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Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis

Hans-Detlev Stahl, Bernd Hubner, Bernd Seidl, Uwe G Liebert, Ineke M van der Heijden, Bert Wilbrink, Maarten C Kraan, Frank Emmrich, Paul P Tak

Abstract

Objective—Viruses have a role in the pathogenesis of various forms of arthritis. This study aimed at determining whether viral DNA can be detected in joint samples in the early stages of idiopathic arthritis.

Methods—Synovial fluid (SF) and synovial tissue (ST) samples were obtained from 73 patients, with undifferentiated arthritis (n=22), rheumatoid arthritis (n=13), spondylarthropathy (n=17), crystal arthropathy (n=8), osteoarthritis (n=7), septic arthritis (n=5), and trauma (n=1). The presence of viral DNA was investigated by polymerase chain reaction analysis.

Results—Cytomegalovirus was present in 25 patients, parvovirus B19 in 15 patients, Epstein-Barr virus in 12 patients, and herpes simplex virus in 16 patients (in ST, SF, or both), respectively. The joint samples were negative for viral DNA from adenovirus and varicella-zoster virus. In ST, eight patients were double positive for parvovirus B19 and another viral DNA, with herpes simplex virus being the most prevalent. Seven patients were double positive for other viruses (cytomegalovirus, herpes simplex virus, Epstein-Barr virus). In SF, four patients were double or triple positive for viral DNA. Paired samples were available in 56 patients. In these, viral DNA was detected in 37 patients in ST, as compared with 19 in SF.

Conclusion—These data show that one or more viruses can be detected in the synovial specimens of patients with early arthritis, irrespective of the clinical diagnosis. This observation might be explained by migration of inflammatory cells harbouring viral DNA into the inflamed joints.

(Diagnosis: crystal arthropathies (eight), rheumatoid arthritis (13), osteoarthritis (seven), spondyloarthropathy (17), septic (bacterial) arthritis (five), and trauma (one). The study was approved by the medical ethics committees of the Leiden University Medical Centre and the University of Leipzig.

Patients, materials, and methods

Patients and clinical specimens

Seventy-three patients with arthritis in at least one knee joint were included in the study. All patients had a disease duration of less than one year. ST was available from 68 patients and synovial fluid (SF) from 61 patients. Twenty-two patients had undifferentiated arthritis, based on the exclusion of other rheumatic diseases (10 patients had a monarthritis, nine polyarthritis, and three oligoarthritis). The other patients presented with the following diagnoses: crystal arthropathies (eight), rheumatoid arthritis (13), osteoarthritis (seven), spondyloarthropathy (17), septic (bacterial) arthritis (five), and trauma (one). The study was approved by the medical ethics committees of the Leiden University Medical Centre and the University of Leipzig.

DNA extraction

SF (61) or ST (68) samples, or both, were collected from swollen knee joints using the Parker-Pearson biopsy procedure with several modifications, as described previously.
samples used for DNA extraction (500 µl of 
SF, and six to eight synovial biopsy specimens) 
were transferred into digestion buffer contain-
ing proteinase K and incubated for 18 hours at 
60°C to release total DNA, as described 
previously. During the collection of the SF 
and ST biopsy samples in the outpatient 
procedure room and also during the transfer of 
SF and ST into digestion buffer in a biosafety 
hood, control vials with digestion buffer were 
opened and closed simultaneously with sample 
collection vials (collection controls). DNA 
extraction was performed in a safety hood, 
which was equipped with ultraviolet germicidal 
lamps, in separate, specifically dedicated, posi-
tive pressure laboratories (>10 Pa) with a lock-
gate. Dedicated pipettes with disposable filter 
tips, disposable gloves and laboratory coats, 
and non-reusable waste containers were used. 
The reagents from different manufacturers or 
different batches from a reagent, or both, were 
tested before application of the PCR on patient 
samples. Negative controls also consisted of 
digestion buffer without patient material, 
which were handled in the same way as the 
samples obtained from the patients. Finally, 
DNA was also obtained from ST of patients 
with non-detectable serum antibodies against 
the viruses under investigation and which were 
negative by PCR analysis in previous studies. 

