CME/SAM

A Six-Color Flow Cytometry Assay for Immunophenotyping Classical Hodgkin Lymphoma in Lymph Nodes

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ABSTRACT

Objectives: *We have recently demonstrated that classical Hodgkin lymphoma (CHL) can be immunophenotyped by flow cytometry (FC), thus obviating the need for immunohistochemistry in many cases. The previously described nine-color assay, however, cannot be used by laboratories that do not have access to a nine- or ten-color flow cytometer. Therefore, a six-color FC tube was designed employing the following combination: CD64-FITC/CD30-PE/CD40-PeCy5.5/CD20-PECy7/CD95-APC/CD3-APC-H7.*

Methods: *To validate this assay, we analyzed 408 tissue specimens (including 55 CHL cases, 26 of which had been previously cryopreserved). Specimen inclusion criteria included the identification of an abnormal population by FC or (if no abnormal population was identified) greater than 50,000 viable events and specimen age less than 4 days. All FC studies were examined blinded to any clinical, laboratory, or histologic information.*

Results: *The diagnostic sensitivity and specificity of the six-color FC assay was 85.4% and 99.7%, respectively.*

Conclusions: *Taken together, these results suggest that the six-color FC assay has acceptable sensitivity and specificity for clinical use, allowing more FC laboratories to immunophenotype CHL by this method.*

Upon completion of this activity you will be able to:

- describe the immunophenotype and light scatter properties of Hodgkin and Reed-Sternberg (HRS) cells in classical Hodgkin lymphoma (CHL) as determined by six-color flow cytometry.
- describe the minimum criteria for concluding that a population is an HRS population by flow cytometry.
- compare and contrast the immunophenotypic findings in CHL with those seen in morphologically similar lymphomas (diffuse large B-cell lymphoma, anaplastic large cell lymphoma, and nodular lymphocyte-predominant Hodgkin lymphoma) using this assay.
- describe the diagnostic utility of the six-color assay, including its limitations.

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Questions appear on p 445. Exam is located at www.ascp.org/ajcpcme.

Classical Hodgkin lymphoma (CHL) is an unusual type of lymphoma that is characterized by scattered large, multinucleate Hodgkin and Reed-Sternberg (HRS) cells (comprising 1% or less of the lymph node cells) embedded in a reactive cellular infiltrate composed of lymphocytes, histiocytes, eosinophils, and plasma cells.[1-5] Another unique feature of this disease is that HRS cells bind T cells that ring around or "rosette" the neoplastic population, resulting from the coordinated interaction of adhesion molecules on T cells and HRS cells.[6-12] While evidence in the past decade has convincingly demonstrated that HRS cells are B cells,[2,3] this neoplastic population has an unusual

immunophenotype (expression of CD15, CD30, and Pax-5 without CD3, CD20, or CD45) that needs to be demonstrated to confirm a diagnosis of CHL.[1,4,13,14]

Flow cytometry (FC) is a multiparametric technique that has proven to be useful in the diagnosis of hematopoietic neoplasms as well as monitoring for minimal residual disease in the posttherapy setting.[15,16] While CHL historically could not be immunophenotyped by FC, we have recently shown that FC can identify and diagnose CHL in lymph nodes.[7,17] An interesting and diagnostically important conclusion that resulted from this work is that T cells can bind HRS cells, and these T-cell–HRS-cell rosettes can be detected directly by FC[7]; their identification is also a characteristic of CHL and proves useful in providing support for a diagnosis of CHL when present.[17,18] In this prior study, a total of 420 tissue specimens were examined, resulting in a diagnostic sensitivity and specificity of 88.7% and 100%, respectively.[17] While this method works well, one limitation of this prior work is the reliance on nine- or ten-color FC. In the current study, we have designed a simplified six-color FC (CD64-FITC/CD30-PE/CD40-PECy5.5/CD20-PECy7/CD95-APC/CD3-APC-H7) tube that can also immunophenotype CHL with high sensitivity and specificity.

Materials and Methods

Fluorescently Labeled Antibodies and Controls

For all experiments, FC was performed on a modified four-laser, ten-color Becton-Dickinson (BD; San Jose, CA) LSRII flow cytometer (same flow cytometer used for clinical cases at the University of Washington Medical Center [UWMC]) using the following laser-fluorochrome combinations: (1) a 488-nm blue laser (four colors), including fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE–cyanine 5.5 (PE-Cy5.5), and PE–cyanine 7 (PE-Cy7); and (2) a 633-nm red laser (two colors), including allophycocyanin (APC) and APC-Cy7/APC-H7. Of note, the yellow and violet lasers on the cytometer were not used in the study.

The fluorescently labeled antibodies used in this study were obtained from Beckman Coulter (BC; Hialeah, FL) or BD. Antibodies used in the single six-color FC tube for all specimens employed (antibody clone designation and supplier) included CD64-FITC (22; BC), CD30-PE (Ber-H83; BD), CD40-PE-Cy5.5 (MAB89; BC), CD20-PE-Cy7 (B9E9; BC), CD95-APC (DX2; BD), and CD3-APC-Cy7 (SK7; BD) or CD3-APC-H7 (SK7; BD). Appropriate fluorescence minus one controls were also used (to determine background fluorescence). Where noted, the results were compared with a nine-color assay for CHL[17,18] (used diagnostically in our laboratory since October 2007).

