Dysfunctional Cytokine Production by Host-Reactive T-Cell Clones Isolated From a Chimeric Severe Combined Immunodeficiency Patient Transplanted With Haploidentical Bone Marrow

By Rosa Bacchetta, Robertson Parkman, Martin McMahon, Kenneth Weinberg, Mike Bigler, Jan E. de Vries, and Maria-Grazia Roncarolo

We have investigated the mechanism of tolerance in a patient with severe combined immunodeficiency (SCID) transplanted with HLA-haploidentical, T cell-depleted bone marrow cells obtained from the mother. At 4 years after transplantation, T cells, natural killer (NK) cells, and a small percentage (2%) of B cells were found to be of donor origin, whereas monocytes and the majority of B cells remained of host origin. In primary mixed lymphocyte cultures (MLC), the engrafted T cells of the donor did not proliferate in response to the host cells, whereas untransplanted donor T cells showed good proliferative responses. However, CD4+ and CD8+ T-cell clones of donor origin with specificity for class II and class I HLA determinants of the host were isolated. CD8+, host-reactive T-cell clones displayed normal cytotoxic activity after stimulation with the host cells, but proliferative responses of CD4+, host-reactive T-cell clones were considerably reduced. In addition, both CD8+ and CD4+, host-reactive T-cell clones produced very low to undetectable levels of interleukin-2 (IL-2), IL-4, IL-5, IL-10, interferon-γ, and granulocyte-macrophage colony-stimulating factor after specific antigenic activation, which may be responsible for their nonresponsive state in vivo. Expression of the CD3ζ subunit of the T-cell receptor (TcR) was normal, and after stimulation via CD3ζ, Raf-1 and p42 mitogen activated protein (MAP) kinase were phosphorylated, indicating that this part of the signaling pathway after triggering of the TcR/CD3 complex is present. These results, together with our previous observation that dysfunctional, host-reactive T-cell clones can be isolated in SCID patients transplanted with fetal liver stem cells, demonstrate that lack of clonal deletion of host-reactive T cells is a general phenomenon after HLA-mismatched stem cell transplantation.

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DIFFERENT MECHANISMS have been proposed to be responsible for the induction of tolerance after allogeneic stem cell transplantation. Clonal deletion, which occurs in the thymus at an early stage of T-cell differentiation, leads to elimination of host-reactive T cells.1 Clonal anergy involves T cells that escape thymic clonal deletion and become unresponsive to the host HLA antigens.2,3 This process can take place in the thymus or in the periphery, where mature T cells are chronically exposed to the host HLA antigens.4,5 Clonal anergy can be due to the interaction of antigen-specific T cells with nonprofessional antigen-presenting cells that do not provide costimulatory signals necessary to induce T-cell effector functions.6,7 Interestingly, induction of unresponsiveness in antigen-specific T cells appears to directly influence the cytokine production profile, suggesting that altered cytokine production may play an important role in the establishment of tolerance.5,8

Patients with severe combined immunodeficiency (SCID) and who are transplanted with allogeneic stem cells comprise a model to study the mechanisms of transplantation tolerance in humans. After transplantation, SCID patients frequently develop a state of split lymphoid chimerism, in which T cells are of donor origin and non-T cells remain predominantly or exclusively of host origin.

Several studies performed in patients undergoing haploidentical bone marrow transplantation (BMT) have shown unresponsiveness of the donor T cells towards the cells of the host in primary mixed lymphocyte cultures (MLC), suggesting that the acquisition of tolerance is due to deletion of host-reactive T cells.9,10 However, our studies in SCID patients undergoing HLA-mismatched fetal liver transplantation (FLT) have demonstrated that, despite the unresponsiveness to the host in primary MLC, donor-derived T cells specific for class II or class I HLA antigens of the host are present at high frequencies in the T-cell repertoire of these chimeric patients.11,12

It is still unclear whether stem cells derived from bone marrow differ from stem cells obtained from fetal liver in their self-renewal and proliferative capacities.13 In addition, the two types of stem cells could have different homing capacities15 and undergo different differentiation processes in the allogeneic host environment, resulting in differences in the mechanisms of tolerance acquisition.

In the present study we analyze, at the clonal level, the host-specific responses of an SCID patient transplanted with haploidentical bone marrow cells. CD4+ and CD8+ T-cell clones, which proliferated to or were cytotoxic against the HLA antigens encoded by the mismatched haplotype of the host, were isolated from the patient. The host-reactive T-cell clones produced low or undetectable levels of cytokines after antigen activation, although one of the major signaling pathways downstream of the TcR/T cell receptor (TcR) was activated in response to stimulation via CD3.

Collectively, these data indicate that tolerance after BMT is not exclusively a consequence of clonal deletion of donor-derived, host-reactive T cells and suggest that clonal anergy, probably mediated by defective interactions with the host HLA antigens, also contributes to the establishment of transplantation tolerance.

