FNA Cytology in the Diagnosis of Lymphoma
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FNA Cytology in the Diagnosis of Lymphoma

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We wish to express our deep appreciation to our friends Professors Mario Rubens Montenegro and Marcello Fabiano de Franco, Brazil, who encouraged one of us (E.T.) throughout the training in pathology and to go to the Karolinska Hospital for training in cytopathology. They represent the best blend of practical and academic pathology.

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The continuous support and help provided by Dr. Svante Orell, the Editor of this series, and the staff of Karger Publishers contributed immeasurably to the completion of this book.
The enlarged lymph node became one of the main targets for fine-needle aspiration (FNA) cytology, and was soon accepted in the diagnosis of various types of lymphadenitis and metastatic disease. The diagnosis of lymphoma by FNA cytology was, however, controversial for many years in spite of early reports, in particular by Lopes Cardoso, which demonstrated the great potential of the technique. The scepticism at that time mainly resulted from the emphasis on growth patterns in the diagnosis and subtyping of lymphomas. Obviously, the growth pattern cannot be discerned from FNA smears. However, the introduction of immunocytochemistry led to new classification systems which put much less emphasis on growth patterns and more on immunologic characteristics. In 1988, Tani and coworkers and Ortel and Ortel described the application of immunocytochemistry in the cytologic diagnosis of lymphoma on FNA material. It now seemed possible to conclusively diagnose a majority of lymphomas, which, together with the excellent clinical performance of FNA sampling, should lead to the spread of the technique. Other ancillary techniques such as FISH and PCR have also been applied successfully to FNA material.

This manual has been divided into two chapters which describe the technical and methodological aspects of lymphoma diagnosis, and seven chapters which focus on the cytologic features of neoplastic and reactive lymphoid lesions. We have followed the most recent (2001) WHO lymphoma classification when describing the various lymphoma subtypes. In addition, a separate chapter has been devoted to lymphoma look-alike lesions. Key cytologic and immunologic features are listed to facilitate a conclusive diagnosis of the different lesions.

It is our strong hope that this book will be in the best interest of the patients and will be of help and support to cytopathologists in their diagnostic work with patients with lymphadenopathy of reactive or neoplastic background.

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Lambert Skoog, Stockholm
Two marine officers, Greig and Gray [1], are recognized as the first to use needle biopsy of lymph nodes. In 1904, they reported that motile trypanosomes could be observed in smears from biopsied nodes. For many years node biopsy was considered a valuable means of demonstrating bubonic plague, trypanosomes and spirochetes. Thus, the technique was only used to identify microorganisms and not to evaluate the cellular components in lymph node disorders.

The first report on lymph node puncture to give a cytomorphologic diagnosis of malignant lymphoma, lymphoblastoma, was published in 1914 by Ward [2]. An attempt to systematically describe cytologic findings in lymph node aspirates from a variety of diseases was presented by Guthrie [3], who as early as 1921 had used air-dried smears stained with Romanowsky. Six years later, Forkner [4] reported on the cytologic presentation of several lymph node disorders in a paper entitled ‘Materials from lymph nodes of man’. From then on several papers and monographs were published but the procedure was only slowly accepted by the medical community. In 1952, Morrison et al. [5] reported a large series of lymph node punctures, which included the sensitivity and specificity of the technique. It is puzzling that such an important study had so little impact on the clinical management of patients with lymph node disorders.

In the 1950s and 1960s the development of lymph node cytology was to a large extent the work of clinicians, in particular, those with an interest in hematology. Among them were Pavlowsky, Lopes Cardozo, Abramov, Söderström, and Franzén who all made invaluable contributions [6–10]. However, the clinical background of these pioneers may have been one reason for the slow acceptance of the technique: the pathologists were often unfamiliar with the interpretation of air-dried cells stained with the Romanowsky technique, and also feared that open biopsies could be replaced by fine-needle aspirates. As a consequence, the method was not adopted at most centers for tumor diagnosis in the 1960s.

Several monographs and atlases were however published in the 1960s and 1970s which documented the diagnostic accuracy and wide applicability of fine-needle aspiration cytology. It is of interest to note that even among the enthusiasts and experts in FNA cytology, there seemed to be a discrepancy in the concept of the accuracy of lymphoma diagnosis using cytomorphology alone. Based on a rather limited study, Zajicek [11] concluded that ‘about 20% of cases of well-differentiated lymphocytic lymphoma cannot at present be recognized in smears of aspirates’. However, in poorly differentiated (high-grade) lymphoma, he believed that a reliable diagnosis could be made by an experienced examiner.

A somewhat more optimistic but at the same time cautious standpoint was presented by Koss et al. [12] in their textbook in which they state that the question ‘Is it or is it not a malignant lymphoma? Can be answered on an aspirate of untreated lesions in most cases’. In fact, the authors believe that ‘a precise identification of subtypes of malignant lymphoma can be made by observers with an adequate experience’.

