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BRIEF REPORT

HLA-Mismatched Renal Transplantation without Maintenance Immunosuppression

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SUMMARY

Five patients with end-stage renal disease received combined bone marrow and kidney transplants from HLA single-haplotype mismatched living related donors, with the use of a nonmyeloablative preparative regimen. Transient chimerism and reversible capillary leak syndrome developed in all recipients. Irreversible humoral rejection occurred in one patient. In the other four recipients, it was possible to discontinue all immunosuppressive therapy 9 to 14 months after the transplantation, and renal function has remained stable for 2.0 to 5.3 years since transplantation. The T cells from these four recipients, tested *in vitro*, showed donor-specific unresponsiveness and in specimens from allograft biopsies, obtained after withdrawal of immunosuppressive therapy, there were high levels of P3 (FOXP3) messenger RNA (mRNA) but not granzyme B mRNA.

LONG-TERM RESULTS OF ORGAN TRANSPLANTATION REMAIN UNSATISFACTORY, mainly because of chronic rejection and complications associated with immunosuppressive medications.^{1,2} Immune tolerance, which has been achieved in animal models, might provide a means for avoiding both of these problems. However, the results of attempts to extend such studies from laboratory animals to humans have been disappointing.³⁻⁷

Tolerance of allografts has been induced in mice⁸ and larger animals⁹ by first transplanting hematopoietic stem cells from the prospective donor into the recipient, thereby creating a lymphohematopoietic chimera in which donor and recipient hematopoiesis coexist (“mixed chimera”). Using a nonmyeloablative perioperative regimen, we were able to induce mixed chimerism and tolerance of renal allografts in major-histocompatibility-complex–mismatched cynomolgus monkeys.¹⁰⁻¹³ Subsequently, we showed the clinical feasibility of this approach in recipients of HLA-mismatched kidneys.¹⁴⁻¹⁶ We have extended our work to HLA-mismatched donor–recipient combinations, and here we report stable renal-allograft function for 2.0 to 5.3 years after complete withdrawal of immunosuppressive drugs in four recipients.

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METHODS

PATIENTS

Five patients with end-stage renal disease, 22 to 46 years of age, were enrolled in this study. All had parent or sibling donors who were mismatched for one HLA haplo-

Table 1. Patient Characteristics and Results of Laboratory Tests.*

| Patient No. | Sex | Age yr | Original Disease | Pretransplantation PRA | Chimerism† | Discontinuation of Immunosuppressive Therapy | Kidney Survival days | Current Serum Creatinine Level mg/dl | Creatinine Clearance ml/min | Antidonor Alloantibody | Banff Score‡ |
|-------------|-----|--------|---------------------------|------------------------|--|--|----------------------|--------------------------------------|---|--------------------------------------|-------------------------|
| | | | | | Day 7 | Day 14 | Day 21 | | | | |
| 1 | F | 22 | Alport's syndrome | 4§ | T cell 5.2%, B cell 4.6%, monocytes 44.5%, GRN 90.6% | Undetectable | Day 240 | 1.2 | 61 | None | i0t0v0g0; ci1ct1cv1cg0 |
| 2 | M | 22 | MPGN type 1 | 0 | T cell 3%, GRN 60% | Undetectable | Day 422 | 1.5 | 75 | None | i0t0v0g0; ci1ct0cv0cg0 |
| 3 | M | 39 | Polycystic kidney disease | 52¶ | T cell 25.6%, monocytes 25.6%, GRN 46% | Undetectable | Not discontinued | 1.0 | Underwent second kidney transplantation | | |
| 4 | M | 25 | Alport's syndrome | 0 | T cell 1.8%, monocytes 40%, GRN 98.1% | Undetectable | Day 244 | 1.5 | 60 | New anti-donor HLA class II antibody | i0t0v0 g1; ci0ct0cv0cg1 |
| 5 | M | 46 | Polycystic kidney disease | 0 | T cell 15%, monocytes 19%, GRN 86.5% | T cell 0.1%, B cell 46%, monocytes 19%, GRN 9.9% | Day 272 | 1.8 | 71 | None | i0t0v0 g0; ci0ct0cv0cg0 |

