J Korean Med Sci 1999; 14: 393-400
ISSN 1011-8934

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Flow Cytometric Immunophenotyping in Fine-needle Aspiration of Lymph Nodes

Fine-needle aspiration (FNA) of lymph nodes has been regarded as a useful method in the diagnosis of lymphadenopathy. However, this procedure has been shown to be of limited value in the diagnosis of low or intermediate grade malignant lymphomas in some studies. Immunophenotyping is an essential adjunct to cytomorphology for the diagnosis of lymphoma by FNA. Immunophenotyping using flow cytometry (FCM) is rapid, objective and reliable. Using FCM, multiparametric analysis of 33 FNA materials from lymph nodes was performed and profiles of surface markers of lymphoid cells were assessed. In reactive hyperplasia, patterns of cell surface markers were quite variable, but disclosed polyclonality. Most of the B-cell lymphomas showed immunophenotypes for B-cell lineages with their $\kappa:\lambda$ or $\lambda:\kappa$ ratio being over 3:1. In T-cell lymphomas, T-cell surface markers were predominantly expressed as well. In conclusion, our results suggest that immunophenotyping of lymph node aspirates is a valuable diagnostic adjunct for lymphoproliferative disorders, particularly in B-cell lymphomas because immunophenotyping can be easily and adequately performed by FCM.

Key Words: *Lymphoproliferative disorders; Flow cytometry; Immunophenotyping; Needle biopsy*

Jaegul Chung, Gyungyub Gong, Jooryung Huh, Shin Kwang Khang, Jae Y. Ro

Department of Pathology, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, Korea

Received: 12 October 1998
Accepted: 19 January 1999

Address for correspondence
Jaegul Chung, M.D.
Department of Diagnostic Pathology, Asan Medical Center, 388-1 Poongnap-dong, Songpa-gu, Seoul 138-736, Korea
Tel: +82.2-224-4560, Fax: +82.2-472-7898

INTRODUCTION

Fine-needle aspiration (FNA) cytology is an accurate, rapid, economic, minimally invasive and reliable procedure that is widely used in the initial evaluation of masses in a variety of organs including thyroid gland, salivary gland, breast as well as lymph node. FNA of lymph nodes has been regarded as a successful method in the diagnosis of lymphadenopathy (1-3). However, its usefulness in diagnosing lymphoproliferative disorders, except for Hodgkin's disease, has been questioned in some studies (4, 5). Actually, differentiation of small cell lymphocytic lymphoma from reactive hyperplasia has been reported to be extremely difficult to do solely based on the cytologic features of aspirates (6).

Since the application of flow cytometry (FCM) to diagnostic pathology (7), there has been a rapid progression of FCM in this field which has influenced virtually every area of diagnostic pathology, including DNA analysis of solid tumors (8), multiparametric analysis of normal lymphoid and myeloid cells as well as phenotyping of leukemia and lymphoma, multiparametric analysis of cells in patients with autoimmune diseases, and functional studies of cells and in the field of transplantations (9, 10). Moreover, earlier studies have compared the results of these parameters to established prognostic factors (histopathology and receptor status, etc), risk of recurrence and survival probability (11, 12). In applying FCM to lymphoid disorders, immunophenotyping and clonal analysis using lymphoid cell surface markers are particularly important (13-17). The accuracy of diagnosing lymphomas by FNA without immunophenotyping has been reported to be 72 to 86% (18-21). Immunophenotyping can often be an essential adjunct to cytomorphology for the diagnosis of lymphadenopathy by FNA (22-24). FCM has several practical advantages in immunophenotyping which includes following; diagnostic accuracy and reliability, requirement of low numbers of lymphoid cells, speed of processing, statistical precision and objectivity, potential for multiparametric studies and automated procedure (23, 25-28). Therefore, it is necessary to establish important diagnostic and prognostic parameters for the many types of lymphoproliferative diseases using both FNA and FCM as the definitive diagnosis and prognostic assessment (8, 29). Using FCM, we analyzed FNA materials for immunophenotypic profiles of lymphoid cells in various lymphadenopathies that were histologically confirmed.

