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Recent data indicated that adaptive immunity is involved in the process of atherogenesis. Oligoclonal recruitment of T lymphocytes has been described in coronary plaques of patients with acute coronary syndrome. However, the nature of immune response remains to be determined. In the present study, we examined the Ab response in six coronary plaques obtained by endoluminal directional atherectomy. The IgG1κ-coding gene repertoire of B lymphocytes present in circulating blood and in coronary plaques were cloned and analyzed. In all of the six plaques, we observed 1) a skewed usage of heavy and light IgG1/κ Ab-coding genes, 2) an oligoclonal distribution of Vκ, Jκ, and VH, Dκ, and Jκ genes with overrepresentation of some rarely used IgG genes, and 3) the unequivocal signs of Ag-driven clonal expansion and evolution of B cells. The data document for the first time the presence of a local Ag-driven clonal evolution of B cells in human atherosclerotic plaques.

Materials and Methods

Coronary plaque samples and peripheral blood sampling

After approval by the local ethics committee and patients’ informed consent to participate in the study, six coronary plaque samples (ID02, ID07, ID09, ID10, ID11, and ID12) were obtained by EDA from patients with ACS or undergoing elective EDA and immediately stored in RNA Later stabilization reagent (Qiagen). RNA extraction was performed within 30–60 min by using Qiagen RNeasy Micro kit (Qiagen). Peripheral blood cells, collected at the time of plaque sampling from each patient, were purified by Ficoll gradient centrifugation and stored at −80°C until RNA extraction was performed using a Qiagen RNeasy kit (Qiagen). None of the patients had autoimmune or systemic diseases, nor had they undergone recent surgery or suffered from recent infectious diseases (Table I).

Amplification, cloning, and sequencing of H and L chain Ig genes

cDNA coding for κ L chain (LC) and VH/C1H regions of the IgG1 H chain (HC) were reverse transcribed and amplified separately from both coronary plaque sample (CPS) and parallel samples of peripheral blood lymphocytes (PBLs) immediately after RNA extraction. Briefly, the first-strand cDNA was synthesized using oligo(dT) as primer and SuperScript III enzyme
(Invitrogen). IgG1 H and L chain genes were amplified by using family-specific primers in separate single PCR reactions (primer sequences are shown in supplemental table S1)\(^5\) (24, 25). Each PCR reaction was conducted in a final volume of 50 \(\mu\)l of reaction buffer containing 2 U of native PFU DNA polymerase (Stratagene) and 50 pmol of primers. PCR was conducted for 45 cycles under standard conditions (denaturation, 1 min at 94\(^\circ\)C; annealing, 2 min at 56\(^\circ\)C; and extension, 1 min at 72\(^\circ\)C). Aliquots of the PCR product were analyzed by electrophoresis in a 2% agarose gel containing ethidium bromide.

Sequence analyses and evaluation of clonally related sequences

PCR products were recovered and ligated into the pGEM T vector (Promega). After bacterial transformation and PCR colony screening, positive colonies were picked and grown overnight in 3 ml of Luria-Bertani medium. The double-stranded DNA template from the colonies containing H or L chain gene inserts was then sequenced using a 3130 automatic sequencer (Applied Biosystems). Sequence analyses were conducted for each patient on HC and LC clones derived from CPS and PBLs. Mutations identified by comparing each sequence with germline sequences (30) (international ImMunoGeneTics database at http://imgt.cines.fr) were defined on the basis of nucleotide changes in the \(V_{\text{H}}\) segment. The human Ig H and L chain CDR3 regions and clonality were determined by Ig VDJ or VJ junction analysis with the software provided by ImMunoGeneTics/JunctionAnalysis. The total numbers of nucleotide substitutions, replacement mutations, and the ratio of replacement and silent mutations (R:S) of framework and CDRs were evaluated as previously described (26) (www-stat.stanford.edu/immunogenetics) (Table II and supplemental Table S2). The CDR3 length and the number of positive charges in each CDR3 were also evaluated (supplemental Table S2). Signs of receptor editing or revision were evaluated as previously described (27, 28).

