isothermal amplification (LAMP), that amplifies DNA with high specificity, efficacy, and rapidity under isothermal conditions. The LAMP reaction requires a *Bst* DNA polymerase with strand displacement activity and a set of four specially designed primers that recognize six distinct sequences on the target DNA, the specificity of which should be extremely high. The amplification products are stem-loop DNA structures with several inverted repeats of the target. The advantage of the LAMP method is that the reaction is performed under isothermal conditions of between 60 and 65 °C. As a result, it requires only simple and cost-effective reaction equipment. The LAMP method has emerged as a powerful tool to facilitate genetic testing for various infectious diseases (Enosawa *et al.*, 2003; Iwamoto *et al.*, 2003; Kuboki *et al.*, 2003; Ihira *et al.*, 2004; Parida *et al.*, 2004; Thai *et al.*, 2004).

The purpose of our work is to identify a species-specific region of *Mycobacterium* sp., and to develop a LAMP assay that can differentiate clinically relevant species.

Materials and methods

Bacterial strains and preparation of genomic DNA

The bacteria used in this study comprised 27 strains and 49 clinical isolates as shown in Table 1. All strains except for *Mycobacterium leprae* were cultured on 1% Ogawa medium (Nissui, Tokyo, Japan) at 37 °C. *Mycobacterium leprae* was prepared from infected nude mouse food pad (Shepard, 1960). Genomic DNA was extracted from mycobacterial strains as follows. Mycobacterial cells were resuspended in 1.8 mL of sterile phosphate-buffered saline (PBS) containing 0.1 mm diameter zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK). The mixture was beaded for 20 s with a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo, Japan), transferred to a 1.5 mL microcentrifuge tube, and the genomic DNA was purified with proteinase K treatment and phenol/chloroform extraction followed by ethanol precipitation, then suspended in 100 μL distilled water.

Table 1. *Mycobacterium* species and strains used in this study and results of the loop-mediated isothermal amplification assay

			Primer set	
Species	Strains	Accession number	Kan32	Gas583
Mycobacterium abscessus	JATA 63-01 (ATCC 19977)	AB087684	–	–
Mycobacterium africanum	KK 13-02 (ATCC 25420)	AB087685	–	–
Mycobacterium avium	JATA 51-01 (ATCC 25291)	AB087686	–	–
	Clinical isolate 22 strains			
Mycobacterium bovis	JATA 12-01 (ATCC 19210)	AB087687	–	–
Mycobacterium chelonae	JATA 62-01 (ATCC 35752)	AB087688	–	–
Mycobacterium fortuitum	JATA 61-01 (ATCC 6841)	AB087689	–	–
Mycobacterium gastri	KK 44-02 (ATCC 15754)	AB087690	–	+
Mycobacterium gordonae	JATA 33-01 (ATCC 14470)	AB087691	–	–
Mycobacterium intracellulare	JATA 52-01 (ATCC 13950)	AB087692	–	–
	Clinical isolate 17 strains			
Mycobacterium kansasii	KK 21-01 (ATCC 12478)	AB087693	+	–
	Clinical isolate 10 strains		+	–
Mycobacterium leprae	Thai-53	AB087694	–	–
Mycobacterium malmoense	JATA 47-01 (ATCC 29571)	AB087695	–	–
Mycobacterium marinum	JATA 22-01 (ATCC 927)	AB087696	–	–
Mycobacterium microti	KK 14-01 (ATCC 19422)	AB087697	–	–
Mycobacterium nonchromogenicum	JATA 45-01 (ATCC 19530)	AB087698	–	–
Mycobacterium parafortuitum	ATCC 25807	AB087699	–	–
Mycobacterium phlei	ATCC 19249	AB087700	–	–
Mycobacterium scrofulaceum	JATA 31-01 (ATCC 19981)	AB087701	–	–
Mycobacterium simiae	KK 23-08 (ATCC 25275)	AB087702	–	–
Mycobacterium smegmatis	JATA 64-01	AB087703	–	–
Mycobacterium szulgai	JATA 32-01	AB087704	–	–
Mycobacterium terrae	KK 46-01 (ATCC 15755)	AB087705	–	–
Mycobacterium triviale	KK 50-02 (ATCC 23292)	AB087706	–	–
Mycobacterium tuberculosis	JATA 11-01 (H37Rv)	AB087707	–	–
Mycobacterium ulcerans	KK 43-01	AB087708	–	–
Mycobacterium vaccae	KK 66-01	AB087709	–	–
Mycobacterium xenopi	KK 42-01 (ATCC 19250)	AB087710	–	–

All strains were kindly donated by Dr Kashiwabara, NIID.

Clinical isolates were identified by Amplicore *Mycobacterium* kit (Roche Pharma, Basel, Switzerland) or conventional biochemical test (Jamal *et al.*, 2000).

Amplification of the region within *dnaA* gene

Highly polymorphic regions flanked by conserved regions were identified by aligning the *Mycobacterium* spp. *dnaA* sequences, which were available in GenBank at the time this study was initiated. These regions were used to design a pair of degenerate primers, U1F 5′-GTS CAR AAC GAR ATC GAR CG-3′ and U1R 5′-CCB GAY TCR CCC CAG ATG AA-3′. A schematic representation of the primer design is shown in Fig. 1a. PCR was performed in a TAKARA Thermal Cycler MP (TAKARA Biomedical, Otsu, Japan) with a reaction mixture consisting of 1 μL of genomic DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.4 μM, 1 × PCR buffer with 1.5 mM $MgCl_2$ (TAKARA Biomedical), and 1.25 U of ExTaq (TAKARA Biomedical), with 10 μL PCRX Enhancer System solution (Gibco BRL, Rockville, MD) in a total volume of 50 μL. The PCR thermocycles were 3 min at 94 °C, followed by 30 cycles of 94 °C for 10 s, 50 °C for 20 s, and 72 °C for 45 s, with a final extension step at 72 °C for 7 min. PCR products were visualized by UV illumination of an ethidium bromide-stained 1.5% agarose gel and cut out to purify with EASYTRAP Ver.2 (TAKARA Biomedical) according to the manufacturer's instruction.

DNA sequencing and sequencing analysis

The ABI Prism BigDye Terminator v3.1 Cycle Sequencing Kit (PE Biosystems, Foster City, CA) was used for the sequencing of the PCR products. The same primers for amplification were used for sequencing. The sequencing reaction was performed in accordance with the instruction of the manufacturer. Sequencing products were purified with a Centrisep column (Princeton Separations, Adelphia, NJ).

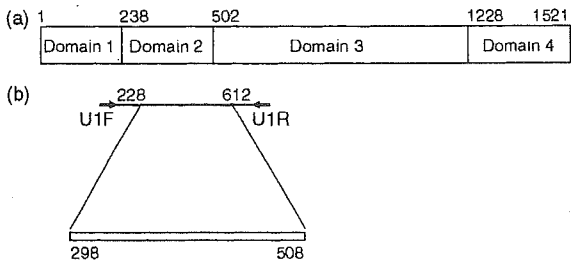

Fig. 1. Schematic representation of the DnaA protein and primer design for the amplification of the partial mycobacterial *dnaA* gene. Number indicates the nucleotide position of *Mycobacterium tuberculosis*, GenBank accession number AL021427. (a) The DnaA protein from *M. tuberculosis* contains four domains. Domain 1 is involved in interaction with DnaB. Domain 2 constitutes a flexible loop. DNA unwinding required Domain 3. Domain 4 is sufficient for specific binding to DNA. Primers U1F and U1R were used to generate about 400 bp fragment from *dnaA* of 27 mycobacterial spp. (b) Analysis and comparison region used in this study are indicated by a bar (298–508 bp).

The sequencing output was analyzed by using the DNA Sequence Analyzer computer software (PE Biosystems). The partial *dnaA* sequences were aligned using the Clustal W algorithm (Thompson *et al.*, 1994) of the software DNASpace ver. 3.5 (Hitachi Software Engineering, Yokohama, Japan), and the alignment was manually corrected. A phylogenetic tree was generated by DNASpace ver. 3.5 (Hitachi Software Engineering) with a total of 1000 bootstraps. Pairwise similarity of the partial *dnaA* sequences was determined by using DNASIS package (Hitachi Software Engineering).

Species-specific LAMP assay for *Mycobacterium kansasii* and *Mycobacterium gastri*

A set of four primers comprising two inner primers and two outer primers that recognized six distinct regions on the target sequence were designed with PrimerExplorer Ver.3 (Fujitu, Tokyo, Japan). The detailed sequences of the primers are shown in Fig. 3. The two inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in late stages. FIP contains the sequence complementary F1 (F1c) and F2. BIP contains the complementary B1 (B1c) and B2. The two outer primers consist of F3 and B3.

The LAMP reaction was carried out in 25 μL of reaction mixture by using the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tochigi, Japan) containing 2.4 μM (each) FIP and BIP, 0.2 μM (each) of the outer primers, F3 and B3, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 1.4 mM (each) of dNTP, 8 U of *Bst* DNA polymerase (New England BioLabs, Beverly, MA), and the template DNA. Amplification was undertaken in 0.5 μL microtubes in a heatblock under isothermal conditions of 63 °C for 60 min, followed by 80 °C for 2 min to terminate the reaction. Positive and negative controls were included in each run, and precautions to prevent cross-contamination were observed. Two microliter aliquots of LAMP products were subjected to electrophoresis on a 4% agarose gel in Tris-borate-EDTA buffer followed by staining with ethidium bromide and were visualized on a UV transilluminator at 302 nm. The specificity of the LAMP-amplified products were further validated by restriction enzyme digestion with *Nae*I and *Hae*II for *M. kansasii* and *M. gastri*, respectively. The diluted genomic DNA was used for determining the sensitivity of the species-specific LAMP assay.

