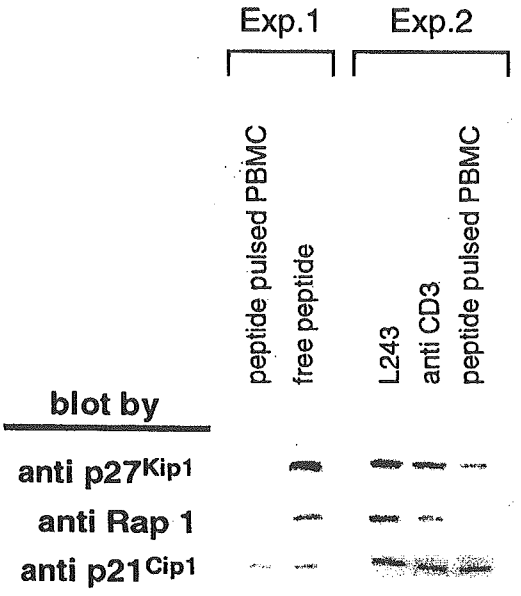

Figure 11. Expression of CDK inhibitors. T cell clone SF36. 16 were stimulated using a soluble form of antigenic peptide (1μM BCGap84-100), immobilized anti-CD3 (OKT3), immobilized anti-DR (L243) or irradiated autologous PBMC prepulsed with the wild-type peptide (5 μM BCGap84-100 for 5 h at 37 °C) at 37 °C. After 7 days of incubation, the T cells were lysed. Cell lysates were directly resolved on 13.5 % SDS-PAGE, transferred to nitrocellulose membranes then blotted using Abs to CDK inhibitor $p27^{Kip1}$ and CDK inhibitor $p21^{Cip1}$.

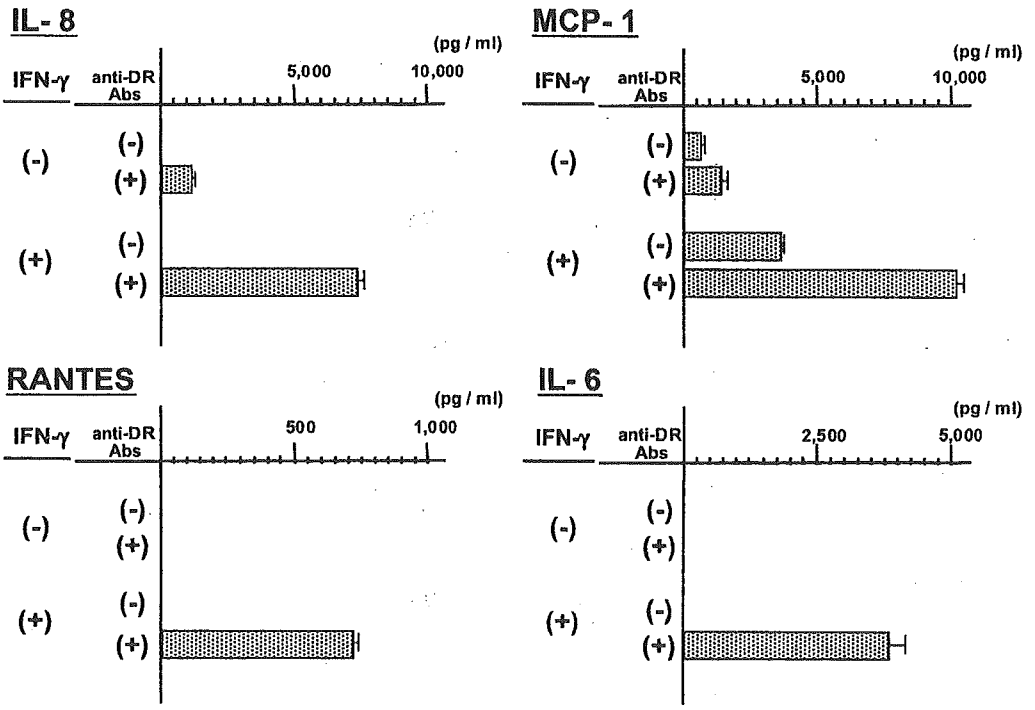

Figure 12. Cytokine productivity of PDL by the stimulus with anti-HLA-DR (L243) mAb. IFN-γ-treated or –untreated PDL ($1x10^4$ cells/well) were cultured with or without L243 in the presence of goat anti-mouse IgG2a Ab. The supernatants were collected following 16 hr culture of the cells, and concentrations of IL-1β, IL-6, IL-8, MCP-1 and RANTES were determined using ELISA kits as in "Materials and Methods".

