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# An Antiproliferative Monoclonal Antibody, 4F9, Reacts with a Subset of Human CD45 Molecules.

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## ABSTRACT

The 4F9 monoclonal antibody (IgM) was established for its ability to induce mild growth inhibition to a human T cell line, SUP-T13. The antibody reacted with all hematopoietic cell lines, but not with nonhematopoietic cell lines. Immunoprecipitation and immunoblotting experiments showed that 4F9 recognized cell surface molecules with apparent molecular weights of between 190 and 220 kDa which varied slightly in size among the cell lines. Therefore, it was strongly suggsted that 4F9 recognizes a subset of human CD45 molecules. In immunohistochemical analysis with paraffin-embedded tissue sections, 4F9 reacted with most lymphocytes except for germinal center B cells in lymph nodes and both medullary and cortical thymocytes. The results of the present study indicated that the anti-CD45, such as 4F9 antibody, affect growth of malignant T cells. The 4F9 antibody is useful in the immunohistochemical analysis of CD45 molecules in paraffin-embedded tissue sections.

**Key words:** CD45, Growth inhibition, Immunohistochemistry

## INTRODUCTION

CD45 molecules are membrane integrated tyrosine phosphatases, which have been shown to play important roles in signal transduction from antigen receptors on both T and B lymphocytes (1, 2). In human leukocytes, there are at least four CD45 isoforms with molecular weights of 210, 205, 190, and 180 kDa, which are the products of different splicing of a single CD45 gene (3). At the Fourth Leukocyte Antigen Workshop, it was decided to designate peptide domains encoded by exon 4, 5, and 6 as CD45RA, CD45RB, and CD45RC, respectively.

Abbreviations:
mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; MTT, 3(4,5)-dimethylthiazol-2, 5-diphenyl tetrazolium bromide; PBS, phosphate buffered saline.

Antibodies, such as UCHL-1, which react with determinants on exon 7 only in the absence of all variable exons, were designated CD45RO. Glycosylations confer further diversity to CD45 molecules (4, 5). Recent studies demonstrated that peripheral blood T lymphocytes are classified into two categories depending on the expression of CD45 molecules (6). One group is CD45RA-positive T cells which are suggested to be naive T cells, the other is CD45RO-positive T cells which are assumed to be memory T cells (7). Therefore, CD45RA+ and CD45RO+ cells appear to be mutually exclusive to each other in peripheral T lymphocytes. These studies suggest that expression of the subsets of CD45 molecules reflects the differentiation state of T lymphocytes (6, 8, 9) The putative ligands for the CD45 molecules are expected to play a regulatory role for activation and function of the T lymphocytes. Recently, it was demonstrated that CD22, one of the B cell markers, is the ligand for CD45RO, suggesting that CD45 may transduce signals produced by cell-to-cell interactions (10). However, little is known about the ligands for the other CD45 isoforms. We demonstrate here that a novel mAb, 4F9, originally isolated for its mild antiproliferative offect on a human leukemia T cell line recognizes a susbset of CD45 isoforms.

## MATERIALS AND METHODS

*Culture conditions and cell lines.*

RPMI 1640 (Sigma Chemical Co., St. Louis, MO, USA) supplemented with heat-treated 10% fetal calf serum and 2 mM L-glutamine (all from Gibco, Grand Island, New York, USA), hereafter referred to as culture media (CM), was used throughout this study unless stated otherwise. Cultures were incubated at 37°C in 5% $CO_2$ with 100% humidity. SUP-T13, Jurkat, CCRF-CEM, Molt-4 are T-cell lines. Raji, Daudi, Reh are B-cell lines. U937 and HL60 are histiocytoid cell lines. K562 and KG-1 are myeloid cell lines.

