B cell-autonomous somatic mutation deficit following bone marrow transplant

B cel-autonoom gebrek in somatische mutatie na beenmergtransplantatie

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Annuska Maria Glas

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Cover: modified human IgG_1 structure. ©Annuska Glas

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"If we knew what we were doing it wouldn't be called research, would it?"
Albert Einstein

I wish to thank everyone who contributed to this thesis.

Stellingen

1. The failure in BMT recipients to accumulate somatic mutations in their V_H region genes in peripheral blood lymphocytes, correlates with an absence of IgD negative cells, and is consistent with a maturational arrest at a fairly late stage of differentiation.

Dit proefschrift

2. The sequence of events that occur during successful BMT can be regarded as a blueprint for immune reconstitution in other clinical settings. However, in the description and interpretation of these events, it is important to realize that immune reconstitution does not appear to recapitulate human fetal ontogeny.

Raaphorst, F.M. 1998. Bone marrow transplantation, fetal B-cell repertoire development, and the mechanism of immune reconstitution [letter]. *Blood* 92:12, 4873

3. Oligonucleotide hybridizations are a useful tool for identifying a large number of genes and to determine the occurrence of somatic mutations among virtually any gene without the need for sophisticated instrumentation.

Dit proefschrift

4. Immunodeficiencies common among BMT recipients are not due to an abnormal usage of V_H gene segments.

Dit proefschrift

5. The inability of B cells from BMT recipients to accumulate somatic mutations appears to be a B cell autonomous deficit.

Dit proefschrift

6. The hematopoietic microenvironment represents a complex network of inductive signals, regulatory molecules, and cell-cell interactions that permit the simultaneous determination of various hematopoietic cell fates. The interaction of notch with multiple components of this regulatory network may allow it to function as a master regulator, integrating various signaling pathways to limit the number of cells that respond to diverse signals.

Milner, L.A. and Bigas, A. 1999. Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood* 93:8 2431

Stellingen behorende bij het proefschrift " B cell autonomous somatic mutation deficit following bone marrow transplantation" van Annuska Glas, Wageningen, 2000

7. The use of HLA-DRB1 genotyping in the early arthritis patient, during the initial clinical and laboratory assessment, should be a useful tool to identify a subset of patients with a high risk of early joint erosions and consequent poor clinical course.

Nepom, G.T. 1998. Major histocompatibility complex-directed susceptibility to rheumatoid arthritis. *Adv. Immunol.* 68:315

8. When everything is coming your way, you are in the wrong lane

Steven Wright

10. Ergert u niet maar verwondert u zich slechts, dan leeft u langer
Don't be aggravated but be amazed, you'll live longer.

12. The generation of random numbers is too important to be left to chance.

Robert R. Coveyou

Stellingen behorende bij het proefschrift " B cell autonomous somatic mutation deficit following bone marrow transplantation" van Annuska Glas, Wageningen, 2000

ter nagedachtenis aan mijn vader

Contents

Chapter 1

Introduction

The bone marrow consists of a sponge-like reticular framework filled with blood cell precursors from stem cells through mature cells of all haematopoietic cell types (except T cells), and a fair amount of adipose tissue. It is the major haematopoietic organ and is critically involved in the production of all formed blood elements in postnatal life. The bone marrow contains rapidly dividing cells from the immune system and therefore is sensitive to DNA damaging agents. In certain types of cancers where a high dose of radiation and chemotherapeutic agents are needed, a bone marrow transplant is necessary to "rescue" the patient from the lethal side effects of radiation and chemotherapy.

More than fifty years have gone by since the first experiments in mice that lead to the wide application of human stem cell transplantation. In 1949 Jacobson et al [1] found that shielding of the spleen of a mouse during otherwise lethal irradiation permitted survival. Further animal studies ranging from mice to dogs and nonhuman primates defined the essentials of transplantation biology. The first attempts of bone marrow transplantation in patients were mostly unsuccessful in the early 1960s. After the discovery of human leukocyte antigens (HLA), which are expressed on the surface of essentially all cells, and are held responsible for organ and tissue graft rejection, a rational approach for the selection of suitable donors could be taken. The first successful HLA-matched marrow transplant was reported in 1968 [2]. Since the first grafts in the late 1960s thousands of transplants have been carried out successfully. According to data compiled by the International Bone Marrow Transplant Registry approximately 45,000 transplants were performed worldwide in 1997 only, of which 30,000 were autologous transplants and 17,000 were allogeneic transplants, in which the donor was a closely matched sibling or unrelated donor [3]. However, marrow recipients who survive the initial post-grafting period do not always become fully immunocompetent; recovery of immune function takes up to several months for autologous transplant recipients and 1 to 2 years for patients receiving allogeneic transplants. The experiments described in this thesis are focused on the recovery of the B cell compartment following BMT. In order to understand the functional deficits that occur after transplant, B cell development in healthy individuals will be discussed first.

Cells of the immune system.

Throughout postnatal life, all blood cells are derived from pluripotent stem cells in the bone marrow. These stem cells have the capacity to self-renew, and are able to differentiate along a number of alternate pathways: the lymphoid, myeloid, thrombocytic and erythroid lineages (Figure 1). Cells that develop along the myeloid lineages differentiate into neutrophils, monocytes/macrophages, eosinophils, erythrocytes, megakaryocytes and mast cells.

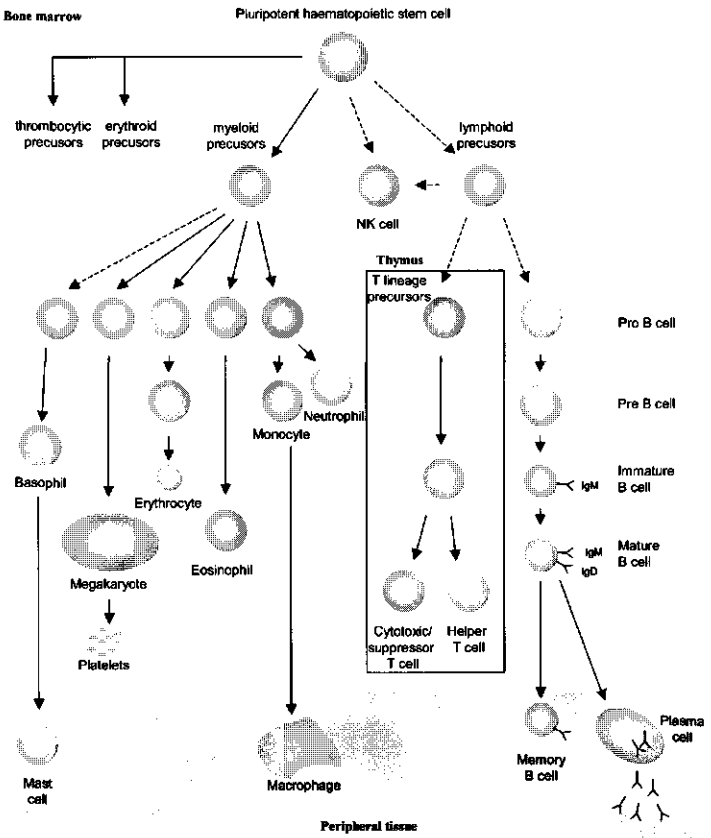

Figure 1. The hypothetical relationship between blood cells of the various lineages. All cells derive from common stem cells. Uncertainties about early steps in the process is indicated by dashed lines. The boxed area shows T cell differentiation in the thymus; the shaded area shows differentiation in peripheral tissue. All other steps take place in the bone marrow.

Lymphocytes are derived from haematopoietic stem cells that have developed along the lymphoid pathway. This differentiation process is only partially understood. Evidence for the existence of a common lymphoid precursor is limited; however, it seems reasonable to believe that there is a common lymphoid precursor cell that gives rise to the different classes of lymphocytes. Two broad classes can be recognized: B- and T-lymphocytes; both classes are part of the specific immune system that is induced or stimulated by exposure to foreign substances. T lymphocytes can be further subdivided into two main, functionally distinct, populations: cytotoxic CD8 T cells that lyse cells which produce foreign antigens, and CD4 helper T cells that allow B cells to make antibody responses to proteins and other T dependent antigens. Further subsets of T cells include γδ T cells, which are a rare population in humans and its function is not exactly known, and suppressor T cells, which can down regulate the immune response. The other class of lymphocytes, the B cells, is responsible for the synthesis and secretion of antibody molecules. A unique feature of B lymphocytes is that all B cells together are pre-programmed to create an enormous number of antibodies. Each individual B cell creates an antibody that has combining sites that differ from antibodies produced by other B cells. This is achieved by creative use of immunoglobulin gene segments, and further fine-tuning by somatic mutation, which will be discussed below.

B cell development

The early maturation steps of B cell development take place in the bone marrow, and are accompanied by specific changes in the immunoglobulin structure and mRNA expression. The first step in the generation of the antibody repertoire includes combinatorial joining of one diversity (D) segment to one joining (J_H) segment with the deletion of the intervening DNA, followed by the joining of one of many

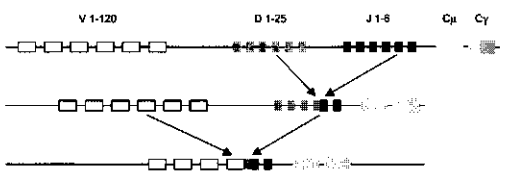

Figure 2. Sequence of human heavy chain rearrangement. V genes are indicated as V 1-120, D genes as D 1-25, and J genes as J 1-6. Gene segments and distances between them are not shown to scale.

variable (V_H) segments to D-J_H segments to form the Ig heavy chain V domain in precursor pro-B cells [4;5] (Figure 2). Cells that have functionally rearranged VDJ regions move on to the pre-B cell stage, where the VDJ forms a pre-B cell receptor with a surrogate light chain [6]. In the transition to the immature B cell stage, the pre-B cell receptor is lost and replaced as surface IgM with a light chain. The cells then emerge from the bone marrow as IgM-expressing, short-lived transitional B cells [7], and home to peripheral lymphoid organs where they continue the sequence of maturational steps that lead to a mature activated B cell stage. However, only a relatively small fraction of the transitional B cells enter the mature long-lived compartment. This process is thought to be competitive and dependent on survival signals that are delivered in secondary lymphoid organs [8;9]. Once the cells are recruited into the long-lived compartment, B cells are subject to an antigen dependent mutation and selection process that is designed to increase the affinity and functional efficiency of the immunoglobulin repertoire. This closely regulated process occurs in a specialized microenvironment, the germinal center, is helper-T dependent and is thought to be initiated in the T cell rich regions of the secondary lymphoid organs [10].

Germinal centers (GC) represent a complex interaction between many secreted and membrane bound molecules and at least three cell types: T lymphocytes, B-lymphocytes and follicular dendritic cells (FDCs) [10]. In this tightly regulated microenvironment, the cells undergo the process of heavy chain class (isotype) switching, and somatic mutation occurs in response to specific signals [11;12] (reviewed in [13]). Isotype switching allows the production of antibodies that are capable of mediating distinct biological functions but retain the same antigen-combining specificity. The final step of germinal center B cell development is the differentiation of high affinity germinal center B cells towards memory B cells and plasma cells. Any self-reactive B cells that are created during B cell development are removed from the repertoire at multiple checkpoints during the B cell maturation steps (reviewed in [8;14]).

Antibody diversity

Several different mechanisms contribute to the pre-immune diversity of the antibody repertoire, such as the random pairing of heavy and light chains, and the use of

multiple germline gene segments to be used in recombination of V, D and J elements. The inclusion of a D region in heavy chain gene assembly creates a significant increase in diversity compared to light chain assembly. Additional diversity is created by flexibility of the recombination site. For example, imprecise DNA rearrangement in which nucleotides at the 3' end of the V_H gene, both ends of the D segment and the 5' end of the J_H gene may be deleted during the recombination event. N-region diversification adds non-germline nucleotides to the junctions of rearranged VJ or VDJ genes, which is catalyzed by the enzyme TdT [15-17]. Less frequently, extra bases are added that are palindromic to the termini of the rearranging gene segments, and are called P-nucleotides [18]. These insertions are the result of the opening of hairpin ends in an asymmetric position during VDJ recombination and contribute to the diversity.

Somatic mutation

After antigenic stimulation, the Ig heavy and light chain genes undergo another type of structural alteration, namely somatic mutation, which contributes to the generation of an even more diverse antibody repertoire. Somatic mutation is a unique feature of B cells. It is triggered by T cell dependent responses and is characteristic of a germinal center reaction [19]. In the somatic mutation process, random mutations are introduced in the variable regions of the immunoglobulin genes to increase the affinity and functional efficiency of the immunoglobulin repertoire.

The somatic mutants undergo affinity selection by antigens presented by FDCs in the germinal center. Cells with high affinity immunoglobulin variants pick up antigen from the FDCs, process and present the antigen to T cells in the germinal center and differentiate into memory B cells and plasma cells. However, random mutations introduced in the V_H genes can cripple the Ig receptor by altering its structural integrity as well. Cells expressing low affinity receptors or crippled receptors fail to acquire sufficient antigen and die by apoptosis [20].

A number of models have been described that involve DNA repair in somatic mutation [21], however the exact mechanism of somatic mutation remains unknown. It is known that the accumulation of somatic mutation is highly focused in rearranged variable genes [22-24], is primarily composed of point mutations [25;26], and seems

to occur without regard to receptor affinity [27]. The rate of somatic mutation in Ig genes is 10^{-3} to 10^{-4} per base pair per generation (reviewed in [28]) and is similar to the range of intrinsic errors when DNA polymerase proofreading and mismatch repair are absent (reviewed in [21]).

Several in vitro models have been described that mimic germinal center reactions [29-33], yet the precise mechanism of activating the somatic mutational process is poorly understood. These models however, are valuable for analyzing extrinsic signals, enzymes and regulatory mechanisms that are responsible for the somatic mutation process. For instance it has been shown that activated T lymphocytes are required for somatic mutation to occur [11;27], and activated T cell-associated molecules, CD40 ligand (CD40L) and CD86, were shown to be important for the formation of germinal centers [34-36].

Genomic organization of immunoglobulin genes.

The organization of rearranging Ig receptor gene segments is important in the generation of antibody diversity. The use of multiple germline gene segments creates a vast number of antibodies with different recombining sites. The human heavy chain variable region locus contains approximately 120 V_H region gene segments [37-39], although haplotypes may vary considerably with respect to numbers of gene segments [40-42]. Approximately 40 V_H gene segments are functional or transcribed [39]. Twenty-five D segments [43] and 6 J_H gene segments [44;45] are located downstream from the V_H gene segments. The estimated combinatorial diversity is approximately 6000 [39]. This number varies because of allelic variation between individuals. The constant regions encoding exons are just downstream of the J regions.

The genes encoding the human immunoglobulin heavy chain comprise the most telomeric locus on the long arm of chromosome 14 (14q32.3) [46-48]. Additional V_H gene segments, all of which are pseudogenes, are located on chromosomes 15 and 16 [49;50]. The human V_H genes can be grouped into seven distinct families based on amino acid [51] or nucleotide sequence similarities [52-57]. Generally, sequence identity is ≥80% within families, but ≤70% between families. Gene segments comprising the human V_H families are highly interspersed [53-55;58-63]. The genes

encoding human immunoglobulin kappa light chains and lambda light chains are situated in multi-gene clusters on chromosomes 2, and 22, respectively.

The expressed repertoire.

The adult repertoire of antibody specificities has been thought to be derived from the random rearrangement (and subsequent selection) of functional V_H and V_L gene segments. The frequency with which members of a gene family were recovered from the repertoire was thought to reflect the complexity (or size) of the family [64-67]. However, analysis of individual V_H3 and V_H4 genes in unselected B cells shows that the adult repertoire represents a markedly biased sample of the germline repertoire [68-80]. Rather than a random assortment, a small number of V_H segments dominate rearrangements in peripheral B cells (reviewed in [81]). The V3-23 gene for example

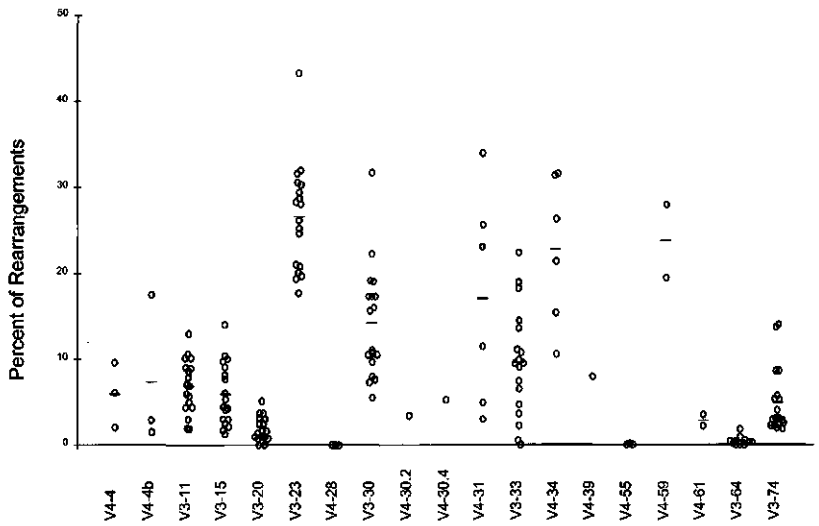

Figure 3. Frequency of occurence of individual V_H3 and V_H4 genes among rearrangements from peripheral blood B cells of healthy adults. Frequencies are calculated independently for V_H3 and V_H4 genes. Circles represent individual subjects. Bars represent the mean percentage of rearrangements for each V_H gene. (From Glas et al, 2000 [81])

is the most commonly expressed V_H gene. Its expression is disproportionate among randomly assessed B cells and comprises as much as 40% of all V_H3 expressing B cells [69;70;73;76-78]. V3-30 and V3-33 are also commonly rearranged [73;76;78]. Four V region genes, V3-11, V3-15, V3-20, and V3-74, made lesser contributions to rearrangements, and the remaining element, V3-64 was rarely represented among rearrangements in a population of 12 subjects [73]. Despite this clear-cut pattern of biased utilization seen on a population basis, there is considerable variation between subjects (Figure 3).

Similar to the utilization of V_H3 gene segments, the utilized V_H4 repertoire includes over-expressed and under-expressed elements (Figure 3). Three genes (V4-4b, V4-31, and V4-34) accounted for more than half of all V_H4 rearrangements in four subjects [70]. A single gene, V4-34, accounted for more than 25 percent of V_H4 rearrangements [70;75], and gene segment V4-59 accounted for 20 to 28 % of all V_H4 rearrangements [82;83]. Three V_H4 gene segments, CH15-8, V4-55, and V4-28 and its allele V4-28b, are not or rarely found in rearrangements. Of these genes, segment V4-55, is classified as a pseudogene because of a stopcodon in CDR1, but this defect should not prevent its rearrangement. It is somewhat surprising, therefore, that V4-55 is not even represented among non-productive rearrangements [70;84]. It is possible that an additional, previously undetected, defect located in the recombination signal sequence of this gene precludes rearrangement. Another of these unrearranged gene segments, CH15-8, is located outside the V_H locus on chromosome 15 [37;50], indicating that CH15-8 can not be rearranged by virtue of being an orphon. The third gene (comprised of two alleles, V4-28 and V4-28b) is located approximately 500 kb 5' to J_H and maps between V3-30 and V3-23. The reason why this gene is not rearranged has not been determined. The sequence of one allele of this locus, including 5' and 3' flanking regions, has been reported [39;85] and appears to encode a fully functional gene. The absence of this gene in the mature B cell pool does not seem to be due to selection processes, since rearranged V4-28 and V4-28b genes are absent from the pre-B and pro-B cell pool as well [82;86].

Stability of the expressed repertoire.

The rearranged V_H repertoire in peripheral B cells should be reasonably constant, because most of B cells in peripheral blood are pre-immune cells. To determine if the repertoire was stable over time, the variability in one donor was assessed by analyzing rearrangements in B cells obtained 8 months and two years apart. The relative stability of the utilized repertoire was striking, little change was observed with representation of most V_H3 or V_H4 genes increasing or decreasing only slightly [70;81].

Although one could imagine that in some pathologic circumstances the pattern of V_H utilization might change, this has not been observed frequently. Some studies have suggested a bias in the repertoire in autoimmune and neoplastic B cells [64;69;79;87-91]. In rheumatoid arthritis (RA) patients, certain heavy chain V genes have been found to be preferentially used for encoding autoantibodies [92-98]. However, while usage of individual V_H genes was highly biased among RA patients, no evidence of a distortion in the bias was observed compared to healthy controls [83]. Systemic lupus erythematosis (SLE) is an autoimmune disease characterized by the production of anti-dsDNA antibodies. In a recent study by Dörner et al [99], no differences were found in the repertoire of an SLE patient when non-productive rearrangements were analyzed and compared to the repertoire in healthy subjects. However, the $V\lambda$ and the V_H repertoires in productive rearrangements were markedly skewed, suggesting that extreme B cell over-activity drives the initial stages of SLE, leading to remarkable changes in the peripheral V_H gene usage that may underlie the failure to prevent the emergence of autoimmunity [99].

Repertoire biases during B cell development.

The developmental stage in which the bias in the repertoire begins was analyzed using pre-B as well as immature B cells from the bone marrow. A strong bias in the usage of certain V_H3 and V_H4 family genes was shown [82], similar to that seen in the peripheral repertoire. In these B cell stages however, the bias may reflect both rearrangement frequency as well as the contribution of selection occurring at later stages of development, e.g. efficiency of μ chain (surrogate light chain) paring at the

pre B cells stage or ligand selection at the immature B cell stage. By studying individual V_H gene segments among out of frame rearrangements from pro-B cells, the frequencies of the individual rearrangements would reflect only processes before translation of the μ-heavy chain and should not be biased by selection mechanisms. Rao et al [86] demonstrated that at the pro-B cell stage some individual V_H gene segments are more frequently rearranged than others, similar to that seen in mature B cells, suggesting that the predominant expression of these genes in peripheral mature B cells is determined to a significant degree by their preferential rearrangement during V-DJ recombination [86].

It was thought that the V_H genes used to encode antibodies expressed earliest in human and murine fetal development comprised a special subset of the total repertoire of V_H genes available to the adult, e.g. proximal to the D locus [55;100-103]. Several studies have suggested that the single member V_H6 family is preferentially used in the fetal repertoire [103-105]. However, we now know that the repertoire is more limited than previously thought, and the bias seen in the human fetal liver repertoire [55;106-109] and fetal bone marrow repertoire [86] is similar to that seen in the adult bone marrow [82;110] and adult peripheral blood. However, it appears that the fetal bone marrow repertoire resembles the adult repertoire more closely than the fetal liver repertoire does [86]. Chromosomal location does not seem to influence rearrangement in the fetal repertoire [108;109;111-113]. Nonetheless, analysis of the adult repertoire does reveal some differences in composition compared to the fetal liver repertoire. For instance, the V_H element V3-30.3 was found to be the predominant element in the fetal repertoire [55;106] but is a relatively minor element in the adult repertoire [70]. Among the V_H4 genes, the genes V4-34 and V4-39, which were not or rarely found in the fetal liver repertoire, are predominant V_H4 genes utilized in the adult repertoire. While the repertoires of the adult subjects differed with respect to a number of genes, some of these differences have been directly attributed to the presence or absence of specific elements in the germline. Similar germline analysis is not generally available for the fetal subjects.

B cell development following bone marrow transplantation.

Shortly after the high dose treatment of chemotherapeutic agents and radiation is

complete, patients receive the replacement marrow through an intravenous catheter. Engraftment of the marrow usually occurs within about 2 to 4 weeks following transplantation. Because BMT recipients have to regenerate their immune system from the transplanted bone marrow, it is not surprising that patients exhibit immunodeficiencies during the first three months after transplant. Both natural and specific immunity are affected. Persistent deficits in patients are often associated with graft versus host disease (GVHD), which is a frequent complication of allogeneic BMT. GVHD is the result of an immune reaction of the engrafted lymphoid cells against tissues of the host, impairing their ability to function and increasing the patients susceptibility to infection.

After transplant, B-lymphocytes recover slowly in number and function (reviewed in [114]) and approach normal levels or even supranormal levels by 1-2 years following transplant [115;116]. Coincident with recovering B cells, serum IgM, IgG_1 and IgG_3 levels but not IgG_2, IgG_4 and IgA levels return to normal by 1 year post transplant [117-124]. Early post-transplant B-lymphopoiesis is mono- or oligoclonal [125-128], and restricted clonality is more frequent in patients with chronic GVHD [129]. Some long-term survivors suffer from opportunistic bacterial infections, especially with encapsulated bacteria such as *Streptococcus pneumoniae* and *Hemophilus influenzae* type b [130;131]. Because immune responses to polysaccharides present in bacterial capsules are typically T-independent, at least a portion of the defect is likely intrinsic to B cells. The complete reconstitution of B cell immunity in recipients can take years.

The nature of the B cell defect(s) leading to the specific humoral immunodeficiency in BMT recipients is uncertain. It has been suggested that B cell development after transplant resembles ontogenic B cell development [115-117;132-134] in that similarities are seen in BMT survivors and normal infants. For example recovering B cell numbers, B cell phenotype, the delay in Ig production, and the use of certain immunoglobulin (Ig) genes[114-117;133-137]. But as shall be discussed in Chapter 6, evidence based on the usage and diversity of the rearranged heavy chains, indicates that post transplant development is different from ontogenic development.

This thesis.

In this thesis experiments are described that are aimed at a further understanding of the nature of the B cell defect(s) that contribute to the immune deficiencies following BMT. Normal levels of serum IgM and IgG [122;123] indicate that immunodeficiency is not due to a general failure to produce immunoglobulin or an overt lack of T cell help in heavy chain class switching. One possibility that is addressed in this thesis is that an abnormal usage of the V_H genes may contribute to the humoral immunodeficiency seen in BMT recipients. The usage of V_H families following BMT appears to mimic usage during B cell ontogeny [135;136]. Thus, to account for the observed immunodeficiencies, it might be expected that during the first year post transplant, BMT patients would utilize a more limited set of V genes than would healthy adult subjects; this is the subject of Chapter 3.

The results indicated that the utilization of V_H genes was normal after transplant, but the accumulation of somatic mutations in peripheral blood B cells was much greater among rearrangements in controls than in BMT recipients. To determine the intrinsic capacity of transplant recipient naïve B cells to acquire somatic mutations, a model was developed that mimics a germinal center reaction in vitro and is described in Chapter 4. Chapter 5 details the utilization of this model to determine if the somatic mutation process in B cells from BMT recipients could be activated in vitro, and to determine if the lack of somatic mutation in BMT recipients in vivo is a consequence of an intrinsic B cell deficit, or a lack of adequate T cell help.

To study the V_H repertoire in multiple individuals, a fast and reliable technique was needed. Nucleotide sequence analysis is useful but provides a relatively small data set. To this end, a highly sensitive hybridization technique [41;63;138-140] was adapted for identification of V_H genes in cDNA libraries and for the analysis of mutation in these genes. The use of motif-specific probes to identify individual genes and to detect somatic mutation is the subject of Chapter 2.

References

1. Jacobson, LO, Marks EK, Robson MJ, Gaston EO, Zirkle RE. Effect of spleen protection on mortality following x-irradiation. J.Lab.Clin.Med. 1949; 34:1538-43.

2. Thomas ED. History of bone marrow transplantation. International Autologous Bone Marrow Registration Newsletter 1993; 7.

3. The Autologous Blood and Marrow Transplant Registry Newsletter. 1998. Vol 5 number 1.

4. Honjo T. Immunoglobulin genes. Annual Review of Immunology 1983; 1:499-528.

5. Tonegawa S. Somatic generation of antibody diversity. Nature 1983; 302:575-81.

6. Melchers F, Karasuyama H, Haasner D *et al.* The surrogate light chain in B-cell development. Immunol.Today 1993; 14:60-8.

7. Carsetti R, Köhler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. J Exp Med 1995; 181:2129-40.

8. Goodnow CC, Cyster JG, Hartley SB *et al.* Self-tolerance checkpoints in B lymphocyte development. Adv.Immunol. 1995; 59:279-368.

9. Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influences peripheral B cell fate. Immunity 1996; 3:691-701.

10. Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. Science 1998; 281:96-9.

11. Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature 1991; 354:389-92.

12. Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell 1991; 67:1121-9.

13. Liu YJ, Arpin C, de Bouteiller O *et al.* Sequential triggering of apoptosis, somatic mutation and isotype switch during germinal center development. Semin Immunol 1996; 8:169-77.

14. Cornall RJ, Goodnow CC, Cyster JG. The regulation of self-reactive B cells. Curr.Opin.Immunol. 1995; 7:804-11.

15. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: Implications from a chromosome with evidence of three D- JH. Proc.Natl.Acad.Sci.USA 1982; 79:4118-22 1982.

16. Desiderio SV, Yancopoulos GD, Paskind M *et al.* Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984; 311:752-5.

17. Blackwell TK, Alt FW. Molecular characterization of the lymphoid V(D)J recombination activity. J.Biol.Chem. 1989; 264:10327-30.

18. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor γδ genes: Implications for γδ T cell lineages and for a novel intermediate of V-(D)-J Joining. Cell 1989; 59:859-70.

19. Rajewsky K, Forster I, Cumano A. Evolutionary and somatic selection of the antibody repertoire. Science 1987; 238:1088-94.

20. Liu YJ, Johnson GD, Gordon J, MacLennan IC. Germinal centres in T-cell-dependent antibody responses. Immunol Today 1992; 13:17-21.

21. Reynaud CA, Quint L, Bertocci B, Weill JC. Introduction: what mechanism(s) drive hypermutation? Semin Immunol 1996; 8:125-9.

22. Lebecque SG, Gearhart PJ. Boundaries of somatic mutation in rearranged immunoglobulin genes: 5' boundary is near the promoter, and 3' boundary is ~1kb from V(D)J gene. J.Exp.Med. 1990; 172:1717-27.

23. Golding GB, Gearhart PJ, Glickman BW. Patterns of somatic mutations in immunoglobulin variable genes. Genetics 1987; 115:169-76.

24. Gorski J, Rollini P, Mach B. Somatic mutations of immunoglobulin variable genes are restricted to the rearranged genes. Science 1983; 220:1179-81.

25. Wilson PC, de Bouteiller O, Liu YJ *et al.* Somatic hypermutation introduces insertions and deletions into immunoglobulin V genes. J Exp Med 1998; 187:59-70.

26. Goossens T, Klein U, Kuppers R. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. Proc Natl Acad Sci U S A 1998; 95:2463-8.

27. Jacob J, Przylepa J, Miller C, Kelsoe G. In situ studies of the primary immune response to (4-hydroxy-3- nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. J.Exp.Med. 1993; 178:1293-307.

28. Storb U. Progress in understanding the mechanism and consequences of somatic hypermutation. Immunol Rev 1998; 162:5-11.

29. Denepoux S, Fournier N, Peronne C, Banchereau J, Lebecque S. T cells can induce somatic mutation in B cell receptor-engaged BL2 Burkitt's lymphoma cells independently of CD40-CD40 ligand interactions. J Immunol 2000; 164:1306-13.

30. Huang S-C, Glas AM, Pinchuk GV *et al.* Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40-ligand. Clin Exp Immunol 1999; 116:441-8.

31. Källberg E, Jainandunsing S, Gray D, Leanderson T. Somatic mutation of immunoglobulin V genes in vitro. Science 1996; 271:1285-8.

32. Denepoux S, Razanajaona D, Blanchard D *et al.* Induction of somatic mutation in a human B cell line in vitro. Immunity 1997; 6:35-46.

33. Razanajaona D, Denepoux S, Blanchard D *et al.* In vitro triggering of somatic mutation in human naive B cells. J.Immunol. 1997; 159:3347-53.

34. Allen RC, Armitage RJ, Conley ME *et al.* CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science 1993; 259:990-3.

35. Ferguson SE, Han S, Kelsoe G, Thompson CB. CD28 is required for germinal center formation. J.Immunol. 1996; 156:4576-81.

36. Han S, Hathcock K, Zheng B, Kepler TB, Hodes R, Kelsoe G. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. J.Immunol. 1995; 155:556-67.

37. Cook GP, Tomlinson IM, Walter G *et al.* A map of the human immunoglobulin V_H locus completed by analysis of the telomeric region of chromosome 14q. Nature Genetics 1994; 7:162-8.

38. Cook GP, Tomlinson IM. The human immunoglobulin V_H repertoire. Immunol.Today 1995; 16:237-42.

39. Matsuda F, Ishii K, Bourvagnet P *et al.* The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. J.Exp.Med. 1998; 188:2151-62.

40. Willems van Dijk K, Sasso EH, Milner ECB. Polymorphism of the human immunoglobulin V_H4 gene family. J.Immunol. 1991; 146:3646-51.

41. Sasso EH, Willems van Dijk K, Milner ECB. Prevalence and polymorphism of human V_H3 genes. J.Immunol. 1990; 145:2751-7.

42. Milner ECB, Hufnagle WO, Glas AM, Suzuki I, Alexander CM. Polymorphism and utilization of human V_H genes. Ann.N.Y.Acad.Sci. 1995; 764:50-61.

43. Corbett SJ, Tomlinson IM, Sonnhammer ELL, Buck D, Winter G. Sequence of the human immunoglobulin diversity (D) segment locus: a systematic analysis provides no evidence for the use of DIR segments, inverted D segments, "minor" D segments or D-D recombination. J.Mol.Biol. 1997; 270:587-97.

44. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. Cell 1981; 27:583-91.

45. Siebenlist U, Ravetch JV, Korsmeyer S, Waldmann T, Leder P. Human immunoglobulin D segments encoded in tandem multigenic families. Nature 1981; 294:631-5.

46. Erikson J, Finan J, Nowell PC, Croce CM. Translocation of immunoglobulin V_H genes in Burkitt lymphoma. Proc.Natl.Acad.Sci.USA 1982; 79:5611-5.

47. Croce CM, Shander M, Martinis J *et al.* Chromosomal location of the genes for human immunoglobulin heavy chains. Proc.Natl.Acad.Sci.USA 1979; 76:3416-9.

48. McBride OW, Battey J, Hollis GF, Swan DC, Siebenlist U, Leder P. Localization of human variable and constant region immunoglobulin heavy chain genes on subtelomeric band q32 of chromosome 14. Nucleic Acids Res. 1982; 10:8155-70.

49. Matsuda F, Shin EK, Hirabayashi Y *et al.* Organization of variable region segments of the human immunoglobulin heavy chain: Duplication of the D_5 cluster within the locus and interchromosomal translocation of variable region segments. EMBO J. 1990; 9:2501-6.

50. Tomlinson IM, Cook GP, Carter NP *et al.* Human immunoglobulin VH and D segments on chromosomes 15q11.2 and 16p11.2. Human Molecular Genetics 1994; 3:853-60.

51. Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest, 5 Edn. U.S. Government printing office, Bethesda, MD: U.S. Department of Health and Human Services, USPHS, NIH, 1991

52. Shen A, Humphries C, Tucker P, Blattner F. Human heavy-chain variable region gene family nonrandomly arranged in familial chronic lymphocytic leukemia. Proc.Natl.Acad.Sci.USA 1987; 84:8563-7.

53. Berman JE, Mellis SJ, Pollock R *et al.* Content and organization of the human Ig V_H locus: definition of three new V_H families and linkage to the Ig CH locus. EMBO J. 1988; 7:727-38.

54. Lee KH, Matsuda F, Kinashi T, Kodaira M, Honjo T. A novel family of variable region genes of the human immunoglobulin heavy chain. J.Mol.Biol. 1987; 195:761-8.

55. Schroeder HW, Jr., Hillson JL, Perlmutter RM. Early restriction of the human antibody repertoire. Science 1987; 238:791-3.

56. Mortari F, Newton JA, Wang JY, Schroeder HW, Jr. The human cord blood antibody repertoire. Frequent usage of the V_H7 gene family. Eur.J.Immunol. 1992; 22:241-5.

57. Willems van Dijk K, Mortari F, Kirkham PM, Schroeder HW, Jr., Milner ECB. The human immunoglobulin VH7 gene family consists of a small, polymorphic group of six to eight gene segments dispersed throughout the VH locus. Eur.J.Immunol. 1993; 23:832-9.

58. Matsuda F, Lee KH, Nakai S *et al.* Dispersed localization of D segments in the human immunoglobulin heavy-chain locus. EMBO J. 1988; 7:1047-51.

59. Kodaira M, Kinashi T, Umemura I *et al.* Organization and evolution of variable region genes of the human immunoglobulin heavy chain. J.Mol.Biol. 1986; 190:529-41.

60. Walter MA, Surti U, Hofker MH, Cox DW. The physical organization of the human immunoglobulin heavy chain gene complex. EMBO J. 1990; 9:3303-13.

61. Matsuda F, Shin EK, Nagaoka H *et al.* Structure and physical map of 64 variable segments in the 3' 0.8-megabase region of the human immunoglobulin heavy-chain locus. Nature Genetics 1993; 3:88-94.

62. Willems van Dijk K, Milner LA, Milner ECB. Mapping of human H chain V region genes (V_H4) using deletional analysis and pulsed field gel electrophoresis. J.Immunol. 1992; 148:2923-31.

63. Willems van Dijk K, Milner LA, Sasso EH, Milner ECB. Chromosomal organization of the heavy chain variable region gene segments comprising the human fetal antibody repertoire. Proc.Natl.Acad.Sci.USA 1992; 89:10430-4.

64. Logtenberg T, Schutte MEM, Inghirami G *et al.* Immunoglobulin V_H gene expression in human B cell lines and tumors: biased V_H gene expression in chronic lymphocytic leukemia. Int.Immunol. 1989; 1:362-6.

65. Logtenberg T, Gmelig-Meyling FHJ, Van Es JH, Berman JE, Alt FW. Frequency of VH gene utilization in human EBV-transformed B cell lines: the most JH-proximal VH segment encoded antibodies. J.Autoimmun. 1989; 7:203.

66. Cuisinier A-M, Fumoux F, Moinier D *et al.* Rapid expansion of human immunoglobulin repertoire (V_H, V_k, V_l) expressed in early fetal bone marrow. New Biol. 1990; 2:689-99.

67. Guigou V, Cuisinier A-M, Tonnelle C, Moinier D, Fougereau M, Fumoux F. Human immunoglobulin V_H and V_K repertoire revealed by *in situ* hybridization. Mol.Immunol. 1990; 27:935-40.

68. Huang C, Stewart AK, Schwartz RS, Stollar BD. Immunoglobulin heavy chain gene expression in peripheral blood lymphocytes. J.Clin.Invest. 1992; 89:1331-43.

69. Stewart AK, Huang C, Stollar BD, Schwartz RS. High-frequency representation of a single V_H gene in the expressed human B cell repertoire. J.Exp.Med. 1993; 177:409-18.