Table 1  Primers used for polymerase chain reaction (PCR) amplification

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Primer</th>
<th>Sequence</th>
<th>Amplified genome region</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus™</td>
<td>A1</td>
<td>5´- TAC GCC AAC TCC GCC CAC GCG CTA (forward)</td>
<td>Hexon region</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>A2</td>
<td>5´- GCC GAG AAG GGC CTC GTG CCG AGG TA (reverse)</td>
<td></td>
<td>161</td>
</tr>
<tr>
<td>Cytomegalovirus™</td>
<td>ex1</td>
<td>5´- CCA AGC GGC CTC TGA TAA CCA AGC C (forward)</td>
<td>Major IE antigen region (external)</td>
<td>435</td>
</tr>
<tr>
<td></td>
<td>ex2</td>
<td>5´- CAG CAC CAT CCT CTC CTT CTT GTG C (reverse)</td>
<td></td>
<td>435</td>
</tr>
<tr>
<td></td>
<td>in1</td>
<td>5´- AGA GTC TGC TCT CCT AGT GTG (forward)</td>
<td>Major IE antigen region (internal)</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>in2</td>
<td>5´- AGA CAC TGG CTC AGA TTG GAC (reverse)</td>
<td></td>
<td>280</td>
</tr>
<tr>
<td>Epstein-Barr virus™</td>
<td>E1</td>
<td>5´- AGG GAT GCC TGG ACA CAA GA (forward)</td>
<td>EBNA2 gene (external)</td>
<td>596</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>5´- TGG TGC TGC TGC TGG TGG CAA T (reverse)</td>
<td></td>
<td>596</td>
</tr>
<tr>
<td>Parvovirus B19™</td>
<td>P1</td>
<td>5´- AAT ACA CTG TGG TTT TAT GGG CCG (forward)</td>
<td>NS1 region (external)</td>
<td>283</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>5´- CCA TTA GAA ACT GGG AGT AGC (reverse)</td>
<td></td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>P5S</td>
<td>5´- CTA AAA TGG CTT TTG CAG CTT CTA C (reverse)</td>
<td>NS1 region (internal)</td>
<td>102</td>
</tr>
<tr>
<td>Varicella-zoster virus™</td>
<td>VZV1</td>
<td>5´- CAG AAC TAC AGC GAG GGC ATC (forward)</td>
<td>Glycoprotein β gene (external)</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>VZV2</td>
<td>5´- CCA TTA GAA ACT GGG AGT AGC (reverse)</td>
<td></td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>VZV3</td>
<td>5´- GCC CAT GAA TCA CCC TCT TGT G (forward)</td>
<td>EBNA2 gene (internal)</td>
<td>497</td>
</tr>
<tr>
<td></td>
<td>VZV4</td>
<td>5´- CCT GCT ATT GAA GTC GTC TGC (reverse)</td>
<td></td>
<td>497</td>
</tr>
</tbody>
</table>

PCR TESTING OF SYNOVIAL DNA AND RNA

PCR experiments were performed, as de-
described previously. DNA extraction, pipet-
ting of each sample, PCR amplification, and 
electrophoresis were performed in separate 
rooms. Table 1 shows the primers used. All 
amplifications except for adenovirus and her-
pes simplex virus were carried out by nested 
PCR, using a thermal cycler 2400 (Perkin-
Elmer Cetus, Weiterstadt, FRG). Positive sam-
ple s were tested a second time and they were 
only classified as positive when the result of 
the first test could be confirmed independently.

The following positive controls were used 
for PCR analysis: herpes simplex virus (types I and 
II); vero cell culture infected with patient 
material, tested by immunofluorescence using 
monoclonal antibodies; varicella-zoster virus: 
human fibroblasts infected with the OKA 
strain; cytomegalovirus: human diploid fibro-
blasts infected with the AD 169 strain; adeno-
virus: HeLa cells, transfected with the AP61 
strain; parvovirus B19 NS1 sequence: positive 
patient serum and VP2 gene segment: baculo-
ivirus recombinant AcB19VP1L expressing 
VP1 protein of human parvovirus B19 (kindly 
provided by Professor W Spaan, Leiden); 
Epstein-Barr virus: Epstein-Barr virus immor-
talised B lymphocytes. As a positive control for 
the PCR procedure the housekeeping gene for

| Viral DNA species in synovial tissue | Internal and external primers were used in nested PCR, according to the respective protocols (see “Methods” for details). | 343 |

Table 2  Detection of various viral DNA in patients for whom both synovial tissue and synovial fluid were available

<table>
<thead>
<tr>
<th>Parvovirus B19 (n=56)</th>
<th>Epstein-Barr virus (n=53)</th>
<th>Herpes simplex virus (n=54)</th>
<th>Cytomegalovirus (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undiff. arthritis</td>
<td>1/16</td>
<td>0/16</td>
<td>1/16</td>
</tr>
<tr>
<td>Spondyloarthropathy</td>
<td>1/14</td>
<td>1/13</td>
<td>2/13</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>0/11</td>
<td>2/10</td>
<td>1/10</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>0/7</td>
<td>1/7</td>
<td>1/7</td>
</tr>
<tr>
<td>Crystal arthritis</td>
<td>0/7</td>
<td>0/6</td>
<td>0/7</td>
</tr>
<tr>
<td>Trauma</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Total (No (%))</td>
<td>2/56 (4)</td>
<td>4/5 (8)</td>
<td>5/5 (9)</td>
</tr>
</tbody>
</table>

