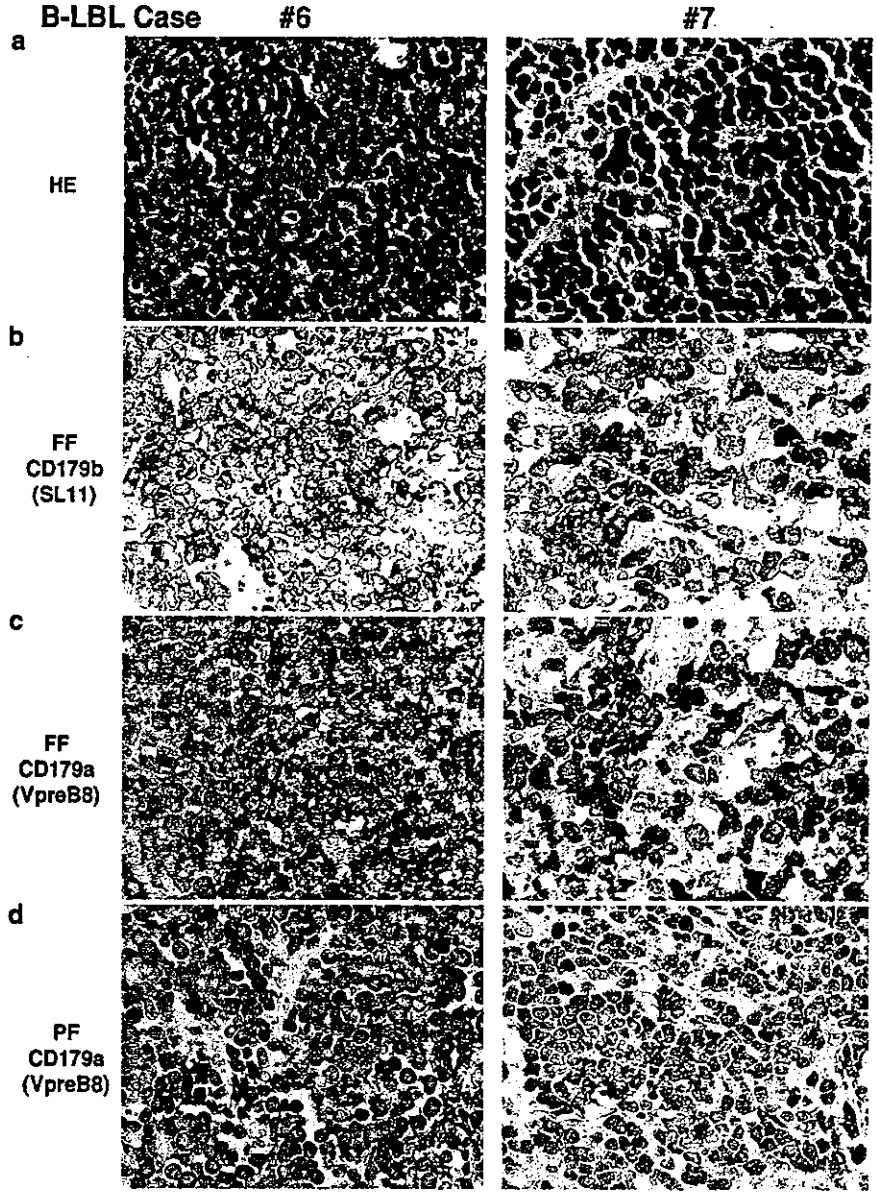

Figure 2 Immunohistochemical detection of CD179a and CD179b. CD179a and CD179b were detected in B-lymphoblastic lymphoma tissues using immunohistochemical staining on acetone-fixed fresh frozen sections ((**b**), (**c**), FF) and formalin-fixed, paraffin-embedded tissue sections ((d), PF) from biopsy tissues. The H&E-staining of formalin-fixed and paraffin-embdded tissues is also shown ((a), HE).

Immunohistochemical Staining of CD179a in Formalin-fixed, Paraffin-embedded Tissues

Next, we examined whether mAbs against CD179a and CD179b could be used in formalin-fixed, paraffin-embedded tissues. When paraffin-embedded tissues prepared from clinical specimens obtained from B-LBL patients were examined using immunohistochemical staining with the heat-induced epitope retrieval treatment, only VpreB8 reacted with the tissue. The staining results were consistent with those obtained from the immunostaining of acetone-fixed frozen sections. None of the other mAbs reacted with the B-LBL samples. Since higher concentrations of VpreB8 resulted in nonspecific nuclear staining in paraffin sections of Burkitt lymphomas, care must be taken when deciding the appropriate conditions for the use of this mAb.

Discussion

In the current study, we clearly presented that both VpreB8 and HSL11 are useful for the immunohistochemical detection of CD179a and CD179b, respectively, in acetone-fixed B-LBL tissues. Furthermore, VpreB8 can also be used in paraffin-embedded sections. The reactivities of these Abs were highly specific for B-LBL. Reactivity was not seen in tissues of Burkitt lymphoma, diffuse large B-cell

Table 1 Detection of CD179a and CD179b in B-lineage lymphoma tissues using immunohistochemical staining in acetone-fixed fresh frozen sections

Case no.	*Age (years)*	*Sex*	*Origin*	*CD179a*	*CD179b*	*TdT*	*CD34*	*CD19*	*CD79a*	*DR*	*CD20*	μ	*LC*	*CD10*	*CD77*
B-LBL															
1	4	M	Bil-CL	+	+	+	−	+	+	+	+P	−	−	+	−
2[a]	9	M	R-testis	+	+	+	−	+	+	+	+M	NT	NT	+	−
3	7	M	L-CL	+	+	+	−	+	+	+	−	+	−	+	−
4	5	F	L-CL	+	+	+	−	+	+	+	−	−	−	+	−
5	7	F	L-CL	+	+	+	+	+	+	+	−	+	−	+	−
6	1	F	R-CL	+	+	+	−	+	+	+	−	−	−	+	−
7	12	M	AT	+	+	−	−	+	+	+	−	−	−	+	−
8	5	F	L-upper arm	+	−	−	−	+	+	+	+M	−	−	+	NT
9	7	M	L-CL	−	−	+	−	+	+	+	+P	−	−	+	NT
10	4	F	R-radius	+	−	−	+	+	+	+	−	−	−	+	−
11	9	M	CNS	NT	+	+	NT	+	+	NT	NT	+	NT	NT	NT
Burkitt															
1	6	F	AT	−	−	−	−	+	+	+	+	+	−	−	+
2	7	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
3	15	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
4	4	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	+
5	6	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	−
6	5	M	AT	−	−	−	−	+	+	+	+	+	Kappa	+	+
7	4	M	AT	−	−	−	−	+	+	+	+	+	Lamda	+	+
B-DL															
1	7	F	R-CL	−	−	−	−	+	+	+	+	+	Lamda	−	−
2	6	M	AT	−	−	−	−	+	+	+	+	+	Lamda	−	−
3	8	M	R-CL	−	−	−	−	+	+	+	+	+	Lamda	+	−

B-LBL, precursor B-cell lymphoblastic lymphoma; DL, diffuse large cell lymphoma; Bil, bilateral; L, left; R, right; CL, cervical lymph nodes; AT, abdominal tumor; LC, light chains; NT, not tested; P, patchy staining pattern; M, membranous staining pattern.
[a]Testicular relapse of precursor B acute lymphoblastic leukemia.

Table 2 Immunohistochemical staining of CD179a and CD179b on acetone-fixed fresh frozen sections of non-B-cell lineage neoplasm tissues

	Positivity	
	CD179a	*CD179b*
T-LBL	0/7	0/7
Extramedullary myeloid tumors		
Granulocytic sarcoma	0/2	0/2
AMoL, skin infiltration	0/1	0/1
Ewing sarcoma	0/2	0/2

T-LBL, precursor T-cell lymphoblastic lymphoma; AMoL, acute monocytic leukemia.

lymphoma, T-LBL, extramedullary myeloid tumors, and Ewing sarcoma.

In pediatrics, the three major types of B-cell lymphoma are B-LBL, Burkitt lymphoma, and diffuse large B-cell lymphoma; the latter two types must be distinguished from B-LBL since the therapeutic protocols for these diseases are quite different from that for B-LBL. In the Berlin Frankfurt Munster (BFM) study group, for example, B-LBL cases were treated using ALL-type protocol with a total therapy duration of at least 24 months.[17] In contrast, mature B-cell lymphoma cases, including Burkitt lymphoma and diffuse large B-cell lymphoma, are treated using a short course of treatment that is usually completed within a year.[18] Each type of B-lineage lymphoma is morphologically unique and distinctive upon histological examination. In the practical pathological diagnosis of lymphomas, however, pathologists may experience difficulties in differentiating B-LBL from other B-lineage lymphomas, especially when only poor-quality biopsy specimens are available.[12] Unfortunately, pathologists are not always familiar with B-LBL because of its rarity among childhood lymphomas; as a result, patients with B-LBL may be misdiagnosed as having mature B-cell lymphoma, such as Burkitt lymphoma. The similarity in marker expression patterns for B-LBL and Burkitt lymphoma is also partly responsible for the risk of misdiagnosis.[11,12]

TdT is considered to be a reliable marker for the diagnosis of cases of precursor lymphocyte origin,[11,12] but TdT is not always positive in B-LBL cases as reported by several different groups.[19–22] For example, Mertelsmann *et al*[20] reported that TdT was absent in approximately 5% of ALL and LBL cases. Orazi *et al*[21] also reported that 6% (two out of 35) of LBL cases was TdT-negative assessed by immunohistochemical staining. On the other hand, CD34 is expressed on human bone marrow progenitor cells and leukemic blasts, and is considered to be an immature marker. Although the expression of CD34 on B-lineage lymphomas suggests their precursor B-cell origin, the positivity of CD34 among the B-LBL cases is approximately 50%. In addition, both TdT

and CD34 are not restricted to the precursor of B cells. In contrast, CD20 is a B-cell-specific marker and its expression increases with B-cell maturation. Therefore, the absence of CD20 expression in B-lineage lymphomas suggests their precursor B-cell origin. However, CD20 expression is variable among cases of B-LBL and approximately 50% of B-LBL cases are CD20-positive, exhibiting sometimes a strong membranous staining pattern.[11] Therefore, it is difficult to specify a B-precursor origin using CD20 expression alone. Based on the above evidences, the development of other markers capable of revealing a precursor B-cell origin is urgently required; in this regard, the results described here are expected to assist in the proper diagnosis of B-LBL among B-cell lymphomas in childhood.