MATERIALS AND METHODS

Clinical samples

FNA of lymph nodes was performed on 33 patients with lymphadenopathy. When malignant lymphoma was suggested, biopsy was done subsequently for histological confirmation. Most lymph nodes were located in neck (19 cases), inguinal area (4 cases) and supraclavicular area (2 cases) and the rest were located at the various sites (8 cases). All the lymph nodes were aspirated by pathologists. Two or more passes were performed with a 23-guage needle, a 10 mL syringe, and a syringe holder to obtain enough cells for the studies. The cells were submitted for both conventional morphologic examination and FCM for immunophenotyping. Air-dried and alcohol-fixed, direct smears were prepared and stained with Diff-Quik® and Papanicolaou stains, respectively. Immunocytochemistry was also performed, if available. For FCM, the remaining aspirate was flushed into cold RPMI 1640 medium and the used needle was also rinsed in it.

Flow cytometry

FCM was performed according to previously well-established methods (23, 24). For immunophenotypic analysis, single or simultaneous dual-color staining techniques were used and approximately 10^6 lymphocytes were incubated with fluorescein isothiocyanate (FITC)- and/or phycoerythrin (PE)-conjugated antibodies. Used antibodies were of B-cell lineage (CD19, CD20), T-cell lineage (CD3, CD5, CD7, CD4, CD8), antihuman κ and λ light chains, and their combination. Ten microliters of each monoclonal antibody were added to cells and incubated at 2-8℃ for 15 min in dark chambers. When contaminated by red blood cells, samples were lysed with ammonium chloride lyse x10 solution for five min and then washed. The cells were resuspended, fixed with 1% paraformaldehyde, and analyzed with consort 30 program run on Epics-XL (Coulter®).

RESULTS

Cytological and/or histologic examination

Thirty-three cases were diagnosed as reactive hyperplasia (16 cases), chronic granulomatous inflammation (CGI) (3 cases), necrotizing lymphadenitis (1 case), leukemic infiltration (1 case), non-Hodgkin's lymphoma (NHL) (10 cases), Hodgkin's disease (1 case) and metastatic carcinoma (1 case). In reactive lymph nodes, there were polymorphous lymphocytic infiltrates with predominantly small lymphocytes, and occasional plasmacytoid lymphocytes and immunoblasts. Tingible body macrophages were easily seen as well as occasional sinus histiocytes. In two of three cases of CGI, there were well-defined granulomas consisting of epithelioid histiocytes and surrounding small lymphocytes. A remaining case of CGI revealed extensive caseous necrosis and numerous acid-fast bacilli without obvious granuloma formation. Aspirates of necrotizing lymphadenitis consisted of phagocytic histiocytes with twisted nuclei and abundant cytoplasm containing karyorrhectic granular debris. Extracellular karyorrhectic debris, lymphocytes and immunoblasts were also prominent. However, neutrophils were strikingly lacking. The patient with leukemia was previously diagnosed as acute myeloblastic leukemia.

Among the 10 cases with NHLs, two cases showed mixed populations of small- to medium-sized cells with irregular nuclear contours, whereas another two cases were characterized by a biphasic population of small cleaved and large atypical lymphocytes without tingible body macrophages. Another three smears were composed of large atypical lymphocytes with irregular nuclei, coarse chromatin, and one or more nucleoli. There were also variable numbers of immunoblasts. In two cases, immunoblasts with prominent central nucleoli were present with a few small mature lymphocytes. Necrosis and mitotic figures were also common. The smears from the last case displayed large monotonous lymphocytes with high nuclear/cytoplasmic ratio. Cells had scanty cytoplasm and large nuclei with fine chromatin and indistinct nucleoli.

Immunocytochemical examination revealed that five were of B-cell lineage, whereas remaining five cases were T-cell lymphomas. By Working Formulation, five B-cell lymphomas were classified as diffuse small cell (2 cases), mixed small and large cell (1 case), diffuse large cell (1 case) and immunoblastic type (1 case). Five T-cell lymphomas were classified as mixed small and large cell (1 case), diffuse large cell (2 cases), lymphoblastic (1 case) and immunoblastic type (1 case). In one Hodgkin's disease, the smears consisted predominantly of small mature lymphocytes and a few histiocytes. There were occasional "popcorn" cells. Diagnostic Reed-Sternberg cells were rarely seen. The case was diagnosed as lymphocyte-predominance type. The metastatic carcinoma was from the breast primary.

