

Gene Conversion and Hypermutation During Diversification of V_H Sequences in Developing Splenic Germinal Centers of Immunized Rabbits

Enrico Schiaffella,^{1*} Devinder Sehgal,^{1*} Arthur O. Anderson,[†] and Rose G. Mage^{2*}

The young rabbit appendix and the chicken bursa of Fabricius are primary lymphoid organs where the B cell Ab repertoire develops in germinal centers (GCs) mainly by a gene conversion-like process. In human and mouse, V-gene diversification by somatic hypermutation in GCs of secondary lymphoid organs leads to affinity maturation. We asked whether gene conversion, somatic hypermutation, or both occur in rabbit splenic GCs during responses to the hapten DNP. We determined DNA sequences of rearranged heavy and light chain V region gene segments in single cells from developing DNP-specific GCs after immunization with DNP-bovine γ -globulin and conclude that the changes at the DNA level that may lead to affinity maturation occur by both gene conversion and hypermutation. Selection was suggested by finding some recurrent amino acid replacements that may contribute increased affinity for antigen in the complementarity-determining region sequences of independently evolved clones, and a narrower range of complementarity-determining region 3 lengths at day 15. Some of the alterations of sequence may also lead to new members of the B cell repertoire in adult rabbits comparable with those produced in gut associated lymphoid tissues of young rabbits. *The Journal of Immunology*, 1999, 162: 3984–3995.

Germinal centers (GCs)³ in secondary lymphoid organs are specialized structures within which somatic diversification of rearranged V genes occurs that lead to affinity maturation of Abs in immune responses to T-dependent Ags. During some stages of GC development, a dark zone and a light zone (LZ) can be defined histologically. B cells in the dark zone (centroblasts) are characterized by intense proliferative activity (1, 2). The rearranged V region genes of the centroblasts undergo somatic hypermutation (3–7). Centroblasts then move to the LZ, become nondividing centrocytes, contact follicular dendritic cells (FDC) with the Ag in a native conformation on their surface, and undergo selection. B cells with high-affinity receptors for the Ag continue to differentiate toward Ab producing cells or memory B cells (8, 9). Cells that fail to bind Ag or that bind with a lower affinity die by apoptosis, or reexpress the recombination-activating genes 1 and 2 (RAG-1 and RAG-2) gene products and undergo receptor editing (10–20). The presence of RAG-1 and RAG-2 proteins in secondary follicles detected by immunohistochemistry (14), the re-expression of RAGs in activated B cells stimulated by LPS and IL-4 (10) and the presence of intermediate products of VJ recom-

bination at the κ locus, (15, 16) reflect GC B cells in which receptor editing or revision occurs.

Emerging evidence suggests that the description of GCs outlined above oversimplifies the actual events that occur. It was recently shown that proliferating cells are present in the LZ in contact with the FDC network at least until day 10 of the response of BALB/c mice to 2-phenyl oxazolone-BSA (21). Successive cycles of hypermutation and selection are thought to occur (22, 23) involving continued proliferation and selection of cells with surface B cell receptor (BCR) near FDC in the T cell-rich LZ (21) rather than by cells recycling between proliferative and selective compartments of the GC (24, 25). Studies in an adoptive transfer model suggest that Ag-specific CD4 T cells interact with dendritic cells in T cell areas. They subsequently participate in cognate T–B cell interactions at B cell follicular borders where lymphokines and signaling between CD40 and its ligand CD154 drive B cell proliferation and development of peanut agglutinin-positive GCs (26). Interactions of CD40 and its ligand remain crucial during much of the GC reaction. Other interactions such as between OX40 and OX40 ligand (27) and between B7–2 and CD28/CTLA-4 are also important. Interference with B7–2 function by administration of Ab interrupts hypermutation and generation of memory populations (28). Affinity-based selection and receptor editing may also be influenced by cognate T–B cell interactions involving CD40 and lymphokine signaling. B cells with low affinity for Ag may be signaled to up-regulate recombinase expression whereas B cells with high affinity receptors may generate BCR-mediated signals that inhibit recombinase reactivation (19). Very recently, the possibility has been raised that some repertoire development may occur in GCs of mice and humans (14, 17, 29) that resembles the primary repertoire development that occurs in tissues such as young rabbit appendix and the chicken bursa of Fabricius (30–34).

GCs are also present in primary lymphoid organs such as the young rabbit appendix (35) and the chicken bursa of Fabricius where they mainly function to produce a broad B cell repertoire rather than to generate a specific immune response (30–34, 36). Unlike mice and humans, rabbits rearrange only a few heavy chain

*Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and [†]U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702

Received for publication October 21, 1998. Accepted for publication December 28, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ E.S. and D.S. contributed equally to this paper.

² Address correspondence and reprint requests to Dr. Rose G. Mage, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N311, 10 Center Drive-MSB 1892, Bethesda, MD 20892-1892. E-mail: address: rm3z@nih.gov

³ Abbreviations used in this paper: GC, germinal center; AP, alkaline phosphatase; BCR, B cell receptor; BGG, bovine γ -globulin; CDR, complementarity-determining region; FDC, follicular dendritic cell; HCDR3, heavy chain complementarity-determining region 3; LZ, light zone; NP, nitrophenyl; RAG, recombination-activating gene; V_H , heavy chain V region.

Table I. Primers used for amplifying rearranged $V_H D_H J_H$ and $V_\kappa J_\kappa$ sequences by single cell PCR

Primer	Sequence	Source
External primers		
DSH1	GCTTCTCCTGGTCGCTGTGCTC	Leader (L_H) ^a
DSH7	GCAGCAAAGGCCCTCAGAGTTG	3' to J_H2^a
DSH8	GCAGAAAATCGCTCAGAGTTG	3' to J_H4^a
DS κ 1	TCCTGCTGCTCTGGCTCCA	Leader (L_κ) ^b
DS κ 7	CAGAAACGGTGAGGAATCAGTG	3' to $J_\kappa2^b$
Internal primers		
DSH2	AGTGACAGTGTC (C/T) TGACCATGT	L_H - V_H intron ^a
DSH5	AGGACTCACCTGAGGAGACGG	$J_H2/3/4^a$
DS κ 2	ATGAGTGTGGTCTCTGTGTTTCT	L_κ - V_κ intron ^b
DS κ 14	GAATCAGTGAGACGGCCACTTAC	$J_\kappa2$ -3' intron junction ^b

^a These primers were described in detail in Ref. 47.

