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Frequent occurrence of identical heavy and light chain Ig rearrangements

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Abstract

Single-cell PCR analyses of expressed Ig H and L chain sequences presented here show that certain rearrangements occur repeatedly and account for a major segment of the well-studied repertoire of B-1 cell autoantibodies that mediate the lysis of bromelain-treated mouse erythrocytes, i.e. antibodies reactive with phosphatidylcholine (PtC). We repeatedly isolated at least 10 different types of V_H region rearrangements, involving three distinct germline genes, among FACS-sorted PtC-binding B-1 cells from three strains of mice (C57BL/6J, BALB/c and C.B-17). The predominant rearrangement, V_H11–DSP–J_H1 (V_H11 type 1), has been previously found in anti-PtC hybridomas in several studies. We show that within each of six mice from two strains (C57BL/6J and BALB/c), unique instances of IgH/IgL pairing arose either from different B cell progenitors prior to IgH rearrangement or from pre-B cells which expanded after IgH rearrangement but prior to IgL rearrangement. Together with other recurrent rearrangements described here, our findings demonstrate that clonal expansion of mature B cells cannot account for all repeated rearrangements. As suggested by initial studies of dominant idiotype expression, these findings confirm that clonal expansion is only one of the mechanisms contributing to the establishment of recurrent rearrangements.

Introduction

Cells expressing identical Ig V_HDJ_H or V_LJ_L rearrangements are frequently treated as originating from a common B cell, and identity of H or L chain rearrangements, alone or as pairs, are taken as markers of clonality (1–5). This common use of Ig structure as an index of clonality assumes that identical rearrangements are extremely rare and therefore that the repeated isolation of the same rearrangement from a given animal is often due to the clonal expansion of a single rearranged B cell.

This assumption is reasonable given that V(D)J recombination confers a vast potential for diversity of the antibody repertoire. One of multiple variable (V_H and V_L) segments combines with one of several D_H and $J_{H/L}$ segments to encode the IgH and IgL variable regions of the expressed antibody molecule. Furthermore, the gain of nucleotides, either template-dependent or -independent, and the loss of nucleotides at the coding junction(s) adds still more diversity to the Ig variable region coding sequence. These processes amplify the potential for expressing a great number of different Ig molecules [10^{18} possible combinations (6)] and hence for generating a population of B cells producing a highly diverse set of antibodies. Thus, independently derived B cells should rarely produce identical antibody molecules, even within the same antibody response (7).

Limited IgH and IgL gene usage, however, is common in immune responses to certain antigens, e.g. α(1–3)dextran, *p*-azophenylarsonate, phenyloxazolone, (4-hydroxy-3-nitrophenyl)acetyl (NP), phosphorylcholine and phosphatidyl-

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choline (PtC), the antigen studied here (8–16). Several studies have shown that the IgH and IgL of anti-PtC antibodies produced by independently isolated hybridomas and neoplasms tend to be encoded by $V_H11–V_\kappa9$ or $V_H12–V_\kappa4$ (9–11,17–23). These anti-PtC antibodies lyse mouse erythrocytes treated with bromelain to expose PtC (a common phospholipid constituent of cell membranes). In normal animals, anti-PtC antibodies are secreted in response to lipopolysaccharide stimulation and are produced exclusively by cells of the B-1 lineage (24,25).

B-1 cells producing PtC-binding surface IgM are readily detectable in the peritoneal cavity and spleen of unstimulated mice by FACS analysis with fluorescein encapsulated in PtC-liposomes (24). In most mouse strains, PtC-liposome-binding cells represent 0.1–0.3% of splenic B-1 cells and 9–25% of peritoneal B-1 cells [where B-1 cells represent 1 and 60% of total splenic and peritoneal lymphocytes respectively (25,26)]. No other specificity has been reported to be shared by such a large number of B cells in unimmunized, normal, nude as well as germ-free mice (27). The actual endogenous or exogenous antigen selecting PtC-binding B-1 cells is unknown.

The anti-PtC repertoire, like the overall B-1 antibody repertoire, is established early in life. *De novo* B-1 development, particularly the development of the $CD5^+$ B-1 (B-1a) cells studied here, terminates after mice reach ~1 month of age. After this time, B-1a cells persist solely by self-replenishment. Thus, it has been suggested that the restricted set of IgH and IgL genes previously identified as a dominant component of the anti-PtC repertoire may be largely due to preferential rearrangement of germline elements, preferential survival of pre-B cells, and of antigen-driven selection and expansion of a relatively small number of B cells (3,28).

Certain V region gene assemblies of anti-PtC antibodies have been repeatedly isolated from different mice (4,10,19, 22,23,29). Collectively, these earlier studies raised the question of whether identical anti-PtC IgH rearrangements are derived from a common progenitor or from multiple progenitors which develop into a limited PtC repertoire (3,9–11,19,24,25).

Various mechanisms may explain how the same rearrangement occurs frequently enough to be isolated so routinely. For example, sequence homology at coding junctions or other mechanistic biases may operate to favor rearrangement of certain combinations of germline gene elements (28,30–34). These considerations, which support a model in which rearrangement mechanism limits diversity, also predict a smaller role for clonal expansion in the development of the adult PtC repertoire.

The FACS-based single-cell PCR studies presented here test this and related hypotheses concerning the origin and structure of the expressed anti-PtC repertoire. Our work extends earlier studies of various antigen responses characterized by dominant idiotypes initially defined by anti-idiotypic antibodies (35), and subsequently by sequencing the involved $V_{H/L}$, D_H and $J_{H/L}$ gene segments (15,16). We demonstrate that at least 25%, and in some cases as many as 60%, of the cells producing anti-PtC antibodies using the same V_H region sequence within a given mouse cannot be explained by clonal expansion. Furthermore, we show that the frequency of these repeatedly isolated IgH sequences is genetically controlled in that it is very similar among PtC-liposome-binding B-1 cells from animals of the same strain but varies from strain to strain. Thus we show by single-cell analysis, evidence for the generation of identical IgH and IgL rearrangements in individual unmanipulated mice by a mechanism other than clonal expansion of mature B cells. We also, for the first time, correlate IgH and IgL sequences with the surface markers expressed by single PtC-binding B cells.

Methods

Cells and animals

Peritoneal cells from four C57BL/6J (C57) (IgH-Cb) female mice at 6 months of age, two separate pools of BALB/cHz (IgH-Ca) female mice at 5.5 months of age [nine BALB/c mice (experiment 1) and five BALB/c mice (experiment 2)] and two female C.B-17 mice at 5.5 months of age were used in this study. Individual mice were studied from C.B-17 and C57 strains to determine more accurately the repertoire of the populations present. Mice were bred and housed at the Herzenberg animal facility, Division of Laboratory Animal Medicine (Stanford University).

FACS reagents and staining

Single-cell suspensions, stained cells and FACS reagents were prepared as described previously (36). FACS reagents consisted of phycoerythrin-conjugated anti-IgM (331.12), allophycocyanin-conjugated anti-CD5 (Ly-1, 53-7) and fluorescein-encapsulated in liposomes as first described by Mercolino *et al.* (24,25) (see below). Dead cells were excluded with propidium iodide monitored in the Texas Red channel. After staining, cells (2.5×10^8/ml) were incubated with 10 ng/ml RNase (Gibco/BRL, Life Technologies, Gaithersburg, MD) for 30 min on ice and then diluted 10-fold (2.5×10^7/ml, 1 ng/ml RNase) for sorting.

Cells were analyzed and sorted on 'Flasher', an extensively modified dual laser (488 and 595 nm excitation) FACS II (37) (Becton Dickinson, Mountain View, CA) interfaced with a VAX 6300 computer (Digital Equipment, Maynard, MA) running FACS/Desk software (W. Moore, Stanford University). Machine calibration was accomplished using standard fluorescent polystyrene microspheres (Spherotech, Libertyville, IL). Machine calibration and fluorescence compensation were done with FACS/Shiva software (M. Bigos, Stanford University).

