Tissue Persistence of Parvovirus B19 Genotypes in Asymptomatic Persons

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Parvovirus B19 (B19V) can persist in immunocompetent symptomatic and non-symptomatic individuals, as demonstrated by the finding of viral DNA in different tissues, in absence of viremia and of anti-B19V IgM. The spread and the nature of this phenomenon have not been clearly determined. In order to investigate the frequency of persistence and the tissue distribution of the three genotypes of B19V, the viral load of the persistent virus and its expression in the affected tissues, 139 tissue samples and 102 sera from 139 asymptomatic individuals have been analyzed by consensus PCRs and genotype specific PCRs for B19V detection and genotyping. Viral load was measured by real time PCR and viral mRNAs were detected by RT-PCR. Altogether, 51% individuals carried B19V DNA, more frequently in solid tissues (65%) than in bone marrow (20%). Genotype 1 was found in 28% tissue samples and 102 sera from 139 asymptomatic individuals have been analyzed by consensus PCRs and genotype specific PCRs for B19V detection and genotyping. Viral load was measured by real time PCR and viral mRNAs were detected by RT-PCR. Altogether, 51% individuals carried B19V DNA, more frequently in solid tissues (65%) than in bone marrow (20%). Genotype 1 was found in 28% tissue samples and 102 sera from 139 asymptomatic individuals have been analyzed by consensus PCRs and genotype specific PCRs for B19V detection and genotyping. Viral load was measured by real time PCR and viral mRNAs were detected by RT-PCR. Altogether, 51% individuals carried B19V DNA, more frequently in solid tissues (65%) than in bone marrow (20%). Genotype 1 was found in 28% tissue samples and 102 sera from 139 asymptomatic individuals have been analyzed by consensus PCRs and genotype specific PCRs for B19V detection and genotyping. 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slower than believed previously, as B19 DNA may be detectable in patient serum for months after infection [Musiani et al., 1995; Candotti et al., 2004; Lindblom et al., 2005]. Viral persistence has been demonstrated first in immunocompromised individuals, in whom it may cause severe chronic anemia [Kurtzman et al., 1987]. However, several reports have documented the ability of B19V to persist also in immunocompetent, symptomatic and non-symptomatic, individuals [Lefrere et al., 2005]. Numerous studies have demonstrated viral persistence in different tissues such as bone marrow, skin, synovium, liver, brain, myocardium [Cassinotti et al., 1997; Söderlund et al., 1997; Cassinotti et al., 1998; Lundqvist et al., 1999; Lundqvist et al., 1999a; Söderlund-Venermo et al., 2002; Vuorinen et al., 2002; Candotti et al., 2004; Lotze et al., 2004; Ohtsuka et al., 2004; Norja et al., 2006; Sanabani et al., 2006; Baskan et al., 2007]. However, the spread and the nature of this phenomenon have not been established and only few studies on the distribution of B19V genotypes in healthy individuals have been performed.

The study of B19V DNA persistence in asymptomatic individuals is fundamental for understanding the persistence of B19V DNA in symptomatic individuals and its possible pathogenic role. In the present study, bone marrow, skin, synovium and myocardium samples from different groups of persons, without signs and symptoms associated with B19 infection, were examined for the presence, the amount and the expression of the genome of the three genotypes of B19 parvovirus.

MATERIALS AND METHODS

Tissue and Serum Samples

Tissue biopsies were collected in 2006, from 120 Italian adult patients, after informed consent (Table I): (Group A) biopsies of bone marrow from 44 patients (mean age 60; range: 22–82; median: 61) with B19-unrelated hematological disorders referred to the Department of Hematology; (group B) biopsies of synovial tissue from 38 healthy individuals (mean age:70, range:36–82, median: 71) who underwent surgery for arthritis or joint trauma, referred to the Department of Orthopedics and Traumatology; (group C) skin biopsies from 38 patients (mean age: 67, range: 33–93, median: 70) with B19-unrelated dermatological lesions, referred to the Department of Dermatological Sciences. Group B and C patients were immunocompetent whereas group A patients where submitted to different chemotherapeutic regimens. A serum sample was collected at the same time of the biopsy from 102 patients. In addition, autopsy samples of the myocardium were obtained from 19 Italian individuals (group D) (mean age: 70, range: 53–84, median: 60) without known viral myocarditis or dilated cardiomyopathy.

