phenotype (18,42,47).

The special markers are the consistent presence of myeloid markers (CD13, CD33) with the absence of myeloperoxidase, a phenotype mimicking AML-M0. Among the NK markers, CD57 and CD16 are negative whereas CD11b, CD11c, and CD56 are positive. Besides, this tumor also expresses CD7 and CD34, and both are markers for precursor NK cells. Therefore, this tumor is considered more immature than the blastic NK-cell lymphoma or leukemia (18,42). Terminal deoxynucleotidyl transferase (TdT) and EBV are negative for all cases studied. One recent report showed that the tumor cells expressed human leukocyte antigen-DR (HLA-DR), CD4, and CD38 (44), but another report did not demonstrate the expression of HLA-DR (43).

The typical immunophenotype of myeloid/NK-cell acute leukemia is CD56+, CD33+, CD11a+, $CD13^{lo}$, $CD15^{lo}$, CD34+, HLA-DR-, CD16-, CD57-, and myeloperoxidase negative (49, 50, 51, 52, 53, 54 and 55). The absence of HLA-DR in a myeloid population and the morphology of the blasts (deeply invaginated nuclear membranes, scant cytoplasm with fine azurophilic granules) are consistent with AML-M3v. However, the characteristic cytogenetic marker of M3, t(15;17), has not been detected in most cases studied, thus excluding the diagnosis of M3. The two cases that showed t(15;17) should have been considered true M3 (55).

The blastic NK-cell lymphoma/leukemia is positive for CD56 but negative for CD57 and CD16 (14, 15, 16, 17, 18, 19, 20, 21, 22 and 23). Both surface and cytoplasmic CD3 are negative. In paraffin sections, tumor cells react to a T-cell marker, CD43, but not to another T-cell marker, CD45RO (17,22). In contrast to other types of NK-cell neoplasms, this tumor is positive for CD4 but negative for CD8. CD2 and HLA-DR are consistently positive, whereas CD5 is negative and CD7 is mostly negative. B-cell and myeloid antigens are all negative, except for cases with myelomonocytic transformation. In all the cases studied so far, cytotoxic proteins (TIA-1 and granzyme B) are usually absent (17, 18, 19 and 20,23), and there is no EBV association with this tumor (16,18,22,23). Recent studies show that additional marker characteristics, such as CD123+, CD68+, are more consistent with a plasmacytoid dendritic cell (pDC) or type 2 dendritic cell (DC2) origin rather than with an NK-cell lineage (24). The detection of two pDC antigens, T-cell leukemia 1 (TCL1) and cutaneous lymphocyte-associated antigen (CLA), in this tumor further support this hypothesis (25,26).

In addition to the above markers, there are two new systems that are involved with T cells and NK cells. The first one is the chemokine

receptors, which control lymphocyte trafficking (60). The CC-chemokine receptor R7 (CCR7) can be used to divide memory T cells into two distinct subsets: the central memory cells (CCR7+) and the effector memory cells (CCR7-). Both NK- and T-cell lymphoproliferative diseases of granular lymphocytes (LDGL) express the CCR7- phenotype (60).

Another one is NK receptors (NKRs), which can be divided into killer Ig-like receptors (KIR), leukocyte Ig-like receptors and/or Ig-like transcripts (LIR/ILT), killer lectinlike receptor (KLR), natural cytotoxicity receptors (NCR), coreceptors and other receptors (60,61). The two major NK receptors are KIR, which recognizes classical major histocompatibility complex (MHC) class I molecules; and C-type KLR, which recognizes nonclassical MHC-related molecules (60). Both NK- and T-LDGL show restricted NKR subsets, and CD94 (a KLR marker) is most commonly expressed. Another study demonstrated dysregulated NK receptor expression in patients with the NK type of lymphoproliferative disease (62).

Based on the expression of two KLR markers (CD94 and CD161) and CD56, NK cells can be divided into three developmental stages (63). The pre-NK cells express CD161, immature NK cells express CD161 and CD56, and mature NK cells express CD161, CD56, and CD94. The study by Mori et al. (63) shows that blastic NK-cell lymphoma/ leukemia has an immunophenotype of CD56+, CD94-, CD161-, but aggressive NK-cell leukemia and/or lymphoma, nasal NK-cell lymphoma, and chronic NK lymphocytosis express both CD56 and CD94 (63). The differences between various NK-cell tumors are summarized in Table 6.32.3.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry plays an important role in distinguishing between NK-cell and NK-like T-cell neoplasms because it can differentiate surface CD3 and cytoplasmic CD3 staining in the tumor cells. In addition, flow cytometry may be used to demonstrate the chemokine and NK receptors. In contrast, cytotoxic proteins can be detected only by immunohistochemical stains.

Molecular Genetics

Cells in NK tumors are characterized by the absence of the TCR gene (both ß- and γ-chains) and Ig gene rearrangements. However, the clonality of NK-cell tumors can be determined by X-linked DNA analysis, which is only applicable to female patients (64). If the tumor contains EBV DNA, the clonality can also be identified by EBV genome rearrangement (65).

The most common genetic abnormalities in NK-cell tumors are loss or gain of genetic materials. Deletion of 6q has the highest frequency (1). Other common abnormalities include del(11q), del(13q), del(17q), i(1q), i(6q), and 3+xp (66). However, the general consensus is that numerical genetic abnormalities usually represent secondary changes. In recent years, several case reports showed chromosome translocations, mainly involving 8p23, including der(8)t(8;8)(p23;q13) in a case of aggressive NK-cell leukemia/lymphoma (37), der(8)t(8;17)(p23;q24) in a case of nasal type NK-/T cell lymphoma (67), and der(8)t(1;8)(q10;p23) in a case of aggressive NK-cell leukemia (67). An add(8)(q23) abnormality was reported in a case of aggressive NK-cell lymphoma (67), and a nasal NK-/T-cell lymphoma (68). Therefore, translocation involving 8p23 appears to be a nonrandom chromosomal change in NK-cell tumors, especially aggressive NK-cell leukemia and nasal/nasal type NK-/T-cell lymphoma.

A report on the loss of material on 16p and 17p may represent the loss of p53 in the NK-cell tumor. In one study of 28 cases of nasal T-/NK-cell lymphomas, 86% had overexpression of p53 (31).

As mentioned before, t(15;17) has been demonstrated in only two cases of myeloid-/NK-cell acute leukemia (55). Two other cases of this leukemia showed chromosome 17q

abnormalities: one with del(17)(q25) and the other with t(11;17)(q23;q21) (65). Neither case responded to *all-trans*-retinoic acid treatment.

TABLE 6.32.3

Difference between Various NK Neoplasms

	Blastic NK-Cell Lymphoma/ Leukemia	*Myeloid/ NK-Cell Precursor Acute Leukemia*	*Myeloid/ NK-Cell Acute Leukemia*	*Nasal/ Extranodal NK/T-Cell Lymphoma, Nasal Type Lymphoma*	*Aggressive NK-Cell Lymphoma/ Leukemia*	*Indolent NK-LGL Leukemia*
$_{S}$CD3	-ve	-ve	-ve	-ve	-ve	-ve
$_{C}$CD3	-ve	ND	ND	+ve	+ve	+ve

CD56	+ve	+ve	+ve	+ve	+ve	+ve
CD57	-ve	-ve	-ve	-ve	-ve	-ve
CD16	-ve	-ve	-ve	-ve	-/+ve	+ve
CD2	-/+ve	+ve	-ve	+ve	+ve	+ve
CD7	-/+ve	+ve	ND	-/+ve	-/+ve	+/-ve
CD13/33	-ve	+ve	+ve	-ve	-ve	ND
HLA-DR	+ve	+/-ve	-ve	+ve	+ve	ND
TCRαß/γδ	-ve	-ve	-ve	-ve	-ve	-ve
TCR rear.	-ve	-ve	-ve	-ve	-ve	-ve
Cytotoxic proteins	-ve	ND	ND	+ve	+ve	+ve
EBV	-ve	-ve	ND	+ve	+ve	-/+ve

CD, cluster of differentiation; $_{s}$CD3, surface CD3; $_{c}$CD3, cytoplasmic CD3; TCR, T-cell receptor; rear, rearrangement; EBV, Epstein-Barr virus; NK, natural killer; ND, not done; -ve, negative; +ve, positive; HLA-DR, human leukocyte antigen-DR; LGL, large granular lymphocyte.

Several studies showed the overexpression of Fas molecule (CD95) and Fas ligand (CD95L) in nasal T-/NK-cell lymphoma (69). In addition, high levels of soluble serum CD95L has also been found in NK-cell lymphoma/leukemia in recent studies. The interaction between CD95 and CD95L may facilitate local tissue invasion, distant metastasis, systemic tissue damage, and immune evasion and is presumably the contributing factor to the marked aggressive behavior of these tumors. The salient features for laboratory diagnosis of NK-cell lymphoma or leukemia are summarized in Table 6.32.4.

Clinical Manifestations

Indolent NK-LGL leukemia is defined by an immunophenotype of CD56+ CD3-. The cutoff of the LGL level in the peripheral blood is 600/µL (13), and the median absolute NK-cell count in a large series is 2300/µL (29). Most of the reported cases may represent reactive lymphocytosis, but the minority of cases may show slow progress with organ involvement. Those cases can be considered true NK-LGL leukemia, if evidence of monoclonality is demonstrated. The clinical presentation may be similar to but milder than that

of T-LGL leukemia. Rheumatoid arthritis, pure red cell aplasia, vasculitis, and cyclic neutropenia are seen in rare cases (29). Neutropenia, if present, is moderate. Splenomegaly is the most frequent physical finding, but it is not as frequently seen as it is in T-LGL leukemia. Hepatomegaly is rare, and lymphadenopathy is not present in this entity. Recurrent bacterial infections are the major clinical presentation in cases with severe neutropenia. Substantial increases in NK cells have been observed in patients with other lymphomas, leukemias, immune thrombocytopenic purpura, or myelodysplastic syndrome (70,71). Therefore, these conditions should be included in the differential diagnosis.

TABLE 6.32.4

Salient Features for Laboratory Diagnosis of NK-Cell Lymphomas

1. Demonstration of azurophilic cytoplasmic granules in tumor cells by Wright-Giemsa stain on smear of peripheral blood or bone marrow, or tissue touch preparation
2. Expression of one or two NK markers (CD56, CD57, CD16)
3. Absence of surface CD3 but presence of cytoplasmic CD3 ($CD3_{\varepsilon}$)
4. Presence of cytotoxic proteins: Perforin, granzyme B, and TIA-1
5. Expression of some myeloid antigens (CD11b, CD11c)
6. Absence of B-cell antigens and other myeloid antigens (except for immature NK-cell lymphoma)
7. No expression of $TCR_{\alpha\beta}$ or $TCR_{\gamma\delta}$ proteins
8. No TCR gene rearrangements
9. Frequent demonstration of EBV genomes in tumor cells or EBV antibodies

EBV, Epstein-Barr virus; NK, natural killer; TCR, T-cell receptor; TIA, T-cell intracellular antigen.

Extranodal NK/T-cell lymphoma, nasal type, is the most common primary nasal lymphoma, especially in cases where the paranasal sinus is not involved. However, the involvement of nasopharynx and paranasal sinuses is seen in 37% to 50% of patients (32). The early clinical presentation is nasal obstruction, nasal discharge, and epistaxis. It finally progresses to a destructive nasal or midline facial tumor, with palatal destruction, orbital swelling, and edema. Although this tumor is usually localized in presentation, it may disseminate to distant sites, such as the skin, gastrointestinal tract, liver, lung, and testis (2,30,32). When an NK tumor is present as a primary tumor in these sites without nasal involvement, it is called nasal type or extranasal NK/T-cell lymphoma (Fig. 6.32.12). The extranasal tumor usually carries a worse prognosis than the primary nasal tumor.

The aggressive NK-cell lymphoma or leukemia is usually presented with systemic dissemination of the disease with bone marrow and late peripheral blood involvement (32,34,36, 37, 38, 39 and 40). Constitutional symptoms including fever, coagulopathy, bleeding tendency, hemophagocytic syndrome, and multiorgan failure are common. Most patients have hepatosplenomegaly, but lymphadenopathy and skin lesions may vary from patient to patient. Most patients are young and succumb within weeks to months after the diagnosis.

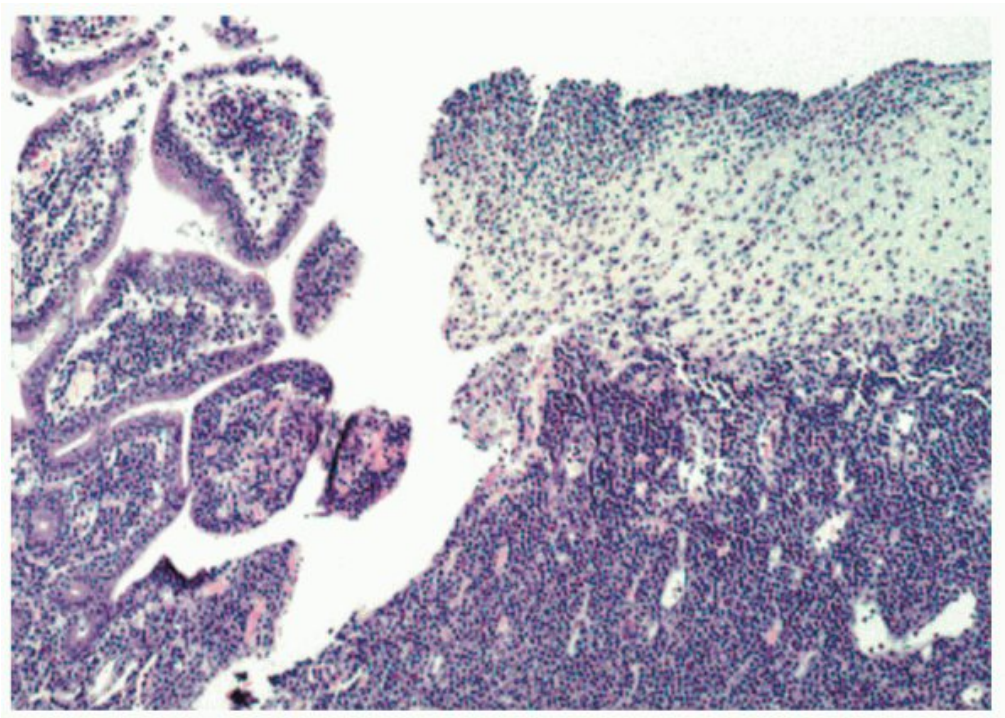

FIGURE 6.32.12 Case of extranodal natural killer (NK)-and/or T-cell lymphoma involving the ileum shows extensive tumor cell infiltration and destruction of normal architecture. The ulcerative mucosal surface is covered with fibrinopurulent exudates. Hematoxylin and eosin, 20× magnification.

Myeloid/NK-cell precursor acute leukemia presents as acute leukemia in the peripheral blood with consistent bone marrow involvement (13,18,42, 43, 44, 45, 46 and 47). Extramedullary lesions, involving mainly lymph nodes and mediastinum, are relatively common. The prognosis of this disease is poor with frequent relapses. This disease can be transformed from other hematologic disorders, such as acute T-lymphoblastic leukemia (46) and essential thrombocythemia (43).

There have been very few cases of myeloid/NK-cell acute leukemia reported in recent years (49, 50, 51, 52, 53, 54 and 55). This disease has both bone marrow and peripheral blood involvement in all cases. Despite the similarity of this leukemia and M3, bleeding diathesis is seldom seen. Extramedullary dissemination is usually not demonstrated, but involvement of the spleen (51) and thymus (52) has been reported. This disease has been presented secondary to T-cell lymphoma (54) and myeloma (53).

In the contrast, there is a plethora of new reports on the blastic NK-cell lymphoma/leukemia (14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26). The initial presentation of this disease is skin lesion, with subsequent bone marrow and lymph node involvement. Leukemic presentation is usually in the late stage of the disease. There are reports of terminal transformation to acute myeloid leukemia (21). Most patients have an aggressive clinical course.

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CASE 33 Adult T-Cell Leukemia/Lymphoma

CASE HISTORY

A 47-year-old heterosexual man was admitted to the hospital because of generalized lymphadenopathy for 2 weeks' duration. The patient was in generally good health until 2 weeks before admission, when he started to notice generalized skin rash, lymphadenopathy, and fever. The skin lesion did not respond to diphenhydramine (Benedryl). The patient was born in Colombia and migrated to this country 10 years previously.

Physical examination revealed generalized discrete and confluent erythematous skin rashes, generalized lymphadenopathy, and hepatosplenomegaly. Laboratory data showed a total leukocyte count of 7,700/μL with 51% neutrophils and 37% lymphocytes. His hemoglobin was 15.3 g/dL, and platelets 201,000/μL. The chemistry profile demonstrated generally elevated enzyme levels, including lactate dehydrogenase 363 U/L, alanine aminotransferase 63 U/L, and alkaline phosphatase 466 U/L, as well as a high serum calcium level (13.5 mg/dL). Viral serologic tests showed positive antibodies for cytomegalovirus, Epstein-Barr virus (IgG), hepatitis B virus, and human T-cell leukemia virus type 1 (HTLV-1).

Chest x-ray examination revealed enlarged hilar lymph nodes, a finding confirmed by computed tomography. The lymph node biopsy was

reported as diffuse mixed large and small cell lymphoma with positive T-cell markers. The bone marrow aspirate was, however, nondiagnostic. The patient responded well to chemotherapy and was discharged 3 weeks after admission to be followed in the outpatient clinic.

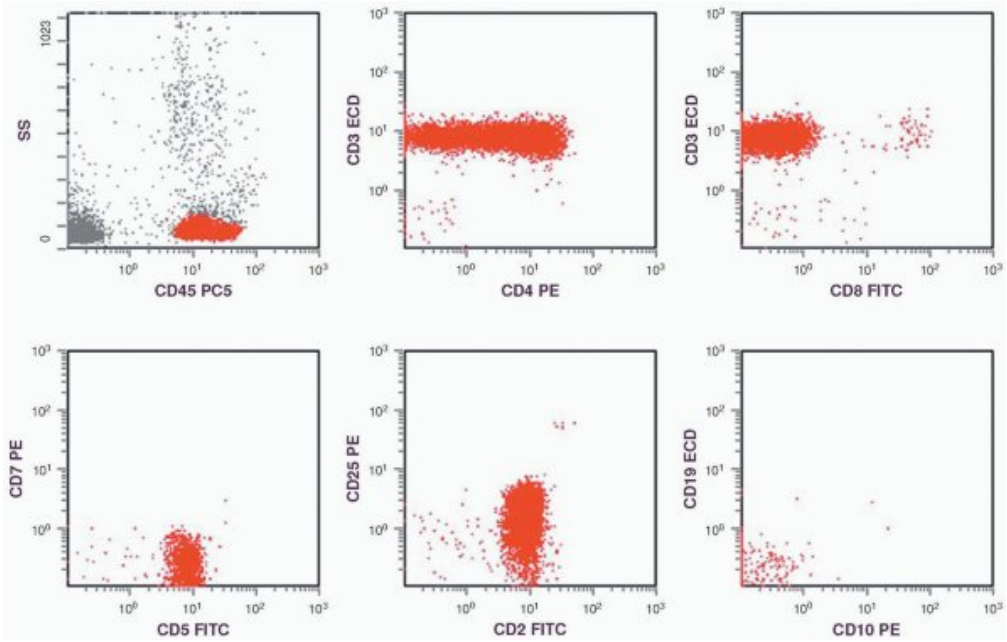

FIGURE 6.33.1 Flow cytometric histograms show positive reactions to CD2, CD3, CD4, CD5, and CD25, but negative reactions to CD7, CD8, CD10, and CD19. This immunophenotype is characteristic of, though not diagnostic for, adult T-cell leukemia/lymphoma (ATCL). These histograms are not from the current case. SS, side scatter; PC5, phycoerythrin-cyanin 5; ECD, phycoerythrin-Texas Red; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

FLOW CYTOMETRY FINDINGS

Peripheral blood: B-cell markers: CD19 1%, CD20 1%, human leukocyte antigen-DR (HLA-DR) 4%. T-cell markers: CD2 98%, CD3 96%, CD4 93%, CD5 95%, CD7 15%, CD8 5%, CD25 (interleukin 2 receptor [IL-2R]) 8%. Monocyte marker: CD147% (Fig. 6.33.1).

CYTOCHEMICAL STAINS

Acid phosphatase (AP): Focal paranuclear staining was sensitive to tartrate treatment. Periodic acid-Schiff (PAS): A few lymphocytes showed fine PAS-positive granules.

DISCUSSION

The first cases of adult T-cell leukemia/lymphoma (ATCL) were reported from the Kyushu Islands in Japan in 1977 (1). The circumscribed geographic distribution of the Japanese patients in southwestern Japan raised the question of a viral cause, which was later proved to be a retrovirus, HTLV-1 (2,3). Subsequently, patients with similar symptoms were reported from the Caribbean Basin (4,5) and from the south-eastern United States (6,7). Seroepidemiologic studies have indicated that HTLV-1 is also endemic in other Asian regions, such as Taiwan and Okinawa. ATCL cases have subsequently been reported from Hawaii, Britain, and the European continent (8). Current epidemiologic studies have confirmed that the major endemic areas for HTLV-1 infection are the Caribbean, southern Japan, Central and South Africa, and South America. Patients found in North America and Europe are mainly confined to certain immigrant groups and intravenous drug users (9). In the endemic areas, the seroprevalence varies between 0.1% and 30% (9). In Japan, the cumulative incidence of ATCL is estimated to be 4% among HTLV-1 carriers (10). In one study in Japan, ATCL accounted for 48% of all T-cell lymphomas (11).

The median age at diagnosis is about 35 years for patients in the United States, 40 years in the Caribbean Basin, and 60 years in Japan. HTLV-1 can be transmitted through sexual intercourse, blood transfusion, and breast feeding. However, most ATCL patients in endemic areas contract the disease through breast milk, and the disease has a long latent period of several decades after the initial infection (12). Transmission requires transfer of HTLV-1-infected cells and not free virus.

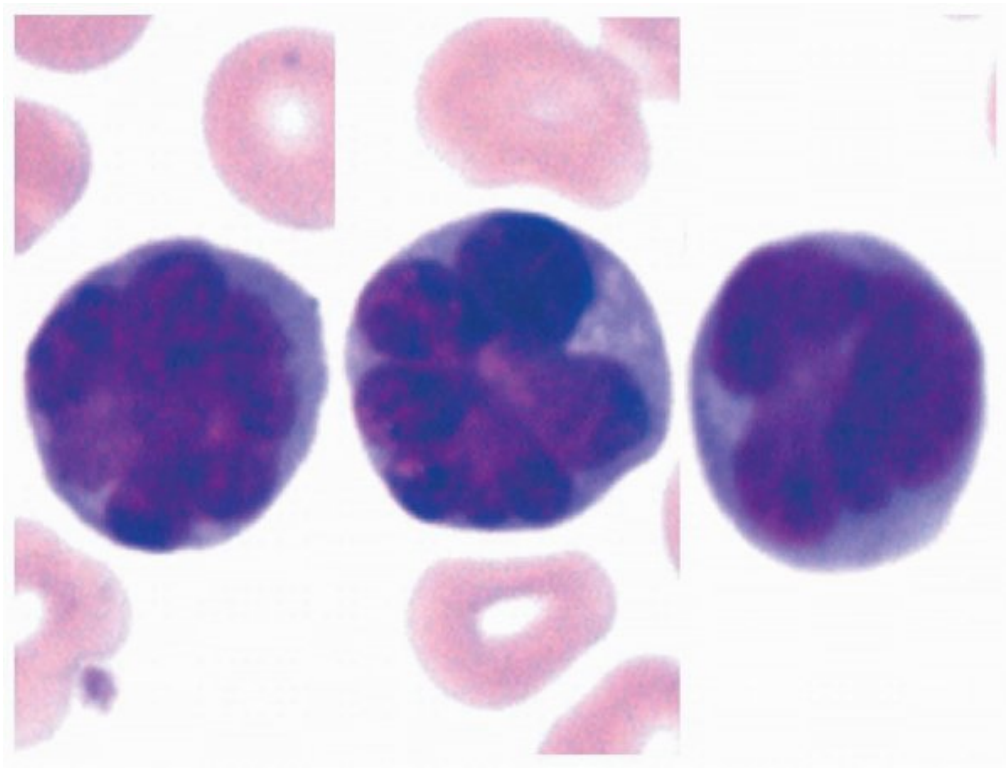

FIGURE 6.33.2 Three adult T-cell leukemia/lymphoma (ATCL) cells with hyperlobated nucleus and basophilic cytoplasm (flower cells) are detected in the peripheral blood of an ATCL patient. Wright-Giemsa, 100× magnification.

Morphology

The pathognomonic feature of ATCL is the presence of polylobated nuclei in the tumor cells (flower cells) (Fig. 6.33.2) (12,13). The nuclear chromatin is moderately condensed, and nucleoli are inconspicuous. The cytoplasm is slightly to moderately basophilic. The percentage of such atypical leukemic cells present in the peripheral blood varies depending on the clinical forms of ATCL. As will be discussed later, there are four clinical forms of ATCL: acute (55%), chronic (20%), smoldering (5%), and lymphomatous (20%) (9).

In the acute form, there are numerous atypical lymphoid cells in the blood, and those cells show marked variation in size and in shape (12). In the chronic form, the tumor cells are relatively uniform in cell size and in nuclear configuration. The percentage of atypical cells in the chronic form is lower than in the acute form. In the smoldering form, there are only 0.5% to 3% atypical cells present in the blood. The tumor cells are relatively large with indented, cleft, or bilobed nuclei (12). These tumor cells are sometimes difficult to distinguish from Sézary cells. The lymphomatous form shows no leukemic cells in the peripheral blood.

Skin lesions are a constant feature of ATCL and may mimic mycosis fungoides/Sézary syndrome (MF/SS) by showing plaque, tumor, and erythroderma forms (14). Only the papular form is specific for ATCL. Skin biopsy may show dermal infiltration by small or large tumor cells, including CD30-positive large cells, mimicking Ki-1 anaplastic large cell lymphoma (Fig. 6.33.3). Pautrier microabscesses are present in >50% of ATCL cases and thus do not constitute an absolute index for distinguishing ATCL from MF/SS (11).

When the lymph nodes are involved, the normal architecture is completely replaced by pleomorphic tumor cells of varying sizes (Fig. 6.33.4) (14). Reed-Sternberg-like cells may be present in some cases. The tumor cells in the lymph node or other tissues show even more variation than the cytology in the peripheral blood (13). The tumor cells can be small cell, medium-sized cell, large cell, mixed type, or pleomorphic type (13). The large cells have ovoid, vesicular nuclei, conspicuous nucleoli, and abundant pyroninophilic but PAS-negative cytoplasm (14).

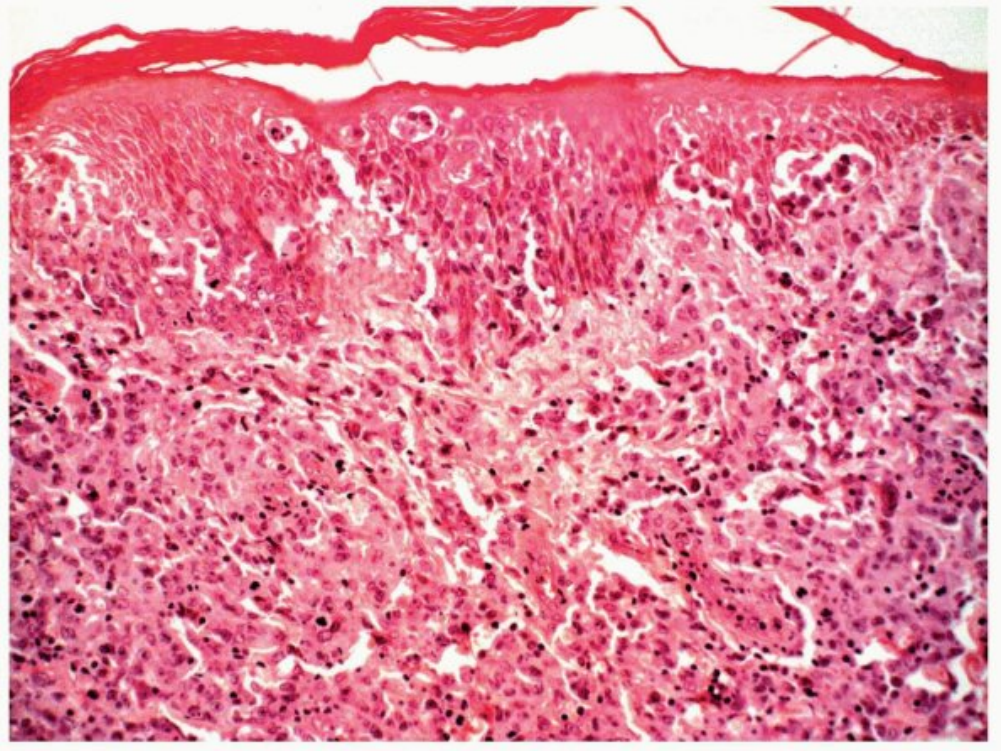

FIGURE 6.33.3 Skin biopsy of an adult T-cell leukemia/lymphoma (ATCL) patient shows multiple Pautrier microabscesses in the epidermis. The dermis is extensively infiltrated by large tumor cells. Hematoxylin and eosin, 20× magnification. (Case contributed by Dr. I. J. Su of the National Taiwan University.)

Immunophenotype

Immunophenotyping in ATCL is characterized by its helper T-cell phenotype (CD4+ CD8-), consistent presence of T-cell activation marker, CD25 (Tac antigen or interleukin 2 receptor [IL-2R]), and frequent loss of CD7, a pan-T-cell antigen

(15,16). Other positive T-cell markers include CD2, CD3, and CD5. The use of CD3/side-scatter gating in flow cytometric study may facilitate the identification of ATCL tumor cells (17).

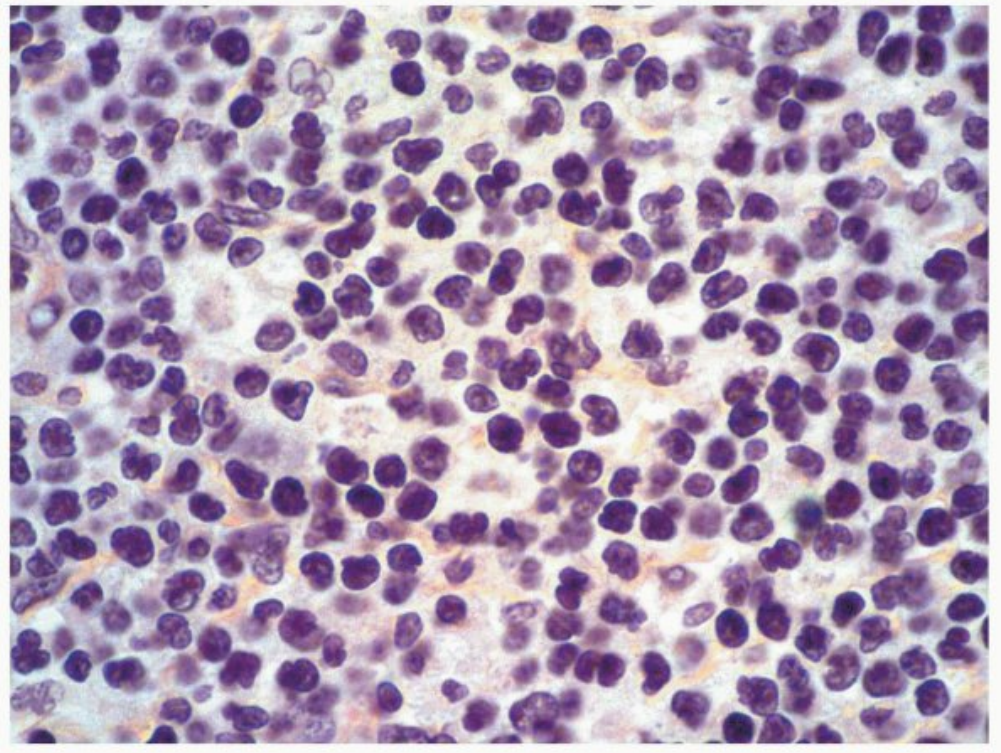

FIGURE 6.33.4 Lymph node biopsy reveals small tumor cells with irregular nuclear configuration, replacing normal architecture. Hematoxylin and eosin, 60× magnification. (Case contributed by Dr. I. J. Su of the National Taiwan University.)

As a peripheral T-cell neoplasm, terminal deoxynucleotidyl transferase (TdT) and CD1 are consistently negative in ATCL. B-cell markers are always negative, but myeloid markers are expressed in an ATCL cell line (18). An interesting finding related to the helper phenotype is that among the CD4 monoclonal antibodies, ATCL cells react only to Leu 3a and OKT-4A but not to OKT-4, a phenomenon that represents an epitope deficiency on the ATCL cells (19).

ATCL cells are also characterized by their frequent expression of activated cell antigens (20). A constant feature is the expression of CD25, the Tac antigen, which is related to the mechanism of leukemogenesis of T cells (21). In addition, high percentages of positivity with CD28, CD38, CD71, and Ki-67 are frequently demonstrated in ATCL cells in the acute stage and, to a much lesser degree, in the chronic stage (20). On the contrary, the expression of HLADR is higher in the chronic than in the acute stage of ATCL. Furthermore, the activated antigens are usually positive in a higher percentage of ATCL cells in the lymph nodes than in the peripheral blood, which is suggestive of a preferential proliferation of ATCL cells in the lymph node (20). The ATCL cells that infiltrate the skin lack CD29 and CD45RA, whereas tumor cells in the peripheral blood and lymph node may be positive for these antigens (14).

The immunophenotype of ATCL overlaps with that of MF/SS, including CD25, which can be present in some MF/SS cases (22). The Leu-8 (CD62 ligand [CD62L]) antibody (which is absent on MF/SS cells, but present on ATCL cells) used to be considered the most helpful marker for differentiation (14,23). However, it is seldom used now in clinical laboratories.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometric analysis may demonstrate a CD4-predominant T-cell phenotype with selective loss of CD7 and positive CD25 staining. Immunohistochemistry is not as convenient as flow cytometry because staining for multiple T-cell markers on the same cells cannot be done and CD25 antibody is not readily available.

Molecular Genetics

In the course of HTLV-1 infection, the viral genome is integrated into the nuclear DNA sequences of the host cells, becoming a provirus that can be replicated along with the host's cellular genome (24). Therefore, although HTLV-1 can be isolated from clinical specimens, it is easier to identify the viral genome in host cellular DNA. Frequently a monoclonal or oligoclonal pattern of HTLV-1 integration into cellular DNA can be demonstrated by Southern blotting technique in DNA extracted from ATCL cells. Currently HTLV-1 can also be detected by the polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR) technique using a probe specific for the HTLV-1 pol sequences or the pX gene (16,25,26).

HTLV-1 does not contain an oncogene, but its gene product, the pX protein, may act via a lymphokine, probably the adult T-cell leukemia-derived factor, to enhance the expression of IL-2R, which responds to the T-cell growth factor (8,21). Another theory is that

the pX region in the HTLV-1 genome encodes two regulatory proteins, Tax and Rex. The Tax protein could activate the transcription of IL-2 and IL-2R α-chain gene in vitro (16,27). Through the above mechanisms, the neoplastic T cells proliferate and the malignant clone expands.

Currently, the Tax protein is widely regarded as the key factor in the tumorigenesis of ATCL, but a unified concept of the detailed mechanism has not yet been established (10,12,28, 29 and 30). One of the theories is that Tax can inactivate p53, a tumor-suppressor protein that has the function of regulating the cell-cycle and apoptosis and maintaining the cellular genome integrity (28). Due to the inactivation of p53, Tax can immortalize the HTLV-1-infected cells and destabilize their genome. Subsequent oncogene involvement or other type of chromosomal aberrations is needed to transform the infected cells into neoplastic cells. This may explain why there is a long latent period between HTLV-1 infection and the development of ATCL. Another theory emphasizes the activation of the nuclear factor-κB (NF-κB) by Tax protein (12). The function of NF-κB includes promotion of cell proliferation, angiogenesis, and resistance to apoptosis. A recent study found overexpression of the CARMA1 gene in lymphoma patients with 7p22 amplification (29). This gene also activates NF-κB and may be complementary to the same function of Tax. However, the function of Tax is multifactorial, including activation of transcription factors, modification of signal transduction pathways, alteration of tumor suppressor protein functions, modulation of cell-cycle checkpoint proteins, interference with DNA repair, and inhibition of apoptosis (10).

Cytogenetic abnormalities are frequently seen in ATCL patients, but a specific cytogenetic marker(s) is lacking. For instance, 53% of the ATCL prodromal group had chromosomally abnormal clones (31). High frequency of allelic loss is seen on chromosomes 6q (41%) and 17p (48%) in patients with acute and/or lymphomatous ATCL (32). The authors suggested that a novel tumor suppressor gene on chromosome arm 6q and the p53 gene on chromosome arm 17p probably have an important role in the development of acute and/or lymphomatous ATCL (32). Indeed, p53 overexpression is observed in almost 50% of aggressive ATCL cases (33). Another study showed that the most common cytogenetic abnormalities in the acute subtype of ATCL were trisomy 3, trisomy 7, and the absence of the X chromosome (8). Chromosomal abnormality is seldom seen in chronic or smoldering ATCL, suggestive of clonal evolution during disease progression.