Flow cytometric immunophenotyping

The results of flow cytometric immunophenotyping are summarized in Table 1 and 2.

In reactive hyperplasia, patterns of cell surface markers were quite variable (Fig. 1). The numbers of cases showing T- and B-cell predominance were nearly equal (9:7)

Table 1. Immunophenotypes of non-neoplastic lymphoid disorders (%)

Case	Diagnosis	CD3	CD5	CD7	CD4	CD8	CD20	CD19	κ/CD19	λ/CD19
1	RH	29.0							29.4/70.7	27.9/70.7
2	RH	47.2	47.1		36.8	7.4		51.1		
3	RH	33.0					63.4		27.3/63.2	20.4/63.2
4	RH	41.5	42.3				57.9	58.7		
5	RH	56.8	42.8		27.1	11.8	12.2		3.9/9.8	4.5/9.8
6	RH	28.9	33.0		27.2	5.5	7.9		5.1/8.8	3.9/8.8
7	RH	60.7			60.4	6.2	41.4	43.6		
8	RH	45.1					56.0		39.5/64.0	24.7/64.0
9	RH	54.1					45.8		23.1/42.5	18.4/42.5
10	RH	10.8			8.3	1.9	13.4		6.2/11.8	3.7/11.8
11	RH	73.3			70.5	5.1	26.5		11.0/20.2	8.1/20.2
12	RH				66.6	11.2	26.9		16.5/27.1	10.4/27.1
13	RH	72.7	73.7						12.6/24.6	13.4/24.6
14	RH	67.2	71.0						16.3/30.6	13.5/30.6
15	RH	36.2	33.4				65.6		32.1/63.4	24.6/63.4
16	RH	67.4	66.4				33.6		18.0/34.6	15.5/34.6
17	NL	38.1	35.5		17.9	16.3	65.7			
18	CGI	90.8			76.5	13.0	8.6			
19	CGI	67.1					31.3			14.4/29.3
20	CGI	74.2			51.9	24.4	21.6	20.1		

RH, reactive hyperplasia; NL, necrotizing lymphadenitis; CGI, chronic granulomatous inflammation

Table 2. Immunophenotypes of neoplastic lymphoid disorders (%)

Case	Diagnosis	CD3	CD5	CD7	CD4	CD8	CD20	CD19	κ/CD19	λ/CD19
21	LEU	10.4			5.2	4.5			1.0/3.7	0.6/3.7
22	MLT		91.0	93.2						
23	MLT	54.4	63.8		19.1	28.0		45.0		
24	MLT	55.9	92.0	93.2	43.2	28.5				
25	MLT	28.9	31.9					28.5		
26	MLT	60.3			33.6	27.5			16.9/39.9	16.3/39.9
27	MLB	19.9	2.2				58.1		57.7/59.0	0.1/59.0
28	MLB	2.8							0.1/95.5	0/95.5
29	MLB	22.1	30.7						47.8/49.3	1.6/49.3
30	MLB	26.3			4.9	20.0	26.9		0.8/24.7	21.6/24.7
31	MLB	33.5			9.3	9.6	17.4		12.8/34.2	7.9/34.2
32	HD	43.3					1.4		2.1/2.6	2.3/2.6
33	MCA	79.7	80.7						9.7/18.3	8.3/18.3

LEU, leukemic infiltration; MLT, T-cell lymphoma; MLB, B-cell lymphoma; HD, Hodgkin's disease; MCA, metastatic carcinoma

and specific expression patterns of surface markers were not found except for polyclonality. The proportions of T-cell markers varied from 10.8 to 73.7%, as well as those of B-cell markers from 7.9 to 70.7%. T-cell subset markers (CD4 and CD8) were examined in seven cases and all of them were CD4+ cell dominant. The ratio of CD4+ to CD8+ cells were 2.3:1 to 13.8:1. Thirteen cases were examined for κ and λ light chain expression. Of those, cells expressing κ light chain were relatively predominant in 11 cases. Their $\kappa:\lambda$ or $\lambda:\kappa$ ratio were polyclonal with less than 2:1 or 1:2, repectively. In two cases, there were only a minority of populations of lymphoid cells expressing surface markers (less than 33%, case 6 and 10).

