Analysis of T-Cell Receptor Variability in Transplanted Patients With Acute Graft-Versus-Host Disease

By Pierre-Yves Dietrich, Anne Caignard, Anita Diu, Catherine Genevée, José-Luis Pico, Michel Henry-Amar, Jacques Bosq, Eric Angevin, Frédéric Triebel, and Thierry Hercend

T lymphocytes play a pivotal role in graft-versus-host disease (GVHD) and largely contribute to the graft-versus-leukemia (GVL) effect. Most mature T lymphocytes specifically recognize antigens through the \( \alpha/\beta \) T-cell receptor (TCR). Each \( \alpha/\beta \) TCR chain includes a constant region and a variable region, the latter being encoded by V-Ja or V-D-Jp rearranged gene segments. To better characterize T cells involved in GVHD, \( V_a \) and \( V_b \) gene segment usage was analyzed, after cDNA amplification, in peripheral blood mononuclear cells (PBMC) and skin samples from three patients with grade II cutaneous GVHD. At time of GVHD diagnosis (days 11, 22, and 25), when first signs of engraftment were detectable, virtually all \( V_a \) and \( V_b \) subfamilies were represented in PBMC RNAs of the three recipients. These results suggest that diversified TCR gene segment expression is observed early after allogeneic bone marrow transplantation (alloBMT). Lymphocytes infiltrating GVHD skin also expressed a large series of \( V_a \) and \( V_b \) subfamily specificities. However, analysis of the \( V_a \) and \( V_b \) amplified products showed substantial differences between PBMC and the skin lymphocyte RNAs. These observations indicate that a large variety of T lymphocytes are present at the disease site, while some of them may be specifically amplified or decreased in response to minor histocompatibility antigens (mHIA). Further characterization of the latter T-cell subpopulations should lead to a better understanding of human in vivo responses directed at mHIA.

MATERIALS AND METHODS

Patients. Between June and December 1990, one woman and two men were treated by HLA-matched related (LC and FG) or unrelated (ML) alloBMT. LC (aged 23 years) suffered from an accelerated phase of chronic myelogenous leukemia (CML), FG (aged 35 years) from a CML in first chronic phase, and ML (aged 43 years) from a \( 5q- \) myelodysplastic syndrome. Conditioning treatment consisted of TAM regimen (total body irradiation [TBI], high-dose cytarabine, and melphalan) for LC, or TBI and cyclophosphamide for FG and ML. Both cyclosporine and a short course of melphotrexate were given for GVHD prophylaxis. FG and his donor were seropositive for cytomegalovirus. Cutaneous grade II acute GVHD was clinically diagnosed at day 11 (ML), 22 (FG), and 25 (LC) after alloBMT.

From the Hemato-Immunology Unit, INSERM U333, Transplantation Unit, Departments of Medicine, Biostatistics and Epidemiology, and Pathology, Institut Gustave Roussy, Villejuif, France.
Submitted February 14, 1992; accepted July 7, 1992.

P.-Y.D. has a European Society of Medical Oncology (ESMO) research fellowship.

Address reprint requests to Pierre-Yves Dietrich, MD, Laboratoire d’Hématologie-immunologie, INSERM U333, Institut Gustave Roussy, 39, rue Camille Desmoulins, 94805 Villejuif Cedex, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/8009-0028$3.00/0


2419
Donor blood samples (30 mL) were collected before alloBMT. Recipient blood and skin samples were collected on the first or the second day of GVHD clinical manifestations, before administering specific treatment. PBMC were obtained and cryopreserved as described. Skin biopsies were performed using a 6-mm biopsy punch (Stiefel, Wooburn Green Bucks, UK) in clinically involved (termed GVH+1) and noninvolved (GVH−) areas. Samples were washed with NaCl 0.9% and immediately frozen in liquid nitrogen. In parallel, a third biopsy sample was obtained for standard histological examination (hematoxylin-eosin) and for immunophenotyping with anti-CD3 (D3, Dakopatts, Glostrup, Denmark) and anti-CD20 (L26, Dakopatts) monoclonal antibodies. Informed consent was obtained from all patients.

**Molecular analysis of TCR V gene segments using PCR.** Skin samples were directly (ie, without any cell separation or in vitro culture) squeezed using a Spex 6700 pulverizer (Spex Industries, Edison, NJ). The procedure used to analyze TCR Va and Vβ gene segment expression has been previously described. Briefly, total RNA was extracted from PBMC and skin samples according to a modified guanidinium isothiocyanate-phenol-chloroform extraction method. Complementary DNA (cDNA) was prepared with oligo(dT) priming and reverse transcription (RT). The TCR transcripts cDNA were amplified in a 30-cycle PCR, using the 29 different Va and Vβ 5′-oligonucleotide primers (Va1-w29/Vβ1-w24), each of them paired with the corresponding Ca or Cβ-specific 3′ primer. Positive α and β controls consisted of constant region amplified products (180 and 190 bp, respectively). Negative controls included reactions without cDNA. To ensure that the conditions used for the amplification reactions were similar from one tube to another, an internal control was added: for each Va-Ca amplification, a defined Cβ region fragment was coamplified, and vice versa. The amplified products were detected by Southern blot analysis using a Cα or Cβ oligonucleotide probe. All experiments were performed at least twice.

