

# A New Murine Model of Islet Xenograft Rejection

## Graft Destruction Is Dependent on a Major Histocompatibility–Specific Interaction Between T-Cells and Macrophages

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**A new murine model of porcine islet-like cell cluster (ICC) xenograft rejection, avoiding interference of unspecific inflammation, was introduced and used to investigate rejection mechanisms. Athymic (*nu/nu*) mice were transplanted with syngeneic, allogeneic, or xenogeneic islets under the kidney capsule. After the original transplantation, immune cells in porcine ICC xenografts undergoing rejection in native immunocompetent mice were transferred to the peritoneal cavity of the athymic mice. At defined time points after transfer, the primary grafts were evaluated by immunohistochemistry and real-time quantitative RT-PCR to estimate cytokine and chemokine mRNA expression. Transfer of immunocompetent cells enabled athymic (*nu/nu*) mice to reject a previously tolerated ICC xenograft only when donor and recipient were matched for major histocompatibility complex (MHC). In contrast, allogeneic and syngeneic islets were not rejected. The ICC xenograft rejection was mediated by transferred T-cells. The main effector cells, macrophages, were shown to be part of a specific immune response. By day 4 after transplantation, there was an upregulation of both Th1- and Th2-associated cytokine transcripts. The transferred T-cells were xenospecific and required MHC compatibility to induce rejection. Interaction between the TCR of transferred T-cells and MHC on host endothelial cells and/or macrophages seems necessary for inducing ICC xenograft rejection. *Diabetes* 52: 1111–1118, 2003**

**A**thymic (*nu/nu*) mice permanently accept fetal porcine islet-like cell cluster (ICC) xenografts (1), whereas rejection occurs within 1 week after transplantation in normal mice (2–7). CD4<sup>+</sup> T-cells have been shown to be the key mediators of cellular xenograft rejection (8,9). Studies on SCID mice rendered immunocompetent through reconstitution with selected subpopulations of lymphocytes have shown that CD4<sup>+</sup> T-cells, without CD8<sup>+</sup> cells or B-cells, can reject xenogeneic skin grafts (10). Similarly, work using an adoptive transfer model in SCID mice demonstrated that CD4<sup>+</sup> T-cells were necessary and also sufficient to induce rat islet xenograft rejection (11). Notably, in these studies, the mice were reconstituted with naive lymphocytes dependent on antigen presentation and activation in the recipient mice at the time of transplantation, making the animal immunocompetent to all antigens, included in the CD4<sup>+</sup> or CD8<sup>+</sup> repertoire. However, the main effector cells during rejection in ICC to rodent xenotransplantation are activated macrophages (12–14), and depletion of macrophages has been shown to delay islet xenograft rejection in mice (13,15). In the present work, a novel experimental transfer model is introduced. Here, athymic (*nu/nu*) mice that had previously received a transplant of an ICC xenograft, were reconstituted with sensitized immune cells present in ICC xenografts undergoing rejection in native immunocompetent mice (Fig. 1). With the use of this approach, it is possible to study components of the acquired immune system exclusively involved in the rejection process of an ICC xenograft, without interference of unspecific inflammatory processes inflicted by surgical trauma and unspecific islet cell loss during the early posttransplantation period. The model enables a selective reconstitution with specific components of the immune system, e.g., immune serum or immunocompetent cells from normal mice or mice with defined genetic defects, to identify the components needed to reconstitute xenoreactivity in an immunodeficient animal otherwise incapable of ICC xenograft rejection.

This transfer model was used to examine the mechanisms by which transferred sensitized T-cells were attracted to the site of the ICC xenograft and to investigate the recruitment and activation of host macrophages in the ICC xenograft. The ICC xenograft rejection in reconsti-

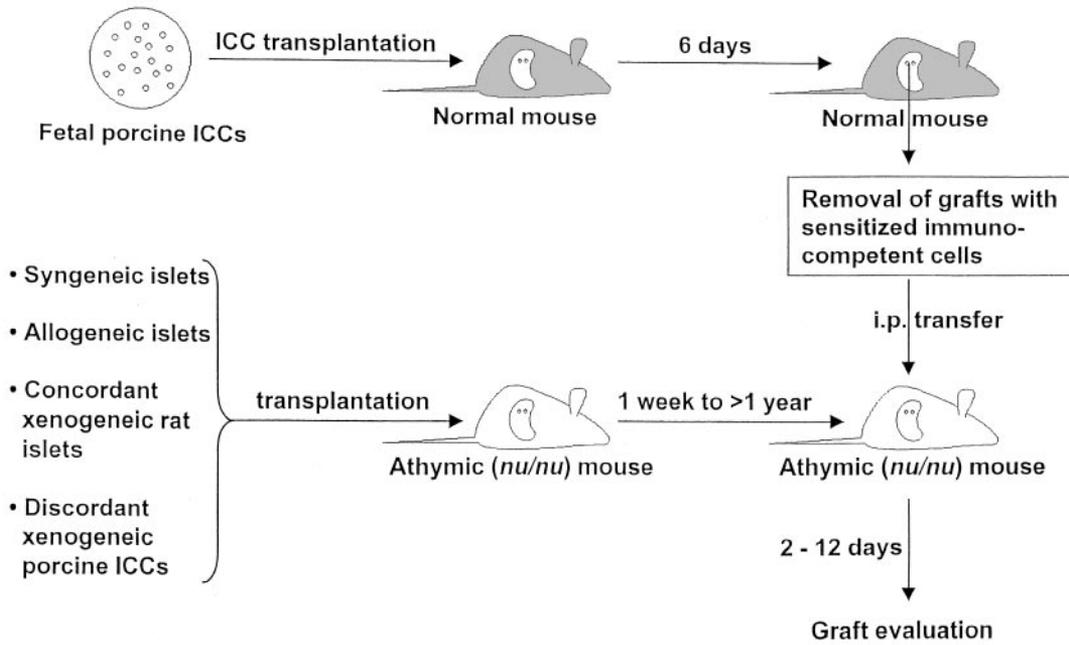
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DTH, delayed-type hypersensitivity; EC, endothelial cell; ICC, islet-like cell cluster; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MHC, major histocompatibility complex; RANTES, regulated upon activation in normal T-cells, expressed, probably secreted; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

