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Infant and Adult Human B Cell Responses to Rotavirus Share Common Immunodominant Variable Gene Repertoires

Jörn-Hendrik Weitkamp,* Nicole Kallewaard,† Koichi Kusuhara,‡ Elizabeth Bures,* John V. Williams,* Bonnie LaFleur,‡ Harry B. Greenberg,§ and James E. Crowe, Jr.*†

Ab repertoires exhibit marked restrictions during fetal life characterized by biases of variable gene usage and lack of junctional diversity. We tested the hypothesis that Ab repertoire restriction contributes to the observed poor quality of specific Ab responses made by infants to viral infections. We analyzed the molecular determinants of B cell responses in humans to two Ags of rotavirus (RV), a common and clinically important infection of human infants. We sequenced Ab H and L chain V region genes (VH and VL) of clones expanded from single B cells responding to RV virus protein 6 or virus protein 7. We found that adults exhibited a distinct bias in use of gene segments in the VH1 and VH4 families, for example, VH1-46, VH4-31, and VH4-61. This gene segment bias differed markedly from the VH3 dominant bias seen in randomly selected adult B cells. Recombinant Abs incorporating any of those three immunodominant VH segments bound to RV-infected cells and also to purified RV particles. The RV-specific B cell repertoires of infants aged 2–11 mo and those of adults were highly related when compared by VH, D, JH, VL, and JL segment selection, extent of junctional diversity, and mean H chain complementarity determining region 3 length. These data suggest that residual fetal bias of the B cell repertoire is not a limiting determinant of the quality of Ab responses to viruses of infants beyond the neonatal period. The Journal of Immunology, 2003, 171: 4680–4688.

Human infants mount relatively poor quality Ab responses of low titer to primary viral infections (1–7). It has been speculated that the B cell repertoire diversity of infants is limited because of residual fetal-like constrained VH and VL usage, and that this bias is a principal mechanism underlying the poor Ab response of infants to foreign Ags early in life (8–11). If the infant B cell repertoire is fundamentally limited in diversity, then attempts to vaccinate at very young ages with novel Ags are unlikely to be effective. In contrast, if an adequate B cell repertoire is present in infants, improved Ags or adjuvants may enhance immunogenicity in this age group. Therefore, it is important to determine whether the Ag-specific B cell repertoire in infants is functionally mature. Virus-specific infant B cell repertoires have not been studied at the single cell level in the past due to technical difficulty. We previously developed a robust method for the generation of human mAbs to rotavirus (RV) (12). The method incorporates several novel features, including physical selection of Ag-specific B cells from human volunteers using fluorescent virus-like particles (VLPs), single B cell expansion, and testing of differentiated cell supernatants against virus particles in an enhanced ELISA, and Fab expression using a novel vector that allows Fv cloning. In the present study, we analyzed human Ab repertoire diversity in RV-specific B cells at the single cell level using fluorescent VLPs to determine whether Ab repertoire restriction in infants is limiting for response to an important childhood pathogen.

RV virus protein (VP) 6 is the major structural component of RV, and it elicits a large portion of the Abs observed following infection in humans. RV VP7 is an outer capsid protein that elicits neutralizing Abs. We used RV VLPs to isolate single RV VP6- or VP7-specific B cells from previously infected or recently infected healthy adults and determined the sequences of the VH regions. We identified five immunodominant H chain variable gene segments used in the adult Ab response to RV. We then compared adult RV-specific Ab sequences with those of single RV-specific B cells derived from RV-infected infants or of randomly selected adult B cells. Our results show that the RV-specific B cell repertoire of infants exhibits adult-like levels of combinatorial and junctional diversity. These data suggest that VH repertoire biases are not limiting for virus-specific Ab responses early in life.

Materials and Methods

Generation of human RV-specific B cell clones

We used a novel method of generating human RV-specific B cell clones in culture after single cell sorting using green fluorescent protein (GFP)-labeled VLP, as recently described (12). For the studies described here, we obtained whole blood from five types of donor groups: group A, infants with acute RV infection; group B, asymptomatic adults with RV exposure;
group C, healthy adults, RV-specific cells; group D, healthy adults, RV-specific IgD- cells; group E, healthy adults, randomly selected B cells. The origins of B cell clones and the number of Ab sequences analyzed for each donor group and RV Ag are shown (see Table I). B cell clones were obtained from four infants, aged 2, 3, 7, or 11 mo (donors 1–4). Blood was obtained from two adult patients (donors 5 and 6) who were naturally exposed to RV and reported symptoms of RV disease within 1 mo prior. Blood for RV-specific B cell analysis from eight healthy blood donors (donors 7–14) was collected from leukocyte reduction filters as described (13). For randomly selected B cell controls, we obtained blood cells from leukocyte reduction filters from three healthy blood donors (donors 15–17) by the same method. We isolated PBMC by density centrifugation on lymphocyte separation medium and separated CD19+ cells with paramagnetic beads according to the manufacturer’s instructions (Dynal Biotech, Lake Success, NY). GFP-VLPs for single cell sorting were produced by coinfection of SP9 insect cells with recombinant baculoviruses as published previously (14). The specificity of binding of RV VLPs to RV-specific B cells was previously established by specific binding to RV-specific (but not nonspecific) murine hybridomas (our unpublished data), to murine B cells induced by RV infection of mice (14, 15), and to human B cells induced by natural RV infection (12, 15). We stained magnetically isolated B cells with anti-CD19-PE, VP2/6 or VP2/6/GFP-VLP, and anti-IgD-PerCP as described (12). We performed flow cytometric analysis and sorting, and single RVVLP+CD19+ cells were collected one cell per well into 96-well culture plates.

