

Many human peripheral VH5-expressing IgM+ B cells display a unique heavy-chain rearrangement

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Abstract

The immunoscope methodology has proven useful to analyze T-cell repertoires in mice and humans. We adapted it to the analysis of VH chains of human peripheral B cells by setting up a quantification of various VH and JH segments and the profiling of IgM-, IgG-, IgA- and IgE-expressing B cells. We then tested the hypothesis that the human B-cell and T-cell repertoires have a similar diversity of VH and V-beta rearrangements. We studied in more detail the VH5 family because it is not abundantly used, which facilitated the analysis. The data showed that the number of distinct VH5 rearrangements in all samples studied is close to the number of cells in the sample. This contrasts with T cells in which we previously showed that distinct V-beta rearrangements amount to a few percent of the number of T cells because each V-beta chain is on the average paired with ~25 alpha chains. Thus, in the VH5 family, the light chains add little quantitative diversity to that produced by the heavy chain alone. Whether this feature can be generalized to other VH chains is discussed.

Introduction

Antibody diversity results from a variety of processes. Genetic rearrangements of V gene segments together with D and J segments for the heavy chain and V genes and J segments for the light chain generate combinational diversity (1, 2). Imprecise junctions of these gene segments, either by nibbling or by random addition of nucleotides by the TdT enzyme, increase the diversity further (3, 4). Antigen-driven affinity maturation introducing somatic hypermutations in the V region, as well as heavy- and light-chain pairing (5) contribute to diversity. Class switch recombination (CSR) results in the production of several antibody isotypes and expands the diversity and functionality of the B-cell repertoire (6). These various mechanisms of antibody diversification have been thoroughly investigated and largely deciphered (7, 8). While V(D)J recombination and TdT activity contribute to diversity, both the T- and B-cell repertoires (9, 10), somatic hypermutation, CSR and diversification by gene conversion are specific to B cells.

How diverse is the antibody repertoire? Early estimates were in the order of 10^6 and 10^7 different antibodies in CBA/H mice (11). The enumeration of mouse B cells able to re-

spond to antigenic stimulation next suggested that the primary repertoire includes more than 10^7 clonotypes (12), a figure also extrapolated from the hybridoma technology (13). In these studies, B-cell diversity was not measured directly and the contribution of somatic mutations to diversity could hardly be assessed. In murine models, the proportion of mutated VH sequences was found to be small (14), and >95% of peripheral blood B lymphocytes from most mice express germ line VH genes (15), while most somatically mutated B cells localize in germinal centers (16, 17). Somatic mutations are rare in the serum IgM, IgG and IgA of young mice. They accumulate with age in response to environmental antigens and in much higher proportions in IgG than in IgM (18).

Human B-cell repertoires have been less extensively studied, and, for obvious ethical reasons, many analyses have dealt with pathological rather than physiological situations. Human blood B lymphocytes display a much higher proportion of cells bearing somatic mutations than mice, reaching up to 40% of all peripheral IgM-expressing B lymphocytes (19, 20). The VH and JH gene usage in normal peripheral

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2 Human B lymphocyte clone size and repertoire

B cells has been studied by single-cell PCR (21). B-cell monoclonal expansions are increased in several autoimmune disorders, such as rheumatoid arthritis (22), and in healthy elderly (23). By and large, none of these studies provides global information on the diversity of IgM+ B cells, on the average B-cell clone size or on the contribution of VH–VL pairing to B-cell diversity generation.

The immunoscope methodology which we developed to analyze T-cell repertoires (24, 25) in mice and humans has proven useful to explore a number of physiological and pathological situations. This methodology (also called by others as spectratyping) allows to explore both global repertoire features and the usage of specific clonotypes. Since this tool was missing for B cells, we developed it for the analysis of human VH chains. We then used it to check whether the diversity of VH chains in a peripheral B-cell population was comparable to that of V-beta chains as previously determined. For the VH5 family, we made the surprising observation that the number of distinct rearrangements is close to the number of cells. The possible generalization of this finding and of its implications is discussed.

Methods

Cell preparations, antibodies and flow cytometry

Blood (~500 ml) from three healthy donors was obtained from the 'Etablissement Français du Sang', Necker Enfants Malades Lecourbe, Paris, France. Donor 789 is a 56-year-old woman, donor 743 is a 55-year-old woman, donor 260 is a 44-year-old woman and donor 522 is a 30-year-old man. PBMCs were isolated by centrifugation over Ficoll-Paque (Amersham Biosciences AB, Uppsala, Sweden) in UNISEPmaxi+ tubes (Novamed, Jerusalem, Israel). B cells from donor 789 were then obtained from the PBMCs by depletion using the B cell negative isolation kit from Dynal (Oslo, Norway). For donors 743 and 522, PBMCs were co-stained with CD19 beads and CD19–PE (PharMingen, San Jose, CA, USA) and purified by positive selection on AutoMACS

using, respectively, Possel or Possel-S programs (Miltenyi Biotec, Bergisch-Gladbach, Germany). The eluted cells from the positive and negative fractions, as well as the total PBMCs, were then labeled with anti-IgM, anti-IgG, anti-IgD, anti-IgE anti-IgA1/IgA2, anti-kappa and anti-lambda antibodies (PharMingen) and rabbit polyclonal anti-human IgM (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Using these different antibodies, the percentage and the absolute number of cells bearing the different antibody isotypes were determined among the PBMC and B cell-positive fractions of donors 743 and 522, as shown in Table 1. The purity of the positive fraction was checked either by CD19 staining or by the sum of anti-kappa and anti-lambda staining.

RNA and cDNA preparation

Cells from both positive and negative fractions were collected and kept frozen at –20°C in the lysis buffer of the RNeasy mini kit (Qiagen, Courtaboeuf, France). Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's specifications, as previously described (26). cDNA was then prepared by RNA reverse transcription with 0.5 g l⁻¹ oligo (dT)17 and 200 U Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France).

Quantitative repertoire

The different VH germ line genes can be clustered in seven families according to their level of homology. Both IMGT (<http://imgt.cines.fr>) (26) and Vbase databases (<http://www.mrc-cpe.cam.ac.uk>) (27) were used to access their sequences and the germinal genes were aligned using the GCG Wisconsin Package Program (http://www.accelrys.com/products/gcg_wisconsin_package). All Taqman MGB probes were designed using the Primer express software program (Applied Biosystems, Courtaboeuf, France). All the primers used are shown in Table 2.