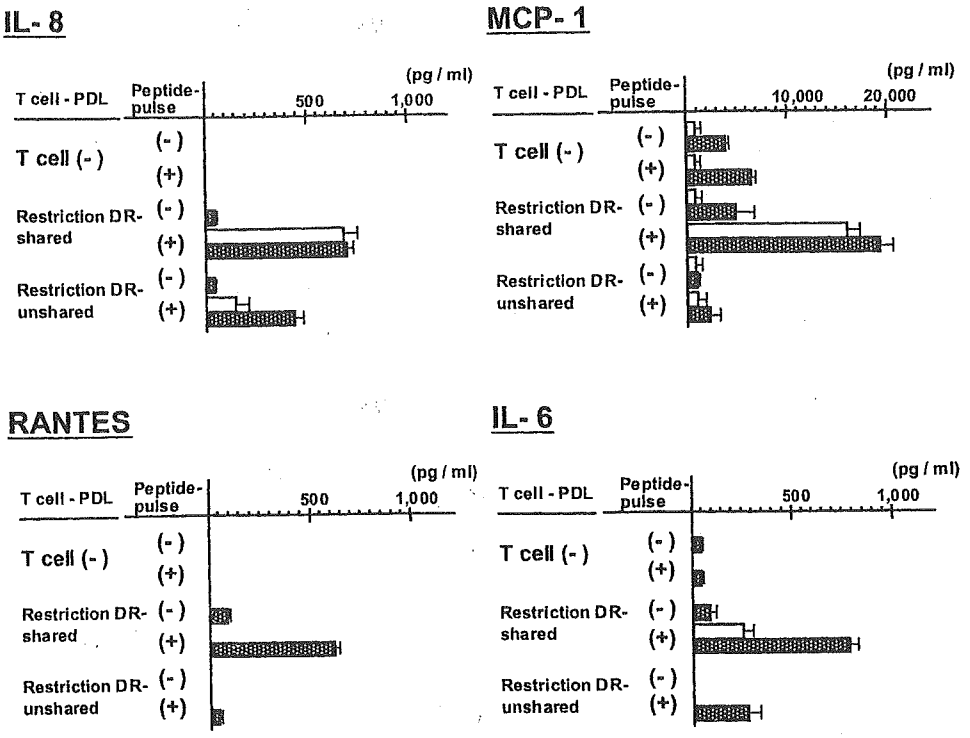

Figure 13. Cytokine productivity of PDL by stimulation via HLA-II molecules when PDL were co-cultured with emetine-treated T cells. PDL ($1x10^4$ cells/well) was pulsed with or without antigenic peptide in the serum free medium for 24 hr following 72 hr culture with or without IFN-γ before the cells were cultured with emetine-treated T cells. The supernatants were collected following 16 hr culture of the cells, and cytokine concentrations were determined. Shaded and open bars indicate the results of IFN-γ-treated and –untreated PDL, respectively.

compared with non-treated and non-pulsed PDL. Moreover, PDL expressing HLA-DRB1*1501 molecules produced the large amounts of IL-8, IL-6, MCP-1 and RANTES when emetine-treated T cells (restricted by DRB1*1501) was co-cultured with PDL. MCP-1 and RANTES were produced only when peptide-pulsed PDL was cultured with restriction DR-shared T cells, whereas IL-6 and IL-8 were produced even when restriction DR-unshared T cells were co-cultured. Although RANTES and IL-6 productivity was relatively higher in IFN-γ treated, peptide-pulsed PDL cultured with DR-restricted T cells, no significant difference was found in IL-8 and MCP-1 productivity between them.

DISCUSSION

The observation that IFN-γ / IL-4 produced by T cells are associated with HLA restriction molecules even in freshly isolated short-term T-cell clonal responses to crude protein antigens or randomized peptide antigens, is evidence that the phenomenon is not limited to three T-cell clones used in this study. Thus, although not being complete as shown in Fig. 3, HLA class II subregions may determine T cell differentiation patterns or IFN-γ / IL-4, probably through monocyte responses. However, one might speculate that DR-peptide complex deliver strongest avidity between TCR, leading to Th1-prone responses (28,29). However, absence of DR-restricted T cell clones

with low IFN-γ / IL-4 rules out this possibility, because low-affinity DR-binding peptides should exist, which should activate Th2-prone responses, if the phenomenon is attributed to avidity alone. Indeed, it is likely that such a phenomenon is attributed to high IL-12 production through DR signaling (Table 1).

PKC and Syk are associated (30) and involved in signaling through MHC molecules. We observed a slight enhancement of monokine production by PKC inhibitor GF109203X and Syk inhibitor piceatannol. The precise mechanisms are yet to be determined, but one might speculate that they negatively regulate monokine production induced by ligating class II HLA.

Because transmembrane and intracellular domains are markedly different among α and β chains of HLA-DR, -DQ and -DP, and MHC molecules have no immunoreceptor tyrosine-based activation motif, it is reasonable to speculate that HLA-DR, -DQ or -DP molecules are associated with distinct signal transduction molecules. Differential endosomal trafficking / recycling (31), differential signaling in monocyte subsets (32), including contaminated dendritic cells, and even differential localization in membrane microdomains would also need to be considered. Study is currently underway, to address these questions, using various monocytic cell lines and mass mapping techniques.

IgM contributes to early defense against microbial infections (33). When B cells are exposed to non-self antigens, such as those of microbial origin, B cells bearing surface IgM specific for the antigen are capable of concentrating the antigen and present it effectively to T cells. We found that cross-linking DR molecules up-regulates not only secretory-type but also membrane-type μ chains, which may indicate that cross-linking DR molecules leads to more effective antigen presentation. It is also important to note that CD40-generated signals arrest B-cell terminal differentiation to produce Igs (34). Although DR-mediated signals appear to up-regulate IgM production in the absence of CD40-CD154 interaction, further investigation is needed to determine whether or not the generation of signals via CD40 under physiological T-B interactions interferes with IgM production induced by DR-mediated signals. In this study, ligation of DR molecules not only with specific Abs (either solid-phase Abs or soluble Abs), but also with HLA-peptide-TCR interaction, induced IgM production, suggesting that signals via DR alone are capable of inducing up-regulation of IgM, which may also occur in physiological T-B interactions. In this relation, DR-mismatched transplantation should be one of rare cases, in which massive T-B interaction via DR occurs *in vivo*. Indeed, when DNA typing of HLA-DR was unavailable, graft-vs-host disease was frequent, and such patients reportedly had deposition of IgM at the dermo-epidermal junction. (35).