CD179a and CD179b are essential for the development of precursor B cells. Although their biological significance is not fully understood, they are believed to serve as surrogate LCs expressed with μ HCs in pre-BCR to determine whether the clone should survive or die. After subsequent rearrangements in κ or λ LC genes, the expression of the surrogate LCs is suppressed.[6–9] The utilization of such functional molecules in the diagnosis of precursor B-cell lymphomas is appropriate if the expression is conserved even in tumor cells. In precursor B-ALL cells, we previously reported that CD179a, CD179b, and the complete form of pre-BCR were detected by HSL96, HSL11, and HSL2, respectively, and were expressed in most of the CD10-positive precursor B-ALL cases,[10] suggesting that these markers may be useful for the further classification of this disease. Consistent with this observation, CD179a and CD179b, detected by VpreB8 and HSL11, respectively, were frequently expressed in B-LBL cases, whose origin is comparable to that of precursor B-ALL. Thus, the successful employment of these functional molecules in the diagnosis of B-cell lymphomas is another important aspect emphasized in this study.

As shown here, CD179a and CD179b immunohistochemistry can identify more than 90% of B-LBL cases. In our series, the positivity of TdT among the B-LBL cases examined was lower (73%) than that of previous reports.[19–22] The reason for this discrepancy is not known, but it is noteworthy that three TdT-negative cases were positive for either CD179a or CD179b or both. Thus, by combining the TdT and CD179 markers, we believe that virtually all B-LBL cases can be properly judged as having a precursor B-cell origin. The absence of CD179a/b reactivity in Burkitt and diffuse large B-cell-type lymphomas further supports the reliability of this marker.

Occasionally, B-LBL may be misdiagnosed as Ewing sarcoma, since these two diseases have similar morphologies and immunostaining patterns.[23] CD99 (MIC2) was previously considered to be a specific marker for Ewing sarcoma, but this molecule has now been shown to be frequently expressed in B-LBL. Bone tumors with a blastic morphology and a CD45−, CD20−, MIC2+ phenotype can be diagnosed as Ewing sarcoma. In such cases, immunostaining for CD179a/b along with TdT and CD79a will lead to a proper diagnosis. In addition, immunostaining for CD179a/b is also useful for distinguishing B-LBL from either T-LBL or extramedullary myeloid tumors, both of which are included in frequent differential for B-LBL.

Diagnostic markers must be usable in paraffin sections for practical diagnostic procedures. In this regard, the utilization of mAb VpreB8 in paraffin sections, as demonstrated in this report, should facilitate its use in daily diagnostics. Caution must be exercised, however, when using VpreB8 because this antibody may produce nonspecific binding. After careful examination, we selected a concentration of 1.25 μg/ml for our system; however, this value should be evaluated for each laboratory in which the mAb is used, since differences in detection systems may affect the results. Other than VpreB8, unfortunately, none of the mAbs against CD179a/b tested in this study was useful for immunohistochemical detection in paraffin-embedded sections. Since the expression of CD179b was always accompanied by that of CD179a in our cases assessed using fresh frozen section staining (Table 1), paraffin section staining with VpreB8 may be sufficient for the diagnosis of B-LBL. However, the generation of novel mAbs against CD179a/b and preBCR that can react in paraffin sections would be useful and may provide more convincing results.

In conclusion, we have demonstrated that mAbs against CD179a/b specifically detect B-LBL tissues. Although an examination of a larger number of lymphoma tissues is required to confirm their reliability, the application of these mAbs in the immunohistochemical examination of lymphoma tissues should contribute to a precise diagnosis of B-lineage lymphomas.

Acknowledgements

This work was supported in part by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan and MEXT, KAKENHI 15019129, JSPS, KAKENHI 15390133 and 15590361. This work was also supported by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation. Additional support was provided by a grant from Sankyo Foundation of Life Science.

We thank M Sone and S Yamauchi for their excellent secretarial works. We also thank Dr S Hakomori (Washington University) and Otsuka Assay Laboratories for gifting CD77 mAb 1A4.

eferences

1 Sakaguchi N, Melchers F. λ5, a new light-chain-related locus selectively expressed in pre-B lymphocytes. Nature 1986;324:579–582.
2 Kudo A, Melchers F. A second gene, VpreB in the λ5 locus of the mouse, which appears to be selectively expressed in pre-B lymphocytes. EMBO J 1987;6: 2267–2272.
3 Bauer SR, Kudo A, Melchers F. Structure and pre-B lymphocyte restricted expression of the VpreB in humans and conservation of its structure in other mammalian species. EMBO J 1988;7:111–116.
4 Hollis GF, Evans RJ, Stafford-Hollis JM, *et al.* Immunoglobulin λ light-chain-related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. Proc Natl Acad Sci USA 1989;86:5552–5556.
5 Schiff C, Bensmanna M, Gulglielmi P, *et al.* The immunoglobulin λ-like gene cluster (14.1, 16.1 and Fl1) contains gene(s) selectively expressed in pre-B cells and is the human counterpart of the mouse λ5 gene. Int Immunol 1990;2:201–207.
6 Karasuyama H, Kudo A, Melchers F. The proteins encoded by the VpreB and λ5 pre-B cell specific genes can associate with each other and with μ heavy chain. J Exp Med 1990;172:969–972.
7 Tsubata T, Reth M. The products of pre-B cell-specific genes (λ5 and VpreB) and the immunoglobulin μ chain form a complex that is transported onto the cell surface. J Exp Med 1990;172:973–976.
8 Karasuyama H, Rolink A, Shinkai Y, *et al.* The expression of VpreB/λ5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. Cell 1994;77:133–143.
9 Lassoued K, Nunez CA, Billips L, *et al.* Expression of surrogate light chain receptors is restricted to a late stage in pre-B cell differentiation. Cell 1993;73:73–86.
0 Tsuganezawa K, Kiyokawa N, Matsuo M, *et al.* Flow cytometric diagnosis of the cell lineage and developmental stage of acute lymphoblastic leukemia by novel monoclonal antibodies specific to human preB cell receptor. Blood 1998;92:4317–4324.
1 Brunning RD, Borowitz M, Matutes E, *et al.* Precursor B lymphoblastic leukaemia/lymphoblastic lymphoma (precursor B-cell acute lymphoblastic leukaemia). In: Jaffe ES, Harris NL, Stein H, Vardiman JW (eds). Pathology & Genetics: Tumours of Haematopoietic and Lymphoid Tissues. IARC Press: Lyon, 2001, pp 111–114.
2 Gatter K, Delsol G. B-cell lymphoblastic lymphoma. In: Gatter K, Delsol G (eds). The Diagnosis of Lymphoproliferative Diseases. Oxford University Press: Oxford, 2002, pp 59–63.
13 Ishii Y, Takami T, Yuasa H, *et al.* Two distinct antigen systems in human B lymphocytes: identification of cell surface and intracellular antigens using monoclonal antibody. Clin Exp Immunol 1984;58:183–192.
14 Ishii Y, Kon S, Takei T, *et al.* Four distinct antigen systems on human thymus and T cells defined by monoclonal antibodies: immunohistological and immunochemical studies. Clin Exp Immunol 1983;53: 31–40.
15 Fujimoto J, Ishimoto K, Kiyokawa N, *et al.* Immunocytological and immunochemical analysis on the common acute lymphoblastic leukemia antigen (CALLA): evidence that CALLA on ALL cells and granulocytes are structurally related. Hybridoma 1988;7:227–236.
16 Ishii E, Fujimoto J, Tanaka S, *et al.* Immunohistochemical analysis on normal nephorogenesis and Wilms' tumor using monoclonal antibodies reactive with lymphohaematopoietic antigens. Virchows Arch A Pathol Anat Histopathol 1987;411:315–322.
17 Neth O, Seidemann K, Jansen P, *et al.* Precursor B-cell lymphoblastic lymphoma in childhood and adolescence: clinical features, treatment, and results in trials NHL-BFM 86 and 90. Med Pediatr Oncol 2000;35: 20–27.
18 Kavan P, Kabickova E, Gajdos P, *et al.* Treatment of children and adolescents with non-Hodgkin's lymphoma (results based on the NHL Berlin–Frankfurt–Munster 90 protocols). Cas Lek Cesk 1999;138:40–46.
19 Kung PC, Long JC, McCaffrey RP, *et al.* Terminal deoxynucleotidyl transferase in the diagnosis of leukemia and malignant lymphoma. Am J Pathol 1978;64:788–794.
20 Mertelsmann R, Moore MA, Clarkson B. Methods and clinical relevance of terminal deoxynucleotidyl transferase determination in leukemic cells. Haematol Blood Transfus 1981;26:68–72.
21 Orazi A, Cattoretti G, Jphn K, *et al.* Terminal deoxynucleotidyl transferase staining of malignant lymphomas in paraffin sections. Mod Pathol 1994;7: 582–586.
22 Kaleem Z, Crawford E, Pathan MH, *et al.* Flow cytometric analysis of acute leukemias. Diagnostic utility and critical analysis of data. Arch Pathol Lab Med 2003;127:42–48.
23 Hsiao CH, Su IJ. Primary cutaneous pre-B lymphoblastic lymphoma immunohistologically mimics Ewing sarcoma/primitive neuroectodermal tumor. J Formos Med Assoc 2003;102:193–197.

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Veterinary Immunology and Immunopathology 103 (2005) 113–127
Veterinary immunology and immunopathology
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Characterization of new monoclonal antibodies against porcine lymphocytes: molecular characterization of clone 7G3, an antibody reactive with the constant region of the T-cell receptor δ-chains

W.-R. Tang[a], N. Shioya[a], T. Eguchi[c], T. Ebata[a], J. Matsui[a], H. Takenouchi[a], D. Honma[c], H. Yasue[c], Y. Takagaki[d], S. Enosawa[b], M. Itagaki[a], T. Taguchi[a], N. Kiyokawa[a], H. Amemiya[b], J. Fujimoto[a,*]

[a]*Department of Developmental Biology, National Research Institute for Child Health and Development, 3-35-31 Taishido, Setagaya-ku, Tokyo 154-8567, Japan*
[b]*Department of Innovative Surgery, National Research Institute for Child Health and Development, 3-35-31 Taishido, Setagaya-ku, Tokyo 154-8567, Japan*
[c]*Genome Research Department, National Institute for Agrobiological Science, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan*
[d]*Department of Molecular Medicine, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara Kanagawa 288-8555, Japan*

Received 14 November 2003; received in revised form 9 July 2004; accepted 26 August 2004

Abstract

A battery of mouse monoclonal antibodies (mAbs) reactive with porcine peripheral blood (PB) leukocytes was generated. Among the mAbs, 6F10 was found to react probably with cluster of differentiation (CD)8 α-chain, while 7G3 and 3E12 were found to recognize γδ T-cells, as revealed by two-color flow cytometric and immunoprecipitation studies. 7G3 was shown to react with the constant (C) region of the T-cell receptor (TCR) δ-chain by the following facts: (1) 7G3 immunoprecipitated full-length TCR δ-chain protein fused with glutathione S-transferase (GST) produced by *Escherichia coli* and (2) 7G3 reacted with TCR δ-chain expressing Cos-7 cells transfected with either full-length or N-terminal deleted mutant cDNA, but did not react with Cos-7 cells transfected with C-terminal deleted mutant TCR δ-chain cDNA. All three mAbs produced high-quality immunostaining results on frozen sections, revealing a distinct distribution of γδ T-cells and $CD8^+$ cells. This report precisely

Abbreviations: CD, cluster of differentiation; PB, peripheral blood; C region, constant region; PE, phycoerythrin; GST, glutathione S-transferase; HRP, horseradish peroxidase

* Corresponding author. Tel.: +81 3 3487 9669; fax: +81 3 3487 9669.
E-mail address: jfujimoto@nch.go.jp (J. Fujimoto).

