Rapid Publication

Prevention of Autoimmune Recurrence and Rejection by Adenovirus-Mediated CTLA4Ig Gene Transfer to the Pancreatic Graft in BB Rat

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Type 1 diabetes is the result of a selective destruction of pancreatic islets by autoreactive T-cells. Therefore, in the context of islet or pancreas transplantation, newly transplanted β-cells are threatened by both recurrent autoimmune and alloimmune responses in recipients with type 1 diabetes. In the present study, using spontaneously diabetic BB rats, we demonstrate that whereas isolated islets are susceptible to autoimmune recurrence and rejection, pancreaticoduodenal grafts are resistant to these biological processes. This resistance is mediated by lymphohematopoietic cells transplanted with the graft, since inactivation of these passenger cells by irradiation uniformly rendered the pancreaticoduodenal grafts susceptible to recurrent autoimmunity. We further studied the impact of local immunomodulation on autoimmune recurrence and rejection by ex vivo adenovirus-mediated CTLA4Ig gene transfer to pancreaticoduodenal grafts. Syngeneic DR-BB pancreaticoduodenal grafts transduced with AdmCTLA4Ig were rescued from recurrent autoimmunity. In fully histoincompatible LEW→BB transplants, in which rejection and recurrence should be able to act synergistically, AdmCTLA4Ig transduced LEW-pancreaticoduodenal allografts enjoyed markedly prolonged survival in diabetic BB recipients. In situ reverse transcription–polymerase chain reaction revealed that transferred CTLA4Ig gene was strongly expressed in both endocrine and exocrine tissues on day 3. These results indicate the potential utility of local CD28-B7 costimulatory blockade for prevention of alloimmune and autoimmune destruction of pancreatic grafts in type 1 diabetic hosts. *Diabetes* 48:XXX–XXX, 1999

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CMV, cytomegalovirus; DP, diabetes-prone; DR, diabetes-resistant; IL, interleukin; MST, mean graft survival time; PCR, polymerase chain reaction; pfu, plaque forming units; RT, reverse transcription; sIg^-, surface immunoglobulin negative; UW, University of Wisconsin.

In spite of recent progress in pharmacological and biological immunosuppressive therapies, the outcome of isolated islet transplantation for treatment of type 1 diabetes has been disappointing, with less than 9% of recipients achieving insulin independence (1). The biological factors responsible for these poor results have been extensively investigated in the spontaneously diabetic BB rat model of human type 1 diabetes (2). Islets transplanted into BB rats rendered tolerant of islet-donor alloantigens, a strategy known to preclude immunologic rejection, uniformly failed, nevertheless, indicating the vulnerability of islets to recurrent anti–β-cell autoimmunity (3,4). These findings firmly established that islet allografts in type 1 diabetic recipients are vulnerable to two biological processes: rejection and recurrent autoimmunity (3–5).

In contrast to the susceptibility of islet allografts to recurrent autoimmunity and rejection, whole pancreaticoduodenal grafts enjoy permanent survival in the spontaneous diabetic BB rats (6). The basis of the resistance of pancreaticoduodenal allografts to immune destruction has been unknown, although graft-derived lymphohematopoietic chimerism has been proposed to contribute to the protection of β-cells from both recurrent autoimmunity and rejection (7,8).

In the present study, we have developed a strategy that uniformly promotes recurrent autoimmunity and rejection of the pancreaticoduodenal grafts in the spontaneous autoimmune diabetic BB rats. This strategy provided a unique opportunity to assess the efficacy of local immunomodulation, achieved by recombinant adenovirus-mediated gene transfer of CTLA4Ig, on the susceptibility of pancreaticoduodenal grafts to both autoimmune and alloimmune processes.

