

Correlation between β cell mass and glycemic control in type 1 diabetic recipients of islet cell graft

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Contributed by Jon van Rood, September 15, 2006

Islet grafts can induce insulin independence in type 1 diabetic patients, but their function is variable with only 10% insulin independence after 5 years. We investigated whether cultured grafts with defined β cell number help standardize metabolic outcome. Nonuremic C-peptide-negative patients received an intraportal graft with $0.5\text{--}5.0 \times 10^6$ β cells per kilogram of body weight (kgBW) under antithymocyte globulin and mycophenolate mofetil plus tacrolimus. Metabolic outcome at posttransplant (PT) month 2 was used to decide on a second graft under maintenance mycophenolate mofetil/tacrolimus. Graft function was defined by C-peptide >0.5 ng/ml and reduced insulin needs, metabolic control by reductions in HbA_{1c}, glycemia coefficient of variation, and hypoglycemia. At PT month 2, graft function was present in 16 of 17 recipients of $>2 \times 10^6$ β cells per kgBW versus 0 of 5 with lower number. The nine patients with C-peptide >1 ng/ml and glycemia coefficient of variation of $<25\%$ did not receive a second graft; five of them were insulin-independent until PT month 12. The 12 others received a second implant; it achieved insulin-independence at PT month 12 when the first and second graft contained $>2 \times 10^6$ β cells per kgBW. Of the 20 recipients of at least one graft with $>2 \times 10^6$ β cells per kgBW, 17 maintained graft function and metabolic control up to PT month 12. At PT month 12, β cell function in insulin-independent patients ranged around 25% of age-matched control values. Thus, 1-year metabolic control can be reproducibly achieved and standardized by cultured islet cell grafts with defined β cell number.

diabetes | islet transplantation | prevention | type 1 diabetes

Islet transplantation can correct type 1 diabetes in both kidney recipients and nonuremic patients (1). However, the degree and duration of clinical success are variable, and only 10% of recipients remain insulin-independent for 5 years (1). Normalization of hyperglycemia is sometimes achieved with a graft from one pancreas (2, 3), but more often requires combined isolates from more donor organs (4–8); these are then injected at one or, more often, two or three time points with different intervals, each involving an additional antibody treatment (2–8). Grafts have not been standardized in terms of cellular composition and β cell mass. This variability in protocol and in implanted β cell mass probably contributes to the variable metabolic outcome and makes it at the same time difficult to undertake comparative protocols in search for a more successful outcome. In the present study we investigated the influence of β cell mass in the implant on β cell function in the recipient during the first year. Data were used to decide whether a second implant would be given and to correlate β cell function in the recipient with metabolic benefit. An immune suppressive protocol was used with one antithymocyte globulin (ATG) course because this was previously found beneficial for survival of a later islet cell allograft while maintaining tolerance to the first graft (9–11). Maintenance immune therapy consisted of a combination of mycophenolate mofetil (MMF) and tacrolimus. At posttransplant (PT) month 12, func-

tional β cell mass was compared with that of age- and body weight (BW)-matched controls, whereas the observed adverse events were analyzed versus those reported in other protocols.

Results

Survival and Function of First β Cell Implant. During PT month 2, 16 of 22 recipients exhibited a surviving graft (Fig. 1) with 6-fold higher median plasma C-peptide [1.32 ng/ml, interquartile range (IQR) 0.93–1.87 ng/ml] than in the six other cases (0.22 ng/ml, IQR 0.12–0.29 ng/ml) ($P < 0.001$). Their mean fasting blood glucose (113 mg/dl, IQR 107–119 mg/dl) was similar (121 mg/dl, IQR 112–137 mg/dl) ($P = 0.18$), but its coefficient of variation (CV) was significantly lower (17%, IQR 12–22% versus 38%, IQR 35–41%) ($P = 0.001$). Comparison of both groups for recipient and graft characteristics demonstrated a significant difference in the number of donor β cells per kilogram of body weight (BW) ($P = 0.002$) (Table 3, which is published as supporting information on the PNAS web site). Plasma C-peptide and CV of fasting glycemia (CVgl) strongly correlated with β cell number per kilogram of BW (Fig. 2). The index of a surviving graft (plasma C peptide ≥ 0.5 ng/ml) was achieved with grafts containing minimally 2×10^6 β cells per kilogram of BW (Fig. 2); all but one patient with 2-month C-peptide <0.5 ng/ml had received fewer β cells per kilogram of BW.

