

# Modulation of Humoral Islet Autoimmunity by Pancreas Allotransplantation Influences Allograft Outcome in Patients With Type 1 Diabetes

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Pancreas transplantation in patients with type 1 diabetes presents allogeneic  $\beta$ -cell autoantigens to the immune system long after the initial  $\beta$ -cell destruction that leads to diabetes has occurred. The aims of this study were to determine whether re-exposure to  $\beta$ -cell autoantigen through transplantation affect the humoral autoimmune response and whether its modulation correlates with graft outcome. Antibodies to the major autoantigens GAD (GADA) and protein tyrosine phosphatase IA-2 (IA-2A) were measured before and after transplantation in patients with type 1 diabetes who received pancreas and kidney allografts. In the 110 cases studied, pancreas graft survival was not significantly associated with the presence of GADA or IA-2A before transplantation. In the 75 patients with sequential follow-up samples up to 11.2 years after transplantation, autoantibodies were persistently undetectable in 44 cases (59%) and remained at stable detectable levels in 13 cases (17%). Substantial changes in antibody levels were found in 18 cases (24%), of which 13 cases (17%) had declining levels and 5 cases (7%) had marked increments after transplantation. Rising GADA and IA-2A levels in these five patients were predominantly of the IgG1 subclass, with progressive spreading of epitope reactivity. Pancreas graft function was lost 0.7–2.3 years after rising autoantibody levels in four of these five patients, and a significantly lower pancreas graft survival was found in patients with major rises in either GADA or IA-2A levels ( $P < 0.0001$  vs. the remainder) and in patients having persistently high levels of IA-2A ( $P = 0.002$  vs. stable antibody-negative patients). Kidney graft survival was not associated with islet autoantibody status. In conclusion, a minority of patients receiving pancreas allografts under generalized immunosuppression show a stimulation of islet autoantibody reactivity characteristic of that found in pre-clinical type 1 diabetes, which is almost invariably followed by graft function failure and resumption of insulin therapy. *Diabetes* 49:218–224, 2000

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ELISA, enzyme-linked immunosorbent assay; GADA, GAD antibodies; IA, insulin antibodies; IA-2A, IA-2 antibodies; IA-2A<sub>JM</sub>, IA-2 JM antibodies; IA-2A<sub>PTP</sub>, IA-2 PTP-like antibodies; IA-2A $\beta$ <sub>PTP</sub>, IA-2 $\beta$ PTP antibodies; ICA, islet cell antibodies; JM, juxta-membrane; OD, optical density; PBS, phosphate-buffered saline; PBST, PBS Tween; PTP, protein tyrosine phosphatase.

**T**ype 1 diabetes is an autoimmune disease (1) characterized by circulating antibodies to pancreatic  $\beta$ -cell autoantigens, which are detectable in up to 90% of cases at the time of disease onset (2,3).

The appearance of circulating autoantibodies may occur very early in life (4) and precedes the clinical onset of the disease by years (5–8). Thereafter, autoantibody levels tend to decline and disappear within a few years in some patients, while in others, they persist at detectable levels indefinitely (9–12). The factors underlying the diversity in the regulation of the humoral autoimmune response are likely to be several, including the quantity of  $\beta$ -cell autoantigen mass present and how available it is to the immune system. Pancreas transplantation, usually performed in association with kidney transplantation, represents an introduction of significant  $\beta$ -cell autoantigen mass. Despite it being an effective cure of type 1 diabetes in the large majority of technically successful cases (13), a recurrence of the type 1 diabetes-associated autoimmunity after human pancreas transplantation, which is characterized by inflammatory islet infiltration and selective  $\beta$ -cell destruction in the pancreatic transplant, has been reported (14–17). In this study, we have characterized the humoral response to the major autoantigens GAD (18) and protein tyrosine phosphatase IA-2 (19,20) before and after pancreas transplantation in a large cohort of patients with type 1 diabetes. The aims of this study were to test whether  $\beta$ -cell specific autoantibodies detected before transplantation may predict the clinical outcome of the pancreatic graft, to what extent exposure to a  $\beta$ -cell autoantigen mass of allogeneic origin introduced by pancreas allotransplantation affects humoral autoimmunity in long-standing patients with type 1 diabetes, and whether changes in autoimmune response relative to pancreas transplantation influence pancreatic graft survival.

## RESEARCH DESIGN AND METHODS

Transplant patients. Between July 1985 and September 1998, 120 pancreas transplants were performed in patients with long-term type 1 diabetes at the San Raffaele Hospital Scientific Institute in Milan, Italy. In 110 of these cases performed in 109 patients (median age 37 years, range 25–56; median duration of diabetes 24 years, range 14–45), pretransplant serum samples obtained no earlier than 3 months before transplantation were available and therefore were included in this study. Pancreas was transplanted simultaneously with kidney in 108 cases and after kidney transplantation in 2 cases. The techniques of pancreas transplantation involved segmental grafting with neoprene injection in 27 cases, whole pancreas and duodenum grafting with urinary drainage in 67 cases, and enteric drainage in 16 cases. Donors were heart-beating cadavers selected for ABO blood compatibility and negative crossmatch. HLA type was not considered a criterion

for donor-recipient matching. Immunosuppression was based on the triple association of cyclosporin, azathioprine, and prednisone with temporary antilymphocyte globulin or OKT3 in case of kidney rejection. At the time of analysis, the median follow-up time after transplantation was 3.1 years (range 0.2–11.2). In 75 of the followed cases, at least two posttransplant serum samples obtained after transplantation were also available (median follow-up time after transplantation 4.6 years; median sample number 4, range 2–15; obtained at a median interval time of 11 months, range 1–48). Levels of antibodies to GAD (GADA) and IA-2 (IA-2A) were measured in all the available samples obtained before and after transplantation. In the cases characterized by major increments of antibody levels, the IgG subclass and epitope specificity of GADA and IA-2A were characterized, and IgG antibody levels to insulin and tetanus were measured.

