10×10^6 cells) using a Quick Prep Micro mRNA Purification kit (Pharmacia Biotech, Uppsala, Sweden) were converted into cDNA using a High Fidelity RNA PCR kit (Takara, Shiga, Japan). The amplification of *A* locus was carried out by using specific primer sets, either Mafa-As/Mafa-Aa or Mafa-A13-s/Mafa-A1013-a (Table 1). Forty cycles of amplification were carried out at 94 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s, followed by an additional extension at 72 °C for 7 min using GeneAmp PCR System 9700 (Applied Biosystem, Norwalk, Conn.). The PCR products were cloned into pCR4-Blunt-TOPO plasmid using Zero Blunt TOPO PCR Cloning kit (Invitrogen, Carlsbad, Calif.). The clones were sequenced with sequencing primers T7, SP6, Ia698, and Is437 (Table 1) by an ABI model 310 DNA Sequencer (Applied Biosystem, Foster City, Calif.).

The multiplex PCR-SSP

The multiplex PCR-SSP was carried out using cDNA from cynomolgus monkeys as the template. The primers used are listed in Table 1. Primer set 1 was a mixture of IA-01s, IA-02s, IA-03s, IA-04s, IA-05s, and IA-a, primer set 2 IA-06s, IA-07s, IA-08s, IA-09s, IA-10s, and IA-a, and primer set 3 IA-11s, IA-12s, IA-13s, IA-

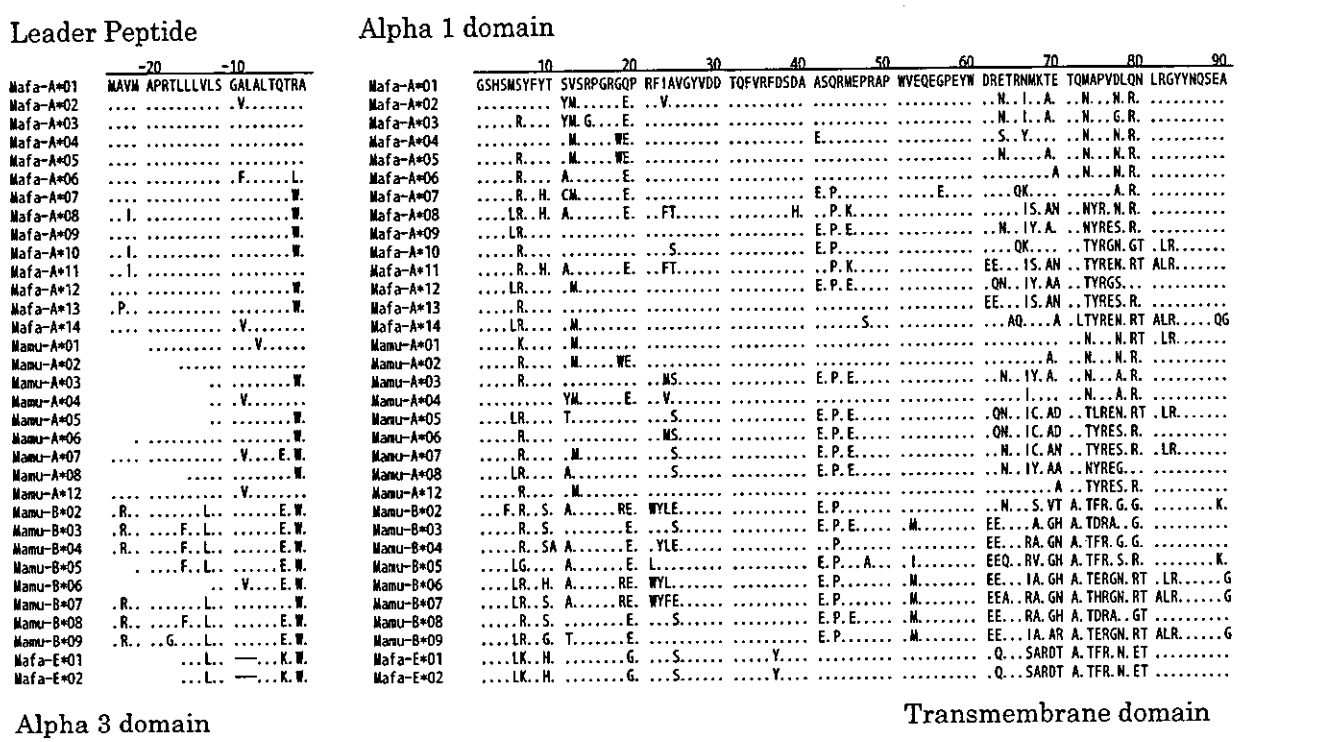

Fig. 1 Alignment of predicted amino acid sequences of *Mafa-A* with previously reported *Mamu-A*, *Mamu-B*, and *Mafa-E* sequences. Identity to predicted amino acid sequence of *Mafa-A*01* is indicated by *dots*, whereas amino acid replacements are depicted by the conventional *one-letter code*. The deletions of amino acid are indicated by *hyphens*

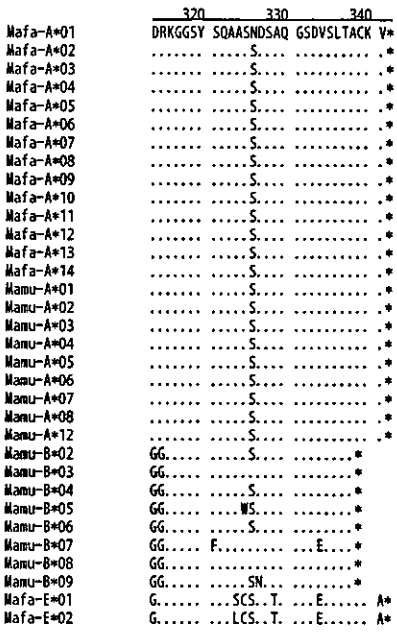

Fig. 1 (continued)

14s, and IA-a. The concentrations of these primers were optimized after preliminary experiments (Table 1). The reactions were heated at 95 °C for 5 min, and then 25 cycles of amplification consisting of denaturation at 95 °C for 30 s, annealing either at 72 °C (for primer set 1 and set 2) or 70 °C (for primer set 3) for 30 s, and extension at 72 °C for 30 s. The amplicons were separated by 1.3% agarose gel electrophoresis and excised bands were purified using Freeze 'N Squeeze Spin Columns (Bio-Rad, Hercules, Calif.). The nucleotide sequences were determined by an ABI model 310 DNA sequencer (Applied Biosystem).

GenBank accession numbers

The *Mafa-A* sequences described in this manuscript have been deposited at GenBank and were assigned accession numbers AB154760–AB154773. The GenBank accession numbers for all sequences used in this studies are as follows: *Gogo-B*0401,* AF157407; *Gogo-B*0501,* AF157408; *Gogo-B*0502,* AF157409; *Gogo-C*0103,* AF157410; *Gogo-C*0204,* AF157411; *HLA-A*0201,* AY365426; *HLA-A*0301,* L77702; *HLA-A*2402,* L47206; *HLA-A*2602,* M98453; *HLA-A*2901,* U83415; *HLA-A*8001,* L18898; *HLA-B*0801,* D83956; *HLA-B*1301,* D50290; *HLA-B*1802,* D25275; *HLA-B*2702,* L38504; *HLA-B*4201,* L76225; *HLA-B*57,* M32318; *HLA-B*7301,* U04787; *Mamu-A*01,* MMU50836; *Mamu-A*02,* MMU50837; *Mamu-A*03,* MMU41379; *Mamu-A*04,* MMU41380; *Mamu-A*05,* MMU41831; *Mamu-A*06,* MMU41834; *Mamu-A*07,* MMU41832; *Mamu-A*08,* AF243179; *Mamu-A*12,* AF157398; *Mamu-B*02,* MMU41833; *Mamu-B*03,* MMU41825; *Mamu-B*04,* MMU41826; *Mamu-B*05,* MMU41827; *Mamu-B*06,* MMU41828; *Mamu-B*07,* MMU41829; *Mamu-B*08,* MMU41830; *Mamu-B*09,* MMU41835; *Patr-A*0301,* AF500288; *Patr-A*0501,* AF500289; *Patr-A*0601,* AF500290; *Patr-A*11,* L47291; *Patr-A*14,* L47292; *Patr-B*01,* L47293; *Patr-B*16,* L47296; *Patr-B*17,* L47348; *Patr-C*03,* L47294; *Patr-C*04,* L47347; *Patr-C*05,* L47298; *Patr-C*06,* L47299; *Popy-A*0302,* AY034115; *Popy-A*0401,* AY034116; *Popy-A*0402,* AY034117; *Popy-A*0501,* AY034114; *Popy-B*0701,* AF118895; *Popy-B*0601,* AF118894; *Popy-B*0801,* AF118896; *Popy-B*0302,* AF118891; *Popy-B*0401,* AF118892; *Popy-B*0501,* AF118893; *Popy-C*0201,* AF118898; *Popy-C*0203,* AF470378; *Popy-C*0301,* AF470379; and *Popy-C*01, 012* AF470376.