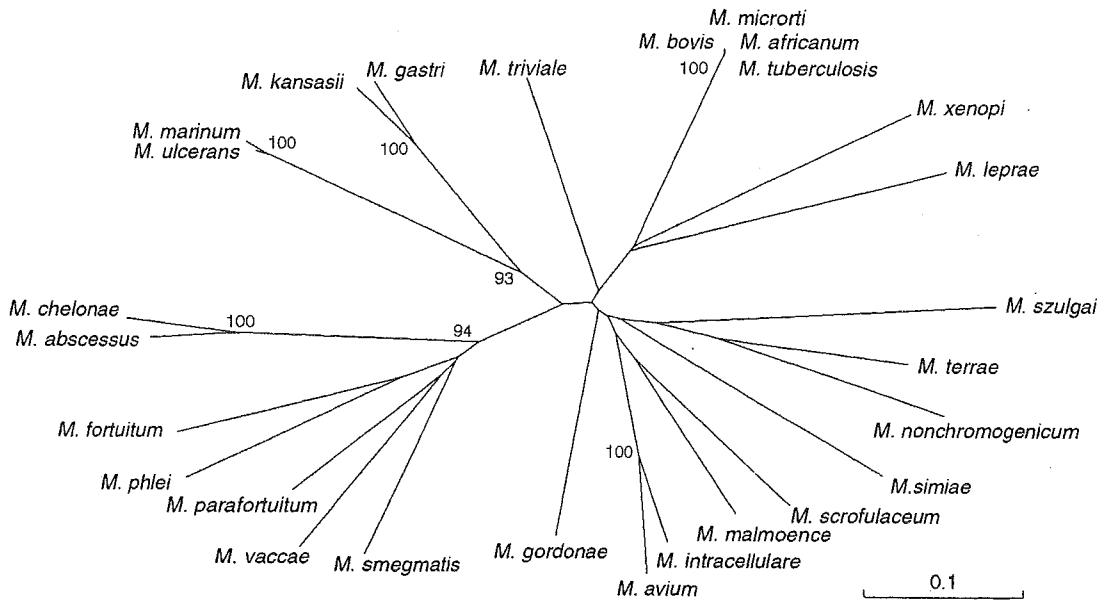

Fig. 2. Phylogenetic relationship of 27 *Mycobacterium* species. Unrooted tree based on the *dnaA* sequences. The tree was generated from DNASpace (Hitachi Software Engineering) with the Clustal W algorithm. The numbers on the dendrogram indicate the percentages of occurrence in 1000 bootstrapped trees; only values of > 90% are shown.

Results

Comparison of partial *dnaA* sequence to identify the *Mycobacterium* species

For the species identification of mycobacterial species, we analyzed some possible variable regions of mycobacterial sequences deposited in the GenBank, and found the 5′ part of the *dnaA* gene as a candidate target for PCR amplification. The PCR products with U1F and U1R, from 27 mycobacterial species, showed the ragged pattern around 400 bp in size (data not shown). Therefore, we determined nucleotide sequences, corresponding to position 228–612 bp of *Mycobacterium tuberculosis*, of all 27 species (Fig. 1a). The alignment of the sequence shows that the region (298–508 bp) in the amplified products had the highest species-specific variability (Fig. 1b). The size of the variable fragment in *dnaA* ranged from 154 bp in *M. triviale* to 232 bp in *M. kansasii*. The variable region exhibits a reasonable number of nucleotide substitution and insertion or deletion sites, which is important for the development of a differential diagnostic tool. The lowest interspecies similarity was 28.2% in *M. leprae* versus *M. vaccae*. The similarity between *M. avium* and *M. intracellulare* was 78.3% and that between *M. marinum* and *M. ulcerans* was 97.7%. Pathogenic *M. kansasii* were easily differentiated from nonpathogenic *M. gastri* (83.6%). The sequences of *M. tuberculosis*, *M. microti*, *M. africanum*, and *M. bovis* were found to be identical, except for one nucleotide substitution that occurred in *M. bovis*. When clinical isolates from clinically relevant mycobacterial strains were analyzed, the following minor variation was found among each species: 97.7–100% (*M. avium*) and 96.0–100% (*M. intracellulare*). We did not find any intraspecies variation in 10 clinical isolates and the standard strain of *M. kansasii*. Because other reports using different systems revealed the existence of more than one sequevar (Yang *et al.*, 1993; Alcaide *et al.*, 1997), we may need to examine a bigger number of clinical isolates.

The unrooted phylogenetic tree showed that the 27 mycobacterial species were resolved by the variable region in the *dnaA* sequence (Fig. 2). All rapidly growing species, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. parafortuitum*, *M. phlei*, *M. vaccae*, and *M. smegmatis*, made a cluster that was clearly separated from those of the other species so far examined. On the other hand, *M. kansasii*, *M. gastri*, *M. avium*, and *M. intracellulare* are clinically relevant species; however, the branch of the former two species was obviously segregated from one of the later two species, which was supported by high bootstrap values. The results indicated that the partial *dnaA* sequence could be useful for the differentiation of NTM (Fig. 2).

Identification of mycobacteria by *dnaA* sequence-targeted species-specific LAMP assay

Several sets of primers designed from the *dnaA* sequence were evaluated for their specificity and sensitivity by the LAMP method. One set of primers named Kan-32 for *M. kansasii* and Gas-583 for *M. gastri* was selected (Fig. 3), and

(b)

Kan 32	
F3	CGATGATTCCGGCCTGGA
B3	GTTGAGGCTGGTTCCGC
FIP	TCTCGTCGGCGTCGTCGGTATGTCACGGGAAACGTCAC
BIP	GACCCGGTTGGCCCACCTAGCAGCGACCGTATCGGC

Gas 583	
F3	AGCCCGGCCTGGAAAT
B3	GTGCGAACCGATTGGAGG
FIP	TGGGCCAATTGGGTCGGGGCCGGGAACCCGAAACCATC
BIP	TCGGGCACCGATACGGTCGGAAGGTGTCGAAGGTGTAGC

Fig. 3. Location of oligonucleotide primer sets Kan 32 and Gas 583, used for the loop-mediated isothermal amplification method. For *Mycobacterium kansasii* partial *dnaA* gene (GenBank accession number AB087693) and for *Mycobacterium gastri* partial *dnaA* gene (GenBank accession number AB087690). A right arrow indicates the sense sequence which is used as the primer. A left arrow indicates that a complementary sequence is used as the primer. The unique restriction enzyme recognition sites in the amplified product are shown with a bold bar. (b) List of each primer sequence.

by using these primer sets, a successful LAMP product appeared as a ladder of multiple bands (Fig. 3a).

The species specificity and intraspecies stability of each primer set were examined with purified DNA from 27 mycobacterial species and 10 clinical isolates of *M. kansasii*. We subjected each sample to amplification using Kan-32 or Gas-583 primer set. The results obtained by electrophoretic examination are summarized in Table 1. Although 200 pg of nontargeted species DNA were not amplified, significant amplification of targeted respective isolates was observed after a 60 min incubation at 63 °C. To confirm that the amplification products had corresponding DNA structures, the amplified products were digested with restriction enzymes and the size of the fragments was analyzed by electrophoresis. *Nae*I cuts between F1 and B1c for the *M. kansasii* amplicon; *Hae*II was used for the *M. gastri* amplicons. The sizes of the fragments generated after digestion were in good agreement with sizes predicted theoretically from the expected DNA structure: 100 and 93 bp by *Nae*I digestion, and 123 and 98 bp by *Hae*II digestion (Fig. 4a). Thus, we concluded that each primer set was species specific.

We next assessed the sensitivity of the assay. Serially diluted *M. kansasii* or *M. gastri* genomic DNA was used. The results of a typical experiment are shown in Fig. 4b. Amplified DNA was readily visible when 500 copies of genomic DNA were present in a 60 min incubation assay. The detection limit did not change with a longer incubation period (data not shown).

