Flow Cytometric Immunophenotyping of Anaplastic Large Cell Lymphoma

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Context.—Anaplastic large cell lymphoma (ALCL) is usually diagnosed by histologic and immunohistochemical analysis. However, fine-needle aspiration is becoming a popular alternative to lymph node biopsy, and flow-cytometric immunophenotyping is often used to analyze fine-needle aspiration specimens.

Objective.—To review our experience using flow-cytometric immunophenotyping to assess cases of ALCL and to evaluate the diagnostic utility of this technique.

Design.—Each case of ALCL was assessed by flow cytometry with 3-color or 4-color antibody panels, and data were reanalyzed by cluster analysis using Paint-a-Gate for cases with retrievable flow cytometry data files. Anaplastic lymphoma kinase (ALK) was assessed by using immunohistochemistry.

Results.—Twenty-three ALCL cases were analyzed by flow cytometry. In 4 cases, neoplastic cells could not be identified. In the remaining 19 cases (11 ALK⁺, 8 ALK⁻), all were positive for CD30 and CD45. Anaplastic large cell lymphoma cells were large and usually CD45 bright, with many or most cells falling in the region of monocytes on the CD45/side scatter plot. The frequencies of T-cell antigen expression in ALK⁺ cases were CD2, 67%; CD7, 60%; CD3, 45%; CD4, 33%; CD5, 14%; and CD8, 14%. In ALK⁻ cases, the frequencies of the T-cell antigen expression were CD2, 100%; CD3, 50%; CD4, 40%; CD7, 40%; CD5, 25%; and CD8, 20%.

Conclusions.—Flow cytometry can be used to immunophenotype ALCL cases. Neoplastic cells may be few, and they may fall outside of the lymphocyte gate. Cluster analysis using software like Paint-A-Gate is often helpful because it allows for flexible, sequential gating strategies to identify and characterize the neoplastic cells.

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nophenotype ALCL cases, and ALK expression is not available for an appreciable subset of the few cases analyzed, perhaps because ALCL is an uncommon neoplasm.\textsuperscript{10-12} The advantages of flow cytometric immunophenotyping are well known. For fluid specimens, including FNA specimens, flow cytometry is ideally suited for immunophenotyping and offers quantitative analysis, high sensitivity, and quick turnaround time. However, there are also disadvantages in assessing neoplasms, which can have few or fragile neoplastic cells, as may be true in ALCL.

In this study, we describe the immunophenotypic findings of 23 cases of ALCL, all with known ALK expression, assessed by multiparameter flow cytometry.

**MATERIALS AND METHODS**

**Study Group**

The files of the Department of Hematopathology at the University of Texas M. D. Anderson Cancer Center (Houston) were searched for cases of ALCL in which a specimen was submitted for flow cytometry. The diagnosis in all cases was established by concurrent, or subsequent, tissue biopsy and/or assessment of ALK and CD30 expression. Clinical data, including demographic information, were obtained from medical records. Flow cytometric immunophenotyping was performed in the Clinical Flow Cytometry Laboratory at our institution or elsewhere for consultation and referral cases.