Results

Detection of 14 MHC class I *A* locus alleles in cynomolgus monkeys

As there was no report on genes coding for class I MHC molecules in cynomolgus monkeys, we attempted to determine the nucleotide sequences of the alleles of the *A* locus in cynomolgus monkeys (*Mafa-A*). The amplicons obtained using *A*-locus-specific primers were cloned into pCR4-Blunt-TOPO plasmid and 8–48 independent clones were sequenced for each animal. When more than two clones from each animal showed the identical nucleotide sequences, the sequence was regarded as a consensus sequence representing a particular allele. Since PCR amplification and cloning procedures as well as sequencing are prone to misincorporation of nucleotides, amino acid sequences deduced from the nucleotide sequences were assessed by phylogenetic analysis using the neighbor-joining method (Saitou and Nei 1987) (data not shown). When the nucleotide sequence variation of the clone was negligible (d<0.025) compared with the consensus sequence, this particular clone was not considered to represent an independent allele. On the other hand, clones showing sequence variation of $d \geq 0.025$ were considered to represent independent alleles.

The deduced amino acid sequences of *Mafa-A* alleles are shown in Fig. 1, together with those published for *Mamu-A* and *-B*. All *Mafa-A* alleles encoded proteins of 365 amino acids. The putative glycosylation site was located at residue 86. In addition, conserved cysteine residues occurred at positions 101 and 164 in α_2 and at positions 203 and 259 in α_3. Other areas of similarity with *Mamu* included a region of variability at residues 77–83 near the C terminus of the α_1 helix, analogous to the site of the Bw4/Bw6 motif in human.

To ascertain that these alleles were indeed derived from cynomolgus MHC class I *A* locus, they were compiled with other primate MHC class I loci (Fig. 2) and a phylogenetic tree was constructed using the amino acid sequences of α_1 and α_2 domains, since both domains were the most polymorphic regions of the molecule. The result showed that the sequences from cynomolgus monkeys aligned within the same cluster as those from other non-human primates, indicating that these cDNA clones were derived from distinct alleles of the MHC class I *A* locus of cynomolgus monkeys, *Mafa-A*.

A family consisting of eight animals was subjected to genetic analysis for better understanding of inheritance of *Mafa-A* alleles (Fig. 3, Table 2). By nucleotide sequence analysis, four *Mafa-A* alleles *(Mafa-A*03, -A*07, -A*11,* and *-A*14*) were found in this family. *Mafa-A*03* was detected in monkeys 2010 and 3005, whereas *Mafa-A*07* was detected in monkeys 8225 and 2010. *Mafa-A*11* was carried by monkeys 9234, 5117, 4045, 5076, and 1102,

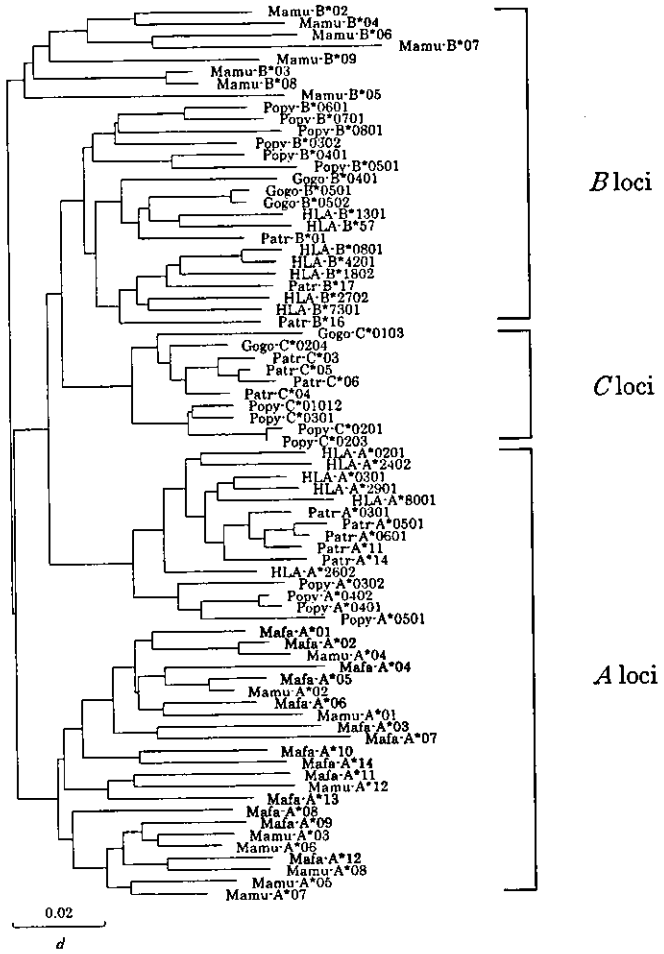

Fig. 2 Phylogenetic analysis of primate class I MHC molecules. The phylogenetic tree of α_1–α_2 was constructed using nucleotide sequences with the neighbor-joining method by DNASIS pro (Hitachi software, Yokohama, Japan)

while *Mafa-A*14* was shared by monkeys 8225, 5117, 4045, 5076, and 3005. By nucleotide sequence analysis, only one allele (*Mafa-A*11*) was detected in 1102.

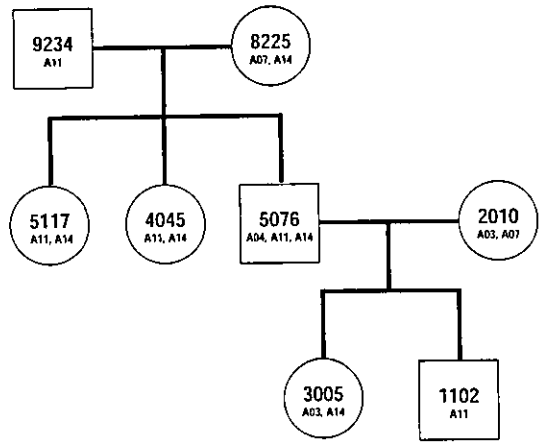

Fig. 3 The family pedigree demonstrating the inheritance of alleles of the MHC class I *A* locus in cynomolgus monkeys. Male or female is denoted by a *square* or *circle*, respectively. The animal number and allele assigned to the animal are shown

The establishment of the multiplex PCR-SSP method

We tried to develop a convenient method for MHC typing among relatively large numbers of animals. We have modified an existing PCR-SSP for detection of multiple allele simultaneously. The concentrations of primers and the annealing temperatures, which appeared critical, were described in Materials and methods in detail. To know whether this method was actually able to detect the *Mafa-A* alleles, plasmids harboring cDNA inserts for respective *Mafa-A* alleles were subjected to multiplex PCR-SSP. As shown in Fig. 4, distinct bands were amplified using appropriate primer sets, although the presence of non-specific bands was also noticed. The cDNAs obtained from members of the family mentioned above were analyzed by multiplex PCR-SSP. *Mafa-A*03* was found in monkeys 2010 and 3005. *Mafa-A*04* was detected in monkeys 5117, 4045, 5076, and 1102, while *Mafa-A*07* was detected in three monkeys: 8225, 2010 and 1102. *Mafa-A*08* was detected only in monkey 8225, while *Mafa-A*11* was shared by monkeys 5117, 4045, 5076, and 1102. *Mafa-A*14* was carried by monkeys 8225, 5117, 4045, 5076, and 3005. The results are summarized in