**MATERIALS AND METHODS**

**Clinical history.** Patient N.J.L. is presently 5 years old. He was admitted to the Children’s Hospital of Los Angeles (Los Angeles, CA) at 3 months of age with recurrent skin infections. Because of a family history of immunodeficiency, he underwent an immunologic evaluation. His initial evaluation showed a markedly reduced number of T lymphocytes (2% CD3+ cells) and an increased percentage of B lymphocytes (77% CD19+ cells). He had low levels of IgG, IgM, and IgA. Based on these laboratory findings, a presumptive diagnosis of X-linked SCID was made. The patient was prepared for a haploidentical, T-lymphocyte-depleted BMT from his mother. He was prepared for transplantation with cyclophosphamide (50 mL/kg) on days -5,-4,-3, and -2 and antithymocyte globulin (ATG; 20 mL/kg) on days -6,-4,-2, and -2. The maternal bone marrow was depleted of T lymphocytes by agglutination with soybean agglutinin and rosette formation with sheep agglutinin and rosette formation with sheep agglutinin. After T-lymphocyte depletion, 9 x 10^9 nucleated cells or 4.1 x 10^9 nucleated cells per kilogram were infused. The infused bone marrow contained 0.7% T cells or 0.9 x 10^9 phenotypic T lymphocytes per kilogram. After transplantation, the patient received steroids (2 mg/kg/d) and ATG (20 mg/kg) starting on day 5 for seven doses on alternative days. After discontinuation of the ATG, the patient developed grade II graft-versus-host disease (GVHD) that was controlled by the initiation of therapy with cyclosporin A and the continuation of steroids. After discharge from the hospital, the cyclosporin A and steroids were slowly tapered and discontinued by 6 months of age. A phenotype of the patient’s peripheral blood mononuclear cells (PBMC) performed 3 years after transplant showed 80% CD3+ cells, 51% CD4+, 10% CD8+, and 8% CD20+. A more recent immunologic evaluation shows 69% CD3+ cells, of which 7% were TcR y6+CD4+CD8-, 41% CD4+, and 23% CD8+. In addition, 17% CD19+ B cells were observed. He has a normal proliferative response to phytohemagglutinin (PHA; Delta, cm, 96,352) and a specific proliferative response to tetanus toxoid and candida. The patient has normal antibody titers to tetanus toxoid and polyribonucleotide phosphate. His primary and secondary antibody responses to PHA stimulation are 2 SD below the mean values. The BMT and subsequent studies were approved by the Committee on Clinical Investigation, the Institute Review Board of the Children’s Hospital of Los Angeles.

**Isolation of host-reactive T-cell clones.** Host-reactive T-cell clones were obtained from PBMC of patient N.J.L. after activation with PHA (CD3+ T-cell clones) or with the patient’s Epstein-Barr virus-transformed lymphoblastoid cell line (EBV-LCL; CD4+ T-cell clones NjL-C), or with the father’s PBMC (CD4+ T-cell clones NjL-Q). PBMC (10^6) were activated with 1 pg/mL of PHA (Wellcome Diagnostic, Beckenham, UK) in 1 mL of Yssel’s medium in Linbro 24-well plates (Flow Laboratories, McLean, VA). Three days later, 20 U/mL of interleukin (IL)-2 was added. After 8 days of culture, the cells were washed, and stained with an anti-CD8 monoclonal antibody (MoAb), and the CD8+ cells were sorted and cloned at one cell per well by a FACStar Plus (BectonDickinson, Mountain View, CA) in round-bottomed, 96-well plates (Costar, Cambridge, MA). Individual cells were collected into wells that contained 100 µL of a feeder cell mixture consisting of 10^6/mL irradiated (40 Gy) syngeneic PBMC, 10^6/mL irradiated (50 Gy) allogeneic EBV-LCL (JY), and 0.1 µg/mL of PHA. After 7 days, 100 µL of medium containing 40 U/mL of IL-2 was added. After 13 days, the growing cultures were screened in cytotoxic assays against donor’s T-cell blasts and host’s EBV-LCL, and the positive clones were expanded for further analysis. Among 480 T-cell clones tested, 15 displayed anti-host reactivity.

To obtain antigen-specific, CD4+ T-cell clones, 10^6/mL patient’s PBMC were stimulated twice with 2 x 10^6/mL host EBV-LCL (cloning NjL-C) or once with 10^6/mL father’s PBMC (cloning NjL-Q), in Linbro flat-bottomed, 96-well plates in a final volume of 200 µL of Yssel’s medium. IL-2 (20 U/mL) was added every 3 to 4 days of culture. After priming, the cells were stained with anti-CD4 MoAb, and CD4+ cells were sorted and cloned at one cell per well as described above. In cloning NjL-C, T-cell clones were screened first for interferon (IFN)-y production against the host’s EBV-LCL, and subsequently for proliferation and cytokine production in response to EBV-LCL obtained from the host, the donor, and the father. Among 120 T-cell clones tested, two were host-reactive, and nine were EBV-specific and restricted by the HLA DR of the donor. In cloning NjL-Q, T-cell clones were tested in parallel for IFN-y and granulocyte-macrophage colony-stimulating factor (GM-CSF) production and proliferation in response to EBV-LCL of the host, the donor, and the father. Among 320 clones screened, two were host-reactive, and seven were reactive to the father’s haplotype, which was not shared with the host. None of the T-cell clones were donor-reactive. Alloreactive and tetanus toxoid-specific, CD4+ and CD8+ T-cell clones and T-cell clones with unknown specificities were isolated from normal donors as previously described.13,17,20 These T-cell clones were used as controls for cytokine production.

