Intravenous Pulse Administration of Cyclophosphamide Is an Effective and Safe Treatment for Sensitized Cardiac Allograft Recipients

Silviu Itescu, MD; Elizabeth Burke, RN; Katherine Lietz, MD; Ranjit John, MD; Donna Mancini, MD; Robert Michler, MD; Eric Rose, MD; Mehmet Oz, MD; Niloo Edwards, MD

Background—The proportion of cardiac transplant recipients with preexisting sensitization to HLA alloantigens has been increasing. Sensitization prolongs duration to obtaining a donor and predicts poorer long-term allograft survival because of increased risk of cellular rejections. We investigated the effect of cyclophosphamide pulse therapy in sensitized cardiac allograft recipients.

Methods and Results—Pretransplant and posttransplant outcomes were compared between 88 cardiac allograft recipients at risk for sensitization and 26 sensitized recipients treated with intravenous cyclophosphamide pulse therapy together with intravenous immune globulin before transplant and as part of a cyclosporine-based triple immunosuppressive regimen after transplant. Preformed IgG anti-HLA antibodies predicted longer duration to transplantation, earlier cellular rejection, and 2.7-fold higher cumulative rejection frequency ($P=0.002$). Before transplant, cyclophosphamide reduced waiting time and mortality to levels in nonsensitized patients. After transplant, cyclophosphamide prevented induction of IgG anti-HLA class II antibodies and interleukin-2 receptor–positive T-cell outgrowth from biopsy sites ($P=0.003$). Multivariable analysis revealed the risk of rejection was 3.7-fold higher in patients treated with mycophenolate mofetil than patients treated with cyclophosphamide ($P=0.019$). There were no differences in infectious or other significant complications.

Conclusions—Immunosuppression incorporating intravenous cyclophosphamide before and after transplantation is safe and highly effective in sensitized cardiac transplant recipients. When used after transplantation as part of triple immunosuppression, cyclophosphamide is superior to mycophenolate mofetil in reducing rejection. The mechanism may involve prevention of diversification of the recipient immune response to donor HLA class II molecules. (Circulation. 2002;105:1214-1219.)

Key Words: transplantation ■ grafting ■ antigens ■ rejection ■ cells

To identify patients at risk of having donor-specific alloreactivity, cardiac transplantation candidates are screened for antibodies reactive with lymphocytes from a panel of volunteers representative of the major HLA allootypes, collectively referred to as panel-reactive antibodies (PRA). $^{1,2}$ Patients with high PRA levels are considered to be sensitized to various alloantigens and require donor-specific T-cell cross-matches before transplantation to exclude the presence of lymphocytotoxic IgG antibodies against donor HLA class I antigens, which can cause early graft failure as a result of complement-mediated humoral rejection. $^{1,2}$ Because a positive donor-specific T-cell cross-match is a contraindication to transplantation, sensitized candidates have longer waiting times and higher mortality rates while waiting for an organ. In addition, the presence of preformed anti-HLA antibodies predicts poorer long-term outcome, including increased cellular rejections, earlier onset of transplant coronary artery disease (TCAD), and decreased long-term graft survival compared with nonsensitized patients treated with standard triple immunosuppressive regimens. $^{3,4}$ These complications seem to be related primarily to the presence of preformed antibodies against allogeneic HLA class II molecules $^{5}$ and may reflect an underlying state of CD4 T-cell allosensitization to class II antigens.

The proportion of highly sensitized patients on cardiac transplant waiting lists has been increasing as a result of both widespread use of left ventricular assist devices (LVADs) and more patients undergoing retransplantation. Whereas alloreactivity in retransplant candidates, recipients of blood products, and multiparous women is a result of repeated B- and T-cell exposure to alloantigens, the high frequency of alloreactivity in LVAD recipients $^{6,7}$ seems to additionally...
result from polyclonal B-cell activation attributable to selective loss of Th1-type T cells through activation-induced cell death and unopposed production of Th2-type cytokines. Previous interventions in sensitized recipients have focused on therapies aimed predominantly at immunoglobulin depletion and B-cell suppression, including plasmapheresis, immunoadsorption, or intravenous immune globulin (IVIg). Because these therapies do not directly impact CD4 T-cell alloreactivity, immunoglobulin-depleting regimens have not demonstrated beneficial effects on rejection or graft survival in sensitized recipients.