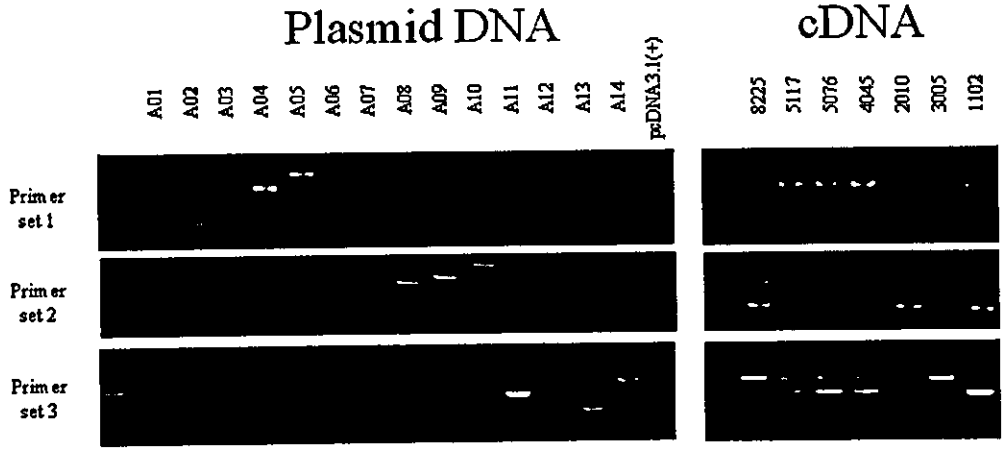

Fig. 4 Multiplex PCR-SSP analyses of *Mafa-A* alleles. Identification of *Mafa-A*01* through *A*14* was conducted using cloned genes inserted into pcDNA3.1(−) vector (*left panel*). Copy DNAs obtained from seven cynomolgus monkeys were similarly subjected to multiplex PCR-SSP (*right panel*)

Table 2 Summary of MHC class I cDNAs isolated from cynomolgus monkeys with RT-PCR and sequencing

Animal	Allele	Number of copies	Primer pair/s
9234	*A11*	8	Mafa-A-s/Mafa-A-a
8225	*A07*	5	Mafa-A-s/Mafa-A-a
	A14	31	Mafa-A-s/Mafa-A-a
5117	*A11*	20	Mafa-A-s/Mafa-A-a
	A14	11	Mafa-A-s/Mafa-A-a
4045	*A11*	35	Mafa-A-s/Mafa-A-a
	A14	7	Mafa-A-s/Mafa-A-a
5076	*A04*	6	Mafa-A13-s/Mafa-A1013-a
	A11	30	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A14	10	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
2010	*A03*	37	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A07	9	Mafa-A13-s/Mafa-A1013-a
3005	*A03*	39	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
	A14	15	Mafa-A-s/Mafa-A-a, Mafa-A13-s/Mafa-A1013-a
1102	*A11*	33	Mafa-A-s/Mafa-A-a

Table 3 Summary of typing alleles with sequencing and multiplex PCR-SSP analysis on cynomolgus monkeys (*ND* not done)

Animal	Sequencing	Multiplex PCR-SSP
9234	*A11*	ND
8225	*A07, A14*	*A07, A08, A14*
5117	*A11, A14*	*A04, A11, A14*
4045	*A11, A14*	*A04, A11, A14*
5076	*A04, A11, A14*	*A04, A11, A14*
2010	*A03, A07*	*A03, A07*
3005	*A03, A14*	*A03*, A14
1102	*A11*	*A04, A07, A11*

Table 3. Since the presence of the *Mafa-A*08* allele was only demonstrated by the multiplex PCR-SSP, the PCR amplicon was subjected to partial nucleotide sequence analysis. The result revealed that the DNA fragment identified as *Mafa-A*08* allele by multiplex PCR-SSP contained, in addition to that of an *Mafa-A* allele per se, the sequence derived from an allele that had not been found before. The results of similar analyses with large numbers of animals were consistent with the view that one to four *Mafa-A* alleles were present in an animal (data not shown). These results strongly suggested that the *A* locus in cynomolgus monkeys had been duplicated.

Discussion

The polymorphic nature of MHC genes is particularly important in antigen recognition because it provides a way of presenting huge numbers of antigens by comparably limited numbers of MHC molecules. Numerous reports on the association of MHC alleles with susceptibility to HIV-1 infection in humans have been published; the prevalence of *HLA-B*14*, *-B*27*, and *-B*57* was shown to be significantly high among slow progressors, while a possible association of *HLA-B*35* and *-B*44* with shorter survival time was also demonstrated in HIV-1 infected patients (Gierowska et al.1999; Hendel et al. 1999; Migueles et al. 2000; Nelson et al. 1997). In SIV-infected rhesus monkeys, *Mamu-A*01* and *-A*1303* have been shown to be associated with not only longer survival time but also lower set-point viral load (Muhl et al.2002). However, as far as we know, there is no literature describing the associations of MHC polymorphism with progression of the disease in SIV-infected cynomolgus monkeys. Moreover, even the nucleotide sequence analyses have not been performed with regard to the class I MHC genes in cynomolgus monkeys. We therefore attempted to determine the nucleotide sequences of the MHC *A* locus genes. *Mafa-A* was amplified using *A*-locus-specific primer pairs. The results showed that at least 14 independent alleles of the class I *A* locus were found in cynomolgus monkeys.

We also have succeeded in developing a multiplex PCR-SSP method which enables us to readily detect several *Mafa-A* alleles simultaneously. When cDNA derived from cynomolgus monkey 8225 was analyzed by this method, however, the presence of a *Mafa-A* allele not detected by the usual RT-PCR was demonstrated. It seemed likely, therefore, that the primer pair designed to amplify *Mafa-A*08* was not specific enough to discriminate the *Mafa-A*08* allele from one with a very similar nucleotide sequence. Similarly, the primer pair designed to detect *Mafa-A*04* amplified a DNA fragment from not only the authentic *Mafa-A*04* allele but also an allele quite similar to *Mafa-A*04*. It seemed, therefore, premature to use only PCR-SSP for detection of either *Mafa-A*04* or *Mafa-A*08*; however, the method could be applied for detection of other alleles. Since we could not rule out completely the possibility that the primers designed to detect other alleles would cross-react with alleles yet to be found, further accumulation of the nucleotide sequences of *Mafa-A* alleles is required.

It was reported that rhesus MHC class I haplotype consisted of at least one *Mamu-A* and at least two *Mamu-B*

(Boyson et al. 1996b). No evidence for the presence of a *HLA-C* homologue was observed, suggesting that the evolution of the *C* locus in gorillas, chimpanzees, and humans is a fairly recent occurrence (Adams et al. 1999, 2000; Boyson et al. 1996b; Cooper et al. 1998; de Groot et al. 2000, Lawlor et al. 1990, 1991). This study showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

The amino acids that composed the Mafa-A pocket were compared with those for the *Mamu-A* pocket. From a crystallographic analysis, a peptide of 8–11 amino acids was demonstrated to bind into the groove of the MHC molecule, which was made up of α_1 and α_2 domains. In the case of the most HLA molecules, the second amino acid of the peptide is thought to play a crucial role in binding to the HLA molecule in conjunction with the C-terminal amino acid of the peptide. In the case of *Mamu-A*01*, however, the most important anchor residue was

Fig. 5 Amino acid residues of pockets B, C, D, and F probably determining the peptide-binding specificity of cynomolgus monkey MHC class I molecule. Amino acids identical with *Mamu-A*01* are indicated by *dashes*

B pocket residue No.

MHC allele	7	9	24	25	34	45	63	66	67	70	99
Mamu-A*01	Y	Y	A	V	V	M	E	N	M	E	V
Mamu-A*1303,NA4	-	-	-	-	-	-	-	-	-	A	-
Mafa-A*01	-	-	-	-	-	-	N	I	-	-	Y
Mafa-A*02	-	-	-	-	-	-	N	I	-	-	Y
Mafa-A*03	-	-	-	-	-	-	S	I	-	-	Y
Mafa-A*04	-	-	-	-	-	-	N	Y	-	-	L
Mafa-A*05	-	-	-	-	-	-	-	-	-	-	Y
Mafa-A*06	-	-	-	-	-	-	-	-	-	A	-
Mafa-A*07	-	H	-	-	-	-	-	K	-	-	Y
Mafa-A*08	-	H	T	-	-	K	N	I	S	N	Y
Mafa-A*09	-	-	-	-	-	E	-	I	Y	-	Y
Mafa-A*10	-	-	S	-	-	-	-	K	-	-	Y
Mafa-A*11	-	H	T	-	-	K	N	I	S	N	F
Mafa-A*12	-	-	-	-	-	E	-	I	Y	A	Y
Mafa-A*13	-	-	-	-	-	-	-	I	S	N	Y
Mafa-A*14	-	-	-	-	-	-	-	-	-	A	Y

C pocket residue No.

