Role of the Thymus in Transplantation Tolerance in Miniature Swine. I. Requirement of the Thymus for Rapid and Stable Induction of Tolerance to Class I–mismatched Renal Allografts

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Summary

The almost uniform failure in transplant patients of tolerance-inducing regimens that have been found to be effective in rodents, has made it necessary to examine large animal models before testing of new approaches clinically. Miniature swine have been shown to share many relevant immunologic parameters with humans, and because of their reproducible genetics, have proved extremely useful in providing such a large animal model. We have previously shown that indefinite systemic tolerance to renal allografts in miniature swine is induced in 100% of cases across a two-haplotype class I plus minor histocompatibility antigen disparity by a 12-d course of Cyclosporine A (CyA), in contrast to irreversible rejection observed uniformly without CyA treatment. In the present study, we have examined the role of the thymus during the induction of tolerance by performing a complete thymectomy 21 d before renal transplantation. This analysis demonstrated a striking difference between thymectomized and nonthymectomized animals. Thymectomized swine developed acute cellular rejection characterized by a T cell ($CD25^+$) infiltrate, tubulitis, endothelialitis and glomerulitis, and anti–donor CTL reactivity in vitro. Nonthymectomized and sham thymectomized animals had a mild T cell infiltrate with few $CD25^+$ cells and no anti–donor CTL response in vitro. These results indicate that the thymus is required for rapid and stable induction of tolerance.

Many methods by which transplantation tolerance can be induced in rodents have failed when applied to large animals or to patients (1–4), making testing in large animals a necessary step before applying new techniques clinically. Miniature swine provide the only large animal model in which one can reproducibly study the effects of selective matching within the MHC on parameters of transplantation (5–7). We have therefore used MHC inbred and recombinant lines of miniature swine extensively for preclinical studies of transplantation tolerance (8–12). Previous studies from this laboratory have demonstrated that tolerance to renal allografts in miniature swine occurs spontaneously in about one-third of animals selectively matched for class II antigens and mismatched for a single class I MHC locus plus minor antigens (8, 13). The induction of spontaneous long-term tolerance was associated with a transient antidonor class I humoral response which has been shown to be almost entirely of the IgM class. Rejector animals developed antidonor class I IgG and promptly rejected their allografts. The failure to switch from IgM to IgG in spontaneous acceptors, suggested that the pathway to tolerance involved a deficiency of T cell help. Studies in miniature swine mismatched for two class I haplotypes were consistent with this hypothesis. Such animals reject renal allografts in 100% of cases without immunosuppression, but when T cell help was limited by the administration of a 12-d course of Cyclosporine A (CyA)[1], 100% of animals developed long-term tolerance (9). Subsequent studies demonstrated that transplants of second renal allografts, MHC-matched to the original donors, were accepted without further immunosuppression if grafted at the time of the transplant nephrectomy (14). These results indicate that long-term graft acceptance is associated with the induction of systemic tolerance.

The role of the thymus has been shown to be critical for systemic central tolerance to self antigens in which poten-

[1]*Abbreviations used in this paper:* CD, cluster of differentiation; CML, cell-mediated lysis; CyA, Cyclosporine A; GIC, graft-infiltrating cell; PAS, periodic acid–Schiff; POD, postoperative day; PSL, percent specific lysis; SLA, swine lymphocyte antigen.

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tially autoreactive T cells are deleted or anergized by exposure to the appropriate self antigens presented by either bone marrow–derived cells or thymic stromal cells (15–19). Similar intrathymic mechanisms may also be important in inducing donor-specific tolerance to alloantigens, and there are recent reports of studies in which donor alloantigens directly injected into the thymus resulted in donor-specific tolerance to the alloantigens in vivo or in vitro (20–23). To determine if the thymus is involved in the induction of tolerance in our two haplotype class I–mismatched renal allograft model, the effect of thymectomy 21 d before renal transplantation was examined. The data from this study demonstrate that the thymus is essential for rapid and stable tolerance induction. However, one graft was accepted by a thymectomized animal, indicating that allograft tolerance may also be achieved by peripheral mechanisms.

Materials and Methods

Animals. Transplant donors and recipients were selected from our herd of partially inbred miniature swine at 5–7 mo of age. The immunogenetic characteristics of this herd and of the intra-MHC recombinant haplotypes available have been described previously (5–7). The haplotypes of miniature swine used in this study are shown schematically in Fig. 1. Recombinant swine lymphocyte antigen (SLA)^{gg} (class I^{c}/II^{d}) animals were used as kidney donors, and SLA^{dd} (class I^{d}/II^{d}) animals were used as recipients to achieve a 2-haplotype class I mismatch. All recipients were tested for cell-mediated lympholysis (CML) reactivity to SLA^{gg} targets before kidney transplantation, and demonstrated significant cytotoxic activity (>20% percent-specific lysis [PSL]).

