



Renal allograft rejection is prevented by adoptive transfer of anergic T cells in nonhuman primates

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Anergic T cells generated ex vivo are reported to have immunosuppressive effects in vitro and in vivo. Here, we tested this concept in nonhuman primates. Alloreactive T cells were rendered anergic ex vivo by coculture with donor alloantigen in the presence of anti-CD80/CD86 mAbs before adoptive transfer via renal allograft to rhesus monkey recipients. The recipients were briefly treated with cyclophosphamide and cyclosporine A during the preparation of the anergic cells. Thirteen days after renal transplantation, the anergic T cells were transferred to the recipient, after which no further immunosuppressive agents were administered. Rejection-free survival was prolonged in all treated recipients, and 3 of 6 animals survived long term (410–880 days at study's end). In the long-surviving recipients, proliferative responses against alloantigen were inhibited in a donor-specific manner, and donor-type, but not third-party, skin allografts were also accepted, which demonstrated that antigen-specific tolerance had been induced. We conclude that anergic T cells generated ex vivo by blocking CD28/B7 costimulation can suppress renal allograft rejection after adoptive transfer in nonhuman primates. This strategy may be applicable to the design of safe clinical trials in humans.

Introduction

Organ transplantation is a therapy of choice for diseases otherwise uncontrollable by medical treatment or surgical procedures. However, daily administration of nonspecific immunosuppressants is presently required to prevent T cell-mediated acute rejection. Use of agents such as calcineurin inhibitors and steroids can lead to the development of opportunistic infections, hypertension, hyperlipidemia, nephrotoxicity, and cancer. For this reason, specific suppression of alloreactive T cells is desirable. One procedure used to induce tolerance in organ transplantation is a short course of treatment with mAbs directed against costimulatory molecules. Blockade of the CD28/B7 or CD40/CD40L pathway alone has been shown to be sufficient to prolong the survival of cardiac allografts in rodents (1–5). On the contrary, some reports have addressed the failure of combined costimulatory blockade in murine transplant models (6, 7). Many methods for reducing the immunosuppressive requirements of allotransplantation have been proposed in large-animal models (reviewed in ref. 8); however, it is commonly accepted that achieving tolerance to organ allografts in primates including humans has been elusive. In rhesus monkeys, it was demonstrated that treatment with anti-CD40L mAb and CTLA-associated antigen-4 (CTLA-4) Ig (9) or anti-CD40L mAb alone (10) can significantly prolong the survival of renal allografts. However, clinical trials with this agent were recently halted because of

unexpected thromboembolic complications (11, 12). The effect of mAbs to human CD40 is also limited in rhesus monkeys (13).

Recently it was reported that anergic T cells have suppressor activities in vitro (14, 15) and in vivo (16). Treatment with anergic T cells was used in human histoincompatible bone marrow transplantation with a relatively low risk of graft-versus-host disease (17). Therefore, it has been proposed that anergic T cells generated ex vivo may also induce indefinite solid organ allograft survival in humans. In the present study, we tested this concept in rhesus monkeys. We show that the anergic T cells, prepared by coculturing with donor alloantigen in the presence of anti-CD80/CD86 mAbs, efficiently prevent renal allograft rejection and induce antigen-specific tolerance in rhesus monkeys.

Results

Characterization of the cultured cells. Proliferative responses of freshly isolated CD4⁺ T cells from rhesus monkeys to allogeneic stimulators were inhibited by mAbs to human CD80/CD86, which indicated that these could be used in monkeys (Figure 1A). Next, we established that recipient splenic T cells cocultured with irradiated donor splenocytes in the presence of anti-CD80/CD86 mAbs for 13 days failed to proliferate in a donor-specific fashion upon restimulation. However, this impaired response was reconstituted by the addition of recombinant IL-2 (Figure 1B). These findings suggest that an anergic state can be attained in this manner, according to the definition of anergy as reversible proliferative blockade (18). Importantly, these anergic cells also suppressed alloproliferative responses in a donor-dominant fashion in vitro (Figure 1, C and D). The purity of T cells enriched by nylon wool columns was 90–93%, which allowed 400–800 million T cells to be obtained from each recipient. In our study, almost

Nonstandard abbreviations used: CP, cyclophosphamide; CsA, cyclosporine A; CTLA-4, CTL-associated antigen-4; MLR, mixed lymphocyte reaction; POD, postoperative day.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 115:1896–1902 (2005). doi:10.1172/JCI23743.

