

Mycophenolate mofetil inhibits lymphocyte binding and the upregulation of adhesion molecules in acute rejection of rat kidney allografts

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Abstract: Mycophenolate mofetil (MMF) interacts with purine metabolism and possibly with the expression of adhesion molecules. In the present study, we analysed the expression of these molecules in transplanted kidney allografts treated with RS LBNF1 kidneys were orthotopically transplanted into Lewis rats and either treated with RS (20 mg/kg/day) or vehicle. Rats were harvested 3, 5 and 7 days following transplantation. For binding studies, fresh-frozen sections of transplanted kidneys were incubated with lymph node lymphocytes (LNL) derived from transplanted rats. Additionally, immunohistology was performed with various monoclonal antibodies. In general, MMF resulted in better preservation of graft structure by 7 days. Cellular infiltration and tubular atrophy were less pronounced. At day 3, macrophages were diminished in MMF-treated animals to a high extent, while the number of T cells was almost identical to that of controls. In addition, the number of cells positive for MHC class II and LFA-1 was reduced in the MMF-treated animals. These findings correlated with the binding results. Three days following engraftment, LNL bound to MMF-treated kidneys to a lesser extent compared to controls. In conclusion, MMF resulted in a markedly reduced leucocytic infiltrate, presumably based on a reduced expression of lymphocytic adhesion molecules and an interaction with macrophages.

Introduction

Much has been learned regarding the process of acute rejection. Recently, interest has focused on the role of adhesion molecules in this process. These molecules play an important role during extravasation, antigen presentation and proliferation of lymphocytes.^{1,2} Upregulation of adhesion molecules may be caused by cytokine-elaborated events such as surgical manipulations, ischemia, cooling and reperfusion. The presence of cytokines increases the expression of several critical molecules on human renal tubular cells, including ICAM-1 and MHC class I and class II.¹ Mycophenolate mofetil (MMF),

on the other hand, is a drug known to act beneficially during acute rejection.^{3,4} *In vitro* experiments indicated its potential to inhibit the proliferation of lymphocytes and to reduce the upregulation of adhesion molecules on several cell types.^{5–8}

Objective

The goal of our study was to elucidate to what extent the beneficial effect of MMF on the incidence of acute rejection is based on a reduced expression of adhesion molecules or other mechanisms.

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Materials and methods

Animals

Naive male 200–250 g inbred rats (Harlan Sprague-Dawley, Indianapolis, IN) were used throughout the experiments; Lewis (LEW) rats acted as graft recipients, Lewis/Brown Norway (LBNF1) as donors.

Kidney grafting

The left donor kidney was removed, cooled and positioned anatomically into the host. Donor and recipient renal artery, vein and ureter were then anastomosed end-to-end with 10–0 prolene sutures. No ureteral stent was used. Recipients ($n = 30$) were either treated with MMF (20 mg/kg/day) or with vehicle.

Tissue preparation

Organs and tissues were harvested 3, 5 and 7 days after transplantation, snap frozen in liquid nitrogen and stored at -70°C , or fixed in formalin or acid formalin for hematoxylin–eosin staining.

Antibodies

Monoclonal antibodies (mAbs) to adhesion molecules included those directed against LFA-1 α (WT.1), ICAM-1 (1A29) (Prof. Miyasaka, Tokyo) and VCAM-1 (Genzyme, Boston). Antibodies against T cells (CD5/OX-19), monocytes/macrophages (ED-1), MHC class II (OX-3) and neutrophils (MOM/3F12/F2) were obtained from Sera-Lab (Accurate Chemicals, Westbury, NY).

Immunohistology

Cryostat sections of frozen tissues were stained with 1A29, WT.1, ED-1, MOM/3F12/F2, OX-3, W3/25, and OX-19 using the alkaline phosphatase/antialkaline phosphatase (APAAP) method. Stained cells were then counted with an ocular grid ($\times 600$, >30 fields counted per specimen); tissue staining was evaluated on a scale of 1–4.

Preparation of cell suspensions

Lymph node lymphocytes (LNL) from cervical and axillary lymph nodes were minced through stainless steel mesh, suspended and washed with phosphate-buffered saline. Cell preparations were evaluated using fluorescence staining with appropriate antibodies and discarded if purification was $<95\%$.

Binding assay

For binding studies, a modified Stamper–Woodruff assay was used.⁸ Aliquots (7×10^8 cells) of LNL from grafted Lewis rats treated with or without MMF were incubated for 30 min at 64 rpm at 25°C with snap-frozen sections of kidney allografts serially harvested from the same animals; LNL from naive Lewis rats served as additional controls. The preparations were fixed in a 1.5% glutaraldehyde solution and counterstained with hematoxylin. Cells, bound to the allografts were then counted and described as cells per field of view. All experiments were performed in quadruplicate. Only sections with the tissue structure unaltered and intact at the time of evaluation were used.