Selection of Specimens

This study was conducted with approval from the University of Washington Human Subjects Review Committee. A total of 408 viable specimens were analyzed by the six-color FC tube to determine the diagnostic sensitivity and specificity of this combination. Residual specimens were initially used in these studies after routine clinical immunophenotyping at the UWMC but without knowledge of previous flow cytometry results, tissue section morphology, or patient clinical history. Morphologic diagnoses were determined by a combination of histologic examination, conventional FC in the UWMC FC laboratory, and immunohistochemistry. To increase the number of Hodgkin cases in the study, we blindly evaluated 26 previously cryopreserved CHL cases in the series.

When possible, between 50,000 and 300,000 events were typically collected for analysis. Criteria for specimen adequacy were as follows: (1) cases ultimately shown to have abnormal populations by FC were included if the abnormal population (either HRS or non-HRS cell population) could be identified in the six-color FC tube. For non-CHL neoplastic cases, after the specimen was evaluated for CHL, the six-color FC tube was correlated with the routine clinical B- and T-cell tube for non-Hodgkin lymphoma to ensure the neoplastic population was identified in the CHL tube. (2) All tissues ultimately shown to not have an abnormal FC population required the presence of greater than 50,000 events and specimen age (time from biopsy to being evaluated by the six-color FC tube) of 3 days or less to be included in the study.

Preparation of Tissue Suspensions

Lymph node suspensions were prepared in the standard manner used for routine clinical immunophenotyping of tissues in our laboratory as described previously.[7] Briefly, tissue was finely minced in 3 to 5 mL of RPMI using a scalpel. The homogenate was then filtered through a 40-µm filter, centrifuged (550 *g* for 10 minutes), washed with phosphate-buffered saline (PBS), and then resuspended in 0.5 mL of RPMI-1640.

Surface Immunophenotyping of Tissue Suspensions

All incubations were performed at room temperature in the dark. Cells were incubated with appropriately titered, fluorescently labeled antibodies for 15 minutes in approximately 100 µL of RPMI. Cell suspensions were then lysed and fixed with 0.15 mol/L of ammonium chloride, pH 4.8, containing 0.25% formaldehyde (Ultra Pure, Polysciences, Warrington, PA) for 15 minutes, washed with 3 mL of PBS–bovine serum albumin, and incubated with 0.1 mL of PBS prior to analysis.

Criteria for Identifying an HRS Population by FC

Criteria to identify an HRS cell population by FC are similar to those described previously.[17] An HRS population must (1) have increased forward and side light scatter

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compared with small lymphocytes; (2) show expression of CD30, CD40 (at the same or greater intensity than a reactive B cell), and CD95; (3) demonstrate significant autofluorescence in the FITC channel without CD64 expression **Image 1**; (4) show no expression of intermediate to bright CD20; and (5) form a distinct cluster of events. Because HRS cells bind T cells and FC studies of CHL frequently demonstrate T-cell–HRS-cell rosettes,[6-12] a putative HRS population may demonstrate expression of CD3.

Data Analysis

Analysis of the data was performed using software (Woodlist) written by one of the authors (B.L.W.).

Results

Demographic Characteristics of CHL Cases

The morphologically confirmed CHL cases demonstrated demographic features expected of this neoplasm. Thirty (55%) of the 55 CHL cases were subclassified as nodular-sclerosis Hodgkin lymphoma, 17 (31%) as "other" (subtype not specified or recurrent), 6 (11%) as mixed-cellularity Hodgkin lymphoma, and 2 (4%) as lymphocyte-rich CHL. The male-to-female ratio and mean age for the patients with CHL were 1.2 to 1 and 32 years, respectively.

Diagnostic Sensitivity and Specificity of the Novel Six-Color FC Assay

Inclusion criteria were established to ensure that (1) preanalytical variables (specimen age and specimen cellularity) did not result in a false-negative result and (2) small abnormal non-Hodgkin lymphoma and CHL populations from relatively paucicellular specimens would be included in the study. Therefore, if an abnormal CHL or non-CHL population was not identified, the specimen had to have greater than 50,000 viable events and be less than 4 days old. Any specimen that had an abnormal FC population was included in this study.

