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HLA-DR8 Subtyping by Polymerase Chain Reaction (PCR) – DNA Conformation Polymorphism (DCP) Analysis : a simple and practical genotyping method

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ABSTRACT

Two hundred Japanese panels were serologically typed for human leukocyte antigen (HLA) – DR to assign 65 HLA-DR8 haplotypes, which were then subdivided into two genotypes, i.e., DRB1*0802 and DRB1*0803, by a polymerase chain reaction (PCR) – based, simple, and practical method. The panels possessing DR8 specificity were firstly subjected to PCR with a couple of primers specifically to amplify their DR52 associated group – DRB1 genes. PCR products were then denatured in the presence of formamide, electrophoresed in a non-denaturing polyacrylamide gel, and visualized by silver staining. The same DRB1 products of these samples were also mixed with the DRB1*1302, and simultaneously analyzed by the same procedure. Electrophoretic mobilities of the samples were compared with those of the typing standards to genotype their DR8-DRB1 alleles by using the characteristic polymorphism in the single-stranded DNAs and the heteroduplexes. This method, designated PCR – DNA conformation polymorphism (DCP) analysis, allowed for genotyping of the DR8-DRB1 alleles without using sequence – specific oligonucleotide probes (SSOP) or restriction endonucleases. The entire process after PCR was completed within a few hours. The tested panels were also genotyped for DRB1 gene by the PCR-SSOP method for comparison with results obtained by the PCR-DCP method. Satisfactory coincidence was achieved and it represented how accurately the new system genotyped DRB1*0802 and DRB1*0803. PCR-DCP analysis was thus shown to be practical and useful for subtyping of serologically defined DR8 specificities.

Key words: *PCR (polymerase chain reaction), Subcloning, HLA (human leukocyte antigen), SSO (sequence-specific oligonucleotide) typing, Sanger's dideoxy sequencing method*

The HLA class II antigens are heterodimers of highly polymorphic glycoproteins expressed on the surface of antigen-presenting cells. Three class II antigens (HLA-DR, DQ, and DP) consist of α and β chains which are separately encoded for by genes on the short arm of chromosome 6. With the exception of the DR α chain, these proteins are highly polymorphic, with variability localized to the distal extracellular domain encoded for by the second exon. The HLA-DR antigens play a key role in immune recognition of foreign antigens by T cells. Allogenic HLA class II antigens are also recognized by T cells in cases of tissue transplantation. Recent studies[23,24,35] have provided substantial evidence that, despite major progress in immunosuppressive therapy, higher success rates of kidney-graft outcome are significantly dependent on optimal HLA-DR matching. Polymorphism of the HLA-DR antigen has been detected using serological reagents that define the polymorphic series DR1-14[6]. In routine typing, however, serological assignment of these polymorphic specificities is not always correct and, in addition, their subtyping is almost

