Original Articles

Transplantation of Allogeneic Islets of Langerhans in the Rat Liver

Effects of Macrophage Depletion on Graft Survival and Microenvironment Activation

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Early impairment of islet function and graft loss limit the success of allogeneic islet transplantation. Nonspecific inflammatory events occurring at the transplant site immediately after grafting, involving the production of cytokines and free radicals and sinusoidal endothelial cell (SEC) activation, may contribute to islet cell damage. To evaluate whether Kupffer cell inactivation would result in prolonged allograft survival in a model system of intrahepatic islet transplantation in rats, we systemically administered either gadolinium chloride ($GdCl_3$) or dichloromethylene diphosphonate (Cl_2MDP) to assess the effects of macrophage inactivation on rejection and on the release of proinflammatory molecules, as well as to assess the functional profile of SEC. The results obtained were compared with those observed in untreated, sham-injected animals and in rats receiving intraportal infusions of microbeads. Transient macrophage inhibition, particularly in hepatic Kupffer cells, is associated with significant prolongation of graft survival after intraportal islet allotransplantation (ITx) in rats: 7.2 days in the control group versus 11.9 days in the $GdCl_3$ group ($P < 0.01$) and 15.6 days in the Cl_2MDP group ($P < 0.0006$), respectively. Although systemic release of inflammatory mediators was observed only when islet transplantations were performed and it could be inhibited by macrophage-targeting treatments, perturbation of the functional profile of endothelial cells was also observed when microembolization was induced by the use of microbeads and could not be prevented by macrophage inhibition. These experiments provide evidence to support the concept that macrophages play a key role in early inflammatory events known to adversely affect islet engraftment and suggest that manipulation of nonspecific immune activation by inhibition of macrophage function may facilitate hepatic engraftment of islet allografts. The mechanisms mediating this effect are likely to include prevention of release of tumor necrosis factor-α, interleukin-1β, and NO and interference with the rate of immune response to the islets. *Diabetes* 47:316–323, 1998

Successful allogeneic transplantation of islets of Langerhans represents a potential cure for type 1 diabetes (1–6). Clinical trials, however, have not so far achieved consistent results, with only a minority of islet recipients retaining partial graft function 1 year after transplantation. Remarkably, the majority of islet grafts are lost early after transplantation (within the first few weeks; 7). Many of these early graft losses are difficult to explain based solely on the occurrence of classic mechanisms of allogeneic immune recognition and recurrence of autoimmunity. If rejection and/or autoimmunity were the only causes of early graft losses, the discrepancy observed between survival rates of whole pancreas (where early graft losses seldom occur) versus islet allografts early after transplantation would not exist. Moreover, early graft losses still occur in syngeneic islet transplants (8) and in experimental models manipulated to suppress T-cell activation (9–13), suggesting that additional mechanisms might contribute to such phenomena. These mechanisms include an inadequate mass of transplanted β-cells, impaired vascularization, and a nonspecific inflammatory reaction in the host microenvironment at the site of transplantation (14). When islet grafts are implanted into the liver, cellular components that serve as mediators of nonspecific inflammatory events, including resident liver macrophages (Kupffer cells) and the vascular endothelial cells (EC), may therefore participate in the induction of early islet graft loss.

Kupffer cells play a central role in the inflammatory

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EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; HA, hyaluronic acid; HBSS, Hanks' balanced salt solution; IEQ, islet equivalent number; IL, interleukin; ITx, islet transplantation; LPS, lipopolysaccharide; SEC, sinusoidal endothelial cell; TNF-α, tumor necrosis factor-α.

responses within the liver because of their ability to recognize and respond to numerous signals from the surrounding environment by secreting an array of substances, including arachidonic acid metabolites, cytokines, and peptides, that can directly affect the survival of intrahepatic islets (15,16). Furthermore, they play a fundamental role in the initiation/amplification of immune responses through the processing and presentation of antigens. In addition, Kupffer cells can induce a response of other non-immune-cell subsets, including EC (17). Additionally, it has been proposed that ischemia/reperfusion phenomena might directly trigger sinusoidal EC (SEC) activation, also contributing to the initiation of a nonspecific inflammatory response (18); ischemia/reperfusion phenomena are likely to occur at the transplant site following embolization of the islet in the presinusoidal capillaries.

Thus, a complex pattern of cell–cell interactions can be envisioned, where Kupffer cells and ECs act synergistically to induce a nonspecific inflammatory response that could be responsible for significant damage to the implanted tissue, as well as promoting efficient immune recognition. There is convincing evidence that islets are highly sensitive to the toxic effects of inflammatory mediators; therefore, perturbation of macrophages and ECs might represent one of the primary triggering events of islet-cell dysfunction and/or death (19,20).

The series of experiments presented in this study was designed to evaluate whether Kupffer cell inactivation would result in prolonged allograft survival in a model system of intrahepatic islet transplantation in rats. Systemic administration of either gadolinium chloride, which leads to short-term macrophage depletion (21), or dichloromethylene diphosphonate (Clodronate), which leads to long-term macrophage depletion (22), was used as a powerful tool to study the effect of macrophage inactivation on rejection following intrahepatic islet transplantation.

RESEARCH DESIGN AND METHODS

Pharmacological induction of diabetes. Sprague Dawley rats (Charles River Laboratories, Portage, MA), 6–8 weeks old, body weight 250–300 g, were rendered diabetic via a single intravenous injection of streptozotocin at 65 mg/kg (Sigma, St. Louis, MO) freshly dissolved in citrate buffer. They received islet allograft after at least 5 days of persistent hyperglycemia (>350 mg/dl).

