Rheumatol Int (1992) 12:147–151

Rheumatology INTERNATIONAL
Clinical and Experimental Investigations

Persistence of B19 parvovirus in synovial membranes of patients with rheumatoid arthritis

J.G. Saal [1], M. Steidle [1], H. Einsele [1], C.A. Müller [1], P. Fritz [2], and J. Zacher [3]

[1] Department of Internal Medicine, University of Tübingen, W-7400 Tübingen, Federal Republic of Germany
[2] Department of Pathology, RBK Hospital, W-7000 Stuttgart, Federal Republic of Germany
[3] Department of Orthopedics, University of Tübingen, W-7400 Tübingen, Federal Republic of Germany

Received February 6, 1992/Accepted May 18, 1992

Summary. Recent clinical observations support the hypothesis that persistent parvovirus B19 is a triggering factor of rheumatoid arthritis (RA) in certain genetically predisposed individuals. If this hypothesis is correct, a number of RA patients may exhibit parvovirus B19 DNA in their synovial membranes. We tested the synovial tissue and peripheral blood leukocytes of 20 patients with RA, 24 patients with other arthritides or osteoarthritis (non-RA), and 34 healthy blood donors for the presence of parvovirus B19 DNA using specific DNA amplification by polymerase chain reaction (PCR). Using this technique, parvovirus B19 DNA was demonstrated in the synovial biopsies of 75% of patients with RA but in those of only 16.7% of patients with non-RA. In autologous peripheral blood mononuclear cells the percentage of PCR-positive patients was about 15% in both RA and non-RA groups and did not differ from that in healthy controls. When the PCR data were correlated with the presence of anti-parvovirus B19 IgG antibodies in serum and synovia all patients with parvovirus B19 DNA in peripheral blood alone or in both peripheral blood and synovial membrane were seropositive. In contrast, about 40% of patients with parvovirus B19 DNA restricted to the synovial membrane were seronegative. These data indicate a highly disease-related persistence of parvovirus B19 in the rheumatoid synovium.

Key words: Rheumatoid arthritis – B19 parvovirus – Polymerase chain reaction

Introduction

In most cases human parvovirus B19 infections remain asymptomatic or cause benign illnesses such as erythema infectiosum in childhood or transient arthritis in the adult [1]. Persistent infection may explain why up to 16% of patients with parvovirus B19 arthritis develop chronic, sometimes recurrent, symmetrical polyarthritis affecting preferentially the small joints of the hands, wrists, knees, and cervical spine [2–6]. Most patients with these symptoms are women [3, 4], and the rheumatoid factor may be present or rise following parvovirus B19 infection [5, 6]. The 1987 American Rheumatism Association (ARA) criteria [7] for diagnosis of rheumatoid arthritis are often met. Therefore, since persistent and latent infections are common with parvovirus [8], one must ask whether persistent parvovirus B19 in susceptible patients can trigger a chronic polyarthritis that is phenotypically identical to rheumatoid arthritis (RA). If this is the case, a number of RA patients should exhibit parvovirus B19 DNA in the synovial membrane, while controls should not. Thus far, parvovirus B19 persistence has not been established as a mechanism for chronic arthropathy using dot blot [9, 10] or in situ [11] nucleic acid hybridization. However, both methods may still be too insensitive to detect persistent or latent infections where only a few copies of viral nucleic acid molecules are present in a very limited number of cells. The most widely used method for increasing the sensitivity of viral DNA detection is the polymerase chain reaction (PCR). In this report a newly developed parvovirus B19 PCR has been used to study the hypothesis of persistent parvovirus B19 infection in patients with RA.

Correspondence to: J.G. Saal

Materials and methods

Patients and clinical specimens

A total of 44 consecutive patients undergoing joint surgery of the knee or hip were studied prospectively: 20 with RA meeting at least five of the seven diagnostic criteria of the ARA [7], 5 with other arthritides (AA; 4 seronegative spondylarthropathies, 1 staphylococcal coxitis), and 19 with osteoarthritis (OA). The diagnosis of OA was based on the radiographic appearance and on the absence of signs, symptoms, and history of arthritis. Synovial tissue, peripheral blood mononuclear cells isolated by Ficoll density gradient centrifugation, and serum and synovial fluid were collected during surgery and cryopreserved at −180 °C immediately thereafter. Peripheral blood mononuclear cells and sera were also obtained from 34 healthy blood donors and used as controls. Clinical, radiological, and histopathological data characterizing the patients' joint disease are presented in Table 1.

