Assessment of the Accuracy of Flow Cytometry

In Immunophenotyping of Lymphoma

Asad Abdella Ahmed Abdella

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Abstract

The present study was conducted to evaluate the role FCM in immunophenotyping of Lymphoma compared with histopathology/Immunohistochemistry phenotyping. The study included 57 freshly excised lymph node specimens obtained from patients, complaining of localized or generalized lymphadenopathy during the period 2013–2015. The mean age of patients was 47.7 (range 5–72 years). The diagnostic distribution of the 50 specimens according to FCI was as follows: 9 cases (18%) were diagnosed as RH, 32 cases (64%) were B-cell NHL, 3 cases (6%) were T-cell NHL, 2 cases (4%) were HL and 4 cases (8%) were diagnosed as non-lymphomatous (NLT) or metastatic tumors (MT). The study concluded that the findings of FCI are consistent with the final tissue diagnosis in the majority of our studied cases (88%). Optimization of flow cytometric procedures and all aspects of the testing is highly recommended.
تقييم دور التدقيق الخلوي في تحديد النمط الظاهري لأورام الغدد الليمفاوية

اسعد عبد الله أحمد عبد الله

ملخص البحث

أجريت هذه الدراسة لتحديد النمط الظاهري المناعي لأورام الغدد الليمفاوية عن طريق تحليل التدفق الخلوي. لتقييم دور تحليل التدفق الخلوي مقارنة بتحليل الأنسجة المريضة والكيمياء المناعية الخلوية في تحديد النمط الظاهري المناعي، شملت الدراسة 57 عينة عدة لتفاوتية أخذت مباشرة من المرضى الذين راجعوا عيادات أمراض الدم في الفترة بين 2013 و2015 وهم يعانون من تضخم عام أو محلي للغدد الليمفاوية. كان متوسط أعمار المرضى 47.7 (من 5 إلى 72 عام). تم تحليل العينات في مختبرات الأنسجة المريضة والكيمياء المناعية الخلوية وتمت الاستفادة من البيانات لأغراض الدراسة. كان التوزيع التشخيصي للمت 50 عينة من أصل 57 عينة حسب تحليل التدفق الخلوي كالآتي: 9 عينات (18%) فرت تنسج تفاعلي، 32 عينة (64%) لمفومة علاً لخلايا ببلاودجكينية، 3 عينات (6%) لمفومة نوع الخلايا غيلودجكينية، 2 عينات (4%) لمفومة غيلودجكينية، 4 عينات (8%) أورام غير الليمفاوية أو أورام انتشارية. أسهم تحليل التدفق الخلوي بصورة فعالة جداً وتطابق مع التشخيص النهائي لأورام الغدد الليمفاوية بواسطة تقنيات الأنسجة المريضة والكيمياء المناعية الخلوية والتي تعتبر المعيار الذهبي في معظم الحالات التي تمتد رأستها بنسبة (88%). أوصت الدراسة بالضبط الأمثل لتقنيات تحليل التدفق الخلوي بما يتعلق بها من التحضيرات والتحليل التي تؤثر على جودة البيانات.
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CHAPTER ONE

1. Introduction

1-1 Background:
Lymphoma represents one of the major health problems all over the world. Abeer M El-Sayed (2008) has described lymphoma as the a common malignancy affecting both children and adults and is continuing to increase rapidly. In the Middle East, non-Hodgkin's lymphoma (NHL) has a high incidence contributing to 7% of total cancer as compared to 4% in USA.
Flow cytometric immunophenotyping has been recommended by International experts met in Bethesda, Maryland in 2006 for the clinical conditions that presented with lymphocytosis, plasmacytosis, organomegaly and tissue masses.
A brief review of the basics of FCM will help in the understanding of the applications of this technique. A flow cytometer is an instrument that evaluates the physical and/or chemical characteristics of single cells and other particles (in the same size range) as the individual cell passes through a measuring device and sensing point at the interrogation point to fluid stream where they are illuminated by an incident light. Various components of the cells, such as surface receptors, intracellular molecules, and DNA, to bind with fluorescent dyes allows their detection and evaluation. Fluorescence is produced when an incident light of one wavelength excites electrons of certain chemicals, fluorochromes, from their ground state to higher energy levels with subsequent return of electrons to lower energy levels by emitting light of a longer wavelength. Flow cytometers detect the characteristics of cells/particles by measuring the amount of incident light reflected by these particles and by detecting the fluorescence produced by fluorochromes conjugated either directly with cell components or conjugated to antibodies directed against various cell components.
The Immunophenotyping of samples from patients with lymphoproliferative disorders has added much to proper diagnosis and classification and better understanding of the pathogenetic mechanisms underlying the development of these disorders. In the context of lymphoma diagnosis, immunohistochemistry (IHC) has the advantage that the cells of interest are identified morphologically and it is applicable retrospectively to fixed tissues, though fixation might lead to loss of some cells and/or cellular antigenicity. However, only a single marker is routinely used on tissue sections, which permits examination of about 100 cells only. Moreover, there is a reported difficulty in demonstrating immunoglobulin light chains.
On the contrary, flow cytometry (FCM) allows a more precise definition of individual cell types since the cells of interest are identified by a combination of physical characteristics and the use of multiple antibodies directly conjugated with different fluorochromes. It also has the ability to assess monoclonality through detection of immunoglobulin light chain expression and the results can be available within few hours after receiving the specimen.

