Oligoclonal IgA Response in the Vascular Wall in Acute Kawasaki Disease

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Oligoclonal IgA Response in the Vascular Wall in Acute Kawasaki Disease

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Kawasaki Disease (KD) is an acute vasculitis of young childhood which may be fatal. KD is the leading cause of acquired heart disease in children in developed nations (1), but little is known about the pathogenesis of the disorder. The clinical features of KD include prolonged high fever, rash, conjunctival injection, erythema of the oral mucosa, swelling and redness of the hands and feet, and cervical adenopathy. These acute features generally resolve within 1–3 wk after the onset of illness; however, ~20% of untreated patients develop coronary artery aneurysms (1). The majority of KD patients who are treated within the first 10 days of illness with i.v. gammaglobulin and aspirin show a rapid resolution of fever and inflammatory signs, and this treatment reduces the prevalence of coronary artery abnormalities to ~5% (2). The mechanism of action of i.v. gammaglobulin in KD is unclear. The symptoms of KD resolve over several weeks, even in untreated patients, and recurrences are unusual, distinguishing the illness from autoimmune vasculitic disorders. The sequelae of undiagnosed KD during childhood appear to account for cases of acute myocardial infarction or sudden death in young adults (3). Because the etiology of KD is unknown, no diagnostic test is available, specific therapy cannot be developed, and prevention is not feasible.

Clinical and epidemiologic features of KD strongly suggest an infectious etiology (1). These include the young age group affected, the clinical features of the illness, the occurrence of epidemics with periodicity, and the geographic wave-like spread of illness during epidemics (1). To date, traditional methods to identify a microbial agent have failed to clarify the etiology of KD. Recently, there has been interest in a superantigen etiology of KD based upon possible selective expansion of Vβ2 and Vγ8 T cell receptor families in peripheral blood in acute KD (4, 5); however, other investigators have been unable to confirm this finding (6–10). Toxic shock syndrome toxin-1 or streptococcal superantigens were proposed to be related etiologically to KD based upon a single study (11) that has not been confirmed by others (7, 8, 12–14).

We have reported the novel finding that IgA plasma cells infiltrate the vascular wall during acute KD (15). In this study, we examined the clonality of the IgA response in KD vascular tissue by sequencing the CDR3 regions of α genes isolated from vascular tissues from three fatal acute KD patients. We report that the IgA produced in acute KD vascular tissue is oligoclonal, consistent with an Ag-driven immune response.

Materials and Methods

Analysis of α genes from a KD vascular cDNA library

KD vascular cDNA library. Vascular tissue (750 mg) from the aorto-iliac junction was used to isolate total RNA by the guanidium thiocyanate-phenol method (16), and an aliquot of the total RNA was used to isolate poly A⁺ RNA using Dynabeads oligo dT (Dynal, Oslo, Norway). The tissue was obtained from patient A, a 10-year-old Caucasian male with acute KD who died from a ruptured coronary artery aneurysm on day 13 of illness (15). An oligo dT-primed, directional cDNA library in the phage expression vector λZAP was synthesized using a cDNA synthesis kit (ZAP-cDNA synthesis kit; Stratagene, La Jolla, CA) as previously reported (15). The primary, unamplified cDNA library was used to isolate α clones for sequencing of the VDJ junctions.

Isolation of α clones. α clones were identified from plates of the primary library by hybridization with an α probe. Initial filter lifts were hybridized with a V₄⁺-Cα probe generated by RT-PCR of control spleen RNA as
Previously described (15), in which products of six separate PCR reactions using primers from the six leader sequences of the human heavy chain variable regions with a primer from the constant region of \( \alpha \) (17, 18) were mixed (Table I). Only five of 11 clones initially identified using this probe contained variable region. Because use of this probe did not obviate the isolation of clones containing the constant region of \( \alpha \) only, we incubated additional filter lifts of primary library with an \( \alpha \) constant region probe derived from a plasmid preparation of a cDNA library clone. PCR amplification of isolated plaques was then performed using a primer in the \( \beta \) variable region (Table I) and a primer in the 5'-3' direction of isolated plaques was then performed using a primer in the constant region of \( \alpha \) DNA sequencing. As a control source for the amplification of a polyclonal population of \( \alpha \) clones, we incubated each of the \( \alpha \) clones with a germline sequence by comparing the sequence data with published human germline sequences. These clones are not included in the list of \( \alpha \) clones identified as they were not isolated from the primary library.