Expansion of single RV-specific B cells into clones

For the expansion of single B cells, we used a B cell culture system that we previously described to amplify and identify RV-specific or randomly selected B cell clones (12). Brieﬂy, 50,000 irradiated EL-4 B5 mouse thymoma cells (kindly provided by Dr. R. H. Zuberb, Geneva University Hospital, Geneva, Switzerland) per well of 96-well culture plates were used as feeder cells immediately following single cell isolation. A combination of 100 U of recombinant human IL-2, 5 nM/mL PA, and 10% (v/v) of supernatant from pokeweed mitogen-activated human T cells (T cell replacing factor) was added. Culture plates were incubated for 7 days, then we removed 100 μL of supernatant, and added 10,000 irradiated fibroblastic L cells stably transfected with human CD154 to each well (16). This cell line was kindly provided by DNAx via the American Type Culture Collection (CRL 12955; Manassas, VA). We also added 5 nM/mL recombinant human IL-4 in addition to the B cell culture media described above. The cultures were kept for another 2 wk with a second addition of CD154 fibroblasts, cytokines, PMA, and T cell replacing factor on day 14. Secreted human Ig or RV-specific Abs were detected by ELISA on day 14 or 21, respectively, to conﬁrm the speciﬁcity of the B cell clone for RV before RT–PCR, as described (12). To maximize the identiﬁcation of human IgG-producing wells secreting RV-specific Abs, we used anti-IgG reagents that detected both heavy and light chains. Therefore, the isotype of the Abs secreted by cell lines derived from single B cells was not determined. Although the VLPS were used to select RV-specific B cells by FACS contained GFP, we used viral particles derived from wild-type RV for the ELISA detection of Abs in B cell supernatants to eliminate the possibility of detection of GFP-specific Abs.

RT–PCR ampliﬁcation

For RT–PCR ampliﬁcation of VH and VL regions we used a commercial oligo-dT based mRNA capture kit (mRNA Capture kit; Roche, Basel, Switzerland) and single tube RT–PCR method (Titan One Tube RT–PCR system) and a pooled primer mix. The VH and VL primer sequences used were designed to amplify all VH and VL gene segments in the VBASE complete database of genomic V gene sequences (17, 18). We further ampliﬁed the ﬁrst-round PCR products using VH and VL nested primers hybridizing to framework regions (FR) 1 (forward) and FR4 (reverse) under the same conditions as above. The primers used and the ampliﬁcation conditions are previously described (12).

DNA sequence analysis

We ligated gel-extracted PCR products into a TA cloning vector (Promega, Madison, WI), generated bacterial clones, then puriﬁed plasmid DNA from overnight bacterial cultures. Plasmid DNA was digested with restriction enzymes to identify clones with proper ligation of V regions. The nucleotide sequences of both strands of plasmid DNAs that contained a VH or a VL insert were determined using an automated DNA sequencer (Applied Biosystems, Foster City, CA).

Criteria for the analysis of Ab sequences

We analyzed Vh4 or Vl4 region sequences using DNAPlot to search VBASE (http://www.mrc-lincoln.ac.uk/immunogenetics/Immunogenetics.html) or the international ImMunoGeneTics database (http://www.ebi.ac.uk/Immunogenetics) (18), reporting results with an up-dated nomenclature of the human Ig genes as recently summarized (19). The characteristics of individual sequences for Vh4 and Vl4 regions are shown in the supplemental material. H chain complementarity determining region 3 (HCDR3) and L chain CDR3 (LCDR3) lengths were determined by counting amino acid residues starting at position 105 according to the ImMunoGeneTics numbering system. D gene segment assignments were performed by database alignment, and conﬁrmed by manual inspection, using the genomic sequences of all D gene segments according to Corbett et al. (20). The assignment of D segments was deﬁned using similarity with D segment germline sequences on the basis of 100% amino acid identity over a stretch of at least three consecutive amino acids. The reading frame was deﬁned relative to the recombination signal sequence (21). For a deﬁnite assignment of a D or J segment, we required at least three consecutive amino acids to be identical to germline sequences. When two different D segment or D segment reading frame assignments were possible, based on these criteria, both segments and reading frames were used to calculate the frequency of use in this study. Two D assignments were possible in sequences from adult donors only in 11 of 100 instances. However, a D segment usage bias was not detected whether these assignments were included in the analysis or not. The number of altered amino acid residues in the N’ or C’ terminus of D segments within the HCDR3 was determined only in HCDR3 sequences in which a deﬁnite D segment assignment was possible. For the enumeration of mutated or added amino acid residues in the HCDR3 or LCDR3 regions, we included both non-templated (N) and palindromic (P) nucleotides if these nucleotides encoded amino acids that differed from germline. Alterations that resulted solely in the loss of amino acids without mutated replacements were not included in the “Altered Amino Acids” category. However, such events would contribute to changes in CDR3 lengths, which were calculated separately. Insertions and deletions within the CDR3 were counted as changes if they resulted in altered amino acids in the CDR3.

