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Identification of an amino acid responsible for the CD3 polymorphism in cynomolgus monkeys (*Macaca fascicularis*)

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Abstract: The FN18 monoclonal antibody (mAb), directed to CD3 molecules, did not react with the lymphocytes of some cynomolgus monkeys (*Macaca fascicularis*), because of the polymorphism of the CD3ε chain. The epitope recognized by the FN18 mAb was successfully expressed on COS7 cells upon transfection of plasmid DNA coding for the CD3ε derived from T cells of a FN18 positive cynomolgus monkey. By construction and expression of plasmid DNA encoding the mutant CD3ε, the amino acid residue at position 67 was demonstrated to be involved in the formation of an epitope recognizable by the FN18 mAb.

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Key words: CD3 – cynomolgus – FN18 – polymorphism

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Introduction

Although FN18 monoclonal antibody (mAb) has been shown to be directed to CD3 molecules, T cells from some rhesus (*Macaca mulatta*) and cynomolgus monkeys (*M. fascicularis*) did not react with the FN18 mAb [3–5, 10]. As CD3 plays a key role in signal transduction within T lymphocytes, it was very important to know the reason why the lymphocytes from some monkeys did not react with FN18 mAb. We previously showed that PBMCs from FN18 negative cynomolgus monkeys appeared functionally normal and that the lack of the reactivity with FN18 mAb was caused by CD3 polymorphism [10].

Nucleotide sequence analysis of the CD3δ, ε, and γ chains suggested that amino acid differences found at positions 67 and 72 of the CD3ε were responsible for the polymorphism [10] (Fig. 1). The present study attempted to express each chain of the CD3 molecule to clarify the molecular basis of the polymorphism in cynomolgus monkey. By expression of mutant proteins of the CD3ε chain, an amino acid at position 67 was shown to be the residue, playing a key role in forming an epitope recognized by the FN18 mAb.

Materials and methods

Animals

All the cynomolgus monkeys under study were raised and reared in the Tsukuba Primate Center for Medical Science, NIID. Both genders were involved and the ages of the monkeys were between 12 and 16 years old. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals in the National Institute of Infectious Diseases.

Cells

COS7 cells established from African green monkey kidney, purchased from the Riken Cell Bank (Tsukuba, Japan), were cultured in Dulbecco's Modified Eagles Minimal Essential Medium

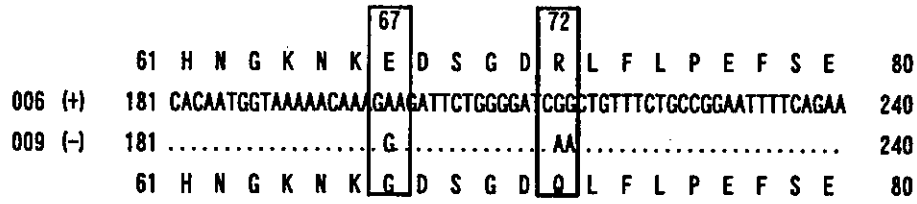

Fig. 1. Nucleotide and deduced amino acid sequence of CD3ε chain. The nucleotide sequence derived from FN18-positive (+) and negative (−) monkeys are shown. Deduced amino acid sequences are shown by one letter. Identical nucleotides are indicated by dots. Different nucleotide are indicated by letters.

supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal calf serum (FCS) in a 5% CO_2 incubator.

Antibodies

FN18 mAb and SP34 mAb were purchased from BioSource (Camlio, CA, USA) and FharMingen (San Diego, CA, USA), respectively.

DNA constructs

PolyA mRNA extracted from PBMCs of FN18 reactive cynomolgus monkey using the QuickPrep micro mRNA purification kit (Amersham, Uppsala, Sweden) was converted into cDNA using high fidelity RNA PCR kit (Takara, Shiga, Japan). cDNAs were amplified by the PCR using pfu DNA polymerase (Promega, Madison, WI, USA) and primers listed in Table 1 as reported before [10]. The PCR products were cut with *Eco*RI and *Bam*HI, and cloned into the pcDNA3.1(−) expression vector (Invitrogen, Carlsbad, CA, USA). These plasmids coding for CD3δ, ε, or γ were designated pcDNAδ, pcDNAε, or pcDNAγ, respectively.

pεE_{67}-G, pεR_{72}-Q, and pεNeg plasmids encoding mutant proteins were generated by the PCR-mediated mutagenesis [7] using mutagenic primers (E_{67}-G, R_{72}-Q, and NEG) and common antisense primer (M-antisense) listed in Table 1. The PCR products containing mutation of E_{67}-G, R_{72}-Q, or Neg were cloned into the pcDNA3.1(−) expression vector, and designated pεE_{67}-G, pεR_{72}-Q, or pεNeg, respectively. The nucleotide sequences of all plasmids were confirmed by sequencing using the model 4000L DNA sequencer (LI-COR, Lincoln, Nebraska, USA).

Transfection of plasmid DNAs

The plasmid DNA was transfected into semi-confluent COS7 cells using LipofectAmine 2000 (Invitrogen) according to the protocol recommended by the manufacturer. Transfected cells were incubated at 37°C for 48 hour in a CO_2 incubator.

Fluorescence-activated cell sorter (FACS) analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood by standard Ficoll-Hypaque gradient centrifugation method. PBMCs were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS at a concentration of 10^6 cells/ml. PBMCs (10^5) were incubated at 4°C

Table 1. Primer sequences used for reverse transcriptase (RT)-PCR and over-lap PCR

Primers for RT-PCR	
δ-sense	5′-CTT CGC GAA ATT CCG CTG GAA GAT GGA ACA TAG CAC GTT T-3′
ε-antisense	5′-ATT CGC GGA TCC GCG TCA TCA CTT GTT CCG AGC CCA GT-3′
ε-sense	5′-CTT CGC GGA ATT CGA AAG AAA GAT GCA GTC GGG CAC TCG C-3′
ε-antisense	5′-ATT CGC GGA TCC GCG TCA GAT GCG TCT CTG ATT C-3′
γ-sense	5′-CTT CGC GGA ATT CAC CAA GAA GAT GGT TCA GGG GAA GAA GGG C-3′
γ-antisense	5′-ATT CGC GGA TCC GCG TCA TCA ATT CAT CCT CAA CTG GT-3′
Primers for over-lap PCR	
R_{72}-Q	5′-CAA CAC AAT GGT AAA AAC AAA GAA GAT TCT GGG GAT CAA CTG TTT CTG-3′
E_{67}-G	5′-CAA CAC AAT GGT AAA AAC AAA GGA GAT TCT GGG GAT CGG CTG TTT CTG-3′
Neg	5′-CAA CAC AAT GGT AAA AAC AAA GGA GAT TCT GGG GAT CAA CTG TTT CTG-3′
M-antisense	5′-TTT GTT TTT ACC ATT GTG TTG-3′

for 1 hour with appropriately diluted antibodies, and washed twice with PBS containing 1% bovine serum albumin. After fixation with 1% paraformaldehyde at 4°C for 30 minutes, FACS analysis was performed (FACS Caliber; Becton–Dickinson, Franklin Lakes, NJ, USA). The data were analyzed with the Cellquest software (Becton-Dickinson).

COS7 cells transfected with plasmid DNAs were dispersed by trypsin-treatment, and then were processed for FACS analysis as described above.

Immunofluorescent assay (IFA)

Cells transfected with respective plasmid DNA were fixed with acetone at room temperature for 20 minutes, and then incubated with 5 or 10 μg/ml of the SP34 or FN18 mAb at 37°C for 1 hour followed by incubation with 1:100 dilution of flourescent isothiocyanate (FITC)-labeled anti-mouse IgG antibody (Cappel, Aurora, OH, USA) at 37°C for 1 hour. Cells were observed under a UV-microscope.

Radio immunoprecipitation assay (RIPA)

Transfected cells were labeled with 100 μCi/ml of the Redivue Pro-mix L-[^{35}S] *in vitro* cell labeling mix (Amersham) for 4 hour, and were lysed with dissociation buffer (10 mM Tris–HCl pH 8.0, 250 mM NaCl, 0.5% Triton-X-100, and 0.5% sodium deoxycholate) supplemented with protease inhibitor of Complete Mini ethylenediaminetetraacetic acid-free (Roche, Mannheim, Germany). Immunoprecipitation with mAbs and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed as described previously [10].

