EBV Gene Expression Not Altered in Rheumatoid Synovia Despite the Presence of EBV Antigen-Specific T Cell Clones

James W. Edinger, Marc Bonneville, Emmanuel Scotet, Elisabeth Houssaint, H. Ralph Schumacher and David N. Posnett

*J Immunol* 1999; 162:3694-3701;
http://www.jimmunol.org/content/162/6/3694

References

This article cites 67 articles, 43 of which you can access for free at:
http://www.jimmunol.org/content/162/6/3694.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
EBV Gene Expression Not Altered in Rheumatoid Synovia Despite the Presence of EBV Antigen-Specific T Cell Clones

James W. Edinger,2* Marc Bonneville,† Emmanuel Scotet,† Elisabeth Houssaint,‡ H. Ralph Schumacher,§ and David N. Posnett2*

T cells infiltrating the rheumatoid arthritis (RA) joint are oligoclonal, implicating an Ag-driven process, but the putative joint-specific Ags remain elusive. Here we examine expression of selected EBV genes in RA synovia and find no abnormal expression in RA. DNA of CMV and EBV was detectable by PCR in the synovial tissue of RA. RNA of several latent and lytic EBV genes was also detectable. However, there were no differences in EBV gene expression in synovial tissues or peripheral blood when comparing RA with osteoarthritis, Gulf War syndrome, and other disease controls. RA synovia with highly expanded CD8 T cell clones reactive with defined EBV peptide Ags presented by HLA class I alleles lacked evidence of abnormal mRNA expression for the relevant EBV Ag (BZLF1) or lacked amplifiable mRNA (BMLF1). Thus, local production of EBV Ags in synovial tissues may not be the cause of the accumulation of T cell clones specific for these Ags. Instead, APCs loaded with processed EBV peptides may migrate to the synovium. Alternatively, EBV-specific T cell clones may be generated in other tissues and then migrate to synovia, perhaps due to cross-reactive joint-specific Ags or because of expression of homing receptors. The Journal of Immunology, 1999, 162: 3694–3701.

It is likely that T cells play a role in the immunopathogenesis of rheumatoid arthritis (RA)1 (1). There is abundant evidence from TCR repertoire studies that T cells in the inflammatory joint of RA contain expanded clones indicative of an Ag-driven process (2, 3). These observations apply to both CD4 and CD8 T cells and to T cells in synovial fluid or synovial biopsy tissues. There are two possible explanations for these findings. First, there may be expression of synovial Ags in RA resulting in local expansion of T cell clones. Second, there may be systemic activation of T cell clones due to expression of Ags in other tissue compartments. These activated T cells might then home to synovial tissues either because of cross-reactivity with joint tissue Ags or because of expression of the correct set of adhesion and homing molecules without a requirement for Ag expression in the joint. Most TCR repertoire studies describe joint-specific accumulation of T cell clones (2, 3), but some reports describe T cell clonal expansions that are more prominent in the peripheral blood (4–8), indicating perhaps that there are extrasynovial Ags driving T cell clonal expansions that do not migrate to synovial tissues.

In considering potential T cell Ags in RA, recent studies have revived an older literature on herpes viruses, in particular EBV (9). These studies showed that in the joints of patients with RA, there were large numbers of CD8 T cells that were specific for EBV transactivator gene products, such as BZLF1 and BMLF1 (10, 11).

In Japanese patients infected with HTLV-1, a clinical disease indistinguishable from idiopathic RA has been described (12). Exposure of synoviocytes to the tax protein (HTLV-1 transactivator) resulted in increased mRNA levels of cytokines, such as IL-1β, IL-6, and TNF-α (13). A mouse transgenic for the HTLV-1 tax gene developed RA-like disease (14). These observations indicate that an intact virus may not be required to develop arthritis. Expression of a viral transactivator protein alone may suffice to induce inflammatory cytokines and an organ-specific autoimmune disease.

By analogy with the HTLV-1 tax model, we postulated that herpes viral transactivators might also play a role in synovial inflammation. The EBV transactivators, BZLF1 and BMLF1, are associated with lytic infection of B lymphocytes. Synovium is not known as a site of lytic EBV infection, but it is at least theoretically possible that transactivators are expressed during abortive viral replication, perhaps in unusual host cells such as synoviocytes (15). Previous studies had addressed the presence of herpes viral DNA in RA synovia by PCR and were inconclusive (16, 17). No studies have focused on transcription of herpes viral mRNAs in synovial tissues.

