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MHC (Major Histocompatibility Complex)-DRB Genes and Polymorphisms in Common Marmoset

Ming-Shiuan Wu,[1] Kenzaburo Tani,[1] Hajime Sugiyama,[1] Hitoshi Hibino,[1] Kiyoko Izawa,[1] Tsuyoshi Tanabe,[1] Yukoh Nakazaki,[1] Hajime Ishii,[4] Jun Ohashi,[2] Hirohiko Hohjoh,[2] Tooru Iseki,[1] Arinobu Tojo,[1] Yusuke Nakamura,[3] Yoshikuni Tanioka,[4] Katsushi Tokunaga,[2] Shigetaka Asano[1]

[1] Division of Molecular Therapy, Advanced Clinical Research Center (ACRC), The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
[2] Department of Human Genetics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan
[3] Human Genome Center, The Institute of Medical Science, The University of Tokyo, Tokyo Japan
[4] Division of Animal Experimentation, Central Institute for Experimental Animals, Kawasaki, Japan

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Abstract. A New World monkey, the common marmoset (*Callithrix jacchus*), will be used as a preclinical animal model to study the feasibility of cell and gene therapy targeting immunological and hematological disorders. For elucidating the immunogenetic background of common marmoset to further studies, in the present study, polymorphisms of MHC-DRB genes in this species were examined. Twenty-one Caja-DRB exon 2 alleles, including seven new ones, were detected by means of subcloning and the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) methods followed by nucleotide sequencing. Based on the alignment of these allele sequences, we designed two pairs of specific primers and established a PCR-SSCP method for DNA-based histocompatibility typing of the common marmoset. According to the family segregation data and phylogenetic analyses, we presumed that Caja-DRB alleles could be classified into five different loci. Southern blotting analysis also supported the existence of multiple DRB loci. The patterns of nucleotide substitutions suggests that positive selection operates in the antigen-recognition sites of Caja-DRB genes.

Key words: Common marmoset, *Callithrix jacchus* — New World monkey — DNA typing — PCR-SSCP — Preclinical animal model — Stem cell transplantation

Introduction

In the development of new drugs including gene therapy vectors, preclinical animals that have similar in vivo conditions to humans are considered to be very helpful. The common marmoset (*Callithrix jacchus*) was officially classified in the *Callitrichidae* family of *Platyrrini* (New World monkey), which has provided animal models for several research fields, such as gene therapy, autoimmune disease, infections, pharmacological study, and organ transplantation (Hibino et al. 1999; Genain et al. 1996; Duboise et al. 1998; Nomoto and Fukuda 1993; Annett et al. 1997). Of particular relevance is that this kind of nonhuman primate is expected to have similar biology to humans, encompassing, for instance, similar reactions to various cytokines and immunoregulatory cells (Hibino et al. 1999; Ryffel et al. 1994; Schmidt et al. 1996; Neubert et al. 1996).

It has been well known that both rejection and graft versus host disease are manifestations of immunological alloreactivities between donor and recipient in the course of organ or stem cell transplantation. In many clinical

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Correspondence to: K. Tani

evaluations, the differences of major histocompatibility antigens associate with post-transplant complications and also with compromised therapeutic results (Burlingham et al. 1998; Sasazuki et al. 1998). To select the most appropriate allogeneic donors for stem cell transplantation, DNA molecular technology has been recently utilized to determine the genotypes of major histocompatibility antigens with relative ease. The introduction of the polymerase chain reaction (PCR) technique into the field of MHC typing has accelerated the identification of more accurate information than the established technique of serologic typing in humans (Kawai et al. 1996; Bannai et al. 1994).

The principal function of the gene products of the major histocompatibility complex (MHC) is to bind an enormous array of possible peptide fragments derived from processed antigens so that T lymphocytes can recognize them optimally. As might be expected from proteins suited for such dynamic function, the hallmarks of MHC antigens are their high degrees of polymorphisms and structural diversity. The MHC antigens can be divided into two groups. Class I molecules consist of one heavy and one associated polypeptide chain, β_2-microglobulin. Class II molecules consist of two polypeptide chains, α-β heterodimers. Products of class I genes are found on all nucleated cells and present the antigenic peptides to cytotoxic T cells (CD 8^+ T cell). In contrast, the products of class II genes are expressed on the surface of activated T cells, B cells, macrophages, and dendritic cells (in short, on cells directly involved with immune function) and are involved in the presentation of antigens to helper T cells (CD 4^+ T cell).

In the human leukocyte antigen (HLA) system, the HLA-DR region contains nine DRB loci (designated as HLA-DRB1 to -DRB9) and a substantially monomorphic DRA locus. Among the HLA-DRB loci, four are functional (-DRB1, -DRB3, -DRB4, and -DRB5) and five are considered to be pseudogenes (-DRB2, -DRB6, -DRB7, -DRB8, and -DRB9). Human DRB haplotypes have been classified into five major groups: HLA-DR1, -DR8, -DR51, -DR52, and -DR53; these individual groups carry different sets of DRB genes (Bergström et al. 1999). In contrast, it is not well known how many different haplotypes exist in nonhuman primates (Klein et al. 1997). A high degree of allelic polymorphisms is known to exist in the second exon of DRB genes both in human and nonhuman primates (Bodmer et al. 1996; O'hUigin 1997).

In the common marmoset, exon 2 of MHC-DR and -DQ genes was characterized recently by nucleotide sequence analysis (Antunes et al. 1998). The results demonstrated that the Caja-DR and -DQ genes were apparently functional, but the Caja-DP region was inactivated. Six loci of the MHC class II molecule, Caja-DQA1, -DQB1, -DQB2, -DRB1*03, -DRB*W12, and -DRB*W16, were proposed in the common marmoset.

To facilitate the introductions of common marmosets, which have been maintained in Japan, into preclinical studies of new drugs or gene therapy vectors for allogeneic hematopoietic stem cell transplantation or immunological disorders, the characterization of MHC class II DRB genes and their polymorphisms was performed. Based on these findings, we established a stable and precise procedures of PCR-based single strand conformation polymorphism (SSCP) analysis and direct DNA sequencing to determine the specific DRB1 genotypes.

