Correlation Between Cellular Rejection of Cardiac Allografts and Quantitative Changes Among T-Cell Subsets Identified by Vβ Epitope Expression

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Background  Cellular rejection of an allograft is mediated in part by peripheral blood T cells. We tested the hypothesis that quantitative changes in T-cell subsets can be detected in the peripheral blood and that these changes correlate with rejection.

Methods and Results  T-cell subset analysis was performed by flow cytometry using monoclonal antibodies recognizing six isotypic epitopes of the T-cell receptor β-chain variable (V) region. These analyses were done at 7-day (mean) time intervals. Fluctuations within a given subset were determined by dividing the number of positive cells observed by the number of positive cells found on the previous analysis. For healthy volunteers observed over a period of 30 days, 119 of 120 subset ratios (99.2%) fell between 0.5 and 2.0. For patients, 57 of 240 subset ratios (23.8%) fell outside of this range (P<.004, $\chi^2$). The occurrence of the abnormal ratios coincided more closely with cellular rejection (mean±SD, 7.7±6.2 days from a positive biopsy; median, 5 days; range, 0 to 28 days) than did the occurrence of normal subset ratios (mean±SD, 14.4±10.9 days from a positive biopsy; median, 11 days; range, 0 to 44 days; $P<.005$ by Mann-Whitney $U$ test). Regression analysis confirmed a significant ($P<.001$, $R^2=.91$) temporal association between cellular rejection and abnormal subset fluctuations. No correlation was found between abnormal subset ratios and either vascular rejection or use of high-dose prednisone.

Conclusions  T-cell subset measurement may be a method of noninvasive monitoring of cellular rejection after transplantation and may provide insights into the physiology of graft rejection with the potential for the development of more specific immunosuppressive therapy. (Circulation. 1994;90:686-693.)

Key Words  • transplantation • T cells • rejection

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shown, we examined serial changes in T-cell subsets and found that weekly variation in the levels of expression of these Vβ epitopes was frequent among transplant recipients but not among healthy controls. Furthermore, we attempted to correlate these fluctuations in subset numbers with clinical events in the posttransplantation period.

Methods

Patients

Patients (n=6) were cardiac transplant recipients in the LDS Hospital/U.T.A.H. (Utah Transplant Affiliated Hospitals) Cardiac Transplant Program. Five patients were men, and one was a woman; the age range was 38 to 56 years, and pretransplantation diagnoses included coronary artery disease (n=5) and valvular heart disease (n=1). Recipients were studied for a mean of 45 days (median, 46.8 days; range, 7 to 105 days) after transplantation.

Immunosuppression

Immunosuppressive protocols have been published elsewhere. Briefly, patients received 14-day OKT3 prophylaxis with cyclosporine, azathioprine, and steroids. After OKT3 prophylaxis, a prednisone pulse evaluation of tissue samples for determination of vascular rejection, one fragment of representative myocardium was obtained unfixed and sectioned at 4 μm onto clear glass slides. Tissues were stained with fluorescein-conjugated antibodies to human IgG, IgM, C3, C1q, fibrinogen, albumin, and mouse immunoglobulin (Cappell Laboratories). Positive staining of vessel endothelium for IgG, IgM, complement, and fibrinogen with endothelial swelling was considered evidence of vascular rejection. Vascular rejection was graded from 1+ to 4+ based on immunofluorescence and light microscopic findings.

Cultures of Lymphocytes From Endomyocardial Biopsy Specimens

The use of a portion of a routinely obtained endomyocardial biopsy specimen for research purposes was approved by the institutional review board of the LDS Hospital, and prior written informed consent was obtained from each patient. A sterile fragment of myocardial tissue was obtained at the time of a regularly scheduled diagnostic biopsy and was placed in HBSS with 5% human type AB serum until processing. Tissue fragments were mechanically disrupted and placed into tissue culture in 35-mm Petri dishes containing 2 mL RPMI 1640 tissue culture medium supplemented with 5% human type AB serum, 0.05 mM/L 2-mercaptoethanol, 50 μg/mL streptomycin, 50 U/mL penicillin, 160 U/mL human interleukin-2 (Pharmacia), 24 mM/L sodium bicarbonate, 1 mM/L sodium pyruvate, and 10 mM/L HEPES buffer. Cultures were incubated at 37°C in 5% CO₂ and observed for growth microscopically.