Estimate of PCR-associated recombination and PCR-induced mutation rates

Finally, PCR-associated recombination and PCR-induced mutation rates in this experimental protocol were estimated. PCR-induced mutation rate was estimated both by sequencing the constant CH1 region of the amplified H chains and by a limiting dilution assay in which a previously cloned Ab was diluted down to one copy, amplified, cloned, and sequenced. In both assays the mutation rate resulted to be between 2.4 \(\times\) 10\(^{-4}\) and 3.2 \(\times\) 10\(^{-4}\).

To estimate the number of PCR-induced recombination events, a mock coronary plaque cDNA was spiked with five plasmids (diluted to 10–100 copies each) containing previously cloned unrelated H chain sequences. The mock cDNA was amplified, cloned, and sequenced. No evidence of PCR-induced recombination was observed between coronary and unrelated H chain sequences (more than 50 full-length sequences were analyzed) (data not shown).

Clonal analysis and lineage tree construction

Clones with different sequences (and thus coming from distinct B lymphocytes) were considered clonally related if they were showing the same VDJ or VJ junctions and identical (or almost identical) CDR3 sequences. A comparable number of clones in plaques and blood were sequenced for each patient. The analysis of clonality was performed as previously described (29) by using tonsil samples as positive control for oligoclonality (data not shown). With our technology, in the positive control samples the ratio between the number of clones with different junctions and the total number of sequenced clones (Table II) was >60% (this indicates that multiple copies of identical sequences are present in the population).

Ig gene lineage trees of clonally related sequences were built as previously described (30) with minor modifications. In brief, the sequences of each clonal group was aligned with the corresponding VC or V\(_k\) germline genes by using ClustalX (www.clustal.org). An initial neighbor-joining tree was generated by using MEGA3 (www.megasoftware.net) and manually corrected on the basis of visual inspection of the mutations in the alignments (supplemental Fig. S2). Each lineage tree was defined, graphically, as a rooted tree where the nodes correspond to BCR sequences (sampled or ancestral) and the root to the germline-encoded sequence. The number of mutations that have occurred during B cell maturation was also displayed in the trees. In particular, since no CDR junction is present in the germline sequence, the number of mutations between the root and the most recent common ancestor was evaluated on the basis of the IG VH or IGVK gene sequences. The distance between each subsequent clone (with identical VDJ or VJ junctions) was evaluated by using the whole sequences (see Fig. 4 and supplemental Fig. S2).

**Statistical analysis**

Fisher’s exact tests were used to analyze dichotomized data. \(I_{\text{K}}\)s were dichotomized into \(I_{\text{K}}\)1-2 vs all the other \(I_{\text{K}}\)s whose usage may involve secondary recombination events. \(V_{\text{K}}\)s were dichotomized into upstream (from \(V_{\text{K}}\)4-1 to \(V_{\text{K}}\)1-17) and downstream (from \(V_{\text{K}}\)4-1 to \(V_{\text{K}}\)1-16). Student’s \(t\) test was used to analyze the differences in proportion of germline sequences in PBL and CPS light chains.

**Results**

Differences in the IgG1/coding gene repertoires of B lymphocytes in the coronary plaques and in the peripheral blood

Six CPS (namely ID02, ID07, ID09, ID10, ID11, and ID12) were obtained by EDA from patients with ACS or undergoing elective EDA. Overall, 259 HC and 252 LC sequences were obtained from CPS using PCR amplification, cloning, and sequencing, with an average of 43 HC and 42 LC sequences per plaque. Otherwise, 214 HC and 243 LC sequences were obtained from PBLs, with an average of 36 HC and 40 LC sequences per sample. Of note, a different distribution of HC and LC family gene usage was observed in all patients between sequences derived from CPS and from matched PBLs (Fig. 1), indicating that two distinct B cell populations were analyzed. Preferential usage of upstream genes of the proximal \(V_{\text{K}}\) locus and a prevalent \(V_{\text{K}}\)-\(J_{\text{K}}\) reassortment with proximal \(I_{\text{K}}\) genes was observed in LC sequences from some CPS (Fig. 2), suggesting, at least in a subset of Abs, a reduced occurrence of secondary recombinations and possibly a reduced control of B cell autoreactivity (31, 32). However, no differences in the usage of proximal or distal IGHV genes were observed between CPS and PBLs except in sample ID07, in which a skewed profile similar to the ones previously observed in systemic autoimmune diseases was documented (33, 34) (Fig. 3). Further analyses of the