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**FIG. 1.** Overview of the experimental transfer model. Normal mice (grey) received a transplant of fetal porcine ICCs under the kidney capsule. After 6 days, the ICC xenografts, at this stage infiltrated with sensitized immunocompetent cells, were transferred into the peritoneal cavity of athymic *nu/nu* mice (white) bearing previously implanted and tolerated syngeneic, allogeneic, or xenogeneic (rat islets or porcine ICCs) islet grafts. Immunohistochemical evaluation of the rejection and analysis of cytokine and chemokine mRNA expression in the grafts were performed at defined time points after transfer.

tuted athymic (*nu/nu*) mice was further characterized by relating the cellular infiltration in the graft to intragraft variations in cytokine and chemokine mRNA expression.

**RESEARCH DESIGN AND METHODS**

All experiments were approved by the Research Ethics Committee of Uppsala University.

**Preparation and culture of fetal porcine pancreas and rodent islets.**

ICC was prepared from porcine fetuses (gestational age 70 ± 5 days) as previously described (16). The ICC were cultured for 4 days in RPMI-1640 (Sigma Chemicals, St. Louis, MO) supplemented with 10 mmol/l nicotinamide (Sigma Chemicals). Human serum (10% [vol/vol] heat inactivated; The Blood Center, Huddinge Hospital, Huddinge, Sweden) was added after 24 h. Male inbred C57BL/6J (B6; H-2<sup>b</sup>) mice and C57BL/KsJ mice (BKs; H-2<sup>k</sup>), originally obtained from The Jackson Laboratories (Bar Harbor, ME), served as donors for syngeneic and allogeneic implantation. Male Sprague-Dawley rats (Biomedical Center, Uppsala, Sweden) were used to obtain islets for concordant xenogeneic transplantation. Pancreatic islets from rodents were prepared by a collagenase (Boehringer Mannheim) digestion method. Groups of ~150 islets were cultured in RPMI-1640 medium (Flow Laboratories, Irvine, U.K.)

supplemented with calf serum (10% [vol/vol]; Statens Bakteriologiska Laboratorium, Stockholm, Sweden). The culture dishes were kept at 37°C in 5% CO<sub>2</sub> in humidified air.

**Animals.** For generating transfer donors, ICC transplantation was performed in 1) male or female inbred B6 mice (H-2<sup>b</sup>; Biomedical Center; Tables 1–3), 2) male homozygous mutant B6 mice with a targeted disruption of the membrane exon of the Ig μ-chain gene (H-2<sup>b</sup>; The Jackson Laboratories; Table 1), 3) male inbred BALB/c mice (H-2<sup>d</sup>; Bomholdtgaard, Ry, Denmark; Table 3), and 4) male outbred NMRI mice (H-2<sup>q</sup>; Bomholdtgaard; Table 3). Transfer experiments were performed in 1) male inbred athymic B6 (*nu/nu*) mice (H-2<sup>b</sup>; Bomholdtgaard; Tables 1–3), 2) male inbred athymic BALB/c (*nu/nu*) mice (H-2<sup>d</sup>; Bomholdtgaard; Table 3), and 3) male outbred athymic NMRI (*nu/nu*) mice (H-2<sup>q</sup>; Bomholdtgaard; Table 3). All rodents had free access to tap water and pelleted food (Type R34; AB AnalyCen, Lidköping, Sweden) throughout the experimental period.

