Transplantation of purified islet cells in diabetic BB rats

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Summary. The ability to prepare purified islet Beta-cell aggregates was used to examine the survival of this cell type after allotransplantation in diabetic BB rats. The aggregates were intraportally implanted in numbers that were previously found to correct a streptozotocin-induced diabetic state in syngeneic or allogeneic Brown Norway recipients. When the grafts were prepared from RT1^u donors, which shared the MHC-class I antigen with the BB recipients (RT1^u), their implant sites became diffusely infiltrated by inflammatory cells and their metabolic function was completely lost within 5 weeks. MHC-class I incompatible islet Beta-cell allografts (RT1ⁿ) exhibited a longer survival, in particular when combined with other islet endocrine cells and/or when covered by a 5-week cyclosporin treatment. In the latter combination, 10 of 12 BB rat recipients remained normoglycaemic over the 10-week observation period, their

liver implants presenting a comparable insulin reserve and similarly discrete mononuclear cell infiltration as streptozotocin-diabetic Brown Norway rats receiving this treatment. However, administration of cyclosporin to diabetic BB rats was associated with a morbidity that was not observed in drug-treated streptozotocin-diabetic Brown Norway animals or in untreated diabetic BB rats. It is concluded that MHC-incompatible islet Beta cells can induce a long-term normalization in diabetic BB rats provided that they are implanted under conditions which allow allograft acceptance. The standardized preparation of purified islet Beta-cell grafts can help assessing the conditions for successful transplantations in diabetes with an autoimmune origin.

Key words: Transplantation, diabetes mellitus, islet cells, BB rats, cyclosporin.

Purified endocrine rat islet cells survive transfer across a major histocompatibility barrier markedly better than unpurified islet preparations [1]. As a result of this property, allografts of purified islet cell aggregates can maintain a long-term normoglycaemia in animals with chemically-induced diabetes [1]. The implants elicit a mononuclear cell infiltration but the immune response is mild and delayed [1] and hence suppressable by short-term cyclosporin treatment [2]. In addition to their lower immunogenicity, purified islet cell aggregates offer the advantage of a standardized preparation, the size and cellular composition of which can be precisely determined in vitro [3]. For these reasons, they appear more appropriate than previously used preparations in assessing the applicability of islet cell transplantation in autoimmune diabetes. In this form of the disease, destructive processes against pancreatic Beta cells can recur as soon as a new pool of islet Beta cells is presented to the immune system [4, 5]. The reappearance and severity of the autoimmune phenomena may, however, depend on the number of grafted Beta cells, on their immunogenicity as well as on the simultaneous transfer of

other cell types. Each of these variables can be tested separately with in vitro constructed islet cell implants of selected size and cellular composition. In the present study, we have examined whether allografted islet Beta cells can survive after implantation in diabetic BB rats, which are known to have developed autoimmune reactions against their own pancreatic Beta cells [6]. The grafts were composed of a fixed number of islet Beta cells that were purified from rat strains with or without disparity in MHC-class I antigens.

Materials and methods

Preparation of donor tissue

Donor tissue was prepared from adult male rats (200–300 g body weight) of the inbred R/A (RT1^p), identical in defined histocompatibility antigens to the RP strain, formerly R1) strain and of the inbred Brown Norway (BN) (RT1ⁿ) strain (colonies of Heverlee, Belgium, [3]). We recently described the methods used for the isolation of islets and the preparation of islet cell aggregates composed of

either purified islet Beta cells or of mixed endocrine islet cells [3]. The cell aggregates were cultured for 4 days in RPMI-1640 containing 8.3 mmol/l glucose, 25 μ mol/l isobutyl methyl xanthine, 25 μ mol/l dibutyryl cyclic AMP, 2 mmol/l L-glutamine, 10% (volume/volume) heat-inactivated fetal calf serum supplemented with penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml). After culture they were filtered through a percoll layer of density 1.04 in order to remove cell debris and then resuspended in albumin-free medium for transplantation [1].

