DNA CONFORMATION POLYMORPHISM ANALYSIS OF DR52 ASSOCIATED HLA-DR ANTIGENS BY POLYMERASE CHAIN REACTION: A SIMPLE, ECONOMICAL AND RAPID EXAMINATION FOR HLA MATCHING IN TRANSPLANTATION

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SUMMARY: HLA-DRB1 and -DRB3 alleles of DR52-associated (DR52ass) HLA-DR antigens were genotyped by a polymerase chain reaction (PCR) - based simple and practical method. Genomic DNAs from two hundred Japanese panels were subjected to PCR with two pairs of primers to separately amplify the DR52ass-DRB1 (DR3, 5, 6, and 8) alleles and DRB3 (DR52) alleles. The specific amplification revealed that 128 and 76 panels possessed DR52ass alleles and DRB3 alleles, respectively. PCR products from these panels were heat-denatured, electrophoresed in a non-denaturing polyacrylamide gel, and visualized by silver staining. Electrophoretic mobilities of the DNA samples were compared with those of the typing standards with known genotypes of DR52ass-DRB1 and DRB3 alleles. This method, designated PCR-DNA conformation polymorphism (DCP) analysis, allowed genotyping of the DR52ass-DRB1 and DRB3 alleles of panels without any sequence-specific oligonucleotide probe (SSOP) or restriction endonuclease, and the entire process after PCR could be completed within a few hours. Because the DR52ass-DRB1 and DRB3 alleles assigned by this method were shown to be iden-
tical to those determined by the PCR-SSOP method, PCR-DCP analysis was suggested to be a simple and practical HLA genotyping method.

INTRODUCTION

Human leukocyte antigen (HLA) complex occupies approximately 40,000 kilobases (kb) of chromosome 6 (1-3). The complex encodes for class I and class II molecules and functionally specialized to present foreign antigens to different subsets of T lymphocytes. HLA-DR, DQ, and DP are three related class II molecules consisting of $\alpha$ and $\beta$ chains which are separately encoded by respective genes. With the exception of the DR $\alpha$ chain, these proteins are highly polymorphic with the variability localized to the distal extracellular domain encoded for by the second exons. The HLA-DR molecules play a key role in immune recognition of foreign antigens by CD4$^+$ T cells. Allogeneic HLA class II antigens are also recognized by CD4$^+$ T cells in cases of tissue transplantation. Recent studies (4-6) have provided substantial evidence showing that, despite recent progress in immunosuppressive therapy, higher success rates of graft outcome are depending on optimal HLA-DR matching between donors and recipients. As the DNA technology is more prevalent, this finding has been more stressed (7-9). Our object is to assess the HLA-DR matching in a tissue transplantation pair in such a simple and precise way as possible at the DNA level.

An unusual feature of the HLA-DR subregion is that it varies in length among individuals. Five major groups of HLA-DR haplotypes have been identified, and each of the major HLA-DR haplotypes contains one functional DRA gene and one or more DRB genes (10). The DR subregions associated with the DR3, DR5 (DR11 and DR12) and DR6 (DR13 and DR14) specificities share a structural motif. The motif is the presence of two functional genes (DRB1 and DRB3) and one pseudogene (DRB2) (11-13). The DRB1 gene for the DR8 specificity shows a relation to those of DR3, DR5, and DR6 specificities, although the DR8 haplotype has only one functional DRB gene (DRB1) (14,15) which is suggested to be generated by a recombination event between DRB1 and DRB3 genes (16). Because the DRB1 alleles encoding for the DR3, DR5, DR6, and DR8 specificities share several residues at the N-terminal side of the DR $\beta$ domain, designated heretofore the DR52-associated (DR52ass) group. The DR52ass group comprises the most difficult alleles to be assigned serologically and most of the serologic typing errors
were found in DR5 and DR6 that have a number of subtypes (17). Therefore, one explanation for the HLA-DR6 effect (18) might be that the serologic designation of HLA-DR6 is not uniform.