Discussion and conclusions

For the identification of species, a target gene must be conserved among strains and species. As the DnaA protein is generally conserved among microbial organisms (Mizrahi *et al.*, 2000), this coding region could be used for the target analysis. Four functional domains of the DnaA protein have been defined (Messer *et al.*, 1998). Domain 1 is involved in oligomerization and interaction with DnaB, Domain 2 constitutes a flexible loop, Domain 3 has ATPase function, and Domain 4 is sufficient for specific binding to DNA. The variable region that we identified in the *dnaA* sequence was equivalent to the Domain 2 coding nucleotide sequence

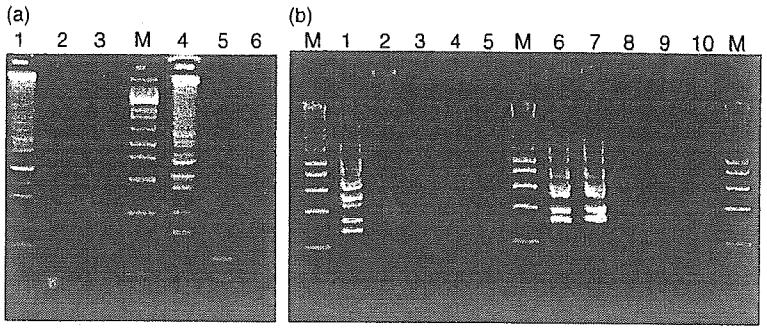

Fig. 4. (a) Four percent agarose gel electrophoresis and restriction enzyme analysis of loop-mediated isothermal amplification (LAMP) products of partial *dnaA* gene of *Mycobacterium kansasii* and *Mycobacterium gastri*. Lanes: M, 100 bp DNA ladder; lanes 1–3, LAMP carried out with *M. kansasii* primer, Kan 32, in the presence of genomic DNA from *M. kansasii* (lanes 1 and 2) and *M. gastri* (lane 3); lane 2, LAMP product from lane 1 after digestion with *Nae* I; lanes 4–6, LAMP carried out with *M. gastri* primer, Gas 583, in the presence of genomic DNA from *M. gastri* (lanes 4 and 5) and *M. kansasii* (lane 6). lane 5, LAMP product from lane 4 after digestion with *Hae* II. (b) Serial dilution of purified *M. kansasii* or *M. gastri* genomic DNA was amplified to determine the sensitivities by LAMP. Lanes: M, 100 bp DNA ladder; lanes 1–5 LAMP carried out with Kan 32 primer set in the presence of genomic DNA of *M. kansasii*, lane 1, 1000 copies; lane 2, 500 copies; lane 3, 100 copies; lane 4, 10 copy; lane 5, distilled water. lanes 6–10 LAMP carried out with gas 583 primer set in the presence of genomic DNA of *M. gastri*, lane 6, 1000 copies; lane 7, 300 copies; lane 8, 100 copies; lanes 9, 10 copy; lane 10, distilled water.

(Fig. 1). This domain is the least conserved region in the *dna A* gene with respect to sequence and length among *M. smegmatis*, *M. tuberculosis*, and *M. leprae* (Fsihi *et al.*, 1996). However, comparative studies of this region using 27 mycobacteria have not been reported and, as far as we know, this is the first report indicating the usefulness of the *dnaA* Domain 2 sequence as a differential diagnostic tool.

An accurate and rapid bacterial identification greatly contributes to this field of medication. Several methods based on molecular biological techniques have been reported. The sequences that have been reported include *hsp65*, 16S rRNA gene, and ITS (Plikaytis *et al.*, 1992; De Smet *et al.*, 1995; Springer *et al.*, 1996; Messer & Weigel, 1997; Roth *et al.*, 1998; Brunello *et al.*, 2001). Each gene has several advantages and disadvantages. An excessive degree of variability is found in the *hsp65* gene (Telenti *et al.*, 1993), which may hinder the development of reliable probes. While 16s rRNA gene sequence is identical in *M. kansasii* and *M. gastri* and shows narrow divergenicity within species (Taylor *et al.*, 1997), ITS sequence can be used to distinguish between *M. kansasii* and *M. gastri* (Roth *et al.*, 1998). While *M. kansasii* is a representative pathogenic mycobacteria, *M. gastri* does not induce an apparent disease. The discrimination between these mycobacteria provides useful information to select the appropriate therapy. The percent similarity of ITS between two species was 93% (Roth *et al.*, 1998), and that of the *dnaA* variable region was found to be 83.6%. These observations may indicate the usefulness of the *dnaA* gene for discrimination of these species, at least in complement with ITS.

The recent trend in genetic testing is to make systems fully automatic with high-throughput analysis. Although this may be an ideal approach, it requires expensive equipment as well as a well-trained person in diagnostic laboratories. The LAMP method could be conducted under isothermal conditions ranging from 60 to 65 °C by a single enzyme. The only equipment needed for LAMP reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature around 63 °C. LAMP does not require a thermal cycling step, and an isothermal reaction for a short time (60 min) is enough to amplify the target DNA to a detectable level. As PCR and other molecular biological techniques are conducted in well-equipped laboratories, these methodologies are often impracticable under a field diagnosis.

In this paper, we demonstrated that the *dnaA* region could be an effective new nucleotide region for the diagnosis of NTM infection and that the LAMP method could be applied for a *dnaA* gene-based differential diagnostic tool.

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ORIGINAL ARTICLE

Polymorphism of the 5′ flanking region of the IL-12 receptor β2 gene partially determines the clinical types of leprosy through impaired transcriptional activity

H Ohyama, K Ogata, K Takeuchi, M Namisato, Y Fukutomi, F Nishimura, H Naruishi, T Ohira, K Hashimoto, T Liu, M Suzuki, Y Uemura, S Matsushita

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Background: Individual differences in T cell responsiveness to interleukin 12 (IL-12), resulting from inherited factors, may be responsible for differences in the intensity of cell mediated immune (CMI) responses in patients with leprosy, a disease with a wide clinical spectrum.
Aim: Polymorphisms in the 5′ flanking region of the IL12RB2 gene were analysed to determine potential immunogenetic factors affecting CMI responses, using leprosy as a model.
Methods: Polymorphisms in the 5′ flanking region of IL12RB2 were examined using direct sequencing techniques, and allele frequencies between patients with lepromatous leprosy and patients with tuberculoid leprosy were compared. The effect of these single nucleotide polymorphisms (SNPs) on IL12RB2 expression was estimated using the dual luciferase reporter gene assay in Jurkat T cells.
Results: Several SNPs, including −1035A>G, −1023A>G, −650delG, and −465A>G, were detected within the 5′ flanking region of IL12RB2. The frequency of haplotype 1 (−1035A, −1023A, −650G, −464A) was high in the general Japanese population, but was significantly lower in lepromatous patients compared with tuberculoid patients and healthy controls. Reporter gene assays using Jurkat T cells revealed that all haplotypes carrying one or more SNP exhibited a lower transcriptional activity compared with haplotype 1.
Conclusion: SNPs within the 5′ flanking region of IL12RB2 affect the degree of expression of this gene and may be implicated in individual differences in CMI responsiveness to mycobacterial antigens, leading to lepromatous or tuberculoid leprosy.

The importance of the cell mediated immune (CMI) response is well established in the host defence to mycobacterial pathogens.[1 2] Leprosy, a chronic disease caused by infection with *Mycobacterium leprae*, shows a wide spectrum of clinical features.[3] Patients with tuberculoid type leprosy (T-lep) show a high CMI response to *M leprae*, with resistance to infection, whereas patients with lepromatous leprosy (L-lep) show a poor CMI response to the pathogen and have a progressive form of the disease. Although a leishmaniasis model using BALB/c and C57BL6 mice has improved our understanding of cellular and genetic control mechanisms for infectious diseases and allergy, no such models have yet been established in humans. We propose leprosy as an alternative model in humans.

> "Polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of the interleukin 12 receptor β2 chain, resulting in individual differences in the intensity of cell mediated immune responses to mycobacteria"

Interleukin 12 (IL-12) is secreted from macrophages and dendritic cells and is a potent inducer of interferon γ production by T helper type 1 (Th1) cells, which is in part dependent upon the degree of expression of the IL-12 receptor (IL-12R) on the cell surface.[4–6] IL-12R is composed of two protein subunits, referred to as the β1 and β2 chains, and expression of the β2 chain is a crucial determinant of Th1/Th2 balance, because STAT4 is activated through interaction with a tyrosine residue on the cytoplasmic domain of the IL-12R β2 subunit.[7–10] It has been shown that the expression of IL-12Rβ2 is greater in tuberculoid lesions than in lepromatous lesions, whereas the expression of IL-12Rβ1 is similar in both.[11] We hypothesise that the susceptibility to several diseases related to mycobacterial pathogens could be determined by the degree of expression of IL-12Rβ2, which might be regulated by genetic factors, including IL12RB2 polymorphism.

One hypothesis is that polymorphisms in the 5′ flanking region of IL12RB2 may affect the expression of IL-12Rβ2, resulting in individual differences in the intensity of CMI responses to mycobacteria. We examined single nucleotide polymorphisms (SNPs) within the 5′ flanking region of IL12RB2 as feasible markers to determine susceptibility to the disease and the effect of these SNPs on the transcription of IL-12Rβ2 molecules.

MATERIALS AND METHODS

Study population

Genomic DNA samples were collected from 176 Japanese patients with leprosy—130 with L-lep and 46 with T-lep—and 68 healthy Japanese donors. Patients were clinically diagnosed, according to the description of Ridley and Jopling, referring to results of the Mitsuda test and their sequelae.[3] Donors were recruited into our study under informed consent guidelines approved by the human ethical committee of Saitama Medical School, Japan.