* GRN denotes granulocytes, MPGN membranoproliferative glomerulonephritis, and PRA panel-reactive antibodies.
 † The percentages of donor chimerism were measured by means of flow-cytometric analysis. To verify the results, polymerase-chain-reaction (PCR) assays for variable short tandem repeats were used. For these latter assays, T-cell and myeloid-cell subpopulations were sorted by means of magnetic beads before PCR assays.
 ‡ The Banff scoring system is used for the grading and classification of short- and long-term changes that occur in the interstitium, tubules, vessels, and glomeruli of a kidney transplant. Scores range from 0 to 3, with higher scores indicating more severe changes. Banff scores are given for the most recent biopsy specimens obtained.
 § This result is class II, not donor-specific.
 ¶ This result is class I, not donor-specific.

type (Table 1). (For detailed HLA types, see Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org.)

CONDITIONING REGIMEN

The conditioning regimen consisted of 60 mg of cyclophosphamide per kilogram of body weight per day administered intravenously on days -5 and -4 with respect to transplantation; 0.6 mg per kilogram per dose of humanized anti-CD2 monoclonal antibody (MEDI 507, MedImmune) on days -1, 0, and 1 (after a test dose of 0.1 mg per kilogram on day -2); and 5 mg of cyclosporine A (Novartis Pharmaceuticals) per kilogram intravenously and thymic irradiation (700 cGy) on day -1. Hemodialysis was performed before and 14 hours after each dose of cyclophosphamide was administered. Kidney transplantation was followed by intravenous infusion of donor bone marrow. Oral cyclosporine A (Neoral) was administered postoperatively at a dose of 8 to 12 mg per kilogram per day, with target trough blood levels of 250 to 350 ng per milliliter; the dose was tapered and discontinued over a period of several months.

The protocol was modified after treatment of the third patient (see the Results section), to include administration of rituximab, 375 mg per square meter of body-surface area on days -7 and -2; and prednisone, 2 mg per kilogram per day starting on the day of transplantation with tapering over the next 10 days.

Written informed consent was obtained from all donors and recipients. All treatment protocols were approved by the institutional review board of Massachusetts General Hospital and developed in collaboration with the Immune Tolerance Network of the National Institutes of Health.

IN VITRO IMMUNOLOGIC ASSAYS

Mixed lymphocyte reaction and cell-mediated lympholysis assays of the recipient's peripheral-blood mononuclear cells were performed sequentially with the use of frozen cells obtained before transplantation to compare responses to the donor's cells before and after transplantation.¹⁶ Direct cross-match of recipient serum on donor lymphocytes was performed by measurement of cytotoxicity, flow-cytometric assays, or both. Antidonor HLA antibodies were detected by means of enzyme-linked immunosorbent

assay (ELISA) on purified HLA antigens (One Lambda).

FLOW-CYTOMETRIC ANALYSIS

Flow-cytometric analysis of blood was performed with the use of an LSR II flow cytometer (BD Biosciences), as described elsewhere.¹⁶ Chimerism was assessed with the use of class I, HLA-specific monoclonal antibodies (One Lambda) to detect donor and recipient leukocytes and also by means of polymerase-chain-reaction (PCR) assays of variable short tandem repeats.^{14,17}

MEASUREMENT OF INTRARENAL mRNA LEVELS

Total RNA was isolated from renal-biopsy specimens and reverse transcribed to complementary DNA (cDNA). Oligonucleotide primers and fluorescent probes were designed and synthesized for measurement of mRNA for FOXP3, granzyme B, and 18S ribosomal RNA (rRNA) (see Table 2 of the Supplementary Appendix). Measurement of mRNA levels was performed with preamplification-enhanced real-time quantitative PCR assays (see the PCR protocol in the Supplementary Appendix), with transcript levels normalized to 18S rRNA copy numbers and log-transformed.¹⁸

SURVEILLANCE BIOPSIES

Surveillance biopsies were performed at the time of transplantation and at regular intervals thereafter (Fig. 1). Biopsy specimens were processed by means of light, electron, and immunofluorescence microscopy including C4d.