The most optimistic view was expressed by Linsk and Franzén [13] who stated that ‘There is no question that the diagnosis can be made with ease by FNA’. Although it may not be all that easy to diagnose lymphomas, one is inclined to believe that it can be made by an experienced examiner after reading the excellent results presented by Lopes Cardozo [14] who accurately diagnosed 1,023 lymphomas on cytology. Moreover, the ‘Atlas of clinical cytology’ by Lopes Cardozo contains an overwhelming series of beautiful color illustrations of various lymphomas which should make most morphologists interested in lymphoma cytology and accept it as a potentially valuable adjunct to histopathology [15].
The application of immunocytochemistry on lymph node aspirates led to an increased interest in utilizing FNA material for lymphoma diagnosis [16–18]. Simultaneously, it was also shown that immunologic phenotyping of lymphoid cells together with their proliferative characteristics in body cavity effusions enhanced the diagnostic accuracy [19]. With the use of immunocytochemistry, it now became possible to conclusively diagnose lymphoma with an accuracy comparable to that of histopathology. Several studies were published over the following years which confirmed that a cytological diagnosis corroborated by an immunological characterization of the lymphoma cells had a diagnostic accuracy which sometimes not only matched that of histopathology but also surpassed it [20–25].

Subtyping of non-Hodgkin’s lymphoma, however, was problematic since the classifications used at this time included growth pattern, i.e. follicular or diffuse, which obviously could not be evaluated on smears.

The situation in the late 1990s has changed dramatically, as the new European American consensus classification (Revised European American classification (REAL) was accepted and replaced all other classifications [26]. This classification is based on clinical, cytologic, immunophenotypic and genetic features and places less emphasis on architectural features. Thus, only follicle center lymphomas are classified with regard to a follicular or diffuse growth pattern. The recognition of pattern is, however, of relative importance in grading but not in diagnosis with one exception, the centroblastic lymphoma. The diffuse subtype is recognized as a variant of diffuse large B cell lymphoma while the follicular variant belongs to the follicle centre lymphoma grade III category. The WHO classification published in 2001 is based on the REAL classification and thus likewise does not place much importance on architectural features [27]. It therefore seems logical that FNA biopsy material, which is an excellent source for cytomorphology, immunology and cytogenetics, could be used not only for diagnosis but also for subtyping of non-Hodgkin lymphoma. This has indeed been confirmed by several studies which all show a high diagnostic accuracy of FNA cytology [28–34].

**General Aspects**

The cytologic interpretation of smears from lymph node aspirates differs in several respects from that of other organs. Thus, the diagnosis of most solid neoplasms is based on the atypia shown by the tumor cells as compared to their normal counterpart. In contrast, low-grade as well as some high-grade variants of non-Hodgkin lymphomas show little or no cellular atypia and the tumor cells cannot be differentiated from their benign counterparts with certainty. Instead, the cytologic diagnosis is based on the overrepresentation of one or several cell types in the smear. Obviously, such an evaluation can only be made if the spectrum of variation of reactive lymph nodes is fully known. However, even the most experienced cytopathologist cannot reliably diagnose and separate some reactive lymphoid populations from variants of low-grade non-Hodgkin lymphoma on routine smears but also requires ancillary techniques.

In some high-grade lymphomas, smears are dominated by blastic cells which may show only mild cellular atypia. Again the lymphoma diagnosis rests on the overrepresentation of the blastic cells as compared to a reactive lesion, but immunophenotyping should always be used for a conclusive diagnosis and subtyping. Not infrequently, however, the smears from high-grade lymphomas show a highly atypical cell population which on routine smears can only be diagnosed as a high-grade malignant tumor NOS. An immunological evaluation is necessary to reveal the origin of these tumor cells.

Finally, some lymphomas are dominated by benign lymphoid cells, granulocytes or histiocytes. Examples are T cell-rich B cell lymphomas, variants of follicular lymphomas, nodular lymphocyte predominant Hodgkin lymphoma and some cases of classical Hodgkin lymphoma. A correct diagnosis of these variants rests on the identification and cytologic evaluation of only a few tumor cells. In such cases, immunocytochemistry will allow a conclusive diagnosis only if a correct antibody panel has been selected on the basis of a tentative cytological diagnosis.

Today it is clear that cytological diagnoses of lymph node disorders should always be corroborated by an immunological evaluation. This approach is mandatory to reach a sufficiently high diagnostic accuracy for FNA cytology to be accepted for the safe clinical management of patients with lymph node disorders. Aspirates from lymph nodes suspended in buffered saline offer an excellent material for immunological characterization. Routine FNA sampling yields material enough for numerous analyses. In contrast, direct smears should not be used for immunocytochemistry since such material will often have high background staining which can be detrimental to a correct immunological evaluation.

Immunophenotyping can be performed using flow cytometry or immunocytochemistry on cytospin preparations. Flow cytometry is a rapid and accurate technique for immunological characterization of lymphoid cells and is the method of choice in diagnosing most reactive lymphadenopathies as well as low-grade lymphomas. However, blastic lymphomas are
often fragile and may be difficult to evaluate using flow cytometry. Moreover Hodgkin lymphomas, T cell-rich B cell lymphomas and nonlymphoid tumors cannot be diagnosed using this technique.

