

# Characterization of Monoclonal Antibodies against Human Leukocyte Common Antigen (CD45)

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**Background:** The leukocyte common antigen (CD45) is a transmembrane-type protein tyrosine phosphatase that has five isoforms. **Methods:** We generated seven murine mAbs against human CD45 by injecting cells from different origins, such as human thymocytes, PBMCs, and leukemic cell lines. By using various immunological methods including flow cytometry, immunohistochemistry, and immunoprecipitation, we evaluated the reactivity of those mAbs to CD45 of thymus as well as tonsil lysates. Furthermore, we transiently transfected COS-7 cells with each of gene constructs that express five human CD45 isoforms respectively, and examined the specificities of the mAbs against the transfected isoforms. **Results:** In case of thymocytes, lymphocytes, and monocytes, all the seven mAbs demonstrated positive reactivities whereas none was reactive to erythrocytes and platelets. The majority of immune cells in formalin-fixed paraffin-embedded thymus and tonsil tissues displayed strong membranous immunoreactivity, and the main antigen was detected near 220 kDa in all cases. Among the mAbs, four mAbs (AP4, DN11, SHL-1, and P6) recognized a region commonly present in all the five isoforms. One mAb, YG27, recognized four isoforms (ABC, AB, BC, and O). Two mAbs, P1 and P14, recognized the isoforms that contain exon A encoded regions (ABC and AB). **Conclusion:** In this study, we confirmed that AP4, DN11, SHL-1, YG27 and P6, are mAbs reactive with the CD45 antigen whereas P1 and P14 are reactive with the CD45RA antigen.

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## INTRODUCTION

The leukocyte common antigen (CD45), the most common hematopoietic lineage marker, belongs to a family of transmembrane-type protein tyrosine phosphatases with high molecular masses of 180 to 220 kDa (1-7). Using many monoclonal antibodies (mAbs) against CD45, it was revealed that CD45 comprises 5~10% of lymphocyte surface proteins, known as one of the most abundant glycoproteins expressed on lymphocytes (1).

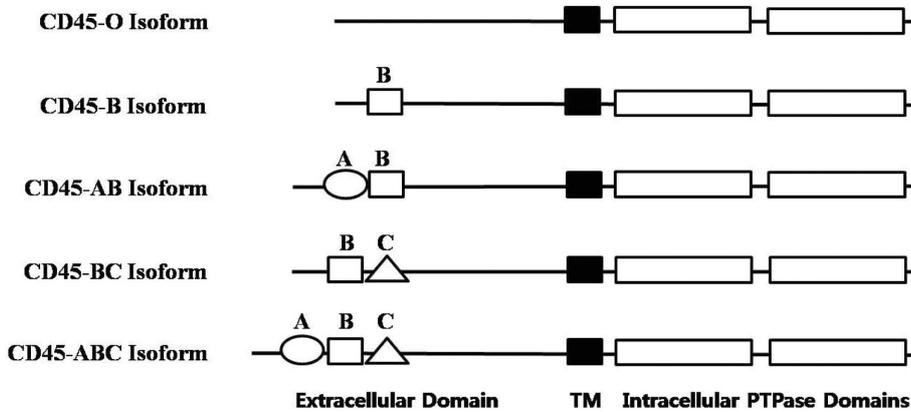
Charbonneau et al. observed a significant sequence similarity between the tandem repeats in the cytoplasmic domains of two proteins, CD45 and protein tyrosine phosphatase (PTP) 1B (8). Subsequent cloning of CD45 at the cDNA and genomic levels revealed several interesting characteristics about the primary structure of this molecule (9-11). The extracellular domain of human CD45 varies in length (391~552 amino acids) depending on which combination of exons are alternatively used to form the CD45 mRNA. The three alternatively used exons of CD45, exons 4, 5, and 6, encode peptide segments designated A, B, and C, respectively. In human, five different isoforms of CD45 mRNAs have been isolated, which contain all three exons (ABC isoform), two of the three exons (AB and BC isoform), only one exon (B isoform), or no exons (O isoform), respectively (9-11). All of the isoforms (schematically shown in Fig. 1) have the same 8 amino acids at their amino-terminus, which are followed by the various combinations of A, B, and C peptides (66, 47,

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**Figure 1.** The structures of the five human CD45 isoforms derived from cDNA cloning.

and 48 amino acids long, respectively). The remaining regions (the 383-amino-acid extracellular region, the 22-amino-acid transmembrane peptide, and the 707 amino-acid-cytoplasmic region) have the identical sequences in the all isoforms. The N-terminal region of CD45 is known to be heavily glycosylated (12). Therefore, alternative mRNA splicing of CD45 can result in a significant degree of heterogeneity in the extracellular domain due to differential O-linked glycosylation as well as the structure changes of the molecule.