### Table I. Probes to identify a variable region size and primers used for sequencing and to perform RT-PCR on paraffin-embedded, formalin-fixed KD tissues

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide Sequence (5'-3')</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co8</td>
<td>CTGGGATTCGTTAGTGGCCCTT</td>
<td>constant alpha primer&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SK</td>
<td>CGCTCTAAGACTTAGGTC</td>
<td>pBluescript SK primer</td>
</tr>
<tr>
<td>CoS</td>
<td>AGGCGTACACCAGCTATCCCAT</td>
<td>constant alpha sequencing primer</td>
</tr>
<tr>
<td>AR1</td>
<td>CTCCACACCTGCTGCTGGA</td>
<td>VH4-group 1 CDR3 probe</td>
</tr>
<tr>
<td>V(_{\alpha1})</td>
<td>CCATGACTGGACCTGG</td>
<td>VH1 and VH7 leader primers&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha2})</td>
<td>ATGGACATCTTTGGTCTCCAC</td>
<td>VH2 leader primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha3})</td>
<td>CCATGGGAATTTGGGCTGAGC</td>
<td>VH3 leader primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha4})</td>
<td>ATGAAACACCTGTTGGTCTT</td>
<td>VH4 leader primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha5})</td>
<td>ATGGGGTCACCCGCGACCT</td>
<td>VH5 leader primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha6})</td>
<td>ATGTCTGTCTTCTCTTCAT</td>
<td>VH6 leader primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V(_{\alpha}93)</td>
<td>MGATTCACCATCTCMAGRG</td>
<td>VH3 framework 3 primer</td>
</tr>
<tr>
<td>V(_{\alpha}94)</td>
<td>CGAATCCACTRCTCMGTAGAC</td>
<td>VH4 framework 3 primer</td>
</tr>
<tr>
<td>J(_{H1,2,4,5})</td>
<td>JH1,2,4,5 ATGTCTGTCTCCTCCTCAT</td>
<td>JH1.2,4,5 primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>J(_{H3})</td>
<td>TACCTGAAGAGACGGTGACC</td>
<td>JH3 primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>J(_{H6})</td>
<td>ACCTGAGGAGACGGTGACC</td>
<td>JH6 primer&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ref. 17.
<sup>b</sup> Ref. 18.

### Table II. Distribution of \( \alpha \) genes in KD cDNA library by \( \alpha \) family

<table>
<thead>
<tr>
<th>( \alpha ) Family</th>
<th>No. of Clones</th>
<th>Percentage of Total</th>
<th>Percent Detected in Adult Peripheral Blood B Cells&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>V(_{H1})</td>
<td>3</td>
<td>7</td>
<td>13-19</td>
</tr>
<tr>
<td>V(_{H2})</td>
<td>9</td>
<td>0</td>
<td>2-4</td>
</tr>
<tr>
<td>V(_{H3})</td>
<td>17</td>
<td>40</td>
<td>56-56</td>
</tr>
<tr>
<td>V(_{H4})</td>
<td>14</td>
<td>33</td>
<td>20-22</td>
</tr>
<tr>
<td>V(_{H5})</td>
<td>2</td>
<td>5</td>
<td>4-6</td>
</tr>
<tr>
<td>V(_{H6})</td>
<td>2</td>
<td>5</td>
<td>1-2</td>
</tr>
<tr>
<td>V(_{H7})</td>
<td>4</td>
<td>10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0-2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Refs. 18 and 22
<sup>b</sup> Significantly different from adult control (\( p = 0.05 \)) as determined by Fisher’s exact test.

**Analysis of \( \alpha \) genes from formalin-fixed, paraffin-embedded coronary artery blocks**

### Tissue blocks.
Coronary artery tissue blocks were obtained from patients B (11-month-old Caucasian male) and C (4-mo-old Caucasian female), two fatal KD cases in which death occurred within 2 mo of onset of the illness. Patient B was not treated with l.v. gammaglobulin because the diagnosis of KD was not made prior to death. Patient C received i.v. gammaglobulin therapy. Ten 8-\( \mu \)m sections from each block were processed for RNA isolation. As a control source for the amplification of a polyclonal population of \( \alpha \) genes, ten 8-\( \mu \)m sections of paraffin-embedded, formalin-fixed spleen tissue from patient A were also obtained for RNA processing.

