Differential Roles of Mac-1+ Cells, and CD4+ and CD8+ T Lymphocytes in Primary Nonfunction and Classic Rejection of Islet Allografts

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Summary

The high rate of persistent hyperglycemia, termed primary nonfunction, after islet allotransplantation in C57BL/6 mice recipients of B10.BR strain islets, as compared with B10.BR recipients of C57BL/6 islets, led to a series of experiments to determine whether islet allograft primary nonfunction was attributable to technical aspects of the transplant procedure or whether it was a consequence of alloimmunity. Primary nonfunction was prevented by systemic pharmacologic immunosuppression of the host with cyclosporine. Selective immunodepletion of host CD4+ and CD8+ T lymphocytes significantly extended the time of classic rejection but did not significantly affect the rate of primary nonfunction. However, modulation of macrophages by administration to the host of silica completely abolished primary nonfunction. These observations, in conjunction with the immunohistological findings of intense macrophage infiltration in islet allografts from recipients exhibiting persistent post-transplant hyperglycemia, support the hypothesis that primary nonfunction results from a cell-mediated host-immune response of rapid onset that is dependent on macrophages or macrophage byproducts as the main effectors.

Immediate and permanent failure of an organ or tissue allograft in which there is never any discernible function (termed primary nonfunction) is usually ascribed to nonimmunologic events. Vascularized organ grafts may fail to function because of technical factors such as prolonged preservaton (1) or arterial or venous thrombosis (2, 3). In free grafts of cells or tissues, such as adult islets of Langerhans, primary nonfunction has been thought to reflect tissue injury during procurement (4) or implantation of an inadequate functional mass of tissue (5, 6).

Allograft primary nonfunction may also be caused by immunologic phenomena. In immediately vascularized solid organs such as kidneys, the predominant immunologically defined cause of primary nonfunction is hyperacute rejection. This phenomenon occurs in recipients with preformed antidonor antibodies directed against MHC or blood group antigens (7, 8). In free tissue grafts requiring neovascularization, such as bone marrow, failure of engraftment (termed allogeneic resistance) appears to be mediated, at least in part, by NK cells (9-12). Except for bone marrow, nonhumoral, cell-mediated immediate destruction or inhibition of primary graft function has not been described.

In clinical and experimental studies of pancreatic islet transplantation, the distinction between immunologic and nonimmunologic causes of allograft primary nonfunction has not been made. In the few clinical trials of adult human islet allotransplantation (13-17), graft function, demonstrated by at least transient achievement of a euglycemic, insulin-independent state, has occurred only rarely, and the cause of islet allograft failure has not been elucidated. Although islet allograft primary nonfunction may be the consequence of technical problems of the transplant procedure itself, such as having an inadequate mass of functional islets, the yields reported from human pancreas islet isolations are theoretically sufficient, and islet autografts after total pancreatectomy for treatment of benign disease have prevented the occurrence of diabetes (18). This observation, based upon a limited number of cases, suggests that an immune mechanism that affects β cells might be responsible for the immediate failure of human islet allografts.

Primary nonfunction also occurs in rodent models of islet allotransplantation. In previous publications, we and others have considered such an outcome to be due to technical factors (19-21), and have excluded recipients that never exhibited graft function from data analysis. A review of our experience with adult islet transplantation in inbred mice, however, showed that primary nonfunction was confined to allograft recipients, and was dependent on the donor-recipient combination used,
varying from an incidence of <10 to >40%. In light of these observations, we sought to determine whether islet allograft primary nonfunction was a consequence of early allosecrepe events, and to determine the roles of Mac-1+ cells and CD4+ and CD8+ T lymphocytes in these events, as well as their role in the ultimate destruction of allogeneic islets.

Materials and Methods

Mice and Induction of Diabetes. Adult male C57BL/6J (H-2b) and B10.BR.SgSnJ (H-2b) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used as both donors and recipients. The strains of mice were genetically disparate over the H-2, Qa, and Tla regions, but otherwise had similar genetic backgrounds. There were no significant differences between the B10.BR and C57BL/6 strains in the normal nonfasting blood glucose values (overall mean value 143 ± 5.2 mg/dl, 16 determinants). Recipient mice were made diabetic by a single intraperitoneal injection of streptozotocin, 220 mg/kg body weight (a gift from Upjohn Laboratories, Kalamazoo, MI). All recipient mice were hyperglycemic for at least 1 wk before islet transplantation, and only those with nonfasting blood glucose levels >350 mg/dl were used. There were no strain differences in the degree of hyperglycemia attained by intraperitoneal streptozotocin.

Islet Isolation and Transplantation. Donor mice were fasted overnight preceding islet isolation. For each transplant, islets were isolated from 8-12 donor pancreases by the sequential collagenase (Type I; Sigma Chemical Co., St. Louis, MO) digestion technique, and were handpicked free of contaminating lymphoid and acinar tissue as previously described (19, 21). This method yielded a range of 75-100 islets per donor pancreas. During ether-induced anesthesia of the diabetic recipient mouse, the kidney was exposed via a subcostal incision, and donor islets were transplanted beneath the kidney capsule using a 23-gauge butterfly needle.

