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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

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 schenckii*) 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
<i>Histoplasma capsulatum</i>	2–5	Small yeast, often within macrophages	Single bud	GMS
<i>Coccidioides immitis</i>	20–200 (spherules) 2–5 (endospores)	Large spherules with or without endospores	Endospores	GMS, PAS, Fontana-Masson
<i>Blastomyces dermatitidis</i>	8–15	Spherical yeasts with thick (double contoured) walls	Single, broad-based bud	GMS, PAS, Congo red
<i>Cryptococcus neoformans</i>	2–15	Variably sized yeasts, thick capsule	Single, narrow-based bud	GMS, PAS, Mucicarmine, Fontana-Masson
<i>Sporothrix shenkii</i>	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. SSC 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

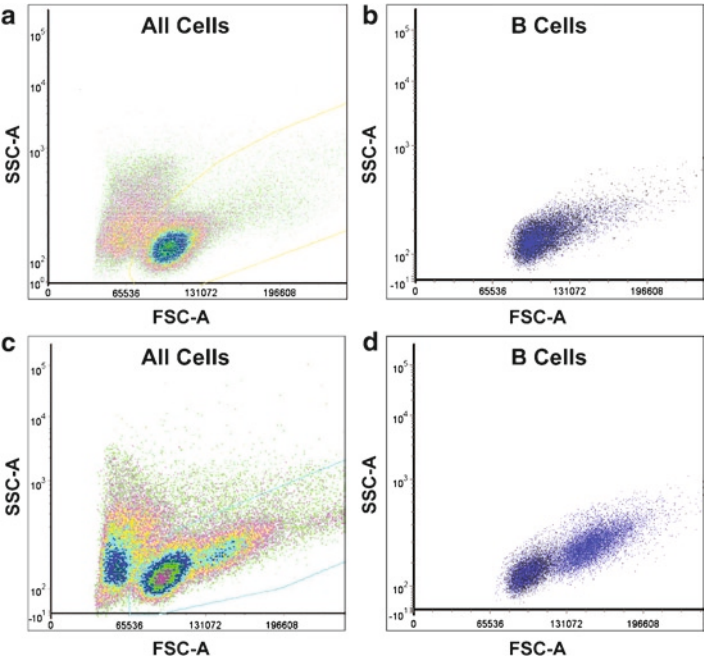


FIGURE 2.1. Histograms of side scatter (SSC) vs. forward scatter (FSC) can help detect the presence of a large cell (neoplastic) lymphoid population. (a, b) Reactive lymph node aspirate: no significant large cell lymphoid population is present when all cells are considered (a) or when gating only on 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.

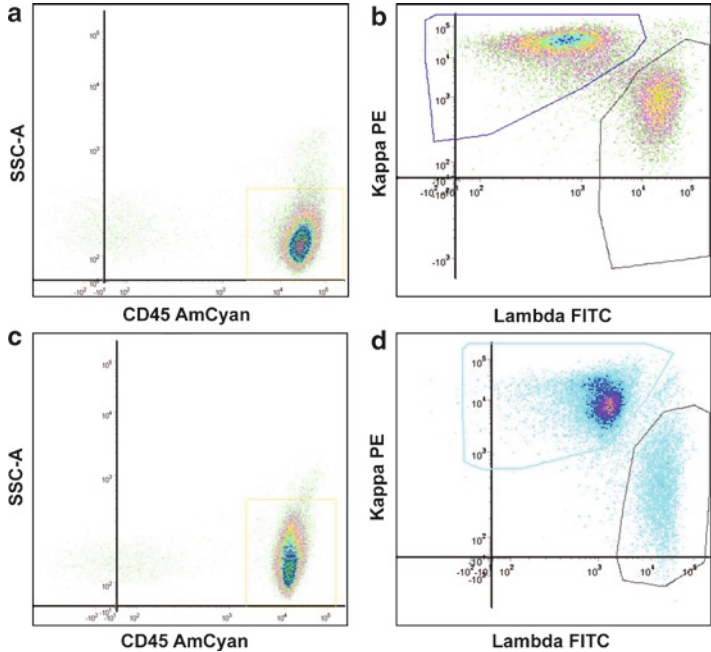


FIGURE 2.2. Histograms of SSC vs. CD45 can help determine if the cells fall in the lymphocyte gate (CD45 bright and low SSC); histograms of kappa vs. lambda can determine the presence of light chain restriction. (a, b) Reactive lymph node aspirate: most cells fall within the leukocyte gate (CD45 bright 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.

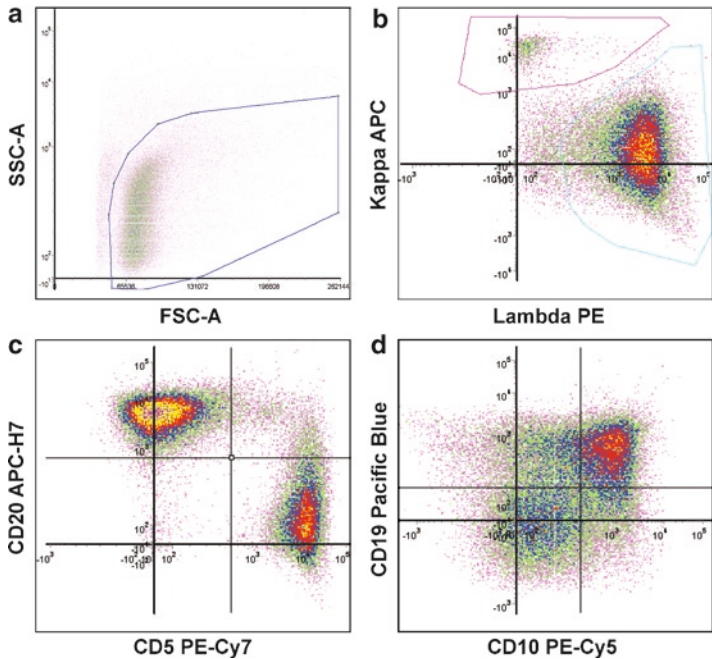


FIGURE 2.3. Flow cytometry (FC) from an aspirate of a patient with grade 1 follicular lymphoma. FC allows the determination of light chain restriction/clonality and the lack of a large cell component on FSC may help excluding a DLBCL. The diagnosis requires clinical and cytologic correlation. (a) SSC 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).