RESEARCH DESIGN AND METHODS

Experimental animals. Male diabetes-prone (DP) and diabetes-resistant (DR) BB/Wor rats ($RT1^u$) were obtained from the University of Massachusetts (Worcester, MA) and maintained at the laboratory animal facilities of the University of Pennsylvania. DP-BB rats spontaneously develop type 1 diabetes at ~10 weeks of age; the cumulative incidence of diabetes by 20 weeks of age reaches 91.1% (n = 157) in our colony. Diabetic DP-BB rats with blood glucose levels >300 mg/dl for three consecutive days were treated with Protamine Zinc insulin before transplantation. Male Lewis (LEW) rats ($RT1^l$) were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed under specific pathogen free conditions with a 12-h light/dark cycle and had free access to food and water. All animal procedures and use of recombinant DNA were approved by the Institutional Animal Care and Biosafety Committees, respectively.

Pancreas transplantation. Pancreaticoduodenal grafts were procured from DR-BB or LEW donors and transplanted immediately into acutely diabetic DP-BB rats (within 2 weeks after the onset of diabetes) as previously described (8). In other experiments, the pancreaticoduodenal grafts were irradiated (1000 rad, Gammacell 1000; Nordian International, Ontario, Canada) ex vivo before transplantation into diabetic BB rats. All surgical procedures were carried out in a sterile field under Metofane anesthesia. Animals were bled every other day from the tail vein, and graft survival was determined by measurement of blood glucose with Accu-Chek Advantage (Boehringer Mannheim, Indianapolis, IN). Recipients were killed when they showed hyperglycemic relapse, defined as blood glucose levels >200 mg/dl for two consecutive days or at other defined intervals. Recurrence of β-cell autoimmunity or rejection was further assessed by histological examination of pancreaticoduodenal grafts.

Construction of recombinant adenovirus. The adenoviral vector, AdmCTLA4Ig, contains an expression cassette encoding the murine CTLA4 gene coupled to the mouse immunoglobulin sequence, replacing the viral E1 and most of the E1B region, and rendering the virus replication defective. Expression is under control of a cytomegalovirus (CMV) promoter. A *Bam*H1 fragment of mouse CTLA4Ig DNA (a gift from Dr. Peter Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) was inserted into the pAcCMV plpa vector. The resulting plasmid pAcCMVmCTLA4Ig was cotransfected with *Cla* 1 large right fragment of adenovirus serotype 5 mutant dl309 into 293 cells by calcium coprecipitation. AdmCTLA4Ig was purified by the method described by Hirt (9). The 293 cells were expanded in a spinner flask, pelleted, frozen, and thawed, yielding whole viral lysates that were titered by plaque assay and expressed as plaque forming units (pfu) per milliliter (10). Adenovirus containing Lac Z (AdLac Z) and CTLA4Ig genes were gifts from Drs. Steven Raper and Andrew Gelman (University of Pennsylvania, Philadelphia, PA).

Delivery of AdmCTLA4Ig to pancreaticoduodenal grafts. Ex vivo gene transfer into the pancreatic grafts was performed via perfusion of the aortic cuff with 1 ml of University of Wisconsin (UW) preservation solution containing 10^{11} pfu of AdmCTLA4Ig. For an additional control group, pancreaticoduodenal grafts were perfused with UW solution containing AdLac Z. The grafts were perfused without clamping the venous outflow (portal vein) with an initial 0.6 ml of virus solution, followed by occlusion of the portal vein during the infusion of the remaining 0.4 ml. Incubation with the recombinant adenovirus was performed at 4°C, and the average cold ischemia time (the duration from virus infusion to graft reperfusion with blood) was 2 h.

Detection of CTLA4Ig gene expression by in situ reverse transcription–polymerase chain reaction. To detect the mRNA gene expression of CTLA4Ig, pancreatic grafts from designated groups were removed on days 3 and 14 after transplantation and immediately frozen in Tissue-Tek OCT compound in preparation for in situ reverse transcription (RT)–polymerase chain reaction (PCR). Briefly, the 5-μm sections were fixed in 3.7–4.0% formaldehyde at room temperature for 10 min, followed by DNase I digestion at 50 U/slide at 37°C for 3 h. In situ RT reactions were carried out using Moloney murine leukemia virus reverse transcriptase and random hexamer primers (all from GeneAmp RNA PCR kit; Perkin Elmer, Norwalk, CT). The reaction was run at 42°C for 30 min, followed by denaturation at 95°C for 5 min and a cooling step at 5°C for another 5 min. The in situ PCR reactions were carried out using GeneAmp In Situ PCR Kit (Perkin Elmer) supplemented with the 5′ fluorescein-labeled mCTLA4Ig-specific sense primer (TGTGCCACGACATTCACAGAG) and the unlabeled antisense primer (CATGAAGTCTGTGACCATGCA). The thermocycle amplifications were completed on the GeneAmp in situ PCR system 1000 (Perkin Elmer, Foster City, CA). RT-PCR products were evaluated by fluorescence microscopy.