In all three cases of CGI, there was a T-cell predominance (67.1-90.8%) (Fig. 2). But, a lymph node diagnosed as necrotizing lymphadenitis consisted of B-cell rich pattern (65.7%).

The case (case 21) with leukemic infiltration revealed low population of background T and B cells. Four of five cases with B-cell NHL showed B-cell surface markers with all of their $\kappa:\lambda$ or $\lambda:\kappa$ ratio being over 3:1 (Fig. 3). A remaining case of B-cell lymphoma (case 31) showed nearly equal numbers of B and T cells with slightly

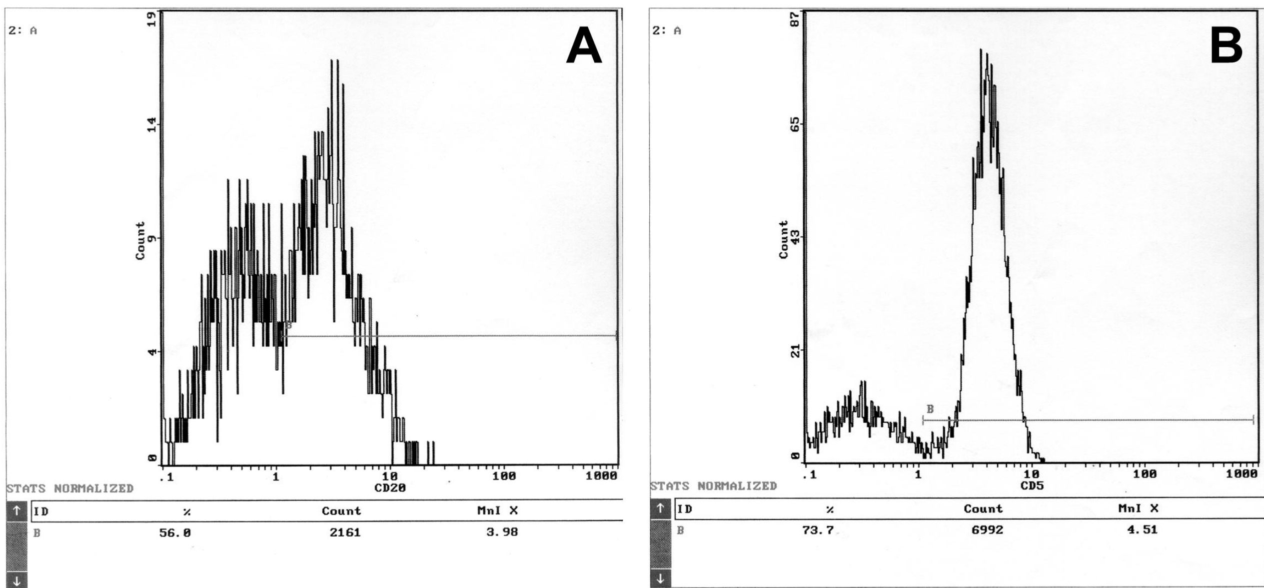

Fig. 1. Histograms of two cases of reactive hyperplasia (case 8, A and case 13, B). Expression patterns of surface lymphocyte markers are variable. In the A, B-cells (CD20+) are predominant (56.0%) while T-cells (CD5+) are predominant (73.7%) in the B.

