

Prolonged Islet Graft Survival in NOD Mice by Blockade of the CD40-CD154 Pathway of T-Cell Costimulation

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Allorejection and recurrence of autoimmunity are the major barriers to transplantation of islets of Langerhans for the cure of type 1 diabetes in humans. CD40-CD154 (CD40 ligand) interaction blockade by the use of anti-CD154 monoclonal antibody (mAb) has shown efficacy in preventing allorejection in several models of organ and cell transplantation. Here we report the beneficial effect of the chronic administration of a hamster anti-murine CD154 mAb, MR1, in prolonging islet graft survival in NOD mice. We explored the transplantation of C57BL/6 islets into spontaneously diabetic NOD mice, a combination in which both allogeneic and autoimmune components are implicated in graft loss. Recipients were treated either with an irrelevant control antibody or with MR1. MR1 administration was effective in prolonging allograft survival, but did not provide permanent protection from diabetes recurrence. The autoimmune component of graft loss was studied in spontaneously diabetic NOD mice that received syngeneic islets from young male NOD mice. In this combination, a less dramatic yet substantial delay in diabetes recurrence was observed in the MR1-treated recipients when compared with the control group. Finally, the allogeneic component was explored by transplanting C57BL/6 islets into chemically induced diabetic male NOD mice. In this setting, long-term graft survival (>100 days) was achieved in MR1-treated mice, whereas control recipients rejected their grafts within 25 days. In conclusion, chronic blockade of CD154 results in permanent protection from allorejection and significantly delays recurrence of diabetes in NOD mice. *Diabetes* 50:270–276, 2001

Successful human islet allotransplantation represents a potential cure for diabetes. Although significant advances have recently been achieved and improved results have been reported in pilot clinical trials (1), islet transplantation is still performed as an alternative to pancreas transplantation in patients with advanced microangiopathy, either in combination with or after kidney transplantation. The prospects of transplanting islets alone in diabetic patients before the occurrence of these disabling complications depend on the development of novel immunosuppressive regimens devoid of the significant immediate or long-term side effects of conventional immunosuppression (2).

Blockade of costimulatory pathways of T-cell activation is currently being tested as the basis of new immunomodulatory strategies, because it has been demonstrated that T-cell receptor ligation in the absence of costimulatory signals can lead to T-cell deletion or anergy and donor-specific tolerance (3–5). Blockade of the CD28-B7 interaction by CTLA4-Ig prolonged graft survival in various models of organ allotransplantation (6), as well as in xenogeneic human-to-mouse and allogeneic nonhuman primate islet transplantation models (7,8).

More recently, data has been obtained on the crucial role of CD40-CD154 interaction in costimulation. Initially, CD154, the ligand to CD40, was identified on activated CD4⁺ T-cells, and shown to trigger B-lymphocyte proliferation on ligation with CD40 (9). Since then, the list of cells expressing CD40 or CD154 and the consecutive effects of their interaction has been steadily expanding (10). CD40-CD154 interaction blockade by the use of a monoclonal antibody (mAb) directed against CD154 has shown efficacy in several models of heart, kidney, aorta, bone marrow, and skin transplantation (10). The interest in anti-CD154 therapy in islet transplantation has been enhanced by its described ability to prevent the release of nonspecific inflammatory mediators (11,12), a phenomenon likely to be involved in early islet graft loss. Furthermore, analysis of the role of CD40-CD154 interaction in the development of several autoimmune diseases (13–16) has recently yielded data demonstrating that its blockade could prevent the initiation of insulinitis and thus prevent diabetes onset in NOD mice (17).

A positive effect of costimulatory blockade on allogeneic islet graft survival via administration of anti-CD154 mAb was recently demonstrated in chemically induced diabetic rodents (18–20) and surgically induced diabetic nonhuman

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FCS, fetal calf serum; HBSS, Hank's balanced saline solution; IEQ, islet equivalent; KLH, keyhole limpet hemocyanin; mAb, monoclonal antibody; MHC, major histocompatibility complex.

primates (21,22). Excellent graft survivals (>200 days) have recently been reported in different models of allotransplantation in primates treated with prolonged CD154 monotherapy (21–23). Long-term graft survival (>100 days) was also obtained in rodent allogeneic and xenogeneic models when the mAb was administered for a short course combined with donor-specific splenocytes, whereas anti-CD154 treatment alone had less dramatic effects on graft survival prolongation (18–20). Furthermore, a brief course of mAb administration was not able to promote islet graft survival in spontaneously diabetic NOD mice (24).

Altogether, these results and the reported efficacy of CD40-CD154 interaction blockade in preventing autoimmune diabetes (17) make anti-CD154 mAb treatment an excellent candidate for studying graft rejection and autoimmunity recurrence in transplants of islets of Langerhans in NOD mice. In this study, we analyzed the effect of anti-CD154 mAb administration on the recurrence of autoimmunity and/or allograft recognition in NOD mice, demonstrating a significant protective effect of mAb chronic monotherapy on both diabetes recurrence and allograft rejection.

