Auto- and Alloimmune Reactivity to Human Islet Allografts Transplanted Into Type 1 Diabetic Patients

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Allogeneic islet transplantation can restore an insulin-independent state in C-peptide–negative type 1 diabetic patients. We recently reported three cases of surviving islet allografts that were implanted in type 1 diabetic patients under maintenance immune suppression for a previous kidney graft. The present study compares islet graft–specific cellular auto- and alloreactivity in peripheral blood from those three recipients and from four patients with failing islet allografts measured over a period of 6 months after portal islet implantation. The three cases that remained C-peptide–positive for >1 year exhibited no signs of alloreactivity, and their autoreactivity to islet autoantigens was only marginally increased. In contrast, rapid failure (<3 weeks) in three other cases was accompanied by increases in precursor frequencies of graft-specific alloreactive T-cells; in one of them, the alloreactivity was preceded by a sharply increased autoreactivity to several islet autoantigens. One recipient had a delayed loss of islet graft function (33 weeks); he did not exhibit signs of graft-specific alloimmunity, but developed a delayed increase in autoreactivity. The parallel between metabolic outcome of human β-cell allografts and cellular auto- and alloreactivity in peripheral blood suggests a causal relationship. The present study therefore demonstrates that T-cell reactivities in peripheral blood can be used to monitor immune mechanisms, which influence survival of β-cell allografts in diabetic patients. *Diabetes* 48:484–490, 1999

Type 1 diabetes results from a T-cell–mediated selective autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans. Successful instances of intraportal islet transplantation restoring metabolic control in type 1 diabetes patients have been reported (1–4). The transplantation procedure involves minor surgery and appears not to be associated with major side effects (2). Nonetheless, the overall outcome thus far, as registered by the International Islet Transplant Registry, is disappointing: <10% of recipients were insulin-independent after 1 year (5). The reasons for this low success rate are unknown but are believed to be due to graft rejection or recurrence of autoimmunity, alone or in combination (6). In immunocompetent type 1 diabetes patients receiving a pancreatic segment from a twin, rapid recurrence of autoimmunity with selective β-cell destruction was noted without evidence of graft rejection (7). Immunosuppressive antirejection therapy may also prevent autoimmune damage of the islet or pancreas graft (8), but not always, since selective autoimmune β-cell destruction in pancreas grafts despite HLA mismatches has been reported in type 1 diabetes patients receiving immunosuppressive therapy (6,9). After this recurrence of diabetes, the donor islets greatly resembled islets in pancreases of long-standing type 1 diabetes patients. In other studies, increases or reappearances of islet cell autoantibodies suggested reactivation of autoimmunity upon islet implantation (10). Failure of immunosuppressive drugs to prevent progressive β-cell dysfunction has also been found in prediabetic subjects and recent-onset type 1 diabetes patients (11). The risk of recurrence of autoimmunity as the cause of graft failure seems to be associated with type 1 diabetes, since islet implantations are usually successful in type 2 diabetes patients and in cases of islet auto- or allotransplantation in patients after pancreatectomy (18% of type 1 diabetes patients insulin independent in <1 year vs. 60% of type 2 diabetes patients and 88 and 80% of auto- or allotransplanted nondiabetic patients, respectively) (5).

Thus far, cellular immunologic studies in islet transplantation trials have not been performed. In the present study, we analyzed immunologic changes in relation to the fate of human allogeneic islet grafts implanted in the liver of immunosuppressed type 1 diabetes patients. Our results demonstrate that transplantation of islets can induce both allo- and autoimmune responses that are associated with graft failure. Absence of signs of auto- or alloreactivity was associated with long-term graft survival.

RESEARCH DESIGN AND METHODS

Patients. Seven human islet allografts were performed in Brussels between October 1994 and September 1995. Patient characteristics and follow-up have been described elsewhere (12), except for patient ML (Table 1). All were C-peptide–negative type 1 diabetes patients with a functioning renal allograft transplanted 2–6 years earlier. Six patients were under triple immunosuppressive therapy, and one patient (ML) only received azathioprine and cyclosporin (Table 1).

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Received for publication 10 July 1998 and accepted in revised form 2 November 1998.

ATG, antithymocyte globulin; BSA, bovine serum albumin; CTL, cytotoxic T-lymphocyte; ICA, islet cell antibody; IL, interleukin; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; SI, stimulation index.

Two hours before islet injection into a portal vein branch, all recipients were given 500 mg methylprednisolone intravenously. No additional antirejection therapy was administered.

The islet cell transplants consisted of a mixture of cultured (70%) and cryopreserved (30%) cells in three patients (VW, DG, LJ), cultured cells in three patients (VC, KV, VM), and cryopreserved cells in patient ML. Cellular composition and in vitro function of all islet grafts were determined before islet cell transplantation. These parameters were found to be comparable and within normal range (12). In patient VW, fewer β-cells were transplanted (0.92 million per kg body wt) than in the other patients (range 1.06–1.75 million per kg body wt). The HLA typing results of the kidney and pancreas donors are shown in Table 2.