In addition, flow cytometric immunophenotyping (FCI) has become a widely used laboratory procedure for diagnosis and sub-typing of lymphoma. It is an objective and quantitative diagnostic tool that allows quick multiparametric analysis of a very large number of cells (20,000–50,000 cells per sample) which could be obtained from small tissue sample (0.1 cm³ or even smaller). Meanwhile, analysis of such small samples is facilitated by applying dual & triple markers that permit in a single experiment, the detection of expression of combination of 2 or 3 antigens respectively on the same cell.

1.2 Justification:
Several studies have supported the usefulness of FCI in diagnosing lymphoma in fine needle aspiration (FNA) samples as well as in staging and follow up of cases. However, only few reports are available regarding the role of FCM in tissue diagnosis and typing of lymphoma.

1.3 Objectives:
1.3.1 General Objective:
To assess the diagnostic role of FCM for immunophenotyping of lymphoma on freshly excised tissue biopsies.

1.3.2 Specific Objectives:
- To identify maturity or immaturity and linages of cells in suspension.
- To detect abnormal cells that express antigens that differ significantly from normal.
- To identify the phenotype of abnormal cells from their normal counterparts.
CHAPTER TWO

2. Literature Review

2.1 Literature Review:
Morse et al. 1994, reported that 9 out of 16 cases (56%) were diagnosed by FCI alone as lymphoma or carcinoma and 4 (25%) were consistent with a final diagnosis of normal or reactive hyperplasia whereas, 3 cases only had histologic evidence of malignancy on biopsies that escaped detection by FCM[3]. Moreover, Dunphy, reported that FCI data contributed significantly to, or was consistent with the final tissue diagnosis in 94% of a large series including 373 cases. Furthermore, Martinez et al. 2003, reported that FCI diagnosed 218 cases of NHL out of 250 cases with negative predictive value 0.52 and positive predictive value 1. Mayall et al. 2000, reported that FCI in combination with touch imprint cytomorphology was useful in excluding diagnosis of lymphoma and non-lymphomatous tumors & showed 100% concordance with histopathologic results.

Regarding Ig light chain detection by FCM, Leers et al. 2000, estimated the clonality of lymphoproliferative disorders by FCI & IHC. They pointed out a major drawback of immunohistochemical detection of monoclonality in B-cell lymphoproliferative disorders which was the lack of contrast between surface-immunoglobulin staining and extracellular immunoglobulin staining. Monoclonality was established in 9 out of 10 NHL cases by FCI while only 6 of 9 cases were conclusive by IHC.

Fadol et al in one of the recent studies done in 2014-2015, in their research work ‘The Role of CD5 and CD23 in Classification of B. Cell Non Hodgkin Lymphoma in patient of Khartoum State Using Flow Cytometer’, concluded that using of co-expression of D5/CD23 had remarkable efficiency in the differentiation between sub types of Non-Hodgkin lymphoma in general as well as in identification of SLL, and using of this marker in scoring system is proved to be useful in lymphoma sub-typing.

Mikhail Roshal, Brent L. Wood, and Jonathan R. Fromm, 2010-2011, in their article ‘Flow Cytometric Detection of the Classical Hodgkin Lymphoma: Clinical and Research Applications’; state that the diagnosis of CHL currently relies on a combination of morphologic findings and immunohistochemical stains. With the exception of rare cases with dramatically increased malignant populations, isolation of pure viable tumor cells has not been historically possible. Recently, a reliable flow cytometric assay for direct detection and isolation of the malignant cells in this disease has been developed. This assay has proven
useful diagnostically and has been clinically validated to have a very high sensitivity and nearly absolute specificity for the diagnosis of CHL in routine clinical samples.

Kaleem 2006 in his article review ‘Flow Cytometric Analysis of Lymphomas- Current Status and Usefulness’ elaborately discussed the status and usefulness of flow cytometry to diagnosis and classification of lymphomas, especially when he described the role of FCM as can both complementary and competitive to IHC. The decision to use either FCM or IHC for the same role (competitive use) is primarily governed by experience with the technique, personal preference, and laboratory resources and expertise in the techniques. In his review he pointed out the distinctive uses and limitations of each technique, however. The FCM has the advantage that it offers the multiparameter evaluation of single cells and the ability to work with very small samples, including fluid samples, whereas the major edge of IHC when compared to FCM, is its ability to work on fixed, paraffin-embedded tissues and provision of morphologic analysis at the same time. The recent availability of antibodies against the variable region of the TCR beta (V beta) chain allows determination of T-cell clonality by FCM; such an evaluation is not possible currently for diagnostic purposes using IHC. Most commonly used antibodies are available for both FCM and IHC, but a few antibodies work better for flowcytometric analysis than paraffin-based use, such as antibodies to immunoglobulin Kappa and Lambda light chains, whereas antibody to cyclin D1 for mantle cell lymphoma works well with IHC and not with FCM.

