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Catalytic RAG1 mutants obstruct V(D)J recombination in vitro and in vivo

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Abstract

To generate severe combined immunodeficient (SCID) livestocks for xenotransplantation, we have attempted to generate a SCID phenotype without gene knockout. Based on the reported mouse RAG1 mutants, we constructed the corresponding rabbit RAG1 mutants by mutagenesis of three residues within the catalytic domain: D602A, D710A, and E964A. As expected, these mutants each exhibited no catalytic activity on artificial substrates and inhibited recombination by the wild type RAG1. Moreover, replacement of the N-terminus of RAG1 with enhanced green fluorescent protein (EGFP) greatly increased protein stability, and the triple mutant RAG1 showed a twofold increase in its ability to inhibit wild type activity in vitro. We generated mice transgenic for the latter mutant to assess its effect on V(D)J recombination in vivo. Serum IgM levels in four out of seven transgenic mice were reduced to approximately 30–50% of control levels in four out of seven transgenic mice. Our results suggest that immunodeficient animals for regenerative medicine could be generated without gene knockout.

Keywords: Xenotransplantation; SCID; RAG1 mutants

1. Introduction

Xenotransplantation is a potential solution to the chronic shortage of human donor organs. Although clinical obstacles to this approach are more formidable than with human-to-human transplantation, progress has been made to prevent the hyperacute rejection (HAR) of donor tissues by the recipient's immune system. In addition to the development of immunosuppressive drugs (Lei et al., 2000), organs from transgenic animals that express human complement regulatory proteins, including decay accelerating factor (DAF), membrane co-factor protein (MCP), and CD59, are expected to be of therapeutic use (Rosengard et al., 1995). However, even if HAR were completely suppressed, xenogeneic organs would still produce large amounts of foreign proteins, especially in the case of liver transplantation, that could result in the activation of the patient's immune system. To address this problem, we aim to create a hybrid liver consisting of human hepatocytes within a framework of blood vessels and bile ducts from the pig. A severe combined immune deficient (SCID) pig that accepts human cells without rejection would be required to grow such organs. Our goal is to use an in utero manipulation technique (IUM) to replace porcine hepatocytes with their human counterparts in a transgenic SCID pig fetus expressing human DAF (Enosawa et al., 2001a,b; Fujino et al., 2001). Although several SCID lineages have been generated by gene disruption in murine embryonic stem (ES) cells (Gao et al., 1998a,b; Gu et al., 1997; Mombaerts et al., 1992; Shinkai et al., 1992; Zhu et al., 1996), the ES cell approach is not feasible in livestock such as pigs and rabbits. Somatic cell nuclear transplantation has been successful in pigs (Onishi et al., 2000; Polejaeva et al., 2000), and piglets with disruption of one allele of the α1,3 galactosyltransferase locus have been produced (Dai et al., 2002; Lai et al., 2002). Nevertheless, homologous recombination in somatic cells is quite inefficient and the technique has

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not been optimized. As a preliminary step towards our goal of creating SCID livestock, we have used transgenic techniques to create small animals with the SCID phenotype.

In vertebrates, V(D)J recombination plays a critical role in the generation of antigen receptor diversity in B and T cells (Tonegawa, 1983). V(D)J recombination is initiated by the association of two proteins, recombination activating gene (RAG) 1 and RAG 2 (Oettinger, 1992; Schatz et al., 1989), at the recombination signal sequences (RSS) (Max et al., 1979; Sakano et al., 1979). This leads to the nicking of one DNA strand between the RSS and the coding sequence. Although both RAG1 and RAG2 are essential for recombination (McBlane et al., 1995), only RAG1 has a direct role in DNA cleavage (Kim et al., 1999; Landree et al., 1999). To date, there have been many efforts to identify catalytic mutants of RAG1 (Aidinis et al., 2000; Kim et al., 1999; Landree et al., 1999; Li et al., 2001; Lin et al., 1999; McMahan et al., 1997; Noordzij et al., 2000; Sadofsky et al., 1993; Schwarz et al., 1996; Steen et al., 1999; Villa et al., 1998, 2001; Yarnell Schultz et al., 2001). We have focused on catalytic mutants that retain normal RSS binding to develop derivatives with dominant negative activity against the wild type protein (Kim et al., 1999; Landree et al., 1999). Seven point-mutant RAG1 proteins were generated and their activities were assessed in vitro using an artificial substrate plasmid that enabled recombination frequency to be reported as luciferase activity.

In this report, we describe catalytic RAG1 mutants that bind normally to DNA, but that inhibit the activity of wild type RAG1. Moreover, the replacement of the N-terminus of RAG1 with the enhanced green fluorescent protein (EGFP) increased the inhibition activity in vitro, and transgenic mice carrying this mutant gene showed low serum IgM levels. These data suggest that immunodeficient animals could be generated without gene disruption and that this strategy is applicable to almost any vertebrate species.

2. Materials and methods

2.1. Cloning of rabbit RAG1 and RAG2 gene

Because the coding regions of both rabbit *RAG* genes were contained in signal exons, they were amplified from genomic DNA by the polymerase chain reaction (PCR). Primer sequences were based on the reported nucleotide sequences (RAG1, M77666; RAG2, M77667) with *Eco*RI or *Not*I sites added to their 5′ ends. The *RAG1* gene was amplified in two segments with the following primers: RAG1S1, 5′-AAGGATCCATCATGGCTGTGTCTTGC-3′; RAG1AS1, 5′-AAGCGGCCGCCTTGACTTGTAACTTC-AGCTCC-3′; RAG1S2, 5′-AAGGATCCCGTCAACATCT-CCTATCG-3′; RAG1AS2, 5′-AAGCGGCCGCATAAGTG-GTTGAACCCTCC-3′. To reconstitute the full-length *RAG1* gene, the subcloned fragments were joined at a unique *Sac*I site in each fragment. The *RAG2* gene was amplified with the following primers: RAG2S1, 5′-AA-GAATTCGAAAACATGTCGCTGCAGATG-3′; RAG2AS1 5′-AAGCGGCCGCAAAATTAGTCAAACAACCGTC-3′.

2.2. Mutagenesis and expression vectors

Three amino acid mutations D602A, D710A, and E964A were introduced into RAG1 by PCR-assisted DNA mutagenesis, and double/triple mutants were also generated. Wild-type and mutant *RAG1* genes were subcloned into the pCDNA3.1(−)/Myc-His expression vector (Invitrogen, Carlsbad, CA, USA), with a myc-His tag sequence fused to the 3′-terminus. To generate truncated RAG1 proteins, core domains (residues 380–1042) were amplified by PCR with the following primers: RAG1S3, 5′-AAGGATCCATGGAA-TCAAGAGATACTTTTGTGCA-3′; RAG1AS3, 5′-TTAA-GCTTAAATTCCATTGAATATTGGC-3′. Amplified fragments derived from both the wild type and point-mutant *RAG1* genes were subcloned into the pCDNA3.1(−)/Myc-His expression vector with the *EGFP* gene from pEGFP-C1 (Invitrogen) inserted in-frame at the *Bam*HI site at the 5′ end of each derivative. The *RAG2* gene was subcloned into the pCDNA3 expression vector (Invtrogen). The fidelity of PCR and recombination was confirmed by DNA sequencing.

2.3. Cell cultures

NIH3T3 and Cos7 cells were cultured in Dulbecco's modified Eagle's medium (GIBCOBRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS).