Table 1. Clinical characterization of patients. ESR = erythrocyte sedimentation rate; RF = rheumatoid factor; ANA = anti-nuclear-antibodies; NSAIDs = nonsteroidal anti-inflammatory drugs; DMARDs = disease-modifying antirheumatic and immunoregulatory agents

	RA	AA	OA
Patients (*n*)	20	5	19
Female sex (%)	70	20	58
Age (years)	51 ± 4	42 ± 4	66 ± 2
Duration of disease (years)	11 ± 2	11 ± 4	NA
ESR (mm/h)	34 ± 4	41 ± 9	21 ± 3
RF positive (%)	59	20	NT
ANA positive (%)	21	NT	NT
Synovial proliferation (%)[a]			
0	0	20	40
1	8	60	20
2	25	0	40
3	67	20	0
Synovial exudation (%)[a]			
0	0	0	17
1	21	40	33
2	29	0	50
3	50	60	0
Anatomical stage (%)[b]			
1	6	20	0
2	12	0	0
3	29	20	29
4	53	60	71
Histology of synovitis (%)[c]			
Serofibrous	28	75	86
Lymphoplasmacellular	28	0	7
Ulcerous	44	25	7
Fibroblast transformation	0	0	0
Therapy (%)			
NSAIDs	100	75	29
Steroids	39	20	0
DMARDs	72	0	0
Arthroplasty	0	60	88
Synovialectomy	100	40	0

[a] Graded from 0 = lacking to 3 = severe
[b] Graded according to [26]
[c] Histomorphological subtyping according to [27]

Serological assay

Testing of anti-parvovirus B19 IgG antibodies in sera and synovial fluids was performed according to standard procedures (parvovirus IgG ELISA Kit, IBL, Hamburg, FRG).

Preparation of target DNA

DNA was extracted from tissue of a parvovirus B19 infected abortion (control) as well as from peripheral blood mononuclear cells, serum, and synovial biopsies after proteinase K digestion using the standard phenol-chloroform-isoamylalcohol extraction and precipitation with ethanol at −70 °C [12].

Slot blot hybridization

For slot blot hybridization a cloned parvovirus B19 DNA probe covering the integrate virus was kindly provided by J. P. Clewley (London). Radiolabeling to a specific activity of at least 2–4 × 10 cpm/μg as well as prehybridization and hybridization of the DNA insert and vector were performed as described elsewhere [12].

In situ hybridization

For in situ hybridization, a 483-bp fragment between nucleotides 2537 and 3022 of parvovirus B19 [28] was synthesized using PCR. After denaturation of 10 pg cloned parvovirus B19 DNA at 94 °C for 4 min and amplification as described below the fragment was purified by preparative gel electrophoresis and after precipitation labeled with digoxigenin 11-dUTP by the random primed labeling technique. Pretreatment of the tissues was carried out as described by Permeen et al. [13]. Prehybridization of the slides was carried out with 50 μl prehybridization solution (6 × SSC, 45% formamide, 5 × Denhardt's solution, and 100 μg/ml heat-denaturated salmon testes DNA) for 1 h at room temperature. After removal of the prehybridization solution, 20 μl hybridization solution containing 6 × SSC, 45% formamide, 10% dextran sulfate, and 20 ng digoxigenin-labeled probe was added on each slide. After 20 h hybridization at 37 °C slides were washed twice for 10 min in 6 × SSC, 45% formamide at 52 °C, twice for 5 min in 2 × SSC at room temperature, and finally twice for 10 min in 0.2 × SSC at room temperature. The color development of the slides was performed according to the instructions of the supplier of the digoxigenin kit (Boehringer, Mannheim, FRG). After 1 day the color precipitation was completed.

