Frequent occurrence of identical heavy and light chain Ig rearrangements

Katherine J. Seid1,4, John D. MacKenzie1, Denong Wang2, Aaron B. Kantor1,3, Elvin A. Kaban2, Leonard A. Herzenberg1 and Leonore A. Herzenberg1

1Department of Genetics, Beckman Center B007, Stanford University School of Medicine, Stanford, CA 94305-6125, USA
2Department of Microbiology, Columbia University, New York, NY 10032, USA
3AmCell Corporation, 1190 Bordeaux Drive, Sunnyvale, CA 94089, USA
4Present address: Children's Hospital, 300 Longwood Avenue, Enders 861, Boston, MA 02115, USA

Keywords: B-1 cells, CD5, clonal expansion, dominant idotype, FACS, Ig sequences, phosphatidylcholine, single cell, V\textsubscript{H}11, V\textsubscript{L} region sequences, V\textsubscript{L} region sequences

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 bromelin-treated mouse erythrocytes, i.e. antibodies reactive with phosphatidylcholine (PC). We repeatedly isolated at least 10 different types of V\textsubscript{H} region rearrangements, involving three distinct germline genes, among FACS-sorted PC-binding B-1 cells from three strains of mice (C57BL/6J, BALB/c and C.B-17). The predominant rearrangement, V\textsubscript{H}11-DSP-J\textsubscript{H}1 (V\textsubscript{H}11 type 1), has been previously found in anti-PC 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 idotype 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\textsubscript{H}D\textsubscript{JH} or V\textsubscript{L}J\textsubscript{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 VDJ recombination confers a vast potential for diversity of the antibody repertoire. One of multiple variable (V\textsubscript{H} and V\textsubscript{L}) segments combines with one of several D\textsubscript{H} and J\textsubscript{H} 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)acectyl (NP), phospholipid...
choline (PIC), the antigen studied here (8-16). Several studies have shown that the IgH and Igl of anti-PIC antibodies produced by independently isolated hybridomas and neoplasms tend to be encoded by V_{H}1-11-V_{\delta}9 or V_{H}12-V_{\lambda}4 (9-
11, 17-23). These anti-PIC antibodies lysed mouse erythrocytes treated with bromelain to expose PIC (a common phospholipid constituent of cell membranes). In normal animals, anti-PIC 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 PIC-binding surface IgM are readily detectable in the peritoneal cavity and spleen of unstimulated mice by FACS analysis with fluorescein encapsulated in PIC-liposomes (24). In most mouse strains, PIC-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 80% of total splenic and peritoneal lymphocytes respectively (25,26)]. No other class of B cells has been shown 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 PIC-binding B-1 cells is unknown.

The anti-PIC repertoire, like the overall B-1 antibody repertoire, is established early in life. De novo B-1 development, particularly the development of the CDS+ 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-PIC repertoire may be largely due to preferential rearrangement of germline elements, preferential survival of pre-B cells, or preferential antigen-driven selection and expansion of a relatively small number of B cells (5,28).

Several regions gene assemblies of anti-PIC 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-PIC IgH rearrangements are derived from a common progenitor or from multiple progenitors which develop into a limited PIC repertoire (3,9-
11,19,24,25).

Various mechanisms may explain how the same rearrange-
ments occur in a frequency high enough to be seen 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 PIC repertoire.

The FACs-based single-cell PCR studies presented here test this and related hypotheses concerning the origin and structure of the expressed anti-PIC repertoire. Our work extends earlier studies of various antigen responses character-
ized by dominant idiootypes initially defined by anti-idiotypic antibodies (35), and subsequently by sequencing the involved V_{H}, D_{H}, and J_{H} gene segments (15,16). We demonstrate that at least 25%, and in some cases as many as 60%, of the cells producing anti-PIC 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 PIC-liposome-binding B-1 cells from animals of the same strain but varies from strain to strain. These data show by single-cell FACs, evidence for the generation of identical IgH and IgI 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 IgI sequences with the surface markers expressed by single PIC-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/c-H2-K (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 C3H/17 mice at 5.5 months of age were used in this study. Individual mice were studied from C3H-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 made as described previously. FACs reagents consisted of phycoerythrin-conjugated anti-IgM (331.12), allo-
phyocyanin-conjugated anti-CDS (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 (2.5x10^6/ml) were incubated with 10 ng/ml RNase (Gibco/BRL, Life Technologies, Gaithersburg, MD) for 30 min on ice and then diluted 10-fold (2.5x10^5/ml, 1 ng/ml Rhase) for sorting.

