Prolonged Survival of Rat Islet and Skin Xenografts in Mice Treated with Donor Splenocytes and Anti-CD154 Monoclonal Antibody


Treatment of C57BL/6 mice with one transfusion of BALB/c spleen cells and a brief course of anti-CD154 (anti-CD40 ligand) antibody permits BALB/c islet grafts to survive indefinitely and BALB/c skin grafts to survive for ~50 days without further intervention. We now report adaptation of this protocol to the transplantation of islet and skin xenografts. We observed prolonged survival of rat islet xenografts in mice treated with donor-specific spleen cell transfusion and anti-CD154 monoclonal antibody (mAb). Challenge islet xenografts placed on these animals indicated that graft acceptance was species-specific but not strain specific. Spleen cells from recipients bearing intact grafts led to rejection of rat islet xenografts in scid mice, suggesting that graft acceptance was not due to complete clonal deletion of xenoreactive cells. We also observed prolonged survival of rat skin xenografts in mice treated with donor-specific transfusion and anti-CD154 mAb. Prolonged survival of skin xenografts was also species specific. We conclude that treatment with appropriately timed donor-specific transfusion and anti-CD154 mAb induces durable survival of both islet and skin xenografts in mice. Because this procedure is targeted directly at CD154, a co-activation molecule expressed predominantly by activated CD4+ T-cells, the results suggest that CD4+ cells have a major role in the cellular immune response to xenografts. *Diabetes* 47:1199-1206, 1998

A llotransplantation to cure human diabetes faces the obstacle of obtaining adequate tissue from human donor pancreata. In the U.S. alone, an estimated 8 million people have been diagnosed with diabetes, and an equal number of individuals are thought to have undiagnosed disease (1). Obtaining adequate numbers of allografts may become an insurmountable problem. The United Network for Organ Sharing scientific registry reported in 1995 that its organ recipient waiting list had grown to approximately 38,000 individuals (2), and an estimated 9 people die each day awaiting a human allograft. An alternative to the human allograft is the xenograft (3-7). Once thought an unlikely modality for treating human disease, experimental transplants of baboon hearts, baboon bone marrow, and porcine neural tissue and islets in humans have already been attempted (2,8).

Those attempts at xenotransplantation have generally employed prolonged immunosuppression. A more attractive option is the induction of immunologic tolerance to xenografts (3,9-13). Several tolerance induction strategies have been applied to rat-to-mouse and human-to-mouse islet xenografts (2,3). These strategies have sought to alter donor islet tissue antigenicity, to modulate the host immune response, or both.

Attempts to reduce the antigenicity of transplanted islets have used in vitro culture to remove graft antigen-presenting cells (APCs). Other strategies have used anti-major histocompatibility complex (MHC) class II monoclonal antibody (mAb) to remove islet APCs or anti-MHC class I mAb to mask donor islet antigens (2,3).

Strategies for modulating the host response have included the use of CTLA4-Ig to block co-stimulatory signals (14), treatment with anti-lymphocyte serum (15), depletion of host CD4+ and/or CD8+ T-cells (16-18), and the intrathymic injection of xenogeneic islet and bone marrow cells (19). Another strategy has been the creation of xenogeneic bone marrow chimeras in advance of subsequent islet transplant (20). None of these strategies has yet been applied to human islet xenotransplantation in the clinic (2,3).

We report here a novel strategy for the modification of the host immune response to xenografts. We have previously demonstrated the induction of long-term survival of murine islet allografts by pretreatment of recipients with a donor-specific transfusion (DST) of splenocytes and injections of anti-CD154 mAb directed against the CD40 ligand (21,22). This form of combination therapy also prolongs the survival of murine skin allografts, with 20% of grafts surviving >100 days (23). In a preliminary communication, we showed that this method of prolonging allograft survival can be extended to LEW rat islet and skin xenografts transplanted to C57BL/6 mice (24). The present study documents that the combination of DST and anti-CD154 mAb induces species-specific acceptance of rat islet xenografts in mice. We also document that...
this protocol is effective in a much more stringent skin transplant system, suggesting that the protocol is robust and may be applicable to tissues other than islets.