Separation of splenic T-cell populations and flow cytometry analysis. Surface immunoglobulin negative(sIg^-) spleen cell populations were prepared as previously described (8). Flow cytometric analysis of $RT6^+$ T-cells was performed by incubation of 1.5×10^6 sIg^- cells with culture supernatant containing anti-RT6.1 mAb (P4/16, rat IgG_{2b}; Serotec, Oxford, U.K.). The cells were washed twice with phosphate-buffered saline and then incubated an additional 30 min with appropriately diluted $F(ab')_2$ fragment of phycoerythrin-conjugated goat anti-rat IgG and fluorescein isothiocyanate–conjugated mouse anti-rat abTCR (R73; PharMingen, San Diego, CA). At least 1×10^4 viable lymphocytes were analyzed using FACS IV (Becton Dickinson, Sunnyvale, CA) with computation of data by a CellQuest program (Becton Dickinson).

RESULTS

Survival of isolated islet and pancreaticoduodenal grafts in BB rats. In initial experiments, isolated islets from DR-BB rats were transplanted into the portal vein of the syngeneic diabetic DP-BB rats. Despite an initial period of normoglycemia, all DP-BB recipients eventually demonstrated relapse of hyperglycemia, confirming the vulnerability of islet grafts to recurrent autoimmune destruction (Table 1). Histological examination of islet-bearing liver revealed mononuclear infiltration of pancreatic islets (data not shown). In contrast to isolated islets, all pancreaticoduodenal grafts from DR-BB rats enjoyed permanent survival in diabetic DP-BB rats (Table 1 and Fig. 1). In accordance with our previous reports (8), all DP-BB recipients of long-surviving DR-BB pancreaticoduodenal grafts demonstrated the chimerism of immunoregulatory $RT6^+$ T-cells derived from the donor pancreas. Figure 2*C* and *D* demonstrate a progressive increase in the level of chimerism of $RT6.1^+$ T-cells in DP-BB recipients of DR-BB pancreaticoduodenal grafts. Since chimerism of $RT6^+$ T-cells has been proposed as the explanation for the resistance of pancreatic grafts to recurrent autoimmunity, we examined the fate of pancreaticoduodenal grafts in which graft-derived passenger leukocytes had been eliminated. This was accomplished by ex vivo irradiation of the pancreaticoduodenal graft to destroy the lymphohematopoietic passenger leukocytes and thus prevent donor chimerism in the recipient. All BB recipients of irradiated DR-BB pancreaticoduodenal grafts demonstrated recurrent diabetes 13–18 days after transplantation (Table 1 and Fig. 1). Furthermore, DP-BB recipients of irradiated DR-BB pancreaticoduodenal grafts had no detectable chimerism of $RT6.1^+$ T-cells (Fig. 2*E*). More importantly, histological examination of pancreaticoduodenal grafts at the time of recurrent hyperglycemia revealed mononuclear infiltration confined to the islets of Langerhans with sparing of the exocrine tissue. That the irra-

TABLE 1
Survival of islet or pancreaticodudenal grafts in BB rats

Donor	Recipient	Graft	Graft treatment	Graft survival	MST (days)
DR-BB	DP-BB	Islet	None	9, 17, 23, 26, 29, 30	22.3
		PD	None	>120 × 7	>120.0
		PD	Irradiation*	13, 14, 14, 16, 16, 18	15.2
		PD	Irradiation + AdmCTLA4Ig†	114, >120 × 5	>119.0
		PD	Irradiation + AdLac Z	8, 8, 9, 11	9.0
DR-BB	DR-BB	PD	Irradiation	>120 × 3	>120.0
LEW	DP-BB	PD	None	11, 19, 23, 25, 25, 27	22.2
		PD	Irradiation	17, 17, 17, 18	17.3
		PD	Irradiation + AdmCTLA4Ig	17, 24, 32, 54, >120 × 2	>61.2