Statistical analysis of genetic data

Fisher’s exact tests were used to examine overall differences in proportions of RV VP6- and VP7-speciﬁc frequencies of VH4, D, Jg, family use, Vh41 gene segments, and VK, Jk, VA and JA family use in random or RV-speciﬁc human B cell clones from adults and infants. Comparisons of LCDR3 and HCDR3 amino acid lengths were performed using ANOVA techniques. Logistic regression was used to examine the proportion of D assignments possible number of VH gene segments and the proportion of an L chain used. Exact logistic regression techniques were required for analysis of the number of Jg segments containing mutations because of the relatively small number of Jg assignments possible. All analyses were performed using SAS version 8.2 (SAS Institute, Cary, NC).

Expression and puriﬁcation of recombinant Abs

Detailed methods for the expression and puriﬁcation protocols used were previously described (12). Brieﬂy, RV-speciﬁc VH4 and VL4 segments were ligated in step-wise fashion into the linearized pcDNA3×A19 vector that contains both CH1 and C regions in-frame with the VH4 and VL4 inserts. The sequences of the constructs were veriﬁed by nucleotide sequence analysis and transformed into nonsuppressor strain HB2151 Escherichia coli cells to produce soluble Fab. To express the Fab Abs, a fresh bacterial colony containing a Fab phagemid was inoculated into 2XYT broth supplemented with 100 μg/ml ampicillin and 2% glucose and grown overnight at 37°C. The overnight culture was then diluted 1/10 into fresh medium and grown until it reached an OD600 of 0.6. Protein expression was induced by resuspension in fresh 2XYT broth supplemented with ampicillin and 50 μM isopropyl β-D-thiogalactoside. The culture was grown at 30°C for 3 h and then cells were collected by centrifugation. To harvest the Fab, the periplasm was extracted by addition of TES buffer (10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 0.5% EDTA, 1% SDS) and measured in the periplasmic extract using a Ni-TA agarose system (Qiagen, Valencia, CA). The eluted puriﬁed Fab products were then dialyzed against PBS. The quantity of puriﬁed Fab in each preparation was determined by a sandwich ELISA in which Fab concentrations were determined from comparison of experimental OD values to that of varying dilutions of a puriﬁed Fab standard (The Jackson Laboratory, West Grove, PA).

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5 The on-line version of this article contains supplemental material.
To verify that the recombinant Fab Abs using recombinant Abs

Specific binding of recombinant Abs to RV-infected cells by

immunofluorescence

We next demonstrated that recombinant human Fab using each of three immunodominant VH gene segments bound to authentic RV proteins in infected cells but not to uninfected cells. Virus-infected cells used in the immunofluorescent staining procedure were obtained by infecting MA104 cell culture monolayers in 96-well tissue culture plates with 100 µl of serially 10-fold diluted suspensions of virus strain RRV after trypsin activation using 20 µg of trypsin/ml. Virus was adsorbed by centrifugation at 1000 × g at room temperature for 1 h. Wells were washed once with serum-free DMEM, and then 100 µl of serum-free DMEM containing 4 µg of trypsin/ml was added. The plates were incubated at 37°C for 15 h, after which the culture medium was removed by aspiration. The cells were fixed with 150 µl/well of ice cold 80% acetone for 20 min at −20°C. Acetone was removed and the wells were dried completely. Mock-infected (negative control) cell monolayers were processed in the same fashion. The staining reagent used as a positive control (RV-specific guinea pig serum, kindly provided by Dr. R. Ward, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH), was diluted 1/800 in 5% nonfat dry milk, added to the wells, and then incubated for 1 h. The wells were washed with PBS and an anti-guinea pig Ig Alexa 568 conjugate (Molecular Probes, Eugene, OR) was added at 1/500 in 5% nonfat dry milk for 1 h. Wells were washed again and fluorescence was visualized. The staining procedure for the recombinant Fabs was similar. The purified Fabs were diluted 1/4 in 5% nonfat dry milk and incubated for 1 h. After washing with PBS, a mouse anti-hemagglutinin tag Ab (Sigma-Aldrich) was diluted 1/500 in 5% nonfat dry milk for 1 h. A similarly prepared recombinant human Ab to respiratory syncytial virus with the same Ag tags was used in replicate wells as a control to rule out nonspecific binding of the Fab preparations (data not shown). Fluorescent foci were visualized on a Nikon Eclipse TE300 fluorescence microscope (Melville, NY). Photomicrographs were obtained at dilutions for each virus strain that resulted in large numbers of separated infectious foci.

Results

The number of RV-specific B cells analyzed

The number of RV-specific B cells sorted and the yields of ELISA and RT-PCR methods are shown in Table I. We calculated the overall efficiency of amplification of variable gene sequences from Ig-positive and Ag-positive ELISA wells as a percentage of total sorted B cells for each Ag and donor population.