Results

Recognition of CD3 components by FN18 mAb

Previous reports suggested that the amino acids at positions 67 and 72 of the CD3ε derived from FN18 positive cynomolgus monkeys were different from those from FN18 negative ones [10]. Although it was strongly suggested that one or both of those amino acid substitutions were responsible for the phenotypic difference among monkeys in terms of the reactivity with the FN18 mAb, there was no direct experimental evidence to prove this possibility. Therefore, an attempt was made to examine the cynomolgus monkey CD3 molecules expressed from cDNAs for the reactivity with FN18 mAb. The coding region of CD3δ, ε or γ chains were cloned into pcDNA3.1(−) plasmid, and each plasmid was designated as pcDNAδ, pcDNAε, and pcDNAγ, respectively. COS7 cells transfected with these plasmid DNAs alone or in combination, were metabolically labeled with the Redivue Pro-mix L-[^{35}S] *in vitro* cell labeling mix. The cell extracts were immunoprecipitated with FN18 or SP34 mAb (Fig. 2). The FN18 immunoprecipitated two proteins from the cells transfected with three plasmids together (Fig. 2, lane δεγ). It was also evident that the CD3ε chain could be detected by the FN18 even when COS7 cells were transfected with pcDNAε alone (Fig. 2, lane ε), indicating that the epitope recognized by the FN18 mAb not only resided on the CD3ε chain but was also formed in the absence of other chains. SP34 mAb, which was produced against denatured CD3ε molecules, immunoprecipitated a single band of CD3ε although cells were transfected with plasmid DNA coding for other CD3 components (Fig. 2, bottom panel). These results suggested that the epitope recognized by FN18 mAb was a mature

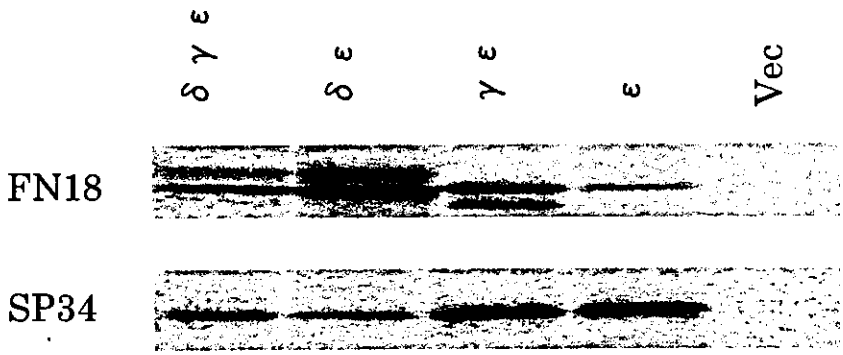

Fig. 2. Reactivity of FN18 mAb to CD3 molecules. COS7 cells transfected with plasmid DNAs in combination shown in the figure were labeled for 4 hour, at 48 hour after transfection. Cell lysates were subjected to immunoprecipitation with FN18 or SP34 mAbs followed by SDS-PAGE analysis.

form of CD3ε that could directly interact with the other chains.

Identification of important amino acids for formation of the epitope recognized by FN18 mAb

Previous reports reveal that the common amino acid changes were found at positions 67 and 72 of the CD3ε-chain of the FN18 negative cynomolgus monkey [10]. It is quite possible that one or both substitutions lead to alteration of the epitope recognized by this particular antibody. To this end, an attempt was made to express three mutants of the CD3ε and examined them for reactivity with the FN18 mAb. Predicted amino acid sequences of the mutants are shown in Table 2. Transfection of COS7 cells with pcDNAε or $p\varepsilon R_{72}$-Q yielded bands corresponding to the CD3 that could be immunoprecipitated by both the FN18 and SP34 mAb (Fig. 3A). However, only faint bands were precipitated with the FN18 mAb from the extracts prepared from COS7 cells transfected with $p\varepsilon E_{67}$-G, although distinct bands corresponding to the CD3ε were precipitated by the SP34 mAb. This result strongly suggested that the replacement of glutamic acid at position 67 by glycine rendered the CD3ε chain irresponsive to the FN18 mAb. To confirm the above observation transfected cells were subjected to IFA and FACS analyses (Fig. 3B,C). As the CD3 molecules were not transported to the cell surface when a single chain was expressed, COS7 cells were transfected with plasmid DNAs coding for the mutant CD3ε chains together with those encoding CD3δ and γ [1, 2, 6]. The cells transfected with pcDNAε or $p\varepsilon R_{72}$-Q were stained with the FITC-conjugated FN18 mAb, whereas cells transfected with neither $p\varepsilon E_{67}$-G nor pNeg gave positive signals when stained with the FITC-conjugated FN18 mAb. The expression of the proteins on the cells transfected with pcDNAε, $p\varepsilon R_{72}$-Q, $p\varepsilon E_{67}$-G, and pεNeg were confirmed by both FACS and IFA analysis using FITC-conjugated SP34 mAb (data not shown).

Table 2. Reactivity of CD3ε mutants

Mutant	Phenotype		FN18 mAb reactivity
		60 67 72 80	
ε	++	QHNGKNEDSGDRLFLPEFDR	+
εR_{72}-Q	+−	———Q——	+
εE_{67}-G	−+	——G———	−
ε-Neg	−−	——G—Q——	−

Expression was analyzed by immunoprecipitation, IFA, and FACS. '+', protein located on cell surface in COS7 cells, and '−' not found on cell surface in COS7 cells.

Discussion

Lymphocytes from some monkey populations, either rhesus or cynomolgus macaques, did not react with the FN18 mAb raised against CD3 [3–5, 10]. A previous study addressed whether CD3 polymorphism in cynomolgus monkey was attributable to the observed phenomenon by determining nucleotide sequences of CD3δ, ε and γ among the FN18-positive and -negative individuals [10]. As amino acid changes found at positions 67 and 72 of the CD3ε were common among the FN18-negative individuals, it was concluded that one or both substitutions led to the alteration of the epitope recognized by this particular antibody.

The present study attempted to identify the amino acid that defines the epitope recognized by FN18 mAb (Fig. 2). Transfection of the plasmid DNA coding for the CD3ε chain of the FN18 positive cynomolgus monkeys alone was sufficient for the production of protein immunoprecipitable by the FN18 mAb. This observation indicated that the epitope recognized by the FN18 mAb resided on the CD3ε chain. It is not clear at present why the γ chain was missing when the cell extract from cells transfected with three components of the CD3 was immunoprecipitated with FN18 mAb. It seems possible, however, that the affinity between the δ and ε chains is stronger than that between the γ and δ or ε chains, and that the formation of the heterodimer between the δ and ε chain further reduce the binding affinity of the γ chain to two other chains. The possible reduction in the affinity might result in dissociation of the δ chain from the complex in the presence of the detergent in RIPA buffer. The above system was used to determine which one of the amino acids at positions 67 and 72 was responsible for the formation of the FN18 epitope. The protein expressed from the $p\varepsilon E_{67}$-G having glycine at amino acid position 67 instead of glutamic acid, lost the reactivity with the FN18 mAb, while the protein with arginine at position 72 expressed from the $p\varepsilon R_{72}$-Q was recognized by FN18 mAb. It is quite likely that the amino acid at position 67 played a key role in determining whether the epitope could be recognized by the FN18 mAb. IFA and FACS analyses also indicated the importance of the amino acid at position 67 in the recognition of the molecule by FN18 mAb. As a trace amount of the CD3ε was detected upon transfection of $p\varepsilon E_{67}$-G, possibility that an amino acid at position 72 might influence the formation of the FN18 epitope cloud not be completely excluded.

