Frequent HLA Class I and DP Sequence Mismatches in Serologically
(HLA-A, HLA-B, HLA-DR) and Molecularly (HLA-DRB1, HLA-DQAI,
HLA-DQB1) HLA-Identical Unrelated Bone Marrow Transplant Pairs

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The rates of graft-versus-host disease (GVHD) and rejection are significantly higher among recipients of unrelated donor marrow (BM) than in recipients of marrow from HLA-identical siblings, even when donors and recipients are mixed lymphocyte culture (MLC) compatible and serologically and Dw identical. It has been hypothesized that phenotypically silent HLA class I and DP sequence mismatches might be associated with these differences, but little is known about their incidence. We have sequenced the HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQA1, and HLA-DPB1 genes expressed by 12 unrelated marrow transplant pairs, 11 of whom were molecularly matched at DRB, DQA1, and DQB1 loci. Nine of these pairs were also HLA-A and HLA-B matched by serology. Six of these nine “HLA-identical” pairs were HLA-A (2 of 6), HLA-B (1 of 6), and HLA-C (6 of 6) mismatched at the sequence level. The mismatched class I alleles of all these pairs had strikingly different sequence motifs in the six specificity pockets of their antigen recognition site, and in five pairs they also had sequence differences at positions implicated in T-cell receptor (TCR) binding. Two of the three pairs who were serologically mismatched for one HLA-A or HLA-B antigen were also sequence mismatched at HLA-C. Finally, 10 of 11 pairs tested expressed different DP sequences. These data indicate that HLA class I, especially HLA-C, and DP sequence mismatches are frequent among unrelated subjects defined as HLA identical by current typing methods. We speculate that these sequence differences may explain, at least in part, the higher incidence of acute GVHD and rejection in unrelated BM transplantation as opposed to transplantation between HLA-identical siblings. Because of their high frequency, the role of HLA-A, HLA-B, HLA-C, and HLA-DP mismatches in transplantation outcome is now amenable to direct study.

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Bone marrow (BM) transplants from genotypically HLA-identical sibling donors have been used successfully to treat patients with leukemia and a wide variety of congenital disorders of lymphohemopoiesis. However, fewer than 40% of eligible patients have an HLA-identical sibling as a potential donor. Serologically matched or partially mismatched unrelated donors have become a treatment alternative for patients who do not have a genetically HLA-identical sibling donor. Unfortunately, the rates of graft-versus-host disease (GVHD) and rejection are significantly higher among recipients of marrow from phenotypically HLA-identical unrelated donors than in recipients of marrow from HLA-identical siblings and appear to increase with the extent of HLA mismatch.

Currently, selection of histocompatible unrelated donors is largely based on serologic HLA identity and negative mixed lymphocyte culture (MLC) between potential donor/recipient pairs. However, DNA sequencing of HLA class I and class II genes has revealed that polymorphism at each HLA locus is much greater than that defined by serology. Thus, although HLA-identical siblings are genotypically HLA identical, many phenotypically matched (HLA-A, HLA-B, HLA-DR) unrelated donor/recipient pairs are likely to be genotypically different, as the same HLA phenotypes can often be expressed by distinct HLA gene combinations. Therefore, serologically silent HLA mismatches may be, at least in part, responsible for the greater risk of acute GVHD and rejection in unrelated marrow versus marrow from HLA-identical siblings. One case report study has demonstrated that even a single amino acid difference in an HLA class I antigen is sufficient for the development of alloreactivity in vivo.

We have previously reported that HLA class II polymorphism can be analyzed by a sequence-based typing (SBT) approach and have recently developed SBT protocols for all classic HLA class I genes. To determine the frequency, nature, and distribution of sequence mismatches between HLA-identical unrelated donor/recipient pairs, we have sequenced the HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DQA1, and HLA-DPB1 genes expressed by a series of unrelated BM transplant pairs molecularly matched at DRB1, DQA1, and DQB1 loci. This study shows, for the first time, that HLA-identical unrelated individuals are frequently HLA class I mismatched at the sequence level and confirms previous studies showing a high incidence of DP mismatches among serologically identical individuals. Application of complete HLA class I and DP-SBT to HLA-A, HLA-B, and HLA-DRB1, HLA-DQA1, and HLA-DQB1 matched unrelated donor/recipient pairs on a large scale will allow us to establish correlations between molecular HLA class I and HLA-DP mismatches and transplantation outcome and eventually determine their degree of clinical permissiveness.