MHC allele	9	22	70	73	74	97	99	114	116
Mamu-A*01	Y	F	E	N	A	R	V	E	Y
Mamu-A*1303,NA4	-	-	A	-	-	-	-	-	-
Mafa-A*01	-	-	-	M	-	T	Y	-	F
Mafa-A*02	-	-	-	-	-	M	Y	D	F
Mafa-A*03	-	-	-	-	-	I	Y	S	F
Mafa-A*04	-	-	-	-	-	-	L	D	H
Mafa-A*05	-	-	-	-	-	K	Y	H	S
Mafa-A*06	-	-	A	-	-	-	-	-	-
Mafa-A*07	H	-	-	M	-	V	Y	S	-
Mafa-A*08	H	-	N	-	Y	K	Y	-	F
Mafa-A*09	-	-	-	-	Y	K	Y	-	S
Mafa-A*10	-	-	-	T	Y	T	Y	-	-
Mafa-A*11	H	-	N	T	Y	-	F	S	-
Mafa-A*12	-	-	A	T	Y	-	Y	S	-
Mafa-A*13	-	-	N	T	Y	-	Y	S	H
Mafa-A*14	-	-	A	T	Y	T	Y	D	S

D pocket residue No.

MHC allele	99	114	155	156	159	160
Mamu-A*01	V	E	S	M	Y	L
Mamu-A*1303,NA4	-	-	R	-	-	-
Mafa-A*01	Y	-	Q	-	-	-
Mafa-A*02	Y	D	Q	H	-	-
Mafa-A*03	Y	S	Q	W	-	-
Mafa-A*04	L	D	-	-	-	-
Mafa-A*05	Y	H	-	F	-	-
Mafa-A*06	-	-	Q	I	-	-
Mafa-A*07	Y	S	Q	H	-	-
Mafa-A*08	Y	-	Q	H	-	-
Mafa-A*09	Y	-	Q	-	-	-
Mafa-A*10	Y	-	Q	W	-	-
Mafa-A*11	F	S	-	F	-	V
Mafa-A*12	Y	S	-	F	-	V
Mafa-A*13	Y	S	Q	I	-	-
Mafa-A*14	Y	D	Q	Q	-	-

F pocket residue No.

MHC allele	77	80	81	116
Mamu-A*01	N	T	L	Y
Mamu-A*1303,NA4	A	N	-	-
Mafa-A*01	D	N	-	F
Mafa-A*02	-	N	-	F
Mafa-A*03	G	N	-	F
Mafa-A*04	-	N	-	H
Mafa-A*05	-	N	-	S
Mafa-A*06	-	N	-	-
Mafa-A*07	A	N	-	-
Mafa-A*08	-	N	-	F
Mafa-A*09	S	N	-	S
Mafa-A*10	-	-	-	-
Mafa-A*11	-	-	A	-
Mafa-A*12	S	N	-	H

identified at the third position of the antigen peptide (Allen et al. 1998; Dzuris et al. 2000; Sidney et al. 2000). On the other hand, anchor motif analysis of *Mamu-B*03* showed that the second as well as the C-terminal amino acids of the peptide was indispensable for binding to the MHC molecule (Dzuris et al. 2000). The second amino acid of the antigenic peptide interacts with the B pocket composed of the amino acids shown in Fig. 5. A close look at the amino acid sequences suggested that a peptide presented by *Mafa-A*06* might have a similar motif to the peptide presented by *Mamu-A*1303*, as amino acids involved in the formation of the B pocket were identical in both MHC molecules. Since it was shown that *Mamu-A*01* and *-A*1303* were associated with longer survival and lower set-point viral load (Nelson et al. 1997) in SIV-infected rhesus monkeys, it would be of interest to look at whether the presence of the *Mafa-A*06* allele in a cynomolgus monkey would affect the regulation of viral replication of SIV.

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JOURNAL OF MEDICAL PRIMATOLOGY

Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP

Uda A, Tanabayashi K, Mukai R, Terao K, Yamada A. Detection of CD3ε polymorphism in cynomolgus monkeys by a method based on RFLP. J Med Primatol 2004; 33:34–37. © Blackwell Munksgaard, 2004

Abstract: We previously reported that peripheral lymphocytes from about 12% of cynomolgus monkeys lacked reactivity with anti-rhesus monkey CD3 monoclonal antibody (FN18). The nucleotide sequence analysis of the genes encoding CD3 component proteins revealed that a single amino acid substitutions found in the CD3ε chain determined the phenotype. In this study, we attempted to develop a method based on the restriction fragment length polymorphism (RFLP) and apply it for determination of the genotypes of individual monkeys. Comparison of the phenotype determined by fluorescence-activated cell sorter analysis with the genotype determined by RFLP analysis revealed that the FN18 -positive trait was dominant over the FN18-negative trait. It was also revealed that allele frequency was significantly different among macaques depending on the geographical region where their ancestors were derived from.

Akihiko Uda[1], Kiyoshi Tanabayashi[1], Ryozaburo Mukai[1], Keiji Terao[1], Akio Yamada[2]

[1]Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Ibaraki, [2]Department of Veterinary Science, National Institute of Infectious Diseases, Tokyo, Japan

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Akio Yamada, PhD, Department of Veterinary Science, National Institute of Infectious Disease, 1-23-1 Toyama, Shinjuku, Tokyo 162-8460, Japan.
Tel.: +81 3 5285 1111;
fax: +81 3 5285 1150;
e-mail: yamada@nih.go.jp

Introduction

Cynomolgus monkeys (*Macaca fascicularis*) are important experimental animals for biomedical research and understanding immunobiology of these animals is essential for interpretation of experimental data. The FN18 monoclonal antibody (mAb), which was raised against CD3 molecules of rhesus monkey (*Macaca mulatta*), is also able to be used for identification of T cells of cynomolgus monkey; however, it was shown that T cells from some cynomolgus monkeys and rhesus monkeys did not react with FN18 mAb [1–3, 7, 8].

The nucleotide sequence analysis of cDNAs coding for CD3 components showed that CD3ε chain from FN18 non-reactive cynomolgus monkeys had two common amino acid substitutions at positions 67 and 72 [8]. We have further shown that the amino acid at position 67 played a key role in determining the FN18 responsiveness by *in vitro* experiments using several mutated CD3ε genes [7].

In this study, we attempted to establish a method for genotyping individual monkeys based on the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method and used the method to determine the allele frequencies among cynomolgus monkeys derived from different geographical regions.

Materials and Methods

Animals

All the cynomolgus monkeys studied here 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 2 and 16 years. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Disease.

RFLP analysis

PolyA mRNA extracted from peripheral blood mononuclear cells (PBMCs) of FN18-reactive

cynomolgus monkeys using the Quickprep micro mRNA purification kit (Amersham, Uppsala, Sweden) was converted into cDNA using high fidelity RNA PCR kit (Takara, Shiga, Japan). Genomic DNA was extracted form PBMCs using the GeneTLE solution (Takara). PCR of CD3ε chain was performed in 20 μl reaction mixture containing ε-sense primer (5'-CTC CAT CTC TGG AAC CAC AGT A-3') and ε-anti-sense primer (5'-CAG GTA GAG ATG ATG GCT CG-3'), 0.6–0.8 μg of genomic DNA or cDNA and 0.6 U of *ExTaq* polymerase (Takara). The size of PCR products was expected to be 207 bp. The reaction mixtures were heated at 95°C for 5 minutes, and then 40 cycles of amplification consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s was carried out followed by an additional extension at 72°C for 7 minutes. About 0.3–0.5 μg of amplified DNA were digested with 2.5 U of *Mbo*II at 37°C for 2 hour followed by agarose gel electrophoresis using 4% gel. As similar results were obtained using both cDNA and genomic DNA as templates for PCR, most part of this study was conducted using the genomic DNA as template.

Fluorescence-activated cell sorter (FACS) analysis

The 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, 0.1 mg/ml streptomycin, and 10% foetal calf serum at a concentration of 10^6 cells/ml. PBMCs (10^5) were incubated at 4°C for 1 hour with FN18 mAb (Biosource, Camlio, CA, USA), and washed twice with PBS containing 1% bovine serum albumin. After fixation with 1% paraformaldehyde at 4°C for 30 minutes, FACS analysis (FACS Caliber; Becton Dickinson, Cockeysville, MD, USA) was performed.

