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World J Gastroenterol 2005;11(18):2733-2738
World Journal of Gastroenterology ISSN 1007-9327

• *BASIC RESEARCH* •

Effects of mycophenolate mofetil *vs* cyclosporine administration on graft survival and function after islet allotransplantation in diabetic rats

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Received: 2004-10-30 Accepted: 2004-12-08

Abstract

AIM: To develop an experimental model of islet allotransplantation in diabetic rats and to determine the positive or adverse effects of MMF as a single agent.

METHODS: Thirty-six male Wistar rats and 18 male Lewis rats were used as recipients and donors respectively. Diabetes was induced by the use of streptozotocin (60 mg/kg) intraperitoneally. Unpurified islets were isolated using the collagenase digestion technique and transplanted into the splenic parenchyma. The recipients were randomly assigned to one of the following three groups: group A (control group) had no immunosuppression; group B received cyclosporine (CsA) (5 mg/kg); group C received mycophenolate mofetil (MMF) (20 mg/kg). The animals were killed on the 12th d. Blood and grafted tissues were obtained for laboratory and histological assessment.

RESULTS: Median allograft survival was significantly higher in the two therapy groups than that in the controls (10 and 12 d for CsA and MMF respectively *vs* 0 d for the control group, $P<0.01$). No difference in allograft survival between the CsA and MMF groups was found. However, MMF had less renal and hepatic toxicity and allowed weight gain.

CONCLUSION: Monotherapy with MMF for immunosuppression was safe in an experimental model of islet allotransplantation and was equally effective with cyclosporine, with less toxicity.

Key words: Diabetes; Islet transplantation; Rats; Mycophenolate mofetil; Cyclosporine

Fotiadis C, Xekouki P, Papalois AE, Antonakis PT, Sfiniadakis I, Flogeras D, Karampela E, Zografos G. Effects of mycophenolate mofetil *vs* cyclosporine administration on graft survival and function after islet allotransplantation in diabetic rats. *World J Gastroenterol* 2005; 11(18): 2733-2738
http://www.wjgnet.com/1007-9327/11/2733.asp

INTRODUCTION

Islet cell transplantation can provide a minimally invasive method of restoring euglycemia and insulin independence early in the course of diabetes mellitus[1]. This alternative treatment comprises a very promising therapeutic approach since the early 1970s[2-4] and recent reports in the literature support this hypothesis[5-9]. However, the results "fall short" compared to the expectations mainly due to the loss of thousands of islets during the three-stage islet isolation process, the need for immunosuppressive agents having significant diabetogenic side effects and the difficulty to detect graft rejection early[10]. Thus, the reported 1-year insulin-independent survival after islet transplantation in 1999 was only 14%[11].

A major issue in transplantation is graft rejection. The administration of immunosuppressive drugs such as cyclosporine (CsA), tacrolimus and corticosteroids is essential in order to prevent this complication[12]. Nevertheless immunosuppressants are not devoid of serious side effects such as the induction of diabetes, nephrotoxicity and carcinogenesis[13-17].

In 1993 mycophenolate mofetil (MMF), the 2-4-morpholino ethyl ester of mycophenolic acid, the biologically active component, was introduced as a novel immunosuppressive agent[18]. MMF reversibly inhibits the enzyme inosine monophosphate dehydrogenase (IMPDH), an important enzyme in de novo synthesis of purine building blocks of DNA, namely guanine and adenine[19]. Lymphocytes, which play a significant role in graft rejection, have no way of producing adequate amounts of purines if IMPDH is not available[20]. Thus, MMF prevents proliferation of both T cells and B cells and thereby inhibits antibody production. Moreover, through depletion of intracellular GTP levels in lymphocytes, MMF suppresses glycosylation and the expression of some adhesion molecules, thereby reducing

lymphocyte migration to the transplant[21]. However, its effect on T cell proliferation received more attention because of the importance of T cells in the allogeneic response[22].

