GENOME METHODS

PCR–LIS–SSCP (Low Ionic Strength Single-stranded Conformation Polymorphism)—A Simple Method for High-resolution Allele Typing of *HLA–DRB1*, *–DQB1*, and *–DPB1*

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We have developed a simple and efficient procedure with which to form single-stranded DNA (ssDNA) and then applied *HLA–DRB1*, *–DQB1*, and *–DPB1* allele typing. This method is referred to as low ionic strength single-stranded conformation polymorphism (LIS–SSCP), and is based on the diversity in the electrophoretic mobility of ssDNA formed by heat denaturation in low ionic strength solutions. This method detected DNA polymorphisms, including point mutations at a variety of positions in the DNA fragments of the *HLA–DRB1*, *–DQB1*, and *–DPB1* genes. Under our experimental conditions, stable ssDNA could be kept at room temperature ≥ 5 hr without having been cooled on ice immediately after heat denaturation. A total of 41 *HLA–DRB1*, 14 *HLA–DQB1*, and 17 *HLA–DPB1* alleles from 220 healthy people were analyzed using a combination of PCR–LIS–SSCP with group-specific amplification. All of the alleles analyzed were discriminated among the *DRB1*, *DQB1*, and *DPB1* groups except for *DPB1*0402* and *0201*. The efficiency of ssDNA formation using the LIS–SSCP procedure was higher than that of the traditional formamide method, and the SSCP profiles were clearer than those of the original SSCP. This procedure is useful for screening new alleles as well as the donor–recipient molecular matching of HLA class II genes. It is simple, rapid, and cost effective, requiring neither radioisotopes nor enzymes to confirm the typing results of other methods.

The HLA class II (*HLA–DR*, *–DQ*, and *–DP*) antigens are heterodimers (α and β chains) of highly polymorphic glycoproteins expressed on the surface of antigen-presenting cells and they play a key role in the immune recognition of foreign antigens (Zinkernagel and Doherty 1974; Babbitt et al. 1985; Bach 1985; Schwartz 1985; Buus et al. 1987; Guillet et al. 1987; Stern et al. 1994). Allologous HLA class II antigens are also recognized by the immune surveillance system in tissue transplantation (Bach and Sachs 1987). The β chains of *HLA–DR*, *–DQ*, and *–DP* antigens show remarkable polymorphism in the first domain, and they are encoded by the second exon of the *HLA–DRB*, *–DQB*, and *–DPB* genes, respectively. *HLA–DRB1*, *–DQB1*, and *–DPB1* have been reported to possess 124, 25, and 62 alleles, respectively (Bodmer et al. 1995).

HLA class II DNA typing has become very im-

portant in the fields of basic and clinical medicine as HLA disease association and donor–recipient matching for transplantations increase. In heterologous bone marrow transplantation, HLA alleles must be typed with precision and efficiency for selecting the most appropriate donor. There are many methods of HLA allele typing. However, all of them have advantages and weaknesses, so at present, there is no perfect method that is easy, accurate, prompt, and cost effective.

Single-stranded conformation polymorphism (SSCP) was developed by Orita et al. (1989) as a means of detecting a mutated sequence. This method is based on the fact that the electrophoretic mobility of a single-stranded nucleic acid in a nondenaturing polyacrylamide gel depends not only on its size but also on its sequence. Heat denaturation in the presence of formamide is the usual means of generating single-stranded DNA (ssDNA). This procedure has been used by Bannai et al. (1994) in *HLA–DRB1* allele typing, and by Hoshino et al. (1992) in *HLA–*

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DPA1 and *-DPB1* allele typing. We have developed a simple and rapid means of forming stable ssDNA by heat denaturation under conditions of low ionic strength (LIS) that requires neither denaturing reagents nor cooling.