Recently the CDKN2 gene on chromosome 9q21 has been considered a tumor suppressor gene, playing an important role in the malignant transformation process induced by HTLV (34). Although alterations in the CDKN2 gene were detected in only 15% to 20% of ATCL patients, most patients with this alteration had a clinically aggressive form. Another study showed that methylation of the CDKN2A gene was more frequently demonstrated in fresh

tumor cells isolated from patients with the clinically aggressive form than from those with a less aggressive clinical course (35). Thus, methylation of CDKN2A was found in 47% of patients with the acute form, 73% of patients with the lymphomatous form, 17% of patients with the chronic form, and 17% of patients with the smoldering form. Recently, BCL11B overexpression has also been found in the acute form of ATCL cases (29). All these cytogenetic aberrations may represent the cumulative events after genome destabilization induced by the Tax protein, finally leading to the development of ATCL.

TABLE 6.33.1

Diagnostic Point System for ATCL

Criteria	*Points*
Hypercalcemia	1
Lymphomatous skin lesion	1
Leukemic phase (>2% abnormal lymphocytes)	1
T-cell lymphoma or leukemia	2
HTLV-1 antibody	2
CD25-positive tumor cells	1
HTLV-1-positive tumors	2

ATCL, adult T-cell leukemia/lymphoma; HTLV-1, human T-cell leukemia virus type 1; CD, cluster of differentiation.

Clinical Manifestations

The characteristic clinical features in ATCL are peripheral lymphocytosis with atypical lymphocytes, skin lesions, lymphadenopathy, and hepatosplenomegaly. Although these features may be seen in other T-cell neoplasms, a positive HTLV-1 serologic test and hypercalcemia are usually more supportive of the diagnosis of ATCL. The international collaborative study on the diagnostic criteria of ATCL was proposed in 1994 (Table 6.33.1) (14). Based on this system, seven diagnostic points indicate a definitive diagnosis, five or six is probable, three or four is possible, and fewer than three points is inconsistent with ATCL (14).

The clinical course of ATCL may follow a temporallyrelated spectrum, including preleukemic, smoldering, chronic, and subacute-to-acute stages (8). In the preleukemic stage, patients are asymptomatic with no organ involvement except for the bone marrow, which may or may not be infiltrated by tumor cells. Diagnosis is usually made by incidental findings of lymphocytosis with atypical lymphocytes. These patients are, however, seropositive for HTLV-1, and Southern hybridization or PCR frequently shows monoclonal integration of HTLV-1 provirus into tumor cell DNA. About half of the preleukemic patients recover spontaneously without further progression, whereas the remaining half progress through smoldering, chronic, and subacute-to-acute stage.

In one study, the median proviral DNA level was 212 copies/10^5 lymphocytes in the first 3 months after HTLV-1 infection (Time 1), 99 copies in 7 to 14 months after infection (Time 2), and 27 copies after 14 months' infection (Time 3) (36). The HTLV-1 antibody titers were low at Time 1, significantly increased by Time 2, and stable by Time 3. Some ATCL patients may have multiple HTVL integrations. When one tumor cell clone carried multiple copies, these patients had an extremely aggressive clinical course with the infiltration of unusual organs. When only one copy of the provirus was carried by each of the multiple clones, patients showed an indolent clinical course with skin lesions (37).

As mentioned before, ATCL can be divided into four clinical subtypes: Smoldering, chronic, acute, and lymphomatous forms, which may also represent different developmental stages of ATCL (Table 6.33.2) (38). The criteria include percentage of abnormal lymphocytes in the peripheral blood; presence or absence of hypercalcemia; levels of lactate dehydrogenase; and tumor involvement of the lymph nodes, liver, spleen, central nervous system, bone, gastrointestinal tract, skin, and lung. A study from Taiwan also included anemia, thrombocytopenia, eosinophilia, hypoproteinemia, hyperbilirubinemia, and elevated alkaline phosphatase levels in the differential criteria (15). The serum IL-2R levels can also help to distinguish the clinical subtypes. One study showed that the mean IL-2R level was 9740 U/mL in the acute and lymphomatous subtypes, 1961 U/mL in the chronic subtype, and 788 U/dL in the smoldering subtype (39). In a study of 124 cases of ATCL, the median survival time was 4 months in the acute subtype, 7 months in the lymphomatous subtype, 14 months in the chronic subtype, and 16 months in the smoldering subtype (40).

It should be emphasized that the characteristic hypercalcemia is seen in the acute stage secondary to oestolytic lesions (34). These patients may have abnormal bone scintigraphy, elevated alkaline phosphatase levels, and increased osteoclastic activity in bone marrow biopsy, but the parathyroid hormone, cyclic adenosine monophosphate (AMP), prostaglandin, and vitamin D levels in serum are normal or low (7,41). Therefore, the release of an osteoclast-activating factorlike substance by the tumor cells is suspected to be the mechanism of hypercalcemia (41). One study demonstrated a parathyroid hormone-related protein in cellular and extracellular sites of neoplastic tissues by immunohistochemical techniques in six of seven ATCL patients with hypercalcemia and suggested that this substance may be responsible for hypercalcemia (42).

Patients with ATCL are immunosuppressed, which may or may not be related to the heightened suppressor function. As a result, opportunistic infections, such as *Pneumocystis carinii* pneumonia, cytomegalovirus pneumonia, *Candida* sepsis, and various bacterial infections, are common and are frequently the cause of death in ATCL patients (7,8,15). More recently, strongyloidiasis has been found to be associated with HTLV-1 infection (33,43). For some unknown reason, patients with a strongyloides hyperinfection showed a high response rate to chemotherapy and prolonged survival (33).

HTLV-1 infection may cause different clinical manifestations: Besides ATCL, there are HTLV-1-associated myelopathy/tropical spastic paresis (HAM/TSP), uveitis, arthropathy, and infectious dermatitis (44). The mechanism for the induction of different clinical entities by HTLV-1 is still unclear. However, it may be related to the defect of the provirus and the dose of infection. Retention of the structural

genes by defective proviruses might be a risk factor for HAM/TSP development; retention of the regulatory genes in the pX region by the defective proviruses could be a risk factor for ATCL development (45). Another study showed that patients with ATCL had a higher percentage of HTLV-1-positive cells in the peripheral blood than did patients with HAM/TSP (8% to 93% vs. 3.1% to 8.5%) (46).

TABLE 6.33.2

Clinical and Laboratory Findings in Various Clinical Subtypes of ATCL

	Smoldering	*Chronic*	*Lymphoma*	*Acute*
Anti-HTLV-1 antibody	+	+	+	+
Peripheral lymphocytosis ($\times 10^6/\mu L$)	<4	≥4	<4	≥4
Abnormal T lymphocytes (%)	≥5	≥5	≤1	≥5
Polylobated lymphocytes	Rare	Rare	No	Frequent
Bone marrow infiltrate	-	+	-	+
Lymphadenopathy	-	+	+	+
Skin lesions	+	+	+	+
Hepatomegaly	-	+	+/-	+
Splenomegaly	-	+	+/-	+
Lytic bone lesions	-	-	-	+
Pulmonary lesions	-	-	+/-	+
Central nervous system lesions	-	-	+/-	+/-
Gastrointestinal tract lesions	-	-	+/-	+/-
Hypercalcemia	-	-	-	+
Elevated lactate dehydrogenase	+/-	+	+	+
Elevated alkaline phosphatase	-	-	-	+
Hypoproteinemia	-	-	+/-	+/-
Hyperbilirubinemia	-	-	-	+/-
Anemia	-	+/-	+/-	-
Thrombocytopenia	-	-	-	+/-

Eosinophilia	-	+/-	-	+/-

ATCL, adult T-cell leukemia/lymphoma; HTLV-1, human T-cell leukemia virus type 1.

The current patient had clinical manifestation of generalized lymphadenopathy, skin rash, laboratory findings of the characteristic lymphoid cells with polylobated nuclei (flower cells) in the peripheral blood, and hypercalcemia; thus, ATCL should be suspected. Immunophenotyping of the peripheral mononuclear cells showed a predominant helper T-cell phenotype with selective loss of CD7, which can be seen in both ATCL and MF/SS. In fact, the morphology of the atypical lymphoid cells in ATCL and SS is similar, and yet the low percentage of CD25-positive cells is more frequently seen in MF/SS. (The percentage of CD25-positive cells in the current case increased gradually to a high level after admission.) However, a positive serology for HTLV-1 and the high serum calcium level are strongly in favor of HTLV-1, even though these features can be seen occasionally in MF/SS. For differential diagnosis of post-thymic T-cell leukemias, the reader is referred to Table 6.16.1 in Case 16. The salient features for laboratory diagnosis of ATCL are summarized in Table 6.33.3.

TABLE 6.33.3

Salient Features for Laboratory Diagnosis of ATCL

1. Positive HTLV-1 antibodies
2. Monoclonal helper-T-cell phenotype
3. Positive for CD2, CD3, CD4, CD5, and CD25
4. Negative for TdT, CD7, CD8, and CD1
5. Identification of HTLV-1 proviral genome in tumor cell DNA by Southern blotting or PCR
6. Isolation of HTLV-1 virus
7. Hypercalcemia

ATCL, adult T-cell leukemia/lymphoma; HTLV-1, human T-cell leukemia virus type 1; CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase; PCR, polymerase chain reaction.

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CASE 34 Hepatosplenic T-Cell Lymphoma

CASE HISTORY

A 28-year-old man was admitted to the hospital because of fever of unknown origin. The patient had had a low-grade fever for 10 days without other symptoms. On admission, he appeared chronically ill with a temperature of 38.4°C. No lymph node was palpable. Complete blood cell count showed a hemoglobin level of 8.1 g/dL, hematocrit 24.4%, and leukocyte count 15,800/µL with 72% segmented neutrophils, 6% bands, 22% lymphocytes, and 1% nucleated erythrocytes. His platelet count was 127,000/µL, and reticulocytes 8.5%. Blood chemistry tests on admission showed an alkaline phosphatase level of 152 U/L, lactate dehydrogenase level of 540 U/L, and alanine amino transferase level of 58 U/L. Results of chest x-ray were within normal limits. Cultures for bacteria and fungi were negative. The result of a skin tuberculin test was negative.

Computed tomography of the abdomen revealed hepatosplenomegaly. Barium enema and upper gastrointestinal series were normal. A bone marrow biopsy showed a hypercellular bone marrow with erythroid hyperplasia and focal infiltration by immature lymphoid cells. Hemoglobin electrophoresis was consistent with diagnosis of thalassemia. Because of persistent fever, bone marrow abnormality, and splenomegaly, the patient underwent splenectomy, and a liver biopsy was performed. A lymph node biopsy was subsequently taken. After the operation, the patient started on chemotherapy, which resulted in normalization of his temperature and liver function. He subsequently had several episodes of recurrent fever, and leukopenia necessitated repeated chemotherapy. The patient was finally referred for allogeneic bone marrow transplantation.

FLOW CYTOMETRY FINDINGS

Splenomegaly specimen: B-cell markers: Immunoglobulin (Ig)G 14%, IgA 9%, IgM 11%, κ 12%, λ 10%, CD19 11%, CD20 10%, human leukocyte antigen-DR (HLA-DR) 19%. T-cell markers: CD3 79%, CD5 12%, CD7 4%, CD4 0%, CD8 0%. Monocyte marker: CD14 11%.

Liver biopsy: B-cell markers: κ 5%, λ 5%, CD19 0%, CD20 0%, HLA-DR 8%. T-cell markers: CD3 80%, CD5 5%, CD7 8%. Monocyte marker: CD11c 16%.

CYTOCHEMISTRY

Tumor cells in the liver biopsy were negative for terminal deoxynucleotidyl transferase (TdT).

IMMUNOGENOTYPING

No rearrangement was demonstrated in the Ig heavy-chain gene, and κ and λ light-chain genes. T-cell receptor (TCR) ß-chain gene rearrangement was also negative. TCR_{γ}-chain gene rearrangement analysis was not performed.

DISCUSSION

On the basis of gene arrangement, T cells can be divided into αß T cells and γδ T cells. In the peripheral blood, only 1% to 5% of lymphocytes are γδ T cells (1). γδ T cells may express a $C_{\gamma}1$-containing TCR or $C_{\gamma}2$-containing TCR. These two receptors can be roughly distinguished by the anti-Vδ1 (A13) and anti-Vδ2 (BB3) antibodies, respectively (2). The Vδ1 subset is preferentially distributed in the spleen and thymus, whereas the Vδ2 subset is preferentially distributed in the peripheral blood, lymph nodes, tonsils, skin, and mucosa (3,4). The highest concentration of γδ T cells is located in the spleen (2). The γδ TCR seems to direct T cells homing to the splenic sinusoids (5,6), which is a characteristic histologic feature of hepatosplenic γδ T-cell lymphoma, as defined by the Revised European American

classification of Lymphoid neoplasms (REAL). This is a rare disease, but approximately 61 cases have been reported (3,7, 8 and 9). Several cases of hepatosplenic αß T-cell lymphoma have also been reported recently, and they are indistinguishable morphologically and clinically from the hepatosplenic γδ T-cell lymphoma (10, 11 and 12). Therefore, the World Health Organization (WHO) classifies these two tumors into a single entity, the hepatosplenic T-cell lymphoma (HSTCL) (13).

γδ T cells are similar to natural killer (NK) cells in several respects. They may assume the morphology of large granular lymphocytes (2), and express both the NK markers (CD56 and CD16) and cytotoxic proteins T-cell intracellular antigen ([TIA]-1). However, γδ T cells can be distinguished from NK cells by the presence of TCR gene rearrangement and by the expression of CD3 and the TCR proteins.

As TCRγδ-chain genes arrange before TCRαß-chain genes do, the γδ T-cell neoplasms are mostly seen in precursor T-cell lymphoma/leukemia; only a small percentage of peripheral T-cell lymphomas are of γδ origin (1). Among the peripheral γδ T-cell lymphomas, hepatosplenic γδ T-cell lymphoma is the prototype, but rare cases can be detected in the nasal, respiratory, gastrointestinal, and cutaneous sites (2).

Morphology

Histologically, HSTCL is characterized by the presence of sinusoidal infiltration of the tumor cells in the spleen, liver, and bone marrow without involvement of the lymph nodes. The most characteristic features are usually demonstrated in the spleen. The spleen is often markedly enlarged, frequently in the range between 1,000 and 3,500 g (1). The cut surface is characteristically a homogenous purple-red color, reflecting the extensive red pulp infiltration. No nodular pattern is present because the white pulp is usually atrophic without tumor cell infiltration. Microscopically, there is extensive tumor cell infiltration in the red pulp cords of Billroth and the sinuses (Fig. 6.34.1). The red pulp sinuses are dilated, containing clusters of monomorphic tumor cells; this feature is characteristic though not diagnostic of this disease.

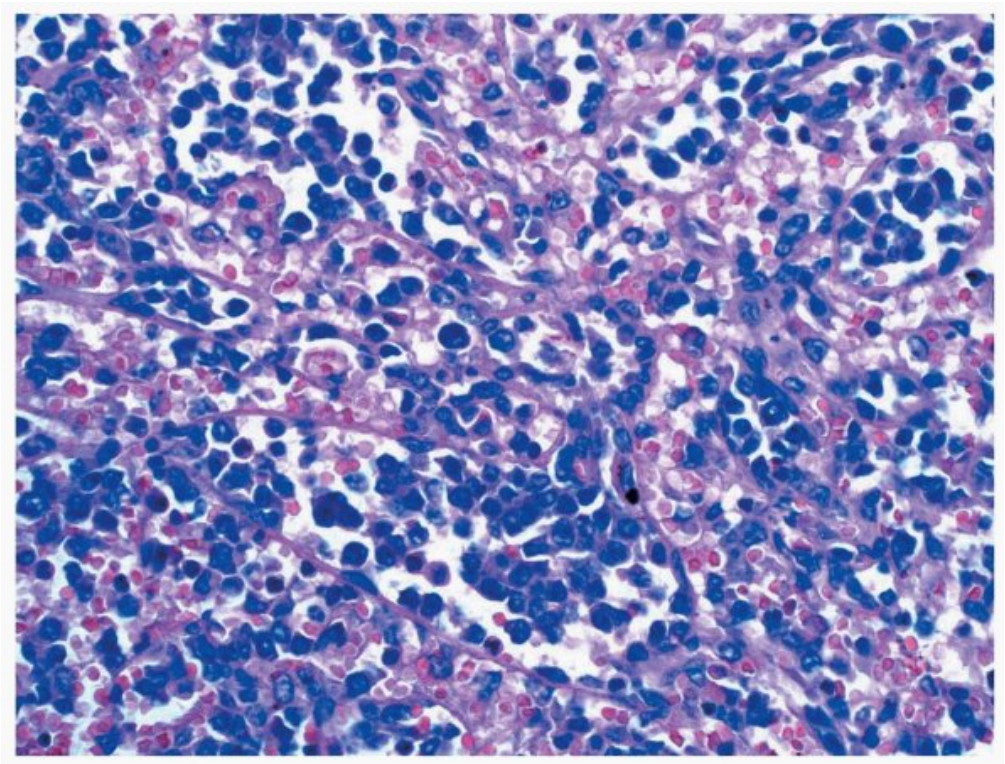

FIGURE 6.34.1 Splenectomy specimen shows dilated sinuses filled with lymphoma cells. Hematoxylin and eosin, 40× magnification.

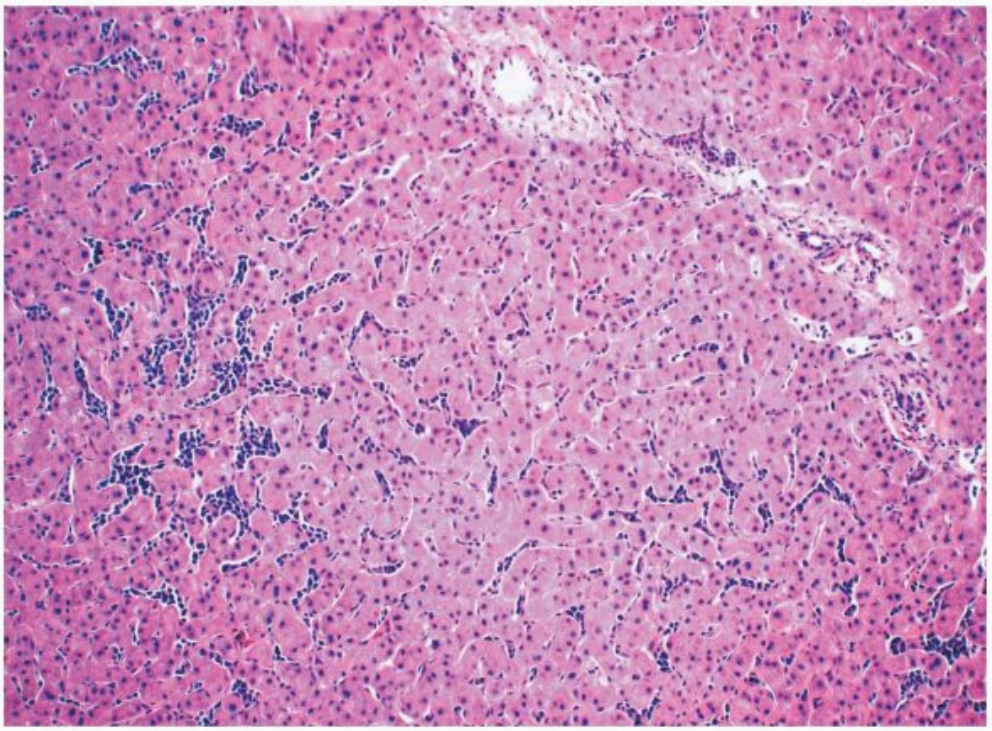

FIGURE 6.34.2 Liver biopsy shows sinusoidal infiltration by lymphoma cells, whereas the portal areas are devoid of tumor cell infiltration. Hematoxylin and eosin, 10× magnification.

The tumor cells are of small to medium size, with a moderate amount of cytoplasm. Although normal γδ T cells contain azurophilic cytoplasmic granules, these granules are seen only in a few cases of HSTCL. The nuclei can be irregular with slightly dispersed chromatin and inconspicuous nucleoli (1,3). When the disease advances, large tumor cells or blast forms may appear in the bone marrow (1,2). Histiocytosis, which may be accompanied by hemophagocytosis, is seen in the spleen in a small number of cases. Hepatomegaly without nodules is also a constant feature in this disease. Similar to the splenic lesion, sinusoidal infiltration by tumor cells is characteristic (Figs. 6.34.2 and 6.34.3). The portal areas are usually spared with tumor cells, or they show mild infiltration.

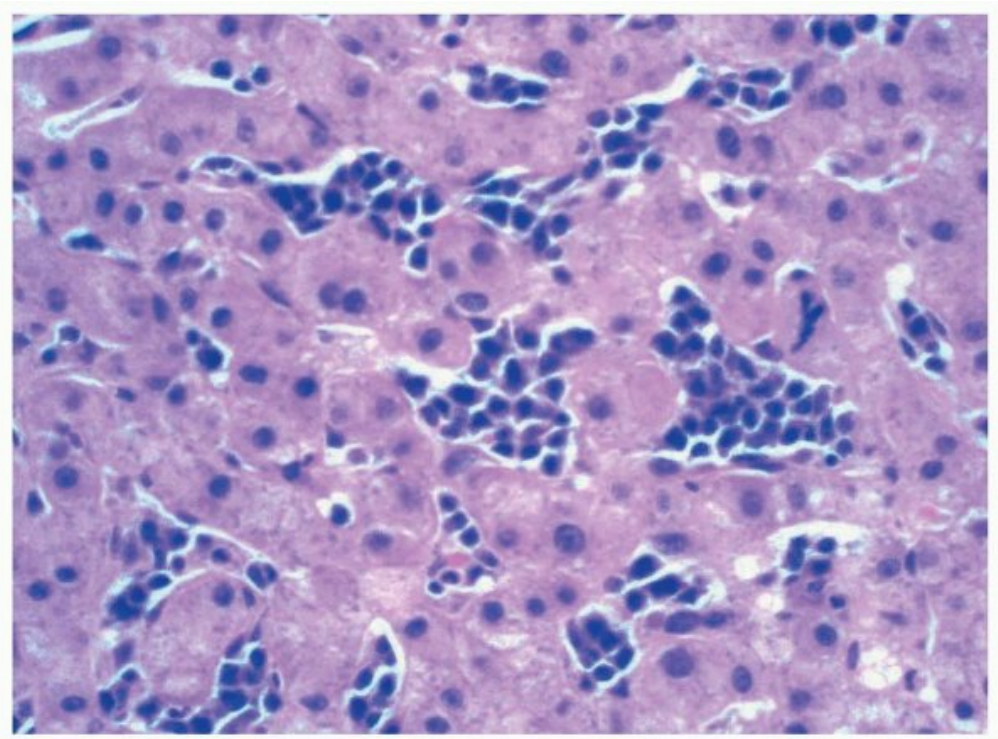

FIGURE 6.34.3 Higher magnification of the liver biopsy shows clusters of tumor cells in the sinuses. Hematoxylin and eosin, 40× magnification.

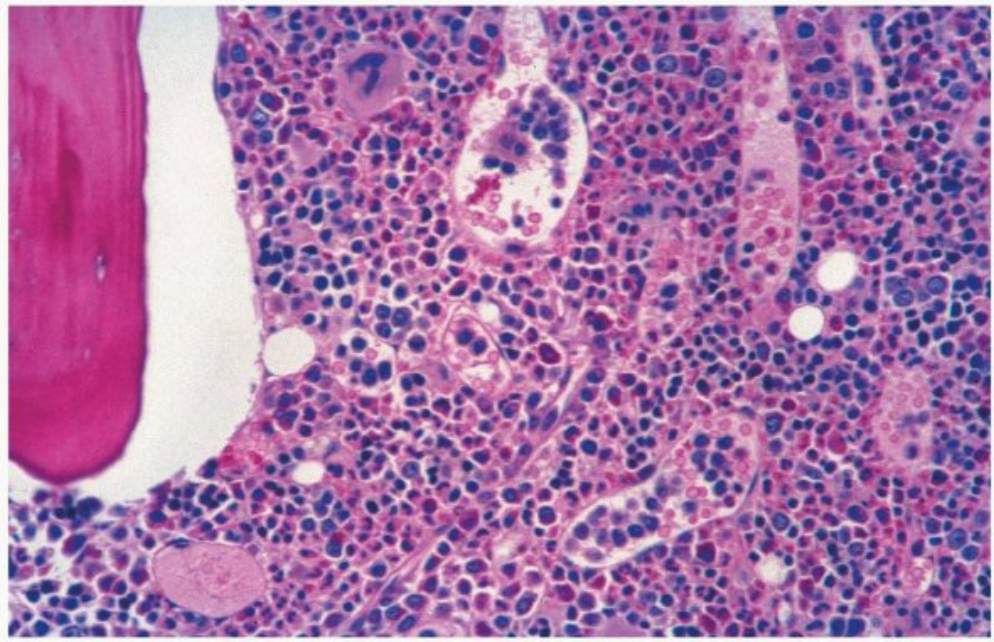

FIGURE 6.34.4 Bone marrow biopsy reveals sinusoidal lymphoma cell infiltration. Hematoxylin and eosin, 40× magnification. (Courtesy of Dr. Judith Brody, North Shore University Hospital, originally from Sun T. *Flow Cytometric Analysis of Hematologic Neoplasms*. Lippincott Williams & Wilkins; 2002.)

The bone marrow infiltration is usually mild, so the lesion is frequent missed (5,6,14). Therefore, immunohistochemical stains with CD3 and other markers are usually needed to accentuate the tumor cells. The sinusoidal infiltration pattern is an important clue to the diagnosis and should lead to further immunologic studies (Fig. 6.34.4).

Lymph nodes, as a rule, are not affected by this tumor (Fig. 6.34.5). Occasionally, the hilar lymph node of the spleen may show mild tumor cell infiltration.

The peripheral blood is usually devoid of lymphoma cells. However, a leukemic picture can be demonstrated in the late stage occasionally (3,15). The common hematologic findings are marked thrombocytopenia accompanied by certain degrees of anemia and leukopenia (1, 2 and 3). The diagnostic morphologic features of HSTCL are summarized in Table 6.34.1.

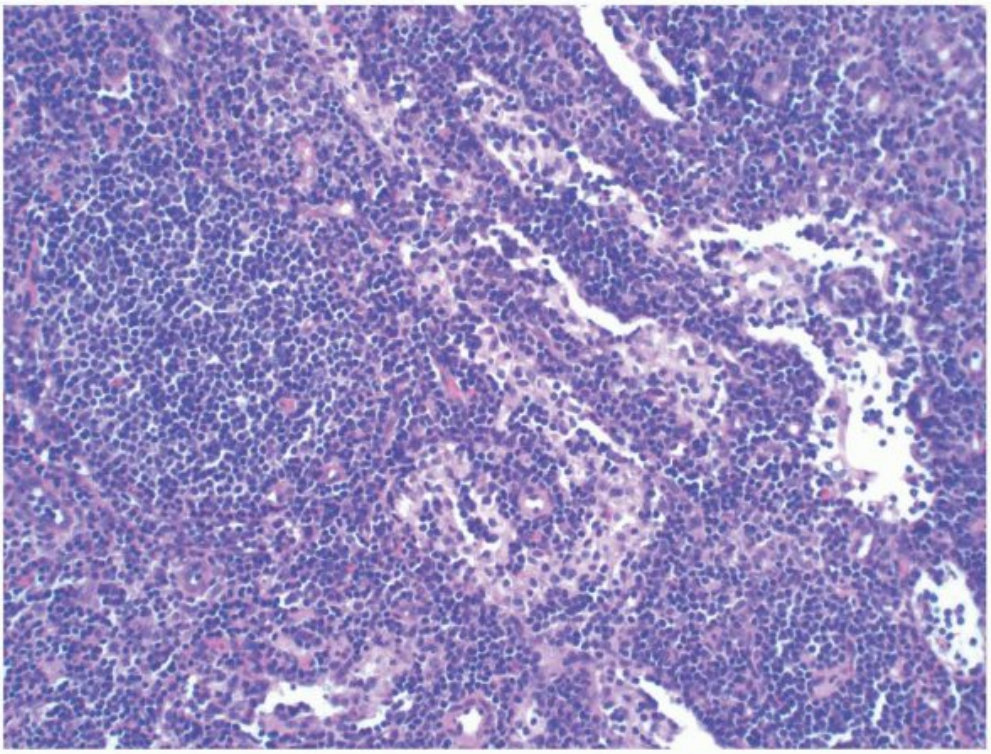

FIGURE 6.34.5 Lymph node from a patient with hepatosplenic T-cell lymphoma (HSTCL) shows only sinusoidal histiocytosis. No tumor cells are seen in the node. Hematoxylin and eosin, 20× magnification.

TABLE 6.34.1

Characteristic Morphologic Features of Hepatosplenic γδ T-Cell Lymphoma

Histologic pattern	Sinusoidal infiltration of tumor cells in the spleen, liver, and bone marrow
Cytology	Monomorphic medium-sized tumor cells with slightly irregular nuclei and abundant cytoplasm
Specific feature	Sinusoidal infiltration in the spleen, liver, and bone marrow without lymph node involvement

Immunophenotype

Because there are so many characteristic morphologic features, a constellation of these findings may reach a reasonable diagnosis. However, recent efforts have been made to diagnose the disease by bone marrow biopsy alone to avoid splenectomy and liver biopsy (3). As bone marrow is usually mildly involved without any pathognomonic features, immunophenotyping becomes most important for the diagnosis.

Antibodies βF1 and TCRδ1 can be used to identify αβ T cells and γδ T cells, respectively. Therefore, in cases of HSTCL, the immunophenotype is βF1-/TCRδ1+. However, during progression, γδCR may become lost, leading to a “TCR-silent” phenotype

(βF1-/TCRδ1-) (1). For the TCRδ variable region, most studies show that the tumor cells are positive only for Vδ1, not for Vδ2 (2,4,16,17). Because Vδ1 has a strong affinity to the splenic tissue, HSTCL always involves the spleen. However, all the above mentioned antibodies can only apply to frozen or fresh tissues. Therefore, in paraffin sections the cell lineage cannot be determined.

T-cell subset studies may help, as γδ T cells usually show a double-negative phenotype (CD3+ CD4- CD8-), whereas αβ T cells are either CD4+ or CD8+ (Fig. 6.34.6).

One of the exceptions is intestinal γδ T-cell lymphoma, in which CD8 is predominant (2). In HSTCL, CD2 is consistently positive, CD5 is characteristically negative, and CD7 can be positive or negative.

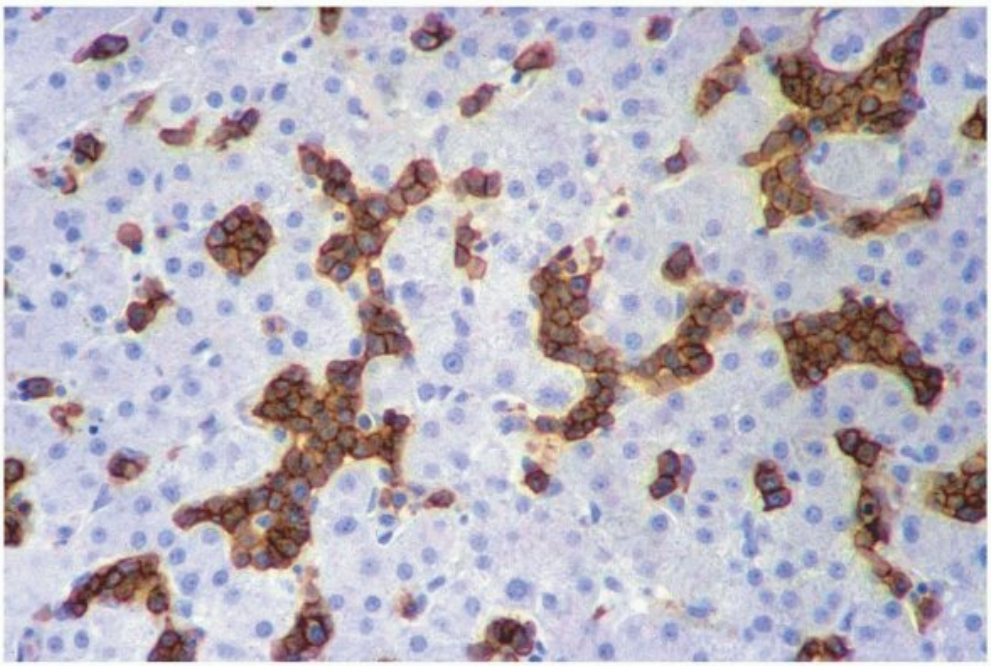

FIGURE 6.34.6 Liver biopsy reveals positive staining of the tumor cells with CD3 antibody. Immunoperoxidase, 40× magnification.

In addition to T-cell markers, the NK-cell and cytotoxic markers are important to establish the diagnosis. Among the NK cell-associated antigens, CD56, CD16, and CD11c are frequently positive, but CD57 is consistently negative (4). As TIA-1 (a cytotoxicity-associated molecule) has been demonstrated in the tumor cells of HSTCL, the neoplastic γδ T cells are considered cytotoxic T cells. However, other cytotoxic molecules such as perforin and granzyme B are frequently negative; HSTCL cells are, therefore, suggested by some authors to be inactivated cytotoxic T cells (1,2,18). Others consider HSTCL cells composed of a heterogenous population of both activated and inactivated cytotoxic cells because perforin and granzyme B can be detected in some cases (2). Besides the T-cell subset, the immunophenotype of hepatosplenic αβ T-cell lymphoma is similar to its γδ T-cell counterpart (10, 11 and 12).

Comparison of Flow Cytometry and Immunohistochemistry

For the diagnosis of HSTCL, immunohistochemistry is more useful than flow cytometry. The former can highlight the tumor cells and is particularly helpful in bone marrow biopsy, where tumor cell infiltration is usually mild. When frozen tissue is available, immunohistochemical stains can distinguish αβ T cells from γδ T cells. When necessary, immunohistochemical stains can also be used to identify Vδ1 and Vδ2 subtypes.

Molecular Genetics

As expected, HSTCL of γδ T-cell origin usually shows rearrangement of both TCRγ- and TCRδ-chain genes. However, TCRβ-chain gene rearrangement is also frequently detected in γδ T-cell lymphomas. It seems that γδ lineage commitment does not exclude TCRβ-chain gene rearrangements, nor do TCRβ gene rearrangements irrevocably incite the cell to αβ TCR expression (2). In contrast, TCRγ and TCRδ rearrangement can also be seen in αβT cells and the corresponding lymphomas (10,16). As mentioned before, HSTCL usually expresses Vδ1 protein, and the Vδ1 gene can also be demonstrated by gene rearrangement analysis using the Vδ1 probe (16).

Isochromosome 7q [i(7)(q10)] is the most common cytogenetic abnormality (2,4,19, 20, 21 and 22). The formation of isochromosome 7q is the result of losing one copy of the TCRγ gene (expressed on 7p15) and duplicating the TCRβ gene (expressed on 7q35) (22). Whereas this abnormality is also demonstrated in other hematologic malignancies (such as acute myeloid leukemia, acute lymphoblastic leukemia, prolymphocytic leukemia, and lymphoblastic lymphoma), its presence in those diseases is usually considered to be a secondary aberration associated with tumor progression (1,4,6). In HSTCL, isochromosome 7q is considered a primary aberration whether it is the

sole abnormality or associated with other abnormal karyotypes.

The second most common chromosomal abnormality in HSTCL is trisomy 8, which is usually seen in acute myeloid leukemia (22). In a review of the literature, 12 of 19 reported cases of HSTCL had trisomy 8, and 11 of these cases presented with concomitant i(7)(q10) (22). Because trisomy 8 is frequently coexistent with isochromosome 7q and often present at the time of disease progression, it is considered to be a secondary event.

Loss of Y chromosome is the third common abnormality in HSTCL. However, this abnormality has been described as an age-related phenomenon in healthy men; therefore, its significance in pathogenesis of HSTCL is unclear. Other occasionally reported nonrandom chromosomal aberrations include t(7;9)(p15;q13), t(7;21)(q11.2;p11.2), t(13q;14q), -11, -22, and 14p+ with t(1;14)(q21;p13) (2,4,16,19,22).

In most reports, molecular identification of viral genomes usually showed negative results (13,21,23). The Epstein-Barr virus (EBV) genome by in situ hybridization and polymerase chain reaction has proven to be negative in many cases (23). Viral studies of human T-cell leukemia virus type 1, human immunodeficiency virus, human herpesvirus-6, and human herpesvirus-8 have also been negative (14,23). However, EBV-encoded small RNAs and EBV genomes have been identified in a nodal γδ T-cell lymphoma (24) and in two Japanese cases of HSTCL (18).

In the current case, the lymph node biopsy was unremarkable. The sections from the splenectomy specimen, liver biopsy, and bone marrow biopsy showed extensive sinusoidal infiltration by medium-sized atypical lymphoid cells, mimicking malignant histiocytosis (25). However, both monocyte-histiocyte markers (CD11c and CD14) used in this study showed a low percentage of positive cells, a result that did not support the diagnosis of histiocytosis. In contrast, the high CD3-positive cell count with the concomitant loss of CD5 and CD7 in this T-cell population is consistent with a T-cell malignancy.

The diagnosis was substantiated by immunostaining the liver and spleen specimens. The immunoperoxidase stains revealed negative reactions to antibodies against cytokeratin, B-cell antigens (CD74, CDw75, and CD20), CD30, and CD15 but positive reactions to T-cell antibodies, UCHL1 (CD45RO), MT1 (CD43), and Leu 22 (CD43). The T-cell subset study showed the absence of CD4 and CD8 markers, which is characteristic of HSTCL. The pattern of sinusoidal infiltration of the spleen, liver, and bone marrow and the absence of lymph node involvement are certainly consistent with HSTCL. Our case did not show TCR gene rearrangement because a TCRγ or TCRδ gene probe was not used. The salient features for laboratory diagnosis of HSTCL are summarized in Table 6.34.2.

