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Evidence for a Gene Controlling the Induction of Transplantation Tolerance

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Abstract

Class I mismatched kidney transplantation in Massachusetts General Hospital MHC-defined miniature swine has been studied extensively as a model for induction of systemic allograft tolerance. In a large series of juvenile swine, long-term graft acceptance has been observed consistently following a 12-day course of cyclosporine. It was therefore surprising when three of five recipients in one of our studies rejected their grafts. Examination of the origins of the rejecting animals revealed that they were derived from a subline of the SLA^{dd} miniature swine herd that was intentionally being inbred toward full homozygosity and had been inbred for eight generations prior to these experiments. A blinded study of additional class I mismatched renal transplants into animals from this subline confirmed the genetic basis of this rejection. We present here preliminary evidence suggesting that a likely explanation for this phenomenon is that the rejectors in this subline are homozygous for a recessive mutant allele of a gene normally involved in the induction of tolerance. Subsequent studies will be directed toward identification and characterization of the gene(s) involved, since existence of a similar genetic locus in humans might have implications for assessing an individual's likelihood of graft rejection versus tolerance induction prior to organ transplantation.

Keywords

MGH miniature swine; MHC; thymus; tolerance/suppression/anergy; transplantation

Introduction

Previous studies from this laboratory have demonstrated that durable tolerance of renal allografts across a class I disparity can be achieved in juvenile miniature swine treated with a 12-day course of high-dose cyclosporine (1). Since the first description of this phenomenon, more than 60 cases have been performed in the class I mismatched haplotype combination swine leukocyte antigen (SLA)^{gg} or SLA^{hh} → SLA^{dd}, all of which have

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confirmed the original observation. Tolerance in this model has been substantiated both by the *in vivo* acceptance of a subsequent donor-matched kidney without further immunosuppression and by *in vitro* assays showing donor-specific hyporesponsiveness. We have postulated that the mechanism underlying the development of tolerance in this protocol involves a relative deficit of T cell help at the time of first exposure to the antigen (1) and have reported evidence for involvement of the thymus (2) and regulatory T cells (Tregs) (3–6) in this phenomenon.

The miniature swine utilized in these studies have been developed in this laboratory over the past 40 years as a model system for experimental transplantation. We currently maintain three MHC homozygous lines (SLA^{aa}, SLA^{cc} and SLA^{dd}) (7), and several intra-MHC recombinant lines (8,9). In addition to these partially inbred lines, since 1991 we have carried out sequential brother–sister matings within the SLA^{dd} herd in order to produce a fully inbred line of miniature swine (10). Animals from this highly inbred line were found to be histocompatible after seven generations of brother–sister matings (10) and they have now reached a coefficient of inbreeding of >96% in the G11 inbred SLA^{dd} herd. Among other potential uses, these animals have facilitated the first adoptive transfer experiments for mechanistic studies in a large animal model (11). In addition, animals from this inbred line were frequently used interchangeably with other noninbred SLA^{dd} animals as recipients of SLA^{gg} or SLA^{hh} kidneys as part of our studies of tolerance across a class I barrier.

It was therefore unexpected when animals from two sublines of the highly inbred SLA^{dd} animals either experienced a rejection crisis or rejected kidney allografts in spite of receiving the standard 12-day course of cyclosporine. After investigating and excluding several potential causes for this aberrant result, we examined the origins of the recipient animals. We report here the results of experiments performed to determine whether these rejection episodes were due to selection of novel genetic characteristics in this herd—A finding that could have important theoretical and practical implications in clinical transplantation. This brief report is intended to document the inherited nature of this phenomenon and thereby lay the groundwork for the future molecular and mechanistic studies that will be needed to establish its functional and genetic basis.