Isolation of rat islets of Langerhans. Lewis (LEW) rats (Charles River Laboratories), 6–8 weeks old, body weight 250–300 g, were used as islet donors. Rat islets were isolated by means of a technique routinely used in our laboratory (23,24). Briefly, general anesthesia was induced using a mixture of ketamine (33.2 mg/kg), xilazine (3.32 mg/kg), and acepromazine (3.32 mg/kg) given intramuscularly; a midline abdominal incision was performed; and the pancreas was exposed and injected via the pancreatic duct with Hanks' balanced salt solution (HBSS; Gibco BRL, Long Island, NY) containing collagenase (Sigma Type V, Sigma) at a concentration of 1.5 mg/ml. After death of the animal, the pancreatic tissue was surgically removed and incubated at 37°C in collagenase solution. After a static incubation of 17 min, the digestion kinetics were sharply slowed by addition of cold HBSS supplemented with 10% bovine calf serum (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 ascertained via observation by microscope. Islet purification, obtained by means of centrifugation on discontinuous density gradients using a Cobe 2991 cell separator (COBE BCT, Lakewood, CO), routinely provided pure islets (purity >90%). Islet purity was assessed by Dithizone (Sigma) staining (25), and the islets were counted and scored for size. An algorithm was used for the calculation of islet equivalent number (IEQ). Before transplant, islets were placed in culture overnight in CMRL medium (Gibco) supplemented with 10% fetal calf serum (Hyclone), 2 mmol/l L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 25 mmol/l HEPES buffer (Mediatech, Herndon, VA) at 22°C, 5% CO_2.

Experimental groups. Six groups of diabetic animals were included in our study. In group 1 were diabetic rats (n = 13) that received intraportal islet allografts without pharmacological treatment. Group 2 consisted of diabetic rats (n = 12) that received intraportal islet allografts preceded by a single administration of gadolinium chloride (30 mg/kg) 2 days before islet transplantation. Group 3 consisted of diabetic rats (n = 8) that received intraportal islet allografts preceded by a single administration of dichloromethylene diphosphonate (1 ml of liposome encapsulated compound, 15–20 µmol/l). Group 4 consisted of diabetic rats that received intraportal glass microcarrier-beads (50–200 µm). Volume and size of injected microcarriers were adjusted to roughly equal and reproduce the microembolization phenomenon that occurs when islets are implanted in the presinusoidal capillaries of the liver (n = 8). Group 5 consisted of diabetic rats (n = 8) that were sham-injected in the portal vein with HBSS alone. Group 6 consisted of untransplanted diabetic rats (n = 8).

Animals were maintained in accordance with U.S. Department of Agriculture regulations. All animal manipulations were conducted and monitored under an approved protocol reviewed by the Institutional Animal Care and Use Committee.

Macrophage depletion. Short- or long-term macrophage inactivation was achieved with single administrations of either gadolinium chloride or dichloromethylene diphosphonate.

As previously described, intravenous injection of gadolinium chloride not only inhibits phagocytosis by rat liver macrophages (Kupffer cells) but also selectively eliminates the large macrophages situated in the periportal zone of the liver acinus (21). Two days before islet transplantation, animals were treated with one intravenous injection of 1 ml of saline solution (pH 7) containing gadolinium chloride (Aldrich, Milwaukee, WI) at a dose of 30 mg/kg (24). Macrophage repopulation was not directly assessed but has been described to begin 4 days after injection (26).

Dichloromethylene diphosphonate is one of the diphosphonates currently used for the treatment of osteolytic bone diseases. The drug needs to be encapsulated into multilamellar liposomes to be delivered with remarkable selectivity to macrophages, where it acts by inducing what has been called "macrophage suicide." Liposomes are phagocytosed and reach the intracellular compartment of the macrophage, where they promote release of enzymes contained in the lysosomes with consequent autodigestion of the macrophage (27). In addition, the multilamellar structure allows them to interact with the next viable macrophage, perpetuating the effect for up to 3 weeks. It has been demonstrated that dichloromethylene diphosphonate is capable of depleting 90% of the larger Kupffer cells and up to 50% of the small Kupffer cells (27). Two days before islet transplantation, rats received one single intravenous injection of 1 ml of solution containing liposome-encapsulated dichloromethylene diphosphonate (15–20 µmol/l) (27).

Preparation of liposomes. Multilamellar dichloromethylene diphosphonate–containing liposomes were prepared as described by Van Rooijen and Sanders (27). Briefly, a mixture of phosphatidylcholine (86 mg L-a-lecithin, Avanti, Alabaster, AL) and cholesterol (8 mg, Sigma) in chloroform (10 ml) underwent low vacuum rotation to form a phospholipid film, after evaporation of the chloroform phase. The phospholipid film was dispersed in aqueous solution containing dichloromethylene diphosphonate (kindly provided by Dr. Barbara Miller of Norwich Eaton Pharmaceuticals, Norwich, NY), leading to the formation of dichloromethylene diphosphonate–containing liposomes. Removal of the free dichloromethylene diphosphonate was achieved by sequential centrifugations of the liposomes and washing in Dulbecco's phosphate buffer solution (Gibco). The concentration of liposome-included dichloromethylene diphosphonate was determined with a method based on the murexide-dichloromethylene diphosphonate competition for Ca^{2+} as described by Claassen and Van Rooijen (28). Dichloromethylene diphosphonate–containing liposomes were stored at 4°C under N_2 and were used within 1 week.

