Thymic Transplantation Across an MHC Class I Barrier in Swine


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Thymic Transplantation Across an MHC Class I Barrier in Swine


Thymic tissue transplantation has been performed previously in adult mice to induce donor-specific tolerance across allogeneic and xenogeneic barriers. We have now attempted to extend this technique to a large animal preclinical model and describe here our initial studies of allogeneic thymic transplantation in miniature swine. Two miniature swine were thymectomized before thymic tissue transplantation, and two remained euthymic. Donor thymic tissue was harvested from SLA class I-mismatched juvenile pigs and placed into recipient sternoclephalicus muscle, kidney capsule, and omentum. A 12-day course of cyclosporin A was started on the day of transplantation. Allogeneic thymic engraftment could only be achieved in euthymic and not in thymectomized miniature swine using this treatment regimen. Both nonthymectomized animals showed good graft development, with evidence of thymopoiesis, as indicated by positive CD1 and host-type SLA class I immunoperoxidase staining of immature graft-infiltrating cells. Both animals also demonstrated donor-specific T cell hyporesponsiveness, as measured by MLR and cell-mediated lympholysis. The thymic grafts continued to develop despite the appearance of high levels of anti-donor specific cytotoxic IgG Abs. Thus, thymic tissue transplanted across an SLA class I barrier can engraft and support host thymopoiesis in euthymic miniature swine. The presence of the host thymus was required for engraftment. These data support the potential of thymic transplantation as part of a regimen to induce donor-specific tolerance to xenogeneic organ grafts. The Journal of Immunology, 1999, 163: 3785–3792.

The thymus is of central importance in T cell development (1, 2). Both thymic epithelial cells and bone marrow-derived APCs participate in the process of selecting immature thymocytes. The thymic epithelium is believed to positively select thymocytes in the cortex of the thymus, so that only cells with a self MHC-restricted TCR are allowed to mature (reviewed in Refs. 3 and 4). The bone marrow-derived APCs, found mainly at the cortico-medullary junction, are largely responsible for negative selection, which leads to deletion of T cells with high affinity for self MHC (reviewed in Ref. 5). Recent studies both in vitro, using thymic epithelial cell cultures (6), and in vivo, using thymic transplantation models of mice (7, 8), suggest that the thymic epithelium may also be involved in the negative selection process. Thus, only thymocytes with a low to intermediate self-reactivity are selected for expansion and exportation to the periphery (6, 9, 10).

Successful transplantation of allogeneic or xenogeneic thymic tissue might induce donor-specific tolerance through the deletion of newly developing T cells by the same mechanism that is used in the thymus for deletion of T cells with high affinity for self. Thus, colonization of the donor thymus with bone marrow precursors would lead to negative selection to donor MHC. Migration of host APCs into the grafts would also assure negative selection to host MHC, leading to a specific state of tolerance to both self and donor-specific non-self.

Thymic tissue engraftment has been achieved in immunocompetent mice after thymectomy, followed by lethal whole body irradiation (11, 12), total lymphoid irradiation (13) or T cell and NK cell depletion with mAbs (8). In a xenogeneic pig-to-mouse model involving host thymectomy as well as T and NK cell depletion, the recovery of a tolerant recipient T cell repertoire was shown to be due in part to intrathymic deletion of donor- and host-reactive recipient thymocytes developing in the xenogeneic thymic graft (8, 14, 15). Of importance for potential clinical applications is the fact that both allo- and xenogeneic thymic grafts have been shown to support essentially normal T cell development with normal host-restricted immunocompetence (16–18).

In an attempt to transfer this technique to large animals, we have initiated a series of experiments in inbred Massachusetts General Hospital (MGH)4 miniature swine. Miniature swine provide the unique opportunity to study the effects of selective MHC matching on parameters of immunity in a reproducible fashion (19). Kidney transplantation across a class I barrier was shown to be successful in 100% of cases when high dose CyA was administered during the first 12 days after transplantation (20). We have begun these studies by using the same immunosuppressive regimen and MHC
mismatch for thymic transplantation in miniature swine. We report here successful transplantation of class I-mismatched juvenile thymic tissue and the ability of such tissue to support host thymopoiesis and confer donor-specific hyporesponsiveness.