Preparation of liposomes

Liposomes were prepared using a modification of previous methods (24,25,38,39). Distearoyl PtC, distearoyl phosphatidylglycerol (both Avanti Polar Lipids, Birmingham, AL) and cholesterol (Sigma) were mixed at a molar ratio of 45:5:50 in chloroform:methanol 4:1 until material was dissolved. The lipid mixture was completely dried in a round-bottom flask by rotary evaporation. The lipid was hydrated at 65°C with a buffer consisting of 50 mM sodium acetate, 50 mM sodium chloride, 1 mM EDTA, containing 5 mM fluorescein sulfonic acid (FSA) (Molecular Probes, Eugene, OR) at a final lipid concentration of 50 mM. After overnight hydration, the multi-

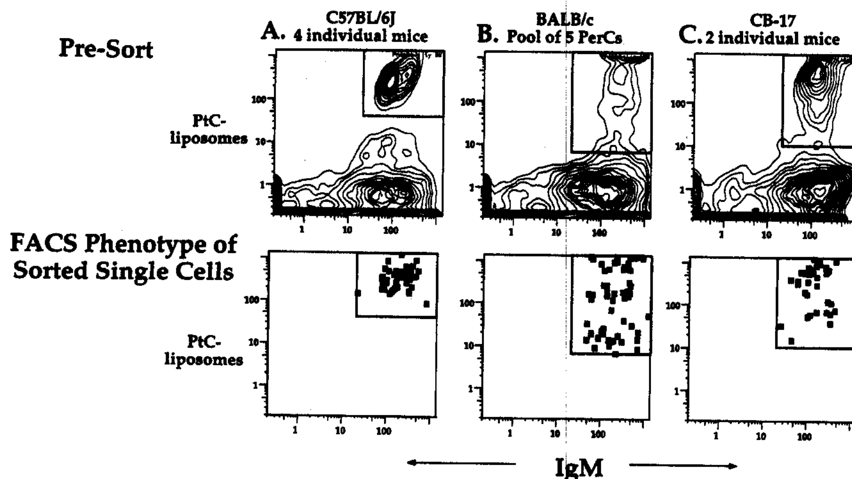

Fig. 1. Single-cell sorting of PtC-binding B-1 cells. PtC-binding cells were identified by FACS using fluorescein encapsulated in PtC-liposomes and surface IgM staining. PtC-binding cells were sorted from four C57 mice (A), a pool of peritoneal cells from nine BALB/c mice (experiment 1), a pool of peritoneal cells from five BALB/c mice (B) (experiment 2) and two C.B-17 mice (C). Gates used for sorting are indicated. The typical FACS profile for a mouse (mice) from each strain is shown. PtC-liposome-binding cells were first bulk sorted, based on size, viability, and expression of PtC-liposomes and IgM. Single cells were sorted into lysis solution and snap frozen on dry ice for future analysis. Each square represents a single sorted cell. Among C57 mice, 93 ± 2% of PtC-liposome-binders are B-1a cells (CD5$^+$). On average, 80% of BALB/c PtC-binders are CD5$^+$ and 72% of C.B-17 PtC-binders are CD5$^+$ (data not shown).

lamellar vesicles were vortexed and freeze–thawed five times (liquid nitrogen/water bath at 65°C) to form oligolamellar vesicles. This preparation was then extruded through capillary-path polycarbonate filters (Nucleopore, Pleasanton, CA). The extrusion was facilitated by a high pressure vesicle extruder (HPVE-10; Sciema Technical Services, Richmond, BC). The entire apparatus was placed in a water bath at 65°C. The liposome preparation was extruded five to 10 times each through filters of sequentially smaller pore sizes (0.4, 0.1 and 0.5 μm). The material was removed from the extruder and allowed to cool to room temperature. FSA not incorporated into the liposomes was removed by gel filtration on a pre-equilibrated Sepharose CL-6B column, and eluted with a buffer of 50 mM MES, 50 mM sodium chloride and 1 mM EDTA, pH 6.0. The PtC-liposomes were titrated on peritoneal cavity cells from BALB/c and C57 mice (used at a final lipid concentration of ~40 μM per 6×10^5 peritoneal cells).

Single-cell sorting

Sorting conditions for single-cell analysis have been described previously (40,41). All populations were first bulk sorted and then reanalyzed to check purity. PtC-liposome-binding cells from either C57, BALB/c or C.B-17 mice were sorted based on PtC-liposome$^+$IgM$^+$ fluorescence staining. Reanalysis demonstrated an average of 81.1 ± 1.3% purity for the four individual C57 mice, 76% purity for the peritoneal cells sorted from BALB/c mice, and 98.7 and 79.4% purity for two individually sorted C.B-17 mice. A second sort was done to deposit single cells. Cells at low concentration (2×10^5/ml) were sorted at a low flow rate (50 cells/s) to ensure very high purity. The PtC-liposome$^+$IgM$^+$ phenotype of the sorted cells was recorded for each cell (Fig. 1). All of the cells described here are CD5$^+$ (data not shown) and are thus B-1a cells.

FACS phenotype collection for each sorted single cell

When sorting single cells, we took advantage of a feature on 'Flasher' which allows the collection of FACS information on the sorted single cells. Data is collected only when the cloner is enabled and only on cells meeting the selection criteria. Once a cell has been sorted, the cloner automatically becomes briefly disabled and data collection ceases. This feature is not provided on most commercially available FACS machines.

During sorting, another cell is occasionally in close proximity to the wanted cell and coincidence detection circuitry aborts sorting. The decision to sort a cell occurs hundreds of milliseconds after it has been determined that the cell meets the selection criteria. Thus FACS data has already been collected on the aborted cell even though it does not get sorted. When an eligible event is finally sorted, the cloner is automatically disabled and data collection ceases. However, data has been collected on both the aborted event and the true sorted event. To distinguish between these events and determine the phenotype for the sorted cell, an additional parameter, time, is collected during the sort. The time period between enabling of the cloner (i.e. the time period between changing collection tubes) is several seconds while the time period between aborted and sorted events is hundreds of milliseconds. Thus time easily distinguishes between aborted and sorted events. FACS data was exported into the program JMP (SAS Institute, Cary, NC) to match the FACS phenotype of selected sorted cells and for further analysis. The ability to collect the FACS phenotype for the individual cells sorted allowed for the subsequent correlation of sequence information with the phenotype as in Figs 1, 3 and 5. Statistical comparisons of the FACS phenotype between cells were calculated using the non-parametric Kruskal–Wallis test. No FACS phenotype information was collected on the single cells sorted in BALB/c (experiment 1).

cDNA construction and PCR amplification of V_H and V_L region genes

The method for V_H region amplification has been described previously (40,42). Briefly, random hexamers were used for cDNA synthesis and 1/10 volume of the cDNA was used for PCR amplifications of either the V_H or V_L region from each sample. Promiscuous primers corresponding to the conserved codons in the framework 1 region of V_H (codons 1–8) or V_L (codons 1–8) and primers complementary to the 5′ portion of C_μ region were used. Primers for the V_H region were (MsV_HE, GGG AAT TCG AGG TGC AGC TGC AGG AGT CTG G) and (MsC_μE ATG GCC ACC GAA TTC TTA TCA GA); those for the V_κ and V_λ regions were (MsV_κM, GAT ATT GTG ATG ACC CAG TCT), (MsC_κ1, ACA CTC ATT CCT GTT GAA GCT CTT), (MsV_λM2, ATG GCC TGG ACT TCA CTT ATA CTC T) and (MsCλM1, GCA GGA GAC AAA CTC TTC TCC ACA). The IgL primers were tested with hybridoma cell lines, showing specific amplification of 14 V_κ genes of seven V_κ subgroups (43) and of two V_λ cell lines. Clones negative by PCR with V_κ primers could be analyzed further with V_λ primers.

Two PCR amplifications were performed for either V_H or V_L regions. The second amplification was semi-nested using the same promiscuous V_H region primer (except for V_λ for which the primer is MsV_λM1, CAG GCT GTT GTG ACT CAG GAA TCT) and an internal constant region primer tagged for sequencing at the 5′ end with M13 sequence; (M13-MsC_μN, TGT AAA ACG ACG GCC AGT CAT TTG GGA AGG ACT GA), (M13-MsC_κ2, TGT AAA ACG ACG GCC AGT TCT AGA TGG TGG GAA GAT GGA) and (M13-MsC_λ2, TGT AAA ACG ACG GCC AGT GAG CTC CTC AGA GGA AGG TGG AAA). Primers were prepared at the Stanford Protein and Nucleic Acid Facility (Stanford University).

Positive samples were identified by ethidium bromide staining in agarose gels. The ability to recover cDNA and amplify Ig PCR products from up to 90% of the cells sorted, along with recovery of transcripts from each of the 14 V_H families, suggests this method is robust and lacks bias for particular V_H families (40,42).