Patients with 2-month C-peptide ≥ 1 ng/ml and CVgl $<25\%$ ($n = 9$) did not receive a second graft (group A). At PT month 12, they all exhibited a surviving graft (C-peptide ≥ 0.5 ng/ml), but only five maintained the 2-month values of C-peptide and CVgl: these five became insulin-independent and remained so up to PT month 12 (Fig. 1). Of the four others in group A, two were transiently insulin-independent (PT months 3–9), but for all four insulin dose at PT month 12 was significantly lower than at start (median 53% lower) with similar HbA_{1c} values than preimplantation (median 7.7%). These four cases did not receive fewer β cells (3.7×10^6 per kilogram of BW; IQR 3.4–4.3; $P = 0.33$) than the five that were insulin-independent at PT month 12 (2.6×10^6 per kilogram of BW; IQR 2.3–4.2).

Survival and Function of Second β Cell Graft. In 13 patients a second graft was implanted during PT month 3, namely in seven patients with 2-month C-peptide 0.5–1 ng/ml and CVgl $>25\%$ (group B),

Author contributions: B.K., C.M., J.v.R., and D.P. designed research; B.K., P.G., C.M., B.M., G.M., G.D., D.Y., E.V., C.H., and Z.L. performed research; B.R., M.M., P.I.V., M.B., F.G., and Z.L. contributed new reagents/analytic tools; B.K., P.G., Z.L., and D.P. analyzed data; and B.K., P.G., and D.P. wrote the paper.

The authors declare no conflict of interest.

Abbreviations: PT, posttransplant; IQR, interquartile range; ATG, antithymocyte globulin; BW, body weight; CV, coefficient of variation; CVgl, CV of fasting glycemia; MMF, mycophenolate mofetil; IEQ, islet equivalent.

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PT Month 2

Plasma C-peptide	≥ 0.5 ng/ml 16/22		< 0.5 ng/ml 6/22	
	Group A	Group B	Group C	
CV-Glycemia $< 25\%$ and C-peptide ≥ 1.0 ng/ml	9/9	0/7	0/6	
Transplant of Graft 2 with Beta Cell Number 10^6 /kg BW	0/9	7/7	6/6	
	--	≥ 2 < 2	≥ 2 < 2	

PT Month 12

Plasma C-peptide ≥ 0.5 ng/ml	9/9	4/4	1/3	3/4	1/2
CV-Glycemia $< 25\%$ and C-peptide ≥ 1.0 ng/ml	5/9	4/4	0/3	2/4	0/2
Insulin-Independence	5/9	4/4	0/3	1/4	0/2

Fig. 1. Flow diagram of islet β cell transplants in 22 nonuremic type 1 diabetic patients. Survival of a β cell implant in C-peptide-negative (< 0.09 ng/ml) recipients was defined by PT plasma C-peptide ≥ 0.5 ng/ml.