DNA and RNA Purification

DNA from tissue and serum samples was extracted with the High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. For RNA extraction the TriPure Isolation Reagent (Roche, Basel, Switzerland) was used. The integrity of DNA and the absence of the Taq DNA polymerase inhibitors were checked by β-globin PCR as previously described [Bauer et al., 1991], and the efficiency of RNA extraction using RT-PCR for cellular β-actin mRNA [Bostic et al., 1999].

B19 DNA Detection and Genotyping

Screening and genotyping of clinical samples was undertaken with two consensus PCR assays using primers 1905f and 1987r located in the NS1 gene (NS1-PCR) and primers e2717f and e2901r specific for the VP1 unique region (VP1u-PCR) [Servant et al., 2002]. Discrimination among the three genotypes was done by restriction fragment length polymorphism analysis (RFLP) after digestion of the PCR products by Mfe I and Apa I [Servant et al., 2002]. Mfe I treatment of the NS1-PCR product gave two fragments of 36 and 67 bp for genotype 1 and an uncleaved fragment, 103 bp long, for genotypes 2 and 3. Similarly, Apa I restriction of the VP1u-PCR product gave two fragments of 149 and 55 bp for the genotypes 1 and 2 and an uncleaved fragment, 204 bp long, for genotype 3.

In addition, the samples were analyzed by two typespecific PCR assays: a genotype 1 specific nested PCR [Zakrzewska et al., 2001; Norja et al., 2006] and a genotype 2 specific PCR with the primers described by Hokynar [Hokynar et al., 2002].

DNA sequencing was used to establish the genotype of 16 B19 DNA sequences not clearly determined by the methods reported previously. Viral DNA for sequencing was prepared by PCR amplification of the NS1 region with the forward primer ATGAAAACCTTTCCATTAAATGATGTAG (nts1499-1525) and the reverse primer TTTGCAATCCAGACAGGTAAG (nts 2411-2431). Sequencing was carried out by the dideoxynucleotide

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<tr>
<th>Group A—bone marrow</th>
<th>60</th>
<th>44</th>
<th>38</th>
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<tr>
<td>Group B—synovium</td>
<td>70</td>
<td>38</td>
<td>33</td>
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<td>Group C—skin</td>
<td>67</td>
<td>38</td>
<td>31</td>
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<tr>
<td>Group D—myocardium</td>
<td>70</td>
<td>19</td>
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TABLE I. Tissue and Serum Samples

chain termination method on an ABI Prism 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were determined on both strands. The sequences were compared with the GenBank database by the BioEdit program; their belonging to one of the three B19V genotypes was assessed on the basis of their percentage of homology with consensus sequences for genotype 1, 2 and 3.

Real Time PCR

A real time PCR, which used Syber Green as intercalating dye, was developed in order to assess the copy number of genotype 1, 2 and 3. One μl (25 pmol) of each primer of the consensus NS1-PCR [Servant et al., 2002] and 5 μl of extracted DNA were added to 18 μl of Quantitec SYBR Green PCR Master Mix (Qiagen, Hilden, Germany). A first step of 10 min at 94°C, to activate the Taq polymerase, was followed by 40 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 60 sec, with a reading step at 75°C for 15 sec in the Rotor-Gene 3000 real time apparatus (Corbett Research, Sydney, Australia).

Melting curve analysis was undertaken to control the specificity of the PCR products.

To construct the calibration curves, serial dilutions of cloned sequences of each genotype were used. For genotype 1, a complete genome sequence cloned in the pGEM3Z, a kind gift of Giorgio Gallinella, was used. For genotype 2 and 3 the products of amplification (103 bp long) of a genotype 2 (chosen among our isolates) and of a genotype 3 (a kind gift of Sabri Sanabani) with the primers of the NS1-PCR were cloned in the pGEM-T vector (Promega, Milano, Italy). The sensitivity of this reaction was of 1 DNA copy per reaction and the range of linearity was 10^0–10^7. The reliability of the PCR was also evaluated by the use of the working reagent B19-DNA ISS 0300 (Istituto Superiore di Sanità, Roma, Italy).

