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Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand

(transplantation/tolerance/islets of Langerhans/diabetes mellitus)

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ABSTRACT **Combined treatment with allogeneic small lymphocytes or T-depleted small lymphocytes plus a blocking antibody to CD40 ligand (CD40L) permitted indefinite pancreatic islet allograft survival in 37 of 40 recipients that differed from islet donors at major and minor histocompatibility loci. The effect of the allogeneic small lymphocytes was donor antigen-specific. Neither treatment alone was as effective as combined treatment, although anti-CD40L by itself allowed indefinite islet allograft survival in 40% of recipients. Our interpretation is that small lymphocytes expressing donor antigens in the absence of appropriate costimulatory signals are tolerogenic for alloreactive host cells. Anti-CD40L antibody may prevent host T cells from inducing costimulatory signals in donor lymphocytes or islet grafts.**

A long-term goal in allotransplantation is to develop methods of inducing antigen-specific tolerance to replace generalized immunosuppression. Recent advances in understanding how antigen-presenting cells (APCs) regulate T-cell activation now provide new strategies for inducing transplantation tolerance.

To proliferate and differentiate into destructive effector cells, T cells depend on multiple signals from APCs. APCs use nonclonal recognition systems to detect tissue damage or infectious organisms (1). These signals are termed costimulation (2). When an antigen receptor is ligated, responding T cells continuously monitor for these signals in the environment. Costimulatory signals include the interaction between CD28 on responding T cells and B7 family members on APCs. This interaction enhances autocrine interleukin 2 synthesis and secretion by the responding T cell (3). Costimulatory signals can also regulate effector functions elicited by antigen (4).

Only dendritic cells constitutively express high levels of costimulatory molecules (5, 6), and their expression can be further up-regulated (6, 7). On macrophages, B lymphocytes, endothelial cells, and keratinocytes, costimulatory molecules can be induced by infection, adjuvant, inflammatory cytokines, or activated T cells (3, 8–11).

T cells become anergic or die when they recognize antigen in the absence of costimulation *in vitro* (2, 12). T cells escaping clonal deletion in the thymus disappear or become anergic when they recognize self-antigens on healthy APCs *in vivo* (13, 14). This is thought to be a major mechanism of self-tolerance.

These observations suggest that tissue allografts should be tolerogenic if care is taken to avoid transplanting cells with constitutive costimulatory activity. The concept of costimulation was first proposed in the context of "passenger leukocytes" and allograft rejection (15), and its importance has been underscored by demonstrations that blockade of B7–CD28 interaction allows survival of cardiac allografts (16) and xenogeneic human islets (17) in mice. Blockade was achieved using CTLA4–Ig fusion protein, CTLA4 being a CD28–like receptor found on activated T lymphocytes (18).

Activated $CD4^+$ T cells express a membrane-bound protein, CD40 ligand (CD40L, gp39), which engages CD40 on resting B cells and accounts for most cell contact-dependent T-cell help for small B cells (19–21). CD40L induces expression of B7 and other costimulatory activities of B cells; it also enhances expression of costimulatory molecules on other APCs (9, 22).

Here we report the survival of allogeneic islet grafts in chemically diabetic mice pretreated with allogeneic small lymphocytes, an antibody to the CD40L to prevent T-cell–B-cell interactions, or both treatments in combination. When anti-CD40L was combined with transfusion of small lymphocytes of donor type, long-term islet graft survival was obtained in nearly all recipients.

MATERIALS AND METHODS

Mice. C57BL/6 (*H-2*^{b}), BALB/c (*H-2*^{d}), (BALB/c × C57BL/6)F_1, and (C57BL/6 × C3H)F_1 mice were obtained from the National Cancer Institute, Frederick, MD. C3H mice are *H-2*^{k}. Animals were housed in microisolators and provided with autoclaved food and water *ad libitum*.