We evaluated 408 tissue specimens to determine the diagnostic utility of the novel six-color assay for the detection of the neoplastic HRS cells of CHL, blinded to any clinical, morphologic, or immunophenotypic information so as to provide an unbiased evaluation of this assay. The tissue specimens included 55 CHL cases **Table 1** (examples shown in **Image 2**), 26 of which had been cryopreserved. The non-CHL cases **Table 2** (examples shown in **Image 3**) included a variety of reactive cases, B- and T-cell non-Hodgkin lymphomas, nonhematopoietic neoplasms, and a number of neoplasms that can be confused morphologically with CHL (diffuse large B-cell lymphoma [DLBCL], nodular lymphocyte-predominant Hodgkin lymphoma [NLPHL], and anaplastic large cell lymphoma [ALCL]). Overall, the sensitivity and specificity of the six-color assay were 85.4% and 99.7%, respectively (Table 1). Of the 55 CHL cases, 47 were correctly diagnosed as CHL (eight false-negative results; of the 353 non-CHL tissu es, one was incorrectly diagnosed as CHL when the diagnosis was peripheral T-cell lymphoma (false-positive result; see Discussion section). Cryopreserved and noncryopreserved CHL cases yielded similar results; 5 of 29 nonfrozen cases and 3 of 26 cryopreserved cases gave false-negative results, suggesting that this manipulation had no effect on the likelihood of a false-negative result.

Our standard nine-color clinical FC assay for CHL[17,18] was run on 52 of the 55 CHL cases; six of the eight

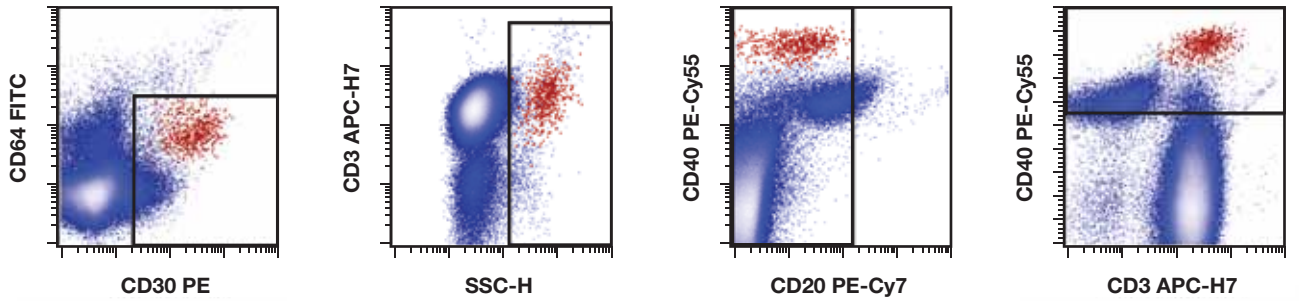

Image 1 Gating strategy to identify Hodgkin and Reed-Sternberg (HRS) cells in classical Hodgkin lymphoma (CHL). HRS cells are in red and are highlighted (emphasized); all other events are in blue. Putative HRS cell events must fall in all four gates and form a distinct population. Specifically, the cells must be CD30+, have increased autofluorescence in the fluorescein isothiocyanate (FITC) channel compared with the immunoblasts and small lymphocytes, and lack expression of CD64 (first panel), have increased side scatter (SSC-H; second panel), express no or low CD20 (third panel), and express CD40 at the same or greater intensity than a reactive B cell (fourth panel). The B cells in the fourth panel are CD3– and CD40+. APC, allophycocyanin; Cy, cyanine; PE, phycoerythrin.

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false-negative six-color cases yielded false-negative results in the nine-color assay. No false positives were identified in the nine-color assay (although the one false-positive case identified using the six-color assay was not evaluated with the nine-color assay).

Immunophenotypes of the CHL Cases

The FC-derived immunophenotypes of the HRS cells were essentially identical to those described previously.[7,17] By definition, all putative HRS populations showed expression of CD30, CD40, and CD95, with increased side and forward