RESEARCH DESIGN AND METHODS

Animals. C57BL/6 (H-2b) and BALB/c (H-2d) mice were obtained from the National Cancer Institute (Frederick, MD). C57BL/6-prkdcscid mice (H-2d, hereafter referred to as C57BL/6-scid mice) were obtained from The Jackson Laboratory (Bar Harbor, ME). DR-BB/Wor rats (RT-1u) were obtained from the colony sponsored by the National Institutes of Health at the University of Massachusetts Medical Center, Worcester, MA. Lewis (LEW) rats (RT-1u) were obtained from Harlan Industries (Indianapolis, IN). All animals were housed under specific pathogen-free conditions in sterile cages with micro-isolator lids and given autoclaved food and water ad libitum. Rats and mice used in these studies were certified to be serologically free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kailman rat virus, HI (Toolan's virus), GD7, Reo-3, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, Myxovirus pneumonia, and encephalitis virus. All animals were maintained in accordance with recommendations in the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training, the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and local institutional guidelines.

Antibodies and flow microfluorimetry. Phycoerythrin (PE)-conjugated mouse anti-rat CD45 (clone OX-1, pan-lymphohemopoietic cells), FITC-conjugated rat anti-mouse H-2K (AF6-85, anti-MHC class I), biotinylated hamster anti-mouse CD3 (145-2C11, pan- T-cells), PE-conjugated rat anti-mouse CD19 (1D3, pan B-cells), and PE-mouse anti-rat CD45RB (HBR4, pan B-cells) mAbs were obtained from Pharmingen (San Diego, CA). Cy-Chrome streptavidin, goat anti-rat Ig, used for the visualization of bound biotinylated anti-mouse CD3 mAb, was obtained from Pharmingen. Anti-rat CD45 and anti-mouse H-2K mAbs were used in dual-label studies to examine the level of rat cell chimerism in DST and anti-CD154 mAb-treated recipients. The level of detection of rat DR-BB/Wor splenocytes in the presence of mouse C57BL/6 splenocytes was determined to be 0.5% in independent mixing experiments (data not shown). Flow microfluorimetry was also used to determine the level of lymphocyte engraftment in C57BL/6-scid adoptive recipients of spleen cells from C57BL/6 donors.

Flow cytometry analysis was performed as described (25,26). Briefly, single cell suspensions were labeled with antibody, rinsed, fixed in 1% paraformaldehyde, and analyzed on a FACScan (Becton Dickinson, Sunnyvale, CA). Dead cells and erythrocytes were excluded by electronic gating. At least 10⁵ events were analyzed for each sample.

Pancreatic islet transplantation. C57BL/6 and C57BL/6-scid recipient mice 8-10 weeks of age were rendered hyperglycemic by a single intraperitoneal injection of 150 or 160 mg/kg of streptozotocin, respectively. Plasma glucose was measured using a Beckman glucose analyzer (Beckman, Fullerton, CA). The presence of diabetes in graft recipients was established by the observation of a plasma glucose concentration >250 mg/dl or at least two different days before transplantation. Pancreatic islets were harvested from donor rats or mice by collagenase digestion. Flow microfluorimetry was also used to determine the level of lymphocyte engraftment in C57BL/6-scid adoptive recipients of spleen cells from C57BL/6 donors.

The animals in group 4 received DST at doses of 5 × 10⁶ DR-BB/Wor rat spleen cells 5-7 days before transplantation. Group 3 (n = 15) was given anti-CD154 mAb (0.25 mg) twice weekly for 7 weeks beginning 5-7 days before transplantation. Group 4 (n = 29) received both anti-CD154 mAb and one transfusion of 5-25 × 10⁶ spleen cells, with the first dose of antibody being administered on the same day as the transplantation. Animals were observed until hyperglycemia occurred or until days 63-72 after transplantation, at which time unilateral nephrectomy was performed to document the function and histology of the islet xenograft. Hyperglycemia was observed to recur in all nephrectomized graft recipients.

