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Latent Epstein–Barr virus (EBV) infection and cytomegalovirus (CMV) infection in synovial tissue of autoimmune chronic arthritis determined by RNA- and DNA-*in situ* hybridization

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In rheumatoid arthritis (RA) viral triggers, especially Epstein–Barr virus (EBV) and cytomegalovirus (CMV), have been suggested. By PCR analysis DNA of several viruses among which EBV, CMV, and parvovirus B19 (B19) has been detected in RA synovial fluid and synovial tissue. In 63 synovial tissues of 29 rheumatoid arthritis (RA), 6 psoriatic arthritis (PsA), 26 reactive arthritis/synovitis (rA/S), and two normal synovial cases, we recently could demonstrate a high percentage of replicative B19 infection within the synovial tissue, being significantly more frequent in autoimmune arthritis. To further investigate the influence of synovial virus infections in rheumatoid arthritis, we now analyzed the same sample of synovial tissues for CMV and EBV infections by DNA-*in situ* hybridization (CMV), EBER1/2-RNA-*in situ* hybridization (EBV), and immunohistochemistry. A significant latent EBV infection of synovial lining cells, synovial fibroblasts, and/or infiltrating lymphocytes was identified in 5/29 (17.2 %) RA, 1/6 (16.7%) PsA, and to a much lower degree in 1/26 (3.8%) rA/S specimens. CMV-DNA was detected in 31% of RA, 3/6 (50%) of PsA, and 11.5% of rA/S. Immunohistochemical analysis of CMV early antigen revealed replicative CMV activity in 20.7% of RA and 2/6 (33.3%) of PsA specimens but not in reactive arthritis synovia. Comparative analyis of the EBV-, CMV-, and published B19-data demonstrated that relevant synovial virus infections in general and furthermore double or multiple infections are far more common in autoimmune arthritis than in rA/S. A triple virus infection was found solely in RA in 10.3% of cases. The evidence of increased synovial persistence of EBV, CMV, or B19 either alone or even more as coinciding infections may further reinforce the notion of a primary role of these viruses in autoimmune arthritis.
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Viral agents have repeatedly been suggested as triggers in the pathogenesis of autoimmune chronic arthritis.[1–3] Especially Epstein–Barr virus (EBV)- and cytomegalovirus (CMV)-infections indicated by a positive serologic response have been shown to be associated with an increased risk for rheumatoid arthritis.[4–7] Compared to single infections with either CMV or EBV a combined seropositivity to both viruses creates an increased risk for rheumatoid arthritis (RA), which is higher than to be assumed by simple additive risk. The EBV protein gp110 (a late-stage protein, also referred to as BALF4) contains a major immunogenic amino-acid sequence (QKRAA), which shows intriguing homology to the third hypervariable region of HLA-DRB1 alleles associated with RA risk.[8,9] The QKRAA sequence was found to be a major immunogenic epitope in RA patients leading to the establishment of a molecular mimicry hypothesis in the pathogenesis of RA.[7,10] Besides serologic data, several PCR studies could demonstrate various viral DNA,

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among which also EBV, CMV, and parvovirus B19 (B19), in the synovial tissue, or fluid of rheumatoid arthritis patients.[2,11–13]

EBV as well as CMV belong to the human herpes virus family with a high seroprevalence in adults in the population of about 95% (EBV) and 50–70% (CMV), respectively.[14] For both viruses persistence or latency, and acute replicative infections are known.[14] The most frequent clinical manifestation of EBV as primary affection is infectious mononucleosis. EBV has a well-documented tumorigenic potential being strictly associated with the African endemic Burkitt lymphoma and the Asian nasopharynx carcinoma.[14] Furthermore, an association of EBV to Hodgkin's lymphoma, other lymphocytic malignancies, and gastric cancer is well known.[14] EBV targets B lymphocytes, which are reservoirs in latent infection;[15] EBV infection of synovial fibroblasts has been described as well.[16]

CMV in healthy adults is supposed to cause mainly subclinical, nonspecific infections. In immunocompromised individuals or as prenatal infection of the fetus, however, severe replicative CMV infections of the CNS (including ophthalmologic affection), and multiple inner organs, for example, lung, liver, kidney, pancreas, and intestine can occur.[17]

In spite of an increasing body of evidence a direct causality between RA and either EBV or CMV could not be proven yet. Most early studies relied on serologic virus testing. More recently, direct analyses of synovial EBV infection in RA joints have been reported revealing conflicting results;[18–22] the extent and acuity of synovial CMV infections apart from PCR data have not yet been documented in chronic arthritis. In a recent immunohistochemistry study of 63 synovial specimens of rheumatoid arthritis (RA; $n=29$), psoriatic arthritis (PsA; $n=6$), reactive arthritis (rA/S; $n=26$), and few normal synovial specimens (nS; $n=2$), we could demonstrate a replicative B19 infection within the synovial tissue in a high percentage (64%) of cases, the frequency as well as the extent being significantly higher in RA and PsA as compared to rA/S cases.[23]

To elucidate the potential importance of other synovial virus infections — explicitly CMV and EBV — in autoimmune chronic arthritis, we analyzed the same study collective of synovial tissues for CMV and EBV infections by DNA-*in situ* hybridization (CMV), EBER1/2-RNA-*in situ* hybridization (EBV) and complementary immunohistochemical virus protein analysis (CMV-early antigen, EBV-LMP, -EBNA1, -BZLF1) in CMV-DNA- or EBER1/2-positive cases.

