Antigen-driven B Cell Differentiation In Vivo

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Summary

The secretion of specific antibodies and the development of somatically mutated memory B cells in germinal centers are consequences of T cell-dependent challenge with the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP). Using six-parameter flow cytometry and single cell molecular analysis we can directly monitor the extent of somatic hypermutation in individual responsive (isotype switched) antigen-specific B cells. The current study provides a direct quantitative assessment of recruitment into the antibody-secreting compartment on the one hand, and the germinal center pathway to memory on the other. Cellular expansion in both compartments is exponential and independent during the first week after challenge. The first evidence of somatic mutation, towards the end of the first week, was restricted to the germinal center pathway. Furthermore, germinal center cells express a significantly shorter third hypervariable region (CDR3), even when unmutated, than their antibody-secreting counterparts, suggesting a secondary selection event may occur at the bifurcation of these two pathways in vivo. By the end of the second week, the majority of mutated clones express a shorter CDR3 and affinity-increasing mutations as evidence of further selection after somatic mutation. These data provide evidence for substantial proliferation within germinal centers before the initiation of somatic mutation and the subsequent selection of a significant frequency of mutated clonotypes into the memory compartment.

Secretion of specific antibody by plasmacytes and the development of germinal centers is an early response to T cell-dependent immunization. After immunization, early B cell blast development is apparent in the periarteriolar lymphoid sheaths (PALS) in T cell zones of the spleen and produce foci of short-lived plasmacytes (1-4). In contrast, early proliferation seen within the follicular B cell regions creates a secondary lymphoid follicle which subsequently differentiates into a germinal center with histologically distinct light and dark zones (5-7). The complex microenvironment of the germinal center reaction is the proposed site of memory cell differentiation. Germinal center B cells are thought to randomly diversify their Ig genes by a process of somatic hypermutation after which high affinity variants are selectively preserved as memory cells for response to secondary antigenic challenge (6, 8-15). The mechanisms that initiate or control these complex differentiative responses to primary antigenic challenge remain elusive.

The response of C57BL/6 mice to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) has been well characterized at both the molecular and cellular level (16-18). B cell clones expressing antibodies using the V\textsubscript{H}186.2 H and V\textsubscript{L}1 L chain combination dominate the early primary NP response (19, 20). There is evidence for somatic mutation in these clonotypes as early as day 6 after primary NP immunization (21, 22). Furthermore, a mutational change in the CDR1 resulting in a tryptophan to leucine change at amino acid position 33 can lead to a 10-fold increase in affinity for NP (23). The change in position 33 is found in about 75% of late primary and memory clones and provides a good molecular marker of selection post-somatic mutation (24, 25). Using immunohistochemical methods, subsequent regional microdissection and molecular analyses, Jacob et al. (3, 12, 26) have analyzed both extrafollicular and germinal center pathways of NP-specific B cell development in vivo. These workers outline the magnitude and kinetics of the cellular response and identify the process of somatic mutation in only the germinal center pathway.

Using six-parameter flow cytometry, we can isolate individual IgG1\textsuperscript{+} NP-specific memory B cells after secondary challenge with NP (25) and IgG1\textsuperscript{+} NP-specific B cells from the primary response (27). Furthermore, the surface expres-
sion of an extracellular matrix receptor, syndecan (28, 29), defines antibody-secreting cells in vivo (27), whereas the lectin peanut agglutinin (PNA) selectively binds to high levels on within the antigen-specific compartment of a primary response. Single cell molecular analysis allows direct assessment of the mutational status within individual members from each of these pathways. The results confirm the early onset of somatic mutation to the end of the first week after challenge (22) and support the restriction of somatic mutation to the germinal center pathway of development (11, 12). The data also provide evidence of substantial proliferation in the germinal center before initiation of somatic mutation. Further, the pattern of CDR 3 usage in germinal centers versus antibody-secreting cells suggests that a secondary selection event may occur at the bifurcation of these pathways in vivo. Finally, a significant frequency of mutated clones bearing affinity-increasing mutations emerges by the end of the second week as evidence of further selection after somatic mutation.

