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Immunology

Predominant V_H genes expressed in innate antibodies are associated with distinctive antigen-binding sites

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ABSTRACT **Antibodies to phosphatidylcholine (PtC), a common constituent of mammalian and bacterial cell membranes, represent a large proportion of the natural antibody repertoire in mice. Previous studies of several mouse strains (e.g., C57BL/6) have shown that anti-PtC antibodies are mainly encoded by the V_H11 and V_H12 immunoglobulin heavy chain variable region gene families. We show here, however, that V_H11 and V_H12 encode only a small proportion of the anti-PtC antibodies in BALB/c mice. Instead, V_HQ52-encoded antibodies predominate in this strain. In addition, two-thirds of the cells expressing V_HQ52 family genes use a single gene (which, interestingly, has been previously shown to predominate in the anti-oxazolone response). We also show here that in anti-PtC antibodies from all strains, the distinctive antigen-binding sites associated with V_HQ52 differ substantially from those associated with V_H11 and V_H12. That is, V_HQ52-containing transcripts preferentially use the joining region J_H4 rather than J_H1 and exhibit more diverse complementarity-determining region 3 (CDR3) junctions with more N-region nucleotide additions at the gene segment junctions. Thus, the V_H gene family that predominates in the anti-PtC repertoire differs among mouse strains, whereas the distinctive V_HDJ_H rearrangements (CDR3, J_H) associated with each V_H gene family are similar in all strains. We discuss these findings in the context of a recent hypothesis suggesting that CDR3 structure, independent of V_H framework, is sufficient to define the specificity of an antibody.**

Consistent with the major role natural antibodies play in the innate defense against invading pathogens, antibodies to phosphatidylcholine (PtC, a ubiquitous membrane phospholipid found in both bacterial and mammalian membranes) have recently been shown to protect against acute peritonitis in a murine cecal ligation and puncture model (1). B-1 cells, which produce many natural antibodies, are the sole producers of natural anti-PtC in the mouse (2, 3). Cells producing these antibodies comprise 5–15% of B-1 cells in spleen and peritoneal cavity and bind to PtC on fluorochrome-containing liposomes (3, 4). Thus, they are readily detectable by flow cytometry with a fluorescence-activated cell sorter (FACS).

Previous studies have shown that anti-PtC antibodies are largely encoded by V_H11 and V_H12 in combination with the $V_\kappa9$ and $V_\kappa4$ light chains, respectively (5–11). Other V_H families, such as V_HQ52, V_HJ558, and V_H7183, have been detected at very low frequencies (10, 12–14). We have revisited this issue by sorting individual PtC-binding cells from several mouse strains and by using a single-cell reverse transcriptase (RT)-PCR method (with a promiscuous primer that amplifies V_H genes from all V_H families) to more precisely define the Ig heavy chain variable region sequences these cells express. Our findings agree with the predominance of V_H11 (5–11, 15) in C57BL/6 mice and with the predominance of V_H12 in other mouse strains (we demonstrate it here in C.B-17). However, we surprisingly find that another gene family, V_HQ52, predominates among anti-PtC in BALB/c mice.

We consider the mechanisms underlying these strain differences elsewhere (J.A.W., K.J.S., and L.A.H., unpublished work) and focus here, instead, on comparison of the full rearrangement sequences [joining region (J_H) and diversity region (D) usage, N-region addition, etc.] of the large number of anti-PtC antibodies isolated in the course of these genetic studies.

Examination of these anti-PtC sequences demonstrates that each of the three variable region gene families has distinctive antigen-binding site characteristics. We show that anti-PtC antibodies encoded by the V_HQ52 gene family are more diverse with respect to N-region addition and other complementarity-determining region 3 (CDR3) features than those encoded by V_H11 and V_H12 gene families. Furthermore, we show that V_HQ52 is mainly associated with J_H4, whereas V_H11 and V_H12 are mainly associated with J_H1. This close association between V_H gene family and CDR3 structure challenges the recently proposed idea that CDR3 structure *per se* is sufficient to define antibody specificity (16). Thus, we discuss our findings in the context of this model as it applies to anti-PtC and other natural antibodies.

MATERIALS AND METHODS

Mice. C57BL/6J (Igh^b), BALB/cnHz (Igh^a), and C.B-17/Hz (BALB/c.Igh^b) females, 5–6 months of age, were bred and housed at Stanford or were a kind gift from R. Riblet (Torrey Pines Institute for Molecular Studies, San Diego, CA).