Infants (donors 1–4). This method generated a total of 11 VP7-specific H chain, 11 VP7-specific L chain, 3 VP6-specific H chain, and 2 VP6-specific L chain V region Ab genes from RV-infected infants for analysis. The overall efficiency for RV VP7-specific VH

Table 1. Origins of RV-specific B cell clones and number of gene segments analyzed for each donor group and RV Ag

<table>
<thead>
<tr>
<th>Donor Group</th>
<th>RV History</th>
<th>Yield of Cell Sort</th>
<th>Wells Positive in ELISA Indicated (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield of Cell Sort</td>
<td>Ig</td>
</tr>
<tr>
<td>A</td>
<td>RV-infected infants&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (2 mo, F, C)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 (3 mo, F, C)</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (7 mo, F, C)</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 (11 mo, F, AA)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total infants</td>
<td>2359</td>
</tr>
<tr>
<td>B</td>
<td>Recently RV-exposed adults&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5 (35 years, M, AA)</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 (28 years, F, C)</td>
<td>0.6</td>
</tr>
<tr>
<td>C</td>
<td>Healthy adult donors&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7–12 (ns)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>0.05 (0.03–0.07)</td>
</tr>
<tr>
<td>D</td>
<td>Healthy adult donors&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13 + 14 (ns)&lt;sup&gt;k&lt;/sup&gt;</td>
<td>0.5&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>Randomly selected B cells&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15–17 (ns)&lt;sup&gt;j&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total healthy adults</td>
<td>6130</td>
</tr>
</tbody>
</table>

<sup>a</sup> Percentage of RV ELISA-positive B cell clones that generated a positive RT-PCR product for the VH and VL region, respectively.
<sup>b</sup> Gender: F = female; M = male. Ethnicity: C = caucasian; AA = African American.
<sup>c</sup> Indicates percentage of cells with positive GFP fluorescence compared to all cells with positive CD19 PE fluorescence during the single cell sort.
<sup>d</sup> Supernatants of clones that were both RV VP6- and RV VP7-positive in the ELISA were considered RV VP6-specific, those that were positive for RV VP7 only were considered RV VP7-specific (see also Materials and Methods).
<sup>e</sup> Indicates the number of B cell clones from which both the VH and VL regions were available for sequence analysis.
<sup>g</sup> Donors 1–4, RV disease was confirmed by positive Ag test and characterized by fever, vomiting, and diarrhea in the time of blood draw.
<sup>f</sup> Donors 5 and 6 were exposed to RV and reported symptoms consistent with RV disease within 1 mo of blood draw; donor 5 is the father of donor 4.
<sup>g</sup> Donors 7–17 were healthy adult blood donors at the American Red Cross.
<sup>j</sup> ns, not specified, because donor-specific identifiers associated with Red Cross donations were not released.
<sup>k</sup> Mean (range).
<sup>l</sup> B cells from two donors were pooled for this cell sorting experiment.
<sup>m</sup> Thirty-eight percent of CD19<sup>+</sup> cells were IgD<sup>+</sup>. Of those, 0.5% was also RV VLP-GFP<sup>+</sup>.
<sup>ae</sup> NA, not applicable.
segments was 11 confirmed clones amplified of 2359 B cells sorted (0.5%) and for RV VP6-specific V_H segments 3 confirmed clones amplified from 2359 sorted B cells (0.1%). The efficiency data for RV VP7- or VP6-specific V_L segments were 11 of 2359 sorted B cells (0.5%) and 2 of 2359 sorted B cells (0.1%), respectively. Infant B cells did not appear to proliferate or differentiate in culture as well as adult B cells in this study. The decreased proliferation likely reflects the fact that some infant samples were rested in vitro for several hours or overnight, because those patients presented to the emergency department at a time of day when sterile flow cytometry sorting services were not immediately available. In contrast, all adult cell selections were performed with cells immediately after phlebotomy. It is possible that the difference in efficiency between adults and infants may be due to intrinsic limitations in the ability of infant B cells to proliferate in culture. However, subsequent experiments for the expansion of neonatal or young infant B cells of another specificity that were performed only with freshly isolated cells showed no difference in efficiency of proliferation and differentiation of infant and adult cells (data not shown).

**Adults (donors 5–17).** The frequency of isolated RV VP6- or VP7-specific sequences is shown in Table I. The frequency of RV-specific B cells was low in healthy adult volunteers who were not recently infected (5 in 10,000 B cells). In contrast, RV-specific cells were found at an ~10-fold higher frequency in infants or adults who were recently infected, or in IgD− cells of healthy volunteers. The recovery of RV VP7-specific V_H and V_L segments following expansion was four to five times more efficient in recently RV-exposed adults than in healthy adult blood donors or RV-infected infants. We analyzed a total of 30 RV-specific V_H and 39 RV-specific V_L segments from recently RV-exposed adults (donors 5 and 6) and 62 RV-specific V_H and 46 RV-specific V_L segments from healthy adult blood donors (donors 7–14). For the control group (donors 15–17), a total of 83 V_H and 46 V_L segments from randomly selected (not virus-specific) B cell clones was analyzed. For the analysis of somatic mutations in the CDR1 and CDR2 and the three FR regions in randomly selected B cell clones, 77 V_H segments were examined.

The repertoire in randomly selected adult B cells was V_H3-dominated

We first expanded randomly selected CD19+ B cells from three healthy donors and determined the nucleotide sequences of V_H segments from 83 B cells. In randomly selected B cells, 50% of all V_H segments belonged to the V_H3 family (Fig. 1, A and B), a frequency that was very similar to the V_H frequencies of directly amplified randomly selected B cells reported by others (20, 22). The distribution of D and J_H gene segment use in randomly selected B cells (Fig. 1, C and D) was similar to that described previously (20, 22). As shown in Fig. 2A, the most frequently observed V_H segment in randomly selected B cells in our study was V_H3–23 (average of 10% (range 3–19%)). These data show that the culture amplification method used does not significantly bias or alter the frequency of V_H segments recovered.