**Antibody measurements.** GADA and IA-2A measurements were performed by radiobinding assay with in vitro translated [<sup>35</sup>S]methionine-labeled GAD65 or IA-2, as previously described (2,20). Results were converted into arbitrary units by extrapolation from a standard curve with a local standard designated at 100 U undiluted and at a 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 dilution in negative serum. The thresholds for positivity were determined from the 99th centile of 178 control subjects (median age 12 years, range 1–40) and corresponded to 3 U for GADA and 1 unit for IA-2A. These GADA and IA-2A assays obtained the following performances at the last Combined Islet Autoantibody Workshop: 88% sensitivity, 98% specificity, and 100% reproducibility for GADA; 70% sensitivity, 99% specificity, and 100% reproducibility for IA-2A (21). Insulin antibodies (IA) were measured by using a competitive protein A Sepharose/protein G Sepharose (protein A/G) insulin radiobinding microassay, as previously described (22). The threshold for positivity, which was calculated according to the levels of 97 control subjects (median age 11 years, range 2–59), corresponded to 4 U. Levels of IgG antibodies to tetanus toxoid were measured by enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well plates were coated with 0.5 limit of flocculation units/ml of antigen in 125 µl of 147 mmol/l NaCl, 1.5 mmol/l KCl, 2.7 mmol/l KH<sub>2</sub>PO<sub>4</sub>, and 81 mmol/l Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4 (phosphate-buffered saline [PBS]) by overnight incubation at 4°C. Plates were washed three times in PBS containing 0.05% Tween 20 (PBST) and blocked in 0.1% gelatin in PBS. Sera were diluted 1/200 in PBST, and 100 µl was added in duplicate and sera were incubated for 2 h at room temperature. Wells were washed three times with PBST, and 100 µl specific biotinylated monoclonal antibody to total IgG (Pharmingen, San Diego, CA) that was diluted 1/500 in PBST was added. Plates were incubated for 2 h at room temperature and were washed three times with PBST, and 100 µl peroxidase-conjugated streptavidin (Pharmingen) that was diluted 1/1,000 in PBST was added. After a 1-h incubation, plates were washed three times with PBST, color substrate (ABTS; Sigma, St. Louis, MO) was added, and the optical density (OD) of each well was measured after a 15-min incubation at room temperature by an ELISA reader at a wavelength of 405 nm. After subtraction of the OD of binding by sera to wells without antigen, results were read from a standard curve constructed from serial dilutions of the World Health Organization reference standard preparation.

**GAD and IA-2 epitope antibodies.** GAD epitope reactivity was measured against GAD65, GAD67, and GAD65/67 chimeras by radiobinding assay. The following GAD constructs were used to measure antibody binding: full-length GAD65 and GAD67, and GAD65<sub>1–95</sub>/GAD67<sub>102–593</sub> (NH<sub>2</sub>-terminal GAD65 epitopes), the GAD67<sub>1–101</sub>/GAD65<sub>96–444</sub>/GAD67<sub>453–593</sub> (central GAD65 epitopes), and the GAD67<sub>1–452</sub>/GAD65<sub>445–585</sub> (COOH-terminal GAD65 epitopes) chimeras. Radiobinding assays were performed as for GADA. Results were expressed as arbitrary units relative to a standard curve prepared by measurement in each assay of a serum

from a patient with Stiff-man syndrome with high autoantibody levels serially diluted in normal serum. The upper value of 50 control sera was used as the threshold of autoantibody detection for each construct, corresponding to 3 U for GAD65, 5 U for GAD67, 6 U for GAD67<sub>1–101</sub>/GAD65<sub>96–444</sub>/GAD67<sub>453–593</sub>, 8 U for GAD67<sub>1–452</sub>/GAD65<sub>445–585</sub>, and 4 U for GAD65<sub>1–95</sub>/GAD67<sub>102–593</sub> antibodies. The following IA-2 constructs were used to measure antibody binding to the different IA-2 epitope regions: IA-2<sub>389–779</sub> for IA-2 juxta-membrane (JM) antibodies (IA-2A<sub>JM</sub>), IA-2<sub>687–979</sub> for IA-2 protein tyrosine phosphatase (PTP)-like antibodies (IA-2A<sub>PTP</sub>), IA-2β<sub>741–1033</sub> for IA-2βPTP antibodies (IA-2Aβ<sub>PTP</sub>), as previously described (23–25). Bacterially expressed recombinant IA-2<sub>JM</sub>, IA-2<sub>PTP</sub>, and IA-2Aβ<sub>PTP</sub> were used for inhibition studies to confirm specificity of reactivity as previously described (23–25).

IgG subclass autoantibodies. For GADA and IA-2A IgG subclass analysis, the protein A/G radiobinding assays were used by substituting the addition of the protein A/G Sepharose with IgG subclass or isotype-specific antibody-bound sepharose beads as previously described (26). Biotin-labeled mouse monoclonal antibodies against human IgG1 (cat. no. 35052D), IgG2 (cat. no. 35072D), IgG3 (cat. no. 35082D), IgG4 (cat. no. 35092D), and rat IgM (cat. no. 10062D) as controls were obtained commercially (Pharmingen). Sepharose 4B streptavidin beads (Zymed, San Francisco, CA) were prepared by washing once with ice-cold PBS (50 mmol/l phosphate buffer, 150 mmol/l NaCl, pH 7.4), followed by incubation of beads with biotinylated monoclonal antibody with rotation at 48°C for 18 h, washing twice in PBS, washing once in assay buffer, and resuspension in assay buffer. The quantity of antiserum and streptavidin beads required to completely capture immunoglobulin in the assay reaction was determined by checkerboard titration under assay conditions. For GADA and IA-2A, this corresponded to 10 µl of Sepharose 4B streptavidin beads with 5 µg of antibody per well of the assay reaction. Results were expressed as SD scores calculated from the mean and SD of results obtained after subtraction of nonspecific binding to the antirat IgM for 44 control subjects. The mean + 3 SD was used as the threshold for detection (26). **Statistical analyses.** Pancreatic graft survival was defined as a condition of normoglycemia in the absence of administration of exogenous insulin. Kidney graft survival was defined as a condition free of hemodialysis or any other renal replacement therapy. The survival rates, projected risks, and 95% CIs were calculated by Kaplan-Meier analysis, and comparison between groups was performed using the log-rank test. For all statistical methods, the Statistical Package for Social Sciences (SPSS, Chicago) was used.