DR-BB recipients were rendered diabetic by streptozocin injection. *Grafts were γ-irradiated with 1,000 rad. †Grafts were perfused with 10^{11} pfu AdmCTLA4Ig after the irradiation. PD, pancreaticoduodenal grafts.

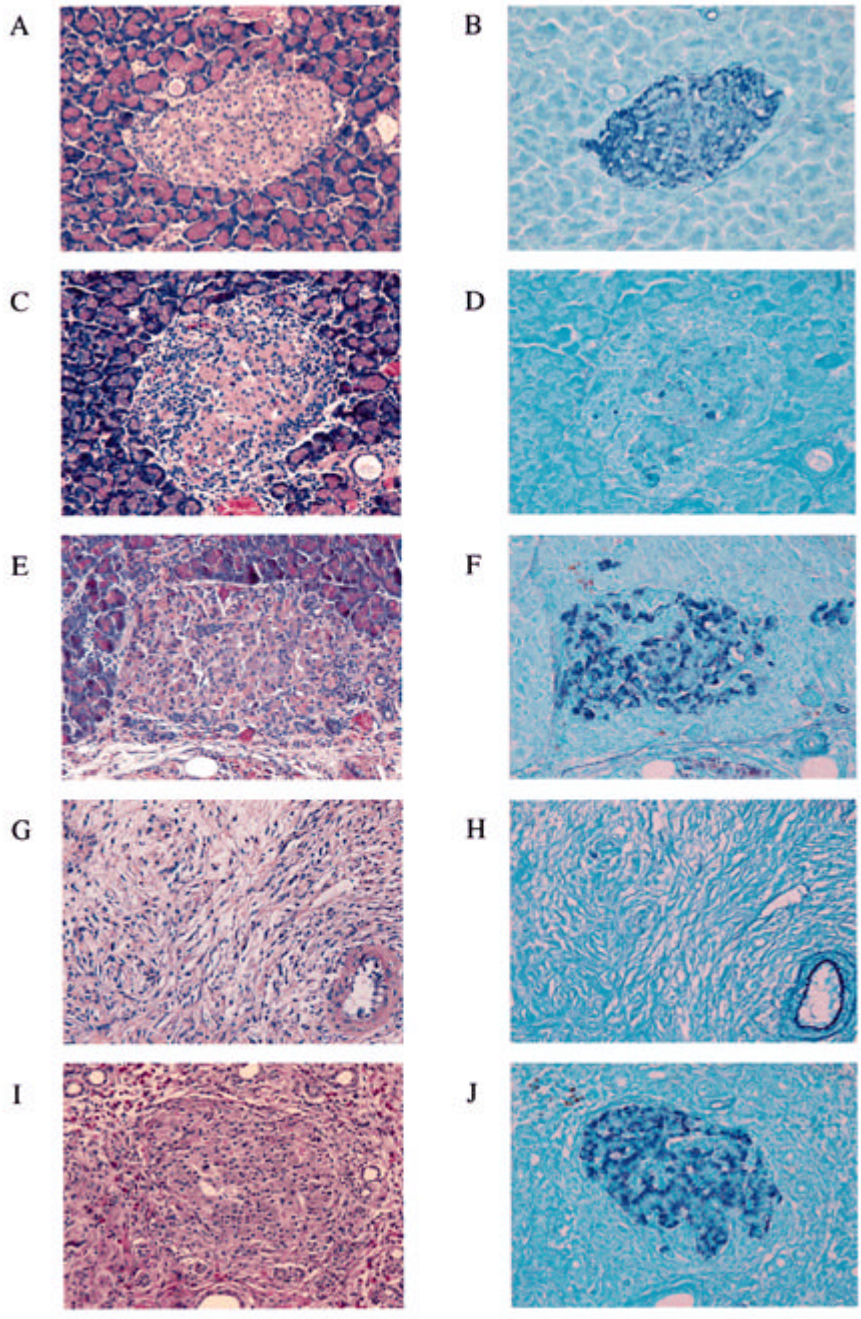

FIG. 1. Representative histological examination of pancreaticoduodenal grafts across DR-BB→DP-BB (*A–F*) and LEW→DP-BB (*G–J*). *A*: Hematoxylin and eosin staining of pancreas graft on day 142 demonstrating normal islet and exocrine tissue without lymphocytic infiltration. *B*: Aldehyde fuchsin (AF) staining revealed well-granulated β-cells. *C*: Irradiated pancreatic graft demonstrating dense mononuclear infiltration confined to islets sparing acinar tissue at the time of relapse of hyperglycemia on day 13 after transplantation. *D*: AF staining of adjacent section revealed virtual absence of β-cells. *E* and *F*: Islets in the long-surviving recipient with AdmCTLA4Ig-treated pancreas graft on day 153 demonstrating minimal peri-islet mononuclear infiltration but well-preserved β-cells. *G* and *H*: Irradiated LEW pancreatic graft showed complete destruction of both islets and exocrine tissues on day 25. *I* and *J*: The normoglycemic BB recipient with AdmCTLA4Ig-treated LEW grafts showing destruction of exocrine tissues and preservation of islets on day 145. Original magnification ×200.

diation-induced structural damage to the pancreas was not the cause of failure was indicated by the permanent survival of irradiated DR-BB syngeneic grafts transplanted into chemically diabetic DR-BB recipients. All of these recipients remained normoglycemic without any evidence of inflammation or damage in the pancreaticoduodenal grafts.