Materials and Methods

Animals and DNA Samples. The animals used in this study were housed at the Division of Animal Experimentation, Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan. These monkeys originated from separately imported populations from Brazil, Britain (H series), and the United States (I series) and have been colonized in this facility for more than 20 years.

Peripheral blood samples were obtained from more than 35 marmosets. Genomic DNA was extracted by using a DNA isolation kit according to the manufacturer's protocol (MicroTurbo Gen, Invitrogen, San Diego, CA). Some genomic DNA samples were isolated from tissues or from cell lines derived from a tissue, using basic enzymatic preparation methods. Briefly, cells or minced tissue samples were suspended in the digestion buffer (10 m*M* Tris-HCl pH 8.0, 10 m*M* EDTA, 150 m*M* NaCl) containing 250 μg/ml proteinase K and 0.5% SDS and were incubated for 16–18 h at 37°C. Then genomic DNA was extracted by using organic solvents (phenol and chloroform). After ethanol precipitation, DNA was suspended into sterile water and incubated with 100 μg/ml RNase to remove any residual RNA for 1 h at 37°C. Then proteinase K digestion, followed by organic solvents extraction and ethanol precipitation steps, was repeated. Finally, extracted DNA was dissolved in sterile water, and the DNA concentration was optically measured.

Polymerase Chain Reaction (PCR). Two 20-mer oligonucleotides were used as a generic primer pair for the PCR amplification of the MHC-DR gene, spanning the boundary of the first intron and the exon 2 of the human gene, and the 3′ end of exon 2 of common marmoset. The nucleotide sequence of the upstream primer DRBAMP-C (Kimura and Sasazuki 1992) was 5′-TCGTGTCCCCACAGCACGT-3′, and the nucleotide sequence of downstream primer MA-DR2 was 5′-CTCTCCGCGGCACTAGGAAC-3′. PCR was carried out in a 30-μl mixture containing 100 ng of template DNA, 1× kit-supplied PCR buffer, 1.5 m*M* $MgCl_2$, 200 μ*M* of each deoxynucleotide triphosphate, 0.5 unit of DNA polymerase (AmpliTaq Gold, Perkin Elmer, Foster City, CA), and 0.2 μ*M* of each primer. The amplification step was carried out using a pre-PCR heat step for 9 min at 96°C, followed by 40 cycles of denaturation at 96°C for 30 s, primer annealing at 60°C for 30 s, extension with Taq polymerase at 72°C for 1 min, and a final incubation at 72°C for 10 min in thermal cyclers (TaKaRa PCR Thermal Cycler MP, Takara Shuzo Co., Kyoto, Japan; GeneAmp 9700, Perkin Elmer, Foster City, CA).

For DNA typing, 0.2 μ*M* of the two specific sense primers MA-DRI (5′-ACAGCACGTTACTTGGAGCAG-3′) and MA-DRIV (5′-GCACGTTTCTTGGAGTATAGC-3′) were added with 0.2 μ*M* of the anti-sense primer MA-DR2 into separated PCR mixtures. The amplification was performed as described above. To confirm the existence of the Caja-DRB*W1201 allele, we designed two kinds of specific sense primers MA-W12-1 (5′-GTTTAAGTATGAGTGTCTTCACC-3′) and

MA-W12-2 (5′-GCTGCACTAGGAACCTCTCA-3′) for amplification.

Subcloning and Sequencing. The PCR products were cloned into plasmid vectors using a TA Cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Positively selected colonies were checked for the appropriate insertion length by PCR, followed by SSCP analysis as described in the next section. At least three clones with identical SSCP patterns from more than two different PCR processes were subjected to cycle sequencing bidirectionally using a commercial kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit, Perkin Elmer). Reactions were performed according to the manufacturer's instructions.

Single Strand Conformation Polymorphism (SSCP) and Direct Sequencing. The amplified product was analyzed for the detection of polymorphism using SSCP method described previously with slight modification (Bannai et al. 1994). Briefly, the PCR product was added with loading solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 10 m*M* Na_2EDTA, pH 7.4) in the ratio of one to four. After denaturation for 3 min at 95°C, sample was applied to 10% polyacrylamide gel (acrylamide:bisacrylamide = 49:1). Electrophoresis was performed in TBE solution (22.5 m*M* Tri-borate pH 8.0 and 0.5 m*M* EDTA) using a minigel electrophoresis apparatus (Resolmax AE-6410, Atto, Tokyo) in an electric field of 20 mA at 20°C for 120 mins.

After electrophoresis, the nucleotide bands were visualized by a silver staining kit (Daiichi Pure Chemicals, Tokyo). Separated bands were directly cut out from the gel with a clean scalpel to be used as DNA templates and were reamplified for 35 cycles using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) following the amplification method as described above. After verifying that the recovered fragment showed the original SSCP pattern, the amplified fragment was used as a template for cycle sequencing. The nucleotide sequence was obtained in both directions using a dye-labeled terminator cycle sequencing kit (Perkin Elmer).

Southern Blotting. To investigate the Caja-DRB genes on genome, we performed Southern blot hybridization. Ten micrograms of genomic DNA was digested with Bam HI, EcoR I, and Hind III and separated electrophoretically on 0.7% agarose gel (SeaKem GTG agarose, FMC, Rockland, ME) containing TAE buffer (40 m*M* Tris-acetate, pH 8.0, and 1 m*M* EDTA). After electrophoresis, DNA was blotted to nylon membrane (Hybond-N^+, Amersham, UK) and hybridized with the P^{32}-labeled probe, which was the amplified fragment with a generic primer pair (DRBAMP-C and MA-DR2). The hybridization was done as described by Church and Gilbert (1984) with slight modification. The filter was placed in hybridization buffer (1 m*M* EDTA, 0.5 *M* $NaHPO_4$, pH 7.2, 7% SDS) at 65°C for 16–20 h. Then, the filter was submerged in washing buffer (40 m*M* $NaHPO_4$, pH 7.2, 1% SDS), followed by three washes at 65°C for 5 min with agitation. The last wash was done for 15 min at 65°C. The hybridized signal on the filter was analyzed by an image Analyzer (FUJI, Tokyo)

Phylogenetic Tree and Statistical Analysis. Phylogenetic trees for the DRB alleles were constructed by the unweighted pair-group method with arithmetic mean (UPGMA) (Sokal and Sneath 1963) and neighbor-joining (NJ) method (Saitou and Nei 1987) based on the pair-wise genetic distances corrected for multiple hits by Jukes-Cantor method (Jukes and Cantor 1969). The amino acid residues involved in the antigen-recognition sites (ARS) were selected as described by Brown et al. (1993). The patterns of synonymous and nonsynonymous nucleotide substitutions were analyzed as described by Nei and Gojobori (1986). All statistical analyses were performed using the software program MEGA (Kumar et al. 1994). The differences between each mean d_s and d_n for each putative allelic comparisons were examined by *t* test with Welch's correction.