Materials and Methods

Animals

Massachusetts General Hospital (MGH) MHC-defined miniature swine with SLA^{gg} (class I^c/II^d) or SLA^{hh} (class I^a/II^d) were used as renal allograft donors. Miniature swine with SLA^{dd} (class I^d/II^d) were used as recipients. The immunogenetic characteristics of these swine have been described previously (7,9). All animals were naïve pigs between 4 and 6 months of age. Animals were housed and treated in accordance with the National Institutes of Health guide for care and use of animals under supervision of the Institutional Animal Care and Use Committee.

Surgery

Recipients underwent a bilateral nephrectomy and received an orthotopic renal transplant, as previously described (12).

Immunosuppression

Cyclosporine (Sandimmune; Novartis Pharmaceuticals Corporation, East Hanover, NJ) 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 days, starting on day 0. Whole blood trough levels were determined by a monoclonal radioimmunoassay.

Monitoring of rejection

Rejection was monitored by plasma creatinine level and by histological examination of biopsy tissue. The clinical end points used in this study were defined as: (1) rejection: animal moribund with uremia, and histologic evidence of rejection; (2) rejection crisis: transient increase in creatinine (>3 mg/dL) after postoperative day (POD) 5, with histologic evidence of rejection; (3) graft acceptance: low stable creatinine (<2.0 mg/dl) for more than 100 days after transplant, without histologic evidence of rejection.

Histology

Sequential wedge kidney biopsies were performed on PODs 18 and 30, or when a rise in plasma creatinine was observed. Tissues were stained using hematoxylin and eosin, and periodic acid-Schiff. Coded samples were examined for histologic evidence of rejection by a renal pathologist (13).

Mixed leukocyte reaction

Responder peripheral blood lymphocytes (PBL) were plated in triplicate in 96-well flat-bottom plates (Costar, Cambridge, MA) at a final concentration of 4×10^5 cells/well in RPMI 1640 supplemented with 6% fetal pig serum and were stimulated by an equal number of irradiated (2500 cGy) stimulator PBL as previously described (14). For tests of mixed lymphocyte reaction (MLR) in the presence of cyclosporine, the drug was added to the medium at a final concentration of 0, 5, 10, 20 and 40 ng/mL.

Primary cell-mediated lympholysis assays

One-way MLRs were performed as previously described (15). Briefly, 4×10^6 responder and 4×10^6 irradiated stimulator PBL were added per well in 2 mL of medium and incubated for 6 days at 37°C in 8% CO₂ and 100% humidity. Target lymphoblasts were phytohemagglutinin stimulated and labeled with ⁵¹Cr. In all experiments, effector cells were tested on three targets (self, SLA^{gg} and SLA^{hh}). The tests were run in triplicate at four different effector:target ratios (100:1, 50:1, 25:1 and 12.5:1) and were incubated for 5.5 h. ⁵¹Cr release was determined on a gamma counter (Micromedics, Huntsville, AL), and maximum lysis was obtained with the nonionic detergent NP-40(BRL, Rockville, MD). The results were expressed as percent-specific lysis.

Secondary coculture cell-mediated lympholysis suppression assay

This assay was performed as described previously (4). Briefly, in the priming phase (day 0–6), cells from experimental or control animals were primed for 6 days with irradiated stimulator cells and then recovered and rested overnight. In the coculture phase (day 7–13), naïve recipient-matched responder cells were cocultured with 2×10^6 cultured cells from the experimental or control animals, and incubated with irradiated stimulator cells. Effectors were subsequently recovered and tested as described in the primary cell-mediated lympholysis (CML) assays above.

Statistical analysis

Statistical analysis was performed using a paired Student's t-test.

Results

Clinical course of inbred animals in the original study of class I mismatched renal transplantation

Of five inbred SLA^{dd} animals receiving class I mismatched kidney transplants, three unexpectedly rejected their allografts within the first postoperative month. One rejection occurred as early as day 10, while the animal was still receiving cyclosporine. All three of these animals showed an abrupt rise in serum creatinine with concomitant histology confirming rejection, which was uncharacteristic of the clinical courses of animals previously treated under this protocol. The two remaining animals had uneventful posttransplant courses, accepting the kidney allografts indefinitely, with normal serum creatinine (Figure 1A).

