Anti-CD8 Abrogates Effect of Anti-CD4–Mediated Islet Allograft Survival in Rat Model

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We studied the effects of anti-CD4 treatment of diabetic ACI rats on the induction of tolerance to allogeneic (Lewis) islet allografts. When given as a 4-day treatment regimen, OX38, a mouse anti-rat CD4 antibody, caused depletion of >80% of $CD4^+$ cells from the peripheral blood of treated rats. After induction of diabetes (a single high-dose bolus of streptozocin) and 3 days after the initiation of anti-CD4 immunotherapy, recipient ACI rats were transplanted with fully allogeneic (Lewis) islets of Langerhans via the portal circulation. These transplanted islets were capable of returning the anti-CD4–treated ACI recipients to normoglycemia, which was maintained indefinitely in the absence of further immunosuppression. In contrast, treatment of recipient rats with OX8, an anti-CD8 monoclonal antibody (MoAb), induced only a slight prolongation of graft survival (≤30 days). Further characterization of the cellular requirements for the induction of long-term transplantation survival revealed that successful pretransplantation anti-CD4 therapy could be ablated by the coincident treatment of recipient rats with depleting levels of anti-CD8 MoAb. These data point to the necessity of a regulator $CD8^+$ cell in the induction of anti-CD4–mediated transplantation survival in this rat model of islet transplantation. *Diabetes* 40:1430–34, 1991

The transplantation of islets of Langerhans has long been considered an option for the treatment and possible cure of insulin-dependent diabetes mellitus (IDDM). Although exogenous insulin therapy can control blood glucose levels, it does not seem to prevent the long-term secondary complications of IDDM (1). It has been suggested that islet transplantation will not only restore physiological control of glucose homeostasis but also might result in prevention of the long-term morbidity associated with IDDM (2–6). It has been shown previously in animal models of IDDM that islet transplantation blocked and sometimes reversed the progression of secondary complications such as nephropathy (2), retinopathy (3), and neuropathy (4). Historically, the major obstacle to islet transplantation in humans has been the difficulty associated with isolating a sufficient quantity of purified islets for transplantation (7). Several recent clinical trials with allogeneic islets from multiple human donors and conventional immunosuppression (cyclosporin A [CSA], antilymphocyte globulin), demonstrated that islet transplantation can be performed in humans despite the technological difficulties inherent in the isolation of sufficient numbers of islets (8). These recent trials also underscored the need for improved methods of immunosuppression for diabetic recipients, because the common side effects of CsA, specifically hypertension and nephrotoxicity, tend to exacerbate the complications of IDDM (9).

Our laboratory demonstrated previously that treatment with depleting levels of anti-CD4 monoclonal antibody (MoAb) allowed indefinite survival of islet allografts in a mouse model (10). In this study, we used a mouse MoAb, OX38, directed against the rat CD4 molecule in a rat model of immunotherapy for islet transplantation. In rats, the CD4 surface glycoprotein is expressed on immature thymocytes, macrophages, and a subset of mature T lymphocytes (11). As with other species, including mice and humans, the subset of mature $CD4^+$ T lymphocytes constitutes the subset that interacts with exogenous antigen presented in the context of MHC class II gene products (12,13). T lymphocytes bearing the $CD4^+$ marker serve as helper-inducer cells during an immune response and are thought to be critical in driving the differentiation of precursor cells to mature effector cells (14–16). It has been demonstrated previously that anti-CD4 MoAbs exert powerful immunosuppressive effects in mice (17–27). Anti-CD4 MoAbs block the development or

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perpetuation of autoimmune diseases (17–21) and induce permanent allograft survival to transplanted tissue in mice (22–27). However, the effect of homologous reagents has been less successful in treatment protocols in humans and primates (28–32), and conflicting results have been obtained in previous rat models (33–38). Such discrepancies might reflect either intrinsic differences between mouse CD4 and other species or, alternatively, may be due to differences in the characteristics of the therapeutic MoAbs utilized. The goal of this study was to elucidate further the potential of anti-CD4 MoAbs in tolerance induction to organ allografts by studying islet transplantation in rats and to investigate the role of $CD8^+$ cells in this induced islet allograft unresponsiveness. CD8 is a cell surface glycoprotein found on mature T lymphocytes reactive with alloantigen and/or processed antigen in the presence of MHC class I gene products (12). In the studies described below, we demonstrated that administration of depleting regimens of anti-CD8 coincident with tolerance-inducing regimens of anti-CD4 ablates the establishment of long-term tolerance to Lewis islet allografts. This finding suggests that a $CD8^+$ regulatory cell is necessary for the induction or maintenance of tolerance in rats undergoing successful anti-CD4–mediated islet allograft tolerance induction.

