Med. J. Kagoshima Univ., Vol. 47, Suppl. 2. 157~162, November, 1995

Case Report

An Analysis of Pre-B Cell Lymphoblastic Lymphoma of the skin by Using 3-Color flow Cytometry

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Summary

A case of CD45-negative, CD10-positive lymphoblastic lymphoma (LBL) with precursor B lymphocyte (pre-B) phenotype, was analyzed by using 3 color flow cytometry (3-FCM) in addition to morphological observation and immunohistochemistry.

The difference of antigen expression as defined by the fluorescent intensity of FCM was significantly clear in CD20 and CD22 between lymphoma cells and normal B lymphocyte. Thus, the lymphoma cell population could be cleary separated on the three dimensional cubic representation using PAINT-A-GATE software. Also, cytoplasmic IgM was easily demonstrated by the combined use of both flow cytometry and immunohistochemistry. The former showed negative and the latter positive for IgM.

For a better clinical application of the detailed phenotypic results examined by 3-FCM, it could be considered that the unusual expression or alternation of B cell-lineage antigens and defectiveness of common antigen may be useful to detect small number of lymphoma cells in peripheral blood or bone marrow.

Key words: Pre-B cell lymphoblastic lymphoma, skin, 3-color flow cytometry

Introduction

Lympoblastic lymphoma (LBL) with a precursor B lymphocyte phenotype (pre-B LBL) is a relatively rare subtype of malignant lymphoma[1,2,3]. It frequently occurs in the skin, but differs from primary cutaneous B cell lymphoma in cellular morphology or phenotype[4]. We applied 3-color flow cytometry (3-FCM) to a case of pre-B LBL and discussed its utility in phenotypic examination and the possibility of better clinical applications.

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Case report

The patient, an 11-year-old girl, was hit on the forehead by the tip of a freind's umbrella, and a hematoma formed there. The lesion did not regress, and gradually increased in size. The lesion was compressed following a clinical diagnosis of "hypertrophic scar", but it gradually increased in size, to 4×4×1 cm. A diagnosis of pre-B LBL was made based on a biopsy specimen from the lesion on the forehead. No atypical cells were identified in the peripheral blood, defined by both morphological observation and 3-FCM analyses. She received combined chemotherapy and has been in complete remission for two years.

Materials and Methods

Freshly obtained biopsy material and peripheral blood were submitted for multiparameter analysis as follows:

Morphological examination

A section of formalin-fixed, paraffin-embedded specimens was stained with routine hematoxylin and eosion for histological diagnosis. A Giemsa stain of touch preparation was used for cytological observation. Small pieces of material were fixed in 2 % glutaraldehyde in a 0.1 mol cacodylated buffer (pH 7.3) and processed for electron microscopic examination.

Three color flow cytometry

Suspended cells separated from the tumor were mixed with three kinds of monoclonal antibodies, each of which was directly conjugated with FITC (fluorescein isothiocyanate), PE (phycoerythrin) and PerC-P(peridinin chlorophyll protein). The combination of antibodies is listed in Table 1. Each sample was washed in PBS and analyzed by FACScan using PAINT-A-GATE software (Becton-Dickinson Immunocytometry Systems USA). Whole peripheral blood was analyzed with a combination of CD10-CD19-CD45, CD20-

CD22-CD45, according to the phenotypic results.

Immunohistochemistry

Frozen sections of material, pre-fixed in 1 % paraformaldehyde, were washed in PBS with 20 % sucrose, then stained with the same kinds of non-labeled antibodies as in Table, using the avidin-biotin peroxidase method. In addition, anti-TdT(terminal deoxynucleotidyl transferase) antibody was also used.

Southern blotting

High-molecular DNA samples were extracted from the tumor and digested with the restriction enzymes BamHI, EcoRI and Hind Ⅲ. They were analyzed by the method, of Southern, using DNA probes for human T-cell receptor beta, gamma and delta, JH, kappa and lambda.