Materials and methods

In all, 63 synovial specimens were obtained from the Department of Pathology and the Department of Orthopedic Surgery of Saarland University, Homburg, the Department of Pathology of the Central Hospital in Augsburg, the Department of Orthopedic Surgery of the Bundesknappschaft's Hospital Püttlingen, and the Department of Rheumaorthopedics of the Rheumazentrum Oberammergau. Synovial tissue had been removed either surgically or by arthroscopy for therapeutic or diagnostic reasons. Normal synovia was gained by diagnostic arthroscopy in traumatic joint lesion or by autopsy (Department of Forensic Medicine of Saarland University, Homburg). In prospectively collected cases, informed consent for research use of the tissues was obtained; in retrospectively analyzed tissue specimens of anonymized archival material, general consent for scientific use of diagnostic material had been ensured. Cases included in this study are 29 rheumatoid arthritis (RA; 20 f/ 9 m), 26 chronic reactive arthritis/synovitis (rA/S; 14 f/12 m), six psoriatic arthritis (PsA; 6 m), and two normal synovial specimens (1f/1 m). Among chronic reactive arthritis/synovitis cases, we compiled synovial specimens of osteoarthritis from arthrotic joints, detritus arthritis, chronic irritation following joint injuries, and unclear chronic synovitis without clinical diagnosis of rheumatoid arthritis. The criteria for the clinical diagnosis of rheumatoid arthritis and psoriatic arthritis were based on international standard convention of rheumatology;[24] the criteria for diagnoses of reactive arthritides were the exclusion of any specific or systemic arthritis, the lack of diagnostic criteria of rheumatoid or psoriatic arthritis, or the clear presence of posttraumatic changes. Synovial tissue, in descending frequencies, was derived mainly from knee, hip, carpal, or tarsal joints.

Serologic data on EBV and CMV (IgM and IgG) with regard to the archival material, in which serologic data could not be obtained, have not been investigated in this study.

Lung tissue of a CMV pneumonia in an HIV-infected individual was used as positive control for CMV immunohistochemistry and CMV-DNA-*in situ* hybridization. Paraffin sections of infectious mononucleosis tonsils and of an EBV-positive Hodgkin's lymphoma served as EBV positive controls in EBV immunohistochemistry and EBER1/2-RNA-*in situ* hybridization.

Immunohistochemistry

For analysis of replicative CMV infection, a monoclonal mouse antibody reactive to CMV early antigen was used (clone CCH2, DAKO, FRG). For assessment of EBV infection, a mouse monoclonal antibody to EBV latent membrane protein (LMP1; clone CS1-4; DAKO, FRG), and a monoclonal mouse antibody to EBV ZEBRA protein (BZLF1; DAKO, FRG) were applied in EBER1/2-positive cases. In cases showing extensive label for LMP1 or EBER1/2, additional EBNA1 immunostaining was performed using a monoclonal rat antibody kindly provided by Friedrich Grässer (Department of Virology, Saarland

University, Homburg). Negative controls were done for CMV CCH2, EBV-LMP1 CS1-4-, and EBNA1-antibodies including elimination of the antibody from the reaction or substitution by diluted normal serum of the primary antibody producing source. Immunohistochemistry was carried out using horseradish peroxidase-mediated AEC (aminoethyl carbazole)- or DAB immunostaining. Endogenous peroxidase activity was quenched by 1.2% H_2O_2 (25 min, RT) followed by a proteolytic pretreatment of tissue sections in 1% pronase/PBS (protease type XIX; Sigma, FRG; 2 min 37°C); primary antibodies were applied in optimized dilution in 1% BSA/PBS and incubated for 1 h at 37°C. The primary antibody binding was visualized employing a two-step detection with a biotinylated secondary antibody combined with sequential application of HRP–AB complex (avidin/horseradish peroxidase). Cells were counterstained with hematoxylin, or not counterstained, and mounted with an aqueous mount for visualization.