0165-2427/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2004.08.018

characterizes mAbs against porcine TCR for the first time, facilitating molecular biological investigations of the porcine immune system.
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Keywords: Pig; T lymphocytes; Antibodies; T-cell receptors

1. Introduction

Considerable interest has been focused on the immunobiology of the pig, since this animal is regarded as a candidate for organ supply in transplantation medicine. From an animal husbandry point of view, understanding the immune systems of livestock animals enables the food supply to be improved through the manipulation of immunity-related genes. In this regard, workshops on porcine cluster of differentiation (CD) have been conducted and reports are published periodically. Several mAbs have been established, but the number of mAbs for porcine leukocyte Ags remains small compared with those for murine and human systems; furthermore, most of the mAbs that have been established for porcine systems require a more detailed characterization (Haverson et al., 2001a,b).

We thus attempted to develop new mAbs that could be used to analyze the porcine immune system. This manuscript describes the development of various mAbs that are reactive with porcine leukocytes. In particular, one mAb designated as 7G3 was confirmed to react with the constant (C) region of the recombinant TCR δ-chain. This report is the first description of a mAb against porcine TCR δ-chain, whose characterization was confirmed using molecular biology techniques. The utilization of the newly developed mAbs in the immunostaining of tissue sections was also demonstrated.

2. Materials and methods

2.1. Animals and tissues

Landrace or (Landrace × Large White) F1 pigs were used in the study. Peripheral blood (PB) and tissues were obtained from anesthetized animals and processed using conventional techniques, PB was collected in tubes containing acid citric buffer to avoid coagulation. Tissues were immediately snap frozen and kept in a deep freezer until use. In some experiments, thymus tissue was minced with scissors to generate a cell suspension. A viable thymocyte suspension was then prepared using Ficoll–Paque (Immuno-Biological Laboratories Co. Ltd., Takasaki-shi, Gunma-ken, Japan) gradient centrifugation.

2.2. Monoclonal antibodies

PB leukocytes were isolated from PB by RBC lysis with an NH_4Cl lysis buffer followed by centrifugation at 1500 rpm for 10 min. After washing twice in PBS, approximately 1×10^8 cells were injected into the abdominal cavity of an 8-week-old female Balb/c mouse. Two boost injections were performed at 2-week intervals. Four days after the last boost injection, splenocytes were fused with P3U1 mouse myeloma cells and incubated in hypoxanthine and thymidine (HAT) medium. Supernatants of growing hybridomas were screened on porcine PB leukocytes using flow cytometry, and clones that secreted Abs which were reactive with porcine PB leukocytes were subcloned twice using limited dilutions. The clones were grown in the abdominal cavity of Pristane-treated Balb/c mice and ascites samples were subsequently obtained. Purification of the mAbs was performed using a Protein-G column (Bio-Rad Laboratories, Hercules, CA). After purification, the mAbs were either biotinylated or conjugated with FITC, as previously described (Fujimoto et al., 1988). Commercially available mAbs against porcine CD2 (clone MSA4), CD3 (8E6), CD4 (74-12-4, also known as PT4), CD5 (PG114A), CD8 α-chain (76-2-11, also known as PT8), CD8 β-chain (PG164A), γδ T-cells (PT79A, PG92A, PG94A, PGBL22A and 86D) and CD21 (BB6-11C9) were obtained from Veterinary Medical Research and Development Inc. (Pullman, WA).

2.3. Flow cytometry and immunohistochemistry

The flow cytometry analyses of the mAbs were performed as follows. Briefly, an aliquot of porcine PB

leukocytes or thymocytes was incubated with an appropriate amount of mAb for 30 min at 4 °C. After washing with PBS, the cells were incubated with either FITC-conjugated or phycoerythrin (PE)-conjugated goat anti-mouse Abs (Jackson Laboratory Inc., West Grove, PA) for 30 min at 4 °C. The cells were then washed with PBS and analyzed using an EPICS XL analyzer (Beckman/Coulter Inc., Westbrook, MA). In some experiments, two-color flow cytometry was performed to distinguish between the newly established mAbs and commercially available mAbs. Briefly, porcine PB leukocytes were incubated first with unconjugated mAbs and then with PE-labeled secondary Abs, as described above. The free-binding sites of the second Abs were blocked with an excess amount of unrelated mouse Ab, and the cells were treated with FITC-conjugated mAbs.

The reactivity of the mAbs in tissues was analyzed using frozen sections and immunohistochemistry. Briefly, porcine tissues were snap-frozen in optimal cutting temperature (OCT) compounds, and frozen sections were made using a cryostat apparatus. The sections were fixed in acetone for 15 min at 4 °C. After washing in PBS and blocking with normal rabbit serum, the sections were incubated with mAbs at appropriate dilutions for 30 min at room temperature. The sections were then washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Abs (Jackson) for 30 min at room temperature. After washing with PBS, color development was performed in a diaminobenzidine solution (10 mM in 0.05 M Tris–HCl, pH 7.5) with 0.003% H_2O_2.

2.4. Immunoprecipitation

Porcine PB leukocytes were biotinylated and lysed in a lysis buffer, as previously described (Takada et al., 1995). After centrifugation for 30 min at 15,000 rpm and 4 °C, the supernatant was removed and used for the immunoprecipitation. Cell lysates were first incubated with Protein-G agarose beads (Boehringer Mannheim Biochemica, Mannheim, Germany) to remove non-specific binding proteins, and aliquots were incubated with the mAbs followed by Protein-G beads. After washing, the immunoprecipitates were loaded onto SDS-PAGE and transferred to a nitrocellulose membrane. The immunoprecipitates were then reacted with HRP-conjugated avidin and washed. Finally, HRP activity was visualized using chemiluminescence (ECL, Amersham Life Science, Buckinghamshire, UK), as described elsewhere.

2.5. Cloning and expression of porcine TCR cDNA

cDNA libraries of $7G3^+$ PB leukocytes and $6F10^+$ PB leukocytes were constructed. Porcine PB lymphocytes labeled with FITC-7G3 Ab were incubated with magnetic-activated cell sorting (MACS) beads conjugated with anti-FITC Ab (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and loaded onto an AutoMACS cell separator (Miltenyi Biotec). $7G3^+$ cells were positively selected, and a cDNA library was constructed using the oligo-capping method (Maruyama and Sugano, 1994) and plasmid vector pME18S-FL3, which contains the SR-α promoter for expression in mammalian cells, FITC-6F10 was added to the $7G3^-$ pass-through fractions and labeled. These cells were also positively selected by AutoMACS and used for the cDNA library construction. Out of several thousand clones sequenced from both cDNA libraries, eight clones (four TCR δ-chain clones [D1 through D4], two TCR α-chain clones [A1 and A2], one TCR γ-chain clone [G], and one TCR β-chain clone [B]) that exhibited homologies to known TCR sequences and contained full-length open reading frames were selected. It was confirmed that all of the cDNAs were full length and error-free in comparison with previously published nucleotide sequence of porcine TCRs (Thome et al., 1993). To generate a mammalian cell expression vector for V region of the TCR δ-chain, a termination codon was introduced at nucleotides 343–345 of the TCR δ-chain coding sequence in pME18S-FL3 TCR D4 by site-directed mutagenesis (Quick Change Mutagenesis Kit, Stratagene) (see Fig. 5). To generate a mammalian cell expression vector for the C region of the TCR δ-chain, an additional *Bam*HI site was introduced at nucleotides 436–441 of the TCR δ-chain coding sequence in pME18S-FL3 TCR D4, and the nucleotide fragment 50–436 of the TCR δ-chain coding sequence was deleted by *Bam*HI digestion followed by self religation (see Fig. 5). For the bacterial expression of the glutathione S-transferase (GST)-fusion recombinant

TCR δ-chain protein, *Bam*HI and the blunt-ended *Sac*I fragment of the TCR δ (D4) cDNA were introduced to the pGEX-6P-1 (Pharmacia Biotech, Uppsala, Sweden) vector at the *Bam*HI and *Sma*I sites.

cDNA coding for the TCRs under the SRα promoter was introduced into Cos-7 cells by lipofection (LIPOFECTAMIN, Invitrogen, Groningen, The Netherlands); after 3 days, the cells were stained with 7G3 mAb. Recombinant TCR proteins with a GST-fusion form were immunoprecipitated with 7G3 mAb and analyzed by SDS-PAGE.