**Flow Cytometry Analysis and Interpretation**

Tissue processing and antibody staining were performed as described previously.\textsuperscript{13} Briefly, fresh tissue-biopsy specimens were manually disaggregated in Roswell Park Memorial Institute (Buffalo, NY) medium; 10\(^6\) cells per tube were incubated with antibodies for 10 minutes at room temperature, followed by a wash with phosphate-buffered saline containing 0.1% sodium azide, using a Sorvall Cell Washer 2 (Thermo Scientific, Waltham, Mass). Specimens were subsequently lysed with PhamLyse ammonium chloride solution (Pharmingen/Becton Dickinson Biosciences, San Jose, Calif). Cells were resuspended for acquisition in phosphate-buffered saline containing 1% formaldehyde. As these cases were analyzed during a period of years, the antibody panels changed throughout time. Each case was assessed with a variable combination of the following antibodies, specific for CD2, CD3 (surface), CD4, CD5, CD7, CD8, CD14, CD16, CD19, CD20, CD25, CD26, CD30, CD38, CD45, CD56, and monoclonal k and \(\lambda\) (all from BD Biosciences, San Diego, Calif, except CD30, which was from Immunotech/Beckman-Coulter, Fullerton, Calif). CD30 is not used in routine lymphoma panels in our laboratory but was added if the morphologic findings (on FNA smears or frozen sections or routinely stained slides of the biopsy specimen) and initial flow cytometry results raised the possibility of ALCL. Cytoplasmic CD3 was assessed in 2 cases. For this purpose, fixation and permeabilization were performed before staining using 4% formaldehyde and 0.25% saponin in phosphate-buffered saline. One case was stained with an acute leukemia antibody panel that included CD3, CD15, and CD33.

Antibodies were conjugated with fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, or allophycocyanin. Anti-CD45 antibody was included in most tubes. A portion of each specimen was stained with isotype-matched fluorescent control monoclonal antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, and allophycocyanin, along with CD45 peridinin chlorophyll protein. Acquisition and initial analysis of flow cytometry data were performed on 4-color FACSCalibur instruments (BD Biosciences) with CELLQuest software (BD Biosciences). Distinct cell clusters were identified on the basis of the composite pattern of forward scatter (FSC) and side scatter (SSC), together with different antibody combinations. Nonviable cells and debris were excluded from analysis based on scatter properties. Cells were considered to be positive for a given antigen if more than 20% of cells in a population expressed the antigen at greater amounts than threshold for the same population in the isotype control tube. In most cases, neoplastic cells were identified by their CD30 expression and back-gated to establish gates on CD45 versus SSC and on FSC versus SSC plots. These CD45 versus SSC and FSC versus SSC gates were then used to analyze expression of T-cell antigens in the remaining tubes. Reactive background T cells could be excluded on the basis of their low FSC and SSC. Monocyte numbers were assessed in each specimen by CD14 staining.

**Histologic and Immunohistochemical Analysis**

Tissue biopsy specimens were fixed in either 10% neutral buffered formalin or B5 and routinely processed. Fine-needle aspiration smears and body fluid–cytocentrifuged preparations were air dried or fixed in alcohol and were stained with Papanicolaou or Wright and/or hematoxylin-eosin stains, respectively.

The diagnoses of ALCL, either ALK\(^+\) or ALK\(^-\), were based on definitions reported by others. The diagnosis of ALK\(^+\) ALCL was based on the definition of the World Health Organization classification.\textsuperscript{2} The diagnosis of ALK\(^-\) ALCL was based on both the World Health Organization classification and the definition of Savage and colleagues.\textsuperscript{2,7} Immunohistochemical staining was performed on tissue biopsy specimens using methods reported previously.\textsuperscript{14} The antibodies used were those expected in the workup of cases suspected to be ALCL, including T-cell and B-cell antigens, CD30, and cytotoxic-associated markers (TIA-1, granzyme B, or both).\textsuperscript{a} Analysis for ALK was performed in every case using the ALK-1 antibody (Dako, Carpinteria, Calif). These results confirmed the diagnosis of ALCL and are not discussed further.

**RESULTS**

**Clinical Features**

Twenty-three cases of ALCL with material sent for flow cytometric immunophenotyping were identified. These cases included 12 excisional biopsy, 10 FNA, and 1 bone marrow aspirate specimens. The biopsy sites for excision and FNA specimens were lymph nodes (\(n = 13\)), soft tissue masses (\(n = 7\)), and skin (\(n = 2\)). The clinical data are summarized in Table 1.