Results

Differentiation of genotype by the PCR-RFLP

As the nucleotide at position 200 of the FN18+ and FN18− cDNA clone was A and G, respectively, and the flanking sequence, GAAGA, gave rise to a recognition site by restriction enzyme *Mbo*II (Fig. 1), it was likely that the nucleotide difference could readily be differentiated by *Mbo*II digestion. As expected, the PCR amplicons derived from cloned FN18+ DNA was digested by *Mbo*II yielding two smaller bands, but that from FN18− clone was resistant to the digestion with *Mbo*II (Fig. 2A). We therefore attempted to apply this technique to determine the genotype of individual monkeys. There should be three distinct electrophoreic patterns of *Mbo*II-digested fragments. The PCR amplicons from homozygotes bearing A/A at position 200 should be resistant to *Mbo*II digestion, whereas those bearing G/G must be cut into two fragments. The PCR fragments from heterozygotes, however, would give rise to three fragments of 207, 113 and 94 bp, if properly digested. As incomplete digestion may be misleading, the PCR amplicons were subjected to complete digestion. In order to accomplish complete digestion, we used sufficient amount (2.5 U) of the enzyme to digest 0.3–0.5 μg of a short DNA fragment containing only one cutting site. Furthermore, the PCR amplicons from presumed heterozygotes were subjected to the nucleotide sequencing to assure that appearance of three fragments were due indeed to the heterozygocity. The results showed that both A and G were present at position 200 (data not shown), indicating that the presence of a 207-bp fragment after *Mbo*II digestion was because of the absence of *Mbo*II recognition site but not of incomplete digestion. These results indicate that RFLP analysis with *Mbo*II could be used as a powerful tool to determine the genotype of macaques.

Inheritance of the polymorphism

To analyse how this polymorphism is inherited, a family consisted of three parents (one sire and two dams) and four offspring were selected and subjected to the PCR–RFLP analysis. They were bred and raised in the Tsukuba Primate Center for Medical Science, NIID. By FACS analysis it was shown that the sire (3028) was FN18 negative while the two dams (3032 and 1159) were positive

```
                                  67          72
          61  H N G  K N K E  D S G  D R L  F L P E  F S E  80
FN18 +/+ 181  CACAATGGTA AAAACAAAGA AGATTCTGGG GATCGGCTGT TTCTGCCGGA ATTTTCAGAA 240
FN18 -/- 181  ---------- ---------G ---------- ----AA---- ---------- ---------- 240
          61  - - - - - - G - - - - Q - - - - - - - - - 80
```

Fig. 1. Nucleotide sequence of CD3ε. The nucleotide and the deduced amino acid sequences around polymorphic region were shown. Dots indicated identical nucleotide or amino acids. Recognition and cleavage site of *Mbo*II are indicated by underline and arrowhead, respectively.

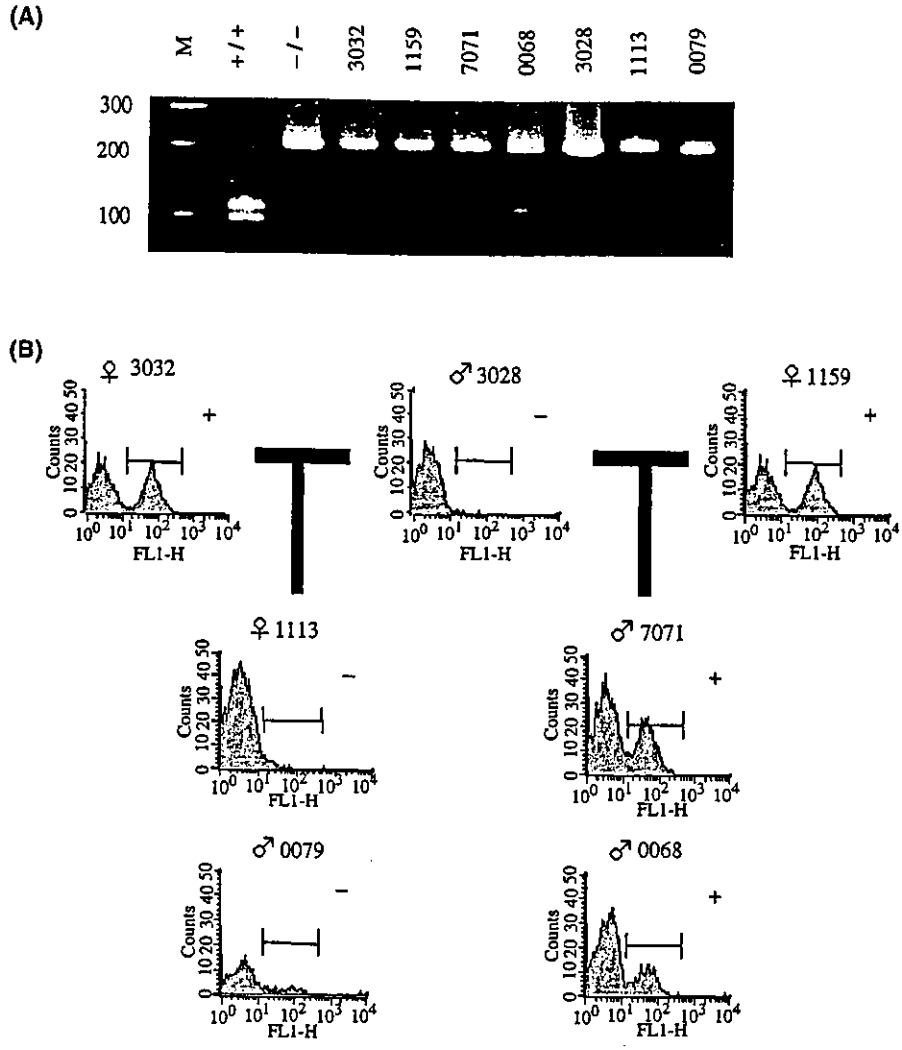

Fig. 2. The family pedigree demonstrating the inheritance of CD3ε genotypes and phenotypes. (A). The PCR products (207 bp) amplified from the genomic DNA of PBMCs were digested with *Mbo*II. The PCR products from cloned FN18 +/+ or −/− were also included as a positive or negative control. (B) The phenotypes regarding the reactivity with FN18 mAb were determined by FACS analysis. 1-H (*x*-axis): the fluorescence intensity of FN18 mAb.

(Fig. 2B). Similarly two offspring (1113 and 0079) were negative whereas the others (7071 and 0068) were positive. As the FACS profile of 0079 was rather ambiguous, we stained PBMC of this monkey with an mAb directed to monomorphic epitope of CD3ε (SP34). It was shown that SP34 positive cells did not react with FN18 confirming that 0079 was FN18 negative. The PCR–RFLP analysis using cDNA as templates showed that three monkeys (3028, 1113 and 0079) were homozygous (−/−) while the other monkeys (3032, 1159, 7071 and 0068) were heterozygous (+/−) (Fig. 2A). This finding indicates that these genes were co-dominantly expressed on RNA level. As there is no antibody available that would react with the protein expressed from the FN18 −/− genotype, the FN18-positive phenotype appeared to be inherited according to the Mendelian rules, and dominant over FN18.

Allele frequency of monkeys from different geographical areas

We then applied the RFLP technique to determine the allele frequency of this particular single nucleotide polymorphism among cynomolgus monkeys whose ancestors were introduced from three different countries – Malaysia, Indonesia and Philippines. As shown in Table 1, the frequency of three genotypes, FN18 +/+, FN18 +/− and FN18 −/− in total, was 0.576, 0.339 and 0.085, respectively. It was noted, however, that the frequency of FN18 −/− was significantly higher (0.208) in the monkeys derived from Philippines

Table 1. The frequency of CD3ε genotype in cynomolgus monkeys

	Country							
	Malaysia		Indonesia		Philippine		Total	
Genotype	Frequency	n	Frequency	n	Frequency	n	Frequency	n
FN18 +/+	0.808	38	0.675	52	0.226	12	0.576	102
FN18 +/–	0.149	7	0.299	23	0.566	30	0.339	60
FN18 –/–	0.043	2	0.026	2	0.208	11	0.085	15
Total	1.000	47	1.000	77	1.000	53	1.000	177

than in those from Malaysia (0.043) and Indonesia (0.026).

Discussion

In this study, we established a simple method for the detection of CD3 polymorphism, and applied the method to analyse the mode of inheritance of the CD3ε polymorphism. We also determined allele frequency among monkeys originated from different countries. We found that the frequency of FN18 –/– genotype was higher in the Philippine population. This might be caused by bottleneck effect as mating was carried out among monkeys of the same origin. Another possibility was that FN18-negative gene had diffused widely into Philippine population as FN18-negative phenotype might be advantageous in reproduction or adaptation, in particular environment of Philippines.