---

**Table I. Clinical characteristics of patients from which coronary samples were obtained**

<table>
<thead>
<tr>
<th>Plaque ID</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Family History</th>
<th>Hypertension</th>
<th>Cholesterol</th>
<th>Smoking</th>
<th>Diabetes</th>
<th>Vessels</th>
<th>Plaque Localization</th>
<th>Clinical Presentation</th>
<th>LVEF (%)</th>
<th>QCA (%)</th>
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<td>2</td>
<td>M</td>
<td>59</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Ex</td>
<td>No</td>
<td>1-D</td>
<td>OM</td>
<td>NSTEMI</td>
<td>55</td>
<td>59</td>
</tr>
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<td>7</td>
<td>M</td>
<td>62</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>3-D</td>
<td>Cx</td>
<td>CSA</td>
<td>47</td>
<td>69</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>47</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Ex</td>
<td>No</td>
<td>2-D</td>
<td>Cx</td>
<td>NSTEMI</td>
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<td>68</td>
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<tr>
<td>10</td>
<td>M</td>
<td>68</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>1-D</td>
<td>Cx</td>
<td>UA</td>
<td>56</td>
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<tr>
<td>11</td>
<td>M</td>
<td>47</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>2-D</td>
<td>LAD</td>
<td>UA</td>
<td>56</td>
<td>88</td>
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<tr>
<td>12</td>
<td>M</td>
<td>29</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>1-D</td>
<td>LAD</td>
<td>NSTEMI</td>
<td>55</td>
<td>56</td>
</tr>
</tbody>
</table>

* LVEF, left ventricular ejection fraction; QCA, quantitative coronary assessment; 1-D, one-vessel disease; 2-D, two-vessel disease; 3-D, three-vessels disease; OM, obtuse marginal artery; Cx, circumflex artery; LAD, left descending artery; NSTEMI, non-ST segment elevation myocardial infarction; CSA indicates chronic stable angina; UA, unstable angina with negative troponin.