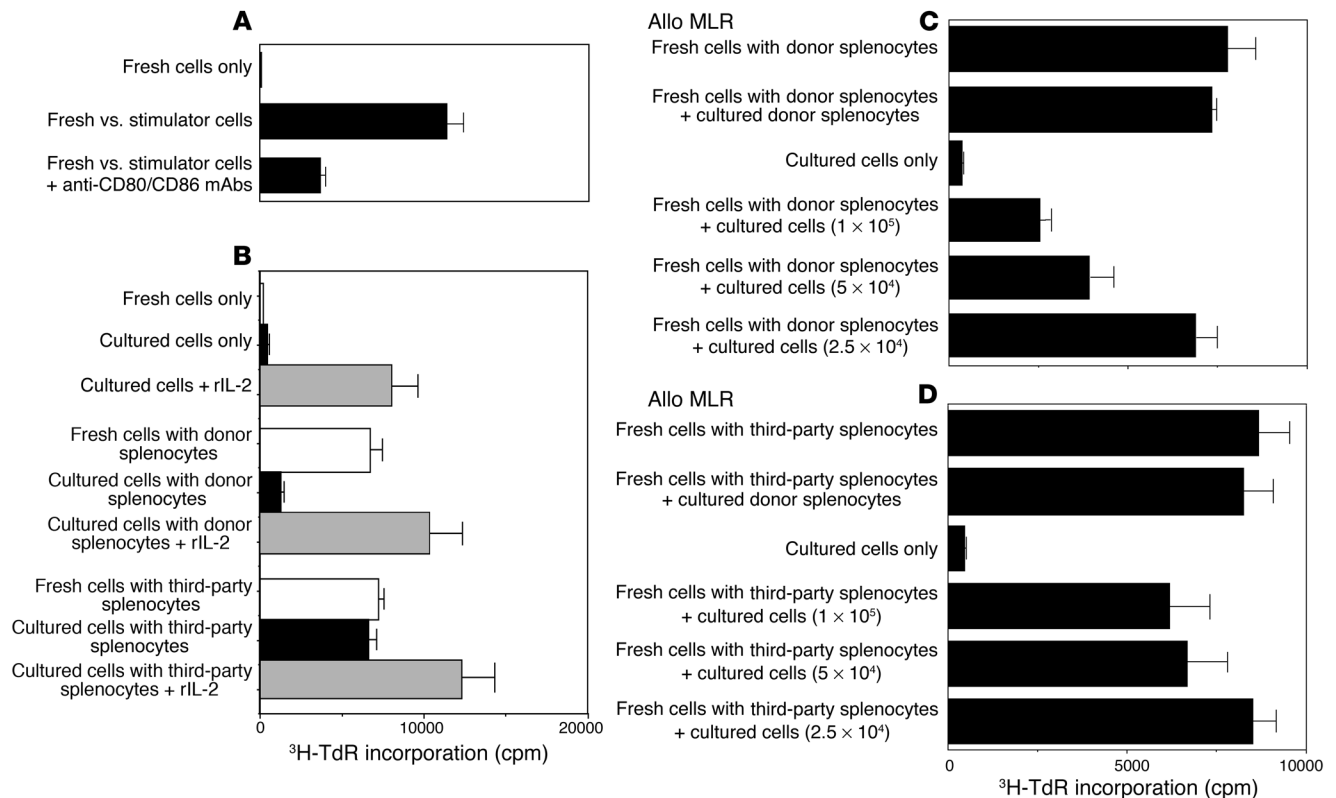


Figure 1

Effect of anti-human CD80/CD86 mAbs on MLR in rhesus monkeys and functional activities of the cultured cells. (A) Freshly isolated CD4⁺ T cells (fresh cells) from peripheral blood of rhesus monkeys were cocultured with irradiated allogeneic PBMCs (stimulator) in the presence or absence of anti-human CD80/CD86 mAbs (10 μg/ml each) for 5 days. (B) Peripheral blood CD4⁺ T cells (fresh cells, white bars) or the cultured cells (black bars) were stimulated with donor or third-party splenocytes (gray bars). After 3 days culture or 5 days (for fresh cells), the responder cells were evaluated for their proliferation. (C and D) Dose-dependent suppression of the alloresponses of peripheral blood CD4⁺ T cells to donor-type stimulator cells by the cultured anergic cells. Cultures were set up with recipient CD4⁺ T cells (10⁵ cells/well) and donor (C) or third-party (D) stimulators (10⁵ cells/well) for 7 days. Cultured donor splenocytes (10⁵) or different numbers of the cultured cells were also added in some wells. In all assays, cells were incubated for 6 days and then pulsed with 10 μCi of [³H]thymidine for the last 18 hours and counted. The bars represent the mean of triplicate values and the brackets indicate the SD. rIL-2, recombinant IL-2.

the entire inoculum consisted of T cells, including 40–48% CD4⁺ cells (Figure 2D), and there were very few B cells (Figure 2B) or monocytes (Figure 2F). CD25 expression was upregulated in these cultured CD4⁺ T cells (Figure 2H) compared with that of the freshly isolated peripheral blood CD4⁺ T cells (Figure 2G). CTLA-4 expression by the CD4⁺CD25⁺ cultured T cell population was also upregulated compared with that of peripheral blood T cells of the same phenotype (Figure 2I). Before transfer, these cells were washed 6 or 7 times until the concentration of residual anti-CD80/CD86 mAbs in the preparation was below the level detectable by ELISA (data not shown).