### RNA isolation.
Ten 8-\( \mu \)m sections were processed for RNA isolation by first deparaffinizing with xylene, and washing the tissue with 95% ethanol. The tissue sections were resuspended in digestion buffer (1% SDS, 0.1 M Tris-HCl, pH 7.3, 25 \( \mu \)M EDTA, and 1 mg/ml proteinase K) and incubated at 50°C for 12 h with agitation. After phenol-chloroform extraction and ethanol precipitation, the nucleic acid pellet was resuspended in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% N-lauryl sarcosine, 0.1 M mercaptoethanol) and incubated at room temperature for 24 h. After phenol-chloroform extraction and ethanol precipitation, the pellet was treated with 20 U of DNase I and incubated at 37°C for 30 min, followed by repeat extraction, precipitation, and resuspension of the pellet in water for use in RT-PCR.

**RT-PCR.** One-tenth volume of the isolated RNA was incubated with 120 pm random hexamers (Amersham Pharmacia, Piscataway, NJ) for 10 min at 70°C, and first-strand buffer (Life Technologies), 500 \( \mu \)M dNTP, 10 mM DTT, 27 U RNAguard (Pharmacia), and 200 U Superscript II (Life Technologies) were added. The reaction was incubated at room temperature for 10 min, followed by 1 h at 42°C. PCR was performed in a standard reaction (19) using a primer in the constant region of \( \alpha \) and a primer in the third framework region of V\(_{\alpha3}\) or V\(_{\alpha4}\) (Table I) for 40 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, and extension at 72°C for 2 min. A semi-nested PCR amplification followed, using the third framework primer and three J region primers (Table I) for 40 additional cycles using the same cycling parameters. Pfu Turbo (Stratagene) was used to generate PCR products instead of Taq polymerase, to minimize copying errors. After performing runoff reactions as described below, PCR products were cloned into PCR Script (Stratagene) according to the manufacturer’s instructions, and clones were sequenced as above.
Runoff reactions. To obtain a rapid overall assessment of the distribution of CDR3 sizes in the population of $\alpha$ genes amplified from the tissues, we adapted a method previously reported for determining the CDR3 sizes in a population of T cell receptor genes (20). This method uses a fluorescent dye-labeled primer for a single cycle of primer annealing and extension of the PCR products previously generated as above.

**FIGURE 1.** Clonally related $\alpha$ sequences from vascular cDNA library of KD patient A, showing evidence of somatic mutation. A, Nucleotide sequences of the five clones that are members of the KD patient A VH4-group 1 set of sequences, and of the related germline sequence VH4.18 (21). Clone E2 is full-length. The other four clones (5-1, 7-1a, A2, and C5v) are not full-length; the length of each is indicated in the figure.

B, Amino acid sequences of the KD VH4-group 1 clones

<table>
<thead>
<tr>
<th>Variable region (FR3)</th>
<th>CDR3 sequence</th>
<th>N</th>
<th>DN1</th>
<th>N</th>
<th>JH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2, 5-1, 7-1a, C5v</td>
<td>STNQVSLNRLRVSVAADTAPYYCAR</td>
<td>RP</td>
<td>AAGG</td>
<td>RG</td>
<td>NRYPRY</td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C, KD VH4-group 2 (clones C2 and 4-5) and related germline sequence DP-65

<table>
<thead>
<tr>
<th>Variable region (FR3)</th>
<th>CDR3 sequence</th>
<th>N</th>
<th>DL2-like N</th>
<th>JH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D, Amino acid sequences of KD VH4-group 2 clones

<table>
<thead>
<tr>
<th>Variable region (FR3)</th>
<th>CDR3 sequence</th>
<th>N</th>
<th>JH4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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the runoff reaction were analyzed on a DNA sequencer (PE Biosystems 377XL, ABI Prism, Genescan 3.0, run mode 36C-2400, well-to-read distance 36 cm). The runoff products migrate by size, which depends on the length of the CDR3 region(s) amplified. Size standards were made by performing runoff reactions on PCR products of α genes of known CDR3 size using a primer labeled with a different fluorescent dye. Size standards were run in the same well with the sample to be assayed. The primers used were designed at the start of the third framework region (Table I). Primers for samples were labeled with 6-FAM dye, and primers for size standards were labeled with HEX dye.