Cyclophosphamide is a cytotoxic agent with suppressive effects on discrete stages of the cell cycle, including proliferation and differentiation, making it a potentially effective agent for concomitant inhibition of both preactivated B and T cells. Long-term use of oral cyclophosphamide has been limited by significant complications, including bone marrow toxicity, hemorrhagic cystitis, gonadal failure, and development of malignancies. However, administration of cyclophosphamide via intravenous monthly pulses for treatment of autoimmune diseases has been shown to significantly reduce the risk of these complications while retaining therapeutic efficacy. In this study, we compared the outcome of cardiac allotransplantation in sensitized recipients treated with triple immunosuppression incorporating either mycophenolate mofetil (MMF) or cyclophosphamide. Our results demonstrate that cyclophosphamide therapy in transplant recipients is extremely effective and safe for reduction of serum anti-HLA alloreactivity, allogeneic T-cell activation, and cellular rejection.

Methods

Patient Population and Study Design

Pretransplantation Studies
During the period of 1990 to 1999, a total of 720 and 92 patients underwent cardiac transplantation and LVAD insertion, respectively. To maintain homogeneity of treatment practice and physician continuity, we performed an analysis of all pretransplant and posttransplant outcome data in sensitized and unsensitized patients seen at our institution only before 1996, because these data provide the natural history of the problem in the absence of any proven therapy. We studied the influence of anti-HLA antibodies on waiting time to cardiac transplantation in 55 previously unsensitized LVAD recipients between 1990 and 1996. Any patient who was sensitized before an LVAD implant was excluded from this analysis, because the influence of sensitization on waiting time and on pretransplant mortality cannot be ascertained without knowing the precise onset of sensitization.

Subsequently, 23 LVAD recipients with anti-HLA antibodies awaiting cardiac transplantation received a treatment regimen consisting of 1 to 3 monthly courses of intravenous cyclophosphamide given in a single-infusion dose of 0.5 to 1.0 g/m² together with IVIg 2 g/kg given in 4 divided daily doses. All 23 sensitized patients who were treated subsequently underwent successful cardiac transplantation. The effect of this immunosuppressive regimen on anti-HLA alloreactivity, waiting time, and pretransplant mortality was then determined for the entire group and compared with untreated LVAD recipients who were either sensitized or unsensitized. Three additional patients who were sensitized because of a prior transplant (1) or multiparity (2) were also evaluated after desensitization using this regimen.

Posttransplantation Studies
The relationship between preformed anti-HLA antibodies and high-grade cardiac allograft rejection was evaluated in 88 recipients at risk for sensitization (55 LVAD recipients, 30 retransplant candidates, and 3 multiparous females) who underwent transplantation between 1989 and 1998. All patients were treated with triple immunosuppression, and patients receiving either azathioprine or MMF were equally distributed among the sensitized and nonsensitized groups. No patients received induction therapy with OKT3, antithymocyte globulin, or monoclonal antibodies against interleukin 2 (IL-2) receptors.

To evaluate the effect of intravenous cyclophosphamide administration on posttransplant outcome, the frequency of high-grade rejection was compared in sensitized cardiac allograft recipients treated with triple immunosuppression incorporating either monthly posttransplant pulses of intravenous cyclophosphamide 0.5 to 1.0 g/m² for 4 months (n=26) or oral MMF 2 to 3 g/day (n=48). All patients were followed for at least 12 months after transplantation. All patients treated for the first 4 months after transplantation with cyclophosphamide subsequently received MMF for the remainder of the follow-up period. Because there is a direct relationship between episodes of cellular rejection and development of immunologic markers of alloreactivity, such as IL-2 receptor–positive T-cell biopsy outgrowth (lymphocyte growth assay) and production of circulating IgG anti-HLA class II antibodies, the effect of each treatment regimen on posttransplant induction of these variables was evaluated in 16 patients from each group who were matched by age, sex, and initial anti-HLA antibody isotype.