BCR-Ag-complex is internalized to supply T-cell epitopes, and subsequent DR-peptide-TCR interaction results in class switching, which eventually leads to decreased IgM production (36). Indeed, our experimental system did not allow BCR to interact with protein antigens, and T cells were treated with emetine (thereby bearing no class switch pressure). Such a system might have up-regulated IgM to be readily detected. However, because the disappearance of surface IgM at antigen presentation (before class switching) is incomplete, one might speculate that signaling through DR supplies new IgM molecules, for a short and critical period of time for T-B interaction before class switching is initiated. Other factors should also be considered, because even with thymus-independent antigens, IgM production from B cells can be induced (37).

In case of the anergy induced by soluble antigenic peptide, signals should be transmitted to T cells not only via TCR but also via class II HLA because T cells express both TCR and HLA-DR. Although anergy induced by the lack of co-stimulation was rescued by signaling via CD28, anergy induced by soluble antigenic peptide was not rescued (38), which is also the case in our present study (data not shown). It is therefore likely that molecular mechanisms differ between anergy induced by the lack of co-stimulation and that induced by anti-DR, even though the behavior of CDK inhibitors are apparently the same.

Fibroblasts are known to participate in the immune system because of their expression of several immunoregulatory molecules on their cell surfaces, as well as many cytokine species. It was reported that fibroblasts produce IL-8 by the stimulus with several inflammatory cytokines (IL-1β and TNF-α; 39,40) and LPS (41,42). A previous study demonstrated that the engagement of MHC class II molecules by the superantigen including SEA and *Mycoplasma arthritidis*-derived superantigen induced the RANTES, MCP-1 and IL-8 mRNA expression in synovial fibroblasts (43). Herein, we demonstrated that PDL produced large amounts of chemokines when cultured with emetine-treated T cells, under restriction DR-shared conditions. Thus, signaling via HLA-II molecules into fibroblasts is not only induced by the engagement of MHC class II molecules with superantigens, but also by making the DR-peptide-TCR complex.

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Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12

HIDEKI OHYAMA,[1] NAHOKO KATO,[2] KAZU TAKEUCHI,[2] YOSHIHIKO SOGA,[2] YASUSHI UEMURA,[1] FUSANORI NISHIMURA[2] and SHO MATSUSHITA[1]

[1]Department of Allergy and Immunology, Saitama Medical School, Moroyama and [2]Department of Patho-physiology/Periodontal Science, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan

Ohyama H, Kato K, Takeuchi K, Soga Y, Uemura Y, Nishimura F, Matsushita S. Monocytes of distinct clinical types of leprosy are differentially activated by cross-linking class II HLA molecules to secrete IL-12. APMIS 2004;112:271–4.

Leprosy is characterized by a wide spectrum of clinical features depending on the individual differences in Th1-type immunity. The objective of this study was to evaluate whether monocyte activation by stimulus via class II HLA molecules would be correlated with the differences in cellular immune responses among diverse clinical forms of leprosy. IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs was estimated in patients with lepromatous- and tuberculoid-type leprosy. We found that monocytes from lepromatous patients produced significantly higher (about 4-fold higher) amounts of IL-12 as compared to in patients with tuberculoid type of leprosy when class II HLA molecules were cross-linked with anti-HLA class II antibodies, whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via class II HLA molecules regardless of the clinical form of leprosy. These results suggest that monocyte activation differs between lepromatous and tuberculoid patients in terms of IL-12 secretion, which might be related to individual differences in the cellular immune responses according to the clinical type of leprosy.

Key words: Class II HLA; IL-12; leprosy patients; cellular immune response.

Hideki Ohyama, Department of Allergy and Immunology, Saitama Medical School, 38 Morohongo, Moroyama 350-0495, Japan. e-mail: imohyama@saitama-med.ac.jp

Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features (1). Tuberculoid type of leprosy is at one end and lepromatous leprosy at the other end of the spectrum. Tuberculoid patients show a high level of cell-mediated immunity (CMI) responses against *M. leprae*, which results in resistance to infection, whereas lepromatous patients show poor CMI responses against the pathogen and progressive form of the disease. This clinical spectrum of leprosy is explained by the differences in responses to *M. leprae* among individuals. The differences of T-cell subsets accumulating in leprosy lesions may account for the diversity of protective patterns to *M. leprae*. In the case of *M. leprae* infection, the clinical type of the disease depends on individual differences in Th1-type immunity. One of the most important cytokines produced by Th1, IFN-γ, promotes activation of macrophages, thus leading the host immunity toward cellular immunity to these bacteria. IL-12, which is produced by antigen-presenting cells (APCs), is

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well-known as a powerful inducer of IFN-γ production from Th1 cells. In leprosy patients, IL-12 productivity is likely to influence the cellular immune responsiveness in patients with lepromatous and tuberculoid type of the disease (2, 3). Toll-like receptor 2 (TLR2) is thought to be a key molecule in inducing the IL-12 production from monocytes in the response against mycobacterial pathogens (4), and the polymorphism of the TLR2 gene is likely to affect the low productivities of IL-12 in the lepromatous type of leprosy patients (5).

Meanwhile, recent studies have suggested that class II HLA (HLA-II) molecules not only act as antigen-presenting molecules, but also as receptor molecules to transduce signals into APCs, resulting in the production of several cytokines, including IL-1β and IL-12 (6). From this point of view, it is likely that the difference in IL-12 production induced by the stimulus via HLA-II molecules will also be a possible factor implicated in determination of the clinical type of leprosy.