\(^5\) The online version of this article contains supplemental material.
Table II. Analysis of HC and LC sequences cloned from CPSs or PBLs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Compartment</th>
<th>Ig Chain</th>
<th>No. Sequenced Clones (clones with different junctions)</th>
<th>Relationship (no. clones with different junctions)</th>
<th>Most Represented V Gene</th>
<th>V Gene Avg. Divergence from GL (clones &gt;99%)</th>
<th>Clones with ( p &lt; 0.05 ) in FRs (%)</th>
<th>Clones with ( p &lt; 0.05 ) in CDRs (%)</th>
<th>Avg. CDR3 Length (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID02</td>
<td>Plaque</td>
<td>HC</td>
<td>39 (19) Clonal (1) Nonclonal (18)</td>
<td>V_H3-11</td>
<td>92.81 (0)</td>
<td>3 (100)</td>
<td>0</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LC</td>
<td>26 (6) Clonal (1) Nonclonal (5)</td>
<td>V_H3-23</td>
<td>92.50 (43)</td>
<td>5 (31.2)</td>
<td>7 (43.7)</td>
<td>10.00</td>
<td></td>
</tr>
<tr>
<td>PBLs</td>
<td>HC</td>
<td>35 (26)</td>
<td>Nonclonal (26)</td>
<td>V_H3-4</td>
<td>92.77 (11)</td>
<td>12 (46.1)</td>
<td>13 (50.0)</td>
<td>15.04</td>
<td></td>
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<tr>
<td></td>
<td>LC</td>
<td>24 (24)</td>
<td>Nonclonal (24)</td>
<td>V_3-20</td>
<td>97.46 (7)</td>
<td>4 (16.6)</td>
<td>3 (12.5)</td>
<td>9.04</td>
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<td>Plaque</td>
<td>HC</td>
<td>41 (23) Clonal (1) Nonclonal (22)</td>
<td>V_H3-21</td>
<td>92.28 (2)</td>
<td>100 (1)</td>
<td>2 (100)</td>
<td>23.00</td>
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<td>LC</td>
<td>75 (36) Nonclonal (32)</td>
<td>V_H3-34</td>
<td>90.96 (6)</td>
<td>5 (23.8)</td>
<td>5 (23.8)</td>
<td>15.15</td>
<td></td>
</tr>
<tr>
<td>PBLs</td>
<td>HC</td>
<td>45 (32)</td>
<td>Nonclonal (32)</td>
<td>V_H3-34</td>
<td>95.42 (2)</td>
<td>11 (42.3)</td>
<td>15 (57.7)</td>
<td>9.18</td>
<td></td>
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<td></td>
<td>LC</td>
<td>62 (57)</td>
<td>Nonclonal (57)</td>
<td>V_K3-39</td>
<td>92.73 (4)</td>
<td>18 (29.0)</td>
<td>20 (32.2)</td>
<td>8.98</td>
<td></td>
</tr>
<tr>
<td>ID09</td>
<td>Plaque</td>
<td>HC</td>
<td>36 (5) Clonal (2) Nonclonal (3)</td>
<td>V_H3-7</td>
<td>91.66 (0)</td>
<td>3 (100)</td>
<td>2 (66.6)</td>
<td>17.33</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>LC</td>
<td>46 (32) Nonclonal (32)</td>
<td>V_3-15</td>
<td>90.56 (0)</td>
<td>7 (21.9)</td>
<td>9 (28.2)</td>
<td>13.89</td>
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</tr>
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<td>PBLs</td>
<td>HC</td>
<td>26 (24)</td>
<td>Nonclonal (24)</td>
<td>V_3-15</td>
<td>95.03 (6)</td>
<td>13 (54.2)</td>
<td>9 (37.5)</td>
<td>11.10</td>
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<tr>
<td></td>
<td>LC</td>
<td>45 (5)</td>
<td>Nonclonal (5)</td>
<td>V_H1-39</td>
<td>96.72 (0)</td>
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<td>3 (100)</td>
<td>19.80</td>
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<tr>
<td>ID10</td>
<td>Plaque</td>
<td>HC</td>
<td>44 (5) Clonal (2) Nonclonal (5)</td>
<td>V_H3-23</td>
<td>92.52 (1)</td>
<td>1 (33.3)</td>
<td>16.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LC</td>
<td>39 (16) Nonclonal (16)</td>
<td>V_K3-39</td>
<td>94.64 (0)</td>
<td>6 (75.0)</td>
<td>6 (75.0)</td>
<td>8.67</td>
<td></td>
</tr>
<tr>
<td>PBLs</td>
<td>HC</td>
<td>31 (24)</td>
<td>Nonclonal (24)</td>
<td>V_K3-4</td>
<td>91.27 (0)</td>
<td>7 (30.4)</td>
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<td>LC</td>
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<td>Nonclonal (38)</td>
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<td>18 (67.2)</td>
<td>9.09</td>
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<td>HC</td>
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<td>V_3-61</td>
<td>92.20 (0)</td>
<td>2 (66.0)</td>
<td>2 (66.0)</td>
<td>15.33</td>
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<tr>
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<td></td>
<td>LC</td>
<td>50 (21) Nonclonal (16)</td>
<td>V_H3-30</td>
<td>91.00 (0)</td>
<td>11 (68.7)</td>
<td>9 (56.2)</td>
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<tr>
<td>PBLs</td>
<td>HC</td>
<td>28 (28)</td>
<td>Nonclonal (28)</td>
<td>V_H1-4</td>
<td>94.17 (0)</td>
<td>8 (42.1)</td>
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<td>8 (20.0)</td>
<td>9.15</td>
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<td>HC</td>
<td>46 (15) Clonal (12) Nonclonal (12)</td>
<td>V_H3-23</td>
<td>91.56 (0)</td>
<td>6 (50.0)</td>
<td>9 (75.0)</td>
<td>15.67</td>
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<td>LC</td>
<td>20 (12) Clonal (3) Nonclonal (9)</td>
<td>V_3-20</td>
<td>94.92 (0)</td>
<td>8 (53.4)</td>
<td>6 (100)</td>
<td>9.33</td>
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<tr>
<td>PBLs</td>
<td>HC</td>
<td>39 (34)</td>
<td>Nonclonal (34)</td>
<td>V_K3-30</td>
<td>95.55 (5)</td>
<td>13 (38.2)</td>
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<td>42 (40)</td>
<td>Nonclonal (40)</td>
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<td>97.08 (15)</td>
<td>10 (27.1)</td>
<td>8 (21.7)</td>
<td>9.06</td>
<td></td>
</tr>
</tbody>
</table>

