MODULATION OF THE T CELL COMPARTMENT BY BLOOD TRANSFUSION

EFFECT ON CYTOTOXIC AND HELPER T LYMPHOCYTE PRECURSOR FREQUENCIES AND T CELL RECEPTOR Vß USAGE

BARBARA J. VAN DER MAST, HENK E. VIÊTOR, ELLEN M.W. VAN DER MEER-PRINS, SIMONE P.M.J. VAN BREE, ANNEKE BRAND, PETER J. VAN DEN ELSN, FRAN H.J. CLAAS

Departments of Immunohematology and Blood Bank, and Obstetrics, University Hospital Leiden, 2300 RC Leiden, The Netherlands

Recent data suggest that the favorable effect of pretransplant blood transfusion (BT) on transplant outcome depends on the HLA match. HLA-DR or haplotype shared transfusions lead to transplantation tolerance, and HLA-mismatched BT leads to immunization. The immunological mechanism involved is still unknown. To investigate the effect of HLA compatibility between blood donor and recipient on the T cell compartment, we determined the frequency of cytotoxic and helper T cell precursors specific for blood donor cells (n=20) and the T cell receptor Vß (TCRBV) repertoire of the CD4- and CD8-positive peripheral blood mononuclear cells before, at 2 weeks after, and at more than 10 weeks after BT (n=10). Patients who received one transfusion of a nonstored (<24 hr after withdrawal) erythrocyte concentrate without buffy coat containing on average 6x10⁶ leukocytes. Eight patients shared an HLA-B and -DR antigen, nine patients shared one HLA-DR antigen, and three patients shared no HLA class II antigens with the blood donor. All patients showed a significant increase in both cytotoxic and helper T cell precursor frequencies against the blood donor 2 weeks after BT. In most patients, the frequencies reached pretransfusion levels again long after BT. In 5 of 10 patients, an expansion of one or more TCRBV families was observed in either the CD4 or CD8 compartment.

This study demonstrates that BT, irrespective of the degree of HLA matching, induces activation of the T cell compartment. The degree of sharing of HLA antigens was not correlated with quantitative changes in cytotoxic T lymphocyte precursor or helper T lymphocyte precursor frequencies, or changes induced in the TCRBV repertoire. Cytotoxic and helper T lymphocyte precursor frequencies and TCRBV repertoire determined after BT do not give an indication for a state of tolerance prior to transplantation.

Induction of donor-specific tolerance is the major goal in organ transplantation. In humans, there is evidence that the administration of blood has a favorable effect on graft survival (1). Although the immunological mechanisms involved are still unknown, it is clear that many factors may influence this effect. The presence of viable leukocytes in the transfused blood seems essential, as infusion of filtered, leukocyte-depleted blood was ineffective in enhancing kidney graft survival (2). However, the number of leukocytes needed, their specific characteristics, time of storage of the transfused blood, the recipient's immune status, HLA matching, the optimal number of transfusions, and the timing of the transfusions in relation to transplantation have yet to be assessed.

Several studies have indicated that the beneficial effect of a pretransplant blood transfusion may be diminished during the last decade. Improved patient care, introduction of cyclosporine, and better rejection diagnosis and treatments have resulted in better graft survival in patients who do not receive transfusions (3-5). However, other studies still show a transfusion effect even in the cyclosporine era (6). The presence of viable leukocytes in the blood donor might be an important factor.

Studies in animal models and in man have led to several hypotheses concerning the mechanism of the BT effect, including clonal deletion of donor-reactive cells (7), induction of suppressor cells (8), veto cells (9), and CD4+ regulatory cells (10), and the formation of anti-idiotypic antibodies (11). The induction of permanent mixed chimerism has been suggested to be essential for induction of transplantation tolerance (12, 13). In the majority of mechanisms proposed, T cells play an important role.