Table 1
Summary of Clinical Features of 55 Morphologically Defined CHL Cases

Case No.	Age, y	Sex	Subtype	Biopsy Location	Six-Color Flow Result	% HRS Cells[a]
1	25	M	CHL (MCHL)	Right neck mass	+	0.04
2	38	F	CHL	Right cervical lymph node	–	NA
3	38	M	CHL (NSHL)	Mediastinal lymph nodes	+	0.02
4	74	M	CHL, favor NSHL	Left inguinal lymph node	+	0.51
5	20	F	Recurrent CHL	Right neck mass	+	0.07
6	23	F	CHL (NSHL)	Left supraclavicular lymph node	+	0.08
7	38	M	CHL	Right neck lymph node	+	0.18
8	22	M	CHL, favor MCHL	Neck mass	+	0.01
9	25	M	CHL, favor MCHL	Lymph node	+	0.01
10	67	M	Recurrent CHL	Retroperitoneal lymph node	–	NA
11	49	F	CHL (NSHL)	Supraclavicular lymph node	+	0.07
12	64	F	CHL (NSHL)	Left groin lymph node	+	0.13
13	63	F	CHL (MCHL)	Axillary lymph node	–	NA
14	40	M	CHL (NSHL)	Anterior mediastinal mass	+	0.04
15	24	F	CHL (NSHL)	Left axillary lymph node	+	0.40
16	22	M	CHL (NSHL)	Left neck lymph node	+	0.01
17	44	M	CHL (LRCHL)	Lymph node, 4R	+	0.01
18	54	F	CHL (NSHL)	Right neck lymph node	–	NA
19	38	F	CHL (NSHL)	Left neck lymph node	+	0.10
20	26	F	CHL, favor NSHL	Cervical lymph node	+	0.01
21	20	M	CHL, NSHL	Right neck lymph node	+	0.26
22	31	F	CHL (NSHL)	Left clavicular lymph node	+	0.02
23	26	M	CHL (NSHL)	Right neck mass	+	0.08
24	26	F	CHL (NSHL)	Supraclavicular lymph node	+	0.08
25	40	M	CHL (MCHL)	Neck lymph node	+	0.04
26	51	M	CHL	Tissue (site unspecified)	+	0.40
27	28	M	CHL (NSHL)	Left axillary lymph node	–	NA
28	30	F	CHL (NSHL)	Left neck lymph node	+	0.03
29	25	M	CHL (NSHL)	Right neck mass	+	0.10
30	57	M	CHL (NSHL)	Left supraclavicular lymph node	–	NA
31	19	M	CHL (NSHL)	Left neck lymph node	+	1.51
32	41	F	CHL, favor NSHL	Right supraclavicular lymph node	+	0.18
33	27	M	CHL	Right interlobar mass	+	4.11
34	58	M	CHL (MCHL)	Right lymph node	+	1.69
35	59	M	CHL	Right cervical lymph node	+	0.05
36	46	F	CHL	Right cervical lymph node	+	1.37
37	46	F	CHL	Tissue, unspecified	+	3.06
38	26	M	CHL	Left iliac lymph node	+	0.17
39	60	F	CHL (NSHL)	Right groin lymph node	+	0.30
40	32	F	CHL	Aortocaval lymph node	+	0.17
41	35	F	CHL	Left cervical lymph node	+	0.05
42	56	M	CHL (MCHL)	Left subclavian lymph node	+	3.17
43	26	M	CHL, NSHL	Right cervical lymph node	+	0.73
44	37	F	CHL, NSHL	Lymph node	+	0.09
45	41	F	Recurrent CHL	Lymph node, 4R	+	0.18
46	21	F	CHL (NSHL)	Right lower neck mass	+	0.43
47	50	M	CHL (LRCHL)	Left neck lymph node	–	NA
48	29	M	CHL (NSHL)	Lymph node	–	NA
49	27	F	CHL	Left cervical lymph node	+	0.25
50	27	M	CHL	Left neck mass	+	10.87
51	32	F	CHL (NSHL)	Right supraclavicular lymph node	+	0.02
52	71	M	CHL, favor NSHL	Left neck lymph node	+	0.10
53	25	M	CHL (NSHL)	Right deep axillary lymph node	+	0.05
54	20	M	CHL	Left axillary lymph node	+	2.60
55	18	F	CHL (NSHL)	Right cervical lymph node	+	0.17

CHL, classical Hodgkin lymphoma; HRS, Hodgkin and Reed-Sternberg; LRCHL, lymphocyte-rich classical Hodgkin lymphoma; MCHL, mixed-cellularity Hodgkin lymphoma; NA, not applicable; NSHL, nodular-sclerosis Hodgkin lymphoma; + or –, HRS population identified or not identified by the flow cytometry assay, respectively.
[a] % HRS cells is the percentage of the viable events that are HRS cells (as determined by the six-color assay).