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impossible[36]. The HLA-DR encoding genes (DRB1 genes), precisely assigned by molecular biological technology that identifies 60 DRB1 alleles[14], have to be matched in clinical renal transplantation[19,26]. They have recently been genotyped by two representative PCR-based typing techniques. The PCR-restriction fragment length polymorphism (RFLP) method[37] is a suitable typing method for a small number of samples. However, multiple endonucleases are indispensable in the analysis, and when more samples are analyzed, the system inevitably becomes more burdensome. On the other hand, a number of samples can be analyzed by the PCR – sequence specific oligonucleotide probe (SSOP) method[29], although hybridization with multiple probes is an obstacle to clinical use. Whichever method is used, it is almost impossible to genotype the DRB1 genes without considerable cost and laborious operations. Recently, T. M. Clay et al reported a simple method to detect mismatches between given DRB alleles. The method does not require oligonucleotide probes or restriction endonucleases[5]. When multiple different HLA-DRB genes are amplified by PCR, subjected to denaturation followed by renaturation, and analyzed by electrophoresis in a polyacrylamide gel, single – stranded DNA products form both homoduplexes (double – stranded DNAs) between the complementary strands and heteroduplexes between heterologous sequences. The formation of the heteroduplexes is representative of allelic polymorphisms because it depends on the sequence variations in the PCR products. Sorrentino et al reported a similar method for genotyping DRB3[32] and DPB1[33] genes by artificially adding PCR products from a given allele to all samples and detecting the characteristic heteroduplexes. On the other hand, we have reported that the single – stranded DNAs from HLA class II genes showed characteristic electrophoretic patterns in a polyacrylamide gel depending on the sequence variations, that is, single – strand conformation polymorphism (SSCP)[12,15]. The PCR-SSCP method was originally developed for the purpose of detection of sequence variations in human genome based on the phenomenon that the electrophoretic mobility of a single – strand DNA in a neutral polyacrylamide gel is dependent on its length and sequence[27,28,34]. We modified the original method so that the SSCP could be detected nonradioactively by using silver staining of DNA fragments, and applied to the matching study of HLA-DRB, -DQB, and -DPB in kidney transplantation[13,17]. The modified method was designated the PCR-DNA conformation polymorphism (DCP) method, because the silver staining allowed for detection of single – stranded DNAs as sensitively as the other DNA fragments, i. e., double – stranded DNAs and heteroduplexes, and simultaneous detection of single – stranded DNAs and heteroduplexes facilitates the detection of allelic differences. In this study, we applied the PCR-DCP method to subdividing of the HLA-DR8 specificities. The DR8 gene products show a relation to those of DR3, DR5, and DR6 (the DR52 group) because they share certain serologic epitopes, although the DR8 haplotype has only one DRB gene (DRB1) while the other DR haplotypes contain two or more DRB genes. It is suggested that the DR8-DRB1 gene is generated by a recombination event between DRB1 and DRB3 genes[1,2,4,10]. Because the DRB1 genes encoding for the DR3, DR5, DR6, and DR8 specificities share several residues at the N terminal side of DRβ domain that differ from the other DR specificities, a given primer corresponding to these residues allows for the specific amplification of these DRB1 genes. DR3, DR5, DR6, and DR8 cand then be designated as the DR52-associated (DR52ass) group. The DR8 is subdivided at DNA level into the DRB1*0801, DRB1*0802, and DRB1*0803, although the DRB1*0801 can not detected in the Japanese population[16]. In fact, the DRB1*0801 was never assigned by our PCR-SSOP analysis of 527 Japanese (data not shown). The serologically assigned DR8 haplotypes were subjected to PCR with a couple of DR52ass group – specific primers, and successfully subdivided into the DRB1*0802 and DRB1*0803 by the PCR – DCP method discriminating their characteristic DCP patterns from those of the other DR52ass group alleles.

MATERIALS AND METHODS

Materials

Two hundred panels were selected from 100 kidney transplantation pairs from operations in the 2nd Department of Surgery, Hiroshima University Hospital or related hospitals. Eighteen B-cell lines from the 10th international HLA workshop panels[18,39] and 20 normal individuals with different DR52ass subtypes[6,16] were also tested. In addition to these panels, the Hi2 cell line[7] was used as the DRB1*0803 standard. Genomic DNA was prepared from peripheral leukocytes by the standard procedure[3].

PCR

Genomic DNA (0.5 μg) was subjected to PCR in a 50 μl reaction mixture of 10 mM Tris – HCl (pH 8.4 at 24°C), 50 mM KCl, 0.1 mg/ml gelatin, 0.02 % NP40 and 1.5 mM $MgCl_2$ to amplify the second exons of HLA-DRB1 gene of the DR52ass group using thermostable DNA polymerase (1 unit, Amplitaq, Perkin Elmer Cetus). The primers (each 15 pmoles) used in PCR were AKDR52ass (5'CACGTTTCTTGGAGTACTCTAC3') and AKDRB2

(5'CCGCTGCACTGTGAAGCTCT3'). PCR was carried out in a Thermal Cycler (Perkin Elmer Cetus Instrument, Norwalk) via 30 cycles of denaturation (96°C, 1 min), annealing (60°C, 30 sec), and extension (72°C, 1 min) steps.

