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Specific tolerance across a discordant xenogeneic transplantation barrier

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ABSTRACT **Successful induction of tolerance across disparate (discordant) species barriers could overcome the organ shortage that presently limits clinical transplantation. We demonstrate here that xenogeneic swine thymic transplants can induce tolerance to swine antigens in mice, while positively selecting functional host CD4+ T cells. Immunologically normal C57BL/10 mice were thymectomized and depleted of T and natural killer cells; then they received transplants of fetal pig thymus and liver fragments. Mature mouse CD4+ T cells developed in the pig thymus grafts and migrated to the periphery. Swine grafts grew markedly and no anti-pig IgG response was produced. Mixed lymphocyte reactions confirmed that the new T cells were functional and were tolerant to pig antigens.**

Thymic epithelial and bone marrow-derived cells determine the T-cell repertoire via positive and negative selection in the thymus, but the role of each cell type in thymocyte selection is controversial. Although the major role of thymic epithelium is thought to be positive selection of developing thymocytes, recent studies utilizing either mice expressing a transgenic T-cell antigen receptor (TCR) specific for a viral peptide or using mice with major histocompatibility complex (MHC)-mismatched thymic stromal and bone marrow-derived cells demonstrate that thymic stroma can also anergize and/or delete developing T cells (1–4), resulting in a state of transplantation tolerance. To our knowledge, no previous studies have examined the potential of thymic stroma to induce tolerance across discordant xenogeneic barriers. We have previously demonstrated that fetal pig thymus and liver (THY/LIV) grafts transplanted into *scid* mice can grow and support pig thymopoiesis *in vivo*, with histologically normal thymic architecture (5). We have now used immunocompetent C57BL/10 (B10) mice to test the ability of pig thymus to induce specific tolerance to discordant pig antigens.

MATERIALS AND METHODS

Transplantation Protocol. All mice were purchased from the Frederick Cancer Research Facility, Frederick, MD. Six- to 12-week-old euthymic or thymectomized (ATX) B10 mice received i.p. injections of monoclonal antibodies (mAbs) GK1.5 (anti-mouse CD4), 2.43 (anti-mouse CD8), 30-H12 (anti-mouse Thy1.2), and PK136 (anti-mouse NK1.1) in depleting doses, as described (6), on days −6 and −1 prior to transplant. On either day −1 or day 0, 7 grays (Gy) of localized mediastinal (thymic) irradiation and 3 Gy of whole-body irradiation were administered to recipients, and second trimester (gestational day 36–72) miniature swine fetal thymic and liver fragments, ≈1 mm^3 in size, were transplanted under the kidney capsule via a midline laparotomy incision. Mediastinal irradiation was found to be unnecessary for mouse T cells to mature in pig thymus grafts and was therefore eliminated from the conditioning regimen in later experiments. After the abdomen was closed in two layers, 10^8 fetal liver cells (FLCs) from the same litter were injected i.p. Recipients were treated on a weekly basis posttransplantation with depleting doses of the same four mAbs for a period of 0–6 weeks. No difference in murine CD4+ T-cell reconstitution or tolerance to pig antigens was observed in mice that were treated with no mAb posttransplant compared to those that received 6 weeks of mAb treatment posttransplant. Some groups of control mice were maintained on chronic mAb treatment until the time of sacrifice, as is detailed below. Animal care was in accordance with the American Association for the Accreditation of Laboratory Animal Care and institutional guidelines. Operations were performed under metofane inhalational anesthesia.