In addition, a real time PCR for the β-globin gene was carried out to calculate the number of cells of the tissues extracted. The primers reported in literature have been used [Bauer et al., 1991]. A first step of 10 min at 94°C, to activate the Taq polymerase, was followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 sec, with a reading step at 76°C for 15 s in the Rotor-Gene 3000 real time apparatus (Corbett Research, Sydney, Australia). The product of amplification of the β-globin sequence was cloned in the pGEM-T vector (Promega, Milano, Italy) and the recombinant plasmid was used to construct the calibration curve for this reaction.

RT-PCR for Viral mRNA Detection

RT-PCR was used to detect spliced parvovirus mRNA species for the structural protein VP1, as previously described [Bostic et al., 1999], with the following modifications: (1) the reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), (2) for PCR the antisense primer was modified slightly (5’CCA CGA TGC AGC TAC AAC TT 3') and 45 cycles of amplification were carried out, using TagGold DNA polymerase (Applied Biosystem, Foster City, CA, USA). To detect the non-structural protein (NS1) mRNA, the cDNA was amplified using the PCR as described previously [Poole et al., 2006].

Statistical Analysis

The χ² test was used to compare the percentages of B19V DNA positive samples in different kind of tissues and the percentages of genotype 1 and 2 positive samples.

The Mann Witney test was used to compare the copy number of genotype 1 and 2 detected in the tissues.

RESULTS

The presence of B19V DNA was demonstrated in 71 out of 139 tissue samples (51%) from adult individuals, by two consensus PCR assays. Specimens were obtained from biopsies from 120 individuals, all without signs of B19 infection, and from 19 autopsy samples of the myocardium from adults, without known viral myocarditis or dilated cardiomyopathy.

B19V-specific immunological status could be evaluated in 102 of the patients participating in the present study: all the sera were anti-B19 IgM negative and anti-B19 IgG were present in 73.5% of the sera; in particular, anti-B19 IgG was present in 68% of group A (bone marrow) patients, in 97% of group B (synovium) patients and in 58% of group C (skin) patients.

As to the distribution of B19V DNA in different tissues, viral sequences were present in 9 out of 44 bone marrow samples (20%), in 21 out of 38 synovium biopsies (55%), in 29 out of 38 skin biopsies (76%) and in 12 out of 19 myocardium samples (63%) (Fig. 1).

Low levels of B19V DNA were present in only 4 out of 102 serum samples (less than 10^3 copies/ml).

![Fig. 1. Frequency of B19V genotypes in different tissues. The frequency is the percentage of positive samples among the total number of samples analyzed in each category. * the difference between the frequency of B19V DNA (all genotypes) in bone marrow and synovium (as well as in the other solid tissues), evaluated by the χ² test, was significant (P < 0.005).](image-url)
RFLP analysis of the consensus PCR products and genotype specific PCRs revealed the presence of genotype 1 in 19 samples (27%), of genotype 2 in 34 (48%) and of genotype 3 in only 2 (3%). In addition, 16 B19V DNA positive samples (22.5%) were not clearly typeable by the methods used in this study and have been analysed by sequencing: fifteen specimens which were positive for both genotype 1 and genotype 2-specific PCR, presenting with the RFLP pattern typical of genotype 2, were shown to be genotype 2 and one, with the RFLP profile of genotype 1, negative for both genotype 1 and genotype 2-specific PCR, showed to be genotype 1. (GenBank accession numbers: EU496865 and EU496866, EU797479, EU797480, EU797481, EU797482, EU797483, EU797484, EU797485, EU797486, EU797487, EU797488, EU797489, EU797490, EU797491, EU797492). Thus, genotype 1 was found in 20 samples (28%) and genotype 2 in 48 (68%), with a statistically significant difference ($P < 0.01$) between the two.

Among viral sequences found in bone marrow, four were identified as genotype 1 and five as genotype 2. In synovium, five were identified as genotype 1, 14 as genotype 2 and one as genotype 3. One synovium biopsy resulted positive for both genotype 1 and 2 sequences. In skin biopsies, eleven genotype 1 sequences and eighteen genotype 2 sequences were found. Finally, in the myocardium biopsies, genotype 1 sequences were not found, eleven sequences were genotype 2 and one genotype 3 (Fig. 1).

