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# Beneficial effect of preoperative mycophenolate mofetil in murine corneal transplantation

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**Abstract** To investigate the effect of preoperative mycophenolate mofetil (MMF) on allograft survival in a murine corneal transplantation model. Corneal grafting was performed from Brown Norway to Lewis rats. Groups were divided as follows: Rats that received syngeneic or allogeneic grafts without therapy served as controls. MMF treatment was either started 7 days prior to transplantation and continued for 14 postoperative days (POD) or started at the day of corneal grafting until POD 14. MMF (20 mg/kg) administered postoperatively had no significant beneficial effect on corneal graft survival when compared with controls. However, the group receiving 40 mg/kg MMF postoperatively showed a statistically significant prolonged graft survival. A 1-week preoperative administration of 20 mg/kg MMF allowed superior graft survival. Priming the immune system of corneal transplant recipients preoperatively with MMF proved to be a beneficial therapeutic regimen for prolonging corneal allograft survival in rats.

**Key words** Immunosuppressive agents · Keratoplasty · Mycophenolate mofetil

## Introduction

The requirement for an effective, minimally toxic immunosuppressive regimen remains a major obstacle to performing high-risk human corneal transplantation. Acute rejection is the major cause of over 50 % of transplant opacifications in immunological high-risk groups [8, 12]. Cyclosporin A (CSA), a macrolide antibiotic which interferes with interleukin (IL)-2 production, is a very potent prophylactic agent for preventing corneal allograft rejection and is used in some specialised centres after high-risk keratoplasty. Although therapy with CSA allows superior graft survival, its use is limited because of a wide range of side effects (i.e. diabetogenicity, arterial hypertension, hyperlipidaemia, nephrotoxicity).

Mycophenolate mofetil (MMF, the morpholinoethylester of mycophenolic acid) is the first immunosuppressant that has been certified for clinical use in the United States for preventing allograft rejection following renal transplantation in the past 10 years. Its safety and effectiveness in combination with CSA following kidney transplantation has already been proven in several clinical studies [3, 11, 13]. Unlike CSA or tacrolimus, mycophenolic acid does not interfere with IL-2 pathways. Mycophenolic acid reversibly inhibits the de novo formation of guanosine nucleotides [1] by inhibiting the enzyme inosine monophosphate dehydrogenase. As T- and B cells are predominantly dependent on the de novo synthesis of guanosine nucleotides, the purine biosynthesis of these cells is selectively inhibited [7]. We have already been able to prove the potency of this

drug and its syngeneic effect to CSA in delaying corneal allograft rejection in the rat keratoplasty model [9]. Gregory et al. showed that, when mycophenolic acid and rapamycin were administered 3 days preoperatively, arterial intimal thickening after balloon-catheter arterial intimal injury was significantly reduced compared with the postoperative treatment [4]. This study focused on the antiproliferative effect of both MMF and rapamycin, but it also favoured the idea that preoperative administration of MMF might be a more effective immunosuppressive therapeutic regimen.

The purpose of this study was to investigate whether preoperative "priming" of the recipients immune system with MMF can prolong graft survival following corneal transplantation in a murine keratoplasty model.

## Methods

An orthotopic perforating keratoplasty was conducted according to the technique of Herbort et al. [5]. Inbred strains used in this experiment were Lewis ($Rt^{le}$) and Brown Norway ($Rt^{ln}$), (Janvier, France), which differ in major and minor histocompatibility antigens. All animals were females weighing between 200 g and 240 g. The animals were obtained and cared for in accordance with the Directives of the European Community and the recommendations of the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication no. 85–23; revised 1985).

### Operative technique

Before surgery, all animals were given phenylephrine (10%) eye drops administered three times at 10-min intervals. During general anaesthesia with diethyl ether, full-thickness corneal grafts were excised from the Brown Norway (BN) donors using a 3.5-mm trepan. Two grafts were obtained from each donor. Donor buttons were stored at room temperature for approximately 20 min in a conservation medium for corneae (Likorol) until implantation.

