Lymph Node Cytopathology

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Series Preface

The subspeciality of cytopathology is 60 years old and has become established as a solid and reliable discipline in medicine. As expected, cytopathology literature has expanded in a remarkably short period of time, from a few textbooks prior to the 1980s to a current and substantial library of texts and journals devoted exclusively to cytomorphology. E*ssentials in Cytopathology* does not presume to replace any of the distinguished textbooks in cytopathology. Instead, the series will publish generously illustrated and user-friendly guides for both pathologists and clinicians.

Building on the amazing success of *The Bethesda System for Reporting Cervical Cytology*, now in its second edition, the *Series* will utilize a similar format, including minimal text, tabular criteria, and superb illustrations based on real-life specimens. *Essentials in Cytopathology* will, at times, deviate from the classic organization of pathology texts. The logic of decision trees, elimination of unlikely choices, and narrowing of differential diagnosis via a pragmatic approach based on morphologic criteria will be some of the strategies used to illustrate principles and practice in cytopathology.

Most of the authors for *Essentials in Cytopathology* are faculty members in The Johns Hopkins University School of Medicine, Department of Pathology, Division of Cytopathology. They bring to each volume the legacy of John K. Frost and the collective experience of a preeminent cytopathology service. The archives at Hopkins are meticulously catalogued and form the framework for text and illustrations. Authors from other institutions have been selected on the basis of their national reputations, experience, and

enthusiasm for cytopathology. They bring to the series complementary viewpoints and enlarge the scope of materials contained in the photographs.

The editor and the authors are indebted to our students, past and future, who challenge and motivate us to become the best that we possibly can be. We share that experience with you through these pages, and hope that you will learn from them as we have from those who have come before us. We would be remiss if we did not pay tribute to our professional colleagues, the cytotechnologists and preparatory technicians who lovingly care for the specimens that our clinical colleagues send to us.

And finally, we cannot emphasize enough throughout these volumes the importance of collaboration with the patient care team. Every specimen comes to us as questions begging an answer. Without input from the clinicians, complete patient history, results of imaging studies and other ancillary tests, we cannot perform optimally. It is our responsibility to educate our clinicians about their role in our interpretation, and for us to integrate as much information as we can gather into our final diagnosis, even if the answer at first seems obvious.

We hope you will find this series useful and welcome your feedback as you place these handbooks by your microscopes and into your book bags.

Baltimore, MD *Dorothy L. Rosenthal*

Acknowledgements

To the memory of my father, who guided my first steps in pathology.
To my wife Corina and my son Felix.
My gratitude to my mentors Harry L. Ioachim, Yener Erozan, and Dorothy L. Rosenthal.

Stefan E. Pambuccian

To my parents, my first teachers.
To Angela, Angie, and Ricky from whom I continue learning.
To my mentors, residents, and fellows.

Ricardo H. Bardales

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1
Introduction

Historical Overview

Lymph nodes are some of the most common targets for fine needle aspiration (FNA) as persistent lymphadenopathy is a common finding. It is therefore not surprising that the first description of what we now call FNA biopsy was made in 1904 by two British marine officers, Captain E.D.W. Greig and Lieutenant A.C.H. Gray who reported their findings on the aspiration of lymph nodes in sleeping sickness. They could demonstrate motile trypanosomes in the "node juice" obtained by aspirating from swollen neck lymph nodes of patients in Uganda with hypodermic needles. The following year, German physicians Fritz Schaudinn and Erich Hoffmann, were able to identify *Treponema pallidum*, which they had previously identified as the causative agent of syphilis, in inguinal lymph node aspirates by using dark-field microscopy.

The first uses of lymph node FNA in the diagnosis of tumors occurred in 1914 by an English physician, Gordon R. Ward who was able to diagnose "lymphoblastomas" (i.e., lymphomas) by this method. In 1921, Dr. C.G. Guthrie, head of the department of Clinical Pathology at the Johns Hopkins Hospital, successfully made the diagnosis of Hodgkin lymphoma by "gland puncture," and the patient received treatment based solely on that diagnostic procedure.

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However, the most influential in establishing needle aspiration of lymph nodes as a valid diagnostic measure was Dr. Hayes E. Martin from the Head and Neck Service of the Memorial Hospital for Cancer and Allied Diseases in New York (currently the Memorial Sloan Kettering Cancer Center). With the help of Edward B. Ellis, the chief histotechnologist and Fred W Stewart, the surgical pathologist responsible for interpreting the smears, Dr. Martin proposed needle aspiration of cervical lymph nodes to replace excisional biopsies in 1926.