RESEARCH DESIGN AND METHODS

Adult male ACI (*RT1*^{a}) and Lewis (*RT1*^{l}) rats weighing 150–200 g were purchased from Charles River (Wilmington, MA) or Simonsen (Gilroy, CA) and housed in viral antigen–free conditions. Injections were administered intravenously in methoxyflurane-(metofane) anesthetized animals.

OX38 was produced from a hybridoma generously provided by A.A. Like (Univ. of Massachusetts, Worcester, MA; obtained from A.F. Williams, Oxford Univ., Oxford, UK). Antibodies were harvested as ascites from hybridomas grown in Pristane (Sigma, St. Louis, MO)-primed Balb/c mice (Jackson, Bar Harbor, ME) and purified via passage over an affinity gel protein A–Sepharose column (Bio-Rad, Richmond, CA). Antibody content was quantified by optical density (Spectronic 1001, Bausch and Lomb, Rochester, NY). MoAbs OX8 (CD8), OX19 (CD5), and OX42 (C3*b* receptor) were purchased as purified preparations from Accurate Chemical and Scientific (Westbury, NY).

Fluorescence-activated cell-sorter (FACS) analysis was performed on peripheral blood lymphocytes (PBLs) to determine lymphocyte-subset frequencies in antibody-treated and control rats. One milliliter of retro-orbital blood was collected into heparinized tubes and diluted 1:4 with Ca^{2+}-Mg^{2+}–free phosphate-buffered saline (PBS, GIBCO, Grand Island, NY). The diluted sample was underlaid with Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and centrifuged at 800 × *g* for 20 min. Further elimination of erythrocytes was carried out by treatment of samples with 4 ml of an ammonium chloride and potassium solution. Cells were then washed in FACS media (PBS, 2% fetal calf serum; 100 U/ml penicillin, and 100 μg/ml streptomycin) and aliquoted into 12 × 75-mm culture tubes. Fifty microliters of pretitrated antibodies was added to the cell suspension. Antibodies were directly conjugated to either fluorescein or biotin. After 30 min of incubation on ice, the cells were washed twice with 3 ml FACS media (200 × *g* for 5 min, 4°C). If necessary, 50 μl of a pretitrated second step of avidin–Texas Red (Becton Dickinson, Mountain View, CA) was added and allowed to incubate for 30 min at 4°C.The cells were washed once more and resuspended in 150 μl FACS media. Ten thousand cells were then analyzed on a modified dual-laser FACS II system (Becton Dickinson Immunocytometry Systems) equipped with logarithmic amplifiers. A predominantly lymphocyte population was obtained by selective gating with forward and obtuse scatter. Elimination of macrophages from the population was achieved by only considering those cells that were negative for the marker OX42.

Recipient rats were made diabetic by a single 65-mg/kg i.v. injection of streptozocin (Sigma). Rats were considered diabetic and suitable for an islet transplantation when plasma glucose values rose >22.2 mM on two separate analyses at least 3 days apart. Grafts were considered to have rejected the transplant when the animals' plasma glucose readings reached a level >11.1 mM for two consecutive testings. Plasma glucose values were obtained from analysis of retro-orbital blood that was analyzed on a Beckman Glucose Analyzer II (Beckman, Fullerton, CA).

Lewis islets were isolated by collagenase digestion and Dextran (Sigma)-gradient purification. Briefly, the rat pancreases were perfused with 20 ml Hank's balanced salt solution (HBSS, Gibco) containing collagenase (Boehringer Mannheim, Indianapolis, IN) at a concentration of 1.25 mg/ml. The intact pancreases were incubated at 37°C for 25 min and gently disrupted with a 10-ml pipette. Resultant cell suspensions were washed twice at 1000 rpm for 1 min. After the second wash, the pellet was resuspended in 5 ml 27% Dextran. The suspension was then underlaid with another 2 ml 27% Dextran and overlaid with 2 ml each of 23 and 11% solutions of Dextran. The gradient was spun for 10 min at 1800 rpm, and the islets migrated to the interface between the 23 and 11% layers. After washing, 1600–1800 free islets were handpicked under a dissecting microscope and cultured overnight (37°C, 5% CO_2) in CMRL-1066 (Gibco) supplemented with 10% fetal calf serum and 1% penicillin-streptomycin.

For islet transplantation, recipient ACI rats were anesthetized with metofane, and the 1600–1800 islets isolated the previous day were resuspended in HBSS and injected into the liver via portal vein cannulation.

