

## CONCISE REPORTS

## Persistence of parvovirus B19 in synovial fluid and bone marrow

S Nikkari, A Roivainen, P Hannonen, T Möttönen, R Luukkainen, T Yli-Jama, P Toivanen

**Abstract****Objectives**—To determine whether parvovirus B19 (B19) persists in rheumatoid arthritis (RA).**Methods**—Polymerase chain reaction (PCR) was used to detect parvovirus B19 genome in the synovial fluid cells or peripheral blood mononuclear cells from 61 patients with early RA; bone marrow from one patient was also studied. The synovium or synovial fluid cells from 28 patients with advanced RA, and synovial fluid cell samples from 18 patients with reactive arthritis (as controls) were studied. Two separate sets of primers and probe were used.**Results**—Parvovirus B19 specific gene sequences were detected in two patients with early arthritis fulfilling the criteria for RA.**Conclusion**—Parvovirus B19 does not play a significant role in the aetiopathogenesis of RA. However, a few cases of a disease indistinguishable from RA may be triggered by parvovirus B19 infection.

(Ann Rheum Dis 1995; 54: 597-600)

Parvovirus B19 (B19) is the only known human pathogenic parvovirus. B19 causes several clinical manifestations, the commonest being erythema infectiosum, the fifth rash disease of children. There is published evidence of chronic B19 infection in association with congenital and acquired immunodeficiency, in fetuses of non-immune women experiencing B19 infection,<sup>1</sup> and in association with certain vasculitides<sup>2-3</sup> and chronic arthropathies.<sup>4-5</sup> Arthritic symptoms after acute B19 are common among adults, especially in women. Several cases of chronic B19 arthro-

pathy fulfilling criteria for rheumatoid arthritis (RA) have been reported.<sup>4-6</sup> In acute B19 arthropathy, the virus has been detected in the synovial fluid<sup>7</sup> and synovial fluid cells.<sup>8</sup> In chronic B19 arthropathy, persistence of B19 in bone marrow was reported recently.<sup>9</sup> Saal *et al*<sup>10</sup> reported the presence of B19 DNA in 75% (15/20) of synovial samples of patients with RA and in 16.7% (4/24) of control patients with other arthropathies. In the peripheral blood mononuclear cells, B19 DNA could be amplified in 15% of RA patients and the controls. As these observations are in contrast to our recent findings,<sup>11</sup> we have repeated essential parts of the study by Saal *et al* in our own patients. In addition to 28 patients with advanced RA, 61 with early RA (duration of disease less than one year) were included. Eighteen patients with reactive arthritis were studied as controls. The positive polymerase chain reaction (PCR) findings were confirmed by using an additional set of primers and a probe.

**Patients and methods**

## PATIENTS AND CLINICAL SPECIMENS

The table shows details of the patients studied. At the time of sample collection, all RA patients fulfilled at least four of the seven American Rheumatism Association (ARA) revised criteria for RA.<sup>12</sup> The samples were collected between April 1989 and November 1993, from Jyväskylä Central Hospital (18 patients), Satalinna Hospital (29 patients), Pikonlinna Hospital (five patients), and Turku University Central Hospital or Paimio Hospital (37 patients). These hospitals are situated in central Finland (Jyväskylä), Southern Finland (Pikonlinna), and South West Finland (Satalinna, Turku and Paimio). Samples from 18 patients with reactive arthritis were

Department of Medical Microbiology, Turku University, Turku, Finland

S Nikkari  
A Roivainen  
P Toivanen

Department of Medicine, Central Hospital, Jyväskylä, Finland  
P Hannonen

Division of Rheumatology, Department of Medicine, Turku University Central Hospital, Turku, Finland  
T Möttönen

Department of Surgery  
T Yli-Jama

Satalinna Hospital, Harjavalta, Finland  
R Luukkainen

Correspondence to: Simo Nikkari, MD, Department of Medical Microbiology, Turku University, Kiinamyllynkatu 13, SF-20520 Turku, Finland.