The recipient Lewis rats were pretreated the same way as the donors. After a brief inhalation anaesthesia with diethyl ether, the rats were anaesthetised with an intraperitoneal mixed injection of ketamine hydrochloride (100 mg/kg bw), midazolam (0.5 mg/kg bw) and atropine (0.5 mg/kg bw), and fixed in a dextral lateral position. The grafts were sutured into a full-thickness, 3.0-mm central corneal bed of the recipients left eye. The transplant was sewn in with eight interrupted sutures (Ethicon 11.0). The anterior eye chamber was restored at the end of the operation by the instillation of balanced saline solution. At the end of the procedure, a tarsorrhaphy was performed with two interrupted sutures (Prolene 6.0) and remained in place for 3 days, and Refobacin (gentamicin) was applied in the palpebral fissure.

The animals were grouped as follows:

Group 1 ($n = 6$). Lewis/Lewis (no therapy, control 1)
Group 2 ($n = 8$). BN/Lewis (no therapy, control 2)
Group 3 ($n = 9$). BN/Lewis (MMF, 20 mg/kg, starting at the day of transplantation)
Group 4 ($n = 7$). BN/Lewis (MMF, 40 mg/kg, starting at the day of transplantation)
Group 5 ($n = 8$). BN/Lewis (MMF, 20 mg/kg, starting at preoperative day 7)
Group 6 ($n = 8$). BN/Lewis (MMF, 40 mg/kg, starting at preoperative day 7)

Medication in the therapy groups was given orally once a day until POD14 with the use of a feeding gavage.

### Clinical evaluation

All rats were subjected to a clinical examination by two independent examiners every third day for the duration of, at most, 8 weeks. Each animal was examined by means of slit-lamp microscopy during a brief inhalation anaesthesia with diethyl ether. The transplants were evaluated by means of the following scoring system, which assessed opacity, oedema and neovascularisation.

Opacity:
0 No opacity
1 Slight opacity – details of iris clearly visible
2 Some details of iris no longer visible
3 Pronounced opacity – pupil still recognisable
4 Total opacity
Edema:
0 No oedema
1 Mild oedema
2 Pronounced oedema with raised transplant
Neovascularization:
0 No vessels
1 Vessels in the periphery
2 Vessels extending to the middle periphery
3 Vessels extending to the centre

The target criterion was complete opacification (i.e. rejection) of the transplant. As soon as the donor cornea had been clinically identified as rejected, the animal was sacrificed through the inhalation of $CO_2$.

### Histological and immunohistological evaluation

After 8 weeks, or following a clinically diagnosed complete opacification of the transplant, the recipient animals were sacrificed by $CO_2$ inhalation. Subsequently, the transplanted eye was enucleated and fixed in a buffered formalin solution (4%). The formalin-fixed eyes were cut into 4-μm-thick preparations and subjected to haematoxylin-eosin or elastica staining for histological assessment. For immunohistological evaluation, the 4-μm-thick preparations were pretreated with a Histosafe-Enhancer (Serotec, Canada) to improve antibody affinity. The preparations were then subjected to an immunohistological examination using the avidin-biotin-peroxidase complex method. The primary monoclonal antibody used was MCA 48 g (Serotec, Canada), which reacts with rat CD8 antigen. The secondary antibody, a biotin-marked rabbit-anti-mouse antibody, and the reagents for the third-phase reaction, were obtained from the Serotec company (Vectastain Elite ABC peroxidase kit). 3–3 Diaminobenzidine (peroxidase substrate kit) was used as the substrate for the peroxidase.

### Statistical analysis

Statistical analysis was performed between the various groups using the Mann-Whitney U test. Times are reported as mean (± SEM).