Materials and Methods

Animals

Two- to three-month-old SLA\(^{05}\) (class I\(^{F}\)/(class II\(^{D}\)) donors and 4- to 5-month-old, class I-mismatched SLA\(^{31}\) (I\(^{D}/\)II\(^{B}\)) recipients were selected from our herd of partially inbred miniature swine. The immunogenetic characteristics of this herd (21, 22) and of the intra-MHC recombinant haplotypes (23) have been described previously and are illustrated schematically in Fig. 1. Each experiment used one thymic tissue donor and two recipients. One of the two recipients in each experiment was thymectomized before thymic transplantation.

Surgical procedures

All animals received preoperative sedation with i.m. xylazine (2 mg/kg), atropine (0.04 mg/kg), and Telazol (1.4 mg/kg; Elkins-Sinn, Cherry Hill, NJ). Isoflurane inhalation anesthesia was administered before endotracheal intubation and was continued throughout the entire operative procedure at a level permitting spontaneous respiration. Perioperatively, each animal received one prophylactic dose of cefazolin (40 mg/kg i.m.).

Thymectomy

In the thymectomized group, a complete thymectomy was performed 3–4 wk before thymic transplantation using a combined midline cervicotomy and partial sternotomy as previously described (24). Briefly, the pretracheal muscles were retracted, and the trachea, internal jugular vein, and carotid artery were exposed from the mandibular area to the ventral pericardium. The entire thymus was then carefully dissected and removed. Adherent fatty tissue was also excised when necessary to ensure completeness.

Thymic transplantation

Partial thymectomy was performed in the thymic donor on day 0 through a midline cervicotomy after exposing the major portion of the cervical thymus. Two pieces of thymus, \(3 \times 3 \text{ cm}^2\) each, were harvested, placed in 0.9% saline solution, and immediately transferred to one euthymic and one thymectomized animal. The thymic tissue was minced into \(10^{-3} \text{ mm}^3\) fragments and implanted into three prepared sites in the recipients:

- **Sternocephalicus muscle.** Ten small pockets, each capable of holding five pieces of tissue, were formed in each sternocephalicus muscle using mosquito clamps. \(1 \times 10^{-3} \text{ cm}^3\) of minced thymic tissue was placed into the pockets, and the sites were closed with 4-0 Prolene (Ethicon, Somerville, NJ), which also served to mark the implantation sites for future biopsies.
- **Omentum.** After midline laparotomy, a volume of \(1 \times 10^{-3} \text{ cm}^3\) of minced thymic tissue was wrapped into an edge of the omentum and closed with 4-0 Prolene (Ethicon, Somerville, NJ). This was done directly opposite the hilum.
- **Kidney capsule.** One cubic centimeter of minced thymic tissue was spread out under both kidney capsules as described previously (25). Briefly, a 1-cm incision was made in the kidney capsule directly opposite the hilum. The capsule was then carefully detached from the underlying cortex using an curved hemostat, and thymic tissue was inserted beneath it.

All recipients received two central venous Silastic catheters (Dow Corning, Midland, MI) placed into each external jugular vein via direct cut down. The catheters were tunneled s.c., exiting dorsally. One catheter was used for CyA administration, and the other for obtaining blood samples.

Skin grafting

Fresh split-thickness skin grafts (40 \(\times 40 \times 2.2 \text{ mm}\)) were harvested from donors using a Zimmer dermatome and placed on graft beds, also prepared with a dermatome, on the lateral thorax as previously described (26). The day of rejection was defined as the time at which <10% of the skin graft showed signs of viability as judged by color, texture, and warmth to touch.

