Immunoglobulin Heavy Chain Variable Region Gene Usage in Bone Marrow Transplant Recipients: Lack of Somatic Mutation Indicates a Maturational Arrest

By Ivy Suzuki, Eric C.B. Milner, Annuska M. Glas, Wendy O. Hufnagle, Sambasiva P. Rao, Laurie Pfister, and Carol Nottenburg

Many recipients of bone marrow transplant (BMT) make normal amounts of serum immunoglobulin but are deficient in generating specific antibody responses to exogenous stimuli. To determine if abnormal usage of VH genes contributes to this immunodeficiency, the usage of VH genes was determined in peripheral blood B cells of four BMT recipients, two of whom had developed chronic graft versus host disease. The pattern of usage of V_{H}3 or V_{H}4 genes assessed at either 90 days or approximately 1 year after transplant was similar to that observed in healthy subjects and was marked by the over utilization of two elements, one V_{H}3 and one V_{H}4. However, the repertoires of each of the four BMT recipients appeared to be less complex than the repertoires of healthy subjects. The differences were a consequence of the accumulation of somatic mutations among rearrangements in the controls but not in the BMT recipients. The failure to accumulate somatic mutations in rearranged VH genes is consistent with a defect in antigen driven B-cell responses. These results indicate that although the VH gene content of the repertoire has normalized by 90 days posttransplant, a maturational arrest in B-cell differentiation associated with antigen activation persists for at least 1 year after BMT.

© 1996 by The American Society of Hematology.

From the Virginia Mason Research Center, Seattle, and the Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA.

Submitted July 20, 1995; accepted October 16, 1995.

Supported in part by National Institutes of Health Grants No. AR39918 and CA18221, and American Cancer Society Grant No. IM485.

Address reprint requests to Eric C.B. Milner, PhD, Virginia Mason Research Center, 1000 Seneca St, Seattle, WA 98101.

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.

© 1996 by The American Society of Hematology.
0006-4971/96/8705-0040$3.00/0


1873
The usage of V\textsubscript{H} families following BMT appears to mimic usage during B-cell ontogeny.\textsuperscript{28,40} Thus, to account for the observed immunodeficiencies, it might be expected that during the first year posttransplant, BMT patients would use a more limited set of V genes than would healthy adult subjects. This hypothesis is supported by the results of Storek et al\textsuperscript{18} who found that the B-cell repertoire is restricted to fetal-type V\textsubscript{H} genes early posttransplant. In this report, a direct assessment of the B-cell repertoire was made by analyzing the occurrence of specific V\textsubscript{H} gene segments in rearrangements in peripheral blood (PB) B cells of BMT recipients at 90 days and 1 year following transplant. The results indicated that the pattern of usage of V\textsubscript{H} genes was similar between BMT recipients and healthy subjects. However, BMT recipients exhibited a markedly reduced level of somatic mutation that is consistent with a maturational arrest.

### MATERIALS AND METHODS

**BMT recipients and control subjects.** Four patients (3 women, 1 man) and their marrow donors were recruited through the BMT program of the Fred Hutchinson Cancer Research Center under Institutional Review Board approval (Table 1). All were white. All received marrow from HLA-identical siblings. Two of the patients were diagnosed with GVHD within the first 100 days following transplant. Two healthy controls recruited from laboratory personnel were studied simultaneously. Analysis of the repertoire of these healthy subjects has been published.\textsuperscript{17}

**Cell isolations.** All patient blood was obtained after approval by the Fred Hutchinson Cancer Research Center Institutional Review Board. Healthy subject blood was obtained after approval by the Virginia Mason Research Center (VMRC) Institutional Review Board. PB mononuclear cells (MC) were isolated from a Ficoll-Hypaque gradient (Pharmacia Biotech, Piscataway, NJ). B cells were stained with fluorescein-conjugated anti-IgM or anti-IgD (Coulter, Hialeah, FL), phycoerythrin (PE)-conjugated anti-CD19 or CD20 (Coulter), or appropriately conjugated mouse Ig isotype control antibodies. Viable lymphocytes were selected on the basis of forward and side-angle light scattering criteria. A Coulter Epic 750 or a Becton Dickinson FACstar flow cytometer (Becton Dickinson, Mountain View, CA) was used to sort live B cells into positively stained fractions. The non-B cell (negative) fraction of each sort was also collected. The purity of the sorted B cells ranged from 94% to 95%. Phenotypic analyses are shown (see Table 2).

