Tracing the Development of Single Memory-Lineage B Cells in a Highly Defined Immune Response

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Summary

To study the development of B lymphocyte memory, we identified and isolated splenic B cells expressing a highly defined antibody variable region that constitutes a reproducible and predominant component of the memory antibody response to *p*-azophenylarsonate (Ars). Isolation was achieved during the primary immune response by surface staining and flow cytometry using a specific anti-idiotypic antibody called E4, which recognizes this canonical V region, encoded by one set of V gene segments. The isolated $E4^+$ cells displayed all of the phenotypic characteristics of germinal center centrocytes, including a low level of surface Ig, a lack of surface IgD, a high level of receptor for peanut agglutinin, and expression of mutated antibody V genes.

$E4^+$ B cells were first detected in the spleen 7–8 d after primary immunization, reached peak numbers from days 10–13, and waned by day 16. Surprisingly, at their peak, $E4^+$ cells comprised only 40,000 of all splenocytes, and half of these failed to bind Ars. Using this number, we estimate the total number of Ars-specific memory-lineage cells in the spleen to be no more than 50,000 (0.1%) at any one time, and presumably far fewer that are committed to the memory pool. Chromosomal copies of rearranged V genes from single $E4^+$ cells were amplified by nested PCR, and the amplified products were sequenced directly without cloning, using standardized conditions that disclose virtually no *Taq* polymerase errors. V gene sequence analyses of $E4^+$ cells isolated from single mice confirmed their canonical nature and revealed that they were derived from few precursors. In the average mouse, the $E4^+$ pool was derived from fewer than five canonical precursors. Somatic mutations were found within the V genes of almost all cell isolates. At day 13, a significant fraction of $E4^+$ cells had mutations known to increase antibody affinity for Ars, suggesting they were products of at least one cycle of post-mutational antigen-driven selection. However, the lack of shared mutations by clonally related cells indicated that the selective expansion of mutant subclones typical of memory responses had not yet taken place. This was supported by the observation that half of the $E4^+$ cells failed to bind Ars. Collectively, our results indicate that the memory compartment is a highly selected entity, even at relatively early stages of the primary immune response when somatic mutation and clonal selection are still in progress. If germinal centers are the source of memory B cells, our data suggest that B cell memory may be derived from only a small fraction of all germinal centers.

The development of memory B cells is strongly influenced by two antigen-dependent processes. One of these is a poorly defined somatic hypermutation mechanism that targets rearranged antibody variable region genes expressed by immune participants (1–12). The second is an intense selection process that recruits into the memory compartment rare B cell mutants expressing antibodies with improved affinity for antigen (reviewed in reference 13). Together, these processes appear to be necessary for the maturation of antibody affinity, which is observed in memory or T cell–dependent antibody responses. However, neither somatic mutagenesis nor the dynamics of cell selection leading to recruitment into the memory B cell compartment are understood in any significant detail.

Recent molecular studies have provided concrete evidence that the anatomic site of somatic hypermutation and affinity-based selection is the germinal center (GC)[1] (14–20). GC B cells have been isolated by microdissection of frozen

[1] *Abbreviations used in this paper:* Ars, *p*-azophenylarsonate; GC, germinal center; PNA, peanut agglutinin.

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spleen sections, and by flow cytometry using labeled antigen and peanut agglutinin. In these studies, hapten-specific GC B cells were found to comprise ~1–2% of all splenocytes during the second week after immunization (18, 21). PCR-based sequencing studies of their expressed V genes indicated that they contained somatic mutations, sometimes including specific mutations associated with affinity improvements (17, 18). In contrast, few or no observable mutations have been seen in V genes of B cells isolated from other anatomic sites in secondary lymphoid tissues (14, 18).

The fraction of GC cells that will ultimately end up in the memory B cell compartment, however, is unknown and has been difficult to assess using model anti-hapten immune responses in which the primary response is dominated by clonotypes that constitute only a minor fraction of the memory response. For example, in both the anti-oxazolone and anti-NP immune responses, B cells expressing particular VH/VL pairs dominate early, but wane in frequency as the response progresses (7, 22). In the primary immune response to NP, V gene sequences derived from germinal center B cells revealed that only one of several germinal centers examined contained B cells with a diagnostic affinity-improving somatic mutation (14–16). This suggests that B cells from most germinal centers are not destined to enter the memory compartment in which mutant cells expressing high-affinity antibodies typically accumulate. In addition, phylogenetic analyses of hybridoma cells have indicated that progeny of relatively few precursor B cells dominate any given memory immune response (10–12, 23, 24). These observations suggest that relatively few of the developing clones in the primary immune response contribute to the pool of memory B cells.

To study the development of presumably rare memory-lineage B cells, we exploited the antibody response of A/J mice to *p*-azophenylarsonate (Ars), which has been extensively characterized (25, 26). Hybridoma sampling studies have revealed that ~50% of anti-Ars memory B cells express a dominant idiotype (IdCR), and the majority of these (~80%) express a defined "canonical" combination of V gene segments (V_HIdCR, $D_{fl16.1}$, J_H2; and V_KIdCR, J_K1) (5, 8, 27–29), with two specific junctional residues that are necessary for Ars binding: serine at position 99 in the V_H-$D_{fl16.1}$ junction (30); and arginine at position 96 in the V_K-J_K1 junction (31) (see Fig. 1). Two variable junctional residues (at the V_H-D and the D-J_H joins) are the only sources of variability in unmutated canonical V regions (Fig. 1). Results of hybridoma sampling studies have shown that canonical Ars-specific B cells are only a minority of all Ars-specific cells participating in the primary immune response, but they emerge to dominate the secondary or memory immune response, when they comprise ~40% of all Ars-specific cells (8). Their emergence appears to be the consequence of an affinity-driven selection process. Most canonical hybridomas isolated late in the primary immune response or in the memory response carry V gene somatic mutations that confer affinity improvements upon the antibody product. In addition, many of them carry specific recurrent somatic mutations that are known to enhance affinity for Ars (32). These include threonine to isoleucine and lysine to threonine changes at positions 58 and 59, respectively, in V_H CDR2 (33). As such, the mutated canonical V region is the most reproducible component of the memory immune response.