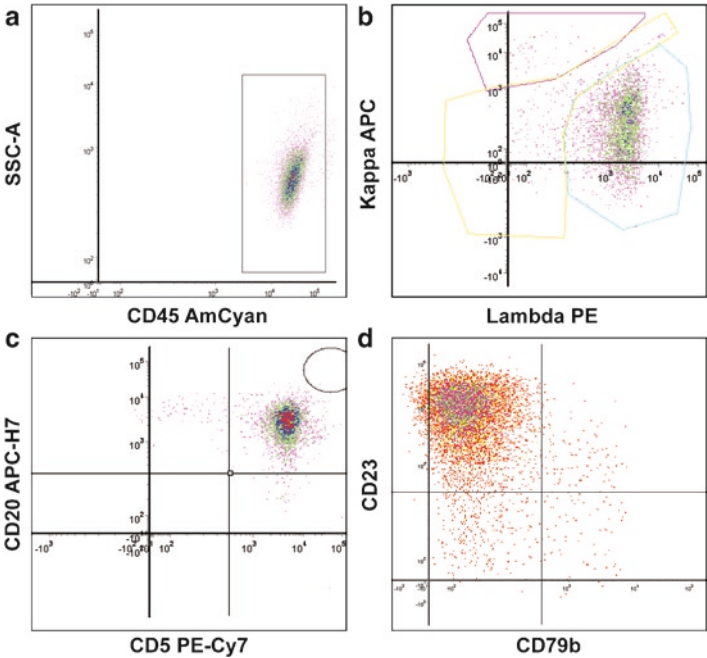


FIGURE 2.4. Flow cytometry from an aspirate of a patient with small lymphocytic lymphoma (CLL/SLL). The flow cytometric findings are characteristic. **(a)** CD45 vs. SSC histogram showing that the cells are hematology (CD45+) and are present within the lymphocyte window. **(b)** Kappa vs. lambda histogram 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).

In about 15-25% of diffuse large B-cell lymphoma (DLBCL) FC results are nondiagnostic due to the presence of nonviable or apoptotic cells, or of fragile cells that are preferentially lost during transportation, storage, or processing. In addition, mechanical factors, such as sclerosis, which is more commonly encountered in DLBCL, can lead to mechanical disruption of neoplastic cells. All of these factors lead to underrepresentation of neoplastic cells in the FC sample (Fig. 2.5).

For similar reasons, lymphomas with low numbers of neoplastic cells such as Hodgkin lymphoma, T-cell-rich B-cell lymphoma, and anaplastic large cell lymphoma (ALCL) frequently lead to nondiagnostic FC results. If the presence of one of these types of lymphoma is suspected during on-site evaluation, it is preferable to submit the specimen remaining after the smears are made for cell block preparation rather than for FC.

Establishing T-Cell and NK Cell Clonality

T-cell clonality is more difficult to determine by FC. The demonstration of an abnormal phenotype on a large percentage of T-cells is a useful, albeit indirect indicator of T-cell clonality. Cytomorphologic correlation is essential for the diagnosis once an aberrant immunophenotype has been found, but additional studies, including cytogenetics, and molecular studies, especially T-cell receptor (TCR) gene rearrangement studies, may sometimes be necessary for the diagnosis.

Normal peripheral T-cells express CD2, CD3, CD5, CD7, and either CD4 or CD8, while thymic T-cells express both CD4 and CD8. In normal T-cells, the percentage of cells expressing CD2, CD3, CD5, and CD7 is similar, and approximates the sum of CD4- and CD8-expressing cells. Peripheral T-cell lymphomas frequently show loss of or dim expression of one or more of these pan-T antigens; absence or presence of both CD4 and CD8 on a large proportion of nodal T-cells; and diminished CD45 (leukocyte common antigen) expression. These abnormalities are frequently associated with increased FSC. Abnormal CD4/CD8 ratios, especially above 15/1, also occur frequently in peripheral T-cell lymphomas, but are not specific as they are also found in Hodgkin lymphomas, viral infections, DLBCLs, dermatopathic lymphadenopathy, and atypical T-cell proliferations associated with phenytoin

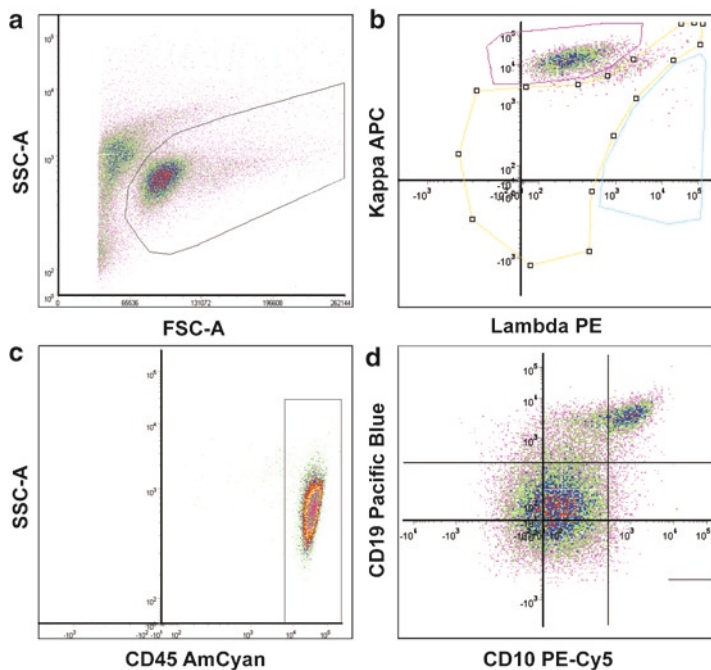


FIGURE 2.5. Flow cytometry from a lymph node aspirate of a patient with DLBCL. FC allows the determination of light chain restriction/clonality but the immunophenotype is not specific, as follicular lymphomas and Burkitt lymphomas may show the same immunophenotype and the diagnosis requires cytologic correlation. (a) SSC vs. FSC histogram showing that most cells show low FSC and SSC consistent with small lymphocytes. A population of larger cells (higher FSC) is also present. Larger cells are underrepresented due to loss during processing. (b) Kappa vs. lambda histogram shows overwhelming predominance of kappa-positive cells, demonstrating light chain restriction. (c) CD45 vs. SSC histogram showing that the cells are hematolymphoid (CD45+) and are present within the lymphocyte window. (d) CD19 vs. CD10 histogram shows CD10 expression in a subset of cells (courtesy of Timothy P. Singleton, M.D. and Dan McKeon, Flow Cytometry Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

treatment. Most T-cell lymphomas have a CD4+ phenotype; some T-cell lymphomas also express other characteristic markers such as CD25 in adult T-cell leukemia/lymphoma, CD10 in angioimmunoblastic lymphoma, and CD30 in ALCL.

T-cell clonality may also be determined by the flow cytometric analysis of the TCR's V β repertoire.

NK cells and their proliferations express markers also expressed by T-cells (CD2, CD7, and CD8) but do not express surface CD3 and CD4, and frequently express CD56, and CD57.

Classification of Lymphoma

According to the current (2008) World Health Organization classification, non-Hodgkin lymphomas are defined by their morphology, immunophenotype, cytogenetics, and sometimes also by their clinical features. Therefore, it is essential to establish the immunophenotype of a neoplastic lymphoid proliferation by FC (preferably) or immunohistochemistry. This immunophenotype, interpreted in the clinical and cytomorphologic context, is essential in the accurate classification of lymphomas on FNA specimens.