MATERIALS AND METHODS

Patients. Twelve unrelated BM transplant donors and recipients of DRB, DQA1, and DQB1 moleculesly matched allogeneic grafts were studied. All transplant pairs were part of the Minnesota

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280

Bone Marrow Transplant Program and were selected at random exclusively on the basis of HLA typing information provided that lymphoblastoid cell lines (LCLs) were available for both donors and recipients. Neither the investigator who selected the samples nor those who performed the molecular analysis had access to clinical data from these pairs at the time the analysis was performed. Neither the phenotype of the patients and unrelated donors nor clinical transplant outcome were used as criteria to select these 12 pairs. All donors and recipients were of Caucasian origin. Recipients (six females and six males, 0.3 to 32 years of age) were transplanted with non-T-cell–depleted marrow because of hematologic malignancies (n = 9), Hurler’s disease (n = 1), aplastic anemia (n = 1), or severe combined immunodeficiency (SCID, n = 1). Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

HLA typing. Serology, Dw typing, restriction fragment length polymorphism (RFLP), and polymerase chain reaction amplification followed by hybridization to sequence-specific oligonucleotide probes (PCR-SSO) were performed at the Histocompatibility Laboratories at the University of Minnesota. Serology was performed by complement-dependent cytotoxicity (CDC) using Xth Workshop, local, and commercial antisera. Dw typing was performed using a panel of local and Xth Workshop homoygous typing cells (HTCs) previously described. Pertinent donor-recipient pairs were those that were novel (allele designations followed by “MN”) in Table 1. Of these 12 pairs, 8 were serologically identical pairs (66.6%) were sequence mismatched at one (n = 1), four at two (n = 2), and five at three (n = 3) loci. Each of these primer combinations allow equal amplification of both allelic transcripts in heterozygotes.

RESULTS

To determine the degree of molecular heterogeneity between unrelated donors and recipients of BM transplants selected on the basis of phenotypic and molecular HLA identity, we studied 12 patients and their corresponding unrelated donors by SBT (Table 1). Of these 12 pairs, 8 were serologically (HLA-A, HLA-B, and HLA-DR), PCR-SSO (HLA-DQB1, HLA-DQA1, or HLA-DPB1), RFLP identical (HLA-DRB, HLA-DQA1 and -DQB1), and Dw identical and thus appeared to carry identical class I and class II HLA alleles at these loci, which is an ideal situation for bone marrow transplantation. One pair was exclusively mismatched at the DRB1 locus (DRB1*0101 v DRB1*0102) and the remaining three were Dw, RFLP, and PCR-SSO matched but serologically mismatched at one class I locus (HLA-A or HLA-B).

Some of the class I sequences expressed by these 12 unrelated transplant pairs were novel (allele designations followed by “MN” in Table 1). Of these, only six encoded novel amino acid sequences (A*0201-MN6; C*0801-MN16; C*0301-MN10; C*0702-MN1; C*0801-MN6; and C*1401-MN12). The last four of these six novel sequences had also been found in Xth Workshop homozygous LCLs (also homozygous by class I SBT). Assignment of the sequences corresponding to A*0201-MN6 and C*0801-MN16 alleles was possible because they paired with a second allele with a match perfectly a known sequence (A*0301 and C*0701, respectively). These novel allelic sequences, confirmed by independent SBT experiments to rule out sequencing artifacts or tag polymerase or reverse transcriptase incorporation errors have been published elsewhere. As shown in Table 1, six of the nine class I serologically identical pairs (66.6%) were sequence mismatched at one (n = 3) or more (n = 3) class I loci on either