RESEARCH DESIGN AND METHODS

Animals. NOD mice were purchased from Taconic Farms (Germantown, NY) and C57BL/6J (B6) were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were certified to be free of the most common laboratory animal pathogens. They were housed in microisolated cages in a virus antibody-free room at the University of Miami animal facilities and were given free access to autoclaved food and water. All animal manipulations were conducted and monitored under protocols reviewed and approved by the Institutional Animal Care and Use Committee. Female NOD mice were obtained at 8–10 weeks of age and monitored for blood glucose until they became diabetic. They were used as islet recipients after at least two consecutive nonfasting blood glucose readings >250 mg/dl. Islets of Langerhans were obtained from either 12-week-old B6 males or from 5- to 7-week-old NOD males. Young NOD males were also used, in selected experiments, as recipients of allogeneic B6 islet transplants.

Induction of diabetes. Male NOD mice recipients were rendered diabetic via a single intravenous injection of 200 mg/kg streptozotocin (Sigma, St. Louis, MO) freshly dissolved in citrate buffer. Diabetes occurrence was defined as two consecutive nonfasting blood glucose readings >250 mg/dl. Only mice with blood glucose levels >350 mg/dl at the time of the transplantation were used as recipients.

Islet of Langerhans isolation. Murine islets were isolated as previously described (25). Briefly, a collagenase (type V, Sigma) solution was prepared at a final concentration of 1.5 mg/ml in Hank's balanced saline solution (HBSS) (Gibco, Long Island, NY). The mice were killed, the abdomen was opened, and the pancreas was exposed and injected with the collagenase solution via the main bile duct until full distension was achieved. The pancreatic tissue was then sterilely removed and pooled in ice-cold collagenase solution. Digestion was started by raising the temperature to 37°C, performed with gentle shaking for 17 min, and terminated by the addition of cold HBSS supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Mechanical disruption of the digested pancreatic tissue was achieved by repeated passages through needles of decreasing gauge until complete release of free islets was observed under the microscope. Islet purification was obtained by centrifugation at 900g for 11 min on discontinuous Euro-Ficoll gradients and routinely provided islets of purity >90%. Islet purity was assessed by dithizone (Sigma) staining, and the islets were counted and scored for size. An algorithm was used for the calculation of the 150 μ m diameter islet equivalent (IEQ) number. Before transplantation, islets were kept in culture overnight at 37°C in a 5% CO₂ atmosphere, in CMRL medium (Gibco) supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 mmol/l HEPES buffer (Mediatech, Herndon, VA).

CD40-CD154 blockade. Hamster anti-murine CD154 monoclonal antibody (MR1) and an irrelevant, isotype-matched hamster anti-keyhole limpet hemocyanin (KLH) monoclonal antibody (Ha4/8) were provided by Biogen (Cambridge, MA,) and used in all of the experiments. The antibodies were injected intraperitoneally at different doses (10–20 mg/kg) and according to selected schedules as detailed elsewhere.

Prevention of diabetes. Female NOD mice were treated with MR1 or Ha4/8 starting at 4 and 8 weeks of age. Mice received weekly intraperitoneal injections of 20 mg/kg of the antibody for 6 weeks (long protocol) ($n = 6$ for both ages). In another group, a total of three doses of 10 mg/kg of the antibody were injected every other day when the mice reached 8 weeks of age (short protocol) ($n = 6$).

Mice were checked twice weekly for glycosuria using Ketodiastix (Bayer, Tarrytown, NY). Positive readings were confirmed by demonstration of hyperglycemia. Mice were killed after three consecutive readings of blood glucose >250 mg/dl.

Transplant combinations. Three separate transplant combinations were used. In the first set of experiments, spontaneously diabetic female NOD mice received an H-2-mismatched islet graft from B6 donors under the kidney capsule. In the second set of experiments, spontaneously diabetic NOD mice were transplanted with syngeneic islets obtained from young (5- to 7-week-old) male NOD mice. In the last set of experiments, chemically induced diabetic male NOD mice were transplanted with allogeneic B6 islets.

Treatment groups. Two different treatment protocols were used. In the first one, an induction dose of 10 mg/kg of MR1 or Ha4/8 was given intraperitoneally on days -1, 0, and 3, day 0 being the day of transplantation. In the second protocol, a higher induction dose (20 mg/kg) of MR1 or Ha4/8 was injected on the same days. The maintenance dose was 20 mg/kg on day 7 and every 7 days thereafter, until diabetes recurrence or at 100 days posttransplantation.

Islet transplantation. Immediately before transplantation, islets were divided in aliquots of 650 IEQ per recipient. Under general anesthesia induced by methoxyflurane (Metofane; Schering-Plough Animal Health, Atlanta, GA), a left lombotomy was performed, and the left kidney was exposed. A breach was made in the kidney capsule, and a polyethylene catheter was introduced through the breach and advanced in the subcapsular space to the opposite pole of the kidney. Islets were slowly injected and allowed to spread at the pole. The catheter was then retrieved, the opening was cauterized, and the kidney was repositioned, followed by suturing of muscle and skin.