Plasma C-peptide levels posttransplantation. Fasting plasma C-peptide was detectable in all patients during the first 2 weeks posttransplantation (12). Patients DG, LJ, and KV became C-peptide–negative in the 3rd week after implantation, while plasma C-peptide levels slowly decreased over a period of 30 weeks in patient ML. Patients VW, VC, and VM remained C-peptide–positive for more than 52 weeks. Patients VC and VM became insulin independent (12).

Islet cell antibodies pre- and posttransplantation. All recipients were negative for islet cell antibodies (ICA) before and after islet cell transplantation (12). Patient LJ was strongly GAD65 antibody–positive before implantation, and patient ML was borderline GAD65 antibody–positive before transplantation. GAD65-negative patients remained negative, and GAD65-positive patients exhibited a rise in titer after transplantation (12).

Autoreactivity

Antigens. Preparations enriched for insulin-secretory granules and crude β-cell membranes were taken from rat insulinoma tissue as described (13,14) and tested at a concentration of 10 μg/ml. Other islet autoantigens proposed to be associated with T-cell–mediated β-cell destruction in type 1 diabetes were tested: insulin (25 μg/ml) (Sigma Chemical, St. Louis, MO); GAD65 (10 μg/ml) (15,16) (purified baculovirus-expressed recombinant human GAD65 [17] provided by Dr. Thomas Dyrberg, Novo Nordisk A/S, Bagsvaerd, Denmark); ICA69 (20 μg/ml) (18,19) (provided by Dr. Stephan Martin, Diabetes Research Institute at the Heinrich-Heine University, Düsseldorf, Germany); imogen-38 (r38kDa) (5 μg/ml) (13,20) (provided by Dr. John C. Hutton, University of Cambridge, U.K., and Barbara Davis Center for Childhood Diabetes, University of Colorado, Denver); and human 38-kDa (h38kDa) enriched autoantigen (25 μg/ml) (provided by Dr. Jacob Petersen, Hagedorn Research Institute, Gentofte, Denmark). Two unrelated control antigens were tested: αB-crystallin (10 μg/ml), which is recognized in a number of neurologic autoimmune disorders unrelated to type 1 diabetes (21) (provided by Dr. Hans van Noort, TNO Health and Prevention, Leiden, the Netherlands) and tetanus toxoid (1.5 *Limes flocculationes*/ml or 12.0 international units/ml) (National Institute of Public Health and Environmental Protection, the Netherlands). Bovine serum albumin (BSA) was included since it might exhibit cross-reactivity with islet antigen (25 μg/ml) (22) (Organon Chemicals, Boxtel, the Netherlands). The antigen concentrations were selected on the basis of either recommendation by the suppliers (GAD65 and αB-crystallin) or previous studies in which optimal concentrations were defined.

Lymphocyte proliferation test. Peripheral blood mononuclear cells (PBMC) were isolated from freshly drawn heparinized blood and tested as described (23). In short, ~150,000 PBMC were cultured in tissue-coated, round-bottomed 96-well plates (Costar, Cambridge, MA) in Iscove's modified Dulbecco's medium with 2 mmol/l glutamine (Gibco, Paisley, Scotland) supplemented with 10% human type AB pool serum, 10 U/ml penicillin, and 10 μg/ml streptomycin (Flow Laboratories, Irvine, Scotland), in the presence of antigen, T-cell growth factor (10% Lymphocult; Biotest, Dreieich, Germany), or medium alone in 150 μl at 37°C, 5% CO_2. After 5 days, 50:l RPMI 1640 (Dutch Modification; Gibco) containing 0.5 μCi [^{3}H]thymidine per well was added, and incubation was continued for 16 h. Cultures were then harvested on glass-fiber filters, and [^{3}H]thymidine incorporation was measured by liquid scintillation counting. The results are expressed as stimulation indexes (SI), that is, the median of triplicates in the presence of stimulus divided by the median of triplicates with medium alone. In previous studies, the proliferative responses in nonimmunosuppressed newly diagnosed type 1 diabetes patients and nondiabetic controls to secretory granules were in the SI range of 0.8–320 (mean 37.0) and 0.6–31.0 (mean 3.3), respectively (23); to tetanus toxoid, 0.7–572 (mean 80.4) and 0.7–394 (mean 75.7) (23); to ICA69, 0.6–110 (mean 8.9) and 0.5–5.0 (mean 1.2) (19); to insulin, 1.0–28.3 (mean 7.1) and 0.3–6.0 (mean 1.1); to GAD65, 0.5–32.4 (mean 9.4) and 0.7–7.6 (mean 3.8); to imogen-38 (r38kDA), 0.4–200 (mean 24.6); to human 38-kDa enriched autoantigen (h38kDA), 1.0–65.0 (mean 18.6); and to BSA, 0.7–9.0 (mean 1.4) and 0.9–5.7 (mean 1.2). The intra-assay coefficient of variation of positive responses was always between 0 and 20%. The interassay variation for tetanus toxoid was previously determined to be in the range of 14–21% (23).