The biological significance of CD3 polymorphism is not clear yet. There are several reports suggesting that CD3 polymorphism might be

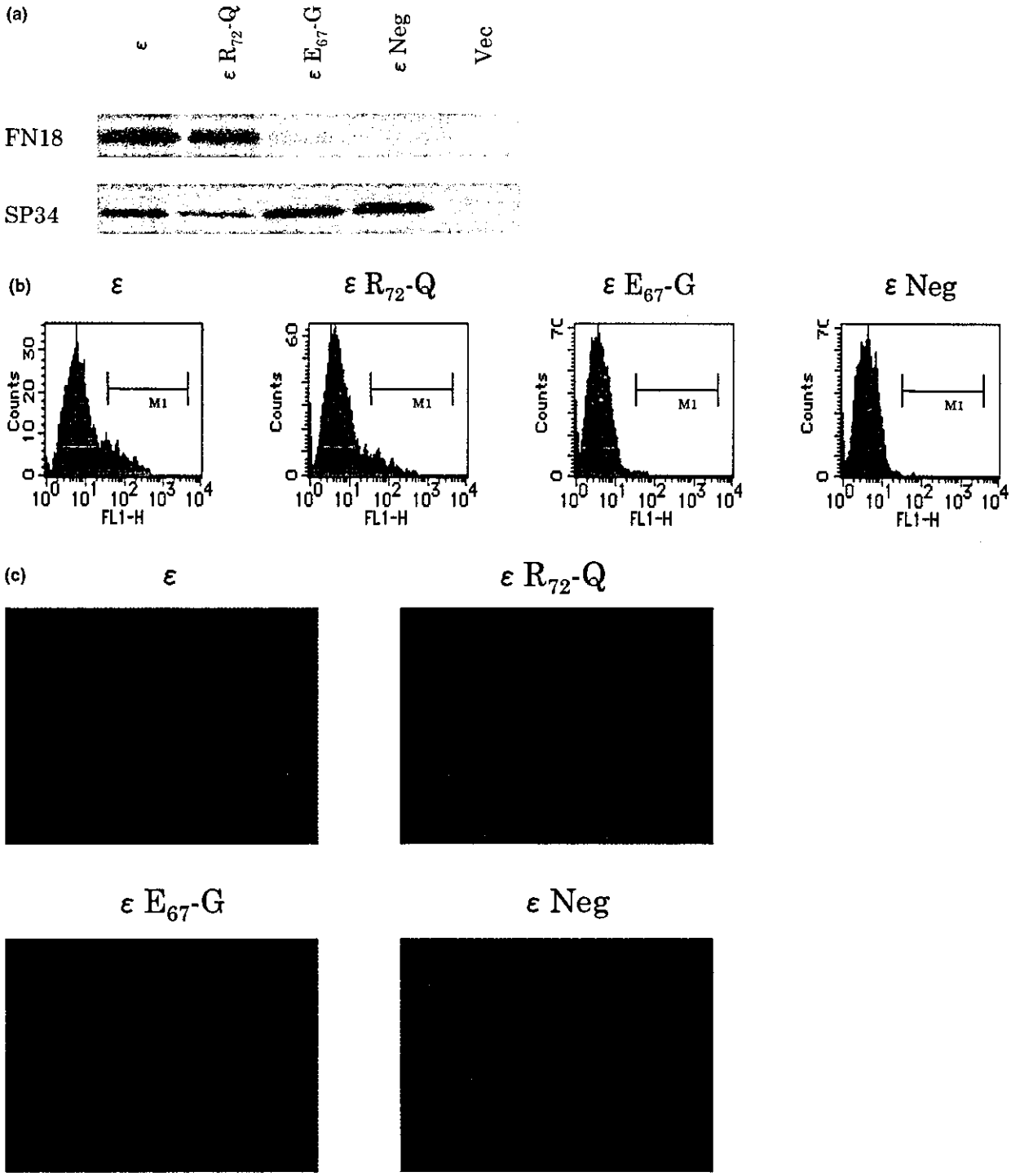

Fig. 3. Reactivity of mutant CD3ε to FN18 mAb analyzed by Radio immunoprecipitation assay (RIPA) (A), Fluorescence-activated cell sorter (FACS) (B), and Immunofluorescent assay (IFA) (C). (A) The cell extracts from COS7 cells transfected with pcDNAε, pεE_{67}-G, pεR_{72}-Q, or pεNeg, were immunoprecipitated with FN18 or SP34 mAb as mentioned in the legend of Fig. 2. (B) COS7 cells transfected on the respective CD3ε mutant plasmids together with both pcDNAδ and γ were incubated with flourescent isothiocyanate (FITC)-conjugated FN18 mAb, and then analyzed by FACS. FL1-H indicates fluorescence intensity of FITC. (C) COS7 cells transfected on the same conditions as FACS analysis were fixed by acetone, incubated with FITC-conjugated FN18 mAb, and observed under the UV-microscope.

related to type I diabetes in human [8, 9, 11]. According to previous studies [3, 10], lymphocytes from FN18-negative monkeys did not show any immunologic abnormality as far as they were examined. It will be of interest to look for any linkage between the CD3 polymorphism and the prevalence of certain diseases recorded for cynomolgus monkeys kept in our facility.

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Modification of the Leukapheresis Procedure for Use in Rhesus Monkeys (*Macaca mulata*)

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One of the most serious problems in applying leukapheresis to human infants is the large extracorporeal blood volume (ECV), resulting in substantial loss of platelets and red blood cells (RBCs). In this study, we developed a safe and effective modified procedure to collect peripheral blood stem cells (PBSCs) from rhesus monkeys (*Macaca mulata*) using a Baxter CS3000+ Blood Cell Separator (Baxter, Deerfield, IL) with several devices that reduced chamber size and shortened the standard apheresis kit to decrease ECV from 130 to 70 ml. Pump speed was controlled by monitoring hematocrit values and platelet counts during leukapheresis. This system makes it possible to perform safe and effective leukapheresis in rhesus monkeys whose body weight is similar to that of human infants. A total of 12 leukapheresis procedures were performed in nine monkeys and resulted in the collection of sufficient numbers of white blood cells (mean, 1.38×10^9 cells/kg), $CD34^+$ cells (mean, 17.80×10^6 cells/kg), mononuclear cells (mean, 3.67×10^8 cells/kg), and colony forming units (mean, 75.02×10^6 cells/kg) in all cases. In addition, no complications, such as anemia or trombocytopenia, occurred after leukapheresis. This modified leukapheresis procedure will be useful to test new approaches in gene therapy, perform organ transplantation using nonhuman primates, and collect PBSCs from human infants in a noninvasive manner. Our nonhuman primate model provides an important framework for such future clinical studies. J. Clin. Apheresis 18:26–31, 2003.

Key words: PBSCs; extracorporeal blood volume; nonhuman primates

INTRODUCTION

Peripheral blood stem cell (PBSC) transplantation has been increasingly used clinically as an alternative to allogeneic bone marrow transplantation [1,2]. The PBSCs and bone marrow (BM) stem cells are prominent targets for stem cell gene therapy [3–6] and recently have been used for induction of immune tolerance in organ transplantations [7–10]. Although numerous clinical trials have demonstrated the safety and effectiveness of leukapheresis for harvesting PBSCs from adults [11] and children [12–14], only one limited trial in human newborn babies has been reported [15]. Clinical trials are lacking partly because there is limited applicability of leukapheresis to the neonatal condition. One of the most serious problems when applying leukapheresis to a human newborn is the large amount of extracorporeal blood volume (ECV) that causes a substantial loss of platelets and red blood cells (RBCs).

The close phylogenetic relationship of macaque monkeys to humans has resulted in their widespread use as a preclinical model for BM transplantation and hematopoietic stem cell (HSC) gene therapy [3,5,6]. The safety and therapeutic efficacy of PBSCs and BM transplantation have been evaluated prior to human trials using monkey models. Since a large ECV frequently causes severe anemia and trombocytopenia and a rapid reduction in hematocrit (Ht) level during leukapheresis, which poses a risk for the patient, it is necessary to evaluate the safety and efficacy of leukapheresis using nonhuman primates as a model for human infants.

