Immunotherapy and beta-cell replacement in type I diabetes mellitus

Linde, P. van de

Chapter 5

Alloreactivity against repeated HLA mismatches of sequential islet grafts transplanted in non-uremic type 1 diabetes patients

C. A. van Kampen, P. van de Linde, G. Duinkerken, J. J.van Schip, D. L. Roelen, B. Keymeulen, D.l G. Pipeleers, F. H.J. Claas, B. O. Roep.

Transplantation 2005 (80), p118-126.

ABSTRACT

Islet transplantation can restore insulin production in type 1 diabetes patients. However, survival of the islet allografts will face rejection or recurrence of autoimmunity or a combination of both. In a study on islet-after-kidney transplants, we previously reported that islet cell recipients presented low T-cell alloresponses for HLA mismatches that were shared by the islet cell graft and the prior kidney graft, that is, repeated mismatch, while vigorous responses were measured against novel HLA mismatches.

We now investigated T-cell alloreactivity to repeated HLA-mismatches in three non-uremic type 1 diabetic patients each receiving three sequential islet cell implants.

These islet-after-islet recipients patients exhibited low or absent responses to repeated mismatches to the first graft which was accompanied by sustained graft function, and reduced responsiveness towards subsequent grafts. In one patient, T-cell responses towards these mismatches were noticed following new mismatches in subsequent grafts, with loss of graft function.

These case reports further support the view that subsequent islet implantations can reduce alloreactivity for repeated HLA mismatches. They demonstrate the usefulness of monitoring T-cell reactivity against islet allografts to correlate immune function with graft survival and to identify conditions for preservation of beta-cell function.

INTRODUCTION

Type 1 diabetes (T1D) is caused by insulin deficiency due to the autoimmune destruction of insulin secreting beta-cells in the pancreas. Islet transplantation can restore the insulin production in T1D patients (1-8). Survival of the grafts will depend on several variables, among which the quality and quantity of the beta-cells in the donor tissue, as well as the allo- and/or autoimmune reactivities of the recipient. The immunosuppressive drugs used to prevent graft rejection may prevent autoimmune damage of the graft (9) although islet grafts may not benefit from such action (10-12). Failure of immunosuppressive drugs to prevent autoimmune beta-cell destruction has also been found in prediabetic individuals and in recent-onset T1D patients (13). In a limited study on islet-after-kidney transplants, we have previously shown that cultured human beta-cells can survive for more than 1 year in T1D patients on maintenance anti-rejection therapy for a prior kidney graft without the need for an increased immunosuppression at time of their implantation (14). Furthermore, the usage of functionally standardized beta-cell grafts in this study helped to identify recipient and graft factors that influence survival and metabolic effects. It was thus found that the cultured islet cell graft did induce allo- and autoimmune responses of variable intensity (15) whereby low responses were associated with long-term islet graft survival. A striking observation was the fact that in all cases low alloreactivity was found against HLA mismatches present on the islets shared with the kidney that was transplanted years before (repeated mismatch), even in case of a high T-cell alloreactivity against novel HLA mismatches only present on the islets (16). This pattern was found even when these novel mismatch and repeated mismatch were present on the same islet allograft. In the present study we investigated whether islets rather than kidney allografts can induce a similar effect on alloreactivity against HLA mismatches shared between sequential islet implantations in non-uremic T1D patients. If this would be the case, it might open selection windows for subsequent islet cell grafts. In order to test this possibility, we undertook a pilot study on three patients each of whom received three consecutive beta-cell implants within an interval of 2 to 6 months. These recipients of a second and third implant were C-peptide positive at the time of implantation but were not insulin-independent. The laborious experimental design for measuring specific T-cell alloreactivity forced us to minimize the number of cases. T-cell responses were measured repeatedly following each implant in order to analyze alloreactivities to repeated HLA mismatches. Although C-peptide levels will be reported, this study was not designed to determine use of alloreacitivity testing as a marker for graft survival. The intention is that data collected from this study can help clarify immune reactivities in T1D recipients of islet cell grafts.

MATERIALS AND METHODS

Patients

The selected patients in this report each received three consecutive beta cell implants with intervals of 2 to 6 months. All patients were insulin-dependent diabetes patients (C-peptide levels <0.02 nmol/l). Before transplantation the patients received ATG (anti-thymocyte globulin) as induction therapy at the time of the first transplantation only and after transplantation MMF (Mycophenolate Mofetil; 2000 mg/days) and tacrolimus (3-4 mg/days; trough levels 4.1 – 5.7 ng/mL) as immunosuppressive therapy. The islets were transplanted via the portal vein (17). The patients in this report had three islet implantations consisting of fresh and cryopreserved islets of different pancreas donors (see Table 1). The number of islet cells per transplant varied between 0.7 and 3.8 million per kilogram bodyweight (see Table 2).