Cytospin preparations allow an immunological evaluation of aspirates from both lymphoid and nonlymphoid lesions of various types. In addition, the equipment used for these techniques is available to most cytology laboratories. The preparation of cytospin material and immunological staining is, however, time consuming, which limits the number of cases that can be processed.

FNA of lymph nodes for primary and follow-up diagnosis with immunophenotyping has been performed at the Division of Clinical Cytology, Karolinska Hospital, Stockholm, since 1986 with an average of 400 lymphoma patients (250 of whom are primary cases) accessioned per year. In this work, immunophenotyping has been performed using either flow cytometry or cytospin preparations.

The excellent performance of FNA cytology in conjunction with immunocytochemistry has been described in several articles. A high rate of both detection and subclassification was demonstrated in a prospective study that included surgical biopsy following FNA of lymph nodes [30]. These results are in agreement with those of others [28, 31–34]. Thus, it is obvious that the diagnostic accuracy of FNA cytology in conjunction with immunophenotyping in trained hands is comparable to that of histopathology.

In situ hybridization and in situ amplification techniques are of importance in both diagnosis and subclassification of some lymphomas [27]. Both techniques are readily applicable to cytologic specimens.

References

27 Zajicek J, Engzell U, Franzen S: Aspiration Biopsy of lymph nodes [27]. Both techniques are readily applicable to cytologic specimens.

Historical Aspects


Techniques

Fine-Needle Aspiration Biopsy and Smear Preparation

The technique of fine-needle aspiration biopsy has been described in detail in several articles as well as previous textbooks [1–4]. The following presentation is therefore condensed and focused on technical details of particular importance in the collection of lymph node aspiration biopsy material.

Aspiration biopsy of lymph nodes or tumorous lesions should be performed with a thin needle, usually 23–27 gauge (0.6–0.4 mm) fitted to a 10-ml syringe in a one hand grip syringe holder (fig. 2.1). The use of a larger needle should be avoided since it often results in an admixture of peripheral blood which may be detrimental both to cytology and to immunological work-up. The procedure is virtually painless if 23–25 gauge needles are used, and local anesthesia is seldom required. However, in children, we use a local anesthetic cream in most cases (fig. 2.2).

A palpable target is fixed by pressing two fingers horizontally towards it which will immobilize the mass (fig. 2.3). The thumb is used to support the syringe facilitating aspiration of small tumors. Large tumors are fixed between the thumb and the index finger (fig. 2.4). In most cases, one or two needle passes with several [5–10] up and down strokes will procure enough cells for several smears as well as a cell suspension for immunophenotyping and molecular biology.

Nonpalpable lesions, e.g. in the abdomen, lung, mediastinum, orbita, sinuses and bone, are biopsied with the aid of ultrasonography, X-ray or CT. Long 0.5–0.6 mm needles with a stylet are required to reach deep-seated lesions.

Local anesthesia is used when the pleura or peritoneum is penetrated. The needling of deep-seated small targets can

Fig. 2.1. Metal pistol-grip syringe holder and the most commonly used thin needles.

Fig. 2.2. Anesthetic cream applied on the site of puncture.
be challenging since long thin needles can be deflected by normal structures. When the needle is in the correct position, the biopsy procedure is similar to that for palpable lesions (fig. 2.5).

One part of the aspirated material is expressed onto a glass slide and smeared carefully with a spreader glass slide. The smears should be even and thin, however care must be exercised not to use too much pressure in preparing thin smears because lymphoid cells are relatively fragile and may easily be crushed (fig. 2.6). The preparation of optimal smears from aspirates of blastic lymphomas, the cells of which are very fragile, is a particularly challenging task.

Several matched smears can be produced from a small drop of aspirated cells using the ‘splitting technique’. This is done by touching the drop of aspirated material with the edge of a second glass slide several times. The small droplets are then smeared onto new slides (fig. 2.7). Using this procedure, it is possible to prepare several matched slides from only one aspirate.

**Fixation and Staining**

Both air-dried and alcohol (ethanol or methanol)-fixed smears should be prepared and stained with May-Grünwald
Techniques

Giemsa (MGG) stain and Papanicolaou, respectively. An air-dried slide is stained with a ‘quick’ MGG stain for immediate assessment of cellularity and cell type. The quick stain takes approximately 1.5 min. The smear is stained in May-Grünwald for 45 s followed by 30 s staining in concentrated Giemsa (1:1 dilution with water). Alcohol-fixed smears can also be used for special stains for mycobacteria or fungi. Air-dried smears are well suited for immunocytochemical detection of nuclear proteins such as TdT, cyclin D1, Alk-1, TTF-1, CDX-2, hormone receptors and proliferation marker (MIB-1) after fixation in buffered formalin [5]. Proliferation markers are assessed on smears from all lymphomas as well as other tumors. In addition, air-dried smears can be used for techniques such as FISH although cytospin material generally offers a better source for such analyses.