Cells were analyzed and sorted on a '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/Sheva software (M. Bigos, Stanford University).

Preparation of liposomes

Liposomes were prepared using a modification of previous methods (24,25,38,39). Distearoyl PC, distearoyl phosphatidylglycerol (both Avanti Polar Lipids, Birmingham, AL) and cholestrol (Sigma) were mixed at a molar ratio of 45:5:5 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 sulfitic acid (FSA) (Molecular Probes, Eugene, OR) at a final lipid concentration of 50 mM. After overnight hydration, the multi-
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 0.05 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 Sephrose CL-6B column, and eluted with a buffer of 50 mM MES, 50 mM sodium chloride and 1 mM EDTA, pH 6.0. The PIC-liposomes were titered on peritoneal cavity cells from BALB/c and C57 mice (used at a final lipid concentration of ~40 μM per 6 x 10^6 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. PIC-liposome-binding cells from either C57, BALB/c or C.B-17 mice were sorted based on PIC-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 x 10^6/ml) were sorted at a low flow rate (50 cells/s) to ensure very high purity. The PIC-liposome* IgM* phenotype of the sorted cells was recorded for each cell (Fig. 1). All of the cells described here are CDS* (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 "Flashe"r 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_{H} were used. Primers for the V_{H} region were (MvV{sub}E, GGG AAT TCG AGG TGC AGC TGC AGG GAT CTC G) and (MscC{sub}E, ATG GCC ACC GAA TTC TTA TCA GA); those for the V_{L} region were (MvM{sub}M, GAT ATT GTG ATG ACC CAG TCT), (MscC{sub}M1, ACA CTC ATT CCT GAA GCT CT1), (MvV{sub}M2, ATG GCC TGG ACT TCA CTC ATA CTC T) and (MscCAM1, GCA GGA GAC AAA TTC TTC ACA). The Igl primers were tested with hybridoma cell lines, showing specific amplification of 14 V_{L} genes of seven V_{L} subgroups (43) and of two V_{H} cell lines. Clones negative by PCR with V_{H} primers could be analyzed further with V_{L} 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_{H} for which the primer is MvV{sub}M1, 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-MscC{sub}N, TGT AAA ACG ACG GCC AGT CAT TGG GGA ACG ACT GA), (M13-MscC{sub}2, TGT AAA ACG ACG GCC AGT TCT AGA TGG TGG GAA GAT GGA) and (M13-MscC{sub}2, TGT AAA ACG ACG GCC AGT GAC 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_{L} families, suggests this method is robust and lacks bias for particular V_{L} families (40, 42).

The method is equally robust for obtaining the Igl sequence. The k Igl primers almost always produced positive bands (98% of cells tested, 45 out of 51) confirming the observation that almost all PIC-liposome-binding cells are k positive by FACScan staining (80-99%, depending on strain measured, data not shown).

To control and detect contamination, negative controls (sorted polysynthetic 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 PIC-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 k Igl primers: 82H (k), 19.1.2 (k), 5.1H11 (k), 4.3F1 (k), 4.26D12 (k), 58.2C10.3 (k) and W3129 (k). The cell lines 26.9.4F (λ) and 17-L2-1F-α (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_{H} subgroups (43). Parts of the package implemented Intelligent 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 PIC-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: AF00029 [96.4]; Igl: AF00032 [94.1], AG00039 [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 MMIVHAB, MMIVH0006 and MUSIGHAEM 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. MUSIG5826 and MUSIGHKJ were previously identified as bona fide IgH germline genes (45,46). Igl germ line genes MUSIGKAA1, MUSIGKC85, MUSIGK51 and MUSIGK5L were considered putative (GenBank LOCUS field designations). MUSIGK5V4 is a bona fide germline gene (47) as is S376635 (48).