FACS Reagents and Staining. Reagents, cell preparation, staining, and FACS sorting methods were described previously (17). For single-cell sorting, cells were stained with fluorescein-encapsulated liposomes (PtC-liposomes) (3, 4, 15), phycoerythrin-conjugated anti-IgM (331.12), and allophycocyanin-conjugated anti-CD5 (Ly-1, 53–7). Dead cells were excluded by propidium iodide.

For FACS measurement of V_H-family frequencies, cells were stained with PtC-liposomes, anti-V_H11 ($V_H11Id.6e9$)

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Abbreviations: PtC, phosphatidylcholine; FACS, fluorescence-activated cell sorter; PFC, plaque-forming cells; RT-PCR, reverse transcriptase–PCR; V_H, immunoglobulin heavy chain variable region; D, diversity region; J_H, joining region; CDR3, V_H complementarity-determining region 3.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF118930 to AF119039).

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(18) (kind gift from G. Haughton, Univ. of North Carolina), anti-V_H12 (CH27Id.5c5) (also from G. Haughton), anti-IgD (11-26), propidium iodide, anti-IgM^a (DS-1), anti-IgM^b (AF6-78), and antibodies specific for T cells, macrophages, and granulocytes conjugated to the same fluorochrome (Dump) (PharMingen). Samples from the latter studies were analyzed on a flow cytometer modified at Stanford to simultaneously detect eight fluorochromes (19).

Single-Cell Sorting and Sequence Analyses. PtC-binding cells were first bulk-sorted either from individual C57BL/6 and C.B-17 mice or from a pool of either 5 or 9 BALB/c mice and then resorted to deposit individual cells into tubes for single-cell sequence analysis, and FACS phenotype information was collected for individually sorted cells. These methods, and methods used for RT-PCR amplification and sequencing of V_H, and the sequence analysis software tools, have all been described (15, 20, 21). Sequences were aligned to a germ-line database that we have assembled (A.B.K., J.D.M., K.J.S., and L.A.H., unpublished work).

Detection of Antibody Production by PtC-Binding Cells. Peritoneal cells pooled from 13 three-month-old BALB/c female mice were stained with PtC-liposomes, anti-CD5, and anti-IgM (see above) and sorted on the basis of level of staining with PtC-liposomes. PtC-bright, -intermediate and -negative sorted populations and mock-sorted total peritoneal cells were put into culture in RPMI medium 1640 supplemented with 10% fetal calf serum, L-glutamine (290 μg/ml), penicillin (100 units/ml), and streptomycin (70 μg/ml)) for 48 hr at a density of 10^6 cells per ml in the presence or absence of *Salmonella typhosa* lipopolysaccharide (LPS) (50 μg/ml). Sort purity was 98% for PtC-bright, 69% for PtC-intermediate, and 68% for PtC-negative cells. Cells were then resuspended at a concentration of 5×10^6 cells per ml and tested for the ability to lyse bromelain-treated mouse erythrocytes (BrMRBC) in a complement-dependent plaquing assay (PFC) as previously described (22).

RESULTS

Anti-PtC Antibodies in BALB/c, C57BL/6, and C.B-17 Use the Same V_H Families, Although at Markedly Different Frequencies. Consistent with previous findings, our single-cell RT-PCR and sequencing studies show that anti-PtC antibodies in C57BL/6 mice are predominantly V_H11-encoded (15). Surprisingly, however, anti-PtCs from BALB/c mice are predominantly encoded by the V_HQ52 family (Fig. 1 *Upper*; Table 1). C.B-17 mice, which are congenic to BALB/c but carry an IgH^b chromosome region similar to C57BL/6, show a third anti-PtC pattern: V_H12 predominates; V_H11 is common; and V_HQ52 is rare. V_HJ558 family genes are used equally by all strains, at frequencies below the other three gene families. Despite these differences, representatives of each V_H family are found among the anti-PtC antibodies in all strains (Fig. 1, Table 1).