Accepted for publication 6 March 1995

**Patients and samples**

Patient group	Sex (F/M)	Age (yr)	Duration of disease	PCR (positive/total)				Circulating IgG antibodies against B19†	
				Synovial tissue	SFMC	PBMC	BMC		
Early rheumatoid arthritis† (n = 61)	61	37/24	56 (16)	5 (3) months	ND	2/41	1/36	1/1	27/33
Advanced rheumatoid arthritis (n = 28)	28	19/9	57 (9)	16 (9) years	0/20	0/8	ND	ND	ND
Reactive arthritis§ (n = 18)	18	7/11	36 (10)	4 (3) weeks	ND	0/18	ND	ND	ND
Total	107	63/44			0/20	2/67	1/36	1/1	27/33

Values are number, or mean (SD). †All patients fulfil at least four of the seven revised ARA criteria; 56, 59, 60, 60, one, 55, and 23 of the 61 patients meet the ARA criteria 1-7, respectively.

§Four triggered by *Chlamydia*, nine by *Salmonella*, five by *Yersinia*. SFMC = Synovial fluid mononuclear cells; PBMC = peripheral blood mononuclear cells; BMC = bone marrow cells; ND = not done.

collected between April 1989 and May 1993, from the same hospitals.

DNA extraction from the synovial and peripheral blood mononuclear cells was performed after Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) centrifugation and proteinase K digestion.<sup>13</sup> A 5 µl aliquot of DNA, equivalent to 50 000 cells, was used as a template in PCR. Cells from the bone marrow aspirate were treated as described above. The synovial tissue samples were collected during surgery of the hand, wrist, foot, ankle, elbow, or knee joints. After removal, the tissue was immediately frozen in liquid nitrogen and stored at -70°C. The frozen synovial tissue was pulverised mechanically and DNA isolation was performed after proteinase K digestion by phenol-chloroform-isoamyl alcohol (25:24:1) extraction, essentially as described by Jackson *et al.*<sup>14</sup> After ethanol precipitation, the DNA concentration was measured spectrophotometrically at 260 nm. For PCR, 200 ng of DNA isolated from the synovial tissue samples was used as template.

#### PCR CONDITIONS

The standard PCR amplification with primers B19I (5'-ATG GGA TAC TCA ACC CCA TGG-3', position 3365-3385) and B19II (5'-CCT GTA GTG CTG TCA GTA ACC-3', position 3564-3544) was performed essentially as described by Saal *et al.*<sup>10</sup> in 0.5 ml polypropylene tubes with a Perkin Elmer Cetus thermal cycler. The total incubation volume was 50 µl, containing 10 mmol/l Tris-hydrochloric acid pH 8.3, 50 mmol/l potassium chloride, 1.5 mmol/l magnesium chloride, 0.1 mg/ml gelatin, 200 µmol/l of each oligonucleotide, 1.25 units Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany), 50 pmol each primer and 5 µl template. The PCR steps 94°C for one minute, 66°C for three minutes were repeated 35 times. The confirmatory PCR procedure for B19 using primers P1 (5'-GTA CGC CCA TCC CCG GGA CCA GTT CAG G-3', position 2060-2087) and P5 (5'-CCC ACA TGG CAG CTA CAT CGC ACC AAA T-3', position 2369-2342) was performed as reported earlier.<sup>11</sup> To exclude possible inhibitory factors from the PCR reaction, the presence of human DNA in all clinical samples was verified by collateral amplification of the human beta-globin gene.<sup>11</sup>

Special care was taken from the beginning of our studies to avoid contamination of samples with amplicons.<sup>11</sup> A negative control consisting of all reagents necessary for PCR, with water as template, was always included. A known B19 positive serum 1:1000 was used as positive control.<sup>11</sup>

#### DETECTION OF AMPLIFIED PRODUCT

Ten microlitres of the amplified product was separated by agarose gel electrophoresis and visualised as ultraviolet fluorescence after staining with ethidium bromide. Southern hybridisation experiments with B19 specific

oligonucleotide probes (probe B19: 5'-ATA TGG TTA CAG TTA AAG CAT CAG GAG CTA TAC TT-3', position 3494-3460;<sup>10</sup> probe P2: 5'-CAG GTA AAC CCC TTA CAC CGT CCC ACA C-3', position 2230-2203<sup>11</sup>) were performed essentially as reported earlier.<sup>11 15</sup>