70. Suzuki I, Pfister L, Glas A, Nottenburg C, Milner ECB. Representation of rearranged V_H gene segments in the human adult antibody repertoire. J.Immunol. 1995; 154:3902-11.

71. Klein U, Küppers R, Rajewsky K. Variable region gene analysis of B cell subsets derived from a 4-year-old child: Somatically mutated memory B cells accumulate in the peripheral blood already at young age. J.Exp.Med. 1994; 180:1383-93.

72. Hufnagle WO, Huang S-C, Suzuki I, Milner ECB. A complete preimmune human V_H3 repertoire. Ann.N.Y.Acad.Sci. 1995; 764:293-5.

73. Huang S-C, Jiang R, Glas AM, Milner ECB. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. Mol.Immunol. 1996; 33:553-60.

74. Rao SP, Huang S-C, Milner ECB. Analysis of the V_H3 repertoire among genetically disparate individuals. Exp.Clin.Immunogenet. 1996; 13:131-8.

75. Kraj P, Friedman DF, Stevenson F, Silberstein LE. Evidence for the overexpression of the *VH4-34* (*VH4.21*) Ig gene segment in the normal adult human peripheral blood B cell repertoire. J.Immunol. 1995; 154:6406-20.

76. Brezinschek HP, Brezinschek RI, Lipsky PE. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J.Immunol. 1995; 155:190-202.

77. Guillaume T, Rubinstein DB, Young F *et al.* Individual V_H genes detected with oligonucleotide probes from the complementarity-determining regions. J.Immunol. 1990; 145:1934-45.

78. Demaison C, David D, Letourneur F, Thèze J, Saragosti S, Zouali M. Analysis of human V_H gene repertoire expression in peripheral $CD19^+$ B cells. Immunogenetics 1995; 42:342-52.

79. Braun J, Berberian L, King L, Sanz I, Govan HL, III. Restricted use of fetal VH3 immunoglobulin genes by unselected B cells in the adult. J.Clin.Invest. 1992; 89:1395-402.

80. Huang C, Stollar BD. A majority of Ig H chain cDNA of normal human adult blood lymphocytes resembles cDNA for fetal Ig and natural autoantibodies. J.Immunol. 1993; 151:5290-300.

81. Glas AM, van Montfort EHN, Milner ECB. The human antibody repertoire: old notions, current realities, and V_H gene-dependent biases. In: Zanetti M, Capra JD, eds. The antibodies, Volume 6. Amsterdam: Harwood Academic Publishers, 2000

82. Kraj P, Rao SP, Glas AM, Hardy RR, Milner ECB, Silberstein LE. The human heavy chain Ig V region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. J.Immunol. 1997; 158:5824-32.

83. Huang S-C, Jiang R, Hufnagle WO, Furst DE, Wilske KR, Milner ECB. V_H usage and somatic hypermutation in peripheral blood B cells of patients with rheumatoid arthritis. Clin.Exp.Immunol. 1998; 112:516-27.

84. Glas AM, Nottenburg C, Milner ECB. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. Clin.Exp.Immunol. 1997; 107 :372-80.

85. Chen PP, Yang P-M. A segment of human Vh gene locus is duplicated. Scand.J.Immunol. 1990; 31:593-9.

86. Rao SP, Riggs JM, Friedman DF, Scully MS, LeBien TW, Silberstein LE. Biased V_H gene usage in early lineage human B cells: Evidence for preferential Ig gene rearrangement in the absence of selection. J.Immunol. 1999; 163:2732-40.

87. Stewart AK, Huang C, Long AA, Stollar BD, Schwartz RS. VH-gene representation in autoantibodies reflects the normal human B-cell repertoire. Immunol.Rev. 1992; 128:101-22.

88. Kohsaka H, Carson DA, Rassenti LZ *et al.* The human immunoglobulin V_H gene repertoire is genetically controlled and unaltered by chronic autoimmune stimulation. J.Clin.Invest. 1996; 98:2794-800.

89. Hauke G, Hadam M, Epplen JT, Gahr M, Hollmann A, Peter HH. Predominant immunoglobulin gene rearrangements in two patients with immunodeficiency: Restricted use of V gene segments and DNA hypermethylation. Immunobiology 1989; 178:449-61.

90. Pascual V, Andris J, Capra JD. Heavy chain variable region gene utilization in human antibodies. Int.Rev.Immunol. 1990; 5:231-8.

91. Pascual V, Capra JD. VH4-21, a human VH gene segment overrepresented in the autoimmune repertoire. Arthritis Rheum. 1992; 35:11-8.

92. Mannik M, Nardella FA, Sasso EH. Rheumatoid factors in immune complexes of patients with rheumatoid arthritis. Springer Semin.Immunopathol. 1988; 10:215-30.

93. Franklin EC, Holman HR, Muller-Eberhard HJ, Kunkel HG. An unusual protein component of high molecular weight in the serum of patients with rheumatoid arthritis. J.Exp.Med. 1957; 105:425-32.

94. Stuart JM, Huffsttutter EH, Townes AS, Kang AH. Incidence and specificity of antibodies to type I, II, III, IV, and V collagen in rheumatoid arthritis and other rheumatic diseases as measured by 125I-radioimmunoassay. Arthritis Rheum. 1983; 26:836-40.

95. Chen PP, Fong S, Carson DA. Rheumatoid factor. Rheum.Dis.Clin.North Am. 1987; 13:545-68.

96. Winchester RJ, Agnello V, Kunkel HG. Gamma globulin complexes in synovial fluids of patients with rheumatoid arthritis: partial characterization and relationship to lowered complement levels. Clin.Exp.Immunol. 1970; 6:689-706.

97. Munthe E, Natvig JB. Characterization of IgG complexes in eluates from rheumatoid tissue. Clin.Exp.Immunol. 1971; 8:249-62.

98. Zvaifler NJ. The immunopathology of joint inflammation in rheumatoid arthritis. Adv.Immunol. 1973; 13:265-336.

99. Dörner T, Farner NL, Lipsky PE. Ig lambda and heavy chain gene usage in early untreated systemic lupus erythematosus suggests intensive B cell stimulation. J.Immunol. 1999; 163:1027-36.

100. Perlmutter RM. Programmed development of the antibody repertoire. Curr.Top.Microbiol.Immunol. 1987; 135:95-109.

101. Perlmutter RM, Kearney JF, Chang SP, Hood LE. Developmentally controlled expression of immunoglobulin VH genes. Science 1985; 227:1597-601.

102. Yancopoulos GD, Desiderio SV, Paskind M, Kearney JF, Baltimore D, Alt FW. Preferential utilization of the most J -proximal VH gene segments. Nature 1984; 311:727-33.

103. Cuisinier A-M, Guigou V, Boubli L, Fougereau M, Tonnelle C. Preferential expression of V_H5 and V_H6 immunoglobulin genes in early human B-cell ontogeny. Scand.J.Immunol. 1989; 30:493-7.

104. Berman JE, Nickerson KG, Pollock RR *et al.* V_H gene usage in humans biased usage of the V_H6 gene in immature B lymphoid cells. Eur.J.Immunol. 1991; 21:1311-4.

105. Van Es JH, Raaphorst FM, Van Tol MJ, Meyling FH, Logtenberg T. Expression pattern of the most J_H-proximal human V_H gene segment (V_H 6) in the B cell and antibody repertoire suggests a role of V_H6-encoded IgM antibodies in early ontogeny. J.Immunol. 1993; 150:161-8.

106. Schroeder HW, Jr., Wang JY. Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. Proc.Natl.Acad.Sci.USA 1990; 87:6146-50.

107. Hillson JL, Oppliger IR, Sasso EH, Milner ECB, Wener MH. The emerging B cell repertoire: influence of developmental stage and interindividual variation. J.Immunol. 1992; 149:3741-52.

108. Raaphorst FM, Timmers E, Kenter MJH, Van Tol MJD, Vossen JM, Schuurman RKB. Restricted utilization of germ-line V_H3 genes and short diverse third complementarity-determining regions (CDR3) in human fetal B lymphocyte immunoglobulin heavy chain rearrangements. Eur.J.Immunol. 1992; 22:247-51.

109. Pascual V, Verkruyse L, Casey ML, Capra JD. Analysis of Ig H chain gene segment utilization in human fetal liver. J.Immunol. 1993; 151:4164-72.

110. Milili M, Schiff C, Fougereau M, Tonnelle C. The VDJ repertoire expressed in human preB cells reflects the selection of *bona fide* heavy chains. Eur.J.Immunol. 1996; 26:63-9.

111. Schutte MEM, Ebeling SB, Akkermans-Koolhaas KE, Logtenberg T. Deletion mapping of Ig V_H gene segments expressed in human CD5 B cell lines. J_H proximity is not the sole determinant of the restricted fetal V_H gene repertoire. J.Immunol. 1992; 149:3953-60.

112. Cuisinier AM, Gauthier L, Boubli L, Fougereau M, Tonnelle C. Mechanisms that generate human immunoglobulin diversity operate from the 8th week of gestation in fetal liver. Eur J Immunol 1993; 23:110-8.

113. Raaphorst FM, Langlois van den Bergh R, Waaijer JL, Vossen JM, Van Tol MJ. Expression of the human immunoglobulin heavy chain VH6 gene element by fetal B lymphocytes. Scand J Immunol 1997; 46:292-7.

114. Storek J, Witherspoon RP. Immunological reconstitution after hemopoietic stem cell transplantation. In: Atkinson K, ed. Clinical Bone Marrow and Blood Stem Cell Transplantation. Cambridge: Cambridge University Press, 2000

115. Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: A recapitulation of normal B cell ontogeny. Blood 1990; 76:1647-56.

116. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? Bone Marrow Transplant. 1993; 12:387-98.

117. Velardi A, Cucciaioni S, Terenzi A *et al.* Acquisition of Ig isotype diversity after bone marrow transplantation in adults. A recapitulation of normal B cell ontogeny. J.Immunol. 1988; 141:815-20.

118. Norhagen EG, Engström P-E, Björkstrand B, Hammarström L, Smith CIE, Ringdén O. Salivary and serum immunoglobulins in recipients of transplanted allogeneic and autologous bone marrow. Bone Marrow Transplant. 1994; 14:229-34.

119. Sheridan JF, Tutschka PJ, Sedmak DD, Copelan EA. Immunoglobulin G subclass deficiency and pneumococcal infection after allogeneic bone marrow transplantation. Blood 1990; 75:1583-6.

120. Zintl F, Prager J, Sauerbrey A, Metzner G, Hermann J, Fuchs D. Immunoreconstitution after human bone marrow transplantation. Folia Haematologica - Internationales Magazin fur Klinische und Morphologische Blutforschung 1989; 116:519-26.

121. Matsue K, Lum LG, Witherspoon RP, Storb R. Proliferative and differentiative responses of B cells from human marrow graft recipients to T cell-derived factors. Blood 1987; 69:308-15.

122. Lum LG, Seigneuret MC, Storb RF, Witherspoon RP, Thomas ED. In vitro regulation of immunoglobulin synthesis after marrow transplantation. I. T-cell and B-cell deficiencies in patients with and without chronic graft-versus-host disease. Blood 1981; 58:431-9.

123. Witherspoon RP, Deeg HJ, Lum LG *et al.* Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-versus-host disease. Transplantation 1984; 37:456-61.

124. Sullivan KM, Storek J, Kopecky KJ *et al.* A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs.-host disease after marrow transplantation: Clinical outcome and effect on subsequent immune recovery. BB&MT 1996; 2:44-53.

125. Näsman I, Lundkvist I. Evidence for oligoclonal diversification of the V_H6-containing immunoglobulin repertoire during reconstitution after bone marrow transplantation. Blood 1996; 87:2795-804.

126. Van Muiswinkel WB, Radl J, van der Wal DJ. The regulatory influence of the thymus-dependent immune system on the heterogeneity of immunoglobulins in irradiated and reconstituted mice. Adv Exp Med Biol 1976; 66:617-21.

127. Mink JG, Radl J, Van den Berg P, Van Muiswinkel WB, Van Oosterom R. Homogeneous immunoglobulins in the serum of irradiated and bone marrow reconstituted mice: the role of thymus and spleen. Immunology 1979; 37:889-94.

128. Gokmen E, Raaphorst FM, Boldt DH, Teale JM. Ig heavy chain third complementarity determining regions (H CDR3s) after stem cell transplantation do not resemble the developing human fetal H CDR3s in size distribution and Ig gene utilization. Blood 1998; 92:2802-14.

129. Mitus AJ, Stein R, Rappeport JM *et al.* Monoclonal and oligoclonal gammopathy after bone marrow transplantation. Blood 1989; 74:2764-8.

130. Winston DJ, Schiffman G, Wang DC *et al.* Pneumococcal infections after human bone-marrow transplantation. Ann.Intern.Med. 1979; 91:835-41.

131. Aucouturier P, Barra A, Intrator L *et al.* Long lasting IgG subclass and antibacterial polysaccharide antibody deficiency after allogeneic bone marrow transplantation. Blood 1987; 70:779-85.

132. Guillaume T, Rubinstein DB, Symann M. Immune reconstitution and immunotherapy after autologous hematopoietic stem cell transplantation. Blood 1998; 92:1471-90.

133. Lum LG. The kinetics of immune reconstitution after human marrow transplantation. Blood 1987; 69:369-80.

134. Storek J, Saxon A. Reconstitution of B cell immunity following bone marrow transplantation. Bone Marrow Transplant. 1992; 9:395-408.

135. Fumoux F, Guigou V, Blaise D, Maraninchi D, Fougereau M, Schiff C. Reconstitution of human immunoglobulin VH repertoire after bone marrow transplantation mimics B-cell ontogeny. Blood 1993; 81:3153-7.

136. Storek J, King L, Ferrara S, Marcelo D, Saxon A, Braun J. Abundance of a restricted fetal B cell repertoire in marrow transplant recipients. Bone Marrow Transplant. 1994; 14:783-90.

137. Uckun FM, Haissig S, Ledbetter JA *et al.* Developmental hierarchy during early human B-cell ontogeny after autologous bone marrow transplantation using autografts depleted of CD19+ B-cell precursors by an anti-CD19 pan-B-cell immunotoxin containing pokeweed antiviral protein. Blood 1992; 79:3369-79.

138. Willems van Dijk K, Schroeder HW, Jr., Perlmutter RM, Milner ECB. Heterogeneity in the human immunoglobulin VH locus. J.Immunol. 1989; 142:2547-54.

139. van der Maarel SM, Willems van Dijk K, Alexander CM, Sasso EH, Bull AB, Milner ECB. Chromosomal organization of the human V_H4 gene family: Location of individual gene segments. J.Immunol. 1993; 150:2858-68.

140. Sasso EH, Willems van Dijk K, Bull A, van der Maarel SM, Milner ECB. V_H genes in tandem array comprise a repeated germline motif. J.Immunol. 1992; 149:1230-6.

Chapter 2

Motif-specific probes identify individual genes and detect somatic mutations

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Motif-specific probes identify individual genes and detect somatic mutations

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Abstract

This report describes the correlation between motif-specific hybridization and necleotide sequence as an approach to the identification of individual human V_H genes using motif-specific oligonucleotide probes, complementary to specific motifs within individual V_H genes. The sensitivity of the hybridization and post washing processes permits discrimination of single nucleotide differences between probe and target. This feature is used both to identify individual genes, as well as to detect mutations in genes by sequential hybridization with multiple probes. In addition to the general strategy, specific details are provided for the identification of 12 V_H3 genes and 14 V_H4 genes. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Oligonucleotide probes; Mutation; Gene detection; V_H genes; B cells

1. Introduction

Analysis of multi-gene families comprised of closely related genes, and discrimination between germline variation and somatic mutation can be prohibitively labor intensive or require sophisticated instrumentation. We have employed an approach which requires no specialized instrumentation and can be performed in any moderately equipped laboratory. Essentially, the method is to hybridize recombinant libraries containing genes of interest with motif-specific, synthetic oligonucleotide probes targeted to unique sequences of individual genes. The use of synthetic oligonucleotides as probes has been well described (Wallace et al., 1979; Wallace et al., 1981; Conner et al., 1983; Verlaan-de Vries et al., 1986; Amar et al., 1987; Gitschier et al., 1986; Guillaume et al., 1990; Macintyre et al., 1990). Under appropriate hybridization and washing conditions, single nucleotide mismatches between probe and target can be readily discriminated from perfect matches (Wood et al., 1985; Sasso et al., 1990). Therefore, a sequence motif detected by one probe will not be detected by another probe targeted to another gene or another region of the gene. The method is useful for the analysis of any multi-gene family, where discrimination between highly similar elements is desired.

Our particular focus has been on the human immunoglobulin heavy chain variable region (V_H) genes, which comprise a complex multi-gene locus containing approximately 100 haploid elements. We have generated and used synthetic oligonucleotides corresponding to human immunoglobulin V_H sequences. By using 21 base pair probes in conjunction with high stringency hybridization and washing conditions, it has been possible to unambiguously identify individual germline V_H genes, based on the occurrence of unique sequence

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motifs, and to identify variation in closely related germline genes (Willems van Dijk et al., 1989; Sasso et al., 1990; Willems van Dijk et al., 1991; Willems van Dijk et al., 1992; Sasso et al., 1992; van der Maarel et al., 1993; Willems van Dijk et al., 1993). An outgrowth of these studies was the establishment of diagnostic criteria for identification of specific V_H elements. These criteria have been used in several studies to analyze V-gene use among peripheral B cells (Suzuki et al., 1995; Glas et al., 1995; Suzuki et al., 1996; Milner et al., 1995; Huang et al., 1996; Glas et al., 1997; Hufnagle et al., 1995; Milner, 1996; Huang et al., 1998; Rao et al., 1996; Kraj et al., 1997). Similar criteria have been used in other studies (Guillaume et al., 1990; Stewart et al., 1993).

Because of the high degree of specificity, the uniqueness of a motif is determined by single nucleotides. Probes can be effectively targeted to single nucleotide substitutions that distinguish otherwise identical sequences. Many of the V_H genes have multiple unique nucleotides that can be targeted, permitting the generation of multiple diagnostic probes, each one of which detects a distinct motif of that particular gene.

Motif-specific probes can be used not only to identify individual genes but to detect somatic mutation as well. By sequential hybridization with multiple oligonucleotide probes, mutations in individual genes can be detected. For example, if a gene carries three unique motifs, the sequence of the particular gene can be detected by a probe directed at any one of the three motifs. If hybridized sequentially, concordant results indicate that the germline sequence has been retained through the target region of each probe. However, genes that have accumulated mutations in the target site of one or more of the probes will display a loss of concordance when hybridized (Suzuki et al., 1996). Loss of concordance is easily scored and provides an index of the acquisition of somatic mutations. In this report we show the specificity of the oligonucleotide hybridization and its correlation with nucleotide sequence. We illustrate the use of the method for identifying individual V_H gene segments in rearrangements, and for the detection of somatic mutations.

2. Materials and methods

2.1. Cell preparations

Peripheral blood mononuclear cells (PBMC) were isolated from a Ficoll-Hypaque gradient. B cells were stained with fluorescein-conjugated anti-IgM or anti-IgD (Coulter), phycoerythrin-conjugated anti-CD19 or CD20 (Coulter), or appropriately conjugated mouse immunoglobulin isotype control antibodies. Viable lymphocytes were selected on the basis of forward and side angle light scattering criteria. A Coulter Epics 750 or a Becton-Dickinson Facstar flow cytometer was used to sort 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%.

2.2. Rearrangement library construction

Purified B cells ($CD19^+$ or $CD20^+$) were lysed in cell lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 0.5% Tween 80, and 200 μg/ml proteinase K) and incubated at 50°C for 30 min. Cell lysates were sonicated for 30 s using a Sonifier 450 (Branson, Danbury, CT) fitted with a cup horn, at 50% duty cycle, 50% output to provide more uniform PCR amplification. Proteinase K was heat-inactivated by incubation for 10 min at 95°C. The rearranged V_H genes were amplified using one of the family-specific 5′ primers, E68 (V_H3-L), 5′-CTGAATTCCATGGAGTTTGGGCTGAG-3′ or E74 (V_H4-L), 5′-CCGAATTCATGAAACACCTGTGGTTCTT-3′, corresponding to the 5′ ends of the leader sequences of V_H3 and V_H4 families respectively, and the 3′ primer E71, 5′-GCTCTAGACT(T/C)ACCTGAGGAGACGGTGA-3′, complementary to the 3′ end of the 6 J_H gene sequences. Restriction sites (*Eco*RI for 5′ primers; *Xba*I for 3′ primers) included in the primers are underlined. An aliquot of lysate containing 15,000–50,000 cell equivalents was amplified by PCR using AmpliTaq DNA polymerase (Perkin Elmer, Fostercity, CA) and using cycle conditions to obtain amplification in the linear range; a denaturation step of 1 min at 95°C, then 30 cycles of 45 s at 94°C, 30 s at 60°C, 1 min at 72°C, and a last extension step of 3 min at 72°C. The DNA polymerase has an error rate of 1×10^{-5} per base pair according to the manufacturer. The linearity of the PCR reactions has been confirmed by electrophoresing [α-^{32}P]dCTP-incorporated PCR products of different cycle points on a 2.0% agarose gel and quantifying the bands by phosphor imaging (data not shown). The PCR products were cloned into *Eco*RI/*Xba*I digested pBS+phagemid vector (Stratagene, La Jolla, CA) and the recombinant plasmids were used to transform *Epicurian Coli*® XL2-Blue MRF′ ultracompetent cells (Stratagene). The transformants, about 400 per library, were toothpicked into wells of a 96-well plate containing Luria-Bertani medium with 75 μg/ml carbenicillin or ampicillin. Single-stranded DNA was recovered by adding (5×10^8 pfu/ml) VCSM13 Interference-Resistant Helper Phage (Stratagene). After a 1 h incubation period at 37°C, kanamycin was added to 75 μg/ml and the cultures were grown for another 16–20 h.

Table 1
Oligonucleotides corresponding to human V_H sequence motifs. The probes are listed in 5′–3′ orientation and are oriented in the sense direction with respect to the coding sequence of the V_H gene segment

Oligonucleotide	Sequence	Gene	Location
E7	TCCATCAGTAGTTACTACTGG	V4-59, V4-4	CDR1
E8	TCCATCAGCAGTGGTAGTTAC	V4-61b	CDR1
E13	TCCGTCAGCAGTGGTAGTTAC	V4-61	CDR1
E22	GCAGTTATATCATATGATGGAAGC	V3-30.3	CDR2
E35	AGTGACTACTACATGAGCTGG	V3-11	CDR1
E36	AGTGGTAGTACCATATACTAC	V3-11	CDR2
E39	GAAATCCATCATAGTGGGAGC	V4-55p	CDR2
E41	GAAATCAATCATAGTGGAAGC	V4-34	CDR2
E42	AGTATCTATTATAGTGGGAGC	V4-39	CDR2
E43	TATATCTATTACAGTGGGAGC	V4-59, V4-61	CDR2
E44	CGTATCTATACCAGTGGGAGC	V4-61b	CDR2
E53	TCAGCTATTGGTGCTGGTGGT	HC16–11	CDR2
E54	TCAGCTATTGGTACTGGTGGT	V3–47p	CDR2
E56	TACATCTATCATAGTGGGAGC	V4-30.2	CDR2
E57	GAAATCTATCATAGTGGGAGC	V4-4, V4-4b, CH15	CDR2
E58	TACATCTATTACAGTGGGAGC	V4-31, V4-31b, V4-30.4	CDR2
E83	AGTAGCAACTACATGAGCTGG	V3-53	CDR1
E87	TCAGCTATTAGTGGTAGTGGT	V3-23	CDR2
H110	CGTATTAAAAGCAAAACTGAT	V3-15	CDR2
H139	TACATCTATTATAGTGGGAGC	V4-28, V4-28b	CDR2
M8	AGCAGCTATGCCATGAGCTGG	V3-23	CDR1
M16	AGTAGCTATGGCATGCACTGG	V3-30	CDR1
M18	GTGAAGGGCCGGTTCACCATC	V3-23	CDR2FR3
M19	GGAAGCAATAAATACTACGCA	V3-30.3	CDR2
M20	GGAAGTAATAAATACTATGCA	V3-30	CDR2
M24	GGTGGTAGCACATACTACGCA	V3-23, V3-53	CDR2
M25	TACGCAGACTCCGTGAAGGGC	V3-23, V3-53	FR3
M41	GGAAGTAATAAATACTACGCA	V3-30b	CDR2
M42	AGCTGTTGGAGTCTGGGGGAG	V3-23	FR1
M69	TGGTGGAGCTGGGTCCGCCAG	CH15	CDR1FR2
M76	GCAGTTATATGGTATGATGGA	V3-33	CDR2
M84	AGTAGCTACTGGATGCACTGG	V3-74p	CDR1
M85	AGTAGCTACGACATGCACTGG	V3-13	CDR1
M86	GGTGGTTACTCCTGGAGCTGG	V4-30.2	CDR1
M91	GATGATTATGGCATGAGCTGG	V3-20	CDR1
M93	GTCCGCCAGGCTCCAGGCAAG	V3-30	FR2
M98	AGTGGTGGTTACTACTGGAGC	V4-31, V4-31b	CDR1
M99	AGTAGTAGTTACTACTGGGGC	V4-39	CDR1
M100	AGCAGTAGTAACTGGTGGGGC	V4-28, V4-28b	CDR1
M101	AGCAGTGGTAACTGGTGAATC	V4-55p	CDR1
M105	AGTGGTGATTACTACTGGAGT	V4-30.4	CDR1
M109	AGTGGTTACTACTGGAGCTGG	V4-34	CDR1
M112	AAGCCTTCGGAGACCCTGTCC	V4-39, V4-80, CH15, V4-34, V4-4, V4-55p, V4-61, V4-59	FR1
M122	GGTAGTTACTACTGGAGCTGG	V4-61, V4-61b	CDR1

2.3. Germline library construction

V_H family-specific germline libraries were similarly generated as above from either the negative fraction collected from the cell sorts or unsorted PBMC. PCR reactions used for constructing V_H3 libraries were carried out using the 5′ primer E68 and either the 3′ primer E46, 5′-GACTCTAGACAATGACTTCCCCTC-ACT-3′, which is complementary to the 3′ flanking recombination signal sequence of V_H3 genes, or E88, 5′-GACTCTAGATCTCAGGCTGTTCATTTG-3′, which is complementary to a conserved V_H3 FR3 sequence. PCR reactions used for constructing V_H4 libraries were carried out using the 5′ primer E74 and either the 3′ primer E45, 5′-AATTCTAGACTGGG-CTCACACTCACCTCC-3′, which is complementary to the 3′ flanking recombination signal sequence of V_H4 genes, or E89, 5′-AATTCTAGACACAGAGC-TCAGCTTCAG-3′, which is complementary to a conserved V_H4 FR3 sequence.

2.4. Library screening

Multiple replicate filters were prepared by dot blot-

ting approximately 10 μl of supernatants containing phage particles in a 96-well grid on Hybond N+nylon membranes (Amersham, Arlington Heights, IL). Filters were denatured in 0.5 M NaOH, 1.5 M NaCl, neutralized in 0.5 M Tris-HCl pH 7.0, 3 M NaCl, and UV cross-linked. Hybridization with a ^{32}P-labeled full length probe allowed a determination of the total number of recombinant clones in each library. The cDNA clone 58p2 (Schroeder et al., 1987; Willems van Dijk et al., 1991) was used for the V_H4 family-specific probe, and cDNA clone 56p2 (Schroeder et al., 1987; Willems van Dijk et al., 1989) was used for the V_H3 family specific probe. The probes were labeled by random hexamer priming (Feinberg and Vogelstein, 1984). Hybridization of the family-specific V_H probes to replicate filters was performed in 50% formamide, 5 × SSC, 1 × Denhardt's, 20 mM $NaPO_4$, pH 7.6, 50 μg/ml salmon testes DNA, 10% Dextransulfate, and 50 μg/ml tRNA (50 × Denhardt's is 1% polyvinylpyrrolidone, 1% Bovine Serum Albumen, 1% Ficoll, all wt/vol) for 16 h at 42°C. Final washes after hybridization were performed in 0.1 × SSC, 0.1% SDS at 60°C for 1 h.

The panel of diagnostic oligonucleotide probes listed in Table 1 were also hybridized to replicate filters. Oligonucleotide probes were end labeled using T4 polynucleotide kinase in the recommended buffer to a specific activity of approximately 1×10^9 cpm/μg. Hybridization of oligonucleotides to replicate filters was performed in 6 × NET, 5 × Denhardt's, 0.1% SDS, 0.05% Nonidet P-40, 5 mM EDTA, 250 μg/ml tRNA and 250 μg/ml salmon testes DNA (12 × NET is 1.2 M NaCl, 0.3 M Tris, pH 8.0, 12 mM EDTA). The filters were prehybridized in the hybridization solution for 1–4 h and were hybridized at 5°C above the melting temperature (T_m) for 30 min and 5°C under the T_m overnight. The T_m is estimated using 2°C for A+T and 4°C for G+C. After hybridization the filters were washed three times at 43°C in 5 × SSC, 0.5% SDS for a total of 2 h. This was followed by a wash in 3.2 M Tetramethylammoniumchloride (TMACL) (Aldrich, Milwaukee, WI), 0.5% SDS, 0.05 M Tris, pH 7.8 for 1 h (modified from Wood et al., 1985). The predicted melting temperatures of oligonucleotides in TMACL as determined by probe length is approximately 62°C for 21 base pair probes (Wood et al., 1985). The TMACL post wash temperatures were empirically determined to be 55°C for probe M99, 57°C for probe H139, 59°C for probes E43 and M69, 61°C for probes E22 and E53, 61.5°C for probe M109 and 62°C for probes M18 and M93. All other probes were post-washed at 60°C. The filters were exposed for a maximum of 3 h to Kodak X-OMAT AR film in metal cassettes at −70°C, with enhancing screens.

Table 2
Accuracy of motif-specific hybridization

Probe		Correct	Incorrect	Percentage correct
VH4	E7	117	0	100.0
	E8	117	0	100.0
	E13	117	0	100.0
	E39	49	0	100.0
	E41	116	1	99.1
	E42	116	1	99.1
	E43	117	0	100.0
	E44	117	0	100.0
	E56	49	0	100.0
	E57	117	0	100.0
	E58	115	2	98.3
	H139	117	0	100.0
	M69	117	0	100.0
	M86	115	2	98.3
	M98	116	1	99.1
	M99	46	3	93.9
	M100	117	0	100.0
	M101	117	0	100.0
	M105	116	1	99.1
	M109	115	2	98.3
	M112	48	1	98.0
	M122	117	0	100.0
	All VH4	2288	14	99.4
VH3	E22	38	0	100.0
	E35	21	0	100.0
	E36	88	0	100.0
	E53	38	0	100.0
	E54	38	0	100.0
	E55	14	0	100.0
	E83	36	0	100.0
	H110	88	0	100.0
	M8	75	1	98.7
	M16	70	1	98.6
	M18	93	0	100.0
	M19	88	0	100.0
	M20	36	2	94.7
	M24	37	1	97.4
	M25	38	0	100.0
	M41	74	0	100.0
	M42	5	0	100.0
	M76	87	1	98.9
	M84	36	0	100.0
	M85	71	0	100.0
	M91	36	0	100.0
	M93	72	2	97.3
	All VH3	1179	8	99.3

2.5. DNA sequence analysis

DNA sequence analysis was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (PE Applied Biosystems) on the Applied Biosystems Model 373A DNA Sequencing System. Sequencing reactions were primed with either a universal primer (T7 or T3 sequencing primers) or oligonucleotide E61, 5′-CTGTTCACAGGGGTCCTGTC-3′, which corresponds to the leader sequence of V_H4 gene segments.

Additional primers were used to determine the complete sequence of rearrangements, E36, M18, M76, E99: 5′-AGTTACACCACAGAATACGCC-3′, M10: 5′-ATATGATGGAAGTAATAAAT-3′, M22: 5′-ACTGATGGTGGGACAACAGAC-3′, M92: 5′-TGCATAACCTGTGCTACCACC-3′, corresponding to motif-specific sequences of V3-11, V3-23, V3-33, V3-72, V3-30, V3-15, and V3-20 respectively.

3. Results and discussion

3.1. Specificity of the oligonucleotide probes

Under the appropriate hybridization and washing conditions single nucleotide mismatches between probe and target can, in principle, be detected (Wood et al., 1985; Willems van Dijk et al., 1989; Sasso et al., 1990). To assess the practical application of this principle, and its utility for the analysis of a complex multi-gene family, we compared motif-specific hybridization results with nucleotide sequences among more than 200 closely related cloned genes. Recombinant libraries were generated as described, and were assessed by hybridization and sequence analysis. The hybridization results were then scored as correct or incorrect hybridization results by comparison to sequence results. Two conditions, corresponding to the two concordant possible outcomes, were scored as a correct hybridization result: (1) when a clone was positively identified by hybridization to a specific probe and this result corresponded to the presence of the target sequence, or (2) the absence of a hybridization signal corresponded to the absence of the exact target sequence. Two conditions were scored as incorrect hybridization: (1) a clone was positively identified by hybridization to a particular probe, but the target sequence contained at least one nucleotide difference from the probe, or (2) there was no hybridization signal but the target sequence was present, as determined by sequence analysis. A total of 210 clones were analyzed by hybridization with 43 probes and by sequence analysis. For each individual probe, scoring of hybridization results are based solely on homologous combinations, i.e., V_H3 probes on V_H3 clones, and V_H4 probes on V_H4 clones. Results are shown in Table 2.

The concordance between hybridization data and sequence analysis is very high. Nearly two thirds of the probes were correct 100% of the time, i.e., yielded hybridization results that were in complete agreement with sequence data. Among the remaining probes, only two were incorrect more than 5% of the time. Overall, the probes were correct 99.4% of the time (Table 2). When the results of all oligonucleotides are taken together, the error incidence was low. Among 3489 hybridization events, 22 errors were detected. Two of these errors lead to incorrect identification of a clone, e.g. sequence analysis identified a different gene than did hybridization analysis. Also two clones were not identified by hybridization, but should have been identified according to sequence data. The other 18 errors did not lead to misidentification of clones. In total, misidentification of clones using oligonucleotides was 1.8%. These results demonstrate that the accuracy of the approach is very high, and that mistaken hybridization results are rare, and are a minor source of error.

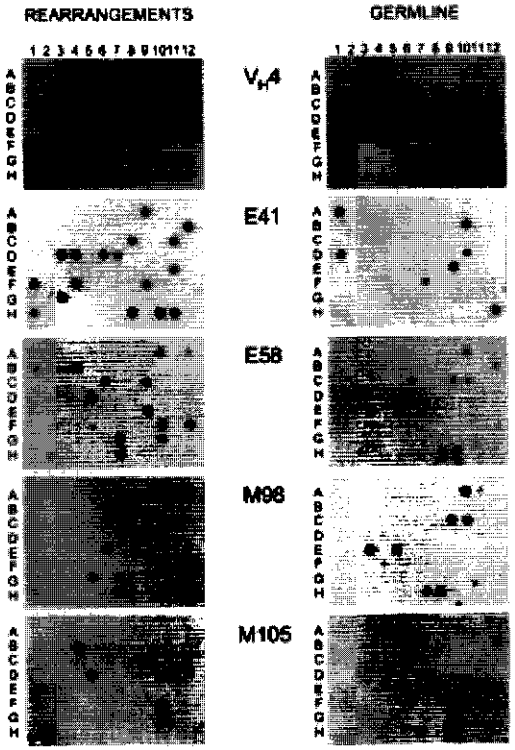

Fig. 1. Identification of V_H4 gene segments in heavy chain gene rearrangement libraries and germline libraries. Recombinant clones were picked into 96 well plates and grown overnight in the presence of helper phage. Aliquots of single stranded template were transferred to nylon membranes for hybridization analysis. The filters were hybridized with a full-length V_H4 probe (top panels) and gene-specific oligonucleotide probes E41, E58, M98, and M105.

3.2. Identification of individual V_H genes contained in rearrangements

We have used these oligonucleotides to analyze immunoglobulin V_H genes in peripheral blood B cells. The frequency of occurrence of individual V_H gene segments in libraries containing rearranged V_H genes

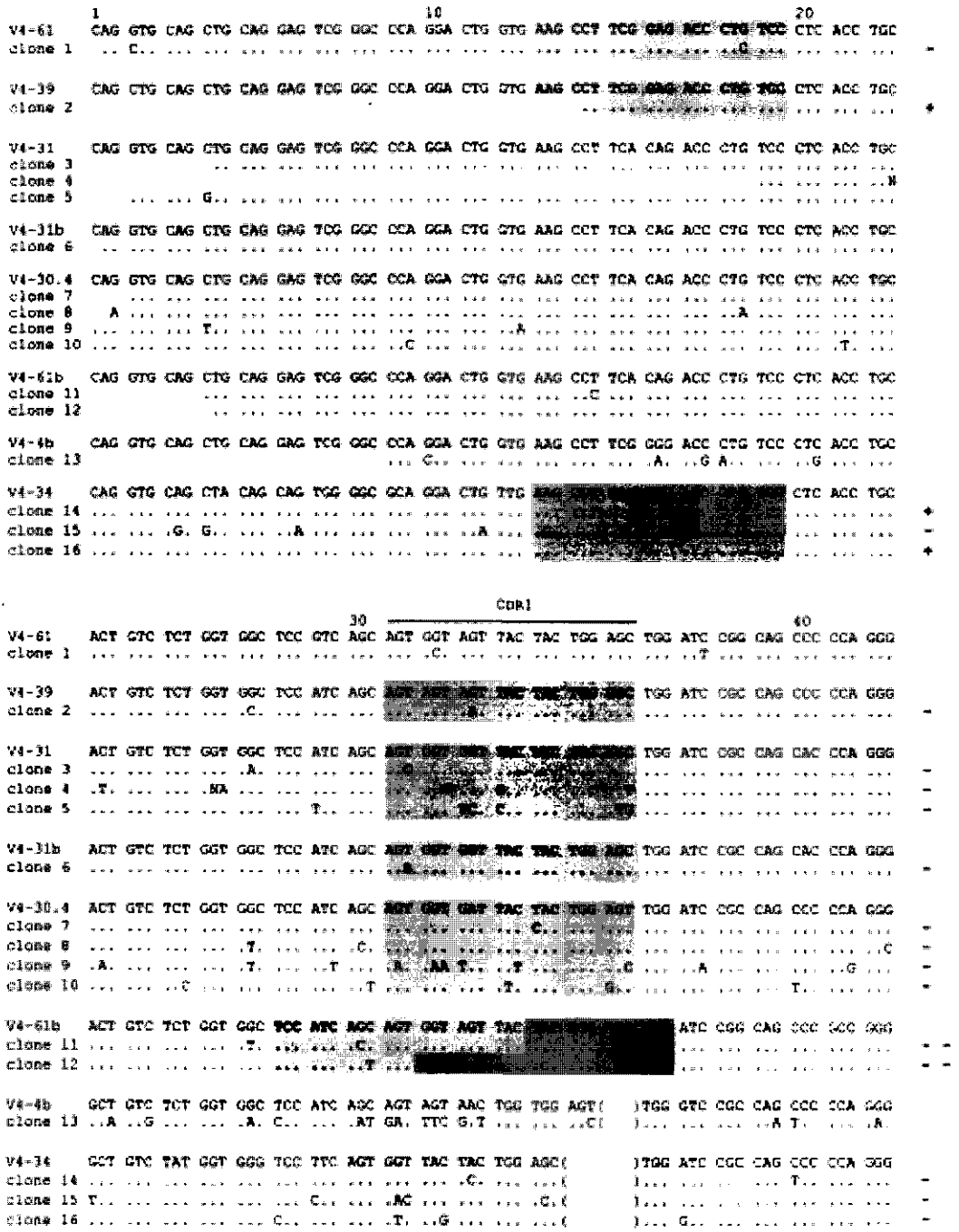

Fig. 2. Sequence analysis of 16 unidentified or partly identified V_H4 gene rearrangements. The clones are aligned with their putative germline sources. Only the V-region sequences are shown, and codons are numbered according to the method of Kabat et al. (1991). Target rshaded, and hybridization results are displayed in the margin as '+' for a positive identification of the clone by the specific oligonucleotide probe, or a '−', which indicates that there was no hybridization to that probe. The sequences reported here are available from the GenBank database under accession numbers AF126264–AF126278, and U80165.