The V_H gene frequencies in anti-PtC in the three strains are mirrored by data from multiparameter FACS studies in cells that were stained with fluorochrome-coupled monoclonal antibodies to V_H11 and V_H12 (Fig. 1). In initial studies confirming the specificity of these antibodies, cells costained with PtC-liposomes and each of the antibodies coupled to a different fluorochrome were FACS-sorted and analyzed by RT-PCR and sequencing. Results showed that all cells stained with anti-V_H11 expressed V_H11 sequences (19/19); all cells stained with anti-V_H12 expressed V_H12 sequences (18/18); and all PtC-binding cells that failed to bind either anti-V_H11 or anti-V_H12 expressed V_H genes from other families. Furthermore, roughly two-thirds of these latter cells, which were sorted from BALB/c mice, expressed V_HQ52. FACS staining and analysis of peritoneal cells from individual BALB/c, C.B-17, and C57BL/6 mice reveal a V_H gene representation pattern among PtC-binding cells that is largely similar to the pattern obtained by single-cell RT-PCR (Fig. 1 *Lower*). The FACS data most likely constitute the more accurate view

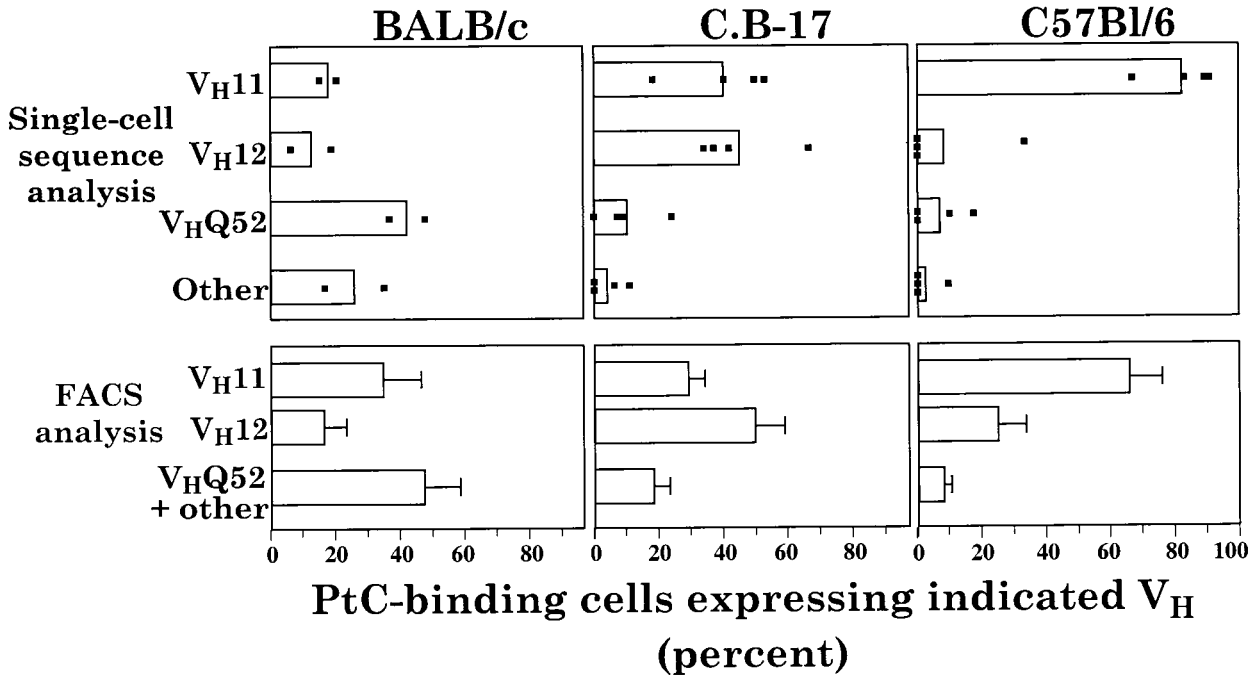

FIG. 1. V_H gene family expression among PtC-binding B cells. (*Upper*) Bars represent the average V_H family representation based on single-cell data from individually sorted PtC-binding cells. V_H genes other than V_H11, V_H12, and V_HQ52 are designated "Other." Number of FACS-sorted individual (single) cells analyzed by RT-PCR and sequencing for each strain: 43 and 58, from two BALB/c pools (5 and 9 animals); 19, 17, 33 and 36, from four C.B-17 mice; and 15, 12, 10, and 10 from 4 C57BL/6 mice. Each point in the figure represents the V_H family frequency found for a single mouse or pool of mice (BALB/c). (*Lower*) Bars with standard deviations represent the V_H family representation among all PtC-binding cells determined by FACS for individual mice and averaged for the figure. Number of mice analyzed: 12 BALB/c, 8 C.B-17, and 6 C57BL/6.