Results
To determine if the IgA response in the KD vascular wall was polyclonal or oligoclonal, we isolated α gene sequences from vascular tissues from three fatal KD cases. First, we isolated α sequences from a primary, unamplified cDNA library derived from nonfixed KD vascular tissue (15). Because additional unfixed KD vascular tissues were not available, we isolated α sequences by RT-PCR from formalin-fixed, paraffin-embedded vascular tissues from two additional fatal KD cases.

Analysis of α gene sequences from the primary, unamplified KD vascular cDNA library
The primary library titer was 2.5 × 10^6 plaques, with >90% recombinants and 0.1% β-actin clones. The library has at least 0.1% immunoglobulin clones detected by immunoscreening with 125I-labeled anti-human Ig (Amersham) as previously reported (15). The dimensions of the tissue piece were approximately 5 × 3 × 30 mm (450 mm^3). The mean number of IgA plasma cells in a 10 mm × 3 mm × 0.005 mm (0.15 mm^3) piece of this tissue was 300, as determined by calculating the average of IgA-positive cells in eight separate FITC-stained sections of the tissue. Thus, a 0.005-mm tissue section contained ~2000 IgA plasma cells/mm^2. However, because the maximal diameter of a large plasma cell is ~0.025 mm, the same plasma cell might appear in up to five successive 0.005-mm sections. Therefore, the number of IgA plasma cells in the tissue more closely approximated 400 per mm^3 (2000 ÷ 5). Thus, in the original 450-mm^3 piece of tissue, there would be ~180,000 IgA plasma cells (400 cells/mm^3 × 450 mm^3).

We isolated 88 α clones from the primary, unamplified library by hybridization with an α probe as described in Materials and Methods. Forty-four of these 88 clones included the CDR3 region. Ten clones were sequenced twice, using excised phagemid and PCR, to ensure that Taq polymerase error did not introduce random mutations into the products; identical CDR3 sequence was obtained by both methods in all cases.

**Distribution of VHα genes in the KD vascular cDNA library by family.** To determine whether expansion or deletion of a heavy chain variable region (VH) family occurred in the IgA genes in the KD tissue, isolated clones were grouped by VH family by examination of the CDR1 and 2 regions and the framework regions and comparing them to published VH family sequences (21). Of the 44 α clones, 42 were members of the VH3-1, VH3-3, VH4-4, VH5-5, VH6-6, and VH7-7 families as shown in Table II. The distribution of VH family members in the α genes in the KD cDNA library was similar to that reported in the literature for adult peripheral blood B cells (18, 22). More VH7 clones were present in the KD vascular cDNA library than would be expected by comparison to published data for adult controls (10 vs 2%, p = 0.05 by Fisher’s exact test); however, VH7 has been reported to be frequently used in the infant cord blood repertoire (23), and data for its use in childhood peripheral blood B cells are lacking. The remaining two α clones did not contain sufficient framework 3 region to allow assignment to a family. Overall, the distribution of VH families in KD vascular tissue appears to be similar to that found in normal peripheral blood B cells.

**Identification of clonally related sequences.** To determine whether α genes in the vascular cDNA library were polyclonal or oligoclonal, we sequenced the CDR3 regions. Sequence identity between B cells in this region indicates clonal relatedness (24, 25); expansion of B cells with the same VDJ rearrangements and with evidence of somatic mutation are characteristic features of an immune response to Ag (24, 25).

Sequence analysis of the 44 α clones revealed two groups of clonally related VH4 sequences (Fig. 1). The VH4-4-group 1 sequences contain five members (clones C5v, E2, 5-1, 7-1a, and A2) and the VH4-4-group 2 sequences contain two members (clones C2 and 4–5). As can be seen in Fig. 1A, the VH4-4-group 1 sequences show evidence of somatic mutation compared to germline sequence Vh4-4.18, and clone A2 shows evidence of somatic mutation compared to clones E2, 5–1, 7–1a, and C5v. Amino acid sequences for this group are seen in Fig. 1B. Similar evidence of somatic mutation is seen in the VH4-4-group 2 sequences (Fig. 1C). Clones C2 and 4–5 show somatic mutation compared to germline DP-65 and to each other. Fig. 1D shows amino acid sequences for this second group. The identification of multiple clones with identical VDJ rearrangements and evidence of somatic mutation strongly suggests an oligoclonal, Ag-driven immune response in acute KD.

