Generation of heterogeneous rabbit anti-DNP antibodies by gene conversion and hypermutation of rearranged V_L and V_H genes during clonal expansion of B cells in splenic germinal centers

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The mechanisms described here account for development of the heterogeneous high-affinity anti-DNP antibodies that rabbits can produce. Rearranged immunoglobulin light and heavy chain genes from single DNP-specific splenic germinal center B cells were amplified by PCR. We found that in clonal lineages, rearranged V_κ and V_H are further diversified by gene conversion and somatic hypermutation. The positive and negative selection of amino acids in complementarity-determining regions observed allows emergence of a variety of different combining site structures. A by-product of the germinal center reaction may be cells with sequences altered by gene conversion that no longer react with the immunizing antigen but are a source of new repertoire. The splenic germinal center would thus play an additional role in adults similar to that of the appendix and other gut-associated lymphoid tissues of young rabbits.

Key words: Rabbit / Gene conversion / Germinal center / Immunoglobulin light chain / Spleen

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1 Introduction

In rabbits, diversity generated by combinatorial mechanisms at the *Igh* locus is more limited than in mice and humans, because 80–90% of the V_HDJ_H rearrangements utilize the 3' most (D-proximal) gene of the V_H cluster, V_H1 [1–3]. In the young developing rabbit, rearranged V_H1DJ_H genes undergo further diversification by somatic gene conversion-like and hypermutation mechanisms in bursa-like organs in gut-associated lymphoid tissues such as the appendix and sacculus rotundus [4–8]. Non-reciprocal exchange has not been formally shown in rabbits whereas studies in chickens [9] showed that donor sequences were unaltered after a gene conversion event. Although the mechanism in rabbits should be referred to as gene conversion-like rather than gene conversion, for brevity we will henceforth refer to the process as gene conversion. Recently we reported that in contrast to the limited rabbit V_H gene repertoire, a diverse set of $V_\kappa J_\kappa$ sequences are expressed as κ L-chains, the major L-chain isotype of normal rabbits [10]. Thus, expression of a diverse set of V_κ genes further compensates for the limited combinatorial diversity at the *Igh* locus. We identified at least 39 rabbit germ-line V_κ genes, of which at least 28 were expressed. This is a conservative estimate and the numbers could be as high as 142 and 80 [10]. With so many V_κ genes to draw upon, it was of interest to see whether rabbit L-chain sequences are further diversified by gene conversion during Ag-driven primary immune responses in secondary lymphoid sites. We and others recently showed that gene conversion of rearranged V_HDJ_H sequences not only occurs in gut-associated lymphoid tissues, but also within germinal centers (GC) of immunized adult rabbits during clonal expansion of Ag-specific B cells [11–13]. At present there are no convincing data documenting whether somatic gene conversion contributes to diversification of rearranged rabbit V_κ sequences, although an earlier study suggested that this may be the case [14]. To look for definitive evidence for gene conversion, we investigated diversification of rearranged V_κ sequences in splenic GC during T cell-dependent immune responses to DNP-bovine γ globulin (DNP-BGG) in adult rabbits by combining microdissection of single Ag-specific B cells with a PCR-based sequencing strategy

[I 20810]

Abbreviations: GC: Germinal center **CDR:** Complementarity-determining region **BGG:** Bovine γ globulin **FR:** Framework region **AP:** Alkaline phosphatase

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that avoids PCR artifacts. We determined genealogical relationships between B cells that were undergoing clonal expansion, based on nucleotide sequences from single cells [11, 12]. Since only a few rabbit germ-line V_κ genes were known, we generated a database of germ-line V_κ genes [10] so that we could determine the V_κ gene that was productively rearranged in the cell that founded the clone as well as identify candidate donor V_κ genes for blocks of changes (gene conversions) that occurred in the rearranged V_κ gene. Here we present definitive evidence that gene conversion and hypermutation alter the sequences of rearranged V_κ genes in developing splenic GC during a primary immune response to the T cell-dependent hapten DNP. In addition we show the development of different potential heavy and light chain pairs through gene conversion that affects amino acids in complementarity-determining regions (CDR). Our observations help to account for the rabbit's known ability to produce heterogeneous high-affinity anti-DNP antibodies [15].

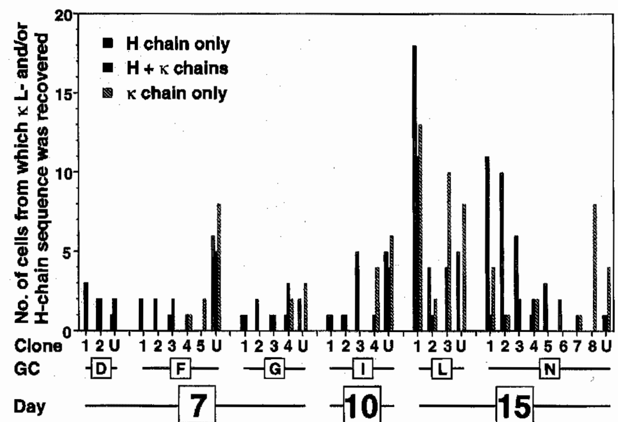

Fig. 1. Summary of clones found in DNP^+ splenic GC at days 7, 10, and 15 of the immune response to DNP-BGG. 'U' denotes clonally unrelated cells from any given GC with unique independent rearrangements. GenBank accession numbers of $V_\kappa J_\kappa$ sequences of GC D: AF211350-AF211356; F: AF211357-AF211375; G: AF211376-AF211385; I: AF211-386-AF211402; L: AF211403-AF211451; N: AF211452-AF211479. GenBank accession numbers of V_HDJ_H sequences of GC D: AF058512-AF058521; F: AF058522-AF-058540; G: AF058541-AF058552; I: AF058584-AF058602; L: AF087712-AF087754; N: AF087755-AF087796 [11, 12].