A second advantage in studying memory development to Ars is the availability of a monoclonal anti-idiotype called E4, which recognizes canonical V regions almost exclusively in a hapten-inhibitable manner (34, 35). In this study, we used E4 to identify and isolate cells belonging to the memory lineage during the primary immune response. V_H and V_K genomic templates were amplified from single isolated cells and directly sequenced to preclude artifacts associated with PCR amplification of multiple homologous templates and with cloning of PCR products (36). The results indicate that, already at 13 d after a primary immunization, the potential pool of memory cells is small, heterogeneous with respect to antigen binding, and derived from very few precursors despite ongoing selection.

Materials and Methods

Immunization, Splenocyte Staining, Flow Cytometry and Cell Sorting. Specific pathogen-free A/J mice (8–12 wk old) were immunized i.p. with Ars-KLH or KLH (100 μg) emulsified in incomplete Freund's Adjuvant. 6–24 d later, erythrocyte-depleted splenocytes were stained with biotinylated E4 (10 μg/ml), followed by Streptavidin-PE (0.1 μg/ml; Becton Dickinson, San Jose, CA). Splenocytes were also stained with FITC-anti-Mac1 (M1/70, 5 μg/ml) and FITC-anti-IgD (JA12.5, 3 μg/ml) to identify and exclude macrophages, antigenically naive B cells, and cells with a propensity to stain nonspecifically. In some experiments, FITC-conjugated to peanut agglutinin (PNA, 1 μg/ml; Sigma) or anti-B220 (RA3-6B2, 10 μg/ml) was used to identify germinal center B cells. Conjugation of antibodies with biotin or FITC was based on described methods (37). During and after staining, cells were kept in an ice-chilled and sterile-filtered buffer (Staining Buffer) of calcium-free Dulbecco's PBS (GIBCO BRL, Gaithersburg, MD) supplemented with heat-inactivated FCS (2%; HyClone, Logan, UT) and sodium azide (0.1%) before cell sorting to minimize surface receptor capping and internalization. To demonstrate the receptor-specific nature of E4 staining, control samples of splenocytes were pretreated with a competitive inhibitor consisting of Ars conjugated to BSA (ArsBSA: 200 μg/ml) for 15 min before E4 staining.

Stained splenocytes were analyzed first for size (forward scatter) and granularity (side scatter), then for color (PE and FITC) using a flow cytometer (EPICS 751; Coulter, Hialeah, FL) equipped with an argon-ion laser (λ = 488 nm), a neutral density filter, and Cicero Data Acquisition software (Cytomation, Fort Collins, CO). In general, 15,000 events in the two-color gate were analyzed for each sample. In some experiments, cells were sorted into an Eppendorf tube containing Dulbecco's PBS supplemented with FCS (15%) and ArsBSA (200 μg/ml) to inhibit cell aggregation and to prolong B cell survival in vitro. Cells were analyzed at a rate of ~600 events/s and sorted at a rate of ~1,500 events/s, yielding about 3,500 collected cells in 2 h with an approximate collection efficiency of 50%.

Determining Cell Size. The diameters of larger and smaller splenocytes, and B-hybridoma cells were determined by photographing cell samples mixed with Latex Microspheres (Coulter

EPICS) on an objective micrometer (10-μm intervals), visualized with a phase contrast microscope (Nikon Diaphot - TMD). The diameter of ~20 cells from each sample was measured.

Single Cell V Gene Amplification and Sequence Analysis. Sorted cells were gently concentrated at 4°C in a microfuge (4,000 rpm × 10 min). Cells were then visualized by phase contrast microscopy in isolated microdrops to confirm their singularity, as described (38). Microdrops containing single cells were aspirated using a P20 Pipetteman (Rainin, Woburn, MA) and subjected to direct V_H and V_K gene amplification using canonical genomic V_HIdCR and V_KIdCR-specific oligonucleotide primers in a nested polymerase chain reaction (PCR), as described (38). Microdrops lacking cells were also picked in each experiment to serve as negative controls. Special precautions were taken throughout these experiments to minimize and assess for any chance of DNA contamination before PCR amplification. Amplification products were visualized by electrophoresis of 1-μl samples through 2% agarose gels. PCR-amplified V_H and V_K gene products from single cells were purified and sequenced with ^{32}P-labeled primers using Version 2.0 of the Sequenase Sequencing Kit (United States Biochemical, Cleveland, OH), with modifications of the manufacturer's recommendations as described (38). PCR and sequencing oligonucleotide primers used for these experiments were described (38).

Statistical Analyses. Mean values and standard errors were calculated with the JMP statistical software program, version 2.0.5 (SAS Institute, Cary, NC).

The total number of precursor clones present in the spleen, from which the sample population of B cells was obtained, was estimated using the mathematical model to the "hidden species" problem, originally developed by RA Fisher in 1943, and applied to the study of B cell precursor numbers by Blier and Bothwell (23). Briefly, the equation for the model is $\lambda = N/S \times (1 - e^{-\lambda})$, where the experimental sample consists of N total cells of S distinct clones, and λ is related to the total number of clones in the sample population (S_t) by the equation $S_t = N/\lambda$. An iterative method was used to estimate λ, and a χ^2 goodness of fit statistic to estimate 95% confidence intervals for S_t. Calculations assumed that each clone was represented in equal numbers, and thus sampling followed a Poisson distribution.

Results

Flow Cytometric Analysis of Memory-lineage B Cells during the Primary Anti-Ars Response. To identify and isolate potential memory-lineage B cells during their development, we exploited a monoclonal anti-idiotype (E4) that recognizes the canonical V region product of the dominant memory population of Ars-specific B cells (Fig. 1). Using flow cytometry, $E4^+$ cells were analyzed in Ars-immunized mouse samples obtained 6, 8, 10, 13, 16, 20, and 24 d after immunization. Splenocytes were initially gated on the basis of size and granularity in order to separately analyze the larger, activated lymphocytes (Region 1 or R1) and the smaller, presumably resting lymphocytes (R2), while excluding macrophages and RBC's (Fig. 2 *a*). R1 and R2 cells were then analyzed to identify $E4^+$ (PE)-stained cells that were $Mac1^-$ and $sIgD^-$ (R5). The R1 gate was determined empirically. Increasing the size of this gate to include additional larger cells only decreased the frequency of $E4^+$ cells. Similarly, the R5 gate was adjusted empirically using the pattern of stained splenocytes from mice immunized only with KLH as a control. A total of 66 mice were studied in this manner, primarily at 10 or 13 d after immunization.