Clinical Manifestations

The clinical features of patients with HSTCL are characterized by hepatosplenomegaly without lymphadenopathy. Bone marrow is often involved at diagnosis; therefore, patients are invariably in stage 4 at presentation (2). For this reason, bone marrow examination should be the first step for the diagnosis of HSTCL. In most circumstances, a diagnostic bone marrow may prevent further invasive procedures such as splenectomy and liver biopsy.

Lymphoma cells are seldom detected in the peripheral blood until the terminal stage, but pancytopenia is a constant

feature in most patients and thrombocytopenia is most striking (5). The mechanism of pancytopenia may be associated with hypersplenism and/or the release of cytokines, such as interferon-γ by neoplastic γδ T cells (3).

TABLE 6.34.2

Salient Features for Laboratory Diagnosis of Hepatosplenic T-Cell Lymphoma

Major phenotype: CD3+ CD4- CD8- TCRγδ + TCRαß-
Minor phenotype: CD3+ CD4- CD8+ TCRγδ+ TCRαß-
Selective loss of CD5 and/or CD7 (some cases)
Presence of NK markers: CD16, CD56, CD11c
TCR δ-chain variable region: Vδ1+ Vδ2-
Cytotoxicity-associated molecules: TIA+, perforin±, granzyme B±

TCR γ/δ-chain gene rearrangement

CD, cluster of differentiation; TCR, T-cell receptor; NK, natural killer; TIA, T-cell intracellular antigen.

In a small group of patients, γδ T-cell lymphoma involved mainly skin or mucosa at various anatomic sites, including the gastrointestinal and respiratory tracts (26). Rarely, lymphadenopathy is the initial clinical presentation, but spleen, liver, and bone marrow involvement appears at a later stage (17).

Most patients are young (15 to 32 years at diagnosis) and predominantly male (5). A few pediatric cases have also been reported (22,27). Individuals usually present with pronounced clinical symptoms, including fatigue, fever, sweats, and abdominal pain (8,21). Hemophagocytic syndrome is an infrequent but serious complication, which often precipitates a rapidly downhill clinical course (2).

Many HSTCL cases have a condition of chronic immunosuppression or prolonged antigenic stimulation that includes transplantation, chemotherapy, hypogammaglobulinemia, T-cell deficiency, human T-cell leukemia virus type 1 infection, cytomegalovirus retinitis, and others (1,2,17,23,24). These predisposing factors are even more frequently encountered in nodal γδ T-cell lymphoma or γδ T-cell lymphoma involving the skin or mucosa. The above conditions may induce proliferation of γδ T cells, and an additional transforming event, such as the acquisition of isochromosome 7, may finally lead to irreversible growth of the malignant clone (2,3).

A recently reported case of HSTCL developed during pregnancy (9). The authors hypothesized that this might be related to decreased immunity or hormonal imbalance during pregnancy. An interesting association is that the pregnant uterus and the decidual lymphocytes contain high concentrations of perforin, which is one of the cytotoxic molecules expressed by the γδ T cells (9).

HSTCL is an aggressive lymphoma. Patients may respond to chemotherapy initially, but most cases show relapse after a few months to years (4). Splenectomy may induce elevated red blood cells and platelet counts, but it does not change the course of the disease (2). The medium survival for HSTCL is 12 to 18 months, with a range of 3 to 42 months (1,4). When patients have blastic large cell transformation, they usually die within a few months (4).

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CASE 35 Mycosis Fungoides and Sézary Syndrome

CASE HISTORY

A 72-year-old man presented with a 2-week history of a whole-body pruritic, erythematous rash with scaling as well as purpuric nodules on the right thigh. The patient also had increased fatigue, bilateral lower extremity swelling, and scrotal edema. He denied any trauma to the leg, or exposure to new medications, soaps, detergents, or perfumes.

A lower extremity ultrasound was negative for deep venous thrombosis. The nodule on the right thigh was aspirated, and Gram stain and cultures were negative. However, physical examination revealed posterior auricular, axillary, and inguinal lymphadenopathy.

A skin biopsy was performed that showed lymphocytic infiltration in the dermis and epidermis. The Oncology Service was consulted, and the oncologist recommended further examination of the peripheral blood and bone marrow. The peripheral blood showed a total leukocyte count of 10,000/μL with 26% neutrophils, 5% bands, and 55% lymphocytes. Many lymphocytes revealed cerebriform nuclei consistent with Sézary syndrome (SS). Atypical lymphocytes were also demonstrated in the bone marrow. A right axillary lymph node biopsy was subsequently performed that demonstrated total replacement of the normal architecture by the tumor cells. A computed tomography (CT) scan of the abdomen and pelvis showed mediastinal and abdominal lymphadenopathy.

The patient was treated with psoralen plus ultraviolet A (PUVA) and interferon-α, but he responded poorly. As the patient also had congestive heart failure, chronic renal insufficiency, and adult onset diabetes mellitus, his condition deteriorated rapidly, and he died approximately 3 months after the diagnosis.

FLOW CYTOMETRY FINDINGS

Peripheral blood: Cluster of CD2 94%, CD3 97%, CD3/CD4 94%, CD3/CD8 3%, CD5 96%, CD7 32%, CD19 1%, CD20 0%, κ 1%, λ 1%, CD14 0%, CD25 18%, CD30 3% (Fig. 6.35.1).

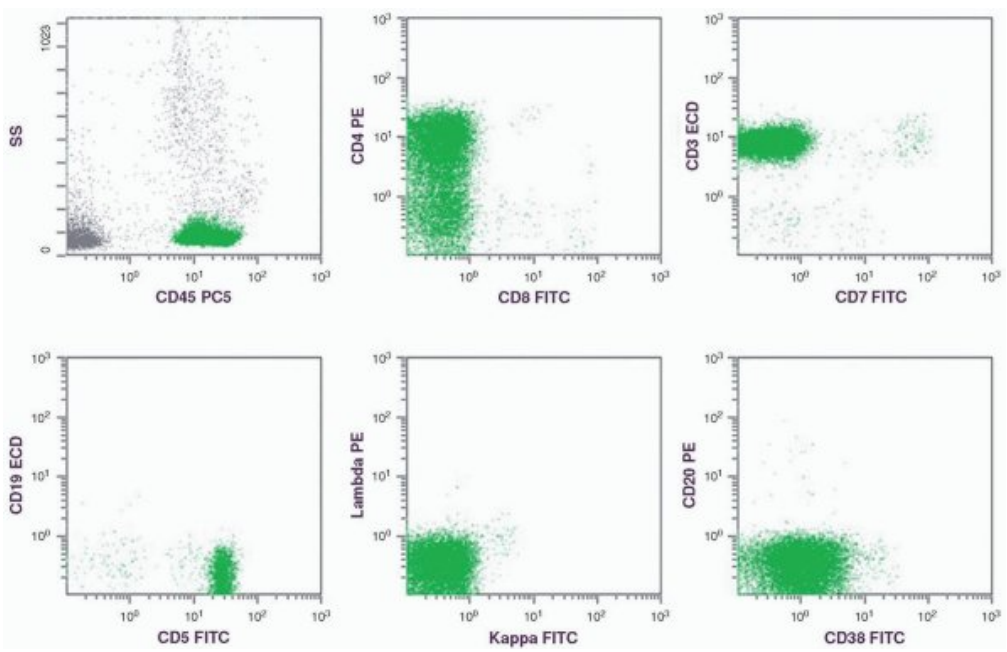

FIGURE 6.35.1 Flow cytometric histograms show positive for CD3, CD4, CD5, and CD38 (activated T-cell antigen), but negative for CD7, CD20, κ, and λ. SS, side scatter; PC5, phycoerythrin-cyanin 5; PE, phycoerythrin; FITC, fluorescein isothiocyanate; ECD, phycoerythrin-Texas-Red.

Bone marrow: CD3 94%, CD3/CD4 85%, CD3/CD8 10%, CD5 90%, CD7 12%, CD19 6%, CD25 0%.

Lymph node biopsy: CD3 90%, CD3/CD4 88%, CD3/CD8 2%, CD5 90%, CD7 19%, CD19 9%, CD25 8%.

IMMUNOHISTOCHEMISTRY FINDINGS

The lymphoid infiltrate in the skin showed positive stain for CD3 and CD4, but negative stain for CD8 and CD20.

DISCUSSION

Mycosis fungoides (MF) is a primary cutaneous T-cell lymphoma (CTCL) with a clinical presentation of patch, plaque, and tumor stages, and an epidermal and dermal skin infiltration by small to medium-sized lymphoid cells with cerebriform nuclei (1, 2 and 3). It is the most common CTCL, accounting for about one half of all primary cutaneous lymphomas (3). The incidence is increased in the United States, with about 0.2 cases per 100,000 population in 1973 to 0.4 cases per 100,000 population in 1984 (4). Thus the absolute number of new cases is about 1,000 per year. In the earlier literature, SS was considered an erythrodermic leukemia variant of MF (1,4). However, the new classifications, including the World Health Organization (WHO)-European Organization for Research and Treatment of Cancer (EORTC) classification for cutaneous lymphomas, generally separate them into two distinct entities, partly because their prognoses are markedly different (2,5,6).

Morphology

The histologic features of MF/SS overlap with other cutaneous lymphomas and even some benign inflammatory conditions. Therefore, clinical correlation is of utmost importance in making a morphologic diagnosis. The histologic pattern varies in different clinical stages of MF and in SS (3,7). A patch lesion may reveal scanty to patchy lymphocytic infiltration in the upper dermis. It is usually perivascular rather than bandlike. Intraepidermal lymphoid infiltration (Pautrier microabscesses) is rare or absent. Cytologic atypia, if present, is minimal. The plaque lesion is characterized by a dense, bandlike infiltrate of atypical lymphoid cells in the upper dermis (Fig. 6.35.2). Pautrier microabscesses are often present. The atypical lymphoid cells show hyperchromatic and convoluted nuclei and scant cytoplasm. The deep dermis and subcutis are seldom involved. The tumor lesion, in contrast, shows a dense infiltrate of atypical lymphoid cells that may extend into the

lower dermis and subcutis. At this stage, the tumor cells increase in number and in size, so that a mixed population of small, medium-sized, and large cerebriform cells as well as blasts with prominent nucleoli is present (4). Epidermal infiltration, in contrast, gradually disappears.

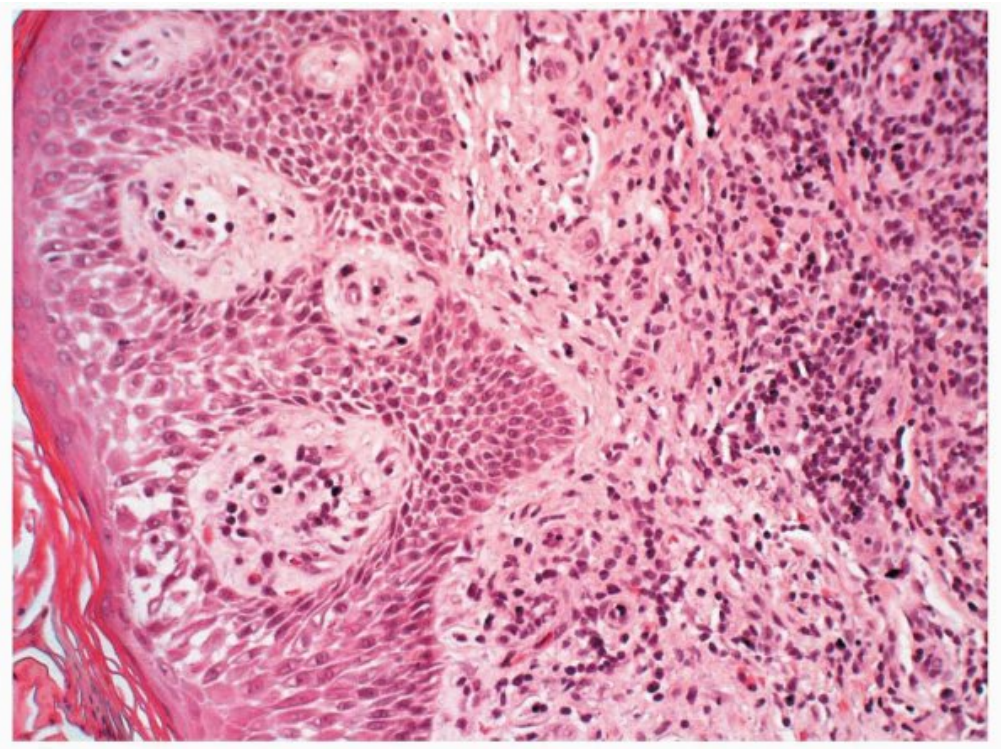

FIGURE 6.35.2 Skin biopsy from a patient with mycosis fungoides shows a few Pautrier microabscesses in the epidermis as well as a bandlike infiltration in the upper dermis. Hematoxylin and eosin, 20× magnification.

The features that are considered most specific for MF include Pautrier microabscesses and the presence of lymphocytes within the epidermis that are larger than those within the dermis (5). The definition of Pautrier microabscesses by the EORTC is “sharply marginated clusters of atypical lymphoid cells … that were closely opposed to one another with uniform cytologic features … with no plasma or fibrin deposition or significant cytopathic changes in the surrounding keratinocytes” (8). The presence of convoluted lymphocytes 7 to 9 microns in diameter, epidermal infiltration with single-haloed lymphocytes (lymphocytes surrounded with vacuoles), lining up of lymphocytes along the basal layer, and a lichenoid infiltrate that spares the dermal epidermal junction are also considered specific diagnostic features for MF (5,8,9).

The International Society for Cutaneous Lymphomas (ISCL) has classified erythrodermic CTCLs into three categories: SS, erythrodermic

MF (secondary erythrodermic CTCL developed in patients with MF), and erythrodermic CTCL, not otherwise defined (10). In this classification, SS consists of one or more of the following features: (i) an absolute Sézary cell count of ≥1000/μL (Fig. 6.35.3); (ii) demonstration of immunophenotypic abnormalities: CD4/CD8 ratio of ≥10 or higher or aberrant loss of pan-T-cell markers by flow cytometry; and (iii) demonstration of a T-cell clone in the peripheral blood by molecular or cytogenetic techniques (10).

Histologically, SS may differ from MF in more obvious monotonous cellular infiltration and less conspicuous epidermotropism (3). The cutaneous features may also be nonspecific in up to one third of cases (3). However, the lymph node usually shows effacement of the normal architecture with a dense, monotonous infiltrate of Sézary cells (3). In equivocal cases, ultrastructural morphometric measurement of the nuclear size and shape, chromatin distribution, and nuclear/cytoplasmic ratio are helpful in differential diagnosis (11). Sézary cells are positive for periodic acid-Schiff, tartrate-resistant acid phosphatase, and ß-glueuronidase, whereas normal lymphocytes are negative for these reactions (12).

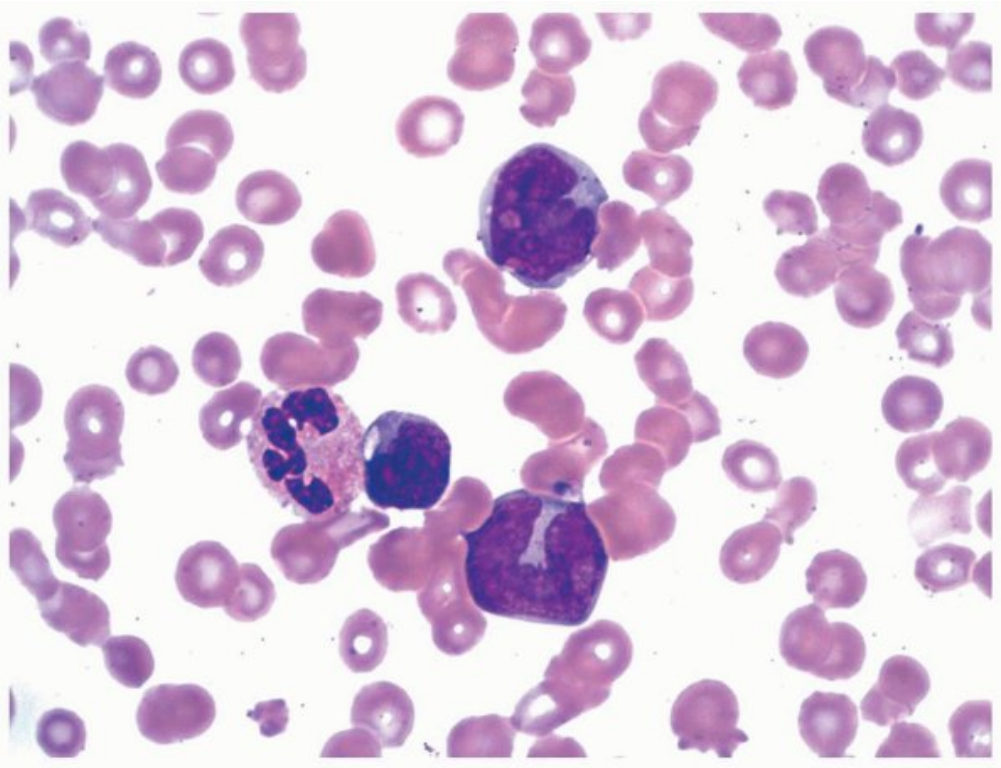

FIGURE 6.35.3 Peripheral blood smear from a case of Sézary syndrome reveals three Sézary cells and one neutrophil. Cell in the upper field shows typical cerebriform nucleus with prominent nucleoli. Wright-Giemsa, 100× magnification.

The histologic features in the lymph node of MF/SS cases are most commonly those of dermatopathic lymphadenopathy. Characteristically, the lymph node contains sinus histiocytosis, an abundance of pigment-laden macrophages, and various numbers of atypical lymphocytes depending on the stage of the disease (1). According to the degree of tumor cell involvement, a grading system divides the lymph node (LN) changes into four categories (13). LN1 shows dermatopathic lymphadenopathy with occasional atypical lymphocytes. LN2 is designated when clusters of <6 atypical lymphocytes are demonstrated. When >15 atypical cells are seen in clusters, it is graded LN3. When the normal architecture of the lymph node is partially or completely effaced, it becomes LN4 (Fig. 6.35.4). The WHO grading system has three categories. Grade I includes LN0 to LN2; grade II is equivalent to LN3, and grade III is equivalent to LN4 (2).

There are several less common presentations of MF (6,14), but only three variants are recognized in the new WHO-EORTC classification (3). Folliculotropic MF is characterized by perifollicular and intrafollicular infiltrates of atypical lymphocytes without epidermotropism and with or without follicular mucinosis (14,15). Pagetoid reticulosis is characterized by the presence of localized patches or plaques with an intraepidermal proliferation of neoplastic T cells (3). The granulomatous slack skin shows MF features in the epidermis and papillary dermis and sarcoidal granuloma or granuloma annulare-like palisading granuloma

in the reticular dermis. Lymphophagocytosis by the multinucleated giant cells is sometimes present (16).

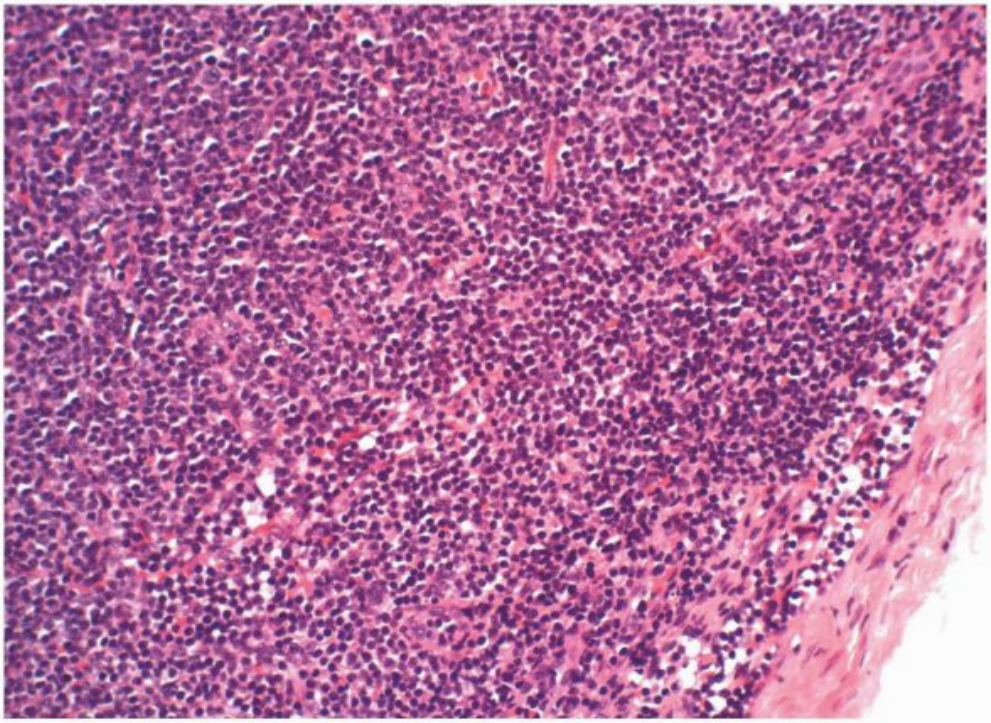

FIGURE 6.35.4 Lymph node biopsy from a case of Sézary syndrome shows total effacement of the normal architecture by the lymphoid tumor cells. The lesion is considered to be in the lymph node (LN)4 grade. Hematoxylin and eosin, 20× magnification.

Transformation of MF (T-MF) to a large T-cell lymphoma has been demonstrated in 8% to 55% of MF patients (17). The current definition of T-MF is the presence of >25% large cells in the infiltrate throughout the biopsy or presence of microscopic nodules of large cells (17,18). The major differential diagnosis is granulomatous MF in which the histiocyte/macrophages may mimic large cells (17). In case of doubt, a CD68 (PG-M1) stain should be performed to identify the histiocytes and/or macrophages. T-MF should also be distinguished from MF coexistent with a CD30+ lymphoproliferative disorder, such as anaplastic large cell lymphoma or lymphomatoid papulosis (17). T-MF carries a poor prognosis, whereas primary cutaneous CD30+ lymphoproliferative disorders have a good prognosis.

In a review of 29 autopsies of MF patients, lymph node was involved in 37.9%; spleen, 34.5%; liver, 31.0%; lungs, 27.6%; bone marrow, 24.1%; and pleura, stomach, and kidneys, 17.2% each (19). Hepatic involvement by MF/SS usually manifests as nodular aggregates of tumor cells in the portal zones or lobules, and bone marrow involvement may show nodular aggregates or diffuse infiltrate by the tumor cells (Fig. 6.35.5) (20). In general, bone-marrow infiltration is more frequently demonstrated in SS than in MF with limited skin disease (1). However, bone marrow can appear uninvolved even if significant numbers of circulating Sézary cells are detected (4).

Immunophenotype

Immunophenotyping is not very specific, but it is highly sensitive for the distinction of MF/SS from other non-neoplastic entities. Weiss et al. (21) found it helpful to distinguish dermatopathic lymphadenopathy from lymph nodes with Sézary cell involvement. MF/SS cells are generally positive for CD2, CD3, CD4, and CD5 but are characteristically negative for CD7, CD8, CD1, and terminal deoxynucleotidyl transferase (22,23). In immunohistochemical staining, CD45RO is usually positive, which makes Sézary cells the memory helper T cells (CD4+ CD45RO+) (4). Suppressor T-cell type (CD8+) has been demonstrated occasionally in MF cases. However, such cases have the same clinical behavior and prognosis as CD4+ cases (3).

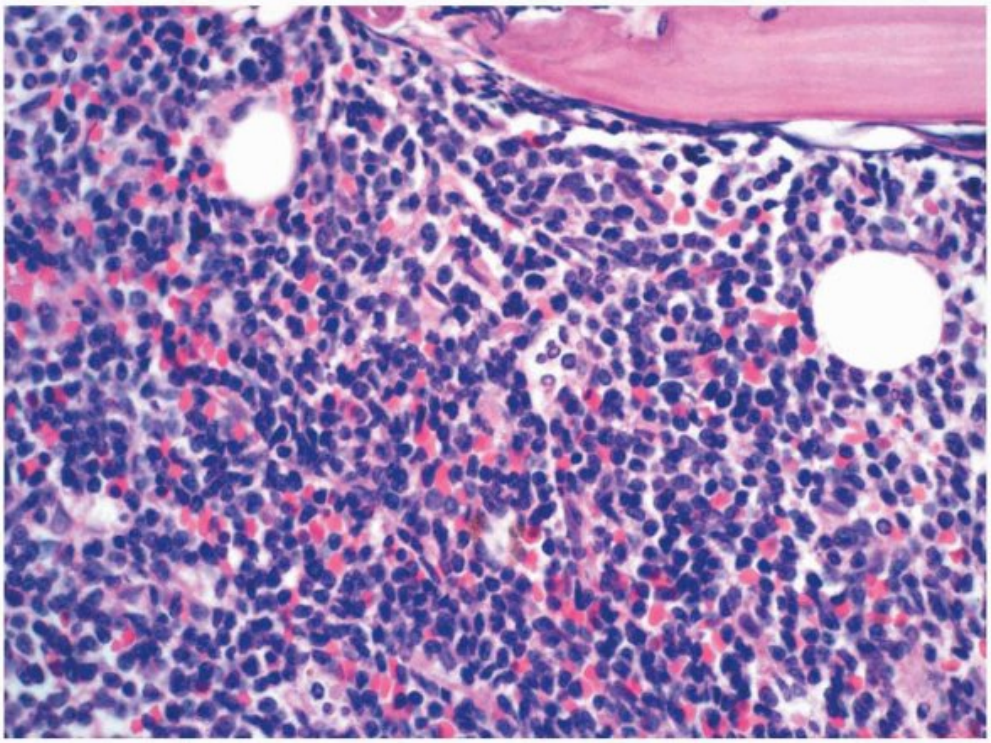

FIGURE 6.35.5 Bone marrow biopsy from a patient with Sézary syndrome reveals extensive interstitial infiltration by the small tumor cells with irregular nuclei. Hematoxylin and eosin, 40× magnification.

Although the absence of CD7 is an important finding in the diagnosis of MF/SS, CD7 is expressed in one third of MF patients (4). In the early stage of MF, the percentage of CD7 may be normal or only partly lost. Bergman et al. (24) found that when the percentage of CD7 was ≤36% by manual counting on immunohistochemical-stained sections, it would be supportive for the diagnosis of CTCL rather than inflammatory skin disorders. Another study used 50% as the cutoff point to define deficiency of CD7 and CD62 ligand (CD62L; L-selectin) in MF/SS (20). The loss of CD7 and CD62L on T-cells is more helpful in identifying lymph node than skin involvement by MF/SS, because this phenomenon is seldom demonstrated in reactive lymph nodes (20). Nevertheless, CD7 loss cannot reliably distinguish CTCL from benign dermatoses in cases with indetermined histology (24).

In some cases, the loss of CD2, CD3, or CD5 is also helpful in identifying neoplastic infiltration (24). Such deficiency is detected in the intraepidermal T cells but not intradermal T cells in 10% of cases of MF/SS (20). This discordance of immunophenotype between the epidermal and dermal T cells can be used to distinguish MF/SS from other skin lesions.

Michie et al. (25) studied the T-cell receptor (TCR) antigens in MF and found that early cases showed the normal CD3+ TCR_{β}+ TCR_{γ}- phenotype, but abnormal CD3/TCR_{β} antigen expression was seen in 50% of tumor stage MF cases (25).

There are several rare, but clinically important markers. CD25 is negative in at least half the cases of MF (4); its

expression may predict large cell lymphoma transformation and poor prognosis (26). The expression of cytotoxic proteins (T-cell intracellular antigen-1 [TIA-1] and granzyme B) has been found to be increased with progression from plaque stage to tumor stage in CD4+ MF patients (27). A few cases with the immunophenotype of CD4- CD8+ CD56+ TIA+, granzyme B+ have also been reported (28). In addition, there are two new markers, CD158k and T-plastin, which are reported to be specific for Sézary cells, but only limited studies are available (29,30).

The epidermotropism of malignant T cells in MF/SS is an intriguing phenomenon involving lymphocyte-keratinocyte interaction. An earlier study suggested that the lymphocyte function-associated antigen 1 (LFA-1) of the T lymphocyte may bind to the intercellular adhesion molecule 1 (ICAM-1), which forms the basis of epidermotropism (31). A current study presented a more complicated feature, which includes the interactions of lymphoma-cell integrin $\alpha_E\beta_7$, CC chemokine receptor (CCR)4, and the CD4 T-cell receptor complex with E-cadherin, CCL22, and major histocompatibility complex class II (MHC-II) molecules of the Langerhan cell, respectively (32). The cutaneous lymphocyte antigen (CLA) and the CCR4 on the lymphoma cell surface are responsible for extravasation from the dermal capillaries (32).

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometric analysis may demonstrate the selective loss of pan-T-cell antigens, particularly CD7, and the alteration of CD4/CD8

ratio that are essential for the diagnosis of MF/SS. However, this immunophenotype can be seen in other peripheral T-cell lymphomas as well. Immunohistochemistry has the advantage of demonstrating separate immunophenotypes in intraepidermal and intradermal T cells, and can be helpful to distinguish MF/SS from other skin lesions. Nevertheless, the calculation of CD4/CD8 ratio in immunohistochemistry is not as accurate as in flow cytometry.

In the current case, the patient had generalized pruritic, erythematous skin lesion and generalized lymphadenopathy. Skin biopsy showed intraepidermal Pautrier microabscesses and bandlike infiltration with cerebriform lymphocytes in the upper dermis. Lymphoid cells with the same morphology were found in the peripheral blood, bone marrow, and lymph nodes. The lymph nodes showed a total effacement of the normal architecture by the tumor cells. Immunohistochemical staining of the skin biopsy identified a helper T-cell phenotype (CD3+ CD4+ CD8-). Flow cytometry, in addition, demonstrated the selective loss of CD7 in the tumor cells in the peripheral blood, bone marrow, and lymph nodes. In conclusion, this patient had disseminated SS that led to his prompt death. Based on the Tumor, Node, Metastasis, Blood (TUMB) staging system, the patient was in stage IVa: T_4, N_3, M_0, B_1.

Molecular Genetics

Molecular biological technique is the most sensitive means used to identify MF/SS cells and distinguish them from reactive lymphoid cells in peripheral blood, lymph nodes, and visceral organs (33). Whittaker et al. (34) detected TCR gene rearrangement in the skin lesion and the peripheral blood in some patients with MF of both early and late stages. However, five patients in early stages showed only germline configuration, suggesting that some cases may start as a polyclonal lymphoproliferative disorder. Six patients with SS also showed no TCR gene rearrangement, but only one of the six patients died of an unrelated cause. On the contrary, five of eight SS patients with clonal disease died during the period of observation (34). Another interesting finding was that a dual TCR and heavy-chain gene rearrangement was detected in 4 of 11 patients with SS, but none of the MF cases showed additional heavy-chain gene rearrangement (34).

In early lesions of MF (patch stage) in which the number of tumor cells is low, Southern blotting may not be sensitive enough to detect clonal T-cell population (4,35). Therefore, polymerase chain reaction, with the capability of amplifying DNA, has gradually replaced Southern blotting. The positive rate of polymerase chain reaction is about 50% to 80% in patients with patch and plaque stage disease (16), whereas it is 100% for those in the tumor stage and 83% in those in the erythroderma stage (36). For indeterminate lesions, the positive rate is only 16% (21). In contrast, the demonstration of a monoclonal band, particularly a low density one, can be seen in benign T-cell proliferation as a result of local expansion of stimulated cells (4). Therefore, clinicopathologic correlation is still the gold standard for the diagnosis of MF/SS.

Clonal identification is probably more helpful for monitoring the disease than for initial diagnosis. Dereure et al. (37) found that clonal rearrangements in the skin disappeared in 8 of 13 patients who showed a complete clinical and histopathologic response, but not in 10 patients with histologically persistent disease. Flow cytometry, using TCR Vβ 14 antibodies, has also been successfully applied to therapeutic monitoring (38).

Many cytogenetic abnormalities have been found in MF/SS, but recurrent specific aberrations have not been identified thus far. The most common karyotypic abnormalities include loss of chromosome 10, deletions of 1p and isochromosome 17q, additions of 17p and 19p, and translocations involving 1p, 10q, and 14q (39). Abnormalities in p15, p16, and p53 tumor suppressor genes are also frequently found in MF cases (40). In addition, SS may also have chromosomal amplification of the JUNB gene, which is a member of the activator protein-1 (AP-1) transcription factor complex involved in cell proliferation and T-helper 2 (Th2) cytokine expression by T cells (41). In early stages of MF, the tumor cells display a predominant Th1 cytokine profile, which shifts to a Th2 profile when the disease progresses to advanced MF/SS (42). The salient features for laboratory diagnosis of MF/SS are summarized in Table 6.35.1.

Clinical Manifestations

MF/SS is usually seen in middle-aged adults with an average age of about 50 years (26). However, the disease can be seen in young adults (<20 years) or pediatric patients (43,44). The clinical course and pathologic findings are similar between early- and adult-onset MF, except that hypopigmentation

is more frequently seen in childhood MF (6,43,44). The male-to-female ratio is 2.2:1, and the black-to-white ratio is 2:1 (4). Clusters of cases within families have been reported, and an association with histocompatibility antigens, such as AW31, AW32, B8, BW35, and DR5, has been described.

TABLE 6.35.1

Salient Features of Laboratory Diagnosis of MF/SS

1. Skin biopsy shows characteristic histologic pattern.
2. Peripheral blood, bone marrow, and lymph node may show characteristic Sézary cells with cerebriform nuclei in SS.

3. Immunophenotype: Positive for CD2, CD3, CD4, and CD5

4. Important negative markers: CD7, CD8, CD1, TdT

5. Immunogenotyping: TCR gene rearrangement in most cases

6. Electron microscopic identification of characteristic nuclei

7. Sézary cells are positive for TRAP, PAS, and ß-glucuronidase.

MF, mycosis fungoides; PAS, periodic acid-Schiff; SS, Sézary syndrome; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase; TRAP, tartrate-resistant acid phosphatase.

The etiology of SS/MF is still not clear, but a retroviral cause has been suggested. The association of human T-cell leukemia virus type I with MF/SS is supported by evidence based on serology, molecular biology, and ultrastructural studies (1,4,45,46). However, other studies failed to substantiate these findings (1,4).

The clinical course of MF/SS is unusually indolent and is frequently preceded by a premalignant phase for several years. Many cases have an orderly progression from limited patches to generalized patches, plaques, and tumors. Patients may also have alopecia, palmoplantar hyperkeratosis, onychodystrophy (thickened nails), or ectropion (drooping eyelid) (1). Nodal and visceral involvement is usually seen in a later stage. Infection is still the major cause of death (47). Transformation into high-grade lymphomas and coexistence with second malignancies (colon and lung cancers) have been reported (18,48).

Lymphadenopathy has been found in 60% of patients at presentation (49). Histologic examination of these lymph nodes usually reveals features of dermatopathic lymphadenopathy. However, when immunophenotypic, cytogenetic, or molecular techniques are used, neoplastic cells can be identified in up to 85% of these nodes (25,50).

The existence of leukemic phase depends on the stage of development: 12% in the plaque stage, 16% in the tumor stage, and 100% in patients with generalized erythroderma (51). When peripheral blood is involved, about 50% of patients have lymphadenopathy (51).

Bone marrow involvement has been demonstrated in 21.7% of specimens in one report (52). An infiltrative pattern in the marrow was related to an advanced stage with 100% peripheral blood involvement, 75% generalized erythroderma, and 65.2% nodal or visceral disease (52). In contrast, a nodular pattern in the marrow was seen in an earlier stage with 20% peripheral blood involvement and 20% nodal or visceral disease.

Visceral dissemination is most commonly seen in the liver, spleen, and lungs and is associated with short survival times (median, 25 months) (26). Epithelial surfaces, such as genitourinary tracts, are preferentially involved (26,53). Most patients with visceral involvement also have lymphadenopathy and circulatory Sézary cells (51).

The standard staging classification system for MF is the tumor, node, metastasis, blood (TUMB) system first proposed by the National Cancer Institute (Table 6.35.2) (1). However, the original criterion of using 5% abnormal lymphocytes in the peripheral blood as the cutoff point for blood involvement is being challenged. The current practice by many MF referral centers is to raise the threshold to the level of 20% lymphocytes or an absolute Sézary cell count of ≥1,000/µL to consider peripheral blood involvement (1,10). This stricter criterion is partly due to the fact that small numbers of Sézary-like cells can be seen in the peripheral blood in other conditions, such as benign inflammatory dermatoses, rheumatic disease, sepsis, and virus-induced lymphocytosis (11).

The treatment and prognosis of MF/SS depend on the stage of the disease. For patients in the T1 and T2 stages, the treatment is limited to skin-targeted phototherapy (e.g., PUVA), topical application of nitrogen mustard or other chemicals, or radiation therapy (1,3,42,54). Multiagent chemotherapy is used only in systemic disease. Extracorporeal photopheresis has been reported to be an effective treatment for SS or erythrodermic MF (3).

The 5-year survival for stage I is 80% to 90%; stage II, 60% to 70%; stage III, 40% to 50%; and stage IV 25% to 35% (2). When transformation to large cell lymphoma occurs, the mean survival time is 22 months because of the frequent presence of extracutaneous progression (19).