DNA-*In Situ* Hybridization

CMV-DNA-*in situ* hybridization was performed on 5 μm synovial paraffin sections using a commercially available biotinylated CMV-DNA probe (Enzo; FRG) as well as a biotinylated plasmid probe of a 1.6 kb CMV pp65 cDNA fragment BamH1-ligated to pcDNA3.1/Hygro (pcDNA3.1/Hygro-pp65) kindly provided by Martina Sester (Department of Internal Medicine, Saarland University, Homburg). After dewaxing in xylene and rehydration in a descending ethanol series the tissue sections were subjected to proteolytic pretreatment in 0.1% pronase in PBS (2 min; RT). In all, 75 ng of virus-specific probe each were hybridized along with 10 μg herring sperm DNA in 20 μl of hybridization mixture (50% formamide, 2 × SSC, 10% dextrane sulphate). DNA denaturation of virus probe and tissue specimens was performed simultaneously at 80°C for 10 min on a heating plate. The slides were allowed to hybridize at 42°C for 16–48 h. The hybridized biotin-labeled probe was visualized by an alkaline phosphatase-mediated New Fuchsin or nitro blue tetrazolium (NBT) staining using a monoclonal mouse anti biotin antibody (DAKO; FRG) combined with a secondary two-step antibody detection according to the APAAP protocol.

EBER1/2-*In Situ* Hybridization

EBER1/2-*in situ* hybridization was performed on routinely processed paraffin-embedded 5 μm synovial tissue sections. Digoxigenin labeled EBER1- and EBER2-antisense transcript probe was created according to the manufacturer's recommendations by *in vitro* transcription (Roche Diagnostics; FRG) of EBER1- and EBER2-cDNA containing plasmid vectors kindly provided by Friedrich Grässer (Department of Virology, Saarland University, Homburg). Negative controls were carried out using digoxigenin-labeled EBER1- and EBER2-sense transcript probes.

Paraffin sections were dewaxed in xylene and rehydrated in a descending ethanol series. Pretreatment steps in the following order included incubation in 0.2% HCl (20 min, RT), proteolytic digestion by Proteinase K (0.03% in Tris-HCl, pH 8.0; 20 min, RT), incubation with 0.2% glycin/PBS (5 min, RT), postfixation in 4% paraformaldehyde/PBS (20 min, RT), and incubation in acetic anhydride (0.25% in 0.1 M triethanolamine, 10 min, RT). About 50–200 ng each of digoxigenin -labeled EBER1- and EBER2-antisense transcript probe in 100 μl hybridization buffer (50% formamide/2.5 × SSC/12.5% dextrane sulphate) were hybridized along with 12.5 μg total yeast RNA, 25 μg tRNA, and 12.5 μg herring sperm DNA at 50°C in a humid chamber overnight. After single-strand RNA digestion by RNAse A (0.002%, 30 min, 37°C) and stringent washes in 2 × SSC at 45°C (2 × 30 min) and 0.2 × SSC at 55°C (2 × 30 min) hybridized probe was detected by alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody and sequential AP-mediated NBT staining.

Results

CMV Analysis (Table 1)

DNA-*in situ*-hybridization revealed CMV-DNA in 15 of 63 (23.8%) synovial specimens (24.6% of 61 arthritic specimens). In detail, CMV-DNA was identified in 9/29 (31%) RA, 3/26 (11.5%) rA/S, and 3/6 (50%) PsA cases. Virus DNA mainly could be localized to either synovial lining cells or infiltrating lymphocytic cells (Figure 1a), but in some cases also to synovial fibroblasts or neighboring connective tissue. To differentiate between latent and replicative CMV infections in CMV-DNA positive cases immunohistochemical analysis of CMV early antigen indicating replicative virus cycle was performed. Thereby, replicative CMV activity could be shown in 6/9 RA (20.7% of 29 RA) and 2/3 PsA (33.3% of 6 PsA) cases but not in two analyzed rA/S specimens (Figure 1b). Notably, all CMV early antigen positive specimens revealed limited replicative activity, showing mainly small numbers of protein-expressing cells.

EBV Analysis (Table 2)

EBER1/2-*in situ* hybridization revealed EBV-positive cells in 23/63 (36.5%) synovial specimens (37.7% of 61 arthritis specimens), in detail 14/29 (48.2%) RA, 3/6 (50%) PsA and 6/26 (23.1%) rA/S cases. Evidence of a local synovial infection indicated by numerous EBER1/2-positive lymphocytes (two RA cases) and/or positive synovial lining cells as well as synovial stroma and connective tissue cells (three RA, one PsA, one rA case mainly

Table 1 CMV-positive cases in synovial specimens of different arthritis entities

Clinical diagnosis	*RA*	*PsA*	*rA/S*	*nS*	*χ^2-test*
Total number of cases	*29*	*6*	*26*	*2*	*(P-values)*
CMV-DNA positive cases (ISH)	9 (31%)	3 (50%)	3 (11.5%)	—	0.083
CMV-EA detection (IHC) in DNA-positive cases	6/9	2/3	—[a]	n.a.	0.24

Human CMV-positive cases in synovial specimens of different diagnostic entities according to CMV-DNA-*in situ* hybridization and immunohistochemical analysis. CMV, cytomegalovirus; RA, rheumatoid arthritis; PsA, psoriatic arthritis; rA/S, reactive chronic arthritis/synovitis; nS, normal synovia; ISH, *in situ* hybridization; IHC, immunohistochemistry; CMV-EA, CMV-early antigen; —, negative; n.a., not analyzed.[a] one CMV-DNA positive rA/S case could not be analyzed by CMV-EA-IHC due to sparse material. *P*-values were evaluated using the exact χ^2-test.