21 cases were assessed for ALK, with 13 (57%) ALK\(^+\) and 10 (43%) ALK\(^-\). For the patients with ALK\(^+\) ALCL, ages ranged from 2 to 71 years (median, 21 years). In the patients with ALK\(^-\) ALCL, males outnumbered females (M/F = 8:5). For the patients with ALK\(^-\) ALCL, ages ranged from 25 to 72 years (median, 51 years). The ratio of men to women (M/F = 7:10) was higher than in the ALK\(^+\) group.

**Flow Cytometric Immunophenotyping**

The flow cytometry findings are summarized in Table 1. In 4 specimens (2 ALK\(^-\) and 2 ALK\(^+\)), a neoplastic population was not identified in the material analyzed by flow cytometry. Two of these cases were analyzed using conventional analysis and gating at outside submitting institutions, and the other 2 were reanalyzed in our institution using Paint-A-Gate software (although the staining panels in the latter 2 cases did not include CD30). Thus, the number of cases with diagnostic flow cytometry results is 19, as shown in Table 1. CD30 and CD45 were positive in all 19 cases (CD30 was shown only by immunohistochemistry in 3 cases, of which 2 were ALK\(^+\)).

Eleven ALCL cases with data files available for cluster analysis were reanalyzed with Paint-A-Gate at our insti-
Of the 11 cases analyzed, 8 expressed ALK. We did not systematically investigate these ALCL cases for myeloid antigens by flow cytometry because they are not a part of the lymphoma panel at our institution. Two cases of ALK− ALCL were assessed for CD13, and all were positive. The frequencies of T-cell antigen expression (in decreasing order) were CD2, 4 of 6 (67%); CD7, 3 of 5 (60%); CD3, 5 of 11 (45%); CD4, 2 of 6 (33%); CD5, 1 of 7 (14%); and CD8, 1 of 7 (14%). Other antigens assessed in a small number of these cases included CD25, 1 of 1 (100%); CD26, 1 of 1 (100%); and CD56, 1 of 2 (50%). B-cell antigens (CD19 or CD20) were negative in all cases. All 4 cases assessed for CD10 were negative (0%).

ALK+ ALCL.—Of the 8 cases analyzed, 6 (75%) expressed at least one T-cell antigen. The frequencies of T-cell antigen expression (in decreasing order) were CD2, 4 of 6 (67%); CD7, 3 of 5 (60%); CD3, 5 of 11 (45%); CD4, 2 of 6 (33%); CD5, 1 of 7 (14%); and CD8, 1 of 7 (14%). Other antigens assessed in a small number of these cases included CD25, 1 of 1 (100%); CD26, 1 of 1 (100%); and CD56, 1 of 2 (50%). B-cell antigens (CD19 or CD20) were negative in all cases. All 4 cases assessed for CD10 were negative (0%).

Myeloid Antigens.—We did not systematically investigate these ALCL cases for myeloid antigens by flow cytometry because they are not a part of the lymphoma panel at our institution. Two cases of ALK+ and 1 case of ALK− ALCL were assessed for CD13, and all were positive. One of these cases, case 8, was initially assessed by FNA and flow cytometry, and the smears and immunophenotypic data were misinterpreted as myeloid sarcoma at the referring hospital. The neoplastic cells were positive for CD2, CD4, CD11c (variable), CD13, CD25 (variable), CD34, CD10, and CD138 and negative for CD14 and myeloid-associated antigens (Figure 2, e). In 3 cases, lymphoma cells overlapped on scatter plots almost entirely with small-sized to intermediate-sized lymphocytes (Figure 3, a through f). In these cases, the neoplastic cells were most easily identified by CD30 staining, and in 2 cases, they also demonstrated aberrant pan-T-cell antigen expression (Figure 3, d through f).