In this work, synovial tissues from patients with RA were examined for the presence of human herpesvirus (HHV) 4 (EBV), HHV5 (CMV), HHV6, HHV7, and HHV8) DNA by sensitive radioactively labeled PCRs. EBV DNA was most prevalent. Synovial tissues were next examined for the presence of viral transcripts that encode potential T cell Ags. Samples of particular interest were from patients with documented synovial T cell clones specific for EBV Ags. We considered two possible outcomes. 1) EBV viral transcripts could be increased in RA compared with controls, indicating that viral Ags might be driving the expansion of synovial T cell clones specific for these Ags. 2) mRNA for the viral Ags might not

---

*Immunology Program, Graduate School of Medical Sciences, and Department of Medicine, Weill Medical College, Cornell University Weill, New York, NY 10021; †Institut National de la Santé et de la Recherche Médicale, Unit 463, Institut de Biologie, Nantes, France; and §Veterans Affairs Medical Center, University of Pennsylvania, Philadelphia, PA 19104

Received for publication June 10, 1998. Accepted for publication December 11, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by U.S. Public Health Service Grants RO-1 AI22333 and RO-131140 (to D.N.P.), a grant from the Association pour la Recherche sur la Polyarthrite (to M.B.), and institutional grants from the Institut National de la Santé et de la Recherche Médicale.

2 Address correspondence and reprint requests to Drs. James W. Edinger or D. N. Posnett, Weill Medical College, Cornell University Weill, 1300 York Avenue, Box 56, New York, NY 10021.

3 Abbreviations used in this paper: RA, rheumatoid arthritis; GW, Gulf War syndrome; SLE, systemic lupus erythematosus; HHV, human herpesvirus; NT, PBL depleted of T cells by rosetting with SRBC; dNTP, deoxynucleotide triphosphate; CDR, complementarity-determining region.
be increased in RA or even absent, indicating that the corresponding synovial T cell clones were cross-reactive with unidentified self-Ags or that they homed to the synovium independent of synovial Ag expression.

Materials and Methods

The synovial tissue samples used were from four sources: Rheumatoid Arthritis Registry, The Hospital for Special Surgery (New York, NY); H. R. Schumacher, Rheumatology Clinic, Veterans Affairs Medical Center (Philadelphia, PA); A. Alam, Toulouse, France (frozen samples) (previously used; Ref. 18); and Nantes, France (frozen samples) (previously used; Refs. 10 and 11). All human samples were obtained in the context of studies approved by institutional review boards. Samples were either synovial tissues obtained at surgery and sometimes by blind needle biopsy or synovial fluids obtained by needle aspiration. RA patients fulfilled at least five of seven ACR criteria as proposed by Arnett et al. (19), except for patient 11, who fulfilled four of seven of the criteria. The diagnosis of RA had been made 5–39 years before study. Patients with Gulf War syndrome (GW) complained of articulargia and myalgias but had no arthritis on examination or by radiographic criteria. Other patients included a group with osteoarthritis (OA) and a single patient with systemic lupus erythematosus (SLE). Note: the frozen synovial DNA samples from RA patients (see Fig. 3) were all from the Veterans Affairs Medical Center (Philadelphia, PA), as were RNA samples from GW patients. OA, SLE, normal controls, RA synovial tissue samples 5–26, and the sample from patient JCM (see Fig. 3) were from New York. RA samples A and B were from New York, A and B were from Toulouse, France. RA1, RA3, RA4, RA19, and RS2 were from Nantes, France. For the samples from Toulouse, T1 and T2 refer to different time points of sampling, while A and B refer to normal samples from different joints. In some cases (6a, 6b, 20a, 20b, 27a, 27b, GW7a, GW7b, GW11a, GW11b, and GW11c), surgically obtained tissue was divided into 1-mm³ samples (designated a, b, or c) and assessed in parallel. Normal samples were either PBL (see Fig. 2), PBL depleted of T cells by rosetting with sheep red blood cells (NT), or NT treated with PHA (see Fig. 3).

Viral infections to obtain PCR control cDNAs

For infections with HHV6 (strain U1102) and HHV7 (strain J1), which infect T lymphocytes, PBL (5 × 10⁶) from cord blood were cultured in media (RPMI 1640, 10% FCS, 0.2 U/ml penicillin, 0.2 mg/ml streptomycin, 2 mg/ml fungizone, and 2 mM l-glutamine) with PHA (50 μg/ml) for 3 days. The activated PBL were infected with a multiplicity of infection 1 with each virus in the presence of Polybrene (2 μg/ml) for 2 h. The cells were washed three times and cultured for 3 wk with the addition of IL-2 (40 IU/ml) every 3–4 days. The cells were harvested at aliquots of 2 × 10⁶ cells each, and genomic DNA was prepared as described. As a source of EBV, a transformed B cell line, B95-8 (courtesy of E. Cesarman, Weill Medical College, Cornell University), was used. For CMV, MRC5 fibroblasts were infected with aliquots of 10–200 μl of viral containing supernatant from cultured by infectious centers. cDNA synthesis was performed with 50 μmol-Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 5 μg RT6 primer (Promega, Madison, WI), 0.3 U/ml RNase inhibitor (Promega); 5 U of Moloney murine leukemia virus RT (Life Technologies, Grand Island, NY), and 0.5 mM deoxynucleotide triphosphates (dNTPs; Pharmacia, Piscataway, NJ) for 1 h at 42°C, followed by 1 min at 95°C.