Table 1. V_H family representation in PtC-binding cells

Strain*	V_H rearrangements		
	Family	Total no.	Unique no.
BALB/c	V_H1 (J558)	11	
(n = 101)	V_H10 (DNA4)	2	
	V_H8 (3609)	1	
	V_H8 (3609N)	1	
	V_H12	14	12
	V_H3 (3660)	3	
	V_H3 (3660b)	2	
	V_H7 (S107)	1	
	V_H11	18	5
	V_H2 (Q52)	44	43
	V_H5 (7183)	4	
C.B-17	V_H1 (J558)	1	
(n = 36)	V_H6 (J606)	1	
	V_H12	13	
	V_H11	17	15
	V_H2 (Q52)	4	3

*Sequence representation in C57BL/6 has been published elsewhere (5–11, 15).

because more cells per animal and more animals per strain were examined.

V_HQ52 PtC-Binders Secrete Anti-PtC Antibodies. V_HQ52 PtC-binding cells tend to bind fewer PtC-liposomes than cells expressing V_H11 anti-PtC (ref. 15 and Fig. 2). To determine whether V_HQ52 PtC-binding cells secrete anti-PtC antibodies, we FACS-sorted three fractions of PtC-binding cells (negative, intermediate, and bright PtC-binding cells) from BALB/c, stimulated the sorted cells *in vitro* with lipopolysaccharide, and tested for anti-PtC secretion in a standard plaquing assay (complement-dependent lysis of bromelain-treated mouse erythrocytes). Results showed that the intermediate and bright anti-PtC populations were equivalent with respect to plaque-forming cell (PFC) activity (3 PFC/100 PtC-binding cells cultured in both cases; equivalent to the overall PtC-binding population).

In addition, as expected, PFC activity in these populations was enriched at least 7-fold in comparison with the activity in the negative PtC-binding population (0.4 PFC/100 cells cultured), which may have included a few very dull PtC-binding cells. Thus, like V_H11 and V_H12 anti-PtCs, anti-PtCs encoded by V_HQ52 are functional antibody-producing cells whose activity is readily detectable in the standard anti-PtC PFC assay.

PtC-Binding Cells Mainly Express a Single Germ-Line Gene from Each of the Predominant V_H Families. Although the V_HQ52 family is the third-largest V_H family (reviewed in ref. 23), a single V_HQ52 germ-line gene, MMU53526 also known as V_HOX-1, predominates among V_HQ52 PtC-binding cells. This gene, which represents 68% of V_HQ52 anti-PtC in BALB/c, has previously been shown to encode the dominant idiotype produced by BALB/c (Igh^a) in response to phenyloxazolone (30) and phthalate (24). Consistent with evidence that V_HOx-1 does not predominate in this response in similarly immunized Igh^b mice, a recent study failed to detect the germ-line gene encoding the V_HOX-1 sequence in C57BL/6 mice (24). However, this finding represents either a technical failure or a genetic difference among Igh^b mice, since V_HOX-1 is present in V_HQ52 anti-PtC antibodies from C.B-17 and C57BL/6, which both carry IgH^b chromosomes. In contrast to the tendency to use a single germ-line gene in each of the predominant V_H gene families in anti-PtC, seven different V_HJ558 genes are used in anti-PtC antibodies.

Within the V_H11 family, which is estimated to have only 1 or 2 germ-line genes, PtC-binding cells from C57BL/6 and BALB/c mice express the same V_H11 germ-line gene (MMIGVHAB) (15). V_H11 PtC-binding cells from C.B-17 mice also use only a single V_H11 germ-line gene; however, it differs slightly in sequence from that used in C57BL/6 and BALB/c mice—i.e., by conserved nucleotide changes in codons 29 [framework region 1 (FR1)] and 84 (FR3). Thus, at the amino acid level, C.B-17, BALB/c, and C57BL/6 mice all express the same V_H11 product.

A single germ-line gene (MUSIGHAAM) is also expressed in V_H12 anti-PtC and, like V_H11, the germ-line gene expressed in C57BL/6 and BALB/c mice differs slightly from that expressed in C.B-17. However, while the nucleotide changes in codons 20 (FR1), 62 (CDR2), and 73, 74, 79, and 80 (FR3) of this V_H12 germ-line gene result in conserved amino acids, a change in codon 21 (FR1) results in a change from alanine (nonpolar hydrophobic) to threonine (polar but uncharged). Therefore, C.B-17 mice express a V_H12 anti-PtC antibody highly homologous to but nevertheless distinct from that expressed by BALB/c and C57BL/6.

