DNA-Based HLA Typing of Nonhematopoietic Tissue Used To Select the Marrow Transplant Donor for Successful Treatment of Transfusion-Associated Graft-versus-Host Disease

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Transfusion-associated graft-versus-host disease (TAGVHD) is a rare and usually fatal complication of blood transfusion. The disease can arise when immunocompetent lymphocytes from the donor of a cellular blood product are transfused into a severely immunocompromised recipient. If the recipient cannot eliminate them, these donor lymphocytes may proliferate in response to the antigens that they recognize as foreign, leading to infiltration and damage of host tissues, most notably the skin, liver, gut, and bone marrow (2, 24). TAGVHD occurs most frequently in patients with congenital and acquired immunodeficiencies, although not in immunodeficiency related to human immunodeficiency virus infection (1). TAGVHD also occurs in otherwise immunocompetent recipients if the HLA antigens on the donor lymphocytes are not recognized by the recipient as foreign (29). Certain other populations, such as low-birth-weight neonates, are also at risk (1, 2, 18). TAGVHD differs from the GVHD which arises in the setting of allogeneic bone marrow transplantation. TAGVHD typically causes profound pancytopenia and bone marrow aplasia and is usually severe; 85% or more of cases are fatal (1, 18). The diagnosis of TAGVHD is often made postmortem, when characteristic lymphocytic infiltration is noted in the skin, lymph nodes, gastrointestinal tract, and other organs at autopsy. The clinical suspicion of TAGVHD usually arises because of fever, rash, gastrointestinal bleeding, and pancytopenia in the appropriate clinical setting. Characteristic histologic findings in a skin biopsy specimen establish the diagnosis. Definitive confirmation of TAGVHD can be obtained by demonstrating the foreign origin of lymphocytes in the circulation or in the affected tissues. Serologic HLA typing can often provide such confirmatory data. However, other methods, including DNA-based HLA class II typing, karyotype analysis, sex chromatin analysis, restriction fragment length polymorphism analysis with probes from HLA and non-HLA regions, and genetic fingerprinting, have successfully been used (4, 6, 9, 14, 19, 32).

TAGVHD can be prevented by gamma irradiation of blood products prior to transfusion (1, 18). Once established, however, the condition responds poorly to medical therapies such as corticosteroids or immunosuppression. Bone marrow transplantation (BMT) has been reported as therapy for GVHD that resulted from in utero materno-fetal transfusion in infants with severe combined immunodeficiency (SCID) (17, 20). In addition, a case of TAGVHD in a patient with SCID was treated successfully with immunosuppression and BMT from a sibling with an identical HLA type (13).

Here we report the case of an 8-month-old male who developed TAGVHD from an irradiated erythrocyte transfusion given before his SCID syndrome was recognized. Severe pancytopenia developed as part of the TAGVHD and was not reversed by immunosuppressive therapy. Therefore, BMT was undertaken as definitive therapy both to correct the underlying immunodeficiency and to treat the TAGVHD. Because the patient’s circulating lymphocytes were of blood donor origin,
his constitutive HLA type could not be determined by standard serologic typing of peripheral blood lymphocytes (PBLs). Instead, DNA-based class I and class II HLA typing was used both to confirm the diagnosis of TAGVHD and to guide the selection of the marrow donor. The child is alive more than 2 years after the onset of TAGVHD, with full hematopoietic engraftment and with clinical and laboratory evidence that the TAGVHD has been eradicated.

**CASE REPORT**

The patient was an 8-month-old Cambodian male scheduled for placement of myringotomy tubes for recurrent otitis media. During the preanesthetic evaluation, pulmonary rales and decreased capillary perfusion were noted, and his chest film, electrocardiogram, and echocardiograph demonstrated an acute dilated cardiomyopathy presumed to be viral in origin.

On admission to intensive care, a focally abnormal neurologic examination was noted in addition to the signs of cardiogenic shock. Within the first 24 h his cardiovascular status had deteriorated, requiring mechanical ventilation, pressor support, and erythrocyte transfusion.