Graft survival analysis. Blood glucose levels were measured daily after transplantation on whole blood samples collected from the tail vein using a strip glucometer (Elite, Bayer). Graft survival was calculated as the number of days before diabetes recurrence. The day of diabetes recurrence was defined as the first of 2 consecutive days of nonfasting blood glucose >250 mg/ml. Mice were killed after confirmation of diabetes recurrence, and the transplanted kidneys were harvested for histology.

Histology and immunohistochemistry. Graft-bearing kidneys were fixed in 10% buffered formalin and embedded in paraffin at the time of diabetes occurrence or at selected time points after transplantation. Sections were stained with hematoxylin and eosin. Insulin staining was performed using a guinea pig anti-insulin primary antibody (Dako, Carpinteria, CA) and a biotinylated rabbit anti-guinea pig (Zymed, South San Francisco, CA) secondary antibody. Glucagon staining was performed on serial sections using a rabbit anti-glucagon primary antibody (Zymed) and a biotinylated goat anti-rabbit secondary antibody. Staining was revealed using a streptavidin-horseradish peroxidase system (Zymed) and a red chromogen aminoethylcarbazole (AEC).

Sections of the graft-bearing kidneys snap-frozen in OCT compound (Sakura Finetek, Torrance, CA) were stained by the use of biotinylated antibodies directed to CD4 (L3T4; Pharmingen, San Diego, CA) or CD8a (Ly-2; Pharmingen) followed by streptavidin-horseradish peroxidase and AEC. Slides were analyzed and scored by four different observers for CD4 and CD8 percentages in a blind manner.

Flow cytometry analysis. Splenocytes and peripheral blood lymphocytes of treated or untreated NOD mice were obtained by purification on ficoll gradients and incubated with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 or phycoerythrin-conjugated rat anti-mouse CD8 (Pharmingen) and analyzed on a Coulter flow cytometer (Beckman Coulter, Fullerton, CA).

Statistical analysis. The Statistica software package (Statsoft, Tulsa, OK) was used for statistical analysis. Data were expressed as median and range or mean \pm SE, wherever appropriate. Kaplan-Meier analysis was performed for diabetes-free survival determination, and differences were assessed with the Mantel-Cox log-rank test. Values of $P < 0.05$ were considered significant. Student's t test was used for comparison of flow cytometry data.

RESULTS

MR1 treatment delays, but does not prevent, diabetes occurrence in NOD female mice. Treatment of female NOD mice with MR1 starting at 4 weeks of age had no effect on the spontaneous incidence of diabetes. On the other hand, some delay in diabetes occurrence was observed when anti-CD154 monoclonal antibody administration was started at 8 weeks of age. Analysis of diabetes prevalence at 32 weeks of

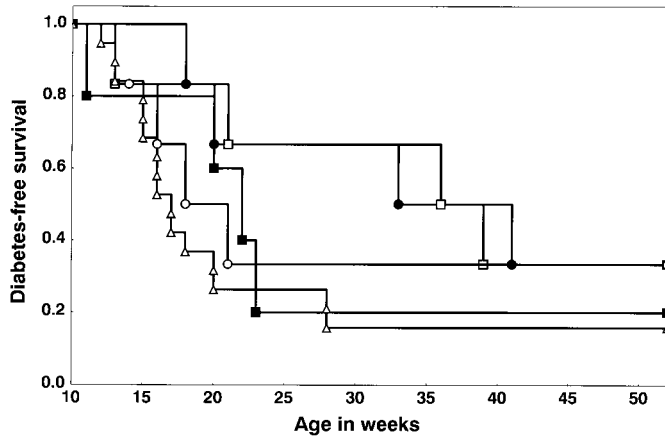


FIG. 1. Diabetes-free survival plot in NOD mice. Weekly intraperitoneal administration of 20 mg/kg MR1 was given for 6 weeks (long protocol) to 4-week-old (○) or 8-week-old (●) female NOD mice, and 10 mg/kg of MR1 was given every other day for 6 days (short protocol) to 8-week-old female NOD mice (□). Control groups received either Ha4/8 (■) or no treatment (△). Mice were checked twice weekly for glycosuria. Diabetes was defined as at least three consecutive blood glucose readings >250 mg/dl. No statistically significant differences were observed between any of the experimental groups (Mantel-Cox log-rank test).

age, when 80% of the control animals had developed diabetes, showed a protective effect of the treatment, with only 33% of the MR1-treated mice having developed diabetes at that age (MR1 vs. nontreated, long protocol $P = 0.02$ and short protocol $P = 0.04$). Importantly, however, longer follow-up of the mice (52–60 weeks of age) revealed that diabetes incidence in the MR1- and the Ha4/8-treated groups was no longer significantly different. Median diabetes-free survival time for the MR1-treated group was 33 weeks for the long protocol and 28 weeks for the short protocol, 21 weeks for the Ha4/8-treated group, and 16.5 weeks for the nontreated group (Fig. 1).