PCR testing

Oligonucleotides: Primers specific to a sequence of the *Pst*1 fragment of parvovirus B19 were synthesized using a DNA synthesizer (Pharmacia, LKB Gene Assembler Plus). Primer I (5′-ATG GGA TAC TCA ACC CCA TGG-3′, nucleotides 3365–3385) was complementary to sense DNA strand. Primer II (5′-CCT GTA GTG CTG TCA GTA ACC-3′, nucleotides 3544–3564) was complementary to the antisense DNA strand. For the hybridization experiments, a 35-bp-long synthetic probe (5′-ATA TGG TTA CAG TTA AAG CAT CAG GAG CTA TAC TT-3′, nucleotides 3460–3494) complementary to the central region of the amplified DNA sequence was synthesized.

Polymerase chain reaction: The examined DNA (200 ng) was amplified, with 35 cycles performed for 3 min at 66 °C (annealing and primer extension) and 1 min at 94 °C (denaturation). The reaction mixture and analysis of amplification products have been described elsewhere [14].

Nonradioactive labeling of the oligonucleotide: The probe was labeled on its 3′terminus with digoxigenin 11-dUTP (Boehringer) and terminal transferase (Boehringer). The filters were prehybridized with 6 × SSPE, 5 × Denhardt's solution, 0.1% sodium sarcosine, and 0.02% SDS at 43 °C for 30 min. For hybridization, 1 pmol digoxigenin 11-dUTP labeled probe per milliliter of hybridization solution was added. Hybridization was carried out at 43 °C for 30 min. Thereafter the filters were washed twice in 2 × SSPE, 0.1% SDS at room temperature for 5 min each, and in 6 × SSPE, 1% SDS at 61 °C for 10 min. The color development was performed as described above. After 2 h, color precipitates were shown. Validation of PCR was performed as described elsewhere [14].

Statistics

For statistical analysis of continuous variables the Mann-Whitney test was used. Nonparametric data were analyzed using Fisher's exact test and χ^2-test.

Results

Development of the PCR assay

The conditions for the amplification reaction were established with cloned parvovirus B19 DNA [28] as a substrate. After 35 cycles of PCR 1.0 fg parvovirus B19 DNA was demonstrated using agarose gel electrophoresis, and 0.1 fg was detectable by hybridization (Fig. 1). All controls examined (reaction mixtures without DNA, herpes simplex virus types 1 and 2, varicella zoster virus, adenovirus 2, cytomegalovirus, pGem2 DNA, pBR 322 DNA, and DNA from parvovirus B19 seronegative donors) were negative both in the agarose gel and the Southern blot assay.

Slot blot hybridization experiments

None of the synovial DNA specimens extracted from the 44 patients with different joint diseases studied showed parvovirus B19 DNA when tested by slot blot hybridization.

Parvovirus B19 PCR

After PCR amplification parvovirus B19 DNA ranging from 1.0 to 50.0 fg was detected in the synovial tissue of 19 of the 44 patients investigated (43.2%). Fifteen of the 19 patients had RA and four OA. Thus, 75% of the RA patients but only 21% of the OA patients and none of the AA patients (i.e., 16.7% of the non-RA patients) harbored minute amounts of parvovirus B19 DNA in their synovium. Statistically the differences between RA and non-RA groups are highly significant (Table 2).

In parallel experiments DNA extracted from autologous peripheral blood leukocytes was screened by parvovirus B19 PCR. As shown in Table 2, only seven patients (15.9%) produced positive signals. There was no statistically significant difference between the detection rates in RA patients, non-RA patients, and controls. Clinically, the RA and OA patients with synovial parvovirus B19 DNA did not differ significantly from the negative cases with regard to sex, age, duration of disease, erythrocyte sedimentation rate, rheumatoid factor positivity, synovial proliferation, synovial exudation, or anatomical stage of the disease. However, the DNA-positive RA synovium revealed more active inflammation, i.e., a higher prevalence of marked lymphoplasmacellular infiltration and an increase in lining cell ulceration and fibrin deposition.

Parvovirus B19 serology

In patients and healthy controls the presence of anti-parvovirus B19 IgG antibodies was analyzed by enzyme-linked immunosorbent assay (ELISA), and the results were compared with the PCR data. In addition, the synovial fluid from 15 RA patients was tested serologically.