For the differential diagnosis among T-cell lymphomas and leukemias, the reader is referred to Case 17. The distinction between MF/SS and other CTCLs is sometimes difficult. However, other CTCLs differ from MF/SS in showing no protracted skin lesions; occasional spontaneous regression; the presence of mixed cellular components, including a large number of monocyte-macrophages, heterogeneous phenotype; and no leukemic phase (55).

TABLE 6.35.2

TUMB Staging System for Cutaneous T-Cell Lymphomas

T (tumor)

- T_1: Cutaneous patches and/or plaques covering <10% of body surface
- T_2: Generalized patches and/or plaques covering >10% of body surface
- T_3: One or more cutaneous tumor nodules
- T_4: Generalized erythroderma

N (lymph node)

- N_0: Lymph nodes clinically normal and histologically negative for tumor cells
- N_1: Lymph nodes clinically enlarged but histologically negative
- N_2: Lymph nodes clinically normal but histologically positive
- N_3: Lymph nodes clinically enlarged and histologically positive

M (metastases, visceral organs)

- M_0: No visceral organ involvement
- M_1: Visceral involvement present

B (blood)

- B_0: Atypical circulating cells not present (≤5%)
- B_1: Atypical circulating cells present (≥5%)

Staging

- Ia: T_1, N_0, M_0, B_0
- Ib: T_2, N_0, M_0, B_0

IIa: T_{1-2}, N_1, M_0, B_0

IIb: T_3, N_{0-1}, M_0, B_0

IIIa: T_4, N_0, M_0, B_0

IIIb: T_4, N_1, M_0, B_0

IVa: T_{1-4}, N_{2-3}, M_0, B_0

IVb: T_{1-4}, N_{0-3}, M_1, B_1

TUMB, tumor, node, metastasis, blood.

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CASE 36 Peripheral T-Cell Lymphoma, Unspecified

CASE HISTORY

A 65-year-old woman presented with low-grade fever, night sweats, and weight loss for several months. Physical examination by her private care physician revealed splenomegaly and multiple lymphadenopathies, involving cervical, retroperitoneal, and mediastinal lymph nodes. No hepatomegaly was found. Peripheral blood examination was essentially normal with a total leukocyte count of 4,600/μL, hematocrit 45.4%, and platelets 156,000/μL. She was admitted to the hospital for further studies. A cervical lymph node biopsy was performed and was diagnosed as peripheral T-cell lymphoma (PTCL). A bone marrow biopsy, however, showed no lymphoma involvement.

She was treated with chemotherapy and did not respond to the treatment at first. After switching to another regimen, she showed symptomatic improvement as well as shrinkage of lymph nodes in the mediastinum and retroperitoneum. However, she suffered neutropenic fever and peripheral neuropathy in the lower extremities.

Two years later, multiple skin nodules were found on her left forearm and both shoulders. Skin biopsy showed B-cell lymphoma, and subsequent skin biopsy 1 year later revealed amyloid deposits. Nevertheless, a mucosal biopsy did not detect amyloidosis, and echocardiogram showed no amyloid cardiac involvement. Serum protein electrophoresis and immunoglobulin quantitation revealed no evidence of myeloma.

She continued to receive chemotherapy as well as local radiation of the skin lesions. The patient was followed in the hematology/oncology clinic for periodic evaluation of her skin lesions, adenopathy, hepatosplenomegaly, complete blood cell counts, and lactate dehydrogenase levels.

FLOW CYTOMETRIC FINDINGS

Lymph node biopsy: T-cell markers: CD2 88%, CD3 82%, CD3/CD4 70%, CD3/CD8 6%, CD5 80%, CD7 51%; B-cell markers: CD19 14%, CD20 12%, κ 8%, λ 4% (Fig. 6.36.1).

IMMUNOHISTOCHEMICAL STAINS

The lymph node biopsy showed positive stains for CD3, CD4, CD5, CD43, and CD45, but negative stains for CD8, CD20, and Alk1 on the tumor cells.

DISCUSSION

PTCL is composed of a heterogenous group of T-cell tumors with different clinical and morphologic features. The Working Formulation (1) and Kiel scheme (2) classify PTCL mainly on the basis of morphology. The Revised European-American classification of lymphoid neoplasms (REAL) and the World Health Organization (WHO) define PTCL by comprehensive clinicopathological features (3,4). Clinically, PTCL can be divided into three groups: nodal, extranodal, and leukemic/disseminated (5), and each clinical subtype contains several well-defined entities (Table 6.36.1). T-cell tumors that cannot be classified into one of those entities is designated as peripheral T-cell lymphoma, unspecified (PTCL-U). PTCL accounts for 7% to 10% of all non-Hodgkin lymphomas (6). However, the frequency of PTCL varies greatly in different geographic regions. One study of PTCL (excluding anaplastic large cell lymphoma) showed that the frequency varied from 1.5% in Canada to 18.3% in Hong Kong (7). Among the PTCLs, the most common entities are PTCL-U, angioimmunoblastic T-cell lymphoma, anaplastic large cell lymphoma, and cutaneous T-cell lymphoma (8). These four entities account for approximately 80% of all PTCLs in the United Kingdom (8).

Morphology

PTCL-U is usually a nodal lymphoma with extensive infiltration of the lymph node and effacement of the normal architecture (4,9,10). There is a great variation of cytologic features. Most cases show a mixture of large and small tumor cells, but predominantly large cells or predominantly small cells can also be found in some cases (Table 6.36.2). The nuclear configuration is usually irregular, and the cytoplasm is frequently transparent or lightly stained (clear cells) (Fig. 6.36.2). Although clear cells can

be seen in B-cell lymphomas, the presence of clear cells should raise the suspicion of a T-cell lymphoma. The chromatin pattern in large tumor cells is often vesicular or dispersed with or without the presence of nucleoli. The small tumor cells usually show a clumped chromatin pattern without nucleoli. Even in the small cell variant, large cells, multinucleated giant cells, or Reed-Sternberg-like cells may be present (Fig. 6.36.3). In addition, eosinophils, plasma cells, and epithelioid histiocytes can be seen in the background.

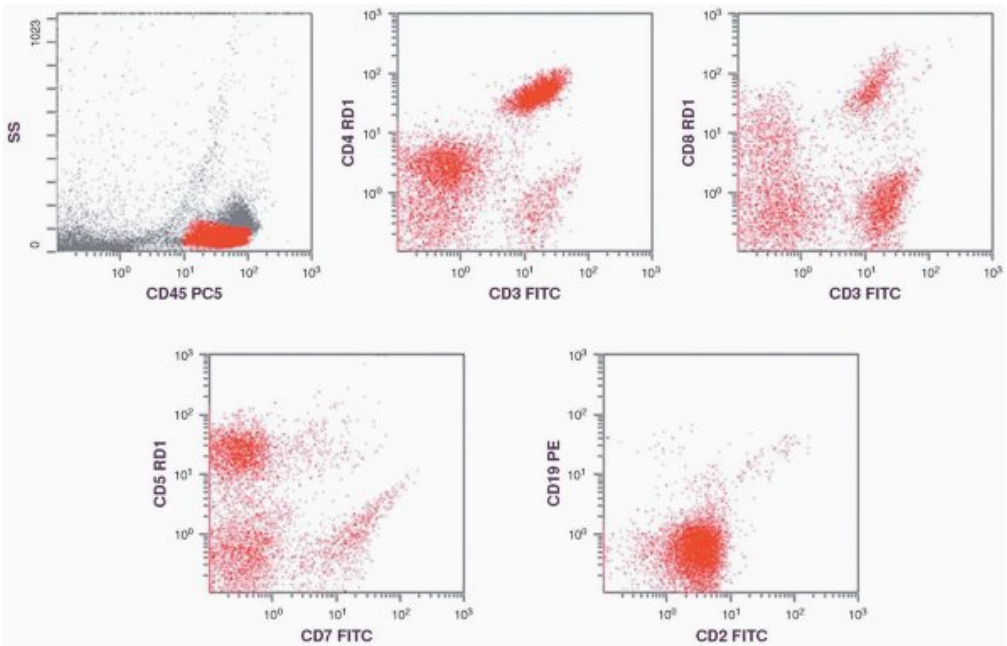

FIGURE 6.36.1 Flow cytometric analysis of the lymph node shows predominance of CD3/CD4 over CD3/CD8 staining. CD2 and CD5 are positive, but CD7 shows a lower percentage than other T-cell markers. SS, side scatter; PC5, phycoerythrin-cyanin 5; RD1, rhodamine; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

TABLE 6.36.1

Classification of Peripheral T-Cell Lymphoma

Clinical Presentation	*Lymphoma Subtype*
Nodal	Angioimmunoblastic T-cell lymphoma
	Anaplastic large cell lymphoma
	Peripheral T-cell lymphoma, unspecified
Extranodal	Mycosis fungoides

	Primary cutaneous anaplastic large cell lymphoma
	Subcutaneous panniculitis-like T-cell lymphoma
	Enteropathy-type T-cell lymphoma
	Extranodal NK/T-cell lymphoma, nasal type
Leukemic/disseminated	T-cell prolymphocytic leukemia
	T-cell large granular lymphocytic leukemia
	Adult T-cell lymphoma/leukemia
	Aggressive NK-cell leukemia

NK, natural killer.

TABLE 6.36.2

Characteristic Morphologic Features of PTCL-U

Histologic pattern	Diffuse or interfollicular infiltration, high endothelial venules may be present.
Cytology	Mixed small and large cells, or predominantly small cells with clumped chromatin pattern and no nucleoli, or predominantly large cells with vesicular chromatin pattern and nucleoli
	Clear cytoplasm is characteristic but not always present.
	Multinucleated giant cells and Reed-Sternberg-like cells may be present.
Special features	Diffuse tumor cell infiltration with epithelioid histiocytes, eosinophils, and/or plasma cells in the background
	The presence of clear cells and high endothelial venules are characteristic.

PTCL-U, peripheral T-cell lymphoma, unspecified.

Cases that show Reed-Sternberg-like cells and an inflammatory background are difficult to distinguish from Hodgkin lymphoma. Careful

inspection of the lymphoid population and the presence of other Hodgkin tumor cells may help to differentiate these two entities, but immunophenotyping or genotyping is frequently required to make the distinction. The T-cell-/histiocyte-rich B-cell lymphoma may also show a mixture of small lymphoid cells and histiocytes mimicking PTCL-U, but the small number of neoplastic B cells can be identified with immunohistochemical staining.

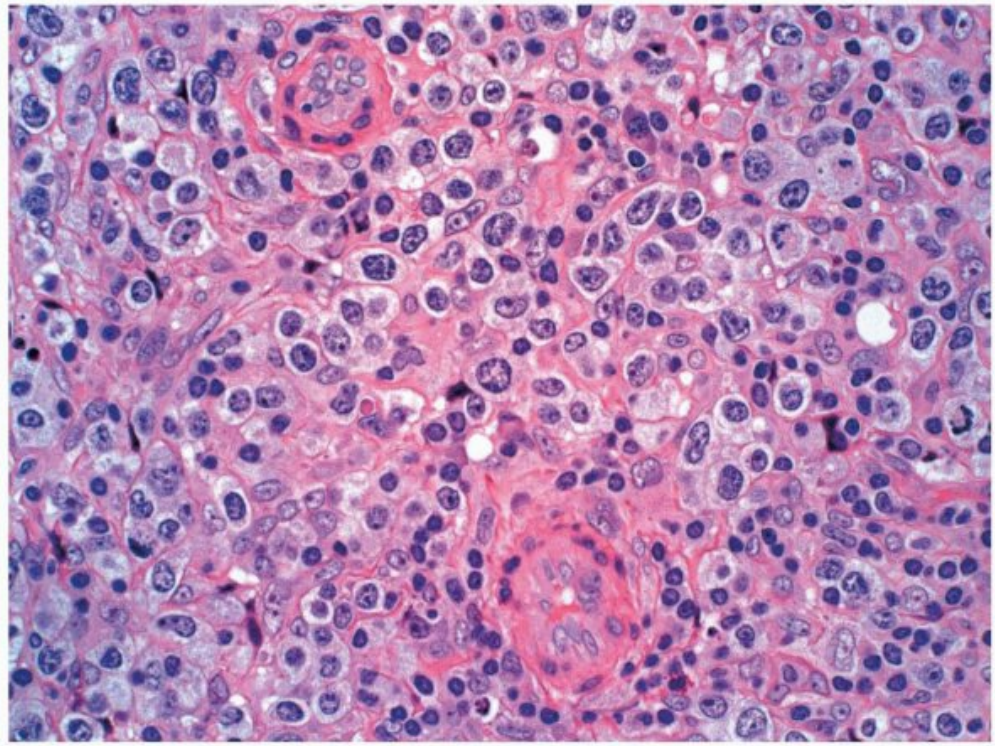

FIGURE 6.36.2 Lymph node biopsy of a peripheral T-cell lymphoma shows tumor cells with clear cytoplasm and pleomorphic nuclei. There are two high endothelial venules present in this field. Hematoxylin and eosin, 40 × magnification.

Although it is not a common feature, high endothelial venules and even arborizing blood vessels can be seen in some cases of PTCL-U (Fig. 6.36.4). These cases have to be distinguished from angioimmunoblastic T-cell lymphoma. The latter may show a prominent angiocentric pattern in the paracortical region with the presence of a follicular dendritic meshwork (Fig. 6.36.5).

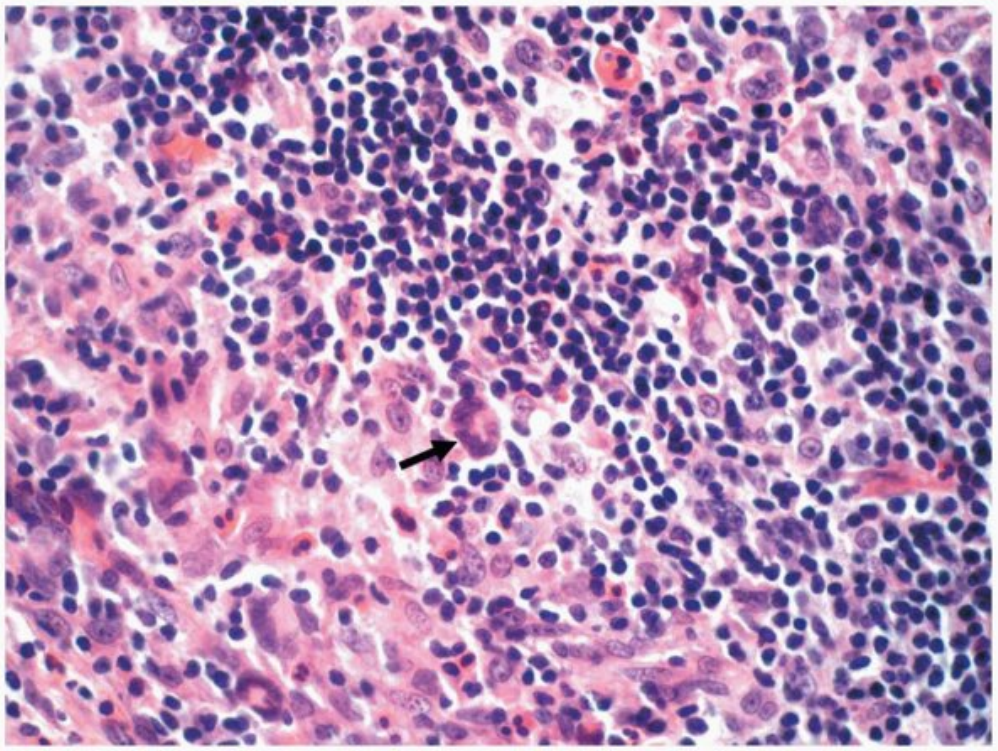

FIGURE 6.36.3 Lymph node biopsy of lymphoepithelioid lymphoma shows a few multinucleated giant cells (*arrow*) in this field. Hematoxylin and eosin, 40× magnification.

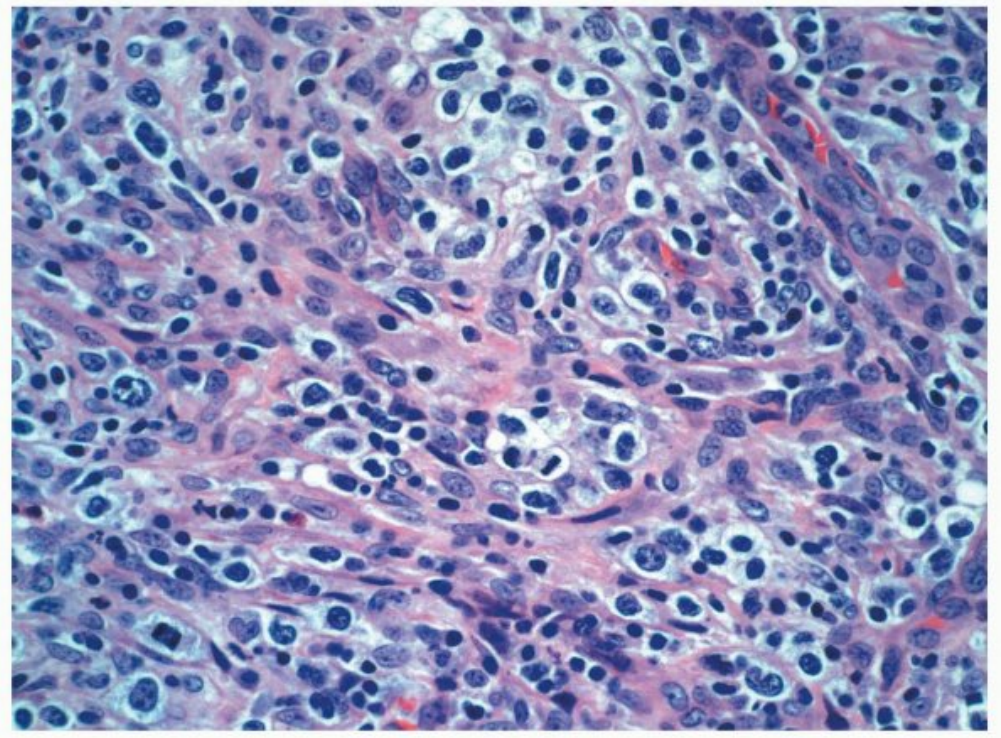

FIGURE 6.36.4 Lymph node biopsy of a peripheral T-cell lymphoma shows arborizing blood vessels. Hematoxylin and eosin, 40 × magnification.

Bone marrow involvement is present in one third of PTCL-U cases (8). The histologic pattern can be interstitial, intrasinusoidal, diffuse, or nodular, and is thus indistinguishable from other lymphomas. Two specific morphologic variants are included in the PTCL-U category, but no specific clinical features are associated with them.

Lymphoepithelioid Cell Variant (Lennert Lymphoma)

Tumor cells in this variant are usually composed of small lymphoid cells with slightly irregular nuclei, clumped chromatin pattern, and absence of nucleoli (10, 11, 12 and 13). There are also small numbers of medium-sized or large cells present. The infiltration pattern can be diffuse or interfollicular. The characteristic feature is an exuberant proliferation of epithelioid histiocytes (Figs. 6.36.6 and 6.36.7), presumably a response to lymphokines released by the tumor cells (10). The epithelioid histiocytes, in turn, may secrete interleukin-6 to stimulate the proliferation of the tumor cells (13).

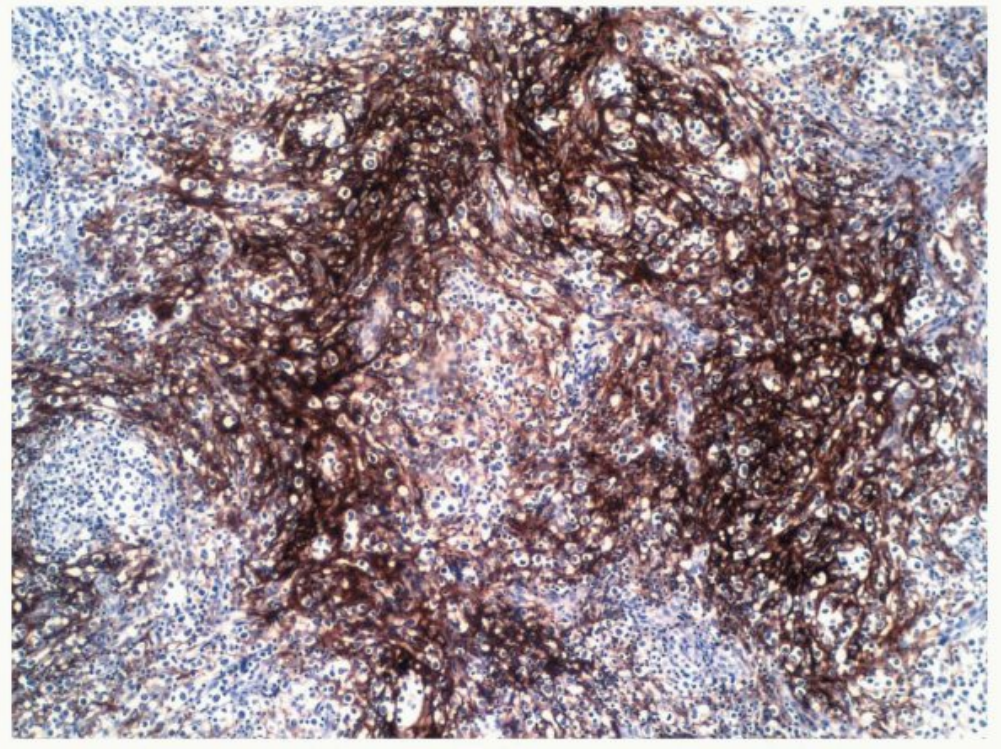

FIGURE 6.36.5 Lymph node biopsy of angioimmunoblastic T-cell lymphoma shows a follicular dendritic meshwork with CD21 stain. Immunoperoxidase, 10 × magnification.

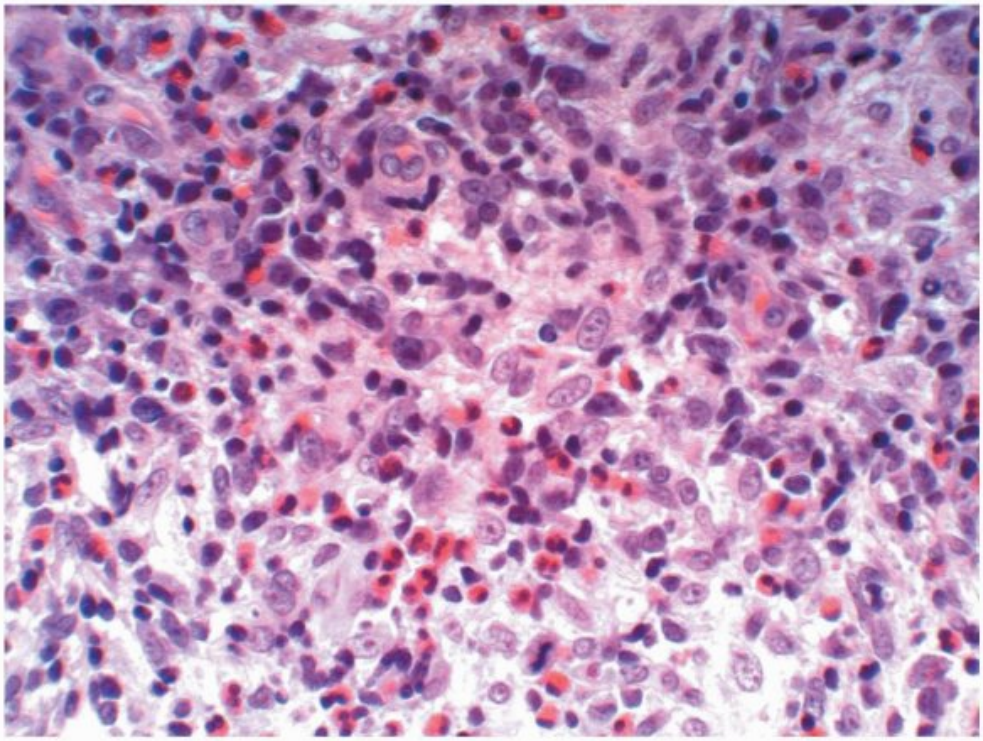

FIGURE 6.36.6 Lymph node biopsy of lymphoepithelioid lymphoma shows large numbers of epithelioid histiocytes and eosinophils intermixing with lymphoma cells. Hematoxylin and eosin, 50 × magnification.

The frequent presence of eosinophils, plasma cells, and Reed-Sternberg-like cells in this entity makes it difficult to distinguish from Hodgkin lymphoma. As mentioned before, immunophenotyping or genotyping is frequently required to make the distinction.

Lymphoepithelial lymphoma is somewhat similar to T-cell-rich B-cell lymphoma in that, as the disease progresses, the tumor cells become more prominent and the epithelial component is gradually diminished (10,11). Lymphoepithelial lymphoma can also transform into

angioimmunoblastic T-cell lymphoma, T-zone lymphoma, or large cell lymphoma (10,12,14).

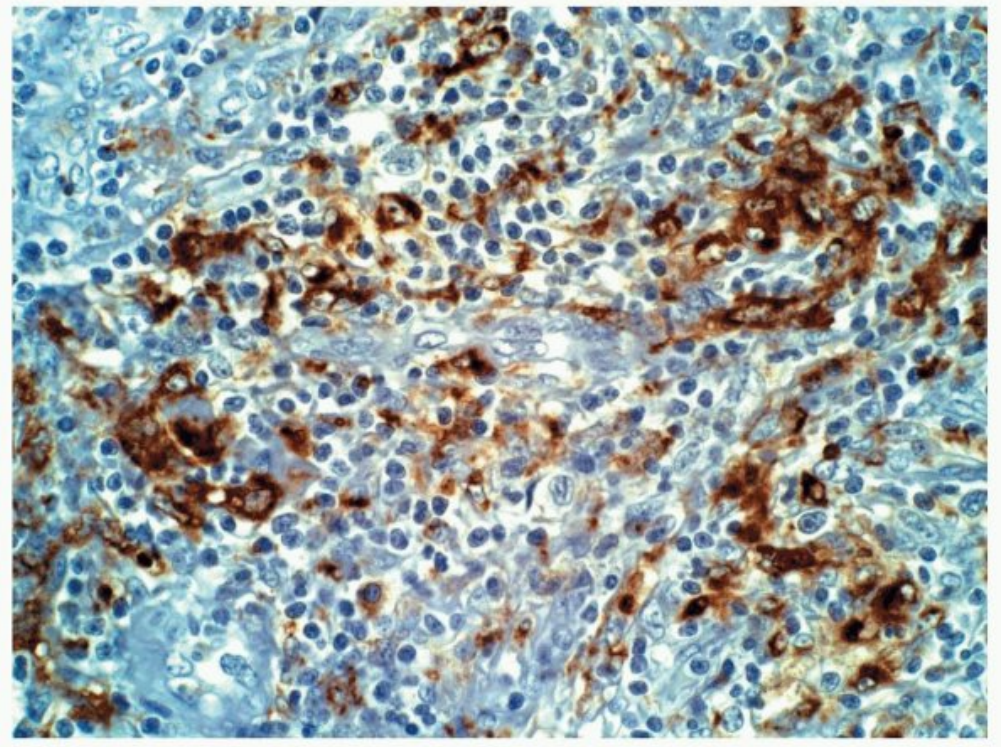

FIGURE 6.36.7 Lymph node biopsy of lymphoepithelioid lymphoma shows numerous histiocytes demonstrated by CD68 stain. Immunoperoxidase, 40× magnification.

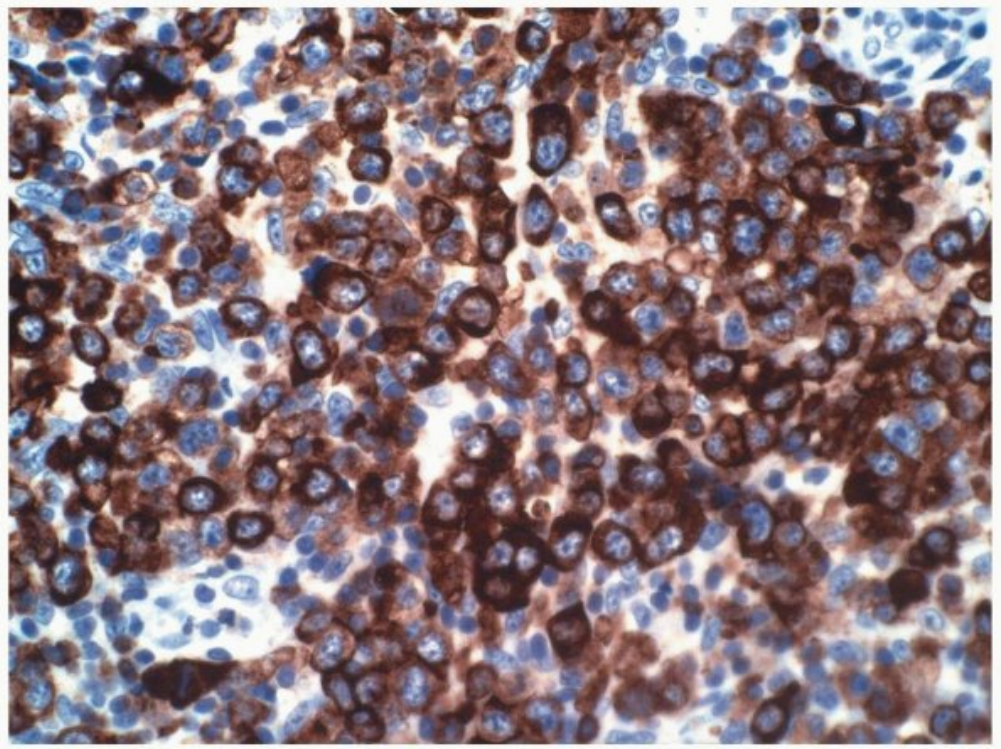

FIGURE 6.36.8 Lymph node biopsy of a peripheral T-cell lymphoma shows exclusive CD4 staining on the tumor cells. Immunoperoxidase, 50× magnification.

T-Zone Variant

In this variant, the tumor cell infiltrate is concentrated in the interfollicular area (T-cell zone) (4,9,12,15,16). The lymphoid follicles are well preserved or even hyperplastic. The tumor cells are usually of small to medium size with slightly irregular nuclear configuration. Clear cells can be seen, and high endothelial venules may be increased in some cases. Those cases should be distinguished from angioimmunoblastic T-cell lymphoma. In the background, epithelioid histiocytes, eosinophils, and plasma cells are frequently present, mimicking lymphoepithelioid lymphoma. When Reed-Sternberg-like cells are present, this tumor should be distinguished from Hodgkin lymphoma.

Immunophenotype

The characteristic immunophenotype is the absence of thymic T-cell markers [CD1 and terminal deoxynucleotidyl transferase (TdT)] (11,17) and presence of peripheral pan-T-cell markers (CD2, CD3, CD5, and/or CD7) (11,18). However, selective loss of one or more pan-T-cell markers is a common finding, and this aberrant immunophenotype helps to identify T-cell neoplasm (19). The only exception is lymphoepithelioid lymphoma, which seldom shows this phenomenon (18). Unlike B-cell lymphomas, there are no monoclonal markers for T-cell tumors. PTCL may be presented with a predominantly CD4 (helper/inducer) or CD8 (suppressor/ cytotoxic) phenotype. For instance, both lymphoepithelioid lymphoma and T-zone lymphoma express predominantly CD4 markers (Fig. 6.36.8). However, the predominance of CD4 or CD8 cells is not a reliable indicator of monoclonality because it may also be demonstrated in reactive lymphoproliferation. Only when both CD4 and CD8 are absent or when there is coexpression of both CD4 and CD8 in the same population should T-cell lymphoma be suspected.

Double staining with Ki-67/CD4 and Ki-67/CD8 may also help to distinguish neoplastic from normal T-helper cells (13). The tumor cells are Ki-67 positive, and normal T-cells are negative. When CD4 and CD8 were applied without Ki-67, a large number of cases showed almost equal numbers of CD4- and CD8-positive cells in one study (12). However, when frozen sections were double stained with Ki-67/CD4 and Ki-67/CD8, all cases showed a Ki-67-/CD4-predominant phenotype.

In immunohistochemical stains, CD2, CD3, CD4, CD5, CD7, CD43, and/or CD45RO can be detected in the tumor cells (18). Selective loss of one or more pan-T-cell markers is frequently found by immunohistochemistry just the same as in flow cytometry. It should be emphasized that the presence of CD43 alone is not sufficient to ascertain a T-cell lineage, as it is also a myeloid marker and can be demonstrated in myeloid sarcoma. CD20 stain can help to diagnose T-cell-rich B-cell lymphoma, but the demonstration of CD20 does not exclude T-cell lymphoma, as a rare type of CD20-positive T-cell lymphoma is present (20,21). Furthermore, lymphoepithelioid lymphoma of B-cell type has been reported (17). Some of these cases may represent histiocyte-rich B-cell lymphomas (22,23).

CD30+ and CD15+ are useful to identify the Reed-Sternberg cells in Hodgkin lymphoma. CD30+ CD15-immunophenotype is characteristic of anaplastic large cell lymphoma, but can also be present in PTCL-U, particularly in the large cell variant (4). In fact, even CD15 can be occasionally demonstrated in PTCL-U and predict a poor prognosis (19). The Reed-Sternberg-like cells are positive for CD15 in 10% of lymphoepithelial lymphoma cases (10). When an immunophenotype of CD30+ CD15- is identified, Alk1 can be used to differentiate PTCL-U from anaplastic large cell lymphoma.

Natural killer (NK) cell markers (CD16, CD56, CD57) and cytotoxic granule-associated proteins T-cell intracellular antigen ([TIA]-1, granzyme B, and perforin) are helpful to exclude NK/NK-like T cell and cytotoxic T-cell lymphoma/leukemia. Epstein-Barr virus (EBV) is usually absent in PTCL-U.

Comparison of Flow Cytometry and Immunohistochemistry

Flow cytometry is not as helpful as immunohistochemistry because there are no specific monoclonal markers for PTCL, and flow cytometric study lacks direct morphologic correlation. Immunohistochemical stains, in contrast, can demonstrate all pan-T-cell markers as well as the CD4 or CD8 subset on the tumor cells. Some special stains can further differentiate the subtype of PTCL, such as CD15, CD30, CD16, CD56, CD57, and Alk1. Ki-67 may help to distinguish the neoplastic T-cell from the normal T-cell and predict a poor prognosis.

The current case presented with B symptoms, enlargement of multiple lymph nodes, and splenomegaly. Lymph node biopsy revealed a diffuse infiltrating small cell lymphoma with numerous clusters of epithelioid histiocytes and eosinophilia. Immunophenotyping by both flow cytometry and immunohistochemistry demonstrated a predominant helper T-cell (CD4+) phenotype. The percentage of CD7 was lower than other pan-T-cell markers, as detected by

flow cytometry, representing selective loss of T-cell antigen. Specific features of anaplastic large cell lymphoma, angioimmunoblastic T-cell lymphoma, and T-zone lymphoma were not present. Therefore, a diagnosis of lymphoepithelioid lymphoma was established. Two unusual findings in this case were the development of a B-cell cutaneous lymphoma and amyloidosis in the skin. PTCL-U may have skin involvement, but a lymphoma of different cell lineage is seldom seen. Amyloidosis is also rarely seen in T-cell lymphomas, but there was no evidence of myeloma in this case.

Molecular Genetics

T-cell receptor (TCR) gene rearrangement is frequently demonstrated in PTCL-U, lymphoepithelioid lymphoma (13) and T-zone lymphoma (16). The positive rate for TCR gene rearrangement in lymphoepithelioid lymphoma varies from 56% to 100% (12,13,24,25). A false-negative result is probably due to the existence of a low percentage of tumor cells, which can be roughly estimated by the percentage of CD4-/Ki-67-positive cells (13). Immunoglobulin heavy-chain gene rearrangement is usually negative in PTCL-U cases. Specific cytogenetic abnormalities have been detected in several PTCL subtypes, such as t(2;5) in anaplastic large T-cell lymphoma and isochromosome 7q in hepatosplenic T-cell lymphoma (9). In PTCL-U cases, trisomy 3, 6q-, trisomy 7q, and monosomy 13 have been

reported (9). In a study of seven cases of lymphoepithelioid lymphoma, six showed cytogenetically abnormal clones, always including aberration of chromosome 3 (26). In those cases, 3q22 is either broken or duplicated. Another study found cytogenetic abnormalities in 13 of 18 cases of lymphoepithelioid lymphoma; most cases showed trisomy 3 or trisomy 5 (27). Trisomy 3 is also the most common aberration observed in T-zone lymphoma cases (28,29).

In a study of 36 PTCL-U cases with comparative genomic hybridization, recurrent chromosomal losses were found on chromosome 13q (36%), 6q and 9p (31% each), 10q and 12q (28% each), and 5q (25%) (30). Recurrent gains were found on chromosome 7q22 (31%) (30). Using DNA microarrays containing 6386 cancer-related genes, Martinez-Delgado et al. (31) were able to identify the specific gene expression profile in PTCL that was distinguished from that of the lymphoblastic lymphoma (31). These authors also found a close relationship between genes associated with survival and those that differentiate the stages of disease and the responses to therapy.

A study of the nuclear factor-κB (NF-κB) using complementary DNA (cDNA) microarrays in a group of 62 PTCL patients found that one third of PL cases showed clearly reduced NF-κB expression, whereas another group showed high expression of these genes (32). Patients with reduced expression of NF-κB genes were associated with shorter survival; this finding was an independent prognostic factor in a multivariate analysis. The salient features of laboratory diagnosis of PTCL are summarized in Table 6.36.3.