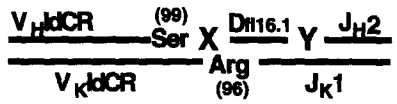

Figure 1. The "canonical" configuration of V gene segments and junctions that encodes the dominant V region of the memory antibody response of A strain mice to *p*-azophenylarsonate. X and Y represent the only codons that vary among unmutated versions.

A very small but reproducible subpopulation of $E4^+$, $Mac1^-$ and IgD^- (R5) cells emerged 7 to 8 d after primary Ars immunization among the larger 10–15% of total splenocytes (R1- Fig. 3 *a*). Their numbers peaked from days 10 (0.28 ± 0.05% SEM) to 13 (0.30 ± 0.06% SEM), and waned substantially by day 16. These cells averaged 10 ± 1 μm in diameter. The antigen specificity of $E4^+$ cells was assessed by competitive inhibition of E4-staining with an excess of ArsBSA. This reduced the percentage of $E4^+$ cells by ~50% in day 10 and day 13 samples, indicating that one-half of the $E4^+$ cells had apparently lost the capacity to bind Ars, perhaps due to the recent acquisition of somatic mutations. Inhibition of E4 staining with ArsBSA was also assessed for KLH-immunized mice and was found to be negligible (data not shown). The smaller 50% of the splenocytes (R2), averaging 6 ± 1 μm in diameter, were similarly analyzed. $E4^+$ cells were ~0.05% of the small splenocytes obtained 10 and 13 d after Ars immunization (Fig. 3 *b*). This is about sixfold less than the percentage of $E4^+$ cells observed among larger splenocytes, indicating their relative enrichment among larger cells.

$E4^+$ B Cells Are PNA_{hi} and $B220^+$. To further characterize $E4^+$ B cell isolates, sorted cell samples were stained with PNA or anti-B220 to identify germinal center B cells (39–44). Sorted $E4^+$ cells or unsorted splenocytes that were stained with FITC-PNA or FITC-anti-B220 were reanalyzed by flow cytometry (Fig. 2 *b*). Only 5% of total splenocytes stained brightly with PNA, while 78% of the large $E4^+$ cells and 65% of the small $E4^+$ cells were PNA_{hi}. Given this enrichment of PNA_{hi} cells among sorted $E4^+$ cells, the low representation of $E4^+$ cells among total large (~0.3%) and small (~0.05%) splenocytes, and intrinsic sorting limitations, we infer that the large majority of isolated $E4^+$ cells were PNA_{hi}.

A similar B220 re-analysis of sorted $E4^+$ cells revealed that 80% of the large $E4^+$ cells and 61% of the small $E4^+$ cells were $B220^+$ (Fig. 2 *b*). Since most splenocytes are $B220^+$ (e.g., 60% of total splenocytes stained with B220), this finding does not indicate a significant enrichment of $B220^+$ cells in the sorted populations. However, it confirms that the majority of sorted cells were $B220^+$ B cells, and correlates well with the percentage of sorted cells that were PNA_{hi}.

Isolated $E4^+$ Cells Carry Rearranged Canonical V_H, V_K, J_H, and J_K genes. To confirm that the cells identified by flow