Results

Histologically, the lesion showed an obvious Grenz zone between the epidermis and the intradermal lesion (Fig 1-a). Atypical cells proliferated diffusely with scattered collagen bundles in the dermis. No relationship between the atypical cells and the glandular epithelia of sweat glands was noted. The cells, ranging

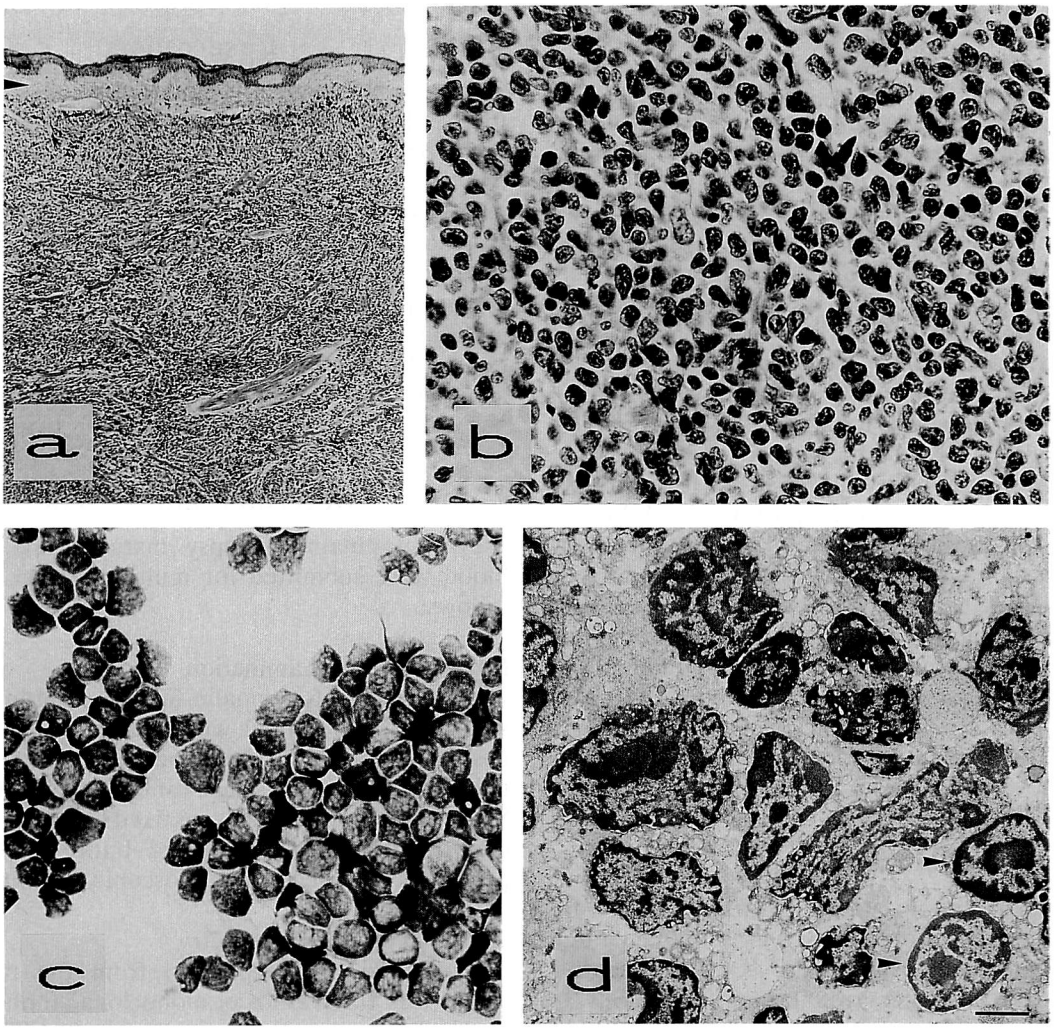

Fig. 1-a. Low power photomicrograph of the tumor. Obvious Grenz zone (arrow head) is identified between the epidermis and the intradermal lesion (H&E stain, 53X).

