High Prevalence of Human Parvovirus B19 DNA in Myocardial Autopsy Samples from Subjects without Myocarditis or Dilative Cardiomyopathy

Thomas Schenk,1 Martin Enders,2 Stefan Pollak,3 Ralph Hahn,2 and Daniela Huzly1*

Department of Virology, Freiburg University Medical Center, Freiburg, Germany1; Labor Enders und Partner and Institut für Virologie, Infektiologie und Epidemiologie e.V., Stuttgart, Germany2; and Department of Forensic Medicine, Freiburg University Medical Center, Freiburg, Germany3

Received 28 August 2008/Returned for modification 26 October 2008/Accepted 1 November 2008

Human parvovirus B19 has been linked to a variety of cardiac diseases, as well as to erythema infectiosum, acute arthropathy, and fetal hydrops. A causal association between viral infection and cardiac disease was frequently postulated following the detection of B19 DNA by PCR in endomyocardial biopsy specimens. Since the lifelong persistence of B19 DNA in bone marrow, skin, synovia, tonsils, and liver was previously reported, the aim of our study was to investigate the possibility of asymptomatic B19 DNA persistence in heart tissue. Myocardial autopsy and postmortem blood samples were prospectively collected from 69 bodies sent to the Department of Forensic Medicine, Freiburg University Medical Center, for inquests. All study subjects were screened for B19-specific antibodies using a commercial enzyme immunoassay. Tissue samples were analyzed by real-time PCR for the presence of viral DNA. Since the presence of B19 genotype 2, known to have been circulating before 1960, would prove long-lasting persistence, the presence of the B19 genotype was retrospectively determined in seven of the study subjects by melting temperature analysis and sequencing of the PCR product. B19 DNA was found in myocardial samples from 46 of 48 seropositive and in none of 21 seronegative individuals. B19 genotype 1 was found in three patients born between 1950 and 1969. Genotype 2 was found in four patients born between 1927 and 1957. Our findings suggest lifelong persistence of B19 DNA in heart tissue. Thus, the detection of B19 DNA in myocardial biopsy specimens alone is not sufficient to postulate a relationship between B19 infection and cardiac disease.

Parvovirus B19, a member of the genus Erythrovirus, is a nonenveloped single-stranded DNA virus which replicates in erythroid progenitor cells of bone marrow. Its role as a human pathogen causing erythema infectiosum (fifth disease) (2), fetal hydrops after infection in utero (1), and transient aplastic crisis in patients with preexistent erythropathy is well established (23).

After the development of PCR technology and its application for the sensitive detection of viral DNA, B19 infection has been linked to a variety of acute and chronic disease processes. Based on the amplification of B19 genome sequences from tissue samples, an association with chronic arthropathy (6, 12) and acute fulminant liver failure in children (13), as well as with several entities of cardiac disease, i.e., acute myocarditis (5, 27), dilative cardiomyopathy or idiopathic left ventricular dysfunction (14, 16, 19), and peripartum cardiomyopathy (3), was suggested.

In the case of chronic arthropathy, the causal relationship of B19 infection has been questioned, since a case-control study showed a higher percentage of B19 DNA-positive synovial tissue samples in the control group than in the cases (28% in children with chronic arthropathy versus 48% in patients without it) (28). Likewise, the role of B19 in inducing acute hepatic failure was doubted when the persistence of viral DNA in histologically normal livers of adults was demonstrated (8). The persistence of parvoviral DNA has also been shown for the bone marrow and skin of healthy adults (4, 29). Recently, B19 DNA was detected in 17 to 64% of skin, synovia, tonsil, and liver tissue samples from heterogeneous sources, including both patients with a variety of symptoms and healthy donors (22). Norja et al. also showed that B19 genotype 2 (Gt-2), which was circulating before 1960, was present in tissue samples of subjects born before 1973, thus proving the long-term persistence of B19 DNA (22). The aim of our study was to investigate whether there is a lifelong asymptomatic persistence of B19 DNA in the heart after primary infection, as was previously shown for other tissues. This finding would be instrumental in the interpretation of PCR results from cardiac biopsy samples.