The Ab repertoire of RV-specific B cells in adults was V_L4-dominated

We found that RV-specific B cells exhibited a strong V_H bias that differed from that of randomly selected B cells. Forty-one percent (11 of 27) of RV VP7-specific B cells from recently RV-exposed and 50% (8 of 16) of RV VP7-specific B cells from healthy blood donors expressed V_H gene segments belonging to the V_H4 family. The frequency of all VP7-specific adult clones belonging to V_H4 was 44% (19 of 43; Fig. 1A). We also observed a marked V_H4 bias for RV VP6-specific B cells with a frequency of 71% (22 of 31; Fig. 1B). This difference was statistically significant for both RV VP7- and VP6-specific clones in comparison with the 20% (17 of 83) V_H4 frequency in randomly selected, nonspecific B cells ($p = 0.030$ and $p < 0.001$, respectively). Therefore, an overall V_H4 family bias was observed in RV-specific clones (Fig. 1, A and B). In addition to the V_H4 family predominance, the most frequently used individual V_H segment in VP7-specific adult B cells overall was segment V_H1–46 (14%, 6/43) (Fig. 2A). The most frequently used V_H segment in RV VP6-specific adult B cells was V_H4–31 (23%, 7/31) (Fig. 2B). These V_H segments were used infrequently in randomly selected B cells. Thus, adult RV-specific B cells

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Table I. Continued

| Number of H Chain or L Chain Ab Sequences Analyzed for Each RV Ag from Indicated Donor |
|------------------------------------------|---------------------------|---------------------------|
| Wells positive by RT-PCR (%) | RV VP7 | RV VP6 |
| V_H | V_L | Pairs' | V_H | V_L | Pairs' |
| 43 | 29 | 4 | 3 | 2 | 0 | 0 | 0 |
| 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| 67 | 100 | 2 | 1 | 1 | 1 | 1 | 1 |
| 47 | 47 | 11 | 11 | 8 | 3 | 2 | 2 |
| 53 | 50 | 17 | 16 | 9 | 0 | 0 | 0 |
| 69 | 54 | 10 | 13 | 7 | 3 | 2 | 2 |
| 50' (8–85) | 61' (9–100) | 2.7' (0–5) | 3.3' (0–10) | 2' (1–4) | 4.7' (1–9) | 3.3' (1–6) | 3' (0–6) |
| 57 | 68 | 16 | 20 | 12 | 28 | 20 | 18 |
| NA | NA | NA | NA | 16 | 8 | 6 | 46 |
| 46 | 38 | 21 |
showed a strong V\textsubscript{H}1/V\textsubscript{H}4 bias that greatly differed from the random V\textsubscript{H}1 usage.

**RV-specific B cell clones from infants also demonstrated a V\textsubscript{H}1/V\textsubscript{H}4 bias and a relative paucity of V\textsubscript{H}3 family usage**

The relative underrepresentation of V\textsubscript{H}3 family members in RV-specific adult B cells compared with randomly selected B cells was also observed in infant B cells. The V\textsubscript{H}4 bias in infants was similar to that in adults, with 44 vs 36% V\textsubscript{H}4 (for RV VP7) or 71 vs 67% V\textsubscript{H}4 (for RV VP6) in adults vs infants, respectively. As shown in Fig. 1A, 55% (6 of 11) of RV VP7-specific V\textsubscript{H}1 segments in infants belonged to the V\textsubscript{H}1 family, a strong bias that was consistent with the highly increased use of segment V\textsubscript{H}1–46 in RV VP7-specific V\textsubscript{H}1 segments in adults (Fig. 2A).

Infants and adults both showed a frequent usage of the V\textsubscript{H}1–46 segment in RV VP7-specific B cells

The V\textsubscript{H}1–46 gene segment, rarely used in randomly selected B cells (0 of 83 randomly selected adult B cells in our study), was highly overrepresented in adult RV VP7-specific B cells. Infants also used the V\textsubscript{H}1 segment 1–46 more often than by chance alone, with 2 of 11 (18%) infant RV VP7-specific B cell clones using that segment (Fig. 2A). The V\textsubscript{H}1–46 segment was not overrepresented in randomly selected B cells in previously reported second trimester liver or cord blood cDNA libraries (9). These data suggest that the V\textsubscript{H}1–46 segment is a dominant component of both adult and infant RV-specific responses. Other dominant V\textsubscript{H}4 gene segments were observed in RV VP6- or VP7-specific B cells in adults, such as V\textsubscript{H}4–31, V\textsubscript{H}4–61, V\textsubscript{H}4–39, and V\textsubscript{H}4–30–4 (Fig. 2).

The distribution of RV-specific D and J\textsubscript{H} gene segments was similar in adults and infants

We analyzed in detail the D and J\textsubscript{H} gene segment usage in all populations, but significant differences between groups were not detected. In nonspecific adult B cells or RV-specific B cells from adults or infants, we observed a uniform bias toward the D3 and J\textsubscript{H}4 families (Fig. 1, C and D). These data are consistent with previously identified usage frequencies of randomly selected B cells in other studies.