### Table 1. Summary of Flow Cytometry Immunophenotypic Results for Anaplastic Large Cell Lymphoma*

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* ALK indicates anaplastic lymphoma kinase; FNA, fine-needle aspiration; NT, not tested; −, negative; +, positive; ↓, decreased intensity of expression compared with normal T-cell findings.
† Positive for cytoplasmic CD3, negative for surface CD3.
‡ Determined by immunohistochemistry.
CD45, and HLA-DR and were negative for CD3, CD5, CD7, CD8, CD10, CD16, CD19, CD20, CD22, CD23, CD33, CD34, CD56, and immunoglobulin light chains (Figure 1). An excisional lymph node biopsy at our institution, assessed by flow cytometry and immunohistochemistry, established the diagnosis of ALK+/ALK- ALCL.

**Discordance Between Flow Cytometry and Immunohistochemistry.**—In almost every case in this study, immunohistochemical analysis was performed on a tissue biopsy specimen or on smears of FNA specimens. In 6 cases, discordances between flow cytometry and immunohistochemistry occurred and involved 3 ALK+ and 3 ALK- neoplasms. In the ALK+ group, in case 2, CD3 was positive by flow cytometry but negative by immunohistochemistry; the converse was true for case 6. In case 7, CD2 was positive by flow cytometry but negative by immunohistochemistry. In the ALK- group, in cases 17 and 19, CD3 was negative by flow cytometry but positive by immunohistochemistry. In case 20, CD5 was negative by flow cytometry but positive by immunohistochemistry.

**COMMENT**

In this article, we review our experience using flow cytometry to immunophenotype 23 cases of ALCL in which material was sent to us for analysis. We believe that this topic is important for at least 2 reasons. First, at many centers flow cytometric immunophenotyping is routinely performed on all tissue biopsy specimens obtained from patients suspected of having lymphoma.8,9,12 Second, FNA is becoming increasingly popular. Fluid samples obtained by using FNA are ideally suited to assessment by flow cytometry, and for some patients, larger tissue specimens may be much more difficult to obtain.15 Thus, specimens from previously undiagnosed cases of ALCL may be received for analysis in the flow cytometry laboratory. A false-negative diagnosis by flow cytometry may lead to an unnecessarily broad immunohistochemical workup, increasing the cost to the patient and potentially wasting precious material from scant tissue core biopsies or scarce cytologic preparations.

There are a number of technical factors and potential pitfalls that make detecting ALCL by flow cytometry particularly challenging. As a testament to these challenges, in 4 (17%) of 23 cases in this study, neoplastic cells could not be identified in cell suspensions analyzed by flow cytometry. Similarly, Kesler and colleagues11 could not identify neoplastic cells by flow cytometry in 4 (14%) of 29 ALCLs. In some cases, the neoplastic cells appear underrepresented in the flow cytometry analysis, likely because of the fragility of the large atypical neoplastic cells, which may be easily disrupted during processing. Sampling issues also may be involved because ALCL can involve lymph nodes only focally. Therefore, in suspected cases of ALCL, careful analysis is warranted because the diagnostic aberrant population may be very small. Acquisition of larger-than-usual numbers of cells can be very helpful in
analysis. In our opinion, using an analysis strategy that examines total ungated cells on plots displaying a variety of combinations of markers and light scatter properties should reduce the potential for false-negative studies and other problems in diagnosis. We find Paint-a-Gate useful for this approach, but appropriate templates using other flow cytometry analysis software packages could accomplish the same aim.

In many cases, ALCL cells show increased SSC and, thus, fall largely or entirely outside the lymphocyte gate, so conventional gating for lymphoma cells with low SSC could lead to a false-negative study. In 7 (64%) of our 11 reanalyzed cases, very few lymphoma cells would be included in a lymphocyte gate (Figure 1, b). In some cases, the level of involvement by ALCL is high enough to draw attention to the significant areas on the FSC versus SSC or CD45 versus SSC plots, as in Figure 1, b and c. In a subset of cases, however, the ALCL cells may effectively masquerade as monocytes on these plots, and monocytes may not even appear increased in number (as in Figure 2). An-

