BRIEF REPORT

Tolerance and Chimerism after Renal and Hematopoietic-Cell Transplantation

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SUMMARY

We describe a recipient of combined kidney and hematopoietic-cell transplants from an HLA-matched donor. A post-transplantation conditioning regimen of total lymphoid irradiation and antithymocyte globulin allowed engraftment of the donor's hematopoietic cells. The patient had persistent mixed chimerism, and the function of the kidney allograft has been normal for more than 28 months since discontinuation of all immunosuppressive drugs. Adverse events requiring hospitalization were limited to a 2-day episode of fever with neutropenia. The patient has had neither rejection episodes nor clinical manifestations of graft-versus-host disease.

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Immune tolerance of organ transplants has been induced in laboratory animals when persistent mixed blood and immune-cell chimerism has been achieved by infusing hematopoietic cells from the organ donor before or after transplantation of the organ.[1-3] The continued presence of the organ donor's immune cells in the recipient's thymus and peripheral lymphoid tissue promotes and maintains immune tolerance by eliminating T-cell clones that can react to alloantigens of the graft.[1-3]

We have attempted to achieve persistent mixed chimerism and tolerance in humans after transplantation of combined HLA-matched kidney and hematopoietic cells, using a low-intensity conditioning regimen of total lymphoid irradiation and antithymocyte globulin. This regimen can induce tolerance of organ allografts in laboratory animals.[4-6] It also provides protection against graft-versus-host disease when used in patients with hematologic malignant conditions who are given HLA-matched hematopoietic-cell transplants.[7] Total lymphoid irradiation can facilitate tolerance of kidney transplants in some patients without the administration of donor hematopoietic cells.[8]

We enrolled six patients in a study in which the conditioning regimen was given for 10 days after kidney transplantation, and cryopreserved donor cells were infused thereafter. The first patient is described here.

CASE REPORT

The patient was a 47-year-old white man with end-stage renal disease of unknown origin at the time of preemptive kidney transplantation. The kidney donor was his 49-year-old brother, who shared the HLA-type A1,26;B38,51;BW4;DRB1*04,12 with the patient. On the day of kidney transplantation (day 0), the patient received the

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first of five daily injections of rabbit antithymocyte globulin (1.5 mg per kilogram of body weight), and on day 1 he received the first of 10 doses of 80 cGy each of total lymphoid irradiation. Treatment with cyclosporine was initiated on day 0, with a target whole-blood trough level of 350 to 400 ng per milliliter. Prednisone was administered as premedication for antithymocyte globulin injections and was discontinued on day 10. The patient was discharged from the hospital on day 6, and he completed total lymphoid irradiation in the outpatient clinic on day 14. An intravenous infusion of cryopreserved donor cells was administered in the clinic immediately thereafter. Donor mononuclear cells had been mobilized into the peripheral blood by the administration of granulocyte colony-stimulating factor. They were highly enriched for CD34+ hematopoietic progenitor cells with the use of an immunomagnetic-bead column. A dose of 1×10^6 CD3+ T cells from the column effluent was injected with 8×10^6 enriched CD34+ progenitor cells per kilogram.

Mycophenolate mofetil (1000 mg twice a day) was administered for 1 month after the intravenous injection of donor cells. The patient was hospitalized on day 21 because of fever with neutropenia, and was discharged on day 23. Blood cultures were negative, but the fever rapidly resolved after antibiotics were administered. The patient had no further hospitalizations. Two kidney-biopsy specimens were obtained according to an outpatient protocol at 3 and 12 months. Cyclosporine was gradually tapered and was discontinued 6 months after the transplantation. During those 6 months, there were neither rejection episodes nor clinical manifestations of acute or chronic graft-versus-host disease, and the chimerism was persistent. The patient returned to work about 3 months after the transplantation, and graft biopsy specimens showed normal kidney tissue; no cellular infiltrates were seen. Antihypertensive medications were withdrawn shortly after the discontinuation of cyclosporine. The patient remains in good health 34 months after transplantation.