Genomic PCR and RT-PCR

The genomic PCRs were performed using the following primers (5′ to 3′) and optimal conditions. In each case, the quantity of primers was 10–100 pmol/reaction.

Primer for EBV (strain B95-8) were the following: sense, AACAT GCTGTATAGCTGCGAGGAC (124,935–124,957), and antisense, AAT TACTGTTGGAATTGTGCCCA (125,109–125,186). Conditions were as follows: 3 mM MgCl₂; 35 PCR cycles at 94°C for 1 min, 58°C for 90 s, and 72°C for 1 min; the product was 175 bp (22).

Primer for CMV (IE1 gene of strain AD169) were the following: sense, TTCTATGCGACACCTGTC (exonon 4), and antisense, TGGAGTCTTGGACAGAGAGA (exon 2). Conditions were as follows: 2.5 mM MgCl₂; 35 PCR cycles at 94°C for 1 min, 60°C for 90 s, and 72°C for 2 min; the product was 581 bp (23).

Primer for HHV6 (strain U1102) were the following: sense, AAGCT TCTGGCAATAAAC (exon 2b, 65°C), and antisense, TAGATTTTTGAAAGATTTAATAA (exon 1 – 171,514). Conditions were as follows: 1.5 mM MgCl₂; 35 PCR cycles at 94°C for 1 min, 55°C for 2 min; the product was 123 bp (24).

Primer for HHV7 (strain J1, primers derived from clone 43L3, 171 nucleotides) were the following: sense, CAGAAATGATAGACAGATGT (exon 1), and the antisense primer was 5′-TGTGGACCTCAGCGG (48,397–48,416); for the Q spliced transcript (238-bp PCR product), the sense primer was 5′-GGTGGTACCTCAGCGG (62,440–62,457); in both cases, the antisense primer was 5′-TATCCACGAGTGTACCT (107,980–107,967) (26).

For the BZLF1 spliced transcript (182-bp PCR product), the sense primer was 5′-TTCACACGCTGCAGCTAGG (102,719–102,700), the antisense primer was 5′-GGCACTGACCAACCTCGAGT (102,330–102,341/102,424–102,433), and the probe was 5′-CCTTAACECTCGGCCAT (27).

For the EBER1 unspliced transcript (167-bp PCR product), the sense primer was 5′-AAACCTACGGGACCCAGC (676–6795) and the antisense primer was 5′-AGGAGCTTGCTGCTCTAGA (6648–6629) (27).

The reaction conditions for each of the above RT-PCRs were as follows: 40 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 1 min; 1.7 mM MgCl₂, and 0.2 mL dNTPs containing [α-32P]dCTP (1 × 10⁶ cpm per reaction, Amersham, Arlington Heights, IL).

For the BMLF1 unspliced transcript (172-bp PCR product), the sense primer was 5′-TAGTCCTCCGCGTTAGAGAAG (83,098–83,118), and the antisense primer was 5′-GGCAGATGTGAAAGAGGC (83,250–83,269). Conditions were 35 PCR cycles at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min; 4 mM MgCl₂, and 0.1 mL of each primer, with radioactive dCTP.

For the BHRF1 unspliced transcript (239-bp PCR product), the sense primer was 5′-TGCCGCTTTCAACAGGG (54,373–54,396), and the antisense primer was 5′-ATCCACTATTCGCTGIGIG (54,596–54,615). Conditions were 36 PCR cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; 1.5 mM MgCl₂, and 0.1 mL of each primer, with radioactive dCTP.

For the actin unspliced transcript with primers in exon 4 (281-bp PCR product), the sense primer was 5′-CTCTGAAATAGCTCCAGC, and the antisense primer was 5′-TTCAGCACTGCTCAG (27). Conditions were 30 PCR cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; 1.5 mM MgCl₂; 12.5 pmol of each primer, with radioactive dCTP.

For the CD19 spliced transcript (170-bp PCR product), the sense primer was 5′-CTCCATATTCAACAGGGGAG (54,377–54,396), and the antisense primer was 5′-ATCCACTATTCGCTGIGIG (54,596–54,615). Conditions were 36 PCR cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; 1.5 mM MgCl₂, and 0.1 mL of each primer, with radioactive dCTP.
implemented with 10% human serum, 1 mM L-glutamine, and rIL-2 (150 ng/ml) as described elsewhere (29). Cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 10% human serum, 1 mM L-glutamine, and rIL-2 (150 ng/ml). Culture supernatants were tested 6 h later for TNF production by a sensitive bioassay (29, 31).