HLA class II genes have recently been analyzed by PCR-sequence-specific oligonucleotide probe (SSOP) method (19,20) and the PCR-restriction fragment length polymorphism (RFLP) method (21). Recently, Clay et al. (22) and Bidwell et al. (23) reported a simple method to detect mismatches between given DRB alleles, which does not require multiple oligonucleotide probes or restriction endonucleases. When multiple different HLA-DRB genes are amplified by PCR and subjected to denaturation-renaturation process, single-stranded DNA products form heteroduplexes between heterologous sequences, and these heteroduplexes are representative of allelic polymorphism. Sorrentino et al. (24,25) reported a similar method of genotyping for DRB3 and DPB genes by artificially adding a given allele to 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 polyacrylamide gel, that is the single-strand conformation polymorphism (SSCP) (26,27). The PCR-SSCP method was originally developed for the detection of sequence variations in human genome (28-30). We have modified this method by using silver staining of DNA fragments and designated herein the PCR-DNA conformation polymorphism (DCP) method, because the silver staining allows detection of single-stranded DNAs as sensitively as double-strand DNAs, and detection of single-stranded DNAs and/or heteroduplexes may reflect the allelic differences (26,27). In this study, we applied the PCR-DCP method to genotyping of DRB1 and DRB3 genes in the DR52ass group, making it possible to use it for assessing the HLA-DR matching in kidney transplantation.

MATERIALS AND METHODS

Materials: Two hundred panels were selected from 100 kidney transplant pairs operated in the 2nd Department of Surgery, Hiroshima University Hospital, or related hospitals. Genomic DNA was prepared from peripheral leukocytes by the standard procedure (31). In addition to these panels, 18 B-cell lines from the 10th International HLA Workshop panels (32,33) and 20 normal individuals with different DR52ass subtypes (34,35) were tested.
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 MgCl₂ to amplify the second exons of HLA-DRB1 and -DRB3 genes of the DR52ass group with thermostable DNA polymerase (1 unit, Ampli-taq, Perkin Elmer Cetus, obtained via Takara Co., Ltd., Kyoto). The primers (each 15 pmol) used in PCR were AKDR52ass (5'-CACGTTTCTTGGAGTACTCTAC-3') and AKDRB2 (5'-CCGCTGCACCTTGAGCTCT-3') for DRB1 gene amplification, and AKDRA3 (5'-CCCAGCACGTTTCTTGAGCT-3') and AKDRA2 for DRB3 gene amplification, respectively. PCR was carried out in a Thermal Cycler (Perkin Elmer Cetus Instrument, Norwalk, Conn.) via 30 cycles of denaturation (96 C, 1 min), annealing (56 C, 30 sec), and extension (72 C, 1 min) steps.

PCR-SSOP analysis: The DRB1 and DRB3 genotypes of each subject were determined by the PCR-SSOP method. Amplified DNAs were spotted onto a nylon membrane (Hybond N plus, Amersham Corp., Arlington Heights, IL) and hybridized with 23 DR52ass group-specific 32P-labeled SSOPs and 12 DRB3-specific SSOPs, respectively (33). The hybridization procedures were described previously (36).

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 were incubated at 96 C for 5 min, chilled in ice-water, and electrophoresed in 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. DNA fragments were detected with a silver staining kit (Daiichi Chemical Co., Ltd., Tokyo) according to the manufacturer's instructions.

RESULTS

PCR-DCP Analysis of DRB1 Gene

The SSCP patterns of DRB1 gene from DR52ass-DRB1 (DR3, 5, 6, and 8) homozygotes and heterozygotes were analyzed with 18 different B-cell lines selected in the 10th International HLA Workshop (Fig. 1a) and 20 healthy individuals (Fig. 1b), respectively. Characteristic patterns of single-stranded DNAs (ss DNA) were reproducibly observed for all the DR52ass alleles tested, i.e., two DR3 (DRB1*0301, and DRB1*0302), six DR5 (DRB1*1101, DRB1*1102, DRB1*1103, DRB1*1104, DRB1*1201, and DRB1*1202), nine DR6 (DRB1*1301, DRB1-
Fig. 1. 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 (36). The DRB1 allele of each subject is indicated at the top of the figure.
Fig. 2. PCR-DCP analyses of DR52ass-DRB1 and DRB3 alleles. Fifteen different cells were analyzed by the PCR-DCP method separately for the DRB1 (upper side) and DRB3 (lower side) genes. In addition to ss DNA and ds DNA, heteroduplexes (hd DNA) are easily recognized in heterozygotes. Both the DRB1 and DRB3 alleles of each subject are indicated at the top of the figure. It should be noted that DR8 samples have no DRB3 gene.

