Allorecognition and Effector Pathways of Islet Allograft Rejection in Normal versus Nonobese Diabetic Mice

LEILA MAKHLOUF,* AKIRA YAMADA,* TOSHIRO ITO,*† REZA ABDI,* MOHAMMED JAVEED I. ANSARI,* CHAU Q. KHUONG,† HENRY J. WINN,† HUGH AUCHINCLOSS, JR.,*‡ and MOHAMED H. SAYEGH*§

*Laboratory of Immunogenetics and Transplantation, Renal Division, Department of Medicine, Brigham and Women’s Hospital, Boston, Massachusetts; †Transplant Laboratory, Department of Surgery, Massachusetts General Hospital, Boston, Massachusetts; and §Nephrology Division, The Children’s Hospital, Harvard Medical School, Boston, Massachusetts.

Abstract. Islet transplantation is becoming an accepted therapy to cure type I diabetes mellitus. The exact mechanisms of islet allograft rejection remain unclear, however. In vivo CD4⁺ and CD8⁺ T cell-depleting strategies and genetically altered mice that did not express MHC class I or class II antigens were used to study the allorecognition and effector pathways of islet allograft rejection in different strains of mice, including autoimmunity-prone nonobese diabetic (NOD) mice. In BALB/c mice, islet rejection depended on both CD4⁺ and CD8⁺ T cells. In C57BL/6 mice, CD8⁺ T cells could eventually mediate islet rejection by themselves, but they produced rejection more efficiently with help from CD4⁺ T cells stimulated through either the direct or indirect pathway. In C57BL/6 mice, CD4⁺ T cells alone caused islet rejection when only the direct pathway was available but not when only the indirect pathway was available. In contrast, in NOD mice, CD4⁺ T cells alone, with only the indirect pathway, could mediate islet and cardiac allograft rejection. These findings indicate that different mouse strains can make use of different pathways for T cell-mediated rejection of islet allografts. In addition, they demonstrate that NOD mice, which develop autoimmunity and are known to be resistant to tolerance induction, have an unusually powerful CD4⁺ cell indirect mechanism that can cause rejection of both islet and cardiac allografts. These data shed light on the mechanisms of islet allograft rejection in different responder strains, including those with autoimmunity.

Type I diabetes mellitus is an important cause of end-stage renal disease, and islet allotransplantation, with or without kidney transplantation, offers a potential cure for diabetes mellitus (1). Different pathways of allorecognition are involved in alloimmune responses mediated by T cells. The direct pathway is defined as the presentation of alloantigen by MHC class II or class I molecules on the surfaces of donor antigen-presenting cells to recipient CD4⁺ or CD8⁺ T lymphocytes, respectively. The indirect pathway is defined as alloantigen presentation by recipient antigen-presenting cells to recipient CD4⁺ or CD8⁺ T lymphocytes (2–6). The roles of these pathways in graft rejection have been studied for different tissues (6,7). The roles of these pathways in islet allograft rejection have also been studied (8–16), but there have been conflicting results.

Transplantation of allogeneic islets is likely to be performed for recipients with autoimmune diabetes mellitus but may also be appropriate for some patients with nonautoimmune diabetes mellitus. For study of the mechanisms of islet allograft rejection, murine models of autoimmune type I diabetes mellitus (the nonobese diabetic [NOD] mouse model) (17) and nonautoimmune diabetes mellitus (chemically induced diabetes mellitus in strains that do not spontaneously develop diabetes mellitus) are available. In both types of murine recipients, rejection of islet allografts involves a T cell-mediated immune response (8–11,18,19).

In NOD recipients, the CD4⁺ T cell direct pathway of allorecognition seems to participate in the process (12,20–24), but the roles of the CD4⁺ cell indirect pathway and CD8⁺ T cells have not yet been clarified (25). In other strains, the mechanisms of islet allograft rejection have been more intensively investigated. CD4⁺ (10,26,27) and CD8⁺ (10,28) T cell subsets have been demonstrated to be important contributors. Furthermore, the CD4⁺ cell direct pathway seems to be critical (14,21–23). However, the relative contributions of the CD4⁺ T cell direct and indirect pathways in various mouse strains and in recipients with autoimmunity remain unclear.

