The Repertoire of Somatic Antibody Mutants Accumulating in the Memory Compartment after Primary Immunization Is Restricted through Affinity Maturation and Mirrors That Expressed in the Secondary Response

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Summary

The anti-(4-hydroxy-3-nitro-phenyl)acetyl (NP) response is dominated by \( \lambda \) chain-bearing antibodies expressing the \( V_\kappa \) gene \( V^{186.2} \) in combination with the D element DFL16.1. \( \lambda \)-positive B cells were isolated from the spleens of mice immunized with NP-chicken gamma globulin 6 wk earlier. Rearranged \( V^{186.2} \) genes were amplified from the genomic DNA of these cells and sequenced. In cases where the rearrangement was typical for secondary anti-NP antibodies, the \( V_\kappa DJ_N \) sequences were generally heavily mutated. The frequency and the nature of the nucleotide exchanges mirrored those of secondary response antibodies. \( V^{186.2} \) genes with other rearrangements and \( V^{186.2} \)-related genes isolated concomitantly were essentially unmutated. These results demonstrate: (a) that somatic antibody mutants are largely restricted to a small compartment of peripheral B cells, namely, that of memory cells; (b) that the memory compartment is strongly selected for high affinity precursors and largely purged from antigen-binding loss mutants; and (c) that the repertoire of binding specificities expressed in the secondary response is established in its final form before secondary immunization.

Present knowledge about the molecular nature of the B cell memory compartment is derived from the phenotypic and genotypic analysis of secondary immune responses. Memory B cells that differentiate into secondary response plasma cells are of different clonal origin than primary response precursor cells. Their variable region (V) genes are extensively somatically mutated. Collectively, they bind the immunizing antigen with higher affinity than primary response antibodies (1). However, it is not known whether the secondary response readout of B cell memory generated in response to a particular antigen reflects the overall composition or a selected subset of the memory cell pool. Considering the high rate of somatic mutation, \( \sim 10^{-3}/\text{bp/cell division} \) (2, 3), one must expect the generation of antibody mutants that do not bind antigen with increased affinity, including low affinity binders, loss variants, and mutants that have acquired specificity for autoantigens. In fact, experimental evidence has been obtained indicating that auto-reactivity can be generated by somatic mutation (4), and that variants with drastically reduced affinities or altered antigen binding specificities do persist in the memory compartment (5). We previously identified the B cell precursor of a secondary response lymphocyte with genotypic characteristics strongly suggesting its original stimulation by the immunizing antigen and subsequent loss of immunogen binding through somatic mutation (5). Its crippling mutation had been identified independently in a hybridoma variant isolated in vitro (6). Interestingly, the loss of binding to the immunizing antigen in this variant was paralleled by the acquisition of binding specificity for a related hapten. Based on these observations, one could hypothesize that the antigen-induced hypermutation mechanism not only serves to increase the efficiency with which an organism deals with a primary immunizing agent, but in addition, acts as a means to increase the overall diversity of the antibody repertoire of peripheral B cells. A prediction resulting from this model would be a discrepancy between the molecular characteristics of the overall population of memory B cells induced by a particular antigen, and the subset expressed after secondary immunization.

With the advent of PCR technology, it has become possible to selectively draw into a molecular analysis the subset of rearranged \( V \) genes known to dominate a particular immune response. This has enabled us to study the composition of the memory compartment directly, independent of its expression triggered by secondary immunization with antigen. As a model system, we used the immune response to...
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independent V186.2-encoded secondary response antibodies
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This response has been well characterized. It is
dominated by λ1-bearing antibodies expressing the H chain V gene V186.2
in association with the D segment DFL16.1. In approximately
half of these antibodies, the 5' part of DFL16.1 consists of
germline sequences encoding three consecutive tyrosines (7–9).
In secondary response antibodies, the 3' part of DFL16.1 is
replaced by heterogeneous N sequences (8, 9). 70% of clonally
independent V186.2-encoded secondary response antibodies
share a mutation at position 33 of the H chain (8, 9). This
tryptophan-to-leucine exchange alone is sufficient to account for
the 10-fold increase in affinity that distinguishes most sec-
condary from primary response anti-NP antibodies (10). Addi-
tional mutations in the H chain gene as well as amino acid
substitutions in the V region of the L chain contribute in
most cases at best marginally to the hapten binding affinity.
We have studied the composition of the memory compart-
ment by isolating and sequencing V186.2 genes joined to
DFL16.1 from λ1-expressing spleen cells of mice that had
received a single immunization with NP 6 wk earlier.