Alloreactivity

Screening for HLA class I alloantibodies. Sera were tested before and after islet transplantation for the presence of HLA class I antibodies in a routine complement-dependent cytotoxicity assay. Islet donors were selected on the basis of a negative serologic cross-match with patient sera from before transplantation.

T-helper and cytotoxic T-lymphocyte precursor frequency determination. In a combined assay, both cytotoxic T-lymphocyte (CTL) precursor and T-helper frequencies were determined against all mismatched HLA antigens of the islet donors. These mismatched antigens were presented by three to four different stimulator cells. The kinetics of the precursor frequency of a particular patient was tested in a single assay. The combination assay was performed as previously described (24). Briefly, twofold-diluted responder PBMC were cultured at 37°C with 50,000 irradiated (5,000 rad) stimulator PBMC in 96-well V-bottomed plates (Greiner, Alphen a/d Rijn, the Netherlands) for 3 days in RPMI 1640 (Gibco) supplemented with glutamine (2 mmol/l) and 10% heat-inactivated pooled human serum. On day 3, supernatant was harvested and frozen at –20°C until further use in the CTLL-2 bioassay to determine T-helper frequencies (described below). The cells were resuspended in culture medium containing 20 U/ml of interleukin (IL)-2 (Cetus) and transferred to 96-well U-bottomed plates (Costar). After a second culture period of 7 days, cytotoxicity was determined by split-well analysis in each well by addition of Europium-labeled target cells (25). PBMC cultured with phytohemagglutinin (PHA) (Wellcome, Dartford, U.K.) were used as target cells (PHA-blasts). Target cells were PHA-blasts of the stimulator cells, expressing all the mismatched islet donor antigens, and PHA-blasts that shared only one or some of the HLA antigens with the stimulator cells (that is, the mismatched antigens with the kidney transplant). As a control, PHA-blasts that had no antigens in common with the stimulator cells were included. After 4-h incubation, Europium release in the supernatant due to target cell lysis was measured. Frequencies of CTL precursors were calculated using the jackknife method according to Strijbosch et al. (26), as published previously (24). Identical precursor frequencies were calculated if maximum likelihood or minimal χ^2 methods were applied.

The amount of IL-2 present in the supernatants was determined using the IL-2–dependent murine CTLL-2 cell line (27). Since T-helper cells are thought to be primarily responsible for IL-2 production (28,29), their precursor frequency can be estimated in this way. Frequencies of T-helper precursors were calculated according to Strijbosch et al., as described above.

RESULTS

Autoreactive T-cells. We analyzed proliferative T-cell responses against a range of islet autoantigens that are candidates for targets of autoreactive T-cells in relation to β-cell autoimmunity. All patients displayed normal proliferative responses to IL-2. Before islet transplantation, no T-cell reactivity was detected to any of the tested autoantigens or control antigens, except in patient ML, who exhibited T-cell reactivity to both groups of antigens. After implantation of the islet allografts, three patterns of reactivity were observed (Figs. 1–3). First, patients VM and KV displayed no T-cell reactivity to islet autoantigens or control antigen after islet implantation, and patients VW, VC, and LJ developed only a slight increase in T-cell reactivity to insulin-secretory granules, insulinoma membranes, and/or human 38-kDa enriched autoantigen at posttransplant week 2–4 (Fig. 1). This reactivity was comparable to that previously described for type 1 diabetes patients several years after disease onset (23). In all three cases, the reactivity declined to baseline after 4 weeks. Responses to tetanus toxoid were completely absent and remained undetectable. In patients VW and LJ, the T-cell responses increased slightly and remained constant up to 52 weeks. No reactivity was detected to ICA69, insulin, or BSA in these patients.

Second, one patient (DG) presented with an acute and very high increase in autoimmune T-cell reactivity to multiple autoantigens within 2 weeks, while her T-cell reactivity to the panel of antigens was low before transplantation (Fig. 2). The autoimmune T-cell responses remained high during follow-up, only reaching baseline levels after 34 weeks. Her T-cell reactivity was most pronounced to insulin-secretory granules, but was also clearly detectable with the human 38-kDa enriched autoantigen. Reactivity to insulinoma membranes reached a maximum at 6 weeks after islet transplan-