and in six with 2-month C-peptide < 0.5 ng/ml (group C) (Fig. 1). At PT month 12, all patients in group B had C-peptide ≥ 0.5 ng/ml, indicating that a second graft implanted under maintenance immune suppression did not result in massive destruction of the first implant. In fact, when the second graft contained $> 2 \times 10^6$ β cells per kilogram of BW (4 of 7 patients), it increased β cell functions in all cases as shown by the higher PT month 12 plasma C-peptide (1.88 ng/ml, IQR 1.70–2.17 ng/ml) and lower CVgl (9%, IQR 8–10%) (Fig. 1); these four patients also became insulin-independent and remained so up to PT month 12. Such an increase in β cell function was not achieved in patients receiving a second graft with $< 2 \times 10^6$ β cells per kilogram of BW (3 of 7 patients in group B) (Fig. 1). In group C patients, a second graft with $\geq 2 \times 10^6$ β cells per kilogram of BW resulted in graft function at PT month 12 in 3 of 4 recipients (Fig. 1) with low CVgl in two and a state of insulin independence in one; one other patient was insulin-independent between months 5 and 10. Comparison of recipient and graft characteristics did not reveal differences between the insulin-independent and the insulin-treated groups or among the patient groups that maintained insulin independence after either one ($n = 5$) or two ($n = 5$) grafts. All four patients who received two grafts with $\geq 2 \times 10^6$ β cells per kilogram of BW were insulin-independent at PT month 12, whereas this was the case for only five of nine receiving one such graft (Fig. 1).

Metabolic Effects of Surviving β Cell Graft. In 18 recipients, β cell function (as evidenced by plasma C-peptide ≥ 0.5 ng/ml) was maintained up to PT month 12 (Fig. 1). This occurrence was associated with beneficial metabolic effects in the form of (i) lower mean fasting glycemia for lower doses of insulin treatment (Fig. 3), (ii) a significantly lower CVgl (Fig. 3), and (iii) fewer hypoglycemic episodes. The percentage of glucose measurements < 70 mg/dl decreased from 11% (IQR 8–16%) before transplantation to 0% (IQR 0–3%) ($P = 0.001$) between 6 and 12 months, and the percentage of severe hypoglycemia (< 50 mg/dl) decreased from 3% (IQR 1–5%) to 0% (IQR 0–0%) ($P < 0.001$). None of the recipients exhibited a hypoglycemic episode requiring assistance.

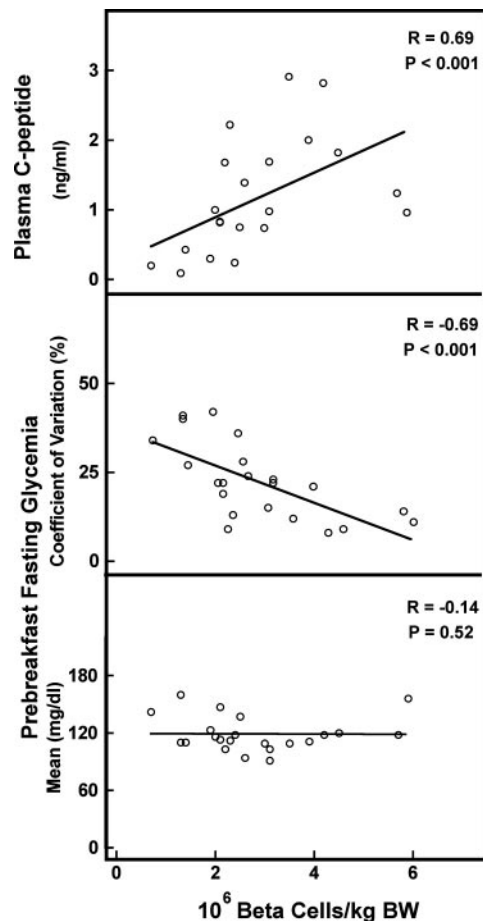


Fig. 2. Correlation between β cell number in the first graft and metabolic outcome at PT month 2 as measured by plasma C-peptide (Top), CV (Middle), and mean (Bottom) prebreakfast glycemia. A positive correlation was found between β cell number and plasma C-peptide ($R = 0.69$, $P < 0.001$) and CVgl ($R = -0.69$, $P < 0.001$) but not with mean glycemia ($R = -0.14$, $P = 0.52$).