In this study, we modified a standard procedure for the collection of PBSCs from rhesus monkeys (*Macaca mulata*) using a blood cell separator (Fenwall CS3000+; Baxter, Deerfield, IL) adapted for a

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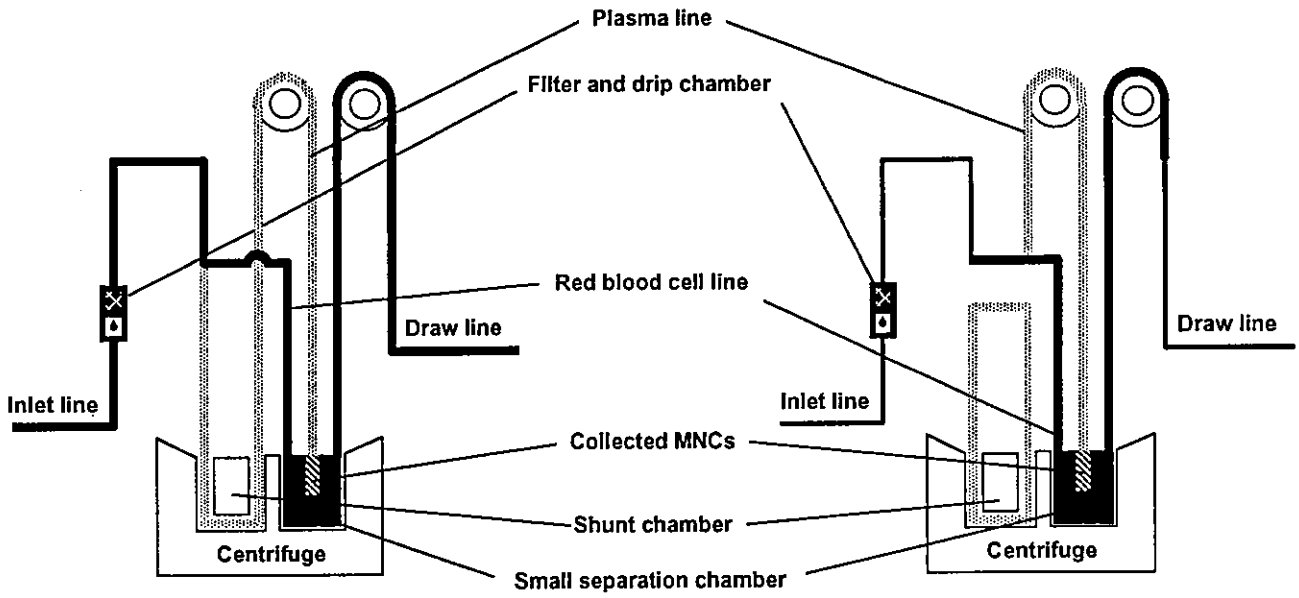

Fig. 1. Diagram of apheresis kit before and after modification. Modifications included the use of a small S25A separation chamber and the shortening of the standard apheresis kit. The plasma line (shadow line) was cut and connected under sterile conditions to the red blood cell line (solid line) using a polypropylene tubing connector. The plasma flowed directly into the inlet line without passing through the shunt chamber. Inlet and draw lines were exchanged for thin lines (2.5 mm in diameter) to reduce extracorporeal blood volume as much as possible. This modification made it possible to reduce the volume of extracorporeal circulation from 130 to 70 ml.

small chamber and short apheresis kit as shown in Figure 1. This modified procedure made it possible to reduce ECV and perform safe and effective leukapheresis with monkeys whose body weight is similar to that of human infants. This study provides information on the modified leukapheresis process for those investigators who want to perform leukapheresis in neonates. This nonhuman primate model will be useful to test new approaches for PBSCs transplantation, HSC gene therapy, and organ transplantation.

MATERIALS AND METHODS

A total of 12 leukapheresis procedures were performed in nine male rhesus monkeys, aged 3 to 6 years, with a mass of 4.2 to 8.5 kg, which is a range that includes the mass of many human newborns. Three of the monkeys (nos. 099036, 000033, and 099030) underwent leukapheresis twice over two consecutive days.

Animals and Husbandry

All of the rhesus monkeys were imported from China (Shin-nihon-kagaku, Tokyo, Japan) and were free of intestinal parasites, and herpes-B and simian varicella viruses. The animals were quarantined for 5 weeks and then kept in the Tsukuba Primate Center (TPC) of the National Institute of Infectious Diseases. They were housed individually in stainless steel cages at a temperature of 23–27°C and humidity level between 50 and 70%. There were 12 air changes per hour in the room and the light/dark cycle was 12 h/12 h. Each day, the animals were fed 70 g commercial monkey chow (Type AS; Oriental Yeast, Chiba, Japan), 200 g of fruit, and tap water ad libitum. They were apparently healthy and showed no abnormal sign in a periodic health check.

This study was strictly subject to the Rules for Animal Care and Management of TPC [16] and the Guiding Principles for Animal Experiments Using Nonhuman Primates formulated by the Primate Society of Japan [17]. The protocol of the experimental procedure was approved by the National Institute of Infectious Diseases (Tokyo, Japan).

Preparative Regimen

Fifty micrograms/kilogram of recombinant human granulocyte colony-stimulating factor (G-CSF; lenograstim: Chugai, Tokyo, Japan) were subcutaneously administered to animals daily for 5 days prior to leukapheresis. For autologous blood donation,

animals received 150 IU/kg recombinant human erythropoietin (EPO; EPOGIN: Chugai) subcutaneously three times a week during the 2 weeks preceding leukapheresis. Autologous blood (20–25 ml) was collected once a week and saline was infused for volume replacement. A total of 60–75 ml of peripheral blood was obtained from each animal and stored in a bag containing anticoagulant (acid-citrate dextrose) at 4°C prior to use for priming the apheresis kit [18]. To provide vascular access for leukapheresis, the right or left femoral artery was cannulated with a 19-gauge catheter. The saphenous vein was catheterized with a 19-gauge intracath. This cannulation was performed under general anesthesia by administration of ketamine hydrochloride (Ketalar; Sankyo, Tokyo, Japan) and xylazine hydrochloride (Seraktar; Bayer, Leverkusen, Germany). Animals received a course of 0.5 mg/kg butorphanol tartrate intramuscularly for 3 days to alleviate any postoperative pain.

Leukapheresis Procedure

The leukapheresis protocol was a modification of the procedure originally developed by Donahue et al. [3]. All procedures were performed under general anesthesia (A.D.S.1000; Shin-ei, Tokyo, Japan) with isoflurane gas. Collection was accomplished using a small S25A separation chamber and a shunt chamber in place of a standard collection chamber. For the purpose of reducing extracorporeal blood volume, the plasma line of a standard apheresis kit was cut and connected under sterile conditions to the red blood cell line using a polypropylene tubing connector (Iuchi, Osaka, Japan) (Fig. 1). The plasma flowed directly into the inlet line without passing through the shunt chamber. In addition, the inlet and draw lines were also exchanged with thin lines (extension tube: 70 cm, 1.4 ml, 2.5 mm diameter; TOP, Tokyo, Japan) to reduce extracorporeal blood volume as much as possible. A blood component inlet set with a 170-μm filter and drip chamber was sterilely connected to the packed red blood cell line using a polypropylene tubing connector (Iuchi). The inlet line was connected to a catheter (Terumo, Tokyo, Japan) that was placed in the saphenous vein of the animal. Hemostats were placed on the unused return line and acid-citrate-dextrose (ACD) line. The apheresis kit was primed with autologous blood that had been collected 3 weeks before leukapheresis. The animal received a dose of 100 U/kg heparin and the draw line was connected to the catheter in the femoral artery immediately before starting the procedure. Blood was processed at the rate of 10–12 ml/min for a total of two to three times the total blood volume of the animal. When the processed blood volume reached 50 ml/kg, a 1-ml blood sample was collected via draw line and the Ht value and platelet count were monitored throughout the procedure for manual control of plasma pump speed [19]. The plasma pump speed was increased when a decrease in Ht value was observed. Conversely, plasma pump speed was decreased when Ht increased. In addition, when an increase in Ht value was observed, saline was infused into the inlet line for volume replacement to prevent blood pressure fluctuations. After the procedure was completed, the remaining cells in the apheresis kit were recovered and used either to prime the blood cell separator for future leukapheresis or to reinfuse into the treated animal. Immediately after leukapheresis, the animals were given an appropriate dose of protamine sulfate.