Prevention of acute renal allograft rejection by adoptive transfer of anergic cells. The 5 monkeys of the control group treated with cyclosporine A (CsA) and cyclophosphamide (CP) but not inoculated with anergic cells (group B) died of acute rejection between postoperative days (PODs) 15 and 28 (Table 1). Anergic cells prepared by coculture with donor alloantigen in the presence of both anti-CD80 and anti-CD86 mAbs for 13 days were adoptively transferred to 6 animals (group A), 3 of which survived indefinitely (410–880 days at study's end). Animals that received the entire regimen (treatment of CsA and CP and inoculation with the anergic cells) but with cells activated by coculture with third-party cells and the

same antibodies (group C) also died of acute rejection, which suggests that long-term survival in half of the recipients seems likely to be dependent on the specifically anergized cells. The mean graft survival time in group A was 416 ± 365 days, which represents a statistically significant prolongation compared with that of group B (22 ± 6.5 days; *n* = 5; *P* < 0.005) or group C animals (47.0 ± 19.0 days; *n* = 5; *P* < 0.005). Serum creatinine levels in group A were slightly elevated within 14 days after transplantation; however, graft function was not compromised at any time after inoculation with the anergic cells (Figure 3). Serum CsA levels were 92–142 ng/ml on the day of inoculation; they then gradually decreased and became undetectable about 45–60 days after the last administration (Figure 4). At study's end, no infections or malignancies had been observed in these monkeys. A recent report indicated that blockade of CD86 alone could induce long-term allograft survival in mice (19). In 5 monkeys transfused with T cells cocultured with donor alloantigen in the presence of anti-CD86 mAb alone (group E; *n* = 4) or anti-CD80 mAb alone (group F; *n* = 1), graft survival was only prolonged to 43–111 days. In these monkeys, serum creatinine levels increased 5–7 days before death, and histological examination of the grafts showed acute cellular rejection characterized by diffuse interstitial lymphocytic infiltration

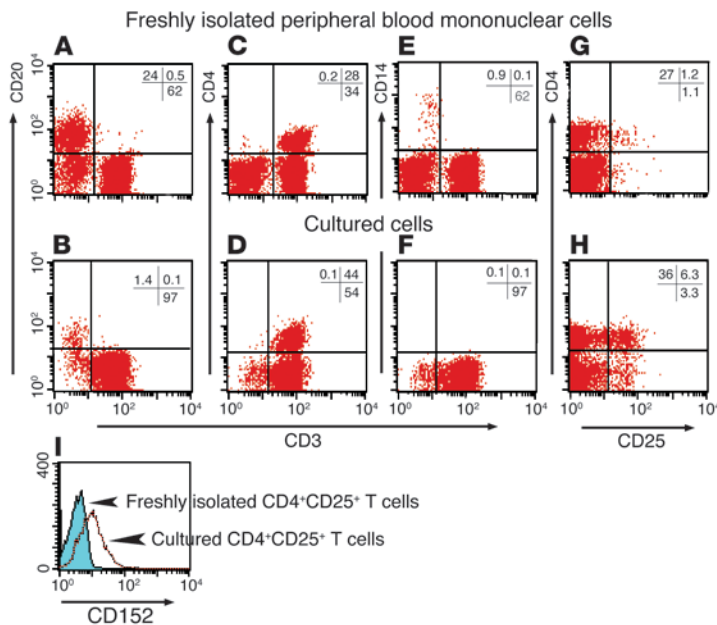


Figure 2

Characterization of the cultured anergic cells. (A–H) Freshly isolated PBMCs (A, C, E, and G) and the cultured anergic cells (B, D, F, and H) were analyzed for cell-surface expression of CD3, CD20, CD4, CD14, and CD25. (I) CTLA-4 (CD152) expression in cultured CD4⁺CD25⁺ T cells (open histogram) and freshly isolated T cells (filled histogram). Results are representative of 3 independent experiments.

In animals in group A receiving more than 6×10^7 anergic cells, the serum creatinine level was 1.2 ± 0.4 mg/dl at POD 7, after which it gradually decreased. In 1 group A animal that received only 4×10^7 cells, the serum creatinine level increased gradually from POD 67, and it died of renal failure at POD 75 (Table 1).

Establishment of donor-specific unresponsiveness in long-surviving recipients. The long-surviving recipients have been studied serially over time. Impaired responses against donor cells were observed 2 months after transplantation, and almost complete nonresponsiveness was evident after 10 months (Figure 5A). In contrast, CD4⁺ T cell responsiveness against third-party alloantigens was mostly preserved in the monkey studied here. In order to determine the status of donor-specific tolerance in vivo, long-surviving recipients were challenged by donor or third-party skin allografting. Consistent with the in vitro results, third-party but not donor skin was rejected (Figure 5B).

Histological characterization of the long-surviving allografts. Renal tissues were obtained from some transplanted kidneys at PODs 75 and 810 or at necropsy. In monkeys in group A, the grafts retained well-preserved renal architecture without evidence of tubulitis or glomerulitis (Figure 6A), and blood vessels were free from intimal hyperplasia or thickening and, in the case of long-surviving animals, occasional inflammatory cells in the cell wall (Figure 6B). Histological examination of all grafts undergoing rejection in group E showed diffuse infiltration by mononuclear cells in the interstitium with tubulitis, loss of tubules, collaptic glomeruli (Figure 6C), and severe endothelialitis with intimal thickening of small arteries (Figure 6D).