2 Results

2.1 General remarks

We report here analyses of sequences recovered from single B cells collected from six DNP^+ splenic GC. The genealogical relationships among the B cells undergoing clonal expansion within these GC were inferred from the nucleotide sequences of the rearranged V_κ and V_H genes recovered from individual cells. The numbers of clonally related cells from which κ L-chain and/or H-chain sequences were recovered from DNP^+ splenic GC at the various time points tested during the immune response to DNP-BGG are summarized in Fig. 1. We analyzed three GC from day 7, one from day 10, and two from day 15.

In some instances, it was difficult to identify with certainty the germ-line V_κ gene that was used in the $V_\kappa J_\kappa$ rearrangement because there are germ-line V_κ gene sequences that differ from each other by only a few base pairs. Nevertheless, when we searched independently rearranged V_κ sequences between codons 1 and 88 against our germ-line V_κ database, 16 of 40 (40%) of the V_κ sequences from days 7 and 10 had zero to nine nucleotide differences from a germ-line V_κ gene. However, by day 15, 18 of 22 (82%) had ten or more nucleotide differences from any of the known germ-line V_κ gene sequences. In previous analyses of rearranged V_H recovered from such DNP^+ GC, we also found more V_H sequences that were close to germ-line on days 7 and 10 than on day 15 [11, 12].

2.2 Gene conversion occurs during clonal expansion of B cells with rearranged $V_\kappa J_\kappa$ that are germ-line in sequence

Analysis of the nucleotide changes occurring in the rearranged V_κ and V_H sequences recovered from clonally expanding GC B cells revealed that changes occur by somatic gene conversion and hypermutation mechanisms in both the rearranged V_κ and V_H genes. Examples of clones recovered from GC at day 7 of the anti-DNP immune response (G4 and D2) where the precursor cell had a rearranged V_κ gene that was germ-line or close to germ-line in sequence are shown in Fig. 2. In clone G4, the precursor cell (Ga 16) had a rearranged $V_\kappa J_\kappa$ (k-151-J_κ2) and V_HDJ_H (V_H1-D2x-J_H4) that were germ-line in sequence. During clonal expansion, nucleotide changes occurred in both rearranged V_κ and V_H genes. The κ L- and H-chain sequences (and changes that occurred during clonal expansion) are indicated in black and grey, respectively. Changes of the sequence of the rearranged V_κ gene in cell Ga 22 are accounted for as a block of gene conversion in part of framework region (FR)2 and LCDR2 (indicated as a horizontal black box); the donor for this block could have been germ-line V_κ genes 1–065 or 1–108 [10] (Fig. 2). In clone D2, the precursor cell had a rearranged V_κ gene that was close to germ-line in sequence. The rearranged V_H gene in the precursor cell had undergone changes by gene conversion (indicated

G4

Ga 17 Ga 16 Ga 22 Ga 14 Ga 12

L + H chains

L chain

Unmutated precursor

Mutated precursor

D2

P Da 12 Da 13

Fig. 2. Examples from day 7 of the anti-DNP immune response showing evidence for gene conversion during clonal expansion of precursor cells that had a rearranged V_κ gene that was germ line or close to germ line in sequence. Deduced genealogical trees for clones G4 and D2 are shown along with the changes [codon position: new amino acid or silent change (lower cases 's')]. Blocks of gene conversion that can be accounted for by known germ-line V_κ and V_H genes are shown within black and grey boxes, respectively. The hypothetical unmutated and mutated precursor are indicated in the schematic diagram. Individual GC are designated by capital letters, sections within a GC are designated by lower case and cells are numbered. A suffixed '-k' or '-H' was added to the cell name to indicate κ L- and H- chain sequence, respectively. L-chain (black) and H-chain (grey) sequence alignment, clonal relationships, and a summary of the base changes, replacements and gene conversions (sequence in horizontal boxes) that occurred during clonal expansion of B-cells are shown. The codon positions (Kabat numbering [16]) are shown above the nucleotide sequences of the rearranging germ-line V_κ and V_H genes. With the exception of CDR3, the FR and CDR codon positions that were invariant are not shown. The amino acid sequence and replacements are shown in single letter code (italicized) below the κ L-chain (in black) and the H-chain sequences (in grey); silent base changes are in lower case. The black dashes (-) and grey dots (.) indicate identity to the corresponding nucleotides in the κ L- and H-chain sequence, respectively. The gene conversion blocks in the κ L- and H-chain are shown within black and grey boxes, respectively. The name of the candidate donor gene(s) for the block of gene conversion is underlined. Point mutations that are superimposed on the blocks of gene conversion are shown as discontinuities in the outline of the box.

by a horizontal grey box) as well as point mutations; many of the changes leading to amino acid replacements occurred in the CDR (Fig. 2). During the process of clonal expansion, cell Da 13 underwent a block of gene conversion in FR1 of the κ L-chain (horizontal black box). A candidate donor gene for the block could be k-062 (Fig. 2). These examples provide evidence that gene conversion occurs in the rearranged L-chain genes during clonal expansion of B cells in Ag-specific GC.