MMF is considered as a safe drug and the most frequently reported side effects are mild and involve mainly the gastrointestinal system (diarrhea, abdominal pain, nausea and vomiting)[23-25]. Its major advantages are the lack of nephrotoxicity and diabetogenic effects, which makes MMF an important agent in renal and islet transplantation. Although its use in renal transplantation has been established[26–28], its use in clinical islet transplantation is still limited[29,30]. There are also a few reports regarding the use of MMF in combination with other regimens in experimental models of islet xeno- and allotransplantation[31-37] but in very few cases as monotherapy.

The aim of this study was to evaluate the efficacy of MMF as monotherapy on islet graft function and development in a period as early as 12 d after allogeneic islet transplantation in chemically induced diabetic rats, with a dose lower than the usually administered and to compare it with the efficacy of a widely used immunosuppressant such as cyclosporine A.

MATERIALS AND METHODS

Animals and experimental groups

Thirty-six male Wistar rats and 18 male Lewis rats weighing 220-300 g were used as recipients and donors respectively (animals were obtained from Pasteur Institute, Athens, Greece and Democretos Research Center, Athens, Greece). All the principles for laboratory animal care were followed according to the European Union Regulations and the Greek law for the use of laboratory animals (Act 160, Volume 64, No. A, May 1991, License Ref. 1267/2570). The recipients were randomly allotted to three groups of 12 animals: group A (control group) had no immunosuppression, group B received CsA (Neoral - Novartis) at a dose of 5 mg/kg, and group C received a low dose MMF (CellCept - Roche) (20 mg/kg), which is half the usual dose.

Induction of diabetes

Diabetes was induced by single intraperitoneal injection of 60 mg/kg streptozotocin (STZ - Sigma - S-0130) freshly resolved in a solution of PBS (phosphate buffer solution Sigma 1000-3) at a concentration of 10 mg/mL (pH 4.5 using citric acid) 7 d prior to transplantation. It has been documented that doses bigger than 50 mg/kg cause irreversible and complete destruction of beta cells in adult rats[38].

Isolation of unpurified islets

Islets were acquired with a modification to the technique previously described by Papalois *et al*[39]. Briefly, after the animals were anesthetized a midline abdominal incision was performed and the common bile duct was recognized and ligated in its middle. Then the duct was catheterized with a fine catheter (polyethylene tubing, PE 10 - ID 0.28 mm and *A* 0.61 mm - Becton Dickinson) and 6 mL of collagenase (Sigma - Type XI - C 7657) solution (0.9 ng/mL) were infused slowly until the pancreas was distended. Subsequently pancreatic resection was performed and the donor was killed. The pancreatic specimen was incubated in water bath (at 37 ℃) for 20 min. After incubation the distended pancreas was washed twice in cold Hanks solution in order to terminate collagenase activity and to wash out collagenase and fat tissue. The product was then filtered through a 400-μL filter in order to retain duct remnants, sutures, lymph nodes and pancreatic capsule remnants. The cell suspension was then considered ready for transplantation. We omitted the separation of the endocrine from the exocrine tissue in order to avoid the loss of islet yield during the purification process. A 100-μL sample was taken and islets were counted by dithizone staining. The mean±SE islet yield for transplantation was 1 812±145.

Transplantation technique

Spleen was used as the site of transplantation[40–42]. The rats were anesthetized as previously described and 0.7 cc of the isolated cell suspension was injected slowly using an insulin syringe into the splenic parenchyma. Leakage was avoided with a 2-0 silk suture tied at the spleen's pole.

Determination of biochemical indices

Blood glucose levels were measured in blood obtained from rat-tails using a Glucometer Elite blood glucose-measuring instrument (Bayer AB, Gothenburg, Sweden). Measurements were performed one week prior to transplantation (d -7), right after transplantation (d 0) and at the 3rd, 5th, 7th, 10th and 12th d post-transplantation. Diabetes was diagnosed when two consecutive glucose readings were over 180 mg/dL. Grafts were considered as functional when blood glucose levels were below 200 mg/dL after the 2nd post-operative d for two consecutive measurements. The 2 d interval was allowed for islets to become functional. Liver enzymes (SGOT, SGPT, γGT) as well as creatinine values were determined from blood samples obtained on d 12 from 11 animals of the two therapy groups and from 5 animals of the control group that were still alive. The normal range of each of the biochemical indices for the Wistar rats used in our laboratory is as follows: SGOT: 116-278 IU/L, SGPT: 29-80 IU/L, γGT: 0.6-2.1 IU/L, creatinine: 0.4-0.8 mg/dL.