Intraportal islet transplantation. Under general anesthesia induced by inhalation of metoxyfluorane (Metofane, Pitman-Moore, Moundelain, IL), the peritoneal cavity of the recipient was accessed through a midline incision. Immediately before infusion, islets were counted and divided in aliquots of 4,000 IEQ per recipient, resuspended in 1 ml of HBSS, and loaded in a 1-ml syringe. After visualization of the portal vein by extra-abdominal repositioning of the bowel, the islets were infused into the portal vein via a 25-gauge needle. After infusion, the syringe was rinsed several times by repeated aspiration and re-infusion of portal vein blood. Manual compression of the injection site followed removal of the needle to minimize the risk of bleeding.

Tumor necrosis factor-α, interleukin-1β, and nitrate/nitrite blood level determination. Blood samples were collected before transplant and at different time points (3, 6, 12, 24, 48, and 72 h) after intraportal infusion of islets, microcarriers, or vehicle. Samples from nontransplanted rats were taken at identical time points, assuming an arbitrary zero time. Blood samples collected from the tail vein were centrifuged to separate plasma or serum and immediately stored at –70°C until used.

Tumor necrosis factor-α (TNF-α) serum concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit with an antibody specific for rat TNF-α (BioSource International, Camarillo, CA). Absorbance was determined at 450 nm on a VMax Kinetic Microplate reader (Molecular Devices, Menlo Park, CA); results were plotted using a linear curve fit (correlation coefficient = 0.999) (29,30).

Interleukin-1β (IL-1β) serum levels were assessed by ELISA using an antibody specific for rat IL-1β (BioSource International). Absorbance was determined at

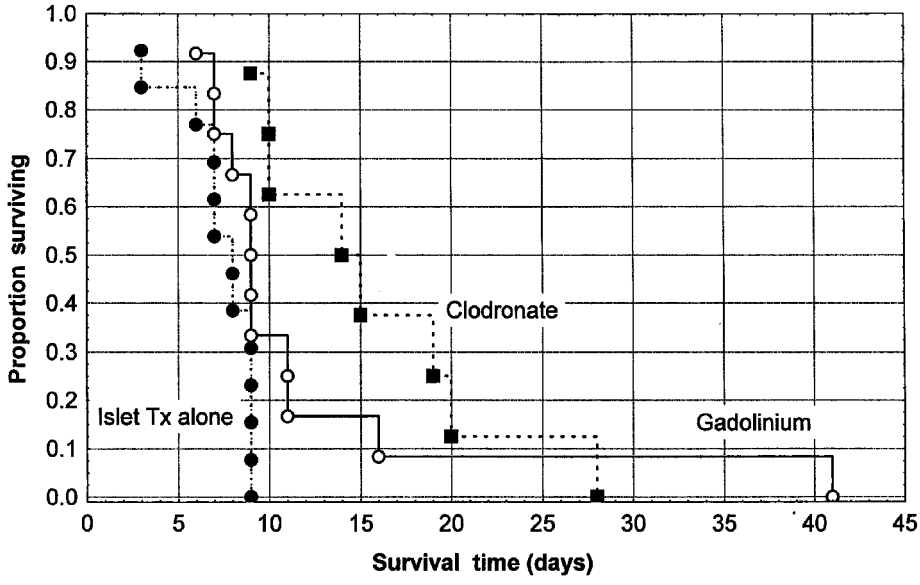

FIG. 1. Survival plots of allogeneic islets transplanted intrahepatically in diabetic recipient rats. Rejection was defined as recurrence of hyperglycemia on two consecutive daily measurements. Unmanipulated recipients (islet transplantation only, ITx; n = 13) are compared with recipients that received either gadolinium (gadolinium chloride; n = 12) or Clodronate (dichloromethylene diphosphonate; n = 8). Both treatments resulted in significant prolongation of graft survival (P < 0.01 for gadolinium vs. ITx; P < 0.006 for Clodronate vs. ITx).

450 nm on a VMax Kinetic Microplate reader plate, and results were plotted using the four-parameter algorithm curve fit (31,32).

NO_2/NO_3 plasma levels were determined using a modification of the Griess method (33,34). Samples were diluted and ultracentrifuged on Ultrafree-MC filters (Millipore) for removal of proteins, followed by stochiometric reduction of nitrate to nitrite by nitrate reductase (Boehringer Mannheim, Indianapolis, IN). After oxidation of NADPH to avoid interference with nitrite determination, total nitrite was measured spectrophotometrically using the Griess reaction.

Graft survival. Graft survival was defined as number of days of persistent normoglycemia (nonfasting blood glucose levels less than 140 mg/dl) and increase in body weight over the corresponding time. The day of rejection was defined as the first day of two consecutive days of nonfasting glucose >250 mg/dl. Blood glucose levels were measured daily on whole-blood (tail vein) samples using an Accu-Check Easy glucose monitor (Boehringer Mannheim).

EC function. EC function was evaluated by assessing the capability of liver EC to take up hyaluronic acid (HA) from the blood through a process of receptor-mediated endocytosis (35,36). Alterations of the normal profile of HA clearance have been associated with endothelial functional damage and in particular with hepatic EC dysfunction, because hepatic ECs are responsible for a large fraction of the observed clearance (36). HA uptake and clearance after exogenous injection in the various experimental groups were compared.

Plasmatic clearance of HA. HA (Sigma) was diluted in sterile saline solution and administeredintravenously (300 μg/kg) to all animals 2 h and 55 min after infusion of islets, microcarriers, or HBSS. The first two blood samples were collected through the tail vein 5 min and 35 min after the HA infusion and thereafter at 3, 9, 21, 45, and 69 h, corresponding to 6, 12, 24, 48, and 72 h after transplant. Serum levels were determined using a commercially available radiometric assay (Pharmacia, Uppsala, Sweden). Briefly, HA from the samples was allowed to bind with ^{125}I-labeled HA binding proteins (isolated from bovine cartilage). Quantitation of the unbound ^{125}I-labeled HA binding proteins was determined by incubation with HA covalently coupled to Sepharose particles of small size and low density. Separation of the unbound ^{125}I-labeled HA binding proteins was performed by centrifugation followed by decanting. The radioactivity detected on the particles was used to calculate the concentration of free HA in the samples (37,38).

