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Effacing of the T Cell Compartment by Cardiac Transplantation in Infancy

Brenda M. Ogle,*† Lori J. West,** David J. Driscoll,‡ Scott E. Strome,†† Raymund R. Razonable,*‡ Marilia Cascalho,*‡# and Jeffrey L. Platt‡*†#

For cardiac transplantation in infants, T cells are depleted and the thymus is removed. These manipulations should cause profound defects in the T cell compartment. To test this concept, 20 subjects who underwent cardiac transplantation in infancy were compared to 20 healthy age-matched subjects. The number of T cells in the blood was nearly normal in all subjects 1–10 years after surgery. However, newly generated T cells were undetectable in 10 recipients and 10-fold less than controls in 10, suggesting absence of thymic function. TCRβ chain diversity, measured by a novel technique, was ~100-fold lower than controls. T cell function, deduced from levels of human herpesvirus 7 and response to hepatitis B immunization, were notably impaired. Yet cardiac transplant recipients were generally free of opportunistic infections. Our findings demonstrate a novel approach to measuring lymphocyte diversity and suggest that understanding how these subjects resist infection could yield important insights into immune fitness.


Each year, ~80 infants <1 year of age undergo cardiac transplantation to correct the devastating physiology of congenital heart disease or cardiomyopathy (1). These infants are usually treated with Tymoglobulin or anti-CD3 Abs to deplete mature T cells to avert rejection, and their thymus is removed, all or in part, to facilitate cannulation of the great vessels (2–4). Although these manipulations performed separately, in conjunction with organ transplantation or nontransplantation cardiac surgery are usually innocuous, the depletion of mature T cells and removal of the thymus together should have a profound impact on the structure of the T cell compartment and on cell-mediated immunity.

Removal of the thymus in infancy (without depleting mature T cells) causes modest defects in the structure of the T cell compartment and has little or no impact on the well-being of human subjects. Rubinstein et al. (5) studied four children who underwent thymectomy in the first year of life for a suspected malignancy of the thymus. Two had a significant reduction of peripheral blood lymphocytes and three responded inadequately to delayed-type hypersensitivity tests. However, the four subjects suffered no apparent increase in the rate or severity of infections as long as 14 years after the surgery. Brearley et al. (6) studied 18 infants who underwent thymectomy in conjunction with cardiac surgery before the third month of life. Nine to 36 mo later, the infants had decreased numbers of T cell subsets (CD4+ and CD8+) in the blood and impaired responses to Con A and PHA, but showed no obvious clinical consequences of thymectomy. Wells et al. (7) studied 25 infants who underwent thymectomy in the course of cardiac surgery in the first 3 mo of life. One year after the procedure, the subjects had decreased proportions of T cells (CD3+) and of CD4+ T cells in the blood. However, the T cells of these subjects exhibited normal responses to Con A and PHA, and none of the subjects had required hospitalization for infections and none even experienced increased numbers of minor infections. Eysteinsdottir et al. (8) studied 19 children who underwent nontransplant cardiac surgery in the first months of life. Five to 16 years later, these subjects had a small decrease in the number of T cells (CD3-) and CD4+ T cells in the blood but no defects in proliferative responses to tetanus toxoid or PHA. None of the subjects had required hospitalization for infection. The lack of clinical consequences is not because the thymus regenerates, as no evidence of regeneration has been observed (7); rather, those who undergo thymectomy in the newborn period remain well because the T cell repertoire of humans is normal at birth and these T cells proliferate as needed to maintain the full size of the compartment (9–11).

Depletion of mature T cells has a profound impact on cell-mediated immunity (which is why depletion is performed in conjunction with transplantation). Chakrabarti et al. (12) found that subjects depleted of T cells before stem cell transplantation develop twice as many adenosivirus infections as subjects who were not depleted, and the incidence of adenosivirus infection was inversely related to the rate of recovery of T cells (12). Recovery of T cells depends on the size of the thymus. Mackall et al. (13) found that after T cells are depleted during induction of chemotherapy, the rate of return of CD4+ T cells is an inverse function of age, owing to progressive thymic atrophy (14–16). After depletion, the T cell compartment is restored in young subjects over 18 mo mainly through production of new T cells and in older subjects by proliferation of residual T cells (a process referred to as homeostatic proliferation) (17).