^b The primers for κ light chain were selected based on sequences bearing accession numbers D38376, K00751, K01360, K01361, K01363, K02131, K02438, K02439, K02440, M14067, M22541, X00032, X00231, X00977, X02336, X02337, X02338, X14364, X14365.

V region (V_H) genes, so that the diversity generated by combinatorial mechanisms is limited (37, 38). Appendix GCs are seeded by B cells that have already rearranged their Ig genes in sites such as fetal liver, omentum, or bone marrow (36, 39). They colonize the GCs of the appendix during the first weeks of life, proliferate in response to Ags and superantigens (36) and their rearranged V_H genes diversify (30) by somatic hypermutation and a gene conversion-like mechanism (38, 40, 41). We refer to the mechanism in rabbits as gene conversion-like because nonreciprocal exchange has not been formally shown in rabbits whereas studies in chickens (42) showed that donor sequences were unaltered.

The objective of this work was to investigate diversification of rearranged V_H and light chain V region sequences in splenic GCs during T cell-dependent immune responses to DNP-bovine γ -globulin (DNP-BGG) in adult rabbits, an Ag chosen because of its previous use in documenting affinity maturation in the rabbit (43, 44). We addressed the question of whether V_H -gene conversion, somatic hypermutation or both also take place during the GC reaction in response to a T cell-dependent Ag in GCs of the rabbit spleen. We combined the techniques of microdissection of single Ag-specific B cells with a PCR-based sequencing strategy that avoids PCR artifacts. This approach is particularly successful in rabbit because the B cells rearrange only a few V_H genes and the majority do not rearrange the second allele (45). Although it was previously reported that V_H sequences in adult rabbit B cells were highly diversified (46) we found that cells collected during early stages of the GC reaction (days 7 and 10) from adult rabbit spleen had rearranged V_H sequences that were identical or nearly identical with germline sequences (47). This report presents our observations, both at the histological and molecular levels, on the progression of the GC reaction in rabbit spleens during the response to the T cell-dependent hapten DNP. We find changes that may lead to affinity maturation occur by both gene conversion and somatic hypermutation.

Materials and Methods

Ag and immunization

DNP₃₇-BGG was prepared as described (47, 48). Rabbits from 1 to 2 years of age, homozygous for $V_H d2$ (haplotype F-I) and $C_\kappa b5$ allotypes, were given a priming immunization of 500 μ g of BGG in CFA injected s.c. Between 7 and 10 days later, 500 μ g of DNP-BGG were given i.v. Control rabbits received BGG at both time points.

Immunohistochemistry

At 7, 10, and 15 days after the second injection, rabbits were killed, and the spleens embedded in OCT and quickly immersed in liquid nitrogen. Serial

(7 μ m) sections 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 an appropriate substrate as detailed below. The preparation of DNP-AP and staining of tissue sections were as described previously (47). 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 GCs, an adjacent section was stained with mouse anti-human Ki-67 mAb that crossreacts with rabbit (Dako, Carpinteria, CA), followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and avidin-biotin complex conjugated to AP (Vector Laboratories). To obtain information about isotype expression we used biotinylated polyclonal goat anti-rabbit IgM (μ heavy chain-specific) and biotinylated polyclonal goat anti-rabbit IgG (γ heavy chain-specific) Abs (Southern Biotechnology Associates, Birmingham, AL). In some experiments for double staining with DNP-AP and anti-IgG, IgM, or CD4 we used VECTOR Blue Substrate kit (Vector Laboratories) in combination with the VECTOR Red Substrate kit.

Microdissection of frozen tissue sections

The stained sections were incubated with 5 mg/ml collagenase H (Boehringer Mannheim, Indianapolis, IN) as described (49). Single cells from GCs 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⁺ GCs that were either DNP⁺ (day 7), Ki-67⁺ (day 10), or both (day 10) (47). From day 15 we collected proliferating cells based on positive staining with anti-Ki-67 from a GC that stained positive for DNP-binding cells.

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

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. First, without a cloning step the effect of *Taq* DNA polymerase errors is minimized (50–52) because *Taq* DNA polymerase-based errors are then detected in sequences only if they occur in the first few cycles of amplification. Second, by amplification of $V_H D_H J_H$ sequences from single cells we avoided producing hybrid $V_H D_H J_H$ sequences that were found especially during the somatic hypermutation stage of the GC reaction (6).

The rearranged $V_H D_H J_H$ and $V_\kappa J_\kappa$ sequences were amplified from single cells of DNP⁺ GCs using a nested PCR strategy. The primers used are shown in Table I. In the first round, the rearranged $V_H D_H J_H$ and $V_\kappa J_\kappa$ sequences were amplified simultaneously. Nested PCR was performed separately for the rearranged heavy and κ light chain using internal primers specific for the rearranged $V_H D_H J_H$ and $V_\kappa J_\kappa$ sequences. The temperature conditions for the first and second round of touchdown PCR were the same (53). There was an initial 95°C 2-min denaturation step. This was followed

by five cycles of denaturation at 94°C for 1 min; for the first cycle annealing was at 66°C for 30 s that dropped down to 56°C at the end of the fifth cycle at the rate of 2°C per cycle; and an extension step at 72°C for 1 min. The annealing temperature for the remaining 29 cycles was 56°C. The PCR ended with a 5-min extension at 72°C. The PCR was performed on a PTC-100 programmable thermal cycler (96-well model with hot bonnet) (MJ Research, Watertown, MA).

For the first round, MgCl₂, Tris-HCl (pH 8.3), Triton X-100, and external primers (DSH1, DSH7, and DSH8 for the heavy chain; and DSκ1 and DSκ7 for the κ light chain) (see Table I) were added to the tube containing the neutralized cell lysate such that the final concentration in a 40 μl volume were 3.75 mM, 10 mM, 0.1% (v/v), and 0.75 μM (of each primer), respectively. The DNA was denatured at 95°C for 10 min. Thereafter, 20 μl of a mix containing 10 mM Tris-HCl (pH 8.3), 0.1% (v/v) Triton X-100, 600 μM of each dNTP, and 5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) was added and the tube setup for PCR. From the first round PCR, 2.5 μl of the product served as the DNA template for the second round touchdown PCRs (53) that were done for the rearranged V_HD_HJ_H and V_κJ_κ sequences separately. The internal primers used for amplifying the rearranged heavy chain were DSH2 and DSH5 (Table I). The corresponding set of internal primers used for amplifying the rearranged κ light chain were DSκ2 and DSκ14 (Table I). In addition to the template DNA, the other components in the 50 μl of PCR were 1× PCR buffer (containing MgCl₂) (Perkin-Elmer), 200 μM of each dNTP, 0.5 μM of each primer (internal to those used in the first round) (DSH2 and DSH5 for the heavy chain; DSκ2 and DSκ14 for the κ light chain) (see Table I) and 2 units of AmpliTaq DNA polymerase. The PCR parameters were the same as for the first round.