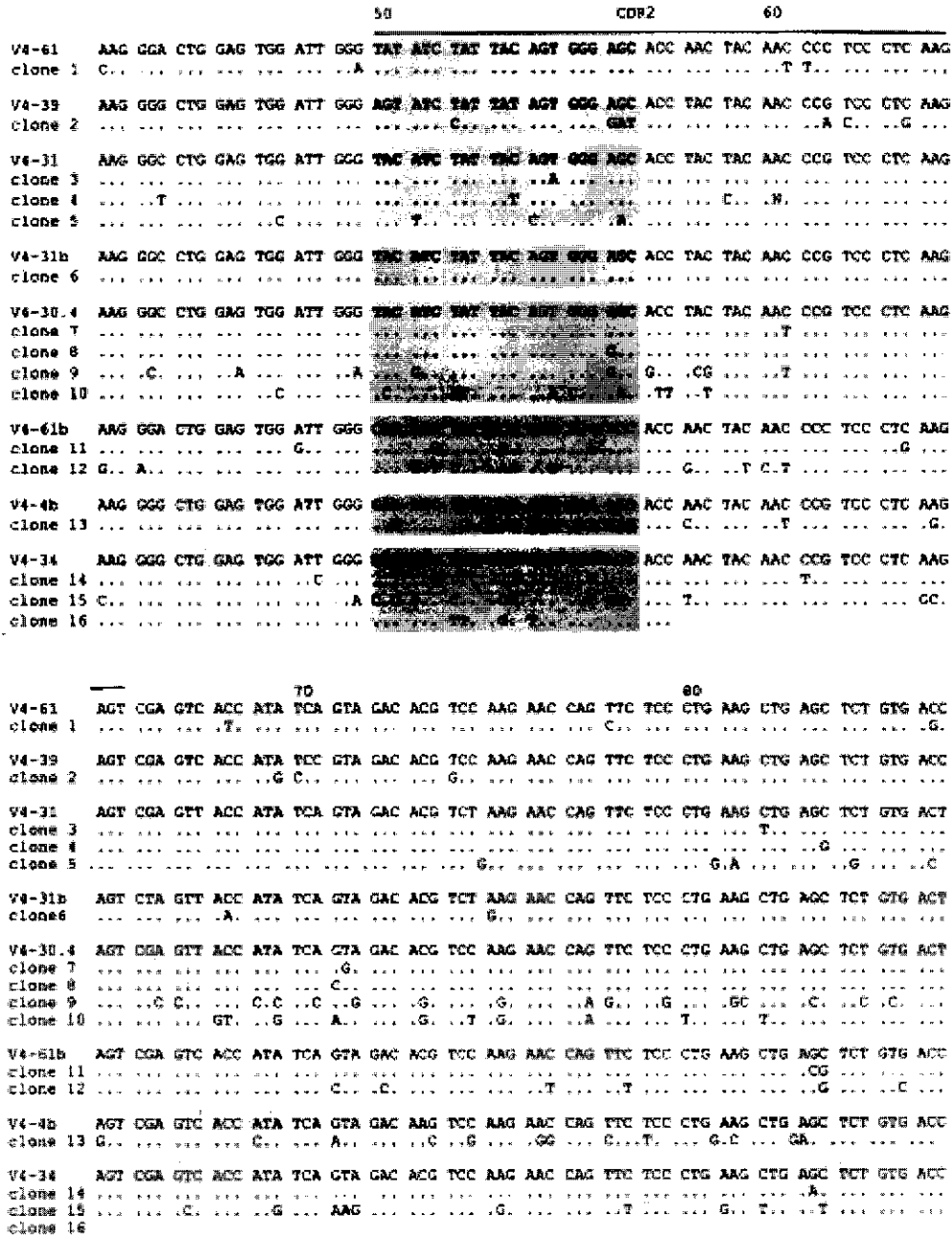

Fig. 2 (*continued*)

```
                                                    90
V4-61     GCT GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
clone 1   ... ... ... ... ... ..T ... ... ... ... ...

V4-39     GCC GCA GAC ACG GCT GTG TAT TAC TGT GCG AGA
clone 2   ... ... ... ... ... ... ... ... ... .T. ...

V4-31     GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
clone 3   ... ... ... ... ... ... ... ..T ... ... ...
clone 4   ... .Y. ... ... ... ... ... .T. ... .T. ...
clone 5   ... ... ... ... ... A.A ... .T. ... ... ...

V4-31b    GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
clone 6   ... ... ... ... ... ... ... ... ... ... ...

V4-30.4   GCC GCA GAC ACG GCC GTG TAT TAC TGT GCC AGA
clone 7   ... ... ... ... ... ... ... ... ... ... ...
clone 8   ... ... ... ... ... ... ... ... ... ... ...
clone 9   ... ... ... ... ... ..T ... ..T ... ... ...
clone 10  ... ... ... ... ... A.. ... ..T ... ... ...

V4-61b    GCC GCA GAC ACG GCC GTG TAT TAC TGT GCG AGA
clone 11  .N. .G. ... ..A ... ... ..C .T. ... ... ...
clone 12  ... .G. ... ... ... ..A ... ... ... ... C..

V4-4b     GCC GCG GAC ACG GCC GTG TAT TAC TGT GCG AGA
clone 13  ... ... ... ... ... ..C ... ..T ... ..C ...

V4-34     GCC GCG GAC ACG GCT GTG TAT TAC TGT GCG AGA
clone 14  ... ... ... ... ... ... ... ... ... ... ...
clone 15  ... ... ... .T. .G. C.A ... .T. ... ... ..C
clone 16              ... ... ... ... ... ... ... ...
```

Fig. 2 (*continued*)

was determined by comparing the number of clones that hybridized to each motif-specific diagnostic probe with the total detected by the family-specific probe. From 300 to 400 individual rearrangements were studied per subject.

Dot blot hybridization results for five probes on two sets of replica filters, one set of rearrangement, and one set of germline libraries from one donor are shown in Fig. 1. The full length probe detects 72 clones on the rearrangements filter. Probe E41 corresponds to a unique motif carried by the V4-34 gene. A positive hybridization result specifically identifies this unique motif and is therefore diagnostic for the presence of the V4-34 gene. Hybridization to E41 identified 17 positive clones on the rearrangement filter. Thus 24% (17/72) of the clones are derived from rearrangement of the V4-34 gene. Probe E58 detects a motif that is shared by two genes, V4-30.4 and V4-31. Fourteen (19%) positive clones are identified on the rearrangement filter, and contain either V4-30.4 or V4-31 genes. To discriminate between V4-30.4 and V4-31, probe M98 is used. Probe M98 corresponds to a unique motif carried by the V4-31 gene and is diagnostic for the presence of this gene. Eight (11%) positive clones are identified. Probe M105 detects a unique motif in the V4-30.4 gene, and is diagnostic for the presence of that gene. The six (8%) clones not detected by probe M98 were detected by probe M105 and are identified as V4-30.4. Clones identified by hybridization to M98 or M105 should be identified by probe E58 as well. However, six clones showed discordant hybridization results, as discussed in the following sections, each of these clones has acquired a substitution, presumably as a result of somatic mutation, in the target region of one of the probes.

3.3. Germline libraries

To compare the germline content of different individuals, and to verify that all germline genes could be identified with the diagnostic probes, libraries were generated containing unrearranged genes. Dotblot hybridization results for five probes are shown in Fig. 1. The full length probe detects 69 clones on the germline filters. Seven clones are identified by hybridization to probe E41, and contain a V4-34 gene segment. Probe E58 detects 9 clones, which contain a V4-30.4 or a V4-31 gene segment. Probe M98 detects 7 clones, which contain a V4-31 gene segment. Probe M105 detects 3 clones, which contain a V4-30.4 gene segment.

The number of positively identified clones by each probe reflects the germline complexity. In previous studies the copy number of the individual genes was determined for this donor (Willems van Dijk et al., 1992; van der Maarel et al., 1993). The hybridization results are consistent with the copy number; the diploid element V4-34 is detected by probe E41 which identified 7 clones (10.1%), the combination of the haploid element V4-30.4 and the diploid element V4-31 is detected by probe E58 which identified 9 clones

(13%), the diploid element V4-31 is detected by probe M98 which identified 7 clones (10.1%), and the haploid element V4-30.4 is detected by probe M105 which identified 3 clones (4.3%). These results are consistent with a germline repertoire of 10–12 diploid elements as previously established (Willems van Dijk et al., 1992; van der Maarel et al., 1993). One discordance (between E58 and M98) is identified and is discussed in Section 3.4.

All clones in the V_H4 germline libraries could be identified using the V_H4 probes, whereas approximately 80% of clones in the V_H4 rearrangement libraries could be identified. The unidentified clones containing V_H4 rearrangements are presumed to have accumulated somatic mutations in the regions of the probes. That no previously uncharacterized V_H4 gene segments were amplified was confirmed by analyzing the nucleotide sequence of 16 unidentified V_H4 rearrangements (Fig. 2).

The sequences in Fig. 2 are compared to the germline counterpart. The target regions of the oligonucleotide probes are shaded. The hybridization results for these clones are indicated in the margin of Fig. 2. With a single exception (clone 8 CDR1 probe), the sequence data confirm the hybridization data, and demonstrate that the abrogation of hybridization is caused by one or more nucleotide substitutions in the target region of the probe.

3.4. System background

Faint hybridization occasionally presents difficulties in interpretation. In instances where a full length probe gives strong hybridization, a very faint signal from an oligo probe has usually been found to represent a contaminant in the well, that is, the motif detected by the probe is present but not as part of the predominant species. Examples on the germline filter are clones A11 and F4 hybridized to M98, and on the rearrangement filter clone E7 hybridized to M105. These clones were not scored as positive clones. In other instances, clones which give a faint signal after hybridization, such as on germline filter clone F7 hybridized to E41, and on the rearrangement filter clones A12 and G10 hybridized to probe E58, are considered positive clones. These scoring decisions require careful judgment; however, the error introduced by faint hybridization is minor when large numbers of clearly hybridizing clones are analyzed.

When germline genes are analyzed by sequential hybridization according to the criteria in Table 3, loss of concordance should not occur. However, in Fig. 1, on the germline filter, clone H7 is positively identified by probe M98, but failed to hybridize to probe E58. Because these filters are made from germline libraries, no mutations were expected. Among the germline clones the loss of concordance is very unli-

Table 3
Diagnostic criteria for identification of individual V_H genes by oligonucleotide probes

Gene		Hybridization profile	Probe target
V_H3	V3-11	$E35^{positive}$ or $E36^{positive}$	CDR1, CDR2
	V3-13	$M85^{positive}$	CDR1
	V3-15	$H110^{positive}$	CDR2
	V3-20	$M91^{positive}$	CDR1
	V3-23	$M8^{positive}$ or $M18^{positive}$	CDR1, CDR2/FR3
	V3-30	$M16^{positive}$ and $M76^{negative}$	CDR2, CDR2
	V3-30b	$M41^{positive}$	CDR2
	V3-30.3	$M19^{positive}$ or $E22^{positive}$	CDR2, CDR2
	V3-33	$M16^{positive}$ and $M76^{positive}$	CDR2, CDR2
	V3-53	$E83^{positive}$	CDR1
	V3-74	$M84^{positive}$	CDR1
V_H4	V4-4	$E7^{positive}$ and $E44^{positive}$	CDR1, CDR2
	V4-4b	$E57^{positive}$ and $M69^{negative}$	CDR2, CDR1/FR2
	V4-28	$M100^{positive}$ and $H139^{positive}$	CDR1, CDR2
	V4-30.2	$M86^{positive}$ or $E56^{positive}$	CDR1, CDR2
	V4-30.4	$M105^{positive}$ and $E58^{positive}$	CDR1, CDR2
	V4-31	$M98^{positive}$ and $E58^{positive}$	CDR1, CDR2
	V4-34	$M109^{positive}$ or $E41^{positive}$	CDR1, CDR2
	V4-39	$M99^{positive}$ or $E42^{positive}$	CDR1, CDR2
	V4-55p	$M101^{positive}$ or $E39^{positive}$	CDR1, CDR2
	V4-59	$E7^{positive}$ and $E43^{positive}$	CDR1, CDR2
	V4-61	$E13^{positive}$ and $E43^{positive}$	CDR1, CDR2
	V4-61b	$E8^{positive}$ and $E44^{positive}$ or $M122^{positive}$	CDR1, CDR2, CDR1
	V4-80	$M69^{positive}$ and $H139^{positive}$	CDR1/FR2, CDR2
	CH15	$M69^{positive}$ and $E57^{positive}$	CDR1/FR2, CDR2

kely to be the result of somatic mutations in the regions of the probe, but rather is likely to be the result of system errors. Errors in the system could be introduced by Taq polymerase, PCR and cloning artifacts, and hybridization errors. Predicted concordance between oligonucleotide probes differed from the observed concordance by 10% on analysis of >15,000 germline clones (results not shown). From these results we consider that the system error introduced by amplification, cloning and hybridizing is approximately 10%. Our procedure assays 42 bp. An error for any one nucleotide yields a discordance, which is effectively an error for all 42. Thus, an error incidence of about 10% is equivalent to a mutation incidence of 0.24% per bp, or approximately one substitution per rearrangement. Other investigators, using different methodologies, have reported similar error incidences. Klein et al. observed five errors among 11 control sequences, Pascual et al. observed 28 errors among 47 transcripts, and Huang et al. reported 1 substitution every 300 bp, which led them to calculate a Taq error rate of 2×10^{-5} per nucleotide incorporated (Pascual et al., 1994; Klein et al., 1993; Huang et al., 1992).

3.5. The use of oligonucleotides to detect somatic mutation in V_H rearrangements

When rearranged genes were analyzed by sequential hybridization according to the criteria in Table 3, discordant hybridization results occurred more frequently among rearranged genes than among germline genes. For example clones identified by hybridization to E58 should be identified by probes M98 or M105 as well. However, three E58 positive clones (clones A12, B1, and E9 on rearrangement blots in Fig. 1) are not positively identified by either M98 or M105. Reciprocally, three M98 positive clones (clones E6, G5, and H12 on rearrangement filters in Fig. 1) are not positively identified by E58. As predicted by this hybridization result, sequence analysis of one of these clones (H12) confirmed the presence of a substitution, presumably acquired as a result of somatic mutation, in the target region of the E58 probe.

The accumulation of somatic mutation in individual rearrangements is addressed by sequential hybridization with multiple probes. We have focused on V3-23, which is the most frequently rearranged V_H3 gene, accounting for approximately 15–40% of the total V_H3 repertoire in peripheral B cells (Logtenberg et al., 1989; Brezinschek et al., 1995; Suzuki et al., 1995; Huang et al., 1996; Rao et al., 1996; Kraj et al., 1997). The germline sequence of V3-23 can be detected by either of two motif-specific probes. Concordant hybridization of both these probes on rearranged V3-23 genes indicates V3-23 genes that have retained the germline sequence through the targeted motifs. However V3-23 genes that have accumulated one or more mutations in the target motifs of one or the other probe will display a loss of concordance when hybridized.

An example of hybridization results using the V3-23 specific probes, M8 (CDR1) and M18 (CDR2/FR3) is shown in Fig. 3. Probe M8 identifies 14 clones. Probe M18 identifies 17 clones including 12 of the M8 positive clones, and five clones (D9, E1, E2, G12, and H4) are identified by probe M18, but not by probe M8. There are 19 clones identified by probe M8, M18, or both. Two clones that gave faint signals (E2 on the M8 filter, and H11 on the M18 filter) are not scored as positive clones. Therefore these results are scored as 19 V3-23 rearrangements, of which seven (clones D9, E1, E2, F7, G12, H1, and H4) are detected by one or other probe, but not both, and are scored as mutated (37% mutation). These clones are presumed to have accumulated one or more mutations in the region of the non hybridizing probe. This number is not an exact measure of mutation frequency, but does provide a very useful, if somewhat arbitrary, index of the

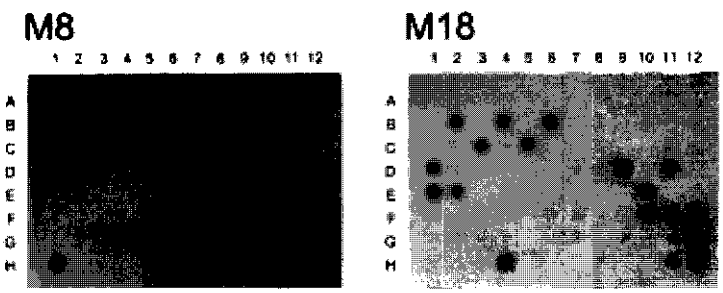

Fig. 3. Detection of somatic mutation in V3-23 rearrangements. Left panel: hybridization of a filter containing rearranged V3-23 gene segments hybridized to oligonucleotide M8, which is targeted to a motif specific sequence in CDR1 of V3-23. Right panel: replicate filter hybridized to oligonucleotide M18, which is targeted to a motif-specific sequence in CDR2/FR3.

amount of mutation among the rearrangements that were studied.

Using this hybridization technique, clones that had acquired mutations in both target regions would not be detected as clones containing V3-23 rearrangements. In such instances, the mutation index as calculated, would be an underestimate of the actual somatic mutation incidence. However, sequence analysis of random clones showed that clones containing V3-23 rearrangements rarely had mutations in the M18 target region.

We have found oligonucleotide probe hybridization to be useful to identify genes and to determine the occurrence of somatic mutation among virtually any gene. Judgment is needed when identifying faintly hybridizing clones; however, a large number of genes can be identified and analyzed in a relatively short time span without sophisticated instrumentation. One person can easily make libraries and analyze from 1500 to 2500 recombinant clones in a week.

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References

Amar, A., Holbeck, S.L., Nepom, G.T., 1987. Specific allelic variation among linked HLA class II genes. Transplantation 44, 831–835.

Brezinschek, H.P., Brezinschek, R.I., Lipsky, P.E., 1995. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J. Immunol. 155, 190–202.

Conner, B.J., Reyes, A.A., Morin, C., Itakura, K., Teplitz, R.L., Wallace, R.B., 1983. Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides. Proc. Natl. Acad. Sci., USA, 80, 278–282.

Feinberg, A.P., Vogelstein, B., 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137, 266–267.

Gitschier, J., Wood, W.I., Shuman, M.A., Lawn, R.M., 1986. Identification of a missense mutation in the Factor VIII gene of a mild hemophiliac. Science 232, 1415–1416.

Glas, A.M., Hufnagle, W.O., Suzuki, I., Pfister, L., Rao S, Nottenburg, C., Milner, E.C.B., 1995. Anomalous diversification of the antibody repertoire following bone marrow transplantation. Ann. NY Acad. Sci. 764, 312–314.

Glas, A.M., Nottenburg, C., Milner, E.C.B., 1997. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. Clin. Exp. Immunol. 107, 372–380.

Guillaume, T., Rubinstein, D.B., Young, F., Tucker, L., Logtenberg, T., Schwartz, R.S., Barrett, K.J., 1990. Individual V_H genes detected with oligonucleotide probes from the complementarity-determining regions. J. Immunol. 145, 1934–1945.

Huang, C., Stewart, A.K., Schwartz, R.S., Stollar, B.D., 1992. Immunoglobulin heavy chain gene expression in peripheral blood lymphocytes. J. Clin. Invest. 89, 1331–1343.

Huang, S-C., Jiang, R., Glas, A.M., Milner, E.C.B., 1996. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. Mol. Immunol. 33, 553–560.

Huang, S-C., Jiang, R., Hufnagle, W.O., Furst, D.E., Wilske, K.R., Milner, E.C.B., 1998. V_H usage and somatic hypermutation in peripheral blood B cells of patients with rheumatoid arthritis. Clin. Exp. Immunol. 112, 516–527.

Hufnagle, W.O., Huang, S-C., Suzuki, I., Milner, E.C.B., 1995. A complete preimmune human V_H3 repertoire. Ann. NY Acad. Sci. 764, 293–295.

Kabat, E.A., Wu, T.T., Perry, H.M., Gottesman, K.S., Foeller, C., 1991. Sequences of Proteins of Immunological Interest. US Department of Health and Human Services, USPHS, NIH, US Government printing office, Bethesda, MD.

Klein, U., Küppers, R., Rajewsky, K., 1993. Human IgM^+IgD^+ B cells, the major B cell subset in the peripheral blood, express V_k genes with no or little somatic mutation throughout life. Eur. J. Immunol. 23, 3272–3277.

Kraj, P., Rao, S.P., Glas, A.M., Hardy, R.R., Milner, E.C.B., Silberstein, L.E., 1997. The human heavy chain Ig V region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. J. Immunol. 158, 5824–5832.

Logtenberg, T., Schutte, M.E.M., Inghirami, G., Berman, J.E., Gmelig-Meyling, F.H., Insel, R.A., Knowles, D.M., Alt, F.W., 1989. Immunoglobulin V_H gene expression in human B cell lines and tumors: biased V_H gene expression in chronic lymphocytic leukemia. Int. Immunol. 1, 362–366.

Macintyre, E.A., D'Auriol, L., Duparc, N., Leverger, G., Galibert, F., Sigaux, F., 1990. Use of oligonucleotide probes directed against T cell antigen receptor gamma delta variable-(diversity)-joining junctional sequences as a general method for detecting minimal residual disease in acute lymphoblastic leukemias. J. Clin. Invest. 86, 2125–2135.

Milner, E.C.B., 1996. *Organization of the human V_H locus and rearrangement patterns of the V_H3 gene family.* In: Zouali, M. (Ed.), Human B-cell Superantigens. R.G. Landes Company, Austin, p. 1.

Milner, E.C.B., Hufnagle, W.O., Glas, A.M., Suzuki, I., Alexander, C.M., 1995. Polymorphism and utilization of human V_H genes. Ann. NY Acad. Sci. 764, 50–61.

Pascual, V., Liu, Y-J., Magalski, A., de Bouteiller, O., Banchereau, J., Capra, J.D., 1994. Analysis of somatic mutation in five B cell subsets of human tonsil. J. Exp. Med. 180, 329–339.

Rao, S.P., Huang, S-C., Milner, E.C.B., 1996. Analysis of the V_H3 repertoire among genetically disparate individuals. Exp. Clin. Immunogenet. 13, 131–138.

Sasso, E.H., Willems van Dijk, K., Bull, A., van der Maarel, S.M., Milner, E.C.B., 1992. V_H genes in tandem array comprise a repeated germline motif. J. Immunol. 149, 1230–1236.

Sasso, E.H., Willems van Dijk, K., Milner, E.C.B., 1990. Prevalence and polymorphism of human V_H3 genes. J. Immunol. 145, 2751–2757.

Schroeder Jr, H.W., Hillson, J.L., Perlmutter, R.M., 1987. Early restriction of the human antibody repertoire. Science 238, 791–793.

Stewart, A.K., Huang, C., Stollar, B.D., Schwartz, R.S., 1993. High-frequency representation of a single V_H gene in the expressed human B cell repertoire. J. Exp. Med. 177, 409–418.

Suzuki, I., Milner, E.C.B., Glas, A.M., Hufnagle, W.O., Rao, S.P., Pfister, L., Nottenburg, C., 1996. Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood 87, 1873–1880.

Suzuki, I., Pfister, L., Glas, A., Nottenburg, C., Milner, E.C.B.,

1995. Representation of rearranged V_H gene segments in the human adult antibody repertoire. J. Immunol. 154, 3902–3911.

van der Maarel, S.M., Willems van Dijk, K., Alexander, C.M., Sasso, E.H., Bull, A.B., Milner, E.C.B., 1993. Chromosomal organization of the human V_H4 gene family: Location of individual gene segments. J. Immunol. 150, 2858–2868.

Verlaan-de Vries, M., Bogaard, M.E., van den Elst, H., van Boom, J.H., van der Eb, A.J., Bos, J.L., 1986. A dot-blot screening procedure for mutated ras oncogenes using synthetic oligodeoxynucleotides. Gene 50, 313–320.

Wallace, R.B., Johnson, M.J., Hirose, R., Miyake, T., Kawashima, E.H., Itakura, K., 1981. The use of synthetic oligonucleotides as hybridization probes. II. Hybridization of oligonucleotides of mixed sequence to rabbit β-globulin DNA. Nucleic Acids Res. 9, 879–895.

Wallace, R.B., Shaffer, J., Murphy, R.F., Bonner, J., Hirose, T., Itakura, K., 1979. Hybridization of synthetic oligodeoxyribonucleotides to phi × 174 DNA: the effect of single base pair mismatch. Nucleic Acids Res. 6, 3543–3557.

Willems van Dijk, K., Milner, L.A., Sasso, E.H., Milner, E.C.B., 1992. Chromosomal organization of the heavy chain variable region gene segments comprising the human fetal antibody repertoire. Proc. Natl. Acad. Sci., USA, 89, 10,430–10,434.

Willems van Dijk, K., Mortari, F., Kirkham, P.M., Schroeder Jr, H.W., Milner, E.C.B., 1993. The human immunoglobulin VH7 gene family consists of a small, polymorphic group of six to eight gene segments dispersed throughout the VH locus. Eur. J. Immunol. 23, 832–839.

Willems van Dijk, K., Sasso, E.H., Milner, E.C.B., 1991. Polymorphism of the human immunoglobulin V_H4 gene family. J. Immunol. 146, 3646–3651.

Willems van Dijk, K., Schroeder Jr, H.W., Perlmutter, R.M., Milner, E.C.B., 1989. Heterogeneity in the human immunoglobulin VH locus. J. Immunol. 142, 2547–2554.

Wood, W.I., Gitschier, J., Lasky, L.A., Lawn, R.M., 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci., USA, 82, 1585–1588.

Chapter 3

Immunoglobulin heavy chain variable gene usage in bone marrow transplant recipients; lack of somatic mutation in rearranged heavy chains

3.1 Anomalous diversification of the antibody repertoire following bone marrow transplant. *Ann.N.Y.Acad.Sci.* (1995) 764:312

3.2 Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: Lack of somatic mutation indicates a maturational arrest. *Blood* (1996) 87:1873

3.3 Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. *Clin. Exp. Immunol.* (1997) 107:372-380

Chapter 3.1

Anomalous diversification of the antibody repertoire following bone marrow transplant.

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

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Anomalous Diversification of the Antibody Repertoire following Bone Marrow Transplantation[a]

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Bone marrow transplantation (BMT) is a recognized treatment for certain leukemias, other blood diseases, and some inborn errors of metabolism, and it has potential as a vehicle for gene therapy. Nevertheless, patients undergoing BMT may remain immunodeficient for several years despite the return to normal IgM and IgG levels.[1,2] These observations suggest continued defects in both T- and B-cell compartments. To determine whether abnormal usage of V_H genes may contribute to this immunodeficiency, the relative usage of V_H genes after marrow transplantation has been determined. As a measure of V_H gene utilization, rearrangements of eight individual V_H3 genes were assessed in peripheral blood B cells of four BMT recipients and compared to similarly obtained rearrangements from two healthy subjects. In addition, rearrangements of 14 individual V_H4 genes were assessed in peripheral blood B cells of one of the BMT recipients and compared to similarly obtained rearrangements from one of the healthy subjects. Heavy chain rearrangements were amplified quantitatively, and the resulting PCR products were cloned into a phagemid vector. Identification of the V_H gene present in an individual clone was established by hybridization with a sequence-specific oligonucleotide probe. More than 700 independent rearrangements from each person were analyzed.

The pattern of usage of V_H genes assessed at 90 days and approximately one year after transplant was similar to that observed in healthy subjects and was marked by overuse of two elements (V3-23 and V4-34).[3] However, the repertoires of the BMT recipients were less diversified than the repertoires of the healthy subjects. Sequence-specific oligonucleotide probes for 14 individual V_H4 genes, composing 100% of the germline repertoire, identified more than 99% of the rearrangements in the BMT recipient, but only 80% of the rearrangements in the healthy subject. Similarly, oligonucleotide probes specific for eight V_H3 genes detected 75% of rearrangements in the BMT recipients, but only 55% of the

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rearranged V_H3 genes in the healthy subjects. The difference in the percentage of identified rearrangements between healthy subjects and BMT patients is presumed to be the result of somatic mutations in the regions of the probe that abrogate hybridization. To assess more directly differences in the occurrence of somatic mutations, we determined the nucleotide sequence of 20 randomly picked V_H3 and 20 randomly picked V_H4 rearrangements from one of the BMT recipients and from one healthy subject. The BMT recipient exhibited fewer mutations in both V_H3 gene segments (FIG. 1) and V_H4 gene segments (FIG. 2) than did the healthy subject. The ratio of replacement to silent substitutions (R/S ratio) in the complementarity-determining regions (CDRs) from the BMT recipient was lower than in the healthy subject. Somewhat unexpectedly, although the absolute number of substitutions was lower in the BMT recipient, the R/S ratio in the framework regions in the BMT recipient was much higher than in the healthy subject. The significance of this high ratio is not certain, but suggests that the mechanism of somatic mutation favors amino acid replacement.

A high R/S ratio and the selective accumulation of somatic mutations in the CDRs is an indicator of antigen-driven responses. The failure in the BMT recipients to accumulate somatic mutations is consistent with a defect in antigen-driven B-cell responses. This is also consistent with the observation that germinal center

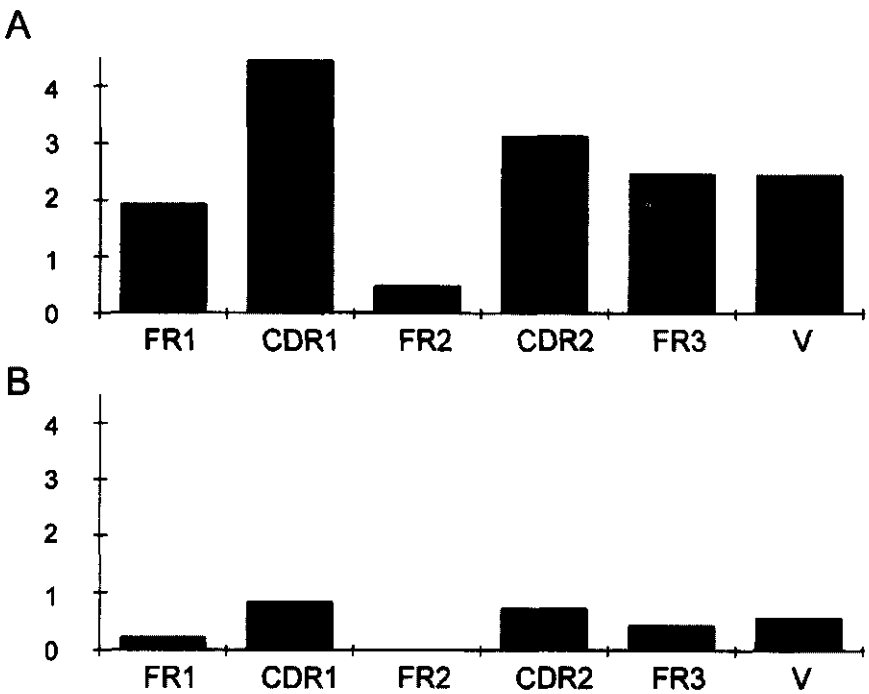

FIGURE 1. Nucleotide substitutions per 100 bp in randomly picked V_H3 rearrangements. Sequences were determined in both orientations to assess differences in the occurrence of somatic mutations. **Panel A,** healthy subject (VMRC3116); **Panel B,** BMT recipient (UPN5012).

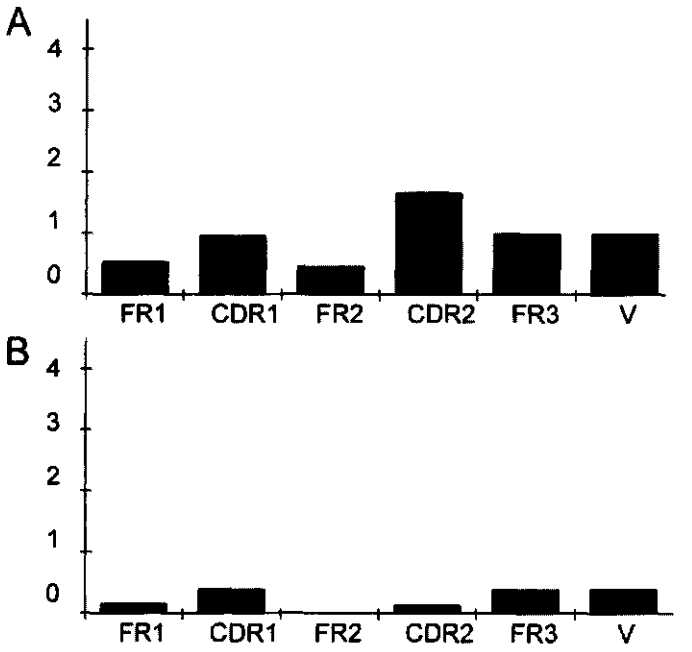

FIGURE 2. Nucleotide substitutions per 100 bp in randomly picked V_H4 rearrangements. Sequences were determined in both orientations to assess differences in the occurrence of somatic mutations. **Panel A,** healthy subject (VMRC3116); **Panel B,** BMT recipient (UPN5012).

formation is defective in BMT recipients months to years post transplant.[4] Together, these data indicate that, although the V_H gene content of the repertoire has normalized by 90 days post transplant, a defect in B-cell differentiation associated with antigen activation persists for at least one year after BMT.

REFERENCES

1. LUM, L. G., M. C. SEIGNEURET, R. F. STORB, R. P. WITHERSPOON & E. D. THOMAS. 1981. Blood **58:** 431–439.
2. WITHERSPOON, R. P., H. J. DEEG, L. G. LUM, H. D. OCHS, J. A. HANSEN, E. D. THOMAS & R. STORB. 1984. Transplantation **37:** 456–461.
3. SUZUKI, I., L. PFISTER, A. M. GLAS, C. NOTTENBURG & E. C. B. MILNER. 1995. J. Immunol. In press.
4. DILLY, S. A., J. P. SLOANE & I. S. M. PSALTI. 1986. J. Pathol. **150:** 213–221.

Chapter 3.2

Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: Lack of somatic mutation indicates a maturational arrest.

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

Blood 87 (1996) 1873-1880

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 V_H genes contributes to this immunodeficiency, the usage of V_H 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_H3 or V_H4 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_H3 and one V_H4. 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 V_H genes is consistent with a defect in antigen driven B-cell responses. These results indicate that although the V_H 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.

ALL MARROW recipients exhibit immunodeficiencies in the first 3 months after bone marrow transplantation (BMT). Both cellular and humoral immunity are affected, reflecting in part the recapitulation of ontogeny. B cells recover slowly in numbers, in frequency, and in function.[1-8] Impaired function of B cells isolated during the first 90 days after transplant is apparent in their reduced ability to proliferate or to secrete Ig in response to mitogens.[3,5,7-9] These in vitro functions usually approach normalcy by 1 year after transplant.[1-3,5-8,10,11] Coincident with recovering B-cell functions, serum Ig levels also return to normal.[5,10,12]

Despite this recovery, marrow recipients who survive this initial postgraft period do not always become fully immunocompetent. Early posttransplant B-lymphopoiesis is mono- or oligoclonal, and restricted clonality is more frequent in patients with chronic graft versus host disease (GVHD). Immune responses to the antigens φX174 and KLH are meager in patients with chronic GVHD.[6] As well, some long-term survivors suffer from opportunistic bacterial infections, especially with encapsulated bacteria such as *Streptococcus pneumoniae* and *Hemophilus influenzae* type b.[13,14] Susceptibility to infections is most severe in recipients with chronic GVHD; the mortality rate for infections in these patients is 15% to 40%.[15] Because immune responses to polysaccharides present in bacterial capsules are typically T-independent, at least a portion of the defect is likely intrinsic to B cells.

The nature of the B-cell defect(s) leading to this specific humoral immunodeficiency is uncertain. Normal levels of serum IgM and IgG[10,12] indicate that immunodeficiency is not because of a general failure to produce Ig or an overt lack of T-cell help in heavy chain class switching (although T-cell function may be impaired to some extent). One possibility is that abnormal restriction of the potential Ig repertoire in B cells from BMT patients, possibly because of effects of chronic GVHD, contributes to humoral immunodeficiency.