Preparation of Lymphocytes and Assays for APC Function. Spleen cells from 8-wk-old female mice were depleted of erythrocytes and fractionated by centrifugal elutriation (23, 24). A small cell fraction (fraction 19) was collected at 19 ml/min at 3200 rpm in a JE-6B rotor or at 77 ml/min at 3200 rpm with a JE-5.0 rotor (Beckman). A second fraction (fraction 20) was collected at 20 ml/min. The fractions were composed of small lymphocytes with a symmetric size distribution (mean cell volume, 115–120 μm^3; Coulter Counter and Multisizer; Coulter). In some experiments, small lymphocyte fractions were depleted of T cells by treatment with anti-T-cell monoclonal antibodies and complement (25). Before T-cell depletion, fraction 19 was reduced in T cells (39% $CD3^+$ in unfractionated spleen; 14% $CD3^+$ in fraction 19; mean of three experiments). The T-depleted fraction 19 cells were 89% $B220^+$, 1.1% $CD3^+$, and 10% null, presumably natural killer, cells. To monitor radioresistant APC function, fractionated cells were exposed to 3000 rads (1 rad = 0.01 Gy) from a ^{137}Cs source and tested for ability to induce proliferation of the *H-2*^{b} alloreactive Th2 line, D10.G4 (American Type Culture Collection). Various numbers of spleen cells were added to 1×10^5 D10.G4 cells in 200 μl of culture medium, and [^{3}H]thymidine incorporation was measured 72 hr later (25).

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Abbreviations: APC, antigen-presenting cell; MHC, major histocompatibility complex.

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Islet Transplantation. Male C57BL/6 mice were rendered diabetic (plasma glucose, >22.2 mM on three occasions within 1 wk) with intraperitoneal streptozotocin (140 mg/kg). Streptozotocin diabetic mice were not ketotic and did not require exogenous insulin for survival. They lost weight after onset of hyperglycemia but uniformly gained weight after successful islet transplantation. At the end of the protocol, pancreatic morphology in all animals revealed shrunken islets that appeared devoid of their normal core of β cells. BALB/c islets were isolated by collagenase digestion (26) and implanted immediately after isolation (30 per g of body weight) beneath the renal capsule of C57BL/6 mice that had been diabetic for 14–25 days. Graft survival was defined as maintenance of plasma glucose <13.9 mM. Mice that died for unknown reasons but normoglycemic at the last measurement are shown in the figures as falling to 0 mM but not included in survival statistics.

Treatment Protocols. Five to 8 days before islet transplantation, diabetic mice were injected once via the tail vein with 40–88 $\times$ 10^6 allogeneic, elutriated small lymphocytes or erythrocyte-depleted unfractionated spleen cells. Beginning on the day of lymphocyte injection, certain mice received 250 μg of the MR1 hamster anti-CD40L monoclonal antibody (19) intraperitoneally twice weekly for 2–7 wk or until graft failure.

RESULTS

Effect of Pretreatment with Allogeneic Lymphocytes on Islet Allograft Survival. We first measured survival of BALB/c (*H-2*^{d}) islet grafts in diabetic C57BL/6 (*H-2*^{b}) mice, some of which were transfused with (BALB/c $\times$ C57BL/6)F_1 small splenic lymphocytes. F_1 lymphocytes were used to avoid graft vs. host reactions. Because the ability of donor lymphocytes to tolerize could depend on their APC activity, this function was determined for each injected cell population. Fraction 19 was devoid of radioresistant APC function as documented by its inability to simulate proliferation of an alloreactive Th2 cell line; fraction 20 was nearly indistinguishable in size but had low APC activity (data not shown). Because the APC activity of activated B cells and other splenic APC is relatively radioresistant, these data suggest that fraction 19 was depleted of cells with constitutive APC activity (27, 28).