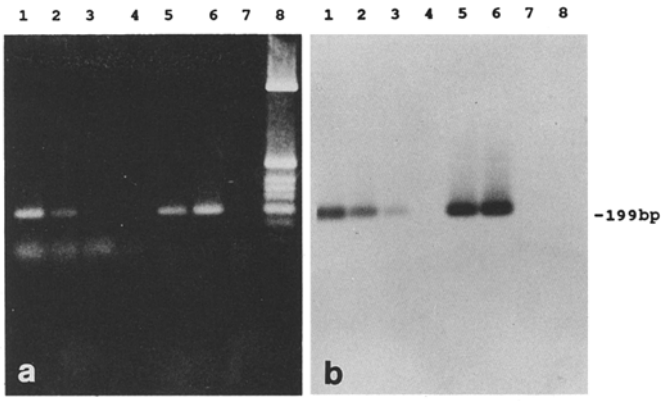

Fig. 1. **a** Agarose gel electrophoresis of PCR-amplified 199-bp fragments from cloned parvovirus B19 DNA. **b** Southern blot hybridization of **a** with a digoxigenin 11-dUTP-labeled oligonucleotide. Lanes 1–3 = 100 fg, 10 fg, 1.0 fg parvovirus B19 DNA; lane 4 = 200 ng embryonal lung fibroblast DNA (negative control); lane 5 = 200 ng DNA from synovial tissue of a patient with RA; lane 6 = 100 ng DNA from hydrops fetalis (positive control); lane 7 = 100 pg herpes simplex virus 1 DNA (negative control); lane 8 = marker (pBR 322/Hinf1)

Table 2. Prevalence of parvovirus B19 DNA in synovial membranes and autologous peripheral mononuclear cells (PBL) of patients and healthy controls as detected by PCR screening: correlation with the presence of anti-parvovirus B19 IgG antibodies (ELISA) in serum and synovial fluid

	n	Parvovirus B19 DNA (PCR)				Anti-parvovirus B19 IgG antibodies (ELISA)			
		Synovium		PBL		Serum		Synovia	
		n	%	*n*	%	*n*	%	*n*	%
RA patients	20	15	75	3	15	11	55	7[a]	47
Non-RA patients	24	4	17	4	17	13	54	NT	–
Controls	34	NT	–	5	15	18	53	NT	–

[a] Only 15 patients were analyzed

There was no significant difference between the percentages of seropositive cases in RA (55.0%), non-RA (54.2%; AA 40.0%, OA 57.9%), and healthy controls (52.9%) (Table 2). However, all the individuals who were parvovirus B19 DNA positive in peripheral blood were also seropositive. When only those 12 RA patients were considered whose parvovirus B19 DNA detection was restricted to the synovial membrane, five (41.7%) were seronegative. Anti-parvovirus B19 IgG antibodies were demonstrated in the synovial fluid of only one of these five patients.

In situ hybridization

In situ hybridization with a digoxigenin-labeled parvovirus B19 probe synthesized by PCR showed positive

signals in only one out of four synovial biopsies selected for these experiments because of their strong PCR signal of about 50 fg. The parvovirus B19 DNA signals were found in the nuclei of cells with marked affinity for synovial blood vessels, most probably endothelial cells.

Discussion

The hypothesis that parvovirus B19 virus is a triggering factor for RA in a genetically predisposed host is based mainly on the following observations. (a) A considerable number of patients with parvovirus B19 induced arthritis develop chronic RA-like polyarthritis [3, 4, 6]. (b) In 4 out of 69 cases of early RA [5] and in one patient with juvenile chronic arthritis (JCA) [15] evidence of recent infection with parvovirus B19 was demonstrated by the presence of anti-parvovirus B19 IgM antibodies. (c) The RA- and JCA-associated HLA haplotypes DR4 and DRw11 are considered risk factors for chronicity of parvovirus B19 induced arthritis by some authors [5, 15–17], although not by others [18]. (d) RA has recently been linked to a new parvoviruslike agent named RA-1 [19]. (e) In chronic parvovirus B19 arthritis persistent B19 DNA was detected in bone marrow [20].

Regarding RA, however, there is no evidence in the literature for persistent parvovirus B19 DNA as a pathogenetic factor. Although parvovirus B19 DNA was detected by slot blot hybridization in the synovial fluid of a patient with febrile nonrheumatoid polyarthritis [21], neither B19 antigen nor DNA was found in any of the synovial fluids tested from patients with RA or non-RA by radioimmunoassay or slot blot hybridization. Similarly, we found no parvovirus B19 DNA in the synovium of the 44 patients of this study or in that of another 101 patients with various rheumatic diseases (data not shown) using slot blot hybridization. This may be due to viral gene copy numbers below the detection limit of the hybridization tests applied.