RESULTS

Our previous studies have shown that maximal depletion of $CD4^+$ T lymphocytes from the peripheral blood of ACI rats can be achieved with administration of MoAb OX38 at a dose of 5 mg/kg body wt i.v. (33). This same treatment regimen of 5 mg/kg on day −3 relative to the day of transplantation, followed by maintenance doses of 1 mg/kg on days −2,

TABLE 1
Lymphocyte subset depletion in rats treated with monoclonal antibodies

Treatment	Peripheral blood leukocytes (% total)			
	$CD5^+CD4^+$	$CD5^+CD8^-$	$CD5^+CD8^+$	$CD5^+CD4^-$
Control	40.0 ± 2.6	42.1 ± 2.9	14.3 ± 1.3	15.1 ± 1.1
OX38	6.0 ± 1.2	7.8 ± 1.9	25.4 ± 3.1	30.0 ± 3.3
OX8	52.3 ± 2.1			2.3 ± 0.2
OX38 + OX8	8.2 ± 0.6			3.6 ± 0.8

−1, and 0, relative to transplantation, was chosen for the studies outlined below. The level of depletion (≥80%) seen with this regimen of treatment with OX38 is in Table 1. MoAb OX35 recognizes an epitope on the CD4 molecule that is distinct from the epitope recognized by OX38 and does not compete in antibody cross-blocking. Therefore, OX35 labels $CD4^+$ cells even in the presence of residual bound OX38. To differentiate actual levels of depletion of $CD4^+$ cells from cell surface modulation of the CD4 molecule, PBLs were also labeled with MoAbs recognizing CD5, a marker for T lymphocytes. The CD8 molecule was labeled with MoAb OX8. Thus, by staining with anti-CD5 in conjunction with anti-CD4 or CD8, it was possible to distinguish between depletion or modulation of CD4- and CD8-bearing T lymphocytes. If modulation of CD4 had occurred, there would be no change in the $CD5^+CD8^-$ population. However, after OX38 treatment, there was a profound decrease in the $CD5^+CD8^-$ population and the $CD5^+CD4^+$ population, demonstrating that CD4 cells had been removed from the peripheral blood of treated rats (Table 1). Transplantation of islets from Lewis donors (*RT1*^{l}) into diabetic ACI (*RT1*^{a}) rats that had been treated with OX38 before engraftment resulted in permanent survival of these islet allografts (Table 2). ACI rats received no additional form of posttransplantation immunosuppression. Graft survival was measured by the ability of the islet transplant to result in and maintain normoglycemia.

To further examine the cellular requirements for anti-CD4–mediated tolerance induction, we treated rats with anti-CD8 MoAb OX8 alone or coincident with anti-CD4 MoAb depletion before islet transplantation. The ability of the OX8 treatment to deplete $CD8^+$ T lymphocytes was studied after intravenous administration of titrated doses of OX8. OX8 is efficient in mediating $CD8^+$ T-lymphocyte depletion, even at levels as low as 2 mg/kg (Fig. 1). A 4-day treatment regimen consisting of an initial dose of 2 mg/kg followed by maintenance doses of 1 mg/kg beginning at day −3 relative to transplantation was chosen. The levels of depletion achieved by administering OX8 alone (≥80%) and those achieved when

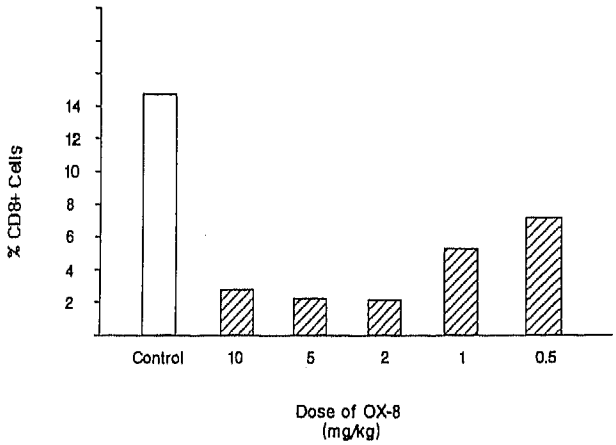

FIG. 1. Effect of OX8 monoclonal antibody treatment on $CD8^+$ cells. Titrated doses of anti-CD8 OX8 were administered to ACI rats by intravenous injection, and relative percentage of $CD8^+$ lymphocytes remaining in peripheral blood was analyzed by fluorescence-activated cell-sorter analysis. *Open bar*, percentage of CD8 subset frequency for untreated control rats. *Hatched bars*, subset frequencies for rats treated with 0.5, 1, 2, 5, or 10 mg/kg OX8. Data are analyses performed 48–72 h after treatment. Each *bar* represents ≥3 rats.

TABLE 2
Survival of Lewis islet grafts in ACI rats treated with anti-CD4 (OX38), anti-CD8 (OX8), or OX8 and OX38 antibodies

Treatment	Days of treatment (relative to transplantation)	Graft survival (days)
Control		8, 8, 10, 13
OX8	−3, −2, −1, 0	17, 27, 30
OX38	−3, −2, −1, 0	>115, >130, >140 >160, >400 (2)
OX8 + OX38	−3, −2, −1, 0	22, 23, 23, 36, 44

OX38 and OX8 were given together, are shown in Table 1. The levels of circulating $CD8^+$ cells in this assay were determined by the $CD5^+CD4^-$ phenotype.

