Oligoclonal B Lymphocyte Expansion in the Synovium of a Patient With Behçet’s Disease

Chang Hee Suh, Yong Beom Park, Jungsik Song, Chan Hee Lee, and Soo Kon Lee

Objective. Plasma cell infiltration is observed in recurrent arthritis associated with Behçet’s disease (BD). The immune mechanism underlying B lymphocyte proliferation in the synovium is unclear. One hypothesis involves nonspecific polyclonal activation and another involves antigen-driven activation. The present study was undertaken to test both hypotheses and identify immunoglobulin genes that are clonally expanded in the synovium.

Methods. Peripheral blood lymphocytes (PBL) and synovial cells from a patient with BD and PBL from a healthy control subject were obtained. Complementarity-determining region 3 (CDR3) fingerprinting analysis and nucleotide sequence analysis of Ig transcripts derived from clonally expanded B lymphocytes were performed in parallel.

Results. Of 44 μ heavy chain clones of the V\(\mu\)4 family identified in the synovial tissue from the BD patient, 8 clones showed identical nucleotide sequences, and therefore, 18.2% were clonally expanded. For γ heavy chain, 4 of 50 clones of the V\(\gamma\)3 family showed nearly identical sequences; therefore, 4–8% were clonally expanded. The κ light chain did not show a dominant band, but a clone with a 12-amino acid CDR3 showed 3% clonal expansion. Somatic mutations were frequently observed, with a high ratio of replacement to silent mutations in the CDRs compared with the framework regions. Three Ig genes expressed in the clonally expanded B lymphocytes were derived from germline gene segments reported to be involved in the production of autoantibodies.

Conclusion. These results support the hypothesis that antigen-driven clonal B lymphocyte proliferation occurs in the synovium in BD. Immunoglobulin transcripts clonally expanded in the synovium were identified.

Behçet’s disease (BD) is a chronic syndrome that causes recurrent oral and genital ulcers, skin rashes, and uveitis. BD also affects various other organs, including the joints. The disease is proposed to be of viral or bacterial origin, but its exact cause is not clear. Several studies suggest that there are abnormalities of the cells involved in cell-mediated immunity, including T lymphocytes, monocyte/macrophages, and neutrophils (1,2). Abnormalities of humoral immunity have also been reported. IgM and C3 have been shown to be deposited in oral ulcers and erythema nodosum–like lesions in BD patients (3). In the peripheral blood, the B lymphocytes that spontaneously secrete immunoglobulins are increased, yet mitogen-induced proliferation is decreased (4). Increases in serum Ig and autoantibodies, such as antinuclear antibodies and anti–endothelial cell antibodies, have also been reported (5).

Plasma cells infiltrate the synovial tissue in BD, and apparent germinal centers are observed in the arthritic tissue (6,7). These findings, coupled with the knowledge that Ig accumulate on the surface of the synovial tissue, suggest that B lymphocytes are involved in the development of arthritis. However, the cause of proliferation and infiltration of B lymphocytes in the synovium is not known.

The third complementarity-determining region (CDR3) of Ig is generated by the rearrangement of germline V–(D)–J gene segments. This allows the length of CDR3 to be used as a clonal marker (8). CDR3 fingerprinting analysis, a 2-stage anchored polymerase
chain reaction (PCR), can be used to investigate the clonality of B and T lymphocytes.

To determine whether the B lymphocytes in the synovial tissues of BD patients with arthritis have undergone clonal expansion, clonality was examined using CDR3 fingerprinting analysis of the Ig heavy and light chains expressed in these tissues. To identify the germline origin, the nucleotide sequences of Ig genes showing clonal expansion were analyzed. Our results suggest that B lymphocytes proliferate in response to an antigen and are involved in the pathogenic mechanism of arthritis in BD.

## PATIENTS AND METHODS

### Study subjects

The patient, a 37-year-old woman, came to our hospital because of a 3-year history of recurrent knee arthritis. Three years previously, she had been diagnosed as having BD, with aphthous orogenital ulcers and erythema nodosum–like skin lesions, according to the diagnostic criteria of the International Study Group (9). Serum rheumatoid factor was negative. Arthroscopic synovectomy of the knee was performed because of her recurrent arthritis, although she had been treated with antiinflammatory drugs. There was mild synovial hypertrophy and some inflammatory cells in the synovium, but no granuloma was seen.

As a control, peripheral blood was obtained from a healthy 32-year-old woman who had no history of orogenital ulcerations or other autoimmune diseases.