TABLE 1
Recipient and graft characteristics

	Patient						
	VW	VC	VM	DG	LJ	KV	ML
History of diabetes							
Age at onset (years)	19	3	7	9	18	11	21
Sex	M	F	M	F	M	M	M
HLA type	DR3, 6	DR1, 4	DR3, 4	DR3, 4	DR1, 3	DR3, 4	DR4, 4
Status at implantation							
Age (years)	52	38	38	39	46	45	48
ICA and GAD65 antibodies	Negative	Negative	Negative	Negative	$GAD65^+$	Negative	$GAD65^+$
Immunosuppressive treatment							
History of ATG treatment	Yes	Yes	Yes	No	No	No	No
Cyclosporin level (ng/ml)	89	126	109	93	47	103	152
Azathioprine dosage ($mg \cdot k^{-1} \cdot day^{-1}$)	1.5	1.7	1.8	0.7	1.1	1.0	0.6
Methylprednisolone dosage ($mg \cdot k^{-1} \cdot day^{-1}$)	0.09	0.14	0.08	0.09	0.05	0.06	—
Graft							
Number of pancreas donors	8	6	8	8	9	7	14
Number of β-cells ($\times 10^6$)	64.4	103.3	94.5	102.8	80.1	100.8	125.5
Percent cryopreserved	30	0	0	27	37	0	100
Graft survival (weeks)	>52	>52	>52	<3	<3	<3	33

Data for all patients except ML are from Keymeulen et al. (12).

tation. Minor increases in T-cell responses to ICA69, imogen-38, and BSA were measured. A second peak in immune reactivity at 22 weeks after transplantation was accompanied by an increase in T-cell proliferation to tetanus toxoid, suggestive of insufficient immunosuppressive therapy at that time point.

Third, patient ML, who displayed unusually high T-cell reactivity to autoantigens as well as reference antigens before islet transplantation (Fig. 3), showed an initial decline of T-cell reactivity upon islet transplantation, which increased rapidly after 2 weeks to reach levels similar to those before islet transplantation. This patient was also exceptional with regard to the high T-cell reactivity to tetanus toxoid, which was not observed in the other six patients. Insulin-secretory granules induced high T-cell proliferative responses, followed by imogen-38 and insulinoma membranes. Interestingly, T-cell reactivity to GAD65 first appeared 22 weeks after transplantation, when C-peptide production started to decline. After 27 weeks, the GAD65 T-cell reactivity declined.

Alloreactive T-cells. T-cell alloreactivity was measured in six of the seven patients. All but one patient (KV) initially displayed low frequencies of alloreactive CTL precursors that were directed to a panel of allogeneic donor PBMC representing the HLA class I mismatches in the islet graft (■, ▼, ◆, and ▲ in Figs. 1*B*, 2*B*, and 3*B*). After islet implantation, the levels of circulating CTL precursors remained negative or low in patients VC, VM, and ML, with a single, slight increase of CTL response to a single stimulator present in one of the islet donors at 2 weeks in the case of patient VM, which rapidly declined. In contrast, patients DG, LJ, and KV exhibited significant increases in CTL precursors specific for alloantigens expressed by two or more allogeneic stimulators, whereas no CTL reactivity was seen to autologous PBMC. In patient DG, the alloreactivity was maximal 4 weeks after implantation (2 weeks after maximal autoreactive T-cell responses) and gradually declined with time, reaching baseline levels 35 weeks after implantation. One of the allodeterminants expressed by two of the stimulators was also expressed by the previously implanted kidney. Specific alloreactivity to this repeated mismatch could be excluded, however, since recognition of one of these two stimulators by the patient's T-cells remained low. The peak of alloreactivity in patient LJ at 4 weeks after transplantation coincided with a slight increase of responses to islet autoantigens.

TABLE 2
Comparison of HLA typing results from type 1 diabetic patients with mismatches of kidney and pancreas donors

Patient	Patient HLA type			Mismatch of kidney donor	Mismatch of islet donors		
VW	A1, 11	B8, 13	DR3, 6	B35	A2, 3, 9, 10	B7, 18, 27, 40, 44, 57, 62	DR1, 2, 4, 5, 7
VC	A2, 28	B45, 62	DR1, 4	B44	A1, 3, 11	B7, 8, 27, 35, 38, 44	DR2, 5, 6, 7
VM	A11, 28	B18, 62	DR3, 4	A2, B44, DR2, 6	A1, 2, 3, 24, 29, 33	B5, 8, 14, 27, 35, 38, 44	DR1, 2, 5, 6, 7
DG	A2, 3	B18, 62	DR3, 4	A24, 25, B41	A1, 11, 23, 24, 36, 66	B5, 7, 8, 27, 41, 44, 47, 57	DR1, 2, 5, 6, 7, 8
LJ	A2, –	B7, 8	DR1, 3	A1	A1, 3, 11, 19, 24, 28	B13, 17, 18, 35, 37, 38, 44, 47, 51, 60, 62	DR2, 5, 6, 7
KV	A1, 3	B8, 12	DR3, 4	A11	A2, 11, 24, 28, 29, 30	B7, 13, 18, 27, 35, 39, 51, 60, 61, 62	DR1, 2, 5, 6, 7
ML	A1, 24	B7, 62	DR4, –	A2, 30, B38, DR6	A2, 3, 11, 23, 25, 26, 28, 30	B8, 13, 27, 35, 37, 44, 47, 49, 51, 60	DR1, 2, 3, 5, 6, 7, 8, 9