**Proliferation and cytotoxicity assays.** MLC were performed by culturing 10^5/mL freshly isolated PBMC in flat-bottomed, Linbro 96-well plates with 10^5/mL irradiated (40 Gy) PBMC as stimulators, in 200 µL of Yssel’s medium plus 10% human serum, in the presence or in the absence of exogenous IL-2 or IL-4. After 4 days of incubation at 37°C in a humidified atmosphere of 5% CO2, the cultures were pulsed overnight with 1 µCi of tritiated thymidine ([H] TdR) and harvested onto fiberglass filters. The [H] TdR incorporation was determined by scintillation spectrophotometry.

Proliferative responses of host-reactive T-cell clones were determined by [H] TdR incorporation as previously described.12,13 Cytotoxic activity of host-reactive T-cell clones was measured in a standard 4-hour [Cr]-radioisotope release assay.2 MoAbs. Fluorescein isothiocyanate- (FITC-) or phycoerythrin (PE-) conjugated anti-CD3 (anti-Leu4), anti-CD4 (anti-Leu3a), anti-CD8 (anti-Leu2b), anti-CD14 (anti-LeuM3), anti-CD16 (anti-Leu11a), and anti-CD19 (anti-Leu12) MoAbs and control MoAbs of the appropriate isotype were purchased from Becton Dickinson. Anti-CD3 (TIA-2) was provided by Dr P. Anderson, Dana-Farber Cancer Institute (Boston, MA). Anti-CD28 (L293) was provided by L. Lanier (DNAX Research Institute). The hybridoma-producing MoAb GAP A3 specific for HLA-A3 was obtained from the American Type Culture Collection (Rockville, MD).

**Immunofluorescence analysis.** For detection of cell surface antigens, 10^6 cells were labeled with MoAbs and FITC-labeled goat anti-mouse antibodies or with FITC-PE-conjugated MoAbs. Cells were incubated for 30 minutes with the appropriate MoAb at 4°C in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 0.02% NaN3. After three washes, the labeled cells were analyzed on a FACScan (Becton Dickinson) in the presence of 0.1% propidium iodide to allow exclusion of dead cells.

For cell sorting, PBMC were labeled according to the same procedure in PBS with 5% fetal calf serum and were sorted by FACStar Plus or Vantage (Becton Dickinson).

To detect the cytoplasmic CD3 proteins, cells were fixed in PBS containing 0.1% paraformaldehyde at 4°C overnight. After two washes in PBS/BSA/Na3, cell membranes were permeabilized by incubation on ice for 30 minutes with PBS containing 0.1% Triton X-100 and then stained with the relevant MoAbs.

**Determination of cytokine production.** Resting T-cell clones were collected 8 to 10 days after activation with feeder cell mixtures, washed, and stimulated with 10^6 irradiated (50 Gy) host-derived EBV-LCL (1:1 ratio) or with concanavalin A (ConA; 10 µg/mL) or with 12-0-tetradecanoylphorbol-13-acetate (TPA; 1 ng/mL) plus anti-CD3 MoAb (0.1 µg/mL) or with TPA (1 ng/mL) plus Ca++ ionophore (A23187; 500 ng/mL) as previously described.15 Superna-
tients were harvested after incubation for 24 hours. The presence of IL-2, IL-4, IL-5, IL-10, IFN-γ, and GM-CSF was quantified by immunoenzymetric assays performed as previously described. Sensitivity of these assays was 20 pg/mL for IL-2, IL-5, and GM-CSF; 40 pg/mL for IL-4; 100 pg/mL for IFN-γ; and 50 pg/mL for IL-10.

Preparation of cell extracts and Western blot analysis of p42 mitogen-activated protein (MAP) kinase and p74ERK1. T-cell clones were collected 6 days after feeding with the feeder cell mixture, washed, and incubated in 15-μL tubes with biotinylated anti-CD3 (Becton Dickinson) for 15 minutes on ice. After washing, the pellet of cells was placed at 37°C for 2 minutes, and streptavidin was added for 30 seconds, 2 minutes, and 5 minutes. The reaction was stopped by adding cold PBS.

After centrifugation, cells were lysed on ice in Gold lysis buffer (GLB) containing 20 mmol/L Tris pH 7.9, 137 mmol/L sodium chloride, 5 mmol/L EDTA Na₂, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L aprotinin, 1 mmol/L leupeptin, 1 mmol/L sodium orthovanadate, 1 mmol/L EDTA, 10 mmol/L sodium fluoride, 1 mmol/L tetrasodium pyrophosphate, and 100 μmol/L β-mercaptoethanol. Insoluble material was removed by centrifugation at 12,000g. The protein concentration of the soluble material was estimated using the BCA protein assay kit (Pierce, Rockford, IL). Cellular proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blots were prepared and probed with an anti-MAP kinase MoAb that recognizes both p42 and p44 MAP kinases (Zymed, San Francisco, CA) and an anti-p74ERK1 polyclonal antiserum (gift of Dr R.C. Schatzman, Syntex Research, Palo Alto, CA). Western blots were incubated with the appropriate primary antibody at a dilution of 1:1,000, then washed in Tris-buffered saline containing 0.5% (vol/vol) NP40. Antigen-antibody complexes were detected using 1:10,000 diluted sheep anti-mouse:horseradish peroxidase (hrp) or goat anti-rabbit:hrp as appropriate and were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL) and x-ray film (Fuji, Burbank, CA).