a In all of the analyses, a unique consensus sequence was used as representative of each clonal group. To avoid bias due to artificial clonal duplications, both the overall number of sequenced clones and the number of different CDR3 junctions (in parentheses) are shown. The most represented IGHV or IGKV genes within clonally related and nonrelated sequences are also shown. The average percentage divergence from germline (GL) sequences for each HC and LC were defined on the basis of nucleotide changes in the IGHV or IGKV sequences. The proportion of clones with a significant ratio of replacement and silent mutations (R:S) of framework (FR) and CDRs and the average number of amino acids (aa) in the CDR3 region within each compartment are described.

b The overall number of sequenced clones and the number of different CDR3 junctions are shown.

c Proportion of sequences that have a significant \( p \) value in FR and CDR.

specificity of these Abs may clarify the exact role of these Abs in the pathogenesis of atherosclerotic disease.

**Ag-driven evolution of B cells in coronary plaques**

Several events reveal Ag-driven clonal expansion of B cells in germinal centers, including somatic hypermutation, affinity maturation, Ig class switching, and B cell receptor revision. We verified whether these events could be identified at the molecular levels in the plaque, in which oligoclonal T cell response has recently been documented (35). First, a difference in LC germline sequences frequency was observed in the two populations, with only 2 LC germline sequences in CPS, compared with 55 LC GLS in PBLs (\( p = 0.014 \)). Second, the percentage of HC and LC sequences bearing a statistically significant ratio of replacement and silent mutations (R:S) of framework and CDRs (Table II) was analyzed (26). Third, an oligoclonal distribution of VK, J\_K, and VH, D\_H families or an overrepresentation of some rarely used IgG genes was documented in most of the CPS (such as V\_H1-2, V\_H3-11, V\_K1-12, V\_K1-13, V\_H1-45, V\_H3-46, V\_H3-64 and V\_H3-64, V\_K1-5, V\_K1-6, V\_K1-9, V\_K1-D16, V\_K3-D20 for H and L genes, respectively), while in the PBLs the frequency of observed HC and LC families was the one expected in this sample derived from healthy patients (34, 36, 37) (Fig. 3). Evidence of receptor editing or revision was observed in some sequences from CPS (supplemental Fig. S1). Whether this finding be associated with an increased number of autoreactive Abs, as it has been suggested previously (28, 34), is still uncertain and will need to be demonstrated in additional experiments. Finally, the presence of clonally related LC and HC sequences was documented in all CPS (Table II) and in none of the matched PBLs, confirming that the result is plaque-specific and not an artifact. HC and LC lineage trees obtained from each CPS are shown in Fig. 4. More importantly, HC sequence analysis identified in 5 out of 6 CPS more than one clonally related group. The same phenomenon was present in LC sequences from four out of six CPS. In many cases, a large number of mutations were observed between clonally related sequences, thus demonstrating the presence of long-lasting Ag-driven stimulation (30). Neither the CDR3 lengths nor the number of positively charged residues in the CDR3 resulted in significantly differences between CPS and PBLs (Table II). In all CPS, mutations from the germline were present in HC and in LC sequences, as expected for most class-switched IgG molecules. Although the number of HC germline sequences present in CPS- and PBL-derived sequences was comparable, the number of LC germlines in PBLs was significantly
higher. This is probably due to the presence in PBLs of a higher number of naive B cells (whose LCs were amplified together with IgG1 HCs). These data indicate that coronary plaque B lymphocytes constitute a positively selected population different from the one present in peripheral blood.