The rapidly accumulated experience with 2,500 tumors aspirated by 1933 allowed Dr. Fred Stewart to formulate the principles of needle aspiration cytology, which are still valid today, emphasizing sample preparation, clinical correlation, histologic correlation, attention to both smear pattern and individual cell cytomorphology, and awareness of the limitations of the method. One of the limitations that he noticed was in the diagnosis of primary lymph node lesion, where the diagnosis was difficult even when tissue sections were available. This skepticism was appropriate at a time when diagnostic criteria and ancillary studies were lacking, and very high rates of histopathologic misdiagnosis were occurring, as demonstrated by the amazing 50% reclassification rate (most often to benign conditions) of cases originally diagnosed histologically as Hodgkin disease found by Dr. W.S.C. Symmers in 1968.

After being almost abandoned in USA, the needle aspiration technique was revived and improved in the 1950s in Europe (especially in the Netherlands and Sweden) by clinical hematologists who used Romanovsky-type stains instead of the hematoxylin and eosin stain used by Dr. Fred Stewart, and smaller needles that were less likely to result in complications or tumor implantation. The technique, which became known as FNA, was used most often for cytologic diagnosis of metastatic lesions, an indication for which it rapidly gained widespread acceptance and worldwide dissemination.

However, skepticism about the possibility of accurate lymphoma diagnosis in aspiration smears persisted in the pathology community despite a 1980 publication documenting the diagnosis of over 1,000 lymphomas diagnosed by FNA by one of the pioneers of FNA, Dr. Lopes-Cardozo, and other papers on the subject.

FNA in the initial diagnosis of lymphomas became more widely accepted only in the 1990s, when ancillary studies (especially immunophenotyping) became routinely used in the diagnosis of lymph node aspirates suspected of lymphoma, and the classification of lymphomas was modified with more emphasis placed on cytomorphology (rather than histologic/architectural pattern), immunophenotypic, and cytogenetic features in the revised European American classification (REAL) of 1994, and the World Health Organization (WHO) classifications of 2001 and 2008.

Indications for Fine Needle Aspiration of Lymph Nodes

Currently, FNA, whether performed on superficial lymph nodes by palpation or performed under ultrasound, transesophageal endoscopic ultrasound (EUS), endobronchial ultrasound (EBUS), or computerized tomography (CT) guidance is used to:

1. Establish the cause of lymphadenopathy
2. Stage a known lymphoid, or nonlymphoid malignancy
3. Monitor for recurrence of lymphoid, or nonlymphoid malignancies, and for potential progression or transformation of lymphoid malignancies

Lymph Node Pathology Diagnosed by Fine Needle Aspiration

In patients presenting with lymphadenopathy without a history of malignancy, more than half of lymph node aspirates represent a variety of reactive, inflammatory, infectious, and granulomatous disorders; about a third are metastatic malignancies and less than 10% are lymphomas. Benign conditions are even more common in pediatric lymphadenopathies, where about 80% of aspirated lymph nodes prove to be benign. The relative frequency of aspirates diagnostic of malignancy varies according to the nodal site biopsied. The most commonly aspirated lymph node region, the cervical lymph nodes is the least likely to be malignant, and the likelihood of a

malignant diagnosis increases for inguinal, axillary, intraabdominal, retroperitoneal, and supraclavicular lymph nodes.

As with fine needle aspirates from other sites, the reported specificity of lymph node aspirates is very high, between 98 and 100% as false positive diagnoses are very rare. The sensitivity of FNA of lymph nodes, on the contrary, depends heavily on the population studied and case mix. The sensitivity for metastatic malignancies causing lymph node enlargement is over 95%, while the sensitivity for lymphomas varies from 80 to 90%, with a typing accuracy of about 70% and higher when flow cytometry is routinely used in fine needle aspirates of lymph nodes. The sensitivity of FNA in lymphoma diagnosis is heavily dependent on the relative representation of T- vs. B-cell lymphomas, and small-cell vs. large-cell lymphomas, the former being more difficult to diagnose than the latter.

Benefits of Lymph Node FNA

Lymphadenopathy is most commonly superficial and palpable, and therefore easily accessible to sampling by palpation or ultrasound-guided FNA, which usually results in a fast, reliable, and relatively inexpensive diagnosis. A variety of ancillary studies (cultures, immunohistochemistry, EM, flow cytometry, cytogenetics, and molecular diagnostics) can be performed on the aspirate and the choice of ancillary studies is determined during the on-site evaluation of the aspirate.

Excisional biopsy is not necessary when inflammatory or reactive conditions, or metastases are diagnosed. The management of patients with lymphoma initially diagnosed by FNA combined with flow cytometry or other immunophenotyping studies is controversial, but in most institutions, an excisional biopsy is performed to confirm the diagnosis after weighing the potential benefits that histologic assessment of the lymph node would give against the risks of the procedure. Excisional biopsy will be performed in most cases diagnosed as lymphoma, or suspicious for lymphoma in FNA of superficial lymph nodes. However, the definitive diagnosis of lymphoma on fine needle aspirates or core biopsies of deep lymph nodes such as retroperitoneal lymph nodes is usually not followed by excisional biopsies due to the higher risks of the surgical procedure. The advantages of needle core biopsies as opposed to fine needle aspirates

obtained by imaging-guided procedures are probably overstated, since the presence of some minimal architectural features has to be weighed against the frequent presence of extensive crush artifact that may render the morphologic interpretation difficult and lower the yield of ancillary studies. When such core biopsies are performed, it is always useful to prepare gentle touch imprints or "roll-preps" of the cores during the procedure to serve for both the evaluation of adequacy and triage of the specimen, and for morphologic correlation with the core biopsy histology, as these cytologic preparations frequently allow better evaluation of morphologic features.