The animals in group 4 received DST at doses of 5 × 10⁶ (n = 10), 10 × 10⁶ (n = 14), or 25 × 10⁶ (n = 5) cells. Median survival times (MSTs) associated with these doses were 71, 72, and 63 days, respectively. Life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.93), and these recipients are considered as one group in subsequent analyses.

Overall life-table analysis (Fig. 1) demonstrated that combined treatment with DST plus anti-CD154 mAb (group 4) was associated with significantly longer islet graft survival (MST = 71 days) than was treatment with anti-CD154 mAb alone (group 3, MST = 41 days, P < 0.05). DST alone (group 2, MST = 8 days, P < 0.001), or no treatment (group 1, MST = 11 days, P < 0.001). In the case of the recipients of combined therapy, 69% of islet grafts were still functioning when the treated animals were nephrectomized on days 62-73 and entered into additional experiments. Although not as effective as the combination of DST plus antibody, treatment with anti-CD154 mAb alone was statistically
Histological analysis of functioning xenogeneic islets obtained at the time of nephrectomy revealed the presence of readily detectable insulin- and glucagon-containing cells. Few, if any, mononuclear cells were observed within the islet grafts, but foci of inflammatory cells were observed at the periphery of the grafted tissue ("peri-insulitis," Fig. 2). No islet tissue was detectable in any of the grafts that had ceased to function (data not shown).

**Abbreviated courses of anti-CD154 mAb plus DST are less effective.** In a separate experiment, diabetic C57BL/6 mice were given either no additional treatment (n = 11) or DST at a dose of $5 \times 10^6$ cells plus anti-CD154 mAb 0.25 mg twice weekly for 2 weeks starting on the day of transfusion (n = 5). All mice were given DR-BB/Wor rat islet grafts; in the case of treated animals, grafts were placed 5–7 days after transfusion. Graft survival in the recipients of DST plus this abbreviated course of anti-CD154 mAb (MST = 20 days) was statistically superior to graft survival in controls (MST = 10 days, P < 0.05) but inferior to that achieved using 14 doses of antibody plus DST (group 4 in Fig. 1).

**Challenge islet xenograft studies.** The C57BL/6 mice described above that had been treated with one transfusion of $5-25 \times 10^6$ DR-BB/Wor rat spleen cells and 14 doses of anti-CD154 mAb, and whose DR-BB/Wor rat islet xenografts had survived 62–73 days, were entered into a challenge graft protocol. This protocol was designed to determine if xenogeneic or allogeneic cells were still present in mice with successful xenografts. The mice first underwent unilateral nephrectomy of the kidney bearing the xenograft. After documentation of recurrent hyperglycemia for 7–14 days, the mice were randomized to receive either a second RT1" DR-BB/Wor rat xenograft (n = 12), an MHC-disparate RT1\(^{b}\) LEW rat islet xenograft (n = 4), or a fully allogeneic H-2d BALB/c mouse islet graft (n = 3).

As shown in Table 1, 83% of DR-BB/Wor and 100% of LEW rat islet challenge grafts were accepted by their hosts and restored normoglycemia for a minimum of 36 days, whereas all mouse islet allotransplants were rejected 18–22 days after implantation. Six of the mice that tolerated their DR-BB/Wor islet grafts were entered into a third protocol (see below) on days 36–60 after challenge grafting. DR-BB/Wor grafts in the remaining four recipients were documented to function through 103 days after challenge. Recipients of LEW islet

![Histology of functioning islet xenografts](image-url)
Survival of challenge islet grafts in C57BL/6 mice that had borne long-term DR-BB rat islet xenografts