Competitive use where FCM analysis works better than IHC:

1. Determination of B-cell clonality by analysis of immunoglobulin Kappa and Lambda light chains.
2. Evaluation of coexpression profiling of antigens on the same cells, such as coexpression of CD5 and CD23, aberrant loss of antigens, and simultaneous evaluations of cytoplasmic and surface antigens.

Competitive use where IHC analysis works better than FCM:

1. Evaluation of nuclear antigens such as cyclin D1 and TdT

Clinical conditions and specimen types where FCM in comparison with IHC, in addition to morphology, plays either an exclusive, preferable, complementary, or limited role in the diagnosis of lymphoma.

1. Evaluations where only FCM and not IHC plays the diagnostic role a. Blood specimens for lymphoma/leukemia involvement.
2. Evaluations where FCM is often diagnostic and more informative than IHC

a) Needle core biopsies of lymph nodes, especially with scant tissue.
b) Lymph node FNAB.
c) Diagnosis of central nervous system lymphoma in the spinal fluid in patients with acquired immune deficiency syndrome.

d) Pleural, peritoneal, and pericardial lymphocytic effusions.

e) Orbital lymphoid proliferations and intraocular fluid analysis.

f) Suspected T-lymphoblastic lymphoma of the thymus, core biopsies.

g) Minimal nodal involvement by Sézary cells in patients with known mycosis fungoides

h) Splenic and nodal involvement by hepatosplenic/T-cell lymphoma

3. Evaluations where FCM and IHC are almost equally informative and diagnostic

a) Excisional lymph node biopsies.

b) Splenectomy specimens.

c) Resection of extranodal masses for suspected lymphoma.

d) Bone marrow for NHL staging.

4. Evaluations where IHC is often diagnostic and more informative than FCM

a) Skin biopsies for suspected B-cell, T-cell, or NK-cell lymphoma.

b) Small biopsies of stomach, intestine, lung, liver, kidney, and brain.

The current World Health Organization Lymphoma Classification Scheme and the Immunophenotyping is one of the integral components in it, and FCM has proven value in the diagnosis and proper classification of most categories.

The multiple roles that FCM plays in lymphoma diagnosis, classification, and prognostication serve the current management plans and treatment protocols based on our current understanding of the biology of the disease. With advances in our understanding of the molecular basis of the disease, however, we are delving more and more into tying and correlating various components of the normal and deranged molecular machinery and specific genetic findings with specific disease categories and predicting clinical outcomes. Examples include determining the mutation status of the IgV(H) genes in B-cell CLL to predict the clinical course of the disease. The biggest move, however, is toward gene expression profiling of various lymphomas and leukemia to understand the molecular derangements and biology of the disease.101 The dissection of the deranged molecular machinery will undoubtedly facilitate better understanding, diagnosis, and ultimately treatment of these malignancies.

From literature review, it is evident that the rapid advances in the flow cytometry instrumentation, more development of markers, and its analytical capabilities made the FCM more preferable to IHC in most situations for lymphoma analysis; FCM can identify conditions that could potentially be missed if IHC were to be used instead, such as partial involvement of the node by marginal zone B-cell lymphoma and composite B-cell and T-cell
lymphomas. Furthermore, analysis by FCM provides a better and more informative immunophenotypic evaluation for certain diagnosis such as hepatosplenic/T-cell lymphoma and hairy cell leukemia. Distinction of a lymphocyte-rich thymoma from T-cell lymphoblastic lymphoma can be very challenging on morphology with or without IHC is another example where FCM has successfully identified the correct diagnosis in most cases. The future of lymphoma FCM in the coming years, not only limited to its ability to detect surface receptors, but also intracellular and intranuclear molecules such as enzymes, nucleic acid, and protein products of specific genes which likely to lead to reinvention of the diagnostic and prognostic applications of this technique for several possible future uses in lymphoma management. Such applications might range from detection of novel specific surface, intracellular, or intranuclear protein products relevant in diagnosis, classification, or prognostication to evaluating enzyme activities for cell kinetics and tumor cell metabolism and response to drugs. Thus, with the identification of each new molecule critical in lymphoma management, new prospective for FCM is shaped.
CHAPTER THREE

3. Materials and Methods

3.1 Study Site:
The study was carried out for patients with generalized or localized lymphadenopathy presented to our clinical hematology clinics during the years 2013 and 2015 and on lymph nodes sent to the laboratory to be processed by Histopathology and immunohistochemistry and flow cytometry workup. The study involved both male and female patients with age ranging from 5-72 years with mean age of 47.7.

3.2 Samples Collection and Storage:
The study included 57 freshly excised lymph node specimens obtained from 57 patients (During 2013-2015). Specimens were immediately suspended in sterile chilled RPMI or similar Tissue Transport Medium, TTM. Each specimen was bisected and touch imprint was prepared, fixed and stained immediately by modified PAP, Giemsa, or Kwik™Diff to assess the presence of malignant lymphoma cells and facilitate diagnosis, one half was fixed in 10% neutral buffered formalin, and processed routinely for histologic diagnosis and immunohistochemistry for lymphoma sub-typing.