Fluid Preparation

The pleural and/or peritoneal serous cavities may be involved in patients with lymphoma. Such effusions often contain clotted material which should be removed before the fluid is carefully mixed to produce a homogenous preparation. After the cells have been sedimented at 2,000 g for 10 min, the supernatant is discarded. One part of the sediment is spread on slides, air dried or wet fixed and stained by MGG and PAP, respectively. The remaining material is suspended in phosphate-buffered saline (PBS) and cellularity of the suspension is checked in a Bürker chamber. The number of cells should be adjusted to 1–2 $\times 10^6$ cells/ml and the resulting suspension used for cytospin preparation and flow cytometry.

Cytospin Preparation

After using one part of the aspirate to make smears for cytologic diagnosis and for the assessment of cell proliferation, the remaining portion is suspended in 1.5 ml of PBS for cytospin preparation or flow cytometry [6]. This is accomplished by gently aspirating the PBS from the tube through the needle into the syringe. The needle is then disconnected from the syringe and the suspension is then slowly ejected into the tube. The yield of a routine aspirate from an enlarged lymph node may vary between 1 and 10 million cells. This suspension can be analyzed by flow cytometry without adjustment of cellularity (see chap. 3). However, for cytospin preparations, the number of suspended cells should be calculated and the concentration adjusted to 1–2 $\times 10^6$ cells/ml before making the cytospin slides. Cell-rich suspensions can be diluted to optimal concentration by adding PBS solution. If the cell concentration is low, a new FNA biopsy is usually performed. If the cell yield still is too low, the cells can be concentrated by centrifugation at 700 rpm for 3–5 min. The resulting pellet is then gently resuspended in a reduced volume of PBS solution and 50–90 $\mu$l of the resulting suspension with adjusted cell concentration are used for each cytospin slide.

In most cases, 4 slides with 3 wells are prepared (fig. 2.8). Cells not used for cytospin slides are pelleted and stored frozen for further investigations such as molecular genetics.

One cytospin should be prepared on a regular slide and stained with MGG and compared with the smears to monitor recovery of all cell components (fig. 2.8).

Occasionally, the suspension contains a rich admixture of red blood cells which may interfere with the immunologic
staining. In this case, it is possible to purify the lymphoid cells by density gradient centrifugation in Ficoll-Hypaque® and then use them for cytospin preparations. However, flow cytometry is less sensitive to contaminating blood and is the technique of preference for this type of material.

**Storage**

Air-dried cytospin preparations can be stored at room temperature for at least 1 week without having a detrimental effect on the immunological staining. Preferably, the cytospins should be stored at −20°C in a plastic box. Under these conditions, lymphoid cells retain their immunological and morphological characteristics for at least several months. For a longer period of storage, −70°C is recommended. Slides which have been stored frozen must be kept in their closed box until fully thawed. Air moisture may otherwise condense on the slides and this will result in poor cell preservation.

**Immunostaining**

Both immuno-alkaline phosphatase and immuno-peroxidase methods are suitable for cytospin preparations. The high sensitivity and distinct red staining produced with alkaline phosphatase when counterstained with the blue of the hematoxylin stain has made it our procedure of choice [6].

Alkaline phosphatase immunostaining is performed in three steps. Immediately before staining the cells are fixed in acetone (−20°C) for 10 min, air dried and rinsed in Tris-buffered (pH 7.4) saline (TBS) for 5 min. The preparations are incubated with monoclonal antibody in a moist chamber at room temperature for 30 min, followed by rinsing in TBS for 10 min. Alkaline phosphatase-conjugated rabbit antimouse Ig is applied for 30 min followed by rinsing. Alkaline phosphatase conjugated swine antiserum to rabbit Ig is then applied for 30 min (fig. 2.9). After rinsing and incubation in alkaline phosphatase developing solution (Vectastain Kit I, Vector Laboratories) for 15–30 min, the slides are washed in water for 5 min and counterstained with Harris hematoxylin. Glycerol gelatin is used as mounting medium.

The selection of the monoclonal antibodies is based on the primary cytomorphologic evaluation and differential diagnoses. In most cases of reactive lymphadenitis and non-Hodgkin lymphoma, a mini-panel of markers is used: anti-kappa, anti-lambda, CD3, CD5, CD10, CD20. A kappa: lambda ratio of 6:1 or a lambda:kappa ratio of 3:1 is considered sufficient to prove monoclonality. Depending on the cytomorphology, monoclonal antibodies are selected from a panel of anti-CD4, CD7, CD8, CD15, CD19, CD23, CD30, CD43, CD45, Bcl-2, pan CK, CK7, CK20, EMA, CEA, HMB45, vimentin, desmin, CD99, NB84, HBME-1, SM actin, chromogranin A, uroplakin and polyclonal antibodies such as S100, PSA, PSAP, thyroglobulin, synaptophysin and calcitonin.

**Cell Proliferation**

The fraction of cell proliferation is routinely analyzed on an air-dried smear which has been fixed within 4 h in buffered (pH 7.4) formalin for 15 min followed by rinsing in PBS and immersion in ice-cold methanol and acetone for 4 and 1 min, respectively. After rinsing, the slides are incubated with the primary antibody (MIB-1) for 30 min and rinsed in
PBS. The next steps are incubation with the secondary antibody followed by the avidin-biotin-peroxidase (ABC) complex and diaminobenzidine (DAB). The slides are rinsed in PBS between each step.