**Transplantation and transfer procedures.** A summary of the different transfer experiments is shown in Fig. 1 and Tables 1–3. Fetal porcine ICCs were transplanted under the kidney capsule as previously described (17). One week to >1 year before transfer, recipient athymic (*nu/nu*) mice received an implant of either 3 μl of fetal porcine ICC alone (Tables 1 and 3) or together with an additional graft composed of 150 B6 islets, BKs islets, or rat islets

**TABLE 1**  
Outline of the experimental transfer model

Donor of transferred material	Transferred material	Observation time after transfer (days)	No. of animals	Rejection after transfer	Evaluation
B6	ICC xenograft	2	5	No	IH, PCR
B6	ICC xenograft	3	9	No	IH, PCR
B6	ICC xenograft	4	8	No	IH, PCR
B6	ICC xenograft	6	12	Yes	IH, PCR
B6	Freeze-thawed ICC xenograft	6	2	No	IH
B6	15 Gy-irradiated ICC xenograft	6	3	No	IH
B6 Ig -/-	ICC xenograft	6	4	Yes	IH
B6	Immune serum	7	2 + 2*	No	IH
B6	ICC xenograft	6 + 42†	6	Yes/Yes	IH

Transfer of immunocompetent cells or immune serum to athymic B6 (*nu/nu*) mice that previously received a transplant of ICC xenografts. IH, immunohistochemistry. \*Mice received an injection of either 0.5 ml of serum at the time of transplantation or 0.1 ml of serum on days 1–5 after transplantation. †Mice on the alternative protocol, i.e., ICC xenografts taken from the removed left kidney on day 6 after transfer and the grafts taken from the right kidney of the same retransplanted mice 6 weeks after transfer.

TABLE 2  
Xenospecificity

Islet grafts in the athymic mice	Observation		Rejection after transfer
	time after transfer (days)	No. of animals	
ICC	12	3	Yes
Syngeneic B6 islets	12		No
ICC	12	5	Yes
Allogeneic BKs islets	12		No
ICC	12	6	Yes
Concordant xenogeneic rat islets	12		No incomplete

Transfer from immunocompetent B6 mice to athymic B6 (*nu/nu*) mice bearing a tolerated ICC xenograft together with syngeneic B6 islets, allogeneic BKs islets, or concordant xenogeneic rat islets placed under the kidney capsule of the same kidney but at different locations.

(Table 2). Animals used for intragraft mRNA analysis received two 3- $\mu$ l ICC grafts (Table 1). Immunocompetent donor mice, used for generating grafts to be transferred, received two 3- $\mu$ l ICC grafts and were killed after 6 days. The grafts, at this stage infiltrated with immune cells, were excised and then left untreated, subjected to two cycles of freeze-thawing, or irradiated (15-Gy) before transfer into the peritoneal cavity of the recipient mice.

In some transfer experiments, the graft-bearing kidney of the recipient athymic (*nu/nu*) mice was removed and the graft was prepared for immunohistological evaluation. Five weeks later, the same mice received another transplant of 3  $\mu$ l of fetal porcine ICCs under the capsule of the remaining kidney. After another 6 days, the animals were killed and the grafts removed and prepared for evaluation. Some of the recipient athymic B6 (*nu/nu*) mice that received an implant of ICC grafts from major histocompatibility complex (MHC)-mismatched donors (Table 3) were NK1.1<sup>+</sup> cell-depleted by means of repeated intraperitoneal injections of an anti-NK1.1 monoclonal antibody before transfer until the end of experiments (3,18,19).

For generating immune sera, male inbred B6 mice received an intraperitoneal injection of 6  $\mu$ l of fetal porcine ICCs at days 0, 7, and 14, and serum was collected on day 21. Intraperitoneal injections of the serum to the recipient athymic (*nu/nu*) mice (Table 1) were given either in daily portions of 0.1 ml on days 1–5 after ICC transplantation or as a single injection of 0.5 ml at the time of transplantation. Control experiments, in which all strains of mice received a transplant of fetal porcine ICC, were performed to evaluate the outcome in a conventional pig-to-mouse transplantation model.

**Immunohistochemistry.** Animals were killed at different time points after transfer (Tables 1–3). The grafts were collected and stored in a transport medium (Histocon, HistoLab, Betlehem Trading, Gothenburg, Sweden) at 4°C until snap-frozen in isopentane and subsequently stored at -70°C. Serial sections (6  $\mu$ m thick) were cut in a cryostat (-20°C), air-dried, and then stored at -70°C. After storage, the slides were fixed in cold acetone, diluted 1:2 in distilled water for 30 s, followed by final fixation in cold acetone (100%) for 5 min. Sections were incubated with antibodies for 30 min, followed by a

5-min wash in PBS between each step. Unspecific antibody binding was blocked by incubation with PBS containing 4% (wt/vol) BSA and 10% (vol/vol) normal rabbit serum (code no. X0902; Dako A/S, Glostrup, Denmark) for 10 min. Immunohistochemical stainings using rat anti-mouse monoclonal antibodies to F4/80, Mac-1, MHC class II, CD3, CD8 (Serotec, Kidlington, U.K.), and CD4 (Pharmingen, San Diego, CA) were performed as previously described (17). The slides were counterstained with hematoxylin and mounted in glycerine gelatin. Control experiments were performed by omitting the primary antibody. Surviving ICC and the frequency of the different cell phenotypes infiltrating the xenograft was assessed semiquantitatively.