*Monoclonal antibodies and reagents.*

2H4 (CD45RA; IgG1) was from Coulter clone (Nikkaki Inc., Tokyo) (11). ZOL03 (CD45; IgG1) was purchased from Nichirei Inc. (Tokyo) (12). 2B11+ PD7/26 (CD45; IgG1) (13) and UCHL-1 (CD45RO; IgG2a) (14) were purchased from Dako Japan Inc. (Kyoto). Fluorescein (FITC)-conjugated goat anti-mouse Ig (G+M) was purchased (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA). For immunohistochemistry of paraffin-embedded tissue, Histofine kit was used as vender's protocol (Nichirei Inc.).

*Immunization and hybridoma production.*

The method of hybridoma production was prviously described (15). Briefly,

4F9 was produced by immunizing Balb/c mice with $2\text{-}4\times10^7$ of SUP-T13 cells intraperitoneally. A similar number of the cells were given i. v. and spleen cells were harvested three days later. Hybridomas were made by fusing the spleen cells with Balb/c myeloma cells, NS-1, using polyethylene glycol (#4000, Kanto Chemical Inc., Tokyo). The cells were resuspended in HAT medium and plated in 96-well flat bottom plates (Falcon #3072, Becton-Dickinson, CA, USA). After partial medium changes with CM several times, supernatants were harvested for screening two weeks post-fusion.

*Immunostaining and flow cytometry.*

Cells ($5\times10^5$) were suspended in 10 $\mu l$ of phosphate buffered saline (PBS) at 4°C and incubated with the indicated antibodies on ice for 30 minutes followed by incubation with FITC conjugated goat anti-mouse Ig (G+M) at 4°C for 30 min. After washing, cells were fixed in PBS containing 1.0% formaldehyde and were analyzed by a fluorescence-activated cell sorter (FACStar, Becton Dickinson). For blocking experiments, cells were preincubated with mAbs for 30 minutes at 4°C followed by biotinylated 4P9 and FITC conjugated streptavidin (Becton Dickinson).

*Cell surface radiolabelling and immunoprecipitation.*

Cell surface proteins were labeled with $^{125}I$ by the lactoperoxidase-glucose oxidase (Sigma Chemical Co.) method (16). They were then solubilized in 1.0% NP-40 in a lysis buffer (150 mM NaCl, 10 mM Tris, 0.02% $NaN_3$, 1 mM PMSF, 1.0 $\mu g/ml$ pepstatin, 1% aprotinin, 10 mM iodoacetamide) and used for immunoprecipitation. These cell lysates were incubated with specific antibodies, which had been preincubated with goat anti-mouse IgG conjugated Sepharose 4B beads (Sigma Chemical Co.). After washing the beads five times with lysis buffer and 0.5% of a single detergent, samples were boiled in sample buffer (62.5 mM Tris, 3.0% SDS, 10% Glycerol) containing 5.0% 2-mercaptoethanol (for reduced samples) or without 2ME (for non-reduced samples) and analyzed by 8% SDS-PAGE. Radiolabelled proteins were visualized by autoradiography using Kodak XAR 5 film and intensifying screens.

*Immunoblotting.*

The method for immunoblotting was previously described (17). Briefly, cell lysates were separated by 8% SDS-PAGE and blotted to a Immobilon membrane (Millipore, Bedford, MA, USA) by a semidry electroblotter (Bio-Rad Japan, Tokyo). After blocking the membrane with blocking buffer (1.0% bovine serum albumin, 0.05% Tween-20, 50 mM Tris, 140 mM NaCl, 0.01% $NaN_3$, pH 8.0),

the membrane was incubated with mAbs as indicated (1 $\mu$g/m*l* each in blocking buffer), followed by biotinylated goat anti-mouse Ig and alkaline phosphatase conjugated streptavidin. The membrane was developed by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (KPL, Gaitherburg, CA, USA).

