Rapid Communication

Evolving Abundance and Clonal Pattern of Human Germinal Center B Cells During Childhood

By Yadira Valles-Ayoub, Herman L. Govan III, and Jonathan Braun

Childhood is a critical period for the development of the memory B-lymphocyte repertoire, necessary in protective humoral immunity. This study addressed the natural history of memory B cells based on the previous identification of germinal center and mantle zone cells as the probable precursor and mature memory cell populations, respectively. Using flow cytometric quantitation of these B-cell subpopulations in human tonsil, we found that germinal center cells were abundant (70% of tonsil B cells) during early childhood (2 to 3 years), but decline by early adolescence (8 to 14 years) to a low level (33%, P = .0003). To study the clonal evolution of these B-cell subpopulations, germinal center and mantle zone B cells were isolated using a preparative magnetic immunobead method, and analyzed using a novel polymerase chain reaction-based quantitative assay to measure the abundance of B-cell clones bearing certain rearranged VH subfamilies. Two VH subfamilies were informative: VH1N clones were uniquely deficient in germinal center B cells at the early age period, but became abundant in later childhood; and VH3L clones were absent among germinal center cells regardless of age. In contrast, B-cell clones bearing each VH subfamily were abundant in the mantle zone subpopulation throughout childhood. These findings suggest that the abundance and clonal pattern of germinal center B cells evolves during childhood, presumably due to changing antigenic or ontogenic processes. Moreover, the distinct clonal pattern of germinal center versus mantle zone B cells suggests that a major phase of clonal selection occurs after germinal center emigration.

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In humans, large numbers of B lymphocytes are produced daily beginning in the eighth week of gestation, and the newborn appears to have a full complement of B cells expressing all immunoglobulin (Ig) isotypes. In contrast, the adult pattern of antibody responsiveness is not acquired until several years after birth, generally attributed to the expansion and diversification of memory B cells through cumulative influence of environmental antigens and the developing T-lymphocyte compartment. Intrinsic ontogenic factors may also play a role in this physiologic immune deficit, but are difficult to assess due to the lack of definitive markers for memory B cells.

This gap has been partially filled by recognition that the germinal center is the site of a critical stage in the development of memory B cells. The germinal center is also considered the major source of precursors for the mantle zone, a site populated by long-lived B cells apparently representing the quiescent memory cell pool. However, the presumed precursor-descendent relationship between germinal center and mantle zone cells is complicated by uncertainty as to whether B cells undergo one or multiple periods of residence in the germinal center, and whether the same or different antigenic and regional factors determine trafficking to and inhabitation of germinal center versus mantle zone sites.

Accordingly, much effort has been devoted to the isolation and characterization of B-cell subpopulations residing in these microanatomic locations based on buoyant density, peanut agglutinin (PNA) binding sites, and expression of a various differentiation-specific membrane proteins. In this report, we have used these phenotypic criteria to determine the abundance of germinal center and mantle zone B cells, and as the basis of a rapid, large-scale fractionation method using magnetic immunobeads.

Another distinction between B-cell subpopulations is the divergent pattern of antigenic and immunoregulatory selection they encounter, which in turn should create characteristic patterns of antibody repertoire and V gene clonal patterns. If local germinal center B cells are indeed the precursors of regional memory cells, and if predominant immunoselection initiates rather than follows memory cell differentiation, then germinal center and mantle zone B cells should display a common clonal pattern. To test this prediction, we have isolated these subpopulations a quantitatively assayed by nonamer utilization at the germline level using a novel polymerase chain reaction (PCR)-based strategy. The present study describes an unexpected difference in clonal pattern between germinal center and mantle zone B cells, and its evolution from early to later periods of childhood.

MATERIALS AND METHODS

Reagents. Purified monoclonal mouse antibodies for human IgD, CD3 (Leu-4), CD20 (Leu-16), and CD38 (Leu-17) were purchased from Becton-Dickinson (Mountain View, CA); PNA was purchased from E-Y Laboratories (San Mateo, CA). Leu-16 was obtained as a phycoerythrin conjugate. The other reagents were obtained as fluorescein conjugates. Monoclonal mouse antibodies to CD44 (HK23) and globotrioseyl ceramide (SB5) were tissue culture reagents.