The Ig repertoire is established by the developmentally regulated usage of V genes[16,17] and the ensuing selection of B cells.[16-19] Variable regions of Ig heavy chains are generated during differentiation of a B cell by combinatorial association of three gene elements, V_H, D_H, and J_H. Approximately 100 V_H genes are organized into seven families based on nucleic acid hybridization and DNA sequence similarities.[20-24] The families range in size from one gene (V_H6 family)[23] to approximately 30 to 35 genes (V_H3 family).[20]

The usage of V_H families is not random during development[25-29] or in phenotypically defined subpopulations of B cells.[30,31] For example fetal B cells derived from 7 weeks of gestation use genes from the V_H5 and V_H6 families, exclusively. Although all V_H families are used by 15 to 18 weeks of gestation, V_H gene usage may still not be totally random. For example, at this time not all genes within the V_H3 family are used with equal frequency.[26,27,29] In adults, although the relative percentage of V_H family usage is approximately equivalent to the proportion of each family in the genome,[31-36] within V_H families certain elements predominate and some elements are underrepresented or absent.[37] During fetal development, unequal usage of the variable region gene families has been hypothesized to account in part for the immunodeficiency of human neonates,[16,27] which lack antibody responses to many antigens, including *H. influenzae*.[38] Concomitant then with the normalization of the repertoire would be the ability to respond to the wide variety of environmental antigens typically encountered. At least in adults, although the repertoire is not totally random within V gene families, immunodeficiencies are not apparent. However, it remains unknown if deviation from the normal representation of V_H

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Table 1. Patients Studied

Patient	Sex	Diagnosis	Onset of GVHD*	Age at Transplant	Age of Donor
UPN5012	F	Chronic myeloid leukemia	88	28	17
UPN5007	F	Acute myeloid leukemia	N	54	63
UPN4986	F	Chronic myeloid leukemia	N	37	33
UPN5403	M	Chronic myeloid leukemia	90	45	39

Abbreviation: N, no GVHD present.
* Number of days following BMT.

families or genes in B cells after BMT accounts, in part, for the observed immunodeficiencies.

The usage of V_H families following BMT appears to mimic usage during B-cell ontogeny.[39,40] 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[40] who found that the B-cell repertoire is restricted to fetal-type V_H genes early posttransplant. In this report, a direct assessment of the B-cell repertoire was made by analyzing the occurrence of specific V_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_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 at a fairly late stage of differentiation.

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 under Virginia Mason Research Center (VMRC) Institutional Review Board approval were studied simultaneously. Analysis of the repertoire of these healthy subjects has been published.[37]

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 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 Epics 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).

Rearrangement library construction by V_H family-specific primer method. Sorted B cells were lysed in cell lysis buffer (50 mmol/L Tris, pH 8.0, 1 mmol/L Na EDTA, 0.5% NP-40, 0.5% Triton X-100 (JT Baker, Phillipsburg, NJ), 0.5% Tween 80, and 200 μg/mL proteinase K) and incubated at 50°C for 30 minutes. Cell lysates were sonicated for 30 seconds using a Sonifier 450 (Branson, Danbury, CT) fitted with a cup horn, at 50% duty cycle, 50% output to provide more uniform polymerase chain reaction (PCR) amplification. Proteinase K was heat-inactivated by incubation for 10 minutes at 95°C. The rearranged V_H genes were amplified using one of the family-specific 5′ primers, V_H3-L, 5′-CTGAATTCCATGGAGTTTGGGCTGAG-3′ or V_H4-L, 5′-CCGAATTCATGAAACACCTGTGGTTCTT-3′, corresponding to the 5′ ends of the leader sequences of V_H3 and V_H4 families, respectively, and the 3′ primer J_HAmp-7, 5′-GCTCTAGACT(T/C)ACCTGAGGAGACGGTGA-3′, complementary to the 3′ end of the 6 J_H gene sequences. Restriction sites (*Eco*RI for 5′ primers; *Xba* I for 3′ primers) included in the primers are underlined. An aliquot of lysate containing 15,000 to 50,000 cell equivalents was amplified by PCR using cycle conditions to obtain amplification in the linear range. The linearity of the PCR reactions has been confirmed by electrophoresing [α-^{32}P]dCTP-incorporated PCR products on a 2.0% agarose gel and quantifying the bands by phosphor imaging (Molecular Dynamics 400A PhosphorImager, Sunnyvale, CA).[37] The PCR products were cloned into *Eco*RI/*Xba*I digested pBS(M13+) phagemid vector and the recombinant plasmids were used to transform competent DH5aF′ (GIBCO-BRL, Gaithersburg, MD) or BSJ72.[41] The transformants were toothpicked into wells of a 96-well plate containing Luria Broth (10 mg/mL bactotryptone, 5 mg/mL bacto-yeast extract, 10 mg/mL NaCl, pH 7) with 100 μg/mL carbenicillin or ampicillin, 4 μg/mL kanamycin, and viruses containing single stranded DNA were rescued by the addition of K07 helper phage.

Germ-line library construction. V_H family-specific germ-line libraries were similarly generated as previously described from either the negative fraction collected from the cell sorts or unsorted PBMC. PCR reactions used for constructing V_H3 libraries were carried out using the 5′ primer VH3-L and either the 3′ primer VH3-RS, 5′-GACTCTAGACAATGACTTCCCCTACT-3′, which is complementary to the 3′ flanking recombination signal sequence of V_H3 genes, or VH3-FR3, 5′-GACTCTAGATCTCAGGCTGTTCATTTG-3′, which is complementary to a conserved V_H3 framework three (FR3) sequence. PCR reactions used for constructing V_H4 libraries were performed using the 5′ primer VH4-L and either the 3′ primer V_H4-RS, 5′-AATTCTAGACTGGGCTCACACTCACCTCC-3′, which is complementary to the 3′ flanking recombination signal sequence of V_H4 genes, or VH4-FR3, 5′-AATTCTAGACACAGAGCTCAGCTTCAG-3′, which is complementary to a conserved V_H4 FR3 sequence.

Library screening. Multiple replicate filters were prepared by dot blotting 10 μL 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.[42,43] Hybridization with ^{32}P-labeled family-specific V_H probes allowed a determination of the total number of recombinant clones in each library. A panel of diagnostic ^{32}P-labeled oligonucleotide probes that identify individual V_H gene segments were also hybridized to replicate filters. The frequency of occurrence of each specific V_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 2. Phenotype of Lymphocytes From BMT Recipients

Patient	Donor			90 d Posttransplant			1 yr Posttransplant		
	CD20	CD5 + CD20	IgM + IgD	CD19	CD5 + CD19	IgM + IgD	CD19	CD5 + CD19	IgM + IgD
	% of Total PBL			% of Total PBL			% of Total PBL		
UPN5012	8.22	3.64	ND*	7.91	4.36	2.17	13.6	6.09	11.7
UPN5007	15.1	5.3	ND	8.94	2.02	4.58	ND	ND	ND
UPN4986	5.05	2.58	9.6	13.13	4.74	10.39	14.54	6.73	10.24
UPN5403	9.68 (CD19)	4.0 (CD19)	4.06	3.70	2.43	1.09	16.62	6.04	12.4

Abbreviation: ND, not determined.

Oligonucleotide probes. The following oligonucleotide probes were used: M8, AGCAGCTATGCCATGAGCTGG[44]; M76, GCAGTTATATGGTATGATGGA[44,45]; M16, AGTAGCTATGGCATGCACTGG[46]; E36, AGTGGTAGTACCATATACTAC; H110, CGTATTAAAAGCAAAACTGAT[42]; M85, AGTAGCTACGACATGCACTGG[44]; M19, GGAAGCAATAAATACTACGCA[46]; M41, GGAAGTAATAAATACTACGCA[44,45]; E42, AGTATCTATTATAGTGGGAGC (reverse complement of M114[47]); M69, TGGTGGAGCTGGGTCCGCCAG[47]; E57, GAAATCTATCATAGTGGGAGC (reverse complement of M115[47]); M109, AGTGGTTACTACTG*GAGCTGG*[47]; *M101, AGCAGTGGTAACTGGTGAATC*[47]; *E8*, TCCATCAGCAGTGGTAGTTAC[47]; E44, CGTATCTATACCAGTGGGAGC (reverse complement of M121[47]); E58, TACATCTATTACAGTGGGAGC (reverse complement of M103[47]); M105, AGTGGTGATTACTACTGGAGT[47]; E13, TCCGTCAGCAGTGGTAGTTAC[47]; E7, TCCATCAGTAGTTACTACTGG[47]; M100, AGCAGTAGTAACTGGTGGGGC[47]; M86, GGTGGTTACTCCTGGAGCTGG.[47]

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.

Table 3. Serum Ig Levels of Marrow Recipients

Patient	IgM		IgG		IgA	
	90 d	1 yr	90 d	1 yr	90 d	1 yr
UPN5012	34*	154	846	1,060	90	28
UPN5007	190	ND	899	ND	93	ND
UPN4986	185	116	705	613	99	79
UPN5403	52	57	784	843	155	125
Normal	56-275		670-1,700		70-350	

Abbreviation: ND, not determined.
* mg/dL.

V_H gene-specific analysis. To assess the usage of individual genes within V_H3 and V_H4 families, libraries of rearrangements were generated using V_H family specific 5′ primers and the consensus J_H 3′ primer as described previously.[37] This system has been found to amplify approximately 25 V_H3 genes and 10 to 12 V_H4 genes,[37] which together account *for more than 75% of the total expressed* V_H *repertoire.*[48] Synthetic oligonucleotide probes that specifically identify both germ line and rearranged individual V_H elements directly in genomic DNA and in libraries of cloned V regions have been described.[37,42,44,46] For this report six V_H3 and 11 V_H4 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_H3 elements assessed here account for approximately 50% to 80% of the V_H3 component, and the V_H4 elements assessed account for virtually 100% of the V_H4 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_H3 genes were assessed in PB B 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_H 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_H3 and V_H4 genes from the same individuals were also performed.

The occurrence of V_H3 genes in rearrangements for all subjects is shown in Fig 1. The occurrence of rearranged V_H3 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.[49] 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

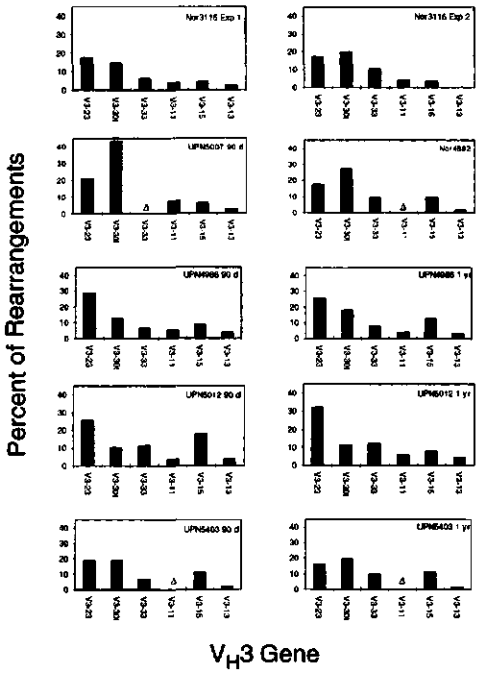

Fig 1. V_H3 repertoire in BMT recipients and normal subjects. The frequency of representation of six V_H3 genes in phagemid clones of quantitatively amplified rearranged V_H3 genes is shown. Data in each panel represent analysis of at least 700 rearrangements. The sample used in experiment 2 of subject Nor3116 was obtained 8 months after the sample used in experiment 1. (Δ) indicates that subject has a germ-line deletion of this V_H element. V_H nomenclature is that of Matsuda et al,[56] except V3-30t which encompasses a complex allelic region and may include V3-30, V3-30b, and/or V3-30.4. Data for Nor3116 and Nor4885 are from Suzuki et al.[37]

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_H4 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_H3 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_H 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_H gene of origin could be assigned by hybridization. Among the V_H3-containing rearrangements (A), there was a slight trend toward increased identification in BMT recipients compared to the two healthy subjects ($p < .005$ for patients v controls). This trend was more pronounced among V_H4-containing rearrangements (B). Sequence-specific oligonucleotide probes to 9 V_H4 loci (11 distinct gene segments) identified more than 99% of the V_H4 rearrangements in the BMT recipient, but only 80% of the V_H4 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_H genes. However, extensive hybridization and sequence analysis have not revealed the presence of additional V_H 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_H3 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

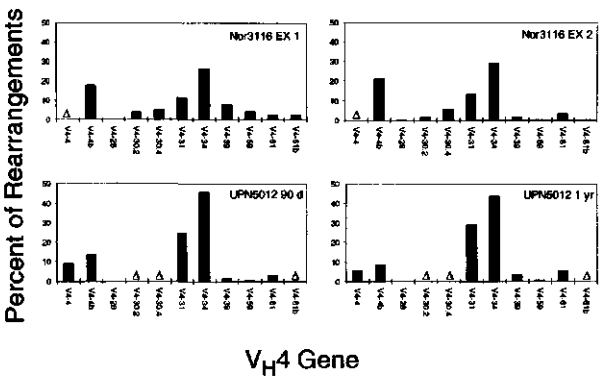

Fig 2. V_H4 repertoire in a BMT recipient and a normal subject. The frequency of representation of nine V_H4 loci (11 elements) in phagemid clones of quantitatively amplified rearranged V_H4 genes is shown. Data in each panel represent analysis of at least 700 rearrangements. The sample used in Nor3116 experiment 2 was obtained 8 months after the sample used in experiment 1. (Δ) indicates that subject has a germline deletion of this V_H element. Data for Nor3116 are from Suzuki et al.[37]

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.[37,49]

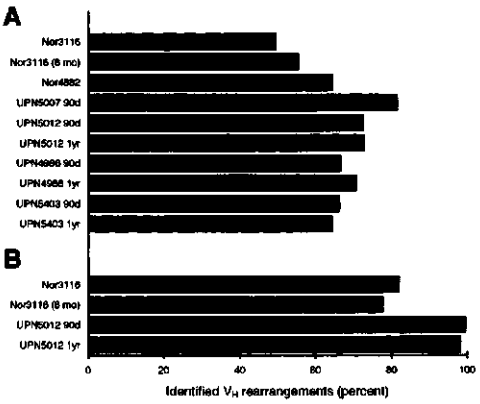

Fig 3. Germ-line complexity of the amplifiable V_H3 (A) or V_H4 (B) repertoires. Bars represent the percent of rearrangements in each library for which the germ-line gene of origin could be assigned by sequence-specific hybridization.

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.

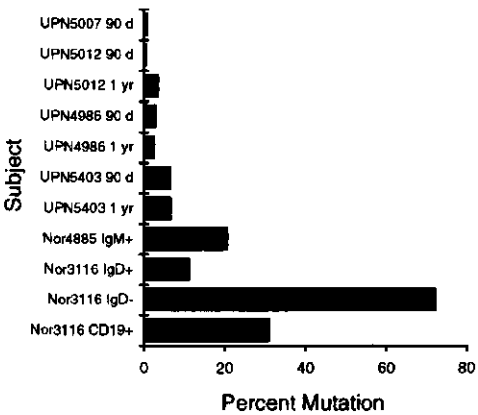

Fig 4. Analysis of somatic mutation in rearranged V_H3 genes from BMT recipients and normal subjects. The accumulation of somatic mutations in one V_H3 gene, V3-23, was assessed by sequential hybridization with the probes, M8 and M18, as described in Materials and Methods. Clones that have accumulated 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 (ie, 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_H 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 phenotypically $CD19^+$, 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.[50] 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_H 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,[51] and that the reconstitution of $CD4^+CD8^-$ T cells (but not $CD4^-CD8^+$ T cells) is similarly delayed.[52] In contrast to BMT recipients, germinal centers are present in neonates from about 1 month of age.[53]

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.[54]

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REFERENCES

1. Witherspoon RP, Lum LG, Storb R, Thomas ED: In vitro regulation of immunoglobulin synthesis after human marrow transplantation. II. Deficient T and non-T lymphocyte function within 3 to 4 months of allogeneic, syngeneic, or autologous marrow grafting for hematologic malignancy. Blood 59:844, 1982

2. Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N: B-cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: A recapitulation of normal B cell ontogeny. Blood 76:1647, 1990

3. Kiesel S, Pezzutto A, Moldenhauer G, Haas R, Körbling M, Hunstein W, Dörken B: B-cell proliferative and differentiative responses after autologous peripheral blood stem cell or bone marrow transplantation. Blood 72:672, 1988

4. Antin JH, Ault KA, Rappeport JM, Smith BR: B lymphocyte reconstitution after human bone marrow transplantation. Leu-1 antigen defines a distinct population of B lymphocytes. J Clin Invest 80:325, 1987

5. Matsue K, Lum LG, Witherspoon RP, Storb R: Proliferative and differentiative responses of B cells from human marrow graft recipients to T cell-derived factors. Blood 69:308, 1987

6. Witherspoon RP, Storb R, Ochs HD, Flournoy N, Kopecky KJ, Sullivan KM, Deeg HJ, Sosa R, Noel DR, Atkinson K, Thomas ED: Recovery of antibody production in human allogeneic marrow graft recipients: Influence of time posttransplantation, the presence or absence of chronic graft-versus-host disease, and antithymocyte globulin treatment. Blood 58:360, 1981

7. Noel DR, Witherspoon RP, Storb R, Atkinson K, Doney K, Mickelson EM, Ochs HD, Warren RP, Weiden PL, Thomas ED: Does graft-versus-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation of 56 long-term survivors. Blood 51:1087, 1993

8. Kagan J, Champlin RE, Saxon A: B-cell dysfunction following human bone marrow transplantation: Functional-phenotypic dissociation in the early posttransplant period. Blood 74:777, 1989

9. Klebanoff SJ, Waltersdorph AM, Rosen H: Antimicrobial activity of myeloperoxidase. Methods Enzymol 105:399, 1984

10. Lum LG, Seigneuret MC, Storb RF, Witherspoon RP, Thomas ED: In vitro regulation of immunoglobulin synthesis after marrow transplantation. I. T-cell and B-cell deficiencies in patients with and without chronic graft-versus-host disease. Blood 58:431, 1981

11. Velardi A, Cucciaioni S, Terenzi A, Quinti I, Aversa F, Grossi CE, Grignani F, Martelli MF: Acquisition of Ig isotype diversity after bone marrow transplantation in adults. A recapitulation of normal B cell ontogeny. J Immunol 141:815, 1988

12. Witherspoon RP, Deeg HJ, Lum LG, Ochs HD, Hansen JA, Thomas ED, Storb R: Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-versus-host disease. Transplantation 37:456, 1984

13. Winston DJ, Schiffman G, Wang DC, Feig SA, Lin C, Marso EL, Ho WG, Young LS, Gale RP: Pneumococcal infections after human bone-marrow transplantation. Ann Intern Med 91:835, 1979

14. Aucouturier P, Barra A, Intrator L, Cordonnier C, Schulz D, Duarte F, Vernant J, Preud'homme J: Long lasting IgG subclass and antibacterial polysaccharide antibody deficiency after allogeneic bone marrow transplantation. Blood 70:779, 1987

15. Sullivan KM, Buckner CD, Sanders JE, Anasetti C, Appelbaum FR, Clark J, Doney K, Meyers J, Sale G, Storb R, Thomas ED, Witherspoon RP: Long-term complications of bone marrow transplantation, in Buckner CD, Gale RP, Lucarelli G (eds): Advances and Controversies in Thalassemia Therapy. Bone Marrow Transplantation and Other Approaches. New York, NY, Liss, 1989, p 367

16. Malynn B, Berman JE, Yancopoulos GD, Bona CA, Alt FW: Expression of the immunoglobulin heavy-chain variable gene repertoire, in Paige CJ, Gisler RH (eds): Current Topics in Microbiology and Immunology. Berlin, Germany, Springer-Verlag, 1987, p 75

17. Perlmutter RM: Programmed development of the antibody repertoire. Curr Top Microbiol Immunol 135:95, 1987

18. Gu H, Tarlinton D, Muller W, Rajewsky K, Forster I: Most peripheral B cells in mice are ligand selected. J Exp Med 173:1357, 1991

19. Valles-Ayoub T, Govan HL III, Braun J: Evolving abundance and clonal pattern of human germinal center B cells during childhood. Blood 76:17, 1990

20. Berman JE, Mellis SJ, Pollock R, Smith CL, Suh H, Heinke B, Kowal C, Surti U, Chess L, Cantor CR, Alt FW: Content and organization of the human Ig VH locus: Definition of three new VH families and linkage to the Ig CH locus. EMBO J 7:727, 1988

21. Lee KH, Matsuda F, Kinashi T, Kodaira M, Honjo T: A novel family of variable region genes of the human immunoglobulin heavy chain. J Mol Biol 195:761, 1987

22. Shen A, Humphries C, Tucker P, Blattner F: Human heavy-chain variable region gene family nonrandomly arranged in familial chronic lymphocytic leukemia. Proc Natl Acad Sci USA 84:8563, 1987

23. Schroeder HW Jr, Walter MA, Hofker MH, Ebens A, Willems van Dijk K, Liao LC, Cox D, Milner ECB, Perlmutter RM: Physical linkage of a human immunoglobulin heavy chain variable region gene segment to diversity and joining region elements. Proc Natl Acad Sci USA 85:8196, 1988

24. Mortari F, Newton JA, Wang JY, Schroeder HW Jr: The human cord blood antibody repertoire. Frequent usage of the V_H7 gene family. Eur J Immunol 22:241, 1992

25. Cuisinier A-M, Guigou V, Boubli L, Fougereau M, Tonnelle C: Preferential expression of V_H5 and V_H6 immunoglobulin genes in early human B-cell ontogeny. Scand J Immunol 30:493, 1989

26. Raaphorst FM, Timmers E, Kenter MJH, Van Tol MJD, Vossen JM, Schuurman RKB: Restricted utilization of germ-line VH3 genes and short diverse third complementarity-determining regions (CDR3) in human fetal B lymphocyte immunoglobulin heavy chain rearrangements. Eur J Immunol 22:247, 1992

27. Schroeder HW Jr, Hillson JL, Perlmutter RM: Early restriction of the human antibody repertoire. Science 238:791, 1987

28. Berman JE, Nickerson KG, Pollock RR, Barth JE, Schuurman RKB, Knowles DM, Chess L, Alt FW: V_H gene usage in humans biased usage of the V_H6 gene in immature B lymphoid cells. Eur J Immunol 21:1311, 1991

29. Schroeder HW Jr, Wang JY: Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. Proc Natl Acad Sci USA 87:6146, 1990

30. Mageed RA, MacKenzie LE, Stevenson FK, Yuksel B, Shokri F, Maziak BR, Jefferis R, Lydyard PM: Selective expression of a V_HIV subfamily of immunoglobulin genes in human CD5+ B lymphocytes from cord blood. J Exp Med 174:109, 1991

31. Schutte MEM, Ebeling SB, Akkermans KE, Gmelig-Meyling FHJ, Logtenberg T: Antibody specificity and immunoglobulin VH gene utilization of human monoclonal CD5+ B cell lines. Eur J Immunol 21:1115, 1991

32. Guigou V, Guilbert B, Moinier D, Tonnelle C, Boubli L, Avrameas S, Fougereau M, Fumoux F: Ig repertoire of human polyspecific antibodies and B cell ontogeny. J Immunol 146:1368, 1991

33. Guigou V, Cuisinier A-M, Tonnelle C, Moinier D, Fougereau M, Fumoux F: Human immunoglobulin V_H and V_K repertoire revealed by *in situ* hybridization. Mol Immunol 27:935, 1990

34. Logtenberg T, Schutte MEM, Inghirami G, Berman JE, Gmeling-Meyling FHJ, Insel RA, Knowles DM, Alt FW: Immunoglobulin V_H gene expression in human B cell lines and tumors: Biased V_H gene expression in chronic lymphocytic leukemia. Int Immunol 1:362, 1989

35. Zouali M, Theze J: Probing V_H gene-family utilization in human peripheral B cells by in situ hybridization. J Immunol 146:2855, 1991

36. Huang C, Stewart AK, Schwartz RS, Stollar BD: Immunoglobulin heavy chain gene expression in peripheral blood lymphocytes. J Clin Invest 89:1331, 1992

37. Suzuki I, Pfister L, Glas A, Nottenburg C, Milner ECB: Representation of rearranged V_H gene segments in the human adult antibody repertoire. J Immunol 154:3902, 1995

38. Lucas AH, Azmi FH, Mink CM, Granoff DM: Age-dependent V region expression in the human antibody response to the *Haemophilus influenzae* type b polysaccharide. J Immunol 150:2056, 1993

39. Fumoux F, Guigou V, Blaise D, Maraninchi D, Fougereau M, Schiff C: Reconstitution of human immunoglobulin VH repertoire after bone marrow transplantation mimics B-cell ontogeny. Blood 81:3153, 1993

40. Storek J, King L, Ferrara S, Marcelo D, Saxon A, Braun J: Abundance of a restricted fetal B cell repertoire in marrow transplant recipients. Bone Marrow Transplant 14:783, 1994

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

42. Willems van Dijk K, Schroeder HW, Jr., Perlmutter RM, Milner ECB: Heterogeneity in the human immunoglobulin VH locus. J Immunol 142:2547, 1989

43. Willems van Dijk K, Sasso EH, Milner ECB: Polymorphism of the human immunoglobulin V_H4 gene family. J Immunol 146:3646, 1991

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

45. Sasso EH, Willems van Dijk K, Bull A, van der Maarel SM, Milner ECB: V_H genes in tandem array comprise a repeated germline motif. J Immunol 149:1230, 1992

46. Sasso EH, Willems van Dijk K, Milner ECB: Prevalence and polymorphism of human V_H3 genes. J Immunol 145:2751, 1990

47. van der Maarel SM, Willems van Dijk K, Alexander CM, Sasso EH, Bull AB, Milner ECB: Chromosomal organization of the human V_H4 gene family: Location of individual gene segments. J Immunol 150:2858, 1993

48. Brezinschek HP, Brezinschek RI, Lipsky PE: Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J Immunol* 155:190, 1995

49. Huang S-C, Jiang R, Glas AM, Milner ECB: Nonstochastic utilization of Ig V regions in unselected human peripheral B cells. Mol Immunol (in press)

50. MacLennan IC: Somatic mutation. From the dark zone to the light. Curr Biol 4:70, 1994

51. Dilly SA, Sloane JP, Psalti ISM: The cellular composition of human lymph nodes after allogeniec bone marrow transplantation: An immunohistological study. J Pathol 150:213, 1986

52. Storek J, Ferrara S, Rodriguez C, Saxon A: Recovery of mononuclear cell subsets after bone marrow transplantation: Overabundance of CD4+CD8+ dual-positive T cells reminiscent of ontogeny. *J Hematother* 1:303, 1992

53. Westerga J, Timens W: Immunohistological analysis of human fetal lymph nodes. Scand J Immunol 29:103, 1989

54. Lum LG: Immune recovery after bone marrow transplantation. Bone Marrow Transplant 4:659, 1990

55. Matsuda F, Shin EK, Nagaoka H, Matsumura R, Haino M, Fukita Y, Taka-ishi S, Imai T, Riley JH, Anand R, Soeda E, Honjo T: Structure and physical map of 64 variable segments in the 3′ 0.8-megabase region of the human immunoglobulin heavy-chain locus. Nat Genet 3:88, 1993

Chapter 3.3

Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient.

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Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient

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SUMMARY

Haematopoietic stem cell transplantation has been used for the treatment of many different malignant and non-malignant diseases. The immune system of transplant recipients must be regenerated from the transplant inoculum, and it is not surprising that many transplant recipients are deficient in generating specific antibody responses to exogenous stimuli. This B cell immunodeficiency in these patients is associated with clinically significant infections, although the underlying mechanism remains unknown. We have previously shown that the pattern of usage of V_H genes was similar between healthy subjects and BMT recipients, indicating that the immunodeficiency was not due to a dramatic imbalance in V_H utilization. However, motif-specific hybridization analysis indicated that the accumulation of somatic mutations was much greater among rearrangements in controls than in BMT recipients. The failure of BMT recipients to accumulate somatic mutations in rearranged V_H genes correlates with an absence of IgD^- B cells, and is consistent with a defect in antigen-driven B cell responses. In the current study, which extends those findings, we have determined the nucleotide sequences of 68 heavy chain rearrangements from one patient as well as 39 rearrangements from a healthy control. Analysis of these sequences made possible a more precise definition of variable region configuration and of the status of somatic mutation in this BMT recipient. The results validate the hybridization data and support the conclusion that, although somatic hypermutation and, by inference, antigen-driven responses are detected in BMT recipients, they are deficient compared with healthy subjects as late as 1 year after transplant.

Keywords somatic mutation immunoglobulin bone marrow transplant diversity segment heavy chain variable region

INTRODUCTION

Haematopoietic stem cell transplant, using either bone marrow or mobilized peripheral stem cells, is characterized by a prolonged period of humoral immunodeficiency, reflecting in part the recapitulation of ontogeny [1]. Results suggest that the humoral immunodeficiency that exists post-transplant is the result of either a B cell defect, a monocyte defect, or a B cell/T cell cooperation defect which, in some patients, may be correctable with the addition of a cytokine [2]. T cell defects, including lack of helper activity and elevated suppresser activity, as well as B cell defects, are commonly observed [2–4]. B cells recover slowly in numbers, possibly because the post-transplant bone marrow microenvironment is deficient in supporting the proliferation and/or differentiation of B cell precursors [5], in frequency, and in function [6–13]. Impaired function of B cells isolated during the first 90 days after transplant is apparent in their reduced ability to proliferate or to secrete immunoglobulin in response to mitogens [8,10,12–14]. These *in vitro* functions usually approach normalcy by 1 year after transplant [3,6–8,10–13,15]. Coincident with recovering B cell functions, serum immunoglobulin levels also usually return to normal [3,10,16], with normal levels of IgM returning at 6 months, IgG at 12 months, and IgA after 2 years [17]. However, some patients who survive the initial post-grafting period do not become fully immunocompetent [18,19].

B cell and CD4 T cell numbers are low for at least 3 months; this is followed by fast recovery of B cell numbers (by 6–12 months) and slower recovery of CD4 T cells [7,20,21]. Between 1 month and 1 year after transplantation, in most patients there is a marked increase in the percentage of B cell precursors in the marrow, which probably accounts for the normalization or even overshooting of blood B cell counts within 1 year after transplant. However, these early emerging blood B cells have some features of immaturity, such as large size, a high surface expression of IgD, IgM and CD38 [7,13,15,20].

Early post-transplant B lymphopoiesis is mono- or oligoclonal,

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and restricted clonality is more frequent in patients with chronic graft-*versus*-host disease (GVHD). Immune responses to the antigens bacteriophage φX174 and keyhole limpet haemocyanin (KLH) are meagre in patients with chronic GVHD, as is antibody formation against pneumococcal polysaccharides [11]. Post-transplant infections are a well documented complication among transplant patients [22]. Some long-term survivors suffer from opportunistic bacterial infections, especially with encapsulated bacteria such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b [23,24]. Susceptibility to infections is most severe in recipients with chronic GVHD; the mortality rate for infections in these patients is 15–40% [25]. In an analysis of 443 patients with chronic myelogenous leukaemia (CML) in chronic phase given human leucocyte antigen (HLA)-matched sibling ($n = 251$) or mismatched/unrelated ($n = 192$) marrow since 1987, 1553 episodes of infection developed after day 120: 60 patients had no episodes; 160 had one to two episodes; 125 had three to five episodes; and 98 had six or more episodes [22]. With the use of routine antibiotic prophylaxis of penicillin and trimethoprim-sulfamethoxazole, encapsulated bacteria are now less frequent isolates than previously reported. Fungal and Gram-negative organisms are now more frequently observed.

It seems likely that a heterogeneous collection of defects is responsible for the humoral deficiencies following marrow transplant. The evidence cited above is consistent with a variety of mechanisms. For example, B cell dysfunction could be secondary to T cell dysfunction, either due to inadequate T cell help or to suppression; accessory cell (e.g. follicular dendritic cell) function could be defective; disturbance of the bone marrow microenvironment could lead to abnormalities of B cell differentiation [5]; the disruption of secondary lymphoid tissue architecture could lead to a failure to supply an adequate microenvironment for peripheral B cell differentiation [26,27]. This latter possibility may be important. Although studies are limited to autopsy, the evidence indicates that germinal centres recover slowly in many patients [28]. In a study of 40 patients 70–458 days following transplant, only two had recovered germinal centres. In another study, no patients recovered mesenteric lymph node germinal centres before day 133 post-transplant (reviewed in [29]).

One possibility, which we have explored, was that disruption of the programme of selection of the antibody repertoire in B cells from BMT patients contributed to humoral immunodeficiency [30,31]. This was based on the observation that usage of V_H families following BMT appears to mimic usage during B cell ontogeny [32,33]. Thus, to account for the observed immunodeficiencies, it might be expected that during the first year post-transplant, BMT patients would utilize a more limited set of V genes than would healthy adult subjects. Additional support for this hypothesis came from the observation that the B cell repertoire is restricted to fetal-type V_H genes in the very early post-transplant period [33].

In a previous study, we analysed the occurrence of specific V_H gene segments by motif-specific hybridization in over 4900 rearrangements in peripheral blood B cells of two healthy subjects and four BMT recipients at 90 days and 1 year following transplant [31]. The results indicated that the pattern of usage of V_H genes was similar between BMT recipients and healthy subjects. These observations suggested that the processes involved in generating and selecting the antibody repertoire are largely functional within the first few months following BMT [31]. Furthermore, these results indicated that the immunodeficiencies common among BMT recipients are probably not due to the failure to utilize appropriate V region genes in generating the pre-immune antibody repertoire. However, the results did provide some indication of possible mechanisms contributing to poor antibody responses in BMT recipients. Using a highly sensitive hybridization technique, we found that rearrangements in BMT recipients exhibited much less somatic mutation than did rearrangements of the same gene, V3–23, obtained from healthy subjects. The failure in BMT recipients to accumulate somatic mutations in rearranged V_H genes in peripheral blood lymphocytes (PBL) correlated with an absence of IgD^- B cells, and is consistent with a maturational arrest at a fairly late stage of differentiation.

In this study, to define more precisely the status of somatic mutation among BMT recipients, and to discriminate between quantitative and qualitative differences, we determined the nucleotide sequence of randomly selected rearrangements representing the spectrum of V_H3 and V_H4 genes utilized in one patient. The results show that the number of somatic mutations in the BMT recipient was significantly lower than in the healthy subject. The complexity of the CDR3 (third complementarity determining region) and D_H and J_H utilization further supports the conclusion that variable region configuration is normal in BMT recipients. The ratio of replacement to silent substitutions, and the finding that the specific pattern of substitutions deviated from the pattern characteristic of unselected mutations induced by the mutator mechanism, suggest that the few observed mutations were the result of antigen-driven responses. The results validate the hybridization data and support the conclusion that, although somatic hypermutation and, by inference, antigen-driven responses are detected in BMT recipients, they are deficient compared with healthy subjects as late as 1 year after transplant.

PATIENTS AND METHODS

BMT recipients and control subjects

We have previously reported V_H utilization and accumulation of somatic mutations among four BMT patients (three female, one male) [31]. One of the patients, UPN 5012, who was diagnosed with GVHD within the first 100 days following transplant, was selected for additional analysis in the current study. One of the healthy controls (VMRC donor 3116) was also studied further. All samples were obtained under Institutional Review Board approved informed consent.

Cell isolations, library construction and oligonucleotide hybridization were performed as described [31,34].

DNA sequence analysis

DNA sequences were determined with Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on the Applied Biosystems Model 373 A DNA Sequencing System. Sequencing reactions were primed with either a universal primer (T7 or T3 sequencing primers) or oligonucleotide E61, 5′-CTGTTCACAGGGGTCCTGTC-3′, which corresponds to the leader sequence of V_H4 gene segments, or additional primers corresponding to the CDR2 and FR3 regions of different genes were used to sequence CDR3 regions: M10, 5′-ATATGATGGAAGTAATAAAT-3′; M18, 5′-GTGAAGGGCCGGTTCACCATC-3′; M22, 5′-ACTGATGGTGGGACAACAGAC-3′; E99, 5′-AGTTACACCACAGAATACGCC-3′; M76, 5′-GCAGTTATATGGTATGATGGA-3′; M92, 5′-TGCATAACCTGTGCTACCACC-3′; E36, 5′-AGTGGTAGTACCATATACTAC-3′. For each

sequence, the V, D, and J germ-line counterparts were identified. Initially 20 randomly drawn clones from each library were studied. Of those, complete VDJ sequences were obtained from 68 heavy chain rearrangements from the BMT recipient and 39 heavy chain rearrangements from the healthy subject.

RESULTS

V_H segment mutation

To determine if there were qualitative differences in number of mutations or differences with respect to distribution and kind of substitutions in BMT recipients, we analysed the complete nucleotide sequences from randomly selected clones from those previously analysed libraries of one BMT recipient and compared these with nucleotide sequences determined for randomly chosen rearrangements obtained from a healthy control. Clones were chosen for analysis by random drawing from each of the V_H3 and V_H4 libraries. A total of 107 clones comprising 99 independent rearrangements were analysed. The results of sequence analysis are summarized in Table 1. The germ-line source of each of the rearrangements was determined by comparison with known germ-line gene sequences, including some sequences from germ-line libraries made from the bone marrow donor, some sequences previously published by us [34,35], and some sequences extracted from GenBank. Comparison of CDR3 sequences was used to establish the independence of rearrangements. In control experiments, in which rearrangements were amplified from B cell lines, we found that as many as two nucleotide substitutions per rearrangement can be introduced by the process of generating the rearrangement libraries (data not shown). The number of substitutions in combined FR and CDR is shown separately for replacement (R) and silent (S) interchanges. The ratio of R/S was calculated for each group of sequences in Table 1. Somewhat surprisingly, R/S was higher in the BMT sequences than in the normal subject sequences. However, the number of mutations in BMT rearrangements was significantly lower when compared with the normal subject ($P < 0{\cdot}001$). As shown in Table 2, the differences are very significant when rearrangements with one, two or three or more mutations are compared between BMT recipient and healthy subject ($P < 10^{-5}$).

Analysis of independent substitutions both in the normal subject and the BMT recipient showed that transitions (≈55% of all substitutions) occurred more often than transversions, although it should be noted that transitions are the most frequent misincorporation events of Taq-polymerase [36,37]. In agreement with previous reports, there were more mutations of purines than pyrimidines in the healthy subject [38–42]. Three clones (BMT clone 9, normal clones 90 and 103) which contained high numbers of mutations were treated separately in the analysis shown in Table 3.