Three additional sets of identical sequences were identified in the KD vascular library (Fig. 2). The VH3-3-group 1 has three members (clones D3, 11–2, and 11–5), the VH3-3-group 2 has two members (clones 2–2 and 13–1), and the VH7-group 1 contains three members (clones 10–1, 7–1b, and 10–4). The members of each group were identical to each other in the regions sequenced. Therefore, these clones likely represent sequences isolated from plasma cells that are highly represented in the vascular tissue and were

**FIGURE 2.** Nucleotide sequences of the CDR3 regions of VH3-group 1 (clones D3, 11–2, and 11–5), VH3-group 2 (clones D2 and 13–1), and VH7-group 1 (clones 10–1, 7–1b, and 10–4) from patient A, showing identical sequences within each group.

**TABLE II.** Percentage of VH α families as shown in Table II. The distribution of VH family members in the α genes in the KD cDNA library was similar to that reported in the literature for adult peripheral blood B cells (18, 22). More VH7 clones were present in the KD vascular cDNA library than would be expected by comparison to published data for adult controls (10 vs 2%, p = 0.05 by Fisher’s exact test); however, VH7 has been reported to be frequently used in the infant cord blood repertoire (23), and data for its use in childhood peripheral blood B cells are lacking. The remaining two α clones did not contain sufficient framework 3 region to allow assignment to a family. Overall, the distribution of VH families in KD vascular tissue appears to be similar to that found in normal peripheral blood B cells.

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derived from the same original B cell that underwent clonal expansion.

**VH4-group 1 sequences.** The VH4-group 1 sequences were investigated further, as this set contained the largest number of members of the five clonally related sets. These clones use a VH4 gene most closely related to the germline VH4.18 gene, as determined by comparing the obtained sequence to published germline sequences (21) and by comparing the sequence to immunoglobulin sequences entered in the Genbank/EMBL database. The CDR3 region contains sequence most similar to the DN1 gene and uses a JH1 gene. JH1 has been reported to be used by only 1% of all human peripheral blood B cells (22, 26). Clones C5v, E2, 5-1, and 7-1a have identical CDR3 sequence, and clone A2 has one nucleotide difference (G>C) in CDR3 that confers an amino acid substitution (G>A) (Fig. 1, A and B). Clone A2 extends only to the start of FR3 and has two additional nucleotide differences in FR3 compared to the other clones. Sequencing of the VH4-group 1 was performed on excised phagemid rather than on PCR-amplified

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** Nucleotide sequences of the CDR3 regions of additional α sequences identified in the KD patient A vascular cDNA library. These clones include members of the VH1, VH3, VH4, VH5, VH6, and VH7 families. Clones 10-11 and 6-4 are shorter clones with insufficient framework 3 region to allow assignment to a VH family. Clones 10-11 and 6-4 are shorter clones with insufficient framework 3 region to allow assignment to a VH family.
product, ruling out PCR as a cause of these mutations. Therefore, the sequence differences in these clones likely represent point mutations introduced during somatic mutation in B cells undergoing clonal expansion.

In a separate experiment, we performed PCR amplification of the amplified cDNA library using a $V_{H}4$ leader primer and a primer in the constant region of $\alpha$, cloned the PCR products, and hybridized clones with the oligonucleotide probe AR1 derived from the $V_{H}4$-group 1 CDR3 sequence. We obtained three full-length clones with the expected CDR3 sequence, designated clones 17PCR, 28PCR, and 39PCR (GenBank accession numbers AF247737–AF247739), all of which had $V_{H}4$ gene sequence more closely related to the germline $V_{H}4.18$ than the clones isolated from the primary library. For example, clone 28PCR demonstrated 95% homology to germline $V_{H}4.18$, whereas the $V_{H}4$-group 1 sequence E2 demonstrates 85% homology to germline $V_{H}4.18$. The CDR3 sequence of the clones identified by PCR was similar to that of the $V_{H}4$-group 1 sequences, except for the last two amino acids (HY in $V_{H}4$-group 1, QH in the PCR clones). This provides further support for the assignment of the $V_{H}4$-group 1 sequences to the germline $V_{H}4.18$, as the PCR clones had the same VDJ rearrangement but higher homology to germline $V_{H}4.18$ than the $V_{H}4$-group 1 set of clones. This indicates the presence of additional clones of the same B cell lineage in the cDNA library and provides further support for clonal expansion with selection by Ag.