Recent studies have shown that the degree of HLA matching between transfusion donor and recipient plays a pivotal role in the immunomodulating effect of BTs. BT with a shared HLA-DR antigen results in better graft survival and a decreased number of rejection episodes in kidney and heart allograft recipients compared with HLA-DR-mismatched BT (14-16). Furthermore, a one-HLA-DR-antigen-shared BT has a decreased risk of inducing alloantibodies compared with a two-HLA-DR-antigen-mismatched BT (14). In vitro studies have shown that nonresponsiveness of cytotoxic T lymphocytes (CTLs) against the blood donor occurs in pa-
MATERIALS AND METHODS

Patients. Twenty-one patients waiting for a kidney transplantation received transfusions between 1989 and 1995 according to a study protocol to improve graft survival. None of these patients had a BT history. Women with previous pregnancies were excluded from this study. All patients received 1 U of nonstored (<24 hr after withdrawal) erythrocyte concentrate, the buffy coat of which was removed. The transfusate contained an average of 6 x 10^6 leukocytes.

Heparinized blood samples from the patient were taken before, 2 weeks after, and at a time varying between 10 weeks and 58 months after transfusion. All samples were taken before transplantation. Blood donor peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat. At the time of transfusion, the patients did not receive immunosuppressive therapy. Patients, blood donors, and healthy (third-party) volunteers were HLA-typed for class I and class II antigens by conventional serological methods, based on complement-dependent cytotoxicity. HLA typing of patients and blood donors and timing of the third blood sample are shown in Table 1.

Isolation and storage of lymphocytes. PBMC were isolated using a Ficoll-amidrotiazol density gradient centrifugation procedure. Heparinized blood was diluted 1:1 with Hanks' balanced salt solution (Gibco, Paisley, UK), layered on the gradient, and centrifuged for 15 min at 600 x g. PBMC isolated from the interface were washed twice with Hanks' balanced salt solution and frozen in RPMI 1640 medium (Gibco) with 20% fetal calf serum (Bodinco, Alkmaar, Holland) and 10% dimethyl sulfoxide (Fluka, Buchs, Switzerland). The cells were frozen at -70°C and then stored in liquid nitrogen until needed.

Limiting dilution assay. The analysis of CTLp frequencies directed against the blood donor was based on the principle of limiting dilution analysis (LDA) (22).Responder lymphocytes (patient PBMC), re-suspended in RPMI 1640 medium with 3 mM L-glutamine supplemented with 10% pooled human serum (referred to as complete medium), were set up in 12 replicate wells at 40,000 cells/well and were diluted twofold (40,000 cells/well down to 625) across the wells of two 96-well U-bottom plates (Costar, Cambridge, MA). Responder cells were excluded from the last 12 wells. Individual sera included in the serum pool and used in the culture medium were tested in a mixed lymphocyte culture assay using three different responder-stimulator combinations. They were excluded from the pool if inhibitory or stimulatory effects on proliferation were present.

Stimulator lymphocytes (blood donor PBMC or third-party PBMC mismatched for HLA class I and II antigens with patient and blood donor) were irradiated with 3,000 rads (137Ca source; Isomedix, Paramus, NJ) and added in complete medium to the responder cells at a concentration of 50,000 cells/well. Responder and stimulator cells were cultured in the presence of 20 U/ml human interleukin (IL)-2 (Cetus, Amsterdam, The Netherlands) for 7 days. After 5 days of culture, half the medium was changed for fresh complete medium containing rIL-2 at 20 U/ml. Lymphocytes to be used as target cells were set up at the beginning of the culture period in complete medium containing 20 U/ml rIL-2 and 2 µg/ml purified phytohemagglutinin (Welcome, Dartford, UK) at a cell density of 1 x 10^6 cells/ml/well in a 24-well plate (Costar). Target cells were refreshed on days 3 and 6 with complete medium containing 20 U/ml of rIL-2. After 7 days of culturing, target cells were labeled with europium-DTPA, as described previously (23). Targets of the stimulator type were added at a concentration of 5,000 cells/well to each responder-stimulator combination that had been split into two identical portions, one of which was incubated for 1 hr at room temperature with an anti-CD8 monoclonal antibody (24) to study the effect of CD8 molecule blockade on CTLp frequencies (25, 26). The plates were then incubated for 4 hr at 37°C in 5% CO2. After centrifugation for 5 min at 600 x g, 20 µl of supernatant of each well were harvested and transferred to 96-well flat-bottom plates with a low background fluorescence (Fluoroomunoplate, Nunc, Denmark). Additionally, 200 µl of enhancement solution (Wallac, Turku, Finland) were added to each well. Fluorescence of the released europium was measured in a time-resolved fluorometer (Arcus 1234, Wallac). As a control for every target cell, spontaneous lysis (target cells + complete medium) and maximal lysis (target cells + 1% Triton X-100; Fluka) was determined. The CTLp frequencies of different bleeding dates from each individual patient were determined in the same experiment.