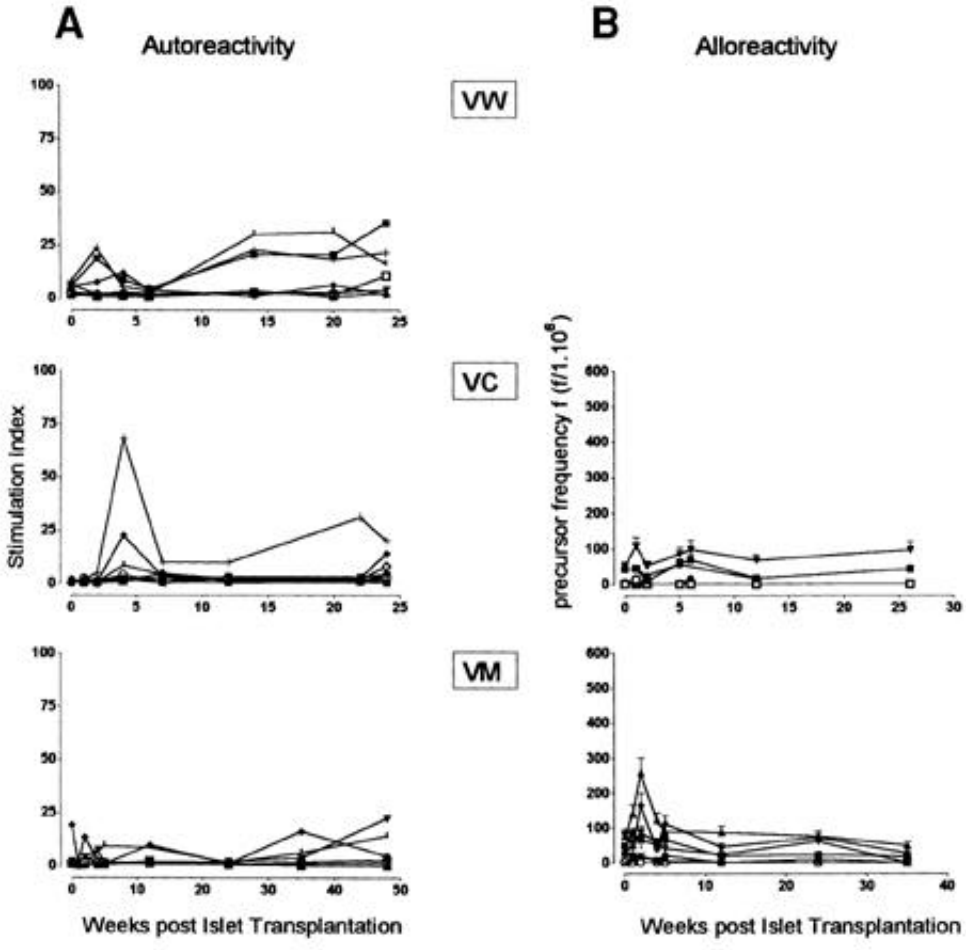

FIG. 1. Longitudinal analyses of auto- and alloreactivity in long-term surviving islet allografts (patients VW, VC, and VM). Autoreactivity (*A*) is depicted as proliferative T-cell responses (stimulation index) to a panel of islet autoantigens and control stimuli. Autoreactive responses against islet autoantigens (GAD65 [○], insulin [▲], insulin-secretory granules [+], ICA69 [◆], h38kD [■, ●], r38kDa [◇], insulinoma membranes [ ]) and control stimuli (BSA [□], αB-crystallin [△], tetanus toxoid [▼]). Alloreactivity (*B*) is depicted as CTL precursor frequencies (responding cells per million ± SE) of serial blood samples directed against mismatched HLA antigens of the islet donors [■, ▼, ◆, ▲] and autologous cells [□], and, if present, against HLA mismatches shared with the kidney transplant (first repeated mismatch [●], second repeated mismatch [X]). Frequencies against a third party (○) were determined in VC and VM.

No changes in CTL precursor reactivity to allogeneic HLA expressed by the kidney allograft, third-party donor leukocytes, or autologous leukocytes were detectable in any of the patients during the duration of our analyses (6 months) (Figs. 1–3).

Patient VW continuously suffered from lymphopenia, so insufficient numbers of PBMC were available for retrospective analyses of alloreactive T-cell activity. A limited series of mixed lymphocyte reactions with a concentration range of responder lymphocytes isolated before and 2, 20 and 26 weeks after islet transplantation indicated a low reactivity to a panel of allogeneic donor PBMC containing mismatches present in the islet graft, which remained unaltered over time (data not shown).