Statistics

Results were evaluated using Student's *t*-test.

Results

Transplanted kidneys of control animals were almost completely destroyed 10 days after transplantation. On the other hand, only a few necrotic areas and a small number of atrophic tubules were observed in kidneys of MMF-treated rats at that time. These results correlated with the extent of cellular infiltration. While in the MMF kidneys macrophages and lymphocytes were found at 10 days postoperation, only minimal cellular infiltration was observed in the almost completely destroyed kidneys of the control animals (Table 1).

The pattern of leucocyte infiltration differed between MMF and vehicle-treated animals. Three days postoperation, infiltration of neutrophils (MMF: 1.2 ± 0.3 vs 3.7 ± 0.1 in controls, $p < 0.01$) and macrophages (MMF: 16 ± 5.8 vs 45.3 ± 17.2 in controls, $p < 0.01$) was considerably lower compared to MMF-treated animals, whereas the number of lymphocytes (MMF: 28.4 ± 11.1 vs 28.4 ± 11 in controls) was almost identical. In both groups cellular infiltration peaked 5 days postoperation. However, the number of infiltrating leucocytes was markedly reduced in MMF-treated animals. Furthermore, while the number of macrophages decreased in control animals in parallel to the degree of destruction and fibrosis, it remained almost constant in MMF-treated animals.

Leucocytes positive for LFA-1 α were significantly reduced in grafts treated with MMF compared to control animals on days 3 and 5. Surprisingly, ICAM-1 and VCAM-1 expression

Table 1 Effects of MMF and vehicle treatment

Days post-transplant	Group	Lymphocytes (c/fv)	Monocytes/macrophages (c/fv)	Neutrophils (c/fv)	MHC II positive leucocytes (c/fv)	ICAM-1 ^a	LFA-1 α positive leucocytes (c/fv)	VCAM-1 ^a
3	MMF	28.4 + 11.1	16 + 5.8*	1.2 + 0.3	18.5 + 5.7	1.3 + 0.3	4.8 + 0.8	0.3 + 0.1
3	Vehicle	28.4 + 11	45.3 + 17.2	3.7 + 0.1	25.2 + 7.5	1.5 + 0.4	7.4 + 0.7	0.3 + 0.1
5	MMF	27.2 + 6.3	67.8 + 5.3	1.9 + 0.3	46.2 + 2.1	3 + 0.4	14.1 + 6.3	0.3 + 0.1
5	Vehicle	56.4 + 4.2	73.2 + 7	2 + 0.2	43.8 + 4	3.3 + 0.1	19.9 + 4.4	0.5 + 0.2
7	MMF	13.5 + 2.8	63.5 + 6.1	1.9 + 0.4	36.8 + 4.9	3.5 + 0.2	10.4 + 3.5	0.3 + 0.1
7	Vehicle	2.4 + 0.9	37.1 + 1.5	6.1 + 3	48.6 + 7.8	3.5 + 0.2	6.1 + 4.3	0.1 + 0.1

Values are given as mean \pm standard deviation.

^aGraded by visual intensity on a scale 0–4 (0, no staining; 4, extremely strong staining).

* $p < 0.01$.

was almost identical in both groups. There was, however, a difference in MHC II expression. The number of MHC II positive infiltrating cells was reduced in the MMF group at day 3 postoperation and continued on the days 5 and 7. This tendency, however, was not statistically significant. Taken together, adhesion molecules on endothelial cells were affected by MMF treatment, whereas those on lymphocytes were reduced.

Thus far, the results of our binding studies revealed similar results. On days 3 and 5, in particular, the number of LNL derived from both naive and recipient animals was lower in the MMF-treated groups. This effect proved to be statistically significant in those grafts incubated with lymphocytes derived from the same animal. In these cases, MMF significantly lowered the number of lymphocytes bound to transplanted grafts on days 3 and 5 postoperation ($p < 0.01$) (Table 2). While there was a tendency towards lower numbers of lymphocytes derived from naive rats bound to MMF-treated grafts compared to grafts treated with vehicle, the results were only significant for day 5 postoperation. Overall, the number of naive lymphocytes bound to transplanted grafts treated with vehicle was lower than that of lymphocytes derived from transplanted animals. In contrast, lymphocytes derived from MMF-treated animals bound to a lower degree than naive lymphocytes to MMF-treated grafts.

We evaluated particularly the binding to the different kidney structures, i.e. interstitial and tubular epithelial cells and endothelial cells. Although there was a difference regarding the number of lymphocytes bound to the kidneys as a whole, the relative distribution remained the same in all groups. Lymphocyte binding was most prominent in and around tubules, followed by binding to endothelial vessel walls, venules in particular. Binding to interstitial structures tended to increase only in parallel with interstitial fibrosis and sclerosis. Lymphocyte binding to glomerular structures was insignificant.