Cyclosporine. Some islet allograft recipients were administered cyclosporine (20 mg/ml dissolved in olive oil) subcutaneously beginning at the time of transplant and daily thereafter for 100 d. Control groups of nontransplanted diabetic mice (n = 5) and recipients of isograft islets (n = 5) were given 20 mg/kg of cyclosporine daily for >100 d. Nonfasting blood glucose levels were determined in experimental and control animals daily for the first 30 d post-transplant and then three times per week.

Immunodepletion of T Cell Subsets. Hybridomas secreting anti-CD4 mAb (22) (GR 1.5, rat anti-mouse IgG2b), and anti-CD8 mAb (23) (2.43; rat anti-mouse IgG2b) were obtained from the American Type Culture Collection (Rockville, MD). The expanded hybridomas were implanted in outbred nu/nu C57BL/6 strain mice. The antibodies were precipitated from ascites fluid with 50% ammonium sulfate and dialyzed against PBS. Some diabetic C57BL/6 mice received 750 μg of anti-CD4 or anti-CD8 mAb intraperitoneally (∼30 μl) on days –1, 0, 1, 3, and 5, relative to the time of islet transplantation.

In vivo efficacy of the mAbs to specifically immunodeplete the T lymphocyte cell subset of interest was assessed in nontransplanted control mice by FACS (FACS IV; Becton Dickinson & Co., Mountain View, CA) analysis of peritoneal and splenic lymphocytes. After administration of antibody to nontransplanted control animals, the CD4+ or CD8+ T cell subsets in the spleen and intraperitoneal compartments were decreased to 15–20% of the normal mean proportions, and remained decreased over a 28-d time course.

Depletion of Macrophages. Silica administered intraperitoneally (1 mg/d for five consecutive days) in a series of control C57BL/6 mice resulted in progressive diminution in the number of Mac-1+, granular, large-cell peritoneal macrophages (as determined by FACS analysis), over the course of 3–4 wk, results consistent with previous reports (24). For subsequent C57BL/6 recipients of B10.BR islets, diabetes was induced 3 wk after silica administration, and islet transplantation was performed thereafter.

Immunohistology. Experiments were designed to correlate the functional outcome of transplanted islets with the immunohistological features of infiltrating cell types. C57BL/6 mice (n = 9) had B10.BR islets transplanted under the renal capsule of both the left (300 islets) and right (500 islets) kidneys. At 5 d post-transplant, a left nephrectomy was performed, and the islet-bearing portion of the kidney was processed for histology as described below. Blood glucose levels were determined daily post-transplant, and post-left nephrectomy, to assess the functional status of the 500 allogeneic islets (a quantity known to be sufficient to induce normoglycemia from the isograft experiments) under the right kidney. This permitted accurate correlation of histological features with graft function at the time of the biopsy, post-transplant day 5, and with the ultimate functional outcome of the transplant as manifested by the islets transplanted in the remaining kidney. Immunocytochemical analysis of graft-infiltrating cells, and Ig and complement deposition, in grafts from recipients with classic rejection and primary nonfunction were compared. The features of islet isograft and allograft biopsies taken at day 5 post-transplant in recipients that were normoglycemic established the baseline level of inflammation of lymphoid cells and macrophages.

Kidney tissue specimens that contained transplanted islets were snap frozen in precooled isopentane and stored at −70°C until sectioned. Frozen tissue sections (4 μm) were cut on a Lipshaw cryostat, air dried, and stored in a constant temperature (25°C) and humidity (60%) facility. Sections used for light microscopic histologic analysis were fixed in buffered 10% formalin and stained with periodic acid-Schiff reagents.

Methods of immunocytochemistry have previously been described (25). Briefly, sections were acetone fixed and washed with PBS, pH 7.4. Specimens evaluated for insulin-containing tissue were reacted with guinea pig anti-human insulin (Ventrex, Inc.) and a second layer of goat anti-mouse IgG (Organon Teknika). Phenytoin analysis of graft-infiltrating cells was analyzed using a second FITC-conjugated reagent, affinity-isolated, mouse IgG (Organon-Teknika). Complement deposition was studied using affinity-isolated goat anti–mouse C3 (Organon-Teknika) stained with affinity-isolated FITC rabbit anti–goat IgG (Organon-Teknika). Phenotypic analysis of graft-infiltrating cells was analyzed using rat mAbs against murine Thy-1.2, CD4, CD8, and Mac-1 from cell lines obtained from American Type Culture Collection. These were overlaid with affinity-purified, mouse serum–adsorbed, FITC-F(ab')2; goat anti–rat IgG (Pel-Freeze Biologicals, Rogers, AR) and washed with PBS. Amplification was achieved by the use of a second FITC-conjugated reagent, affinity-isolated, mouse serum–adsorbed, F(ab')2; rabbit anti–goat IgG (Organon-Teknika). After a final PBS wash, ethidium bromide was applied to stain nuclei, and p-phenylenediamine to retard fluorescence fading (26). Control sections of each tissue were prepared as above, omitting primary antibody, and using appropriate second and third layers of FITC-labeled antibodies and ethidium bromide and p-phenylenediamine.