**Real-time quantitative RT-PCR.** Athymic (*nu/nu*) mice that received a transplant of two ICC grafts were used in transfer experiments or left untreated as controls. The animals were killed at different time points after transfer, and one of the ICC xenografts was used for immunohistochemistry and the other for mRNA analysis (Table 1). This graft was peeled off the kidney with minimal amounts of underlying kidney tissue and immediately snap-frozen in liquid nitrogen and stored at -70°C until use.

mRNA from the grafts was extracted, immobilized onto oligo(dT)-coated manifold supports (20), and reverse-transcribed to cDNA according to a previously described protocol (16). 5' nuclease assays for quantitative analysis of the obtained cDNA were performed in triplicate using the iCycler iQ RealTime PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

The cDNA sequences for murine  $\beta$ -actin, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , IL-4, and IL-10 were obtained from GenBank, and the primer and probe sequences, specified in Table 4, were designed using PrimerExpress software (Applied Biosystems, Foster City, CA). To avoid amplification of genomic DNA, the primer-probe sets were designed to span exon-exon boundaries. The probes were labeled with 6-carboxyfluorescein at the 5' end and 6-carboxytetramethylrhodamine at the 3' end. PCR amplifications were performed in a total volume of 25  $\mu$ l, containing 5  $\mu$ l of cDNA sample, 800 nmol/l of each primer, 100 nmol/l of the corresponding probe, 200  $\mu$ mol/l dNTP, 0.625 units of AmpliTaq Gold (Applied Biosystems), 50 mmol/l KCl, 10 mmol/l Tris HCl (pH 8.3), 10 mmol/l EDTA and MgCl<sub>2</sub> (1 mmol/l for  $\beta$ -actin, 2 mmol/l for TNF- $\alpha$ , 3 mmol/l for IL-10, and 5 mmol/l for IL-1 $\beta$  and IL-4). For each reaction, the polymerase was activated by preincubating at 95°C for 10 min, and amplification was then performed by alternating between 95°C for 15 s and 58°C for 60 s for 50 cycles. For analysis of murine IL-2, IL-12p40,  $\gamma$ -interferon (IFN- $\gamma$ ), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 $\alpha$ , and RANTES (regulated upon activation in normal T-cells, expressed, probably secreted), PCR amplifications were performed using Predeveloped TaqMan Assay Reagents (Applied Biosystems) designed not to amplify genomic DNA. For each 25- $\mu$ l reaction, containing 5  $\mu$ l of cDNA sample, 6.25  $\mu$ l of water, 12.5  $\mu$ l of 2 $\times$  TaqMan Universal PCR Master Mix, and 1.25  $\mu$ l of 20 $\times$  Target Primers and Probe, the polymerase was activated by preincubating at 95°C for 10 min, and amplification was performed through 50 cycles of switching between 95°C for 15 s and 60°C for 60 s.

Known amounts of amplicons, generated by the different primer pairs, were diluted and run in all PCR amplifications. Standard curves, created by plotting C<sub>t</sub> values versus the log of the amount of cDNA template in the respective dilution, were then used to calculate the initial quantity of cDNA template in the tissue samples. No template controls, i.e., cDNA substituted with water, and RT controls, where reverse transcriptase had been left out in the cDNA synthesis, were run with the samples in all PCR amplifications to

TABLE 3  
MHC restriction

Donor of transferred material	Recipient of transferred material	Observation time after transfer (days)	No. of animals	Rejection after transfer
B6 (H-2 <sup>b</sup> )	B6 (H-2 <sup>b</sup> )	12	5	Yes
B6 (H-2 <sup>b</sup> )	BALB/c (H-2 <sup>d</sup> )	12	5	No/Yes*
B6 (H-2 <sup>b</sup> )	NMRI (H-2 <sup>q</sup> )	12	5	No
BALB/c (H-2 <sup>d</sup> )	BALB/c (H-2 <sup>d</sup> )	12	5	Yes
BALB/c (H-2 <sup>d</sup> )	B6 (H-2 <sup>b</sup> )	12	9	No†
BALB/c (H-2 <sup>d</sup> )	NMRI (H-2 <sup>q</sup> )	12	5	No
NMRI (H-2 <sup>q</sup> )	NMRI (H-2 <sup>q</sup> )	12	5	Yes
NMRI (H-2 <sup>q</sup> )	B6 (H-2 <sup>b</sup> )	12	9	No†
NMRI (H-2 <sup>q</sup> )	BALB/c (H-2 <sup>d</sup> )	12	5	No

Transfer of immunocompetent cells from donor mice MHC matched or mismatched with the recipient mice that previously received a transplant of ICC xenografts. \*Rejection was induced in two of five recipient mice. In one of these two recipient mice, the previously tolerated graft was found to be fibrotic. †Four of the animals in these groups were NK cell-depleted during the experiment after repeated intraperitoneal injections of an anti-NK 1.1 monoclonal antibody.