Cultures for Cytomegalovirus

Cultures for cytomegalovirus (CMV) identification were done by the shell vial culture technique without centrifugation. MRC-5 human embryonic lung cells were obtained routinely (Viromed) and passed serially for approximately 8 to 10 passages. Cells were split two times a week (at confluency) and maintained in minimal essential medium with nonessential amino acids, 10 μg/mL gentamicin, 50 μg/mL streptomycin, 50 U/mL penicillin, 4 μg/mL amphotericin B, 15 mM/L HEPES, 9.5 mM/L sodium bicarbonate, and 2% fetal calf serum.

For isolation of CMV, specimens were diluted 1:1 with tissue culture medium, and 0.3 mL was placed into a shell vial containing a coverslip with adherent, freshly cultured (24 to 72 hours) MRC-5 cells. The total volume was adjusted to 2.0 mL with medium, and cultures were incubated at 37°C in 5% CO₂. Cultures were stained at 24 and 48 hours for CMV immediately early and early antigens using an indirect monolocal antibody mixture with an Evans blue counterstain (Siva). Characteristic nuclear staining was considered positive.

Correlation Between Abnormal Changes in TCR Expression and Clinical Events After Transplantation

An attempt was made to determine whether abnormal test results correlate with the occurrence of clinically important events. The events studied were acute cellular rejection, vascular rejection, and increased corticosteroid immunosuppressive therapy. The data for all patients were pooled, and
the time interval in days (absolute value) from the occurrence of each individual test result (normal and abnormal) to the nearest biopsy positive for cellular rejection and also the nearest biopsy positive for vascular rejection was measured. (When multiple tests were performed on a given day relative to rejection, the mean number of normal and abnormal findings was used to avoid undue weighting of that time interval in the results.) Similarly, the time intervals from the beginning of high-dose prednisone therapy and abnormal changes in T-cell subset numbers were measured.

To compare the distributions for normal and abnormal results, the nonparametric Mann-Whitney U test was used. Linear regression analysis was used to test for correlation between the frequency of abnormal subset ratios and the time interval to a clinically significant event. All statistical analyses used STATVIEW II (Abacus Concepts).

Results

Comparison of Changes in TCR Expression Over Time Between Transplant Recipients and Controls

Changes in the number of Vβ-expressing T-cell subsets were determined for cardiac allograft recipients in the early posttransplantation period and for healthy subjects. Five healthy volunteers were followed for a period of 1 month with blood specimens obtained weekly and subsets quantified. Serial changes in Vβ expression were determined by comparing the number of positive cells at 7-day intervals. A total of 120 ratios (using the panel of six monoclonal antibodies) were calculated for the healthy controls. These ratios are given in Fig 1. Mean and median ratios were 1.03 and 1.0, respectively. A weekly ratio of 1.0 indicates no detectable change in Vβ expression. Thus, for controls, essentially no quantitative changes occurred among these T-cell subsets during the study period.

The entire range of calculated ratios for the healthy controls was 0.4 to 1.8. The values 0.5 and 2.0 (which correspond to a twofold decrease or twofold increase in cell count, respectively) contained 119 of the 120 calculated ratios (99.2%) for controls and were chosen to be the lower and upper limits of the normal range. A single control value (0.4) fell outside of these limits.