**Library screening.** Multiple replicate filters were prepared by dot blotting 10 \(\mu L\) of supernatants containing phage particles in a 96-well grid on Hybond N+ nylon filters (Amersham, Arlington Heights, IL). Filters were denatured in 0.5 mol/L NaOH, 1.5 mol/L NaCl, neutralized in 0.5 mol/L Tris-HCl, 1.5 mol/L NaCl, and UV cross-linked. Replicate filters prepared from the libraries were probed as previously described.\textsuperscript{24,41} Hybridization with \textsuperscript{3}P-labeled family-specific V\textsubscript{H} probes allowed a determination of the total number of recombinant clones in each library. A panel of diagnostic \textsuperscript{3}P-labeled oligonucleotide probes that identify individual V\textsubscript{H} gene segments were also hybridized to replicate filters. The frequency of occurrence of each specific V\textsubscript{H} gene segment was calculated by dividing the number of clones hybridizing with an oligo probe by the total number of clones hybridizing with the family-specific probe.

### Table 1. Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Onset of GVHD*</th>
<th>Age at Transplant</th>
<th>Age of Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN5012</td>
<td>F</td>
<td>Chronic myeloid leukemia</td>
<td>88</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td>UPN5007</td>
<td>F</td>
<td>Acute myeloid leukemia</td>
<td>N</td>
<td>54</td>
<td>63</td>
</tr>
<tr>
<td>UPN4986</td>
<td>F</td>
<td>Chronic myeloid leukemia</td>
<td>N</td>
<td>37</td>
<td>33</td>
</tr>
<tr>
<td>UPN5403</td>
<td>M</td>
<td>Chronic myeloid leukemia</td>
<td>90</td>
<td>45</td>
<td>39</td>
</tr>
</tbody>
</table>

Abbreviation: N, no GVHD present.

* Number of days following BMT.
**Table 2. Phenotype of Lymphocytes From BMT Recipients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD20</th>
<th>CDS + CD20</th>
<th>IgM + IgD</th>
<th>CD20</th>
<th>CDS + CD20</th>
<th>IgM + IgD</th>
<th>CD20</th>
<th>CDS + CD20</th>
<th>IgM + IgD</th>
</tr>
</thead>
<tbody>
<tr>
<td>UPN5012</td>
<td>8.22</td>
<td>3.64</td>
<td>ND*</td>
<td>7.91</td>
<td>4.36</td>
<td>2.17</td>
<td>13.6</td>
<td>6.09</td>
<td>11.7</td>
</tr>
<tr>
<td>UPN5007</td>
<td>15.1</td>
<td>5.3</td>
<td>ND</td>
<td>8.94</td>
<td>2.02</td>
<td>4.58</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>UPN5403</td>
<td>9.68</td>
<td>4.0 (CD19)</td>
<td>4.06</td>
<td>3.70</td>
<td>2.43</td>
<td>1.09</td>
<td>16.62</td>
<td>6.04</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

**RESULTS**

**Regeneration of B cells following BMT.** The number and percentage of B cells found in PB were determined at intervals after marrow transplant for the marrow recipients and before marrow donation for the marrow donors (Table 2). Mononuclear cells were stained with a fluorescein-conjugated anti-CD20 or anti-CD19, and in separate analysis, with fluorescein-conjugated anti-IgM and PE-conjugated anti-IgD, and analyzed by flow cytometry. For all patients at both timepoints, the percent of total lymphocytes that were B cells was within normal range. Serum Ig levels were assayed at approximately 90 days and 1 year after transplant (Table 3). By 90 days, serum Ig levels were largely within the normal range. In patient UPN5012, IgM was below normal at 90 days and IgA was below normal at 1 year. In patient UPN4986, IgG was slightly low at 1 year, and in patient UPN5403, IgM was slightly low at 90 days after transplant.