RESULTS

CLINICAL COURSE

The nadir of absolute neutrophil counts (mean [±SD] value, 36±20 per cubic millimeter) occurred about 1 week after the infusion of donor bone marrow cells; an absolute neutrophil count of more than 500 per cubic millimeter was reached within 14±3 days. Multilineage blood-cell chimerism was detectable in all five recipients by means of flow-cytometric analysis on day 7 (Table 1) but not after day 21, even with the use of PCR assays to detect polymorphisms of short tandem repeats.

Patient 1 was a 22-year-old woman who had received a renal transplant from her father in 1993 at 12 years of age for end-stage renal disease due to Alport's syndrome. Her course was

complicated by human papillomavirus infections, which caused disabling disseminated warts, requiring reduction of immunosuppressive therapy, which in turn resulted in rejection of the graft. She received a combined kidney and bone marrow transplant from her mother in September 2002. Consistently good renal function ensued, with no evidence of rejection or graft-versus-host disease. The dose of cyclosporine was gradually tapered (Fig. 1A), and treatment with the drug was discontinued in May 2003 (on day 240). The disseminated warts disappeared. Allograft function has remained stable (current creatinine level, 1.2 mg per deciliter [106.1 μ mol per liter]; creatinine clearance, 61 ml per minute) for more than 5.3 years, with no antidonor alloantibodies detected by means of either ELISA or flow-cytometric cross-match assays. The surveillance biopsy specimen obtained on day 1135 showed no infiltrate (Fig. 1B) and no C4d deposition, and the results of electron microscopy were normal.

Patient 2 was a 22-year-old man with end-stage renal disease due to membranoproliferative glomerulonephritis. He received a combined kidney and bone marrow transplant from his father in June 2003. The early postoperative course was complicated by the capillary leak syndrome, which was treated with corticosteroids and hemofiltration. Persistent elevation of the patient's serum creatinine level prompted a kidney biopsy on day 45. The biopsy specimen showed deposition of C4d and neutrophils in peritubular capillaries, but no antidonor HLA alloantibodies were detected in his serum by means of either direct cross-match or ELISA. He was treated for suspected humoral rejection with plasmapheresis and intravenous immunoglobulin; cyclosporine was discontinued (Fig. 1A), and treatment with tacrolimus and mycophenolate mofetil was begun. Renal function recovered, and all immunosuppressive medications were tapered and then discontinued on day 422. Allograft function has remained stable for more than 4.5 years (Fig. 1A); the current creatinine clearance is 75 ml per minute (Table 1). No antidonor alloantibodies have been detected. Protocol biopsy specimens obtained on days 92, 265, 364, 735, 787, and 1087 showed only transient mononuclear-cell infiltrates on days 735 and 787. No C4d deposition was detected after day 92, and there were no infiltrates and mini-

Figure 1 (facing page). Clinical Course of Patients with Tolerance of Kidney Transplants.

