

# Anti-CD154 (CD40L) Prevents Recurrence of Diabetes in Islet Isografts in the DR-BB Rat

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**Islet transplantation for the treatment of autoimmune diabetes is more difficult because of the additional barrier presented by the autoimmunity. We tested the ability of hamster anti-rat CD154 to prevent recurrence of diabetes in renal subcapsular islet isografts in DR-BB (RT1uu) rats with established autoimmune diabetes. Experimental animals with established diabetes received intravenous injections of 15 mg/kg anti-CD154 on a specified schedule starting 2 days before renal subcapsular transplantation of an islet isograft. Control animals received either saline or hamster IgG. Plasma glucose levels >250 mg/dl over 3 days were used to indicate the recurrence of diabetes. Rats that received saline ( $n = 5$ ) or control antibody ( $n = 3$ ) had a recurrence of diabetes 6–11 days after transplantation. Histological examination of islet isografts from these rats showed complete destruction of the insulin-producing portion of the isograft with residual cells positive for glucagon. Recipient rats that received anti-CD154 at the 15-mg/kg dosage ( $n = 6$ ) did not have a recurrence of diabetes for 308–461 days after transplantation. Islet isografts removed from the rats showed low levels of insulin immunoreactivity, high levels of insulin mRNA, and focal infiltration with lymphocytes but no evidence of islet destruction. Mean peak antibody concentration was 266  $\mu\text{g/ml}$  and returned to undetectable levels by 67–88 days after transplantation. Rats that received anti-CD154 starting at 4–7 days after transplantation had a recurrence of diabetes within 11 days of the isograft transplantation. Therefore, anti-CD154 as the sole immunomodulator prevented the recurrence of diabetes in islet isografts in rats with established autoimmune diabetes. This suggests that CD40/CD154 blockade is effective in preventing the insulinitis or the effector phase of autoimmune diabetes. *Diabetes* 49:1666–1670, 2000**

**A**utoimmune diabetes in animal models and humans results from the development of autoreactive T-cells that are targeted to destroy the  $\beta$ -cells, which results in the insulinitis characteristic of type 1 diabetes. Therefore, the initiation of the loss of self-tolerance and the effect of the loss of tolerance in autoimmune diabetes both appear to be T-cell dependent (1,2). Even though the exact mechanism for the development of autoreactive T-cells is unclear, the initiation of the autoimmune response probably involves interaction between the major histocompatibility complex (MHC) protein of antigen-presenting cells (APCs) and the T-cell receptor on T-cells. In addition to the MHC-T-cell receptor interaction, other receptor-ligand interactions between APCs and T-cells are important in determining the outcome of the APC-T-cell interaction. The interaction of CD40 on APCs and of CD154 on T-cells represents one of the important costimulatory pathways (3–6).

Regarding the insulinitis of autoimmune diabetes, the CD40/CD154 costimulatory pathway may also be important at the effector phase of the autoimmune response through interactions between CD154<sup>+</sup> T-cells and CD40<sup>+</sup> endothelium and/or macrophages at the site of inflammation. Therefore, modification of the CD40/CD154 costimulatory pathway's interactions could potentially prevent or modify not only the T-cell responses that initiate autoimmune diabetes but also the T-cell functions that result in the insulinitis. This is of special importance in islet transplantation for the treatment of autoimmune diabetes because the autoimmune response is already established at the time of transplantation, and recurrence of insulinitis depends on the effector phase of the immune response involving constitutively expressed components of  $\beta$ -cells.

We have demonstrated that blockade of CD40/CD154 prevents rejection of islet allografts in streptozotocin-induced diabetic rats by modifying both the initiation and effector phases of the alloimmune response (7). This suggested that CD40/CD154 blockade may be successful in preventing the recurrence of diabetes in islet isografts by preventing the effector phase of the autoimmune response. Anti-CD154 treatment of autoimmune diabetic NOD mice did not prevent destruction of isografts or allografts with or without donor-specific transfusion (8). Because the effect of anti-CD154 was different in rats and mice regarding islet allografts, we tested the ability of hamster anti-rat CD154 (AH.F5 clone, Biogen, Cambridge, MA) to prevent the recurrence of diabetes in renal subcapsular islet isografts in DR-BB rats with established autoimmune diabetes.