As a result of the variability of the N-terminal region of CD45, mAbs raised against the CD45 protein recognize either all of the CD45 isoforms (CD45 mAb), or only a subset of the isoforms ("restricted" CD45R mAb). Thus, the suffix RA, RB, or RO indicates the requirement of the amino acid residues corresponding to exon A (RA), exon B (RB), or a lack of amino acid residues corresponding to exon A, B and C (RO) for the CD45 epitope expression, respectively. Accordingly, CD45 mAb binds to all isoforms, whereas CD45RA mAb binds to ABC and AB isoforms, CD45RB mAb binds to ABC, AB, BC, and B isoforms, and CD45RO mAb binds only to the 180 kDa isoform, which lacks any of the alternatively used exons (O isoform). In this report, we analyzed the characteristics of seven murine mAbs raised against the human leukocyte common antigen (CD45) (AP4, DN11, SHL-1, YG27, P1, P6, and P14). By using these antibodies, we were able to not only immunoprecipitate the CD45 isoforms, but also differentiate cellular expression profiles of the isoforms. In addition, by transiently transfecting COS-7 cells with the plasmids expressing CD45 isoforms, we examined the binding specificities of the mAbs to those isoforms.

## MATERIALS AND METHODS

### Production of monoclonal antibody

Seven murine mAb (AP4, DN11, SHL-1, YG27, P1, P6, and P14) were developed by immunizing Balb/c mice with various native human cellular immunogens. Briefly, AP4 was raised by immunizing PHA-activated human peripheral blood mononuclear cells (PBMC), and other antibodies were raised with cells from various origins: thymic stromal cells (for DN11), Jurkat (for SHL-1), thymocytes (for YG27), and resting human PBMCs (for P1, P6, P14). After 6 week-old Balb/c mice were immunized i.p. with each immunogen ( $10^7$  cells), the spleens were removed, and  $10^8$  spleen cells were fused with  $10^7$  SP2/0-Ag14 mouse myeloma cells using polyethylene glycol (PEG 4000, Rahway, NJ). The hybrids were cultured in flat-bottom microculture trays at  $37^\circ\text{C}$  in an atmosphere of humidified air conditioning 5%  $\text{CO}_2$ , and selected in HAT media. After 10 days, culture supernatant was harvested and tested for reactivity to human lymphocytes by indirect immunofluorescence method using flow cytometry. Seven of the resulting hybridoma clones, whose supernatant was reactive to human lymphocytes, were named AP4, DN11, SHL-1, YG27, P1, P6, and P14. Cells from a microculture well were subcloned by limiting dilution, and the culture supernatant of the clones was tested for antibody production. For production of the mAb in ascites form, pristane-treated Balb/c mice were injected i.p. with  $5 \times 10^6$  hybridoma cells, and the ascites were collected after 1 week of injection. mAb was purified from ascites.

### Determination of antibody isotype

The isotype of AP4, DN11, SHL-1, YG27, P1, P6, and P14

were determined by enzyme immunoassay using mouse monoclonal subtyping kit EK-5050 (Hyclone, Utah, USA). Isotyping was performed with rabbit anti-murine isotype-specific antisera (IgG1, IgG2a, IgG2b, IgG3, IgM, Kappa, Lambda) followed by peroxidase-labeled goat anti-rabbit IgG as the secondary antibody. with the addition of o-phenylene diamine and hydrogen peroxide substrate, positive samples turned an intense yellow.

### Cells and cell lines

We screened the expression of the AP4, DN11, SHL-1, YG27, P1, P6, and P14 antigen on the freshly isolated peripheral blood erythrocytes, lymphocytes, monocytes, granulocytes and platelet from the healthy volunteers. The tumor cell lines were purchased from American type culture collection (atcc, Rockville, MD) and cultured in RPMI-1640 medium supplemented with 10~20% fetal calf serum.