Fig. 1-b. High power photomicrograph of the lymphoma cells. They ranged from 6 to 8 microns in diameter, and have hyperchromatic, indented or irregular nuclei with high N/C ratio (H&E stain, 264X).

Fig. 1-c. A touch preparation of the tumor. The scant cytoplasm and an incomplete nuclear cleavage, and a course chromatin distribution are seen. No granular component are identified in the cytoplasm (Giemsa stain, 264X).

Fig. 1-d. Electron micrograph of the lymphoma cells. The nuclear features are better defined than those of the histological section. The cells with arrows are considered to be normal lymphocyte (bar=2micrometer).

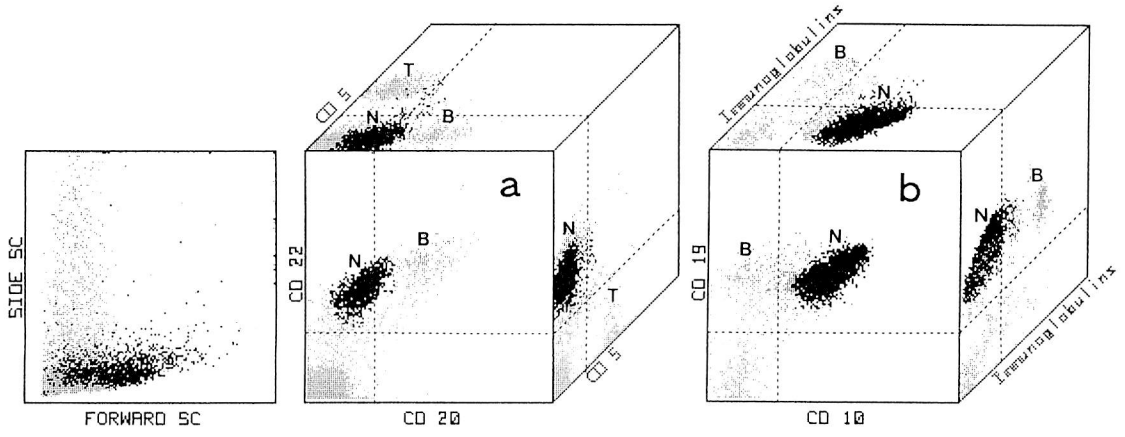

Fig. 2. A scattergram (left) and cubic representations of 3-color flow cytometry. The scattergram shows the population of dark black dots are mainly located in the middle of the forward scattergram and low side scattergram. a) The X-axis represents CD10, the Y-axis represents CD19 and the Z-axis represents immunoglobulins. The dark black dots, $CD10^{+}CD19^{+}Igs^{-}$cells, are recognized as an unusual cell population apart from normal Blymphocyte (B), $CD10^{-}$ $CD19^{+}Igs^{+}$. This is because the double positive cells for CD10 and CD19 are not identified in the skin of a normal indivedual, except for germinal center cells if there were lymphoid follicles. b) In this cubic representation, the X, Y and Z-axis represent CD20, CD22 and CD5, respectively. The dark black dots are completely negative for CD5, very slightly duller in CD22, and much duller in CD20 than those of normal B lymphocytes (B) which have almost the same fluorescent intensity of CD20 and CD22. Thus, the dark black populations in a) and b) are considered to be lymphoma cells, and they are also clearly demonstrated to be separated from the normal B lymphocyte in this representation.