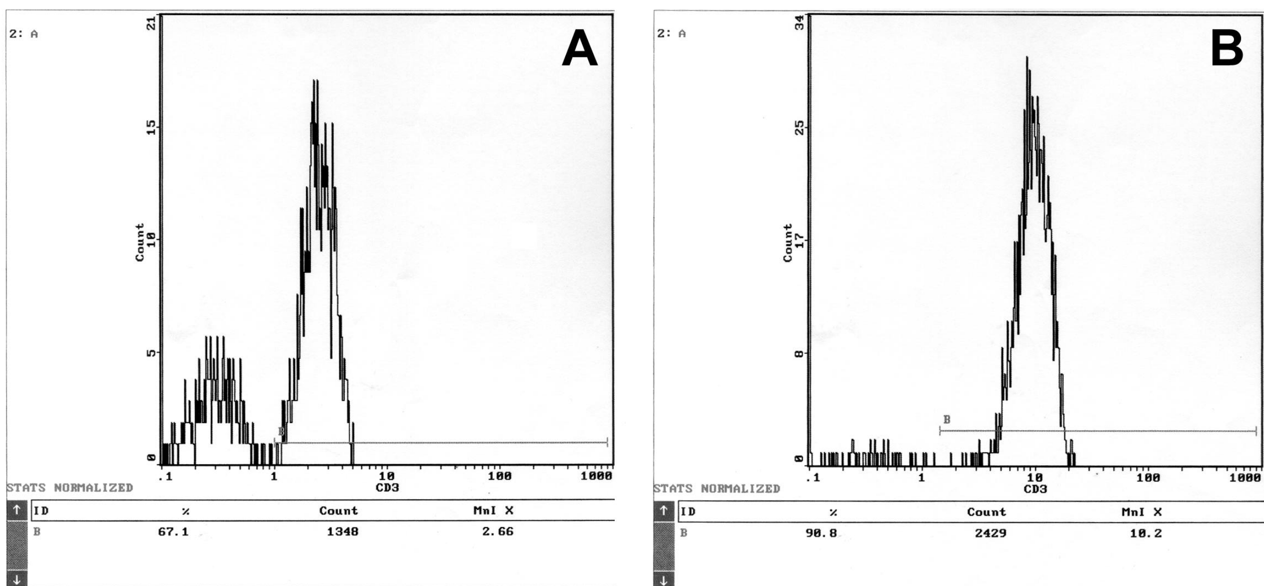

Fig. 2. Histograms of two cases of chronic granulomatous inflammation (case 18 and 19). Note T-cell (CD3+) predominant immunophenotype (90.8 and 67.1%).

dominant CD4+ cells and its $\kappa:\lambda$ ratio was less than 2:1. Four of five cases with T-cell NHL revealed predominant T-cell surface markers (54.4-93.2%) (Fig. 4A). Their CD4/CD8 ratio was variable, ranging from 2:1 to 1:2, and no dominant T-cell subset was found. The last case of T-cell NHL (case 25) showed relatively low and equal population of B- and T-cells (Fig. 4B). A Hodgkin's disease showed relative T-cell rich population (43.3%). The background lymphoid population of metastatic carcinoma largely consisted of T cells (80.7%).

DISCUSSION

FNA provides a convenient and rapid alternative to more invasive surgical approaches for the diagnosis of many neoplastic and non-neoplastic disorders including lymphoproliferative diseases. However, there have been split reports regarding its accuracy, reliability, and usefulness compared with biopsy (18, 19, 30-39). Some lymphoproliferative disorders may be difficult to differentiate from benign lymphoid or other neoplastic processes using