*1302, DRB1*1401, DRB1*1402, DRB1*1403, DRB1*1405, DRB1*1406, DRB1*1407, and DRB1*1408), and three DR8 (DRB1*0801, DRB1*0802, and DRB1*0803) alleles. Although each allele generated more than three single-stranded DNAs, these ssDNAs should have been originated from a single gene and not from nonspecific amplified products, because no unexplicable DNA fragments were detected by the PCR-RFLP analysis and because multiple slow migrating bands were observed even when a cloned DRB1 gene was used as a tem-
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.
plate in PCR (data not shown). It is suggested that one ssDNA may have taken multiple secondary structure under our condition. The PCR-SSCP analysis was then applied to the other DR52ass-DRB1 heterozygotes of a given allele representing a common SSCP pattern, and the characteristic SSCP pattern of each heterozygote was identical to a mixed SSCP pattern of the two corresponding alleles (Figs. 1b and 2). In addition, polymorphic heteroduplexes (hd DNA) were recognized in almost all combinations of DR52ass-DRB1 alleles.

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 efficiently than did the other DR52ass alleles (data not shown). As shown in Fig. 3a and 3b, the heteroduplex pattern and ssDNA pattern of a heterozygote were identical to those formed by mixing two corresponding homozygous alleles prior to gel electrophoresis. These heteroduplexes were informative especially in discriminating DRB1*1101, DRB1*1302, DRB1*1403, DRB1*0802, and DRB1*0803, although these heterozygous alleles showed distinct but similar SSCP patterns especially when the migration time was short. In contrast, ssDNAs were useful to discriminate DRB1*1401 and DRB1*1407 which showed a similar heteroduplex between DRB1*1302 and themselves. Therefore, comparison of both single-stranded DNAs and heteroduplexes was needed to allow discrimination of the DR52ass-DRB1 alleles.

PCR-DCP Analysis of DRB3 Gene

As shown in Fig. 2, three DRB3 (DR52) alleles, i.e., DRB3*0101, DRB3*0202, and DRB3*0301, were recognized in the Japanese panels. All of them were clearly distinguished by SSCP, and the heterozygotes showed a simple summation pattern of the corresponding homozygotes, while the heteroduplex was detected only between DRB3*0101 and DRB3*0202. Although the DRB3 gene is in strong linkage disequilibrium with the DRB1 gene (11), this analysis was helpful in the HLA-DR matching because there are some DR-haplotypes carrying the same DRB1 allele and a different DRB3 allele (33).

PCR-DCP Genotyping of DR52ass-DRB1 and DRB3 Genes

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 then analyzed by the PCR-DCP method for genotyping their alleles. Electrophoretic mo-
Fig. 4. DRB1 and DRB3 genotyping of the DR52ass alleles by the PCR-DCP method. Sample 1 (S1) and sample 2 (S2) were genotyped for the DRB1 and DRB3 genes by the PCR-DCP method. Two samples were subjected to the DRB1- and DRB3-specific PCR and were separately analyzed for each locus by subsequent electrophoresis. Three heterozygotes indicated by asterisks were used as typing standards; DRB1*1101/*1201 (DRB3*0202/*0101), DRB1*0802/*0803, DRB1*1401/*1403 (DRB3*0202/*0101) for the SSCP study, and DRB1*0802/*1302 and DRB1*1101/*1302 for the heteroduplex study.
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<th>1401</th>
<th>1403</th>
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<td>10</td>
<td>4</td>
<td>4</td>
<td>26</td>
<td>43</td>
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DRB1 and DRB3 alleles in the 128 DR52ass panels from the Japanese kidney transplant pairs were assigned by the PCR-DCP method. Numbers of panels of each haplotypic combination determined by the current analysis is shown.
Table II. Comparison of the assignment of DR52ass antigens by three typing method; the PCR-SSOP, the PCR-DCP, and the serologic methods