RESULTS

Detection of chimerism. The HLA phenotype of the patient before transplantation was HLA-A3,23; B44,35; Cw4; DR4,w13. HLA typing performed on the patient after transplantation showed the presence of a second population of cells derived from the haploidentical bone marrow donor (HLA A23,1; B35,18; Cw4,2; DRw13).

To evaluate the extent of chimerism, a double immunofluorescence analysis was performed of PBMC of the patient 4 years after transplantation, using an anti-HLA A3 MoAb as a marker for host cells. Donor PBMC were tested in parallel as control. Results in Fig 1 show that 99% of CD3+ T cells in the patient and 100% in the donor did not stain with the anti-HLA A3 MoAb, indicating that the T cells present in the peripheral blood of the patient are of donor origin. Both TCR αβ and TCR γδ T cells were HLA-A3− (data not shown). In the CD19+ B-cell subset, the majority were of host origin (HLA-A3−; positive); however, a small proportion (2%) of B cells of donor origin (HLA-A3−) was detected. All the CD14+ monocytes were HLA-A3− and, thus, of host origin. No CD16+ bright natural killer (NK) cells were detected, and the 1% CD16− dim cells were of donor origin (HLA-A3−). Overall, these data indicate that the T and NK subsets are of donor origin and that donor cells only represent a small percentage of the B cells.

Proliferative responses of pretransplant and engrafted donor PBMC to donor, host, and third-party allogeneic PBMC. To investigate whether the antithost reactivity of engrafted donor T cells differed from that of pretransplanted donor T cells, we tested the proliferative responses of PBMC of the donor and the transplanted patient in primary MLC. The PBMC of the father (HLA-A3, 2; B44, 50; DR4, 1) were used as control. Antidonor reactivity and alloreactivity were also evaluated. Results in Table 1 show that untransplanted T cells of the donor displayed proliferative responses to the patient's HLA-haploidentical PBMC. In contrast, the donor-derived T cells engrafted in the patient did not proliferate in response to the patient cells, indicating that they lost their reactivity to the host. The engrafted donor T cells showed comparable proliferative responses to the host and to the donor (self). Therefore, no antidonor (self) reactivity was found in either pretransplanted or engrafted donor cells. T cells of both the donor and the patient proliferated normally in response to the father's PBMC and to HLA-unrelated cells. As expected, the father's PBMC proliferated vigorously to all allogeneic cells.

The effects of exogenous IL-2 and IL-4 on the proliferation of the patient's PBMC in response to cells of the host or the donor were investigated. Results in Table 2 show that, in the presence of different concentrations of IL-2, the proliferation of patient's PBMC to the host and the donor cells did not significantly increase as compared with the proliferation observed in response to IL-2 alone. The minimal increase in proliferation to the host PBMC in the presence of IL-2 has to be considered not antigen-specific, because it was also observed when PBMC of the donor (Table 2) or unrelated PBMC (data not shown) were used as stimulators. These data indicate that the unresponsiveness of the donor-derived T cells of the patient to the host cells could not be reversed by exogenous IL-2, despite the fact that these cells proliferated well in response to IL-2 alone. Similarly, IL-4, which has been shown to have T-cell growth-promoting activity, was ineffective. We also investigated whether addition of anti-CD28 MoAbs to the MLC could reverse the host-specific unresponsiveness, but no increase in proliferation was observed under these culture conditions (data not shown), suggesting that the unresponsiveness is not due to a defect in the interactions between CD28/CTLA-4 and their ligand.

Characterization of CD4+ and CD8+, host-reactive T-cell clones. We previously demonstrated that unresponsiveness to mismatched host cells in primary MLC of patients, who are split lymphoid chimeras after allogeneic fetal stem cell transplantation, is not due to the clonal deletion of donor-derived, host-reactive T cells. To address the question of whether host-reactive T cells are present in PBMC of chimeric patients after haploidentical BMT, we established T-cell clones from the patient's PBMC after they had been primed with host cells or after polyclonal activation. CD4+ T-cell clones were obtained after priming the PBMC of the patient with either the host's EBV-LCL or the father's PBMC. To isolate both low and high proliferative T-cell
clones, host-reactive CD4+ T-cell clones were selected based on their capacity to produce IFN-γ after antigen-specific stimulation. Their host-specific proliferative responses, tested subsequently or in parallel to the cytokine production, are shown in Table 3. All the T-cell clones were tested in medium without fetal calf serum to avoid the isolation of clones specific for serum components. Host-reactive T-cell clones proliferated in response to host and father cells, which shared HLA antigens. Proliferative activities of the T-cell clones to the host PBMC were lower compared with those in response to the father PBMC. Furthermore, none of the CD4+ T-cell clones showed cytolytic activity against host EBV-LCL or paternal T-cell blasts (data not shown).

The cytotoxic activity of the donor-derived, CD8+, host-reactive T-cell clones is shown in Table 4. They were isolated from PHA-activated T-cell blasts of the patient. These T-cell clones had variable cytotoxic activities against both EBV-LCL of the host and PHA-activated T-cell blasts of the father; none of them lysed mother’s (donor) PHA-activated T-cell blasts. In addition, none of the CD8+ T-cell clones tested displayed proliferative activity to the EBV-LCL of the host (data not shown).