Immunosuppression

The only immunosuppressive treatment was a 12-day course of CyA, administered once per day to all animals beginning on day 0. The first dose on day 0 was 13 mg/kg body weight i.v. given intraoperatively. Animals were either maintained on i.v. solution or switched to an oral CyA solution (Neoral) on day 5, which was given by a gastrostomy tube inserted on the day of thymic transplantation. CyA trough levels were measured in whole blood by a mAb-based RIA. CyA dosage was adjusted daily to maintain a level between 400 and 800 ng/dl. The i.v. and oral (Neoral) preparations of CyA were provided by Novartis Pharmaceuticals (Hanover, NJ).

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication 85-23, revised 1985) and were approved by the Massachusetts General Hospital animal research committee.

Isolation of PBL

Freshly drawn, heparinized whole blood was diluted with HBSS (Life Technologies, Gaithersburg, MD), and mononuclear cells were obtained by gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). The mononuclear cells were washed once with HBSS before the contaminating red cells were lysed with ACK lysing buffer (BioWhittaker, Walkersville, MD). Cells were then washed in HBSS and resuspended in tissue culture medium (see below). Cell suspensions were kept at 4°C until they were used in cellular assays.

Mixed lymphocyte reaction

MLR cultures, to test for proliferative response to alloantigen, have been described previously (28). Briefly, \(4 \times 10^5\) responders and an equal number of irradiated (25 Gy) stimulators were incubated in 200 \(\mu\)l of standard MLR medium using flat-bottom 96-well plates (Costar, Cambridge, MA). After 5 days of incubation, 1 \(\mu\)Ci of \([\text{H}]\)-thymidine was added to each well, followed by an additional 5-h incubation. \(^{3}\)H incorporation was determined in triplicate samples by liquid scintillation.

CML assays

CML assays were performed as described previously (27). Briefly, lymphocyte cultures containing \(4 \times 10^6\) responder and \(4 \times 10^6\) irradiated (25 Gy) stimulator PBL were incubated in 2 ml of medium for 6 days at 37°C in 7% CO\(_2\) and 100% humidity. These effector cells were harvested, and 5 \(\times 10^5\) cells were added to the first three wells of a 96-well round-bottom well before being serially diluted in triplicate (E:T cell ratio, 100:1, 50:1, 25:1, and 12.5:1). \(^{51}\)Cr (5 \(\times 10^3\) rates) were added to the wells, and the plates were incubated at 37°C for 2 h. The supernatant was removed and 100 \(\mu\)l of standard M199 was added to each well. After 5 h of incubation at 37°C the supernatants were harvested with the Skatron collection system (Skatron, Sterling, VA), and \(^{51}\)Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). Maximum lysis was obtained with a 5% solution of the nonionic detergent, Nonidet P-40 (BRL, Rockville, MD). Baseline levels were measured as the rate of spontaneous release of \(^{51}\)Cr from 5 \(\times 10^5\) targets. The results were expressed as the percentage of specific lysis: [(experimental release (cpm) – spontaneous release (cpm)) / (maximum release (cpm) – spontaneous release (cpm))] \(\times\) 100%.

Targets were matched to the stimulators, which included thymus and skin donor-matched PBL (SLA\(^{05}\); class I\(^{F}\)/(class II\(^{D}\)), and skin donor-only-matched PBL (SLA\(^{31}\); class I\(^{D}\)/(class II\(^{B}\))). TBL. Targets SLA matched to the effectors were used as negative controls in all assays.

Humoral responses

Animals were tested for the presence of anti-donor cytotoxic Abs using a two-stage, complement-dependent lymphocytotoxicity assay as described.
Controls included omission of primary Ab or horse anti-mouse Ab. Dipping slides into distilled water. Sections were then fixed in 4% paraformaldehyde containing 3-amino-9-ethyl carbazole for 2–5 min. Staining was stopped by washing twice, cells were resuspended in 0.5 ml of flow cytometry buffer. Analysis was performed using a Becton Dickinson FACScan II (San Jose, CA).