The method is equally robust for obtaining the IgL sequence. The κ IgL primers almost always produced positive bands (88% of cells tested, 45 out of 51) confirming the observation that almost all PtC-liposome-binding cells are κ positive by FACS staining (80–99%, depending on strain measured, data not shown).

To control and detect contamination, negative controls (sorted polystyrene beads or water instead of sample) were added at the cDNA synthesis, first amplification and second amplification steps, and PCR tubes were maintained in the same order throughout the reactions. Since one particular rearrangement was observed with great frequency in C57 mice, in some cases we separated each cell sample with a negative control or with PtC-liposome-binding B-1 cells from a more heterogeneous population (BALB/c mice), while maintaining all sample tubes in the same order throughout all steps of the method. Negative controls remained negative and B-1 cells from the heterogeneous population expressed different V_H region gene sequences than those derived from the C57 mice.

Cell lines

The following cell lines (D. Wang and E. A. Kabat) were used to test the κ IgL primers: 82H (κ), 19.1.2 (κ), 5.1H11 (κ), 4.3.F1 (κ), 4.2.6D12 (κ), 58.2C10.3 (κ) and W3129 (κ). The cell lines 26.9.4F (λ) and 17-L2-1F-a (λ) were used to test the λ primers.

Sequencing of PCR products

Sequencing reactions were done using the PRISM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems). Most sequencing gels and analyses were done at the Stanford Protein and Nucleic Acid Facility (Stanford University). Some IgL sequencing and analysis was performed at Columbia University.

Sequence analysis

Analysis of IgH sequences was accomplished using sequence analysis software tools (J. D. MacKenzie, unpublished) and by alignment of V_H genes to a germline database that we have assembled (A. B. Kantor *et al.*, in preparation) (40). The software tools assisted in the process of alignment and display of both IgH and IgL genes. The analysis package also performed pairwise comparison by percent identity to germline sequences in a germline V_H database (A. B. Kantor *et al.*, in preparation) and to consensus sequences for the six V_κ subgroups (43). Parts of the package implemented Intelligenetics gene analysis software (Mountain View, CA). Partial analysis of IgL sequences was accomplished using Seqhunt (Kabat Database of Sequence of Proteins of Immunological Interest, Northwestern University). D element, reading frame, N region insertion, P nucleotide addition, nucleotide loss and CDR3 length were evaluated manually. Heavy chain CDR3 length was defined as extending between codon 94 of the V element and codon 103 of the J element. Only repeatedly isolated IgH rearrangements observed in PtC-liposome-binders from BALB/c and C.B-17 mice are presented here.

Percent identity to V_H germline genes was defined as a match of 98% and above within the V_H segment (framework region 1 minus the primer region through all but the last two codons of framework region 3) (44). Three cells (IgH: AF0002 [96.4]; IgL: AF0032 [94.1], AG0039 [92.3]) matched germline genes at an identity (shown in brackets) <98% but were not considered new germline genes due to sequence quality. Germline genes MMIGVHAB, MMVH0006 and MUSIGHAAM were considered 'putative' (GenBank LOCUS field designations). 'Putative' was defined as sequences derived from IgM antibodies and observed multiple times from separate experiments and/or sources. MMU53526 and MUSIGHXJ were previously identified as *bona fide* IgH germline genes (45,46). IgL germline genes MUSIGKAA1, MUSIGKC85, MMIGVKV4 and MUSIGKCLM were considered putative (GenBank LOCUS field designations). MUSIGKVQ is a *bona fide* germline gene (47) as is S37663S2 (48).

Results

A limited set of V_H germline genes are used by PtC-liposome-binding cells from C57BL/6J mice

PtC-binding cells in C57BL/6J mice (C57) are largely found within a relatively homogeneous population of B cells that stain brightly both with anti-IgM and PtC-liposomes (see Fig. 1). In contrast, PtC-liposome-binding populations from

Cell # or IgH Rearrangement Type[b]	VH Germline Gene[c]	# of cells[a] Ms 1	Ms 2	Ms 3	Ms 4	VH (3')	N/P	D Segment Sequence	N/P	JH (5')	D Segment	JH	RF[d]	CDR3[e]
C57BL/6J PtC-binding IgH Rearrangements														
VH11 n=38 (81%)														
Type 2	MMIGVHAB	0	0	0	2	TGT ATG AG		G TAT GGT AAC TAC		TGG TAC	Dsp2.8	1	1	9
AF0223/Type 3	MMIGVHAB	0	0	0	1	TGT ATG AGA TA		C GGT AGT AGC TAC		TGG TAC	DFL16.1	1	1	10
Type 1	MMIGVHAB	9	9	9	6	TGT ATG AGA TA		T GGT AAC TAC		TGG TAC	Dsp2.8,2.7,2.1	1	1	9
AF0019	MMIGVHAB	1	0	0	0	TGT ATG AGA TA		T TCT GG		C TAC TGG TAC	?	1	NA	9
AF0072	MMIGVHAB	0	1	0	0	TGT ATG AGA TA	C	GGT AAC TAC		TGG TAC	Dsp2.7,2.8,2.1	1	1	9
VH12 n=5 (11 %)														
AF0010	MUSIGHAAM	1	0	0	0	TGT GCA GGA GAC A		AC TGG G	G	C TAC TGG TAC	DQ52	1	1	10
AF0025	MUSIGHAAM	1	0	0	0	TGT GCA GGA GAC A		AC TAC GGC TAC		TGG TAC	DFL16.2	1	1	10
AF0026	MUSIGHAAM	1	0	0	0	TGT GCA GGA GAC AGA		GAT GGT TAC		TGG TAC	Dsp2.9	1	1	10
Type 1	MUSIGHAAM	2	0	0	0	TGT GCA GGA GAC AGA		TAC GGC TAC		TGG TAC	DFL16.2	1	1	10
3660 n=1 (2%)														
AF0185B	MUSIGHXJ	0	0	0	1	TGT GCA AGA	AGT	TAT GAT GGT TAC TAC	TCCT	AC TAC	Dsp2.9	2	1	12
Q52 n=3 (6%)														
AF0112	MMU53526	0	1	0	0	TGT GCC AGA	G	TT TAT TAC TAC GGT AGT A	G	T GCT	DFL16.1	4	1	11
AF0128	MMU53526	0	0	1	0	TGT GCC AGA	GATGGGAGA	TAT AGG TAC GAC	GTAGGGGGAT	AT TAC TAT GCT	Dsp2.11,2.10	4	1	17
AF0116	MMU53526	0	1	0	0	TGT GCC AGA	GGCTC	T TAT TAC TAC GGT AGT AGC TAC	GT	T GCT	DFL16.1	4	1	14
BALB/c Repeated PtC-binding IgH Rearrangements		Expt 1		Expt 2										
VH11														
Type 1	MMIGVHAB	2		5		TGT ATG AGA TA		T GGT AAC TAC		TGG TAC	Dsp2.8,2.7,2.1	1	1	9
Type 3	MMIGVHAB	2		0		TGT ATG AGA TA		C GGT AGT AGC TAC		TGG TAC	DFL16.1	1	1	10
Type 6	MMIGVHAB	0		2		TGT ATG AGA TA	TGGA	AGC TAC		TGG TAC	DFL16.1	1	1	9
Type 7	MMIGVHAB	0		2		TGT ATG AGA TA		C GGT AGT		TAC TGG TAC	DFL16.1	1	1	9
VH12														
Type 1	MUSIGHAAM	0		2		TGT GCA GGA GAC AGA		TAC GGC TAC		TGG TAC	DFL16.2	1	1	10
Type 2	MUSIGHAAM	0		2		TGT GCA GGA GA		T TAC TAC GGC TAC		TGG TAC	DFL16.2	1	1	10
Q52														
Type 1	MMU53526	0		2		TGT GCC AGA	G	AT TAC TAC GGT AGT AGC TAC		TGG TAC	DFL16.1	1	1	12
Type 2	MMU53526	2		0		TGT GCC AGA	GGG	TAC TAC GGC TAC	G	AT TAC TAT GCT	DFL16.2	4	1	12
C.B-17 Repeated PtC-binding IgH Rearrangements		Ms 1		Ms 2										
VH11														
Type 4	MMVH0006[f]	2		0		TGT ATG AGA TA		T AGT AAC TAC		TGG TAC	Dsp2.X	1	1	9
Type 5	MMVH0006	2		0		TGT ATG AGA TA		T GGT AAC	CA	C TAC TGG TAC	Dsp2.7,2.8,2.1	1	1	10
Q52														
Type 3	MMU53526	0		2		TGT GCC	CGCCC	C TAT GGT AAC TAC	G	AT GCT	Dsp2.7,2.1	4	1	10