<table>
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<th>PCR — DCP and PCR — SSOP</th>
<th>freq. 1)</th>
<th>serology 2)</th>
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<tr>
<td>DRB1 * 1101</td>
<td>13</td>
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<td>DRB1 * 1302</td>
<td>4</td>
<td>DR6 (2), NA (2)</td>
</tr>
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<td>DRB1 * 1401</td>
<td>6</td>
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</tr>
<tr>
<td>DRB1 * 1406</td>
<td>4</td>
<td>DR5 (2), NA (2)</td>
</tr>
<tr>
<td>DRB1 * 1407</td>
<td>3</td>
<td>DR13 (1), NA (2)</td>
</tr>
<tr>
<td>DRB1 * 0802</td>
<td>16</td>
<td>DR8 (13), NA (3)</td>
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<tr>
<td>DRB1 * 0803</td>
<td>19</td>
<td>DR8 (19)</td>
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</table>

In 46 panels, serologic data were available. The typing results by three methods are shown.

1) Number of panels with DR52ass-DRB1 alleles assigned by the PCR-DCP and PCR-SSOP methods that showed identical results.

2) Serologic assignments of DR52ass-DRB1 alleles. Several panels were subtyped to have DR11 (a subtype of DR5) or DR13 (a subtype of DR5). NA indicate that the alleles were not assigned serologically, and figures in brackets represent the numbers of panels.

abilities of single-stranded DNAs from the samples were compared with those from three typing standards which are the heterozygotes of DRB1*1101/*1201, DRB1*0802/0803, and DRB1401/*1403. The heteroduplexes of the same samples artificially added with DRB1*1302 were also analyzed along with two typing standards, DRB1*0802/*1302 and DRB1*1101/*1302 heterozygotes. Representative results are shown in Fig. 4. DRB1 genotyping of sample 1 was made by comparing the migration of single-stranded DNAs between the sample and the typing standards, while DRB1 alleles of sample 2 was assigned by analysis of both the single-stranded DNAs and the heteroduplexes. The DRB3 genotyping was also
possible as mentioned above. By this simple method, DR52ass-DRB1 alleles of 128 Japanese panels were genotyped. Some of these panels possessed no DRB3-specific PCR products, and it could then be identified that their DRB1 alleles were of the DR8 group. The DRB1 and DRB3 genotypings of the panels were also carried out by the PCR-SSOP method. When the data were compared between the PCR-DCP method and the PCR-SSOP method, it was revealed that the genotype-assignments of all panels were completely identical, indicating that 10 DR52ass-DRB1 and three DRB3 alleles were precisely genotyped by the PCR-DCP method, as shown in Table I. Forty-six panels were also assigned for DR52ass-DR specificities by serologic typing, and the data were compared with genotypes of the HLA-DRB1 determined by the molecular techniques, i.e., the PCR-SSOP method and the PCR-DCP method. Table II shows the comparison of typing results by the three methods. It was found that the assignment of DR52ass alleles were difficult by serologic typing. It was then suggested that DR52ass alleles should be assigned by genotyping, and the genotyping was possible by the PCR-DCP method at least for Japanese panels.