In a variation on this protocol, BALB/c nude mice were depleted of natural killer (NK) cells with an i.p. injection of 50 μl of rabbit anti-mouse asialo-GM1 serum (Wako Chemicals USA, Richmond, VA) on day −2 and received 3 Gy of whole-body irradiation on day 0. Fetal swine THY/LIV grafts were then placed under the kidney capsule, and 10^8 FLCs were injected i.p.

mAbs and Flow Cytometry (FCM). Mice were tail bled at regular intervals posttransplant to obtain peripheral white blood cells (WBCs), which were treated with hypotonic shock to remove red blood cells (RBCs). Cells were stained with a fluoresceinated rat anti-mouse CD4 mAb (PharMingen; green fluorescence) versus biotinylated hamster anti-mouse αβ-TCR mAb (PharMingen) or rat anti-mouse CD8 mAb (PharMingen) plus phycoerythrin streptavidin (orange fluorescence) and analyzed by two-color FCM using either a FACScan or FACSort flow cytometer (Becton Dickinson). Fluorescein isothiocyanate- or biotin-conjugated murine mAb HOPC1, with no known reactivity to pig or mouse cells, was used as the negative control mAb in both the green and orange fluorescence. Nonviable cells were excluded using the vital nucleic acid stain propidium iodide. Percentages of positive cells were determined by subtracting the percentage of cells staining with the control mAb HOPC1 from the percentage of cells staining with the anti-mouse mAbs in the same quadrant on a two-color dot plot.

Ammonium chloride potassium-lysed splenocytes or peripheral WBCs (RBCs were removed by hypotonic shock) were collected 13–19 weeks posttransplant and analyzed by FCM for $V_\beta 11$ T-cell deletion. Murine Fc receptors were blocked using rat anti-mouse Fc receptor mAb 2.4G2, and cells were then stained with either fluoresceinated hamster

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Abbreviations: TCR, T-cell antigen receptor; MHC, major histocompatibility complex; THY/LIV, thymus and liver; ATX, thymectomized; mAb, monoclonal antibody; NK, natural killer; FCM, flow cytometry; MLR, mixed lymphocyte reaction; FLC, fetal liver cell; RBC, red blood cell; WBC, white blood cell.

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anti-mouse V_β8.1/8.2 TCR mAb (PharMingen) or rat anti-mouse V_β11 TCR mAb (PharMingen; green fluorescence) followed by phycoerythrin-conjugated rat anti-mouse CD4 and CD8 mAbs (PharMingen; orange fluorescence) and analyzed by two-color FCM. Fluoresceinated murine mAb HOPC1, with no known reactivity to mouse cells, or rat anti-mouse IgG1 (Zymed) was used as the negative control mAb in the green fluorescence. Phycoerythrin-conjugated mAb Leu-4 (Becton Dickinson) was used as the negative control mAb in the orange fluorescence. Approximately 5000 gated $CD4^+$ and $CD8^+$ cells were usually collected for analysis of V_β families. Nonviable cells were excluded using the vital nucleic acid stain propidium iodide. Percentages of positive cells were determined as described above.

Mixed Lymphocyte Reactions (MLRs). Murine spleen cell suspensions were prepared, RBCs were lysed with ammonium chloride potassium, and the remaining cells were washed and reconstituted in RPMI medium supplemented with 15% (vol/vol) controlled processed serum replacement (CPSR-2; Sigma), 4% nutrient mixture (L-glutamine, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin), 1% Hepes, and 10 μM 2-mercaptoethanol. Swine peripheral blood lymphocytes were prepared by centrifugation over a Ficoll/Hypaque layer. Triplicate wells containing 4×10^5 responders with either 4×10^5 murine stimulators (30 Gy irradiated) or 1×10^5 swine stimulators (30 Gy irradiated) in a total volume of 0.2 ml of medium were incubated at 37°C for 4 days in 5% CO_2. Cultures were pulsed with 1 μCi (1 Ci = 37 GBq) of ^3H on the third day, harvested on the fourth day with a Tomtec automated harvester, and assayed in a Pharmacia LKB liquid scintillation counter. MLRs for all mice tested ($n = 6$) were set up in duplicate and pulsed on days 3 and 4 and harvested on days 4 and 5 with similar results.