Clinical Manifestations

A study of 108 cases of lymphoepithelioid lymphoma showed that the median age was 60 years, with the range from 21 to 87 (33). The male/female ratio of the same series was 1.3:1. The initial clinical symptoms included lymphadenopathy (100%), splenomegaly (43%), hepatomegaly (23%), B symptoms (60%), pruritus (18%), skin rash (7%), fatigue (55%), and lymph node pain (17%) (14,33). At presentation, most patients were in advanced stages (stage I, 9%; stage II, 19%; stage III, 35%; and stage IV, 37%). However, bone marrow was rarely involved (5%) in this series, whereas five of nine cases had bone marrow involvement in other series (11). Extranodal manifestation, which is characteristic for other peripheral T-cell lymphomas (11), was not common in lymphoepithelioid lymphoma. For instance, only 4% involved the skin, 4% soft tissue, 3% lung, and 1% each for pleura and stomach (33). In hematologic workup, anemia, lymphocytopenia, eosinophilia, and monocytosis were seen in 26% to 37% of cases (14,33). About one half of the cases showed hypoproteinemia and hypoalbuminemia. Hypogammaglobulinemia and hypergammaglobulinemia were seen in about one fourth of the cases, but no monoclonal gammopathy was detected in the series of 108 patients (33).

TABLE 6.36.3

Salient Features for Laboratory Diagnosis of PTCL-U

1. Most tumor cells are of helper T-cell type (CD3+ CD4+ CD8-).
2. Selective loss of pan-T-cell antigens (seldom seen in lymphoepithelioid lymphoma)
3. No immature T-cell markers (CD1-, TdT-)
4. The Reed-Sternberg-like cells are usually negative for both CD15 and CD30, but can be positive for CD15 or CD30 in occasional cases.
5. TCR gene rearrangement is seen in most cases except for those with low percentage of CD4/Ki-67 cells.
6. Aberration of chromosome 3, especially trisomy 3, is characteristic.

PTCL-U, peripheral T-cell lymphoma, unspecified; CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase; TCR, T-cell receptor.

Patients with T-zone lymphoma are mostly in the fifth and sixth decade of life, with a male/female ratio of 1.5:1 (16). The clinical presentation is a rapid enlargement of lymph nodes, spleen, and liver (16). Institutional symptoms include malaise, fever, and sometimes skin rashes (15). However, lymphomatous infiltrate of the skin is seldom seen (16). Lung and pleura are often affected, but peripheral blood involvement is rare.

The overall survival for patients with lymphoepithelioid lymphoma was 16 months (range, 1 to 49 months), with a 3-year survival of 36%

(33). Those patients in stage I/II had a median survival of 18 months and a 3-year survival of 49% (33). The PTCL-U group, as a whole, has one of the lowest overall and failurefree survival rates (34). Patients with PTCL-U generally fare worse than those with diffuse large B-cell lymphoma in several studies (35). In a study of 385 PTCL-U cases, age >60 years, increased serum lactate dehydrogenase, ECOG performance status >2, and bone marrow involvement were found to be associated with a worse overall survival on multivariate analysis (36). Another group used age, performance status, lactate

dehydrogenase and Ki-67 marking >80% tumor cells to stratify three groups of patients with different responses to therapy and life expectancy (19).

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CASE 37 Anaplastic Large Cell Lymphoma

CASE HISTORY

A 14-year-old boy with fever and sore throat for a week was admitted to the hospital for evaluation. The patient was treated with antibiotics before admission but showed no response. His mother claimed that the patient had apparent weight loss in the last 2 weeks. Physical examination on admission showed lymphadenopathy involving the cervical and axillary regions. His sedimentation rate was elevated. A monospot test was negative. Serologic examination for antibodies against toxoplasma, cytomegalovirus, and Herpes simplex were all negative. Peripheral blood examination showed mild lymphocytosis, but the morphology of the lymphocytes was normal. His hemoglobin was 12 g/dL, and hematocrit 36%. The platelet count was 190,000/μL. A lymph node biopsy was performed and revealed morphologic evidence of lymphoma. A subsequent bone marrow biopsy also showed tumor involvement.

FLOW CYTOMETRY FINDINGS

Lymph node biopsy: B-cell markers: CD10 0%, CD19 10%, CD20 4%, CD23 1%, κ 6%, λ 4%. T-cell markers: CD3/CD4 22%, CD3/CD8 72%, CD5 69%, CD7 9%. Monocyte marker: CD14 2%. Activation antigen: CD30 76%.

Bone marrow aspirate: B-cell markers: CD19 12%, CD20 6%, CD23 0%, κ 8%, λ 4%. T-cell markers: CD3/CD4 16%, CD3/CD8 64%, CD5 58%, CD7 7%. Monocyte marker: CD14 1%. Activation antigen: CD30 96% (Fig. 6.37.1).

IMMUNOHISTOCHEMISTRY

Immunohistochemical staining showed that the tumor cells were positive for CD30, epithelial membrane antigen (EMA), anaplastic lymphoma kinase (ALK)1, CD3, and CD8, but negative for CD15 and CD20.

MOLECULAR GENETIC FINDINGS

Cytogenetic karyotyping revealed t(2;5)(p23;q35) translocation.

DISCUSSION

In the search of Reed-Sternberg cell-specific antigens, a new antigen, designated Ki-1 antigen, was discovered in 1982 and was later clustered as CD30. Subsequently, CD30 was identified in a group of diffuse large cell lymphomas; this group of tumors was then called Ki-1+ anaplastic large cell lymphoma (ALCL) or CD30+ ALCL (1). However, because CD30 is also expressed in other tumors, including Hodgkin lymphoma, embryonal carcinoma, body cavity lymphoma, mediastinal B-cell lymphoma, and cutaneous T-cell lymphoma (2), the descriptive term Ki-1 or CD30 was dropped from the designation. This group of tumors is now called ALCL in the Revised European-American classification of lymphoid neoplasms (REAL) and World Health Organization (WHO) classification (3,4).

Morphology

In recent years, several variants of ALCL have been identified. As the subclassification has become so complicated, the term “anaplastic large cell lymphoma” is probably no longer appropriate. In early reports, ALCL was divided into several types: pleomorphic (common or classic), monomorphic, small cell, lymphohistiocytic, Hodgkin-like, and mixed (5, 6, 7, 8, 9 and 10). In addition, there are reported cases that show large numbers of fibroblastlike spindle cells (sarcomatoid variant) (11), neutrophils (neutrophil-rich) (12), eosinophils (eosinophil-rich) (13), and signet-ring cells (signet-ring appearance) (14), but these rare morphologic forms are not considered to be distinct subtypes by most authors. In the WHO classification, there are only three variants: (i) common, (ii) small cell, and (iii) lymphohistiocytic (5).

The *common variant* consists of a wide spectrum of large tumor cells with different morphologic features and was originally designated the polymorphic variant. The most common cell type has chromatin-poor horseshoe- or kidney-shaped nuclei with multiple nucleoli (Fig. 6.37.2). Ring-shaped nuclei (doughnut cell) can also be seen. Some authors emphasize the presence of a perinuclear eosinophilic region, which probably represents a prominent Golgi apparatus (9). These tumor cells have a moderate amount of mildly basophilic cytoplasm, which may contain vacuoles that are only visible in touch preparations (6,7). Cells with these features have been called hallmark cells because they are present in all ALCL variants in various proportions and their presence is the major diagnostic criterion.

Multinucleated giant tumor cells are frequently demonstrated in the common type; when giant cells are predominant, it is designated the giant cell-rich variant (10). However, this designation is no longer recognized in the new classification. The monomorphic variant, which is a rare morphologic manifestation, is also currently included in the common variant (6,7).

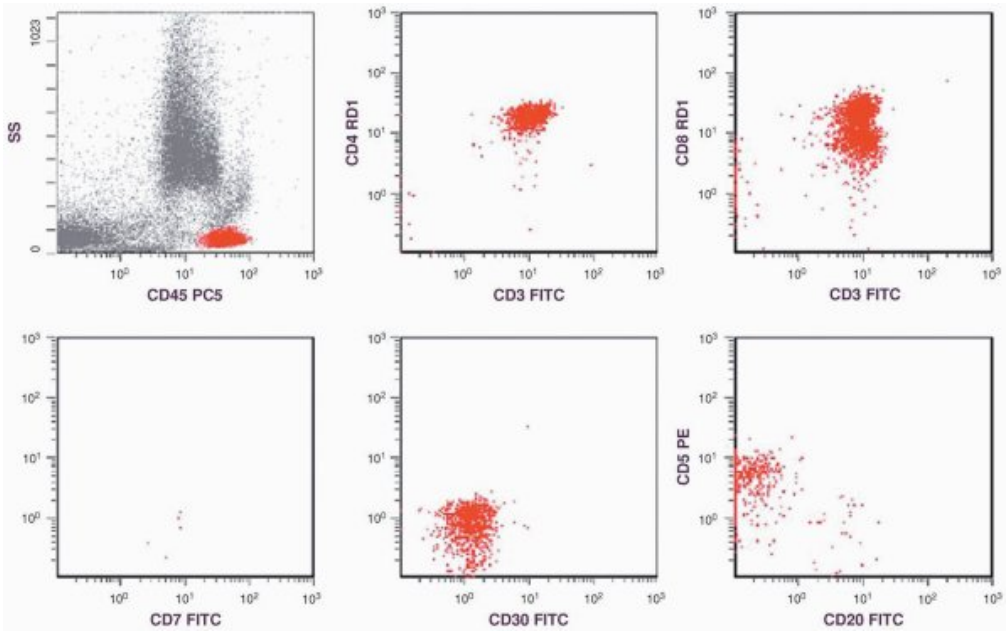

FIGURE 6.37.1 Flow cytometric analysis of bone marrow shows the presence of CD3-/ CD4-, CD3-/CD8-, CD5-, and CD30-positive cells but absence of CD7- and CD20-positive cells. SS, side scatter; PC5, phycoerythrin-cyanin 5; RD1, rhodamine; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

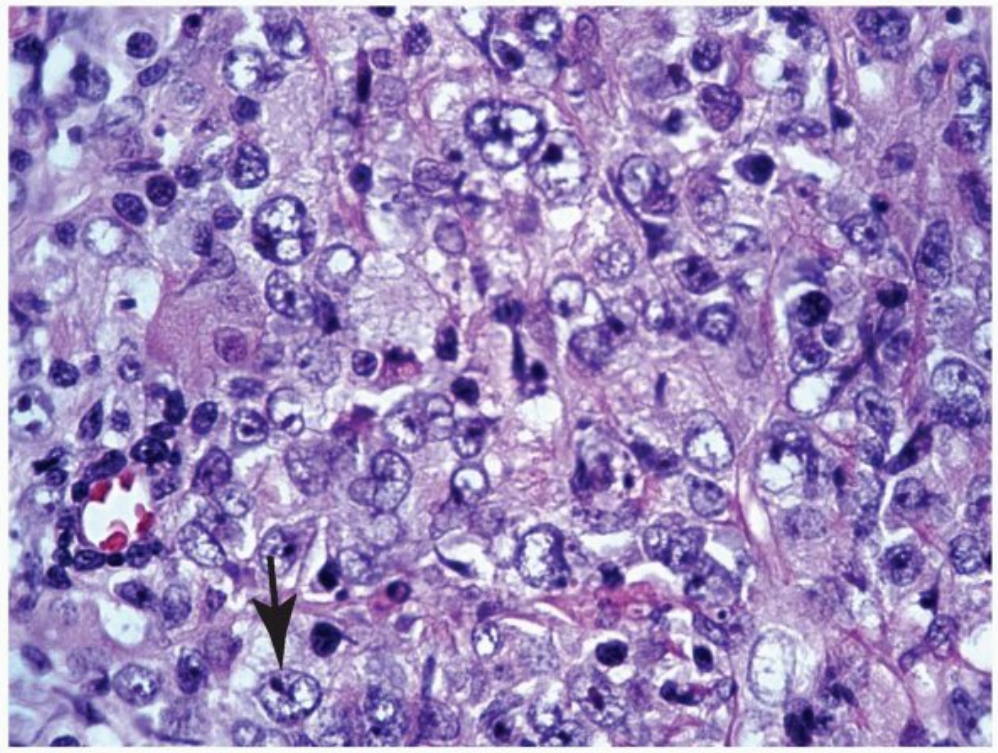

FIGURE 6.37.2 Case of the common variant of anaplastic large cell lymphoma (ALCL) shows cohesive clusters of large tumor cells with chromatin-poor nuclei and prominent nucleoli. Some tumor cells reveal the characteristic kidney-shaped nucleus (*arrow*). Hematoxylin and eosin, 40× magnification.

The so-called Hodgkin-like variant contains a nodular sclerosis pattern and large numbers of packed or dispersed tumor cells with features resembling those of classic Hodgkin and Reed-Sternberg cells (Fig. 6.37.3). However, the current thinking is that this variant may represent a tumor cell-rich variant of classic Hodgkin lymphoma and not a true ALCL (6,7). Therefore, this entity has not been included in the WHO classification (5).

The *small cell variant* is characterized by the presence of predominantly small to medium-sized tumor cells (15) (Fig. 6.37.4). Therefore, it is frequently misdiagnosed as peripheral T-cell lymphoma unspecified if CD30 and other special stains are not performed (5). However, the hallmark cells are invariably identifiable around the blood vessels, particularly with the help of CD30 staining.

The *lymphohistiocytic variant* is defined by the presence of a large number of histiocytes, which may occasionally show signs of erythrophagocytosis (16). As in the small cell variant, a diagnosis depends on the finding of perivascular infiltration of the large tumor cells.

In approximately 10% of cases, more than one variant can be present in the same patient (5,17). In addition, in

case of relapses, a variant different from that seen in the initial diagnosis may appear (5,17).

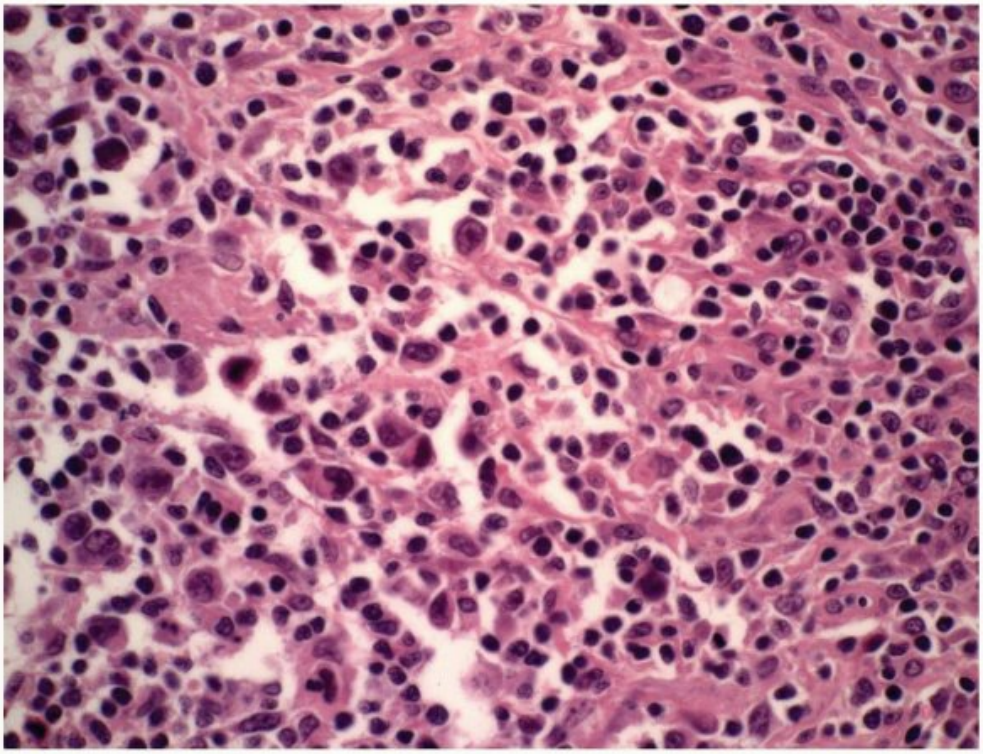

FIGURE 6.37.3 Another case of the common variant reveals scattered large tumor cells on a background of non-neoplastic small lymphocytes, mimicking Hodgkin lymphoma. Note a sclerotic band (*upper*). Hematoxylin and eosin, 40× magnification.

The general histologic features are characterized by a sheetlike cohesive growth pattern with the tendency of sinusoidal infiltration (Fig. 6.37.5), a phenomenon mimicking carcinoma. When the lymph node is not completely obliterated, a perifollicular pattern may be demonstrated. As mentioned before, another special feature is the perivascular infiltration by the large tumor cells, which is especially striking in the small cell and lymphohistiocytic variants.

In primary cutaneous ALCL, the tumor cells are usually large and anaplastic (Fig. 6.37.6). Sheets of tumor cells may infiltrate collagen fibers, skin appendages, and subcutaneous fatty tissue (2). Mitoses and apoptosis are frequent features. The demonstration of vascular invasion may help to distinguish it from lymphomatoid papulosis. The distinction between these two entities is usually difficult morphologically, and observation of the clinical course is usually needed for a definitive diagnosis (18). The so-called neutrophil-rich variant can be seen in cutaneous ALCL in which neutrophilic infiltration may mask the tumor cells (19). The characteristic morphologic features of ALCL are summarized in Table 6.37.1.

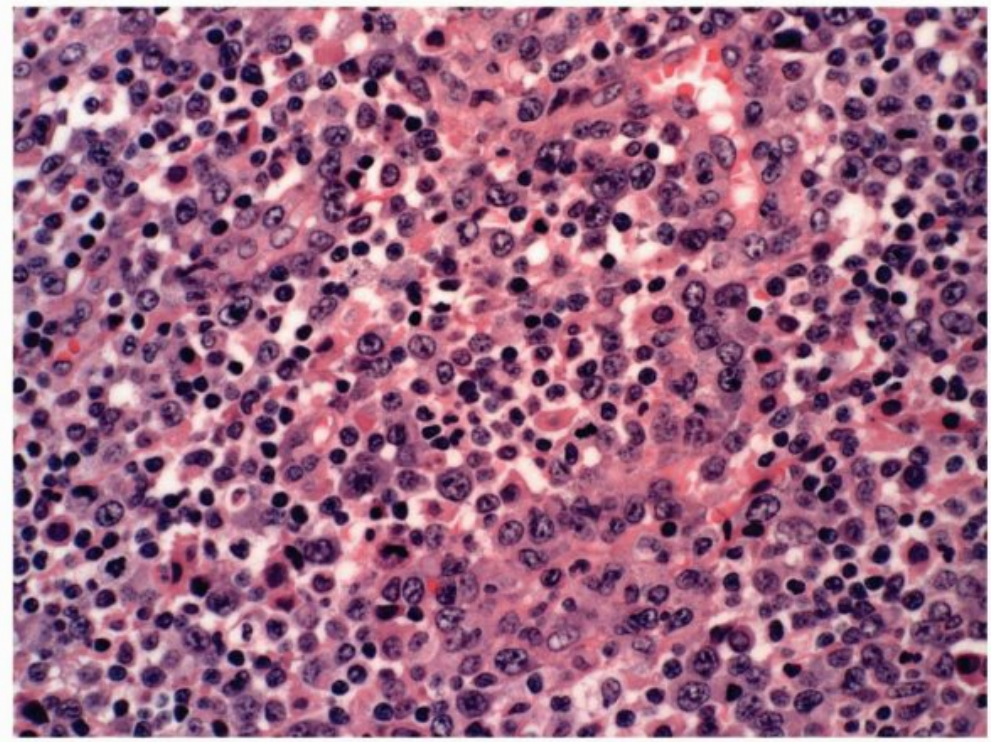

FIGURE 6.37.4 Case of the small cell variant shows predominantly small tumor cells. Large tumor cells are concentrated around the blood vessels. Hematoxylin and eosin, 40× magnification.

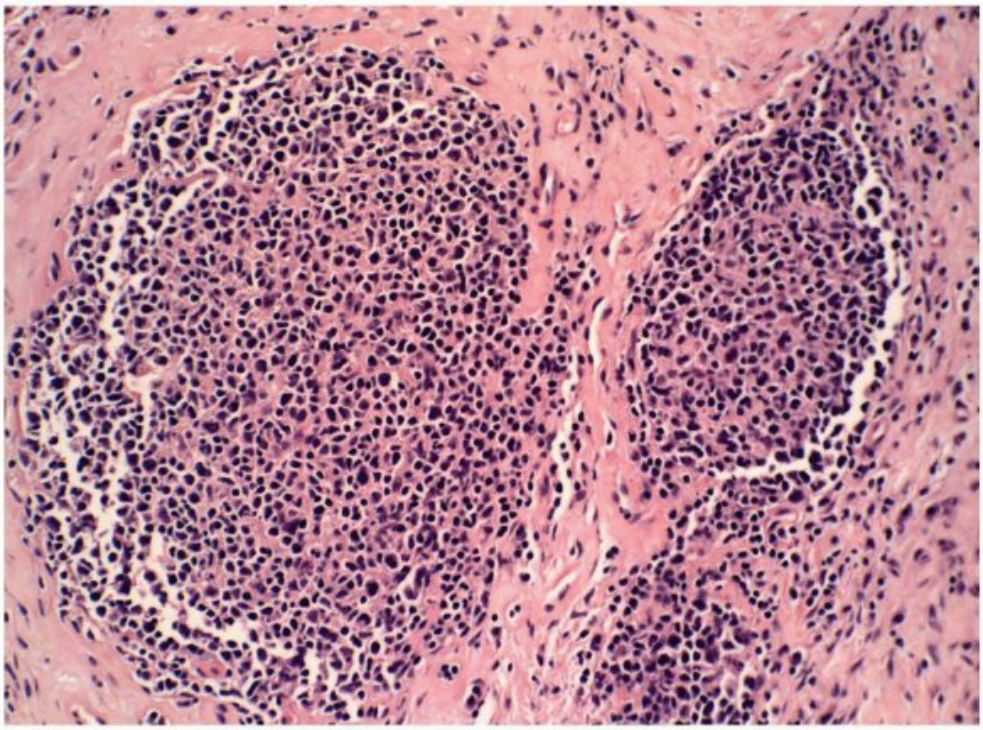

FIGURE 6.37.5 Case of common variant demonstrates a sinusoidal infiltration pattern. Hematoxylin and eosin, 20× magnification.

Immunophenotype

In the REAL and WHO classifications, only T-cell and null cell tumors with positive CD30 are included as ALCL. In the Kiel classification, a B-cell ALCL is recognized, but the general consensus is that B-cell ALCL is closer to diffuse large B-cell lymphoma than to ALCL because it differs from the

T-cell and null cell types in clinical, cytogenetic, and molecular aspects (20). In addition, the B-cell tumor is frequently associated with Epstein-Barr virus, whereas the T- and null cell tumors are not (6,7).

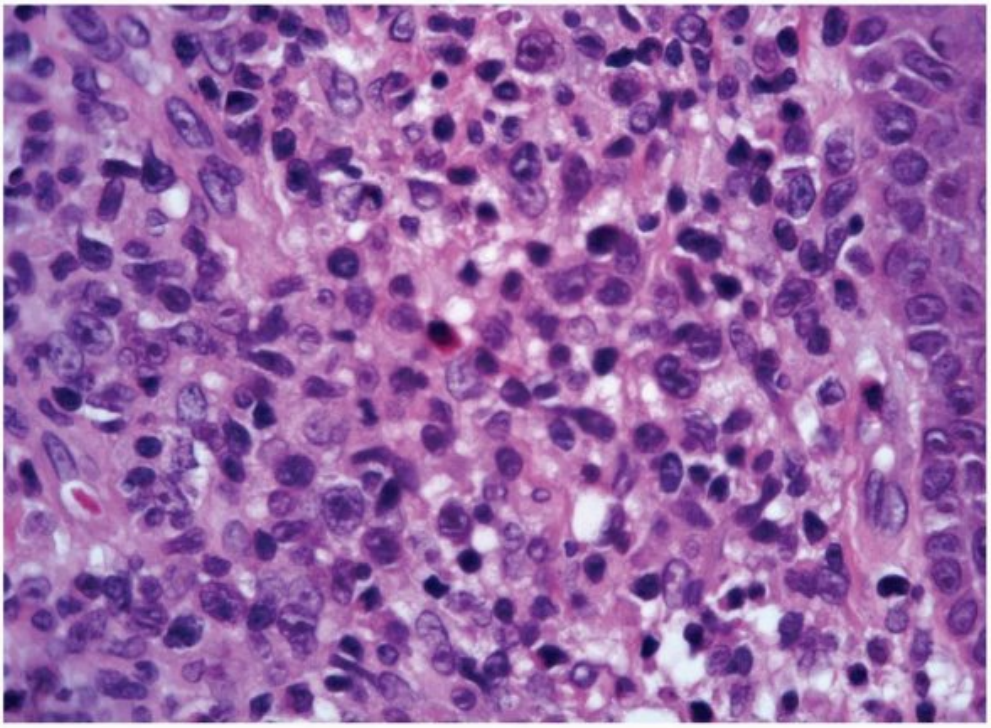

FIGURE 6.37.6 Case of primary cutaneous anaplastic large cell lymphoma (ALCL) shows large tumor cell infiltration intermixing with small lymphocytes. Hematoxylin and eosin, 60× magnification.

TABLE 6.37.1

Characteristic Morphologic Features of Anaplastic Large Cell Lymphoma

Histologic pattern	Diffuse cohesive proliferation of tumor cells, preferentially infiltrating the sinusoids Perifollicular pattern in early lesions Perivascular large cell infiltration in small cell and lymphohistiocytic variants
Cytology	Hallmark cells have chromatin-poor horseshoe-shaped nuclei with multiple nucleoli and a perinuclear eosinophilic region. Moderate amount of mildly basophilic cytoplasm that may show vacuoles in touch preparations
Specific features	Demonstration of diffusely infiltrating hallmark cells or perivascular infiltration of anaplastic large cells with admixture of other cell components (small tumor cells, histiocytes, neutrophils, eosinophils)

Immunohistochemistry is the mainstay for immunophenotyping of ALCL. There are several important characteristic markers for the diagnosis of ALCL—namely, CD30, anaplastic lymphoma kinase (ALK), clusterin, and epithelial membrane antigen (EMA)—that can be

demonstrated by immunohistochemical staining.

CD30, by definition, should be present in every case of ALCL. However, the reaction to CD30 staining differs in various cell types (6,7). CD30 stains strongly for the large tumor cells or hallmark cells (Fig. 6.37.7). It stains weakly or negatively for small tumor cells in the small cell variant. The histiocytes in the lymphohistiocytic variant are negative for CD30 but positive for CD68. The characteristic staining pattern for CD30 is the presence of both membranous and Golgi staining.

The immunohistochemical demonstration of the ALK protein is not only specific for the diagnosis of ALCL but also a reliable prognostic predictor (8,9,21). Therefore, it has been used to divide the clinical cases into ALK-positive and ALK-negative subtypes. The availability of ALK antibodies helps to detect more histologic variants, which could have been misdiagnosed on a morphologic basis (9).

The distribution of ALK differs depending on the karyotypes (2). When the karyotype is t(2;5), which represents ALK/NPM (nucleophosmin) translocation, both nuclear and cytoplasmic staining for ALK occur. Because NPM is a nucleolar shuttling protein, any variant without NPM shows only cytoplasmic staining (2). In terms of cytology, ALK is usually present in both nucleus and cytoplasm of the anaplastic large cells but only in the nucleus of the small tumor cells (Fig. 6.37.8).

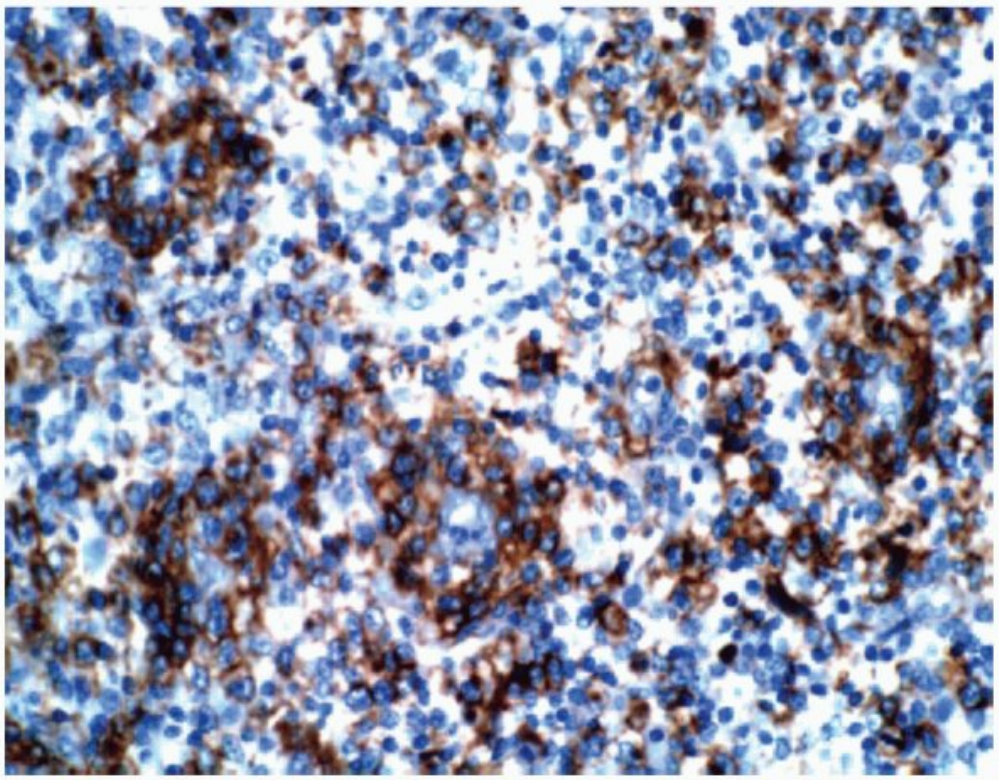

FIGURE 6.37.7 Case of small cell variant reveals that CD30 preferentially stains for large tumor cells around the blood vessels. Immunoperoxidase, 40× magnification. (Case provided by Dr. Xiyuan Liang, The Children's Hospital, Denver, CO.)

Under normal conditions, ALK is only demonstrated in a few scattered cells in the nervous system, including some glial cells, a few endothelial cells, and some pericytes. In diseases, ALK has been reported in rhabdomyosarcoma, inflammatory myofibroblastic tumors, neuroblastomas, and rare large B-cell lymphomas (6).

Clusterin is a relatively new marker for ALCL, but it is highly specific and thus important in differential diagnosis

(22). Clusterin is not present in Hodgkin lymphoma and in most cases of primary cutaneous ALCL cases (2,5,18).

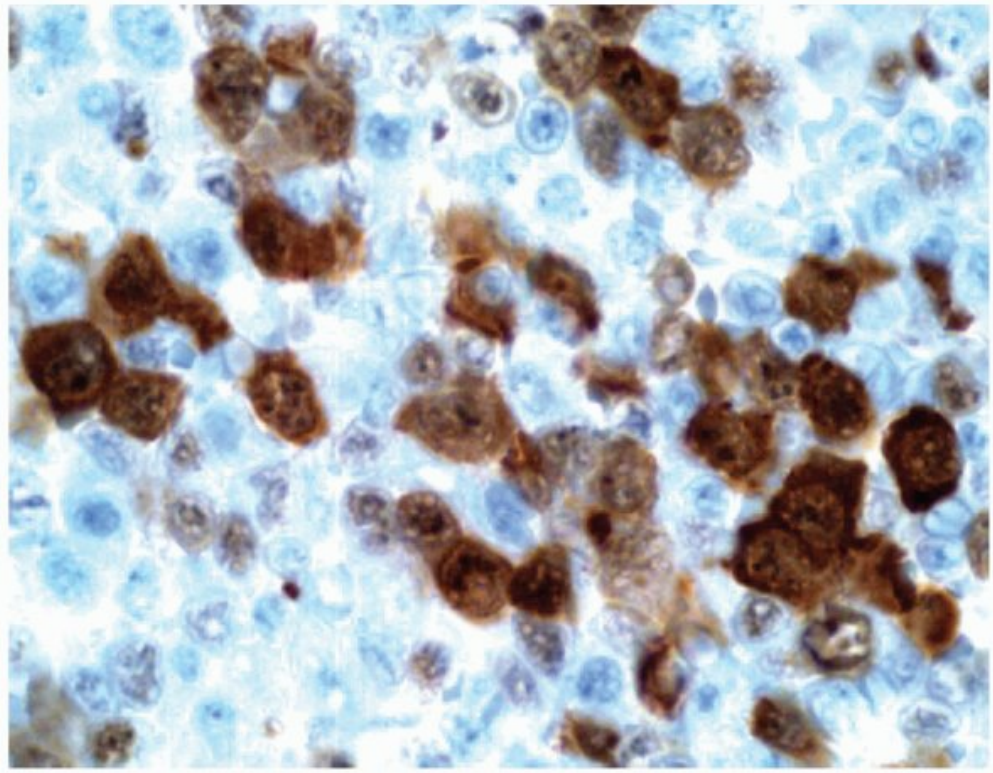

FIGURE 6.37.8 Case of small cell variant reveals anaplastic lymphoma kinase (ALK)1 staining of both large and small tumor cells. Large cells stain for both nucleus and cytoplasm, whereas small cells only express weak nuclear staining. Many small cells are negative. Immunoperoxidase, 100× magnification.

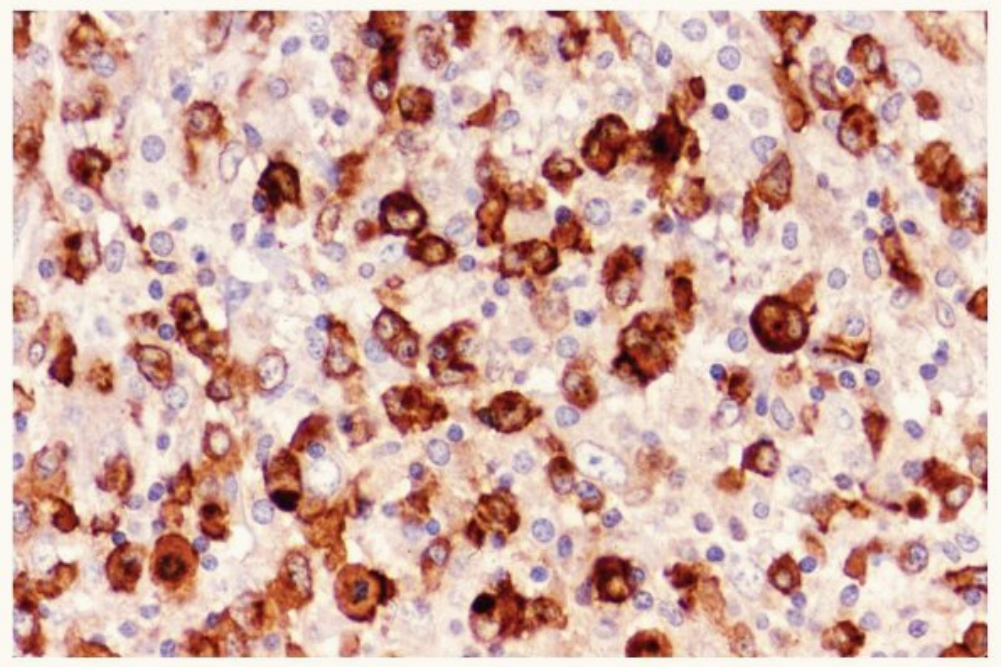

FIGURE 6.37.9 Case of small cell variant shows epithelial membrane antigen staining in most of the tumor cells. Immunoperoxidase, 60× magnification.

EMA is considered a marker of carcinoma, and is rarely seen in lymphoma. However, EMA has been demonstrated in most cases of the common variant, small cell variant, and lymphohistiocytic variant of ALCL (6,7) (Fig. 6.37.9). EMA is negative in primary cutaneous ALCL and in Hodgkin lymphoma (2,18). Because one third of ALCL cases may not express CD45 (LCA) (Fig. 6.37.10), the positive EMA reaction may mislead the diagnosis to carcinoma (23).

As mentioned before, ALCL is mainly a T-cell tumor. However, ALCL is characterized by an aberrant T-cell phenotype, lacking one or more pan-T-cell markers (2,9). Therefore, if a large panel of T-cell markers is not used, the tumor may be mislabeled as null cell type. Nevertheless, even in those so-called null cell cases, T-cell lineage can still be identified at the genetic level (5). CD3 is most frequently negative, followed by CD5 and CD7 (5). CD2 and CD4 are more often positive and should be used for the identification of cell lineage. Other T-cell markers, such as CD43 and CD45RO, are variably positive (5). The demonstration of these markers can help rule out the null cell phenotype.

