Effect of Mycophenolate Mofetil Therapy on Lymphocyte Activation in Heart Transplant Recipients

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Background: Mycophenolic acid is reported to provide effective immunosuppression by inhibiting inosine monophosphate dehydrogenase. In an attempt to monitor the effects of therapy with mycophenolate mofetil, we measured the expression of the activation markers CD25, CD38, CD69 and HLA-DR on lymphocytes of patients after heart transplantation.

Methods: Thirty-six patients enrolled in the study were randomly assigned to one of two groups. Patients in the control group (n = 15) received cyclosporine, azathioprine and prednisone. Patients in the study group (n = 21) were switched from azathioprine to mycophenolate mofetil (MMF) 3 months after heart transplantation. The expressions of the activation markers CD25, CD38, CD69 and HLA-DR on B cells, T cells and natural killer (NK) cells in peripheral blood were determined by flow cytometry.

Results: In patients treated with MMF a significant reduction of the B-cell count was observed in comparison to a healthy control group and patients under therapy with azathioprine. The decline of B cells in the MMF group started 3 months after onset of therapy and, after 1 year, was nearly halved. In addition, the percentages of CD38-positive B cells, activated T cells (CD4+/CD25+, CD8+/CD38+) and HLA-DR–expressing NK cells were reduced during therapy with MMF.

Conclusions: Our studies have shown administration of MMF to be associated with a reduction of B lymphocytes and a downregulation of activation markers on B cells. In contrast to in vitro findings, our data indicate that the immunosuppressive effect of MMF in vivo is exhibited mainly on B cells. J Heart Lung Transplant 2002;21:1074–1079.

The immunosuppressive agent, mycophenolic acid (MPA), an active metabolite of the pro-drug mycophenolate mofetil (MMF), shows specific activity in lymphocytes where the target enzyme, inosine monophosphate dehydrogenase (IMPDH), is blocked and the production of GTP is inhibited.
Although most cell lines are capable of recycling purine nucleotides via the salvage pathway, lymphocytes rely predominantly on the de novo synthesis so that treatment with MPA leads to the inhibition of DNA synthesis. In addition to their role in DNA synthesis guanine nucleotides are required for the transfer of sugar structures to cell-surface glycoproteins. The selective inhibition of lymphocyte proliferation by MPA has been demonstrated in allo-stimulated mice injected with tritium-labeled thymidine. Gummert et al showed that MPA inhibits mitogen-stimulated lymphocyte proliferation as well as the expression of cell-surface cytokine receptors in rat whole blood. However, the published data on the in vitro effects of MPA on the expression of cell-surface antigens are controversial. Despite the valuable information these studies have provided, the in vivo mechanism of action of MPA is poorly understood and its effect on surface markers that characterize lymphocyte activation is less clear.

In the pathogenesis of graft rejection CD4 T-cell activation plays an important role. Upon activation these cells proliferate, express interleukin-2 (IL-2) receptors on their surface and secrete cytokines including IL-2. They can be identified in the graft, where they regulate cytotoxic T cells, B cells and macrophages, which causes destruction of the graft via cell- and/or antibody-mediated lysis of target cells. The locally infiltrating lymphocytes are recruited from other lymphoid tissues and presumed to circulate through the peripheral blood, thereby reflecting the local immune response within the graft. CD25 (IL-2 receptor α chain) is expressed in the early phase after T-cell activation and the clonal proliferation of activated T cells depends on the expression of this receptor. CD38 and CD69 are not expressed on resting lymphocytes, but they do appear upon activation on T cells, natural killer (NK) cells and B cells.

In recent years, the application of flow cytometry has progressed rapidly, and there have been attempts to correlate post-operative immunophenotypic monitoring of peripheral blood mononuclear leukocytes with rejection episodes in transplant recipients. In addition, immunophenotyping analyses of blood have been applied to the diagnosis of viral infection and cyclosporine toxicity in an attempt to distinguish these processes from rejection.

The purpose of this in vivo study on patients after heart transplantation was to assess in peripheral blood by flow cytometry whether MMF immunomodulation affects the expression of the early activation markers CD25, CD38 and CD69 as well as that of HLA-DR on lymphocytes, which may contribute to the immunosuppressive efficacy of MPA in vivo.

**PATIENTS AND METHODS**

**Patients**

Thirty-six patients were enrolled in this study after they underwent orthotopic heart transplantation. All patients provided informed consent for the switch from azathioprine to mycophenolate mofetil. Demographic data for both groups are shown in Table I. There were no differences between groups in recipient age, gender, diagnosis for transplantation, donor age and ischemic time. Randomization was performed so that every odd-numbered patient was included in the study group (mycophenolate mofetil) and every even-numbered patient received the control medication (azathioprine). Initially, all patients received the same immunosuppressive therapy, which consisted of anti-thymocyte globulin as induction therapy over a period of 7 days and maintenance therapy that consisted of cyclosporine,