Table 1. Features of heavy chain rearrangements analysed*

Donor	Clone	V_H	D_H	J_H	Frame†	Substitutions FR (R/S)	Substitutions CDR (R/S)
BMT V_H3 90 days	1	V3–07	ND‡	4	+	0/1	0/0
	2	V3–11	$D_{XP}1$	4	+	0/0	0/0
	3	V3–11	$D_{XP}1$	3	+	0/1	6/0
	4	V3–13	D_N1b	4	–	1/0	0/0
	5	V3–13	ND	4	–	0/0	0/0
	6	V3–15	D_H21S10	4	+	0/0	1/0
	7	V3–23	D_K1	4	+	1/0	2/0
	8	V3–30	$D_{LR}2$	4	–	0/0	0/0
	9§	V3–30	$D_{LR}2$	4	+	16/10	14/8
	10–15¶	V3–30	$D_{LR}5$, D_H21S9, DIR4**	4	+	1/1	3/0
						1·0‡‡	12·0‡‡
BMT V_H3 1 year	16	V3–11	D_N1	4	+	0/0	0/0
	17	V3–11	$D_{LR}2$	4	+	0/0	0/0
	18	V3–15	D_H21S10	4	+	0/0	0/0
	19	V3–20	$D_{XP}'1$	4	+	1/0	0/0
	20	V3–23	ND	4	+	0/1	0/0
	21	V3–23	$D_{LR}2$, *D_N1inv*††	6	–	2/0	4/1
	22	V3–23	D_H21S10	4	+	0/0	0/0
	23	V3–23	D_N1b	2	+	0/0	0/0
	24	V3–23	$D_{XP}4$	4	–	0/0	0/0
	25	V3–30	$D_{XP}'1$	6	+	0/0	0/0
	26	V3–30	ND	3	–	0/0	0/0
	27	V3–30	D_N1b	4	+	0/0	0/0
	28	V3–30	D_N1	4	+	0/0	0/0
	29	V3–30	ND	3	+	0/0	0/0
	30	V3–33	D_N1b	4	–	0/1	0/0
	31	V3–33	D_N1	4	+	0/0	0/0
	32	V3–72	D_{NEW}	4	+	0/0	0/0
						1·5‡‡	4·0‡‡

Table 1. Cont.

Donor	Clone	V_H	D_H	J_H	Frame†	Substitutions FR	Substitutions CDR (R/S)
BMT V_H4 90 days	33	V4–30.2	D_N1b, DIR5inv	4	+	1/0	0/0
	34	V4–31	D_H21S9	1	+	0/0	0/0
	35	V4–31	D_{XP}4	4	–	1/0	0/0
	36	V4–31	DIR, D_{XP}	4	–	1/1	0/0
	37	V4–31	D_{XP}4	4	–	0/1	0/0
	38	V4–34	D_H21S9	4	+	0/0	0/0
	39–40¶	V4–34	D_{XP}'1, D_{XP}	4	+	0/0	0/0
	41	V4–34	ND	3	+	0/0	0/0
	42	V4–34	D_N1	4	+	1/1	1/1
	43	V4–34	ND	4	+	0/0	0/0
	44	V4–34	D_H21S9	6	+	1/0	1/0
	45	V4–34	D_{XP}'1	4	+	0/0	0/0
	46–47¶	V4–34	D_N1	4	+	0/0	0/0
	48	V4–39	D_H21S9	5	+	1/0	0/0
	49	V4–4	D_N1b	4	+	0/0	0/0
	50	V4–4b	DK4, D_{LR}1inv	4	–	1/0	0/0
	51	V4–55	D_{XP}'1, D_{NEW}	4	–	0/1	0/0
						1·75‡‡	2.0‡‡
BMT V_H4 1 year	52	V4–31	D_{XP}1	6	+	0/0	0/0
	58	V4–31	D_H21S9	3	+	0/0	0/0
	54–55¶	V4–31	D_N1b	4	+	0/0	0/0
	56	V4–31	D_{XP}	4	+	0/0	0/0
	57	V4–31	D_A1b	4	+	0/0	0/0
	58	V4–34	D_N1b	4	+	0/0	0/0
	59	V4–34	D_N1b	6	+	0/0	0/0
	60	V4–34	D_{XP}'1	4	+	1/0	1/0
	61	V4–34	D_{LR}4	4	+	1/0	0/0
	62	V4–34	D_A1b	4	+	0/0	0/0
	63	V4–34	ND	4	+	0/0	0/0
	64	V4–4	D_H21S9	1		0/0	0/0
	65	V4–4	D_N1	4	+	0/0	2/0
	66	V4–4b	D_N1b	4	+	0/0	0/0
	67	V4–4b	ND	6		2/1	0/0
	68	V4–61	D_A1b	6	+	5/0	2/0
						9·0‡‡	5·0‡‡
Normal V_H3	69	V3–11	D_{LR}4	6	+	0/0	0/0
	70	V3–15	D_{XP}4	4	+	0/1	0/0
	71	V3–20	D_N1b	5	+	0/0	0/0
	72	V3–20	D_{XP}4	6	+	1/2	0/0
	73	V3–23	D_H21S9	4	–	2/0	2/0
	74	V3–23	D_{NEW}	6	–	1/0	0/0
	75	V3–23	D_N4	4	–	0/0	0/0
	76	V3–23	D_{NEW}	5	+	0/0	0/0
	77	V3–23	D_K4, D_H21S9inv	4	+	4/4	5/3
	78	V3–23	D_H21S9	4	–	0/0	0/0
	79	V3–23	ND	4	+	2/0	4/2
	80	V3–23	D_{XP}4	4	+	0/0	0/0
	81	V3–30	D_H21S9	4	+	0/2	0/0
	82	V3–30	D_{LR}2, D_{XP}	5	+	9/3	2/1
	83	V3–30	D_N1b	4	+	3/5	1/3
	84	V3–30	D_N1	3	+	0/0	0/0
	85	V3–33	D_{LR}1, D_{XP}1/4	3	+	3/3	3/0
	86	V3–33	D_K1	4	+	2/2	0/0
	87	V3–33	D_{XP}4, D_N1b	4	–	1/0	0/0
	88	V3–74	ND	4	+	4/2	7/4
						1·33‡‡	1·85‡‡

Table 1. Cont.

Donor	Clone	V_H	D_H	J_H	Frame†	Substitutions FR (R/S)	Substitutions CDR (R/S)
Normal V_H4	89	V4–30.2	D_N1b, $D_{XP}1$	4	–	11/1	3/1
	90§	V4–30.4	$D_{XP}1$	1	+	12/15	8/5
	91	V4–30.4	D_{NEW}	4	–	0/0	0/0
	92	V4–31	D_H21S9	1	+	1/0	0/0
	93	V4–31	ND	4	+	3/2	3/0
	94	V4–31b	$D_{LR}3$	6	–	3/0	1/0
	95	V4–34	D_{LR}	6	+	1/0	1/0
	96	V4–34	$D_{LR}2$, D_K4	4	+	0/0	0/0
	97	V4–34	$D_A1/4$	3	+	0/0	0/0
	98	V4–34	$D_{LR}4$	6	+	1/0	0/0
	99	V4–34	D_H21S9	3	+	0/0	0/0
	100	V4–39	$D_A1/4$inv, $D_N1$inv	4	+	2/1	3/1
	101	V4–39	D_H21S9	4	+	1/0	1/0
	102	V4–39	$D_{XP}4$	6	+	0/0	0/0
	103§	V4–39	$D_{XP}'1$, $D_A1/4$	3	+	11/13	11/6
	104	V4–4b	$D_A1/4$	4	+	0/0	0/0
	105	V4–4b	$D_{LR}4$	4	–	0/0	0/0
	106	V4–61b	D_{NEW}	4	+	0/0	0/0
	107	V4–61b	$D_{LR}4$	6	+	0/0	0/0
						5·75‡‡	6·0‡‡

*Sequences are available in Genbank: accession nos. μ80083–80182.
†Productive rearrangement shown as +, non-productive rearrangements shown as −.
‡Not determined.
§Highly mutated clones not included in total R/S calculation.
¶Identical clones counted as one rearrangement.
**In some rearrangements multiple D segments were used.
††Inverted D segments in the rearrangements are indicated by 'inv'.
‡‡Total R/S ratio for each group.

The distribution of substitutions of coding strand nucleotides generated by the intrinsic action of the hypermutation mechanism in the absence of selection by antigen has been found to be asymmetric [42,43]. In the absence of selection, the proportion of all substitutions involving each nucleotide is: T = 0·13, C = 0·21, A = 0·29, and G = 0·37 [42]. Deviations from these proportions can be indicative of selection. In our analysis, substitutions in rearrangements from the BMT recipient did deviate from the predicted proportions in that there was a relative increase in substitutions of T (which are less commonly substituted by the hypermutation mechanism) and a relative decrease in substitutions of A, on the coding strand (Table 3). This deviation was significant whether raw data or normalized data were compared with the predicted values. None of the other comparisons revealed significant differences from the values predicted for the hypermutation mechanism.

The distribution of substitutions among the highly mutated clones (BMT clone 9, normal clones 90 and 103) was generally typical of the distribution generated by the antibody hypermutation mechanism in the absence of selection, although there was a slight increase in transversions (Table 3). Compared with the cumulative BMT or normal sequences, BMT clone 9 and normal clone 90 had an increase in A to C substitutions, and normal clone 103 had increased T to A substitutions (Table 3).

Table 2. Numbers of mutations among rearrangements

Number mutations	Normal*	BMT
0	16	35
1	5	13
2	3	4
3 or more	15	8

*The number of rearrangements with the indicated number of somatic mutations is given. For all arrangements, $P < 0{\cdot}000\,01$ for BMT *versus* normal

CDR3 complexity

D segments were identified (according to the criteria of Klein *et al.* [44]), either (i) by identity to a known D_H or DIR region over a stretch of seven consecutive basepairs, or as (ii) having a single basepair difference at least two basepairs from the 5′ or 3′ end in a stretch of eight basepairs otherwise identical to a known D_H or DIR element [45–48]. By these criteria, the germ-line source of D segments in ≈92% of rearrangements from the BMT recipient and 85% of rearrangements from the healthy subject could be identified.

Table 3. Distribution of coding strand substitutions among rearrangements

		Distribution of substitutions*				Cumulative substitutions		
BMT†	From$^{TO:}$	T	C	A	G	n‡	Normalized§	Predicted¶
	T	–	0·53	0·29	0·18	17	20.29	7.41
	C	0·42	–	0·25	0·33	12	11.00	11.97
	A	0·13	0·13	–	0·75	8	8.95	16.53
	G	0·10	0·35	0·55	–	20	16.76	21.09
				P versus predicted		0·00075	6.4×10^{-6}	
BMT	From$^{TO:}$	T	C	A	G			
Clone 9	T	–	0·6	0·2	0·2	5	5·72	5.2
	C	0·5	–	0·2	0·3	10	8·36	8·4
	A	0	0·42	–	0·58	12	13·56	11·6
	G	0·23	0·31	0·46	–	13	12·32	14·8
				P versus predicted		0·9088623	0·8497228	
Normal**	From$^{TO:}$	T	C	A	G			
	T	–	0·44	0·25	0·31	16	19·448	17·68
	C	0·60	–	0·25	0·15	40	38·488	28·56
	A	0·27	0·09	–	0·64	33	38·216	39·44
	G	0·06	0·43	0·51	–	47	39·712	50·32
				P versus predicted		0·1109961	0·1164651	
Normal	From$^{TO:}$	T	C	A	G			
clone 90	T	–	0·6	0·2	0·2	5	5·72	5·2
	C	0·5	–	0·2	0·3	10	8.36	8·4
	A	0	0·42	–	0·58	12	13·56	11·6
	G	0·23	0·31	0·46	–	13	12·32	14·8
				P versus predicted		0·9088623	0·8497228	
Normal	From$^{TO:}$	T	C	A	G			
clone 103	T	–	0·57	0·43	0·00	7	8·487	5·33
	C	0·31	–	0·23	0·46	13	11·111	8·61
	A	0·11	0·11	–	0·78	9	10·742	11·89
	G	0·00	0·25	0·75	–	12	10·66	15·17
				P versus predicted		0·2481294	0·2563223	
Preferences††	From$^{TO:}$	T	C	A	G			
	T	–	0·52	0·31	0·17			
	C	0·74	–	0·09	0.17			
	A	0·29	0·19	–	0·52			
	G	0·09	0·32	0·59	–			

*Proportion of total substitutions of each germ-line base contributed by each of the other three bases.

†Includes all BMT sequences from Table 1 except clone 9.

‡Total number of coding strand substitutions for each base.

§Number of coding strand substitutions normalized for base composition, calculated as normalized $=(s/t)(n/4m)$, where s = number of substitutions of m, t = total number of substitutions, n = total number of nucleotides analysed, and m = total occurrence of each nucleotide.

¶Predicted number of substitutions calculated as: predicted $=f_N t$, based on intrinsic substitution frequency (f) where $f_T = 0{\cdot}13, f_C = 0{\cdot}21, f_A = 0{\cdot}29$, and $f_G = 0{\cdot}37$ [42], and t = total number of substitutions.

**Includes all sequences from the normal subject from Table 1, except clones 90 and 103.

††Intrinsic base substitution preferences of the somatic mutational mechanism [42].

Some of the D families were frequently used in both subjects, e.g. D_{XP}. Other families were less frequently used or not used at all. No rearrangements of DQ52 were observed in either subject. Overall, D_H gene usage in both subjects was consistent with previous reports [49–52]. Rearrangements involving D–D joining or D–DIR joins accounted for 6% of rearrangements in the patient 1 year after transplant, and 23% of the rearrangements 90 days after transplant. In the healthy subject, rearrangements involving D–D joining accounted for 19% of rearrangements. No DIR segments were used in rearrangements from the healthy subject.

In both the healthy subject and the BMT recipient, all three reading frames of the D segments were used, and about 7·5% of the D segments used were inversions, which is consistent with previous results [50,51]. Furthermore, the healthy subject and the BMT recipient generally used similar D segment reading frames, although some individual V genes exhibited an apparent preference for reading frame 2; in the 14 clones containing V4–34, 13 used a D segment in reading frame 2. Also, consistent with earlier results [50,51], some of the D segments used a specific reading frame, e.g. D segments from the D_N family preferentially used reading frame 2.

Novel D segments

Three different sequence motifs were identified in both subjects which could not be assigned to any of the published D_H or DIR region element. The first motif, a 20 bp sequence, GTATAGCAGTGGCTGGTACG, which includes 7 bp previously identified by Yamada *et al.* [51], was found in 12 independent rearrangements. This sequence belongs to the D_N family, differing by two nucleotides from D_N1, and is designated D_N1b. The second motif, a 16 bp sequence, ATAGTGGGAGCTACTT, was found in seven rearrangements. The first seven nucleotides of this sequence are identical to bases 7–13 of D_K1. This new segment could be part of a novel D_H or DIR region element. Part (13 bp) of this D segment is also used in rearrangements reported by Klein *et al.* [44], where it was designated D_{NEW}. The third motif, a 12 mer, GACTACGGTGAC, was found in five rearrangements. This D belongs to the D_A family, differing from D_A1 and D_A4 by two nucleotides, and is designated D_A1b.

J_H distribution

The most frequently used J_H segments were J_H4 (56·8% healthy subject, 66·7% BMT recipient) and J_H6 (21·6% healthy subject, 16·7% BMT recipient) in both BMT recipient and control.

Productive versus *non-productive rearrangements*

In the healthy subject, 75·7% of the randomly picked sequences were in frame, compared with 76·3% in the BMT recipient. The length of CDR3 from productive rearrangements ranged from 13 bp to 69 bp in the healthy subject (mean 42 bp) and from 15 bp to 91 bp in the BMT recipient (mean 39·4 bp).

DISCUSSION

In this study, qualitative differences with respect to distribution and kind of substitutions resulting from somatic mutation of immunoglobulin variable region genes in recipients of bone marrow transplant were evaluated by sequence analysis. To this end, the complete nucleotide sequences from randomly chosen rearrangements from a BMT recipient were compared with nucleotide sequences from randomly chosen rearrangements obtained from a healthy control. As predicted from prior hybridization data [31], rearrangements from this BMT recipient exhibited far fewer somatic mutations than did rearrangements from the healthy subject, although the difference was more dramatic in the V_H3 gene family than in the V_H4 gene family. The complexity of CDR3 regions was similar in the BMT recipient and healthy subject, further supporting the conclusion that the antibody repertoire is generated normally following BMT.

The generation of high-affinity, somatically mutated antibodies in response to antigenic challenge is the hallmark of a protective, secondary antibody response. In the absence of somatic mutation, high-affinity antibodies are rarely produced. Individuals who have a compromised ability to generate somatically mutated antibodies will have a compromised ability to clear viral and microbial pathogens and opportunistic infectious agents. Antibodies that have been antigen-driven may have numerous mutations (20–40 substitutions per variable region are not uncommon). They usually display a ratio of replacement/silent (R/S) substitutions in the CDRs that is greater than that expected of random mutation, whereas R/S in FR is frequently less than that expected of random mutation [53,54]. In general, $R/S > 4·7$ is taken as indicating antigen-driven mutation, and $R/S < 1·5$ [54] or $< 1·9$ [53] is taken as indicating unselected (random) mutation. In rearrangements obtained from the BMT recipient, R/S within CDRs was elevated ($R/S = 11·5$ for all BMT sequences). By comparison, R/S in rearrangements obtained from the healthy subject was 2·4, which is similar to the values for the framework regions in both subjects (2·0 in healthy subject and 2·2 in BMT recipient). These values are close to the value of 1·5 predicted by Shlomchik *et al.* [54], or 1·9 predicted by Reynaud *et al.* [53] for unselected substitutions. The relatively low R/S in CDRs in the healthy subject is somewhat unexpected, but has been reported before [55]. The high mutation frequency of thymidines, and the high R/S in the BMT recipient should be cautiously interpreted, because most of the rearrangements displayed only one or two nucleotide substitutions from the germ-line. Because the overall incidence of mutation in the BMT recipient fell in the range of system background, the high incidence of thymidine substitutions could be a consequence of an *in vitro* bias. To the extent that this is true, the data represent an overestimation of the amount of somatic mutation.

The pattern of the observed mutations was suggestive of antigenic selection and differed from the expected pattern for the intrinsic hypermutation mechanism in immunoglobulin V regions [42], in that there was a high incidences of thymidine substitutions, and an elevated R/S ratio in CDR1. This pattern provides evidence that at least some antigen-driven B cells are present in the circulation of BMT recipients. It is uncertain if these B cells resulted from immune responses generated in the patient or if they represent donor-derived memory cells. The observation that there were more mutations at 90 days than at 1 year post-transplant suggests the persistence of donor-derived memory B cells. In this regard, several studies have shown that adoptively transferred immunity persists in recipients for years post-grafting [1,56], and may be a consequence of peripheral blood contamination of bone marrow preparations [57].

The B cell phenotype following BMT is consistent with several, not necessarily mutually exclusive, functional deficits. For example, B cell dysfunction could be secondary to T cell dysfunction, either due to inadequate T cell help or to suppression. Alternatively, the disruption of secondary lymphoid tissue architecture could lead to a failure to supply an adequate microenvironment for peripheral B cell differentiation. The last possibility is intriguing. It is thought that recruitment of naive B cells into a long-lived preimmune B cell pool depends upon receipt of survival signals delivered in secondary lymphoid organs [58]. The disruption of secondary lymphoid architecture may preclude efficient delivery of these signals. Thus, BMT recipients may have an elevated short-lived B cell compartment and a reduced long-lived compartment. The failure to recruit and maintain a long-lived B cell population could account for an overall reduced ability to mount an effective antibody response.

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REFERENCES

1 Storek J, Saxon A. Reconstitution of B cell immunity following bone marrow transplantation. Bone Marrow Transplant 1992; 9:395–408.
2 Benkerrou M, Wara DW, Elder M *et al.* Anti-tetanus toxoid antibody

production after mismatched T cell-depleted bone marrow transplantation. J Clin Immunol 1994; **14**:98–106.
3 Lum LG, Seigneuret MC, Storb RF, Witherspoon RP, Thomas ED. *In vitro* regulation of immunoglobulin synthesis after marrow transplantation. I. T-cell and B-cell deficiencies in patients with and without chronic graft-vs.-host disease. Blood 1981; **58**:431–9.
4 Shiobara S, Lum LG, Witherspoon RP, Storb R. Antigen-specific antibody responses of lymphocytes to tetanus toxoid after human marrow transplantation. Transplantation 1986; **41**:587–92.
5 Dittel BN, LeBien TW. Reduced expression of vascular cell adhesion molecule-1 on bone marrow stromal cells isolated from marrow transplant recipients correlates with a reduced capacity to support human B lymphopoiesis *in vitro*. Blood 1995; **86**:2833–41.
6 Witherspoon RP, Lum LG, Storb R, Thomas ED. *In vitro* regulation of immunoglobulin synthesis after human marrow transplantation. II. Deficient T and non-T lymphocyte function within 3–4 months of allogeneic, syngeneic, or autologous marrow grafting for hematologic malignancy. Blood 1982; **59**:844–50.
7 Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: a recapitulation of normal B cell ontogeny. Blood 1990; **76**:1647–56.
8 Kiesel S, Pezzutto A, Moldenhauer G *et al.* B-cell proliferative and differentiative responses after autologous peripheral blood stem cell or bone marrow transplantation. Blood 1988; **72**:672–8.
9 Antin JH, Ault KA, Rappeport JM, Smith BR. B lymphocyte reconstitution after human bone marrow transplantation. Leu-1 antigen defines a distinct population of B lymphocytes. J Clin Invest 1987; **80**:325–32.
10 Matsue K, Lum LG, Witherspoon RP, Storb R. Proliferative and differentiative responses of B cells from human marrow graft recipients to T cell-derived factors. Blood 1987; **69**:308–15.
11 Witherspoon RP, Storb R, Ochs HD *et al.* Recovery of antibody production in human allogeneic marrow graft recipients: influence of time posttransplantation, the presence or absence of chronic graft-vs.-host disease, and antithymocyte globulin treatment. Blood 1981; **58**:360–8.
12 Noel DR, Witherspoon RP, Storb R *et al.* Does graft-vs.-host disease influence the tempo of immunologic recovery after allogeneic human marrow transplantation? An observation of 56 long-term survivors. Blood 1978; **51**:1087–105.
13 Kagan JM, Champlin RE, Saxon A. B-cell dysfunction following human bone marrow transplantation: functional-phenotypic dissociation in the early posttransplant period. Blood 1989; **74**:777–85.
14 Klebanoff SJ, Waltersdorph AM, Rosen H. Antimicrobial activity of myeloperoxidase. Methods Enzymol 1984; **105**:399–403.
15 Velardi A, Cucciaioni S, Terenzi A *et al.* Acquisition of Ig isotype diversity after bone marrow transplantation in adults. A recapitulation of normal B cell ontogeny. J Immunol 1988; **141**:815–20.
16 Witherspoon RP, Deeg HJ, Lum LG *et al.* Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-vs.-host disease. Transplantation 1984; **37**: 456–61.
17 Pedrazzini A, Freedman AS, Andersen J *et al.* Anti-B-cell monoclonal antibody-purged autologous bone marrow transplantation for B-cell non-Hodgkin's lymphoma: phenotypic reconstitution and B-cell function. Blood 1989; **74**:2203–11.
18 Gerritsen EJ, Van Tol MJ, Van 't Veer MB *et al.* Clonal dysregulation of the antibody response to tetanus-toxoid after bone marrow transplantation. Blood 1994; **84**:4374–82.
19 Dror Y, Gallagher R, Wara DW *et al.* Immune reconstitution in severe combined immunodeficiency disease after lectin-treated, T-cell-depleted haplocompatible bone marrow transplantation. Blood 1993; **81**:2021–30.
20 Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? Bone Marrow Transplant 1993; **12**:387–98.
21 Storek J, Witherspoon RP, Storb R. T cell reconstitution after bone marrow transplantation into adult patients does not resemble T cell development in early life. Bone Marrow Transplant 1995; **16**:413.
22 Sullivan KM, Agura E, Anasetti C *et al.* Chronic graft-vs.-host disease and other late complications of bone marrow transplantation. Semin Hematol 1991; **28**:250–9.
23 Winston DJ, Schiffman G, Wang DC *et al.* Pneumococcal infections after human bone-marrow transplantation. Ann Intern Med 1979; **91**: 835–41.
24 Aucouturier P, Barra A, Intrator L *et al.* Long lasting IgG subclass and antibacterial polysaccharide antibody deficiency after allogeneic bone marrow transplantation. Blood 1987; **70**:779–85.
25 Sullivan KM, Buckner CD, Sanders JE *et al.* Long-term complications of bone marrow transplantation In: Buckner CD, Gale RP, Lucarelli G, eds. Advances and controversies in thalassemia therapy. Bone marrow transplantation and other approaches. New York: Alan R. Liss, Inc., 1989:367–74.
26 Horny HP, Ruck M, Kaiserling E, Ehninger G. Immunohistology of the human spleen after bone marrow transplantation for leukemia with special reference to the early post-transplantation period. Pathology, Research & Practice 1990; **186**:775–83.
27 Sale GE, Alavaikko M, Schaefers KM, Mahan CT. Abnormal CD4:CD8 ratios and delayed germinal center reconstitution in lymph nodes of human graft recipients with graft-vs.-host disease (GVHD): an immunohistological study. Exp Hematol 1992; **20**:1017–21.
28 Dilly SA, Sloane JP, Psalti ISM. The cellular composition of human lymph nodes after allogeniec bone marrow transplantation: an immunohistological study. J Pathol 1986; **150**:213–21.
29 Sale GE. Pathology of the lymphoreticular system. In: Sale GE, Shulman HM, eds. The pathology of bone marrow transplantation. Chicago: Year Book Medical Publishers, 1984:171–91.
30 Glas AM, Hufnagle WO, Suzuki I *et al.* Anomalous diversification of the antibody repertoire following bone marrow transplantation. Ann NY Acad Sci 1995; **764**:312–4.
31 Suzuki I, Milner ECB, Glas AM *et al.* Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood 1996; **87**: 1873–80.
32 Fumoux F, Guigou V, Blaise D, Maraninchi D, Fougereau M, Schiff C. Reconstitution of human immunoglobulin V_H repertoire after bone marrow transplantation mimics B-cell ontogeny. Blood 1993; **81**:3153–7.
33 Storek J, King L, Ferrara S, Marcelo D, Saxon A, Braun J. Abundance of a restricted fetal B cell repertoire in marrow transplant recipients. Bone Marrow Transplant 1994; **14**:783–90.
34 Suzuki I, Pfister L, Glas A, Nottenburg C, Milner ECB. Representation of rearranged V_H gene segments in the human adult antibody repertoire. J Immunol 1995; **154**:3902–11.
35 van der Maarel SM, Willems van Dijk K, Alexander CM, Sasso EH, Bull AB, Milner ECB. Chromosomal organization of the human V_H4 gene family: location of individual gene segments. J Immunol 1993; **150**:2858–68.
36 Tindall KR, Kunkel TA. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. Biochemistry 1988; **27**:6008–13.
37 Keohavong P, Thilly WG. Fidelity of DNA polymerases in DNA amplification. Proc Natl Acad Sci USA 1989; **86**:9253–7.
38 Pascual V, Liu Y-J, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med 1994; **180**:329–39.
39 Golding GB, Gearhart PJ, Glickman BW. Patterns of somatic mutations in immunoglobulin variable genes. Genetics 1987; **115**:169–76.
40 Rogerson B, Hackett J Jr, Peters A, Haasch D, Storb U. Mutation pattern of immunoglobulin genes compatible with a model of somatic mutations which targeting of the mutator is linked to the direction of DNA replication. EMBO J 1991; **10**:4331–41.
41 Weber JS, Berry J, Manser T, Claflin JL. Position of the rearranged Vk and its 5′ flanking sequences determines the location of somatic mutation in the Jk locus. J Immunol 1991; **146**:3652.
42 Betz AG, Neuberger MS, Milstein C. Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes. Immunol Today 1993; **14**:405–11.

43 Betz AG, Rada C, Pannell R, Milstein C, Neuberger MS. Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: clustering, polarity, and specific hot spots. Proc Natl Acad Sci USA 1993; **90**:2385–8.

44 Klein U, Küppers R, Rajewsky K. Variable region gene analysis of B cell subsets derived from a 4-year-old child: somatically mutated memory B cells accumulate in the peripheral blood already at young age. J Exp Med 1994; **180**:1383–93.

45 Ichihara Y, Matsuoka H, Kurosawa Y. Organization of human immunoglobulin heavy chain diversity gene loci. EMBO J 1988; **7**:4141–50.

46 Buluwela L, Albertson DG, Sherrington P, Rabbitts PH, Spurr N, Rabbitts TH. The use of chromosomal translocations to study human immunolobulin gene organization: mapping DH segments within 35 kb of the Cμ gene and identification of a new DH locus. EMBO J 1988; **7**:2003–10.

47 Matsuda F, Shin EK, Hirabayashi Y *et al.* Organization of variable region segments of the human immunoglobulin heavy chain: duplication of the D_5 cluster within the locus and interchromosomal translocation of variable region segments. EMBO J 1990; **9**:2501–6.

48 Lisitsyn N, Wigler M. Cloning the differences between two complex genomes. Science 1993; **259**:946–51.

49 Huang S-C, Jiang R, Glas AM, Milner ECB. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. Mol Immunol 1996; **33**:553–60.

50 Sanz I. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. J Immunol 1991; **147**:1720–9.

51 Yamada M, Wasserman R, Reichard BA, Shane S, Caton AJ, Rovera G. Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult human peripheral blood B lymphocytes. J Exp Med 1991; **173**:395–407.

52 Brezinschek HP, Brezinschek RI, Lipsky PE. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J Immunol 1995; **155**:190–202.

53 Reynaud C, Garcia C, Hein WR, Weill J. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. Cell 1995; **80**:115–25.

54 Shlomchik MJ, Aucoin AH, Pisetsky DS, Weigert MG. Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. Proc Natl Acad Sci USA 1987; **84**:9150–4.

55 Braun J, Berberian L, King L, Sanz I, Govan HL III. Restricted use of fetal V_H3 immunoglobulin genes by unselected B cells in the adult. J Clin Invest 1992; **89**:1395–402.

56 Lum LG, Noges JE, Beatty P *et al.* Transfer of specific immunity in marrow recipients given HLA-mismatched, T cell-depleted, or HLA-identical marrow grafts. Bone Marrow Transplant 1988; **3**:399–406.

57 Shepherd DM, Noelle RJ. The lack of memory B cells in immune bone marrow. Transplantation 1991; **52**:97–100.

58 Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. Immunity 1996; **3**:691–701.

Chapter 4

Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40 ligand

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Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40 ligand

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SUMMARY

The acquisition of somatic mutations in the rearranged immunoglobulin V regions in B cells occurs within the tightly regulated microenvironment of a germinal centre. The precise mechanism responsible for turning on the mutational process is unknown. To dissect the role of different components of the germinal centre in this mechanism, we have used *in vitro* cultures of normal human IgD^+ peripheral blood B lymphocytes co-cultured with activated $CD4^+$ T cells, or with resting $CD4^+$ T cells, or with CD40 ligand and IL-4. We observed that if the cultures included activated $CD4^+$ T cells, then up to 100% of V_H transcripts on day 14 were somatically mutated. Transcripts were found to carry from one to 36 substitutions (median five). In contrast, in the absence of activated T cells, transcripts contained only background levels of somatic mutation irrespective of the presence of resting T cells or CD40 ligand and IL-4. Cell–cell contact was required for mutation because mutations were not detected when B cells were separated from activated T cells by a membrane.

Keywords B lymphocytes T cell–B cell collaboration antibodies generation of diversity

INTRODUCTION

Somatic mutation of rearranged immunoglobulin V region genes is a powerful diversifier of the antibody repertoire and an important requisite in the formation of memory B cells. Although the underlying mechanism remains to be fully elucidated, somatic mutation has been found to be highly focused on rearranged variable regions (V) of immunoglobulin genes [1–3]. The possible start site for immunoglobulin mutator activity has been suggested to be located in the vicinity of the promoter or leader sequence [4,5]. Both the 3′-enhancer and the intron-enhancer/matrix attachment region seem to be necessary for effective hypermutation [6–8], but the requirement for a specific promoter sequence is less certain [8].

In vivo, somatic mutation appears to involve an active process that occurs normally in response to specific signals transmitted within the tightly regulated microenvironment of a germinal centre (reviewed in [9,10]). Although germinal centres appear by day 4 following immunization, mutation is generally not observed in germinal centre B cells until day 8 post-immunization; thereafter, point mutations favouring asymmetrical transversions accumulate until day 14 [11]. Activated T lymphocytes were shown to be required for the somatic mutation to occur [11,12], and an activated T cell-associated molecule, CD40 ligand (CD40L), was shown to be important for the formation of germinal centres [13]. The mechanism responsible for 'switching on' somatic mutation, however, remains enigmatic.

An *in vitro* system of studying the mutation progression in murine B cells has been established, and it has been found that T cell-derived signals are necessary for progression of somatic mutation [14]. In the human system, Denepoux *et al.* [15] have reported that somatic mutations can be induced in a lymphoma B cell line *in vitro* upon surface immunoglobulin cross-linking by anti-IgM and co-culturing with an activated T cell clone. Evidence showing the

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induction of somatic mutation in human tonsillar B cells by activated T cells was provided by Lebecque and colleagues [16].

Here we show, using an *in vitro* culture system originally described by Lipsky and colleagues [17], the accumulation of somatic mutations among freshly isolated human B cells from peripheral blood. B cells ($CD19^+/IgD^+$) were isolated from peripheral blood lymphocytes (PBL) of a normal individual and were co-cultured with either activated autologous $CD4^+$ T cells, or with resting autologous $CD4^+$ T cells, or with CD40L and cytokines. After 2 weeks of culture, the occurrence of somatic mutations was examined among immunoglobulin V_H transcripts containing the V3–23 gene, the most frequently represented V_H gene in PBL B cells [18–21].

MATERIALS AND METHODS

Reagents and cell line

Murine monoclonal anti-human CD19 (IgG1), goat polyclonal anti-human IgD and goat preimmune IgG, unconjugated or conjugated to FITC, and goat anti-human IgM (unconjugated), were purchased from Becton Dickinson (Mountain View, CA). Purified CD40L (a soluble trimeric form [22]) was a kind gift of Drs R. J. Armitage, M. K. Spriggs and W. C. Fanslow (Immunex Corp., Seattle, WA). Recombinant human IL-4 was purchased from R&D Systems (Minneapolis, MN). CD_w32L (L cells), a murine fibroblast line stably transfected with human FcγRII [23], was obtained from Dr E. Clark (University of Washington, Seattle, WA) and cultured as described [24].

Lymphocyte separation

PBL were isolated from peripheral blood of a healthy adult male Caucasian, VMRC donor 3116. B cells were isolated from PBL by anti-CD19 coated immunomagnetic beads, and detached using 'detachabead' CD19 reagent (DynaBeads; Dynal, Inc., Lake Success, NY). Naive B cells ($CD19^+/IgD^+$) were further purified from the $CD19^+$ B cells either by anti-IgD-coated immunomagnetic beads according to the manufacturer's instructions (Dynal) or by anti-IgD (FITC-conjugated) sorting on a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA). The resulting population was >95% $CD19^+IgD^+IgM^+$. Autologous $CD4^+$ T cells were isolated using anti-CD4-coated immunomagnetic beads (Dynal). The $CD4^+$ T cells were detached from the beads using 'Detachabead' reagent (Dynal) according to the manufacturer's protocol. The purity of T cells as determined by FACS analysis was >99% following isolation. Isolated cells were resuspended in RPMI 1640 (GIBCO, Grand Island, NY), 10% fetal bovine serum (FBS; GIBCO), 2 mM glutamine, 1% sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI).

Cell culture

B cells (10^3 per well) were cultured in flat-bottomed microplates (Costar, Cambridge, MA), in RPMI supplemented with IL-4 (100 U/ml). Other additions depending on the experiment were anti-IgM (1 μg/ml), CD40L (1 μg/ml), CD_w32L cells (L cells) (10^4 per well), resting or activated $CD4^+$ T cells (10^5 per well). L cells and T cells were irradiated before initiation of the cultures (70 and 30 Gy, respectively). To activate T cells, wells were pre-coated as described [17] with 64.1 antibody, a murine monoclonal anti-human CD3 (IgG1) which was a generous gift of Dr E. Vitetta (University of Texas South-western Medical Center, Dallas, TX). Some 64.1 antibody was also obtained through Dr E. A. Clark (University of Washington, Seattle, WA). In some experiments, B cells were separated from T cells by a membrane (3 μm pore size, Transwell; Costar). Cell growth was monitored daily by light microscopy. Cumulative immunoglobulin production in the cultures was detected by ELISA essentially as described [25].

Construction of cDNA libraries containing rearranged V_H3 genes

Total RNA was extracted from the cells using RNAzol (Tel-Test, Friendswood, TX) and cDNA was made as described [26]. V_H3-containing transcripts were amplified in a 31-cycle polymerase chain reaction (PCR) with AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA), using a V_H3-specific 5′ leader primer and either a consensus J_H 3′ primer, a Cγ primer, or a Cμ primer as described [20,21,26]. The PCR-amplified transcripts containing V_H3 genes were cloned into a phagemid vector and libraries were constructed as described [20,21]. In some experiments, colonies were lifted directly onto nylon filters. In other experiments, single strand phages were rescued in 96-well plates by addition of K07 helper phage (Stratagene, La Jolla, CA) and 10 μl of supernatant from each well were dot-blotted on multiple replicate nylon filters [20,21]. Dot blotted filters were used in the hybridization analysis for gene identification and detection of mutation.

Detection of somatic mutation by differential oligonucleotide probe hybridization

The V3–23 gene is the most frequently rearranged V_H3 gene, accounting for 20–40% of the total V_H3 repertoire in peripheral B cells [18–21,27]. Identification of clones carrying transcripts of the V3–23 gene and occurrence of somatic mutations were estimated by differential hybridization using oligonucleotide probes M8 and M18 as described [20,28,29]. M18 (GTGAAGGGCCGGTT CACCATC) is targeted at a region overlapping FR3/CDR2 of V3–23 [29], and M8 (AGCAGC TATGCCATGAGCTGG) is targeted at CDR1 of V3–23 [30]. Hybridization was conducted by an overnight incubation of the filters with ^{32}P-labelled M8 or M18 at a temperature of 61°C. Filters were washed with 5× SSC, 0·5% SDS, at 43°C once for 1 h, then twice for 30 min each, followed by 3.2 M tetramethylammonium chloride (TMACL; pH 7·8) containing 0·5% SDS for 1 h at 60°C (M8) or at 63°C (M18) [20,31]. Hybridization results were visualized by autoradiography. Occurrence of somatic mutations was estimated by loss of concordant hybridization of the V3–23-specific probes as described [29,32].

DNA sequence analysis

Supernatant (5 μl) from selected phage clones carrying V_H3 cDNA was used for PCR amplification of the insert by M13(-20) and M13-reverse primers (Stratagene). PCR products were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) and sequences were determined using the Dye Terminator cycle sequencing kit (Perkin Elmer) on the Applied Biosystems model 373A DNA sequencing system using universal T3 and T7 primers. The comparison and alignment of DNA sequences of the rearranged V_H3 genes with the germ-line sequence of the donor was performed using the Lasergene analysis package (DNASTAR Inc., Madison, WI).