Endomyocardial Biopsies and Lymphocyte Growth Assay
Endomyocardial biopsies were performed to a schedule as previously described. In addition to histologic examination, one biopsy fragment was placed in medium supplemented with recombinant IL-2, and lymphocyte growth assay was performed as previously described.

Detection of Anti-HLA Antibodies
After transplantation, sera were obtained from all patients at risk for sensitization on the day of initial listing as UNOS status 1 for transplantation and then every 2 weeks until transplantation. After transplantation, sera were obtained from all patients with each biopsy. Sera were screened for the presence of lymphocytotoxic antibodies against a panel of HLA class I and II antigens, as previously described.

Statistical Analyses
Univariate analyses were performed by Kaplan-Meier log-rank statistics, Wilcoxon’s rank sum test, and Student’s t test. Cumulative high-grade rejections were modeled by the method of Wei et al. Cox Proportional Hazard model was used for the multivariable analysis of factors conferring protection against high-grade rejection in sensitized patients. These factors included immunosuppression consisting of either cyclophosphamide or MMF, donor and recipient age, sex, and race, donor and recipient HLA matching, and ischemic time. All data were analyzed using SAS system software (SAS Institute Inc).

Results

Presence of Preformed IgG Antibodies Increases Waiting Time to Cardiac Transplantation
As shown in Figure 1, whereas the mean duration to transplantation in unsensitized LVAD recipients (n=18) was 75 days (range, 7 to 143), this was significantly increased to 120 days (range, 23 to 217) in LVAD recipients with anti-HLA IgG antibodies (n=37) (P=0.002). Among non-LVAD recip-
After transplantation, the mean duration to transplantation was 69 days (range, 50 to 88).

**Presence of Preformed IgG Antibodies Is Associated With Shorter Duration to High-Grade Cellular Rejection and Higher Cumulative Rejection Frequency**

The presence of preformed IgG antibodies at the time of transplantation was highly predictive of early high-grade cellular rejection. By 3 months after transplantation, 53% of patients with IgG anti-HLA class II antibodies (n=29) had developed high-grade rejection compared with only 27% of those without these antibodies (n=51) (P<0.05) (Figure 2). Moreover, the cumulative annual rejection frequency was 2.7-fold higher in patients with preformed IgG anti-HLA class II antibodies (1.29 rejections/year) than in those without (0.48 rejections/year, P=0.02) (Table 1). Although the presence of preformed IgG anti-HLA class I antibodies (n=48) was also associated with both earlier rejection and higher rejection frequency compared with patients without these antibodies (n=38), this was not statistically significant. Preformed IgM antibodies against either class I or II antigens had no influence on high-grade cellular rejection (P>0.5).

**Pretransplant Immunosuppression in Sensitized Recipients Reduces IgG Anti-HLA Alloreactivity and Shortens Waiting Time to Transplantation**

To evaluate the overall effect of the treatment course (1 or more cycles), the mean reduction in IgG anti-HLA alloreactivity per cycle relative to baseline was determined for 16 patients who received 1 to 3 monthly cycles (total, 28 cycles), then data for all patients were pooled. IgG anti-HLA class I alloreactivity was reduced by 14% to 52% (mean, 33%) (P<0.01) and class II alloreactivity was reduced by 14% to 52% (mean, 33%) (P<0.01). In 23 sensitized treated LVAD recipients, the mean waiting time was significantly reduced to 3.3 months (range, 0.3 to 6.2 months) (P<0.05), and no patient was transplanted across a positive donor-specific IgG T-cell cross-match. The 3 additional non-LVAD–sensitized patients (1 retransplant and 2 multiparous) demonstrated a similar reduction in anti-HLA alloreactivity and were all successfully transplanted within 3 months of protocol initiation. None of the 23 sensitized treated LVAD recipients died awaiting transplantation compared with 6 of 44 (14%) sensitized untreated LVAD recipients (P=0.08). These data indicate that shortening the transplant waiting time in sensitized treated recipients may reduce the mortality associated with delay because of persistent sensitization and repeated positive cross-matches.

