Does parvovirus B19 have a role in rheumatoid arthritis?

Simo Nikkari, Reijo Luukkainen, Timo Möttönen, Olli Meurman, Pekka Hannonen, Mikael Skurnik, Paavo Toivanen

Abstract

Objectives—To determine whether parvovirus B19 (B19) infection is associated with rheumatoid arthritis (RA).

Methods—The polymerase chain reaction was applied to serum, cells isolated from synovial fluid, and synovial fluid. Enzyme immunoassay technique was used to detect antibodies against B19.

Results—Of 142 patients with early RA (onset of disease under one year) and 67 control patients, serological evidence of recent parvoviral infection was found in 4/135 and 2/62, respectively. However, no evidence for the presence of parvoviral DNA was observed in 18 synovial fluids, 21 samples of synovial fluid granulocytes or 40 sera, all obtained from 65 patients diagnosed with early RA.

Conclusion—Although there is published evidence of chronic rheumatoid-like arthropathy following acute parvovirus infection, our findings do not support the involvement of B19 in the aetiopathogenesis of RA.

Parvovirus B19 (B19) is a small single stranded DNA virus. B19 is the only known pathogenic human parvovirus. It is known to cause erythema infectiosum, aplastic crisis in patients with haematological disorders or immunosuppression, transient erythroid aplasia of childhood, hydrops fetalis, and may be associated with other conditions such as fibromyalgia. Especially in adults, arthropathies following primary infection with B19 are common, and cases of chronic rheumatoid-like arthropathy following parvovirus infections have been reported. B19 DNA has been detected in the synovial fluid of a patient with serologically proven parvovirus B19 infection using dot blot hybridisation and in synovial fluid cells of a patient with 'reactive arthritis' using in situ hybridisation. In a recent report B19 DNA could be amplified by PCR from the synovial tissue, but not from the serum of two patients with chronic B19 arthropathy.

Parvovirus laboratory diagnostics is based primarily on serology. Because parvovirus infections are difficult to culture in vivo, lack of native antigen has limited the availability of the test. Antigen purified from viraemic human sera, synthetic peptides and recombinant antigens have been used. Methods based on the detection of B19 DNA have been described in several reports. As a routine clinical test, dot-blot hybridisation and DNA amplification by polymerase chain reaction (PCR) have been reported to be useful in acute B19 infection, before seroconversion, in association with haematological disorders, PCR in chronic B19 infection in immunocompromised patients, and in screening of blood products B19.

In the present work, we have studied the role of B19 in the aetiopathogenesis of RA. For this purpose, samples from patients with early RA (onset of disease under one year) were analysed by using serological assay of B19 antibodies and DNA amplification by PCR. As controls, patients with reactive arthritis (ReA) or other arthropathies and healthy blood donors were used.

Patients and methods

Patients

Samples from 62 patients initially diagnosed with RA were studied by PCR for the presence of B19 virus and for serological evidence of recent B19 infection. The detailed characteristics of the patients are given in table 1. The final diagnosis was made according to the American College of Rheumatology criteria. Forty patients were examined in the Turku University Central Hospital, 15 in Satalinna Hospital (situated also in south west Finland) and seven in Jyväskylä Central Hospital in central Finland. The samples were collected between April 1989 and December 1991.

The patients were primarily examined by general practitioners and sent to the rheumatologist for verification of the diagnosis and treatment. The samples were collected during the rheumatological examination and taken within one year from onset of disease [mean (SD) time from onset of disease 5.7 (3.2) months]. The diagnosis was confirmed approximately a year after the sample collection.

An additional group of 80 patients [mean (SD) age 51.2 (15.4) years, 51 females, 29 males] diagnosed with RA, with disease duration of one year or less [mean (SD) time from onset of disease 5.2 (2.6) months], was included. Paired sera taken at 3 month intervals were available from 54 of these patients, and only the first sample from 26. All patients of this group were examined in Jyväskylä Central Hospital during the years 1988 and 1989. In this group, the serum samples were analysed for B19 antibodies and only samples of patients with serological
evidence of recent B19 infection (detectable anti-B19 IgM antibodies or a rise in anti-B19 IgG antibody titre) were studied by PCR (table 2).

