Flow Cytometric Immunophenotyping of Adult T-Cell Leukemia/Lymphoma Using CD3 Gating

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Key Words: Adult T-cell leukemia/lymphoma; Flow cytometry; Immunophenotyping; CD3 gating

Abstract

Adult T-cell leukemia/lymphoma (ATLL) is a lymphoproliferative neoplasm of helper T lymphocytes caused by human T-cell leukemia virus type-1 (HTLV-I). The disease was first described in Kyushu, in southwestern Japan, and most frequently occurs in endemic areas, such as Japan, the Caribbean basin, West Africa, Brazil, and northern Iran. ATLL is essentially a disease of adults, characterized clinically by generalized lymphadenopathy, hepatosplenomegaly, skin lesions, and hypercalcemia. The prognosis of most patients is quite poor, with a median survival time of only 13 months, even if multiagent combination chemotherapy is given.

In the present study, flow cytometric immunophenotyping with CD3 gating was performed on 30 samples from 26 patients who had been given a diagnosis of ATLL. The records of these patients also were reviewed retrospectively. In 14 of the 30 samples, an abnormal CD3low T-cell population was distinguishable from the normal T-cell populations by flow cytometric analysis. Herein we report a novel strategy for flow cytometric immunophenotyping of ATLL facilitated by CD3low gating.

Materials and Methods

Case and Control Samples

We retrospectively reviewed all cases of ATLL diagnosed at the Osaka Medical College, Osaka, Japan, from January 1998 to January 2004. Diagnosis of ATLL was based on the presence of an antibody positive for anti-HTLV-1, histologic identification of lymphoproliferative disease of peripheral T-cell origin, and confirmation of monoclonal integration of the HTLV-1 proviral genome by Southern blot. This analysis yielded 30 samples from 26 patients, consisting of 20 peripheral blood samples, 3 bone marrow aspirate samples, 5 lymph node samples, 1 pleural effusion sample, and 1 bronchoalveolar lavage fluid (BAL) sample. The 26 patients (14 men and 12 women) had a mean ± SD age of 60.3 ± 11.8 years. Because ATLL is a clinically heterogeneous disease, the cases were classified in 4 subtypes1: acute-type ATLL, 19 (73%); chronic-type ATLL, 1 (4%); lymphoma-type ATLL, 3 (12%); and smoldering ATLL, 3 (12%). All cases exhibited 5% or more abnormal ATLL cells in each specimen, including cells with hyperlobated nuclei (flower cells) or irregular nucleolar contours.

For comparison of CD3 fluorescence intensity of normal T cells with that of ATLL cells, 15 normal peripheral blood samples obtained from healthy volunteers were studied. The mean ± SD age of the healthy volunteers was 41.5 ± 10 years (range, 26-58 years).

Flow Cytometry

Sample Preparation and FACS Acquisition

For preparation of the cell suspension, lymph node samples were minced with scissors and filtered through a 50-µm
mesh nylon filter. Peripheral blood samples, bone marrow aspirates, and pleural effusion and BAL samples were treated with EDTA or heparin for anticoagulation and processed using a lysed whole blood technique using fluorescence-activated cell sorting (FACS) lysing solution (Whole Blood Lysis Reagent, Coulter, Fullerton, CA). All samples (regardless of origin) were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS at a concentration of approximately $2 \times 10^6$ cells per milliliter. Samples were stained dually with fluorescein isothiocyanate (FITC)-conjugated CD2 (MT910, DAKO, Carpenteria, CA), CD4 (T4, Coulter), CD28 (COLT2, Nichirei, Tokyo, Japan), and phycoerythrin-cyanine 5.1 (PC5)-conjugated CD3 (UCHT1, Coulter) for 30 minutes at 4°C. Negative control samples were stained with FITC-conjugated rat antihuman immunoglobulin (LODNP1, Coulter) for 30 minutes at 4°C.

Case samples also were stained dually with phycoerythrin-conjugated CD5 (UCHT-2, Pharmingen, San Diego, CA), CD7 (My7, Coulter), CD8 (DK25, DAKO), CD25 (2A3, Becton Dickinson, San Jose, CA), CD45RO (UCHL-1, Becton Dickinson), CD45RA (2H4, Coulter), CD62L (TQ1, Coulter), HLA-DR (L243, Pharmingen), and PC5-conjugated CD3 (UCHT1, Coulter) for 30 minutes at 4°C. Negative control samples were stained with phycoerythrin-conjugated rat antihuman immunoglobulin (LODNP1, Coulter) for 30 minutes at 4°C.