J_H Segment Usage and CDR3 Characteristics Differ According to the Associated V_H. Although there are strain-specific differences in the V_H expression frequencies among PtC-binding cells, characteristic J_H and CDR3 regions are associated with each of the predominant V_H gene families. The characteristics of these rearrangements are documented in the sections that follow, in which we analyze sequences obtained for each V_H gene family independent of strain of origin. Repeated rearrangements occur frequently among PtC-binding cells (15); however, these are counted only once in the

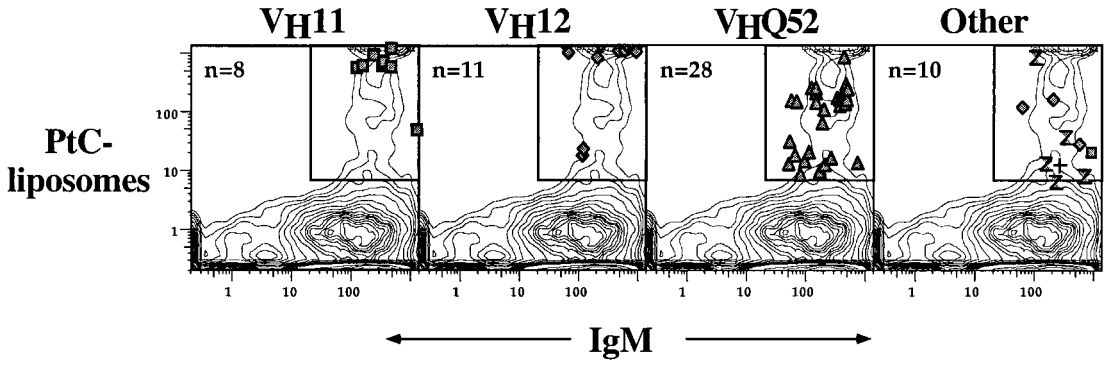

FIG. 2. V_HQ52 expression is enriched among intermediate PtC-binders. The FACS phenotype of individual BALB/c PtC-binding cells is overlaid on the FACS plot for the B cell population from which the cells were sorted. The PtC-binding cells were sorted from a pool of 9 BALB/c mice. The three most frequently expressed V_H families (Q52, V_H11, and V_H12), are displayed separately from other V_H families (V_HJ558, V_H10, V_H3660, V_H7183). Specialized FACS hardware and software developed at Stanford were used to determine the precise phenotype of cells that were sorted and subsequently analyzed by single-cell RT-PCR and sequencing.

following analyses, which are based on data from 48 V_HQ52, 27 V_H11, 28 V_H12, and 12 V_HJ558 anti-PtC sequences.

J_H Usage. V_H11 and V_H12 preferentially use J_H1 (89% and 100%, respectively), whereas V_HQ52 preferentially uses J_H4 (68%). In contrast, V_HJ558 uses all four J_H segments in roughly equivalent amounts (17–33%) (Fig. 3). D segment usage also differs among the families, with V_H11 and V_HQ52 being more similar than V_H12 (Fig. 3).

CDR3 Diversity. Most of the anti-PtC antibodies that have N-region additions at one or both junctions are encoded by V_HQ52 genes, whereas V_H11 and V_H12 often have no insertions at all (Table 2; Fig. 4). Consistent with this observation, V_HQ52 CDR3 length is extremely heterogeneous, whereas V_H11 and V_H12 have homogeneous CDR3 lengths (Fig. 4).

CDR3 Homology. The conserved CDR3 amino acid sequence (codons 95–102) in V_H11 and V_H12 anti-PtC differ as previously reported (18, 25). V_HQ52 sequences differ from those in either V_H11 or V_H12 and are conserved only from positions 100H to 102 [Y100H Y100I (A/Y)100J M100K D101 (Y/V)102], which are derived from the J_H4 gene segment. V_HJ558 sequences differ from those in V_HQ52 but are also conserved from positions 100H to 102 [Y100H W100I Y100J F100K D101 (V/Y)102]. The positions 100H to 102 are conserved among the V_HJ558, V_H11, and V_H12 families because of their predominant use of J_H1 (Fig. 3) and may represent a PtC-binding motif. However, the amino acids conserved at these positions are not the same as those conserved among the V_HQ52 (J_H4) sequences.