Discussion

The present study documents, for the first time to our knowledge, that anergic cells generated ex vivo can suppress renal allograft rejection in nonhuman primates. In the long-surviving recipients,

with severe tubulitis, glomerulitis, and endothelialitis. In group A, only 1 animal died of acute renal failure due to cellular rejection, at POD 75. One animal died of uncontrolled bleeding after renal biopsy at POD 81. In 1 monkey, which died at POD 212, the serum creatinine level was elevated before death but the cause of death was not considered likely to have been classical acute or chronic rejection and might have been associated with hydronephrosis probably due to ureteral stenosis. There was no evidence of tubular infiltration, glomerular damage, or parenchymal necrosis in these latter 2 animals (data not shown).

Treatment with CP and inoculation of adequate numbers of the anergic cells are critical for the success of this regimen. Next we examined the effect of lymphocyte depletion by treatment with CP. In 6 monkeys so treated, the lymphocyte count was 200–800 cells/mm³ at the time of anergic cell inoculation (40–220 million cells were inoculated). In these monkeys, neutrophil and platelet counts were $1,540 \pm 470$ cells/mm³ and $37.4 \times 10^4 \pm 4.0 \times 10^4$ cells/mm³, respectively. In contrast, in animals of the control group not treated with CP (group D, $n = 2$), the lymphocyte count was $3,920 \pm 120$ cells/mm³. Although $9 \times 10^7 \pm 0.5 \times 10^7$ anergic cells were inoculated into these monkeys, they died of acute rejection at POD 27 and 28.

Table 1
Treatment and outcome of transplanted monkeys

Group	Origin of the stimulators in culture	mAbs added in the culture medium	Administration of CP	Lymphocyte cell count (/mm ³)	Number of inoculated cells ($\times 10^6$)	Survival (d)
A	Donor	anti-CD80/CD86 mAbs	(+)	662 ± 239	102 ± 67	75, ^{A,B} 81, ^C 212, ^D >410, >840, >880
B	(-)	(-)	(+)	550 ± 192	0	15, ^A 16, ^A 26, ^A 28, ^A 28 ^A
C	Third-party	anti-CD80/CD86 mAbs	(+)	718 ± 132	79 ± 22	28, ^A 33, ^A 41, ^A 60, ^A 73 ^A
D	Donor	anti-CD80/CD86 mAbs	(-)	3920 ± 120	90 ± 5	27, ^A 28 ^A
E	Donor	anti-CD86 mAbs	(+)	465 ± 150	155 ± 55	43, ^A 67, ^A 69, ^A 111 ^A
F	Donor	anti-CD80 mAbs	(+)	300	150	43 ^A

Values are expressed as mean \pm SD. All recipients were splenectomized. Numbers in Survival column represent values for each animal in the group. ^ADied of acute rejection; ^Breceived an inoculation of 4×10^6 cells; ^Cdied of bleeding after renal biopsy; ^Ddied of hydronephrosis due to urethral stenosis.

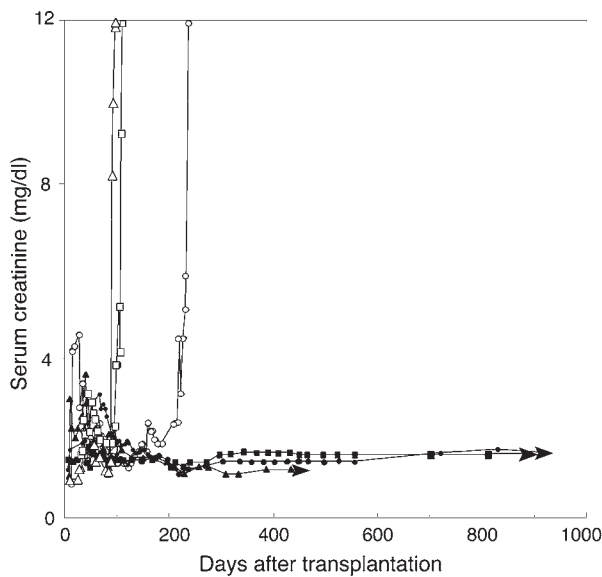


Figure 3 Renal function was determined by serum creatinine level (mg/dl) after transplantation in group A ($n = 6$). Solid arrowheads indicate continued survival without rejection.

donor-specific unresponsiveness was observed *in vitro*, and donor but not third-party skin was accepted, which suggests that allospecific tolerance had indeed been induced.