In this study, we tested the hypothesis that different allorecognition and/or effector pathways mediate islet allograft rejection among recipients with autoimmune diabetes mellitus (NOD mice) and recipients without autoimmunity (BALB/c and C57BL/6 mice with chemically induced diabetes mellitus). We used T cell-depleting strategies and genetically altered
mice that did not express MHC class I or class II antigens. Cardiac allograft transplantation was performed with NOD mice, to compare the rejection of islet allografts with that of an organ not susceptible to tissue-specific autoimmune destruction.

The study indicates that different mouse strains can make use of different pathways for T cell-mediated rejection of islet allografts. In addition, it demonstrates that NOD mice, which develop autoimmune diabetes mellitus and are known to be resistant to tolerance induction (29,30), have an unusually powerful CD4+ cell indirect mechanism that can cause rejection of both islet and cardiac allografts.

Materials and Methods

Mice

Diabetic female NOD/Lt (H-2^d) mice (referred to as NOD mice) were used as recipients. Eight- to 12-wk-old BALB/c (H-2^b) and C57BL/6 (H-2^b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice lacking MHC class II antigen expression (I/-) (Abb targeted mutation mice, 12th generation backcross) were purchased from Taconic (Germantown, NY). Previous characterization of the class II-deficient mice demonstrated that no class II antigen expression could be detected. These mice demonstrate substantial depletion of CD4+ peripheral T cells (31), although 3 to 5% of peripheral Thy1+ cells are CD4+. C57BL/6 mice lacking MHC class I antigens (I/-) (B2m^mice, homozygous for the B2m^m targeted mutation and exhibiting little, if any, MHC class I protein expression at the cell surface) were purchased from The Jackson Laboratory.

Mice expressing MHC class II antigens only on their thymic epithelium were generated by breeding class II-deficient mice with the B6-transgenic strain 36.5 (a gift from Dr. D. L. Sperber, Scripps Research Institute, La Jolla, CA), which expresses an Ea transgene only on thymic epithelium. These mice are class II-deficient on all cells other than thymic epithelium, but the presence of class II molecules in the thymus allows them to generate normal numbers of peripheral CD4+ cells (32,33). We have referred to these as II/-. 4+ mice and have used them as recipients for transplantation experiments to examine responses in the absence of an indirect pathway for CD4+ T cells (7,31).

Murine Models for Islet Transplantation

C57BL/6 and BALB/c mice were made diabetic by treatment with streptozotocin (225 mg/kg, administered intraperitoneally), and 400 islets were transplanted under the renal capsule. NOD mice were allowed to develop autoimmune diabetes mellitus spontaneously, and 700 islets were transplanted under the renal capsule. Diabetes mellitus was defined on the basis of blood glucose levels of >250 mg/dl on at least 2 d consecutively. Reversal of diabetes mellitus was defined on the basis of blood glucose levels of <200 mg/dl on at least 2 d consecutively. Islet graft function was assessed with blood glucose measurements (Accu-Check Advantage; Boehringer Mannheim, Indianapolis, IN) twice each week (30).

Isolation of Pancreatic Islets

Pancreatic islets were isolated by collagenase digestion followed by Histopaque 1077 (Sigma Chemical Co., St. Louis, MO) density gradient separation and then hand-picking, as described elsewhere (34).

Cardiac Transplants

Cardiac transplantation was performed as described previously (35). Cardiac graft survival was assessed by graft palpation. Islet and cardiac grafting procedures were performed independently.