Images

Image 1. All samples were rinsed twice in PBS and analyzed on a FACScan flow cytometer (EPICS XL, Beckman Coulter, Fullerton, CA) using System II software, version 3.0 (Beckman Coulter). At least 10,000 cells in total were analyzed.

FACS Analysis

A CD3 gating strategy was used to identify ATLL cells. All samples were analyzed in the side scatter (SS) vs CD3 histogram mode. In the abnormal cell samples, CD3 peak fluorescence intensity below 10 on the log scale was defined as CD3low, an intensity between 10 and 100 as CD3medium, and an intensity more than 100 on the log scale as CD3high. The cells in the CD3low and CD3medium population regions were acquired (gate A), and this procedure was defined as CD3low and CD3medium gating, respectively. Cells with overlap of less than 50% with the isotype control or with more than a 30% positive distinct population above the isotype control were defined as positive. The expression levels of T cell–associated antigens (CD2, CD3, CD4, CD5, CD7, CD8, CD45RA, CD45RO, and CD62L), activated antigen (CD25), and HLA-DR in positive cells were evaluated.

Immunohistochemical Analysis

Lymph node slides were obtained from tissue samples fixed with 10% buffered formalin and embedded in paraffin. Peripheral blood samples and bone marrow aspirates were smeared manually. BAL samples were processed by application of Cytospin (Shandon, Pittsburgh, PA) to the fluid. Immunohistochemical analysis of the peripheral blood, bone marrow aspirate, lymph node, and BAL slides was performed using CD7 (titrated at 1:20; DK24, DAKO), CD4 (titrated at 1:20; MT310, DAKO), and CD8 (titrated at 1:20; DK25, DAKO) monoclonal antibodies, using a modified avidin-biotin-peroxidase procedure with 3,3’-diaminobenzidine (Sigma Chemical, St Louis, MO) as the chromogen. A negative control experiment was performed for each sample using normal rabbit immunoglobulin fraction (DAKO), and a positive control experiment was performed using lymphocytes from peripheral blood smears from healthy volunteers, using the CD7, CD4, and CD8 monoclonal antibodies as described. The percentage of abnormal cells, such as ATLL cells, was determined from the CD7, CD4, and CD8 immunohistochemical analysis.

Statistical Analysis

Associations between the gating procedure and CD3 fluorescence intensity were tested using a Kruskal-Wallis test and a Bonferroni-corrected Mann-Whitney U test for unrelated variables. The mean values and their standard deviations, the median, 25th and 75th percentiles, and range were calculated for each variable. P values of .017 or less were considered statistically significant.

Associations between the gating procedure and immunophenotyping also were tested using a Fisher exact test, as determined by expected frequencies of variables in 2 × 2 tables. A P value of less than .05 was used as the criterion for statistical significance. Statistical analyses were performed using SPSS 12J software (SPSS, Tokyo, Japan).

Results

CD3 Populations

CD3 expression was positive in abnormal cells, but the CD3 fluorescence intensity showed 2 patterns. Of the 30 samples...
from 26 patients, 14 samples contained a CD3low population and 16 contained a CD3medium population. Figure 1 shows the fluorescence intensity for the CD3low (3.94 ± 2.6) and CD3medium (26 ± 12) populations and for the normal T-cell population from healthy volunteers (15.6 ± 2.8, control population). Statistically significant differences in the mean fluorescence intensity were observed between the CD3low population and either the CD3medium or control population (P < .001). The mean fluorescence intensity of the CD3low population was significantly lower than that of the control population (P < .001), but no statistically significant difference was observed between the mean fluorescence intensities of the control and CD3medium populations (P = .037).

CD3 Gating

The histogram containing the CD3medium population was separated into 3 categories: CD3medium population (gate A), granulomonocytes (gate B), and mature B lymphocytes and precursor cells (gate C). The CD3medium population showed the highest CD3 fluorescence intensity and a low SS signal. Granulomonocytes showed negative CD3 fluorescence intensity and the highest SS signal, and the mature B lymphocytes and precursors showed negative CD3 fluorescence intensity and a low SS signal. Hence, the CD3medium population was easily distinguishable from granulomonocytes and mature B lymphocytes and precursor cells based on the CD3 and SS signals. By using this approach, the CD3medium population was identified by gating, and immunophenotyping was performed.