Abbreviations: CMI, cell mediated immunity; IL-12, interleukin 12; IL-12R, interleukin 12 receptor; L-lep, lepromatous leprosy; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; Th, T helper; T-lep, tuberculoid type leprosy

Table 1 Allelic distribution in patients with leprosy

SNPs	Clinical form	Allele frequency	OR	RR	p Value
−1035A>G	L-lep	24.6%	3.97	3.24	<0.001
	T-lep	7.6%			
−1023A>G	L-lep	24.2%	2.95	2.48	<0.01
	T-lep	9.8%			
−650delG	L-lep	28.8%	3.74	2.95	<0.001
	T-lep	9.8%			
−464A>G	L-lep	23.1%	3.64	3.03	<0.01
	T-lep	7.6%			

Frequencies of four single nucleotide polymorphisms (SNPs) were compared between lepromatous (L-lep) and tuberculoid (T-lep) patients. The χ^2 test was used to compare differences in the distribution of clinical phenotypes and allele frequencies. The odds ratio (OR) and relative risk (RR) were also calculated by comparing the frequency of variant alleles between two clinical types of leprosy.

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Genomic DNA was sequenced for polymorphisms in the 5′ flanking region of IL12RB2 using direct sequencing. Briefly, a fragment spanning −1247 to +55 of IL12RB2 was polymerase chain reaction (PCR) amplified and subsequently sequenced using the ABI 3730 DNA sequencer (PerkinElmer Life Sciences, Wellesley, Massachusetts, USA). The sequence data obtained were compared with the GenBank database (GenBank accession number AL389925) to determine the SNPs on IL12RB2. Numbers of base positions were defined as the distance from the start point of the reported cDNA sequence.[12] The haplotypes were determined by cloning the PCR products into the pGEM-T Easy plasmid (Promega, Madison, Wisconsin, USA) and sequencing.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

For transfection studies, the dual luciferase reporter gene assay system (Promega) was used with the IL12RB2–pGL3 plasmid constructs. The plasmids comprised a 1.3 kb NheI/HindIII digested PCR fragment of IL12RB2 ligated to the pGL3 basic vector (Promega). Jurkat T cells (1×10^7) were electroporated with 25 μg of plasmid DNA (IL12RB2–pGL3 constructs) and a control *Renilla* luciferase reporter plasmid (pRL–TK, 25 ng; Promega) using a Gene Pulser (Bio-Rad Laboratories, Hercules, California, USA), as described previously.[13] Cells were then cultured for 48 hours in the presence of antihuman CD3 monoclonal antibody (PharMingen, San Diego, California, USA), and antihuman CD28 monoclonal antibody (PharMingen) for the final 24 hour period. Cell lysates were prepared, and both firefly and *Renilla* luciferase activities were evaluated using a dual luminometer Fluoroskan Ascent FL (Thermo Electron Oy, Vantaa, Finland).

Table 2 Haplotypes of the 5′ flanking region of IL12RB2

Haplotype	−1035	−1023	−650	−464	Frequency (%)
1	A	A	G	A	41.2
2	G	G	del	G	32.4
3	A	A	del	A	11.8
4	G	G	G	A	8.3
5	A	A	del	G	5.9

Haplotypes were determined by sequencing 34 subcloned polymerase chain reaction amplified DNA fragments from 17 donors with heterozygous alleles in at least in one of the positions −1035, −1023, −650, and −464, but not from donors carrying homozygous alleles at all positions.

RESULTS

Analysis of polymorphism in the 5′ flanking region of IL12RB2

Twelve SNPs located within the 5′ flanking region of IL12RB2 (−1247 to +55), comprising −1047delT, −1035A>G, −1033T>C, −1023A>G, −650delG, −568A>C, −557T>C, −550T>C, −464A>G, −464A>C, −202T>C, and −188A>C, were identified. The surveillance study of 176 patients (130 with L-lep and 46 with T-lep) revealed significant differences in frequencies of SNPs between these patients groups; in particular: −1035A>G, −1023A>G, −650delG, and −464A>G (table 1).

We randomly selected 17 healthy donors heterozygous in at least one of the positions −1035, −1023, −650, and −464 to determine linkage disequilibrium. Five haplotypes were determined by sequencing 34 subcloned PCR amplified DNA fragments from the 17 heterozygous donors. Haplotype 1 consisted of −1035A, −1023A, −650G, and −464A, and haplotype 2 consisted of −1035G, −1023G, −650del, and −464G, and accounted for 73.6% of the haplotypes detected in our study (table 2).

Haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView (SAS Institute, Cary, North Carolina, USA) statistical software program, by comparing the frequency of haplotype 1 in patients with L-lep leprosy, those with T-lep leprosy, and healthy controls. The frequency of haplotype 1 was significantly lower in patients with L-lep compared with those with T-lep and healthy donors (fig 1), suggesting that haplotype 1 might contribute to the intensity of CMI responses to mycobacteria.

Evaluation of transcriptional activity of the 5′ flanking region of IL12RB2

The transcriptional activity of each reporter construct was determined using the dual luciferase reporter gene assay. The transcriptional activity of haplotype 1 was significantly higher than haplotypes 2, 3, 4, and 5 (fig 2). Each experiment was performed using triplicate wells and was repeated four times. Because each SNP is a genetic factor potentially able to reduce the expression of IL-12Rβ2 molecules, a poor CMI response to mycobacteria may occur.

DISCUSSION

It was recently reported that the lack of IL-12Rβ1 expression caused by mutations in IL12RB1 resulted in human immunodeficiency, thereby demonstrating the essential role

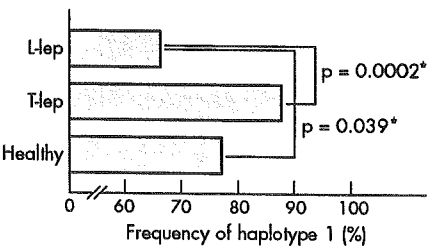

Figure 1 Frequency (%) of haplotype 1 in the three groups: patients with lepromatous leprosy (L-lep), patients with tuberculoid type leprosy (T-lep), and healthy controls. The haplotype frequency was calculated based on the assumption that each group would be in accordance with the Hardy–Weinberg equilibrium because subjects were selected from the Japanese Wajin population of mainland Japan (Honshu). p Values were calculated using the StatView statistical software program, by comparing the frequency of haplotype 1 in the L-lep leprosy, T-lep leprosy, and healthy control groups.

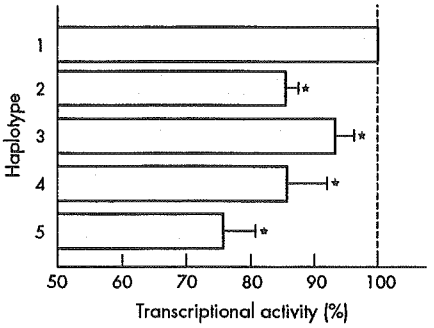

Figure 2 Basal promoter activity of reporter constructs containing −1247 to +55 of IL12RB2 harbouring each haplotype. The transcriptional activity of each haplotype is indicated by the percentage of relative luciferase units compared with the haplotype 1 construct. Each experiment was assayed using triplicate wells and was repeated four times and similar results were obtained. *$p < 0.05$; χ^2 test.

of IL-12 in resistance to infections caused by intracellular bacteria.[14–16] However, no differences in the expression of IL-12Rβ1 on T cells were detected between donors, including patients and healthy subjects (data not shown). It has been reported that IL-12Rβ2 is absent in freshly isolated peripheral blood mononuclear cells, whereas up to 72% of resting peripheral blood mononuclear cells from normal volunteers express IL-12Rβ1 molecules, and that IL-12Rβ2 is expressed selectively in Th1 cells but not in Th2 cells.[9 17] Moreover, because IL-12Rβ2 has tyrosine residues in the cytoplasmic domain that play a role in signal transduction, we hypothesised that IL-12Rβ2, but not IL-12Rβ1, could be important in explaining the low CMI responses induced by IL-12 in T cells from patients with leprosy.

> "Individual differences in the intensity of the cell mediated immune response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes"

Alternatively spliced mRNA with the absence of IL12RB2 exon 15 leads to the loss of induction of interferon γ production.[18] Similarly, we have previously found several coding SNPs of IL12RB2, but could not determine an effect on CMI response intensity or on alternatively spliced mRNA (data not shown). Epidemiological studies recently performed by two independent groups demonstrated that there was no influence of IL12RB2 coding SNPs on susceptibility to mycobacterial infection.[19 20] Therefore, individual differences in the intensity of the CMI response to mycobacteria are probably regulated primarily by the degree of expression of IL-12Rβ2, rather than possible conformational changes caused by the coding SNPs detected in the Japanese population. Bleharski *et al* showed differences in gene expression profiles according to the clinical type of leprosy,[21] and the IL-12R gene was included among those differentially expressed between patients with L-lep and T-lep.

The differences in transcriptional activity between haplotype 1 and other haplotypes are marginal, except for that between haplotypes 1 and 5. These findings suggest that haplotype 5 might be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4. To investigate this issue, it will be necessary to analyse the frequency of all haplotypes in the patient population. Moreover, the functional effects of SNPs −650 and −464 on the transcriptional mechanism should also be elucidated.