TTGCGTACGTGGACGCA-3'; A108, 5'-AGAGATACG- GGT-GTG-GCT-3'; A105, 5'-AGGGGCTTGGACGCA- CTCA-3'; B104, 5'-AGGGGCTTCGGACGACCTCA-3' and C103, 5'-AGGGGCTTGGACGACCTCA-3' for 35 cycles (1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C). Direct sequencing of the amplified products was performed as described before for class II products but using different sequencing primers (primer sequences: DPB-12-5, 5'-TACTG ATGGTGGCTGCT-ACAT-3'; DPA88/94, 5'-CTTTGGAAAACACGGTGACCTC-3'; A108, 5'-AGAGATACGCTTTGGTGGTGTCT-3'; B1579, 5'-TCC- CCACTGGCCGCGAGGTCGACT-3' and Z133, 5'-GTCACAGGGCGCGGCTC-3'). Each of the two reactions per locus was designed to provide overlapping sequence information. Reactions 1, 4, and 6 provide sequence information from the 5' end of exon 4 through most of exon 3; reactions 2, 3, and 5 provide sequence information for exon 2 and most of exon 3 (overlapping with reactions 1, 4, and 6). Each of these primer combinations allow equal amplification of both allelic transcripts in heterozygotes. Interpretation of each allelic sequence in heterozygotes was done essentially as described previously; a unique pattern is found for every particular heterozygote combination in the same way that certain RFLP banding patterns correspond to certain heterozygote combinations. Once two candidate alleles are identified, the overlapping ladder is carefully read to confirm presence of both sequences. The presence of unexpected bands or absence of expected bands for a particular allele or allelic combination is suggestive of sequence heterogeneity, that is, new alleles.
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* Dw typing using HTCs, DRB1, DOA1, and DOB1 results were obtained by PCR-SSO unless indicated (f1, it1). DOA1, and DOB1 typing performed by RFLP; allelic types were deduced according to well-established correlations between SBT and RFLP in HTCs and in cell lines carrying recombinant HLA class II haplotypes. ** DPB1 and DPA1 SBT results were confirmed by PCR-SSO, except in pair no. 1 where DPB-SBT was not performed with the donor LCL. Underlining is to indicate mismatches between pairs. Novel DNA sequences are designed with the official WHO nomenclature corresponding to the most closely related allele known followed by "MN" and an arbitrary number. These sequences, which with the exception of C*0801-MN16 and A*0201-MN6 also were found in homozygous cell lines, have been published elsewhere. **Partial sequence information. **C: 123-188, *C: 83-188, *C: 7-86, --, not determined.
one (n = 5) or both (n = 1) chromosomes. Surprisingly, all six of these pairs were HLA-C mismatched (C*0701 v C*0702-MN4 in pair no. 1; C*0801-MN16 v C*02022 in pair no. 2; C*0701 v C*0702-MN1 in pair no. 3; C*02022 v C*0701 and C*0101 in pair no. 4; C*02022 v C*0102 in pair no. 5; and C*0801-MN16 v C*1401-MN5 in pair no. 6). Two of these pairs were also HLA-A mismatched (A*0201 v A*0206 in pair no. 1; and A*0101 v A*0302 in pair no. 3) and one was HLA-B mismatched as well (B*2702-MN13 v B*2701-MN12). These results indicate that molecular HLA class I mismatches, especially at the HLA-C locus, are frequent among unrelated BM transplant pairs even when donors and recipients are serologically matched for HLA-A and HLA-B antigens and express molecularly identical HLA-DRB1, HLA-DQA1, and HLA-DQB1 genes.

Three additional pairs were studied to determine presence of HLA-C mismatches in DRB1-, DQA1-, and DQB1-matched transplant pairs with one serologic class I mismatch (HLA-A or HLA-B). As expected on the basis of the data presented above, HLA-C sequence mismatches also were found in two of these three pairs (C*0302 v C*0702-MN1 in pair no. 10; and C*0102 v C*0302 in pair no. 11).

Comparison of the sequences of the mismatched class I alleles of these six pairs (Fig 1) indicates that the number and type of mismatches varied considerably among pairs (from 3 in pair no. 1 to 40 in pair no. 10). All but one pair (pair no. 1) had mismatches at several positions directly implicated in T-cell receptor (TCR) binding (Fig 1). Most interestingly, sorting of class I residues according to their location in each of the six pockets within the antigen-binding groove that accommodate peptide side chains showed clearly distinct sequence motifs between mismatched alleles of all pairs (Fig 2). The peptides bound by these alleles are therefore expected to be different and may be able to trigger allogeneic responses in vivo.

Finally, sequence analysis of DP genes showed that 10 of 11 pairs (91%) were mismatched at DPA1 (n = 6) or DPB1 (n = 10) loci. No novel DP sequences were identified. Two of eight pairs carried mismatches on only one chromosome (pairs nos. 5 and 9), whereas the remaining were mismatched on both chromosomes (pairs nos. 2, 3, 4, 6, 7, and 8). One of the three class I serologically mismatched pairs was matched at DP loci (pair no. 11); the other two were only partially mismatched for DPB1 (pairs nos. 10 and 11). Considering class I and DP loci, all 12 pairs (100%) were sequence mismatched (Table 1).

