Intramyocyte Detection of Epstein-Barr Virus Genome by Laser Capture Microdissection in Patients With Inflammatory Cardiomyopathy

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Background—The causal role of Epstein-Barr virus (EBV) in inflammatory cardiomyopathy (IC) is still unclear, because this virus is present in latently infected circulating B lymphocytes in 90% of adults. Laser capture microdissection (LCM) has been applied on endomyocardial biopsy samples from patients with IC to assess the presence of EBV genome in separately dissected lymphocytes and myocytes.

Methods and Results—Among 142 patients with cardiac dilation and dysfunction and a histological and immunohistochemical diagnosis of myocarditis, 44 had a myocardial viral infection detected by polymerase chain reaction on frozen endomyocardial biopsy samples. In 9 of them, the virus detected was EBV. LCM was performed on 5-μm-thick paraffin sections of EBV-infected hearts. Lymphocytes and myocytes were microdissected and analyzed separately by polymerase chain reaction analysis on DNA extracted from the collected cells. Blood and myocardial samples from patients with positive and negative serology for EBV were used as controls. EBV genome was detected in myocytes but not in infiltrating lymphocytes of patients, nor in myocardial samples from controls. Despite full conventional antifailure therapy, a progressive cardiac dilation and dysfunction was documented in patients with EBV-related IC at a mean of 31±14 months of follow-up.

Conclusions—Intramyocyte detection of EBV can be obtained by LCM in up to 6.3% of patients with IC. This supports a cytopathic EBV role and suggests the opportunity for an antiviral/immunomodulatory therapy. (Circulation. 2004;110:3534-3539.)

Key Words: cardiomyopathy ■ myocarditis ■ viruses ■ heart failure ■ microdissection

Heart failure is a leading cause of morbidity and mortality, reaching 50% of cases within 1 year from the clinical diagnosis.1 Idiopathic dilated cardiomyopathy is the underlying cause in ~50% of patients, and growing evidence links this disease to myocarditis, because the incidence of biopsy-diagnosed myocarditis in patients with unexplained congestive heart failure can reach 67%.2,3 Cardiac dilation and dysfunction associated with evidence of inflammation in myocardial tissue has been defined recently as inflammatory cardiomyopathy and included in the World Health Organization’s classification of cardiomyopathies.4 Several studies demonstrated a high prevalence of viral genomes in the heart of patients with inflammatory cardiomyopathy.5 Enterovirus and adenovirus have been investigated extensively, and their relevance in the pathogenesis of inflammatory cardiomyopathy has been established. Conversely, the role of Epstein-Barr virus (EBV) in inflammatory cardiomyopathy is still unclear.

EBV is a ubiquitous human herpesvirus that infects >90% of adults worldwide and persists for life in B lymphocytes of the infected host.6,7 Primary EBV infection occurs in childhood and is usually asymptomatic, but some adolescents and young adults develop infectious mononucleosis, which can be complicated by myocarditis in up to 6% of cases, occasionally with a fatal course.8 Although EBV has been detected by polymerase chain reaction (PCR) analysis in the myocardium of patients with myocarditis,9-12 a possible source of viral DNA from EBV-infected bystander B lymphocytes has not been ruled out. This difficulty makes uncertain the role of EBV in the pathogenesis of myocarditis in patients with a past viral infection. The understanding of this process may have important consequences for the patient’s treatment and
prognosis, because the presence of a viral agent in the myocardium has been linked to the progression of cardiac dysfunction, and antiviral or immunomodulating drugs are now available as therapeutic options. The aim of the present study was to clarify the role of EBV in patients with inflammatory cardiomyopathy by use of laser capture microdissection (LCM) to localize the viral genome in EBV-infected hearts.