To further determine the specificity of the CD4+ and CD8+, host-reactive T-cell clones, their activities were tested against a panel of EBV-LCL or T-cell blasts of unrelated donors that shared the same HLA antigens with the host. Results showed that the CD4+ T-cell clones NJL-C34 and NJL-C87 are HLA-DR4-specific; the CD8+ T-cell clones NJL-296 and NJL-409 are specific for HLA-A3 antigen; and

Table 1: Proliferative Responses of PBMC of the Donor and of the Transplanted Patient to Mother (Donor), Host, Father, and Allogeneic Cells

<table>
<thead>
<tr>
<th>Stimulators</th>
<th>Medium</th>
<th>NM (donor)</th>
<th>NJL (host)</th>
<th>NF (father)</th>
<th>ND (allogeneic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>1.8 ± 0.3</td>
<td>1.7 ± 0.1</td>
<td>24.7 ± 3.4</td>
<td>43.2 ± 0.8</td>
<td>41.9 ± 1.1</td>
</tr>
<tr>
<td>NJL</td>
<td>0.9 ± 0.1</td>
<td>1.7 ± 0.9</td>
<td>1.2 ± 0.3</td>
<td>21.6 ± 2.0</td>
<td>25.0 ± 0.9</td>
</tr>
<tr>
<td>NF*</td>
<td>1.1 ± 0.1</td>
<td>35.7 ± 1.6</td>
<td>17.2 ± 2.4</td>
<td>1.3 ± 0.1</td>
<td>20.0 ± 3.0</td>
</tr>
</tbody>
</table>

Freshly isolated PBMC were used as stimulators. Data are cpm × 10^3 [3H]Tdr incorporation and are expressed as mean of triplicate cultures ± SD.

Abbreviations: NM, patient’s mother; NJL, patient; NF, patient’s father; ND, normal donor.

*PBMC of the father tested in parallel.

Table 2: Effects of IL-2 and IL-4 on Proliferation of the Patient’s PBMC in Response to Host and Donor Cells

<table>
<thead>
<tr>
<th>Stimulators*</th>
<th>Responder</th>
<th>Medium</th>
<th>Host</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1: NJL-PBMC</td>
<td>0.9 ± 0.4</td>
<td>2.3 ± 0.9</td>
<td>5.1 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>+ IL-2, 0.02 U/mL</td>
<td>1.2 ± 0.4</td>
<td>1.9 ± 0.5</td>
<td>4.5 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>+ IL-2, 0.2 U/mL</td>
<td>2.9 ± 1.1</td>
<td>2.6 ± 0.8</td>
<td>9.7 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>+ IL-2, 2 U/mL</td>
<td>3.4 ± 0.6</td>
<td>6.7 ± 1.6</td>
<td>10.1 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>+ IL-2, 20 U/mL</td>
<td>30.5 ± 1.2</td>
<td>29.5 ± 1.2</td>
<td>28.8 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>+ IL-2, 200 U/mL</td>
<td>33.1 ± 1.2</td>
<td>37.2 ± 2.5</td>
<td>33.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Experiment 2: NJL-PBMC</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>+ IL-4, 0 U/mL</td>
<td>11.2 ± 0.4</td>
<td>10.6 ± 0.2</td>
<td>10.9 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>+ IL-4, 20 U/mL</td>
<td>12.7 ± 1.5</td>
<td>12.2 ± 0.5</td>
<td>12.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>+ IL-4, 2,000 U/mL</td>
<td>12.5 ± 0.2</td>
<td>11.6 ± 0.2</td>
<td>12.4 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Data are cpm × 10^3 [3H]Tdr incorporation and are expressed as mean of triplicate cultures ± SD.

*NJL-PBMC were used as host cells, and NM-PBMC were used as donor cells.
produce IL-4, after stimulation with the host EBV-LCL (Table 5 and 6). The levels of cytokine production were considerably lower than those detected in the supernatants of T-cell clones isolated from normal donors (Table 5). The cytokines in response to the antigen and to various modes of polyclonal activation (Tables 5 and 6).

CD4⁺, host-reactive T-cell clones were able to produce IL-2, IL-5, IL-10, IFN-γ, and GM-CSF, but they did not produce IL-4, after stimulation with the host EBV-LCL (Table 5). The levels of cytokine production were considerably lower than those detected in the supernatants of T-cell clones isolated from normal donors (Table 5). However, cytokine production, including the production of IL-4, was enhanced when the CD4⁺, host-reactive T-cell clones were polyclonally activated with ConA, TPA plus anti-CD3 MoAb, or TPA plus Ca²⁺.

CD8⁺, host-reactive T-cell clones did not secrete IL-2, IL-4, or IL-5 after optimal stimulation with the host EBV-LCL (Table 6), whereas IL-10, IFN-γ, and GM-CSF were only detectable at very low levels. After polyclonal activation, the CD8⁺, host-reactive T-cell clones secreted levels of IL-10, IFN-γ, and GM-CSF that were in the same range as those produced by T-cell clones isolated from normal donors (Table 6). No IL-4 or IL-5 production was observed after polyclonal activation, whereas IL-2 was present in low amounts after stimulation with ConA or TPA plus anti-CD3 MoAb and at higher levels after TPA plus Ca²⁺ activation. The T-cell clones did not secrete any detectable cytokines in the absence of stimulation or in the presence of donor-derived cells (data not shown).