Genotype 1 was present in three sera with B19V DNA, one from a subject with genotype 1 positive biopsy and two from subjects with genotype 2 positive biopsy. The genotype 2 was present in a serum sample from a patient with genotype 2 positive biopsy.

Eight anti-B19V antibody-negative individuals had B19V DNA in the tissue sample: three were group A patients (bone marrow), two with genotype 1 sequences and one genotype 2 positive, one was a group B patient (synovium) with both genotype 1 and 2 sequences and four were group C patients (skin) genotype 2 positives.

The age of persons with genotype 1 varied from 40 to 85 years (mean: 60.5, median: 63), and that with genotype 2 from 32 to 91 years (mean: 66.8, median: 71).

The amount of viral DNA in the positive tissues was investigated by a real time PCR.

The copy number of B19V DNA ranged from less than 10 copies per 10^6 cells to 7 x 10^4 copies per 10^6 cells, except for two myocardium samples where about 10^6 copies per 10^6 cells were found. About 50% of the samples had less than 10^3 copies of B19V genomes per 10^6 cells. Figure 2 shows a comparison of the number of genome copies in 1 million of cells. The virus load is expressed as number of genome copies in 1 million of cells. The difference between genotype 1 and 2 copy number in these tissues was not significant by the Mann Witney test.

In order to evaluate the activity of the virus in the infected tissues, viral mRNA detection for structural and non structural proteins was carried out by RT-PCR. Eight samples of bone marrow, 8 samples of synovium and 8 cutaneous biopsies were analyzed. For each tissue 4 samples with genotype 1 and 4 samples with genotype 2 were analyzed. All these samples were positive by RT-PCR for cellular β-actin mRNA. Viral mRNA, for the capsid proteins, was demonstrated in two, genotype 2 DNA positive, bone marrow samples only. Viral messengers were not detected in solid tissue samples.

**DISCUSSION**

The aim of this study was to assess the frequency of B19V DNA persistence in asymptomatic individuals and to determine the sites of persistence and the condition of the persistent virus.

B19V DNA sequences were present in a large proportion (51%) of the asymptomatic individuals examined.

The B19V occurrence was highest in the skin (76%), followed by the myocardium and synovium (63% and 55%, respectively). The occurrence in bone marrow samples (20%) was significantly lower ($\chi^2$ test, $P < 0.005$) than that in solid tissues. Such data suggest that bone marrow, which is the known site of viral replication, is less apt to sustain B19 persistence in asymptomatic individuals. The finding of mRNA for B19V capsid proteins in 2 of 8 bone marrow samples, not in association with the highest viral load (data not shown) represents a marker of virus activity not found in any of the solid tissues examined in this study and could be related to the immunocompromised status of the hematological patients.

To assess the persistence and tissue distribution of the three B19V genotypes, B19V genotyping was carried out by RFLP analysis of two consensus PCR products and by two genotype specific PCRs, described in the literature. Using these methods, the genotype of 77.5% of the isolates was determined. The remaining 22.5% isolates (16 samples) gave ambiguous results, characterized by RFLP pattern typical of genotype 2 and positivity for both genotype 1 and genotype 2-specific PCR (15 samples) or by RELP pattern typical of genotype 1 and negativity by both genotype 1 and genotype 2-specific PCR (1 sample). After sequencing, fifteen samples showed to belong to genotype 2 and one to genotype 1. As the genotype 1 specificity of the nested PCR mainly depends on four to five nucleotide substitutions in the forward outer primer relative to the genotype 2 and 3 sequences, it is possible that few nucleotide changes in those positions decrease the specificity of the assay for genotype 1.

As recently described in the literature [Schneider et al., 2008], the presence of mixed infections by genotype 1 and 2 was also demonstrated in this study: in one biopsy sample, DNA of both genotypes was shown and two other patients had genotype 1 in the serum sample and genotype 2 in the tissue sample.

In this study, the similar occurrence of genotype 1 and 2 in bone marrow is at variance with the finding in solid tissues where genotype 2 was more frequent relative to the genotype 2 and 3 sequences. This indicates that genotype 2 is, or was [Norja et al., 2006], widely spread within the population, and suggests a preferable tropism of genotype 2 virus for solid tissues and its tendency to endure persistence.