Comparison of derived amino acid sequences of the entire length of the V_HJ558, V_H11, V_H12 and V_HQ52 germ-line genes (including the CDR1, CDR2 and FW regions) did not reveal any unexpected amino acid motifs shared among all PtC-binding antibodies.

Table 2. N-region addition in PtC-binding cells

V_H in BALB/c, C.B-17, and C57BL/6 PtC-binding cells		N-region addition at the V–D/D–J junction, % of sequences			
Family	no.	0/0	0/>1	>1/0	>1/>1
V_H11	27	67	15	19	0
V_H12	28	54	7	32	7
V_HJ558	12	50	8	25	17
V_HQ52	48	8	0	60	31
Total PtC-binders	115	37	6	40	17

Junctional Sequence Overlap. Sequences lacking N-region nucleotides often exhibit sequence overlap (homology) such that either coding end participating in a joint could have contributed the observed nucleotides at the joint. Consistent with this, V_H11 and V_H12 commonly lack N-region insertions (Table 2) and tend to have more joints with sequence homology—e.g., of the V_H11 N-less sequences, two or more nucleotides of sequence overlap occurs at 68% (15/22) of the V–D junction, and 70% (16/23) of the D–J junctions.

DISCUSSION

Recent studies have shown directly that the innate production of antibodies to PtC plays a central role in protection against acute peritonitis in a murine cecal ligation and puncture model (1). These antibodies, which also react with PtC revealed on mouse erythrocyte membranes by protease treatment, are produced by B-1 cells that constitute a major fraction (5–15%)