**DISCUSSION**

We applied the PCR-DCP method to the DRB1 genotyping of DR52ass alleles. PCR samples were analyzed by the non-denaturing polyacrylamide gel electrophoresis. Allelic polymorphisms in the single-stranded DNAs and heteroduplexes, both of which were useful for identification of each allele, were detected by the silver staining. The entire procedure could be accomplished within a few hours after PCR. Recently, Olerup et al. (37) developed another simple and PCR-based HLA-DR typing technique as an alternative to serologic DR typing by using primers allowing for specific amplifications, and this technique is recommended as a suitable method for donor screening of cadaveric kidney transplantation (38). However, 19 pairs of primers are needed, inevitably making the procedure complicated and expensive, despite the inability to subtype some DRB alleles at the DNA level. It was reported that the HLA-DR matching at the DNA level was significant in clinical renal transplantation (39). In fact, mixed lymphocyte reaction (MLR) is elicited by a DRB mismatch between subtypes to the same degree as that by a serologic DR mismatch. For example, even when both a donor and a recipient of a renal transplant pair are assigned for the same serologic DR8,
strong MLR is recognized between the donors and the recipients lymphocytes if they are different for the DR8 subtype, that is DRB1*0802 or DRB1*0803. Therefore, a simple subtyping method is apparently needed to evaluate the DR matching. Because the PCR-DCP method described here is simple, rapid, and applicable to other DR groups which are amplified with one of the DR1, DR2, DR4, DR7, DR9, and DR10 specific 5' side primers in combination of a common DRB 3' side primer (27,33,36), this method should further facilitate the evaluation of DR matching even in cadaveric renal transplantation. We have already reported the applications of PCR-DCP analysis to the allelic discrimination of DPA and DPB (26,40,41) and DQB (27,42) genes.

In the current study, 18 DR52a ss DRB1 alleles were tested, and all of them were distinguished from one another by their characteristic patterns in the single stranded DNAs (ss DNA) although five alleles showed similar SSCP patterns. Heteroduplexes formed between tested DR52a ss alleles and the DRB1*1302 were useful for allelic identification of these alleles especially in heterozygous cases. This heteroduplex approach might be an alternative tool for the study of the DR6 effect (18), because Giphart et al. suggested that the DR6 effect in renal transplantation was associated with the DRB1*1302. Sorrentino et al. (25) have discussed that highly such polymorphic loci as DRB1 and DQB1 were hardly amenable to DNA heteroduplex analysis because of the number of different heterozygous combinations which might give complicated and partially overlapping band patterns. Although it may be impossible to analyze genomic PCR products of DRB genes and to genotype for the DRB alleles with one gel electrophoresis, the DRB1 genotyping could be achieved by subgrouping of DRB1 alleles by using PCR with group-specific primers. Although there are some DR52a ss alleles not tested in the current study, they may be able to be discriminated from other alleles, because the DR52a ss specific primers flank the DRB exon 2 so that all the allelic variable regions are included (43), and because almost all the one base mismatches were clearly detected by the PCR-DCP method (26). In addition, this procedure is powerful for the detection of new alleles as was reported for the DPA1 and DPB1 alleles (26,41). In this analysis, however, no unusual DCP patterns were detected in the 128 Japanese DR52a ss panels.

Tiercy et al. (44) have reported that comparison between serologic and oligotyping data showed an excellent correlation in only 66.3% of the 110 renal transplant pairs. At least one HLA-DR antigen was discrepant in 26.4% of the patients, and the oligotyping resolved uninterpretable serology in 7.3% of the cases. They reported that almost all of the discrepancies were due to errors in the as-
signmet of alleles belonging to the DR52ass group. We have also found that the discrepancy of serologic and oligotyping data was in the DR52ass group (Table II). Therefore the PCR-DCP analysis presented here will provide an alternative tool to analyze this toughest antigen group without using the oligoprobes or endonucleases. DR4 is also composed of many subtypes at the DNA level. We have reported that all the subtypes showed distinctive SSCP patterns (27), while DRB1*0401 and DRB1*0405 were similar in the SSCP patterns but were clearly discriminated by the definite heteroduplex formation. In addition, the DR2 subtypes in our panels, DRB1*1501, DRB1*1502, and DRB1*1602 were found to be easily genotyped by the PCR-DCP method (data not shown). These observations suggest that all DRB1 alleles might be discriminated by the group-specific amplification and subsequent PCR-DCP analysis, although many other samples from different ethnic groups should be tested to establish the complete PCR-DCP-based genotyping of the DRB1 alleles.

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