HTLP/CTLp combination test. In four patients, both HTLP and CTLp frequencies were determined in a single assay (27). Responder cells were set up in 24 wells at 20,000 cells/well and diluted twofold across the wells of two 96-well V-bottom plates (Greiner, Apen aan de Rijn, The Netherlands). Twenty-five thousand stimulator cells, irradiated with 5,000 rads, were added per well. After 5 days of culture at 37°C in 5% CO2, supernatant was harvested and transferred to U-bottom 96-well plates (Costar). The supernatants were stored at −20°C until needed. The remaining cells were transferred to U-bottom plates (Costar) and were cultured for another 7 days at 37°C in 5% CO2 in the presence of 20 U/ml rIL-2. At day 7, half the medium was replaced with fresh complete medium containing rIL-2 at 20 U/ml. Cell-mediated lymphotoysis was performed as described above. To determine the amount of IL-2 present in the supernatant, we used the IL-2-dependent murine CTLL-2 cell line. This CTLL-2 cell line was kept in culture continuously in RPMI 1640 medium supplemented with 3 mM L-glutamine and 10% fetal calf serum (Bodinco) and 10 U/ml rIL-2. The assay was performed as described by Schanz et al. (28). In brief, the supernatants were thawed and CTLL-2 cells that had been put in IL-2-free medium for 20 to 24 hr were added at 3,000 cells/well to the plates. After incubation for 3 days at 37°C in 5% CO2, lysing-staining-quenching medium containing Triton X-100 (Fluka), ink (Leitz, Wetzlar, Germany), and propidium iodide (Sigma, St. Louis, MO) in EDTA buffer was added. The plates were then read with an automated fluorescence microscope (Leica-Patimed, Wetzlar, Germany), which measures photometer values (mV) to determine the number of propidium-iodine-stained nucleated cells and thus proliferation of the CTLL-2 cells. Since proliferation of the CTLL-2 cells is dependent on the amount of IL-2 present in the supernatants, this LDA can be used as a measure of the number of IL-2-secreting cells present in the responder cell fraction. Since IL-2 production is dependent on the IL-2-secreting cells that are present in the responder cell fraction. As mainly T helper cells are considered responsible for IL-2 production (29, 30), the HTLP frequency can be estimated.

Analysis of TCRBV usage. In 10 patients, the TCRBV gene usage was determined before and after BT.

After thawing, PBMC were stained with anti-CD4 (fluorescein isothiocyanate-conjugated) and anti-CD8 (phycoerythrin-conjugated) monoclonal antibodies (Becton Dickinson, Mountain View,
Table 1. HLA typing of patients and blood donors

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<th>HLA-B</th>
<th>HLA-DR</th>
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* HLA typing was based on serological typing methods.
* >10 wk = timepoint long after BT.
* HLA-shared antigens between patient and blood donor are underlined.
**RESULTS**

