Reconstitution of Human Immunoglobulin VH Repertoire After Bone Marrow Transplantation Mimics B-Cell Ontogeny

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Bone marrow transplantation (BMT) is now widely used as a standard and effective therapy for patients with hematologic malignancies. The transplanted marrow contains a mixture of stem cells and lymphoid cells at various stages of development. After transplantation, stem cells start to differentiate in the BM and colonize the periphery after a period of 2 to 3 weeks.1

Before reconstitution of both the B and T compartments, a period of profound cellular and humoral immune deficiency is observed for several months. So far, impaired reconstitution of the B-cell lineage has been mainly documented through the ability of producing specific antibodies against defined antigens such as tetanus toxoid.2,3 or, alternatively, through the ability of cells to respond to polyclonal activators.4 Levels of serum IgG can be easily measured as well as the percentage of the various isotypes expressed. IgM and IgG—and more precisely, IgG2 and IgG4 subclasses—are produced at a very low level for several months and IgA does not reach normal values until several years.5,6 This progressive shaping of serum Ig isotypes pattern follows roughly that described in normal young children.7 In addition, several investigators have reported an increased percentage of the CD5+ B-cell subpopulation compared with the situation of normal adults,8,9,10 which is suggestive of values closer to that reported for fetal tissues, such as spleen and cord blood, in which CD5+ B cells are predominant.

The human IGVH locus located on chromosome 14 extends approximately over 2,000 kb. The number of VH genes may be evaluated to less than 100, including a substantial proportion of pseudogenes.11,12 These genes can be grouped into six families based on sequence homologies. The size of each family varies from 1 member (VH6) to around 30 for VH3. We have recently defined the different VH family usage in normal peripheral blood lymphocytes (PBLs) and in a large number of Epstein-Barr virus (EBV) clones derived from fetal or adult tissues.13 Our results14 and those from others15 indicate that the expression pattern of the different families in the adult PBL merely reflects their respective sizes. So, VH3, which is the largest family, accounts for about 60% of the heavy chains, whereas the VH6 single-member family represents less than 5% of the VH expressed repertoire. Early in ontogeny, VH6 and VH5 have been shown to occur first and to be transiently overexpressed.16,18 This seems, at least in part, to correlate with the localization of VH6 and one of the VH5—a small family composed of 2 or 3 members—at the 3’ end of the VH locus.11,18,20

Analysis of the expression pattern of the six VH families therefore provides a precise basis to evaluate the acquisition of the Ig repertoire in the course of reconstitution after allogeneic or autologous BMT. In this report, the kinetics of reconstitution of the Ig repertoire is evaluated by following the relative expression of the six VH families. Our results indicate that during the early posttransplantation period (ie, about 3 months), the expressed repertoire of VH is dramatically affected. Kinetics of reconstitution mimics normal repertoire development described in ontogeny, although normalization, as compared with the adult pattern, may take as long as 1 year.

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Materials and Methods

Normal donors. Marrow samples were obtained from five normal adult donors.

Patients. Twelve patients suffering from hematologic malignancies (acute myelogenous leukemia [AML], 5; chronic myelogenous leukemia [CML], 3; ALL, 3; M6 lymphoma, 1) were analyzed in this study. All patients received a preparative regimen,21 including total body irradiation (TBI), followed by infusion of an unmanipulated BM. In seven cases, the graft was harvested from an HLA-identical sibling and patients received an association of methotrexate and cyclosporine A as postgraft immunosuppression.22 Six patients developed an acute graft-versus-host disease (GVHD), followed in five cases by a chronic GVHD. Five patients received an autologous graft: three of them were treated either with recombinant interleukin-2 (IL-2) (n = 2) or interferon alpha (INFα) (n = 1) 1 to 3 months after BMT.25,24 BM samples were collected at days 30, 60, and 90 after transplantation (and over 1 year for one allogeneic grafted patient). All normal donors and patients gave informed consent before the procedure.