In MHC-mismatched rhesus monkeys, it is known that renal allografts are rejected within 10 days in the absence of immunosuppression (9, 10, 20). Long-term survival has occasionally been achieved after cessation of conventional immunosuppression. This seems to be more frequent in cases where recipients had been treated for an extended period prior to withdrawal (8, 21). In our study, recipients were treated with a low dose of CsA for only 2 weeks after transplantation, and all animals treated with CsA and CP died within 28 days after transplantation. One might suspect that the residual mAbs contained in the medium at the time the anergic cells were transferred could themselves have prolonged survival. However, this seems unlikely, as recipients treated with 0.25–0.5 mg/kg of anti-CD80/CD86 mAbs for 14 days after transplantation still rejected renal allografts (22). Therefore, in our study, long-term survival in half of the recipients seems likely to be dependent on the cultured, specifically anergic, cells. Although the inoculated cells may well have contained donor cells that survived in culture, it was demonstrated that cultured donor-type splenocytes alone could not inhibit alloproliferative responses *in vitro* (Figure 1C). Therefore, the donor-specific immunoregulatory activity in this study is most likely due to the anergized recipient T cell population and not to the presence of contaminating donor-type cells or residual antibody. Koenen et al. demonstrated that alloreactive T cells that were anergized by costimulation blockade with anti-CD86/CD40 mAbs in the presence of exogenous IL-15 were powerful suppressors (23). The suppressive activity of *ex vivo*-expanded CD4⁺ T cells against primary mixed lymphocyte reaction (MLR) *in vitro* was almost as powerful as that of the cultured cells that we report here. The suppressive activity of anergic cells depends on direct cell-cell contact but not on the release of inhibitory cytokines (15). Splenic T cells, tolerized to alloantigen *ex vivo* by coculturing with anti-CD80/CD86 mAbs, caused sec-

ondary MLR hyporesponsiveness when added at ratios of 1:1 to 1:10 anergic/responder cells (16). Thus, the frequency of alloreactive cells relative to inoculated regulatory cells is critical for the outcome. One plausible cause of the failure of tolerance induction in 1 monkey treated according to the full regimen may have been that there were insufficient numbers of inoculated anergic cells.

Recent studies have demonstrated a pivotal role for CD4⁺, particularly CD4⁺CD25⁺, regulatory T cells in the induction and maintenance of allograft tolerance (24, 25). Taylor et al. demonstrated that CD4⁺CD25⁺ regulatory T cells are required for *ex vivo* induction of tolerance to alloantigen via costimulatory blockade. Moreover, these CD4⁺CD25⁺ regulatory T cells suppressed the response to alloantigen (24). CD25⁺ cells represent a minor population (5–10%) of freshly isolated CD4⁺ T cells, and they are enriched in the cells inoculated here, which had been cocultured with donor cells in the presence of anti-CD80/CD86 mAbs. It is very likely that the CD25⁺ population is heterogeneous, i.e., containing CD4⁺ T cells that constitutively express CD25 and CD25[−] cytotoxic T cells that express CD25 after *in vitro* stimulation even under costimulation blockade. CD25[−] cells that acquire CD25 after *in vitro* activation are not anergic and can proliferate in response to alloantigen (26). Both would be present in the CD25⁺ population at the end of culture; thus, it is difficult to distinguish regulatory and effector T cells by the marker CD25. However, it is thought that CTLA-4 is associated with the inhibitory properties of CD4⁺CD25⁺ regulatory T cells (27, 28). In this study, CTLA-4 expression was consistently upregulated in CD4⁺CD25⁺ cells after culture compared with that in freshly isolated peripheral blood CD4⁺CD25⁺ cells. Accordingly, in our study, cultured cells of this phenotype did suppress alloproliferative responses in a donor-dominant fashion *in vitro*. Therefore, CD4⁺CD25⁺ regulatory T cells, which were responsive to the donor alloantigen, might have become dominant in the inocula used in the present study. If our assumption that the anergic cells have suppressive activity on rejection processes is true, continuing administration of high-dose CP after inoculation

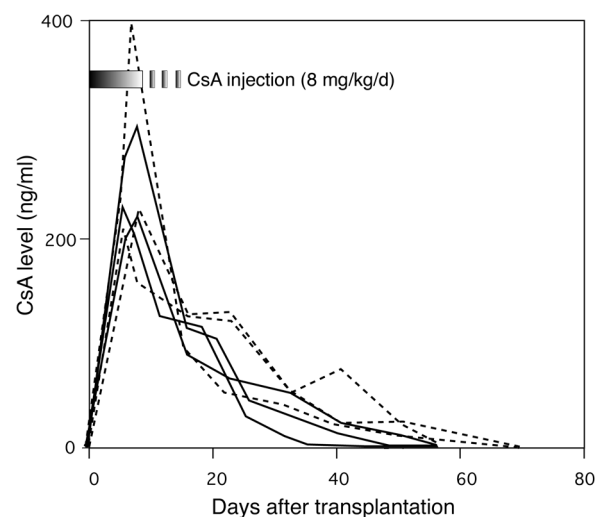


Figure 4 CsA whole blood levels (ng/ml) in recipients (group A). CsA (8 mg/kg) was injected intramuscularly daily from the day of operation to 7 days after transplantation and thereafter on days 9, 11, and 13. Solid lines, animals surviving more than 1 year ($n = 3$); dotted lines, animals dying within 1 year ($n = 3$).