METHODS

CONDITIONING

Total lymphoid irradiation (irradiation of the supradiaphragmatic lymph nodes and thymus and the subdiaphragmatic lymph nodes and spleen) was performed as described previously.[9] Rabbit antithymocyte globulin (Thymoglobulin, Genzyme) was given intravenously, starting with an intraoperative injection. The patient received prophylactic medications against fungal, bacterial, and viral infections. The protocol was approved by the institutional review board at Stanford University School of Medicine, and all the patients and donors provided written informed consent.

DONOR CELLS

Six weeks before transplantation, the donor received a 5-day course of subcutaneous injections of granulocyte colony-stimulating factor at a dose of 16 mg per kilogram per day, and mononuclear cells were harvested by means of leukopheresis. CD34+ cells were enriched with the use of an Isolex column (Baxter) and cryopreserved until they were infused.

ASSESSMENT OF CHIMERISM

Chimerism was determined by means of DNA genotyping of simple sequence-length polymorphic markers that encode short tandem repeats, as described previously.[10] Chimerism was assessed by analysis of T cells, B cells, natural killer cells, and granulocytes after enrichment of blood mononuclear cells on immunomagnetic beads (Dynabeads, Dynal) coated with monoclonal antibodies against CD3, CD19, CD56, and CD15, respectively.

IMMUNOFLUORESCENCE STAINING AND ANALYSIS OF T-CELL SUBGROUPS

Blood mononuclear cells were stained with fluorochrome-conjugated monoclonal antibodies against CD3, CD4, CD8, CD62L, CD45RA, CD45RO (BD Pharmingen), and $V_\alpha 24$,$V_\beta 11$ (Beckman Coulter).[11] Four-color analyses were performed by means of flow cytometry with the use of standard techniques and equipment (LSR and FACS Vantage cytometers, BD Biosciences).

T-CELL–RECEPTOR EXCISION CIRCLE ANALYSIS

Sorted subgroups of T cells obtained by means of flow cytometry were cryopreserved and sent to the Human Vaccine Institute Immune Reconstitution Core Facility at Duke University for T-cell–receptor excision-circle (TREC) analysis with the use of a polymerase-chain-reaction–based assay.[12]

T-CELL RESPONSES TO ANTIGENS

The mixed leukocyte reaction was performed by culturing peripheral-blood mononuclear cells as responder cells with irradiated allogeneic mono-