Flow cytometry

Flow cytometry was used to follow the course of serum Abs sampled serially before and after thymic transplantation. Polyclonal, fluorescence-activated, goat anti-swine IgG (γ-chain) or IgM (μ-chain) Abs (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were used as second reagents after incubation for 30 min with the specific test sera. All steps were performed at 4°C using flow cytometry buffer (HBSS containing 0.5% BSA and 0.5% sodium azide). The total bound Abs were measured as the median fluorescence.

Macrophomimer was determined by one-color indirect Ab staining. Mouse anti-pig mAbs reactive with SLA class I (2.27.3a: IgG1), class F (16.7.E4.2; IgM), and class I (2.12.3a; IgM) as previously described (29) were added in separate tubes and incubated for 30 min at 4°C. Nonreacting anti-mouse class I mAbs (36-7-5, IgG2a and 12-2-2, IgM) were used as negative control Abs. All Abs were FITC conjugated. After washing twice, cells were resuspended in 0.5 ml of flow cytometry buffer. Analysis was performed using a Becton Dickinson FACScan II (San Jose, CA).

Immunoperoxidase staining

Frozen sections of sequential thymic graft biopsies were analyzed using the avidin-biotin-HRP complex technique reported previously (30). Donor and recipient cells were stained with murine anti-pig mAbs. An SLA class F-specific mAb (16.7.E4.2) was used to identify donor tissue, whereas an SLA class I mAb (2.12.3a) served as a marker for graft-infiltrating cells. Immature thymocytes were stained with anti-CD1 mAb (76-7-4: IgG2a) (31), whereas mature thymocytes were stained with anti-CD3 mAb (2-6-15: IgG2a) (24). Briefly, 2- to 4-μm sections were incubated with 1% normal horse serum to inhibit nonspecific binding of horse IgG and with avidin to block endogenous biotin. After the wash, the tissue was covered with optimally diluted primary Ab (mouse anti-pig mAb) and incubated for 60 min at room temperature. Sections were rinsed with PBS and incubated in a solution of biotin (1 mg/100 ml in PBS) with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. The biotinylated secondary Ab (horse anti-mouse) was added and incubated for 30–45 min. After a PBS wash, sections were incubated in an optimal dilution of avidin-biotin-peroxidase complex for 60 min, rinsed in PBS, and stained in a solution containing 3-amino-9-ethyl carbazole for 2–5 min. Staining was stopped by dipping slides into distilled water. Sections were then fixed in 4% paraformaldehyde with Glutaraldehyde and counterstained with Gill’s single-strength hematoxylin. Controls included omission of primary Ab or horse anti-mouse Ab.

Results

Engraftment and importance of thymectomy

To evaluate the optimal conditions required for thymic tissue engraftment and development, four animals received class I-mismatched miniced thymic tissue placed into three distinct sites: sternocleidomastoideus, muscle, kidney capsule, and omentum. Two animals were thymectomized, and two were left euthymic before thymic transplantation to investigate the role of the recipient thymus in allogeneic thymic engraftment. CyA, given during the first 12 postoperative days, was the only immunosuppression used in these animals. The same immunosuppressive regimen has previously been shown to induce long term tolerance to SLA class I-mismatched kidney grafts in euthymic recipients, whereas untreated recipients uniformly rejected (20).

The animals in the first experiment (12311 Thx and 12364 EuThy) received a 12-day course of i.v. CyA. Their CyA trough levels were within the desired range between 400 and 800 ng/dl throughout this period. In the second experiment, the two animals (12578 Thx and 12579 EuThy) were switched to Neoral on day 5. Both animals had supratherapeutic trough levels and suffered from neurologic side effects (tremor and somnolence), but suffered no permanent sequelae.

Thymectomy before allogeneic thymic transplantation (12311 Thx and 12578 Thx) did not support engraftment in this model. In contrast, both euthymic animals showed thymic graft development with evidence of host thymopoiesis (12364 EuThy and 12579 EuThy). Initial graft biopsies performed on these animals in all three sites at 2 and 4 wk showed complete loss of thymic architecture, whereas untreated animals reject (20).

