Prevalence of TTV DNA and GBV-C RNA in patients with systemic sclerosis, rheumatoid arthritis, and osteoarthritis does not differ from that in healthy blood donors

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Abstract
Objective—To determine the prevalence of GB virus-C (GBV-C) RNA and TT virus (TTV) DNA in patients with systemic sclerosis (SSc), rheumatoid arthritis (RA), and osteoarthritis (OA) as well as to compare the autoantibody pattern in patients with SSc with and without evidence of viral infection.

Patients and methods—The study included 168 patients (84 SSc, 41 RA, and 43 OA) diagnosed according to the American College of Rheumatology criteria and 122 volunteer blood donors. The presence of GBV-C RNA and TTV DNA in serum was assessed by nested reverse transcriptase-polymerase chain reaction (RT-PCR) and semi-nested PCR, respectively. Autoantibodies in patients with SSc were determined by enzyme linked immunosorbent assay (ELISA) and Hep-2 immunofluorescence.

Results—TTV-DNA was detected in 10/84 (12%) patients with SSc, 9/41 (22%) patients with RA, 3/43 (7%) patients with OA, and 16/122 (13%) blood donors. GBV-C RNA was present in 48/84 (58%) patients with SSc, 2/43 (5%) patients with OA, and 5/122 (4%) blood donors. No patient with RA was positive for GBV-C RNA. One patient with SSc and one patient with OA showed a double infection with GBV-C and TTV. 74/84 (88%) patients with SSc were positive for at least one autoantibody species tested: 18/84 (21%) showed anticytokeratin autoantibodies, 55/84 (66%) a speckled (36/84 (43%) fine, 19/84 (23%) coarse), and 20/84 (24%) a homogeneous nuclear Hep-2 pattern, and 21/84 (25%) had antinuclear autoantibodies. Anti-Scl-70 antibodies were found in 31/84 (37%) and anti-RNP antibodies in 5/84 (6%) patients with SSc. No differences in the autoantibody pattern in patients with SSc with or without viral infection could be detected.

Conclusion—The prevalence of GBV-C RNA and TTV DNA in serum samples from patients with SSc, RA, and OA was low and comparable with that in blood donors. A continuing infection with TTV and or GBV-C was not associated with a significant change in the autoantibody pattern in patients with SSc. These data provide no evidence for an association between GBV-C and/or TTV infections and SSc and/or arthritis (RA and OA).

The cause of systemic sclerosis (SSc) is still unknown, though the involvement of viral infections in this autoimmune disorder is suspected. Thus Pandey and Le Roy proposed that latent human cytomegalovirus (CMV) infection and its downstream effects on immune, vascular and repair mechanisms might serve as an accelerating factor in the abnormalities of SSc. A direct link between CMV infection and SSc is the high titre of antibodies to the polypeptide motifs of human CMV in comparison with those of Epstein-Barr virus in the serum of several types of rheumatic and connective tissue diseases. Our group described a higher prevalence of IgA and IgG antibodies against human CMV in patients with SSc. The pattern of autoantibodies in the patients with SSc with anti-CMV was also different.

Ferri et al demonstrated a slightly higher prevalence of anti-B19 NS1 antibodies and parvovirus B19 DNA in SSc than in osteoarthritis (OA). Intriguingly, Lunardi et al obtained evidence that chronic parvovirus B19 infection induces the production of anti-virus antibodies with autoantigen binding properties in patients with chronic symmetrical arthritis.

The hypothesis of a possible involvement of viruses in the development of autoimmune diseases is attractive, though direct proof is still missing. In particular, we do not know which mechanisms initially trigger the autoimmune responses. One of the open questions remains, how to identify those viruses which play a part in autoimmune processes, and how to differentiate between true anti-self immunoreactivity and immunopathology induced by unknown viruses or viruses not yet identified. Moreover, it remains unclear, whether the observed changes in autoantibody expression patterns illustrate an association between a viral infection and initiation or progression of an autoimmune disease or if they are just non-specific consequences of influence of the viral infection on the immune response of the infected subject. Finally, a viral infection might be not a cause, but a consequence of an autoimmune disease.
To answer these questions additional studies investigating the possible involvement of different viruses in the development of particular autoimmune disorders should be undertaken. Recent progress in molecular biology has led to the identification of two new viruses of humans, GB virus-C (GBV-C) and TT virus (TTV). GBV-C is a single stranded RNA virus with a genome length of about 10 kb and causes a persistent infection in humans. It belongs to the group of the Flaviviridae, which includes pestiviruses and flaviviruses. TTV is a small unclassified virus with a single stranded circular DNA of about 3900 nucleotides. Both viruses were detected with a high frequency in serum of patients with non-A-E hepatitis, but also in serum from blood donors and subjects at risk of parenterally transmitted infections. The pathological potentials of GBV-C and TTV and clinical consequences of GBV-C and TTV infections are not yet clear, though the association of these infections with some of the autoimmune disorders cannot be excluded. In this paper we report the prevalence of GBV-C and/or TTV infections in SSc and compare the infection rate with that of patients with rheumatoid arthritis (RA) and OA matched for age and sex. In addition, we ask the question, whether a continuing infection with TTV and GBV-C is associated with a different autoantibody pattern in patients with SSc.