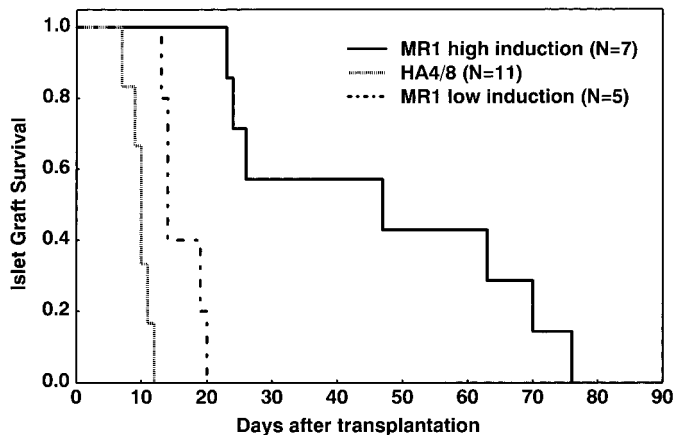


FIG. 2. Graft survival of allogeneic B6 islets transplanted into spontaneously diabetic NOD mice. Overtly diabetic NOD mice were transplanted with 650 IEQ under the kidney capsule and treated with 10 mg/kg (low induction dose) or 20 mg/kg (high induction dose) of MR1 or Ha4/8 on days -1, 0, 3, and 7 after transplantation, and every 7 days thereafter, until recurrence of diabetes (see RESEARCH DESIGN AND METHODS). Significant differences exist between MR1- and Ha4/8-treated groups (high-induction dose group $P = 0.0002$ and low-induction dose group $P = 0.002$). A significant difference was also observed when the two MR1 treatment groups were compared ($P = 0.0003$).

MR1 treatment significantly prolongs survival of islet allografts in spontaneously diabetic NOD female mice.

First, we explored a B6-into-NOD combination in which allogeneic and autoimmune components were involved in graft loss. With low-dose MR1-induction therapy (10 mg/kg), a small but significant prolongation of graft survival ($P = 0.002$) was observed (median 14 days, range 13–18 days) compared with the Ha4/8-treated group (median 10 days, range 7–12 days). When a higher induction dose of MR1 (20 mg/kg) was used, the graft survival difference between treated and control mice was markedly increased. Median survival was 46 days for the MR1 group (range 23–77 days) and 10 days for the Ha4/8 group (range 9–12 days) ($P = 0.0002$). A significant difference in graft survival times was also observed when the groups treated with high and low induction doses of MR1 were compared with each other ($P = 0.0018$) (Fig. 2).

MR1 treatment delays recurrence of autoimmunity in diabetic NOD female mice.

To analyze the effect of MR1 treatment on the autoimmune component of the observed graft loss, spontaneously diabetic (autoimmune) female NOD mice received syngeneic islet grafts from young male NOD mice. Different groups of recipients either were untreated or were treated with the irrelevant antibody (Ha4/8) or the MR1 antibody in the same manner as described in the B6-to-NOD group. Graft loss occurred between days 9 and 17 (median 12 days) in the untreated group and between days 10 and 35 (median 21 days) in the control group treated with the irrelevant antibody (Ha4/8) (no statistically significant difference). On the other hand, a significant delay in graft loss was observed in the group of mice treated with the low-induction dose of MR1 (median 38, range 24–54 days) when compared with either control group (MR1 vs. untreated group $P = 0.007$; MR1 vs. Ha4/8 $P = 0.016$) (Fig. 3). No measurable additional prolongation was observed when 20 mg/kg MR1 was used as an induction dose (median 22, range 7–50 days).

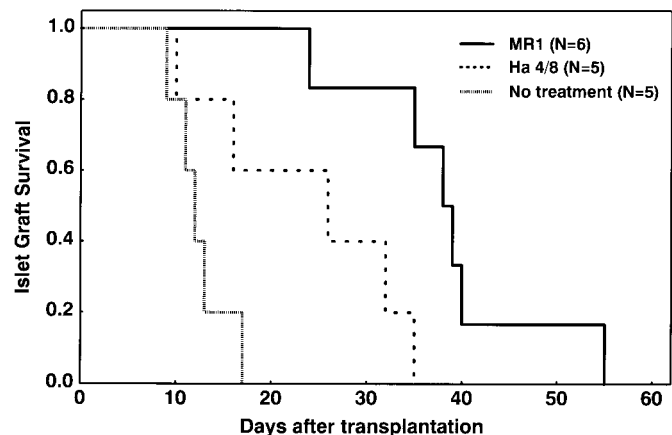


FIG. 3. Graft survival of syngeneic islets transplanted into spontaneously diabetic NOD mice. Male NOD islets (650 IEQ) were transplanted in the renal subcapsular space of overtly diabetic NOD mice. Recipients were untreated or were treated with either MR1 or Ha4/8 at 10 mg/kg (low induction dose) on days -1, 0, and 3 and at 20 mg/kg on day 7 and every 7 days thereafter, until recurrence of diabetes. Significant differences were observed between MR1- and Ha4/8-treated groups ($P = 0.016$) and between the MR1-treated and the untreated groups ($P = 0.007$).