It has been calculated that FNA of lymph nodes avoids at least 86% of lymph node excisional biopsies. Cases in which FNA fails to establish a definitive diagnosis, as well as cases in which the lymph node enlargement persists 1–3 months after the FNA was interpreted as benign/reactive, should undergo excisional biopsy to rule out a potentially missed pathology.

Difficulties and Limitation of FNA Diagnosis

FNA biopsy of lymph nodes is arguably one of the most difficult areas of cytopathology as lymph nodes can harbor a bewildering array of benign and malignant conditions Over 50 types of lymphomas are included in the latest (2008) WHO classification (Table 1.1), some of them are so rare that they may be encountered only once in a pathologist's career. In addition, lymph nodes can be the site of metastases from virtually any malignancy. The difficulty of correctly interpreting lymph node FNAs is increased by the minimal degrees of cytologic atypia that some low-grade lymphomas exhibit and the potential overlap between the cytologic features of some high-grade lymphomas and metastatic malignancies. This makes the use of ancillary techniques (immunohistochemistry, flow cytometry, fluorescence in situ hybridization, and molecular techniques) more important to achieve an accurate FNA diagnosis than in any other site. Accurate subtyping of lymphoid malignancies according to the current classification of lymphoid neoplasms, the 2008 WHO classification that relies heavily on immunophenotypic, cytogenetic, and molecular features, is virtually impossible without the use of ancillary studies. We will follow the 2008 WHO

TABLE 1.1. WHO classification of lymphoid neoplasms (2008), including only neoplasms potentially encountered in fine needle aspiration of lymph nodes and mediastinum.

Precursor lymphoid neoplasms
B lymphoblastic leukemia/lymphoma NOS
T lymphoblastic leukemia/lymphoma
Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
Lymphoplasmacytic lymphoma/Waldenstrom macroglobulinemia
Plasma cell myeloma
Extraosseous plasmacytoma
Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) type
Nodal marginal zone lymphoma
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma, NOS
T-cell/histiocyte-rich type
Diffuse large B-cell lymphoma with chronic inflammation
Primary mediastinal large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK+ large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma associated with HHV8+ Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, Burkitt-like
B-cell lymphoma, unclassifiable, Hodgkin lymphoma-like
Mature T-cell & NK-cell neoplasms
Adult T-cell lymphoma/leukemia
Extranodal T-cell/NK-cell lymphoma, nasal type
Mycosis fungoides
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
Anaplastic large cell lymphoma, ALK+ type
Anaplastic large cell lymphoma, ALK– type
Hodgkin lymphoma
Nodular lymphocyte-predominant Hodgkin lymphoma
Classic Hodgkin lymphoma
Nodular sclerosis Hodgkin lymphoma
Lymphocyte-rich classic Hodgkin lymphoma
Mixed cellularity Hodgkin lymphoma
Lymphocyte depletion Hodgkin lymphoma

(continued)

Table 1.1. (continued)

Posttransplant lymphoproliferative disorders (PTLD)
Plasmacytic hyperplasia
Infectious mononucleosis-like PTLD
Polymorphic PTLD
Monomorphic PTLD (B & T/NK cell types)
Classic HD-type PTLD
Histiocytic and dendritic cell neoplasms
Histiocytic sarcoma
Langerhans cell histiocytosis
Langerhans cell sarcoma
Interdigitating dendritic cell sarcoma
Follicular dendritic cell sarcoma
Fibroblastic reticular cell tumor
Indeterminate dendritic cell sarcoma
Disseminated juvenile xanthogranuloma

Classification of Tumors of Hematopoietic and Lymphoid Tissues throughout this book, unless otherwise stated.

The purpose of this book is to describe the application of FNA to the assessment of lymphadenopathy, with particular emphasis on the utility, limitations, and potential pitfalls of FNA. It will adopt an algorithmic diagnostic approach, starting from the cytomorphologic pattern of the lymph node aspirate, focusing on the appropriate and effective use of ancillary studies, and integration of their results into the final diagnosis.

The book will present the cytopathologic features and differential diagnoses for the major cytologic patterns in lymph node FNA. The entities typically falling within each of these patterns will be discussed with illustration of the spectrum of cytologic features, differential diagnoses, and pitfalls. The cytologic diagnosis of lymph node aspirates, even when combined with ancillary studies, has definite limitations in the diagnosis of some conditions. Awareness of these limitations helps avoid some potential diagnostic pitfalls. Lymph node aspirates should be interpreted in the clinical context; aspirates where the cytomorphology does not explain the clinical findings or where the cytomorphologic findings or ancillary test results are not entirely characteristic are best diagnosed descriptively with a recommendation for excisional biopsy.