Their specificity was tested by specifically amplifying each VH family from a PBMC mix of three healthy donors, using

Table 1. Proportion and absolute numbers of cells expressing different isotypes in the blood of two healthy donors

Donor 743			Donor 522	
Type of positive cells among all cells	Non-purified fraction, % (absolute number × 10 ⁶)	CD19-purified fraction, % (absolute number × 10 ⁶)	Non-purified fraction, % (absolute nb × 10 ⁶)	CD19-purified fraction, % (absolute number × 10 ⁶)
CD19	3.8 (19.8)	ND	3.7 (17.8)	67.4 (14.2)
IgM	3.2 (16.7)	64.5 (16.3)	2.3 (11)	42.3 (8.9)
IgD	2.6 (13.5)	49.6 (12.5)	2.3 (11)	51 (10.8)
IgG	0.6 (3.1)	12 (3)	0.2 (1)	4.6 (2.1)
IgA	0.8 (4.2)	16.1 (4.1)	0.5 (2.4)	10.1 (2.1)
IgE	1 (5.2)	1.1 (0.3)	0.3 (1.4)	1.3 (0.3)
Kappa	4 (20.8)	44.2 (11.1)	2.8 (13.4)	41.9 (8.8)
Lambda	3.3 (17.2)	40.1 (10.1)	2.3 (11)	29.8 (6.3)
Sum kappa and lambda	7.3 (38)	84.3 (21.2)	5.1 (24.4)	71.7 (15.1)
Kappa and lambda	ND	ND	4.8 (23)	68.4 (14.4)
Total cell number	520	25.2	480	21

Blood cells from donor 743 and 522 were stained before and after B cell enrichment with the different antibodies according to Materials and Methods and analyzed in a FACScalibur (Becton Dickinson). For each staining, results are expressed as % of total cells. Corresponding absolute numbers are shown in parenthesis. The sum of distinct stainings for kappa and lambda light chains is shown for both donors, as well as double kappa and lambda light chains staining for donor 522.

Table 2. Specific primers for B cell heavy chain quantitative immunoscope analysis

VH, JH and C μ , γ , ϵ , and α specific primers			
Primer name	Sequence	Specificity	Primer localization
IGHV subgroup			
VH1	HUMVH1a	AGTGAAGGTCTCCTGCAAGGC	VH1—02, 08, 18, 58, 69, 2
	HUMVH1b	AGTGAAGGTTTCCTGCAAGGC	VH1—03, 45, 46
	HUMVH1c	AGTGAARRTCTCCTGCAAGGT	VH1—f, 24
VH2	HUMVH2	AACCCACASAGACCCTCAC	VH2—05, 70, 26
VH3a	HUMVH3aa	GCAGATTCACCATCTCAAGAGATG	VH3—15, 49, 72
	HUMVH3ab	GCAGGTTACCATCTCCAGAGATG	VH3—73
VH3b	HUMVH3ba	GCCGATTCACCATCTCCAGAGA	VH3—07, 09, 13, 20, 21, 30, 30, 3, 33, 43, 48, 53, 74
	HUMVH3bb	GCAGATTCACCATCTCCAGAGA	VH3—d, 64, 66
	HUMVH3bc	GCCGATTCACCATCTCCAGGGA	VH3—11
	HUMVH3bd	GCAGGTTACCATCTCCAGAGA	VH3—23
VH4	HUMVH4a	CTACAACCCGTCCTCAAGAGT	VH4—04, 28, 30—2, 30—4, 31, 34, b
	HUMVH4b	CTACAACCCCTCCCTCAAGAGT	VH4—59, 61
VH5	HUMVH5	GTGAAAAAGCCCGGGGAG	VH5—51, a
VH6	HUMVH6	TCCGGGGACAGTGTCTCT	VH6—01
VH7	HUMVH7	GGTGCAATCTGGGTCTGAGT*T*	VH7—04, 1
IGJH subgroup			
JH1	IGJH1	CCCTGGCCCCAGTGCTG	JH1
JH2	IGJH2	CCACGGCCCCAGAGATCG	JH2
JH3	IGJH3	CCCTTGGCCCCAGAYATCAAAAG	JH3a, b
JH4	IGJH4-1	GGTTCCTTGGCCCCAGTAG	JH4a
	IGJH4-2	GGTTCCTTGGCCCCAGTAG	JH4b
	IGJH4-3	GGTCCCTTGGCCCCAGTAG	JH4d
JH5	IGJH5	TGGCCCCAGGRGTCAAC	JH5a, b
JH6	IGJH6-1	CCTTGCCCCAGACGTCCAT	JH6a
	IGJH6-2	CCTTGCCCCAGACGTCCAT	JH6b
	IGJH6-3	CCTTGCCCCAGACGTCCAT	JH6c
IGH mu chain			
	HIGCM	CAGCCAACGGCCACGC	IGHM—01, 02, 03
	HCM-Fam	6Fam-GGAGACGAGGGGAAAAGG	CH1
	HCM-MGB	6Fam-CCGTGCGATACGAGC-MGB	CH1
IGH gamma chain			
	HIGCG	TCAG(A/G)GCGCCTGAGTTCCA	IGHG—1, 2, 3, 4
	HCG-Fam	6Fam-AAGTAGTCCTTGACCAAGGCAGC	CH1
	HCG-MGB	6Fam-CCGGTGACGGTGTG-MGB	CH1
IGH epsilon chain			
	HIGCE	GTGGTGGCTGGTAAGGTCATAG	IGHE—01, 02, 03
	HCE-Fam	6Fam-CGGGTCAAGGGGAAGACGG	CH1
	HCE-MGB	6Fam-CTCCCTCAACGGGAC-MGB	CH1
IGH alpha chain			
	HIGCA	TTTCGCTCCAGGTCACACTG	IGHA—1, 2
	HCA-Fam	6Fam-TCAGCGGGAAGACCTTGGG	CH1
	HCA-MGB	6Fam-TTCCCCCAGGAGCCA-MGB	CH1

This table shows the list of primers which were used to quantify VH and JH utilization by real time PCR. Each primer specifically amplifies one or several VH, as indicated. The primer localization in the FR1 or FR3 is also shown. 3 end phosphorothioate chemical modification of some primers is mentioned by asterisk. Only three primers display degenerate sites, with R meaning A or G and S meaning G or C.

VH family-specific primers and HIGC primers, for PCR cloning and sequencing. Only one VH family, the VH7 family, did not meet full specificity since a few amplified products belonged to the VH1 family.

Quantitative analysis was performed as described (28). PCRs were carried out by combining primers and a specific fluorophore-labeled probes for the constant region (CH ϵ , CH μ or CH γ) with one of eight primers covering the different VH1–7 chains.

Real-time PCRs were subsequently carried out in a 25- μ l reaction mixture with a final concentrations of 400 nmol l⁻¹ of each oligonucleotide primer, 200 nmol l⁻¹ of the fluorogenic probe and 1 $\times$ TaqMan Universal PCR Master Mix

(Applied Biosystems). Thermal cycling conditions comprised an AmpliTaq Gold DNA Polymerase activation at 95°C for 10 min, and then subjected to 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min.