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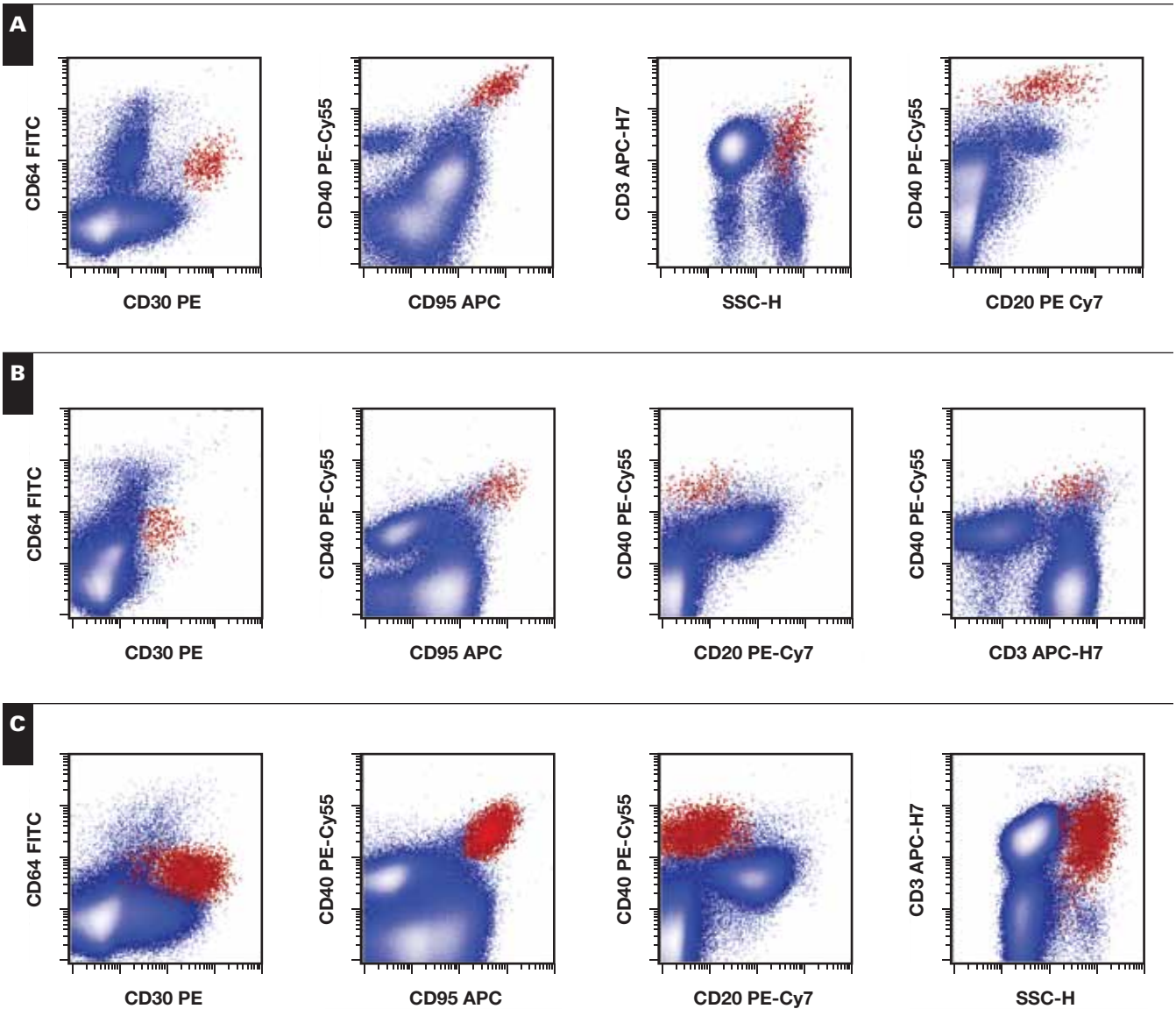

Image 2 Representative examples of flow cytometric studies of morphologically confirmed classical Hodgkin lymphoma (CHL) cases using the novel six-color flow cytometry assay. Hodgkin and Reed-Sternberg (HRS) cells (shown in red and emphasized) are identified by their expression of CD30, CD40, and CD95; significant autofluorescence in the fluorescein isothiocyanate (FITC) channel; absence of expression of CD64; and increased side light scatter (SSC-H) compared with normal lymphocytes (see Image 1 and Materials and Methods section). All remaining viable events are in blue. When present, the expression of CD3 suggests the presence of HRS-cell–T-cell rosettes. **A**, Case 39, Table 1. HRS cells have increased forward (not shown) and side light scatter, expression of intermediate to bright CD30, bright CD40 and CD95, intermediate CD3, and variable CD20, without expression of CD64 (position of negative determined by fluorescence minus one control experiment; data not shown). While the level of expression of CD20 is greater than seen in a typical case of CHL, the apparent coexpression of CD3 suggests the presence of HRS-cell–T-cell rosettes, supporting a diagnosis of CHL. In the paraffin section, the HRS cells had expression of CD30 and variable CD20, without expression of CD3. **B**, Case 11, Table 1. HRS cells have increased forward and side light scatter (not shown), expression of low to intermediate CD30, intermediate CD40, intermediate to bright CD95, and intermediate CD3, without expression of CD64 or CD20. In the paraffin section, the HRS cells had expression of CD30. **C**, Case 46, Table 1. Neoplastic HRS cells have increased forward (not shown) and side light scatter, expression of intermediate to bright CD30 and CD95, intermediate CD40, and intermediate CD3, without expression of CD64 or CD20. A prominent population of nonneoplastic CD30+ immunoblasts with lower autofluorescence compared with the HRS population can be seen on the plot of CD64 vs CD30. In the paraffin section, the HRS cells had expression of CD30, without expression of CD20 or CD3.