These beneficial effects were significantly higher in patients who were insulin-independent at PT month 12 than in those who were on insulin treatment (Fig. 3); they never presented a glycemia < 70 mg/dl (0%, IQR 0–0%) whereas this occurred in 3% (IQR 2–8%) ($P = 0.03$) of insulin-treated patients; their HbA_{1c} level was also lower (Fig. 3). No correlation was noticed between the degree of metabolic normalization at PT month 12 and the total β cell number that was transplanted at one or at two occasions; however, the small number of patients per subgroup limits the significance of this analysis. For the same reason we could not assess whether a positive correlation existed between long-term metabolic function and the number of cytokeratin-19 positive cells, as recently reported by Street *et al.* (12).

Comparison of Functional β Cell Mass in Insulin-Independent Recipients with That in Matched Normal Controls. At PT month 12, fasting glycemia was slightly higher in insulin-independent recipients than in age- and BW-matched controls (107 mg/dl versus 83 mg/dl; $P < 0.001$) whereas the corresponding plasma C-peptide levels were slightly lower (Table 1). The difference in functional β cell mass was quantified by expressing C-peptide release during a glucose clamp test, first in the absence and then in the presence of glucagon. In insulin-independent graft recipients, no secretory response was detected during the first 10 min of glucose stimulation; it was clearly present after prolonged hyperglycemia (120–150 min) but corresponded only to 26% of the values in the normal controls (Fig. 4 and

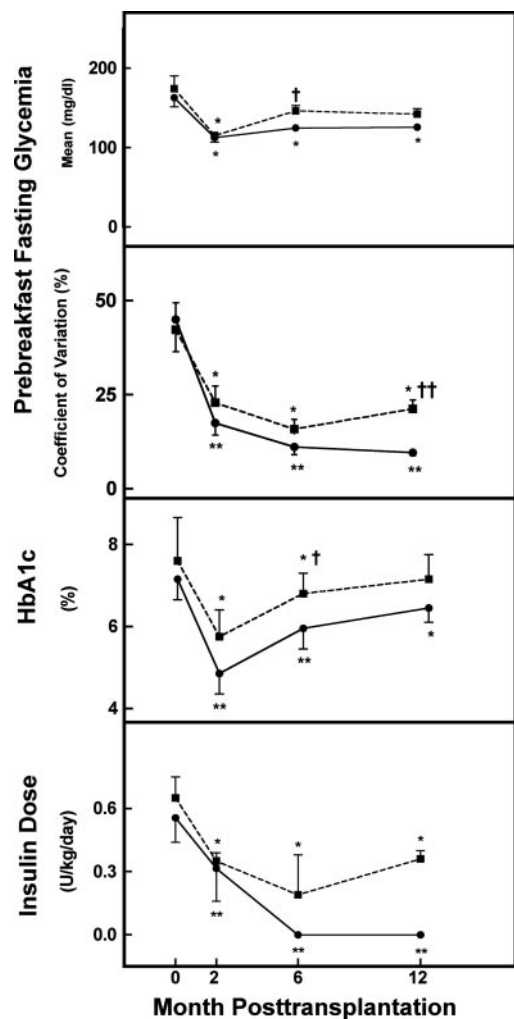


Fig. 3. Longitudinal follow-up of mean and CVgl, HbA1c, and daily insulin dose in the 18 type 1 diabetic patients with 1 year surviving grafts. Patients are divided in two groups according to their state of insulin (in)dependence at PT month 12: $n = 10$ insulin-independent (solid line) and $n = 8$ insulin-dependent (dashed line) patients. Data are means \pm SEM. Statistical difference versus pretransplantation: *, $P < 0.01$; **, $P < 0.001$. Statistical difference between groups: †, $P < 0.01$; ††, $P < 0.001$.

Table 1); administration of glucagon did further increase C-peptide levels but not their relative proportion with respect to the control levels (Table 1). When individual values were plotted, all recipients were found to exhibit a markedly lower β cell secretory capacity than controls, both in the absence and in the presence of glucagon (Fig. 4). Nine of the 10 recipients were clustered at 20% of the median control and one was at 60% (Fig. 4). These data can be considered as an index for a markedly lower functional β cell mass in insulin-independent recipients at PT month 12.