To quantify JH usage, a cDNA aliquot was amplified using the VH5int-specific primer (5'-AGCCCGGGGAGTCTCTGA-3') on the 5' side and each of the six JH-specific primers on the 3' side together with the TM-MGB-VH5int probe (5'-ACCCCTACAGGAGATCT-3'), a Taqman MGB/FAM-labeled nested probe specific for the VH5 family. PCRs were carried out in 25- μ l total volume. For all these different reactions, real-time quantitative PCR was then performed on an ABI 7300 system (Applied Biosystems). The relative usage of

4 Human B lymphocyte clone size and repertoire

each VH family or each JH segment was calculated according to the formula:

$$U(\lg \text{ VH}_y) = \sum_{x=1}^{x=8} 2(\text{Ct}(x) - \text{Ct}(y)) \text{ and} \\ U(\lg \text{ JH}_y) = \sum_{x=1}^{x=8} 2(\text{Ct}(x) - \text{Ct}(y)),$$

In which Ct(x) is the fluorescent threshold cycle number measured for VH(y) family or JH(y). In this case, the VH family primer pair displays a mean efficiency of 0.95 ± 0.08 and the JH segment primers display a mean efficiency of 0.91 ± 0.08 .

Immunoscope profiles

PCR products were then subjected to 'run-off' reactions with a nested fluorescent primer specific for the constant region (Fc ϵ , Fc μ , Fc α or Fc γ) or JH region for four cycles. The fluorescent products were separated and analyzed using an ABI PRISM 3730 DNA analyzer. The size and intensity of each band were analyzed with 'Immunoscope software' (29, 30), which has been adapted to the capillary sequencer. Fluorescence intensities were plotted in arbitrary units on the y-axis and CDR3 lengths (in amino acids) on the x-axis.

CDR3 band purification

In the VH5–JH2 amplification experiments, bands corresponding to a CDR3 length of eight amino acids were purified from the acrylamide gel stained with silver staining system as previously described (Promega, Madison, WI, USA) (25, 31). The band of interest was cut from the gel, disrupted in 50 μ l of Tris-EDTA and the PCR product was recovered. A second 33-cycle PCR using the VH5int and the JH2 primers was then conducted with 1 μ l recovered product, followed by cloning as follows.

VH transcript sequencing and analysis

Either the total VH5–JH1 amplified products from different donors or the two CDR3 bands treated as above were cloned using the TOPO Blunt PCR cloning kit (Invitrogen). Direct sequencing was performed as previously described (24) using VH5int primer and the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The sequencing reactions were run in an ABI PRISM 3730 DNA analyzer (Applied Biosystems). Sequences were analyzed using Taps1.1 software written by Emmanuel Beaudoin (25).

Calculation of the VH/B-cell repertoire size

The size of the VH repertoire can be estimated from the number of distinct sequences found for a given rearrangement after sequencing to saturation. This figure is then divided by the product of the considered VH frequency with the considered JH frequency. When starting from a purified CDR3 band, the percentage of this band among all rearrangement was introduced in the above calculations. The diversity due to somatic mutations can be evaluated, thanks to the Taps software.

Results

The immunoscope methodology

The immunoscope was initially developed in our laboratory as a means of assessing characteristics of T-cell repertoires.

Its core principle is to analyze the size of the hypervariable CDR3 regions of the V-alpha, -beta, -gamma or -delta chains in a variety of V(J)C combinations. Experimentally, it uses cDNA samples prepared from mRNA extracted from a mixture of cells and specific PCR primers to specify the V(J)C combinations. Most of the work has been performed so far with the V-beta chains of mouse and human T cells, but the operational principles can be generally applied to others. Additional experimental 'modules' have been added to the core approach to make immunoscope more reliable and ultimately usable in the clinics. One module is a precise quantification of the V(J) segment usage in each particular sample. Another is based upon the isolation of a given CDR3 size peak in a certain V(J)C combination and its extensive sequencing. When the size peak corresponds to a clonal expansion, this opens the way to the synthesis of a clonotypic primer, which can serve to follow the clone of interest both prospectively and retrospectively (if samples are available). When the size peak corresponds to a mixture of clones, the sequencing, under conditions which have been carefully evaluated and controlled (32), allows to evaluate the diversity within that size peak, but also, importantly, to extrapolate to the entire repertoire. This set of procedures constitutes a powerful approach to analyze T-cell repertoires but it has several limitations. Contrary to single-cell PCR, it usually provides no information on the two chains of the receptor heterodimer expressed by a given cell. Also, it does not in itself provide functional clues. Finally, it measures mRNA, not gene frequencies. Extrapolation to genes is possible but requires caution.

With these limitations in mind, we have adapted the immunoscope methodology to the analysis of human B cells, with the following modules: (i) the quantification of VH and JH transcript and gene usage in human (blood) samples, (ii) the profiling of CDR3 sizes in a variety of VHJHCM combination and (iii) the sequencing of selected size peaks.

Details are provided in Methods. In this paper, these various steps were sequentially used to analyze some features of the human peripheral blood B-cell repertoire, mostly that of IgM+ B cells expressing the VH5 chain.

Quantification of VH and JH gene segment usage in human PBMCs

Representative FACS analysis of the PBMC of the two donors, before and after B-cell enrichment as well as the proportion of cells expressing antibodies of different isotypes and the absolute number of corresponding cells are shown for both donors in Table 1. As expected, IgM+ B cells represent the vast majority of the purified B cells, quantification of Ig transcripts with three healthy donors showed that the blood B cells express mainly of IgM (95.5%, see Fig. 1A) as compared with 2–3% for IgG and IgA.

cDNAs prepared from the blood of several donors were amplified using VH family-specific primers and a C μ primer (specific for the constant region of IgM). The TM-MGB-HCM fluorescent probe, nested in the constant region close to the C μ primer was used for quantification by real-time PCR (see Methods). Reproducibility was verified by testing two samples of the same donor (donor 743). The quantification of VH family transcript usage is shown (Table 3A). For a given donor, VH family usage varies widely. The two donors show

Table 3. VH and JH quantification by real-time PCR

	Donor 789	Donor 743		
	% means	Sample W %	Sample Z %	% means
A. VH Families				
VH1	5.9 ± 1	4.9	4.5	4.7 ± 0.2
VH2	9.5 ± 1.5	4.7	4.4	4.5 ± 0.2
VH3a	5.8 ± 0.1	6.7	7.3	7 ± 0.4
VH3b	52.5 ± 2.1	65.7	65.5	65.6 ± 0.2
VH4	20.2 ± 1.4	13.2	13.3	13.2 ± 0.1
VH5	3.2 ± 0.3	1.1	1	1 ± 0.1
VH6	2.6 ± 0.9	1.3	1.3	1.3 ± 0.1
VH7	0.3 ± 0.2	2.5	2.8	2.6 ± 0.2
B. JH Families				
JH1	0.2 ± 0.05	1 ± 0.2	1 ± 0.1	
JH2	2.1 ± 0.4	2.5 ± 0.1	2.9 ± 0.5	
JH3	10.2 ± 0.5	14.1 ± 2.2	15.5 ± 1.2	
JH4	41 ± 2.3	42.1 ± 1.8	40.7 ± 1	
JH5	34.6 ± 2	16.1 ± 0.8	18.5 ± 1.8	
JH6	11.9 ± 0.8	24.2 ± 0.8	21.4 ± 1.3	