We next examined the fate of fully mismatched LEW pancreaticoduodenal allografts in diabetic DP-BB rats. As shown in Table 1 and Fig. 1, BB recipients of LEW pancreaticoduodenal grafts demonstrated relapse of hyperglycemia 11–27 days after transplantation. This was due to the damage from the combined effect of rejection and recurrent autoimmunity, evidence of which was clear from mononuclear infiltration of both endocrine and exocrine elements of the grafts, as well as marked global fibrosis of the pancreatic grafts. The survival of irradiated LEW pancreaticoduodenal grafts was comparable to that of nonirradiated cohorts (mean graft survival time [MST] = 17.3 vs. 22.2 days).

Fate of AdmCTLA4Ig-transfected pancreaticoduodenal grafts in BB rats. The uniform susceptibility of irradiated pancreaticoduodenal grafts to recurrent autoimmunity and rejection provided an opportunity to assess the efficacy of local immunomodulation on these two biological barriers. Ex vivo perfusion of irradiated DR-BB pancreaticoduodenal grafts with AdmCTLA4Ig resulted in their indefinite survival in diabetic DP-BB recipients (Table 1 and Fig. 1). However, despite the indefinite survival of AdmCTLA4Ig-transduced DR-BB pancreaticoduodenal grafts, DP-BB recipients remained lymphopenic and did not demonstrate RT6.1$^+$ T-cell chimerism (Fig. 2*F*). The survival of a control