Results

A limited set of V_{H} germline genes are used by PIC-liposome-binding cells from C57BL/6J mice

PIC-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 PIC-liposomes (see Fig. 1). In contrast, PIC-liposome-binding populations from
Repeated Ig rearrangements 693

Fig. 2. VDJH rearrangements of IgH genes expressed by PIC-binders in three strains of mice. When multiple cells express an identical VDJH rearrangement a rearrangement type is designated. For each unique rearrangement we report a cell identifier number and/or a rearrangement type, the VDJH family, the bona fide or putative germline gene, from which mouse or experiment the cells are derived, the most 3' nucleotides of the VDJH (starting with the Cys - TGG (codon 62, Kabat numbering system)), the D segment sequence, the most 5' end of the Jκ 3-5 regions at either the V-D or D-J junctions, the name of the D segment, the number of the Jκ, the reading frame of the D segment, and the DORS length. Sequences 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 Vκ11, five are Vκ12, one is 3660 and three are Q52. MMVGHAB, MUSGHAA and MMVV0006 are putative germline genes. MMUS3526 and MUSGHOU are bona fide germline genes. We observed one JS58 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 AF00002 (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 PIC-liposome staining (Fig. 1). We used the gates shown in Fig. 1 to sort individual PIC-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 Vκ genes, each from a different family (Fig. 2). The most frequent gene, a putative Vκ11 germline gene (MMVGHAB, Fig. 2), was expressed in 38 of the 47 cells analyzed (81%). The other three genes were the putative germline genes MUSGHAA (Vκ12, five of 47, 11%) and MMUS3526 (Vκ52; three of 47, 6%). One sequence was also isolated from the Vκ1 family JS58. The sorting gates chosen here tend to somewhat overemphasize the homogeneity of the C57 PIC-binders in that studies with BALB/c, where Vκ52 predominates (K. J. Seidt et al., in preparation), demonstrate that Vκ52 PIC-binders stain less brightly than Vκ11 and Vκ12. Thus, if the gates were set to include the dull PIC-binding region, additional Vκ52-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 Vκ52-expressing cells that fell within the sort gates are relatively dull for PIC-liposome staining. The cells that express Vκ11 and Vκ12, in contrast, tend to stain more brightly and thus tend to be more similar to each other than to cells expressing Vκ52. These differences may reflect differences in the affinity of PIC-liposome binding or the presence of endogenous antigen in the combining site (49).

The possible failure to recover some dull PIC-staining cells expressing Vκ52 or other Vκ genes does not detract from the overall conclusion that the C57 PIC repertoire tends to be relatively homogeneous and to include a high proportion of cells expressing Vκ11. Light chain analyses discussed below further underscore the relative homogeneity of this C57 anti-PIC repertoire.
Table 1. \( V_{\mu 11} \) rearrangement type 1 frequency in three mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mouse or experiment no.</th>
<th>Total cells sequenced (no.)</th>
<th>( V_{\mu 11} ) sequences (no.)</th>
<th>( V_{\mu 11} ) RT-1(^{*}) sequences (no.)</th>
<th>( V_{\mu 11} ) RT-1(^{*}) percent of total</th>
<th>RT-1 Percent of total</th>
<th>Percent of ( V_{\mu 11} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>1</td>
<td>15</td>
<td>10</td>
<td>9</td>
<td>67</td>
<td>60</td>
<td>90</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>2</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>83</td>
<td>75</td>
<td>90</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>3</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>90</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>4</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>90</td>
<td>60</td>
<td>67</td>
</tr>
<tr>
<td>BALB/c</td>
<td>1(^{b})</td>
<td>43</td>
<td>8</td>
<td>2</td>
<td>19</td>
<td>9</td>
<td>25</td>
</tr>
<tr>
<td>BALB/c</td>
<td>2(^{a})</td>
<td>56</td>
<td>9</td>
<td>5</td>
<td>16</td>
<td>9</td>
<td>56</td>
</tr>
<tr>
<td>C.B-17</td>
<td>1</td>
<td>18</td>
<td>9</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C.B-17</td>
<td>2</td>
<td>18</td>
<td>7</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*RT-1 denotes rearrangement type 1 (rearrangement type is defined by identity of V, D and J segments, and N/P additions).