PCR-SSOP analysis

The DRB1 genotypes of all subjects amplified with the above DR52ass group – specific primers were determined by the PCR-SSOP method. Amplified DNAs were spotted onto a nylon membrane (Hybond N plas, Amersham) and hybridized with the 23 DR52ass group – specific ^{32}P-labeled SSOPs[18]. The hybridization procedures were described in the protocol from the DNA component of the 11th International HLA Workshop[14].

PCR-DCP analysis

PCR products (5 μl) were mixed with 8 μl of formamide dye (80% formamide, 20 mM EDTA, 0.01% bromophenol blue, pH 8.0). Samples, incubated at 96°C for 5 min, and chilled in ice – water, were then electrophoresed in an 8 % polyacrylamide gel (14 × 14 × 0.1 cm, 0.5 × TBE, acrylamide : bisacrylamide = 50 : 1) in 0.5 × TBE (1 × TBE : 89 mM Tris – borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 13 V/cm at room temperature until the bromophenol blue reached the bottom of the gel (it usually takes about two hours). DNA fragments were detected using a silver staining kit (Daiichi Chemical Co. Ltd., Tokyo) following the manufacturer's instructions. For the heteroduplex study with the DRB1*1302, PCR products (each 3 μl) of both a given sample and the DRB1*1302 were mixed with 9.6 μl of formamide dye in a microtube, and subjected to the same analysis.

DNA sequencing

The PCR amplified DNA, purified by the agarose gel cutting using stripes of DEAE – cellulose paper (DE81, Whatman Paper Ltd., Maidstone, UK), was made blunt ended by T4 DNA polymerase (Takara Shuzo, Kyoto, Japan). The sample was then phosphorylated by T4 polynucleotide kinase, and cloned into M13mp18 which was cleaved by restriction endonucleases Pst I and Xbal I, made blunt ended. The competent cells were transformed with the ligated DNA, and exponentially growing E. coli TG1, transferred into a microtube with both isopropyl-thio-galactoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal), were combined with the transformed cells[20,21,38]. The saturated culture of E. coli TG1 was diluted with TY Medium, and a translucent plague was transferred to the culture. The M13 phage was propagated by shaking vigorously at 37°C for 5 hr, and the single – stranded DNA was refined with Solution I, II, and III11) to obtain the double – stranded DNA which was analyzed by an agarose gel electrophoresis for screening the presence of insert fragments. Each suitable clone DNA (showing a slower migration band in gel electrophoresis) was subjected to dideoxy chain termination sequencing according to the Sanger's method[30].

RESULTS

The entire 2nd exon gene of the Hi2 cell line was subjected to DNA sequencing and confirmed to be the DRB1*0803 so that it could be used as the typing standard in PCR-DCP analysis of the HLA-DR8. The PCR-DCP method analyzes entire base sequences in toto, while the PCR-SSOP and -RFLP methods are based on the detection of sequence polymorphisms in a relatively narrow range. Then the typing standard had to be confirmed at first by DNA sequencing (Fig. 1). The nucleotide sequence of five clones was completely identical to that of the DRB1*0803[8].

The SSCP patterns of DRB1 genes from DR52 associated group (DR52ass) cells were analyzed using 18 different B-cell lines selected in the 10th International HLA Workshop and the Hi2 cell line (Fig. 2a), and 20 healthy individuals (Fig. 2b), respectively. Characteristic single – stranded DNAs (ss DNA) were reproducibly observed for each DR52ass allele or allelic combination. The alleles tested were two DR3 (DRB1*0301, and DRB1*0302), six DR5 (DRB1*1101, DRB1*1102, DRB1*1103, DRB1*1104, DRB1*1201, and DRB1*1202), and nine DR6 (DRB1*1301, DRB1*-1302, DRB1*1401, DRB1*1402, DRB1*1403, DRB1*1405, DRB1*1406, DRB1*1407, and DRB1*-1408) in addition to three DR8 (DRB1*-0801, DRB1*0802, and DRB1*0803). Although each allele generated more than three ss DNAs, these ss DNAs should have originated from a single gene and not from nonspecific amplified products because no unexplicable DNA fragments were detected by PCR-RFLP analysis, and also

```
     1
[1]  CCCCACAGCACGTTTCTTGGAGTACTCTACGGGTGAGTGTTATTTCTTCAATGGGACGGA
[2]  ------------------------------------------------------------
     61
[1]  GCGGGTGCGGTTCCTGGACAGATACTTCTATAACCAAGAGGAGTACGTGCGCTTCGACAG
[2]  ------------------------------------------------------------
     121
[1]  CGACGTGGGGGAGTACCGGGCGGTGACGGAGCTGGGGCGGCCTAGCGCCGAGTACTGGAA
[2]  ------------------------------------------------------------
     181
[1]  CAGCCAGAAGGACATCCTGGAAGACAGGCGGGCCCTGGTGGACACCTACTGCAGACACAA
[2]  ------------------------------------------------------------
     241
[1]  CTACGGGGTTGGTGAGAGCTTCACAGTGCAGCGG
[2]  ---------------
```