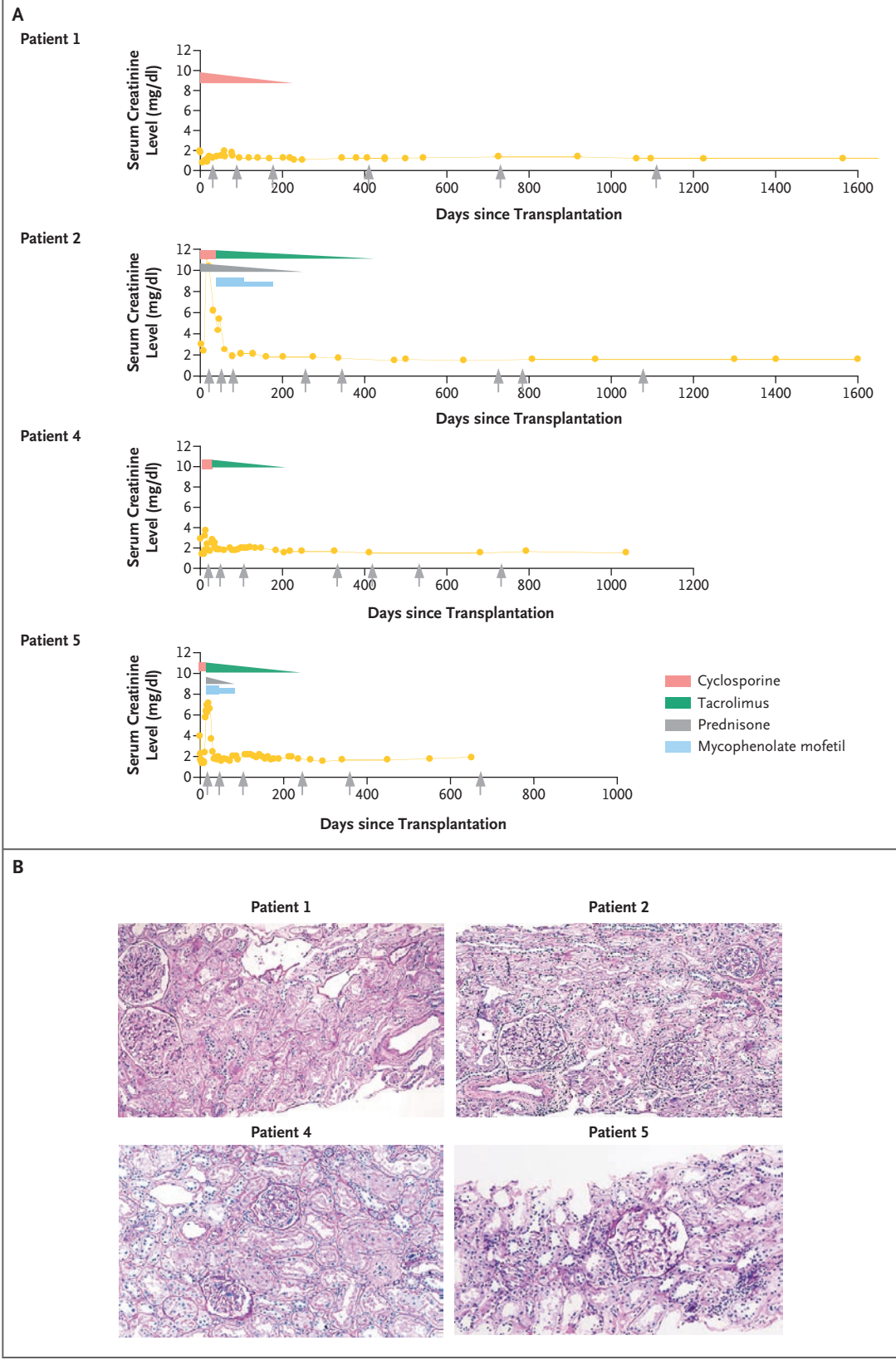
In Panel A, the serial creatinine levels in Patients 1, 2, 4, and 5 are indicated in yellow, and the withdrawal of immunosuppressive medications is shown as tapering bars above each creatinine curve. Arrows indicate the time at which allograft-biopsy specimens were obtained from these four patients. To convert the values for creatinine to micromoles per liter, multiply by 88.4. Panel B shows the recent protocol biopsy specimens from each of these four patients (obtained on days 1135, 1087, 731, and 666, respectively) (periodic acid–Schiff). The findings in all of the specimens were within normal limits, except for the specimen from Patient 4, which showed segmental glomerular basement membrane duplication and C4d deposition (see Fig. 1 in the Supplementary Appendix for representative glomeruli from protocol biopsy specimens).

mal fibrosis in the biopsy specimen obtained on day 1087 (Fig. 1B).