Results

In view of prior reports on both EBV and CMV in RA synovial tissues, initial studies were performed to screen DNA from 11 RA synovial tissue biopsies for viral sequences using sensitive DNA PCR methods. Data on HHV6, HHV7, and HHV8 DNA in RA synovial tissues have not yet been reported. As shown in Fig. 1, specific bands were not observed with primers for HHV6, HHV7, and HHV8. Thus, these viruses are not present in RA synovia at the detection limits of the PCR assays used. However, 10 of 11 samples contained EBV DNA, and in most samples weak bands corresponding to CMV DNA were also observed. Because of the prominence and frequency of the EBV PCR bands, we next examined transcriptional activity of selected EBV genes.

RT-PCR for EBER1 was the most sensitive assay used. EBER RNAs are small, nonpolyadenylated viral RNAs that are by far the most abundant RNAs in latently infected cells (32). Fig. 2 demonstrates the degree of reproducibility between two experiments with this PCR showing the same patterns of band intensities. Samples from normal PBL (NL1–6), RA PBL, and RA synovial tissue were analyzed. The results were compared with RT-PCR using cDNA from the indicated cell number equivalents of an EBV-transformed cell line. The experimental samples, both PBL and synovial tissue, which contained on average 105–106 cell equivalents per PCR, yielded bands in the intensity range below 1 EBV-transformed cell. This indicates that cells transcribing EBER1 are rare and is consistent with estimates of a low frequency of EBV-infected B cells in latently infected individuals, 10–10^4 (33). Fig. 2 further demonstrates the sensitivity of the assay. By analyzing 10-fold dilutions of the cDNA templates, it was shown that most samples contained EBER1 RNA at 1–2 logs above the detection limits of the assay.

A series of similar RT-PCR experiments were next performed to assess transcription of several EBV genes: the immediate early transactivator, BZLF1, and the early transactivator, BMLF1 (32), as well as the latency-associated EBER1, EBNA1 Q, and EBNA1 Y3 (Fig. 3 and data not shown). Each sample analyzed was also tested for the presence of mRNA of a constitutive cellular housekeeping gene (actin). In addition, the samples were tested for the presence of CD19 mRNA as an internal control for the presence of B cells in the tissue sample. CD19 was chosen because of the consistent expression of mRNA throughout differentiation of the B cell lineage (34). Each experiment included the same set of controls: dilutions of template cDNA ranging from 1 to 1000 cell equivalents.
Previous studies have suggested that EBV transcripts may have derived from cell types other than B cells. There were two possible explanations. The CD19 RT-PCR reactions were conducted with RNA from control samples; titered B lymphoblastoid BSKS2— cells (cell equivalents indicated); normal PBL (see Fig. 2), non-T cells in normal PBL (nt), or PHA-treated non-T cells from normal PBL (pha); PBL from a patient with systemic lupus erythematosus (SLE) or from patients with GW; and synovial tissues (st) from disease controls with GW syndrome or with OA. The controls were compared with synovial tissues (st), synovial fluid (sf), or PBL from RA patients. Patients 6 (a through c) and 7 had juvenile RA (JRA). An additional control was RNA from PBL of patient JCM with (+rt) or without (−rt) RT added during cDNA synthesis. Samples from patients P and C (18) and patients RA1 and RA19 (10) have been used in previous publications. Arrowheads indicate that T cells specific for the EBV Ag were present in the same tissue samples (see Table I). Asterisks denote samples of cDNA that were diluted 1/10 before analysis by PCR. All PCRs were repeated three times, and results were generally reproducible within the limits shown in Fig. 2.

A consistent finding was the variable presence of CD19 mRNA in all of the synovial tissue samples. Separate samples dissected from the same surgical tissue (i.e., samples 20a and 20b) yielded variable intensities of the RT-PCR CD19 bands. This is consistent with previous findings on the focal nature of B cell infiltrates in the synovium (35). In some cases, a heavy CD19 band appeared to correlate with up-regulation of EBER1 message, as in samples of normal NT PBL cells stimulated with PHA and in a patient with SLE. However, in most synovial tissue samples there was little correlation between the presence of CD19 and EBV gene expression. Some samples contained EBV transcripts but no detectable CD19. There were two possible explanations. The CD19 RT-PCR may have been less sensitive than the EBV PCRs. Alternatively, viral transcripts may have derived from cell types other than B lymphocytes.