3. Results and discussion

3.1. Development of mAbs reactive with porcine PB leukocytes

From one hybridization experiment, 45 hybridoma clones were established. The mAbs produced by these clones reacted differently to the porcine PB leukocytes, as revealed by flow cytometry, and were classified into several groups according to their pattern of reactivity (Table 1). Among them, mAbs 6F10, 7G3 and 3E12, which reacted with lymphocyte subpopulations were extensively analyzed.

mAb 6F10 reacted with 49.0% (range 40.3–60.5%; $n = 5$) of the PB lymphocytes (Table 2). The histogram profiles always displayed bright and dull peaks (Fig. 1). Since this pattern is typical of CD8, a two-color analysis was performed using previously established CD3 and CD8 mAbs. As shown in Fig. 1A, most of the cells stained positive for both 6F10 and CD3, but a small population of $CD3^-$ $6F10^+$ cells were also identified. A two-color analysis using the CD8 α-chain mAb showed that 6F10 and CD8 α-chain reacted in identical cell populations, since the histograms could be aligned in a diagonal fashion. On the other hand, a two-color analysis with CD8 β-chain indicated that the mAb against CD8 β-chain reacted only with a bright population of $6F10^+$ cells. In thymocytes, a similar 6F10 staining pattern was obtained (Fig. 1B). Thus, $6F10^+$ cells were mostly found in $CD3^+$ cells, and the two-color histograms for 6F10 and CD8 α-chain were aligned in a diagonal fashion. From these results, we concluded that 6F10 probably recognizes the porcine CD8 α-chain.

mAb 7G3 reacted with 24.7% (range 14.2–32.7%; $n = 5$) of the PB lymphocytes (Table 2). As shown in Fig. 2, a two-color analysis demonstrated that nearly all the $7G3^+$ cells were found in $CD2^+$ and $CD3^+$ cells. On the other hand, the $7G3^+$ cells were always $CD4^-$. The relationship between 7G3-positivity with CD8 α-chain-positivity varied depending on the nature of the sample that was being examined. $7G3^+$CD8 α-$chain^+$ and $7G3^+$CD8 α-$chain^-$ cells were always present, but their proportions differed significantly from sample to sample (see samples #1 and #2 in Fig. 2). Since the $7G3^+$ cells were identified as $CD3^+$ T-cells, we next examined whether $7G3^+$ cells were related to γδ T-cells using previously established mAbs against porcine TCR δ-chain (Fig. 2, right panel). Using five

Table 1
Classification of new monoclonal antibodies defined by their specificities to peripheral blood leukocytes using flow cytometry

Specificity	Clones
Pan-leukocyte	1B4, 1B8, 4G8, 5B6, 5B11, 6F1, 6F2, 7D8, 7G12
Granulocyte	1H2, 3C5, 3F5, 3F11, 5A10, 5E6, 5H7, 6B4, 6B8, 6E10, 6G1, 7H12, 8F2
Lymphocyte subset	2E2, 2H3, 3E12, 5D8, 5G8, 6E12, 6F10, 7G3
Granulocyte and lymphocyte subset	1F4, 2F12, 3D10, 4H9, 5E11, 6A8, 6D10, 6F11, 7C7, 7G1, 7G11, 8A5, 8C1, 8C3, 8H7

Table 2
Reactivities of monoclonal antibodies against porcine peripheral blood lymphocytes

	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5	Mean (%)	S.D.
T-cell							
CD3	73.0	63.9	55.9	52.5	71.4	63.3	9.1
CD4	24.8	24.8	18.0	19.1	25.7	22.5	3.6
CD8α	46.4	54.3	39.1	44.3	42.6	45.3	5.7
CD8β	15.4	23.3	12.7	11.9	17.1	16.1	4.5
6F10	47.5	60.5	40.3	45.3	51.5	49.0	7.6
γδ TCR							
PT79A	15.3	16.5	18.8	12.1	23.5	17.2	4.3
PG92A	10.7	16.5	9.1	4.9	17.1	11.7	5.2
PG49A	13.0	5.2	12.6	12.7	13.4	11.4	3.5
PGBL22A	29.7	16.1	27.0	19.3	32.1	24.8	6.9
86D	4.7	6.2	6.7	8.8	12.9	7.9	3.2
7G3	30.5	14.2	27.2	19.0	32.7	24.7	7.9
3E12	16.2	9.3	16.1	11.6	20.4	14.7	4.3
B cell							
CD21	6.0	10.5	8.1	8.9	5.4	7.8	2.1

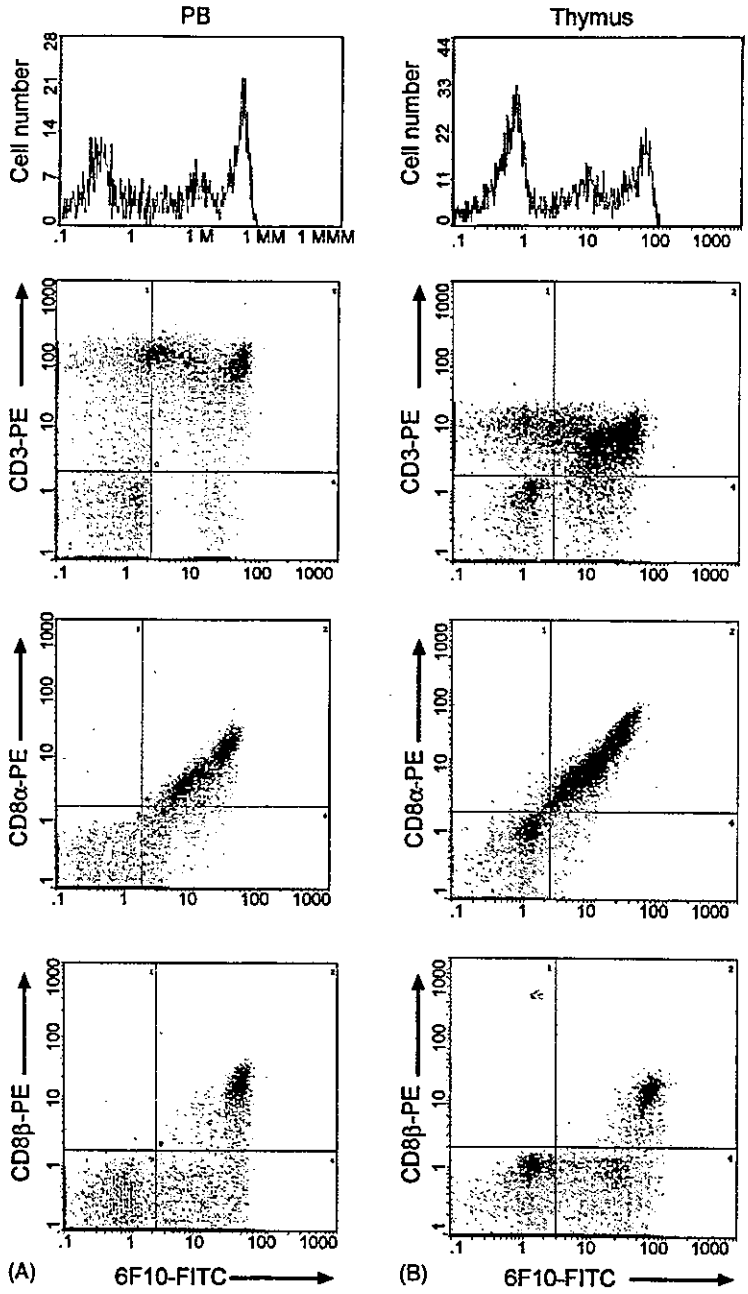

Fig. 1. Two-color staining of porcine peripheral blood lymphocytes and thymocytes using the 6F10 monoclonal antibody and other T-cell markers. Peripheral blood lymphocytes (A) and thymocytes (B) were stained with combinations of FITC-conjugated 6F10 (*X*-axis) and other PE-labeled antibodies (*Y*-axis).

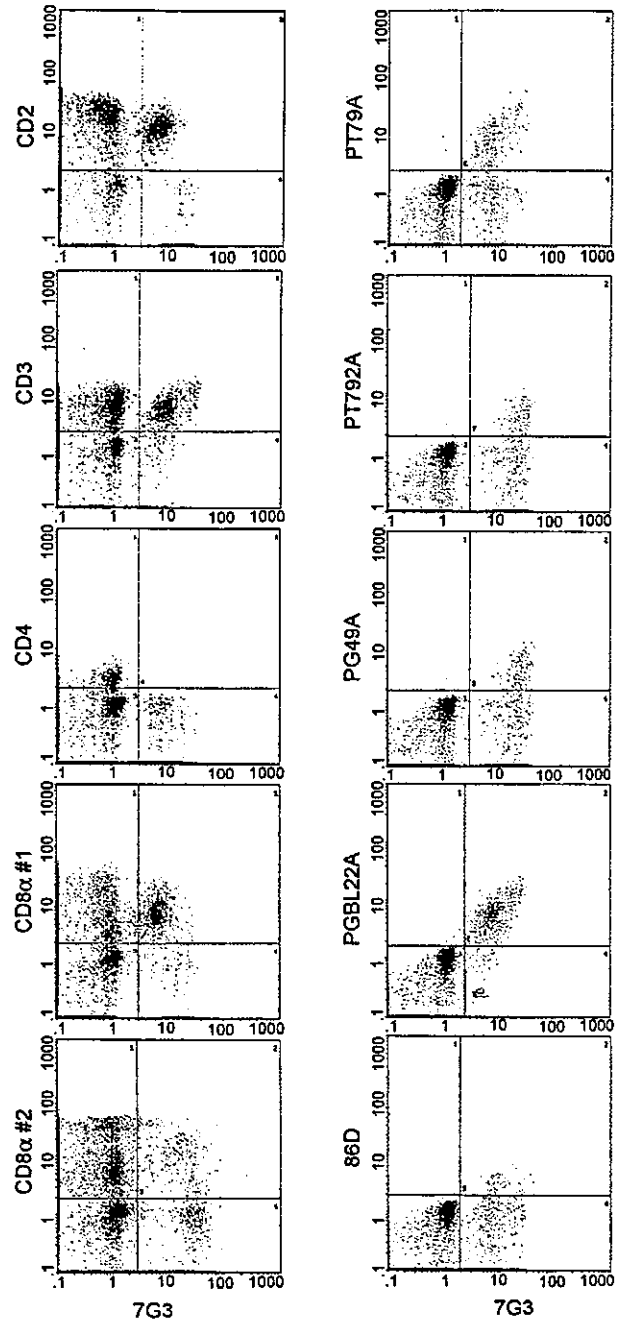

Fig. 2. Two-color staining of porcine peripheral blood lymphocytes using 7G3 and other T-cell markers. Peripheral blood lymphocytes were stained with combinations of FITC-conjugated 7G3 (*X*-axis) and other PE-labeled antibodies (*Y*-axis).