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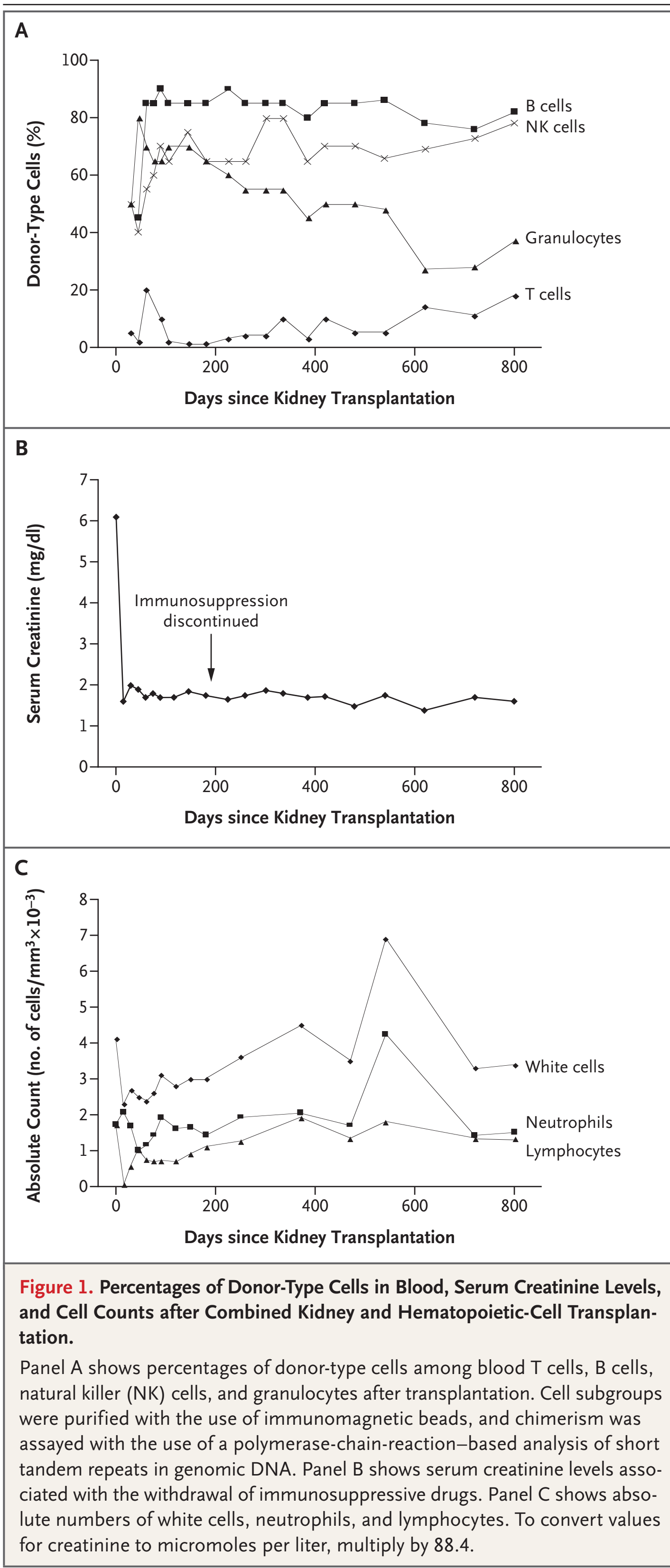

Figure 1. Percentages of Donor-Type Cells in Blood, Serum Creatinine Levels, and Cell Counts after Combined Kidney and Hematopoietic-Cell Transplantation.

Panel A shows percentages of donor-type cells among blood T cells, B cells, natural killer (NK) cells, and granulocytes after transplantation. Cell subgroups were purified with the use of immunomagnetic beads, and chimerism was assayed with the use of a polymerase-chain-reaction–based analysis of short tandem repeats in genomic DNA. Panel B shows serum creatinine levels associated with the withdrawal of immunosuppressive drugs. Panel C shows absolute numbers of white cells, neutrophils, and lymphocytes. To convert values for creatinine to micromoles per liter, multiply by 88.4.

nuclear cells or purified dendritic cells as stimulator cells and measuring ^{3}H-thymidine incorporation.[13,14] Dendritic cells were purified with the use of metrizamide gradients.[15] Stimulation of mononuclear cells with tetanus toxoid and influenza virus antigens has been described previously.[13,14] Concentrations of interleukin-2 and interferon-γ in culture supernatants were measured with the use of commercial kits (BD Pharmingen).

RESULTS

Figure 1A shows that mixed chimerism developed during the first month after transplantation and persisted until the last analysis, according to testing of short tandem repeats of DNA from purified white-cell subgroups. B cells and natural killer cells in blood contained the highest percentages of donor cells (70 to 85%). Granulocytes ranged between 30 and 70%. The lowest levels of chimerism were detected among T cells, and these levels remained in the range of 5 to 20% for more than 2 years after the transplantation. The level of serum creatinine decreased from about 6 mg per deciliter (530 μmol per liter) before transplantation to a stable level that has been below 2 mg per deciliter (177 μmol per liter) for more than 2 years after the transplantation (Fig. 1B). Immunosuppressive drugs were discontinued 6 months after the transplantation.