The birth history, growth, and development were normal. He had received routine diphtheria and tetanus toxoids and pertussis vaccine and oral polio vaccine immunizations at 2 and 4 months of age. He had an extensive eccemoid rash which was treated with a topical corticosteroid, and he received cefaclor for otitis media. The family history was unremarkable for immunodeficiency or early childhood deaths, and there were no siblings.

Abnormal results of the initial immunologic evaluation (see Results) together with the history of eczema and recurrent otitis media suggested an immunodeficiency syndrome affecting both humoral and cellular immune response, and the decision was made to irradiate all blood products. However, he had received a single unirradiated erythrocyte transfusion.

An endocardial biopsy was performed, from which an enterovirus grew rapidly. The isolate was demonstrated to be poliovirus type 2 by serological typing at the Pennsylvania State Public Health Laboratories. Restriction fragment length polymorphism analysis in Roland Sutter’s laboratory at the Centers for Disease Control and Prevention, Atlanta, Ga., demonstrated that the isolate was vaccine-strain poliovirus.

The patient’s cardiovascular function improved, but his neurologic function remained severely impaired. Two months after admission he developed persistent fever, desquamation and extension of his rash, hepatomegaly, eosinophilia and lymphopenia, and later, total leukopenia with an absolute neutrophil count of less than 50 cells per mm³. A bone marrow aspirate and biopsy specimen were hypocellular, and a skin biopsy specimen revealed lymphocytic infiltration suggestive of GVHD. HLA typing of the patient, the family, and the donor of the unirradiated blood confirmed the diagnosis of TAGVHD (Table 1).

Two weeks after the initial signs of TAGVHD, methylprednisolone at doses of 1 to 2 mg/kg of body weight per day was begun and was continued for more than 8 weeks. The rash and hepatomegaly improved, but severe pancytopenia persisted, as illustrated in Fig. 1. BMT was therefore considered to treat both the underlying immunodeficiency and the TAGVHD.

Since the child’s true HLA type could not be determined from PBLs, scrapings of buccal mucosal cells were taken as a source of nonhematopoietic tissue for DNA-based HLA typing. In addition, in vitro fibroblast cultures were established from a thymic biopsy specimen taken during an open lung biopsy for culture and histology to assess a chronic pulmonary infiltrate prior to transplantation.

Myeloablative conditioning to eliminate the engrafted blood donor lymphocytes and to ensure engraftment of marrow donor cells was given with busulfan (16 mg/kg/day) on days 8 to 5, cyclophosphamide (60 mg/kg) on days 4 to 2, and antithymocyte globulin (20 mg/kg/day) on days 3 and 2. Maternal bone marrow, depleted of T cells by a two-step procedure with soybean agglutinin and then sheep erythrocyte rosetting (12), was infused on day 0, 3 months after the onset of TAGVHD. Cyclosporin A was administered after transplantation for GVHD prophylaxis.

There was evidence of hematopoietic engraftment with erythroid, myeloid, and megakaryocytic lineages within 3 weeks, and engraftment has been stable for more than 24 months (Fig. 1). Immunologic reconstitution was incomplete, with reduced peripheral blood lymphocytes and reduced immunoglobulin levels in the serum, which required the ongoing administration of intravenous gamma globulin therapy. The child has significant but static residual neurologic abnormalities.

Finally, since a specific diagnosis for the child’s congenital SCID syndrome could not be reached on the basis of studies obtained at the time of presentation, maternal lymphocytes were studied as described previously (7, 23) for their pattern of X chromosome inactivation. The pattern of X inactivation in maternal lymphocytes was found to be random, suggesting that this may be an autosomal rather than an X-linked SCID syndrome.