<table>
<thead>
<tr>
<th>Treatment before placement of primary DR-BB rat islet xenograft</th>
<th>Challenge islet graft</th>
<th>Challenge islet graft survival (days)</th>
<th>n</th>
<th>MST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD154 mAb + 5–25 × 10^6 BB rat spleen cells</td>
<td>DR-BB rat</td>
<td>14, 33, ≥36, ≥36, ≥36, ≥40, ≥40, ≥40, ≥40, ≥103, ≥103, ≥103, ≥103</td>
<td>12</td>
<td>&gt;40</td>
</tr>
<tr>
<td>Anti-CD154 mAb + 10 × 10^6 BB rat spleen cells</td>
<td>LEW rat</td>
<td>≥95, ≥110, ≥111, ≥111</td>
<td>4</td>
<td>&gt;110.5</td>
</tr>
<tr>
<td>Anti-CD154 mAb + 10 × 10^6 BB rat spleen cells</td>
<td>BALB/c mouse</td>
<td>18, 20, 22</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

C57BL/6 mice were treated with one transfusion of 5–25 × 10^6 DR-BB/Wor rat spleen cells and 14 doses of anti-CD154 mAb and given DR-BB/Wor rat islet xenografts. After these primary xenografts had survived 62–73 days (see Fig. 1), the original graft was removed. After recurrence of hyperglycemia for 7–14 days, challenge rat and mouse islet grafts were transplanted as indicated in the table. Six of the mice that tolerated their DR-BB/Wor islet grafts were entered into another protocol (Table 2) on days 36–60 after challenge grafting. DR-BB/Wor grafts in the remaining four recipients were documented to function through 103 days after challenge. Recipients of LEW islet xenografts were followed for 95–111 days before being used in the third protocol.

Adoptive transfer studies. In a final experiment, spleen cells were obtained from the C57BL/6 mice that 1) had been treated with DST and anti-CD154 mAb, 2) had accepted a primary DR-BB/Wor rat islet xenograft, and 3) had then accepted either a DR-BB/Wor or LEW islet challenge xenograft. The spleen cells (25 × 10^6) were adoptively transferred to chemically diabetic C57BL/6-scid mice (n = 32) that had received a DR-BB/Wor rat islet xenograft 10–14 days earlier. Control C57BL/6-scid mice bearing DR-BB/Wor islet xenografts received either no treatment (n = 5) or a single transfusion of 25 × 10^6 spleen cells obtained from normal, untreated C57BL/6 donors (n = 5).

As shown in Table 2, adoptive transfer of spleen cells from normal C57BL/6 donors was associated with prompt rejection (MST = 13 days after transfusion) of the DR-BB/Wor islet grafts in the scid recipients. Adoptive transfer of cells from the experimental mice was also associated with rejection of the DR-BB/Wor rats in the scid mice, but rejection was delayed (Table 2). Overall MSTs were 68 days in the case of spleen cells from mice bearing DR-BB/Wor challenge xenografts and 49 days in the case of spleen cells from mice bearing LEW challenge grafts. Control xenografts in C57BL/6 mice were followed for 95–111 days before being used in the third protocol.
Prolonged survival of rat skin xenografts in mice were randomized into six groups, each of which received a DR-BB/Wor rat skin graft. Group 1 received no further treatment. The anti-CD154 mAb–only group was treated twice weekly for 7 weeks beginning 7 (group 2) or 14 (group 3) days before transplantation. The combined treatment groups received anti-CD154 mAb and one transfusion of spleen cells, with the first dose of antibody being administered on the day of the transfusion, either 0 (group 5), 5–7 (group 6), or 14 (group 4) days before transplantation. The dose of spleen cells was 10 × 10^6 except for 11 animals in the 5–7-day group that received 5 × 10^6 cells. Preliminary life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.53) in this group, and these recipients were pooled in the overall analysis. Overall life-table analysis showed that combined treatment with DST plus anti-CD154 mAb begun on days −5 to −7 (group 6) was associated with significantly longer skin xenograft survival (MST = 36 days) than was treatment with anti-CD154 mAb alone started on day −7 or day −14 (groups 2 and 3, MST = 9 and 6 days, respectively, P < 0.001 for both), combined treatment begun on days −14 or 0 (groups 4 and 5, MST = 11 and 10 days, respectively, P < 0.001 for both), or no treatment (group 1, MST = 7 days, P < 0.001). No grafts in any group survived indefinitely.