## RESULTS

**Pretransplant GADA and IA-2A and relationship to graft survival.** Elevated levels of GADA were found in 33 cases (30%, median units in positives 12.0, range 3.7 to >100) and IA-2A in 18 cases (16%, median units in positives 3.2, range 1.1–27.6) of 110 cases before pancreas transplantation. In nine cases (8%), elevated levels of both antibodies were found (Table 1). The overall 5-year survival was 65% (58–72) for pancreatic grafts, 80% (72–89) for kidney grafts, and 86% (78–94) for patients. The survival of pancreas allografts was not significantly different between patients with or without elevated levels of GADA, IA-2A, or both antibodies, although those with IA-2A and with both

**TABLE 1**  
Pancreas and kidney transplant survival according to the autoantibody status before transplantation

Pretransplant antibodies	Cases (n)	5-Year pancreas transplant survival	5-Year kidney transplant survival	Median pancreas transplant survival (years)	Median kidney transplant survival (years)	P value
Total	110	65 (58–72)	80 (72–89)	6.3	9.2	—
GADA +	33	59 (41–78)	76 (57–95)	5.2	9.2	—
GADA –	77	67 (56–79)	82 (72–91)	6.9	9.1	0.6
IA-2A +	18	44 (19–69)	81 (58–100)	2.4	9.2	—
IA-2A –	92	69 (59–75)	80 (71–89)	6.9	9.1	0.2
Antibodies						
0 Ab	69	69 (57–80)	80 (69–90)	6.9	9.1	—
1 Ab	32	65 (46–83)	86 (71–100)	6.3	9.1	0.7*
2 Ab	9	40 (6–74)	62 (21–100)	2.4	9.2	0.3*

Data are % (95% CI), unless otherwise indicated. \*Versus no antibodies.