2.3 Analyses of the largest expanded clone from day 15

By day 15 of the anti-DNP response, we found clones with larger numbers of members (Fig. 1) and both L- and H-chain sequences were more diversified from germ line. Fig. 3 shows the clonally related cells in clone L1 from which either both L- and H-chain (black squares) or only L-chain sequences (red circles) were recovered. We recovered 42 cells from GC L that belonged to clone L1. Of the 29 members based on H-chain sequences [12], 11 also yielded V_κ sequences. Ten of these sequences encoded functional L-chain; one contained a stop codon. In addition, we obtained 13 clonally related cells from which we only recovered L-chain sequences. Because the $V_\kappa J_\kappa$ sequences were highly diversified and we were unable to definitively identify the germ-line V_κ gene that rearranged, the $V_\kappa J_\kappa$ sequences were compared to that of the key cell La 55 at the hub of the pedigree (Fig. 3). The clonal relationships previously deduced based on $V_H DJ_H$ sequences [12] were fully compatible with the $V_\kappa J_\kappa$ data. Five other cells in addition to cell La 55 had an identical κ L-chain sequence (underlined in red). Three postulated precursor cells (P) and cell Lb 92 (one of five cells with identical $V_H DJ_H$ sequence) were inferred to also have the same kappa amino acid sequence (dotted red underline). There were only two replacement changes in HCDR3 and few in LCDR3. Although cells with the combination of LCDR and HCDR amino acids found in La 55 predominated in the GC, additional modifications, some of which affected LCDR or HCDR sequence, were still occurring. Again in clone L1 some of the changes found in sequences from the other cells were accounted for as blocks of gene conversion substitutions that could have been derived from known candidate donor sequences (symbolized by red and black boxes, for L- and H-chains, respectively).

2.4 L- and H-chain amino acids of the anti-DNP combining site

Early affinity-labeling studies [17], confirmed in subsequent investigations [18, 19] showed that rabbit anti-DNP combining sites contained tyrosine residues. Studies of spectral shifts upon hapten binding to anti-DNP suggesting that tryptophan was present in the combining sites [20] were also confirmed in later studies [21, 22]. A 2.9 Å resolution structure of a mouse anti-DNP-spin-label mAb [22, 23] Fab fragment with bound hapten showed the hapten sandwiched between tryptophan residues at HCDR3 position 96 and LCDR3 position 91 [24]. In addition, Tyr 34 L and Gln 89 L made contact with the 2-nitro group of the DNP ring. Table 1 summarizes the amino acids in this and other DNP-binding Ig that provide contacts and/or play important roles in stabilizing the combining sites [25–28]. Our studies suggest that many CDR structures are able to yield DNP binding sites in rabbits. Nevertheless, we found some recurrent amino acids in the HCDR of different anti-DNP clones including Tyr at position H32, Tyr (Phe or Ile) at H50, H99 or H100 and Ile (Trp, Ser or Phe) at H100 or H100A [12] (Table 1). In the largest clone studied (clone L1, Fig. 3), L- and H-chain sequences found in cell La 55 predominated. Tyr residues that could contribute to interaction with the DNP hapten were found in LCDR3 (amino acid position 92) and HCDR3 (positions 96 and 99). Antigen may have selected cells with particular CDR sequences for entry into and/or expansion within DNP-specific GC [29].

2.5 Selection of amino acids in an expanding day 15 clone

Another clonal group from GC L was L3 [12]. In addition to providing further evidence for the active diversification of $V_\kappa J_\kappa$ sequences by gene conversion, analyses of the amino acid changes introduced in clone L3 were particularly informative because some amino acids that are implicated as important for anti-DNP binding site structures (Table 1) were introduced into the rearranged L- and H-chain sequences and clonal expansion was observed. Although this clone had yielded only four cells with $V_H DJ_H$ sequences, we recovered a $V_\kappa J_\kappa$ sequence from all four cells plus ten additional clonally related $V_\kappa J_\kappa$ sequences. Thus we recovered 14 cells belonging to clone L3. Genealogical relationships were determined by comparing the $V_\kappa J_\kappa$ sequences with the closest germ-line V_κ sequence (1–010) (Fig. 4).