A single cell suspension is prepared from the other half of lymph node by mechanical disaggregation for FCI before staining and analysis. An aliquot of the single cellsuspension is incubated with different mixtures of directly conjugated monoclonal antibodies. Red cells, if any, are lysed with the VersaLyse “Fix-andlyse” mixture and the remaining white cells are washed in PBS with 0.1% formaldehyde and analyzed by flow cytometry.

Samples to be kept at 4°C overnight and must not be frozen if testing is not started immediately. The samples must be analyzed within 24 hours of collection.
3.3 Reagents:
  - Directly conjugated monoclonal antibodies From ENOVA BIOSCIENCE DIAGNOSTICS; CD45, CD45RO, CD 20, CD19, CD3, CD10, CD5, D23, CD30, CD15, bcl-2, anti-κ, anti-λ, CD4 and CD8.
  - Versalyse Lysing Solution.
  - IO Test3 Fixative Solution.
  - PBS (Phosphate buffered saline).
  - PBS with 0.1% formaldehyde Heat inactivated Fetal Calf Serum (HI-FCS).
  - Sampling tubes and materials necessary for sampling.
  - Scalpel blade.
  - Petri dish.
  - Automatic pipettes with disposable tips.
  - Centrifuge.
  - Automatic Agitator(Vortex type).
  - CBC analyser

3.4 Equipment:
Flow cytometry Analysers- Beckman Coulter CYTOMICS FC 500 and Beckman Coulter NAVIOUS with tetra CXP systems software.

3.5 Quality Control:
Flowcheck /FlowcheckPro for instrument verification of alignment and fluidics
  - Flowset / FlowsetPro for monitoring instrument performance.
  - The CD4/CD8/CD3 tube is used as an internal QC check for compensation.
  - Appropriate isotype-matched negative controls are used in the panel of monoclonal antibodies to assess background fluorescence intensity.
  - Internal consistency of each run is examined and tube-to-tube variability assessed.
  - Unusual findings are re-stained and rerun with alternate antibody clones and fluorochromes.
3.6 Procedure (33):

3.6.1 Precautions
- Do not use the reagents beyond the expiry date.
- Do not freeze.
- Minimize exposure to light.
- Avoid microbial contamination of the reagents or false results may occur.

3.6.2 Reagent Preparation:
- Prepare Buffer A (by adding 6mL of HI-FCS to 294 mL of PBS). The buffer can be stored for several weeks at 2-8°C.
- Prepare Buffer C (By adding 12.5 μL of IO Test 3 fixative to 1mL of PBS). A volume of 1mL is used per test tube. The buffer has to be prepared fresh.
- Prepare the VersaLyse “Fix-and-lyse” mixture by adding 25μL of IO Test 3 fixative to 1mL of VersaLyse. A volume of 1mL is used per test tube. The solution has to be prepared fresh.

3.6.3 Sample Preparation:
- Obtain a single cell suspension of the received node by manual disaggregation with 1.5ml of phosphatebufferedsaline (PBS).
- Centrifuge the suspension at 150xg for 5 minutes at RT. Discard supernatant, vortex and add 4mL of Buffer A.
- Centrifuge again at 150xg for 5 minutes at RT.
- Discard supernatant and re-suspend the pellet with 1mL of Buffer C and assess the cellularity on a CBCanalyzer.
- Dilute the suspension with PBS if required, such that the number of cells is approximately 5x10^9 leucocytes/ L with optimum acceptable percent of viable cells of about 80%
- Label test tube according to patient’s ID and combination of antibodies taken.
- For each sample analyzed, an additional control tube is required in which the cells are mixed in the presence of the negative control corresponding to the specific stain selected.
- Add 10μL of monoclonal antibodies (specific for a membranous antigenic determinant) to the corresponding test tube, including the control tube. Add 5 μL of corresponding PC5 and PC7 antibodies.
- Add 50 μL of corresponding sample to the test tubes.
- Vortex and incubate for 20 minutes at RT, protected from light.
- Add 1 mL of VersaLyse "Fix-and-lyse" mixture to each test tube.
- Vortex immediately each tube for 1 second and incubate for 5 mins at RT.
- Centrifuge each tube at 150xg for 5 minutes at RT.
- Discard supernatant, vortex and add 4mL of Buffer A.
- Centrifuge each tube at 150xg for 5 minutes at RT.
- Discard supernatant and re-suspend the pellet with 1mL of Buffer C.
- Analyze cells and Acquire 50,000 cells.
- Immunophenotypic analysis is done using a CD45 gating strategy, multi-color staining, and an extensive panel of monoclonal antibodies.
- PDF’s are sent to the hematopathologist for review and interpretation. Results are compared with patient history or clinical information and correlated with morphologic/IHC evaluation in Histopathology and/or haematology.

3.6.4 Antibody panels design:

- First run Isotype control IgG1-FITC/IgG2a-PE was used at the start of each run.
- Followed by Screening Triple Marker; CD3-FITC/CD19-PE/CD45-PerCP.
- Further analysis with the appropriate antibodies was performed on the remaining of the sample based on the initial results of both touch imprints and the screening triple marker. The markers used in this study (All from BD Bioscience, USA) were: 1) The dual markers (CD5-FITC/CD19-PE; Anti kappa-FITC/CD19-PE; Anti lambda-FITC/CD19-PE; CD4-FITC/CD8-PE), and 2) The single markers [CD20-FITC; CD10-FITC; CD45RO-PE; CD23-PE; CD30-FITC; CD15-FITC; Anti-BCL2-FITC].