Materials and Methods

Immunization, Spleen Cell Suspensions, and FACS® Staining. 8–10-wk-old specific pathogen-free, male and female C57Bl/6 mice were immunized with 200 μg of alum-adsorbed NP-KLH together with 10⁵ inactivated Bordetella pertussis as a blocking reagent. Mice were killed and spleens removed on the stated days after immunization and processed individually with erythrocytes and dead cells removed as previously described (31). Spleen cells were stained on ice for 20 min with biotin-goat anti-IgG1 (Southern Biotechnology Associates, Birmingham, AL) was washed, and resuspended in 50 μg/ml of purified MOPC2145T (an IgG1 myeloma) as a blocking reagent followed by staining with Texas red-avidin (TR-av), NP and NIP (4-hydroxy-5-iodo-3-nitrophenyl)acetyl-coupled allophycocyanin (NP/NIP-APC), PE conjugates of 331.12 (anti-IgM), 8C5 (anti-GR1) and M1/70 (anti-MAC1/CD11b) (331.12/8C5/M1/70-PE), and fluoresceinated JCS (anti-λ L chain; kindly provided by Dr. L. A. Herzenberg, Stanford University, Stanford, CA) or fluoresceinated PNA (Vector Laboratories Inc., Burlingame, CA). Alternatively, cells were stained with fluoresceinated anti-IgG1 (Southern Biotechnology Associates), blocked with MOPC245T, and stained with biotin-281.2 (anti-syndecan; 28), washed, and costained with TR-av, NP/NIP-APC, and 331.12/8C5/M1/70-PE. Cells were finally resuspended in propidium iodide (1 μg/ml), analyzed by use of a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA), and sorted using an automated cell deposition unit. All reagents and staining procedures have been described in more detail previously (27).

In Vitro Culture and Assay for Specific Antibody. Single cells were sorted directly into 200-μl 96-well microtiter plates with 2,500 BALB/c 3T3 fibroblasts per well, 40 μg/ml of Escherichia coli 011:B4 LPS (Difco Laboratories Inc., Detroit, MI), 50 U/ml rIL-4 (kindly provided by Dr. S. Gerendais, Walter and Eliza Hall Institute), 20 U/ml rIL-2 (Perkin Elmer Cetus Corp., Emeryville, CA), and 2% rIL-5-containing supernatant (vol/vol) for 8 d at 37°C in 10% CO₂ and air (32). ELISA for detection of total IgG1 and NP-specific IgG1 were performed as previously described (33, 34). Briefly, flexible U-bottomed 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight with 50 μl/well of either sheep anti–mouse Ig (4 μg/ml; Silenus Laboratories, Melbourne, Australia) or NP-BSA conjugate (25 mg/ml), washed, and samples added in PBS with 0.1% Tween 20, 0.3% (wt/vol) skim milk powder, and 1% newborn calf serum, held at room temperature for at least 4 h, washed before the addition of 50 μl/well horse-radish peroxidase conjugates of sheep anti–mouse IgG1 (Silenus Laboratories), and held again overnight at room temperature before washing and the addition of 100 μl/well of enzyme substrate 2,2'-azoniobis(3-ethylbenzthiazoline) (Sigma Chemical Co., St. Louis, MO) at 0.55 mg/ml in 0.1 M sodium citrate, pH 4.5, and 0.1% H₂O₂. Absorbance was read 1 h later at 414 nm (reference wavelength 492 nm) on a Titertek Multiscan (MCC/340; Flow Laboratories, McLean, VA).

Clonal V₅ Gene Analysis. Individual cells with the desired phenotype were sorted directly using the automatic cell deposition unit on the FACStar Plus® as two or four cells per well into 96-well microtiter plates containing 10 μg of carrier E. coli tRNA (Boehringer Mannheim Corp., Indianapolis, IN) and 200 μl/well of a protein denaturating solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.2% sarsosyl, 0.2 M 2-ME) followed by an acid phenol–chloroform extraction and ethanol precipitation of the RNA as described (35). First strand cDNA was synthesized from one fifth of each sample using AMV reverse transcriptase (Promega, Madison, WI) with oligo dT priming (Boehringer Mannheim Corp.) in standard reaction conditions. One quarter of the cDNA mixture was processed through two separate sets of the PCR (36) using ampli-Taq (1 U/30 μl reaction) (Perkin Elmer Cetus Corp.) in standard buffer containing 2 mM MgCl₂, 0.2 mM mixed dNTPs and 1 μM oligomer primers. First set primers were GGATGACCTCAGGGGTCAATGGAGTC for Ch₂/1 and GGTGTATCAITCTCCTTG for V₅/186.2 with the second set “nested” medially with CCAGGCGCGTGAGGAC for Ch₂/1 and GGTGTCATTCCAGTGTCACA for V₅/186.2. 35 cycles were performed with melting at 94°C for 45 s, annealing at 55°C for 60 s and elongation at 72°C for 90 s, 5 ml of a 1/500 dilution of the first round PCR reaction mixture was used as DNA source for 35 cycles of the second round using identical conditions. The PCR products were purified by low melt agarose gel electrophoresis and isolated by phenol–chloroform extraction. DNA sequencing was performed by the dideoxy chain termination method (37) with modifications for double stranded DNA template as described (38) using the same primers as the second set for PCR. Positive samples that contained more than one species of DNA (from starting cell numbers of two and four this was a possibility) were readily discernible by two distinct lengths of template (resolvable to single base differences) and smearing through CDR 3 on the autoradiographs, in these infrequent cases (1/18 for 281* and λ*, 1/20 for the PNA*) the sequence was not included in the analysis. When two sequences were identical through the CDR 3 and were unmutated, only one sample was reported because of the inability to verify the origins as independent events.