Statistical analysis. Data were analyzed with Statistica for Windows software, version 5.0 (Statsoft, Tulsa, OK) and are expressed as means ± SE. Relations among variables of interest were analyzed with the Student's t test for independent samples. Repeated measurements of the same values at different time points were analyzed by ANOVA. The Cox F test was utilized to determine the significance of difference in overall allograft survival; P < 0.05 was considered significant.

RESULTS

Graft survival. Monitoring of blood glucose levels was used to assess graft survival. The mean graft survival of untreated diabetic recipients of allogeneic islet transplant (group 1) was 7.2 days (Fig. 1). The treated groups of recipient rats consisted of animals that were pretreated with either gadolinium chloride or dichloromethylene diphosphonate 2 days before intrahepatic transplantation. Rats treated with gadolinium chloride or dichloromethylene diphosphonate showed prolonged graft survival. Islets in the gadolinium chloride group survived, on average, 11.9 days, and islets in the dichloromethylene diphosphonate group had a mean survival of 15.6 days. Graft survival was statistically different from group 1 in both treated groups, with P values of <0.01 and <0.0006, respectively. No significant difference was observed in the survival of the grafted islets between the two treated groups (gadolinium chloride vs. dichloromethylene diphosphonate). Figure 1 depicts the survival in the three groups (Kaplan Meyer plots).

Effects of islet transplantation with or without macrophage depletion on systemic increments of proinflammatory mediators. Transient alterations of the levels of selected cytokines and inflammatory mediators have been reported to occur after intrahepatic islet transplantation (15,16). We wanted to evaluate whether the pretreatment of recipient rats with antimacrophage agents might modify these responses. The kinetics of alterations in the levels of TNF-α, IL-1β, and the endproducts of the nitric oxide catabolism are shown in Figs. 2*A–C* for transplanted untreated animals (group 1) and compared with those obtained in the two experimental groups treated with either dichloromethylene diphosphonate or gadolinium chloride (groups 2 and 3).

Figure 2*A* shows the levels of TNF-α at 3 and 6 h after islet transplant (ITx) in untreated and macrophage-depleted animals. Incremental levels over the basal values were significantly different at 3 h posttransplant in the gadolinium chloride group versus the control group (1.05 ± 0.70 vs. 16.2 ± 6.7; P = 0.022). Similarly, a reduction of the increment at 3 h was observed in the dichloromethylene diphosphonate group, although it did not reach statistical significance (4.5 ± 3.3, P = 0.07). Comparison of the incremental levels of TNF-α at 6 h also revealed differences in the treated groups compared with the untreated, transplanted recipient group. In rats pretreated with multilamellar dichloromethylene diphosphonate–containing liposomes, TNF-α levels were 1.0 ± 0.9 vs. 5.0 ± 2.1 in the control group (P = 0.035). Values in the group