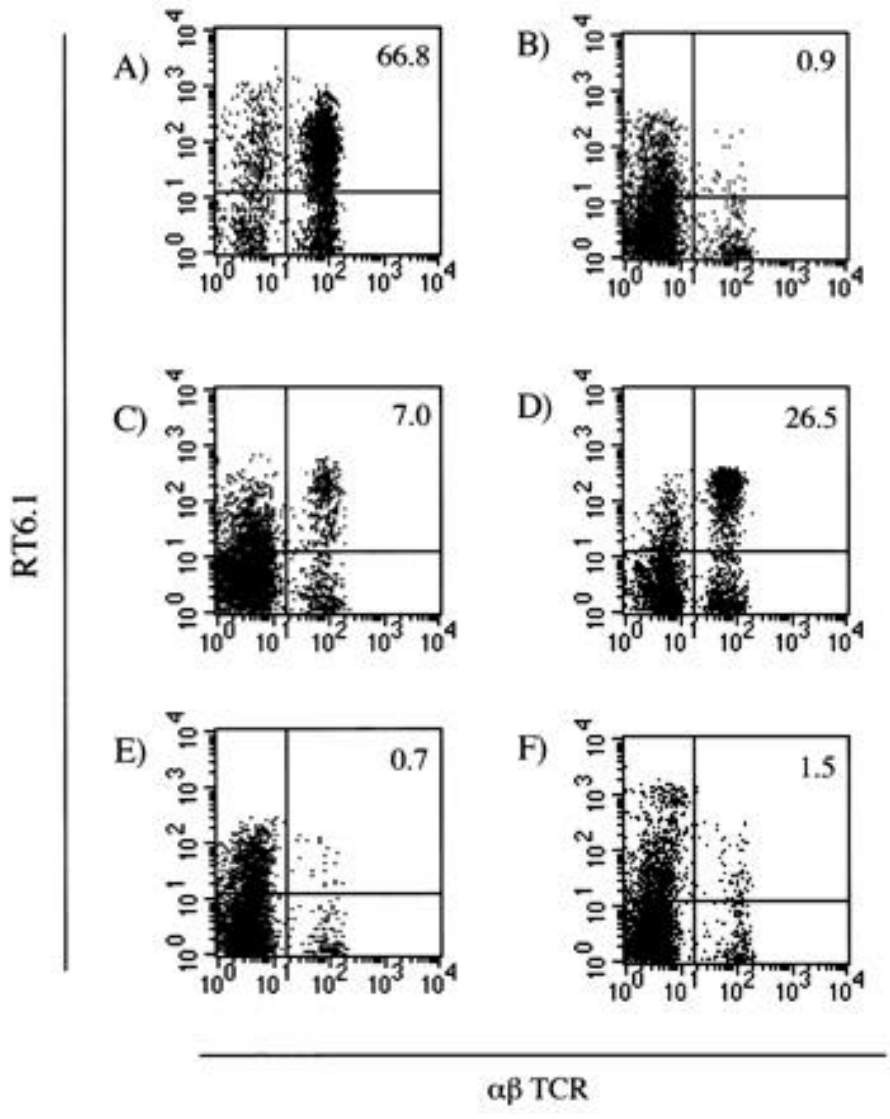

FIG. 2. Representative flow cytometry analysis of splenic T-cells double-stained with dβTCR and RT6.1 antigen. *A*: Naive DR-BB rat demonstrating normal complement of T-cells. *B*: Naive DP-BB rat demonstrating the well-characterized T-cell lymphopenia. *C* and *D*: The level of RT6.1$^+$ T-cell chimerism in the lymphopenic DP-BB recipients on days 14 and 139 after transplantation with DR-BB pancreaticoduodenal grafts, respectively. *E*: The level of chimerism of RT6.1$^+$ T-cells in a DP-BB recipient of irradiated DR-BB pancreatic graft on day 14. *F*: The level of RT6.1$^+$ T-cell chimerism in normoglycemic DP-BB recipient 159 days after transplantation with Adm-CTLA4Ig-transduced pancreatic graft from DR-BB rat.

group consisting of irradiated DR-BB pancreaticoduodenal grafts perfused with AdLac Z was comparable to that of a group with irradiated grafts that were infused only with UW solution (MST = 9.0 vs. 15.2 days). The survival of fully mismatched irradiated LEW pancreaticoduodenal graft perfused with AdmCTLA4Ig was markedly prolonged compared with that of the nontransduced group (MST = 61.2 vs. 17.3 days); only two recipients, however, demonstrated permanent normoglycemia. Histological examination of long-surviving grafts revealed fibrosis and mononuclear infiltration of acinar tissue with intact islets (Fig. 1).