RESULTS AND DISCUSSION

We treated B10 mice with a nonmyeloablative conditioning regimen that permits induction of tolerance to rat xenoantigens in mice (6). Euthymic or ATX mice received depleting doses of anti-T cell and anti-NK cell mAbs, 7 Gy of mediastinal irradiation and 3 Gy of whole-body irradiation, and then fetal swine THY/LIV transplants under the kidney capsule followed by administration of 10^8 FLCs i.p. Mice either received no further anti-T cell and anti-NK cell mAb treatments after 0 to 6 weeks posttransplant or were maintained on chronic mAb treatment for the duration of the experiment.

Swine THY/LIV grafts grew initially in treated euthymic mice but stopped growing after T cell- and NK cell-depleting mAbs were discontinued, and these mice developed anti-pig IgG responses (data not shown). When euthymic mice were maintained on chronic mAb treatment, the grafts enlarged markedly (Fig. 1*A*), and no T cell-dependent anti-pig IgG_1, IgG_{2a}, or IgG_{2b} response was observed. Pig thymopoiesis was supported for at least 32 weeks during chronic mAb administration, although no pig T cells were detected in the periphery by FCM. Percentages of intragraft $CD4^+/CD8^-$, $CD4^-/CD8^+$, $CD4^+/CD8^+$, and $CD4^-/CD8^-$ pig thymocyte subsets were similar to those in normal pig thymus.

In contrast, swine THY/LIV grafts grew markedly in adult ATX mice that received only a short (<6 weeks) course of mAb treatment posttransplant (Fig. 1*B*). FCM analysis of peripheral WBCs in these mice 6 weeks after discontinuing mAb treatment revealed the presence of mature ($\alpha\beta$-TCR^{hi}) mouse T cells. Unlike T cells in euthymic grafted mice, these cells were tolerant to pig antigens, as evidenced by the growth of swine THY/LIV grafts (Fig. 2) and the absence of anti-pig T cell-dependent IgG antibody responses in seven of nine mice. The majority (>90%) of the $\alpha\beta$-TCR^{hi} T cells were $CD4^+/CD8^-$. FCM analyses 13–26 weeks posttransplant

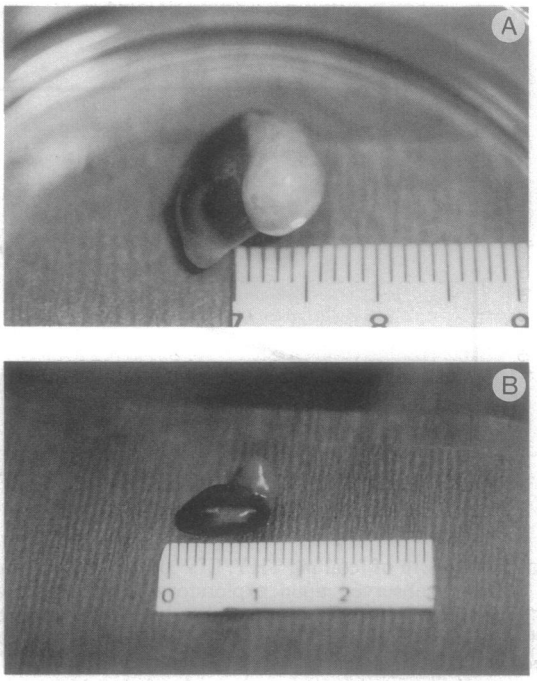

FIG. 1. Swine THY/LIV grafts in euthymic and ATX B10 mice depleted of T cells and NK cells. (*A*) Graft from a euthymic mouse chronically treated with mAbs until the time of sacrifice 32 weeks posttransplant. (*B*) Graft from an ATX B10 mouse 14 weeks after mAbs were discontinued and after murine peripheral $CD4^+$ T cells had recovered (20 weeks posttransplant).

demonstrated normal mouse thymocyte subsets in swine thymi. For example, 7.9% $CD4^+/CD8^-$, 2.9% $CD4^-/CD8^+$, 85.5% $CD4^+/CD8^+$, 3.7% $CD4^-/CD8^-$, and 11.6% $\alpha\beta$-TCR^{hi} thymocytes were found in a swine THY/LIV graft by FCM 17 weeks posttransplant compared to 3.5% $CD4^+/CD8^-$, 3.2% $CD4^-/CD8^+$, 87.8% $CD4^+/CD8^+$, 5.5% $CD4^-/CD8^-$, and 10.0% $\alpha\beta$-TCR^{hi} thymocytes in a normal B10 thymus. Fetal swine liver grafted without a thymus fragment did not grow in control mAb-treated ATX B10 mice, and $\alpha\beta$-TCR^{hi} T cells did not appear in the periphery. Thus, the pig thymus was required for the development of mature mouse T cells.