Furthermore, noncommon mutations shared between clones with the same VDJ or VJ junctions were observed in all CPS, indicating a stepwise B cell evolutionary process. In many cases, especially in clonally related sequences, the analysis of the ratio of silent and nonsilent mutations in frameworks and CDRs (26) showed the presence of a local Ag-driven selection (Table II and supplemental Table S2). This selection was confirmed by the study of sequences from sample ID10. In this case, the HC of the clonal groups showed one insertion and several deletions occurring in multiples of three within the CDR3 region, maintaining the original reading frame and an intact IgG receptor downstream of the recombination and mutation site, thus preserving the functional activity of the mutated Ab needed for the survival of this B cell clone (supplemental Fig. S2).

Discussion

The data presented herein demonstrate that coronary atherosclerotic plaques are sites of B lymphoid proliferation in patients with ACS and strongly suggest the hypothesis of the local presence of an activating or recruiting Ag in these lesions. Indeed, Walton et al. failed to detect a restricted usage of VH genes in B cells infiltrating the wall of atherosclerotic abdominal aneurysms, obtaining a pattern similar to what we observed in the peripheral blood of our ACS patients (38). However, both the difference in size between coronary and abdominal atherosclerotic lesions and the different

![FIGURE 1. IGHV, IGHD, IGHJ, IGKV, and IGKJ family gene usage. Inner ring: coronary plaque samples. Outer ring: PBL samples. The numbers of sequences analyzed are indicated for each sample in the center or in the upper right corner, respectively, for L or H chains (clones with identical sequences were considered only once to avoid bias due to artificial clonal duplications).](http://www.jimmunol.org/)

![FIGURE 2. Ig gene usage. Patients’ upstream VK usage frequency was plotted against that of downstream J3-4-5 usage. ID09 and ID02 samples were not included due to the very limited number of LC sequences in plaque samples (six and three distinct clones, respectively). Proximal and distal genes were dichotomized according to Lossos et al. (26). A preferential usage of upstream VK genes in CPS compared with PBL derived sequences was statistically significant in patients ID07 (p = 0.0226), ID11 (p = 0.0016), and ID12 (p = 0.0235), and a tendency was observed in ID09 (p = 0.0752). A preferential usage of VJ proximal genes was also statistically significant in ID07 (p = 0.016). The number of sequences used in the analysis is indicated in Table I (only clones with different junctions were considered).](http://www.jimmunol.org/)
FIGURE 3. IGHV and IGKV gene distributions. Genes are ordered by their relative position in the chromosome (the IGHV6-1 is the most proximal of the IGHV cluster; the IGKV4-1 is the most proximal of the IGKV cluster). The number of sequences evaluated for each patient is shown in Table I. In all of the analyses, a unique consensus sequence was used as representative of each clonal group. To avoid any bias due to artificial clonal duplications, each distinct VDJ or VJ junctions were sampled once.
approaches used to analyze B cells may have led to only apparently contrasting results. More interestingly, tertiary lymphoid organogenesis was observed in the aorta adventitia of aged apolipoprotein E<sup>−/−</sup> mice just beneath intimal plaques (39). Erosion of internal lamina and adjacent elastic membranes as well as destruction of medial structure observed in advanced lesions (39) may have allowed the migration of B cells directly into the endoluminal plaque portion.