Serial changes in the number of Vβ-expressing T-cell subsets were determined for transplant recipients. These are also given in Fig 1. In total, 246 ratios were calculated; the mean was 1.3 and the median was 1.0, with a range of 0.03 to 10. Although the entire sample of patient subset ratios tended to distribute centrally around 1.0, there was much more variability for the transplant recipients than for the healthy controls. The ratio of the variance between controls and transplant recipients (F test) indicated a significant difference between these populations (F = 19.5, P < .001).

For transplant recipients, 57 of the 240 calculated subset ratios (23.8%) fell outside of the defined normal limits (27 were above and 32 were below the control limits) compared with 1 of 120 (0.8%) for the controls (P < .004, χ²).

Correlation Between Abnormal Changes in TCR Expression and Clinical Events After Transplantation

An association between abnormal T-cell subset ratios among transplant recipients and clinical events in the posttransplantation period was examined. The events studied were acute cellular rejection, vascular rejection, and high-dose corticosteroid immunosuppressive therapy. A correlation between test results and infection was also attempted. Patient 4 had a diagnosed infection (CMV ischemic colitis) approximately 57 days after transplantation. This patient was culture positive for CMV for a period of 17 days, during which time abnormal T-cell changes were observed for four of the six subsets examined. A second recipient (patient 5) was culture positive (throat) for CMV 20 days after transplantation; abnormal T-cell subset ratios were seen subsequent to this infection. These limited observations suggest that there may be a relation between CMV infection and perturbations in T-cell subsets (although confirmation will be required). Because of a suspected relation between CMV infection and alterations in T-cell subsets, the abnormal test results obtained for these patients during the period of virus shedding were not included in this data analysis.

The temporal distributions of normal and abnormal serial subset ratios are given in Fig 2A through 2C for the clinical events of interest. As shown, there were no differences between normal and abnormal T-cell subset ratios relative to either vascular rejection or the initiation of high-dose prednisone immunosuppression (Fig 2B and 2C). However, a significant difference (P < .005) between the temporal distributions of normal and abnormal T-cell ratios was seen with respect to the occurrence of biopsy-confirmed cellular rejection (Fig 2A). The abnormal T-cell subset ratios were found to occur a mean (± SD) of 7.7 ± 6.2 days from a biopsy positive for cellular rejection (median, 5 days; range, 0 to 28 days). In contrast, normal test results occurred a mean of 14.4 ± 10.9 days from a biopsy positive for cellular rejection (median, 11 days; range, 0 to 44 days). This observation suggests that abnormal changes in T-cell subset numbers coincide with cellular rejection.

As a further test for correlation between abnormal subset ratios and clinical events, the frequency of occur-
The serial variations in TCR expression are presented for four of the graft recipients as a function of time after transplantation (Fig 4). In addition to subset ratios, the results of histological assessments for cellular and vascular rejection, CMV isolations, and steroid pulses are shown. Of the 57 total abnormal T-cell subset ratios, 54 were observed for these four recipients; these recipients also experienced 9 of the 11 total cellular rejection episodes that occurred during the study.

For patient 3, abnormal T-cell subset ratios were observed over a period of 27 days; 6 of 30 calculated ratios (20%) were abnormal between days 14 and 35 after transplantation. During this same period, four normal biopsies were obtained. A biopsy positive for cellular rejection was obtained on day 41 concurrently with 3 of 6 abnormal T-cell subset ratios (50%).

For patient 4, abnormal subset ratios (9 of 18; 50%) occurred over a period of 10 days, 5 days preceding and 5 days following a biopsy positive for both cellular and vascular rejection. Subset ratios and biopsies were normal for 31 days, at which time subset ratios (3 of 6; 50%) became abnormal 3 days before positive cultures for CMV. CMV cultures continued to be positive, and this patient underwent a subtotal colectomy for ischemic necrosis subsequent to cytomegalic inclusion disease. This patient also received three steroid pulses for vasculitis resulting from OKT3 hypersensitivity.