We reasoned that those who undergo both T cell depletion and removal of the thymus for cardiac transplantation in infancy...
should have profound and lasting defects in the T cell compartment and on cell-mediated immunity. Unless the thymus regenerates, these infants should resemble older subjects described above. To test this hypothesis, we studied a series of subjects who had undergone thymectomy and T cell depletion in conjunction with cardiac transplantation in infancy. We asked whether, and to what extent, these subjects exhibit T cell deficiency and whether and how the T cell compartment is restored over time. What we found will have implications for the management of infants with cardiac disease and the usefulness of TCR diversity as a measure of immune fitness.

Materials and Methods

Patient demographics

Twenty children (1–10 years of age; 12 male, 8 female) who underwent cardiac transplantation in the first year of life and who were on maintenance immunosuppression were studied. Eight subjects had congenital cardiomyopathy, eight had hypoplastic left heart syndrome, two had refractory heart failure after previous cardiac surgery, one had right atrial isomerism and one had endocardial fibroelastosis and aortic stenosis. One healthy control subject matched in age with each transplanted subject was studied. Written consent was obtained from the parents of each child, and the study was conducted with the approval of the Mayo Foundation Institutional Review Board and the Research Ethics Board at the Hospital for Sick Children, Toronto. Information on original diagnosis, the operation, the number of postoperative infections, and hospital stay of each subject was retrieved from hospital charts. Subjects with known immunologic disorders before cardiac transplantation were excluded from the study. A peripheral venous blood sample (2–5 ml according to the age of the subject) was collected from all subjects.

Hematological studies

White blood cell counts were determined by Coulter Counter (Coulter) and confirmed using a hemacytometer.

FACS analysis

Leukocytes were isolated from whole blood by Ficoll-Hypaque (Amer sham Biosciences) gradient. The leukocytes were washed with PBS supplemented with 0.3% BSA to block nonspecific binding of Abs. Approximately 100,000 leukocytes were resuspended in 100 μl of PBS/BSA solution to which 2.5 μl of fluorochrome-conjugated mAbs (BD Pharmingen; CD3-FITC, SK-7; CD4-PerCP, SK-3; CD8-allophycocyanin, SK-1; CD45RA-FTC, L48; CD45RO-PE, UCHL-1) were added. The leukocytes were incubated for 30 min at 4°C and then washed twice with the PBS/BSA solution. Fluorescence was measured using a FACScalibur instrument (BD Biosciences) and the data were analyzed using CellQuest software (BD Biosciences).

Measurement of TCR excision circle (TREC)1 DNA in humans

TREC levels were determined by real-time PCR as follows. DNA was isolated from 106 leukocytes using the DNeasy Tissue Kit (Qiagen). Real-time PCR was conducted on 100 ng of DNA with the following primers: 5′-CACATCCCCCTTCAACCATGCTG-3′ and 5′-GCCAGCTGAGGTT TAGG-3′ corresponding to the TCRβ-chain signal joint. Each 20 μl of PCR contained 10 μl of Quantitect SYBR Green PCR Master Mix (Qiagen) and 500 nM of each primer. Reactions were heated to 95°C for 15 min, then 95°C for 15 s, 60°C for 25 s, and 72°C for 25 s for 40 cycles on a LightCycler (Roche). Reactions containing known amounts of TREC molecules were amplified simultaneously and ranged from 101–105 TREC molecules. Samples were also amplified using GAPDH primers (5′-CCCAACACAGTGAGCT-3′ and 5′-CTAGTCCAGGCTTGA ATT-3′) to ensure a consistent amount and integrity of DNA. Melting curve analysis was conducted on all products to ensure a single product of appropriate size.