As a control we also conducted a blind experiment where we collected a group of 23 CD4⁺ cells from the T cell area along with 69 Ki-67⁺ cells from within the GC. In the PCRs run at the same time, we amplified 48 of 69 V_HD_HJ_H sequences from the cells collected from the GC; none of the 23 T cells gave a PCR product.

Recovery of PCR products for sequencing was done as described (47). 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 Applied Biosystems model 377 automated sequencer (54). The sequences were analyzed using AUTOASSEMBLER version 1.3 (Applied Biosystems) and MACVECTOR versions 5.0 and 6.0 (Kodak Scientific Imaging Systems, Rochester, NY; Oxford Molecular Group, Campbell, CA).

Results and Discussion

Immunohistochemical characterization of splenic GCs in the rabbit

For histological characterization of GCs in the spleens of immunized rabbits, we stained frozen sections with the DNP-AP in combination with other Abs such as monoclonal mouse anti-human Ki-67 that crossreacts with rabbit, mouse anti-rabbit CD4, polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. In rabbits that were carrier-primed 7–10 days before i.v. immunization with DNP-BGG, GCs were present at day 7 and their appearance and staining were similar at day 10. Fig. 1, A–F shows staining of tissue sections with polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. DNP-AP staining (blue) in combination with anti-IgM (red; Fig. 1, A–C) and in combination with anti-IgG (red; Fig. 1, D–F), suggests that isotype switching had not yet occurred at day 7 because all the GCs stained positively for IgM, but little or no IgG staining was evident (Fig. 1, A and D). By day 15 (Fig. 1, B, C, E, and F) the cells in many GCs had undergone isotype switching. The GC shown in Fig. 1, B and E, show evidence of recent isotype switching as adjacent sections stained positively with anti-IgM (B) and IgG (E). Fig. 1, C and F, show another GC at day 15 that lost most of its IgM expression (C) and stained with anti-IgG (F). Fig. 1, G–J, shows staining of adjacent sections at days 10 and 15 with anti-Ki-67 that identifies a nuclear Ag in proliferating cells (G and H) and of DNP-AP in combination with anti-CD4 (I and J). At day 15 (Fig. 1, H and J), proliferating cells were more concentrated in the areas of the GC where cells down-regulated BCR expression, and staining with DNP-AP was less

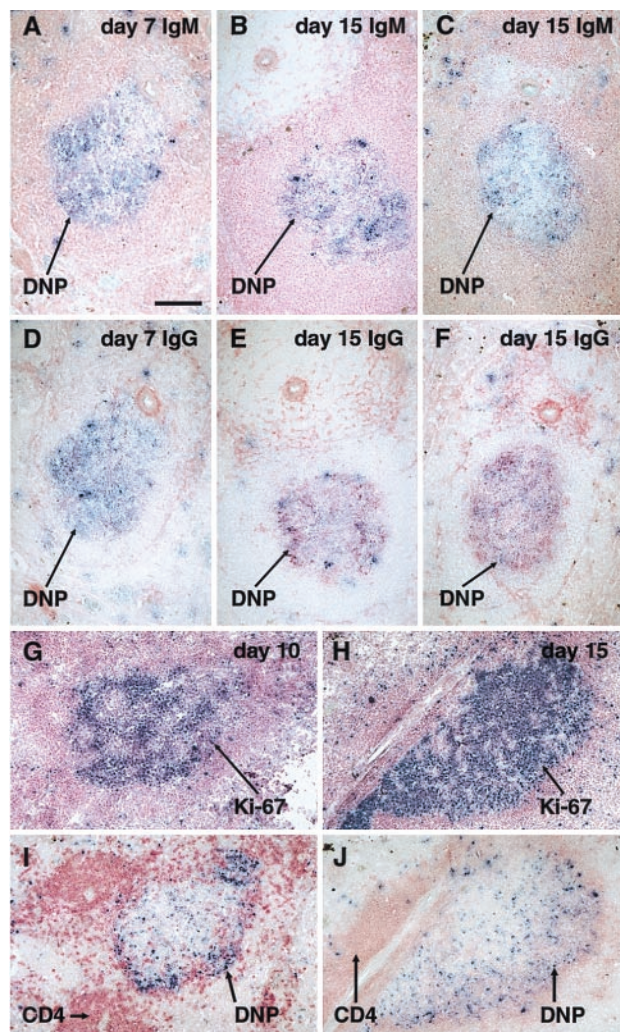


FIGURE 1. Progression of rabbit splenic GC reactions between days 7 and 15 after i.v. immunization of BGG primed rabbits with DNP-BGG. A–F, Staining of rabbit splenic follicles and GCs for IgM and IgG on days 7 (A and D) and 15 (B, C, E, and F) of the immune response to DNP-BGG. A/D, B/E, and C/F are serial sections. The sections in A–C are double-stained with anti-IgM (red) and DNP-AP (blue). In D–F, anti-IgG (red) in combination with DNP-AP (blue) was used. G–J, Proliferating and DNP-binding cells in rabbit splenic follicles and GCs at days 10 and 15 of the response to DNP-BGG. G/I and H/J are serial sections at days 10 and 15, respectively. (G) and (H), anti Ki-67 (blue), counterstained with nuclear fast red. I and J, Double staining with DNP-AP (blue) and anti-CD4 (red). Bar = 100 μm.

intense. CD4⁺ T cells constituted a major population in the periarteriolar lymphoid sheath area. Within the GC, they were found interspersed with DNP-binding cells.