Animal sacrifice

The overall time of observation was 12 d. On the last day the animals were killed and blood as well as grafted tissues were obtained for laboratory assessments and histological examination.

Histological examination of the grafted tissues

Spleens carrying the transplanted islet grafts were removed and fixed in formalin saline. Paraffin tissue sections (3-μm thick) were stained with hematoxylin-eosin. Intracellular content of insulin was demonstrated immunohistochemically using the peroxidase technique by means of polyclonal anti-insulin antibodies (rabbit anti-insulin, Monosan, Netherlands) and with an indirect biotin streptavidin detection kit (*i*View™ DAB detection kit, Ventana Medical Systems, SA, France), which detects mouse IgG, mouse IgM, and rabbit polyclonal primary antibodies. Pancreas tissue was used as positive control. We estimated the intensity of staining as weak and intense. Evaluation was performed using light microscopy

(HE ×200). The presence of insulin positive cells and intact islets was assessed using a semi-quantitative scoring system: none (0-1), occasional (1-8), many (9-18) and plentiful (>18) (numbers representing count of islets per 10 big optic fields). The presence of infiltrating inflammatory cells was also assessed using a semi-quantitative method (0 = occasional infiltrating cells, + = few, ++ = moderate, +++ = many, ++++ = massive infiltration)[35]. Eleven out of the twelve grafted sites were available for histological examination from the CsA and MMF groups (one rat from each group died during the transplantation procedure). All grafted tissues from the control group were obtained for histological assessment regardless of the animal's day of death.

Statistical analysis

All values were presented as mean±SD. Statistical analysis was performed using the Student's *t* test comparing group means. To evaluate the differences in graft survival, the Kaplan-Meier survival analysis was performed and a log rank test was used for the evaluation of differences. *P* values less than 0.05 were considered significant. Comparisons among time measurements of each variable during treatment period for each study group were analyzed using one factor repeated measures ANOVA (pair wise multiple comparisons were performed using Tukey critical difference). Mann-Whitney *U* test (exact significance) was used for the comparisons between groups.

RESULTS

The operation-related mortality in the three groups was as follows: no rat died in the control group, 1 out of 12 rats died in group B (8.3%) and 1 out of 12 died in group C (8.3%). All rats in CsA and MMF groups that survived the operation were alive at the 12th d (the day of killing). In the control group, however, mean survival was 8.1 d and only five rats reached the day of killing (41.7%). This difference was statistically significant (P<0.01, Table 1).

Table 1 Islet allograft survival and animal survival in recipients treated with CsA and MMF

Variable	Group A Controls n = 12	Group B CsA n = 11	Group C MMF n = 11	P x^2
3rd-d graft survival (%)	3 (25)	9 (82)	9 (82)	<0.01
Graft survival in days after Tx (median SD)	0 (1.2)	10 (4.9)	12 (5.1)	Kruskal Wallis <0.01
Animal survival in days after Tx (median SD)	8.1 (3.7)	12 (0.0)	12 (0.0)	Kruskal Wallis <0.01

Tx: transplantation, CsA: cyclosporine A, MMF: mycophenolate mofetil.

The functional outcome of islet allografts was evaluated after d 3 (Table 1). In the control group, 3-d allograft survival was significantly less than both cyclosporine and MMF groups (25% *vs* 82%, P<0.01, Table 1). Median allograft survival was also significantly higher in the two therapy groups than that in the controls (10 and 12 d for CsA and MMF respectively *vs* 0 d for the control group, P<0.01, Table 1). Actuarial allograft survival was calculated for all three groups and the Kaplan-Meier curves were constructed (Figure 1). In both therapy cases the differences in allograft survival in comparison to the control group were statistically significant (log rank test, P<0.01 for MMF *vs* control and P<0.01 for CsA *vs* control). In contrast, the difference in actuarial allograft survival between the CsA and MMF groups was not statistically significant (log rank test, P = 0.505).