Fig. 1. The sequencing study of the Hi2 cell line. Comparison of the nucleotide sequence of the DRB1 gene of the Hi2 cell line [1] with the published sequence of the DRw8.3 cell line [2]. A dash indicates that each base pair coincides. The DRw8.3 sequence was derived from cell line TAB 089[8].

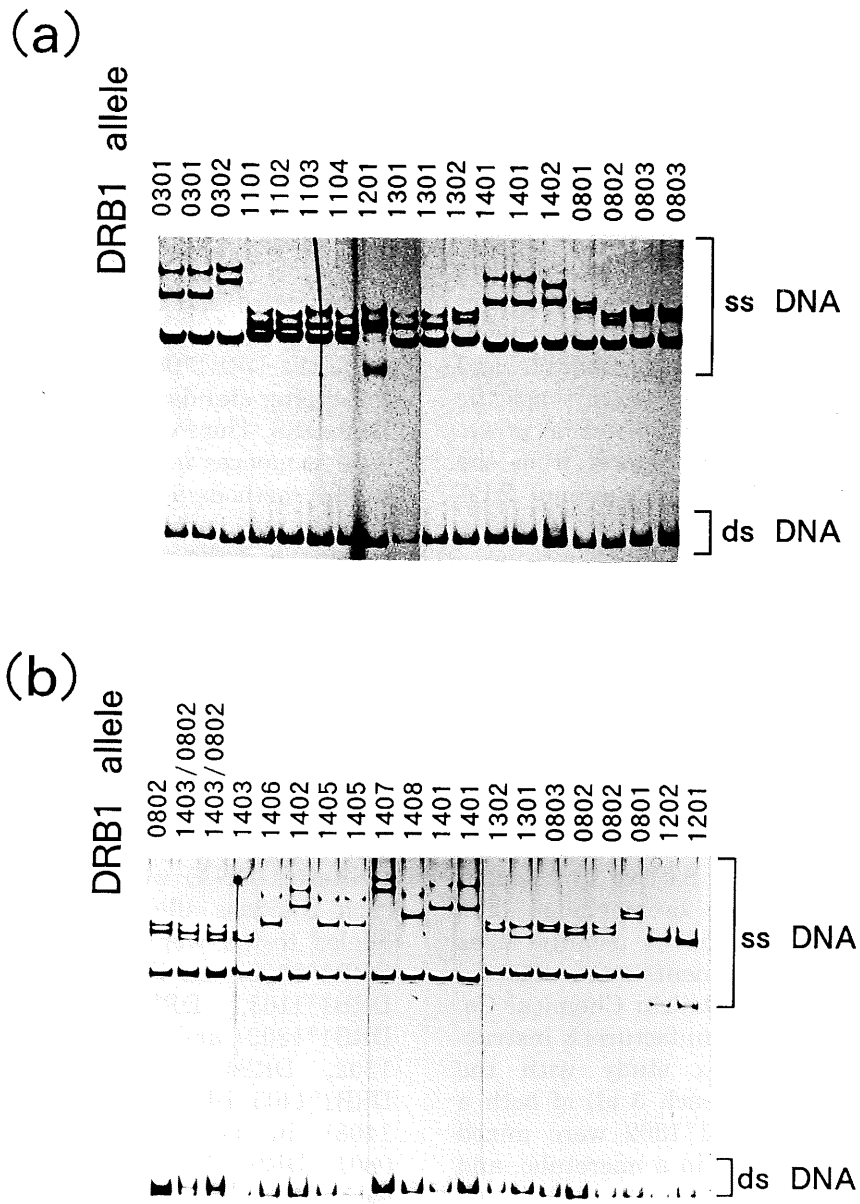

Fig. 2. PCR-DCP analysis of DR52 associated (DR52ass)-DRB1 alleles.
Eighteen different DR52ass-DRB1 alleles from B-cell lines homozygous for HLA selected in the 10th International HLA Workshop (a) and those from 20 individual cells heterozygous for HLA (b) were analyzed by the PCR-DCP method. The second exon of DRB1 gene was amplified by PCR with the primers AKDR52ass and AKDRB2. After electrophoresis in a neutral polyacrylamide gel, DNA fragments of single-stranded form (ss DNA) and double-stranded form (ds DNA) were visualized by silver staining. All of these cells were genotyped for the DRB1 gene by the PCR-SSOP method[14] The DRB1 allele of each subject is indicated at the top of the figure.

because multiple slow migrating bands were observed even when a cloned DRB1 gene was used as a template in PCR (data not shown). The objective two alleles, i.e., DRB1*0802 and DRB1*0803 were shown to represent the distinguishable ss DNAs from those of the other DR52ass alleles. However, DRB1*1101, DRB1*1302, and DRB1*1403 which are common in the Japanese showed similar ss DNAs to DRB1*0802 and DRB1*0803, and it was difficult to distinguish these heterozygous alleles by polymorphism of the ss DNAs.

The heteroduplexes were then analyzed between DR52ass-DRB1 alleles and DRB1*1302. DRB1*1302 was chosen for an artificially added allele, because it formed polymorphic heteroduplexes more significantly than the other DR52ass alleles. As shown in Fig. 3, the heteroduplex pattern and ss DNA pattern of a heterozygote were identical to those formed by mixing two corresponding homozygous alleles prior to gel electrophoresis. These heteroduplexes were helpful