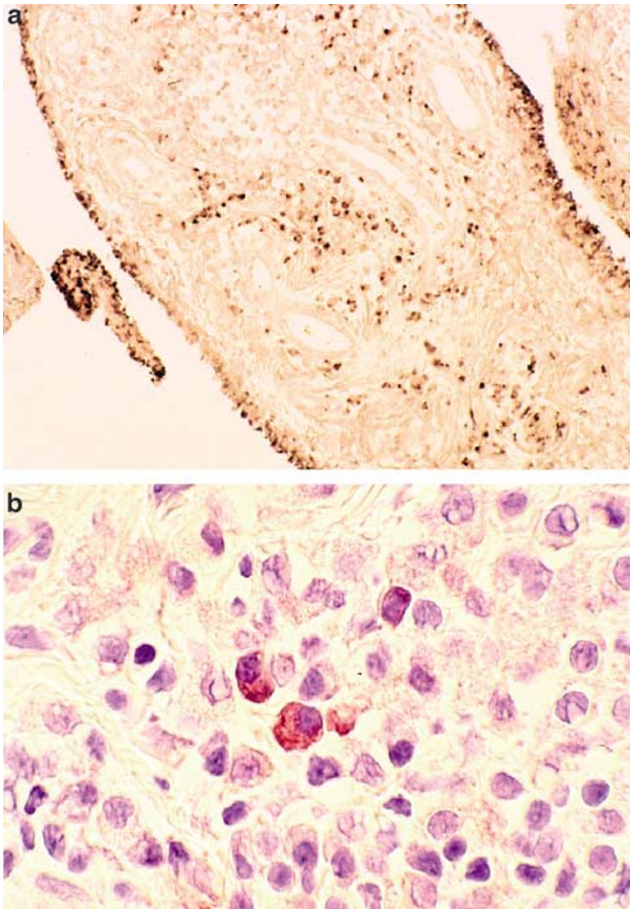

Figure 1 CMV detection in formalin-fixed paraffin-embedded synovial tissue in PsA, case 31. (**a**) CMV-DNA-*in situ*-hybridization using a biotinylated CMV-pp65 probe detected by NBT-staining (purple black) employing the APAAP system (× 100). A high number of synovial lining cells and areas of lymphocytic cells within the synovial stroma show clear distinct signals for CMV-pp65 DNA. (**b**) Immunohistochemistry targeting CMV-early antigen detected by AEC-staining (red) revealing several positive cells, which partly show CMV-typical morphological features (× 400).

restricted to synovial lining cells), however, was found in only 7/63 (11.1%) cases (7/61 arthritic specimens; 11.5%), in detail 5/29 (17.2%) RA, 1/6 (16.7%) PsA, and 1/26 (3.8%) rA/S (Figure 2a–c). Loosely scattered single EBER1/2-positive cells were identified within the synovial stroma in 16/63 (25.4%) cases (16/61 arthritic specimens; 26.2%), in detail in 9/29 (31%) RA, 2/6 (33.3%) PsA, and 5/26 (19.2%) rA/S tissues, being consistent with immigrated latently EBV-infected lymphocytes of the B strain.

EBER1/2-positive cases were further characterized by immunohistochemistry targeting EBV-latent membrane protein 1 (LMP1) indicative for latent EBV-infection as well as EBV-BZLF1-protein as lytic cycle protein. Thereby, in all cases LMP1 expression could be demonstrated in comparable cell types and partly in an even higher number of affected cells as previously identified by EBER1/2 detection (Figure 3a–c). In contrast, merely very few single BZLF1-expressing cells were identified within the synovial stroma in three cases (two RA, one PsA) harboring a high number of EBER1/2-positive cells. Additional EBNA1 immunostaining in the strongly LMP1- or EBER1/2-expressing cases revealed EBNA1 positive synovial lining and stromal cells confirmimg latent EBV infection in all analyzed specimens.

Multiple Virus Infections

To evaluate the prevalence of relevant coincidal double or multiple synovial virus infections the CMV- and EBV- data were compared and completed with the previously published B19 results[23] of the same sample collective (Table 3).

Evidence for a relevant infection of the respective virus was defined by the presence of CMV-DNA in synovial lining cells for CMV, the presence of EBER1/2 in synovial lining cells or in a high number of infiltrating cells for EBV, and expression of VP1/VP2 capsid protein for B19 in more than single cells (several B19-positive cells or numerous B19-positive cells).[23] In both normal synovial tissues according to the defined limits no relevant virus infection was observed. A relevant B19 infection as defined by the above-mentioned criteria was detected most frequently in 68.9% (20/29) of RA, 33.3% (2/6) of PsA, and 11.5% (3/26) of rA/S cases.[23] A relevant synovial virus infection with at least one virus could be detected in 50.8% (31/61) of arthritis specimens being more frequent in RA (21/29; 72.4%), and PsA (4/6; 66.7%), than in rA/S (6/26; 23.1%) cases (Table 3).