The $V_{H}4$-group 1 sequences have undergone significant somatic mutation; the R (replacement)/S (silent) ratio in these sequences compared to germline is 12:1 in the CDR regions compared to 13:7 (1.9) in the FR regions. This compares to the calculated R/S ratios for the $V_{H}4$-group 1 $V_{H}4$ gene of 4.5 for the CDR regions and 2.6 for the FR regions (27); observed number of CDR R mutations is significantly higher than expected, $p < 0.001$ using formula in Ref. 27. These data collectively indicate that the $V_{H}4$-group 1 sequences have undergone significant somatic mutation and have been clonally expanded, consistent with an Ag-driven immune response.

**Other VDJ Rearrangements among isolated $\alpha$ genes.** CDR3 sequence data for additional $\alpha$ genes isolated from the vascular cDNA library are given in Fig. 3. They consist of three $V_{H}1$ genes, 12 $V_{H}3$ genes, seven $V_{H}4$ genes, two $V_{H}5$ genes, two $V_{H}6$ genes, and one $V_{H}7$ gene.

**Summary of clonally related sequences in KD vascular library.** Overall, five sets of clonally related sequences were identified. The $V_{H}4$ group 1 family, consisting of clones E2, A2, C5v, 5-1, and 7-1a; and the $V_{H}4$ group 2 family, consisting of clones C2 and 4–5, show evidence of somatic mutation within the group. We also identified clonally related $V_{H}3$ group 1, consisting of clones D3, 11-2, and 11-5; $V_{H}3$ group 2, consisting of clones 2-2 and 13-1; and $V_{H}7$ group 1, consisting of clones 10-1, 7-1b, and 10-4. These five groups of CDR3 sequences accounted for 15 of 44 (34%) of the $\alpha$ clones (five $V_{H}4$-group 1, one $V_{H}4$-group 2, three $V_{H}3$-group 1, two $V_{H}3$-group 2, and three $V_{H}7$-group 1) in the primary cDNA library. None of the clonally related CDR3 regions had been entered previously into the GenBank/EMBL database. All 44 sequences are now entered into the GenBank/EMBL database under accession numbers AF064878–AF064921.

Additional unfixed tissues were not available from other fatal KD cases to allow for construction of other KD vascular libraries. Therefore, methodology was developed to isolate and analyze $\alpha$ gene sequences from formalin-fixed, paraffin-embedded coronary artery tissues from other fatal KD cases.

**Analysis of formalin-fixed, paraffin-embedded KD coronary artery tissue blocks**

We chose to amplify $V_{H}3\alpha$ and $V_{H}4\alpha$ gene sequences because these families are most represented in the peripheral blood B cell repertoire and consistently gave polyclonal CDR3 size distribution...
patterns when RT-PCR and runoff reactions were performed on α genes from peripheral blood B cell RNA from controls (data not shown). Moreover, RT-PCR amplification of RNA isolated from paraffin-embedded, formalin-fixed spleen tissue from KD patient A yielded polyclonal VH 3α and V H 4α CDR3 size profiles (Fig. 4) as expected. Sequencing of the spleen V H 3α and V H 4α PCR products revealed a diverse repertoire of CDR3 genes (Fig. 5). These experiments demonstrate that our procedure will detect a polyclonal distribution of α sequences if such sequences are present in the sample.

**FIGURE 5.** Nucleotide and amino acid sequences of CDR3 regions of VH 3α and V H 4α genes in paraffin-embedded, formalin-fixed spleen from patient A.
IgA plasma cells were counted in 5-μm tissue sections from these blocks. Using the same calculation as described for the un-fixed vascular tissue and assuming that the same plasma cell might appear in five successive sections, we calculated that there would be at least three times as many plasma cells in ten 8-μm tissue sections as in one 5-μm tissue section. Sections from blocks from both patients had ~400 IgA plasma cells in a 5-μm tissue section or, at a minimum, 1200 IgA plasma cells in the ten 8-μm tissue sections.

RT-PCR amplification of the coronary artery block from patient B did not yield any visible V_{H3}α product after reverse transcription and nested PCR, but a reproducible V_{H4}α product was obtained. The runoff reaction products of this V_{H4}α product are seen in Fig. 6; one single prominent band corresponding to a CDR3 size of ~33 nucleotides is seen. Nine clones obtained from this PCR product were sequenced in the CDR3 region and were identical (Fig. 7).