As control groups, 27 patients with reactive arthritis (ReA) and 15 patients with other arthropathies were studied (table 1). The samples were taken during March 1990–January 1992. Plasma samples from 25 healthy blood donors were also screened for B19 specific antibodies and DNA; the samples were taken during April–June 1991. Several serum samples taken during 1984–1991 from patients with serological evidence of acute B19 infection were screened by PCR as well.

**Clinical Specimens**

Synovial fluid (SF) was aspirated from knee joints and mixed immediately with heparin (50 IU/ml). The synovial fluid cells were pelleted by low speed centrifugation (400 g) and the supernatant was stored at -40°C. Mononuclear and polymorphonuclear cells were separated by Ficoll-Histopaque gradient centrifugation. Red blood cells were lysed using 0.83% NH₄Cl, pH 7.0. After washing twice with Hanks' buffered saline the cells were counted and cryopreserved in fetal calf serum containing 10% DMSO at -153°C.

**DNA extraction from the polymorphic nucleic cells was performed after proteinase K digestion.** A 5 µl aliquot of DNA equivalent to 50 000 cells was used as a template in PCR.

For PCR, serum, plasma and synovial fluid supernatant samples were treated as described earlier, with slight modifications; 500–1000 µl of sample was heated at 95°C for 10 minutes and centrifuged for 1 hour in 20 000 g at +4°C (Beckman TL-100 ultracentrifuge, Palo Alto, CA, rotor type TLA-100, 3). The supernatant was collected and 5 µl used as a template in PCR. Additionally, supernatants of plasma samples and synovial fluid were further diluted 1:10 in PCR buffer (Boehringer Mannheim, Mannheim, Germany) before the analysis. Inhibition of the polymerase enzyme was excluded by the ability of parallel amplification of a small amount of known B19 positive serum mixed with the clinical sample. The sample serum from the additional RA group were diluted 1:10 in PCR buffer and 5 µl was used as such for PCR analysis.

## Table 1  The clinical specimens studied

<table>
<thead>
<tr>
<th>Patients</th>
<th>No</th>
<th>Female/Male</th>
<th>Age Mean (SD) (years)</th>
<th>Results (positive*tested)</th>
<th>Serology</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early rheumatoid arthritis</td>
<td>62**</td>
<td>42/20</td>
<td>54-3 (14-7)</td>
<td>19/55</td>
<td>0/37***</td>
<td>0/18</td>
</tr>
<tr>
<td>Reactive arthritis†</td>
<td>27</td>
<td>12/15</td>
<td>42-9 (14-5)</td>
<td>1/27</td>
<td>0/16</td>
<td>0/16</td>
</tr>
<tr>
<td>Other arthropathies</td>
<td>15</td>
<td>9/6</td>
<td>42-7 (16-8)</td>
<td>0/10</td>
<td>0/8</td>
<td>0/5</td>
</tr>
<tr>
<td>Healthy blood donors</td>
<td>25</td>
<td>9/16</td>
<td>40-0 (10-1)</td>
<td>1/25</td>
<td>0/255§</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*Evidence of recent parvovirus infection; see table 2.
**48/62 were RF positive and 17/62 had erosive disease at the time when the first clinical speciments were taken.
***From 12 patients both serum and synovial fluid specimens were studied. In addition serum samples of 3 patients from the reactivity group of patients with RA were negative.
†In addition, three patients from the additional group of 80 patients with RA were positive.
§Seven triggered by Salmonella, 6 by Yersinia, 5 by Chlamydia, 1 by Chlostridium difficile and 8 by non-defined micro-organisms.
§§Plasma instead of serum studied.