Diabetic mice given only islets rejected allografts within 13 days (9±2 days; range, 5–13 days; n = 23; Fig. 1). Mice transfused with higher doses of fraction 19 lymphocytes (75–88 $\times$ 10^6) showed longer graft survival (15±5 days; range, 7–21 days; n = 13), but the effect was temporary, and all islet grafts were rejected by day 21. Animals treated with smaller numbers of fraction 19 lymphocytes (40–44 $\times$ 10^6), with fraction 20 lymphocytes (75–88 $\times$ 10^6), or with unfractionated spleen cells rejected grafts with the same kinetics as did untreated recipients.

Pretreatment with Allogeneic Small Lymphocytes in Combination with Anti-CD40L. We expected allogeneic small lymphocytes to induce graft tolerance. We hypothesized that their failure to do so resulted from host T cells inducing or enhancing expression of costimulatory signals in the transfused leukocytes. To test this, we prevented the interaction of CD40L on host T cells with CD40 on donor leukocytes using an antibody against CD40L (19). Antibody was used in combination with allogeneic small lymphocytes to allow the presentation of alloantigen while preventing T cells from inducing costimulatory activity.

Again, all untreated recipients rejected islet allografts (n = 22; Fig. 2*A*). Treatment with anti-CD40L antibody by itself produced indefinite graft survival in 40% of recipient animals (n = 25; Fig. 2*B*) and delayed rejection in the remaining recipients (mean failure at 24±30 days; range, 10–125 days). This result suggests that anti-CD40L may modulate islet cell graft rejection in the absence of transfused allogeneic lymphocytes.

When anti-CD40L was injected in combination with allogeneic lymphocytes, we observed 96% long-term survival of islet grafts (n = 23), the one failure occurring on day 118 (Fig. 3*A*).

In additional experiments, 5 of 8 mice given one injection of small lymphocytes plus four injections of anti-CD40L antibody over 2 wk retained their grafts for the duration of the 6-wk experiment. Mice receiving unfractionated spleen cells and anti-CD40L antibody for 2 wk (n = 6) or 7 wk (n = 5) were similar in outcome; 2 mice in each group rejected grafts within 40 days. These results suggest that shorter courses of anti-CD40L antibody combined with small lymphocytes promote islet graft survival and that fractionated small lymphocytes may be more effective than unfractionated spleen cells.

Effect of Allogeneic Lymphocytes in Combination with Anti-CD40L Is Donor Antigen-Specific. Transfusion of (C57BL/6 $\times$ C3H)F_1 lymphocytes in combination with anti-CD40L was not more effective than anti-CD40L antibody alone in prolonging BALB/c islet graft survival (5 of 9 mice rejected grafts; time to failure, 20±13 days; range, 10–40 days;

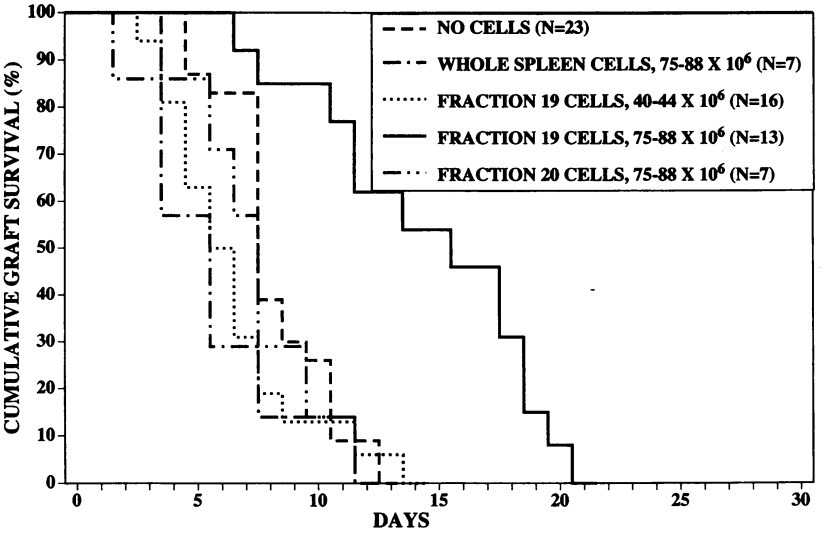

FIG. 1. Islet allograft survival (defined as plasma glucose <13.9 mM). Streptozotocin diabetic recipients were transfused with F_1 allogeneic spleen cells or small lymphocyte fractions of F_1 allogeneic spleen cells bearing islet donor alloantigens 5–8 days before transplantation.