The percentages of CD1- and class I d-positive host thymocytes were stained with anti-CD1 mAb (76-7-4: IgG2a) (31), whereas mature thymocytes were stained with anti-CD3 mAb (2-6-15: IgG2a) (24). Briefly, 2- to 4-μm sections were incubated with 1% normal horse serum to inhibit nonspecific binding of horse IgG and with avidin to block endogenous biotin. After the wash, the tissue was covered with optimally diluted primary Ab (mouse anti-pig mAb) and incubated for 60 min at room temperature. Sections were rinsed with PBS and incubated in a solution of biotin (1 mg/100 ml in PBS) with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. The biotinylated secondary Ab (horse anti-mouse) was added and incubated for 30–45 min. After a PBS wash, sections were incubated in an optimal dilution of avidin-biotin-peroxidase complex for 60 min, rinsed in PBS, and stained in a solution containing 3-amino-9-ethyl carbazole for 2–5 min. Staining was stopped by dipping slides into distilled water. Sections were then fixed in 4% paraformaldehyde and counterstained with Gill’s single-strength hematoxylin. Controls included omission of primary Ab or horse anti-mouse Ab.

FIGURE 2. Histologic appearance (H & E staining) of thymic grafts transplanted into the sternocleidomastoideus muscle of a nonthymectomized animal (12579 EuThy). a. Marked mononuclear cell infiltrate without thymic structure was evident at wk 2 after transplantation. b. At wk 4 after thymic transplantation showing an area of central necrosis. c. Eight weeks after transplantation the areas of mature thymocytes are organized, and immature thymocytes are seen to be forming a neocortex (C) and medulla (M) with Hassall’s corpuscles (arrow). d. Thymic grafts then progressively repopulated with immature thymocytes (wk 51 is shown here).
the size of the i.m. grafts increased to histologically from native thymus except by size. Macroscopically, the size of the i.m. grafts increased to ~3–4 times that of the original implants. The omentum grafts also increased in size, but the kidney capsule grafts did not. Histology obtained on grafts from the omentum and kidney capsule revealed the same findings as those from the sternocephalicus muscle (data not shown). How-

ever, because the procedure for taking biopsies from the muscle was technically easier and less invasive, muscle grafts were biopsied preferentially at later time points. The histologic appearance of the thymic grafts was evaluated at 4-wk intervals, and with each biopsy performed, at least one graft was excised. Therefore, the total amount of thymic grafts continuously decreased. The euthymic animals were followed over a period of 12 mo after transplantation, until the majority of the grafts were removed. In one of the animals (12579 EuThy), the remaining grafts were structurally intact (Fig. 2d). The remaining grafts of the other animal (12364 EuThy) may have been rejected after the recipient was sensitized with donor skin grafts (see below).

In contrast to the animals that remained euthymic, both thymectomy animals showed a loss of thymic tissue with scarring and no evidence of thymic engraftment on any scheduled protocol biopsy. These results suggested that, as for class I-mismatched renal allografts transplanted with 12 days of cyclosporin (24), the host thymus may be crucial for engraftment of class I-mismatched thymic tissue.

### Decrease in donor-specific T cell response in thymic-grafted animals

MLR responses in naive animals were mainly directed against SLA class II, whereas the SLA class I proliferative responses were generally low (SI = 2–10). In contrast, CML responses in naive animals directed against SLA class I were always vigorous (32).

Rejection of the thymic grafts in the thymectomized animals led to a marked increase in anti-donor MLR (12311 Thx: SI = 35; 13578 Thx: SI = 43; measured at wk 8), consistent with sensitization (Fig. 4). In contrast, euthymic animals that accepted their thymic grafts continued to have low anti-donor responses, suggesting that sensitization did not occur.