Fig. 3 contains two sets of RA samples analyzed in detail in previous studies. First, samples from patients P and C had been shown to contain well-characterized clonal T cell expansions (18). These samples were different from other RA samples in the expression of EBV genes (Fig. 3). Second, patients RA19 and RA1 had previously been shown to contain CD8 clonal expansions in synovial fluid. These clones were then found to recognize antigenic peptides derived from the EBV transactivator proteins BZLF1 and BMLF1 (10, 11). Patient RA3 also had CD8 T cells derived from the synovial fluid that reacted with BMLF1 and BZLF1 (Table I). Samples from these three patients (Fig. 3, arrowheads) gave RT-PCR bands that were similar to those of all other patients and controls. Thus, in RA joints with well-documented infiltration by CD8 T cell clones specific for BMLF1 and BZLF1, either mRNA for the same viral Ags was not detectable (BMLF1) or there was no difference in expression compared with disease control samples (BZLF1).

A striking finding was the presence of detectable BZLF1 transcripts in 47 of 56 samples, while BMLF1 was below the detection limit in all samples (Fig. 3), even though titrations of positive controls indicated that the BMLF1 PCR was the more sensitive PCR. The primer pairs selected for BZLF1 straddle two introns between exons 1 and 3 (27). A detectable 182-bp PCR product is dependent on a correctly spliced BZLF1, including exon 2, which encodes the DNA binding domain of BZLF1 (36), and which is detected by the primer probe in a Southern blot of the PCR product (see Materials and Methods). A PCR artifact is therefore unlikely. The BZLF1 transcript was originally reported to be undetectable in PBL of normal donors (27), but a more sensitive PCR protocol detected transcripts in 72% of normal PBL (37). BZLF1 is thought to be the key transcriptional regulator that triggers lytic EBV infection by transactivating early genes that share BZLF1 response elements in their promoters, including BMLF1 (38, 39). The presence of low levels of BZLF1 transcripts without BMLF1 is compatible with an aborted lytic infection. Indeed, it has been suggested that critical concentrations of BZLF1 protein are required for target gene transcription (40, 41). Moreover, the BZLF1 protein may be inactivated by serine phosphorylation (42) and by interactions with both viral and cellular proteins such as p53 and

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

**FIGURE 3.** PBL and synovial tissue samples from RA and controls, tested by RT-PCR for EBV gene transcription. The indicated RT-PCR reactions were conducted with RNA from control samples; titered B lymphoblastoid BSKS2—cells (cell equivalents indicated); normal PBL (see Fig. 2), non-T cells in normal PBL (nt), or PHA-treated non-T cells from normal PBL (pha); PBL from a patient with systemic lupus erythematosus (SLE) or from patients with GW; and synovial tissues (st) from disease controls with GW syndrome or with OA. The controls were compared with synovial tissues (st), synovial fluid (sf), or PBL from RA patients. Patients 6 (a through c) and 7 had juvenile RA (JRA). An additional control was RNA from PBL of patient JCM with (+rt) or without (−rt) RT added during cDNA synthesis. Samples from patients P and C (18) and patients RA1 and RA19 (10) have been used in previous publications. Arrowheads indicate that T cells specific for the EBV Ag were present in the same tissue samples (see Table I). Asterisks denote samples of cDNA that were diluted 1/10 before analysis by PCR. All PCRs were repeated three times, and results were generally reproducible within the limits shown in Fig. 2.
that were tested in the COS transfection assay. A total of 10 lytic and 8 latent EBV genes were tested. Patient RS2 has Reiter’s syndrome. CD8

combinations of an EBV gene and an HLA I allele. The HLA genotype of each patient is given in the left-hand columns. We have underlined the HLA alleles of each patient

assay by measuring TNF-

reactivity was scored in a sensitive

expressing an HLA class I allele of the patient and one of several

tatient (RS2). The cell lines were then tested against cotransfectants generated from four RA patients and one Reiter’s syndrome pa-

found in synovial samples from RA patients, CD8 T cell lines were

specific EBV gene transcription in synovial samples.

the presence of these CD8 T cells in synovial tissues of

are unique to RA. In each of the five cases represented in Table I, different RT-PCRs failed to show evidence of overexpressed EBV transcripts in synovial tissues, despite the presence of the EBV Ag recognizing T cells (Fig. 3 and Table I). Thus, the presence of these CD8 T cells in synovial tissues of various patients with arthritis is not due to the production of viral Ags in the synovium.

their lytic infection in normal PBL (33, 47).

Because BMLF1 mRNA levels were barely detectable, we selected two EBV genes that are usually prominently expressed during lytic infection: BALF2 and BHRF1 (32). There was no trans-

cription of BHRF1 and only rare, low levels of BALF2 in both RA and control samples (Fig. 4). These results lend further support to the idea that lytic infection is abortive in these ex vivo tissues.

PCR products for EBER1 (Fig. 3), EBNA1 Y3, and EBNA1 Q transcripts (not shown) also failed to demonstrate any differences between RA and control samples. EBNA1 Q transcripts were faintly detectable in 5 of 14 and EBNA1 Y3 in 6 of 14 samples. Thus, by the most sensitive of RT-PCRs, we found no evidence of RA-specific EBV gene transcription in synovial samples.