Results

The Emergence of NP-specific B Cells In Vivo. We directly monitored the emergence of NP-specific B cells that had switched to IgG1 surface isotype in response to T cell–dependent immunization with NP. Fig. 1 A clearly demonstrates the specificity of the reagents used in the FACScan analysis of the NP response. The population of (IgM; Mac1: GR1*) IgG1* λ* NP* cells only emerges after immunization with NP-KLH and is absent with carrier only (Fig. 1
Furthermore, the population of NP-binding cells was negative for staining with an anti-κ L chain antibody (187.1). The majority (>90%) of (IgM; Mac1; GR1)+ IgG1+ NP+ B cells expressed the λ L chain (Fig. 1 B). The population of small resting B cells displays the level of background staining for the NP and anti-λ reagents demonstrating a reassuring signal to noise ratio at all stages of the response. Between 2 and 4% of the resting B cells display 4–25 fluorescence units of binding to the anti-λ reagent, but cannot be seen on the contour representation in Fig. 1 B. Interestingly, a population of cells binding higher levels of NP (>15 U) and expressing higher levels of λ (>25 U) (NP2+ λ2+) emerges over time to comprise up to 60% of the total IgG1+-specific B cell population by day 14.

During the first week of the primary response, the frequency of λ+ isotype-switched antigen-specific cells expands exponentially after a 2–3 d lag phase (Fig. 1 C). The 100-fold increase in cell numbers between days 4 and 7 corresponds to an approximate 10-h doubling time of cell accumulation within this compartment. The extent of the response is remarkable, reaching 1,900 ± 330 (± SEM, n = 6) per 10^6 spleen cells at its peak on days 7–8. The frequency of NP-specific cells remains ~1% of total splenocytes during the second week. There is evidence of cell death in vivo during
this latter phase (6), suggesting that proliferation and death appear balanced during this second week of the primary NP response.

**The Onset of Somatic Mutation in Responding NP-specific B Cells.** We next assessed the level of point mutation in the V\_186.2 genes of individual cells sorted according to the unique phenotype described above. To obtain enough DNA to sequence directly without subcloning, we needed to subject newly synthesized cDNA to two separate sets of 35 cycles of PCR. 50% of single micromanipulated hybridoma cells produced enough DNA for sequence analysis that remained faithful to the expected germline sequence in 10 out of 10 separate cases. With independent PCR products sequenced in both directions, this represents a background of <1/6,000 introduced errors “seen” by this method. The lack of background is further evidenced in Fig. 2. None of the 18 unique NP-specific cells bearing V\_186.2 sequence contained evidence of mutation (<1/10,000 bp). This approach renders

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| Day 6 | Day 7 |
|-----------------|-----------------|
| **CDR 1** | **CDR 2** | **CDR 3** |
| 27 28 29 30 | 31 32 33 34 | 35 36 37 38 39 40 41 |
| 42 43 44 45 | 46 47 48 49 | 50 51 52 53 54 55 56 |
| 57 58 59 60 | 61 62 63 64 | 65 66 67 68 69 70 71 |
| **V\_186.2** | **NF** | **GR** |
| **VH** | **JH** | **DM** |
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the system operationally free of background. Whereas the
introduced Taq polymerase error is not ablated, the de-
tection of molecules containing these errors is limited be-
cause of the relative inefficiency of direct double stranded DNA
sequencing. The efficiency of producing sufficient DNA for
direct sequence analysis after immediate ex vivo purification
RNA extraction, cDNA synthesis, and two separate rounds
of PCR was 12 and 20% for day 6- and day 7-specific cells,
respectively (Fig. 2).

Using this approach, we are able to analyze DNA sequences
from individual cells that are expressing the λ L chain, have
isotype switched in response to antigen, and are able to bind
NP. All IgM⁻ IgG1⁺ λ⁺ NP-binding cells isolated from day
6 of the primary response expressed unmutated V₅₈6.2 H
chain genes (Fig. 2). By day 7, 30% of NP-specific cells ex-
pressed a somatically varied V₅₈6.2 gene with an average of
1.6 mutations per V₅ gene and a 1:7 silent to replacement
ratio. A characteristically retained tyrosine at position 95 on
the V₅-D border was expressed in 24 out of 27 NP-specific cells
from this early stage of the primary response. An intact GSS
motif at the D-J₅ border was expressed by only 13 of the
27 cells, and evidence of N sequence insertion at either the
V₅-D or D-J₅ border was present in all but two cells ana-
lyzed. The average number of amino acids in CDR 3 from
responders at this early phase of the response was 9.9 ± 0.27
(± SEM; n = 25). Joining region J₅ was expressed in 22
out of 25 cells assigned and the DFL16.1 in 22 of 27 cells.