a. denotes from which mouse the sequence was obtained and the number of times sequences were observed

b. Cell # denotes the cell identifier or the Rearrangement Type for each family if there are repeated sequences: VH11 TYPE 1=AF0006, AF0007, AF0008, AF0009, AF0011, AF0024, AF0032, AF0038, AF0057, AF0066, AF0067, AF0069, AF0070, AF0071, AF0075, AF0094, AF0097, AF0109, AF0124, AF0125, AF0126, AF0127, AF0129, AF0130, AF0137, AF0171, AF0178, AF0182, AF0183, AF0186, AF0188, AF0189, AF0190; n=33 (C57), AG0031,AG0035,AE0008, AE0013,AE0020B,AE0024,AE0131; n=7 (BALB/c); VH11 TYPE 2= AF0187, AF0221; n=2 (C57); VH11 Type 3=AG0038 and AG0039; n=2 (BALB/c) identical to AF0223; VH11 Type 4=AH0003 and AH0078; n=2 (C.B-17); VH11 Type 5=AH0034 and AH0076; n=2; VH11 Type 6=AE0004 and AE0041; n=2; VH11 Type 7=AE0006 and AE0011; n=2; VH12 TYPE 1= AF0001, AF0002; n=2 (C57), AE0017 and AE0022 n=2 (BALB/c); VH12 Type 2 =AE0023 and AE0045; n=2 (BALB/c); Q52 Type 1=AE0075 and AE0076; n=2 (BALB/c); Q52 Typ2 2=AG0015 and AG0037; n=2 (BALB/c); Q52 Type 3= AH0318 and AH0367; n=2 (C.B-17)

c. see figure legend for *bona fide* or putative designation

d. RF denotes Reading Frame of D segment

e. CDR3 length in amino acids

f. encodes for same amino acid sequence as the putuative germline VH gene MMIGVHAB

Fig. 2. V_HDJ_H junctions of IgH genes expressed by PtC-binders in three strains of mice. When multiple cells express an identical V_HDJ_H rearrangement a rearrangement type is designated. For each unique rearrangement we report a cell identifier number and/or a rearrangement type, the V_H family, the *bona fide* or putative germline gene, from which mouse or experiment the cells are derived, the most 3′ nucleotides of the V_H [starting with the Cys = TGT (codon 92, Kabat numbering system)], the D segment sequence, the most 5′ end of the J_H, any N regions at either the V–D or D–J junctions, the name of the D segment, the number of the J_H, the reading frame of the D segment, and the CDR3 length. Sequence in bold can be from either of two germline elements. Potential P elements are underlined. A total of 47 transcripts are reported for C57 mice; 38 are V_H11, five are V_H12, one is 3660 and three are Q52. MMIGVHAB, MUSIGHAAM and MMVH0006 are putative germline genes. MMU53526 and MUSIGHXJ are *bona fide* germline genes. We observed one J558 sequence which predicted an out of frame IgH V region sequence in the CDR3 region (data not shown). Repeated IgH rearrangements observed in BALB/c mice and C.B-17 mice are also shown. All sequences are 100% identical to the reported germline genes except AF0002 (96.4%) and AG0038 (99.2%). These data are available from GenBank under accession nos U64356–U64429.

BALB/c and C.B-17 mice tend to be heterogeneous both with respect to IgM and to PtC-liposome staining (Fig. 1). We used the gates shown in Fig. 1 to sort individual PtC-binding cells for PCR analysis to determine the IgH and IgL sequences expressed by each cell.

Analysis of 47 individual cells sorted from C57 mice yielded only four germline V_H genes, each from a different family (Fig. 2). The most frequent gene, a putative V_H11 germline gene (MMIGVHAB, Fig. 2), was expressed in 38 of the 47 cells analyzed (81%). The other three genes were the putative germline genes MUSIGHAAM (V_H12; five of 47, 11%) and MUSIGHXJ (V_H3660; one of 47, 2%), and the germline gene MMU53526 (V_HQ52; three of 47, 6%). One sequence was also isolated from the V_H family J558.

The sorting gates chosen here tend to somewhat overemphasize the homogeneity of the C57 PtC-binders in that studies with BALB/c, where V_HQ52 predominates (K. J. Seidl *et al.*, in preparation), demonstrate that V_HQ52 PtC-binders stain less brightly than V_H11 and V_H12. Thus, if the gates were set to include the dull PtC-binding region, additional V_HQ52-expressing cells would likely be found. In Fig. 3, which shows the FACS phenotype of each of the cells analyzed for IgH expression, the three V_HQ52-expressing cells that fell within the sort gates are relatively dull for PtC-liposome staining. The cells that express V_H11 and V_H12, in contrast, tend to stain more brightly and thus tend to be more similar to each other than to cells expressing V_HQ52. These differences may reflect differences in the affinity of PtC-liposome binding or the presence of endogenous antigen in the combining site (49).

The possible failure to recover some dull PtC-staining cells expressing V_HQ52 or other V_H genes does not detract from the overall conclusion that the C57 PtC repertoire tends to be relatively homogeneous and to include a high proportion of cells expressing V_H11. Light chain analyses discussed below further underscore the relative homogeneity of this C57 anti-PtC repertoire.

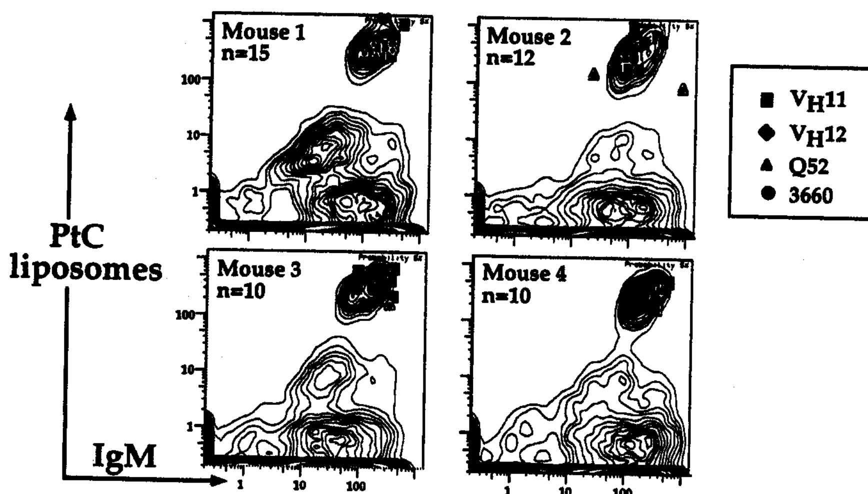

Fig. 3. Overlay of V_H family information and the FACS phenotype of each sequenced cell onto the pre-sort overall population of each mouse. The PtC-liposome binding cells were sorted from four different 6-month-old C57 mice. As single cells are sorted, their FACS profile is collected as described in Methods. Each symbol represents a single sorted cell.

Table 1. V_H11 rearrangement type 1 frequency in three mouse strains

Strain	Mouse or experiment no.	Total cells sequenced (no.)	V_H11 sequences (no.)	V_H11 RT-1[a] sequences (no.)	V_H11 percent of total	RT-1	
						Percent of total	Percent of V_H11
C57BL/6J	1	15	10	9	67	60	90
C57BL/6J	2	12	10	9	83	75	90
C57BL/6J	3	10	9	9	90	90	100
C57BL/6J	4	10	9	6	90	60	67
BALB/c	1[b]	43	8	2	19	5	25
BALB/c	2[b]	58	9	5	16	9	56
C.B-17	1	18	9	0	50	0	0
C.B-17	2	18	7	0	39	0	0

[a]RT-1 denotes rearrangement type 1 (rearrangement type is defined by identity of V, D and J segments, and N/P additions).
[b]1 denotes Expt 1, a pool of peritoneal cells from nine mice; 2 denotes Expt 2, a pool of peritoneal cells from five mice.

Identical rearrangements containing a single V_H11 gene occur repeatedly among PtC-binding cells

Since the CDR3 region is generated by joining V, D and J germline segments with an accompanying gain or loss of junctional nucleotides, it is more diverse than the CDR1 and CDR2 regions encoded by the V_H gene itself. In fact, prior to somatic mutation, the CDR3 region provides the only sequence that uniquely identifies a rearranged IgH utilizing a particular V_H gene. However, although identity for both the V_H germline gene and the CDR3 region among IgH sequences is commonly considered to be an index of clonality, our findings demonstrate that identical rearrangements frequently arise independently in the same animal.