From each patient blood samples were collected at several time points after transplantation. Fresh PBMC were used for autoreactivity testing and the remaining part of the cells were frozen in RPMI 1640 (Gibco, Scotland, UK) with 20% Fetal Calf Serum (Greiner, Frickenhausen, Germany) and 10% DMSO (dimethylsulfoxid, Fluka, Buchs, Switzerland). Cells were frozen in -70˚C and stored in liquid nitrogen until they were used for alloreactivity testing. For alloreactivity, all samples of a patient taken at different time points were analyzed within one test.

Alloreactivity

The Cytotoxic T-Lymphocytes precursor (CTLp) assay was used to study the presence of alloreactive cytotoxic T lymphocytes (CTL) specific for the HLA mismatches present on the islets. Different combinations of stimulator cells and target cells were used in a limiting dilution assay to quantify the absolute number of allospecific CTLs. The combinations were designed such that we could test whether suppression of the alloresponse specific for a repeated HLA mismatch was present and whether this suppression could influence the response against novel HLA mismatches (linked suppression) (Table 3). For example, in combination I of patient KN, PBMC were stimulated with PBMC expressing the repeated mismatch HLA-B44 (stimulator 1), which was present on all three islet implants. After 7 days of stimulation, the effector cells of the patient were tested for their cytotoxic capacity against target cells expressing HLA-B44 together with transplantation-unrelated HLA antigens (target 1). The effector cells were also tested against target cells expressing HLA-B44 and HLA-A1, which was present on the islets of the second and third transplantation (target 2). In this way it was possible to look at the effect of the presence of HLA-A1, which acted in the samples before the second implant as third party and in the samples

Table 1. HLA typings of the patients and islet donors.

patient	patients HLA typing			tx	number of donors	HLA mismatch of islet donors [a]		
RI	A2, A1	B7, B15	DR4	tx1	3	**A3**, A11, A24	B27, B35, B37, **B44**, **B51**	DR1, DR2, **DR3**, DR10, **DR13**
				tx2	1		**B8**, B50	**DR3**, DR7
				tx3	4	**A3**, A25, A26, A31	**B8**, B18, B38, **B44**, B45, **B51**, B60	DR7, **DR3**, DR11, **DR13**
VVK	A26, A32	B27, B44	DR3, DR7	tx1	3	**A1**, A23, **A25**, A28, A29	**B8, B18**, B58	**DR1, DR4**
				tx2	4	**A2**, **A1**, **A3**, **A25**, A68	B7, **B8, B18**, B39, B51, **B60**	**DR1, DR4**, **DR11**, **DR13**, **DR15**
				tx3	4	**A2**, **A3**, A11, A24, **A25**	B13, B15, **B18**, B35, **B60**	**DR11**, **DR13, DR15**
KN	A2, A11	B8, B51	DR3, DR4	tx1	3	A3, A68	B35, **B44**, B60, B62	DR1, DR8, **DR11**, **DR13**
				tx2	5	**A1**, A26, **A29**, A30, A33	B7, **B44**, B57	**DR7,** DR15, DR16
				tx3	3	**A1**, A24, **A29**	B15, B18, **B44**, B49	**DR7**, DR10, **DR11**, **DR13**

[a] The repeated HLA mismatches between the islet implantations are "bold-typed"

Table 2. C-peptide production before and after islet transplantation.

pat.	time after first tx	islets (10^6/ kg BW)	C-peptide 6-10 wks after tx (ng/ml)	C-peptide 52 wks after tx1 (ng/ml)
RI	0 wks	3.4	0.89	
	22wks	0.7	0.67	
	26 wks	2.4	0.19	
				0.06
VVK	0 wks	0.7	0.11	
	8 wks	1.3	0.32	
	33 wks	3.8	0.95	
				1.35
KN	0 wks	2.0	0.94	
	22 wks	1.1	0.96	
	33 wks	1.2	1.03	
				0.26

between the second and third implant as novel mismatch. Combination II tested the reverse: stimulator cells expressing HLA- A1 and target cells expressing HLA-A1 or HLA-A1 and –B44 were used. Combination III tested whether the presence of both antigens during the stimulation phase influences the cytotoxic capacity of the effector cells against target cells which express only one or a combination of these HLA mismatches.

Limiting Dilution Assay (LDA)

The analysis of CTLp frequencies was performed as described (18). Briefly, PBLs of the patient (from 40,000 cells/well down to 625 cells/well in two-fold dilutions, plated with a Biomek automated workstation; Beckman Instruments, Mijdrecht, The Netherlands) were cultured in 96-well, round-bottom plates (Costar, Cambridge, MA) together with 50,000 irradiated (3,000 rad) stimulator cells. The stimulator cells were PBLs from a healthy donor. Limiting dilution cultures were incubated for 7 days at 37°C, 5% CO2. Lymphocytes to be used as target cells were set-up at day 0 in RPMI 1640 with 3mM glutamine, 20 U/ml IL-2, 10% pooled human serum and 2µg/ml PHA (purified phytohemagglutinin, Welcome, Dartford, UK) in a 24-well plate (Costar) at $1x10^6$ cells/well. After 7 days of culture, the LDA-plates were split and the wells were tested for cytolytic activity against 5,000 Europium (Eu)-labeled target cells/well. Lysis was measured by Europium release in the supernatant. A well was scored positive if the counts in that well exceeded the spontaneous release (mean of the wells with stimulator cells and corresponding target cells only) plus 3 standard deviations. CTLp frequencies were calculated using a computer program developed by Strijbosch et al. (19).