To assess how commonly EBV-specific T cell responses are found in synovial samples from RA patients, CD8 T cell lines were generated from four RA patients and one Reiter’s syndrome patient (RS2). The cell lines were then tested against cotransfectants expressing an HLA class I allele of the patient and one of several candidate EBV proteins (29). Reactivity was scored in a sensitive assay by measuring TNF-α release. A summary of these data is given in Table I. The results show that patient RA1, e.g., had synovial T cells recognizing seven different EBV proteins. In the case of BZLF1, three different HLA class I-presenting alleles (A*02, B*61, and Cw*01) could be used, indicating that there were probably several different BZLF1 peptides recognized. In each patient, CD8 T cells reactive with at least two EBV proteins were detected. BZLF1 and EBNA3A were the most commonly recognized Ags. The Ags included both EBV lytic proteins and latent proteins.

Concerning CD8 T cells reactive with EBV Ags, patients with Reiter’s syndrome (such as RS2), psoriatic arthritis, and ankylosing spondylitis gave results similar to those of RA patients (Table I and Ref. 29). Therefore, it is unlikely that EBV-specific CD8 T cells in synovial tissues are unique to RA. In each of the five cases represented in Table I, different RT-PCRs failed to show evidence of overexpressed EBV transcripts in synovial tissues, despite the presence of the EBV Ag recognizing T cells (Fig. 3 and Table I). Thus, the presence of these CD8 T cells in synovial tissues of various patients with arthritis is not due to the production of viral Ags in the synovium.

Discussion

In addressing the role of EBV in RA, we first showed that EBV DNA can be detected in RA synovial tissues. Compared with other nonneurotropic herpes viruses, EBV and CMV DNA were most readily and frequently detected. However, no differences were observed for mRNA transcripts of seven different EBV genes in RA vs control synovial tissues. Even in well-characterized samples known to contain expanded CD8 T cell clones with specificity for EBV gene products, such as BZLF1 and BMLF1, either the corresponding mRNA for the viral protein Ag was scarce or the bands were comparable in RA patients and disease controls, e.g., patients with OA or GW.

Table I. Summary of synovial EBV responses

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA Phenotype</th>
<th>HLA Restriction Element</th>
<th>EBV Lytic Proteins</th>
<th>EBV Latent Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA1</td>
<td>A2 B27, B61</td>
<td>A2 B27</td>
<td>63/53 62/23</td>
<td>45/4 15/2</td>
</tr>
<tr>
<td>RS2</td>
<td>A29, A30 B27 B49</td>
<td>A2 A22 11/2</td>
<td>65/7 2/1</td>
<td></td>
</tr>
<tr>
<td>RA3</td>
<td>A1, A2 B8, B44 Cw5, Cw7</td>
<td>A1 B27                  35/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA4</td>
<td>A2, A32 B27, B44 Cw5, Cw7</td>
<td>A1 B27                  11/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RA19</td>
<td>A24, A31 B35, B60 Cw3, Cw7</td>
<td>A2 A24                  51/17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS2</td>
<td>A29, A30 B27 B49 Cw5, Cw7</td>
<td>A24 B60                 187/36</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Synovial T cell lines (see Materials and Methods and Ref. 29) were tested for responses against EBV Ags presented by various HLA class I alleles. TNF-α release (in pg/ml) by 10⁵ and 10⁴ cells (left and right numbers, respectively) was measured after a 6-h coculture with transfected COS cells. The COS cells were transfected 48 h earlier with various combinations of an EBV gene and an HLA I allele. The HLA genotype of each patient is given in the left-hand column. We have underlined the HLA alleles of each patient that were tested in the COS transfection assay. A total of 10 lytic and 8 latent EBV genes were tested. Patient RS2 has Reiter’s syndrome. CD8 T cells derived from patient’s PBL were tested in parallel and yielded a weaker or no response against COS cells transfected with EBV/HLA gene combinations in almost all cases (for further details, see Ref 29).

FIGURE 4. Expression of the EBV lytic genes BALF2 and BHRF1 in selected control and RA samples. The BALF2 PCR yields a 203-bp product, and the BHRF1 PCR a 239-bp product. Titered B lymphoblastoid BSKS2– cells (cell equivalents indicated) demonstrate the sensitivity of the PCRs. Right lanes contain the alternative PCR products as size markers: the 239-bp BHRF1 product (top) and the 203-bp BALF2 product (bottom).
There is a long history of studies on the role of EBV in RA. The original excitement about EBV was due to the description, in sera from RA and Sjögren’s syndrome patients, of Abs that were reactive with EBV-transformed B cell lines (48). Later studies gave conflicting results in support of a role of EBV in RA pathogenesis (49, 50), but it is still thought that RA patients have higher numbers of EBV-infected B cells than controls. Most normal adults have a controlled, latent infection by EBV. The estimate for the frequency of EBV-infected cells among total B cells during latent infection is $10^{-5}$ to $10^{-6}$, and in acute infectious mononucleosis it may be as high as $10^{-1}$ (33). Compared with latently infected controls, RA patients may have higher titers of Abs against EBV Ags (51), and at least a 10-fold higher frequency of EBV-infected B cells in the peripheral blood (52, 53). IgG anti-viral capsid Ag titers to EBV were reported to correlate with high titers of IgM RF in RA (54). Serum Abs to lytic-phase proteins BMLF1 and BHRF1 were also described in some patients (55).