Surgery. The surgical procedures used for kidney transplants have been described in detail previously (24, 25). A semipermanent indwelling Hickman silastic central venous catheter was placed surgically into the external jugular vein. The catheter facilitated CyA administration and frequent blood sampling for monitoring of renal function, whole blood CyA levels, and in vitro assays. A complete thymectomy was carried out 21 d before kidney transplantation in the thymectomized group. The pretracheal muscles were retracted exposing the trachea from the cervicothoracic junction to the mandibular area, as well as the thymus. The cervical portion of the thymus was then dissected and excised. To achieve a complete thymectomy, a partial sternotomy was then performed, and the thoracic portion of the thymus was exposed. In the chest, the thymus consisted of nonencapsulated tissue adherent to the pericardium, the pleura, and the large vessels, and therefore careful dissection of this portion of the thymus was required to complete the thymectomy (Fig. 2). Removed thymic tissue was examined histologically and showed active thymopoiesis (data not shown). Sham thymectomy was performed in an identical fashion to complete thymectomy; however, thymic tissue was not removed.

Immunosuppression. CyA (Sandimmune) was provided by Novartis Pharmaceutical Corp., (Hanover, NJ) and was mixed and administered as an intravenous suspension according to specifications of the manufacturer. CyA was given daily as a single infusion at a dose of 10–13 mg/kg (adjusted to maintain a blood level of 400–800 ng/ml) for 12 consecutive d, starting on the day of kidney transplantation. Whole blood trough levels were determined by a monoclonal radioimmunoassay, and the results were expressed in ng/ml.

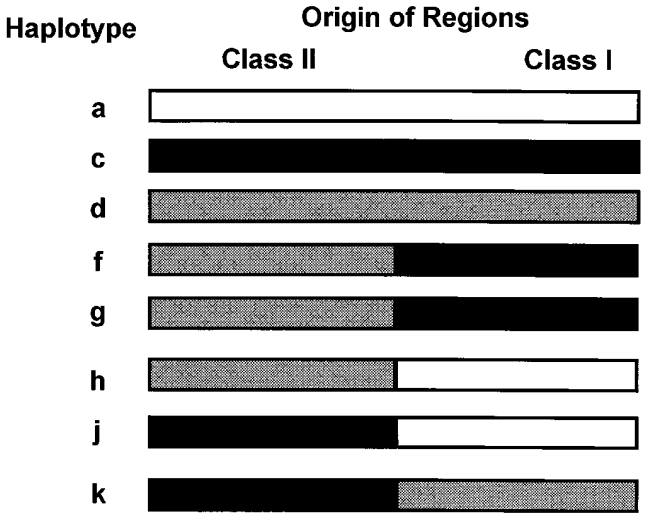


Figure 1. Schematic diagram of the origin of available homozygous porcine MHC haplotypes. Partially inbred SLA^{aa}, SLA^{cc}, and SLA^{dd} haplotypes were derived from the original founder miniature swine. Recombination events between the MHC class I and class II haplotypes have been identified and maintained as homozygous, recombinant haplotypes SLA^{ff}, SLA^{gg}, SLA^{hh}, SLA^{jj}, and SLA^{kk}.

Rejection Monitoring. Rejection was monitored by plasma creatinine and histological examination of biopsy tissue. The clinical endpoints used in this study were (*a*) death or euthanasia from terminal uremia, and (*b*) survival >100 d after kidney transplantation with a stable plasma creatinine.

Histology. Sequential wedge kidney biopsies were performed on postoperative day (POD) 8, 11, 18, 30, 60, and >100 through a flank incision. Tissues were stained using hematoxylin and eosin and periodic acid–Schiff (PAS), and coded slides were examined. Rejection was scored according to standard pathologic criteria (26). Frozen sections for immunoperoxidase staining of kidney biopsies were analyzed using the avidin-biotin–horseradish-peroxidase complex technique (27). A murine anti–pig mAb (K231-3B2) recognizing the low affinity chain (p55, α chain) of the pig IL-2R was used to assess cluster of differentiation (CD) 25 expression (28). 4-μm sections were incubated with 1% normal horse serum and avidin (100 μg/ml in PBS) to inhibit nonspecific binding of horse IgG and endogenous biotin, respectively. After 20 min, the tissue was covered with optimally diluted primary mAb (mouse anti–pig mAb) and incubated for 60 min at room temperature. Sections were rinsed in PBS and incubated in a solution of biotin (10 μg/ml in PBS) with 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase. The biotinylated secondary Ab (horse anti–mouse IgG) was added and incubated for 45 min. After a further PBS wash, sections were incubated in an optimal dilution of avidin-biotin–peroxidase complex (Dako Corp., Carpinteria, CA) for 60 min, rinsed in PBS, and visualized by staining with 0.02% hydrogen peroxide containing 0.3 mg/ml 3,3′-diaminobenzidine in 0.05 M Tris buffer. Staining was stopped by dipping the slides into distilled water. Sections were then counterstained with Gill's single strength hematoxylin. Controls included omission of primary Ab, horse anti–mouse Ab, and an irrelevant primary mAb (36.7.5, murine anti–mouse K^{k}; reference 29).