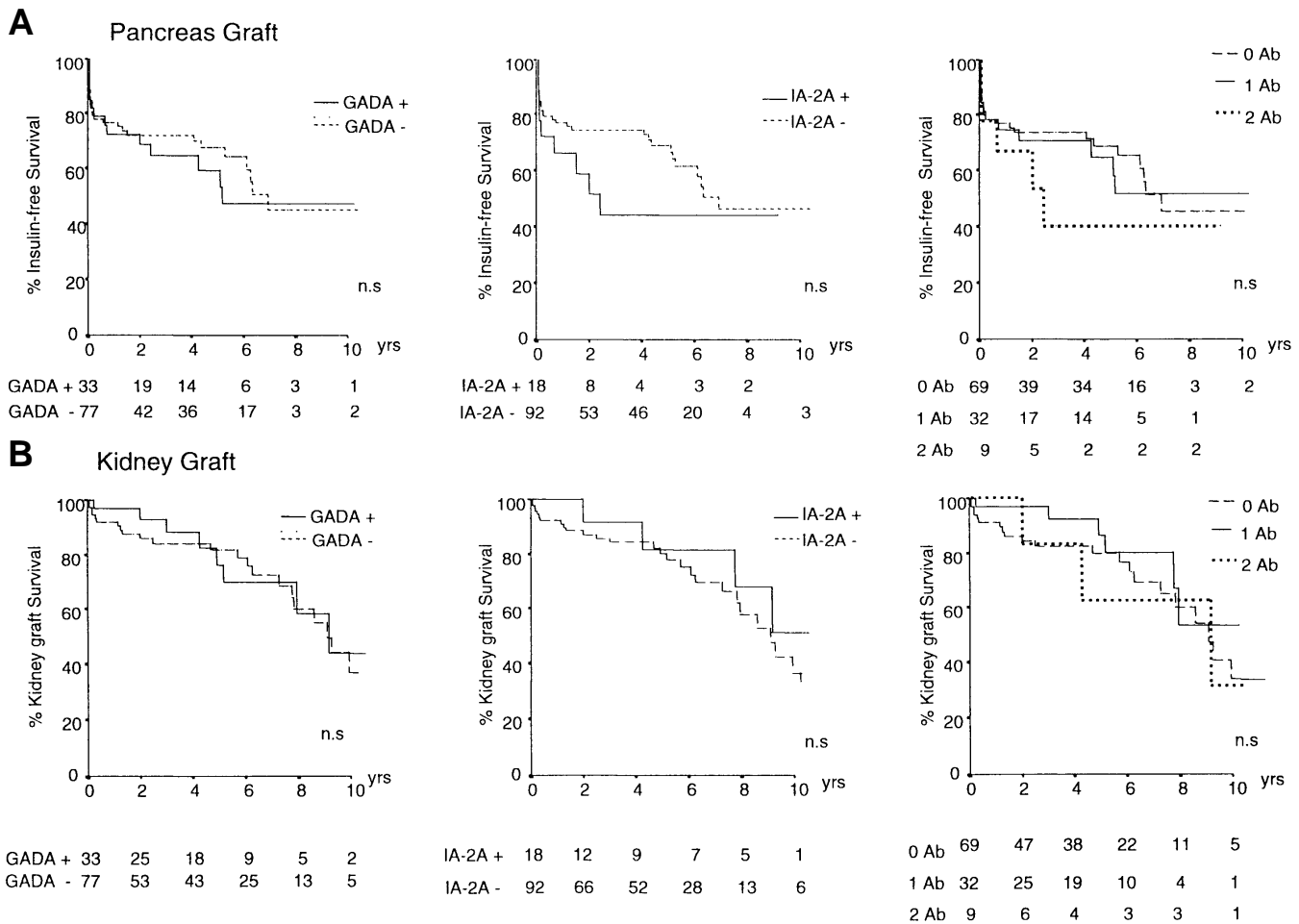


FIG. 1. Kaplan-Meier analysis of pancreas (A) and kidney (B) transplant survival according to autoantibody status before transplantation.

GADA and IA-2A had a tendency to lose graft function earlier than those without IA-2A. Patient and kidney graft survival were not affected by the presence of GADA, IA-2A, or both antibodies (Table 1 and Fig. 1).

**Posttransplant GADA and IA-2A.** In the majority (76%) of the 75 patients with follow-up samples, autoantibody levels remained unchanged after transplantation. Antibodies persisted to be undetectable on follow-up in 44 of 47 patients in whom neither GADA nor IA-2A were found before transplantation. Antibody levels also remained relatively stable in 13 of 28 patients in whom either GADA or IA-2A were detected at relatively high levels before transplantation. In these patients, the median level of GADA before transplantation was 23.7 U, while it was 17 U on the last posttransplant sample; the median level of IA-2A was 4.4 and 4.9 U, respectively (Table 2 and Fig. 2).

Substantial changes in antibody levels were found in 18 patients (24%) (Fig. 2). In 13 of these patients (9 patients with GADA only, 2 with IA-2A only, and 2 with both), elevated antibody levels were detected before transplantation with a subsequent progressive decline thereafter: GADA levels decreased from a pretransplant median of 24.8 to 9 U on the last posttransplant sample and became undetectable in five cases, while IA-2A levels declined from 3.2 to 0.2 U. In contrast, five patients (6.7%) had major increments in levels of GADA (three cases), IA-2A (one case), or both antibodies (one

case) after transplantation: GADA levels increased from a pretransplant median of 4 U to a posttransplant median peak of 16 U, while levels of IA-2A in two patients increased from 0.5 and 2 U to 16 and >100 U, respectively (Tables 2 and 3).

Kaplan-Meier analysis showed a significantly lower survival of functioning pancreas transplant in the five patients in whom major rises in antibody levels were observed compared with the remaining 70 patients ( $P < 0.0001$ ) (Fig. 3). Of these five patients, four lost pancreatic graft function from 0.9 to 4.3 years after transplantation and from 0.7 to 2.3 years after antibody levels started to rise (Table 3). Kidney graft survival in these patients was not different from that observed in the remaining patients (Fig. 3). When GADA and IA-2A were examined separately, pancreas transplant survival was also significantly lower in the six patients who had persistently high levels of IA-2A compared with those with undetectable ( $P = 0.003$ ) or declining ( $P = 0.01$ ) IA-2A levels (Fig. 4). Of these six patients, four lost pancreatic graft function within 2 years after transplantation. Kidney graft survival was not influenced by IA-2A detected in the posttransplant period. Patients with persistently high GADA levels did not have a significantly reduced pancreas graft survival.

Antibody characteristics in patients with rising autoantibodies. Antibody levels, epitope reactivity, and subclass for each of the five patients with major rises in autoantibody levels after transplantation are shown in

TABLE 2  
Pancreas and kidney survival relative to posttransplant autoantibody status

Posttransplant antibodies	Cases (n)	Median titer before transplant (U)	Median titer after transplant (U)	Survival 5 years after pancreas transplant	Survival 5 years after kidney transplant
Stable negative	44			95 (89–100)	92 (82–100)
GADA	49	0.9	0.7	89 (80–98)	93 (85–100)
IA-2A	63	0.2	0.2	89 (80–97)	84 (73–96)
Declining titers	13			92 (78–100)	83 (61–100)
GADA	11	24.8	9	91 (73–100)	79 (54–100)
IA-2A	4	3.2	0.2	100	100
Stable positive	13			54 (22–85)	80 (55–100)
GADA	11	23.7	17	64 (30–98)	75 (44–100)
IA-2A	6	4.4	4.9	33 (0–71)	62 (21–100)
Rising titers	5			100	100
GADA	4	4.1	16.0	0	100
IA-2A	2	0.5; 2*	16; >100*	0	100

Data are % (95% CI), unless otherwise indicated. \*Single values instead of medians.

Table 3. In case number 4, GADA levels were high (49 U) and IA-2A levels were weak (2 U) before transplantation. After 9 months, both GADA and IA-2A levels declined, and 4 months later, both were markedly increased (GADA levels >100 U; IA-2A levels, 16 U). The pancreatic graft was lost 16 months later. IA levels were below the threshold for positivity before transplantation and remained negative during follow-up. GADA were predominantly of the IgG1 subclass, and IA-2A were predominantly of the IgG2 subclass. GADA recognized middle- and COOH-GAD65 epitopes before transplantation and NH<sub>2</sub>-GAD65 and -GAD67 epitopes in peak samples after transplantation. Similarly, IA-2 epitope reactivity spread from the IA-2 PTP epitope region only to include reactivity against the IA-2 JM and IA-2 $\beta$  regions after transplantation. Antibodies to tetanus toxoid were undetectable before and after transplantation.