#### SEROLOGY

Parvovirus B19 specific IgG and IgM antibodies were determined from the serum using an enzyme linked immunosorbent assay test based on recombinant antigen (Progen Biotechnik GMBH, Heidelberg, Germany). Calculation of the test specific cut off was performed as described by the manufacturer (absorbance of the positive control + absorbance of negative control × lot specific constant = cut off). IgM antibodies were determined only from the two patients with molecular evidence of B19 infection (patients 1 and 2).

#### Results

The sensitivity of the PCR with primers B19I and B19II was tested using cloned plasmid<sup>16</sup> as template; after Southern hybridisation of the PCR products, about 0.2 fg of whole B19 DNA per reaction was detectable. This is in agreement with the sensitivity reported by Saal *et al.*<sup>10</sup>

B19 specific DNA was detected in two patients diagnosed initially as having early RA, from the total of 61 studied. Concordant results were obtained using both sets of primers and probe. A slight discrepancy was observed only with the peripheral blood mononuclear cells (PBMC) of patient 2; B19 DNA was detectable in small numbers after Southern hybridisation of the P1P5 primer set products, but not in those obtained with the B19I, B19II primer set. This reflects a difference in the sensitivity of the two sets of primers and probe. No B19 specific DNA was present in the samples from 28 patients with advanced RA, or from 18 control patients with reactive arthritis; the samples represented synovial tissue and synovial fluid cells (table).

B19 specific IgG antibodies were detected in 27 of 33 patients with early RA, indicating past B19 infection and the rate of immunity in the population studied. The two patients with positive PCR findings had high levels of B19 specific IgG antibodies in paired serum samples; no B19 specific IgM antibodies were detectable. This is in agreement with the clinical findings, as B19 IgM antibodies normally persist for three months or less after acute B19 infection.

The disease histories of the two patients with positive B19 PCR findings were as follows.

#### PATIENT 1

A 53 year old male from Central Finland had been treated for arterial hypertension for 20 years. Both parents and an older brother had RA. In early June 1993, this HLA-DR4 positive patient had an influenza like illness, with arthralgia and transient purpura in both

legs. Two weeks later he developed symmetric arthritis of the large joints. Rheumatoid factor was positive (253 IU/ml by nephelometry). At the Rheumatology Department of Jyväskylä Central Hospital on 6 July, the patient was found to have symmetric aggressive polyarthritis involving most small joints of the hands and feet, wrists, elbows, shoulders, knees, and ankles. No crystals were observed in synovial fluid taken from the knee. In addition to local corticosteroid injections, treatment with sulphasalazine and systemic prednisolone was initiated. After four weeks, the patient developed significant eosinophilia (32% of leucocytes). Sulphasalazine was discontinued and treatment with intramuscular aurothiomalate was started. The synovial fluid drawn on 19 August was positive for B19 by PCR. Despite the aggressive treatment, RA remained active, and at the end of November 1993 weekly methotrexate was added to the drug regimen. During these five months, rheumatoid nodules had developed on the elbows and buttocks, with erosions in several small joints of the hands and feet. PCR analysis of bone marrow aspirate drawn on 1 December revealed the presence of B19 sequences. IgG antibodies to B19 were detectable in sera drawn on 19 August and 1 December. No B19 specific IgM antibodies to B19 were detected. On 21 February 1994 the clinical symptoms and signs had subsided slightly, but RA remained active, necessitating intra-articular corticosteroid injections. In May, proteinuria was observed and gold treatment was discontinued. Otherwise, the patient's condition had improved, although there was still some activity in both wrist joints.