In this study, we measured IL-12 production from APCs by stimulus via HLA-II molecules in humans with leprosy, in order to find the differences in CMI activities in vitro between the patients with lepromatous and tuberculoid types of leprosy.

MATERIALS AND METHODS

Study population

Ten leprosy patients, including 5 LL patients and 5 TT patients, and 7 healthy individuals were enrolled in this study. Patients were clinically diagnosed as having each type of leprosy based on the description of Ridley & Jopling (1) and with reference to their results in the Mitsuda test and their sequelae.

Cell preparation

Mononuclear cells were prepared by the Ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) gradient solution method. Peripheral blood mononuclear cells (PBMC) were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human sera (HS) and placed on HS-pretreated plastic dishes (Falcon, Becton Dickinson, Lincoln Park, NJ). These dishes were incubated for 2 h at 37°C. After the dish-nonadherent cells were harvested and extensive washes, the adherent cells were removed by washing with ice-cold PBS containing 0.04% EDTA and by scraping them with a rubber policeman. This fraction was served as a monocyte fraction. More than 90% of the adherent cell fraction was CD14-positive.

Inducing the monokine production from monocytes

Mouse anti-HLA-DR (L243: Leinco Technologies Inc., Ballwin, MO), anti-DQ (1a3: Leinco Technologies Inc.) monoclonal antibodies (mAbs) and isotype-matched control antibodies (mouse IgG2a) were coated onto 96-well flat-bottomed culture plates for a day at 10 μg/ml PBS. After extensive washing of the plates with PBS, monocytes were added at 2×10^4 cells/well, and incubated for 16 h at 37 °C in a CO_2 incubator (7).

Quantitation of cytokines

Culture supernatants of monocytes stimulated via HLA-II molecules were collected. The hIL-1β and hIL-12 (p40 & p70) ELISA kits (Endogen Inc. Woburn, MA) were used for the quantitation of cytokines in the supernatants. The detection limit of both these cytokines is 5 pg/ml.

RESULTS AND DISCUSSION

We first estimated IL-1β and IL-12 productivity in monocyte preparations obtained from PBMCs of lepromatous, tuberculoid, and healthy Japanese subjects. Monocytes from lepromatous patients produced significantly higher amounts of IL-12 as compared with those from patients with tuberculoid type leprosy (Mann-Whitney *U* test; $p<0.05$), whereas almost equal amounts of IL-1β were produced from each monocyte preparation by stimulus via HLA-II molecules regardless of the clinical form of leprosy. In particular, monocyte preparations from three tuberculoid patients produced extremely low amounts of IL-12. A small amount of IL-β and IL-12 was detected when monocytes were cultured in a dish coated with isotype-matched antibodies as control. There were no differences between DR-induced and DQ-induced cytokine productivity from monocytes.

In this regard, the present findings are not in agreement with those of some previous studies showing that IL-12 mRNA is more highly expressed in tuberculoid lesions as compared to lepromatous lesions (2, 3). According to the results of these previous studies, tuberculoid patients are supposed to allow *M. leprae* infection although they have the ability to produce a suf-

TABLE 1. *IL-1β and IL-12 production from monocytes by signaling via HLA-II molecules*

Clinical type	IL-1β (pg/ml)	IL-12 (pg/ml)	IFN-γ/IL-12
L-lep	322±107	402±299 †	2.93±2.89 ‡
T-lep	264±138	110±119 †	28.03±8.71 ‡
HC	418±340	304±225	N.D

The amounts of cytokines are given as the mean value of duplicate cultures with L243, after subtraction of the mean value obtained from cultures with control antibodies in each subject. L-lep, T-lep and HC represent patients with lepromatous type of leprosy, tuberculoid type of leprosy, and healthy controls, respectively.
†; Mann-Whitney *U* test, $p<0.05$.
‡; Mann-Whitney *U* test, $p<0.01$.

ficient volume of IL-12 to induce CMI responses. Moreover, *M. leprae* has the longest doubling time of all known bacteria and has massive gene decay, including the genes coding virulence determinants (8). From this viewpoint, we believe that tuberculoid patients are also susceptible to *M. leprae*. Thus, we speculate that the low productivities of IL-12 by stimulus via HLA-II molecules in tuberculoid patients might have an influence on the host defense function in early stage *M. leprae* infection.

We previously reported the lymphokine productivity of T-cell lines established from PBMCs in response to Major Membrane Protein II (MMPII), one of the outer membrane protein species derived from *M. leprae* (9). In such studies, however, it was impossible to classify leprosy patients based on IFN-γ productivity in T-cell lines. We thus considered that the balance between IL-12 from monocytes produced by stimulus via HLA class II molecules, and IFN-γ from Th cells might explain individual differences in CMI activities of clinical types of leprosy. The IFN-γ/IL-12 ratio in each subject was calculated and revealed that the ratio of lepromatous patients was significantly lower than that of tuberculoid patients ($p<0.01$). These findings indicate that insufficient IFN-γ production was induced by IL-12 in lepromatous patients, and this might reflect the abnormality of cellular immune responses observed in the lepromatous type of leprosy.

We speculate that this phenomenon might reflect the abnormality in cellular immune responses seen in the lepromatous type of leprosy. In this study, however, we did not evaluate the cytokine productivity from monocytes using antigen-specific stimuli. Further studies are needed to clarify whether the difference of IFN-γ/IL-12 ratio between lepromatous and tuberculoid patients depends upon the antigen-specific immune responses.

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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

Key words: allele – CD3 – cynomolgus – FN18 – polymorphism

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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.