Materials and Methods

Immunizations. 10-wk-old female C57BL/6 mice from our
colony were primed by intraperitoneal injection of 100 μg of alun-
precipitated NP-CG together with 2 × 10^9 inactivated pertussis
bacteria.

Isolation of Spleenic λ1-Expressing B Cells. Spleen cells of 12
mice were pooled and depleted of erythrocytes by treatment with 0.08%
NH₄Cl. The cells were stained on the surface with the biotiny-
lated anti-λ1 antibody Ls136 (11), and streptavidin-FITC. Ls136-
positive cells comprised 1.8% of the population. λ1+ cells were en-
riched to a level of 45% using a magnetic cell sorter (12), and
subsequently enriched to 97% by fluorescence-activated cell sorting
on a FACS 440. Dead cells and cells other than lymphocytes were
excluded by the addition of propidium iodide and appropriate
gating.

Preparation of Cellular DNA and PCR for Amplification. Genomic
DNA of 4.5 × 10^4 sorted cells was purified (13). The following
oligonucleotide primers were used for PCR amplification: V186.2
5'CTGACCCAGATGCTCCCTCCAGCGG 3' (this primer hy-
bridizes to genomic DNA 5' of the transcription start
site of V186.2); intron j, 5'GGGTCTAGACCGGTTTCCA-
AGTAATGTCGAG 3'; intron j, 5'GGGTCTAGAGGGTGTCG-
ACCAGGG 3' (the j primers include the recog-
nition sequence for XbaI). V186.2 genes rearranged to Jλ1 and Jλ2,
respectively, were amplified in separate reactions. Each PCR reac-
tion contained 10% of total purified DNA, 20 pmol of each primer,
10 mM Tris-Cl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 200 μM
of each dNTP, 0.01% gelatine, and 7 U Taq polymerase (Perken-
Elmer Corp., Hayward, CA) in a total volume of 50 μl. PCR was
performed for 50 cycles: each cycle consisted of 96°C for 1.4 min
and 70°C for 2 min.

Cloning of Amplified DNA and Bacterial Transformation. Am-
plified DNA was extracted once with phenol-chloroform, precipi-
tated with NaAc/ethanol, digested with the restriction enzymes
NcoI and XbaI, and subjected to agarose gel electrophoresis. The
DNA fragment corresponding to the expected size was purified
and ligated into a modified pUC19 vector which contains an addi-
tional multisite cloning cassette derived from the plasmid vector
pGEM-5Zf (+) (Promega Biotec, Madison, WI) (kind gift of Steffen
Jung, Institute for Genetics, Köln, FRG). DH-5xr bacteria were
transformed by electroporation (gene pulser; Bio-Rad Laborato-
ries, Munich, FRG).

 Colony Hybridization and DNA Sequence Determination. Bacterial
colony hybridization with a 32P end-labeled oligonucleotide was
performed as described (14). The probe had the following sequence:
5'GTAGTATATCTTGCC 3'; it hybridized to the terminal six
nucleotides of the Vh16.2 gene plus the initial nine nucleotides of
DFL16.1 when joined directly to the Vh1 gene in reading frame
1 (15). Vh gene sequences were obtained by direct plasmid
sequence using the Sequenase TM kit (United States Biochemical
Corp., Cleveland, OH).

Expression Vector Construction and Transfection into Myeloma
Cells. The mutated V186.2 genes designated K3 and K11 in Fig.
3 were excised from modified pUC19 and inserted into an Ig γ1
expression vector (pEVCcyl). The vector pEVCcyl was constructed
by inserting the enhancer cassette of pEV₈ (16) into the vector
pMSLP (17). The resulting constructs were linearized with PvuI
and transfected into J558L myeloma cells (18) by electroporation.
J558L cells were grown in RPMI 1640 supplemented with 10%
FCS, penicillin (100 μg/ml), streptomycin (100 μg/ml), and 2 ×
10⁻³ M 2-ME. Transfected cells were seeded into 48-well plates
48 h after transfection and selected for expression of the bacterial
gpt marker in supplemented RPMI containing 5 μg/ml myco-
phenolic acid, 250 μg/ml xanthine, and 50 μg/ml hypoxanthine.
lg-producing cell lines were identified by screening culture super-
natants in an avidin-biotin ELISA (19) using goat anti–mouse IgG₁
antibodies (Southern Biotechnology Associates, Birmingham, AL)
to coat the plastic plates. Cells supernatants were used for serologi-
cal assays.