**V<sub>H</sub> gene-specific analysis.** To assess the usage of individual genes within V<sub>H</sub>3 and V<sub>H</sub>4 families, libraries of rearrangements were generated using V<sub>H</sub> family specific 5' primers and the consensus J<sub>H</sub> 3' primer as described previously. This system has been found to amplify approximately 25 V<sub>H</sub>3 genes and 10 to 12 V<sub>H</sub>4 genes, which together account for more than 75% of the total expressed V<sub>H</sub> repertoire. Synthetic oligonucleotide probes that specifically identify both germ line and rearranged individual V<sub>H</sub> elements directly in genomic DNA and in libraries of cloned V regions have been described. For this report six V<sub>H</sub>3 and 11 V<sub>H</sub>4 gene segments were selected for analysis based on two criteria: (1) the gene could be amplified quantitatively from the germline in control experiments, and (2) specific, diagnostic oligonucleotide probes were available for the gene. The six V<sub>H</sub>3 elements assessed here account for approximately 50% to 80% of the V<sub>H</sub>3 component, and the V<sub>H</sub>4 elements assessed account for virtually 100% of the V<sub>H</sub>4 component of the expressed repertoire (discussed later). Therefore, we estimate that the 17 elements assessed comprise from 45% to 60% of the total expressed repertoire.

Rearrangements of the six V<sub>H</sub>3 genes were assessed in PB cells of four BMT recipients and compared to similarly obtained rearrangements from two healthy subjects. Heavy chain rearrangements were amplified quantitatively and the resulting PCR products were cloned into a phagemid vector. Identification of the V<sub>H</sub> gene present in an individual clone was established by hybridization with a sequence-specific oligonucleotide probe. More than 700 independent rearrangements from each individual were analyzed. For controls, amplifications, and subsequent identification of nonrearranged V<sub>H</sub>3 and V<sub>H</sub>4 genes from the same individuals were also performed.

The occurrence of V<sub>H</sub>3 genes in rearrangements for all subjects is shown in Fig 1. The occurrence of rearranged V<sub>H</sub>3 genes assessed at 90 days and approximately 1 year after transplant is similar to that observed in the healthy controls. The variation between individuals is similar to that seen previously in a healthy population. One of the healthy subjects (Nor4882) and one of the BMT recipients (UPN5403) have a deletion of the V3-11 gene and one of the BMT recipients (UPN5007) has a deletion of the V3-33

**Table 3. Serum Ig Levels of Marrow Recipients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgM</th>
<th>IgG</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 d</td>
<td>1 yr</td>
<td>90 d</td>
</tr>
<tr>
<td>UPN5012</td>
<td>34*</td>
<td>154</td>
<td>846</td>
</tr>
<tr>
<td>UPN5007</td>
<td>190</td>
<td>ND</td>
<td>899</td>
</tr>
<tr>
<td>UPN4986</td>
<td>185</td>
<td>116</td>
<td>705</td>
</tr>
<tr>
<td>UPN5403</td>
<td>52</td>
<td>57</td>
<td>784</td>
</tr>
<tr>
<td>Normal</td>
<td>56-275</td>
<td>670-1,700</td>
<td>70-350</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

* mg/dL.
gene (data not shown). These deletions account for the failure to detect rearrangement of these genes in these subjects (Fig 1).

In addition, rearrangements of individual V\(\mu\)4 genes were assessed in PB B cells of one of the BMT recipients and from one of the healthy subjects (Fig 2). As was the case for V\(\mu\)3 rearrangements, no significant difference was observed between the patient and the control. To the extent that these results are representative of the entire B-cell repertoire, they indicate that the repertoire following BMT contains substantially the same assortment of V\(\mu\) genes as that found in nontransplanted controls.