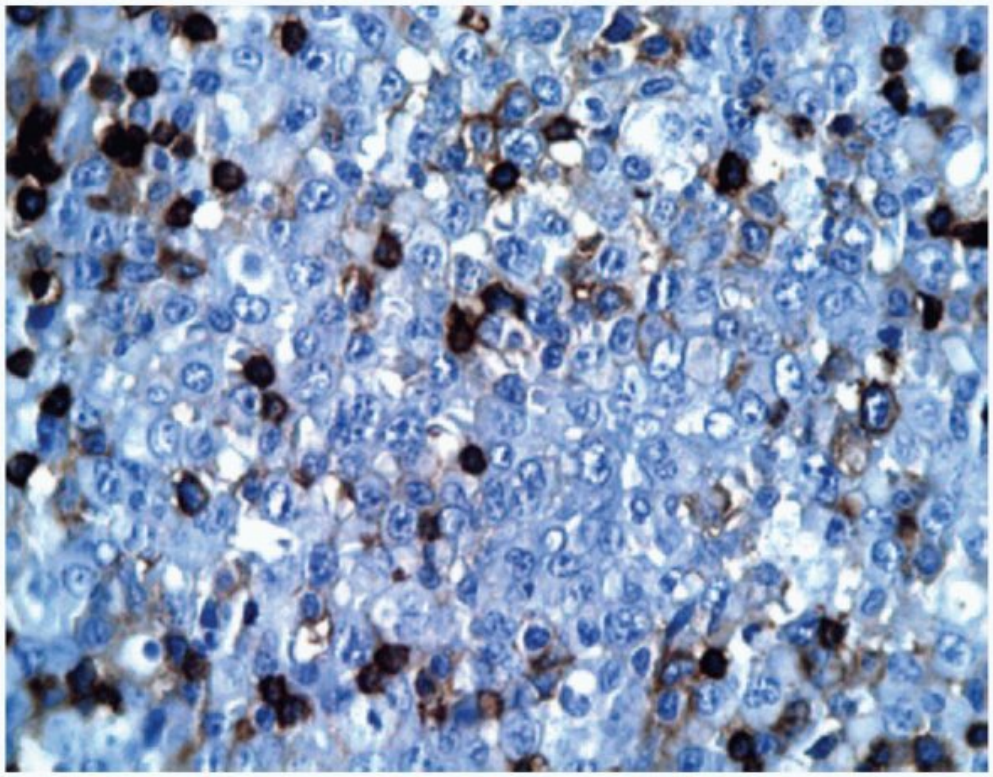

FIGURE 6.37.10 Case of small cell variant reveals CD45 staining mainly in small tumor cells. Large tumor cells show negative staining. However, large tumor cells can be positive for CD45. Immunoperoxidase, 60× magnification.

An unexplainable phenomenon is the absence of T-cell receptor (TCR) protein on ALCL cells despite the presence of TCR gene rearrangement (24). The defective TCR expression in ALCL is probably analogous to the defective immunoglobulin expression in Hodgkin lymphoma (24).

A flow cytometric study of 19 cases of ALCL showed the expression of CD2 in 71% of cases, CD3 in 32%, CD4 in 63%, CD5 in 26%, CD7 in 32%, and CD8 in 21% (25). No matter whether CD4 or CD8 is predominant in a case, cytotoxic proteins, namely, T-cell-restricted intracellular antigen-1 (TIA-1), granzyme B, and perforin, are frequently expressed, particularly in ALK-positive systemic ALCL (6,7,18). As CD56 is also expressed in a subset of ALCL cases and 10% of ALCL cases have no TCR gene rearrangement, there is a possibility that a minority of ALCL cases is derived from natural killer (NK) cells (11). Those CD56+ cases are associated with a worse prognosis (26). In addition to CD30, there are a few more activation antigens identified in ALCL, which include CD25 (interleukin-2 receptor), CD71 (transferrin receptor), and human leukocyte antigen-DR (HLA-DR) (2).

CC-chemokine receptor 4 (CCR4) is expressed in primary cutaneous ALCL but not in systemic ALCL (18). In contrast, primary cutaneous ALCL does not express ALK, EMA, or clusterin in most cases. The use of CD15, BNH.9 (blood group antigen H and Y) EMA, ALK, and cytotoxic proteins may help to distinguish ALCL from Hodgkin lymphoma, although overlapped results can be demonstrated in these two entities (2,6,7,18). Recently, the B-cell-specific activation protein (BSAP or PAX5) has been found to be expressed only by Reed-Sternberg cells but not by cells of T-cell or null cell type ALCL (27). The distinctions between systemic and primary cutaneous ALCL and Hodgkin lymphoma is listed in Table 6.37.2 (6,7,18).

Comparison of Flow Cytometry and Immunohistochemistry

The diagnosis of ALCL depends mainly on immunohistochemistry because most markers are available for this technique. Flow cytometry can demonstrate CD30 only when large numbers of tumor cells express this antigen. In addition, flow cytometry can show the selective loss of some T-cell markers. ALK protein can also be demonstrated by flow cytometry, as shown in a recent study (25).

Molecular Genetics

TCR gene analysis has shown that 90% of cases of ALCL have TCR ß- and γ-chain gene rearrangement, including those cases with a null cell immunophenotype (6,7). Some of the 10% of cases without TCR rearrangement may be of NK-cell origin, as suggested by the positive reaction to CD56 and the presence of cytotoxic proteins.

The recently discovered clusterin gene is specific to ALCL, and is seen only in a minority of B-cell lymphomas (28). This gene is not found in Hodgkin lymphoma or other T-cell lymphomas.

TABLE 6.37.2

Distinction between Systemic ALCL, Primary Cutaneous ALCL, and Hodgkin Lymphoma

Features	*Systemic ALCL*	*Primary Cutaneous ALCL*	*Hodgkin Lymphoma*
Gene rearrangement	TCR	TCR	Ig
Cytotoxic proteins	+	±	-
ALK	+	-	-
CD15	-	-	+
Clusterin	+	-	-
EMA	+	-	-
BSAP (PAX5)	-	-	+
BNH.9	+	-	-
EBV	-	-	±
CCR4	-	+	-

ALCL, anaplastic large cell lymphoma; ALK, anaplastic lymphoma kinase; BNH.9, blood group antigen H and Y; BSAP, B-cell-specific activation protein; CCR4, CC-chemokine receptor 4; EBV, Epstein-Barr virus; EMA, epithelial membrane antigen; Ig, immunoglobulin gene; TCR, T-cell receptor gene; CD, cluster of differentiation.

In the late 1980s, a recurrent, reciprocal, balanced translocation, t(2;5)(p23;q35), was found in most cases of ALCL. This translocation leads to the fusion of the *ALK* gene at 2q23 and *NPM* at 5q35 (6,7). As a result, a chimeric NPM-ALK protein is produced. ALK is a novel tyrosine kinas, whereas NPM carries the newly synthesized protein from the cytoplasm to the nucleolus (7).

Although NPM-ALK fusion protein is demonstrated in 72.5% of cases of ALCL, other proteins are detected fusing with ALK as a result of other cytogenetic abnormalities (6,7,29,30). For instance, t(1:2)(q21:p32) produces nonmuscle tropomyosin (TPM3)-ALK fusion protein. Translocation t(2;3)(p23;q21) leads to the product of tropomyosin receptor kinase-fusion gene (*TFG)*-ALK fusion protein. Inversion (2)(p23; q35) encodes 5-amino-imidazole-4-carboxamide-1-beta-D-ribonucleotide transformylase/inosine monophosphate cyclohydrolase (ATIC)-ALK fusion protein. Translocation t(2;17)(p23;q11) leads to the production of clathrin heavy polypeptide-like gene (CLTCL)-ALK fusion protein (5). Finally, t(2:X)(p23;q11-12) produces moesin (MSN)-ALK fusion protein (31). These various fusion proteins other than NPM contain no nuclear localization signals, so they are absent in the nucleus and have only cytoplasmic distribution. The frequency of these cytogenetic variants and their products and distribution are summarized in Table 6.37.3 (10,11). A recent study of 21 pediatric

cases of ALCL showed that 7 cases had t(2;5), 6 cases had variants, and 7 cases had uncharacterized rearrangements (32). The authors suggested that the frequency of variants in ALCL is probably higher than what has been conceived. The constant presence of the *ALK* gene in ALCL cases indicates its importance in the pathogenesis of ALCL.

TABLE 6.37.3

Characteristics of Various ALK Fusion Proteins

Frequency	*Genetic Abnormality*	*Fusion Proteins*	*Staining Patterns*
72.5%	t(2;5)	NPM-ALK	Cytoplasmic and nuclear
17.5%	t(1;2)	TPM3-ALK	Cytoplasmic and membrane
2.5%	t(2;3)	TFG-ALK	Cytoplasmic
2.5%	inv(2)	ATIC-ALK	Cytoplasmic
2.5%	t(2;17)	CLTCL-ALK	Granular cytoplasmic
Rare	t(2;X)	MSN-ALK	Membrane

ALK, anaplastic lymphoma kinase; NPM, nucleophosmin; TPM, tropomyosin; TFG, tropomyosin receptor kinase-fusion gene; ATIC, 5-aminoimidazole-4-carboxamide-1-beta-D-ribonucleotide trans-formylase/inosine monophosphate cyclohydrolase; CLTCL, clathrin heavy polypeptide-like gene; MSN, moesin.

TABLE 6.37.4

Salient Features of Laboratory Diagnosis of Systemic Anaplastic Large Cell Lymphoma (ALCL)*

1. T-cell or null cell phenotype with predominance of T-helper cell subtype
2. Positive immunohistochemical staining for CD30, clusterin, EMA, cytotoxic proteins, and in most cases, ALK1
3. Negative for B-cell markers, BSAP and CD15
4. Cytogenetic or molecular demonstration of t(2;5) or NPM-ALK genes or their variants
5. T-cell receptor-β- or γ-chain gene rearrangement is seen in most cases.

* See Table 6.37.2 for differences between systemic ALCL and primary cutaneous ALCL. ALK,

anaplastic lymphoma kinase; CD, cluster of differentiation; BSAP, B-cell-specific activation protein; EMA, epithelial membrane antigen; NPM, nucleophosmin.

The NPM-ALK translocation can be demonstrated by various techniques, including Southern blotting, reverse transcriptase-polymerase chain reaction (PCR), RNA in situ hybridization, and genomic DNA-PCR (33, 34 and 35). These techniques are either time-consuming, difficult to apply to paraffin sections, or prone to artifact. Therefore, immunohistochemical staining with monoclonal or polyclonal antibodies against the fusion protein, NPM-ALK, appears to be the most desirable technique for a prompt diagnosis (7). However, molecular genetic techniques are important to identify the variants and additional cytogenetic aberrations.

The current case is typical for a small cell variant of ALCL in morphology showing predominantly small tumor cells with a minority of large tumor cells infiltrating the perivascular areas. Immunohistochemical studies are diagnostic by demonstrating positive staining of CD30, ALK, and EMA. The demonstration of t(2:5) translocation further confirms the diagnosis. Flow cytometry demonstrated CD30 and the selective loss of CD7. The low percentages of B-cell markers are helpful in substantiating the diagnosis of a T-cell lymphoma. The salient features of laboratory diagnosis of ALCL are summarized in Table 6.37.4.

Clinical Manifestations

Clinically, ALCL can be subdivided into primary and secondary forms (6,7). The primary form is further divided into systemic and cutaneous subforms. The primary form can be rarely induced by human immunodeficiency virus (HIV) infection, but those cases are usually of B-cell lineage. The clinical manifestation of ALCL varies greatly from case to case in early studies. The variation may be due to the differences of diagnostic criteria, the inclusion of cutaneous and systemic forms, the variation of patient age, and the immunophenotype. However, current studies have found that the most important factor that influences the clinical outcome is the presence or absence of ALK protein (14,21,36, 37, 38 and 39). There are no clinical differences among various fusion proteins, whether it is NPM-ALK or its variants (2,6). Therefore, it appears that the variation of cytogenetics does not affect the clinical course of ALCL. Accordingly, primary systemic ALCL is subdivided into ALK-positive and ALK-negative subforms.

Anaplastic Lymphoma Kinase-Positive Primary Systemic Anaplastic Large Cell Lymphoma

ALK-positive systemic ALCL is usually seen in children and younger populations in their second and third decades (6,7). Its frequency is about 13% of childhood non-Hodgkin lymphoma and 2% of adult cases (2). The male/female ratio is 6.5:1. At diagnosis, the lymphoma is usually at stage III or IV. The noncontiguous distribution of lymphadenopathy, low frequency of splenomegaly, and high frequency of extranodal involvement help to distinguish ALCL from Hodgkin lymphoma (2).

Systemic B symptoms (fever, night sweats, and/or weight loss) are present in about 75% of patients. Extranodal involvement is commonly present in the skin, bone, soft tissues, lung, and liver (2,6,7). Bone marrow involvement is detected in about 11% of cases by examining hematoxylin and eosin-stained sections. With immunohistochemical stain, the positive rate increases to 30%. Peripheral blood involvement is uncommon in ALCL. Cases with leukemic presentation usually show a poor response to therapy or early relapse (40).

The 5-year survival rate is markedly different between the ALK-positive and ALK-negative groups. In three studies, the differences were 79.8% versus 32.9% (37), 71% ± 8% versus 15% ± 11% (38), and 79% versus 46% (39), respectively.

Anaplastic Lymphoma Kinase-Negative Primary Systemic Anaplastic Large Cell Lymphoma

ALK-negative systemic ALCL occurs in older individuals aged 46 to 61 years (6,7). The male/female ratio is 0.9:1. One study showed that this clinical form has a lower incidence of stage II to IV disease and extranodal involvement (38), but these findings were not confirmed by another study (39). However, both studies revealed a poor prognosis in this clinical form. Nevertheless, more recent studies revealed that this subtype has variable clinical behavior and that new parameters are needed to stratify this group of patients. The prognostic factors that are suggested include the International Prognostic Index (composed of age, disease stage, number of extranodal sites, lactate dehydrogenase [LDH] level, and performance status) and the number of tumorinfiltrating activated cytotoxic T lymphocytes (18).

Primary Cutaneous Anaplastic Large Cell Lymphoma

This form is invariably ALK negative. It is seen in older patients with a median age of about 60 years (6,7). The

most common clinical presentation is a solitary, asymptomatic cutaneous or subcutaneous reddish-violet mass with or without superficial ulceration. The lesion is often located in the extremities and trunk (2). In contrast to the general rule, the ALK-negative primary ALCL has a better prognosis than does the ALK-positive primary ALCL with skin involvement. Approximately 25% of patients show partial or complete spontaneous regression. Patients have long-term survival after local excision with or without radiation therapy.

The histologic features of primary cutaneous ALCL overlap with those of lymphomatoid papulosis, and their distinction frequently depends on clinical follow-up. Almost 100% of cases of lymphomatoid papulosis have spontaneous regression (2). Therefore, no treatment is required for this entity, even with clinical relapse.

Secondary Anaplastic Large Cell Lymphoma

Secondary ALCL represents a transformation from other lymphomas (mycosis fungoides, peripheral T-cell lymphomas), Hodgkin

lymphoma, or lymphomatoid papulosis (6,7). It is usually seen in older individuals with the absence of ALK protein expression. The clinical prognosis is generally poor.

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CASE 38 Hodgkin Lymphoma

CASE HISTORY

A 63-year-old man came to the clinic for a prostate biopsy because his prostate serum antigen was elevated. During the subsequent workup, he was found to have hilar and paratracheal adenopathy by chest x-ray. Computed tomography (CT) scan further identified bulky disease in porta hepatis lymph node with compression of the stomach, multiple liver nodules, and adenopathies in the mesenteric, periaortic, and paratracheal regions. At that time, the patient also had low-grade fever, drenching night sweats, and pruritus. He had lost 30 pounds in the past 6 months.

Physical examination on admission revealed hepatosplenomegaly but no superficial lymphadenopathy. His total leukocyte count was 4,500/μL, hematocrit 37%, hemoglobin 12 g/dL, and platelets 479,000/μL. The serum lactate dehydrogenase was 378 U/dL.

Because no lymph nodes were accessible, a bone marrow biopsy was done; it showed features that were suspicious of Hodgkin lymphoma (HL). The subsequent mediastinal lymph node biopsy confirmed that diagnosis. The patient was treated with HL regimen and showed clinical improvement, including gaining 5 pounds and having fewer night sweats and less skin rash.

FLOW CYTOMETRY FINDINGS

Lymph node biopsy: CD3/CD4 67%, CD3/CD8 25%, CD5 87%, CD19 11%, CD19/κ 6%, CD19/λ 4%, CD20 13%, CD23 8%, FMC-7 12%, CD10 1%, CD45 100%.

DISCUSSION

HL is a unique tumor in that the tumor cell is in the minority of the cellular composition (about 1%), whereas the major component in the background histology is reactive inflammatory cells (1,2). The comprehensive review by Taylor and Riley (3) describes the interesting history of longstanding efforts to elucidate the nature of the tumor cell (Reed-Sternberg [RS] cell), with various techniques, including microbiology, serology, immunohistochemistry, cell culture, animal inoculation, and finally molecular genetics. At the beginning, HL was considered an infectious disease; when it became evident that it is a malignancy, the cell lineage of the tumor cells had been elusive for many years until recently. Only when a micromanipulative technique is applied in combination with the polymerase chain reaction (PCR) to single RS cells are the RS cells finally

identified as clonal B cells. As a result, Hodgkin disease is now considered a lymphoma, thus the name has been changed to HL (1,2).

TABLE 6.38.1

Comparison of World Health Organization (WHO) Classification and Rye Classification

WHO Classification	*Rye Classification*

Nodular lymphocyte predominant HL	Lymphocyte predominance, nodular subtype
Classical HL	
Nodular sclerosis classical HL	Nodular sclerosis
Mixed cellularity classical HL	Mixed cellularity
Lymphocyte-rich classical HL*	Lymphocyte predominance, diffuse subtype
Lymphocyte-depleted classical HL	Lymphocyte depletion

* Includes some cases of mixed cellularity and cellular subtype of nodular sclerosis. HL, Hodgkin lymphoma.

Since the Rye Conference in 1966 (4), the classification of HL went unchanged until 1994 when the International Lymphoma Study Group updated the classification (5). The original classification divided HL into lymphocyte predominance, nodular sclerosis, mixed cellularity, and lymphocyte depletion. In the Revised European-American classification of lymphoid neoplasms (REAL), the lymphocyte predominance type is redefined to include only the nodular subtype of lymphocyte predominance. A lymphocyte-rich classical Hodgkin disease (HD) type has been created to include the diffuse subtype of lymphocyte predominance, some cases of cellular phase of nodular sclerosis, and some cases of mixed cellularity in the Rye classification (5). The World Health Organization (WHO) classification separates the lymphocyte predominance type from the classical HL and designates it as nodular lymphocyte predominance (NLP) HL (6). The remaining four types are classified as classical HL (Table 6.38.1).

Morphology

The most important criterion for the diagnosis of HL is the identification of a small number of large mononucleated and multinucleated tumor cells, designated Hodgkin and RS cells (HRS cells) (7,8). RS cells are characterized by their large size, bilobed or polylobated nuclei, or multinucleation, with prominent eosinophilic inclusion-like nucleoli and abundant amphophilic cytoplasm (Figs. 6.38.1 and 6.38.2). There are several variants of RS cells. The presence of the variants alone is not diagnostic, but it should be an indication of the need to search further for diagnostic cells. The mononuclear variant is a large cell with a single nucleus that contains a prominent eosinophilic nucleolus. This cell is called a mononucleated Hodgkin cell. A cell with similar features, but multinuclear, is called a multinucleated Hodgkin cell. Mononucleated and multinucleated Hodgkin cells are not diagnostic in an unknown case, but their presence is sufficient for the diagnosis of extranodal HL in patients with known nodal HL. The degenerated form of a tumor cell is a dark smudged cell, which is called a “mummified cell.”

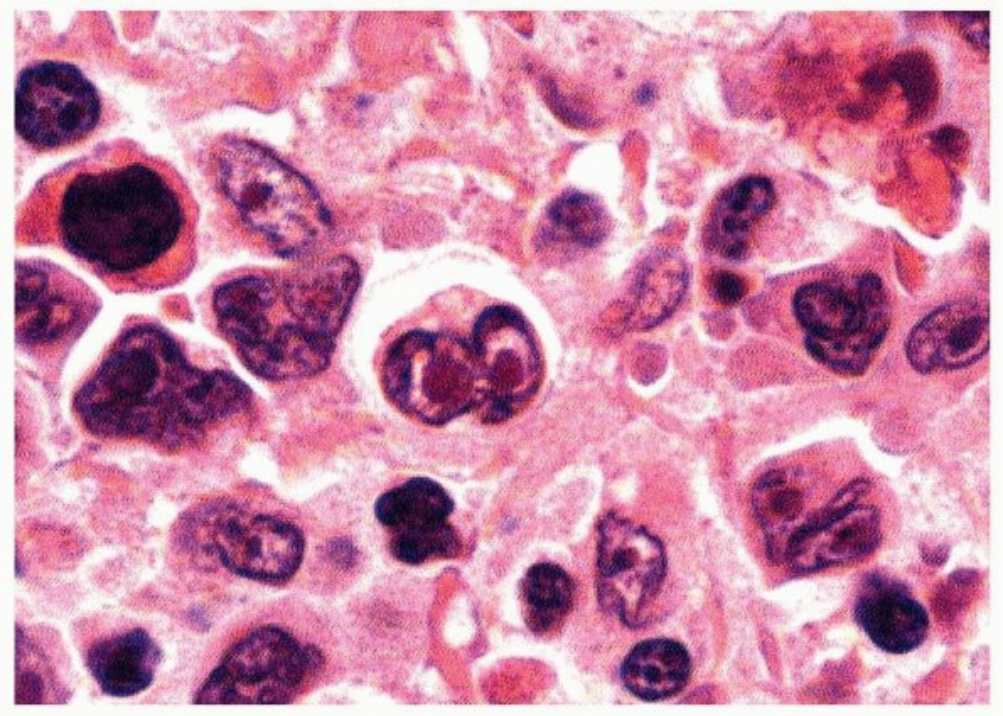

FIGURE 6.38.1 Lymph node biopsy shows a Reed-Sternberg cell in the center, surrounded by a few mononuclear and multinuclear Hodgkin cells. Hematoxylin and eosin, 200× magnification.

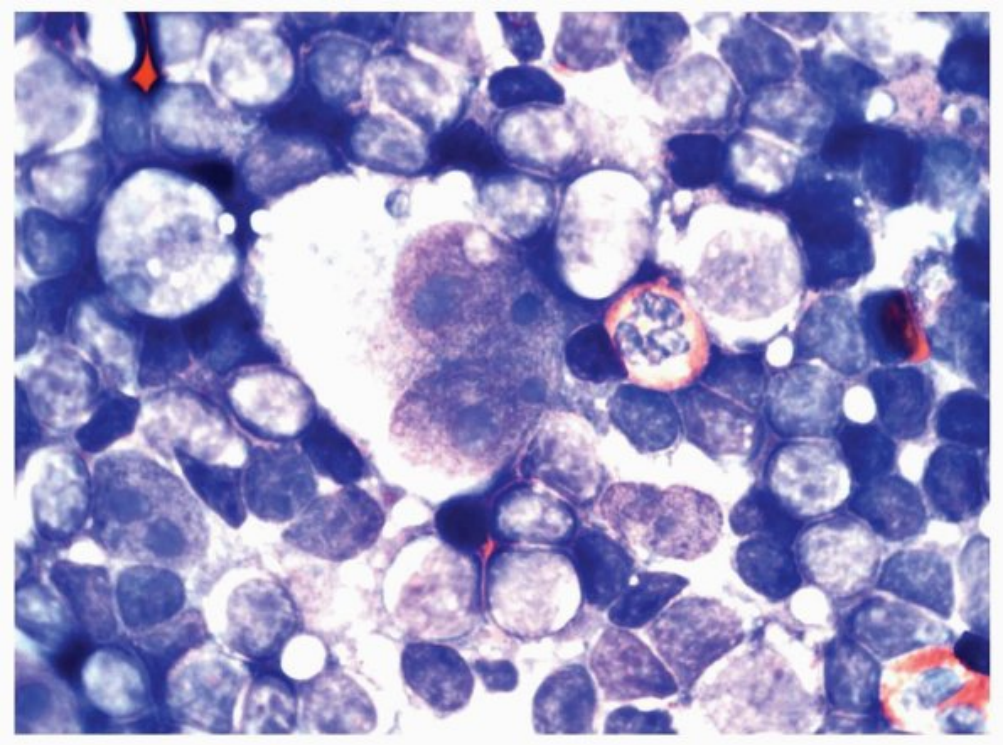

FIGURE 6.38.2 Lymph node imprint demonstrates a Reed-Sternberg cell in the center, surrounded by lymphocytes, granulocytes, and a few eosinophils. Wright-Giemsa, 200× magnification.

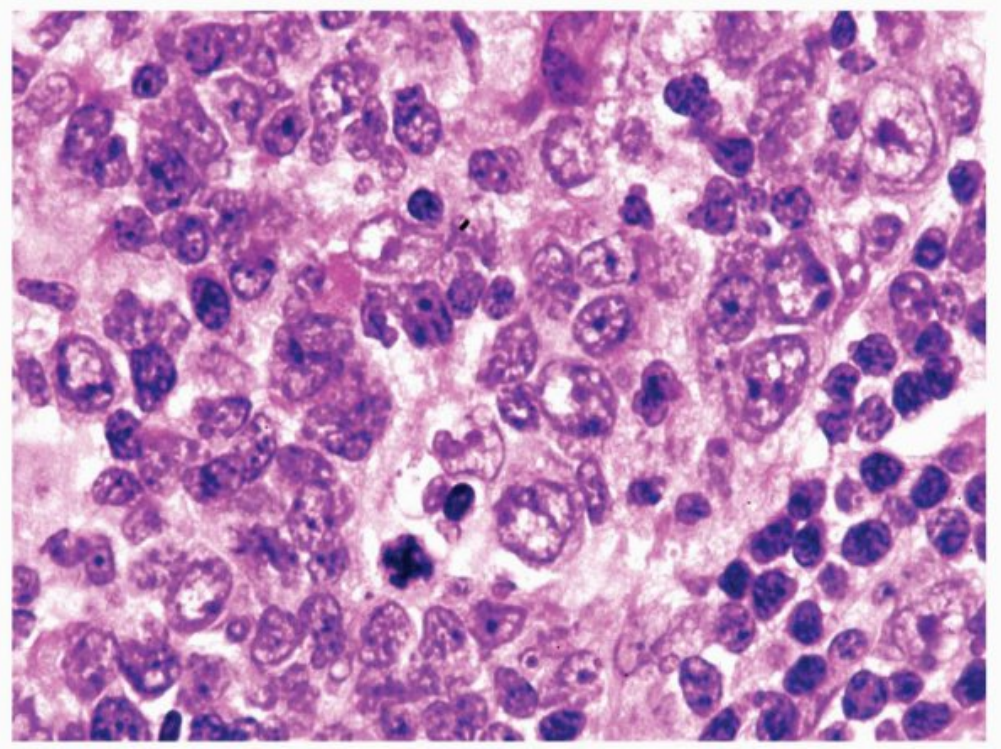

FIGURE 6.38.3 Lymph node biopsy reveals a cluster of lymphocytic and/or histiocytic (L & H) cells with folded, convoluted, or lobated nuclei; thin nuclear membrane, vesicular chromatin pattern, and inconspicuous nucleoli. Hematoxylin and eosin, 100× magnification.

The other two variants are also not diagnostic of HL, but are characteristic of certain types of HL. The lymphocytic and/or histiocytic (L&H) cell variant or popcorn cells are large cells with folded, convoluted, or lobated nuclei; thin nuclear membrane; vesicular chromatin pattern; and inconspicuous nucleoli (Fig. 6.38.3). The cytoplasm of these cells is usually abundant and pale staining. The lacunar variant is a large cell located in a lacunalike space. This phenomenon is considered to be an artifact due to formalin fixation, because the lacunalike space is not seen in other forms of fixation. The lacunar cells have monolobated or polylobated nuclei, delicate nuclear chromatin, small nucleoli, and an abundant water-clear or pale eosinophilic cytoplasm (Fig. 6.38.4).

The identification of these tumor cells in the lymph node frequently leads to a definitive diagnosis. When the tumor cells are seen in extranodal tissues, however, a diagnosis of HL cannot be established unless the patient has a history of HL diagnosed by lymph node biopsy. In the bone marrow, the characteristic feature is focal fibrosis or focal increase of cellularity (Fig. 6.38.5). The cellular component usually includes eosinophils, plasma cells, histiocytes, and a few Hodgkin tumor cells, but classical RS cells are seldom present. It is important to use immunohistochemical stains to confirm the diagnosis.

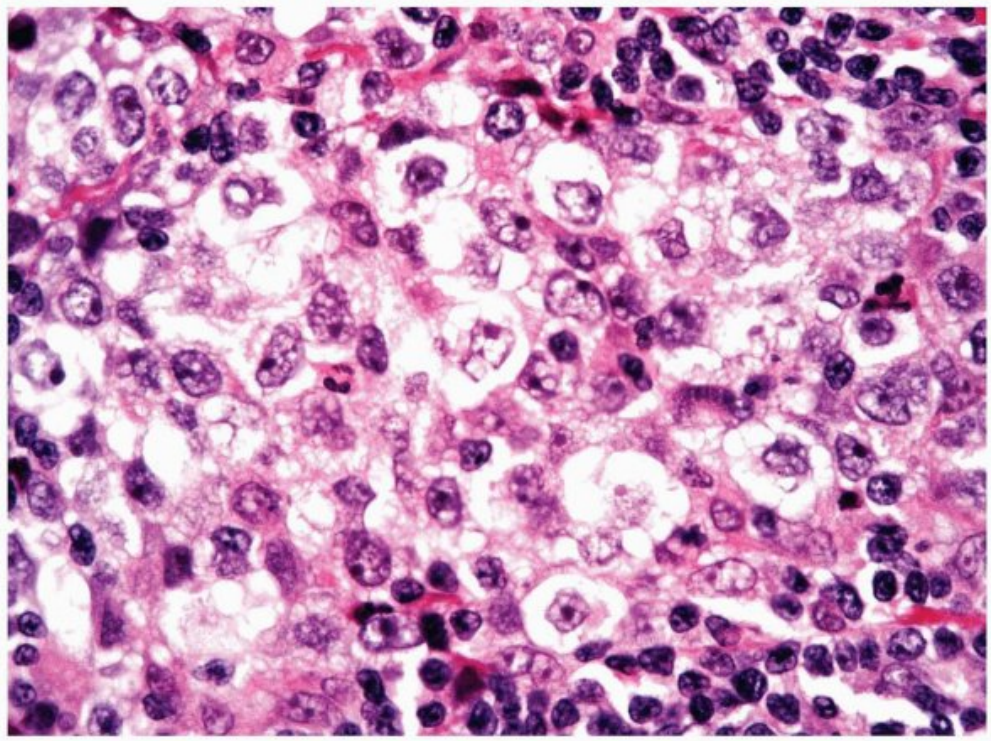

FIGURE 6.38.4 Syncytial variant of nodular sclerosis shows sheets of lacunar cells. Hematoxylin and eosin, 60× magnification.

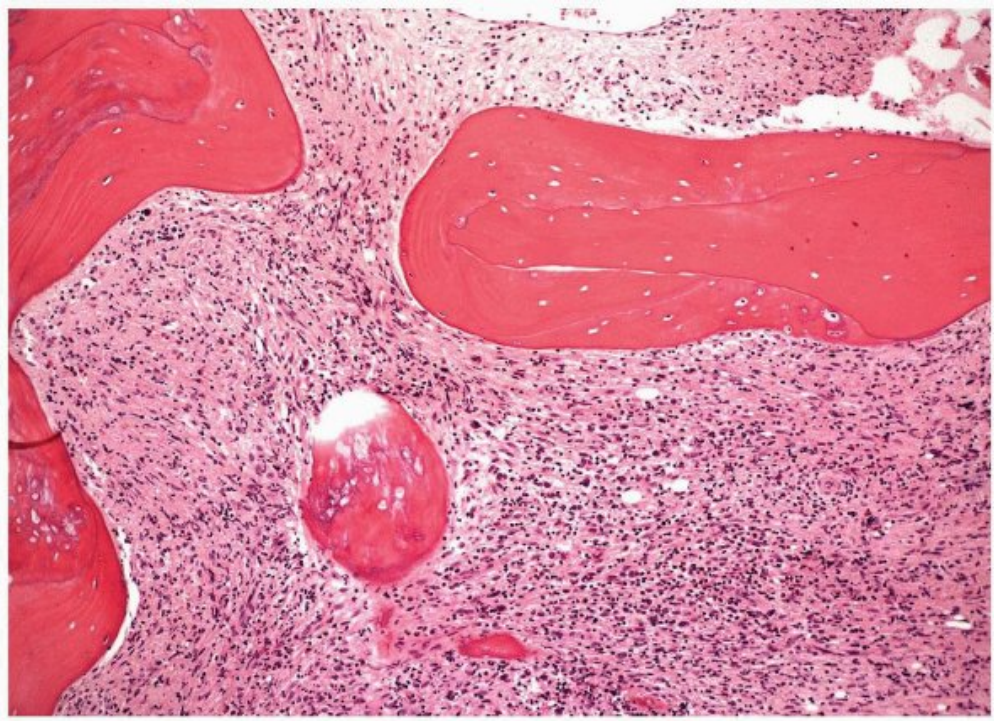

FIGURE 6.38.5 Bone marrow biopsy reveals a cellular area with fibrosis. There are scattered tumor cells with an eosinophilic background. The diagnosis of Hodgkin lymphoma involvement is proved by immunohistochemistry. Hematoxylin and eosin, 10× magnification.

Nodular Lymphocyte Predominance Hodgkin Lymphoma (NLPHL)

This type is characterized by a vague or partially nodular pattern with diffuse areas. Reticulin stain may accentuate the nodular configuration. The background is composed of small lymphocytes, histiocytes, and epithelioid histiocytes. Eosinophils, neutrophils, plasma cells, and necrosis are rarely seen. The tumor cells are mainly L&H cells, with accompanied mononuclear Hodgkin cells, mummified cells, and abnormal mitosis. However, classical RS cells are hard to find. In rare cases, follicular hyperplasia with progressive transformation of germinal centers (PTGC) may be present (9). These germinal centers contain various proportions of small lymphocytes of mantle zone type (Fig. 6.38.6). This lesion may be similar to T-cell-rich B-cell lymphoma, but the latter does not contain nodular pattern.

Nodular Sclerosis Hodgkin Lymphoma (NSHL)

The characteristic histologic pattern of NSHL is the presence of interconnecting bands of collagen fibers separating lymphoid tissue into cellular nodules (Fig. 6.38.7). The collagen bands can be identified by their birefringent character when examined under polarized light. The lymph node capsule may also become thickened. The characteristic tumor cells are the lacunar cells, which have subtle differences in number and in morphology among various subtypes.

The number of classical RS cells also varies in different subtypes of NSHL. The background cellular components may be predominantly lymphocytes or mixed populations of lymphocytes, eosinophils, and neutrophils. Plasma cells and histiocytes may also be present. When eosinophils account for >5% of the background cells, a poor prognosis is expected (10). On the basis of the cellular background of the nodules, NSHL can be further divided into subtypes of lymphocytic predominance, mixed cellularity, and lymphocyte depletion. The syncytial variant of NSHL is the diagnosis when large aggregates of lacunar cells are present. Focal necrosis is common in NSHL.

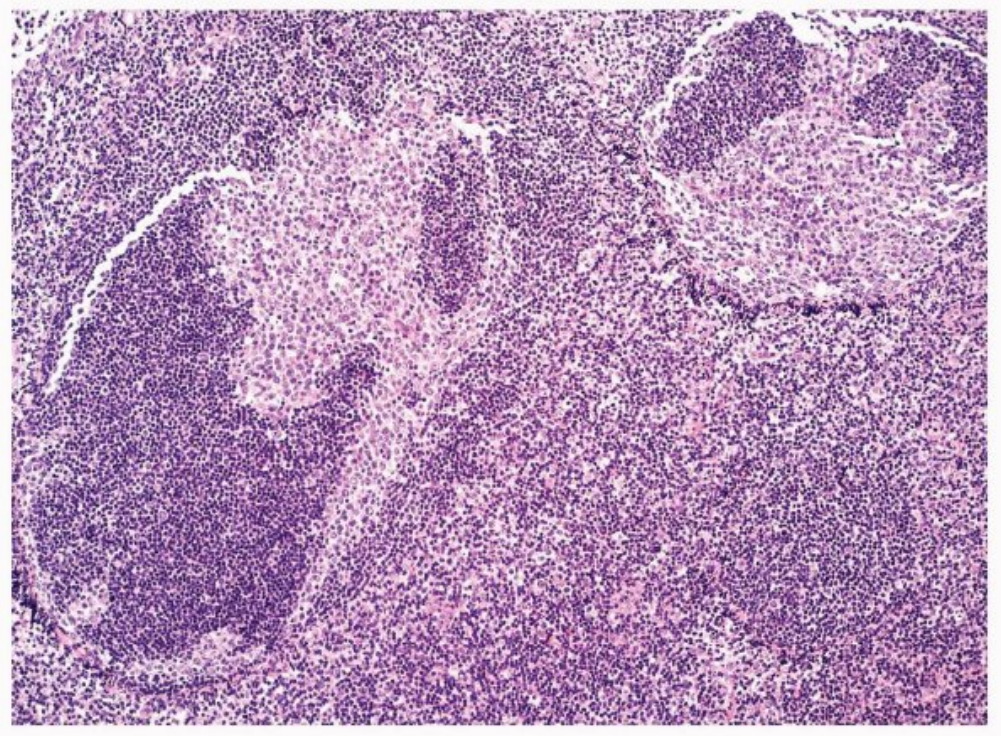

FIGURE 6.38.6 Hyperplastic lymph node shows small lymphocytic infiltration in two germinal centers, consistent with progressive transformation of germinal centers. Hematoxylin and eosin, 10× magnification.