3.6.5 Cells Acquisition:

- Acquisition and analysis of stained suspension was performed by FC500 flow cytometry systems with tetra CXP systems software; tetra CXP in conjunction with quality control reagents, provide automated standardization of light scatter and fluorescence intensities and automated adjustment of color compensation setting.
- All tetra CXP protocols and panels are fully created and reside in the common\tetraCXP protocol and panels folders. All tetraCXP and User-Modified Protocols; they are used in the Lymphocyte Subset Application (LSA). AutoSetup protocols used in the AutoSetup II applications reside in the common protocol folder.
- Acquisition and analysis of stained suspension was performed by FACScan flow cytometer (Becton Dickinson, USA) acquiring at least 50,000 cells at a high rate of 400–500 cells/second for each marker. Negative isotype control was run first to identify the position of the negative and the positive populations. At least two plots.
were drawn during the acquisition of each tube; one of them displayed forward scatter (FSc) on X axis versus side scatter (SSc) on Y axis to identify the size and granularity of cells and to exclude debris and dead cells. The second plot displayed the antibody marker on X axis versus FSc or the other marker in case of dual markers on Y axis.

- Data was stored in the list-mode where the raw data for each parameter on every analyzed cell were sequentially stored in a list to allow reanalysis at any time including redrawing gates, different population gating and new histogram drawing.

- Analysis of sample tubes was performed as follows: setting cursors for differentiating positive and negative populations (Isotype control plot analysis) so that \( \geq 98\% \) of the cells are negative. The tube containing CD45 (gating reagent) was analyzed first to set a gate around lymphocyte clusters using FSc and SSc patterns and fluorescence staining based on low FSc and SSc patterns and bright stain of lymphocytes with CD45.

- Light-scattering patterns were examined on each sample tube and the remaining sample tubes were analyzed with the cursors previously set based on the isotype control. The data was reported as a percentage of the total lymphocytes and/or percentage of gated population. Absolute numbers of positive and negative populations were also reported

3.6.6 Method limitations:

- Flow cytometry may produce false results if the cytometer has not been aligned perfectly, if fluorescence leaks have not been correctly compensated for and if the regions have not been carefully positioned.

- Accurate and reproducible results will be obtained as long as the procedures used are in accordance with the technical insert leaflet and compatible with good laboratory practices.

- Verify the preparations using the naked eye to assess the efficacy of lysis. If they are cloudy or if the light diffraction histograms are unusual, lysis may be incomplete.

- The erythroblasts may be incompletely lysed and appear on a light diffraction histogram in the same location as the leucocytes.

- Acetazolamide, an inhibitor of carbonic anhydrase can completely inhibit the action of VersaLyse.

- In certain disease states, such as severe renal failure or haemoglobinopathies, lysis of red cells may be slow, incomplete or even impossible. In this case, it is recommended to incubate with Versalyse for a longer period of time.
3.6.7 Interpretation of Data:

- The Size of cells was defined depending on FSc as: small (FSc 200–400), medium (FSc 400–600) or large (FSc> 600) [21]. The fluorescence intensity was determined by using a logscale of dot plot as follows: the population which lies in region 10⁰-10¹ was considered negative, region 10¹-10² was considered weak, region 10²-10³ was considered moderate and region > 10³ was considered strong.

- A negative (isotype) reagent control was used with each specimen to determine non-specific binding of the mouse monoclonal antibody to the cells and to allow setting of markers for distinguishing fluorescence-negative and fluorescence-positive cell populations. The marker set on the negative control plot was copied on each analyzed plot of dual markers plots, so dividing it into 4 quadrants. Analysis of the population in each quadrant was as follows: the lower left quadrant; double negative to both markers, the lower right quadrant; positive for X axis marker only, the upper left quadrant; positive for Y axis marker only, and the upper right quadrant; positive to both markers.

- Detection of B-cell monoclonality was determined by overlay of histograms of both anti-kappa and anti-lambda and measuring the difference between them using the Cell Quest software of the machine. Light chain restriction (LCR) was considered when κ/λ was < 0.5 or κ/λ was > 2.5 or if there is absence of both κ & λ expression by tumor cells.

3.6.8 Statistical Methods:

Sensitivity and specificity of FCI as compared to histopathologic findings were calculated. Sensitivity is defined as the probability of a positive test among patients with disease. Specificity is the probability of a negative test among patients without disease. The positive predictive value PPV and the negative predictive value NPV were also measured as in Table 3.1

\[
\text{Sensitivity} = \frac{\text{No. of true positive cases}}{\text{No. of true positive cases} + \text{false negative cases}} \times 100
\]

\[
\text{Specificity} = \frac{\text{No. of true negative cases}}{\text{No. of true negative cases} + \text{false positive cases}} \times 100
\]
### Table 3.1 Calculation of PPV and NPV