Patient 3 was a 39-year-old man with end-stage renal disease due to polycystic kidney disease. He had antibodies that were reactive in an ELISA assay to 52% of a panel of different HLA antigens (panel-reactive antibodies). Despite this high level of panel-reactive antibodies, he had a negative direct cross-match with his sister, from whom he received a combined bone marrow and kidney transplant in October 2003. Acute humoral rejection with C4d deposits in the kidney biopsy developed on day 10. Antidonor HLA class II antibodies were detected in his serum, and he was treated with plasmapheresis, intravenous antithymocyte globulin (Thymoglobulin, Genzyme), intravenous immune globulin, and rituximab. The patient's renal function did not recover, and hemodialysis was resumed 5 months after transplantation. He received a second kidney transplant, from his cousin, in August 2004, and he has had stable renal function while receiving conventional immunosuppressive therapy since then.

PROTOCOL MODIFICATION

After this experience with Patient 3, the protocol was modified, with the consent of the Immune Tolerance Network, the data and safety monitoring board, and our institutional review board, to include two doses of rituximab before transplantation (375 mg per square meter on days -7 and -2) to prevent humoral rejection and prednisone (2 mg per kilogram per day starting on the day of transplantation with tapering over the next 10



days) to mitigate the capillary leak syndrome. Pretransplantation sensitization, as determined by the presence of panel-reactive antibodies, was made an exclusion criterion for enrollment.

Patient 4 was a 25-year-old man with end-stage renal disease due to Alport's syndrome who received a combined kidney and bone marrow transplant from his mother in February 2005, under the modified protocol. The immunosuppressive therapy was switched from cyclosporine to tacrolimus on day 30 because of acute nephrotoxic effects of cyclosporine. Otherwise, the postoperative course was uneventful, with only minor evidence of the capillary leak syndrome. The dose of tacrolimus was tapered, and the drug was discontinued on day 244. Low levels of antidonor HLA class II antibodies in the peripheral blood were detected 2 months later, and C4d deposits appeared in four subsequent biopsy specimens of the renal allograft, but the creatinine level has remained stable at 1.5 mg per deciliter (132.6 μmol per liter) (Fig. 1A and Table 1), and there has been no proteinuria. Protocol biopsy specimens obtained on days 328 and 412 were histologically normal, despite C4d deposits in peritubular capillaries. Biopsy specimens obtained on days 542 and 731 (Fig. 1B) showed mild segmental duplication in the glomerular basement membrane, with no worsening during the 6 months between these biopsies (i.e., duplication was seen in 36% of glomeruli on day 542 and 26% of glomeruli on day 731).

Patient 5 was a 46-year-old man with end-stage renal disease due to polycystic kidney disease, who received a combined kidney and bone marrow transplant from his sister in January 2006, with the use of the modified protocol. The capillary leak syndrome developed on day 12, at which time antibodies reactive to donor HLA antigens appeared, and diffuse deposits of C4d were seen in the renal-biopsy specimen. He was treated with thymoglobulin, plasmapheresis, and rituximab. Cyclosporine was discontinued, and tacrolimus and mycophenolate mofetil were begun. The serum creatinine level decreased to 1.8 mg per deciliter (159.1 μmol per liter) by day 35, and antidonor antibodies disappeared. Subsequent biopsy specimens, obtained on days 91, 234, 360, and 666 (Fig. 1B), showed no C4d and no signs of rejection. Glomeruli and peritubular capillaries were normal on electron microscopy. The doses of tacrolimus and mycophenolate

Figure 2 (facing page). In Vitro Assays for Tolerance of Kidney Transplantation.