There was transient neutropenia and prolonged lymphopenia (Fig. 1C). The absolute number of CD8+ T cells approached pretransplantation levels within 3 months after transplantation and remained above 300 cells per cubic millimeter thereafter (Fig. 2A). The absolute number of CD4+ T cells remained considerably reduced during the first year, however (Fig. 2A). The percentages of naive, central memory, effector memory, and effector T cells were also monitored (Fig. 2B).[16,17] The percentage of naive CD4+ T cells decreased from 40% before conditioning to 10 to 20% for at least 2 years after conditioning (Fig. 2C). A rapid decrease in the percentage of naive CD8+ T cells after conditioning was followed by a rapid increase, and 3 months after transplantation, the naive CD8+ T-cell population had exceeded pretransplantation levels (Fig. 2D).

Recent emigrants from the thymus to the peripheral blood contain DNA fragments of TRECs.[11] Figure 2E shows that in healthy subjects, the number of TRECs in purified naive CD4+ and

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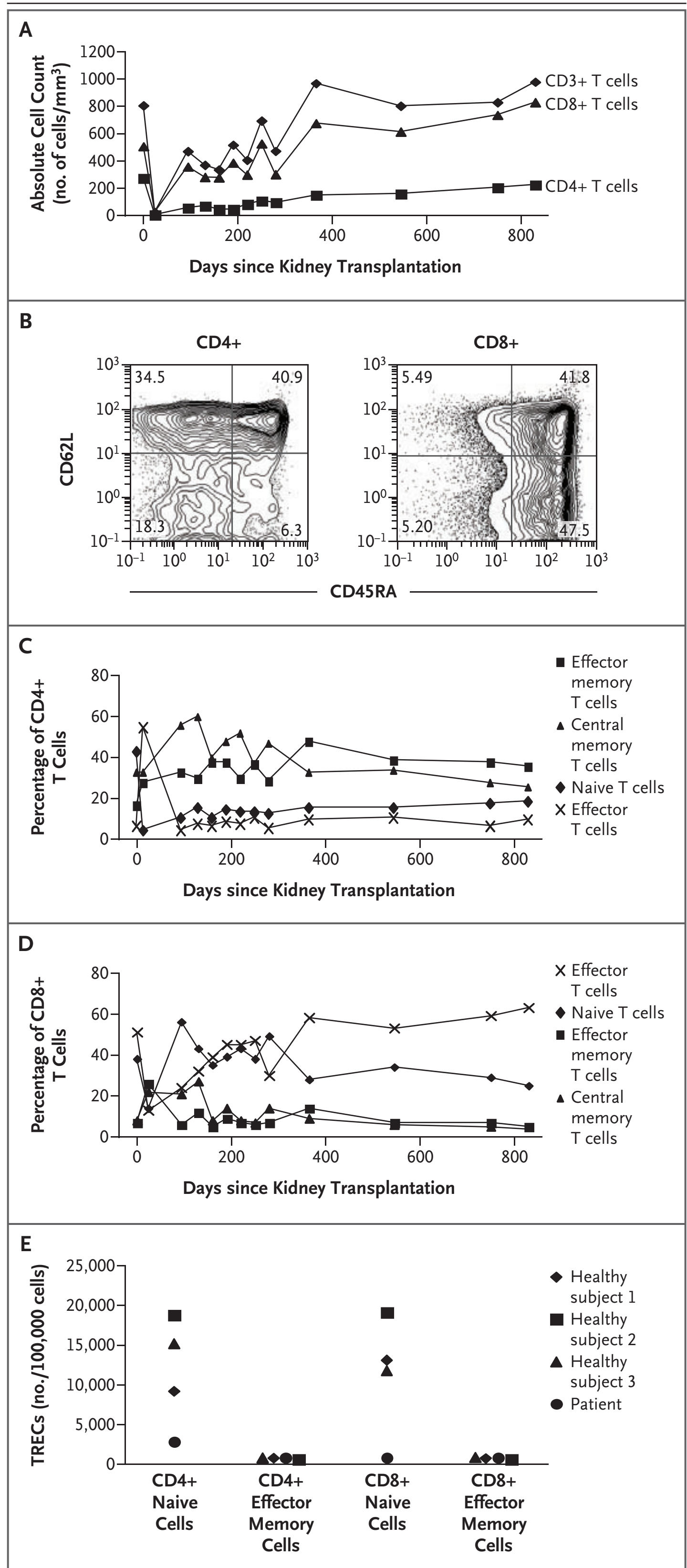
Figure 2. **CD4+ and CD8+ T-Cell Subgroups after Transplantation.**