Take home messages

- Single nucleotide polymorphisms (SNPs) within the 5′ flanking region of the IL12RB2 gene could affect the expression of the interleukin 12 receptor β2 chain (IL-12Rβ2) and result in the individual differences in the intensity of cell mediated immune responses that lead to the lepromatous and tuberculoid types of leprosy
- Haplotype 5 appears to be more closely associated with susceptibility to lepromatous leprosy than haplotypes 2, 3, and 4, but further investigations are necessary
- These SNPs may also affect susceptibility to allergy because IL-12Rβ2 is involved in the allergic response

An SNP at position −464 of IL12RB2 was found to have high transcriptional activity compared with the wild-type allele, possibly because of disruption of a GATA site.[22] This appears to contradict our present data, although the effects of SNPs on the binding affinity with GATA-3 were not directly tested in this report. It was determined that the main target of GATA-3 is not IL-12Rβ2, but rather STAT4.[23] Further studies are needed to determine the precise molecular mechanism.

Taken together, we conclude that SNPs within the 5′ flanking region of IL12RB2 could affect the expression of IL-12Rβ2, thus causing individual differences in the intensity of CMI responses leading to the lepromatous and tuberculoid types of leprosy. It is probable that these SNPs also affect susceptibility to allergy, because IL-12Rβ2 is implicated in the Th1/Th2 balance in the allergic response. A study investigating this hypothesis is currently under way.

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ORIGINAL ARTICLE

Positional Effect of Amino Acid Replacement on Peptide Antigens for the Increased IFN-γ Production from CD4T Cells

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ABSTRACT

Background: Based on the fact that site-specific amino acid replacement on peptide antigens stimulated T cell clones to produce increased amount of IFN-γ, we investigated this structure-function relationship, using various peptide analogues.

Methods: We used three human Th0 clones (BC20.7,BC33.5 and BC42.1) that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand ; *i.e.*, the same framework of peptide antigen BCGa p84-100 in the context of DRB1*1405. These T cells were stimulated with various peptide analogues, followed by determination of proliferative responses and IFN-γ production.

Results: Replacement of Leu at peptide position 2 (P2) by amino acids which are less hydrophobic than the wild type (Val, Ala) or those with similar structural or neutral charge (Thr, Ser), induced increased IFN-γ production from T cells. This phenomenon was associated with structural features of TCR, especially the length of CDR3 region of TCRα. Amino acid replacement at the other positions did not induce increased IFN-γ production.

Conclusions: Amino acid substitution at P2 frequently induces increased IFN-γ production in a clone-specific manner, which is associated with the structure of CDR3 in TCRVα chains.

KEY WORDS

analogue peptide, complementarity determining region 3, interferon gamma, peptide antigens, T-cell antigen receptor

INTRODUCTION

Recent studies showed that T cell activation is not an all-or-none type of event ; rather, qualitative changes in T cell responses can be induced by amino acid substitutions by either MHC molecules or antigenic peptides, *i.e.*, TCR ligands. Flexibility in recognition results in T-cell activation in the absence of a proliferative response, which is designated by the following terminology as demonstrated in previous studies by our group and others : partial agonism,[1] TCR antagonism,[2] anergy,[3] survival[4] and cytokine-specific up-regulation.[5,6]

Amino acid residues on antigenic peptides have been roughly divided into two groups ; one that is important for binding to TCR (T cell epitope), and the other that is important for binding to MHC (MHC anchor). However, the crystal structure of the human class II HLA-DR1 complexed with the influenza peptide reported by Stern *et al.*[7] demonstrated that all the amino acid residues of the influenza virus peptide physically made contact with both HLA and TCR, with the exception of only one residue at the amino terminus which is buried deeply in the groove of class II, hence, there is no possibility for interaction with TCR.

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In our previous studies, single amino acid substitutions on a group I allergen in the *Cryptomeria japonica* -derived peptide resulted in a significant increase in IFN-γ production, with no remarkable changes either in proliferative response or IL-4 production.[5] In this study, by using various analogue peptide species, we stimulated three human Th0 clones that express distinct TCRα and TCRβ chains, but recognize the same TCR ligand, and tried to determine the structure-function relationship that leads to increased IFN-γ production from T cells.

METHODS

SYNTHESIS OF PEPTIDES

The wild-type BCGa p 84-100 (EEYLILSARDVLAVVSK) and its analogue were synthesized using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu Corp., Kyoto, Japan), and were purified by C18 reverse-phase HPLC (Millipore).

T CELL CLONES

BCGa p84-100-specific T cell lines were established as described.[8] Three human CD4+ T cell clones (BC 20.7,BC33.5 and BC42.1) specific to BCGa p84-100+ DRA/DRB1*1405, yet bearing distinct TCRβ (BV13S3 ,BV6S1 and BV5S4, respectively ;)[8] established from PBMC of a BCG-primed healthy individual as described elsewhere,[4] were used throughout the study. T cells were fed 50 U/ml human rIL-2 and irradiated autologous PBMC prepulsed with the wild-type BCGa p84-100 on a weekly basis.

ASSESSMENT OF T-CELL RESPONSES

Antigen-induced proliferation of the T cell clones were assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottomed culture plates in the presence of a peptide(s) and irradiated PBMC (1.5×10^5/well), using RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated autologous plasma. For the proliferation assay, cells were cultured for 72 hr in the presence of 1 μCi/well of[^{3}H]thymidine during the final 16 hrs. Culture supernatants collected immediately before the addition of[^{3}H]thymidine were used to determine lymphokine concentrations, using hGM-CSF ELISA kits (R&D systems) and hIFN-γ ELISA kits (Otsuka,Tokyo,Japan).

DETERMINATION OF TCRA GENE USAGE BY T CELL CLONES

To determine TCRVA gene usage of the T cell clones that were cultured with irradiated autologous PBMC, RNA were extracted from the cell mixture, and converted to cDNA. TCRA variable region cDNA were amplified with anchored PCR as described previously for amplification of TCRA variable region cDNA.[9] A primary PCR was followed by two sequential nested PCR. TCRAC-specific primers used for primary PCR, nested PCR, and final PCR were CA4 (5'-CAG AAT CCT TAC TTT GTG AC), CA3 (5'-ATC GGT GAA TAG GCA GAC AG), and biotinylated CA5 (5'- CAC

Table 1 A panel of labeled TCRAV-specific oligonucleotide probes

AV gene	sequence	pool
AV4, 20	TGCTAAGACCACA/CCAGCC	A
AV11	TCTTCAGAGGGAGCTGTG	A
AV2	ATCCTTGAGAGTTTTACT	B
AV8a	CCATTCGAGCTGTATTTA	B
AV8b	GCATTCGAGCTTTATTTA	B
AV15	CATTTGCTGGATTTTCGT	B
AV17	GATCTTAGGAGCATCATT	B
AV21	TGGGGGCATCAGTGCTGA	B
AV3	GAGAAGAGGATCCTCAGG	C
AV5	ACTATTCTCCAGCATACT	C
AV10	CCGTGTCCATTCTTTGGA	C
AV13	GAGAGGAATACAAGTGGA	C
AV19	CAATTTTTGTTGGCTATT	C
AV24	AGCATCTGACGACCTTCT	C
AV25	TCCTTGAACATTTATTAA	C
AV26	CCTAGGGATATTGGGGTT	C
AV27	GAAAAAACTATACCATCT	C
AV29	CAGGCACCTTGTTGTGGC	C
AV32	ACTCATCACATCAATGTT	C
AV18	CTTTGGCAGCCCCATTAC	D
AV23	GAGACCCTCTTGGGCCTG	D
AV28	ACTAACTTTCGAAGCCTA	D
AV30	GGAGTGTGCATTCATAGT	D
AV7	GGAGGCACTA/GCAGGACAA	E
AV6	ACAGCTTCACTGTGGCTA	F
AV12	TGCCAGCCTGTTGAGGGC	F
AV14	GTGA/GTCTCCACCTGTCTT	F
AV1a	CTCCTGTTGCTCATACCA	G
AV1b	CTCCTGCTGCTCGTCCCA	G
AV1c	CTCCTGGAGCTTATCCCA	G
AV9	AAGCCCACCCTCATCTCA	G
AV16	GCCTCTGCACCCATCTCG	G
AV22	CTGATACTCTTACTGCTT	G
AV31	CCTCTCTGGACTTTCTAA	G

Oligomucleotide probes specific to TCRAV family genes. Degenerated probes were used to specify AV4 and AV20, AV7, and AV14 families. Three probes for AV1 family, and two probes for AV8 family were required to specify all members of each family. These probes were grouped into seven pools (pool A to G) depending on sequence similarity.

Table 2 TCRVA and TCRVB usage of BC clones

BC clone	TCRVA	TCRVB
20.7	(AV25S1)FCAGHNAG(AJ14S3)	(BV12S3)CASRQAGTAYE(BJ2S7)
33.5	(AV3S1)FCATERGQ(AJ13S2)	(BV6S1A1)CASSPTGTANT(BJ1S1)
42.1	(AV8S1A1)FCAASLDNY(AJ126)	(BV5S1A1)CASRRSTGE(BJ2S2)

TCRVA and VB usage are shown, with amino acid sequences in the N(D)N region.

TGG ATT TAG AGT CTC TC), respectively. A panel of labeled TCRAV-specific oligonucleotide probes (Table 1) were used to study TCRAV gene usage with PCR-ELISA.[10] First, seven pools of the AV-specific probes were hybridized with immobilized PCR products in microplates to find out positive wells. Then, the products were hybridized with individual AV probes in another set of plates to pin-point the AV genes predominantly used by the cDNA. To clone the entire variable region cDNA, cDNA were amplified with CA4 and reamplified with a nested primer, CA2 (5'-ACG CGT CGA CAC TGG ATT TAG AGT CTC TC). The products were subcloned into pBluscript II SK+ (Stratagene, La Jolla), and recombinant clones with the dominant VA gene were selected with dot blot DNA hybridization using corresponding VA-specific oligonucleotides. After sequence determination of these clones, dominant clones were selected as cDNA for the T cell clones.