*MTT assay.*

An assay for viable cells using MTT (3(4, 5)-dimethylthiazol-2, 5-diphenyl tetrazolium bromide) (Sigma Chemical Co.) has been described (18). Briefly, cells ($1\text{-}4\times10^4$) suspended in CM containing 4F9 (10 $\mu$g/m*l*) in 96-well flat bottom plates (Falcon #3072, Becton-Dickinson) were incubated at 37°C. After three days, MTT was added. A purple formazan product is formed by the action of mitochondrial enzymes in living cells. This product is solubilized by the addition of acidic isopropanol. The absorbance of each well was quantified by a multiwell scanning photometer (MicroELISA MR600, Dynatech Laboratories, INC., Alexandria, VA, USA) in OD units. The test wavelength was 570 nm and the reference was 630 nm. Percent growth inhibition was calculated according to the formula:

% growth inhibition = (1-O. D. of experimental mAb/O. D. of control medium) $\times$ 100

## RESULTS

*A mAb (4F9) induced mild antiproliferative effect on a human T leukemia cell line (SUP-T13)*

We have tried to isolate mAbs for their ability to induce growth inhibition on T cells to elucidate the growth-regulatory mechanism in T cells. The spleen cells from mice immunized with human T leukemia cells, SUP-T13, were fused with myeloma cells, NS-1, and the hybridoma cells were screened for their antiproliferative effects on the immunized T cells in a proliferative assay using MTT. A mAb, 4F9 with IgM subclass, was successfully isolated. This mAb demonstrated relatively mild antiproliferative effect, up to 30% growth inhibition, on SUP-T13 at a concentration of 10 $\mu$g/m*l* (Table 1). This antiproliferative effect seemed rather cytostatic than cytocidal, because the T leukemia cells kept growing in the presence of 4F9 mAbs. In a panel of cell lines, reactivity and antiproliferative effects were analized. 4F9 failed to react with epitheloid cell lines (data not shown). All of the hematopoietic cell lines reacted with 4F9 mAb, however, the antiproliferative effects of the mAb were insignificant in most of the cell lines. Activation of peripheral blood T cells by mitogens was not apparently affected either (data not shown).

**Table 1** *Reactivity of 4F9 with various hematopoietic cell lines*

| Cell type | Reactivity | % Growth inhibition |
|---|---|---|
| T cell | | |
| SUP-T13 | + | 20-30% |
| Jurkat | + | <10% |
| CCRF-CEM | + | <10% |
| Molt-4 | + | <10% |
| B cell | | |
| Raji | + | <10% |
| Daudi | + | <10% |
| Reh | + | <10% |
| Myelomonocytoid cell | | |
| U937 | + | <10% |
| HL60 | + | <10% |
| K562 | + | <10% |
| KG-1 | + | <10% |

*4F9 reacts with a subset of CD45 molecules*

Immunoprecipitation and western blotting experiments were undertaken to define the antigen molecules of 4F9 with various cell lines. Immunoprecipitaion of 4F9 with the lysates from surface labeled cells of SUP-T13 demonstrated a band of approximately 190-200 kDa in both non-reducing and reducing conditions (Fig. 1A and data not not shown). A mixture of anti-CD45 mAb, 2B11+PD 7/26, demonstrated broad bands between 190 and 220 KDa. Anti-CD45RA (2H4) showed similar band with approximately 200 kDa. In western blotting analysis, comparable bands were detected by 4F9 with the lysates of another T cell line, Molt-4 (Fig. 1B). Anti-CD45 mAbs demonstrated bands between 190 and 220 kDa in a similar fashion with immunoprecipitation. However, 4F9 demonstrated different bands with approximately 200 kDa from a B cell line, Reh, as well as the other anti-CD45 mAbs (Fig. 1B). Because of the common reactivity of 4F9 among hematopoietic cell lines and the antigen molecules of the mAb varies around 200 kDa, 4F9 most likely reacts with a subset of CD45 molecules (9, 19, 20). Next we examined to see whether the subfamily-specific anti-CD45 mAbs can block the binding of 4F9. As shown in Fig. 2, flow cytometric analysis demonstrated that the binding of 4F9 was blocked by anti-CD45 (both ZCL03 and 2B11+PD7/26) and anti-CD45RO (UCHL-1), but not by anti-CD45RA (2H4). These data indicate that 4F9 recognizes an epitope commonly shared among various CD45 molecules and that 4F9 epitope is, at least, not identical to CD45RA.