Table 3. Cytotoxic T lymphocyte precursor assay design; the "bold-typed" HLA antigens are the target antigens.

Patient KN		A2	A11	B51	B8	DR17	DR4
combination I	Stimulator 1	A2	A32	**B44**	B61	DR11	DR13
	Target 1	A11		**B44**	B51	DR15	DR12
	Target 3	A1		**B44**	B8	DR15	DR17
combination II	Stimulator 2	**A1**	A2	B50	B37	DR1	DR11
	Target 2	**A1**		B8		DR17	DR4
	Target 3	**A1**		B44	B8	DR15	DR17
combination III	Stimulator 3	**A1**	A2	**B44**		DR11	DR13
	Target 1	A11		**B44**	B51	DR15	DR12
	Target 2	**A1**		B8		DR17	DR4
	Target 3	**A1**		**B44**	B8	DR15	DR17
Patient RI		A2	A1	B7	B15	DR4	
combination I	Stimulator 1	A1	A32	**B51**	**B8**	DR17	DR13
	Target 1	A2		**B51**		DR8	
	Target 2	A1		**B8**		DR17	
combination II	Stimulator 2	A2		**B8**	**B44**	DR17	DR13
	Target 1	A2		**B8**		DR17	
	Target 2	A2		B7	**B44**	DR1	
	Target 3	A1		**B8**	**B44**	DR15	DR17
combination III	Stimulator 3	A2	A30	**B44**	**B13**	DR7	DR8
	Target 1	A2		B7	**B44**	DR1	
	Target 2	A2		**B13**	B60	DR4	DR7
Patient VVK		A26	A32	B27	B44	DR17	DR7
combination I	Stimulator 1	**A2**		**B8**	B39	DR8	
	Target 1	A66	A11	**B8**	B41	DR17	DR4
	Target 2	**A2**	A26	B44	B27	DR4	DR13
	Target 3	**A2**		**B8**		DR17	
combination II	Stimulator 2	**A2**		B62	B41	DR4	DR7
	Target 1	**A2**	A26	B44	B27	DR4	DR13
combination III	Stimulator 3	**A68**		**B60**	B72	DR17	DR13
	Target 1	A66	A32	**B60**		DR15	DR13
	Target 2	A26	**A68**	B51	**B60**	DR4	DR13
	Target 3	**A68**		B27	**B60**	DR17	DR7

Cells from each patient were incubated with stimulator cells mismatched for an HLA-A and/or -B of the donor. We did not deliberately match for HLA-DR in the stimulation phase, but this occurred due to linkage disequilibrium between HLA class I and II (Table 3). We made an effort to avoid repeating HLA class II incompatibility between stimulators and target cells in the CTLp analysis, to stay focussed on alloreactivity to HLA class I, as our read-out was CTL alloreactivity. Stimulator and target cells were selected on basis of matching on the HLA "allele" level in the majority of cases (when HLA typing on DNA was available), and sometimes on the HLA "antigen" level (when HLA was typed serologically only). Homozygosity for a particular HLA allele was confirmed by DNA analysis and/or family studies. The target cells were selected on basis of sharing only one HLA-A or -B antigen with the stimulator. The presence of cross-reactive antigens and other donor antigens on the target cells was avoided as described (20). In this way the CTLp frequency for one individual HLA class I antigen can be determined.

Europium release assay

After 7 days of culture, the target cells were washed twice with 0.9% NaCl solution and labeled with Eu chloride as described before in detail (18). After the labeling 5000 target cells per well were added to the responder-stimulator combinations in the LDA-plates and incubated at 37°C, 5% CO2 for 4 hours. As a control, spontaneous lysis (target cells with RPMI/10% human serum) and maximal lysis (target cells with 1% Triton X-100; Fluka) were determined for each target cell.

Alloantibodies

The sera from each patient were tested for the presence of donor-specific anti-HLA antibodies in a complement dependent cytotoxicity assay (CDC) using the standard NIH method. Additional screening was done using the ELISA (LAT class I & II, One Lambda, CA).