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APC, antigen-presenting cell; MHC, major histocompatibility complex; poly-IC, polyinosinic-polycytidylic acid.

## RESEARCH DESIGN AND METHODS

**Animals.** DR-BB rats (RT1<sup>uu</sup>) were obtained from Biomedical Research Models (Rutland, MA). Diabetes in the recipient rats was induced by treatment with RT6.1 T-cell-depleting antibody and polyinosinic-polycytidylic acid (poly-IC). Plasma glucose levels were determined with a One Touch II glucose meter (Johnson & Johnson, Milpitas, CA) using plasma from blood samples obtained from the tail vein. Plasma glucose levels >250 mg/dl over 3 consecutive days were considered diagnostic of diabetes. The DR-BB rats developed diabetes by 21 days after initiation of the depletion. The rats were maintained for an additional 28 days after the development of diabetes by implanting an Alzet minipump loaded with U500 pork insulin calibrated to deliver 4 to 5 U insulin/day. This was done to allow repletion of the RT6.1 T-cell population (9). The insulin is not given until after clear development of diabetes, at which time the  $\beta$ -cells of the islets have been completely destroyed. Therefore, the insulin should not have any effect on the immune process leading to the onset of diabetes. Anti-CD154 alone does not have any effect on T-cell number. We have observed an effect of circulating anti-CD154 on mixed lymphocyte reaction in the rats bearing islet allografts (7), so the time after transplantation was extended beyond the time when anti-CD154 concentrations become negligible. Plasma glucose levels were determined daily until resolution of the diabetes after transplantation and then either weekly until termination of the experiment or daily if diabetes recurred.

**Islet isolation and transplantation.** Islets were isolated from the pancreases of DR-BB rats using a modification of a method of collagenase digestion (10). The pancreases of 300-g adult rats were cannulated in situ via the common bile duct and distended using 20 ml of a solution of collagenase in Hank's balanced salt solution. The pancreases were removed by blunt dissection and were placed in a sterile petri dish on ice for removal of fat. The flasks with pancreases were incubated for ~14 min in a stationary water bath at 37°C. The incubated pancreases were placed in a 50-ml tube with 25 ml cold RPMI with 5% newborn calf serum and were shaken vigorously for 15 s. The tubes were centrifuged for 40 s at 1,000 rpm to sediment the contents. The pellet was resuspended in 20 ml cold RPMI and was sedimented for another wash. The resuspended pellet was filtered through a stainless steel screen with a 0.6-mm pore size. The filtrate was sedimented for 2 min at 1,000 rpm, and the supernatant was discarded. The pellet was resuspended in 10 ml Histopaque with a specific gravity of 1.109 by repeated pipetting (20 times) with a 10-ml pipette. Cold RPMI was layered on top, and the gradient was centrifuged for 10 min at 2,400 rpm at 4°C. The supernatant was collected and centrifuged at 1,000 rpm for 5 min to sediment the particulate material. The pellet was resuspended in HF12 medium with 10% fetal calf serum, and 10-ml aliquots were allowed to settle for 1 min at unit gravity. Then 8 ml of the supernatant was transferred to another 15-ml tube and allowed to settle for 3 min. A total of 7 ml of this supernatant was transferred to another tube and allowed to settle for 5 min. The pellet from each sedimentation representing highly purified islets was cultured overnight at 37°C in HF12 plus 10% fetal calf serum. Each recipient received 1,400 EIN (islet equivalent number) to the renal subcapsular site on a kidney delivered into the incision in the left flank.