Recipients

Donor tissue was implanted by intraportal injection in diabetic male BB/Hagedorn rats ($RT1^{u/u}$). The animals were born at the Hagedorn Institute and shipped to Brussels after 60 days of age. Clinical onset of diabetes occurred between the ages of 60 and 120 days. All animals were treated with insulin (zinc protamine insulin, a gift of Nordisk, Gentofte, Denmark) from the day of clinical onset until the day of transplantation, 4 to 20 weeks later; insulin administration was discontinued after transplantation. Recipients were kept in metabolic cages to measure weekly urine volumes over 24 h. Urine volume and body weight were measured weekly, plasma glucose levels monthly. A state of normalization was defined by a daily urine volume under 16 ml and plasma glucose levels under 11 mmol/l. For each experimental group, the number of normalized recipients was taken as an index for the graft survival at a particular time after implantation [1].

Three groups of transplanted rats and one group of diabetic control rats were treated with cyclosporin for 5 weeks. The drug (donated by Sandoz, Basel, Switzerland) was solubilized in olive oil (10 mg/ml) and administered by gavage. One dose of 10 mg/kg was given 16 h before transplantation; during subsequent days, the animals received one daily dose of 5 mg/kg. Previous experiments had shown that the administration of olive oil alone did not influence graft survival [2]. Cyclosporin levels in plasma were determined using the Sandoz radioimmunoassay kit [7].

Analysis of liver and pancreas

Liver and pancreas of recipients and controls were examined 10 weeks after transplantation, or earlier in the case of spontaneous death. Fragments were taken for histology and the remaining tissue was extracted for the insulin assay. A detailed description of the methodology has been given previously [1]. The histologic examination was conducted on 5 μ m thick sections which were stained with haematoxylin-eosin. Sections with endocrine tissue were im-

Table 1. Survival of purified islet cell grafts ($RT1^{u/l}$) in diabetic BB recipients ($RT1^{u/u}$)

Graft		Number of recipients						
		Total	With surviving grafts					
Post-transplant week			1	2	3	5	10	
Aggregates purified	no Cy	9	9	5	3	1	0	
Islet Beta cells	Cy	7 ^a	7	5	1	0	—	
Aggregates purified	no Cy	5	5	2	1	1	0	
Islet endocrine cells								

^a not included is one recipient that died in oliguria at week 3. Cy: Cyclosporin A 5 mg $\cdot$ kg⁻¹ $\cdot$ day⁻¹ for 5 weeks

munocytochemically stained for insulin and glucagon. In animals which had died spontaneously, tissue from the heart, lung and kidney was also histologically examined.

Results

Metabolic capacity of $RT1^{n/n}$ donor tissue

Aggregates of islet Beta cells isolated from BN rats ($RT1^{n/n}$) corrected, within 3 days, daily urine volume and 4 h fasting plasma glucose levels in streptozotocin-diabetic BN rats (data not shown). The grafts were the same size (100–200 μ m in diameter), cell number (a total of 10^6 cells) and cellular composition (more than 95% islet Beta cells) as those prepared from RA rats ($RT1^{u/l}$) [3]. A mixture of 10^6 islet Beta cells with 8.10^5 islet endocrine non-Beta cells induced the same rapid normalization when isografted in diabetic BN rats (data not shown). Both groups of recipients remained normal for over 20 weeks.

Survival of ($RT1^{u/l}$) Beta cells in diabetic ($RT1^{u/u}$) BB rats

Intraportal transplantation of ($RT1^{u/l}$) Beta cell aggregates in diabetic BB rats corrected the diabetic state within 3 days (Table 1). Without any insulin treatment, all recipients remained oliguric for at least 1 week. However,

Fig. 1a,b. Light microscopy of intraportal implants consisting of $RT1^{u/l}$ purified Beta cells. After reversal to the diabetic state, several graft sites were still identified as aggregates containing insulin-positive cells and surrounded by a dense mononuclear infiltrate (**a** im-

munoperoxidase stain for insulin, $\times 286$). When the recipient BB rat had been treated with cyclosporin, the liver often showed focal intra-lobular necrosis and dense inflammatory infiltrates (**b** HES, $\times 86$)

all animals reversed to a diabetic state during the following 5 weeks (Table 1). One to two weeks after reversal, the liver content in insulin was low ($< 1 \mu\text{g}$ per organ), but sites of graft implantation were still recognizable from the presence of a few insulin-containing cells surrounded by mononuclear cell infiltrations (Fig. 1 a).