Functional Outcome and Analysis of Data. Long-term acceptance of an islet graft was defined as induction and persistence of normoglycemia (nonfasting blood glucose <200 mg/dl) for >100 d post-transplant in the recipient, classic rejection as reversion to glu-
cose >250 mg/dl after a period of transient normoglycemia, and primary nonfunction as glucose persistently >200 mg/dl. In mice with long-term acceptance, confirmation that the grafts were responsible for normoglycemia was accomplished by demonstrating hyperglycemia after nephrectomy of the islet-bearing kidney. Mean values are given with SDs and numbers of animals in groups, and student's t test was used to determine the significance of the differences in mean values. The Wilcoxon test was used to determine the significance of differences in overall allograft functional survival times between groups. The χ² test was used to determine the significance of differences in proportions of the various outcomes between groups.

Results

Number of Islets Required and Time Course for Reversal of Streptozotocin-induced Diabetes. We determined the minimal number of islets required to reverse streptozotocin-induced diabetes, and the dose-response relationship between the number of islets implanted and the time post-transplant for amelioration of the diabetic state (glucose <200 mg/dl) (Fig. 1, A and B). Beyond a threshold of 300 islets, the number of islets transplanted did not influence the ultimate outcome in isograft recipients, and failure to reverse the diabetic state due to technical problems did not occur with implantation of >300 islets. The time between transplantation and graft function producing normoglycemia varied inversely with the number of islets transplanted up to 600 islets. Transplantation of 600–900 (n = 16) islets brought about normoglycemia in all syngeneic recipients between 1 and 5 d (mean 2.4 ± 1.7 d), and all became permanently normoglycemic. Therefore, in the experiments described below, each recipient was transplanted with between 600 and 900 islets.

Functional Outcomes of Islet Allotransplantation. The functional outcomes of allogeneic islet transplants in both directions between the B10.BR and C57BL/6 strains were classified into three groups: primary nonfunction, classic rejection, and spontaneous long-term functional acceptance. Primary nonfunction was defined as glucose persistently >200 mg/dl, classic rejection as reversion to glucose >250 mg/dl after a period of transient normoglycemia (nonfasting blood glucose <200 mg/dl for at least 2 d), and spontaneous long-term acceptance as permanent (>100 d) normoglycemia. The frequency distributions of the various outcomes (Table 1) was compared between a series of 26 islet transplants from C57BL/6 donors to B10.BR recipients (mean 669 ± 57 islets/recipient), and 39 transplants from B10.BR donors to C57BL/6 recipients (mean 722 ± 94 islets/recipient). The mean number of islets per recipient was similar in both directions (p > 0.41).

In the C57BL/6 (donor) to B10.BR (recipient) combination, primary nonfunction occurred in two (8%), classic rejection in six (23%) at a mean time of 23.8 ± 7.8 d post-transplant, and spontaneous long-term graft acceptance in 18 (69%). The number of islets transplanted were similar in the three recipient subgroups exhibiting the various outcomes: 700 ± 0 (n = 2), 667 ± 75 (n = 6), and 667 ± 54 (n = 18), respectively (p > 0.35 for all comparisons). The mean functional survival time for C57BL/6 islets in all B10.BR recipients was 74.7 ± 39.3 d (primary nonfunction counted as 0 d of function, and long-term acceptance counted as 100 d).

For islet transplants from B10.BR donors to C57BL/6 recipients, the mean functional survival time was 28.3 ± 40.8
Recipent mice were made diabetic by a single intraperitoneal injection of streptozotocin, and donor islets were transplanted beneath the left kidney capsule. Blood glucose levels were determined twice weekly (see Materials and Methods). Long-term survival was defined as permanent (>100 d) normoglycemia (fasting blood glucose <200 mg/dl for at least 2 d), rejection as reversion to glucose >250 mg/dl after a period of transient normoglycemia, and primary nonfunction as glucose persistently >200 mg/dl for mean survival time, primary nonfunction was counted as 0 d of function, long-term acceptance was counted as 100 d, and for those undergoing classic rejection, the time to rejection was counted as the survival time.

\(^*\) p < 0.001, student's t test.

In addition, logistic regression analysis of the combined influences of the number of islets transplanted and the recipient pre-transplant glucose level showed no statistically significant effect on the incidence of primary nonfunction.