In case number 38, both GADA and IA-2A were undetectable before transplantation. GADA remained below the threshold for positivity throughout the posttransplant follow-up, whereas IA-2A were found at very high levels (>100 U) 3.2 years after transplantation, and pancreatic graft function was lost and insulin therapy resumed 9 months later, after which IA-2A levels declined. IA were detected at high levels before transplantation and, after an initial decline at 3 months, they returned to above pretransplant levels at 3.2 years and paralleled IA-2A levels. IA-2A were constantly IgG1 and included low levels of IgG2 and IgG3 at the time of pancreatic graft loss and spread from an initial epitope reactivity limited to the IA-2 PTP epitope region to also include the IA-2 JM and IA-2 $\beta$  regions throughout the posttransplant follow-up. Tetanus toxoid antibodies remained low before and after transplantation.

In case number 10, GADA increased from undetectable levels to 18 U 2 years after transplantation with a subsequent slight tendency to decline but remained elevated until pancreas graft was lost 2.3 years later. GADA epitope reactivity was detected only to the middle GAD65 region. IA-2A were around the threshold for positivity and were reactive against the IA-2A JM region. IA were present at the time of transplantation and decreased markedly after transplantation. Tetanus toxoid antibodies were low before transplantation and also became undetectable after transplantation.

In case number 103, GADA levels rose from 5.5 U before transplantation to 15 U 2 months after transplantation and 7 months before pancreas graft failure. IgG epitope reactivity was limited to the COOH-terminal region. IA were undetectable before transplantation and rose significantly at the same time as GADA 2 months after transplantation. Weak IA-2A were detected with spreading to IA-2 $\beta$  by the time of graft failure. Tetanus toxoid antibodies declined 2 months after transplantation and rose again to pretransplant levels at the time of graft failure.

Finally, in case number 89, an IgG1 GADA peak was observed in a single sample 1 month after pancreas transplantation with a subsequent drop below the threshold for positivity at 2.2 years. IA were found before transplantation but also became undetectable at 2.2 years. The pancreas transplant was still functioning 3.2 years after transplantation.

#### DISCUSSION

Pancreas transplantation is a therapeutic procedure that has now reached a reasonable level of safety to be offered as a realistic option to diabetic patients receiving kidney grafts (13). Pancreas transplantation also represents a model of potential

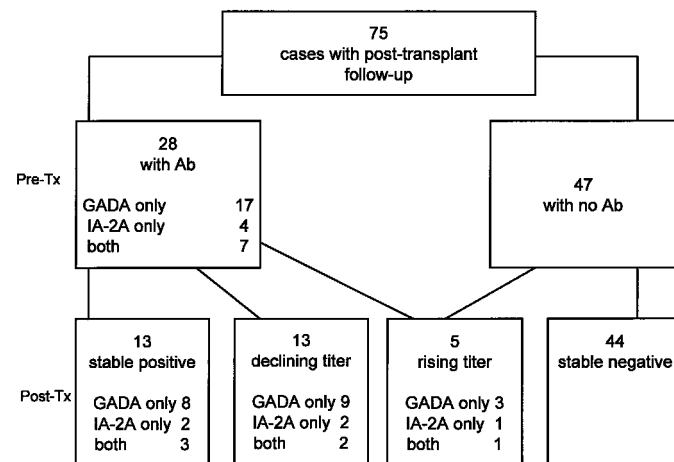


FIG. 2. Posttransplant patient profile according to autoantibody behavior. Ab, antibody; Tx, transplant.

**TABLE 3**

Posttransplant antibody profile, IgG subclass, epitope reactivity, and pancreas transplant outcome in patients with major rises in autoantibody titer

Case	Sample (years posttransplant)	Antibodies				IgG subclass		Autoantibody epitope reactivity							Pancreas transplant outcome	
		GADA (U)	IA-2A (U)	IA (U)	Tetanus OD	GADA	IA-2A	GADA				IA-2A				
								-NH <sub>2</sub>	Middle	-COOH	GAD67	JM	PTP	PTPβ		
4	Basal sample	50*	2*	3	0.08	IgG1	†	-	++	++	-	-	-	++	-	IT
	0.1	32*	<1	2	0.08	NT		NT	NT	NT	NT					
	0.9	31*	<1	2	0.07	IgG1		+	++	+	-					
	1.1	>100*	16*	3	0.08	IgG1	IgG1, IgG2	++	+++	+++	+	+	+++	-		
	2.4	>100*	5*	NT	NT	NT	NT	NT	NT	NT	NT	+	-	NT		
	2.6	>100*	6*	3	0.07	NT	IgG2	NT	NT	NT	NT	+	+++	-		
38	Basal sample	1	<1	404*	0.27	IgG1, IgG2									IT	
	0.3	<1	2*	40*	0.23		†						-	+		-
	3.2	<1	>100*	832*	0.29		IgG1						++	+++		+++
	3.4	<1	>100*	908*	0.12		IgG1						++	+++		+++
	4.3	<1	>100*	798*	NT		IgG1, IgG2, IgG3						+++	+++		+++
	5.0	1	38*	73*	NT		IgG1						++	+++		NT
10	Basal sample	<1	1	43*	0.28										IT	
	2.0	18*	<1	5	NT	†							+	-		-
	3.0	13*	<1	3	0.26	†		-	+	-	-					
	3.6	10*	2*	4	0.10	†	†	-	+	-	-					
	4.3	4*	1	2	0.10	†	†	†	†	†	†	+	-	-		
	4.9	3	<1	3	0.11											
103	Basal sample	6*	1	3	0.34	†		-	-	+	-		+	+	-	IT
	0.2	15*	1	91*	0.08	†										
	0.9	5*	2*	NT	0.28	†	†	NT	NT	NT	NT		-	+	++	
89	Basal sample	3	<1	29*	0.13											Functioning at 3.2 years
	0.1	15*	<1	21*	0.12	IgG1		-	-	-	-					
	2.2	2	<1	1	0.08											

IT, insulin therapy; NT, not tested. +, ++, and +++ represent low, medium, and high levels, respectively. \*Positive levels; †not tested because of low antibody titer; ‡undetectable because of low antibody titer.

pathogenetic interest, in which the patient immune system is exposed to an autoantigen mass of allogeneic origin many years after the initiation of the autoimmune process at a time when it is expected that endogenous β-cell destruction has totally, or almost totally, been accomplished. In this study, we show that graft function and survival were not associated with the presence of islet autoantibodies before transplantation but that large increases in autoantibody levels after trans-

plantation seen in a minority of patients were a specific marker of subsequent loss of pancreatic graft function.