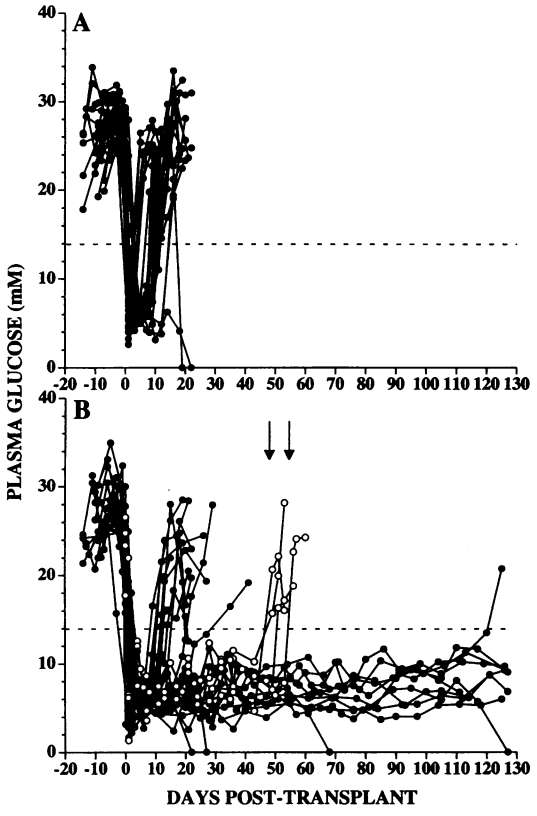

FIG. 2. Anti-CD40L by itself enhances islet allograft survival. Transplantation was done on day 0, and blood glucose of individual mice is shown. (*A*) Rejection of islet allografts in untreated control animals (n = 22, a separate control group from that shown in Fig. 1). (*B*) Animals treated with 250 μg of anti-CD40L antibody twice weekly for 1 wk before and 6 wk after transplantation (n = 25). ○, Mice from which functioning islet allografts were removed by unilateral nephrectomy to confirm allograft survival on the days indicated by arrows (n = 4). Normoglycemic animals dead of unknown cause are shown as dropping to 0 mM glucose.