## Table 2  Patients with serological evidence of recent parvovirus infection.

<table>
<thead>
<tr>
<th>Patient No</th>
<th>Age at onset (years)</th>
<th>Sex</th>
<th>RF</th>
<th>Characterisation of disease</th>
<th>Duration of follow up (years)</th>
<th>Duration of disease at drawing of sample (months)</th>
<th>Anti-parvovirus B19 antibodies (ELISA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgM (OD 405 nm)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35</td>
<td>F</td>
<td>-</td>
<td>Parovirus B19 arthropathy. Fully recovered in 3 years. Non-erosive. Treated as seronegative, oligoarticular RA.</td>
<td>3</td>
<td>6</td>
<td>1:019</td>
</tr>
<tr>
<td>25</td>
<td>38</td>
<td>F</td>
<td>-</td>
<td>Parovirus B19 arthropathy. Symptoms preceded by sore throat. Total remission in 6 months.</td>
<td>4</td>
<td>3</td>
<td>0:187</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>M</td>
<td>+</td>
<td>Rheumatoid arthritis. Seropositive, polyarticular, erosive disease. Almost total remission with gold treatment. Serological evidence of B19 infection unclear.</td>
<td>3</td>
<td>4</td>
<td>0:213</td>
</tr>
<tr>
<td>5</td>
<td>38</td>
<td>F</td>
<td>+</td>
<td>Parovirus B19 arthropathy. Initially diagnosed as self-limiting reactive arthritis. Symptoms during follow up.</td>
<td>1</td>
<td>3 weeks</td>
<td>0:673</td>
</tr>
<tr>
<td>6</td>
<td>54</td>
<td>M</td>
<td>-</td>
<td>Healthy blood donor.</td>
<td>–</td>
<td>–</td>
<td>0:776</td>
</tr>
</tbody>
</table>

1 Mean value of duplicate samples.
2 Mean value of negative controls.
3 Diagnostic rise in I, II and III sample.
4 Patients 2-4 are from the additional RA group of 80 patients; from these only serum samples were available.

ND = not done.

SF = synovial fluid.
SFC = synovial fluid cells.
The total incubation volume was 50 µl, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.1 mg/ml gelatin, 200 µM each dNTP, 2.5 units Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), 50 pmol each primer and 5 µl template. The PCR steps were: 94°C for 45 s, 63°C for 1 min, 72°C for 2 minutes repeated 30 times and only 1:25 units of polymerase enzyme per reaction were used.

Special care was taken to avoid contamination of samples with amplicons. Clinical material was handled whenever possible in laminar flow hoods. The preparation of PCR reaction mixture and pipetting of samples were performed in laminar flow hoods situated in separate rooms with restricted passage of personnel and decontamination barriers, including changing of protective clothing on entering and positive air pressure of the area. The rooms and hoods were illuminated by UV irradiation lamps, when not in use. The amplification procedure and analysis of PCR products were performed in another laboratory room. Special emphasis was placed on preventing carryover of amplicons from post-PCR to pre-PCR facilities. Positive displacement pipettes (Tri-Continent Scientific, Grass Valley, CA) were used in pipetting of specimens.

PrIMeRS AND CONTROLs USeD IN PCR

The oligonucleotide primers used were selected according to previously published sequences and analysed using Oligo (Version 3-4, MedProbe, Oslo, Norway) primer analysis software. Primer P1 (5'-GTA CGC CCA TCG CGG GGA CCA GTT CAG G-3') is situated in position 2060-2087 and P5 (5'-CCC ACA TGG CAG CTA CAT CGC ACC AAA T-3') in position 2369-2342 of the sequence published by Shade et al. The length of the amplified product is 310 bp. P2 (5'-CAG GTA AAC CCC TTA CAC CGT CCC ACA C-3', position 2230-2203) was used as a probe in hybridisation experiments. Five µl of a known B19 positive serum sample diluted 1:1000 in water was used in all assays as positive control. A negative control consisting of all reaction reagents necessary for PCR, with water as template, was always included.