### Effect of Cyclosporine on Islet Allograft Primary Nonfunction and Long-term Acceptance

Cyclosporine was administered to recipient mice to probe for a possible role of the alloimmune response as a cause of primary nonfunction. A dose-response study was conducted whereby C57BL/6 recipients of B10.BR islets were given cyclosporine at doses of 5–10 mg/kg/d (low dose, \(n = 16\)), or 20–60 mg/kg/d (high dose, \(n = 14\)), for 100 d.

A nonimmunosuppressed control group of 30 C57BL/6 recipients of B10.BR islets demonstrated a mean islet allograft functional survival time of 31.6 ± 42.5 d, similar to that observed for the 39 C57BL/6 recipients of B10.BR islets in the previous series (mean functional survival time 28.3 ± 40.8 d; \(p = 0.581\)). In the controls, the proportions of recipients with each of the three functional outcomes were nearly identical to the proportions observed in the initial islet allograft experiments using the B10.BR donor to C57BL/6 recipient combination. In the controls of the present experiment, primary nonfunction occurred in 11 (37%), classic rejection in 11 (37%) at a mean of 13.5 ± 4.9 d post-transplant, and spontaneous long-term acceptance in eight (26%). Again, the mean of the number of islets was similar in the three outcome groups, 673 ± 70 (\(n = 11\)), 698 ± 72 (\(n = 11\)), and 679 ± 42 (\(n = 8\)) islets per recipient, respectively (\(p > 0.65\)).

Islet allografts in recipients given high-dose cyclosporine (\(n = 14\)) had a significantly longer (\(p < 0.03\)) overall duration of function (mean survival 57.1 ± 45.0 d) than the nonimmunosuppressed control recipients (mean survival 31.6 ± 42.5 d).
Long-term acceptance

Classical rejection

Primary nonfunction

Outcome

| No. of islets transplanted* | 600–700 | 701–900 |
|---------------------------|---------|---------|
| (n = 23)                  | (n = 16) |
| % Primary nonfunction     | 52 (12) | 37 (6)  |
| % Classical rejection     | 26 (6)  | 37 (6)  |
| % Long-term acceptance    | 22 (5)  | 26 (4)  |

Pre-transplant blood glucose value†

| mg/dl | >451 mg/dl |
|-------|------------|
| (n = 20) | (n = 19) |
| % Primary nonfunction | 45 (9) | 47 (9) |
| % Classical rejection | 35 (7) | 26 (5) |
| % Long-term acceptance | 20 (4) | 26 (5) |

* Overall distribution: χ², 0.88; p = 0.64. Incidence of primary nonfunction: p = 0.54 (student’s t test).
† Overall distribution: χ², 0.42; p = 0.81. Incidence of primary nonfunction: p = 0.89 (student’s t test).

The overall duration of function was also significantly longer (p < 0.002) in the high-dose than in the low-dose cyclosporine group (mean survival 11.7 ± 24.6 d), while the survival time of the low-dose and the control group did not differ significantly (p > 0.09).

Primary nonfunction occurred in only one (7%) of the 14 recipients given high-dose cyclosporine, an incidence significantly lower (p = 0.041) than the 37% incidence in the control group, as well as that of the low-dose cyclosporine group (Table 3). In the latter group, 8 (50%) of 16 recipients exhibited primary nonfunction, not significantly different from the incidence in the control group. Classic rejection occurred in six (43%) of the recipients given high-dose cyclosporine, at a mean of 16.5 ± 6.9 d after transplantation, similar to the incidences of classic rejection and mean times of rejection in the control group (incidence 37%, at a mean of 13.5 ± 4.9 d post-transplant), and in the low-dose cyclosporine group (incidence 44%, at a mean of 12.4 ± 5.4 d post-transplant). Spontaneous long-term acceptance occurred in seven (50%) of the recipients given high-dose cyclosporine vs. one (6%) of the low-dose cyclosporine group (p < 0.05), and vs. eight (26%) in the untreated controls (p = 0.134).

Cyclosporine did not influence the course of diabetes in nontransplanted animals, or functional survival of islets in isograft recipients. Diabetic mice administered 20 mg/kg/d cyclosporine and not transplanted remained hyperglycemic.

Histological examination on day 5 of islet grafts from recipients with primary nonfunction showed a moderate inflammatory infiltrate.

Biopsy samples taken within 3 d of reversion to hyperglycemia in C57BL/6 recipients of B10.BR islet allografts with classic rejection demonstrated intense infiltration of lymphoid cells that disrupted the architecture of the islets, leaving only small remnants of insulin-containing tissue (Fig. 2, A and B). The infiltrate consisted of Thy-1+ and IL-2-R+ cells and a relative paucity of Mac-1+ cells (Fig. 2, C and D). The concentration of CD4+ and CD8+ cells infiltrating grafts taken from recipients exhibiting classic rejection was nearly double that observed in grafts from recipients with primary nonfunction (Table 4). Histological examination on day 5 of islet grafts from recipients with primary nonfunction showed a moderate inflammatory cell infiltrate surrounding large clusters of insulin-positive cells within what appeared to be viable islets. The infiltrate was composed of approximately equal proportions of Thy-1+ lymphoid cells and large Mac-1+ mononuclear cells (Fig. 3, A–D). The infiltrate in recipients exhibiting primary nonfunction was more intense than that observed in recipients of isografts or in recipients of allografts that were normoglycemic on day 5 post-transplant. The concentration of Mac-1+ cells in the infiltrate of grafts with primary nonfunction on day 5 was also higher than that observed in the grafts removed from recipients undergoing classic rejection.