### Flow cytometry

Fresh cell suspension from patients and healthy donors were examined for the AP4, DN11, SHL-1, YG27, P1, P6, and P14 antigen expression by flow cytometric analysis using AP4, DN11, SHL-1, YG27, P1, P6, and P14 mAb. Cells ( $10^6$ ) were incubated with saturating amounts of the purified AP4, DN11, SHL-1, YG27, P1, P6 and P14 or a control immunoglobulin for 30 min at 4°C. Thereafter, cells were washed two times with phosphate-buffered saline (PBS), incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse as secondary antibody for 20 min at 4°C and washed two times with PBS. Cells were analyzed on a FACS (COULTER, USA). Samples containing over 20% AP4, DN11, SHL-1, YG27, P1, P6, and P14-positive cell were regarded as positivity.

### Immunohistochemical study

We screened tonsil and thymus obtained from the surgical pathology files of Chungbuk National University Hospital. All tissues had been fixed in 10% formalin and embedded in paraffin, and indirect immunoperoxidase technique and antigen retrieval method by microwave heating were employed (13,14).

Chromogen used as 3,3'-diaminobenzidine. Counterstain was not performed and the reaction pattern was analyzed based on serial hematoxylin-eosin stained sections. We defined positive cells by staining pattern along cell membrane.

### Immunoprecipitation

Fresh cell suspensions of tonsil from patient of chronic tonsillitis and of thymus from patient of congenital heart disease were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40 and 1 mM PMSF [phenyl methyl sulfonyl fluoride]). Lysates were precleared successively with protein A-Sepharose and rabbit anti-mouse IgG bound to protein A-Sepharose at 4°C for 4 h. 20  $\mu$ l of seven mAb coupled to protein A-Sepharose were used for immunoprecipitation with precleared cell lysates at 4°C overnight. Immune complexes were washed with a buffer containing 0.25% NP-40, 5 mM PMSF, 10 mM Tris, pH 8.0, 150 mM NaCl, 5 mM KI, and 5 mM EDTA. After extensive washing, the immunoprecipitates were eluted by boiling for 5 min in SDS sample buffer and analyzed on 6% SDS-PAGE under non-reducing condition, with appropriate molecular weight markers. After electrophoretic transfer of immune complex, the nitrocellulose was blocked with 5% skimmed milk in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing 0.05% Tween-20 (TBST). The bound peroxidase was visualized using the ECL chemoluminescence detection system (Amersham).

### Production of transfectants

The cDNAs encoding the five CD45 isoforms, which were inserted into the cloning site of a slightly modified version of the pcDL-SR  $\alpha$  296 expression plasmid (15), termed pSP65-SR  $\alpha$  2. The resultant plasmids were used to transiently transfect the COS-7 cells by the DEAE-dextran method (16). COS-7 cells were harvested by trypsinization and were seeded into 10 cm plate at a final density of  $1 \times 10^6$  cells/plate. After 12 h, the cells were washed twice with prewarmed phosphate-buffered saline (PBS) (138 mM NaCl, 2.6 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4) and were fed with transfection mixture (DME, 0.25 mg/ml DEAE dextran, 0.1 M Tris, pH 7.3 containing DNA), using 4 ml/plate for 12 h. After 12 h, the cells were washed with prewarmed PBS and were incubated with 10% DMSO reagent at room temperature. 3 min after addition of the 10% DMSO reagent, the medium was removed by aspiration and the monolayers were washed with prewarmed PBS. The cells were subsequently incubated in culture medium containing 0.1 mM chloroquin for 2 h 30 min. The cells were washed with prewarmed PBS, and incubated with fresh culture medium in CO<sub>2</sub> atmosphere at 37°C for 48 h. Resulting cells express each of the CD45 isoforms. The binding specificities of seven mAb were characterized by flow

**Table I.** Flow cytometric analysis of seven mAbs in hematopoietic cells

mAb	Cell				
	Lymphocyte	Monocyte	Granulocyte	Erythrocyte	Platelet
AP4	+(88.2%)	+(96.0%)	+(93.7%)	—	—
DN11	+(87.2%)	+(98.6%)	+(86.7%)	—	—
SHL-1	+(85.7%)	+(99.2%)	+(94.5%)	—	—
YG27	+(81.8%)	+(90.9%)	+(37.2%)	—	—
P1	+(82.2%)	+(82.7%)	—	—	—
P6	+(82.9%)	+(98.9%)	+(93.5%)	—	—
P14	+(83.8%)	+(90.5%)	—	—	—

cytometric analysis using the COS-7 cells that expressing the individual human CD45 isoforms. The anti-CD45/FITC (T29/33; DAKO, Denmark) was used as a positive control. Samples containing over 10% AP4, DN11, SHL-1, YG27, P1, P6, and P14-positive cell were regarded as positivity.