To exclude possible false negative results caused by inhibitory factors, the presence of human DNA in the synovial fluid cell samples was verified by collateral amplification of human beta-globin gene using primers H1 (PC04, 5'-CAA CT T CAT CCA CGT TCA CC-3', position 54-73) and H2 (GH20, 5'-GAA GAG CCA AGG ACA GGT AC-3', position 195-176), which amplify a 268 bp segment of human beta-globin encoding genome.

DETECTION OF AMPLIFIED PRODUCT

Ten µl of the amplified product was analysed on 1.5% agarose gel electrophoresis and visualised by UV fluorescence after staining with ethidium bromide. After electrophoresis the DNA was transferred overnight to a Hybridisation Transfer Membrane (Gene-ScreenPlus, Du Pont, Boston) with 0.4 N NaOH using the capillary blot procedure. The filter was washed with 0.2 M Tris-HCl, pH 7-5, 2 × SSC (0.3 M NaCl and 0.03 M sodium citrate) before drying. The amplified 268 bp human beta-globin gene product was visualised after agarose gel electrophoresis and staining with ethidium bromide.

The hybridisation experiments with B19 specific oligonucleotide probe (P2) were performed as reported earlier.

SeRology

B19 specific IgG and IgM antibodies were determined by enzyme linked immunosorbent assay using Parvoscan-B19™ (Ferring diagnostic, Malmö, Sweden). The synthetic-peptide based assay has been described by Fridell et al. From most patients serum was available, if not, synovial fluid was used (in 15 patients with RA, 13 with ReA, 4 with other arthropathies). From the healthy blood donors plasma was tested. Nonspecific anti-B19 IgM antibody-assay results caused by rheumatoid factor were eliminated by GullSORB (Gull laboratories, Salt Lake City, Utah) inactivation reagent or RF-Absorbant (Behring, Marburg, France).

Results

PCR

The sensitivity of the assay was about 70 molecules per assay when purified PCR product was used as template (figure). With the known B19 positive serum sample used as positive control, B19 DNA was detectable by Southern hybridisation of the PCR product to a serum dilution of 1:10⁴. Addition of human DNA equivalent to 50 000 synovial fluid granulocytes per assay did not affect sensitivity of the PCR. No B19 DNA was detectable from the patients with different forms of arthritis, including 65 patients with early RA (table 1). Conversely, B19 DNA could be amplified from 6/21 serum samples taken from patients with serologically proven acute B19 infection as well as in cases of hydrops fetalis and transient erythroblastopenia. Prolonged B19 viraemia in a child with aplastic crisis was detectable by our assay. Human beta-globin gene was amplified from all the synovial granulocyte samples, indicating that significant inhibitory agents were not present and that DNA isolation had been properly carried out.

Serology

The patients with serological evidence of recent B19 infection are shown in table 2. Four patients of 135 initially diagnosed with early RA had serological evidence of recent B19 infection (patients 1–4). Three of these patients seem to be true B19 arthropathies.
Sensitivity of the assay using different amounts of amplified parvovirus B19 DNA fragment as template. The 310 bp amplified B19 DNA fragment was purified from a preparative agarose gel using an Ultrafree-MC Filter Unit with 0.45 μm membrane (Millipore Corporation, Bedford, MA) as recommended by the manufacturer. The DNA concentration was measured by UV \textsubscript{260} spectrophotometry. (A) Agarose gel electrophoresis of amplified product after 40 cycles of polymerase chain reaction (PCR) with primers P1 and P5. Lane M, molecular weight marker; lane 1, 7 × 10\textsuperscript{5} molecules; lane 2, 7 × 10\textsuperscript{5} molecules; lane 3, 7 × 10\textsuperscript{5} molecules; lane 4, 7 × 10\textsuperscript{5} molecules; lane 5, 7 × 10\textsuperscript{5} molecules; lane 6, 7 × 10\textsuperscript{5} molecules; lane 7, 7 × 10\textsuperscript{5} molecules; lane 8, 7 × 10\textsuperscript{5} molecules; lane 9, 7 × 10\textsuperscript{5} molecules; lane 10, 7 × 10\textsuperscript{5} molecules; lane 11, known parvovirus positive serum, dilution 1:1000; lane 12, negative control; (B) Southern blot hybridisation of the same agarose gel, using parvovirus B19 specific probe (P2).