Results

Caja-DRB Alleles

A total of 21 Caja-DRB alleles, including 7 new alleles, were detected in this study. All alleles detected were designated according to the nomenclature system reported previously (Antunes et al. 1998; Trtková et al. 1993). The locus designations are assumed to be orthologous to those reported in common marmoset. The predicted amino acid sequences along with those previously reported are shown in Fig. 1. Nucleotide sequence homology with HLA-DRB genes ranged between 80 and 90%.

Sequence alignment demonstrated that these genes could be divided into two major groups according to the shared different motifs at the N-terminal region. The first group (Caja-DRB1*03) was characterized by the YSTS motif at positions 10–13, whereas the other group (Caja-DRB*W12 and Caja-DRB*W16) had different sequences. Based on the finding, we designed two kinds of marmoset-specific upstream primers (MA-DRI and MA-DRIV) for DNA typing. Several Caja-DRB*W16 alleles were characterized by an arginine at position 77, as well as a single amino acid deletion at position 78.

Antunes et al. (1998) reported that all marmosets shared a monomorphic Caja-DRB*W1201 allele. In contrast, this allele was not detected in some individuals in the present study. For further confirmation, we designed two specific sense primer, MA-W12-1 and MA-W12-2, and performed PCR with common antisense primer, MA-DR2. We again observed some marmosets lacked Caja-DRB*W1201.

As for the gene copy number of Caja-DRB, we found multiple distinct fragments by Southern blot hybridization (Fig. 2). There were at least four major fragments that hybridized with a Caja-DRB exon 2 probe in three genomic DNA samples from two individuals. Thus there may be a moderate numbers of fragments in marmosets by comparison with other primate species (Sullivan et al. 1991; Grahovac et al. 1991). On the other hand, essentially equal numbers of fragments were observed in genomic DNAs from two different samples—one is muscle cells and the other is a cell line derived from thymus—of the same individual.

DNA Typing

For the histocompatibility typing of common marmoset, we established a novel PCR-SSCP method, using two

	10	20	30	40	50	60	70	80	
Consensus	FLEQ	AKSECHFFNG	TERVRFLDRY	FYNQEEYVRF	DSDVGEYRAV	TELGRPDAEY	WNSQKDILEQ	KRAEVDTYCR	HNYGVVE
Caja-DRB1*0301 (J)	---Y	ST----------	----------	-F-----NL--	-------F---	----------S	-------F--D	R-------V--	---EILD
Caja-DRB1*0302 (J)	---Y	ST----------	-----L-E--	IH----NL--	-------F---	----------S	-------F--D	R--A---F--	---EIS-
Caja-DRB1*0303 (J)	---Y	ST----------	----------	-F-----NL--	-------F---	----------S	-------F--D	R--A---F--	---EILD
Caja-DRB1*0304	---Y	ST----------	----------	-F-----NL--	-------F---	----------S	-------L---	R-------F--	---EILD
Caja-DRB1*0305 (J)	---Y	ST----------	-----L-E--	IH----------	----------	----------S	-------F---	R----------	---EIS-
Caja-DRB1*0306 (J)	---Y	ST----------	-----L-----	-F-----L----	-------F---	----------S	-------F--D	R--A---F--	---EIS-
Caja-DRB1*0307	---Y	ST----------	----------	-F-----NL--	-------F---	----------	-------L---	R----------	---EIS-
Caja-DRB1*0308 (J,N)	---Y	ST----------	----------	-F-----NL--	-------F---	----------S	-------F--D	R--A---V--	---EILD
Caja-DRB1*0309 (J,N)	---Y	ST----------	----------	-F-----NL--	-------F---	----------S	-------F--D	R-------F--	---EILD
Caja-DRB1*0310 (J,N)	....	.T----------	-----L-E--	IH----------	----------	----------S	-------F--D	R--A---F--	---EILD
Caja-DRB1*0311 (J,N)	....	.T----------	----------	-F-----NL--	-------F---	----------S	-------L---	R--A---F--	---EILD
Caja-DRB*W1201 (J)	-----	F-Y---LHL--	--G--L-V--	I----------	-------F---	-------A---	F--L--YM--	T--A---V--	----IF-
Caja-DRB*W1601 (J)	Y----	V-F----H----	------Y-E--	----------	----------	----------	----------	---A--R*--	---EGF-
Caja-DRB*W1604 (J)	Y----	--C----H----	------Y-E--	-------NL--	----------	----------	----------	---A--R*--	---EIS-
Caja-DRB*W1605 (J)	Y----	V-F----H----	-----L-E-R	IH------A--	----------	----------	-------L---	R--A-------	---EILD
Caja-DRB*W1606	-----	V-Y----H----	--------F-D	I------VL--	-------F---	----------	----------D	R--A---V--	---EILD
Caja-DRB*W1607	-----	V-Y----H----	--------F-D	I------VL--	-----W-F---	----------	----------D	R--A---V--	---EILD
Caja-DRB*W1608 (J)	Y----	H-A----H----	----------	--------VL--	----------	-------S--K	L----------	T------R*--	---EIF-
Caja-DRB*W1609 (J)	Y----	--C----H----	------Y-E--	----------	----------	----------	----------	-------R*--	---E-S-
Caja-DRB*W1610 (J)	Y----	--C----H----	------Y-E--	----------	----------	----------	----------	---A--R*--	---E-S-
Caja-DRB*W1611	Y----	R-A----H----	------L-E--	-------F---	-------F---	-------V--S	---R------	R--A---F--	---EGF-
Caja-DRB*W1612 (J)	Y----	--C----H----	------Y-E--	-------L----	----------	-------S--K	L------L---	R-------V--	---EIS-
Caja-DRB*W1613 (J)	Y----	V-F----H----	------L-E-R	IH------A--	----------	----------	----------	R--A-------	---EILD
Caja-DRB*W1614	Y----	--C----H----	------Y-E--	-------L----	----------	-------S--K	L------L---	-------R*--	---E-S-
Caja-DRB*W1615	Y----	V-F----H----	------L-E-R	IH------A--	----------	----------	-------L---	R----------	---EIS-
Caja-DRB*W1616 (J,N)	Y----	--C----H----	------Y-E--	----------	----------	----------	-------L---	T-------V--	---EIF-
Caja-DRB*W1617 (J,N)	Y----	--C----H----	------Y-E--	----------	----------	----------	----------	---A--R*--	---EIF-
Caja-DRB*W1618 (J,N)	....	V-F----H----	------L-E-R	IH------A--	----------	----------	----------	-------R*--	---E-S-