The more common nodal B-cell non-Hodgkin lymphomas can be divided according to their expression of CD5 and CD10 into the following:

1. CD5-positive, CD10-negative
 - (a) Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)
 - (b) Mantle cell lymphoma (MCL)
 - (c) Diffuse large B-cell lymphoma, including Richter transformation of CLL
 - (d) Lymphoplasmacytic lymphoma
2. CD5-negative, CD10-positive
 - (a) Follicular lymphoma (FL)
 - (b) Diffuse large B-cell lymphoma (DLBCL)
 - (c) Burkitt lymphoma (BL)
3. CD5-negative, CD10-negative
 - (a) Marginal zone lymphoma (MZL) (Fig. 2.6)
 - (b) Lymphoplasmacytic lymphoma

While these are the typically encountered immunophenotypes, follicular lymphoma, DLBCL, and MCL lymphoma can also have a CD5+/CD10+, or a CD5-/CD10- phenotype, Burkitt lymphoma can be CD5+/CD10+, and MZL can be positive for CD5.

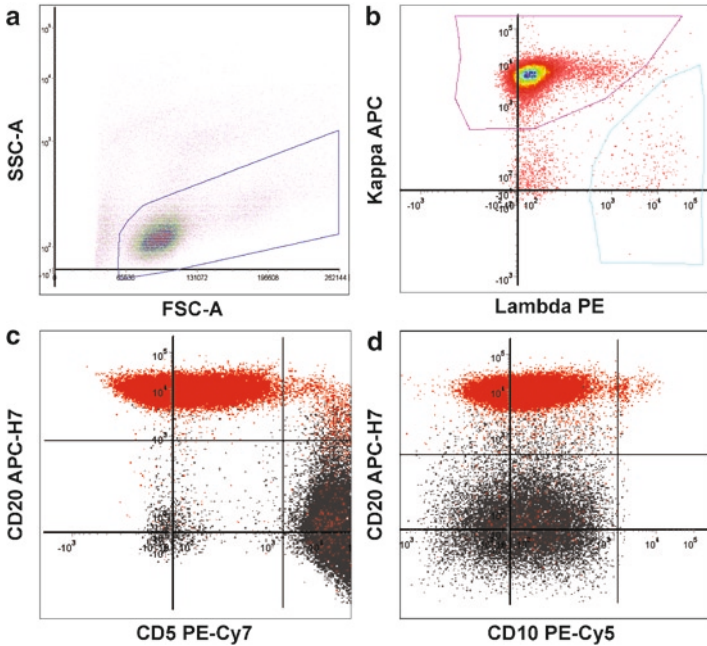


FIGURE 2.6. Flow cytometry from an EUS-guided fine needle aspirate of a perigastric lymph node of a patient with marginal zone lymphoma. FC allows the determination of light chain restriction/clonality but there are no specific surface markers for this lymphoma and the diagnosis is based on the absence of immunophenotyping features characteristic for other lymphomas, clinical and cytologic correlation. **(a)** SSC vs. FSC histogram showing that most cells show low FSC and SSC consistent with small lymphocytes. **(b)** Kappa vs. lambda histogram shows overwhelming predominance of kappa-positive cells, demonstrating light chain restriction. **(c)** CD20 vs. CD5 histogram shows lack of CD5 expression on the kappa monotypic B-cells. **(d)** CD20 vs. CD10 histogram shows lack of CD10 expression on the kappa 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).

The typical phenotype of each lymphoma will be presented in the following chapters together with their characteristic cytomorphology and cytogenetic findings.

Immunocytochemical and Immunohistochemical Methods

Immunocytochemistry on Cytospin Preparations

In some laboratories, immunophenotyping is performed on cytospin preparations. This technique has some advantages over FC, since it requires fewer cells, and the presence and intensity of markers can be correlated with cytomorphology. It may also allow immunophenotyping in cases where FC is unsuccessful due to the fragility of the neoplastic cells. However, the disadvantage of this technique is that it does not allow the simultaneous assessment of multiple markers on the same cell.

Immunohistochemistry on Cell Block Specimens

Immunohistochemical stains for a variety of markers can also be performed on cell block preparations. These immunohistochemical stains can be useful in the classification of lymphoid proliferations, and the differential diagnosis between lymphoid and nonlymphoid neoplasms.

In the diagnosis of lymphoid proliferations, this method has some definite disadvantages over FC, because the number of antibodies available is smaller than for FC, the simultaneous assessment of multiple markers on the same cell is not possible, and kappa and lambda stains are difficult to interpret, thus limiting its usefulness in the determination of clonality. It is therefore important to submit routinely the needle rinses from 2 to 3 passes for FC and possibly submit the majority of cells obtained in an additional FNA pass for cell block preparation, since immunohistochemistry also has some advantages. Apart from the possibility to correlate the stains with the cytomorphology, immunohistochemical stains frequently react with nonviable “ghost” cells. It also allows the use of some markers that are not routinely available for FC, such as cyclin D1. In addition, immunohistochemistry is the method of choice for lymphoid proliferations showing rare neoplastic cells such as Hodgkin lymphoma, ALCL, and T-cell-rich B-cell lymphomas, despite recent advances with multicolor FC in these conditions.

Because each immunohistochemical stain is performed on a separate section and the material available is limited, panels of

immunostains are sequentially performed to reach a complete characterization of the lymphoid proliferation.

A general panel that can be used when non-Hodgkin lymphoma is suspected is composed of CD20, which identifies most B-cells and their neoplasms; CD3, which identifies most T-cells (membranous staining) and natural killer cells (cytoplasmic staining); CD5 and CD43, T-cell markers frequently aberrantly expressed in B-cell lymphomas; CD10 and Bcl6, markers of germinal center B-cells; and Bcl2, which marks many neoplastic B-cells but not normal germinal center B-cells and Cyclin D1.

As normal lymph node aspirates show a marked predominance of T-cells, a suspicion of B-cell lymphoma is raised if a large proportion of the aspirate is composed of CD20+ B-cells. Coexpression of CD5 and/or CD43 on a large proportion of these cells is an indicator of lymphoma (CLL/SLL), as is the presence of Bcl2 staining on CD10 or Bcl6-staining cells (follicular lymphoma), or staining for Cyclin D1 of a large proportion of lymphoid cells (MCL).

In addition to markers also available for FC, there are a number of unique immunohistochemical markers that may be useful in characterizing lymphoid proliferations. Pax-5, a B-cell marker expressed in normal B-cells (but not plasma cells) and most B-cell lymphomas, is useful especially in situations when CD20 is negative in recurrences of B-cell lymphomas after rituximab therapy. Awareness of the fact that Pax-5 is also expressed in small cell carcinomas, Merkel cell carcinomas, and alveolar rhabdomyosarcomas that may enter the differential diagnosis is essential to prevent pitfalls. Other potential pitfalls are the expression of CD5 in smooth and skeletal muscles and their tumors, and the expression of TdT in Merkel cell carcinoma.