**CTLp frequencies.** CTLp frequencies were determined before and at two time points after BT. Patients were divided into three groups: (1) patients who received an HLA class II-mismatched (nonshared) transfusion (n=3); (2) patients who received a transfusion with one HLA-DR antigen in common with the blood donor (one HLA-DR shared) and mismatched for HLA class I antigens (n=9); and (3) patients who received a transfusion with at least one HLA-B and one HLA-DR antigen in common with the blood donor (HLA-B+DR shared, n=8). Matching of patient and blood donor for HLA class I was based on serologically defined antigens, and for class II on HLA-DR broad antigens. CTLp frequencies against blood donor and third-party cells for the different patient groups are shown in Figures 1, 2, and 3. In group 1, an increase in CTLp frequencies against the blood donor was seen 2 weeks after BT (Fig. 1A). In two of three patients, the CTLp frequency subsequently decreased after an interval of more than 10 weeks after transfusion. In one patient (Dur), the frequency remained high (215/10^6 at 2 weeks, 235/10^6 at 10 weeks). Against third-party cells, no dramatic changes were seen in this group (Fig. 1B). In group 2 (one-HLA-DR-shared BT), a significant increase in CTLp frequencies was observed against blood donor cells 2 weeks after BT (P=0.0039). These frequencies decreased to pretransfusion levels long after BT (Fig. 2A). Only in patient Ar did the frequency decrease to a level lower than the one observed before transfusion. This decrease was specific for blood donor cells, since the frequency against third-party cells did not decrease in this patient. Against third-party cells, no dramatic changes were seen in this group (Fig. 2B). In group 3 (at least one-HLA-B+DR-shared BT), a significant increase in CTLp frequencies against blood donor cells was also observed 2 weeks after BT (P=0.0156). Long after BT, the frequencies almost decreased to pretransfusion levels (Fig. 3A). Against third-party cells, no dramatic changes were seen in this group (Fig. 3B). In conclusion, in the total group of patients, the CTLp frequencies never reached levels below the pretransfusion values (with the exception of patient Ar in group 2), irrespective of HLA disparity. However, the range of the >10-week samples was highly variable, which might explain why we could not detect the donor-specific CTLp nonresponsiveness observed by others after a HLA-B+DR-matched BT (17). To test whether CTLp nonresponsiveness is a time-dependent phenomenon, the CTLp frequencies of all patients against the BT donor tested more than 10 weeks after BT were grouped according to the sampling time after BT (Fig. 4). No significant down-regulation of the donor-specific CTLp could be observed in one of these different time periods. Furthermore, no differences were observed between the CTLp frequencies measured in these separate time intervals.

**Inhibition with anti-CD8 antibodies.** To study the effect of CD8 molecule blockade on CTLp frequencies, each responder-stimulator combination was incubated with a noncytotoxic anti-CD8 monoclonal antibody 1 hr before target cells were added. In the total group of patients, the patterns of (anti-CD8 resistant) CTLp frequencies were similar, as described above (Fig. 5). A significant increase in frequencies of anti-CD8-resistant CTLp was seen 2 weeks after BT (P<0.0001). No differences were observed among the three groups of patients. When percentage of inhibition of CTLp by anti-CD8 was calculated long after BT, lysis of blood donor-derived target cells could be less inhibited with anti-CD8 than lysis of third-party target cells (mean inhibition: 43% vs. 70%, P=0.0297).
HTLp frequencies. In four patients from group 2, who had received an HLA-DR-antigen-shared BT, the HTLp frequency was investigated before, 2 weeks after, and >10 weeks after BT (Fig. 6, A and B). In three of four patients, BT resulted in an increase of HTLp frequency against the blood donor 2 weeks after BT, which confirms earlier data obtained in 10 other patients (33). In contrast, such an increase was not observed against third-party cells. No correlation between changes in CTLp and HTLp frequencies was observed in these patients.

Analysis of TCRBV gene usage. Analysis of the TCRBV repertoire of CD4+ and CD8+ T cells revealed that in 5 of 10 patients the overall repertoire remained stable after BT. However, in the remaining five patients a modulation of the TCRBV gene usage was observed in either the CD4 or CD8 population ($R^2<0.75$), whereas the other subset remained relatively unaffected (Table 2). Changes in the repertoire of the CD4 or CD8 subset are displayed in Figure 6. In these subsets, BT resulted in expansions or contractions of one or more distinct TCRBV gene families. These changes seemed patient specific, and general patterns with respect to these

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**Figure 2.** CTLp frequencies (CTLpf) of patients (n=9) who received an HLA-DR-shared BT measured before, 2 weeks after, and long after BT against blood transfusion donor (BTD) cells (A) or against third-party cells (B). *P=0.0039.