RESULTS

TCRVA AND VB SEQUENCES

TCRVA and VB sequences of three T-cell clones BC 20.7, BC33.5 and BC42.1 are shown in Table 2. The N (D)N region sequences are shown as one-letter codes for amino acids, between V and J segments in parentheses. As described in our earlier studies, these T-cell clones recognize BCGa p84-100 (EEYLILSARDVLAVVSK; with first anchor underlined), in the context of HLA-DRB1*1405.[4] It is especially important to note that N(D)N region consists of 8 and 11 residues at TCRVA and VB of BC20.7 and BC33.5, respectively, whereas that of BC42.1 consists of 9 and 9 residues, repectively.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOG PEPTIDE L87V TO BC20.7

To evaluate the effects of single amino acid substitutions, proliferation and lymphokine production in response to analogue peptides were determined and findings were compared with those seen with the wild-type peptide. Most of the analogue peptides that stimulated BC clones showed a pattern of lymphokine production similar to that for the wild-type peptide (not shown). However, IFN-γ production of BC20.7 was increased in response to several analogue peptides at high concentration (16 μM), especially peptide L87V in which Leu is replaced by Val at the 87 th residue of the peptide BCGa p 84-100, whereas neither T cell proliferation nor production of other lymphokines, showed any remarkable change; *i.e.*, only the production of IFN-γ was affected for recognition of the analog peptide L87V. As shown in Figure 1, to determine whether or not the change of IFN-γ production was due to differences in the HLA-peptide or TCR-TCR ligand avidity between L87V and the wild-type peptide, responses of BC20.7 to several different concentrations of L87V were compared with those of the wild-type peptide. In the range of concentrations from 0.016 μM up to 16 μM, IFN-γ production in response to L87V constantly exceeded that of the wild-type peptide. Moreover, the plateau level of L87 V-driven IFN-γ production was significantly higher. Mean IFN-γ production of BC20.7 for L87V increased significantly in comparison to the wild-type, whereas no statistical differences were noted in proliferative responses between R21K and the wild-type at a range of 0.16 μM to 16 μM. The IL-4 production of BC20.7 for each analogue peptide was proportional to the proliferative response to each peptide, at a range of 0.0016 to 16 μM (not shown). In contrast, production of GM-CSF gradually increased, in a dose-dependent manner throughout the range of 0.016 to 16 μM. These data indicate that the plateau responses and proliferation of IFN-γ are not due to saturation of the TCR ligand on the APC surface.

STIMULATORY ACTIVITIES OF BCGA P84-100-DERIVED ANALOGUES TO THREE BC CLONES

All three T-cell clones were stimulated with analogues at 16 μM, with replacements at P1 (=86Y) through P9 (=94V). Table 3 summarizes the results, regarding proliferative responses and IFN-γ production. P1 (=86Y) replaced by Ala (A) indicates a peptide species EEALILSARDVLAVVSK. Relative IFN-γ responses are shown, where IFN-γ production was divided by proliferation. P1 replaced by A gave values of 96/100/98, indicating that BC20.7,BC33.5 and BC 42.1 exhibited 96%, 100% and 98% responses respectively, as compared with the wild-type. Asterisks indicate peptide species that did not exert full agonistic activity; *i.e.*, peptide stimulation even at 16 μM did not give a plateau response.

Most of analogues that exhibited full agonistic activity, stimulated IFN-γ production at levels roughly similar to the wild-type peptide, *i.e.*, at around 100%. However, it is important to note that L87T,L87S,L87A, and L87V significantly ($p<0.01$) induced increased levels of IFN-γ production of BC20.7 and BC33.5, but not of BC42.1. Such a clone-specific phenomenon was

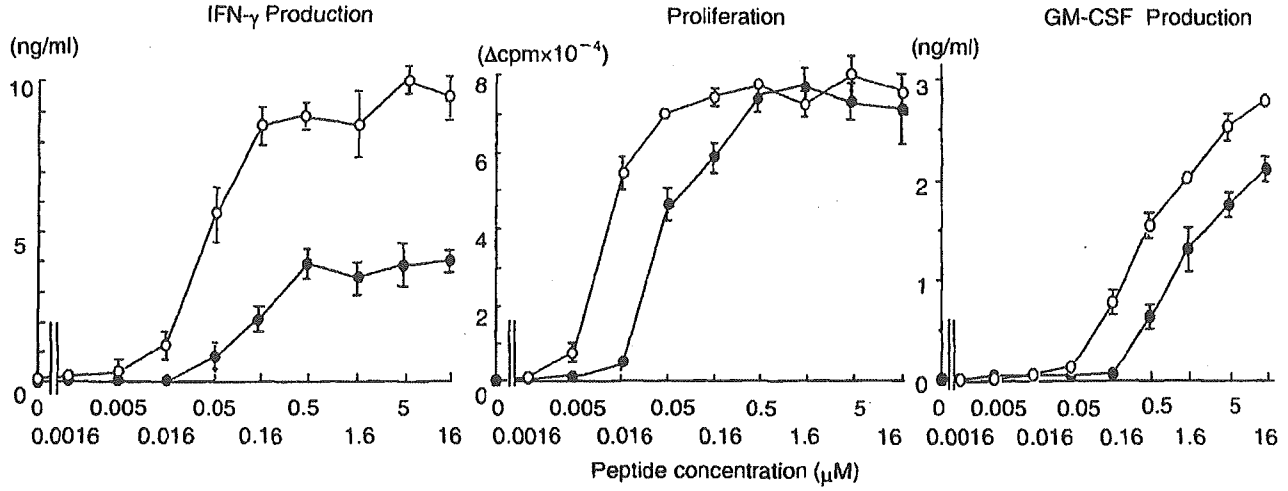

Fig. 1 IFN-γ production, GM-CSF production and proliferation of BC20.7 in recognition of either the wild-type peptide or L87V, at different concentrations. BC20.7 cells were cultured in triplicate with peptides and irradiated autologous PBMC, at the indicated concentrations. After 48-h incubation, supernatant fluids of triplicate cultures were collected. The remaining cells were pulsed with [^{3}H]-thymidine, harvested after 16h, and subjected to liquid scintillation counting. Closed circle, wild type peptide; open circle, L87V. Results are expressed as the geometric means ± standard error. IFN-γ production induced by L87V was significantly (p<0.01) higher than that induced by the wild-type peptide, at peptide concentrations ranging from 0.016 to 16 μM. On the other hand, plateau level of proliferation did not exhibit a significant difference, between 0.16 and 16 μM (p>0.05). GM-CSF production did not reach a plateau response even at 16 μM, without any statistical difference between L87V and the wild-type peptide, at 16 μM.

also observed when P5- and P8-substituted analogues were tested. Thus, S90E,S90G,S90M,D93Q,D93T and D93Y exhibited full agonism, in a clone-specific manner.

DISCUSSION

It is not very easy to identify TCR genes used by T cell clones, since they are usually cultured with irradiated autologous PBMC that includes polyclonal T cells. Random cloning of TCR cDNA derived from the cultured cells is minimally helpful in the identification, unless a large number of clones are examined. This problem was circumvented by the use of PCR-ELISA that was developed for TCRBV use,[9] and established in the present report for TCRVA usage. This technique allowed us to quantitate TCRV gene usage in the cDNA samples, and thus to identify the TCRV gene used by the T cell clones.

Three T-cell clones used in the present study recognize the same TCR ligand, as proven in our previous study. This is based on the fact that these clones recognize BCGa p84-100(^{84}EEYLILSARDVLAVVSK^{100}) in the context of DRB1*1405, and react to truncated peptides in a similar fashion.[11] Both BC20.7 and BC33.5 have 8 and 11 residues at N(D)N region of TCRVA and VB, respectively, whereas BC42.1 alone exhibits a different pattern, *i.e.*, 9 residues at N(D)N regions of TCRVA and VB. When peptide antigen is presented by class II MHC molecules, the N-terminal half of antigenic peptide is recognized mainly by CDR3 of TCRVA, whereas the C-terminal half is recoginized by CDR3 of TCRVB, which corresponds to N(D)N regions.[12] Interestingly, certain amino acid replacements on P2 induced increased IFN-γ production in BC20.7 and BC33.5 but not in BC42.1 cells, whereas those on P8 exhibited full agonism in BC 42.1 cells alone. It is thus likely that structural features of VACDR3 and VBCDR3 are responsible for specific responses induced by P2 and P8 analogues, respectively. Shuffling of N(D)N sequences between BC 42.1 and BC 20.7, or between BC 42.1 and BC 33.5 is underway to address this point.