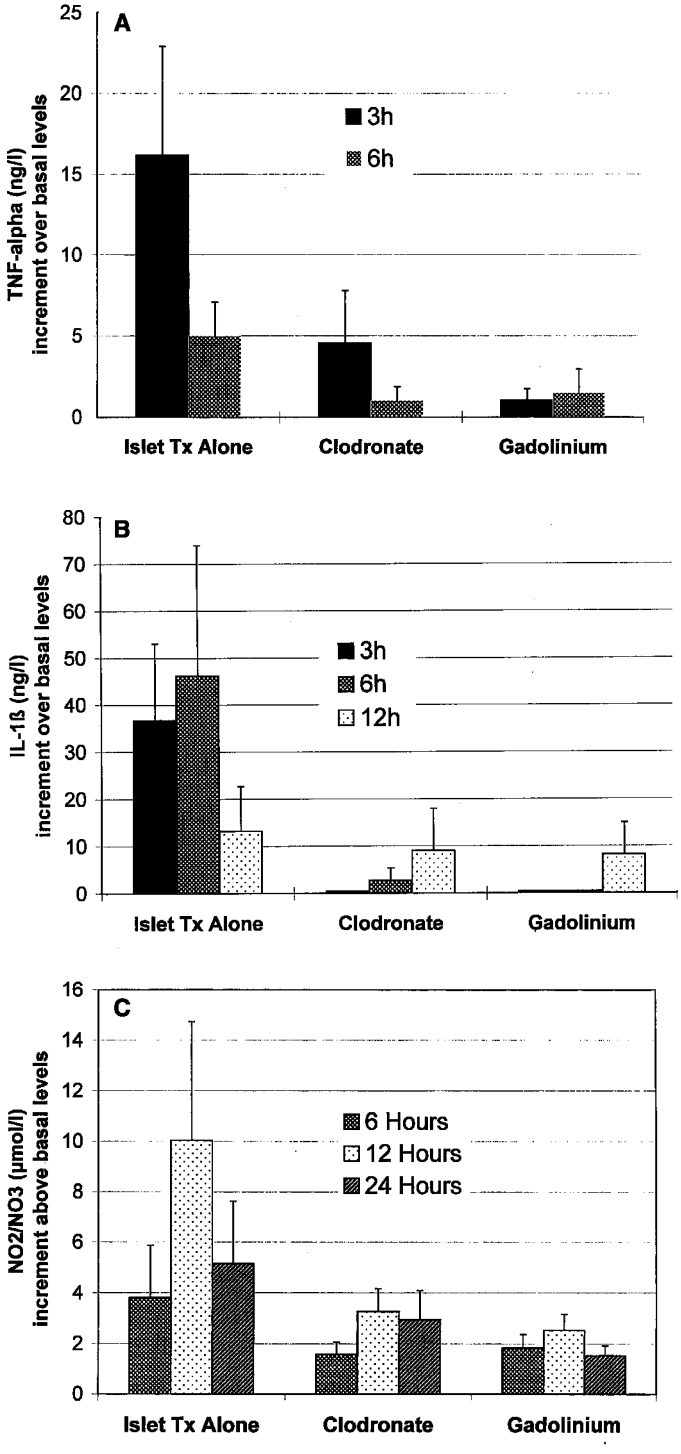

FIG. 2. Measurements of the levels of TNF-α (*A*), IL-1β (*B*), and stable products of NO catabolism (NO_2/NO_3; *C*) in unmanipulated islet recipients and in recipients pretreated with either gadolinium or Clodronate (dichloromethylene diphosphonate). Data were collected at the indicated time points and are expressed as means ± SE.

treated with gadolinium chloride averaged 1.5 ± 1.5, approaching but not reaching statistical significance (P = 0.06). No differences were observed when macrophage-depleted groups were compared with untransplanted (results not shown) or HBSS sham-injected (Figs. 2*A* and 4*A*) rats. No differences were observed when macrophage-depleted groups were compared with each other (Fig. 2*A*).

Incremental levels of IL-1β over baseline values (Fig. 2*B*) in both groups of macrophage-depleted ITx recipient animals were significantly decreased at 3 and 6 h when compared with controls. IL-1β levels in the gadolinium chloride group at 3 and 6 h were 0.40 ± 0.02 and 0.40 ± 0.016 and yielded P values of 0.018 and 0.01, respectively, versus control (values in the control group were 36.7 ± 16.3 and 46.17 ± 27.75). IL-1β values in the dichloromethylene diphosphonate group were 0.40 ± 0.001 and 2.69 ± 2.69 and yielded P values of 0.01 and 0.04 at 3 and 6 h versus the control group. No differences among the three groups were noted at 12 h.

Significant suppression of the increase in the systemic levels of NO_2/NO_3 was detected at 12 h in macrophage-depleted animals compared with recipients of ITx alone (gadolinium chloride vs. dichloromethylene diphosphonate vs. ITx alone 2.53 ± 0.62 vs. 3.25 ± 0.90 vs. 10.03 ± 4.70; P = 0.002 for gadolinium chloride vs. ITx alone; P = 0.006 for dichloromethylene diphosphonate vs. ITx alone; Fig. 2*C*). No differences among the three groups were noted at 6 or 24 h. When NO_2/NO_3 systemic levels in macrophage-depleted animals were compared with untransplanted (results not shown) or HBSS sham-injected rats, once again no statistical differences were observed (Figs. 2*C* and 4*C*).

SEC function. By utilizing a technique that takes advantage of the peculiar capability of EC to take up HA via the surface-bound receptor CD44, we attempted to assess the degree of EC functional impairment after islet cell transplantation. EC function was assessed by measuring HA clearance in plasma samples obtained from animals that received intraportal islet infusion and comparing it to clearance observed in animals that received ITx after macrophage inactivation. Figure 3 shows the kinetics of disappearance of plasma HA as a function of time after exogenous injection of a single dose. Untransplanted diabetic rats (group 6) were used to assess the normal clearance profile in our experiments. In untransplanted diabetic animals, HA concentration peaked 5 min after exogenous administration, followed by a rapid decrease that approached baseline values 35 min after injection. Plasma concentrations at later time points were not statistically different from baseline values. At variance, values obtained from rats receiving islet transplants demonstrated a return to baseline values 3 h after injection, followed by a late increase in HA plasma concentrations. Pretreatment of recipient rats with either gadolinium chloride or dichloromethylene diphosphonate (groups 2 and 3, respectively) did not prevent the apparent alterations of the pattern of HA clearance observed in group 1. Both the delay in clearance and the occurrence of the subsequent rebound were unaffected by either drug. HA concentrations differed significantly between the untransplanted animals and the gadolinium chloride group at 9 and 21 h (115 ± 19 and 88 ± 20 vs. 436 ± 46 and 507 ± 104 µg/l). HA concentrations in the dichloromethylene diphosphonate group were significantly different from those in the control group at every time point examined, with values of 440 ± 109 at 9 h, 656 ± 142 at 21 h, 450 ± 61 at 45 h, and 481 ± 186 µg/l at 69 h.

Effects of microembolization on microenvironment perturbation. The observed results of both cytokine and NO production as well as the alterations observed in the capacity of the EC to clear exogenously injected HA in the recipients of