Autoreactivity

Lymphocyte proliferation test

Peripheral blood lymphocytes were isolated from heparinized blood of the patient and freshly tested as described before (21). Briefly, 150,000 PBLs were cultured in round-bottom 96 well plates (Costar, Cambridge, MA) in Iscove's Modified Dulbecco's medium with 10% pooled human serum in the presence of antigen, 10% T-cell growth factor (Biotest, Dreieich, Germany) or medium alone in a total volume of 150µl at 37°C, 5% CO2. After 5 days of incubation, each culture was pulsed for 16 hours with 0.5µCi/well of 3H-thymidine. The proliferation is expressed as stimulation index (SI = median of triplicates in presence of stimulus divided by the median of triplicates with medium alone), and was scored positive when the SI ≥3.0.

Antigens

Several autoantigens were tested in the lymphocyte stimulation test. Preparations enriched for insulin-secretory granules and beta-cell membranes were taken from rat insulinoma cell lines (RIN) as described (22;23) and tested at a concentration of 20µg/ml. Furthermore, insulin (25µg/ml, Sigma Chemical, St. Louis, MO), glutamic acid decarboxylase 65 (GAD65) (10µg/ml, Diamyd, Stockholm, Sweden) (24;25), insulin peptide β-chain amino acid 9-23 (10µg/ml , produced by dr.J.W. Drijfhout, Leiden, The Netherlands) and protein tyrosine phosphatase (IA-2) (10µg/ml, provided by dr. E. Bonifacio, Milan, Italy) were tested. As unrelated control antigen tetanus toxoid (1.5 LF/ml or 12.0 IU/ml, National Institute of Public Health and Environmental Protection, The Netherlands) was tested. Recombinant human IL-2 (35U/ml, Cetus, Amsterdam, The Netherlands) was used as positive control.

Autoantibodies

The sera of the patients were tested for the presence of autoreactive antibodies. ICA were measured by indirect immunofluorescence and endpoint titers were expressed as Juvenile Diabetes Foundation Units (26). IA-2 and GAD65 antibodies were determined by liquid radiobinding assays and expressed as percent tracer bound in haemolysis-free sera (27). Cut-off values were determined as the 99th percentile of antibody levels obtained in 783 non-diabetic control subjects after the omission of outlying values, and amounted to ≥12 JDF Units for ICA, ≥2.6% for GAD antibodies, and ≥0.4% for IA-2 antibodies (28).

RESULTS

Autoreactivity

All patients were negative for autoantibodies against GAD65, IA-2 and ICA, except for patient KN who became positive for GAD65Ab after the second islet implantation (Table 4).

All patients had normal T-cell responses against recombinant human IL-2 (data not shown). Responses to tetanus toxoid were low and then absent in patient RI, while higher and remaining present in patient VVK and KN. This varies with our data in islet cell after kidney transplantation where all islet cell recipients exhibited a complete absence of the tetanus toxoid response (15). Under immune suppression, the three patients lacked T-cell responses against insulin and RIN, two of them also lacked responses against GAD65 while one developed a response only after the second implant. Two patients (VVK and KN) presented strong pre-transplant IA-2 reactivities which were reduced after the first implant but which remained clearly present.

Table 4. T-cell autoreactivity and autoantibodies before and after islet transplantation

		autoantibodies [a]			T-cell autoreactivity [a]				
pat		**GAD65**	**IA-2**	**ICA**	**GAD 65 (SI) [b]**	**IA-2 (SI)**	**Insulin (SI)**	**RIN (SI)**	**TT (SI)**
RI	pre-tx	neg	neg	neg	**4.1**	**3.4**	<3	<3	**3.2**
	max. after tx1	neg	neg	neg	<3	**4.6**	<3	<3	**6.2**
	max. after tx2	neg	neg	neg	<3	<3	<3	<3	<3
	max. after tx3	neg	neg	neg	<3	<3	<3	<3	<3
VVK	pre-tx	neg	neg	neg	**8.9**	**14.9**	<3	**52.8**	**18.8**
	max. after tx1	neg	neg	neg	<3	<3	<3	<3	<3
	max. after tx2	neg	neg	neg	<3	**6.8**	<3	<3	**24.0**
	max. after tx3	neg	neg	neg	<3	**20.2**	<3	<3	**28.4**
KN	pre-tx	neg	neg	neg	**5.4**	**22.8**	<3	<3	**51.5**
	max. after tx1	neg	neg	neg	<3	**7.1**	<3	<3	**47.5**
	max. after tx2	**pos**	neg	neg	**4.2**	**10.8**	<3	<3	**32.1**
	max. after tx3	**pos**	neg	neg	<3	**9.1**	<3	<3	**23.5**

[a] Peak values after transplantation (max. after tx) are presented in this table.
[b] SI = stimulation index .

Alloreactivity

In patient RI, alloreactivity was low after the first islet implantation which was associated with sustained C-peptide release (Fig. 1). After the second implant, an increased response was seen against the new mismatch HLA-B8 but not to mismatch HLA-B51 present in the first implant, suggesting that this alloreactivity was specifically reactive against the second islet implant; the patient remained C-peptide positive (Table 2). However, the third implant was followed by an evolution to C-peptide negativity (Table 2); it was now noticed that this third implant, containing both HLA-B8 and B51, strongly increased CTL responses against both repeated mismatches. No signs of autoreactivity were measured (Table 4). It is conceivable that, in this patient, T-cell responses to repeated mismatches correlate to survival of the grafts carrying these antigens: thus, implant 1 may have survived throughout the second post-implant period but not following the third implant; the data are also compatible with alloreactivities against all grafts following the third implant.