Table 2 Detection of EBV gene products in 24 of 61 synovial tissues of RA, PsA, and rA/S cases*

Case no.	*Age (years)/sex (f/m)*	*Clinical diagnosis*	*EBER1/2 ISH S/L*	*LMP1 IHC S/L*	*EBNA1 IHC S/L*	*BZLF1 IHC S/L*
2	44/m	RA	−/+	−/+	n.a.	−/−
4	13/f	RA	−/+	−/+	n.a.	−/−
5	50/f	RA	−/+	−/+	n.a.	−/−
6	71/f	RA	−/+	−/+	n.a.	−/−
8	74/f	RA	+/++	++/+++	++/++	−/+
10	69/f	RA	−/+	−/+	n.a.	−/−
13	33/m	RA	−/+	−/+	n.a.	−/−
18	63/m	RA	++/++	+++/+++	++/++	−/+
19	68/f	RA	−/++	−/++	n.a.	−/−
20	40/m	RA	−/+	−/+	n.a.	−/−
22	71/f	RA	−/++	−/++	+/++	−/−
24	29/f	RA	++/++	+++/+++	++/++	−/−
25	64/m	RA	−/+	−/+	n.a.	−/−
27	75/f	RA	−/+	−/+	n.a.	−/−
30	34/m	PsA	++/++	+++/+++	++/++	−/+
31	33/m	PsA	−/+	−/+	n.a.	−/−
35	54/m	PsA	−/+	−/+	n.a.	−/−
39	60/m	RA/S	−/+	−/+	n.a.	−/−
41	75/f	RA/S	++/+	++/+	+/+	−/−
44	18/m	RA/S	−/+	−/+	n.a.	−/−
46	20/f	RA/S	−/+	−/+	n.a.	−/−
49	69/m	RA/S	−/+	−/+	n.a.	−/−
56	54/f	RA/S	−/+	−/+	n.a.	−/−

EBV-positive cases in synovial specimens of different diagnostic entities according to EBER1/2-RNA-*in situ* hybridization and immunohistochemical analysis of EBV proteins. EBV, Epstein–Barr virus; RA, rheumatoid arthritis; PsA, psoriatic arthritis; rA/S, reactive arthritis/synovitis; f, female; m, male; EBER1/2, Epstein–Barr virus encoded small nuclear RNA1 and 2; LMP1, latent membrane protein 1; EBNA1, EBV nuclear antigen 1; ISH, *in situ* hybridization; IHC, immunohistochemistry; S, synovial cells; L, lymphocytic cells; +++, numerous positive cells exceeding the number of EBER1/2-labeled cells; ++, significant number of positive cells; +, scattered few single positive cells; −, negative; n.a., not analyzed.
*In two analyzed normal synovial specimens, no EBV gene products were detected.

A relevant synovial infection with only one virus type was found in 29.5% (18/61) of arthritis cases, in detail in 11/29 (37.9%) RA, 2/6 (33.3%) PsA, and 5/26 (19.2%) rA/S specimens (Table 3). Thereby, CMV appeared as single virus infection in 1 RA, 1 PsA, and 2 rA/S specimens; a single synovial EBV infection was observed in only 1 rA/S case. B19 as relevant single virus infection showing several or numerous positive cells according to our previously published data[23] had been detected in 21.3% (13/61) of arthritic cases, namely 10/29 RA (34.5%), 1/6 PsA (16.7%), and 2/26 rA/S (7.7%) specimens.

A relevant synovial infection by two different viruses could be shown in 16.4% (10/61) of arthritis cases, being detected in 7/29 (24.1%) RA including five samples with CMV and B19, and two with EBV and B19, 2/6 (33.3%) PsA consisting of one case with CMV and B19 and another with CMV and EBV, and 1/26 (3.8%) rA/S specimens with CMV and B19 (Table 3). Relevant triple virus infection by CMV, EBV, and B19 was observed solely in 3 RA specimens (3/61 arthritis cases; 4.9%) corresponding to 10.3% of RA cases (Table 3).

Discussion

Synovial CMV infection was detected in 24.6% of 61 arthritic synovial specimens (23.8% of 63 total synovial specimens) affecting as well infiltrating lymphocytes as synovial lining cells in all affected cases. CMV-positive cases, however, were more frequent in RA (31%) and PsA (3/6; 50%) as compared to rA/S (11.5%). Notably, CMV early antigen indicating virus replication was detected in at least two-thirds of CMV-DNA-positive RA (6/9) and PsA (2/3) showing a low to moderate replicative activity. CMV is known to persist in various human tissues.[14] Apparently, CMV can also persist in synovial tissue. In both normal synovial specimens, CMV was not detected. The prevalence of latent CMV infection in normal synovial tissues, though, has not yet been analyzed in a representative number of cases. Thus, it remaines indecisive, whether persistent synovial CMV infection is associated with arthritic symptoms in general. The higher frequency of CMV infections in RA and PsA as compared to rA/S cases, however, may suggest a causative role in the pathogenesis of autoimmune arthritis as supposed by serologic data. Likewise, synovial CMV replication as identified in RA and PsA cases could indicate a primary situation attributing to chronic inflammation; on the other hand, CMV reactivation as a result of immunosuppressive therapy was conceivable.[17]

