Molecular Typing Shows a High Level of HLA Class I Incompatibility in Serologically Well Matched Donor/Patient Pairs: Implications for Unrelated Bone Marrow Donor Selection

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In comparison with HLA-matched sibling bone marrow transplants, unrelated donor transplants are associated with increased graft-versus-host disease and graft failure. This is likely in part due to HLA incompatibilities not identified by current matching strategies. High resolution DNA-based typing methods for HLA class II loci have improved donor selection and treatment outcome in unrelated donor bone marrow transplantation. By using DNA-based typing methods for HLA-A and -B on a cohort of 100 potential bone marrow donor/patient pairs, we find that serological typing for HLA class II is limited in its ability to identify incompatibilities in unrelated pairs. Furthermore, the incompatibilities identified are associated with the presence at high frequency of alloreactive cytotoxic T-lymphocyte precursors. DNA typing also indicates that HLA-C mismatches are common in HLA-A and -B serologically matched pairs. Such mismatches appear to be significantly less immunogenic with respect to cytotoxic T-lymphocyte recognition, but are expected to influence natural killer cell activity. Thus, improved resolution of HLA class I shows many previously undisclosed mismatches that appear to be immunologically functional. Use of high resolution typing methods in routine matching is expected to improve unrelated donor selection and transplant outcome.

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llytic response, this interaction has been mapped to a dimorphic epitope at residues 77 and 80 on the α1 domain of HLA-C, each epitope interacting with a distinct killer inhibitory receptor (KIR). Those cells expressing HLA-C molecules with a motif shared with HLA-Cw*0303 (Ser77/Lys80) inhibits lysis by NK clones expressing KIR2DL2/3, whereas expression of a motif shared with HLA-Cw*0401 (Asn77/Lys80) are able to reject parental bone marrow20 that lacks expression of DRB1 and similarly 95 of 100 for DQB1, as previously described. DNA-based typing. All patient/donor pairs were also analyzed for HLA-A and -B serotypes to varying levels of resolution. The resolution offered by these techniques was dependent on the combination of alleles present, because heterozygosity often hindered allele-specific assignment.

**Results**

Identification of serologically undetected HLA-A and -B incompatibilities. To determine the level of HLA-A and -B undetected mismatching in serologically matched patient/donor pairs, samples were analyzed using higher resolution DNA-based methods. Patient/donor pairs were initially fully matched for HLA-A in 97% of cases (Fig 1). DNA typing of the patient/donor pairs confirmed the mismatches identified by serology. Two further patient/donor pairs were found to be incompatible using oligotyping for HLA-A, one indicating an HLA-A*02 subtype mismatch and the other an HLA-A*0301 versus -A*0302 mismatch (Table 1). Specific subtyping confirmed the HLA-A*02 incompatibility as HLA-A*0201 versus -A*0205 and that 55 further HLA-A2 seropositive pairs were matched at the subtype level. That 91 of 92 HLA-A2 seropositive individuals were encoded by HLA-A*0*201 reflects the dominance of this subtype in North European Caucasoids. RSCA matching indicated two further mismatches at A locus, an HLA-A*30 subtype mismatch and an HLA-A*03 heterozygote donor (A*0301, *03v) and HLA-A*0301 homozygous patient.
Original serological testing showed that 91% of patient/donor pairs were matched for HLA-B. A greater level of mismatching was found for HLA-B using DNA-based typing techniques. Discrepancies may occur between serological techniques due to the cross-reactivity of alloantisera, limitations in the alloantisera used, or the lack of expression of an allele. HLA-B SSOP found 3 samples to be misassigned by serology. In one case, HLA-B58 was missed and in another the same antigen was misassigned HLA-B57. In both cases, HLA-B62 was the second serotype expressed and errors in serological typing were likely due to serological cross-reactivity for HLA-B15 and -B17 groups. In the third, an HLA-B38 was not originally identified by serology. However, retyping by serology confirmed the presence of HLA-B38 as being expressed and not a null allele.

