CONCISE REPORT

Potential relationship between herpes viruses and rheumatoid arthritis: analysis with quantitative real time polymerase chain reaction

R Álvarez-Lafuente, B Fernández-Gutiérrez, S de Miguel, J A Jover, R Rollin, E Loza, D Clemente, J R Lamas

Ann Rheum Dis 2005;**64**:1357–1359. doi: 10.1136/ard.2004.033514

Objective: To determine whether the human herpes viruses, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpesvirus 6 (HHV-6), are detectable in serum and peripheral blood mononuclear cells (PBMCs) of patients with rheumatoid arthritis (RA).

Methods: 133 PBMC samples (61 RA, 72 healthy donors) and 136 serum samples (59 RA, 77 healthy donors) were analysed by quantitative real time polymerase chain reaction for DNA prevalence and viral load of HHV-6, EBV, and CMV.

Results: For PBMC samples significant differences were found for EBV in DNA prevalence (56% in RA *v* 33% in controls, p = 0.009) and viral load (copies/μg DNA 0–592.3 for RA *v* 0–40.4 for controls, p = 0.001). For serum samples a significant difference was found for HHV-6 DNA prevalence (10% in RA *v* 0% in controls, p = 0.006) and viral load (copies/μg DNA 0–529.1 for RA *v* 0 for controls, p = 0.007).

Conclusions: Herpes viruses may have a role in RA, although alternative explanations are possible: (*a*) defects in cellular immunity in patients with RA may result in a relatively high viral load; (*b*) patients with RA may be more prone to infection/reactivation. The usefulness of monitoring the DNA viral load in patients with RA is questioned by these data.

Rheumatoid arthritis (RA) is an inflammatory disease; although it is known that genetic, immunological, and environmental factors contribute to disease development, the aetiology remains uncertain. It has long been suspected that viral infections may have an important role in the aetiology and pathogenesis of RA[1] through direct joint tropism that causes tissue damage, or their ability to activate immune responses directed at joint tissues.[2] However, a major difficult in establishing a correlation between a viral infection and an autoimmune disease is the long lag period that often precedes the onset of the disease. Previous studies have examined the presence of herpes virus DNA in RA synovium, but the results were inconclusive[3–5]; other reports have supported the presence of certain viruses (human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV), human cytomegalovirus (CMV), parvovirus B19, rubella virus, human T cell leukaemia virus, and hepatitis B virus) as potential triggers of RA, on the basis of epidemiological evidence and abnormal immune responses to these viruses.[1 6] However, the role of viral infections in RA remains unresolved.

To assess the relevance of viral infection in RA we studied the presence and viral load of HHV-6, EBV, and CMV genomes by a quantitative real time polymerase chain reaction (PCR) assay in peripheral blood mononuclear cells (PBMCs) and serum of patients with RA and compared it with that of healthy controls.

PATIENTS AND METHODS

Patients and controls

Patients who fulfilled the American College of Rheumatology revised criteria[7] for the diagnosis of RA were included. All had established disease, and disease duration was more than 2 years. The study group comprised 61 patients with RA who were positive for rheumatoid factors (mean age 67.5 years, range 23–91; 41 female) and 77 healthy volunteers used as controls (mean age 32.6 years, range 18–57; 29 female); all of them were white. Among the patients with RA, 10 were not taking any drugs and 51 were receiving non-steroidal anti-inflammatory drugs or prednisone (<10 mg/day): 20 were also treated with methotrexate alone, and 14 in combination with intramuscular gold salts, 2 with leflunomide, and 5 with anti-tumour necrosis factor α monoclonal antibodies; 10 patients were taking other combinations of drugs without methotrexate. The local ethics committee granted approval for the study and all patients gave informed consent.

Samples and DNA isolation

DNA of PBMCs and serum samples was extracted from patients with RA and healthy blood donors, using Qiagen columns (QIAamp Blood Kits; Qiagen Inc), according to the manufacturer's protocol; finally, DNA concentration was determined by spectrophotometry.