### CDR3 fingerprinting analysis

Total RNA was extracted from the peripheral blood lymphocytes (PBL) and synovial cells (SC) obtained from the BD patient, as well as from the PBL obtained from the control subject. First-strand complementary DNA (cDNA) was prepared with an oligo(dT) primer. The first-stage PCR was performed with a set of family-specific sense and antisense primers (Table 1) to amplify the variable regions of the Ig. The conditions of the first-stage PCR were 1 cycle of denaturation at 94°C for 3 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute, and a final extension at 72°C for 7 minutes. The predicted PCR products were ~500 bp and were confirmed by 1.5% agarose gel electrophoresis.

Second-stage PCR was performed with the first-stage PCR products to amplify the CDR3 with a set of primers. The forward internal primer included LSK56, LSK64, or LSK65; the reverse internal primer included LSK57 or LSK66 (Table 1). The conditions of the second-stage PCR were 1 cycle of denaturation at 94°C for 3 minutes, 15 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute, and a final extension at 72°C for 7 minutes. Polyacrylamide gel (6%) electrophoresis was done with the second-stage PCR products. The distribution of the CDR3 length was observed visually. A predominant band was defined as the only dominant band in a specific family.

As a control, peripheral blood was obtained from a healthy 32-year-old woman who had no history of orogenital ulcerations or other autoimmune diseases.

### Table 1. Primers used for the first- and second-stage polymerase chain reaction of the third complementarity-determining region fingerprinting analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene sequence</th>
<th>Location within gene of origin</th>
<th>Orientation</th>
<th>Sequence, 5′→3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSK44</td>
<td>V_H1 Leader</td>
<td>Sense</td>
<td>CGGAATTCATGGAGCTGGAA(GA)(GC(TC(T)TC(T)</td>
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<td>LSK45</td>
<td>V_H2 Leader</td>
<td>Sense</td>
<td>CGGAATTCATGGAGCTGGAA(GA)(GC(TC(T)TC(T)</td>
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<td>LSK46</td>
<td>V_H3 Leader</td>
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<tr>
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<td>V_H4 Leader</td>
<td>Sense</td>
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<tr>
<td>LSK48</td>
<td>V_H5 Leader</td>
<td>Sense</td>
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<tr>
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<td>V_H6 Leader</td>
<td>Sense</td>
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<tr>
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<td>V_L1 Leader</td>
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<td>LSK53</td>
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<td>LSK55</td>
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<td>LSK56</td>
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<tr>
<td>LSK57</td>
<td>J_H6 Leader</td>
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<td>LSK58</td>
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<td>LSK60</td>
<td>C_L Proximal</td>
<td>Antisense</td>
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* Nucleotides in parentheses indicate sites of degeneracy. FR3 = framework region 3.
RESULTS

CDR3 fingerprinting analysis of PBL from the control subject demonstrated a lack of dominant bands of the μ heavy chain, but 1 dominant band in the V_{H4} family of the γ heavy chain was expressed. The PBL from the Behçet’s disease (BD) patient, however, expressed dominant bands in the V_{H1} and V_{H4} families of the μ chain and in the V_{H3}, V_{H4}, V_{H5}, and V_{H6} families of the γ chain. In synovial cells (SC) from the BD patient, the μ chain showed dominant bands in the V_{H1} and V_{H4}, V_{H5}, and V_{H6} families and the γ chain showed dominant bands in the V_{H1}, V_{H2}, V_{H3}, V_{H5}, and V_{H6} families (A and B). In the SC, a predominant band was observed in the V_{H4} family of the μ chain and the V_{H3} family of the γ chain (white asterisk). In terms of the κ light chain, the control PBL and the BD PBL and SC showed a similar pattern of distribution, consisting of 8–11–amino acid CDR3, and dominant bands were not observed visually. A CDR3 band (white asterisk) consisting of 12 amino acids was observed in SC from the BD patient (C), but not in PBL from either the control subject or the BD patient. a.a. = amino acid.