TABLE 4  
Oligonucleotides and probes used in 5' nuclease assays of IL-1 $\beta$ , IL-4, IL-10, TNF- $\alpha$ , and  $\beta$ -actin

Gene	Accession number GenBank	Forward primer sequence	5' nuclease probe (labeled with 5'-FAM and 3'-TAMRA)	Reverse primer sequence	PCR product (bp)
IL-1 $\beta$	NM_008361	5'TGT CCT GTG TGA TGA AAG ACG G3'	5'ACA CCC ACC CTG CAG CTG GAG AGT3'	5'TTG GGT ATT GTT TGG GAT CCA3'	69
IL-4	M25892	5'ACA GGA GAA GGG ACG CCA T3'	5'TCC TCA CAG CAA CGA AGA ACA CCA CA3'	5'GAA GCC CTA CAG ACG AGC TCA3'	95
IL-10	M37897	5'TGT GAA AAT AAG AGC AAG GCA GTG3'	5'AGC AGG TGA AGA GTG ATT TTA ATA AGC TCC AAG ACC3'	5'CAT TCA TGG CCT TGT AGA CAC C3'	85
TNF- $\alpha$	M11731	5'GAC CCT CAC ACT CAG ATC ATC TTC T3'	5'TAG CCC ACG TCG TAG CAA ACC ACC AA 3'	5'CGC TGG CTC AGC CAC TCC3'	105
$\beta$ -actin	X03672	5'GCT CTG GCT CCT AGC ACC3'	5'TGA AGA TCA AGT CAT TGC TCC TCC TGA GC3'	5'CCA CCT ATC CAC ACA GAG TAC TTG3'	73

screen for possible contamination and genomic amplification. Data are given as mean  $\pm$  SE (Fig. 2).

## RESULTS

Results from the different transfer experiments described below are summarized in Tables 1–3. All donor and recipient mice tolerated the transplantation, immunization, and transfer procedures without any visible signs of infirmity. At the point of transfer, an abundant number of fetal porcine ICC, arranged in chords and duct-like structures, or mouse or rat islets were seen in the athymic (*nu/nu*) recipient mice. No inflammatory reaction was observed in these grafts.

**Transfer to athymic B6 (*nu/nu*) mice that had previously received a transplant of fetal porcine ICC.** After transfer, ICC xenografts in athymic (*nu/nu*) mice were rejected with no or only a few remaining endocrine cells, irrespectively of whether the transfer was from normal or Ig-deficient B6 mice. Mice that received another transplant with an ICC xenograft 6 weeks after transfer also rejected this graft. ICC xenograft rejection was not induced after freeze-thawing or 15-Gy irradiation of the transferred tissue before transfer. Also, intraperitoneal injections of immune serum did not produce any signs of ICC xenograft destruction (Table 1).

In rejecting animals, the pattern of cellular infiltration resembled that seen in normal B6 mice after ICC transplantation. The majority of the infiltrating cells were large, macrophage-like cells expressing the macrophage-specific phenotype marker F4/80, as well as Mac-1 and MHC class II. A few CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T-cells were accumulated mainly in the peripheral parts of the xenograft. When compared with fetal porcine ICC xenograft rejection in normal B6 mice, athymic B6 (*nu/nu*) mice after transfer exhibited reduced numbers of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> cells within the rejected xenograft. The process was somewhat faster after transfer when compared with ICC xenograft rejection in normal mice.

**Evaluation of intragraft cytokine and chemokine mRNA expression.**

**Controls (no transfer).** ICC xenografts were tolerated and no infiltrating cells were detected in the graft corresponding to low levels of  $\beta$ -actin gene expression. No or only low mRNA expression of all of the studied cytokines and chemokines was detected.

**Day 2 after transfer.** Transcripts of  $\beta$ -actin, IL-1 $\beta$ ,

MCP-1, and RANTES were detected in all animals at low levels. IL-2, IL-4, IL-10, IL-12p40, TNF- $\alpha$ , IFN- $\gamma$ , and MIP-1 $\alpha$  mRNAs were detected in only one of five animals or not at all.

**Day 3 after transfer.** An elevated gene expression of  $\beta$ -actin correlated with the appearance of the first immune cells within the grafts. Gene expression of TNF- $\alpha$ , IFN- $\gamma$ , IL-2, IL-4, IL-10, and IL-12p40 was detected in the majority of the animals. IL-1 $\beta$  demonstrated a marked elevation of mRNA expression compared with day 2. MIP-1 $\alpha$  was detected in the grafts of all animals, and MCP-1 gene expression was increased compared with day 2. RANTES mRNA levels remained low.

**Day 4 after transfer.** Peaking  $\beta$ -actin mRNA levels reflected a further increase of infiltrating immune cells into the grafts. Gene expression of IL-2 and particularly IL-12p40 showed a marked increase, and both IFN- $\gamma$  and TNF- $\alpha$  mRNA levels also increased by day 4, whereas mRNA levels of IL-4, IL-10, and IL-1 $\beta$  were comparable with those of day 3. RANTES demonstrated a fivefold increase in gene expression compared with day 3, and mRNA levels of MIP-1 $\alpha$  and MCP-1 remained elevated.

**Day 6 after transfer.** Almost all endocrine cells were rejected 6 days after transfer, and at this stage the grafts were infiltrated by a massive number of immune cells. However, expression of  $\beta$ -actin mRNA was decreased by  $\sim$ 75% compared with day 4. A marked decrease in gene expression compared with day 4 of all cytokines and chemokines analyzed was observed (grafts illustrated in Fig. 2).