<table>
<thead>
<tr>
<th>True condition</th>
<th>Predicted condition</th>
<th>Predicted Condition positive</th>
<th>Predicted Condition negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>condition positive</td>
<td><strong>True positive</strong></td>
<td><strong>False Negative</strong> (Type II error)</td>
<td></td>
</tr>
<tr>
<td>condition negative</td>
<td><strong>False Positive</strong> (Type I error)</td>
<td><strong>True negative</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Accuracy (ACC)**

\[
\text{Accuracy (ACC)} = \frac{\Sigma \text{True positive} + \Sigma \text{True negative}}{\Sigma \text{Total population}}
\]

**Positive predictive value (PPV), Precision**

\[
\text{Precision} = \frac{\Sigma \text{True positive}}{\Sigma \text{Test outcome positive}}
\]

**False omission rate (FOR)**

\[
\text{False omission rate (FOR)} = \frac{\Sigma \text{False negative}}{\Sigma \text{Test outcome negative}}
\]

**False discovery rate (FDR)**

\[
\text{False discovery rate (FDR)} = \frac{\Sigma \text{False positive}}{\Sigma \text{Test outcome positive}}
\]

**Negative predictive value (NPV)**

\[
\text{Negative predictive value (NPV)} = \frac{\Sigma \text{True negative}}{\Sigma \text{Test outcome negative}}
\]
CHAPTER FOUR

4. Results and Discussion

4.1 Results:

The results of 50 lymph nodes out of 57 were evaluated by both FCM and IHC was in three phase; the results of touch imprint which used along with the first FMC screening antibodies for the designing of antibodies panel; the Results of FCM immunophenotyping; and the results of histopathology/IHC as the gold standard for comparison.

Table 4.2. Results and of touch imprint:

<table>
<thead>
<tr>
<th>Group</th>
<th>Finding</th>
<th>Possible Diagnosis</th>
<th>Number of Cases</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Small Mature lymphocytes</td>
<td>Reactive Hyperplasia (RH)</td>
<td>7 (14%)</td>
<td>14%</td>
</tr>
<tr>
<td>2</td>
<td>Large a typical lymphoid cells with vesicular nuclei and prominent nucleoli associated with atypical small to medium sized cells</td>
<td>Non-Hodgkin’s lymphoma NHL</td>
<td>26 (52%)</td>
<td>52%</td>
</tr>
<tr>
<td></td>
<td>Atypical small lymphoid cells</td>
<td></td>
<td>8 (16%)</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>Atypical lymphocytes of varying size with irregular nuclear membranes, necrotic debris and multinucleated cells</td>
<td>Non-Hodgkin’s lymphoma NHL</td>
<td>3 (6%)</td>
<td>6%</td>
</tr>
<tr>
<td>3</td>
<td>Few large atypical mononuclear cells and large bi-nucleated cells with prominent nucleoli in a background of small lymphocytes</td>
<td>Hodgkin’s lymphoma (HL)</td>
<td>2 (4%)</td>
<td>4%</td>
</tr>
<tr>
<td>4</td>
<td>non-lymphomatous malignant cells with scattered or few lymphocytes, suggestive of non-lymphomatous or</td>
<td>Metastatic tumor (MT)</td>
<td>4 (8%)</td>
<td>8%</td>
</tr>
</tbody>
</table>
4.2 Results and of Touch Imprint:
The stained touch imprints were evaluated as regards their contribution to the FCI data and their role in facilitating the diagnosis of cases. The cases were divided into 4 groups: 1) 7 cases (14%) showed small mature lymphocytes, suggestive of reactive hyperplasia (RH); 2) 37 cases (74%) showed variable features suggestive of NHL of which, 26 (52%) showed large atypical lymphoid cells with vesicular nuclei and prominent nucleoli associated with atypical small to medium sized cells, 8 (16%) revealed atypical small lymphoid cells and 3 (6%) revealed atypical lymphocytes of varying size with irregular nuclear membranes, necrotic debris and multinucleated cells; 3) Two cases (4%) revealed few large atypical mononuclear cells and large bi-nucleated cells with prominent nucleoli in a background of small lymphocytes suggestive of Hodgkin's lymphoma (HL) and 4) 4 cases (8%) showed non-lymphomatous malignant cells with scattered or few lymphocytes, suggestive of non-lymphomatous or metastatic tumor (MT) as shown in table 2.

4.2 Flow cytometric (FCI) findings.
Positive reaction of cells to monoclonal antibodies was detected as fluorescent dots in the dot plot quadrants. The distribution of dots differed depending on the number of markers used and the status of positivity to each marker. The diagnostic distribution of the 50 specimens according to FCI was as follows: 9 cases (18%) were diagnosed as RH, 32 cases (64%) were B-cell NHL, 3 cases (6%) were T-cell NHL, 2 cases (4%) were HL and 4 cases (8%) were diagnosed as non-lymphomatous (NLT) or metastatic tumors (MT).