Quantitative real time PCR

Quantitative real time PCR was used to detect HHV-6, EBV, and CMV DNA in a Real-Time Cycler (Rotor-Gene 2000; Corbett Research, Sydney). Primers and TaqMan probes were located in the putative immediate early 1 region of the HHV-6 genome,[8] in the BAM HI-K region of EBV sequence,[9] and in the glycoprotein B gene of the CMV genome.[10] As amplification control, a set of primers, and an exonuclease probe located in the human β-globin gene were used.[11] The reaction mix was performed in a final volume of 25 μl containing 10×TaqMan buffer, 200 μmol/l each dATP, dGTP, dCTP, and dTTP, primers at 0.25 μmol/l, TaqMan probe at 0.125 μmol/l, 1 U AmpliTaq gold DNA polymerase (PE Applied Biosystems, Foster City, CA), and 250 ng of the extracted DNA. The TaqMan PCR cycling conditions were 15 minutes at 95°C, 45 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 58°C for HHV-6 and 60°C for EBV and CMV,

Abbreviations: CMV, cytomegalovirus; EBV, Epstein-Barr virus; HHV-6, human herpesvirus 6; PBMCs, peripheral blood mononuclear cells; PCR, polymerase chain reaction; RA, rheumatoid arthritis

Table 1 DNA prevalence of HHV-6, EBV, and CMV in patients with RA and controls

	PBMCs			Serum		
	HHV-6	EBV	CMV	HHV-6	EBV	CMV
Patients with RA						
Positives/n	20/61	34/61	15/61	6/59	0/59	1/59
DNA prevalence (%)	33	56	25	10	0	2
Controls						
Positives/n	21/72	24/72	15/72	0/77	0/77	0/77
DNA prevalence (%)	29	33	21	0	0	0
p Value	0.65	0.009	0.6	0.006	1	0.4

Data were analysed by the χ^2 test for PBMC samples and by Fisher's exact test for serum samples.

for 50 seconds. The β-globin gene was amplified at both temperatures.

Each sample was analysed in duplicate and this was repeated twice for each virus, and once for β-globin. With these primers and conditions, we were able to detect as little as one viral copy. To exclude the possibility of contamination during the PCR, one negative control was amplified for every 10 samples in each experiment, consisting of all reagents except sample DNA. Each PCR run included dilution series of the quantified viral DNA, equivalent to 5000, 500, 50, and 5 copies (Advanced Biotechnologies, Inc, Columbia, MD); each point of the standard curve was analysed in triplicate. The final quantification of the DNA was performed by the software provided by the manufacturer.

Statistical analysis

Quantitative data are reported as median and range, and were compared using the Mann-Whitney U test. For qualitative data analysis, χ^2 was used unless an expected cell value was <5, when Fisher's exact test was preferred. Values of $p<0.05$ were considered to be significant.

RESULTS

DNA prevalences and viral loads in PBMCs

Table 1 shows prevalences of HHV-6, EBV, and CMV DNA in PBMCs of patients with RA; statistical analysis of the results showed a significant difference for EBV ($p = 0.009$). When we analysed the viral load (table 2), we again found a significant difference for EBV ($p = 0.001$). None of the controls were positive for more than one virus, whereas 28% of RA samples were positive for two or three viruses.

DNA prevalences and viral loads in serum

When we studied the serum samples, we found a DNA prevalence of 10% for HHV-6 and 2% for CMV in patients with RA (table 1); no positive samples were detected in samples from controls. We found a significant difference only for HHV-6 ($p = 0.006$). Viral loads were analysed by their median value (table 2), and a significant difference was found only for HHV-6 ($p = 0.007$).

Relationship between treatment, age, and sex in patients with RA and herpes viruses

We analysed the results for the 10 patients who had received no treatment and compared them with results obtained from the 51 patients treated with different disease modifying antirheumatic drugs. We compared the detection of some viral DNA in both groups. We found that 60% of non-treated patients had viral DNA compared with 76% in treated patients. These results were not significant ($p = 0.279$). In addition, no statistically significant differences were seen when different forms of treatment were compared (data not shown).