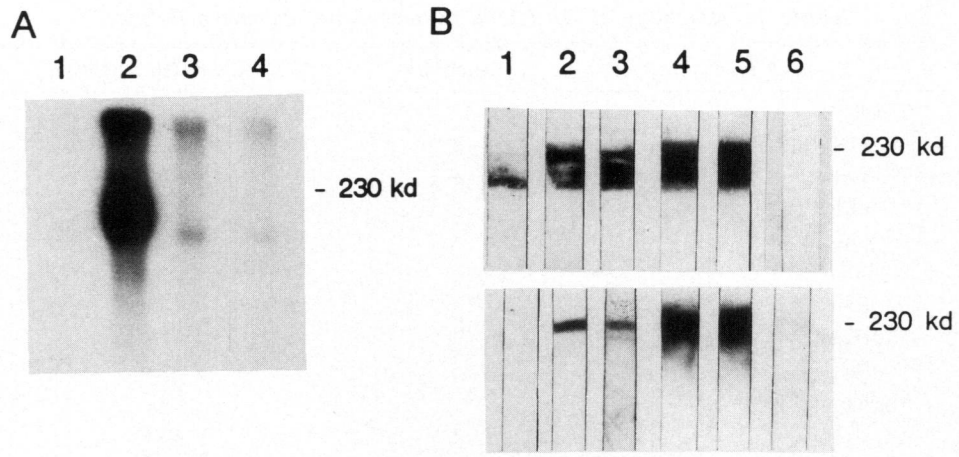


**Fig. 1** Reactivity of 4F9 with cell lines by immunoprecipitation (A) and western blotting (B). (A) Immunoprecipitation of $^{125}$I-labeled lysate of SUP-T13 with irrelevant control mAb, lane 1: 2B11+PD7/26 (CD45), lane 2: 4F9, lane 3: 2H4 (CD45RA), lane 4. (B) Western blotting anallysis of T cell line, Molt-4 (top), and B cell line, Reh (bottom), with UCHL-1 (CD45RO), lane 1: 2H4 (CD45RA), lane 2: 4F9, lane 3: ZCL03 (CD45), lane 4: 2B11+PD7/26 (CD45), lane 5: irrelevant control mAb, lane 6.

*Immunohistochemical analysis of 4F9 mAb*

For further analysis of the specificity of 4F9 mAb, tissue distribution of the 4F9 mAb was compared with the other anti-CD45 mAbs immunohistochemically. 4F9 showed exclusive reactivity with lymphoid cells in formalin-fixed paraffin-embedded tissue sections, as well as frozen sections (data not shown). In normal lymph nodes, 4F9 reacts with paracortical T cells and B cells in mantle zone, but not with most germinal center cells except for a few scattered T cells (Fig. 3C). In contrast, anti-CD45 (2B11+PD7/26) stained all lymphocytes in the lymph node (Fig. 3A). Although anti-CD45RA (2H4) showed similar bands to 4F9 in western blotting experiments, 2H4 demonstrated different reactivity from 4F9 in tissue staining, as 2H4 was positive in mantle zone and germinal center and was largely negative in the parafollicular area (Fig. 3D). UCHL-1 (CD45RO) stained T cells in the paracortex, but not most of the mantle zone or germinal center except for the T cells in the light zone of the germinal center (Fig. 3B).

In the immunohistochemical analysis of thymus, 4F9 stained both cortical and medullary thymocytes (Fig. 4C). Anti-CD45 (2B11+PD7/26) also stained