Amplification and clonal relationships of rearranged Ig genes from GC B cells

Using PCR, we amplified the rearranged Ig genes in single cells obtained from several DNP-reacting splenic GCs of DNP-BGG immunized rabbits. Table II summarizes the numbers of cells collected from individual GCs at days 7, 10, and 15 and the numbers of sequences that we were able to amplify and analyze. In the seven different GCs studied at days 7 and 10, 55% and 63% of the sequences respectively, had unique complementarity-determining region 3 (CDR3) and were unrelated. At these time points we found one to four different clones per GC with only between two

Table II. Summary of PCR amplification, sequence analyses, and clones in splenic DNP⁺ GCs during immune responses of adult rabbits to DNP-BGG

Days Post-immunization	No. of GCs Studied	Cells Collected (VDJs amplified)	Successful Sequences ^a	Independent Unique Sequences (%)	Groups of Clonally Related Sequences			
					No. of clones	No. of members	Average no. of members/clone	Average no. of clones/GC
7	5	239 (97)	78	43 (55)	12	2–6	2.9	2.4
10	2	230 (79)	62	39 (63)	7	2–7	3.3	3.5
15	2	335 (134)	85	8 (9.5)	9	2–29	8.6	4.5
Total	9	804 (310)	225	90	28	2–29		

^a Refers to the subset of amplified V_HD_HJ_H sequences from which reliable nucleotide sequences were obtained. The GenBank accession numbers are AF058506–AF058645 (days 7 and 10; Ref. 47) and AF087712–AF087796 (day 15; this report).

and seven members (47). The proportions of independent unique sequences decreased from more than half (82/140) at days 7 and 10 to 9.5% (8/85) at day 15. Conversely, groups of clonally related sequences predominated at day 15 and on average contained larger numbers of members. Although it is generally accepted that GCs develop oligoclonally (55–57), we observed a predominance of unique clonally unrelated sequences at days 7 and 10, many with 10 or fewer changes from the germline sequence of the rearranged V_H gene. For the other small groups of clonally related sequences we could also readily identify the germline V_H, D_H, and J_H genes that rearranged in their precursors. At day 10, the population of proliferating cells still showed considerable heterogeneity (47). However, by day 15, most sequences were clonally related, the V region portion of every heavy chain sequence had nine or more differences from germline sequences and there were also on average more changes in the D_H and J_H sequences. In GCs from mice immunized with the hapten nitrophenyl (NP), clonal diversity also decreased with time but at 4–6 days postimmunization most GCs had only between three and six different CDR3 region sequences and by day 8, when hypermutation had initiated, most GCs had only one or two clones (6). In chickens immunized with FITC-BSA, there were also numerous unique light chain sequences and great clonal complexity in individual GCs at day 7 but complexity markedly diminished by day 11. The data suggest that at the later time, more hypermutations and fewer gene conversions were occurring (58).

Table III summarizes the results of analyses of heavy and light chain sequences from cells of GC L and N collected on day 15. All but 7 of the 85 successfully sequenced heavy chains were grouped into clones, 3 in GC L and 6 in GC N. Identification of clonally related light chain sequences showed correspondences with the heavy chain clones. In addition, there was one group of light chain sequences from cells in GC N where we obtained no heavy chain

sequences and another pair of cells with related light chain sequences one of which also yielded a heavy chain sequence. Thus GC N had at least eight different clones and GC L had at least three. The emergence of expanding, diversifying dominant clones by day 15 correlates with the switching from IgM to IgG (Fig. 1, A–F). Analyses of clonally related sequences from day 15 show that gene conversion as well as hypermutation was occurring during clonal expansion.

Gene conversion and hypermutation during clonal expansion of B cells in GCs

Fig. 1H shows GC L stained for Ki-67 and Fig. 1J shows its adjacent section stained for DNP. Proliferating cells in GC L were collected from two serial sections (La and Lb) and rearranged V_HD_HJ_H and V_κJ_κ genes were amplified. GC L had a predominant clone (L1) with 29 heavy chain members and an additional 13 cells from which we recovered only light chain sequences related to those in cells with heavy chains. A summary of this largest clone is shown in Fig. 2. Fig. 2A shows the approximate locations of the cells collected on a diagram of GC L based on the stained sections shown in Fig. 1, H and J. Cells belonging to clones L1, L2, and L3 are shown as green, red, and black dots, respectively. Unique members are shown as black squares. We generated genealogical trees based on the changes that occurred in clonally related sequences. For L1, this is shown in Fig. 2B and the corresponding sequences in Fig. 2C. The cells of clone L1, recovered from the two serial sections appeared to share a common precursor sequence derived from rearrangement of V_H1a2, Df and J_H4 that had already undergone several sequence alterations involving blocks of sequence that are best explained as arising by gene conversion from known (V_H4, shaded orange) and unknown donors (UD, black boxes). The sequence of the rearranged V_HD_HJ_H of cell La 55 appears closest to the precursor sequence from which the cells

Table III. Summary of PCR amplification, sequence analyses of rearranged V_H and V_κ, and clonally related groups of cells from DNP⁺ GCs at day 15 of the immune response of adult rabbits to DNP-BGG

Days Post-immunization	GC	Cells Collected (VDJs, V _κ J _κ amplified)	Successful Sequences (VDJs, V _κ J _κ)	Independent Unique Sequences (VDJ, V _κ , V _κ J _κ)	Groups of Clonally Related Sequences (no. of members) (VDJ; V _κ J _κ)
15	L	148 (71, 86)	43, 49	5 ^a , 8	3 (29, 5, 4); (24, 3, 14)
	N	187 (63, 69)	42, 28	2 ^b , 5	8 (12, 11, 8 ^c , 3, 3, 2, 1 ^d , 0); (5, 2, 2, 4, 0, 0, 2, 8)
Total		335 (134, 155)	85, 77	7, 13	

^a GenBank accession numbers AF087750–AF087754.

^b GenBank accession numbers AF087795–AF087796.

^c GenBank accession numbers AF087778–AF087785.

^d GenBank accession number AF087794.

arose. A single point mutation in the sequence of cell La 01, replaced Thr with Asn at amino acid position 79 (Kabat numbering; Ref. 59) and the remaining sequences acquired a silent g in the Lys⁸¹ codon. Cells with identical sequences were found in serial sections and at some distance from each other in a given section (see Fig. 2A). Lb 92 and four other cells (La 02, La 21, La 39, and La 58) had the same V_HD_HJ_H sequence (indicated by a plus after the cell name in Fig. 2A). The remaining cells diverged from this sequence by acquiring additional point mutations and gene conversions, but only two sequences (La 11 and Lb 22) had replacement changes in CDR3. The rearranged germline sequence of this CDR3 may have already been well suited for binding to DNP. Cell La 40 acquired a stretch of sequence in FR1 and CDR1 that matches a likely gene-conversion-donor gene sequence present in V_H6 (boxed in dark blue); in La 04, a smaller tract only affected framework 1 (FR1). Both La 40 and Lb 83 underwent replacements of Gly¹⁶ with Asp that is found in many potential donor sequences including V_H2, V_H4, V_H8, and V_H9 (pink shading). Cells Lb 29 and Lb 30 exhibited the commonly observed replacements of Leu⁷⁵ with Glu that are likely to have occurred through gene conversion from donor sequences present in both V_H7 and V_H9 (red and green box). This tract of changes extended at least through the silent g in Lys⁸¹. Lb 82 acquired a Ser codon TCT at position 50 that involved a two-base change plus acquisition of an adjacent silent base change. Another sequence, La 71 also acquired a Ser AGC at position 50 but it was via a single-base change from the Thr codon ACC. Ser⁶¹ was changed to Thr independently in several branches of the genealogical tree. This might reflect some selective advantage (e.g., for improved affinity). We also found two different Thr⁶¹ codons, ACA in Lb 17 and La 49 and ACC in five other sequences (Fig. 2C). Of 24 successful V_κJ_κ sequences, 11 were from the group of 29 cells with successfully sequenced V_HD_HJ_H. This is indicated in Fig. 2B by underlining the cell name. As regards the V_κ sequences, deduced V_κJ_κ protein sequences from four cells (La 55, La 64, Lb 22, and Lb 29) were identical; the others had between one and eight replacements. Definition of the complete database of germline V_κ sequences (unpublished manuscript) will allow us to reconstruct the events leading to the sequence changes with confidence. Now we can only conclude that the light chain sequences generally complement the heavy chain data (data not shown).