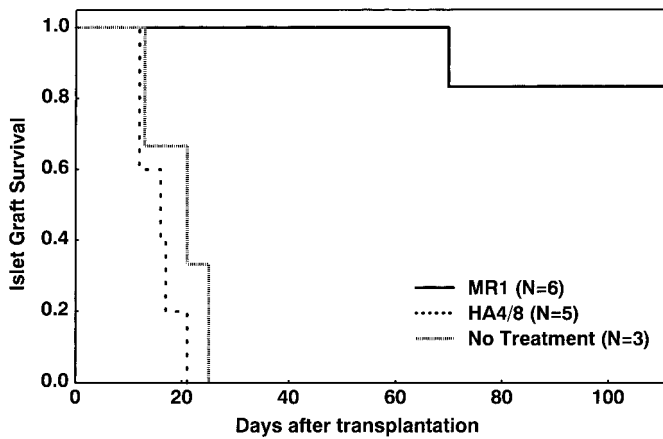


FIG. 4. Allograft survival in chemically induced diabetic male NOD mice. Male 5- to 7-week-old NOD mice were rendered diabetic via a single injection of streptozotocin (200 mg/kg) and transplanted with 650 IEQ under the kidney capsule. Recipients were treated with 20 mg/kg of MR1 or Ha4/8 on days -1, 0, 3, and 7 posttransplant and once a week thereafter, until rejection or until day 98 posttransplantation. Graft survival in the MR1-treated group was significantly longer than in the Ha4/8-treated recipients ($P = 0.00067$) or in the untreated controls ($P = 0.00018$).

MR1 induces long-term survival of islet allografts in NOD mice. This set of experiments was aimed at analyzing the effect of MR1 administration on graft performance in a major histocompatibility complex (MHC)-mismatched setting in the absence of the autoimmune component. In this case, chemically induced diabetic male NOD mice were used as recipients of B6 islets and treated with MR1 or Ha4/8 at the doses previously mentioned.

Male NOD mice treated with Ha4/8 rejected their grafts between 12 and 21 days after transplantation (median 16.5 days), whereas MR1-treated mice remained normoglycemic for >100 days (Fig. 4). Graft failure occurred in only one of the recipients (71 days after transplantation), with histological features suggestive of autoimmune destruction. A second recipient died of unrelated causes 90 days after transplantation, bearing a fully functional graft.

Histology and immunohistochemistry. Hematoxylin and eosin staining of islet grafts obtained 10 days after transplantation showed a strong mononuclear infiltration of the site of transplantation in both MR1-treated (Fig. 5A) and control (Fig. 5D) group in the B6 to female NOD transplant combination. Despite mononuclear infiltration in both groups (treated and control), islet architecture was preserved in MR1-treated mice (Fig. 5A), whereas in the control recipients, islets were mostly destroyed (Fig. 5D).

Immunohistochemical analysis of the graft insulin and glucagon staining in the pure autoimmune setting (NOD to NOD) showed absence of insulin staining and presence of glucagon-positive cells. These characteristics were observed in both MR1-treated (Fig. 5B and C) and control Ha4/8-treated mice (not shown). Mononuclear cell infiltration was also observed in mice of both groups (not shown).

When analysis was performed at the time of diabetes recurrence on the spontaneously diabetic NOD mice that received B6 islets and the control antibody Ha4/8 (Fig. 5D, E, and F), the heavy mononuclear cell infiltration observed (Fig. 5D)

was paralleled by islet architecture derangement and minimal or absent glucagon and insulin staining (Fig. 5E and F).

Female NOD mice that received MR1 after the allotransplant examined at the time of graft loss also showed heavy mononuclear infiltration, with some remaining islet tissue (Fig. 5G). Whereas glucagon staining was often observed in the insular tissue, insulin staining was minimal or absent.

Histological and immunohistochemical analysis, performed at the time of diabetes recurrence in male NOD mice that received a B6 allograft and Ha4/8, showed mononuclear infiltration and absence of both glucagon and insulin staining (not shown).

MR1-treated NOD male mice with a functioning B6 graft showed a lack of infiltration (Fig. 5J), well-preserved glucagon (Fig. 5K), and insulin (Fig. 5L) staining.

The one male NOD mouse that developed hyperglycemia 71 days after transplant had no insulin staining but measurable glucagon staining (not shown).

MR1-induced protection is not associated with T-cell subset alteration. To determine whether the therapeutic effect obtained by the treatment with anti-CD154 was related to a measurable alteration in the relative percentages of the main T-cell subsets, CD4⁺ and CD8⁺ T-cell populations were analyzed in peripheral blood, spleen cells, and graft-infiltrating cells of treated (MR1) and control (Ha4/8) allograft recipients 10 days after transplantation. Immunohistochemistry of the graft site revealed similar mononuclear accumulation around the grafted tissue in both groups, although preservation of the islet structure was evident in the tissues obtained from MR1-treated mice and not in the control groups. The proportion and distribution of CD4⁺ and CD8⁺ cells in the graft site was comparable in MR1- and Ha4/8-treated recipients (Table 1). In addition, phenotype characterization of T-cells purified from peripheral blood or spleens showed no significant differences in the percentages of CD4⁺ and CD8⁺ cells, and the CD4⁺/CD8⁺ ratios remained comparable in all female NOD recipient mice, regardless of the treatment (Table 1).