These data differ from the results obtained with CD8⁺ T-cell clones isolated from normal donors (Table 6) which produce IL-2 and high levels of IFN-γ and GM-CSF after antigen-specific activation. Despite the undetectable/low levels of cytokine production, the specific cytotoxic activity of the CD8⁺, host-reactive T-cell clones was in the normal range. Their antigen specificity was corroborated by the presence of cytotoxic Ca²⁺ that was 10% and 15% above the baseline at 2 and 6 minutes, respectively, after stimulation with cells of the host or the father bearing the relevant HLA-haploidentical antigens.
Table 6. Cytokine Production by CD8+ T-Cell Clones of Patient N.J.L. and of Normal Donors

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stimulation</th>
<th>Patient (n = 18)</th>
<th>Normal Donors (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2 (pg/mL)</td>
<td>Ag</td>
<td>&lt;20</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>307</td>
<td>6,000</td>
</tr>
<tr>
<td></td>
<td>TPA + anti-CD3</td>
<td>25</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>6,814</td>
<td>9,567</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>Ag</td>
<td>&lt;40</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>&lt;40</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>TPA + anti-CD3</td>
<td>&lt;40</td>
<td>&lt;40</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>IL-5 (pg/mL)</td>
<td>Ag</td>
<td>&lt;20</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>&lt;20</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>TPA + anti-CD3</td>
<td>&lt;20</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>Ag</td>
<td>173</td>
<td>418</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>210</td>
<td>324</td>
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<tr>
<td></td>
<td>TPA + anti-CD3</td>
<td>670</td>
<td>485</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>68</td>
<td>86</td>
</tr>
<tr>
<td>IFN-γ (ng/mL)</td>
<td>Ag</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>ConA</td>
<td>46.0</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>TPA + anti-CD3</td>
<td>5.1</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>52.0</td>
<td>56.0</td>
</tr>
<tr>
<td>GM-CSF (ng/mL)</td>
<td>Ag</td>
<td>0.07</td>
<td>0.5</td>
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<tr>
<td></td>
<td>ConA</td>
<td>1.2</td>
<td>3.5</td>
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<tr>
<td></td>
<td>TPA + anti-CD3</td>
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<td>1.5</td>
</tr>
<tr>
<td></td>
<td>TPA + Ca++</td>
<td>18.0</td>
<td>12.4</td>
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</table>

EBV-LCL of the host were used as stimulator cells for host-reactive T-cell clones of the patient; allogeneic EBV-LCL were used for activating T-cell clones of normal donors (see Materials and Methods). Data are median values.

Abbreviation: NT, not tested.

Analysis of signal transduction pathway in CD8+, host-reactive T-cell clones. We studied whether CD8+, host-reactive T-cell clones had defective signal transduction pathways that could account for their lack of or markedly reduced levels of cytokine production after antigen-specific activation. We analyzed the expression of CD3ζ and the phosphorylation of Raf-1 and p42 MAP kinase after stimulation via CD3.

The TcR complex plays a central role in directing the initiation of antigen-specific responses. The αβ chains are responsible for the recognition of the different peptide antigens and the major histocompatibility complex (MHC) molecules, whereas the associated invariant CD3 subunits (γ, δ, ε) and the CD3ζ subunit serve to couple these molecules to intracytoplasmic signaling transduction structures, triggering T-cell activation after receptor engagement. In particular, it has been demonstrated in ζ chain-deficient cells that the intracytoplasmic domain of ζ is important for the reconstitution of signaling.

Intracytoplasmic staining indicated that the levels of CD3ζ and CD3ε in the CD8+, host-reactive T-cell clone NJL-350 were in the same range as those in CD8+ T-cell clones isolated from normal donors (Fig 2). In addition, no significant differences in surface CD3ε expression were observed.

The Raf-1/MEK/MAP kinase cascade is believed to be an essential component in the response of T cells to either antigen or IL-2 stimulation. This pathway can be activated either through activated Ras proteins or through activation of protein kinase C. To determine whether NJL CD8+ T cells were defective in this signaling pathway, extracts were prepared from stimulated cells. The phosphorylation of Raf-1 and MAP kinases was analyzed by probing a Western blot with appropriate antibodies. These cells express p42 but not p44 MAP kinase (Fig 3). A slower migrating form of p42 MAP kinase appeared after 30 seconds of anti-CD3 stimulation and remained present at both 2 and 5 minutes after stimulation. This slower migrating form of p42 MAP kinase has previously been demonstrated to be the phosphorylated, enzymatically activated form of the enzyme. In addition, the p74Raf-1 protein also showed a phosphorylation-induced mobility shift after stimulation, with similar kinetics to that observed for p42 MAP kinase. These results indicate that the clones have not lost the capacity to respond to TcR-mediated signals.