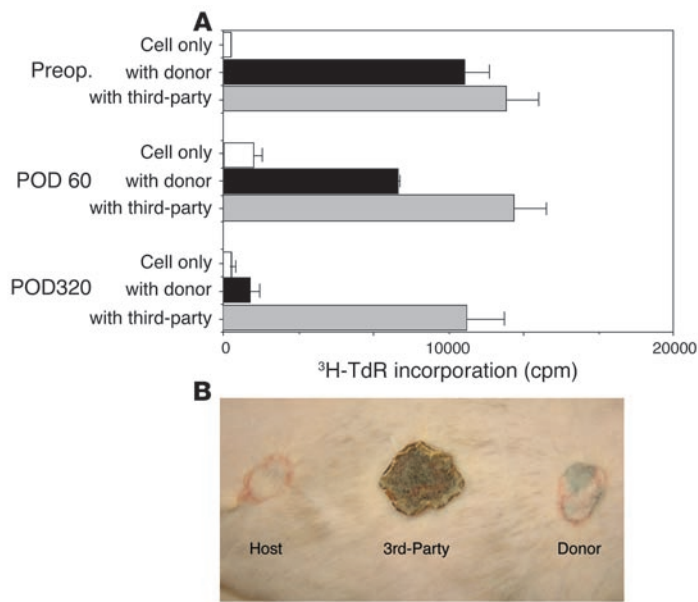


Figure 5

Assessment of the responses against alloantigens in tolerance-induced recipients. **(A)** Proliferative response of peripheral blood CD4⁺ T cells against alloantigens. Peripheral blood CD4⁺ T cells from a recipient with a long-surviving renal allograft were cocultured with irradiated donor (black bars) or third-party splenocytes (gray bars) preoperatively (Preop.) or 60 and 320 days after transplantation. After 7 days of culture, [³H]thymidine incorporation during the final 18 hours of culture was counted. Data are expressed as mean ± SD of triplicate samples. **(B)** Challenge with skin allografts. A long-surviving recipient was transplanted with autologous skin (host; autograft), donor skin (allograft), or third-party skin. Forty days after skin transplantation, the third-party skin was completely rejected, although both host and donor skin grafts remained intact, with no signs of rejection.

of the anergic cells may lead to a higher rate of rejection (29). To clarify these issues, further studies, such as those directed toward breaking the induction of tolerance, are needed.

Splenectomy might have profound effects on allograft rejection. We performed splenectomy in order to reduce the number of potentially alloreactive cells and provide a source of autologous cells for in vitro induction of donor-specific regulatory cells. However, human CD4⁺ regulatory T cells generated by costimulation blockade can be expanded in vitro in the presence of IL-15 (23). Therefore, it might be possible to obtain sufficient regulatory T cells from the peripheral blood, and not from spleen, for rejection treatment in humans. Even in nonhuman primates without splenectomy, it is to be expected that tolerance would still be induced, as reported in a previous article (8). In contrast, Kawai et al. reported that monkeys receiving nonmyeloablative regimens, but without splenectomy, developed alloantibody and rejected their allografts acutely or chronically (30). Blood vessels were free from intimal hyperplasia or thickening and did not even show the occasional inflammatory cell in the wall in our long-surviv-

ing animals. This suggests that chronic rejection was completely avoided. Additionally, the grafts retained well-preserved renal architecture without evidence of tubulitis or glomerulitis. In our study, the recipient monkeys were 2–3 years old, i.e., relatively young.

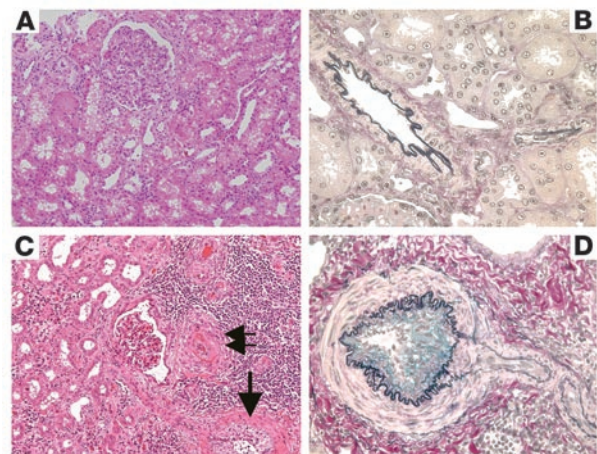
In clinical kidney transplantation, the adverse effect of splenectomy on mortality appeared to be more pronounced in younger than in older patients (31). Therefore, our regimen may have benefited from splenectomy. However, even when we can prepare sufficient T cells from a source other than spleen, splenectomy may be one option for achieving a better outcome with the protocol of ex vivo costimulation blockade.