For patient 5, T-cell subset ratios were abnormal on day 30 after transplantation, 10 days after isolation of CMV. Ratios were normal on day 36 with a biopsy negative for cellular rejection but positive for vascular rejection. Between days 55 and 106, 10 of 48 ratios (21%) were abnormal and 4 of 6 biopsies were positive for cellular rejection (2 were positive for vascular rejec-
Figure 3. A, Plot of the number of abnormal subset ratios (in 3-day intervals) plotted as a function of time from a biopsy positive for cellular rejection. Regression analysis of this plot indicated a significant correlation ($P<.001, R=.91$) between these occurrences. B, Plot of the number of abnormal subset ratios plotted as a function of time from a biopsy positive for vascular rejection. Regression analysis of this plot indicated no correlation between these occurrences. C, Plot of the number of abnormal subset ratios plotted as a function of time from the start of high-dose prednisone immunosuppressive therapy. Regression analysis of this plot indicated no correlation between these occurrences.

Subsection 3.1: Correlation Analysis

For patient 6, biopsies were positive for cellular rejection on days 23 and 30; 14 of 36 subset ratios (39%) were abnormal for a period surrounding these biopsies (days 15 to 37). The remaining abnormal subset ratios (6 of 24; 25%) occurred within a 13-day period surrounding a biopsy positive for both cellular and vascular rejection (10 days preceding and 3 days following). Subset ratios ($n=18$) were normal and biopsies ($n=3$) were negative for cellular rejection (although positive for vascular rejection) for the next 14 days.

The two patients not shown each experienced a biopsy-proved rejection episode in the time intervals during which they were observed (7 and 17 days, respectively). For these recipients, abnormal subset ratios ($n=3$) were observed concurrently with the positive biopsy.

**Vβ Expression by Graft-Infiltrating T Cells**

Preliminary investigation of Vβ expression among graft-infiltrating lymphocytes was undertaken in an attempt to understand the mechanisms underlying the above observations. In vivo activated lymphocytes were expanded in vitro by treatment with interleukin-2 and analyzed for Vβ expression. T lymphocyte cultures were generated from five myocardial specimens obtained by endomyocardial biopsy from three transplant recipients. The Vβ distributions for these five cultures (presented as a percentage of the total) are given in the Table. The normal Vβ distributions for blood specimens from healthy donors ($n=15$) are also included in the Table. These normal peripheral blood subset distributions were used as control values for analyzing the biopsy-derived lymphocyte cultures. The use of peripheral blood as a control was necessitated by the fact that T cells are not recovered from normal myocardium by this method. Therefore, a more appropriate control was unavailable.

For each of the T-cell cultures, at least one subset was increased in its expression beyond the normal range (greater than the control mean±2 SD), and the remaining subsets were generally reduced in number; one subset in each culture was reduced below the established normal range. This pattern is most clearly seen for the culture generated from the biopsy specimen obtained from recipient 1, 25 days after transplantation. For this culture, 84.7% of the cells were of either the Vβ5a or Vβ5b subset; each of the remaining subsets was markedly reduced below its normal level of expression (3.2% to 4.9% of the total). It is of interest that in addition to the overexpressed subset found in each culture of graft-infiltrating cells, for three of the five cultures derived from biopsy tissue, one of the six Vβ subsets was completely absent. These findings suggest that the entire T-cell compartment did not proliferate in response to the alloantigens expressed on the graft but rather that proliferation occurred among selected clones belonging to unique Vβ-expressing subsets.

These results also indicate that there may be differences in subset expression within the graft at different times. The three cultures originated from serial biopsy specimens obtained from recipient 1 show such differences in subset expression. For all these cultures,
Vβ5b is elevated in number, and Vβ5a is increased for day-25 and -50 cultures but is normally expressed in the culture begun at day 37 after transplantation. The Vβ8 subset is reduced at day 25 but is normally expressed in the remaining cultures; Vβ5b is reduced in the day-25 culture and is completely absent in the remaining cultures. Thus, activation of T cells within the graft (as suggested by these interleukin-2-expanded, graft-derived cultures) appears to be a dynamic process in which the levels of expression of these T-cell subsets is in continuous flux regulated by a presently unknown mechanism(s).