Acceptance of thymic grafts was also accompanied by donor-specific hypo- or nonresponsiveness in CML (Table II). Anti-third-party CML responses were generally normal, but anti-SLA<sup>hh</sup> responses were diminished, suggesting possible cross-tolerance to determinants shared by class I of SLA<sup>h</sup> and SLA<sup>a</sup> (Fig. 1). Stimulators from fully allogeneic outbred (Yucatan) animals produced vigorous responses in nearly all experiments. Representative data

<table>
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<tr>
<th>Week</th>
<th>% CD1&lt;sup&gt;+&lt;/sup&gt; recipient cells</th>
<th>Expt. 1</th>
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<th>12364 EuThy</th>
<th>Expt. 2</th>
<th>12578 Thx</th>
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* Assay performed after recipient rejected donor skin graft.
* Assay performed after recipient rejected donor and third-party class I disparate skin grafts.
* Specimen obtained from sternocephalicus muscle.
* Specimen obtained from kidney capsule.
* ND, not determined.
* —, Animal had been sacrificed.
for one of the euthymic animals (12579 EuThy) at wk 0, 4, and 8, demonstrating the development of donor nonresponsiveness, are illustrated in Fig. 5.

The mechanism of the observed donor-specific hyporesponsiveness in CML was addressed by providing T cell help to the effectors through addition of IL-2 to the CML cultures. Alternatively, effectors were generated by culture with stimulators fully mismatched to the responder, but SLA class I matched to the thymic graft donor. In both experiments, anti-donor responses could not be restored (data not shown), suggesting that these T cells in the thymic graft recipients were either anergic or deleted.

Thymic grafted animals did not show humoral tolerance

Anti-donor cytotoxic Abs were detected 4 wk after thymic transplantation in both thymectomized and euthymic animals, as measured on donor-type PBL. Serum treatment with DTT did not diminish the cytotoxicity, suggesting that Abs of IgG class were responsible for at least part of the complement-mediated cytotoxicity. Representative data for one of the two engrafted animals (12579 EuThy) are shown in Fig. 6.

Attempts to detect chimerism

Flow cytometry was performed to assess macrochimerism in a thymectomized and a euthymic animal (12311 Thx and 12364 Eu-Thy). PBL, gated on lymphocytes, were stained for donor-type SLA class I. Samples were obtained from both animals on days 0, 4, 7, 10, 16, and 30 and again on day 126 in the euthymic pig only (12364 Eu-Thy). In addition, the host thymus was analyzed in the same animal on days 0, 16, and 126. No SLA class I-positive cells could be detected at a detection level of 0.05% (data not shown).

Influence of skin graft rejection on thymic graft survival

In an attempt to determine whether tolerance to the thymic donor might have been induced, skin grafts obtained from the original thymic donor were transplanted onto each thymic graft recipient, 8 wk after thymic transplantation. Naive animals from our inbred herd of miniature swine generally reject class I-mismatched skin grafts within 8–10 days (33). Both animals that were thymectomized before thymic transplantation rejected the skin grafts between 9 and 10 days (12311 Thx and 12578 Thx, respectively). One of the euthymic animals (12364 EuThy) also rejected in 10 days, while the other (12579 EuThy) showed a prolonged skin survival of 18 days. After skin graft rejection, subsequent thymic

Table II. Sequential CML assays performed in both experiments

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<th>SLA&lt;sup&gt;gg&lt;/sup&gt;</th>
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<td>36</td>
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<td>42.2</td>
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<sup>a</sup> Each experiment included one euthymic and one thymectomized animal. Effectors were tested on targets that were matched to the stimulators. Data are expressed as percent specific lysis at an effector:target ratio of 100:1.

<sup>b</sup> Assay performed after recipient rejected donor-specific skin graft.

<sup>c</sup> Assay performed after recipient rejected donor-specific and third-party class I disparate skin grafts.

<sup>d</sup> ND, not determined.

<sup>e</sup> —, Animal had been sacrificed.
graft development was impaired in both of these animals, as measured by the relative levels of immature CD1-positive thymocytes compared with the mature T cells in the grafts (Table I). In addition, both euthymic animals experienced an immediate increase in their levels of cytotoxic Abs (Fig. 6), and an increase several weeks later in their anti-donor CML responses (Table II). This apparent rejection process was seen only transiently in the animal with prolonged skin graft survival (12579 EuThy). The other animal (12364 EuThy) may have rejected its remaining thymic grafts between 28 and 40 wk after skin grafting (i.e., 36 and 48 wk after thymic transplantation).