 Autoradiographs were scanned by computerized quantitative densitometry (Chromoscan 3, Joyce Loelbl, Gateshead, UK) providing an absolute value for each autoradiographic spot. Each Va or Vβ spot was expressed as a percentage of the sum of all Va or Vβ signals detected on the autoradiogramm, respectively. However, it should be pointed out that these calculations were not performed to compare the level of one gene segment expression (eg, Va1) to another (eg, Vα2) in an “horizontal analysis.” Indeed, amplification efficacy is known to be variable from a primer pair to another. Densitometric evaluation allowed intrasample comparisons of a given V gene segment, because the same Vα-Cα or Vβ-Cβ primer pair was always used to perform the amplification reaction of a given V gene segment.

Variation in signal intensities was estimated by studying the ratio of each V spot observed in recipient PBMC to that of donor PBMC (Table 1A), or the ratio of that observed in the skin sample (GVH+) to that of the donor PBMC (Table 1C). A third comparison was made within the recipient material (Table 1B). The distribution of the logarithmic transformation of the ratios was studied to select those that significantly differ from the observed mean with a risk of 10%. Values less than 0.5% were considered as missing values in the analysis, because the biological assay was difficult to interpret in this range.

### RESULTS AND DISCUSSION

**Histological examination.** The histological analysis of the involved skin in the three patients showed epidermal damage characterized by vacuolization of basal cells and several single necrotic keratinocytes in the basal layer (data not shown). These characteristic features were accompanied by a T-lymphocyte infiltrate (CD3+, CD20−) present mostly in upper dermis, lower epidermis, and around dermal vessels. Skin samples taken in clinically noninvolved fields were virtually normal with little, if any, T-lymphocyte infiltration.

**Analysis of TCR α/β V gene segment expression.** TCR Va and Vβ gene segment usage was analyzed for each patient (LC, FG, ML) in three distinct RNA samples: donor PBMC, recipient PBMC at onset of GVHD, and clinically involved skin areas (GVH+). RNAs extracted from these samples were used to synthesize cDNAs, which served subsequently as template of DNA amplification reaction. Coamplification of a Cα or a Cβ fragment was used as an internal positive control (data not shown). The amplified material was revealed by autoradiography. Each amplified Va and Vβ gene product was obtained with the expected size (deduced from the positions of the V and C primers on the cDNA sequence), varying from 250 to 355 bp and a single band was observed in virtually all cases. As shown, donor and recipient PBMC, as well as lymphocytes infiltrating the skin (GVH+ samples), appeared to express virtually all Va and Vβ gene segments (Figs 1 and 2).

Film exposure differences may induce misinterpretation when one compares the relative representation of a unique Va or Vβ subfamily in the different samples derived from the same patient. Using computerized densitometry, we thus expressed each Va or Vβ signal as a percentage of the sum of all Va or Vβ spots detected on the autoradiogram.

### Table 1. Differences in TCR Va/Vβ Gene Segment Subfamily Expression

<table>
<thead>
<tr>
<th>Patient</th>
<th>Va</th>
<th>Vβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Recipient PBMC and donor PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. LC</td>
<td>3.23, 26, 26, 26, 25, 25</td>
<td>18, 22</td>
</tr>
<tr>
<td>FG</td>
<td>9, 10, 12, 14, 17, 19</td>
<td>9, 11, 12</td>
</tr>
<tr>
<td>ML</td>
<td>6, 20</td>
<td>5, 10</td>
</tr>
<tr>
<td>2. LC</td>
<td>18</td>
<td>17, 21</td>
</tr>
<tr>
<td>(B) Recipient skin biopsy and recipient PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. LC</td>
<td>9, 15, 17, 22, 24</td>
<td>21</td>
</tr>
<tr>
<td>FG</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>ML</td>
<td>7, 23, 24, 25, 26</td>
<td>4, 9, 12, 18</td>
</tr>
<tr>
<td>4. LC</td>
<td>3, 11</td>
<td>23</td>
</tr>
<tr>
<td>FG</td>
<td>12</td>
<td>8, 9, 12</td>
</tr>
<tr>
<td>ML</td>
<td>10, 11</td>
<td>16</td>
</tr>
<tr>
<td>(C) Recipient skin biopsy and donor PBMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. LC</td>
<td>18</td>
<td>24</td>
</tr>
<tr>
<td>FG</td>
<td>13</td>
<td>—</td>
</tr>
<tr>
<td>ML</td>
<td>12</td>
<td>21, 23</td>
</tr>
</tbody>
</table>