Identical rearrangements containing a single \( V_{\mu 11} \) 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 \( V_{\mu} \) gene itself. In fact, prior to somatic mutation, the CDR3 region provides the only sequence that uniquely identifies a rearranged IgH utilizing a particular \( V_{\mu} \) gene. However, although identity for both the \( V_{\mu} \) 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 \( V_{\mu 11} \) rearrangements shown in Fig. 2, 33 out of 38 were identical. Over two-thirds of the \( V_{\mu 11} \) rearrangements isolated from each of the four mice analyzed expressed this IgH (\( V_{\mu 11} \) 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 \( V_{\mu 11} \) 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 \( V_{\mu 11} \) type 1 within each mouse analyzed here are not due to clonal expansion of mature B cells.

IgL sequences were obtained for the \( V_{\mu 11} \) 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: \( V_{\mu 9-J_{\mu} 2} \) was used in 14 of 31 cells (45%) and \( V_{\mu 9-J_{\mu} 4} \) was used in 16 of 31 cells (52%). All of the \( V_{\mu 9-J_{\mu} 2} \) rearrangements were identical as were all of the \( V_{\mu 9-J_{\mu} 4} \). An additional IgL rearrangement, \( V_{\mu 21-E-J_{\mu} 2} \), 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

<table>
<thead>
<tr>
<th>Heavy chain rearrangement typea</th>
<th>Strain</th>
<th>Mouse (Ms) no./Experiment (Expt) no.</th>
<th>Light chain</th>
<th>No. of cellsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>V\textsubscript{H}11 type 1</td>
<td>C57BL/6J</td>
<td>Ms 1</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ms 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ms 3</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ms 4</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>Expt 1</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>1</td>
</tr>
<tr>
<td>V\textsubscript{H}11 type 3</td>
<td>C57BL/6J</td>
<td>Ms 4</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>Expt 1</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}1</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}11 type 4</td>
<td>C.B-17</td>
<td>Ms 1</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}2\textsubscript{\textalpha} J\textsubscript{\textalpha}4</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}12 type 1</td>
<td>C57BL/6J</td>
<td>Ms 1</td>
<td>NA\textsuperscript{c}</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>BALB/c</td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}2</td>
<td>2</td>
</tr>
<tr>
<td>Q\textsubscript{\textgamma}2  type 1</td>
<td>BALB/c</td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}2\textsubscript{\textalpha} J\textsubscript{\textalpha}4</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Rearrangement type is defined by identity of V, D and J segments, and N/P additions within a heavy chain family.

\textsuperscript{b}NA, not available.

\textsuperscript{c}Germline gene is S376632 as opposed to MMIGFWV4. See Fig. 4.

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

<table>
<thead>
<tr>
<th>Heavy chain rearrangement typea</th>
<th>Strain</th>
<th>Mouse (Ms) no./Experiment (Expt) no.</th>
<th>Light chain</th>
<th>No. of cellsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>V\textsubscript{H}11 type 2</td>
<td>C57BL/6J</td>
<td>Ms 4</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}11 type 5</td>
<td>C.B-17</td>
<td>Ms 1</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}1</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}11 type 6</td>
<td>BALB/c</td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}2</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}11 type 7</td>
<td>BALB/c</td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}4\textsubscript{\textalpha} NA\textsuperscript{c}</td>
<td>2</td>
</tr>
<tr>
<td>V\textsubscript{H}12 type 2</td>
<td>BALB/c</td>
<td>Expt 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}5\textsubscript{\textalpha} NA\textsuperscript{c}</td>
<td>2</td>
</tr>
<tr>
<td>Q\textsubscript{\textgamma}2  type 2</td>
<td>BALB/c</td>
<td>Expt 1</td>
<td>NA</td>
<td>2</td>
</tr>
<tr>
<td>Q\textsubscript{\textgamma}2  type 3</td>
<td>C.B-17</td>
<td>Ms 2</td>
<td>V\textsubscript{\textgamma} J\textsubscript{\textgamma}4\textsubscript{\textgamma}5\textsubscript{\textalpha} NA\textsuperscript{c}</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Rearrangement type is defined by identity of V, D and J segments, and N/P additions within a heavy chain family.

\textsuperscript{b}NA, not available.

\textsuperscript{c}Germline gene is S376632 as opposed to MMIGFWV4. See Fig. 4.