Within the group of B-cell NHL (32 cases), 24 cases (75%) were diffuse large B-cell lymphoma (DLBCL) showing positive reaction to CD45, CD20, CD19 and large FSc (Fig. 1), 2 cases (6.25%) were follicular lymphoma (FL) showing positive reaction to CD45, CD20, CD19, CD10, anti-BCL-2 and small to medium FSc. Three cases (9.4%) were small lymphocytic lymphoma (SLL) showing positive reaction to CD45, CD19, CD5, CD23 and small FSc, 2 cases (6.25%) were mantle cell lymphoma (MCL) showing positive reaction to CD45, CD19, CD5, negative reaction to CD23 and small FSc (Fig. 2), and a single case (3.12%) was diagnosed as T-cell rich B-cell lymphoma (TCRBCL) showing numerous T-cells which were CD45RO+ve, CD3+ve with small FSc. The less common B-cells in this case were CD20+ve and CD19+ve. The B-cells demonstrated large FSc and light-chain restriction.

The 3 cases which were diagnosed as T-cell NHL were further subdivided into 2 cases (66.7%) of peripheral T-cell lymphoma (PTCL) showing positive reaction to CD45, CD3, CD45RO and a predominance of CD4+ve cells and a single case (33.3%) of anaplastic large T-cell lymphoma (ALTCL) with positive CD45, CD3, CD45RO and CD30.
Light chain restriction (LCR) was detected in the 32 FCI-diagnosed B-cell lymphoma cases, where the ratio of $\kappa/\lambda$ determined by FCM ranged from 0.14 to 15.67. Two out of these 32 cases (6.25%) showed absence of both $\kappa$ & $\lambda$ expression. A total of 20 cases (62.5%) revealed monoclonality of $\kappa$-light chain with a mean of 5.73. The remaining 10 cases (31.25%) showed monoclonal expression of $\lambda$-light chain with a mean of 0.36. On the other hand, the $\kappa/\lambda$ in the 9 RH cases ranged from 0.57 to 1.09 with a mean of 0.91 and they were all polyclonal Figure (4.1) and (4.2).
Figure 4.1: (A) Touch imprint of DLBCL case showing large atypical lymphoid cells with vesicular nuclei and prominent nucleoli associated with atypical small to medium sized cells (modified PAP, 400×), (B): DLBCL case showing diffuse infiltration by large atypical lymphoid cells with vesicular nuclei and prominent nucleoli. (H & E, 400×), (C): The same case showing positive reaction to CD 20 (400 ×), (D): FCM dot plots of a case of DLBCL showing (i) Large FSc, (ii) Negative isotype control (iii) Atypical large population showing positive reaction to CD45, CD 19, (iv) CD20 positive cells show large FSc (v&vi) Small population of CD 3 positive cells, (vii&viii) Absence of light chain expression by B-cells (LCR).
**Figure 4.2:** MCL case showing (A): Diffuse infiltration by monotonous atypical small lymphoid cells with dark nuclei and inconspicuous nucleoli (H & E, 400×), (B): Positive reaction to CD 20 (400×), (C): Positive reaction to CD 5 (400×), (D): Negative reaction to CD23 (400×), (E): FCM dot plots of a case of MCL showing (i) Small FSc, (ii) Negative isotype control (iii, iv, v) Triple marker showing atypical population showing positive reaction to CD45, CD 19, negative reaction to CD 3 (vi) B-cells show positive reaction to D5, CD19 (vii) Negative reaction to CD 23 (viii) Monoclonality for λ light chain (ix) Overlapping histogram showing B-cell monoclonality expressing λ light chain (arrow)
4.3 Result and Discussion of Histopathologic/IHC data:

The frequency distribution of the 50 cases evaluated by histopathology/IHC was as follows: 4 cases (8%) were diagnosed as RH, 34 (68%) were B-cell NHL, 3 (6%) were T-cell NHL and 5 (10%) were HL. However, 4 cases (8%) were confirmed as metastatic duct carcinoma of breast origin (Table 1). Accordingly, NHL represented 88.1% of the diagnosed malignant lymphoma cases and HL represented 11.9% only with a ratio of 7.4:1.

As regards to sub-typing by IHC of the 34 cases of B-cell NHL: 23 cases (67.65%) were DLBCL, 3 (8.82%) were FL, 2 (5.88%) were MCL, 3 (8.82%) were SLL and 3 cases (8.82%) were diagnosed as TCRBCL. On the other hand, the 3 cases diagnosed as T-cell NHL by IHC were subdivided into one case (33.3%) of PTCL and 2 cases (66.7%) were ALCL.

Light chain expression by B-cell NHL cases was not evaluated by immunohistochemistry, hence, it was not reported in patients' records.

4.4 The correlation between FCI, histopathology and IHC:

Seven groups were identified in the present study according to the data obtained from FCI, histopathology/IHC: A) 4 cases were diagnosed as RH by FCI and were morphologically benign by histopathology; of which 3 were proved to be follicular hyperplasia and one case was diagnosed as sinus histiocytosis, B) 2 cases were diagnosed as RH by FCI but they were categorized as NHL by histopathology/IHC (both cases proved to be TCRBCL), C) 3 cases were diagnosed as RH by FCI and were proved to be HL by histopathology/IHC, D) 4 cases showed NLT by FCI and were diagnosed as metastatic tumors by histopathology/IHC, E) 32 cases were accurately diagnosed as B-cell NHL lymphoma by both FCI and histopathology/IHC, F) 3 cases were clearly defined as T-cell NHL lymphoma by both FCI as well as histopathology/IHC, and G) 2 cases were diagnosed as HL by both FCI and histopathology/IHC. The overall concordance between FCI and histopathology/IHC was 88% (44/50 cases).