In patient VVK, implantation of the first islet graft containing HLA-B8 did not lead to increased CTL alloreactivity against this determinant (Fig. 1). Shortly after the second implant, a strongly increased reactivity against the new allodeterminants HLA-A2 was observed. Target cells containing both HLA-A2 and B8 were also lysed. However, targets selectively expressing HLA-B8 in the absence of HLA-A2 were spared, indicating that the alloreactivity was selectively directed against the new mismatch HLA-A2 in the second graft, despite the presence of HLA-B8 in both islet grafts. After the third islet implantation containing the HLA-A2 mismatch that was also present in the second islet implant, but

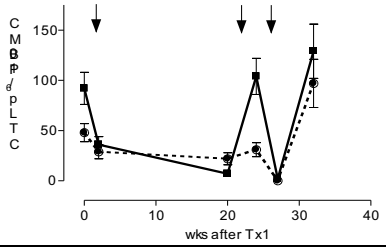
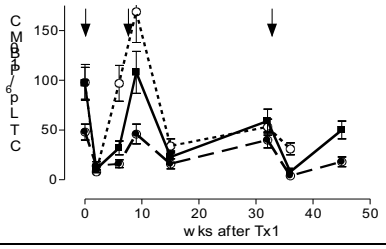
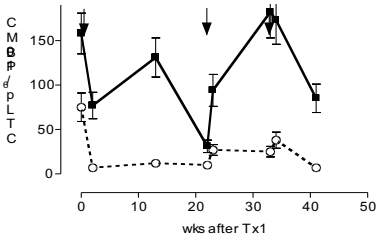
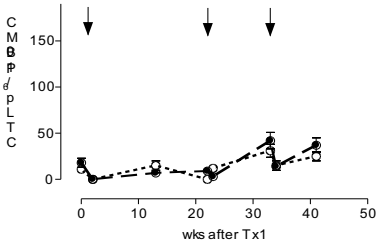
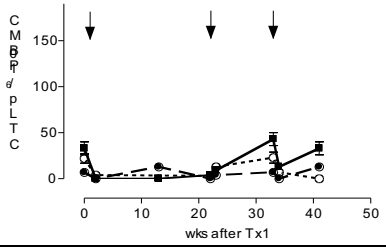
Patient	Stimulator	Target (presence)	Alloreactive CTLp frequency
RI	B8, B51	● B51 (Tx1, Tx3) ■ B8 (Tx2, Tx3)	
VVK	A2, B8	● B8 (Tx1, Tx2) ■ A2 (Tx2, Tx3) ○ A2 (Tx2, Tx3) B8 (Tx1, Tx2)	
KN	B44	■ B44 (Tx1, Tx2, Tx3) ○ A1 (Tx2, Tx3) B44 (Tx1, Tx2, Tx3)	
	A1	● A1 (Tx2, Tx3) ○ B44 (Tx1, Tx2, Tx3)	
	A1, B44	■ B44 (Tx1, Tx2, Tx3) ● A1 (Tx2, Tx3) ○ A1 (Tx2, Tx3) B44 (Tx1, Tx2, Tx3)	

Figure 1. Monitoring of cellular alloreactivity against repeated HLA mismatches in sequential islet implants**.** Indicated are the precursor frequencies of alloreactive cytotoxic T lymphocytes before and following implantation of islet allografts. Details on the HLA typings of stimulator and target cells are indicated in Table 3. *Arrows* indicate the time points of islet implantation. Tx1, Tx2 and Tx3 refer to first, second and third transplant, respectively.

not the HLA-B8 mismatch repeated present in the first and second implant, a markedly reduced CTL alloreactivity was demonstrated against both HLA-A2 and B8. The recipient presented sustained C-peptide positivity, with levels increasing in time (Table 2). These data indicate induction of operational tolerance against these HLA determinants.

In patient KN, similar results suggestive of induction of low alloreactivity to repeated islet allodeterminants could be demonstrated with a correlation to sustained beta-cell function (Fig 1). In this case, very low T-cell responses were found for HLA-B44 in the presence of HLA-A1 on either the stimulator cells or the target cells. This very low CTL response against B44 persisted after twice-repeated in vivo challenge with islet allografts containing HLA-B44, despite a significant precursor frequency of CTLs reactive against targets cells exclusively expressing HLA-B44 obtained after selective stimulation of patient's leukocytes with HLA-B44 after the second, but not the third islet implantation. In contrast, stimulation of patient's cells with cells expressing HLA-A1 alone or in combination with HLA-B44 in all cases resulted in low precursor frequencies of alloreactive CTLs against either HLA-A1 or B44. These results are indicative for the induction of low responsiveness against islet allodeterminants that persists after in vivo re-challenges, and that is dependent of the co-expression of different allodeterminants.