Administration of cyclosporin did not prolong the metabolic effects of the islet Beta cell grafts (Table 1). During the treatment period, one animal died in oliguria. Extraction of the livers, one to two weeks after reversal, indicated a low insulin content ($< 1 \mu\text{g}$ per organ). Several graft implantation sites were still histologically recognized as foci of mononuclear cells. Most livers showed spotty necrosis and diffuse sinusoidal infiltrations with mononuclear cells, suggestive of hepatitis (Fig. 1 b).

Mixtures of endocrine islet cells were also capable of normalizing the tested metabolic parameters for at least one week, but in none of the recipients was this effect long-lasting. By the 10th post-transplantation week, all animals were again diabetic (Table 1).

Survival of ($RT1^{n/n}$) Beta cells in diabetic ($RT1^{u/u}$) BB rats

Implantation of ($RT1^{n/n}$) Beta cell aggregates corrected the diabetic state in all BB recipients (Table 2). Three weeks later, seven out of ten recipients were still normoglycaemic. However, at the end of the 10th post-transplantation week, only three animals presented the signs of a surviving graft. Mixing the donor Beta cells with other endocrine islet cells did not prevent reversal to a diabetic state but restricted this phenomenon to the first three post-transplantation weeks (Table 2). With $RT1^{n/n}$ allografts, more recipients remained normalized up to week 10 (Table 2) than with $RT1^{u/l}$ allografts (Table 1).

Analysis of liver tissue illustrated a longer survival of the $RT1^{n/n}$ donor tissue than of the $RT1^{u/l}$ implants:

In animals which reversed to a diabetic state within 2 to 5 weeks, the liver still contained donor Beta cells as evidenced by immunoassay of tissue extracts and by immunocytochemistry of tissue sections; the hepatic insulin content was markedly higher (2 to $5 \mu\text{g}$) than that in $RT1^{u/l}$ recipients shortly after reversal ($< 1 \mu\text{g}$).

In recipients which had maintained a normalized state for 10 weeks, the mean liver content in insulin was comparable to that in normalized streptozotocin-diabetic rats (Table 3, [2]). On the other hand, their pancreatic insulin

Table 2. Survival of purified islet cell grafts ($RT1^{n/n}$) in diabetic BB recipients ($RT1^{u/u}$)

Graft		Number of recipients					
		Total	With surviving grafts				
Post-transplant week			1	2	3	5	10
Aggregates purified Islet Beta cells	no Cy	10	10	8	7	6	3
	Cy	7 ^a	7	7	7	7	5
Aggregates purified Endocrine islet cells	no Cy	12	12	10	6	6	5
	Cy	5 ^b	5	5	5	5	5

not included are the ^a4 and ^b2 recipients that died in oliguria between weeks 3 and 10. Cy: cyclosporin A $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 5 weeks

Table 3. Organ insulin content in oliguric BB rats ($RT1^{u/u}$) 10 weeks following transplantation of purified islet cells ($RT1^{n/n}$)

Graft			Insulin content	
			Liver	Pancreas
(n)			$\mu\text{g}/\text{organ}$	
Aggregates purified Endocrine islet cells	no Cy	(3)	23.0 ± 0.3	< 1
	Cy	(4)	13.8 ± 4.4	< 1

Cy: cyclosporin A $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 5 weeks

content was markedly lower than in the streptozotocin transplants (Table 3, [2]). The liver tissue presented insulin-positive cell aggregates, one third of which was free of mononuclear cell infiltration, one third was peripherally infiltrated and one third diffusely (Fig. 2). The Beta cells varied in their individual intensity of insulin staining (Fig. 2).

