

Independent Diagnostic Accuracy of Flow Cytometry Obtained From Fine-Needle Aspirates

A 10-Year Experience With 451 Cases

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Abstract

Although the topic is somewhat contentious, fine-needle aspiration (FNA) is frequently used in conjunction with flow cytometry (FC) to evaluate lymphoid proliferations. Despite the fact that the FNA and FC are often analyzed independently, no previous large-scale study has independently analyzed FC of FNA specimens. FC reports of 511 FNAs were retrospectively reviewed and FC diagnoses categorized as monoclonal, atypical, normal/reactive, or insufficient cellularity (3.9%). Abnormal immunophenotype was considered a positive test result. "Gold standard" diagnoses were established by histologic examination, treatment based on FNA, or clinical features. In 92.2% (451/489), there was adequate follow-up. The diagnostic accuracy of FC was 88.4%, sensitivity was 85.8%, and specificity was 92.9%. In addition, FC accuracy for classes of non-Hodgkin lymphoma was assessed. We conclude that FC is an independently accurate ancillary test in the evaluation of FNA. However, the presence of false-negative and false-positive cases supports the common practice of correlating FC with cytomorphologic findings even if performed independently.

The advent of modern classification systems for lymphoproliferative disorders that emphasize morphologic and immunophenotypic characteristics has ushered in a new era of increased potential for the use of fine-needle aspiration (FNA) in the evaluation of lymphoid proliferations. Ancillary studies such as flow cytometry (FC) can be combined with classic cytomorphologic features and create the potential for accurate classification of lymphoproliferative disorders.¹⁻³ Interpretation of ancillary studies, however, depends on the performance characteristics of the given test or procedure.

Although the topic is somewhat contentious, a number of recent studies have highlighted the usefulness of FC in conjunction with FNA in the assessment of lymphoid proliferations.³⁻¹⁶ However, to date, no large-scale study has evaluated the independent diagnostic accuracy of FC obtained from FNA samples, with most studies limited to the diagnostic accuracy of FNA when combined with FC results. Although this integrated approach is clearly preferable, the reality is that FC is often analyzed by noncytopathologists and may be performed in a separate laboratory or institution. At times, the FC results may be reported entirely separately from the FNA morphologic results. Thus, the independent diagnostic accuracy of FC from FNA samples is of interest to cytopathologists and clinicians who must correlate FC results with cytomorphologic findings. The current study was conducted to evaluate the independent performance characteristics of FC performed on samples obtained by FNA.

Materials and Methods

Between January 1997 and December 2006, 511 fine-needle aspirates were analyzed by FC at the University of Iowa,

Iowa City. The average patient age was 56 years (range, 2-96 years; SD, 19), and the male/female ratio was 0.92. FNAs were performed under ultrasound guidance by a radiologist or, alternatively, at the bedside or in the FNA clinic at the University of Iowa by a cytopathologist. Usually, a 22- to 25-gauge needle was used to collect specimens. Aspiration was facilitated by a 10-mL syringe and syringe holder for bedside and clinic FNA samples; however, those obtained under ultrasound guidance by radiologists were often performed without aspiration. Typically, a minimum of 2 passes were made per site to provide adequate material for analysis. A cytopathologist evaluated all FNA samples on site using a modified Wright-Giemsa stain (rapid Romanowsky), and, based on clinical suspicion or morphologic abnormalities, the pathologist determined whether to send material for flow cytometric analysis. Material sent for FC was placed in RPMI medium and sent immediately to immunopathology for flow cytometric analysis. FNA smears and FC reports were performed independently without knowledge of the diagnosis of the other.