Mouse anti-pig MLRs were performed to determine whether or not mouse T cells that matured in pig thymus grafts were tolerant to pig antigens. Four of six ATX B10 mice ($H\text{-}2^b$) transplanted with pig THY/LIV grafts mounted no anti-B10 or anti-pig responses but demonstrated normal responses against a fully MHC-mismatched allogeneic stimulator, B10.BR ($H\text{-}2^k$) (Fig. 3). Additional experiments demonstrated that fetal THY/LIV grafts were sufficiently immunogenic to induce strong MLR responses to pig antigens in similarly conditioned, grafted euthymic control mice (data not shown).

To determine if host bone marrow-derived cells were participating in negative selection of the developing mouse thymocytes, we transplanted fetal pig THY/LIV grafts into both $I\text{-}E^+$ (BALB/c nude) and $I\text{-}E^-$ (ATX B10) recipients. $I\text{-}E^+$ mice delete V_β11 T cells because of presentation in the thymus of an endogenous superantigen in association with I-E (7), whereas $I\text{-}E^-$ mice do not delete this T-cell family. The percentages of V_β11 T cells were therefore compared between $I\text{-}E^+$ and $I\text{-}E^-$ recipients of fetal pig thymus grafts in which murine T cells developed in pig thymi. ATX B10 recipients were treated as above. BALB/c nude mice were depleted of NK cells and irradiated with 3 Gy of whole-body irradiation prior to transplant. These mice also developed large numbers of mature $CD4^+$ T cells that migrated to the