Measurement of T cell repertoire diversity by a gene chip method

The diversity of T cells was measured using gene chips as we recently described (18). To measure the repertoire of peripheral blood T cells, total RNA was isolated from the leukocytes in 1.5–5.0 ml of whole blood using the Qiagen RNeasy kit (Qiagen). First-strand cDNA was generated using a primer designed to hybridize to the constant region of the human TCRβ gene plus the T7 polymerase promoter and SuperScript II Reverse Transcriptase (Invitrogen) 5′-GGCCAGTGAATTTGATACGACTCAC CATAGGGAGGCCGGCTGCTCTTTGAGGGGCTGC3′. Second-strand synthesis was generated via nick translation. The double-stranded cdNA product was biotinylated with the BioArray High Yield RNA Transcript Labeling Kit (Enzo Life Sciences). The in vitro transcription product (cRNA) was purified using RNeasy spin columns (Qiagen). The purified product was assayed by spectrophotometry, resuspended at 1 μg/μl in nuclease-free water and then fragmented to 50- to 200-bp sizes to facilitate binding to the chip. Equal amounts of cRNA (5 μg) from different samples were hybridized to U133B Affymetrix gene chips (Affymetrix). Each experimental sample yielded intensity data at specific probe locations on a gene chip. Data were arranged in order of ascending hybridization intensity and the number of locations with intensity above background, i.e., the number of hits, was summed. Samples with known diversity were used to generate a standard curve of hits vs diversity from which samples with unknown diversity could be deduced. Results were expressed as number of different TCRβ per 5 μg of cRNA.

Real-time PCR for human herpesvirus 7 (HHV-7)

DNA was extracted from 200 μl of whole blood using the Isoquick (Roche Applied Science) and 5 μl of the 200-μl extraction volume was used for each PCR. Primers were developed for the HHV-7 amplification corresponding to the structural phosphoprotein in the U10 and U11 genes and are available from Roche Molecular Biochemicals as ASRs referenced as LC HHV-7, primer/hybridization probes; LC HHV-7, template DNA (positive control); and LC HHV-7, recovery template (internal amplification control). Each 20 μl of PCR contained 15 μl of amplification master mix containing the primers and probes, 0.03 μl/μl platinum Taq, 0.2 nM dNTP, 0.2% uracil-DNA glycosylase, and 4 nM MgCl2. Reactions were heated to 95°C for 180 s, then 95°C for 1 s, 60°C for 12 s, and 72°C for 12 s for 45 cycles on a LightCycler (Roche). Standards containing 101–106 HHV-7 molecules were reacted simultaneously; the assay could detect as few as 10 copies of HHV-7 DNA (19).

ELISA for hepatitis B Ab

Subjects were vaccinated with the Recombivax H-B-Vax II (Merck). The levels of anti-hepatitis surface Ag in serum of subjects were quantified using an Abbott quantitation panel (AUSBAB Quantitation Panel; Abbott Laboratories). A positive control and a standard for sensitivity and specificity were derived from analysis of age-matched healthy children.

Statistical methods

For comparison of normally distributed variables between groups, two-sided t test and one-way ANOVA were used. Nonparametric methods were used to compare groups when the distribution of the dependent variable was not normal (Wilcoxon rank-sum test and Kruskal-Wallis test). To estimate the degree of association between two variables, Pearson product moment correlation, simple linear regression, and χ2 (Pearson’s) tests were used. Data were analyzed with JMP 5.0.1 for Windows (SAS Institute).