Methods

Patient Population
From January 1997 to May 2003 at our institutions, 142 patients (79 males, 63 females, mean age 43.5 ± 16 years) who presented with idiopathic left ventricular dilation and dysfunction (ejection fraction < 40%) received a histological and immunohistochemical diagnosis of myocarditis. PCR and reverse transcription–PCR analysis, performed on frozen endomyocardial biopsy tissue, enabled the detection of a myocardial viral infection in 44 of these patients. The viruses detected were adenovirus in 13 patients (9.1%), enterovirus in 11 (7.7%), parvovirus B19 in 6 (4.2%), hepatitis C virus in 4 (2.8%), influenza A virus in 1 (0.7%), and EBV in 9 (5 males, 4 females, mean age 44±14 years). These 9 subjects constituted the patient population of the present study.

Clinical Studies
Cardiac studies included both noninvasive (resting ECG, Holter monitoring, 2D Doppler echocardiography) and invasive (cardiac catheterization, left and right ventriculography, coronary angiography, and biventricular endomyocardial biopsy) examinations. All invasive cardiac studies were performed after informed consent was obtained and approval was given by the ethics committee of our institution. Endomyocardial biopsies (6 to 8 for each patient) were performed and samples collected as described previously. At the time of cardiac catheterization, blood samples were collected and stored at −80°C.

Serological Studies
All patients underwent routine laboratory tests, serological tests for the most common cardiotropic viruses, and immunological studies. In particular, anti-EBNA (EBV nuclear antigen) and anti-VCA (viral capsid antigen) IgM and IgG antibodies and anti-human immunodeficiency virus antibodies were tested. Anti-EBNA and anti-VCA antibodies were also tested in controls.

Immunohistological Studies
Histological and immunohistochemical studies were performed according to previously published protocols. Dallas criteria were adopted for histological diagnosis of myocarditis and implemented by the characterization of inflammatory infiltrates, performed with the following antibodies: CD45 (1:20), CD43 (1:40), CD45RO (1:100), CD20 (1:100), CD68 (1:50), CD4 (1:100), and CD8 (1:100; all from Dako). Myocytes were labeled by α-sarcomeric actin antibody (clone 5C5, Sigma), diluted 1:50.

Laser Capture Microdissection
Formalin-fixed, paraffin-embedded endomyocardial biopsy samples from EBV-infected hearts were subjected to LCM with the MMI NIKON UV-CUT System as described previously, according to the suggestions given by manufacturer’s protocols. Briefly, serial 5-μm-thick cut sections were mounted on a polyethylene foil slide (SL Microtest), immunostained with CD45RO antibody or with α-sarcomeric actin antibody by the avidin-biotin-peroxidase method, and counterstained for 1 minute with hematoxylin. Sections were allowed to dry further for 15 minutes at 37°C. Selection of cells for LCM was guided by immunohistochemical detection of lymphocytes, marked by CD45RO, and of myocytes, marked by α-sarcomeric actin. Myocytes and lymphocytic infiltrates were dissected separately from serial tissue sections by means of an ultraviolet laser that performs circumferential dissection of selected tissue areas following precisely a drawn incision path. By this cold ablation, the material to be extracted is never directly exposed to the laser. The microdissected tissue areas were measured, documented, and collected on an adhesive cap of nanotubes for nucleic acid extraction. A minimum of 100 cells, pooled on the same cap, were collected for each cell population. Lymphocytes and myocytes were randomly selected from different areas on serial sections. For each cell population, the procedure was repeated 3 times in each case. Specificity of selected cells was evaluated by direct microscopic visualization.

Molecular Biology Studies
DNA from laser microdissected tissue fractions was extracted and isolated with the Pico Pure DNA Extraction kit according to instructions given by the manufacturer (Arcturus). Briefly, 155 μL of extraction buffer was pipetted into each vial of proteinase K, and 10 μL of the solution was dispensed into each nanotube used during microdissection. Caps with collected isolated microdissected samples were inserted on the tubes, and they were immediately inverted. To ensure that the extraction buffer was covering the caps, the tubes were incubated for 16 to 18 hours at 65°C and, after incubation, the proteinase K was inactivated by heating at 95°C for 10 minutes. The samples, cooled at room temperature, were immediately used for PCR analysis according to previously described protocols. Appropriate positive and negative controls were added to each reaction. In addition, to ensure that no aspecific transfer of cells to the cap could happen, the extraction procedure was performed with 2 caps that were placed on the tissue sample without laser activation and 2 caps with a cut nude area adjacent to the stained tissue. The primers used for PCR amplification targeted DNA specific for the EBNA-1 protein of the EBV, which amplifies a fragment of 268 base pairs (bp). Amplified products were separated electrophoretically on 2% agarose gel stained with ethidium bromide. The purified PCR products were sequenced directly on an automated ABI model 310 DNA sequencer as described previously. Sequence data were analyzed with Sequence Analysis 2.1.2 and Sequence Navigator for sequence comparison. BLAST search was used to identify homology between sequences and all published DNA reported in the Gene Bank Data Base. Viral type was identified when nucleotide comparisons revealed an identity of >95% with the known type.