DISCUSSION

Allogeneic stem cell transplantation in children with SCID frequently can result in the stable engraftment of T cells of donor origin, whereas the non-T cells remain of host origin. Our studies in such split lymphoid chimeras after fetal liver stem cell transplantation demonstrated that tolerance is not due to the clonal deletion of host-reactive T cells. Despite the in vivo tolerance and the in vitro unresponsiveness of the donor-derived T cells to the host HLA antigens in primary MLC, host-reactive T cells were present at high frequencies in the PBMC of these patients.

In the present study, we extended our investigations to a patient transplanted with T cell-depleted, haploidentical bone marrow stem cells derived from his mother. In this patient, all T cells and the few detectable NK cells are of donor origin, whereas all the monocytes and most of the B cells are of host origin. The patient is now fully reconstituted, and complete tolerance has been achieved in vivo between the partially mismatched lymphoid populations. Tolerance between donor and host cells was also observed in vitro, as the engrafted donor T cells were unresponsive to the host cells in primary MLC. Tolerance was acquired during maturation of the donor T cells in the mismatched environment of the host, as T cells isolated from the original donor showed proliferative responses to the host cells.

As previously demonstrated in patients transplanted with fetal liver, the in vitro unresponsiveness of donor-derived T lymphocytes to host cells could not be reversed by addition of exogenous IL-2 or IL-4. Furthermore, stimulation of the CD28 signaling pathway with an anti-CD28 MoAb was ineffective. Analysis at the clonal level detected CD4+ and CD8+ T-cell clones that specifically proliferated in response to or were cytotoxic for cells bearing the HLA antigens of the host. These findings were comparable with those previously reported for patients transplanted with fetal liver. Host-reactive T-cell clones were isolated from PBMC that had been activated polyclonally, or in an antigen-specific fashion. Both CD8+ and CD4+ T-cell clones recognized the HLA
antigens shared by the host and the father. None of the T-cell clones tested showed specific antidonor reactivity, but many of the CD4+ T-cell clones obtained after in vitro priming with the host EBV-LCL were EBV-specific and HLA-restricted by the mother class II antigens (data not shown). These results in a BMT patient, together with our previous observations in FLT patients, indicate that in human chimeras, clonal deletion is responsible for tolerance to the donor, whereas tolerance to the host is not due only to clonal deletion of host-reactive T cells. Furthermore, the present data suggest the existence of a general mechanism responsible for the acquisition of tolerance after allogeneic stem cell transplantation, irrespective of the source of stem cells used.

The mechanisms underlying the induction of tolerance in SCID patients reconstituted with mismatched bone marrow stem cells have been investigated by several groups.9,11,35,37 In these studies, the presence of antihost reactivity has been mainly investigated using bulk cultures. Under such culture conditions, engrafted donor T cells were consistently unresponsive to the host alloantigens, suggesting the absence of host-reactive T cells in the T-cell repertoire of the chimeric patients. Using cell cloning techniques, we have demonstrated that donor-derived, host-reactive T cells were detectable. In particular, CD8+ T-cell clones with antihost reactivity were isolated at high frequency. These findings are quite unique for transplanted patients, as autoreactive CD8+ T-cell clones have not been found in normal individuals.13 However, as the host-reactive T-cell clones had low specific activities, it can be hypothesized that they are representative of a portion of host-reactive T cells with low affinity for the host antigen. These cells could have been rendered anergic in vivo and only detectable at the clonal level after repetitive stimulations in the presence of mitogen and IL-2 in vitro, whereas the high-affinity T cells could have been deleted. On the other hand, the presence of suppressor cells or suppressor factors produced by the PBMC of the patient can not be excluded. In favor of this hypothesis, is the observation that the proliferative responses of the host-reactive T-cell clones...
were higher when the father's PBMC, instead of the patients's PBMC, were used as stimulators.

Controversial data about the presence of antidonor reactivity have been reported. Reactivity of engrafted donor T cells versus pretransplanted donor cells has been detected either in bulk cultures or at the clonal level in SCID patients reconstituted with haploidentical bone marrow. It has been proposed that antidonor reactivity can vary depending on the type of chimerism and that the engraftment of donor-derived HLA class II antigens expressing cells can induce donor specific tolerance. This could explain the absence of donor versus donor reactivity in our BMT patient, because a small proportion of B cells of donor origin expressing high levels of class II HLA antigens was still present 4 years after transplantation.

The CD4+, host-reactive T-cell clones produced IL-2, IL-5, IL-10, IFN-γ, and GM-CSF, but they did not secrete IL-4. These results, which are in accord with previous observations, indicate that host-reactive T-cell clones cannot be considered as TH1- or TH2-like cells. The amounts of cytokines produced by the host-reactive T-cell clones after antigen-specific stimulation was low compared with those measured in supernatants of host-reactive and alloreactive T-cell clones isolated from other patients or from normal donors. In addition, unlike host-reactive T-cell clones isolated from FLT patients, which produced high levels of IL-10, NJI CD4+, host-reactive T-cell clones secreted levels of IL-10, which were in the normal range.