Many of the mice analyzed expressed the same Igh/H\textalpha{}L pair. Nevertheless, unique instances of Igh/H\textalpha{}L pairing in each of the individual mice show that at least two to three of the V\textsubscript{H}11 type 1-expressing cells have arisen from different B cell progenitors, either prior to Igh1 rearrangement or after light rearrangement but prior to IGL rearrangement (51,52). In total, at least 12 of the 31 V\textsubscript{H}11 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/H\textalpha{}L 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/H\textalpha{}L pairs that we observed. The vast majority (30 of 31) of the Igl rearrangements found in association with V\textsubscript{H}11 type 1 were V\textsubscript{\textgamma}J\textsubscript{\textgamma}2 and V\textsubscript{\textgamma}J\textsubscript{\textgamma}4. Since the same V\textsubscript{\textgamma}9 germline gene is used in both of these rearrangements, the V\textsubscript{\textgamma}J\textsubscript{\textgamma}2 and V\textsubscript{\textgamma}J\textsubscript{\textgamma}4 light chains could not have derived from one another unless a recombination signal sequence exists in the V\textsubscript{\textgamma}9 germline gene segment. Such an event would predict a strikingly different coding joint of V\textsubscript{\textgamma}J\textsubscript{\textgamma}4 compared to V\textsubscript{\textgamma}J\textsubscript{\textgamma}2. However, a similar coding joint is maintained in cells expressing either of these rearrangements with differences between the two contributed by the J\textsubscript{\textgamma} 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

<table>
<thead>
<tr>
<th>Rearrangement Type</th>
<th>95</th>
<th>100</th>
<th>A</th>
<th>R</th>
<th>S</th>
<th>E</th>
<th>102</th>
</tr>
</thead>
<tbody>
<tr>
<td>VH11 Type 1</td>
<td>Cys Met Arg Tyr Gly Ame</td>
<td>TOP TAC AGA TAT GGT AAC</td>
<td>TAC TGC TAC TCC GAT TGC TGG GGC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 2</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 3</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 4</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 5</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 6</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH11 Type 7</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH12 Type 1</td>
<td>Cys Ala Gly Arg Tyr Srs</td>
<td>TOP GAC GGA GAC AGA TAC</td>
<td>TAC GAT GGC GGG GGG ACC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VH12 Type 2</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q5 Type 1</td>
<td>Cys Ala Arg Arg Tyr Srs</td>
<td>TOP GAC GGA GAC AGA TAC</td>
<td>TAC GAT GGC GGG GGG ACC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Q5 Type 2</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td>--- --- --- --- --- ---</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### CDR3 Regions of Light Chains

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Subgroup</th>
<th>germine gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(\lambda)</td>
<td>J(\lambda)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
<tr>
<td>V(\kappa)</td>
<td>J(\kappa)</td>
<td>VKV</td>
</tr>
</tbody>
</table>