(A) The mean percentage of VH utilization determined by real-time PCR for B cells from donor 789 and 743. For donor 743, this determination was done separately on the two samples W and Z, as displayed. Samples W and Z come from the same sample containing 5.4×10^6 cell IgM, it was divided into two equal shares. The quantifications are carried out separately on each sample, the averages and the standard deviations are calculated. (B) JH segment usage in association with the VH5 gene family. VH5 gene family, JH1 and JH2 gene segments are highlighted.

differences but, in both of them, VH3 and VH4 represent the majority of all rearrangements (60–70% and 13–20%, respectively). This fits the complexity of the VH3 and VH4 families, which contain 27 and 10 genes, respectively. These results agree with the data obtained by single-cell PCR (54% of VH3 and 23% of VH4 rearrangement out of 491 analyzed cells) (21, 32). The dominant VH segment was always VH3b followed by VH4 and VH3a for each isotype (IgM, IgG, IgA and IgE), Fig. 1A.

We then quantified the JH usage for the VH5 family, which we selected because it is not overused (Table 3A) and because it contains only two genes (VH5 and VH5-S1). For JH quantification, the VH5-C μ amplification products from the two donors were subjected to a second PCR amplification using the VH5int primer (specific for the two VH5 genes) and each of the six JH-specific primers, respectively. The TM-MGB-VH5int fluorescent probe, nested in the vicinity of the VH5int primer and specific for the VH5 genes, was used for quantification (see Methods and Table 2). The reproducibility was assessed by measuring two samples of the same donor. When two donors were compared, the patterns were similar. JH4 was overutilized, but there were significant variations (Table 3B).

The extrapolation from the distribution of transcripts in the IgM-bearing B cells to the distribution of genes is based upon the assumption that genes in the various B cells are equally transcribed, a point which is discussed below. For the rest of this study, we focused on the least abundant rearrangements JH1 and JH2 in order to facilitate sequencing studies.

Immunescope profiles of human peripheral blood IgM+ B lymphocytes

As for T cells, the cDNA amplification products were submitted to a run-off reaction, which records the size of the CDR3

regions (30). For B cells, primers specific for the various constant regions can be used in order to analyze and compare the various isotypes. A sample of profiles obtained for IgM, IgG, IgA and IgE, using a variety of VH primers, is displayed in Fig. 1(B). The patterns are often quite different, the IgM profiles being almost always very regular with as many as 20–25 size peaks. In contrast, IgG, IgA and IgE profiles are usually irregular and often dominated by a few peaks, sometimes over a regular 'background'.

The most plausible interpretation of these observations is that IgG, IgA and IgE patterns depart from regularity for two convergent reasons. One is that clonal expansions impact B-cell distributions as they do for T cells. The other is that, contrary to T cells, activated B cells synthesize very large amounts of specific mRNA (a B-cell blast-secreting IgG makes 1000 more mRNA than the corresponding resting B cell). But previous studies (33) have demonstrated that human circulating plasma cells (CD38 high cells) obtained from healthy donor showed very low numbers of cells (<0.003% of blood mononuclear cells).

Other groups have studied the diversity of heavy-chain rearrangements at the DNA level. The profiles of CDR3 sizes thus generated are, in many instances, similar to the ones shown here. In particular, the IgM profiles are very much alike. This supports the above statement that, for IgM, the transcriptional patterns reflect the gene patterns. For other isotypes, the more contrasted profiles are likely to reflect the fact that transcriptional activation, when cumulated with clonal expansion, over-increases the size of certain peaks.

Compared with T cells (24), IgM B-cell profiles differ in two main characteristics: (i) the number of size peaks is much larger (up to 25 as opposed to up to 12) and (ii) the mean size peak is close to 13 amino acids per CDR3, instead of nine for T cells. As for T cells, most patterns display a regular distribution, often Gaussian or Gaussian like (showing a certain degree of asymmetry). This agrees with the previously reported profiles of mouse splenic B cells (34). Nevertheless, irregular patterns are occasionally observed with the smallest VH families (e.g. VH2, VH6 and VH7 for donors 743 and 789) (Fig. 2A).

As with T cells, the resolution power can be increased by profiling specific VH–JH pairs within a given VH–CH-amplified cDNA subset. An example is shown in Fig. 2B, where VH5–JH2 and VH5–JH1 patterns are displayed. As observed with T cells, the patterns tend to become less regular as minor subsets are profiled. This may be interpreted to mean that, as they contain fewer members, they are statistically less 'buffered' than more abundant subsets. With the Immunescope software, the surface of each peak can easily be quantified, and we demonstrated earlier that, as expected, the surface is proportional to the number of transcripts reflected in the peak. For example, the eight-amino acid CDR3 peak in the VH5–JH2 profile of donor 789, which we shall use below, represents 1.84% of all VH5–JH2 rearrangements.

Estimation of the number of distinct peripheral blood VH5-expressing IgM+ B cells

We next followed the same approach which we used for T cells, that is to extensively sequence selected size peaks in given V–J combinations and then to extrapolate back to the entire repertoire (24). Numerous controls have been made to validate this