**TABLE I  Patient characteristics**

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Mycophenolate Mofetil</th>
<th>Azathioprine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>57.0 ± 11.5</td>
<td>52.0 ± 10.4</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>20 /1</td>
<td>14 /1</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic cardiomyopathy</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>Dilative cardiomyopathy</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Donor age ± SD (years; mean ± SD)</td>
<td>39.4 ± 13.6</td>
<td>36.2 ± 9.9</td>
</tr>
<tr>
<td>Ischemic time ± SD (minutes; mean ± SD)</td>
<td>192.5 ± 47.1</td>
<td>202.7 ± 43.3</td>
</tr>
</tbody>
</table>
azathioprine (2 mg/kg per day) and prednisone (1 mg/kg per day, which was then decreased to a maintenance dose of 0.15 to 0.2 mg/kg per day) from the first day up to 3 months after cardiac transplantation. Cyclosporine target levels, as measured with an Abbott monoclonal fluorescence polarization immunoassay, varied between 150 and 250 ng/ml during Months 2 to 6, and between 100 and 150 ng/ml thereafter.

After 3 months, patients were separated into groups according to the randomization scheme. The control group (n = 15) received cyclosporine, azathioprine and prednisone. Patients in the study group (n = 21) were switched from azathioprine to mycophenolate mofetil (1 g twice daily). When leukopenia occurred (white blood cell count <4 G/liter), therapy with azathioprine or mycophenolate mofetil was stopped until the leukocyte count recovered.

Sample Collection

Venipuncture was performed on a biweekly basis during the first 3 months after the switch from azathioprine to mycophenolate mofetil. Thereafter, blood specimens were collected in the outpatient clinic every second month for follow-up. Blood samples of patients that were drawn within 6 days before the diagnosis of infection or rejection were excluded retrospectively. Blood samples from 15 age-matched healthy subjects (mean age 53 ± 9 years) served as controls.

Flow Cytometric Analysis

Analyses of leukocyte surface antigens were performed within 8 hours after venipuncture. All antibodies used were monoclonal mouse anti-human antibodies and fluorescently labeled with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin-chlorophyll (Per-CP). They were purchased from Becton Dickinson (USA). One hundred microliters of whole blood was incubated with the appropriate antibody combination (10 μl of each antibody). After 15-minute incubation, the red blood cells were lysed with a lysis solution (Becton Dickinson) and the remaining cells were washed three times using phosphate-buffered saline (PBS; CellWash, Becton Dickinson, San Jose, CA). At least 10,000 leukocytes were analyzed on a FACSscan flow cytometer (Becton Dickinson). Results are expressed as percentages of positive cells (mean ± SE). Lymphocytes positive for CD19 were identified as B cells, cells co-staining for CD3 and CD4 as helper T cells, cells co-staining for CD3 and CD8 as suppressor/cytotoxic T cells and those staining positive for CD16 and CD56 as natural killer cells (NK cells).

Statistical Analysis

Values are expressed as mean ± SE. Wilcoxon’s signed rank test was used and p < 0.05 was considered statistically significant.

RESULTS

Clinical Outcome

Survival in both groups was 100% at 1 year. Thereafter, there were 3 deaths in the mycophenolate mofetil group (pulmonary embolism = 1, graft arteriosclerosis = 2) and 1 death in the azathioprine group (graft arteriosclerosis). Freedom from rejection was 81% at 3 months, 81% at 12 months and 76% at 36 months in the mycophenolate mofetil group; freedom from infection was 86% at 3 months, 62% at 12 months and 57% at 36 months.

In the azathioprine group, freedom from rejection was 80% at 3 months, 67% at 12 months and 67% at 36 months; freedom from infection was 67% at 3 months, 40% at 12 months and 27% at 36 months. The differences in rejection and infection rates between the two study groups did not reach statistical significance.

Expression Patterns

In patients given long-term treatment (>11 months) with MMF a significant reduction in B-cell count was observed in comparison to healthy control patients and patients under therapy with azathioprine (Table II). The decline of B-cells in the MMF group started 3 months after onset of therapy (Figure 1), and after 1 year was nearly halved. No significant differences in T-cell percentages were found between healthy control subjects and patients under therapy with either MMF or azathioprine (AZA) (Table II). In contrast, a slight percentage increase in CD3-positive T cells (p < 0.038) was observed in the MMF group when compared with patients under therapy with AZA (Table II).

From the activation markers investigated on B cells, the percentage of CD38-positive B-cells was significantly lower in patients treated with MMF when compared with healthy subjects or patients treated with AZA (Table II). In contrast, AZA therapy led to a statistically significant reduction of both CD25- and CD69-positive B-cells compared with patients in the control group (Table II).
The expression of HLA-DR on B cells in both groups (95.1 ± 4.9% of total B-cells in the MMF group vs 96.3 ± 3.7% in the AZA group) was not significantly different, whereas both MMF and AZA dramatically reduced the percentages of activated (CD16/CD25 double-positive) NK cells.