Results

Dimensions of the T cell compartment in infants with cardiac transplants

To determine whether, and to which extent, T cell depletion and thymectomy performed with cardiac transplantation in infancy change the number of T cells in the blood, peripheral blood leukocytes were studied by flow cytometry for T cell-specific surface Abs (Fig. 1a and Table I). Most cardiac transplant recipients had normal numbers of CD3+ cells in the blood (0.2–3.7 × 109 cells/ml). 1.0 ± 0.8 × 106 cells/ml and none had lymphopenia. Most cardiac transplant recipients also had normal numbers of CD4+ T cells (range, 0.1–2.3 × 109 cells/ml; 0.8 ± 0.6 × 106 cells/ml) and CD8+ T cells (0.1–1.8 × 109 cells/ml; 0.7 ± 0.5 × 106 cells/ml). Because treatment with thymoglobulin depletes 99% and treatment with anti-CD3 depletes 90% of T cells (20), the nearly normal numbers of T cells in subjects with cardiac transplants suggests that the compartment had been largely replenished.

1 Abbreviations used in this paper: TREC, TCR excision circle; HHV-7, human herpesvirus 7.
functions; solid bar, normal range.

normal or nearly normal numbers of T cells 

had normal numbers of white blood cells 

recipients 

and cardiac transplant recipients studied was nearly 10-fold lower than that of age-

100 ng of DNA). The average level of TREC of all cardiac trans-

tion in infancy had profoundly decreased levels of TREC, below 

the lower limit of detection of the assay (<10 copies of TREC per 

100 ng of DNA). The average level of TREC of all cardiac trans-

plant recipients studied was nearly 10-fold lower than that of age-

matched controls (310 ± 520 TREC per 100 ng DNA and 2500 ± 1700 TREC per 100 ng DNA, respectively; \( p < 0.001 \)) (Fig. 2a and Table I). Thus, some subjects with cardiac transplantation had 

no evidence of thymic function, and the T cell compartment must 

have been restored by proliferation of residual T cells.

T cell diversity in cardiac transplant recipients

To the extent that the T cell compartment of recipients of cardiac transplants is restored by homeostatic proliferation, rather than production of new T cells, the diversity of those T cells should be contracted. To test that possibility, we measured the diversity of TCR\( \beta \) genes in the peripheral blood using a method we developed to determine genomic diversity directly (18). Using a sequence homologous to CDRs, we previously generated random point mutations to yield \( 10^5 \)–\( 10^9 \) sequences. After hybridization to gene chips, the natural log of the number of hits varied linearly 

with the natural log of the theoretical diversity of the synthesized sequences (18). Based on this, we used oligonucleotide samples generated from CDR and containing 5 and 15 point mutations (corresponding to diversity of \( 10^5 \) and \( 10^9 \), respectively) as standards for each analysis. The diversity of TCR\( \beta \) genes in the blood of subjects who had cardiac transplantation in infancy was contract

ed by 100-fold, compared with age-matched controls (\( 5.7 \times 10^4 \pm 1.1 \times 10^4, 5.9 \times 10^5 \pm 8.4 \times 10^6 \), respectively; \( p < 0.001 \)) (Fig. 2, b and c; Table I). Moreover, the diversity of TCR \( \alpha \) genes correlated with the level of TREC in the blood (\( r^2 = 0.76; r = 0.31 \); Fig. 2d).

The correlation of TCR\( \beta \) diversity with levels of TREC in these unique subjects validates this novel approach to measuring lymphocyte receptor diversity. The results also show in two independent tests that thymic function is deficient in those who undergo cardiac transplantation in infancy; indeed, most recipients of cardiac transplants with detectable levels of TREC had decreased TCR\( \beta \) diversity. As an additional control, we studied two subjects with nontransplant cardiac surgery in infancy. These subjects underwent thymectomy in the course of surgery and suffered some level of T cell depletion as a consequence of cardiopulmonary bypass (25, 26), but were not treated with T cell-depleting agents or immunosuppression. One subject, studied 2 mo after surgery had normal TCR\( \beta \) diversity (\( 4.1 \times 10^4 \)) and normal levels of TREC (1925 TREC per 100 ng DNA); however, the other subject, studied 1 year after surgery, had decreased TCR\( \beta \) diversity (\( 1.7 \times 10^5 \)) and decreased levels of TREC (177 TREC per 100 ng DNA) (Fig. 2d). These controls, although few, show that surgical manipulations, and not ongoing immunosuppression, determined defects in thymic function.