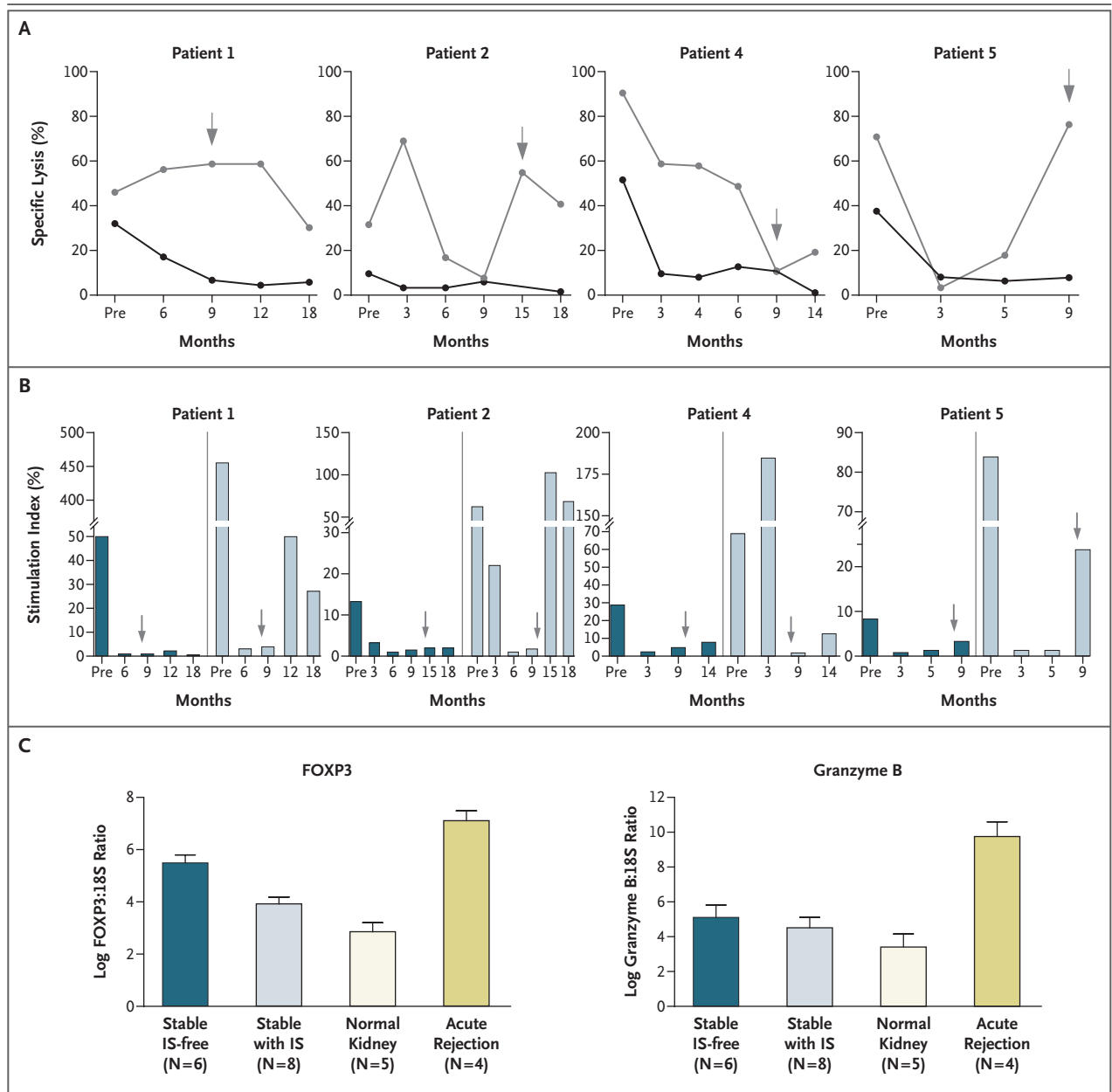
Sequential cell-mediated lympholysis assays in each patient against the donor (black circles) and third-party persons (gray circles) showed specific unresponsiveness to the donor at most post-transplantation time points tested (Panel A). Sequential assays of mixed-lymphocyte reactions in each patient against the donor (dark-blue bars) and third-party persons (light-blue bars) showed specific unresponsiveness to the donor, as indicated by return of anti-third-party, but not antidonor, responses after discontinuation of immunosuppressive therapy (Panel B). Arrows indicate the time of complete discontinuation of immunosuppressive therapy. Panel C shows intragraft levels of mRNA in renal allografts. Total RNA was isolated from renal-allograft biopsy specimens and reverse transcribed to cDNA, and levels of mRNA were measured with the use of pre-amplification enhanced real-time quantitative polymerase-chain-reaction assays. A total of 23 biopsy specimens were examined for intragraft levels of FOXP3 mRNA, granzyme B mRNA, and housekeeping gene 18S ribosomal RNA (18S rRNA). Of the 23 biopsy specimens, 6 were obtained from four patients with stable renal-allograft function who were not receiving immunosuppressive therapy (IS) (stable IS-free group). Eight biopsy specimens were obtained from eight patients with stable renal-allograft function and normal protocol biopsy results; these patients were receiving maintenance immunosuppressive drug therapy comprising tacrolimus and mycophenolate mofetil (stable-with-IS group). Five biopsy specimens were obtained from five kidney donors (normal-kidney group), and four biopsy specimens were obtained from four patients with biopsy-confirmed acute rejection (acute-rejection group). The mRNA copies were normalized with the use of 18S rRNA copies and log-transformed. The log-transformed mean (\pm SE) ratio of FOXP3 mRNA copies to 18S rRNA copies was 5.48 \pm 0.31 in the stable IS-free group, 3.90 \pm 0.27 in the stable-with-IS group, 2.85 \pm 0.34 in the normal-kidney group, and 7.09 \pm 0.38 in the acute-rejection group. The log-transformed mean ratio of granzyme B to 18S rRNA was 5.12 \pm 0.69 in the stable IS-free group, 4.53 \pm 0.59 in the stable-with-IS group, 3.41 \pm 0.75 in the normal-kidney group, and 9.76 \pm 0.84 in the acute-rejection group. Pre denotes pretransplantation.