Fig. 1. Alignment of the predicted amino acid sequences of exon 2 of the Caja-DRB genes. A dash indicates identity with the top consensus sequence. An asterisk indicates deletion of amino acid. Unavailability of sequence data is indicated by a dot. Sequences obtained in this study are indicated by J, and the unreported data is indicated by N in parenthesis. The numbering of codons is according to the amino acid position in the β1 domain.

specific primer sets to amplify the second exon of Caja-DRB genes. Analysis involved 18 marmosets originated from five individual families. As a reference, the typical result of a silver-stained SSCP pattern is shown in Fig. 3. After the SSCP analysis, different bands were isolated and used as DNA templates for subsequent PCR amplification. The reamplified fragments were then determined for their nucleotide sequences. The segregation patterns of the Caja-DRB alleles in family samples are demonstrated in Fig. 4. A number of alleles were detected in each individual as previous report (Antunes et al. 1998). Furthermore, the sharing of the same alleles among twins in families 1, 4, and 5 was observed. This finding could support that marmosets are born carrying bone marrow chimeras because they shared blood cells through vascular anastomoses in the placenta during gestation period (Benirchke et al. 1962).

Phylogenetic Analysis

Both of UPGMA and NJ trees based on the nucleotide sequences and predicted amino acid sequences demonstrated that at least three major clusters exist in these DRB genes, which were interpreted as three groups comprised of Caja-DRB1*03, Caja-DRB*W12, and Caja-DRB*W16 groups (data not shown), as previously reported (Antunes et al. 1998). For further investigation, the Caja-DRB exon 2 alleles were compared with the nucleotide sequences of DRB genes from the cotton-top tamarin (*Saginus oedipus*), humans, and other species in the NJ tree (Fig. 5).

While performing this comparison, it was noted that the Caja-DRB1*03 alleles clearly form one major cluster; however, the alleles within the Caja-DRB*W16 lineage showed more extensive diversification and could be divided into several groups. Among those groups, Caja-DRB*W1606 and Caja-DRB*W1607 were separated from the other Caja-DRB*W16 alleles and close to the Caja-DRB*W12 branch. Moreover, Caja-DRB*W1611 was separated from the other Caja-DRB*W16 clusters and joined to a Soae-DRB cluster. In contrast, the Caja-DRB*W1201 was isolated from the other Caja-DRB genes, but close to the Saoe-DRB*W12 group. Thus, trans-species polymorphisms were found in the DRB genes of New World monkey. Furthermore, MHC-DRB alleles in New World monkeys were clearly separated for those in apes and Old World monkeys.

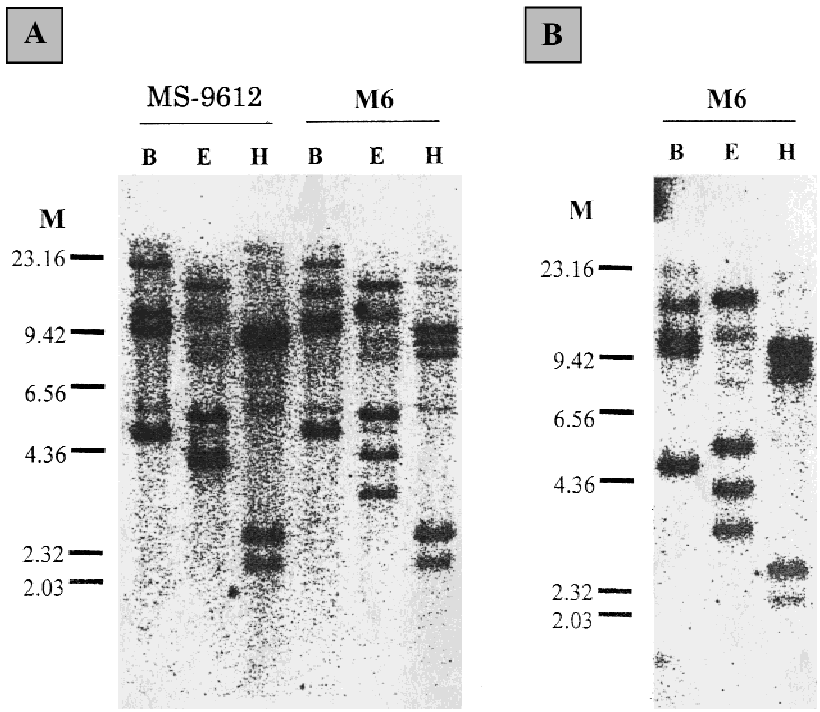

Fig. 2. Southern blotting analysis for Caja-DRB genes. Genomic DNA samples derived from the stromal cell line (MS-9612), the cell line derived from thymus (M6) in panel A, and muscle (M6) in panel B, were digested with three kinds of restriction endonucleases, B: Bam HI; E: EcoR I; H: Hind III, completely. The blot was hybridized with a probe that is the amplified fragment by generic primer set (DRBAMP-C and MA-DR2). Marker sizes are given in kilobases on the left of each panel.