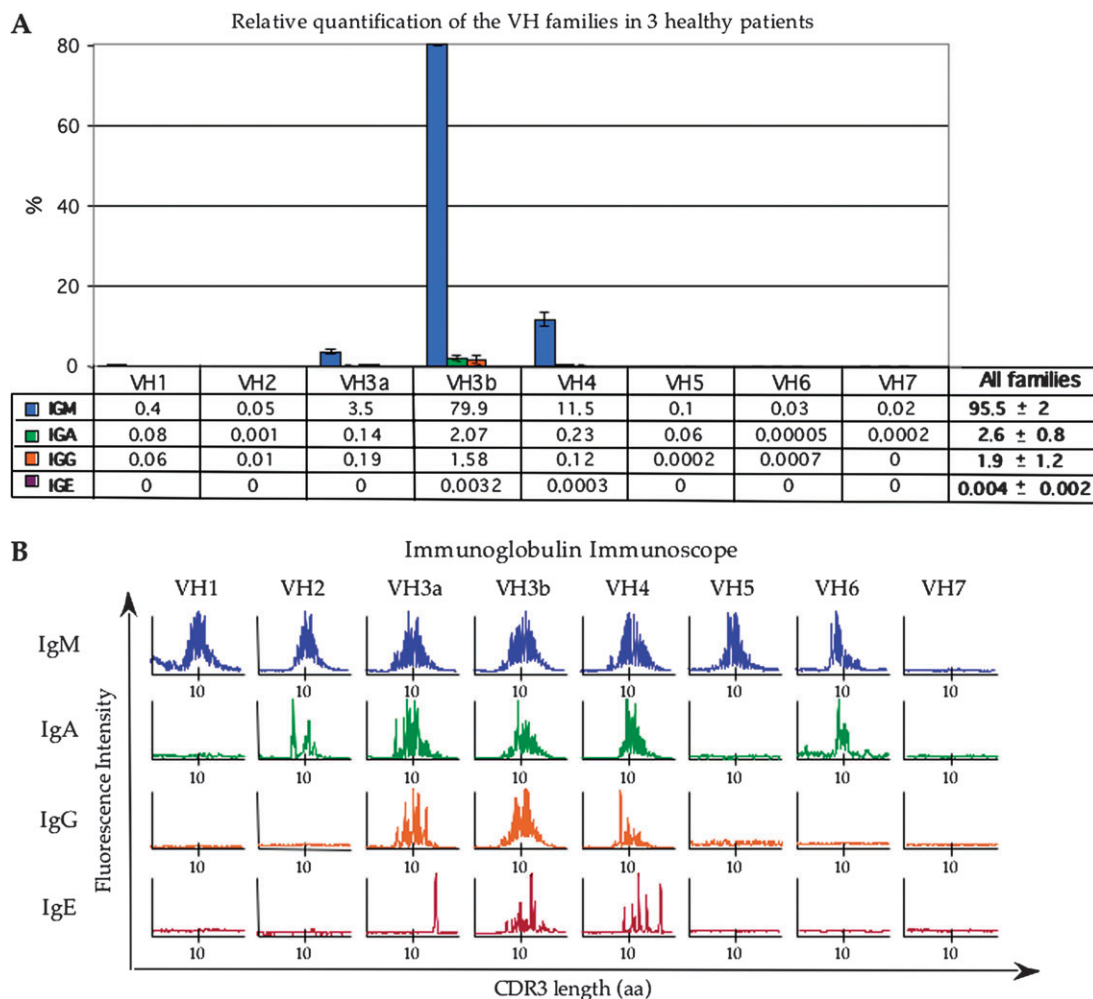


Fig. 1. Quantification of VH families and immunoscope profiling for IgM, IgA, IgG and E. (A) Sample from three healthy donors was subjected to VH family-specific real-time PCR amplification as detailed in Methods using specific primers for the constant region (CH μ , CH α , CH γ or CH ϵ) with one of eight primers covering the different VH1–7 chains. IgM transcripts represented the majority of Ig transcripts ranging from 95 to 98%. (B) PCR amplification was followed by a run-off reaction with specific fluorophores-labeled probes for the constant region (CH μ , CH α , CH γ or CH ϵ).

experimental design with T cells. Only the most critical one, namely the parallel analysis of two identical samples from the same donor, will be reported in detail. Given the large number of different sequences, which are to be anticipated, it is practical to focus on VH and JH gene segments that are poorly utilized. As measured earlier by quantitative PCR, VH5–JH1 and JH2 fulfill this requirement (VH5: 3.2% of all VH; JH1: 0.2% and JH2: 2.1% of all VH5 rearrangements) (Table 3). In addition, the VH5–JH1 and VH5–JH2 profiles of donor 789 were quasi-Gaussian, and only slightly perturbed by a few presumptive clonal expansions (activations).

The JH1 usage among VH5 rearrangements in donor 789 is such that it turned out possible to achieve exhaustive sequencing of the whole VH5–JH1 subset (within the VH5–C μ one) without cutting out a size peak to further decrease the complexity (see below). Once, ~1000 sequences were acquired, a quasi-plateau of 346 distinct sequences (Table 4) was reached as further sequencing did not bring new ones (a mathematical treatment of the data is described in ref.

25). Similarly, in donor 743, where VH5 and JH1 usage are slightly different, a quasi-plateau of ~230 sequences were reached after ~650 sequences were acquired (Fig. 4A).

These two figures were surprising to us because the estimated numbers of VH5–JH1-expressing IgM+ B cells in the two samples were only slightly higher. In donor 789, the VH5–JH1 subset represents $(3.2 \times 10^{-2}) \times (0.2 \times 10^{-2}) = 6.4 \times 10^{-5}$ of total (Table 3), that is 640 cells in the 10^7 B-cell sample. In donor 743, the VH5–JH1 subset represents $(1.1 \times 10^{-2}) \times (1 \times 10^{-2}) = 1.1 \times 10^{-4}$ of total (Table 3), that is 240 cells in the 2.2×10^6 B-cell sample. These 640 and 240 cells in donors 789 and 743 have to be compared with 346 and 230 distinct sequences, respectively.

We next used another, more represented JH segment, namely JH2 and analyzed the VH5–JH2 subset in the entire B-cell population, irrespective of the expressed isotype. We, therefore, PCR amplified cDNA from donor 789 with a VH5 primer and a JH2-specific primer. A run-off reaction with a JH2 fluorescent primer was performed and the size