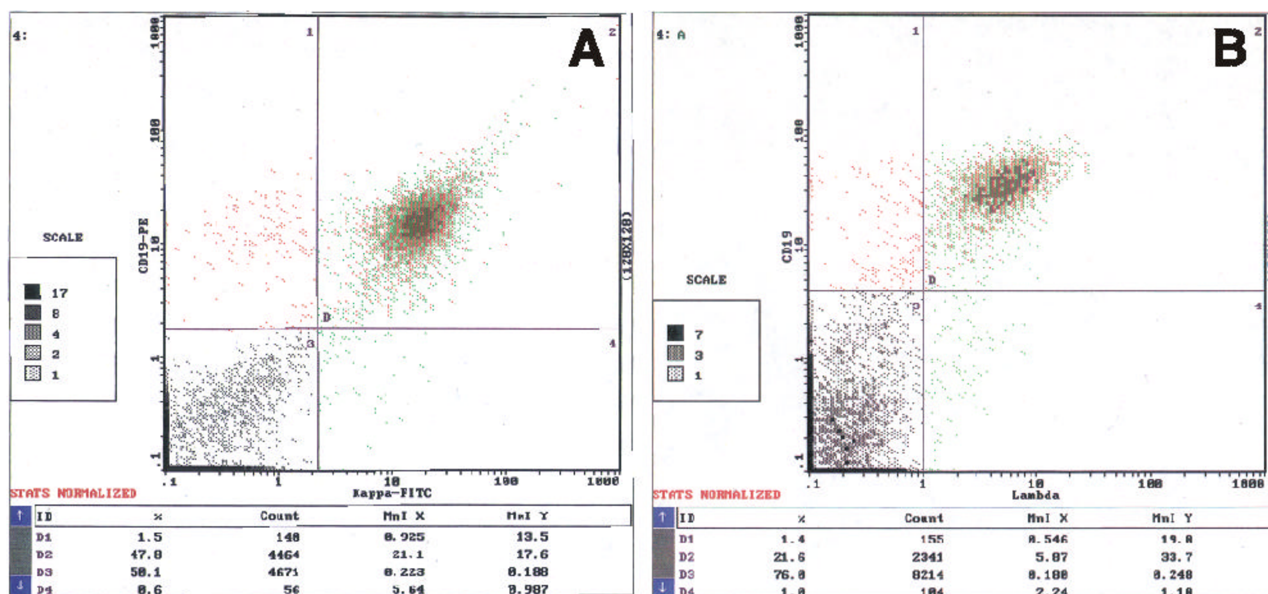

Fig. 3. Dual-fluorescence, flow cytometric analysis of κ- (case 29, A) and λ- (case 30, B) expressing B-cell lymphomas. Red and green fluorescences are shown on vertical axis (CD19) and on horizontal axis (κ or λ light chain), respectively. Note κ/CD19 (zone D2/D1+ D2=47.8/49.3%) in A and λ/CD19 ratio (zone D2/D1+D2=21.6/24.0%) in B.