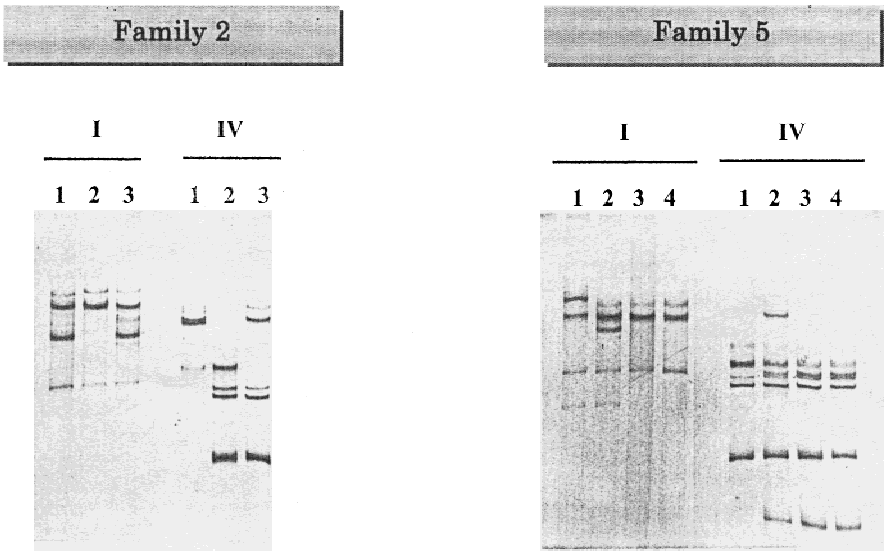

Fig. 3. PCR-SSCP analysis for Caja-DRB genes. Genomic DNA from blood samples underwent PCR-SSCP analysis for detecting the polymorphism of Caja-DRB exon 2 genes. Two kinds of upstream specific primers were used. I indicates primer MA-DRI, and IV indicates primer MA-DRIV. There are three samples from Family 2, 1: H215; 2: I8512; 3: IH406, and four samples from Family 5, 1: H0120, 2: H087, 3: H283, 4: H284. All individuals showed multiple bands, suggesting the existence of multiple loci.

Analysis of Nucleotide Substitution

The patterns of nucleotide substitutions between Caja-DRB alleles were examined in the region of the putative antigen recognition sites (ARSs) and the other region. Twenty-two amino acid residues were selected as the putative ARS sites (Brown et al. 1993). The mean values of the numbers of nucleotide substitutions per synonymous site (d_s) and per nonsynonymous site (d_n) based on pairwise comparisons among the sequences are shown in Table 1. The means of d_s and d_n based on all Caja-DRB alleles were analyzed as well as those within Caja-DRB1*03 and -DRB1*03 and -DRB*W16. In the ARSs, the mean d_n is greater than the mean d_s for all pair-wise comparisons, and also the d_n is greater than the d_s for the comparison within each group. This result suggests that positive selection operates in the ARS of Caja-DRB lineages.

Discussion

Our preliminary experiment demonstrated that serological typing trays for humans could not be applied to marmosets (data not shown). Furthermore, current PCR-

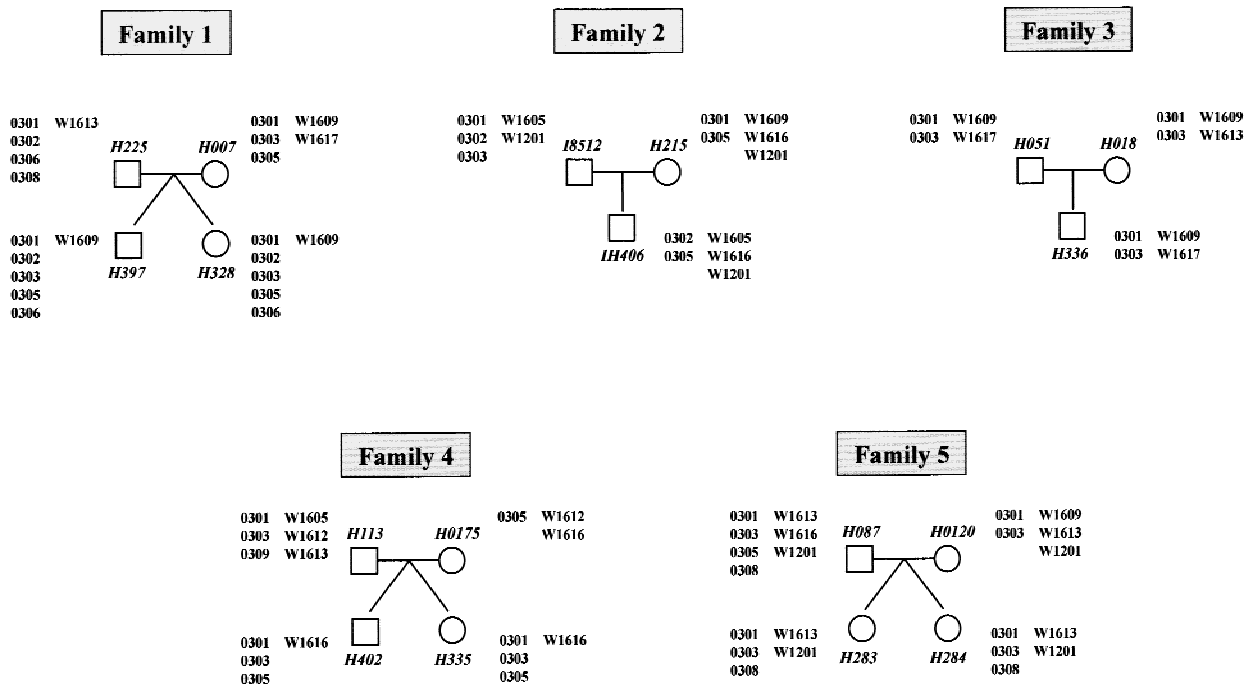

Fig. 4. Pedigree pattern of Caja-DRB genes. Eighteen common marmosets bear relations to five family trees. Distribution of alleles in each individual has been obtained from DNA typing by using PCR-SSCP analysis. The Caja-DRB alleles of each individual are shown in last part of name. Pairs (H328, H397), (H335, H402), and (H238, H284) are twins.

based typing kits for HLA genes (Kawai et al. 1996; Bannai et al. 1994) could not be directly applicable to the analysis of common marmoset MHC either because of the nucleotide sequence differences. Therefore, in the present studies, we investigated Caja-DRB genes and their polymorphisms in common marmoset bred in Japan and established a DNA-based histocompatibility test system for allogeneic transplantation approaches using this species.