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supernatants\(^\text{18}\) (generously provided by Dr M.H. Nahm, Washington University School of Medicine), and detected with fluorescein- \(\text{Fab}^\text{2}\)-goat anti-mouse IgG (Tago, South San Francisco, CA). For cell separations, monoclonal mouse antibodies for human IgD, TA4-1,\(^\text{21}\) and CD38, THB-7\(^\text{22}\) were produced as mouse ascites and tissue culture supernatant, and used without further purification. Sheep anti-mouse IgG magnetic immunobeads (Dynabeads, Dynal, Inc) were purchased from Robbins Scientific (Mountain View, CA).

**Cell separation.** Tonsil tissue was obtained as fresh tonsillectomy specimens from patients ranging in age from 2 to 14 years, and processed in culture medium (RPMI-1640, 20% fetal calf serum [Hyclone, Logan, UT], 1 mmol/L sodium pyruvate, 50 mmol/L 2-mercaptoethanol, and antibiotics). Enriched B cells were prepared by Ficoll-Hypaque mononuclear cell isolation and sheep erythrocyte-T-cell depletion.\(^\text{24}\) In some cases, mantle zone and germinal center B-cell subpopulations were isolated. B cells, \(2 \times 10^6\), were incubated with anti-CD38 or anti-IgD for 30 minutes on ice, then washed, resuspended at \(6 \times 10^7\) cells/mL with anti-mouse IgG magnetic immunobeads (5 mg/mL), and incubated in a 15-mL plastic conical tube on a rocker for 45 minutes at 4°C. Immunobead-bound cells were separated from unbound cells (CD38-depleted and IgD-depleted cells, respectively) by twice decanting the solution in the presence of a strong magnet on the wall of the tube. The different cell fractions were washed and used without further efforts to remove free or cell-bound immunobeads.

**Flow cytometry.** Flow cytometry was performed on a FACS 440 (Becton-Dickinson) with 3.5 decade logarithmic amplification; list mode data analysis was performed using a Digital MiniVAX system (Boston, MA). For quantitation studies, fluorescence channel gates were selected by setting a level of -3% positive cells with negative control reagents (for single-step staining, an isotype-matched, fluorochrome-conjugated irrelevant monoclonal antibody; for two-step staining [5B5 and HK23 fluorescein anti-IgG alone). Pokeweed mitogen (PWM)-induced Ig secretion. CD38- and IgD-depleted B-cell fractions and autologous T cells were each adjusted to \(1 \times 10^6\) cells/mL in culture medium, and 50 mL of B- and T-cell suspensions were added to U-bottom tissue culture microtiter plates with 50 mL PWM (GIBCO, Grand Island, NY, 1:100 in tissue culture supernatant, and used without further purification. Sheep anti-mouse IgG magnetic immunobeads (Dynabeads, Dynal, Inc) were purchased from Robbins Scientific (Mountain View, CA). Enriched tonsil B cells were labeled with antibodies detecting surface antigens known to distinguish between germinal center and mantle zone B cells, and analyzed by flow cytometry (Fig 1). Each of these antigens (Leu-16, IgD, Leu-17, and PNA) divided the tonsil B cells into bright and dim populations. As expected, in each specimen there was a correlation between the abundance of cells with phenotypes characteristic of the germinal center (bright for Leu-16, Leu-17, and PNA; dim for IgD) and mantle zone (dim for Leu-16, Leu-17, and PNA; bright for IgD). However, after examination of a series of patients, we were surprised to find that the proportion of germinal center to mantle zone cells differed between individual patients, but was consistent according to age (Table 1). In young children (2 to 4 years old), the ratio of germinal center to mantle zone cells was 2:1, whereas in older children (8 to 14 years old), the ratio was reversed. In contrast, there was no age effect on the fraction of B and T cells in the total mononuclear cell population (Table 1) or the total number of tonsil lymphocytes recovered (data not shown).

To directly analyze germinal center and mantle zone B cells, we isolated these two populations by magnetic immunobead fractionation. We expected that germinal center B cells would be enriched in the IgD-depleted and CD38-bound fraction. Conversely, mantle zone B cells would be enriched in the CD38-depleted and IgD-bound fraction. In fact, the relative yield of cells in each fraction corresponded to the ratios predicted by flow cytometry, and were nearly quantitative (greater than 90% recovery) (data not shown). The purity of the fractionated populations was assessed by flow cytometry (Table 2, top) using several subpopulation-specific markers for germinal center (CD20\(^\text{30b}\); 5B5, and Leu-17) or mantle zone (CD44 or IgD) cells. Expression of these antigens was heterogenous: while all the markers demonstrated enrichment of the appropriate subpopulation, estimates of purity were generally greater than 90%, but ranged from 75% to 95%.