There are several reports suggesting that the polymorphism found in human CD3 might be related to type I diabetes [4–6], but controversial results are also reported. It would be interesting to see whether there are any differences in biological properties between macaques of Philippine and other places. It also seems important to look at whether there are linkages between this polymorphism and certain diseases in cynomolgus monkeys.

In conclusion, we established a simple method to identify the polymorphism of CD3ε by genotyping using RFLP. The RFLP analysis of a large number of monkeys demonstrated that the frequency of the genotype of the CD3ε differed among cynomolgus monkeys of different origin of country.

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RESEARCH ARTICLE

Collection and Analysis of Hematopoietic Progenitor Cells From Cynomolgus Macaques (*Macaca fascicularis*): Assessment of Cross-Reacting Monoclonal Antibodies

HIROAKI SHIBATA[1], YUTAKA HANAZONO[2], NAOHIDE AGEYAMA[3], TAKEYUKI NAGASHIMA[4], YASUJI UEDA[4], MAMORU HASEGAWA[4], KEIYA OZAWA[2], YASUHIRO YOSHIKAWA[5], AND KEIJI TERAO[1]*

[1]*Tsukuba Primate Center, National Institute of Infectious Diseases, Ibaraki, Japan*
[2]*Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan*
[3]*Corporation for Production and Research of Laboratory Primates, Ibaraki, Japan*
[4]*DNAVEC Research, Inc., Ibaraki, Japan*
[5]*Department of Biomedical Science, Graduate School of Agriculture and Life Science, University of Tokyo, Tokyo, Japan*

Previous studies have shown that hematopoietic progenitor cells can be isolated from human or nonhuman primate bone marrow (BM) cells. In the present study, we studied the cross-reactivity of 13 anti-human CD34, two anti-human c-Kit, and one anti-human CD133 monoclonal antibodies (mAbs) with cynomolgus macaque (*Macaca fascicularis*) BM cells, using flow cytometric analysis, cell enrichment, and clonogenic assay. Among the 13 anti-human CD34 mAbs assessed, six cross-reacted as previously reported by other groups. However, only three of these six mAbs (clones 561, 563, and 12.8) recognized cynomolgus $CD34^+$ cells that formed progenitor colonies when grown in methylcellulose culture. Similarly, of the two anti-human c-Kit mAbs (clones NU-c-kit and 95C3) that were previously reported to cross-react with cynomolgus BM cells, only one (clone NU-c-kit) resulted in a similar outcome. The anti-human CD133 mAb (clone AC133) also cross-reacted with cynomolgus BM cells, although these cells did not give rise to colonies when grown in culture. These results suggest that antibodies that cross-react with nonhuman primate cells may not identify the hematopoietic cells of interest. In addition, while the CD34 mAb (clone 561) results in the selection of hematopoietic progenitor cells of all lineages when assessed in methylcellulose culture, the c-Kit^{high} fraction (NU-c-kit) exclusively identifies erythroid-specific progenitor cells after growth in culture. It is important to consider these findings when selecting cross-reacting mAbs to identify cells of hematopoietic lineages in macaque species. Am. J. Primatol. 61:3–12, 2003.

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*Correspondence to: Keiji Terao, Director, Tsukuba Primate Center, National Institute of Infectious Diseases, 1 Hachimandai, Tsukuba, Ibaraki, 305-0843, Japan. E-mail: terao@nih.go.jp

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Key words: cynomolgus macaques; hematopoietic progenitor cells; monoclonal antibodies; cross-reactivity; CD34; c-Kit; CD133

INTRODUCTION

The CD34 antigen is widely used as a marker for the positive selection of human and macaque hematopoietic stem/progenitor cells, both in research and in clinical hematopoietic stem cell (HSC) transplantation and gene therapy [Berenson et al., 1991; Dunbar et al., 1995; Andrews et al., 1999; Cavazzana-Calvo et al., 2000; Ageyama et al., 2002]. Several anti-human CD34 monoclonal antibodies (mAbs) have previously been shown to cross-react with macaque bone marrow (BM) cells. Clones 12.8, 561, 563, 581, and QBEnd10 have been reported to cross-react with rhesus BM cells [Guadernack & Egeland, 1995; Sopper et al., 1997; Rosenzweig et al., 2001; http://research.bidmc.harvard.edu/v_path/v_pathogens.asp], whereas clones 12.8, 563, 581, QBEnd10, and NU-4A1 have been shown to cross-react with cynomolgus BM cells [Yoshino et al., 2000; http://research.bidmc.harvard.edu/v_path/v_pathogens.asp]. Among the cross-reacting CD34 mAbs, only two (clones 12.8 and 561) have been successfully used for the purpose of hematopoietic progenitor cell enrichment and HSC transplantation in macaques [Donahue et al., 1996; Banerjee et al., 1997; Ageyama et al., 2002]. The other mAbs have been evaluated for cross-reactivity by immunophenotyping. However, results from flow cytometry alone may be misleading as a result of the methods used for "gating," or the positive and negative controls used.

The c-Kit (CD117) antigen is a transmembrane tyrosine kinase receptor, the ligand of which is stem cell factor (SCF). c-Kit is expressed in immature hematopoietic cells [Kawashima et al., 1996; D'Arena et al., 1998; Ratajczak et al., 1998]. Yoshino et al. [2000] have shown by flow cytometric analysis that three anti-human c-Kit mAbs (clones 95C3, 104D2, and NU-c-kit) cross-react with macaque BM cells, whereas Rosenzweig et al. [2001] reported that clone 95C3 does not cross-react with macaque BM cells. Thus, there is some discordance among assessments by flow cytometry alone. The CD133 mAb is another candidate for the positive selection of hematopoietic stem/progenitor cells [Yin et al., 1997; Miraglia et al., 1997]. To our knowledge, however, the cross-reactivity of human CD133 mAb with macaque cells has not yet been reported.

In the present study, we examined the cross-reactivity of human CD34, c-Kit, and CD113 mAbs with cynomolgus macaque cells derived from BM using flow cytometry, cell selection and sorting, and subsequent hematopoietic progenitor clonogenic assay. In contrast to previous studies, the current results show that some cross-reaction mAbs with cynomolgus cells may not necessarily identify the cell of interest.

METHODS

Animals

Sixty-six healthy cynomolgus macaques (22 males and 44 females, 2–15 years old, 2.1–6.4 kg body weight) were reared at the Tsukuba Primate Center, and housed in accordance with the rules for animal care and management set forth by the Tsukuba Primate Center [Honjo, 1985] and the *Guiding Principles for Animal Experiments Using Nonhuman Primates* formulated by the Primate

Society of Japan [1986]. The animals were free of intestinal parasites and were seronegative for simian type-D retrovirus (SRV), herpes virus B, varicella-zoster-like virus, measles virus, and simian immunodeficiency virus (SIV) [Buchl et al., 1997]. BM collection (see below) was performed under general anesthesia by intramuscular injection of ketamine hydrochloride (Ketalar, 10 mg/kg; Sankyo, Tokyo, Japan). After the BM was harvested, the animals were administered butorphanol tartrate (0.5 mg/kg IM) daily for 3 days to alleviate any discomfort associated with the procedure.

Preparation of BM Cells

BM aspirates (10–20 ml) were collected once from the femur, iliac crest, or ischial tuberosity of each animal. Aspirates were collected into syringes with attached needles (Illinois bone marrow aspiration/intraosseous infusion needle 18G; Baxter, Deerfield, IL) containing preservative-free heparin (Sigma, St. Louis, MO). Mononuclear cells (MNCs) were isolated by density-gradient centrifugation using Ficoll Paque (1.077 g/ml; Pharmacia, Piscataway, NJ) followed by red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA; Wako, Osaka, Japan). MNCs were washed twice with phosphate-buffered saline (PBS; Sigma) containing 2% human serum type AB (Sigma) and 100 μg/ml DNase I (Sigma), and were suspended in α(–)-minimum essential medium (α(–)-MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS; Intergen, Purchase, NY) and antibiotics (100 U/ml penicillin (Banyu, Tokyo, Japan) and 0.1 mg/ml streptomycin (Meiji, Tokyo, Japan)). Nonadherent cells were collected after 1-hr incubation in tissue-culture flasks at 37°C under a 5% CO_2 condition.