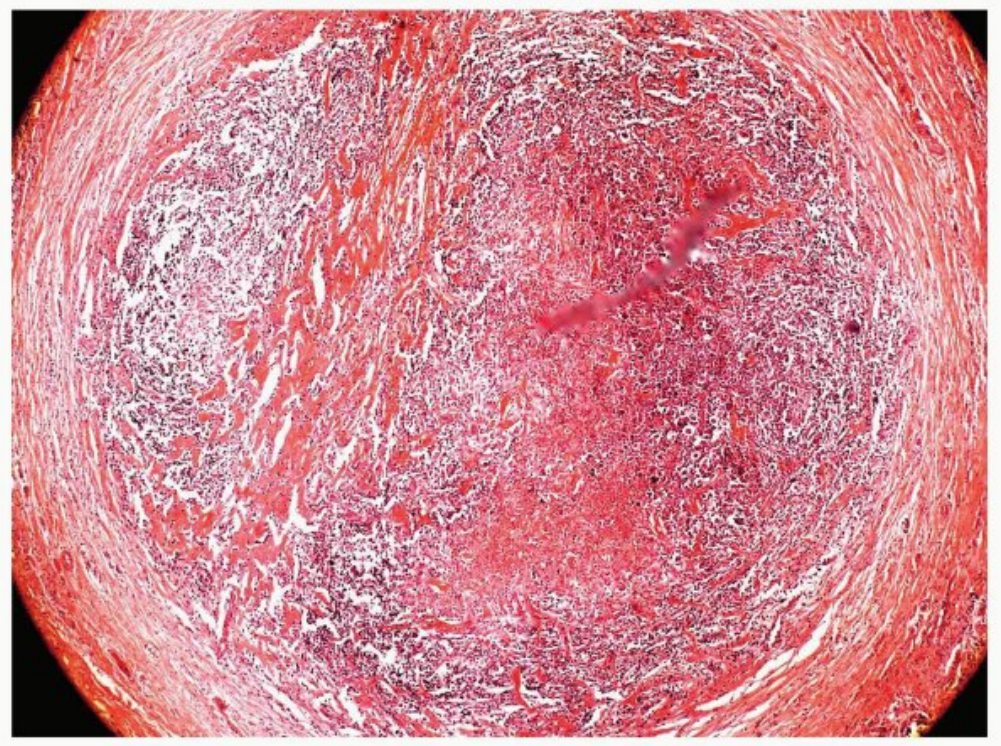

FIGURE 6.38.7 Lymph node biopsy in a case of nodular sclerosis Hodgkin lymphoma reveals a cellular nodule surrounded by a thick band of collagen fibers. There is focal necrosis in the center, and scattered lacunar cells are barely recognizable. Hematoxylin and eosin, 4× magnification.

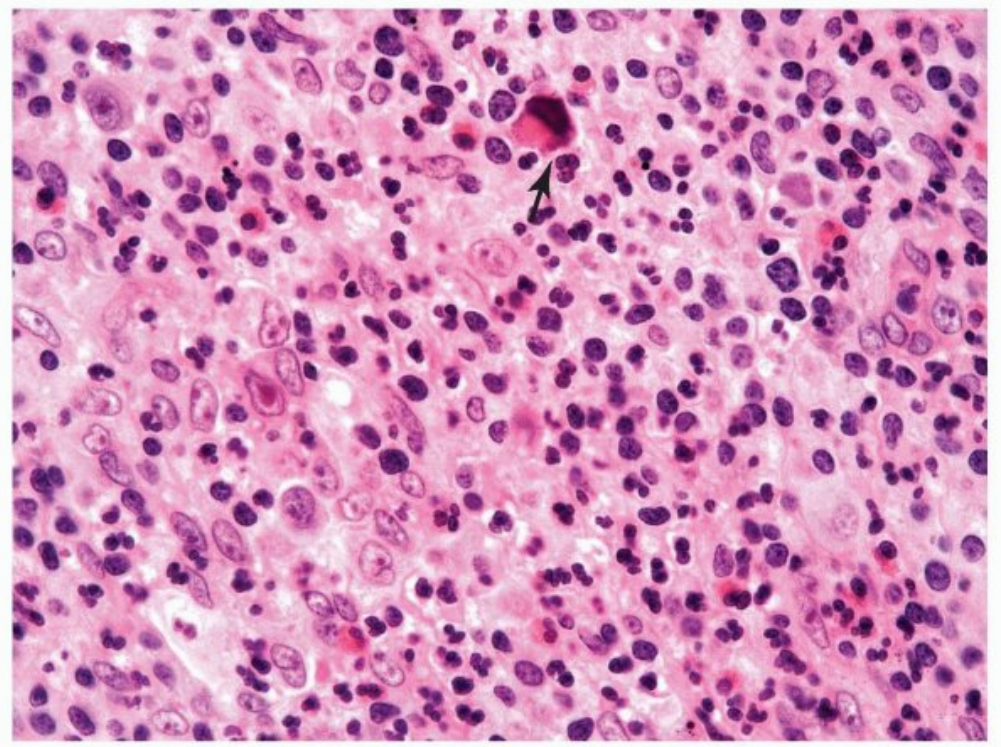

FIGURE 6.38.8 Lymph node biopsy in a case of mixed cellularity Hodgkin lymphoma shows a

background of lymphocytes, eosinophils, neutrophils, and histiocytes. A few Hodgkin cells and a mummified cell (*arrow*) are present. Hematoxylin and eosin, 60× magnification.

Mixed Cellularity Hodgkin Lymphoma (MCHL)

This type was considered to be a wastebasket of unclassifiable cases in the Rye classification, but is now recognized as a true subtype of HL. It is characterized by diffuse mixed cellular infiltration with easily identifiable RS cells but without a nodular sclerosing pattern (Fig. 6.38.8). The number of classical RS cells and mature lymphocytes in MCHL falls between that of LRHL and LDHL (7). The background cells include lymphocytes, eosinophils, neutrophils, histiocytes, and plasma cells, but one of the cell types may be more prominent. When the epithelioid histiocytes are predominant, granuloma-like clusters may be present.

Lymphocyte-Rich Classical Hodgkin Lymphoma (LRCHL)

In this type, the growth pattern can be nodular or diffuse. In the nodular subtype, the nodule is composed mainly of small lymphocytes and may contain regressed germinal centers. Eosinophils and neutrophils are seldom seen. The HRS cells are usually present in the expanded mantle zone. They are mostly L&H cells or mononuclear lacunar cells. In the diffuse subtype, histiocytes and epithelioid histiocytes may be abundant (Fig. 6.38.9). Sometimes LRCHL can only be distinguished from NLPHL by immunophenotyping (11).

Lymphocyte-Depleted Hodgkin Lymphoma (LDHL)

LDHL is considered to be the last histologic phase of HL, in which tumor cells predominate and reactive lymphocytes diminish (6). Thus, the classical RS and Hodgkin cells become abundant and lymphocytes are depleted along with fibrosis. On the basis of variations in RS cells and the fibrotic pattern, LDHL is subdivided into the reticular and diffuse fibrous subtypes.

The reticular subtype is characterized by the presence of abundant classical RS cells or of bizarre multinucleated

RS cells, referred to as sarcomatous RS cells (Fig. 6.38.10). In this variant, there are occasional mononuclear Hodgkin cells, mummified cells, and scattered aberrant mitoses. In the background lymphocytes are scarce, and plasma cells, eosinophils, histiocytes, and neutrophils are also rare or absent. The fibrosis is diffuse, patchy, irregular, and fibrillar in nature. The fibrous tissue is not birefringent and thus not collagenous. Focal necrosis is common in this subtype. Many cases previously diagnosed as this subtype may well be non-Hodgkin lymphoma (NHL) particularly anaplastic large cell lymphoma. In the subtype of diffuse fibrosis, lymphocytes are depleted, fibrosis is diffuse and disorderly, the number of RS cells is variable, and focal necrosis is frequently present. If a nodular sclerosing pattern is present, it should be classified as NSHL.

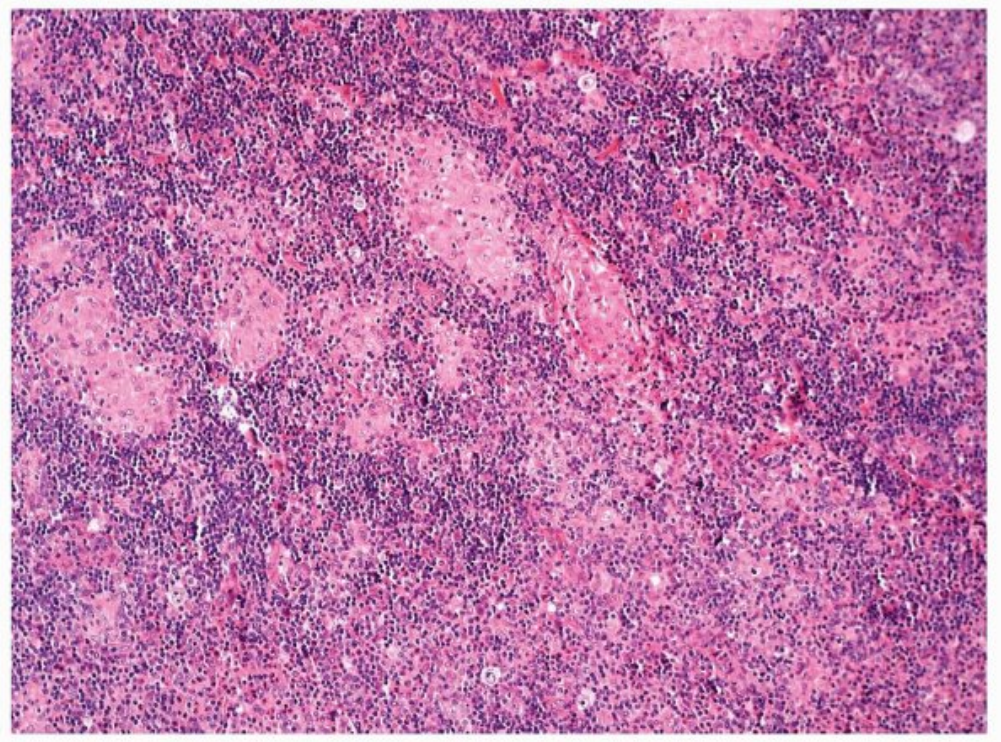

FIGURE 6.38.9 A case of the diffuse subtype of lympho-cyte-rich Hodgkin lymphoma shows

several clusters of epithelioid histiocytes mimicking granulomas. Hematoxylin and eosin, 10× magnification.

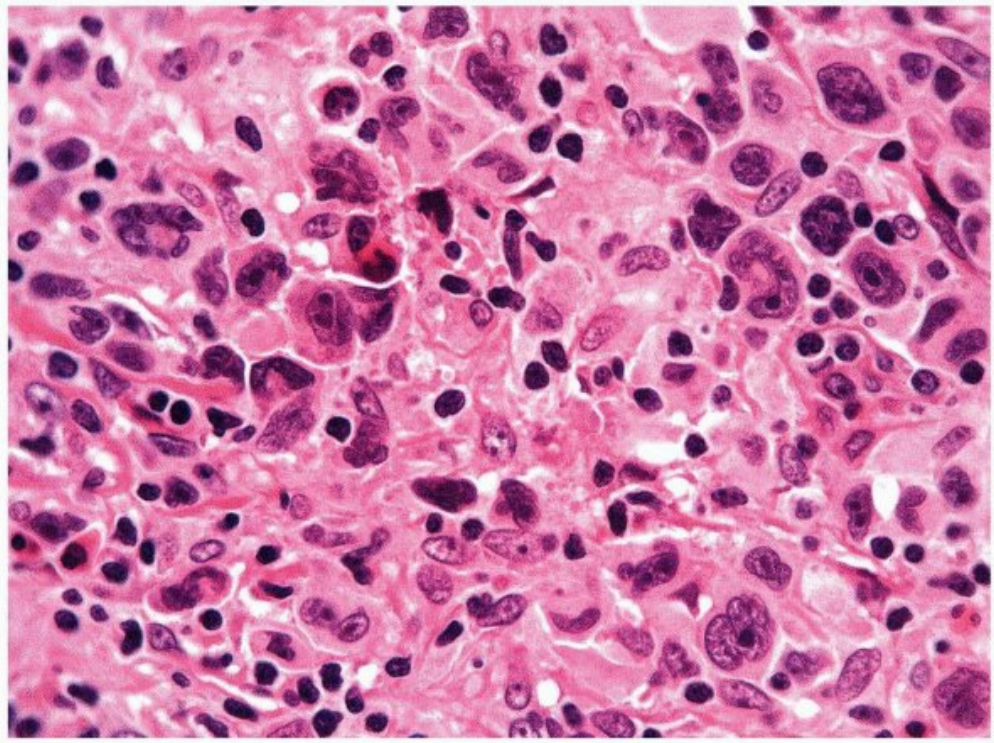

FIGURE 6.38.10 Lymph node biopsy in a case of lymphocyte-depleted Hodgkin lymphoma shows multiple bizarre multinucleated Hodgkin and Reed-Sternberg cells assuming a sarcomatous feature. The background contains very few lymphocytes, eosinophils, and neutrophils. Hematoxylin and eosin, 60× magnification.

Differential Diagnosis

Several NHLs mimic HL and are called the gray zone lymphomas, and one benign condition should also be distinguished from HL (12).

The major differential diagnosis is T-cell-/histiocyte-rich B-cell lymphoma (TCRBCL), because the large lymphoma cells mimic HRS cells, whereas the background cells are normal mature T lymphocytes and histiocytes, similar to those seen in HL. Their distinction depends on immunophenotyping (1,2,12).

Another lymphoma that is sometimes difficult to distinguish from HL is ALCL. The tumor cells may resemble mononuclear or multinucleated HRS cells. However, the ALCL cells frequently form cohesive sheets and involve lymph node sinuses, features that are not seen in HL (1,2,12). HL can transform into ALCL, and an intermediate form between ALCL and HL has been reported (13).

When HL is present in the mediastinum, it should be distinguished from primary mediastinal B-cell lymphoma. The latter may show a nodular sclerosing pattern with the presence of RS-like cells and occasionally an immunophenotype of CD30+ CD15+ (14).

In PTGC, the follicles may, in some cases, resemble the nodules in NLPHL. These two entities may coexist, and PTGC may occasionally develop into NLPHL; however, PTGC usually involves only a small number of lymphoid follicles. In addition, in PTGC, the normal architecture is wellpreserved in most parts of the lymph node, whereas in NLPHL, the entire nodal architecture is effaced (15). The major difference between these two entities is that the nodules of NLPHL contain L&H and RS cells, whereas the follicles in PTGC contain follicular center cell aggregates (16).

Immunophenotype

Because the HRS cells in HL cases are usually <1% of the cellular components, flow cytometry offers no help in identifying HRS cells and in diagnosing HL. The role of flow cytometry in HL is to exclude the possibility of NHLs, such as diffuse large B-cell lymphoma and some cases of TCRBCL (when the number of neoplastic B lymphocytes is high enough for clonal identification). In addition, flow cytometry

also can identify the T-cell background in classical HL, which usually shows a predominance of CD4 cells.

Immunohistochemistry, in contrast, plays a very important role in the diagnosis and classification of HL. The minimal panel for immunophenotyping includes CD30, CD15, and CD45 (Figs. 6.38.11, 6.38.12, 6.38.13). In classical HL, the HRS cells are positive for CD30 and CD15, but negative for CD45. B-cell lymphomas, in contrast, should be negative for CD30 and CD15 but positive for CD45. However, some RS-like large cells in B-cell lymphomas may pick up CD30 staining, but they are usually CD15 negative.

In NLPHL, the L&H and RS cells have an immunophenotype similar to that of NHL, such as negative for CD30 and CD15 but positive for CD45 and other B-cell-associated antigens (CD20 and CD79a). Unlike B-cell lymphoma,

NLPHL is positive for epithelial membrane antigen (EMA) and is generally negative for immunoglobulin (Ig) light chains (1,2), although one group reported light-chain restriction in the L&H cells (17). However, J chain has been demonstrated in many cases (1,2), and clonal light-chain messenger RNA (mRNA) has been detected by the in situ hybridization technique (18). Furthermore, the bcl-6 protein and two activation-associated molecules, CD40 and CD86, are also expressed by the L&H cells (1,2). Oct2 is a transcription factor that activates the promoter of the Ig genes in conjunction with its coactivator BOB.1. These two markers are positive in NLPHL cases but negative in classical HL (19).

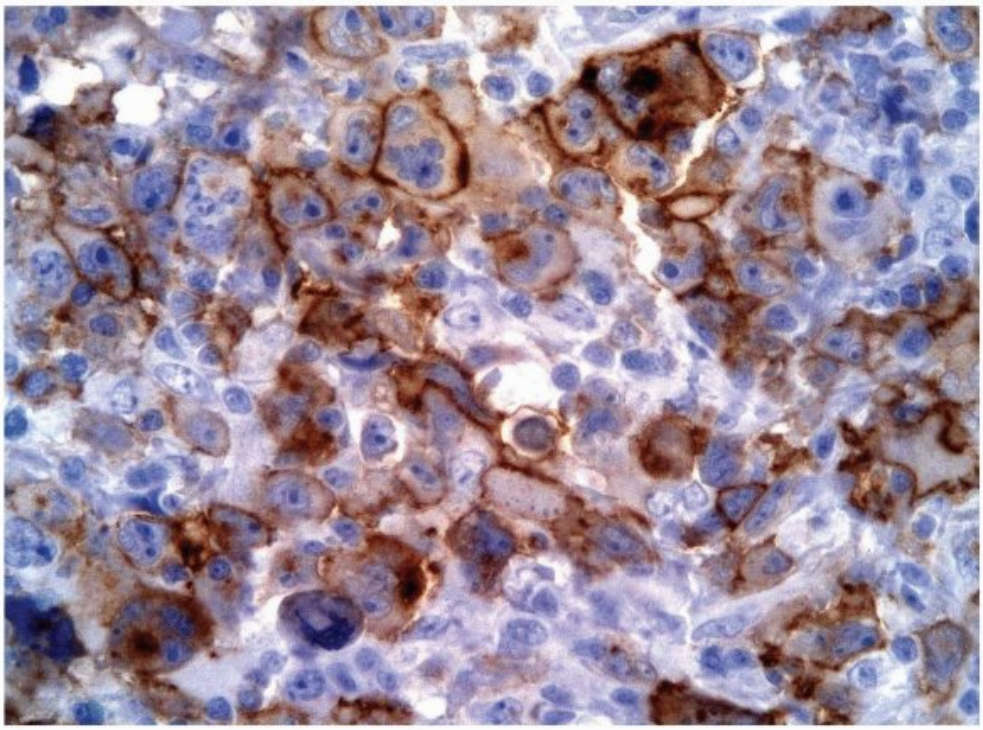

FIGURE 6.38.11 Lymph node biopsy of the same case as in Fig. 6.38.9 shows multiple Hodgkin and Reed-Sternberg cells stained with CD30. Note the membrane and Golgi staining pattern. Immunoperoxidase, 60× magnification.

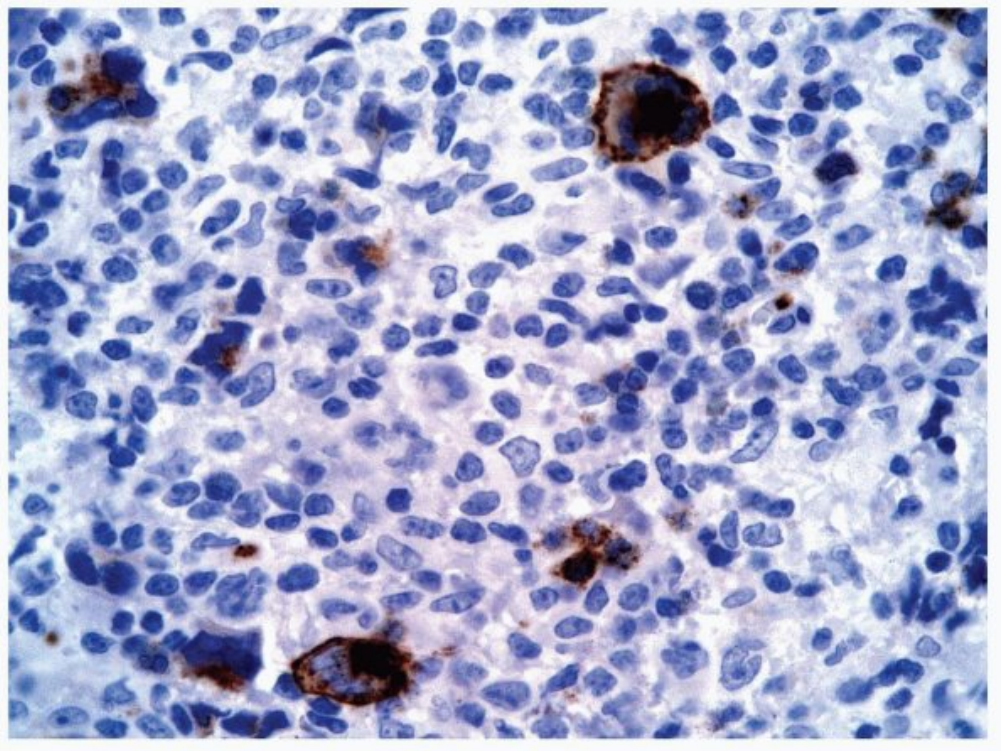

FIGURE 6.38.12 Lymph node biopsy of the same case as in Fig. 6.38.9 shows two Hodgkin and Reed-Sternberg cells with CD15 stain. Immunoperoxidase, 60× magnification.

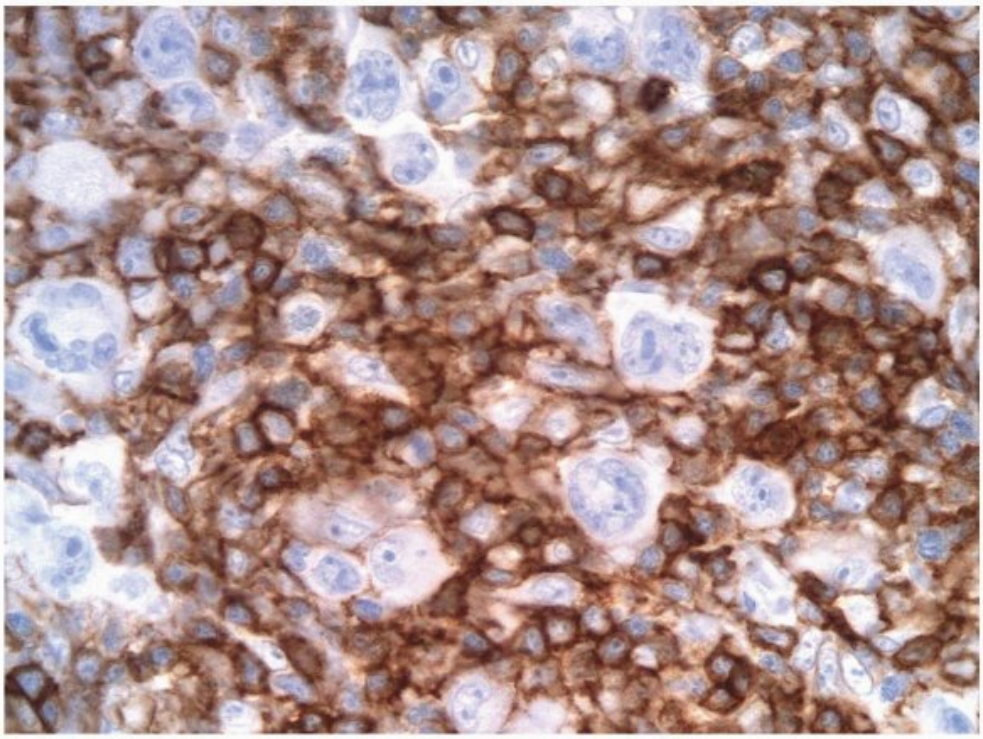

FIGURE 6.38.13 Lymph node biopsy of the same case as in Fig. 6.38.9 shows CD3 staining of the background lymphocytes. The Hodgkin and Reed-Sternberg cells are negative for CD3 staining. Immunoperoxidase, 60× magnification.

The small lymphocytes in the NLPHL nodules are a mixture of polyclonal B cells and T-cells. Many of the T-cells bear CD57 similar to those seen in the normal germinal centers. Some of the T-cells also coexpress CD57 and bcl-6 (20). These T cells are characterized by forming small aggregates and rosetting around the neoplastic B cells. The nodules also contain meshwork of follicular dendritic cells demonstrated by CD21 or CD35 (1,2).

In the LRHL, the HRS cells show the typical immunophenotype of CD30+ CD15+ CD45-. B-cell antigens are usually not expressed on the HRS cells, but 3% to 5% of LRHL cases show positive CD20 (1,2). Weak expression of one or more T-cell antigens is encountered in a minority of cases (21). In addition, HRS cells also express bcl-6 (20). In the background, CD57-positive T-cells are present and form rosettes with HRS cells (1,2). In one study, the background T-cells expressed mainly bcl-6 but rarely CD57 (20). The cellular nodule in LRHL cases may show extensive CD20 staining (Fig. 6.38.14) and the follicular dendritic cell meshwork as highlighted by CD21 staining (9).

In other classical HLs, including NSHL, MCHL, and LDHL, the HRS cells show an immunophenotype similar to that mentioned in the LRLH type, namely CD30+ CD15+ CD45-, with CD20 demonstrated in 5% of cases (22). In a few studies, CD20 was demonstrated in 10% to 20% of cases in paraffin material and in 87% of cases in frozen sections (8). CD20 staining is usually variable in intensity and present only on a minority of the tumor cells (23,24). CD79a is seldom present. The B-cell-specific activation protein (BSAP), a product of the PAX5 gene, is present in approximately 90% of cases, but is usually weaker than that of reactive B cells (25). HRS cells also express CD25, human leukocyte antigen-DR (HLA-DR), intercellular adhesion molecule (ICAM)-1, CD95, CD40, CD86, vimentin, and fascin (8,12). The T-cells surrounding the HRS cells are positive for CD40 ligand and CD28, the ligand for CD86 (1,2). In contrast to

NLPHL, the HRS cells in classical HL (except for LRHL) cases do not express bcl-6 protein (1,2).

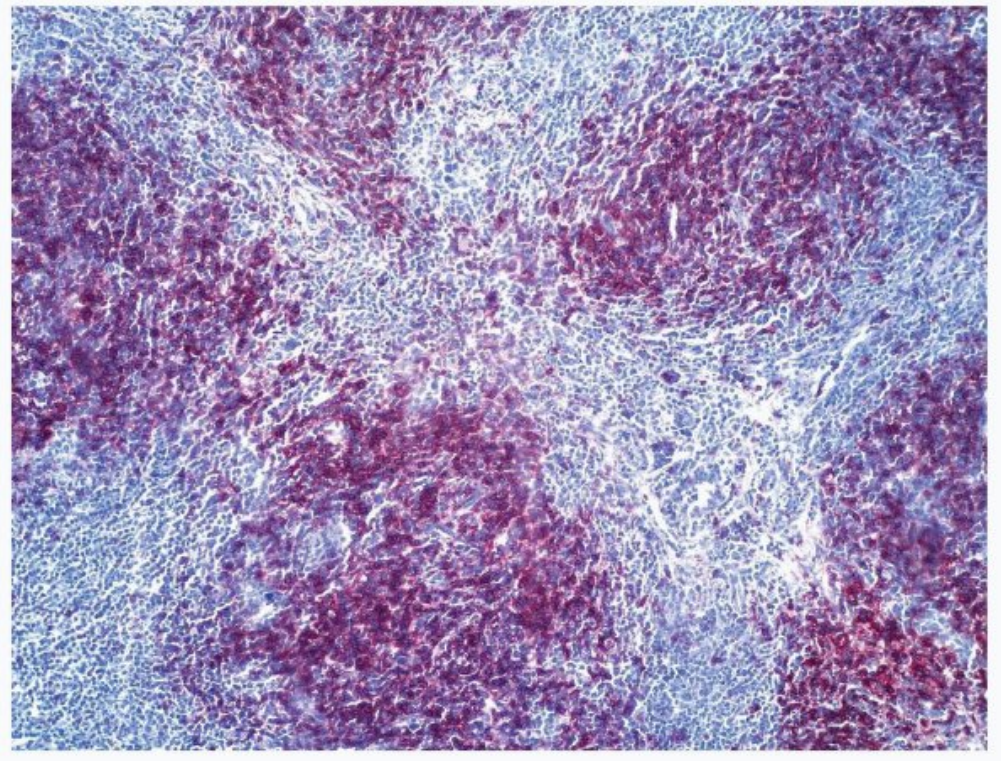

FIGURE 6.38.14 Lymph node biopsy in a case of lymphocyte-rich Hodgkin lymphoma shows multiple nodules highlighted by CD20 staining. Immunoperoxidase, 10×magnfication.

In TCRBCL, the tumor cells stain for pan-B-cell markers and are often EMA positive, but they are negative for CD30, CD15, and vimentin (1,2,26). The background lymphocytes usually show T-cell staining but are CD57 negative (12). However, a recent study showed that, in a subset of TCR-BCL, the background lymphocytes were CD57 positive or coexpressed CD57 and bcl-6 (20). The large tumor cells were bcl-6 positive. The authors suggested that this subset might be an architectural variant of NLPHL.

ALCL is usually CD30+ CD15- EMA+. A subset of ALCL is also positive for anaplastic lymphoma kinase (ALK) protein (1,2,12). Most cases of ALCL are positive for pan-T-cell markers. The CD30+ large cell lymphoma of B-cell lineage is classified as a diffuse large B-cell lymphoma (5,6).

The immunophenotype of PTGC is similar to that of NLPHL. They both contain large nodules of B cells with follicular dendritic cell meshworks and many CD57-positive T cells (27). However, the T cells in NLPHL often form clusters, and those in PTGC are evenly distributed as in normal germinal centers. T-cell rosetting with large B cells is only seen in NLPHL (1,2).

In Epstein-Barr virus (EBV)-positive cases, the RS cells show EBV-latent membrane protein and Epstein-Barr nuclear antigen 1 (EBNA1) but not EBNA2. EBV can be detected by showing latent membrane protein in paraffin sections, but the more sensitive technique for its identification is in situ hybridization for EBV latency-associated small RNAs (1,2). Using these methods, EBV is found in approximately 50% of classical HL, but it is rarely found in NLPHL (28). In addition, some patients with HL have high titers of antibodies against EBV. Therefore, EBV may play a role in the pathogenesis of some types of HL. However, in populations in whom HL is common, the tumor cells seldom show EBV, whereas in populations in whom HL is rare, the tumor cells are frequently positive. These findings may argue against the role of EBV in the pathogenesis of HL, and EBV infection may be merely an epiphenomenon in HL (1,2).

In addition, classical HL is frequently associated with overexpression of large numbers of cytokines and chemokines and/or their receptors in HRS cells (9,29). These proteins may be responsible for the presence of abundant inflammatory cells in classical HL cases (9,29). For instance, the overexpression of eotaxin probably accounts for the eosinophilia in the background. Transforming growth factor-ß may be responsible for the fibrosis. The CC chemokine, (TARC), may contribute to the predominance of thymus and activation-regulated chemokine Th2 cells in the infiltrating T-cell population in classical HL cases.

Comparison of Flow Cytometry and Immunohistochemistry

The diagnosis and differential diagnosis of HL depend on immunohistochemistry. Flow cytometry plays no role in the diagnosis of HL.

In the current case, flow cytometry showed mainly T lymphocytes with a normal CD4/CD8 ratio. The B-lymphocyte population was in the minority and revealed no monoclonality by the κ/λ ratio. Thus, the flow cytometric result helped to exclude B-cell lymphoma. The diagnosis was initially made by a bone marrow biopsy, but bone marrow involvement is only seen in about 5% of HL cases and is the result of hematogenous spread. Because of bone marrow infiltration and prominent B symptoms, the patient was in stage IVb. A lymph

node biopsy is necessary for the confirmation of the diagnosis of HL and for subclassification. In this case, the lymph node showed a nodular sclerosing pattern, which generally carries a favorable prognosis. However, because the patient was already in stage IVb with a bulky disease, his clinical course became rapidly progressive and he died 2 years after the diagnosis. The salient features for laboratory diagnosis of HL are summarized in Table 6.38.2.

Molecular Genetics

Monoclonality of HRS cells has never been convincingly demonstrated by immunophenotyping or by molecular biologic techniques (Southern blot and PCR) done in whole-tissue DNA. Because HRS cells are usually composed of <1% of the cellular population in HL and because polyclonal B cells may be present in the same sections, it is understandable why the old techniques failed to draw a conclusion in terms of clonality. It has been only after the use of the sophisticated single-cell micromanipulation techniques in combination with PCR that the monoclonal nature of HRS cells has been finally established (16,30,31).

The second question is the origin of the HRS cells. The presence of follicular dendritic cells, CD57+ T cells in the tumor nodules or in the background of HL, and the expression of bcl-6 by the tumor cells and some background T cells are all suggestive of the tumor cell being a centroblast in the germinal center (31). This hypothesis has now been proved by the use of molecular biology techniques.

In all instances, sequence analysis of RS cell-derived Ig gene rearrangements demonstrated high loads of somatic mutations, which were associated with the presence of

stop codons and deletions in some cases (30). Somatic mutation in the variable region of immunoglobulin heavychain (VH) genes takes place in the germinal center; thus the somatically mutated V_H genes are specific markers for germinal center B cells. Signs of typical ongoing mutations were missing in the classical HL cases studied (30). However, cases of NLPHL not only showed a high load of somatic mutations but also intraclonal mutations, indicating continued mutation after establishment of the clone (16).

TABLE 6.38.2

Salient Features of Laboratory Diagnosis of Hodgkin Lymphoma (HL)

Classical HLs	
	Specific markers for HRS cells: CD30+ CD15±
	Other important positive marker for HRS cells: BSAP
	Important negative marker for HRS cells: CD45
NLPHL	
	Positive markers for L&H cells: CD45+ CD20+ CD79a+ BSAP+ J chain ± Ig±
	Negative markers for L&H cells: CD30, CD15
Immunophenotype for background lymphocytes	
	Classical HL: Polyclonal CD4+ cells and CD57+ cells
	NLPHL: Polyclonal B cells and T-cells with CD57+ cells

HRS, Hodgkin and Reed-Sternberg; CD, cluster of differentiation; Ig, immunoglobulin; BSAP, B-cell-specific activation protein; L&H, lymphocytic and/or histiocytic; NLPHL, nodular

lymphocyte predominant Hodgkin lymphoma.

Differing from the deleterious mutations found in classical HL, NLPHL does not contain mutations that would prevent their translation into functional proteins. The crippling mutations in the Ig gene in HRS cells lead to the absence of κ and λ gene transcripts by in situ hybridization studies (16). The absence of Ig transcripts is caused by the inactivation of the Ig promoter by the absence of octamer-dependent transcription factor (Oct2) and/or its coactivator (BOB.1) (32,33). However, B cells acquiring crippling mutations are usually efficiently eliminated within the germinal center by apoptosis; therefore, HRS cells must be rescued by some transforming events so that they can survive.

There are many possible antiapoptotic mechanisms that involve the constitutively expressed transcription factors, such as nuclear factor-κB (NF-κB), Stat3, Notch1, and highly expressed cFlip molecules (34,35). The involvement of NF-κB is suggested by the fact that its inactivation restores the sensitivity of HRS cells to apoptosis in vitro (36). Most of the classical HL cases express tumor necrosis factor receptor (TNFR) family members and their ligands, leading to the activation of NF-κB (29,37). Another possibility is that EBV infection induces the expression of latent membrane protein (LMP)1, which possesses antiapoptotic potential (38).

In several cases of HL associated with NHL or chronic lymphocytic leukemia (either simultaneously, progressing, or subsequently), the RS cells were found by sequence analysis to share a common B-cell precursor with other B-cell neoplasms (38, 39, 40 and 41). However, the transforming event totally changes the morphology and immunophenotype of HL, whereas the transforming event for NHL or chronic lymphocytic leukemia maintains the features of the precursor B cell (30). In addition, the rearrangements of the NHL cells (but not of the RS cells) show signs of continuing mutation (30).

HL cell line studies identify distinct gene expression profiles, which are similar to those of EBV-transformed B cells and cell lines derived from diffuse large cell lymphomas (42).

Clonal cytogenetic abnormalities are found in most cases of classical HL by conventional karyotyping, but they are not recurrent or specific (1,2). Many cases show 14q abnormalities but not t(14;18). With the fluorescence in situ hybridization technique, clonal numeric aberrations in all cases of HL are shown to fall into two groups (1,2). Comparative genomic hybridization shows recurrent gains of the chromosomal subregions on chromosomal arms 2p, 9p, and 12q and distinct high-level amplifications on chromosomal bands 4p16, 4q23-q24, and 9p23-p24 (43).

Clinical Manifestations

NLPHL is seen in approximately 5% of cases of HL (7,8). It is characteristically seen in young (<35 years of age) males. Most patients are asymptomatic. About 80% of patients are stage I or II at presentation. The most frequently involved lymph nodes are in the cervical and axillary regions, followed by the inguinal region. The prognosis of NLPHL is very favorable, but it may transform into MCHL or LDHL with subsequent widespread extranodal disease. NLPHL also frequently transforms into NHL (44). However, because 80% of untreated NLPHL patients have achieved 10-year survival, some authors suggest a reduction therapy or a watch and wait strategy (44).

LRCHL comprises 6% of HL (22). It is also seen predominantly in male patients with an average age higher than that of patients with NLPHL. Most patients are in stage I or II at presentation. However, mediastinal mass is more frequently encountered in LRCHL than in NLPHL but not as frequent as seen in NSHL (1,2,44). Although relapses are frequent, the prognosis is generally good in this group of patients.

NSHL is the most common type of HL, accounting for 60% of the total cases (7,8). This is the only type of HL with female predominance. Patients are usually <50 years of age, and 60% are stage I or II at presentation. The clinical manifestation is often cervical or supraclavicular lymphadenopathy or a mediastinal mass. NSHL has a greater histologic stability than other types of HL. The prognosis is usually good, except for the lymphocyte depletion subtype, which is more frequently seen in men with symptoms and advanced stage of disease.