By using group-specific oligotyping methods, we identified heterogeneity within the HLA-B35 and -B44 serotypes. Four HLA-B*35 subtypes were identified at varying frequencies within the 27 individuals tested (Table 1). Importantly, 40% of the HLA-B35 seropositive patient/donor pairs tested were mismatched at the subtype level. HLA-B*44 subtyping indicated two common subtypes, HLA-B*4402 and -B*4403, in a 3:2 ratio. Despite the presence of these two common subtypes, only 4 of 25 (16%) of HLA-B44 seropositive pairs were mismatched at the subtype level. This low percentage is likely due to the HLA-B*44 subtypes commonly segregating on different haplotypes and matching for other loci fortuitously results in frequent HLA-B*44 subtype compatibility.

Oligotyping was limited in its resolution by the number of probes used and the heterozygosity exhibited by most samples. Two PCR primer mixes were used for HLA-B SSOP, which enabled the separate typing of each allele in a proportion of allelic combinations. However, RSCA was useful in identifying polymorphisms not detected by oligotyping and also in confirming homogeneity in potentially heterogeneous serotypes. Three HLA-B*51 subtypes and two commonly occurring HLA-B*39 subtypes were identified by this technique (Table 1) as well as those HLA-B*35 and -B*44 subtypes identified by SSOP. In contrast, only one subtype was detected in 45 HLA-B7 and 40 HLA-B8 seropositive individuals. Furthermore, HLA-B62, a serotype shown to be encoded by many distinct alleles, was identified as B*1501 in all 16 cases tested.

After molecular typing methods were applied, mismatching for HLA-A and -B increased to 7% and 27% of pairs, respectively. In total, those matched for HLA-A and -B decreased from 89% as initially detected by serology to 70% of pairs (Fig 2). The higher level of HLA-B mismatching reflects the limitations in serological resolution at this locus.

Molecular HLA-C typing. Unlike HLA-A and -B, no account was taken of HLA-C match status in the selection of donors for final stage testing. However, because HLA-B and HLA-C are in strong linkage disequilibrium, some level of matching was expected. To measure the level of matching at HLA-C and so determine its impact on the match status of patient donor pairs, PCR-SSP and PCR-SSOP typing methods were used. DNA-based typing identified HLA-C mismatching in 33 of 84 pairs tested (39%), a higher level than that seen for HLA-A or -B (Fig 2), with 8 pairs mismatched for both HLA-C alleles. Importantly, 24 of the 33 (73%) HLA-C mismatched pairs had further HLA-A and/or -B incompatibilities identified at the molecular level, indicating that HLA-C is a useful marker for HLA-A and -B.

Table 1. Frequency of Mismatching Is Serotype Dependent

<table>
<thead>
<tr>
<th>HLA Serotype</th>
<th>A2†</th>
<th>A3†</th>
<th>A30†</th>
<th>B35†</th>
<th>B39†</th>
<th>B44†</th>
<th>B51†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor/patient pairs</td>
<td>56</td>
<td>24</td>
<td>4</td>
<td>15</td>
<td>8</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>Mismatches detected</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Subtypes found</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

*Mismatch detected by PCR-SSOP.
†Mismatch detected by RSCA.

Fig 2. HLA matching for bone marrow donor selection. DNA-based typing (■) showed an increased level of mismatching for HLA-A and -B over that defined by serology (●). Although no HLA-C serology was performed for original typing, DNA-based typing indicated a high level of incompatibility.
for other mismatches on the haplotype. The utility of HLA-C type as an indicator of HLA-B compatibility was allele dependent. HLA-B*4402 and B*4403 were predominantly associated with restricted HLA-C locus alleles. Cw*0501 and Cw*1601, respectively. In contrast, HLA-B*5101 was associated with a wide range of HLA-C alleles, as reported previously. Thus, mismatching at HLA-C was not predictive of further mismatches for all haplotypes. Overall, 22 of 25 potential donor/patient pairs mismatched at HLA-B were also mismatched at HLA-C.

Mismatching for the HLA-C encoded motifs that interact with KIRs, thus influencing NK cell allorecognition, was next assessed. Each individual was classified as being positive for the Asn77 and Lys80 (group 1) and/or Ser77 and Asn80 (group 2) HLA-C molecules. We then calculated whether the HLA-C mismatched donor/patient pairs were also mismatched for this motif. Twenty-two of the 33 (67%) HLA-C mismatched pairs were also mismatched at the level of KIR binding motif. However, no mismatched pairs were homozygous for opposite KIR binding motifs, and so NK cell allorecognition would be expected to be unidirectional. Nine of the 22 mismatches may be expected to influence allorecognition in the graft-versus-host direction and 13 in the host-versus-graft direction.