Functions of the T cell compartment

The function of the T cell compartment and hence the competence of cell-mediated immunity is thought to depend, to a large extent, on the availability of a diverse repertoire of T cells capable of responding to a range of environmental challenges (27). Mice engineered to express a single TCR \( \alpha \beta \) chain specific for OVA-\( \alpha \pmb{3} \pmb{3} \), thereby reducing diversity to that contributed by the TCR\( \alpha \) chain alone, do not reject allogeneic bone marrow (28). Similarly, subjects with complete DiGeorge syndrome, a disease characterized by thymic aplasia and decreased thymic output of >50% (29–32), and AIDS, a disease characterized by a 10-fold decrease in thymic output (33–36), have striking “distortion” of the T cell repertoire and are highly susceptible to opportunistic infection (30, 37). However, diversity of TCR per se has not been directly measured.

To determine whether contraction of T cell diversity per se impairs protective immunity, we asked whether the level of thymic function predicts control of replication of endogenous viruses and

Thymic function and restoration of the T cell compartment

After depletion, the T cell compartment is thought to be restored by production of new T cells by the thymus and/or by proliferation of residual T cells (21). Because the thymus had been removed in whole or in part in infancy with cardiac transplantation, restoration should reflect expansion of residual T cells. To test this concept, we measured the ratio of CD45R0+ to CD45RA+ T cells in cardiac transplant recipients and age-matched controls. The ratio of CD45R0+ to CD45RA+ was significantly increased in recipients of cardiac transplants, compared with controls consistent with expansion of residual T cells following thymectomy and T cell depletion (\( p < 0.001 \)).

![FIGURE 1.](http://www.jimmunol.org/) White blood cells and T cells in the blood after cardiac transplantation in infancy. The number of white blood cells and T cells of subjects who underwent cardiac transplantation in infancy was studied 1–10 years after the procedure. a, White blood cell and T cell counts in cardiac transplant recipients and age-matched controls. The white blood cell count was determined by Coulter Counter and confirmed by hemacytometer, and the numbers of T cells (CD3+), and T cell subsets (CD4+, CD8+) were determined by FACS. Although T cells had been depleted and the thymus was removed at the time of transplantation, cardiac transplant recipients (C) had normal numbers of white blood cells (\( p = 0.52 \)) and normal or nearly normal numbers of T cells (\( p < 0.001 \)). b, Age-matched controls; solid bar, normal range. b, The ratio of CD45RO+ to CD45RA+ T cells in cardiac transplant recipients and age-matched controls. The ratio of CD45RO+ to CD45RA+ was significantly increased in recipients of cardiac transplants, compared with controls consistent with expansion of residual T cells following thymectomy and T cell depletion (\( p < 0.001 \)).
after transplantation, and who received their final boost concentration of hepatitis B IgG in five subjects first immunized 3). To ascertain T cell-dependent Ab responses, we measured the higher levels of HHV-7 than 3 subjects with thymic function (Fig. 3). To determine whether the defects of function of the T cell compartment impair protective immune responses, we examined the records of subjects with cardiac transplantation in infancy for evidence of opportunistic infection or autoimmune disease seen in complete DiGeorge syndrome and HIV (30, 37, 40–43). Contrary to what might be expected, only 1 of 10 subjects with defective function of the thymus experienced unusual complications (frequent respiratory infections requiring hospitalization). Thus diversity of the peripheral TCR repertoire per se is not needed to prevent opportunistic infections at least over a period of a few years.