2.4. In vitro recombination assay

The recombination substrate vector, pRSSLuc, carried the puromycin^r (*puro*^{r}) gene and the PGK polyadenylation signal flanked by two synthetic recombination signal sequences, 12-RSS and 23-RSS. Expression of the *puro*^{r} gene was driven by the chicken β-actin (CAG) promoter originated from pCAGGS vector (Miyazaki et al., 1989) placed upstream of the 12-RSS. The firefly luciferase (*Luc*) gene, derived from pGV-B2 vector (Toyo inki, Tokyo, Japan), was placed downstream of the 23-RSS, followed by the rabbit β-globin polyadenylation signal. RAG-mediated joining of the two RSSs removes the puro^r and PGK polyadenylation signal by DNA deletion, and places the *Luc* gene immediately downstream of the CAG promoter (Fig. 2). Plasmids were transiently transfected into NIH3T3 cells using LIPOFECTAMINE Plus Reagent (GIBCO-BRL) following the manufacturer's protocols. Briefly, 0.2 μg of pRSSLuc, 0.1 μg of the RAG2 expression vector, 0.1 μg of the wild type-RAG1 expression vector, 0.1 μg of pRL-TK (*Renilla* luciferase expression vector, Promega, Madison, WI, USA), and 0.1–0.2 μg of the mutant RAG1 expression vector were co-transfected into cells on 24-well plates. The amount of total plasmid DNA was made equivalent by the addition of pcDNA3.1(−)/MycHis/lacZ vector DNA

(Invtrogen) because the *Escherichia coli, β-galactosidase* gene was almost equivalent in size to the *RAG1* gene. Cells were harvested after 40–48 h and firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity.

2.5. Western blot analysis

Cos7 cells were rinsed with PBS and harvested by scraping in cold RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40) containing protease inhibitors (Complete EDTA-free, Roche Molecular Biochemicals, Mannheim, Germany). Protein samples (50 μg per lane) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were electrophoretically transferred onto PVDF membranes. Proteins on the membranes were probed with anti-myc antibody (Clontech), and bound antibodies were visualized with the ECL Western detection system (Amersham Biosciences, Piscataway, NJ, USA).

2.6. Generation of transgenic mice

GFP fused to the mutant *RAG1* gene was inserted at the *EcoR*I site of the pCAGGS vector. A purified DNA fragment containing the GFP-mutant RAG1 fusion gene, CAG promoter and rabbit β-globin polyadenylation signal, but lacking the vector backbone was dissolved in PBS to 10 ng/μl. Matured female F1 (DBA × C57Bl/6J) mice (Charles River Japan, Kanagawa, Japan) were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotrophin (SEROTOROPIN, TEIKOKU HORMONE MSG, Tokyo, Japan) followed 48 h later by 5 IU of human chorionic gonadotrophin (PUBEROGEN, Sankyo, Tokyo, Japan), and then mated with ICR male mice (Charles River Japan). Fertilized eggs were collected from the fallopian tubes 20 h after hCG injection, and were microinjected with ~2 pl of the DNA solution into the male pronucleus. Eggs were cultured in M16 medium (Sigma, St. Louis, MO, USA) for 16–20 h at 37 °C in a 5% CO_2/air, and then transplanted into the fallopian tubes of pseudopregnant ICR mothers.

2.7. Enzyme linked immunosorbent assay (ELISA)

F16 MaxiSorp plates (NUNC, Roskilde, Denmark) were coated with 40 ng/well of rabbit anti-mouse IgM (61-6800, Zymed Labolatories, CA, USA) and incubated with blocking buffer (3% bovine serum albumin containing PBS). Diluted mouse sera were added to the ELISA plate wells, and incubated for 1 h at room temperature. After washing with buffer (0.02% Tween-20 containing PBS), 1:2000 diluted horseradish peroxidase (HRP) conjugated goat anti-mouse IgM (62-6820, Zymed Labolatories) was added. After 1 h incubation and washing, bound antibodies were detected by *o*-phenylenediamine (OPD). Serum IgM concentrations were calculated using purified mouse IgM (02-6800, Zymed Labolatories) as a standard.

3. Results

3.1. Construction of RAG1 mutants

We first created RAG1 catalytic mutants that retained DNA-binding activity and maintained their interaction with protein partners involved in DNA recombination. Landree et al. (1999) and Kim et al. (1999) previously described mutants in the mouse RAG1 protein with intact DNA binding activity, but lacking DNA cleavage activity. Based on these reports, we mutated three residues in the rabbit RAG1 protein: D602A, D710A, and E964A, located in the catalytic domain, and evolutionarily conserved between fish and mammals (Fig. 1A and B). All seven mutants, including double/triple mutants (D602A/D710A, D602A/E964A, D710A/E964A, and D602A/D710A/E964A), and wild-type RAG1 were introduced a myc-His tag at the C-terminus.

3.2. RAG1mutants inhibited recombination by wild type RAG1 in vitro

To measure the catalytic activity of RAG1 protein, we established an in vitro recombination assay system using the pRSSLuc artificial substrate vector, which contains two RSSs (Fig. 2). We confirmed the RAG-dependent DNA recombination of the two RSSs by direct sequencing of PCR products recovered from transfected cells (data not shown). Myc-His tagged RAG1 protein showed similar activity to non-tagged, wild type RAG1 in this assay, indicating that the tag did not affect RAG1 recombination activity. In contrast, D602A, D710A, E964A, and double/triple mutant RAG1 proteins were completely inactive (Fig. 3). Furthermore, co-transfection of equivalent amounts of mutant and wild type *RAG1* genes decreased the recombination frequency of the RSS substrate vector to 50% of the control value.

Myc-His tagged wild type RAG1 protein was barely detectable (Fig. 4A) by Western blot analysis with anti-myc antibody, suggesting that the half-life of RAG1 proteins was quit short in vivo. There are reports that N-terminal truncation of RAG1 protein increases its stability (Silver et al., 1993; McMahan et al., 1997; Steen et al., 1999). To prolong the half-life of RAG1 proteins, we constructed a RAG1 variant comprising N-terminal residues, 1–379, fused to the GFP protein at the N-terminus, allowing easy detection of expression (GFPwtRAG1, see Fig. 1A). We clearly detected the GFPwtRAG1 protein by Western blot analysis (Fig. 4A), and found that it produced twofold greater recombination of the RSS substrate vector than the wild type RAG1 protein (Fig. 4B). In co-transfection experiments, GFPmutRAG1 (GFP fused to the triple mutant RAG1) inhibited the recombination in a dose-dependent manner and exhibited a twofold increase in inhibition activity compared to non-truncated RAG1 mutants (Fig. 4C).

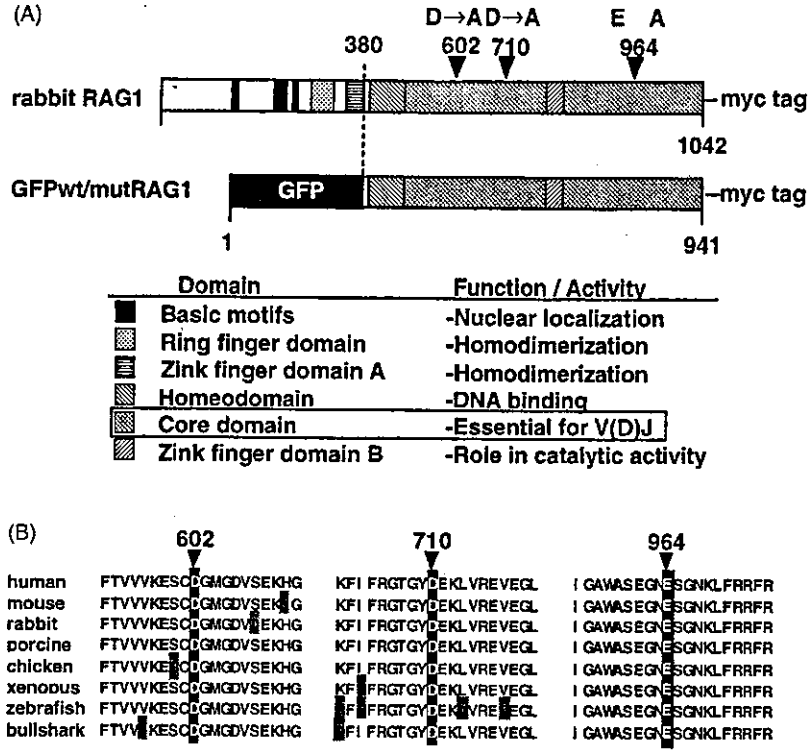

Fig. 1. (A) Schematic diagram of the rabbit RAG1 protein indicating the positions of the three mutations used in this study. GFPwt/mutRAG1 is shown below, and is a derivative whose N-terminal domain (1–379) has been replaced with GFP protein. The various domains and their functions are indicated below the diagram (Villa et al., 2001). (B) Alignment of the catalytic residues at the core domain of rabbit RAG1 with other vertebrates. Highlighted amino acid residues show the position of mutations.