**Transfer to athymic B6 (*nu/nu*) mice that previously received a transplant of fetal porcine ICC together with syngeneic B6 islets, allogeneic BKs islets, or concordant xenogeneic rat islets.** Twelve days after transfer, the ICC xenograft was rejected in all recipients and the cellular infiltration resembled that seen in the grafts of rejecting immunocompetent mice. In contrast, the concordant xenogeneic rat islet grafts were completely intact in some mice, whereas in other mice, the rat islet grafts were incompletely rejected and exhibited large areas of remaining intact endocrine tissue divided by septa of inflammatory cells, characterized as F4/80<sup>+</sup>, Mac-1<sup>+</sup>, and MHC class II<sup>+</sup> cells. Large numbers of CD3<sup>+</sup> T-cells, predominantly consisting of CD8<sup>+</sup> cells, were also present.

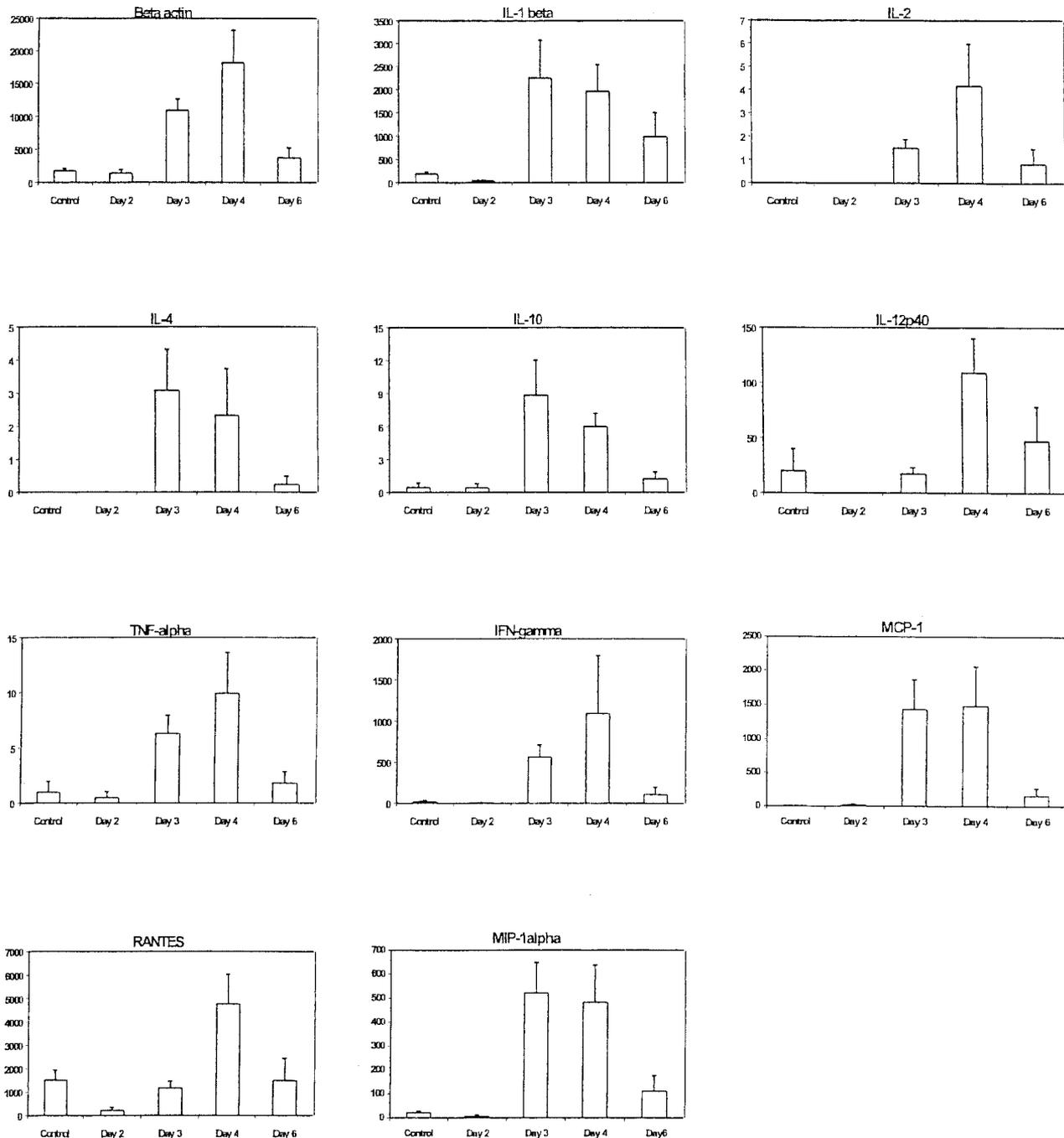


FIG. 2. Graphs representing the absolute levels of mRNA copies (divided by  $10^3$ ) ( $y$ -axis) at different times after transfer, ( $x$ -axis). Values are expressed as mean  $\pm$  SE. Controls did not receive any transferred graft. The variation in expression of the different gene transcripts does not necessarily reflect their relative importance. Differences in mRNA degradation, translation efficiency, and receptor properties for the cytokines and chemokines remain to be fully characterized. Moreover, both the cDNA synthesis methods and the real-time PCR protocol applied to this experiment could differ in efficiency from one target molecule to another. As a consequence, rather than comparing absolute levels of different gene transcripts, the interpretation should be restricted to relating variations of each cytokine and chemokine transcript level at different time points.