Together with the direct frequency estimate of NP-specific
cells, 0.3–0.9% of total cells in the spleen would have initi-
ated a process of somatic mutation known to proceed in the
germininal center environment of the primary response (12,
26). At this early stage of the response, the majority (70%)
of NP-specific responders remain unmutated. This could be
due to their location in the PALS-associated foci as unmu-
tated antibody-forming cells (3, 26) or their presence in ger-
minal centers in an unmutated form.

The Emergence of Antigen-specific Antibody-secreting Cells In
Vivo. We have recently defined surface expression of syn-
decan as a sensitive marker of antibody-secreting cells (27),
and therefore can purify NP-specific cells in this phenotypi-
cally and functionally distinct developmental pathway. Syn-
decan-expressing, antigen-specific, isotype-switched B cells
are a subset of cells whose primary response to antigenic chal-
lenge is the production of non-IgM antibody. Fig. 3 A out-
lines a representative series of FACS® profiles depicting the
emergence of syndecan⁺ cells within the IgM⁻ NP-binding
compartment of the spleen during a primary response to NP-
KLH. The small IgM⁺ NP⁻ cells serve to display the back-
ground staining for syndecan. The phenotype profile of the NP-binding cells also identifies a sizable non-IgM, non-IgG1 component in these animals, both secreting and nonsecreting (Fig. 3 A). This fraction of the response appears before the IgG1 component and can remain sizable up to 10 d after initial antigen exposure. Other IgG isotypes could contribute to this subset with clones that have switched to isotypes downstream of IgG1 appearing later. There is still a need for detailed analyses of this unique and heterogeneous subset.

Emergence of the NP-specific IgG1+ secreting compartment parallels of the total isotype-switched compartment. A lag phase of 3–4 d precedes an exponential rise that peaks on days 7–8 and declines noticeably thereafter (Fig. 3 B). A 500-fold increase in cell numbers between days 4 and 7 represents an approximate doubling time of 8 h for this population at the proliferative phase of the response. At its height, the NP-specific secreting population comprises 720 ± 180 (± SEM; n = 6) cells per 10^6 spleen cells that represents approximately one third of the total NP-specific compartment. Furthermore, by day 10 of the primary response 75–90% of these cells are lost. The rapid decline in cell numbers may be due to the short life span of these cells, consistent with the 3–5-d life span of the short-lived plasmacyte of the PALS-associated foci (4). Alternatively, the syndecan+ cells may be rapidly exported from the spleen at the later stage of the response. In either case, syndecan expression defines the primary response B cell as one that secretes antibody as a response to initial encounter with antigen.

**Early Antibody-secreting Cells Remain Unmutated.** Next, we assessed the mutational status of the antigen-specific cells in this primary response B cell compartment. Single cells from the early primary antibody-secreting pathway were purified by six-parameter flow cytometry for molecular analyses of their V_{\text{V}186.2} genes as described. On average, 36% of cells sorted immediately ex vivo produced sufficient DNA for direct sequence analysis after RNA extraction, cDNA synthesis, and two separate cycles of PCR. Fig. 4 displays a panel of sequences from antibody-secreting cells at the end of the first week after primary immunization. All 18 members express an unmutated V_{\text{V}186.2} H chain gene. Of the 15 V gene sequences through to the J_{\text{J}13} retained the tyrosine at position 95. Only 6 out of the 15 expressed an intact GSS motif at the D-J_{\text{J}5} border, previously thought to be characteristic of the early primary NP response (39). Furthermore, all 11 cells expressing the DFL16.1 diversity element showed evidence of N sequence insertion on either the V_{\text{V}D} or D-J_{\text{J}} borders, unlike the typical unmutated primary NP hybridomas. Usage of J_{\text{J}2} dominated with only three cells using J_{\text{J}4}. The length of CDR3 varied from 8 to 13 amino acids with an average length of 10.4 ± 0.37 (± SEM; n = 15).

Overall, the syndecan+ antigen-specific compartment correlates well with the PALS-associated foci regarding functional and mutational status (3, 26). Direct in situ staining is yet to be done with the anti-syndecan reagent. Furthermore, the profile of CDR3 diversity appears somewhat different from what was seen in the primary response NP

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**Figure 4. Antibody-secreting cells of the early primary response remain unmutated.** The nucleotide sequence and predicted amino acid assignment obtained from individual cells with the phenotype of antibody-secreting cells from days 6 and 7 as described. The sequences are compared to the germline sequence of V_{\text{V}186.2} displaying only those positions in which changes were seen. Where obtained, sequence to the fourth codon in the J_5 region is displayed and the predicted size of CDR3 is listed in the last column. (Dashes) Identity with the germline sequence, where possible J_5 assignment is included in parentheses at the end of each sequence. (*) DFL16.1 not used.
hybridomas and may reflect the different selection bias imposed by the fusion procedure.