**Figure 3.** CTLp frequencies (CTLpf) of patients (n=8) who received a BT that shared at least one HLA-B and one HLA-DR antigen measured before, 2 weeks after, and long after BT against blood transfusion donor (BTD) cells (A) or against third-party cells (B). *P=0.0156.

**Figure 4.** CTLp frequencies (CTLpf) of all patients against blood transfusion donor (BTD) cells grouped according to sampling time. In every column, the mean frequency is depicted with a horizontal line.
alterations were not observed. In some patients, the effect was limited to a few families (Fig. 7, A and D), whereas in other cases (Fig. 7, B, C, and E) the BT had a broader effect on the TCRBV repertoire. This is exemplified by patient Ve, who showed an increase in TCRBV22 from 5% before BT to more than 45% in the CD8 subset after receiving a one-DR-matched BT (Fig. 6A). When TCRBV22 was not included in the analysis of changes in the overall TCRBV repertoire, the R² increased from 0.035 to 0.831, which suggests that these changes were mainly the result of an (oligo)clonal expansion of this gene family. This effect was still present, but less pronounced, at 41 months after BT. At the same time, a contraction of TCRBV2 occurred in this subset. Almost similar results were observed in patient Op, in whom 2 weeks after receiving a B+DR shared transfusion more than 90% of the TCRBV repertoire of CD4+ PBMC consisted of TCRBV8 (Fig. 7D). After 39 months, the usage frequency of this T cell receptor gene family decreased below pretransfusion values. Fewer restricted changes of the TCRBV repertoire were observed in the other three patients, in whom BT resulted in an increase in the usage of certain TCRBV families, or a decrease in the usage of other TCRBV families (Figs. 7, B, C, and E). The contraction of several TCRBV families was not just the result of an expansion of other TCRBV, since the relative decrease of other TCRBV families was not distributed equally among the nonexpanding TCRBV families. Furthermore, no correlation was observed between the degree of HLA sharing of the BT and the effect on TCRBV gene usage. In general, the impact of BT on the composition of the TCRBV repertoire of the CD4 and CD8 subset of T cells was independent of HLA sharing and exhibited an individual-specific character.

**DISCUSSION**

This study was performed to analyze possible mechanisms involved in the beneficial effect of BT on transplant survival. Because T cells are likely to play an important role in almost all immunoregulatory mechanisms proposed and are responsible for graft rejection, we investigated the T cell compartment in patients who received a pretransplant BT. The results of our study show that, in the total group of patients, CTLp frequencies against the blood donor initially increase
after BT (34) and eventually almost always reach (except for one patient in group 2) pretransfusion levels again long after BT (median, 19 months). The patterns of HTLp frequencies were comparable to those observed in CTLp frequencies. Two weeks after BT, increased levels of HTLp frequencies were found in three of four patients, confirming earlier data obtained from another group of patients (33). An increased number of anti-CD8-resistant CTLp against the blood donor were found long after BT, indicating that BT leads to high-avidity CTLs directed against antigens of the blood donor.

In contrast to previous studies (17), we were not able to show any T cell nonresponsiveness against the donor, neither after HLA-B+DR-shared BT nor after HLA-DR-shared BT. Our results are in concordance with those of van Prooijen et al. (35), who reported that in 14 patients a single transfusion of HLA haplotype-matched, buffy coat-depleted blood generally did not induce donor-specific down-regulation. After transfusion of HLA haplotype-matched whole blood, no donor-specific down-regulation was seen at all (H.C. van Prooijen, Department of Immunohaematology, University Hospital Utrecht, The Netherlands, personal communication, 1996). Different composition and storage conditions of blood products may account for the discrepancy between the results. Fresh blood containing viable leukocytes is necessary for the immunosuppressive effect in vivo (37) or in vitro (38). Characterization of blood products before administration is essential to monitor the immunological effects of BT in transplantation candidates.

In 5 of 10 patients, an expansion or contraction of one or more TCRBV families was observed after BT. These changes were limited in four cases to the CD8 T cell compartment and in one case to the CD4 T cell compartment. In the other five patients, the TCRBV repertoire remained stable after BT. In most cases, the alterations concerned a limited number of TCRBV gene families and displayed an individual-specific pattern. No correlation was seen between the degree of HLA sharing and the CTLp or HTLp frequencies or in changes induced on TCRBV repertoire.