3.3. Transgenic mice carrying the GFPmutRAG1 gene showed low serum IgM levels

Next, we generated mice transgenic for the GFPmutRAG1 gene to examine the ability of this mutant RAG1 to inhibit V(D)J recombination in vivo. The CAG promoter was used to confer ubiquitous and strong expression to the mutant RAG1 protein. Although no pups exhibited strong green fluorescence under UV light, seven transgenic mice were identified by PCR analysis of genomic DNA extracted from

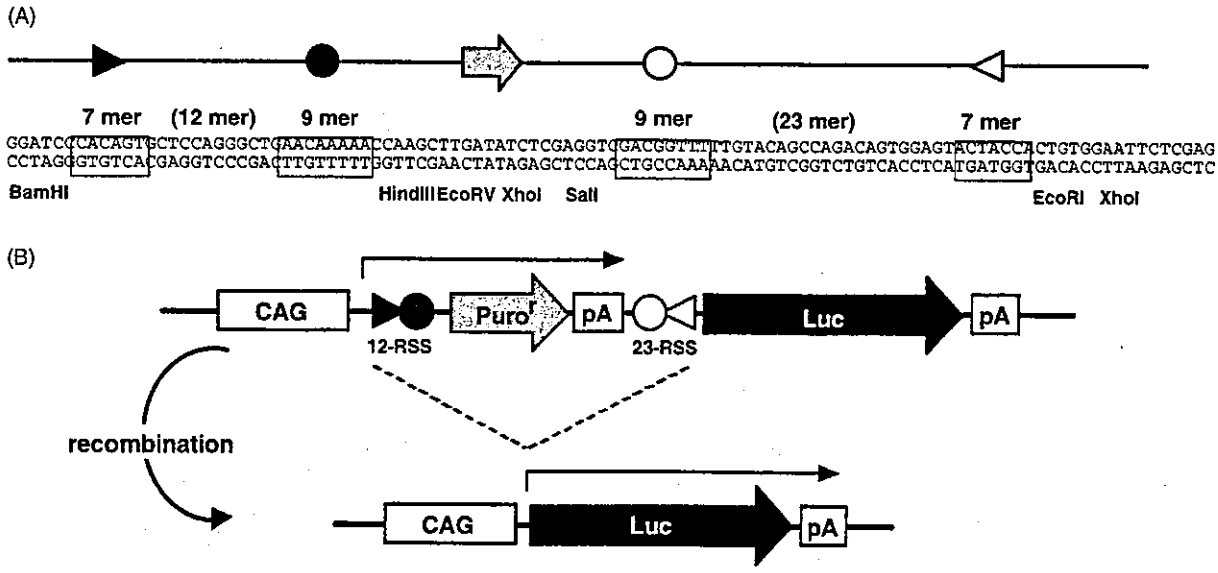

Fig. 2. Artificial recombination substrate used in this study. When RAG-dependent recombination occurs, the *puromycin*^{r} gene cassete flanked by two synthetic RSSs become deleted and the luciferase gene is relocated immediately downstream of the CAG promoter, leading to luciferase expression.

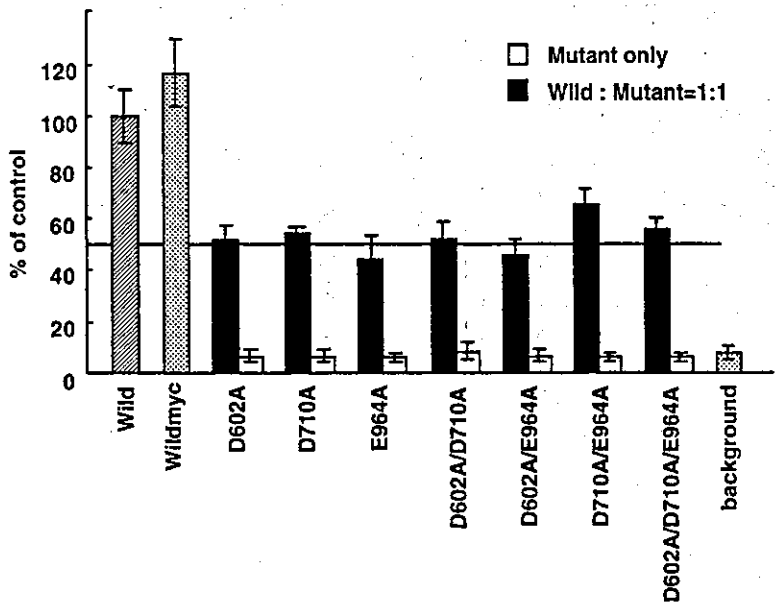

Fig. 3. Catalytic RAG1 mutants inhibit wild type RAG1. The recombination frequency is shown as a percentage of the value of wild type (shaded bar) and data are means ± S.D. from three independent experiments.

tail biopsies. The transgenic mice were healthy and indistinguishable from their littermates. If the mutant RAG1 protein inhibited V(D)J recombination during the process of B lymphocyte maturation, the production of antibodies might decrease in transgenic mice. To test this idea, we measured serum IgM concentrations, because IgM is the first type of antibody produced by the humoral immune system and maternal IgMs are not transferred to pups, as are other types of immunogloblins. The mean serum IgM levels of transgenic mice was significantly lower than that of the non-transgenic littermates at 5 weeks of age (314.5 ± 81.6 versus 227.8 ± 56.6 μg/ml, respectively, Fig. 5A) and this lower level was sustained at least until 8 weeks (485.6 ± 130.3 versus 292.2 ± 76.4 μg/ml, respectively, Fig. 5B). One of these transgenic lineages, no. 4, produced viable litters and F1 mice inherited the *GFPmutRAG1* gene and also showed low serum IgM levels compared to their littermates (Fig. 5C).

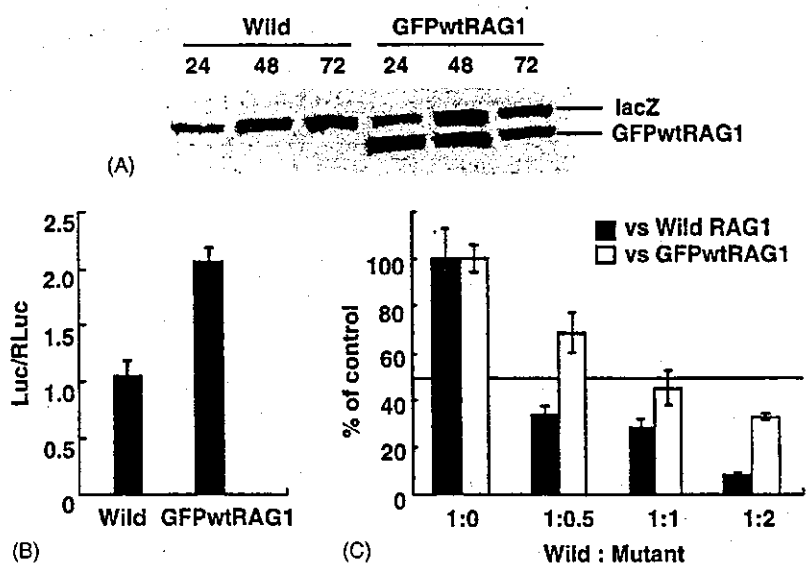

Fig. 4. (A) Protein expression of wild type RAG1 and GFPwtRAG1 obtained by transient expression in Cos7 cells. Fifty micrograms of total protein were electrophoresed per lane and the protein was detected with anti-myc antibody. (B) Recombination activty of GFPwtRAG1. Recombination frequency is shown as firefly lucifererase activity values normalized to *Renilla* luciferase activity. Data are means ± S.D. from three independent experiments. (C) Inhibition activity of GFPmutRAG1 against wild type RAG1 (solid bar) and GFPwtRAG1 (open bar). Data are means ± S.D. from three independent experiments.