We previously reported that the reappearance of islet cell antibodies (ICA) or their persistence at high levels as their complement-fixing variant after transplantation were predictive of the subsequent pancreatic graft failure in a small cohort of patients undergoing pancreas transplantation in Lyon, France (27). Subsequent reports, based on observations

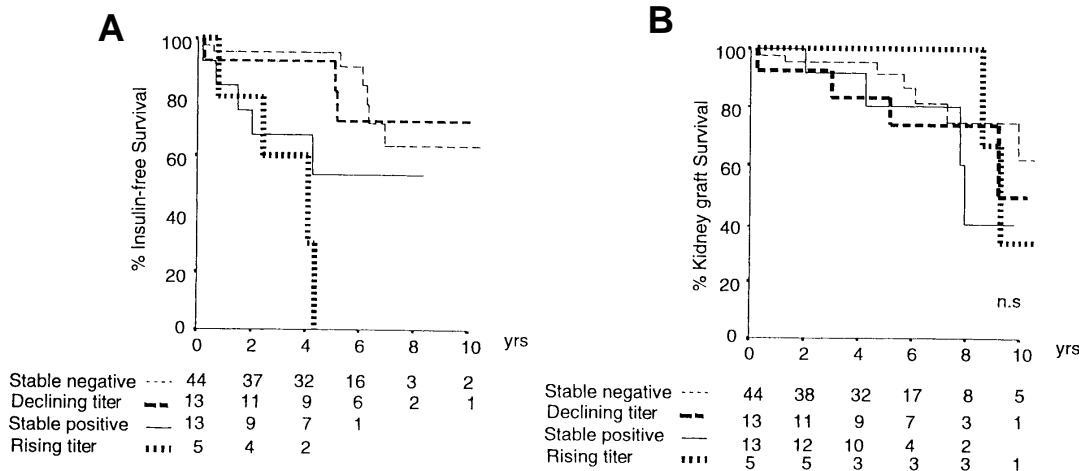


FIG. 3. Kaplan-Meier analysis of pancreas (A) and kidney (B) transplant survival according to posttransplant autoantibody behavior. The 75 patients with follow-up measurements of GADA and IA-2A have been classified as having stable negative antibody titers for both GADA and IA-2A, stable titers of both GADA and IA-2A, at least one of which was positive before transplant, declining titers of GADA and/or IA-2A, and rising titers of GADA and/or IA-2A according to Fig. 2. Pancreas transplant survival was lower in patients with rising titer antibodies ( $P < 0.0001$  vs. all the remainders;  $P < 0.0001$  vs. stable antibody-negative;  $P = 0.002$  vs. declining titer antibodies; NS vs. stable antibody-positive). Kidney transplant survival was not influenced by autoantibody status.