Flow cytometric immunophenotyping was performed by using an extensive lymphoma panel, which included the following antibodies: CD8-fluorescein isothiocyanate (FITC)/CD4-phycoerythrin (PE)/CD3-Texas red (ECD)/CD7-PE cyanine 5 (PC5), κ -FITC/CD19-PE/CD10-ECD/CD5-PC5, λ -FITC/CD19-PE/CD10-ECD/CD5-PC5, FMC7-FITC/CD103-PE/CD20-ECD/CD19-PC5, CD38-FITC/CD23-PE/CD19-ECD/CD138-PC5, and CD16+CD56-FITC/CD25-PE/CD3-ECD/CD14-PC5; all tubes contained CD45-PE cyanine 7 (PC7). The panel was altered, if necessary, based on clinical data, limited material, concurrent morphologic assessment, or previously known lymphoma subtype. A limited flow panel was evaluated in 88 cases (18.0%), usually owing to limited material, and typically consisted of 1 tube to analyze B-cell clonality (κ -FITC/ λ -PE/CD19-ECD/CD5-PC5).

Appropriate isotype-matched negative controls were used to assess background staining. Abnormal T cells included populations with aberrant loss of typical T-cell markers (CD3, CD5, and CD7) or a markedly altered CD4/CD8 ratio. Monoclonality of B cells was defined as a substantial predominance of κ or λ light chain. We used a threshold κ/λ ratio of 3.0 or more or 0.7 or less to establish a significant light chain predominance.¹⁷⁻¹⁹ In a subset of cases, a small clone was diagnosed by the intensity of expression of a specific light chain unique to a small population of lymphoid cells, even if the overall κ/λ ratio was within the normal range. Aberrant expression of non-B-cell markers, defined as expression of non-lineage-specific markers, was also used to identify small B-cell clones. Data acquisition was performed on a Beckman Coulter (Brea, CA) FC500 flow cytometer and analyzed by CXP Software or on FACSCalibur (BD Biosciences, San Jose, CA) using CellQuest Software. Fluorochrome-labeled antibodies and reagents were purchased from Beckman Coulter

or BD Biosciences when using the FC500 or FACSCalibur, respectively.

In our laboratory, FC is initially analyzed independently of the FNA smears. The FC report is generated by noncytopathologists and incorporated into the cytology report, allowing for retrospective, independent assessment of FC. The final cytologic diagnosis, however, integrates cytomorphologic with FC results.

FC reports were retrospectively reviewed, and the final diagnosis for FC was categorized as monoclonal/"consistent with lymphoma" (M; 44.6%), atypical/"suspicious for" or "suggestive of lymphoma" (A; 6.7%), normal or reactive (44.8%), or insufficient cellularity for analysis (3.9%). Nondiagnostic samples (2 [0.4%]) and cases with insufficient cellularity for FC (20 [3.9%]) were excluded from further analysis regarding the diagnostic accuracy of FC, leaving 489 samples for subsequent study. Of these, 88 (18.0%) were evaluated with a limited panel of antibodies, which was defined as fewer than 6 antibodies owing to limited cellularity.

A positive test consisted of an abnormal immunophenotype (M + A) by FC. The "gold standard" was established by patient follow-up with subsequent excisional biopsy in 65.5%, treatment based on FNA + FC diagnosis in 15.7%, and clinical course in 18.8%. Clinical follow-up of greater than 6 months was required for a negative flow cytometric study without subsequent biopsy. The median clinical follow-up of samples included in this study was 35 months (average, 45 months; SD, 35 months; range, 6-118 months). Adequate follow-up to establish a gold standard diagnosis, as previously defined, was obtained in 92.2% of cases (451/489). These 451 cases were used for all further analysis of diagnostic accuracy.

Statistical analysis was performed on data entered in a 2 × 2 table to arrive at diagnostic accuracy, sensitivity, specificity, positive predictive values, and negative predictive values. The 95% confidence intervals (CIs) were calculated using the modified Wald method and the Newcombe efficient-score method. Two-tailed *P* values were calculated by using the Fisher exact test. *P* values of less than .05 were considered statistically significant.