For example, among the C57 V_H11 rearrangements shown in Fig. 2, 33 out of 38 were identical. Over two-thirds of the V_H11 rearrangements isolated from each of the four mice analyzed expressed this IgH (V_H11 type 1, see Table 1). PtC-binding cells from BALB/c mice also expressed this rearrangement, albeit much more rarely (seven of 101). Furthermore, although we failed to isolate V_H11 type 1 from C.B-17, others have found it within unseparated populations of B cells from C.B-17 (50) and other mouse strains (4,10,19,22,23).

Light chain analysis indicates that at least two or three cells expressing V_H11 type 1 within each mouse analyzed here are not due to clonal expansion of mature B cells

IgL sequences were obtained for the V_H11 type 1-expressing cells isolated from the four C57 mice and the two pools of cells from BALB/c (see Table 2). Within each mouse or pool, two predominant IgL rearrangements were found: $V_\kappa 9$–$J_\kappa 2$ was used in 14 of 31 cells (45%) and $V_\kappa 9$–$J_\kappa 4$ was used in 16 of 31 cells (52%). All of the $V_\kappa 9$–$J_\kappa 2$ rearrangements were identical as were all of the $V_\kappa 9$–$J_\kappa 4$. An additional IgL rearrangement, $V_\kappa 21E$–$J_\kappa 2$, which has not been reported previously among PtC-binding cells, was detected in one cell. Thus, like the IgH usage in PtC-liposome-binding cells, the IgL usage is relatively restricted.

Table 2. Cells expressing recurrent heavy chain rearrangements distinguishable by light chain usage, mouse or strain

Heavy chain rearrangement type[a]	Strain	Mouse (Ms) no./ Experiment (Expt) no.	Light chain	No. of cells[b]
V_H11 type 1	C57BL/6J	Ms 1	$V_\kappa9\ J_\kappa2$	2
			$V_\kappa9\ J_\kappa4$	2
			$V_\kappa21E\ J_\kappa2$	1
		Ms 2	$V_\kappa9\ J_\kappa2$	5
			$V_\kappa9\ J_\kappa4$	3
		Ms 3	$V_\kappa9\ J_\kappa2$	3
			$V_\kappa9\ J_\kappa4$	3
		Ms 4	$V_\kappa9\ J_\kappa2$	3
			$V_\kappa9\ J_\kappa4$	3
	BALB/c	Expt 1	$V_\kappa9\ J_\kappa4$	1
		Expt 2	$V_\kappa9\ J_\kappa2$	1
			$V_\kappa9\ J_\kappa4$	4
V_H11 type 3	C57BL/6J	Ms 4	$V_\kappa9\ J_\kappa1$	1
	BALB/c	Expt 1	$V_\kappa9\ J_\kappa1/V_\kappa2\ J_\kappa1$	2
V_H11 type 4	C.B-17	Ms 1	$V_\kappa9\ J_\kappa2/V_\kappa9\ J_\kappa4$	2
V_H12 type 1	C57BL/6J	Ms 1	NA[b]	2
	BALB/c	Expt 2	$V_\kappa4/5\ J_\kappa2$	2
Q52 type 1	BALB/c	Expt 2	$V_\kappa45\ J_\kappa5$[c]$/V_\kappa20\ J_\kappa4$	2

[a]Rearrangement type is defined by identity of V, D and J segments, and N/P additions within a heavy chain family.
[b]NA, not available,
[c]Germline gene is S376632 as opposed to MMIGVKV4. See Fig. 4.

Table 3. Cells expressing recurrent heavy chain rearrangements not distinguishable by light chain usage, mouse or strain

Heavy chain rearrangement type[a]	Strain	Mouse (Ms) no./ Experiment (Expt) no.	Light chain	No. of cells[b]
V_H11 type 2	C57BL/6J	Ms 4	$V_\kappa9\ J_\kappa4$	2
V_H11 type 5	C.B-17	Ms 1	$V_\kappa9\ J_\kappa1$	2
V_H11 type 6	BALB/c	Expt 2	$V_\kappa9\ J_\kappa4$/NA[b]	2
V_H11 type 7	BALB/c	Expt 2	$V_\kappa9\ J_\kappa4$/NA	2
V_H12 type 2	BALB/c	Expt 2	$V_\kappa4/5\ J_\kappa5$/NA	2
Q52 type 2	BALB/c	Expt 1	NA	2
Q52 type 3	C.B-17	Ms 2	$V_\kappa4/5\ J_\kappa2$[c]/NA	2

[a]Rearrangement type is defined by identity of V, D and J segments, and N/P additions within a heavy chain family.
[b]NA, not available,
[c]Germline gene is S376632 as opposed to MMIGVKV4. See Fig. 4.

Many of the mice analyzed expressed the same IgH/IgL pair. Nevertheless, unique instances of IgH/IgL pairing in each of the individual mice show that at least two to three of the V_H11 type 1-expressing cells have arisen from different B cell progenitors, either prior to IgH rearrangement or after IgH rearrangement but prior to IgL rearrangement (51,52). In total, at least 12 of the 31 V_H11 type 1 cells studied for IgL expression in our data set are not due to clonal expansion of mature B cells because they either are derived from different mice or express different light chains within the same mouse (Tables 2 and 3).

Varied IgH/IgL pairings may also be accounted for by IgL editing after B cells reach maturity (53–55). However, it is unlikely that such editing accounts for very many of the unique IgH/IgL pairs that we observed. The vast majority (30 of 31) of the IgL rearrangements found in association with V_H11 type 1 were $V_\kappa9$–$J_\kappa2$ and $V_\kappa9$–$J_\kappa4$. Since the same $V_\kappa9$ germline gene is used in both of these rearrangements, the $V_\kappa9$–$J_\kappa2$ and $V_\kappa9$–$J_\kappa4$ light chains could not have derived from one another unless a recombination signal sequence exists in the $V_\kappa9$ germline gene segment. Such an event would predict a strikingly different coding joint of $V_\kappa9$–$J_\kappa4$ compared to $V_\kappa9$–$J_\kappa2$. However, a similar coding joint is maintained in cells expressing either of these rearrangements with differences between the two contributed by the J_L only (see Fig. 4B).