## RESULTS

### Determination of antibody isotype

The isotype of AP4, DN11, SHL-1, YG27, P1, P6, and P14 mAb were determined by enzyme immunoassay. The isotype of AP4 was IgG2b whereas the isotypes of the other remaining mAbs were all categorized into IgG1.

### Reactivity of AP4, DN11, SHL-1, YG27, P1, P6, and P14 to hematopoietic cells

To evaluate the recognition profile of the mAbs in various hematopoietic cells, normal human hematopoietic cells from peripheral blood were analyzed by flow cytometry. Five monoclonal antibodies (AP4, DN11, SHL-1, YG27, and P6) were reactive with lymphocytes, monocytes, and granulocytes, but not with erythrocytes or platelet. Two monoclonal antibodies (P1 and P14) were reactive with lymphocytes and monocytes, but not with granulocytes, erythrocytes, or platelets (Table I).

### Reactivity of AP4, DN11, SHL-1, YG27, P1, P6, and P14 to various leukemic cell lines

Similarly to the above experiment, we examined the recognition profile of the mAbs in leukemic cells, U937 (Histiocytic lymphoma), K562 (Erythroid leukemia) and Molt-4 (T-ALL) cells. AP4, DN11, SHL-1, YG27, and P6 mAb were reactive with all of the tested leukemic cell lines. P1 and P14 were reactive with U937, but not with K562 or Molt-4 (Table II).

**Table II.** Flow cytometric analysis of seven mAbs in leukemic cell lines

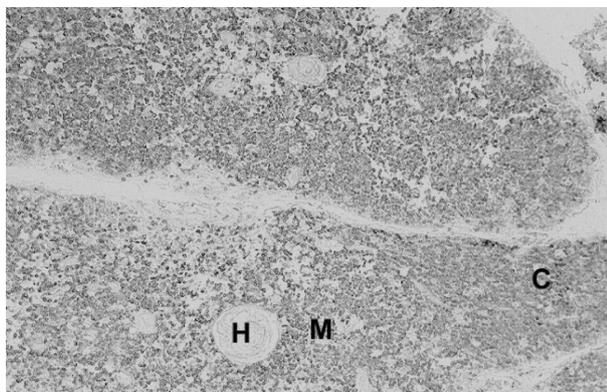
mAb	Cell line (Origin)		
	U937 (Histiocytic lymphoma)	K562 (Erythroid leukemia)	Molt-4 (T-ALL)
AP4	+(96.7%)	+(39.4%)	+(98.6%)
DN11	+(95.0%)	+(30.9%)	+(97.2%)
SHL-1	+(98.6%)	+(35.5%)	+(92.0%)
YG27	+(93.9%)	+(89.8%)	+(78.8%)
P1	+(82.5%)	—	—
P6	+(97.9%)	+(37.4%)	+(99.2%)
P14	+(77.2%)	—	—

### Reactivity to formalin fixed thymus and tonsil

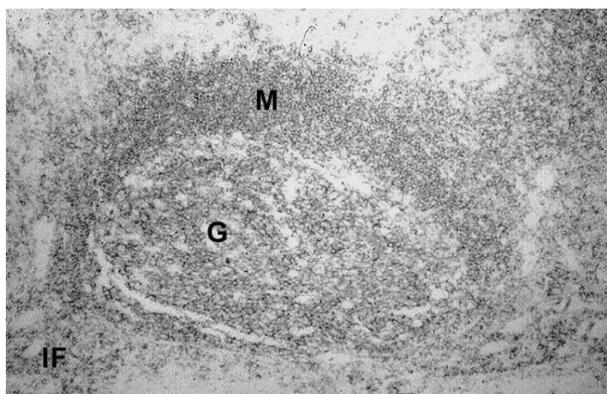
To validate their clinical usage, the immunoreactivity of mAbs on formalin-fixed paraffin-embedded human tissue was checked. All the seven mAbs displayed strong membranous immunoreactivity in nearly all cases of immune cells in formalin fixed paraffin embedded thymus (Fig. 2) and tonsil (Fig. 3) samples.