Results

FC Compared With Gold Standard Diagnoses

The overall diagnostic accuracy of FC was 88.4% (95% CI, 85.2%-91.1%) with a sensitivity of 85.8% and specificity of 92.9% (Table 1). When a limited panel of antibodies (<6) was used, the diagnostic accuracy of FC declined to 81.8%, compared with 90.1% when a more extensive antibody panel was used (*P* = .039). Of the 451 specimens, there were 242 true-positives (53.7%), 157 true-negatives (34.8%), 40 false-negatives (8.9%), and 12 false-positives (2.7%). Of the

negative FC results, 20.3% were falsely negative, and 67.5% of these had abnormal cytomorphologic findings. Of the true-negatives, 10% had abnormal cytomorphologic findings ($P \leq .0001$), which might prompt further diagnostic studies despite negative FC results.

The gold standard diagnoses for false-negative FC results are shown in **Table 2**. Hodgkin lymphoma, both classical and nodular lymphocyte predominant, was the most common of the gold standard diagnoses, accounting for 35% of false-negative results (14/40), followed by diffuse large B-cell lymphoma (9/40 [23%]), peripheral T-cell lymphoma (5/40 [13%]) and follicular lymphoma (5/40 [13%]).

Of the 242 true-positive FC results, 69.0% were followed up by tissue biopsy. Of the true-positives, 36.0% were recurrent lymphomas, and recurrent lymphomas were less likely to be followed up by tissue biopsy than a primary diagnosis (47% vs 81%, respectively; $P \leq .0001$). The patients receiving confirmatory follow-up biopsies for a primary diagnosis were younger than patients who did not (mean age, 61 vs 73 years; $P = .0002$) and were more likely to have superficial lesions accessible to biopsy (77% vs 47%; $P = .0017$). Patients who were older than 75 years with a deep lesion ($n = 13$) were much less likely to receive follow-up biopsy for a primary diagnosis (15% vs 85%; $P < .0001$).

False-positive FC results were due primarily to surface immunoglobulin-negative B-cell populations ($n = 4$) or small populations of light chain-restricted B cells ($n =$

Table 1
Performance Characteristics of Flow Cytometry*

Characteristic	Value (95% Confidence Interval)
All fine-needle aspirates	
Diagnostic accuracy	88.4 (85.2-91.1)
Sensitivity	85.8 (81.1-89.6)
Specificity	92.9 (87.6-96.1)
Positive predictive value	95.3 (91.7-97.4)
Negative predictive value	79.7 (73.3-84.9)
B-cell non-Hodgkin lymphoma	
Diagnostic accuracy	93.6 (90.9-95.5)
Sensitivity	91.8 (87.3-94.8)
Specificity	95.5 (91.6-97.7)
Positive predictive value	95.5 (91.6-97.7)
Negative predictive value	91.7 (87.2-94.8)
T-cell non-Hodgkin lymphoma	
Diagnostic accuracy	98.2 (96.5-99.2)
Sensitivity	36.4 (12.4-68.4)
Specificity	99.8 (98.5-99.9)
Positive predictive value	80.0 (29.9-98.9)
Negative predictive value	98.4 (96.6-99.3)

* Values and 95% confidence intervals are given in percentages.

6). Gold standard diagnoses for false-positive samples are shown in **Table 3**. FC diagnoses categorized as “A” were more likely to be falsely positive (9/33 [27%]) than those categorized as “M” (3/221 [1.4%]; $P \leq .0001$). If only FC cases diagnosed as “M” were considered as positive results the diagnostic accuracy would be 89.7%, with a sensitivity of 84.5% and a specificity of 98.1%.