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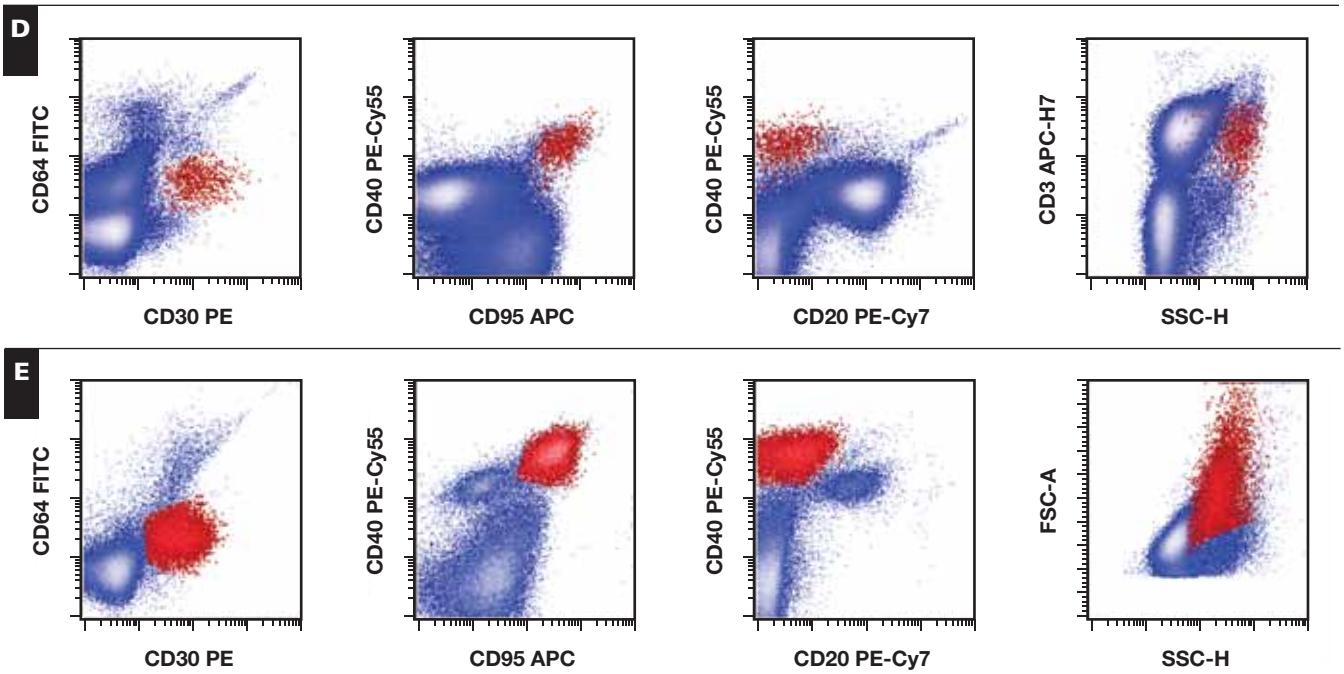

D, Case 32, Table 1. HRS cells have increased forward (not shown) and side light scatter; expression of intermediate CD3, CD30, and CD40; and intermediate to bright CD95, without expression of CD64 or CD20. **E**, Case 50, Table 1. HRS cells have increased forward and side light scatter, expression of low to intermediate CD30, and intermediate to bright CD40 and CD95, without expression of CD3 (not shown), CD64, or CD20. While this was favored to represent CHL, the numerous HRS cells suggested the possibility of a "gray zone lymphoma" (B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and CHL). In the paraffin section, the HRS had expression of CD30 without expression of CD3 or CD20, consistent with CHL. APC, allophycocyanin; Cy, cyanine; PE, phycoerythrin.

scatter, without expression of CD64. Apparent expression of CD3 was variable and, when present, was likely due to the presence of T-cell–HRS-cell rosettes. As expected given the above criteria defining a putative HRS population by FC, CD20 expression was usually negative; when present, CD20 expression was variable and low.

Discussion

Previous work in our laboratory has demonstrated that the HRS cells of CHL can be identified by nine-color FC,[17] allowing this unique type of B-cell neoplasm to be clinically immunophenotyped by this methodology. The work herein describes a six-color FC tube to immunophenotype the neoplastic HRS cells of CHL. When compared with the nine-color FC assay, the six-color assay demonstrates a similar diagnostic sensitivity and specificity. While the nine-color assay showed a perfect specificity of 100%,[17] the six-color assay incorrectly classified a single case (of peripheral T-cell lymphoma) as CHL (resulting in a 99.7% specificity). It is important to note that (for this false-positive case) HRS-like cells were present by immunohistochemistry (demonstrating expression of Pax-5, CD15, CD30, and variable weak CD20) and almost certainly represents the population identified by FC. The presence of HRS cells in T-cell lymphoma has been documented in the literature[19]; although their significance is uncertain, these cells are thought to be an epiphenomenon, unrelated to the T-cell lymphoma.[19] While this case was not analyzed by our nine-color assay that we use for routine clinical immunophenotyping of CHL, a prominent abnormal

Table 2
Morphologic Diagnoses of 408 Tissues Samples

No. of Cases	Diagnosis
133	Reactive changes of various types
55	Classical Hodgkin lymphoma
51	Follicular lymphoma
50	Large B-cell lymphoma (various morphologies)
25	Chronic lymphocytic leukemia/small lymphocytic lymphoma
23	Nonhematopoietic neoplasm
21	B-cell lymphoma, not otherwise specified
10	Marginal zone lymphoma
8	Mantle cell lymphoma
7	Miscellaneous T-cell non-Hodgkin lymphomas
6	Nodular lymphocyte-predominant Hodgkin lymphoma
5	Peripheral T-cell lymphoma, unspecified
4	Angioimmunoblastic T-cell lymphoma
3	Burkitt lymphoma
3	Composite lymphoma
2	Anaplastic large cell lymphoma
1	Lymphomatoid granulomatosis
1	Mycosis fungoides