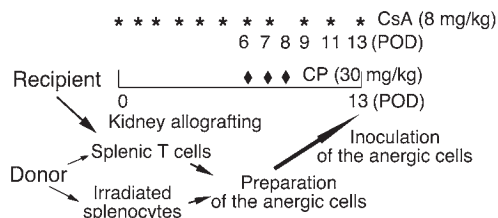
How is this tolerance maintained in vivo? CD4⁺ T cells from tolerant mice disable naive lymphocytes so that they cannot reject the graft in vivo (32). Also in vitro, coactivation of CD25⁺ cells with other cells depleted of regulatory T cells results in anergized CD4⁺ T cells that in turn inhibit the activation of conventional, freshly isolated CD4⁺ T helper cells (33). Once a state in which suppressor cells are dominant against alloreactive cells is established, it is probable that any naive, potentially alloreactive cells, newly generated by the thymus, would be inactivated in the grafts or even mediate suppressor activity themselves.

An optimal clinical protocol for induction of allograft tolerance without persistent immunosuppression needs to be developed. Experimentation in large-animal models is the best way to gain

Figure 6

Representative pathological findings of kidney allografts in groups A and E. **(A and B)** Histology of the surviving allograft on POD 810 in group A. **(A)** There was minimal cellular infiltrate in evidence around small arteries but no tubulitis, glomerulitis, or endothelialitis (H&E stain; magnification, ×200). **(B)** Medium and small-sized arteries with elastic fiber stain showed intact architecture without intimal hyperplasia (Movat pentachrome stain; magnification, ×400). **(C and D)** Histology of the rejected allograft on POD 67 in group E. Diffuse and marked cell infiltration occurred. **(C)** The small arteries showed acute endothelialitis with fibrinoid necrosis (double arrow) and chronic allograft vasculopathy with intimal fibrous thickening and cell infiltration (single arrow), which indicated that severe and prolonged rejection developed in the graft (H&E stain; magnification, ×200). **(D)** Chronic allograft vasculopathy developed in medium- and small-sized arteries (Movat pentachrome stain; magnification, ×400).



**Figure 7**

Schematic diagram of the regimen (group A). CsA (8 mg/kg/d) was administered intramuscularly on the days indicated by asterisks. CP (30 mg/kg) was administered intramuscularly at PODs 6, 7, and 8. During the operation, the spleen was removed from both donor and recipient. Splenic T cells from the recipient were cocultured with irradiated donor splenocytes for 13 days in the presence of anti-CD80/CD86 mAbs and injected into the recipient. No further immunosuppression was given thereafter.

sufficient experience to initiate ethically acceptable human trials. The protocol described here is very simple and easy to perform, requiring only a single adoptive transfer of anergic T cells prepared ex vivo to leukocyte-depleted recipients after renal transplantation. The safety and efficacy of this regimen makes it promising for clinical application to human transplantation.

Methods

Animals, MHC typing, and donor-recipient selection. Outbred male juvenile rhesus monkeys, *Macaca mulatta* (age 2–3 years, weighing 3–4 kg), seronegative for simian immunodeficiency virus and hepatitis B virus, were purchased from Hamri Co. All surgical procedures and postoperative care of the animals were in accordance with the Guiding Principles for Animal Experiments Using Nonhuman Primates (Primate Society of Japan) and were approved by the Juntendo University Subcommittee on Animal Research. Donor-recipient combinations and third-party animals were selected based on genetic nonidentity at both MHC class I and class II loci. Class I disparity was defined serologically and later established by 1-dimensional isoelectric focusing as previously described (34). T cell responsiveness of the recipient toward the donor was confirmed in vitro by the MLR assay. Each animal was tested against all potential donors so that responder pairs with the greatest degree of incompatibility for transplantation could be identified. In addition, disparity at the DRB loci was verified by denaturing gradient gel electrophoresis and direct sequencing of the second exon of DRB as previously described (35).

Antibodies. mAbs against human CD80 (2D10) (36) and CD86 (IT2.2) (37) were kind gifts of eBioscience Inc. Rhesus CD3-FITC (SP34), rhesus CD4-PE (L200), human CD20-PE (2H7), and rhesus CD14-PE (M5E2) were purchased from BD Biosciences – Pharmingen. CD25-FITC (M-A251) and CD152-PE (14D3) were from eBioscience Inc.

Preparation of anergic cells. The spleen was aseptically removed, mechanically minced, and suspended in AIM-V medium (Invitrogen Corp.) containing 1% autologous serum. Cell suspensions were filtered through nylon mesh and treated with Gey solution to remove red blood cells. T cells from the recipient were obtained after the cell suspensions were passed through a nylon column. To generate anergic cells, 2×10^8 recipient splenic T cells were cocultured with 2×10^8 30 Gy-irradiated donor splenocytes in the presence of anti-CD80/CD86 mAbs (10 μ g/ml each) in large dishes (catalog 430599; Corning Inc.) containing 50 ml of AIM-V medium supplemented with recipient serum. After a 6-day culture at 37°C in a humidified 5% CO₂ atmosphere, viable cells were collected and counted. These cells were restimulated with the same number of irradiated donor splenocytes in the pres-

ence of anti-CD80/CD86 mAbs for an additional 6 days. The cells were then washed 3 times and cultured in mAb-free medium for 1 more day. Viable cells were then collected and suspended in 50 ml of saline for intravenous administration to the recipients.