#### PATIENT 2

A previously healthy 44 year old man from South West Finland, with no history of rheumatic diseases in the family, suffered an influenza like disease, followed by several attacks of upper respiratory tract symptoms diagnosed as tonsillitis, at the end of December 1991. Other family members suffered similar symptoms. At the beginning of January 1992, this HLA-B27 and DR4 positive patient developed joint pains, joint swellings, and morning stiffness. In the middle of March, sulphasalazine and prednisolone treatment was begun. The patient was referred to the Rheumatology Department of Satalinna Hospital at the end of March. At this stage his general condition was good. There was swelling in both wrist joints, in the right metacarpophalangeal joints II and III, in the right elbow, and in the left knee. The rheumatoid factor was positive (243 IU/ml by nephelometry). Erosions or bony decalcification were not demonstrable. The hydropic left knee joint was aspirated on 31 March 1992, and B19 sequences were detected in the synovial fluid cells of this sample. High levels of circulating B19 specific IgG antibodies were present, with no detectable B19 specific IgM antibodies. The patient's condition improved

and prednisolone treatment was discontinued in August 1992. In October, almost complete remission was observed; only extremely slight arthralgias occurred. In April 1993, only heavy exercise induced arthralgia, and sulphasalazine treatment was discontinued. In April 1994, the clinical condition was similar to that one year earlier.

#### Discussion

We report two HLA-DR4 positive cases of B19 infection associated with early seropositive arthritis, fulfilling the criteria for RA. In patient 1, B19 DNA sequences were detectable two and a half and six months from the beginning of disease in cells of the synovial fluid and bone marrow, respectively. The disease of the patient greatly resembled classic, erosive RA. The possibility that this patient also suffers from a coincidental, serendipitous B19 infection cannot be excluded because chronic parvovirus arthropathy has been reported previously to differ from classic RA typically by the absence of rheumatoid nodules, joint destruction, and rheumatoid factor.<sup>4</sup> In contrast with that of patient 1, the disease in patient 2 was self limiting. He had a high level of circulating B19 IgG antibodies and a positive PCR finding from joint fluid aspirated three months after the beginning of the disease. A similar case of acute erosive polyarthritis associated with B19 infection was recently reported by Tyndall *et al.*<sup>5</sup> All these findings suggest that the differentiation between classic RA and chronic B19 arthropathy is not as clear as reported earlier. It is possible that not all patients with chronic arthritis after B19 infection have a benign course of the disease.

To our knowledge, this is the first report indicating persistence of B19 virus at the site of inflammation in early arthritis fulfilling the criteria for RA. In previous studies,<sup>7,8</sup> B19 was detected in the synovial fluid within a few weeks of arthritic symptoms and the disease resolved during three months or less. Persistence of B19 infection in seronegative chronic arthropathy has been documented in bone marrow aspirates obtained from four patients 24–42 months after acute infection.<sup>9</sup> Until now, only preliminary evidence is available indicating persistence of B19 sequences at the site of inflammation in chronic B19 arthropathy.<sup>4</sup> There are recent reports of chronic B19 infection associated with systemic vasculitis.<sup>2,3</sup> Therefore, it is attractive to speculate that certain genetically predisposed individuals are susceptible to autoimmune diseases when exposed to B19 infection.

Detection of two different sequences of the B19 genome in our two patients suggests the presence of the entire B19 virus. In addition to strict laboratory standards,<sup>11,15</sup> the use of two different sets of primers and probe further excludes the possibility of false positive PCR results caused by laboratory contamination. We have previously suggested possible cross reaction with other parvovirus sequences as an explanation for the difference between our

findings<sup>11</sup> and those of Saal *et al.*<sup>10</sup> The experiments have now been repeated using the same primer sequences with approximately the same sensitivity, and with different results. Therefore, only technical errors or an occurrence of local B19 epidemics remain as potential explanations. The incidence of B19 infections in Finland during the study period is not known. However, according to serological data obtained at the Turku University Department of Virology, a major B19 epidemic lasting for several months was experienced from the beginning of the year 1993; otherwise, only sporadic cases of erythema infectiosum were seen in the Turku area.<sup>11</sup> The disease of patient 1 might be associated with this epidemic, although the possible causal or casual relationship of the arthritis and B19 infection in this patient still remains open.