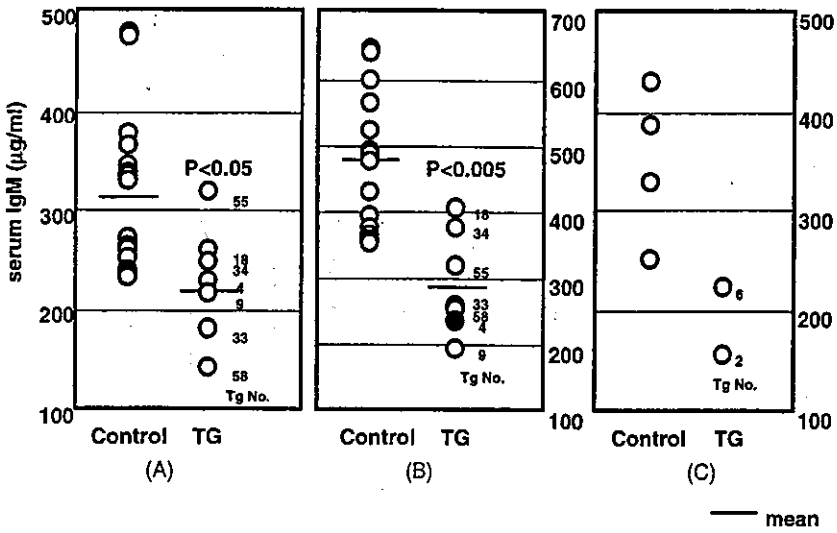

Fig. 5. Serum IgM levels in GFPmutRAG1 mice at 5 (A) and 8 weeks (B). (C) Sera from TG and their littermates from lineage no. 4 F1 mice were tested by ELISA for IgM (shown in μg/ml).

4. Discussion

4.1. RAG1 mutants inhibit recombination by wild RAG1 in vitro

To inhibit the V(D)J recombination, we generated catalytic RAG1 mutants with normal DNA binding activities as competitors for the endogenous RAG1 protein. Landree et al. (1999) and Kim et al. (1999) used site-directed mutagenesis to identify three RAG1 mutants in mice that bound DNA normally, but lacked DNA cleavage activity. We constructed seven RAG1 mutants making use of these previously identified mutations (D602A, D710A, and E964A). As expected, our mutants exhibited no recombination activity and inhibited recombination of an artificial substrate by wild type RAG1 in co-transfection experiments. These results suggest that RAG1 mutants bind to RSS together with the RAG2 protein and other protein partners but prevent the cleavage step of recombination. Unfortunately, the presence of equivalent amount of mutants and the wild type only inhibited recombination by 50%, indicating that mutants were not acting in a dominant negative fashion against wild type RAG1.

Sadofsky et al. (1993) reported that RAG1 protein was transported to the nucleus and rapidly degraded, with a half-life of 15 min. We also observed weak expression of full-length RAG1 protein in transiently transfected cells (Fig. 4A). Thus, we tried to prolong the half-life of mutants to provide them a functional advantage over wild type RAG1. RAG1 core protein (mouse residues 384–1008) truncated at both the N- and C-termini, has been reported to be highly stable (McMahan et al., 1997; Steen et al., 1999), and deletion of amino acid residues 15–79 (in human) increased protein levels (Silver et al., 1993). In agreement with previous studies, the steady state level of GFPwtRAG1, with its N-terminal domain (residues 1–379) replaced by GFP protein, was apparently higher than that of the wild type (Fig. 4A), which might account for its twofold greater ability to recombine the artificial substrate (Fig. 4B). Likewise, the GFPmutRAG1 triple point mutant showed a twofold increase in its recombination inhibition activity (Fig. 4C). RAG1 protein contains a Ring finger domain at residues 292–330 and many Ring finger proteins act as E3 ubiquitin protein ligases that bind to both target proteins and ubiquitin E2 conjugating enzymes (Joazeiro et al., 1999; Lorick et al., 1999; Waterman et al., 1999; Yokouchi et al., 1999). While ligase activity of RAG1 protein has not been reported, the Ring finger domain of RAG1 protein might promote the ubiquitination of RAG1 and/or other components of recombination complexes.

Previous studies have shown that the truncation of the N-terminus of RAG1 reduced its recombination activity (McMahan et al., 1997; Roman et al., 1997; Steen et al., 1999). Although the catalytic activity of GFPwtRAG1 was not determined in this study, the large increase in GFPwtRAG1 protein stability was not associated with a similar increase in its effect on recombination, suggesting that its catalytic activity might be reduced compared to the wild type protein. A zinc-finger motif (residues 292–330) is believed to participate in RAG1 dimerization (Bellon et al., 1997; Rodgers et al., 1996) and N-terminal truncation of RAG1 could conceivably affect the disassembly of the intermediate complexes and cording joint formation (Steen et al., 1999). The truncation of the N-terminus of RAG1 might alter its affinity for the recombination complex and/or DNA.

4.2. Transgenic mice carrying GFPmutRAG1 gene showed low serum IgM levels

Among seven transgenic mice carrying the GFPmutRAG1 gene, four (no. 4, 9, 33, and 58) showed a particularly

significant reduction of serum IgM levels, to approximately 33–50% of control levels. In lineage no. 4, progeny that inherited the GFPmutRAG1 gene also showed low serum IgM levels. These data suggested that the mutant RAG1 protein competed with the endogenous RAG1 protein for binding to recombination signal sequences at the immunoglobulin gene loci, resulting in the partial blockage of V(D)J recombination. However, the immunodeficient phenotypes were less dramatic than expected from the data on the mutant obtained in the in vitro assays. The serum IgG levels of the transgenic mice were indistinguishable from their littermates. The size of spleen and thymus of transgenic mice was equal to that of wild mice, and flow cytometric analysis of these tissues using antibodies recognizing lineage markers demonstrated normal lymphocyte profiles (data not shown). Although amino acid sequence of rabbit RAG1 shows high homology (89.2%) with a mouse RAG1, heterologous proteins might be due to the inefficiency of the inhibition. We are currently attempting to generate mouse expressing high levels of the mutant RAG1 protein and rabbit transgenic lines. In combination with the RAG1 mutant, other mutant proteins involved in V(D)J recombination, such as RAG2 and DNA-PK, might allow us to produce more severe immunodeficiency.

Evidence for the existence of somatic stem cells for most tissues has recently been described (Jiang et al., 2002; Krause et al., 2001). As with ES cells, such cells were expected to have therapeutic utility for tissue repair and organ regeneration. However, it is quite difficult to generate functional solid organs from stem cells in vitro. Our strategy to generate human-livestock animal hybrid organs by IUM will be applicable for stem cell therapy in the future.

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Development of Novel Monoclonal Antibody 4G8 against Swine Leukocyte Antigen Class I α Chain

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ABSTRACT

A mouse monoclonal antibody (MAb) was generated against swine leukocyte antigen (SLA) class I α chain. A newly developed series of MAb clones that react with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. Among them, MAb 4G8 was characterized by the following features: (1) 4G8 reacted with Cos-7 cells transfected with SLA class I α chain from the *d* haplotype, (2) 4G8 recognized epitopes that were different from those of commercially available anti-SLA class I MAbs 74-11-10 and PT85A, and (3) 4G8 could be used to immunostain frozen sections of thymus, spleen, lymph node, kidney, and liver tissues with good results.