DISCUSSION

Our results demonstrate that islet allografts can be associated with low T-cell responses to HLA mismatches, and this for several weeks after the first implant. We also show that this low T-cell alloreactivity can persist or even be reduced for mismatches that are repeated in subsequent islet implantations and that this is correlated with sustained C-peptide levels while the later appearance of T-cell reactivity to repeated mismatches was associated with loss of graft funtion.

Our earlier study of patients transplanted with islets after a previous kidney transplantation suggested that islet transplantation in T1D patients can lead to recurrent autoimmunity and/or islet graft rejection, despite the maintenance immunosuppressive therapy for the previous transplanted kidney (15). However, the same study also demonstrated that islet transplantation can lead to successful islet graft function in patients with a history of ATG treatment at the time of kidney transplantation, while patients without a history of ATG treatment frequently lost their graft function early after transplantation. In the successful patients, ATG might have depleted autoreactive memory T-cells which play a role in the pathogenesis of T1D (15). Another remarkable aspect in this study was the low alloreactive response against repeated HLA mismatches that were present on the prior transplanted kidney and again present on the implanted

islets. None of the repeated mismatches induced alloreactivity, even though the islet implantation resulted in alloreactivity that, in some cases, caused rejection of the islets (16). This was in accordance with the absence of rejection episodes of the kidney in all patients. It appears that the kidney allograft has induced a state of tolerance, which is not abrogated by a subsequent islet implantation with a repeated HLA mismatch.

The present study examined whether implantation of an islet allograft in the absence of a kidney allograft can invoke a similar state of low responsiveness from which a subsequent islet implant can benefit. It was conducted in three patients each of whom received three islet grafts in sequence whereby the first was implanted under ATG. In all three patients a low auto- and alloreactivity was observed after the first implantation, indicating that ATG treatment has wiped out almost all T-lymphocytes. However, the effect of this treatment was not the same in all patients, as illustrated by the persistence of immune responses against tetanus toxoid in patients VVK and KN. Conflicting data are present in literature whether the tetanus response is useful to monitor the effect of immunosuppressive therapy (29;30). Nonetheless, in patients with a persisting tetanus response more T-cell autoreactivity for IA-2 was present compared with patients where the tetanus response was absent. This might be partly related to differences in immunosuppressive regimen after transplantation: the "islets-after-kidney-group" received azathioprine, cyclosporin A and methylprednisolone as immunosuppressive therapy while the "islets-alone-group" received MMF and tacrolimus.

We obtained evidence for the potential of islet allografts to induce low responsivness towards their allodeterminants from which sequential islet implants may benefit. However, no firm rules exist that predict whether new islet implants are covered by the reduced alloreactivity or invoke alloreactivity that may jeopardize survival earlier functioning islet implants. Although the present study was not designed for this purpose, our immune monitoring studies proved useful to correlate immune function with clinical fate of islet allografts implanted in T1D patients.

The pattern of immune reactivities following islet implantations in patient RI, closely mirror beta-cell function of the sequential islet implants. Although no alloreactivity against the HLA mismatch of the first implant was observed, a vigorous alloimmune response against a new mismatch on the second mismatch was detected shortly after transplantation. Since no improvement of beta-cell function was measurable, this could be interpreted as a selective rejection of islets in the second implant; absence of T-cell reactivity to the repeated mismatch would be compatible with further survival of the first graft. When the mismatches of the first and second implant were repeated on the third implant, an alloresponse developed against both repeated mismatches. We attribute this observation to a lack of induction of tolerance against the initial islet allograft. The CTL alloreactivity developed against the second islet allograft led to failure to further increase C-peptide production by rejection of the second implant, and conceivably trig-

gered an alloimmune response in the third graft that expressed mismatches present in both the first and second implant. Consequently, any tolerance established against the first implant was lost, and the first implant was rejected. Since our data did not provide evidence for tolerance to a preexistent HLA mismatch here, this unfortunate case argues for a determination of CTL alloreactivity against previous implants to avoid the risk to jeopardize earlier successful islet implantations. An explanation for the response against the repeated mismatch of the second implant after the third implant could be that the time between the implants, which was only four weeks, was too short to induce a state of tolerance against the novel mismatch. In addition to immunosuppressive regime and kinetics of islet implantation, other factors may affect the chance of islet allograft to induce tolerance in the recipient, such as graft composition and purity (14). Although repeated islet implantations have shown to be feasible and successful, we also demonstrate the risk for induction of immune reactivity after repeated exposure to allodeterminants that can affect graft function of earlier implants. A factor that may affect function of repeated islet implants unrelated to immunology involves hepatic steatosis (31).