In L3, an initial gene conversion event [labeled (a) in Fig. 4A] led to replacements of H-chain codons at positions 32 in HCDR1 through 57 in HCDR2 including H32 (Asn to Tyr) and H50 (Ala to Phe) that may be important for hapten binding (Table 1). Single base changes also led to replacements at H99, 100A and 100C (Gly, Ala, Ala to Asp, Ile, Gly). In the expanding clone, H100 Tyr was replaced by Arg in Lb 23 and H50 Ala/Phe by Tyr in Lb15 and by Ile in La 68. Amino acid replacements were nota-

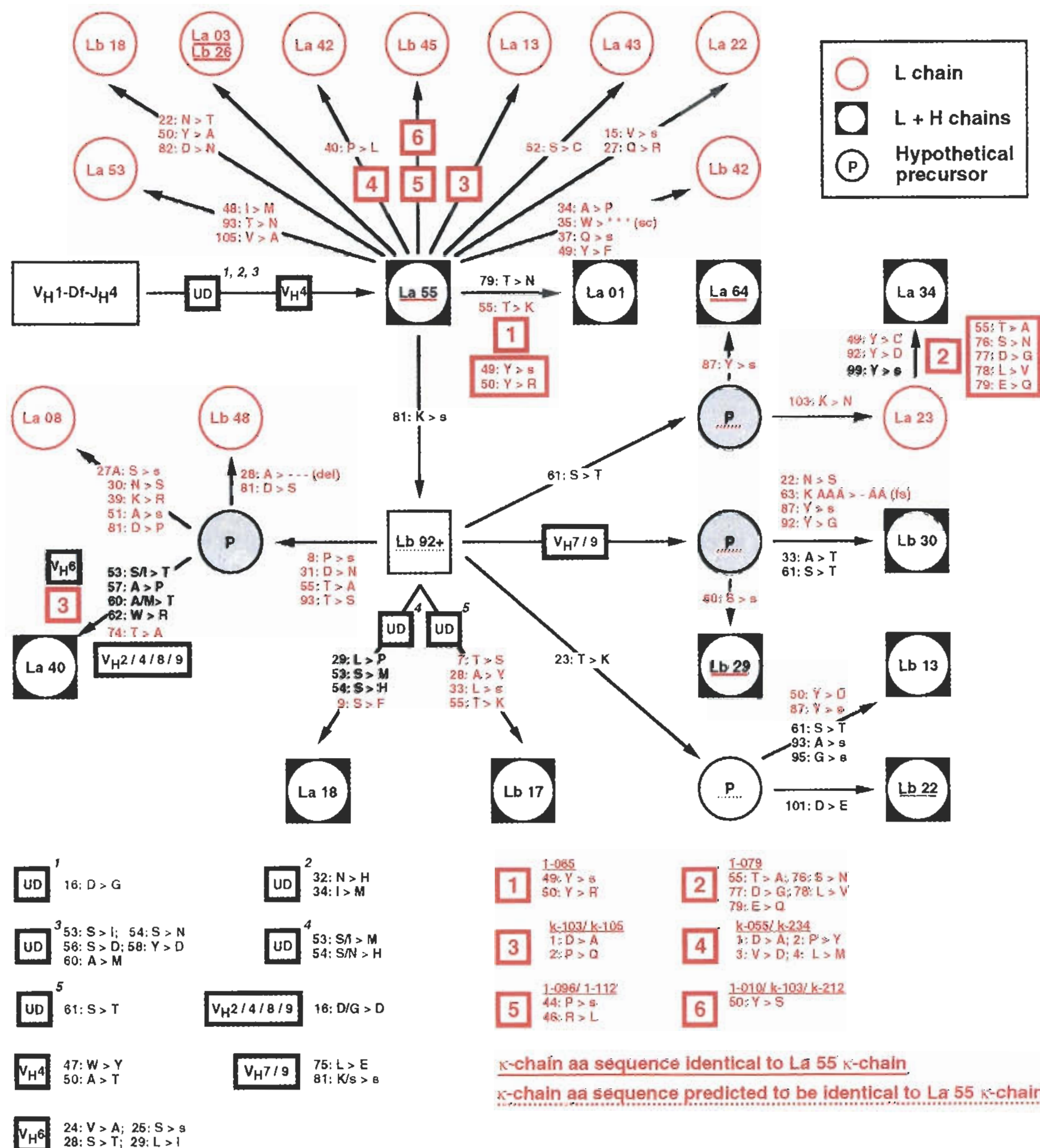

Fig. 3. Genealogical relationships between those members of clone L1 from which sequences of both the L- and H-chain or only the L-chain were recovered. One additional cell (Lb 92) from which only H-chain sequence was recovered is shown because of its key position in the genealogical tree. In addition to Lb 92, there were four other cells with the same V_HDJ_H sequence. The tree showing genealogical relationships of all 29 cells from which V_HDJ_H sequence was obtained can be found in [12]. Changes occurring in the L- and H-chain are color coded in red and black, respectively. The details about the blocks of gene conversion are shown below the tree in red (L-chain) and black (H-chain). UD, unknown donor; del, codon deletion; fs, frameshift.