RESULTS

B cells co-cultured with activated T cells but not with CD40L accumulate somatic mutations

Although activated T cells were shown to induce Ig V hypermutation in human B cells [15,16], the exact role of T cells, as well

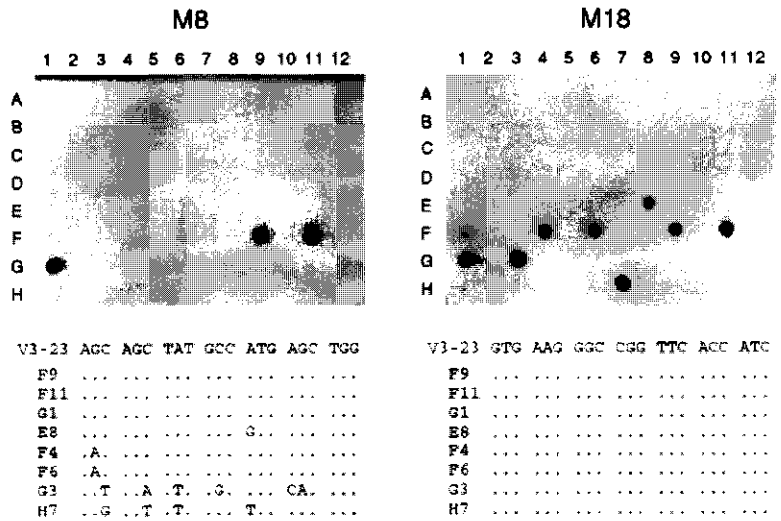

Fig. 1. Detection of somatic mutation in V3–23 rearrangements. Upper left panel: hybridization of a blot containing V_H3 cDNA clones with oligonucleotide probe M8, which is targeted to a sequence motif spanning CDR1. Upper right panel: replicate blot hybridized to probe M18, which is targeted to a sequence motif which extends from CDR3 into FR2. Concordant hybridization is scored as unmutated; discordant hybridization is scored as mutated. Bottom panels: nucleotide sequences of M8 (left) and M18 (right) targeted motifs corresponding to clones identified in upper panels.

as of other components of the germinal centre, in the induction of somatic hypermutation in normal B cells is not yet established. To address this role, we cultured highly purified IgD^+ peripheral blood B cells as described above, and estimated the accumulation of somatic mutations among B cells expressing the V3–23 gene. We took advantage of motif-specific oligonucleotide probe hybridization to screen a large number of cDNA clones containing V3–23 transcripts and to obtain an estimate of the number of transcripts carrying mutations in the probe-targeted sequences.

V_H3 cDNA clones were screened for hybridization to the probes M8 and M18. Each instance of discordant hybridization of these probes was scored as one mutation. The specificity of this scoring is illustrated in Fig. 1. The left panel of Fig. 1 shows hybridization of a 96-dot blot array of cloned V_H3 cDNAs with the probe M8, which is targeted to a sequence motif which spans CDR1 of V3–23. A replica of the filter is shown in the right panel, hybridized to the probe M18 which is targeted to a sequence motif that extends from CDR2 into FR3. DNA sequences of the targeted motifs are shown below each filter. Clones that hybridize to both probes are scored as unmutated (e.g. F9, F11, and G1). The nucleotide sequences (bottom panel) verify the nucleotide identity between probe and target for all hybridizing clones, and reveal at least one substitution in the targeted motif, for all non-hybridizing clones.

Similar analysis was performed on a large number of recombinant V_H3 clones and is summarized in Fig. 2. As shown in Fig. 2, an extremely high frequency of discordance (i.e. mutation) was observed among V3–23 clones obtained from cultures that contained B cells, activated T cells and anti-IgM. In contrast, a low frequency of discordance, comparable to background levels of mutation found in pre-culture B cells, was observed in cultures that contained B cells, purified CD40L and CD_w32L but not activated T cells.

To estimate more accurately somatic mutations, nucleotide sequences were analysed among independent V3–23 transcripts selected from T cell-supported B cells and from CD40L-supported B cells (Fig. 3). T cell-supported cultures produced at least five-fold more mutations (0–36 substitutions, median = 5, mean = 7·1) than did CD40L-supported cultures (0–6 substitutions, median = 1, mean = 1·1). All mutations were single nucleotide substitutions, no insertions or deletions were observed. The ratios of replacement to silent substitutions (R/S) in CDRs and FRs from T cell-supported cultures were 6·5 and 1·2, respectively, and from CD40L-supported cultures were 6·0 and 1·8, respectively. Individual substitutions occurred largely at sites comprised of the motif

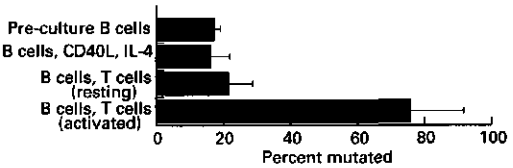

Fig. 2. Mutations accumulate in activated T cell-supported B cell cultures. Bars indicate percentage of rearrangements carrying mutations (± s.d. of three independent experiments). Libraries were made from pre-culture, purified B cells (top bar), or from 14 day B cell cultures which included either CD40L and IL-4 (second bar), resting T cells (third bar), or anti-CD3-activated T cells (bottom bar). V3–23 cDNA transcripts were identified by hybridization to probes M8 and/or M18. Mutation was scored as discordant hybridization to these probes. Each bar represents the analysis of at least 1000 V3–23 transcripts.

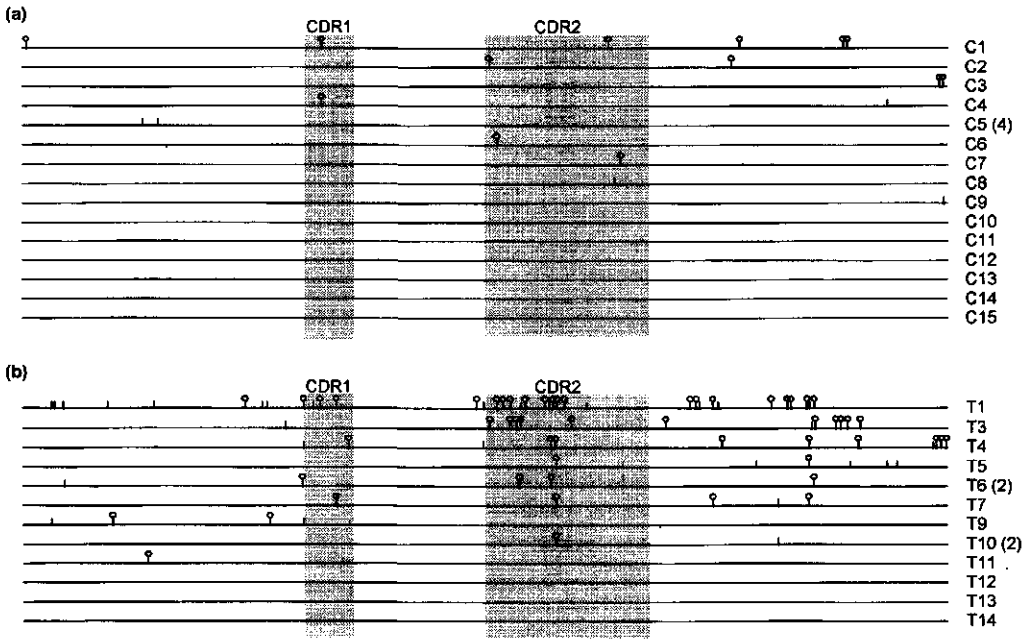

Fig. 3. DNA sequence analysis of V3–23 transcripts from B cell cultures supported by CD40L (a) and from B cell cultures supported by activated T cells (b). Only differences from the germ-line are indicated. Silent mutations are depicted as plain vertical bars and replacement mutations are depicted as capped vertical bars. Repeated isolation of identical cDNA clones is indicated by number in parentheses. Nucleotide sequences are available from GenBank under accession numbers AF123351–AF123379.

RGYW (where R = purine, G = guanine, Y = pyrimidine, W = adenine or thymine).

A temporal increase in the proportion of mutated B cells

To determine the kinetics of accumulation of somatic mutations, wells were harvested for analysis 7, 10 and 14 days after initiation of culture. At day 7, the percentage of transcripts that had acquired somatic mutations was similar to that seen in pre-culture B cells (Table 1). However, by day 10 the percentage of transcripts that had acquired somatic mutations had increased to twice background, and in one experiment was more than four-fold over background on day 14.

Table 1. Somatic mutations among V_H transcripts on different days of culture

	Experiment 1		Experiment 2	
Day	*n*	Percent mutated	*n*	Percent mutated
0	2037	16·3	1455	16·7
7	1393	20·0	229	16·6
10	599	31·6	288	32·3
14	165	66·1	2533	42·5

Cultures contained RPMI 1640, 10% fetal bovine serum (FBS), 100 U/ml IL-4, 1 μg/ml CD40L, anti-IgM, 1×10^3 B cells, and 1×10^5 irradiated (30 Gy) $CD4^+$ T cells. Cultures also contained anti-IgD-coated immunomagnetic beads. Plates were coated with anti-CD3 antibody to activate T cells.

n, Number of transcripts assayed.

Somatic mutations did not accumulate in B cells co-cultured with resting T cells

To determine if activation of $CD4^+$ T cells is required for the induction of somatic mutations in B cells, the accumulation of somatic mutations was compared between activated T cell-supported cultures and resting T cell-supported cultures. Initial experiments showed that resting T cells could not support B cell growth, presumably because resting T cells do not express CD40L [33] and secrete little, if any, IL-4. To test if addition of IL-4 and CD40L, *per se*, would modulate the accumulation of somatic mutations in activated T cell-supported cultures, experiments were performed in which somatic mutations were compared in activated T cell-supported cultures in the presence or absence of additional added IL-4 and CD40L. Results from two independent experiments showed no difference in the accumulation of somatic mutations (not shown). In subsequent experiments, CD40L and IL-4 were added to cultures supported by resting T cells. As shown in Fig. 2, the accumulation of somatic mutations was significantly higher among activated T cell-supported cultures compared with resting T cell-supported cultures (76% *versus* 21%, respectively; $P < 0.001$). This difference was not compensated by the addition of CD40L

Table 2. Somatic mutation is not induced across a Transwell membrane

Chamber		Experiment 1		Experiment 2	
Upper	Lower	*n*	Percent mutated	*n*	Percent mutated
B cells	Activated T cells	161	16·1	16	18·8
B cells	–	153	13·1	36	13·9
Pre-culture B cells		95	15·0	138	19·5

Cultures contained RPMI 1640, 10% fetal bovine serum (FBS), 100 U/ml IL-4, 1 μg/ml CD40L, anti-IgM, 5×10^3 B cells, and if present, 5×10^5 irradiated (30 Gy) $CD4^+$ T cells. Cultures also contained anti-IgD-coated immunomagnetic beads. T cells were activated by coating plates with anti-CD3 antibody.

n, Number of transcripts assayed.

plus IL-4 (not shown). Somatic mutations were not significantly elevated in cultures supported by resting T cells compared with pre-culture B cells ($P = 0.23$).

Induction of somatic mutation probably requires cell–cell contact

It has been suggested that cross-linking of B cell antigen receptor and T cell-derived signals as well as soluble factors from T cells are important for progression of somatic mutation [14]. To determine if cell–cell contact between B and T cells was necessary for the induction of somatic mutations in human B cells, we utilized a culture system in which B cells were separated from activated T cells by a Transwell membrane. IgD^+ B cells (5×10^3) were plated in the upper chamber and the activated $CD4^+$ T cells (5×10^5) were plated in the bottom chamber. CD40L and IL-4 were added to the medium to promote B cell proliferation. Results from two independent experiments demonstrated that if the B and T cells were separated by a Transwell membrane, the level of mutation in the B cells was low and similar to that from B cells cultured in the absence of activated T cells (Table 2).

Table 3. Soluble products from cellular interaction between B and T cells are not sufficient for induction of somatic mutations

Conditions		Upper chamber		Lower chamber	
Upper chamber	Lower chamber	*n*	Percent mutated	*n*	Percent mutated
B cells	Activated T cells	556	8·5	–	–
B cells	–	1700	10·9	–	–
B cells	B cells, activated T cells	1195	9·1	1777	52·3
–	B cells, activated T cells	–	–	2325	53·9

Cultures contained RPMI 1640, 10% fetal bovine serum (FBS), 100 U/ml IL-4, 1 μg/ml CD40L, anti-IgM, 5×10^3 B cells, and if present, 5×10^5 irradiated (30 Gy) $CD4^+$ T cells. B cells were sorted with anti-IgD antibody and the antibodies were left undetached. The percentage of mutations in pre-culture B cells was 16·7%. T cells were activated by coating plates with anti-CD3 antibody.

n, Number of transcripts assayed.

An additional experiment was performed to determine if soluble products generated as a result of B cell–T cell contact might be responsible for the induction of somatic mutations. To address this question, B cells were plated in both chambers of the Transwell plate, while activated T cells were plated only in the lower chamber. The results, shown in Table 3, demonstrated that B cells that had direct contact with activated T cells in the lower chamber acquired somatic mutations, but that B cells in the upper chamber did not. Thus, physical contact between activated T and B cells appears to be necessary. If soluble factors are important for the induction of somatic mutation, they function only in the context of cell–cell contact.

Somatic mutations accumulate in both IgM and IgG transcripts

To address the possibility that the mutator mechanism is more active during IgG production than during IgM production, IgM and IgG transcripts were assayed independently following *in vitro* culture. Results from the analysis of >660 IgG and >2400 IgM transcripts from two independent experiments (each performed in duplicate) are presented in Fig. 4. The proportion of transcripts with detectable mutation in the 14 day cultures was largely independent of isotype, but was clearly dependent on culture conditions (Fig. 4). Thus, in the CD40L-supported cultures, the proportion of transcripts with detectable mutation was not above background in either IgM or IgG transcripts. In the activated T cell-supported cultures, however, the proportion of transcripts with detectable mutation ranged from 35·1% to 48·7% among IgMs and from 51·4% to 53·1% among IgGs. These results indicate that neither selective mutation of IgG nor selective expansion of mutated IgG-expressing cells can explain the accumulation of somatic mutations in these B cells.

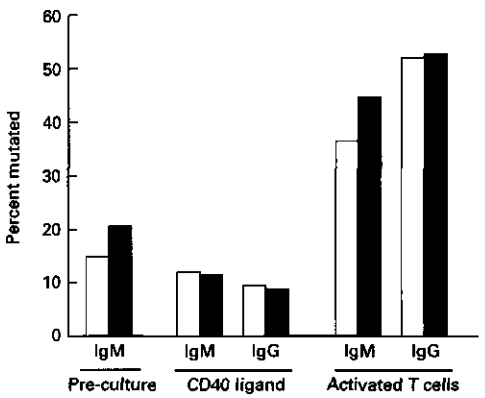

Fig. 4. Somatic mutations in IgM and IgG transcripts. Results from two independent experiments are presented. IgM and IgG transcripts were separately amplified from the same total RNA samples in independent reverse transcriptase-polymerase chain reaction (RT-PCR) reactions. Analysis was performed on >3000 V3–23 transcripts (1201 IgM transcripts from pre-culture B cells, 674 IgM transcripts and 89 IgG transcripts from activated T cell-supported cultures, 568 IgM transcripts, and 577 IgG transcripts from CD40L-supported cultures). As expected, no IgG transcripts were recovered from pre-culture IgD^+ B cells. □, Experiment 1; ■, experiment 2.

DISCUSSION

In this study we describe the accumulation of somatic mutations in cultures of freshly isolated human naive B cells. In all, > 10 000 V_H transcripts were assayed for mutation by differential hybridization with motif-specific oligonucleotide probes. Among B cells obtained from cultures containing activated autologous $CD4^+$ T cells, 43–100% of the analysed transcripts had acquired somatic mutations by the end of the 14-day culture, suggesting that the mutator mechanism had been turned on in these B cells. In contrast, B cells cultured in the presence of purified CD40L, but without activated T cells, proliferated vigorously and isotype-switched but showed only a background level of mutation. These data further support the notion that a combination of CD40 and IL-4 signalling is able to induce clonal expansion and isotype switching in naive B cells but is unable to induce somatic mutation [34–36].

Our laboratory has exploited a method of detection of individual V_H genes by hybridization with oligonucleotide probes targeted to unique motifs contained in CDRs and FRs of V_H genes [20,21,30,37–42]. The stringency of the analysis is such that a single substitution at any position of the probe target results in virtually complete loss of signal. We have used this approach to study the accumulation of somatic mutations in rearrangements of V_H3 genes among peripheral blood B cells [29,32]. By comparison of hybridization profiles of multiple, non-overlapping gene-specific oligonucleotide probes, the presence of somatic mutations within probe-targeted regions can be revealed by the loss of concordance (relative to the hybridization profile on germ-line clones) of one or more probes. In our analysis, each instance of discordant hybridization was scored as a mutation. The results are expressed as the percentage of transcripts that exhibited discordant hybridization.

The reliability of this assay has been confirmed in numerous experiments comparing results from DNA sequence analysis and motif-specific hybridization [32]. No discrepancies between the sequence analysis and hybridization analysis were found among > 120 clones analysed. In all instances of hybridization, the target sequence was identical to the probe sequence. In each instance of discordan hybridization or non-hybridization, however, clones had one or more nucleotide substitutions in the discordant probe-targeted region. No erroneous false-positive hybridization patterns were identified. We have previously demonstrated the exquisite specificity of motif-specific hybridization and sensitivity to single nucleotide substitutions within the targeted sequences, by modified Southern analysis on restriction-digested genomic DNA [30,37–42].

In this study we have used both differential hybridization and DNA sequence analysis to estimate somatic mutations. To date, $CD19^+$ IgD^+ B cells have been used. This population, which is > 95% pure, is primarily comprised of naive, unmutated B cells [43]. Analysis of somatic mutation performed on this population prior to culture is taken as system background. System background includes artefacts from all sources as well as a potential contribution from any pre-existing mutated B cells in the population. Contaminating IgD^- cells (which are not necessarily B cells) comprise < 5% of the population and cannot account for the background. In the CD40/IL-4 cultures the frequency of mutated rearrangements does not increase over this background, and often shows a slight decrease. The background level of mutations as detected by differential hybridization in this study was about 15%, which is similar to that found in our previous studies [29,44,45]. Because 42 bp/transcript are actually assayed, a 15% background calculates to approximately three mutations per 1000 bp, or one mutation per complete VDJ transcript. This is a little high compared with two mutations per 1000 bp estimated by Pascual *et al.* [43], but is not excessive considering that this frequency includes all sources of background, including Taq polymerase error, PCR-mediated cross-overs (which are common among PCR products of V_H genes) and hybridization scoring errors, in addition to *bona fide* somatic mutations pre-existing in the population.

A population of IgD^+IgM^- B cells that are highly mutated has been identified in human tonsillar lymph node germinal centres [46]. This population was not found in the peripheral blood. However, it cannot be excluded that the system background that we observe is, in part, due to the presence of a population of somatically mutated IgD^+ B cells present in peripheral blood.

Results from the Transwell experiments showed that physical contact of B cells with activated T cells appeared to be necessary for the increase in mutations to occur, indicating that a signal for V gene somatic mutation is delivered to B lymphocytes by molecule(s) associated with activated T cells distinct from CD40 signalling. Engagement of CD40 by CD40L can not substitute for the T cell contact in this pathway. Further, our data (Table 3) suggest that soluble products generated as a result of the interaction between B and T cells are not enough to trigger the mutational process.

Results from DNA sequence analysis showed that R/S in CDRs and FRs from the B cells co-cultured with activated T cells were 6·5 and 1·2, respectively, and from B cells co-cultured with CD40L, 6·0 and 1·8, respectively. Based on several analyses [47,48], these findings might be interpreted as suggesting that antigen-driven processes are involved in both culture conditions. However, individual substitutions mostly occurred within Ser codons AGT or AGC, which are more common in CDRs than in FRs. Substitutions in these codons have been considered as a result of intrinsic action of hypermutation mechanism in the absence of antigenic selection [49,50]. Furthermore, the pattern of substitution among non-productive rearrangements, which cannot have been antigen-driven, has been found to be similar [51].

It has been suggested that IgG rearrangements are a better substrate for the mutator mechanism than are IgM rearrangements [52,53]. This suggests the hypothesis that an increased rate of isotype switching is responsible for the high level of accumulation of somatic mutation among B cells cultured in the presence of activated T cells, compared with the low-level accumulation among B cells cultured in the presence of CD40L. The prediction would be that IgG transcripts would have more mutation than IgM transcripts regardless of culture conditions, but the fraction of transcripts that were IgG would be significantly higher in activated T cell-supported cultures. The results are incompatible with this prediction, because no increase over background, or over IgM transcripts, was detected among IgG transcripts from CD40L-supported cultures. Initial populations were highly purified IgD^+ cells (> 95% purity), and, as expected, there was essentially no IgG mRNA obtained from these cells. Therefore, the background level of mutation is a reflection primarily of IgM-expressing B cells, along with artefacts from all sources. Thus, neither selective mutation of IgG, nor selective expansion of mutated IgG-expressing cells, can explain the accumulation of somatic mutations in these B cells. Therefore the only tenable hypotheses remaining are that either (i) T cells regulate the differential outgrowth of mutated (or mutating)

B cells compared with unmutated B cells, or (ii) T cells induce activation of the mutator mechanism in B cells that grow in these cultures.

Differential outgrowth might account for the observed accumulation of somatic mutations *in vitro*. For this to be true, we have to postulate that a small population of B cells, which previously acquired mutations *in vivo*, or is constitutive for the mutational mechanism, is selectively permitted to proliferate in the T cell-supported cultures, but has no growth advantage in the CD40/IL-4 cultures. Calculations based on our current results suggest that this is an unlikely explanation for the accumulation of mutations in culture. In most experiments, 1000 B cells were plated. The plating efficiency ranged from 10% to 30% ([54] and G. V. Pinchuk and E. C. B. Milner, unpublished data), therefore 100–300 B cell clones would grow out. As many as 50% of B cells express a V_H3 heavy chain, therefore there would be 50–150 V_H3-expressing clones. From 20% to 40% of V_H3 are derived from the V3–23 gene, therefore 10–60 V3–23 clones should grow out. If, as seems likely based on the known purity of the starting B cell population, mutations were confined to a minor subset (e.g. 1%) of B cells, then from 0·1 to 0·6 (i.e. not more than one) mutated V3–23 clone should be found to account for the entire set of mutated rearrangements.

The results are inconsistent with this prediction. When nucleotide sequences were determined for V3–23 transcripts, 9/12 independent clones carried mutations, substantially exceeding the number predicted by the differential outgrowth hypothesis (Fig. 3). The only scenario under which this number of mutated clones does not substantially exceed the number predicted by the differential outgrowth hypothesis would be if the entire 15% background were due to pre-existing somatic mutation. In view of the fact that a background of approx. 15% mutation is obtained in a variety of control experiments, this explanation is very unlikely.

In view of these results and the demonstration that somatic mutation can be induced in tonsillar B cells by activated T cells [16], it is very likely that the first hypothesis is incorrect and that the second hypothesis is correct, i.e. T cells induce activation of the mutator mechanism in B cells that grow in these cultures.

The burning question that remains, of course, is what is the signal(s) that results in activation of the mutator mechanism by B cells? At this juncture the answer remains unknown, although the prospects of solving one of the remaining great immunologic mysteries are bright. *Signalling through CD40/CD40L is required* but not sufficient. CD28 signalling is required for the formation of germinal centres [55] and is therefore at least indirectly required for somatic mutation. Whether a heretofore unknown receptor–counter receptor is required is a possibility but not a certainty. Perhaps the answer will lie among familiar players acting in a novel context.

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REFERENCES

1 Lebecque SG, Gearhart PJ. Boundaries of somatic mutation in rearranged immunoglobulin genes: 5′ boundary is near the promoter, and 3′ boundary is ~1kb from V(D)J gene. J Exp Med 1990; **172**: 1717–27.

2 Golding GB, Gearhart PJ, Glickman BW. Patterns of somatic mutations in immunoglobulin variable genes. Genetics 1987; **115**:169–76.

3 Gorski J, Rollini P, Mach B. Somatic mutations of immunoglobulin variable genes are restricted to the rearranged genes. Science 1983; **220**:1179–81.

4 Rogerson BJ. Mapping the upstream boundary of somatic mutations in rearranged immunoglobulin transgenes and endogenous genes. Mol Immunol 1994; **31**:83–98.

5 Motoyama N, Miwa T, Suzuki Y, Okada H, Azuma T. Comparison of somatic mutation frequency among immunoglobulin genes. J Exp Med 1994; **179**:395–403.

6 Sharpe MJ, Milstein C, Jarvis JM, Neuberger MS. Somatic hypermutation of immunoglobulin kappa may depend on sequences 3′ of C kappa and occurs on passenger transgenes. EMBO J 1991; **10**:2139–45.

7 Sharpe MJ, Neuberger M, Pannell R, Surani MA, Milstein C. Lack of somatic mutation in a kappa light chain transgene. Eur J Immunol 1990; **20**:1379–85.

8 Betz AG, Milstein C, Gonzalez-Fernandez A, Pannell R, Larson T, Neuberger MS. Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. Cell 1994; **77**:239–48.

9 Berek C, Berger A, Apel M. Maturation of the immune response in germinal centers. Cell 1991; **67**:1121–9.

10 Liu Y-J, Arpin C, de Bouteiller O *et al.* Sequential triggering of apoptosis, somatic mutation and isotype switch during germinal center development. Semin Immunol 1996; **8**:169–77.

11 Miller C, Stedra J, Kelsoe G, Cerny J. Faculative role of germinal centers and T cells in the somatic diversification of IgV_H genes. J Exp Med 1997; **181**:1319–31.

12 Lahvis GP, Cerny J. Induction of germinal center B cell markers in vitro by activated $CD4^+$ T lymphocytes. J Immunol 1997; **159**:1783–93.

13 Allen RC, Armitage RJ, Conley ME *et al.* CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. Science 1993; **259**:990–3.

14 Källberg E, Jainandunsing S, Gray D, Leanderson T. Somatic mutation of immunoglobulin V genes in vitro. Science 1996; **271**:1285–8.

15 Denepoux S, Razanajaona D, Blanchard D *et al.* Induction of somatic mutation in a human B cell line in vitro. Immunity 1997; **6**:35–46.

16 Razanajaona D, Denepoux S, Blanchard D *et al.* In vitro triggering of somatic mutation in human naive B cells. J Immunol 1997; **159**:3347–53.

17 Hirohata S, Jelinek DF, Lipsky PE. T cell-dependent activation of B cell proliferation and differentiation by immobilized monoclonal antibodies to CD3. J Immunol 1988; **140**:3736–44.

18 Logtenberg T, Schutte MEM, Inghirami G *et al.* Immunoglobulin V_H gene expression in human B cell lines and tumors: biased V_H gene expression in chronic lymphocytic leukemia. Int Immunol 1989; **1**:362–6.

19 Brezinschek HP, Brezinschek RI, Lipsky PE. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. J Immunol 1995; **155**:190–202.

20 Suzuki I, Pfister L, Glas A, Nottenburg C, Milner ECB. Representation of rearranged V_H gene segments in the human adult antibody repertoire. J Immunol 1995; **154**:3902–11.

21 Huang S-C, Jiang R, Glas AM, Milner ECB. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. Mol Immunol 1996; **33**:553–60.

22 Fanslow WC, Srinivasan S, Paxton R, Gibson MG, Spriggs MK, Armitage RJ. Structural characteristics of CD40 ligand that determine biological function. Semin Immunol 1994; **6**:267–78.

23 Stuart SG, Trounstine ML, Vaux DJT *et al.* Isolation and expression of cDNA clones encoding a human receptor for IgG ($Fc\gamma RII$). J Exp Med 1997; **166**:1668–84.

24 Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science 1991; **251**:70–72.

25 Nutman TB. Measurement of polyclonal immunoglobulin synthesis

using ELISA. In: Coligan JE, Kruisbeek AM, Margulies DH *et al.*, eds. Current protocols in immunology. New York: Wiley & Sons, 1994; 7.12.1–7.12.3.

26 Pinchuk GV, Alexander CM, Glas AM, Armitage RJ, Milner ECB. VH4 repertoire in human B lymphocytes stimulated by CD40 ligand and IL-4: evidence for positive and negative selection mechanisms coupled to CD40 activation. Mol Immunol 1996; **33**:1369–76.

27 Rao SP, Huang S-C, Milner ECB. Analysis of the V_H3 repertoire among genetically disparate individuals. Exp Clin Immunogenet 1996; **13**: 131–8.

28 Glas A, Huang S-C, van Montfort EHN, Milner ECB. Motif-specific probes identify individual genes and detect somatic mutations. (UnPub) 1998.

29 Suzuki I, Milner ECB, Glas AM *et al.* Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood 1996; **87**:1873–80.

30 Willems van Dijk K, Milner LA, Sasso EH, Milner ECB. Chromosomal organization of the heavy chain variable region gene segments comprising the human fetal antibody repertoire. Proc Natl Acad Sci USA 1992; **89**:10430–4.

31 Wood WI, Gitschier J, Lasky LA, Lawn RM. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc Natl Acad Sci USA 1985; **82**:1585–8.

32 Huang S-C, Jiang R, Hufnagle WO, Furst DE, Wilske KR, Milner ECB. V_H usage and somatic hypermutation in peripheral blood B cells of patients with rheumatoid arthritis. Clin Exp Immunol 1998; **112**: 516–27.

33 Armitage RJ, Fanslow WC, Strockbine L *et al.* Molecular and biological characterization of a murine ligand for CD40. Nature 1992; **357**:80–82.

34 Kelly PJ, Pascual V, Capra JD, Lipsky PE. Anti-CD3-stimulated T cells induce the production of multiple Ig H chain isotypes by individual human peripheral B lymphocytes. J Immunol 1992; **148**: 1294–301.

35 Galibert L, Van Dooren J, Durand I *et al.* Anti-CD40 plus interleukin-4-activated human naive B cell lines express unmutated immunoglobulin genes with intraclonal heavy chain isotype variability. Eur J Immunol 1995; **25**:733–7.

36 Armitage RJ, Macduff BM, Spriggs MK, Fanslow WC. Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. J Immunol 1993; **150**: 3671–80.

37 Willems van Dijk K, Schroeder HW Jr, Perlmutter RM, Milner ECB. Heterogeneity in the human immunoglobulin VH locus. J Immunol 1989; **142**:2547–54.

38 Sasso EH, Willems van Dijk K, Milner ECB. Prevalence and polymorphism of human V_H3 genes. J Immunol 1990; **145**:2751–7.

39 Willems van Dijk K, Sasso EH, Milner ECB. Polymorphism of the human immunoglobulin V_H4 gene family. J Immunol 1991; **146**:3646–51.

40 Willems van Dijk K, Milner LA, Milner ECB. Mapping of human H chain V region genes (V_H4) using deletional analysis and pulsed field gel electrophoresis. J Immunol 1992; **148**:2923–31.

41 van der Maarel SM, Willems van Dijk K, Alexander CM, Sasso EH, Bull AB, Milner ECB. Chromosomal organization of the human V_H4 gene family: Location of individual gene segments. J Immunol 1993; **150**:2858–68.

42 Milner ECB, Hufnagle WO, Glas AM, Suzuki I, Alexander CM. Polymorphism and utilization of human V_H genes. Ann NY Acad Sci 1995; **764**:50–61.

43 Pascual V, Liu Y-J, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med 1994; **180**:329–39.

44 Glas AM, Hufnagle WO, Suzuki I *et al.* Anomalous diversification of the antibody repertoire following bone marrow transplantation. Ann NY Acad Sci 1995; **764**:312–4.

45 Glas AM, Nottenburg C, Milner ECB. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. Clin Exp Immunol 1997; **107**:372–80.

46 Liu Y-J, de Bouteiller O, Arpin C *et al.* Normal human IgD^+IgM^- germinal center B cells can express up to 80 mutations in the variable region of their IgD transcripts. Immunity 1996; **4**:603–13.

47 Shlomchik MJ, Aucoin AH, Pisetsky DS, Weigert MG. Structure and function of anti-DNA autoantibodies derived from a single autoimmune mouse. Proc Natl Acad Sci USA 1987; **84**:9150–4.

48 Garcia C, Hein WR. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. Cell 1995; **80**:115–25.

49 Betz AG, Neuberger MS, Milstein C. Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes. Immunol Today 1993; **14**:405–11.

50 Betz AG, Rada C, Pannell R, Milstein C, Neuberger MS. Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: clustering, polarity, and specific hot spots. Proc Natl Acad Sci USA 1993; **90**:2385–8.

51 Dörner T, Brezinschek H-P, Brezinschek RI, Foster SJ, Domiati-Saad R, Lipsky PE. Analysis of the frequency and pattern of somatic mutations within nonproductively rearranged human variable heavy chain genes. J Immunol 1997; **158**:2779–89.

52 Green NS, Rabinowitz JL, Zhu M, Kobrin BJ, Scharff MD. Immunoglobulin variable region hypermutation in hybrids derived from a pre-B- and a myeloma cell line. Proc Natl Acad Sci USA 1995; **92**: 6304–8.

53 Zhu M, Green NS, Rabinowitz JL, Scharff MD. Differential V region mutation of two transfected Ig genes and their interaction in cultured B cell lines. EMBO J 1996; **15**:2738–47.

54 Amoroso K, Lipsky PE. Frequency of human B cells that differentiate in response to anti-CD3-activated T cells. J Immunol 1990; **145**:3156–61.

55 Ferguson SE, Han S, Kelsoe G, Thompson CB. CD28 is required for germinal center formation. J Immunol 1996; **156**:4576–81.

Chapter 5

B cell-autonomous somatic mutation deficit following bone marrow transplant

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B-cell–autonomous somatic mutation deficit following bone marrow transplant

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Hematopoietic stem cell transplantation is characterized by a prolonged period of humoral immunodeficiency. We have previously shown that the deficiencies are probably not due to the failure to utilize the appropriate V regions in the pre-immune repertoire. However, a striking observation, which correlated with the absence of immunoglobulin IgD$^-$ cells and was consistent with a defect in antigen-driven responses, was that rearrangements in bone marrow transplant (BMT) recipients exhibited much less somatic mutation than did rearrangements obtained from healthy subjects. In this paper, we present evidence suggesting that naive B cells obtained from BMT recipients lack the capacity to accumulate somatic mutations in a T-cell–dependent manner compared with healthy subjects. This appears to be a B-cell–autonomous deficit because T cells from some patients, which were not able to support accumulation of mutations in autologous naive B cells, were able to support accumulation of mutations in heterologous healthy-subject naive B cells. (Blood. 2000;96:1064-1069)

Introduction

Hematopoietic stem cell transplantation is a recognized treatment for certain leukemias, other blood diseases, and some inborn errors of metabolism and has potential as a vehicle for gene therapy. However, hematopoietic stem cell transplant, using either bone marrow or mobilized peripheral stem cells, is characterized by a prolonged period of immunodeficiency affecting both B-cell and T-cell compartments (reviewed in Storek et al[1]). B-cell counts are low but usually approach normalcy by 1 year after transplantation.[1] Coincident with recovering B cells, serum immunoglobulin IgM, IgG1 and IgG3 levels but not IgG2 and IgA levels return to normal by 1 year posttransplantation.[2-5] Thus, marrow recipients who survive the initial postgrafting period do not always become fully immunocompetent. Many recipients are deficient in generating specific antibody responses to exogenous stimuli. The complete reconstitution of B-cell immunity in recipients can take years.

The nature of the B-cell defect(s) leading to this specific humoral immunodeficiency is uncertain. Normal levels of serum IgM, IgG1, and IgG3[5] indicate that immunodeficiency is not due to a general failure to produce immunoglobulin or an overt lack of T-cell help (although impaired T-cell function may be an important factor). Evidence indicates that the processes involved in generating and selecting the primary antibody repertoire are largely functional within the first year following bone marrow transplant (BMT) and that the immunodeficiencies common among BMT recipients are probably not due to the failure to utilize appropriate V region genes in generating the pre-immune antibody repertoire.[6,7] The complexity of the CDR3 (third complementarity determining region) and D_H and J_H utilization is similar in BMT recipients and healthy subjects 1 year posttransplantation, further supporting the conclusion that the primary antibody repertoire is generated normally following BMT.[8,31] However, a striking observation was that rearrangements in BMT recipients exhibited much less somatic mutation than did rearrangements obtained from healthy subjects.[6-8] The failure in the BMT recipients to accumulate somatic mutations in rearranged V_H genes is consistent with a maturational arrest at a fairly late stage of differentiation. This deficit could be a consequence of either an intrinsic B-cell deficit or a lack of adequate T-cell help. In this paper, we present evidence suggesting that, in contrast to healthy-subject B cells, B cells obtained from transplantation patients 1 year posttransplantation lack the capacity to accumulate somatic mutations in a T-cell–dependent manner. This appears to be a B-cell–autonomous deficit, because T cells from some patients were able to support accumulation of mutations in heterologous healthy-subject B cells but not in autologous B cells.

Patients, materials, and methods

Patients and donors

Blood samples were obtained under Institutional Review Board–approved protocols, and written consent was always obtained. Blood mononuclear cells were separated by density-gradient centrifugation, with the use of Ficoll-Hypaque (1.077 kg/L) (Amersham Pharmacia Biotech, Piscataway, NJ). We studied 9 recipients of allogeneic hematopoietic cell transplants at approximately 1 year after grafting (median, 378 days; range, 354-432 days) in combination with 6 healthy subjects. The median age at transplantation was 42 years (range, 29-52 years). No patient had a history of splenectomy. All patients were transplanted for hematological malignancies. They were usually conditioned with cyclophosphamide (120 mg/kg) and fractionated total body irradiation (12.0 to 13.2 Gy). The hematopoietic cell donors for BMT1, 2, 3, 7, 8, and 9 were siblings matched for HLA-A, HLA-B, and HLA-DR, and the donors for patients BMT4, 5, and 6 were unrelated volunteers matched for HLA-A, HLA-B, and HLA-DR. Seven patients (BMT1, 4, 5, 6, 7, 8, 9) received unmodified marrow; 1 patient

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(BMT3) received unmodified filgrastim-mobilized blood progenitor cells; and 1 patient (BMT2) received filgrastim-mobilized blood progenitor cells positively enriched for $CD34^+$ cells. Graft-versus-host disease (GVHD) prophylaxis typically consisted of methotrexate (day 1, 3, 6, and 11) and cyclosporine (day −1 through 180).[9] Grade 2 to 3 acute GVHD occurred in 7 patients (BMT2, 3, 5, 6, 7, 8, and 9); it was usually treated with oral prednisone (1 to 2 mg/kg/d). Prior to the 1-year posttransplantation evaluation, clinical limited chronic GVHD developed in 4 patients (BMT2, 5, 6, and 8) and clinical extensive chronic GVHD in 3 patients (BMT1, 3, and 9).