### Table 1. HLA typing summary

<table>
<thead>
<tr>
<th>Specimen source</th>
<th>Serologic typinga</th>
<th>DNA-based typinga</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father’s PBLs</td>
<td>A11, A29, B46, B7</td>
<td>DR2, DR12, DQ6, DQ7</td>
</tr>
<tr>
<td>Mother’s PBLs</td>
<td>A29, B7</td>
<td>DR10, DQ5</td>
</tr>
<tr>
<td>Blood donor’s PBLs</td>
<td>A1, A3, B8, B35</td>
<td>DR1, DR3</td>
</tr>
<tr>
<td>Child’s PBLs during TAGVHD</td>
<td>A1, A3, B8, B35b</td>
<td>DR1, DR3</td>
</tr>
<tr>
<td>Child’s nonhematopoietic cells</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Child’s PBLs after BMT</td>
<td>Not tested</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

a The serologic and DNA-based HLA typing results of each specimen are summarized by using the World Health Organization nomenclature (5). The two digits to the right of the asterisk in allele notation are the same as the number of the corresponding HLA serotype, with the exception of DQB1*0301, which corresponds to DQ7.

b Repeated on three specimens because of high background.
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were serologically performed assays phorylase the serum for phy by magglutinin of toxoid, clinical manifestations of TAGVHD. Fluctuations in the neutrophil count after engraftment reflect intercurrent illnesses.

MATERIALS AND METHODS

Clinical immunology testing. Quantitative determination of immunoglobulin M (IgM), IgG, and IgA was performed by nephelometry (25), measurement of the subclasses of IgG was performed by enzyme-linked immunosorbent assay (ELISA), and measurement of IgA was performed by radial immunodiffusion (Microbiology Reference Laboratory, Cypress, Calif.). Testing for specific antibodies to diphtheria toxoid, tetanus toxoid, and pneumococcal antigens was performed by ELISA (Microbiology Reference Laboratory). Flow cytometry for immunophenotyping of peripheral blood B- and T-cell subsets was performed (15, 16) by using the Coulter EPICS-C cytometer (Coulter Cytometry, Hialeah, Fla.). Lymphocyte responses to stimulation with pokeweed mitogen and phytohemagglutinin were measured as described previously (26). Erythrocyte adenosine deaminase and purine nucleoside phosphorylase assays were performed by thin-layer chromatography by using radiolabeled nucleosides in the laboratory of Michael Hershfield (3).

Tissue from the thymic biopsy specimen was dispersed, and 10⁶ fibroblasts were cultured in RPMI 1640 with 10% fetal bovine serum and 1% L-glutamine until they were confluent. The fibroblasts were removed from the T75 flasks with scrapers and were frozen until use.

HLA typing. The patient, his parents, and the blood donor were serologically typed for HLA class I and class II molecules by the long-incubation microlymphocytotoxicity technique (11). The patient's and the blood donor's PBLs and the patient's buccal mucosal scrapings and cultured fibroblasts were also typed by DNA-based technology by using PCR and sequence-specific oligonucleotide probe (SSOP) methodology as described in several publications (21, 31, 33) and modified by us. Briefly, our approach was as follows. Genomic DNA was extracted from PBLs or nonlymphoid cells by using a standard phenol-chloroform method for high-molecular-weight DNA (27). For the preparation of DNA from nonlymphoid cells, a cell pellet of 10⁶ cells was lysed with 0.5 ml of buffer consisting of 10 mM Tris (pH 7.6)–10 mM EDTA (pH 8.0)–50 mM NaCl. Molecular typing of HLA class I was performed to distinguish HLA A11 from HLA A29 as described by Oh et al. (21). The amplification step spanned exon 2, intron 2, and exon 3. HLA B7 was distinguished from HLA B46 as described by Yoshida et al. (33). The amplification step at the HLA B locus involved exon 2, and the primers CG4 and CG3 were used. The cycling parameters were modified for the Thermal Cycler 9600 (Perkin-Elmer Cetus), with denaturation at 96°C for 20 s, annealing at 62°C for 10 s, and extension at 72°C for 65 s for a total of 35 cycles. Locus- and allele-specific amplifications were performed for the DRB1, DRB3, and DQB1 genes by using the primers and procedure described previously (31).

