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Relationship Between Chimerism and Tolerance in a Kidney Transplantation Model

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The persistence of donor leukocytes in recipients of organ allografts has been associated with long-term graft acceptance. However, it remains unclear whether this peripheral donor cell microchimerism plays an active role in graft acceptance or is simply a consequence of the maintenance of sufficient immunosuppression to avoid rejection. A model of kidney transplantation between swine leukocyte Ag (SLA)-matched miniature swine, in which tolerance can be established with or without immunosuppressive treatment, has been used to study the correlation between donor leukocyte chimerism and kidney graft acceptance. SLA-identical kidney transplants were performed from animals positive for an allelic pig leukocyte Ag to animals negative for this marker. SLA-identical kidney transplant recipients given a 12-day course of cyclosporine (CyA) (n = 3) became tolerant, showing stable serum creatinine levels (1–2 mg/dl) after cessation of CyA treatment. Donor cell chimerism (0.2–0.7%) was present by FACS in all three animals with peak levels detected at 3 wk. Two control animals receiving SLA-identical kidney grafts without CyA also showed stable serum creatinine levels and became tolerant. However, in neither of these animals could donor leukocytes be detected in the peripheral blood beyond 1 wk following transplantation. In one additional control animal, ureteral obstruction occurred at day 10, and was associated with additional peripheral chimerism, presumably related to inflammation rather than to immune status. These results indicate that the persistence of donor cell chimerism is not a requirement for the maintenance of tolerance to organ allografts in this model. The Journal of Immunology, 1999, 162: 5704–5711.

Transplantation tolerance, the long-term acceptance of grafted tissue in the absence of continuous immunosuppression, remains an important goal in human transplantation. Proposed mechanisms influencing tolerance include peripheral anergy or clonal deletion of donor-reactive cytotoxic T cells and suppression of alloreactive clonotypes. With the reports in recent years of small percentages of donor-derived dendritic or hematopoietic cells detected in various tissues in long-term kidney (1), liver (2–4), and heart recipients (4), a novel theory to elucidate graft tolerance has been proposed. The theory contends that long-lived donor-derived cells play an important role in generating long-term graft acceptance (5, 6). However, consensus has not emerged regarding the causal relationship between such chimerism and tolerance.

Current limitations in clinical transplantation technology necessitate the use of continuous posttransplant immunosuppression in allograft recipients to prevent graft rejection. Thus, the persistence of donor hematopoietic cells in recipient tissues may be caused by the inability of an immunosuppressed host to effectively eliminate these cells. Indeed, rather than inducing tolerance itself, donor chimerism may be an epiphenomenon accompanying immunosuppression-induced graft acceptance.

Using the partially inbred miniature swine developed in this laboratory as a large animal model of organ transplantation (7), we have studied the possible role of chimerism in transplantation acceptance and the factors that influence this chimerism. Earlier studies in this laboratory have demonstrated that tolerance can be established in swine leukocyte Ag (SLA)6-matched kidney grafts, with or without immunosuppression (8, 9). This model has allowed us to assess the potential role of chimerism in graft acceptance, independent of immunosuppression. Using an allelic non-histocompatibility marker, pig allelic Ag (PAA), previously described in this laboratory,7 we have been able to identify the presence of chimeric cells in kidney graft recipients.

We performed SLA-identical kidney transplants using donor animals positive for PAA and recipient animals negative for this marker, with and without immunosuppression. We also performed an SLA-mismatched kidney transplant with immunosuppression. We evaluated the relationship between donor chimerism, immunologic response, and graft acceptance in these animals.

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 intra-MHC recombinant haplotypes have previously been described (7, 10).

6 Abbreviations used in this paper: CyA, cyclosporine; GIL, graft infiltrating leukocytes; PAA, pig allelic Ag; SLA, swine leukocyte Ag.

Surgery

The details of the surgical procedures have been previously described (8, 11). Placement of an indwelling central venous silastic catheter into an external jugular vein facilitated cyclosporine (CyA) administration and frequent blood sampling for monitoring of renal function (blood urea nitrogen, serum creatinine) and whole blood CyA levels.

Immunosuppression

An i.v. preparation of CyA was generously provided by Novartis Pharmaceuticals (Hanover, NJ). CyA was given each morning as a single daily infusion at a dose of 10–13 mg/kg (adjusted according to blood levels) for 12 consecutive days posttransplant. The first dose was administered preoperatively, before the unclamping of vessels to the kidney allograft.