RESULTS

The Efficiency of ssDNA Formation

The efficiencies of ssDNA formation by heat denaturation with LIS solution and with formamide are compared in Figure 1. Equivalent amounts of amplified product were resolved. Lanes 1–3 show untreated, heat-denatured profiles in the presence of formamide and in LIS, respectively. The double-stranded DNA (dsDNA) bands are shown in lanes 1 and 2. However in lane 3, the dsDNA band disappeared. Therefore, LIS was more efficient for ssDNA formation than formamide.

DR1 Alleles (5'R1/3'R)

Three types of DR1 *DRB1* alleles (*DRB1**0101, 0102, and 0103) were examined (data not shown). The migration rates of each allele differed, so it was easy to distinguish among them.

DR2 Alleles (5'R2/3'R)

Four types of DR2 *DRB1* alleles (*DRB1**1501, 1502, 1601, 1602) were examined (data not shown). The electrophoresis at 22°C discriminated *DRB1**1501, 1502, 1601, or 1602. *DRB1**1601 and 1602 were migrated close together. However, a parallel run of DR1601 and 1602 enabled their discrimination. Profiles of *DRB1**1503 and 1505 were also discriminated from other DR2 *DRB1* alleles (data not shown).

DR4 Alleles (5'R4/3'R)

Ten alleles included in the DR4 group (*DRB1**0401, 0402, 0403, 0404, 0405, 0406, 0407, 0408, 0410, 0411) were tested (Fig. 2A). The migration rates of each allele band differed (8% gel, 22 °C), so these alleles were distinguishable. *DRB1**0404, 0406, and 0410 migrated close together. However, electrophoresis at 22°C in 12.5% gels discriminated among the three alleles (Fig. 2B). *DRB1**0407 and *DRB1**0408 were also migrated close together. They also were easily dis-

tinguishable by electrophoresis at 22°C in 12.5% gel (data not shown).

DR8, DR12 Alleles (5'R8/12/3'R)

Seven alleles in this group (*DRB1**0801, 0802, 0803, 0804, 0806, 1201, 1202) were examined. Five alleles included in the DR8 group migrated at different rates in 12.5% gels at 4°C (data not shown). Two alleles included in the DR12 group migrated at different rates in 15% gels at 32°C (data not shown).

DR3, 5, 6 Alleles (5'R3/5/6/3'R)

There were 15 alleles in this group (*DRB1**0301, 1101, 1102, 1103, 1104, 1301, 1302, 1303, 1304, 1305, 1401, 1402, 1403, 1405, 1406). All of them migrated at different rates in 12.5% gels at 22°C (data not shown).

DQI Group Alleles (5'QBKY02/QB202)

Eight alleles in this group (*DQB1**0501, 0502, 05031, 0601, 0602, 0603, 0604, 0609) were examined (Fig. 3). *DQB1**05031 and *DQB1**0601, or *DQB1**0602 and *DQB1**0603, or *DQB1**0604 and

Figure 1 The efficiency of ssDNA formation under conditions of LIS. (Lane 1) Untreated dsDNA; (lane 2) heat denatured with formamide; (lane 3) heat denatured with LIS. The amplified PCR product was *HLA-DRB1**1502 and applied in equivalent volume to each lane. Electrophoresis was carried out on a 12.5% gel at 22°C for 1 hr.

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*DQB1*0609* migrated close together. However, a parallel run of mixtures enabled their discrimination (e.g., *DQB1*0604* and *DQB1*0609* are shown in Fig. 3, lanes 8–10).

DQ2, 3, and 4 Group Alleles (5'QBKY02/QB204)

Six alleles in this group (*DQB1*0201*, *0301*, *0302*, *0303*, *04001*, *0402*) were tested. Each allele migrated at different rates in 12.5% gels at 32°C (data not shown).

Figure 3 PCR-LIS-SSCP analysis of HLA-DQ1 group alleles. Eight alleles were examined. Only DQ1 group alleles were amplified and analyzed by LIS-SSCP. (Lane 1) *DQB1*0501*; (lane 2) *DQB1*0502*; (lane 3) *DQB1*05031*; (lane 4) *DQB1*0601*; (lane 5) *DQB1*0602*; (lane 6) *DQB1*0603*; (lane 7) *DQB1*0604*; (lane 8) *DQB1*0609*; (lane 9) *DQB1*0604*; (lane 10) *DQB1*0604* + *0609*. Electrophoresis was carried out on a 12.5% gel at 32°C.