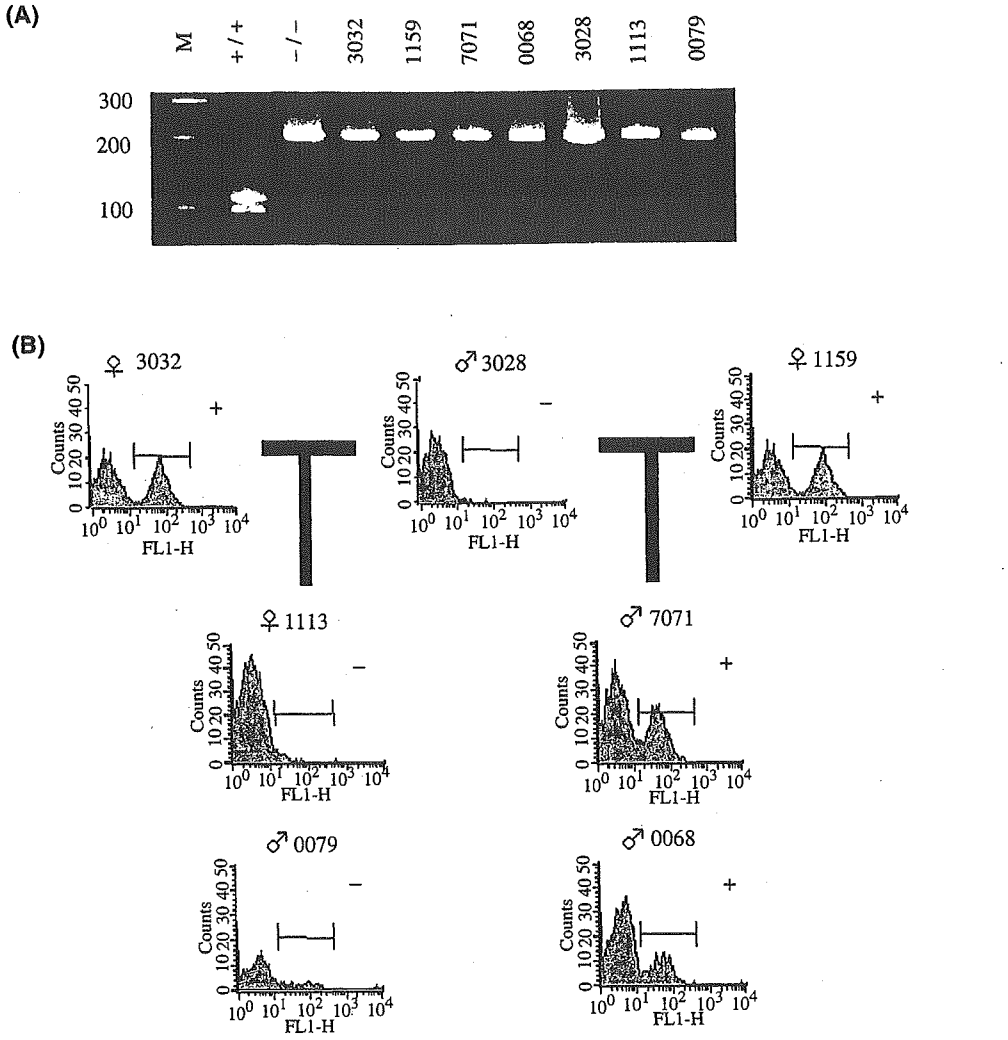

Fig. 2. The family pedigree demonstrating the inheritance of CD3ε genotypes and phenotypes. (A). The PCR products (207 bp) amplified from the genomic DNA of PBMCs were digested with *Mbo*II. The PCR products from cloned FN18 +/+ or −/− were also included as a positive or negative control. (B) The phenotypes 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.

Acknowledgments

We thank Mr K. Ono, A. Hiyaoka, and other staff in The Corporation of Production and Breeding of Primate for animal care and blood collection. This study was supported by the Program of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan, and by the Health Science Research Grants from the Ministry of Health and Welfare of Japan.

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ORIGINAL PAPER

Akihiko Uda · Kiyoshi Tanabayashi · Yasuko K. Yamada · Hirofumi Akari · Young-Jung Lee · Ryozaburo Mukai · Keiji Terao · Akio Yamada

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

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Abstract A basic understanding of the major histocompatibility complex (MHC) class I, which, together with T-cell receptors, is a key player in antigen recognition by cytotoxic T lymphocytes, is necessary to study the cellular immune response to intracellular pathogens. The MHC has hardly been reported in cynomolgus monkeys (*Macaca facicularis*), although cynomolgus monkeys have been frequently used as the surrogate animal model. We attempted to determine the nucleotide sequences of the MHC class I *A* locus of cynomolgus monkeys (*Mafa-A*) and eventually 34 independent sequences of *Mafa-A* were obtained from 29 cynomolgus monkeys. These 34 sequences were classified into 14 *Mafa-A* alleles according to the results of phylogenetic analyses using the neighbor-joining method. One to three *Mafa-A* alleles were obtained from a single animal. We also tried to establish a multiplex PCR-SSP method for convenient typing of *Mafa-A* alleles. cDNA from a family of cynomolgus monkeys, which is composed of four sirs and four dams, were examined by multiplex PCR-SSP. The result of multiplex PCR-SSP showed that an individual cynomolgus monkey had two or three *Mafa-A* alleles, suggesting that the *A* locus of cynomolgus monkeys might be duplicated.