**Patients and methods**

**PATIENTS**

Serum samples were obtained from patients with SSc (n=84, mean (SD) age 59 (10) years, 14 men), RA (n=41, age 56 (11) years, 8 men), and OA (n=43, age 56 (11) years, 8 men) matched for age and sex. The patients with SSc and RA were diagnosed according to the criteria of the American College of Rheumatology, and patients with OA according to the criteria of Altmann et al. Serum samples were also obtained from 122 healthy volunteer blood donors (age 39 (13) years, 73 men).

**DETECTION OF GBV-C RNA**

GBV-C RNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) as described earlier, with a few modifications. RNA was extracted from 140 µl of serum with the QIAamp Viral RNA Kit (Qiagen), suspended in 40 µl of distilled water, and 5 µl aliquots were analysed by PCR according to Okamoto et al with a few modifications. The first round of PCR was run with primers p59 (5'-ACA GAC AGR GGM GRA GGN AAY ATG) and p63 (5'-CAT YTT WCC RTT TCC AAA RTT) for 30 cycles, each consisting of one minute at 94°C, one minute at 50°C, and two minutes at 72°C. The second round of a 35 cycle PCR was run with primer p61 (5'-GGN AAY ATG YTR TGG ATA GAC TGG) and p63 for 35 cycles, each consisting of one minute at 94°C, one minute at 57°C, and two minutes at 72°C. An elongation step at 72°C for seven minutes was added to both rounds of PCR. Amplified DNA was separated by agarose gel electrophoresis and the 267 base pair product was visualised by ultraviolet fluorescence after staining with ethidium bromide.

**DETECTION OF TTV DNA**

DNA was extracted from 200 µl of serum with QIAamp Blood Kit (Qiagen), suspended in 40 µl of distilled water, and 5 µl aliquots were analysed by PCR according to the manufacturer's instructions with the primers YK-876 (5'-CTT TAC AGT CCT TAT TGC TTC CTC) and YK-1183 (5'-CAG AAC CAT ACA GCC TAT TGT GAC). All primer sequences were derived from the NS5 region of the GBV-C genome. Each cycle included denaturation at 94°C for one minute, annealing at 60°C for one minute, and extension at 72°C for two minutes. An elongation step at 72°C for seven minutes was added to both rounds of PCR. The 354 bp PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining.

**DETERMINATION OF THE AUTOANTIBODIES IN SSc**

Serum levels of IgG anti-topoisomerase 1 (Scl-70) and anti-RNP were measured in patients with SSc using Quanta Lite Scl-70 and RNP enzyme linked immunosorbent assay (ELISA) kits (Inova Diagnostics, San Diego, CA). Protein concentrations of more than 20 U/ml were considered positive. The Hep-2 immunofluorescence kit (Nova Lite; Inova) and monospecific autoantibody controls (Auto AB Panel) were used for detection of various autoantibodies in the serum of patients with SSc. Detected autoantibody patterns were classified into antinuclear, antinucleolar, and the antinuclear antibody into fine speckled, coarse speckled, and homogeneous patterns, correspondingly. Serum samples with autoantibody titres of more than 1/80 were considered positive.

**STATISTICAL ANALYSIS**

Statistical analysis was performed with the Mann-Whitney U test for non-parametric variables.

**Results**

GBV-C RNA was detected in 4/84 (5%) patients with SSc and in 2/43 (5%) patients with OA (table 1). No patient with RA had this viral marker. The differences between these three groups were not significant (p>0.05). Overall, GBV-C RNA was detected in six (4%)
Table 2  Prevalence of autoantibodies in the serum of patients with systemic sclerosis (SSc) in presence or absence of TTV-DNA and/or GBV-C RNA infection