Detection of CTLA4Ig gene by in situ PCR in pancreaticoduodenal grafts. To assess the expression of CTLA4Ig gene, mRNA detection by in situ RT-PCR was established. In both combinations, CTLA4Ig mRNA was abundantly detected in islets as well as exocrine tissue on day 3 after transplantation (Fig. 3). However, the level of expression of the gene was markedly diminished on day 14, and in situ RT-PCR failed to detect CTLA4Ig mRNA expression in pancreaticoduodenal grafts functioning for >120 days. On day 3, in situ RT-PCR also revealed the expression of CTLA4Ig gene in the liver of the recipients; however, the gene was undetectable on day 14 after transplantation (data not shown).

DISCUSSION

The present study demonstrates that, in contrast to isolated islets, whole pancreaticoduodenal grafts are resistant to recurrent β-cell autoimmunity after transplantation to BB rats. Because donor chimerism has been implicated in the protection of pancreatic grafts, we reasoned that inactivation of graft-derived lymphohematopoietic cells might prevent chimerism and abrogate the resistance to autoimmunity. This contention was confirmed by the uniform development of recurrent β-cell autoimmunity (insulitis) in the irradiated pancreaticoduodenal grafts. Furthermore, recipients of irradiated syngeneic DR-BB grafts suffering from recurrent diabetes failed to demonstrate donor RT6$^+$ T-cell chimerism. To our knowledge, this is the first demonstration of a uniform model of recurrent autoimmunity affecting pancreaticoduodenal grafts in autoimmune BB diabetes. The model is reminiscent of the recurrence of diabetes seen in identical twin recipients of segmental pancreatic isografts (11).

Pancreatic allografts transplanted to diabetic recipients are threatened by both rejection and recurrent autoimmunity. The prompt destruction of fully mismatched LEW-pancreaticoduodenal allografts in BB rats indicates a synergistic impact of these processes on transplanted β-cell mass.