Rat-to-mouse skin xenografts

Prolonged survival of rat skin xenografts in mice treated with appropriately timed DST and anti-CD154 mAb. We next tested the hypothesis that treatment with DST and anti-CD154 mAb treatment would also prolong rat skin xenograft survival in mice. C57BL/6 mice were randomized into six groups, each of which received a DR-BB/Wor rat skin graft. Group 1 (n = 18) received no other treatment. Groups 2 and 3 were given anti-CD154 mAb (0.25 mg) twice weekly for 7 weeks beginning 7 (group 2, n = 9) or 14 (group 3, n = 6) days before transplantation. Groups 4 and 5 received both anti-CD154 mAb using the same dosing schedule and one transfusion of 10^7 spleen cells; the transfusion and the first dose of antibody were administered either 14 days before (group 4, n = 5), or on the day of transplantation (group 5, n = 7). Group 6 (n = 25) received both anti-CD154 mAb and one transfusion of 5 × 10^6 or 10 × 10^6 spleen cells, with the transfusion and the first dose of antibody being administered 5–7 days before transplantation. The animals in group 6 received DST at doses of 5 × 10^6 (n = 11) or 10 × 10^6 (n = 14) cells. A preliminary life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.53), and these recipients are considered as one group in subsequent analyses.

Overall life-table analysis is shown in Fig. 3. Combined treatment with DST plus anti-CD154 mAb begun on day −7 (group 6) was associated with significantly longer skin xenograft survival (MST = 36 days) than was treatment with anti-CD154 mAb alone begun on day −7 or day −14 (groups 2 and 3, MST = 9 and 6 days, respectively, P < 0.001 in each case), combined treatment begun on day −14 (group 4, MST = 11 days, P < 0.02), or combined treatment begun on 0 (group 5, MST = 10 days, P < 0.001). No grafts in any group survived indefinitely.

**An abbreviated course of anti-CD154 mAb plus DST is comparably effective.** We also tested the efficacy of combined treatment with DST and a briefer course of anti-CD154 mAb in prolonging rat skin xenograft survival in mice. C57BL/6 mice were randomized into three groups, each of which received a DR-BB/Wor rat skin graft. Group 1 (n = 15) received no other treatment. Group 2 (n = 15) was given anti-CD154 mAb (0.25 mg) twice weekly for 2 weeks beginning 7 days before transplantation. Group 3 (n = 25) received both anti-CD154 mAb on the same dosing schedule and one transfusion of 5–50 × 10^6 spleen cells, with the transfusion being administered with the first dose of antibody. The animals in this last group received DST at doses of 5 × 10^6 (n = 10), 25 × 10^6 (n = 10), or 50 × 10^6 (n = 5) cells. Preliminary life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.25), and these recipients were pooled in the overall analysis. The combination of DST plus four doses of anti-CD154 mAb (group 3) significantly prolonged skin xenograft survival (MST = 29 days) compared with either no treatment (group 1, MST = 7 days, P < 0.01) or anti-CD154 mAb treatment alone (group 2, MST = 7 days, P = 0.01).