Panel A shows CD4+, CD8+, and CD3+ T cells in the recipient's blood. Panel B shows flow-cytometric analysis performed with a fluorescence-activated cell sorter before transplantation on gated CD4+ and CD8+ T cells for CD62L and CD45RA markers. The upper right quadrants enclose naive (CD62L+CD45RA+) T cells, the upper left quadrants enclose central memory (CD62L+CD45RA–) T cells, the lower right quadrants enclose effector (CD62L–CD4RA+) T cells, and the lower left quadrants enclose effector memory (CD62L–CD45RA–) T cells. The percentage of cells in each quadrant is shown. CD45RA– cells were all CD45RO+ (data not shown). Panel C shows percentages of naive, central memory, effector, and effector memory CD4+ T cells. Panel D shows percentages of naive, central memory, effector, and effector memory CD8+ T cells. Panel E shows numbers of copies of T-cell–receptor excision circles (TRECs) in CD4+ and CD8+ naive and effector memory T cells from three healthy subjects and from the patient 19 months after the transplantation.



CD8+ T cells is about 10,000 to 20,000 per 100,000 cells, and that the number in effector memory T cells is less than 200 per 100,000 cells. Nineteen months after transplantation, both naive and effector memory CD8+ T cells from the patient contained less than 200 TRECs per 100,000 cells. This result indicates that, instead of emigrating from the thymus, the naive CD8+ T cells had undergone homeostatic expansion.[11]

The culture of mononuclear cells obtained from the patient before the transplantation and 24 months after the transplantation with irradiated allogeneic mononuclear cells from two healthy subjects resulted in vigorous cellular proliferation as measured by means of ^{3}H-thymidine incorporation (approximately 12,000 to 42,000 cpm) stimulated by cells from both healthy subjects (Fig. 3A). Background incorporation without stimulator cells was less than 1000 cpm. A comparison of pretransplantation and post-transplantation responses of the recipient's T cells to dendritic cells from the donor showed that there was a significant reduction in the post-transplantation response after subtraction of background counts per minute ($P=0.04$, by Student's t-test) (Fig. 3B). Figures 3C through 3F show that the patient's post-transplantation mononuclear cells were capable of responses to graded concentrations of influenza and tetanus toxoid antigens, as assessed by the secretion of the cytokines interferon-γ and interleukin-2, respectively, into the culture supernatants.