Effect of cyclosporin on the survival of ($RT1^{n/n}$) Beta cells in diabetic ($RT1^{u/u}$) BB rats

Diabetic BB rats in which implantation of ($RT1^{n/n}$) purified islet cells was combined with cyclosporin treatment ($5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ orally) also normalized within the first 3 days following transplantation (Table 2). They reversed, however, markedly less frequently than untreated recipients, even after ceasing the treatment on post-transplantation day 35 (Table 2). Over the 10-week observation period, reversal was noticed in only 2 out of 11 recipients of purified islet Beta cells, and in none of the seven recipients of purified mixed islet endocrine cells (Table 2). However, 6 of the 18 cyclosporin-treated animals died in an oliguric state, two during the last 2 weeks of cyclosporin administration and four shortly after discontinuation of the drug.

Microscopically, no difference was noted in the intra-portal grafts of killed or spontaneously dead recipients. During the period of cyclosporin treatment, all implants were free from mononuclear cell infiltrates. At the 10th post-transplantation week, this was still the case for two thirds of the grafts, whereas the others presented only a peripheral infiltration (Fig. 3). At all time points numerous insulin-positive cells were present but their individual staining intensity varied (Fig. 3). Some aggregates presented foci of cell necrosis (Fig. 3).

In long-term oliguric animals, tissue extraction indicated a negligible pancreatic insulin content and a substantial hepatic insulin store (Table 3). The mean hepatic insulin levels were lower than those measured in recipients without cyclosporin treatment but this observation remains without statistical meaning until more data are available (Table 3).

Toxicity of cyclosporin in diabetic BB rats

The occurrence of spontaneous deaths in the series of cyclosporin-treated transplants led us to examine the effect of the drug on the survival of diabetic controls. Five diabetic BB rats were kept on insulin injections and re-

Fig. 2a-c. Light microscopy of intraportal implants consisting of RT1^{n/n} purified Beta cells. The analysis was conducted on oliguric BB recipients (RT1^{u/u}), 10 weeks after transplantation. Between 20 and 30% of sectioned implants were infiltrate-free; 40% exhibited a peripheral infiltration (**a** HES, $\times 130$). The endocrine tissue appeared histologically intact (**b** HES, $\times 260$) and was composed of insulin-positive cells (**c** immunohistochemistry for insulin – $\times 260$)

ceived, in addition, a daily oral dose of cyclosporin $5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$. All five died between day 18 and day 32 of treatment, whereas none of the 10 diabetic controls without cyclosporin were lost (Table 4). Plasma levels of cyclosporin varied between 50 and 500 ng/ml. Autopsy of the drug-treated BB rats revealed extensive bronchopneumonia and disseminated abscesses, in par-

Fig. 3a-c. Light microscopy of RT1^{n/n} purified Beta-cell grafts in BB recipients which were treated for 5 weeks with cyclosporin. The animals were oliguric at the time of analysis, 10 weeks after implantation. Less than 10% of sectioned implants presented a diffuse infiltration; other graft sites were not, or only peripherally, infiltrated (**a** HES $\times 230$). Densely stained cells were abundant after immunohistochemistry for insulin (**b** $\times 230$). A few grafts presented signs of focal necrosis (**c** HES $\times 230$)

ticular in the kidney; the number of germ centres in the spleen was markedly reduced as compared to untreated controls. The same pathology was observed in cyclosporin-treated transplants which had died spontaneously. The mortality in the latter group was lower (6 of 18) than in the drug-treated diabetic controls (5 of 5). In the group of transplants without cyclosporin, none of the animals

Table 4. Effect of cyclosporin on survival of BB rats

Animal group	Number of survivors at week		
	0	5	10
Diabetic BB controls on insulin treatment			
no Cy	10	10	10
Cy	5	0	0
BB recipients of RT1 ^{n/n} graft ^a			
no Cy	22	22	22
Cy	18	14	12

^a transplantation carried out on first day of treatment. Cy: cyclosporin A 5 mg · kg⁻¹ · day⁻¹ during first 5 weeks.

died spontaneously during the 10-week observation period (Table 4).