Reagents, Antibodies, and In Vivo T Cell Depletion

For anti-CD8 ascites, 116-13.1 (anti-Lyt2.1, IgG2a) was used for NOD mice and 2.43 (anti-Lyt2.2, rat IgG2b) was used for BALB/c and C57BL/6 mice. For anti-CD4 ascites, GK1.5 (anti-L3T4, rat IgG2b) was used for all strains of mice. Ascites were obtained from the American Type Culture Collection (Rockville, MD). Anti-CD8-treated and anti-CD4-treated C57BL/6 and BALB/c mice received 0.1 ml of unpurified ascites, administered intraperitoneally. NOD mice received 0.2 ml of unpurified ascites of the appropriate antibody, administered intraperitoneally. This treatment was approximately equivalent to 100 or 200 mg of purified antibody, respectively, on day 1, day 0, and days 1, 2, and 3 after transplantation and then twice each week until rejection or day 60 after transplantation (for animals with surviving grafts). Depletion was confirmed at the beginning of the experiments and was monitored every second or third week until the end of the experiments. Depletion was confirmed with fluorescence-activated cell-sorting analysis. We achieved depletion of >98% of targeted T cells. Control groups were untreated. Fluorescence-activated cell-sorting analyses were performed as follows. Peripheral blood was obtained from NOD mice 1 wk after transplantation and then every 3 to 4 wk under the depletion regimen with GK1.5 or 116-13.1. Lymphocytes in single-cell suspensions were directly labeled with rat anti-mouse GK1.5 conjugated with FITC or rat anti-mouse 116-13.1 conjugated with phycoerythrin (PharMingen, San Diego, CA), rinsed, fixed in 1% paraformaldehyde, and analyzed with a FACScan analyzer (Becton Dickinson, San Jose, CA). Data were analyzed by using Cellquest software (Becton Dickinson).

Statistical Analyses

Kaplan-Meier survival graphs were constructed, and log-rank comparisons of the groups were used to calculate P values. Differences were considered significant at P < 0.05.

Results

Mechanisms of Islet Allograft Rejection in Nonautoimmune Recipients

Contributions of Recipient CD4+ and CD8+ T Cells to Rejection of Islet Allografts in BALB/c and C57BL/6 Mice.

First, we studied the roles of CD4+ and CD8+ T cells in islet allograft rejection by BALB/c mice. Depletion of either CD4+ or CD8+ cells prolonged islet allograft survival in most cases (Figure 1A). Therefore, in BALB/c mice, both subpopulations of T cells are required for islet allograft rejection. In accord with this conclusion, we also observed that MHC class I-deficient islets were not rejected (Figure 1A).

Next, we studied the roles of CD4+ and CD8+ T cells in C57BL/6 recipients (Figure 1B). Whereas chronic depletion of CD8+ T cells resulted in long-term allograft survival in all recipients, all grafts in chronically CD4+ T cell-depleted recipients were rejected within the 60-d follow-up period. These data suggest an important contribution of CD8+ T cells in this strain, which is capable of mediating islet allograft rejection with no help from CD4+ T cells (36,37).
Pathways of CD4\(^+\) Cell-Derived Help for CD8\(^+\) Cell-Mediated Islet Allograft Rejection in C57BL/6 and BALB/c Mice. Although CD8\(^+\) cells in C57BL/6 mice could eventually reject islet allografts in the absence of CD4\(^+\) cells, the aforementioned results indicated that CD8\(^+\) cells in both C57BL/6 and BALB/c mice caused islet rejection more efficiently when help from CD4\(^+\) cells was available. We next determined which pathway was necessary for the generation of CD4\(^+\) help. When class II-deficient islet allografts were transplanted into BALB/c mice, rejection occurred within 2 wk (Figure 2A). In addition, when normal islets were transplanted into recipients that had CD4\(^+\) cells but no class II molecules on their antigen-presenting cells, rapid rejection occurred (Figure 2B). These results indicate that CD4\(^+\) help for CD8\(^+\) T cells can be generated through either the direct or indirect pathway of allorecognition.