A. Uda · H. Akari · Y.-J. Lee · R. Mukai · K. Terao
Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases,
1 Hachimandai, Tsukuba,
305-0843 Ibaraki, Japan

K. Tanabayashi · A. Yamada (✉)
Department of Veterinary Science, National Institute of Infectious Diseases,
1-23-1 Toyama, Shinjuku,
162-8640 Tokyo, Japan
e-mail: yamada@nih.go.jp
Tel.: +81-3-52851111
Fax: +81-3-52851150

Y. K. Yamada
Division of Experimental Animals Research, Institute of Infectious Diseases,
1-23-1 Toyama, Shinjuku,
162-8640 Tokyo, Japan

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Introduction

The major histocompatibility complex (MHC) class I consists of heavy chain, β_2-microglobulin (β_2m), and antigen peptide (Hennecke et al. 2001). Human cells are known to express three highly polymorphic MHC heavy chains (*HLA-A*, *-B*, and *-C*) and three conserved MHC heavy chains (*HLA-E*, *-F*, and *-G*). *HLA-A*, *-B*, and *-C* present antigen peptides to cytotoxic T lymphocytes (CTL) and the CTL are then activated (Flynn et al. 1992; Hou et al. 1992; York and Rock 1996). These classical molecules, especially *HLA-C*, also provide both stimulatory and inhibitory signals to natural killer (NK) cells through killer cell immunoglobulin-like receptors (KIR) (Valiante et al. 1997).

The gene encoding the class I heavy chain is composed of eight exons. Exon 1 encodes the signal peptide, exons 2–4 specify the extracellular domains α_1–α_3, exon 5 codes for the transmembrane domain, and exons 6–8 code for the cytoplasmic domain. The α_1 and α_2 domains are the most polymorphic, while the α_3 domain contributes to the β_2m association (Hebert et al. 2001) and interaction with the CD8 molecule (Salter et al. 1990).

The rhesus MHC has been extensively studied among non-human primates because rhesus monkeys are most frequently used as the surrogate animal model (Allen et al. 2001; Horton et al. 2001; Mothe et al. 2002) for HIV infection in human. Rhesus MHC (*Mamu*) class I *A* (Boyson et al. 1996b; Miller et al.1991; Urvater et al. 2000a; Voss and Letvin 1996; Watanabe et al. 1994), *B* (Boyson et al. 1996b; Voss and Letvin 1996; Yasutomi et al. 1995), *E* (Boyson et al. 1995), *F* (Otting and Bontrop 1993), *G* (Boyson et al. 1996a), *AG* (Slukvin et al. 1999), and *I* (Urvater et al. 2000b) have already been reported. Rhesus monkeys were shown to carry at least one *A* and two *B* loci, because three *Mamu-A* and five *Mamu-B* alleles have been identified in a single animal (Boyson et

al. 1996b). *HLA-C* homologues have been identified in the common chimpanzee, bonobo, gorilla, and orangutan (Adams et al. 1999, 2000; Cooper et al. 1998; de Groot et al. 2000; Lawlor et al. 1990, 1991), while no evidence of an *HLA-C* homologue was observed in old and new world monkeys (Adams and Parham 2001). Although SIV infection in cynomolgus monkeys is also used as the animal model for human HIV infection (McClure et al. 1990; Putkonen et al. 1992), there are few reports about cynomolgus MHC (*Mafa*) except for class II loci (Gaur and Nepom 1996; Kriener et al. 2000; Otting et al. 1992), class I *E* (Alvarez et al. 1997; Boyson et al. 1995), and *I* loci (Urvater et al. 2000b).

In this study, we have determined the nucleotide sequences of the genes coding for the cynomolgus MHC class I *A* molecules and found 14 *Mafa-A* alleles. In addition, we established a convenient method to detect the *Mafa-A* alleles.

Materials and methods

Animals

All the cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases. Both genders were involved and the cynomolgus monkeys were between 2 and 23 years old. This study was conducted in accordance with the Guide for Animal Experiments Performed at the National Institute of Infectious Diseases.

RT-PCR and nucleotide sequencing

Peripheral blood mononuclear cells (PBMC) were isolated from the fresh blood of 29 cynomolgus monkeys by a standard Ficoll-Hypaque gradient method. PBMC were washed twice with PBS and suspended in 5 ml of RPMI-1640 (Sigma, St. Louis, Mo.) containing 100 U/ml penicillin (Meiji Seika Kaisha, Tokyo, Japan), 10% FCS (GIBCO-BRL, Grand Island, N.Y.), and 5 μg/ml concanavalin A (ConA; Pharmacia, Cleveland, Ohio) at a concentration of 10^5 cells/ml. PBMC were cultured at 37 °C for 3–4 days. Messenger RNA extracted from the cultured PBMC (2–

Table 1 Primers used for the amplification and sequencing of MHC class I cDNAs from cynomolgus monkeys