DISCUSSION

In this study, we explored the effect of long-term anti-CD154 monotherapy on the outcome of islet transplantation. We demonstrated the differential efficacy of this treatment in preventing rejection of islet allografts and in delaying the autoimmune process that leads to type 1 diabetes recurrence in overtly diabetic mice receiving a syngeneic islet transplant.

In addition, a set of experiments was performed to assess the effects of the anti-CD154 antibody administration on occurrence of spontaneous diabetes in NOD mice. In the type 1 diabetes prevention experiments, no beneficial effect was observed when treatment was started at 4 weeks of age. Treatment started at 8 weeks of age delayed but did not permanently prevent the occurrence of diabetes in NOD mice. In fact, when the analysis of diabetes occurrence was performed after 41 weeks of age, no significant differences were detected. Surprisingly, even chronic MR1 administration did not result in further delay of type 1 diabetes occurrence or in reduced incidence at later time points (data not shown). These observations are at variance with previous reports of prevention of diabetes using different MR1 treatment regimens (17,24). Notably, in one study reporting total prevention of diabetes occurrence, the follow-up of the experimental mice was interrupted at 24–31 weeks of age (17).

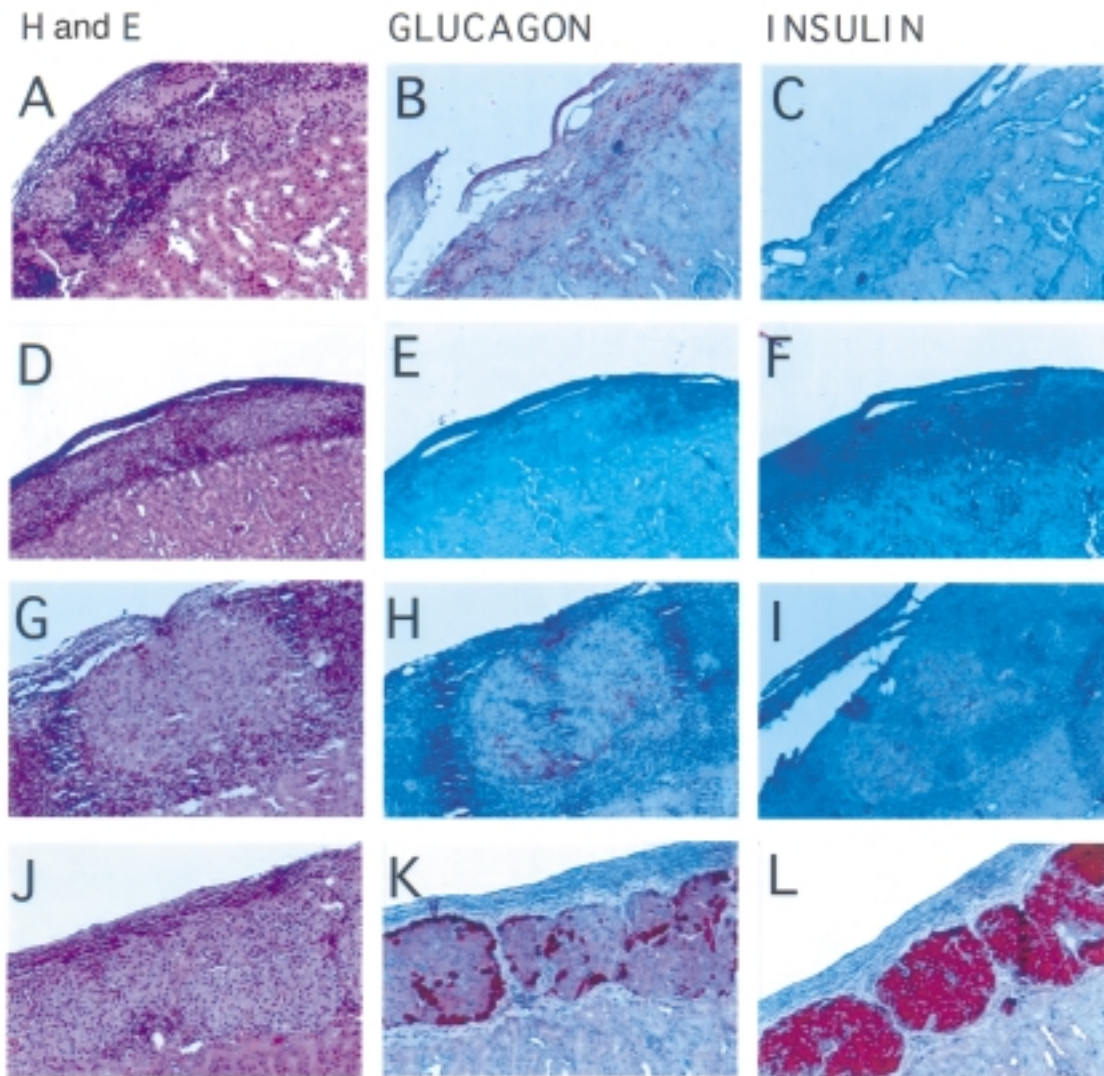


FIG. 5. Histological analysis of islet grafts in NOD mice. *A*: Staining of functioning allogeneic (B6) islets 10 days after transplantation into an MR1-treated female NOD recipient shows mononuclear infiltration with preservation of islet architecture (hematoxylin and eosin, 200 \times). *B* and *C*: Immunohistochemistry of syngeneic islets transplanted into a female NOD mouse treated with MR1, at the time of diabetes recurrence, shows presence of glucagon-producing cells (*B*, 200 \times) and absence of insulin staining in the graft (*C*, 200 \times). *D–F*: Representative graft from a female NOD recipient of B6 islets and Ha4/8, at the time of rejection (10 days), shows heavy mononuclear infiltration, disruption of islet architecture (*D*; hematoxylin and eosin, 100 \times), and absence of both α - (*E*; anti-glucagon, 100 \times) and β -cells (*F*; anti-insulin, 100 \times). *G–I*: hematoxylin and eosin (*G*, 200 \times), glucagon and insulin staining of an islet graft from an MR1-treated female NOD recipient of allogeneic B6 islets, at the time of diabetes recurrence, shows a heavy mononuclear cell infiltrate surrounding residual islets; presence of glucagon-producing cells (*H*, 200 \times) and nearly complete absence of insulin staining (*I*, 200 \times). *J–L*: Staining of a functional B6 islet allograft 90 days after transplantation in a male NOD treated with MR1 shows preservation of islet graft structure and minimal cellular infiltration (*J*; hematoxylin and eosin, 200 \times); glucagon-producing cells confined to the periphery (*K*, 200 \times) and insulin-containing cells accounting for ~80% of the islet cell mass (*L*, 200 \times).

Doses and schedule of antibody administration were also different in the three studies, suggesting that they are critical parameters for achieving a protective effect, and that CD40-CD154 interaction may play a measurable role in delaying the occurrence of spontaneous type 1 diabetes. On the other hand, a permanent effect has not yet been demonstrated by long-term follow-up in either study.