mofetil were reduced, and the drugs were discontinued completely on day 272. Renal function has remained stable for more than 2.0 years (Fig. 1A and Table 1).

IN VITRO STUDIES

Post-Transplantation Assays

Assays for functions mediated by CD8+ and CD4+ T cells were performed when sufficient numbers of peripheral-blood T cells were available. These assays showed patterns consistent



with specific unresponsiveness to donor antigens in cell-mediated lympholysis (CD8+) (Fig. 2A) and mixed-lymphocyte reactions (CD4+) (Fig. 2B).

Intragraft mRNA Levels

Levels of mRNA for transcription factor FOXP3, a marker for regulatory T cells,¹⁹ and for cytotoxic attack protein granzyme B (released by CD8+ T cells) were measured in 23 renal-allograft biopsy specimens. Six of these specimens were obtained from the four patients who were not

receiving immunosuppressive therapy (the stable IS-free group), eight were obtained from eight patients with stable renal-allograft function and normal protocol biopsy specimens while they were receiving maintenance immunosuppressive therapy (tacrolimus and mycophenolate mofetil) (the stable-with-IS group), five were obtained from five normal kidney donors (the normal-kidney group), and four were obtained from patients with biopsy-confirmed acute rejection (the acute-rejection group).

Intra-graft levels of FOXP3 mRNA were about six times higher in the stable IS-free group than in the stable-with-IS group, whereas the granzyme B mRNA levels were similar in the two groups (Fig. 2C). Intra-graft levels of mRNA for granzyme B and FOXP3 were highest in the acute-rejection group and lowest in the normal-kidney group (for additional data, see the Supplementary Appendix).