The expression of HLA-DR on B cells in both groups (95.1 ± 4.9% of total B-cells in the MMF group vs 96.3 ± 3.7% in the AZA group) was not significantly different, whereas both MMF and AZA dramatically reduced the percentages of activated (CD16/CD25 double-positive) NK cells.
Although the T-cell count increased slightly in the MMF group, expression levels of activation markers CD25, CD38 and CD69 on T cells were not statistically significantly altered when compared with healthy controls (Table II). In contrast, treatment with azathioprine did not prevent upregulation of CD25 and CD38 on CD4 T cells and CD8 T cells, respectively (Table II).

A significant increase in HLA-DR expression on T cells was seen in both (MMF and AZA) groups. Treatment with MMF upregulated HLA-DR on all CD3-positive cells, whereas AZA affected the CD4- and CD8-positive T-cell subsets only (Table II). Therefore, when comparing HLA-DR/CD3 double-positive cells between the two groups, a statistically significantly higher percentage of HLA-DR-positive CD3 cells was found in the MMF group (Table II). A similar effect was observed for HLA-DR expression on NK cells in the AZA group (Table II), which led to a significant reduction of HLA-DR-positive cells in the MMF group ($p < 0.0077$) (Table II).

**DISCUSSION**

Until now, very few reports have dealt with the activation of lymphocytes after organ transplantation. Most were in vitro studies wherein isolated white blood cells were incubated with one or more immunosuppressive agents for rather short periods. The aim of this study was to monitor the expression of activation markers such as CD69, CD25 and CD38 as well as HLA-DR. B-, T- and NK-cells were analyzed in heart-transplant patients receiving triple therapy consisting of CyA, corticoids and azathioprine or MMF. Thereby, we demonstrated that MMF therapy not only reduces the percentage of total B cells but also induces a tenacious reduction of B-cell activation markers. Azathioprine decreased the expression of the activation marker CD69 on B-cells, but did not influence the percentage of B-lymphocytes. The decrease of CD25 expression (except on CD4-positive cells) seen in both groups might be partially attributable to calcineurin inhibition, because all patients received CyA. In contrast to MMF, treatment with azathioprine did not prevent the upregulation of CD25 on CD4-positive and of CD38 on CD8-positive T lymphocytes. In both groups, an upregulation of HLA-DR was seen, an effect usually observed after organ transplantation. Although neither the triple therapy including MMF nor the combination therapy with azathioprine could suppress the enhanced expression of HLA-DR on T-lymphocytes in patients after heart transplantation, the percentages of HLA-DR-positive T-lymphocytes were lower in the azathioprine group. In contrast to azathioprine, MMF prevented HLA-DR expression on NK cells. In MMF-treated patients, the increase of ready-to-activate HLA-DR-positive T lymphocytes seems to have been compensated by a decrease in total and CD38-positive B cells as well as in activated CD4 and CD8 T cells. Azathioprine led to a reduction of the T-cell count as well as to a reduction of B-cells expressing CD69. When comparing the effects of MMF and azathioprine, it is obvious that MPA was more effective in reducing the frequencies of CD38-positive B-cells, CD25-positive CD4 T cells and CD38-positive CD8 T-cells. In contrast to Eugui et al, who reported that in vitro the expression of the IL-2 receptor (CD25) on mitogen-stimulated human T lymphocytes is not affected by MPA, we found a significant reduction of this activation marker in vivo. This is in accordance with the observations made by Thomson et al, who reported an MPA dose-dependent inhibition of CD25 expression on human lymphocytes after allogeneic stimulation. The effects of MMF on CD38 expression seem to be of special importance because this surface marker is predominantly expressed on hematopoietic cells, where its expression correlates with differentiation and proliferation. Kilmartin et al showed a reduction of CD69-positive CD4 T cells in patients with active uveitis treated with MMF. We did not observe this effect in our patients, which is probably due to the fact that blood samples of patients that were drawn within 6 days before the diagnosis of infection or rejection were excluded retrospectively.

The expression of CD69 on B-lymphocytes in heart-transplant recipients treated with MMF increased by >50% in comparison to patients treated with azathioprine. GTP-binding proteins (G proteins) have been shown to be involved in the expression of activation markers such as CD69.$^{18}$ Recently, it was shown that MMF therapy leads to an increase in intracellular GTP levels in red blood cells.$^{17}$ Whether a transient or permanent increase of GTP in B cells can explain the enhanced CD69 expression requires further investigation.

In summary, we have shown, for the first time, that MPA not only leads to a reduction in the percentage of B-cells but also to a downregulation of activation markers in patients after heart transplantation. The immunosuppressive effect of MPA in clinical transplantation may therefore be related not only to inhibition of lymphocyte proliferation but also to the inhibition of cell-surface antigen expression, which is in line with the findings of Gummert et al in a rat
model. Although in vitro studies have shown that MPA also affects the function of T cells, our in vivo data provide evidence that, in patients given long-term treatment with MMF, the immunosuppressive effect is exhibited mainly on B-cells. Russell et al. provided evidence that inhibition of the humoral response after transplantation must be considered for controlling chronic allograft vasculopathy, and our findings support a potential role of MMF for preventing graft sclerosis.

REFERENCES