**Discussion**

Cardiac transplantation is an effective treatment for infants with severe congenital heart disease or cardiomyopathy. More than 80% of those who undergo cardiac transplantation in infancy survive the first year after transplantation, and the overall half life of survival is 13.3 years (1). These favorable outcomes might be surprising if one considers the impact of cardiac transplantation on the structure of the T cell compartment. We report that those who

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**Table I. Dimensions of the T cell compartment of subjects with cardiac transplantation in infancy and controls**

<table>
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<tr>
<th>Number</th>
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<th>Age at Transplant</th>
<th>Age at Sample</th>
<th>WBC</th>
<th>CD3 × 10⁶/ml</th>
<th>CD4 × 10⁶/ml</th>
<th>CD8 × 10⁶/ml</th>
<th>CD45 RO/RA</th>
<th>Diversity/5 ng cRNA</th>
<th>TREC/100 ng DNA</th>
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<td>5.4</td>
<td>0.7</td>
<td>1.3</td>
<td>0.4</td>
<td>1.9</td>
<td>270</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
<td>Transplant</td>
<td>0.5</td>
<td>3.0</td>
<td>5.5</td>
<td>0.5</td>
<td>0.6</td>
<td>0.2</td>
<td>4.2</td>
<td>520</td>
<td>10</td>
</tr>
</tbody>
</table>

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*Individual subjects are listed by number. Multiple samples from the same subject are demonstrated by lowercase letters. WBC, White blood cells.*

T cell-dependent Ab responses to vaccines. To ascertain the capacity to control viral replication, we measured the levels of HHV-7, which usually infects children during the first 2 years of life (38, 39). Twelve transplant recipients, all receiving immunosuppressive therapy and ranging from 1.5 to 10 years of age, were tested. Six of the 12 had been previously infected with HHV-7; of these, 3 subjects lacking evidence of thymic function had 100-fold higher levels of HHV-7 than 3 subjects with thymic function (Fig. 3). To ascertain T cell-dependent Ab responses, we measured the concentration of hepatitis B IgG in five subjects first immunized after transplantation, and who received their final boost <3 years before sampling. Three subjects, lacking evidence of thymic function, had undetectable or low concentrations of HBs-specific IgG (<1, <1, and 3). Two subjects, with evidence of thymic function, had concentration of HBs-specific IgG within the normal range (9 and 11 mIU/ml). Although only a small number of subjects could be evaluated, the results show that thymic function predicts the capacity to mount protective immune responses in infants who undergo cardiac transplantation ($\chi^2$, 11; $p < 0.01$).
undergo surgical removal of the thymus and T cell depletion in conjunction with cardiac transplantation in infancy have a profound decrease in the diversity of the T cell repertoire. In subjects with no evidence of thymic function T cell diversity may be 10,000-fold less than controls. Subjects lacking thymic function appear less able to control replication of HHV-7 and less able to respond to vaccination than recipients with residual thymic function or normal controls. Although these responses are undoubtedly impaired in part by immunosuppressive therapy, the defect in thymic subjects far exceeds the defect in those with residual thymic function.

Despite changes in the structure and function of the T cell compartment, individuals with cardiac transplantation in infancy are remarkably healthy. The subjects experienced none of the opportunistic infections observed in those with complete DiGeorge syndrome and AIDS (30, 37, 40–43). One explanation for sustained well-being is that unlike individuals with complete DiGeorge syndrome, subjects with cardiac transplantation had normal thymopoiesis during fetal and newborn life and hence a full T cell repertoire before surgery. Those with AIDS have normal thymopoiesis before infection, but presumably must suffer selective loss of critical T cell clones.

Although those who undergo cardiac transplantation in infancy are generally in good health, they are not inured to thymectomy and T cell depletion. It will be interesting to determine whether these subjects suffer from opportunistic infections and manifestations of autoimmunity as they age. Should long-term complications be observed, it may be necessary to reevaluate the value of T cell depletion and thymectomy in conjunction with cardiac transplantation in infancy. Clearly, this group of subjects merits further study and consideration; knowing how they remain healthy, whereas those with complete DiGeorge syndrome or AIDS do not, may provide important insights to the clonal basis of immune competence. Besides characterizing a unique set of human subjects, our findings point to the potential value of the novel approach we used to measure lymphocyte diversity. This direct measure is simpler than spectratyping and not biased by CDR families. Moreover, because the output is a single value for which a normal range can be defined, the assay may be more easily adopted for clinical use.

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References