Discussion

Diabetic BB rats probably develop their disease as a result of an immune-mediated destruction of their pancreatic Beta cells [6]. This Beta-cell-specific autoreactivity may also interfere with the survival of islet Beta-cell grafts [8], and hence, with the prospects of transplantation as a cure for diabetes with an autoimmune origin. However, if this autoreactivity is MHC-restricted, it may become irrelevant for implants which do not express the RT1^{u/u} haplotypes that are pathognomic for the BB rat [9]. This possibility can be tested by assessing the survival of MHC-incompatible islet Beta-cell grafts provided they are not rejected by the alloreactivity of the host. Several ways exist to prevent rejection of allogeneic islet tissue in streptozotocin-diabetic rodents: implantation in an immunoprivileged site [10, 11], pre-treatment of the donor tissue with the purpose of reducing its immunogenicity [12, 13] and immunosuppression of the recipient [14]. Each of these approaches, either alone or in combination, have been applied in diabetic BB rats [15–25]. They have given controversial answers to the question as to whether the allogeneic tissue survives in BB rat hosts or whether it undergoes recurrence of an autoimmune destructive process. The observed discrepancies can be attributed to various explanations, each of which is difficult to experimentally support or exclude. The present study addresses this complex issue in a more simple and standardized experimental model than those which have been used so far. Rat islet Beta cells are purified and reaggregated into particles with documented *in vitro* [26, 27] and *in vivo* [3] viability and function. The purified Beta-cell aggregates are transplanted in diabetic BB rats and their survival is followed over a 10-week-period. All grafts were prepared from MHC-incompatible donors, with or without disparity in MHC-class I antigens. Similar grafts had been previously found to induce long-term metabolic normalization in syngeneic or allogeneic recipients with streptozotocin-induced diabetes [1–3].

When purified Beta-cell aggregates were prepared from RT1^{n/n} or RT1^{u/u} donors, they were capable of correcting the diabetic state in syngeneic streptozotocin-diabetic rats (present study and [3]). The normalized condition was

maintained for longer than 20 weeks and the intraportal grafts remained free of mononuclear cell infiltrations during this period [3]. Purified Beta-cell grafts were also successful in allogeneic combinations (RT1^{u/l}→RT1^{n/n}), in particular when they were composed of a mixture of islet endocrine Beta and non-Beta cells or when their implantation was temporarily covered with cyclosporin [1, 2]. The allogeneic implants exhibited a mild but persistent infiltration of mononuclear cells which did not become more prominent with time [1, 2]. We have now examined whether allografts of purified endocrine islet cells can establish a normalization in diabetic BB rats (RT1^{u/u}). The results of this study are compared to those obtained in allografted streptozotocin-diabetic rats.

In an allogeneic combination with MHC-class I homology (RT1^{u/l}→RT1^{u/u}), none of the previously successful conditions induced a long-lasting normoglycaemia in the BB transplants: neither the addition of other islet endocrine cells nor the administration of cyclosporin prevented massive infiltration of the purified Beta-cell grafts and their subsequent destruction. The failure of the RT1^{u/l} Beta-cell grafts in RT1^{u/u} rats may result from the fact that these recipients generate a stronger alloreactivity towards the donor Beta cells than the previously tested RT1^{n/n} strain [1]. Since purified endocrine cell preparations are devoid of MHC-class II expressing cells [3], it is unlikely that the disparity in class II or Ia antigens is responsible for this phenomenon. There is, on the other hand, the possibility that the homology in class I antigens reactivated host T cells to attack donor islet Beta cells via an autoimmune process. Treatment with antibodies against T cell subsets could have assessed this possibility [24]. That cyclosporin did not suppress such autoimmune destruction of the graft may be related to the mode of its administration. The drug was given in a regimen that was shown to suppress alloreactivity towards purified Beta-cell grafts [2], but that differed markedly from the treatments used to prevent or delay autoimmune destruction of pancreatic Beta cells [28, 29]. Under the latter conditions, cyclosporin has been shown to prolong survival of pseudoisogenic islet grafts in diabetic BB rats [25].