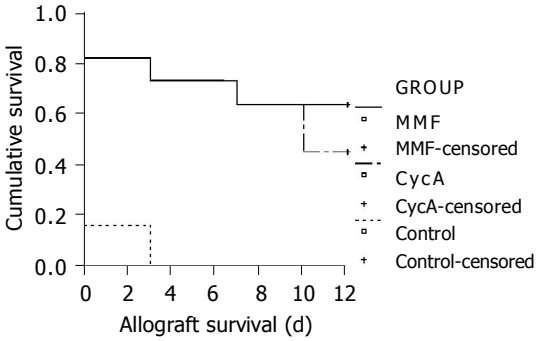

Figure 1 Actuarial allograft survival curves in the three study groups based on serum glucose levels, showing significantly prolonged survival in the two therapy arms in comparison to the controls.

Glucose changes from baseline were recorded (Figure 2). Significant overall differences between the three groups were observed at the 3rd, 5th, 7th, 10th and 12th post-transplantation day (ANOVA, Figure 2). These differences, at all times but one, were attributed to higher glucose levels in the control groups (Tukey). The difference between the CsA and the control group was not significant only on the 3rd post-transplantation day and consequently the overall difference was attributed to lower glucose levels in the MMF group. The glucose values over time tended to be at lower levels in the MMF group compared to the CsA group although this observation was not statistically significant (P = 0.747).

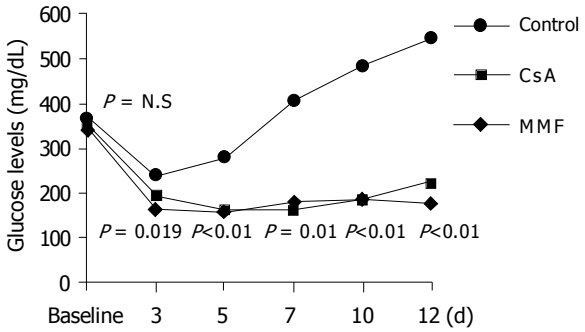

Figure 2 Effect of MMF and CsA on the blood glucose levels of the recipients over time after transplantation.

Weight changes were also recorded for all recipients (Figure 3). Statistically significant differences in the proportion of weight change from baseline were found between the three groups after the 7th post-operative day. These changes were significant also in the 10th and the 12th d. The MMF

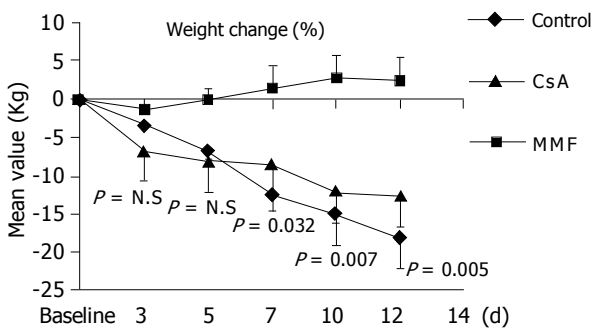

Figure 3 Effect of MMF and CsA on the percentage of weight change of the recipients.

group (Figure 3) was the only one in which weight gain was recorded, although this observation was not statistically significant.

Post-transplantation biochemical data (SGOT, SGPT, γGT and creatinine) in the two therapy cases and in the control group were also recorded. All the post-transplantation biochemical parameters in the control group and in the MMF group were within normal range. The same observation was made for the CsA group, except for γGT values (Table 2). All biochemical data recorded, except for SGOT values, were significantly lower in the MMF group in comparison to corresponding values in the CsA group (Table 2). Compared with the control group creatinine levels were significantly lower in the MMF group ($P<0.027$) and γGT levels were significantly elevated in the CsA group ($P<0.01$, Table 2).