Primer	Sequence	Concentration (pmol/sample)	Annealing temperature (°C)
Primers used for RT-PCR			60
Mafa-A-s	5′-GCAGGATCCGAATCTCCCCAGACGCGCA-3′	10	
Mafa-A-a	5′-GCTCTAGACCTCACAAGGCAGCTGTC-3′	10	
Mafa-A13-s	5′-CGAACCCTCCTCCTGG-3′	10	
Mafa-A1013-a	5′-CTGAGAGTAGCTCCCTCCTTTTCTAT-3′	10	
Primers used for multiplex PCR			
Primer set 1			72
IA01-s	5′-GCAGCGGGATGGAGAGGAA-3′	20	
IA02-s	5′-GCTGTGGTTGTGCCTTCTGGAAAA-3′	10	
IA03-s	5′-ACGCTGCAGCGCGCA-3′	2	
IA04-s	5′-GCGGCGGATGTGGCGGAGAG-3′	2	
IA05-s	5′-CTGCGACCTGGGGCCG-3′	2	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 2			72
IA06-s	5′-GGGCCTGTGCGTGGAGTCCCTG-3′	10	
IA07-s	5′-CACACTGACCTGGCAGCGT-3′	10	
IA08-s	5′-CTGCGACCTGGGGCCA-3′	10	
IA09-s	5′-CTACAACCAGAGCGAGGCCA-3′	10	
IA10-s	5′-GCAGCCCCGCTTCATCT-3′	10	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primer set 3			70
IA11-s	5′-ACACATGTGACCCATCACCCT-3′	5	
IA12-s	5′-GCCGGAGTATTGGGACCA-3′	20	
IA13-s	5′-GGCCTGCAGGAGATGGAAA-3′	20	
IA14-s	5′-CGGACCTGGGGGCTCAA-3′	15	
IA-a	5′-CCTGGGCACTGTCACTGCTT-3′	20	
Primers used for sequencing			
T7 primer	5′-TAATACGACTCACTATAGGG-3′	3	55
SP6 primer	5′-ATTTAGGTGACACTATAG-3′	3	55
Ia698	5′-TAGAAGCCCAGGGCCCAGGC-3′	3	55
Is437	5′-ATTACATCGCCCTGAACGAG-3′	3	55

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-

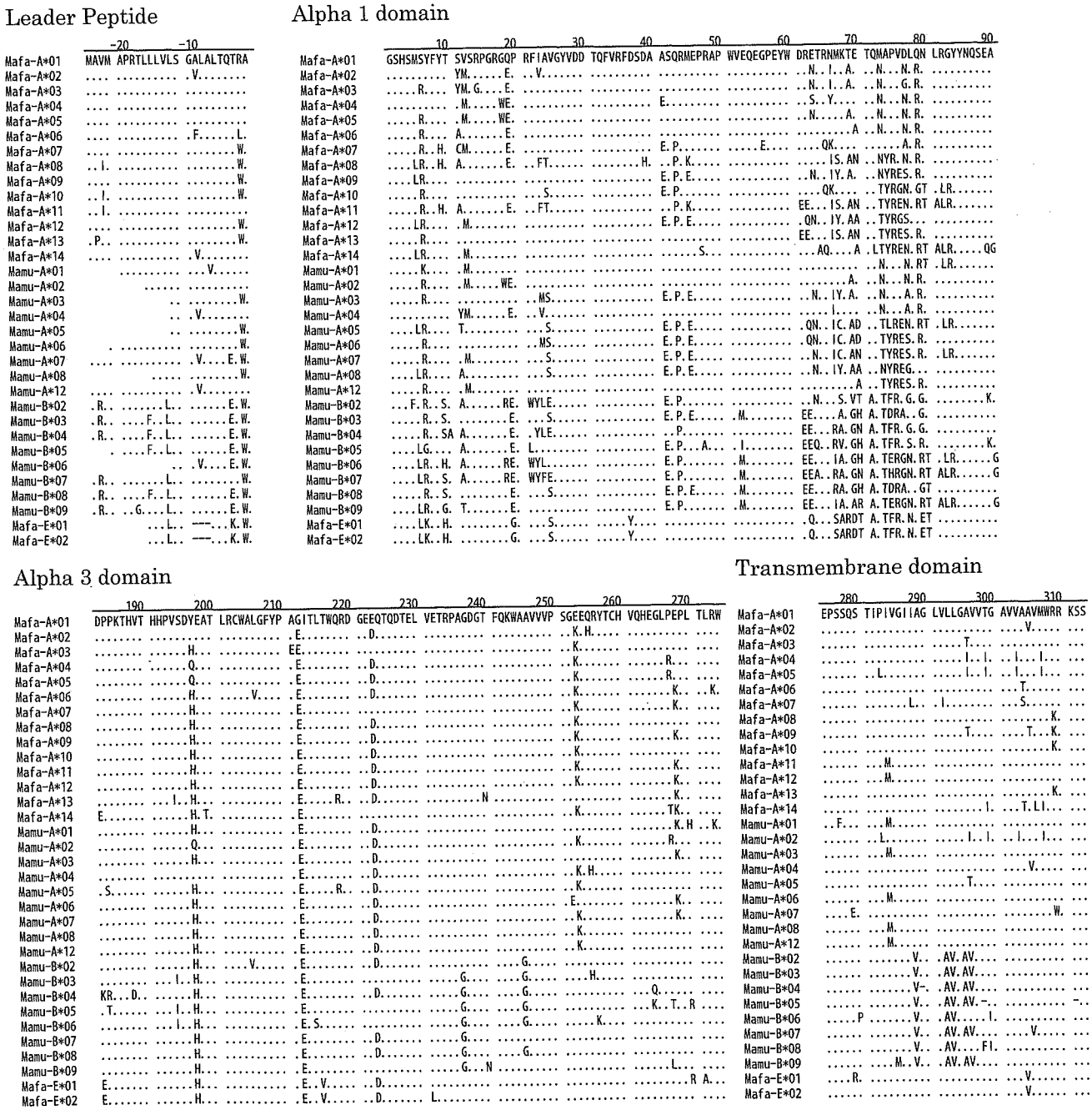

Fig. 1 Alignment of predicted amino acid sequences of *Mafa-A* with previously reported *Mamu-A*, *Mamu-B*, and *Mafa-E* sequences. Identity to predicted amino acid sequence of *Mafa-A*01* is indicated by *dots*, whereas amino acid replacements are depicted by the conventional *one-letter code*. The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