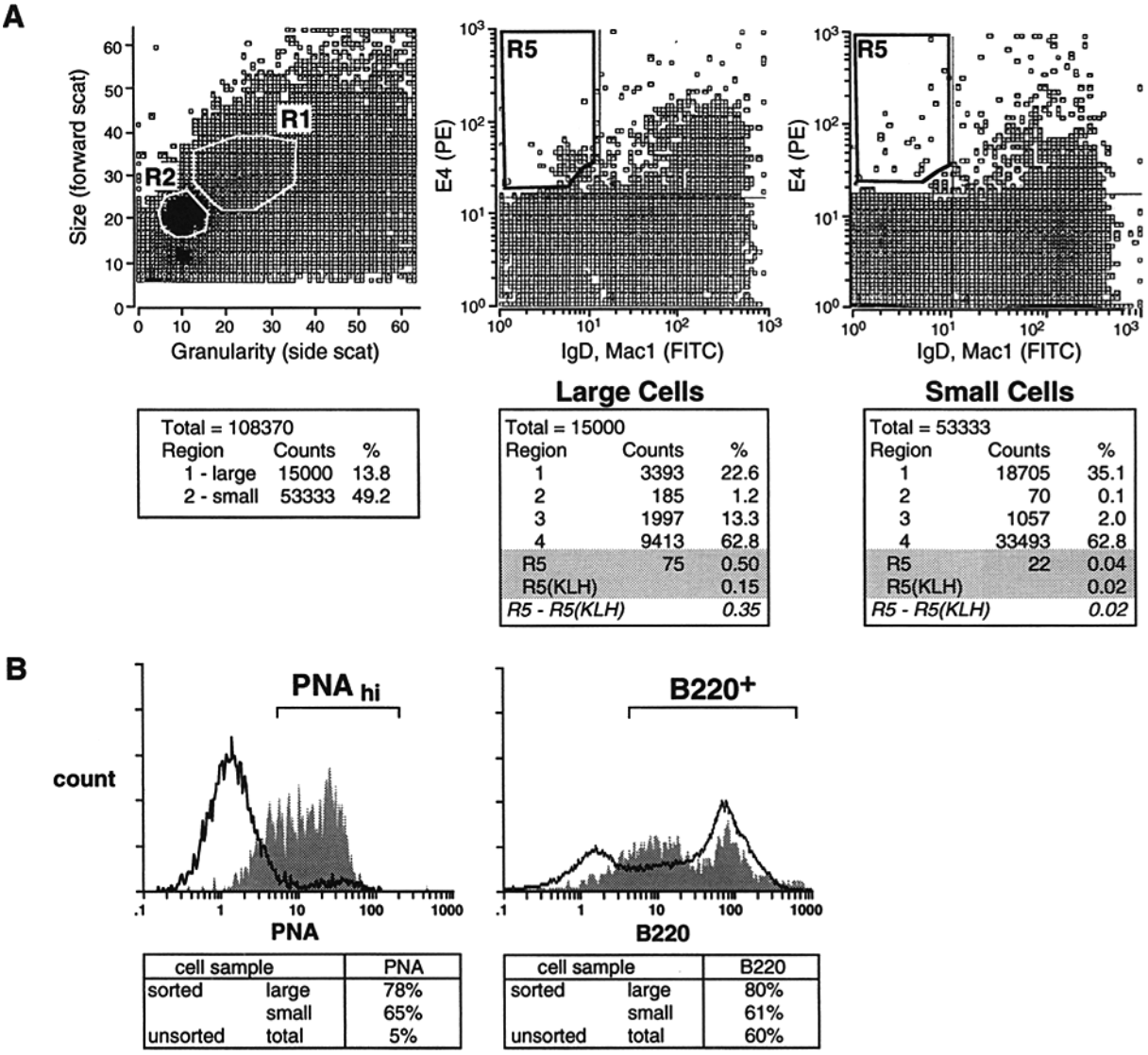

Figure 2. Flow cytometric analysis of $E4^+$ splenocytes during the primary antibody response to Ars. (*A*) A representative flow cytometric analysis of splenocytes obtained from A/J mice 13 d after primary immunization with ArsKLH. Splenocytes were first assessed by size (forward scatter) and granularity (side scatter) to identify larger (*R1*, 13.8% of total splenocytes) and smaller (*R2*, 49.2%) cells for separate two-color analyses. Of particular interest in the two-color gates are the $E4^+$ (*PE*), $Mac1^-$ and $sIgD^-$ (FITC) cells of region 5 (*R5*). In this example, R5 cells comprise 0.5% of the larger splenocytes of R1, and 0.04% of the smaller splenocytes of R2. To determine the background for E4 staining, a cell sample from a carrier (KLH)-only immunized mouse was simultaneously analyzed; the R5 percentage for this sample is shown as R5(KLH). $E4^+$ cell frequencies adjusted for background were reported as R5 - R5(KLH). (*B*) PNA and B220 staining of $E4^+$ splenocytes. Sorted large $E4^+$, Mac1 and IgD-negative splenocytes were stained with FITC-PNA or FITC-anti-B220 and reanalyzed by flow cytometry. In each diagram, the stained sorted sample (*filled graph*) is compared with a simultaneously stained, but unsorted sample (*solid line*) from the same mouse. Results pertaining to the bracketed regions of positively stained cells are tabulated below each flow cytometric diagram.

cytometry carried canonical V gene segments characteristic of anti-Ars memory B cells, we isolated $E4^+$ cells from four mice immunized with Ars-KLH. Larger cells were obtained from 2 mice at day 13, and one mouse at day 10; smaller and larger cells were also obtained from one mouse at day 13. Single $E4^+$ cells were visualized microscopically and manually aspirated into tubes using strict conditions and controls to prevent and assess for contamination. Chromosomal V_H and V_K genes from single $E4^+$ cell isolates were individually amplified ($\sim 10^{14}$-fold) in a nested PCR protocol specific for rearranged canonical V genes (38). Aliquots of PCR reactions were analyzed by agarose gel electrophoresis.

From these four mice, a high frequency of $E4^+$ B cells carried rearranged V_HIdCR and/or V_KIdCR genes. 53/175 (30%) single cells yielded one or both canonical PCR products: 48 V_HIdCR, 34 V_KIdCR, 28 both V_HIdCR and V_KIdCR (Table 1). Furthermore, the amplified V_HIdCR and V_KIdCR products were ~650 and 780 bp long, respectively, which is consistent with rearrangements to ca-

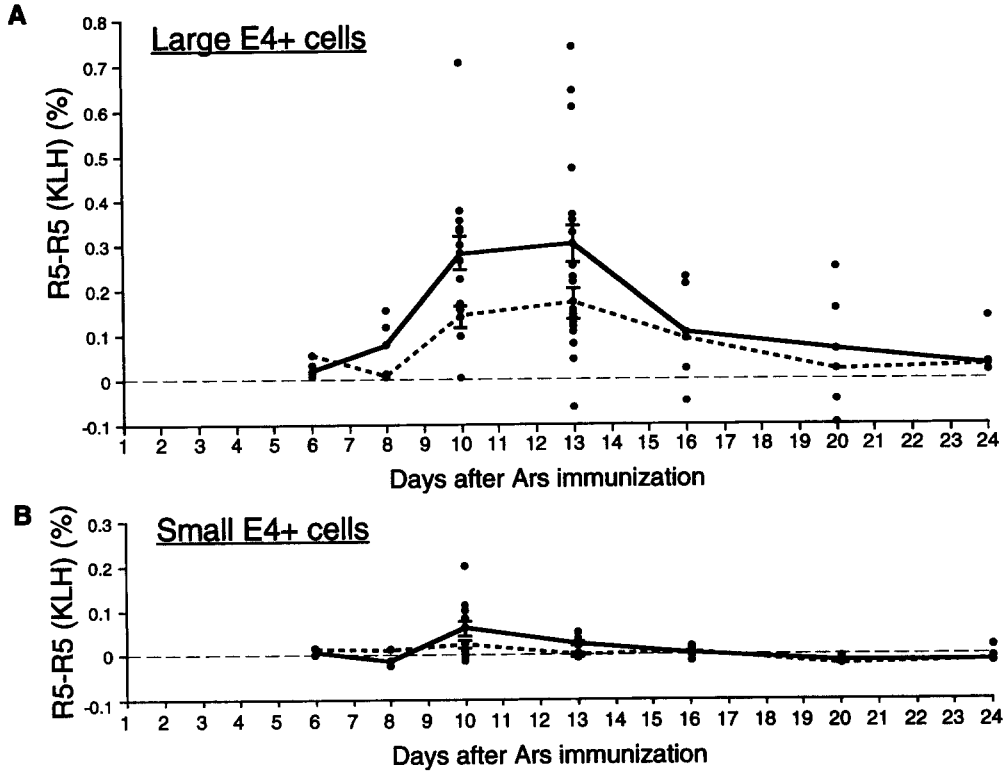

Figure 3. Emergence of $E4^+$ splenocytes during the primary antibody response to Ars. (*A*) Timecourse demonstrating the peak emergence and duration of the subpopulation of large $E4^+$ R5 cells (10 ± 1 μm in diameter). The percentages of these cells in the spleen were adjusted for background E4 staining and reported as R5-R5(KLH) (see Fig. 2 *A*). Mean values are shown (*solid line*). To quantify the Ars-specific fraction of $E4^+$ cells, similar calculations were derived for samples pretreated with ArsBSA to block E4 staining. Mean values for these samples adjusted for background [R5(ArsBSA) − R5(ArsBSA/KLH)] are also represented (*dashed line*). SEMs for day 10 and day 13 are shown. (*B*) Timecourse for small $E4^+$ R5 cells (6 ± 1 μm in diameter), using calculations and terminology described above.

nonical J_H2 and J_K1 gene segments. Only one PCR product was smaller than expected, consistent with a non-canonical V_KIdCR-J_K2 rearrangement (Table 1). No PCR products were obtained from 37 negative control microdrops in these experiments. It is not surprising that some of the cell isolates yielded no PCR products, given limitations on the efficiency of sorting such a small fraction of spleen cells.

