ARTHRITIS & RHEUMATISM
Vol. 44, No. 7, July 2001, pp 1707–1712

Published by Wiley-Liss, Inc.

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_H4 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_H3 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

Supported by grant HMP-97-M-2-0041 from the Ministry of Health and Welfare of South Korea.

Chang Hee Suh, MD, PhD, Yong Beom Park, MD, Jungsik Song, MD, Chan Hee Lee, MD, PhD, Soo Kon Lee, MD, PhD: Yonsei University College of Medicine, Seoul, South Korea.

Address correspondence and reprint requests to Soo Kon Lee, MD, PhD, Division of Rheumatology, Department of Internal Medicine, Yonsei University College of Medicine, CPO Box 8044, Seoul, South Korea.

Submitted for publication November 2, 2000; accepted in revised form February 19, 2001.

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

Primer	Gene sequence	Location within gene of origin	Orientation	Sequence, 5′→3′
LSK44	V_H1	Leader	Sense	CGGAATTCATGGACTGGACCTGGAGG(A/G)TC(C/T)TCT
LSK45	V_H2	Leader	Sense	CGGAATCATGGACA(C/T)ACTTTG(C/T)TCCACGCTCC
LSK46	V_H3	Leader	Sense	CGGAATTCATGGAGT(C/T)TGGGCTGAGCTGG(C/G)TTT
LSK47	V_H4	Leader	Sense	CGGAATTCATGAA(A/G)CA(C/T)CTGTGGTTCTTCCT(C/T)C
LSK48	V_H5	Leader	Sense	CGGAATTCATGGGGTCAACCGCCATCCTCGCCC
LSK49	V_H6	Leader	Sense	CGGAATTCATGTCTGTCTCCTTCCTCATCTTCC
LSK50	C_μ	$C_H1(5')$	Antisense	GCTCTAGAAGACGAGGGGGAAAAGGGTT
LSK51	C_γ	$C_H1(5')$	Antisense	GCTCTAGAGGAGGAGGGTGCCAGGGG
LSK58	$V_\kappa1$	Leader	Sense	CGGAATTCATGGACATGAGGGTCC(C/T)CGCTCAGC
LSK59	$V_\kappa2$	Leader	Sense	CGGAATTCATGAGGCTCCCTGCTCAGCTCCTGG
LSK60	$V_\kappa3$	Leader	Sense	CGGAATTCATGGAA(A/G)CCCCAGC(G/T)CAGCTTCTCT
LSK61	$V_\kappa4$	Leader	Sense	CGGAATTCATGGTGTTGCAGACCCAGGTCTTCA
LSK62	C_κ	C(5′)	Antisense	GCTCTAGATTTCAACTGCTCATCAGATGG
LSK56	V_H1-6	FR3	Sense	CGGAATTCACAC(C/G/T)GC(C/T)(A/G)TGTATTACTGTGC
LSK57	J_H1-6	J_H	Antisense	GCTCTAGATGA(A/G)GAGAC(A/G)GTGACC
LSK64	$V_\kappa1,3,4$	FR3	Sense	CGGAATTCACTCTCACCATCAGCAG
LSK65	$V_\kappa2$	FR3	Sense	CGGAATTCACACTGAAAATCAGC
LSK66	C_κ	Proximal to LSK62	Antisense	GCTCTAGACAGATGGTGCAGCCAC

* Nucleotides in parentheses indicate sites of degeneracy. FR3 = framework region 3.

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, extension at 72°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 γ^{32}P-dATP–labeled 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, extension at 72°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.

Colony hybridization. To identify the CDR3 sequence of the predominant band, we cut the band and eluted the DNA. The second-stage PCR was performed again to amplify the eluted DNA, and subsequent ligation into a pT7 Blue T vector (Novagen, Madison, WI) and transformation into *Escherichia coli* (DH5α) were performed. After sequencing, a CDR3 probe was synthesized to find the whole Ig variable-region sequence. After ligating the first-stage PCR product of the same family and transforming it into *E coli*, colony hybridization was performed with the γ^{32}P-dATP–labeled CDR3 probe. A probe-positive colony was identified and then sequenced.