CyA levels

Whole blood trough levels were determined by a monoclonal radioimmunoassay technique. Daily trough levels between 500 and 800 ng/ml were achieved by adjusting the dose between 10 and 13 mg/kg, since these blood levels have previously been shown to be effective for induction of tolerance to renal allografts across selective SLA class I and minor Ag disparities (12–14).

FIGURE 1. Clinical course of recipients of an SLA-matched kidney allograft: with CyA (A) and without CyA (B).

FIGURE 2. Chimerism in peripheral blood in recipients of SLA-identical kidney allografts with CyA treatment (A), and without CyA treatment (B), shown as percent donor cells in PBMC. Percent donor cells was defined as: 100 × (PAA-positive PBMCs/total PBMCs) by FACS.
Histology

Sequential wedge kidney biopsies were performed on postoperative days 8, 11, 18, 30, and 60 through a flank incision. Tissues were stained using hematoxylin and eosin and periodic acid–Schiff, and coded slides were examined.

Isolation of PBMC

Freshly drawn, heparinized whole blood was diluted with HBSS (Life Technologies/BRL, Grand Island, NY), and 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 ACK buffer (B&K Research Laboratories, Fiskeville, RI). Cells were washed a second time with HBSS and resuspended in tissue culture cell-mediated lympholysis medium. Cell suspensions were kept at 4°C until used in cellular assays.

Preparation of renal cell suspensions

Kidney biopsies (100–500 mg) were minced and then suspended in HBSS buffer, using the flat end of a syringe plunger. The cell suspension was then filtered on a coarse mesh and centrifuged at 1600 rpm for 10 min at 4°C. Cells were resuspended in 1 ml of flow cytometry analysis medium (HBSS buffer, 0.1% of BSA, and 0.1% sodium azide).

Identification of graft-infiltrating leukocytes (GIL) within renal cell suspensions

Purification of GIL from small wedge biopsies of kidney has been difficult to achieve because GIL represent <3% of total renal cells at any given time point. Selective gating of these cells was therefore performed as previously described (15).

Flow cytometry as a measure of chimerism

To determine the level of peripheral donor cell chimerism, 1 × 10⁶ PBMC were incubated with FITC-conjugated 1038H-10-9 (IgM mAb) specific for swine PAA. FITC-conjugated 12-2-2 was used, as an IgM isotype-matched control. Staining was performed using FACS medium consisting of HBSS with Ca²⁺, 0.1% BSA, and 0.1% Na₂₃. Flow cytometry was performed using a Becton Dickinson (Sunnyvale, CA) FACSscan Il. Flow cytometry data were analyzed using Winlist mode software (Verity Software House, Topsham, ME).

For two-color analysis, incubation with biotinylated mouse mAbs specific for porcine CD2 (MSA4), CD3 (2-6-15), CD4 (74-12-4), or CD8 (76-2-11) (16–18) were added, followed by staining with PE-conjugated streptavidin (Becton Dickinson). Because the levels of chimerism in the peripheral blood were low, we used a statistical analysis to assure the presence of chimeric cells. The mean percent chimerism among all four two-color tubes from experimental animals was calculated and compared with the mean percent background staining for the two-color tubes of naive PAA-negative animals. The statistical significance of the mean donor cell chimerism for each experimental animal was determined relative to the mean background staining of the naive PAA-negative control animals. The biotinylated anti-mouse-class I Ab 36.7.5 (IgG2a) served as a negative isotype-matched control. Propidium iodide (PI) was added immediately before acquisition, and dead cells were excluded from analysis based on PI staining.

Results

Effects of CyA on renal function and renal allograft survival

Three SLA-matched, PAA-mismatched allografts were performed with CyA treatment, and three were performed without CyA treatment. Serum creatinine levels were measured to assess kidney function. Results are shown in Fig. 1. All three animals with CyA treatment maintained stable serum creatinine levels (1–2 mg/dl), even after cessation of CyA treatment, and two of three animals without CyA treatment maintained stable serum creatinine levels (1–2 mg/dl). One animal showed a transient increase in creatinine due to ureteral obstruction (day 10), but levels rapidly returned to normal after relieving the obstruction, and the kidney allograft was accepted long-term (shown below). All three animals survived long-term (>2 yr) with normal renal function and normal kidney histology. Thus, normal kidney function and long-term graft acceptance were established with or without transient CyA immunosuppression.

Effects of CyA on donor cell chimerism in peripheral blood and graft

Peripheral chimerism. Two-color FACS analysis of PBMC was performed at periodic intervals posttransplant to evaluate donor cell chimerism in peripheral blood. Donor chimerism (0.2–0.7%, p < 0.005 relative to naive controls) was detected in all three animals with CyA treatment, with peak levels around 3–4 wk and a gradual decrease thereafter. Chimerism disappeared by day 40–60 (Fig. 2A), except in one animal (no. 11870, shown later). In two of the three animals without CyA treatment, donor chimerism could not be detected in the peripheral blood beyond 1 wk following transplantation (Fig. 2B).