B-cell light chain restriction by kappa and lambda light chain immunoperoxidase stains, or in-situ hybridization studies can only rarely establish clonality; however, they may be useful in characterizing aspirates with a prominent plasma cell component.

A variety of other immunohistochemical markers may be used, including Ki67 (MIB1), either as diagnostic or as prognostic markers. Almost 100% expression of Ki67 in Burkitt lymphoma is helpful in its differentiation from other high-grade lymphomas.

A general first panel that can be used when Hodgkin lymphoma is suspected is composed of CD20, CD3, CD15, CD30, CD45RB

(LCA), EMA, and ALK-1. Their use in the differential diagnosis between Hodgkin lymphoma and non-Hodgkin lymphomas and in the classification of Hodgkin lymphoma will be addressed in Chap. 11.

Immunoperoxidase stains for LMP-1 and in situ hybridization for EBER can also detect EBV, which is present in infectious mononucleosis, immunosuppression-associated lymphoproliferations, Burkitt lymphoma, immunoblastic/plasmablastic tumors, Hodgkin lymphoma, angioimmunoblastic T-cell lymphoma, and NK-cell neoplasms. Immunohistochemical detection of HHV8 with an antibody against the latent nuclear antigen (LNA-1) may be useful in the diagnosis of multicentric Castleman disease (MCD), plasmablastic lymphoma (PBL) arising in MCD, and primary effusion lymphoma (PEL).

FISH and Conventional Cytogenetics

Both conventional cytogenetics (G-banding) and FISH can be used to demonstrate the characteristic recurrent cytogenetic abnormalities found in some lymphomas (see Table 2.2).

Conventional cytogenetic studies can also demonstrate additional abnormalities that may be of prognostic importance. For the evaluation of FNA biopsy samples, FISH is preferable to conventional cytogenetics because it is faster, does not require a fresh sample (although fresh tissue is preferred), and can be performed on smears, cytopsins, or cell block preparations. Because FISH is usually performed on interphase (nondividing) nuclei, low proliferative rates do not preclude analysis.

Several types of FISH probes can be used in the evaluation of lymphoid proliferation. Probes that hybridize to specific centromeres are useful for detection of polysomies such as trisomy 12 in CLL/SLL (Fig. 2.7a–c), and fusion or break-apart probes are useful in the detection of translocations.

Break-apart probes hybridize to a specific region on a single chromosome, flanking the breakpoint region with a green and a red probe (i.e., the 3' region is labeled in either red or green, and the 5' region is labeled in the other color). In a fluorescence microscope, overlapping or closely apposed red and green signals appear yellow; thus, the pattern in a normal interphase nucleus is two yellow signals (the red and green components of this yellow fusion signal

TABLE 2.2. Characteristic cytogenetic abnormalities in lymphomas.

Lymphoma	Cytogenetic abnormalities	Frequency (%)	Genes involved	Diagnostic or prognostic use	FISH type
SLL/CLL	Trisomy 12	25		Diagnostic	Centromeric enumeration
SLL/CLL	del 13	40–60	<i>RB1</i>	Positive prognostic	Locus specific
SLL/CLL	del 11	10–20	<i>ATM</i>	Negative prognostic	Locus specific
SLL/CLL	del 17	10	<i>p53</i>	Negative prognostic	Locus specific
MCL	t(11;14)	Over 95	<i>IGH/BCL1</i>	Diagnostic	Break-apart, dual-fusion
FL	t(14;18)	70–80	<i>IGH/BCL2</i>	Diagnostic	Break-apart, dual-fusion
MZL	t(11;18)	15–30	<i>API2/MALT1</i>	Diagnostic	Break-apart, dual-fusion
BL	t(8;14)	80	<i>IGH/CMYC</i>	Diagnostic	Dual fusion, break-apart
DLBCL	t(14;18)	30	<i>IGH/BCL2</i>	Diagnostic	Break-apart, dual fusion
ALCL	t(2;5)	70	<i>ALK/NPM</i>	Diagnostic Positive prognostic	Break-apart, Multicolor

SLL/CLL small lymphocytic lymphoma/chronic lymphocytic leukemia; *MCL* mantle cell lymphoma; *FL* follicular lymphoma; *MZL* marginal zone lymphoma; *BL* Burkitt lymphoma; *DLBCL* diffuse large B-cell lymphoma; *ALCL* anaplastic large cell lymphoma

are sometimes visible). A translocation will break apart the 3' and 5' probes flanking the breakpoint, thus resulting in the following signal pattern: one yellow (the normal chromosome homolog), one red, and one green signal (representing the now separated 3' and 5' portions of the involved gene) (Fig. 2.8a–c).

Break-apart probes are useful in determining rearrangement of “promiscuous” genes that have a number of different partner genes. Although such a signal pattern indicates the presence of a translocation, it does not identify the other partner chromosome. For example, the *IGH* gene is involved in translocations with several different partners in a large percentage of B-cell lymphomas. Using an *IGH* break-apart probe will identify the presence of an *IGH*

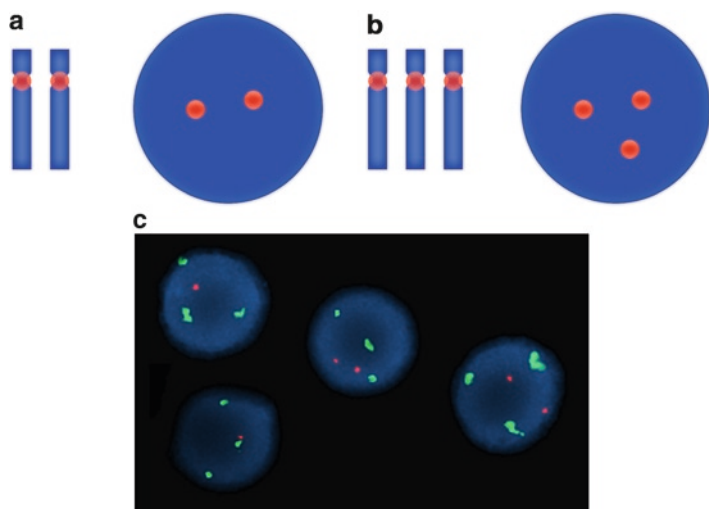


FIGURE 2.7. FISH with chromosome enumeration probes to determine trisomy. (a, b) Schematic representation with normal (a) and abnormal (b) results of FISH. (c) FISH result in a case of CLL/SLL with trisomy 12 in all four cells (*green signals*) and loss of D13S319 (13q14) (*red signals*) in two cells (courtesy of Michelle Dolan, M.D., Cytogenetics Laboratory, and Jonathan Henriksen, Department of Laboratory Medicine and Pathology, University of Minnesota).

gene rearrangement, but a specific dual-fusion translocation probe (e.g., *IGH/BCL2* or *IGH/CCND1*) would be needed to identify the partner chromosome.