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**Fig. 3.** Skin xenograft survival. C57BL/6 mice were randomized into six groups, each of which received a DR-BB/Wor rat skin graft. Group 1 received no further treatment. The anti-CD154 mAb–only group was treated twice weekly for 7 weeks beginning 7 (group 2) or 14 (group 3) days before transplantation. The combined treatment groups received anti-CD154 mAb and one transfusion of spleen cells, with the first dose of antibody being administered on the day of the transfusion, either 0 (group 5), 5–7 (group 6), or 14 (group 4) days before transplantation. The dose of spleen cells was 10 × 10^6 except for 11 animals in the 5–7-day group that received 5 × 10^6 cells. Preliminary life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.53) in this group, and these recipients were pooled in subsequent analyses.

**Fig. 4.** Skin xenograft survival after abbreviated treatment with anti-CD154 mAb. C57BL/6 mice were randomized into three groups, each of which received a DR-BB/Wor rat skin graft. Group 1 received no other treatment. Group 2 was given anti-CD154 mAb only (0.25 mg) twice weekly for 2 weeks beginning 7 days before transplantation. Group 3 received both anti-CD154 mAb on the same dosing schedule and one transfusion of 5–50 × 10^6 spleen cells, with the transfusion being administered with the first dose of antibody. The animals in this last group received DST at doses of 5 × 10^6 (n = 10), 25 × 10^6 (n = 10), or 50 × 10^6 (n = 5) cells. Preliminary life-table analysis showed that DST dose had no statistically significant effect on graft survival (P = 0.25), and these recipients were pooled in subsequent analyses. The combination of DST plus four doses of anti-CD154 mAb (group 3) significantly prolonged skin xenograft survival (MST = 29 days) compared with either no treatment (group 1, MST = 7 days, P < 0.01) or anti-CD154 mAb treatment alone (group 2, MST = 7 days, P = 0.01).

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**Cumulative Graft Survival (%)**

**Skin Xenograft Survival (Days)**

- No Treatment (Group 1, N=18)
- Anti-CD154 mAb Only on Day -7 (Group 2, N=9)
- Anti-CD154 mAb Only on Day -14 (Group 3, N=6)
- DST + Anti-CD154 mAb on Day -14 (Group 4, N=5)
- DST + Anti-CD154 mAb on Day 0 (Group 5, N=7)
- DST + Anti-CD154 mAb on Day -7 (Group 6, N=25)

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**Cumulative Graft Survival (%)**

**Skin Xenograft Survival (Days)**

- No Treatment (Group 1, N=15)
- Anti-CD154 mAb Only (Group 2, N=15)
- DST + Anti-CD154 mAb (Group 3, N=25)
SURVIVAL OF RAT ISLET AND SKIN XENOGRAFTS

C57BL/6 mice that had been treated with a DST and 14 doses of anti-CD154 mAb and whose DR-BB/Wor rat skin xenografts had survived 50–70 days received the challenge grafts indicated in the table.

treatment (group 1, MST = 7 days, P < 0.01) or anti-CD154 mAb treatment alone (group 2, MST = 7 days, P < 0.01). The degree of prolongation achieved with DST plus the abbreviated (4-injection) course of anti-CD154 mAb treatment (MST = 29 days, group 3 in Fig. 4) was statistically similar (P = 0.17) to that achieved using the standard 14-injection regimen (MST = 36 days, group 6 in Fig. 3).

Challenge skin xenograft studies. The C57BL/6 mice described above that had been treated with one transfusion of 5–25 × 10⁶ DR-BB/Wor rat spleen cells and 14 doses of anti-CD154 mAb, and whose DR-BB/Wor rat skin xenografts had survived 50–70 days, were entered into a challenge graft protocol. This protocol was designed to determine if xenoreactive cells were still present in mice at a time when skin xenografts were healed and free of any sign of rejection. The mice were randomized to receive either a second RT1<sup>u</sup> DR-BB/Wor rat skin xenograft (n = 5) or a fully allogeneic H-2<sup>d</sup> BALB/c mouse islet graft (n = 2).