Analyses of Leukapheresis Products

The leukapheresis products were collected in a small S25A separation chamber. The product (40–45 ml) was collected and mixed with 7 ml of ACD. The numbers of recovered white blood cells (WBCs) and mononuclear cells (MNCs) were counted with a Sysmex K-4500 instrument (Toa-iyoudenshi, Kobe, Japan). The blood cells were collected after centrifugation at 1,200 rpm for 10 min and treated with ammonium chloride for lysis of erythrocytes. The $CD34^+$ cells were then isolated as a fraction containing HSCs with immunomagnetic beads (Dynal, Lake Success, NY) conjugated to a monoclonal antibody (clone 561) that reacts to both human and monkey CD34 [20,21]. CD34 is a cell-surface marker of undifferentiated HSCs, and $CD34^+$ cell transplantation is widely performed in patients with cancer or other disorders [22]. The purity of the $CD34^+$ cells obtained using this technique ranged from 90 to 95%, a value similar to that reported by other investigators [23]. Progenitor cell enrichment was assessed from colony-forming progenitor assays performed before enrichment. Cells were suspended in α-minimum essential medium (MEM; Gibco) containing 1.2% methylcellulose (Shinetsu Kagaku, Tokyo, Japan) supplemented with 2 U/ml recombinant human erythropoietin (EPO; Roche Diagnostics, Mannheim, Germany), 100 ng/ml recombinant human interleukin-3 (PeproTech, London, U.K.), 100 ng/ml interleukin-11 (IL-11; PeproTech), 100 ng/ml recombinant human stem cell factor (SCF; Biosource, Camarillo, CA), 20% fetal calf serum (FCS; Intergen, Purchase, NY), 1% bovine serum albumin (Sigma, St. Louis, MO), $5 \times 10\text{-}5$M 2-mercaptethanol (Sigma), and antibiotics (100 U/ml penicillin, Banyu, and 0.1 mg/ml streptomycin, Meiji Seika). On day 14 of the culture, colonies containing more than 50 cells were counted as colony-forming units (CFUs). The total numbers of WBCs, MNCs, $CD34^+$ cells, and

TABLE I. Characteristics of Rhesus Monkeys Subjected in Leukapheresis

			Processed blood		Harvested cells			
Animal ID	Body weight (kg)	Estimated total blood volume (ml)[a]	Total volume (ml)	ml/kg	Total nucleated cells ($\times 10^9$/kg)	MNCs ($\times 10^8$/kg)	$CD34^+$ cells ($\times 10^6$/kg)	CFU ($\times 10^6$/kg)
099033	4.9	310	1,000	200	2.99	10.45	27.17	—
099038	4.9	300	1,000	210	1.41	10.17	26.44	98.97
000026	7.5	420	1,600	210	1.12	1.90	—	—
000030	7.3	410	1,600	220	0.94	1.69	—	—
000031	8.5	470	1,300	150	0.47	1.66	—	—
000029	6.5	380	1,000	150	2.21	1.46	—	—
099036[b]	4.5	290	1,000	220	1.68	0.55	1.44	14.44
099036[b]	4.2	270	900	210	1.29	6.22	16.16	247.96
000033[b]	7.4	420	1,150	150	0.83	2.10	—	9.57
000033[b]	7.4	420	1,150	150	0.75	1.13	—	4.15
099030[b]	4.3	400	1,050	150	0.89	3.78	—	13.75
099030[b]	4.3	400	1,100	160	1.61	4.06	—	7.19
Average	6.5	380	1,200	180	1.38	3.67	17.80	75.02

[a]The total blood volume was estimated by the following formula: Y (total blood volume) = 44.07 × (body weight) + 90.25 [Ref. 26].
[b]Leukapheresis was performed in these three animals on two consecutive days.

CFUs in the apheresis products were calculated by multiplying the percentage of lymphocytes, $CD34^+$ cells, and CFUs by the total blood cell count in the leukapheresis products.

RESULTS

We administered G-CSF to monkeys for 5 days prior to leukapheresis. Administration of G-CSF increased peripheral WBC counts to 42,000 (24,000–66,000) cells/ml on the average, and did not produce any adverse effects such as fever or anorexia. After the 5-day administration of G-CSF, leukapheresis was performed.

It is difficult to perform leukapheresis and autologous blood donation in small animals and human infants because of the large ECV involved [24,25]. Modification of the leukapheresis procedure involved installing a small chamber and shortening the extracorporeal blood line in a standard apheresis kit, which made it possible to reduce ECV from 130 to 70 ml. The amount of autologous blood needed is only 60–75 ml, and can be collected safely without any adverse effects for the donor. Before using this modified apheresis kit in monkeys, we circulated the pooled blood to determine the correlation between tubing length and ECV in vitro, and demonstrated that ECV can be reduced safely when shortening the tube.

The presence of platelets and RBCs in the leukapheresis products was observed when the equipment was operated in automatic mode. However, manual adjustment of plasma pump speed by monitoring Ht values and platelet counts during apheresis effectively prevented the overdraw of extracorporeal blood. Saline was infused into the inlet line for volume replacement when an increase in Ht value was observed. Leukapheresis was performed safely without any adverse effects such as blood pressure reduction, and sufficient numbers of $CD34^+$ cells, MNCs, and CFUs could be collected. Leukapheresis was performed safely and efficiently on all nine monkeys weighing 4.2 to 8.5 kg, a range similar to that of human infants. As shown in Table I, mean processed blood volume was 1,200 ml (180 ml/kg), which was approximately three times the estimated total blood volume of an individual animal [26].

No complications, such as severe anemia or trombocytopenia, developed in the three monkeys that underwent leukapheresis on two consecutive days. Serial changes in circulating WBC counts, Ht levels, and platelet counts were monitored throughout the leukapheresis. A transient drop in Ht occurred during leukapheresis, but a blood transfusion was not necessary. Platelet counts also fell briefly during the process but did not require treatment. In contrast, WBC counts decreased to below the target value in all monkeys before leukapheresis was completed.

No viral or fungal organisms were isolated from culture of the leukapheresis products after cutting and sterile docking of the tube, and no microbial contamination occurred in colony-forming progenitor assays. In addition, we performed successful transplantations using these leukapheresis products without any complications such as the development of infectious disease (data not shown).

DISCUSSION

No problems were encountered in the animals or with the function of the blood cell separator throughout the process of leukapheresis in rhesus

monkeys. We succeeded in reducing ECV, but still needed autologous blood for priming. However, reduction of the ECV allowed priming with autologous blood in these nonhuman primates. Therefore, the modified procedure enables safe and effective leukapheresis in nonhuman primates and other small animals. Sufficient numbers of $CD34^+$ cells, MNCs, and CFUs could be collected for stem cell transplantation as shown in Table I. In addition, an adequate number of cells were also collected from monkeys (Monkey nos. 099036, 000033, and 099030) that underwent leukapheresis on two consecutive days.

This is the first report of a leukapheresis procedure with a blood cell separator modified for use in nonhuman primates. The results clearly indicate that this modification enables the safe and effective application of the blood cell separator for leukapheresis in rhesus monkeys. Although the minimum blood volume (150 ml/kg) was processed in leukapheresis, sufficient numbers of cells were harvested without complications in either the animals or the system. Since processing time was short, the procedure was less invasive. Decreases in platelet counts and Ht levels after leukapheresis were minimal in all animals, indicating that this procedure allows the collection of PBSCs with negligible contamination of platelets and RBCs in the apheresis products. The decrease in Ht value observed after leukapheresis was a complication of the cannulation operation, but did not necessitate treatment with a transfusion. Also, Ht and platelet counts fell transiently during leukapheresis but a blood transfusion was not necessary.

Although clinical trials in humans using this modified procedure would be difficult, it is clear that our modified procedure makes it possible to perform safe and effective leukapheresis in nonhuman primates or other small animals. This animal model will be useful for testing new approaches to PBSC transplantation, HSC gene therapy, and organ transplantation. Despite the need to modify the tube each time in a clinical setting, we believe this modification will be helpful to researchers who may need a noninvasive method of collecting PBSCs from human newborn infants [27,28]. We are now applying this modified leukapheresis procedure to juvenile cynomolgus monkeys (*Macaca fascicularis*) that weighed approximately 2 kg, a mass equivalent to that of a human newborn infant. Our nonhuman primate model will provide an important framework for such future clinical studies.