**Discussion**

We have previously demonstrated that fetal pig thymic tissue transplanted into T and NK cell-depleted thymectomized mice engrafts and supports host thymopoiesis. The developing mouse T cells were immunocompetent and demonstrated in vitro and in vivo tolerance to porcine xenoantigens (8, 16, 34, 35). Because of the potential of this methodology as a means of inducing tolerance clinically, it was deemed important to determine the feasibility of extending these findings to a large animal model. The present study was therefore undertaken to evaluate the requirements for allogeneic thymic engraftment in miniature swine before proceeding to a xenogeneic large animal system.

Class I-mismatched kidney grafts are uniformly accepted in euthymic pigs when transplantation is followed by a 12-day course of CyA. In contrast, such kidney grafts are rejected when transplanted without immunosuppression (20). Therefore, we adopted the same CyA treatment regimen and SLA disparity for development of a thymic transplantation model. We compared animals who underwent thymectomy before thymic transplantation to those who remained euthymic. Because previous studies in rodent models have demonstrated host thymectomy to be an absolute requirement for allogeneic or xenogeneic thymic tissue engraftment, it seemed possible that the same might be true in miniature swine (13, 35). In theory, the host thymus might be expected to continuously produce allo-reactive T cells and cause rejection of the thymic graft, while removal of the host thymus in conjunction with T cell depletion might allow the transplanted thymus to survive and sustain T cell development after engraftment.

Contrary to this expectation, not only was removal of the host thymus found to be unnecessary to permit engraftment of class I-mismatched thymic tissue, but such thymectomy was also found to be detrimental. Thus, after host thymectomy and class I-mismatched thymic transplantation followed by a 12-day course of CyA, sequential graft biopsies revealed that as early as 2 wk and
continuing throughout the entire observation period, there was no evidence for viable thymic graft tissue. In contrast, the nonthymecto-
mized animals successfully engrafed, and by 8 wk post-trans-
plant the grafts had differentiated into tissue that fulfilled all the
morphologic criteria of a functional thymus. The grafts were re-
populated with immature, host-type thymocytes and showed nor-
mal thymic architecture, including a neocortex and medulla. In
subsequent weeks, the relative number of immature thymocytes
continuously increased, and Hassall’s corpuscles developed.

This requirement for the presence of the host thymus is probably peculiar to the use of our CyA treatment regimen for induction of
tolerance across an MHC class I barrier (20). Under these circum-
stances, mature T cells that would otherwise react with the graft
are apparently inactivated by a peripheral mechanism, currently
under investigation in our renal transplantation studies. In those
clones L-mismatched renal transplantation studies, prior thymectomy
was also found to be detrimental to the induction of tolerance (24).
On the other hand, when T cell depletion is used to permit engraft-
ment, it is likely that host thymectomy may not be detrimental
and may even be required, as was the case in murine studies (8).
We are now attempting to extend our thymic transplantation stud-
ies to more extensive histocompatibility barriers in our miniature
swine, where, on the basis of our previous studies of renal trans-
plantation, we anticipate that CyA alone will not be sufficient to
induce long term graft acceptance (26). We therefore plan to use T
cell depletion in these studies and will again examine the effects of
thymectomy (G. W. Haller et al., work in progress).

There are additional reasons why thymectomy may have been
detrimental to successful engraftment in these studies. These in-
clude recent evidence that the host thymus may be involved in the
development of donor-specific regulatory cells (36, 37). In addi-
tion, in murine models, CyA treatment has been shown to lead to
thymic medullary involution with destruction of the medullary
dendritic cells. After CyA treatment is stopped, the thymus rapidly
repopulates with dendritic cells (38, 39). It is therefore possible
that in the present studies either donor dendritic cells or host cells
presenting donor peptides may repopulate the thymus early after
CyA treatment. This repopulation, which would of course require
the presence of the host thymus, could be responsible for subse-
quent deletion of developing donor-reactive thymocytes.