RESULTS

Initial evaluation of immunodeficiency. Immunoglobulin levels in the patient's serum at the time of presentation

FIG. 1. Clinical course of TAGVHD reflected by neutrophil counts. The patient's leukocyte count (WBC) and absolute neutrophil count (ANC) are plotted over time, beginning with the transfusion of unirradiated erythrocytes. The period of profound neutropenia coincided with other clinical manifestations of TAGVHD. Fluctuations in the neutrophil count after engraftment represent intercurrent illnesses.
revealed markedly decreased IgG levels (less than 9 mg/dl; normal range, 383 to 1,070 mg/dl), decreased IgM levels (less than 7 mg/dl; normal range, 43 to 113 mg/dl), and normal IgA levels (43 mg/dl; normal range, 27 to 169 mg/dl). The levels of IgG subclasses were all markedly reduced, but both IgA1 and IgA2 were detectable. An intermediate-strength tuberculin test with intradermal purified protein derivative and an anergy panel consisting of *Trichophyton* and *Candida* antigens were all nonreactive. Additional immunologic testing revealed nonprotective levels of antibodies to diphtheria, tetanus, and pneumococcus and no serologic evidence of past infection with Epstein-Barr virus, hepatitis B virus, echovirus or coxsackievirus. Antibody to human immunodeficiency virus type 1 was undetectable by ELISA, and PCR amplification of a segment of the human immunodeficiency virus p24 gene from PBL DNA gave negative results. Coculture of patient PBLs with activated normal mononuclear cells yielded no evidence of replication of human immunodeficiency virus. Flow cytometry revealed a reduction in the number of peripheral blood B cells, and lymphocyte stimulation studies revealed markedly reduced responses to phytohemagglutinin and pokeweed mitogen. Levels of erythrocyte adenosine deaminase and purine nucleoside phosphorylase measured 2 weeks after an erythrocyte transfusion were within normal limits. The clinical and laboratory findings suggested a SCID syndrome affecting both humoral and cellular immunities, but no specific SCID defect could be established.

Serologic HLA typing. Serologic HLA typing of the patient's PBLs revealed HLA antigens not derived from either parent but identical to those of the blood donor (Table 1). PBL HLA typings were repeated on three separate specimens from the child and yielded the same results, although some of the typings were suboptimal because of low cellular viability and, in addition, because they appeared to show some additional reactivities. Thus, serologic methods demonstrated the presence of blood donor lymphocytes in the child but were unable to determine the child's underlying HLA type.

In addition, the serologic typing of the parents (Table 1) revealed two unusual findings. First, the mother demonstrated a single specificity at each HLA locus: A29, B7, DR10, and DQ5. She either is homozygous for this haplotype or has unknown or undetectable blank alleles at one or more of the four loci tested. Second, the parents share the HLA A29 and B7 antigens. The sharing of these class I antigens by the parents presented an interesting dilemma in deciding which parent should be the bone marrow donor for the child, whose HLA type was unknown.

Figure 2 illustrates this dilemma by showing that some of the HLA types possible for the offspring of these parents have greater histocompatibility with the father, while others have greater histocompatibility with the mother. If the child inherited HLA A11, HLA B46, or both from the father, then the mother's lymphocytes in a bone marrow graft would recognize these antigens on the child's tissues as foreign but the father's lymphocytes would not. Thus, if the child had genotypes III to VIII in Fig. 2, the father would be the preferred bone marrow donor.
donor. On the other hand, if the child had genotypes I or II, then neither parent would recognize the child's class I HLA antigens as foreign, and by the typing results, either parent could be the bone marrow donor.

For simplicity, Fig. 2 shows only those genotypes which would be possible if the mother were homozygous at all four HLA loci; the presence of blank loci in the mother would introduce additional possibilities. However, if such blank class I or class II alleles were present and inherited in the child, they would be recognized as foreign by the father's lymphocytes but not the mother's. Thus, the possibility that the mother has blank alleles would favor the use of her as the donor.

Whether she is HLA homozygous or not, the sharing of A29 and B7 by the parents makes it important to determine the class I antigens which the child actually inherited in order to select the bone marrow donor. In particular, if paternal alleles A11 and B46 can be excluded from the child's HLA alleles (Fig. 2, genotypes III to VIII), then the remaining possibilities (Fig. 2, genotypes I and II and genotypes with maternally inherited blank alleles) would all be compatible with the mother as the bone marrow donor.