Lytic-phase EBV Ags are expressed by productively infected cells. Indeed, RA patients may have higher levels of EBV and HHV6 shedding in the oropharynx (53, 56, 57). Whether the greater degree of productive EBV infection in RA extends to other tissues is still an unsettled question. In the joint, the most obvious source of EBV is B lymphocytes resident in the synovium. However, it is not yet clear that EBV+B cells are infected in the synovium or that they are productively, rather than latently, infected.

Other synovial cell types might also be infected with EBV. Koide et al. have recently established an RA synovium-derived fibroblastoid cell line (with features of the synoviocyte type I), which expresses the latency genes EBNA1, EBNA2, and LMP1 and also expresses early Ag and viral capsid Ag in a small percentage of cells (15). However, it is not yet clear whether fresh ex vivo synoviocytes are infected with EBV. EBV can also infect endothelial cells in vitro (58), follicular dendritic cell lines in vitro (59), T cells in hemophagocytic syndrome (60), and smooth muscle tumor cells in immunosuppressed hosts in vivo (61, 62). These cell types are clearly present in inflamed synovial tissue. Samples from the GW patients contained very little CD19 mRNA, and yet EBV transcripts were demonstrated in both the GW and in the RA patients. This result might mean that cells other than B cells are transcribing EBV genes in these samples.

With all of the prior suggestive results, it is perhaps conspicuous that PCR studies measuring genomic DNA of EBV within synovial tissues, or in blood, have been quite inconclusive. For example, the percentage of positive samples for EBV DNA in RA synovial fluids was 19%, vs 33% for reactive arthritis (16). The percentage of samples containing detectable EBV genomic DNA in the peripheral blood was similar among several patient groups: RA (39%), reactive arthritis (39%), other arthropathies (27%), and normal controls (31%). In one study, EBV DNA was found in synovial tissue of 23% of RA patients and in no OA synovial tissues as measured by in situ hybridization (17), but yet another study using the same technique with BHLF1 and EBER probes failed to detect EBV DNA in RA synovial tissue (63).

By analyzing RNA for EBV genes by RT-PCR, our data extend previous work on the presence of EBV genes. The results, however, provided no evidence for EBV Ag expression in joint tissues that was any different in RA vs controls or in joint vs blood. The most striking finding was that joints, heavily infiltrated with EBV-specific CD8 T cells, lacked evidence of abnormal expression of the relevant EBV Ag. For instance, in fresh ex vivo synovial fluid of patient RA1, Vβ2 CD8 T cells were expanded to 15%, vs 6.9% in the PBL (10). The majority of these cells expressed a TCR Vβ2 with a CDRIII region of eight amino acids in length with predominant usage of a Jβ1.2 segment. Among these cells, the most common TCRβ CDRIII sequence was RDRIGNY, representing an estimated 22% of the Vβ2 CD8 cells or 3.3% of all CD8 cells. A CD8 T cell clone derived in vitro from the synovial fluid T cells (A2.10) and expressing the same TCRβ-chain (10) was then used to clone the Ag recognized by this TCR (11). This was a 9-mer peptide of BMLF1 (GLCTLVAML) presented by HLA-A*0201. Yet, the data in Fig. 3 of this paper clearly show that BMLF1 mRNA was below the limits of detection in the same synovial tissue from patient RA1. The same arguments can be made for BMLF1-specific T cells in RA3 and BZLF1-specific T cells in RA1, RA19, and RA3 (Table I), in that the T cells appear to be enriched in the synovium in the absence of abnormal expression of these EBV genes.

The lack of detectable EBV lytic-phase Ag expression could simply be due to efficient removal of the productively infected cells by cytotoxic T cells. However, patients RA1 and RA3 had increased synovial CD8 T cells specific for BZLF1 (Table I), and these T cells were apparently unable to remove all of the cells producing BZLF1, since this transcript was detectable in the same synovial samples (Fig. 3, arrowheads). Moreover, productively infected cells in the oropharynx can generate viral transcripts and proteins despite the presence of specific CTL. Thus, we do not favor this possibility.

Overall, the results suggest several possibilities, as detailed below.