Ig and complement were not observed in islet allografts from recipients exhibiting either primary nonfunction or rejection. Additionally, when sera from C57BL/6 recipients with primary nonfunction (n = 3) were layered on freshly isolated B10.BR islets, the presence of antisera antibody deposition could not be detected.

These findings suggested that islet allograft primary nonfunction is associated with a prominent infiltration of Mac-1+ cells, whereas in classic rejection, Thy-1+, CD4+, CD8+, and IL-2-R+ T cells dominated.
Table 3. *Outcome of B10.BR Islet Allografts Transplanted to Normal and to Immunosuppressed C57BL/6 Mice*

| Immunosuppression | Duration of function | Islet survival | No. with primary nonfunction | No. rejecting (n) | No. surviving long term |
|--------------------|----------------------|---------------|-------------------------------|------------------|------------------------|
|                    | mean d ± SD          | d             |                               |                  |                        |
| None               | 30                   | 31.6 ± 42.5   |                               | 11 (37%)         | 8 (26%)                |
|                    |                      |               |                               | (13.5 ± 4.9)     |                        |
| CsA (5–10 mg/kg)   | 16                   | 11.7 ± 24.6   | 8 (50%)                      | 7 (44%)          | 1 (6%)                 |
|                    |                      |               |                               | (12.4 ± 5.4)     |                        |
| CsA (20–60 mg/kg)  | 14                   | 57 ± 45       | 1 (7%)                       | 6 (43%)          | 7 (50%)                |
|                    |                      |               |                               | (16.5 ± 6.9)     |                        |
| Silica†            | 10                   | 36.1 ± 34.4   | 0 (0%)                       | 8 (80%)          | 2 (20%)                |
|                    |                      |               |                               | (20.1 ± 7.8)     |                        |
| Anti-CD4           | 12                   | 22.5 ± 25.8   | 2 (17%)                      | 9 (75%)          | 1 (8%)                 |
|                    |                      |               |                               | (18.4 ± 4.7)     |                        |
| Anti-CD8           | 9                    | 61.2 ± 39.4   | 2 (22%)                      | 4 (45%)          | 3 (33%)                |
|                    |                      |               |                               | (62.8 ± 11.5)    |                        |

The p values were as follows: a, 0.05; b, 0.02; c, 0.003; d, 0.002; e, 0.041; f, 0.024; g, 0.05; h, 0.024; i, 0.017; j, 0.03; k, 0.03; l, 0.03; m, 0.001; n, 0.134; o, 0.05 (student's t tests).

* C57BL/6 allograft recipients were treated with cyclosporine (20 mg/ml dissolved in olive oil) administered subcutaneously (20-60 mg/kg/d) or 5–10 mg/kg/d) beginning at the time of transplant and daily thereafter for 100 d.
† C57BL/6 allograft recipients were treated with silica (10 mg/ml dissolved in saline solution) administered intraperitoneally (1 mg/d for 5 d) beginning 4 wk before transplantation. Induction of diabetes and subsequent islet transplantation were conducted thereafter.

Effect of Macrophage Depletion on Islet Allograft Primary Nonfunction and Classic Rejection. Immunofluorescence analysis suggested an association between the presence of macrophages and allograft primary nonfunction. To address the functional role of macrophages in primary nonfunction, C57BL/6 recipients of B10.BR islet allografts were administered silica to deplete that cell subset. Depletion of host peritoneal macrophages several weeks after administration of silica was assessed by FACS analysis of peritoneal exudate cells from nontransplanted controls (Fig. 4). The peritoneal exudate cell population consisted of two major populations of cells distinguished by size: the smaller Thy-1+ cells and a granular, large-cell population that consisted mostly of Mac-1+ cells. Administration of silica depleted the large Mac-1+ cells and changed the Mac-1/Thy-1 ratio from ~1:1 to 1:8. Furthermore, the function of splenic C57BL/6 T cells as assessed by generation of allocytotoxicity in a cell-mediated lympholysis assay (51Cr release by labeled B10.BR blasts) was similar in treated and untreated mice.