We have attempted to cover the full spectrum of benign and malignant primary conditions of the lymph nodes, with emphasis on common disorders. Discussion of metastatic conditions will be restricted to those that are relevant to the differential diagnosis of primary lymphoid disorders.

Suggested Reading

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2
Overview of Ancillary Methods in Lymph Node FNA diagnosis

Ancillary tests are critically important tools in the assessment of lymph node aspirates, as they can help establish the etiology of infectious lymphadenopathies, determine the type and primary site of metastatic malignancies, diagnose and subclassify lymphomas, and exclude malignancy in reactive lymphadenopathies. In addition, ancillary tests can also be used to provide prognostic information in certain neoplasms and help predict treatment response.

Clinical correlation is *always* essential when employing ancillary tests and their results should *only* be interpreted in the cytomorphologic context to prevent potential pitfalls.

The decision on which of the ancillary tests are available to use in the individual case depends on the clinical context, characteristics of the lymph node aspirated, and the on-site examination of the aspirate. Some of the ancillary studies that are most useful in the evaluation of lymph node FNA, such as cultures, flow cytometry (FC), and cytogenetic testing, require fresh (not fixed) samples. FC and cytogenetics also require the presence of viable neoplastic cells. It is therefore important that the specimen is triaged and aliquots are taken during the on-site evaluation of lymph node fine needle aspirates. Aspirates showing predominantly neutrophils and/or granulomas should be submitted for the appropriate microbiologic cultures, whereas aspirates from enlarged lymph nodes showing a predominantly lymphoid population in adults should be submitted to immunophenotyping by FC. Care should be taken

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that sufficient sample is available for performing adequate studies and the appropriate medium (culture or transport media and RPMI) is used. If no on-site evaluation was performed and no fresh sample is available, cell block preparations should be routinely performed, as studies not requiring fresh samples, such as immunohistochemistry and fluorescent in situ hybridization, can frequently be performed on such cell blocks.

The following is a brief overview of ancillary techniques that are useful in the diagnosis of lymph node aspirates. The use of some of these methods will be further discussed in the following chapters. The suggested references contain a more extensive discussion of these ancillary methods. Ancillary tests used in the diagnosis of lymph node aspirates can be divided according to their usefulness into the following:

1. Ancillary methods useful for establishing the etiologic agent of lymphadenitis;
2. Ancillary methods useful for establishing the clonality of a lymphoid process and characterizing the clonal proliferation;
3. Ancillary methods useful for establishing the nature and potential site of origin of a metastatic malignancy.

Ancillary Methods Useful for Establishing the Etiologic Agent of Lymphadenitis

Special Stains and Immunohistochemical Stains

Special stains, such as the Gram stain for bacteria, acid-fast stains (Ziehl–Neelson, Fite, auramine-rhodamine) for mycobacteria, Gomori's methenamine silver (GMS), PAS, and mucicarmine stains for fungi, Warthin Starry stain for cat-scratch disease (*Bartonella henselae*) and spirochetes can be applied to either the FNA smears or cell block preparations. However, the sensitivity of these stains is, rather low, especially for mycobacteria (40–60%, depending on the mycobacterial species), and the interpretation of the stains may be difficult and time consuming. Immunoperoxidase stains are commercially available against some infectious agents that are otherwise difficult to identify, such as *Bartonella henselae, Listeria monocytogenes, Mycobacterium tuberculosis, Aspergillus, CMV, Herpes*

simplex, HHV8, Pneumocystis jiroveci, and Toxoplasma gondii, and can be used when clinical and/or morphologic findings suggest these organisms. Since immunohistochemical stains for *M. tuberculosis* are superior in sensitivity and specificity to conventional acid-fast stains and are easier to interpret, they can be used as an alternative method to the conventional stains.

Special stains are useful in the diagnosis of fungi identified in lymph node aspirates (Table 2.1).

As the morphologic identification of fungi may sometimes be difficult, in situ hybridization can be used to determine definitively the species of fungi identified on GMS stains. This method allows the specific identification of yeasts (*Blastomyces dermatitides, Coccidioides immitis, Cryptococcus neoformans, Histoplasma capsulatum, and Sporothrix schenkii)* based on the sequence differences of their 18S and 28S rRNA, and of filamentous fungi (*Aspergillus, Fusarium, and Pseudoallescheria*) based on the sequence differences of their 5S, 18S, and 28S rRNA.

Cultures

Cultures of lymph node aspirates are performed if the clinical or imaging findings suggest an infection, or if neutrophilic or granulomatous inflammation is identified during the on-site evaluation. Depending on the clinical presentation and the presence or absence of granulomas, aerobic and anaerobic, mycobacterial and fungal cultures are submitted in the appropriate media.