The evaluation of proliferation by MIB-1 is based on the percentage of positive tumor cells with nuclear staining. If there is a rich admixture of non-neoplastic cells, the rate of proliferation should be corrected accordingly. Two hundred cells are counted and even faint staining is considered positive (fig 2.10). The rate of cell proliferation has been shown to vary both between and within the various subtypes of lymphoma [5, 7–9] and is therefore useful when determining potential aggressiveness.

**Molecular Techniques**

Molecular analyses supplementary to cytomorphology and immunocytochemistry may be required for diagnosis, subtyping or staging in selected cases (table 2.1). In addition, the molecular analyses will provide information about prognosis and treatment [10–16]. In most cases, fine-needle aspirations provide sufficient material for both cytomorphology, immunocytochemistry and molecular analyses such as polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH) or gene expression profiling by DNA microarray.

Table 2.1. Chromosomal changes in lymphomas

<table>
<thead>
<tr>
<th>Lymphoma</th>
<th>Cytogenetics</th>
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<tr>
<td>B cell tumors</td>
<td></td>
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<tr>
<td>Small cell lymphocytic</td>
<td>trisomy 12</td>
</tr>
<tr>
<td>Mantle cell</td>
<td>t(11;14)</td>
</tr>
<tr>
<td>Marginal zone</td>
<td>trisomy 3, t (11;18)</td>
</tr>
<tr>
<td>Follicular</td>
<td>t(14;18)</td>
</tr>
<tr>
<td>Burkitt/Burkitt like</td>
<td>t(8;14), t (2;8) or t (8;22)</td>
</tr>
<tr>
<td>T cell tumors</td>
<td></td>
</tr>
<tr>
<td>Anaplastic large cell</td>
<td>t(2;5)</td>
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It should be stressed that these techniques although relatively easy to set up, need to be meticulously standardized to provide reliable results. In addition, it is important that the results should be integrated with cytologic and immunologic findings.

The PCR technique allows amplification of nucleic acid sequences and can be used to detect monoclonal rearrangements of the IgH or T cell receptor genes [13]. The PCR technique is sensitive and can be used even if the amount of cells is limited. A single FNA sample often contains sufficient material for both cytology and for a cell suspension to be used for immunocytochemistry and PCR. Approximately half the cell suspension can be used to make a cell pellet which is either prepared immediately for PCR or stored frozen for later use.

FISH uses specific probes to detect numerical and structural chromosomal abnormalities in interphase nuclei of tumor cells. This technique is presently used to identify the t(2; 5) in anaplastic large cell lymphoma of the Ki-1 type, t(11; 14) in the mantle cell lymphoma, t(14; 18) in follicle center cell lymphoma, and t (2; 8), t(8; 14) and t(8; 22) in Burkitt lymphoma (table 1). Cytospin material offers an almost ideal cell preparation for FISH analysis.

Gene expression profiling using mRNA hybridization to gene chips will allow a simultaneous analysis of the expression of many genes in neoplastic cells. Results from such studies are likely to allow a more precise subtyping as well as predicting the response to chemotherapy and overall survival. FNA biopsy material has been used successfully to study the gene expression in non-Hodgkin lymphomas [16–18].

**References**


Fig. 2.10. MIB-1 staining of proliferating cells is shown as brown nuclear staining (immunoperoxidase, high-power view).


Flow Cytometry in Fine-Needle Aspiration Diagnosis of Lymphomas

Immunophenotyping is essential in fine-needle aspiration (FNA) diagnosis of lymphoma. It can easily be accomplished via multiparameter flow cytometry (FC), which is a rapid and sensitive method to evaluate lymphoid markers. FC appears to be especially useful in FNA specimens from lymphoid tissues and other infiltrates suspected of lymphoma, from which single-cell suspensions can be easily obtained. It is also often applied in fluid samples where cells are naturally suspended (blood, bone marrow, cerebrospinal fluid, ascites, pleural fluid) [1, 2]. Cells in suspension are stained with antibodies conjugated with fluorescent markers that emit light at different wavelengths, thus allowing detection of multiple marker expression in each cell. At the moment four-color FC is a standard method in immunophenotyping of lymphomas, but polychromatic FC where up to 17 fluorochromes can be employed is developing rapidly [3]. Evaluation of scattering of light (forward scatter – FSC, and side scatter – SSC) properties allows elimination of dead and apoptotic cells as well as granulocytes. Combination of scatter characteristics and lineage-associated markers (CD19 and/or CD7 or CD3) makes it possible to investigate subpopulations of B and T cells, and to achieve subclassifications of lymphomas.

FC requires only a small sample, gives quantitative results and can detect small abnormal cell populations in a reactive background. Moreover, if necessary, FC results can be obtained within a couple of hours from the time of biopsy. The usual turnaround time is 1 working day.