**Administration of anti-CD154.** Hamster anti-rat CD154 was administered intravenously by the tail vein daily starting 2 days before and on the day of transplantation at a dosage of 15 mg · kg<sup>-1</sup> · dose<sup>-1</sup>. Doses were then given 3 times/week for a total of 12 doses. Another group of rats received daily doses of anti-CD154 starting at 4–7 days after transplantation. Control animals received either saline or nonspecific hamster antibody on the same schedule. Levels of hamster antibody were determined on a specified schedule for 20–30 days and then weekly after initiation of treatment.

**Histology.** Grafts were removed either at the time of or before recurrence of diabetes and were fixed in formalin for paraffin imbedding and sectioning to determine the grade of inflammation. As previously described for allograft rejection (11), grade 0 indicates no cellular response, grade 1 indicates peritransplant infiltration, grade 2 indicates infiltration of the allograft with min-

imal islet destruction, grade 3 indicates infiltration with significant islet destruction, and grade 4 indicates complete destruction of the allograft with fibrosis of the transplant site. The sections were also stained immunohistochemically for insulin and glucagon. Insulin mRNA was determined by in situ hybridization.

## RESULTS

Recipient DR-BB rats that received either saline ( $n = 3$ ) or hamster control antibody ( $n = 5$ ) injections had a recurrence of diabetes within 6–11 days after transplantation (Table 1). Histology of the islets in these animals showed grade 3 to 4 inflammation with no residual staining for insulin but residual staining for glucagon.

All of the rats ( $n = 6$ ) that received anti-CD154 had prompt resolution of the diabetes after islet transplantation without recurrence of the diabetes for up to 461 days after transplantation (Fig. 1). The rats have not required additional anti-CD154 treatment to prevent recurrence of the diabetes. Islet isografts obtained from the treated rats at 40 days after transplantation showed focal accumulations of lymphocytes or grade 1 inflammation. These focal accumulations of lymphocytes persisted and were observed in an isograft removed 267 days after transplantation (Fig. 2). This isograft stained positive for glucagon, whereas the immunostaining for insulin was limited to the subcapsular rim of the isograft. Insulin mRNA detected by in situ hybridization, however, was strongly positive throughout the isografts. This suggests that the insulin is being secreted toward the parenchyma of the kidney rather than the capsule and that the full productive capacity of the isograft is being used to maintain normoglycemia.

The mean peak anti-CD154 concentration was 266  $\mu$ g/ml during antibody treatment. Antibody levels were undetectable at ~66–88 days after transplantation (Fig. 3). The rate of disappearance of the antibody was greater in the autoimmune diabetic rats than in the streptozotocin-induced diabetic rats (7).

## DISCUSSION

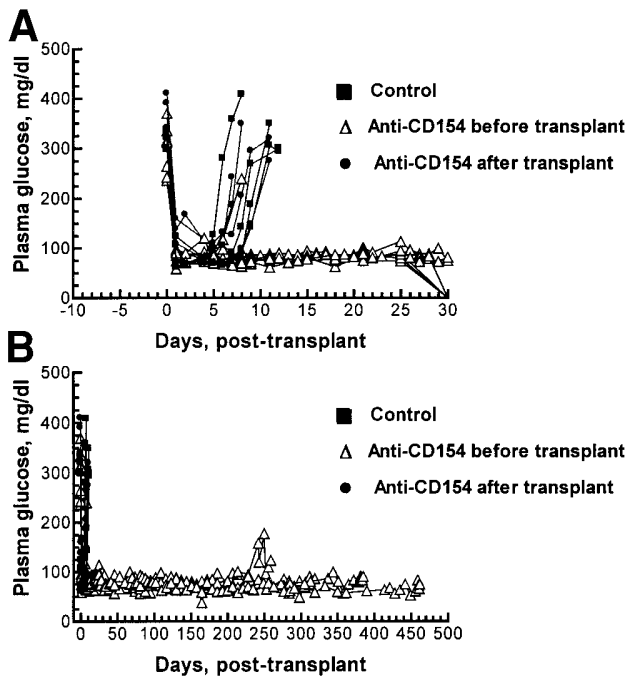
The understanding that antigen presentation resulting in complete T-cell activation requires two different classes of receptor–ligand interactions has led to the development of specific therapies that modify the T-cell response to antigen presentation. Antibodies specific for CD154 (CD40 ligand) on the T-cell are one example of therapies that modulate these costimulatory receptor–ligand interactions. Anti-CD154 monoclonal antibodies developed for humans and mice have been useful in defining the role of this costimulatory pathway. Anti-CD154 has been tested either alone or in combination with other immunomodulators to prevent rejection of allografts and/or xenografts in mice and nonhuman primates,