DPB1 Group 2 Alleles (DPB1OIN/DPB3'6)

Five alleles in this group (*DPB1*0201*, *0202*, *4701*, *0401*, *0402*) were tested (data not shown). *DPB1*0201* and *0402* were indistinguishable. We examined various acrylamide concentrations (8%, 10%, 12.5%, and 15%) with or without 10% glycerol at various temperatures (4°C, 22°C, and 32°C) of running buffer. However, we failed to discriminate between these two alleles. Therefore, amplified DNAs were digested with restriction endonucleases (*Eco*NI) and then resolved by electrophoresis to detect allele-specific restriction profiles (*DPB1*0201* = 174 bp, 71 bp, 30 bp; *DPB1*0402* = 245 bp, 30 bp; data not shown).

Allele Detection Rates

We examined 41, 14, and 17 known alleles of *DRB1*, *DQB1*, and *DPB1*, respectively, and could detect the mutations of 41 (100%), 14 (100%), and 15 (88%) alleles. Overall, we succeeded in distinguishing among a total of 70 (97%) of the 72 alleles examined by LIS-SSCP.

DISCUSSION

The SSCP method was first developed by Orita et al. (1989), and Hoshino et al. (1992) have used silver staining in recognition of the genotyping of *HLA-DPA1* and *-DPB1*. This method requires a small quantity of sample DNA compared with

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Figure 4 PCR-LIS-SSCP analysis of HLA-DPB1 group 1 alleles. Twelve alleles were examined. Only DPB1 group 1 alleles were amplified and analyzed by LIS-SSCP. (Lane 1) *DPB1*0101*; (lane 2) *DPB1*0301*; (lane 3) *DPB1*0501*; (lane 4) *DPB1*0901*; (lane 5) *DPB1*1101*; (lane 6) *DPB1*1401*; (lane 7) *DPB1*1701/0101*; (lane 8) *DPB1*1901*; (lane 9) *DPB1*2701/0101*; (lane 10) *DPB1*0601/0101*; (lane 11) *DPB1*1001*; (lane 12) *DPB1*2101*. Electrophoresis was carried out on a 12.5% gel at 32°C.

ethidium bromide staining, and the electrophoretic resolution was improved. However, in their method, Hoshino et al. (1992) have observed several bands for a single allele. Bannai et al. (1994) have reduced the proportion of amplified DNA in the application mixture for SSCP electrophoresis and have observed only two bands per allele, which correspond to sense and antisense strands appearing for the *HLA-DRB1* gene. The procedure generating the ssDNA described here is more efficient than that using the formamide. Additionally, the banding profiles of LIS-SSCP were clearer than that of the original SSCP. The formation of ssDNA was very stable in LIS, even at room temperature. Therefore, it is not necessary to cool the mixture on ice immediately after heat denaturation. Because formamide, as a denaturing reagent, makes hydrogen bonds between adenine and thymine unstable, renaturing after heat denaturation is prevented. We assumed that the ssDNA stability in the absence of formamide was based on two kinds of effects. The primary effect was the decline in the melting temperature (T_m) of the DNA under low ionic conditions and the secondary effect was that of the dilution that resulted from the decreased chance of hybridization between sense and antisense strands after heat denaturation, by adding a large volume of LIS solution (amplified PCR product/LIS solution = 1:20).