### Characterization of seven mAb target antigen

To identify the antigen immunoprecipitated with these seven mAbs, lysates of thymus and tonsil were analyzed. The molecular weights of proteins recognized by AP4, DN11, SHL-1, YG27, and P6 were examined by immunoprecipitation using thymus lysates. AP4 recognized two proteins of 220 and 205 kDa whereas DN11, SHL-1, YG27, and P6 recognized three proteins of 220, 205, and 190 kDa (Fig. 4). The molecular weight of the antigen recognized by P1 and P14 was determined by immunoprecipitation using tonsil lysates. Two discrete bands of the antigen corresponded to two molecules



**Figure 2.** Reactivity of AP4 on formalin fixed thymus. All T cells of cortex (C) and medulla (M) were reactive with AP4. but, squamous cells of Hassall's corpuscle (H) were not reactive with AP4.

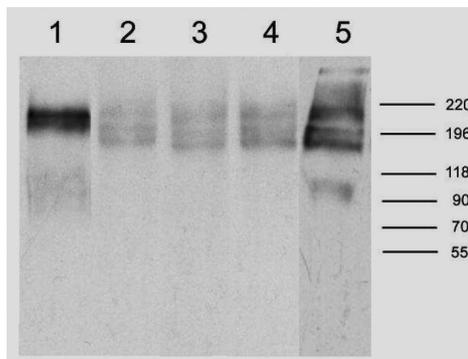


**Figure 3.** Reactivity of P14 on formalin fixed tonsil. All lymphocytes of germinal center (G), mantle (M) and interfollicular (IF) area were reactive with P14.

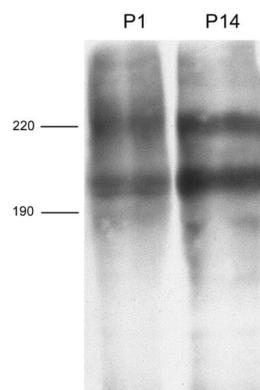
of approximately 225 and 200 kDa (Fig. 5).

### Transfection study

To confirm which isoform of CD45 is recognized by each of these seven mAbs, transfection study was performed. In transfection study, four mAb (AP4, DN11 (Fig. 6), SHL-1, and P6) bound to all five isoforms (the ABC isoform, AB isoform, BC isoform, B isoform and O isoform), one mAb (YG27) bound to four isoforms (the ABC isoform, AB isoform, BC isoform and O isoform) and two mAb (P1 (Fig. 7) and P14) bound to two isoforms that include exon A encoded sequences (the ABC isoform and AB isoform). In these results, we confirmed that AP4, DN11, SHL-1, YG27 and P6 are conventional CD45 mAb and P1 and P14 are CD45RA mAb (Table III).