Table 2 SGOT, SGPT, γGT and creatinine measurements in the controls as well as in the two therapy groups

Variable	Group A Controls	Group B CsA	Group C MMF
SGOT (mean, SD)	144 (27.6)	119 (47)	131 (46)
SGPT (mean, SD)	48.4 (12.9)	66 (27)	33 (15)[f]
γGT (mean, SD)	1.14 (0.42)	2.8 (0.5)[b]	0.7 (0.5)[f]
Creatinine (mean, SD)	0.6 (0.16)	0.6 (0.1)	0.4 (0.1)[d,f]

[b]$P<0.01$ *vs* controls, [d]$P<0.027$ *vs* controls, [f]$P<0.01$ *vs* CsA.

Light microscopy histological assessment of the grafts from the three different groups revealed the appearance of exocrine tissue, islets clumped together or free-lying in the surrounding exocrine tissue, peri-islet tissue inflammatory infiltration and areas of necrosis (Table 3). In untreated rats a massive infiltrate covered most of the allografts and completely destroying most of them (Table 3). Twelve days after transplantation various numbers of islets and various degrees of infiltration were observed in allografts removed from animals treated with CsA or MMF alone. Immunohistochemical staining for insulin in the control group was relatively weak (Figure 4A), whereas in both treatment groups a marked number of cells had more intense insulin staining (Figures 4B and C). Bigger and better developed islets were found in the MMF-treated group (Figure 4C).

Table 3 Histologic evaluation after transplantation

Group Animal	CsA Inf/End	MMF Inf/End	Controls Inf/End
1	0/Occasional	0/Occasional	0/Occasional
2	0/Occasional	+/Occasional	+++/None
3	0/Many	+/Occasional	++++/None
4	++++/Occasional	+/Plentiful	++++/None
5	++++/Occasional	+++/Plentiful	++++/None
6	++++/Many	++++/Occasional	++/Occasional
7	++++/Many	++++/Occasional	++/Occasional
8	++++/Many	++++/Occasional	+++/Occasional
9	Necrosis	++++/Occasional	++++/Occasional
10	Necrosis	++++/Occasional	Necrosis
11	Necrosis	Necrosis	Necrosis
12	–	–	Necrosis

Inf: infiltrating cells, End: endocrine cells. Sections with abundant necrosis were not evaluated.

DISCUSSION

In the present study we compared the efficacy of MMF and CsA in an experimental model of islet allotransplantation. The present study was based on a previous one[45] in which the efficacy of two different doses of MMF (12 and 23 mg/kg) with CsA (5 mg/kg) was compared. In the previous study MMF in the dose of 23 mg/kg was equally effective with CsA in maintaining graft function. However, graft survival in the group of 12 mg/kg was not satisfactory. Consequently it was decided to omit the MMF group that was not effective, trying to have higher MMF levels and at the same time to test a dose that is half of that presented in many reports.

Immunosuppression with cyclosporine (5 mg/kg) maintained graft function for a median of 10 d, while administration of MMF at a dose of 20 mg/kg was equally effective prolonging graft survival for a median of 12 d. The present results are in accordance with those reported by other researchers demonstrating that treatment with CsA and MMF alone or in combination with other immunosuppressants reduces allograft and xenograft rejection. However, in these studies higher doses were employed (10-30 mg/kg for CsA and 40 mg/kg for MMF)[32-37]. Previously reported data have shown that CsA increases insulin resistance and has nephrotoxic and hepatotoxic effects, especially when combined with other agents such as glucocorticoids and sirolimus[41-43]. In clinical studies MMF has been shown to be a safe drug and furthermore there is evidence that it not only maintains the graft but improves renal function as well[26-28].