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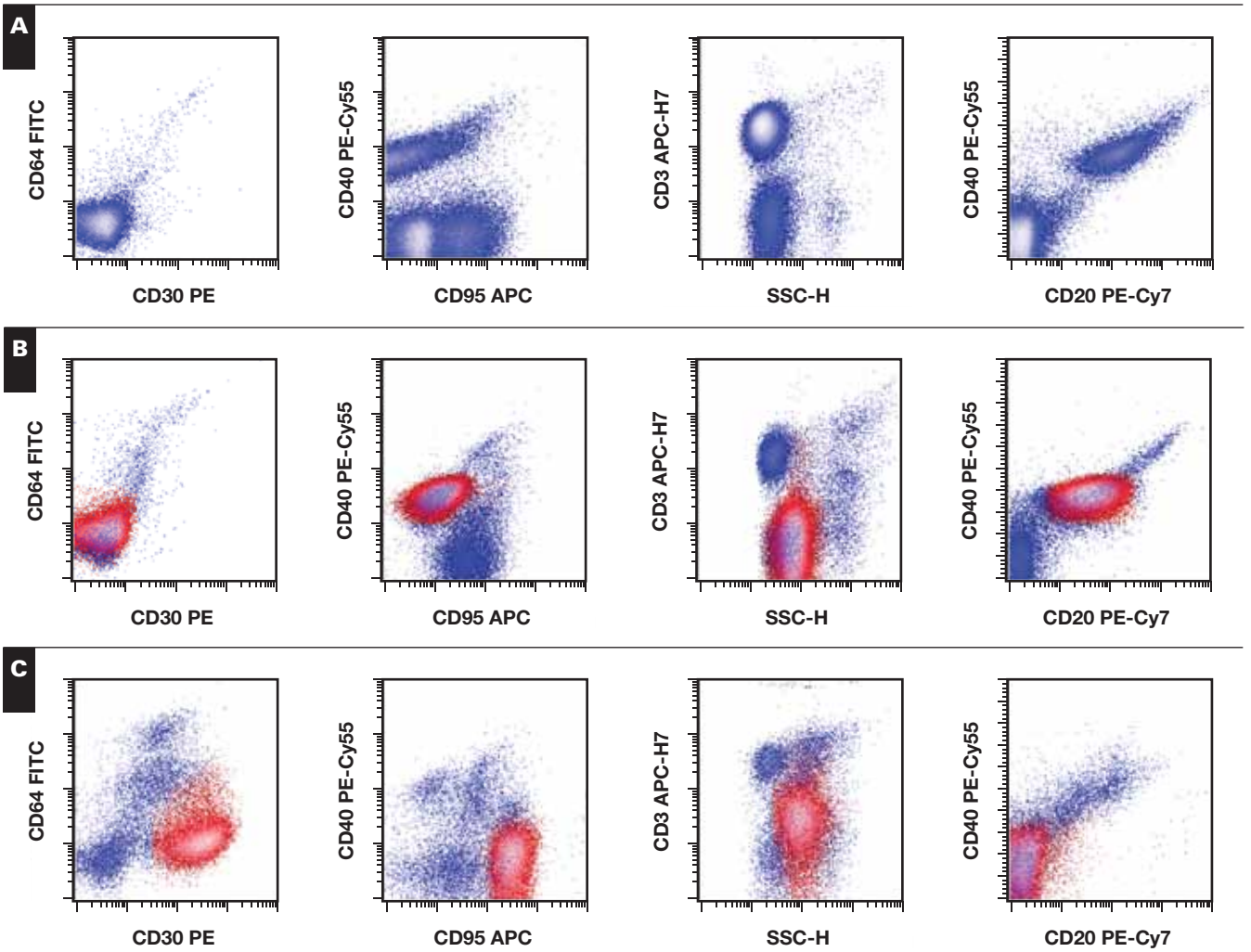

Image 3 Representative examples of flow cytometric studies of reactive tissues and non–classical Hodgkin lymphoma (CHL) neoplasms using the six-color flow cytometry assay. Neoplastic cells are shown in red; all other events are in blue. **A**, A reactive lymph node without a population showing increased side scatter (SSC-H) and expression of CD30, CD40, and CD95, excluding a diagnosis of CHL. **B**, Diffuse large B-cell lymphoma. The neoplastic population has expression of CD40, CD20, and CD95 (very low or absent) without expression of CD3, CD30, or CD64. The absence of CD30, very low CD95, and uniform CD20 exclude a diagnosis of CHL. **C**, ALK1– T-cell lymphoma, favoring peripheral T-cell lymphoma. The neoplastic population has increased forward (not shown) and side light scatter and intermediate to bright CD30 and CD95, without CD3, CD20, CD40, or CD64. The lack of CD40 expression excludes a diagnosis of CHL. The autofluorescence in the fluorescein isothiocyanate (FITC) channel is lower than that seen in most CHL cases. APC, allophycocyanin; Cy, cyanine; PE, phycoerythrin.