At the time of the 1-year posttransplantation evaluation, the patients were thoroughly tested for potential relapse of the hematological malignancy and for chronic GVHD status. Eight patients were in complete remission; 1 patient (BMT2) was in early relapse (patient with IgG-lambda multiple myeloma who had 12% plasma cells in marrow and 16.0 g/L monoclonal IgG-lambda in serum at 1 year posttransplantation). All 9 patients were complete chimeras defined by more than 99% donor cells in marrow and/or blood, with the use of Y-chromosome in situ hybridization or variable nucleotide tandem repeats.[10,11] Two patients had clinical extensive chronic GVHD (BMT3 and 9), and the remaining 7 patients had no clinical GVHD at the 1-year posttransplantation blood draw. One patient (BMT1) was on oral prednisone (50 mg/d), and the remaining 8 patients were on no systemic immunosuppressive drugs. Patients had received no biological response modifiers such as interferon and no intravenous immunoglobulin (IVIG) within 2 months prior to the 1-year posttransplantation evaluation, except for 1 patient (BMT2) who received IVIG approximately 6 weeks prior to the 1-year posttransplantation evaluation.

Each patient sample was tested in parallel with a volunteer control (age 20 to 50) recruited from employees of the Virginia Mason Research Center or the Fred Hutchinson Cancer Research Center.

Flow cytometry and sorting

The enumeration of B cells and CD4 T cells was done with the use of 3-color flow cytometry as described.[12,13] For sorting, blood mononuclear cells were stained with fluorescein isothiocyanate (FITC)–conjugated goat-antihuman IgD antibody ($F(ab')_2$) (Caltag, Burlingame, CA) and phycoerythrin (PE)-conjugated mouse-antihuman CD4 antibody (Becton Dickinson, Franklin Lakes, NJ, or Coulter-Immunotech, Fullerton, CA) and sorted on a FACS Vantage (Becton Dickinson). Forward- versus side-scatter gate was set to encompass primarily lymphoid cells and only a small fraction of monocytoid cells. CD4 T cells were defined as $CD4^{high}$ cells; IgD^+ B cells were defined as IgD^{high} cells. Sorted fractions were more than 91% pure.

For the major histocompatibility complex (MHC) control experiments, B cells were isolated from peripheral blood lymphocytes (PBL) by anti-CD19–coated immunomagnetic beads (DynaBeads, Dynal, Lake Success, NY) according to the manufacturer's protocol. Naive human B-cells ($CD19^+/IgD^+$) were further purified from the $CD19^+$ B cells by anti-IgD (FITC-conjugated) sorting on a FACS Vantage. The resulting population was more than 95% $CD19^+IgD^+IgM^+$. Autologous $CD4^+$ T cells were isolated with the use of anti-CD4–coated immunomagnetic beads (Dynal) according to the manufacturer's protocol. The purity of CD4 T cells as determined by FACS analysis was greater than 99% following isolation.

Cell culture

In vitro cultures for analysis of somatic mutation were performed as described.[14] In brief, B cells (10^3 per well) were cultured in flat-bottomed microplates in RPMI 1640 medium supplemented with interleukin (IL)–4 (100 U/mL) and anti-IgM (1 μg/mL). Other additions, depending on the experiment, were CD40L (1 μg/mL), CD_w32L cells (L cells) (10^4 per well), resting or activated $CD4^+$ T cells (10^5 per well). L cells and T cells were irradiated before initiation of the cultures (70 and 30 Gy, respectively). To activate T cells, wells were precoated with 64.1 antibody, a murine monoclonal antihuman CD3.

Complementary DNA library construction

Complementary DNA (cDNA) libraries were constructed as described.[14,15] Polymerase chain reactions (PCRs) used for constructing the libraries were performed with the use of the family-specific 5′ primers, E310 (V_H3-L), 5′-CTGAATTCCATGGAGTTTGGGCTGAGCTG-3′, corresponding to the 5′ ends of the leader sequence of V_H3 family, and a 70:15:15 mixture of the 3′ primers E311, 5′-GACTCTAGACT(CT)ACCTGAGGAGACGGTGACC-3′, complementary to the 3′ ends of J_H1, 4, 5, and 6 gene sequences; E312, 5′-GACTCTAGACT(CT)ACCTGAGGAGACAGTGACC-3′, complementary to the 3′ end of J_H2 gene sequence; and E313, 5′-GACTCTAGACT(CT)ACCTGAAGAGACGGTGACC-3′, complementary to the 3′ end of J_H3 gene sequence. Restriction sites (*Eco*RI for 5′ primers; *Xba*I for 3′ primers) included in the primers are underlined. For amplification of V_H3 transcripts Pfu or Taq DNA polymerase (Promega Corp, Madison, WI) was used in a 30-cycle program.

Detection of somatic mutation

Replicate dot blot filters were made from the libraries and were hybridized as described.[14,15] Occurrence of somatic mutations was estimated by loss of concordant hybridization to V_H-specific probes and DNA sequence analysis as described.[7,14-16]

Semiquantitative PCR

cDNA was amplified with the use of Taq DNA polymerase (Promega) and primers for V_H3 expressing IgM (E310 and E213, 5′-AATTCTAGATCACAGGAGACGAGGGGGAAAAG- 3′) and IgG transcripts (E310 and E212, 5′-AATTCTAGAGGGGAAGTAGTCCTTGACCAGGCA-3′). As an internal control, β-actin primers were included (E376, 5′-GGTGGGCATGGGTCAGAAGGATT-3′ and E377, 5′-CCAGAGGCGTACAGG-

Table 1. Lymphocyte subsets by flow cytometry 1 year following transplantation

Sample identification	Abs MNC ($\times10^6$/L)	% B cells	Abs B ($\times10^6$/L)	% IgD^+	% T cells	% CD4	% CD8	Abs CD4 ($\times10^6$/L)	Abs CD8 ($\times10^6$/L)
BMT1	3070	7	215	91	57	13	53	227	927
BMT2	550	3	17	87	30	48	23	79	38
BMT3	2750	22	605	93	46	53	44	670	557
BMT4	1120	26	291	95	50	47	42	263	235
BMT5	1940	33	640	95	30	57	34	332	198
BMT6	810	18	146	98	36	53	30	155	87
BMT7	1510	14	211	93	57	31	51	267	439
BMT8	2010	28	563	96	41	54	25	445	206
BMT9*									
Median	1725	20	253	94	44	51	38	265	221
Normal range†			94-561	62-95				400-1313	

Abs, absolute; MNC, mononuclear cells.

*No flow data available for BMT9.

†Normal ranges were defined as 5th to 95th percentiles of at least 91 adult healthy volunteers analyzed at the Fred Hutchinson Cancer Research Center.

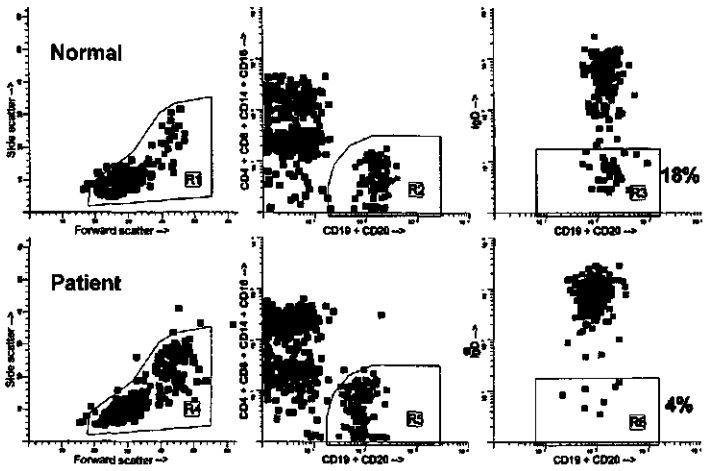

Figure 1. Flow cytometric analysis and gating of blood B cells showing the relative lack of IgD⁻ B cells in a BMT recipient 1 year after grafting. Both specimens, normal and patient, were processed concurrently. Ficoll-isolated mononuclear cells (MNCs) were stained with anti-IgD–FITC (goat $F(ab')_2$ antihuman delta chain), anti-(CD4, CD8, CD14, CD16)–PE, and anti-(CD19, CD20)–peridinin chlorophyll protein. Data were acquired on FACSCAN cytometer. For analysis, first the MNC gate (R1, R4) was drawn on the forward- versus side-scatter dot plots (left). Then, B cells were gated on the CD19/CD20 versus CD4/CD8/CD14/CD16 dot plots (R2, R5), excluding non-B cells binding anti-CD19/CD20 nonspecifically, "B cell + non-B cell" doublets, and $CD20^{low}$ T cells (middle). Finally, CD19/CD20 versus IgD dot plots were created exclusively of the cells falling within the MNC gate and the B-cell gate, ie, R1 and R2 in the normal and R4 and R5 in the patient (right). To calculate the percentage of IgD⁻ B cells, regions R3 and R6 were set so as to encompass the B cells showing only background FITC fluorescence. In this example, 18% of B cells in the normal were IgD⁻ versus 4% of B cells in the patient.

GATAGCAC-3′). The PCR products were size-fractionated on a 1.5% agarose gel and stained with ethidium bromide.

Results

It was not certain if the previously reported lack of somatically mutated B cells in peripheral blood of transplant recipients was a result of an actual deficit in the somatic mutational process or if it was the product of population dynamics. Our approach to investigating this issue was suggested by the observation that a high incidence of mutations was observed in V_H transcripts obtained from healthy-subject B cells following a 14-day coculture with activated $CD4^+$ T cells.[14] We hypothesized that if there was an actual deficit in the mutational process, then BMT B cells would not accumulate mutations in cocultures with activated $CD4^+$ T cells. Furthermore, we reasoned that if an intrinsic B-cell deficit existed, then neither healthy-subject T cells nor BMT-recipient T cells would support the accumulation of somatic mutations in BMT-recipient B cells in culture. Alternatively, if the deficit was only in the T-cell compartment, then healthy-subject T cells should support the accumulation of mutations among BMT-recipient B cells. To test these hypotheses, B cells and $CD4^+$ T cells obtained from transplant recipients and from healthy subjects were cocultured, and accumulation of somatic mutations was assessed.

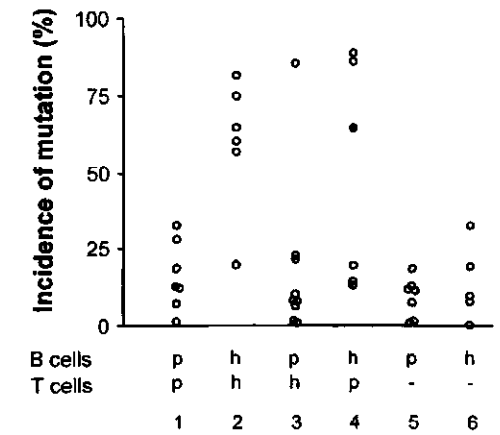

Figure 2. Incidence of somatically mutated V_H transcripts in lymphocyte cocultures is dependent primarily on the source of B cells. Accumulation of somatic mutation was assessed by sequential hybridization as described.[7,14-16] The results are presented as the percentage of V_H transcripts that have acquired 1 or more mutations in either of two 21-base-pair (bp) target sequences.[7,14,15] In the Figure, h indicates healthy subject; p, BMT patient; (−), T cells replaced by CD40 ligand and L cells. Closed circles indicate sources of transcripts selected for sequence analysis (see also Figure 4).

In this study, 9 recipients of allogeneic bone marrow transplants were studied. At the time of the 1-year posttransplantation evaluation, the number and percentage of B cells and T cells in peripheral blood were determined (Table 1). The median $CD4^+$ T-cell count was 265×10^6/L, which was below the normal range. The B-cell counts were normal or supranormal, except for 1 patient in whom the B-cell count was subnormal; the median count was 253×10^6/L, which fell in the normal range. The percentage of cells expressing membrane IgD was determined as described in Figure 1; it tended to be above normal, although the median fell within the normal range (Table 1).

Autologous combinations of BMT-recipient lymphocytes had poor cell growth, and as shown in Figure 2 (Group 1), yielded a low incidence of somatic mutation. As expected, autologous combinations of healthy-subject lymphocytes yielded a high incidence of mutation (Figure 2, Group 2). These results indicate a deficit among BMT-recipient lymphocytes that might be a consequence of poor growth or a failure to activate BMT-recipient T cells.

Heterologous combinations of BMT-recipient B cells and healthy-subject T lymphocytes yielded background levels of

Table 2. HLA-class II typing of healthy donors

Sample ID	DRB1*	DQA1*	DQB1*	DPB1*
HS1	0101, 0404	0101, 0301	0501, 0302	0402, 0501
HS2	0701, 0701	0201, 0201	0201, 0201	0402, 1101
HS3	0701, 0101	0201, 0101	0201, 0501	0201, 0402
HS4†				
HS5†				
HS6†				

*HLA-class II loci.
†MHC typing unavailable.

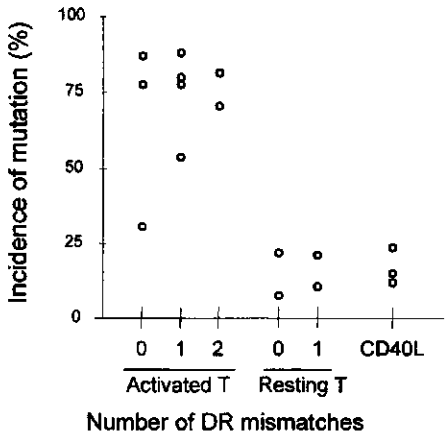

Figure 3. Incidence of somatically mutated V_H transcripts in lymphocyte cocultures of healthy subjects with disparate MHC loci. Each data point represents the average of duplicate cultures. For each point, an average of 724 V_H transcripts were analyzed (range 476-885, median 731). Accumulation of somatic mutation was assessed as in Figure 2.

mutation in all but 1 experiment (Group 3). Thus, neither healthy-subject T cells nor BMT-recipient T cells were consistently able to induce the accumulation of somatic mutation in BMT-recipient B cells, suggesting that BMT B cells had an intrinsic inability to be driven to accumulate somatic mutations. The inability of healthy-subject T cells to induce mutation in heterologous culture combinations is probably not due to MHC mismatch between BMT-recipient B cells and healthy-subject T cells; B cells in heterologous combinations of lymphocytes from HLA-disparate healthy donors, shown in Table 2, accumulate mutations as well as B cells in autologous combinations (Figure 3). That resting T cells, even if supplemented with CD40L, did not induce somatic mutations in these cultures suggests that the accumulation of somatic mutation in this system is not driven by an allogeneic reaction.

In 3 of 6 experiments, T cells from BMT recipients were able to support mutation in healthy-subject B cells, indicating that T lymphocytes from some BMT recipients can give adequate T-cell help (Figure 2). This finding is consistent with previous reports that posttransplantation CD4 T cells appear qualitatively normal.[17-20]

To estimate the accumulation of somatic mutation more accurately, we analyzed nucleotide sequences among transcripts selected from 2 B-cell cultures (Figure 4). In 1 culture, BMT-recipient B cells (BMT5) had been cocultured with autologous T cells (Table 3, exp 4). In the other culture, healthy-subject B cells (HS4) had been cultured with BMT-recipient T cells (BMT5) (Table 3, exp 4). These cultures are also indicated by filled circles in Figure 2. The sequences from the first set have an average of 0.2% mutation (median 0.17%). In contrast, the sequences from the second set have on average 3.1% mutation (median 1.7%), more than 10-fold higher. The different percentages of mutated V_H transcripts seen in Figure 2 reflect both quantitative differences in the number of mutations among the V_H transcripts and differences in the incidence of transcripts with any mutation. The correlation between mutation detected by hybridization and that detected by nucleotide sequence analysis has been reported.[14,15]

To determine if the lack of somatic mutation in BMT recipients correlated with the inability to differentiate in culture, IgG messenger RNA (mRNA) production (an indicator of differentiation[21]) was estimated with the use of a semiquantitative PCR (Figure 5). Except for lane 1 in Figure 5A and 5B, in which poor cell growth was observed, an IgG PCR product was obtained from all healthy subjects as well as all BMT recipients, suggesting that in these cultures isotype switching has occurred. As expected, no IgG PCR product was obtained from preculture IgD^+ cells (data not shown). In addition, secreted IgG was detected by enzyme-linked immunosorbent assay in culture supernatants from T-cell–supported cultures irrespective of somatic mutation (data not shown).

Discussion

We have previously shown that BMT recipients fail to acquire somatic mutations in rearranged V_H genes in PBL[6-8] and have low memory B-cell counts.[22] In the current study, we wished to determine the intrinsic capacity of transplant recipient naive B cells to acquire mutations. Several models have been described that mimic germinal center reactions in which somatic mutation takes place.[14,23-26] We used an in vitro system in which activated $CD4^+$ T cells drive B-cell differentiation over a 14-day culture period. In this system, healthy-subject B cells accumulate large numbers of V-segment mutations, presumably as a consequence of activation of the somatic mutator mechanism.[14] In contrast, we found that B cells obtained from BMT recipients 1 year posttransplantation failed to accumulate mutations. This deficit could not be overcome by coculture with healthy-subject T cells, although T cells from certain patients were able to drive the accumulation of mutations in healthy-subject B cells.

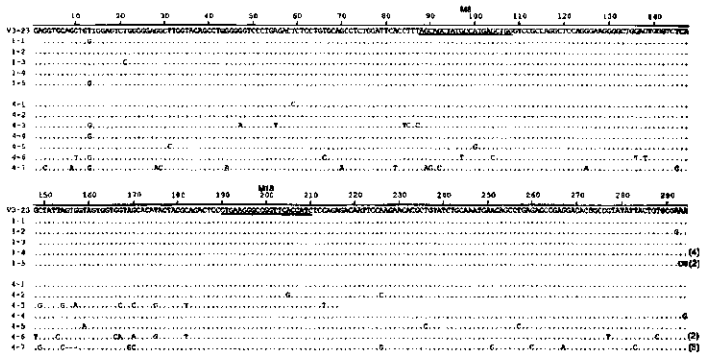

Figure 4. DNA sequence analysis of V3-23 transcripts from T-cell–activated B-cell cultures. B-cell cultures from BMT-recipient B cells supported by autologous activated T cells (sequences 1-1-1-5) and healthy-subject B cells supported by BMT-recipient T cells (sequences 4-1-4-7). Repeated isolation of the same cDNA clone is indicated by parentheses. Sequences 1-1-1-5 are taken from BMT5; sequences 4-1-4-7 are taken from HS4 (indicated by the filled circles in Figure 2).

Table 3. Incidence of somatically mutated V_H transcripts in lymphocyte cocultures is dependent primarily on the B-cell donor

Experiment	Source of B cells	Source of T cells	Number of transcripts		Mutated transcripts* (%)	Nucleotide substitutes† (%)
			V_H3	V3-23		
Exp 1	BMT1	BMT1	nd§	161	12	
	BMT1	HS1	nd	100	8	
	HS1	BMT1	nd	138	13	
	HS1	HS1	nd	155	57	
Exp 2	BMT2	HS1	344	151	8	
Exp 3	BMT3	BMT3	nd	53	15	
	BMT3	HS3	374	99	1	
	BMT4	HS3	>354	54	86	
	HS3	BMT3	712	37	86	
	HS3	HS3	>375	11	55	
	BMT3	—	726	206	1	
	BMT4	—	482	59	1	
	HS3	—	381	20	10	
Exp 4	BMT6	BMT6	461	74	1	
	BMT5	BMT5	922	254	13	0.2
	BMT6	HS4	412	109	10	
	BMT5	HS4	>742	98	1	
	HS4	BMT5	620	273	64	3.1
	HS4	HS4	491	74	60	
	BMT6	—	570	155	11	
	BMT5	—	789	163	12	
	HS4	—	86	12	0	
Exp 5	BMT7	BMT7	647	152	7	
	BMT7	HS5	751	212	6	
	HS5	BMT7	601	171	14	
	HS5	HS5	754	137	19	
	BMT7	—	745	139	11	
	HS5	—	820	168	7	
Exp 6	BMT8	BMT8	353	90	28	
	BMT8	HS6	664	87	21	
	HS6	BMT8	681	90	88	
	HS6	HS6	736	164	75	
	BMT8	—	784	130	18	
	HS6	—	683	153	19	
Exp 7	BMT9	BMT9	345	58	33	
	BMT9	HS4	331	70	23	
	HS4	BMT9	288	142	20	
	HS4	HS4	>1320	194	82	
	BMT9	—	684	98	7	
	HS4	—	697	176	33	

Nd indicates not determined; BMT, bone marrow transplant; HS, healthy subject. Dash indicates that no T cells were added but that B-cell cultures were supported by L cells supplemented with CD40L (1 μg/mL).

*Percentage of V3-23 transcripts with mutation detectable by hybridization.

†Incidence (%) of nucleotide substitution per base pair (bp) based on 290 bp/transcript of sequence shown in Figure 4.

Among the patients studied here, the only striking difference was in the capacity of patient T cells to support mutation in healthy-subject B cells. All 3 of these patients (BMT3, 5, 8) had normal levels of $CD4^+$ T cells and supranormal levels of B cells, suggesting that recovery of the immune system was more robust in these patients.

Unmutated, naive B cells have the phenotype $CD19^+$ IgM^+ IgD^+. This phenotype is exhibited among healthy subjects by approximately 80% of B cells, and among BMT recipients by more than 95% of B cells.[12] Thus, by both cell-surface phenotype and the extent of somatic mutation, the B-cell repertoire post-BMT resembles the pre-immune component of the B-cell repertoire of a healthy adult. Simply because of population dynamics, it would seem logical that the B-cell repertoire post-BMT would be primarily naive. However, our finding that these B cells cannot be driven to acquire somatic mutations suggests that additional processes are at work. The failure of the cells to accumulate

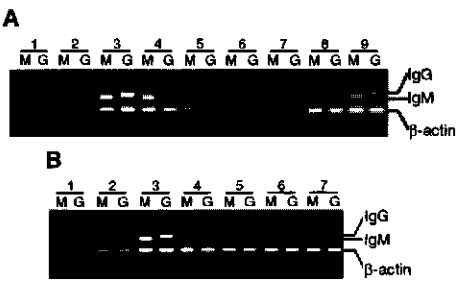

Figure 5. Presence of IgM and IgG mRNA in 14-day B-cell cultures of BMT recipients and healthy subjects. cDNA from B-cell cultures from 9 recipients and 7 healthy subjects was amplified with the use of 5′ primers specific for V_H3 and 3′ primers specific for either IgM or IgG. Results are representative of analysis of triplicate cultures. As an internal control, β-actin mRNA was used. Lanes M were amplified with the use of Cμ primers, lanes G with the use of Cγ primers. (A) BMT recipients. (B) Healthy subjects.

somatic mutations did not seem to parallel a failure to switch class. Taken together, the data are most easily explained by postulating that there is a deficit in the capacity of BMT-recipient B cells to respond to signals to activate the somatic mutator mechanism.

Because the marrow donors are themselves healthy subjects whose B cells are capable of acquiring mutations, this posttransplantation deficit must be developmentally determined. All patients received cyclosporine for the first 180 days after transplantation, and this immunosuppressive treatment might be expected to delay recovery of immunocompetency. However, ongoing immunosuppressive therapy cannot explain the results because cyclosporine was terminated at least 180 days prior to our studies for all but 1 patient (BMT1), who was receiving immunosuppressive therapy at the time of this study.

Another possibility is that during the pretransplantation conditioning regimen, a critical cellular function is disrupted and is not restored by marrow transplant. One such function, for example, could be the delivery of survival signals. In normal B-cell differentiation, newly formed transitional B cells are recruited into a long-lived pre-immune B-cell pool, in a process thought to be dependent on survival signals delivered in secondary lymphoid organs.[27,28] Because the pretransplantation conditioning regimen may disrupt lymphoid tissue architecture (particularly follicular dendritic cells),[29] the survival signals may be missing in the transplant recipients. As a result, BMT recipients may fail to select a long-lived naive B-cell compartment. The implication of this is that the B-cell compartment in BMT recipients is composed primarily of transitional B cells, which are short lived and have newly emerged from the bone marrow.[30] We postulate that this transitional B-cell population can participate in primary immune responses and can be driven to differentiate into plasma cells, but does not participate in a germinal center reaction and does not acquire mutations.

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References

1. Storek J, Witherspoon RP. Immunological reconstitution after hemopoietic stem cell transplantation. In: Atkinson K, ed. Clinical Bone Marrow and Blood Stem Cell Transplantation. Cambridge, England: Cambridge University Press; 2000:111-146.
2. Matsue K, Lum LG, Witherspoon RP, Storb R. Proliferative and differentiative responses of B cells from human marrow graft recipients to T cell-derived factors. Blood. 1987;69:308-315.
3. Lum LG, Seigneuret MC, Storb RF, Witherspoon RP, Thomas ED. In vitro regulation of immunoglobulin synthesis after marrow transplantation. I. *T-cell and B-cell deficiencies in patients with and* without chronic graft-versus-host disease. Blood. 1981;58:431-439.
4. Witherspoon RP, Deeg HJ, Lum LG, et al. Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-versus-host disease. Transplantation. 1984;37:456-461.
5. Sullivan KM, Storek J, Kopecky KJ, et al. A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs.-host disease after marrow transplantation: clinical outcome and effect on subsequent immune recovery. Biol Blood Marrow Transplant. 1996;2:44-53.
6. Glas AM, Hufnagle WO, Suzuki I, et al. Anomalous diversification of the antibody repertoire following bone marrow transplantation. Ann N Y Acad Sci. 1995;764:312-314.
7. Suzuki I, Milner ECB, Glas AM, et al. Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood. 1996;87:1873-1880.
8. Glas AM, Nottenburg C, Milner ECB. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. Clin Exp Immunol. 1997; 107:372-380.
9. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. N Engl J Med. 1986;314:729-735.
10. Przepiorka D, Thomas ED, Durnam DM, Fisher L. Use of a probe to repeat sequence of the Y chromosome for detection of host cells in peripheral blood of bone marrow transplant recipients. Am J Clin Pathol. 1991;95:201-206.
11. Ugozzoli L, Yam P, Petz LD, et al. Amplification by the polymerase chain reaction of hypervariable regions of the human genome for evaluation of chimerism after bone marrow transplantation. Blood. 1991;77:1607-1615.
12. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? Bone Marrow Transplant. 1993;12: 387-398.
13. *Storek J, Dawson MA, Maloney DG. Comparison* of two flow cytometric methods enumerating CD4 T cells and CD8 T cells. Cytometry. 1998;33:76-82.
14. Huang S-C, Glas AM, Pinchuk GV, et al. Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40-ligand. Clin Exp Immunol. 1999;116:441-448.
15. Glas AM, Huang S-C, van Montfort EHN, Milner ECB. Motif-specific probes identify individual genes and detect somatic mutations. Mol Immunol. 1999;36:599-610.
16. Huang S-C, Jiang R, Hufnagle WO, Furst DE, Wilske KR, Milner ECB. V_H usage and somatic hypermutation in peripheral blood B cells of patients with rheumatoid arthritis. Clin Exp Immunol. 1998;112:516-527.
17. Storek J, Dawson MA, Maloney DG. Normal anti-CD3 stimulated proliferation of CD4 T cells at one year after allogeneic marrow transplantation. Transpl Immunol. 1999;7:123-125.
18. Jin NR, Lum LG, Buren EV, Lerman SP, Walker AL, June CH. Signal transduction by B and T cells early after bone marrow transplantation: B cell calcium flux responses are intact whereas lack of CD4 cells accounts for impaired T cell responses. Bone Marrow Transplant. 1995;16:103-109.
19. Lum LG, Seigneuret MC, Orcutt-Thordarson N, Noges JE, Storb R. The regulation of immunoglobulin synthesis after HLA-identical bone marrow transplantation. VI. differential rates of maturation of distinct functional groups within lymphoid subpopulations in patients after human marrow grafting. Blood. 1985;65:1422-1433.
20. Lum LG, Orcutt-Thordarson N, Seigneuret MC, Storb R. The regulation of Ig synthesis after marrow transplantation. IV. T4 and T8 subset function in patients with chronic graft-vs-host disease. J Immunol. 1982;129:113-119.
21. Banchereau J, Rousset F. Human B lymphocytes: phenotype, proliferation, and differentiation. Adv Immunol. 1992;52:125-262.
22. Storek J, Witherspoon RP, Storb R. Reconstitution of membrane IgD$^-$ (mIgD$^-$) B cells after marrow transplantation lags behind the reconstitution of mIgD$^+$ B cells. Blood. 1997;89:350-351.
23. Denepoux S, Fournier N, Peronne C, Banchereau J, Lebecque S. T cells can induce somatic mutation in B cell receptor-engaged BL2 Burkitt's lymphoma cells independently of CD40-CD40 ligand interactions. J Immunol. 2000;164:1306-1313.
24. Källberg E, Jainandunsing S, Gray D, Leanderson T. Somatic mutation of immunoglobulin V genes in vitro. Science. 1996;271:1285-1288.
25. Denepoux S, Razanajaona D, Blanchard D, et al. Induction of somatic mutation in a human B cell line in vitro. Immunity. 1997;6:35-46.
26. Razanajaona D, Denepoux S, Blanchard D, et al. In vitro triggering of somatic mutation in human naive B cells. J Immunol. 1997;159:3347-3353.
27. Goodnow CC, Cyster JG, Hartley SB, et al. Self-tolerance checkpoints in B lymphocyte development. Adv Immunol. 1995;59:279-368.
28. Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate. Immunity. 1995;3:691-701.
29. Sale GE, Alavaikko M, Schaefers KM, Mahan CT. Abnormal CD4:CD8 ratios and delayed germinal center reconstitution in lymph nodes of human graft recipients with graft-versus-host disease (GVHD): an immunohistological study. Exp Hematol. 1992;20:1017-1021.
30. Carsetti R, Köhler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. J Exp Med. 1995;181:2129-2140.
31. Gokmen E, Raaphorst FM, Boldt DH, Teale JM. Ig heavy chain third complementarity determining regions (H CDR3s) after stem cell transplantation do not resemble the developing human fetal H CDR3s in size distribution and Ig gene utilization. Blood. 1998;92:2802-2814.

Chapter 6

Discussion

During the decade prior to the mapping of the V_H locus and identification of essentially all of the of V_H genes, multiple reports showed biased utilization of specific V_H genes in particular situations, ranging from biases in the fetal repertoire to association of particular V_H genes with autoantibodies and predominant utilization of individual genes in specific antibody responses. We now know that some of these apparent biases were a reflection of the fact that the V_H repertoire was more limited than previously thought. Although one could imagine that in some disease situations the pattern of V_H utilization might change, this has not always been the case. In the study described in this thesis, bone marrow transplant (BMT) recipients were studied because the recipients regenerate their immune system from the grafted haematopoietic stem cells, and exhibit immunodeficiencies following marrow transplantation. The exact nature of the B cell defects contributing to the humoral immunodeficiency is unknown.

V_H gene usage after BMT

One of the possibilities that we have explored is that an abnormal restriction of the potential antibody repertoire in B cells from BMT recipients contributes to the humoral deficiency. It has been suggested that the reconstitution of the humoral immune system follows ontogenic development [1-6]. The usage of V_H families following BMT appears to mimic usage during B cell ontogeny [7;8], and was thought to be restricted to fetal type V_H genes early post transplant [6-9].Thus, to account for the observed immunodeficiencies, it was to be expected that during the first year post transplant, BMT patients would utilize a more limited set of V genes than would healthy adult subjects. This hypothesis was supported by the fact that early post transplant B cell lymphopoiesis is mono- or oligoclonal [10-13], and thus might have a limited diversity. However, analysis of rearrangements in peripheral blood B cells of healthy subjects [14-16] and BMT recipients at 90 days and 1 yr following transplant indicated that the pattern of usage of V_H genes was similar between BMT recipients and healthy subjects [Chapter 5; 17-19]. Thus the processes involved in generating the antibody repertoire are largely functional within the first few months following transplant. Furthermore, these results indicated that the immunodeficiencies common among BMT recipients probably are not due to the failure to utilize appropriate V region genes in generating the pre-immune antibody repertoire. The complexity of the CDR3 (third complementarity region) and D_H and

J_H utilization is similar in BMT recipients and healthy subjects 90 days and one year after transplant [Chapter 3; 19] and [10], and thus further support the conclusion that the primary antibody repertoire is generated normally following BMT by 90 days [Chapter 3; 17;18].

B cell development after BMT vs ontogenic development

As mentioned above, it has been suggested that the immune reconstitution after BMT resembles the developing repertoire. This is based on similarities between long-term survivors without graft versus host disease (GVHD) and normal infants. For example, compared to adult B cells, B cells from both neonates and BMT recipients are larger in size [3;20]. In both infants and BMT recipients B cell numbers are low at first, but then tend to overshoot normal adult values and return to normal [5;6;21-25]. After BMT as well as in neonates the B cells have an immature phenotype, IgD^{high},IgM^{high} and $CD38^{high}$ [3;22;24;26;27]. In fetal and cord blood and BMT recipients the accumulation of somatic mutation in rearranged V_H gene segments is low or absent [Chapter 3; 17-19;28-31] (and AM Glas and E.C.B. Milner unpublished results). In BMT recipients and infants the sequence of achieving normal adult levels of serum isotypes is similar, first IgM, IgG_1 and IgG_3, then IgG_2, IgG_4 and IgA [6]. In vitro function of BMT recipients' B cells and B cells from neonates is abnormal in the production of IgG and IgA when compared to adult B cells [5;32-36]. Both neonates and BMT recipients are susceptible to encapsulated bacteria [37]. There are a few differences after BMT and normal B cell ontogeny such as most cord blood cells are L-selectin negative, while a high proportion of BMT recipient B cells are L-selectin positive. In fetal spleen, liver and bone marrow and in neonates CD5 B cells are abundant [38-41], however the percentage of CD5 B cells after BMT is inconsistent. Some studies report high percentages of CD5 B cells [5;22], while in other reports the number is low or similar to adult levels of CD5 B cells [38;42;43]. Some discrepancies were also found in the expression of CD21, CD23, and CD38 [3-5;22;42]. These differences may be due to the differences in the flow cytometry techniques [3].

The restriction to fetal type V_H genes early post transplant suggested that the V_H repertoire parallels ontogenic development as well. The current understanding that the spectrum of V_H gene segments used in the fetal repertoire resembles the adult

repertoire contradicts the old notion that the fetal repertoire was comprised of a special subset of the repertoire available to adults. The repertoire is more limited than once thought, and a few genes dominate both the fetal repertoire and adult repertoire (reviewed in [44]). Hence, the repertoire following BMT is similar to that seen in adults [Chapter 3; 17;18].

B cell development after BMT follows many patterns that are similar to ontogenic development; however immune reconstitution does not appear to recapitulate human fetal ontogeny based on the diversity of rearranged heavy chains. The molecular characteristics that typify fetal repertoires, such as the use of D_HQ52 and the paucity of N nucleotides [28;45-52], are more reliable markers for the distinction between fetal and adult repertoires [53]. In our studies [Chapter 3; 19] and others [10;11;54], the CDR3s exhibit none of these fetal characteristics, but rather resemble CDR3s from healthy adults.

Somatic mutation deficit

The hypothesis that an abnormal restriction of the antibody repertoire contributed to the observed immunodeficiencies proved to be wrong. However, the results described in Chapter 3 provide some indication of possible mechanisms contributing to poor antibody responses in BMT recipients. Rearrangements in BMT recipients exhibited much less somatic mutation than did rearrangements from healthy subjects [Chapter 3; 17-19]. The failure in the BMT recipients to accumulate somatic mutations in rearranged V_H genes in PBL correlated with a restricted diversity in expressed IgG CDR3s [10], and an absence of IgD^- B cells [55] and suggest that the prolonged deficiency of humoral immunity after transplant may be due in part to the slow reconstitution of memory B cells.

The accumulation of somatic mutations is a characteristic of T cell dependent antigen driven responses [56]; the absence of mutation is consistent with a defect in T cell dependent antigen driven responses. The successful recovery of secondary B cell responses after transplant will therefore depend in part on the recovery of the T cell compartment. The B cell phenotype following BMT is consistent with several, not necessarily mutually exclusive, functional deficits. For example, B cell dysfunction could be secondary to T cell dysfunction, either due to inadequate T cell help or to

suppression. Alternatively, the B cells could be unable to respond to signals that induce mutation. However, normal levels of serum IgM, IgG_1 and IgG_3 one year post transplant indicate that T cells are able to provide at least some help [36;57-59], although the reconstitution of $CD4^+$ T cells is delayed post transplant [60]. As detailed in Chapter 5, T lymphocytes from some patients are able to provide adequate T cell help to healthy subject B cells in vitro [61], which is consistent with earlier reports that post transplant $CD4^+$ T cells are qualitatively normal [62-65], and suggests that the B cell defect seen in BMT patients is not exclusively the result of defects in the T cell compartment. Moreover, B cells from BMT recipients one-year post transplant failed to accumulate mutations in vitro. This could not be overcome by co-cultures with healthy subject T cells, suggesting that the defect seen in BMT recipients is intrinsic to the B cells [Chapter 5; 61]. The inability of the healthy subject T cells to induce mutation in BMT B cells is probably not due to an MHC mismatch between recipient B cells and healthy T cells, since heterologous cell combinations of healthy HLA disparate individuals accumulate mutations as well as autologous combinations do [Chapter 5; 61]. MHC mismatched resting T cells did not drive accumulation of somatic mutation suggesting that the mutation seen in this culture system is not driven by an allogeneic reaction [Chapter 5; 61].

In vitro model of germinal center reactions

Several in vitro models have been described that mimic germinal center reactions [66-69]. In the system reported in Chapters 4 and 5, we used a system in which freshly isolated, activated $CD4^+$ T cells drive naïve B cell differentiation over a 14 day culture period [61;70]. In this system, healthy subject B cells accumulate large numbers of V segment mutations, presumably as a consequence of activation of the somatic mutator mechanism. However, there are other possible mechanisms that might account for the observed accumulation of somatic mutation. Therefore the question as to whether mutation is actually occurring during the culture period must be addressed. One of the possibilities is that T cells regulate differential outgrowth of mutated (or mutating) B cells compared to unmutated B cells by selectively driving the previously mutated B cells to grow out, or that T cells are selectively killing naïve (i.e. unmutated) B cells allowing the mutated cells to grow out. Several studies of tonsillar B cells [71-73] as well as of peripheral blood B cells [74-78] have indicated that somatically mutated IgM or IgD memory cells occur, albeit at a low frequency.

Recently, a rare population of cells has been described that is $CD27^{+}IgD^{+}$ [79], and may be comprised of a novel population of highly mutated memory cells [80].