Flow Cytometric Analysis and Sorting

All of the antibodies (except clone H-140) used in the present study were mouse anti-human mAbs (see Table I). The anti-CD34 antibody, H-140, is a rabbit polyclonal antibody. All antibodies were obtained from Becton Dickinson (Franklin Lakes, NJ), PharMingen (San Diego, CA), Beckman Coulter (Miami, FL), CellPro (Bothell, WA), Nichirei (Tokyo, Japan), Medical & Biological Laboratories (MBL; Nagoya, Japan), Santa Cruz Biotechnology (Santa Cruz, CA), or Miltenyi Biotec (Bergisch Gladbach, Germany), as shown in Table I. Unlabeled mAbs, ICO115 (anti-CD34) and H-140 (anti-CD34) were detected with fluorescein isothiocyanate (FITC)-conjugated goat $(Fab')_2$ anti-mouse IgG/M mAb (Biosource, Camarillo, CA) and FITC-conjugated anti-rabbit immunoglobulins antibody (Dako, Copenhagen, Denmark), respectively. To block nonspecific binding via Fc receptors, aggregated human IgG (Sigma) was included at a concentration of 100 μg/ml in the blocking buffer. Biotinylated anti-CD34 mAb (clone 12.8) was detected with phycoerythrin (PE)-conjugated streptavidin (Beckman Coulter). Isotype-matched, irrelevant mAbs (Dako) served as controls. The cells were incubated with each antibody in the washing medium (PBS with 2% FCS and 0.1% NaN_3) for 30 min at 4°C, and were washed with the washing medium twice followed by fixation with 1% paraformaldehyde (Wako)-PBS. Flow cytometric analysis was performed using a FACS Calibur flow cytometer (Becton Dickinson) equipped with an argon-ion laser set at 488 nm. Data acquisition and analysis were performed using the CellQuest software (Becton Dickinson). Each antibody was evaluated for cross-reactivity using MNCs from at least three

TABLE I. Cross-Reactivity of Anti-Human Monoclonal Antibodies With Cynomolgus Macaque Bone Marrow Cells

Antigen	Clone	Source	Cross-reactivity[a]
CD34	My10	Becton Dickinson	−
	8G12	Becton Dickinson	−
	QBEnd10	Beckman Coulter	±
	Immu-133	Beckman Coulter	−
	Immu-409	Beckman Coulter	−
	12.8	CellPro	+
	561	Dynal	+[b]
	563	PharMingen	+
	581(formerly ICH-3)	Beckman Coulter	±
	NU-4A1	Nichirei	−
	H-140[c]	Santa Cruz	−
	ICO115	Santa Cruz	−
	AC136	Miltenyi Biotec	−
CD117 (c-kit)	NU-c-kit	Nichirei	+
	95C3	MBL	−
CD133	AC133	Miltenyi Biotec	±

[a]Evaluated by flow cytometry: +, positive reaction; ±, weak staining; −, negative reaction.
[b]Evaluated by the immuno-magnetic separation method (see Methods).
[c]Rabbit polyclonal antibody.

different animals. To ensure that small populations were reliably detected, more than 10,000 events were acquired for analysis.

For cell sorting, nonadherent MNCs were incubated with each mAb (anti-CD34, anti-c-Kit, or anti-CD133) for 1 hr at 4°C. They were then washed and resuspended in PBS containing 2% human serum type AB and 0.1% NaN_3. All of the cells were stained with propidium iodide (PI; 5 μg/ml) for 5 min at 4°C so that viable cells could be enumerated prior to sorting. Experiments were performed using MNCs from two or three different animals. The cells were sorted using a FACS Vantage (Becton Dickinson) or EPICS ELITE (Beckman Coulter) cell sorter, each of which was equipped with an argon-ion laser. Data acquisition and analysis were performed using CellQuest or EXPO2 software (Beckman Coulter), respectively.

Immunomagnetic Cell Selection

BM cells were rosetted with Dynabeads M450 directly coated with the anti-CD34 mAb clone 561 (Dynal, Oslo, Norway) for 45 min at 4°C on an apparatus that provided tilting and gentle rotation (Dynal). A cell density of 1–2 × 10^8 cells/ml (beads to cell ratio = 1:1) was found to be optimal. The rosetted cells and beads were suspended in a tube containing 8 ml of chilled PBS with 0.5% bovine serum albumin (BSA; Sigma) and 5 mM EDTA, and the tube was attached to a magnet stand (Dynal). Non-rosetted ($CD34^-$) cells were removed by aspiration, and rosetted cells that were retained in the tube were washed five times. The beads were detached from rosetted cells by incubation with 100 μl DETACHaBEAD (Dynal) in a final volume of 300 μl for 15 min at 37°C with gentle shaking. After incubation, 8 ml of the above-mentioned buffer was added and beads were removed by the magnets (repeated five times). To completely remove the beads,

resuspended cells were passed through the MACS separation column (Miltenyi Biotec). $CD34^+$ cells were washed with the buffer and counted.

Clonogenic Hematopoietic Progenitor Assay

The cells (100–1,000 sorted cells by each mAb) were plated in a 35-mm petri dish in 1 ml of α(–)-MEM containing 1.2% methylcellulose (Shin-Etsu Chemicals, Tokyo, Japan) supplemented with 2 U/ml recombinant human erythropoietin (Roche, Basel, Switzerland), 100 ng/ml recombinant human interleukin-3 (PeproTech, Rocky Hill, NJ), 100 ng/ml recombinant human interleukin-11 (PeproTech), 100 ng/ml recombinant human SCF (Biosource, Camarillo CA), 20% FCS, 1% deionized BSA, 5×10^{-5} M 2-mercaptoethanol (Sigma), and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). After incubation for 10–14 days at 37°C with 5% CO_2, colonies containing >50 cells were counted using an inverted light microscope (Nikon, Tokyo, Japan). Experiments were performed in triplicate. The average and the standard deviation (SD) of colony numbers per 500 cells were calculated.

RESULTS

Antibody Cross-Reactivity Assessed by Flow Cytometry

We first examined the cross-reactivity of anti-human CD34, c-Kit, and CD133 mAbs with the cynomolgus macaque samples by flow cytometric analysis. Isotype-matched mAb staining served as the negative control. As shown in Table I, although four anti-human CD34 mAbs (QBEnd10, 12.8, 563, and 581) cross-reacted with cynomolgus BM cells, the intensity of cross-reactivity varied considerably among the mAbs. Clones 12.8 and 563 cross-reacted strongly, while clones QBEnd10 and 581 showed weak or inconsistent results. Clone NU-4A1 did not cross-react with cynomolgus BM cells. Clone 561 was examined for cross-reactivity by immunomagnetic separation only, since this mAb is not commercially available for use in flow cytometric analyses.

Of the two anti-human c-Kit mAbs, clone NU-c-kit was shown to cross-react with cynomolgus BM cells by flow cytometry, but clone 95C3 resulted in a negative outcome. The anti-human CD113 mAb, clone AC133, was shown to cross-react with cynomolgus BM cells, albeit weakly.

Clonogenic Assay of Cynomolgus BM Cells Sorted or Immunoselected by Anti-Human CD34 and CD133 mAbs

We examined whether clonogenic progenitor cells could be grown after being sorted by the CD34 and CD133 mAbs that were shown to cross-react by flow cytometric analysis or immunomagnetic cell selection. Experiments showed similar results between animals (two or three animals per antibody), as demonstrated in Table II. Although the 561^+ and 563^+ cells showed a significant growth of clonogenic progenitor cells (colony-forming units (CFUs)) in culture, the 581^+, $QBEnd10^+$, and $AC133^+$ cells resulted in no detectable CFUs. These results suggest that antibodies that cross-react with macaque cells may not identify the hematopoietic progenitor cells of interest.

Some differences in CFU numbers between $CD34^+$ fractions (561^+ and 563^+ fractions) were found and attributed to individual differences between monkeys. We were not able to examine whether the 12.8^+ cells included CFU, since this clone is no longer commercially available.

TABLE II. Colony Formation From Sorted or Selected Bone Marrow Cells

Antigen	mAb[a]	Fraction	CFU/500 cells[b]
CD34	QBEnd10	−	160±5
		+	0
	561	−	0
		+	325±40
	563	−	10±5
		+	460±95
		−	115±10
		+	0
	581	−	55±10
CD133	AC133	+	0

[a]Sorted by flow cytometry, except the clone 561$^+$ cells that were immunoselected by magnetic beads.
[b]Experiments were conducted in triplicate and repeated two or three times using samples from different animals. Data represent the mean ± SD of a representative experiment.

TABLE III. Colony Formation From Cynomolgus Bone Marrow CD34$^+$ Cells

	Colonies per 500 sorted cells[a]			
Cell fractions[b]	CFU-GM[c]	BFU-E[d]	GMEmix[e]	Total
$CD34^{negative}$	1.5±1.0	0	0	1.5±1.0
$CD34^{positive}$	48.0±5.2	13.7±1.5	1.0±1.7	62.7±3.1

[a]Experiments were conducted in triplicate and repeated three times using samples from different animals. Data represent the mean ± SD of a representative experiment.
[b]Sorted by clone 563.
[c]Colony dorming unit-granulocyte, macrophage.
[d]Burst forming unit-erythroid.
[e]Granulocyte, macrophage, and erythroid.