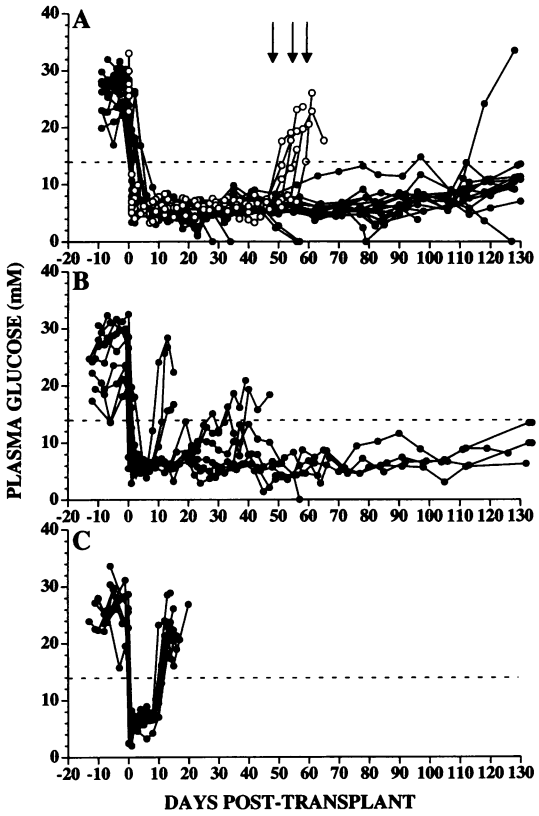

FIG. 3. Islet allograft survival. (*A*) Animals injected with 75–88 × 10^6 small lymphocytes (fraction 19) from (BALB/c × C57BL/6)F_1 mice 5–8 days before transplantation in combination with anti-CD40L antibody twice weekly for 7 wk beginning on the day of lymphocyte injection (n = 17). ○, Mice from which functioning islet allografts were removed by unilateral nephrectomy to confirm allograft survival on the days indicated by arrows (n = 6). (*B*) Animals injected with (C57BL/6 × C3H)F_1 small lymphocytes (fraction 19) lacking BALB/c islet donor antigens in combination with anti-CD40L (n = 9). (*C*) Animals injected with (C57BL/6 × C3H)F_1 small lymphocytes (fraction 19) lacking BALB/c islet donor antigens without anti-CD40L (n = 8).

Fig. 3*B*). Transfusion of (C57BL/6 × C3H)F_1 lymphocytes without coadministration of anti-CD40L resulted in rapid rejection of BALB/c islets in all C57BL/6 recipients (Fig. 3*C*).

T Cells Are Not Required in the Spleen Cell Inoculum for Graft Survival. The small lymphocyte fraction includes T cells that might act as "veto" (29, 30) or suppressor cells. However, T-cell-depleted F_1 small lymphocytes were as effective as complement-treated control F_1 small lymphocytes for inducing allograft survival in combination with anti-CD40L (Fig. 4).

Recipient Antigens on Transfused Lymphocytes Are Not Required. Some models of tolerance induction by donor-specific transfusion require donor and recipient antigens on the same tolerizing cell (31, 32). To test this requirement, we next studied fully allogeneic lymphocytes, avoiding graft vs. host reactivity by T-depleting the donor inoculum. T-depleted, allogeneic lymphocytes in combination with anti-CD40L resulted in long-term graft survival (one failure at 128 days; Fig. 5*A*). These results were comparable to those obtained using F_1 lymphocytes (Fig. 4). In contrast, T-depleted allogeneic lymphocytes without anti-CD40L resulted in rapid rejection of islet allografts in all recipients (Fig. 5*B*).

Survival of Islet Allografts after Discontinuation of Anti-CD40L Injections. To determine if anti-CD40L acts as a transiently immunosuppressive agent, mice were monitored after antibody was discontinued (Figs. 2–5). In all groups, with or without allogeneic lymphocyte injections, most animals whose grafts were functional when antibody was stopped remained normoglycemic thereafter.

Confirmation of Islet Allograft Function. Because all animals that became nondiabetic after transplantation gained weight, decreased metabolic demands cannot explain the observed normoglycemia. To confirm islet graft function and the absence of insulin secretion by residual native islets, the kidneys bearing islet implants were removed after 6–8 wk from 10 normoglycemic animals (Figs. 2*B* and 3*A*, ○). In every case, removal of the grafted islets resulted in diabetes recurrence (3.3 ± 1.9 days; range, 2–8 days).

Histology of Transplanted Islets. Islet allografts were studied histologically in these 10 animals and in 58 instances of graft failure. BALB/c islet allografts from normoglycemic mice given F_1 small lymphocytes and anti-CD40L antibody appeared intact, showed no mononuclear infiltration, and contained well-granulated insulin- and glucagon-positive cells (data not shown). Graft sites from all 58 hyperglycemic animals evidenced either no residual islet tissue or only small remnants of islet tissue heavily infiltrated by mononuclear cells. In selected cases we performed immunohistochemical analyses for insulin, and none was detected.