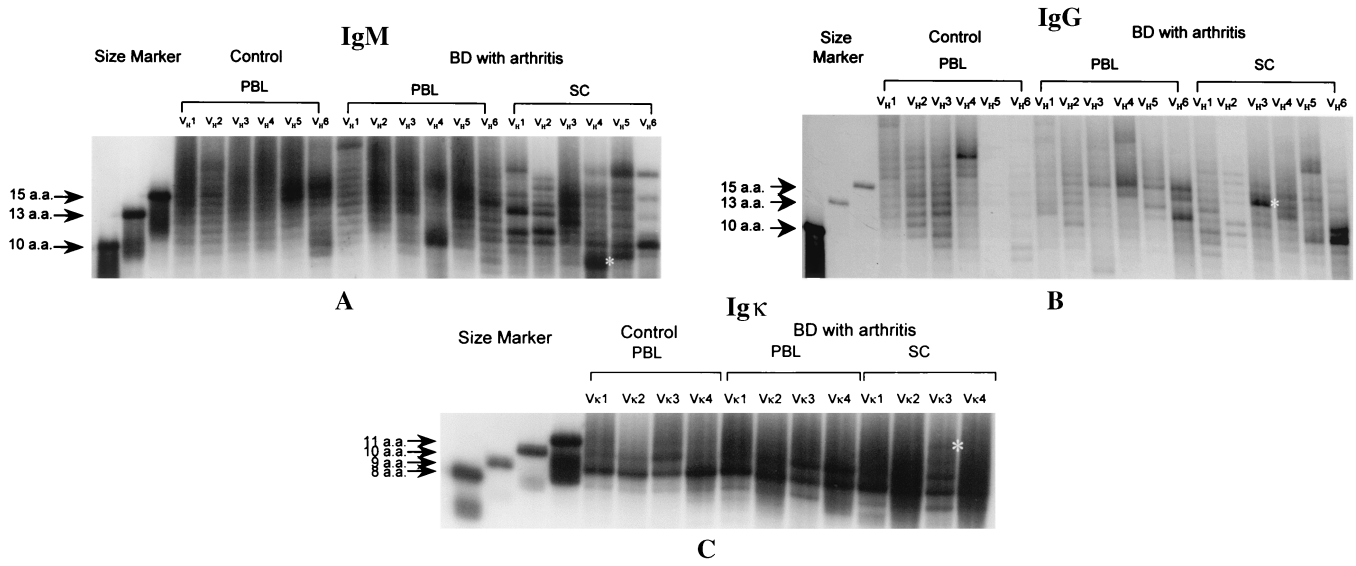

Figure 1. Third complementarity-determining region (CDR3) fingerprinting analysis of immunoglobulin μ heavy, γ heavy, and κ light chains. Using complementary DNA, we amplified the Ig variable region in the first-stage polymerase chain reaction (PCR) and the CDR3 in the second-stage PCR, and then performed 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.

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 BD patient, however, demonstrated dominant bands in several families of the μ and γ chains. The SC from the BD patient showed dominant bands in nearly all families of the μ and γ chains (Figures 1A and B). The dominant bands of each family in the SC from the BD patient differed in length from those of the same family in the patient's PBL. Clonal predominance was observed in the SC: the V_H4 family of the μ chain and the V_H3 family of the γ chain. No dominant band was observed in κ light chains, but in the SC, a 12–amino acid CDR3 band appeared in the $V_\kappa3$ family (Figure 1C).

After sequencing the predominant band of μ and γ heavy chains and the band with the 12–amino acid CDR3 of the κ light chain, we synthesized CDR3 signature probes. Each of the μ chain (4m9, 5′-CTC-GGG-ATG-GGA-GCT-TTT-GAT-TTC-3′), γ chain (3g15, 5′-GCT-CGG-GGA-GTT-ACC-CCT-GAT-GCT-3′), and κ light chain (3k12, 5′-AAT-AAC-TGG-CCT-CCG-GAG-GAG-CTC-3′) signature probes was synthesized as a 24-mer.

After colony hybridization with the 4m9 probe, 8 of 44 colonies that had a μ-chain variable-region insert showed identical sequences throughout the whole variable region (7m4-9). In particular, in the V_H4 family, 7m4-9 showed 18.2% clonal expansion. This clone consisted of 450 bases and was generated as a result of recombination of the germline gene V4-39 (94.1% homology) with a part of the DXP′1 gene and the J_H3 gene (Figure 2A). Nongermline-encoded nucleotides were observed between the V_H and D genes and between the D and J_H genes. There were 10 mutations in framework regions (FRs), and 5 of them were replacement mutations; the ratio of replacement to silent mutations (R:S) was therefore 1. In CDRs, 8 of 10 mutations were replacement mutations, and the R:S ratio was 4.