INTRODUCTION

THE PORCINE SYSTEM has received much attention as a suitable model for transplantation medicine. Therefore, an accurate understanding of human immune responses to porcine tissues has become increasingly important. However, the details of the porcine immune system, especially those features that are novel to the pig, remain unclear. We thus attempted to develop new MAbs that could be used to analyze the porcine immune system.

The immune response to foreign antigens is determined by the expression of specific major histocompatibility complex (MHC) molecules that can bind and present peptide fragments of that protein to T cells. There are two different types of MHC gene products, termed Class I and Class II MHC molecules, and any given T cell recognizes foreign antigens bound to only one Class I or Class II MHC molecule. Antigens associated with Class I molecules are recognized by $CD8^+$ cytolytic T cells, whereas class II–associated antigens are recognized by $CD4^+$ helper T cells. Class I molecules are located on every nucleated cell surface, except those of neurons and trophoblasts. In contrast, the expression of Class II molecules is limited to certain cell types. In pigs, MHC molecules are known as swine leukocyte antigens (SLA). All SLA class I molecules contain two separate polypeptide chains: an MHC-encoded α chain of 45 kD and a non–MHC-encoded β chain of 12 kD.

Recently, the profound involvement of SLA Class I molecules in human anti-porcine cell reactions has been described. Several studies have shown that human T cells can directly recognize porcine MHC molecules and that such recognition can lead to the killing of the porcine cells. Porcine cells have recently been shown, moreover, to be targets for human NK cells. Since human MHC class I molecules deliver a negative signal to human NK cells, protecting syngeneic cells from lysis, we surmised that differences in the gene sequences of porcine MHC class I molecules may be responsible for the lack of recognition by human NK cell receptors and subsequent cytolysis of the porcine cells. In addition, it was reported that a single treatment with a monoclonal antibody (MAb) directed against the SLA class I provides an attractive approach to the induction of T cell tolerance, possibly enabling long-term graft survival in porcine-to-human cell transplantations.[1] These studies indicate that SLA class I molecules play critical roles in transplantation medicine.

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Here, we report a novel MAb 4G8 against the SLA class I α chain that was proven to be different from four commercially available anti-SLA class I MAbs. The utilization of 4G8 in tissue sections was also examined.

MATERIALS AND METHODS

Animals and tissues

Landrace or (Landrace × Large White) F1 pigs were used in this study. Peripheral blood (PB) and tissues were obtained from anesthetized animals and were processed. PB was collected in acid citric buffer to avoid coagulation. Tissues were immediately snap frozen and kept in the deep freezer until use.

Monoclonal antibodies

PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. After washing twice in phosphate-buffered saline (PBS), approximately 1×10^8 cells were injected into the abdominal cavity of 8-week-old female Balb/c mice. Boost injections were performed twice at 2-week intervals. At 4 days after the last boost, 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 by flow cytometry and clones secreting antibodies reactive with porcine PB leukocytes were subcloned twice by limiting dilution. Clones were grown in the abdominal cavity of Pristane-treated Balb/c mice, and ascites were obtained. Purification of MAbs was performed by Protein-A or Protein-G column (Bio-Rad Laboratories, Hercules, CA). After purification, MAb was fluorescence isothiocyanate (FITC) conjugated as described previously.[2] Commercially available MAbs against SLA class I 74-11-10, PT85A, H17A*, H58A* (* indicates known as cross-reactive with pig and other species) were obtained from Veterinary Medical Research and Development (Pullman, WA).

Flowcytometry and immunohistochemistry

Flowcytometrical analysis of MAbs was carried out as follows. Briefly, aliquot of porcine PB was incubated with appropriate amount of MAb for 30 min at 4°C. After washing with PBS, cells were incubated with FITC-conjugated (Jackson Laboratory, West Grove, PA) for 30 min at 4°C. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter, Westbrook, MA).

Reactivity of MAbs on tissues were analyzed by immunohistochemistry on frozen sections. Briefly, porcine tissues were snap-frozen in optimal cutting temperature (OCT) compounds and frozen sections were made by cryostat apparatus. Sections were fixed by acetone for 15 min at 4°C. After washing in PBS and blocked with normal rabbit serum, sections were incubated with MAbs at appropriate dilutions for 30 min at room temperature. Sections were then washed with PBS and incubated with horseradish peroxidase (HRP)–conjugated rabbit anti-mouse antibodies (Jackson Laboratory) for 30 min at room temperature. After washing with PBS, color development was done in diaminobenzidine solution (10 mM in 0.05 M Tris-HCl, PH 7.5) with 0.003% H_2O_2.

Binding competition assay

Binding competition assay was carried out as follows. Briefly, after aliquot of porcine PB leukocytes were incubated with 2ug saturated amount of commercially available MAbs for 30 min at 4°C. The cells saturated with these commercially available MAbs were stained with FITC-4G8 for 30 min at 4°C. Then, PB leukocytes were treated using RBC lysis with NH_4Cl lysis buffer followed by centrifugation at 1,500 rpm for 10 min. FITC-mouse immunoglobulin (MsIg) was used as control antibody. Cells were washed with PBS and analyzed by EPICS XL analyzer (Beckman/Coulter).

Cloning and expression of porcine cDNA library

As another purpose for analysis of $\gamma\delta$TCR against MAb (7G3) and CD8 against MAb (6F10), cDNA libraries of 7G3-positive as well as 6F10-positive PB leukocytes were first constructed. A brief description is shown below. Porcine PB labeled with FITC-7G3 antibody was incubated with magnetic-activated cell sorting (MACS) beads conjugated with anti-FITC antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) and was loaded onto AutoMACS cell separator (Miltenyi Biotec). 7G3-positive cells were positively selected and a cDNA library was constructed using the oligo-capping method[3] and plasmid vector pME18S-FL3, which contains the SR-α promoter for expression in mammalian cells. To 7G3-negative pass-through fractions, FITC-6F10 was added 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, one clone was selected which exhibited homologies to known porcine MHC class I sequences from d haplotype and contained full-length open reading frames.

Complementary DNA coding for porcine MHC class I under SRα promoter was introduced into COS7 cells by lipofec-

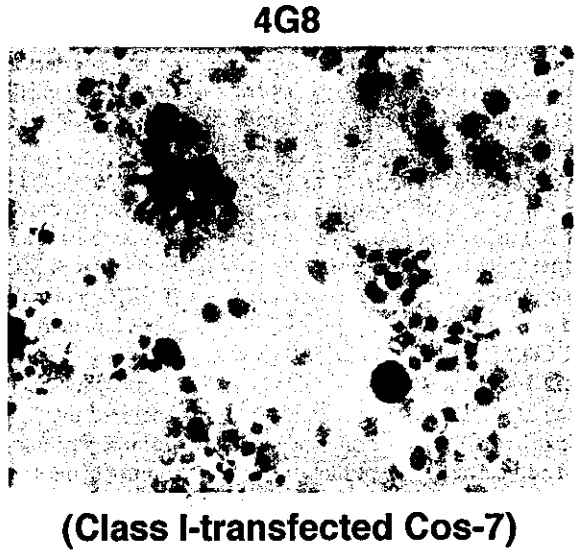

FIG. 1. Reactivity of 4G8 on Cos-7 cells transfected with SLA class I PD1. Mammalian expression vectors containing SLA class I PD1 were introduced into Cos-7 cells, and the cells were stained with 4G8 using immunohistochemistry.

tion (LIPOFECTAMIN, Invitrogen, Groningen, Netherlands) and cells were stained with 4G8 MAb after 3 days.