Cultures obtained on fine needle aspirates of lymph nodes may be positive for a variety of microorganisms, especially for pyogenic organisms such as *Staphylococcus aureus* or *Streptococcus pyogenes*, and the culture may be useful in choosing the right antibiotic, especially due to the increasing prevalence of methicillin-resistant *S. aureus*. Anaerobes such as *Peptostreptococcus* and *Bacteroides* species may also be cultured singly or in mixed cultures with aerobes. Other organisms may rarely be the cause of suppurative lymphadenitis (*Actinomyces israeli, Francisella tularensis, Yersinia* spp*, Corynebacterium* spp*, Brucella* spp*, Listeria monocytogenes, and Bacillus anthracis*).

Bartonella henselae, the causative agent of cat-scratch disease, is a small, Gram-negative bacillus, detectable by silver stains

TABLE 2.1. Differential diagnostic findings in FNA of fungal lymphadenitis.

Fungus	Size (μm)	Morphology	Budding	Stains typically positive
Histoplasma capsulatum	2–5	Small yeast, often within macrophages	Single bud	GMS
Coccidioides immitis	20–200 (spherules) 2–5 (endospores)	Large spherules with or without endospores	Endospores	GMS, PAS, Fontana-Masson
Blastomyces dermatitidis	8–15	Spherical yeasts with thick (double contoured) walls	Single, broad-based bud	GMS, PAS, Congo red
Cryptococcus neoformans	2–15	Variably sized yeasts, thick capsule	Single, narrow-based bud	GMS, PAS, Mucicarmine, Fontana-Masson
Sporothrix shenkii	2–8	Pleomorphic round, oval, or elongate, cigar-shaped yeasts	Narrow-based, "teardrop" buds; may be multiple	GMS, PAS

(Warthin–Starry silver impregnation). Although the organism can be identified by culture, it is more commonly identified by special stains, immunohistochemistry, or molecular methods as culture is slow and lacks sensitivity.

Cultures from lymph node FNAs may also be positive for mycobacteria, especially for nontuberculous mycobacteria (*M. avium-intracellulare, M. scrofulaceum, and M. kansasii*) in young children and patients who are immunosuppressed or have hematologic malignancies, and *M. tuberculosis* mostly in nonimmunosuppressed adults

Cultures for fungal diseases are rarely positive but should be undertaken in granulomatous lymphadenitis, especially in mediastinal granulomata.

The limitations of cultures are twofold: they are slow and final results may be available only after 4–6 weeks as in the case of mycobacteria, and they may be falsely negative. This may occur either due to the submission of nonrepresentative samples, especially if multiple passes were performed and one pass was entirely submitted for cultures. Performing a single smear from the aspirate submitted for cultures with care not to contaminate the specimen may be useful. Other causes of falsely negative culture results are the compromise of bacterial, mycobacterial, or fungal viability due to delays, inappropriate transportation media, and empiric antibiotic treatment received by the patient prior to the FNA. Finally, the significance of some cultured organisms requires clinical correlation, as they may represent skin contaminants.

Molecular Tests for Microorganisms

Molecular diagnoses for infectious agents can be useful if no specimen was sent for culture, if cultures are negative but the clinical suspicion for infection is high, or clinical therapeutic decisions have to be made before final culture results. Tests based on PCR, real-time PCR, or alternative exponential amplification methodologies can be used for many causative agents of lymphadenitis such as viruses, *Bartonella henselae, F. tularensis, Tropheryma whipplei, M. tuberculosis*, fungi, and protozoa (*T. gondii, Leishmania, etc.*).

Compared to culture, these tests are fast and have a high sensitivity and specificity; however, the sensitivity is frequently

lower in fixed specimens. A limitation is the inability to perform susceptibility testing.

Establishing the Clonality of a Lymphoid Process and Characterizing the Clonal Proliferation

Flow Cytometry

FC is probably the ancillary method that is most helpful in the diagnosis of lymph node aspirates. Its increasing use during the last two decades in conjunction with the cytomorphologic evaluation of fine needle aspirates has made lymph node FNA more acceptable in the primary diagnosis of lymphomas by increasing the sensitivity and accuracy of the subclassification of lymphomas on aspirates. As submitting all FNA samples from enlarged lymph nodes may be impractical, some institutions have developed guidelines on which samples should be submitted for immunophenotyping. One of these is the "rule of twos": aspirates from lymph node, which have been enlarged for over 2 months, measure over 2 cm in patients over 20 years of age should be submitted for FC in addition to any aspirates in which the lymphoid population appears atypical during on-site evaluation.

Aspirates from lymph nodes placed in RPMI are an ideal sample for FC, since FC is performed on single cell suspensions. FC usually detects surface antigens, but may also detect cytoplasmic or nuclear antigens after permeabilization of the cell membranes. A relatively high number of cells are needed for accurate FC results, in the range of 300,000–1,000,000; however, this number is easily achieved by placing needle rinses from three successful cellular FNA passes in RPMI or other media.