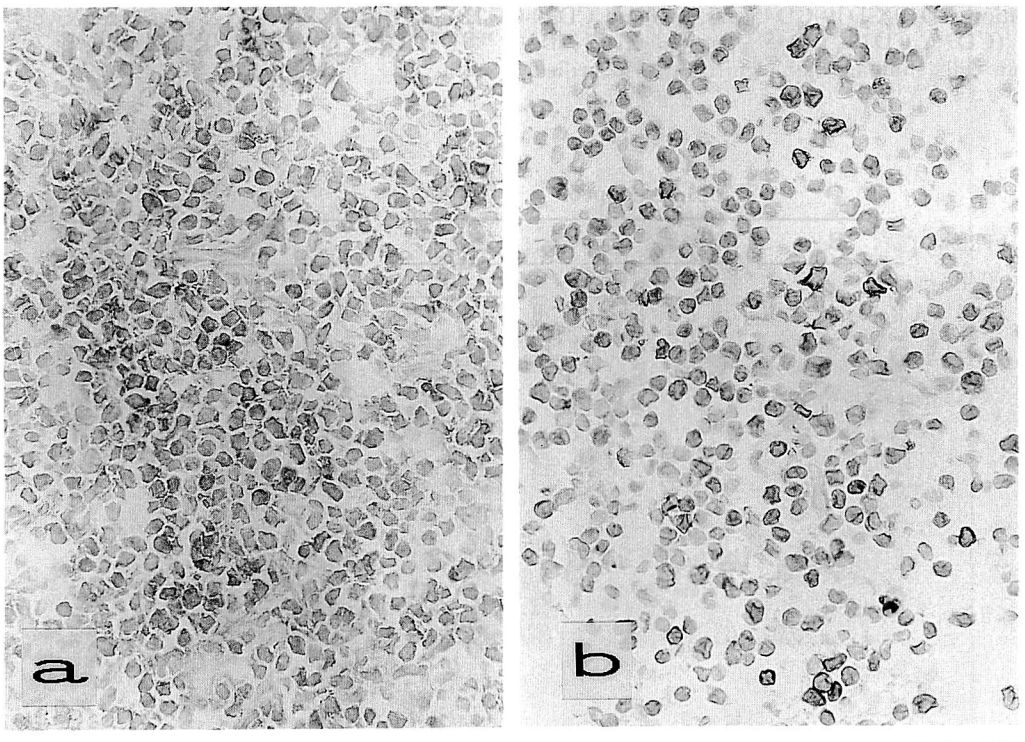

Fig. 3. Immunohistochemical demonstration of CD19(a) and Ig M(b). a) Reactivity in lymphoma cells for CD19 are linear in general. b) Although a positive reaction for IgM is not always identified in lymphoma cells, and its reactivity is recognized along the nuclear shape. This is apparently different from that of CD19. The intensity of the reaction is weak in almost all lymphoma cells and is strong in some ones. These findings suggest the existence of cytoplasmic IgM, since no surface immunoglobulins were detected by flow cytometry.

in size from 6 to 8 microns in diameter, were small and had a high N/C ratio (Fig 1-b). This feature was better defined by electron microscoopy (Fig.1-c). The shape of the unclei was a small cleaved one, and the lymphoma cells of the Giemsa stain on the touch preparation were categorized in L2 according to the FAB classification (Fig.1-d).

CD 10 and CD 19 were found to be positive for the same cell population and simultaneously negative for surface immunoglobulin (Fig. 2-a), under 3-FCM analysis using a three dimensional cubic representation on PAINT-A-GATE software. Lymphoma cells tested positive for CD20 and CD22, but negative for CD45, kappa, lambda, IgG, IgA and IgD, any T cell-lineage antigens and myeloid antigen, on both 3-FCM and immunohistochemistry (Table and Fig.-a). However,