Preparation of PBL. For separation of PBLs, freshly heparinized whole blood was diluted ~1:2 with HBSS (GIBCO BRL,

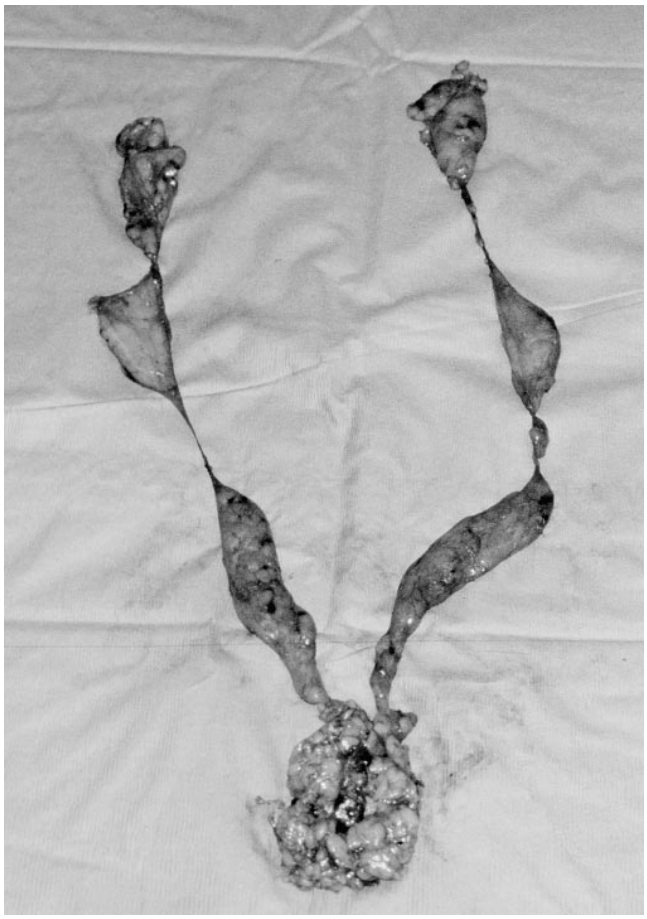


Figure 2. Macroscopic findings of a completely resected thymus from a thymectomized animal.

Gaithersburg, MD) and the mononuclear cells were obtained by gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC). The mononuclear cells were washed once with HBSS, and contaminating red cells were lysed with ammonium chloride potassium buffer (B&B Research Laboratory, Fiskeville, RI). Cells were then washed with HBSS and resuspended in tissue culture medium. All cell suspensions were kept at 4°C until used in cellular assays.

Preparation of Graft Infiltrating Cells. Kidney biopsy specimens (100–500 mg) were finely minced with a scalpel blade and then dispersed with the tip of a syringe plunger in HBSS buffer. The cell suspension was then filtered through nylon mesh, pelleted by centrifugation, and resuspended in flow cytometry media (see below).

Flow Cytometry. Flow cytometry of PBL and graft infiltrating cells (GICs) was performed using a Becton Dickinson FACScan® (San Jose, CA). Cells were stained using directly FITC-labeled or biotinylated mAbs and two-color analysis, as previously described (30). The T cell content of PBLs and GICs was evaluated with mAbs 74-12-4 (IgG2b, anti–swine CD4), 76-2-11 (IgG2a, anti–swine CD8), MSA4 (IgG2a, anti–swine CD2), and K231-3B2 (IgG2a, anti–swine CD25, α chain) (28, 31–33), which were the same antibodies used for immunohistochemistry. For staining, cells were resuspended in flow cytometry buffer (HBSS containing 0.1% BSA, and 0.1% NaN_3) and incubated for 1 h at 4°C with saturating concentrations of a FITC-labeled mAb and a biotinylated second mAb. After two washes, phycoerythrin-streptavidin was added and incubated for 10 min. Cells were then washed and analyzed using propidium iodide gating to exclude dead cells.

CML Assay. Tissue culture media used for CML assays consisted of RPMI 1640 (GIBCO BRL) supplemented with 6% FCS (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, and 135 μg/ml streptomycin (GIBCO BRL), 50 μg/ml gentamicin (GIBCO BRL), 10 mM Hepes (Fisher Scientific, Pittsburgh, PA), 2 mM l-glutamine (GIBCO BRL), 1 mM sodium pyruvate (BioWhittaker, Inc., Walkersville, MD), nonessential amino acids (BioWhittaker, Inc.) and 5×10^{-5} M β2 mercaptoethanol (Sigma Chemical Co.). The effector phase of the CML assay was performed using Basal Medium Eagle (GIBCO BRL) supplemented with 6% controlled processed serum replacement 3 CPSR-3 (Sigma Chemical Co.), and 10 mM Hepes (Fisher Scientific).

CML assays were performed as previously described (24, 25). In brief, lymphocyte cultures containing 4×10^6 responder and 4×10^6 stimulator PBLs (irradiated with 2,500 rads) were incubated for 6 d at 37°C in 7.5% CO_2 and 100% humidity. Bulk cultures were harvested and effectors tested for cytotoxic activity on ^{51}Cr (Amersham Corp., Arlington Heights, IL)-labeled targets generated from lymphocytes stimulated for 24 h with a 1:500 dilution of phytohemagglutinin (M-Form; GIBCO BRL) previously titrated to give optimal proliferation. Effector cells were incubated for 5.5 h with a negative control target (i.e., target PBL matched to the effectors) and targets matched to the stimulators which included donor-matched PBL (SLA^{gg}: class I^{cc}, class II^{dd}) and third party stimulators (SLA^{aa}: class I^{aa}, class II^{aa}). E/T ratios of 100:1, 50:1, 25:1, and 12.5:1 were tested. Supernatants were harvested using the Skatron collection system (Skatron, Sterling, VA) and ^{51}Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as PSL, calculated as:

$$\text{PSL} = [\text{experimental release (cpm)} - \text{spontaneous release (cpm)}/\text{maximum release (cpm)} - \text{spontaneous release (cpm)}] \times 100.$$

Statistical Analysis. Statistical analyses were performed by using the Student's *t* test. A *P* value of <0.05 was considered significant.