The ability of MMF to preserve islet allo- and xenograft function is not only due to the selective antiproliferative effect on B and T cells, but also due to the protection of the microvasculature from the immune response, even from the 10th d after transplantation. As a consequence the grafts' nutritive microcirculation[44] is preserved. In the present study, despite the limited observation period, the fact that larger and better-developed islets were found in the MMF group in comparison to the cyclosporine group suggests an immediate beneficial effect of MMF in the preservation of islet architecture. Interestingly, in our previous report

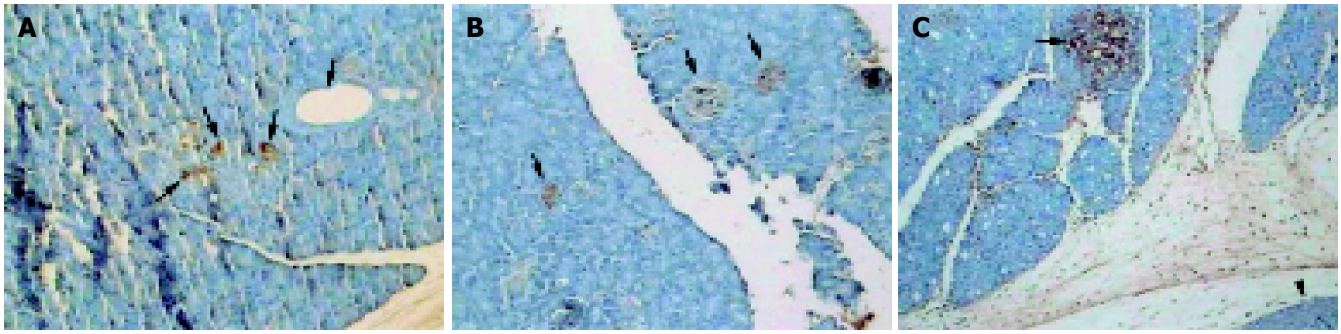

Figure 4 **A**: Three weak stained islets (long arrows) located into the exocrine tissue 12 d after transplantation without immunosuppression. A dilated pancreatic duct (short arrow) is also present (anti-insulin, ×200); **B**: Intact islets into the exocrine tissue (long arrows) 12 d after engraftment and immunosuppression with CsA. The insulin staining is positive and intense (anti-insulin, ×200); **C**: A well-developed and large islet (long arrow) into the exocrine tissue 12 d after transplantation and immunosuppression with MMF. Anti-insulin stain shows beta cell granulation within islets. At the lower right corner of the figure splenic parenchyma is present (short arrow) (anti-insulin, ×200).

such beneficial effect on islet architecture was observed even when approximately half of the present dose of MMF was used[45].

It was observed that transplantation of allogeneic islet tissue without immunosuppression resulted in 100% rejection within few days. It has been well established that the islet allografts survive about 5 d in diabetic rats without immunosuppression[37]. The fact that very few of the grafts in the control group became functional after the 3rd d of transplantation is probably due to the presence of exocrine tissue. Exocrine tissue contamination of freely transplanted pancreatic islets deteriorates the process of graft revascularization and induces additional injury by provoking a deleterious inflammatory response and consequently leading to graft destruction[46,47]. Taking into account the massive infiltration detected in the grafts of untreated recipients in the present study, this might be the reason for the low graft survival in this group.

In this experimental model of islet transplantation creatinine, SGPT and γGT values were found to be lower in the MMF group compared to those found in the CsA group and with the exception of γGT values all other biochemical data in the CsA group did not exceed the normal range. The significantly increased γGT values found in the CsA case are indicative of a cholostatic effect of the specific drug, a well-documented side effect[48]. Interestingly, creatinine values were found to be lower in the MMF group compared to controls. This finding is in accordance with the published data that MMF may protect from and even reverse nephrotoxicity caused by other immunosuppressant agents, such as CsA[26,49]. The fact that the MMF group was the only one in which weight gain was recorded indicates that this agent is well tolerated without serious side effects.

In conclusion, low dose MMF provided effective immunosuppression in an experimental allograft islet transplantation model and compared favorably to CsA in terms of islet morphology and side effects. Given the fact that complications of immunosuppressive therapy continues to be one of the major hurdles to successful islet transplantation, management of immunosuppression requires careful risk *vs* benefit assessment. Favorable benefit/side effects ratio for the biochemical and histological parameters with the low dose monotherapy of MMF was observed in the present study, compared to data presented in other reports. This drug might represent a standard suitable immunosuppressive agent for improving the out come of pancreatic islet allo-transplantation.

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