**DISCUSSION**

This study was undertaken to determine the frequency, nature, and distribution of HLA-A, HLA-B, HLA-C, HLA-DPA1, and HLA-DPB1 sequence mismatches between unrelated BM transplant donors and recipients who were typed as HLA-identical by current methods. We show for the first time that HLA class I sequence mismatches, especially at the HLA-C locus, are frequent between unrelated subjects carrying phenotypically (HLA-A and HLA-B) and molecularly (HLA-DRB1, HLA-DQA1, and HLA-DQB1) identical HLA types. We also show that most of these HLA-identical transplant donor/recipients also are HLA-DPA1 or HLA-DPB1 nonidentical.

It is well documented that the degree of donor/recipient HLA matching has an important effect on transplant outcome. Case-control studies comparing results of marrow transplants from HLA-identical sibling donors and unrelated donors have found that the rates of acute GVHD and rejection were significantly higher in recipients of unrelated donor marrow even when donors and recipients appeared to be well matched by current methodologies (ie, MLC compatible and serologically and Dw identical). Because molecular HLA-DR and HLA-DQ mismatches are unusual among Dw- and RFLP-identical haplotypes, it has been hypothesized that serologically silent HLA class I and HLA-DP mismatches may account, at least in part, for these differences. Earlier DNA-RFLP and PCR-SSOP studies had already revealed that the incidence of HLA-DPB1 mismatches in HLA-A, HLA-B, HLA-DR, HLA-DQ, and HLA-Dw matched BM transplant pairs is very high (81.3% to 95.8%). On the other hand, very little is known about the frequency, nature, and distribution of HLA class I mismatches among serologically identical individuals.

Six of the nine HLA-identical pairs studied here (66.6%) were mismatched at HLA-A (2 of 6), HLA-B (1 of 6), and HLA-C (6 of 6) loci and eight of eight pairs tested (100%) also were HLA-DP mismatched. Of special note is the fact that these pairs were molecularly matched at HLA-DRB1, HLA-DQA1, and HLA-DQB1 loci and serologically matched for HLA-A and HLA-B antigens, which are conditions that minimized the chances of identifying further heterogeneity at the sequence level; frequency of class I sequence mismatches may be higher among individuals carrying serologically distinct class I haplotypes. Analysis of three additional transplant pairs, also molecularly matched for DRB1, DQA1, and DQB1 but serologically mismatched for one HLA-A or HLA-B antigen suggests that the incidence of HLA-C mismatches is at least as high among the latter. Because the number of new alleles identified is relatively large considering the small number of samples analyzed, it may be argued that some of these novel sequences may correspond to sequencing artifacts or Taq polymerase or reverse transcriptase incorporation errors. As discussed previously, we believe this to be unlikely for four main reasons. First, direct sequencing of PCR products greatly reduces detectable errors in Taq incorporation during PCR. Second, four of the six novel sequences identified in this study also were found in homozygous LCLs, which provided good positive controls for SBT. Third, new allelic sequences were mostly found at the HLA-C locus and were much less frequent at the HLA-A and HLA-B loci. Fourth, these sequences were reproduced in repeated experiments.

The high incidence of HLA-C mismatches in these pairs is particularly surprising. The HLA-C locus has been mapped at approximately 80 kb from the more centromeric HLA-B locus, and at approximately 1,420 kb from the more telomeric HLA-A locus. In these HLA class II–mismatched pairs, one would expect a higher frequency of HLA-A locus mismatches than HLA-B or HLA-C mismatches, loci closer to the centromeric HLA-D region, assuming a correlation between interlocus physical distance and the frequency of recombination. One possible explanation for this unexpected finding is that relatively frequent HLA-B/HLA-C
Fig 1. Amino acid sequences of HLA class I alleles of mismatched transplant pairs. The sequence shown at the top is the consensus sequence used by Zemmour and Parham. Only the sequences corresponding to the mismatched alleles in each pair are shown. Dashes indicate homology to the consensus sequence. Mismatched residues are boxed. Mismatched positions putatively involved in TCR binding also are shown (Φ).
terlocus recombination events may have generated extensive HLA-B/HLA-C haplotypic diversity during evolution. Subsequently, more frequent recombination events between the HLA-C and HLA-A loci would have generated haplotypes sharing HLA-B and HLA-A alleles, yet carrying distinct HLA-C alleles. Alternatively, some of these pairs may carry very similar, but not identical, DR-DQ haplotypes. Although unlikely, mismatching at DRB1, DRB3-5, DQA1, and DQB1 loci in these pairs can not be definitively excluded in the absence of direct sequence information and solely on the basis of PCR-SSO, Dw, and RFLP typing results.