Cellular recognition. It has been reported that HLA-A and -B mismatches detected by serology and at the DNA level are recognized in vitro by high frequency CTL. The limit of sensitivity of this assay is 1 patient-specific donor T cells are found at a lower frequency than those at HLA-A and -B. However, no mismatched pairs were homozygous for opposite KIR binding motifs, and so NK cell allorecognition would be expected to be unidirectional. Nine of the 22 mismatches may be expected to influence allorecognition in the graft-versus-host direction and 13 in the host-versus-graft direction.

Overall level of matching. Donors were selected on the basis of being closely matched for HLA-A and -B by serological methods and -DRB1 (and DQB1) by molecular methods. Using molecular typing methods for HLA-A and -B and including HLA-C typing data, the level of matching was seen to be greatly reduced. Of 89 pairs (89 donors for 76 patients) studied, 63% were originally regarded as fully matched. After DNA-based typing, this figure was reduced to 46%. However, single detected mismatches indicate mismatched haplotypes and increase the chances of there being incompatibilities at other loci.

Fig 3. The effect of HLA-C mismatching on CTLp frequency. CTLp frequencies of 30 pairs with patient HLA-C locus incompatibilities are shown above. Mean CTLp frequencies differed significantly between those with only HLA-C mismatches (1:316,500) and those with further HLA class I mismatches (1:47,110). This indicates that HLA-C alloreactive T cells are found at a lower frequency than those at HLA-A and -B. The limit of sensitivity of this assay is 1 patient-specific donor CTLp/10^5 or higher. Mean CTLp frequencies differed significantly between groups according to the Mann-Whitney test.

Fig 4. DNA-based typing shows many HLA class I mismatches in the high CTLp frequency group not identified using serological methods. [■] Matched pairs; [□] those with detected HLA class I mismatches. Forty-seven donors had high (>1:10^5) patient-specific CTLp frequencies. Only 8 (13%) of these had HLA class I mismatches detected by serological typing, reflecting the low overall number of HLA class I serological mismatched pairs. The proportion of HLA class I mismatched pairs in the high CTLp frequency group increased to 23 (49%) after DNA-based typing, with 21 pairs mismatched at the HLA-B and/or -A locus. However, 24 pairs (51%) with high CTLp frequencies appear to be compatible for HLA class I.
By using high resolution class I typing, we have found 43% of donor patient pairs with two or more mismatches and 17% with three or more (Fig 5).

**DISCUSSION**

Unrelated donor BMT has only been made practical by the establishment of large volunteer registries and implementation of HLA typing techniques with sufficient resolution for matching. Although HLA-matched siblings can be identified by a combination of a mixed lymphocyte reaction (MLR) assay and serological typing, this combination of tests has not proven effective in predicting GVHD in the unrelated donor setting. DNA-based typing methods have therefore been developed to allow accurate matching of HLA class II loci and are now used routinely for selection of matched unrelated donors, with improved transplant results. In contrast, HLA-A and -B specificities have until recently only been defined using classical serological methods. We have implemented DNA typing methods for HLA-A, -B, and -C to study the deficiencies of current HLA class I typing in accurately matching unrelated pairs. It is only by identifying the precise level of matching in BMT that the importance of serologically undefined differences can be assessed.

By using DNA-based typing for HLA class I, we have been able to identify many more serologically undetected mismatches in HLA-B than HLA-A. It appears that most HLA-A serotypes in our population are encoded for by one dominant allele as indicated by HLA-A2 subtyping (Table 1). In contrast, several HLA-B serotypes were encoded by multiple alleles. Although the incompatibilities identified by DNA-based typing involved molecular differences of as little as one amino acid, these are likely to be in positions on the HLA molecule expected to interact with bound peptide and/or T-cell receptor. Such small differences have been shown to generate vigorous alloreactive CTL responses in the BMT setting leading to severe complications. To further emphasize the functional relevance of these mismatches, high CTLp frequencies were detected in 86% of HLA-A, -B mismatched pairs.