To our knowledge, the study presented here is the first systematic attempt to detect synovial parvovirus B19 DNA in RA patients using specific DNA amplification by PCR. The PCR assay detected about 1.0 fg parvovirus B19 DNA, which is in good agreement with other published PCR assay data [6, 9, 10, 22, 23]. Using this test, parvovirus B19 DNA was demonstrated in synovial biopsies of 75% of patients with RA but in those of only 17% of patients with non-RA. In autologous peripheral blood mononuclear cells, however, the percentage of PCR-positive cases was 15%–17% in both RA and non-RA groups and did not differ from healthy controls. Thus, in most cases with RA the synovial parvovirus B19 DNA appeared not to be due to passenger lymphocytes or monocytes from the patients' peripheral blood. Histologically, RA patients with parvovirus B19 DNA-positive synovial membranes seemed to suffer from more active and advanced disease compared with negative cases.

Since particular efforts have been made to ensure the validity of PCR data [14], these results can be interpreted as evidence for a highly disease-related persistence of parvovirus B19 in the rheumatoid synovium. However, the synovial presence of parvovirus B19 DNA does not per se implicate parvovirus B19 as a causative agent in the pathogenesis of RA. Alternatively, rheumatoid synovium may only provide a microenvironment especially suitable for persistence or latency of the virus after intercurrent parvovirus B19 infection in RA patients. Parvovirus replication depends on certain cellular helper functions which are expressed during the late S or early G_2 phase of mitosis. As a consequence virus replication is relatively extensive in rapidly dividing cells. Furthermore, infection is related to particular stages of cellular differentiation [1]. Therefore, rheumatoid synovium with its proliferating synovial cells as well as various kinds of differentiating lymphoid and myeloid cells might meet a number of requirements for parvovirus infection and persistence. In fact, cryptic, persistent, and latent infections are common with parvovirus, also in the parvovirus B19 species [1, 2, 8, 24].

In situ hybridization experiments were performed to determine the cell type in the parvovirus B19 positive synovial membranes that might harbor small amounts of viral nucleic acid [11]. In this study the positive cells were most likely endothelial cells. Serological studies using ELISA showed that the prevalence of anti-parvovirus B19 IgG antibodies did not differ significantly between RA patients, non-RA patients and healthy controls. The percentage of about 55% were compatible with data reported in the literature for healthy individuals and RA patients [25].

A correlation of the PCR data with parvovirus B19 serology revealed that all patients with parvovirus B19 DNA in peripheral blood alone or in both peripheral blood and synovial membrane were seropositive. In contrast, about 40% of the patients with parvovirus B19 DNA restricted to the synovial membrane were antibody negative in both serum and synovial fluid. Whether these findings point to defective antibody production [2] as a cause of synovial parvovirus B19 persistence in certain individuals requires further study. Other explanations, such as effects of disease-modifying therapy or sensitivity problems of the antibody test system [5], should also be considered. Furthermore, epitope differences between parvovirus B19 strains not detectable by the ELISA used or inappropriate stimulation of the humoral immune system by latent synovial infection cannot be ruled out.

The synovial presence of parvovirus B19 DNA in RA patients first demonstrated in this study supports the hypothesis that parvovirus may play a pathogenetic role in RA.

Acknowledgements. This research was supported by the Bundesministerium für Forschung und Technologie (01ZU8601), Bonn, FRG. It is dedicated to Prof. Dr. Dr. h. c. H. D. Waller on the occasion of his 65th birthday. The excellent technical assistence of Mrs. K. Fenneberg, Mrs. S. Koch, and Ms. C. Schmidt is gratefully acknowledged.