RESULTS AND DISCUSSION

Anti-SLA Class I MAb 4G8 recognizes a distinct epitope from those of commercially available antibodies

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 (data not shown). To determine whether a MAb against SLA class I α chain was included among these clones, MAb clones that reacted with pan leukocytes were selected and tested by immunohistochemistry using SLA class I α chain expressing Cos-7 cells. As shown in Figure 1, when a mammalian expression vector of SLA class I PD1 from d haplotype was transfected into Cos-7 cells, clone 4G8 was found to stain the cells, whereas control MsIg failed to stain the cells (data not shown). Therefore, 4G8 was considered to recognize the SLA class I α chain, including the d haplotype.

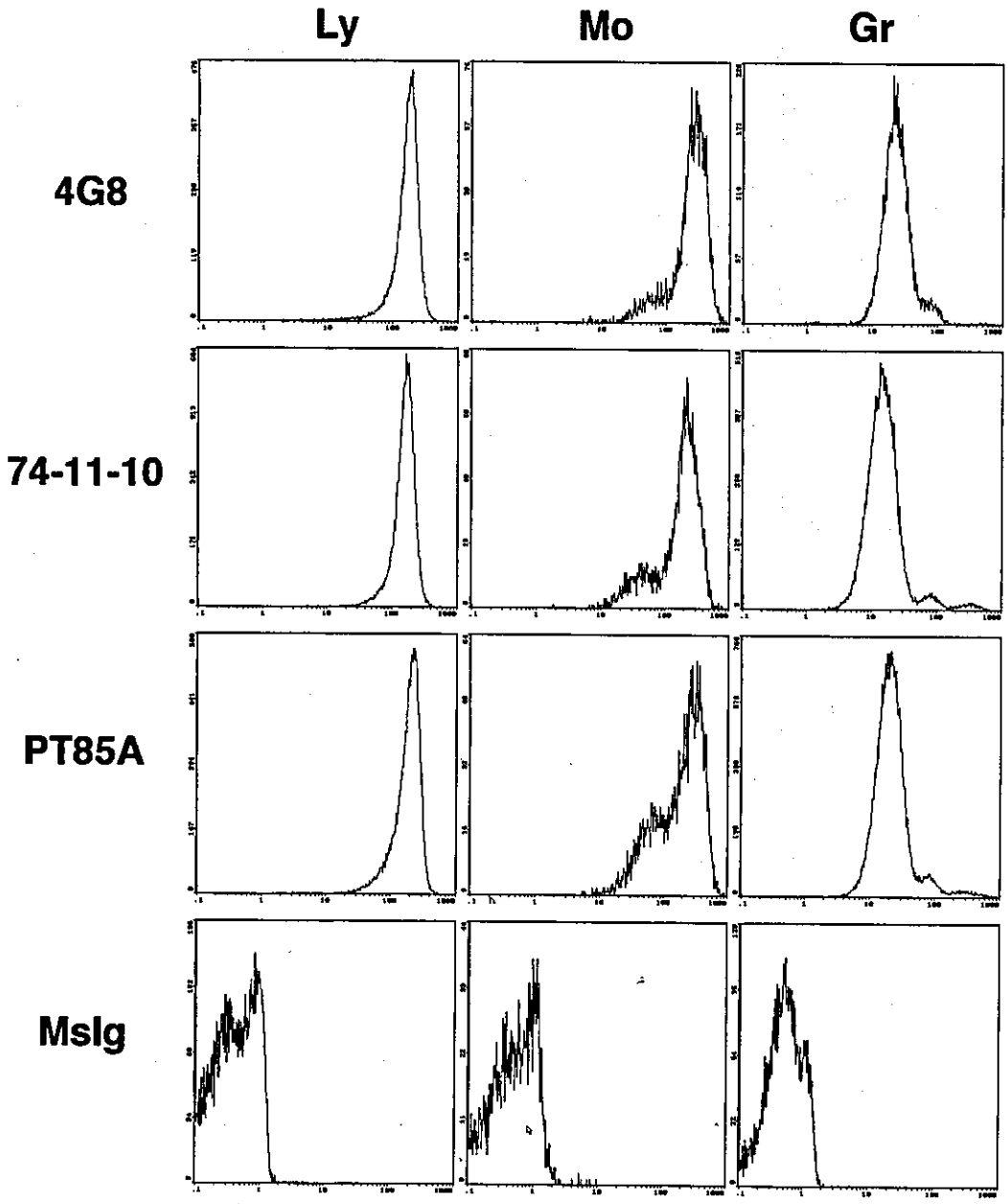

FIG. 2. Comparison of reactivity profiles of 4G8 and commercially available anti-SLA class I monoclonal antibodies. Porcine PB leukocytes were stained with 4G8 and commercially available anti-SLA class I MAbs, 74-11-10, and PT85A, using flow cytometry. MsIg was used as a control antibody.

Next, we compared the reactivity of 4G8 with commercially available anti-SLA class I Abs 74-11-10, PT85A, H17A, H58A to PB from outbred domestic pigs. As shown in Figure 2, flow-cytometrical analysis demonstrated that the reactivity of 74-11-10 and PT85A to the porcine PB was similar to that of 4G8. Although multiple samples from individual animals were tested, all of these MAbs always revealed a pan leukocyte positive staining pattern. In contrast, H17A did not react with the domestic porcine PB samples (data not shown), indicating that H17A is polymorphic in pigs. H58A, exhibited variable reactivity from one animal to another (data not shown). This data indicates that the reactivity of H17A and H58A is different from that of 4G8.

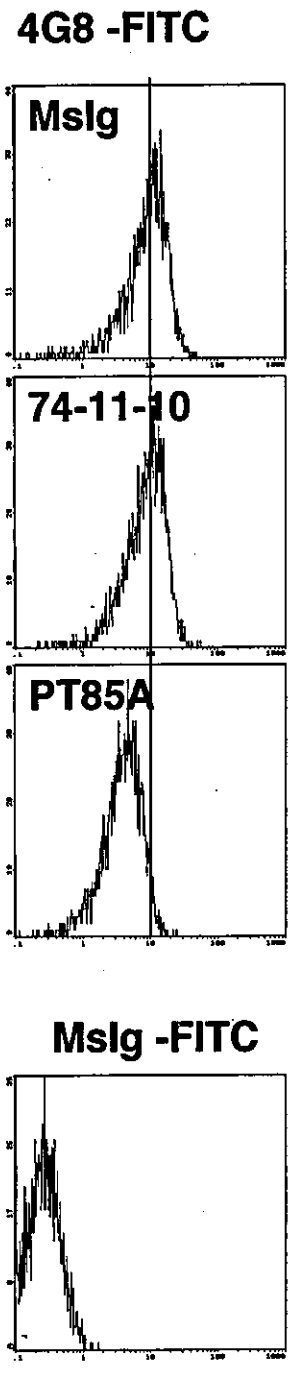

FIG. 3. Competition binding assay using flow cytometry. 4G8 continued to react with porcine PB leukocytes that had been saturated with commercially available MAbs (74-11-10 and PT85A). MsIg-FITC was used as a negative control.

To determine whether the epitope recognized by 4G8 was the same as that of 74-11-10 and PT85A, we examined whether 4G8 could still bind to the cells after the cells had been saturated with 74-11-10 or PT85A. As shown in Figure 3, 4G8 continued to react with PB leukocytes that had been saturated with 74-11-10 or PT85A. These results indicate that 4G8 recognizes a novel epitope distinct from those recognized by 74-11-10, PT85A, H17A and H58A. MHC class I molecules are extremely polymorphic, and polymorphism often occurs in the $\alpha 1$ domain, or the $\alpha 2$ domain, but the $\alpha 3$ domain is nonpolymorphic.[4] 74-11-10 and PT85A have been reported to require the PD1 $\alpha 1/\alpha 2$ domains, but not the $\alpha 3$ domain, to exhibit reactivity.[1] A precise analysis to clarify which domain is recognized by 4G8 is now underway.