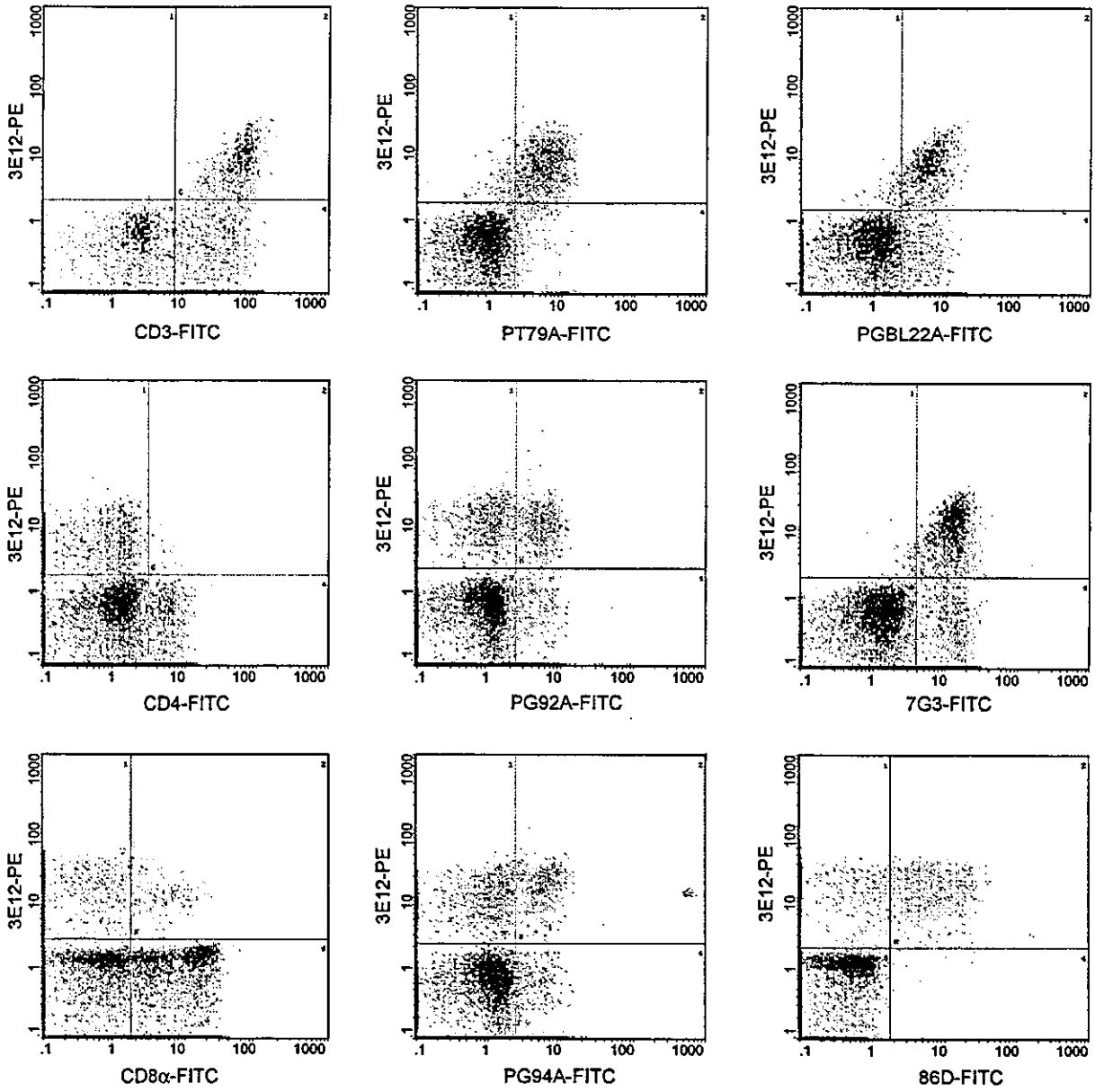

Fig. 3. Two-color staining of porcine peripheral blood lymphocytes using 3E12 and other T-cell markers. Peripheral blood lymphocytes were stained with combinations of PE-conjugated 3E12 (*Y*-axis) and other FITC-labeled antibodies (*X*-axis).

mAbs against γδ T-cells (PT79A, PG92A, PG94A, PGBL22A and 86D), we found that 7G3 always labeled a population of cells that overlapped the population stained by these mAbs against porcine γδ T-cells. Among them, the $7G3^+$ cells were almost identical to those detected by PGBL22A. The reactivity of 7G3 was also examined in porcine thymocytes. Consistent with the results obtained for PB lymphocytes, the staining pattern for 7G3 was almost identical to that for PGBL22A (data not shown).

mAb 3E12 reacted with 14.7% (range 9.3–20.4%; $n = 5$) of the PB lymphocytes (Table 2). As shown in

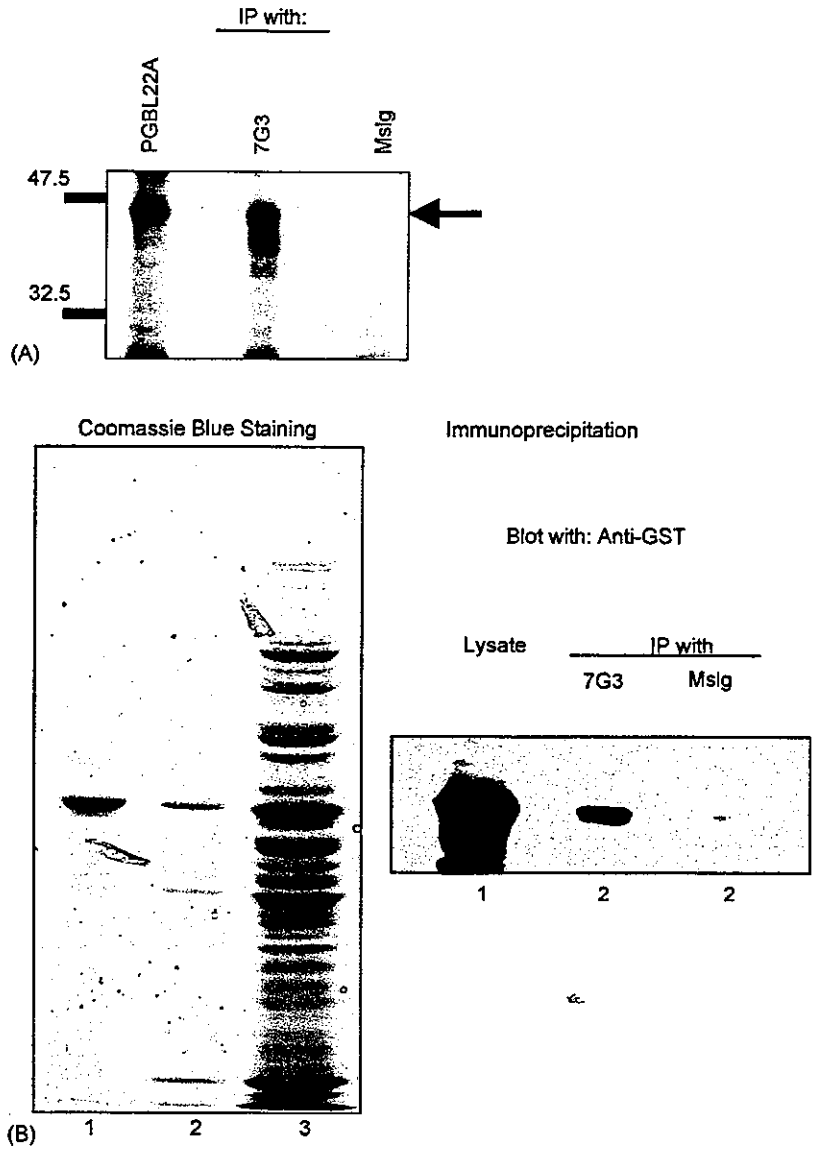

Fig. 4. Immunoprecipitation study using 7G3. (A) Immunoprecipitation was performed using 7G3 or PGBL22A in cell lysates extracted from biotinylated porcine peripheral blood leukocytes. After separation on an SDS-PAGE gel, the proteins were transferred onto a nitrocellulose membrane and probed with peroxidase-conjugated avidin. An unrelated mouse monoclonal antibody (MsIg) was used as a control. (B) GST fusion proteins of the full-length porcine TCR δ-chain were produced in *Eschericha coli* (lane 3). After purification with glutathione sepharose (lane 1), the proteins were eluted with reduced glutathione (lane 2). Each protein was separated by SDS-PAGE in a 10% acrylamide gel and visualized using Coomasie blue-staining. (C) Immunoprecipitation was performed on eluted purified GST fusion TCR δ-chain proteins as described in A followed by immunoblotting with an anti-GST antibody. Lysates of full-length porcine TCR δ-chain were produced in *E. coli* and used as a positive control for the anti-GST antibody. As a negative control, an unrelated mouse monoclonal antibody (MsIg) was used.

Fig. 3, a two-color analysis demonstrated that the staining pattern of 3E12 was similar to that of 7G3. All $3E12^+$ cells were included in the $CD3^+$ cells, but not in the $CD4^+$ cells, and a portion of the $3E12^+$ cells overlapped with the CD8 α-chain$^+$ cells. Further analysis revealed that the $3E12^+$ cells were always included in both $7G3^+$ and $PGBL22A^+$ cells, with approximately two-thirds of the $7G3^+$ cells being

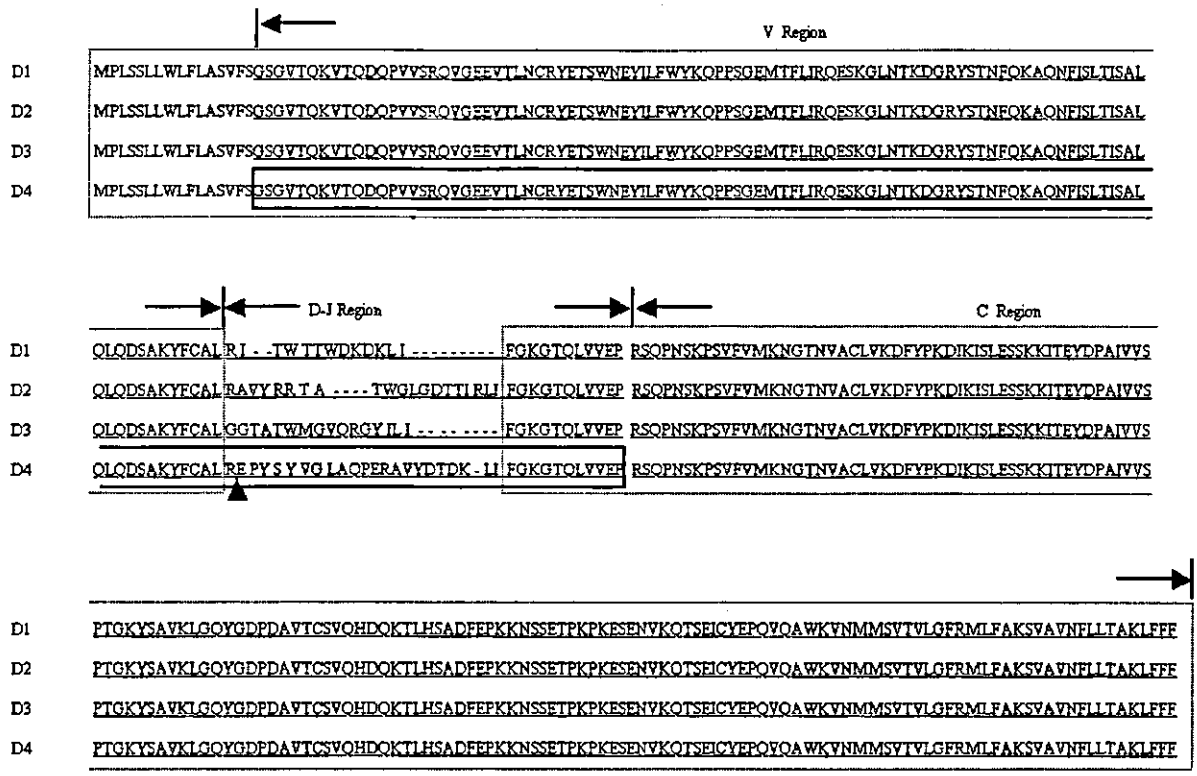

Fig. 5. Schematic presentation of TCR δ-chain cDNA. The nucleotide sequences of the TCR δ-chain cDNAs that were cloned from the 7G3$^+$ cell cDNA library are shown. The arrow-head indicates a termination codon introduced in TCR D4 to generate the TCR D4 V region construct. The solid box indicates the deleted site in the TCR D4 C region construct.