Latently EBV-infected cells were observed in 37.7% of 61 arthritic specimens, being detected considerably more frequent in about half of RA (48.2%) and PsA (3/6; 50%) cases as compared to

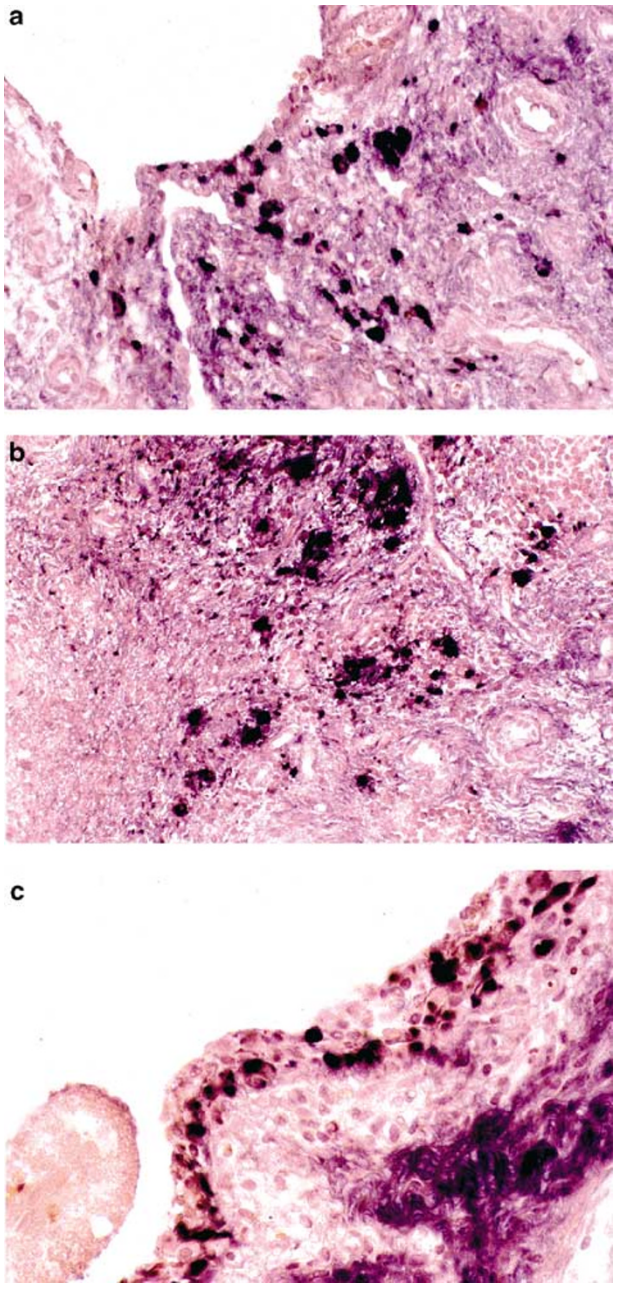

Figure 2 EBER1/2-RNA-*in situ*-hybridization on formalin-fixed paraffin-embedded synovial tissue detected by alkaline phosphatase-mediated NBT-staining (purple black). (**a**) RA-synovia, case 18, showing EBER1/2-positive synovial lining cells and positive cells within the synovial stroma (× 400). (**b**) RA-synovia, case 24, revealing EBER1/2-staining in lymphocytic infiltration (× 200). (**c**) rA/S-synovia, case 41, EBER1/2-positive cells are mainly localized in the synovial lining cell area (× 400).