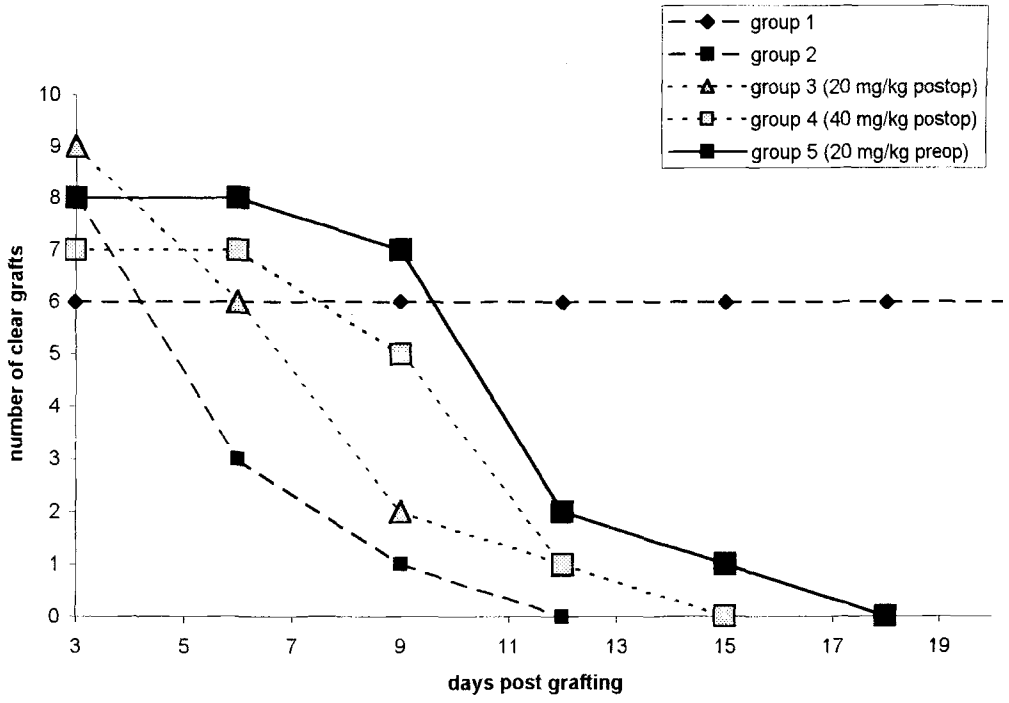


**Fig. 1** Transplant survival rate in the control and therapy groups; clear grafts are defined as corneas that do not meet the clinical rejection score (opacity = 4). *Group 1* Syngeneic control; *group 2* allogeneic control; *group 3* mycophenolate mofetil (MMF; 20 mg/kg) starting at the day of transplant; *group 4* MMF (40 mg/kg) starting at the day of transplant; *group 5* MMF (20 mg/kg) starting 1 week preoperatively

## Results

### Clinical evaluation

Figure 1 shows the transplant survival rate in the control and therapy groups. Transplants that opacified in the first 3 days postoperatively represented errors of operative technique (bleeding, suture dehiscence, lens opacification) and were omitted from the study (two animals in group 1 and one animal in group 4). Cornea transplantation in the syngeneic combination group (group 1, Lewis/Lewis) led to slight perioperative stromal transplant oedema, which was no longer detectable from the sixth postoperative day onwards. Throughout the entire period of the examination, the transplants remained clear. Neovascularisation was detectable only in the area of the sutures. The average transplant survival rate in the allogeneic combination group (group 2, Brown Norway/Lewis) was 7.9 days (SEM 1.1). The postoperative low-dose (20 mg/kg) therapy with MMF (group 3) showed no beneficial effect on graft survival (8.7 days, SEM 0,8). The postoperative therapy with high-dose MMF (40 mg/kg, group 4) led to a statistically significant prolongation of transplant survival to 11.6 days (SEM 0.9, $P < 0.05$ compared with control group 2). When this high-dose MMF was applied to the recipients preoperatively (group 6), therapy had to be discontinued because of drug toxicity, which manifested itself as severe weight loss after the operative procedure. MMF (20 mg/kg) started 1 week prior to operation extended graft survival to 12.4 days (SEM 0.7, group 5). This was statistically significant compared with the allogeneic control (group 2, $P < 0.05$) but not to the high-dose postoperative therapy group (group 4).