 Farr Assay. The method used for determining antibody binding
affinity has been described (20). It is based on the inhibition of
binding antibody to N[125I]caproate by increasing concentrations
of unlabeled NP-caproate. The concentration of hapten resulting
in 50% inhibition of N[125I]caproate binding approximates the
binding constant for the hapten.

Results

Experimental Design. C57BL/6 mice were immunized in-
traperitoneally with a single dose of NP-CG. Six wk later
spleen cells expressing λ1 L chains on their surface were iso-
lated as described above, using a biotinylated λ1-specific mAb,
and magnetic and fluorescence activated cell sorting. Genomic
DNA was prepared, and V186.2 genes rearranged to Jλ1 and
Jλ2 were amplified in separate PCRs. In most (57%) of the
secondary response anti-NP antibodies that express the Vh
gene V186.2, it is joined to Jλ2, but rearrangements to Jλ1,
Jλ3, and Jλ4 were also seen. After digestion with appropriate
restriction enzymes, the amplified DNA was cloned into a
derivative of the plasmid vector pUC19. Bacterial colonies
expressing V186.2 genes were identified by direct DNA se-
quence analysis or after screening with a radioactively labeled
oligonucleotide. Since the Vh gene specificity of the am-
plication reaction rests on the 5' PCR primer, and V186.2
belongs to a large family of related Vh genes (group 1 in the
classification of Dildrop [reference 21]), it was not surprising
to find that the majority (75%) of genes recovered by direct
sequence analysis were genes closely related to V186.2 but
not V186.2 itself. To facilitate the identification of rearranged
V186.2 genes derived from NP-induced memory cells, we
designed an oligonucleotide homologous to the Vh-to-D
junction of approximately half of the antibodies that had pre-
viously been recovered after immunization with NP (7-9).
It encompasses the six most 3' bp of V186.2 and the nine
most 5' bp of DFL16.1. We cannot rule out that the selec-
tion associated with this screening procedure introduces a
bias in the scope of the analysis. However, it is unlikely that
the focus on Vh-to-D junctions characteristic for rearranged
genes known to participate in the response against NP should
selectively exclude those genes from the analysis that encode
antibodies which, through hypermutation, have lost the ability
to respond to a secondary immunization with NP and/or
have acquired new antigenic specificities.

The Vh Gene Repertoire of Splenic B Cells Includes Germ-
line and Somatically Mutated Vh Genes. The nucleotide se-
dequences of altogether 49 rearranged Vh genes were deter-
mined. They fall into two classes: genes that differ from the
germline at most at two positions, and genes that are exten-
sively mutated. The first class includes V186.2-related genes
joined to heterogenous D elements, V186.2 genes with heter-
egenous Vh-to-D junctions, and V186.2 genes joined
directly to DFL16.1 in reading frame 1. The second class con-
ists exclusively of V186.2 genes in direct association with
DFL16.1.

Most of the Rearranged Vh Genes in the Peripheral B Cell Pool
of the Mouse Are Unmutated. Taking into account the avail-
able sequence information encompassing the Vh region but
excluding possible nucleotide exchanges in the D region and
at the Vh-D and D-Jh joins, we observe three mutations in
1,827 nucleotides derived from 8 V186.2-related genes, eight
mutations in 5,258 nucleotides derived from 17 V186.2 genes
with heterogenous Vh-to-D junctions (data not shown), and
three mutations in 2,127 nucleotides derived from 7 V186.2
genesis joined directly to DFL16.1 (Fig. 1). This corresponds
to mutation frequencies of 1 in 609, 1 in 657, and 1 in 709,
respectively. The 14 exchanges are distributed over 13 of the
32 Vh gene sequences present in this class. We believe that
these mutations were introduced not in vivo but in vitro for
the reasons outlined below.