CD8+ T-cell clones isolated from patient N.J.L did not secrete IL-2, IL-4, or IL-5 after antigen-specific activation, whereas IL-10, GM-CSF, and IFN-γ were detectable at very low levels. Although host-reactive CD8+ T-cell clones isolated from FLT patients also did not produce IL-4, the majority of these T-cell clones, as well as CD8+ T-cell clones isolated from normal donors, were able to secrete IL-2 and produce considerable amounts of IFN-γ and GM-CSF. Therefore, the low/undetectable levels of cytokine production by host-reactive T-cell clones isolated from the BMT patient are a specific property of these cells when they are triggered via their TcR. With the exception of IL-4 and IL-5, the CD8+ T-cell clones produced variable levels of the other cytokines after polyclonal activation with ConA, TPA plus anti-CD3 MoAb, or TPA plus Ca++, indicating that lack of production after antigen-specific activation is not due to an intrinsic defect of the cells.

The reason for the low cytokine production by host-reactive T-cell clones remains to be clarified. It may reflect a state of antigen-specific unresponsiveness of host-reactive T cells induced by the immunosuppressive treatment that the patient received to control a mild GVHD that developed shortly after transplantation. At that time, donor-derived cells were likely primed to the host alloantigens, which resulted in the development of host-reactive cells. The effects of immunosuppressive drugs during the T-cell priming could have determined a long-lasting functional inactivation of these cells that can only partially be overcome in vitro after polyclonal stimulation in the presence of IL-2. This would explain why it has been possible to isolate these T-cell clones under the present culture conditions and to detect their specific cytotoxic or proliferative activity, despite the fact that these cells are dysfunctional in terms of cytokine production.

The administration of immunosuppressive drugs after transplant differs between BMT and FLT patients. In FLT patients, immunosuppressive treatment after transplant is usually not necessary because the fetal liver cell suspensions are virtually devoid of mature T cells capable of provoking a GVHD. Therefore, the use of posttransplant immunosuppression could account for the differences in the cytokine production patterns observed in the host-reactive T-cell clones obtained from BMT and FLT patients. On the other hand, the differences in the cytokine production profiles between host-reactive T-cell clones obtained from BMT and FLT patients could be due to the different origin of host-reactive T cells. In FLT patients, host-reactive cells derive from T cells that differentiated in the allogeneic host environment and escaped negative selection in the thymus. Similarly, in BMT patients, host-reactive T cells may have been generated in vivo during the maturation of donor-derived stem cells into T cells; however, they may also derive from the residual mature T cells present in the bone marrow inoculum. In this latter case, the cytokine profiles of host-reactive T cells isolated from BMT patients may reflect the induction of anergy in the periphery rather than in the host thymus.

Despite their defect in cytokine production, the NJI T-cell clones were specifically activated by the host alloantigens, as demonstrated by increase in [Ca++], after contact with cells of the host or father that expressed the relevant HLA antigens. Therefore, these T-cell clones could become activated via triggering the TcR, even though the activation did not result in cytokine production. It has been reported that, in anergic T cells, intracellular activation events, such as increase in [Ca++], occur after TcR occupancy by the antigen. However, TcR occupancy is not sufficient to induce anergic cells to proliferate and produce IL-2. For induction of complete T-cell effector functions, an appropriate costimulatory signal is necessary. Such second signals can be provided by stimulation of the CD28/CTLA-4 pathway, which strongly synergizes with the TcR signal. The fact that the unresponsiveness to the host in the MLC was not reversed by the addition of anti-CD28 MoAb, suggests that the impaired responses of host-reactive T-cell clones are not due to abnormalities in the CD28/CTLA-4 ligand-receptor interaction.

NJI host-reactive T-cell clones have normal intracytoplasmic expression of the CD3ε and CD3ζ subunits of the TcR complex and display phosphorylation of molecules involved in the signal transduction cascade, such as Raf-1 and p42 MAP kinase. Recent biochemical and genetic studies have indicated that, besides the TcR and costimulatory molecules, intracellular tyrosine and serine kinases play a major role in propagation of antigen receptor-derived signals. The CD3ζ subunit of the TcR complex is particularly important in coupling the TcR subunit to the signal-transducing structures. Raf-1 and MAP kinases are serine/threonine kinases that can be activated by many different stimuli, including activation of T lymphocytes via CD3. In the signal transduction cascade, they can be regulated by protein kinase C, which has been shown to synergize with rise in [Ca++], and costimulatory signals in the induction of T-cell
activation and effector functions. Our data indicate that the biochemical events initiated by stimulation via CD3 are present in the host-reactive T-cell clones. However, quantitative differences between signal transduction pathways in T-cell clones isolated from transplanted patients and from normal donors cannot be excluded at present. In addition, it remains to be determined whether antigen activation of host-reactive T cells results in other, more downstream, intracellular events, such as induction of proteins that suppress regulatory molecules responsible for cytokine production.

Collectively, our data show that the nondeletional type of tolerance is a common finding after allogeneic stem cell transplantations and that a peripheral mechanism of tolerance is likely to be responsible for the functional inactivation of non-deleted, host-reactive T cells. These data provide additional evidence that the state of antigen-specific unresponsiveness is associated with alteration of cytokine production pathways, suggesting that cytokine imbalances can play a major role in the induction and maintenance of transplantation tolerance.

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Dysfunctional cytokine production by host-reactive T-cell clones isolated from a chimeric severe combined immunodeficiency patient transplanted with haploidentical bone marrow

R Bacchetta, R Parkman, M McMahon, K Weinberg, M Bigler, JE de Vries and MG Roncarolo