Infants and adults shared a similar distribution of Ab L chain gene segments in RV-specific B cells

We analyzed 11 RV VP7- and 2 RV VP6-specific L chain sequences from RV-infected infants. These sequences were compared with 53 RV VP6- or VP7-specific L chain sequences from recently RV-exposed or healthy adults, 24 RV VP6-specific L chain sequences from adult memory (IgD\textsuperscript{−}) B cells, and 46 L chain sequences from randomly selected B cells. The distribution

**FIGURE 1.** Frequency of V\textsubscript{H}, D, or J\textsubscript{H} family use in random adult cells (●, donor group E) or RV-specific clones from adults (□, donor groups B, C, and D combined) or infants (■, donor group A). The number of B cell clones and donors for each of the groups analyzed is shown in Table I. Comparisons in proportions for these figures were done by using the Fisher’s exact test statistic for RxC contingency tables: comparison between randomly selected B cells and RV VP7-specific (A) B cells from adults p = 0.03. Comparison between randomly selected B cells and RV VP6-specific (B) B cells from adults p < 0.001. RV VP6-specific B cells from infants and randomly selected B cells p = 0.01. There was no statistically significant difference between groups in D family (C) or J\textsubscript{H} family (D) use for RV VP7- or VP6-specific B cells. C and D, Combined sequence data from RV VP7- or VP6-specific B cells are shown. Individual V\textsubscript{H} sequences are shown in the supplemental material.

**FIGURE 2.** Dominant V\textsubscript{H} gene segment use in RV-specific human B cell clones from adults or infants. The relative frequency of three dominant V\textsubscript{H} gene segments in RV VP7-specific (A) or RV VP6-specific (B) B cells from adults (□) or infants (■) are plotted in comparison with the frequency of use of these V\textsubscript{H} gene segments in random B cells (●). The frequency of use of the V\textsubscript{H}3–23 segment (the dominant segment in randomly selected B cells) is shown for comparative purposes. Individual V\textsubscript{H} sequences are shown in the supplemental material.

**FIGURE 3.** L chain use in random or RV VP6- or VP7-specific human B cell clones from adults or infants. Relative frequencies of the V\textsubscript{L} (A), J\textsubscript{L} (B), V\textsubscript{A} (C), and J\textsubscript{A} (D) family usage in RV VP6- or VP7-specific B cells from adults (□) or infants (■) were plotted against randomly selected B cells from three unrelated donors (●). Comparisons in proportions for these figures were done by using the Fisher’s exact test statistic for RxC contingency tables. The only statistically significant difference found was in C in the comparison between randomly selected B cells and RV VP6/VP7-specific B cells from adults (p = 0.018). All other comparisons, p ≥ 0.05. Individual V\textsubscript{L} sequences are shown in the supplemental material.
of Vκ, Jκ, and JA families in randomly selected B cells did not differ from that of RV-specific B cells from infants or adults (all p values ≥ 0.05). RV-specific B cells from infants showed a trend toward overrepresentation of the Jκ family 4 in contrast to RV-specific B cells from adults and randomly selected B cells that demonstrated a Vκ family 1 bias. The VA distribution was different between randomly selected and RV-specific B cells from adults (Fig. 3C, p = 0.018), suggesting a possible minor selection bias associated with particular VA segments but a specific segment association could not be discerned.

**Junctional diversity, D segment usage, and CDR3 lengths were similar in adults and infants**

We analyzed the adult and infant RV-specific V-D and D-J junctional sequences for evidence of the functional maturity of insertion or excision mechanisms in infants (Table II). The mean frequency of mutations at the N- and C-terminal ends of D segments was similar in all donor groups. Templated P insertions were observed in both infant and adult junctional sequences. The mean amino acid length of D segments did not differ between adult and infant sequences. Similarly, we found that the mean total HCDR3 length and the mean frequency of the hydrophobic reading-frame usage for D segments did not differ between adults and infants, when analyzed as a group (all p values ≥ 0.05; Table II). Interestingly, however, all three assignable D segments of B cells derived from the youngest infant (donor 1, 2-mo-old) were encoded by the hydrophobic D reading frame, which is less commonly used in functional Abs in the expressed repertoire (23).

Similar results were found in the LCDR3 (Table III). Both the frequency of definite Jκ segment assignment and the mean amino acid length of the LCDR3 were similar in infant and adult RV-specific IgD-unselected B cells. The mean length of infant

**Table II. Comparison of RV-specific and random HCDR3s from infants and adults**

<table>
<thead>
<tr>
<th>Type of B Cells</th>
<th>Donor Group (n)</th>
<th>VH Gene Segments Analyzed</th>
<th>D Assignment Possible (%)</th>
<th>Mean Amino Acid Length of D Segments (Range)</th>
<th>Mean Number of Amino Acids Altered on N or C terminus of D (Range)</th>
<th>Hydrophobic RF Used (%)</th>
<th>Mean Amino Acid Length of HCDR3 (Range)</th>
<th>Number of JH Segments Containing Mutations (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomly selected</td>
<td>Healthy adults (3)</td>
<td>83</td>
<td>53 (64)</td>
<td>5.4 (3–10)</td>
<td>5.5 (0–15)</td>
<td>19 (36)</td>
<td>13.9 (7–21)</td>
<td>10 (12)</td>
</tr>
<tr>
<td>RV-specific</td>
<td>RV-infected infants (3)</td>
<td>14</td>
<td>13 (93)</td>
<td>5.3 (3–9)</td>
<td>5.0 (2–9)</td>
<td>4 (31)</td>
<td>14.4 (8–28)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Recently RV-exposed adults (2)</td>
<td>30</td>
<td>16 (53)</td>
<td>4.4 (3–6)</td>
<td>5.7 (4–8)</td>
<td>3 (19)</td>
<td>13.0 (7–19)</td>
<td>6 (20)</td>
</tr>
<tr>
<td></td>
<td>Healthy adults (6)</td>
<td>44</td>
<td>26 (59)</td>
<td>4.5 (3–8)</td>
<td>5.3 (2–12)</td>
<td>8 (14)</td>
<td>14.3 (8–18)</td>
<td>10 (23)</td>
</tr>
<tr>
<td></td>
<td>Healthy adults IgD⁺ B cells (2)</td>
<td>18</td>
<td>6 (33)</td>
<td>5.8 (3–9)</td>
<td>7.2 (3–8)</td>
<td>3 (50)</td>
<td>14.1 (7–19)</td>
<td>6 (33)</td>
</tr>
</tbody>
</table>