Table 1. *Rearranged Canonical V Genes in Single $E4^+$ B Cells*

Mouse*	Single cell isolates	Single cells yielding: V_H or V_K product	V_H product	V_K product	Both V_H and V_K	No cell controls‡
1-day 10	28	12	11	10§	8	8
2-day 13	30	10	8	9	7	10
3-day 13	70	16	15	6	5	10
4-day 13	47	15	14	9	8	9
Total	175	53	48	34	28	37

*PCR results from single $E4^+$ cells obtained from four mice on the indicated days following immunization. Cells that utilized rearranged V_HIdCR and V_KIdCR genes gave single amplified bands 647 and 782 base pairs long, which are consistent with canonical V_HIdCR-J_H2 and V_KIdCR-J_K1 rearrangements, respectively.

‡Numbers in this column indicate control microdrops without cells that were subjected to the same PCR tests. None of them yielded PCR products.

§One of these 10 products was a smaller size, consistent with a noncanonical V_KIdCR-J_K2 arrangement.

Few Splenic Memory Progenitors Two Weeks Post-immunization. We can estimate the size of the hapten-specific memory cell compartment in the spleen based on flow cytometric analyses of E4-specific cells. At day 13, the size of the large $E4^+$ cell compartment was ~0.30% × 10% (larger splenocytes) × (5 × 10^7) cells/spleen = ~15,000 large $E4^+$ B cells/spleen. A similar calculation reveals an equivalent estimate for the number of small $E4^+$ B cells/spleen (~0.05% × 50% × [5 × 10^7] = ~15,000). Considering that our flow cytometric analyses excluded ~25% of all B cells, we adjusted the estimate to ~40,000 total $E4^+$ B cells/spleen. Finally, since E4 identifies ~40% of the memory response to Ars, as observed in serologic and hybridoma sampling studies (8, 26), the size of the complete Ars-specific memory cell compartment presumably does not exceed 100,000 cells/spleen. This is an upper-end estimate for actual memory cells because only half of the $E4^+$ cells bound Ars, and an unknown fraction presumably bound Ars with reduced affinity due to somatic mutagenesis. Such cells are almost certainly destined for exclusion from the memory pool, based on hybridoma sampling studies (8, 26).

Since our sorting procedure excluded any cell that stained specifically with anti-IgD or anti-Mac1 or that might have stained nonspecifically (e.g., via Fc receptors), we were concerned that the low frequencies of canonical cells observed might be misleading. For example, our $E4^+$ Ars^+ B cell frequencies were at least 10-fold less than the hapten-specific frequencies described by McHeyzer-Williams et al. (18) in the anti-NP response. To test our numerical conclusions, we estimated the number of cells in unfrac-

tionated spleen from immunized mice that could possibly be expressing the canonical set of V gene segments. Total spleen cell suspensions were prepared 13 d following immunization with either Ars-KLH or KLH. PCR amplifications for rearranged canonical V_HIdCR genes were performed on lysates derived from groups of 100 or 150 splenocytes (Table 2). From the ArsKLH and the KLH-immunized control mice, we found that ~1/1,000 and ~1/850 cells yielded a V_H PCR product of the appropriate size for a V_HIdCR-J_H2 rearrangement, respectively. This is in good agreement with random hybridoma sampling studies of Manser et al. (45) who found that ~1/350 LPS-generated hybridomas expressed the V_HIdCR gene with any J_H gene segment. To further assess whether these V_H gene PCR products were indeed canonical, we sequenced five products each from ArsKLH and KLH-only immunized mice. All 10 products encoded a J_H2 gene segment; however, only 5 of 10 used the canonical V_HIdCR gene segment, while the other five encoded identical V_H gene segments similar to V_HIdCR. Furthermore, 0 of 10 used the canonical $D_{fl16.1}$ gene segment, reinforcing our view that canonical B cells do not dominate the immune response because of precursor frequency considerations, and that the cells we are examining represent a highly selected subset that are preferentially expanded as the response progresses.

$E4^+$ B Cells Carry a Rearranged Canonical D Gene Segment, Have Critical Junctional Residues and Somatically Mutated V Genes. PCR-amplified V gene products of single $E4^+$ cells were directly sequenced using a method that was previously standardized with hybridomas (38). For the four mice described above, 78 of 82 PCR products (95%) obtained from 53 $E4^+$ cells were entirely canonical (Table 3). Two exceptions (sample no. 3-26H and no. 4-S27H) contained a D gene segment other than the canonical $D_{fl16.1}$ (Fig. 5); a third PCR product did not contain the canonical arginine codon at the V_K-J_K junction (sample no. 2-22K has a V_K junctional TGG codon encoding a tryptophan residue); a fourth product described in Table 1 was smaller than expected and consistent with a non-canonical V_KIdCR-J_K2 rearrangement. These data demonstrate that the large majority of the isolated $E4^+$ cells producing at least one V_H or V_K amplification product were using entirely canonical V gene segments and junctions. We interpret this result to indicate that most of the $E4^+$ cells visualized by flow cytometry were also expressing the canonical V region of the anti-Ars memory response.