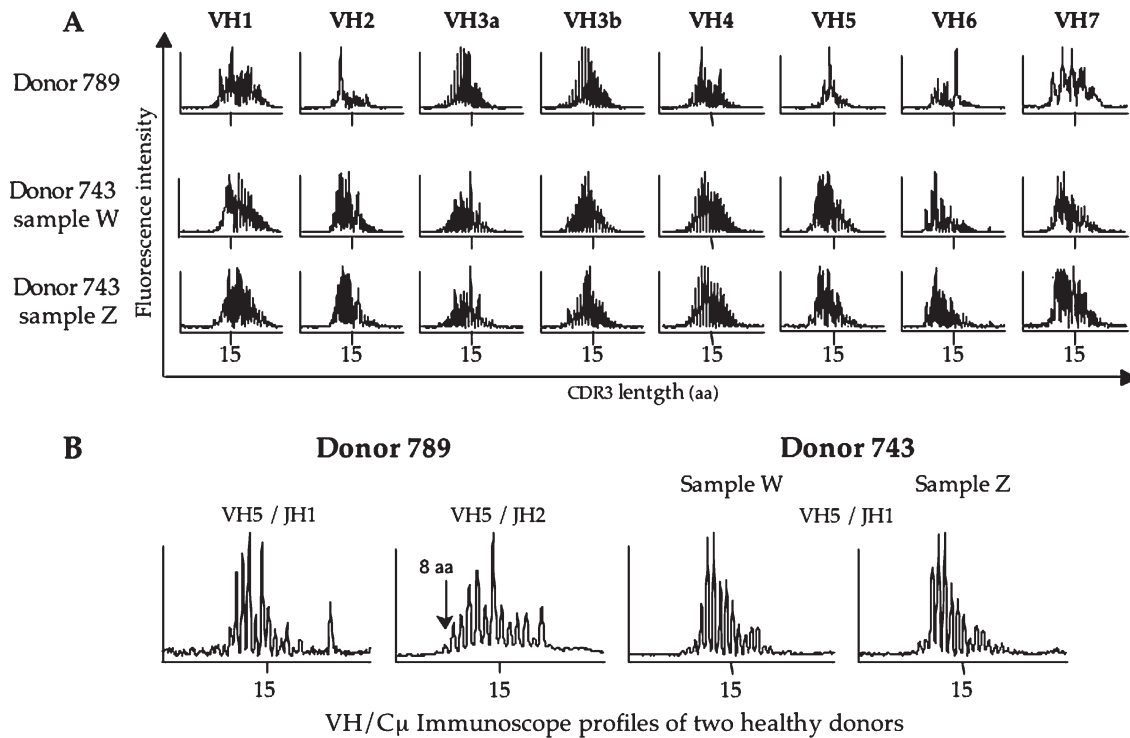


Fig. 2. Immunoscope profiles from two healthy donors. The IgM, IgA, IgG and IgE immunoscope profiles were obtained as described in Methods. (A) Purified B cells from two healthy donors, 789 and 743, were subjected to VH family-specific PCR amplification as detailed in Methods using VH-specific primers (see Table 2) and C μ primer, followed by a run-off reaction with a C μ -Fam probe. For donor 743, immunoscope profiles were obtained from two separate samples, samples W and Z, harboring the same initial number of B cells. (B) Immunoscope profiles were realized for two given rearrangements, VH5-JH1 and VH5-JH2. For both 789 and 743 donors, VH5-JH1 rearrangements have been chosen as prototype rearrangements for diversity determination and subjected to exhaustive sequencing. In addition, purified CDR3 band from the VH5-JH2 rearrangement of donor 789 (shown by arrow) was also used for the same purpose. This purified band had a CDR3 length of eight amino acids.

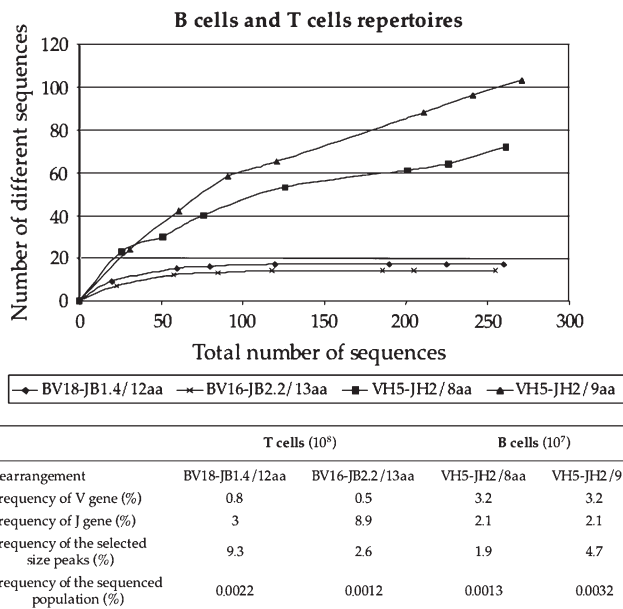
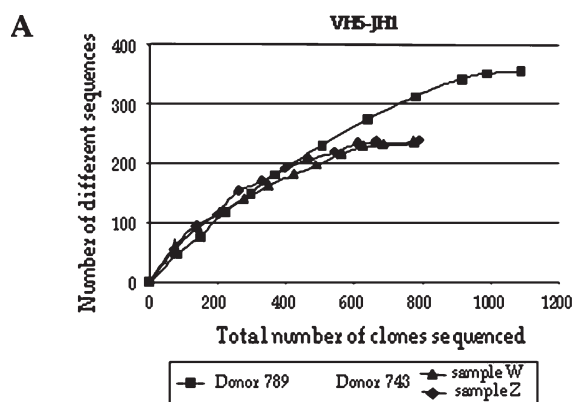


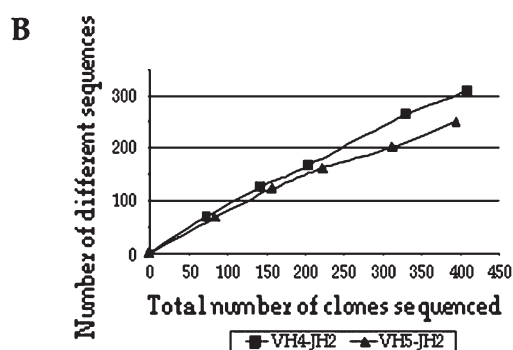
Fig. 3. Comparison of sequence acquisition in T- and B-cell subsets of similar sizes. The number of different sequences (in ordinates) is plotted against the total number of sequences acquired. For T cells, data originate from ref. 24.

pattern was displayed (Fig. 2B). The total number of individual sequences included in that profile was too high to allow direct sequencing. The eight-amino acid CDR3 band was, therefore, cut-out, re-amplified (with the VH5int and JH2 primers, see Methods) (note that this experiment, alike the above ones, involves a total of two PCRs, which, as we experienced in our T-cell studies, do not distort significantly the sequence distributions). When the eight-amino acid band was sequenced, a quasi-plateau of 72 sequences was reached after some 261 sequences. Given the relative area of eight-amino acid band in the profile (1.84%), an elementary calculation allows to extrapolate back to 3900 distinct VH5-JH2 sequences in the sample studied. Two other bands (corresponding to 9- and 17-amino acid long CDR3s) were purified and analyzed with similar results.

These figures have to be compared with the estimated number of VH5-JH2-expressing B cells, irrespective of their isotype, present in the sample of 13.9×10^6 cells, namely 9300 cells. Therefore, any given individual sequence is, on the average, present two to three times. It must be noted that this mixture of B cells contains a number of B-cell blasts, which are both, activated and expanded. Their presence can be read out in the sequences because many bear somatic mutations. Once sequences with somatic mutations are set apart, figures with total B cells become close to those obtained with IgM+ B cells. It seems likely that other



Donors : Bcells (1-3.10 ⁷)	789	743	
		Sample W	Sample Z
Rearrangement	VH5-JH1	VH5-JH1	VH5-JH1
Frequency of V gene (%)	3.2	1.1	1
Frequency of J gene (%)	0.2	1	1
Frequency of the sequenced population (%)	0.006	0.01	0.01



	Bcells (10 ⁷)	
Rearrangement	VH4-JH2	VH5-JH2
Frequency of V gene (%)	20.2	3.2
Frequency of J gene (%)	5.4	2.1
Frequency of the sequenced population (%)	1.1	0.07

Fig. 4. (A) Exhaustive sequencing corresponding to a subset of VH5-JH1-expressing IgM+ B cells of donor 743 and donor 789. Individual sequences are indeed found several times and further sequencing reveals no new one. (B) Comparison of sequence acquisition in VH5 and VH4 subsets of B cells. The number of different sequences (in ordinates) is plotted against the total number of sequences acquired. See text for explanation.