The pattern of frequencies of alloreactive T-helper precursor cells in all patients before and after islet implantation completely reflected the pattern observed with CTL precursors (data not shown). Alloantibody titers were undetectable and did not appear over time in any of the type 1 diabetes patients transplanted with islet grafts.

DISCUSSION

Three trends were noticed in this series of seven patients. First, minor immunoreactivity occurred in three patients (VW, VC, and VM): the islet graft did not induce alloreactivity and produced only occasional minor and delayed increases in islet autoreactivity. These three patients maintained a surviving and functioning graft for longer than a year (12). Patients VC and VM remain insulin independent. The number of β-cells implanted in patient VW were insufficient for insulin independence (12).

A second pattern was characterized by strong alloreactivity after islet implantation, which was associated with rapid graft failure (patients DG, LJ, and KV). An exceptional increase in islet graft–specific alloimmunity in patient DG was preceded by an immediate increase in autoreactive T-cell responsiveness. We conclude that the islet graft was destroyed by allograft rejection (patients LJ and KV). A combination of recurrent autoimmune islet destruction and allomediated graft rejection, perhaps precipitated by the autoimmune reactivity to the islets, may explain the failure in patient DG.

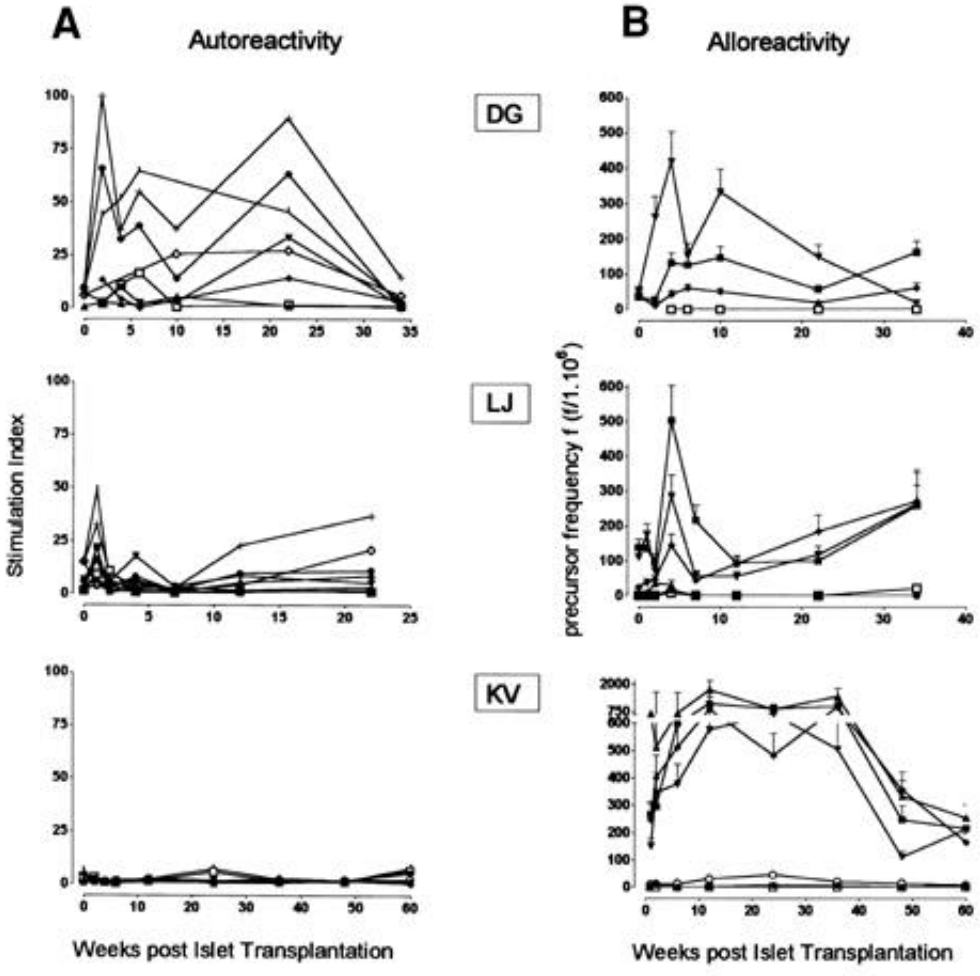

FIG. 2. Longitudinal analyses of auto- and alloreactivity in rapidly rejected islet allografts (patients DG, LJ, and KV). Autoreactivity (*A*) is depicted as proliferative T-cell responses (stimulation index) to a panel of islet autoantigens and control stimuli. Autoreactive responses against islet autoantigens (GAD65 [○], insulin [▲], insulin-secretory granules [+], ICA69 [◆], h38kD [■, ●], r38kDa [◇], insulinoma membranes [ ]) and control stimuli (BSA [□], αB-crystallin [△], tetanus toxoid [▼]). Alloreactivity (*B*) is depicted as CTL precursor frequencies (responding cells per million ± SE) of serial blood samples directed against mismatched HLA antigens of the islet donors (■, ▼, ●, ▲), autologous cells (□), and, if present, HLA mismatches shared with the kidney transplant (first repeated mismatch [●], second repeated mismatch [X]). Frequencies against a third party (○) were determined in KV.