In patient VVK, a decrease in the alloresponse against the repeated mismatches was observed after a peak of alloreactivity. After the third implant a slight increase in the T-cell autoreactivity for IA-2 was observed. However, this appears to have no influence on the graft function because C-peptide production persisted.

In the third patient in this report, KN, a remarkable phenomenon was observed. In this patient the presence of a particular HLA mismatch (HLA-A1) on the stimulator cells or on the target cells seems to have a protective effect on the T-cell alloresponse against another HLA mismatch (HLA-B44). When we studied the HLA typings of the islet donors for this patient in more detail, both mismatches were indeed present on the same donor in three out of seven donors expressing HLA-B44. It might be that a part of the islets is protected in this way against the HLA-B44 specific alloresponse that was observed. On the other hand, T-cell autoreactivity for IA-2 and GAD65 and GAD65-autoantibodies were present after the second implant. Part of the transplanted beta-cells could be damaged by alloreactivity and/or autoreactivity because the patient still needs insulin but not as a high dose as before the islet transplantation. In the group of non-uremic patients transplanted with islets alone there were also patients present that could stop their insulin injections after transplantation without showing any signs of either allo- or autoreactivity at all (data not shown).

Inducing a state of reduced immune reactivity against the repeated mismatches as present in patients that were transplanted with islets after a prior kidney transplantation (15) might be different in our situation of non-uremic patients repeatedly transplanted with islets. In patients with a prior kidney transplantation the tolerizing HLA mismatch is persistently present, while in patients transplanted with islets alone it is not known whether the islets of each pancreas donor survive after the first implantation.

In this study, we did not explicitly study the contribution of HLA-DR, although it is conceivable that HLA-DR matching could contribute to both indirect presentation of alloantigen and possibly regulation of the immune response. In our previous studies on allo- and autoreactivity against islet allografts in 'islet-after-kidney' recipients, we performed split-well analyses in each case to determine the contribution of CD4 T-cells (15). The concordance between CTL and T-helper alloreactivity was remarkable, and made us decide to chose a different experimental design excluding T-helper analyses to allow testing of more specific allodeterminants, in multiple combinations. In the present series, we tried to avoid repeating HLA class II incompatibility between stimulators and target cells in the CTLp analysis, to stay focussed on alloreactivity to HLA class I.

How would our study advance the field? Our data demonstrate that repeating islet implantation once does not increase alloreactivity. Repeating a mismatch between the first and second implant did not jeopardise islet function of the first implant, and the second implant may even benefit from the preexistent state of low responsiveness towards to first implant. However, our results also suggest that multiplying donors and repeating HLA class I mismatches in a third implantation can be adverse and lead to islet graft loss, due to immunization and loss of low responsiveness to earlier implants. As a consequence, we decided to limit implantations to two time points, regardless of the state of insulin-dependence after the second implantation. Our immunological results obtained thus far are perfectly correlating with the in vivo fate of islet allografts. Therefore, CTLp analyses may guide selection and exclusion of future donors. Nonetheless, we believe that continuing our immunological monitoring blinded from clinical outcome is critical to avoid premature changes in protocol based on limited series of transplantations.

Although it is tempting to speculate that the reduced immune reactivity observed against two repeated islet implants results from the induction of a state of operational tolerance, our present data do not allow to draw any conclusions on the mechanism of reduced immune responsiveness.

In conclusion, repeated islet implantation in non-uremic patients can successfully install reduced immune reactivity towards the islet grafts. Our results suggests that reduction of alloreactivity can be induced by islet allograft for a particular mismatch from which subsequent islet implants may benefit. However, this reduction does not occur in all patients and the reasons for the lack of regulation in these cases are not yet known. Monitoring the auto- and alloreactive responses may provide prove of the existence of operational tolerance and may help to determine parameters that improve the rate of immune modulation induced. Moreover, our immunological studies may provide insight in the mechanisms behind the success or failure of the islet transplantations. More research is required to determine which immunosuppressive treatment will provide the best results and what time course for the subsequent islet implantation should be followed for clinical success.