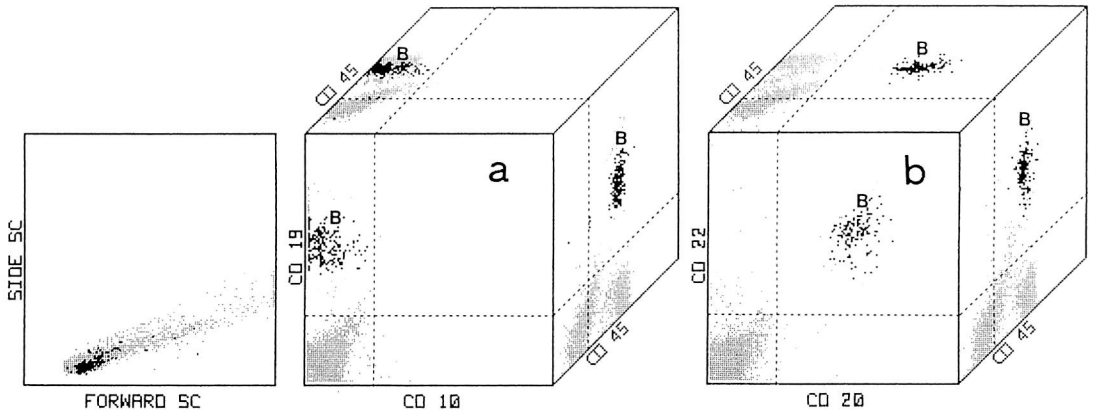

Fig. 4. The scattergram and cubic representation of the peripheral blood by 3-FCM. Analyzed cells were gated on cells only with a lymphoid appearance on the scattergram (left) in order to devoid non-specific fluorescence signals and to raise the sensitivity. Dark black dots are recognized as normal B lymphocytes in both cubic representations with antibody combination CD10-CD19-CD45(a:7.1%) and CD20-CD22-CD45(b:5.8%), X-Y-Z axes respectively. Cells with an unusual ($CD10^{+}CD19^{+}CD45^{-}$) or defective ($CD20^{+very\ dull}CD22^{+dull}CD45^{-}$) expression are considered to be lymphoma cells, but are not significantly identified in both.

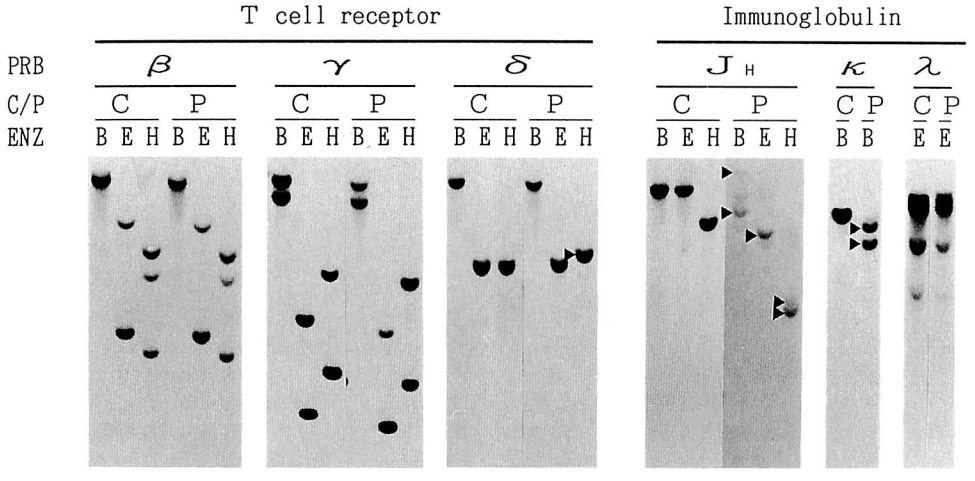

Fig. 5. The results of the Southern blot analysis. The rearranged fragments were seen in the heavy chain and the kappa chain as non-germ line bands. There were no rearranged bands in the lambda chain gene or the T cell receptor subunit gene, except a Hind Ⅲ digestion band in the TCR delta. PRB:probes, C:negative control, P:patient, ENZ:restriction enzymes.

since the fluorescence intensity of CD22 was slightly weak and that of CD22 was much weaker, the lymphoma cell population could clearly be separated by normal B lymphocytes on the cubic representation (Fig 2-a).

Although the surface IgM was negative in the lymphoma cell population using flow cytometry, IgM was demonstrated by immunohistochemistry (Fig.3-b). This suggested the existence of cytoplasmic IgM (cIgM). TdT was negative in lymphoma cells.

No atypical cells with defective or unusual phenotypic expression were detected by 3-FCM (Fig 4) and morphologic observation of the peripheral blood film.