After the cells have been conjugated with fluorochrome-tagged antibodies, FC evaluates for the simultaneous presence and absence of multiple specific antigens on each individual cell that passes in front of a laser beam. The emitted immunofluorescence signals from all cells in suspension are captured and presented as histograms by the instrument's software. In addition, FC gives information about the size of the cells in the form of forward scatter (FSC) and the complexity of the cells (including the granularity of the cytoplasm and nuclear shape) in the form of side scatter (SSC).

The number of "colors" (fluorochromes) of a FC denotes the maximal number different antibodies that can be applied simultaneously to the cells. Most FC laboratories use at least three, allowing five-parameter analysis (three colors plus forward and side scatter), but 4-color, 8-color, and even 11-color flow cytometers are used by some laboratories. Fewer cells are needed when more colors are used, since the cells can be marked simultaneously with more antibodies, thus reducing the number of tubes used.

FC not only allows the simultaneous assessment of multiple antigens on a cell population, but can also quantify the intensity of antigen expression (e.g., dim vs. moderate vs. bright), a feature that may be important in the classification of some lymphoid proliferations.

In addition to determining what markers the cells express, FC can be helpful by allowing an objective measurement of the abnormal lymphoid population by its FSC, and by allowing ploidy and S-phase measurements.

The most important first step in the flow cytometric immunophenotyping analysis of a lymph node aspirate is *gating* the populations of interest. Gating refers to the selections of subsets of cells based on their levels of expression of one or more markers and/or their light scatter properties. The most important gating strategies employed in fine needle aspirates of lymph nodes are as follows:

1. By cell size in the FSC vs. SSC histograms (FSC vs. SSC) (Fig. 2.1). Lymphoid populations usually have low FSC and low SSC as they are small, and show little nuclear or cytoplasmic complexity. Large cell lymphoma cells usually show intermediate FSC and higher SSC than reactive lymphocyte populations and may be gated on the FSC vs. SSC histogram for further analysis.
2. By cell distribution in the CD45 vs. SCC histogram (Fig. 2.2). This gating strategy is more useful in specimens that include mixed cell populations like bone marrow samples, but can be useful in the assessment of lymph node aspirates. Lymphocytes are usually brightly CD45 positive and show low SSC. Almost all lymphoid malignancies, except Hodgkin lymphoma, plasmablastic lymphomas, and plasma cell neoplasms express CD45, although in some the expression may

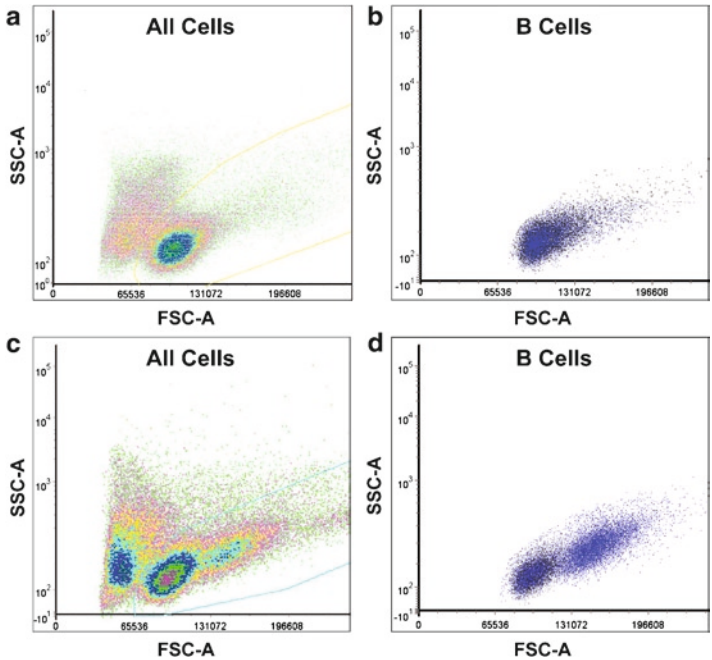

FIGURE 2.1. Histograms of side scatter (SSC) vs. forward scatter (FSC) can help detect the presence of a large cell (neoplastic) lymphoid population. (**a**, **b**) Reactive lymph node aspirate: no significant large cell lymphoid population is present when all cells are considered (**a**) or when gating only on CD19+ B-cells (**b**). (**c**, **d**) Diffuse large B-cell lymphoma (DLBCL) aspirate showing the presence of a larger cell population (increased FSC) when all cells are considered (**c**) or when gating only on CD19+ B-cells (**d**) (courtesy of Timothy P. Singleton, M.D. and Dan McKeon, Flow Cytometry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

be reduced. This strategy is also useful to identify and exclude nonhematopoietic cell populations such as metastatic malignancies which do not express CD45.