Mononuclear cell preparation. Human adult BM mononuclear cells were obtained from 1 to 5 mL of heparinized BM by centrifugation on FICOLL-Hypaque. After extensive washing with phosphate-buffered saline (PBS) (1 U heparin/mL), the cells were directly used for in situ hybridization.

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Fig 1. In situ hybridization of normal BM cells with the VH3-specific probe using the hybridizing (right) and nonhybridizing (left) strands. Counterstaining was done with 10% Giemsa for 30 seconds. Original magnification × 63.

In situ hybridization. DNA probes were subcloned in M13 bacteriophage as previously described. They were [3H]labeled by the primer extension technique and yielded a specific activity of 3 to 4 × 10^6 cpm/μg. Probes were purified on 6% polyacrylamide gel before use.

In situ hybridization using VH1 to VH6, Cμ, Cγ, probes using the hybridizing and the nonhybridizing strands was performed as previously described. Some minor modifications were introduced: microscope slides were siliconized, immersed into a solution of 3-aminopropyl triethoxysilane (Fluka) in toluene for 30 minutes at 90°C, washed in toluene, and finally air dried. To obtain active aldehyde groups, the slides were then incubated in 4% paraformaldehyde, 10 mmol/L Na-phosphate, pH 7.0 for 30 minutes, washed in the same buffer, and rinsed with water. Cells in a 5-μL drop at 2 × 10^6 cells/mL (5 μL heparin/mL) were allowed to sediment onto treated glass slides for 1 hour. The slides were immersed for fixation into freshly prepared 4% paraformaldehyde in PBS for 20 minutes and washed four times for 5 minutes in PBS.

Prehybridization was performed at 35°C for 3 to 5 hours with 0.1 mL of a solution of 50% formamide, 0.6 mol/L NaCl, 2 mmol/L EDTA, 10 mmol/L TRIS-HCl, pH 7.5, 1.6 × Denhardt’s, 150 mg/mL sonicated salmon sperm DNA, and 500 mg/mL tRNA. Hybridization was made with 9 μL of the same buffer containing 6,500 dpm/μL of the DNA probe. Slides were covered with a coverslip and incubated for 20 hours at 35°C in a humid chamber containing 50% formamide and 0.6 mol/L NaCl. Washings were performed over 24 hours first in 50% formamide, 1 mmol/L EDTA, 10 mmol/L TRIS-HCl, pH 7.5, with various concentrations of NaCl (0.6 or 2.4 for CH and VH probes, respectively), and then in PBS. Slides were air dried and dipped in a Kodak nuclear track emulsion (NTB-2, Eastman, Kodak, Rochester, NY). Emulsion-coated slides were exposed for a minimum of 3 weeks at 4°C. Development was performed in D19 (Kodak) solution for 4 minutes and fixed (Kodafix). Counterstaining was done with 10% Giemsa for 30 seconds.

A total of 2,000 cells per slide were screened for each probe using a Leitz (Heidelberg, Germany) laborlux K microscope. Cells bearing 10 or more silver grains were considered positive. Relative expression of the various families was given in percentages of average values. Statistical analysis was performed using the Mann-Whitney U test.

Specificity of probes was previously checked on a panel of IgM-secreting EBV clones: No cross hybridization between the discrete VH families was observed. Controls also included usage of the nonhybridizing strand.

RESULTS

VH family usage in normal adult BM cells. Normal BM cells from five healthy donors were analyzed by in situ hybridization. A typical pattern is given in Fig 1, using the VH3 probe (hybridizing and nonhybridizing strands). Hybridization with the Cμ and Cγ probes yielded an average of 0.3% to 11.0% of mononuclear cells. Cells expressing the γ isotype were twice as numerous as those of the μ isotype.

A clear pattern of VH expression (Table 1) could be defined according to the following gradient: VH3 > VH1 > VH4 > VH5. These values were similar to those reported earlier for peripheral blood lymphocytes, as analyzed by in situ hybridization on adult B-cell clones. Expression of VH2 and VH6 in the BM samples was below the detection level.