The third pattern of reactivity was detected in only one patient (ML). Hyperautoreactivity was measured in the absence of any detectable alloreactivity at any time point, in combination with chronic progressive islet graft failure. The high (auto)antigen-specific T-cell reactivity was measured before islet implantation and declined upon islet transplantation, but returned to the initial high levels. Interestingly, C-peptide levels and responses to glucose appeared to be normal in the first 3 months but became gradually distorted. Increased levels of T-cell autoimmunity accompanied this process. Patient ML became C-peptide–negative after 33 weeks. In this patient, the islet graft was probably lost due to chronic, progressive autoimmune T-cell–mediated β-cell destruction.

Although we have no indication that our methods failed to detect relevant immunologic responses, it is certainly possible that we may have missed in vivo islet-related T-cell auto- or alloreactivity. Nonetheless, the T-cell responses detected fit remarkably well with the metabolic and clinical results (12). Moreover, our interpretation of the data on T-cell responses was corroborated by the serological results on auto- and alloantibodies, which can be measured with great sensitivity and specificity. We therefore believe that the results, although not conclusive, strongly suggest that loss of islet graft function was due to autoreactivity, alloreactivity, or a combination of both, while persistent graft function was associated with low auto- as well as alloreactivity.

Our results indicate that maintenance immunosuppressive treatment for an established kidney graft can prevent immune responses to an islet graft, but only in some type 1 diabetes patients. The absence of auto- and alloreactivity to the islet implant was only noticed in the three patients with a history of antithymocyte globulin (ATG) therapy, namely at the time of the prior kidney transplantation. We hypothesize that ATG might have depleted autoreactive memory T-cells developed in the pathogenesis of type 1 diabetes in patients VW, VC, and VM. Our data suggest that this depletion need not occur immediately before the islet transplantation. This implies that the apparent tolerance to previously implanted kidney grafts should not be jeopardized by lymphocyte depletion in relation to the islet implantation. Further stud-

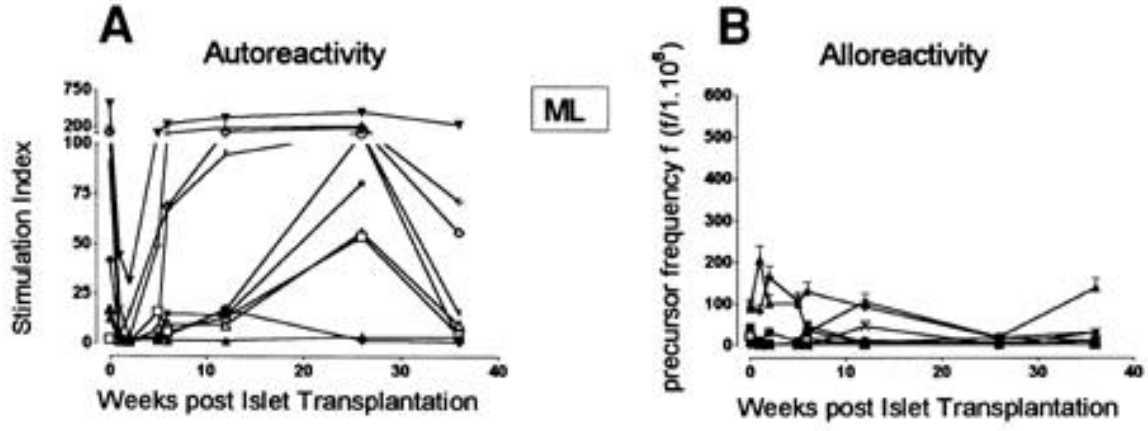

FIG. 3. Longitudinal analyses of auto- and alloreactivity in slowly failing islet allografts (patient ML). Autoreactivity (*A*) is depicted as proliferative T-cell responses (stimulation index) to a panel of islet autoantigens and control stimuli. Autoreactive responses against islet autoantigens (GAD65 [○], insulin [▲], insulin-secretory granules [+], ICA69 [◆], h38kD [■, ●], r38kDa [◇], insulinoma membranes [ ]) and control stimuli (BSA [□], αB-crystallin [△], tetanus toxoid [▼]). Alloreactivity (*B*) is depicted as CTL precursor frequencies (responding cells per million ± SE) of serial blood samples directed against mismatched HLA antigens of the islet donors (■, ▼, ◆, ▲), autologous cells (□), and, if present, HLA mismatches shared with the kidney transplant (first repeated mismatch [●], second repeated mismatch [X]). Frequencies against a third party (○) were determined in ML.

ies are in progress to evaluate the efficacy of prior ATG treatment on the outcome of islet allograft acceptance.