**TABLE 1. Influence of Preformed Anti-HLA Antibodies in Cardiac Allograft Recipients at Risk for Sensitization (n=88) on Cumulative Annual Rejection Frequency After Transplantation**

<table>
<thead>
<tr>
<th>Preformed Antibody Type</th>
<th>Positive</th>
<th>Negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG anti-HLA class II</td>
<td>1.29</td>
<td>0.48</td>
<td>0.02</td>
</tr>
<tr>
<td>IgG anti-HLA class I</td>
<td>0.611</td>
<td>0.291</td>
<td>0.09</td>
</tr>
</tbody>
</table>
Posttransplant Intravenous Cyclophosphamide Pulse Therapy in Sensitized Cardiac Transplant Recipients Reduces Immunologic Markers of Alloreactivity

Compared with MMF, treatment for 4 to 6 months with intravenous pulses of cyclophosphamide protected against IL-2 receptor–positive T-cell outgrowth from biopsy sites during the first year after transplant (*P*/H11021 0.01) (Table 2). Moreover, cyclophosphamide prevented the posttransplant induction of IgG antibodies against HLA class II but not class I (defined as increase by >10% above pretransplant values). Whereas 9 of 16 (56%) MMF-treated patients produced increased levels of anti-HLA class II IgG antibodies, only 2 of 16 (13%) cyclophosphamide-treated patients showed an increase in anti-HLA class II IgG antibodies (*P*/H11005 0.009).

Posttransplant Intravenous Cyclophosphamide in Sensitized Recipients Prolongs Rejection-Free Interval and Decreases Cumulative Rejection Frequency

As shown in Figure 3, immunosuppression using intravenous pulses of cyclophosphamide in sensitized recipients for 4 to 6 months after transplantation significantly prolonged the rejection-free interval compared with MMF. Overall, only 4 of 26 (15%) cyclophosphamide-treated patients developed 1 or more high-grade rejections within the first posttransplant year compared with 22 of 48 (46%) patients treated with MMF (*P*/H11005 0.009). Moreover, treatment with cyclophosphamide reduced the cumulative annual rejection frequency by 63%, from 0.94 rejections/year for sensitized patients treated with MMF to 0.35 rejections/year (*P*/H11005 0.03) (Table 3). The latter value is within the same range as the annual rejection frequency in nonsensitized patients at our institution. Again, similar results were found irrespective of whether sensitization was attributable to IgG anti-HLA class I or II antibodies (Table 3).

Posttransplant Intravenous Cyclophosphamide in Sensitized Recipients Is the Only Protective Factor Against Rejection

By multivariable analysis, the only significant protective factor against development of high-grade cellular rejection in sensitized patients was treatment with cyclophosphamide (Table 4). Compared with cyclophosphamide, MMF treatment conferred a 3.7-fold higher risk of rejection (*P*/H11005 0.009).