Although most of the V_HD_HJ_H sequences found in cells collected from GC L belonged to a single clone, two other smaller sets of clonally related sequences (clones L2 and L3; Fig. 3) and five independent unique sequences were found. Clone L2, with five members provided additional examples of gene conversions that appear to have occurred during expansion of the clone within the GC. The initial precursor appears to have rearranged V_H1 to Df and J_H4. A common precursor (P1) underwent a large gene conversion that extended from FR1 through the first codon of CDR2 with the likely donor being V_H7 (green) and a change involving V_H9 (red) extending from CDR2 through position 75 of FR3. Precursor P1 also acquired a silent change in Ile¹⁰² of the J region and two amino acid replacements in CDR3. Cells Lb 24 and Lb 31 then underwent additional changes that may have been due to point

mutations. Cells Lb 12, La 20, and Lb 35 share a common precursor P3 that arose after a second gene conversion involving V_H9 and a silent change in Ser³⁰ that may have been due to V_H6.

Although clone L3 had only four heavy chain members, we obtained rearranged heavy and light chain sequences from all four cells and there were 10 additional light chain members. The light chain data complemented the heavy chain data and strongly favored the clonal tree we show compared with an alternative tree (data not shown). The presence of a replacement of Ser⁹⁷ by Gly on two branches of the tree may indicate a selective advantage for improved affinity.

Based on heavy chain sequences, GC N yielded six groups of clonally related cells. One group of eight cells (N3) all had a frameshift mutation at the V_H to D_H junction and will not be considered in detail (sequences deposited in GenBank and given accession numbers AF087778–AF087785). Analyses of the other sequences allowed construction of clonal trees shown in Figs. 4 and 5. In clone N1 (Fig. 4), rearrangement of V_H1-D2a-J_H4 then led to a common precursor (P1) with a gene conversion that was likely due to V_H4, four replacement changes in CDR3, and a silent change in J4. Cell Na 73 branched off and 11 other cells shared a set of gene conversions that could have involved V_H9 (red box) and either V_H2, V_H5, or V_H8 (pink shading). Na 16, Nb 49, and Nb 56 from two serial sections had the same heavy chain sequence and two additional cells Nb 37 and Nb 72 had only a silent change in Ser³¹. Nb 09 acquired replacements in FR1, CDR1, and CDR3. The change of Val³⁴ to Met and the reversions of Tyr³², Gly³³, and Ile³⁵ to Asn, Ala, and Ser can all be accounted for by a block derived from V_H6 (dark blue box). Similarly, the loss of the silent base change in Ser³ (red box) in cells Na 11 and Nb 58 can be explained by a gene conversion block from V_H2 or V_H7 (green box). The remaining few silent and replacement changes may have been due to point mutations. Fig. 4 also shows diagrammatically the three small clones N4, N5, and N6. Clones N5 and N6 both had precursors with changes in CDR1 that can be explained by two different gene conversions from the V_H4 sequence. All three sequences from clone N5 also had the frequently observed replacements of Leu 75 with Glu that can be explained by conversion involving either V_H7 or V_H9 (red and green box). In N6 this replacement occurred in the sequence of Na 65 after divergence from Na 77.

The sequences and clonal tree derived for clone N2 are shown in Fig. 5. During expansion of clone N2 from precursor P1, several additional gene conversion events occurred involving a donor that could have been V_H6 (dark blue boxes), and an unknown donor (UD, boxed in black) to generate the 10 functional heavy chain sequences that we recovered. An eleventh sequence (Nb 69) acquired a frameshift at position 6. The common precursor, P1, already showed evidence for gene conversions involving V_H3 or V_H4 (orange), V_H2, V_H5, or V_H8 (pink), and V_H7 or V_H9 (red and green). Although studies of V_H and/or V_L sequences in B cells of splenic GCs from chickens immunized with NP-BSA (61) or FITC-BSA (58) also found somatic gene conversion had occurred during clonal expansion in spleen, the precursor cells with between

FIGURE 2. (continued)

same cell. Known germline V_H genes that can account for blocks of gene conversion are shown as colored boxes. RVH indicates five different potential germline donor sequences (RVH700, 701, 714, 720, and 755) found in an a2 homozygous rabbit by Roux et al. (41). UD, unknown V_H donor. (C) Heavy chain sequence alignment showing clonal relationships and a summary of the base changes, replacements and gene conversions (horizontal boxes) that occurred during clonal expansion of these cells. Amino acid replacements are shown above the sequences, silent base changes are in lowercase. The numbers above the reference sequence indicate codon positions according to Kabat et al. (59). Most framework region positions that were invariant are not shown. The V_HD_HJ_H sequences of members of clone L1 have been assigned GenBank accession numbers AF087712–AF087740.

Table IV. CDR3 lengths of potentially functional sequences obtained from days 7, 10, and 15

Day Post-immunization	No. of Rearrangements ^a	CDR3 Length (in codons)	
		Range	Mean \pm SD
7	49	4–18 (H)	12.88 \pm 2.93
10	39	4–19 (H)	12.00 \pm 3.59
15	14	8–14 (H)	11.00 \pm 1.56
	22	10–14 (κ)	11.27 \pm 0.96

^a Included are only one representative of each clone plus the unique independent anti-DNP sequences. Those that were rendered nonfunctional because of a stop codon or frameshift mutation were excluded from these analyses.

near-germline sequences at days 7 and 10 that underwent gene conversions in the spleen (47).