Blood samples of the 9 patients (500 μL each) and surgical ventricular endomyocardial specimens from (1) 5 age-matched patients undergoing valve replacement for mitral stenosis with serological positivity for anti-EBNA and anti-VCA IgG antibodies and with normal histology (normal seropositive controls) and (2) 3 pediatric patients undergoing tetralogy of Fallot surgical repair with negative serology for EBV (seronegative controls) were used.

Detection of EBV Protein in Nucleus of Myocytes
Colocalization of α-sarcomeric actin, a myocyte-specific marker, and EBV nuclear antigen (EBNA-1, 1:10; Chemicon), which is essential for maintenance of viral episome and for its replication, was assessed. The sections were examined by confocal microscopy. EBV-1 staining was performed according to the manufacturer’s protocols. In particular, a 3-step amplification procedure was applied, with goat anti-mouse IgG followed by rabbit anti-goat FITC-conjugated antibody. Tetramethylrhodamine isothiocyanate–conjugated anti-mouse IgM was used as secondary antibody for α-sarcomeric actin. Nuclei were stained with propidium iodide. Sections of known EBV-positive classic Hodgkin’s disease were used as positive controls for EBNA-1. Positive staining for EBNA-1 was interpreted as nuclear or granular nuclear. Sections with omission of the primary antibodies were used as negative controls. Surgical ventricular endomyocardial specimens from seropositive and seronegative controls were also stained.
Statistical Analysis
Data were analyzed by paired and unpaired Student’s t test. All values are expressed as mean±SD. A value of P<0.05 was considered statistically significant.

Results

Patient Population
Clinical and echocardiographic data of patients with EBV myocarditis are summarized in the Table. In all 9 patients, the clinical manifestation was heart failure (New York Heart Association class II to IV) lasting ≥6 months. All patients had been undergoing full conventional antifailure therapy, including digitalis, diuretics, ACE inhibitors, and carvedilol, for at least 3 months before admission and did not show an improvement in cardiac dimensions and function. During follow-up (mean 31±14 months; range 10 to 48 months), left ventricular diameters further increased despite treatment and were associated with worsening of left ventricular function (Table), although no fatal event or major arrhythmias occurred.

No patient had a history of familial cardiomyopathy, autoimmune disease, recent pregnancy, or alcohol abuse. No patient referred to a history of infective mononucleosis, but all of them had a past EBV infection as documented by the antibody profile. No EBV-related disease, hematologic disorder, or malignancy was present at the time of patient selection. Nevertheless, during follow-up, patient 3 developed a Burkitt lymphoma.

Serological Studies
The serological profile was consistent with a past EBV infection in all 9 cases. Specifically, anti-VCA IgM and anti-EBNA IgM scored negative, whereas anti-VCA and EBNA IgG scored positive (anti-VCA IgG=98.2±40.9 UA/mL, anti-EBNA IgG=80.6±45.1 UA/mL, positive value >20 UA/mL). No differences were found in these parameters compared with normal seropositive controls (anti-VCA IgG=106.8±46.7, anti-EBNA IgG=88.4±60.1, P=NS versus EBV myocarditis patients). Patients did not have immunologic abnormalities, and the serology for anti-human immunodeficiency virus antibodies was negative in all cases.