Safety Profile of Pretransplant and Posttransplant Immunosuppressive Therapy

Treatment with intravenous cyclophosphamide has proven to be extremely safe. Systemic fungal infections occurred before transplant in 24% of sensitized LVAD recipients who did not receive cyclophosphamide and in 22% of those in the immunosuppression regimen group. Of particular interest, all

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**Table 2. Intravenous Cyclophosphamide Therapy Is Superior to Mycophenolate Mofetil in Reducing Serum and T-Cell Alloreactivity in Matched Cohorts of Sensitized Recipients**

|                      | Mycophenolate Mofetil (n=16) | Intravenous Cyclophosphamide (n=16) | *P*
|----------------------|------------------------------|--------------------------------------|-----
| **Demographics**     |                              |                                      |     
| Preformed IgG anti-HLA class II | 4/16                     | 4/16                               | NS  
| Preformed IgG anti-HLA class I  | 12/16                     | 12/16                              | NS  
| Male:female           | 14:2                       | 12:4                               | NS  
| Mean age, y           | 49                         | 43                                 | NS  
| 1 to 2 HLA-DR matches | 4/16                       | 4/16                               | NS  
| **Immunological variables** |                            |                                      |     
| Median duration to first positive LGA | <1 month                | >12 months                         | 0.01  
| Induction of IgG anti-HLA class II, n (%) | 9/16 (56)               | 2/16 (13)                          | 0.009  
| Induction of IgG anti-HLA class I, n (%) | 9/16 (56)                | 7/16 (44)                          | NS  

NS indicates not significant.
fungal infections in the immunosuppression regimen group occurred before initiation of cyclophosphamide treatment. The incidence of CMV disease after transplant (defined as clinical disease together with virologic culture confirmation) was actually lower in cyclophosphamide-treated patients (3 of 26 [12%]) than in those treated with MMF (10 of 54 [19%]). No other systemic viral, bacterial, or fungal infections were seen in patients treated with cyclophosphamide. Cyclophosphamide therapy was frequently (>80%) accompanied by transient nausea and vomiting, which responded to antiemetic therapy. Mesna was coadministered with cyclophosphamide and may have contributed to the absence of any cases of hemorrhagic cystitis. No malignancies have developed after 53.5 patient-months of follow-up (range, 6 to 38 months). IVIg therapy was associated with immune complex disease in 4 of 27 (15%) monthly courses, as evidenced by fevers, arthralgias, and maculopapular rashes. Reversible renal insufficiency (defined as >50% increase in serum creatinine level) occurred in 4 cases, all of which resolved spontaneously over the ensuing 3 weeks after infusion.

Discussion

These results demonstrate that intravenous pulse cyclophosphamide therapy together with IVIg pretransplantation and as part of a cyclosporine/steroid-based regimen in sensitized cardiac allograft recipients is extremely effective and safe for decreasing recipient serum and cellular alloreactivity, shortening transplant waiting time, reducing allograft rejection, and decreasing cumulative rejection frequency to levels seen in nonsensitized recipients. Posttransplantation use of cyclophosphamide significantly reduced IL-2 receptor–positive T-cell outgrowth from biopsy sites and prevented the posttransplant induction of IgG antibodies against HLA class II molecules. Because there is a direct relationship between IL-2 receptor–positive T-cell outgrowth, production of circulating anti-HLA class II antibodies, and episodes of acute cellular rejection, this provides a mechanism for the significant reduction in cellular rejection episodes observed after treatment with cyclophosphamide.

It is likely that the principal component of our immunomodulatory regimen responsible for pretransplant reduction in anti-HLA alloreactivity is IVIg. The mechanism by which IVIg transiently reduces anti-HLA serum reactivity is not well defined but may be related to the presence in the IVIg preparation of soluble HLA class I molecules that bind circulating anti-HLA antibodies or of noncomplement-fixing antibodies against HLA class I molecules. Although IVIg stimulates the production of IgM anti-idiotypic–blocking antibodies to HLA in recipient serum, this immunomodulatory mechanism is unlikely to account for the rapid, transient, and nonsustained clinical effect on reduction in anti-HLA alloreactivity that has been observed when using IVIg in sensitized cardiac transplant recipients. In contrast, our combined IVIg/intravenous cyclophosphamide regimen seemed to have a prolonged inhibitory effect on CD4 T-cell activation, as defined by sustained prevention of both T-cell–mediated allograft rejection and induction of anti-HLA class II antibodies after transplantation. This immunomodulatory effect suggests that the principal component of the regimen responsible for these effects is cyclophosphamide.