Several studies have evaluated FC immunophenotyping in FNA material showing a high detection rate of NHL (table 3.1) [4–20]. All these studies showed good agreement with the histopathological diagnoses on surgical biopsies. The proportion of samples correctly diagnosed and classified by FC has been increasing from 70–80% in the early studies and in cases of high-grade or T cell lymphomas to almost 100% in the latest publications.

Methodological Considerations

Sample Preparation

The four-color FC method applied in our department has been previously described in detail [20]. A stain and then lyse/wash technique is used. The optimal number of cells per tube is approximately $1 \times 10^6$, which may be difficult to achieve in everyday practice. At minimum, approximately $50 \times 10^3$ cells can be stained to acquire and analyze 5–10 $\times 10^3$ ‘events’.

Antibody Panel

It is evident from the literature that the sensitivity and specificity of lymphoma detection increase with the number of fluorochromes applied. Four four-color monoclonal antibodies extensively tested by us are presented in table 3.2 and illustrated in figure 3.1. Recently, we have replaced this panel with a one four-color/seven MAB combination (table 3.3; fig. 3.2). This panel is very efficient in samples with only few cells and quickly provides information on the presence of a monoclonal B cell population in the sample. When a population suspect for lymphoma is present, an additional tube(s) (usually Bcl-2 FITC/CD10PE/CD20 PerCP/CD5 APC) is analyzed to further determine the immunophenotype of monoclonal B cells.

In the Bcl-2 expression analysis, the staining for surface markers is followed by a fixation and permeabilization with Intrastain (Dako), according to the manufacturer’s recommendation [20]. We found that inclusion of Bcl-2 into the FC panel was useful, since, in most samples, malignant B cells had higher levels of Bcl-2 expression than reactive B cells. In cases of FL or high-grade NHL where malignant B cells often showed overexpression of Bcl-2, it was possible to detect a malignant population in a substantial reactive background (fig. 3.1, row 4, right plot).
Data Analysis

Most FNA aspirates from lymph nodes or other lymphatic tissue contain normal lymphocytes, which serve as a reference for scatter and staining analysis. Small reactive T cells have low FSC and SSC characteristics (fig. 3.1, row 2, left plot). Both reactive germinal center B cells and neoplastic B cells of low-grade lymphomas can be larger in size (fig. 3.1, row 1, middle plot and row 3, left plot). Especially in diffuse large B cell lymphomas, diagnostic information may be obtained by gating on large B cells separately (fig. 3.3).

The most important part of the analysis is assessing clonality of B cells (fig. 3.1, 3.2). The light chain expression should be assessed in CD19 versus SSC gate, corrected for adequate FSC (fig. 3.1, row 3, left and middle plot, fig. 3.2, upper right and lower left plot). In our study, the median kappa/lambda ratio in reactive lymphatic tissue was 1.6 (range 0.4–4.7). In practice, a ratio under 0.6 and above 6.0 is considered suspicious. Additional analyses can be performed looking for clonal B cells in CD10+/CD19+ or CD5+/CD19+ cells or CD19/large cell FSC/SSC gate or for Bcl-2 overexpressing B cells. However, in rare lymph nodes with reactive follicular hyperplasia, light chain restriction may occur within a CD10+ B cell population without overexpression of bcl-2 or t(14,18) [21]. Another aberrant finding commonly encountered in lymphomas is the presence of...
Fig. 3.1. FC diagnostics of lymphomas using a panel of four 4-color MAB combinations listed in table 3.2. Example of analysis of FNA from a lymph node involved by a follicular lymphoma. Row 1 shows analysis of the first tube where a large population of CD19/CD20-positive cells (left plot, blue dots) was found. These cells are larger than T lymphocytes (middle plot) and positive for CD10 and CD23 (right plot). Row 2 shows analysis of tube 3 where T cells present in the sample are smaller than B cells (left plot, red dots) and show normal expression of CD3 and CD7 (middle plot, blue dots), and normal CD4/CD8 ratio (right plot: CD4+ red dots, CD8+ green dots). Row 3 shows analysis of tube 2. After CD19/SSC gating (left plot) there is a dominance of kappa+ B cells (red dots), but a population of lambda+ B cells is also present (green dots). Note that kappa+ have different scatter compared to lambda positive ones (left plot). Only a minimal population of CD5+ B cells is present (mantle zone cells, right plot, cyan dots). Row 4 shows Bcl-2 analysis (tube 4) where CD10+ cells (green dots) have stronger Bcl-2 expression when compared to CD10− B cells (blue dots) and CD3+ T cells (red dots).
a B cell population lacking surface light chain expression. This can be seen in B precursor leukemia/lymphoma, CLL, DLCB or plasma cell proliferations. In the latter category of cases, additional analysis of cytoplasmic light chain expression may show a clonal B cell population [22].