DISCUSSION

On the basis of the hypothesis of Starzl and Demetris²⁰ regarding the interrelationship between chimerism and tolerance, there have been previous attempts to induce allograft tolerance in clinical kidney transplantation by infusing donor bone marrow into recipients of solid organ transplants receiving conventional immunosuppressive therapy.^{21,22} This approach, however, has led to very low levels of chimerism, generally detectable only by means of PCR, and subsequent withdrawal of immunosuppressive therapy has seldom been accomplished. Combined kidney and bone marrow transplantation has been attempted with the use of conditioning with total lymphoid irradiation by Millan and colleagues⁵; transient mixed chimerism developed in three of four recipients, but rejection occurred when withdrawal of immunosuppressive therapy was attempted.

In this study of combined HLA-mismatched bone marrow and kidney transplantation in five patients, we were able to discontinue all immunosuppressive therapy in four patients, and they have retained the graft and maintained stable renal function for 2.0 to 5.3 years after the transplantation (i.e., 1.2 to 4.6 years after complete withdrawal of immunosuppressive therapy).

The mechanism responsible for stable graft function without exogenous immunosuppressive therapy in these four recipients remains under investigation. In studies in mice, the immune tolerance that follows the induction of chimerism by bone marrow transplantation has been associated with elimination of cells in the thymus that are reactive to donor antigen (called "central deletion"). The specific loss of *in vitro* reactivity of the recipient's T cells against the donor's cells in all four of these patients (Fig. 2A and 2B) is consistent with this mechanism, but several other mechanisms are also possible.

By contrast with the permanent chimerism that can be achieved with nonmyeloablative regi-

mens in patients with malignant conditions or other diseases, the chimerism detected in monkeys^{10,12,13} and in the recipients described here has been transient. Although nonmyeloablative conditioning has achieved long-term chimerism when it has been used for the treatment of hematologic malignant conditions in humans, this kind of conditioning has generally been available only for HLA-matched donor–recipient combinations, since graft-versus-host disease has been a major limitation in the use of HLA-mismatched donors. In the present study, transient chimerism was achieved without the development of graft-versus-host disease, despite HLA mismatches between the donor and the recipient.

Evidence from our studies in monkeys¹² suggests that the mechanism of tolerance may switch from central deletion to a peripheral mechanism that may include regulatory T cells. After this switch, the kidney allograft may be essential to the maintenance of the tolerant state.²³ High levels of FOXP3+, a marker of regulatory T cells, without a concomitant inflammatory response (as indicated by decreased granzyme B expression) in the renal allografts of these patients suggest such a regulatory mechanism, and they may indicate active involvement of intra-graft regulatory T cells for the maintenance of unresponsiveness, as has been shown in mice.²⁴

We did not anticipate the early development of antidonor antibodies, since all of the patients initially received maintenance regimens that suppress T cells, and since anti-HLA antibody production is thought to be a T-cell–dependent response.²⁵ This complication had not been seen in our studies involving nonhuman primates,¹⁰ nor in the HLA-identical patients treated with our previous nonmyeloablative regimen for multiple myeloma.¹⁶ As a result of this finding and because of the possibility that B cells might have been activated during the capillary leak syndrome, which occurred during the second post-transplantation week in all patients, we modified the protocol to include B-cell depletion with the use of rituximab before the transplantation procedures. Since the capillary leak syndrome is usually reversed by corticosteroids,²⁶ we added a short course of corticosteroids during the post-transplantation period.

The fourth patient had a low titer of antidonor HLA class II antibodies 2 months after complete withdrawal of immunosuppressive medications, and there were C4d deposits in the allograft,

with segmental duplication of the glomerular basement membrane in some glomeruli (see Fig. 1 in the Supplementary Appendix). The clinical significance of C4d deposition in the absence of other evidence of rejection remains unclear.^{27,28} Since the minor histologic changes in this patient have not progressed and there is no other evidence of rejection, we have chosen an approach of watchful waiting. Our observations show that stable graft function after planned, complete withdrawal of immunosuppressive drugs is feasible in recipients of HLA-mismatched grafts.

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No potential conflict of interest relevant to this article was reported.

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