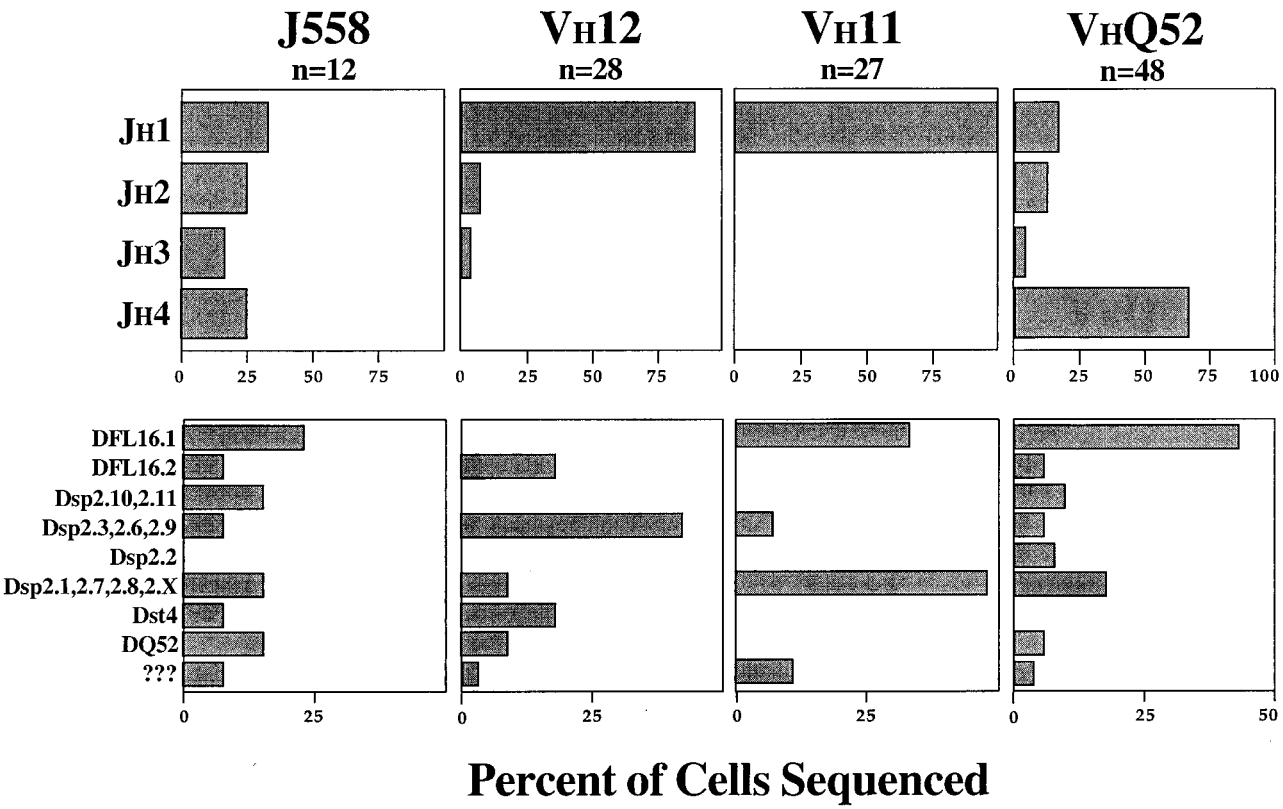

FIG. 3. J_H and D gene usage differ for the four most frequently expressed V_H families among anti-PtC antibodies. Analyses shown are based on unique sequences combined from all three strains of mice (BALB/c, C57BL/6, C.B-17). Data are expressed as percentage of cells sequenced within each V_H family. Some D segments are at times indistinguishable from each other and are therefore grouped; several could not be identified and are grouped as "???". Reading frame usage is not significantly different among the V_H families (not shown). There is no significant difference between V_H12 and V_H11 for J_H usage (Kruskal–Wallis, $P = 0.09$). J_H usage of V_H11 and V_H12 significantly differs from V_HJ558 and V_HQ52 (Kruskal–Wallis, $P < 0.0002$ for all combinations). The usage of V_HQ52 and V_HJ558 significantly differ from one another ($P = 0.02$).