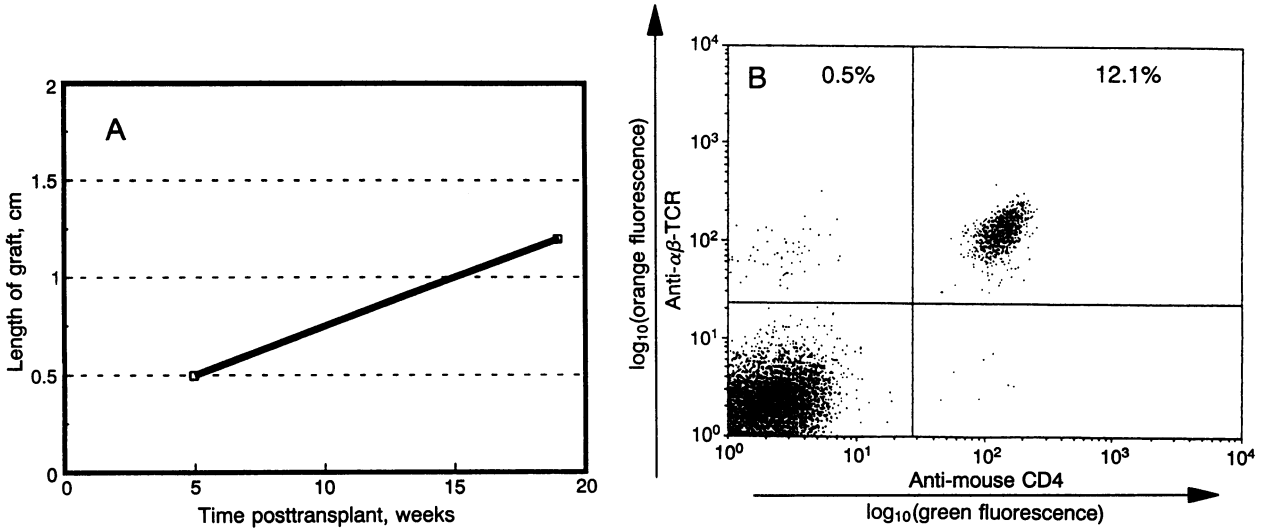

FIG. 2. Growth of fetal pig THY/LIV graft in the presence of mature mouse T cells in the periphery. An increase in fetal pig THY/LIV graft size was observed upon exploratory laparotomy performed at 5 and 19 weeks posttransplant, despite the presence of mature CD4$^+$/αβ-TCR^{hi} T cells in the peripheral blood (shown 16 weeks after mAbs were discontinued). Peripheral WBCs contained 12.1% CD4$^+$/αβ-TCR^{hi} T cells and 0.5% CD8$^+$/αβ-TCR$^+$ T cells. Control ATX mice that received fetal swine liver grafts without a thymus fragment did not maintain their grafts and developed <5% αβ-TCR$^+$ T cells in the periphery. ATX mice were conditioned as described above. (*A*) Exploratory laparotomies were performed at 5–6 and 15–19 weeks posttransplant to measure graft size. (*B*) A dot plot analysis of live peripheral WBCs of a representative animal 16 weeks posttransplant (16 weeks after mAbs were discontinued) is shown. Overall, 57% (27/47) of ATX mice treated with this protocol maintained swine grafts and reconstituted their CD4$^+$ T-cell compartment. We currently achieve this result in 90% (9/10) of mice treated with this regimen.

periphery. Complete deletion of V_β11 T cells was observed in the periphery of BALB/c nude recipients of fetal swine thymus grafts (Table 1), suggesting that mouse I-E also participated in negative selection of mouse T cells developing in pig thymi. Negative selection is most likely carried out by murine Ia$^+$ dendritic cells, which were detected predominantly in the cortico-medullary junction of swine thymus grafts by immunoperoxidase staining (data not shown). In the ATX B10 recipients of swine THY/LIV grafts, a reduction in the percentage of V_β11$^+$ T cells was observed compared to normal B10 mice (mean ± SD: 2.8% ± 0.8%, ATX B10; 5.2% ± 0.5%, normal B10, $P < 0.005$), suggesting that the pig SLA-DR class II, which shares significant homology with mouse I-E class II, may participate in negative selection of mouse T cells developing in the pig thymus graft (Table 1).

These studies demonstrate that discordant xenogeneic thymic stroma is capable of supporting mouse thymopoiesis and that CD4$^+$/CD8$^-$/αβ-TCR^{hi} T cells that are released into the periphery are phenotypically normal, functional, and tolerant to donor xenoantigens and to host antigens. The lack of CD4$^-$/CD8$^+$/αβ-TCR^{hi} repopulation in the periphery may be due to failure of mouse CD8 to interact with pig MHC class I molecules (9), as has been demonstrated for mouse anti-human responses (10), thereby preventing positive selection of CD8$^+$ thymocytes by swine thymic epithelium. Since human CD8$^+$ T cells are able to interact with pig MHC class I directly (11), human CD8$^+$ T cells might mature effectively in swine fetal thymus grafts if this model were to be applied clinically to induce donor-specific tolerance to xenoantigens for clinical organ transplantation.