positive for 3E12. The 3E12$^+$ cells were almost identical to those detected by PT79A, and only partially overlapped with either PG92A$^+$ or PG94A$^+$ cells. The above data indicates that 3E12 recognizes a subpopulation of γδ T-cells.

3.2. Reactivity of 7G3 with TCR δ-chain protein

From the biotinylated cell lysates of PB lymphocytes, 7G3 immunoprecipitated a protein with a molecular weight of approximately 40 kDa under reducing conditions (Fig. 4A). A side-by-side comparison revealed that mAb PGBL22A, which has been reported to detect the TCR δ-chain, produced a band with an identical molecular weight.

To confirm that 7G3 reacted with TCR δ-chain, 7G3 was challenged to react with recombinant forms of the TCR δ-chain protein. In our cDNA libraries prepared from 7G3$^+$ cells and 6F10$^+$ cells, one TCR β-chain clone (TCR B), one TCR γ-chain clone (TCR G), two TCR α-chain clones (TCR A1 and A2) and four TCR δ-chain clones (TCR D1 through D4) (Fig. 5) full-length cDNAs were identified. These cDNAs were inserted into mammalian expression vector pME18S-FL3 and transfected into Cos-7 cells. After culturing for three days, the cells were stained with 7G3 using an immunohistochemical method. No staining was observed when the TCR β-chain (Fig. 6A), TCR α-chain or TCR γ-chain cDNAs were transfected (data not shown), but the Cos-7 cells that were transfected with one of the four different TCR δ-chain cDNAs stained positive for 7G3 (Fig. 6A, Table 3). An identical staining pattern to that of 7G3 was obtained when mAb PGBL22A was used (Table 3).

The above results prompted us to examine the reactivity of 7G3 with TCR δ-chain recombinant protein. For this purpose, we first produced a full-

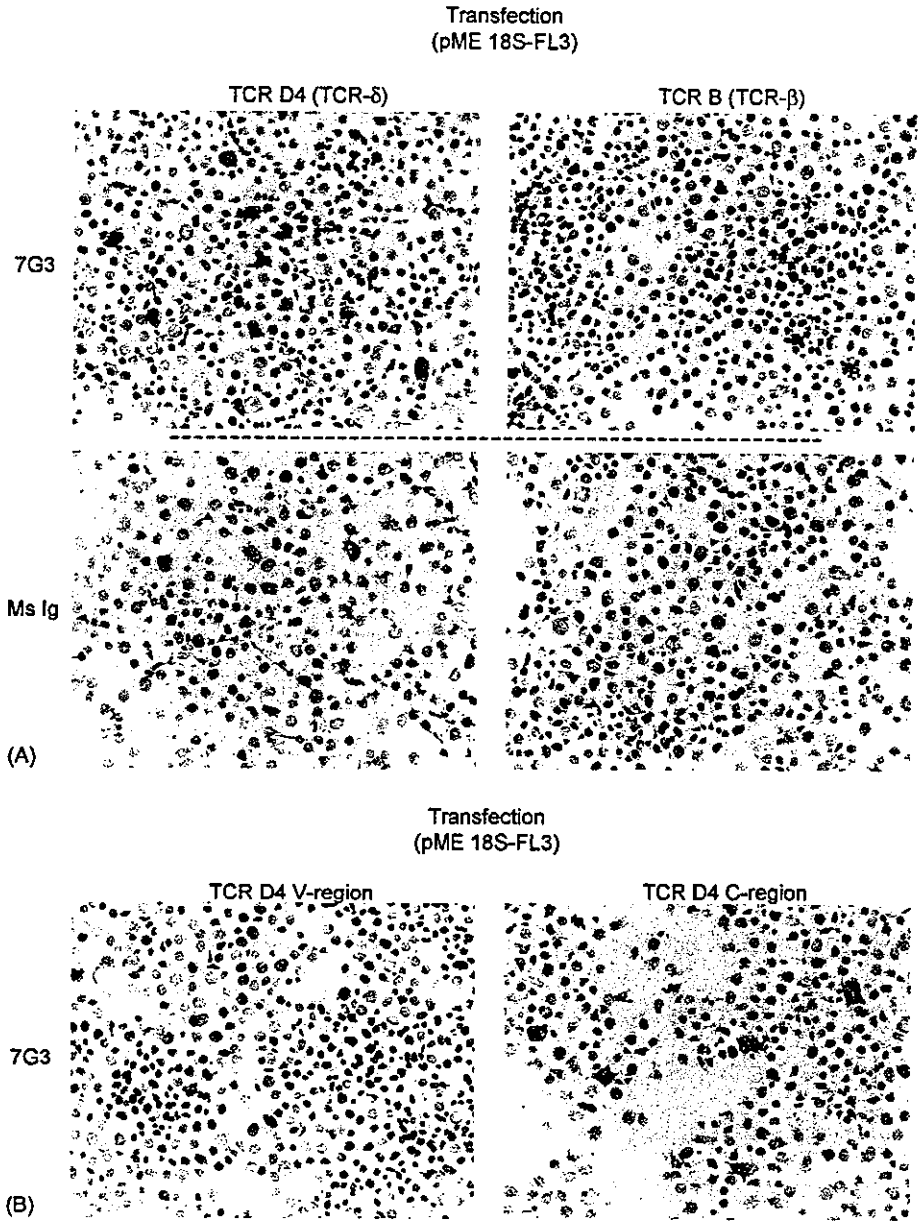

Fig. 6. Reactivity of 7G3 on Cos-7 cells transfected with TCR δ-chain cDNA. (A) Mammalian expression vectors containing either TCR δ-chain (TCR D4) or TCR β-chain (TCR B) were introduced into Cos-7 cells, and the cells were stained with 7G3 or a control antibody. (B) Expression vectors for the TCR δ-chain mutant that completely lack either the C region (TCR D4 V region) or the V region (TCR D4 C region) were introduced into Cos-7 cells, and the cells were stained with 7G3 or a control antibody.

length TCR δ-chain recombinant protein fused with GST in bacteria. As shown in Fig. 4B, a full-length TCR δ-chain-GST fusion protein with a molecular weight of 60 kDa was effectively produced (lane 3) and purified using glutathione beads (lanes 1 and 2). An immunoprecipitation experiment using 7G3 and the recombinant full-length TCR δ-chain-GST fusion protein, followed by immunoblotting with anti-GST

Table 3
Reactivities of monoclonal antibodies against Cos-7 cells transfected with various types of porcine TCR cDNA

	TCR D1	TCR D2	TCR D3	TCR D4	TCR A1	TCR A2	TCR G	TCR B
7G3	+	+	+	+	–	–	–	–
PGBL22A	+	+	+	+	–	–	–	–
PT79A	–	–	–	–	–	–	–	–
PG92A	–	–	–	–	–	–	–	–
PG94A	–	–	–	–	–	–	–	–
86D	–	–	–	–	–	–	–	–
3E12	–	–	–	–	–	–	–	–

Ab, clearly indicated that 7G3 reacted with the full-length TCR δ-chain protein (Fig. 4C).

Next, we determined whether 7G3 reacts with the C region or the V region of the TCR δ-chain. GST protein fused with the V region of the TCR δ-chain was successfully produced in bacteria, but was not reactive with 7G3 (data not shown). Since we failed to produce the C region of the TCR δ-chain protein in bacteria, we attempted to use a mammalian expression system and a deletion mutant of the TCR D4 clone. As shown in Fig. 6B, 7G3 reacted with the Cos-7 cells in which the N-terminal deletion mutant of the TCR D4 clone, containing the C region of the TCR δ-chain, was expressed. On the other hand, 7G3 did not react with Cos-7 cells in which the C-terminal deletion mutant of the TCR D4 clone, containing the V region of the TCR δ-chain, was expressed. Based on the above data, we concluded that 7G3 recognizes the C region of the TCR δ-chain.

3.3. Reactivity of 6F10 (CD8), 7G3 (TCR δ-chain) and 3E12 (γδ T-cells) in tissue sections

We next examined whether the newly established mAbs (6F10, 7G3, and 3E12) could be used for immunostaining on frozen sections. As shown in Fig. 7, all of the mAbs produced high-quality immunostaining results when used on frozen sections. In the thymus, 6F10 stained a significant number of both cortical and medullary thymocytes (Fig. 7). The strong staining pattern produced by 6F10 was striking when compared with the staining pattern produced by the commercially available CD8 α-chain mAb, the latter of which only weakly stained the thymocytes. Both 7G3 and 3E12 also stained small subpopulations of the thymocytes. $7G3^+$ cells were found mainly in the medulla, but a significant number of $7G3^+$ cells were also identified in the cortex of the thymus. In the medulla of the thymus, the $7G3^+$ cells tended to be found around Hassal's corpuscle. The $3E12^+$ cells were distributed in a similar manner as the $7G3^+$ cells, but the number of positive cells was lower than the number of $7G3^+$ cells. Commercially available mAbs against γδ T-cells were also examined, but none of these mAbs produced a satisfactory staining intensity. For example, mAb PGBL22A weakly stained only a small number of thymocytes. Similarly, the staining pattern produced by CD3 was not distinct on the frozen sections.