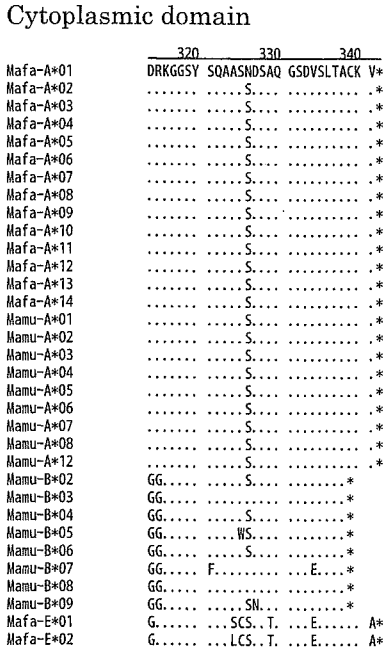

Fig. 1 (continued)

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,

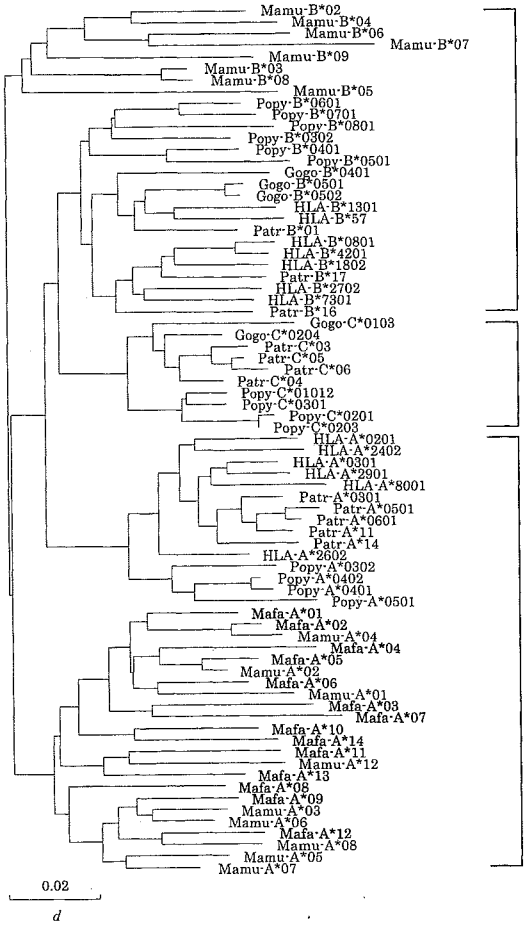

Fig. 2 Phylogenetic analysis of primate class I MHC molecules. The phylogenetic tree of α_1–α_2 was constructed using nucleotide sequences with the neighbor-joining method by DNASIS pro (Hitachi software, Yokohama, Japan)

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.

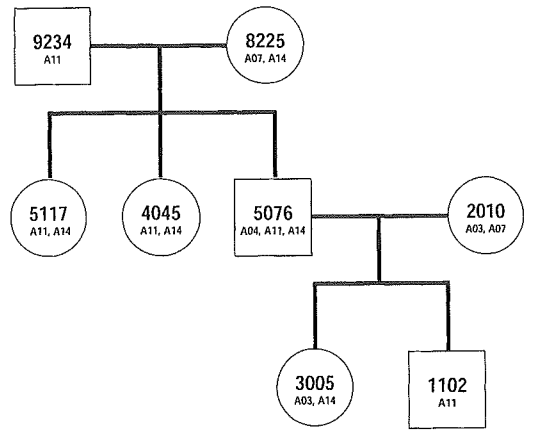

Fig. 3 The family pedigree demonstrating the inheritance of alleles of the MHC class I *A* locus in cynomolgus monkeys. Male or female is denoted by a *square* or *circle*, respectively. The animal number and allele assigned to the animal are shown

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

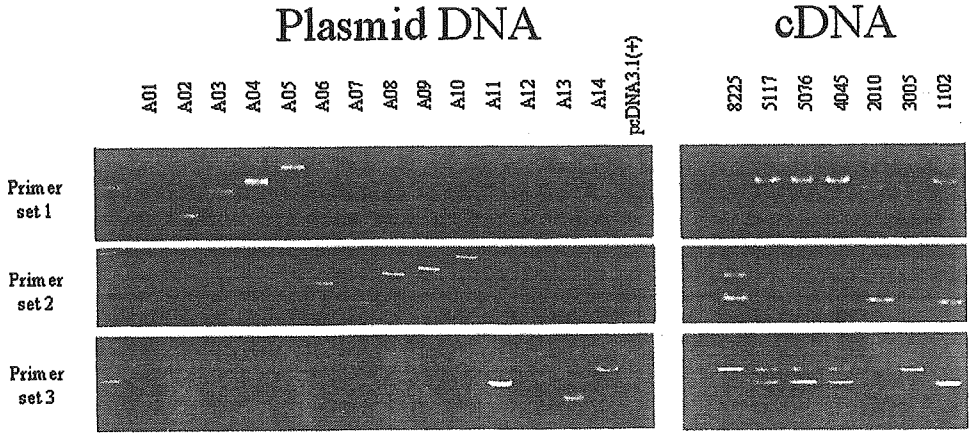

Fig. 4 Multiplex PCR-SSP analyses of *Mafa-A* alleles. Identification of *Mafa-A*01* through *A*14* was conducted using cloned genes inserted into pcDNA3.1(−) vector (*left panel*). Copy DNAs obtained from seven cynomolgus monkeys were similarly subjected to multiplex PCR-SSP (*right panel*)

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