Figure 1. Sequences of the V186.2
genesis that are functionally rearranged
to the D element DFL16.1 in reading
frame 1 and show no evidence for hyper-
mutation. The sequences are compared
with the germline Vh gene V186.2,
the DFL16.1 segment, and the Jh seg-
ments Jh1 and Jh2. Nucleotides identical
to the reference sequence are indicated
by dashes; blanks indicate uncertainty.
Blanks at the 5' end of the D and Jh
segments denote the absence of those
codon from the sequenced gene. Marked
in black circles are the amino acid sub-
stitutions relative to the reference se-
quence. The codons are numbered ac-
cording to Kabat et al. (22). The amino
acid length of CDR3 is indicated. g1,
germline.

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It has been shown that the Taq polymerase used for PCR amplification has a misincorporation rate of $\sim 10^{-4}$ (23, 24). From the number of PCR cycles our DNA isolates were subjected to before sequence analysis (i.e., 50) and the observed mutation frequency of overall 1 in 658, we calculate a misincorporation rate of $6 \times 10^{-3}$ (23). This mutation rate lies somewhat below the Taq polymerase error rate described by others (23, 24). This difference could be due to slightly varied reaction conditions during PCR amplification. The mutations apparent in this set of genes resemble in their molecular nature typical Taq polymerase errors in that there is a strong bias for purine transitions (86%), most of which result in the replacement of a TA by a C-G bp (67%). Taken together, the frequency and the nature of the mutations make it likely that they were generated in vitro, and that the peripheral B cells from which these gene sequences were derived expressed antibody receptors encoded by germline genes.

7 of the 8 V186.2-related and 16 of the 17 V186.2 genes of this essentially unmutated subset are productively rearranged to D elements differing widely in length and sequence, and are characterized by extensive junctional variability. This is depicted in Fig. 2. Because of this variability, we consider these Vn region genes representative of the genes expressed by the population of naive peripheral B cells. This sequence analysis therefore gives direct evidence for the predominant expression of germline-encoded antibodies in the peripheral B cell pool of the mouse.

The seven V186.2-DFL16.1-Jn gene combinations depicted in Fig. 1 resemble the H chain V genes of typical anti-NP antibodies in that they encode three tyrosines at the 5′ end of CDR3. However, three of the seven genes in this group have retained all or most of the germline sequence of the 3′ half of DFL16.1. In particular, downstream of the codons specifying the three tyrosines, they encode a glycine followed by two serines. In all secondary response antibodies that have been obtained from hybridomas so far, the amino acid sequence deviates from the germline-encoded gly-ser-ser sequence (8, 9). These codons are substituted by N sequences generating a truncated CDR3 with an average length of nine amino acids. Curiously, with few exceptions, all primary anti-NP antibodies studied to date do express the germline-encoded gly-ser-ser sequence (7). Thus, as judged by the sequence and length of their CDR3s, at least three of the gene sequences in this set, i.e., K112, K59, and K9, encode H chain structures that appear to be incompatible with those required for channeling the parent B cells into the memory compartment. The CDR3s specified by the other four Vn gene sequences in this set have also not been seen in secondary response antibodies. It is not clear whether these genes originate from B cells with antibody receptors that did not fit the prerequisites for memory cell precursors or else from B cells that were seeded into the peripheral immune system after termination of the antigen-stimulated recruitment of memory cell precursors into the hypermutation pathway.