1) Expression of EBV viral Ags may not be quantitatively increased as assessed by RT-PCR, but instead there may be qualitative changes. For instance, viral proteins may be differently processed in the setting of synovial inflammation. Perhaps the APCs are unusual, i.e., dendritic cells, endothelial cells, or infected synoviocytes (15, 58, 59), rather than EBV-infected B cells. Perhaps protein/protein interactions, such as those reported with BZLF1 (43–46), influence Ag processing. In this way, Ag processing was altered when tetanus toxoid was bound to specific Ab (64) or when CD4 was bound to HIV-1 gp120 (65). This can lead to presentation of cryptic neoepitopes, a possible source of autoimmunity (66).

2) An alternative possibility, which does not require qualitative changes in Ag presentation in the synovium, is that dendritic cells take up EBV Ag at a distant site. This might be a mucosal epithelium at which productively EBV-infected epithelial cells are taken up and Ags processed for “cross-presentation.” Dendritic cells would then migrate to inflamed synovial tissues, possibly just because they contain organized secondary lymphoid aggregates indistinguishable from lymph nodes. These dendritic cells might be preferentially loaded with EBV peptides, since herpesvirus shedding in the oropharynx is increased in RA (53, 56, 57).

3) T cell clones in RA synovial tissues may be cross-reactive with unidentified synovial Ags. It is well recognized that CTL specific for an EBV-encoded Ag can be cross-reactive with other Ags (67). One example is the response of CD8 T cells to the immunodominant peptide of EBNA3A (FLRGRAYGL) presented by HLA-B*0801, which generates CTL using highly similar TCRs (68) that were also cross-reactive with an unknown endogenous peptide presented by HLA-B*4402 (69). A cross-reactive peptide derived from a joint-specific protein would be less likely to induce complete deletion of potentially autoreactive T cells, because there are many examples of peripheral autoantigens that do not delete specific T cells. In animal models, T cells with autoreactive potential can be induced to initiate autoimmunity, e.g., in the setting of viral infection (70). Therefore, EBV-specific CD8 T cells may be infiltrating synovia in RA due to a cross-reaction with unidentified joint-specific self-Ags.

4) T cell clones expanded in RA joints may have preferentially homed to the synovium independent of any antigen. For instance,
very late Ag-1 (αβ1), which binds collagen and laminin, is expressed by RA synovial T cells and by T cells associated with respiratory mucosa (71). Synovial T cells in RA also express very late Ag-4 (αβ2), which mediates T cell adhesion to VCAM-1 (or to fibronectin) expressed by synovial endothelial cells. Another integrin of interest is lymphocyte Peyer’s patch adhesion molecule (LPAM-1) (αβ3). In patients with RA, 25% of synovial fluid T cells express αβ3 compared with only 7% of blood T cells (72). This adhesion molecule also binds to VCAM-1 but has additional specificity for the high endothelial venules in Peyer’s patches and thus mediates homing to gut-associated lymphoid tissue in mice (73). Finally, the integrin α4β7, specifically expressed on gut-associated T lymphocytes, is increased in synovial T cells and the ligand for α4β7, E-cadherin, is abundantly expressed in the synovium (74). Therefore, there are apparent similarities between the adhesion molecules expressed by T cells of the gut/respiratory mucosa and synovial T cells.

In human RA, we speculate that synovial T cells could have been activated in the gut (or respiratory) mucosal tissue before finding their way to the synovium. It is well appreciated that intermittent productive EBV infection occurs in epithelial cells of the oropharyngeal mucosa. Perhaps productive EBV infection also occurs in epithelial cells at other levels of the gastrointestinal or respiratory tract. It is known that in Sjögren’s syndrome, productive EBV infection occurs in the epithelial cells of the salivary glands (75). Often, these patients also have arthritis symptoms similar to those of patients with RA. Perhaps EBV-specific T cells from the salivary glands home to synovia in Sjögren’s syndrome, also.

There is an interesting precedent of RA-like disease in a transgenic mouse in which the apparent autoantigen is not joint specific, as described by Kouskoff et al. (76). These mice have a transgenic TCR reactive with an autoantigen presented by MHC class II I-A
t. The autoantigen is expressed in the thymus and in the spleen of these animals. The autoreactive T cells are omnipresent, including in the synovial fluid, but for some unknown reason the clinical manifestation of autoimmunity in this is an RA-like disease.

Because of the genetic linkage with HLA-DR4 alleles, many investigators favor a pathogenic role for CD4 T cells in RA, especially early in the disease. The abundance of clonal EBV-reactive CD8 T cells in RA, particularly in the synovial fluid compartment, is suggestive that these T cells may also play a role (2). Perhaps they serve primarily to perpetuate long term chronic inflammation, long after the initial insult is cleared, rather than in initiation of the disease.

Acknowledgments

We thank Drs. M. Crow and A. Asch for critical reading of the manuscript and Dr. Antoine Alam for providing RA patient samples.

References