The most sensitive FISH probes to detect reciprocal translocations are dual fusion probes. Each of the genes or loci of interest is labeled in either red or green, and each probe extends both proximal and distal to the breakpoint (i.e., the labeled region spans the breakpoint). In normal cells, two distinct red and two distinct green signals are present. In a reciprocal translocation, the chromosomal regions distal to the breakpoints are exchanged, thus bringing together red and green probes on each of the involved derivative chromosomes. Thus, the signal pattern in a cell with a translocation is one red and one green (representing the normal, uninvolved chromosomes) signal, and two yellow fusion signals (representing

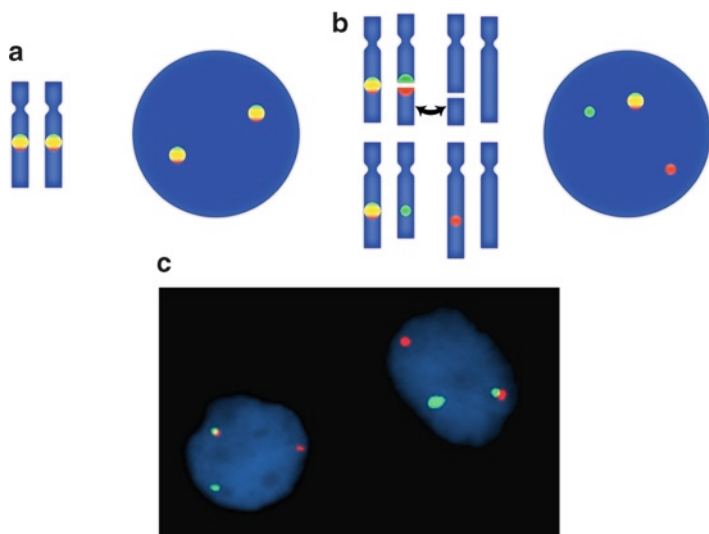


FIGURE 2.8. FISH with break-apart probes to determine the presence of a translocation involving a specific region. (a, b) Schematic representation with normal (a) and abnormal (b) results of FISH. (c) FISH result in a case of anaplastic large cell lymphoma (ALCL) with t(2;5) translocation. The normal (nontranslocated) 2p23 ALK region is seen as two immediately adjacent *orange-red/green* signals or as a fused *yellow* signal; the 2p23 ALK region that has suffered a t(2;5) (or another translocation) is seen as one *orange-red* and one *green* signal (courtesy of Michelle Dolan, M.D., Cytogenetics Laboratory, and Jonathan Henriksen, Department of Laboratory Medicine and Pathology, University of Minnesota).

each of the partner chromosomes involved in the translocation) (Fig. 2.9a–c).

In contrast to a conventional G-banded cytogenetic analysis in which all chromosomal abnormalities can be identified, FISH can identify only the translocation specifically targeted by the probe set used. Therefore, FISH tests should be ordered based on the differential diagnosis generated by the cytomorphologic or immunophenotypic data, especially if the entities considered in the differential (e.g., FL vs. MZL, FL vs. MCL, etc.) are characterized by different translocations. Because of considerable cytomorphologic and immunophenotypic overlap, one of the most difficult differential diagnoses is between DLBCL and Burkitt lymphoma.

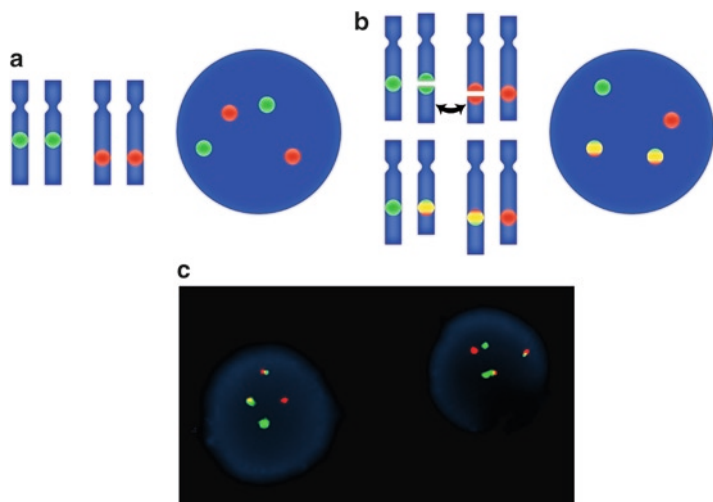


FIGURE 2.9. FISH with dual color, dual fusion probes to determine the presence of a specific translocation. (a, b) Schematic representation with normal (a) and abnormal (b) results of FISH. (c) FISH result in a case of follicular lymphoma (FL) with t(14;18) IGH/BCL2 translocation. The nuclei show one *orange-red* signal and one *green* signal representing the normal (nontranslocated) chromosomes and two *orange-red/green* or *yellow* fusion signals representing the two chromosomes resulting from the reciprocal translocation (courtesy of Michelle Dolan, M.D., Cytogenetics Laboratory, and Jonathan Henriksen, Department of Laboratory Medicine and Pathology, University of Minnesota).

In this situation, determining the presence of a translocation involving *MYC* using a break-apart probe may be diagnostically useful.

FISH studies can also be helpful in determining prognosis. For example, in CLL/SLL, a FISH panel test for B-CLL may include, among others, probes for the demonstration of the deletion of markers with prognostic implications such as *MYB* (6q23), *ATM* (11q22.3), 13q14.3, and *TP53* (17p13.1) (Fig. 2.10a–c).

Molecular Studies

Molecular studies are rarely used in the work-up of lymph node fine needle aspirates, but may be helpful in cases where other

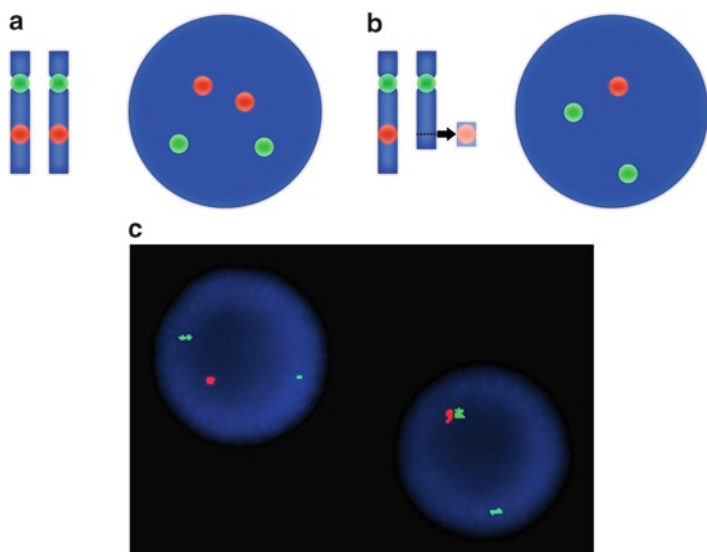


FIGURE 2.10. FISH with locus-specific probes to determine the presence of a specific deletion. (a, b) Schematic representation with normal (a) and abnormal (b) results of FISH. (c) FISH result in a case of small lymphocytic lymphoma (CLL/SLL) with del 17p13.1 (TP53), associated with worse prognosis. Only one *orange-red* signal is present, while the two *green* signals signify the presence of both centromeres (courtesy of Michelle Dolan, M.D., Cytogenetics Laboratory, and Jonathan Henriksen, Department of Laboratory Medicine and Pathology, University of Minnesota).