MATERIALS AND METHODS

Samples. Sixty-nine myocardial tissue and blood samples were randomly collected in three periods between November 2002 and July 2004. Corpses with putrefactive changes were excluded. The cause of death was unknown except in those cases showing massive signs of traumatization. In no case was acute viral infection suspected on the basis of information available. Heart tissue samples of approximately 1 by 1 by 0.5 cm in size from left and right ventricular myocardia were transferred into sterile empty containers for DNA analysis. Heart or femoral vein blood was drawn for serological analysis. All samples were immediately frozen at −20°C and tested in a blind manner in the virology laboratories (Freiburg University Medical Center and Labor Enders und Partner) as well as in the histology laboratory of the Freiburg University Medical Center.
Serology. B19 immunoglobulin M (IgM) and IgG antibody titers were measured by commercial enzyme immunoassay (Biotrin, Dublin, Ireland) in an automated BEP device (Dade Behring, Marburg, Germany). In addition, seropositive samples were subsequently tested by a parvovirus recomBlot assay (Mikrogen, Munich, Germany) to identify recent primary infections. These are characterized by a prominent IgG immune response against the viral protein (VP) C-terminal linear epitopes within VP2, usually detectable only within the first months after acute infection.

DNA analyses. For DNA analysis, myocardial tissue fragments of approximately 2 mm³ were digested overnight with 20 μl (±0.6 U/μl) proteinase K (Roche Applied Science, Mannheim, Germany) in 180 μl ATL lysis buffer (Qiagen, Hilden, Germany) at 56°C. Automated DNA extraction was performed with a total nucleic acid isolation kit in a MagnNA Pure liquid chromatography (LC) instrument (Roche Applied Science, Mannheim, Germany). Five microliters of these nucleic acid preparations was used for amplification with the FastStart DNA master hybridization probe kit in a LightCycler 1.0 instrument (Roche Applied Science, Mannheim, Germany). Five microliters of the amplified nucleic acid was used for amplification with the FastStart DNA master hybridization probe kit in a LightCycler 1.0 instrument (Roche Applied Science, Mannheim, Germany).

To demonstrate the long-term persistence of B19 Gt-2 DNA in heart tissue, the seven available samples from individuals born before 1973 were retrospectively subjected to a LightCycler consensus PCR (PCR-2). PCR-2 was performed as described previously with primers that bind within the VP1 coding sequence, resulting in an amplicon size of 212 bp (26). Immediately after PCR-2, an additional analytical cycle for melting temperature (Tm) analysis was included. Melting curves were automatically converted by LC software version 3.5 (Roche) into melting peaks. Isolates with a Tm value differing from that determined for B19 Gt-1 are considered to belong to Gt-2 or -3. The 212-bp amplicons were directly removed from the sequencing reaction mixtures using terminators were directly removed from the sequencing reaction mixtures using a DyeEx 2.0 spin kit (Qiagen, Hilden, Germany). Bidirectional sequencing of the 212-bp amplicons was performed with a Dual CyDye terminator sequencing kit (Amersham Biosciences Europe GmbH, Freiburg, Germany). Unincorporated dye terminators were directly removed from the sequencing reaction mixtures using a DyeEx 2.0 spin kit (Qiagen, Hilden, Germany). Sequence analysis was performed by the Trugene genotyping system (Siemens Medical Solutions Diagnostics GmbH, Bad Nauheim, Germany). Recombinant plasmid constructs carrying B19 DNA of the different genotypes (Gt-1, B19-NAN; Gt-2, B19-LaLi; Gt-3, B19-V9) were used as controls. Gt-1 and Gt-2-containing B19 plasmid constructs (11) were kindly provided by Maria Soderlund-Venermo and Klaus Hedman, Haartman Institute, University of Helsinki, Finland. The cloned genome of the V9 prototype (Gt-3) was obtained from the Collection Nationale de Cultures de Microorganisms (identification reference number PCD.V9/C22, accession number C5CM 1-206).

Histology. For histological evaluation of the myocardium, samples from the right and left ventricles were taken at standardized locations of each heart. Neutral phosphate-buffered formaldehyde (pH 7.0) was used as a fixative for up to 48 h. Sections (5 μm) of the paraffin-embedded tissue were stained with hematoxylin-eosin and investigated for myocarditis according to the Dallas criteria.