Evidence for selection of CDR amino acid replacements and heavy chain CDR3 (HCDR3) lengths

In the clonal anti-DNP populations present on day 15 we observed slightly shorter CDR3 lengths. Although differences in the mean lengths were not statistically significant, there appeared to be selective narrowing of the range of CDR3 lengths compared with the earlier time points. These results are summarized in Table IV. By day 15 the range of HCDR3 lengths narrowed to 8–14 codons from 4–18 and 4–19 codons at days 7 and 10, respectively. Our results are consonant with those of Brezinschek et al. (62) who recently reported that human memory B lymphocytes have slightly shorter (11.9 ± 3.4 codons vs 14.3 ± 4.3 codons) and a more limited range of HCDR3 lengths than naive B cells and suggested this may facilitate more effective Ag binding. Significantly shorter HCDR3 were also found in sequences that also had affinity-increasing mutations at day 12 of the response to NP in C57BL/6 mice (63).

By day 15, some D_HJ_H regions had and were continuing to accumulate mutations. Because in rabbit there are no known donor sequences for D_H regions, it is likely that the replacements in the D_H regions were the result of point mutations and selection. Replacement changes in the body of the V region are more complex

to analyze because many changes appear to have resulted from gene conversion events. The donors that are the proposed sources of the altered sequences themselves contain base changes that mainly result in replacements. Thus the strikingly low proportions of silent changes in the V regions may reflect both the highly efficient gene conversion mechanism for introducing templated replacements (64) and selection for favorable replacements during the clonal expansion and selection stages of the GC reaction.

In an extensive analysis of serum anti-DNP Abs produced by rabbits in our National Institutes of Health allotype-defined breeding colony (44), anti-DNP affinities and concentrations were found to increase most between days 14 and 28. Analyses of affinities of serum Abs indicated that some molecules of the highest affinity species were present in all rabbits by day 42. It is likely that in rabbits as in mice, high-affinity precursors of anti-hapten Ab-producing cells exit GCs and undergo further selection for high-affinity Ab forming cells in the bone marrow (65, 66). The selection that may occur in bone marrow would involve competition between independently evolved cells with high affinity. In addition to narrowing of the HCDR3 lengths in the dominant clones found at day 15, comparisons of the CDR sequences in the separately evolved clones revealed certain recurrent amino acid replacements in the dominant clones that may contribute to favorable increases in affinity for Ag, or on rates of binding to hapten (67). The recurrences shown in Table V are particularly striking in view of the variations occurring in parallel in the light chain CDRs and the likelihood that many CDR structures can yield high affinity anti-DNP Abs in rabbits. Although only seen in 2 of 29 sequences from L1 (Lb 81 and Lb 84; Fig. 2) the replacement of Asn³² by Tyr was seen in 30 of the 31 sequences from clones L2, L3, N1, N2, and N5. The largest clone from day 10, M1 (47), had two sequences with this replacement (Ma 28 and Ma 34). None of the five sequences in the other two small clones N4 and N6 had this change. In CDR2, there were recurrent replacements of Ala⁵⁰ by Tyr (or Phe, Ile, and Val) and of Tyr⁵⁸ by Asp (or Asn in clone N2). Interestingly, the smaller clones (N4, N5, and N6) did not have the replacements in CDR2 seen in the larger clones. In CDR3 we observed a recurrence of Tyr-Ile at positions 99 and 100 (or 100

Table V. Recurrent amino acids in the HCDRs of different clones

Clone Name ^a	HCDR1	HCDR2		HCDR3		D Gene Used
	Asn ³² to Tyr	Ala ⁵⁰ to Tyr (Phe, Ile)	Tyr ⁵⁸ to Asp (Asn)	Tyr ⁹⁹ or 100	Ile ¹⁰⁰ or Ile ^{100A} (Trp, Ser, Phe)	
L1	2/29	0/29	28/29	29/29 ^b	29/29	Df
L2	5/5	3/5	3/5	5/5 ^b	0/5	Df
		Phe (1/5) Ile (1/5)			Trp (5/5)	
L3	4/4	1/4	0/4	0/4	0/4	D2x
		Phe (2/4) Ile (1/4)		100 (3/4) ^b	100A (4/4)	
N1	8/9	8/9	8/9	0/9 100 (9/9)	0/9 100A Ser (8/9) Phe (1/9)	D2a
N2	10/10 ^c	0/10 Phe (1/10) Ile (8/10)	0/10 Asn (7/10)	0/10	0/10	D5
N4	0/3	0/3	0/3	0/3	0/3	D?
N5	3/3	0/3	0/3	0/3	0/3	D5
N6	0/2	0/2	0/2	0/2	0/3	D2x
M1	2/7	0/7	0/7	5/5 ^{b,c}	0/5	D2x

^a These are from GCs at day 15 of the anti-DNP immune response with the exception of M1, which is from day 10 (Ref. 47).

^b Maintained from germline.

^c One nonfunctional sequence was not included.

and 100A) in all L1 and three of four L3 sequences. Whereas in L1, Tyr-Ile was retained from the germline sequence, L3 acquired Ile by a 2-bp change from the germline sequence. Clone L2 maintained Tyr⁹⁹ from the germline sequence and had a two-base change to yield Trp at position 100. Clone N1 maintained germline Tyr¹⁰⁰ and replaced Ala^{110A} with Ser (8/9) or Phe (1/9).

The overall pattern that is documented by this and the previous study (Figs. 2–5 and Ref. 47) is one of splenic precursor cells whose germline or near germline sequences changed both by gene conversion and point mutations during early divisions and largely by point mutations during later divisions. Because our preliminary data show that within the same expanding clonal populations, considerable diversification of light chain sequences occurred in parallel with the changes in the V_H sequences, these events may produce the diverse sequences that serve as substrates for further affinity maturation by selection either within GCs or later among emigrant cells in sites such as bone marrow. The reappearance of gene conversion in rabbit splenic GCs provides an important example of re-expression of immature functions (neoteny) (14, 17, 29). It remains to be determined whether “fine-tuning” of anti-DNP combining site structures occurs by both gene conversions and point mutations or whether the gene conversions are largely an alternative means of receptor editing. Future studies of expressed heavy and light chain pairs should allow us to evaluate this as well as the contributions of observed recurrent replacements in CDRs (Table V) to affinities for DNP.