Overall, the number of IgL transcripts which could have arisen from IgL editing in our study is at most one, i.e. the

```
CDR3  Regions of Heavy Chains                                                   A

                                                            100
                                                    ________________
Rearrangement Type              95                  100 H   I   J   K       102
                    Cys Met Arg Tyr Gly Asn             Tyr Trp Tyr Phe Asp Val Trp Gly
VH11 Type 1         TGT ATG AGA TAT GGT AAC             TAC TGG TAC TTC GAT GTC TGG GGC

VH11 Type 2         --- --- --G --- --- ---             --- --- --- --- --- --- --- ---
                                        Ser Ser
VH11 Type 3         --- --- --- --C --- -GT AGC         --- --- --- --- --- --- --- ---
                                    Ser
VH11 Type 4         --- --- --- --- A-- ---             --- --- --- --- --- --- --- ---
                                            His
VH11 Type 5         --- --- --- --- --- --- CAC         --- --- --- --- --- --- --- ---
                                        Ser
VH11 Type 6         --- --- --- --- --A -G-             --- --- --- --- --- --- --- ---
                                        Ser
VH11 Type 7         --- --- --- --C --- -GT             --- --- --- --- --- --- --- ---

                    Cys Ala Gly Asp Arg Tyr         Gly Tyr Trp Tyr Phe Asp Val Trp Gly
VH12 Type 1         TGT GCA GGA GAC AGA TAC         GGC TAC TGG TAC TTC GAT GTC TGG GGC
                                    Tyr
VH12 Type 2         --- --- --- --T TAC ---         --- --- --- --- --- --- --- --- ---

                    Cys Ala Arg Asp Tyr Tyr Gly Ser Ser Tyr Trp Tyr Phe Asp Val Trp Gly
Q52 Type 1          TGT GCC AGA GAT TAC TAC GGT AGT AGC TAC TGG TAC TTC GAT GTC TGG GGC
                                Gly             Cys Asp     Tyr Ala Met     Tyr
Q52 Type 2          --- --- --- -GG --- --- --C TAC GAT --- -AT GCT A-G --C TA- --- --T
                                Pro     Gly Asn             Asp Ala Met     Tyr
Q52 Type 3          --- --- C-C CCC --T GGT AAC         --- GAT GCT A-G --C TA- --- --T
```

```
CDR3 Regions of Light Chains                                                                B

Gene Family Jk    Subgroup  germline gene         90                  95  95A                 100
                                          Cys Leu Gln His Gly Glu Ser Pro     Tyr Thr Phe Gly Gly Gly Thr
Vk9         Jk2   vkV       MUSIGKAA1     TGT CTA CAG CAT GGT GAG AGC CCG     TAC ACG TTC GGA GGG GGG ACC
                                                                              Phe             Ser
Vk9         Jk4   vkV       MUSIGKAA1     --- --- --- --- --- --- --- --A     -T- --- --- --C TC- --- --A
                                                                              Trp
Vk9         Jk1   vkV       MUSIGKAA1     --- --- --- --- --- --- --- ---     -GG --- --- --T --A --C ---
                                              Gln His Ser Trp     Ile
Vk21E       Jk2   vkIII     MUSIGKVQ      --- -AG --C AG- T-G --- -TT ---     --- --- --- --- --- --- ---
                                              Trp     Gly Thr His Phe
Vk2         Jk1   vkII      MUSIGKCLM     --C TGG --A GG- ACA C-T TTT         XGG --- --- --T --A --C ---
                                              Gln     Trp Ser Ser Asn         Leu             Ala
Vk4/5       Jk5   vkVI      S37663S2      --- CAG --- TGG A-- AGT -A- --A     CTC --- --- --T -CT --- ---
                                              Gln     Trp Ser Ser Asn
Vk4/5       Jk2   vkVI      S37663S2      --C CAG --- TGG A-- AGT -A- ---     --- --- --- --- --- --- ---
                                              Gln     Gly Ser Ser Ile         Arg
Vk4/5       Jk2   vkIV      MMIGVKV4      --- CAG --- GGT A-- AGT ATA --A     CGC --- --- --- --- --- ---
                                              Gln     Gly Ser Ser Ile         Arg             Ala
Vk4/5       Jk5   vkIV      MMIGVKV4      --- CAG --- GGT A-- AGT ATA --A     CGC --- --- --T -CT --- ---
                                              Leu     Ser Asp Asn             Ser             Ser
Vk20        Jk4   vkV       MUSIGKC85     --- T-G --A AG- -A- A-C  ATGCCTCT   AGC --- --- --C TC- --- --A
```

Fig. 4. Amino acid alignment of IgH and IgL CDR3 regions from cells expressing repeated heavy chains. Nucleotide sequences and derived amino acid sequences are indicated in the top sequence of each IgH subgroup and the top sequence of the light chains. A dash indicates identity to the top sequence. (A) Alignment of CDR3 regions for repeated IgH sequences from C57, BALB/c and C.B-17 mice. Heavy chain rearrangements are categorized by V_H family into rearrangement types based on V_H, D, J_H and CDR3 region identity. Codons 95–102 represent the CDR3 region [numbering according to Kabat (43)]. One cell (AB0196) from a separate study of the general BALB/c repertoire is identical to V_H11 type 3 (42). Other information about rearrangement types can be found in Fig. 2. (B) Alignment of CDR3 regions for sequenced light chains. We analyzed 31 of the cells expressing rearrangement V_H11 type 1 from C57 and BALB/c mice and 25 of the cells expressing other repeated rearrangements for IgL expression. An example of each IgL type (based on CDR3 region) observed is given. The percent identity to the V_κ germline gene of sequences that match with <100% identity is indicated in brackets. Cells expressing $V_\kappa9$–$J_\kappa2$ IgL sequences and matching the putative germline gene MUSIGKAA1 are AE0013, AF0024, AF0057, AF0066, AF0067, AF0070, AF0097, AF0109, AF0127, AF0129 [99.6], AF0137, AF0182, AF0183, AF0190 and AH0078. Cells expressing $V_\kappa9$–$J_\kappa1$ are AG0039 [92.3], AH0034, AH0076, AF0223 and match the putative V_κ germline gene MUSIGKAA1. Cells expressing $V_\kappa9$–$J_\kappa4$ are AE0004, AE0008, AE0011 [99.2], AE0020, AE0024, AE0131, AG0031, AF0032 [94.1], AF0038, AF0071, AF0075, AF0094, AF0124 [99.6], AF0171 [99.6], AF0178, AF0186 [99.2], AF0187, AF0188, AF0189, AF0221, AH0003 and also match MUSIGKAA1. Cell AF0008 expresses $V_\kappa21E$–$J_\kappa2$ [99.5] and matches the germline gene MUSIGVKQ. Cell AG0038 expresses $V_\kappa2$–$J_\kappa1$ and matches putative germline gene MUSIGKCLM. Cell AE0075 expresses $V_\kappa4/5$–$J_\kappa5$ and matches germline gene S37663S2. Cell AE0076 expresses $V_\kappa20$–$J_\kappa4$ and matches the putative germline gene MUSIGKC85 [98.3]. This sequence predicts a non-functional IgL protein but distinguishes the origin of AE0076 and AE0075, the other Q52 type 1 sequence. Cells AE0017 and AE0022 express $V_\kappa4/5$–$J_\kappa2$ and match the putative germline gene MMIGVKV4. Cell AE0045 expresses $V_\kappa4/5$–$J_\kappa5$ and also matches MMIGVKV4. Cell AH0367 expresses $V_\kappa4/5$–$J_\kappa2$ and uses the germline gene S37663S2. Numbering and subgroup assignments according to Kabat (43). Family assignments according to Kofler *et al.* (58). Codons 89–97 define the CDR3 region. The J_κ gene segment starts at codon 96 but can sometimes start at 95 depending on the amount of nucleotide loss. These data are available from GenBank under accession nos U54503–U54550.

$V_\kappa 21E$–$J_\kappa 2$ which could have arisen by a mechanism of inversion from a $V_\kappa 9$–$J_\kappa 4$ rearrangement. Eliminating this rearrangement from consideration decreases the number of cells expressing $V_H 11$ type 1 not due to clonal expansion by one cell. This decrease does not alter the fundamental conclusion that identical IgH (and IgL) rearrangements occur frequently in the same and different animals and are not all a result of clonal expansion of mature B cells.

All of the repeated $V_H 11$ rearrangements code for strikingly similar IgH

In addition to $V_H 11$ type 1, we isolated six $V_H 11$ IgH rearrangements that each occurred at least twice in our data set (Figs 2 and 4, Tables 2 and 3; $V_H 11$ types 2–7). Nucleotide sequences for several of these rearrangements are different; however, they encode IgH with similar or identical amino acid sequences and CDR3 lengths (Fig. 4A). $V_H 11$ types 1 and 2 code for exactly the same protein as do $V_H 11$ types 6 and 7; $V_H 11$ types 3–7 encode proteins that differ at only one or two amino acid residues. These differences occur at positions 96, 97 or 98 at the V–D joint and result from the use of either of two D elements, DSP or DFL16.1 (Fig. 2), and the addition or loss of nucleotides from coding ends. Thus these nucleotide sequence changes maintain the CDR3 region length of nine or 10 amino acids and most likely preserve the shape of the combining site for PtC (28).

Identical rearrangements expressing single $V_H 12$ and $V_H Q52$ germline genes also occur repeatedly

Although $V_H 11$ type 1 was the most frequently repeated IgH rearrangement among PtC-liposome-binding cells, identical heavy chains using germline genes other than the putative $V_H 11$ (MMIGVHAB) germline gene were also found repeatedly (Tables 2 and 3). Among the 32 PtC-binding cells that expressed $V_H 12$ from all three strains, four were identical ($V_H 12$ type 1; two of five from C57 and two of 14 from BALB/c) and expressed the MUSIGHAAM putative germline gene (Fig. 2). BALB/c $V_H 12$ PtC-binding cells also yielded a second repeated $V_H 12$ rearrangement ($V_H 12$ type 2).

$V_H 12$ types 1 and 2 utilize a similar amino acid sequence in the CDR3 region (Figs 2 and 4) although they differ substantially from the $V_H 11$ rearrangement types. The CDR3 region lengths of $V_H 12$-expressing sequences differ only slightly in comparison to $V_H 11$-expressing sequences. Both repeated and non-repeated $V_H 12$-expressing sequences presented here ($n = 9$) adopted a restricted CDR3 length of 10 amino acids compared to nine or 10 amino acids of the $V_H 11$-expressing sequences. Previous studies also reported a 10 amino acid restriction in length for $V_H 12$-expressing PtC-binding IgH (4,56,57).