The repertoires of the BMT recipients are less complex than are the repertoires of healthy subjects. Figure 3 shows the percent of rearrangements in which the germline V\(\mu\) gene of origin could be assigned by hybridization. Among the V\(\mu\)3-containing rearrangements (A), there was a slight trend toward increased identification in BMT recipients compared to the two healthy subjects (\(p < 0.005\) for patients v controls). This trend was more pronounced among V\(\mu\)4-containing rearrangements (B). Sequence-specific oligonucleotide probes to 9 V\(\mu\)4 loci (11 distinct gene segments) identified more than 99% of the V\(\mu\)4 rearrangements in the BMT recipient, but only 80% of the V\(\mu\)4 rearrangements in the healthy subject (\(p < 10^{-5}\) for patients v control). This observed difference in the percentage of identified rearrangements between healthy subjects and the BMT patients could be because the BMT recipients used fewer V\(\mu\) genes. However, extensive hybridization and sequence analysis have not revealed the presence of additional V\(\mu\) genes rearranged in healthy subjects but not rearranged in BMT recipients (A.M. Glas and E.C.B. Milner, unpublished observations, 1994). Alternatively, and more likely, these results suggest that the abrogation of hybridization resulted from the accumulation of somatic mutations in the target regions of the probes in healthy subjects but not BMT recipients. Therefore the accumulation of somatic mutations was assessed in the two groups.

Detection of somatic mutations by sequence-specific hybridization. The accumulation of somatic mutations in BMT recipients and healthy subjects was addressed directly in the following manner. Somatic mutations in one V\(\mu\)3 gene, V3-23, can be detected by sequential hybridization with multiple probes. The germ line sequence of V3-23 can be detected by either a CDR1 probe or a FR3 probe. Hybridization of both of these probes on rearranged V3-23 genes indicate which have retained the germ-line sequence through the target regions. However, rearranged V3-23 genes that have accumulated one or more mutations in the target site of one or the other probe will display a loss of concordance when hybridized. Figure 4 shows the percent of V3-23 rearrangements that have lost concordance for one of the probes in the four BMT patients and the two healthy subjects. Among the BMT recipients, the percent of V3-23 rearrangements isolated from CD19+ or CD20+ B cells that have acquired mutations ranged from <1% to approximately 10%. For patients UPN5403 and UPN4986 the percentage was similar between samples taken 90 days and 1 year posttransplant. The distribution of somatic mutations in the healthy subjects was assessed in different B-cell populations. In one of the healthy subjects, rearrangement libraries were constructed from three B cell populations: (1) CD19+ B cells (all B cells), (2) CD19+, IgD+ B cells (preimmune B cells), and (3) CD19+, IgD- B cells (antigen-driven B cells). Among CD19+ B cells, approximately 30% had acquired mutations in CDR1 of V3-23 (Fig 4). When the CD19+ B cell population was further fractionated on the basis of IgD expression, it was found, as expected, that the vast majority of mutations could be attributed to the IgD+ population. More than 70% of V3-23-containing rearrangements from IgD- cells had detectable mutations. In contrast, approximately 10% of rearrangements from IgD+ cells had detectable mutations, a value that is comparable to that observed among the BMT recipients. In a second experiment, B cells from another healthy subject were sorted for IgM. Approximately 20% of V3-23-containing rearrangements from IgM+ cells in this subject had acquired mutations. This value is approximately
twice the frequency of occurrence of mutations among the BMT recipients. Together, these results indicate that utilization and diversification of $V_H$ genes in the peripheral B-cell repertoire of BMT recipients is similar to the utilization and diversification of $V_H$ genes in the preimmune component of the peripheral B-cell repertoire of healthy subjects.

DISCUSSION

In this report, the usage of $V_H3$ and $V_H4$ genes in four BMT recipients was assessed. In this regard, the frequency of rearrangements that contained each of 6 distinct $V_H3$ loci, and 9 $V_H4$ loci (11 gene segments) was determined among PB B cells of these subjects. We have previously found that healthy adult subjects exhibit a biased but highly reproducible pattern of $V_H$ gene utilization in rearrangements.\(^{17,19}\) The results reported here indicate that, compared to healthy subjects, there were no apparent differences in the spectrum of genes used at either 90 days or approximately 1 year after transplant among the BMT recipients. We interpret these observations to mean that the processes involved in generating the antibody repertoire are largely functional within the first few months following BMT. Furthermore, these results indicate that the immunodeficiencies common among BMT recipients are not likely because of the failure to use appropriate V region genes in generating the preimmune antibody repertoire.