As shown in Table 3, 100% of DR-BB/Wor challenge skin xenografts were eventually rejected by their hosts. Rejection of the challenge grafts correlated temporally with rejection of the primary xenografts; three of the five mice rejected both the challenge and primary grafts within 9 days of challenge. In contrast, the remaining two mice maintained both the challenge and the primary grafts for 34–41 days. The total duration of primary skin xenograft survival in these two mice was 111 days.

The two C57BL/6 recipients that had borne DR-BB rat skin xenografts for 50 days and were then challenged with allogeneic BALB/c skin rejected both the challenge allograft and the primary xenograft within 9 days of challenge (Table 3).

Survival of C57BL/6 mouse skin xenografts on DR-BB/Wor rat skin DST and anti-mouse CD154 mAb. In our rat-to-mouse transplantation protocol, treatment with anti-CD154 mAb could have mediated its effect on CD154<sup>+</sup>cells present in the recipient, CD154<sup>+</sup>cells present in the DST, or both. To help discriminate among these possibilities, reciprocal mouse-to-rat skin xenografts were performed. DR-BB rats were given no treatment (n = 9),hamster anti-mouse CD154 mAb (n = 10), or the combination of anti-CD154 mAb and one transfusion of 20 × 10⁶ C57BL/6 mouse spleen cells (n = 9). The first dose of antibody was administered on the same day as the transfusion, 5–7 days before transplantation. Antibody treatment was continued biweekly throughout the experiment. Rejection of skin xenografts occurred rapidly in all three groups (MST = 6, 7, and 10 days, respectively).

**DISCUSSION**

These data document that the combination of a single transfusion of donor spleen cells and a brief course of anti-CD154 mAb markedly prolongs the survival of concordant rat islet xenografts in mice. More than 75% of islet grafts were still functional 70 or more days after implantation. At that point, the grafts were removed for studies of the mechanism of tolerance induction, but given our published preliminary observations (24), it is likely that most of these grafts could have survived much longer. In that preliminary study, which used a very similar protocol, 6 of 9 rat islet xenografts were still functional 175 days after transplantation. Taken together, these two data sets suggest that combined treatment with DST and anti-CD154 mAb induces durable islet xenograft survival.

In the present study, the combination of DST and anti-CD154 mAb also prolonged the survival of rat skin xenografts. Some skin xenografts survived >100 days, a remarkable result given the strong immunogenicity of skin. The outcome of the challenge graft studies suggests that, in some treated animals, levels of xenoreactive cells are low for long periods of time.

Skin xenograft survival in the present study is superior to that reported in our preliminary communication (24). In that study, the DST was manipulated to remove fibronectin adherent splenocytes. The superior outcome in the present study may reflect both the use of nonmanipulated spleen cells for the DST and refinement of our technique. Based on theoretical considerations, our original implementation of the two-element protocol used elutriated small B-cells (21). Subsequent work has demonstrated, however, that unfractionated spleen cells are equally efficacious in prolonging allograft survival (23,33), and the present study extends this observation to the xenograft system. Studies to identify those specific cell populations that mediate the effect of DST in our two-element protocol are in progress in our laboratory.

It is worth noting that the outcomes for both skin and islet grafts were in part dependent on the dosage and timing of treatment, suggesting that additional refinement of the protocol may further improve outcomes.

The present data also suggest that the prolongation of xenograft survival induced by DST and anti-CD154 mAb is

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**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment of recipient primary skin graft</th>
<th>Duration of primary skin graft survival at the time of challenge grafting (days)</th>
<th>Challenge graft</th>
<th>Graft survival after challenge grafting (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DST (5 × 10⁶), anti-CD154 mAb</td>
<td>70</td>
<td>DR-BB rat</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>DR-BB rat</td>
<td>8</td>
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<tr>
<td></td>
<td>50</td>
<td>BALB/c mouse</td>
<td>9</td>
</tr>
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</table>

Survival of primary skin xenografts after challenge with a second skin graft.
These observations are consistent with previous reports of species-specific xenotolerance induced by the combination of islet culture, intrathymic injection of islets, and treatment of the host with anti-lymphocyte serum (34).