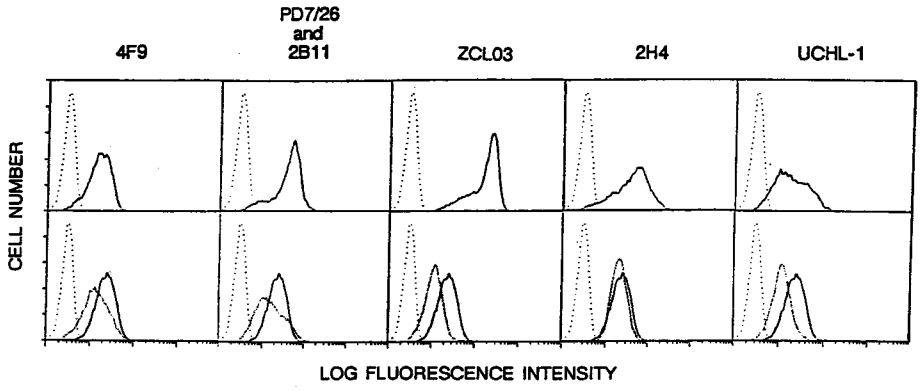


**Fig. 2** Flow cytometric analysis of SUP-T13 cells with 4F9 and various anti-CD45 mAbs. In immunostaining analysis (top histgram), SUP-T13 cells stained with the mAb (solid lines) noted on the top of each histogram followed by FITC conjugated goat anti-mouse Ig (G+M). Irrelevant negative control was indicated as dotted lines. All of the tested mAbs demonstrated positive staining on SUP-T13 cells. In blocking of 4F9 by various anti-CD45 mAbs (bottom histgram), SUP-T13 cells were preincubated with mAbs (dense dotted lines) noted with the top of the histgram followed by biotinylated 4F9 and FITC conjugated streptavidin. Positive staining of biotinylated 4F9 (solid lines) and negative control staining (faint dotted lines). Anti-CD45 (both PD7/26+2B11 and ZCL03) and anti-CD45RO (UCHL-1), but not anti-CD45RA (2H4), demonstrated apparent blocking of 4F9 binding.

**Table 2** *Reactivity of anti-CD45 mAbs, 4F9, ZCL03, 2H4, and UCHL-1 with lymphoid cells*

| Tissue | 4F9 | ZCL03[@] (CD45) | 2H4 (CD45RA) | UCHL-1 (CD45RO) |
|---|---|---|---|---|
| Thymus | | | | |
| Cortical | + | + | − | + |
| Medullary | + | + | + | ± |
| Lymph | | | | |
| Parafollicular T | + | + | − | + |
| Mantle B | + | + | + | − |
| Germinal center B | − | + | + | − |
| Germinal center T | + | + | − | + |

[@] A mixture of mAbs, 2B11+PD7/26, demonstrated essentially same staining pattern with ZCL03.

virtually all of the thymocytes (Fig. 4A). Reactivity of anti-CD45RA (2H4) was largely confined to the medulla and only a few cells were positive in the