ancillary studies could not be performed, were nondiagnostic, or yielded ambiguous results. Molecular studies are helpful in determining the clonality of lymphoid proliferations through DNA-based antigen receptors assays (Southern blot or PCR based) and in aiding the correct classification of lymphomas by demonstrating translocations characteristic for certain lymphomas by PCR, as in the case of t(14;18) of follicular lymphomas, or their fusion gene transcript by RT-PCR, as in the case of the t(2;5) of ALCL.

Antigen Receptor Assays by Southern Blot Analysis or PCR

Southern blot analysis is the most specific of the molecular methods used but it is not commonly used because of the need for fresh

samples, large amounts of high quality DNA, slow turn-around time, and high cost. PCR-based techniques, on the contrary, are fast, require very small amounts of cellular sample, and can be performed on material from cell blocks or from cells scraped from smears.

B-cells and T-cells recognize antigens by structurally similar heterodimer proteins composed of both variable and constant regions linked by disulfide bonds, the B-cell surface immunoglobulin receptor (*IG*), and the *TCR*. These are encoded by the *IG* and *TCR* genes, which belong to the same antigen receptor supergene family. Both *IG* and *TCR* genes undergo somatic rearrangements in the earliest stages of lymphoid differentiation and are, therefore, present in the vast majority of immature and mature lymphoid cells.

The *IGH* gene at chromosome 14q32 contains multiple *V*, *J*, *D*, and *C* gene segments that rearrange by somatic recombination to achieve the enormous diversity of antigen receptors. Rearrangements occur sequentially in an orderly fashion; *D* is first rearranged to *J* and then *V* is rearranged to *DJ*. These rearrangements occur with deletion of the intervening DNA and, therefore, result in variably sized DNA segments that are different from the initial nonrearranged DNA referred to as *germline configuration* and between different rearranged cells.

Similar rearrangements occur in the *TCR* genes. The *TCR* is composed of either one alpha and one beta polypeptide chain ($\alpha\beta$ *TCR*) or one gamma and one delta polypeptide chain ($\gamma\delta$ *TCR*). The corresponding four *TCR* genes are rearranged sequentially: First *TCR* δ (14q11), then *TCR* γ (7p15), *TCR* β (7q34), and finally *TCR* α (14q11). The *TCR* γ gene is usually targeted for clonality studies because it is less complex than *TCR* β . *TCR* γ rearrangements are present in both $\alpha\beta$ and $\gamma\delta$ T-cells and the vast majority of T-cell neoplasms.

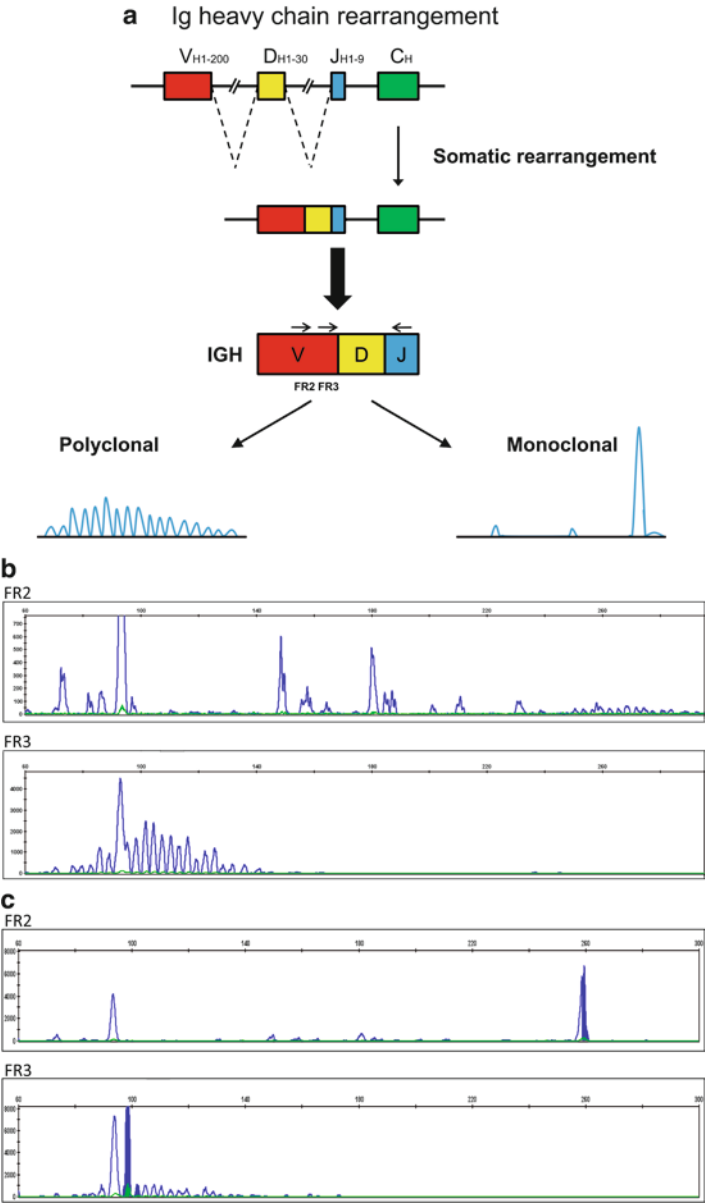
The diversity of sizes of the rearranged *IG* or *TCR* genes is diagnostically useful in determining clonality, as DNA amplified from reactive, polyclonal lymphoid populations shows a wide range of gene sizes, whereas neoplastic lymphoid cells, which are in principle all derived from a common clone, show identically rearranged *IG* or *TCR* genes.

Southern blot analysis uses restriction endonuclease enzyme digestion of the DNA extracted from the lymphocyte population. The fragments are then separated by electrophoresis and immobilized on a nylon membrane, and then hybridized to a radiolabeled

fragment of the gene of interest (*IGH* or *TCR β*). A band is detected if the sample contains more than about 5–10% clonal, presumably neoplastic cells admixed with polyclonal cells. Polyclonal cells appear as a background smear because of the variable size of the fragments resulting from endonuclease digestion.