VH family usage after BMT. VH expression pattern was followed in BM-grafted patients over a period of 30, 60, and 90 days and compared with that defined above. The average percentage of cells detected upon hybridization increased to
Table 1. VH Family Usage in Normal and Allografted BM Cells

<table>
<thead>
<tr>
<th>VH Family</th>
<th>Normal BM (n = 5)</th>
<th>After Allogeneic BMT (n = 7)</th>
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<tr>
<td></td>
<td>d 30</td>
<td>d 60</td>
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<tr>
<td>1</td>
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<td>6</td>
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<td>2 ± 1</td>
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Results are expressed as average values ± SE.

* Significant at 5%.
† Significant at 1%

3% (days 60 and 90), a value that is indicative of a higher proportion of activated cells in the analyzed population. Although individual variations were observed, it can be seen on Table 1 that the average values were greatly modified within the first months following allogeneic BMT (seven cases). There is no obvious difference between patients who did or did not develop a chronic GVHD. Essentially, the relative contribution of the VH3 major subgroup was deeply decreased by day 30, to reach one third of the original value by day 60 (P < 1%) and was balanced by a correlative increase in the expression of VH2, VH4, VH5, and VH6 (P < 5%).

On day 90, slopes were inverted for most families (VH2, VH3, VH4, and VH6) suggesting that an evolution toward a normal adult pattern had started. In one case, representative of the average values, VH family expression could be followed for 1 year. It can be seen in Fig 2 that by this time, expression of the various families had indeed reached values close to those of the donor cells (day 0 in Fig 2) except for VH5.

The kinetics of reconstitution of the VH repertoire was also analyzed in five patients who received an autograft. It can be seen in Table 2 that the same general pattern was obtained: a twofold decrease of the VH3 family (P < 1%) was observed and appeared mostly compensated by an increase of VH5 (P < 5%).

DISCUSSION

Reconstitution of the immune system in patients grafted with BM has been followed by multiple criteria, including phenotypic approaches based on the identification of some of the B- and T-lymphocyte CD markers, identification of Ig isotypes, or functional restoration of the immune response to well-defined antigens. On these bases, it was suggested that B-cell differentiation in BM grafted patients could be considered a "recapitulation of normal B-cell ontogeny."16

In this report, we used a different approach based on the analysis of the kinetics of expression of the various VH families over several months following the graft, both in autologous and allogeneic conditions, using in situ hybridization with VH family-specific DNA probes.

Despite individual variations, a clear evolution pattern was obtained for the VH families (Tables 1 and 2). In all cases, expression of the VH3 family, which accounts for over 60% of the VH repertoire in the adult, was profoundly decreased, with a correlative increase of expression of the other families. As the BM of the grafted patients does not start to repopulate before 2 to 3 weeks, this relative increase is mostly the result of expansion of new B-cell clones expressing the "minor" VH families (VH2, VH4, VH5, and VH6) and suggests that an active process of reconstitution of the Ig repertoire is taking place. We have previously shown that in early ontogeny (ie, between 7 and 9 weeks of gestation), VH5 and VH6 families occurred first and were overexpressed as compared with the adult pattern.16 VH expression analysis was also previously performed on 41 EBV clones derived from 8- to 13-week-old embryos.13 The expression pattern of VH gene evolves rapidly and at week 13 is very close to that described in the adult; ie, VH3 remains the major subgroup. Moreover, we have also previously compared the VH family usage in normal adult PBLs by in situ hybridization and in a large sampling of EBV clones. Our results clearly indicated that EBV transformation does not introduce a bias in the repertoire utilization.14 It is therefore tempting to compare both situations. The diagram in Fig 3 clearly illustrates that during the reconstitution period, the expression pattern of the VH families

![Fig 2. Kinetics of reconstitution of the VH repertoire in an allografted patient. Values at day 0 are those taken from the donor BM.](https://www.bloodjournal.org/)