Table 2
“Gold Standard” Diagnoses of False-Negative Flow Cytometry Samples

Gold Standard Final Diagnosis	No. of Cases	No. (%) With Abnormal Fine-Needle Aspiration Diagnosis
Hodgkin lymphoma	14	11 (79)
Classical	10	8 (80)
Nodular lymphocyte predominant	4	3 (75)
Diffuse large B-cell lymphoma	9	5 (56)
Peripheral T-cell lymphoma	5	5 (100)
Follicular Lymphoma	5	1 (20)
Anaplastic large cell lymphoma (lineages, 1 T-cell, 2 B-cell)	3	1 (33)
Extramedullary myeloid tumor	1	1 (100)
Mucosal-associated lymphoid tissue lymphoma	1	1 (100)
Plasmacytoma	1	1 (100)
Posttransplant lymphoproliferative disorder	1	1 (100)
Total	40	27 (68)

Table 3
“Gold Standard” Diagnoses of False-Positive Flow Cytometry Samples

Gold Standard Diagnosis	No. of Cases	No. (%) With Abnormal Fine-Needle Aspiration Findings
Follicular, interfollicular, or paracortical hyperplasia	7	4 (57)
Atypical paracortical hyperplasia	1	0 (0)
No diagnostic abnormality	3	1 (33)
Sjögren syndrome	1	1 (100)
Total	12	6 (50)

Diagnostic Accuracy of FC for Non-Hodgkin Lymphoma

When FC was analyzed based on its ability to correctly diagnose B-cell non-Hodgkin lymphoma (NHL), the diagnostic accuracy was 93.6% (95% CI, 90.9%-95.5%), with a sensitivity of 91.8% and a specificity of 95.5% (Table 1). Of the 451 specimens, there were 212 true-positives (47.0%), 210 true-negatives (46.6%), 19 false-negatives (4.2%) and 10 false-positives (2.2%).

Of the B-cell NHL cases, 26 were also given a specific subclassification diagnosis by FC. Of these, 20 (77%) were correctly identified as mantle cell lymphoma (n = 7) or diffuse large B-cell lymphoma (n = 13). Gold standard subclassification diagnoses are given in **Table 4**. Of the cases given a specific classification of NHL, 2 (8%) of 26 were false-positive results.

It is interesting that the diagnostic accuracy for T-cell NHL was 98.2% (95% CI, 96.5%-99.2%) with a specificity of 99.8%. However, the sensitivity of FC for T-cell NHL was poor, at 36.4% (Table 1). Not surprisingly, the number of T-cell lymphomas was low: of the 451 specimens, there were only 4 true-positives (0.9%), 7 false-negatives (1.6%), and 1 false-positive (0.2%).

Discussion

The present study evaluated the independent performance characteristics of FC obtained from FNA samples, totaling 451 cases during the span of 10 years. Overall sensitivity, specificity, and diagnostic accuracy were 85.8%, 92.9%, and 88.4% respectively, indicating that FC is an accurate independent ancillary technique in the evaluation of FNA samples. When only cases of NHL were considered, the accuracy for B-cell NHL improved to 93.6% with a sensitivity of 91.8% and a specificity of 95.5%.

While no large-scale studies have evaluated the independent diagnostic accuracy of FC for FNA samples,

El-Sayed and colleagues²⁰ reported a small study examining the correlation between FC on samples from excised tissue and subsequent histopathologic diagnoses. Their results showed an 88% concordance between FC and the final histopathologic diagnoses, with respective sensitivities and specificities for various subcategories as follows: 94.9% and 100% for NHL, 40% and 100% for Hodgkin lymphoma, and 100% and 89.1% for reactive hyperplasia. Our results are similar to those of El-Sayed and colleagues,²⁰ as both had similar sensitivities and specificities with values greater than 90% in cases of NHL.

The high accuracy rates noted for FC performed on FNA samples, however, is tempered slightly by the 20.3% false-negative rate (40 of 197 negative FC samples). The rate initially appears relatively high but can be explained by further analysis of the contributing specimens. Roughly one third (35%; 14 of 40 samples) had gold standard diagnoses of Hodgkin lymphoma. Diagnosis of Hodgkin lymphoma rests on identification of characteristic Reed-Sternberg cells with CD15 and CD30 positivity on immunohistochemical staining in the appropriate cellular background. These cells are not detected by FC, and it is nearly universally acknowledged that FC has little use in the diagnosis of Hodgkin lymphoma.^{7,11,21-23} When these cases are excluded from the analysis, the false-negative rate drops to 14.2% (26 of 183 samples), and the accuracy of FC improves to 91.3% with a sensitivity of 90.3% and a specificity of 92.9%.