Synovial tissue

<table>
<thead>
<tr>
<th>Parvovirus B19 (n=56)</th>
<th>Epstein-Barr virus (n=52)</th>
<th>Herpes simplex virus (n=54)</th>
<th>Cytomegalovirus (n=55)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiff. arthritis</td>
<td>2/16</td>
<td>4/15</td>
<td>3/16</td>
</tr>
<tr>
<td>Spondyloarthropathy</td>
<td>2/14</td>
<td>1/13</td>
<td>3/13</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>2/11</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>1/7</td>
<td>1/7</td>
<td>2/7</td>
</tr>
<tr>
<td>Crystal arthritis</td>
<td>0/7</td>
<td>0/7</td>
<td>1/7</td>
</tr>
<tr>
<td>Trauma</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Total (No (%))</td>
<td>11/56 (20)</td>
<td>9/52 (17 %)</td>
<td>11/54 (20)</td>
</tr>
</tbody>
</table>

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Table 3  Detection of various viral DNA in synovial tissue or synovial fluid

<table>
<thead>
<tr>
<th>Synovial fluid</th>
<th>Parvovirus B19 (n=5)</th>
<th>Epstein-Barr virus (n=5)</th>
<th>Herpes simplex virus (n=5)</th>
<th>Cytomegalovirus (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiff. arthritis</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>Septic arthritis</td>
<td>1/4</td>
<td>1/4</td>
<td>3/4</td>
<td>2/4</td>
</tr>
<tr>
<td>Total (No (%))</td>
<td>1/5 (20)</td>
<td>1/5 (20)</td>
<td>3/5 (60)</td>
<td>2/5 (40)</td>
</tr>
</tbody>
</table>

Table 4  Detection of multiple viral DNA in synovial tissue of patients with different rheumatic diseases

<table>
<thead>
<tr>
<th>Parvovirus B19 and herpes simplex virus (n=12)</th>
<th>Parvovirus B19 and cytomegalovirus (n=5)</th>
<th>Epstein-Barr virus and cytomegalovirus (n=5)</th>
<th>Number of positive patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated arthritis</td>
<td>1</td>
<td>0</td>
<td>4/12 (33)</td>
</tr>
<tr>
<td>Spondyloarthropathy</td>
<td>2</td>
<td>1</td>
<td>1/4 (25)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>1</td>
<td>1</td>
<td>1/3 (33)</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>1</td>
<td>0</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Crystal arthritis</td>
<td>0</td>
<td>0</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Septic arthritis</td>
<td>1</td>
<td>0</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Trauma</td>
<td>0</td>
<td>0</td>
<td>0/1 (0)</td>
</tr>
</tbody>
</table>

GAP-DH was amplified. Randomly chosen samples were used for photometric determination of DNA concentration, which varied from 300 to 600 ng/µl. For PCR analysis a total amount of at least 300 ng was used. The laboratory investigators in Germany were blinded to the clinical diagnoses of the patients from the Netherlands.

Results

All positive controls were positive and the negative controls were negative. PCR analysis of ST was positive for cytomegalovirus in 23 patients, parvovirus B19 in 12 patients, Epstein-Barr virus in 11 patients, and herpes simplex virus in 12 patients (tables 2 and 3). DNA from adenovirus or varicella-zoster virus was not detected in the ST of any of the patients. There was no correlation between the presence of viral DNA and the clinical diagnosis or the serum levels of acute phase reactants (data not shown). As shown in tables 2 and 3, there were some small differences in patient numbers, because in some cases there was insufficient DNA for all PCR analyses.

Fifteen patients were double positive for different viral DNA species. Also, these double positives were scattered evenly over the diagnostic groups (table 4).

Paired samples of ST and SF were available from 56 patients. SF analysis showed essentially the same pattern as ST analysis, but with a lower incidence of positive results (tables 2 and 3). Viral DNA was detected in 37 patients in ST compared with 19 positive SF samples. Some of these specimens were double or triple positive for viral DNA. If SF samples were positive, ST biopsy specimens were also positive, except for one case with parvovirus B19 DNA only in the SF. This patient was positive for Epstein-Barr virus and cytomegalovirus DNA in the ST. In three patients Epstein-Barr virus was detected in both SF and ST. However, parvovirus B19 was found in the SF in only one patient, whereas ST was positive in 11 cases. Twenty patients were positive for cytomegalovirus in ST and, of these, 11 were positive in SF as well. Herpes simplex virus was found in the SF of five of the 11 patients with a positive result in ST.