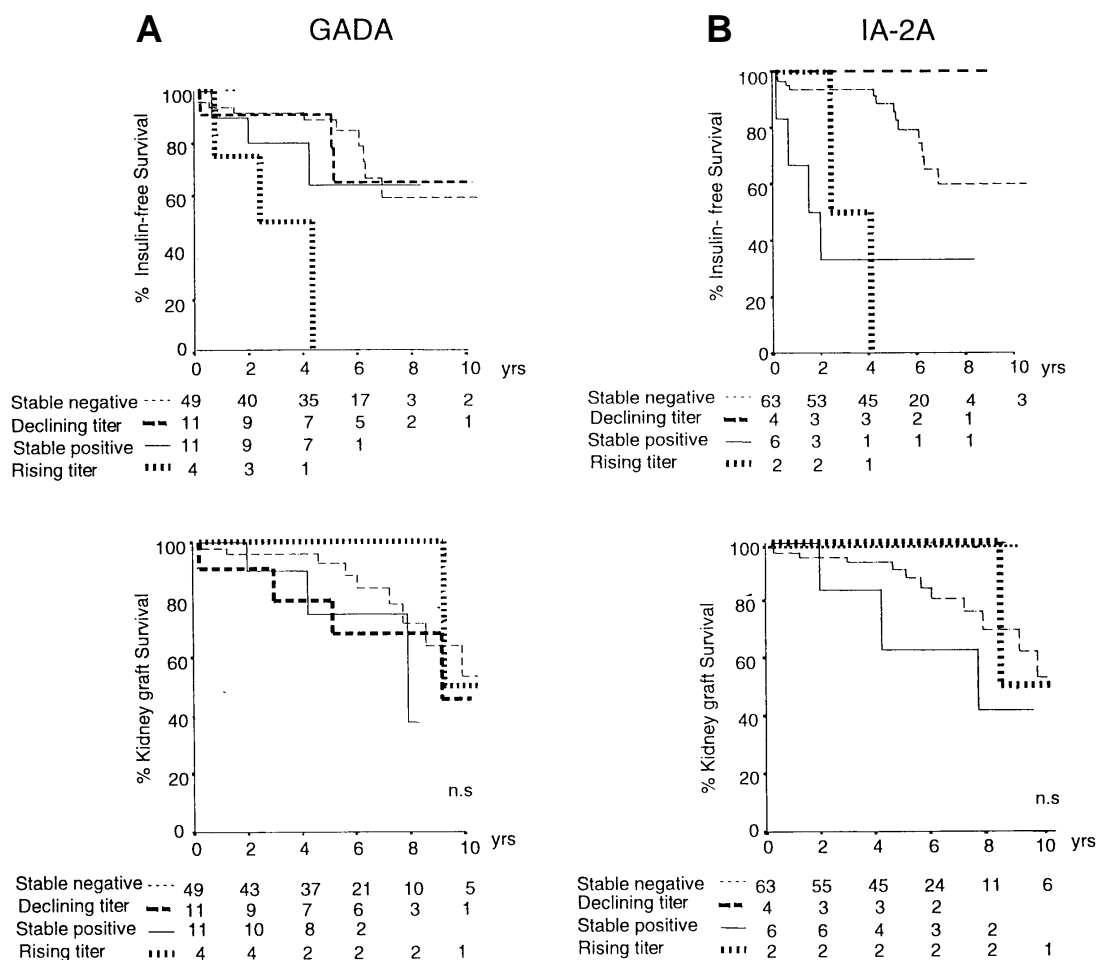


FIG. 4. Kaplan-Meier analysis of pancreas and kidney transplant survival according to posttransplant GADA (A) and IA-2A (B) behavior. Patients have been classified as having stable negative, stable positive, declining titer, and rising titer autoantibody. Upper panels indicate pancreatic graft survival, and lower panels indicate kidney graft survival. Pancreas transplant survival was lower in patients with rising GADA titers ( $P = 0.0003$  vs. stable GADA-negative;  $P = 0.01$  vs. declining GADA titers; NS vs. stable GADA-positive), and in patients with rising levels of IA-2A ( $P < 0.001$  vs. stable IA-2A-negative;  $P < 0.04$  vs. declining IA-2A titers) and stable IA-2A-positive titers ( $P < 0.002$  vs. stable IA-2A-negative;  $P < 0.07$  vs. declining IA-2A titers).

of few cases, were unable to clarify the role of autoantibodies as possible markers of recurrent autoimmunity in pancreas transplantation (17,28,29). Few studies have been performed using antigen-specific autoantibody markers. In a cohort of 23 patients undergoing islet transplantation, the presence of ICA and/or GADA before or soon after transplantation was associated with a poor transplant prognosis (30). Our study does not indicate that the detection of GADA or IA-2A before transplantation correlates with the outcome of pancreas transplantation but does confirm that the reappearance or stimulation of islet antibodies, and their persistence at high levels in the case of IA-2A are associated with subsequent graft loss.

In our cohort, the majority of patients who underwent pancreas transplantation either stayed antibody-negative or, as previously reported in patients treated with cyclosporin (31), gradually lost islet autoantibodies. However, in 24% of patients, autoantibody levels either markedly increased (6.7% of cases) or remained elevated (17.3%) despite immunosuppressive treatment. In the few cases with marked increments, the increases were not usually immediate and occurred up to 3 years after transplantation. Rises were

observed in both GADA and IA-2A, but, as found in prediabetes (4,7,8), some patients had increases in only one of these antibody specificities. IA also showed similar increases in two of the patients, but an effect on antibodies to the recall antigen tetanus was not observed, suggesting that the antibody rises were not merely secondary to a general condition of immune hyperreactivity. Also typical of what has been found in prediabetes, autoantibodies in these patients were of the IgG1 subclass, and rises were predominantly in this subclass with peak antibody samples occasionally having other subclasses (26), although in one patient IA-2A were atypically predominantly IgG2. Epitope reactivity was also similar to that seen in early prediabetes (24,25) with spreading to new epitopes in the first years after the appearance of antibodies. These rises are remarkably similar to what has been found in the early phase of autoimmunity that precedes the onset of type 1 diabetes (4,26) and suggest the recurrence of autoimmunity induced by the introduction of substantial  $\beta$ -cell mass. The recurrence of diabetic insulinitis and of diabetes itself, consequent to relapsing autoimmunity, has been reported after transplantation from identical twins, HLA-identical siblings, and, in some cases, cadaveric donors

(14–17), thus indicating that a second prediabetic phase can be replicated in the same patient after pancreas allotransplantation. Our study suggests that autoantibodies may be a marker of such a recurrence.

In conclusion, this study demonstrates that in a small proportion of patients, pancreas allotransplantation can be associated with a stimulation of islet autoantibody reactivity characteristic of that found in preclinical type 1 diabetes, which is almost invariably followed by a progressive decline of  $\beta$ -cell function and resumption of insulin therapy; this finding suggests recurrent autoimmunity as the cause of pancreas transplant failure in these cases. Clinical monitoring of pancreas transplant patients by systematic measurement of islet-specific autoantibodies should help in the early identification of these cases, and graft biopsy may further clarify their pathogenesis.