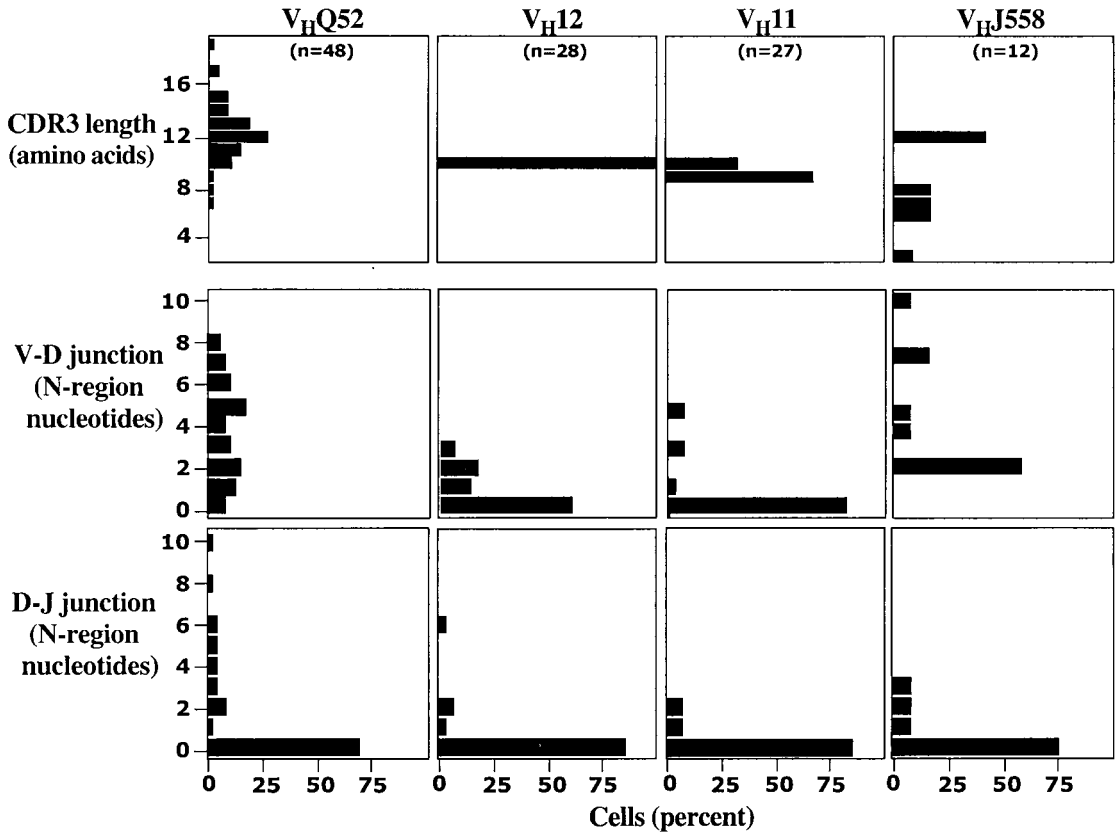

FIG. 4. CDR3 length and N-region addition differ among the four predominant V_H families expressed in the anti-PtC repertoire. Frequency distributions for N-region nucleotides at the V–D and D–J junctions, and CDR3 length (number of amino acids) are shown. Differences in CDR3 length among the V_H families are all significant (Kruskal–Wallis, $P < 0.001$). The only significant differences ($P < 0.01$) in number of N-region insertions were between V_HQ52 and both V_H11 and V_H12 at the V–D junction ($P < 0.0001$).

of the B-1 population in the peritoneal cavity and the spleen (3). Two V_H gene families, V_H11 and V_H12, have been shown to participate or predominate among anti-PtC antibody produced in the mouse strains studied thus far (C57BL/6-related mice, SM/J, NZB, BALB/c) (5, 7–12, 26). These genes are expressed in anti-PtC hybridomas and B cell tumors originating in these animals. The IgH they encode have largely been characterized from these sources.

Here, we use FACS-sorting and single-cell RT-PCR and sequencing methods to characterize the IgH expressed by B-1 cells that bind PtC. We confirm the V_H11 predominance in C57BL/6 and demonstrate that V_H12 predominates among anti-PtC in C.B-17 (BALB/c.Igh^b). Further, we introduce a third gene family to the anti-PtC repertoire, V_HQ52, and show that it predominates among anti-PtCs in BALB/c mice. However, despite this dramatic differential predominance, we find all three V_H gene families represented among anti-PtCs in each strain. In addition, we find that each V_H family is associated with a characteristic J_H, D_H, and CDR3 region regardless of whether it is the major or minor component of the anti-PtC repertoire in a particular strain.

An earlier study identified V_HQ52 as well as the other V_H genes as components of the anti-PtC repertoire in BALB/c mice (5) but examined the relative frequencies of these genes indirectly, by hybridoma analysis. This previous study failed to recognize the predominance of V_HQ52 described here, either for methodological reasons or perhaps because the substrains of BALB/c or the maintenance conditions of the mice differ from our own. The genetic, immune, and developmental mechanisms underlying the differential V_H predominance that we observe will be considered elsewhere, in the context of evidence that bears directly on these issues. We report these finding here mainly to show that, in all strains, a given V_H is typically associated with a characteristic CDR3 region.

Specifically, although V_H representation in anti-PtC differs among mouse strains, in all strains we find that V_H11 and V_H12 anti-PtCs are mainly associated with J_H1 and with CDR3 regions that are homogeneous with respect to length. In contrast, V_HQ52 in anti-PtC is mainly associated with J_H4 and with heterogeneous CDR3s that vary substantially in length. This finding suggests that, to be successful as PtC-binding antibodies, anti-PtC encoded by V_H11 and V_H12 must associate with substantially different J_H and CDR3 structures than anti-PtC encoded by V_HQ52.

Taken at face value, these findings conflict with a recent proposal by Davis *et al.* (16) that V_H genes contribute very little to antibody specificity. These authors point out that the antigen-binding capabilities of the diverse CDR3 structures generated in association with a single V_H should be sufficient, in combination with somatic mutation and antigen selection, to generate the entire range of antibody specificities in the murine repertoire. We do not disagree in principle; however, the selective association of V_H and CDR3 structures in anti-PtC that we have demonstrated suggests more complexity than this model allows.

This contradiction is readily resolved by recognizing that innate antibodies to antigens such as PtC must exist prior to antigen encounter and hence cannot necessarily rely on somatic mutation and selection as a means of defining specificity (for an antigen yet to be encountered). Thus, because B-1 cells are the primary producers of innate antibodies present in circulation (29), we suggest that the model of Davis *et al.* is suitable for B-2 cells, whereas a model that allows germ-line V_H genes to influence antibody specificity is more appropriate for B-1 cells. Evidence that V_H usage (20, 27) and repertoire-

shaping B cell development mechanisms (28, 29) differ between B-1 and B-2 cells is consistent with this dual model, as is the idea that many V_H genes expressed in B-1 cells have evolved to provide innate protection against invading pathogens (29).

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