RESULTS

Study population and histopathology. Sixty-nine subjects—29 (42%) females and 40 (58%) males—with a mean age of 51.4 years (range, 15 to 85 years; median, 53 years) were investigated. In 35 cases (51%), death was caused by multiple traumas, craniocerebral injury, exsanguination, or suffocation in consequence of an accident, suicide, or homicide. Cardiovascular disease, i.e., thrombosis of coronary arteries, cardiac decompensation, and pulmonary embolism, were established in 18 cases (26%). Causes of death are summarized in Table 1. In a 19-year-old man, histological analysis of heart tissue revealed diffuse, prominent lympho-monocytic infiltrations of the myocardium, with scattered destruction of myocytes, and myocarditis of unknown origin was diagnosed. All other myocardial samples did not show any signs of acute or chronic inflammatory disease and did not fulfill the strict criteria of dilative cardiomyopathy.

Serology. The overall rate of B19 seropositivity was 69.6%. B19 IgG antibodies were detected in 72% of cases with a cardiovascular cause of death versus 69% of cases without (Table 1). None of the serum samples was positive for B19 IgM. Subsequent testing of IgG-seropositive samples using the parvovirus recomBlot test (Mikrogen, Munich, Germany) suggested one possible recent primary infection on the basis of a prominent band for the VP C-terminal linear epitopes in a 29-year-old man, who had died of progressive Becker’s muscular dystrophy. In all other seropositive cases, the immunoblot results showed antibody patterns consistent with past infection.

DNA analyses. B19 DNA was detected by PCR-1 in the myocardial tissue samples of 46 (96%) of 48 seropositive and in 0 of 21 seronegative study subjects. There was no significant difference in the prevalences of B19 DNA in tissue samples obtained from subjects with and without cardiac disease (Table 1). Estimated viral loads ranged between 2 × 10⁴ and 5 × 10⁴ genome equivalents (GE)/μg extracted DNA from heart tissue. The patient diagnosed with acute myocarditis was also positive for B19 DNA, with a viral load of 3 × 10⁵ GE/μg myocardial DNA. Results of the Tm analysis obtained with PCR-2 products of seven myocardial tissue samples and different recombinant B19 plasmid constructs are demonstrated in Fig. 1. DNA of Gt-1 was found in three patients born between 1950 and 1969. DNA of Gt-2 was found in four patients born between 1927 and 1957.

| TABLE 1. Age distribution and seropositivity rates grouped by different causes of death |
|-----------------------------|-----------------|-----------------|-----------------|
| Cause(s) of death           | No. of subjects | Mean age (yr) (range) | % PVB19 seropositivity | % B19 PCR positivity |
| Craniocerebral injury       | 6               | 43.2 (22–77)       | 100                      | 100                      |
| Multiple traumas with a blunt instrument | 14             | 47.1 (25–83)       | 78.6                      | 71.4                      |
| Suffocation, drowning, stabbing, or gunshot injury | 15             | 48.3 (15–77)       | 60.0                      | 60.0                      |
| Intoxication                | 10              | 44.0 (23–65)       | 50.0                      | 50.0                      |
| Natural death other than cardiovascular disease | 6              | 63.7 (29–82)       | 66.7                      | 50.0                      |
| Cardiovascular disease      | 18              | 59.2 (19–83)       | 72.2                      | 72.2                      |
DISCUSSION

B19 infection has been linked to a variety of human diseases, based on the detection of viral DNA by PCR in affected tissue (6, 12, 13). In chronic arthropathy and juvenile hepatic failure, the causative role of viral infection was questioned when healthy controls showed the frequent persistence of B19 DNA in synovial membranes and liver tissue (8, 28). In addition, viral DNA was detected in the bone marrow (4), skin (29), and tonsils of unaffected adults (22).

Several authors have postulated a causative role of B19 in cardiac disease, such as acute myocarditis (5, 17, 27), dilative cardiomyopathy or idiopathic left ventricular dysfunction (7, 14, 16, 19), and peripartum cardiomyopathy (3). A search of the Internet on “parvovirus, human, cardiac disease” yields more than 100 hits in 29 publications since 2005. Most of these studies suggest the causal link between B19 infection and cardiac disease, based on the detection by PCR or in situ hybridization of B19 DNA, while data on serostatus were scarce and sufficient control populations were rarely included. Moreover, demonstration of parvoviral protein expression in situ was missing.