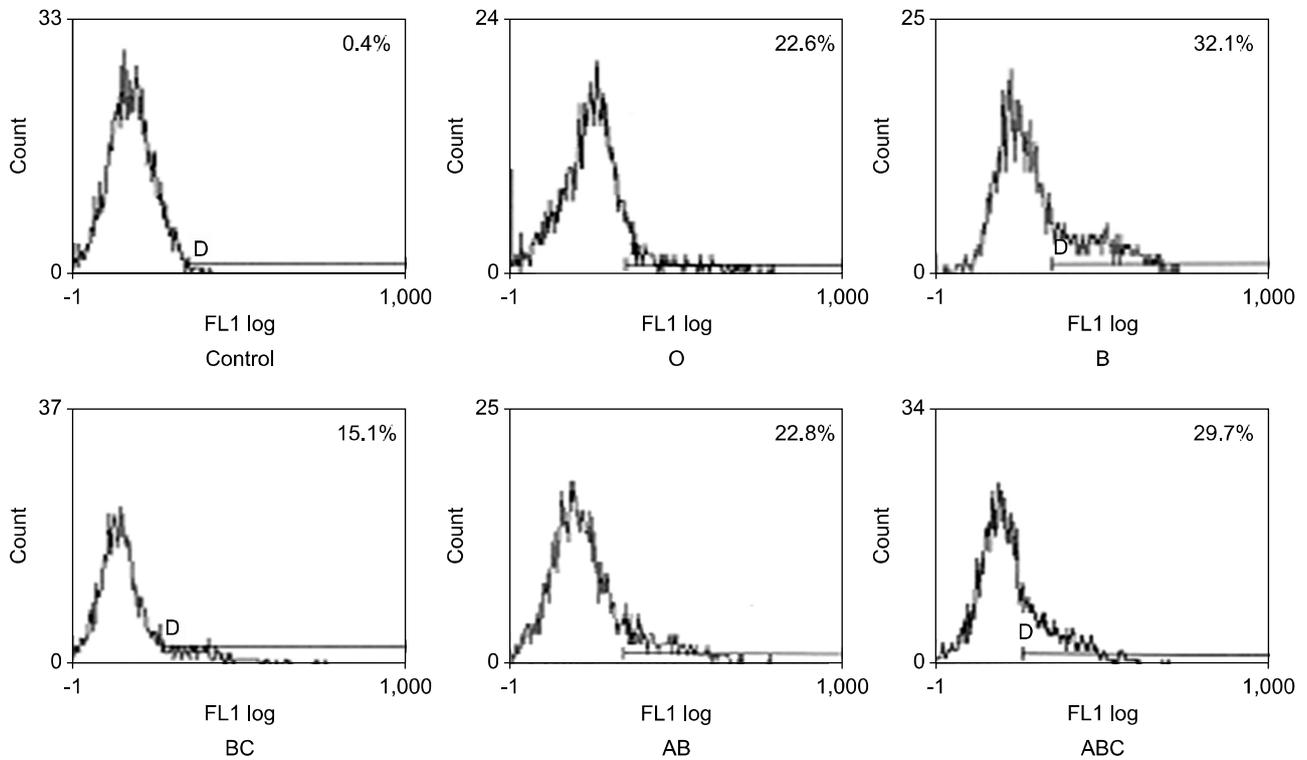
**Figure 4.** Immunoprecipitation of thymus lysates with AP4, DN11, SHL-1, YG27 and P6 mAb. Lane 1; AP4 lane 2; DN11 lane 3; SHL-1 lane 4; YG27 lane 5; P6. Cells were lysed in lysis buffer containing 1% NP-40. precleared cell lysates were immunoprecipitated with AP4, DN11, SHL-1, YG27 and P6 mAb. The immunoprecipitates were analyzed by SDS-PAGE (6%) under nonreducing condition.



**Figure 5.** Immunoprecipitation of tonsil lysates with P1 and P14 mAb. Cells were lysed in lysis buffer containing 1% NP-40. Precleared cell lysates were immunoprecipitated with P1 and P14 mAb. The immunoprecipitates were analyzed by SDS-PAGE (6%) under nonreducing condition.

### DISCUSSION

In this report, we examined the specificities of seven mAbs raised in our lab by using transiently transfected COS-7 cells that express each of five CD 45 isoforms. Based on the binding patterns, the seven mAbs are classified into two groups: AP4, DN11, SHL-1, YG27, and P6 are CD45 mAbs whereas P1 and P14 are CD45RA mAbs. Because of their abundant expression of hematopoietic cells, we were easily able to obtain mAbs against CD45 by immunizing conventional cellular immunogen, such as thymocytes, PBMCs and leukemic cell lines, but mAb against CD45RB or CD45RO could not be