Fig. 4. Amino acid alignment of IgH and IgK 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 light sequences from C57, BALB/c, and C.B-17 mice. Heavy chain rearrangements are categorized by VH family into rearrangement types based on V\(\kappa\), D\(\kappa\), and J\(\kappa\) region identity. Codons 95--102 represent the CDR3 region (numbering according to Kabat (43)). One cell (ABO196) from a separate study of the general BALB/c repertoire is identical to VH11 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 VH11 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 VH germine gene of sequences that match with <100% identity is indicated in brackets. Cells expressing V\(\kappa\)-J\(\kappa\) IgL sequences and matching the putative germine gene MUSIGKA1 are AE0013, AE0024, AE0057, AE0066, AE0067, AE0070, AE0097, AE0109, AE0127, AE0129 [99.6], AE0137, AE0182, AE0183, AE0190 and AE0078. Cells expressing V\(\kappa\)-J\(\lambda\) are AE0039 [92.3], AH0034, AH0076, AF0023 and match the putative VH germine gene MUSIGKA1. Cells expressing V\(\kappa\)-J\(\kappa\) are AE0004, AE0005, AE0011 [99.2], AE0020, AE0024, AE0134, AE0031, AE0032 [94.1], AF0038, AE0075, AF0094, AF0124 [99.6], AF0171 [99.6], AF0178, AE0168 [99.2], AF0187, AE0189, AE0211, AE0003 and also match MUSIGKA1. Cell AE0008 expresses V\(\kappa\)-J\(\kappa\) [95.5] and matches the germine gene MUSIGKV4. Cell AE0038 expresses V\(\kappa\)-J\(\kappa\) and matches putative germine gene MUSIGKLM. Cell AE0075 expresses V\(\kappa\)-J\(\kappa\) and matches germine gene MUSIGKLM. Cell AE0076 expresses V\(\kappa\)-J\(\kappa\) and matches the putative germine gene MUSIGKCB5 [96.3]. This sequence predicts a non-functional IgL protein but distinguishes the origin of AE0076 and AE0075, the other Q5 type 1 sequence. Cells AE0017 and AE0022 express V\(\kappa\)-J\(\kappa\) and the putative germine gene MUSIGKV4. Cell AE0495 expresses V\(\kappa\)-J\(\kappa\) and also matches MUSIGKV4. Cell AH0367 expresses V\(\kappa\)-J\(\kappa\) and uses the germine gene MUSIGKLM. Numbering and subgroups assignments according to Kabat (43). Family assignments according to Koller et al (58). Codons 89--97 define the CDREGION region. The Jk 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 US46503-US46550.
Additional repeated rearrangements now bring 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 $V_{\gamma}12$ and $V_{\gamma}Q52$ repeated IgH rearrangements, only the light chains expressed by cells utilizing $V_{\gamma}Q52$ 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 PIC-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 $V_{\gamma}11$ and $V_{\gamma}12$ IgH described here exhibit sequence identity at the coding junctions (Fig. 2). Rearrangement $V_{\gamma}11$ type 1 utilized a two nucleotide sequence identity (TA) between the $V_{\gamma}$ and DSP segments. In addition, this IgH used a four nucleotide sequence identity (CTAC) between the DSP and J$_{\gamma}$ segments. Seven of 11 ($64\%$) $V_{\gamma}11$-expressing sequences shown here, other than $V_{\gamma}11$ type 1, expressed the TA identity between the V and D segments. Of these same 11 $V_{\gamma}11$-expressing sequences, eight (73%) expressed the CTAC identity between the D and J$_\gamma$ gene segments. Seventy-eight percent (seven of nine) of the $V_{\gamma}12$-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 $V_{\gamma}11$ 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 $V_{\gamma}11$ type 1 rearrangements.

In addition, if the IgH structure encoded by $V_{\gamma}11$ type 1 is particularly well adapted for binding PIC, 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-PIC 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 PIC-ipsosome staining correlate with IgL expression.**

Surprisingly, C57 cells expressing $V_{\gamma}11$ type 1 paired with particular light chains were distinguishable by the amount of surface IgM and PIC staining (Fig. 5). In each C57 mouse, these cells clustered into two fairly distinct groups, according to whether they expressed $V_{\delta}J_\delta_4$ or $V_{\delta}J_\delta_2$. The clusters overlay larger clusters, visible in the contour plot showing
IgM versus Pic 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 different staining levels.

In the C57 mice, V_{4}11 type 1 cells expressing V_{4}9-J_{2}4 always stained brighter for IgM than those expressing V_{4}9-J_{2}4 (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 oblique 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 Pic-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-idiotypic 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 Pic 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-idiotypic 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_{4}11 type 1). This rearrangement has also been detected by analysis of Pic-binding hybridomas in three different strains of mice (BALB/c, B10.H-2^{H}+4^{P}Wts and NZB) (4.10.19.22.23.28). In fact, of 11 previously published hybridomas expressing V_{4}11 type 1, eight expressed the V_{4}9-J_{2}4 IgL and one expressed the V_{4}9-J_{4}4 IgL that predominate in our data set. Identical V_{4}11-expressing IgH rearrangements (not selected for their ability to bind Pic) 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. (22) at 131 is identical to V_{\alpha}11 type 3. However, unlike what we report here for V_{\alpha}11 type 1, no more than two sequences expressed any given repeated rearrangement.

The preference for the V_{\alpha}11 type 1 (V_{\alpha}11-DSR-P_{\alpha}1) rearrangement that we have detected in C57 mice could reflect directed recombination (32,34,50) 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 PIC 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_{\alpha}11 type 1 rearrangements, it is not, as is the case for the other repertoire.