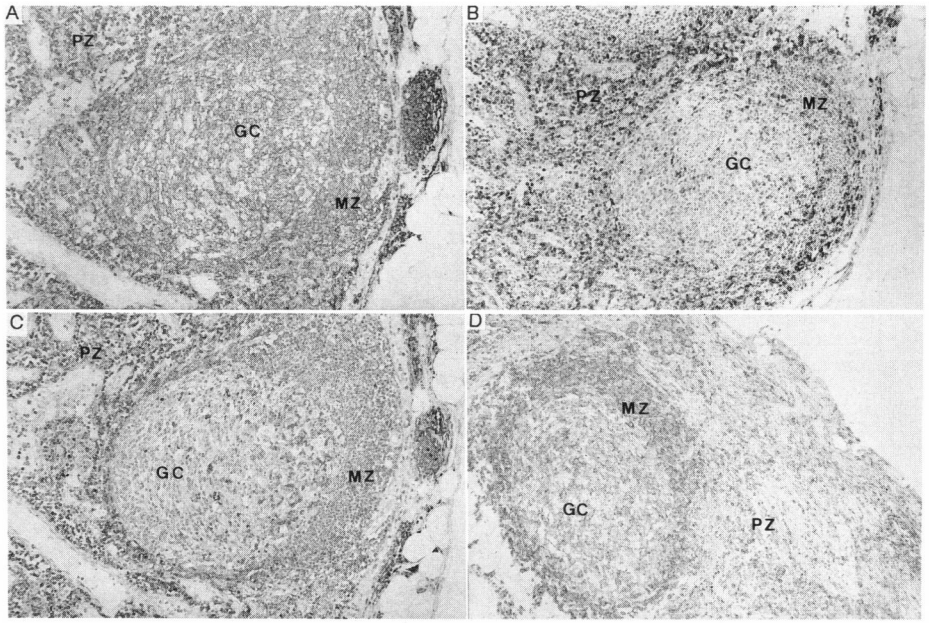


**Fig. 3** Immunohistochemistry of lymph nodes with various anti-CD45 mAbs. PD7/26+2B11 (CD45) stains virtually all lymphoid cells (A). UCHL-1 (CD45RO) stains most of the paracortical T cells as well as T cells at the border of germinal center and mantle zone (B). 4F9 stains most of the paracortical T cells and mantle zone B cells, but not germinal center B cells (C). 2H4 (CD45RA) stains germinal center and mantle zone (D). Abbreviations: GC, germinal center; MZ, mantle zone; PZ, paracortical zone.

cortex (Fig. 4D). Anti-CD45RO (UCHL-1) demonstrated reciprocal expression with 2H4 (CD45RA) in the thymus as well as lymph nodes, as UCHL-1 reacts all cortical thymocytes and a few medullary thymocytes (Fig. 4B). The results from immunohistochemical analysis of the lymphoid tissue are summarized in Table 2. These results indicate that 4F9 reacts with a determinant on the common region of CD45, but not with CD45RA or CD45RO.

## DISCUSSION

We established a mAb, 4F9 (IgM), for its mild antiproliferative effect on SUP-T13 cells. In immunoprecipitation analysis, 4F9 reacted with heterologous molecules of about 200 kD which varied among hematopoietic cell lines. In immunohistochemical analysis, 4F9 showed restricted reactivity to T cells in paracortical areas and B cells in mantle zones in normal lymph nodes. Thus we concluded that 4F9 reacts with a subset of CD45 molecules.

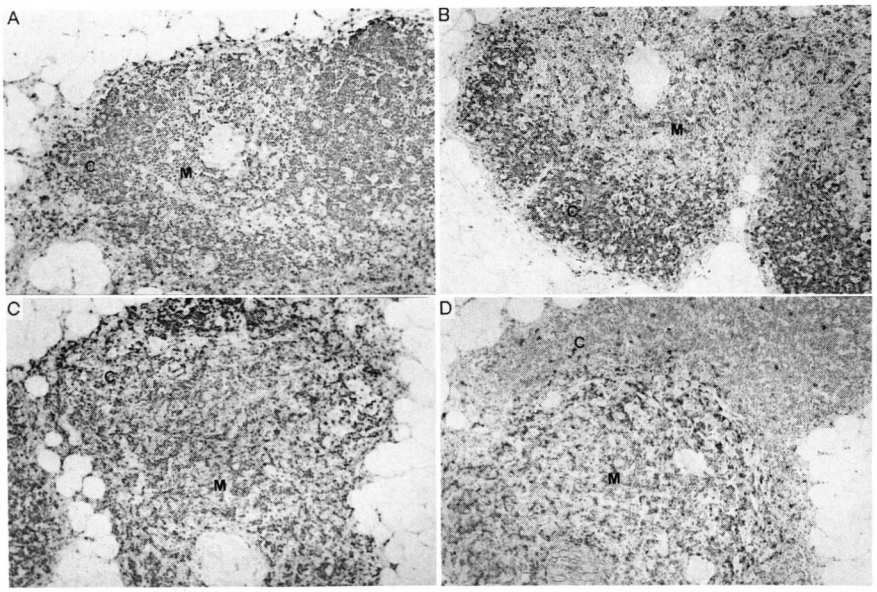


**Fig. 4** Immunohistochemistry of thymus with various anti-CD45 mAbs. PD7/26+2B11 (CD45) stains both cortical and medullary thymocytes (A). UCHL-1 (CD45RO) stains most of the cortical thymocytes and a few medullary thymocytes (B). 4F9 stains medullary thymocytes and cortical thymocytes slightly intensely (C). 2H4 (CD45RA) stains medullary thymocytes and a few cortical thymocytes (D).
Abbreviations: C, cortex; M, medulla.