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(patients 1–3), and one fulfils ACR criteria for RA (patient 3). The serological diagnosis of patient number 1 is based on a positive anti-B19 IgM antibody level, which together with the low anti-B19 IgG suggests a possibility of subclinical infection after the beginning of arthritic symptoms. Patient number 2 is clinically a typical B19 arthropathy patient. The joint manifestations were preceded by symptoms of upper respiratory tract infection in concordance with acute B19 infection. The serological diagnosis is based on slow development of anti-B19 IgG antibodies during several months, a phenomenon we have not encountered before in the diagnosis of acute B19 infection in otherwise healthy individuals. The anti-B19 IgM antibodies detected in patient 3 are consistent with a recent infection within two months. Unfortunately, paired sera were not available from this patient, nor from patient number 1. In patient number 4 a slight rise in anti-B19 IgG antibodies between the first two serum samples is observed, but on the basis of the clinical picture, B19 arthropathy seems unlikely.

In the control group two possible cases of recent B19 infection were found. Patient number 5 was initially diagnosed as having self-limiting reactive arthritis, the triggering agent being uncertain. The IgM and IgG antibody responses against B19 are consistent with recent infection; the patient most probably had B19 arthropathy. In addition, anti-B19 IgM antibodies were observed in one healthy blood donor (patient 6), from which no clinical information is available.

Discussion

Acute B19 infections are quite commonly followed by arthropathies, especially in adults. Chronic conditions resembling RA associated with B19 infection have also been reported.\textsuperscript{6,7} Parvovirus DNA has been observed in synovial fluid, synovial fluid cells and synovium of patients with B19 arthropathy.\textsuperscript{8–10} In acute B19 infection virus can be detected in high concentrations in serum and for a longer period in circulating polymorphonuclear cells.\textsuperscript{24} For these reasons we have studied the serum, synovial fluid and synovial fluid cells from patients with RA and from control patients.

In a recent prospective study the presence of B19 DNA was reported in 75% of synovial biopsy samples taken from patients with RA and 16–7% of biopsies taken from patients with osteoarthritis.\textsuperscript{25} In our study B19 DNA was not detectable in clinical samples taken from any of the patients with chronic arthritis; parvoviral DNA, however, was amplified from several control sera of patients with serological evidence of acute B19 infection, including individual cases of aplastic crisis in a child with an underlying haematological disorder and transient erythroleukemia of an otherwise healthy child.\textsuperscript{3} B19 could also be detected in the amniotic fluid of a case of hydrops fetalis, caused by intrauterine B19 infection.\textsuperscript{4} The sensitivity of our PCR assay was approximately 70 B19 DNA particles, which is the same as that reported by Saal \textit{et al}.\textsuperscript{25} We do not have a good explanation for the discrepancies between these two studies. In our study synovial biopsies were not studied, although this does not explain the different results, as Saal \textit{et al} found B19 in peripheral blood mononuclear cells of 15% of patients in all groups. The duration of RA of the patients described by Saal \textit{et al} was approximately 11 years, whereas our patients suffered from recent onset RA. In our opinion it is preferable to study patients with disease duration as short as possible when the aetiological agent is being sought. We also studied by PCR samples taken from 9 patients with advanced RA [8 sera, 2 synovial fluids, 1 synovial fluid cell sample; mean (SD) from onset of disease 5·1 (4·5) years]; B19 DNA was not present in these
samples either. One possible explanation in the report by Saal et al is a cross-reaction of the primer sequences with other parvoviruses. In 1984 Simpson et al suggested that parvoviruses are associated with RA, but the origin of their RA-1 virus has remained unclear. With PCR, initially three samples of our patients with arthritis (two synovial fluid and one serum sample, all from different patients and patient groups) were weakly positive after hybridisation experiments of PCR products with B19 specific probe. They were negative when PCR was repeated from the original samples; the contamination source of these false positive results was not found. The risk for contamination is great when the amplification method is optimised to yield high sensitivity.