By day 13, 37 of 41 $E4^+$ cells (90%) had acquired point substitution mutations in their rearranged canonical V genes, while 10 of 12 (83%) cells isolated on day 10 had acquired mutations. The mutation frequency calculated from data on all cells was 255/30917 = 0.82%. This was ~80-fold higher than the previously determined error frequency of our V gene amplification and sequencing method (1/7590 or 0.01%) (38), supporting the interpretation that the vast majority of observed mutations were not artifacts of amplification. The mutation frequency at day 13 for V_H and V_K genes was 212 mutations/22705 sequenced bases or 0.93%. The day 10 mutation frequency of 43/8212 or 0.52% was slightly lower than that of day 13.

The pattern and idiosyncrasies of the observed mutations was consistent with the interpretation that they are products of the physiologic V gene mutation mechanism. 57% of the mutations were transition substitutions, especially G to A, while 43% were transversions (random substitution mutations would favor transversions to transitions 2:1). These biases, which are typical of hybridoma V gene mutations (46, 47), were maintained when only non-coding mutations (i.e., silent and flanking regions), that are presumably free of selection pressures, were examined. High frequencies of somatic mutations were observed at positions 31 and 105 in canonical heavy chain genes, as is also true of V_H genes expressed by sampled hybridomas (data not shown). Position 31 appears to be an intrinsic "hotspot" for the mutation mechanism as described by Betz et al. (47), and recent work from our laboratory has identified position 105 as intrinsically highly mutable, based on an analysis of nu-

Table 2. *Frequency of Canonical Heavy Chain Rearrangements in Unsorted Splenocytes*

	Ars 1*	Ars2*	Ars3*‡	KLH1§	KLH2§	KLH3‡§
Number of groups tested‖	16	16	48	16	16	36
Number of groups with an appropriate size PCR fragment¶	2	1	7	3	1	6
Number of PCR products/ number of splenocytes	2/1600	1/1600	7/7200	3/1600	1/1600	6/5400
Number of canonical V_HDJ_H genes/ number of PCR products sequenced			0/5			0/5

*Splenocytes prepared from three A/J mice immunized with Ars-KLH 13 d earlier.
‡For these two mice, each tested group contained 150 unsorted splenocytes. Five PCR products from each mouse were sequenced to determine the frequency of canonical gene segment usage.
§Splenocytes prepared from three A/J mice immunized with KLH-only 13 d earlier.
‖Each group containing 100 unsorted splenocytes were subjected to PCR amplification to detect B cells using rearranged V_HIdCR-J_H2 genes.
¶The V_HIdCR-J_H2 rearrangement produces a single amplified fragment of ~650 base pairs.

Table 3. *Canonical V Gene Segment and Junction Usage by Single E4⁺ B Cells*

Mouse*	V_HIdCR	Pos. 99 serine	$D_{\text{fl16.1}}$	J_H2	V_KIdCR	Pos. 96 arginine	J_K1
1-day 10	11/11‡	11/11	11/11	11/11	9/9§	9/9	9/9
2-day 13	8/8	8/8	8/8	8/8	9/9	8/9	9/9
3-day 13	15/15	15/15	14/15	15/15	6/6	6/6	6/6
4-day 13	14/14	14/14	13/14	14/14	9/9	9/9	9/9
Total	48/48	48/48	46/48	48/48	33/33	32/33	33/33

*Sequence analysis of E4⁺ B cells from four mice (described in Fig. 2 and Table 1) revealed that V gene segment and junction usage in 78 of 81 V gene products derived from 53 cells was restricted to the specific canonical combination illustrated in Fig. 1.
‡Numerator indicates number of canonical PCR products. Denominator indicates total number of PCR products.
§One cell that yielded a smaller than expected V_K PCR product, consistent with a non-canonical V_KIdCR-J_K2 rearrangement (see Table 1), was not sequenced and was excluded from this table.

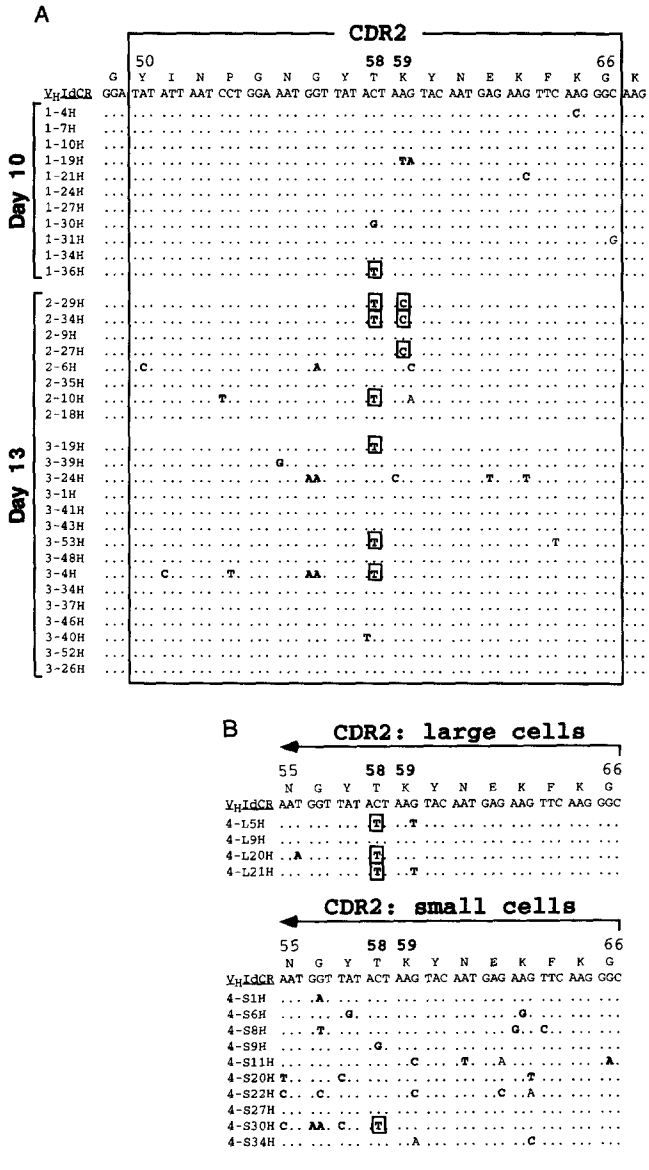

Figure 4. V_H CDR2 mutations in E4⁺ B cells. (*A*) Nucleotide sequences of V_H CDR2 regions of large E4⁺ B cells obtained 10 or 13 d post-immunization. Dots indicate identity with the reference germline-encoded sequence (5). Mutations resulting in amino acid changes (replacement mutations) are in bold. The boxed nucleotides produce amino acid changes (position 58 ACT to ATT, Thr to Ile; and position 59 AAG to ACG, Lys to Thr) that are known to increase binding affinity for Ars (32). Sample designations are consistent with other figures and tables (e.g., *1-4H*: mouse no. 1, sample no. 4, H chain). (*B*) Nucleotide sequences of V_H CDR2 regions from small and large E4⁺ B cells obtained from the same mouse, 13 d post-immunization. Parameters used to describe these samples and their sequences are the same as in the previous figure. All large and small cells from this mouse were apparently derived from the same germline precursor, based on junctional sequences in V_H CDR3 (see Fig. 5).

merous hybridoma V gene sequences (unpublished data). Thus, the V gene mutations observed in our sampled cells had the signature features of the hypermutation mechanism previously defined in hybridoma sequencing studies.