T-cell population was noted on routine immunophenotyping of the T cells, consistent with involvement of this lymph node by T-cell lymphoma. In addition, we have identified one further case in which a lymph node showed both abnormal T-cell and HRS-cell populations by T-cell and nine-color CHL FC immunophenotyping (not evaluated by the six-color assay); immunohistochemistry studies of this case also showed a T-cell lymphoma (peripheral T-cell lymphoma with features of angioimmunoblastic T-cell lymphoma) with the presence of HRS cells. Finally, two cases of chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) with Hodgkin cells on morphologic and immunohistochemical examination (see work by Momose et al[20] and Ohno et al[21]) have been identified in our laboratory with the nine-color assay in which both the CLL/SLL cells and the HRS cells were identified by multicolor FC (data not shown). These rare cases highlight the importance of incorporating the results of immunophenotyping for CHL with the other FC-derived immunophenotypic data to prevent misdiagnosis in routine clinical practice.

Eight of 55 morphologically confirmed CHL cases were misclassified by the six-color FC assay as negative for CHL,

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resulting in a sensitivity of 85.3% (sensitivity for the nine-color FC is 88.7%). Of these eight false-negative cases, one was mistakenly misclassified by the reviewer, since a CHL population was present when reviewed again, while a second case demonstrated an HRS-like population with bright CD40, CD30, and CD95 but with uniform intermediate CD20, thus arguing against this population as a putative HRS cell population according to the criteria described earlier. Interestingly, this case was correctly diagnosed as CHL using the routine clinical nine-color assay for CHL. It is important to note, however, that the reviewer of the nine-color assay had other information (clinical history, results from the FC analysis for B- and T-cell non-Hodgkin lymphoma, morphology of the cell suspension, etc) that may have influenced the interpretation. A third false-negative case was also correctly diagnosed as CHL using the nine-color assay; more important, this HRS population had very high expression of CD71, essential for identifying the population. The absence of CD71 in the six-color tube likely prevented this HRS population from being identified. For the other five false-negative results, the reason for the error in classification is unclear. Anecdotal evidence in our laboratory suggests that HRS cells degrade with time, potentially resulting in false-negative results. We cannot exclude other preanalytical factors, such as the inability to produce single HRS cell suspensions from areas of dense fibrosis, resulting in false-negative results, although there is no direct evidence to support this hypothesis.

As with the nine-color CHL assay, the six-color assay can correctly distinguish between CHL and other neoplasms with which it is frequently confused by morphology, such as DLBCL, NLPHL, and ALCL . None of the cases of DLBCL (n = 50), NLPHL (n = 6), or ALCL (n = 2) showed an HRS population, suggesting this assay is useful for helping to separate these differential diagnostic possibilities when an abnormal, FC-identified population is present. Specifically, DLBCL neoplastic populations identified in this six-color assay lack expression of uniform CD30, do not show increased side scatter to the extent of HRS cells (see examples in Image 2C and Image 3B), and usually show uniform CD20, all features that argue against the diagnosis of CHL. The neoplastic cells of NLPHL cannot be detected reliably by this assay; however, these cells should lack expression of CD30 and have uniform CD20,[14,22] which strongly argue against the conclusion that these are HRS cells. Finally, ALCL cells in this study and in our prior work express uniform CD30, lack expression of CD20, and often express bright CD95, similar to CHL. However, all ALCL cases that we have immunophenotyped with our six- or nine-color CHL FC tubes have had no or very low expression of CD40 (similar to that observed in tissue section[23-25]), always less than that of mature small B cells, excluding the diagnosis of CHL. Indeed, this six-color assay also readily immunophenotypes ALCL, a neoplasm that can be missed by conventional FC.

The six-color combination described above was selected since it should be able to be employed by FC laboratories using a six-color instrument with only minimal validation. Advantages of this (and the nine-color) assay over immunohistochemistry include turnaround times of a few hours at a lower cost. Our experience with the nine-color assay also suggests that morphology with immunohistochemistry does not always identify HRS populations.[17] Consequently, a useful diagnostic approach would be to immunophenotype lymph nodes by FC for CHL when (1) that diagnosis is likely on clinical grounds (ie, young patient age, clinician suspicion, etc), (2) numerous eosinophils and/or scattered very large atypical cells are seen on a cytopreparation made from the lymph node suspension, or (3) the T cells from the lymph node show increased CD45 and CD7 coexpression, a finding that has been shown to suggest CHL.[26,27] If the CHL tube shows a distinct and convincing HRS population, B- and T-cell tubes for non-Hodgkin lymphoma show no abnormalities, and the H&E tissue section morphology suggests CHL, a diagnosis of CHL can be made in the absence of immunohistochemistry. If the CHL tube shows no HRS population but the tissue section morphology suggests the possibility of CHL, the specimen can still be immunophenotyped by immunohistochemistry. This approach will ensure that any false-negative results by FC or morphology do not result in an erroneous diagnosis. Finally, if a putative HRS population is identified and sufficient material is available, a customized additional FC tube can be run containing CD15 and possibly CD123[28] to confirm a diagnosis of CHL if necessary.

Herein, we report on a six-color FC tube that can be used to immunophenotype CHL with high sensitivity and specificity in laboratories equipped with a six-color flow cytometer. Further work is ongoing in our laboratory to identify new immunophenotypic markers that improve the diagnostic sensitivity of this clinical assay.

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