Our histological findings in xenogeneic islets are also consistent with previous observations (34). It appears that xenografted islets continue to function despite the presence of focal mononuclear cell infiltrates near the islets. The identity and functional characteristics of these infiltrating cells are unknown and the subject of ongoing study in our laboratory.

In the present study, we observed that treatment with anti-CD154 mAb alone was somewhat effective in prolonging the survival of islet xenografts, but completely ineffective when applied to skin xenografts. This observation is consistent with our earlier reports that anti-CD154 mAb by itself can prolong the survival of allogeneic islet (21,22) but not skin (23) grafts. The data suggest the possibility that there could exist co-stimulatory pathways in skin that are not dependent on the interaction of CD40 with CD154. Such pathways might be mediated by APCs found in skin but not islets, perhaps epidermal Langerhans cells. The data we have obtained using anti-CD154 mAb alone in both allograft and xenograft systems are consistent with this interpretation and suggest that such co-stimulatory activity may be much more robust in skin than in islet tissue. More generally, the data suggest that prolongation of xenograft survival using anti-CD154 mAb alone is likely to be achievable only in favorable species using only favorable tissues.

The present data also provide additional insight into the nature of the immune response to xenogeneic tissues. The cellular immune response to xenografts is known to be distinct from the cellular immune response to allografts (7). Studies of xenograft rejection suggest that MHC class II is the predominant antigenic target and that MHC class II–restricted CD4+ T-cells are the primary mediators of graft destruction (16–18). It has been suggested, in fact, that xenograft rejection may use a CD4+, cytotoxic T-cell–independent pathway (3,35). Lafferty and colleagues (35–37) have proposed the concept of “indirect” rejection based on the interaction of recipient CD4+ T-cells with recipient APCs presenting xenogeneic antigens. The subsequent release of cytokines and free radicals in the vicinity of grafted islets may then lead to β-cell cytotoxicity or apoptosis. Studies of cytokine gene expression are consistent with this view. For example, Morris et al. (38), studying the rejection of xenogeneic porcine islets in CBA/H mice, have observed that Th2-like CD4+ T-cells appear to be differentially activated by exposure to xenogeneic antigens.

The results of the present study support the hypothesis that CD4+ cells may, in fact, play a major role in the cellular immune response to xenografts. This interpretation is based on the fact that the protocol used in the present study is targeted directly at CD154, a co-activation molecule that is expressed predominantly by activated CD4+ T-cells (39,40).

Our working hypothesis for the prolongation of xenograft survival by DST and anti-CD154 mAb is that foreign antigen presented in the absence of co-stimulation (which is blocked by anti-CD154 mAb) leads to T-cell nonresponsiveness. When applied to the induction of long-term allograft survival, the anti-CD154 mAb component of the protocol could be exerting its effect on either donor or recipient cells. In the rat-to-mouse xenograft system we have studied, it is clear that the effect of the anti-mouse CD154 mAb is directed at host CD154+ cells. In our reciprocal study of mouse-to-rat skin xenografts, we further observed that tolerance could not be induced in rat recipients of mouse xenografts, mouse DST, and anti-mouse CD154 mAb. These data suggest that neither “passenger” nor DST CD154+ cells of donor origin are required for xenograft rejection; they are consistent with the view that “indirect” antigen presentation may mediate the rejection of xenogeneic tissues (35–37).

We conclude that combination therapy with DST plus anti-CD40L mAb in mice can induce durable survival of concordant rat islet xenografts and can prolong the survival of rat skin xenografts. This treatment strategy is of particular interest because it affects only cells that are activated by antigen to express CD154, avoiding the potential complications of therapies that lead to generalized immunosuppression.

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