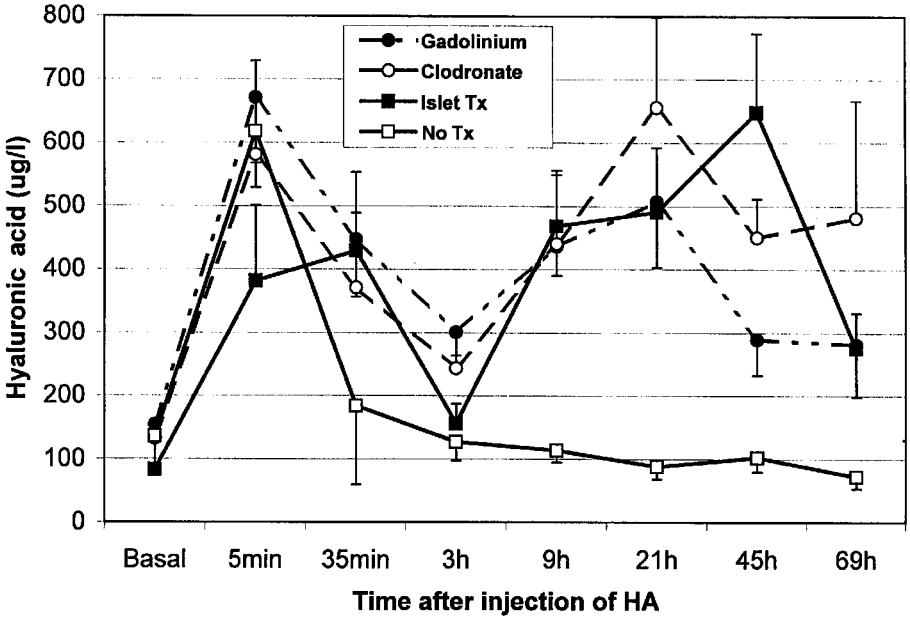

FIG. 3. HA clearance from the systemic circulation after exogenous intravenous administration. Measurements of the systemic concentration of HA were performed at the indicated time points. The results plotted in the figure were obtained in the indicated experimental groups and compared with those obtained in untransplanted animals (Tx, transplantation).

islet alone could be explained on the basis of the mechanical insult of islet grafting, which includes multiple microembolization, or it could be at least partially related to the biological processes triggered by the delivery of islets. To determine whether the microembolization of the pre-sinusoidal capillaries could be responsible for the entire spectrum of observed alterations, we analyzed the same parameters (TNF-α, IL-1β, NO, and HA clearance) in two additional experimental groups of animals: one received glass microbeads that were infused via portal vein, and the other was sham injected (vehicle only). The total volume of the infusion and the size of the beads were chosen to roughly reproduce islet size and volume, thus simulating the embolic events occurring after islet transplantation. Figure 4 (*A–C*) summarizes the results obtained in this series of experiments: neither glass micro-bead injection (group 4) nor vehicle injection (group 5) resulted in significant elevation of systemic levels of TNF-α, IL-1β, or NO_2/NO_3. The incremental levels over baseline of TNF-α at 3 and 6 h were compared between the ITx group and the recipients of microbeads and HBSS, and the results are shown in Fig. 4A. Values of TNF-α in recipients of ITx at 3 and 6 h were 16.2 ± 6.7 and 5.0 ± 2.1. respectively, compared with 0.1 ± 0.001 (both at 3 and 6 h) in the microbead recipients and 0.10 ± 0.01 and 1.1 ± 0.6 ng/l at 3 and 6 h in the sham-injected group. Statistical analysis at 3 h showed $P = 0.006$ for ITx vs. micro-beads and $P = 0.003$ for ITx vs. sham injected. At 6 h, $P = 0.008$ for ITx vs. micro-beads and $P = 0.01$ for ITx vs. sham injected.

IL-1β levels were also studied in these three groups. Clear elevations were seen at 3 and 6 h in the recipients of islets, which significantly differed from the values obtained in rats receiving microcarriers. Values at 3 h in the three groups (ITx, microbeads, and sham-injected) were 36.67 ± 5.48, 0.40 ± 0.01, and 0.40 ± 0.01, respectively ($P = 0.04$ for ITx vs. micro-beads; $P = 0.04$ for ITx vs. sham). At 6 h, values were 46.2 ± 27.7, 0.40 ± 0.01, and 11.5 ± 11.5 ng/l, respectively ($P = 0.04$ for ITx vs. micro-beads; NS for ITx vs. sham-injected).

Furthermore, the systemic NO_2/NO_3 elevation detected at 6 and 12 h after intraportal islet transplant differed significantly from the values observed in recipients of microcarriers (ITx vs. micro-carriers 3.80 ± 2.1 vs. 0.93 ± 0.36 at 6 h, $P = 0.04$, and 10.03 ± 4.70 vs. 1.31 ± 0.53 mmol/l at 12 h, $P = 0.001$, respectively). Significant differences between recipients of ITx and sham-injected rats were seen at 12 h (10.03 ± 4.70 vs. 2.59 ± 0.98 mmol/l, $P = 0.003$).

In contrast, HA clearance was impaired similarly in recipients of glass microcarriers and islets. As shown in Fig 5, values in the untransplanted group and in the microbeads group differed significantly at 9, 21, and 45 h, with values of 114 ± 19 vs. 322 ± 46 at 9 h; 88 ± 20 vs. 577 ± 116 at 21 h; and 103 ± 22 vs. 488 ± 94 μg/l at 45 h. Sham-injected animals were characterized by a slight delay in the first phase clearance and by a small rebound at the late time points. Neither alteration reached statistical significance when compared with untransplanted animals.

DISCUSSION

The aim of the present work was to understand the effects of intrahepatic islet transplantation on microenvironment activation at the site of transplantation.

We were able to demonstrate that islet transplantation in the liver is characterized by release in the systemic circulation of proinflammatory mediators such as TNF-α, IL-1β, and NO. Also, functional impairment of the EC in the liver, as assessed by analysis of the clearance of exogenously administered HA, was demonstrated.