The Emergence of Antigen-specific Germinal Center Cells. In contrast to the antibody-secreting effector arm of the primary response, there is a subset of antigen-activated B cells that proceeds through the germinal center pathway of development after antigenic challenge. The binding of a lectin, PNA, in tissue section identifies these germinal center cells (30). Whereas cells in the antibody-secreting pathway bind higher levels of PNA than small resting lymphocytes, the IgM IgG1+ syndecan− subset of cells binds still 2–10-fold higher PNA levels (Fig. 5). This is also true for the IgM− NP+ cellular subset where syndecan− cells bind higher levels of PNA than the syndecan+ ones (data not shown).

Previously, using a sensitive Elispot assay for detecting the secreted antibody of individual cells, we demonstrate that >90% of specific IgG1+ syndecan+ cells of the early primary response secrete antibody in vitro with no stimulus (27). In contrast, <2% of the syndecan− component of this response were capable of secretion. In the current study, we use an in vitro cloning system to assess the ability of individual antigen-specific cells to form clones that are capable of secreting >1 ng of antibody over the 8-d culture period (Table 1). Greater than 90% of the responding clones from the λ+ population secreted antibody with the isotype and specificity predicted from its phenotype (Table 1). The sorting purity and cloning efficiency were comparable to that achieved with the early secondary response (25). At this early stage of the in vivo response, it was the syndecan− component that responded to culture (Table 1). Whereas only 15% of the syndecan+ cells were capable of producing over 1 ng of specific IgG1, 90% of these clones secreted the predicted specificity and isotype. On the other hand, the syndecan− compartment (the majority of which are PNA+ρ) largely

Table 1. In Vitro Response from Isotype-switched Antigen-binding Primary B Cells

| Phenotype                                  | Experiment 1 |          | Experiment 2 |          |
|--------------------------------------------|--------------|----------|--------------|----------|
|                                            | Total IgG1   | Anti-NP IgG1 | Total IgG1   | Anti-NP IgG1 |
| (IgM, Mac1, GR1)−NP+λ+IgG1*               | 170          | 170       | 140          | 130       |
| (IgM, Mac1, GR1)−NP+λ+syndecan+           | 150          | 140       | 130          | 120       |
| (IgM, Mac1, GR1)-NP+λ+syndecan−           | 35           | 20        | 25           | 10        |

Filler cell–supported cultures in the presence of LPS, rIL-2, rIL-4, and rIL-5 as described. Frequencies were calculated from data for 300–1,000 cells of each selected population directly deposited into individual wells of a 96-well microwell culture plate using the automatic cell dispensing unit on the FACStar Plus®. Supernatants were assayed at day 8 of culture to determine isotype and specificity by ELISA.

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Figure 5. The early antibody-secreting and germinal center are separate pathways of development.
(A) The syndecan and (IgM, Mac1, GR1) levels on IgG1+ cells in the spleen of animals 7 d after immunization. Cells were costained with biotin-anti-IgG1 followed by TR-avidin, fluoresceinated PNA, APC–28.12, and PE–331.12/8C5/M1/70 and resuspended in PI before analysis. (Top inset) The boundaries chosen to signify cells as IgG1+ (IgM, Mac1, GR1)+ syndecan− cells within the spleen; (bottom inset) IgG1+ (IgM, Mac1, GR1)+ syndecan− cells. (B) PNA binding vs. forward light scatter (as an index of cell size) of isotype-switched antibody-secreting cells (syndecan+); (top) and isotype-switched nonsecreting cells (syndecan−); (bottom) in the early primary response (days 6 and 7 after immunization). All plots are 10% probability density contours.
failed to form antibody after stimulation in culture. These
data further support the classification of these cells as ger-
minal center cells known to die rapidly in vitro without the
appropriate accessory cell support or costimulation (14, 40).

By using very high levels of PNA binding (>20 fluores-
cence units) the emergence of isotype-switched antigen-specific
germinal center cells can be monitored. Fig. 6 A displays a
typical series of FACS® profiles used to discriminate the popu-
lation of large forward scatter (an index of a cell's cross-sectional
area) germinal center cells. By day 12, many of the cells are
smaller again (Fig. 6 A). The intermediate staining of PNA
by the syndecan § cells can also be seen on these plots, and
in general these cells are slightly larger than their germinal
center counterparts. Low PNA staining and forward light
scatter typical of the resting IgM + IgG1 - NP + changes only
minimally over the immunization period (Fig. 6 A).