References

1. Pattison JR (1990) Parvoviruses. In: Fields N, Knipe DM (eds) Virology. Raven, New York

2. Kurtzman GJ, Cohen BJ, Field HM et al (1989) Immune respone to B19 parvovirus and an antibody defect in persistent viral infection. J Clin Invest 84:1114–1123
3. White DG, Woolf AD, Mortimer PP et al (1985) Human parvovirus arthropathy. Lancet I:419–421
4. Reid DM, Reid TMS, Brown T et al (1985) Human parvovirus-associated arthritis: a clinical and laboratory description. Lancet I:422–425
5. Cohen BJ, Buckley MM, Clewley JP et al (1986) Human parvovirus infection in an early rheumatoid and inflammatory arthritis. Ann Rheum Dis 45:832–838
6. Naides SJ, Scharosch CC, Foto T et al (1990) Rheumatologic manifestations of human parvovirus B19 infection in adults. Arthritis Rheum 33:1297–1309
7. Arnett FC, Edworth SM, Bloch DA et al (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
8. Frickhofen N, Young NS (1989) Persistent parvovirus B19 infections in humans. Microb Pathog 7:319–327
9. Koch WC, Adler SP (1990) Detection of human parvovirus B19 DNA by using the polymerase chain reaction. J. Clin Microbiol 28:65–69
10. Sevall JS (1990) Detection of parvovirus B19 by dot blot and polymerase chain reaction. Mol Cell Probes 4:237–246
11. Hassam S, Briner J, Tratschin JD et al (1990) In situ hybridization for the detection of human parvovirus B19 nucleic acid sequence in paraffin embedded specimens. Virchows Arch [B] 59:257–261
12. Einsele H, Vallbracht A, Kandolf R et al (1989) Hybridization techniques provide improved sensitivity for HCMV detection and allow quantitation of the virus in clinical specimens. J Virol Methods 26:91–93
13. Permeen A, Sam CK, Pathmanthan R et al (1990) Detection of Epstein-Barr virus DNA in nasopharyngeal carcinoma using a non-radioactive digoxigenin-labelled probe. J Virol Methods 27:261
14. Einsele H, Steidle M, Vallbracht A et al (1991) Early occurrence of human cytomegalovirus infection after bone marrow transplantation as demonstrated by the polymerase chain reaction technique. Blood 77:1104–1110
15. Schwarz TF, Roggendorf M, Suschke H et al (1987) Human parvovirus B19 infection and juvenile chronic polyarthritis. Infection 15:264–265
16. Klouda PT, Corbin SA, Bradley BA et al (1986) HLA and acute arthritis following human parvovirus infection. Tissue Antigens 28:318–319
17. Woolf AD, Campion GV, Klouda P et al (1986) HLA and the clinical manifestation of human parvovirus infection. Br J Rheumatol 25 [Suppl 1]:35
18. Dijkmans BAC, Breedveld FC, Vries RRP de (1986) HLA antigens in human parvovirus arthropathy. J Rheumatol 13:1192–1193
19. Simpson RW, McGinty L, Simon L et al (1984) Association of parvoviruses with rheumatoid arthritis of humans. Science 223:1425–1428
20. Naides SJ, Foto F, Scharosch LL et al (1991) Detection of parvovirus B19-specific DNA sequences in bone marrow aspirates from chronic B19 arthropathy patients. Cell Biochem 45 [suppl 15E]:157
21. Dijkmans BAC, Elsacker-Niele AMW van, Salimans MMM et al (1988) Human parvovirus B19 DNA in synovial fluid. Arthritis Rheum 31:279–281
22. Salimans MMM, Holsappel S, Rijke FM van et al (1989) Rapid detection of human parvovirus B19 DNA by dot blot hybridization and the polymerase chain reaction. J Virol Methods 23:19–28
23. Clewley JP (1989) Polymerase chain reaction assay of parvovirus B19 DNA in clinical specimens. J Clin Microbiol 27:2647–2651
24. Srivastava A, Bruno E, Briddell R et al (1990) Parvovirus B19 induced perturbation of human megakaryo-cytopoiesis in vitro. Blood 76:1997–2004
25. Lefrere JJ, Meyer O, Menkes CJ et al (1985) Human parvovirus and rheumatoid arthritis. Lancet I:982
26. Steinbrocker O, Traeger CH, Batterman RC (1949) Therapeutic criteria in rheumatoid arthritis. JAMA 140:659–662
27. Fritz P, Laschner W, Saal JG et al (1989) Histological classification of synovitis. Zentralbl Allg Pathol 135:729–741
28. Shade RO, Blundell MC, Cotmore SA et al (1986) Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J Virol 58:921–936