bly frequent in L-chains at positions 32 in LCDR1 and 50 in LCDR2. L32 Asp to Tyr in La 46 recurred in two related cells, La 48 and La 57, on a different branch where two other closely related cells, La 19 and Lb 20, acquired L32 Glu and Arg, respectively. In another cluster of cells (Lb 10, La 68, Lb 39 and La 10) we found Trp 32 that could have been due to a gene conversion from a germ-line donor such as 1–096. This change was followed by clonal expansion on a branch that included the four recovered cells plus at least three intermediates (each identified as P in Fig. 4). A similar expansion resulting in cells Lb 20, La 19, La 57 and La 48 occurred after Tyr replaced Ser at L50, although in La 57 the Tyr apparently reverted to Ser. Another group (Lb 23, La 24, Lb 15 and La 44) expanded from a common precursor that acquired Asp 50 and three cells (Lb 23, La 24 and Lb 15)

Table 1. Amino acids in rabbit L- and H-chain sequences and in several mouse antibodies that may contribute to DNP binding

L-CHAINS

Region	Rabbit sequences[a)]	Mouse sequences	Protein	Comments
LCDR1		His 27D	MMLB4ILEV4[b)]	MCR[b)]
	Ala 28[c)]			
	Ile 29[c)]			
	Asn 31	Tyr 31	AN02[e)]	Affects Trp 91L stacking
	Tyr 32[c)]	Tyr 32	MMLA2ILEV[b)] MMLB4ILEV4[b)]	MCR[b)]
	Asp 32[c)]		MOPC 315[d)]	Potential H-bonds
	Asn 32			
	Trp 32			
	Ala 34	Tyr 34	AN02[e)]	Contacts 2-nitro group Potential H-bonds
		Asn 34	MOPC 315[d)]	
LCDR2	Tyr 50[c)]	Asp 50	AN02[e)]	
	Gly 50[c)]			
	Thr 53[f)]			
	Thr 55			
	Lys 55			
LCDR3	Gln 89[f)]	Gln 89	AN02[e)]	Contacts 2-nitro group
	Gly 91[f)]	Trp 91	MOPC 315[d)]	Contacts DNP ring
	Ala 91[c)]		AN02[e)]	
		Gly 91	MMLB4ILEV4[b)] MMLA2ILEV[b)]	MCR[b)]
		Tyr 91		MCR[b)]
	Tyr 92	Tyr 92	MMLA2ILEV[b)]	MCR[b)]
	Tyr 93[c)]			
	Gly 94[c)]	Val 94	MLB4IHEV4[b)]	MCR[b)]
	Leu 95A[c)]			
	Trp 95B			
	Asp 95B[c)]			
	Ala 95B			
		Phe 96	MOPC 315[d)]	
		Tyr 96	MMLA2ILEV[b)]	MCR[b)]

H-CHAINS

Region	Rabbit sequences[a)]	Mouse sequences	Protein	Comments
HCDR1	Tyr 32	Gly 32	MMLB4IHEV4[b)]	MCR[b)]
		Tyr 33	MMLB4IHEV4[b)]	MCR[b)]
			MOPC 315[d)]	Contacts DNP ring
		Phe 34	MOPC 315[d)]	Contacts DNP ring
HCDR2	Tyr 50	Tyr 50	MMLB4IHEV4[b)]	MCR[b)]
	Phe 50	Phe 50	MOPC 315[d)]	
	Ile 50			
		Lys 52	MOPC 315[d)]	Potential H-bonds MCR[b)]
		Tyr 52	MMLB4IHEV4[b)]	
		Tyr 56	MMLB4IHEV4[b)]	MCR[b)]
	Asp 58	Glu 58	MMLB4IHEV4[b)]	MCR[b)]
	Asn 58			
HCDR3	Trp 95	Trp 95	MMLB4IHEV4[b)]	MCR[b)]
	Tyr 95	Ala 95	MMLA2IHEV[b)]	MCR[b)]
	His 95	Asp 95	MOPC 315[d)]	Potential H-bonds
	Tyr 96	Asn 96	MMLA2IHEV[b)]	MCR[b)]
	Leu 96	Trp 96	AN02[e)]	Contacts DNP ring
	Phe 96			
		Leu 97	MMLB4IHEV4[b)]	MCR[b)]
		Trp 97	MMLA2IHEV[b)]	MCR[b)]
		Pro 97	AN02[e)]	
		Asp 98	MMLA2IHEV[b)]	MCR[b)]
		His 98	MOPC 315[d)]	Potential H-bonds
	Tyr 99	Leu 99	MOPC 315[d)]	Potential for vander Waals contacts
	Tyr 100	Tyr 100	MOPC 315[d)]	
	Ile 100			
	Trp 100			
	Ser 100			
	Ile 100A			
	Ser 100A			
	Phe 100A			

a) Includes all the functional $V_\kappa J_\kappa$ sequences reported here and all the functional $V_H DJ_H$ sequences reported in [11, 12]. Position numbers are according to Kabat et al. [16].

b) Major contacting residues (MCR) were identified in a series of docking searches [27] in studies of mouse IgE hybridoma monoclonal anti-TNP antibodies La2 and Lb4 [28] [GenBank locus names MMLA2IHEV, MMLA2ILEV (accesion nos. X65772 and X65774) and MMLB4IHEV, MMLB4ILEV (X65773 and X65775)].