Because the study group was older than the controls, we analysed a subset of 19 patients with RA under 57 years (the median value in our RA population), age matched with controls; we found that the prevalences (table 3) and viral loads (data not shown) in PBMCs and serum were unchanged relative to previous results.

The RA group had more women than men, but similar results were seen when we compared their viral loads and prevalences (data not shown).

DISCUSSION

In this paper we analysed by quantitative real time PCR, the hypothesis of an association of HHV-6, EBV, and CMV with RA. We showed a significant difference in DNA prevalence for EBV in PBMCs, and for HHV-6 in serum; for viral loads, we again found significant differences for EBV in PBMCs and for HHV-6 in serum, when we compared patients with RA and controls. All patients with RA who were positive for a given virus in serum, were positive for the same virus in PBMCs. Although it is has been disputed, it is generally accepted that the detection of cell-free DNA in serum samples is a marker of active infection; this fact probably suggests that the virus found in serum is replicating in PBMCs. These results are in agreement with an increasing number of

Table 2 HHV-6, EBV, and CMV viral load in patients with RA and controls

	PBMCs			Serum		
	HHV-6	EBV	CMV	HHV-6	EBV	CMV
Patients with RA						
Range	0–92.3	0–592.3	0–240.9	0–529.1	0	0–45.9
Median	0	0	0	0	0	0
Controls						
Range	0–13.8	0–40.4	0–22.7	0	0	0
Median	0	0	0	0	0	0
p Value	0.451	0.001	0.337	0.007	1	0.277

Viral load are in copies/μg DNA. Data were analysed by the Mann-Whitney U test.

Table 3 DNA prevalence of HHV-6, EBV, and CMV in patients with RA under 57 years and controls

	PBMCs			Serum		
	HHV-6	EBV	CMV	HHV-6	EBV	CMV
Patients with RA <57 years						
Positives/n	6/19	13/19	5/19	3/19	0/19	0/19
DNA prevalence (%)	32	68	26	16	0	0
Controls						
Positives/n	21/72	24/72	15/72	0/77	0/77	0/77
DNA prevalence (%)	29	33	21	0	0	0
p Value	0.83	0.006	0.6	0.0005	1	1

Data were analysed by the χ^2 test for PBMCs samples and by Fisher's exact test for serum samples.

reports demonstrating frequent detection of cell associated and cell-free viral DNA in patients with RA.[6 12–14] However, other studies have reported conflicting results, as suggested by either infrequent detection of herpes virus DNA in PBMCs and serum of patients with RA, or insignificant differences between patients with RA and controls.[3–5 15] These conflicting results may be due to the different laboratory techniques used (DNA extraction, PCR method, amount of template in the reaction), different groups of patients with RA, different controls, and different study designs.

These results suggest that herpes viruses may have a role in RA, or perhaps, an alternative and more likely possibility is that (*a*) RA is characterised by defects in cellular immunity and this may result in a relatively high viral load; (*b*) patients with RA may be more prone to infection/reactivation of these viruses as a consequence of drug treatment. However, it is highly unlikely that these viruses can directly cause RA. A more likely explanation for the potential role of these or other viruses in RA is that they contribute to the development of autoimmune disease in genetically predisposed people through, for example, molecular mimicry or dysregulation of leucocyte functions.

All these data raise the question of the usefulness of monitoring the DNA viral load in patients with RA: among other topics, it would be of interest to study whether the exacerbation of clinical symptoms in infected patients correlates with increases in viral load. In conclusion, the possible involvement of these herpes viruses in RA should be investigated further.

ACKNOWLEDGEMENTS

Grant supporter: Fondo de Investigaciones Sanitarias (FIS 01/0587).