Intragraft chimerism. In all three animals with CyA treatment, donor chimerism (20–30%) was present in the graft at 2–3 wk and, thereafter, gradually disappeared (Fig. 3A). In two of the three animals without CyA treatment, donor chimerism could not be detected in the graft beyond 2 wk following transplantation (Fig. 3B).

Representative animals. Peripheral and intragraft chimerism in representative animals is shown in Fig. 4. On day 18 after kidney transplantation, CyA-treated animal no. 11784 showed 0.51% (p < 0.005) donor chimerism in peripheral blood (Fig. 4A) and 20% chimerism in graft (Fig. 4C). On the other hand, CyA-untreated animal no. 11892 showed no significant chimerism in peripheral blood (Fig. 4B) and only 0.4% chimerism in graft (Fig. 4D). Thus, there was a positive correlation between CyA treatment and transient donor chimerism in both PBMC and graft.

Histology

Serial kidney biopsies were performed on days 18, 30, and 60. At histologic examination, all three CyA-treated pigs showed a minimal focal mononuclear interstitial infiltration, without tubulitis and endothelialitis on days 18 and 60 (Fig. 5, A and C). In comparison, despite the stability of graft function, CyA-untreated pigs showed a patchy and mild mononuclear cell infiltrate with focal tubulitis on day 18 (Fig. 5B). The mononuclear cell infiltrate decreased spontaneously and remained only minimal on day 60 (Fig. 5D).

Effect of ureteral obstruction

One CyA-untreated animal showed a transient increase of creatinine around day 10 due to ureteral obstruction.
in this animal rose simultaneously with the creatinine increase, and, likewise, dropped rapidly with the creatinine decrease upon relieving the ureteral obstruction. The chimerism gradually disappeared, with a peak level at around 1.8% at day 11 (Fig. 6, A and B). This level was around four times the average of the CyA-treated model.

Class I-mismatched, PAA-mismatched kidney graft

Unlike SLA-matched grafts, which are accepted with or without CyA treatment, class I-mismatched grafts require immunosuppression for 12 days posttransplant to induce tolerance (12). In this experiment, one animal received a class I-mismatched renal

FIGURE 4. Chimerism in peripheral blood and graft in representative CyA-treated (A and C) and untreated (B and D) animals that received SLA-identical kidney allografts on day 18 is shown by FACS analysis. The x-axis shows PAA (1038H-10-9) staining; y-axis shows pig CD2 (MSA4) staining.

FIGURE 5. Renal biopsies taken on day 18 and day 60 from representative CyA-treated (A and C) and untreated (B and D) animals that received SLA-identical kidney allografts (hematoxylin and eosin).
allograft with CyA treatment. Despite the use of immunosuppression, donor chimerism could not be detected in the peripheral blood and in the graft beyond day 10 after kidney transplantation (Fig. 7). However, the animal demonstrated normal kidney functions and no signs of rejection. Similar studies attempting to detect chimerism have been performed in two additional class I-mismatched animals, with the same results.

Donor chimerism in the rekidney transplantation model
Animal no. 11892 received an SLA-identical kidney graft without CyA treatment, and, on day 105, received a second kidney graft from the same donor, this time with CyA treatment. Surprisingly, after the second kidney graft, even with CyA treatment, no donor chimerism could be detected in the peripheral blood and none could be detected in the graft beyond 2 wk (Fig. 8).

Long-term chimerism in peripheral blood after kidney transplantation
Animal no. 11870 received an SLA-identical kidney allograft with CyA treatment. Donor chimerism in the peripheral blood was present (0.1–0.5%; p < 0.05) with a peak level detected at around 4 wk. Chimerism decreased to 0.1% at around 2 mo, however, it then increased to 0.15% at 4 mo, and 0.25% at 11 mo after transplantation (Fig. 9). At this time, donor chimerism could be detected in the peripheral blood and in the graft, but not in the thymus or mesenteric lymph nodes (data not shown).