TABLE 1

Effect of anti-CD154 on prevention of recurrence of diabetes in islet isograft in rats with established autoimmune diabetes

Doses (number of animals)	Days of isograft survival after transplantation	Day of undetectable antibody concentration	Day of recurrence of diabetes
Saline control ( $n = 3$ )	—	—	6, 11, 11
Hamster Ig control ( $n = 5$ )	—	—	6, 9, 11, 11, 11
$12 \times 15$ mg/kg ( $n = 6$ )	267*, 308, 319, 322, 385, 461	88, 88, 81, 74, 74, 67	—

\*Killed at 267 days for histology of the isograft.

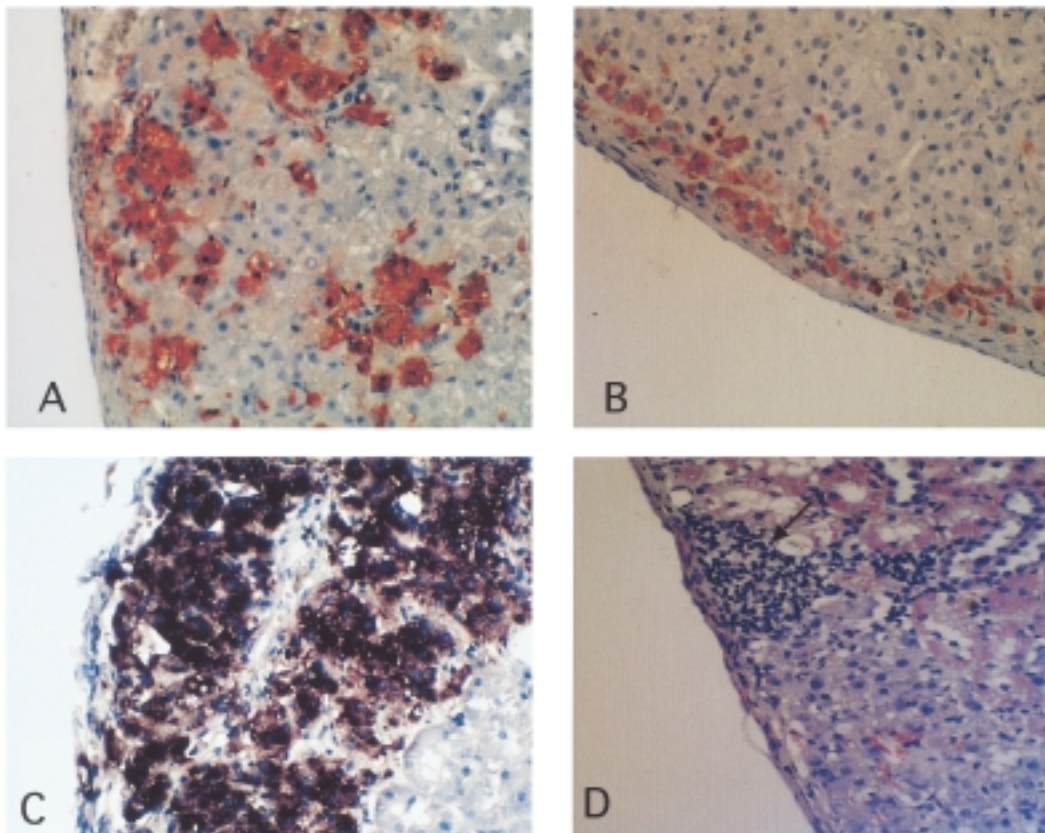


**FIG. 1.** The plasma glucose concentrations in the rats after islet isograft transplantation to the renal subcapsular site. **A:** The first 30 days show the difference between the control and treatment groups. **B:** The long-term effect of the anti-CD154 in preventing recurrence of the diabetes.

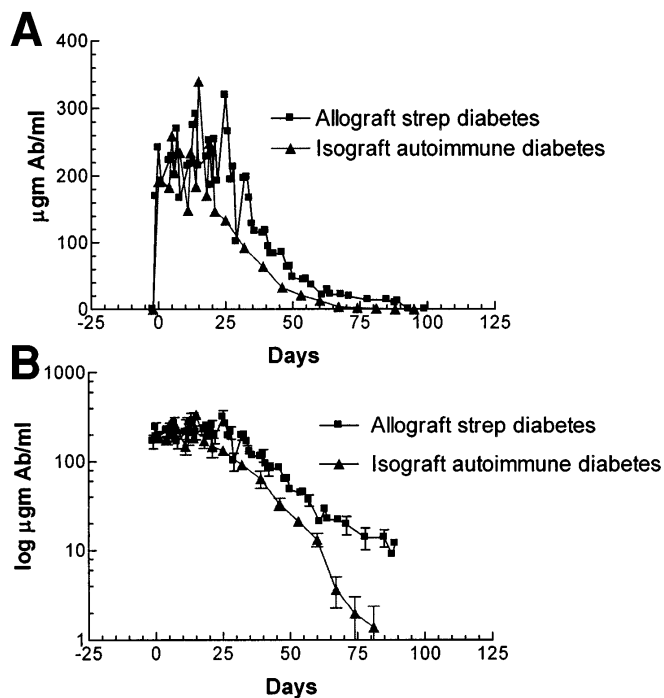
including organs or tissues such as heart (12–15), kidney (16), aorta (17), bone marrow (18), islets (19–23), and skin (6).

We have chosen the DR-BB rat as the model of autoimmune diabetes that parallels autoimmune diabetes in humans (9). The DR rat is not lymphopenic like the DP rat. The forms of autoimmune diabetes in rats and humans share a genetic susceptibility locus in class II of the MHC. The DR rats develop autoreactive T-cells that result in a susceptibility to diabetes that is expressed in response to perturbation (e.g., viral infections or poly-IC) that mimics a viral infection. After