Role of CD8\(^+\) Cell Direct Pathway of Allorecognition. MHC class I-deficient donor islets survived indefinitely in BALB/c mice (mean survival time [MST], 80 ± 18 d; n = 4) (Figure 1A). This finding demonstrates that the CD8\(^+\) cell direct pathway is critical, and it suggests that CD4\(^+\) T cells activated through the direct and indirect pathways together are not able to reject islet allografts in this strain. For further study of the role of the CD8\(^+\) T cell direct pathway, BALB/c islets were transplanted into class II-deficient recipients, which could reject grafts only through CD8\(^+\) T cells. All grafts were rejected (MST, 30 ± 2 d), whereas transplantation of BALB/c islets into class II-deficient recipients depleted of CD8\(^+\) T cells resulted in indefinite graft survival (MST, >80 d) (Figure 2A). The latter finding is not surprising, because recipients had no peripheral CD4\(^+\) (MHC class II-deficient) or CD8\(^+\) (antibody-depleted) T cells. Although the CD8\(^+\) cell direct pathway is critical, it is possible that CD8\(^+\) cells can mediate rejection through the CD8\(^+\) cell indirect pathway (38). In the case of islets, the role of the CD8\(^+\) cell indirect pathway must be limited, compared with that of the direct pathway, because elimination of the direct pathway completely prevented rejection (Figure 1A).

Function of CD4\(^+\) Cells Alone in Islet Allograft Rejection in BALB/c and C57BL/6 Mice. Depletion of CD8\(^+\) cells in either BALB/c or C57BL/6 mice almost always led to prolonged islet allograft survival (Figure 1). We next studied the role of CD4\(^+\) cells alone when only one pathway or the
other was available. CD4\(^+\) cells alone, without CD8\(^+\) cells, were unable to cause rejection of class II-deficient islets (MST, 60 d; \(n = 5\)), indicating that a CD4\(^+\) cell indirect allorecognition/effecter mechanism could not cause islet rejection in BALB/c mice (Figure 2A).

The role of the CD4\(^+\) T cell direct pathway was investigated in II\(^{-/-}\) 4\(^+\) recipients (Figure 2B). These mice lack MHC class II but have peripheral CD4\(^+\) T cells. Therefore, after CD8\(^+\) T cell depletion, these mice can recognize alloantigens only via the CD4\(^+\) cell direct pathway. BALB/c islets transplanted into II\(^{-/-}\) 4\(^+\) recipients treated with depleting anti-CD8 monoclonal antibody (mAb) demonstrated significantly prolonged graft survival but were all ultimately rejected (MST, 22 ± 10 d; MST for control animals not treated with anti-CD8 mAb, 9.75 ± 1.2 d; \(P < 0.05\)). These data suggest that the CD4\(^+\) cell direct pathway alone can mediate islet allograft rejection. These results are in contrast to the prolonged survival of islets when both allorecognition pathways were present (Figure 1B).

**Mechanisms of Islet Allograft Rejection in NOD Recipients**

**Contributions of CD8\(^+\) and CD4\(^+\) T Cells in Islet Allograft Rejection.** C57BL/6 islets were transplanted into diabetic NOD mice. Depletion of CD4\(^+\) cells led to prolonged survival of allogeneic islets, but rejection eventually occurred at approximately the same time as in C57BL/6 recipients of allogeneic islets (MST, 68.4 ± 22 d; \(P < 0.001\), compared with the untreated control group) (Figure 3A). CD4\(^+\) cells alone in NOD mice caused islet rejection with a slight delay (MST, 23 ± 4 d). Class II-deficient islets were rapidly rejected by untreated NOD mice (MST, 13.5 ± 3 d) (Figure 3B). These results indicate that CD4\(^+\) cells in NOD mice can mount more vigorous rejection of allogeneic islets than can CD4\(^+\) cells in C57BL/6 or BALB/c mice. In this case, the CD4\(^+\) cell indirect and direct pathways of allorecognition are both involved.