The administration of gadolinium chloride ($GdCl_3$) or dichloromethylene diphosphonate (Cl_2MDP) as a single dose, 2 days pretransplant, was capable of significantly prolonging the survival of allogeneic grafts in the absence of any additional immunosuppressive therapy. It must be stressed, however, that the encouraging results observed in the two experimental groups reflect a very reproducible prolongation of graft survival in the dichloromethylene diphosphonate group, but the prolongation in the gadolinium chloride group is characterized by a higher variability. Although the prolongation of mean survival time in the latter group seemed to be influenced by the presence of one animal that

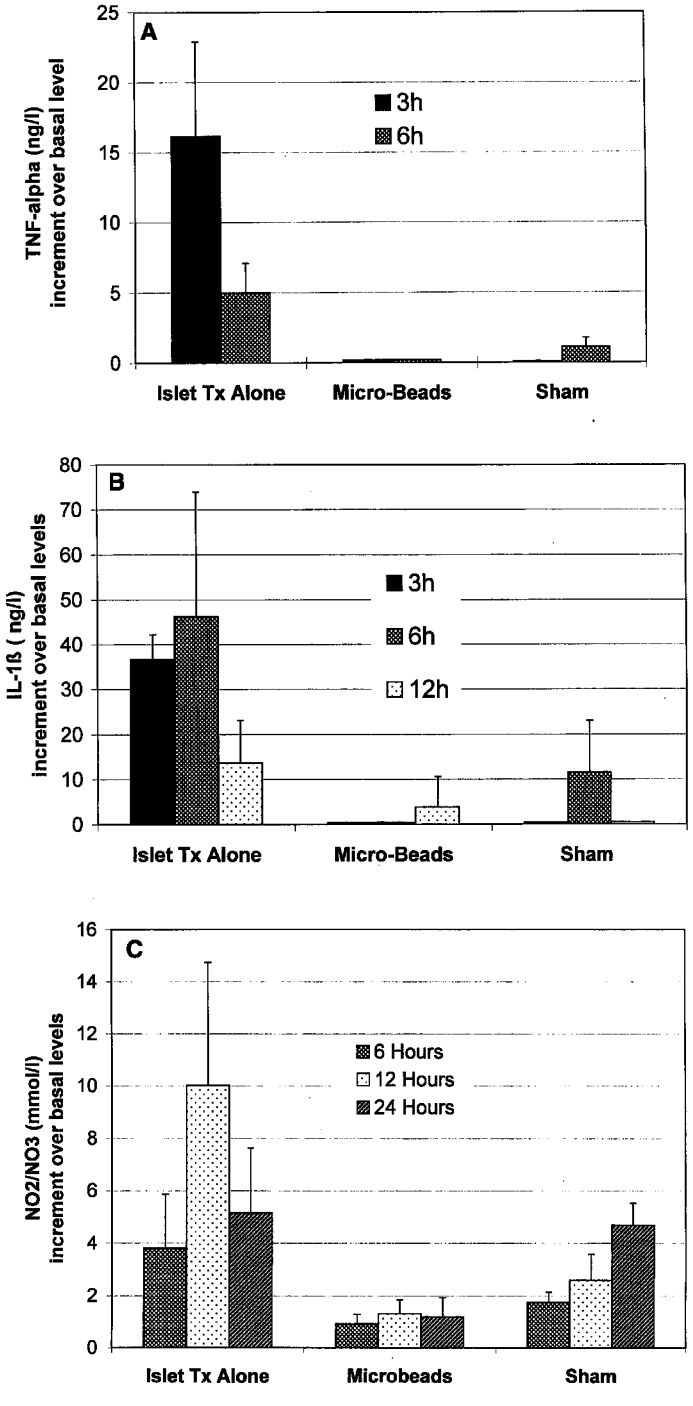

FIG. 4. Measurements of the levels of TNF-α (*A*), IL-1β (*B*), and stable products of NO catabolism (NO_2/NO_3) (*C*) in normal untransplanted rats and in rats receiving intraportal injection of glass microspheres or sham-operated rats. Data were collected at the indicated time points and are expressed as means ± SE.

carried a functioning graft for >40 days, analysis of the data after exclusion of this animal still resulted in statistical significance.

These data suggest that impairment of macrophage function, particularly the hepatic Kupffer cells, induces a significant prolongation of islet allografts in the absence of specific immunosuppressive drugs. There are several possible explanations for this finding. First, the prevention of macrophage activation and the consequent release of inflammatory mediators known to exert a direct toxic effect on the islets might preserve a larger mass of tissue for the initial period that follows transplantation. Allograft rejection may appear to be delayed as the β-cell threshold for return to hyperglycemia is strongly prolonged by an initially higher β-cell mass. Second, in the absence of macrophage activation, immune recognition proceeds at a slower pace, because the intrinsic immunogenicity of the tissue is not amplified by the presence of inflammatory cytokines (39) or by efficient presentation by macrophages. These two hypotheses (preservation of the mass and lack of amplification of the immune response) are, of course, not mutually exclusive; both are consistent with the observation that inhibition of macrophage activation by gadolinium chloride and dichloromethylene diphosphonate prolonged islet allograft survival, when either compound was administered. In addition, in the current study, serum or plasma, instead of focal (hepatic), levels of TNF-α, IL-1β, and NO catabolites were measured. Thus, the observed difference as reported might not reflect the actual magnitude of changes that might have occurred at the microenvironment surrounding the islet allografts. The high concentrations of TNF-α, IL-1β, and NO generated at the site of the allograft may have direct toxic effects to islets. Furthermore, although both drugs appear to exert comparable effects on preventing transplantation-induced elevations of cytokines and NO, they exert quite different effects on graft survival. These observations suggest that other mechanisms (besides cytokine release and NO production) are likely to contribute to islet graft loss, possibly including a macrophage-mediated role on the amplification of the immune response. The long-lasting effect of Clodronate on macrophage depletion (up to 3 weeks) compared with the much shorter action of gadolinium are consistent with the observed difference in graft survival.