The weak immunogenicity and poor expression of HLA-C molecules on cell surfaces, together with limited availability of specific antisera and complex crossreactivities among different HLA-C allelic products, have hindered our understanding of the genetic polymorphism at this locus. Indeed, our recent analysis of 26 HLA-A, HLA-B, HLA-C homozygous LCLs, including 23 Tenth Histocompatibility Workshop cell lines has confirmed the relatively high frequency of serologically silent sequence heterogeneity at the HLA-C locus; 14 of these cell lines expressed novel sequences. Furthermore, as opposed to serological HLA-A and HLA-B typing, class I-SBT has revealed that serologic HLA-C typing frequently mistakes heterozygous samples as homozygous. Therefore, serologic HLA-C typing may not always reveal the presence of HLA-C locus mismatches among unrelated individuals. For example, both the donor and the recipient of pair no. 5 were typed serologically as Cw5 (not shown); however, SBT revealed expression of Cw8 (Cw5) and Cw7 alleles by both individuals. One-dimensional isoelectric focusing (IEF) is an effective method for identifying serologically undetectable variants or subtypes for the HLA-A and HLA-B antigens. Indeed, one-dimensional IEF would have identified most of the HLA-A and HLA-B alleles in these transplant pairs. However, this technique has

![Fig 2. Amino acid sequences of the six specificity pockets of the antigen binding site of mismatched class I alleles. Dashes indicate identity between mismatched alleles of each pair. (The residues involved in each of these pockets are from refs 27 and 31.)](image-url)
not resolved the problems associated with HLA-C typing. Because of these technical problems, the level of diversification and immunologic role of HLA-C may have been underestimated. Different studies have shown that HLA-C molecules can be recognized by cytotoxic T lymphocytes both in vitro and in vivo, and that polymorphism at the HLA-C locus is much more extensive than previously thought with 40 different alleles. This high level of diversification suggests that HLA-C molecules may be as functional as HLA-A and HLA-B, as has been recently proposed. HLA-C mismatches may therefore have a role in the outcome of unrelated bone marrow transplantation.

Detailed analysis of the mismatched HLA-A, HLA-B, and HLA-C alleles of our non-HLA-identical transplant pairs showed strikingly different sequence motifs in the six specificity pockets of their antigen recognition site, as well as in several positions implicated in TCR binding. Mutations of HLA-A and HLA-B genes at positions mismatched in these pairs (residues 9, 24, 74, 77, 80, 97, 99, 114, 116, 152, and 156, involved in antigen-binding, or residues 62, 69, 76, 107, 152, and 163 putatively involved in TcR-binding) have all been shown to affect recognition by cytotoxic T lymphocytes in vitro and in vivo. Therefore, an effect of these molecular mismatches on transplantation outcome would not be surprising and has been observed previously for two HLA-B antigens differing by one amino acid at position 156. In view of the high frequency of class I mismatches in HLA-identical pairs, it is not surprising that the role of HLA-DP in the incidence of acute GVHD after unrelated BM transplantation remains controversial; in the absence of molecular HLA class I information, the role of DP mismatches in transplantation outcome may be difficult to evaluate. Although no conclusions regarding correlation between clinical transplantation outcome and mismatching can be reached on analysis of only 12 pairs (clonally heterogeneous), a trend toward higher risk of severe acute GVHD (grades III and IV) was observed in the patients who received mismatched allografts. None of the three patients who received a class I moleculeally mismatched transplant developed severe acute GVHD (grades III and IV) (patients 1213, 1340, and 1049), whereas six of six patients who suffered from severe acute GVHD had received class I-mismatched grafts (patients 0596, 1101, 0534, 1276, 1066, and 1283). However, despite receiving class I-mismatched grafts, two patients did not develop severe acute GVHD (patients 1098 and 0818). Future prospective studies controlling the many other factors that influence transplant outcome may allow to reach conclusions of clinical significance.

In summary, this study has shown that HLA class I, especially HLA-C, and HLA-DP mismatches are frequent among unrelated, phenotypically identical subjects. The association of HLA class I and DP mismatches detected by SBT with GVHD is not known but can now be tested. Although the practical usefulness of using SBT for selecting donors for unrelated BM transplantation remains questionable, its application to HLA-A, HLA-B, and HLA-DRB, HLA-DQA1, and HLA-DQB1–matched unrelated donor/recipient pairs on a large scale may help establish correlations between molecular HLA class I and HLA-DP mismatches and transplantation outcome and eventually determine their degree of clinical permissiveness.

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HLA SEQUENCE MISMATCHES IN BM TRANSPLANTATION

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