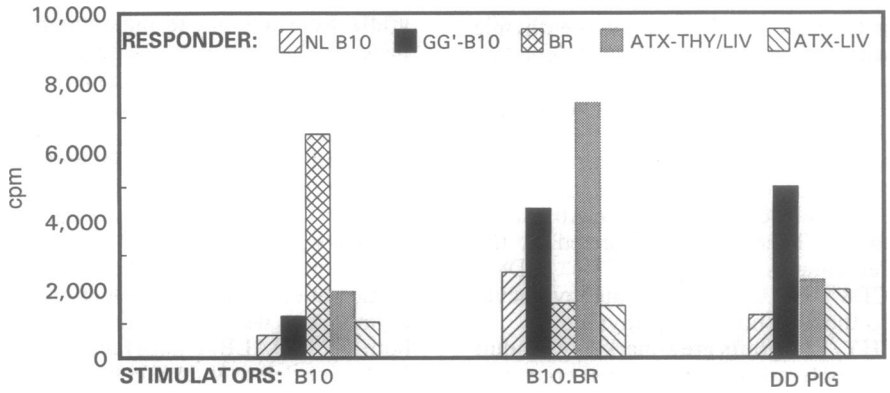

FIG. 3. Specific unresponsiveness of B10 mice transplanted with fetal pig THY/LIV grafts to pig antigens in MLRs. ATX B10 (H-2^b) mice transplanted with pig THY/LIV grafts (ATX-THY/LIV) demonstrated specific unresponsiveness to pig antigens despite the presence of normal responsiveness to a fully MHC-mismatched allogeneic B10.BR (H-2^k) stimulator. Control ATX B10 mice that received a swine liver graft without a thymus fragment (ATX-LIV) mounted no responses to any stimulator, demonstrating the importance of the pig thymus graft in the development of functional mouse T cells. Positive control anti-pig MLR is from a mouse immunized with a swine skin graft, since mice do not mount primary anti-pig MLR responses (8, 9). Sterile splenocyte suspensions from normal B10 (NL B10), normal B10 grafted with GG (SLA-I^c/SLA-II^d) pig skin 12 weeks earlier (GG'-B10), normal B10.BR (BR), or ATX B10 mice conditioned with the nonmyeloablative regimen described above and transplanted with either a DD fetal pig (SLA-I^d/SLA-II^d) THY/LIV graft (ATX-THY/LIV) or a fetal pig liver graft only (ATX-LIV) were used as responders with the indicated 30 Gy irradiated stimulator cell populations.

Table 1. $V_\beta 11$ deletion in mice transplanted with swine THY/LIV grafts

n	Strain	THY/LIV graft	% $V_\beta 8.1/8.2$*	% $V_\beta 11$*
4	Normal C57BL/10	−	16.3 ± 2.2	5.2 ± 0.5
4	Normal BALB/c	−	20.8 ± 0.3	0.2 ± 0.1
4	C57BL/10†	+	16.7 ± 3.0	2.8 ± 0.8
5	BALB/c nude‡	+	20.0 ± 3.7	0.4 ± 0.3

*Ammonium chloride potassium-lysed splenocytes or peripheral WBCs (RBCs were removed by hypotonic shock) were collected 13–19 weeks posttransplant, and gated $CD4^+$ and $CD8^+$ T cells were analyzed by FCM for $V_\beta 11$ T-cell deletion. Results are presented as the mean ± SD of results obtained for individual mice. $P < 0.005$ for the percentage of $V_\beta 11$ in normal B10 mice compared to THY/LIV-grafted B10 mice. $P > 0.20$ for the percentage of $V_\beta 11$ in normal BALB/c mice compared to THY/LIV-grafted BALB/c nude mice.

†B10 recipients were treated as described in *Materials and Methods*.

‡BALB/c nude mice were depleted of NK cells by using rabbit anti-asialo-GM1 serum, received 3 Gy of whole-body irradiation, and received fetal swine THY/LIV grafts under the kidney capsule, followed by injection of 10^8 FLCs i.p. on day 0.