Adverse Events. None of the recipients presented complications of the graft infusion (i.e., no bleeding, thrombosis, or sepsis) or life-threatening adverse events. Only one of 24 patients developed HLA antibodies. Immune suppressive treatment caused a series of side effects (listed in Table 4, which is published as supporting information on the PNAS web site) of which the following are the most prevalent or serious: during the ATG course, 8 of 24 patients presented fever (37.5–39°C) for 1 or 2 days. MMF caused gastrointestinal symptoms (mainly pyrosis, 9 of 24) that were reduced or disappeared after lowering the dose; they caused the dropout of one

Table 1. β cell secretory function of insulin-independent recipients at PT month 12

β cell secretory function	Controls ($n = 12$)	Transplants ($n = 10$)	P value
Basal			
Glycemia	83 (79–88)	107 (100–123)	<0.001
AUC C-peptide	0.94 (0.88–1.17)	0.72 (0.64–0.83)	0.008
Glucose-induced clamp, 0–10 min			
Glycemia	144 (128–151)	173 (159–185)	0.001
AUC C-peptide	1.52 (1.34–1.83)	0.70 (0.62–0.79)	<0.001
120–150 min			
Glycemia	191 (176–198)	199 (168–213)	0.81
AUC C-peptide	7.32 (7.14–8.11)	2.08 (1.00–2.28)	<0.001
150–160 min (plus glucagon)			
Glycemia	199 (178–208)	196 (178–214)	0.97
AUC C-peptide	15.80 (11.93–18.15)	3.71 (2.72–4.39)	<0.001

Data represent median glycemia (mg/dl) and AUC C-peptide (ng/ml per min) with IQR in parentheses. AUC, area under curve.

patient. One patient was hospitalized for cerebellar ataxia that disappeared after reducing the tacrolimus dose.

Alanine transaminases were increased in 8 of 24 recipients 1–3 weeks after the first graft (2 of 12 after laparoscopy and 6 of 12 after transcatheter injection); this was not the case after the second graft (0 of 14) ($P = 0.03$). One patient developed CMV hepatitis (18-fold rise in alanine transaminases at PT week 6 with a progressively increasing number of CMV DNA copies and CMV seroconversion) that was treated with ganciclovir. Red blood cells transiently decreased in 18 of 24 recipients of the first graft; six of them received a blood transfusion at week 2. Leucopenia ($<3,000/\text{mm}^3$) was seen in 17 of 24 patients at PT month 3 but in only 6 of 24 patients at PT month 12 ($P = 0.003$), presumably because the MMF dose was reduced. Creatinine clearance was 16% lower from PT week 2 on without further decrease during the 1-year follow-up; none presented a serum creatinine >2 mg/dl. Albuminuria decreased in 8 of 8 patients with pretransplant micro- or macroalbuminuria: i.e., from 108 (47–368) mg every 24 h (median, IQR) at start to 29 (22–65), 21 (15–48), and 25 (21–86) mg every 24 h at PT months 2, 6, and 12, respectively; corresponding values for estimated glomerular filtration rate (Cockcroft–Gault formula) were 87 (78–100) ml/min at start and 71 (61–91), 70 (61–85), and 72 (64–82) at PT months 2, 6, and 12, respectively. Total cholesterol was significantly reduced at PT month 12 and also in the six patients with pretransplant cholesterol >190 mg/dl. Twenty-two patients lost weight during the first 6 PT months.