JH segments would yield similar results to JH1 and JH2. Therefore, the data suggest that in naive B cells expressing VH5, each individual sequence is present about once or twice in the sample. The analysis of the somatically mutated sequences will be reported in another publication and will not be discussed further.

Sizing the VH5 B-cell repertoire in the entire blood

In samples of a limited size (typically the equivalent of 400 ml of human blood), the size of the clones of individual VH5 B cells, characterized by a unique VH sequence, is in the order of one to two. Can one extrapolate this conclusion to 5 l of blood? For the entire blood, the clone size must lie

between two extremes: close to 1 (option A) or close to 10 (option B).

The mere analysis of the Poisson distributions of sequences cannot convincingly discriminate between the two hypotheses, especially since the occurrence of a number of relatively abundant and/or somatically mutated sequences (corresponding to activated or/and expanded B-cell clones) complicate the issue. A better and more direct answer is obtained by analyzing two samples of the same donor with the same volume in order to evaluate the degree of redundancy. If sequences are highly redundant, hypothesis B will prevail. If there is little redundancy, hypothesis A will be strongly favored.

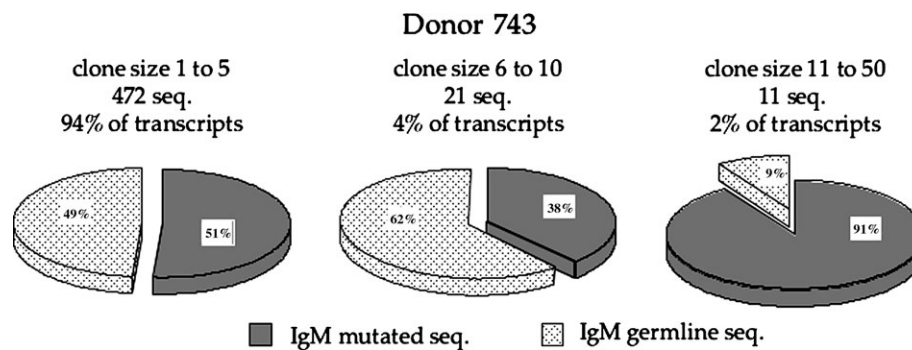
Two samples (W and Z) containing 2.7×10^6 IgM+ B cells (one-sixth of all purified B cells from donor 743) were subjected to amplification with VH5 and C μ primers, and then to a second amplification with VH5 and JH1 primers (as above). The size patterns were identical. Exhaustive sequencing involved 655 and 675 sequences and yielded 232 and 228 distinct sequences, corresponding to a subset of ~ 255 VH5-JH1-expressing IgM+ B cells, estimated, as above, from the VH5 and JH1 usage of the donor (Table 3). On 467 analyzed distinct sequences (in W and Z samples), 37 sequences are common showing an overlap of 8%. We calculated the diversity of donor 743 based on the summation of the two samples (W and Z), and obtained a diversity of 4.4×10^6 rearrangements for a sample of 5.4×10^6 B cell IgM. These results are entirely consistent with those obtained in the previous sections.

In the sequencing process, individual sequences are indeed found several times, and exhaustion is claimed when further sequencing reveals no new one. To calculate the global contribution of somatic mutations, we compared all the VH sequences obtained from exhaustive sequencing of the different rearrangements to the germ line VH5, irrespectively of the CDR3 sequences. Results are shown in Table 4 and Fig. 5. For both donors and for both rearrangements (VH5-JH2 and VH5-JH1), approximately half of the VH sequences bear one or several somatic mutations. To analyze the data, of donor 743, we classified individual sequences into three categories: those found 1-5 times, 6-10 times and 11 times or more. If we analyze the sequences obtained at the beginning of the 'plateau' (Fig. 4A), we observe that out of 504 different sequences analyzed, 94% of the transcripts are accounted for one to five times and half of the sequences are germ line (probably corresponding to the naive IgM B cells) and other half carrying somatic changes which probably correspond to population CD27 memory B cells as previously described (35). Four percent of the transcripts are accounted for 6-10 times and only 11 sequences are represented between 11 and 50 times, 91% of these last are mutated. The simplest interpretation is that apart from a few individual sequences which correspond to expanded and/or activated clones, most sequences are indeed distinct in the two samples. Therefore, the data favor hypothesis A and support the conclusion that, in the entire blood, for the VH5-JH1 subset and more generally for the VH5 family, the clone size is close to 1. It may be emphasized that, when a similar experiment was performed with mouse T cells, for which the individual clone size is ~ 10 ,

Table 4. Estimation of the number of distinct peripheral blood VH5-expressing IgM+ B cells

Rearrangement	Donor 789		Donor 743 VH5-JH1-CM	
	VH5-JH2	VH5-JH1-CM	Sample W*	Sample Z*
Amplified isotype	All	IgM		IgM
CDR3 length	8 aa	All peaks		All peaks
Number of B cells after purification ($\times 10^6$)		13.9		8.4
Number of IgM* cells ($\times 10^6$)		10.8		5.4
Total number of sequences analyzed	261	981	655	675
Number of different sequences	72	346	232	228
Size of CDR3 repertoire ($\times 10^6$)	5.8	5.4	2.1	2.4
Frequency of mutated sequence	42%	24%		55%
Size of sample total repertoire ($\times 10^6$)	10	6.7		4.4
Clone size		-1		-1

Global estimate of B-cell diversity. For donor 789, two re-arrangements were studied. The VH5-JH2 rearrangement is a representative of the entire B-cell population, irrespective of the expressed isotype, while VH5-JH1 only allows the calculation of IgM+ VH5 diversity. For donor 743, only the VH5-JH1 rearrangement was studied, but twice on two samples (W and Z) containing 2.7×10^6 IgM+ B cells. The diversification factor due to mutations in the VH genes and the global clone size are highlighted.

**Fig. 5.** Rate of mutated sequences. We analyze the mutated sequences of 504 individual sequences classified into three categories, those found 1–5, 6–10 and 11 times or more.

a 50% redundancy was found (25), confirming that the absence of overlap is meaningful.

Data obtained with the VH4 family

The above data were all acquired with the VH5 family which represents ~3% of all B cells. It is, therefore, a minor family, which we chose precisely for that reason. Figure 3 illustrates the difference between B-cell and T-cell analyses. In the previously published experiments involving human T cells and demonstrating that their average clone size is in the order of 50, we extracted sequencing data obtained with subsets of a size similar to the subsets of B cells used here. It is seen that, with T-cell subsets, plateaus are reached after ~100 sequences. For B-cell subsets, the plateaus are not seen because they are reached when 700–1000 sequences are accumulated (Fig. 4A).

While Fig. 3 clearly confirms the difference between B-cell and T-cell subsets, it also emphasizes the practical limitation which we faced in extending our analysis to more abundant VH families. It would require thousands and even tens of thousands of sequences, an undertaking which was out of reach for us. We nevertheless approached the question with the following experiment.