Cohen et al observed evidence for recent B19 infection more often in patients with RA than in the controls, using antibody detection based on a radioimmunoassay with monoclonal antibodies. In their study, four patients out of 69 had evidence of B19 infection at the onset of RA; the prevalence of anti-B19 IgG was also significantly higher (92-3%) in RA patients than in the controls (60-9%). Lefrere et al using counterimmuno-electrophoresis, did not find significant differences in B19 seropositivity between patients with RA (33-3%) and the adult healthy population (25-3%). The assay applied by us for detection of B19 antibodies is based on the use of synthetic peptides as the antigen. We could find no significant differences in the mean values for anti-B19 IgM and IgG antibodies in the different patient groups (data not shown).

The clinical specimens from patients with arthritis studied in this work were taken during the years 1988-1992. According to local paediatricians, during the study period only sporadic cases of erythema infectiousum were seen in the Turku area (Jussi Mertsola, Department of Paediatrics, Turku University Central Hospital, personal communication). Serological data of B19 infections from the Turku area are available from the beginning of year 1991. During our arthritis investigations, no B19 epidemics were observed in the community, despite some serologically proven infections, and a few B19 viraemic (PCR positive) patients (unpublished findings).

The epidemiological situation may account for the differences between our findings and those reported by Cohen et al. The earliest serum samples analysed from the RA patients with RA studied by Cohen et al were taken within five months from the onset of arthritic symptoms. The first sample taken from our 62 patients with RA was taken within one year from the beginning of arthritic symptoms (average 5-7 months). From 18 of these 62 patients with RA the serum sample was available within three months from the onset of disease; this is a reasonable window for diagnosis of acute B19 infection based on B19 anti-IgM serology. In chronic arthropathy, however, a prolonged B19 anti-IgM response could occur, as has been shown in arthritis associated with rubella virus infection. In one of our B19 arthropathy patients (number 1, table 2) B19 anti-IgM antibodies were detectable 6 months after the beginning of the arthritic symptoms; the patient did not present any symptoms of an infectious febrile illness, so the true timing of her B19 infection remains unsolved. Among the additional 80 patients with RA the first serum sample was taken from 22 patients within three months from the beginning of the symptoms (average 5-2 months), and convalescent sera were available from 54 patients for detection of IgG anti-B19 antibodies.

It has been proposed that a significant proportion of patients diagnosed as seronegative RA and a lesser proportion of seropositive RA patients actually have B19 arthropathy. In the present study we observed serological evidence of recent B19 infection in only four of 135 patients with early RA. In two cases the finding was based only on high IgM antibody concentrations; paired sera were not available. Evidence of recent B19 infection was also found in one patient initially diagnosed as self-limiting reactive arthritis and in one healthy blood donor. The discrepancy between our findings and those published earlier might be explained by the low prevalence of B19 infection circulating in the community during our study.

According to our PCR and serological data, the association of B19 infection with RA is a rare phenomenon. Nevertheless, chronic B19 arthropathy should be kept in mind in the differential diagnosis of RA. Our data do not exclude the possibility of the virus persisting at the site of inflammation after acute infection, due to the small number of patients in our study who actually had chronic B19 arthropathy. The present findings do not support the involvement of B19 in the aetiopathogenesis of RA. However, its role as a triggering factor in a genetically or otherwise predisposed host should not be ruled out.

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2 Young N. Hematologic and hematopoietic consequences of B19 parvovirus infection. Seminars in Hematology 1988; 25: 159-72.
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