An affinity-based selection process had apparently operated on mutant precursors of the isolated cells. 10 of 27 (37%) large cells isolated on day 13 had acquired one or two specific V_H CDR2 replacement mutations known to improve Ars-binding affinity three- to eightfold (V_H pos. 58: Thr to Ile; pos. 59: Lys to Thr) (32) (Fig. 4 *a* and *b*). At day 10, only one in 11 cells or 9% had acquired one of these specific affinity-improving mutations (Fig. 4 *a*). Interestingly, only 1 of 10 small cells that were isolated on day 13 had acquired one of these mutations (Fig. 4 *b*). Although selection had evidently operated on precursors of large cells expressing mutant antibody products by day 13, there were few shared silent mutations or noncoding mutations (located 3′ of V exons) among cells derived from common precursors (see below). Hence, sequential rounds of selection, characteristic of advanced stages of memory development, apparently had not yet occurred.

Memory-lineage Cells Derived from Few Precursors. Sampled E4⁺ cells were assigned to lineages by inspection of junctional codons at positions 100 and 107 (X and Y in Fig. 1) and the third base of the serine 99 codon in canonical H chain V genes. Sequenced V_H gene products from E4⁺ cells obtained 10 and 13 d after immunization revealed that these junctions were limited in diversity, suggesting common

precursor origins. In Fig. 5, sequences most likely derived from common precursor cells are grouped together. Two samples that did not contain the canonical $D_{fl16.1}$ segment were not included in this analysis. Sampled cells from mice no. 1 through no. 4 in Fig. 5 were apparently derived from 2, 5, 4, and 1 clonal precursor(s), respectively. Based on the frequency of repeat sequences, we used a mathematical model ("hidden species") to obtain an estimate of the total number of canonical precursors from which the sampled memory-lineage cells were derived (23). With 95% confidence, we estimate the number of canonical precursors was 2, 8 (range 5 to 11) , 7 (range 6 to 9), and 1 precursor(s) in the four mice, respectively. This indicates that, as early as day 13, memory-lineage cells are derived from a limited number of precursors that had undergone extensive clonal expansion. Taking 11 as the upper estimate for canonical precursors (from mouse no. 2), and assuming that 40% of the Ars-specific memory compartment is derived from canonical precursors, we estimate the total hapten-specific memory compartment at day 13 to be composed of no more than 28 clones.

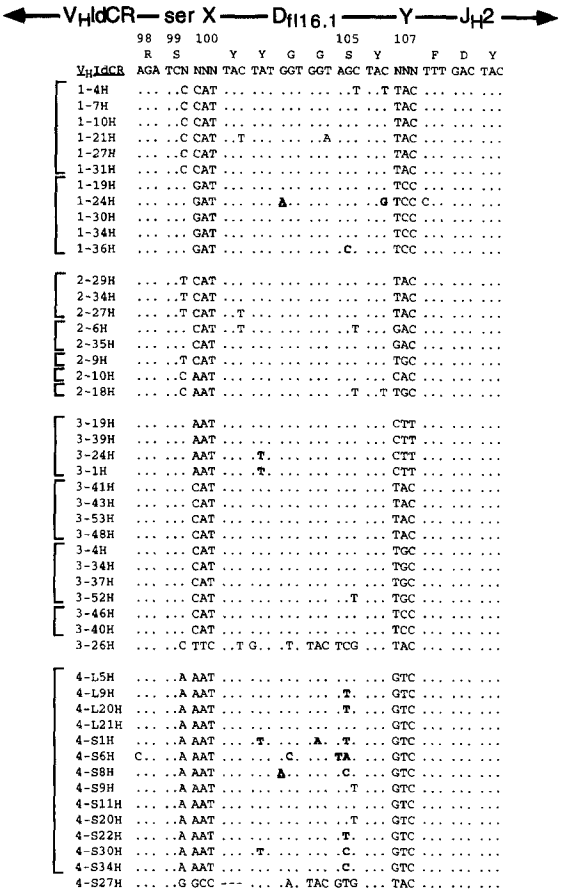

Figure 5. Lineage assignments based on V_H CDR3 junctional sequences. Nucleotide sequences obtained from E4$^+$ cells of four mice are shown. The variable junctional nucleotides are denoted "N" in the reference sequence (5, 27). Dots indicate identity with the reference sequence. Mutations resulting in amino acid changes (replacement mutations) are in bold. Two ambiguous bases are underlined. Sample nomenclature is consistent with other figures and tables (e.g., *1-4H*: mouse no. 1, sample no. 4, H chain). Cells from a given mouse were assigned to a common lineage when their junctional sequences were identical. Two cells utilized noncanonical D segments.