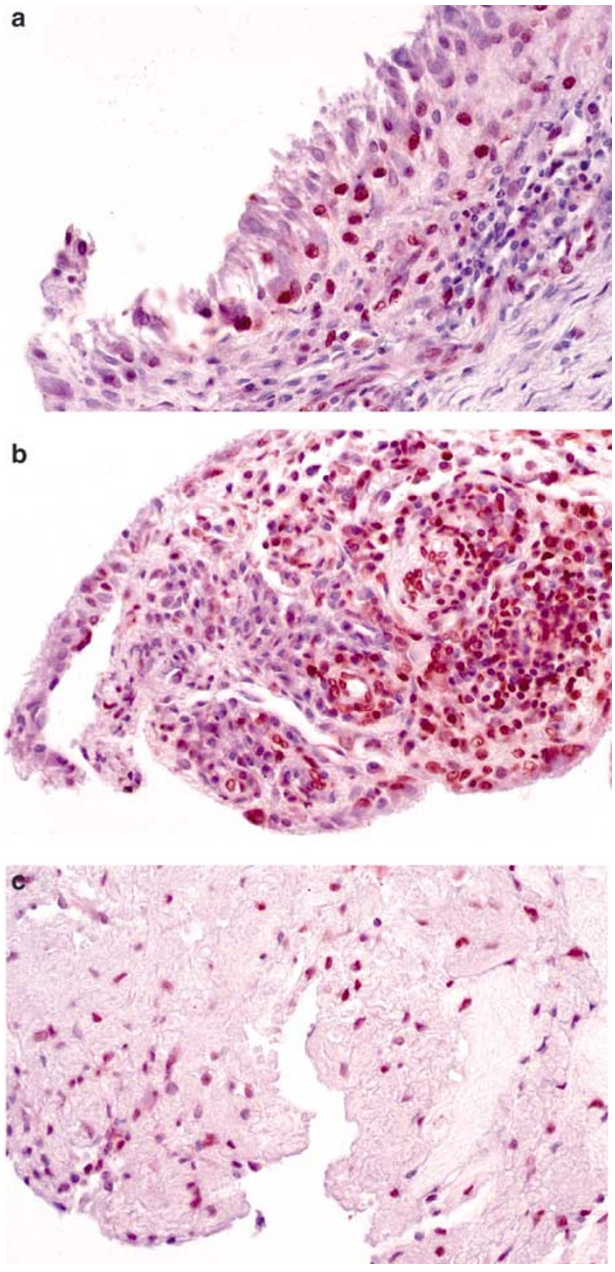

Figure 3 LMP1-immunohistochemistry on formalin-fixed paraffin-embedded synovial tissue of a RA patient (case 18) detected by horseradish peroxidase-mediated AEC-staining (red); hematoxylin counterstain. (**a**) LMP1 labeling in synovial lining cells and infiltrating lymphocytic cells (× 400). (**b**) Few LMP1-positive synovial lining cells beside an extensive number of LMP1-positive lymphocytes in follicular infiltration (× 400). (**c**) LMP1 staining in synovial fibroblasts in a RA case with striking LMP1 and EBER1/2 detection (× 200).

23% of rA/S specimens. Thereby, rarely scattered EBV-positive cells being consistent with immigrated EBV-positive B lymphocytes were discovered in 31% of RA and 33.3% (2/6) of PsA specimens opposed to 19.2% in rA/S cases. EBV seropositivity is prevalent in about 95% of the adult population being accompanied by a lifelong persistence of latently infected cells of the B-strain.[14,15] In RA and PsA characteristically marked synovial infiltration by lymphocytic cells occurs.[25,26]

A relevant latent synovial EBV infection, as defined by the above-mentioned criteria, affecting either synovial lining and stromal cells, or high numbers of infiltrating lymphocytes were identified in 11.5% of arthritic specimens showing clear predominance in RA and PsA making up for 6/7

Table 3 Analysis of isolated or coincidal relevant synovial infections by EBV, CMV, and B19[a] in arthritis cases of different entities

Clinical diagnosis (Total number)	*Any virus infection (%)*	*Synovial virus infection by*			*Coincidal infections*		
		EBV (%)	*CMV (%)*	*B19[a] (%)*	*SVI (%)*	*DVI (%)*	*TVI (%)*
RA	21	5	9	20	11	7	3
($n=29$)	(72.4)	(17.2)	(31)	(68.9)	(37.9)	(24.2)	(10.3)
PsA	4	1	3	2	2	2	—
($n=6$)	(66.7)	(16.7)	(50)	(33.3)	(33.3)	(33.3)	
rA/S	6	1	3	3	5	1	—
($n=26$)	(23.1)	(3.8)	(11.5)	(11.5)	(19.2)	(3.8)	
χ^2-test (P-values)	<0.001	0.32	0.083	<0.001	0.33	0.084	0.25

Detection of relevant synovial virus infections defined by EBER1/2-positive synovial cells or a high number of infiltrating lymphocytic cells for EBV, CMV-DNA detection in synovial cells for CMV, and detection of a relevant number of B19-VP1/VP2-protein positive cells (several or numerous as opposed to single positive cells) for B19. EBV, Epstein–Barr virus; CMV, human cytomegalovirus; B19, parvovirus B19; RA, rheumatoid arthritis; PsA, psoriatic arthritis; rA/S reactive arthritis/synovitis; SVI, single virus infection; DVI, double virus infection; TVI, triple virus infection.
[a]The reported B19 data were obtained from the same sample collective by an immunohistochemistry study targeting B19 VP1/2 structural proteins and have been previously published by our group.[23] *P*-values regarding RA, PsA, and rA/S data were evaluated using the exact χ^2-test (exact χ^2-tests regarding RA and rA/S data revealed similar results).