In contrast, the histogram containing the CD3low population was separated clearly into 4 categories: the CD3low population (gate A), granulomonocytes (gate B), mature B lymphocytes and precursor cells (gate C), and T lymphoblasts and T lymphocytes (gate D). The CD3low population showed low CD3 fluorescence intensity and a low SS signal, the granulomonocytes showed negative CD3 fluorescence intensity and the highest SS signal, the mature B lymphocytes and precursor cells showed negative CD3 fluorescence intensity and a low SS signal, and the mature T lymphocytes and T lymphoblasts showed the highest CD3 fluorescence intensity and a low SS signal. Hence, similar to the CD3medium population, the CD3low population was easily distinguishable from other cells based on the CD3 and SS signals. Following identification, immunophenotyping of the CD3low population was performed.

Flow Cytometric Analysis

Table 1 and Table 2 give the results of flow cytometric analysis of each specimen. This analysis showed aberrant T-cell antigen loss or reduced expression in 6 cases (38%) in the CD3medium population and in 14 cases (100%) in the CD3low population. A reactive or inconclusive phenotype was apparent in 12 cases (75%) in the CD3medium population and in 1 case (7%) in the CD3low population. Loss of CD7 was the most common single T-cell antigen abnormality, occurring in 6 cases (38%) in the CD3medium population and in all cases (100%) in the CD3low population. Coexpression of CD4 and CD8 occurred in 8 cases (50%) in the CD3medium population but did not occur in the CD3low population. Coexpression of CD45RO and CD45RA occurred in 6 cases (43%) in the CD3medium population and 1 case (7%) in the CD3low population.

Regarding differences between the CD3medium and CD3low populations, there was no significant difference in the expression of CD2, CD4, CD5, CD28, CD62L, CD45RA, CD45RO, and HLA-DR. However, the expression levels of CD7 and CD8 in the CD3low population were significantly lower than those in the CD3medium population (P < .001 and P < .04, respectively) and expression of CD25 in the CD3low population was significantly higher than that in the CD3medium population (P < .01) Figure 2.
Immunohistochemical Analysis

Immunohistochemical analysis using CD7, CD4, and CD8 was performed for all samples. The ATLL cells in all specimens were not immunoreactive with CD7. In all specimens but one, the ATLL cells were immunoreactive with CD4 and were not immunoreactive with CD8. In the 1 aberrant case, for which flow cytometric analysis indicated the presence of a CD3\textsuperscript{low} population, the ATLL cells were immunoreactive with CD8 and were not immunoreactive with CD4. Because CD8\textsuperscript{+} and CD4\textsuperscript{−} ATLL has been reported,\(^2,3\) we did not conclude that this case should be categorized as having an inconclusive phenotype.

The results of immunohistochemical analysis with CD7, CD4, and CD8 were consistent with immunophenotyping performed by flow cytometric analysis with CD3\textsuperscript{low} gating. In contrast, immunohistochemical analysis performed with CD7, CD4, and CD8 was inconsistent with the flow cytometric analysis using the CD3\textsuperscript{medium} gating procedure, suggesting that this procedure might not exclude normal T cells from ATLL cells.

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**Table 2**

**Summary of Immunophenotyping Using CD3\textsuperscript{low} and CD3\textsuperscript{medium} Gating**

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* Data are given as number of positive cases/total number of cases (percentage of positive cases).

Discussion

ATLL is a distinct clinicopathologic syndrome characterized by a mature T-cell surface marker profile, an association with HTLV-1, the presence of abnormal lymphocytosis with pleomorphic nuclei,\(^4,6\) and clinical and cytogenetic diversity.\(^3,7,9\) The abnormal lymphocytes typically express CD2, CD3, CD4, CD5, and CD25; rarely express CD8; and lack CD7 expression in immunophenotypic analysis.\(^10,11\) Because the morphologic
features of ATLL are diverse, confirmation of the presence of the
disease is obtained through use of a specific antibody panel for
immunocytochemical analysis, and flow cytometry can be used
to help distinguish ATLL from other peripheral T-cell lympho-
proliferative diseases, such as T-cell prolymphocytic leukemia,
certain kinds of peripheral T-cell lymphoma, and mycosis fun-
goides/Sézary syndrome.10 The expression of CD2, CD3, CD4,
CD8, CD25, CD28, CD45RA, CD45RO, and HLA-DR on
ATLL cells has been reported, but these data are based on
immunophenotyping combined with flow cytometric analysis
using a conventional gating step for forward scatter and SS or on
immunohistochemical analysis.12-16