especially for discrimination of DRB1*0802 and DRB1*0803 from DRB1*1101, DRB1*1302, and DRB1*1403. In contrast, ss DNAs were useful to discriminate DRB1*0802 and DRB1*0803 from the other DR52ass-DRB1 alleles (Fig. 3). Therefore, comparison of both single – stranded DNAs and heteroduplexes was needed to allow for genotyping of DRB1*0802 and DRB1*0803.

Two hundred Japanese panels from 100 kidney transplant pairs were subjected to the DR52ass-DRB1 group specific amplification. One hundred and twenty eight panels possessed DR52ass alleles, and these panels were analyzed by the PCR-DCP method for genotyping DRB1*0802 and DRB1*0803. Electrophoretic mobilities of single – stranded DNAs from the samples were compared with those from three typing standards that were DRB1*1101/*1201, DRB1*0802/*0803, and DRB1*-1401/*1403 heterozygotes. The heteroduplexes of the same samples artificially added with DRB1*1302 were also analyzed using a typing standard that was the DRB1*0802/*1302 heterozygote. Representative results are shown in Fig. 4. At first, comparison of single – stranded DNAs was made between the samples and the typing standards so that it could be decided whether the samples possessed the five alleles with similar single – stranded DNAs that were DRB1*0802, DRB1*0803, DRB1*1101, DRB1*1302, and DRB1*1403. In the cases in which these alleles were recognized, the heteroduplexes with DRB1*1302 were then analyzed. In donor 1, DRB1*1302 was assigned because it never formed any heteroduplexes. Donor 2 and recipient 2 of a transplant pair had been assigned for the same serologic DR8, while strong mixed lymphocyte reaction (MLR) was recognized between the donor's and the recipient's lymphocytes.

