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

Objective: To determine whether the human herpes viruses, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and human herpes virus 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 0–592.1 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.

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, χ² 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 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 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 χ² test for PBMC samples and by Fisher’s exact test for serum samples.

| 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.
between patients with RA and controls. 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 leukocyte 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.

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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 Martin, Lagos s/n, 28040 Madrid, Spain

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

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

<table>
<thead>
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<th></th>
<th>PBMCs</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HHV-6</td>
<td>EBV</td>
</tr>
<tr>
<td>Patients with RA &lt;57 years</td>
<td>6/19</td>
<td>13/19</td>
</tr>
<tr>
<td>DNA prevalence (%)</td>
<td>32</td>
<td>68</td>
</tr>
<tr>
<td>Controls</td>
<td>21/72</td>
<td>24/72</td>
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<tr>
<td>DNA prevalence (%)</td>
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<td>33</td>
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<tr>
<td>p Value</td>
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<td>0.006</td>
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</tbody>
</table>

Data were analysed by the χ² test for PBMCs samples and by Fisher’s exact test for serum samples.

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