Acknowledgments

We thank Drs. S. Bauer, J. Dasso, M. G. Mage, N. A. Mitchison, and M. Shapiro for helpful comments about this paper. We thank Ms. Shirley Starnes for expert editorial assistance, and Ms. G. O. Young-Cooper and Mr. C. Alexander for outstanding technical assistance.

References

- MacLennan, I. C. 1994. Germinal centers. *Annu. Rev. Immunol.* 12:117.
- Kelsoe, G. 1996. Life and death in germinal centers (redux). *Immunity* 4:107.
- Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* 67:1121.
- Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intracloonal generation of antibody mutants in germinal centres. *Nature* 354:389.
- Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165.
- Jacob, J., J. Przylepa, C. Miller, and G. Kelsoe. 1993. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. III. The kinetics of V region mutation and selection in germinal center B cells. *J. Exp. Med.* 178:1293.
- Kimoto, H., H. Nagaoka, Y. Adachi, T. Mizuochi, T. Azuma, T. Yagi, T. Sata, S. Yonehara, Y. Tsunetsugu-Yokota, M. Taniguchi, and T. Takemori. 1997. Accumulation of somatic hypermutation and antigen-driven selection in rapidly cycling surface Ig⁺ germinal center (GC) B cells which occupy GC at a high frequency during the primary anti-hapten response in mice. *Eur. J. Immunol.* 27:268.
- Burton, G. F., D. H. Conrad, A. K. Szakal, and J. G. Tew. 1993. Follicular dendritic cells and B cell costimulation. *J. Immunol.* 150:31.
- Tew, J. G., J. Wu, D. Qin, S. Helm, G. F. Burton, and A. K. Szakal. 1997. Follicular dendritic cells and presentation of antigen and costimulatory signals to B cells. *Immunol. Rev.* 156:39.
- Hikida, M., M. Mori, T. Kawabata, T. Takai, and H. Ohmori. 1996. Reexpression of RAG-1 and RAG-2 genes in activated mature mouse B cells. *Science* 274:2092.
- Hikida, M., M. Mori, T. Kawabata, T. Takai, and H. Ohmori. 1997. Characterization of B cells expressing recombination activating genes in germinal centers of immunized mouse lymph nodes. *J. Immunol.* 158:2509.
- Hikida, M., and H. Ohmori. 1998. Rearrangement of lambda light chain genes in mature B cells in vitro and in vivo: function of reexpressed recombination-activating gene (RAG) products. *J. Exp. Med.* 187:795.
- Han, S., B. Zheng, J. Dal Porto, and G. Kelsoe. 1995. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. IV. Affinity-dependent, antigen-driven B cell apoptosis in germinal centers as a mechanism for maintaining self-tolerance. *J. Exp. Med.* 182:1635.
- Han, S., B. Zheng, D. G. Schatz, E. Spanopoulou, and G. Kelsoe. 1996. Neoteny in lymphocytes: Rag1 and Rag2 expression in germinal center B cells. *Science* 274:2094.
- Han, S., S. R. Dillon, B. Zheng, M. Shimoda, M. S. Schissel, and G. Kelsoe. 1997. V(D)J recombinase activity in a subset of germinal center B lymphocytes. *Science* 278:301.
- Papavasiliou, F., R. Casellas, H. Suh, X. F. Qin, E. Besmer, R. Pelanda, D. Nemazee, K. Rajewsky, and M. C. Nussenzweig. 1997. V(D)J recombination in mature B cells: a mechanism for altering antibody responses. *Science* 278:298.
- Tarlinton, D. 1997. Germinal centers: a second childhood for lymphocytes. *Curr. Biol.* 7:R155.
- Melamed, D., R. J. Benschop, J. C. Cambier, and D. Nemazee. 1998. Developmental regulation of B lymphocyte immune tolerance compartmentalizes clonal selection from receptor selection. *Cell* 92:173.
- Hertz, M., V. Kouskoff, T. Nakamura, and D. Nemazee. 1998. V(D)J recombinase induction in splenic B lymphocytes is inhibited by antigen-receptor signaling. *Nature* 394:292.
- Meffre, E., F. Papavasiliou, P. Cohen, O. de Bouteiller, D. Bell, H. Karasuyama, C. Schiff, J. Banachereau, Y.-J. Liu, and M. C. Nussenzweig. 1998. Antigen receptor engagement turns off the V(D)J recombination machinery in human tonsil B cells. *J. Exp. Med.* 188:765.
- Camacho, S. A., M. Kosco-Vilbois, and C. Berek. 1998. The dynamic structure of the germinal center. *Immunol. Today* 19:511.
- Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* 96, 23.
- Kepler, T. B. and A. S. Perelson. 1993. Cyclic re-entry of germinal center B cells and the efficiency of affinity maturation. *Immunol. Today* 14:412.
- Kepler, T. B. and A. S. Perelson. 1995. Modeling and optimization of populations subject to time-dependent mutation. *Proc. Natl. Acad. Sci. USA* 92:8219.
- Oprea, M. and A. S. Perelson. 1997. Somatic mutation leads to efficient affinity maturation when centrocytes recycle back to centroblasts. *J. Immunol.* 158:5155.
- Garside P., E. Ingulli, R. R. Merica, J. G. Johnson, R. J. Noelle, and M. K. Jenkins. 1998. Visualization of specific B and T lymphocyte interactions in the lymph node. *Science* 281:96.
- Flynn, S., K. M. Toellner, C. Raykundalia, M. Goodall, and P. Lane. 1998. CD4 T cell cytokine differentiation: the B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, Blnr-1. *J. Exp. Med.* 188:297.
- Han, S., K. Hathcock, B. Zheng, T. B. Kepler, R. Hodes, and G. Kelsoe. 1995. Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers. *J. Immunol.* 155:556.
- Rajewsky, K. 1998. Burnet's unhappy hybrid. *Nature* 394:624.
- Weinstein, P. D., A. O. Anderson, and R. G. Mage. 1994. Rabbit IgH sequences in appendix germinal centers: V_H diversification by gene conversion-like and hypermutation mechanisms. *Immunity* 1:647.
- Weinstein, P. D., R. G. Mage, and A. O. Anderson. 1994. The appendix functions as a mammalian bursal equivalent in the developing rabbit. *Adv. Exp. Med. Biol.* 355:249.
- Thompson, C. B., and P. E. Neiman. 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell* 48:369.
- Reynaud, C. A., V. Anquez, H. Grimal, and J. C. Weill. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell* 48:379.
- Reynaud, C. A., A. Dahan, V. Anquez, and J. C. Weill. 1989. Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell* 59:171.
- Archer, O. K., D. E. R. Sutherland, and R. A. Good. 1963. Appendix of the rabbit: a homologue of the bursa in the chicken? *Nature* 200:337.
- Pospisil, R., and R. G. Mage. 1998. Rabbit appendix: a site of development and selection of the B cell repertoire. In *Current Topics in Microbiology and Immunology. Somatic Diversification of Immune Responses*, G. Kelsoe and M. Flajnik, eds. Springer-Verlag, Heidelberg, p. 59.
- Knight, K. L., and R. S. Becker. 1990. Molecular basis of the allelic inheritance of rabbit immunoglobulin VH allotypes: implications for the generation of antibody diversity. *Cell* 60:963.
- Allegrucci, M., G. O. Young-Cooper, C. B. Alexander, B. A. Newman, and R. G. Mage. 1991. Preferential rearrangement in normal rabbits of the 3' VHα allotype gene that is deleted in Alicia mutants; somatic hypermutation/conversion may play a major role in generating the heterogeneity of rabbit heavy chain variable region sequences. *Eur. J. Immunol.* 21:411.
- Knight, K. L., and M. A. Crane. 1994. Generating the antibody repertoire in rabbit. *Adv. Immunol.* 56:179.
- Becker, R. S., and K. L. Knight. 1990. Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. *Cell* 63:987.
- Roux, K. H., P. Dhanarajan, V. Gottschalk, W. T. McCormack, and R. W. Renshaw. 1991. Latent α1 VH germline genes in an a² rabbit: evidence for gene conversion at both the germline and somatic levels. *J. Immunol.* 146:2027.
- Carlson, L. M., W. T. McCormack, C. E. Postema, E. H. Humphries, and C. B. Thompson. 1990. Templated insertions in the rearranged chicken IgL V gene segment arise by intrachromosomal gene conversion. *Genes Dev.* 4:536.
- Eisen, H. N., and G. W. Siskind. 1964. Variations in affinities of antibodies during the immune response. *Biochemistry* 3:996.
- Werblin, T. P., Y. T. Kim, R. Mage, B. Benacerraf, and G. W. Siskind. 1973. The generation of antibody diversity. I. Studies on the population distribution of anti-DNP antibody affinities and on the influence of allotype on antibody affinity and concentration. *Immunology* 25:17.
- Tunyaplin, C., and K. L. Knight. 1997. IgH gene rearrangements on the unexpressed allele in rabbit B cells. *J. Immunol.* 158:4805.