Of 50 colonies having γ-chain variable regions, 2 (one of which is 7g3-15) showed identical sequences and 2 others differed from 7g3-15 by only 1 or 2 nucleotides (Figure 2B). It is probable that these 4 colonies were

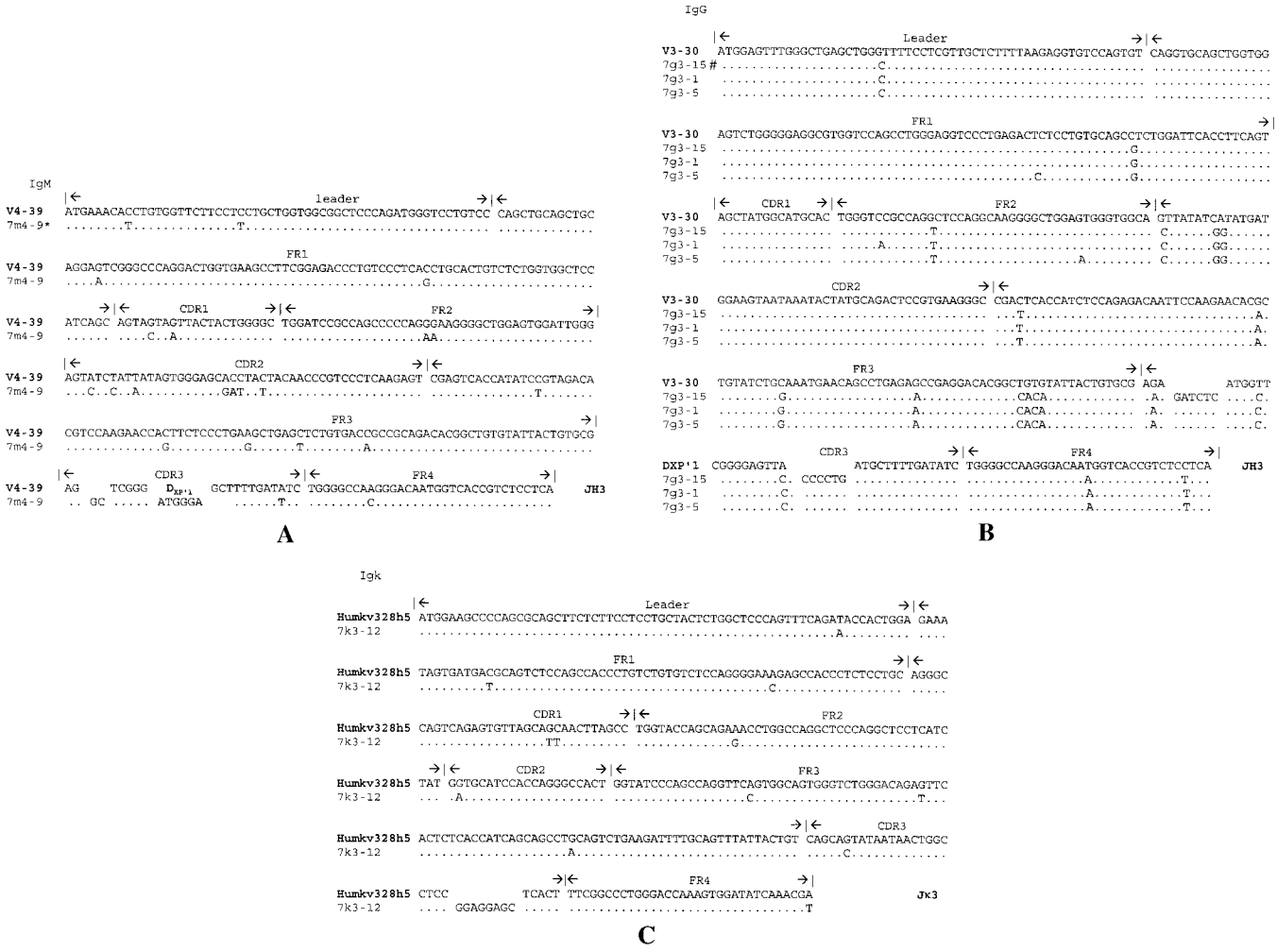

Figure 2. The nucleotide sequence of immunoglobulin μ heavy, γ heavy, and κ light chain transcripts, showing clonal expansion in the synovial cells from the patient with Behçet's disease. The top row is the nucleotide sequence of the germline Ig gene. If the nucleotide sequence of the transcripts was 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′1 gene, and J_H3 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′1 gene, and J_H3 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 Igκ 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.

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 V_H3 family. This clone, which consisted of 470 bases, was constructed by recombination of the germline gene V3-30, a part of the DXP′1 gene, and the J_H3 gene. The sequence of the V_H 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 κ 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 $V_\kappa3$ family. A 7k3-12 clone consisting of 456 nucleotides was made by recombination of the germline gene Humkv328h5 with the $J_\kappa3$ gene. The

sequence of the V_κ gene segment showed 96.8% similarity to the Humkv328h5. Interestingly, the 7k3-12 had a nongermline-encoded nucleotide (GGAGGAGC) between V_κ and J_κ 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 μ and γ 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 V_H4 family of the μ chain and the V_H3 family of the γ 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 κ light chain. In the κ 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 $V_\kappa3$ family of the SC. To our knowledge, this is the first κ 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 V_H4 family of the μ chain, there was 18.2% clonal expansion in the synovium. In the V_H3 family of the γ chain, 4–8% clonal expansion was observed. In the $V_\kappa3$ 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 γ chain recombinants were found to contain nearly identical V_H–D_H–J_H joins and to share somatic mutations, showing 3% clonal expansion (14). Of 64 κ 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 μ chain revealed that the R:S ratio was 1 in the FRs but 4 in the CDRs. This phenomenon was also observed with the γ 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 7κ3-12, 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.