The VH4 family is much more abundant (~20% in donor 789) than VH5 (~3%). We sequenced ~400 VH4-JH2 rearrangements, and the curve of acquisition of distinct sequences was compared with that of VH5-JH2 rearrangements. Figure 4 shows that the two curves are quite similar. Comparing data shown in Figs 3 and 4, it can easily be deduced that, if the clone size in the VH4 family was in the order of 50, as it is for T cells, the curves would have diverged. This allows us to conclude that the clone size in the VH4 family cannot be high, but does not permit to demonstrate that it is close to 1. The mathematical analysis of the available part of the curve (according to the treatment described in ref. 25) agrees with the conclusion of a small clone size, possibly close to 1, but provides in our view an indirect argument, weakened by the small number of available sequences.

Discussion

In this report, we have adapted the immunoscope methodology, which was initially developed for the analysis of mouse and human T cells, to the study of the VH repertoire of human B cells. Quantification of VH and combined VH-JH usage was performed in a couple of donors, and the profiling

of IgM-, IgG-, IgA- and IgE-expressing B cells has been shown.

We were keen to learn whether the diversity of VH chains in B cells would match that of the V-beta chains of T cells. To approach this question, we analyzed in more detail the VH5 family and were surprised to find that, in all samples studied with two donors and two subsets (VH5-JH1 and VH5-JH2), the number of cells only marginally exceeded the number of individual VH rearrangements identified by sequencing. Since this was observed with two JH segments, we assume that this holds for all therefore, in those samples, the clone size is close to 1. The largest sample which we analyzed was the equivalent of 500 ml of blood, i.e. ~10% of PBL, so that in the VH5 family, the average clone size in total PBL cannot exceed 10. We next showed that the processing of two independent samples from the same donor yields very little overlap in the individual sequences, once a number of sequences presumably contributed by activated and/or expanded clones have been removed. This strongly suggests that in total blood, the clone size is also close to 1. Our experiments have not been performed with purified resting B cells, but the data show that even if activated B cells were not subtracted other isotypes, the clone size would remain quite small, around two. We shall keep this conservative figure for discussion.

First, if rearranged VL chains are associated with the rearranged VH5 chains, our result implies that they only have a marginal quantitative contribution to the diversity of VH5-expressing B cells since the latter is already close to maximum. This would be in sharp contrast with what we know about human alpha-beta T cells since in the latter case an average of ~25 distinct V-alpha chain associate with each rearranged V-beta chain, thus increasing the diversity considerably (24).

However, one may also wonder whether we have been dealing with B cells which express surrogate light chains (36), a subset of B cells with possible self-reactivity. However, these V-pre-B⁺L⁺ B cells represent only 0.5–1% of circulating B cells (37). They include a significant proportion of VH5-expressing B cells, but VH1, VH3 and VH4 are also used. Since VH5-expressing B cells represent ~3% of total, it is likely that only a minor fraction belongs to the V-pre-B⁺L⁺ B-cell subset, especially as certain biases in JH6 usage and CDR3 size found in the latter have not been observed here. Furthermore, antibodies using VH5 are commonly found in antibody databases, and trees of somatically mutated VH5 chains can be identified in the bulk of VH5 sequences (data not shown), while immunoscope profiles reveal no qualitative differences between VH5 and other, more abundant, VH5 families (Figs 1 and 2 and data not shown). Therefore, apart from its relatively low usage, we are not aware of a feature that would discriminate VH5 from the other families.

Such a feature may, however, exist. For example, it could be argued that VH5 does not pair properly with surrogate light chains. Pre-B cells using VH5 could differentiate but would not be clonally expanded. One could also imagine that the size of the various families is regulated by some kind of process, for example positive selection, which would increase family size by increasing the number of different clones, the size of the clones or both. For practical reasons (the amount

and cost of required sequencing), we could not address this issue directly. Our limited inspection of the VH4 family which is seven times more abundant (20%) than VH5 shows high diversity because out of 400 VH4-JH2 sequences performed, >300 were different in a sample estimated to comprise 1680 cells. These data rule out a large size clone in the range of 50 as found for alpha-beta cells (and even 10) but not prove that is as small as in the VH5 family. Whether the entire peripheral naive B-cell repertoire is characterized by a very small clone size, thus appears as a possible but somewhat speculative hypothesis. If that generalization happened to be valid, it would raise several interesting issues.

First, irrespective of somatic mutations, the diversity of B cells would be close to maximum. May be this is one way to optimize the chances of recognizing foreign antigen, being granted that somatic diversification and affinity maturation can follow. Second, the VH usage is so high that the light chains would not add much to IgM+ B-cell diversity, even if, obviously, they would contribute to specificity. Third, what about the ontogeny of human IgM+ B cells in the human bone marrow and the selective constraints which they undergo in and out of the bone marrow? Although several VL chains might get associated with one VH chain, the finding that only one combination bearing the VH signature is found in the periphery might simply be the consequence that only a small fraction of B cells produced in the bone marrow reaches the periphery. Do only a few cell divisions (or none) take place in the bone marrow prior to antigenic selection? All these issues would require further investigations.

It should be emphasized that the situation in other species, particularly the mouse, might be different. Indeed, the mouse and human T-cell repertoires are not completely similar in structure (24, 25).

As an answer to the question which we initially asked, our results suggest that the human B- and T-cell repertoires differ in several ways. If the small clone size assigned to the VH5-expressing IgM+ B cells is unique to this subset, there is no obvious equivalent within alpha-beta peripheral T cells. If the observation can be generalized to the entire repertoire of B cells in the blood, and since the diversity of T-cell repertoire is in the order of 2.5×10^7 , the B-cell repertoire would be four or five times more diverse. The average clone size for T cells was estimated to be in the order of 50, as opposed to 1, or a few, for B cells. We showed that each TCR V-beta chain would pair, on the average, with 25 distinct V-alpha chains. Thus, the diversity of V-beta chains would be lower than that of VH chains, and V-alpha chains are a major contributor to diversity, while VL chains would not. Ontogeny and selection processes are quite different in thymus and bone marrow and involve recognition constraints of standard antigens with respect to peptide-MHC complexes that are quite different. Also, the data would not support the 'Protecton theory' proposed by Langman and Cohn some 20 years ago, and according to which that B-cell repertoire could be divided into identical sub-fractions or functional units composed of some 10^7 B lymphocytes bearing 10^5 rearrangements (38). The structure of the T-cell repertoire would be more compatible with their hypothesis, but we earlier found that the clone size is ~50-fold higher in man than in mouse (24, 25). Therefore, even for T cells, there is nothing alike a universal protecton module.

Finally, the quantitative approach used here is applicable to the analysis of many pathological situations in which B-cell repertoires are perturbed and may hopefully contribute to clinical research. Data obtained with atopic disease (39, 40) and autoimmune lymphoproliferative syndrome will be reported elsewhere.

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Abbreviation

CSR class switch recombination

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12 Human B lymphocyte clone size and repertoire

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