In addition to $V_H 11$ and $V_H 12$, a few $V_H Q52$-expressing rearrangements from BALB/c mice using the germline gene MMU53526 were found repeatedly ($V_H Q52$ types 1 and 2; Figs 2 and 4). Two C.B-17 PtC-binding $V_H Q52$ sequences, expressing MMU53526, were also identical ($V_H Q52$ type 3). The $V_H Q52$ rearrangements showed more CDR3 diversity than either the $V_H 11$ or $V_H 12$ rearrangements, largely due to the use of different D and J segments and variable numbers of N-region nucleotide addition. Consideration of the animal origin and the IgL expression of cells expressing these additional repeated rearrangements now brings the total number of recurrent identical rearrangement events observed in this study not due to mature B cell clonal expansion to 20 and overall, including those in the literature, to 41. Of these $V_H 12$ and $V_H Q52$ repeated IgH rearrangements, only the light chains expressed by cells utilizing $V_H Q52$ type 1 could be derived from each other by a mechanism of IgL editing involving inversion (58). This possibility cannot be excluded from our data.

Sequence homology at coding ends is common among PtC-binders

Nucleotide sequence homology at the V–D and D–J coding joints within the CDR3 region, may reflect a constraint of rearrangement outcome that could bear on the mechanism responsible for repeated IgH and IgL rearrangements (30–34). In fact, many of the $V_H 11$ and $V_H 12$ IgH described here exhibit sequence identity at the coding junctions (Fig. 2). Rearrangement $V_H 11$ type 1 utilized a two nucleotide sequence identity (TA) between the V_H and DSP segments. In addition, this IgH used a four nucleotide sequence identity (CTAC) between the DSP and $J_H 1$ segments. Seven of 11 (64%) $V_H 11$-expressing sequences shown here, other than $V_H 11$ type 1, expressed the TA identity between the V and D segments. Of these same 11 $V_H 11$-expressing sequences, eight (73%) expressed the CTAC identity between the D and J gene segments. Seventy-eight percent (seven of nine) of the $V_H 12$-expressing sequences contained the CTAC identity as well.

The importance of sequence homology in directing these repeated rearrangements is unclear. Two of the 10 repeated rearrangements, representing four of 50 repeated sequences, use no sequence homology. Four of the 10 rearrangements, representing 10 of 50 repeated sequences, show homology at one of the two joints, principally the D–J joint; and the remaining four of 10 repeated rearrangements, representing 36 of the 50 repeated sequences (largely $V_H 11$ type 1), show homology at both coding joints. If sequence homology at the coding joints reflects a constraint of rearrangement outcome, this double homology could explain the high proportion of $V_H 11$ type 1 rearrangements.

In addition, if the IgH structure encoded by $V_H 11$ type 1 is particularly well adapted for binding PtC, the high proportion of repeated rearrangements that use sequence homology at both coding joints would also reflect selection of these rearrangements and/or their retention in the anti-PtC repertoire. Such selection, however, is likely to come from a precursor pool with a relatively high frequency of this rearrangement. Selection alone does not account for the high frequency of repeatedly isolated rearrangements in our data set.

Levels of IgM surface expression and PtC-liposome staining correlate with IgL expression

Surprisingly, C57 cells expressing $V_H 11$ type 1 paired with particular light chains were distinguishable by the amount of surface IgM and PtC staining (Fig. 5). In each C57 mouse, these cells clustered into two fairly distinct groups, according to whether they expressed $V_\kappa 9$–$J_\kappa 4$ or $V_\kappa 9$–$J_\kappa 2$. The clusters overlay larger clusters, visible in the contour plot showing

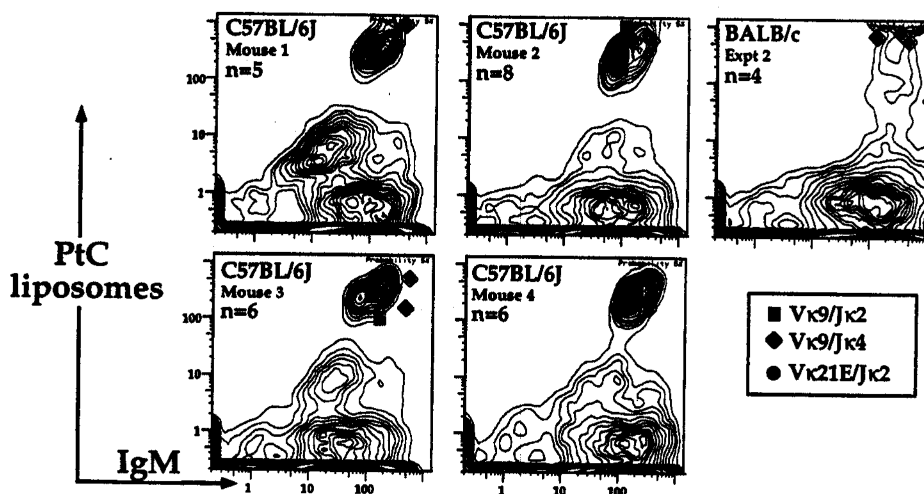

Fig. 5. FACS phenotype of cells expressing the most common IgL rearrangement, V_H11 type 1, in association with $V_\kappa9$–$J_\kappa4$, $V_\kappa9$–$J_\kappa2$ or $V_\kappa21E$–$J_\kappa2$. Median fluorescence for C57 cells: $V_\kappa9$–$J_\kappa2$ (n = 12, four individual mice), IgM = 58, PtC = 201; $V_\kappa9$–$J_\kappa4$ (n = 12), IgM = 198, PtC = 406. Fluorescence range for BALB/c cells (n = 4, pool of five mice): IgM, 73–817; PtC, 36–608. Cell phenotypes are overlaid on FACS phenotypes of peritoneal cells from the appropriate strains.

IgM versus PtC levels of cells in the overall population from which the sorted cells were drawn. Cells expressing the same IgH/IgL pairs in BALB/c mice, however, exhibited diverse staining levels.

In the C57 mice, V_H11 type 1 cells expressing $V_\kappa9$–$J_\kappa4$ always stained brighter for IgM than those expressing $V_\kappa9$–$J_\kappa2$ (median values for IgM were 198 and 58 respectively, $P < 0.0001$), although the cells of the two populations are equal in size (forward and obtuse scatter) and express the same amount of CD5 (data not shown). The difference in amount of surface IgM suggests that IgL expression may regulate or limit the amount of expressed total surface Ig.

Discussion

We have demonstrated that particular IgH and IgL rearrangements occur repeatedly and are detectable by single-cell PCR analysis of FACS-sorted PtC-liposome-binding B cells. We detect cells expressing repeated rearrangements within a single animal, among different animals and among different mouse strains. There is no question that the identical variable region sequences isolated from separate animals rearranged independently. Within an individual animal, however, distinguishing the origin of cells expressing the same IgH rearrangement is more difficult. Cells expressing identical IgH and IgL rearrangements could result from clonal expansion. However, cells expressing identical IgH but different IgL rearrangements must be derived either from independently rearranged B cell progenitors or from clonally expanded pre-B cells that have the same IgH rearrangement but independently rearranged different IgL. Thus the identical IgH rearrangements we have observed in cells with different IgL, like the identical IgH expressed by cells from different animals, cannot be explained by clonal expansion of mature B cells.

Other studies based on anti-idiotype analyses, hybridoma work and bulk sequencing analyses have reported the occurrence of identical rearrangements or common idiotypes and proposed a series of mechanisms to explain these, i.e. independent rearrangements, pre-B cell expansion after IgH rearrangement and clonal expansion of the mature B cell (28,35,51). Our data confirm that not all identical IgH are generated by clonal expansion of mature B cells. Within each individual mouse that we analyzed, at least 25% (and sometimes as high as 60%) of the repeated sequences we isolated did not arise as a result of clonal expansion of mature B cells.

These findings raise question as to whether IgH identity alone is sufficient proof of clonal expansion, particularly regarding B-1 cell antigen responses. In fact, even cells expressing both identical IgH and IgL, normally taken as evidence for clonal expansion, could actually have independent origins. This is probably not the case for most antigens. However, for antigens like PtC where the repertoire is very restricted, expression of the same IgH and IgL may reflect an independent origin of the cells expressing these identical rearrangements. This idea is consistent with the perceptive suggestion that a fair proportion of anti-phosphorylcholine binding cells arise independently (35). These conclusions were based on anti-idiotype analyses of the response to phosphorylcholine, where a large fraction of cells produce a prototypical IgH and IgL pair (16).