**CD4\(^+\) Cell Indirect Mechanisms in NOD Mice.** We transplanted class II-deficient islets into NOD recipients treated chronically with anti-CD8 mAb, to determine whether CD4\(^+\) cells alone could reject allogeneic islets when only the indirect pathway was available. NOD recipients promptly rejected islet allografts by using only the CD4\(^+\) cell indirect pathway (MST, 16.5 ± 2.5 d) (Figure 4A). Untreated control animals also rejected the grafts (MST, 10 ± 0.5 d) (Figure 4A). We initially assumed that the unusually powerful indirect
The effector mechanism of NOD recipients was in some way related to their autoimmune responses to the islet transplants. To test this assumption, we transplanted class II-deficient cardiac allografts into NOD mice that had not developed diabetes mellitus, in which NOD heart isografts survived indefinitely (MST, 150 d; n = 4) (30). When recipients were treated chronically with anti-CD8 mAb, cardiac allografts were rejected (MST, 1 d; n = 4). To confirm the important role of the CD4 cell indirect pathway in allograft rejection, we transplanted C57BL/6 islets or hearts into NOD mice and depleted recipients of both CD4 and CD8 T cells (Figure 4). Cardiac (MST, >55 d; n = 5) and islet (MST, >45 d; n = 8) allografts demonstrated prolonged survival (P < 0.01, compared with the groups that rejected grafts only through the CD4 cell indirect pathway). Therefore, the unusually powerful indirect effector mechanism of NOD mice can cause rejection of allografts that are not subject to tissue-specific autoimmunity.

Discussion

The main goal of this work was to investigate the mechanisms of islet allograft rejection in several different mouse strains, including autoimmunity-prone NOD mice. Although there were some differences for all of the strains we examined, the most striking difference was that NOD mice exhibited an unusually powerful indirect pathway for allograft rejection, irrespective of the contribution of tissue-specific autoimmunity.

Other investigators have examined the roles of T cell subsets and pathways involved in islet rejection by nonautoimmune strains. The general conclusion has been that CD4 (10,26,27) and CD8 (10,28) T cell subsets are critical for islet allograft rejection, although CD8 T cells have been viewed as especially important (27,28). Depletion of CD8 T cells provided long-term survival of islet allografts (10), expression of donor MHC class I molecules was demonstrated to be critical for the rejection process (25,39,40), and transplantation of islet allografts into CD8-deficient recipients yielded prolonged survival (16). A critical contribution by the CD4 cell direct alloimmune response was suggested in some experiments (13–15,23,27). However, transplantation of MHC class II-deficient donor islets only marginally prolonged islet allograft survival (20), suggesting that the CD4 cell indirect pathway could participate in the presence of CD8 T cells. Therefore, although the importance of CD4 T cells is clear, the relative contributions of the CD4 cell direct and indirect pathways remain unclear.

Our results are largely consistent with previously reported findings. In general, rapid islet allograft rejection requires CD4 and CD8 T cells working together. Our findings suggest that CD4 cells can provide help for CD8 cells through
either the direct or indirect pathway. CD8\(^+\) cells functioning alone generally do not cause rapid graft rejection, but they could slowly cause rejection in some strains. An interesting finding was that CD4\(^+\) cells alone could cause rejection when only the direct pathway was available but not when both pathways were available. Of course, this observation involves a comparison between two different types of recipients (normal mice versus II\(^{-/-}\) 4\(^+\) mice), and CD4\(^+\) T cells may be more aggressive in the II\(^{-/-}\) 4\(^+\) strain. However, the fundamental feature of II\(^{-/-}\) 4\(^+\) mice is that they lack an indirect pathway. The rejection of islets by CD4\(^+\) cells in this strain suggests that indirect CD4\(^+\) responses in normal mice might regulate direct CD4\(^+\) effector mechanisms that would otherwise cause islet injury. Such an indirect inhibitory effect would have to be specific for CD4\(^+\) responses, because we demonstrated that CD4\(^+\) cell indirect responses in normal mice could provide help for CD8\(^+\) cells. A second interpretation is that the indirect pathway might be required to achieve tolerance, as previously indicated by our group (7) and others (41).