Discussion

MMF is capable of preventing or reducing the incidence of acute and chronic rejection in *in vivo* experiments.^{3,9,10} This was demonstrated for acute rejection episodes not only in animal experiments but also in human kidney transplantation.⁴ *In vitro* experiments identified an antiproliferative effect of MMF, based on interaction with inosine monophosphate dehydrogenase—an enzyme involved in purine synthesis—and the expression of mannose-rich glycoproteins.^{7,8} As adhesion

molecules are among these mannose-containing glycoproteins, it was not unexpected that MMF additionally affected the expression of adhesion molecules.⁸ Given that adhesion molecules are important for the infiltration of leucocytes into transplanted organs,¹ we investigated whether this inhibition of adhesion molecule expression influences organ transplantation *in vivo*.

Our results were twofold. First, we could demonstrate the beneficial effect of MMF upon acute rejection. Grafts in animals treated with MMF survived longer than those treated with vehicle. This can be attributed to a markedly reduced cellular infiltrate. Most interesting was the striking reduction of macrophages and the comparatively lower reduction of lymphocytes, especially at day 3 postoperation. This implies a significant influence upon macrophages which has not been demonstrated previously, although others observed similar findings in other models of animal transplantation.¹¹ At 3 days postoperation, the start of lymphocytic infiltration, the numbers of lymphocytes observed in the graft were almost identical between MMF- and vehicle-treated rats. This is surprising as the number of lymphocytes bound to the graft at that time differed markedly among these groups. Lymphocyte binding to MMF-treated grafts was significantly lower than in vehicle-treated rats. Additionally, the number of lymphocytes derived from naive animals that bound to MMF-treated animals was higher than that derived from MMF-treated animals. Taken together with the lack of difference between MMF- and vehicle-treated grafts regarding the expression of ICAM-1 and VCAM-1, these findings imply a primary site of action on lymphocytes and not on endothelial or epithelial cells, as has been suggested by previous works.^{7,8} These findings are additionally supported by the reduced expression of LFA-1 on lymphocytes in MMF-treated animals compared to placebo-treated ones.

On the other hand, at day 3 postoperation, binding of naive lymphocytes to endothelial cells of allografted kidneys treated with MMF was markedly reduced compared to control animals, while the number of lymphocytes in the grafts was almost identical. Although VLA-4 is suspected to be responsible for this lymphocyte adhesion, our results do not support this hypothesis, because the expression of VCAM-1, the ligand of VLA-4, was not reduced in our experiments. Based on these findings we would hypothesize that fewer lymphocytes enter the graft of MMF-treated hosts, but remain in the graft for a longer time, thus prolonging recirculation to lymph nodes. The interaction between macrophages and lymphocytes may be important in this setting. Previous studies reported a variety of chemokines and cytokines promoting the interaction of macrophages and lymphocytes, inducing the production of extracellular matrix proteins along which lymphocytes are directed.^{1,12,13} The lack of these molecules, a result of the small number of macrophages inside the grafts, may thus decrease the motility of lymphocytes once they have entered the graft. This would also explain the beneficial effects of MMF on chronic rejection and arterial intimal thickening produced by mechanical injury.^{10,13} Taken together, MMF seems to interact primarily with expression of adhesion molecules on lymphocytes and not on endothelial cells. If there is an additional effect on macrophages, there could be a potential for this drug to influence chronic rejection as has been suggested by previous studies.¹⁰

In conclusion, the beneficial effects of MMF on the process of acute kidney allograft rejection result from an inhibition of leucocyte infiltration into the transplanted kidney. These

Table 2 Results of the binding assay of lymph node lymphocytes (LNL) derived from grafted and naive Lewis rats

Day post-operation	Animals treated with: ^a		Grafts treated with: ^b	
	MMF	vehicle	MMF	vehicle
3	184 ± 37	387 ± 45**	243 ± 26	283 ± 34
5	262 ± 21	480 ± 59**	386 ± 33	443 ± 43
7	211 ± 19	234 ± 33	223 ± 24	301 ± 53*

Cells bound to grafted kidneys expressed as cells per field of view (c/fv).

^aLNL derived from hosts.

^bLNL derived from naive Lewis rats.

* $p < 0.05$; ** $p < 0.01$.

effects are due at least in part to a reduction of lymphocyte adhesion to endothelial cells. Whether the reduced adhesion is based solely on the inhibition of lymphocyte activation and the resulting reduced secretion of inflammatory substances such as cytokines, or whether there is an additional direct effect on macrophages, remains elusive.

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