The majority of the repeated IgH rearrangements that we isolated have identical variable region sequences (V_H11 type 1). This rearrangement has also been detected by analysis of PtC-binding hybridomas in three different strains of mice (BALB/c, B10.H-2^aH-4^bp/Wts and NZB) (4,10,19,22,23,28). In fact, of 11 previously published hybridomas expressing V_H11 type 1, eight expressed the $V_\kappa9$–$J_\kappa2$ IgL and one expressed the $V_\kappa9$–$J_\kappa4$ IgL that predominate in our data set. Identical V_H11-expressing IgH rearrangements (not selected for their ability to bind PtC) were also detected from 18 day fetal liver cells from different mice, from the same mice where allotype

markers could distinguish the two alleles (28) and from adult mice (1). One sequence from Arnold *et al.* (2C-131) is identical to V_H11 type 3. However, unlike what we report here for V_H11 type 1, no more than two sequences expressed any given repeated rearrangement.

The preference for the V_H11 type 1 (V_H11–DSP2–J_H1) rearrangement that we have detected in C57 mice could reflect directed recombination (32,34,59) based on sequence identities at the V–D and D–J junctions. While the identity at the V–D junction only involves two nucleotides, recent data indicate that this is sufficient to influence rearrangement (60,61). On the other hand, regions of sequence overlap could be observed frequently merely because they provide a necessary protein structure for binding to PtC and for that reason are selected into the repertoire. Thus, while sequence homology currently offers perhaps the best explanation for the increased frequency of V_H11 type 1 rearrangements, it is not an altogether satisfying hypothesis.

The repeated isolations of V_H11 type 1, the same rearrangements that we have described here, predominate in C57 mice. Recurrent rearrangements occur in BALB/c and C.B-17 mice as well, but represent a more minor component of the PtC repertoire. In other studies, we have shown that vastly different anti-PtC repertoires are expressed by PtC-liposome-binding cells from BALB/c mice, which carry the a-allotype (Igh^a) Ig chromosome, and from C.B-17 mice, which carry the Igh^b chromosome on the BALB/c genetic background. The anti-PtC repertoires of all three strains predominantly consist of three germline V_H genes; however, their expression frequency differs from strain to strain. This result suggests that there is a different kind of selection for these PtC-binding B-1 cells than is typically observed in an immune response. Furthermore, anti-PtC B-1 cells from both BALB/c and C.B-17 tend to express many unique IgH sequences rather than the few recurrent rearrangements reported here (K. J. Seidl *et al.*, in preparation). Since C.B-17 and BALB/c mice have different IgH chromosomes on the same genetic background and C57 mice have roughly the same Igh^b chromosome as C.B-17 on a different genetic background, these differences among the anti-PtC repertoires in the three strains suggest that the PtC-repertoire is influenced by complex genetically controlled mechanisms that maintain and increase the frequency of cells expressing certain V_H germline genes and dictate the occurrence of repeated rearrangements.

Some of the recurrent identical IgH rearrangements paired with different IgL rearrangements could be due to expansion of pre-B cells, which have rearranged IgH but not IgL (51,52). However, developmental differences between conventional and B-1 cells, which include all PtC-binding cells, suggest that B-1 pre-B cells may not expand extensively. Rothstein and colleagues have shown that signaling through the IgM receptor differs substantially between B-1 and conventional B cells (62). Furthermore, B-1 pre-B cells lack MHC class II expression whereas conventional pre-B cells express class II (63,64). Thus pre-B cell signaling, necessary for expansion, might also be expected to differ between conventional pre-B cells and B-1 pre-B cells. Additional evidence that the dominant paradigm of pre-B cell expansion may not apply to B-1 progenitors comes from gene targeting experiments inactivating Vav (65,66), the Cr2 locus (67), CD19 (68), IL-5 (69) or λ5 (70,71) which resulted in different developmental defects for conventional B cells and B-1 cells.

IgL editing, occurring subsequent to the original derivation of the mature B cell (53, 54), could also account for some of the unique IgH/IgL pairs expressing identical IgH rearrangements. However, as indicated in the Results section, the IgL sequences expressed in PtC-liposome-binding cells basically rule out any significant contribution by this mechanism. Thus the available evidence argues for the occurrence of independently derived identical IgH rearrangements in PtC-binding cells.

The restriction of the anti-PtC repertoire in C57BL/6J mice to a few IgH variable region sequences is similar to that observed in other well-defined T-dependent and T-independent responses. Highly similar (or identical) IgH and IgL sequences have also been demonstrated in the dominant, serologically defined idiotype responses to phosphorylcholine (16) dextran, (12), NP (13,14), *p*-azophenylarsonate (15) and 2-phenyl-oxazolone (72). Indeed, the repeated generation of identical IgH rearrangements is a common characteristic in many antibody responses, e.g. in the responses to α(1–6)dextran (12) and phosphorylcholine (16). The unbiased single-cell analyses presented here confirm and extend this previous evidence, which was predominantly obtained from hybridoma studies. Thus the combined data from our studies and from earlier work suggests that, in addition to clonal expansion, recurrent identical rearrangements may be a major contributory mechanism in dominant idiotype (or clonotype) expression, at least in B-1 antibody responses.

The question of whether recurrent rearrangements are common in B-2 antibody responses is difficult to resolve at present for two reasons. First, most of the sequence data on immune responses in the literature are based on hybridoma studies which may introduce major bias (42). Second, for most of the dominant idiotype responses for which sequence data exists, there is no definitive evidence indicating whether the response is made by B-1 or B-2 cells.

For example, it is not known whether B-1, B-2 or both B cell populations produce the dominant idiotypes observed in the responses to *p*-azophenylarsonate, NP and 2-phenyl-oxazolone. All three of these responses are restricted; however identical repeated IgH are found only in the response to *p*-azophenylarsonate (13–15,72). For two antigens to which B-1 cells are known to respond, α(1–3)dextran (73) and phosphorylcholine (74), the former does not appear to utilize identical IgH and IgL rearrangements (75) and the latter does (16).

Data analyzing the overall repertoires of B-1 and B-2 cell populations from unimmunized mice indicates that repeated rearrangements are observed among B-1 cells but not among B-2 cells (1,34,42,76). In the case of B-1 cells, more repeated rearrangements were found among peritoneal B-1a cells than among B-1b cells in a study by Tornberg and Holmberg where genomic DNA from bulk B cell populations was analyzed (1). Single-cell analyses of randomly sampled peritoneal B cell populations done in our laboratory revealed three sets of two B-1a cells (n = 64) with identical V_HDJ_H rearrangements and two out of 70 B-1b cells (A. B. Kantor *et al.*, in preparation) (42). These two studies agree that recurrent identical IgH are detectable in B-1a and B-1b cells. Similarly, all laboratories

that have tested the B-2 repertoire agree that B-2 cells have very few repeated rearrangements, at least when sampled randomly rather than according to antigen-binding capability.

The demonstration that identical rearrangements recur for certain antibody specificities, raise a caution with respect to the methods used to define clonality in human tumor systems. The homogeneous populations of B-1 cells examined in the C57 mice in this study are analogous to the expanded neoplastic CD5$^+$ B cell clones in human B cell chronic lymphocytic leukemia (B-CLL) (77–79). Mouse hybridomas derived from homogeneous B-1 cell populations were shown to be clonally related since they expressed identical IgL and IgH (both productive and unexpressed alleles) as well as clonally unrelated since they expressed identical IgL but distinct IgH (78). Similarly, our observations indicate that B-1 cells express recurrent IgL rearrangements in addition to repeated IgH rearrangements (this paper, and J. D. MacKenzie and K. J. Seidl, unpublished results). Identical IgH rearrangements have not been reported in human B-CLL; however, Kipps *et al.* have noted the repeated occurrence of a particular IgL CDR3 in several patients (80). If repeated rearrangements of certain IgH or IgL arise independently in human B cell ontogeny, these could confound attempts to assign all cells in a given neoplasm to a single progenitor on the basis of IgH or IgL identity (5,81–83), because some tumors could be considered clonal when in fact they arose from more than one progenitor.

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Abbreviations

B-CLL	B cell chronic lymphocytic leukemia
C57	C57BL/6J mice
FSA	fluorescein sulfonic acid
NP	(4-hydroxy-3-nitrophenyl)acetyl
PtC	phosphatidylcholine

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Note added in proof

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