Figure 1. Third complementarity-determining region (CDR3) fingerprinting analysis of immunoglobulin μ heavy, γ heavy, and κ light chains. Using complementary DNA, we amplified the Ig variable region in the first-stage polymerase chain reaction (PCR) and the CDR3 in the second-stage PCR, and then performed polyacrylamide gel electrophoresis. Peripheral blood lymphocytes (PBL) from the control subject lacked dominant bands in the μ chain but expressed 1 dominant band in the V_{H4} family of the γ chain (A and B). PBL from the Behçet’s disease (BD) patient, however, expressed dominant bands in the V_{H1} and V_{H4} families of the μ chain and in the V_{H3}, V_{H4}, V_{H5}, and V_{H6} families of the γ chain. In synovial cells (SC) from the BD patient, the μ chain showed dominant bands in the V_{H1}, V_{H2}, V_{H4}, V_{H5}, and V_{H6} families and the γ chain showed dominant bands in the V_{H1}, V_{H2}, V_{H3}, V_{H5}, and V_{H6} families (A and B). In the SC, a predominant band was observed in the V_{H4} family of the μ chain and the V_{H3} family of the γ chain (white asterisk). In terms of the κ light chain, the control PBL and the BD PBL and SC showed a similar pattern of distribution, consisting of 8–11–amino acid CDR3, and dominant bands were not observed visually. A CDR3 band (white asterisk) consisting of 12 amino acids was observed in SC from the BD patient (C), but not in PBL from either the control subject or the BD patient. a.a. = amino acid.
derived from the same clone because we did not use a proofreading DNA polymerase. In other words, 7g3-15 showed 4–8% clonal expansion in the VH3 family. This clone, which consisted of 470 bases, was constructed by recombination of the germline gene V3-30, a part of the DXP\textsubscript{9}1 gene, and the JH3 gene. The sequence of the VH gene segment was 95.4% homologous with the V3-30 sequence. Also, nongermline-encoded nucleotide sequences were observed. In the FRs, 7 of 12 mutations were replacement mutations (R:S ratio 1.4). All 6 mutations found in CDRs were replacement mutations.

Of 31 colonies having a \kappa light chain variable region, only 1 (7k3-12) had a 12-amino acid CDR3 and nucleotide sequences that were identical to the 3k12 probe (Figure 2C). Therefore, 7k3-12 showed 3% clonal expansion in the V\textsubscript{k}3 family. A 7k3-12 clone consisting of 456 nucleotides was made by recombination of the germline gene Humkv328h5 with the J\textsubscript{k}3 gene.

Figure 2. The nucleotide sequence of immunoglobulin \mu heavy, \gamma heavy, and \kappa light chain transcripts, showing clonal expansion in the synovial cells from the patient with Behc\text{"}et's disease. The top row is the nucleotide sequence of the germline Ig gene. If the nucleotide sequence of the transcripts was identical to that of the germline, a dot was printed; if the sequence differed, a letter indicating the mutation was printed. A, Nucleotide sequence of 7m4-9 IgM transcript clone (GenBank accession no. AF135164). This clone was synthesized by a recombination of germline gene V4-39, DXP\textsubscript{9}1 gene, and JH3 gene. A total of 22 somatic mutations were observed. \# = 7m4-9 and 7 other clones all showed identical nucleotide sequences. B, Nucleotide sequence of the 7g3-15 IgG transcript clone (GenBank accession no. AF103795). This clone was made by recombining germline gene V3-30, DXP\textsubscript{9}1 gene, and JH3 gene. Nineteen somatic mutations were observed. \# = 7g3-15 and 1 other clone showed identical nucleotide sequences. 7g3-1 and 7g3-5 differed from 7g3-15 by only 1 and 2 nucleotide sequences, respectively. C, Nucleotide sequence of the 7k3-12 Igk transcript clone (GenBank accession no. AF103873). Recombining the germline gene Humkv328h5 and the Jk3 gene generated this clone. A total of 12 somatic mutations were observed. FR = framework region; CDR = complementarity-determining region.

were replacement mutations (R:S ratio 1.4). All 6 mutations found in CDRs were replacement mutations.
sequence of the \( V_\kappa \) gene segment showed 96.8% similarity to the Humkv328h5. Interestingly, the 7k3-12 had a nongermline-encoded nucleotide (GGAGGAGC) between \( V_\kappa \) and \( J_\kappa \) gene. There were 7 mutations in FRs, and 4 of these were replacement mutations (R:S ratio 1.3). In CDRs, there were 3 replacement mutations in a total of 4 mutations (R:S ratio 3).