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Possible Role of Genetic Factor(s) on Age-related Increase of Peripheral CD4⁺CD8⁺ Double Positive T Cells in Cynomolgus Monkeys

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Abstract: *Mature TCR αβ T cells in peripheral blood are generally classified into either CD4 single positive (sp) T cells or CD8sp T cells. Several studies demonstrated that considerable amounts of CD4⁺CD8⁺ double positive (DP) T cells exist in peripheral blood of human and several animals. In particular, we previously reported that peripheral DP T cells increase in an age-related manner in cynomolgus monkeys (Macaca fascicularis), but the finding that DP T cells in some aged monkeys were maintained at a low proportion (under 5%), suggests that the increase in peripheral DP T cells might be genetically controlled in cynomolgus monkeys. To test this hypothesis, 24 families were randomly selected and used in a formal genetic analysis of the proportion of DP T cells. Parents and offspring in selected families were classified into DP-High and DP-Low groups based on a 5% cutoff level of DP T cells. The cutoff value was set by analysis of the distribution of the proportion of DP T cells. Nine out of 13 offspring (69.2%) with DP-High × DP-High parents belonged to the DP-High group, whereas three out of nine offspring (33.3%) belonged to DP-High group in the case of DP-High × DP-Low mating pairs. No offspring (0%) of two offspring with DP-Low × DP-Low parents belonged to the DP-High group. In addition, heritability (h^2: narrow sense) obtained from the regression coefficient of offspring on mid-parent values was 0.54 ± 0.19. Both findings suggest that increases in DP T cells in cynomolgus monkeys may be genetically controlled.*

Key words: *cutoff value, cynomolgus monkeys (Macaca fascicularis), Double positive (DP) T cells, heritability, mid-parent values*

Introduction

Mature circulating T cells are generally divided into two subsets: CD4 single positive (sp) and CD8sp T cells. The CD4sp T cells interact with MHC class II/antigen complex and then function as helper/inducer T cells, whereas CD8sp T cells recognize antigen in combination with MHC class I and have cytotoxic functions

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[19, 28]. In addition to the two major subsets, a low proportion (under 5%) of $CD4^-CD8^-$ double negative (DN) and $CD4^+CD8^+$ double positive (DP) T cells are also observed in the peripheral blood of healthy individuals [3, 14].

A number of studies have recently reported on transient and/or persistent increases of circulating peripheral DP T cells in chicken, swine, macaque monkeys and humans [1, 5, 6–8, 10, 16, 17, 20, 24–26, 29, 30]. In contrast to the immature $CD4^+CD8\alpha\beta^+$ DP subset in the thymus [3, 14, 19, 28], these peripheral DP T cells are a mature memory T cell subset with strong cytotoxic activity and are considered to be of extrathymic T cell lineage having a distinct origin and maturation pathway due to their phenotype with $CD8\alpha\alpha$ homodimers [21, 25, 29]. The origin and functional significance of this subset has remained largely unclear.

Our previous studies demonstrated that $CD4^+CD8\alpha\alpha^+$ DP T cells in cynomolgus monkeys: 1) significantly increase at over 11 years of age; 2) have a phenotype of resting memory T cell and distribute mainly in peripheral blood and spleen but rarely in lymph nodes; and 3) have both helper and cytotoxic functions, and some of their clones share the same TCR $V\beta$ with CD4sp T cells, suggesting that they are derived from the same origin [1, 20, 21].

Interestingly, our cross-sectional data on 195 cynomolgus monkeys also showed that in some aged monkeys (over 11 years of age), the DP T cell subset was maintained at a low proportion [under 5% in the peripheral blood lymphocytes (PBL)][20]. Some investigators have reported that genetic factor(s) have an influence on the sizes of T cell subsets in humans and mice [2, 4, 13, 27]. From these findings, it occurred to us that levels of peripheral DP T cells might be genetically controlled in cynomolgus monkeys. With regard to this, it is important to note that the appearance of DP T cells in chicken was found to be an inherited dominant trait [16, 17]. Like cynomolgus monkeys, $CD4^+CD8\alpha\alpha^+$ DP T cells in chicken also increases in an age-dependent manner [8].

To examine the effect of genetic background on the increase in peripheral DP T cells, a familial study on 24 cynomolgus monkey families was carried out and heritability (h^2) was estimated.

In the present study, we report that the increase in peripheral DP T cells is genetically controlled in cynomolgus monkeys and the heritability (h^2) obtained by regression of offspring on mid-parent values is 0.54 ± 0.19 ($\pm$ standard error).

Materials and Methods

Animals

All cynomolgus monkeys used in this study were bred and reared in our indoor facilities at the Tsukuba Primate Center (TPC), National Institute of Infectious Diseases. The indoor facilities were maintained under conditions of 25 ± 2°C (temperature), 60 ± 5% (relative humidity), and 14 h light per day (from 05:00 to 19:00) [12]. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institute of Infectious Diseases.

In the present study, offspring over 11 years of age were selected because the proportion of DP T cells becomes stable after 10 years of age [20]. To select a suitable family, information on the parentage and breeding group was obtained from the database that is maintained at the TPC. The 24 selected families underwent random mating and all forty-one monkeys in these selected families were born in the TPC. There were no infectious outbreaks that they had been exposed to and all monkeys had no apparent clinical symptoms at the time of blood collection. They were anesthetized with 10 mg/kg ketamine HCl and 2 ml of heparinized blood was collected from the femoral vein at around 10:00 a.m. To establish the cutoff value for classification, data on 195 healthy cynomolgus monkeys from our previous cross-sectional study [20] was re-assessed.

Preparation of cells

The leukocytes were isolated as previously described [20]. Briefly, heparinized blood was treated with ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM of Na_2EDTA, pH 7.2) to lyse red blood cells for 5 min at 37°C. After lysis, leukocytes were washed and resuspended with RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS; Sigma, St. Louis, MO), 50 IU/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma), and 2 mM L-glutamine (Sigma)[hereafter RPMI-10%]. The leukocytes were stored at 4°C until use.

Monoclonal Antibodies (mAbs) and Flow Cytometry Analysis

Cell surface antigens were analyzed by three-color flow cytometry as previously described [1]. Typically, 2×10^5 leukocytes were stained with the following antibodies: FITC (Fluorescein isothiocyanate)-labeled anti-CD3 (clone FN18; Biosource, Camarillo, CA), phycoerythrin (PE)-labeled anti-CD4 (NU-TH/1; Nichirei, Tokyo, Japan), anti-CD8β (2ST8.5H7; Immunotech, Westbrook, ME), and R-phycoerythrin (RPE)-Cy5-labeled anti-CD8 (DK25; DAKO, Glostrup, Denmark). For negative controls, fluorchrome-labeled isotype-matched mouse Abs were used. After staining for 1 h at 4°C, leukocytes were washed with RPMI-10%, fixed with CellFIX (Becton Dickinson, Mountain View, CA), and then were kept at 4°C. The fluorescence of the stained samples was analyzed by a FACSCalibur (Becton Dickinson). Lymphocytes were gated on the forward and side scatter pattern and 10,000–20,000 events were collected. The analysis of the fluorescence intensity was performed with CellQuest software (Becton Dickinson) and markers were based on negative controls.

Statistics

The relationship between variables was tested by Student's *t*-test and differences were accepted as significant at $P<0.05$. Estimate of heritability (h^2) was obtained from the regression coefficient of offspring on mid-parent (average of the two parents) values. The slope of this regression line was deemed to be the heritability (h^2), especially, the narrow-sense heritability [9, 18, 23].

Results

Distribution of peripheral DP T cell subset in selected families

To examine whether the proportion of peripheral DP T cells is genetically controlled, we selected 24 families from our cynomolgus monkey colony. Because our previous study demonstrated that peripheral DP T cells significantly increased over 11 years of age [20], only families in which offspring were over 11 years of age were selected so the number of selected families was very limited. The DP T cell proportion in peripheral blood was measured for all members of the families by three-color flow cytometry analysis as described in materials and methods.

Similar to many traits expressed in an out-bred mating colony, such as non-human primates, the proportion of peripheral DP T cells in cynomolgus monkeys didn't show a simple discontinuous distribution. To evaluate the contribution of genetic background to the proportion of DP T cells in selected families, we first needed to set a cutoff level for classification. For this purpose, data on 195 healthy cynomolgus monkeys from our previous cross-sectional study [20] was re-plotted.