3. By cell lineage-specific antigens. This strategy is useful in the determination of clonality.

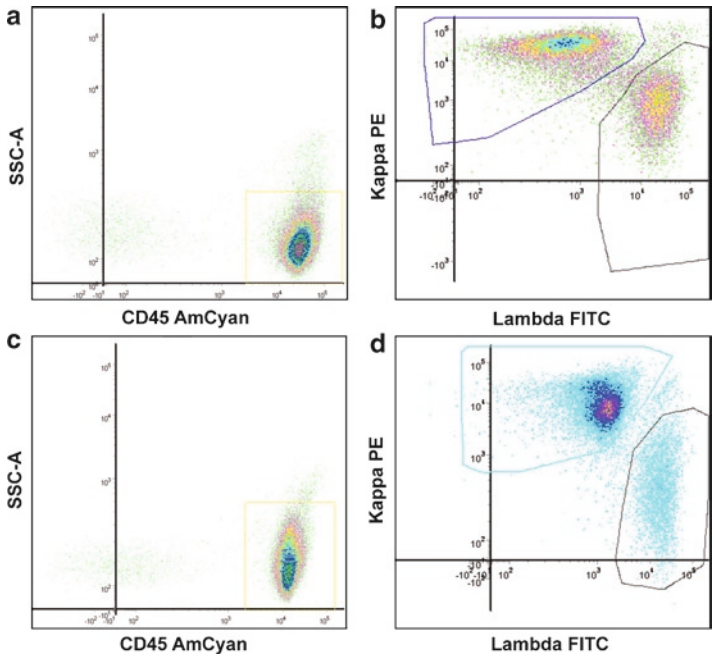

FIGURE 2.2. Histograms of SSC vs. CD45 can help determine if the cells fall in the lymphocyte gate (CD45 bright and low SSC); histograms of kappa vs. lambda can determine the presence of light chain restriction. (**a**, **b**) Reactive lymph node aspirate: most cells fall within the leukocyte gate (CD45 bright and low SSC) and show both kappa and lambda expression (kappa to lambda ratio of 2/1). (**c**, **d**) DLBCL aspirate most cells fall within the leukocyte gate. The cells show light chain restriction (kappa to lambda ratio of 11/1) (courtesy of Timothy P. Singleton, M.D. and Dan McKeon, Flow Cytometry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

Establishing Clonality

Establishing B-Cell Clonality

B-cells express immunoglobulins on their surface, except in very early phases of their differentiation and when terminally differentiated (plasma cells). The latter express only cytoplasmic immunoglobulins. Since individual B-cells express *either* kappa or lambda light

chains, clonal populations of B-cells (i.e., cells derived from the same progeny) show light-chain restriction, i.e., their cells express only one type of light chains (kappa or lambda), in contrast to polyclonal populations, where some cells express kappa and some lambda light chains.

In lymph nodes, blood, and other tissues, kappa-light chain expressing cells usually outnumber lambda-expressing cells by a mean of 2/1 (range 1/1 to 3/1). Kappa/lambda ratios higher than 4/1 or lower than 1/3 are rarely found in reactive lymph nodes and these numbers are frequently used as cut-off points to determine clonality when all B-cells are analyzed. However, cut-off values for kappa/lambda ratios are determined by each laboratory by trying to achieve the best balance between sensitivity and specificity.

When adequately *gated* on the *abnormal* B-cell population showing larger size (higher FSC), inappropriate marker coexpression, or abnormal intensity of marker expression, most lymphomas show much higher light chain ratios (kappa/lambda or lambda/kappa), and values lower than 6/1 should be accepted with care as indicators of clonality. Rare reactive B-cell populations, especially those from reactive germinal centers and Hashimoto thyroiditis, may have light chain ratios over 6/1. In such cases, correlation with other markers determined by FC and with cytomorphologic findings will establish the correct diagnosis.

Some neoplastic B-cell proliferations fail to mark for surface immunoglobulins due to abnormal immunoglobulin synthesis; sometimes applying different antibodies directed against another epitope will successfully determine the presence of the immunoglobulin and the light chain restriction. However, in some cases, no expression of immunoglobulins can be detected and the identification of an abnormal B-cell proliferation has to rely on the demonstration of inappropriate coexpression of differentiation or activation antigens, such as the expression of myeloid antigens (CD13 or CD33) in lymphoplasmacytic lymphoma or of Bcl-2 by CD10-positive B-cells in follicular lymphoma.

Normal B-cells express CD19, which is the most sensitive marker and defines their B-cell lineage. CD20 and CD22 are also expressed by all but the very early B-cells. However, terminally differentiated plasma cells do not express any of these markers, but usually express CD38 and CD138. CD79a may also be used to determine B-cell lineage.