In lymph node (data not shown) and spleen tissues (Fig. 7), 6F10-stained cells were mainly distributed in the parafollicular area, which was rich in $CD2^+$ T-cells. In addition, a few $6F10^+$ cells were identified in the lymphoid follicles, which were visualized by the B-cell marker CD21 (data not shown). In the lymph node and spleen tissues, the $7G3^+$ and $3E12^+$ cells were scattered mainly in the parafollicular area.

4. Conclusions

In this paper, we report the development of a battery of murine mAbs that were reactive with porcine lymphocytes, including one Ab probably recognizes CD8 (6F10) and two anti-γδ T-cell Abs (7G3 and 3E12). All three of the newly characterized mAbs produced high-quality results when used for immunostaining on frozen sections, compared with the results obtained using commercially available anti-CD8 and anti-γδ T-cells mAbs.

The pig is unique with regard to the distribution of TCR in its peripheral organs. In mice and humans, from which a modern immunological scheme has been developed, the number of γδ T-cells in

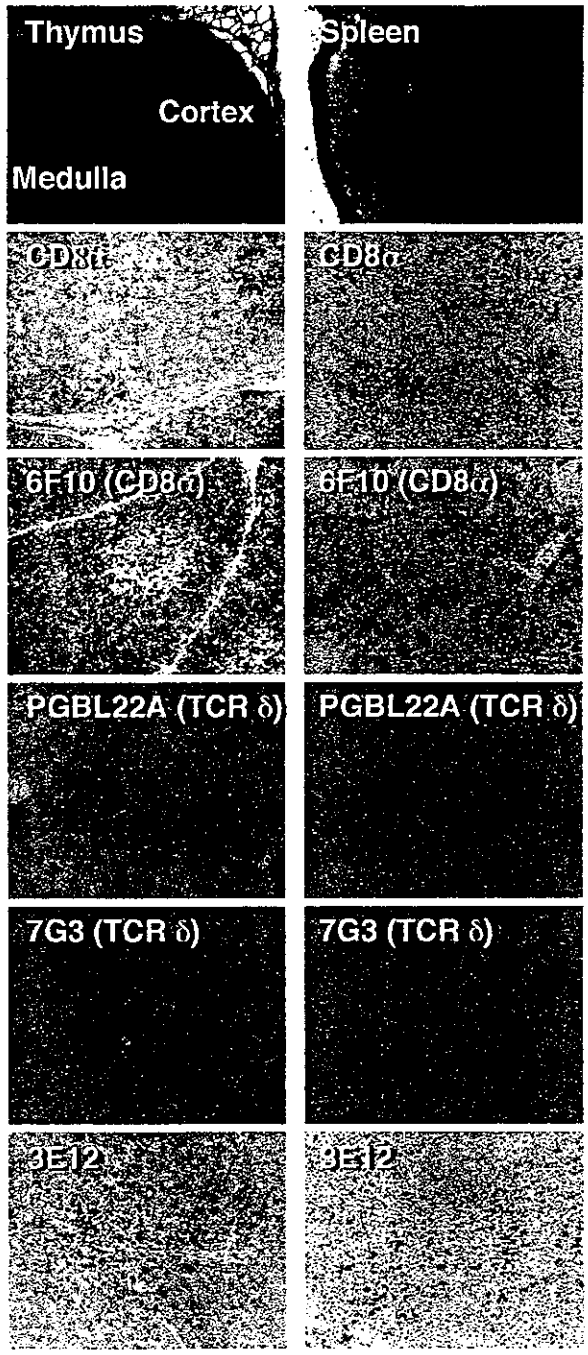

Fig. 7. Reactivity of 7G3, 3E12 and 6F10 on frozen sections of porcine tissues. Frozen sections of porcine thymus (left panels) and spleen (right panels) were stained with the monoclonal antibodies, as indicated.

peripheral lymphoid organs is very small, usually less than a few percent of the lymphocyte population (Carding and Egan, 2002). Instead, γδ T-cells tend to reside in the epithelia of various organs, such as the intestine, uterus and skin, that are often directly subjected to external Ag stimuli. Artiodactyls, including pig, however, are known to be abundant in γδ T-cells in their PB (Hein and Mackay, 1991). Davis et al., previously showed by using mAb PGBL22 that about 30–50% of porcine PB mononuclear cells are γδ T-cells (Davis et al., 1996, 1998, 2001). Indeed, five pigs we investigated also gave high proportion of γδ T-cells with an average of 24.7% with our new mAb 7G3, ranging from 16.1% to 32.7% and the value with PGBL22A was essentially the same with 7G3 in each pig. This striking feature prompted us to reconsider the biological role of γδ T-cells in defense immunity against direct Ag attacks from the external world. For this purpose, a precise characterization of porcine γδ T-cells, as well as αβ T-cells, is indispensable, and the data described here will serve to improve our understanding of the porcine immune system.

mAb 7G3 was selected based on its limited reactivity in PB lymphocytes and was found to react with a subpopulation of $CD3^+$ T-cells using two-color flow cytometry. Since this reactivity was quite similar to the previously reported proportion of γδ T-cells in porcine PB (Davis et al., 1998) we decided to compare our results with those obtained using established, commercially available mAbs. Among the mAbs against γδ T-cells that were tested, 7G3 exhibited a nearly identical distribution to that of mAb PGBL22A. The evidence that 7G3 immunoprecipated a protein with an identical molecular weight (approximately 40 kDa) to that produced by PGBL22A under reduced conditions further indicated that 7G3 and PGBL22A recognized the same molecule. In a previous report by another group, a biochemical analysis revealed that one TCR δ-chain with a molecular weight 40 kDa and three distinct TCR γ-chains with molecular weights of 37, 38, and 46 kDa were distributed in different subsets of porcine γδ T-cells (Hirt et al., 1990; Saalmuller et al., 1990; Thome et al., 1994). Based on the similarity of the identified molecular weights, we hypothesized that 7G3 reacts with the TCR δ-chain. To confirm this idea, we employed a molecular

biology approach. Fortunately, we were able to identify full-length cDNA clones of all TCR α-, β-, γ- and δ-chains in our cDNA libraries generated from 7G3$^+$ cells and 6F10$^+$ cells. Using a mammalian expression system, 7G3 was shown to react with Cos-7 cells in which all four TCR δ-chains cDNA had been introduced. Furthermore, 7G3 was shown to bind with a recombinant full-length TCR δ-chain protein fused with GST. These results confirmed that 7G3 reacts with the porcine TCR δ-chain.

Next, we investigated whether 7G3 reacts with the V or the C region of the porcine TCR δ-chain. Among the mAbs previously shown to react with γδ T-cells, PGBL22A seemed to have a wider range of reactivity than the other mAbs. PGBL22A was known to react with the TCR δ-chain, as proved using immunochemistry, but whether the mAb would react with a recombinant protein and the region of the TCR δ-chain that it recognized where uncertain. Using flow cytometry and various mAbs against porcine TCR γ-, δ-chains, the reactivities of 7G3 and PGBL22A were shown to be identical and were wider than those of the other mAbs. Thus, the possibility that both 7G3 and PGBL22A recognize a common framework in the TCR δ-chain (Davis et al., 1998, 2001) was considered, although the exact determinants are clearly different since these two mAbs can be used to perform a two-color analysis. The pig Vδ region sequences can be grouped into five families (Vδ1–5); one family, Vδ1, consists of a large number of members, whereas the other families have a limited number of members, as determined by cDNA sequencing as well as genomic Southern blotting (Yang et al., 1995). The TCR Vδ1 family was seen in 25 out of 34 cDNA clones in γδ T-cells obtained from a 1-month-old, germfree pig. In fact, four of the cDNAs for the TCR δ-chain found in the 7G3$^+$ cell cDNA library contained an identical V region (Vδ1), and the only differences were seen in the joint (J) region (Fig. 5). Therefore, 7G3 may recognize Vδ1, and not the C region, of the porcine TCR δ-chain. Consequently, we conducted an experiment to determine the region of the TCR δ-chain to which 7G3 reacts. Ideally, recombinant proteins of both the V region and the C region should have been produced, but we were only able to produce a GST-fusion TCR δ-chain mutant, in which the C region was completely deleted; this protein was unreactive with 7G3. These results does not necessary mean, however, that 7G3 does not bind TCR δ-chain V-domain because the folding of the V region peptide might be different from GST-fused full length TCR δ-chain in *Escherichia coli*.

We therefore attempted to use a mammalian expression system. Consistent with the results obtained using recombinant TCR δ-chain V-domain protein fused with GST, 7G3 did not reacted with the Cos-7 cells in which the expression vector for V region of the TCR δ-chain was transfected. It should be noted that the TCR δ-chain V region construct lacked membrane anchoring domain, and the peptides produced might be secreted out of Cos-7 cells. This experiment, therefore, cannot rule out the possibility of 7G3 binding to TCR δ-chain V-domain. We finally showed, however, that 7G3 clearly reacted with the Cos-7 cells in which the C region of the TCR δ-chain was expressed and the strength of staining with this construct was comparable to the Cos-7 cells with full length TCR δ-chain constructs. Taken together, the present evidence strongly suggest that at least a part of the epitope recognized by mAb 7G3 resides within the peptide coded by TCR δ-chain C region. Indeed, the published structure of a human γ δ TCR suggests a portion of TCR δ-chain C region to be accessible to antibody.

In this study, we also developed another mAb, 3E12, that reacts with a subset of γδ T-cells. Since the 3E12$^+$ subpopulation was always included in the population of 7G3$^+$ cells, namely the TCR δ-chain-expressing cells, and was never found in 7G3$^-$ cells, 3E12 likely recognizes a molecule expressed specifically on γδ T-cell subpopulation, but not the common framework of TCR γδ-chains. Furthermore, 3E12 did not react with the four TCR δ-chain proteins (TCR D1–4) or the one TCR γ-chain protein (TCR G) examined in this study. Comparing the reactivity of 3E12 and 7G3, it seems possible that 3E12 may recognize one of the three distinct TCR γ-chains other than one we cloned (Hirt et al., 1990; Saalmuller et al., 1990; Thome et al., 1994). Alternatively, 3E12 may recognize a Vδ family other than Vδ1 or a novel molecule related to the TCR γ- and δ-chain complex that is specifically expressed in a subset of γδ T-cells. A detailed investigation of the 3E12 Ag, including whether 3E12 recognizes the same molecule as that recognized by mAb PT79A, is now underway.

mAb 6F10 was found to react probably with the CD8 α-chain using flow cytometry. The major benefit of using 6F10 in research on the porcine immune system is its applicability to immunohistochemical analyses. Compared with the commercially available CD8 mAb, 6F10 reacts strongly on frozen sections. This benefit is also seen with the mAbs against γδ T-cells. mAb 7G3 distinctly stains TCR δ-chain$^+$ cells on frozen sections. Similarly, mAb 3E12 strongly stains a subset of γδ T-cells. Immunohistochemical analyses are particularly important for studying pathological conditions; thus, the mAbs developed in this study are expected to improve our understanding of not only the fundamental aspects of immune systems in general, but the immunological reactions involved in various porcine diseases.