Although the generic primer set of DRBAMP-C and MA-DR2 enabled to amplify the highly polymorphic Caja-DRB genes in each individual, patterns of the SSCP bands were too complex to recognize differences between individuals (data not shown). For easier accomplishment of the histocompatibility test, we designed two sets of specific primers that could classify all alleles into two major groups and differentiated each allele by means of PCR-SSCP. In addition, our PCR-SSCP analysis demonstrated that the fraternal twin marmosets, which were seen in more than 85% of the delivery (Benirchke, et al. 1962), had the same Caja-DRB alleles (Figs. 3, 4). This finding would bring us important information in our applying the common marmoset for preclinical studies, including hematopoietic stem cell transplantation. We adopted the PCR-SSCP method rather than popularly used methods, such as PCR-SSO (sequence specific oligonucleotide probing) and PCR-SSP (sequence specific primer), since the former method is applicable to unknown alleles and the donor-recipient matching for transplantation can be achieved by simple comparison of SSCP banding patterns. This is also advantageous for testing chimeric twins, who have at most four different alleles in each locus.

Antunes et al. (1998) reported that the Caja-DRB genes could be grouped into three clusters, denoted as Caja-DRB1*03, -DRB*W12, and -DRB*W16. However, our Southern blot analysis demonstrated that there were at least four major hybridized bands with a Caja-DRB exon 2 probe (Fig. 2). This observation supported that the Caja-DRB genes could be tentatively grouped into at least four allelic clusters. Compared with the RFLP analyses of several kinds of primate (Sullivan et al. 1991; Grahovac et al. 1991), common marmosets seemed to have moderate numbers of MHC class II DRB genes at the lineage level. According to our results, including the Southern blotting results, the findings on family segregation and phylogenetic analyses, we presumed that Caja-DRB genes could be divided into at least five different loci. Namely, each of the current Caja-DRB1*03 and Caja-DRB*W16 group could be classified into two groups, respectively, whereas the Caja-DRB*W12 is single. More precise gene mapping studies, however, are required to obtain conclusion results.

Substitution rates in a certain gene and in different DNA regions of a gene, can help us understand the mechanism of nucleotide substitution in evolution (Li 1997), like genetic polymorphism of MHC. In principle, if balancing selection operates in a certain gene, the rate of nonsynonymous substitution should exceed that of synonymous substitution at selection operating sites. In contrast, if the synonymous nucleotide substitution is

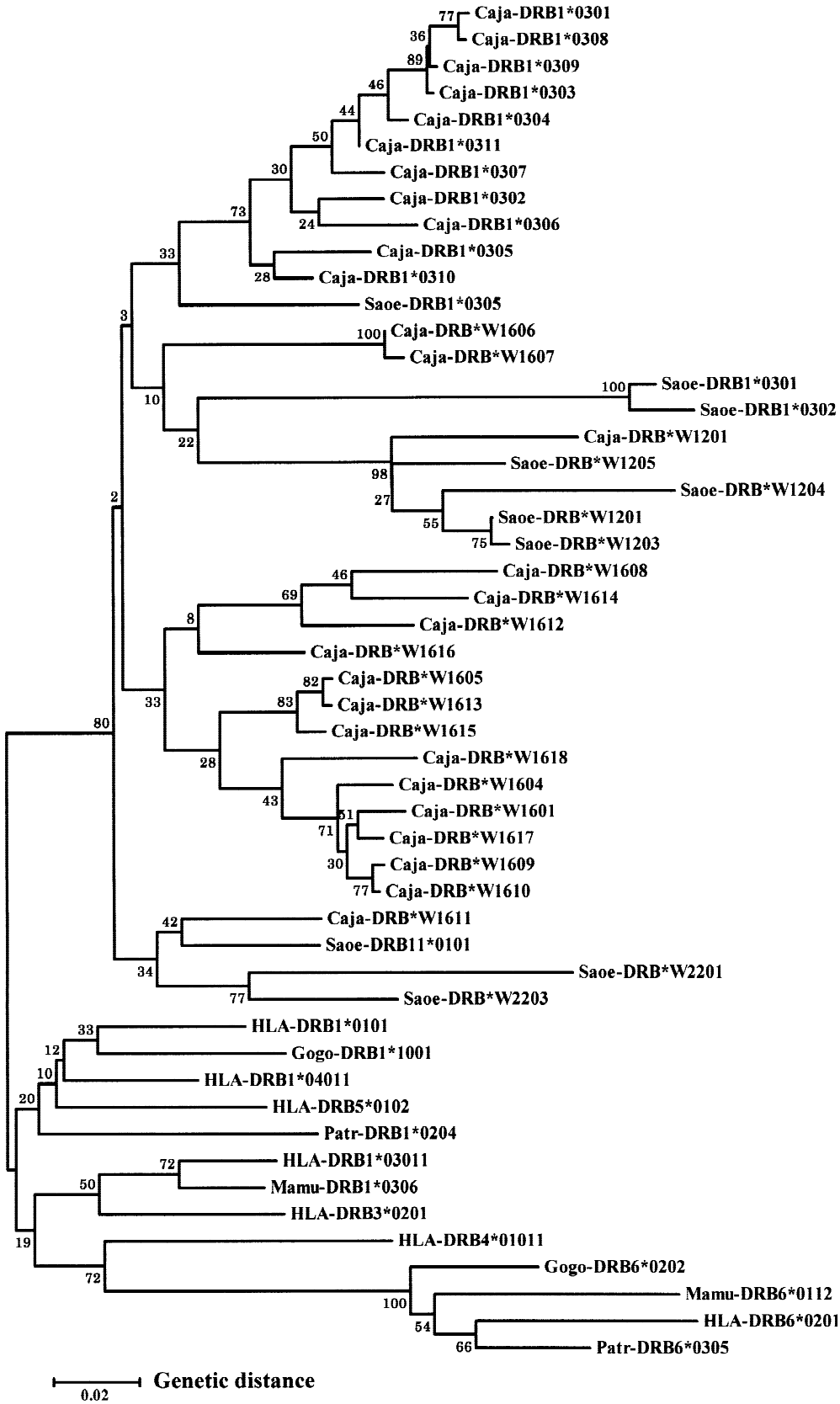

Fig. 5. Phylogenetic tree for MHC-DRB alleles. The nucleotide sequences of all alleles found in common marmoset (*Callithrix jaccus*, Caja) and selected alleles found in the cotton-top tamarin (*Saguinus oedipus*, Saoe), human (*Homo sapiens*, HLA), chimpanzee (*Pan troglodytes*, Patr), gorilla (*Gorilla gorilla*, Gogo), and rhesus macaque monkey (*Macaca mulatta*, Mamu) were subjected to the analysis. The tree was constructed by neighbor-joining method from the pair-wise genetic distances calculated by using the Jukes-Cantor correction for multiple hits and rooted by the midpoint method. Numbers on the nodes indicate the percentage recovery of that node in 500 bootstrap replicates.