The direct frequency analysis displayed in Fig. 6 B indi-
cates the emergence of isotype-switched, antigen-binding ger-
minal center cells during the primary response to NP-KLH.
The kinetics are remarkably similar to those for the antibody-
secreting compartment. A 2–3 d lag period is followed by
an exponential growth phase reaching a peak at days 7–8.
This population increase represents an approximate doubling
time of 10 h at this proliferative phase of the response. At
its height, this population comprises $810 \pm 130$ (± SEM;
$n = 6$) cells per $10^5$ spleen cells and is very similar to the
extent of the syndecan § population. Unlike the latter sub-
set, the germinal center cells persist at a plateau frequency
for the duration of the second week. This plateau may repre-
sent the selection phase of the response where proliferation
of positively selected cells balances the extensive cell death
seen in the germinal centers (14, 41, 42).

Early Germinal Center Cells Express an Unmutated V₅₈.2
Gene with a Short CDR 3. Next, we assessed the mutational
status of the antigen-specific cells in this germinal center
pathway to memory cell differentiation. Single isotype-
switched NP-binding B cells that bound high levels of PNA
(>20 U) were purified by six-parameter flow cytometry for
molecular analyses of their V₅₈.2 genes as described. On aver-
age, 8% of cells sorted immediately ex vivo produced
sufficient DNA for direct sequence analysis after RNA ex-
traction, cDNA synthesis, and two separate cycles of PCR.
We believe the process efficiency relates to the reduced amount
of V gene mRNA present in germinal center cells compared
with antibody-secreting cells as seen by in situ hybridization
(3). Whereas carrier cRNA is essential to reduce loss of specific
mRNA at the extraction step, cDNA synthesis is the most
inefficient process of the preparative phase.

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Fig. 7 displays the V<sub>n</sub>186.2 sequence from a panel of germinal center cells from the end of the first week after primary immunization. Only 2 of the 14 cells analyzed expressed a mutated H chain gene. It must be emphasized that these cells have already switched Ig isotype to IgG1 and are also able to bind antigen. By correlation with immunohistochemical analyses, these cells would most closely correspond to slg<sup>-</sup> centrocytes, the cells emergent from the slg<sup>+</sup> early B cell blast (or later centroblast) phase of the germinal center reaction (3, 43). Therefore, the earliest products of the germinal center reaction appear largely unmutated.

The tyrosine at position 95 was retained by 10 of the 14 cells analyzed implicating the importance of this residue to NP binding even in this differentiation pathway. Only three of the cells expressed an intact GSS motif at the D-J<sub>n</sub> border, a similar pattern to that seen in the antibody-secreting compartment. Furthermore, of the nine cells using the DFL16.1 diversity element, eight displayed N sequence insertions at either the V<sub>n</sub>-D or D-J<sub>n</sub> borders in a similar pattern to the antibody-secreting cells. It was the length of the CDR 3 region that appeared most strikingly different between the two functionally distinct populations. Of the 12 cells characterized through to J<sub>n</sub> assignment, 9 expressed a CDR 3 of nine amino acids or less. Only 4 out of 15 antibody-secreting cells used a CDR 3 of nine amino acids or less. The average CDR 3 length of the germinal center cells was 9.3 ± 0.26 (± SEM, n = 12). \(\chi^2\) statistical analysis of the distributions of CDR 3 length across the two populations confirms that a significant difference exists (\(p = 0.001\)).

The germinal center pathway supports somatic variation in the V<sub>n</sub> genes of antigen-responsive B cells, confirming the predictions of MacLennan and Gray (6) and the data from more recent studies (11, 12). Furthermore, the majority of the earliest products of the germinal center pathway emerge initially unmutated, but appear to be a more restricted subset of V<sub>n</sub>186.2/V<sub>n</sub>186.2-bearing NP-specific cells than their antibody-secreting counterparts. These data indicate a branch point in antigen-driven B cell development and implicate antibody fine specificity in the decision to pursue a pathway of memory cell differentiation.

Late Germinal Center Cells Express V<sub>n</sub>186.2 with a Short CDR 3 and Affinity Increasing Mutations. By day 12 of the primary response, many (63%) NP-specific cells express a V<sub>n</sub>186.2 gene with numerous somatically introduced mutations (3.4 per mutated clone) (Fig. 8) in agreement with recent studies on rearranged genomic DNA from animals in the primary NP response (22). Once again, the tyrosine at position 95 was conserved in 17 out of 19 cells analyzed. The GSS motif at the D-J<sub>n</sub> border was only expressed in six of the cells and N sequence insertions seen in all but four cells, displaying a similar level of CDR 3 diversity as seen in the previous groups.