Flow cytometric analysis has been introduced to study
hematologic malignancy based on surface antigen levels,
because malignant cells often display an aberrant phenotype,
which may involve antigen loss or changes in combinations of
surface markers, compared with normal hematologic cells.
However, most T-cell antigens are expressed on normal and
abnormal T lymphocytes. Hence, it is difficult to discriminate
between normal and abnormal T cells by a conventional
immunophenotyping strategy17 because the conventional gat-
ing step for forward scatter and SS does not discriminate well
between such cells.

In acute myeloid leukemia, good discrimination can be
achieved between the blast cell population and normal cells
through the systematic use of leukocyte common antigen
(CD45) expression and SS.18 This discrimination is based on the
fact that the precursor cells express low and intermediate levels
of CD45, whereas mature lymphocytes express a high level of
CD45.19 However, because ATLL is a lymphoproliferative dis-
ease of peripheral T-cell origin, the ATLL cells partially overlap
with the gated normal lymphocytes, and, therefore, this proce-
dure cannot discriminate between normal and ATLL cells.

CD3 is part of the T-cell receptor complex and is involved
in transducing stimulatory signals after antigen-specific recog-
nition.20 It is expressed in the membrane at the late stage of
thymic differentiation and in mature (peripheral) T lympho-
cytes,21 after which CD3 can be detected only in the cytoplasm
at the earliest stages of T-cell differentiation. The expression of
CD3 is down-regulated after antigen recognition and activa-
tion.22 Similarly, ATLL cells are activated T cells that mostly
express interleukin-2 receptor (CD25), and it has been suggest-
ed that CD3 is down-regulated in ATLL cells after activation
and infection with HTLV-1.23,24 Therefore, we anticipated that
good discrimination between normal T cells and ATLL cells
might be achieved through the use of CD3 expression.

The revised European-American Lymphoma (REAL)
classification is in widespread use. This system emphasizes
immunophenotyping analysis with clinical features and mor-
phologic criteria. In the REAL classification, ATLL is placed
in the category of peripheral T-cell and natural killer cell neo-
plasms. Tumor cells typically express CD2, CD3, CD4, CD5,
and CD25; rarely express CD8; and usually lack CD7, based
on immunophenotypic analysis.10,11 Immunophenotyping
performed by flow cytometric analysis with CD3low gating is
largely consistent with the immunophenotypic criteria sug-
gested in the REAL classification,10 as previous reports also
have stated.12-16

We studied the immunophenotyping of ATLL by flow
cytometric analysis with CD3 gating. Because statistically sig-
nificant differences in immunophenotyping were apparent
between CD3low and CD3medium gating, we compared the results

![Figure 2](https://example.com/figure2.png)

**Figure 2** There was no significant difference in the expression of CD2, CD4, CD5, CD28, CD62L, CD45RA, CD45RO, and HLA-
DR. The expression of CD7 and CD8 in the CD3low gating population (black bars) was significantly lower than that in the
CD3medium gating population (white bars), and CD25 expression in the CD3low gating population was significantly higher than that
in the CD3medium gating population. * P < .001, † P < .04, ‡ P < .01.
of flow cytometric analysis with immunohistochemical data for the expression of CD7, CD4, and CD8. Flow cytometric analysis is easy to perform, reliable, and inexpensive, and clinically relevant data can be obtained soon after completion of flow cytometry measurements. The CD3<sup>low</sup> gating procedure is more sensitive than conventional or CD45/SS gating and seems to be an appropriate method of choice. The clinical course of ATLL is very aggressive, but recently it has been suggested that the disease might be cured by allogeneic stem cell transplantation.

We also believe that minimal residual disease monitoring is clinically useful during follow-up after therapy. If specific surface markers in ATLL cells are determined, flow cytometric assays can efficiently complement techniques for minimal residual disease monitoring, particularly in combination with studies of T-cell receptor gene rearrangement.

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