Figure 2. Case 5. Anaplastic large cell lymphoma; axillary lymph node fine-needle aspiration, anaplastic lymphoma kinase positive. Lymphoma cells (green) are gated using CD30 (a), small T cells (blue in d through f) using CD5 versus side scatter (SSC) plots, and monocytes (yellow in d through f) using CD14 versus SSC plots. Lymphoma cells could be mistaken for debris, based on relatively high SSC compared with forward scatter (FSC) plots (b), and they also overlap with monocytes (c). Gating on CD45 bright cells with moderate SSC (d), in combination with CD14 staining (e), shows that most cells in the monocyte region do not show monocyte markers, which could help lead to an initial suspicion for lymphoma. The lymphoma cells show very bright CD5 expression (f). PE indicates phycoerythrin; PerCP, peridinin chlorophyll protein; and APC, allophycocyanin.
aplastic large cell lymphoma cells can also overlap substantially with lymphocytes (as in Figure 3). In addition, many ALCL cases show an aberrant absence of expression of multiple pan–T-cell antigens and, thus, a limited marker panel may yield a “null-cell” phenotype—which may be particularly difficult to detect, given the other complicating factors.\textsuperscript{12,16}

In our opinion, the most effective means to detect ALCL cells by flow cytometry is by staining for CD30. We found bright staining in most of our cases using a phycoerythrin-conjugated reagent. Identifying CD30$^+$ large cells, and then back-gating onto CD45 versus SSC and FSC versus SSC plots, was a useful approach in many cases. We were able to avoid background reactive T cells in most cases by excluding cells in the lymphocyte gate with low forward and side scatter. The resulting gates did potentially include monocytes, so we used CD14 staining to determine the number of monocytes in each specimen and to ensure that ALCL cells outnumbered monocytes. We found that CD4 expression was particularly difficult to assess be-
cause monocytes in the same gate showed moderate positivity for CD4. Therefore, we did not believe we could reliably score very dim/partial CD4 expression, unless the staining tube also included either CD14 or CD30 to distinguish monocytes from ALCL cells.

As valuable as CD30 staining is, given the uncommon frequency of ALCL cases, many laboratories may not find it an effective use of resources or of limited cell numbers to stain every potential lymphoma case for CD30. Therefore, a high index of suspicion must be maintained, with timely information gathered from examination of aspirate smears, touch imprints, frozen sections, or cytocentrifuged slides. The initial flow cytometry workup may also provide potential clues to the diagnosis, such as finding a large number of cells in the monocyte gate that are negative for expected monocyte markers like CD14 and CD4. In some of our ALCL cases with low SSC, most cells in the lymphocyte gate were negative for CD3, CD19, and CD56. Once ALCL is suspected, CD30 should certainly be included in the staining panel. If there are sufficient available cells, inclusion of CD30 in multiple tubes could enhance the accuracy of phenotyping the lymphoma cells for pan-T-cell, and other, markers.

It could be argued that the events in the CD30⁺ gate in our study are a result of cell aggregates of CD30⁺ T cells. However, we believe that this is not the case. CD30⁺ T cells represent less than 1% of all cells in the peripheral blood. Their percentage in bone marrow is not well defined, but this percentage is also very low. Lymph nodes can have increased numbers of CD30⁺ cells because CD30 is an activation marker. However, if this were the case, then CD30⁺ cells would have a normal immunophenotype, which was not the case in this study.

Regarding the expression of other antigens in ALCL, we observed discordances in T-cell antigen expression between flow cytometry and immunohistochemistry in 6 (32%) of 19 ALCLs, most often (4/6; 67%) involving CD3. In this study, the anti-CD3 antibody used specifically to detect the CD3-ε chain. CD3 was positive by flow cytometry and negative by immunohistochemistry in 1 case, and the converse was true in 3 cases. In the first situation, we believe that the discordance is explained by the ability of flow cytometry to detect dim CD3 expression, not detectable by immunohistochemistry. In the second scenario, a likely explanation is expression of cytoplasmic CD3 without surface CD3. In this case, CD3 would be detectable only by immunohistochemistry, unless cell permeabilization is performed during flow cytometry. Another possible explanation is related to the functional status of CD3.