perturbation and RT6.1 depletion, the DR rats develop insulinitis that progresses to overt diabetes in a predictable and rapid manner that is essential in defining the mechanism. The insulinitis is similar to that observed in humans with targeted destruction of  $\beta$ -cells. Insulin is required for the survival of the diabetic rat. In contrast with the NOD mouse, the forms of autoimmune diabetes in the DR and DP rats and humans are relatively resistant to prevention strategies. The window of opportunity for the development of diabetes in the DR rat is limited to 30–40 days of age, so perturbation after that time does not result in diabetes. No predilection is evident for either sex in the DR-BB rat.

Regarding allogeneic islet transplantation in the mouse (12–15), anti-CD154 antibody treatment alone results in long-term survival of the allogeneic islets in 20–40% of the streptozotocin-induced diabetic mice. Mice treated with donor small lymphocytes 1 week before transplantation and anti-



**FIG. 2.** Histological examination of an islet isograft 267 days after transplantation. Immunohistochemical staining showed many glucagon<sup>+</sup> cells (red) throughout the graft (**A**), whereas insulin<sup>+</sup> cells (red) were localized at the periphery of the graft (**B**). In situ hybridization showed the presence of insulin mRNA throughout the graft (**C**). Hematoxylin and eosin staining showed focal accumulation of mononuclear cells (arrow) at the edge of the graft (**D**).