Only L87T,L87S,L87A,and L87V induced IFN-γ enhancement. These arrangements are either smaller hydrophobic (A and V), or structurally similar neutral amino acids (T and S), indicating that close contact between P2 and TCRVA is taking place. Indeed, such a phenomenon is also seen in B-cell somatic hypermuation.[13] Thus, B-cell V region mutation in immunoglobulin heavy chain genes shows higher affinity than the germ-line sequence, usually associated with Gly, Ala, Val, Ser, Thr, or Cys, *i.e.*, small hydrophobic or small neutral residues. Apparently these mutations are not associated with static charges, but can affect either hydrogen bonding, van der Waar's force, or hydrophobic interactions.

In our previous studies using cedar pollen-derived peptides, T to V replacement on P2 also induced IFN-

Table 3 Increased IFN-γ production induced by peptide analogues

Replaced by	Relative IFN-γ response (BC20.7 / BC33.5 / BC42.1)								
	P1 =86Y	P2 =87L	P3 =88I	P4 =89L	P5 =90S	P6 =91A	P7 =92R	P8 =93D	P9 =94V
K	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	108/115/90	*/*/*	*/*/*
E	*/*/*	*/*/*	*/*/*	*/*/*	*/105/94	*/*/*	*/*/*	*/*/*	*/*/*
Q	*/*/*	88/79/90	*/*/*	92/79/81	*/*/*	*/*/*	*/*/*	*/*/87	*/*/*
N	*/*/*	105/97/95	*/*/*	88/92/97	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
T	*/*/*	177/210/86	110/92/81	*/*/*	77/97/108	*/*/*	*/*/*	*/*/95	85/96/91
S	*/*/*	155/187/90	95/95/99	*/*/*	100/100/100	81/87/97	*/*/*	*/*/*	93/75/99
G	*/*/*	110/98/79	90/100/92	*/*/*	*/*/115	77/69/93	*/*/*	*/*/*	94/99/100
A	96/100/98	189/202/94	105/94/83	107/93/83	88/104/110	100/100/100	*/*/*	*/*/*	80/81/92
V	91/91/85	271/259/92	91/84/86	105/96/86	99/100/101	90/76/85	*/*/*	*/*/*	100/100/100
L	93/88/102	100/100/100	100/90/101	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*
Y	100/100/100	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/98	*/*/*
M	89/93/91	*/*/*	96/99/103	89/70/85	*/91/*	*/*/*	*/*/*	*/*/*	*/*/*
W	90/103/109	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*	*/*/*

Positions 1 – 9 (P1 – P9) of BCGa p84-100 (EEYLILSARDVLAVVSK; with P1 underlined), was replaced by indicated amino acids. T cells were stimulated with peptide species at 16 μM. To obtain relative IFN-γ response values, plateau responses of IFN-γ (pg/ml) were first divided by plateau responses of proliferation (cpm). Then, the following calculation was performed: relative IFN-γ responses=100×[IFN-γ/proliferation to analogues]/[IFN-γ/proliferation to the wild-type BCGa p84-100]. The denominator was 0.0533. *Peptide that did not induce fully agonistic proliferation.

γ enhancement, whereas proliferation remained the same. Therefore, although not generalized, mutual replacement on G, A, V, L, S, or T at P2, tends to induce IFN-γ-specific enhancement. Such observations also have been reported in another study with different peptide species.[14] In this sense, analogue-induced clonal anergy is often observed, especially when residue replacement is made on P7 or P8.[11] Moreover, truncation of the C-terminal moiety of antigenic peptides, in general, exhibit TCR antagonism.[15] In other words, if a rule that applies to altered polyclonal novel responses induced by peptide analogues is established, it will lead us to novel therapeutic interventions using peptide analogues. Our observations on P2 replacement which is associated with increased IFN-γ production are imperative to furthering our understanding.

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ORIGINAL PAPER

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Identification of the MHC class I *B* locus in cynomolgus monkeys

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Abstract By determining the nucleotide sequences of more than 700 cDNA clones isolated from 16 cynomolgus monkeys, we identified 26 *Mafa-B* alleles. In addition, nine sequences with similarity to *Mamu-I* alleles were identified. Since multiple *Mafa-B* alleles were found in each individual, it was strongly suggested that the cynomolgus MHC class I *B* locus might be duplicated and that the *Mafa-I* locus was derived from the *B* locus by gene duplication, as in the case of the *Mamu-I* locus of rhesus monkeys.

Keywords Cynomolgus · MHC · *Mafa* · Allele

Introduction

It is well established that $CD8^+$ T-cell activation is triggered through recognition of the MHC class I molecule loaded with an antigenic peptide by an antigen-specific T-cell receptor. The MHC molecules of the mammals including primates are known to influence the outcome of many diseases such as infectious diseases, cancer, and metabolic disorders. HLA class I genes are divided into three different categories, classical (*HLA-A*, *-B*, and *-C*), non-classical (*HLA-E*, *-F*, and *-G*), and pseudogene (*HLA-H*, *-J*, *-K*, and *-L*), according to their degree of polymorphism and cell surface expression, and the presence of orthologues of the human *HLA-A*, *-B*, *-E*, *-F*, and *-G* genes were identified in several species of the Old World monkeys (Alvarez et al. 1997; Boyson et al. 1996a,b; Evans et al. 2000; Lafont et al. 2004; Otting and Bontrop 1993; Prilliman et al. 1996; Sidebottom et al. 2001; Uda et al. 2004). Cynomolgus monkeys as well as rhesus monkeys are preferentially used for biomedical research; however, cynomolgus MHC class I was not extensively studied compared with those in rhesus monkeys. We have previously reported the nucleotide sequences of cynomolgus MHC class I *A* locus and have shown that at least 14 *Mafa-A* alleles were present in cynomolgus monkeys (Uda et al. 2004). Although the MHC class I *B* locus is the most polymorphic MHC locus in primates, little information is available concerning the MHC class I *B* locus of cynomolgus monkeys. In this study, therefore, we have expanded our analysis on cynomolgus MHC class I genes and identified 26 *B* locus alleles by analyzing 16 monkeys. We have also found the presence of a novel locus that is very similar to MHC class I *I* locus recently identified in rhesus monkeys.

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Materials and methods

Animals

All cynomolgus monkeys were raised and reared in the Tsukuba Primate Center for Medical Science, the National Institute of Infectious Diseases (NIID). Both genders were involved, and the cynomolgus monkeys were between 5 and 24 years old. This study was conducted in accordance with the Guides for Animal Experiments Performed at the NIID.

RT-PCR and nucleotide sequencing

Preparation of mRNA from peripheral blood mononuclear cells (PBMC) and RT-PCR were performed as described before (Uda et al. 2004). Primers used in this study are listed in Table 1. 5′ MBS and 3′ MBS primers designed to amplify the gene products of the rhesus MHC class I *B*

Table 1 Primers used for the amplifications and sequencing of MHC class I cDNA from cynomolgus monkeys

Primers	Binding region (position)	Sequence[a]
Primers for amplification		
5′ Beta 3 XMO[b]	All loci exon 1 (–27-1)	5′-CGC TCG AGG ACT CAG AAT CTC CCC AGA CGC CGA G-3′
Mafa-B1a	*B* and I loci exon 8 (1089–1117)	5′-CCA CTT AAG ACA GTT TCA GGC TTT T-3′
5′ MBS[b]	*B* and I loci exon 1 (10–34)	5′-GCC TCG AGA ATT CAT GGC GCC CCG AAC CCT CCT CCT GC-3′
3′ MBS[b]	*B* and I loci exon 8 (1095–1116)	5′-GCA AGC TTC TAG ACC ACA CAA GAC AGT TGT CTC AG-3′
Primers for sequencing		
T7 primer	pCR4Blunt-TOPO vector (328–347)	5′-TAA TAC GAC TCA CTA TAG GG-3′
T3 primer	pCR4Blunt-TOPO vector (243–262)	5′-AAT TAA CCC TCA CTA AAG-3′
Ia698	All loci exon 4 (680–698)	5′-TAG AAG CCC AGG GCC CAG C-3′
Is437	All loci exon 3 (437–456)	5′-ATT ACA TCG CCC TGA ACG AG-3′

[a] *Xho*I, *Sal*I, and *Hin*dIII sites of 5′ beta 3 XHO, 5′ MBS, and 3′ MBS primers, respectively, are *underlined*
[b] From Boyson et al. (1996b)

locus by Boyson et al. (1996b) were also used to amplify the cynomolgus MHC class I *B* locus. PCR amplification was performed at least twice for each animal. PCR products were cloned into pCR4Blunt-TOPO plasmids (Invitrogen, Carlsbad, Calif., USA) and 48 clones were sequenced by 310 Capillary DNA Sequencer (Applied Biosystems, Foster City, Calif., USA) or 3100-Avant Capillary DNA Sequencer (Applied Biosystems). The *Mafa-B* nucleotide sequences were assembled with the Contig Manager of the DNASIS pro (Hitachi Software, Yokohama, Japan). The Clustal W algorithm provided in DNASIS PRO was used to align sequences.

Phylogenetic analysis

The full-length nucleotide sequences of *Mafa-B*, *Mafa-I*, *Mafa-A*, *Mamu-A*, *Mamu-B*, *Mamu-I*, *HLA-A*, and *HLA-B* were aligned using Clustal W provided online by the DNA Data Bank of Japan [(DDBJ) http://www.ddbj.nig.ac.jp]. A phylogenetic tree of these nucleotide sequences was constructed by the neighbor-joining method of the Molecular Evolution Genetics Analysis, version 2.1 (MEGA 2.1). Genetic distances were estimated using the method of Jules-Canter. At the sites in which alignment indicated a gap, nucleotides at this position in all the sequences were deleted. The reliability of the tree topology was tested by the bootstrap method. Thousand relationships and 64,238 random seeds were used for determining bootstrap values (Fig. 2a, b). Since the bootstrap values of less than 50% were unreliable, the values of less than 50% were not shown in Fig. 2a, b.