The identity of the isolated subpopulations was further
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Leu-16  IgD  Leu-17  PNA

2 year old

8 year old

Fig 1. Phenotype of tonsil B cells during childhood. T-cell-depleted mononuclear cells from tonsil specimens of a 2- or 8-year-old patient were labeled with antibodies to the indicated antigens or with the PNA lectin, and analyzed by single-color flow cytometry.

characterized by testing for PWM-induced Ig secretion (Table 2, bottom). CD38-depleted (mantle zone) cells responded with high level secretion for all tested Ig isotypes. In contrast, IgD-depleted (germinal center) cells were completely unresponsive. These findings are in accordance with previous studies indicating the distinctive unresponsiveness of germinal center (but not mantle zone) B cells to PWM-induced Ig secretion," and suggest a high level of purity.

The V_H clonal patterns of the highly purified germinal center (CD38-bound) and mantle zone (IgD-bound) B cells were analyzed using a quantitative, V_H subfamily-specific PCR assay to characterize the relative abundance of index B-cell clones. The rationale for this type of analysis has been presented previously. Briefly, oligonucleotide probes have been selected to detect subsets ("subfamilies") of several V_H gene families. When 5'-3' V_H subfamily and 3'-5' J_H oligonucleotides are used to amplify genomic DNA, product occurs only with fully rearranged V_H subfamily segments (ie, juxtaposed to a J_H segment by VDJ rearrangement). In addition, the concentration of genomic DNA is reduced to a range where the amount of amplified product (judged by incorporated radioactivity) is proportional to the amount of genomic DNA included in the reaction mixture. In the case of tonsil B-cell DNA, titration studies showed a linear relationship between genomic DNA and amplified product when genomic DNA was between 20 and 200 ng/0.1 reaction (~200 to 2,000 cell equivalents). Finally, because human B cells usually bear only one fully rearranged allele, the abundance of VDJ segments for a particular V_H subfamily closely approximates the abundance of B cells in the population expressing that V_H subfamily. For these reasons, this PCR assay allows a quantitative comparison of the relative number of V_H subfamily B cells in different sample populations.

The results of a typical analysis of a young and old child are shown in Fig 2. B cells bearing rearranged VH1N or VH3L were abundant in mantle zone B cells of both young and old children. In contrast, while germinal center B cells bearing rearranged VH1N were abundant in older children, they were rare in younger children. Moreover, germinal center cells bearing rearranged VH3L were rare in both age groups. These findings were consistent for three additional children in each age group (Fig 3). The data from these groups were compiled to obtain the following quantitative description of these differences.

<table>
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<tr>
<th>Table 1. Abundance of Follicular B-Cell Subpopulations in Young and Old Children</th>
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<td>Patient</td>
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Paired, two-tailed t-test (ranked)

P = .0018  P = .0004  P = .0003  P = .0141

Human tonsillar lymphocytes were analyzed by flow cytometry for the fraction of T cells (CD3+ lymphocytes), and the fraction of B cells either bright (germinal center) or dim (mantle zone) for Leu-16. 
Abbreviations: GC, germinal center; MZ, mantle zone.

*Arithmetic mean ± SE.
mantle zone B cells, respectively; the ratio (germinal center: mantle zone) was 3.1% ± 0.4%. In contrast, B cells bearing two other rearranged V<sub>H</sub> subfamilies (VH46 and VH5N) were abundant in both B-cell subpopulations, regardless of age (Fig 3).

**DISCUSSION**

In this study of chronic tonsillitis specimens, germinal center and mantle zone B-cell subpopulations were found to contain distinct clonal patterns that consistently recurred in different age-matched individuals. In particular, VH1N clones were rare among the germinal center cells of young but not old children, and VH3L clones were rare regardless of age. In contrast, all index clones were abundant among the mantle zone cells throughout childhood.

This study introduces a preparative magnetic immunobead method for separating germinal center and mantle zone subpopulations, based on the reciprocal expression by these subpopulations of CD38 (Leu-17) and IgD. In addition to the criteria of CD38 and IgD expression used in the separation procedure, the purity of fractionated cells was confirmed by three criteria: flow cytometry for a panel of subpopulation-specific markers; responsiveness or anergy to PWM-induced Ig secretion in mantle zone and germinal center cells, respectively; and restriction of the unique deficit of VH3L clones to the germinal center cell fraction. These findings verify the identity of the fractionated populations with a level of purity comparable with other preparative methods. In our hands, the flexibility, speed, and high yield of the immunobead fractionation is an improvement over panning or antibody-rosette procedures. Greater purity can be achieved by fluorescence-activated cell sorting, but the drawbacks include long separation times, low yield, and dependence on sophisticated instrumentation.