Table 1. Nucleotide substitution patterns at MHC class II DRB loci in common marmoset

Comparison	No.	ARS (N = 22)[a]			Non-ARS (N = 59)[a]			All sites (N = 81)		
		d_s	d_n	d_n/d_s	d_s	d_n	d_n/d_s	d_s	d_n	d_n/d_s
Caja-DRB										
All	28	13.17	32.4#	2.460	3.28	6.12	1.866	5.67	12.15#	2.143
		(5.72)	(4.65)		(1.55)	(1.27)		(1.80)	(1.47)	
*03	11	3.95	12.98	3.286	1.60	1.43	0.894	2.16	4.37	2.023
		(3.79)	(2.98)		(1.24)	(0.66)		(1.25)	(0.91)	
*W16	16	9.68	32.26#	3.333	1.54	4.19	2.721	3.62	10.44##	2.884
		(4.79)	(4.91)		(1.24)	(1.07)		(1.41)	(1.30)	
Saoe-DRB										
All[b]	38	34.6	40.33	1.166	11.13	13.14	1.181	16.07	18.91	1.177
		(11.38)	(6.44)		(3.21)	(2.03)		(3.46)	(2.05)	

Mean changes and standard errors (shown in parenthesis) are displayed as percentage of substitution per synonymous site (d_s), and nonsynonymous site (d_n) at common marmoset (*Callithrix jaccus*) and cotton-top tamarin (*Saginus oedipus*) class II DRB locus (exon 2) at codons 7–87 with pair-wise comparison in lineages. The difference between mean d_s and mean d_n is significant at 1% level (#), 0.1% level (##).

[a] The amino acid positions involved in the putative antigen-recognition site (ARS) are 9, 11, 13, 28, 30, 32, 37, 38, 47, 56, 60, 61, 65, 68, 70, 71, 74, 78, 81, 82, 85, and 86. The calculated positions of non-ARS are all compared region (codons 7–87) excluding the positions of ARS.

[b] All allele sequences except for those possessing stop codons were analyzed.

much more frequent than nonsynonymous substitution, purifying selection is considered to operate in the gene. Bergström and Gyllensten (1995) had presented no evidence of selection at the Caja-DRB*W16 lineage, however, we analyzed the Caja-DRB alleles including our new sequences and found that the number of nonsynonymous substitutions exceeded that of synonymous substitutions at the putative ARS in β1 domain (Table 1). Therefore, like human, nonhuman primate, and mouse class II genes (Hughes and Nei 1989; Bergström and Gyllensten 1995), positive natural selection or balancing selection is considered to operate in the ARS of Caja-DRB genes.

Some HLA-DR molecules have long been linked to rheumatoid arthritis (Gregersen et al. 1987; Watanabe et al. 1989). Sequence analysis of these alleles revealed that they encoded similar amino acid sequence within the residues 67–74 in the DR β–chain, termed the shared epitope (Winchester 1994). Recently, in a case-control study, the association of alleles containing the shared epitope QKRAA/QRRAA/RRRAA, and rheumatoid arthritis susceptibility has been reported (del Rincón and Escalante 1999). As shown in Fig. 1, some Caja-DRB alleles also had the shared epitope QKRAA (Caja-DRB*W1601, *W1604, *W1610, *W1617) or QRRAA (Caja-DRB*W1605, *W1611, *W1613) motif at positions 70–74. Compared with the nucleotide sequences of nonhuman primates (O'hUigin 1997), these motifs were observed mostly in the chimpanzee, but only a few in the cotton-top tamarin, a New World monkey of *Callitrichidae*. Nonhuman primate arthritis models had been developed (Shimozuru et al. 1998). The observed Caja-DRB allele data and established typing method in this work might contribute to study the etiology, pathophysiology, and therapeutic strategies for MHC-DR associated diseases using this species as an animal model in the future.

In summary, the detection of Caja-DRB genes and their polymorphisms as described in this study will be helpful to open important areas for immunological and hematological research in the clinical medicine. This investigation would result in the ultimate goal of developing new and successful therapeutic approaches, including cell and gene therapy.

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References

Annett LE, Torres EM, Clarke DJ, Ishida Y, Barker RA, Ridley RM, Baker HF, Dunnett SB (1997) Survival of nigral grafts within the striatum of marmosets with 6-OHDA lesions depends critically on donor embryo age. Cell Transplant 6:557–569

Antunes SG, Groot NG, Brok H, Doxiadis G, Menezes AAL, Otting N, Bontrop RE (1998) The common marmoset: a New World primate species with limited Mhc class II variability. Proc Natl Acad Sci USA 95:11745–11750

Bannai M, Tokunaga K, Lin L, Kuwata S, Mazda T, Amaki I, Fujisawa K, Juji T (1994) Discrimination of human HLA-DRB1 alleles by PCR-SSCP (single-strand conformation polymorphism) method. Eur J Immunogen 21:1–9

Benirchke K, Anderson JM, Brownhill LE (1962) Marrow chimerism in marmosets. Science 138:513–515

Bergström T, Gyllensten U (1995) Evolution of Mhc class II polymorphism: the rise and fall of class II gene function in primates. Immunol Rev 143:13–31

Bergström TF, Erlandsson R, Engkvist H, Josefsson A, Erlich HA, Gyllensten U (1999) Phylogenetic history of hominoid DRB loci and alleles inferred from intron sequences. Immunol Rev 167:351–365