ACKNOWLEDGMENT

We thank Dr. Philip L. Cohen (Division of Rheumatology, University of Pennsylvania, Philadelphia) for critical reading of the manuscript.

REFERENCES

1. Sakane T, Suzuki N, Ueda Y, Takada S, Murakawa Y, Hoshino T, et al. Analysis of interleukin-2 activity in patients with Behçet's disease: ability of T cells to proliferate and respond to interleukin-2. Arthritis Rheum 1986;29:371–8.
2. Emmi L, Brugnolo F, Salvati G, Marchione T. Immunopathological aspects of Behçet's disease. Clin Exp Rheumatol 1995;13: 687–91.
3. Kaneko F, Takahashi Y, Muramatsu Y, Miura Y. Immunological studies on aphthous ulcer and erythema nodosum-like eruptions in Behçet's disease. Br J Dermatol 1985;113:303–12.
4. Suzuki N, Sakane T, Ueda Y, Tsunematsu T. Abnormal B cell function in patients with Behçet's disease. Arthritis Rheum 1986; 29:212–9.
5. Taylor PV, Chamberlain MA, Scott JS. Autoreactivity in patients with Behçet's disease. Br J Rheumatol 1993;32:908–10.
6. Yurdakul S, Yazici H, Tuzun Y, Pazarli H, Yalcin B, Altac M, et al. The arthritis of Behçet's disease: a prospective study. Ann Rheum Dis 1983;42:505–12.
7. Gibson T, Laurent R, Highton J, Wilton M, Dyson M, Millis R. Synovial histopathology of Behçet's syndrome. Ann Rheum Dis 1981;40:376–81.
8. McCarty KP, Sloane JP, Wiedemann LM. Rapid method for distinguishing clonal from polyclonal B cell populations in surgical biopsy specimens. J Clin Pathol 1990;43:429–32.
9. International Study Group for Behçet's Disease. Criteria for diagnosis of Behçet's disease. Lancet 1990;335:1078–80.
10. Moyes SP, Maini RN, Mageed RA. Differential use of immunoglobulin light chain genes and B lymphocyte expansion at sites of disease in rheumatoid arthritis (RA) compared with circulating B lymphocytes. Clin Exp Immunol 1998;113:276–88.
11. Patki V, Monteiro J, Gregersen PK, Chiorazzi N. Evidence for B cell oligoclonality in the blood and joints of patients with rheumatoid arthritis. Ann N Y Acad Sci 1997;815:472–4.
12. Monteiro J, Hingorani R, Choi IH, Silver J, Pergolizzi R, Gregersen PK. Oligoclonality in the human CD8+ T cell repertoire in normal subjects and monozygotic twins: implications for studies of infectious and autoimmune diseases. Mol Med 1995;1:614–24.
13. Lee SK, Song CH, Kim JB, Chwae YJ, Choi IH, Bridges SL Jr, et al. Enhanced expression of immunoglobulin light chains with unusually long CDR3 regions in patients with rheumatoid arthritis. J Rheumatol 1998;25:1067–71.
14. Clausen BE, Bridges SL Jr, Lavelle JC, Fowler PG, Gay S, Koopman WJ, et al. Clonally-related immunoglobulin VH domains and nonrandom usage of DH gene segments in rheumatoid arthritis synovium. Mol Med 1998;4:240–57.
15. Lee SK, Bridges SL Jr, Kirkham PM, Koopman WJ, Schroeder HW Jr. Evidence of antigen receptor-influenced oligoclonal B lymphocyte expansion in the synovium of a patient with longstanding rheumatoid arthritis. J Clin Invest 1994;93:361–70.
16. Shlomchik MJ, Nemazee DA, Sato VL, van Snick J, Carson DA, Weigert MG. Variable region sequences of murine IgM anti-IgG monoclonal autoantibodies (rheumatoid factors): a structural explanation for the high frequency of IgM anti-IgG B cells. J Exp Med 1986;164:407–27.
17. Cairns E, Kwong PC, Misener V, Peter IP, Bell DA, Siminovitch KA. Analysis of variable region genes encoding a human anti-DNA antibody of normal origin: implications for the molecular basis of human autoimmune response. J Immunol 1989;143: 685–91.