c) Amino acids that were most common in anti-DNP V_K and not in the consensus V_K germ-line sequence (see *below*; Fig. 5, filled circles and squares).

d) MOPC 315 is a mouse IgA λ plasma cell tumor product that binds DNP [25, 26].

e) GenBank Locus names MUSIGHXX (accesion numbers M19893 and J03832) and MUSIKGCNC (accession numbers M19906 and J03832) for H- and L-chain V-regions of a mouse monoclonal anti-DNP spin-label antibody [22, 23]. The structure of its Fab fragment complexed with hapten was solved at 2.9 A [24].

f) Selection for more frequent expression than in consensus V_K suggested from the analyses shown below in Fig. 5.

had a point mutation in codon L95C leading to replacement of Ile with Val. There were some other recurrent changes on different branches including L95 Ser to Asn in Lb 39 and in the cluster of related cells Lb 23, Lb 15, La 24 and L94, Ser to Asn in La 68 and La 44. It is likely, though not proven, that the observed expansion of B cells with specific replacement changes in CDR of L- and H-chain pairs reflects the development of increased affinity for the DNP hapten.

2.6 Extensive use of V_κ CDR-encoded variability and selection for and against amino acids at some LCDR positions

In keeping with previous studies showing that rabbits produce highly heterogeneous antibodies in response to DNP coupled to large protein carriers [15, 30, 31], we found that a variety of different V_κ gene sequences can contribute to DNP-specific GC B cells. Whereas Ag-selected amino acids could be identified by inspection of independent V_H sequences [12] the task of identifying Ag-selected V_κ amino acids was much more difficult

A

B

Fig. 4. Selection of amino acids in an expanding day 15 clone. (A) Genealogical relationships between members of clone L3 from which sequences of both the L- and H-chain (boxes) or only the L-chain (circles) were recovered. For the L-chain, amino acid changes in positions that may affect combining site structures are shown in uppercase letters and highlighted with colors corresponding to those shown in (B) below; for the H-chain such amino acids are colored red. Other changes occurring in the L-chain are in black and the H-chain changes are in grey. The amino acid positions that are a part of gene conversion event are shown in parenthesis. Gene conversion events are designated by letters for H-chains and numbers for L-chains; fs, frameshift. Donors for gene conversions of rearranged V_H1a2 were: (a) V_H3 (32: Asn>Tyr; 33: Ala>Gly; 34: Ile>Val; 47: Trp>Tyr; 50: Ala>Phe; 52: Gly>Ser; 54: Ser>Gly; 57: Ala>Thr) (b) unknown donor (52: Gly/Ser>Tyr; 53: Ser>Thr) and (c) V_H9 (3: Ser>s). The genealogical relationships based on H-chain sequences and further details can be found in [12]. (B) Amino acid sequences of L-chains from cells recovered from clone L3 compared with the sequence of the probable rearranging V_κ gene 1–010. The gene conversion events (horizontal boxes) numbered in (A) are marked. Key amino acid changes that may affect the combining site (Table 1) are capitalized and highlighted in color. Candidate donors for L-chain gene conversions [10] are: (1) k-244; (2) 1–096 or 1–112; (3) k-222; (4) 1–065; (5) k-116; (6) 4–034; (7) 1–091; (8) k-098; (9) k-116; (10) 1–144.

because of the diverse set of germ-line V_κ that were used. In order to further identify amino acids in LCDR that may have been Ag selected, we generated a germ line-encoded LCDR consensus sequence based on the most commonly used amino acid at each LCDR codon position of the previously cloned and sequenced 142 "germ-line" V_κ sequences [10]. The frequencies of occurrence of the consensus amino acid in the germ-line genes ranged from 26.8% (position 50) to 100% (positions 24, 27A, 33, 51 and 54). We then calculated the frequency of occurrence of the germ-line amino acid at LCDR codon positions of all functionally rearranged sequences from DNP$^+$ GC B cells (53 from days 7 and 10; 73 from day 15). The frequencies of occurrence in the germ-line and expressed V_κ were similar at the majority of the LCDR codon positions. Thus during the anti-DNP response, there was extensive use of the κ L-chain germ line-encoded variability onto which were superimposed blocks of sequence derived from other V_κ genes. Most of the codon positions that were invariant (24, 27A, 33, 51, 54) or almost invariant (26, 27B, 52, 56) in the germ line remained so in the expressed and diversified sequences (Fig. 5), suggesting that they probably play a structural role.