Analysis of 4G8 using immunohistochemistry

We examined whether 4G8 could be used to immunostain frozen sections. As shown in Figure 4, 4G8 produced high-quality immunostaining results when used on frozen sections. In thymus tissues, 4G8 produced a dense and diffuse staining pattern in the medullar region and a lighter, scattered staining pattern in the cortex, suggesting that SLA class I molecules are mainly expressed on mature thymocytes in the medulla, but not on immature thymocytes in the cortex. In spleen, lymph node, kidney and liver tissues, 4G8 produced an ubiquitous staining pattern, as shown in Figure 4. These results demonstrate that 4G8 can be effectively used to immunostain frozen sections. Therefore, 4G8 may be a useful reagent for immunopathology studies and improving our general understanding of the porcine immune system.

In conclusion, a novel MAb, 4G8, that recognizes the SLA class I α chain has been identified and used to produce high-quality immunostaining results on tissues sections. 4G8 is expected to become a useful tool for investigating the immune system of domestic pigs.

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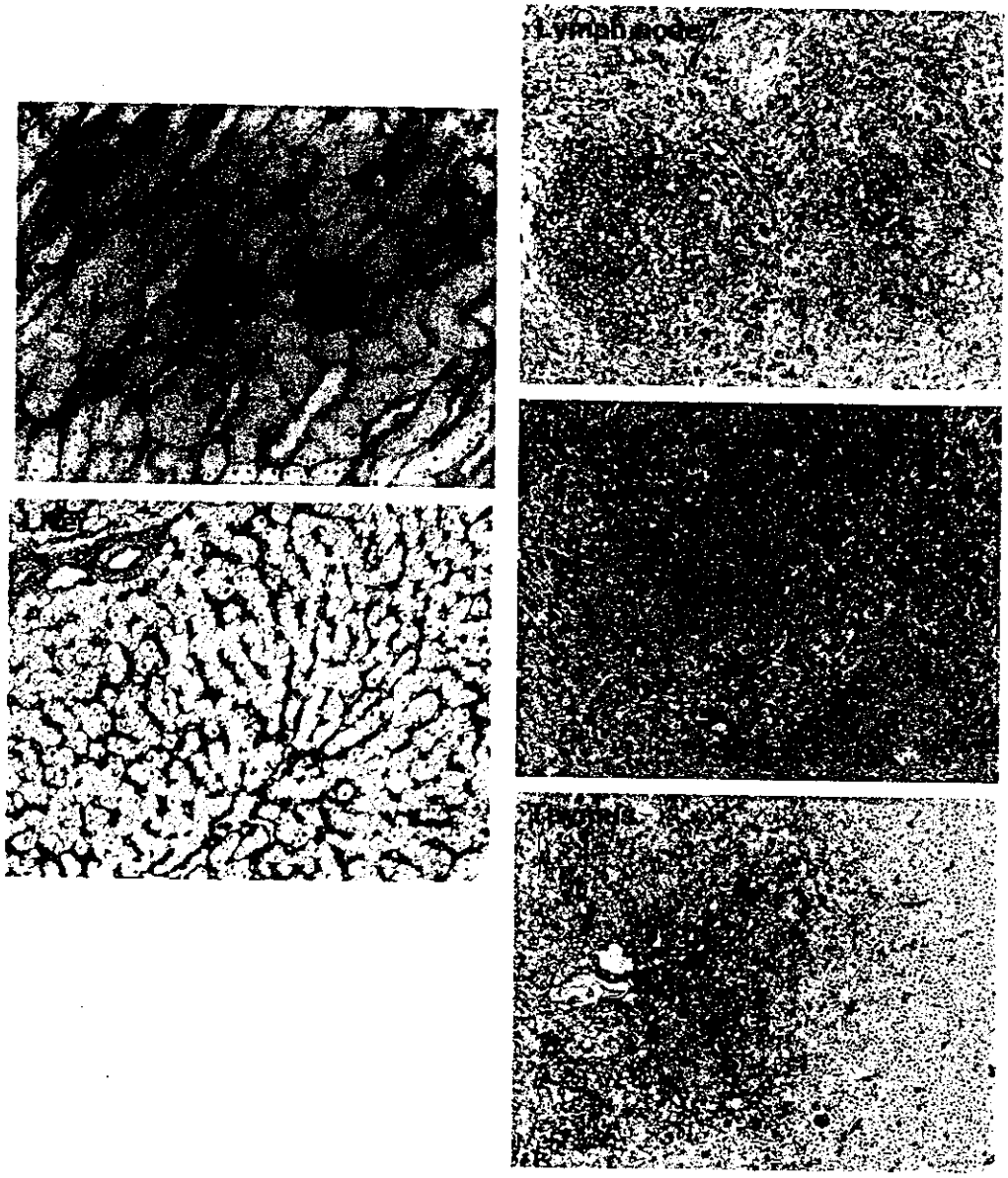

FIG. 4. Reactivity of 4G8 on frozen sections of porcine tissues. Frozen sections of porcine thymus, spleen, lymph node, kidney, and liver tissues were stained with 4G8 using immunohistochemistry.

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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

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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

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characterizes mAbs against porcine TCR for the first time, facilitating molecular biological investigations of the porcine immune system.

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

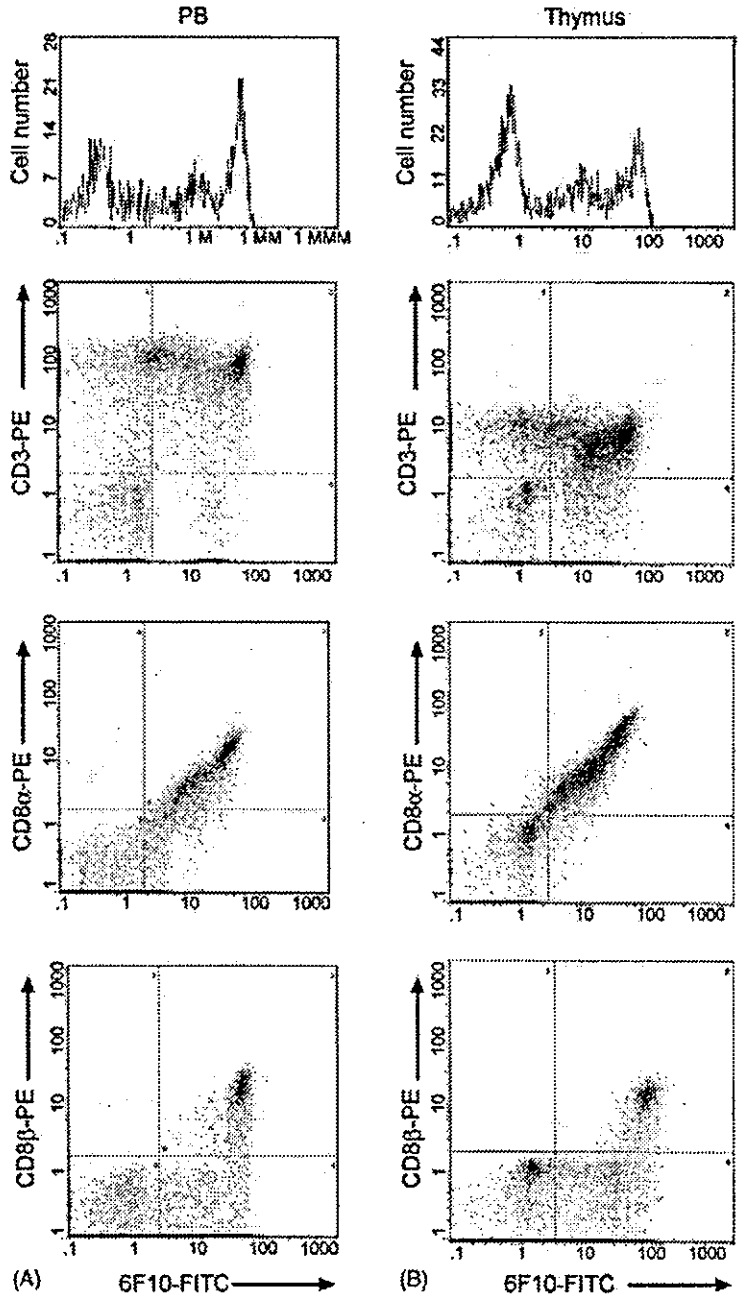

Fig. 1. Two-color staining of porcine peripheral blood lymphocytes and thymocytes using the 6F10 monoclonal antibody and other T-cell markers. Peripheral blood lymphocytes (A) and thymocytes (B) were stained with combinations of FITC-conjugated 6F10 (*X*-axis) and other PE-labeled antibodies (*Y*-axis).