Results

Effect of Thymectomy on the Clinical Course of CyA-treated Recipients of Class I–mismatched Kidney Transplants. We have previously reported that SLA^{dd} swine receiving SLA^{gg} kidney transplants and a 12-d course of CyA uniformly accepted renal allografts (9). In the present study, we reproduced these results in four additional SLA^{dd} animals (No. 11468, 11561, 11574, and 10349). Two animals (No. 11561 and 11574) were removed from this experiment on POD 42, since they were thymectomized at that time to examine the possible effect of posttransplant thymectomy on the maintenance of tolerance (to be reported elsewhere). Two control animals (No. 10418 and 12019) underwent sham thymectomy 21 d before transplantation to assess the effect of this procedure on renal function. All nonthymectomized animals, including the sham-thymectomized control animals, had minimal renal dysfunction and subsequent stable renal function after cessation of CyA (Fig. 3 *b*). The five thymectomized animals demonstrated a markedly different clinical course (Fig. 3 *a*). Four animals (No. 10770, 10549,

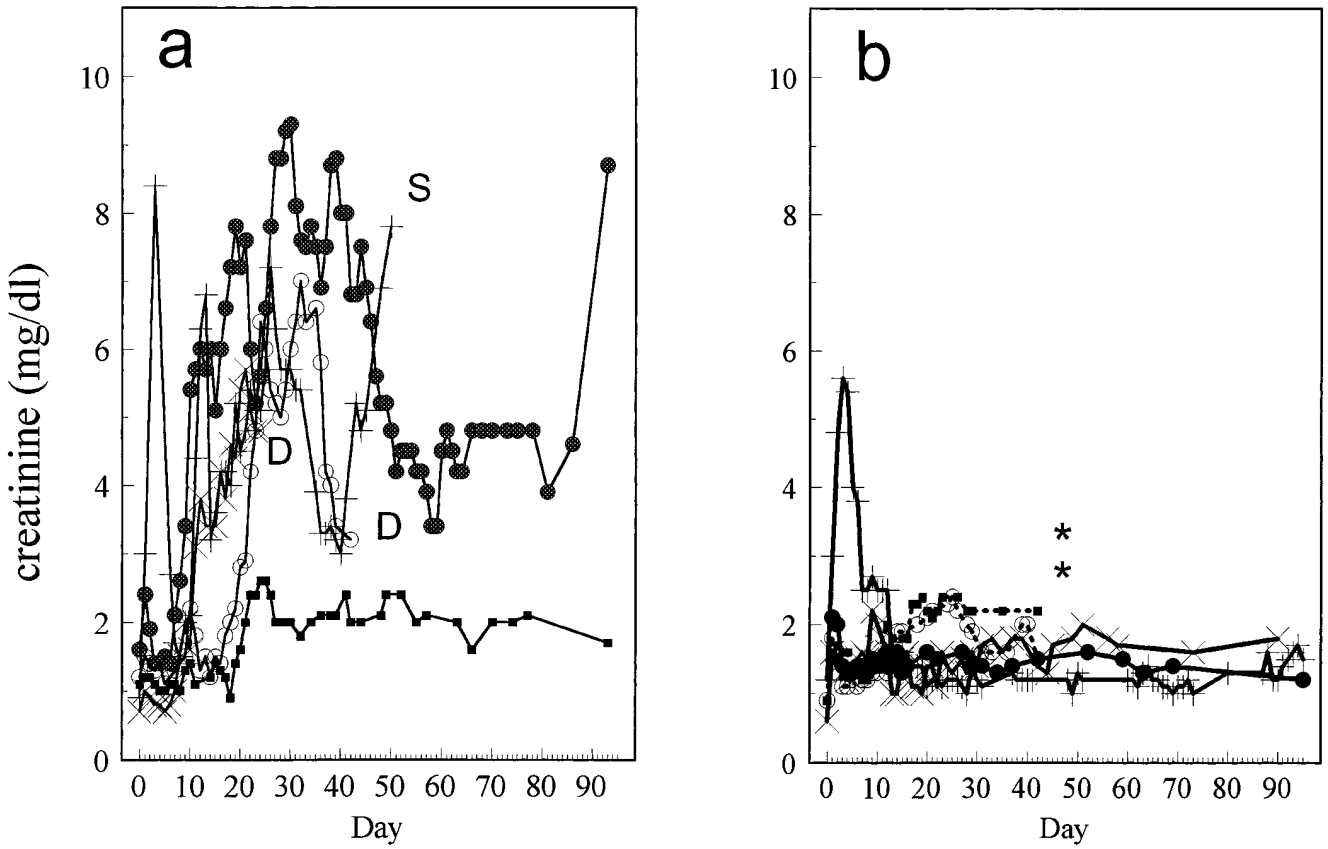


Figure 3. Clinical course of thymectomized animals (*a*) and nonthymectomized animals (*b*). *D*, died; *S*, killed due to progressive uremia; *, No. 11561, 11574. These pigs were excluded from this study on POD 42 (see text).