Immunohistological Studies
Histological and immunohistochemical analysis showed a focally active myocarditis with inflammatory infiltrates mainly represented by T lymphocytes (CD3+, CD45RO+, CD8+) and focal necrosis of the adjacent myocytes, which met the Dallas criteria15 (Figure 1). Areas of interstitial and focal replacement fibrosis were also present. No CD20+ B lymphocytes were detected. As illustrated in Figure 2, immunohistochemically guided microdissection enabled the collection on different caps of a discrete number (≥100) of CD45RO-positive T lymphocytes and of α-sarcomeric actin–labeled myocytes. The immunostaining markedly improved the visualization of cellular targets and allowed a precise microdissection of cell groups. The homogeneity of the captured material was confirmed under microscopic visualization before processing for DNA extraction.

Table: Clinical and Echocardiographic Characteristics of 9 Patients With EBV Myocarditis at Baseline and at Intermediate-Term Follow-Up

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NYHA indicates New York Heart Association; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; M, male; and F, female.

*P<0.001 vs baseline value.

Figure 1. Left ventricular endomyocardial biopsy from patient 3 showing active myocarditis, depicted by clusters of lymphocytic infiltrates adherent to myocyte border and associated with focal cell necrosis. Hematoxylin and eosin, magnification ×250.
Molecular Biology Studies
In all cases, the presence of a sufficient target DNA for PCR analysis was confirmed by amplification of \( \beta \)-globin. EBV genome was detected in microdissected myocytes of all patients and was absent in infiltrating lymphocytes (Figure 3). This result was confirmed on 3 repeated microdissection procedures. All the negative controls, including reagents without template and caps without microdissected cells, always showed no PCR product. EBV genome was not detected in the peripheral blood of the 9 patients and was also absent both in blood and in myocardial samples of normal seropositive and seronegative controls. Sequencing analysis of the PCR amplimers showed a high homology (>97%) with human EBV sequences (accession number V01555). PCR-product sequencing showed a minor variability among the isolates pertaining to different patients, which indicates that no false-positives caused by cross-contamination were amplified. Different single-point mutations and, in 3 cases, the additional presence of a single-nucleotide deletion were found.

Detection of EBV Protein in Nucleus of Myocytes
Immunostaining for EBNA-1 revealed a granular nuclear staining in rare myocytes from all patients (Figure 4). No labeling for EBNA-1 was seen in cardiac cells other than myocytes or in myocytes of controls.

Discussion
The definition of myocardial inflammation as virus-caused requires unequivocal demonstration of the viral genome or virus gene products in myocardial cell populations of affected hearts and their absence in unaffected hearts. Modern molecular biology techniques, in particular PCR analysis, are extremely sensitive and capable of detecting as few as 1 target molecule. When applied on the myocardial tissue, PCR enabled the identification of several viral agents that cause myocarditis. EBV has been detected occasionally in myocardial tissue of patients with myocarditis, but no conclusive data concerning the causal role of this virus are actually available. Indeed, because this virus persists for life in circulating B cells after a primary infection (which occurs in 90% of adults), contamination of blood could be the source of the identified viral DNA. In the present study, we tried to overcome this problem using LCM to ascertain the presence of EBV genome in cardiomyocytes.

LCM on Endomyocardial Biopsy Tissue
LCM is a rapid, reliable method that allows, under direct microscopic visualization, a 1-step selection of specific cells from a section of complex, heterogeneous tissue. It can be routinely used to collect pure populations of targeted cells for subsequent DNA, RNA, or protein extraction. Compared with LCM, in situ techniques are time consuming and sometimes technically difficult, whereas immunohistochemistry may lack sensitivity and specificity.

The present study demonstrated, for the first time, that this technique can be used on small human endomyocardial biopsy samples to selectively analyze a single cardiac cell population, without any loss of sensitivity compared with PCR performed on the whole tissue. This easy and fast method may find several fields of application in cardiology, including the selective study of the gene-expression profile of...
different cells, either resident or injected in the heart, properly identified by histochemical markers.