As shown in Fig. 1, the proportion of peripheral DP T cells in cynomolgus monkeys shows an obvious bimodal distribution in both males (Fig. 1A) and females (Fig. 1B). When the cutoff value was considered as about 5%, the cynomolgus monkeys tested could be classified into two populations: high and low populations. The percentages of monkeys, which belonged to the high population, were very similar between male and female monkeys (34.3% and 32.3%, respectively). These findings suggest that gender differences have no relation to the distribution of DP T cell proportions in cynomolgus monkeys.

We previously reported that the proportion of peripheral DP T cells in cynomolgus monkeys significantly increased in an age-dependent manner [20]. To examine the age-related change in distribution of peripheral DP T cell proportions, the data of the 195 monkeys shown in Fig. 1 were divided into two age groups, young (under 10 years of age) and aged (over 11 years of age) groups, and were plotted according to their peripheral DP T cell proportions. As seen in Fig. 2B, most monkeys (111/119) in the young group exhibited low DP T cell proportions (under 5% in the PBL), whereas monkeys in the aged group showed two populations similar to Fig. 1 (Fig. 2A). By setting 5% as a cutoff value, only 25% (19/76) of monkeys in the aged group belonged to the low population (under 5%). However, this bimodal distribution in the aged group was not attributable to age-related increases in DP T cells because there was no significant difference in the mean age of monkeys between the low (17.65 ± 5.48 years, n=19) and high populations (16.59 ± 4.69 years, n=57). On the basis of these results, the cutoff value of peripheral DP T cell proportion was set at 5% and all monkeys tested were classified into DP-High (DP T cells over 5%) or DP-Low (under 5%) groups by this

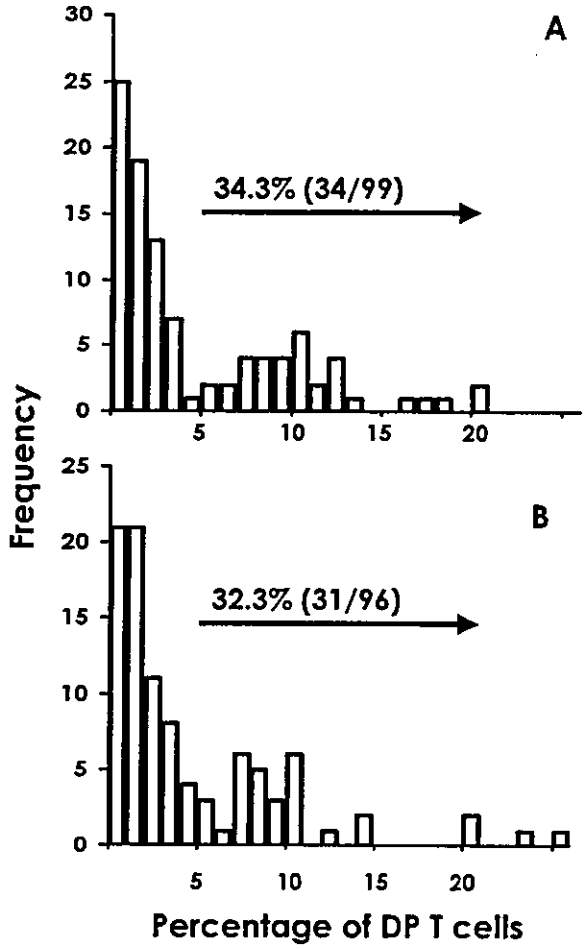

Fig. 1. Distribution of peripheral DP T cell proportions. Data on 195 healthy cynomolgus monkeys from our previous cross-sectional study was re-assessed [20]. The proportion of peripheral DP T cells showed an obvious bimodal distribution in both males (A: n=99, aged from 0 to 22 years old) and females (B: n=96, aged from 0 to 23 years old). By setting the cutoff value at 5%, cynomolgus monkeys tested were classified into two populations, DP T cell high and low populations. Arrows and percentages indicate proportions of monkeys that belonged to the high population with over 5% of DP T cells (DP-High group).

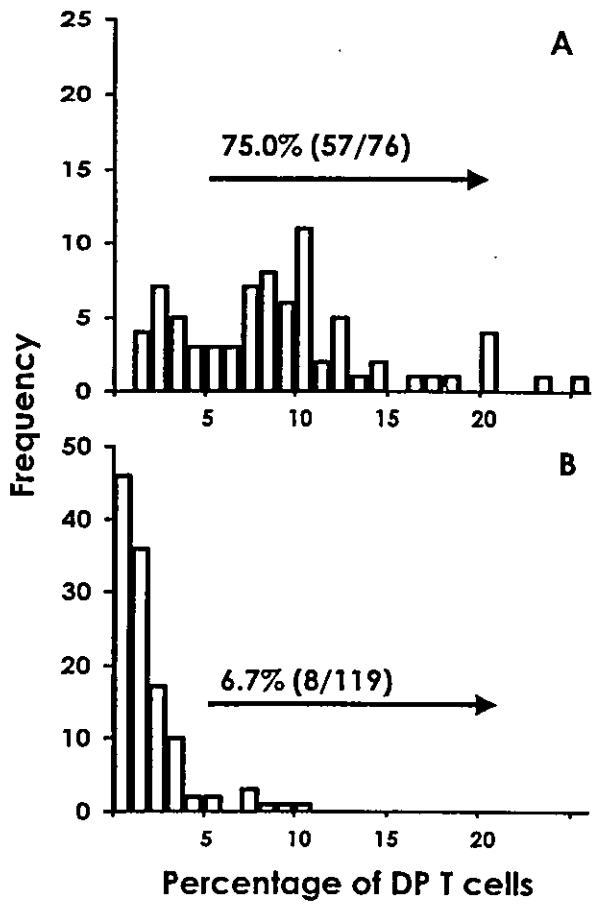

Fig. 2. Distribution of peripheral DP T cell proportions in young and aged monkey groups. The monkey data shown in Fig. 1 were divided into two groups according to age, young (under 10 years of age) and aged (over 11 years of age) groups. In the aged group (A, n=76), by setting 5% as cutoff value, only 25% (19/76) monkeys belonged to the population in which peripheral DP T cells were under 5% (DP-Low group). No significant difference in age was observed between the two populations, DP T cell high and low populations, in the aged group. As shown in Fig. 2B, most young monkeys (93.3%; 111/119) belonged to the DP-Low group (under 5%). The proportion and number of monkeys belonging to the DP-High group is also shown.

cutoff value.

Next, the familial study was performed by comparing DP T cell proportions between parents and offspring in the 24 families selected. Consistent with published reports [1], most of increased DP T cells in this familial study exhibited $CD4^{+}CD8^{dim}$ phenotype and multicolor flow cytometry analysis clearly showed that they exclusively expressed the $CD8\alpha\alpha$ homodimer, but not the $CD8\alpha\beta$ heterodimer (data not shown). Figure 3 shows the distribution of peripheral DP T cells in the offspring of 24 families in which parents were classified into DP-High or DP-Low group by the 5% cutoff value. In the case of families with DP-High × DP-High parents (Fig. 3A), 69% of offspring (9/13) belonged to the DP-High group, whereas only 33% offspring (3/9) (Fig. 3B) and none (0/2) (Fig. 3C) from DP High ×

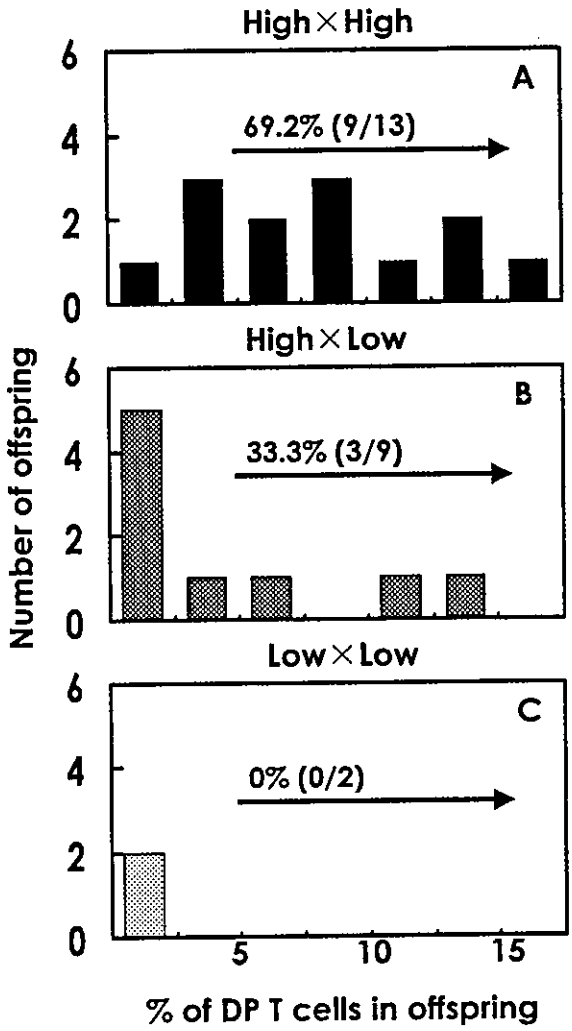

Fig. 3. Distribution in proportions of peripheral DP T cells in offspring of 24 families. According to the proportion of DP T cells, the offspring were divided into two populations, DP-High and DP-Low groups. DP-High × DP-High (A), DP-High × DP-Low (B), and DP-Low × DP-Low (C) indicate the DP T cell proportions in parents. The proportion and number of offspring belonging to DP-High group are shown.