(five RA, one PsA) positive cases. Only one rA/S specimen from the hip joint of a 76-year-old woman after TEP following coxarthrosis of unknown origin showed a synovial EBV infection similar to the affected RA/PsA cases but more selectively of synovial lining cells. Thus, synovial EBV infection may still be responsible for nonspecific chronic arthritis in rare instances. The observed results of synovial lining cells being targets of EBV infection are consistent with few reports in the literature. Synovial lining cells do not express the EBV receptor CD21.[27] Thus, as shown in human epithelial cells, EBV probably enters synovial cells via cell-to-cell-contact with EBV-infected lymphocytes.[28]

EBER1 and EBER2 are small nontranslated EBV-encoded nuclear RNA, which are transcribed in very high copy number (10^7 copies per cell) in as well latent as lytic cycle of EBV-infection.[14] EBER1/2-labeling usually is considered to be the most sensitive procedure to identify EBV infections.[29] Interestingly, in most autoimmune arthritis cases with striking EBV infection reported here, LMP1-labeling revealed more EBV-positive cells than EBER1/2-RNA-*in situ* hybridization partly showing marked expansion of latently EBV-infected cells. LMP1 is a latent phase EBV-protein located at the cytoplasm membrane, which has been suggested as constitutionally active growth factor receptor and has been demonstrated as potential oncogene leading to changes in cell proliferation and morphology inducing synthesis of adhesion molecules (LFA-1, LFA-3, ICAM), transferrin receptor, and proto-oncogene BCL-2, which suppresses apoptosis.[14,15] RA synovia shares some similarities with tumor tissues showing as well proliferation as infiltration of neighboring joint structures.[30] A relevant latent EBV infection with high expression of LMP1 was detected in 17.2% (5/29) of RA and 16.7% (1/6) PsA cases. Thus in at least part of autoimmune arthritis cases, LMP1 expression could be primarily responsible for proliferative transformation of synovial tissue.

In RA synovia, lytic EBV-infection has been reported.[27] By analysis of BZLF1 in EBER1/2-positive cases in this study, however, only very few single BZLF1-positive cells in rare foci could be identified in three cases beside extensive expression of LMP1 in numerous cells suggesting, that lytic EBV-infection is a rare event.

Summarizing the CMV-, EBV-, and former B19 data in synovial tissue, the three different viruses each are far more frequent in RA and PsA than in rA/S. Compared to the high frequency of relevant B19 infections in 69% of RA cases (2/6, 33.3% of PsA), synovial CMV and EBV infections appeared in lower percentages of 31 and 17.2% of RA (3/6, 50% and 1/6, 16.7% of PsA) cases, respectively. EBV as CMV, however, in this study were demonstrated as well to infect primary synovial cells designated as synovial lining cells or synovial fibroblasts in the synovial stroma, whereas B19 was found exclusively in infiltrating lymphocytes.[23]

Thus, the way how the three analyzed viruses could influence arthritic changes seems to be different. On the one hand, there may be effects of viral antigen-dependent immune reactions within the synovial tissue; on the other hand, virus infections of original synovial cells possibly can cause changes in cellular gene expression and proliferative behavior.

Antigen-driven immune response to either virus protein could start or at least deteriorate chronic inflammation by cytokine release. One feature of CMV infection is the upregulation of a number of cellular proteins, among them transcription factors and DNA replicating enzymes.[31,32] Several human CMV encoded proteins have been shown to inhibit apoptosis,[33,34] and CMV infection enhances the transactivation function of NFkappaB, which activates genes involved in apoptosis inhibition.[31] Molecular

mechanisms of latent CMV infection, though, are not well analyzed.[14] In various tumors, persistent CMV was recently identified possibly being associated.[35,36] Likewise also in synovial tissue, changes in cellular gene regulation or expression are thinkable to be induced by persistent CMV infection. Synovial EBV infection was found by us to be mainly latent, expressing EBNA1 and high levels of LMP1 proteins. Latent EBV infection possibly could initially induce RA-specific synovial changes and proliferation by the effects of LMP1 expression. Taking in consideration the molecular mimicry hypothesis [7,10] apart from direct effects of synovial infection, systemic EBV infection in yet another way could contribute to RA pathogenesis initiating the autoimmune reaction. The complexity of possibly combinatorial effects of EBV thus further supports the notion of an important role of EBV in the development and course of autoimmune arthritis.

Beside the individual effect of the respective viruses, multiple virus infections in an additive manner might participate in arthritis pathomechanisms. In nearly all (5/6) virus-positive rA/S cases, a single virus infection was identified and only one case showed a simultaneous double infection. Although the majority of virus infections in RA and PsA as well appeared as single virus infection making up for 37.9% of RA and 2/6 (33.3%) of PsA cases, double infections by two viruses were observed definitely more frequently in RA (24.1%) and PsA (2/6, 33.3%) than in rA/S. A relevant simultaneous triple virus infection finaly was identified solely in RA representing at least 10.3% of RA specimens. In the development of autoimmune arthritis as found by serologic risk analyses for CMV and EBV, several virus infections in combination might add up to finally exceed an individual disease threshold.

Although an influence of immunosuppressive therapy mostly applied in RA and PsA cannot be excluded, the increased prevalence of also latent or persistent synovial virus infections as identified in EBV and CMV, however, rather hints to a primary effect of these viruses in autoimmune arthritis.

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