HLA-DRB1, *-DQB1*, and *-DPB1* reportedly possess 124, 25, and 62 alleles, respectively (Bod-

mer et al. 1995), and 40, 14, 16 alleles among them were examined here. All of *HLA-DRB1*, *-DQB1* alleles were discriminated. However, in *HLA-DPB1* alleles, *HLA-DPB1*0201* and *0402* were indistinguishable. This indicated that not all of the sequence variations can be easily detected by SSCP. Hoshino et al. (1992) have solved this problem by using additional PCR amplification with different primer pairs as well as SSCP. The *HLA-DPB1*0201* and *0402* sequences differed by only one nucleotide substitution at position 205, which led to an amino acid substitution Lys → Glu at position 68. A smaller amplified PCR product that included this substitution could have affected the electrophoretic mobility of ssDNAs. It has been reported that the banding profiles vary according to the electrophoretic conditions, especially the acrylamide concentration, presence or absence of glycerol, and the temperature (Orita et al. 1989). However, repeated PCR and conducting electrophoresis trials were complicated, so RFLP analysis was used in this study, because it was not only simple, but the same amplified product could be used for RFLP. In general, it might be useful for accurate HLA class II allele typing to combine various methods to maximize their advantages (Blasczyk et al. 1995; Rosenberg et al. 1995; Thonnard et al. 1995).

The method described here might be somewhat complex for routine typing of HLA class II alleles, in terms of group-specific amplification and a requirement for reference DNAs in the electrophoretic gels. However, when generic HLA groups are determined beforehand by simple DNA typing methods, such as reverse sequence-specific oligonucleotide (SSO) on microtiter plates described by Kawai et al. (1994) and Maekawajiri et al. (1994), our method is useful for discriminating HLA class II alleles as well as for screening hitherto unknown alleles. Moreover, SSCP is the most applicable method for the detection of mutations. LIS-SSCP, which is an improved version of SSCP, can be used as a more practical mutation detection method for the diagnosis of genetic aberrations in oncology and other areas. The LIS-SSCP method can be applied to donor and recipient matching for bone marrow transplantation.

METHODS

DNA Samples and References

Genomic DNAs were obtained from a total of 20 healthy individuals: 100 Japanese, 50 Caucasians, 40 Hispanics,

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Table 1. PCR Primers for Amplification of HLA Class II Genes and Electrophoretic Conditions

Gene	Group	Name of sense primer	Name of antisense primer	Sequences (5' → 3')	Products	Alleles	PCR annealing temperature (°C)	SSCP gel concentration (%)	SSCP electrophoretic temperature (°C)
DRB1	for DR2	5' R2	3' R	CCGCTGCACTGTGAAGCTCT	261bp	1501~03, 1601~02	59	12.5	22
	for DR4	5' R4		TTCCTGTGGACCCCTAAGAGG	263bp	0401~12	59	8/12.5	22
	for DR9	5' R9		GTTCITGGAGCAGGTAAAC	225bp	901	59		
	for DR1	5' R1		AACGGGACGGAGCGGTGCGGTAT	206bp	0101~03	59	12.5	22
	for DR7	5' R7		GGTTGCTGGAAGATGCATCT	206bp	0701~02	59		
	for DR10	5' R10		AGTTCTGGAAAGACTCTTCT	206bp	1001	59		
	for DR8, 12	5' R8/12		GGTTGCTGGAAGACCGCTCC	256bp	0801~06, 1201~02, 1404	68	12.5/15	32/4
	for DR3, 5, 6	5' R3/5/6		GGAGTACTCTACGGGTGAGTGT	262bp	0301~03, 1101~04, 1301~05, 1401~03, 1405~09	63	12.5	22
DQB1	for DQ1	5' QBKY02		TTCCTGGAGTACTCTACGTC	263bp	0501~03, 0601~05	59	12.5	32
	for DQ2, 3, 4	QB202		GGATTGCGTGACCCAGTTTAAGG	259bp	0201, 0301~03, 0401~02	59	12.5	32
DPB1		DPB101N		CACCTGCAGTCCCGCGGTACGCCACCTC		0101, 0301, 0501, 0601, 0801, 0901, 1001, 1101, 1301, 1401, 1601, 1701, 1901, 2101, 2701			
	for Group 1	DPB3' 4		CACCTGCAGTCCCGCGGTACGCCACCTC	272bp		59	12.5*	32/4
	for Group 2	DPB3' 6		CAGGGTCACGGGCTCGT	275bp	0201, 0202, 0401, 0402, 1501, 1801	59	12.5*	4
(*) Including 10% glycerol.									