PCR-based assays for B-cell clonality use a variety of V and J specific or consensus primers that amplify the *IGH* V-(D)-J region, also known as the CDR3 (complementarity determining region 3) or N region; the amplicon is then detected using conventional electrophoresis in agarose, nondenaturing polyacrylamide gel electrophoresis with ethidium bromide staining, or the more sensitive capillary electrophoresis. If a polyclonal lymphoid population is present, each lymphocyte will have an N region of a different size, and no bands or peaks will be seen on gel electrophoresis and capillary electrophoresis, respectively. In contrast, clonal populations will show a definite band or peak (Fig. 2.11a–c).

FIGURE 2.11. *IGH* rearrangement studies. (a) Schematic representation of the *IGH* rearrangements, location of PCR framework 2 (FR2) and framework 3 (FR3) primers and the common antisense primer that anneals in the J region of the *IGH* gene and possible results of the PCR reaction on capillary electrophoresis. Expected size range for FR2 PCR products is 220–250 bp and that of FR3 products is 70–150 bp. Polyclonal lymphoid populations show a Gaussian distribution of sizes of the PCR products while clonal B-cell populations show a discrete peak. Our laboratory defines a peak as clonal if it meets the following requirements: an electropherogram peak height of >1,500 fluorescent units; and a peak height three times the height of the polyclonal background. (b) Results of PCR using FR2 and FR3 primers for determination of *IGH* gene rearrangement in a case of reactive lymphadenopathy. This is an example of a negative B-cell gene rearrangement test that shows multiple small bands in the FR2 reaction between 220 and 250 bp (*top panel*) and a polyclonal bell shaped curve in FR3 reaction between 70 and 150 bp (*bottom panel*). Note the absence of a discrete peak. (c) Results of PCR using FR2 and FR3 primers for determination of *IGH* gene rearrangement in a case of B-cell lymphoma. This is an example of a positive B-cell gene rearrangement test that shows positive clonal peaks in the FR2 (*top panel*) and FR3 (*bottom panel*) regions. Both these clonal peaks are >1,500 RFU and the peak heights are three times the height of the polyclonal background (courtesy of Bharat Thyagarajan, M.D., Molecular Diagnostics Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).



PCR-based assays to determine T-cell clonality employ various combinations of V family and J region primers on the *TCR* γ with amplicon detection and results interpretation similar to those described above. A clone can be detected if there are about 5% of clonal T-cells in a background of polyclonal cells in nondenaturing polyacrylamide gels, with higher sensitivities for capillary electrophoresis (Fig. 2.12a–c).

The sensitivity of PCR-based methods for determining B-cell or T-cell clonality is significantly lower in cell populations obtained from fixed and paraffin-embedded specimens compared to that in fresh specimens.

For detection of *IGH* rearrangements in B-cell lymphoid proliferations, the false negative rate may be as high as 30% in germinal center/postgerminal center lymphomas, such as follicular lymphoma and DLBCL. This occurs due to the fact that B-cells undergo somatic hypermutation in the *IGH* V region in response to antigen exposure in the germinal center, and lymphomas occurring beyond this stage of B-cell development may show high degrees of somatic hypermutation which may modify the PCR primer-binding sites. No equivalent somatic hypermutation process occurs in rearranged *TCR* genes and mispriming is therefore not a problem in T-cell lymphoma clonality determination.

If only a small number of lymphocytes are available for the test, PCR-based antigen receptor assays may give false-positive results due to pseudoclonality caused by the presence of different rearrangements of identical length.

It should be stressed again that detection of clonality is not in and for itself equivalent with malignancy (lymphoma). Apart from technical problems such as contamination or pseudoclonality, real clonal expansions of lymphoid populations may occur in benign disorders such as immunodeficiency states, autoimmune diseases (Sjögren syndrome and Hashimoto thyroiditis), and viral infections (HIV, EBV, and HHV8).

Antigen receptor rearrangements are not entirely lineage specific, as the clonal *IGH*, or *TCR* gene rearrangement detected may be discordant with the B-cell or T-cell lymphoma immunophenotype, i.e., *TCR* rearrangements may be detected in B-cell lymphomas and *IGH* rearrangements in B-cell lymphomas. This occurs especially in lymphoblastic lymphomas but can rarely occur in other lymphomas.

DNA-Based Tests for Identifying Translocations

In addition to conventional cytogenetics and FISH, translocations and their products can also be identified by various molecular techniques including Southern blot, PCR, and RT-PCR.

However, FISH is more commonly used to identify translocations. The very high sensitivity, with capabilities of detection of less than 1 in 1,000 cells showing the translocation makes some of these molecular methods suitable for detection of minimal residual disease.

Differentiating Lymphoid from Nonlymphoid Neoplasms and Establishing the Nature and Potential Site of Origin of a Metastatic Malignancy

Lymph nodes are the most common site of metastasis and metastatic malignancies outnumber primary lymphoid neoplasms in most lymph node locations. When the primary malignancy is known, metastases are frequently diagnosed by the cytomorphologic features of the lymph node aspirate and comparison with the morphology of the primary tumor. However, about 10–15% of all malignancies first present with metastases and the primary site may be difficult to determine even after a thorough clinical evaluation. In addition, second nonlymphoid or lymphoid primaries may occur in patients with known malignancies. Even when the primary malignancy is not known, cytomorphologic features usually allow a diagnosis of metastatic carcinomas or of a metastasis from another malignancy. However, in cases of small cell malignancies (small cell carcinoma and small blue cell tumors) that are very poorly differentiated, the differential diagnosis with lymphoid malignancies may be difficult or impossible based on cytomorphologic features alone. Since an accurate diagnosis is crucial in determining the best management of the patient, a panel of immunohistochemical stains will usually allow distinction of lymphomas from metastatic carcinomas, melanomas, and other metastatic malignancies. A first panel of antibodies usually includes CD45RB (LCA) as a marker of hematolymphoid neoplasms, keratins (usually a cocktail of cytokeratins such as cytokeratin AE1/AE3 and cytokeratin 8/18) and epithelial membrane antigen (EMA) as epithelial markers, S100 as