4.5 Correlation between the diagnosis of B-cell NHL and T-cell NHL by FCI and IHC:

Within the group of B-cell NHL, it was possible to reach an accurate final diagnosis by FCI in 31/34 B-cell NHL cases representing 91.2% concordance. FCI showed 100% concordance with histopathology/IHC in diagnosing all cases of SLL and MCL (Table 3) and (Fig-3). However, there was a difference in the other subtypes where FCI diagnosed 24 cases as DLBCL instead of 23 cases by histopathology/IHC. The extra case was proved to be FL by histopathology. Also, 2 cases were diagnosed as FL by FCI instead of 3 cases by IHC representing 66.7% concordance, and 1 case was diagnosed as TCRBCL by FCI instead of 3
cases by IHC representing 33.3% concordance. Within the group of T-cell NHL, there was 66.6% concordance between FCI and IHC (one case was diagnosed as PTCL and one case was diagnosed as ALTCL by both FCI and IHC). However, one case was diagnosed as PTCL by FCI and was proved to be ALTCL by IHC.

Figure 4.3: Frequency distribution showing correlation between diagnostic groups in FCI and histopathology.
Table 4.3: Correlation between NHL sub-typing as characterized and diagnosed by FCI, routine histology/ immunohistochemistry.

<table>
<thead>
<tr>
<th>B-cell lymphoma subtypes</th>
<th>FCM diagnosed cases (%)</th>
<th>Histopathology &amp; IHC No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>24 (75)</td>
<td>23 (61.8)</td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>2 (6.25)</td>
<td>3 (8.8)</td>
</tr>
<tr>
<td>Small lymphocytic lymphoma</td>
<td>3 (9.38)</td>
<td>2 (5.9)</td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>2 (6.25)</td>
<td>3 (8.8)</td>
</tr>
<tr>
<td>T-cell rich B-cell lymphoma</td>
<td>1 (3.12)</td>
<td>3 (8.8)</td>
</tr>
<tr>
<td>Total</td>
<td>32 (100)</td>
<td>34 (100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>T-cell lymphoma subtypes</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral T-cell lymphoma</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Anaplastic large T-cell lymphoma</td>
<td>1 (33.3)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>Total</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
</tbody>
</table>

4.6 Statistical evaluation of FCI technique:

Statistical evaluation of FCI technique included calculation of sensitivity, specificity, positive predictive value and negative predictive value. These calculations were performed to evaluate the application of FCI in diagnosis and characterization of NHL, HL, RH and NLT. Within the group of NHL the estimated sensitivity and specificity of FCI were 94.6% and 100%; respectively. The positive predictive value for diagnosis of NHL by FCI was 1 and the negative predictive value was 0.87. Within the group of HL, the sensitivity of FCI was 40% only (2 out of 5 HL cases were identified by FCI). However, the specificity was 100%. The positive predictive value was 1 and the negative predictive value was 0.94. Within the group of RH, the estimated sensitivity was 100% and the specificity was lower (89.1%). Positive predictive value was 0.44 and the negative predictive value was 1. As for the NLT, both sensitivity and specificity were 100%. Similarly, both positive predictive and negative predictive values were 1.
From the above results, FCI technique is 100% reliable in identifying NHL, HL and non-lymphomatous tumors as it gave no false positive results (specific) and to a lesser extent (89.1%) in diagnosing reactive hyperplasia. In addition, FCI technique is 100% reliable in excluding the diagnosis of reactive hyperplasia and non-lymphomatous tumors as it gave no false negative results (sensitive) and to a lesser extent (94.6%) in excluding NHL. However, FCI technique is not reliable in excluding the diagnosis of HL as its sensitivity is only 40%. This could be attributed to the small number of studied HL cases.
5.1 Conclusion:
The study concluded that, the FCI contributes significantly to and are consistent with the final tissue diagnosis in the majority of our studied cases (88%). The false negative results of FCI could be attributed to the presence of heterogeneous populations of lymphocytes that might be present in special situations such as partial involvement of the lymphoid tissue by lymphoma cells, the presence of a follicular lymphoma with normal lymphoid cells in-between the neoplastic follicles, or the presence of numerous residual non-neoplastic lymphocytes among the neoplastic cells of diffuse lymphomas as in T-cell-rich B-cell lymphoma. Thus, in highly suspicious cases IHC is still required if no FCI abnormalities were detected. However, FCI has a definite role in detection of monoclonality (light chain) of NHL.

5.2 Recommendations:
Adoption of these highly sophisticated techniques has reinforced the need for optimization of flow cytometric procedures and for interpretation by individuals who are familiar with all aspects of the testing that may affect the quality of the data. In addition, it is important that interpreters of flow cytometric data have a thorough knowledge of the phenotypes of diverse normal cell populations, can recognize deviations from normal, and are able to discuss the potential clinical significance of the flow cytometric findings.
References


**Kaleem** 2006- Flow Cytometric Analysis of Lymphomas Current Status and Usefulness, Arch Pathol Lab Med—Vol 130, December 2006