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and 30 Blacks. The samples had been typed for *DRB1*, *DQB1*, and *DPB1* by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP; Uryu et al. 1990; Nomura et al. 1991; Ota et al. 1991). The DNAs of the Terasaki DNA Exchange Program (Los Angeles, CA) were used as references. A total of 72 alleles were included in this study. They are as follows: DR1 (*DRB1**0101, 0102, 0103), DR2 (*DRB1**1501, 1502, 1503, 1505, 1601, 1602), DR3 (*DRB1**0301), DR4 (*DRB1**0401, 0402, 0403, 0404, 0405, 0406, 0407, 0408, 0410, 0411), DR5 (*DRB1**1101, 1102, 1103, 1104, 1201, 1202), DR6 (*DRB1**1301, 1302, 1303, 1304, 1305, 1401, 1402, 1403, 1405, 1406), DR8 (*DRB1**0801, 0802, 0803, 0804, 0806), DQ5 (*DQB1**0501, 0502, 0503), DQ6 (*DQB1**0601, 0602, 0603, 0604, 0609), DQ2 (*DQB1**0201), DQ3 (*DQB1**0301, 0302, 0303), and DQ4 (*DQB1**0401, 0402) *DPB1**0101, 0201, 0202, 0301, 0401, 0402, 0501, 0601, 0901, 1001, 1101, 1401, 1701, 1901, 2101, 2701, 4701.

PCR Amplification

Genomic DNA (100 ng) was amplified by PCR with 1.25 units of *Taq* DNA polymerase (Perkin-Elmer Cetus) (Saiki et al. 1988). The reaction mixture (50 μ l) [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pM of each amplification primer] was initially denatured at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 59°C for 1 min, and a final incubation at 72°C for an additional 5 min to complete the final extension step after the last cycle, using an automated PCR thermal sequencer (GeneAmp PCR system 9600, Perkin-Elmer Cetus).

The second exon of the *DRB1* gene was amplified using seven sets of group-specific primers (Table 1). The second exon of the *DQB1* gene with the DQ1 specificity was amplified by the DQ1 group-specific primers, 5'-QBKY02/QB202; and the second exon of the *DQB1* gene with DQ2, DQ3, or DQ4 specificity was amplified by the group-specific primers, 5'-QBKY02/QB204 (Table 1). The second exon of the *DPB1* gene was amplified using two sets of group-specific primers (Table 1). DNA amplification was confirmed by 2% agarose gel electrophoresis and ethidium bromide staining.

LIS-SSCP

One microliter of PCR product was added to 20 μ l of LIS solution (10% saccharose, 0.01% bromophenol blue, and 0.01% xylene cyanol FF) and mixed well. The mixture was incubated for 2 min at 97°C, and 4–10 μ l of the mixture was applied to a 8%–15% polyacrylamide gel (acrylamide/bisacrylamide = 49:1) on a minigel electrophoresis apparatus with a constant temperature control system (90 $\times$ 80 $\times$ 1 mm, AE-6410 and AE-6379, Atto). Electrophoresis was carried out in 45 mM Tris-borate (pH 8.0)/1 mM EDTA at an optimal running temperature and 15 mA for 2 hr. SSCP in the gel was detected by silver staining (Daiichi Pure Chemicals, Tokyo, Japan). To evaluate the optimal electrophoresis conditions for gel concentrations and running temperatures, we performed the electrophoresis on a 12.5% polyacrylamide gel at 22°C and modified the conditions to 8% or 15%, at 32°C or 4°C.

SSCP Using Formamide

One microliter of the amplified product was mixed with 7 μ l of denaturing solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The mixture was heated at 95°C for 5 min, cooled immediately on ice, and 2–4 μ l of the mixture was resolved by electrophoresis as described above.

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