Evaluation of Bcl-2 expression is helpful in lymphoma cases with partial involvement and presence of reactive germinal centers, which makes evaluation of light chain restriction difficult [20, 23, 24]. In the applied MAB combination (table 3.2) Bcl-2 expression is evaluated simultaneously in B and T cells and, if present, in CD10+ B cells. In this approach, nonmalignant T cells present in the sample serve as internal control for the comparison of the levels of Bcl-2 expression (fig. 3.1, row 4). The presence of CD10+ B cells with high Bcl-2 expression is highly predictive for follicular lymphoma. In contrast, CD10+ B cells in reactive lymphatic tissue show a lower level of Bcl-2 expression than T cells and CD10-negative B cells (fig. 3.4). Increased Bcl-2 expression is also found in most CD10-negative low-grade B cell lymphomas [20]. In DLCB lymphomas, Bcl-2 expression is not as informative since malignant B cells may be Bcl-2-negative [25].

The immunophenotypic criteria for diagnosis of various B cell lymphoma subtypes are summarized in table 3.4 and discussed in detail in the respective chapters. Our FC panel is very useful in detecting low-grade B cell lymphomas (96% of cases diagnosed and classified accurately) [20].

**Table 3.3.** One-tube four-color panel of seven monoclonal antibodies for FNA lymphoma diagnostics*

<table>
<thead>
<tr>
<th>Tube</th>
<th>FITC</th>
<th>PE</th>
<th>PE Cy5/PerCP</th>
<th>APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lambda and CD8</td>
<td>kappa and CD56</td>
<td>CD19 and CD4</td>
<td>CD3</td>
</tr>
</tbody>
</table>

*According to Costa et al. [31].
Flow Cytometry in Fine-Needle Aspiration Diagnosis of Lymphomas

**Fig. 3.3.** FC immunophenotyping of fine-needle aspirate from a lymph node with diffuse large B cell lymphoma. Four-color FC was used. Upper left plot shows forward scatter/side scatter image of the sample with kappa + B cells (red dots) being considerably larger in size than CD3+ T lymphocytes (blue dots). Upper right plot shows a dominance of large kappa + B cells within the B cell population (gating on CD19 = side scatter; red dots = kappa + large cells; violet dots = kappa + small cells; green dots = lambda + cells). Lower left plot shows that CD20+ B cells (green dots) were less numerous than T cells (blue dots) (30 and 50%, respectively) and that B cells were negative for CD5. Lower right plot shows that most B cells (green dots) were negative for CD10 and that Bcl-2 expression was similar in B and T cells (blue dots).

**Fig. 3.4.** FC immunophenotyping of fine-needle aspirate from a lymph node with a reactive follicular hyperplasia. Four-color FC was used. Upper left plot shows forward scatter/side scatter image of the sample with CD20+ B cells (red dots) being similar in size to CD5+ T lymphocytes (blue dots). Upper middle plot shows that B cells were polyclonal with a presence of kappa + B cells and lambda + B cells (gating on CD19 = side scatter; red dots = kappa + cells; green dots = lambda + cells). Upper right plot shows a presence of a small CD5+ population of B cells corresponding to normal mantle-zone cells (cyan dots). Lower left plot shows that most CD20+ B cells (red dots) had similar Bcl-2 expression to CD5+ T cells (green dots), but there is a subpopulation of CD20+ cells with higher CD20 expression and lower Bcl-2 (violet dots). These cells correspond to CD10+ germinal center B cells with low Bcl-2 expression as illustrated on the right lower plot.
Flow Cytometry in Fine-Needle Aspiration Diagnosis of Lymphomas

CD10 negative follicular lymphomas are the only problematic category. Grade III and interfollicular infiltrating cells in other FC may lack CD10 and in these cases the lymphoma subtype may be misdiagnosed by FC [26]. Approximately 10% of FLs are reported to be CD10−.

T cell lymphomas are usually more difficult to analyze by FC than B cell lymphomas due to their very variable patterns of antigen expression. The most common aberrant findings are summarized in table 3.5. Most often, T cell lymphomas show imbalance in CD4/CD8 ratio and/or aberrant expression.
of one or more ‘pan-T cell’ markers as CD2, CD3, CD5 or CD7 [27]. Aberrant expression of CD10 is found in T cells in angioimmunoblastic T cell NHL [28]. It has to be noted that increased CD4/CD8 ratios, sometimes with an activated pattern (CD25+), and very low frequency of polyclonal B cells can be found in FNA samples from Hodgkin lymphomas which could lead to a misdiagnosis of T cell lymphoma [2].

Recently, a direct analysis of T cell clonality by FC analysis of T cell receptor V-beta chain expression has been made possible [29]. However, even with this approach, some obvious T cell lymphoproliferations were negative for clonality and in some reactive cases dominant T cell populations with polyclonal background were found.

Advantages and Disadvantages of FC

Immunophenotyping by FC has several advantages. FC is rapid, sensitive, gives quantitative results and allows antigens to be assessed simultaneously. Therefore, various subpopulations of lymphocytes can be analyzed separately with high sensitivity. Small abnormal cell populations can be detected in a reactive background. FC allows detection of antigen expression on the cell surface, which is of importance when planning antibody-based therapy such as Rituximab, Campath or Daclizumab, as the antigens (CD20, CD52, CD4 respectively) have to be expressed on the cell surface for the therapy to be effective.

However, it may be difficult to assess which cells in cytologic preparations correspond to different populations detected by FC. Staining for intracellular markers (intracytoplasmic and nuclear) may produce high levels of background and analysis may need a high level of expertise. Inadequate sampling, fibrosis, and necrosis may result in nonrepresentative samples.