46. Crane, M. A., M. Kingzette, and K. L. Knight. 1996. Evidence for limited B-lymphopoiesis in adult rabbits. *J. Exp. Med.* 183:2119.
47. Sehgal, D., E. Schiaffella, A. O. Anderson, and R. G. Mage. 1998. Analyses of single B cells by PCR reveals rearranged V_H with germline sequences in spleens of immunized rabbits: Implications for B cell repertoire maintenance and renewal. *J. Immunol.* 161:5347.
48. Eisen, H. N. 1964. Preparation of purified anti 2,4-dinitrophenyl antibodies. *Methods Med. Res.* 10:94.
49. Kuppers, R., M. Zhao, M. L. Hansmann, and K. Rajewsky. 1993. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. *EMBO J.* 12:4955.
50. Liu, A. H., G. Creadon, and L. J. Wysocki. 1992. Sequencing heavy- and light-chain variable genes of single B-hybridoma cells by total enzymatic amplification. *Proc. Natl. Acad. Sci. USA* 89:7610.
51. Liu, A. H., P. K. Jena, and L. J. Wysocki. 1996. Tracing the development of single memory-lineage B cells in a highly defined immune response. *J. Exp. Med.* 183:2053.
52. McHeyzer-Williams, M. G., G. J. Nossal, and P. A. Lalor. 1991. Molecular characterization of single memory B cells. *Nature* 350:502.
53. Hecker, K. H., and K. H. Roux. 1996. High and low annealing temperatures increase both specificity and yield in touchdown and stepdown PCR. *Biotechniques* 20:478.
54. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463.
55. van Rooijen, N., N. Kors, and R. van Nieuwmegen. 1984. Double immunocytochemical evidence for a clonal development of specific antibody-containing cells in the rabbit spleen. *Anat. Rec.* 209:385.
56. Kroese, F. G., A. S. Wubbena, H. G. Seijen, and P. Nieuwenhuis. 1987. Germinal centers develop oligoclonally. *Eur. J. Immunol.* 17:1069.
57. Berek, C. 1992. The development of B cells and the B-cell repertoire in the microenvironment of the germinal center. *Immunol. Rev.* 126:5.
58. Arakawa, H., K. Kuma, M. Yasuda, S. Furusawa, S. Ekino, and H. Yamagishi. 1998. Oligoclonal development of B cells bearing discrete Ig chains in chicken single germinal centers. *J. Immunol.* 160:4232.
59. Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller. 1991. *Sequences of Proteins of Immunological Interest*, 5th Ed. Dept. of Health and Human Services, Washington, DC.
60. Chen, H. T., C. B. Alexander, F. F. Chen, and R. G. Mage. 1996. Rabbit DQ52 and DH gene expression in early B-cell development. *Mol. Immunol.* 33:1313.
61. Arakawa, H., S. Furusawa, S. Ekino, and H. Yamagishi. 1996. Immunoglobulin gene hyperconversion ongoing in chicken splenic germinal centers. *EMBO J.* 15:2540.
62. Brezinschek, H. P., S. J. Foster, T. Dorner, R. I. Brezinschek, and P. E. Lipsky. 1998. Pairing of variable heavy and variable κ chains in individual naive and memory B cells. *J. Immunol.* 160:4762.
63. McHeyzer-Williams, M. G., M. J. McLean, P. A. Lalor, and G. J. Nossal. 1993. Antigen-driven B cell differentiation in vivo. *J. Exp. Med.* 178:295.
64. Mage, R. 1998. Diversification of rabbit V_H genes by gene-conversion-like and hypermutation mechanisms. *Immunol. Rev.* 162:49.
65. Smith, K. G. C., A. Light, G. J. V. Nossal, and D. M. Tarlinton. 1997. The extent of affinity maturation differs between the memory and antibody-forming cell compartments in the primary immune response. *EMBO J.* 16:2996.
66. Takahashi, Y., P. R. Dutta, D. M. Cerasoli, and G. Kelsoe. 1998. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. V. Affinity maturation develops in two stages of clonal selection. *J. Exp. Med.* 187:885.
67. Foote, J. and C. Milstein. 1991. Kinetic maturation of an immune response. *Nature* 352:530.