**DISCUSSION**

The clonal dominance of B lymphocytes was rare in the PBL from the control subject, yet in both the PBL and the SC from the BD patient, it was abundant. Several families belonging to \( \mu \) and \( \gamma \) chains expressed various dominant bands in the PBL and the SC from the BD patient. If it is accepted that the clonal expansion of B lymphocytes is taking place systemically, this suggests that the B lymphocyte is involved in the pathogenic mechanism of BD. This finding coincides with the high levels of IgG, IgM, and IgA in the blood of patients with BD (5).

The predominant band in the VH4 family of the \( \mu \) chain and the VH3 family of the \( \gamma \) chain in the SC from the BD patient showed differences in CDR3 length compared with the same families in the PBL. These results suggest that the PBL and SC proliferated in response to different antigenic epitopes in the BD patient. Patients with rheumatoid arthritis (RA) have different patterns of clonal expansion in the PBL and synovial fluid based on CDR3 fingerprinting analysis (10), and this difference is more marked in the synovium (11). It is of interest that clonal predominance was observed in the PBL from the healthy control subject. The significance of this finding is not clear, but in a similar fingerprinting analysis using T lymphocytes from monozygotic twins, the investigators reported that clonal expansion might have occurred because of environmental factors, such as bacterial infection (12).

Unlike the heavy chains, clonal dominance was not observed for the \( \kappa \) light chain. In the \( \kappa \) light chain, a 9–amino acid CDR3 was mainly expressed, and the pattern of distribution did not differentiate between the BD patient and the control subject. This is presumed to be because the functional genes in the germline are less abundant and there is no D gene. What is interesting to us is that a band corresponding to CDR3 with 12 amino acids was observed in the \( V_\kappa \)3 family of the SC. To our knowledge, this is the first \( \kappa \) light chain transcript that has a 12–amino acid CDR3. The significance of this unusually long CDR3 in a patient with BD is not known; however, a study by Lee et al (13) demonstrated 11–amino acid CDR3 expressed abundantly in the synovial tissue of RA patients.

That the clonal dominance observed in the CDR3 fingerprinting analysis is in fact due to clonal expansion was demonstrated by the results of colony hybridization. In the VH4 family of the \( \mu \) chain, there was 18.2% clonal expansion in the synovium. In the VH3 family of the \( \gamma \) chain, 4–8% clonal expansion was observed. In the \( V_\kappa \)3 family, there was 3% clonal expansion. This study is a PCR-based analysis, so it cannot be directly compared with a synovial cDNA library. However, it is comparable in terms of the percentage of clonal expansion in RA. In the synovium of RA patients, 2 of 67 \( \gamma \) chain recombinants were found to contain nearly identical \( V_{H4^-}D_{H4^-}J_{H4} \) joins and to share somatic mutations, showing 3% clonal expansion (14). Of 64 \( \kappa \) light chain recombinants in the cDNA libraries, 3 (5%) had identical nucleotide sequences (15).

B cells that are bound to antigen proliferate and acquire somatic mutations. These mutations cause the affinity of the antigen-binding sites to change. In the case of antibody, the R:S ratio of the FRs must be \( \leq 1.5 \) in order to conserve function. However, the R:S ratio is \( > 2.9 \) in the CDR3, which means that the clone with high affinity for antigen is selected (16). Clone 7m4-9 of the \( \mu \) chain revealed that the R:S ratio was 1 in the FRs but 4 in the CDRs. This phenomenon was also observed with the \( \gamma \) chain (7g3-15). In the FRs, the R:S ratio was 1.4, but all 6 mutations in the CDRs were replacement mutations. In the clone 7k3-12, the R:S ratio was 1.4 in the FRs but 3 in the CDRs. This pattern is understood to be a result of antigen-specific clonal expansion.

It is interesting that the 3 Ig genes that showed clonal expansion in the SC were derived from the germline gene involved in the production of autoantibodies. It has been reported that V4-39 was used for making an autoantibody against thyroglobulin and V3-30 was used for making a rheumatoid factor and anti–double-stranded DNA antibody (17). Humkv328h5 is very similar to humkv328, which is commonly used in rheumatoid factors. Although these data were derived from only 1 BD patient and the specificity of the cloned Ig is not known, they do suggest that B lymphocytes showing clonal expansion have been selected by an autoantigen(s) and further suggest an autoimmune mechanism in BD. In the synovial tissue of RA patients, evidence of clonal expansion has been reported and was shown to be used in the production of autoantibodies (14,15). In future studies in which we recombine the Ig that showed clonal expansion and study the antigen
specificity, we should be able to elucidate the pathogenesis of BD.

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REFERENCES