We conclude that B19 does not play a significant role in the aetiopathogenesis of RA. However, a few cases of a disease indistinguishable from RA may be triggered by B19 infection. Determination of B19 at the site of inflammation in a disease resembling classic RA should provoke further studies on the role of B19 and other microorganisms in the aetiopathogenesis of RA.

We are grateful to Dr Timo Hyypiä (Department of Virology) for valuable advice in preparation of this manuscript. Päivi Alenius, Tiina Haarala, Marju Niskala, and Anne Peippo are thanked for technical assistance.

This work was supported by the Academy of Finland, the Finnish Cultural Foundation (Varsinais-Suomen Kulttuurirahasto), the Finnish Medical Foundation, the Emil and Blida Maunula Fund, the Sigrid Jusélius Foundation, and the Finnish Rheumatology Foundation.

- 1 Frickhofen N, Young N S. Persistent parvovirus B19 infections in humans. *Microb Pathog* 1989; 7: 319-27.
- 2 Finkel T H, Török T J, Ferguson P J, et al. Chronic parvovirus B19 infection and systemic necrotising vasculitis: opportunistic infection or aetiological agent? *Lancet* 1994; 343: 1255-60.
- 3 Nikkari S, Mertsola J, Korvenranta H, Vainionpää R, Toivanen P. Wegener's granulomatosis and parvovirus B19 infection. *Arthritis Rheum* 1994; 37: 1707-8.
- 4 Naides S J. Parvovirus B19 infection. *Rheum Dis Clin North Am* 1993; 19: 457-75.
- 5 Tyndall A, Jelk W, Hirsch H H. Parvovirus B19 and erosive polyarthritis. *Lancet* 1994; 343: 480-1.
- 6 White D G, Woolf A D, Mortimer P P, Cohen B J, Blake D R, Bacon P A. Human parvovirus arthropathy. *Lancet* 1985; i: 419-21.
- 7 Dijkmans B A, van Elsacker-Niele A M, Salimans M M, van Albada-Kuipers G A, de Vries E, Weiland H T. Human parvovirus B19 DNA in synovial fluid. *Arthritis Rheum* 1988; 31: 279-81.
- 8 Kandolf R, Kirschner P, Hofschneider P H, Vischer T L. Detection of parvovirus in a patient with 'reactive arthritis' by in situ hybridization. *Clin Rheumatol* 1989; 8: 398-401.
- 9 Foto F, Saag K G, Scharosch L L, Howard E J, Naides S J. Parvovirus B19-specific DNA in bone marrow from B19 arthropathy patients: Evidence for B19 virus persistence. *J Infect Dis* 1993; 167: 744-8.
- 10 Saal J G, Steidle M, Einsele H, Müller C A, Fritz P, Zacher J. Persistence of B19 parvovirus in synovial membranes of patients with rheumatoid arthritis. *Rheumatol Int* 1992; 12: 147-51.
- 11 Nikkari S, Luukkainen R, Möttönen T, et al. Does parvovirus B19 have a role in rheumatoid arthritis? *Ann Rheum Dis* 1994; 53: 106-11.
- 12 Arnett F C, Edworthy S M, Bloch D A, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
- 13 Zhang L, Nikkari S, Skurnik M, et al. Detection of herpesviruses by polymerase chain reaction in lymphocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 1993; 36: 1080-6.
- 14 Jackson D P, Lewis F A, Taylor G R, Boylston A W, Quirke P. Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J Clin Pathol* 1990; 43: 499-504.
- 15 Nikkari S, Merilahti-Palo R, Saario R, et al. Yersinia-triggered reactive arthritis. Use of polymerase chain reaction and immunocytochemical staining in the detection of bacterial components from synovial specimens. *Arthritis Rheum* 1992; 35: 682-7.
- 16 Anderson M J, Jones S E, Minson A C. Diagnosis of human parvovirus infection by dot-blot hybridization using cloned viral DNA. *J Med Virol* 1985; 15: 163-72.