In the allogeneic combination with a disparity in MHC-class I antigens (RT1^{n/n}→RT1^{u/u}), the survival of purified islet Beta-cell grafts was markedly longer than that with class I homology. Ten weeks after transplantation, 30% of the BB recipients were still oliguric and normoglycaemic, a percentage which is slightly lower than in similarly treated streptozotocin-diabetic animals [1]. Addition of islet endocrine non-Beta cells to the Beta-cell aggregates increased the success rate, but not to the rate seen in rats with chemically-induced diabetes [1]. We have not yet investigated the reasons for the lower efficacy of the mixed endocrine graft in diabetic BB rats. Possible explanations are a higher immunogenicity of allografted RT1^{n/n} islet Beta cells or a lower beneficial effect of the supplemented non-Beta endocrine cells. Other reasons may be strictly metabolic such as the higher body weight of the BB recipient at the time of transplantation or its lack of any pancreatic insulin reserve. A variation in graft composition and a combination with insulin therapy may clarify some of these issues.

In further analogy to our previous allograft data (2), a 5-week cyclosporin treatment suppressed the mononuclear cell infiltration of allografts in diabetic BB rats and preserved their function in virtually all recipients, even after discontinuation of the drug. Administration of cyclosporin was, however, markedly less tolerated in diabetic BB rats, causing death in one third of the BB transplants; no morbidity was noticed in cyclosporin-treated BN transplants or in untreated BB transplants. Although no immunologic analysis was performed, it seems likely that the BB rats – which are already T cell deficient – developed a severe immunodeficiency upon cyclosporin treatment. This could explain the generalized infection that was found at autopsy. This serious side-effect was not observed in cyclosporin-treated BN rats [2]; it was unrelated to the implantation, as it was also observed – with even higher mortality (100%) – in cyclosporin-treated diabetic BB-controls. The diabetic state may thus accelerate the development of a cyclosporin-induced morbidity in BB rats. This could explain why no mortality has been reported for cyclosporin administrations to non-diabetic BB rats [28, 29]. The mean circulating cyclosporin concentrations in diabetic BB rats were not significantly different from those in streptozotocin-diabetic rats [2], but presented wider fluctuations, sometimes reaching the levels which were found to be cytotoxic for islet cells in vitro [2]. That the drug may have damaged a number of islet Beta cells in vivo is suggested by the lower hepatic insulin content in cyclosporin-treated recipients.

The present experiments indicate that MHC incompatible islet Beta cells can survive in rats with diabetes of autoimmune origin, provided that they are implanted under conditions which allow allograft acceptance. This requirement can be met by composing grafts of purified islet endocrine cells and by covering their implantation by a short-term cyclosporin administration. Treatment with cyclosporin can, however, cause toxic effects in diabetic BB rats, stressing the need to adjust the dose, route and period of drug therapy in these animals. The use of other immunosuppressive agents, such as antilymphocyte serum, should also be considered. It is concluded that the standardized preparation of islet cell grafts with selected cellular composition can help assessing the conditions for successful transplantation in diabetes with an autoimmune origin. Future studies should examine the immunologic and metabolic status of the purified Beta-cell grafts over longer periods than those covered in the present work. It will be interesting to compare these data with the survival of other tissues in the same donor-recipient combination. These control studies with non-Beta-cell grafts can help distinguish between Beta-cell specific destruction mechanisms and alloreactivity. They will, however, require the use of purified cell grafts in order to make comparison with Beta-cell grafts possible. The availability of purified Alpha-cell preparations [30] could provide a useful control tissue which can be carried through the same preparation and implantation procedures as the Beta-cell grafts.

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