Transplantation of allogeneic islets into spontaneously diabetic NOD mice represents a model of the situation most frequently found in clinical islet transplantation in diabetic patients, in which largely or fully MHC-mismatched islets are exposed to graft rejection and to autoimmune recurrence of diabetes. In this model, anti-CD154 therapy was effective in

prolonging islet graft survival, but did not provide permanent protection from diabetes recurrence.

In a previous study, Markees et al. (24) reported that MR1 administration alone or in combination with donor-specific transfusion had no effect on allograft or isograft survival in spontaneously diabetic NOD mice, whereas the same treatment led to permanent allograft survival in chemically diabetic recipients. In that study, a high rate of graft failure immediately after transplantation, similar to that described in primary nonfunction, was observed in treated and untreated NOD recipients. The authors attributed the early graft loss to a generalized defect of the NOD mice to respond to transplantation tolerance induction. In our study, we did not observe the

TABLE 1
Distribution of CD4⁺ and CD8⁺ cell subsets in female NOD mice recipient of C57BL/6 islet grafts

Group	CD4 ⁺ :CD8 ⁺		CD4:CD8%
	Peripheral blood	Spleen	Graft
MR 1	2.2 ± 0.5 (8)	1.9 ± 0.3 (5)	40:60 (3)*
Ha4/8	2.2 ± 0.2 (5)	2.0 ± 0.2 (3)	52.5:47.8 (3)*
Untreated	2.1 ± 0.1 (2)	1.7 ± 0.0 (2)	ND
Untransplanted, untreated	3.0 ± 0.4 (8)	2.0 ± 0.5 (2)	ND

Data are means ± SE (*n*) or ratio (*n*). Lymphocytes from female NOD recipients of C57BL/6 islets or untransplanted NOD controls were obtained from peripheral blood or spleens 10 days after transplantation. Ratios were calculated based on percentage of CD4⁺ and CD8⁺ cells analyzed on a fluorescence-activated cell sorter after staining with specific monoclonal antibodies. No statistically significant differences were observed in CD4 and CD8 percentages in all groups. *Histological sections were stained with antibodies specific for CD4 and CD8 and then analyzed and scored by four different observers in a blind fashion. Average results are expressed in percentages. ND, not determined.

occurrence of this phenomenon, and engraftment was achieved in all of the transplants. Several differences in the technical procedures, the most relevant being the greater islet graft mass transplanted in our study, could be responsible for the divergent observations.

Histology of functioning grafts, obtained from MR1-treated mice at early time points (10 days after transplantation) showed degrees of mononuclear infiltration comparable with those observed in rejecting control mice, demonstrating that MR1 treatment did not prevent mononuclear cell migration to the graft site. Furthermore, analysis of the infiltrating cells revealed no alteration in the CD4⁺/CD8⁺ subset distribution between MR1- and control Ha4/8-treated mice. However, the general islet architecture was preserved in treated mice, as opposed to control mice, in which islets were completely infiltrated with a highly disrupted architecture. This observation coincides with previous studies in which anti-CD154 treatment did not prevent mononuclear infiltration of transplanted kidneys and hearts, despite its protective effect on graft function (10,26,27).