Anti-CD8 MoAb alone can prolong the survival of islet allografts (Table 2). However, long-term tolerance was not achieved with this regimen, because all three animals treated with anti-CD8 MoAb rejected their grafts within 30 days of transplantation. More striking are the data that demonstrate that the addition of OX8 MoAb (which depletes $CD8^+$ peripheral T lymphocytes) to the previously tolerogenic dose of anti-CD4 MoAb eliminates the tolerance induction seen with anti-CD4 given as a single reagent. All five animals treated with both antibodies rejected their grafts within 44 days (Table 2). Animals receiving coincident OX8 and OX38 were as efficiently depleted of $CD4^+$ cells as those that received the same level of anti-CD4 MoAb (OX38) alone (Table 1). Thus, the lack of survival of these grafts cannot be attributed to inadequate doses of anti-CD4 MoAb. This rejection implies that persisting $CD8^+$ cells are essential for the induction or retention of the tolerogenic effects of anti-CD4 immunotherapy.

DISCUSSION

These data demonstrate that a short course of treatment with a depletive regimen of anti-CD4 MoAb allowed permanent survival of fully mismatched Lewis islet allografts into streptozocin-induced diabetic recipient ACI rats. The mechanisms by which anti-CD4 MoAbs allow long-term graft survival are not understood. We recently demonstrated that depletive regimens of anti-CD4 MoAbs, used for islet transplantation in mice, are followed by clonal anergy in new thymic migrants as they repopulate the PBL pool (22; S.A., unpublished observations). In this mouse model, clonal anergy was characterized by the following criteria. *1*) There was no clonal deletion of the potentially alloreactive T lymphocytes during repopulation of peripheral $CD4^+$ cells after anti-CD4 depletion. *2*) The potentially alloreactive T lymphocytes that might have recognized donor alloantigens on the transplanted islets showed greatly reduced responses as determined by receptor cross-linking studies with immobilized MoAbs reactive with particular T-lymphocyte receptors known to be involved in allospecific responses across the MHC disparity of this transplantation model. *3*) Finally, the unresponsiveness detected by receptor cross-linking could be partially overcome in vitro by the addition of recombinant interleukin 2. In the rat model, we do not have access to homologous anti-T-lymphocyte–receptor reagents to analyze the induction of anergy and/or dominant deletion elements in the development of the peripheral T-lymphocyte

repertoire. However, taken together with data presented in this report, it is possible that elimination of CD4+ cells at the time of transplantation allowed repopulation of CD4+ cells from thymic precursors that were incapable of inducing allospecific responses against the transplanted tissue.

Depletion of CD8+ T lymphocytes was insufficient to generate long-term organ allograft survival (Table 2). The fact that the addition of a depleting regimen of anti-CD8 MoAb to the previously successful anti-CD4 treatment eliminated the tolerogenic effect of anti-CD4 immunotherapy highlights the important role that a CD8+ cell must play in the induction or maintenance of graft survival. At the time animals are depleted of CD4+ cells, a CD8+ cell must play an important role in rendering or maintaining potentially alloreactive cells unresponsive. Such regulation could exist at several different levels for maintenance of allograft unresponsiveness. The kinetics of anergy in thymic migrant cells and the function and phenotype of the CD8+ cell apparently involved in the induction or perpetuation of allograft unresponsiveness remain undetermined.

The selective immunosuppression of the recipient of an islet allograft remains a viable option to be considered as a treatment for human IDDM. Anti-CD4–mediated transplantation tolerance induction, which allows permanent survival of human islet allografts, would eliminate many of the side effects of current immunosuppression that plague islet transplantation in diabetic hosts. This course of therapy is short term and requires no posttransplantation immunosuppression. However, one of the attractive features of this form of induced unresponsiveness for allograft transplantation tolerance induction may serve as a major obstacle to adapting this form of immunotherapy to islet transplantation. Previously, we demonstrated that depleting regimens of anti-CD4 MoAb leave behind immunologic memory for various immune responses, including the CD4-mediated help for antibody production in response to soluble antigen (42), the CD4+ help required for the induction of cytotoxic T lymphocytes to previous viral infections (43), and the CD4 help required for the induction of organ allograft transplantation rejection (33). It is conceivable, due to the autoimmune nature of IDDM, that this form of immunotherapy might be successful for the induction of organ allograft unresponsiveness but would leave behind the potential for autoimmune responsiveness against the transplanted islets. Whether such autoimmune response requires MHC restriction and might be circumvented by allografting procedures remains unanswered.

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