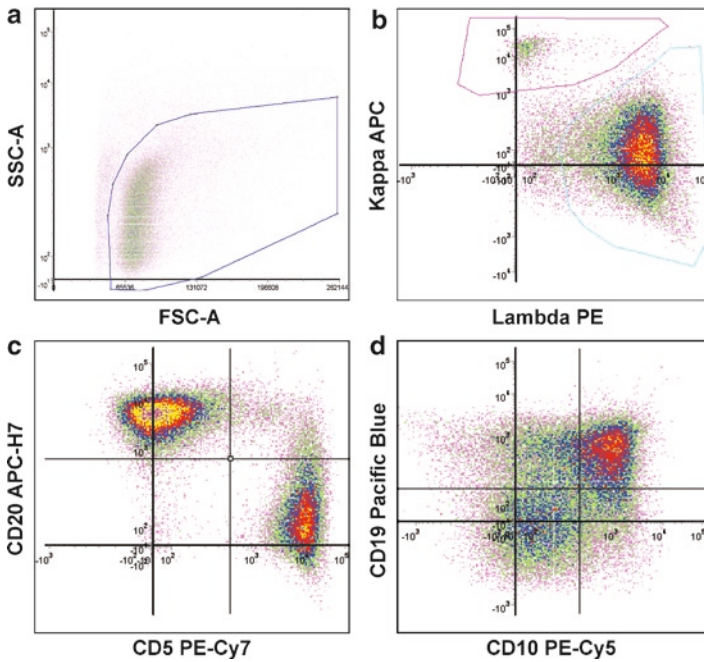

FIGURE 2.3. Flow cytometry (FC) from an aspirate of a patient with grade 1 follicular lymphoma. FC allows the determination of light chain restriction/clonality and the lack of a large cell component on FSC may help excluding a DLBCL. The diagnosis requires clinical and cytologic correlation. (**a**) SSC vs. FSC histogram showing that most cells show low FSC and SSC consistent with small lymphocytes. No large cell population is present. (**b**) Kappa vs. lambda histogram shows overwhelming predominance of lambda-positive cells, demonstrating light chain restriction. (**c**) CD20 vs. CD5 histogram shows lack of CD5 expression on the lambda monotypic B-cells. (**d**) CD20 vs. CD10 histogram shows CD10 expression on the lambda monotypic B-cells (courtesy of Timothy P. Singleton, M.D. and Dan McKeon, Flow Cytometry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

CD10 is expressed by both B-cell and T-cell lymphoid progenitor cells, and on follicular germinal center B-cells; however, CD10 expression on a large percentage of B-cells needs to be further investigated, as it may represent follicular lymphoma (Fig. 2.3).

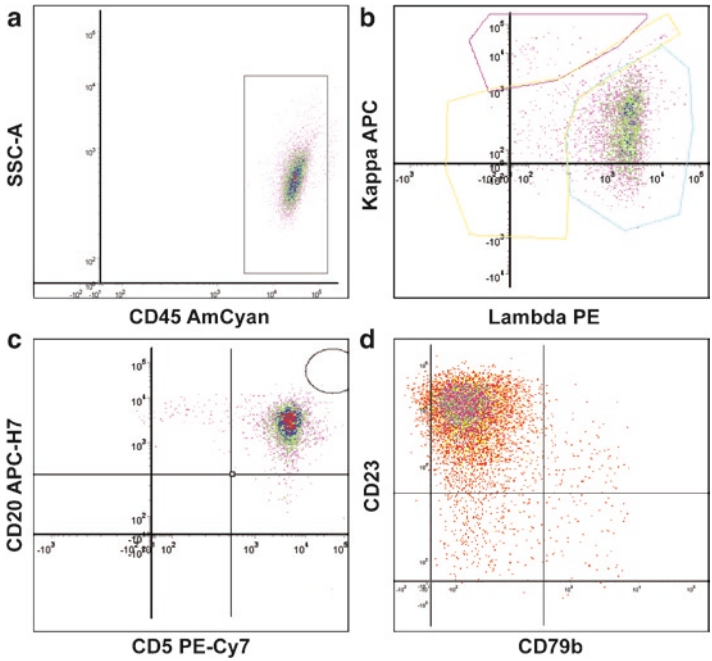

FIGURE 2.4. Flow cytometry from an aspirate of a patient with small lymphocytic lymphoma (CLL/SLL). The flow cytometric findings are characteristic. (**a**) CD45 vs. SSC histogram showing that the cells are hematolymphoid (CD45+) and are present within the lymphocyte window. (**b**) Kappa vs. lambda histogram shows overwhelming predominance of lambda-positive cells, demonstrating light chain restriction. (**c**) CD20 vs. CD5 histogram showing coexpression of CD5 on B-cells. CD20 expression is usually low (dim) in CLL/SLL. (**d**) CD23 vs. CD79b histogram showing expression of CD23 and absence of CD79b expression, which are characteristic for CLL/SLL, as is the absence of FMC7 expression (courtesy of Timothy P. Singleton, M.D. and Dan McKeon, Flow Cytometry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

The T-cell markers CD5 and CD43 may normally be expressed in a small percentage of B-cells; however, their expression on a large percentage of B-cells is abnormal and may be seen in low-grade B-cell lymphomas (Fig. 2.4).