Presumably, tolerance to pig antigens is not induced in euthymic mice that receive swine THY/LIV grafts because mouse T-cell progenitors mature in the host thymus, which lacks the pig cells necessary to tolerize developing mouse thymocytes. The nonmyeloablative conditioning regimen used in this study permits engraftment of rat marrow and induction of donor-specific tolerance in murine recipients (6). Tolerance is thought to be induced in this model by rat dendritic cells detected at the cortico-medullary junction of the thymus of chimeric animals (L.A.L., D.H.S., and M.S., unpublished results). In the present study, failure of pig hematopoietic stem cells, present in the FLC suspension administered on day 0, to migrate to the mouse thymus may be due to failure of homing and differentiation, possibly reflecting species specificity of cytokines and adhesion molecules. In ATX recipients, on the other hand, mouse T-cell progenitors home to the pig thymus graft and are tolerized to pig antigens. No mouse thymic epithelium is present in ATX hosts, but mouse dendritic cells are detectable in THY/LIV grafts and probably mediate the observed clonal deletion of cells reactive to host antigen. Although the decreased percentage of $V_\beta 11^+$ T cells in ATX B10 recipients of swine THY/LIV grafts suggests clonal deletion by swine cells, it is possible that there is a defect in the positive selection of this V_β family on swine thymic stroma. However, the normal percentages of $V_\beta 8.1/8.2$ T cells in both ATX B10 and BALB/c nude recipients of swine THY/LIV grafts compared to those in normal B10 and BALB/c mice suggest that no defect in positive selection is present. To explain the observed tolerance, we hypothesize that swine thymic cells of hematopoietic and/or nonhematopoietic origin either clonally delete or anergize developing mouse thymocytes reactive to donor xenoantigens.

Further definition of the cell types responsible for positive selection of mouse thymocytes in pig THY/LIV grafts will be essential to a full understanding of immunocompetence in these mice. A similar study in which fetal human THY/LIV grafts were transplanted into NIH *bg nu xid* mice demonstrated that mouse T cells that matured in these grafts were able to mount protein-specific responses (12), suggesting that the mouse T cells were able to recognize antigen presented in the context of the murine MHC. MHC restriction studies in our transplanted mice should resolve the issue of whether pig or mouse MHC is positively selecting the mouse T cells.

Although tolerance has previously been induced across concordant xenogeneic barriers using bone marrow transplantation (6) and across fully allogeneic MHC barriers using thymic stromal transplants, this, to our knowledge, is the first demonstration that donor-specific tolerance can be induced across widely disparate (discordant) species barriers. This model should allow increased understanding of thymocyte development and interactions between xenogeneic molecules and might be applicable to the induction of tolerance in humans to discordant species such as swine for clinical transplantation. The successful induction of tolerance to allow xenografting across discordant species barriers could overcome the severe organ shortage that currently limits the advancement of clinical transplantation.

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1. Speiser, D. E., Pircher, H., Ohashi, P. S., Kyburz, D., Hengartner, H. & Zinkernagel, R. M. (1992) *J. Exp. Med.* **175**, 1277–1283.
2. Salaun, J., Bandeira, A., Khazaal, I., Calman, F., Coltey, M., Coutinho, A. & Le Dourarin. (1990) *Science* **247**, 1471–1474.
3. Gao, E.-K., Lo, D. & Sprent, J. (1990) *J. Exp. Med.* **171**, 1101–1121.
4. Kosaka, H. & Sprent, J. (1993) *J. Exp. Med.* **177**, 367–378.
5. Gritsch, H. A., Glaser, R. M., Emery, D. W., Lee, L. A., Smith, C. V., Sablinski, T., Arn, J. S., Sachs, D. H. & Sykes, M. (1994) *Transplantation* **57**, 906–917.
6. Sharabi, Y., Aksentijevich, I., Sundt, T. M., III, Sachs, D. H. & Sykes, M. (1990) *J. Exp. Med.* **172**, 195–202.
7. Bill, J., Kanagawa, O., Woodland, D. L. & Palmer, E. (1989) *J. Exp. Med.* **169**, 1405–1419.
8. Moses, R. D., Pierson, R. N., Winn, H. J. & Auchincloss, H. (1990) *J. Exp. Med.* **172**, 567–575.
9. Moses, R. D., Winn, H. J. & Auchincloss, H. (1992) *Transplantation* **53**, 203–209.
10. Irwin, M. J., Heath, W. R. & Sherman, L. A. (1989) *J. Exp. Med.* **170**, 1091–1101.
11. Lucas, P. J., Shearer, G. M., Neudorf, S. & Gress, R. E. (1990) *J. Immunol.* **144**, 4548–4554.
12. Kollmann, T. R., Goldstein, M. M. & Goldstein, H. (1993) *J. Exp. Med.* **177**, 821–832.