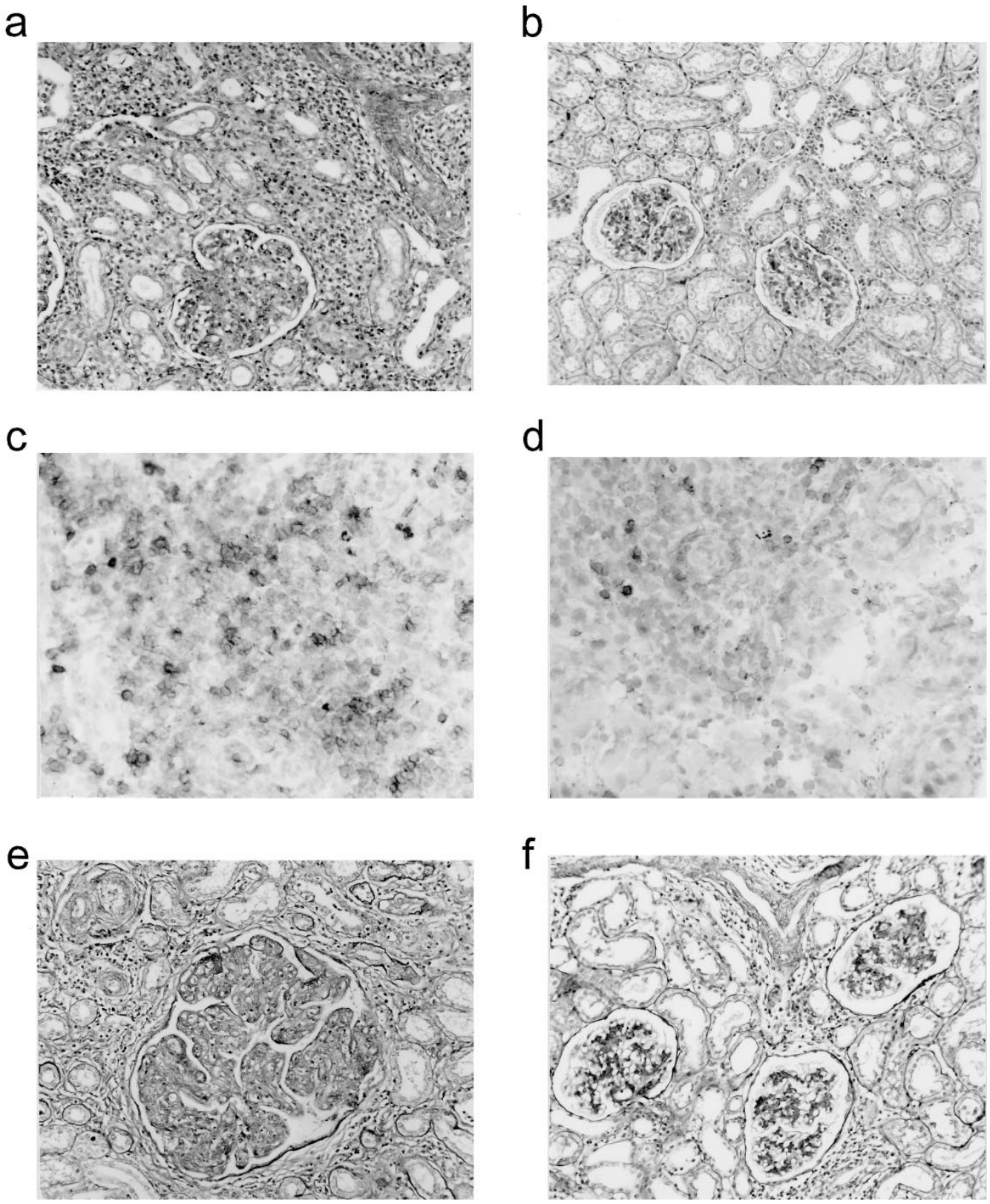
11573, and 11809) developed severe renal dysfunction. Two of these animals (No. 10770 and 10549) died from uremia on POD 26 (creatinine 4.8 mg/dl), and POD 51 (creatinine 7.8 mg/dl) respectively. One animal (No. 11573) demonstrated a marked rise in creatinine for 3 wk after cessation of CyA, and then died from cachexia on POD 42, probably related to the massive proteinuria caused by transplant glomerulopathy. The fourth animal (No. 11809) also demonstrated severe renal dysfunction caused by an initial acute rejection crisis. Its creatinine then stabilized at an elevated value for several weeks, but the renal allograft was lost eventually to chronic rejection. The last thymectomized animal (No. 11301) developed a mildly elevated plasma creatinine (peak 2.6 mg/dl) for a prolonged period, which was a pattern not observed in the nonthymectomized animals, but which, nevertheless, led to long-term acceptance (>100 d). The mean peak creatinine level of thymectomized versus nonthymectomized animals was significantly different (6.5 ± 2.5 mg/dl versus 2.3 ± 0.6 mg/dl, respectively, $P < 0.02$). At autopsy, thymectomized animals were examined for residual thymic tissue and, despite an extensive dissection of the neck and upper mediastinum, no macroscopic thymic tissue was detected in any of the five animals. Histological analysis of multiple biopsy samples taken at autopsy confirmed the absence of thymus.