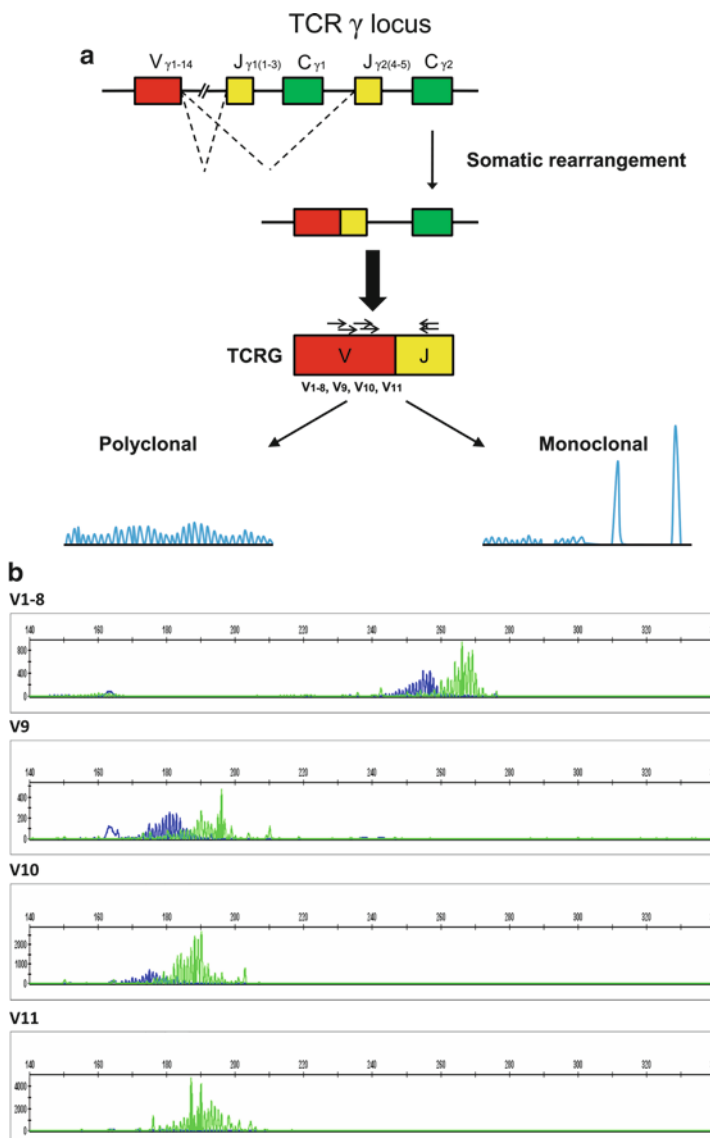


FIGURE 2.12. TCRG rearrangement studies. **(a)** Schematic representation of the TCRG rearrangements, location of PCR primers and results of the PCR reaction on capillary electrophoresis. Primer VG1 binds to regions V1–V8, VG9 binds to V9, VG10 binds to V10 and VG11 binds to V11 paired with two antisense primers JG and JP (labeled with different fluorescent dyes, JG1/2 with blue and JP1/2 with green) that recognize different J regions. The expected size range of PCR products of the VG1/JG1/2 reaction is 250–300 bp, while that of the remaining reactions is 160–210 bp.

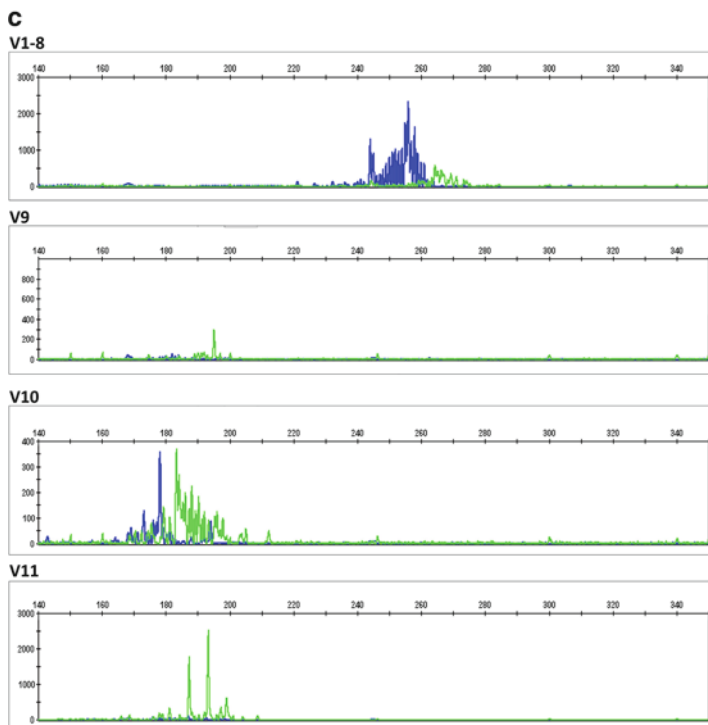


FIGURE 2.12. (continued) Polyclonal lymphoid populations show a Gaussian distribution of sizes of the PCR products while clonal T-cell populations show one or two discrete peaks. Our laboratory defines a peak as clonal if it meets the following requirements: an electropherogram peak height of 1,500 fluorescent units; and a peak height three times the height of the polyclonal background. **(b)** Results of PCR using VG1, VG9, VG10 and VG11 primers for the determination of TCRG gene rearrangement in a case of reactive lymphadenopathy. This is an example of a negative T gene rearrangement test. The VG1 reaction (*top panel*) shows a polyclonal background between 250 and 300 bp. The VG9 (*second panel*), VG10 (*third panel*) and VG11 reactions (*bottom panel*) show a polyclonal background between 160 and 210 bp. Though the VG11 panel shows clonal peak heights that are >1,500 RFUs, none of the peak heights are three times the height of the polyclonal background. **(c)** Results of PCR using VG1, VG9 VG10 and VG11 primers for the determination of TCRG gene rearrangement in a case of T-cell lymphoma. This is an example of a positive T gene rearrangement test. Two positive clonal peaks are detected in the VG11 reaction (*bottom panel*). The height of both peaks is >1,500 RFUs and the peak height three times the height of the polyclonal background. The other three reactions are negative and show a polyclonal background (courtesy of Bharat Thyagarajan, M.D., Molecular Diagnostics Laboratory, Department of Laboratory Medicine and Pathology, University of Minnesota).

a melanocytic marker, and vimentin is frequently used to determine if immunoreactivity of the cells is preserved. Based on the results of this initial panel, additional stains will be selected to confirm the epithelial, hematolymphoid, or melanocytic nature of the metastatic lymphadenopathy, or to rule out less common metastatic malignancies, such as sarcomas and germ cell tumors.

If the immunostains confirm a metastatic carcinoma and the primary site is not known, an attempt is made to determine the primary site by using panels of immunostains that are either “organ specific” such as thyroglobulin, calcitonin, HepPar1, renal cell carcinoma antigen, uroplakin, TTF1, and CDX2, or are differentially expressed by different metastatic carcinomas, such as cytokeratins 7 and 20. It should be noted that no antibody is actually organ specific and that cross-reactivity with other tissues is increasingly reported with the use of these antibodies.

Finally, if a metastasis is suspected but cannot be confirmed by cytomorphology as in the case of cystic or necrotic metastases, chemical determination of thyroglobulin or calcitonin in the lymph node aspirate may be diagnostic of a metastasis from the thyroid. Similarly, the presence of HPV DNA in a cystic lymph node aspirate from the neck usually indicates a cystically degenerated metastasis from a squamous cell carcinoma.

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