RT-PCR amplification of the coronary artery block from patient C yielded visible V_{H3}α and V_{H4}α products; runoff reaction products are seen in Fig. 8. Eleven V_{H3} clones and 12 V_{H4} clones were sequenced; each set of sequences within the amplified V_{H}α family was identical in the CDR3 region (Fig. 7). The CDR3 sequences from patients B and C are entered into the GenBank database under accession numbers AF237658 –AF237660. Thus, in patients B and C, the usage of predominant V_{H3} and V_{H4}α sequences provides further evidence of an oligoclonal response in the KD vascular wall.

One CDR3 sequence cloned from the V_{H4}α PCR products from paraffin-embedded, formalin-fixed spleen from patient A was identical to CDR3 sequence E1, which was isolated from the primary, unamplified vascular cDNA library from KD patient A (Figs. 3 and 5). Isolation of clones from the vascular library and from the formalin-fixed, paraffin-embedded tissues were performed by different methods as described above, at different times, and in different laboratories.

### Discussion

The presence of IgA plasma cells as a prominent component of the vascular immune response in young infants with KD is interesting. Systemic IgA responses appear to be immature in young infants (28). However, mucosal IgA responses in this age group are mature (29), and infants presented with mucosal Ag such as poliovirus mount excellent Ag-specific IgA Ab responses (30). If IgA plasma cell infiltration in KD is the result of stimulation of the systemic IgA immune response, the IgA Abs produced may be oligoclonal as a result of Ag stimulation, or polyclonal as a result of generalized B cell activation or a response to a superantigen. In young infants, polyclonal stimulation of peripheral blood induces B cells to secrete predominately IgM, with fewer cells secreting IgG and only very few secreting IgA (28, 31). A predominant IgA immune response is also unlikely to be the result of stimulation of B cells by a superantigen; B cell superantigens appear to bind preferentially to the framework regions of germline immunoglobulins, and are thus more likely to bind to the IgM repertoire because it generally contains less somatic hypermutation than does the IgG and IgA repertoire (32). Therefore, it seems most plausible that the IgA response in KD is Ag-driven, likely as a result of stimulation of B cells by a superantigen; B cell superantigens appear to bind preferentially to the framework regions of germline immunoglobulins, and are thus more likely to bind to the IgM repertoire because it generally contains less somatic hypermutation than does the IgG and IgA repertoire (32). Therefore, it seems most plausible that the IgA response in KD is Ag-driven, likely as a result of stimulation of the mucosal IgA system by a specific etiologic agent. If this hypothesis is correct, the IgA response in KD is likely to be oligoclonal, directed primarily at various epitopes of the etiologic agent.

We have demonstrated in this study that clonally related α genes are present in a primary, unamplified vascular cDNA library from patient A, a child who died of fatal acute KD. Use of the primary, unamplified cDNA library avoided potential amplification bias that might occur with amplification of the primary cDNA library or copying errors that might occur with use of PCR methods to generate the population of α genes to be studied. Strikingly, five clonally related sets of α sequences account for 34% of the α sequences characterized from the primary, unamplified vascular

<table>
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<tr>
<th>Patient</th>
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<th>FR3</th>
<th>CDR3</th>
<th>GenBank Acc#</th>
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<td>ATGGATGAGCCAGCTGACGATACAAACTAC</td>
<td>AF237660</td>
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</table>
The most frequently isolated sequence, the V\textsubscript{H}4-group 1, shows evidence of extensive somatic mutation, with a R/S ratio of 12 in the CDRs and 1.9 in the FR regions. It uses a J\textsubscript{H}1 gene, which has been reported to be used by only 1% of human peripheral blood B cells (22, 26). The presence of these clones, which have undergone somatic mutation and clonal expansion, supports an Ag-driven B cell immune response in the vascular wall in acute KD.

In addition, we have shown an oligoclonal pattern of V\textsubscript{H}3 and V\textsubscript{H}4\textsubscript{a} gene usage in paraffin-embedded, formalin-fixed coronary arteries from two additional fatal KD patients in whom one CDR3 sequence predominated in the VH\textsubscript{3}\textsubscript{a} or V\textsubscript{H}4\textsubscript{a} genes in the coronary artery tissues. Thus, evidence of an oligoclonal IgA response was detected in vascular tissue of all three KD patients. Using the same method, a polyclonal profile of V\textsubscript{H}3\textsubscript{a} and V\textsubscript{H}4\textsubscript{a} gene usage in paraffin-embedded, formalin-fixed KD spleen was demonstrated. However, it is possible that the clonality of V\textsubscript{H} genes in KD spleen is somewhat more restricted than are those in a spleen from a healthy patient; this may be the case in spleen from any patient with an overwhelming infection. To support this point, one identical CDR3 sequence was obtained from 44 clones isolated from the unamplified vascular cDNA library, and from 20 VH\textsubscript{4}\textsubscript{a} sequences cloned from a paraffin-embedded, formalin-fixed spleen sample from the same patient. The fact that an identical CDR3 sequence was identified in a small number of VH genes examined from vascular tissue and spleen from the same patient implies that the KD spleen is producing some of the same oligoclonal IgA Ab that are being made in the vascular wall.