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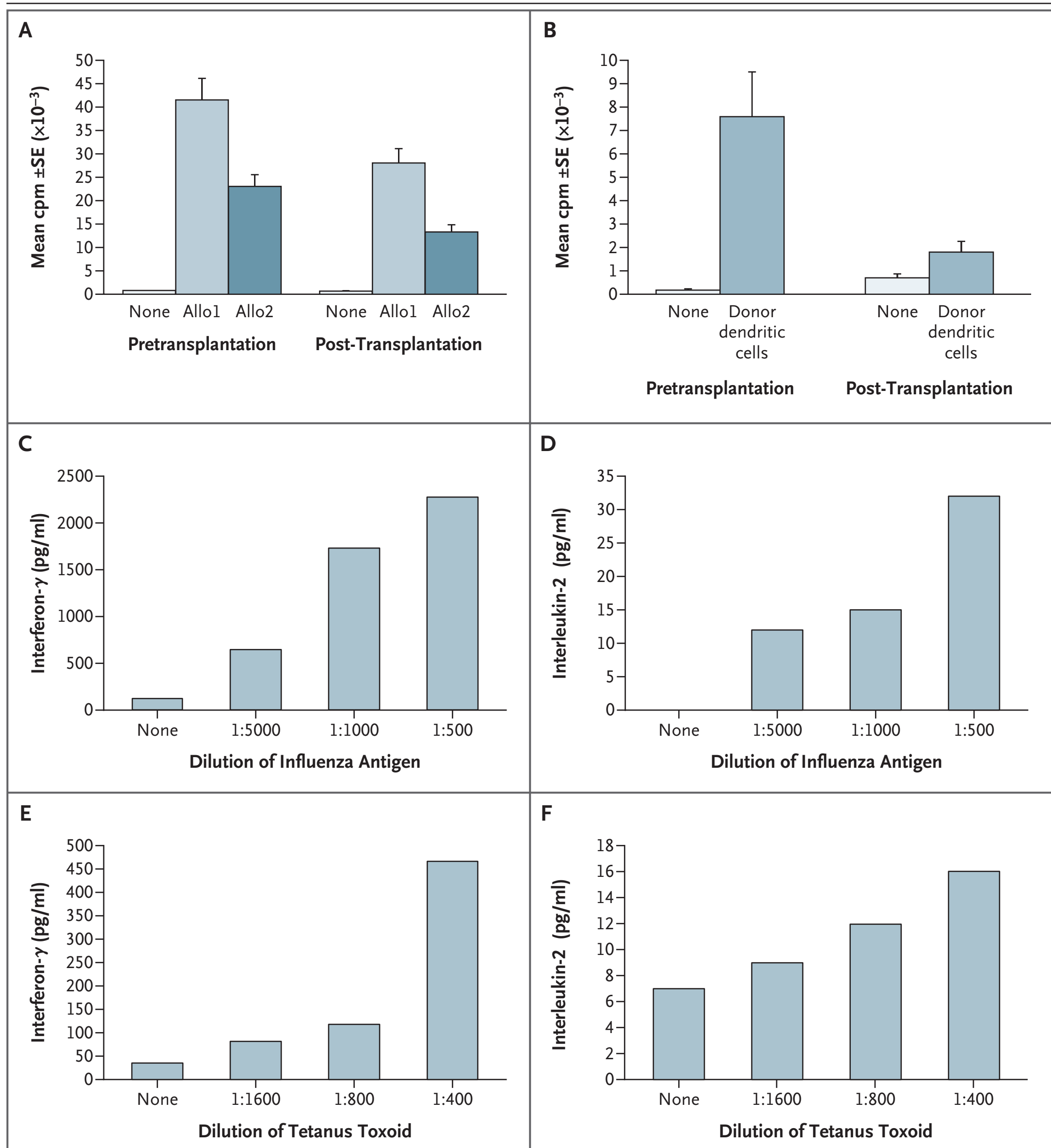

Figure 3. In Vitro Responses of the Patient's Mononuclear Cells to Stimulation by Alloantigens or Microbial Antigens, before Transplantation and 24 Months after Transplantation.

A total of 50,000 mononuclear cells from the patient were cultured for 7 days with or without 50,000 irradiated mononuclear cells (5000 cGy) from unrelated healthy subjects (alloantigen 1 [Allo1] and alloantigen 2 [Allo2]). ^{3}H-thymidine was added 18 hours before harvesting cells on day 7; incorporation of ^{3}H-thymidine into cells was measured with the use of a Microbeta plate scintillation counter. The mean (±SE) response, expressed as counts per minute, from triplicate cultures is shown in Panel A. A total of 100,000 mononuclear cells from the patient were cultured for 7 days with or without 30,000 purified donor dendritic cells; the incorporation of ^{3}H-thymidine was measured as above. The results are shown in Panel B. Post-transplantation mononuclear cells were incubated for 6 days with graded dilutions of influenza antigen. Undiluted antigen (Fluarix, GlaxoSmithKline) was obtained at a concentration of 90 μg per milliliter. Concentrations of interferon-γ and interleukin-2 in supernatants harvested at the end of the culture period were measured by means of enzyme-linked immunosorbent assay. Values, shown in Panels C and D, are from single cultures. Post-transplantation mononuclear cells were incubated for 6 days with graded dilutions of tetanus toxoid antigen. Undiluted antigen was obtained from the University of Massachusetts Biologics Laboratories at a concentration of 490 flocculation units (Lf) per milliliter. Concentrations of interferon-γ and interleukin-2 were measured as described above; the results are shown in Panels E and F.