CD45, also known as leukocyte common antigen, is a family of glycoproteins exclusively expressed on the cells of hematopoietic lineage. There are at least four CD45 isoforms with molecular weights of 180 kDa, 190 kDa, 205 kDa, and 220 kDa, which are produced from a single structural gene by alternate splicing of the mRNA (3, 20, 21, 22). Recent biochemical analysis demonstrated that CD45 is a membrane integrated tyrosine phophatase, which is involved in the activation of both T and B cells (1, 2, 8, 23, 24, 25, 26, 27, 28). Little is known about its ligand except for CD45RO, which has recently been demonstrated to interact with CD22, a cell surface antigen of B cells (10). It has been shown that anti-CD45 mAbs may have various effects on T cell activation, including inhibitory effects on mitogen stimulation of peripheral blood T cells. However, it is not well known whether the anti-CD45 mAbs affect the growth of cultured malignant hematopoietic cells or not. Although, 4F9 demonstrated mild anti-proliferative effects on SUP-T13 cells, the antibody failed to exert consistent growth inhibition in the other hematopoietic cell lines tested. The antiprolifer-

ative effects of 4F9 seemed to be cytostatic rather than cytocidal, but the selective antiproliferative effects of 4F9 on SUP-T13 might be associated with the high sensitivity of the cell line to the apoptotic stimuli as demonstrated previously (18). It is also possible that the crosslinking effect of IgM subclass may be attributable to the effect. 4F9 induced cell aggregation being similar to a mAb, anti-TAPA-1 (17). As SUP-T13 cells were also sensitive to anti-TAPA-1, cell aggregation induced by 4F9 may have a role in the growth inhibition of SUP-T13, At present, the mechanism of mild antiproliferative effect of 4F9 on SUP-T13 remains unknown.

In flow cytometric analysis, the binding of 4F9 to SUP-T13 cell is blocked by anti-CD45 mAbs and by anti-CD45RO, but not by anti-CD45RA. Therefore, it is likely that 4F9 reacts with a determinant on the common extracellular domain of the CD45 between variable region and transmembrane peptide sequence (21, 22). The fact that the specificity of 4F9 was largely identical with that of anti-CD45 mAbs in immunohistochemical analysis of lymph nodes and thymus, except for the negative staining of germinal center of lymph nodes, may support the idea. Clone 2B11, which reacts with carbohydrate determinants on the CD45, has been classified as anti-CD45 mAb and is also known to be unreactive with most of the germinal center cells (5, 13). In contrast to anti-CD45 mAbs, anti-CD45RA and anti-CD45RO stained mutually exclusive areas to each other in lymph nodes: UCHL-1 (CD45RO) stained paracortical T cells and a few T cells at the border of the germinal center and the mantle zone. The results were consistent with the findings obtained by other investigators which showed that CD45RA+ and CD45RO+ T cells may reflect naive T cells and memory T cells, respectively (11). Immunohistochemical analysis suggested that 4F9 recognizes CD45 molecules, but not CD45RA or CD45RO (5, 12, 20). Although 4F9 demonstrated a similar reactivity to 2H4 (CD45RA) in western blotting experiments, it seemed difficult to define the isoforms solely based on the molecular weights, because it has been shown that 205 kDa band, such as 2H4 antigen, seen in SDS-PAGE analysis is composed of at least two different isoforms of CD45 (29). Alternatively, it was considered to be the results of denaturation of CD45 molecules in the presence of a detergent and thus altered the reactivity of 4F9.

In conclusion, the 4F9 (IgM) antibody which was established for its mild antiproliferative effect on SUP-T13 cells, is shown to react with a subset of CD45 molecule, 4F9 antibody has a certain advantage to be useful for the immunohistochemical studies of CD45 molecules in paraffin-embedded tissues.

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