### Histology and immunohistology

In the eighth postoperative week, the transplants of the syngeneic control group (group 1) showed isolated foreign-body giant cells and a low level of mononuclear infiltrate only in the area of the interrupted sutures. The central periphery and the centre of the transplant revealed no cellular infiltration whatsoever, and the configurations of epithelium, stroma and endothelium were normal. Allografts from control rats (group 2) taken at the point of maximum rejection, showed a pronounced oedema recognised by an augmentation in the thickness of the stroma and the development of vacuoles, with the histological picture of a bullous keratopathy. This was particularly evident in the area of the basal membrane of the epithelium. Mononuclear cell infiltration was present in all layers of the transplants, but was most pronounced in the area of the stroma and the deeper layers of the epithelium. An extremely dense mononuclear infiltrate was located at the graft margin and in proximity to the sutures, where an accumulation of macrophages was also to be found.

Immunohistological staining with MCA 48 g, a monoclonal antibody which reacts with rat CD8 antigen, revealed that approximately one-third of the infiltrating lymphocytes were CD8 + at the time of complete rejec-

tion. The composition of the inflammatory cell population was the same in all therapy groups.

### Compatibility of MMF

All rats treated with 40 mg/kg MMF preoperatively developed diarrhoea during the first postoperative days; therefore, therapy had to be discontinued due to severe weight loss. When therapy was started postoperatively, two rats in the high-dose groups suffered diarrhoea during the first postoperative week. However, this clinical symptom was no longer evident in the second postoperative week and did not require a reduction in dosage. Low-dose therapy (20 mg/kg) with MMF was well tolerated, even when given preoperatively. There was no evidence of gastrointestinal side effects, weight loss, perioperative infections or interference with wound healing in the preoperative low-dose therapy group.

## Discussion

MMF has been shown to be a safe and effective agent in inhibiting solid organ rejection both clinically and experimentally. However, to our knowledge, all experimental and clinical studies of the effectiveness of MMF to suppress allograft rejection have been designed solely with postoperative treatment protocols. In this study, we have shown superior graft survival when applying low-dose MMF preoperatively compared with the postoperative high-dose therapy, albeit not to a statistically significant extent. This low-dose therapeutic regimen was well tolerated as there were no signs of gastrointestinal side effects compared with the postoperative high-dose therapy, where two of seven animals developed diarrhoea. However, it is fair to mention that graft survival is still significantly better with a postoperative 10 mg/kg CSA treatment protocol [9].

One concern with preoperative immunosuppression is that it might lead to a higher incidence of perioperative infections or interference with wound healing; however, this did not seem to be the case in this study. Rats that received high-dose MMF preoperatively suffered severe gastrointestinal toxicity and became emaciated in the first postoperative week leading to the sacrifice of these animals, which was not seen in the low-dose group. We speculate that the general anaesthesia led to increased susceptibility to MMF toxicity in the preoperative high-dose group. Gastroenterotoxicity (i.e. diarrhoea) has also been the most common adverse effect in clinical studies following renal transplantation (12.7–36.1 %) [10]; in most cases it was transient and therefore discontinuation of therapy was not indicated. The gastroenterotoxicity can be explained partially by the enterohepatic circulation of MMF [2]. We are conducting a randomised clinical trial with 2 g/day MMF monotherapy following high-risk keratoplasty (19 patients with a mean follow up of 6 months) and have, until now, not seen any gastrointestinal side effects (unpublished data).

Histological and immunohistological evaluation carried out after rejection had taken place revealed that approximately one-third of the infiltrating lymphocytes were CD8 + in both the pre- and postoperative treated grafts. This is consistent with previous findings in this model of corneal transplantation [6]. We did not find a different composition of the inflammatory cell population in the grafts of the different treatment protocols.

In conclusion, conditioning the corneal transplant recipients' immune system preoperatively with low-dose MMF is a well-tolerated therapeutic regimen and has been shown to have a beneficial effect on delaying acute rejection following corneal transplantation.

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