**Table III. Comparison of RV-specific and random LCDR3s from infants and adults**

<table>
<thead>
<tr>
<th>Type of B Cells</th>
<th>Donor Group (n)</th>
<th>VH Gene Segments Analyzed</th>
<th>Jκ Segment Assignment Possible (%)</th>
<th>Mean Number of Amino Acids Altered in LCDR3 (Range)</th>
<th>Mean Amino Acid Length of LCDR3 (Range)</th>
<th>k Chain Used (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Randomly selected</td>
<td>Healthy adults (3)</td>
<td>46</td>
<td>40 (87)</td>
<td>0.8 (0–3)</td>
<td>8.8 (7–11)</td>
<td>21 (46)</td>
</tr>
<tr>
<td>RV-specific</td>
<td>RV-infected infants (4)</td>
<td>13</td>
<td>11 (85)</td>
<td>0.8 (0–2)</td>
<td>9.5 (8–11)</td>
<td>5 (38)</td>
</tr>
<tr>
<td></td>
<td>Recently RV-exposed adults (2)</td>
<td>31</td>
<td>27 (87)</td>
<td>1.1 (0–3)</td>
<td>8.8 (7–12)</td>
<td>18 (58)</td>
</tr>
<tr>
<td></td>
<td>Healthy adults (6)</td>
<td>22</td>
<td>19 (86)</td>
<td>2.3 (0–6)</td>
<td>9.5 (7–14)</td>
<td>9 (41)</td>
</tr>
<tr>
<td></td>
<td>Healthy adults IgD⁺ B cells (2)</td>
<td>24</td>
<td>22 (92)</td>
<td>2.3 (0–6)</td>
<td>8.5 (7–11)</td>
<td>17 (71)</td>
</tr>
</tbody>
</table>

a Comparisons between proportions of Jκ assignments were performed using logistic regression techniques. There were no statistically significant differences between infants and any of the adult groups.

b Statistical comparisons of mean amino number of amino acids altered in LCDR3 were performed using ANOVA techniques. Comparison between RV-specific B cells from RV-infected infants and RV-specific B cells from healthy adults p < 0.001, RV-specific B cells from RV-infected infants and RV-specific IgD⁺ B cells from healthy adults p < 0.001.

c Statistical comparisons of mean LCDR3 lengths were performed using ANOVA techniques. Comparison between RV-specific B cells from RV-infected infants and recently RV-exposed adults p = 0.063, RV-infected infants and RV-specific IgD⁺ B cells from healthy adults p = 0.018.

d Using logistic regression, we did not find differences in the k chain usage between infants and any of the adult groups.
The highly significant association of particular gene sequences with RV-specific B cells, which differed from those of random B cells, suggested that these segments encoded RV-specific Abs. To verify this association, we expressed and purified recombinant Fab Abs from representative adult clones (donor groups C or D) for each of three immunodominant V_H gene segments, V_H1–46, V_H4–31, and V_H4–61 and tested whether they bound to RV Ags in infected cells or to virus particles. We found that each of the Abs bound specifically to purified RV particles, in a dose-dependent manner, using ELISA (Fig. 4). These Abs also bound to RV-infected cell monolayers in an immunofluorescent assay (Fig. 4) but not to uninfected cell monolayers (Ref. 12 and data not shown); similarly prepared Abs to another virus did not bind RV-infected monolayers (Ref. 12 and data not shown). These studies showed that the physical sorting, ELISA binding confirmation, and molecular cloning methods used to determine immunodominant gene segments that were amplified from putative RV-specific B cells actually did specify RV-specific Abs.

### Discussion

It is of major interest to determine whether infant Ab repertoire diversity remains limited during the first year of life, when exposure to many wild-type virus infections or vaccines occurs. A limited amount of information is available on human Ab repertoires in infants. The bulk of studies to date have examined unselected immune repertoires. This study is the first to define an antiviral Ab repertoire in human infants at a detailed molecular level with sufficient numbers of Ag-selected Ab genes to perform rigorous statistical analysis. Our finding is that infant virus Ag-specific Ig repertoires exhibit a high level of combinatorial and junctional diversity similar to that of adults.