1 and 3: Absent or underexpressed V gene segment specificities; 2, 4, and 5: overexpressed V gene segment specificities. The underexpression or overexpression has been determined from the distribution analysis as described in Methods. Asterisks denote signals that were faintly detectable or undetectable. The corresponding values (always <0.5%) were not incorporated into the calculations of the distribution analysis because the biological assay was difficult to interpret in this range.
Figure 3 illustrates these relative densitometric values obtained in donor PBMC, in recipient PBMC, and in skin sample for each of the three patients.

The first part of the analysis relates to the blood TCR diversity after alloBMT. As shown, recipient PBMC were characterized at GVHD onset by the expression of nearly all Vα and Vβ gene segments, whose overall relative usage appeared comparable to that of their respective donor. However, some V subfamilies were clearly less expressed (ie, at one of the extremes of the V signal ratio distribution) or even not detected in the recipient PBMC (Table 1A); these data may reflect either an imbalance of the TCR repertoire secondary to the alterations of environmental conditions or a simple delay in reappearance of some V subfamily specificities, or both. Reciprocally, certain V subfamilies displayed a stronger signal in recipient PBMC (Table 1A), such as, for example, Vα18 and Vβ17 (LC) or Vα16 (ML). This relative increase may relate to the expansion of some T-cell subsets in response to antigenic stimulations.

The major objective of this study was to determine Vα and Vβ gene segment usage in lymphocytes infiltrating the skin (GVH+ samples). Avoiding the potential selection induced by cell separation and in vitro culture, the methodology described here allows an in situ analysis of TCR Vα and Vβ gene segment expression. Again, a large variety of Vα and Vβ subfamilies was represented in these samples, with no evidence for a highly preferential usage. As a control for GVH+ samples, clinically noninvolved skin areas (GVH−) were assessed to eliminate the possibility that the GVH+ tissue was heavily contaminated by blood. Little, if any, lymphocytes were detectable on GVH− histological sections (data not shown). After PCR using the same experimental protocol, no signals (except for Vα2, of uncertain significance) were detected on autoradiograms (data not shown).

Some subfamily specificities appeared decreased or increased in GVH+ samples compared to the one observed in the corresponding recipient PBMC (Table 1B). The decreased representation or the absence of some V subfami-
lies in GVH+ samples may reflect either impaired homing or local deletion of the corresponding T-cell subpopulations. Alternatively, certain V gene segments appeared overexpressed in skin, such as for example Vα3 and Vβ23 (LC), Vβ9 (FG), or Vα11 (ML) (Table 1B). These overexpressions may reflect the expansion of T-cell subpopulations following antigenic in situ stimulation.

Finally, certain V gene segments were overexpressed in both GVH+ and recipient PBMC samples when compared with donor PBMC (Table 1C), reflecting possible T-cell expansion in response to a systemic antigenic stimulation.

CONCLUDING REMARKS

The present data show that most Vα and Vβ subfamily specificities are expressed in the three patients’ PBMC collected 11 to 25 days after alloBMT. Whether these lymphocytes derive from actual T-cell precursors or simply reflect the expansion of mature T lymphocytes present in the graft cannot be determined with the unmanipulated alloBMT procedures used in the present study. In any case, these findings support the view that powerful mechanisms operate to maintain homeostasis of TCR repertoire.

We report here the first molecular analysis of α/β TCR chain variability in lymphocytes infiltrating a GVHD target organ. GVH+ skin lymphocytes do not apparently display a restricted TCR Vα or Vβ chain expression. This may reflect that a nonspecific inflammatory response is ongoing locally. Alternatively, it may be related to T-cell stimulation by multiple miHA. Perhaps more likely, a specific response to a limited set of miHA could be masked within an inflammatory infiltrate. Sequencing the TCR transcripts should help
to address these questions. In this regard, particular attention will have to be devoted in further studies to the analysis of those Vα and Vβ subfamilies which display an altered expression in the skin compared with the blood. Such an approach may lead to characterize the TCR chains expressed in T cells that are expanded in vivo. It should guide subsequent in vitro functional studies focused on the more relevant T-cell subpopulations involved in GVHD.

ACKNOWLEDGMENT

We thank Valérie Morand for excellent technical assistance, and all the staff in the Transplant Unit.

REFERENCES


Analysis of T-cell receptor variability in transplanted patients with acute graft-versus-host disease

PY Dietrich, A Caignard, A Diu, C Geneveee, JL Pico, M Henry-Amar, J Bosq, E Angevin, F Triebel and T Hercend