Discussion
We have previously demonstrated that SLA-identical renal allografts are accepted permanently in approximately two-thirds of untreated miniature swine (8, 19) and in 100% of animals treated with a 12-day course of CyA (20, 21). Similarly, class II-matched renal allografts are accepted in approximately one-third of cases across a single haplotype class I-mismatch (22), but are uniformly rejected across a two-haplotype class I-mismatch (12). In both cases, 100% of such class I-mismatched transplants are accepted if the animals are treated with a 12-day course of CyA (12, 14). Thus, this model provides the possibility for studying tolerance induction to renal allografts with or without the use of exogenous immunosuppression.
There has been considerable debate recently in the literature concerning the importance of lymphohematopoietic chimerism following vascularized organ allografts (6, 23, 24). Most investigators agree that such chimerism can be detected systemically following allografts of this kind. There is controversy, however, concerning whether the chimerism detected is the result of immunosuppression or the cause of allograft acceptance (23). In the case of vascularized organ allografts following the induction of lymphohematopoietic chimerism by bone marrow transplantation, it is clear that the tolerance persists even if the organ allograft is removed (25, 26). However, in the case of organ allografts, which are maintained through the use of exogenous immunosuppression, the tolerance generally disappears when the organ is removed (27). Thus, some investigators have reasoned that the chimerism is merely a reflection of cells that have escaped from the organ, but have not been rejected due to the immunosuppressive drugs, which maintain a state of hyporesponsiveness (23).

In the present study, we have attempted to determine the relevance of chimerism to acceptance of renal allografts in MHC-matched miniature swine. The transplants were performed either utilizing a short course of CyA as an immunosuppressive agent, or in the absence of exogenous immunosuppression. In both cases, the kidney grafts were accepted long term. The fact that this acceptance was due to the induction of true immunologic tolerance has previously been demonstrated in this model by the findings that: 1) skin (as opposed to vascularized) grafts across the same histocompatibility barrier are uniformly rejected (8), leading to sensitization and rapid rejection of subsequent kidney transplants (9); and 2) the survival of skin grafts on animals that have accepted renal allografts is markedly prolonged (8). The data reported here reveal no relationship between the persistence of chimerism and the presence of such tolerance in these animals. Thus, the findings support the contention that such chimerism is incidental to, rather than the cause of, long-term allograft acceptance.

In the case of a class I-mismatched renal allograft, chimerism was not detectable (day 8), even in the presence of CyA as an exogenous immunosuppressive agent. The reason for this lack of
detectable chimerism is not clear, but could reflect the increased strength of the immune reactivity to this class I disparity before tolerance induction, or could be the effect of NK cell activity, which would only be detectable in the face of a class I mismatch (28–30).

Second renal allografts showed no detectable chimerism, although they were accepted long term. The most plausible explanation for the failure to detect such chimerism following the second transplant would be sensitization of the immune system to Ags present on the lymphohematopoietic cells, which escape from the graft. This hypothesis would require that there be Ags, in addition to those expressed on the kidney, which are expressed only on the surface of such lymphohematopoietic cells. Such differential Ag expression would not be unique, since tissue- and organ-specific Ags have previously been described (31–34).

One surprising finding in these studies was the large increase in chimeric cells detectable following an incidental ureteral obstruction during the postoperative course of one animal. The fact that this chimerism disappeared promptly after the ureteral obstruction was relieved surgically suggests that the chimerism was incidental to inflammation and/or another physiologic disturbance in the kidney, but once again, not related to the immune status.

It is interesting to note that one CyA-treated kidney recipient did exhibit persistent, long-term donor chimerism in the peripheral blood. The clinical course of this animal was unremarkable for the first few months after transplant, as the chimeric cell levels followed the same trends as other CyA-treated animals. However, while chimerism disappeared in the other animals at 40–60 days, it began to increase steadily in this animal, to a maximum of 0.25%. This result suggests that among the passenger leukocytes in the kidney graft was a population of hematopoietic stem cells, which apparently survived and proliferated in the immunosuppressed animal (35, 36). In addition, the increase and persistence of these cells even after discontinuation of the immunosuppressive treatment indicates that the animal was tolerant, not only to the kidney graft, but also to any tissue-specific Ags expressed on the hematopoietic stem cells.

Previous examples of such long-term chimerism in graft recipients are well-documented (1, 2, 37) and have been used as evidence that chimerism causes the induction of graft tolerance. Our data would indicate that while long-term chimerism can exist in organ transplant recipients, it is not essential for graft survival.

In summary, these data support the likelihood that chimerism may be associated with long-term organ allograft survival, but that it is not essential for the induction or maintenance of the tolerant state. It is, of course, impossible to ever prove the absence of cells below a detectable level. Nevertheless, the clear presence of such cells in animals prepared by bone marrow transplantation (25, 38), and in animals on immunosuppression, and the absence of such cells in animals that become tolerant without immunosuppression provides no evidence for a relationship between such chimerism and graft acceptance.

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