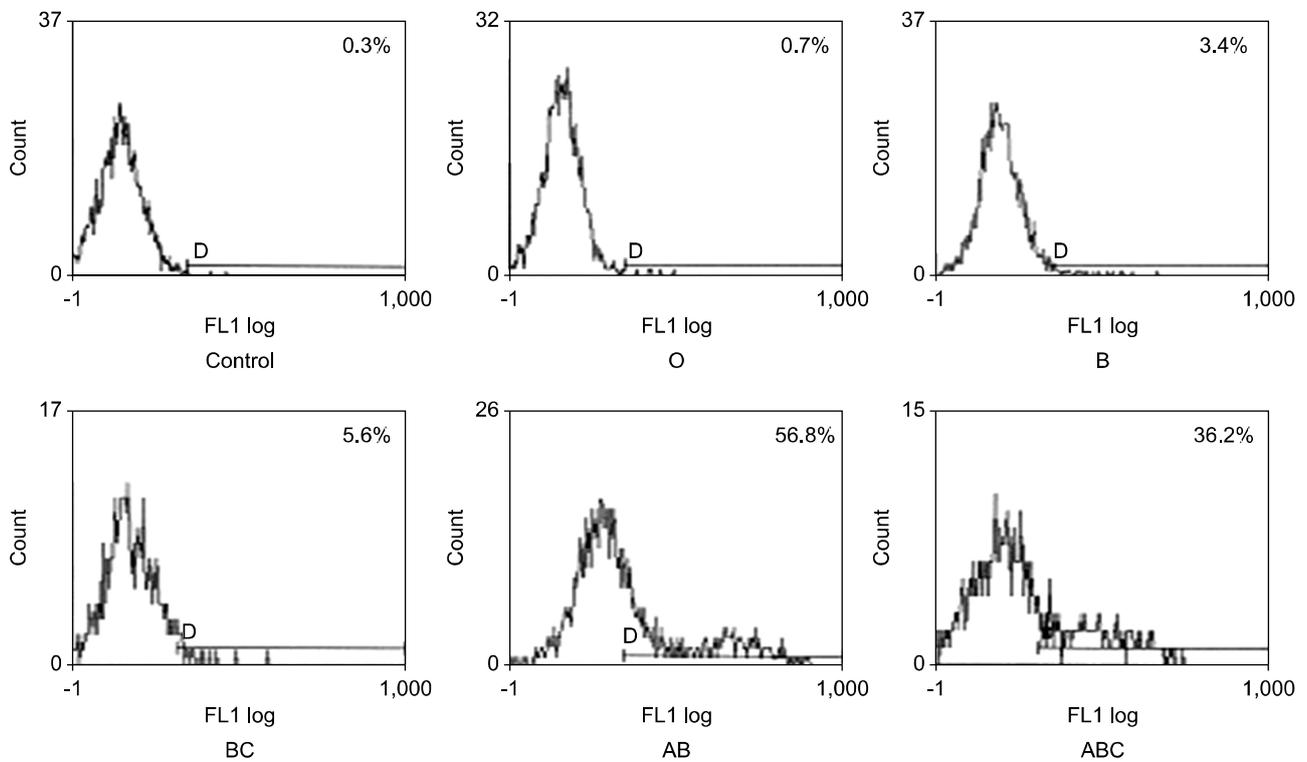
**Figure 6.** Transfection study of DN11 mAb. Flow cytometric analysis of DN11 using COS-7 cells expressing the individual human CD45 isoforms. DN11 bound to all five isoforms (the ABC isoform, AB isoform, BC isoform, B isoform and O isoform).

obtained.

Based on the expression profiles of CD45 mAbs that were previously studied and reported, we classified the generated mAbs by their flow cytometric patterns. In flow cytometric analysis of the seven mAb with hematopoietic cells, five monoclonal antibodies (AP4, DN11, SHL-1, YG27 and P6) were reactive with lymphocytes, monocytes and granulocytes but not with erythrocytes and platelets (Table I). Two monoclonal antibodies (P1 and P14) were reactive with lymphocytes and monocytes but not with granulocytes, erythrocytes and platelets (Table I). According to Leukocyte Typing V (17), CD45RB mAb is reactive with erythrocytes and CD45RA mAb is not reactive with granulocytes. Therefore, in terms of CD45 antigen expression pattern recognized by seven mAbs, there is no CD45RB mAb at least and we supposed that P1 and P14 should be a CD45RA mAb. U937 (Histiocytic lymphoma), K562 (Erythroid leukemia), Molt-4 (T-ALL) were analyzed by flow cytometry. AP4, DN11, SHL-1, YG27 and P6 mAb were reactive with these all leukemic cell lines. P1 and P14 only were reactive with U937, but not with K562 and Molt-4 (Table

II). According to Leukocyte Typing V (17), CD45RO mAb is not reactive with U937. Therefore, there is no CD45RO mAb among seven mAbs. CD45RA mAb was not reactive with K562 and Molt-4. Therefore, those data also support that P1 and P14 would be a CD45RA mAb. In terms of molecular weight analysis AP4, P1, and P14 were immunoprecipitated with two proteins of 220 and 205 kDa (Fig. 5). But DN11, SHL-1, YG27 and P6 recognized three proteins of 220, 205, and 190 kDa (Fig. 4). Above expression profiles and molecular results also support that AP4, DN11, SHL-1, YG27 and P6 would be a conventional CD45mAb and P1 and P14 would be a CD45RA mAb.