**The Majority of Rearranged Vn Genes Likely to Encode NP Binding Specificity in Peripheral B Cells of NP-primed Mice Are Mutated.** 24 V genes specifying the gene segment combination known to dominate the anti-NP response, i.e., V186.2 joined to the D element DFL16.1, were isolated in the course of this study. The majority of them, i.e., 17, carry between three and 15 substitutions (excluded are possible nucleotide exchanges in the D region and at the Vn-D and D-Jn joins) (Fig. 3). Within 5,565 nucleotides sequenced altogether, we identified 108 exchanges, corresponding to a mutation frequency of 1 in 52. We have classified these genes as somatic variants of V186.2 rather than representatives of related germline genes based on the following: (a) a comparison of the nucleotide sequences of the Vn genes depicted in Fig. 3, with all members of the group 1 gene family, shows that each is more homologous to V186.2 than to any other one member of this family. 63 different germline Vn genes can presently be assigned to Vn group 1 in strain C57BL/6 on the basis of nucleotide sequence homologies (Gu, H., I. Förster, W. Müller, and K. Rajewsky, manuscript in preparation). (b) In contrast, eight V186.2-related genes can clearly be identified as known germline genes (Fig. 2). (c) The tryptophan-to-leucine exchange at codon 33, which is specified by 70% of the genes depicted in Fig. 3, is typical for V186.2-encoded antibodies of the secondary response against NP. The genomic DNA of six secondary response hybridomas secreting antibodies that contain leucine at position 33 has previously been analyzed by restriction enzyme analysis and found to be consistent with the predictions of V186.2 rearrangements to different Jn segments (8).

The mutation frequency of 1 in 52 is 13-fold higher than the frequency of errors introduced by Taq polymerase in the course of PCR. Since both classes of genes in the present collection — those with few and those with many mutations — had been amplified together, it is fair to assume that they were mutated by Taq polymerase to the same extent. Therefore, the great majority (≈90%) of the mutations present in the 17 genes depicted in Fig. 3 cannot be attrib-

| Vn gene | 3′ end of D element and N additions |
|--------|-----------------------------------|
| Q1     | AGA TAT AGT GCT AGC ACA G          |
| Q2     | AGA TA CTC GCC GCT TCC             |
| Q3     | AGA TAT CCC GAC TCC                |
| Q4     | AGA AAT GAC TCT GAG                |
| Q5     | AGA TAT AGT GCT A                 |
| Q6     | AGA TTC GCT ACT AGT GTC ACA A    |
| Q7     | AGA GAT GCT TAC GGC T             |
| Q8     | AGA TAT AGC GAT GGG               |
| Q9     | AGA GGA GGG AGT TGG               |
| Q10    | AGA TTC TAT TT                    |
| Q11    | AGA AGT ACC CAG GDD               |
| Q12    | AGA AAA TAT TGG GCT CCG C         |
| Q13    | AGA TTC GCT TAC GCT GGT AGT TCC  |
| Q14    | AGA GAG GGA TTA CCA CC            |
| Q15    | AGA CCG TTA ACT GGC AC            |
| Q16    | AGA CCG TTA ACT GGC AC            |

**Figure 2. VnDn** junctional sequences of V186.2 genes functionally rearranged to D elements other than DFL16.1 (designated Q1 to Q16), and of V186.2-related genes (designated R1 to R7). The Vn germline genes corresponding to R1 to R7 are as follows: R1, V 165.1 (reference 5); R2, V 751.5.6 (5); R3, V 165.1 (5); R4, V CH14 (Dildrop, R., unpublished results); R5, V 24.8 (8); R6, V CH140 (reference 25); R7, V 3 (reference 26).
Figure 3. V region sequences of somatically mutated V186.2 genes joined to the D element DFL16.1 in reading frame 1. Replacement mutations (filled circles), and the amino acid length of CDR3 are indicated. For further description see legend to Fig. 1.

Apart from the extensive mutagenesis characterizing these genes, two additional features make their derivation from memory cells likely: (a) the CDR3s they specify have an average length of nine amino acids as is typically seen in secondary anti-NP antibodies (8, 9); and (b) in all cases, the germ-line sequence encoding gly-ser-ser in the 3' half of DFL16.1 has been replaced by N sequences. As pointed out above, the absence of the germline-encoded sequence gly-ser-ser distinguishes all V186.2-DFL16.1-encoded secondary response anti-NP antibodies from the great majority of the corresponding primary response antibodies (7-9).