Mixed lymphocyte reaction. Peripheral blood was obtained from the monkeys, and PBMCs were prepared by centrifugation over Separate-L (Muto Pure Chemicals Co., Ltd.). CD4⁺ T cells were purified by negative selection with the Untouched CD4⁺ T cell Isolation Kit for untouched CD4⁺ T cells (Miltenyi Biotec). CD4⁺ T cells or the cultured anergic T cells (10^5 cells) prepared as described above were cocultured with the same number of 30 Gy-irradiated donor splenocytes in 96-well round-bottomed plates (catalog 3799; Corning Inc.) in the presence or absence of 10 μ g/ml anti-CD80/CD86 mAbs or 10 units of recombinant IL-2 (Shionogi & Co.). The cells were cultured for 3–7 days, then pulsed with 10 μ Ci of [³H]thymidine for the last 18 hours. The incorporated radioactivity was measured on a 1450 MicroBeta counter (PerkinElmer).

Renal transplantation. Renal transplantation was performed between histoincompatible donor-recipient pairs as determined by the MHC and MLR analysis described above. Donor and recipient animals were anesthetized with 1 mg/kg of intramuscular ketamine (Sankyo Inc.) and 1 mg/kg of xylazine (Bayer AG). Both donors and recipients were splenectomized during the operation. The donors were heparinized (100 U/kg) before the renal graft was harvested. Allografting was carried out using standard microvascular techniques to create an end-to-side anastomosis between the donor renal artery and the recipient distal aorta as well as the donor renal vein and the recipient vena cava. A primary ureteroneocystostomy was then created. Bilateral naive nephrectomy was completed prior to closure. Animals were treated with intravenous fluid for approximately 48 hours until oral intake was adequate, and Cefmetazone (Sankyo Inc.) was administered for 3 days as surgical antibiotic prophylaxis.

Treatments after transplantation. 8 mg/kg of CsA (Sandimmune; Novartis Pharma AG) in oil was administered intramuscularly to the recipients daily from the day of operation to 7 days after transplantation and thereafter on days 9, 11, and 13. EDTA blood samples were taken twice weekly to determine CsA levels using a radioimmunoassay after alcoholic extraction (BML Inc.). To deplete the recipient leukocytes, we administered 30 mg/kg of CP (Shionogi Inc.) intramuscularly 6–8 days after transplantation. The indicated number of anergic cells was intravenously inoculated on POD 13, and no further immunosuppression was given thereafter (Figure 7). The main regimen included administration of CP, intramuscular injection of CsA, and inoculation of the anergic cells against donor prepared in the presence of anti-CD80/CD86 mAbs (group A). The following regimens were also performed for comparison: group B ($n = 5$), no anergic cells were inoculated after transplantation; group C ($n = 5$), the inoculated cells were cocultured with third-party alloantigens; group D ($n = 2$), CP was not administered after transplantation; groups E ($n = 4$) and F ($n = 1$), only anti-CD86 mAb (group E) or anti-CD80 mAb (group F) was added to the culture. Serum creatinine, blood urea nitrogen, total protein, and hemoglobin levels, hematocrit, leukocyte count, and platelet count were determined 2 times a week until 3 months after transplantation and thereafter once every 2 weeks. To test for tolerance, we placed full-thickness skin grafts, consisting of ventral abdominal skin from donor or third-party monkeys, onto the dorsal upper back of the recipient. Grafts were evaluated daily by inspection.

Flow cytometry. Cells (10^5) were incubated with 1 μ g each of the indicated mAbs for 15 minutes at 4°C in PBS, washed twice, and analyzed on a FAC-Scan equipped with CellQuest software version 3.3 (BD). Expression of CTLA-4 was determined by intracytoplasmic staining after the cells were fixed with 2% formaldehyde and the membrane was permeabilized with 0.5% saponin (Sigma-Aldrich) and 2% BSA in PBS.



Histology. Renal biopsies were obtained from some animals for histological evaluation using a 20-gauge need-core device (Biopsy-Cut; Bard). Monkeys were euthanized at the time of renal failure as determined by progressively rising serum creatinine or if a weight loss of 15% over pretransplant body weight occurred, in accordance with American Association for Accreditation of Laboratory Animal Care (AAALAC) standards. Complete gross and histopathological analysis was done at necropsy on all monkeys sacrificed. For light microscopic examination of renal biopsy or autopsy samples, tissue was fixed in 10% buffered formalin and embedded in paraffin. H&E and PAS stains were performed for histological examination. Elastic fiber staining using Movat pentachrome was employed to confirm the vascular findings.

Statistical analysis. Data are expressed as mean \pm SD, and values from the 2 treatment groups were compared by the nonparametric Mann-Whitney *U* statistic, using StatView 4.5 for Macintosh (SAS Institute Inc.). Two-tailed values of $P < 0.05$ were considered significant.

Acknowledgments

This work was supported by grants-in-aid from the Ministry of Health and Welfare and 21st Century Center of Excellence Research and by a Research on Immunology, Allergy and Organ Transplantation grant.

Received for publication October 26, 2004, and accepted in revised form May 3, 2005.

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