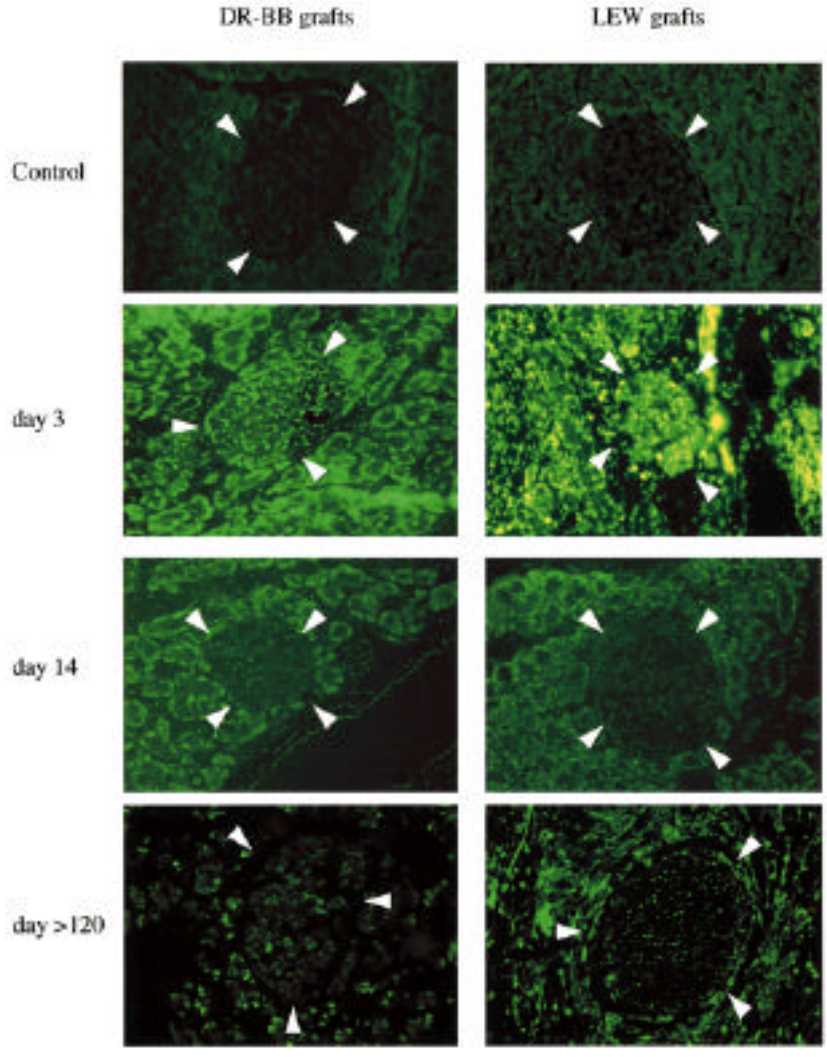

FIG. 3. Representative expression of CTLA4Ig mRNA in DR-BB or LEW pancreatic grafts by in situ RT-PCR. Control: both the exocrine and islets (arrow) lack transgene expression in irradiated pancreatic grafts perfused by UW solution. Day 3: diffuse expression of CTLA4Ig gene in exocrine and islets of AdmCTLA4Ig-transfected pancreatic grafts on day 3 after transplantation into DP-BB rat. Progressive diminution of transgene expression was seen on day 14 and >120 days after transplantation. Original magnification ×200.

Recently, recurrence of diabetes in human recipients of HLA-mismatched cadaveric pancreas allografts has been reported (12). In these cases, histological examination of pancreatic allografts revealed selective destruction of islet β-cells despite standard immunosuppressive therapy.

Costimulation via CD28/CTLA4-B7 signaling pathway plays a critical role in promoting the optimal proliferation and differentiation of T-cells (13). Blockade of CD28-B7 by competitive inhibitor CTLA4Ig has been demonstrated to inhibit T-cell–dependent immune responses in vivo (14). In this regard, costimulatory blockade induced by systemic treatment of recipients with CTLA4Ig protein has been shown to prolong the survival of islet allo- and xenografts (15,16).

The strategy of transferring genes that encode proteins capable of suppressing immune responses within the local microenvironment of the graft is of considerable advantage for avoiding the morbidity of systemic immunosuppression. In this regard, the most promising approach involves recombinant adenovirus mediated gene transfer of the immunomodulatory lymphokines (interleukin [IL]-4, IL-10, and transforming growth factor-β) to achieve local immune modulation for prevention of rejection (17–19). Interestingly, despite the efficacy of adenovirus-mediated IL-4 and IL-10 to prevent the rejection of islet allografts, they failed to prevent the destruction of syngeneic islet grafts by recurrent autoimmunity in NOD mice (17). In the present study, a single infusion of AdmCTLA4Ig via the aorta resulted in the complete protection of pancreaticoduodenal grafts from recurrent autoimmunity and markedly prolonged their survival in the fully allogeneic diabetic hosts. Furthermore, in situ RT-PCR detected a global expression of CTLA4Ig transgene within both the endocrine and exocrine pancreas that progressively diminished in long-surviving pancreaticoduodenal grafts. The mechanism(s) underlying the protection of AdmCTLA4Ig-transfected pancreatic grafts from rejection and recurrence is unknown. It is tempting, however, to speculate that the protection might be mediated by induction of anergy/Th2-polarization of autoreactive (islet-reactive) and alloreactive T-cells after blockade of CD28-B7 costimulatory signals (20,21). In this context, transfection of the intragraft antigen presenting cells and endothelium with CTLA4Ig would render these populations ideal candidates to provide the tolerogeneic signal for T-cells. Since we observed a progressive diminution of CTLA4Ig transgene expression, as revealed by in situ RT-PCR, it is logical to assume that even a transient window of costimulatory blockade may be sufficient to accomplish immunomodulation of T-cells and to promote unresponsiveness.

In summary, pancreaticoduodenal grafts in BB rats regain an exquisite sensitivity to recurrent autoimmunity and rejection after inactivation of lymphohematopoietic cellular passengers of the graft. Furthermore, local immunomodulation by CTLA4Ig-mediated costimulatory blockade provides long-term protection from rejection and recurrent anti–β-cell autoimmunity. This strategy has potential utility for the prevention of autoimmune and alloimmune responses to pancreatic allografts in type 1 diabetic patients.

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