Testing of potential explanations for rejection

Among the possible reasons considered for this unexpected result were: (1) inadequate levels of cyclosporine; (2) reduced effect of cyclosporine; (3) a problem with the particular lot of cyclosporine utilized; (4) differences among the donors due to mistaken SLA typing or to a mutation segregating in the donor pool; (5) fixation of a loss mutation in class II of the rejectors; and (6) fixation of a previously rare recessive allele in the recipients.

All five animals in the original study reached the intended therapeutic target range of 400–800 ng/mL (Figure 1B) making it unlikely that inadequate levels of cyclosporine were the cause of rejection. As is frequently the case, three animals in this series transiently had levels >800 ng/mL during the 12-day course. Of these, two went on to accept their kidney allografts, while one rejected, suggesting that this elevated cyclosporine level was also not the cause of rejection.

MLRs in the presence of varying concentrations of cyclosporine showed no differences in suppression of proliferation between the putative acceptors and rejectors (data not shown). The possibility that the particular lot of cyclosporine might be responsible for the rejection was ruled out by chemical assays performed at Novartis (Dr. Henk Schuurman, personal communication).

We performed serologic and MLR typing on PBL from two of the donors whose kidneys were rejected, and confirmed that both were unequivocally SLA^{gg} animals serologically and that neither was capable of responding to nor stimulating naïve SLA^{gg} cells in MLR assays (data not shown).

Last, in MLRs using cells from acute rejection crisis (ARC) and standard DD line animals reciprocally as responders and stimulators, we did not observe stimulation above background, making it unlikely that a mutation in class II had occurred. In addition, partial sequencing of the DRB, DQA and DQB regions of rejectors, acceptors and standard SLA^{dd} animals did not show any sequence differences (LeGuern, unpublished data).

Pedigree analysis of the recipients of rejecting grafts

After excluding these likely potential explanations for the aberrant rejection episodes, the pedigreed breeding of the recipient animals involved in this study was examined. As seen in Figure 2, the two animals that accepted class I mismatched kidneys came from one subline, while the three rejecting animals came from other sublines. This subline-specific pattern of rejection suggested that the rejections had a genetic basis.

Prospective blinded study of additional recipients

Six SLA^{dd} animals were entered into a blinded, prospective study, in which three were from the same inbred SLA^{dd} subline as the previous inbred rejectors, and three from the ordinary SLA^{dd} herd. Class I mismatched kidney transplants were performed in these recipients using the standard 12-day course of cyclosporine and monitoring for 100 days posttransplant. The investigators remained blinded to the lineage of these animals until POD 30 was reached for the last animal.

All animals maintained cyclosporine levels above the minimum therapeutic threshold. As seen in Figure 3, three animals accepted their kidney grafts with stable creatinine levels and three experienced either a rejection crisis or loss of the graft. One animal (15294) required placement of a ureteral stent on POD 4 due to obstruction and was subsequently euthanized on POD 10 due to pyelonephritis. Although its course until sacrifice was consistent with early rejection, this animal was excluded from the statistical analysis prior to unblinding the study. Another animal (15292) showed a gradual rise in serum creatinine beginning on POD 10 while on cyclosporine, which progressed to uremia, requiring euthanasia on POD 26. One animal (15279) suffered a severe rejection crisis beginning on POD 18, but recovered spontaneously and accepted the kidney graft long-term, but with abnormal histology (discussed below).

At the completion of this study, the specific lineage of each animal was unblinded. The three animals that accepted without evidence of rejection all came from the noninbred lines. The two animals with clear evidence for rejection were from the same subline as the previous rejectors. This difference was statistically significant ($p < 0.02$).