Of the 12 NP-specific cells expressing a mutated receptor, 11 carried the position 33 tryptophan to leucine change typical of the mature NP response (9, 19) and shown to lead to a 10-fold increase in affinity (26). One set of repeat clones expressing identical CDR 3 (Fig. 8, Nos. 3-5) all contain the same position 33 change but also carry unique mutations identifying them as independent cellular events. A silent to replacement mutation ratio of 1:9 for the CDRs (1:6 expected) and 1:2.7 for the framework regions (1:3.1 expected) (24) demonstrates a preferential localization for substitutions in the CDR. Further, a 2:8 distribution of silent mutations for CDR:framework regions reflects the size of the respec-
tive area of the \( V_{\text{n}}186.2 \) gene. Therefore, mutations are randomly introduced, but clones expressing certain substitutions in the CDR (very specifically a position 33 leucine) are positively selected, confirming many studies using both hybridomas and genomic DNA (9, 11, 19, 22).

More interestingly, 10 out of the 12 cells expressing a mutated Ig also bore a short CDR 3 of nine amino acids or less. These data support the hypothesis that cells entering the memory cell pathway are selected initially for antigen binding and a shorter CDR 3 and then later in an iterative fashion for affinity increasing mutations (predominantly a change in CDR 1 position 33).

### Discussion

**The Antigen-specific Response to Immunization.** Using six-parameter flow cytometry we can directly estimate the numbers of splenic B cells involved in the primary response to NP-KLH immunization. We chose to monitor NP-specific cells that had responded to immunization by switching from IgM to IgG1 expression in vivo. The emergence of antigen-specific B cells assessed by flow cytometry in the current study correlates well with many immunohistochemical studies of in vivo immune responses (for reviews see references 1, 2, 44-46).

The lag phase may indicate the time needed for cognate activation involving T and B cells in the microenvironmental context of antigen-presenting cells in secondary lymphoid organs. An extensive proliferative phase soon follows in both T and B cell-rich areas of these organs with evidence of Ig isotype switch in both regions (3).

During the second week, most of the proliferative activity appears localized to the germinal center reaction of secondary follicles in the B cell zones (6). There appears to be a great deal of cell death associated with these structures together with the production of long-lived memory B cells (6, 42).

Evidence for somatic hypermutation (12) and survival of higher affinity mutants within the germinal center (11) indicate its central role in positive selection and the maturation of affinity which is typically associated with T cell–dependent immune responses.

The extent of the isotype-switched NP response observed is remarkable, reaching an average of 1–2% of total spleen cells during the plateau phase of the second week. These data support the extensive immunohistochemical analysis of the response to NP-CG by Jacob et al. (3). In situ analysis by this group estimated the B cells in the PALS-associated foci to reach 1% of spleen volume at their 8-d peak and ~300–500 germinal centers per spleen at their peak by day 12 (55% of which were \( \lambda^+ \) NIP+). Previous flow cytometric analysis of an antigen-specific response estimated a far lower frequency of specific cells (41, 47). In those studies, the fluorophore PE was used as an antigen, and direct binding on the flow cytometer of B220+ (IgM, IgD) was used as a criterion of a memory cell. Animals primed 3 mo previously carried a PE-specific memory compartment comprising 0.02–0.05% of total spleen cells (47). Even around day 12 of this response, frequency estimates were similar and appeared to remain constant for over the following 200 d (41). These differences from the numbers of NP-specific cells found in the present study may reflect differences in the mature of the antigen, the existing T and/or B cell repertoire, or the antigen-presenting capacity of the system. Furthermore, a kinetic difference in the establishment of the PE response might have contributed to the differences seen.

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**Figure 8.** Mutation and selection in the primary NP response. The nucleotide sequence and predicted amino acid obtained from individual (IgM; Mac1; GR1) IgG1+ NP+ \( \lambda^+ \) cells sorted directly from the spleens of animals 12 d after primary immunization with NP-KLH. The sequences are compared to the germline sequence of \( V_{\text{n}}186.2 \) displaying only those positions in which changes were seen. Where obtained, sequence to the fourth possible J assignment is included in the parentheses at the end of each sequence. (*) DFL16.1 not used.
The ability to purify NP-specific cells allows molecular assessment of individual cells shown to be directly involved in the ongoing primary response in vivo. Our approach does not suffer the undefined bias introduced by hybridoma fusion. Furthermore, by using single cells as the starting substrate and finally direct sequencing of PCR products avoids the need for subcloning or screening. This combination of processes renders the system operationally free of background and offers a clear view of the onset of somatic mutation towards the end of the first week after primary challenge. These data align well with recent studies on rearranged genomic DNA from animals in the primary NP response (22). Studies on other hapten responses also describe the appearance of mutated progeny during the first week (48) or early in the second (15). Unlike the response to a T-dependent form of phosphorycholine, the NP responders accumulate more mutations during the second week after immunization, appearing more like the response to the hapten 2-phenyl-oxazalone.