The Pattern of Mutations Suggests the Selective Accumulation of High Affinity Mutants in the Memory Compartment. A comparison of the 17 heavily mutated V186.2 genes depicted in Fig. 3 with the genes encoding previously isolated secondary response antibodies reveals striking similarities in terms of frequency and nature of the mutations. In our present collection of genes derived from NP-primed mice, we observe an average of 6.3 exchanges per rearranged V\(_{\mu}\)DJ\(_{\mu}\) gene, spread over a range of 3 to 15. Secondary response antibodies isolated in two independent studies showed an average of 5.5 and 6.7 mutations per gene falling into a range of 2 to 12 and 3 to 12, respectively (8, 9). The ratio of replacement to silent (R/S) mutations in the genes of secondary response antibodies typically diverges from the value expected in a model of random mutagenesis in that replacement mutations are overrepresented in the complementarity-determining regions and underrepresented in the framework regions. This bias is apparent also in the V\(_{\mu}\) gene sequences depicted in Fig. 3. In the framework regions we observe an R/S ratio of 1.6:1. Taking into account the codon composition of V186:2, we would have expected an R/S ratio of 3.1:1 for random mutagenesis. The observed ratio of 1.6:1 correlates with the R/S ratio of mutations in the frameworks of V\(_{\mu}\) from 29 previously analyzed secondary anti-NP antibodies, i.e., 1.5:1 (references 8, 9), and indicates that amino acid exchanges were counterselected in the region defining the structural backbone of an antibody. In contrast, in CDR1 we observe an R/S ratio of 25:1 in the present gene collection that compares with a ratio of 21:1 in previously isolated secondary anti-NP antibody V\(_{\mu}\) genes (8, 9). The R/S ratio in CDR1 exceeds the expected value of 14:1, indicating that a fraction of the replacement mutations are positively selected in this region (see also below).

In the NP system, the secondary immune response differs from the primary one by an ~10-fold increase in affinity (27).
We have previously shown in the analysis of in vitro-engineered V186.2-DFL16.1-encoded antibodies that a single replacement in CDR1, a tryptophan-to-leucine exchange at codon 33, raises the binding affinity 10-fold (3, 10). This particular mutation is shared by 70% of all clonally independent secondary response antibodies derived from hybridomas (8, 9). Also 70%, i.e., 12 of the 17 mutated genes that we isolated from NP-primed mice and analyzed in the present study, encode this particular amino acid substitution (Fig. 3).

To confirm the association of the position 33 mutation with an increase in affinity to the hapten NP in the present V₄ gene collection, we subcloned two of the V₄ sequences into an expression vector carrying the γ1 C region gene. One of the two, K3, encodes nine replacement mutations, including the tryptophan-to-leucine exchange at position 33. The other, K11, encodes four amino acid replacements, and specifies the germline tryptophan at position 33. The expression vector constructs were transfected into the myeloma cell line J558L, which synthesizes germline A1 L chains. The endogenous L chains pair with the H chains encoded by the transfected gene and antibodies are secreted. (The mutation rate for the A1 L chain is severalfold lower than for the H chain, and studies with engineered antibodies indicate that mutations in the A1 chain generally do not contribute to an increase in the binding affinity in the NP system [8–10].) The affinities to NP were measured by competitive inhibition of radio-labeled hapten (Table 1). As expected, the antibody formed with the H chain mutant K3 (which contains the tryptophan-to-leucine exchange at position 33) exhibits an eightfold higher affinity than the prototype primary antibody NIG9 which expresses the germline form of V₄186.2 (0.36 vs. 2.7 μM). It thus lies within the range of affinities displayed by most secondary response antibodies (0.3–1 μM). In contrast, the four amino acid exchanges in the antibody formed with the H chain mutant K11, which does not contain the position 33 mutation, do not effect an increase in affinity. A few low affinity binders have also been found among hybridoma-derived secondary response antibodies (8).

In addition to the key mutation specifying the amino acid exchange at position 33, other amino acid residues are recurrently mutated in the genes depicted in Fig. 3. Within the regions of CDR1 and CDR2, codons 31, 34, 53, 60, 61, 64, and 65 are mutated in more than one gene. We believe that most of these mutations are the result of mutational hotspots and reflect intrinsic properties of the hypermutation mechanism rather than selective forces for hapten binding affinity for the following reasons. Although an amino acid replacement can usually be brought about by an exchange in the first or second nucleotide position of a codon, in six of the seven codons considered here the same nucleotide is replaced. The restriction characterizing the target of mutagenesis is not paralleled by a similar restriction concerning the nature of the inserted amino acid. In the two codons affected the most, codon 31 (mutated in 8 of 17 genes) and codon 60 (mutated in 6 of 17 genes), the mutated nucleotide is seen replaced with any one of the three possible alternatives, leading to the insertion of three different amino acids.