In the allogeneic BK islet grafts and syngeneic B6 islet grafts, no signs of rejection was seen (Table 2).

**Transfer to MHC-matched or -mismatched athymic ( $nu/nu$ ) mice that previously received a transplant of fetal porcine ICC.** Twelve days after transfer of grafts from MHC-matched donors, ICC xenograft rejection was evident in all three strains of mice. Even though xenografts were heavily infiltrated with immune cells, some intact ICC were still seen in BALB/c ( $nu/nu$ ) and NMRI

( $nu/nu$ ) recipient mice. The pattern of cellular infiltration resembled that seen in the corresponding normal mice after ICC transplantation (Table 3).

Transfer of grafts from MHC-mismatched donors did not produce any signs of ICC xenograft destruction in the athymic ( $nu/nu$ ) recipients, including mice depleted of NK1.1+ cells, apart from two of five mice in one of the groups (B6 to BALB/c [ $nu/nu$ ]). In one of these two recipient mice, the previously transplanted ICC xenograft

was found to be fibrotic. Some MHC-mismatched groups exhibited CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T-cells within the perigraft area, whereas some were completely devoid of T-cells. Occasional F4/80<sup>+</sup>, Mac-1<sup>+</sup>, and MHC class II<sup>+</sup> cells were also seen in the peripheral parts of the graft.

## DISCUSSION

In this experimental transfer model, the athymic (*nu/nu*) mice seemed to be reconstituted with xenoreactivity exclusively, because allogeneic mouse islets remained unaffected by the transfer up to 12 days after transfer and concordant xenogeneic rat islets induced only incomplete rejection. This notion is also supported by the fact that no graft-versus-host reaction was detected when the transfer was performed between MHC-mismatched animals.

Still 6 weeks after transfer of sensitized immune cells, the athymic (*nu/nu*) mice were capable of rejecting a newly implanted porcine ICC xenograft. This finding suggests that a state of memory was induced at the time of transfer. The only cells capable of mediating this type of immunological memory are sensitized lymphocytes. Injection of hyperimmune serum failed to induce ICC xenograft rejection in the athymic (*nu/nu*) mice, and transfer of sensitized immunocompetent cells obtained from Ig-deficient mice, lacking mature B-cells, readily induced rejection. Taken together, these observations demonstrate that the ability to induce xenograft rejection in this experimental model is dependent on the transfer of sensitized T-cells. Morphologically, the pattern of cellular infiltration in the rejected grafts of the reconstituted athymic (*nu/nu*) mice resembled that seen in native immunocompetent mice after ICC xenotransplantation. Activated host macrophages infiltrated the ICC xenograft, and T-cells were seen accumulating in the periphery, much like the immune response associated with a delayed-type hypersensitivity (DTH) reaction. Because no inflammatory reaction is triggered by the transplantation procedure, we conclude that the macrophages were recruited to the ICC graft as a crucial component in a specific immune response. As demonstrated by Wolf et al. (21), xenogeneically transplanted islets largely provoke the indirect pathway of antigen presentation, i.e., sensitized T-cells recognize pig xenoantigens in association with host (murine) MHC molecules. However, this combination of murine MHC class II and porcine peptide is not expressed on the transplanted porcine cells, thereby allowing the xenograft to escape direct T-cell-mediated killing. Instead, the CD4<sup>+</sup> T-cells recruit massive numbers of monocytes/macrophages to the site of the graft. The macrophages are activated and function as effector cells in the rejection (12,13).

It has been demonstrated that sensitized T-cells are unable to transfer DTH to naive mice when donor and recipient are mismatched with regard to MHC class II molecules (22), indicating that the capacity of the CD4<sup>+</sup> T-cells to induce a DTH response is MHC class II-restricted. In the present study, an MHC mismatch between the donor of the immunocompetent cells and the recipient athymic (*nu/nu*) mice rendered the transferred cells unable to induce ICC xenograft rejection. NK cell-mediated elimination of the transferred MHC-mismatched T-cells did not account for this observation because also

the grafts of recipients depleted of NK cells remained intact. The findings in this study, together with the previously reported dependence of CD4<sup>+</sup> T-cells in islet xenograft rejection (9,21), strongly indicate that direct interaction between the T-cell receptor on transferred xenospecific CD4<sup>+</sup> cells and MHC class II on host cells is required to induce ICC xenograft rejection. Morphological studies on ICC xenografts removed from athymic (*nu/nu*) mice in the various MHC-mismatched groups indicate that the requirement of MHC compatibility might be present on two levels. Some groups displayed grafts completely devoid of T-cells, indicating a failure of the CD4<sup>+</sup> T-cells to find the ICC xenograft lined with MHC-mismatched host endothelial cells (ECs), whereas grafts in other groups displayed CD4<sup>+</sup> T-cells within the xenograft area apparently unable to activate MHC-mismatched monocytes/macrophages. All mouse strains in the experiment were equally mismatched with regard to MHC class I (H-2K, -D, and -L) as well as to MHC class II (I-A and I-E) molecules; thus, differences in MHC mismatch do not provide an explanation for these observations.