Discussion

E4$^+$ B Cells Belong to Memory Lineages. Our interpretation that E4$^+$ splenocytes belong to lineages destined for the memory compartment is based in part on previous serologic and hybridoma sampling studies. A large fraction of Ars-specific hybridomas isolated during the secondary (memory) immune response express mutated versions of a highly defined canonical set of V gene segments. In this study, more than 90% of primary-response E4$^+$ cells that were subjected to sequencing analysis carried V genes with all of the canonical structural hallmarks including: (*a*) a specific set of 5 V gene segments; (*b*) codons for two specific junctional residues; (*c*) a uniform V_H CDR3 length; and (*d*) acquisition of point mutations. In addition, large E4$^+$ cells had a high frequency of specific affinity-improving mutations. The kinetics of emergence of this population of cells carrying mutated antibody V genes was also consistent with the kinetics of germinal center development and with results of other investigators (18, 21), who have shown that somatic hypermutation appears to be confined to PNA_{hi} germinal center B cells. E4$^+$ cells also displayed other phenotypic characteristics of germinal center centrocytes, including a low level of surface Ig (40, 43, 48), an absence of surface IgD (43, 49, 50), a high level of receptors for PNA (39–44), and enrichment among larger cells (10 μm in diameter). We have also recently observed that E4$^+$ cells do not secrete antibody, as determined using an ELISPOT assay (data not shown). The basis of the bimodal staining with B220 is unknown. Presumably the stained cells do not include plasma cells which are PNA_{lo} (18, 42), centroblasts which are reported to be sIg$^-$ (50a), or plasmablasts of periarteriolar lymphoid sheaths, which do not mutate their V genes (14). But we cannot exclude the possibility that they are recent sIg$^+$ emigrants from the GC that are in the process of differentiating into plasma cells.

Estimating Frequencies of Memory Progenitors. Our estimates detailed above indicate that fewer than 40,000 E4$^+$ cells and 50,000 total hapten-specific memory-lineage cells per spleen were present at the time of their peak frequencies in primary-immunized A/J mice. E4$^+$ cells that have lost their ability to bind Ars, presumably because of deleterious mutations, account for half of the canonical cells observed and are presumably destined for exclusion from the memory compartment.

Our estimate is at least 10-fold lower than the number of splenic B cells observed to participate in the primary NP-specific immune response as described by others (18, 21). The numerical discrepancy between the two studies could be due to differences in antigens, adjuvants or strains of mice used in the two systems. However, the primary anti-NP response appears to be dominated by λ1-expressing B

cells, whereas the secondary immune response to NP is dominated by kappa-expressing B cells (22). Further, relatively few lineages of NP-specific B cells dominate the secondary immune response in a given animal (23). This indicates that the lambda-expressing clonotypes are superseded by minor kappa-bearing populations that are preferentially recruited and expanded into the memory pool. Thus, the early domination by λ1-bearing B cells could be due to an inordinately high frequency of precursor cells.

We calculated that the $E4^+$ cells isolated 13 d after immunization originated from only 1 to 11 clonal precursors, and thus the complete Ars-specific memory cell compartment was probably derived from 1 to 28 precursors. This indicates that clone sizes varied from ~4,000 to ~40,000 cells at day 13. This precursor estimate is in agreement with the observation, based on hybridoma sampling studies, that late primary or secondary immune responses are generally dominated by relatively few clones of B cells (10–12, 23, 24). However, it is substantially lower than the 300–500 germinal centers estimated to develop in response to the hapten NP (21). Since germinal centers are the sites of memory B cell development (16, 18–20, 43, 44, 54), our estimate suggests one of two possibilities to reconcile these differences: only a small minority of germinal centers ultimately contribute to the memory B cell repertoire, or a small number of clonal precursors populate numerous germinal centers in a given animal.

$E4^+$ Cells Are Phenotypically Diverse and Represent an Intermediate Stage in Memory Development. $E4^+$ B cells sampled 13 d after Ars immunization manifested an intermediate degree of hypermutation and clonal expansion when compared with their secondary response counterparts. Anti-Ars memory B cells typically reveal a frequency of V gene mutations in the range of 2–8% when sampled after secondary exposure to antigen (reviewed in 13, 51). Furthermore, silent or noncoding mutations are often shared by memory B cells in a way that reveals the genealogy of their clonal development (10, 11). In contrast, the frequency of somatic mutations in our E4-specific cells was only ~0.5–1.0%. While some replacement mutations and, in particular, affinity-improving mutations, were shared, there was no sharing of silent mutations among clonally related members. Thus, the $E4^+$ cells had apparently sustained an intermediate degree of somatic mutagenesis and selection by 13 d after immunization.

Evidence for ongoing selection was also supported by heterogeneity within the $E4^+$ population. We found that the presence of ArsBSA in the staining buffer reduced the frequency of cells that stained with E4 by ~50%. This suggests that many of the canonical cells had acquired V gene mutations that diminished or abolished the capacity of their antibody products to bind Ars, and that antigen-based selection was in progress at the time of cell sampling. We infer that these presumed deleterious somatic mutations were recently acquired because antigen binding loss variants are short-lived without the benefit of antigen-based selection (8, 52, 53, 55).

Evidence for heterogeneity could also be seen between large and small $E4^+$ cells. Clonally related $E4^+$ cells from one mouse were found among both large and small cells. Interestingly, the small cells had V_H CDR2 and CDR3 that were more heavily mutated than those of the large cells, yet they were nearly devoid of the specific mutations associated with affinity improvements (for binding to Ars) and positive selection. Perhaps the small $E4^+$ cells, without the benefit of affinity-improving mutations, were undergoing apoptosis, an early sign of which is a contraction in cell volume (56).

It is possible that some of the $E4^+$ cells were mutating their antibody V genes at the time of isolation. Of those that produced amplified canonical gene products (derived from chromosomal DNA), ~50% yielded only a heavy or a light chain product. In contrast, the concordance between amplified heavy and light chain gene products was ~90% for hybridoma cells (38). One possible explanation for this difference is that some of the $E4^+$ cells may have sustained cuts within their V gene templates, perhaps due to the process of somatic mutagenesis. Any interruption between primer sites would preclude successful amplification. Jacob et al. (16) observed a high frequency of PCR hybrid products derived from groups of sampled germinal center cells and also suggested that this might be an indication of DNA modification. DNA nicking has been proposed to be necessary in several models of V gene hypermutation, although no direct evidence for this has been reported to date (51).

Finally, the 255 observed mutations included six ambiguous bases where signals were seen in two lanes of sequencing ladders. Five of the six mutations were transition substitution mutations, which is consistent with the pattern of somatic hypermutation. While this frequency is close to the expected frequency of *Taq* polymerase errors (~4 *Taq* errors/30917 sequenced bases), four of the five ambiguous transition mutations were G to A (three) or C to T (one), while *Taq* Polymerase misincorporations tend to be A to G or T to C transitions (57–59). Thus, the observed single base differences between complementary DNA strands of the same V gene product could be the result of newly acquired point mutations.

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