MCHL is the second most common type of HL, accounting for approximately 30% of the total cases (7,8). This is considered to be an intermediate form between lymphocyte

predominance (including NLPHL and LRCHL) and LDHL. Therefore, the age range of patients, the clinical course, and the prognosis are all between those of lymphocyte predominance and LDHL. The stage at presentation is usually II or III. MCHL frequently transforms into LDHL. It involves the abdominal lymph node and spleen more often, but involvement of the mediastinum is less frequent than the NSHL type (1,2).

LDHL is a rare type of HL seen in only 1% to 5% of total cases (7,8). It is usually encountered in elderly patients with a median age of 50 to 57 years in various reports. Male patients are predominant in this type. Most patients have constitutional symptoms, namely, night sweats in 30% of patients, fever in 60%, and weight loss in 67% (8). Patients may also have peripheral and abdominal lymphadenopathy, hepatosplenomegaly, bone marrow involvement, lymphopenia, or subdiaphragmatic disease (7). Vascular invasion and extranodal spread are common autopsy findings in these cases. About 80% of the patients are found to have stage IIIB or IVB at presentation. The prognosis is very poor, especially for patients with the reticular subtype.

In human immunodeficiency virus-infected patients, the clinical and pathologic presentations differ from those of non-acquired immunodeficiency syndrome (non-AIDS) patients. In AIDS patients, 41% to 100% have either MCHL or LDHL (45). Systemic B symptoms are seen in 70% to 100%, stage III or IV disease in 75% to 90%, and bone marrow involvement in 45% to 70% of AIDS patients (45). The median

survival in this group of patients is approximately 18 months.

The prognosis of HL is strongly associated with the stage of the disease. The Ann Arbor classification is universally adopted (Table 6.38.3) (46). Essentially, a single lymph node region or extranodal site involvement is classified as stage I; two or more lymph node regions on the same side of the diaphragm, stage II; lymph node regions on both sides of the diaphragm, stage III; and disseminated disease, stage IV. In addition, on the basis of absence or presence of constitutional symptoms, such as fever, night sweats, and/or weight loss of 10% of body weight, the disease is further divided into substages A and B, respectively.

TABLE 6.38.3

Ann Arbor Staging System of Hodgkin Lymphoma

Stage	*Degree of Involvement*
I	Involvement of a single lymph node region (I) or a single extralymphatic site (IE)
II	Involvement of two or more lymph node regions on the same side of the diaphragm (II) or localized involvement of an extralymphatic site (IIE)
III	Involvement of lymph node regions on both sides of the diaphragm (III) or localized involvement of an extralymphatic site (IIIE), the spleen (IIIS), or both (IIISE)
IV	Disseminated involvement of one or more extralymphatic sites with or without lymph node involvement

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CASE 39 Thymoma

CASE HISTORY

A 61-year-old man had a resection of the descending colon because of colon cancer 3 months prior to the present admission. In a follow-up computed tomography (CT) scan of the thorax, he was found to have an anterior mediastinal mass, radiologically consistent with a thymoma, and was admitted for removal of the tumor. The patient had no chest pain, cough, dyspnea, superior vena cava syndrome, or signs of myasthenia gravis (MG). Physical examination on admission was unremarkable. At operation, a mass approximately 1.7 cm at its greatest diameter was found in the thymus, and the thymus with the encapsulated mass was resected en bloc. Postoperatively, he had bilateral pneumothoraces, which were promptly resolved after a chest tube was placed on the right side. The patient was discharged 4 days after the operation.

FLOW CYTOMETRY FINDINGS

Thymectomy specimen: B-cell markers: CD19 0%, κ 0%, λ 1%. T-cell markers: CD3 19%, CD5 97%, CD7 97%, CD4/CD8 96%. Myeloid markers: CD13-CD33 0%, CD14 0%. Immature cell markers: CD10 48%, CD34 0%, terminal deoxynucleotidyl transferase (TdT) 65% (Fig. 6.39.1).

IMMUNOHISTOCHEMICAL STAINS

The lymphoid cells are positive for CD1a, CD3, and CD10. The tumor cells stain for pancytokeratin (Fig. 6.39.2).

MOLECULAR GENETICS

T-cell receptor (TCR) gene rearrangement analysis by polymerase chain reaction shows a germline configuration.

DISCUSSION

Thymoma is an epithelial tumor of the thymus. However, various numbers of lymphocytes are often present and intermingle with the tumor cells. These lymphocytes are, therefore, used as a surrogate marker for the diagnosis of thymoma. The stage of maturation of the lymphocytes also affects the grade of thymoma. The thymic function depends on the interaction between thymocytes and epithelial cells (1). The epithelial tumor cells in a thymoma retain the normal thymic “nursing” function by producing a lymphopoietic environment that attracts uncommitted thymocytes (2,3). Therefore, a small group of thymomas with undifferentiated epithelial tumor cells showed essential absence of lymphocytes (4).

Morphology

Thymoma is characterized by the presence of a lobular pattern separated by broad fibrous bands (5). Each lobule is composed of a dense population of epithelial tumor cells intermixing with varying proportions of lymphocytes or thymocytes. The normal division of cortex and medulla is usually not discernable, except for the organoid thymoma (World Health Organization [WHO] classification type B1) (6). Hassall corpuscles are only seen in organoid thymoma and occasionally in cortical thymoma (WHO classification type B2).

The epithelial tumor cells are either polygonal (round or oval) or spindle-shaped. The nuclei of the tumor cells are usually vesicular with a small inconspicuous nucleolus. The cytoplasm is of variable amount and pale acidophilic. Most cases contain lymphocytes, but <4% of thymomas may show no lymphocytes at all. Although the immunophenotype of the accompanying lymphocytes may help the diagnosis, it is the identification of the epithelial tumor cells by cytokeratin stain that confirms the diagnosis.

There are many classifications of thymoma. The early classification of Kuo and Lo divides thymomas into predominantly lymphocytic (Fig. 6.39.3), predominantly epithelial (Fig. 6.39.4), mixed lymphocytic and epithelial, and predominantly spindle cells (7). Levine and Rosai (8) proposed dividing thymomas into benign and malignant on the basis of the presence of capsular invasion. The thymoma with capsular invasion but no prominent cytologic atypia is designated malignant thymoma type I, and those with overt cytologic evidence of malignancy are designated malignant thymoma type II. Marino and Müller-Hermelink (9) introduced a histogenetic classification that divides thymomas into medullary, mixed, predominantly cortical, cortical, and well-differentiated thymic carcinoma. More recently, Suster and Moran (10,11) advocated a simplified classification on the basis of cytologic differentiation. The well-differentiated cytology is designated thymoma; the moderately well differentiated, atypical thymoma, and the poorly differentiated, thymic carcinoma.

Because there is no consensus about the nomenclature of thymic epithelial tumors, the WHO Committee has chosen a noncommittal alphabetic system for classification (Table 6.39.1) (6,12,13). Type A is for tumor cells with a spindle or oval shape, whereas type B is for tumor cells with a dendritic or epithelioid appearance. Tumors with both features are called type AB. Type B thymomas are

further divided into B1, B2, and B3 on the basis of an increasing epithelial cell/lymphocyte ratio and emergence of atypical neoplastic epithelial cells. Type C is designated for nonorganoid thymic carcinomas.

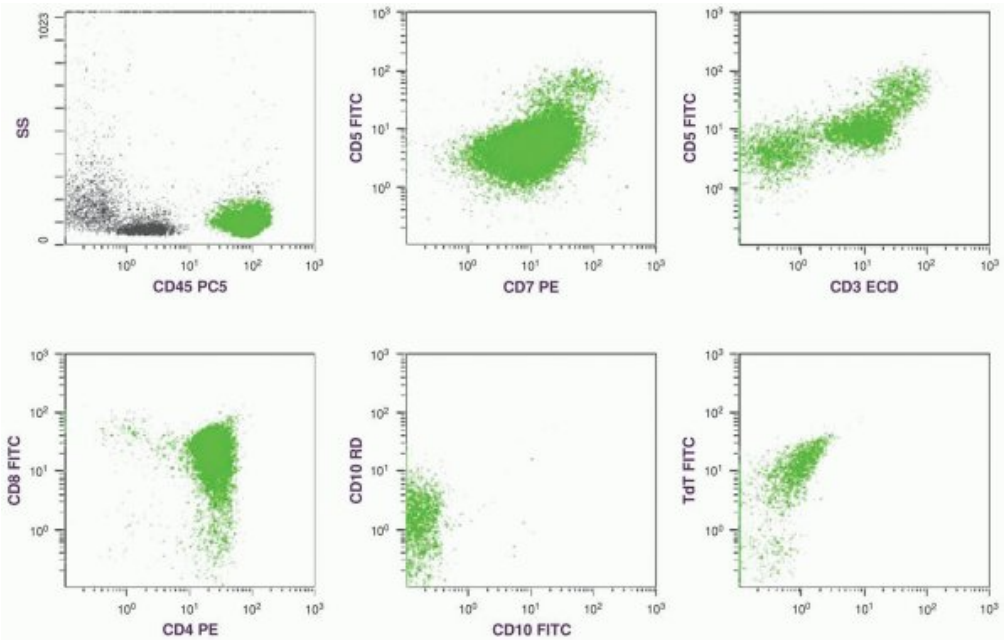

FIGURE 6.39.1 Flow cytometric analysis of a thymus specimen shows positive reactions to CD3, CD4, CD5, CD7, CD8, CD10 (partial), and terminal deoxynucleotidyl transferase (TdT). The presence of a major population of CD4+ CD8+ cells coexistent with small populations of CD4+ CD8- and CD4- CD8+ cells is characteristic. The smearing pattern of other pan-T-cell markers is also a characteristic feature of thymoma. SS, side scatter; PC5, phycoerythrin-cyanin 5; FITC, fluorescein isothiocyanate; PE, phycoerythrin; ECD, phycoerythrin-Texas Red; RD, rhodamine.

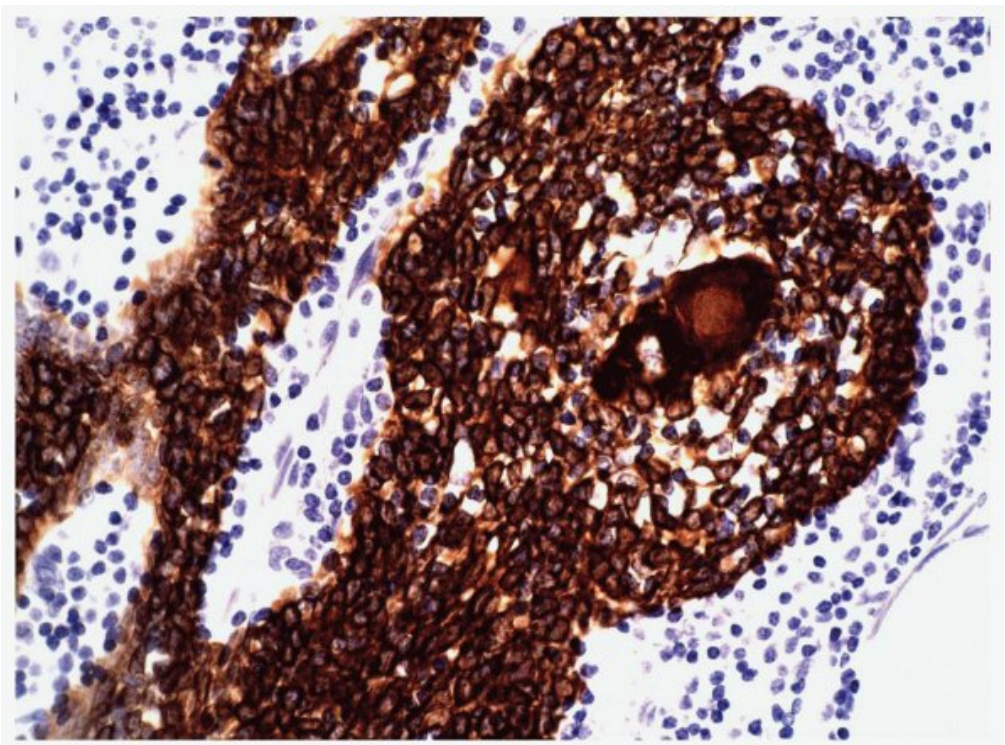

FIGURE 6.39.2 Network of tumor cells stains with cytokeratin, whereas the lymphocytes are negative. Note that a Hassell corpuscle shows strong cytokeratin stain. Immunoperoxidase, 40×

magnification.

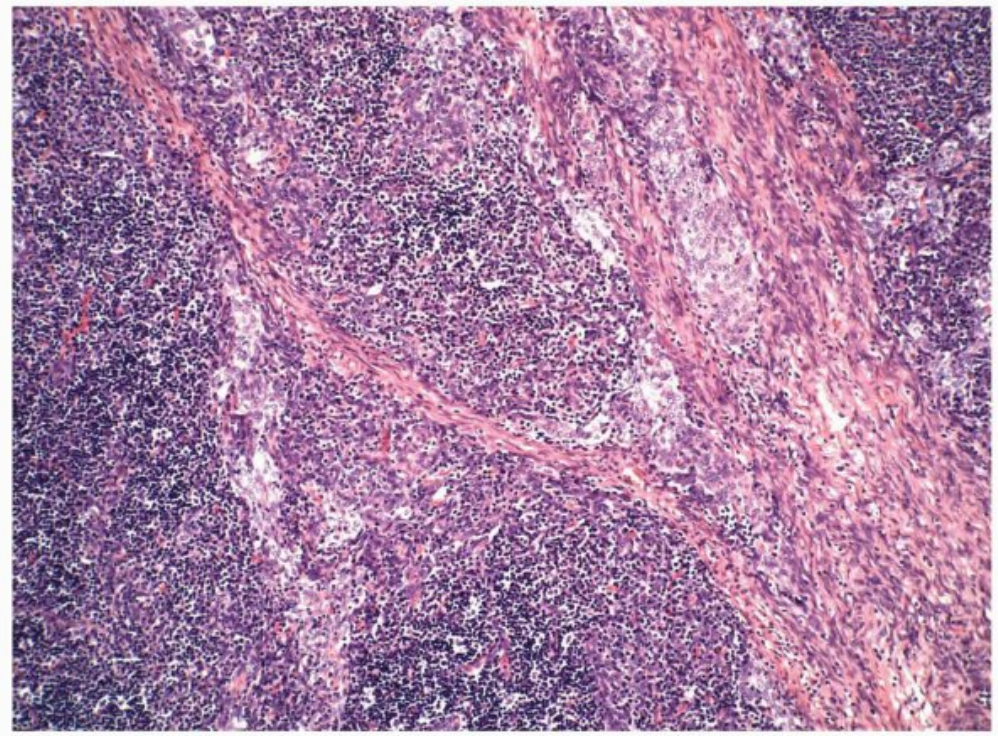

FIGURE 6.39.3 Predominantly lymphocytic thymoma shows a lobular pattern separated by broad fibrous bands. The predominant cell population is lymphocyte with several small clusters of pale-stained epithelial cells. Hematoxylin and eosin, 10× magnification.

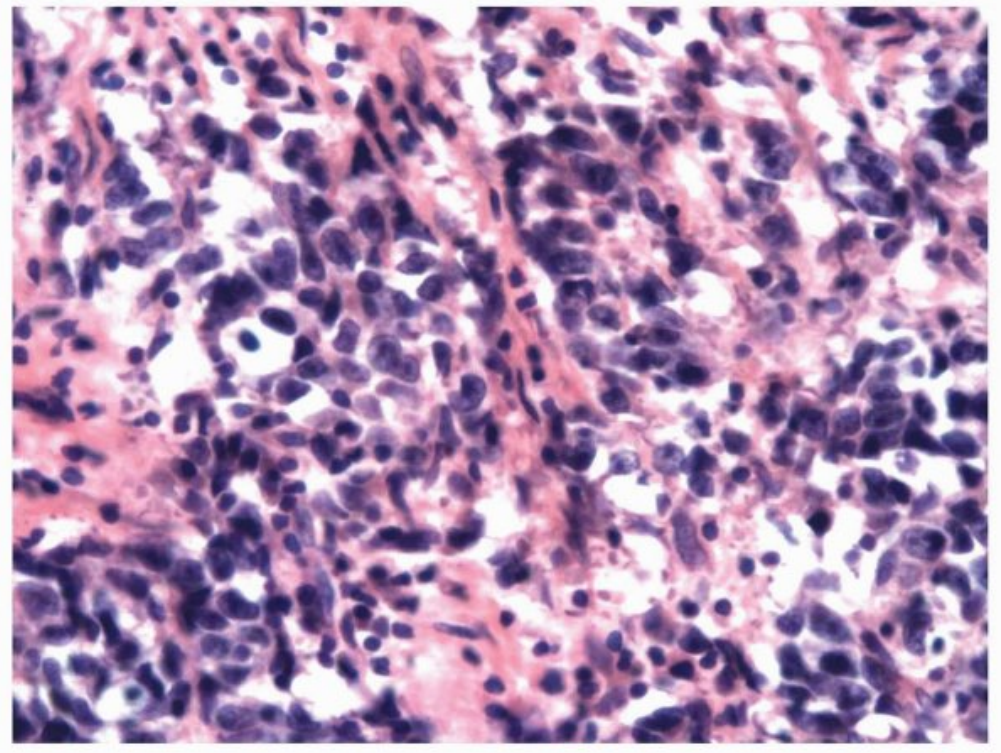

FIGURE 6.39.4 Predominantly epithelial thymoma shows mainly epithelial cells. Hematoxylin and eosin, 60× magnification.

Type C thymoma is composed of various kinds of carcinomas that are similar to nonthymic carcinomas, except that the thymic carcinoma may be accompanied by mature lymphocytes. These tumors include keratinizing and nonkeratinizing epidermoid (squamous) carcinomas, lymphoepithelioma-like carcinomas, sarcomatoid carcinoma, clear cell carcinoma, basaloid carcinoma, mucoepidermoid carcinoma, and undifferentiated carcinoma (12). In most studies, epidermoid carcinoma and lymphoepitheliomalike carcinoma are most common (14). In a small European study series, neuroendocrine tumors (carcinoid included) are as common as epidermoid carcinoma (15). However, the WHO scheme excludes neuroendocrine tumors from thymic carcinoma.

TABLE 6.39.1

WHO Classification of Thymoma

WHO Type	*Histogenetic Type*	*Morphologic Description*
A	Medullary thymoma	Predominantly spindle cells
AB	Mixed thymoma	Mixed spindle and epithelial cells
B1	Predominantly cortical	Lymphocyte rich, no atypical epithelial cells
B2	Cortical	Higher epithelial cell/lymphocyte ratio, more atypical tumor cells

B3	Well-differentiated thymic carcinoma	Atypical epithelial cells and some immature lymphocytes
C	Nonorganoid carcinoma	Various types of carcinoma

WHO, World Health Organization.

There is a rare type of thymoma, which is composed of extensive areas of hyalinized fibroconnective tissue, constituting about 85% to 90% of the tumor mass. It is designated as either sclerosing thymoma (16) or ancient thymoma (17). This rare type cannot be classified in any of the above schemes, and the diagnosis can be easily missed if only a small mediastinoscopic biopsy is obtained.

Electron microscopy can be helpful in identifying desmosomes and tonofilaments in the epithelial tumor cells (Fig. 6.39.5). However, because epithelial tumor cells can be readily identified by immunohistochemical stain, electron microscopic examination is seldom needed.

Immunophenotype

In normal thymus, thymocytes first develop in the cortex with the first markers CD2 and CD7 (Table 6.39.2) (18). This is called the early cortex stage. After proliferation in the cortex (late cortex stage), thymocytes start to express CD1, CD5, and finally CD3. As the thymocytes traverse the corticomedullary junction, CD4 and CD8 are expressed together on the cell surface. Therefore, late cortical thymocytes are also called common thymocytes. When in the medulla stage, thymocytes differentiate into helper T cells (CD4+ CD8-) and suppressor T cells (CD4- CD8+). At this stage, CD1 disappears.

In normal thymus, 80% of thymocytes are of the late cortical phenotype, 10% are of the early cortical phenotype, and the other 10% are of the medullary phenotype (3). Accordingly, most lymphocyte-predominant thymomas are of the late cortical phenotype (3).

Ichikawa et al. (1) suggested that the higher proportion of immature thymocytes (CD1+ CD3-, or CD4+ CD8+) and the lower proportion of mature thymocytes (CD4+ CD8 -/ CD4- CD8+, CD1- CD3+) in thymomas than in the normal thymus represent functional deficiency of the epithelial cells in thymoma, which failed to promote further

differentiation of immature thymocytes into mature thymocytes. Because the function of a normal thymus also includes the elimination of autoreactive T cells, a functionally deficient thymoma may finally lead to an autoimmune disorder, myasthenia gravis (MG).

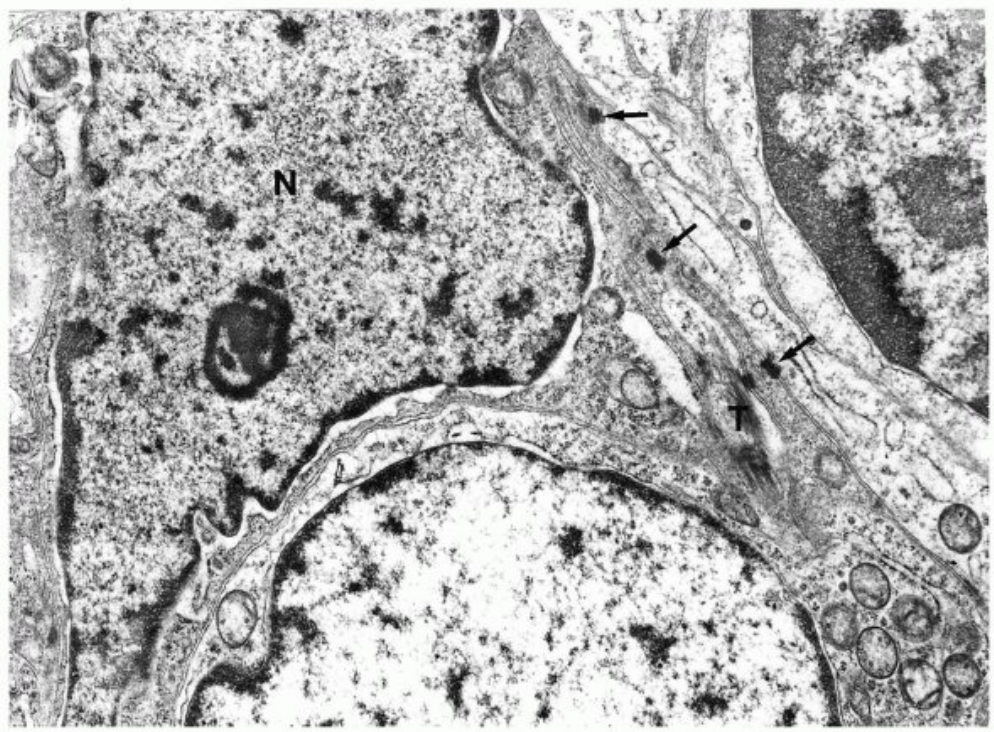

FIGURE 6.39.5 Electron micrograph of a thymoma demonstrates desmosomes (*arrows*) and

bundles of tonofilaments (T). N, nucleus. 23,000× magnification. (Courtesy of Saul Teichberg, Ph.D., North Shore University Hospital, New York.)

Despite the predominance of late-cortical-stage thymocytes (CD4+ CD8+) in most thymomas, small populations of CD4+ CD8- and CD4- CD8+ cells are frequently coexistent (19). The mixed population leads to a flow cytometric pattern that is highly characteristic of thymoma. In histograms with dual CD45 and a T-cell marker (CD2, CD3, CD5, CD7) staining, 3 dot clusters are consistently demonstrated. A smear pattern is also characteristically demonstrated with the staining of CD3, CD4, and CD8, due to the presence of heterogeneous populations (19). Another study emphasized the discriminative value of surface CD3 expression versus forward scatter in flow cytometric histograms (20).

These patterns of histogram are very helpful in distinguishing thymoma from lymphoblastic lymphoma. Lymphoblastic lymphoma is the major differential diagnosis, because it is a thymic tumor, frequently located in the mediastinum. The immunophenotypes of these two tumors are very similar. The major differences are that lymphoblastic lymphoma may express CD10 and CD34 and may have selective loss of some T-cell markers (19). Theoretically, thymoma of the early cortical stage may also express CD10 and CD34 (19), but, in reality, this aberrant phenotype is seldom seen. Selective loss of pan-T-cell markers is usually not seen in thymoma. In addition, because lymphoblastic lymphoma is composed of a homogenous tumor population, the flow cytometric histogram shows a tight cluster pattern instead of a smear pattern as seen in thymomas.

TABLE 6.39.2

Immunophenotype of Thymoma at Various Stages

Stage	*TdT*	*CD1*	*CD2*	*CD3*	*CD4*	*CD5*	*CD7*	*CD8*
Early cortex	+	-	+	-	-	-	+	-
Late cortex	+	+	+	±	+	+	+	+
Medulla	+	-	+	+	±	+	+	±

TdT, terminal deoxynucleotidyl transferase; CD, cluster of differentiation.

Although CD20+ B cells are frequently found in the medulla of the normal thymus and in areas of medullary differentiation in thymomas by immunohistochemical techniques (20,21), flow cytometry often shows fewer B cells in thymomas than in lymphoblastic lymphoma. Our empirical cutoff point for B cells is 10% in distinguishing these two conditions.

A final diagnosis of thymoma requires immunohistochemical staining. A cytokeratin staining highlighting the network of tumor cells is usually diagnostic. Lymphoblastic lymphoma should be negative for cytokeratin. The presence of CD1-positive cells in a carcinoma at the site of the thymus denotes thymic carcinoma and distinguishes it from metastatic carcinoma to the thymus (21). However, in a study of 19 cases of thymic carcinoma, the lymphocytes in the tumor were all negative for CD1a, and most cases also showed negative reaction to CD99, indicating that those lymphocytes were in the mature stage (15).

An extensive immunohistochemical study of thymomas showed that cytokeratin was positive in 94% of cases, epithelial membrane antigen in 75%, neuron-specific enolase in 11%, CD57 in 80%, and human leukocyte antigen-DR (HLA-DR) in 58%, but it was essentially negative for chromogranin and carcinoembryonic antigen (22). However, when neuroendocrine carcinoma was included in the thymoma series, either chromogranin or synaptophysin was demonstrated in these tumor cells (15).

A study of epithelial membrane antigen demonstrated a quantitative difference between thymic carcinoma, invasive thymoma, and noninvasive thymoma; a higher concentration was exhibited in the higher grade malignancy (23). Additional markers, such as bcl-2 and p53, were also expressed in various kinds of thymic carcinomas (15).

It should be clarified that the negative CD3 reaction in early or common thymocytes is based on the results by flow

cytometry, which detects only surface membrane CD3. When frozen sections are examined by immunohistochemical techniques, about 80% of cases of thymoma show cytoplasmic CD3 (24). Furthermore, T-lymphoblastic lymphoma/leukemia may coexist with or transform from thymomas (25,26); this condition makes the distinction between these two tumors more complicated.

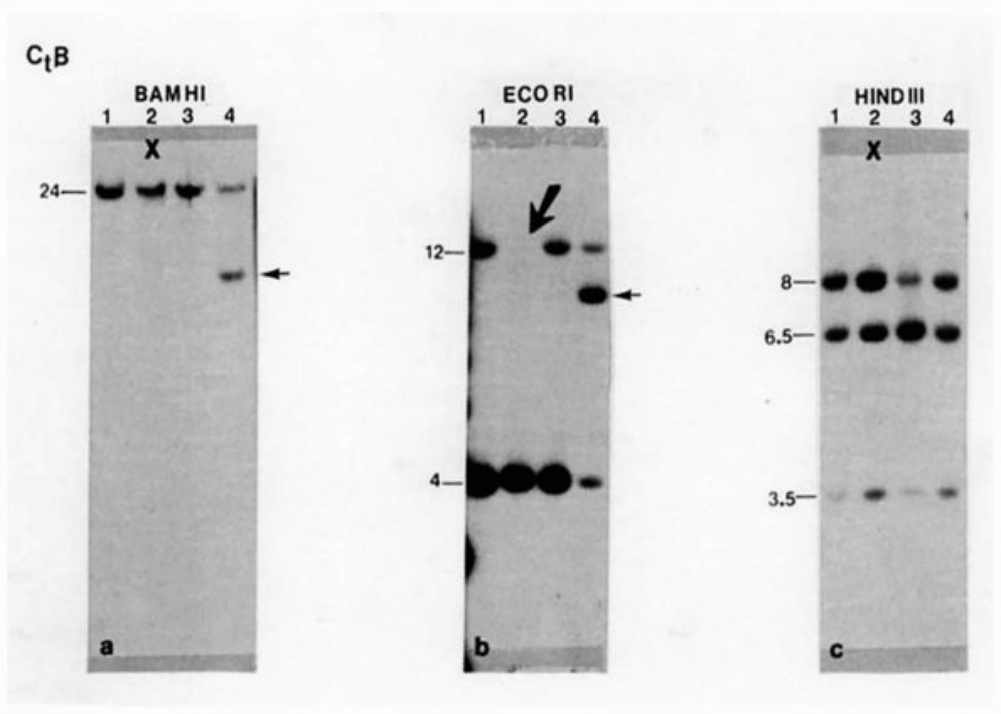

FIGURE 6.39.6 Southern blot analysis of the T-cell receptor ß-chain gene (C_tB) shows a biallelic deletion of the 12-kb fragments (*large arrow*) in the thymoma case (*lane 2*) demonstrated in the *Eco*R1 digest. (From Sun T, Eisenberg A, Benn P, et al. Comparison of phenotyping and genotyping of lymphoid neoplasms. *J Clin Lab Anal*. 1989;3:156-162, with permission.)

Comparison of Flow Cytometry and Immunohistochemistry

Immunohistochemistry is far more helpful than flow cytometry for the diagnosis of thymoma. The cytokeratin stain is most important in defining the epithelial nature of this tumor. However, flow cytometry can help to identify the developmental stage of the thymocyte in the tumor, which may be of clinical significance.

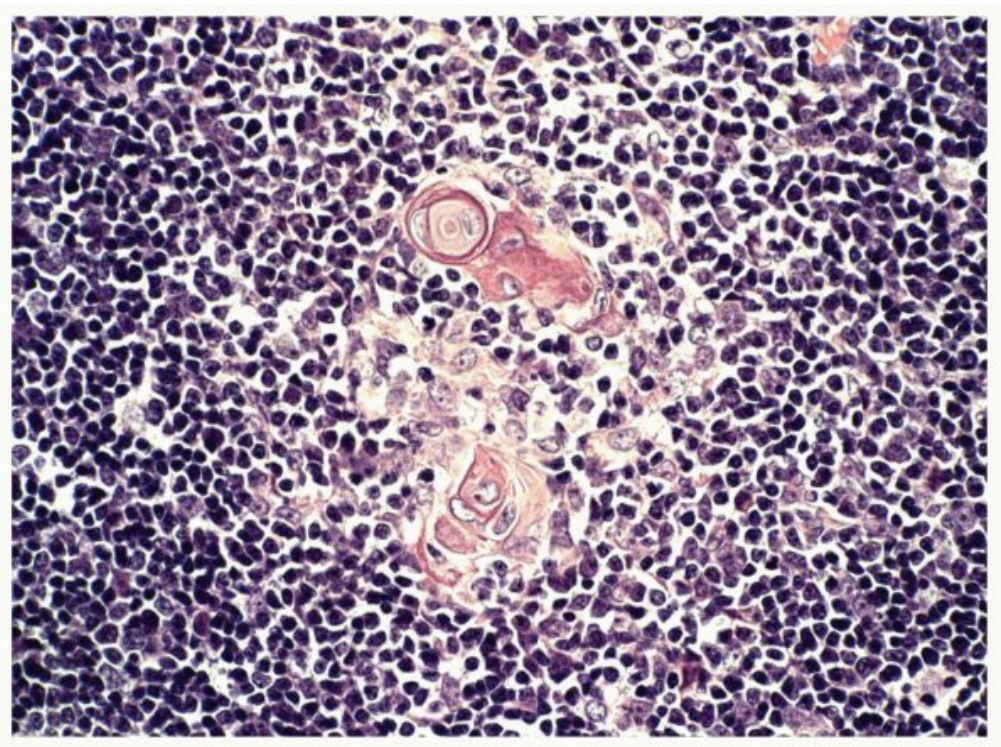

FIGURE 6.39.7 Organoid thymoma shows a pale-stained area that contains Hassall corpuscles, recapitulating the thymic medulla. Hematoxylin and eosin, 20× magnification.

Molecular Genetics

Immunoglobulin and TCR gene analyses in thymomas usually reveal a germline configuration, but germline deletion of the TCR gene may be occasionally encountered (Fig. 6.39.6) (2). Two cases of thymoma were reported to show TCR gene rearrangement for lymphocytes in pleural effusion. Whether this phenomenon represents the malignant nature of these "metastasized" lymphocytes or an aberrant immunologic response to the thymomas is not clear (27).

The biopsy of the current case shows an encapsulated mass composed of densely populated lymphoid cells. There are scattered sparsely populated areas in the mass that contain Hassall corpuscles, representing the recapitulation of the thymic medulla (Fig. 6.39.7). A starry-sky appearance is seen in most of the tumor. This appearance, together with the positive staining of CD10 in the lymphocytes, is suggestive of a lymphoblastic lymphoma. However, the presence of Hassall corpuscles, the extensive cytokeratin stain of the epithelial cells, and the flow cytometric pattern confirm the diagnosis of thymoma. Because of the striking resemblance to the normal thymus, this tumor is classified as organoid thymoma or the WHO type B1 thymoma. The salient features of laboratory diagnosis of thymoma are summarized in Table 6.39.3.

Clinical Manifestations

The sex distribution of patients with a thymic tumor is roughly equal, but patients with accompanying MG are more frequently female (18). Although the age of patients ranges from <1 year to >90 years, most patients with MG are around the age of 30 to 40 years, and those without MG are around 60 to 70 years old (14).

Approximately one third of the patients are asymptomatic (14). The symptomatic patients show either local symptoms due to the intrathoracic mass and/or the paraneoplastic (parathymic) syndrom. The chest symptoms include cough, chest pain, dysphagia, dyspnea, hoarseness, and recurrent respiratory infections. Other major clinical manifestations that have been reported include the

superior vena cava syndrome, Horner syndrome, cardiac tamponade, disseminated intravascular coagulation, and neck mass (18,28).

TABLE 6.39.3

Salient Features for Laboratory Diagnosis of Thymoma

1. TdT is positive in all stages.
2. Reactions to T-cell markers (CD1, CD2, CD3, CD4, CD5, CD7, CD8) depend on the stage.
3. Common thymocyte stage (CD1+, CD4+, CD8+) is most common.
4. Immunohistochemistry: Positive for cytokeratin and negative for LCA (CD45)
5. Electron microscopy: Presence of desmosomes and tonofilaments
6. T-cell receptor gene rearrangement analysis: Germline or deletion

LCA, leukocyte common antigen; TdT, terminal deoxynucleotidyl transferase; CD, cluster of differentiation.

There are several parathymic syndromes that are probably autoimmune in nature. The better known entities are MG, with a frequency

of about 45%, pure red cell aplasia (2% to 5%), and hypogammaglobulinemia (2% to 5%) (14). Cushing syndrome is only seen in patients with thymic carcinoid tumors (14). Additional conditions that have been associated with thymic tumors, including polymyositis, systemic lupus erythematosus, rheumatoid arthritis, thyroiditis, Graves disease, Sjögren syndrome, and ulcerative colitis, are less frequently seen, and their relationship is not fully established (14,29). Several reports also noted the high incidence of secondary malignant tumors in patients with thymoma (14,18).

The prognosis in thymoma patients is mainly related to its stage. The Masaoka staging system is generally accepted as a reliable predictor (Table 6.39.4) (30). Whether the histologic classification is clinically relevant is controversial. Several reports claimed that the WHO scheme is useful clinically (31, 32 and 33). Two studies concluded that the percentage of invasive tumors was seen in the ascending order in A, AB, B1, B2, B3 and C type tumors (31,33). Other studies considered that the Marino and Müller-Hermelink system is superior in predicting the aggressive potential of thymomas (7,34). However, multivariate analyses have generally shown that the histologic type was not of independent prognostic value (14). All thymic tumors, regardless of their grades, can manifest malignant behavior, namely, recurrence. The long-term survival after complete resection in patients with a stage III or IV thymoma is similar to that in patients with a stage I thymoma (14). Therefore, Detterbeck and Parsons (14) considered that the subclassification of bland tumor is of questionable prognostic value; it is only useful to distinguish thymic carcinoma, well-differentiated thymic carcinoma (atypical thymoma) from bland thymomas (typical thymomas), as Suster and Moran suggested. Thymic carcinomas are typically not associated with MG, and the parathymic conditions do not affect the treatment and the prognosis of thymic tumors (14).

TABLE 6.39.4

Masaoka Staging System*

Stage	*Definition*
I	Macroscopically encapsulated tumor, with no microscopic capsular invasion
IIa	Macroscopic invasion into surrounding fatty tissue or mediastinal pleura
IIb	Microscopic invasion into the capsule
III	Macroscopic invasion into neighboring organs
IVa	Pleural or pericardial metastases
IVb	Lymphogenous or hematogenous metastasis

* From Masaoka A, Monden Y, Nakahara K, et al. (30).

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