Bodmer JG, Marsh SGE, Albert ED, Bodmer WF, Bontrop RE, Char-

ron D, Dupont B, Erlich HA, Fauchet R, Mach B, Mayr WR, Parham P, Sasazuki T, Schreuder GMT, Strominger JL, Svejgaard A, Terasaki PI (1996) Nomenclature for factors of the HLA system, 1996. Human Immunol 53:98–128

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33–39

Burlingham WJ, Grailer AP, Heisey DM, Claas FH, Norman D, Mohanakumar T, Brennan DC, de Fijter H, van Gelder T, Pirsch JD, Sollinger HW, Bean MA (1998) The effect of tolerance to noninherited maternal HLA antigens on the survival of renal transplants from sibling donors. N Eng J Med 339:1657–1664

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995

Duboise SM, Guo J, Czajak S, Desrosiers RC, Jung JU (1998) STP and Tip are essential for herpesvirus saimiri oncogenicity. J Virol 72: 1308–1313

Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Linington C, Raine CS, Hauser SL (1996) Late complications of immune deviation therapy in a nonhuman primate. Science 274:2054–2507

Grahovac B, O'hUigin C, Tichy H, Mayer WE, Klein J (1991) Mhc class II genes of new world monkeys and their relationship to human genes. In: Klein J, Klein D (eds) Molecular evolution of the major histocompatibility complex. NATO ASI series, vol. H 59, Springer-Verlag, Berlin, pp 201–212

Gregersen PK, Silver J, Winchester RJ (1987) The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum 30:1205–1213

Hibino H, Tani K, Ikebuchi K, Wu MS, Sugiyama H, Nakazaki Y, Tanabe T, Takahashi S, Tojo A, Suzuki S, Tanioka Y, Sugimoto Y, Nakahata T, Asano S (1999) Common marmoset as a target preclinical primate for cytokine and gene therapy. Blood 93:2839–2848

Hughes AL, Nei M (1989) Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc Natl Acad Sci USA 86:958–962

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York, NY, pp 21–123

Kawai S, Maekawajiri S, Tokunaga K, Kashiwase K, Miyamoto M, Akaza T, Juji T, Yamane A (1996) Routine low and high resolution typing of the HLA-DRB gene using the PCR-MPH (microtitre plate hybridization) method. Eur J Immunogen 23:471–486

Kimura A, Sasazuki T (1992) Eleventh International Histocompatibility Workshop reference protocol for the HLA DNA-typing technique. In: Tsuji K, Aizawa M, Sasazuki T (eds) HLA 1991, vol. 1. Oxford University Press, Oxford, pp 397–419

Klein J, Satta Y, Gongora R (1997) Evolution of length variation in the primate Mhc DR subregion. In: Blancher A, Klein J, Socha WW (eds) Molecular biology and evolution of blood group and MHC antigens in primates. Springer, Berlin, pp 372–385

Kumar S, Tamura K, Nei M (1994) MEGA: molecular evolutionary genetics analysis, version 1.0. Comput Appl Biosci 10:189–191

Li WH (1997) Molecular evolution. Sinauer Associate, Sunderland, MA

Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutes. Mol Biol Evol 3:418–426

Neubert R, Foerster M, Nogueira AC, Helge H (1996) Cross-reactivity of antihuman monoclonal antibodies with cell surface receptors in the common marmoset. Life Sci 58:317–324

Nomoto M, Fukuda T (1993) A selective MAOB inhibitor Ro19-6327 potentiates the effects of levodopa on parkinsonism induced by MPTP in the common marmoset. Neuropharmacology 32:473–477

O'hUigin C (1997) Nonhuman primate MHC class II sequences: a compilation. In: Blancher A, Klein J, Socha WW (eds) Molecular biology and evolution of blood group and MHC antigens in primates. Springer, Berlin, pp 507–551

del Rincón I, Escalante A (1999) HLA-DRB1 alleles associated with susceptibility or resistance to rheumatoid arthritis, articular deformities, and disability in Mexican Americans. Arthritis Rheum 42: 1329–1338

Ryffel B, Car BD, Woerly G, Weber M, DiPadova F, Kammuller M, Klug S, Neubert R, Neubert D (1994) Long-term interleukin-6 administration stimulates sustained thrombopoiesis and acute-phase protein synthesis in a small primate—the marmoset. Blood 83: 2093–2102

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sasazuki T, Juji T, Morishima Y, Kinukawa N, Kashiwabara H, Inoko H, Yoshida T, Kimura A, Akaza T, Kamikawaji N, Kodera Y, Takaku F (1998) Effect of matching of class I HLA alleles on clinical outcome after transplantation of hematopoietic stem cells from an unrelated donor. Japan Marrow Donor Program. N Eng J Med 339:1177–1185

Schmidt S, Neubert R, Schmitt M, Neubert D (1996) Studies on the immunoglobulin-E system of the common marmoset in comparison with human data. Life Sci 59:719–730

Shimozuru Y, Yamane S, Fujimoto K, Terao K, Honjo S, Nagai Y, Sawitzke AD, Terato K (1998) Collagen-induced arthritis in nonhuman primates: multiple epitopes of type II collagen can induce autoimmune-mediated arthritis in outbred cynomolgus monkeys. Arthritis Rheum 41:507–514

Sokal R, Sneath PHP (1963) Principles of numerical taxonomy. WH Freeman, San Francisco, CA

Sullivan J, Kennedy C, Trejaunt J, Dunckly H (1991) Polymorphisms of the major histocompatibility complex in old and new world primates. In: Klein J, Klein D (eds) Molecular evolution of the major histocompatibility complex. NATO ASI series, vol. H 59, Speinger, Berlin, pp 193–200

Trtková K, Kupfermann H, Grahovac B, Mayer WE, O'hUigin C, Tichy H, Bontrop R, Klein J (1993) Mhc-DRB genes of platyrrhine primates. Immunogenetics 38:210–222

Watanabe Y, Tokunaga K, Matsuki K, Takeuchi F, Matsuta K, Maeda H, Omoto K, Juji T (1989) Putative amino acid sequence of HLA-DRB chain contributing to rheumatoid arthritis susceptibility. J Exp Med 169:2263–2268

Winchester R (1994) The molecular basis of susceptibility to rheumatoid arthritis. Adv Immunol 56:389–466