After ICC xenograft transplantation into an athymic (*nu/nu*) mouse, the graft induces revascularization of recipient origin and the process is morphologically completed within 1–2 weeks (23). Still, in this experimental model, a number of xenospecific T-cells are able to wander from the transferred ICC xenograft in the peritoneal cavity into the circulation and by some mechanism identify the ICC graft (with no ongoing inflammation) and cross through the endothelium to initiate rejection. The rapidity and the reduced numbers of T-cells involved in the process (as compared with native immunocompetent mice) suggests that this mechanism is highly effective. It may be speculated that after transplantation, porcine antigens are constantly shed and expressed together with MHC class II on the host ECs and that an interaction between the transferred T-cells and the MHC class II/xenoantigen complex on the host ECs allows the antigen-specific CD4<sup>+</sup> T-cells to home to the ICC xenograft. This would provide an explanation for the apparent requirement of an MHC match between the transferred cells and the host. Enhanced transendothelial recruitment and activation of sensitized T-cells induced by activated human ECs has previously been reported (24,25). It is interesting that in this study, the recruitment of xenospecific T-cells to the site of the ICC xenograft was also highly effective in athymic (*nu/nu*) mice carrying the ICC graft for >1 year. Hence, it seems that the host ECs in the ICC graft do not necessarily have to be activated by a local inflammatory reaction to home xenospecific T-cells to the site of the graft.

Macrophages present within the xenograft engulf xenoantigens and present pig peptides in relation to host MHC class II. This will allow for an interaction between the T-cell receptor on the xenospecific CD4<sup>+</sup> T-cells and the MHC class II together with porcine peptide on host macrophages. This antigen presentation will restimulate the CD4<sup>+</sup> T-cells and enhance their capacity to activate the macrophages into DTH effector cells. This may be achieved by secreted cytokines with macrophage-activating properties, such as TNF- $\alpha$  and IFN- $\gamma$ .

The recruitment of massive numbers of effector mono-

cyte/macrophage cells across the endothelium to the ICC xenograft is likely to be dependent on chemotactic factors. In this study, we analyzed the mRNA expression of MCP-1, MIP-1 $\alpha$ , and RANTES, three CC-chemokines that are known to be induced in a variety of inflammatory conditions (26–29), including transplant rejection (30). These chemokines are potent chemoattractants for monocytes/macrophages and have been shown to upregulate  $\beta$ -integrins on monocytes and enhance the transendothelial migration of these cells (31). In this experiment, we observed a marked increase in mRNA expression of all three chemokines in the grafts, MCP-1 and MIP-1 by days 3 and 4 and RANTES by day 4, which was in parallel with the appearance of infiltrating T-cells. Initially, the source of these chemokines is most probably the transferred sensitized xenospecific CD4<sup>+</sup> T-cells within the graft, but in the later phases of the rejection process, activated ECs and effector macrophages may also be contributing.

That there was a concurrent upregulation of all cytokine transcripts analyzed, including both Th1-associated (IL-12p40 and IFN- $\gamma$ ) and Th2-associated (IL-4 and IL-10) cytokines, by day 4 after transfer indicates that the mechanism of rejection in the transfer model is complex and likely to consist of several components. The cellular infiltration of ICC xenograft rejection in rodents resembles that of a DTH reaction, the prototype of a Th1 response. Previous findings in this laboratory using real-time quantitative RT-PCR to characterize cytokine kinetics during ICC xenograft rejection in rats have implicated a major role of Th1 cells in the initial stages of ICC xenograft rejection followed by Th2-associated responses in the later phases (16). Notably, in this model, the transferred T-cells were harvested from the donor animals on day 6 after ICC transplantation, and xenograft rejection in the athymic (*nu/nu*) mice was completed after an additional 4–6 days. Hence, the T-cells mediating rejection in the athymic (*nu/nu*) mice may be phenotypically and functionally similar to T-cells present in a primary xenograft 10–12 days after transplantation, and at this stage, both Th1- and Th2-associated cytokines are expressed in the xenograft.

In summary, a new experimental model of ICC xenograft rejection in mice was introduced. Transfer of immunocompetent cells, removed from the site of ongoing fetal porcine ICC xenograft rejection in normal mice, enabled athymic (*nu/nu*) mice to reject a previously tolerated ICC xenograft. It was demonstrated that the rejection is dependent on the transfer of viable cells and that these cells are homing to and induce rejection of the xenograft exclusively. Moreover, for the rejection to be initiated, MHC compatibility between the donor and the recipient was required. Finally, the mechanism of graft rejection in this model is highly efficient and is likely to be complex as illustrated by the parallel upregulation of mRNA expression in the ICC xenografts of both Th1- and Th2-associated cytokines.

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