In conclusion, we report the development of three mAbs that recognize distinct subsets of porcine T lymphocytes, all of which produced high-quality immunostaining results on tissue sections. In particular, mAb 7G3, which was confirmed to recognize the C region of the porcine TCR δ-chain, should facilitate the study of porcine γδ T-cells not only in the field of porcine immunity, but also in the field of comparative immunological evolution.

Acknowledgements

We thank M. Sone and S. Yamauchi for their excellent secretarial works. This work was supported in part by Health and Labour Sciences Research Grants and the Research Grant for Cardiovascular Disease from the Ministry of Health, Labour and Welfare of Japan and MEXT. KAKENHI 15019129, JSPS. KAKENHI 15390133 and 15590361. This work was also supported by a grant from the Japan Health Sciences Foundation for Research on Health Sciences Focusing on Drug Innovation. Additional support was provided by a grant from Sankyo Foundation of Life Science.

References

Carding, S.R., Egan, P.J., 2002. γδ T-cells: functional plasticity and heterogeneity. Nat. Rev. Immunol. 2, 336–345.

Davis, W.C., Brown, W.C., Hamilton, M.J., Wyatt, C.R., Orden, J.A., Khalid, A.M., Naessens, J., 1996. Analysis of monoclonal antibodies specific for the γδ TcR. Vet. Immunol. Immunopathol. 52, 275–283.

Davis, W.C., Zuckermann, F.A., Hamilton, M.J., Barbosa, J.I., Saalmuller, A.R., Binns, M., Licence, S.T., 1998. Analysis of monoclonal antibodies that recognize γδ T/null cells. Vet. Immunol. Immunopathol. 60, 305–316.

Davis, W.C., Haverson, K., Saalmuller, A., Yang, H., Lunney, J.K., Hamilton, M.J., Pescovitz, M.D., 2001. Analysis of monoclonal antibodies reacting with molecules expressed on γδ T-cells. Vet. Immunol. Immunopathol. 80, 53–62.

Fujimoto, J., Ishimoto, K., Kiyokawa, N., Tanaka, S., Ishii, E., Hata, J., 1988. Immunocytological and immunochemical analysis on the common acute lymphoblastic leukemia antigen (CALLA): evidence that CALLA on ALL cells and granulocytes are structurally related. Hybridoma 7, 227–236.

Haverson, K., Saalmuller, A., Alvarez, B., Alonso, F., Bailey, M., Bianchi, A.T., Boersma, W.J., Chen, Z., Davis, W.C., Dominguez, J., Engelhardt, H., Ezquerra, A., Grosmaire, L.S., Hamilton, M.J., Hollemweguer, E., Huang, C.A., Khanna, K.V., Kuebart, G., Lackovic, G., Ledbetter, J.A., Lee, R., Llanes, D., Lunney, J.K., McCullough, K.C., Molitor, T., Nielsen, J., Niewold, T.A., Pescovitz, M.D., de la Lastra, J.M., Rehakova, Z., Salmon, H., Schnitzlein, W.M., Seebach, J., Simon, A., Sinkora, J., Sinkora, M., Stokes, C.R., Summerfield, A., Sver, L., Thacker, E., Valpotic, I., Yang, H., Zuckermann, F.A., Zwart, R., 2001a. Overview of the third international workshop on swine leukocyte differentiation antigens. Vet. Immunol. Immunopathol. 80, 5–23.

Haverson, K., Saalmuller, A., Chen, Z., Huang, C.A., Simon, A., Seebach, J., Boersma, W.J., Zwart, R., Niewold, T.A., Thacker, E., Llanes, D., de la Lastra, J.M., Engelhardt, H., Ezquerra, A., Alonso, F., Dominguez, J., Ledbetter, J.A., Grosmaire, L., Lee, R., Nielsen, J., Salmon, H., Valpotic, I., Sver, L., Lackovic, G., Summerfield, A., Khanna, K.V., 2001b. Summary of the first round analyses of the third international workshop on swine leukocyte differentiation antigens. Vet. Immunol. Immunopathol. 80, 25–34.

Hein, W.R., Mackay, C.R., 1991. Prominence of γδ T-cells in the ruminant immune system. Immunol. Today 12, 30–34.

Hirt, W., Saalmuller, A., Reddehase, M.J., 1990. Distinct γ/δ T-cell receptors define two subsets of circulating porcine CD2–CD4–CD8–T lymphocytes. Eur. J. Immunol. 20, 265–269.

Maruyama, K., Sugano, S., 1994. Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. Gene 138, 171–174.

Saalmuller, A., Hirt, W., Reddehase, M.J., 1990. Porcine γ/δ T lymphocyte subsets differing in their propensity to home to lymphoid tissue. Eur. J. Immunol. 20, 2343–2346.

Takada, K., Saito, M., Kaneko, H., Iizuka, K., Kokai, Y., Fujimoto, J., 1995. Novel monoclonal antibody reactive with thrombin-sensitive 74-kDa glycoproteins present on platelets and megakaryocytes both from mouse and rat. Hybridoma 14, 361–367.

Thome, A., Saalmuller, A., Pfaff, E., 1993. Molecular cloning of porcine T-cell receptor alpha, beta, gamma and delta chains using polymerase chain reaction fragments of the constant regions. Eur. J. Immunol. 23, 1005–1010.

Thome, M., Hirt, W., Pfaff, E., Reddehase, M.J., Saalmuller, A., 1994. Porcine T-cell receptors: molecular and biochemical characterization. Vet. Immunol. Immunopathol. 43, 13–18.

Yang, Y.-G., Ohta, S., Yamada, S., Shimizu, M., Takagaki, Y., 1995. Diversity of T-cell receptor δ-chain cDNA in the thymus of a 1-month-old pig. J. Immunol. 155, 1981–1993.

Modern Pathology (2004) 17, 423–429
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Diagnostic importance of CD179a/b as markers of precursor B-cell lymphoblastic lymphoma

Nobutaka Kiyokawa[1], Takaomi Sekino[1], Tsubasa Matsui[1], Hisami Takenouchi[1], Kenichi Mimori[1], Wei-ran Tang[1], Jun Matsui[1], Tomoko Taguchi[1], Yohko U Katagiri[1], Hajime Okita[1], Yoshinobu Matsuo[2], Hajime Karasuyama[3] and Junichiro Fujimoto[1]

[1]*Department of Developmental Biology, National Research Institute for Child Health and Development, Japan;* [2]*Fujisaki Cell Center, Hayashibara Biochemical Labs Inc, Okayama, Japan and* [3]*Department of Immune Regulation, Tokyo Medical and Dental University, Graduate School of Medicine, Tokyo, Japan*

Surrogate light chains consisting of VpreB (CD179a) and λ5 (CD179b) are expressed in precursor B cells lacking a complete form of immunoglobulin and are thought to act as substitutes for conventional light chains. Upon differentiation to immature and mature B cells, CD179a/b disappear and are replaced with conventional light chains. Thus, these molecules may be useful as essential markers of precursor B cells. To examine the expression of the surrogate light-chain components CD179a and CD179b in precursor B-cell lymphoblastic lymphoma, we analyzed tissue sections using immunohistochemistry techniques. Among a number of monoclonal antibodies for the surrogate light chains, VpreB8 and SL11 were found to detect CD179a and CD179b, respectively, in acetone-fixed fresh frozen sections. Moreover, we also observed VpreB8 staining in formalin-fixed, paraffin-embedded sections. Using these antibodies, we found that CD179a/b were specifically expressed in precursor B-cell lymphoblastic lymphomas, but not in mature B-cell lymphomas in childhood. Furthermore, other pediatric tumors that must be included in a differential diagnosis of precursor B-cell lymphoblastic lymphoma, including precursor T-cell lymphoblastic lymphoma, extramedullary myeloid tumors, and Ewing sarcoma, were also negative for both CD179a and CD179b. Our data indicate that CD179a and CD179b may be important markers for the immunophenotypic diagnosis of precursor B-cell lymphoblastic lymphomas.
Modern Pathology (2004) **17,** 423–429, advance online publication, 20 February 2004; doi:10.1038/modpathol.3800079

Keywords: CD179a/b; lymphoblastic lymphoma; precursor B cells; immunohistochemistry; diagnosis

B cells are characterized by the surface expression of immunoglobulin (Ig), consisting of a heavy chain (HC) and conventional κ or λ light chains (LCs). The Ig expressed in B cells is associated with the Igα/Igβ (CD79a/b) complex and forms a B-cell antigen receptor complex. In contrast to these mature B cells, precursor B cells do not express Ig, although they do contain Ig-related components. For example, more primitive pro-B cells already express the Igα/Igβ complex and contain surrogate LCs, consisting of VpreB (CD179a) and λ5 (CD179b).[1–5] Through the successful rearrangement of HC genes, pro-B cells undergo differentiation into pre-B cells and start to express a premature antigen receptor, namely the pre-B-cell receptor (pre-BCR), consisting of μ HC, CD179a/b and the Igα/Igβ heterodimer.[6–9] Upon further differentiation from pre-B cells to B cells, CD179a/b disappear and are replaced with conventional LC.

The stage-specific developmental expression of Ig-related molecules is an essential characteristic of B-lineage cells and is conserved not only in normal cells but also in neoplastic cells of B lineage. Indeed, precursor B acute lymphoblastic leukemias (ALL), which originate from precursor B cells and lack the complete form of Ig, are known to express CD179a/b, while mature and Ig-expressing B-cell ALLs do not.[10] Precursor B-cell lymphoblastic lymphoma (B-LBL) is a disease in which neoplastic precursor B cells proliferate without the obvious involvement of blood or bone marrow and thus exhibits immunophenotypic characteristics that are similar to those of precursor B-ALL.[11,12] Neoplasms of precursor B cells most commonly present as a form of ALL during childhood, and the presentation of B-LBL is infrequent, but may occur in patients of any

Correspondence: N Kiyokawa, MD, PhD, Department of Developmental Biology, National Research Institute for Child Health and Development, 3-35-31, Taishido, Setagaya-ku, Tokyo 154-8567, Japan.
E-mail: nkiyokawa@nch.go.jp
Received 21 August 2003; revised 26 November 2003; accepted 26 December 2003; published online 20 February 2004