In the four patients without history of ATG treatment, β-cell graft function was completely lost. In three of them, this failure occurred within 3 weeks after transplantation and was associated with peripheral signs of alloreactivity, with or without autoreactivity. It has been shown that cyclosporin did not delay insulin dependence in asymptomatic type 1 diabetes patients (11). Our findings of increased autoreactivity despite immunosuppression with cyclosporin are in accordance with this finding. When PBMC were cultured with antigens, addition of cyclosporin in vitro did not alter the T-cell reactivity (data not shown). Cyclosporin affects both primary and memory responses of T-cells, as was illustrated by severely reduced T-cell reactivity to the recall antigen tetanus toxoid. The in vitro responses of lymphocytes to tetanus toxoid were rarely increased and were always accompanied by a general increase in T-cell proliferation. Shearer et al. (30) proposed that increases in T-cell reactivity to tetanus toxoid can be used to monitor the efficacy of immunosuppression, in relation with kidney graft rejection episodes, but this concept is still disputed (31).

Higher doses of azathioprine in combination with cyclosporin or prednisolone, or both, were associated with prolonged islet graft function (that is, insulin production and secretion). Interestingly, azathioprine has been reported to prevent development of insulitis and diabetes in NOD mice (32). Clinical trials in humans have been less successful (33), but a beneficial effect with increased remission rates in older subjects in particular has been reported (34,35). It can thus be concluded that additional immune modulation is needed in these patients to prevent immune destruction of the β-cell graft.

The relation between allograft rejection and alloreactive CTL precursor frequencies has not always been as clear as in this study. Increases in allo-CTL precursors have been described in patients with stable graft function (36). Nonetheless, we only measured such increases in combination with loss of C-peptide production. Allo-CTL precursors with high-affinity receptors for donor antigens have been demonstrated to be a prognostic marker for graft failure (36).

Absence of islet autoantibodies in the islet graft recipients may be another factor that improves stable graft function (12,37). Autoantibodies to GAD65 are frequently detectable many years after the clinical onset of type 1 diabetes. Sensitization to donor HLA antigens has been shown in islet recipients with failing transplants (38). Two of our patients with low titers of autoantibodies to GAD65 developed increased titers after islet transplantation (12). One of these two patients lost β-cell graft function at posttransplant week 33. He did not present any alloreactivity throughout the follow-up period. The increase of GAD65 antibody titers suggests that loss of function is not restricted to primed cytotoxic or T-helper cells, but may involve an entire immune response with B-cells, perhaps acting as antigen-presenting cells.

Even though the grafts transplanted in our study were composed of multiple donor pancreases to obtain sufficient numbers of β-cells, our results show no indications that the islet transplant procedure could jeopardize the kidney graft. The absence of signs of rejection episodes of the kidney accords with the lack of alloreactivity to repeated HLA mismatches (between the kidney and islet donors and the recipient) after islet implantation and emphasizes the specificity of immune responses to the islet allograft. In another islet transplantation study, two type 1 diabetes patients experienced rejection episodes of the previously transplanted kidney 3 weeks after islet implantation (39). Our results showed that indeed the first few weeks after islet implantation generally indicate induction of immune reactivity to the islet graft, which suggests that the kidney rejection episodes in the Milan trial (39) were precipitated by the induction of immune reactivity to the islet graft.

In conclusion, our study of seven long-term type 1 diabetes patients receiving an implant of highly purified human islet preparations demonstrates successful islet graft function beyond 1 year, accompanied by absence of T-cell auto- and alloreactivity to islets in three patients. In four patients, loss of β-cell function was associated with appearance of auto- or

alloreactivity, suggesting a complex reaction between the graft and the immunosuppressed immune system in the recipient.

ACKNOWLEDGMENTS

This study was supported by grants from the Juvenile Diabetes Foundation International (95-00); the Royal Academy of Arts and Sciences, the Netherlands; the J.A. Cohen Institute (IRS); and the Child Health and Wellbeing Fund, and it was performed within the framework of a multicenter program supported by the European Union (Biomed CT92-0805 and CT95-1561), the Vlaamse Gemeenschap (93/019), the Belgian Ministry of Science (D.P.W.B. CE/03/001), and the Belgian Fonds Wetenschappelijk Onderzoek (3.0057.94).

We thank Elvira Timmermans for expert technical assistance, Drs. Thomas Dyrberg, John Hutton, Jacob Petersen, Hans van Noort, and Stephan Martin for the supply of autoantigens, and Christel Hendrieckx, the Eurotransplant Foundation, and BioImplant Services (Theo de By) for their invaluable contributions.

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