	Clone /animal	Leader peptide −20 −10
HLA-B*2702		MRVT APRTLLLLLW GAVALTETWA
HLA-B*5701		....V..... .....
Mamu-B*02		...M .....S ..L.....
Mamu-B*03		...M ....F....S ..L.....
Mamu-B*04		...M ....F....S ..L.....
Mafa-B*01	7/2	...M .....S ..L.....
Mafa-B*02	3/1	.Q.M .....S ..L.....
Mafa-B*03	13/3	...M .....S ..L.....
Mafa-B*04	9/2	...M .....S .TLS.....
Mafa-B*05	36/2	...M ...I.....S .TLS.....
Mafa-B*06	6/1	...M ...I.....S .TLS.....
Mafa-B*07	28/1	...M .....S ..L.....
Mafa-B*08	2/1	...M .....S .TLV.....
Mafa-B*09	21/2	...M .....S ..L.....
Mafa-B*10	4/1	---- --------.S ..L.....
Mafa-B*11	16/1	---- --------.S ..L.....
Mafa-B*12	17/2	...M .....S ..L.....
Mafa-B*13	4/1	...M .....S ..L.....
Mafa-B*14	33/2	...M .....S .PL.....
Mafa-B*15	10/1	---- --------.S ..L.....
Mafa-B*16	25/4	...M .....S A.L...K...
Mafa-B*17	45/3	.Q.M .....S ..LS.....
Mafa-B*18	3/1	...M .....S ..L...Q...
Mafa-B*19	20/2	.QTM .....S ..L.....
Mafa-B*20	6/2	..DM .....S ..L.....
Mafa-B*21	22/2	.QIM .....S ..L.....
Mafa-B*22	36/2	...M .....S ..L.....
Mafa-B*23	22/2	...M .....S ..L.....R.
Mafa-B*24	10/3	...M ..G.....S ..L.....
Mafa-B*25	7/3	..FM .....S ..L...Q...
Mafa-B*26	5/1	...M ..G.....S ..L.....
Mamu-I*04		---- --------HS ..L.....
Mamu-I*07		.M ..G.....S ..L.....
Mamu-I*08		---- --------.S ..L.....
Mafa-I*01013	13/1	...M .....S ..L.....
Mafa-I*02	12/1	...M ..G.....S ..L.....
Mafa-I*03	33/3	...M ..G.....S .TL.....
Mafa-I*04	2/1	...M ..G.....S ..L.....
Mafa-I*05	11/1	...M ..G.....S ..L.....
Mafa-I*06	16/2	...M ..G.....S ..L.....
Mafa-I*07	3/1	...M ..G.....S ..L.....
Mafa-I*08	5/1	...M ..G.....S ..L.....
Mafa-I*09	51/3	...M ..G.....S ..L.....
HLA-A*0201		.A.M .....V...S ..L...Q...
Mamu-A*01		---- .....V.S ..LV..Q.R.
Mamu-A*02		---- ----...V.S ..L...Q.R.
Mafa-A*01		.A.M .....V.S ..L...Q.R.
Mafa-A*02		.A.M .....V.S .VL...Q.R.
Mafa-A*06		.A.M .....V.S .FL...Q.L.

Fig. 1 Deduced amino acid sequences of *Mafa-B* and *Mafa-I* alleles. ▶ Amino acid sequences of *HLA-A*, *HLA-B*, *Mamu-A*, *Mamu-B*, *Mamu-I*, *Mafa-A*, and *Mafa-E* alleles were also included. Amino acids identical to those of *HLA-B*2702* are indicated by *dots*. The deletions of amino acids are indicated by *hyphens*. The total numbers of clones obtained and the numbers of animals having the allele were indicated *after the allele name*

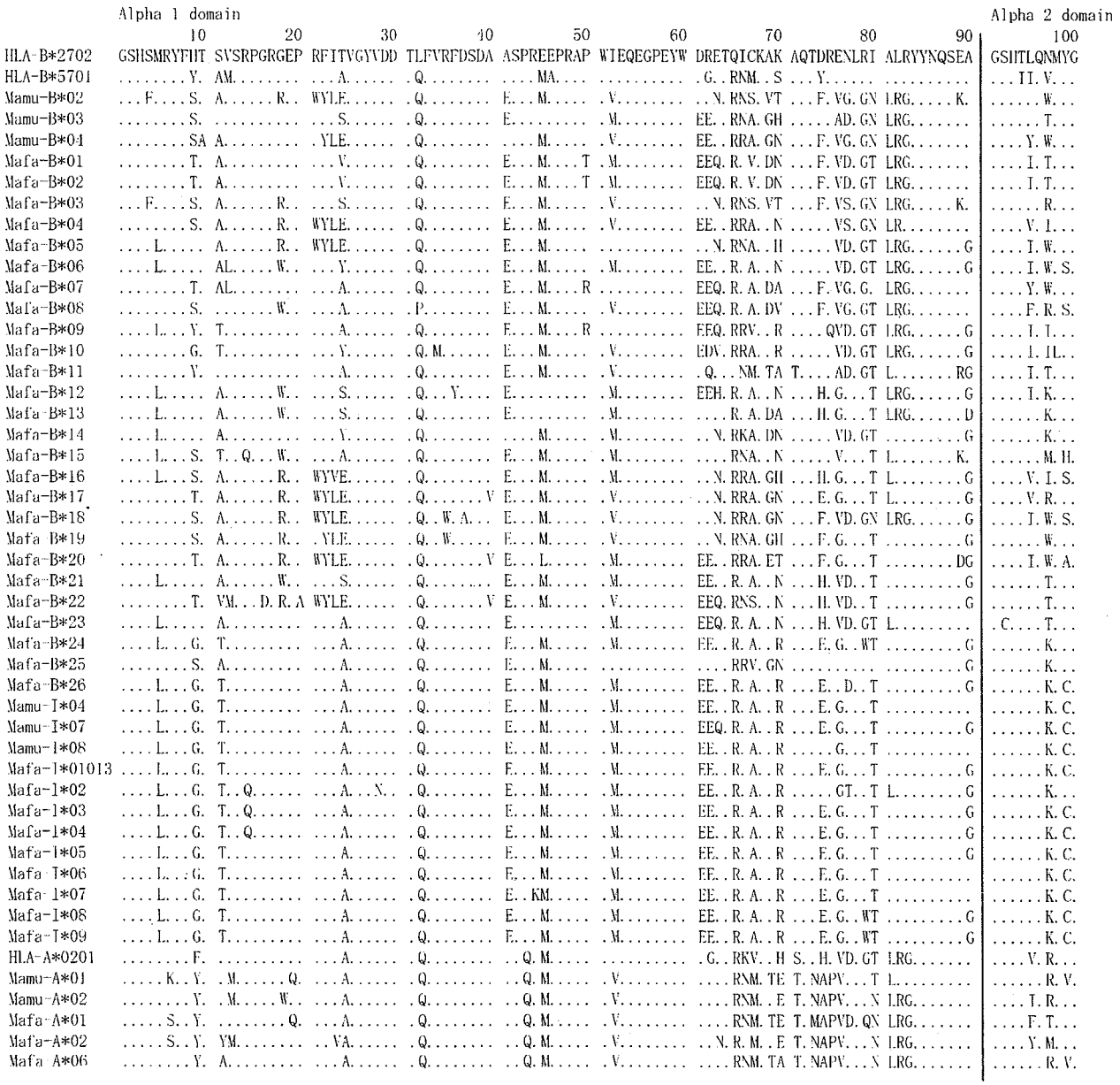

Fig. 1 (continued)

GenBank accession numbers

The *Mafa-B* and *Mafa-I* sequences described in this manuscript had been deposited in the DDBJ and were assigned accession numbers AB195431 to AB195465. We previously deposited *Mafa-A* alleles in the DDBJ, and these alleles were assigned accession numbers AB154760 to AB154773. The GenBank accession numbers for other sequences used in this study are as follows: *HLA-A*0201*, U07161; *HLA-B*2702*, L38504; *HLA-B*5701*, AJ458991; *Mafa-E*01*, U02976; *Mamu-A*01*, U50836; *Mamu-A*02*, U50837; *Mamu-A*03*, U41379; *Mamu-A*04*, U41380; *Mamu-B*02*, U41833; *Mamu-B*03*, U41825; *Mamu-B*04*, U41826; *Mamu-B*05*, U41827; *Mamu-B*06*, U41828; *Mamu-B*07*, U41829; *Mamu-B*08*, U41830; *Mamu-B*36*, AJ556886; *Mamu-I*01011*, AF161865; *Mamu-I*02012*, AF161869, *Mamu-I*04*, AF4161874; *Mamu-I*07*, AF161875; *Mamu-I*08*, AF161876; *Mamu-I*09*, AF161877; *Mamu-I*10*, AF161878; and *Mamu-I*11*, AF161879.