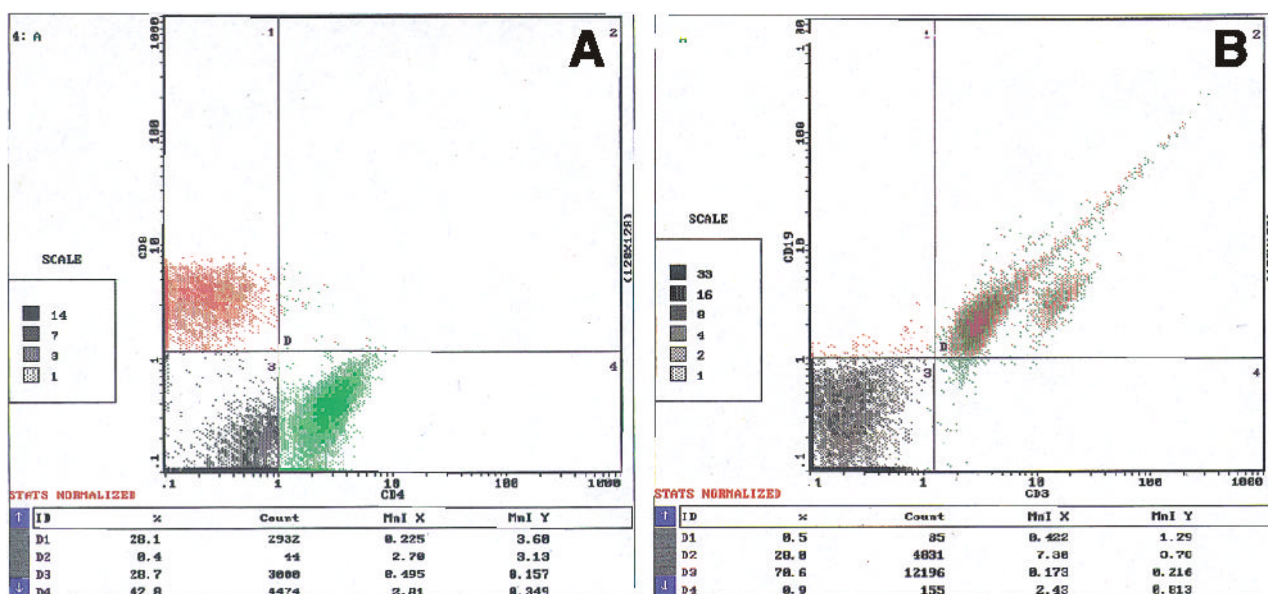

Fig. 4. Dual-fluorescence, flow cytometric analysis of two cases of T-cell lymphomas. In the A (case 24), red fluorescence is shown on vertical axis (CD8) and green fluorescence is on horizontal axis (CD4). Note dominant T-cell population (CD8+ plus CD4+ cells, zone D1+D2+D4+D2=71.7%). In the B (case 25), CD19+ cells are shown on vertical axis as red fluorescence and CD3+ cells are shown on horizontal axis as green fluorescence. Note nearly equal numbers of B- (zone D1+D2=28.5%) and T-lymphocytes (zone D4+D2=28.9%).

conventional cytologic techniques alone, because of lack of overtly abnormal morphologic features. Although these problems have been addressed in some studies by the application of immunohistochemistry, the subjectivity occasionally interfers by staining artifacts and the semi-quantitative nature of this approach have been criticized because of the utility of this technique (1, 40-45).

Recently, FCM analysis has shown that it is a more objective diagnostic tool to the workup of FNA of lymphoid diseases (8, 13-17, 23, 29). Because cells in a suspension are analyzed using physical characteristics and multiple markers, the data can be provided in an objec-

tive fashion. Because a large number of cells are studied, small populations of malignant cells can be detected. The main disadvantage of immunotyping with FCM is difficulty in direct visual correlation with morphology as well as needs for expensive equipment and highly trained personnel. Therefore, the conventional cytomorphologic analysis is essential for diagnosis. Using these multiparametric assessment, FCM analysis can complement traditional morphological assessment of FNA of lymphoid diseases and correct diagnosis may be maximized if a coordinated effort is made to integrate morphology and immunophenotyping fully if possible (23, 25-28). We analyzed the immunophenotypic profiles of various lymphoid diseases, which had been previously diagnosed.

In reactive hyperplasia, expression patterns of surface markers in lymphoid cells were quite variable. There were nearly equal numbers of cases showing T- or B-cell predominance. The proportions of T-and B-cell markers ranged from 10.8 to 73.7% and from 7.9 to 70.7%, respectively. These features represent random proliferation of lymphocytes in reactive conditions. The hallmark of cytomorphology of reactive lymph nodes is the polymorphic cell population. Small lymphocytes always predominate and may comprise the majority of cells in a smear from benign lymph nodes. There can be also other lymphoid cells including plasmacytoid lymphocytes, large immunoblasts, plasma cells, and tingible body macrophages. Atypical lymphoid cells were not predominant in any reactive lymph nodes. This polymorphous nature of reactive lymph nodes is compatible with immunophenotypes by FCM. In seven cases T-cell subsets (CD4 and CD8) revealed CD4+ cell dominant. The ratio of CD4+ to CD8+ cells were from 2.3:1 to 13.8:1. Thirteeen cases were examined for κ and λ light chain expression. Among them, more κ light chain expressed in 11 cases with their ratio being less than 2:1 or 1:2. Results of reactive lymph nodes in this study were similar to those of normal lymph nodes. Ratio of CD4+ to CD8+ cells in reactive lymph nodes suggested dominant T-helper cell population in reactive conditions. According to the previous criterion (23, 24, 44), $\kappa:\lambda$ or $\lambda:\kappa$ ratio greater than 3:1 was regarded as monoclonal. If more than 70% of morphologically atypical cells expressed one or more T-cell antigens, manifested aberrant marking patterns, or expressed subset (helper or suppressor) predominance, a diagnosis of T-cell lymphoma was suggested. So, $\kappa:\lambda$ or $\lambda:\kappa$ ratio in this group proved these lesions being reactive or polyclonal and morphologically atypical cells were not found in lymph nodes with T-cell predominance. In morphologically equivocal cases, variable expression of cell markers and subset patterns of T- or B-cells may help differentiate reactive hyperplasia from lymphoreticular malignancy, particularly by $\kappa:\lambda$ or $\lambda:\kappa$ ratio.