In DNA analysis, rearranged fragments were seen in a heavy chain and the kappa chain. There were no rearranged bands in the lambda chain gene of the T cell receptor subunit gene, except a Hind Ⅲ digestion band in TCR delta (Fig 5). Karyotypic examination failed because no mitotic figures were obtained from the culture.

Table. Panel of monoclonal autibody conbinations for 3-FCM and the results of reactivity in lymphoma cells difined by 3-FCM.

	FITC	PE	PerCP
1.	EMA$^-$	CD33$^-$	CD45$^-$
2.	Kappa$^-$	Lambda$^-$	CD19$^+$
3.	IgA$^-$	IgG$^-$	CD19$^+$
4.	IgD$^-$	IgM$^-$	CD19$^+$
5.	CD10$^+$	CD19$^+$	Igs(μ, γ, α)$^-$
6.	CD5$^-$	CD22^{+dull}	CD20$^{+very\ dull}$
7.	CD7$^-$	CD2$^-$	CD3$^-$
8.	TCR$\alpha\beta^-$	CD3$^-$	TCR$\gamma\delta^-$
9.	CD1a$^-$	CD4$^-$	CD8$^-$
10.	CD45RA$^-$	CD29$^+$	CD4$^-$
11.	CD11a$^-$	CD11b$^-$	CD8$^-$
12.	CD16$^-$	CD56$^-$	CD57$^-$
C	Control$^-$	Control$^-$	Control$^-$

Discussion

A multiparameter examination indicated that this case was lymphoblastic lymphoma of precursor B-cell phenotype, although it was difficult to classify based only on morphological observation. The most important phenotypic findings were the simultaneous expression of both CD10 and CD19 and the negativeness of surface immunoglobulins. It was much easier to identify by 3-FCM on the same cell population than with double or triple immunohistochemistry. In addition, the presence of cytoplasmic IgM was also helpful. These findings strongly suggest that the lymphoma cells are derived from precursor B lymphocyte.

Cytoplasmic IgM (cIgM) usually was objectively detected by the combined use of both flow cytometry and immunohistochemistry. cIgM is not considered easy to distinguish, whether the immunohistochemical localization of IgM in lymphoma cells is surface or cytoplasm because of their high N/C ratio. However, even in immunohistochemistry, it might be possible to identify cytoplasmic IgM in some lymphoma cells by careful examination of the perinuclear localization.

The weak fluorescent intensity of CD20 and CD22 in the lymphoma cells of this case separates the lymphoma cell population from the normal B lymphocyte on a cubic representation of 3-FCM. In addition, the lymphoma cells in this case tested positive for CD10 and negative for CD45. Usually the B lymphocyte in the peripheral blood and bone marrow is CD10 negative and positive for CD45. Therefore, the combinations of CD10-CD19-CD45 or CD20-CD22-CD45 by 3-FCM might be useful to detect a small number of lymphoma cells to diagnose the early stage of leukemic change or minimal residual disease, as individual marker-combinations (IMC). A detailed phenotypic examination as such seemed to be necessary to make the best use of 3-FCM although single color FCM is not available for this kind of examination[5,6,7,8]. It is also well known that often it is dangerous to evaluate the intensity of reactivity or the amount of antigen by immunohistochemistry.

A rearranged fragment with Hind III digestion in TCR delta chain was observed in this case. It has been reported that this kind of fragment is identified occasionally in B cell lymphoma and that it does not always represent a true rearrangement or a clonal proliferation of T lymphocyte[9]. Although there might be some relationship between the fragment and the immaturity of lymphoma cells as precursor B-cell phenotype, the reason has not been clarified. The existence of rearranged fragments in a kappa chain gene and no phenotypic expression of the immunoglobulin light chain is not always controversial, because there may be some problems in the process from DNA to protein synthesis.

Finally again, it should be emphasized that the future use of phenotypic analyses in malignant lymphoma using flow cytometry is not merely for cell-lineage classification, but also should based on a more useful and practical application for patient's evaluation or follow-up system using such as IMC.

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