Although the overall duration of graft function was not significantly different between the recipients treated with silica (mean 36.1 ± 34.4 d) and the untreated C57BL/6 control recipients of B10.BR islets, the functional outcomes occurred in significantly different proportions (p = 0.03). Primary nonfunction was completely abolished in recipients given silica, an incidence of 0% (0:10) vs. 37% in controls (p = 0.024) (Table 3). Classic rejection, however, still occurred in silica-treated mice; the incidence was 80% (8:10) at a mean of 20.1 ± 7.8 d post-transplant, compared with the incidence of 37% (p = 0.017) at a mean of 13.5 ± 4.9 d (p = 0.034) in controls. Long-term acceptance occurred with an incidence of 20% (2:10) in silica-treated recipients, compared with the incidence of 26% (p = 0.843) in controls. Thus, silica prevented primary nonfunction, but the changes in proportions suggested that grafts destined to fail from primary nonfunction were shifted into the classic rejection category. The total failure rate (primary nonfunction plus classic rejection) was 74% in the controls, similar to the classic rejection rate of 80% in the silica-treated mice.

Immunodepletion of CD4 and CD8 T Lymphocytes with mAbs. To address the role of CD4 and CD8 T lymphocytes in allogeneic injury of islets, C57BL/6 recipients of B10.BR islet allografts were administered either anti-CD4 mAb (GK 1.5, rat anti-mouse IgG2b) or anti-CD8 mAb (2.43, rat anti-mouse IgG2b). Recipient mice were given the mAb in vivo (750 µg i.p., on days −1, 0, 1, 3, and 5 relative to time of islet transplantation).

In CD4 T cell–depleted islet allograft recipients (n = 12), the overall duration of graft function (mean 22.2 ± 25.8 d) was similar to that of the normal C57BL/6 controls. The
CD4 T cell–depleted islet allograft recipients had an incidence of primary nonfunction of 17% (2:12), a lower incidence than that of 37% in controls, but not statistically significantly different (p = 0.215) (Table 3). Classic rejection occurred in 9 (75%) of 12 of the CD4 T cell–depleted recipients at a mean of 18.4 ± 4.7 d, significantly later (p = 0.033) than in controls (Table 3). Spontaneous long-term acceptance occurred in 1 (8%) of 12 of the CD4 immunodepleted recipients vs. 26% in nonimmunosuppressed controls (p = 0.024). Thus, immunodepletion with anti-CD4 did not significantly alter the rate of primary nonfunction; however, among those mice that classically rejected the islets, the mean time to rejection was significantly extended.

In CD8 T cell–depleted islet allograft recipients (n = 9), the overall duration of graft function was significantly (p < 0.02) longer (mean 61.2 ± 39.4 d) than that of CD4 T cell–depleted islet allograft recipients (mean 22.2 ± 25.8 d), as well as significantly (p = 0.05) longer than that of the normal C57BL/6 controls (Table 3). However, primary nonfunction occurred in two (22%) recipients depleted of CD8+ T lymphocytes, an incidence lower than the 37% primary nonfunction rate in control animals, but not statistically significant (p = 0.433), and similar to the 17% primary nonfunction rate in CD4 T cell–depleted recipients (p = 0.215) (Table 3). Thus, immunodepletion with anti-CD8 did not significantly alter the rate of primary nonfunction.

In CD8 T cell–depleted mice, classic rejection occurred in four (45%) of nine, and at a median of 57.5 d (mean of 62.8 ± 11.5 d) post-transplant. The incidence of classic rejection in the CD8 T cell–depleted recipients was not significantly

Figure 2. (A) Period acid-Schiff-stained nephrectomy specimen from recurrent hyperglycemic C57BL/6 strain recipient of rejected B10.BR strain allogeneic islets, day 15 post-transplant; (B) insulin-containing cells reactive with guinea pig anti-human insulin and a second layer of goat anti-guinea pig FITC-conjugated IgG (H and L chains); (C) Thy-1.2+ graft-infiltrating cells of the rejected islet allograft; and (D) Mac-1+ graft-infiltrating cells of the rejected islet allograft. All tissues are from adjacent sections (×300).
The methods used for enumeration of mononuclear cell populations by indirect immunofluorescence are similar to those described in detail for kidney (25). The method described herein was developed specifically for analysis of inflammation in islet allograft tissue. Cells were considered “positive” that have an apple-green fluorescent plasma membrane surrounding a red-orange nucleus. The relative number of positive cells are expressed as: 

volumedensityforeachtissue-

mean numberofpositive cells/numberof grid points in microscopic fields viewed (×500) with a 10 × 10-mm indexing grid.

different from the incidence of 37% (p = 0.684) in controls, and of 75% (9:12) in CD4 T cell-depleted recipients. However, the mean time to rejection among the recipients that underwent classic rejection was statistically significantly prolonged in CD8 T cell–depleted animals (mean of 62.8 ± 11.5 d) vs. CD4-immunodepleted animals (mean of 13.5 ± 4.9 d [p < 0.001]), and in the untreated controls (mean 18.4 ± 4.7 d [p < 0.03]). In fact, allogeneic islets transplanted to CD8 T cell–depleted recipients were not rejected before day 56 post-transplant (Fig. 5). Thus, among those mice that classically rejected the islets, the mean time to rejection was significantly extended compared with controls, as well as with CD4 T cell–depleted recipients.