### Table 1. Affinities of Somatically Recombinant Antibodies to NP

| Antibody     | $K_{NP}$ (μM) |
|--------------|---------------|
| NIG9*        | 2.7           |
| aK3          | 0.36          |
| aK11†        | 12.0          |

Affinities of the antibodies for NP-caproate are shown; for experimental details see Materials and Methods.

* Antibody NIG9 is an unmutated primary response anti-NP IgG1 antibody carrying A1 L chains and expressing V186.2 in combination with DFL16.2 (reference 7).
† aK3 and aK11 are recombinant antibodies formed by association of the germline A1 L chain expressed in J558L cells with the H chains encoded by the expression vectors pEV₄-K3-γ1 and pEV₄-K11-γ1, respectively.

The center base of codon 64 which is affected less often (mutated in 4 of 17 genes) has been exchanged for two different bases specifying two different amino acids. If the amino acid exchanges were driven by selective forces, one would expect to observe a trend pointing in the opposite direction, i.e., to find a preponderance of parallel amino acid exchanges encoded by different triplets. (For example, the strongly selected leucine that replaces tryptophan at position 33 constitutes the only alternative amino acid inserted in this position in the anti-NP response, and has been seen encoded by any one of three different triplets; i.e., TTG, TTA, and CTG [reference 3, and Fig. 3]). The isoleucine replacing methionine in codon 34 is specified by two different triplets in 4 of the 17 genes depicted in Fig. 3. Previous studies with engineered antibodies, however, argue against the involvement of selective forces also in this exchange. The presence of isoleucine at position 34 in a secondary response antibody in which the position 33 mutation had been reverted to germline did not suffice to rescue the antibody from a 10-fold loss in affinity, thereby returning it to the affinity range of primary response antibodies (10). The consistent replacement of methionine by isoleucine at codon 34 may therefore result from the wobble nature of the base constituting a mutational hotspot at this position. Mutations presumably resulting from hotspot sites have also been described for other antigenic systems (28, 29). The suggestion has been made that mutational hotspots are associated with special features of the primary sequence or secondary structure of a DNA template that induce the polymerizing enzyme to pause and concomitantly relax its fidelity (30).

The recurrent mutation of particular nucleotides in codons 31, 34, 60, 61, 64, and 65 in the genes depicted in Fig. 3 is evident also in the genes of hybridoma-derived secondary response anti-NP antibodies (8, 9). Of the overall 21 different replacement mutations that fall into CDR1 and CDR2 in the present set of V₄ sequences, 13 have also been identified in the NP-binding population of secondary response hybridomas.
Discussion

Predominance of Germline-encoded Specificities in the Peripheral B Cell Pool. It had been shown in earlier work that mitogen-activated peripheral B cells of the mouse, including cells of the Ly1 B subset, as well as B cells driven into primary antibody response, express germline-encoded V region genes (1, 31, 32). This suggested that the preimmune antibody repertoire in the mouse is essentially germline encoded. However, the earlier experiments invariably involved activation of subsets of B cells that might not be representative for the overall B cell population, the major fraction of which consists of long-lived, presumably selected cells (33). This problem is overcome in the present study in which Vn region genes are isolated from peripheral B cells independent of their state of activation. We find evidence for somatic hypermutation exclusively in the subset of V region genes with VnDJn combinations characteristic for anti-NP antibodies. In contrast, 23 functionally rearranged genes with extensive combinatorial and junctional diversity (Fig. 2), and unrelated to the anti-NP response, are virtually free of mutations. We consider these 23 sequences a representative sample of the V genes expressed in the peripheral B cell pool and conclude that most cells in this population express germline-encoded antibody specificities. This conclusion is further supported by recent experiments in which 32 different, functionally rearranged Vn genes were cloned directly from splenic B cells of unimmunized mice and found to be virtually unmutated (Gu, H., I. Förster, W. Müller, and K. Rajewsky, manuscript in preparation).