The representation of different VH family members in our vascular cDNA library is similar to that reported for human peripheral blood B cells (18, 22). We did not observe a significant expansion of a VH family without clonality nor depletion of a VH family, findings that might have suggested stimulation by a superantigen (32). Rather, we found evidence of clonally related sequences in the VH\textsubscript{3}, VH\textsubscript{4}, and VH\textsubscript{7} families, consistent with an oligoclonal Ab response directed at various antigenic epitopes of an etiologic agent.

Although the presence of a set of two clonally related sequences from our 44 clones isolated from the unamplified vascular cDNA library might simply be due to isolation of the sequence from the same plasma cell by chance, we believe this to be unlikely. First, the cDNA library was prepared from total RNA originally isolated from tissue that contained 180,000 IgA plasma cells, as described in Materials and Methods. Second, sequence data clearly indicate that the VH\textsubscript{4}-group 2 set consists of two different members, as shown in Fig. 1C and D. These clones were sequenced as excised pBluescript phagemids; we performed no PCR amplification with its potential to introduce nucleotide replacement by Taq polymerase error. Clone C2 is full length and clone 4-5 includes most of FR3. The clones differ in sequence by four nucleotides in FR3 and one in CDR3, indicating that they were produced by two different plasma cells (Fig. 1C). We cannot rule out that VH\textsubscript{3}-group 2 clones 2-2 and 13-1 are from the same plasma cell; clone 13-1 extends only to the end of CDR2 and has the same nucleotide sequence as clone 2-2 throughout FR3.

We cannot exclude that a superantigen-driven T cell response occurs concurrently with an Ag-driven B cell response in acute KD, but we believe this to be unlikely. Data indicate that clonal expansion of CD8\textsuperscript{+} T cells occurs in acute KD (6, 9). As previously suggested (6), an apparent polyclonal increase of VB2 and VB8 T cells in acute KD, suggesting activation by a superantigen (4), may have actually resulted from failure to separate CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, with the presence of CD4\textsuperscript{+} T cells obscuring clonal expansion of CD8\textsuperscript{+} cells. The data presented in this paper support an Ag-driven immune response in KD. We hypothesize that the KD infectious agent enters the host through the respiratory route.
and is recognized and processed in the bronchus-associated lymphoid tissues. Epidemiologic data in KD are consistent with a respiratory portal of entry of the etiologic agent (1). B cells then switch to IgA and enter the general circulation. The pathogen may enter the bloodstream, resulting in the multisystem involvement characteristic of KD (1), and also enter vascular tissues. IgA B cells may enter the vascular walls with other inflammatory cells and there differentiate into IgA plasma cells, probably under the influence of locally produced cytokines. Our findings strongly support the hypothesis of an Ag-driven immune response in KD, because we found both clonally related α sequences and evidence of somatic mutation within the related clones in the vascular cDNA library. These findings are characteristic of an Ag-driven immune response in a germinall center (25). By probing the KD vascular cDNA library with an oligonucleotide probe corresponding to the CDR3 sequence of the Vj4-4 group 1 set of α genes, we identified additional sequences of the same β cell lineag in the library, findings again typical of an Ag-driven immune response in a germinall center. Because germinall centers are not present in KD vascular tissue (15), this suggests that IgA cells are undergoing somatic mutation in the bronchus-associated lymphoid tissue or other lymphoid tissue in response to Ag prior to migrating to the vascular tissue.

Our finding of an oligoclonal IgA response in the vascular wall in acute KD supports an Ag-driven immune response. The production of oligoclonal Abs in human tissues that are directed at the disease-causing pathogen is a feature of many infectious processes (33, 34). Our data support the hypothesis of a conventional Ag in the pathogenesis of KD.

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References