Discussion

PCR analysis was used to investigate the presence of DNA from several viruses in joint samples from patients with various forms of early arthritis. DNA from either parvovirus B19, Epstein-Barr virus, herpes simplex virus, or cytomegalovirus could be detected in the ST from 13 of the 22 patients with undifferentiated arthritis. The findings were not specific for any type of arthritis: viral DNA was present in joint samples from some patients with undifferentiated arthritis, spondyloarthropathy, RA, inflammatory osteoarthritis, crystal arthropathy, and bacterial arthritis. Fifteen patients were double positive for different viral DNA species. DNA from adenovirus and varicella-zoster virus was not found in any patient. The findings confirm and extend previous studies. New observations are the presence of viral DNA in undifferentiated arthritis, the simultaneous presence of DNA from several viral species, and the finding that DNA from some viruses can be detected more often in synovial tissue than in synovial fluid.

According to the protocols used in this study the sensitivity of the PCR reaction can be estimated on at least 1–100 genome equivalents—that is, a sensitivity sufficient for the analysis of at least 600 ng total DNA, as used here. Paired samples of ST and SF were available in 56 patients. Overall, if SF samples were positive, ST samples were also positive. In one patient, however, parvovirus B19 DNA was found in...
the SF but not in the ST, even after several replicate analyses with the corresponding probe. Degradation of DNA and the presence of inhibitory substances were excluded by GAP-DH PCR and contamination was excluded by the negative controls, as described. This suggests that, in rare cases, the distribution of viral DNA is not homogeneous in the ST.

On the other hand, viral DNA was detected in 37 patients (total 51 cases) in ST as compared with 19 (total 22 cases) in SF. The difference in the detection of viral DNA between ST and SF depends on the type of virus under investigation. For Epstein-Barr virus it seemed to be of little importance whether ST or SF was used. However, ST samples were substantially more often positive for parvovirus B19, cytomegalovirus, and herpes simplex virus than SF samples. Thus it may be advantageous to use biopsy specimens rather than fluid aspirates for the detection of viral DNA in joint samples.

In this study as many as two thirds of the patients with various forms of early inflammatory arthritis harbour viral DNA in their joints. In addition, about 20% of the patients were positive for DNA of two viral species. The detection of viral DNA in the joints of patients with arthritis, however, does not necessarily indicate the presence of infectious viruses. Obviously, PCR analyses do not allow any conclusion to be drawn about the completeness of the viral genome or the potential of viral replication. Moreover, the fact that viral DNA was not only detected in undifferentiated arthritis, RA, and spondyloarthropathy but also in arthropathies with diverse pathogeneses—for example, crystal induced arthropathy and osteoarthritis, does not substantiate a significant aetiological role for the viruses investigated in the clinically earliest phases of chronic arthritides of unknown cause. Of course this does not exclude a role for these viruses as initiating agents in the earliest phases of the disease. We have previously shown in patients with RA that so-called early arthritis may already represent a chronic phase of the disease.

A tempting speculation is that the presence of viral DNA in various arthropathies, as described here, is secondary to the migration of macrophages or other cells, harbouring viral DNA, into the synovial compartment. Similarly, this mechanism might explain the presence of bacterial DNA in joint samples from arthritic patients. Other mechanisms may also play a part. For instance, herpes simplex virus is known to be activated in neuronal axons by inflammatory cytokines, such as interleukin 1 and tumour necrosis factor α. Thus the inflamed synovium, which produces a variety of proinflammatory cytokines, might activate latent viruses. These mechanisms could also explain the presence of DNA from two or more viruses in the joint samples. Even when the presence of viruses is a secondary phenomenon, they may still play a part in the pathogenesis of arthritis as viral antigens could further enhance synovial inflammation. In addition, partially breaking tolerance is not necessarily sufficient to cause autoimmunity. A second step of conditioning of the inflammatory milieu by other agents was also required, for example, by variable activated T cells to cause tissue damage.

Taken together, viral material of one or more viruses can be detected in ST and SF samples of some patients with early arthritis by PCR analysis, irrespective of the clinical diagnosis, indicating that detection of viral DNA in joint samples has limited diagnostic potential. This phenomenon is presumably the result of the non-specific migration of inflammatory cells containing viral particles into the synovial compartment, where they can promote synovial inflammation.

This work was supported by the Bundesministerium für Bildung, Forschung und Technologie (BMBF+F), Interdisziplinäres Zentrum für Klinische Forschung (IZKF) at the University of Leipzig (Project A1), an HSP-grant by the Ministerium für Wissenschaft und Kunst (SMWK) of Saxony, and The Dutch Arthritis Foundation (Het Natioaal Reumafonds) grant number 94–637.