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The pattern of responses to alloantigens, influenza, and tetanus antigens was similar 18 months after transplantation (data not shown).

DISCUSSION

In our patient, the persistence of mixed chimerism after combined kidney and hematopoietic-cell transplantation, the withdrawal of all immunosuppressive therapy, and the uninterrupted normal functioning of the kidney graft are all consistent with the phenomenon of induced immune tolerance in laboratory animals.[5,6] We used the same conditioning regimen of total lymphoid irradiation and antithymocyte globulin that has been used in patients with hematolymphoid malignant conditions who received hematopoietic grafts,[7] but we reduced the number of donor CD3+ T cells administered from about 200×10^6 to 300×10^6 cells per kilogram to 1×10^6 cells per kilogram in order to reduce the risk of graft-versus-host disease and increase the likelihood of achieving persistent mixed rather than complete chimerism.

The conditioning regimen was not associated with notable adverse events. The patient was discharged from the hospital 6 days after transplantation, and the donor-cell infusion was given in the outpatient clinic. Evidence of adequate immune reconstitution was the absence of opportunistic infections, normal in vitro T-cell responses to tetanus toxoid and influenza antigen, and a vigorous mixed leukocyte reaction to third-party stimulator cells. By contrast, there was a weak response of post-transplantation T cells from the recipient to dendritic cells from the donor.

We examined changes in T-cell subgroups after transplantation to elucidate the source, extent, and kinetics of naive and memory T-cell reconstitution after the initial severe lymphopenia. TREC analysis showed that naive CD8+ T cells returned more rapidly than naive CD4+ T cells, probably because of expansion in the periphery in response to T-cell depletion rather than generation of new cells in the thymus.

The second patient enrolled in the study had a biopsy-confirmed relapse of focal segmental glomerulosclerosis, and severe proteinuria developed during the first week after transplantation. Treatment included a prolonged course of plasmapheresis, antithymocyte globulin, and rituximab. Chimerism was not detected. The patient is receiving cyclosporine and mycophenolate mofetil, and there have been no rejection episodes in 26 months; the current serum creatinine level is 1.2 mg per deciliter (106.1 μmol per liter).

Transient chimerism developed in the third patient, with donor-type cells accounting for a peak of 35 to 65% of natural killer cells, granulocytes, and B cells but only 2% of T cells. The chimeric state was gradually lost in all lineages by the fifth month. A mild rejection episode that developed while the cyclosporine dose was being tapered was reversed by a brief course of corticosteroids, and the current serum creatinine level is 1.1 mg per deciliter (97.2 μmol per liter) 20 months after transplantation while the patient is receiving cyclosporine therapy alone.

The fourth, fifth, and sixth patients were enrolled in the past 6 months, after the protocol was changed to deliver 10 doses of 120 cGy each, instead of 80 cGy each, of total lymphoid irradiation to enhance chimerism. Levels of early peak chimerism were as high as the level in the first patient, with donor-type cells accounting for 39 to 86% of T cells and 31 to 93% of natural killer cells, B cells, and granulocytes.

In a series of seven patients with multiple myeloma given HLA-matched combined kidney and bone marrow transplants, five had transient rather than persistent chimerism, yet all immunosuppressive drugs were discontinued in the five patients.[18] These drugs could not be discontinued in two patients, both of whom had persistent complete chimerism, because of the development of graft-versus-host disease.[18] Our study shows that it is feasible to achieve persistent mixed chimerism and organ-transplantation tolerance in humans, without the development of graft-versus-host disease.

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