The occurrence of genotype 2 in solid tissues in this study was higher than that in the Finnish population reported by Norja and coll. [Norja et al., 2006] who analyzed the distribution of genotype 1, 2 and 3 in a large number of solid tissues from healthy subjects born between 1913 and 2000. The pattern of distribution of the three genotypes among elderly and young individuals led to hypothesize that genotype 2 had circulated widely from the 1930s to the 1950s; after disappearing from wide circulation around the 1970s it remained absent thereafter. In the present study, the mean age of patients was relatively high and only few young subjects were included. This could explain, at least in part, the higher frequency of genotype 2 found in this study, in comparison to the data of the literature [Norja et al., 2006]. As expected, the presence of genotype 3 was rare.

In asymptomatic persistent B19V infections viral loads are lower than, or sometimes around, 10^4 DNA copies per 10^6 cells. The DNA load of genotype 2 in bone marrow, synovium and skin samples tended to be slightly lower in comparison with genotype 1, however the difference was not statistically significant. This result seems to be in agreement with the recent study of Ekman et al. [2007], showing that the three genotypes have a similar biological pattern.

B19V DNA was present in 63% of 19 myocardium autopic samples from persons that died for different causes, without been affected apparently by viral myocarditis or dilated cardiomyopathy; genotype 1 sequences were not found in the 19 autopsy samples whereas genotype 2 was demonstrated in 58% and genotype 3 in 5% of the samples.

The B19V DNA occurrence in myocardium reported in the literature ranges from 11% to 51.4% in patients with myocarditis or idiopathic left ventricular cardiomyopathy [Klein et al., 2004; Kühler et al., 2005; Kühler et al., 2008], with viral DNA loads from 30 to 900 copy number per μg of total DNA (corresponding approximately to 1.5 × 10^6–4.5 × 10^6 copy number per 10^6 cells) and from 7% to 40% in the control groups [Pankuweit et al., 2003; Klein et al., 2004; Lotze et al., 2004]. In patients with isolate left ventricular diastolic dysfunction the prevalence of cardiac B19V infection increases to 84–100%, with viral load of 10^2–10^3 genome equivalents per μg of total DNA (corresponding, approximately, to 5 × 10^9 – 5 × 10^10 copies per 10^6 cells) [Tschöpe et al., 2005]. Likewise, in the present study the viral load in myocardium, determined by a real-time PCR, varied from about 10^5 to 5 × 10^7 copies per 10^6 cells except for two samples, including the one of genotype 3, in which 10^6 copies per 10^6 cells were measured. Unfortunately, these autopsy samples were not suitable for mRNA detection and histological data and serum samples to evaluate the presence of viremia and the anti-B19 antibody status were not available. Most studies concern genotype 1 only, as can be inferred from the assays used for viral DNA detection. However, a higher frequency of genotype 2 (71.5%) in comparison to genotype 1 (28.5%) in the myocardium of patients with dilated cardiomyopathy has been reported recently [Kühler et al., 2008]. The results of the present study show that genotype 2 sequences are present frequently in the myocardium of persons without known viral myocarditis or dilated cardiomyopathy, and the viral load is of the same order as that reported in symptomatic patients.

Surprisingly, 8 (26%) of anti-B19V antibody negative individuals had B19V DNA in their tissues. Three were hematological patients and the lack of specific antibody response could be due to their immunocompromised status. It is possible that the B19V DNA persisted without expression in the other immunocompetent patients, or with very low, sub-immunogenic expression of viral proteins.

In conclusion, the low viral load and the lack of viral expression observed in this study suggest that the virus, persisting in solid tissues of asymptomatic persons, does not replicate. These conditions can allow the virus to persist a long time, evading the immune response, without cell/tissue damage.

In symptomatic patients, B19V persistence could be characterized by higher viral load and/or different levels of viral expression in comparison with asymptomatic persons. In addition, it is known that immune mediated mechanisms, triggered by the infection, [von Landenberg et al., 2007] and host genetic conditions [Kerr, 2005], could play a role in the pathogenesis of B19V-related diseases.

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