REFERENCES

1. Ricordi C, Tzakis AG, Carroll PB, et al. Human islet isolation and allotransplantation in 22 consecutive cases. Transplantation 1992:53:407.
2. Gores PF, Najarian JS, Stephanian E, et al. Insulin independence in type I diabetes after transplantation of unpurified islets from single donor with 15-deoxyspergualin. Lancet 1993:341:19.
3. Secchi A, Socci C, Maffi P, et al. Islet transplantation in IDDM patients. Diabetologia 1997:40:225.
4. Hering BJ, Browatzki CC, Schultz A, et al. Clinical islet transplantation- registry report, accomplishments in the past and future research needs. Cell Transplant 1993:2:269.
5. Soon-Shiong P, Heintz RE, Merideth N, , et al. Insulin independence in a type I diabetic patient after encapsulated islet transplantation. Lancet 1994:343:950.
6. Scharp DW, Lacy PE, Santiago JV, et al. Results of our first nine intraportal islet allografts in type I, insulin-dependent diabetic patients. Transplantation 1991:51:76.
7. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000:343:230.
8. Warnock GL, Kneteman NM, Ryan EA, et al. Long-term follow-up after transplantation of insulin-producing pancreatic islets into patients with type I (insulin -dependent) diabetes mellitus. Diabetologia 1992:35:89.
9. Shapiro A, Hao EG, Lakey JRT, et al. Novel approaches toward early diagnosis of islet allograft rejection. Transplantation 2001:71:1709.
10. Stiller CR, Dupre J, Gent M, et al. Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset. Science 1984:223:1362.
11. Tyden G, Reinholt FP, Sundkvist G, et al. Recurrence of autoimmune diabetes mellitus in recipients of cadaveric pancreatic grafts. New England Journal of Medicine 1996:335:860.
12. Sibley RK, Sutherland DE, Goetz F, et al. Recurrent diabetes mellitus in the pancreas iso-and allograft. A light and electron microscopic and immunohistochemical analysis of four cases. Laboratory Investigation 1985:53:132.
13. Rakotoambinina B, Timsit J, Deschamps I, et al.Cyclosporin A does not delay insulin dependency in asymptomatic IDDM patients. Diabetes Care 1995:18:1487.
14. Keymeulen B, Ling Z, Gorus FK, et al. Implantation of standardized beta-cell grafts in a liver segment of IDDM patients: graft and recipient characteristics in two cases of insulin-independence under maintenance immunosuppression for prior kidney graft. Diabetologia 1998:41:452.
15. Roep BO, Stobbe I, Duinkerken G, et al. Auto- and alloimmune reactivity to human islet allografts transplanted into type I diabetic patients. Diabetes 1999:48:484.
16. Stobbe I, Duinkerken G, van Rood JJ, et al. Tolerance to kidney allograft transplanted into type I diabetic patients persists after in vivo challenge with pancreatic islet allografts that express repeated mismatches. Diabetologia 1999:42:1379.
17. Movahedi B, Keymeulen B, Lauwers MH, et al. Laparoscopic approach for human islet transplantation into a defined liver segment in type-1 diabetic patients. Transplant International 2003:16:186.
18. Bouma GJ, van der Meer-Prins EMW, et al. Determination of cytotoxic T-lymphocyte precursor frequencies using europium labeling as a non-radioactive alternative to labeling with chromium-51. Human Immunology 1992:35:85.
19. Strijbosch LWG, Buurman WA, Does RJMM, et al. Limiting dilution assays; experimental design and statistical analysis. Journal of Immunological Methods 1987:97:133.
20. Zhang L, Li SG, Vandekerckhove B, et al. Analysis of cytotoxic T-cell precursor frequencies directed against individual HLA-A and -B alloantigens. Journal of Immunological Methods 1989:121:39.

21. Roep BO, Kallan AA, Duinkerken G, et al. T-cell reactivity to beta-cell membrane antigens associated with beta-cell destruction in IDDM. Diabetes 1995: 44:278.
22. Roep BO, Arden SD, de Vries RRP, et al. T-cell clones from a type-1 diabetes patient respond to insulin secretory granule proteins. Nature 1990:345:632.
23. Roep BO, Kallan AA, Hazenbos WL, et al. T-cell reactivity to 38 kD insulin-secretory-granule protein in patients with recent-onset type 1 diabetes. Lancet 1991:337:1439.
24. Atkinson MA, Kaufman DL, Campbell L, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 1992:339:458.
25. Worsaae A, Hejnaes K, Moody A, et al. T-cell proliferative responses to glutamic acid decarboxylase-65 in IDDM are negatively associated with HLA DR3/4. Autoimmunity 1995:22:183.
26. Vandewalle CL, Decraene T, Schuit FC, et al. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1* 0301-DQB1*0302 haplotype at clinical type I (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. The Belgian Diabetes Registry. Diabetologia 1993:36:1155.
27. Gorus FK, Goubert P, Semakula C, et al. The Belgian Diabetes Registry: IA-2-autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. Diabetologia 1997:40:95.
28. Vandewalle CL, Falorni A, Lernmark A, et al. Associations of GAD65 - and IA-2 autoantibodies with genetic risk markers in new-onset IDDM patients and their siblings. The Belgian Diabetes Registry. Diabetes Care 1997:20:1547.
29. Muluk SC, Clerici M, Via CS, et al. Correlation of in vitro CD4+ T helper cell function with clinical graft status in immunosuppressed kidney transplant recipients. Transplantation 1991:52:284.
30. van Besouw NM, Vaessen LM, Daane CR, et al. Peripheral monitoring of direct and indirect alloantigen presentation pathways in clinical heart transplant recipients. Transplantation 1996:61:165.
31. Bhargava R, Senior PA, Ackerman TE, et al. Prevalence of hepatic steatosis after islet transplantation and its relation to graft function. Diabetes 2004:53:1311.