One or class II alleles were present and inherited in the child, the donor. The child would have one matched and one unmatched class II haplotype with the bone marrow donor, regardless of which parent gave the marrow. Thus, the distribution of class II alleles does not affect the choice of bone marrow donor.

To summarize, in the most likely scenario in which the mother is HLA homozygous, six of eight possible HLA types for the child favor the use of the father as donor, and two of eight possible HLA types are neutral, favoring neither parent. If the mother has either one or two blank class I alleles, genotypes in addition to those shown in Fig. 2 would be possible, but all of the additional possibilities would favor the use of the mother or would be neutral. In any case, there will be one class II haplotype mismatched between the child and the donor. The choice of parental bone marrow donor for this child thus depends on knowing the child's class I HLA type.

**DNA-based HLA typing of nonhematopoietic cells.** To determine the child's true HLA type, genomic DNA was extracted from the sources given in Table 1 and was used as the template for PCR amplification of specific coding regions of HLA A, B, DR, and DQ genes. The amplified DNA was hybridized with SSOPs, which distinguish HLA alleles on the basis of differences in the nucleotide sequence. DNA-based typing of the child's fibroblasts and epithelial cells revealed the antigens A*29, B*07, DRB1*1001, DRB1*12, DQB1*0301, and DQB1*0501, which are consistent with serologic specificities A29, B7, DR10, DR12, DQ7, and DQ5, respectively. No alleles corresponding to the A11 or B46 serotypes were detected in the child's DNA, while appropriate controls were positive. These results confirmed the parentage of the affected child and established his most likely HLA type as that of genotype I in Fig. 2. With this genotype, either parent's bone marrow would be equally compatible with the child, having only one class II haplotype mismatched. The possibility that the child has maternally inherited blank alleles has not been ruled out by the limited DNA-based typing approach of excluding the inheritance of paternal alleles. However, the results of the DNA-based HLA typing definitively exclude the father as the preferred bone marrow donor.

For these reasons, and because of the additional tolerance considerations discussed below, the mother was in fact selected as the donor.

**DNA-based HLA typing of PBL.** DNA-based class II typing of PBLs during TAGVHD detected both DRB1*1001 and DRB1*12, the child's inherited DR alleles, as well as DRB1*01 and DRB1*0501, the DR alleles acquired from the blood donor. This result confirmed the presence of the two expected populations of lymphocytes in the child's PBLs, a finding that could be inferred only from the background reactivity in the microlymphocytotoxicity assays. The blood donor's DR and DQ types and both parents' DR types were also determined from their PBLs by the DNA-based method and agreed with the serologic results.

**Analysis of chimeric engraftment.** Peripheral blood karyotype studies obtained 2 months after maternal bone marrow infusion revealed chimeric engraftment. Of 29 metaphase spreads examined for karyotype, 6 of 29 were male and 23 of 29 were female. Since the blood donor was male, it was of interest to determine if the male metaphases were derived from the donor or from the child's original hematopoietic system. DNA-based HLA class II typing of the posttransplant PBL DNA (Table 1) revealed maternal (DRB1*1001) and paternal (DRB1*12) but not blood donor (DRB1*01 or DRB1*0501) allele donors. All four alleles were detectable by PCR-SSOP in the pretransplant specimens. Thus, DNA-based HLA class II typing suggested that the male karyotypes were derived from the child's original (birth) hematopoietic system and not from the blood donor. The allogeneic cells responsible for the TAGVHD were eliminated by the transplant, at least to frequencies below the limits of detection by PCR.