The repeated isolations of V_{\alpha}11 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 PIC repertoire. In other studies, we have shown that vastly different anti-PIC reagents are expressed by PIC-liposome-binding cells from BALB/c mice, which carry the a-allyotype (Igh\textsuperscript{a}) Ig chromosome, and from C.B-17 mice, which carry the Igh\textsuperscript{b} chromosome on the BALB/c genetic background. The anti-PIC reagents of all three strains predominantly consist of three germline V_{\alpha} genes; however, their expression frequency differs from strain to strain. This result suggests that there is a different kind of selection for those PIC-binding B-1 cells that are typically observed in an immune response. Furthermore, anti-PIC 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 chromosome as C.B-17 on a different genetic background, these differences among the anti-PIC reagents in the three strains suggest that the PIC repertoire is influenced by complex genetically controlled mechanisms that maintain and influence the frequency of cells expressing certain V_{\alpha} 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 PIC-binding cells, suggest that B-1 pre-B cells may not expand extensively. Rothstein and colleagues have shown that signaling through the Igh 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 C2r locus (67), CD19 (68), IL-5 (69) or A5 (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 PIC-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 PIC-binding cells.

The restriction of the anti-PIC 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), NF (13,14), p-azaphosphorylarsanate (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 unobscured 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-azaphosphorylarsanate, NP and 2-phenyl-oxazolone. All three of these responses are restricted; however identical repeated Igh are found only in the response to p-azaphosphorylarsanate (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 peripheral B-1a cells than among B-1b cells in a study by Tombrek 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_{\alpha}C_{\delta}D_{\mu} 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 and 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. MacKen- zie and K. J. Seidt, 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.

Acknowledgements
The authors thank Dr Rachel Gerstein, Dr Mario Roederer and Jennifer Winten for helpful discussion and criticism of this manuscript, Omcred Human (Stanford University) and Mr. Jerry Liau (Columbia University) for excellent technical assistance, Dr Tsai-Tsu Wu and George Johnson at Northwestern University for the use of Sequunet and the KABAT database, George Yeltzie, Mai Nguyen and Alan Smith at the Stanford Protein and Nucleic Acid Facility for sequencing, the Stanford FAC5 facility development group for FAC5 maintenance, and the Stanford Program for Molecular and Genetic Medicine computer facility. This work was supported by grants CA 42509 and AI 34762 from the National Institutes of Health. K. S. was supported by NIH EY 07106.

Abbreviations
B-CLL  B cell chronic lymphocytic leukemia
C57  C57BL/6 mice
FSA  fluorescein sulfonic acid
NP  (4-hydroxy-3-nitrophényl)acetil
RC  phosphatidylcholine

References
24. Mercolino, T. J., Arnold, L. W. and Haughton, G. 1986. Phosphatidylcholine is recognized by a series of Ly-1 murine
Repeted Ig rearrangements 701