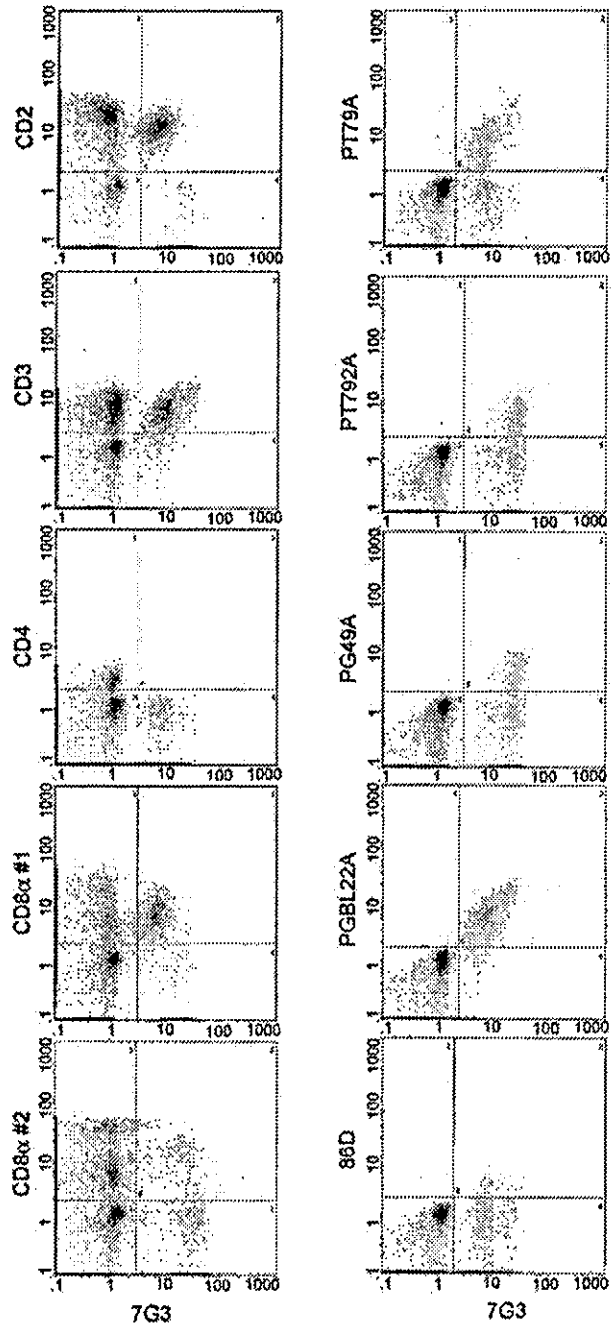

Fig. 2. Two-color staining of porcine peripheral blood lymphocytes using 7G3 and other T-cell markers. Peripheral blood lymphocytes were stained with combinations of FITC-conjugated 7G3 (*X*-axis) and other PE-labeled antibodies (*Y*-axis).

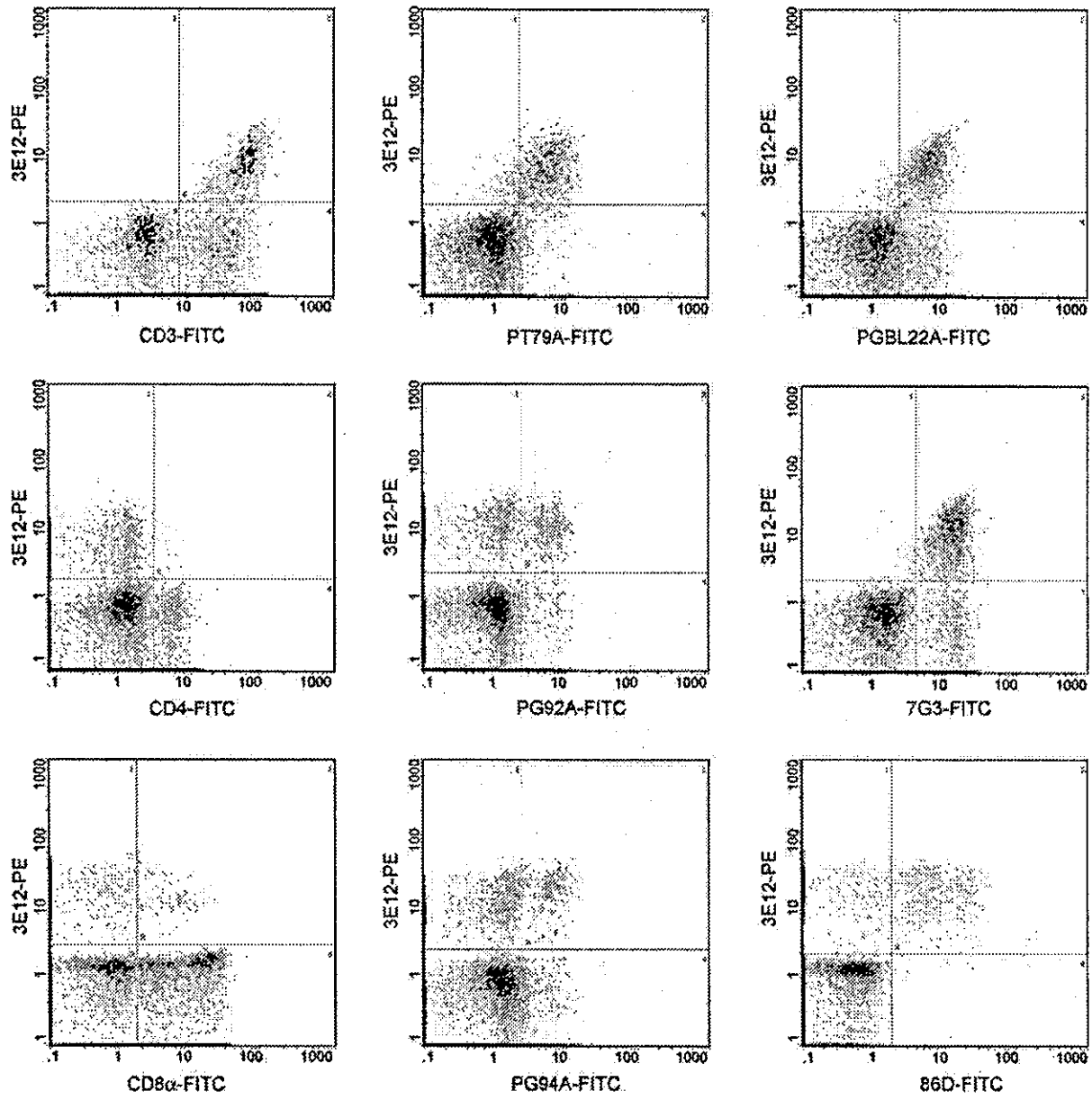

Fig. 3. Two-color staining of porcine peripheral blood lymphocytes using 3E12 and other T-cell markers. Peripheral blood lymphocytes were stained with combinations of PE-conjugated 3E12 (*Y*-axis) and other FITC-labeled antibodies (*X*-axis).

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