Authors' affiliations
R Álvarez-Lafuente, B Fernández-Gutiérrez, S de Miguel, J A Jover, R Rollin, E Loza, D Clemente, J R Lamas, Service of Rheumatology, Hospital Clínico San Carlos, Profesor Martín, Lagos s/n, 28040 Madrid, Spain

Correspondence to: Dr B Fernández-Gutiérrez, bfernandez.hcsc@salud.madrid.org

Accepted 7 February 2005

REFERENCES

1 **Harris**, ed. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* 1990;**322**:1277–89.
2 **Horwitz MS**, Sarvetnick N. Viruses, host responses, and autoimmunity. *Immunol Rev* 1999;**169**:241–53.
3 **Takei M**, Mitamura S, Fujiwara T, Horie J, Ryu S, Osaka S, *et al*. Detection of Epstein-Barr virus-encoded small RNA 1 and latent membrane protein 1 in synovial lining cells from rheumatoid arthritis patients. *Intern Immunol* 1997;**9**:739–43.
4 **Zhang L**, Nikkari S, Skurnik M. Detection of herpes viruses by polymerase chain reaction in lymphocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 1993;**36**:1080–6.
5 **Balandraud N**, Meynard JB, Auger I, Sovran H, Mugnier B, Reviron D, *et al*. Epstein-Barr virus load in the peripheral blood of patients with rheumatoid arthritis. *Arthritis Rheum* 2003;**48**:1223–8.
6 **Newkirk MM**, Watanabe Duffy KN, Leclerc J, Lambert N, Shiroki JB. Detection of cytomegalovirus, Epstein-Barr virus and herpes virus-6 in patients with rheumatoid arthritis without Sjögren syndrome. *Br J Rheumatol* 1994;**33**:317–22.
7 **Arnett FC**, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988;**31**:315–24.
8 **Nitsche A**, Müller CW, Radonic A, Landt O, Ellerbrok H, Pauli G, *et al*. Human herpesvirus 6A DNA is detected frequently in plasma but rarely in peripheral blood leukocytes of patients after bone marrow transplantation. *J Infect Dis* 2001;**183**:130–3.
9 **Wagner HJ**, Wessel M, Jabs W, Smets F, Fischer L, Offner G, *et al*. Patients and risk for development of posttransplant lymphoproliferative disorder: plasma versus peripheral blood mononuclear cells as material for quantification of Epstein-Barr viral load by using real-time polymerase chain reaction. *Transplantation* 2001;**72**:1012–19.
10 **Guiver M**, Fox AJ, Mutton K, Mogulkoc N, Egan J. Evaluation of CMV viral load using TaqMan CMV quantitative PCR and comparison with CMV antigenemia in heart and lung transplant recipients. *Transplantation* 2001;**71**:1609–15.
11 **Shibata D**. PCR-based detection of the herpesviruses and the papillomaviruses. In: Ehrlich GD, Greenberg SJ, eds. *PCR-based diagnostics in infectious disease*. Boston: Blackwell Scientific, 1994:455–81.
12 **Blaschke S**, Schwarz G, Moneke D, Binder L, Muller G, Reuss-Borst M. Epstein-Barr virus infection in peripheral blood mononuclear cells, synovial fluid cells, and synovial membranes of patients with rheumatoid arthritis. *J Rheumatol* 2000;**27**:866–73.
13 **Stahl HD**, Hubner B, Seidl B, Liebert UG, van der Heijden IM, Wilbrink B, *et al*. Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis. *Ann Rheum Dis* 2000;**59**:342–6.
14 **Takeda T**, Mizugaki Y, Matsubara L, Imai S, Koike T, Takada K. Lytic Epstein-Barr virus infection in the synovial tissue of patients with rheumatoid arthritis. *Arthritis Rheum* 2000;**43**:1218–25.
15 **Mousavi-Jazi M**, Boström L, Lövmark C, Linde A, Brytting M, Sundqvist VA. Infrequent detection of cytomegalovirus and Epstein-Barr virus DNA in synovial membranes of patients with rheumatoid arthritis. *J Rheumatol* 1998;**25**:623–8.