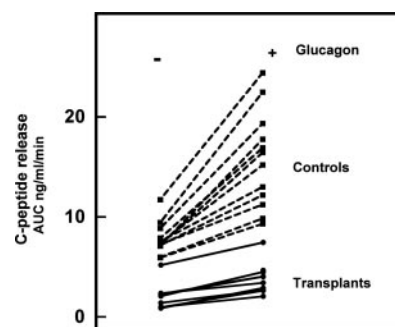


Fig. 4. Glucose-clamp induced C-peptide release in the absence and presence of glucagon. Data represent individual values of area under the curve (AUC) (ng/ml per min) measured in 10 insulin-independent graft recipients at PT month 12 (solid lines) and in 12 age- and BW-matched nondiabetic controls (dashed lines).

with the Edmonton protocol using sirolimus plus tacrolimus (8, 26). It was associated with a weight loss and with gastrointestinal side effects that were reduced after lowering the dose of MMF. Use of tacrolimus may impair kidney function, but this was not detected during the first year; a longer follow-up is needed.

Methods

Graft Recipients. Potential graft recipients (age 18–65 years) were recruited at participating hospitals according to the following inclusion criteria: (i) type 1 diabetes with plasma C-peptide <0.09 ng/ml at glycemia of 120–200 mg/dl, both in the absence and the presence of i.v. glucagon stimulation; (ii) large within-person between-day variation in fasting self-monitored plasma glucose (as defined by CV $\geq 25\%$) together with episodes of hypoglycemia (27, 28); (iii) HbA_{1c} >7% despite intensive insulin therapy; (iv) one or more signs of chronic diabetes lesions (hypoglycemic unawareness, microalbuminuria despite optimal dose of ACE inhibitor, retinopathy). Exclusion criteria were as follows: BW >90 kg; active smoking; pregnancy; disturbed liver function tests; history of hepatic disease, thrombosis, pulmonary embolism, tuberculosis, or malignancy; presence of HLA antibodies; and EBV antibody negative status. After signing an informed consent, candidate recipients were listed at Eurotransplant and selected on the basis of listing date, blood group compatibility with available graft, and status of physical and mental health at the time of implant. Twenty-four patients were consecutively enrolled for the present protocol; two of them were removed from the 12-month metabolic analysis, one after developing CMV hepatitis at PT week 5 and the other by his own decision to drop out at PT week 26 because of gastrointestinal side effects; they were not removed from the safety analysis. None of these patients were treated with metformin or insulin sensitizers either before or during the study. Their baseline characteristics are shown in Table 2.

Immune Suppression and Monitoring. The immune suppression protocol was comparable to that found to sustain survival of cultured β cell grafts when implanted in patients who were on maintenance immune therapy for a prior kidney transplantation (9). It consisted of a short course of ATG and a maintenance treatment with an antimetabolite and a calcineurin inhibitor (9). ATG (Fresenius HemoCare, Redmond, WA) was started 1–4 days before transplantation at a dose of 9 mg/kg and continued at 3 mg/kg for 6 days except when T lymphocyte count was <50/mm³. Total cumulative dose of ATG ranged from 18 to 31 mg/kg (median 24 mg/kg). MMF (2 g/day; gift of Roche, Brussels, Belgium) was started together with the first ATG injection and maintained at this dose. Tacrolimus was started the day before the last ATG administration, and its dose was adjusted to maintain blood trough levels of 8–10 ng/ml during the first 3 months and 6–8 ng/ml afterward. In case a second β cell graft was implanted, no additional course of ATG was given and the MMF dose was not changed, but tacrolimus levels were maintained at 8–10 ng/ml for the subsequent 3 months. Three hours before a β cell implant, one dose of 500 mg of methylprednisolone was given i.v.