In vitro cellular assays revealed that the thymic graft recipients
had developed donor-specific hyposensitiveness. Antidonor pro-
liferative responses as well as cell-mediated cytotoxicity were
markedly reduced or absent as early as 4 wk after transplantation.
Surprisingly, however, the animals not only produced an antici-
donor IgM response, but also demonstrated a class switch to IgG
4 wk after thymic transplantation. Because such class switching is
thought to require T cell help, we speculate that T cell responsivi-

ty may have been present transiently after cessation of CyA
treatment on day 11. This T cell response may have disappeared or
been suppressed by the time at which the MLR was first measured
(4 wk after transplantation). However, the thymic grafts continued
to develop, and endothelial cells remained positive for donor SLA
cells in the thymus. Consistent with the latter possibility, failure of
mAbs to deplete mature T cells in the thymus has previously been
reported in murine studies (40).

Despite successful engraftment, we speculate that the immuno-
suppressive treatment selected for these experiments was insuffi-
cient to prevent sensitization. However, it is remarkable that these
grafts continued to develop and remained viable as long as 1 yr
after transplantation in the presence of alloantibodies. Even after
sensitization with donor skin, which was originally performed with
the intention of assessing donor-specific tolerance, one of the
animals was able to recover donor-specific hyposensitiveness in
CML. These results demonstrate the potential of allogeneic thymus
to induce tolerance in large animals and suggest that thymus may
be resistant to both cellular and humoral rejection. These findings
may be of particular importance in xenotransplantation, where pre-
formed natural Abs are present (reviewed in Ref. 41).

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References

1. Zinkernagel, R. M., A. Althage, E. Waterfield, B. Kindred, R. M. Welsh,
G. Callahan, and P. Pincell. 1980. Restriction specificities, alloreactivity,
and allotolerance expressed by T cells from nude mice reconstituted with H-2-com-
patible or -incompatible thymus grafts. J. Exp. Med. 151:376.
2. Sprent, J. 1993. T lymphocytes and the thymus. In Fundamental Immunology,
tolerance. J. Exp. Med. 177:1155.
F. Hansen. 1997. Quantification of the cell surface level of Ld resulting in
positive versus negative selection of the 2C transgenic T cell receptor in vivo.
Immunity. 7:235.
10. Tomita, Y., A. Khan, and M. Sykes. 1994. Role of intrathymic clonal deletion and
peripheral anergy in transplantation tolerance induced by bone marrow transplan-
tation in mice conditioned with a non-myeloablative regimen. J. Immunol. 153:
1087.
11. Houaka, N., M. Nose, M. Kayogoku, N. Nagata, S. Miyashima, R. A. Good,
and S. Behara. 1996. Thymus transplantation, a critical factor for correction of
transplantation tolerance in mice across major histocompatibility barrier by using
145:499.
Positive and negative selection of functional mouse CD4+ T cells by porcine MHC
positive selection of mouse CD4+ T cells with a mouse MHC-restricted TCR in
158:1641.
17. Markert, M. L., D. D. Kostyu, F. E. Ward, T. M. McLaughlin, T. J. Watson,
R. H. Buckley, S. E. Schiff, R. M. Ungerleider, J. W. Gaynor, K. T. Oldham, et
al. 1997. Successful formation of a chimeric human thymus allograft following
18. Salain, J., A. Bandeira, I. Khazaal, F. Calman, M. Coutinho, A. Coutinho, and
N. M. Le Douarin. 1990. Thymic epithelium tolerizes for histocompatibility an-
Selective breeding of miniature swine leads to an increased rate of acceptance of
149:1099.
Induction of specific tolerance to class I disparate renal allografts in miniature
swine with cyclosporine. Transplantation 54:490.
Transplantation in miniature swine. I. Fixation of the major histocompatibility
Biomedical Research, 1st Ed. M. M. Swindle, D. C. Moody and L. D. Phillips,
eds. Iowa State University Press, Ames, p. 3.