Before and after BMT, the relative increase is mostly the result of expansion of new B-cell clones expressing the "minor" VH families (VH2, VH4, VH5, and VH6) and suggests that an active process of reconstitution of the Ig repertoire is taking place. We have previously shown that in early ontogeny (ie, between 7 and 9 weeks of gestation), VH5 and VH6 families occurred first and were overexpressed as compared with the adult pattern.16 VH expression analysis was also previously performed on 41 EBV clones derived from 8- to 13-week-old embryos.13 The expression pattern of VH gene evolves rapidly and at week 13 is very close to that described in the adult; ie, VH3 remains the major subgroup. Moreover, we have also previously compared the VH family usage in normal adult PBLs by in situ hybridization and in a large sampling of EBV clones. Our results clearly indicated that EBV transformation does not introduce a bias in the repertoire utilization.14 It is therefore tempting to compare both situations. The diagram in Fig 3 clearly illustrates that during the reconstitution period, the expression pattern of the VH families
Table 2. VH Family Usage in Normal and Autografted BM Cells

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† Significant at 1%.

is much closer to the fetal pattern than to that of the adult. There is no statistical difference between the VH repertoire expressed during allograft transplantation on day 60 and the VH repertoire expressed by EBV clones between 8 and 13 weeks of gestation, especially for VH1, VH2, VH3, VH5, and VH6. Therefore, the emergence of the new Ig repertoire in BM-grafted patients seems to follow mainly the pattern defined in ontogeny. However, the kinetics are much slower in BM reconstitution. After 3 months, the expression pattern is still not of the adult type with a downexpression of the VH3. We could follow the kinetics of Ig expression over 1 year in one single allografted patient (Fig 2). After that period, the VH family usage indeed resembled very closely that defined in normal adult BM, a conclusion consistent with the long delay previously reported to ensure restoration of a normal response to a selected number of well-defined antigens. According to previous reports, our data therefore suggest that in grafted patients, transplanted stem cells reconstitute an immune system following a kinetics that mimics ontogeny. Reconstitution seems to proceed similarly in allografted and autografted patients. A careful analysis should nevertheless take into account that some of the latter were treated with IL-2 (two cases) or INFα (one case), a situation that may influence noticeably the activation state. On the other hand, the development of a GVHD in most allografted patients may have similar effects on lymphocyte activation and on selection of the VH repertoire. Transient preferential expression of VH families in grafted patients and in ontogeny might result either from a topologic effect in relation to their localization at the 3’ end of the IGVH locus or be linked to the selection of a particular repertoire implicated in learning of self–nonself discrimination or both. Destruction of the immune system on irradiation abolishes the regulation networks that control the continuous emergence of the immune repertoire in a physiologic situation of permanent seeding of new B-cell clones from BM stem cells. In this regard, it may be not surprising that “relearning” of the self–nonself discrimination in grafted patients uses the same pathway in autologous and allogeneic grafts. Therefore, mimicry of the ontogenetic development may be caused by the same absence of regulatory control. Use of the “minor” VH subgroups might also be more involved in self-recognition, a possibility supported by the analysis of the repertoire of some autoimmune diseases, whereas most of the “conventional” antiforeign antigens would pertain to VH1 and VH3.

A preliminary analysis of the reconstitution of the Vk chain repertoire has been made (data not shown). No clear characteristics of the reconstituted repertoire, as compared with that of the adult BM, could be detected. Indeed, this is not
really surprising in the view of our earlier observations that indicated that as soon as κ chains started to be expressed in ontogeny, they rapidly expanded to reach the adult pattern. This difference in the "maturation" of the VH and κ repertoire remains puzzling. As previously discussed, this might suggest that the selective pressure that drives the emergence of the Ig repertoire operates in a two-step manner. The first would select the VH repertoire in the context of a mainly "pre-B environment," in the fetal liver and/or in the BM, whereas the light chain repertoire would be selected only in a VH-κ association context that would be largely driven, at the periphery, by the "classic" foreign antigens.

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Reconstitution of human immunoglobulin VH repertoire after bone marrow transplantation mimics B-cell ontogeny

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