### RV-specific repertoire in infants

We showed that human infants use sufficiently diversified Ab repertoires to make adult-like Ag-specific B cell responses. The combinatorial diversity of infant RV-specific clones was similar to that of adults, in that they exhibited a V_H1 and V_H4 dominance. Infants shared a high frequency of use of the dominant V_H segment used by adults, V_H1–46. Thus, the analysis of the V_H family and V_H segment use suggests that infants share the strong Ag selection bias seen in adults. It was previously speculated that infant combinatorial diversity was restricted, reflecting repertoire skewing similar to that noted in fetal repertoires. Such repertoire restriction has been hypothesized to limit the structural and functional quality of human infant Abs. Our data show that combinatorial diversity did not appear to be limiting for RV-specific responses in these four infants who ranged from 2 to 11 mo of age. This finding suggests that improved immunogens or multiple antigenic exposures might induce Ab responses with enhanced protective capacity in this age group. Previous work on random B cell repertoires suggests that variable gene repertoire biases noted during fetal life may resolve during the first few months of life. Therefore, future studies of virus-specific responses following fetal or neonatal infection are warranted. Nevertheless, given that disease caused by RV infection occurs predominantly in the age group that we studied, the lack of the repertoire limitations in the RV-specific response that we describe in the age group studied is significant.

### RV-specific repertoire in adults

To compare infant and adult Ag-specific repertoires, we first determined the dominant V_H gene segments used by adults in the response to RV VP6 and VP7 proteins, which were found to be V_H1 and V_H4 family members. For RV VP7-specific B cells, the predominant gene segment used was V_H1–46 in both adults and infants. Segment V_H1–46 was observed in only 1 of 31 RV VP6-specific IgD-unselected B cell clones from adults and was not observed in any of the nonspecific B cell clones, and thus may represent an important variable gene for RV VP7-specific Ab
responses. However, 6 of 18 IgD− RV-specific clones obtained by sorting with VP2/6 VLPs also used the V_{41}−46 segment. This finding suggests that the use of this segment may also be associated with VP6 binding if somatic hypermutations are present. This possibility is supported by the apparent binding of an Fab expressed from cDNA obtained from a VP2/6-sorted V_{41}−46 Ab to VP2/6 double-layered particles in ELISA (see the V_{41}−46 Fab in Fig. 4). There is limited previous data to support the association of particular V_{41} segments with preferential binding to Ags, such as the *Haemophilus influenzae*, *Streptococcus pneumoniae*, or *Cryttopoccus neoformans* polysaccharides (24–28). All of these associations of polysaccharide Ags with particular V_{41} gene segments were made with V_{32}−origin Abs, mostly with the V_{32}−23 segment that is also the dominant segment in randomly selected B cells.

We found a strong association of RV specificity with V_{41} and V_{44} segments. The only other published sequences of human RV-specific Abs were derived from phage display combinatorial libraries (29). In that work, investigators described two RV VP6-specific libraries with distinct epitope recognition, one of which derived from the 4−31 germline gene. Although our methods were different, it is interesting that we also identified V_{41}−31 as a dominant V_{41} segment in RV VP6-specific B cell clones. In contrast to our finding of V_{41} and 4 associations with RV specificity, we did not find a particular association of D, J_{H}, V_{κ}, J_{κ}, V_{λ}, or λ segment usage in RV-specific clones that was shared by adults and infants.

**Junctional diversity is similar in adult and infant RV-specific Abs**

Junctional diversity, representing P or N insertions, the latter mediated by TdT, or excision events, were observed at similar frequencies in infants and adults. Both populations used CDR3s of similar lengths to make RV-specific Abs, consistent with recent analyses of random B cell HCDR3 length in which premature infant B cell repertoires showed progression of HCDR3 length during the third trimester (30). Infants appear to achieve maturation of HCDR3 length in random B cells by 2 mo of age (11), and we show the same applies to Ag-selected B cells. Marked developmental differences have been noted in the past between fetuses and adults in these characteristics. Adults use larger Ag binding site structures (specified by longer HCDR3s) than those used in fetal tissues or early in infancy (11, 31, 32). Fetal B cells use short D segments and a paucity of N additions (11, 33, 34). In human fetal or cord blood B cells, N-region addition can be observed but only at low frequency, and these additions increase with age (11, 32, 35, 36). CDR3 length is of interest because longer D and J_{H} segments lead initially to more complex HCDR3 regions (9), and previous studies suggest high affinity is linked to optimal CDR3 length (37). Previous investigators showed that human fetuses and preterm infants preferentially use short D segments (11, 31, 32, 35). Infants did not use unusually short D segments or high frequencies of the hydrophobic or germline stop reading frames to encode virus-specific Abs in our study, except for the clones derived from the youngest infant (donor 1, see supplemental Table S II). We also analyzed junctional diversity in RV-specific L chain genes. We found that infant sequences differed from those of RV-specific adult IgD− B cells only in that the infant sequences were one amino acid longer, a minimal difference. Thus, maturity of the mechanisms mediating junctional diversity, D segment selection, and CDR3 diversification were not limiting in infants. Our studies cannot rule out limited diversity mediated by these mechanisms in neonates (the first month of life), because the infant B cell clones we obtained were from subjects of at least 2 mo of age. Nevertheless, given that disease caused by RV infection occurs predominantly in the age group that we studied, the lack of these repertoire limitations in this age group is significant.

In conclusion, we determined the molecular basis of human B cell responses to RV, and found an unusual V_{41} and V_{44} family dominance, with particular segments within these families especially preferred. We demonstrated that human infant antiviral responses are not limited by repertoire skewing or lack of junctional diversity. In this regard, our data show that infant B cell repertoires, in contrast to previous speculation, are much closer in character to adult repertoires than fetal repertoires.

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**References**