Renal allografts in additional inbred SLA^{dd} animals

In order to determine the distribution of the rejection phenomenon within this subline of the herd, additional inbred animals were tested with the same tolerance protocol. As seen in Figure 4, the pedigree of all animals tested revealed that all rejectors and acceptors could be accounted for in a subline that separated from acceptors at the G5 generation.

Further evidence against involvement of donor factors

Among the kidney donors in these prospective studies, two animals had donated both of their kidneys. By chance, one kidney from each donor was transplanted to an animal that accepted its graft and the other to an animal that rejected. Therefore, it appears unlikely that a donor factor not detected by MLR or serologic typing might be contributing to rejection.

Histology of kidney allografts

Upon histological analysis, rejector (15292) demonstrated severe lymphocytic infiltrate and vasculitis beginning on POD 18, which culminated in architectural distortion and hemorrhage on POD 26 at sacrifice (Figure 5A). Rejector (15279), which suffered a rejection crisis and recovered, showed glomerulopathy on biopsy at POD 34, indicating parenchymal injury induced during the crisis. In contrast, kidneys from the inbred acceptors were no different from the noninbred controls, demonstrating very mild lymphocytic infiltrates, consistent with accepted grafts (Figure 5B).

In vitro assays

In an attempt to study the difference between acceptors and rejectors *in vitro*, standard CML and secondary coculture suppression assays were performed. Animals that had accepted class I mismatched kidneys and animals that had rejected these kidneys demonstrated donor-specific hyporesponsiveness in standard CML (Figure 6A). Similarly, in secondary coculture assays, responders from both acceptors and rejectors, preincubated with donor-type stimulators in phase I of the assay, showed comparable suppression of generation of cytotoxicity by naïve SLA^{dd} responders when challenged with donor-type stimulators in phase II of the assay (Figure 6B). Therefore, neither *in vitro* assay appeared to be capable of detecting the same immune parameter(s) that affected the *in vivo* response.

Discussion

Previous studies from this laboratory have demonstrated that a 12-day course of cyclosporine A uniformly induces tolerance of class I mismatched kidney grafts in MGH miniature swine. This observation has been reproduced in more than 60 donor/recipient pairs. For the purpose of describing this new phenotype, in which rejection and/or rejection crises are observed, we propose to call the previous common phenotype, in which tolerance is consistently observed, the “stable posttransplant (SPT) phenotype” and the new, rejecting phenotype the “ARC phenotype.”

We have previously hypothesized a competition between alloreactive and regulatory cells as responsible for the outcome in this model, and have demonstrated how, in the presence of adequate T cell help, tolerance succumbs to rejection (16). Consistent with this hypothesis,

cyclosporine early posttransplant may blunt an alloaggressive response while permitting a down-regulatory response to persist, resulting in tolerance (1). Therefore, in assessing potential causes for the sudden appearance of the ARC phenotype, we first examined the role of cyclosporine, and determined that neither resistance to the effects of the drug nor differences in the half-life ($t_{1/2}$) of its elimination from the blood (data not shown) could explain the new phenotype.

One element involved in this tolerance model that has been the subject of several previous studies from this laboratory is the role of Tregs (3,4,11,14). In the present study, we attempted to detect Tregs *in vitro* for both theoretical and practical reasons. Theoretically, a defect in either the generation or function of Treg could result in rejection and might therefore be a clue to the mechanism responsible for the new ARC phenotype. From a practical viewpoint, if a difference in Tregs could be demonstrated between the two phenotypes, it would provide a much easier means for distinguishing these phenotypes for breeding purposes than the laborious and lengthy process of evaluating *in vivo* rejection versus tolerance induction. We found no apparent difference between SPT and ARC phenotype animals in either their CML activity or their ability to mount suppression in secondary coculture CML (Figure 6). However, since the assays utilized measure activities of PBL rather than of cells within the tolerated grafts, which are likely most important (5,17,18), it remains possible that differences in intra-graft regulatory components may still explain the phenotype. For example, failure of homing of Tregs to the graft could be responsible for the ARC phenotype. In this regard, assessment of possible differences in chemokines or cytokines within the grafts of the two sublines are among the studies in progress.