A PCR-based assay was introduced to measure the abundance of B cells bearing rearranged VDJ segments of selected V<sub>H</sub> subfamilies. This approach differs from the typical assessment of V<sub>H</sub> utilization patterns based on levels of V<sub>H</sub>-specific Ig transcripts. While the latter approach is a fair representation of the expressed repertoire, it does not necessarily give an accurate estimate of the clonal abundance of V<sub>H</sub>-expressing cells because it systematically overestimates the abundance of secretary (high-transcript level) B cells and underestimates that of quiescent (low-transcript level) cells. To overcome this issue, several strategies have been developed, most commonly generation of hybridoma or Epstein-Barr virus (EBV)-transformed B-cell libraries. The present assay, which was developed in parallel with other PCR-based studies of V gene usage, has several advantages: (1) it detects only genomic VDJ segments, which are a direct measure of clonal abundance; (2) it allows direct analysis of small numbers (500 to 5,000 cell equivalents) of fresh B lymphocytes; and (3) it obviates the technical requirements for primary cell tissue culture and the low efficiency and potential clonal skewing of hybridoma/EBV transformation.

The absence of VH1N and VH3L clones in the germinal center B-cell subpopulation was a striking and unexpected
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Fig 3. $V_{H}$ subfamily quantitation in fractionated germinal center (CD38-bound) and mantle zone (IgD-bound) B cells from six different patients of the indicated age in years.

finding. A simple interpretation stems from the fact that individual germinal centers are composed of oligoclonal or monoclonal B-cell populations specific for the ongoing response to local antigens. Accordingly, germinal centers from chronic tonsillitis specimens would be expected to be composed of B cells specific for the pathogens responsible for the inflammatory state. Following this reasoning, the VH46 and VH5N subfamilies presumably include $V_{H}$ genes suitable for the generation of relevant antibodies to these pathogens, whereas the VH3L subfamily does not. Similarly, the age-dependent occurrence of VH1N germinal center cells may reflect a shift in pathogens causing tonsillitis during childhood, the VH1N subfamily presumably suitable only for pathogens prevalent during late childhood.

There are certain limitations and discordances with this explanation. (1) The microbiologic premise is uncertain: while the decline in tonsillitis due to *Hemophilus influenzae* coincides with the appearance VH1N germinal center cells, the major pathogens for chronic tonsillitis (*Streptococcus pneumoniae* and adenovirus) do not show an age-specific pattern. (2) It is surprising that subsets of the very small VH4, VH6, and VH5 families are abundant in the presumed antigen response, whereas subsets of the large and immunodominant VH1 and VH3 families are almost completely unrepresented. However, we note that each subfamily may not be functionally representative of its aggregate family. (3) A recent examination of fractionated B-cell subpopulations from mesenteric nodes of a 40-year-old with ulcerative colitis demonstrated the same clonal patterns of the older-child tonsil, including the absence of germinal center VH3L clones (Valles et al, unpublished observations, October 1989). This suggests that the germinal center clonal pattern may be generated by factors independent of childhood and the tonsil antigenic environment.

Alternatively, the evolving abundance and $V_{H}$ clonal pattern of germinal center cells during childhood may be a
manifestation of B-cell ontogeny. In humans, ontogenic shifts have been observed for VH \text{ utilization}^{20,28,38} and patterns of antigen responsiveness.\textsuperscript{4,5} Notably, the late appearance of germinal center VH1N clones corresponds to the period of childhood in which serum IgA\textsuperscript{7} and humoral responses to \textit{H influenzae} infection\textsuperscript{4} shift to adult levels. In this line of thinking, the early deficit of VH1N and VH3L clones would suggest that these clones are uncommon among germinal center cell precursors (such as recent bone marrow emigrants). Accordingly, their abundance in mantle zone cells would indicate that strong positive selection for these clones occurs after germinal center emigration. If so, the continued absence of these clones in the germinal center cell subpopulation would imply that most germinal center colonization involves immature precursors, rather than returning mantle zone cells. This issue is meaningful because it remains uncertain whether germinal center emigrants ever return to the germinal center. A key prediction of this scenario is the deficit of VH1N and VH3L clones in germinal center cell precursors; efforts to test this prediction are a current laboratory focus.

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