Although treatment with anti-CD8 significantly prolonged islet allograft survival in recipients that ultimately rejected the islets, it did not significantly increase the rate of long-term (>100 d) acceptance as compared with untreated recipients. Long-term acceptance occurred in three (33%) of nine of the CD8 T cell–depleted recipients, compared with a long-term acceptance rate of 26% (p = 0.324) in controls, and 8% (p < 0.001) in CD4 T cell–depleted recipients. It is interesting to consider whether or not some islet allografts might be destined to survive permanently in any circumstance. A previous report showed that in untreated islet allograft recipients, the variability of allograft outcome, including long-term acceptance, appeared to be determined to greater extent by host factors (19), rather than immunogenic heterogeneity of islet preparations due to fortuitous inclusion or exclusion of nonislet nodal or ductal tissue (27).

Discussion

Persistent hyperglycemia after transplantation of allogeneic islets in rodent models has been attributed to technical problems with the transplant procedure, such as implantation of an insufficient islet mass or other nonimmunological causes (19, 28, 29). If technical problems were the sole explanation of primary nonfunction, then the incidence should be the same for isologous islet transplants carried out within the same donor and recipient strains. Our experiments showed that primary nonfunction of islet allografts placed under the renal capsule in C57BL/6 and B10.BR mice cannot be explained by technical factors. First, in 28 consecutive transplants of 300–900 syngeneic islets to diabetic recipients of both strains, not a single instance of primary nonfunction was observed. Primary nonfunction occurred only in situations where an alloimmune response was possible. Second, histological examination at day 5 of allogeneic islets with primary nonfunction showed viable islets containing insulin-positive cells; engraftment occurred, but the islets simply did not function to the extent that hyperglycemia could be ameliorated. A further argument against primary nonfunction being due to technical factors, such as variability in day-to-day quality of islet preparations, comes from our previous observations that when groups of two to four recipients were transplanted with clean, hand-picked islets derived from large batch isolations, the incidence of primary nonfunction within groups did not differ from that observed in experiments in which allogeneic islets were prepared individually for each mouse (19).

Rather, our experiments suggested that the phenomenon of islet primary nonfunction was a manifestation of the host alloimmune response. First, in B10.BR recipients of C57BL/6 islet allografts, there was a high incidence (69%) of spontaneous long-term acceptance and a low incidence (8%) of primary nonfunction, whereas, in C57BL/6 recipients of B10.BR islets, the incidence of spontaneous long-term acceptance was significantly lower (23–26%; Tables 1 and 2) and the incidence of primary nonfunction was significantly higher (37–46%, Tables 1 and 2). The contrast in the incidences is much more likely to be due to a difference in the nature of the immune responses of the two strains, or differences in the inherent immunogenicity of islets derived from the two strains, than to technical problems that occur in one direction but not in the other. Second, the high incidence of islet allograft primary nonfunction was not a solitary finding. A high rate of primary nonfunction in the B10.BR to C57BL/6 combination occurred in the two control groups as well as the group.

### Table 4. Immunocytochemical Analysis of Infiltrating Cell Types in Islet Grafts

| Graft combination outcome | Glycemia (post-transplant time of biopsy) | n | Mean no. of positive cells |
|---------------------------|------------------------------------------|---|---------------------------|
|                           |                                          |   | Thy-1+ | CD4+ | CD8+ | IL-2-R+ | Mac-1+ | Ig+ |
| Isograft                  | Normoglycemia (day 5)                    | 3 | 50     | 22   | 17   | 5       | 62     | -   |
| Allograft                 | Normoglycemia (day 5)                    | 3 | 49     | 32   | 18   | 7       | 49     | -   |
| Allograft primary nonfunction | Hyperglycemia (day 5)                  | 3 | 67     | 33   | 27   | 6       | 81     | -   |
| Allograft classical rejection | Hyperglycemia (day 15-23)             | 3 | 97     | 62   | 51   | 21      | 24     | -   |
| Allograft long-term acceptance | Normoglycemia (day 100)                 | 3 | 12     | 9    | 5    | 1       | 9      | -   |
receiving low-dose cyclosporine (5–10 mg/kg/d), demonstrating the reproducibility of the phenomenon. Third, immunosuppression of the C57BL/6 recipients with high-dose cyclosporine (20–60 mg/kg/d) reduced the incidence of primary nonfunction from 37 to 7% (p < 0.05), while also increasing the long-term functional graft survival rate from 26 to 50%. Anti-CD4 and anti-CD8 treatment reduced the rate of primary nonfunction, though not to the degree that cyclosporine did. Finally, the use of the antimacrophage agent, silica, completely abolished primary nonfunction, suggesting a dominant role for macrophages or activated macrophage byproducts, that either directly injured the allogeneic islets, or inhibited function until ultimate destruction was mediated by the CD4+ and CD8+ T cells that are operative in classic rejection.