Other authors have also studied subsets and pathways of allore cognition used by NOD mice, but fewer published data are available. Investigations have probably been hampered by the complexity of the process in the NOD mouse strain, because possible recurrence of the autoimmune response (12,18,21,42) and the unique type of immunologic responses mounted by these mice (29) are additional factors to be considered. The role of T cells has been confirmed in several studies; immunotherapy with short-term depletion of CD4\(^+\) T cells (18) or treatment with nondepleting anti-CD4 mAb (43) prolonged islet allograft survival, suggesting an important role for the CD4\(^+\) T cell subpopulation. Studies have also suggested a role for the CD4\(^+\) T cell direct pathway in islet allograft rejection, by demonstrating that transplantation of MHC class II-deficient islets (20) or oxygen-precultured islet allografts (21–24) resulted in marginal but significant prolongation of islet allograft survival (12). The role of CD8\(^+\) T cells remains unclear. One study demonstrated that transplantation of class I MHC-deficient donor allogeneic islets into NOD recipients did not prolong graft survival (25), suggesting a minor role for class I MHC molecules in this context.

One of the important novel findings of our study was the observation of a very powerful indirect CD4 mechanism. Because NOD mice develop autoimmunity, we originally assumed that such autoimmunity might be responsible for the powerful indirect effector mechanism. However, we determined that was not the case. Other authors reported that NOD mice develop autoimmunity, but the mechanism of tolerance in NOD mice is more complex.

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**Figure 4.** Contribution of the indirect pathway of allore cognition to islet allograft destruction in NOD mice. (A) II\(^{-/-}\) islets transplanted into NOD recipients were rejected at a MST of 10 ± 0.5 d (n = 3). II\(^{-/-}\) islets transplanted into diabetic NOD recipients treated chronically with anti-CD8 mAb were rejected at a MST of 16.5 ± 2.5 d (n = 4). C57BL/6 islets transplanted into diabetic NOD recipients treated chronically with anti-CD4 and anti-CD8 mAb exhibited significantly prolonged survival (MST, >55 d; n = 8), compared with II\(^{-/-}\) islets transplanted into NOD recipients treated chronically with anti-CD8 mAb. (B) II\(^{-/-}\) heart allografts transplanted into NOD recipients were rejected at a MST of 7 ± 1 d (n = 3). II\(^{-/-}\) heart allografts transplanted into NOD recipients treated chronically with anti-CD8 mAb were rejected at a MST of 13 ± 1 d (n = 3) (in treated groups, cardiac versus islet allograft MST, P < 0.04). C57BL/6 islets transplanted into NOD mice treated chronically with anti-CD4 and anti-CD8 mAb demonstrated a MST of >40 d (n = 4). C57BL/6 cardiac allografts transplanted into nondiabetic NOD recipients treated chronically with anti-CD4 and anti-CD8 mAb demonstrated significantly prolonged survival (MST, >50 d; n = 5), compared with II\(^{-/-}\) cardiac allografts transplanted into NOD recipients treated chronically with anti-CD8 mAb.
mice exhibited unusual transplantation features with tissues other than islets (29). Resistance to tolerance induction with strategies that are effective in other mouse strains (18,29,30,42) is becoming a well established trait of this mouse strain, as confirmed in this study by resistance to chronic anti-CD4 treatment. It has not yet been determined whether the transplant traits of NOD mice are associated with their tendency to develop autoimmunity. One hypothesis is that the mice have a general defect in their peripheral regulatory mechanisms, which applies to more than just islet responses (44,45). Such a defect would be expected to alter responses through the indirect pathway, because that pathway is analogous to autoantigen presentation. However, whether the resistance to tolerance observed in NOD mice is definitely related to the findings of this study regarding a powerful indirect response requires further investigation.

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References