Vascular ECs are also influenced by transportal embolization of islets. While the analysis of the functional performance of ECs in this study was based solely on an assay that measures their capability of clearing exogenously administered HA, the results parallel data previously obtained in animals with gram-negative sepsis induced by exogenous injection of lipopolysaccharide (LPS). LPS injection induces a manifold, long-lasting increase in plasma HA concentration, a significant decrease of HA removal from the blood, and a considerable diminution of HA uptake, demonstrating a clear impairment in the hepatic SEC function (40). The rebound observed in plasma HA concentrations at later time points in our experiments is consistent with an anatomical damage of SEC with consequent release of HA into the circulation, and similar profiles were indeed described in LPS-induced EC injury (40).

An interesting observation derives from the series of control experiments included in this study. While the systemic release of inflammatory mediators was observed only when islet transplantations were performed, and this release could be inhibited by macrophage-targeting treatments, the perturbation of the functional profile of EC was also observed when microembolization was induced by the use of microbeads. Similar results in terms of HA clearance obtained in rat recipients of islet transplantation and recipients of intraportal infusion of microbeads demonstrated that the impairment in the function of the EC is a consequence of the microembolization of

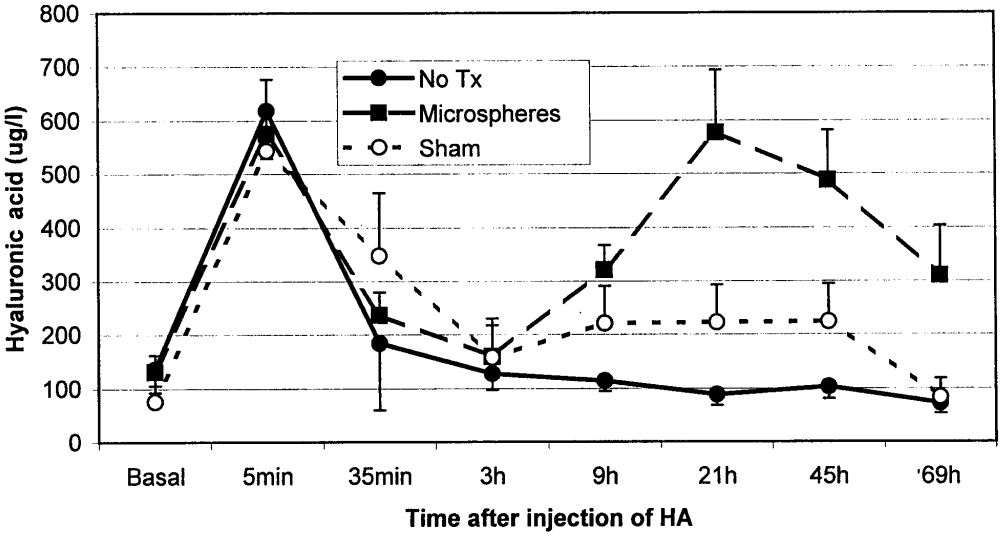

FIG. 5. HA clearance from the systemic circulation in untransplanted animals, animals receiving intraportal microspheres, and sham-operated animals. Measurements were obtained at the indicated time points after intravenous injection of HA, and data are expressed as means ± SE. Statistically significant differences were observed between untransplanted animals and microbead-injected animals at 9, 21, and 45 h. No statistically significant differences were observed between untransplanted and sham-injected animals.

the pre-sinusoidal space in the liver. Further data that support these results were observed in animals that were depleted of macrophages before islet transplantation. Even though a significant decrease in TNF-α, IL-1β, and NO release was observed in those depleted animals, the HA clearance revealed a profile similar to both treated animal recipients of islet transplantation and those recipients of intraportal microbeads. We cannot determine whether similar alterations found in the capacity of the ECs to clear exogenously injected HA induced by the infusion of microbeads or islets reflect entirely similar mechanisms. Perhaps some other functional parameters of EC might be modified differently in the case of microbeads compared with rat recipients of islets. Similarly, these studies do not allow us to determine whether EC dysfunction as a solitary phenomenon significantly contributes to islet cell dysfunction/death.

Furthermore, the lack of cytokine and NO release in animals that received intraportal infusion of microbeads suggests that the microembolization per se does not provide a sufficient stimulus for the activation of cell types capable of releasing inflammatory mediators. Dynamic interactions between transplanted islets and the microenvironment therefore appear to be a necessary condition for the release of nonspecific inflammatory mediators. The release of TNF-α, IL-1β, and NO catabolic products seems to be a direct consequence of the activation of the Kupffer cells, which is only observed when islets, but not microbeads, are infused. The presence of chemicals and biological substances that are necessary in the separation and isolation of islets might provide at least a partial explanation of the observed phenomenon, since we have observed that the carry-over of endotoxins is a more-than-likely event (41). Alternatively, and regardless of the presence of potentially harmful chemicals and biological reagents, the administration of islets per se could be the only event needed to induce activation of the Kupffer cells.

Gadolinium chloride and dichloromethylene diphosphonate have not been previously tested to evaluate the effect of macrophage depletion on allogeneic intraportal islet transplantation in rodents. Previous well-described in vitro studies have demonstrated that biphosphonate products can prevent the release of macrophage-derived cytokines and free radicals (42). In addition, it has been demonstrated that gadolinium chloride modulates the expression of TNF-α mRNA in hepatic nonparenchymal cell after endotoxin-induced liver injury in rats (43). Diminished levels of macrophage-derived inflammatory mediators lead to a prolongation of the islet allograft survival. Taken together, the experiments of the present study suggest that manipulation of nonspecific immune activation may facilitate hepatic engraftment of islet allografts and protect the islets by inhibition of TNF-α, IL-1β, and NO production, perhaps with reduced direct toxic effect and less efficient immune recognition.

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