The use of an antimacrophage treatment in islet allograft recipients was preceded by the histologic and immunopathologic findings suggesting that primary nonfunction was a cell-mediated phenomenon dominated by macrophages. Immunofluorescence analysis of grafts with primary nonfunction showed the concentration of T lymphocytes in the cellular infiltrate to be lower, and of macrophages to be higher, relative to the proportions of T lymphocytes and macrophages infiltrating grafts from recipients that underwent classic rejection. The term “nonfunction” appeared apt since the islets contained insulin despite hyperglycemia. The presence of mac-

Figure 3. (A) Period acid-Schiff-stained nephrectomy specimen from persistently hyperglycemic C57BL/6 strain recipient exhibiting primary nonfunction of B10.BR strain allogeneic islets, day 5 post-transplant; (B) insulin-containing cells reactive with guinea pig anti-human insulin and a second layer of goat anti-guinea pig FITC-conjugated IgG (H and L chains) in an islet allograft specimen from a recipient exhibiting primary nonfunction; (C) Thy-1.2+ graft-infiltrating cells of an islet allograft with primary nonfunction; and (D) Mac-1+ graft-infiltrating cells of an islet allograft with primary nonfunction. All tissues are from adjacent sections (×300).
operative in classic rejection.

In other cases, delayed onset, CD4+ and CD8+ T cells, that are ultimately destroyed by those mediators of rejection may injure the islets, or inhibit function until the islets cannot function any longer. The cascade of events culminating in rejection may also differ in individual animals. Anti-CD8 antibody treatment markedly extended survival of some grafts before onset of classic rejection occurred. Indeed, the earliest time of classic rejection in animals treated with anti-CD8 antibody was day 56 post-transplant. This interval should give sufficient time for at least temporary engraftment of all islet allografts, or recovery from the insulin-inhibiting function. However, even in anti-CD8-treated recipients, primary nonfunction occurred with an incidence of 22%, suggesting that the mechanism resulting in primary nonfunction can be sustained for a long time, or that early destruction and not merely inhibition of function is responsible for some cases of primary nonfunction.

Overall, the statistical analysis and functional/histological correlations demonstrate that islet allograft primary nonfunction is a consequence of host alloimmunity, rather than being technical in nature. This phenomenon may be a basic biological feature of murine allotransplantation of islets to the renal subcapsular space, and perhaps of islet allotransplantation in other species. The experimental findings have clinical implications as well. To date, there has been only one documented

Similar observations of the association of macrophage infiltration of islets in animal models of spontaneous autoimmune diabetes have been reported (30, 31). If macrophage infiltration causes islet graft dysfunction in autoimmune diabetes, then our observations would provide correlative evidence of macrophage-associated injury in the allograft situation. Injury of allogeneic islets might be caused by secretion of monokines, such as IL-1 and TNF, which perturb insulin biosynthesis in and secretion from rodent islets in vitro (32-35). Macrophages have also been reported to cause spontaneous cytotoxicity of islets in vitro (36, 37). Moreover, IL-1 secretion from macrophages has been shown in some models (38-40) to be inhibited by cyclosporine, an observation that would appear to fit with our own in the mouse islet allograft model. However, the complex effects of cyclosporine on macrophage function are incompletely known, and others have shown IL-1 production by macrophages to be unaffected by cyclosporine (41).

Figure 4. FACS analysis of peritoneal exudate cells in normal C57BL/6 mice (A) and mice administered intraperitoneal silica 3-4 wk previously (B). Cells were analyzed according to size (forward scatter) and granularity (side scatter) on a FACS IV (Becton Dickinson & Co.) equipped with an argon laser, and the data were analyzed on a Digital PDP 11/73 computer with specialized software jointly developed by Becton Dickinson & Co. and the NIH. Encircled areas demonstrate RBC (a), T lymphocyte (b), and macrophage (c) populations. The relative proportion of each cell population in a representative normal control animal was: a, 2.9%; b, 52.6%; and c, 44.5%; in a silica treated animal: a, 6.2%; b, 82.4%; and c, 11.4%.

Figure 5. Percent survival of islet allografts among the subsets of recipients exhibiting classic rejection. Recipient groups include non-treated controls, and recipients immunodepleted of CD4 and CD8 T lymphocytes, or treated with silica to modulate macrophage function.

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Reasons offered for the failures have included transplantation to allow sustained withdrawal of exogenous insulin therapy. A report of a human islet allograft attaining sufficient function of an inadequate mass of viable islets (5, 6), or islet injury by exocrine enzymes of contaminating pancreatic acinar cells (42). Although it may be more difficult to make a distinction between technical and immunologic causes of failure in the clinical situation, we believe it is likely that some of the adult human islet allografts have failed because of immune-mediated primary nonfunction as well as classic rejection.

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