We have confirmed that HLA-C incompatibilities are frequent in serologically HLA-A, -B matched unrelated pairs. However, due to the distribution of polymorphic residues within HLA-C antigens and their reduced cell surface expression, it has been suggested that HLA-C may not be as immunologically relevant as the other classical class I loci. It has previously been suggested that HLA-C incompatibilities do correlate with high CTLp frequency, although in that study HLA-A and -B typings had not been determined using high resolution methods. In contrast, we have demonstrated that, whereas HLA-A and -B mismatches correlate with a high CTLp frequency, this was not so for HLA-C mismatching. By using high resolution matching techniques, we show that, of all the C locus mismatched pairs with high CTLp frequency, 89% had other class I differences. Furthermore, 82% of those with no other HLA class I mismatch were found to have a low or negative CTLp frequency (Fig 4). This supports the view that HLA-C may be of less immunological relevance than HLA-A and -B with respect to CTL surveillance.

Whereas HLA-C incompatibilities may not be a major target for alloreactive CTLs, certain mismatches will result in differential expression of the motifs responsible for modulating alloactive NK cell activity. Mismatching for the HLA-C encoded NK resistance motifs was seen in 67% of those mismatched at this locus and 26% of all pairs studied. Because HLA phenotype appears to play an important role in defining the KIR repertoire of an individual, potentially alloreactive NK cells are likely to be present in a significant proportion of unrelated donor transplants and their possible impact should be considered. A phenomenon in the mouse known as hybrid resistance in which bone marrow from an MHC homozygous parent is rejected by its F1 hybrid offspring has been shown to be mediated by NK cells. It is now known that the lack of self-MHC molecules on murine donor bone marrow cells leaves them vulnerable to recipient NK-mediated lysis. An analogous situation has been demonstrated in vitro in the human, with HLA-C playing a key role in the protection from NK lysis. The detection of high CTLp frequency has been hypothesized to be a useful indicator of HLA class I incompatibilities not identified by serological methods. However, the range of CTLp frequencies for the same HLA mismatch may be large between unrelated individuals (Breur-Vriesendorp et al and unpublished data). Whether the absolute frequency or the qualitative properties of alloreactive CTLs calculated in vitro is important is not clear. Evidence suggests the CTLs involved in GVHD are not the same as those generated in vitro. Despite the use of high resolution HLA matching techniques, a significant number of pairs in the high CTLp frequency group (51%) had no detected mismatches in the GVHD direction. In such cases, full HLA-A, -B, and -C sequencing would be useful in confirming patient/donor HLA matching. A possible explanation for such CTL activity is that the peptide repertoire varies sufficiently between unrelated individuals to induce a strong alloreactive CTL response in vitro. In fact, serological reagents have been used to distinguish HLA-B antigens in the absence of allelic differences. CTLp frequency is often used as a
criterion for selection of a suitable unrelated donor. It will therefore be important to assess the functional significance of high CTLp frequencies in the absence of HLA incompatibilities.

The term minor HLA mismatch is commonly used in reference to an HLA-A, -B mismatch of the same serological cross-reactive group or HLA class II allele encoding a product of the same serological group. Matching for HLA-B serological splits has been shown to be of little benefit in renal transplantation. However, evidence suggests that these incompatibilities are of major importance to the outcome of allogeneic BMT. Serologically undetected HLA class I mismatches are recognized efficiently by alloreactive CTL correlating with poor transplant outcome.27,40,56,57

By improving the resolution of HLA class I matching, the number of perfectly matched pairs has significantly decreased. Furthermore, many pairs were found to have multiple mismatches, which has been shown to increase the risk of posttransplant complications. Thus, the problem created by high resolution typing of HLA loci is a reduction in the number of matched donors that can be provided. However, this study has demonstrated that many of the HLA mismatches identified are on haplotypes frequent in the Caucasoid population. Therefore, the likelihood increases that several unrelated donors will be matched at the serological level at HLA class I. The development of techniques such as RSCA will allow the convenient, rapid, and accurate screening of a large number of such potential donors. By using such a strategy, HLA matching levels should improve. However, there will remain a significant number of patients without fully HLA matched donors and we need to assess carefully what level of HLA mismatching can be acceptable for a beneficial outcome to BMT. Retrospective analyses of unrelated donor transplants using high resolution typing have been performed by several groups in an attempt to identify those mismatches best tolerated, and indications that mismatched transplants can be successful, especially for younger patients, are encouraging. The hope is that such studies will allow the rational selection of the most appropriate mismatched bone marrow donor.

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