Cumulative experimental data suggest that initiation of allograft rejection is predominantly a CD4 T-cell–dependent process and that long-term graft acceptance is associated with reduced direct and indirect recognition of donor HLA class II alloantigens by recipient CD4 T cells. Recurrent rejection episodes, as well as the onset of TCAD, are accompanied by intermolecular and intramolecular spreading and CD4 T-cell recognition of multiple donor HLA-DR alloantigenic determinants. This diversification of the immune response has been postulated to result from activation of antigen-specific B cells by soluble myosin heavy chain class II products, particularly HLA-DR molecules, and the subsequent efficient presentation of multiple HLA-DR allopeptides by self B cells to CD4 T cells. Because cyclophosphamide has selective suppressive effects on discrete stages of the cell cycle, it is an effective agent for the inhibition of both B-cell functions (antigen uptake, processing, and presentation) and alloreactive CD4 T-cell responses (recognition of allo-HLA class II molecules). Consequently, its efficacy in reducing allograft rejection may lie in its ability to interrupt the diversification of the recipient immune response to allogeneic HLA class II molecules. Moreover, because a high rate of fungal infection in LVAD recipients seems to be attributable to progressive defects in cellular immunity attributable to excessive CD4 T-cell activation and

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient±SEM</th>
<th>P</th>
<th>Risk Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycophenolate mofetil (relative to cyclophosphamide)</td>
<td>1.27±0.55</td>
<td>0.019</td>
<td>3.9</td>
<td>(1.23, 10.47)</td>
</tr>
<tr>
<td>HLA-DR mismatch</td>
<td>0.27±0.55</td>
<td>0.63</td>
<td>1.3</td>
<td>(0.45, 3.82)</td>
</tr>
<tr>
<td>Ischemic time (&gt;90 minutes)</td>
<td>−0.05±0.62</td>
<td>0.96</td>
<td>0.97</td>
<td>(0.28, 3.29)</td>
</tr>
</tbody>
</table>
apoptosis, the notable lack of infections in such patients treated with cyclophosphamide might be a result of the drug’s effect on cycling CD4 T cells.8

The intravenous cyclophosphamide regimen used in our study was adapted from protocols used in the treatment of SLE and systemic vasculitides, where intermittent low-dose pulse therapy has been shown to significantly reduce the incidence of complications compared with oral cyclophosphamide while maintaining efficacy.18–20 The sustained efficacy of intermittent intravenous pulses of cyclophosphamide compared with daily oral use may be attributable to the high peak dose after each intravenous administration, whereas the reduced short-term toxicity of the regimen lies in the reduction of cumulative dose, with adequate interdose interval allowing for bone marrow recovery. Reduction in long-term side-effects, notably gonadal toxicity and malignancies, is directly related to reduction in cumulative cyclophosphamide dose using intermittent pulse therapy.

The principal limitations of this study arise from the small size of the patient groups and the lack of randomization. Efficacy of this protocol will need to be validated in future multicenter, randomized studies with sufficient numbers and power to compare with other immunosuppressive regimens. Moreover, effects of this regimen on transplant waiting time will need to take into account differences among centers with respect to geographical region as well as patient variables such as blood group and UNOS status.9 Presently, we advocate that all patients at risk for sensitization before transplantation should be specifically screened for the presence of antibodies against both HLA class I and class II antigens. On the basis of our results, immunosuppressive therapy for sensitized patients should commence before transplantation, because initiation of a standard triple-therapy regimen after transplant is not effective at preventing recurrent allograft rejection. Initiation of an immunosuppressive protocol using intravenous cyclophosphamide pulses before and after transplantation is a safe and effective modality for reducing donor-specific B- and T-cell alloreactivity, thereby increasing the likelihood of obtaining a cross-match-negative allograft, reducing pretransplant mortality and posttransplant allograft rejection, and improving long-term allograft function.

References
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