Donoso Mantke et al. (7) showed the presence of B19 DNA in tissue samples in 7% (4 of 56) of explanted hearts and no viral DNA in healthy control samples. Interestingly, in the same study, this was contradicted by the detection of B19 DNA in 9% (5 of 54) of the healthy donor samples. Kühl et al. examined heart tissue samples from 172 patients with suspected myocarditis and found B19 DNA in 37% (17). However, DNA of other viruses was also detected frequently, whereas the patient’s serostatus was not evaluated, samples
showed no histological signs of inflammation, and control subjects were not tested. Klingel et al. compared the viral loads of cases with acute myocarditis and controls. They found up to $4 \times 10^5$ GE in patients, whereas viral titers of controls were below $10^3$ GE per µg myocardial nucleic acid (15). Viral loads of subjects investigated in our study ranged between $2 \times 10^4$ GE and $5 \times 10^4$ GE per µg DNA, with the majority of samples containing between $2 \times 10^2$ and $5 \times 10^2$ GE/µg. There was no difference in the viral loads of subjects with different causes of death. The subject with the highest concentration ($5 \times 10^4$ GE/µg) was a 26-year-old suicide victim. Thus, differences in viral load alone are not sufficient to postulate a pathogenic role of B19 in cardiac disease. Bultmann et al. demonstrated equal rates of B19 PCR positivity in endomyocardial biopsy samples taken from women with peripartum cardiomyopathy (4 of 26 patients [15%]) and controls with nonviral cardiomyopathies (5 of 33 [15%]), indicating viral persistence in the absence of inflammatory disease (3). Those authors also found higher viral loads in cases than in controls and postulated viral reactivation following an autoimmune process to explain a pathogenic role of B19 in cardiac inflammatory disease. However, although the persistence of B19 DNA in peripheral blood after acute primary infection has been shown in human immunodeficiency virus-infected patients as well as organ or stem cell transplant patients, the reactivation of B19 was never demonstrated in patients under immunosuppression (9, 10, 25).

Analyses of B19 genotypes in tissue samples from Finland, Scotland, and Germany revealed the presence of B19 Gt-1 in all examined age groups, whereas the detection of Gt-2 was predominantly confined to those born before 1973 (16, 20, 22). Those authors suggested that Gt-2 DNA originated from primary infection at times of Gt-2 predominance and supported the concept of lifelong asymptomatic persistence of B19 DNA in various tissues. Furthermore, Norja and colleagues recently postulated Gt-2 being the ancestor of Gt-1 in the late 1950s (21). Consistent with these reports, we found four B19 Gt-2 infections in patients born between 1927 and 1957, while two of three Gt-1 infections were from patients born after 1960 (Table 2). These results confirm the lifelong persistence of B19 DNA in myocardial tissue. Since various normal tissues from B19-seropositive individuals were shown to contain B19 DNA, it is hard to prove a causal role of the virus in diseased organs.

To our knowledge, this is the first study to examine B19 DNA positivity in myocardial samples from an unselected population. In none of the cases did the recent medical history suggest viral infection. The rate of seropositivity matched published data on IgG positivity in the local healthy population (24). In only one of 48 seropositive samples could recent infection not be excluded by the Western blot band pattern. However, B19 DNA was detected by PCR in more than 96% (46 of 48) of the myocardial samples taken from seropositive donors. The high rate of DNA positivity in our study might in part be explained by efficient DNA extraction methods and the use of sensitive real-time PCR for detection. We could show differences of up to $2 \log$s in the levels of recovery of viral DNA by different extraction methods (data not shown). Furthermore, autolytic processes in cadavers might contribute to more-efficient lysis of viral particles. In addition, we were able to use large postmortem tissue samples for our study, whereas most published data depend on the use of smaller biopsy samples taken in vivo. Cross contamination can be ruled out, as samples were analyzed in a blind manner without knowledge of serostatus and PCR was negative with all seronegative samples.

In conclusion, our data strongly suggest the lifelong asymptomatic persistence of B19 DNA in myocardial tissue after primary infection. The detection of B19 DNA by a sensitive PCR technology has to be interpreted carefully and is not a proof of a causal relationship between B19 infection and cardiac disease.

**REFERENCES**