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REFERENCES

1. Bach JF: Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15:516–542, 1995
2. Bonifacio E, Genovese S, Braghi S, Bazzigaluppi E, Lampasona V, Bingley PJ, Rogge L, Pastore MR, Boggetti E, Bottazzo GF, Gale AEM, Bosi E: Islet autoantibody markers in insulin-dependent diabetes: risk assessment strategies yielding high sensitivity. *Diabetologia* 38:816–822, 1995
3. Hagopian WA, Sanjeevi CB, Kockum I, Landin-Olsson M, Karlens AE, Sundkvist G, Dahlquist G, Palmer J, Lernmark A: Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 95:1505–1511, 1995
4. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* 48:460–468, 1999
5. Bonifacio E, Bingley PJ, Dean BM, Shattock M, Dunger D, Gale EAM, Bottazzo GF: Quantification of islet cell antibodies and prediction of insulin-dependent diabetes. *Lancet* 335:147–149, 1990
6. Riley WJ, Maclaren NK, Krischer J, Spillar RP, Silverstein JH, Schatz DA, Schwartz S, Malone J, Shah S, Vadheim C, Rotter JI: A prospective study of the development of diabetes in relatives of patients with insulin-dependent diabetes. *N Engl J Med* 323:1167–1172, 1990
7. Dittler J, Seidel D, Schenker M, Ziegler AG: GADIA2-combi determination as first-line screening for improved prediction of type 1 diabetes in relatives. *Diabetes* 47:592–598, 1998
8. Pastore MR, Bazzigaluppi E, Bonfanti R, Dozio N, Sergi A, Balini A, Belloni C, Meschi F, Bonifacio E, Bosi E: Two-step islet autoantibody screening for risk assessment of type 1 diabetes in relatives. *DiabetesCare* 21:1445–1450, 1998
9. Lendrum R, Walker G, Cudworth AG, Theophanides C, Pyke DA, Bloom A, Gamble DR: Islet-cell antibodies in diabetes mellitus. *Lancet* ii:1273–1276, 1976
10. Kolb H, Dannehl D, Zielasek J, Bertrams J, Hubinger A, Gries FA: Prospective analysis of islet cell antibodies in children with type I (insulin-dependent) diabetes. *Diabetologia* 31:189–194, 1988
11. Bosi E, Andreotti AC, Girardi AM, Bottazzo GF, Pozza G: The long-term per-

- sistence of islet cell antibodies in type 1 diabetic patients is unrelated to residual  $\beta$ -cell function. *Diabetes Nutr Metab* 4:319–323, 1991
12. Savola K, Sabbah E, Kulmala P, Ilonen J, Knip M: Autoantibodies associated with type 1 diabetes mellitus persist after diagnosis in children. *Diabetologia* 41:1293–1297, 1998
13. Robertson RP, Sutherland DER, Lanz KJ: Normoglycemia and preserved insulin secretory reserve in diabetic patients 10–18 years after pancreas transplantation. *Diabetes* 48:1737–1740, 1999
14. Sibley RK, Sutherland DER, Goetz F, Michael AF: Recurrent diabetes mellitus in the pancreas iso- and allograft: a light and electron microscopic and immunohistochemical analysis of four cases. *Lab Invest* 53:132–144, 1985
15. Sibley RK, Sutherland DER: Pancreas transplantation: an immunohistologic and histopathologic examination of 100 grafts. *Am J Pathol* 128:151–170, 1987
16. Sutherland DER, Goetz FC, Sibley RK: Recurrence of disease in pancreas transplants. *Diabetes* 38 (Suppl. 1):85–87, 1989
17. Tyden G, Reinholdt FP, Sundkvist G, Bolinder J: Recurrence of autoimmune diabetes mellitus in recipients of cadaveric pancreatic grafts. *N Engl J Med* 335:860–863, 1996
18. Bækkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347:151–156, 1990
19. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, Goldstein DE, Rae PMM: Islet cell antigen 512 is a diabetes-specific autoantigen related to protein tyrosine phosphatases. *J Immunol* 152:3183–3188, 1994
20. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E: Identification of protein tyrosine phosphatase-like IA-2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 155:5419–5426, 1995
21. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, Eisenbarth GS, participating laboratories: Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, and cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 47:1857–1866, 1998
22. Naserke HE, Dozio N, Ziegler AG, Bonifacio E: Comparison of a novel microassay for insulin autoantibodies with the conventional radiobinding assay. *Diabetologia* 41:681–683, 1998
23. Lampasona V, Bearzatto M, Genovese S, Bosi E, Ferrari M, Bonifacio E: Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosin phosphatase-like IA-2 autoantigen. *J Immunol* 157:2707–2711, 1996
24. Bonifacio E, Lampasona V, Bingley PJ: IA-2 (islet cell antigen 512) is the primary target of humoral autoimmunity against type 1 diabetes-associated tyrosine phosphatase autoantigens. *J Immunol* 161:2648–2654, 1998
25. Naserke HE, Ziegler AG, Lampasona V, Bonifacio E: Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. *J Immunol* 161:6963–6969, 1998
26. Bonifacio E, Scirpoli M, Kredel K, Fuchtenbusch M, Ziegler AG: Early autoantibody responses in pre-diabetes are IgG1 dominated and suggest antigen-specific regulation. *J Immunol* 163:525–532, 1999
27. Bosi E, Bottazzo GF, Secchi A, Pozza G, Shattock M, Saunders A, Gelet A, Touraine JL, Traeger J, Dubernard JM: Islet cell autoimmunity in type 1 diabetic patients after HLA-mismatched pancreas transplantation. *Diabetes* 38 (Suppl. 1):82–84, 1989
28. Esmatjes E, Rodriguez-Villar C, Ricart MJ, Casamitjana R, Martorell J, Sabater L, Astudillo E, Fernandez-Cruz L: Recurrence of immunological markers for type 1 (insulin-dependent) diabetes mellitus in immunosuppressed patients after pancreas transplantation. *Transplantation* 66:128–131, 1998
29. Sundkvist G, Tyden G, Karlsson FA, Bolinder J: Islet autoimmunity before and after pancreas transplantation in patients with type 1 diabetes mellitus. *Diabetologia* 41:1532–1533, 1998
30. Jaeger C, Brendel MD, Hering BJ, Eckhard M, Bretzel RG: Progressive islet graft failure occurs significantly earlier in autoantibody-positive than in autoantibody-negative IDDM recipients of intrahepatic islet allografts. *Diabetes* 46:1907–1910, 1997
31. De Filippo G, Carel JC, Boitard C, Bougnères PF: Long-term results of early cyclosporin therapy in juvenile IDDM. *Diabetes* 45:101–104, 1996