In order to confirm those findings, we transiently transfected COS-7 cells with each of 5 isoform cDNA constructs of CD45, and examined the specificities of the seven mAbs against the isoforms. Although the percentage of the positive ones against these mAbs among the transfected COS-7 cells appeared to be relatively low, we were able to classify their specificities by comparison of commercially available anti-CD45 Abs with known specificity (Table III). Of the seven



**Figure 7.** Transfection study of P1 mAb. Flow cytometric analysis of P1 using COS-7 cells expressing the individual human CD45 isoforms. P1 bound to two isoforms that include exon A encoded sequences (the ABC isoform and AB isoform).

**Table III.** Flow cytometric analysis of seven mAbs using transiently transfected COS-7 cells that express individual human leukocyte common antigens

Transfectant	mAb (%)							
	CD45Ab	AP4	DN11	SHL-1	YG27	P1	P6	P14
Control	1.7	0.3	0.4	0.2	0.6	0.3	0.3	0.4
O	13.7	13.5	22.6	10.3	21.3	0.7	10.8	0.5
B	31.2	20.8	32.1	10.9	4.2	3.4	17.4	2.2
BC	29	24.9	15.1	19.3	16.5	5.6	19.9	2.3
AB	24.9	26.3	22.8	19.4	20.0	56.8	25.2	37
ABC	23.5	30.2	29.7	40.8	22.6	36.2	31.3	19.1

mAbs tested, four mAb (AP4, DN11 (Fig. 6), SHL-1, and P6) recognized a sequence common to all of the five isoforms (ABC, AB, BC, B, and O) (Table III). One mAb (YG27) recognized four isoforms (ABC, AB, BC and O), and two mAb (P1 (Fig. 7) and P14) recognized isoforms that include exon A encoded sequences (ABC and AB) (Table III). Thus, these results indicate that AP4, DN11, SHL-1, YG27 and P6 are anti-CD45 mAbs whereas P1 and P14 are anti-CD45RA mAbs, YG27 mAb unusually revealed negative reactivity to COS-7

cells transfected with B construct, but remaining four constructs revealed positive reactivity we categorized YG27 mAb as a CD45 mAb.

Although the development of various CD45 mAbs has been previously reported, only a few antibodies against CD45RB and CD45RO were successful. As expected, the majority of the mAbs that we developed recognized all isoforms, and only two mAbs (P1 and P14) recognized both ABC and AB isoforms. None of mAbs recognizes B and O isoforms. It is

because the epitopes required for the generation of CD45RO or CD45RB mAbs might be heavily glycosylated, which process is essential for the biological function of thymocytes, lymphocytes and leukemic cells (17-20). In case of using CD45 antigens overproduced in *E. coli*, antibodies recognizing only the epitope composed of the polypeptide backbone will be produced, resulting in failure of antibody production that recognize the natural structure of the antigen. Accordingly, we utilized whole immune cell extract for immunization to produce practical antibodies for diagnostic or therapeutic usage in this study, but the generation of CD45RO or CD45RB mAbs was not successful.

In immunohistochemical study, all the seven mAb showed strong membranous staining patterns in case of all the hematopoietic cells in formalin-fixed paraffin-embedded thymus and tonsil samples (Figs. 2 and 3). This result indicates that these mAbs may be used singly or together for the detection and the differential diagnosis of hematopoietic malignancies in surgical pathology (13,14).

Several key issues related to the physiological role of CD45 isoforms remain mostly unsolved, such as the identification of its extracellular ligands as well as intracellular substrates, and the mechanism responsible for regulation of CD45 catalytic activity, albeit a few limited published data (17-21). These questions are currently being investigated using a wide range of technical approaches and cell systems, which seems to be more advanced by the development of various CD45 mAbs. Currently, the modulation of CD45 function by mAbs has been suggested as an optional treatment to control autoimmune diseases, transplant rejection, and even cancer (22-25). Therefore, the possibility of therapeutic significance of these seven mAbs should be evaluated in the near future.

## CONFLICTS OF INTEREST

The author have no financial conflict of interest.

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