Size of the NP-specific Memory B Cell Compartment in NP-primed Mice. Given that most peripheral B cells in the mouse express germline-encoded antibodies, memory B cells, known to express somatic antibody mutants, must represent a minor B cell subset. The consideration of known parameters of the splenic B cell repertoire in C57BL/6 mice in conjunction with the data obtained in the course of this study permit a rough estimate of the size of the NP-specific memory B cell pool. Approximately 3% of the 4 × 107 splenic B cells in C57BL/6 mice express λ1 L chains (reference 34; and this paper). We assume that 64% of these cells, as of LPS-reactive splenic B cells (35), express Vn genes of the group 1, and 1–2% of these 64% express the Vn gene V186.2. This latter estimate is based on our recent analysis of group 1 Vn genes and their expression in strain C57BL/6 through cDNA amplification and sequencing (Gu, H., et al., manuscript in preparation). The number of splenic B cells expressing λ1 L chains in association with V186.2 should then be ~104. When we transformed bacteria with the DNA of amplified rearranged Vn genes from λ1-positive spleen cells and sequenced randomly picked clones, we found that ~20% (4/19) of the V186.2 genes were linked to the D element DFL16.1 and were hypermutated. All four of these hypermutated genes encoded three consecutive tyrosines at the Vn-D border, as is typical of NP-specific antibodies (data not shown). Based on this observation one arrives at an estimated number of 2 × 109 memory cells that bear λ1 L chains and express the H chain gene V186.2 joined to the D element DFL16.1 per spleen. From the analysis of hybridoma-derived secondary anti-NP antibodies, it is known that approximately half of the memory B cell precursors express λ1 L chains in association with Vn186.2 H chains that are joined to DFL16.1 and express three consecutive tyrosines at the beginning of CDR3. Taking this into account, the number of memory cells induced by the hapten NP in the spleen of primed mice should be on the order of 2–4 × 109. This frequency compares well with the frequency of antigen-specific memory cells that was measured late after a single immunization with the (multideterminant) protein PE. In two independent studies it was found to lie in the range of 1–5 × 104 per spleen (36, 37).

The Antigen-induced Memory B Cell Compartment Is Dominated by Antigen-selected High Affinity Binders. The somatically mutated, rearranged V186.2 genes, which were isolated from λ1 L chain-expressing spleen cells of NP-primed mice, strikingly resemble those expressed in secondary response anti-NP antibodies in terms of both rearrangement (i.e., CDR3) and frequency and characteristics of somatic mutations. Most importantly, the amino acid exchange, which represents the key mutation in the affinity maturation of the anti-NP response, is shared by the same proportion of genes (70%) in both collections. We conclude from this result that in a first approximation, the secondary anti-NP response is a direct readout of the somatic antibody mutants that have accumulated in the memory compartment.

This result supports our earlier demonstration that memory B cells recruited into the secondary response do not undergo further somatic mutation and produce a stable response of high affinity antibodies (5). Whether memory B cells can, alternatively, propagate themselves by reentering the pathway of hypermutation and selection (38) remains an open question.

The efficient and selective accumulation of cells expressing somatic antibody mutants with a high affinity for the immunizing antigen in the memory compartment has important biological implications. It is clear from the high rate of somatic mutation that memory cell generation must be accompanied by the production of antibody mutants that have changed their specificity away from the antigen. The present study demonstrates that the memory compartment is efficiently purged from cells expressing such antibodies. Apparently, antigenic selection is the key element in memory cell generation, and consequently, antigen-binding loss mutants only rarely make it to the stage of a long-lived memory cell. The persistence of such mutants in the memory compartment (5) is probably a rare event, triggered perhaps by the acquisition of a particular binding specificity.

The receptor repertoire of the B cells in the memory compartment thus focuses on the immunizing antigen, and high affinity binding to the immunizing antigen is its common denominator. However, the repertoire is also characterized by an extreme heterogeneity, due to the extent to which the antibodies are diversified by somatic mutation. Because of the high rate at which the hypermutation mechanism operates in the cells, affinity-selected mutations are almost invariably introduced in the context of other replacement mutations. Overall, this might be an efficient way for the systems to...
acquire a broad range of crossreactivities as it would seem desirable in the defense against repeated infections by closely related microbes. Still, immunity to a crossreactive agent would be carried by antibodies with a better fit to the original immunogen, quite in the sense of “original antigenicsin” (39).

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