**DISCUSSION**

TAGVHD usually progresses rapidly, responds poorly to immunosuppressive therapy, and is most often fatal. Most of the reported survivors are leukemia patients whose underlying immunosuppression may have improved after the cessation of chemotherapy (1). Among other patient groups, the mortality from TAGVHD is greater than 90%, and children with congenital immunodeficiency rarely survive TAGVHD (13, 26). One previous case of TAGVHD (13), also in a patient with SCID, was successfully treated with immunosuppression with antithymocyte globulin, no myeloablation, and bone marrow transplantation from an HLA-identical sibling. In the patient described here, myeloablation was given in addition to antithymocyte globulin because of the clinical severity of the TAGVHD and because the bone marrow donor was not fully HLA identical with the recipient. In both of these patients, success in controlling TAGVHD may have been related to the fact that they survived longer than expected even prior to transplantation. Both lived with severe pancytopenia from TAGVHD for 3 months, whereas the median survival from the time of diagnosis of TAGVHD is 21 days (1). Thus, these cases may represent attenuated or more indolent examples of TAGVHD. Nevertheless, they demonstrate that TAGVHD can be treated successfully by allogeneic BMT.

The use of DNA-based PCR-SSOP genotyping of HLA class I and class II alleles for marrow donor selection is a novel feature of the case described here. DNA-based PCR-SSOP HLA typing provided three distinct advantages. First, the method can be applied readily to nonhematopoietic cells as well as PBLs. This permits accurate determination of the patient's true HLA type from fibroblasts or epithelial cells when the circulating PBLs do not originate from the patient, as in TAGVHD. Second, the DNA-based PCR-SSOP typing method is less affected by low cell numbers, decreased cell viability, and activation of lymphocytes in the sample, factors which contribute to inconsistency in results and high back-
ground reactivity in microlymphocytotoxicity assays. In TAGVHD and other disease processes which may yield suboptimal specimens, DNA-based PCR-SSOP typing may be more reliable than serologic methods. Third, the DNA-based PCR-SSOP HLA typing method can define minor subpopulations of lymphocytes in a mixed specimen, as illustrated by the typing of the patient's PBLs before and after transplantation, in which the typing demonstrated chimeric engraftment and eradication of blood donor cells. The technical strengths of the DNA-based PCR-SSOP HLA typing method, which were of great value in the present study, may also prove to be advantageous in forensic, archival, and tissue typing applications.

When no HLA-identical sibling is available, patients with SCID may be offered BMT from parents or siblings who are, in general, haploidentical with the patient. In the case described here, the fact that the HLA type of the patient could not be determined serologically and the fact that the parents shared HLA class I antigens suggested that the father should serve as the bone marrow donor (Fig. 2). However, DNA-based HLA typing of the child's nonhematopoietic cells provided unique information which demonstrated that the mother could provide donors as closely matched as that of the father. In the situation in which the mother and the father are equally well HLA matched with the child and no other consideration favors one over the other, the mother is usually selected as the bone marrow donor for her infant with SCID. The mother is chosen because maternal lymphocytes are frequently already present in the circulations of infants with SCID (8, 22, 30) and often do not cause GVHD. Thus, a maternal graft may be more likely to become tolerant and less likely to cause GVHD after the transplant. In the case presented here, information from DNA-based HLA typing permitted the choice of the mother as the bone marrow donor.

The possibility that the mother is not HLA homozygous but has undetected blank alleles at one or more HLA loci was not resolved in the present study because the choice of amplification primers and hybridization probes for the DNA-based class I typing of the child was limited to those which would distinguish the two possible paternal alleles. Putative blank alleles inherited from the mother would not have been identified. However, this limited approach provided sufficient information to us so that we could choose the marrow donor by showing that the paternal alleles A11 and B46 were not inherited. The issue of blank alleles in the mother could be resolved by performing complete DNA-based HLA A and B typing on the maternal specimen, but this result would not have affected the choice of bone marrow donor.

Parental homozygosity for HLA alleles and haplotypes and parental sharing of HLA alleles, as found in the family described here, have been reported previously in families with infants with SCID (8, 13, 17, 20, 22, 30). There is also a higher than expected rate of HLA compatibility for BMT in families of patients with SCID (10). The significance of these observations in the pathogenesis of SCID is not known. Typically, bone marrow as donor material is selected by showing that the maternal alleles A11 and B46 were not inherited. The issue of blank alleles in the mother could be resolved by performing complete DNA-based HLA A and B typing on the maternal specimen, but this result would not have affected the choice of bone marrow donor.

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