We conducted clonogenic assays with cynomolgus BM cells fractionated using clone 561. Three separate experiments showed that this clone almost exclusively results in the growth of progenitor cells of all hematopoietic lineages (CFU-GM, BFU-E, and GMEmix) from the CD34$^+$ fraction (Table III).

Clonogenic Assay of Cynomolgus BM Cells Sorted by Anti-Human c-Kit mAbs

Cynomolgus BM cells were analyzed for the expression of c-Kit using the clone NU-c-Kit (see Fig. 1). In Fig. 1A, a gate was set on live (PI$^-$) mononuclear cells. In Fig. 1B, the gated cells were divided into three subgroups according to the expression of c-Kit; c-Kit^{high} (8.0% ± 4.2%; mean ± SD, n = 5), c-Kit^{low} (8.0% ± 2.6%), and c-Kit$^-$ (84.0% ± 4.8%). Figure 1C–E show profiles of sorted c-Kit$^-$, c-Kit^{low}, and c-Kit^{high} cells, with a purity of 97%, 90%, and 90%, respectively. Clonogenic assays were conducted on each sorted subgroup. Experiments were repeated using BM cells from three different animals, and similar results were obtained (see Table IV). The c-Kit$^-$ fraction included no detectable CFUs. On the other hand, both c-Kit^{low} and c-Kit^{high} fractions resulted in significant numbers of CFUs, although there were more CFUs formed from the c-Kit^{high} fraction than from the c-Kit^{low} fraction. Of note, the c-Kit^{high} fraction included only erythroid

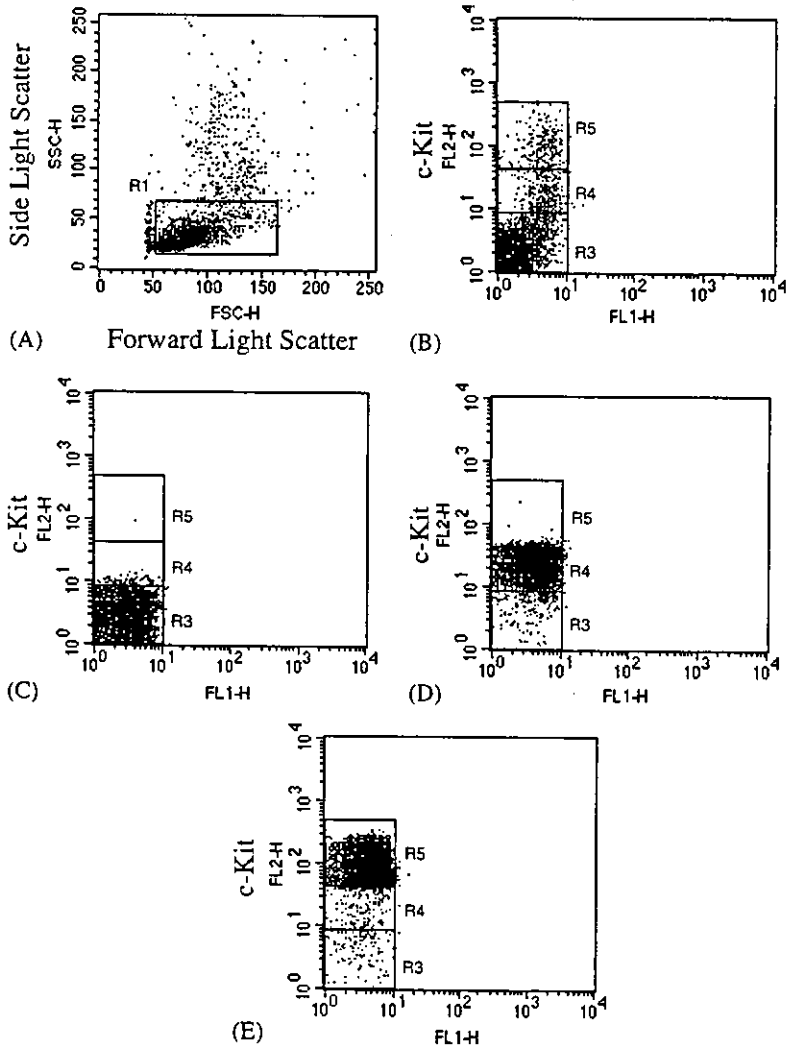

Fig. 1. Flow cytometric analysis of c-Kit expression in cynomolgus BM cells. Three independent experiments were conducted, and one representative dot-plot profile is shown. A: R1 indicates the gate for mononuclear cells. B: R3, R4, and R5 indicate the gates for the c-Kit$^-$ fraction (85%), c-Kit^{low} fraction (8%), and c-Kit^{high} fraction (7%), respectively, derived from R1 and PI$^-$ cells. BM cells were sorted based on c-Kit expression: (C) c-Kit$^-$, (D) c-Kit^{low}, and (E) c-Kit^{high} cells. The purity was 97%, 90%, and 90%, respectively.

progenitor cells (BFU-E), and multipotential CFUs (GMEmix) were detected only in the c-Kit^{low} fraction.

DISCUSSION

Although six anti-human CD34 mAb clones have been reported to cross-react with macaque BM cells, as assessed by flow cytometry, we have shown that one of these antibodies does not result in CFU growth when assessed in culture. Thus, immunophenotyping alone may be misleading. These findings may be a result of the gating methods used, or may be due to inappropriate controls. We have thus confirmed that three anti-human CD34 mAbs (clones 561, 563, and 12.8) truly recognize cynomolgus CD34$^+$ cells, which, when grown in methylcellulose

TABLE IV. Colony Formation of Cynomolgus Bone Marrow Cells Separated on the Basis of c-Kit Expression

Cell fractions[b]	Colonies per 500 sorted cells[a]			
	CFU-GM[c]	BFU-E[d]	GMEmix[e]	Total
c-Kit negative	0	0	0	0
c-Kit^{low}	50±10	30±10	5±5	85±25
c-Kit^{high}	0	135±20	0	135±20

[a]Experiments were conducted in triplicate and repeated three times using samples from different animals. Data represent the mean±SD of a representative experiment.
[b]Sorted by clone NU-c-kit.
[c]Colony forming unit-granulocyte, macrophage.
[d]Burst forming unit-erythroid.
[e]Granulocyte, macrophage, and erythroid.

culture, result in erythroid and myeloid progenitor colonies. Among these clones, only clones 561 and 563 are commercially available.

The CD34 molecule has many O- and N-linked glycosylation sites that give rise to different epitopes [Sutherland & Keating, 1992]. These epitopes can be grouped into three classes [Greaves et al., 1992]. Glycosylation of the macaque CD34 is considerably different from that of the human CD34 antigen (unpublished data), and this may be why only a few anti-human CD34 mAbs cross-react with the macaque CD34 antigen. In fact, the cross-reacting CD34$^+$ mAbs clones 561 and 563, which give rise to colonies in culture, recognize the same epitope class (group III) [Gaudernack & Egeland, 1995]. The tertiary structure of this particular epitope may be similar in humans and macaques.

When we used cross-reacting c-Kit mAbs (NU-c-kit), we found that only erythroid-specific progenitor cells could be grown in methylcellulose culture from the sorted cynomolgus c-Kit^{high} fraction. Although some groups have also reported that erythroid progenitor cells are mostly included in the c-Kit^{high} fraction in humans [Sakaba et al., 1997], others concluded that erythroid progenitor cells are mainly found in the c-Kit^{low} fraction in humans [Gunji et al., 1993]. The discordance among these results may be explained by species differences and differences in the antibodies chosen. In addition, because some c-Kit mAbs may inhibit the growth of c-Kit$^+$ cells [Broudy et al., 1992; Gunji et al., 1993], it is possible that the c-Kit$^+$ cells did not develop adequately in culture post selection.

In conclusion, the current results suggest that mAbs used for immunophenotyping may not necessarily identify the cells of interest. When cynomolgus BM cells were sorted or immunoselected using defined cross-reacting CD34 and c-Kit mAbs, different hematopoietic progenitor cell populations resulted after growth in methylcellose culture. It is important to consider these findings when selecting cross-reacting mAbs to identify cells of hematopoietic lineages in macaque species.

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