In the purely autoimmune combination, insulin staining at the time of graft failure was undetectable, with well-preserved glucagon staining and a significant perigraft mononuclear infiltrate.

Similarly, in the B6-to-autoimmune diabetic NOD combination, the few islets that were still present at the site of implant had measurable glucagon staining with minimal or absent insulin staining. It is important to note the presence of unstained cells, likely representing degranulating β-cells. It is conceivable that degranulation of the residual β-cell mass might derive from the hyperglycemic state that follows destruction of a large part of the graft. Glucagon cells are not subjected to a similarly increased secretory demand, and therefore maintain the intracellular hormone content. An additional possibility is that the production of cytokines by the infiltrating cells might contribute to the β-cell degranulation (28–30). Although in the pure autoimmune combination

it is safe to assume that only β-cells are targeted by the destructive process, it is important to understand the relative contribution of the autoimmune recurrence in the loss of B6 islets in NOD female mice. It is conceivable that autoimmunity plays a measurable role in the failure of the grafts in the B6-to-female NOD combination, in view of the survival and microscopic appearance of the B6 grafts implanted in male NOD mice, where an identical allogeneic mismatch exists but autoimmunity does not. The histological data in the B6-to-female NOD combination, on the other hand, does not allow us to definitively conclude that autoimmunity alone is responsible for the graft loss. We might speculate that autoimmune recurrence, which indeed occurs as shown in the NOD-to-NOD combination, might contribute to overcoming the CD154 blockade-mediated protection from allorecognition by amplifying afferent mechanisms that can contribute to graft failure via allorecognition.

The median time to recurrence observed with MR1 therapy in the B6-into-NOD group was substantially longer than that observed in the NOD-to-NOD combination, in which autoimmunity is the only cause of islet loss. This could be interpreted as the result of a higher fragility or susceptibility of NOD islets to immunological or nonspecific inflammatory insults, leading to a faster destruction of the graft after transplantation. Another possible explanation for the shorter protective effect observed in the autoimmune setting might be the requirement of at least partial MHC matching for the development of a faster immune response against islets autoantigens. Indeed, immune infiltration and destruction of islet grafts by autoreactive β-cell-specific T-cell clones of NOD origin have been shown to be an MHC-restricted phenomenon (31).

Interestingly, a measurable effect of the administration of the irrelevant antibody Ha4/8 was observed in a selected condition (NOD-to-diabetic female NOD). The delay in autoimmunity recurrence did not reach but clearly approached statistical significance. At variance, the administration of the same antibody in the B6-to-diabetic female NOD mice and in the B6-to-male NOD groups did not lead to a measurable delay in graft failure. Irrelevant hamster immunoglobulin has been used as a control antibody in several studies, and no effect on islet graft survival or on T-cell dependent immune responses has been previously reported, although it was not tested in the NOD-to-NOD combination (18). However, a biological effect of a control hamster antibody has been previously reported in a murine model of collagen-induced autoimmune arthritis (13).

In the pure allogeneic combination (B6 to chemically diabetic NOD), long-term survival of islet grafts was achieved in a majority of treated mice. Our results differ from previous studies in which MR1 monotherapy was insufficient to prevent islet allograft rejection in mice (18,32). However, the dosage and timing of therapy used in these studies were different, and these parameters seem to be critical for the success of treatment (10,20,33,34). In this group, the only mouse to return to a hyperglycemic state showed a histological pattern similar to that observed in the NOD-to-female NOD combination, suggesting a role for autoimmunity in the destruction of the graft. Occurrence of autoimmunity is expected in <5 to 65% of male NOD mice, depending on origin of the colony and environmental factors (35). Interestingly, the incidence of autoimmunity in the treated group (1 in 6) was much lower than that of control untransplanted litter-

mates (44%). The rest of the mice (except for the one that died of unrelated causes 90 days after transplantation bearing a functional graft) were normoglycemic for >100 days. At that time, therapy was discontinued, and mice remained normoglycemic for >30 days thereafter. We did not test shorter MR1 administration protocols, but it is conceivable that earlier discontinuation of therapy could still have been protective, as shown in other transplantation models in which the beneficial effect of the antibody persisted despite therapy discontinuation (13,21,23).

In conclusion, our data indicate that blockade of the CD40-CD154 pathway by the use of anti-CD154 monoclonal antibody might represent a promising immunosuppressive strategy to promote survival of islet grafts. Anti-CD154 monotherapy leads to long-term islet allograft survival in mice, as has already been reported in nonhuman primate studies (21,22). CD40-CD154 blockade also delays autoimmune graft destruction in NOD mice, although it appears insufficient to permanently prevent it. Therefore, strategies combining anti-CD154 monoclonal antibody and other immunosuppressive agents must be explored to overcome this obstacle.

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