Our analyses of LCDR used in the anti-DNP response (Fig. 5 and Table 1) indicate that the rabbit anti-DNP com-

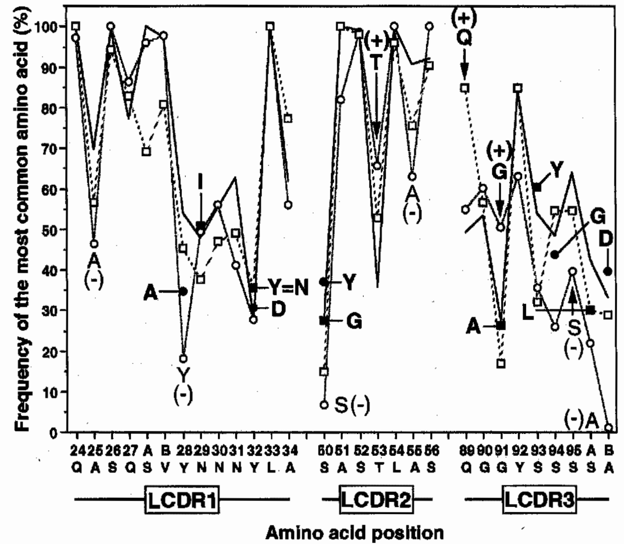

Fig. 5. Positive and negative selection of amino acids at V_κ CDR positions and CDR-encoded variability. The frequency of the most common amino acid in the LCDR1, LCDR2 and LCDR3 of 142 germ-line V_κ sequences from rabbit (black line), V_κ sequences from days (7+10) (open squares) and day 15 (open circles) are shown. Where amino acids that were most common in the expressed sequences deviated from the germ-line-encoded consensus sequences they are indicated by filled squares (days 7+10) and circles (day 15). The consensus germ-line amino acid for each amino acid position is shown below the x-axis. Amino acids that were positively and negatively selected are indicated by (+) and (-).

bining site can accommodate a variety of V_κ sequences. Fig. 5 provides an overview of analyses of LCDR positions from the functionally rearranged sequences. There were 11 LCDR positions at which the amino acid found most frequently in expressed sequences was different from the most frequently found amino acid in the germ line (filled circles and squares in Fig. 5; Table 1; Ala 28, Ile 29, Asp 32, Tyr 32, Tyr 50, Gly 50, Ala 91, Tyr 93, Gly 94, Leu 95A and Asp 95B). Also suggesting positive selection were three LCDR positions where the frequency of the amino acid present in the germ-line consensus was elevated (Thr 53 at all days, Gly 91 and Gln 89 at day 10 [marked with (+) and downward arrows in Fig. 5]). These analyses indicate that during the anti-DNP immune response, the L-chain CDR structures mainly reflect the diversity of the germline V_κ pool further diversified by gene conversion; some negative and positive selection of amino acids occurs at certain LCDR codon positions [identified with (–) or (+) in Fig. 5].

3 Discussion

3.1 General remarks

We show here that although much of the rabbit B cell repertoire is generated early in life in gut-associated lymphoid tissues, cells expanding in response to immunization with DNP-BGG in adult splenic GC undergo remarkable diversification of rearranged L- and H-chain sequences by gene conversion and somatic mutation. We believe our observation that gene conversion and hypermutation occurs during clonal expansion of B cells in Ag-specific splenic GC could help account for the rabbit's known ability to produce highly specific heterogeneous high-affinity Ab against a very wide variety of Ag.

3.2 Does gene conversion contribute to affinity maturation and/or new repertoire?

Although the mechanisms for receptor revision (reviewed in [32]) and gene conversion may differ, one could view gene conversion as a special case of receptor revision where the sequence alteration is restricted to one or more segments of the full-length portion of the rearranged V region gene. An attractive recent proposal of a role for receptor editing (revision) during affinity maturation is particularly relevant for understanding the diversification process in rabbit GC [33]. The BCR on cells undergoing somatic mutation and selection within a GC could become "stuck on a relatively low local optimum" on an affinity landscape. Gene conversion in the rabbit could offer the ability to escape from local optima by allowing a combining site structure to take large leaps through the affinity landscape. Although most changes would land the Ab in a locale where the affinity is lower, occasionally leaps through the affinity landscape could "rescue immune responses stuck on relatively low local optima" and permit the rabbit's immune system to generate the anti-DNP antibodies with very high affinities [15] (9-11 kcal/mol) known to be produced in response to DNP-BGG. Cells with lowered affinities may either be eliminated by apoptosis or could perhaps be a source of new B cell repertoire in adult rabbits. In the latter case, maintenance of B cells for survival may be mediated via superantigen-like interactions of V_H FR regions of surface Ig with endogenous ligands such as CD5. In earlier studies we showed that CD5 interacts with V_H FR regions and suggested this contributes to survival and positive selection of B lymphocytes in young rabbit appendix [34–36].

A recent report of receptor revision of human H-chain V region genes in tonsillar lymphocytes also showed both replacement and point mutations occurring during clonal expansion [37]. We speculate that if cells with sequences altered by gene conversion or receptor revision that no longer react with the immunizing Ag are a by-product of the GC reaction, the splenic or human tonsillar GC could produce new members of the B cell repertoire and thus play a role in adults similar to that of the gut-associated lymphoid tissues of young rabbits.