Our data do not confirm those of Munson et al. (19), who observed deletions in TCRBV families in patients who received an HLA haplotype-shared BT, whereas a mismatched transfusion did not induce such deletions. Also, others have studied the effect of allore cognition on the T cell receptor gene (39). Mixed lymphocyte cultures revealed a restricted but heterogeneous TCRBV gene usage of alloreactive T cells (40-43). Carlquist et al. (44) demonstrated quantitative change of the TCRBV repertoire in PBMC of cardiac recipients just before rejection crisis. In healthy individuals who had not received transfusions, the TCRBV repertoire remained stable over a period of 21 months (45). However, in vivo expansions of TCRBV families can be observed in healthy young individuals and might be the result of exposure to environmental antigens (46, 47). Since the observed changes of one or more TCRBV families appeared within 2 weeks after BT, they were most likely induced by the transfusion and probably not a result of exposure to environmental antigens. In one patient (patient Op), changes in the TCRBV repertoire mimicked a response seen after exposure to superantigens (48). The expansion of TCRBV8 in CD4+ cells to more than 85% of the total repertoire observed 2 weeks after BT and a subsequent decrease below pretransfusion values are in line with this reasoning. This expansion could also represent proliferation of CD4+ cells that have a down-regulating effect on donor-reactive cells in a subsequent transplanted organ (10, 49). The question of whether the expanded TCRBV families specifically concern alloreactive T cells directed against donor antigen(s) is a subject of future studies.

The present study strongly suggests that BT results in activation of the recipient T cell compartment against donor antigens rather than in induction of T cell tolerance. It is unlikely that this activation of the T cell repertoire was due to natural variations in time, since it was shown that precursor frequencies to alloantigens do not alter significantly within normal individuals over several months (50). Our findings support the view of Terasaki (7) that BTs always immunize the recipient and do not induce tolerance. The transplant could act as a second stimulus, and in the pres-

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA sharing</th>
<th>Time after BT</th>
<th>CD4</th>
<th>CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ma</td>
<td>Mismatched</td>
<td>2 wk</td>
<td>0.882</td>
<td>0.897</td>
</tr>
<tr>
<td>5 Bu</td>
<td>1DR</td>
<td>6 mo</td>
<td>0.885</td>
<td>0.735</td>
</tr>
<tr>
<td>6 Sl</td>
<td>1DR</td>
<td>4 mo</td>
<td>0.902</td>
<td>0.899</td>
</tr>
<tr>
<td>8 Ka</td>
<td>1DR</td>
<td>3 mo</td>
<td>0.936</td>
<td>0.933</td>
</tr>
<tr>
<td>9 Ve</td>
<td>1DR</td>
<td>2 wk</td>
<td>0.920</td>
<td>0.941</td>
</tr>
<tr>
<td>13 Ra</td>
<td>1A 1B 1DR</td>
<td>46 mo</td>
<td>0.905</td>
<td>0.617</td>
</tr>
<tr>
<td>14 Ko</td>
<td>1B 1DR</td>
<td>41 mo</td>
<td>0.910</td>
<td>0.045</td>
</tr>
<tr>
<td>18 Du</td>
<td>1A 1B 1DR</td>
<td>2 wk</td>
<td>0.857</td>
<td>0.829</td>
</tr>
<tr>
<td>20 Op</td>
<td>1A 1B 1DR</td>
<td>13 mo</td>
<td>0.886</td>
<td>NT b</td>
</tr>
<tr>
<td>21 Vo</td>
<td>2A 1B 2DR</td>
<td>2 wk</td>
<td>0.927</td>
<td>0.949</td>
</tr>
</tbody>
</table>

a Patient number corresponds to those used in Table 1.
b NT, not tested.
Figure 7. The TCRBV gene usage in CD4 or CD8 cells in patients who revealed an $R^2 < 0.75$ after BT. Frequencies of individual TCRBV families are given as a percentage of the total TCRBV gene usage. The expansion of some TCRBV families resulted in a relative decrease in usage of the other families.
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