Histological Analysis. Serial kidney biopsies were performed on POD 8, 11, 18, 30, 60, and >100. Marked differences in histology were noted between thymectomized and nonthymectomized animals. Nonthymectomized animals and the sham-thymectomized controls demonstrated a patchy and mild mononuclear cell infiltrate with focal tubulitis between POD 8 and 18. Attachment of a few mononuclear cells to the endothelium in small arteries and glomerular capillaries was also observed (Fig. 4 *b*). The mononuclear

Figure 4. Representative histological findings of thymectomized animals and nonthymectomized animals. (*a*) A thymectomized animal on POD 8; diffuse and moderate mononuclear cell infiltration is seen with diffuse tubulitis. Glomeruli show typical acute allograft glomerulopathy (PAS ×200). (*b*) A nonthymectomized animal on POD 8; mild and focal mononuclear cell infiltration is seen with mild focal tubulitis (PAS ×200). (*c*) Immunohistochemistry for CD25 on POD 8 in a thymectomized animal, and (*d*) a nonthymectomized animal. Many infiltrating mononuclear cells are seen expressing CD25 in the thymectomized animal (*c*), whereas only a few of these cells are seen in nonthymectomized animal (*d*) (×600). (*e*) Thymectomized animal on POD 60 shows chronic transplant glomerulopathy with diffuse interstitial fibrosis (PAS ×200), and (*f*) nonthymectomized animal show a normal glomerular structure on POD 60 (PAS ×200).

Thymectomized animals

Non-thymectomized animals



cell infiltrate decreased by POD30, and remained minimal (<5% of the cortex) throughout the remainder of the experiment. In contrast, thymectomized animals showed acute cellular rejection, with a diffuse and marked mononuclear cell infiltration, tubulitis, and endothelialitis starting on POD 8 (Fig. 4 *a*). The glomeruli also showed a mononuclear cell infiltrate. By immunoperoxidase staining, both groups showed an infiltrate of CD4 and CD8 cells. However, many infiltrating cells expressed CD25 in the thymectomized animals (Fig. 4 *c*), whereas only a few infiltrating cells expressed CD25 in nonthymectomized animals, indicating more activation of infiltrating cells in thymectomized animals (Fig. 4 *d*). In late biopsies, the thymectomized animals developed chronic rejection, as manifested by allograft glomerulopathy, consisting of the duplication of glomerular basement membrane, marked mesangial proliferation, and segmental mesangial sclerosis, observed on POD 60 and 94 (Fig. 4 *e*). In addition, interstitial fibrosis and tubular atrophy were present. The glomerular changes correlated with proteinuria and weight loss in the thymectomized animals, and were not seen in nonthymectomized animals or the sham-thymectomized controls (Fig. 4 *f*).

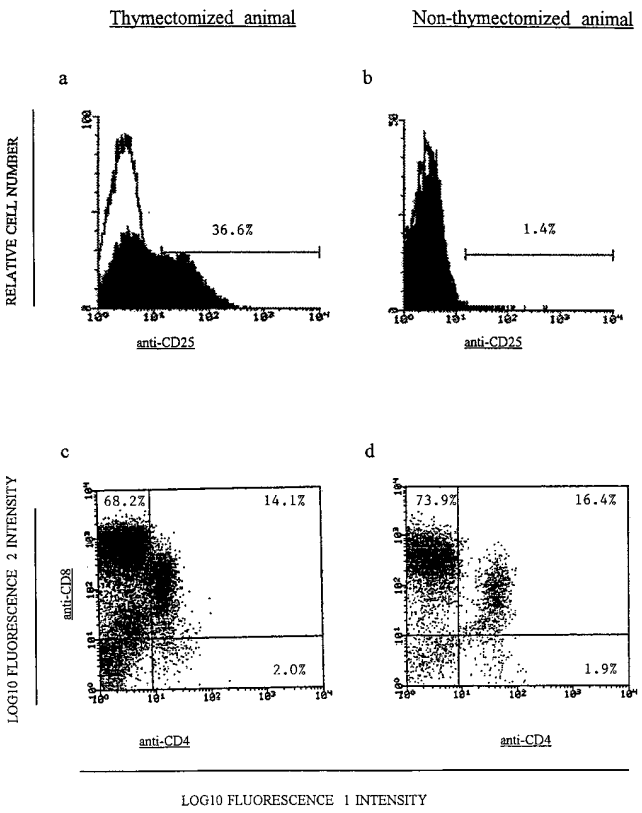


Figure 5. Phenotype of GIC from typical thymectomized and nonthymectomized animals. Flow cytometric analysis of the CD25 expression (*closed histogram*) on GIC on POD 8 prepared from renal biopsies taken from thymectomized (*a*) and nonthymectomized (*b*) animals is represented. The negative control antibody staining is also shown (*open histogram*). The CD8 versus CD4 dot-plot analysis of the GIC on POD 8 is shown for a thymectomized (*c*) and nonthymectomized animal (*d*). The analysis of the phenotype shows the large number of CD8 single positive and CD4/8 double positive GIC in both animals.