<table>
<thead>
<tr>
<th></th>
<th>All patients with SSc</th>
<th>TTV and GBV-C negative</th>
<th>TTV and/or GBV-C positive</th>
<th>TTV positive</th>
<th>GBV-C positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>84</td>
<td>71</td>
<td>13</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Age (years, SD)</td>
<td>59.0 (9.7)</td>
<td>58.7 (9.6)</td>
<td>60.8 (10.8)</td>
<td>57.3 (10.3)</td>
<td>69.1 (3.5)</td>
</tr>
<tr>
<td>No of male/female patients</td>
<td>14/70</td>
<td>12/59</td>
<td>2/11</td>
<td>2/8</td>
<td>0/4</td>
</tr>
<tr>
<td>ELISA positivity, No (%) of patients [mean (SD) in U/ml]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG anti- RNP</td>
<td>5 (6) [6.0 (6.3)]</td>
<td>5 (7) [6.3 (7.0)]</td>
<td>0 [4.1 (3.4)]</td>
<td>0 [5.4 (3.7)]</td>
<td>0 [1.3 (1.1)]</td>
</tr>
<tr>
<td>IgG anti-isotopoisomerase 1 (Scl-70)</td>
<td>31 (37) [39.0 (42.9)]</td>
<td>26 (37) [37.2 (40.5)]</td>
<td>5 (38) [49.1 (54.9)]</td>
<td>5 (50) [62.9 (57.5)]</td>
<td>0 [3.1 (0.85)]</td>
</tr>
<tr>
<td>HEP-2 cell immunofluorescence findings, No (%) of patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centromeric</td>
<td>18 (21)</td>
<td>13 (18)</td>
<td>5 (38)</td>
<td>2 (20)</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Nucleolar</td>
<td>21 (25)</td>
<td>17 (22)</td>
<td>4 (31)</td>
<td>4 (40)</td>
<td>0</td>
</tr>
<tr>
<td>Fine speckled</td>
<td>16 (21)</td>
<td>12 (17)</td>
<td>4 (31)</td>
<td>4 (40)</td>
<td>0</td>
</tr>
<tr>
<td>Coarse speckled</td>
<td>19 (23)</td>
<td>17 (24)</td>
<td>2 (15)</td>
<td>2 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Speckled (fine and coarse)</td>
<td>55 (66)</td>
<td>49 (69)</td>
<td>6 (46)</td>
<td>6 (60)</td>
<td>0</td>
</tr>
<tr>
<td>Homogeneous</td>
<td>20 (24)</td>
<td>15 (21)</td>
<td>5 (38)</td>
<td>5 (50)</td>
<td>0</td>
</tr>
<tr>
<td>At least one positive feature</td>
<td>74 (88)</td>
<td>64 (90)</td>
<td>11 (85)</td>
<td>8 (80)</td>
<td>3 (75)</td>
</tr>
</tbody>
</table>

Of all 168 patients included into the study. This figure was only slightly lower than that for blood donors (5/122 (4%)).

The detection rate of TTV DNA in sera was higher (table 1). TTV DNA was detected in 10/84 (12%) patients with SSc, in 9/41 (22%) patients with RA, and in three (7%) of the 43 patients with OA (table 1). The frequency of the GBV-C RNA detection in all groups of patients 6/168 (4%) was not significantly different, and the overall prevalence of this marker was about the same as that for blood donors (5/122 (4%)). This figure is within the range of 1 to 5% reported for donors from other developed countries, and is close to the prevalence of GBV-C RNA (2.2%) found in patients with RA from Japan.

The overall prevalence of TTV DNA in our patients (13%) was the same as in blood donors but much lower than that in patients at high risk of blood borne infections—for example, in patients with end stage liver disease (44.2%) or in patients with haematological disorders who have received multiple transfusions (55.3%). The observed differences in the detection rates of TTV DNA in our three groups of patients were not significant (p>0.05). The frequency of TTV viraemia in our patients with RA (22%) was similar to the TTV infection rates of 26% and 28% registered in patients with SSc, RA, and OA (table 1). The frequency of the GBV-C RNA detection in all groups of patients 6/168 (4%) was not significantly different, and the overall prevalence of this marker was about the same as that for blood donors (5/122 (4%)). This figure is within the range of 1 to 5% reported for donors from other developed countries, and is close to the prevalence of GBV-C RNA (2.2%) found in patients with RA from Japan.

TTV is characterised by a high genome heterogeneity and the choice of primers to a significant extent influences the PCR results. For the current study we applied the primer set which had been extensively used in numerous studies during the past two years, in order to obtain comparable results. A series of recent reports has indicated that viral infections can influence the pattern of autoantibody expression in patients with autoimmune diseases. Neidhart and coworkers demonstrated differences in the autoantibody pattern of patients with SSs with antibodies to cytomegalovirus. Other investigations showed a significantly lower prevalence of rheumatoid factors in patients with RA infected with TTV in comparison with non-infected patients. Hajeer et al reported a negative correlation between anti-parvovirus B19 antibodies and rheumatoid factors in patients with RA. In the current investigation the autoantibody pattern found in all patients with SSs was similar to...
those reported earlier, including a comprehensive meta-analysis comprising data for more than 1000 patients with SSc from 30 publications. Comparison of the autoantibody patterns in virus infected and non-infected patients with SSc from the current study showed that continuing GBV-C or TTV infection, or both, have no evident effect on the manifestation of these antibodies. In this respect recent data are of interest, indicating the absence of changes in autoantibody patterns in GBV-C infected and non-infected patients with SSc. Therefore, these data provided no evidence for an association between GBV-C and/or TTV infections and SSc.

In conclusion, the obtained results showed neither a higher prevalence of GBV-C RNA and/or TTV DNA, nor changes in the pattern of expression of autoantibodies in patients with SSc. Therefore, these data provided no evidence for an association between GBV-C and/or TTV infections and SSc.

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