In the experiments described in Chapters 4 and 5, 1000 B cells were plated. Plating efficiency in this system is approximately 10% (G.V. Pinchuk and E.C.B. Milner unpublished results), therefore roughly 100 clones would grow out in these cultures. Nearly 40 percent of all the B cells express a V_H3 containing Ig (E.H.N. van Montfort and E.C.B. Milner, unpublished results), which would lead to 40 V_H3 expressing B cell clones. Twenty to 35% of the V_H3 expressing B cell clones are derived from the V3-23 gene, therefore 8 to 14 V3-23 clones would grow out. Our sequence data are consistent with this prediction [70] and (A.M. Glas, E.H.N. van Montfort and E.C.B. Milner, unpublished results), supporting the conclusion that mutation occurs in the cultures. However, it cannot be excluded that the $IgD^{+}CD27^{+}$ memory cells may be selectively permitted to proliferate in the T cell supported cultures, but not the CD40L-supported cultures. However, preliminary results show that sorted $IgD^{+}CD27^{-}$ cells, and cord blood cells, which are $IgD^{+}CD27^{-}$ [79], accumulate mutation in this system, suggesting that $IgD^{+}CD27^{+}$ memory cells are not the sole contributor of the mutations seen in this system (A.M. Glas, E.H.N. van Montfort and E.C.B. Milner, unpublished results).

Origins of B cell dysfunction following transplant

Two results described in this thesis, lack of somatic mutation in vivo and inability to accumulate mutations in vitro, are consistent with the hypothesis that B cells from BMT recipients have an "intrinsic" inability to acquire somatic mutations. Unmutated, naïve B cells have the phenotype $CD19^{+}$ IgM^{+} IgD^{+}. This phenotype is exhibited among healthy subjects by approximately 80% of B cells, and among BMT recipients by more than 95% of B cells [3]. Thus, by both cell surface phenotype and the extent of somatic mutation, the B cell repertoire post-BMT resembles the pre-immune component of the B cell repertoire of a healthy adult. Simply because of population dynamics it would seem logical that the B cell repertoire post BMT would be primarily naïve. However, the finding that these B cells cannot be driven to acquire somatic mutations suggests that additional processes are at work. The failure of the cells to accumulate somatic mutations did not seem to parallel a failure to class switch

[Chapter 5; 61]. Taken together the data are most easily explained by postulating that there is a deficit in the capacity of BMT recipient B cells to respond to signals that activate the somatic mutator mechanism.

Because the marrow donors are themselves healthy subjects whose B cells are capable of acquiring mutations, this post-transplant deficit must be developmentally determined. GVHD is a strong suppressor of immune function, however, the lack of somatic mutation cannot be attributed directly to GVHD because nine of the 13 subjects studied were free of GVHD [Chapters 3 and 5; 17;18]. There were no obvious differences in the diversification of the repertoire between patients with or without GVHD. The patients are usually treated with immunosuppressive drugs, such as cyclosporine, to prevent GVHD; the immunosuppressive treatment might be expected to delay recovery of immunocompetency. However, in most patients studied [Chapter 5; 61], the therapy was terminated at least 180 days prior to our studies, and is unlikely to explain the lack of mutation in these patients.

Memory responses are dependent on germinal center formation. The kinetics and quality of restructuring the architecture of the secondary lymphoid organs after transplant will have a significant impact on reconstitution of the B cell repertoire. One hypothesis is that during the pre-transplant conditioning regimen a critical cellular function may be disrupted that is not restored by marrow transplant. Irradiation and immunosuppression could affect each of the cell types in germinal center formation: T cells, B cells, and follicular dendritic cells (FDCs). As described above, T cells are unlikely to be the sole contributor to the inability to acquire mutations. Consistent with this hypothesis is the fact that histological analysis of spleen and lymph nodes from non-surviving patients of allogeneic BMT revealed a reduced number of B lymphocytes, and absence of lymph node follicles [81-84].

In normal B cell differentiation, newly formed transitional B cells are recruited into a long-lived preimmune B cell pool. The lymphocytes are selected in a process that is thought to be competitive and is dependent on survival signals delivered in secondary lymphoid organs [85;86]. The majority of the newly formed B lymphocytes however, do not enter the mature repertoire. The critical molecular interactions and micro

environments that select which clones will be successfully recruited are poorly understood. It is hypothesized that B lymphocytes move away from the T cell zone towards the follicles in response to a chemotactic gradient that originates in the follicular stroma or in the marginal zone [85]. This process is thought to be competitive; the chemotactic gradient is reduced when the follicles are saturated, either because abundant receptors consume the gradient or because of reduced production [85]. The involvement of chemokines in the migration of the B cells towards the follicles has been recognized recently (reviewed in [87;88]). It is thought that the survival of the B cells that have migrated towards the follicles depends on rescue from apoptotic death by trophic factors produced by the FDCs [85].

Irradiation and immunosuppression could affect the ability of the B cells to receive the appropriate signals (trophic factors), the generation of such signals by other cells, such as the FDCs, or the generation and maintenance of the chemotactic gradient. The lymphoid architecture may be disrupted because of the pre-transplant conditioning [81-83], and it has been shown that the FDCs are missing [84;89]. The absence of FDCs could possibly disrupt the chemotactic gradient in BMT patients and as a result the B cells would be unable to migrate towards the follicles to receive survival signals. Alternatively, if the gradient is unaffected by the pretransplant conditioning, the survival signals could be missing and the cells die by apoptosis. If one or more of these possibilities were true, then BMT recipients may fail to select a long-lived naïve B cell compartment. The implication of this is that the B cell compartment in BMT recipients is comprised primarily of transitional B cells. Transitional B cells are newly emerged from the bone marrow and are thought to be short lived [90]. We postulate that this transitional B cell population can participate in primary immune responses and can be driven to differentiate into plasma cells, but cannot participate in a germinal center reaction and cannot acquire mutations.

Concluding remarks

The B cell deficits in BMT recipients are hypothesized to be due to a failure to recruit mature cells into the long-lived B cell pool, and the failure to generate a memory response through a germinal center reaction. Immunohistological analysis of lymph

nodes and spleen at multiple time points after transplant would provide insight in the recovery of germinal centers after transplant. However, such a procedure is invasive and not justifiable. A more preferable analysis would be to determine the time point at which BMT recipients have regained their ability to somatically mutate.

Finally, the question remains: what is the signal (or signals) that B cells need in order to activate their mutational mechanism? Do well known ligands and receptors play a role in a novel context, or is one as yet unknown ligand and receptor involved in the activation of the somatic mutation process? Revealing the deficit in BMT recipients could provide insight into the somatic mutation process in healthy subjects. Conversely, elucidation of the mechanism that activates the somatic mutation process in normal B cells would be of help in understanding the B cell deficit after BMT.

References

1. Lum LG. The kinetics of immune reconstitution after human marrow transplantation. Blood 1987; 69:369-80.

2. Guillaume T, Rubinstein DB, Symann M. Immune reconstitution and immunotherapy after autologous hematopoietic stem cell transplantation. Blood 1998; 92:1471-90.

3. Storek J, Ferrara S, Ku N, Giorgi JV, Champlin RE, Saxon A. B cell reconstitution after human bone marrow transplantation: recapitulation of ontogeny? Bone Marrow Transplant. 1993; 12:387-98.

4. Velardi A, Cucciaioni S, Terenzi A *et al.* Acquisition of Ig isotype diversity after bone marrow transplantation in adults. A recapitulation of normal B cell ontogeny. J.Immunol. 1988; 141:815-20.

5. Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B cell differentiation following autologous, conventional, or T-cell depleted bone marrow transplantation: A recapitulation of normal B cell ontogeny. Blood 1990; 76:1647-56.

6. Storek J, Saxon A. Reconstitution of B cell immunity following bone marrow transplantation. Bone Marrow Transplant. 1992; 9:395-408.

7. Fumoux F, Guigou V, Blaise D, Maraninchi D, Fougereau M, Schiff C. Reconstitution of human immunoglobulin VH repertoire after bone marrow transplantation mimics B-cell ontogeny. Blood 1993; 81:3153-7.

8. Storek J, King L, Ferrara S, Marcelo D, Saxon A, Braun J. Abundance of a restricted fetal B cell repertoire in marrow transplant recipients. Bone Marrow Transplant. 1994; 14:783-90.

9. Uckun FM, Haissig S, Ledbetter JA *et al.* Developmental hierarchy during early human B-cell ontogeny after autologous bone marrow transplantation using autografts depleted of CD19+ B-cell precursors by an anti-CD19 pan-B-cell immunotoxin containing pokeweed antiviral protein. Blood 1992; 79:3369-79.

10. Gokmen E, Raaphorst FM, Boldt DH, Teale JM. Ig heavy chain third complementarity determining regions (H CDR3s) after stem cell transplantation do not resemble the developing human fetal H CDR3s in size distribution and Ig gene utilization. Blood 1998; 92:2802-14.

11. Näsman-Björk I, Lundkvist I. Oligoclonal dominance of immunoglobulin VH3 rearrangements following allogeneic bone marrow transplantation. Bone Marrow Transplant 1998; 21:1223-30.

12. Van Muiswinkel WB, Radl J, van der Wal DJ. The regulatory influence of the thymus-dependent immune system on the heterogeneity of immunoglobulins in irradiated and reconstituted mice. Adv Exp Med Biol 1976; 66:617-21.

13. Mink JG, Radl J, Van den Berg P, Van Muiswinkel WB, Van Oosterom R. Homogeneous immunoglobulins in the serum of irradiated and bone marrow reconstituted mice: the role of thymus and spleen. Immunology 1979; 37:889-94.

14. Huang S-C, Jiang R, Glas AM, Milner ECB. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. Mol.Immunol. 1996; 33:553-60.

15. Rao SP, Huang S-C, Milner ECB. Analysis of the V_H3 repertoire among genetically disparate individuals. Exp.Clin.Immunogenet. 1996; 13:131-8.

16. Suzuki I, Pfister L, Glas A, Nottenburg C, Milner ECB. Representation of rearranged V_H gene segments in the human adult antibody repertoire. J.Immunol. 1995; 154:3902-11.

17. Glas AM, Hufnagle WO, Suzuki I *et al.* Anomalous diversification of the antibody repertoire following bone marrow transplantation. Ann.N.Y.Acad.Sci. 1995; 764:312-4.

18. Suzuki I, Milner ECB, Glas AM *et al.* Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. Blood 1996; 87:1873-80.

19. Glas AM, Nottenburg C, Milner ECB. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. Clin.Exp.Immunol. 1997; 107 :372-80.

20. Durandy A, Thuillier L, Forveille M, Fischer A. Phenotypic and functional characteristics of human newborns' B lymphocytes. J Immunol 1990; 144:60-5.

21. Kotylo PK, Baenzinger JC, Yoder MC, Engle WA, Bolinger CD. Rapid analysis of lymphocyte subsets in cord blood. Am J Clin Pathol 1990; 93:263-6.

22. Bengtsson M, Smedmyr B, Festin R, Oberg G, Simonsson B, Totterman TH. B lymphocyte regeneration in marrow and blood after autologous bone marrow transplantation: increased numbers of B cells carrying activation and progression markers. Leukemia Research 1989; 13:791-7.

23. Reichert T, DeBruyère M, Deneys V *et al.* Lymphocyte subset reference ranges in adult caucasians. Clin.Immunol.Immunopathol. 1991; 60:190-208.

24. Hannet I, Erkeller-Yuksel F, Lydyard P, Deneys V, DeBruyère M. Developmental and maturational changes in human blood lymphocyte subpopulations [see comments]. Immunol Today 1992; 13:215, 218-5, 218.

25. Caldwell CW, Poje E, Helikson MA. B-cell precursors in normal pediatric bone marrow. Am J Clin Pathol 1991; 95 :816-23.

26. Tedder TF, Clement LT, Cooper MD. Discontinuous expression of a membrane antigen (HB-7) during B lymphocyte differentiation. Tissue Antigens 1984; 24:140-9.

27. Macardle PJ, Weedon H, Fusco M *et al.* The antigen receptor complex on cord B lymphocytes. Immunology 1997; 90:376-82.

28. Raaphorst FM, Timmers E, Kenter MJH, Van Tol MJD, Vossen JM, Schuurman RKB. Restricted utilization of germ-line V_H3 genes and short diverse third complementarity-determining regions (CDR3) in human fetal B lymphocyte immunoglobulin heavy chain rearrangements. Eur.J.Immunol. 1992; 22:247-51.

29. Ridings J, Dinan L, Williams R, Roberton D, Zola H. Somatic mutation of immunoglobulin V_H6 genes in human infants. Clin.Exp.Immunol. 1998; 114:33-9.

30. van Dijk-Härd I, Söderström I, Feld S, Holmberg D, Lundkvist I. Age-related impaired affinity maturation and differential D-JH gene usage in human VH6-expressing B lymphocytes from healthy individuals. Eur.J.Immunol. 1997; 27:1381-6.

31. Ridings J, Nicholson IC, Goldsworthy W, Haslam R, Roberton DM, Zola H. Somatic hypermutation of immunoglobulin genes in human neonates. Clin Exp Immunol 1997; 108:366-74.

32. Storek J, Witherspoon RP, Luthy D, Storb R. Low IgG production by mononuclear cells from marrow transplant survivors and from normal neonates is due to a defect of B cells. Bone Marrow Transplant. 1995; 15:679-84.

33. Andersson U, Bird AG, Britton BS, Palacios R. Humoral and cellular immunity in humans studied at the cell level from birth to two years of age. Immunol Rev 1981; 57:1-38.

34. Wedgwood JF, Weinberger BI, Hatam L, Palmer R. Umbilical cord blood lacks circulating B lymphocytes expressing surface IgG or IgA. Clin Immunol Immunopathol 1997; 84:276-82.

35. Yachie A, Konno A, Ohta K *et al.* Delineation of producing ability of IgG and IgA subclasses by naive B cells in newborn infants and adult individuals. Clin Exp Immunol 1995; 102:204-9.

36. Matsue K, Lum LG, Witherspoon RP, Storb R. Proliferative and differentiative responses of B cells from human marrow graft recipients to T cell-derived factors. Blood 1987; 69:308-15.

37. Atkinson K, Farewell V, Storb R *et al.* Analysis of late infections after human bone marrow transplantation: role of genotypic nonidentity between marrow donor and recipient and of nonspecific suppressor cells in patients with chronic graft-versus-host disease. Blood 1982; 60:714-20.

38. Waddick KG, Uckun FM. CD5 antigen-positive B lymphocytes in human B cell ontogeny during fetal development and after autologous bone marrow transplantation. Exp.Hematol. 1993; 21:791-8.

39. Erkeller-Yuksel FM, Deneys V, Yuksel B *et al.* Age-related changes in human blood lymphocyte subpopulations. J Pediatr 1992; 120:216-22.

40. Antin JH, Emerson SG, Martin P, Gadol N, Ault KA. Leu-1+ (CD5+) B cells. A major lymphoid subpopulation in human fetal spleen: phenotypic and functional studies. J.Immunol. 1986; 136:505-10.

41. Westerga J, Timens W. Immunohistological analysis of human fetal lymph nodes. Scand.J.Immunol. 1989; 29:103-12.

42. Kagan JM, Champlin RE, Saxon A. B-cell dysfunction following human bone marrow transplantation: functional-phenotypic dissociation in the early posttransplant period. Blood 1989; 74:777-85.

43. Drexler HG, Brenner MK, Wimperis JZ *et al.* CD5-positive B cells after T cell depleted bone marrow transplantation. Clin Exp Immunol 1987; 68:662-8.

44. Glas AM, van Montfort EHN, Milner ECB. The human antibody repertoire: old notions, current realities, and V_H gene-dependent biases. In: Zanetti M, Capra JD, eds. The antibodies, Volume 6. Amsterdam: Harwood Academic Publishers, 2000

45. Schroeder HW, Jr., Hillson JL, Perlmutter RM. Early restriction of the human antibody repertoire. Science 1987; 238:791-3.

46. Shiokawa S, Mortari F, Lima JO *et al.* IgM heavy chain complementarity-determining region 3 diversity is constrained by genetic and somatic mechanisms until two months after birth. J.Immunol. 1999; 162:6060-70.

47. Cuisinier AM, Gauthier L, Boubli L, Fougereau M, Tonnelle C. Mechanisms that generate human immunoglobulin diversity operate from the 8th week of gestation in fetal liver. Eur J Immunol 1993; 23:110-8.

48. Schroeder HW, Jr., Wang JY. Preferential utilization of conserved immunoglobulin heavy chain variable gene segments during human fetal life. Proc.Natl.Acad.Sci.USA 1990; 87:6146-50.

49. Hillson JL, Oppliger IR, Sasso EH, Milner ECB, Wener MH. The emerging B cell repertoire: influence of developmental stage and interindividual variation. J.Immunol. 1992; 149:3741-52.

50. Pascual V, Verkruyse L, Casey ML, Capra JD. Analysis of Ig H chain gene segment utilization in human fetal liver. J.Immunol. 1993; 151:4164-72.

51. Sanz I. Multiple mechanisms participate in the generation of diversity of human H chain CDR3 regions. J.Immunol. 1991; 147:1720-9.

52. Nickerson KG, Berman J, Glickman E, Chess L, Alt FW. Early human IgH gene assembly in Epstein-Barr virus-transformed fetal B cell lines. Preferential utilization of the most JH-proximal D segment (DQ52) and two unusual VH-related rearrangements. J.Exp.Med. 1989; 169:1391-403.

53. Raaphorst FM. Bone marrow transplantation, fetal B-cell repertoire development, and the mechanism of immune reconstitution [letter]. Blood 1998; 92:4873-4.

54. Näsman I, Lundkvist I. Evidence for oligoclonal diversification of the V_H6-containing immunoglobulin repertoire during reconstitution after bone marrow transplantation. Blood 1996; 87:2795-804.

55. Storek J, Witherspoon RP, Storb R. Reconstitution of membrane IgD^- ($mIgD^-$) B cells after marrow transplantation lags behind the reconstitution of $mIgD^+$ B cells. Blood 1997; 89:350.

56. MacLennan IC. Somatic mutation. From the dark zone to the light. Current Biology 1994; 4:70-2.

57. Sullivan KM, Storek J, Kopecky KJ *et al.* A controlled trial of long-term administration of intravenous immunoglobulin to prevent late infection and chronic graft-vs.-host disease after marrow transplantation: Clinical outcome and effect on subsequent immune recovery. BB&MT 1996; 2:44-53.

58. Lum LG, Seigneuret MC, Storb RF, Witherspoon RP, Thomas ED. In vitro regulation of immunoglobulin synthesis after marrow transplantation. I. T-cell and B-cell deficiencies in patients with and without chronic graft-versus-host disease. Blood 1981; 58:431-9.

59. Witherspoon RP, Deeg HJ, Lum LG *et al.* Immunologic recovery in human marrow graft recipients given cyclosporine or methotrexate for the prevention of graft-versus-host disease. Transplantation 1984; 37:456-61.

60. Storek J, Ferrara S, Rodriguez C, Saxon A. Recovery of mononuclear cell subsets after bone marrow transplantation: overabundance of CD4+CD8+ dual-positive T cells reminiscent of ontogeny. Journal of Hematotherapy 1992; 1:303-16.

61. Glas, A. M., van Montfort, E. H. N., Storek, J., Green, E. N., Drissen, R. P., Bechtold V.J., Reilly, Z. J., Dawson, M., and Milner, E. C. B. B cell-autonomous somatic mutation deficit following bone marrow transplant. Blood. 2000. In Press

62. Storek J, Dawson MA, Maloney DG. Normal anti-CD3 stimulated proliferation of CD4 T cells at one year after allogeneic marrow transplantation. Transplant Immunology 2000; in press.

63. Jin NR, Lum LG, Buren EV, Lerman SP, Walker AL, June CH. Signal transduction by B and T cells early after bone marrow transplantation: B cell calcium flux responses are intact whereas lack of CD4 cells accounts for impaired T cell responses. Bone Marrow Transplant. 1995; 16:103-9.

64. Lum LG, Seigneuret MC, Orcutt-Thordarson N, Noges JE, Storb R. The regulation of immunoglobulin synthesis after HLA-identical bone marrow transplantation: VI. Differential rates of maturation of distinct functional groups within lymphoid subpopulations in patients after human marrow grafting. Blood 1985; 65:1422-33.

65. Lum LG, Orcutt-Thordarson N, Seigneuret MC, Storb R. The regulation of Ig synthesis after marrow transplantation. IV. T4 and T8 subset function in patients with chronic graft-vs-host disease. J Immunol 1982; 129:113-9.

66. Källberg E, Jainandunsing S, Gray D, Leanderson T. Somatic mutation of immunoglobulin V genes in vitro. Science 1996; 271:1285-8.

67. Denepoux S, Razanajaona D, Blanchard D *et al.* Induction of somatic mutation in a human B cell line in vitro. Immunity 1997; 6:35-46.

68. Razanajaona D, Denepoux S, Blanchard D *et al.* In vitro triggering of somatic mutation in human naive B cells. J.Immunol. 1997; 159:3347-53.

69. Denepoux S, Fournier N, Peronne C, Banchereau J, Lebecque S. T cells can induce somatic mutation in B cell receptor-engaged BL2 Burkitt's lymphoma cells independently of CD40-CD40 ligand interactions. J Immunol 2000; 164:1306-13.

70. Huang S-C, Glas AM, Pinchuk GV *et al.* Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40-ligand. Clin Exp Immunol 1999; 116:441-8.

71. Nicholson IC, Brisco MJ, Zola H. Memory B lymphocytes in human tonsil do not express surface IgD. J.Immunol. 1995; 154:1105-13.

72. Pascual V, Liu Y-J, Magalski A, de Bouteiller O, Banchereau J, Capra JD. Analysis of somatic mutation in five B cell subsets of human tonsil. J.Exp.Med. 1994; 180:329-39.

73. Klein U, Küppers R, Rajewsky K. Variable region gene analysis of B cell subsets derived from a 4-year-old child: Somatically mutated memory B cells accumulate in the peripheral blood already at young age. J.Exp.Med. 1994; 180:1383-93.

74. Klein U, Küppers R, Rajewsky K. Human IgM^+IgD^+ B cells, the major B cell subset in the peripheral blood, express V_k genes with no or little somatic mutation throughout life. Eur.J.Immunol. 1993; 23:3272-7.

75. Liu Y-J, de Bouteiller O, Arpin C *et al.* Normal human IgD^+IgM^- germinal center B cells can express up to 80 mutations in the variable region of their IgD transcripts. Immunity 1996; 4:603-13.

76. Paramithiotis E, Cooper MD. Memory B lymphocytes migrate to bone marrow in humans. Proc.Natl.Acad.Sci.USA 1997; 94:208-12.

77. Van Es JH, Gmelig-Meyling FHJ, Logtenberg T. High frequency of somatically mutated IgM molecules in the human adult B cell repertoire. Eur.J.Immunol. 1992; 22:2761-54.

78. Klein U, Küppers R, Rajewsky K. Evidence for a large compartment of IgM-expressing memory B cells in humans. Blood 1997; 89:1288-98.

79. Agematsu K, Nagumo H, Yang FC *et al.* B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. Eur J Immunol 1997; 27:2073-9.

80. Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (Memory) B cells. J Exp Med 1998; 188:1679-89.

81. Dilly SA, Sloane JP. Cellular composition of the spleen after human allogeneic bone marrow transplantation. J.Pathol. 1988; 155:151-60.

82. Dilly SA, Sloane JP, Psalti ISM. The cellular composition of human lymph nodes after allogeniec bone marrow transplantation: An immunohistological study. J.Pathol. 1986; 150:213-21.

83. Horny H-P, Horst H-A, Ehninger G, Kaiserling E. Lymph node morphology after allogeneic bone marrow transplantation for chronic myeloid leukemia: a histological and immunohistological study focusing on the phenotype of the recovering lymphoid cells. Blut 1988; 57:31-40.

84. Horny HP, Ruck M, Kaiserling E, Ehninger G. Immunohistology of the human spleen after bone marrow transplantation for leukemia with special reference to the early post-transplantation period. Pathology, Research & Practice 1990; 186:775-83.

85. Goodnow CC, Cyster JG, Hartley SB *et al.* Self-tolerance checkpoints in B lymphocyte development. Adv.Immunol. 1995; 59:279-368.

86. Cyster JG, Goodnow CC. Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influences peripheral B cell fate. Immunity 1996; 3:691-701.

87. Loetscher P, Moser B, Baggiolini, M. Chemokines and their receptors in lymphocyte traffic and HIV infection. Adv Immunol. 2000; 74:127-180

88. McHeyzer-Williams MG, Ahmed R. B cell memory and the long-lived plasma cell. Curr Opin Immunol 1999; 11:172-9.

89. Sale GE, Alavaikko M, Schaefers KM, Mahan CT. Abnormal CD4:CD8 ratios and delayed germinal center reconstitution in lymph nodes of human graft recipients with graft-versus-host disease (GVHD): an immunohistological study. Exp.Hematol. 1992; 20:1017-21.

90. Carsetti R, Köhler G, Lamers MC. Transitional B cells are the target of negative selection in the B cell compartment. J Exp Med 1995; 181:2129-40.

ABSTRACT

The bone marrow is the major haematopoietic organ and is critically involved in the production of all formed blood elements in postnatal life. The bone marrow contains rapidly dividing cells and therefore is sensitive to DNA damaging agents. In certain types of cancers where a high dose of radiation and chemotherapeutic agents are needed, a bone marrow transplant is necessary to "rescue" the patient from the lethal side effects of radiation and chemotherapy. However, the immune system of transplant recipients must be regenerated from the transplant inoculum, and it is not surprising that many transplant recipients are deficient in generating specific antibody responses to exogenous stimuli. In this thesis experiments are described which are aimed at a further understanding of the nature of the B cell defects leading to the humoral deficiency in bone marrow transplant (BMT) recipients.

The possibility that an abnormal restriction of the potential antibody repertoire in B cells from BMT recipients could contribute to the deficiency seen in BMT patients is explored in Chapter 3. The results showed that the processes involved in generating and selecting the antibody repertoire are largely functional within the first few months following BMT, and the immunodeficiencies common among BMT recipients probably are not due to the failure to utilize appropriate V region genes in generating the pre-immune antibody repertoire. Rearrangements in BMT recipients, however, exhibited much less somatic mutation than did rearrangements obtained from healthy subjects.

In Chapter 2, the correlation between motif-specific hybridizations and nucleotide sequence variation is described as an approach to identify individual V_H genes. This method is highly specific and allows for the detection of somatic mutation in these genes by sequential hybridization. By using this method, a large number of genes can be analyzed without sophisticated instrumentation.

Somatic mutation of rearranged immunoglobulin genes is a powerful diversifier of the antibody repertoire and an important requisite in the formation of memory B cells. The precise mechanism responsible for turning on the mutational process is unknown.

To dissect the role of different components of the germinal center in this mechanism, we have used a system in which freshly isolated, activated $CD4^+$ T cells drive naïve B cell differentiation over a 14 day culture period. In this system, healthy subject naïve B cells accumulate large numbers of V segment mutations, presumably as a result of activation of the somatic mutation mechanism (Chapter 4). This system is used in Chapter 5 to determine whether the lack of somatic mutation seen in BMT recipients is an intrinsic B cell deficit or secondary to a T cell deficit. The results showed that BMT recipient B cells lack the capacity to accumulate somatic mutation in a T cell dependent manner. This appears to be a B cell autonomous deficit because T cells from some patients were able to support accumulation in heterologous healthy subject B cells, however, they were unable to support the accumulation of mutations in autologous naïve cells.

SAMENVATTING

Het beenmerg is het voornaamste orgaan dat zorgt voor de productie van alle bloed cellen. Het beenmerg bevat cellen die zich snel delen en is daarom zeer gevoelig voor DNA-beschadigende agentia. Bij bepaalde kankertherapieën waarbij gebruik gemaakt wordt van een hoge doses straling en cytostatica is een beenmergtransplantatie (BMT) noodzakelijk om de patiënt te redden van de fatale neven effecten. Het immuun systeem van de patiënten moet echter geregenereerd worden van het getransplanteerde inoculum, en het is daarom niet verrassend dat vele patiënten geen specifieke antilichamen kunnen maken tegen lichaamsvreemde binnendringers. In dit proefschrift worden experimenten beschreven die er op gericht zijn om de aard van de gebreken in BMT patiënten te verhelderen.

De mogelijkheid dat een abnormale beperking van het potentiële antilichaam repertoire zou kunnen bijdragen aan de afwijking in BMT patiënten is onderzocht in hoofdstuk 3. De resultaten laten echter zien dat, binnen de eerste paar maanden na de transplantatie, het proces van het genereren en selecteren van het antilichaam repertoire functioneel is en dat de afwijkingen niet voort komen uit een verkeerd gebruik van V_H gen segmenten. Opvallend was dat de gerecombineerde V_H genen minder somatische mutaties bevatten in vergelijking met gerecombineerde V_H genen in gezonde mensen.

In hoofdstuk 2 is de relatie tussen motief-specifieke hybridisaties en de variatie in nucleotide sequenties beschreven, om de V_H gen segmenten te bepalen in gerecombineerde V_H genen. Deze methode is zeer specifiek en kan ook gebruikt worden voor het bepalen van somatische mutaties in de V_H genen. Tevens heeft deze methode als voordeel dat in korte tijd vele gerecombineerde V_H genen geanalyseerd kunnen worden zonder geavanceerde apparatuur.

Somatische mutatie in gerecombineerde immunoglobuline genen zorgt voor een uitgebreid repertoire van antilichamen specifiek voor vele lichaamsvreemde binnendringers, en is een belangrijke vereiste voor het generen van de zogenaamde "memory" cellen. Het precieze mechanisme dat verantwoordelijk is voor het aanzetten van het mutatie-mechanisme is nog onbekend. Om het mutatie-mechanisme

te onderzoeken is, in hoofdstuk 4, een *in vitro* systeem ontwikkeld waarin vers geïsoleerde en geactiveerde $CD4^+$ T cellen de differentiatie van naïeve B cellen bevorderen in een culture van 14 dagen. In dit systeem hopen zich vele mutaties op in de gerecombineerde V_H genen van B cellen van gezonde mensen, waarschijnlijk als resultaat van de activatie van het mutatie-mechanisme. Dit systeem is in hoofdstuk 5 gebruikt om te bepalen of het defect in het somatische mutatie-mechanisme in BMT patiënten een gevolg is van een afwijking specifiek voor B cellen of dat het een direct gevolg is van een defect in de T cellen. De resultaten laten zien dat de B cellen van BMT patiënten een afwijking in het T cel afhankelijke somatische mutatie mechanisme hebben. Dit blijkt een afwijking specifiek voor B cellen te zijn, omdat T cellen van sommige patiënten niet in staat zijn om mutaties te ondersteunen in hun eigen B cellen, maar wel in B cellen van gezonde personen.

ABBREVIATIONS

BMT	bone marrow transplant
CD40L	CD40 ligand
CDR	complementarity determining region
CML	chronic myelogenous leukemia
FDC	follicular dendritic cell
FHCRC	Fred Hutchinson Cancer Research Center
FITC	fluorescein isothiocyanate
FR	framework region
GVHD	graft versus host disease
H	heavy chain
HLA	human leucocyte antigen
HS	healthy subject
Ig	immunoglobulin
IVIG	intravenous immunoglobulin
L	light chain
MC	mononuclear cells
MNC	mononuclear cells
NK	natural killer cells
PB	peripheral blood
PBL	peripheral blood lymphocytes
PCR	polymerase chain reaction
PE	phycoerythrin
RA	rheumatoid arthritis
R/S ratio	ratio of replacement to silent substitutions
SLE	systemic lupus erythematosis
TMACL	tetramethylammoniumchloride
V	variable region
V_H	immunoglobulin heavy chain variable region
VMRC	Virginia Mason Research Center

LIST OF PUBLICATIONS

Glas, A.M., van Montfort, E.H.N., Storek, J., Green, E-G.N., Drissen, R.P.M, Bechtold, V.J., Reilly, Z.J., Dawson, M., Milner, E.C.B. 2000. B cell-autonomous somatic mutation deficit following bone marrow transplant. *Blood 96:*1064

Glas, A.M., E.H.N. van Montfort, and E.C.B. Milner. 2000. The human antibody repertoire: old notions, current realities, and V_H gene-dependent biases. In *The antibodies, Volume 6*. M. Zanetti and J.D. Capra, eds. Harwood Academic Publishers, Amsterdam.

Glas, A.M., S.-C. Huang, E.H.N. van Montfort, and E.C.B. Milner. 1999. Motif-specific probes identify individual genes and detect somatic mutations. *Mol.Immunol. 36*:599

Huang, S.-C., A.M. Glas, G.V. Pinchuk, E.H.N. van Montfort, S.P. Rao, R. Jiang, and E.C.B. Milner. 1999. Human B cells accumulate immunoglobulin V gene somatic mutations in a cell contact-dependent manner in cultures supported by activated T cells but not in cultures supported by CD40-ligand. *Clin Exp Immunol 116*:441

Glas, A.M., C. Nottenburg, and E.C.B. Milner. 1997. Analysis of rearranged immunoglobulin heavy chain variable region genes obtained from a bone marrow transplant (BMT) recipient. *Clin.Exp.Immunol. 107*:372.

Kraj, P., S.P. Rao, A.M. Glas, R.R. Hardy, E.C.B. Milner, and L.E. Silberstein. 1997. The human heavy chain Ig V region gene repertoire is biased at all stages of B cell ontogeny, including early pre-B cells. *J.Immunol. 158*:5824.

Suzuki, I., E.C.B. Milner, A.M. Glas, W.O. Hufnagle, S.P. Rao, L. Pfister, and C. Nottenburg. 1996. Immunoglobulin heavy chain variable region gene usage in bone marrow transplant recipients: lack of somatic mutation indicates a maturational arrest. *Blood 87*:1873.

Huang, S.-C., R. Jiang, A.M. Glas, and E.C.B. Milner. 1996. Nonstochastic utilization of Ig V region genes in unselected human peripheral B cells. *Mol.Immunol. 33*:553.

Pinchuk, G.V., C.M. Alexander, A.M. Glas, R.J. Armitage, and E.C.B. Milner. 1996. VH4 repertoire in human B lymphocytes stimulated by CD40 ligand and IL-4: evidence for positive and negative selection mechanisms coupled to CD40 activation. *Mol.Immunol. 33*:1369.

Glas, A.M., W.O. Hufnagle, I. Suzuki, L. Pfister, S. Rao, C. Nottenburg, and E.C.B. Milner. 1995. Anomalous diversification of the antibody repertoire following bone marrow transplantation. *Ann.N.Y.Acad.Sci. 764*:312.

Milner, E.C.B., W.O. Hufnagle, A.M. Glas, I. Suzuki, and C.M. Alexander. 1995. Polymorphism and utilization of human V_H genes. *Ann.N.Y.Acad.Sci. 764*:50.

Suzuki, I., L. Pfister, A. Glas, C. Nottenburg, and E.C.B. Milner. 1995. Representation of rearranged V_H gene segments in the human adult antibody repertoire. *J.Immunol. 154*:3902.

Abstracts:

Glas, A.M., van Montfort, E.H.N., Storek, J., E-G.N. Green, and E.C.B. Milner. 2000. Somatic mutation deficit following bone marrow transplant. *FASEB J. 14:6* 94.16

Sasso, E.H., Suzuki, L.A., Martinez, M and Glas, A.M. 1999. Two Ig V_H3 gene loci have alleles found only in African Americans that share a novel arginine containing CDR2 sequence, and is frequently expressed. *Autoantibodies and autoimmunity, an international conference. Oslo, Norway. June 24-28, 1999*

Rao, S.P., P. Kraj, A.M. Glas, R.R. Hardy, E.C.B. Milner, and L.E. Silberstein. 1997. The human VH family gene repertoire is biased at all stages of B cell ontogeny including early pre B cells. *Keystone Symposia* 18.

Huang, S.-C., G.V. Pinchuk, S.P. Rao, R. Jiang, E.H.N. van Montfort, A.M. Glas, and E.C.B. Milner. 1997. Somatic mutation in human B cells cultured *in vitro* . *J.Allergy Clin.Immunol. 99*:LB70.

Milner, E.C.B., S.P. Rao, P. Kraj, S.-C. Huang, G.V. Pinchuk, A.M. Glas, R. Jiang, R.R. Hardy, and L.E. Silberstein. 1997. The antibody repertoire in human B cell development: early, late, and persistent biases. *J.Allergy Clin.Immunol. 99*:459.

Huang, S.-C., W.O. Hufnagle, A. Glas, S. Rao, I. Suzuki, and E.C.B. Milner. 1994. Analysis of the V_H repertoire in RA patients reveals an elevated antigen-driven component and a markedly diminished preimmune component. *Arthritis Rheum. 37*:S287.

Suzuki-Jaecks, I., A.M. Glas, L. Pfister, C. Nottenburg, and E.C.B. Milner. 1993. V_H contribution to the human antibody repertoire. *FASEB J.(supplement)*

CURRICULUM VITAE

Annuska Maria Glas werd geboren op 17 mei 1968 te Alkmaar. Na het behalen van haar VWO diploma aan het C.S.G. Jan Arentsz te Alkmaar in 1987, ging zij Biologie aan de Landbouwuniversiteit in Wageningen studeren. Deze studie werd in 1993 afgesloten met als doctoraal onderwerpen: Moleculaire Genetica (Dr. Ir H.W.J. van den Broek, Landbouwuniversiteit Wageningen), Moleculaire Biologie (Dr W.J.T. Zabel, Landbouwuniversiteit Wageningen) en Immunologie (Dr. E.C.B. Milner, Virginia Mason Research Center, Seattle, WA, USA). Van 1993 tot september 2000 was ze werkzaam bij Virginia Mason Research Center in Seattle waar ze, onder leiding van Dr E.C.B. Milner het in dit proefschrift beschreven onderzoek verrichtte. Tijdens deze periode was ze van januari tot juli 1994, "visiting graduate student" aan de University of Washington, Seattle, WA. In juli 1996 heeft ze aan de University of California San Diego, La Jolla, CA de AAI Advanced Course in Immunology gevolgd.

Annuska Maria Glas was born on May 17th 1968 in Alkmaar, The Netherlands. After graduating from the CSG Jan Arentsz high school in Alkmaar in 1987, she entered the M.S. program in Biology at the Wageningen Agricultural University, Wageningen, The Netherlands. The study was completed in 1993, with majors in Molecular Genetics (Dr. Ir H.W.J. van den Broek, Wageningen Agricultural University), Molecular Biology (Dr W.J.T. Zabel, Wageningen Agricultural University) and Immunology (Dr. E.C.B. Milner, Virginia Mason Research Center, Seattle, WA, USA). She has been working on the project described in this thesis at the Virginia Mason Research Center in Seattle, WA in the group of Dr. E.C.B. Milner from 1993 till September 2000. From January till July of 1994, she attended the University of Washington, Seattle, WA as a visiting graduate student, and in July of 1996 she participated in the AAI Advanced Course in Immunology, at the University of California San Diego, La Jolla, CA.