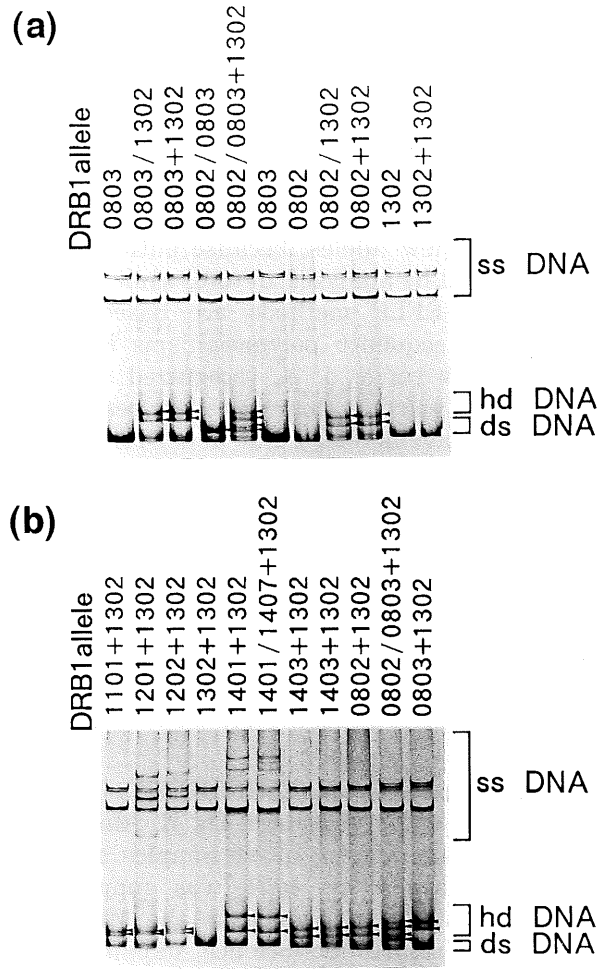

Fig. 3. Analysis of heteroduplexes between DR52ass-DRB1 alleles and DRB1*1302.
Arrowheads indicate the characteristic heteroduplexes. (a) The DRB1*0802, DRB1*0803, and DRB1*1302 which showed distinct but similar SSCP patterns were analyzed by the PCR-DCP method to reveal that the DRB1*1302 was useful as an artificially added (+1302) allele to discriminate these alleles by the presence of heteroduplex. (b) Each PCR sample was artificially added with that from DRB1*1302 and subjected to gel electrophoresis for the PCR-DCP analysis.

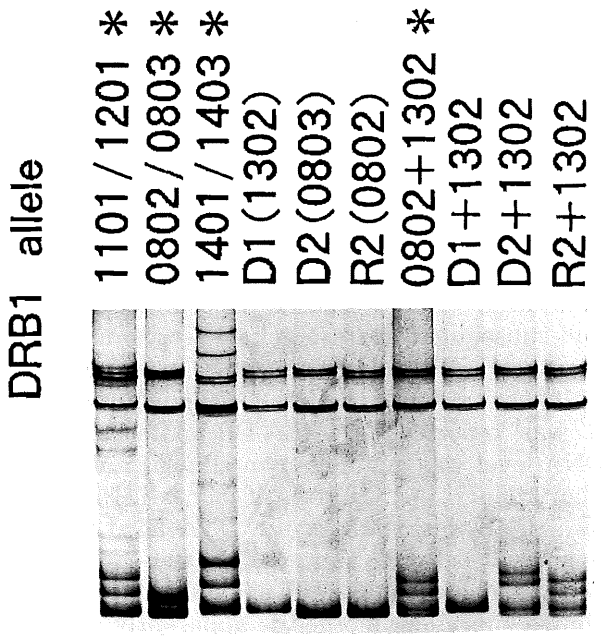

Fig. 4. Genotyping of the DRB1*0802 and DRB1*0803 by the PCR-DCP method.
Out of two renal transplant pairs, three representative samples were selected, i.e., Donor 1 (D1), Donor 2 (D2) and Recipient 2 (R2), that possessed DR52ass alleles amplified by the DR52ass-DRB1 group specific primers. All of the three samples were proved to be assigned for one of the five DR52ass alleles with similar single-stranded DNAs by comparison with the typing standards indicated by asterisks in three left side lanes. D2 and R2 were assigned to be DRB1*0803 and DRB1*0802 by the heteroduplex comparison with a typing standard (0802+1302*).