**Dual Differentiation Pathways and Their Precursors.** We have recently described the use of syndecan expression as a definitive marker of antibody-secreting cells within the antigen-specific compartment in vivo (27). Comparing syndecan expression and the binding of the lectin PNA it was possible to delineate and quantitate directly the recruitment of antigen-specific precursors into the antibody-secreting compartment and the germinal center pathway to memory. In both cases, the cells expressed isotype-switched surface Ig that was capable of binding NP. Therefore, the IgM + antibody-secreting cells first apparent in the PALS-associated foci (3) are not included in this analysis, nor are the slgM + B cell blasts of the early follicular response or the slgM + centroblasts of the germinal center reaction (2). We find that both populations emerge with very similar kinetics. These data suggest that isotype switch was coordinated simultaneously in these two phenotypically and functionally distinct cell compartments. Either control for the gene rearrangement event is at a systemic level (3), or the initiation of this event occurs at a common time point before the functional branching of these two pathways.

The similar doubling time of NP-specific precursors in the proliferative phase of both pathways is close to the more direct estimations of doubling times for cells in secondary follicles (6–7 h) (49, 50). The noticeable difference between the two populations is seen during the second week after immunization. Whereas antibody-secreting cells decline rapidly, the germinal center cell numbers persist at plateau throughout the second week. The former parallels the observed life span of the PALS-associated foci previously described (3). The steady plateau in the latter population suggests that during the second week, cell renewal equals cell death in this microenvironment.

Molecular analyses of single cells from either pathway confirms two features of more recent studies. The process of somatic mutation proceeds within a germinal center (11, 12) and is absent in the antibody-forming cells of PALS-associated foci (12, 26). In the current study, somatic mutation was seen in the germinal center compartment and not in early antibody-secreting cells. Furthermore, it is evident that the early products of the germinal center reaction can switch Ig isotype without evidence of somatic mutation and favors independent mechanisms for these two processes. In fact, the majority of early germinal center cells were also unmutated. These data support a model in which the germinal center environment both initiates the mutational mechanism and subsequently selects and expands the desired specificities at the cellular level (46).

The length of the CDR 3 within the germinal center cells was significantly shorter than those of the antibody-secreting pathway at the end of the first week after challenge. Although this feature has been noted in previous studies, it was associated with the secondary NP-clonotype in cells already containing many mutations and evidence of selection postaffinity maturation (24). Our data suggests that selection of a shorter CDR 3 may occur before somatic mutation and that further selection occurs after diversification by mutation. It is possible that the distinction on CDR 3 length was imposed before entry into the memory differentiation pathway, however, it is equally likely to be the outcome of an initial round of selection within the germinal center itself. Indeed, it remains to be seen whether this difference is preassorted in the naive repertoire as would be predicted by recent studies on the capacity of J11d- B cells to initiate germinal centers and memory responses (51, 52).

**Maturation and Memory Cell Generation.** The sustained germinal center pathway dominates the second week of the response giving rise to cells bearing the hallmarks of an NP-specific memory cell. A tryptophan to leucine change at position 33 in CDR 1 can by itself lead to a 10-fold affinity increase for NP (23). By day 12 of the primary response, the majority of cells (63%) in the current study were somatically mutated. 11 of the 12 mutated clonotypes carried the position 33 leucine and 10 of these also expressed a CDR 3 of nine amino acids in length. These data suggest clones selected into the pathway of somatic mutation are further selected for the presence of affinity-increasing mutations. In the studies by Weiss et al. (22, 24) by day 10 of the primary, 8 out of 13 clones carrying a position 33 leucine also had a nine-amino-acid length CDR 3. By day 14 the frequency was 6 out of 6 and by day 42, 11 out of 12 clones also carried the shorter CDR. Further, in our previous work on the early memory response, >75% of single cells analyzed expressed the shorter CDR 3 predominantly associated with the position 33 change to leucine (25). Together, the data support the stepwise model of affinity maturation during the primary immune response (53–56).

**Conclusions.** The resolution of six-parameter flow cytometry and the capacity to analyze individual members of rare cell populations, at the molecular and functional level, offer unique approaches to the study of in vivo immune responses. The current study further characterizes the development of antibody secretion and memory cell generation as independent early outcomes of a primary encounter with antigen. The data indicate many branch points in the developmental pathway of a primary response B cell and the recruitment of a significant number of antigen-specific responders. The differences in phenotype, cellular function, and molecular mechanisms within antigen-specific cells were divided into
two developmental pathways: antibody secretion without somatic mutation as one early response and germinal center formation; somatic mutation and selection of high affinity variants into the memory compartment as a second. The events that control the outcome of encounter with antigen remain elusive. Using our approach, one is able to retrieve antigen-specific cells from either pathway at any stage of the ongoing response. The cells are in a normal physiological state and can be transferred into appropriate hosts or cultured in vitro to reveal their needs for growth and their capacity for differentiation.

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