**Distribution of T-Cell Receptor Subsets in Interleukin-2-Expanded Lymphocyte Cultures From Endomyocardial Biopsy Tissue**

<table>
<thead>
<tr>
<th>Days After Transplant</th>
<th>Vβ5a</th>
<th>Vβ5b</th>
<th>Vβ5c</th>
<th>Vβ8</th>
<th>Vβ8</th>
<th>Vβ12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recipient 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>64.2↑*</td>
<td>20.5↑</td>
<td>3.4</td>
<td>3.2</td>
<td>15.5</td>
<td>4.9↑†</td>
</tr>
<tr>
<td>37</td>
<td>24.8</td>
<td>21.1↑</td>
<td>0.0↓</td>
<td>16.0</td>
<td>22.6</td>
<td>3.9</td>
</tr>
<tr>
<td>50</td>
<td>35.2↑</td>
<td>20.3↑</td>
<td>0.0↓</td>
<td>10.2</td>
<td>25.8</td>
<td>8.6</td>
</tr>
<tr>
<td>Recipient 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>37.0↑</td>
<td>14.6</td>
<td>7.4</td>
<td>0.0↓</td>
<td>26.1</td>
<td>15.4</td>
</tr>
<tr>
<td>Recipient 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>23.0</td>
<td>21.0↑</td>
<td>7.3</td>
<td>24.3</td>
<td>13.2↓</td>
<td>11.2</td>
</tr>
<tr>
<td>Controls (mean±SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.3±3.4</td>
<td>7.9±4.2</td>
<td>17.1±8.3</td>
<td>17.0±7.3</td>
<td>24.9±5.6</td>
<td>11.9±5.4</td>
<td></td>
</tr>
</tbody>
</table>

*Increased above normal range for peripheral blood (mean±2 SD).
†Decreased below normal range for peripheral blood (mean±2 SD).
The observations reported here indicate that large weekly variations (twofold or greater) in the number of T cells expressing one or more TCR isotypes occur almost exclusively among cardiac transplant recipients compared with healthy controls. Furthermore, these variations were found to correlate with the occurrence of cellular rejection; no correlation was noted between the subset fluctuations and the occurrence of vascular rejection or immunosuppression.

Discussion

In the present study, we used a panel of monoclonal antibodies that identify six unique TCR isotypes; each of these subsets individually constitutes between only 1% and 5% of the total peripheral blood lymphocytes. During the course of the study, twofold or greater changes in one or more of these subsets occurred exclusively among organ recipients compared with healthy controls. Moreover, these alterations in T-cell subsets corresponded in time to the occurrence of cellular rejection.

Perturbations in the lymphoid compartment of the peripheral blood could result from the differential trafficking patterns of lymphocytes that occur during periods of inflammation compared with normal conditions. Under normal conditions, naïve T cells enter lymphoid tissue, and memory T cells enter nonlymphoid tissues with return of these cells to the circulation by way of efferent lymphatics. Inflammation alters normal trafficking patterns and markedly increases T-cell entry into both lymphoid and nonlymphoid tissues. This process is initiated by cytokines and is facilitated by expression of adhesion molecules on the T-cell and vascular endothelium membranes. Interferon-\(\gamma\), for example, has been shown to cause marked disappearance of lymphocytes from the blood and retention of these cells within tissues. Withdrawal of interferon-\(\gamma\) corresponds to the reentry of these cells into the circulation. In addition, clonal proliferation subsequent to T-cell activation could contribute to fluctuations in cell number in the peripheral blood.