Additional diagnoses common among false-negative results were diffuse large B-cell and anaplastic large cell lymphomas, comprising 23% and 8%, respectively. The tendency of large cell neoplasms to be falsely negative by FC has been attributed to a disruption in the cells in which bare nuclei result following the stripping of the fragile cytoplasm by the FNA procedure and/or subsequent processing.^{22,24} These abnormalities in the cells are readily visible on FNA smears and underscore the importance of cytomorphologic studies in cases of negative FC. With respect to T-cell NHL (which

Table 4
Subclassification of B-Cell Non-Hodgkin Lymphoma Samples

Gold Standard Diagnosis	No. of Flow Cytometry Cases	Correct Diagnoses
Mantle cell lymphoma		
Correctly identified	7	—
Incorrectly identified	3	Diffuse large B-cell lymphoma, 2; small lymphocytic lymphoma with plasmacytoid differentiation, 1
Diffuse large B-cell lymphoma		
Correctly identified	13	—
Incorrectly identified	3	Follicular and diffuse large B-cell lymphoma, 1; follicular and interfollicular lymphoid hyperplasia, 1; high-grade lymphoma, unclassifiable, 1
Overall		
Correct	20 (77%)	
Incorrect	6 (23%)	

accounted for 15% of false-negative samples), FC analysis of the V_{β} repertoire for clonality in mature T-cell receptor $\alpha\beta$ T-cell lymphoproliferative disorders is rapid and sensitive.¹⁹ However, this approach requires use of an additional 24 to 28 anti- V_{β} antibodies, which is often not feasible using FNA specimens. In the present study, 67% of false-negative FC results were associated with abnormal cytomorphologic results on FNA; therefore, abnormal cytomorphologic findings should prompt additional diagnostic studies, even in the face of a negative FC report.

Aside from false-negatives, cytopathologists seeking to correlate FC results with FNA smears should also be cognizant of the small, but real false-positive rate of 4.7% (12 of 254 positive FC samples) seen in this study. The gold standard diagnosis for the majority of false positive cases (7/12 [58%]) was benign lymphoid hyperplasia, with follicular, interfollicular, or paracortical patterns noted on follow-up. Further examination of the flow characteristics of these samples demonstrated that they were due to immunoglobulin-negative B-cell populations, a characteristic that can be seen in reactive follicle center cells,²² or very small populations of light chain-restricted B cells. Further review of the immunophenotypic descriptions of several of these cases suggests that some of these interpretations were rather aggressive and may have been viewed by other pathologists as negative. Nevertheless, cytopathologists must be aware of the potential for overinterpretation of abnormalities noted in relatively small populations of cells. Based on these findings, positive FC results should be viewed with caution in FNA cases that have a low suspicion of malignancy based on cytomorphologic and clinical features. Special caution is urged in the face of reactive cytomorphologic findings with abnormal FC noted in small populations of cells. Treatment in these cases should never be based solely on FC findings, and incongruent results between FC, cytomorphologic studies, and clinical findings should trigger additional investigational studies.

The independent accuracy of FC for lymphoproliferative disorders, based on the present study, is high but is not meant to replace morphologic examination. Rather, these results should instill confidence in the technique of FC in settings where FC and cytomorphologic studies are performed separately. Based on the independent performance characteristics of FC, cytopathologists can use the information from an independent FC report and apply it to a given case, cognizant of the potential false-positive and false-negative results. We want to reiterate the importance of interpreting the FC in the context of the cytomorphologic findings and clinical picture. As has been demonstrated here and elsewhere, it is this integrated approach, further enabled by the knowledge of FC's independent diagnostic characteristics, that allows optimization of FNA in the assessment of lymphoid proliferations.

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