Preparation of β Cell Grafts. Pancreases from brain-dead heart-beating donors were procured by hospitals affiliated with the Eurotransplant Foundation (Leiden, The Netherlands) according to local medical, legal, and ethical guidelines for organ donation. Organs were transported to the Beta Cell Bank in Brussels for preparation of cultured β cell suspensions according to standardized procedures (9); the Beta Cell Bank is recognized and monitored as a transplant tissue bank by the Belgian Ministry of Social Affairs, Health, and Environment. Islet cell-enriched fractions were cultured as described previously (29) by using serum-free Ham's F10 medium/0.5% human albumin/135 mg/dl glucose/2 mM glutamine (50 μ l of tissue in 45 ml of medium suspended in a T175 Starsted culture flask with

Table 2. Recipient and graft characteristics

Recipients	
Age, years	43 (39–49)
Gender	13 M/9 F
Body weight, kg	70 (64–75)
BMI, kg/m ²	24 (22–26)
Diabetes	
Age at clinical onset, years	19 (12–24)
Duration of disease, years	25 (18–33)
Positivity for ICA/GADA/IA2-A/(A)A	7/12/9/19
HbA _{1c} , %	7.6 (7.0–8.1)
Insulin dose, units/kg/day	0.7 (0.5–0.8)
Fasting glycemia	
Mean, mg/dl	174 (145–188)
CV, %	43 (40–47)
<70 mg/dl, %	7 (4–12)
<50 mg/dl, %	1 (0–2)
All glycemia	
<70 mg/dl, %	10 (8–16)
<50 mg/dl, %	3 (1–5)
Retinopathy	17
Microalbuminuria	7
Donor tissue	
Donor age, years	50 (43–56)
Pancreas cold ischemia time, h	9 (7–13)
Pancreas weight, g	89 (70–107)
Grafts	
Culture time, days	6 (3–11)
Cellular composition, %	
β cells	33 (26–42)
α cells	9 (5–13)
Nongranulated cells	45 (37–53)
Acinar cells	1 (1–4)
Dead cells	7 (5–11)

Data represent median for 22 recipients with IQR in parentheses. All recipient data were measured before transplantation, except glycemia, which represents measurements at home during the preceding year. The CVgl correlated with the percentage of fasting hypoglycemia <70 mg/dl ($R = 0.71$, $P < 0.001$) and <50 mg/dl ($R = 0.73$, $P < 0.001$). None of the recipients had a CVgl < 25%. M, male; F, female.

a vented cap). After 2–20 days (median 6 days; IQR 3–11 days) the preparations were analyzed for their β cell number and purity (9, 29). Data were used to select preparations that, after combination, would constitute a graft with $0.5\text{--}5 \times 10^6$ β cells per kilogram of recipient BW suspended in 40–85 ml of Ham's F10 medium with 0.5% human albumin. The final cellular composition of each β cell graft was determined on samples that were taken just before implantation (9). For each preparation, whether taken at the start of culture or during culture, or from the final graft, triplicate samples for DNA assay were taken, each being assayed in duplicate; when calculated for 30 consecutive grafts, the CV among these aliquots was 9% (5–14%), and that among duplicate samples was <5%. The total number of cells in a fraction was calculated by dividing its DNA content (in picograms) by 6.5 pg per cell, the average cellular DNA content measured in sorted single human adult β cells and duct cells. The number of β cells was then determined on the basis of the percentage of insulin-positive cells counted in duplicate samples of this fraction. The characteristics of the 35 grafts used in this study are listed in Table 2; the number of donors per graft was four (median; IQR of three to five). Compared with freshly isolated islet fractions (4), these preparations exhibit a higher percent β cells and contain virtually no acinar cells. Culture led to fragmentation of large tissue particles and reassociation of small particles. In view of these characteristics and the selected criteria for quantifying the grafts, we define them as β cell preparations.

Transplantation. Nine patients received one graft. The thirteen others received a second graft 3 months later (IQR 2–5 months). The graft suspension was infused into the portal vein over 5–6 min. Access to the portal vein occurred through a catheter inserted in the umbilical vein after laparoscopic repermeabilization ($n = 20$) (9, 22) or through s.c. transhepatic puncture under ultrasound guidance ($n = 15$) (23).