Clonal proliferation could have a profound influence on the quantitation of the V\(\beta\)-expressing subsets. As we observed for interleukin-2–expanded cultures from myocardial specimens, increase in one or several subsets was accompanied by a corresponding decrease in the remaining subsets. This pattern, although less dramatic, was also seen for blood specimens from the patients we examined. This inverse proportionality arises from the fact that in these investigations, a fixed number of cells were analyzed for V\(\beta\) expression. An increase in one subset under these conditions must therefore be compensated for by an accompanying decrease in another subset(s). This may provide another explanation for the occurrence of abnormally low T-cell ratios.

Thus, there are physiological mechanisms that predict that changes should be identifiable in the peripheral blood at times surrounding the onset of acute graft rejection. However, to date there has been limited success in identifying such changes. Failure to identify changes in the entire T-cell population or the CD4 and/or CD8 subsets does not preclude alterations in smaller T-cell subsets. In the present study, it is believed that the ability to identify alterations in cellular content of the peripheral blood was due to the small fraction of cells identified by each monoclonal antibody; quantitative alterations in these small subsets could be detected, whereas changes in total cell numbers did not occur.

Although a correlation between cellular rejection and perturbation within T-cell subsets was observed, it appears that immune activation of multiple etiologies can result in the kinds of alterations in TCR expression that were observed for transplant recipients. In the present study, for example, alterations in subset ratios corresponded in time to infections with CMV. In a few instances, subset alterations occurred without an apparent cause. These alterations could have been due to mild, undiagnosed rejection or could have resulted from undiagnosed infection with CMV or another agent. The contribution of additional factors affecting T-cell subsets is also possible. We have observed abnormalities in the number of TCR-expressing subsets in two cases of myocarditis (which returned to normal after therapy) and in a case of systemic lupus erythematosus. Thus, it appears that immune activation of multiple etiologies can result in the kinds of alterations in TCR expression observed for transplant recipients undergoing rejection. However, the fact that similar subset alterations did not occur among the volunteers suggests that these initiating factors are not normally encountered by a healthy individual.

An apparent correlation between increased steroid immunosuppression and subset variability was not observed despite the fact that cellular rejection is often treated with a steroid pulse. This probably can be attributed to facts that only 6 of the 11 episodes of cellular rejection associated with subset alterations were treated with a steroid pulse and that episodes of vascular rejection (not associated with subset alterations) were also treated with high-dose prednisone. Thus, there was no association (real or apparent) between abnormal subset variability and increased immunosuppression.

Finally, the recovery of activated lymphocytes from myocardial tissue from several of the grafts provided information about the expression of the T-cell subsets infiltrating the graft. Although limited, these observations do not support the hypothesis that involvement of a single subset occurs in response to a particular HLA mismatched allograft. In each case, more than one subset was recovered by interleukin-2 expansion. However, receptor expression among these graft-derived T cells was different in each case than the receptor distribution observed for the peripheral blood T cells of healthy controls. Thus, the predominance of a few subsets among T cells isolated from the graft suggests that there is a biased T-cell response to the allograft rather than the occurrence of global activation of all subsets. Similar findings of a restricted repertoire of allospecific T cells generated by several responders against a particular HLA class II allele have recently been reported.

In the present study, concurrent evaluation of peripheral blood findings and the pattern of receptor expression among graft-derived T cells was not done because paired blood and biopsy specimens were obtained in only one case. In this instance, however, a single subset (V\(\beta\)5b) was markedly elevated among the graft-derived cells; similarly, for the concurrently obtained blood specimen, this same subset achieved its highest level of expression of all the serially tested peripheral blood
specimens from this same patient (n=11). Further investigation will be required to establish whether there is a relation between receptor subset expression in the peripheral blood and T-cell subsets that infiltrate the graft. Although much remains to be learned about the physiological mechanisms that govern these observations, it appears that changes in TCR expression in the blood correlate with events that are associated with T-cell activation. This approach holds potential for the noninvasive monitoring of graft stability after transplantation and may provide insights into the physiology of graft rejection with the potential for the development of more specific tailoring of immunosuppressive therapy in individual patients.

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