4 Materials and methods

4.1 Antigen and immunization

DNP_{37}-BGG was prepared as described [11, 12]. Rabbits were from our NIAID allotype-defined colony that are descendants of those whose anti-DNP Ab responses and affinities were extensively studied by Werblin et al. [15]. The immunized rabbits were 1-2 years of age, homozygous for C_κb5 and V_Ha2 (haplotype F-I) allotypes. They were given a priming immunization of 500 μg BGG in CFA injected s.c. Seven to ten days later 500 μg DNP-BGG were given i.v. Control rabbits received BGG at both time points. Seven, 10 and 15 days after the second injection, rabbits were killed and the spleens removed. All animal experiments were in compliance with relevant laws and institutional guidelines and were reviewed and approved by the animal care and use committees of NIAID and Spring Valley Laboratories, Inc.

4.2 Immunohistochemistry

The spleens were embedded in OCT and quickly immersed in liquid nitrogen. Serial sections (7 μm) were cut from the spleens in a cryostat microtome, kept for 30 min at room temperature, fixed at 4°C in acetone for 10 min and stored at –70°C. DNP-binding cells from splenic GC were revealed by incubation with DNP-alkaline phosphatase (DNP-AP) followed by incubation with the VECTOR Blue Substrate kit (Vector Laboratories, Burlingame, CA) containing levamisole (to inhibit endogenous AP activity) and counterstained with VECTOR Nuclear Fast Red (Vector Laboratories). The preparation of DNP-AP and staining of tissue sections were as previously described [11, 12]. Spleens from BGG-immunized control animals were also stained with DNP-AP to exclude any nonspecific binding. As an additional control, splenic sections from DNP-BGG-immunized rabbits were stained with unconjugated AP. To identify proliferating cells within GC, adjacent sections were stained with mouse mAb to the human nuclear proliferation Ag Ki-67 that cross-reacts with rabbit (Dako, Carpinteria, CA), followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and avidin-biotin complex conjugated to AP (ABC-AP; Vector Laboratories).

4.3 Microdissection of frozen tissue sections

Serial sections (7 μm) stained as previously described [11, 12] were incubated with 5 mg/ml collagenase H (Boehringer Mannheim, Indianapolis, IN) as described [38]. Single cells from GC obtained at days 7, 10, and 15 were collected from

the sections using a hydraulic micromanipulator (Narishige, Greenvale, NY) assembled on an inverted microscope (Olympus, Lake Success, NY). From days 7 and 10 we collected cells from DNP^+ GC that were either DNP^+ (day 7), Ki-67^+ (day 10) or both (day 10) [11]. From day 15, we collected proliferating cells based on positive staining with anti-Ki-67 from GC that stained positive for DNP-binding cells. Individual GC were designated by capital letters, sections within a GC by lower case letters and cells were numbered. A suffix '-k' or '-H' was added to the name of the cell to indicate κ L- or H-chain sequence.

Individual cells were each transferred to 0.2-ml microfuge tubes containing 5 μl of an alkaline lysing solution (200 mM KOH/50 mM DTT). The tubes were incubated at 65°C for 10 min before adding 5 μl neutralizing solution (900 mM Tris-HCl, pH 8.3/ 300 mM KCl/200 mM HCl).

4.4 DNA amplification and sequencing

We elected to use direct sequencing of PCR products from single cells without a cloning step to avoid two potentially serious artifacts, namely Taq DNA polymerase errors and *in vitro* recombination during PCR [11, 12]. Even with single-cell PCR there could theoretically be some artifactual recombinants between a germ-line V gene sequence and the rearranged V_LJ_L or V_HDJ_H sequence.

In contrast to mouse and human, which have only one Igκ isotype, rabbits have two isotypes (K1 and K2) [39, 40] and complex allelic variants of K1 (b4, b5, b6 and b9 allotypes; reviewed in [6]). In normal rabbits, approximately 70–90% of the expressed Ig is of the K1 isotype, the remainder consists of both λ and traces of K2 L-chain (reviewed in [6]). We chose to only amplify the rearranged $V_\kappa J_\kappa$ sequences because in homozygous b5 rabbits approximately 85% of B cells have surface Ig bearing κ L-chain. Throughout this report the amino acid positions are numbered according to the Kabat numbering system [16].

The rearranged $V_\kappa J_\kappa$ and V_HDJ_H sequences were amplified from single cells of DNP^+ GC using primers and nested PCR strategies described previously [11, 12]. Briefly, in the first round of PCR, sequences were amplified using a mixture of external primers; one set specific for the rearranged $V_\kappa J_\kappa$ and one set for V_HDJ_H. A 5-μl aliquot of the first round PCR product served as the template for the second round of nested PCR which was performed separately for the rearranged $V_\kappa J_\kappa$ and V_HDJ_H sequences using internal primers [11, 12]. The temperature conditions for the first and second round of touchdown PCR were the same as described previously [11, 12]. The PCR was performed on a PTC-100 programmable thermal cycler (96-well model with hot bonnet; MJ Research, Inc., Watertown, MA).

Recovery of PCR products for sequencing was done as described [11, 12]. The Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used following the manufacturer's instructions to sequence both strands using the second round primers on an ABI model 377 automated sequencer (Applied Biosystems). The sequences were analyzed by using Autoassembler version 1.4.0 (Applied Biosystems) and MacVector software version 6.0.1 (Kodak Scientific Imaging Systems, Rochester, NY; Oxford Molecular Group, Campbell, CA).

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