The demonstration of lymphoid neogenesis or of B cell recruitment in endoluminal plaque samples is intriguing, as the same has been observed in an number of autoimmune and inflammatory diseases with apparently unrelated pathogenesis and distinct histological presentations (34, 40). The data presented herein document the accumulation of mutated IgG sequences with unrelated CDR3 junctions in human samples. Despite the very small sample size (1 × 2 mm<sup>2</sup> of endoluminal material), more than one clonal group was observed in CPS; this is in agreement with the slow process of plaque formation and progression with analogy to what has been observed in many chronic autoimmune diseases (40, 41). Clonal expansion, somatic hypermutation, affinity maturation, Ig class switching, and B cell receptor revision, all demonstrated in the CPS we studied, occur within tertiary lymphoid organs after B cell and T cell priming only in the presence of a specific Ag. The data shown herein indicate that the B lymphocytes present in the coronary plaque encounter activating Ag(s) and may enter a germinal center-like reaction, strongly suggesting the presence of molecules playing an antigenic role inside the plaque.

In the past decades, several studies have investigated the role of infectious agents in the pathogenesis of ACS by analyzing the serum level of specific Abs, a parameter that does not necessarily demonstrate the presence of a chronic or latent infection in coronary plaques. PCR strategies for detecting the pathogens in serum or in the plaques could be useful, but the absence of nucleic acid in serum or tissue does not rule out the possible role of infectious agents in atherogenesis. In fact, infectious agents can contribute to the formation of the atheroma starting a process that can continue after elimination of the microorganism with a “hit-and-run” mechanism.

Moreover, while secondary lymphoid organs disappear rapidly after formation and Ag clearance, tertiary lymphoid organs (as seen in peripheral germinal centers) are stable and support, at least in a subset of patients a chronic inflammatory condition or a continuous reactivation of the inflammatory process in case of subsequent encounters with the pathogen (40, 41).

More interestingly, reduction of atherosclerosis in hyperlipidemic apolipoprotein E<sup>−/−</sup> mice with a lack of functional T and B cells has shown a proatherogenic effect of adaptive immunity (42).

**FIGURE 4.** Ig lineage trees of clonally related sequences. The root of each tree corresponds to the germ-line gene sequence. Empty circles correspond to unknown ancestors. Nucleotide changes between the original sequence (germline) and the first common ancestor are evaluated on the basis of IGHV or IGKV differences. Starting from the most recent common ancestor mutations were evaluated also on the basis of the CDR3 and FR4 sequences.
However, other data indicated that activation of an adaptive immune response to self Ags like heat shock protein 60 and β2 glycoprotein may be proatherogenic, whereas the adaptive immune response to oxidized LDL might be both proatherogenic or athero-protective (2, 43–46). Passive immunization, using a mAb to epitopes identified in apolipoprotein B100, has also been shown to reduce atherosclerosis in hyperlipidemic mice (47). However, before clinical testing of a vaccination strategy based on apolipoprotein B100 several questions related to the pathogenic role of adaptive immune response need to be addressed. The most appropriate epitopes and the immunological determinant that will trigger an antiatherogenic immune response avoiding proatherogenic effects of adaptive immune response observed in some animal models will have to be identified (20).

Overall, the data presented in this study document the presence of a process of clonal evolution of B cells driven by local Ags present in the atherosclerotic plaque, being the process at the basis of generation of extensively mutated IgG against specific Ag(s). An alternative explanation could be an active recruitment from the periphery of B cells within coronary lesions, which can be accounted for only by the local presence of a specific Ag. The nature and the abundance of the Ag, as well as its availability to immune cells localized within the plaque, may indeed determine the heterogeneous evolution of the atherogenic lesions even in distinct anatomical sites of the same patient. A further point that will need to be clarified in the future is whether B cells have a pathogenetic role also in the early formation and chronic state of the inflammatory process or if their influx in the late stages of plaque evolution is mainly involved in the exacerbation of the disease leading to its acute manifestations. Our results are in agreement with recent reports on T cell clonality on this compartment indicating an active cross-talk between cells of the immune system at this level. Finally, besides the indirect demonstration of the presence inside the plaque of Ag(s) stimulating or recruiting specific B cell clones, the availability of genes coding for locally maturated Abs allows the generation of recombinant Ag-binding fragments and the construction of combinatorial Ab libraries for studying B cell specificities, thus opening new avenues for the identification of Ags potentially involved in atherogenesis being of microbial origin or self Ags generated or modified during the pathogenetic process (2).

Disclosures

The authors have no financial conflicts of interest.

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