DISCUSSION

Combined treatment with allogeneic small lymphocytes and a blocking antibody to CD40L allows permanent islet allograft survival between mouse strains that differ in major histocom-

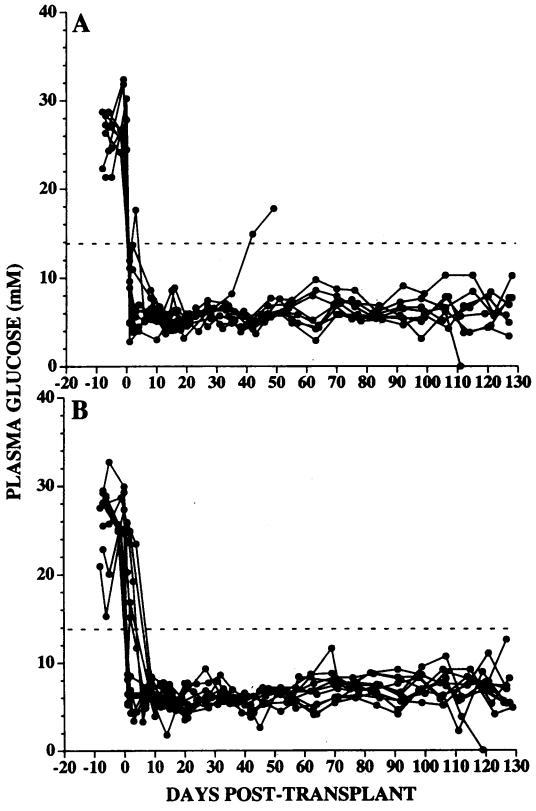

FIG. 4. T-cell-depleted F_1 small lymphocytes are effective in combination with anti-CD40L. (*A*) Animals injected with anti-CD40L and (BALB/c × C57BL/6)F_1 complement-treated small lymphocytes (n = 8). (*B*) Animals treated with anti-CD40L and (BALB/c × C57BL/6)F_1 small lymphocytes treated with anti-T-cell antibodies and complement (n = 10).

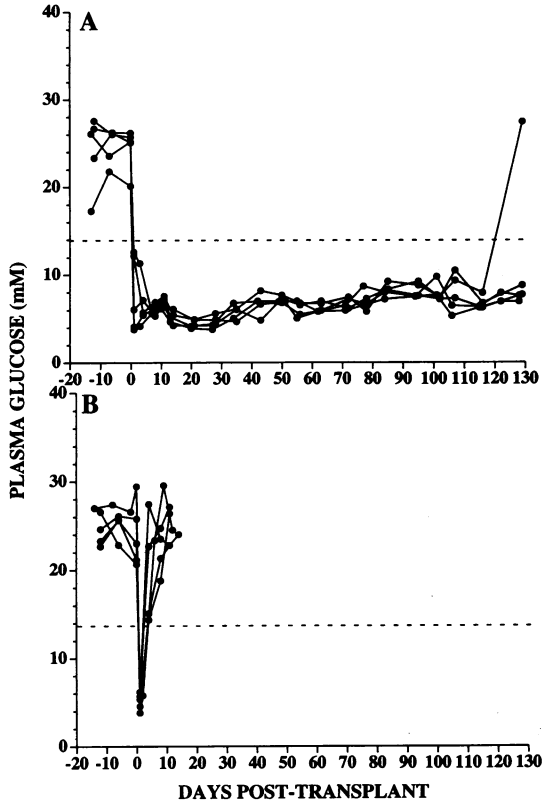

FIG. 5. Fully allogeneic small lymphocytes permit islet allograft survival in combination with anti-CD40L. (*A*) Animals treated with anti-CD40L and T-depleted BALB/c small lymphocytes (n = 5). (*B*) Animals treated with T-depleted BALB/c small lymphocytes without anti-CD40L (n = 5).

patibility complex (MHC)-encoded and minor transplantation antigens. Of 40 treated mice, 37 had permanent islet allograft survival, and 3 displayed delayed rejection (days 50, 127, and 130). In contrast, grafts were promptly rejected in all untreated animals. There was no evidence of graft rejection in the groups given anti-CD40L antibody and small lymphocytes after anti-CD40L was discontinued.