Flow Cytometry. To assess the number of activated T cells in the graft and PBLs semiquantitatively, GICs and PBLs were examined by flow cytometry with the anti-CD25 mAb. Fig. 5 shows CD25 expression on GICs on POD8 in representative thymectomized and nonthymectomized animals. CD25 was expressed on 36.6% of CD2-positive GICs in the thymectomized animal, whereas only 1.4% of CD2-positive GICs expressed CD25 in the nonthymectomized animal (Fig. 5, *a* and *b*). These results were consistent with the immunohistological findings (Fig. 4, *c* and *d*). CD25 expression in the GICs of thymectomized animals decreased over time (data not shown). Representative CD8 versus CD4 staining of GICs from a thymectomized animal (Fig. 5 *c*) and a nonthymectomized animal (Fig. 5 *d*) demonstrated that the majority of the GICs were CD8 single positive or CD4/8 double positive cells, with very few CD4 single positive cells. Two-color flow cytometric analysis indicated that the CD25-positive cells in the thymectomized animals were observed in both the CD8-positive and CD4-positive cells, which would correspond mainly to the CD8 single positive and CD4/8 double positive cells since the CD4 single positive cell population comprised <2% of the GICs. In addition, for both thymectomized and nonthymectomized animals, most of GICs were likely to be T cells since >80% of GICs were CD2-positive cells (data not shown) and >80% of GICs were also CD4 and/or CD8-positive cells (Fig. 5, *c* and *d*). No major differences were observed in the expression of CD25 in the PBLs when comparing thymectomized and nonthymectomized animals (data not shown).

CML. No significant difference in maximum antidonor cell PSL was demonstrated between the nonthymectomized and thymectomized groups before kidney transplantation (37.4 ± 8.5%, thymectomized animals versus 34.1 ± 7.6%, nonthymectomized animals, $P > 0.5$). Posttransplantation, thymectomized animals developed strong antidonor cell reactivity, in contrast to nonthymectomized animals and the sham-thymectomized animals which developed specific unresponsiveness to the donor class I antigens. The difference in PSL was significant (thymectomized animals, 29.5 ± 10.6% versus 3.5 ± 3.8% nonthymectomized controls, on POD 30, $P < 0.004$). Third-party reactivity was maintained in both groups of animals (data not shown). Fig. 6 shows CML reactivities of animals in both groups against donor-matched target cells, at an E/T ratio of 100:1, on POD 30.

Discussion

Miniature swine have been developed in this laboratory over the past 20 yr as a large animal model for studies of transplantation. Availability of animals inbred for SLA loci, as well as of intra-MHC recombinant animals, make these miniature swine the only large animal model in which one can reproducibly study the effect of selective matching for MHC class I or II loci. They also share many immunologic

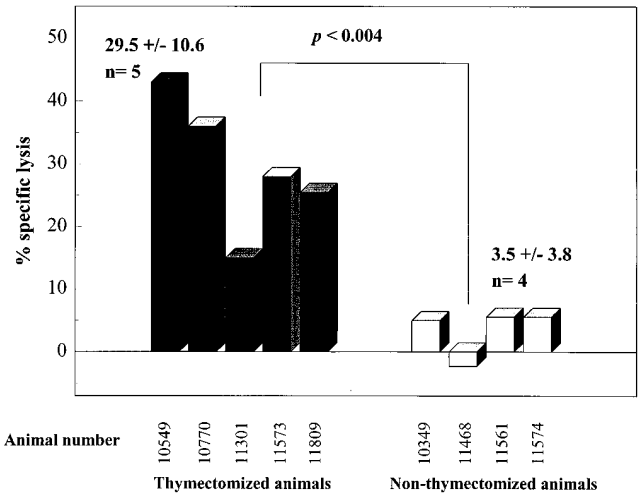


Figure 6. CTL antidonor reactivity on POD 30 in thymectomized animals and nonthymectomized animals. PSL at an E/T ratio of 100:1 was significantly higher in thymectomized animals when compared to nonthymectomized animals.

and physiologic properties with humans, making them extremely useful as a preclinical model for transplantation. Of particular relevance to the present studies is the fact that most large animals, including human beings and swine, express class II antigens constitutively on the vascular endothelium of their organs (34, 35), whereas rodents do not (36, 37). We have postulated that this difference may be relevant to the apparent unimportance of class II matching to the outcome of vascularized transplants in rodents (38–42), which stands in sharp contrast to the overwhelming importance of class II matching to the outcome of such transplants in humans (43) and in swine (8). Consistent with this hypothesis, we have previously demonstrated that at least one of the means by which tolerance to vascularized organ allografts has been induced in rodents, i.e., by a short course of CyA, is also uniformly efficacious in swine, but only if one uses pairs of animals matched for class II antigens (9).