B cell lymphomas specific for erythocyte membranes. J. Exp.
25 Merculino, T. J., Arnold, L. W., Hawkins, L. A. and Haughton, G.
1968. Normal mouse peritoneum contains a large population of
ly-1+ Derb+ B cells that recognize phototoxic, polyclonal
relationship to cells that secrete hemolytic antibody specific for
27 Curnan, M., Spencer, D. H., Clarke, S. H. and Haughton, G.
1993. Mechanisms that limit the diversity of antibody tree:
three sequentially acting mechanisms that favor the spontaneous
production of an IgM encoded anti-phosphatidyl choline. Int.
Immunol. 5:1363.
28 Arnold, L. W. and Haughton, G. 1992. Autoantibodies to
phosphatidylcholine. The murine aberrant B cells response.
29 Ishora, S., Mombaerts, P., Lafaille, J., Iacomini, J., Nelson, A.,
T cell receptor delta gene mutant mice: independent generation of
alpha beta T cells and programmed rearrangements of gamma
delta TCR genes. Cell 32:337.
heavy chain gene segments: implications from a chromosome
USA 79:4118.
all 14 junctions of sequence homology results in limited junctional
Only one D to JF and DSF2 originate from the same primordial DII H.
33 Gu, H., Forster, I. and Rajewsky, K. 1990. Sequence homologies in
NI sequence insertion and JH gene utilization in VH/DJH joining:
implications for the joining mechanism and the ontological timing
of Ly1 B cell and B-CLL progenitor generation. EMBO J. 9:2133.
34 Kanoto, A. B., Stahl, A. M., Adams, S., Watanabe, K. and
Herzenberg, L. A. 1995. De novo development and self-
cytometry and fluorescence activated cell sorting. In: Weir, D. M.,
The Handbook of Experimental Immunology, p. 251. Blackwell
Scientific, Edinburgh.
Aggregation of different-bearing liposomes mediated by specific
37 Kanoto, A. B. 1986. Monoclonal antibody interactions with hapten-
bearing liposomes. Ph.D. Dissertation, University of California
at Berkeley.
38 Kanoto, A. B., Merrill, C. E., Mackenzie, J. D., Herzenberg, L. A.
as revealed by single cell PCR of FACS-Sorted B cell subsets.
39 Kanoto, A. B., Merrill, C. E. and Hillson, J. L. 1996. Construction of
cDNA from Single Unstimulated B Lymphocytes: Method
Application to the Study of Expressed Antibody Repertoire in
FACS-sorted B Cell Subsets. The Handbook of Experimental
Immunology, Vol. II. Immunchemistry and Molecular Immunology.
Blackwell Scientific, Boston, MA, in press.
40 Kanoto, A. B., Merrill, C. E., Herzenberg, L. A. and Hillson, J. L.
1996. The first unabased analysis of VH-D-JH Sequences from
B-1a, B-1b, and conventional B cells. J. Immunol. 156:1175.
41 Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. and Foeller,
C. 1991. Sequences of Proteins of Immunological Interest, 5th
National Institutes of Health, Bethesda, MD.
42 Gu, H., Tarlinton, D. M., Muller, W., Rajewsky, K. and Forster, I.
1991. Most peripheral B cells in mice are ligand selected. J. Exp.
Med. 173:1257.
43 Winter, D., Diamond, M., Abu-hadid, M., Falkenberg, S. and
Bankert, R. 1995. Allelic differences in the VHH-1 gene explain
the absence of a B cell clonal expansion in the primary responses
of C57BL/6 mice to phtalate. J. Immunol. 155:2446.
44 Ottersen, A., Janeway, C. A., Richardson, N. and Milthorpe, A.
The structure of two highly homologous VH genes used to produce
mation creates diversity in the major group of mouse
46 Ewen, J., Griffiths, G. M., Berek, C. and Milstein, C. 1985. Light
chain germ-line genes and the immune response to 2-phenoxyphenoate.
EMBO J. 4:3349.
47 Hayakawa, K., Camack, C. E., Shinton, S. A. and Hardy, R. R.
VH11 bias and normal V-D-J junctions in SCID B lymphocytes
of nonproductive rearrangements of VH81X gene segments
induces a dependence of B cell clonal maturation on the
editing in self-reactive bone marrow B cells. J. Exp. Med.
177:1257.
immunoglobulin light chain genes are replaced by ongoing rearrangements in germ line kapa genes. Identification of
chain editing in kappa-deficient animals: a potential mechanism
positive selection of murine VH12-expressing B cells in the
55 Pennell, C., McCray, S. and Clarke, S. 1992. VH12 rearrangements in
variable-region-gene families: complexity, polymorphism and use
57 Feeney, A. J. 1992. Comparison of junctional diversity in the neonate and
58 Zhang, Y., Cado, D., Asarnow, D. M., kmori, T., Att, F., Rault, D.
and Allison, J. P. 1995. The role of short homology repeats and
Tra in generation of the invariant gamma delta antigen
receptor repertoire in the fetal thymus. Immunity 3:438.
59 Garstein, R. M. and Lieder, M. R. 1993. Extent to which homology can
constrain coding exon junctional diversity in VDJ recombination.
60 Moms, R. E. and Rothstein, D. L. 1994. Decreased surface IgM
receptor-mediated activation of phospholipase C gamma 2 in B-I
class II expression distinguishes two distinct B cell developmental
62 Tarlinton, D. 1991. Direct demonstration of MHC class II surface
63 Takahashi, A., Turner, M., Schall, S., Moe, P. J., Dudley, L. P.,
receptor-mediated proliferation of B and T cells in the absence
64 Zhang, R., Att, F. W., Davidson, L., Orkin, S. H. and Swat, W.
1995. Defective signalling through the T- and B-cell antigen
702 Repeated Ig rearrangements


Note added in proof