The main disadvantage of FC is its unawareness of cytomorphology. The size of cells can only be assessed approximately. Also, if neoplastic cells are fragile as in many high-grade NHL and in Hodgkin lymphoma, they may be destroyed during FC analysis. Grading of follicular lymphoma and detection of transformation to DLCB is possible only by morphology. For that reason close cooperation between cytopathologists and FC laboratory is required.

Comparison between FC and Immunocytochemistry on Cytospins

Three large studies have compared the results of immunocytochemistry (IC) on cytospins and FC [4, 20, 30]. All point out the excellent correlation of obtained results (85–97%). The main advantage of IC over FC is that it requires lower numbers of cells and that staining pattern, intensity of staining and background can be assessed by morphology. Fragile cells that disappear during FC preparation can usually be assessed in cytospins. However, preparation artifacts, necrosis, increased blood contamination and background staining can render an accurate evaluation of cytospin preparation difficult. Also, immunocytochemistry is relatively time consuming (approx. 3 times longer technician time is required). Moreover, routinely it is not possible to evaluate multiple antigen expression and scoring is semiquantitative.

How to Get the Best Results in FC Diagnostics of FNA

Based on our experience, we recommend quick staining of one smear from the FNA sample for immediate evaluation. If small- to medium-sized cells predominate, indicating low-grade lymphoma or a reactive process, FC should be the method of choice for immunophenotyping. When large cells predominate, IC is preferable since FC has a high false-negative rate. Hodgkin lymphoma, anaplastic large-cell lymphoma and some high-grade NHL-like T cell-rich B cell lymphomas cannot be reliably detected by FC. Close cooperation and communication between the cytopathologist and FC laboratory is a prerequisite for a high diagnostic accuracy. It is also of importance that adequate material is saved (frozen cells or cytospins) for FISH or molecular genetics studies.

References


Flow Cytometry in Fine-Needle Aspiration Diagnosis of Lymphomas


Flow Cytometry in Fine-Needle Aspiration Diagnosis of Lymphomas
B Cell Neoplasms

WHO Histological Classification of B Cell Neoplasms

**Mature B Cell Neoplasms**
- Chronic lymphocytic leukemia/small lymphocytic lymphoma
- B cell prolymphocytic leukemia
- Lymphoplasmacytic lymphoma
- Splenic marginal zone lymphoma
- Hairy cell leukemia
- Plasma cell myeloma
  - Monoclonal gammopathy of undetermined significance
- Solitary plasmacytoma of bone
- Extraosseous plasmacytoma
- Primary amyloidosis
- Heavy chain diseases
- Extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
- Nodal marginal zone B cell lymphoma
- Follicular lymphoma
- Mantle cell lymphoma
- Diffuse large B cell lymphoma
- Mediastinal (thymic) large B cell lymphoma
- Intravascular large B cell lymphoma
- Primary effusion lymphoma (see chap. 9 ‘Extranodal lymphomas’)
- Burkitt lymphoma\(^1\)/leukemia\(^2\)
- Lymphomatoid granulomatosis

**Precursor B Cell Neoplasm**
- Precursor B lymphoblastic leukemia\(^1\)/lymphoma\(^2\)

(\* Indicates subtypes described)

Small Lymphocytic Lymphoma/Chronic Lymphocytic Leukemia

**Clinical Features**
Mostly middle aged to elderly patients. The patients may be asymptomatic but anemia, spleno-hepatomegaly and nodal enlargement are frequently observed. Bone marrow involvement is found in a majority of cases with chronic lymphocytic leukemia (CLL) but not seen in the early phase of small lymphocytic lymphoma (SLL). Rare CLL patients have only nodal involvement at diagnosis.

The clinical course is indolent and the median survival is 7 years. Transformation to high-grade B cell lymphoma (Richers lymphoma) is relatively rare. Approximately 7% of non-Hodgkin lymphomas (NHL) are of the SLL/CLL type [1].

**Cytology** (fig. 4.1a, b)
The smears are dominated by small lymphocytes (6–12 µm) with round nuclei which have clumped chromatin (cellules grumelées). The cytoplasm is sparse except in the plasmacytoid variant. In most cases, larger cells such as prolymphocytes with a large pale cytoplasm and para-immunoblasts which are of intermediate size with a grey-blue cytoplasm and a large nucleus can be found [2–6]. Incipient transformation is indicated by an increased number of immature cells.

**Differential diagnoses:** Indolent lymphadenitis, lymphoplasmocytoid lymphoma, CLL of T cell type, follicular lymphoma (low grade), mantle cell lymphoma.

**Immunocytochemistry** (fig 4.1c, d): The cells are CD19, CD20, CD79a, CD5, CD23 positive. Surface Ig expression is usually weak. A low (<10%) MIB-1 positivity is typical (table 4.1). Higher MIB-1 values indicate an aggressive variant.

**Genetics:** Trisomy 12, (20%), deletion 13 q (50%), deletion 11 q (20%) (table 4.1).