Bonzeheim and colleagues have shown that CD3 is often absent or dimly expressed in ALCL, and they hypothesized that T-cell receptor signaling is impaired. The CD3 antigen consists of at least 5 invariable/constant membrane proteins and is associated with 2 variable immunoglobulin-like glycoproteins, the T-cell receptor α and β chains. The T-cell receptor must be complete to be expressed on the cell surface and detectable by flow cytometry without permeabilization. In contrast, immunohistochemistry can detect CD3 in the cell cytoplasm whether CD3 is functional or nonfunctional.

Assessment of ALK status, by immunologic methods or fluorescence in situ hybridization studies, is critical for assessing the prognosis of patients with ALCL. Direct staining for ALK by flow cytometry has been described in a small series of ALCL cases. However, we note that maintaining quality control and proficiency in a clinical flow cytometry laboratory is difficult for antigens, such as ALK, which are positive in only very rare specimens. In common with another recent study, we did not assess ALK by flow cytometry in our cases.

Anaplastic lymphoma kinase expression, assessed by immunohistochemistry on paraffin sections or by FNA cytospins, was known for all cases of ALCL in this study. We combined these cases with other cases of ALCL reported in the literature for which ALK expression was known. We then compared T-cell antigen expression in ALK⁺ ALCL and ALK⁻ ALCL. Using the Fisher exact test, only CD2 expression was significantly more frequent in ALK⁺ (15/22; 68%) versus ALK⁻ (16/17; 94%) ALCL (P = .047). The other comparisons for ALK⁺ and ALK⁻ ALCLs were as follows: CD3, 11 of 27 (41%) versus 10 of 22 (45%; P = .21); CD4, 18 of 22 (82%) versus 12 of 19 (63%; P = .11); CD5, 7 of 23 (30%) versus 5 of 22 (23%; P = .22); CD7, 8 of 21 (38%) versus 5 of 19 (26%; P = .19); and CD8, 1 of 23 (4%) versus 2 of 19 (11%; P = .34).

A limitation of our study is that 8 cases had 3 or fewer T-cell antigens assessed by flow cytometry. This is related to the fact that ALCL is an uncommon disease at a predominantly adult medical center, such as our own, and these cases were assessed by flow cytometry during a long time interval. Also, the specimen quantity and cell viability can be poor in limited specimens obtained with FNA techniques. Furthermore, our standard lymphoma panels to exclude B-cell lymphoma were, in some cases, performed up front, leaving little remaining specimen for an ALCL workup.

In summary, we believe that flow cytometric immunophenotyping is a good method to analyze ALCL cases, particularly for FNA or body fluid specimens, if certain potential pitfalls are recognized and addressed. Neoplastic cells are relatively fragile and may be underestimated in cell suspensions. On SSC, versus CD45 plots, in most cases, ALCL cells fall entirely in the monocyte/histiocyte gate or they overlap with both the monocyte/histiocyte and lymphocyte gates. Hence, conventional CD45/SSC analysis gating on lymphocytes can miss the neoplastic cells. These problems, however, may be overcome by careful examination of ungated cells using a program like Paint-A-Gate. CD30 is an effective marker for identifying ALCL by flow cytometry, but often, it is not included in the routine lymphoma panel. Therefore, a high index of suspicion is required after review of cytologic preparations or frozen section to ensure that CD30 is added to the panel and, possibly, included in multiple tubes. It is important to remember that ALCL cases, and especially ALK⁺ ALCL, can express myeloid-associated antigens, adding to the potential for misdiagnosis. Flow cytometry, in combination with appropriate morphology and immunostaining or fluorescence in situ hybridization studies to determine ALK status, can contribute to a definitive diagnosis of ALCL by FNA.

References