When these two samples were analyzed by the PCR-DCP method, they showed clearly different heteroduplexes, and were assigned for DRB1*0802 and DRB1*0803, respectively. By this simple method, 18 and 33 alleles were assigned for DRB1*0802 and DRB1*0803, respectively, and two DRB1*0802/*0803 heterozygotes were recognized. The alleles assigned by this method were completely identical to those determined by the PCR-SSOP method.

DISCUSSION

We have applied the PCR-DCP method to the subdivision of the serologic DR8 specificity into DRB1*0802 and DRB1*0803. PCR samples were directly analyzed by the non – denaturing polyacrylamide gel electrophoresis. Allelic polymorphism in the single – stranded DNAs and heteroduplexes, both of which were useful for identification of each DR52ass-DRB1 allele, were detected by the following silver staining. The entire procedure could be accomplished within a few hours after PCR. It was also revealed that the other DR52ass alleles including DRB1*0801 could probably be assigned by this simple and economical method, although only a limited number of DR52ass alleles were recognized in our Japanese panels by the PCR-SSOP method. Recently, O. Olerup et al developed a simple and PCR-based HLA-DR typing technique as an alternative to serologic DR typing by using primers allowing specific amplifications[22]. This technique is recommended as a suitable matching method for donor screening of cadaveric kidney transplantation[25]. The method is composed of PCR and subsequent agarose gel electrophoresis. However, each DRB allele can not be subtyped at the DNA level despite the use of 19 pairs of primers. It was reported the HLA-DR matching at the DNA level was significant in clinical renal transplantation[26]. As a matter of fact, MLR is elicited by a DRB mismatch at the DNA level that is a mismatch between subtypes, to the same degree as that by a serologic DR mismatch. Therefore, a simple subtyping method is apparently needed to evaluate the DR matching in a transplant pair. The PCR-DCP method described here is simple, rapid, and applicable to such a purpose. In this study, we applied PCR-DCP analysis exclusively to the subtyping of the DR8 specificity that was difficult to be serologically subtyped because of a lack of effective serous antibodies. The combination of PCR-DCP analysis and specific amplifications should, however, allow the other alleles to be precisely subtyped, and such a trial is being undertaken[31].

Heteroduplexes formed between tested DR52ass alleles and the DRB1*1302 were useful for allelic identification of these alleles especially in heterozygous cases. When the electrophoretic migration time is short (within 1 hr or so), the SSCP patterns tend to be low – resolutionable compared to the distinctive heteroduplex patterns, although ss DNAs are useful to discriminate three DR8 alleles distinctively after the long migration time (usually more than 2 hrs). Heteroduplex studies were also conducted by Clay et al [5] and Sorrentino et al [32,33]. They only detected the heteroduplex forms because of the usage of ethithiumbromide that can not detect ss DNAs. We, on the other hand, can detect not only the heteroduplex forms but also the single stranded forms by the PCR-DCP method because of usage of silver staining. Comparison of both single – stranded DNAs and heteroduplexes is especially needed to allow discrimination of the DR52ass-DRB1 alleles that are the toughest antigen group for the serological assignment (data not shown). The heteroduplexes are more informative in discriminating some DR52ass-DRB1 alleles whose single – stranded DNAs are less resolutionable, or vice versa in the other several alleles.

The PCR-DCP method is also powerful for the detection of new alleles, because entire base sequences could be analyzed in toto, while the PCR-SSOP and -RFLP methods are based on the detection of sequence polymorphisms in a relatively narrow range, i. e., recognized sequences by SSOP or enzyme cleavage sites, respectively. In fact, we have detected four new DPA1 and one new DPB1 alleles by the PCR-DCP analysis[12], and confirmed them by sequencing analysis[9]. In this DR8 analysis, however, no unusual DCP patterns were recognized in the 49 Japanese panels. Furthermore, the PCR-DCP analysis is suitable for the matching study in a kidney transplantation from the aspect of cross matching, because all of the genes could be detected at the same time.

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