Function of β Cell Implants and Metabolic Control. Graft recipients were followed for their fasting blood glucose level and its CV (home monitoring), their plasma C-peptide levels at glycemia 120–200 mg/dl, and their blood HbA_{1c} concentration. Hypoglycemic episodes were documented by home glucose monitoring during the year before and after transplantation and expressed as percentage of all measurements. They are tabulated as all measurements <70 mg/dl as proposed by the American Diabetes Association Workgroup on Hypoglycemia (30) and as all measurements <50 mg/dl, which is considered as a higher risk for acute complications (31, 32). Survival of a β cell implant in C-peptide-negative (< 0.09/ml) recipients was defined by plasma C-peptide ≥ 0.5 ng/ml measured in postbreakfast samples (33). A state of C-peptide negativity was based on measurements in postbreakfast samples as well as after glucagon injection. Graft recipients who were insulin-independent at PT month 12 were submitted to a glucose clamp test in which C-peptide release was measured first at basal glycemia (median 107 mg/dl; IQR 99–120 mg/dl) and then during sustained glucose stimulation (180 mg/dl, 15–160 min), first in the absence of glucagon (at minutes 120, 135, and 150) and then over 5 and 10 min after a glucagon injection at minute 150. Plasma C-peptide levels were used to calculate the area under the curve per minute. The same test was conducted in 12 age- and BW-matched normal volunteers. It was also recently used to follow the insulin secretory capacity in type 1 diabetic patients during the first 18 months after diagnosis (20).

Daily insulin dose was adjusted to keep blood glucose levels between 70 and 180 mg/dl without frequent episodes of symptomatic hypoglycemia. Although the plan was to taper insulin dose only after PT month 2, this was started earlier when patients with graft

function presented hypoglycemic episodes (<70 mg/dl). Insulin treatment was increased or reintroduced after two consecutive HbA_{1c} measurements >7.0% (normal HbA_{1c} < 6.0%).

Medical Follow-Up. Patients were consulted weekly until PT week 6, every two weeks between PT week 6 and 12, and monthly thereafter. They underwent a systematic interrogation by questionnaire and general physical examination for adverse events. Blood was taken for hematology, kidney, and liver tests and CMV PCR. Adverse events were graded with the National Cancer Institute Common Toxicity Criteria system version 3.0 (34). In the safety analysis we included grade two to five events. Plasma C-peptide (C-peptide TRFIA; PerkinElmer, Turku, Finland) and corresponding glycemia, as well as HbA_{1c} concentrations, were measured in the central laboratory of the Belgian Diabetes Registry. Tacrolimus trough levels (Tacrolimus II, IMx Abbott; Abbott Laboratories, Wiesbaden, Germany), lymphocyte subsets (Epics XL flow cytometer; Beckman Coulter, Miami, FL), and HLA antibodies (Lambda Antigen Tray Elisa, Mixed Class 1 and 2; One Lambda, Canoga Park, CA) were also measured centrally.

Statistical Methods. Data are presented as median (quartile range). Comparison of patient subgroups used the Mann–Whitney test for quantitative variables and Fisher's exact test for binary variables. Statistical differences between repeated measurements were examined with the Friedman test and, if significant, with Wilcoxon's signed-rank test. Analysis of correlations was performed by using the Spearman rank-correlation test. Differences were considered significant for $P < 0.05$.

We thank Koen Verbeeck and Sabrina Uytendhove for their excellent collaboration in organizing this project, and we acknowledge the loyal support of the Beta Cell Bank, the Belgian Diabetes Registry, the Diabetes Research Center of Vrije Universiteit Brussel, the Clinical Biology Department of Academisch Ziekenhuis (Vrije Universiteit Brussel), the physicians and nurses of the patients included in this study, and the Eurotransplant Foundation and its transplant surgeons and coordinators. This study was supported by a Center Grant from the Juvenile Diabetes Research Foundation, Fonds voor Wetenschappelijk Onderzoek (FWO)—Flanders, and Vrije Universiteit Brussel. B.K. and C.M. are Senior Clinical Investigators of the FWO.

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