In the present study, LCM allowed the detection of EBV in myocytes of 6.3% of patients with inflammatory cardiomyopathy. The intramyocyte viral localization was confirmed by the detection of Epstein-Barr nuclear antigen in the myocyte nuclei. The presence of the viral genome in myocytes suggests a possible causal role of the virus in inflammatory cardiomyopathy, because EBV genome was absent in the normal myocardium of seropositive controls and in the heart of patients who were not infected by the virus. In addition, the intramyocyte localization of EBV indicates a likely cytopathic mechanism of damage, similar to that established for adenovirus and enterovirus, which enter the myocytes through a specific receptor and exert their cell injury through the release of specific proteolytic enzymes (ie, protease 2A for enterovirus). The negativity of EBV genome in infiltrating lymphocytes can be related to the lack of B lymphocytes in the myocardial inflammatory infiltrate and to the very low tropism of EBV for T lymphocytes in seropositive but not immunocompromised individuals.

Characteristics of Patients With EBV-Related Inflammatory Cardiomyopathy
Each of the 9 patients affected by EBV-related inflammatory cardiomyopathy had a past infection, as demonstrated by the antibody pattern, but they had no history of infectious mononucleosis or of cardiac diseases. None of them showed a serological reactivation of the infection or symptoms of active infection. The same antibody pattern was present in normal seropositive controls, in whom the virus was absent from the myocardial tissue. Thus, as for most of the cardiotropic viruses, the presence of serum antibodies for EBV is not indicative of viral infection in the heart. Moreover, even the presence of the virus in the blood does not allow any conclusion to be drawn about myocardial infection, because EBV persists for life in circulating B lymphocytes of the host after a primary infection and can be detected in 0.5 to 50 B cells for every million B cells in the peripheral blood. This low viral load in previously infected subjects explains our failure to detect the virus in 500 µL of blood from patients and seropositive controls and indicates that the percentage of infected circulating cells in EBV-related inflammatory cardiomyopathy is as low as in normal seropositive subjects.

In all patients, the clinical manifestation was chronic heart failure, with a slow, progressive evolution. This unfavorable trend was not halted or improved by the administration of a full conventional antifailure therapy regimen.

Pathogenetic Considerations of EBV-Related Inflammatory Cardiomyopathy
Up to 50% of patients with infectious mononucleosis show nonspecific ECG abnormalities that usually resolve completely within a few weeks, which demonstrates a possible asymptomatic cardiac localization of the virus. It can be hypothesized that in some patients, a subclinical myocarditis occurs during the primary EBV infection, and that subsequently the viral genome might persist latently in myocytes for years. Nevertheless, under the influence of unknown factors, it can reactivate and induce myocarditis, with a mechanism similar to other cardiotropic viruses, such as parvovirus B19. Moreover, it cannot be ruled out that even in the latent phase, the virus may cause myocardial inflammatory damage, because latent membrane protein 1, a protein that is expressed in latent EBV-infected cells, resembles proteins of the superfamily of tumor necrosis factor receptors and interacts with the family of tumor necrosis factor receptor–associated factors, which are signal-transducing molecules. This interaction results in activation of the nuclear factor-κB transcription factor, which plays a key role in the induction of proinflammatory gene expression and has been implicated in the pathogenesis of several acute and chronic inflammatory diseases.

Clinical Implications
A recent report from Kuhl et al demonstrates disease progression in patients with inflammatory cardiomyopathy who present with adenovirus and enterovirus persistence. On the other hand, viral myocardial clearance, obtained by interferon-β administration, is followed by systematic improvement in cardiac dimensions and function. On this
basis, even EBV-related inflammatory cardiomyopathy might potentially benefit from the administration of antiviral/immunomodulatory agents, particularly when, as in the present patient population, antifailure therapy is unable to halt the disease progression.

Conclusion
LCM may allow the identification of intramyocyte EBV genome in up to 6.3% of patients with inflammatory cardiomyopathy. This indicates a cytopathic EBV role and suggests the opportunity for an antiviral/immunomodulatory therapy.

Acknowledgments
This study was supported by the MURST project, “Myocarditis/Inflammatory Cardiomyopathy: efficacy of a therapeutic approach tailored on patients’ virologic and immunologic profile.”

References

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_Circulation_. 2004;110:3534-3539; originally published online November 22, 2004;
doi: 10.1161/01.CIR.0000148823.08092.0E

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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