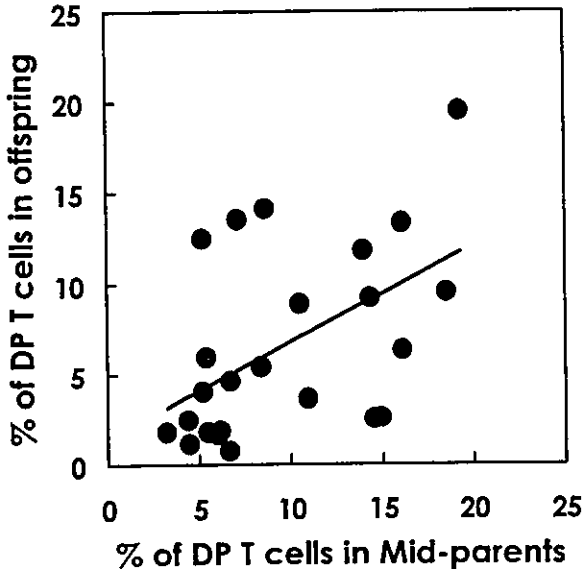

Fig. 4. Regression of offspring on mid-parent values for the proportion of peripheral DP T cells in 24 selected families. Mid-parent values in the x-axis signify the mean value of proportion of DP T cells of the two parents. The slope of this regression line estimates the heritability (h^2), especially, the narrow-sense heritability and has the value: $h^2=0.54 \pm 0.19$ (± standard error).

DP-Low parents and DP-Low × DP-Low parents, respectively, belonged to the DP-High group.

Heritability

Many traits in out-bred populations, such as humans and monkeys, are controlled by single or multiple genes. However, environmental factors also frequently influence them [9, 18, 23]. Therefore, these traits show quantitative or continuous properties rather than qualitative or discontinuous ones [2, 23]. To analyze the relative contributions of genes and environment to a variation in a specific trait, the concept of heritability is generally used. Particularly, narrow-sense heritability (h^2) indicates the proportion of observed phenotypic variation that is attributable to additive or average genetic effects [2, 9, 18, 23].

Figure 4 represents a regression plot of the offspring phenotype and mid-parent phenotype (average of the two parents). In this plot, the magnitude of heritability is defined as the slope of the regression line. The heritability in the present study, analyzed by parent-offspring regression, was estimated as 0.54 ± 0.19.

Discussion

Contrary to current immunological dogma stating that mature T cells are generally classified into CD4sp or CD8sp T cells [19, 28], evidence is accumulating of transient and/or persistent increases in peripheral DP T cells in humans and several species of animals [1, 5, 6–8, 10, 16, 17, 20, 24–26, 29, 30]. Although the origin and functional significance of this population subset

are controversial, a number of studies on human DP T cells suggest that the CD8 molecule, particularly the CD8α chain, is expressed on the surface of activated memory CD4sp T cells, rarely *vice versa*, and subsequently the $CD4^+CD8^+$ phenotype is maintained permanently in certain physiological circumstances such as chronic viral infection, autoimmune diseases, leukemia and lymphoma, and even in apparently healthy individuals [5, 10, 15, 24–26].

Compared with the frequency of the DP T cell subset in humans, considerable proportions of peripheral DP T cells were observed in chicken, swine, and macaque monkeys [1, 6–8, 16, 17, 20, 29, 30]. In particular, features of DP T cells in cynomolgus monkeys are very similar to those in humans, that is, peripheral DP T cells in the two species: 1) increase in older individuals [1, 5, 15, 20]; 2) exhibit a $CD4^+CD8\alpha\alpha^+$ phenotype known as a characteristic of extrathymic T cell lineage [1, 20, 25]; 3) provide help for B cell differentiation and have strong cytolytic activity [21, 22, 25, 29]; and 4) some of their clones may be derived from CD4sp T cells by clonal expansion [5, 21, 25]. Therefore, research on peripheral DP T cells in monkeys may have implications of biological significance for human DP T cells.

In a previous cross-sectional study, we described that in the process of age-dependent remodeling of peripheral DP T cells in cynomolgus monkeys, individual differences in proportions of this subset became broader. Furthermore, some aged monkeys (over 11 years of age) still had a DP T cell subset with a low proportion (under 5%) [20]. Because all monkeys tested in this cross-sectional study were born and reared in the same indoor facility, it is reasonable to assume that age-related individual differences in DP T cell proportion are attributable to factor(s), other than environmental factors. With regard to this, it should be noted that the appearance of DP T cells in chicken was found to be an inherited dominant trait [16, 17]. These findings led us to examine whether the increase in peripheral DP T cells is genetically controlled in cynomolgus monkeys.

As shown in Figs. 1 and 2, re-assessment of data published in our previous cross-sectional study allowed us to decide 5% as the proportion of DP T cells for the cutoff value for classification. At this cutoff value, the proportion of DP T cells was characterized by a bimodal distribution comprised of DP-High (over 5%) and DP-Low (under 5%) groups. Furthermore, the 3:1 ratio of DP-High to DP-Low group in the aged group (Fig. 2A) suggested that the increase in peripheral DP T cells might be simply controlled by one locus system with two alleles in which dominance is complete. In many cases, the pattern of genetic transmission, such as polygene, single-gene recessive or dominant and co-dominant effects, can be demonstrated by complex segregation analysis [2, 4]. Although the number of selected families in the present study was small, familial studies suggest a genetic transmission pattern. It is clear Fig. 3 that the proportion of DP T cells in offspring was affected by genetic factor(s), and the fact that there were no offspring with over 5% of peripheral blood DP T cells from DP-Low × DP-Low parent mating pairs, suggests that allele(s) contributing to the DP-Low phenotype are recessive. Additionally, a high level of heritability ($h^2=0.54 \pm 0.19$), as shown in Fig. 4, supports the conclusion that the DP T cell proportion is affected by genetic factor(s).

In the present study, this genetic control is unlikely to be caused by modulation and/or block of CD8α chain expression on memory CD4sp T cells after activation due to normal CD8α chain expression on CD8sp T cells. It is more easily conceived that this finding is related to certain MHC types associated with resistance and susceptibility to infectious agents that may become recall antigen to DP T cells.

Several studies reported genetic control on the proportions of T cell subsets in humans, inbred mice, and chickens [2, 4, 11, 13, 16, 17, 27]. In particular, it was evident from statistical analysis based on the large-scale familial studies that the numbers of CD4 and CD8 T cells, and consequent CD4/CD8 ratio, were controlled by an autosomal recessive gene in humans [2, 4]. By accumulating a number of proper families, the pattern of genetic transmission of DP T cell phenotype can be made clear in cynomolgus monkeys.

This is the first report to describe the genetic factor(s) affecting the increase of peripheral DP T cell proportions in cynomolgus monkeys. We propose that the proportion of peripheral DP T cells is genetically controlled with high heritability [h^2: 0.54 ± 0.19 (± standard error)]. Considering the similarity of characteristics between human and monkey DP T cells, it is possible that increase of peripheral DP T cells in humans is also under genetic control.

Acknowledgments

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