In all three cases with CGI, there was T-cell dominance. It has been reported that T-lymphocytes play a major role in the formation of granulomas (46-47). In our FCM studies, T-cell predominance was also observed. Since the presence of overt granuloma is essential for the diagnosis of CGI, the importance of these results might be attenuated. In extensively necrotic specimen, however, the immunophenotyping and/or special stain such as acid-fast stain may help the diagnosis of CGI without granuloma. One necrotizing lymphadenitis in this study largely showed CD20+ cells. The importance of this finding is unclear yet and would be assessed further with more cases.

One lymph node of leukemic infiltration showed few cells expressing T- or B-surface markers. The original diagnosis was acute myeloblastic leukemia. By cytologic examination, the aspirates mostly consisted of neoplastic myeloblasts and scattered mature lymphocytes. The finding of low cellularity of T- and B-cells merely suggests that most neoplastic myeloblasts displayed neither T- nor B-cell differentaition and only small mature lymphocytes expressed lymphocytic surface markers. To generalize the findings, studies of more samples would be neccessary. Four of five cases with B-cell NHL showed dominant population of B-cell surface markers (49.4-95.5%) and all their $\kappa:\lambda$ or $\lambda:\kappa$ ratios were over 3:1 (from 27:1 [21.6: 0.8] to infinite [0.1:0]). In accordance with previously set criterion, a $\kappa:\lambda$ or $\lambda:\kappa$ ratio greater than 3:1 was considered as monoclonal (23, 24, 44). By this criterion, these cases were regarded as monoclonal and were consistent with B-cell lymphoma. In addition to usual cytological analysis, these immunophenotypic features with $\kappa:\lambda$ ratio may help diagnose B-cell lymphoma. Only one B-cell lymphoma (mixed small and large cell type) showed nearly equal and small numbers of B- and T-cells and its $\kappa:\lambda$ ratio was less than 2:1. Four of five cases with T-cell NHL elaborated predominant T-cell surface markers (54.4-93.2%). These findings were consistent with the diagnosis of T cell lymphoma. So, FCM immunophenotypic results might be also characteristic with preliminary cytomorphometric examination. However, their CD4/CD8 ratios ranged from 2:1 to 1:2 and no dominant T-cell subset was found. These findings suggested that FCM immunophenotyping could not differentiate T-cell NHL from reactive hyperplasia. Moreover, one case of T-cell NHL (lymphoblastic lymphoma) showed relatively low and equal population of both B- and T-cells. In the diagnosis of T-cell lymphomas, therefore, careful cytomorphologic examination may be more important than that of B-cell lymphoma. Once the diagnosis has already been established, however, the FCM can be performed adequately for immunophenotyping of the tumors. One case of Hodgkin's disease showed a rela-

tively abundant T-cell rich population (43.3%). The lymphoid population of metastatic carcinoma largely consisted of T-cells (79.7-80.7%). In host defense mechanism against tumor, the role of cytotoxic T-lymphocytes is well established. They seem to act as protective antitumor effector. In current case, T-cells in metastatic lymph node appear to exhibit cytotoxicity against metastatic tumor cells. In these limited number of cases, however, the significance of these results cannot be accurately assessed. More cases may be needed for further studies.

In conclusion, immunophenotypic profiles using FCM were analyzed in lymph node aspirates and by FCM, immunophenotyping can be performed easily and adequately. In reactive hyperplasia, there were various patterns of lymphoid cell surface markers. The immunophenotyping of kappa and lambda light chains showed that it could be a valuable method for the diagnosis of B cell lymphoma from the reactive processes. Most T-cell lymphomas were also T-cell predominant. Our results suggest that immunophenotyping is a valuable adjunctive technique for the diagnosis of lymph node aspirates.

ACKNOWLEDGEMENTS

This work was supported by a research grant (1997) from the Asan Institute for Life Sciences.

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