**FIG. 3. A:** The mean antibody (Ab) concentrations ( $\mu\text{g/ml}$ ) during and after treatment with anti-CD154 at a 15-mg/kg dosage versus time after transplantation of either isografts in the autoimmune diabetic DR-BB rat or allografts in the streptozotocin-induced diabetic DR-BB rat (7). **B:** The log concentration of mean antibody concentration versus time to compare the rate of disappearance of anti-CD154 in DR-BB rats with either streptozotocin-induced or autoimmune diabetes.

CD154 at the time of transplantation had long-term survival of the islet allografts. These observations, a similar requirement for donor splenocytes in skin allografts (12), and a synergistic effect of simultaneous administration of CTLA4-Ig and anti-CD154 (6,9,10) led to the hypothesis that anti-CD154 induced tolerance by preventing upregulation of B7 on donor cells that express alloantigen but no costimulatory signal. Although this may be true as a mechanism for induction of tolerance in the mouse, findings with islets and renal allografts in rat and primate models suggest a separate mechanism of action for anti-CD154 when rejection is prevented by the sole administration of anti-CD154. The rescue of longstanding islet allografts after early indication of recurrence of the diabetes would be consistent with an effect of CD40/CD154 blockade on T-cell effector mechanisms such as trafficking of inflammatory cells or expression of inflammatory cytokines within the allograft (7,23–25). This observation is of special importance when considering islet transplantation in autoimmune diabetes because the autoimmunity is already established and persists indefinitely in humans (26).

Anti-CD154 treatment in mice with relapsing experimental autoimmune encephalomyelitis suppressed relapses, which indicates a blockade of Th1 differentiation and effector function (27). In contrast, anti-CD154 (MR1) treatment of NOD mice at 3 weeks of age but not at 9–10 weeks of age prevented onset of diabetes by 24–31 weeks of age, indicating that anti-CD154 treatment impaired antigen-specific Th1 response but was not sufficient to inhibit the effector phase of the T-helper cell response (28).

We have demonstrated that treatment of recipient DR-BB rats with anti-CD154 as the sole immunomodulating agent results in long-term acceptance of islet isografts in animals with established autoimmune diabetes. In contrast with islet allografts in the streptozotocin-induced diabetic rat and primate models that require additional courses of anti-CD154 treatment to establish unresponsiveness, the islet isografts did not have a recurrence of insulinitis beyond a time when the level of anti-CD154 was negligible after the initial and only course of treatment. The results also differ from findings with the NOD mouse in which anti-CD154 did not prevent destruction of isografts or allografts in the autoimmune diabetic animal. The effect of the anti-CD154 was not modified by donor-specific transfusion before the transplantation (8). The differences between the mouse and rat could be explained by the nature of the disease. Other possible explanations for the differences between the effect of anti-CD154 in the NOD and DR-BB rat model could include differences in the nature of the antibody, timing, dosage, and route of administration. The MR1 and anti-rat CD154 (AH.F5) are clearly different, even though they share similar blocking activity of CD40/CD154 interactions. We have observed that the effect of anti-rat CD154 on allograft survival in the streptozotocin-induced diabetic DR-BB rat depends on dosage, timing, and the route of administration (7). Our experience with the effect of the route of administration is not reported, but we did not observe a similar effect of the anti-CD154 when given subcutaneously. Regardless, the response in the rat islet allograft model parallels the nonhuman primate models. A closer parallel with human islet transplantation is allotransplantation of islets into the autoimmune diabetic rat, which is a work in progress.

In the rat, anti-CD154 (AH.F5) alone prevented recurrence of insulinitis by preventing the effector phase of the autoimmune response and creating a state of unresponsiveness that persisted beyond the time when anti-CD154 could be exerting a direct effect on the immune response.

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