The effect of allogeneic small lymphocytes was donor antigen-specific. Small lymphocytes from F_1 animals of the donor and recipient strains and fully allogeneic, T-depleted small lymphocytes of the donor strain were equally effective when used in combination with anti-CD40L antibody. This suggests that non-T cells (B cells) in the donor splenocytes permitted permanent islet allograft survival when administered in combination with anti-CD40L antibody. In support of this hypothesis, we have recently shown that treatment with the anti-CD40L antibody prevents development of alloreactive cytolytic T lymphocytes after injection of allogeneic T-depleted spleen cells and induces specific tolerance to those alloantigens as measured by subsequent mixed lymphocyte responses *in vitro* (33).

We did not test for the nonspecific effects of hamster immunoglobulin on islet graft survival in these experiments, but in other studies nonspecific immunoglobulin had no effect on islet graft survival (unpublished data). Additionally, hamster immunoglobulin has no immunosuppressive effects on other T-dependent immune responses (34, 35), despite reports that it accelerates collagen-induced arthritis (36).

How do small allogeneic lymphocytes plus anti-CD40L enhance graft survival? Anti-CD40L may have a direct, inactivating effect on T cells responding to injected lymphocytes and allograft. CD40L is expressed on activated but not resting T lymphocytes (19, 37, 38). Injected allogeneic cells would induce CD40L expression on alloreactive T cells, rendering them susceptible to anti-CD40L. We think this is unlikely. (*i*) The antibody is not cytotoxic and does not clear $CD40L^+$ cytokine-producing cells *in vivo* (39). (*ii*) Although it blocks primary antibody responses, primed T cells can be recovered from animals immunized in the presence of anti-CD40L (34). (*iii*) As reported here, unfractionated spleen cells were nearly as effective as small lymphocytes in blocking graft rejection when combined with anti-CD40L. Unfractionated spleen cells would be expected to be more effective at inducing CD40L on alloreactive T cells, rendering them more susceptible to anti-CD40L antibody elimination.

We hypothesize that anti-CD40L blocks interaction of CD40L on alloreactive T cells with CD40 on donor leukocytes, preventing the induction or enhanced expression of costimulatory signals on those cells. The host T cells require CD40-dependent costimulatory signals to proliferate and differentiate into effector cells capable of graft rejection or at least to avoid inactivation as a result of antigen recognition on small B lymphocytes that lack costimulatory activity. To test this hypothesis, a disrupted CD40 gene (40) must be bred onto BALB/c mice to study CD40/CD40L interactions in our transplantation model.

The partial but substantial effect of anti-CD40L by itself on islet graft survival suggests that CD40L/CD40 interactions between donor islets and the host immune system may be important. Recent data show that dendritic cells (a passenger leukocyte in islet grafts) can be activated through CD40 (7). Alternatively, because CD40 is also on T cells (41) and macrophages (42), and induces secretion of chemotactic cytokines from monocytes and dendritic cells (7), CD40L/CD40

interactions could be important in inflammation and graft destruction by alloreactive effector T cells.

Our limited success in prolonging graft survival across a complete MHC mismatch with small lymphocytes alone contrasts with earlier reports. Skin graft survival has been observed across an isolated class II difference (43) and the male minor transplantation antigen (44) by pretreatment with APC-depleted spleen cells or purified B cells, respectively. We have also been able to induce tolerance to a transgene-encoded antigen expressed in B cells by transferring transgenic B cells into nontransgenic littermates without anti-CD40L (V. Yuschenkoff and D.C.P., unpublished). The difference between those results and our observations here may be due to the high frequency of alloreactive T cells in complete MHC mismatches. Some of these alloreactive T cells may be primed or recently activated by crossreacting environmental antigens (45) and therefore difficult to tolerize (44). They may also be more effective than naive T cells in inducing costimulatory activity and converting transfused lymphocytes into immunogenic APCs. Others have reported tolerance induction across MHC barriers by injecting spleen cells without removing nonlymphoid APCs (32, 46), but we find this possible only if CD40L/CD40 interaction is blocked.

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