Previous studies in this model have suggested that rejection and tolerance induction involve related but distinct immunologic processes, which may be occurring simultaneously during the response to the allograft. Obviously, for tolerance to be achieved, one must avoid rejection during the critical period required for tolerance induction after the transplant. Presumably, this prevention of rejection is effected by 12 d of CyA administration in this model. The data presented here confirm the requirement for additional immunologic events related to tolerance induction to occur during this period, since the immune response to the allograft after CyA was discontinued was affected markedly by the absence of a thymus. The nature of the immunologic events for which the thymus is required to induce tolerance in this system remain unclear.

Two categories of explanations seem plausible. (*a*) Cells (possibly dendritic cells) from the kidney graft may migrate to the thymus and may be responsible for a central component in both the induction and maintenance of tolerance in this system. A variation of this explanation would involve host cells picking up antigen in the graft and migrating to the thymus, with a similar effect. A recent study from our laboratory has used a sensitive PCR assay that was able to distinguish the host class I allele (SLA^d) from the donor class I allele (SLA^c) (Consorti, R., K. Yamada, S. Germana, D.H. Sachs, and C. Le Guern, manuscript in preparation). Although this assay was able to detect chimerism in skin and lymph node of some animals in a different protocol, no chimerism was observed in the thymus of swine that received a class I disparate renal allograft with CyA and were thymectomized on POD 8 or 42. This finding supports the hypothesis that if antigen presentation in the thymus is involved, donor class I peptides, rather than donor cells, may be the source of such antigen. Since hosts and donors are class II matched in these studies, processed class I antigens presented by class II antigens in the thymus would be expected to be identical regardless of whether the migrating cell were from donor or host. Thus, tolerance at the level of CD4 helper cells recognizing class I peptides through the indirect pathway, might be expected in both cases. The main difference between migration of donor cells versus host cells bearing donor antigens would involve tolerization of the direct pathway, which would only be possible if intact class I antigen, such as that expressed on donor cells, were involved in the intrathymic deletion. Since we have evidence for persistence of anti–class I CTLp in tolerant animals (44), tolerance at the level of helper cells may be sufficient to explain the intrathymic component of tolerance induction.

(*b*) Thymic emigrants may be responsible for permitting tolerance induction peripherally for alloreactive cells in the graft. Such peripheral tolerance could be mediated by a change in cytokine milieu or by regulatory responses including suppressive mechanisms. Recent studies have demonstrated that autoreactive T cells associated with syngeneic graft versus host disease induced by CyA may facilitate acceptance of MHC disparate cardiac allografts by the elimination of alloreactive lymphocytes (45). It was postulated that the T cells responsible for the prolongation of graft survival arose through inhibition of intrathymic clonal deletion of MHC class II autoreactive T cells. Suppressor mechanisms have also been reported in rodent models, in which one group identified a CD4-positive cell as the regulatory cell population (46–51). Additional studies have indicated that CD4-positive cells are capable of downregulating specific immune responses by local secretion of cytokines such as IL-10 and IL-4, and selective activation of such cells may occur (52–54). The apparent suppression may be explained by changes of cytokine milieu, which could result from a thymic-dependent distribution of helper cell types peripherally. Thus, Th1 cells produce IL-2 and IFN-γ, whereas the Th2 cells secrete IL-4 and IL-10. Regulatory pathways are likely to exist since in vitro IL-4 and IL-10 suppress IFN-γ production by Th1 cells, and Th2 cytokine production can be inhibited by IFN-γ. It is likely that alteration in cytokine production plays an important role in the induction of tolerance in our class I mismatch renal allograft

model since inhibition of T cell help (IL-2) by CyA leads to long-term tolerance (9), and furthermore, altered cytokine production consistent with differential activation of Th1 and Th2 cells has been demonstrated in renal tissue from allografts (55, 56). The latter studies demonstrate that renal biopsies from tolerant animals show high levels of IL-10 and low levels of IFN-γ gene transcription, whereas rejecting animals show a marked upregulation of IFN-γ gene transcription. Additional studies of renal tissue from thymectomized animals demonstrated that these animals express high levels of IFN-γ during a rejection crisis (Blancho, G., K. Yamada, F.L. Ierino, P.R. Gianello, I. McMorrow, S. Germana, A. Shimizu, R.B. Colvin, C. LeGuern, and D.H. Sachs, manuscript in preparation). However, it remains to be determined if this dysregulated cytokine production is a cause or effect of the induction and maintenance of tolerance in this swine model.

One animal in this study became tolerant without a thymus, albeit with a less stable clinical course, and this phenomenon has been confirmed in additional animals as part of ongoing studies on the timing of thymectomy (to be presented elsewhere). Such results imply that both central and peripheral mechanisms for induction of tolerance must exist. It seems likely that nonthymectomized animals use both peripheral and central mechanisms of tolerance, which would explain tolerance to the numerous minor antigens that would most likely escape thymic mechanisms. These peripheral mechanisms may include anergy and/or suppression. As noted above, peripheral mechanisms regulating alloresponses are influenced by cells arising from the thymus so that peripheral and central mechanisms may be interdependent. Additional studies in this model will investigate the effects on tolerance induction of the timing of thymectomy and of other manipulations known to affect the thymus such as administration of steroids, thymic biopsy, and the aging process.

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