One main feature of inbred animals is that they eventually become homozygous for all autosomal genes (19). During the process of inbreeding, recessive genes are fixed, and if they are neither lethal nor advantageous, they may not be noticed until a selective pressure is applied. The fact that the ARC phenotype was observed after considerable inbreeding of the SLA^{dd} line and it has not been observed in the standard herd strongly suggests that: (1) the rejection phenomenon was caused by fixation of a recessive allele to homozygosity; and (2) this recessive allele must have been present at a low frequency within the herd prior to inbreeding.

In the clinical arena, the inability to know which patients can successfully be weaned from immunosuppression is a major problem for treatment protocols directed toward tolerance induction (20). The availability of both SPT and ARC phenotypes in our herd might represent an important instrument to address this problem. A process of selective breeding of acceptors and rejectors is currently under way directed toward isolation of two homozygous sublines from the current inbred subline. Availability of such sublines should make it possible to identify the genetic element responsible for the ARC versus SPT phenotypes, which could, in turn, provide a marker for a comparable gene in human populations. Potentially, one might use such a marker to identify which transplant patients would likely benefit from a tolerance-induction protocol. Subsequent studies will therefore be directed toward establishing ARC and SPT homozygous sublines and then identifying

and characterizing the gene(s) and the functional mechanisms by which they are distinguished.

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Abbreviations

ARC	acute rejection crisis
CML	cell-mediated lympholysis
MGH	Massachusetts General Hospital
MLR	mixed lymphocyte reaction
PBL	peripheral blood lymphocytes
POD	postoperative day
SLA	swine leukocyte antigen
SPT	stable post-transplant
Tregs	regulatory T cells

A Clinical course of initial inbred animals **B** Cyclosporine blood levels **Figure 1. Initial inbred animals**

Five inbred SLA^{dd} animals were recipients of a class II matched/class I mismatched kidney transplant treated with 12 days of cyclosporine; (A) kidney function as determined by daily creatinine levels; (B) cyclosporine (CyA) blood levels (target therapeutic levels = 400–800 ng/mL). SLA, swine leukocyte antigen.

Figure 2. Pedigree of initial inbred animals

Partial pedigree of the initial inbred animals that received a class II matched/class I mismatched kidney transplant treated with 12 days of cyclosporine. Acceptors (*italic*), and rejectors (**bold**).

Figure 3. Clinical course

Creatinine levels in animals from a blinded, prospective study involving three animals from the subline that had rejected grafts in the initial study [indicated with (i)], and three from the standard SLA^{dd} herd [indicated with (s)]. SLA, swine leukocyte antigen.

Figure 4. Pedigree of all inbred rejectors and acceptors

Composite pedigree of animals from both the initial studies and the blinded prospective studies. Acceptors (*italic*) and rejectors (**bold**).

Figure 5. Kidney histology of inbred rejectors and acceptors

(A) H&E histologic stain of a kidney biopsy taken at POD 18 and 26 from an inbred animal that rejected a class I mismatched kidney transplant following treatment for 12 days with cyclosporine; (B) H&E histologic stain of a kidney biopsy taken at POD 31 and 42 from an inbred animal that accepted a class I mismatched kidney transplant following treatment for 12 days with cyclosporine. H&E, hematoxylin and eosin; POD, postoperative day.

A Standard CML

B Suppressor CML

15336 = acceptor of graft
15292 = rejector of graft

Figure 6. Standard CML and suppressor CML of acceptor versus rejector
(A) Standard CML assay; and (B) coculture suppressor CML assay; both using PBL as responders from an inbred animal that accepted (15336) and an inbred animal that rejected (15292) a class I mismatched kidney transplant following treatment for 12 days with cyclosporine. CML, cell-mediated lympholysis; PBL, peripheral blood lymphocytes.