Fig 4. Analysis of somatic mutation in rearranged $V_H3$ genes from BMT recipients and normal subjects. The accumulation of somatic mutations in one $V_H3$ gene, V3-23, was assessed by sequential hybridization with the probes, M8 and M18, as described in Materials and Methods. Clones that have accumulated one or more mutations in the target site of one or the other probe will display a loss of concordance when hybridized. Each instance of discordant hybridization is recorded as a single mutation. The results are presented as the percent of V3-23 rearrangements that have acquired a mutation (i.e., lost concordance for one of the probes).
As for the specific immunodeficiency of BMT recipients, our results provide indications of possible mechanisms contributing to poor antibody responses. Strikingly, rearranged \( V_\mu \) genes exhibited much less somatic mutation in BMT recipients than did similar rearrangements obtained from healthy subjects. In contrast to healthy subjects, more than 90% of peripheral B cells from BMT recipients are phenotype typically CD19^+, IgM^+, IgD^-. This phenotype is characteristic of preimmune B cells. When we stratified the peripheral B cell compartment from the healthy subjects, as expected, somatic mutations were found preferentially among the IgD^+ population, a population that is all but nonexistent among BMT recipients. The frequency of mutations among the IgD^+ population, and, to a lesser extent, the IgM^+ population, was similar to that seen in the BMT patients (Fig 3). Thus, by both cell-surface phenotype and extent of somatic diversification, the B-cell repertoire of BMT recipients resembles the preimmune component of the B-cell repertoire of a healthy adult, but lacks features of a mature B-cell repertoire.

The accumulation of somatic mutations is a characteristic of T cell-dependent antigen-driven responses. Conversely, the absence of a somatically diversified B-cell population suggests an absence of antigen-driven processes. The failure in the BMT recipients to accumulate somatic mutations in rearranged \( V_\mu \) genes is consistent with a defect in antigen-driven B-cell responses. However, not all aspects of antigen-driven responses are defective in BMT recipients. For example, BMT recipients are able to make high titers of antibodies and to exhibit class switching, processes that are also dependent on the presence of functional CD4^+ T cells. As such, the data are most consistent with a maturational arrest, which may be limited to the stage of B-cell differentiation during which somatic mutation occurs.

As somatic mutation and affinity maturation are thought to occur primarily in lymph node germinal centers, one attractive hypothesis is that a failure of germinal center processes prevents the normal accumulation of somatic mutations following immunization in BMT recipients. Consistent with this hypothesis are the observations that germinal centers are generally absent from lymph nodes on histologic analysis for months to years in BMT recipients, and that the reconstitution of CD4^+CD8^- T cells is delayed. In contrast to BMT recipients, germinal centers are present in neonates from about 1 month of age.

Although GVHD is a potent suppressor of immune function, the presence of GVHD cannot be the direct cause of the apparent maturational arrest observed in these studies because two of the patients were free of GVHD. Significantly, there were no observable differences in the diversification of the antibody repertoire between patients with or without GVHD.

Although the data presented here provide an explanation for the specific immunodeficiencies after BMT, further study is needed to determine the parameters of recovery of the capacity to mount an effective antibody response. In this regard, analysis of the cause and effect relationship between ineffective antibody responses and germinal center formation in BMT recipients is likely to be especially illuminating.

It may be, for example, that the pretransplant conditioning regimen disrupts a critical cellular function, or destroys a critical population of cells, that is not restored by marrow transplant. In addition, a more comprehensive analysis of recovery of T-cell function might provide insights. Neither T-cell phenotype nor functional analysis was available for the patients studied here. In general, recovery of normal numbers of CD4^+ T cells is slow and may not be achieved for more than 1 year posttransplant.

ACKNOWLEDGMENT

We are grateful to Dr Jan Storek for help with flow cytometry, and Bonnie MacGregor for assistance in obtaining patient clinical data.

REFERENCES


41. Nottenburg C, Rees G, St. John T: Isolation of mouse CD44 cDNA. Structural features are distinct from the primate cDNA. Proc Natl Acad Sci USA 86:8521, 1989


44. Willems van Dijk K, Milner LA, Sasso EH, Milner ECB: Chronosomal organization of the heavy chain variable region gene segments comprising the human fetal antibody repertoire. Proc Natl Acad Sci USA 89:10430, 1992


54. Lum LG: Immune recovery after bone marrow transplantation. Bone Marrow Transplant 4:659, 1990
Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest [see comments]

I Suzuki, EC Milner, AM Glas, WO Hufnagle, SP Rao, L Pfister and C Nottenburg